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Supervisor: Dr. Gianni M. Di Guglielmo, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Physiology

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Transforming Growth Factor Beta Receptor Partitioning: Molecular Mechanisms and Functional Consequences

(Spine title: TGFbeta Receptor Partitioning Signalling Outcomes)

(Thesis format: Integrated Article)

by

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Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
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The thesis by

Sarah Elizabeth Anne McLean

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Transforming Growth Factor Beta Receptor Partitioning: Molecular Mechanisms and Functional Consequences

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ABSTRACT

The transforming growth factor beta (TGFβ) pathway has been conserved throughout evolution and plays important roles in tissue homeostasis. Dysregulation of the TGFβ pathway has been implicated in a number of disorders, including cancer, fibrosis, and vascular conditions. The signalling potential of the TGFβ pathway is regulated by the route of internalization of its cell-surface receptors: Receptors internalized by clathrin-mediated endocytosis propagate signal transduction while those internalized by membrane rafts are targeted for degradation. Given the importance of trafficking of the TGFβ receptors to signal propagation, this thesis focuses on evaluating proteins which direct TGFβ receptor internalization and trafficking. Initial work in this thesis shows that the extracellular domain of the type II TGFβ receptor (TβRII) and the glycosylation state of the cell are important factors in permitting membrane-raft localization of TβRII. Using this information I assessed the ability of TβRIII, a glycosylated cell surface protein, to direct TβRII internalization. I found that TβRIII increases membrane-raft independent internalization of TβRII, increases TβRII/TβRI complex half-life, and basal TGFβ signalling. I next assessed the role of βarrestin2, a protein which interacts with TβRIII, in regulating TβRII trafficking and signalling. I show that βarrestin2 interacts with TβRII and traffics with TβRII to the early endosome to increase Smad-dependent signalling. Also, I show that depletion of βarrestin2 increases Smad-independent signal transduction. In the last data chapter of this thesis, I evaluate the role of TGFβ1 and TGFβ3 to direct TGFβ trafficking and signalling. I found that TGFβ3 is less potent than TGFβ1 at propagating TGFβ signalling. I also show that TGFβ3 induces a different binding ratio of TβRII/TβRI cell-surface complexes, which could explain its decreased potency. Overall my studies highlight the role of receptor-interacting proteins in directing TGFβ receptor trafficking and signal transduction. Since this pathway is dysregulated in a number of pathologies, my studies suggest that TGFβ receptor trafficking is an important avenue to modifying TGFβ signal transduction.

KEYWORDS

Transforming growth factor beta

Membrane raft

Endocytosis

Smad signal transduction

Intracellular trafficking

βarrestin

Rab GTPase

CO-AUTHORSHIP

Chapter 2 of this thesis was previously published in the Biochemical Journal on April 8th, 2009 (Biochem J.,421: 119-131). The peer-reviewed paper is titled 'The extracellular domain of the TGFβ type II receptor regulates membrane raft partitioning'. The sources of all plasmids and reagents that were used in the studies are outlined in the Materials and Methods section of the chapter. We especially thank Dr. E. Leof (Mayo Clinic, Rochester, MN, U.S.A.) for providing the cDNA for the GMCSF-TβRII hybrid receptor and anti-CD131 Fab fragments. We also thank Dr. I.M. Chaiken (Drexel University College of Medicine, Philadelpha, PA, U.S.A.) for the generous gift of GMCSF2RB construct. Figures 2.2, 2.3A, and 2.4 in this thesis were performed by Valbona Luga from Dr. J. Wrana's lab (University of Toronto, Toronto, Canada). All other experiments were performed by Sarah Elizabeth Anne McLean.

Chapter 3 of this thesis was previously published in the Biochemical Journal on April 20, 2010 under 429: 137-145. The peer-reviewed paper is titled 'TGFβ (transforming growth factor β) receptor type III directs clathrin-mediated endocytosis of TGFβ receptor types I and II'. All the experiments in Chapter 3, except those in Figure 3.11, were performed by Sarah Elizabeth Anne McLean. The experiment shown in Figure 3.11 was performed by Dr. Gianni M. Di Guglielmo (Western University, London, Canada).

All of the experiments in Chapters 4 and 5 were performed by Sarah Elizabeth Anne McLean, except for those in Fig. 4.7C, which was performed by Dr. Gianni M. Di Guglielmo.

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This thesis is dedicated in memory of my mother,

Laura Jean Margaret McLean.

(1958-2006)

I know that you are still watching over me

Guiding me with the quietness and stillness

Of my heart.

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
Act-RIIA	Activin A receptor type IIA
Act-RIIB	Activin A receptor type IIB
ADAM12	A disintegrin and metalloprotease protein 12
ALK5	Activin receptor-like kinase 5
AMHR-II	Anti müellerian hormone receptor type II
ANOVA	Analysis of variance
AP-2	Adaptor protein 2
ARE	Activin response element
Arf6	ADP-ribosylation factor 6
ATCC	American Type Culture Collection
β-gal	Beta-galactosidase
βarr2	Beta arrestin 2
BMP	Bone morphogenetic protein
BMPRI	Bone morphogenetic protein receptor type I
BMPRII	Bone morphogenetic protein receptor type II
BSA	Bovine serum albumin
Cav-1	Caveolin 1
CDK4	Cyclin dependent kinase 4

CDK6	Cyclin dependent kinase 6
cDNA	Complementary deoxyribonucleic acid
cPML	Cytoplasmic promyelocytic leukemia protein
CTGF	Connective tissue growth factor
CTLA-4	Cytotoxic T-lymphocyte antigen 4
Cy3	Cyanine 3
Cy5	Cyanine 5
Dab2	Disabled homolog 2
DAP kinase	Death associated protein kinase
DAPI	4',6-diamidino-2-phenylindole
DISC	Death inducing signalling complex
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
Dol-P	Dolichol-phosphate
DSS	Disucinimidyl suberate
Ecad	Epithelial cadherin
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early endosomal autoantigen 1
EGFR	Epidermal growth factor receptor

EMT	Epithelial to mesenchymal transition
Eps15	Epidermal growth factor receptor substrate 15
ERK	Extracellular signal-related kinases
FITC	Fluorescein isothiocyanate
FoxH1	Forkhead box protein H1
GAP	GTPase activating protein
GDF	Growth and differentiation factor
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescence protein
GMCSF	Granulocyte monocyte colony stimulating factor
GPCR	G protein coupled receptor
GPI	Glycophosphatidyl-inositol
Grb2	Growth factor receptor-bound protein 2
GRK	G protein receptor kinase
GTP	Guanosine triphosphate
h	hours
HA	Hemagglutinin
HEK	Human embryonic kidney
HRP	Horse radish peroxidase

HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
I-Smad	Inhibitory smad
Inhba	Inhibin, beta A
IP	Immunoprecipitate
JNK	Jun kinase
KRH	Krebs Ringers Hepes
LAP	Latency associated peptide
LTBP	Latent TGFβ binding protein
Lux	Luciferase
mAb	Monoclonal antibody
MAP	Mitogen activated protein
MAPK	MAP kinase
MAP2K	MAP kinase kinase
MAP3K	MAP kinase kinase kinase
MEM	Modified eagle medium
Mgat5	mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase
MH1	Mad homology 1
MH2	Mad homology 2

MIS	Müellerian inhibiting substance
MMP14	Matrix metalloproteinase-14
Mv1Lu	Mink lung cells
Ncad	Neuronal cadherin
NEAA	Non-essential amino acids
N-t	Amino terminus
pAb	Polyclonal antibody
Par6	Partitioning defective 6
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDZ	Post synaptic density protein drosophila large disc tumour suppressor zona-occludens 1 protein
PEI	Polyethylenimine
PKC	Protein kinase C
PM	Plasma membrane
PMSF	Phenylmethanesulfonylfluoride
PtdIns3P	Phosphatidyl-inositol-3-phosphate
R-Smad	Receptor regulated smad
Rb	Retinoblastoma

RhoA	Ras homolog gene family member A
Rin1	Ras and rab interactor 1
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
SARA	Smad anchor for receptor activation
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shc	Src homology 2 domain containing
siRNA	Small interfering RNA
SMA	Smooth muscle actin
Smad	Small phenotype mothers against decapentaplegic homolog
Smurf1	Smad ubiquitin regulatory factor-1
Smurf2	Smad ubiquitin regulatory factor-2
SNAI1	Snail
SNAI2	Slug
Sos	Sons of sevenless
TAK1	TGFβ activated kinase 1
TβRI	Transforming growth factor beta receptor I

TβRII	Transforming growth factor beta receptor II
TβRIII	Transforming growth factor beta receptor III
TβRII-ΔCyt	Cytoplasmically truncated transforming growth factor beta receptor II
TβRII-ΔEX	Extracellularly truncated transforming growth factor beta receptor II
TBS-T	Tris buffered saline with tween
TGFβ	Transforming growth factor beta
Traf6	Tumor necrosis factor receptor associated factor 6
Ub	Ubiquitin
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor 2
WT	Wild-type
ZO-1	Zona occludens 1

CHAPTER 1

INTRODUCTION

1 Introduction

1.1 TGFβ Overview

The transforming growth factor beta (TGFβ) signalling pathway is essential for numerous cell functions and was thought to arise with the development of metazoans. In development TGFβ plays numerous roles, including induction of epithelial-to-mesenchymal transition (EMT) in endocardial cells which is necessary for normal heart development (1). TGFβ also has several roles in normal tissue homeostasis, regulating such diverse functions as cellular differentiation, apoptosis, cell-cycle arrest, extracellular matrix production, and cellular migration. Partly owing to its pleiotropic effects in numerous cell-types, TGFβ has also been implicated in several pathologies including cancer and fibrosis. In cancer, TGFβ appears to have a dual role: On one hand, it is a tumour-suppressor, promoting cell-cycle arrest and apoptosis; on the other hand, TGFβ can increase cancer cell migration, invasion, and immune evasion (2). In wound healing, TGFβ promotes wound closure and resolution through the production of extracellular matrix (ECM) proteins and inhibition of matrix metalloproteinases. However, in fibrotic diseases, excessive TGFβ production and signalling promotes extensive tissue fibrosis which can compromise normal tissue function (3). Given the numerous roles of TGFβ in both homeostasis and pathology, understanding the regulation of this pathway is critical.

1.1.1 *TGFβ Cytokines*

The TGFβ superfamily consists of structurally and functionally related cytokines that interact with serine/threonine kinase receptors to mediate downstream transcriptional events. The TGFβ superfamily contains over 30 ligands, including the TGFβ/Activin/Nodal subfamily and the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF)/ Müellerian inhibiting substance (MIS) subfamily (4).

In the canonical TGFβ signalling cascade, there are three TGFβ cytokines which have been conserved throughout evolution- TGFβ1, TGFβ2 and TGFβ3; indeed, orthologs to human TGFβ can be found in *D. melanogaster* and *X. laevis* (reviewed in (1)). The three TGFβ ligands are produced by a number of different cell types and the production of all three occurs during development, although TGFβ1 is the predominant type in adults (5,6). Each TGFβ ligand has relatively specific, non-overlapping functions *in vivo*. The TGFβ ligands share significant sequence homology; together they have greater than 76% identity in their active domains (7).

TGFβ ligands are secreted as inactive, homodimeric pro-proteins (8). The activation of TGFβ1 is the best characterized of the three ligands, and latent TGFβ1 is found in one of three forms: a small latent complex, a large latent complex, or a form that is associated with α2-macroglobulin (9). In its small complex form TGFβ is synthesized as a pro-protein dimer, which is cleaved intracellularly by furin convertase and then associates with two precursor chains,

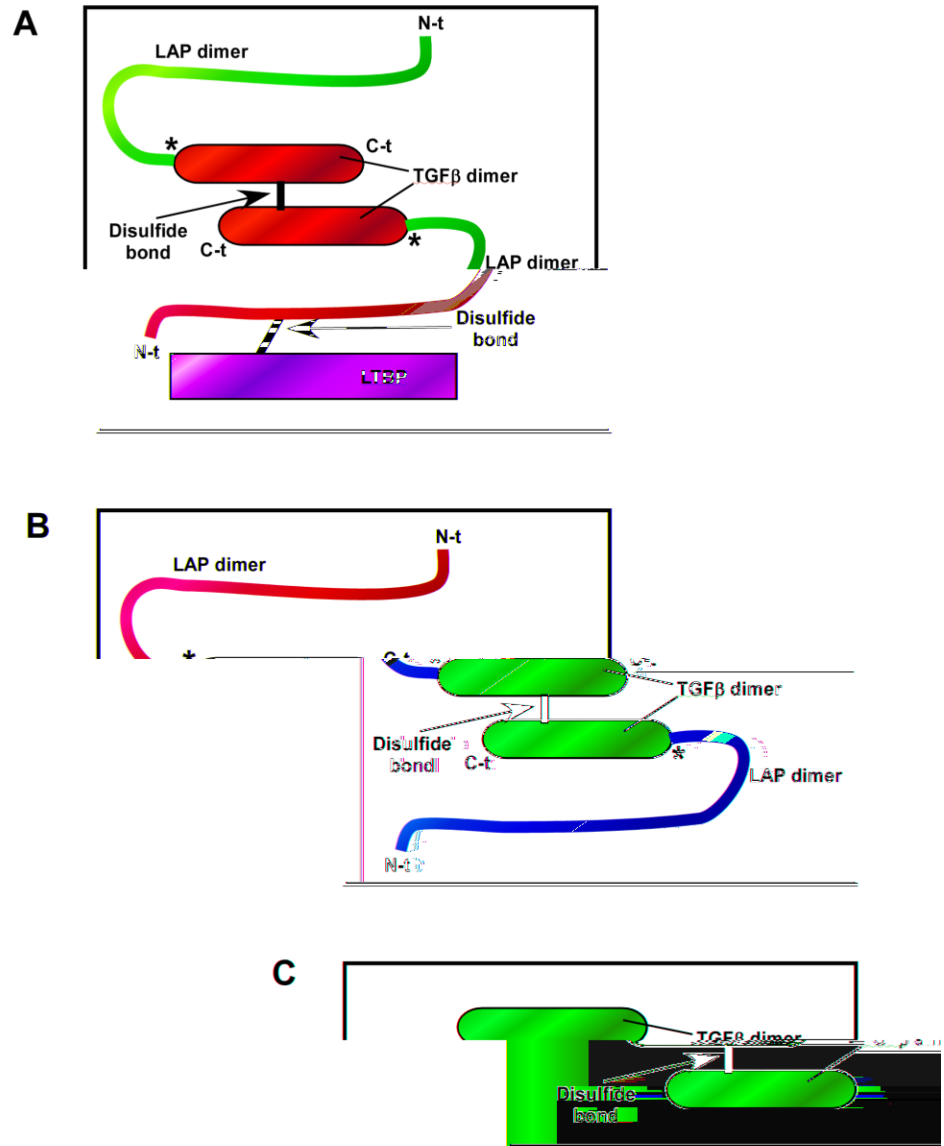
the latency associated peptides (LAPs) (9,10) (Figure 1.1). The large complex also consists of latency associated peptides, dimerized TGFβ, and a third protein, the latent TGFβ binding protein (LTBP), which is essential for proper secretion of TGFβ (11). In the extracellular matrix TGFβ is activated by molecules, such as matrix metalloproteinase 2, thrombospondin-1, plasmin or *in vitro* in acidic conditions (12-14). It has been shown that TGFβ2 and TGFβ3 also exist in latent complexes (15,16), which suggests that the activation of TGFβ in the extracellular matrix may represent an important regulatory mechanism. In their active form, all three TGFβ ligands are homodimers stabilized by disulfide bridges and hydrophobic interactions when in their active form (17).

Despite structural similarities, TGFβ ligands have distinct affinities for TGFβ receptors. The type II TGFβ receptor (TβRII) is able to bind both TGFβ1 and TGFβ3, with slightly higher affinity for TGFβ3 (18,19). TGFβ2 on the other hand, requires betaglycan (TβRIII) in order to bind to TβRII (20). Furthermore, mice containing deletions of the genes encoding the three TGFβ ligands illustrate that these ligands have non-overlapping functions. *Tgfb1*$^{-/-}$ mice develop significant problems *in utero* including vasculogenic and hematopoietic defects (21). Mice that survive gestation develop a severe wasting inflammatory syndrome (21). *Tgfb2*$^{-/-}$ mice have a myriad of developmental defects, including skeletal, cardiovascular, pulmonary and visual problems (22). Interestingly,

Figure 1.1 Latent and active forms of TGFβ ligand

TGFβ can be found in active and inactive forms. In order for proper secretion of TGFβ1 to occur, it must associate with latent TGFβ binding protein (LTBP) **(A)**, and this, along with two latency associated peptides (LAPs) forms the large latent complex. In its small latent complex form **(B)** TGFβ exists as a homodimer following its cleavage by furin convertase, and is associated with two latency associated peptide proteins (furin convertase cleavage site indicated by *). TGFβ is converted to its active form following secretion into the extracellular matrix by proteases or acidic conditions **(C)**

Figure 1.1



Tgfb3$^{-/-}$ null mice have the least defects and die after birth due to an inability to suckle caused by cleft palate (23).

Similar to their non-redundant roles in development, the TGFβ ligands have different effects in various disease states. For example, in the wound microenvironment, there are a large variety of growth factors that promote production of extracellular matrix and wound-closure. In adults, TGFβ1 is found at very high levels in the wound microenvironment and promotes myofibroblast differentiation, extracellular matrix production, and fibroblast chemotaxis (reviewed in (24)). Overall, TGFβ1 promotes the formation of a scar during adult wound healing. Surprisingly, injuries obtained *in utero* heal scar-free.This may be due to the relative ratios of TGFβ1 *vs*. TGFβ3. It has been shown that the embryonic wound microenvironment contains high levels of TGFβ3 and low levels of TGFβ1 (24). Furthermore, adding exogenous TGFβ3 to an adult wound also promotes scar-free healing in rats, possibly through decreasing inflammation (25). Currently, a topical cream, called Avotermin, containing TGFβ3 as its active ingredient is being promoted as a therapy for the improvement of scar appearance in humans (26).

In tumourigenesis, TGFβ1 is well-established as playing a dual role in cancer progression: in pre-malignant states TGFβ1 is anti-tumourigenic and induces cell-cycle arrest and apoptosis; in advanced tumours TGFβ1 correlates with a more aggressive phenotype and induces EMT, migration, and invasion of tumour cells (27). Similarly, it has been shown that TGFβ2 is highly over-

expressed in malignant gliomas and correlates with advanced disease state (5). Inhibitors of TGFβ2, such as the antisense oligonucleotide AP 12009, have been shown to decrease glioma and pancreatic cancer cell migration (5,28). However, there is a lack of information regarding the role of TGFβ3 in the tumour microenvironment. Studies have illustrated that TGFβ3 is highly expressed in breast cancer samples (29), and other studies have shown that high levels of TGFβ3 are associated with good prognosis in breast cancer (30). Overall, many of the roles of TGFβ3 in the tumour microenvironment are assumed to be the same as TGFβ1. If one were to consider that the tumour microenvironment has many of the same cellular players as the wound microenvironment, and TGFβ1 and TGFβ3 have vastly different outcomes in the wound microenvironment, it is unlikely that these two ligands share the same function in tumour growth.

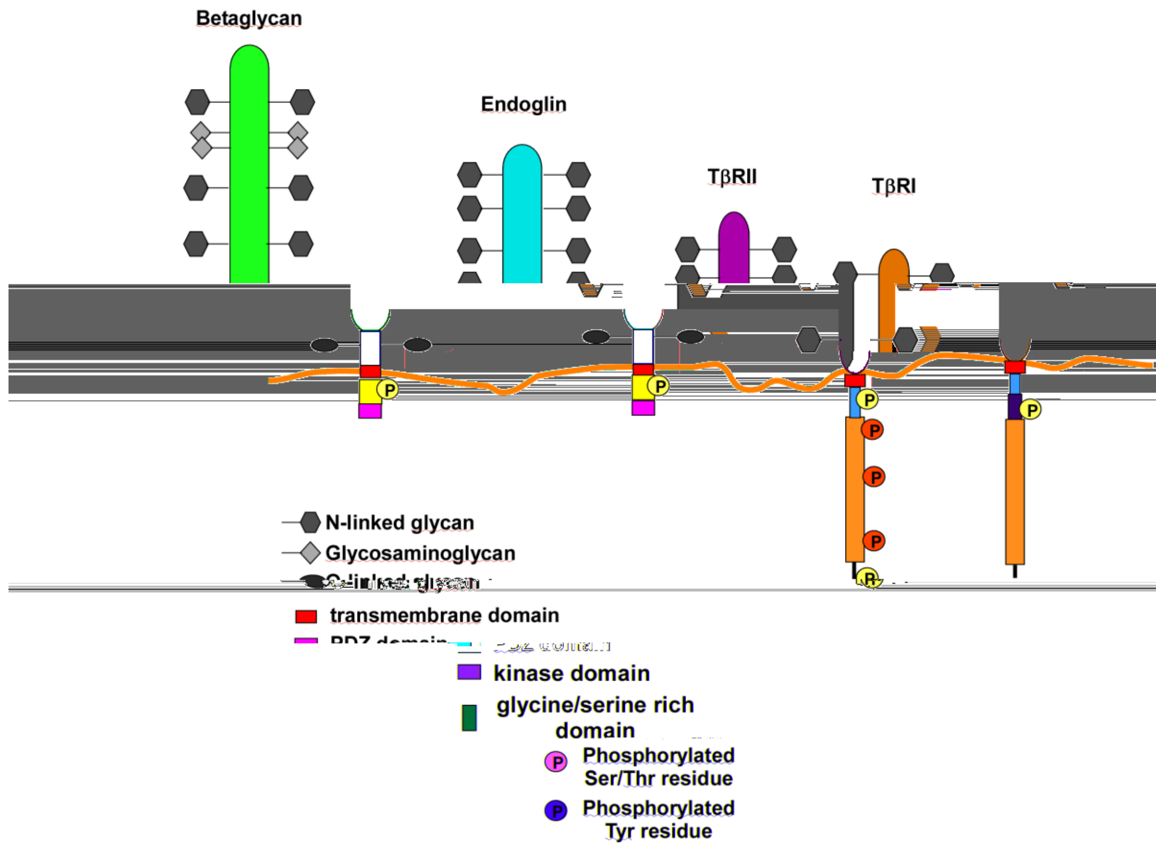
1.1.2 TGFβ Receptors

There are three principal receptor subtypes in the classical TGFβ pathway: TGFβ receptor I (TβRI), TGFβ receptor II (TβRII) and TGFβ receptor III (TβRIII). TGFβ receptor I (TβRI) and TGFβ receptor II (TβRII) are structurally related glycoproteins which contain serine-threonine kinase domains, whereas TβRIII is a large, membrane-bound proteoglycan lacking kinase activity (17) (Figure 1.2). Together, these receptors function to activate cell-type specific signalling programmes through the activation of a family of transcription factors called the Smads. TGFβ signalling can also activate non-Smad mediated pathways such as the MAPK pathway. Each TGFβ receptor type has specific,

Figure 1.2 TGFβ Receptors

In the canonical TGFβ pathway there are three receptor types: TβRIII (which consists of two different receptors- betaglycan and endoglin), TβRII and TβRI. TβRII and TβRI possess Ser/Thr kinase activity and are the signalling receptors in the pathway. The role of TβRIII is primarily ligand presentation to the TβRII and TβRI complex. While all of the receptors are primarily found as homodimers at the cell surface, they have been drawn as single receptors for the sake of simplicity in this diagram. Structural differences are indicated and described in the figure.

Figure 1.2



non-overlapping functions that are crucial to signal transduction.

In the TGFβ superfamily there are five type II TGFβ receptors which can form homomeric complexes to bind ligand: Act-RIIA, Act-RIIB, BMPR-II, AMHR-II and TβRII (31). In the classical TGFβ pathway, TβRII is the primary type II receptor. TβRII is a 62 kDa protein containing a short cysteine-rich, N-glycosylated extracellular domain, a single transmembrane domain and a serine-threonine kinase intracellular domain (reviewed in (18)).The cytoplasmic domain of TβRII is also serine-threonine rich, which is lacking in TβRI (32). At the cell surface, TβRII exists as a homodimer in the absence and presence of ligand (33). TβRII binds TGFβ1 and TGFβ3 with relatively high affinity (34,35), but is unable to bind TGFβ2 without TβRIII (18). In the absence of ligand, TβRII is capable of autophosphorylation on serine residues Ser_{549}, Ser_{551}, Ser_{223}, Ser_{226} and Ser_{227} (34,36). Interestingly, TβRII also has tyrosine kinase activity and its cytoplasmic tyrosine residues are subject to both autophosphorylation or Src phosphorylation leading to signalling cross-talk with the MAP kinase family (37). In response to TGFβ binding the receptor forms a heterotetrameric complex with and phosphorylates TβRI. TβRII function is tightly regulated by post-translational modification through ubiquitination, sumoylation, and/or phosphorylation, all of which result in specific signal transduction events (reviewed in (38)).

In the TGFβ superfamily there are 7 type I receptors called activin linked kinases (or ALKS) 1 through 7. The type I receptor in the classic TGFβ signalling pathway is TβRI, also known as ALK5. TβRI and TβRII are structurally similar,

though TβRI contains a shorter extracellular domain than TβRII and cannot bind ligand in the absence of TβRII (39). Akin to TβRII, TβRI also contains a serine/threonine kinase intracellular domain and exists as a homodimer at the cell surface (18). However, TβRI contains a unique intracellular GS (glycine/serine rich) region that is highly conserved between type I receptor isoforms, and that is phosphorylated by TβRII (17). Once phosphorylated, the GS domain of TβRI acts as a docking platform for receptor-regulated Smad proteins (40). The receptor-regulated Smads are then phosphorylated by TβRI, initiating a Smad signal cascade that culminates in transcription. Mutations of the GS domain have highlighted the importance of this region to TGFβ signal transduction: Mutations of two or more glycine or serine residues in the GS domain impairs TGFβ signalling activity (41). Mutation of threonine 204 to aspartic acid increases TGFβ signal transduction in the *absence* of ligand, as it generates a constitutively active TβRI (41). These mutational studies confirm that TβRI is the key player in Smad signal transduction. Furthermore, SB-431542, a specific inhibitor of TβRI, prevents TGFβ-induced Smad-mediated transcription, but does not affect the cross-talk of TGFβ with the MAP kinase family, highlighting the role of TβRI in Smad-dependent TGFβ signalling (42).

There are two type III TGFβ receptors: endoglin and betaglycan. These receptors are considered accessory receptors with roles in ligand presentation, as no enzymatic activity has been identified for either receptor. Betaglycan and endoglin are structurally related, with large, heavily glycosylated extracellular

domains, and a short cytoplasmic region with high sequence similarity (43-45). Both receptors can be phosphorylated on serine/threonine residues in their cytoplasmic domain (46-48). At the cell surface, endoglin and betaglycan form homodimers (49) (50), as well as complexes with TGFβ receptors I and II (46,51). Though similar, these receptors differ in their ligand-binding ability and expression. Betaglycan can bind all three TGFβ ligand isoforms with high affinity (50); while it has been reported that endoglin requires complex formation with the TβRII/TβRI complex to bind ligand, and even in complex can only bind TGFβ1 and TGFβ3 (46,47). Betaglycan is the most widely-expressed TGFβ receptor, and is expressed in a number of adult and fetal tissues (18), whereas endoglin is primarily expressed on proliferating endothelial cells (52). Future studies to examine compensatory effects of the two type III TGFβ receptors would be interesting, as both *Tgfbr3* $^{-/-}$ (betaglycan) and *Eng* $^{-/-}$ (endoglin) mice die mid-gestation due to cardiovascular defects (52,53).

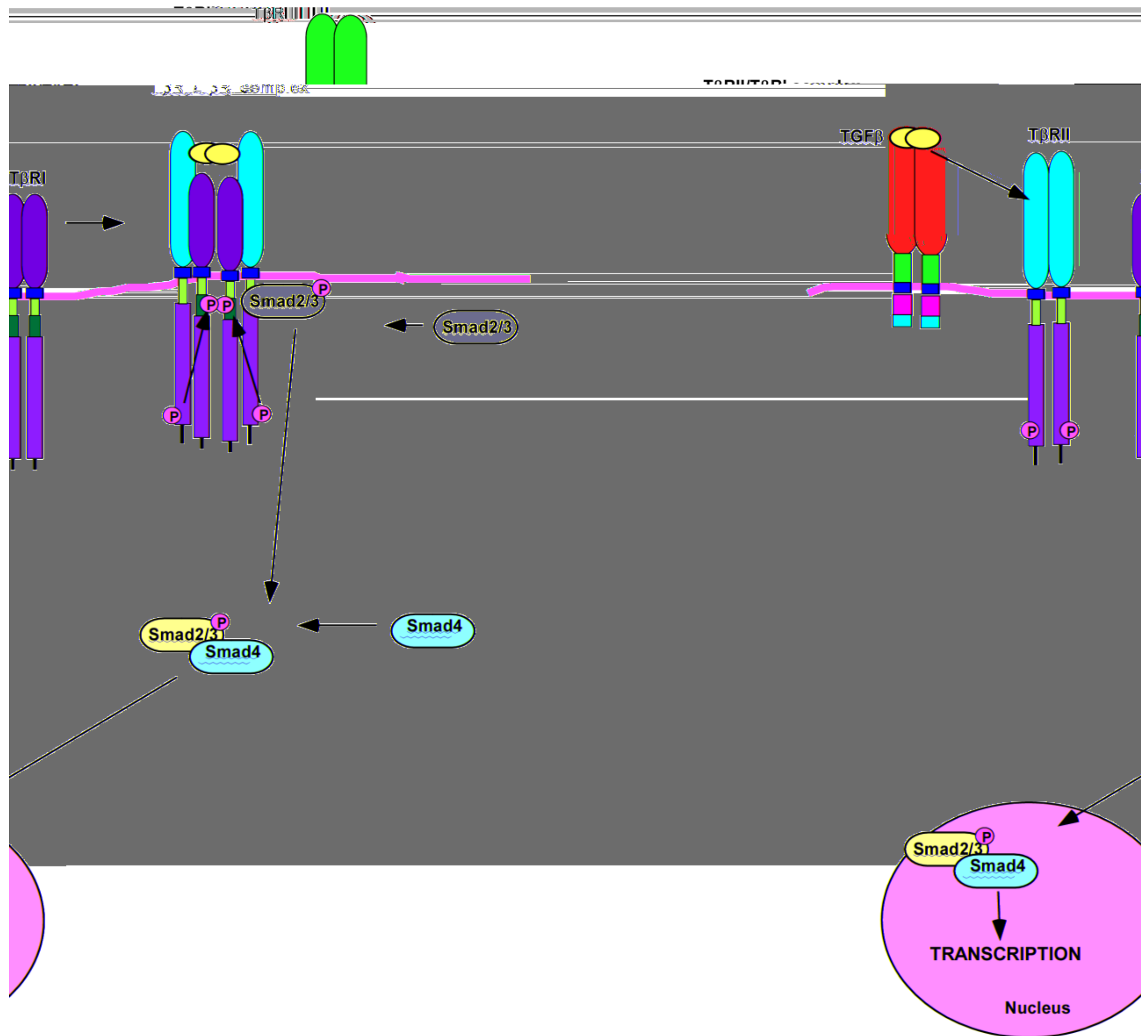
1.2 **TGFβ Signal Transduction**

To propagate TGFβ signalling, homodimeric TGFβ is presented by betaglycan (TβRIII) to TβRII (54) (Figure 1.3). The binding of ligand to TβRII recruits TβRI to the ligand-receptor complex, forming a receptor complex of two TβRII and two TβRI. TβRII then phosphorylates TβRI at serine-threonine residues in its GS domain (55). Phosphorylated TβRI is essential in driving TGFβ signal transduction, and works to activate a group of transcription factors known as Smads. There are three classes of Smads which are activated by the

Figure 1.3 Smad Signal Transduction Pathway

In the canonical TGFβ signalling pathway, the ligand-bound, activated receptor complex propagates Smad signal transduction. Following ligand binding by TβRII, TβRI becomes active and phosphorylates Smad2/3 on its SSXS motif (where S=serine and X= any amino acid). This phosphorylation promotes its disengagement from TβRI and promotes the association of Smad2/3 with the common Smad, Smad4. The Smad complex then translocates to the nucleus to activate transcription.

Figure 1.3



TGFβ superfamily of ligands: the receptor-regulated Smads (or R-Smads, Smads 1, 2, 3, 5 and 8), which are able to interact with the type I receptor; the co-mediator Smad (Smad4), which can associate with R-Smads; and the inhibitory Smads (or I-Smads, Smads 6 and 7) which compete with R-Smads for receptor binding and target TGFβ receptors for degradation (17). In the classical TGFβ signalling pathway, the R-Smads are Smads 2 and 3, whereas the inhibitory Smad is Smad7. Smads typically consist of two domains separated by a variable linker region. The amino MH1 (Mad homology 1) domain has DNA binding capabilities in some Smad sub-types, while the carboxy MH2 (Mad homology 2) domain has been shown to mediate interactions with a variety of proteins (56). The activated GS domain of TβRI serves as a docking site for Smad2 via its MH1 domain (55). The specificity of R-Smad binding is determined by the L45 loop, a nine amino acid sequence between the kinase subdomains IV and V of TβRI (57). TβRI phosphorylates R-Smads on the conserved SSXS motif located at the C-termini of Smads 2 and 3 (serine residues 465 and 467 in the MH2 domain of Smad2, for e.g.) (58-60). The phosphorylated serine residues of Smad2 serve as a docking site for Smad4, and promote the dissociation of Smad2 from TβRI and the formation of a heteromeric complex with Smad4 (31,59). Smad2 is generally located cytoplasmically in the absence of ligand, but upon ligand stimulation translocates to the nucleus with Smad4, which in the absence of ligand is found distributed equally between the nucleus and the cytoplasm (4). Smad4 is able to translocate to the nucleus due to its interactions with nucleoporins; the interaction of Smad4 with the nucleoporin importin-1α is

thought to mediate the translocation of the Smad heteromeric complex to the nucleus (61). In the nucleus the heteromeric complex binds to promoters or enhancers of TGFβ target genes, such as the Smad binding element, via its MH1 domain and interacts with transcriptional co-activators and co-repressors in order to induce cell-specific transcriptional programmes (4,17) (Figure 1.3).

1.3 Membrane Trafficking of TGFβ Receptors

Endocytosis refers to the process whereby cell-surface associated molecules enter the cell without passing through the plasma membrane. Essentially, the plasma membrane invaginates, budding off and forming a vesicle containing the internalized cargo. Internalization of cell-surface receptors is important in the control of signal transduction, functioning either to down-regulate signalling or trafficking receptors to specific endocytic compartments. There are several methods of endocytosis of cell-surface receptors, including membrane-raft dependent endocytosis, caveolin-dependent endocytosis, Arf6-dependent endocytosis, and clathrin-mediated endocytosis (reviewed in (62)). As clathrin-mediated endocytosis and membrane-raft/caveolin-mediated endocytosis are implicated in the TGFβ pathway (63), these processes will be the focus of this introduction.

1.3.1 *Clathrin-mediated Endocytosis*

Clathrin-mediated endocytosis is a highly conserved mechanism implicated in the internalization of many receptor types. Clathrin-mediated endocytosis occurs when clathrin from the cytosol is recruited to the plasma

membrane and aggregates to form pits (62). Protein motifs of cargo play a role in the development of clathrin-coated pits, as di-leucine and tyrosine motifs in the cytoplasmic domains of receptors are detected by adaptor protein 2 (AP-2) and promote clathrin polymerization (64,65). The AP-2 complex is critical in the formation and function of clathrin-coated pits. AP-2 along with Eps15 (epidermal growth factor pathway substrate 15), aid in the polymerization of clathrin into lattices increasing plasma membrane curvature (62). Upon sufficient membrane curvature, dynamin forms a helix around the neck of the clathrin-coated pit and with GTP hydrolysis promotes scission of the clathrin-coated pit from the plasma membrane (66). These excised pits then form clathrin-coated vesicles, lose their clathrin-coat and become endosomes. Endosomes may be routed to the cell membrane for recycling, or may mature and go on to form other compartments. Ligand-binding is not a requirement for clathrin-mediated endocytosis: while some receptors are internalized following ligand stimulation, such as the epidermal growth factor receptor, other receptors such as the transferrin receptor and the T-cell receptor CTLA-4 internalize *independently* of ligand stimulation (67,68).

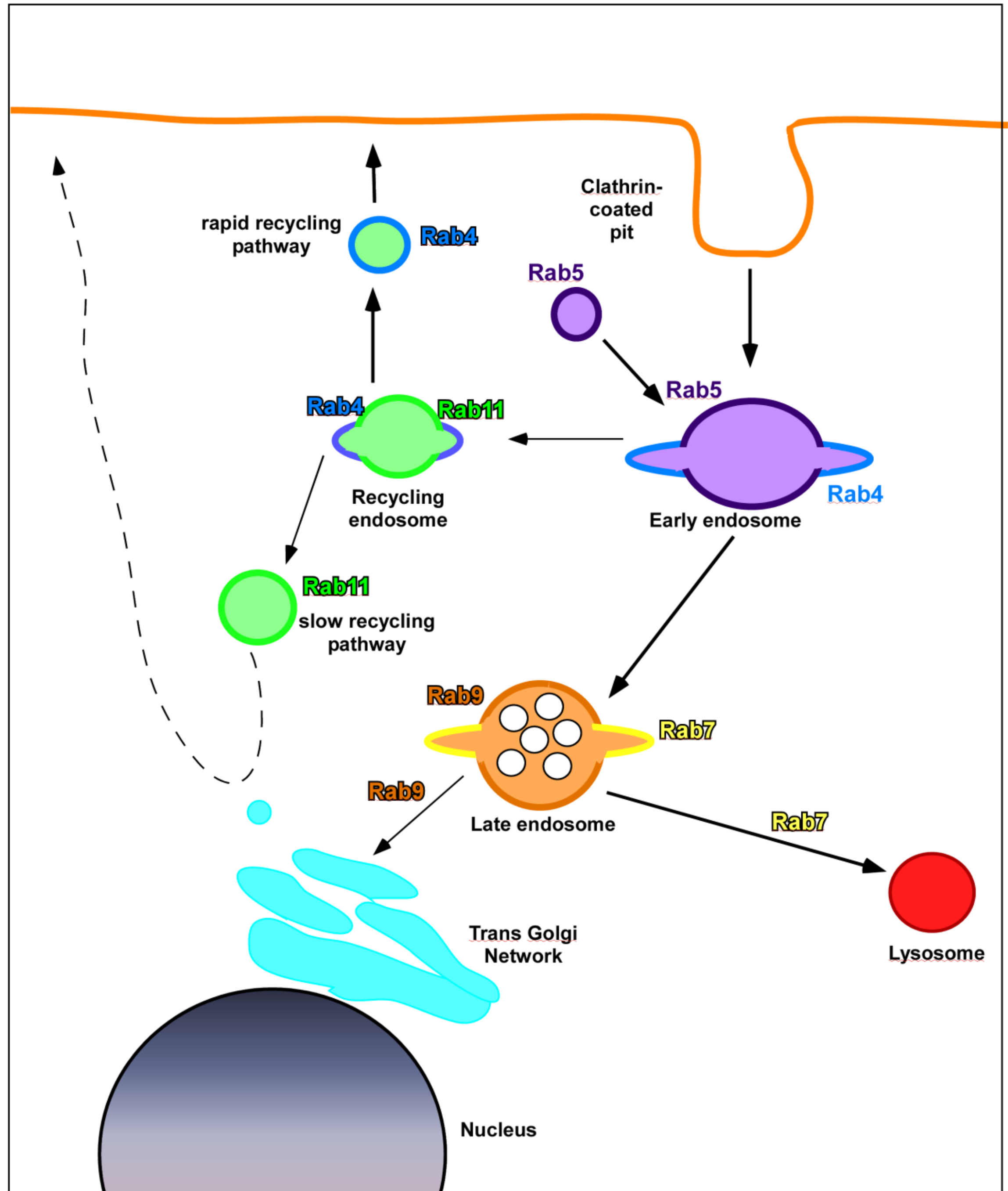
1.3.2 *The role of Rab GTPases following endocytosis*

Following receptor internalization, receptors are directed into distinct endocytic components by a large family of small GTPases called the Rab GTPases (Figure 1.4). There are over 60 members of the Rab GTPase family which function as molecular “on and off” switches- in their “on” state, they are

Figure 1.4 Rab GTPases in vesicular trafficking following endocytosis

The Rab small GTPase family is important in mediating trafficking of intracellular vesicles. Different endosomal compartment are enriched in distinct Rab GTPases. Rab4 and Rab5 are found enriched in the early-endosome. From the early endosome, cargo may traffic to the recycling endosome, which is enriched in Rabs 4 and 11, or to the late endosome, which are enriched in Rabs 7 and 9.

Figure 1.4



bound to GTP, and in their “off” state, they are bound to GDP (69). Rab proteins are activated by Rab GEFs (GDP-GTP exchange factors), which promote the GTP bound form, and are inactivated by GAPs (GTPase activating proteins) (70). Rab GTPases can associate with membranes, vesicular coat components, and molecular motors to direct vesicular traffic by regulating the process of docking and tethering between compartments (Figure 1.4) (69,70).

One of the best-studied Rab proteins, Rab5, has been shown to play a crucial role in the formation of clathrin-coated pits and the internalization of transferrin receptors *via* clathrin-mediated endocytosis (71). GTP-bound Rab5 is also involved in early-endosome fusion (72) and has been used extensively as a marker for the early endosome. Following trafficking into the early endosome, it has been proposed that cargo can either be recycled to the plasma membrane, or progress to the late endosome. A study by Rink *et al.* elegantly illustrated that progression from the early to late endosome is mediated by the loss of Rab5 occurring simultaneously with the acquisition of Rab7 (73). Rab5 replacement with Rab7 depends on the GTP hydrolysis activity of Rab5, as a Rab5 mutant lacking hydrolytic activity recruited Rab7 but was not replaced by Rab7 (73). Following trafficking to the late endosome, cargo can be trafficked to the lysosome, which is mediated by Rab7, or to the *trans*-Golgi network, which is mediated by Rab9 (69). As mentioned, instead of progressing to the late endosome, cargo may traffic to a recycling endosome, which is enriched in Rab 4 and 11 in distinct domains that do not intermix (69,74). These proteins are proposed to have different functions in the recycling pathway: Rab4 has been

implicated in “fast” recycling of cargo from to the cell-surface, whereas cargo in Rab11- positive vesicles has been proposed to take a “slow” recycling route, and can transition through the *trans*-Golgi network and secretory pathways (74).

Overall, the regulation of cargo trafficking within the cell is essential for cellular function. Rab dysregulation can occur in a number of cancers, such as breast and ovarian cancer, which have been found to have over-expression of Rab25 (69).

1.3.3 *Membrane-raft mediated endocytosis*

Clathrin-independent endocytosis through membrane rafts is also a common mechanism for the uptake of signals and nutrients from the extracellular environment. First introduced in 1997, the membrane raft model proposes that cholesterol-sphingolipid-protein complexes form in the plasma membrane to make a tightly packed, liquid-ordered phase mediating endocytosis and signal transduction (75). Importantly, the lipid composition of membrane rafts is distinct from the rest of the plasma membrane as they are enriched in cholesterol and sphingolipids and are therefore more rigid and less fluid than the surrounding plasma membrane (76). Membrane rafts have been shown to be especially important in the endocytosis of proteins with glycophosphatidyl-inositol (GPI) binding domains (77). It is thought that clustering of GPI-containing receptors may increase the affinity of the receptor complex for membrane rafts and increase membrane raft stability (78). Furthermore, it has been suggested that cytoskeletal proteins, such as actin, play an important role in forming membrane

rafts (79). Disrupting the actin cytoskeleton has major effects on the clustering of raft proteins (80). In a feed-forward mechanism, the clustering of proteins enriched in membrane rafts enhances the concentration of actin, which then further stabilizes membrane raft formation (81).

The study of membrane rafts has been limited at times due to previous methodology used to isolate rafts. Membrane rafts have been isolated by their detergent-insolubility. The tight packing of lipids in the liquid-ordered phase of membrane rafts prevents detergent incorporation and therefore disruption by detergents (82). Following detergent extraction of membrane rafts, cell lysates are frequently subjected to sucrose-density ultracentrifugation, as the enrichment of membrane rafts with cholesterol and sphingolipids increases their buoyancy relative to the rest of the plasma membrane. Unfortunately, using detergent-insolubility as the sole defining characteristic is laced with inconsistency. Different results can be obtained depending on the type of detergent and the duration of extraction (83). As it is possible to isolate membrane rafts using a detergent-free method with sodium carbonate (51,63,84,85), using this method may decrease extraction-based artifacts of membrane raft isolation.

1.3.4 *The role of Caveolae in endocytosis*

Another important mediator of TGFβ endocytosis are caveolae. Caveolae are a subset of membrane rafts which are composed of flask-shaped invaginations approximately 60-80 nm in diameter (77). Caveolae are enriched in a protein called caveolin-1. The caveolin family consists of three proteins:

caveolin-1, caveolin-2, and caveolin-3. Caveolin-1 and caveolin-2 are found in non-muscle cells, while caveolin-3 is primarily expressed in muscle (86,87). Caveolin-1 and caveolin-3 can form caveolae, whereas loss of caveolin-2 does not affect caveolae formation (88). As caveolin-3 is only expressed in muscle, caveolin-1 is the primary contributor to caveolae formation in most cells (89). Caveolin proteins have a unique hairpin structure. Their N and C termini are cytoplasmic, whereas the hairpin structure is embedded in the plasma membrane, associating with approximately 1-2 cholesterol molecules (90). Caveolae are formed by the oligomerization of caveolin-1 molecules and association with cholesterol-rich membrane rafts. This oligomerization results in a liquid-ordered, stable domain in the plasma membrane enriched in cholesterol, sphingolipids, and caveolin-1(89). Caveolin-1 has been shown to be important for clathrin-independent, membrane-raft dependent endocytosis through its interactions with the actin cytoskeleton (91). Caveolae-dependent endocytosis has been implicated in the uptake of viruses, nutrients, and cell-membrane receptors (92).

Disruption of the caveolin-1 gene has provided insight into the many potential roles of this membrane protein. In the initial characterization of *Cav1*$^{-/-}$ mice it was shown that the loss of caveolin-1 disrupted caveolae formation in the lung, adipose tissue, kidney, and heart (93). Overall, these mice are viable but have significant vascular defects, and have increased deposition of extracellular fibrillar matrix in the lungs, suggesting that loss of caveolin-1 may initiate fibrosis in the lungs (93). However, loss of caveolin-1 expression also induced the hyper-

proliferation of angioblastic cells (93). Further studies illustrated that loss of caveolin-1 induced many changes consistent with promoting tumourigenesis such as the spontaneous progression through the epithelial-to-mesenchymal transition in epithelial cells (94), increasing the susceptibility of mouse embryonic fibroblasts to transformation (95), and increasing beta-catenin transcriptional activation (96). Overall, these studies illustrate that caveolin-1 may have greater roles in signal transduction than simply in the internalization of cell-surface receptors.

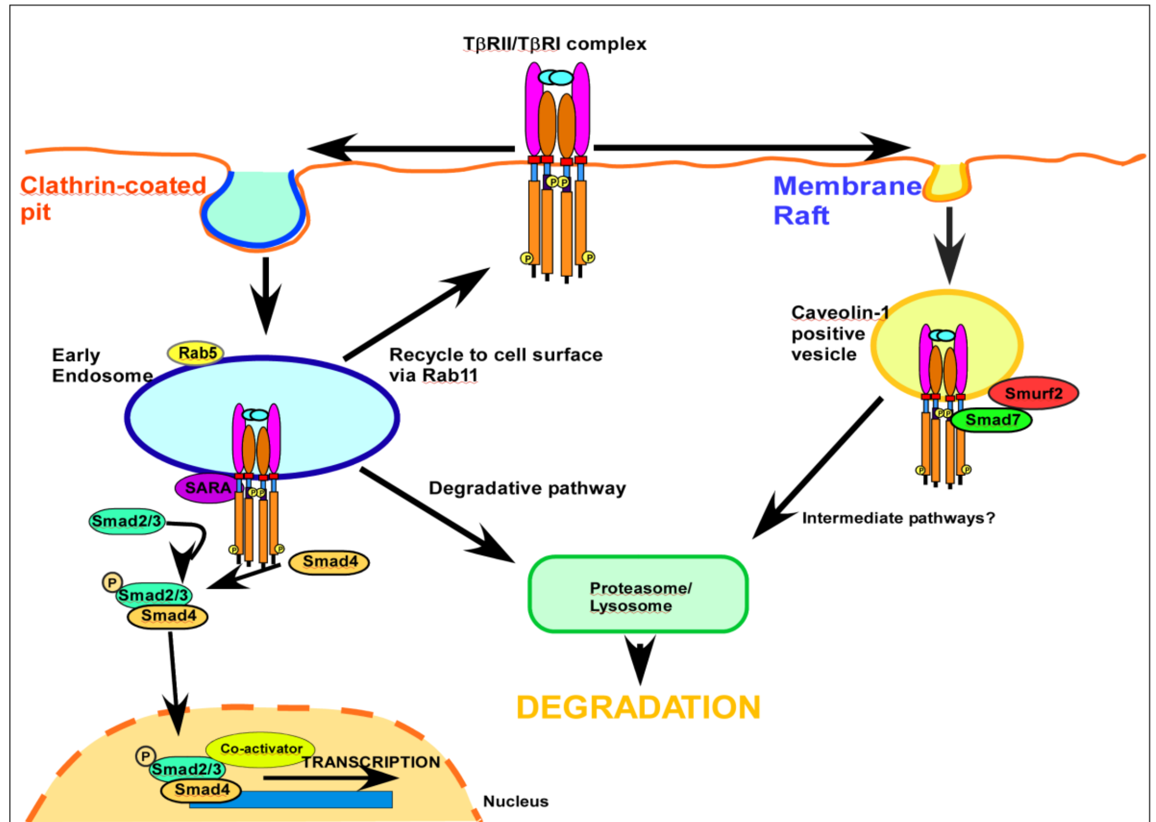
1.4 Endocytosis in TGFβ Signal Transduction

Intriguingly, a role for membrane-raft/caveolar mediated signal transduction has been identified for TGFβ signalling by Di Guglielmo and colleagues. At the cell surface, TGFβ receptor complexes can access both clathrin-coated pits and membrane rafts (63) (Figure 1.5). Inhibition of clathrin-coated pit internalization through the use of a dominant-negative Eps15 mutant shifted receptors into membrane raft fractions; similarly, inhibition of membrane raft formation through cholesterol depletion shifted receptors back into non-membrane raft fractions (63). TGFβ receptors internalized *via* clathrin-mediated endocytosis access the early endosome, a signalling endosome, which propagates TGFβ signal transduction through the recruitment of R-Smads (63,97). Membrane-raft mediated endocytosis, however, promotes ubiquitin-dependent receptor degradation (63,98,99). The landmark paper by Di Guglielmo and colleagues illustrated an important principle regarding TGFβ signal

Figure 1.5 Regulation of TGFβ Signalling by Clathrin-dependent and -independent endocytosis

TGFβ receptors can be internalized by clathrin-dependent mechanisms and clathrin-independent, membrane-raft dependent mechanisms. Receptors internalized via clathrin-coated pit mediated endocytosis traffic to the early endosome and propagate signal transduction. Receptors internalized by membrane-raft dependent endocytosis traffic to the caveolin-1 positive vesicle where they are targeted for degradation and prevented from signalling.

Figure 1.5



transduction: endocytic route plays a powerful role in dictating TGFβ receptor intracellular trafficking and signal transduction (Figure 1.5).

1.4.1 *The role of the early endosome in TGFβ Signal Transduction*

While the classic paradigm of signal transduction suggests that following receptor endocytosis signal transduction is terminated, it has been shown in many different systems that signalling continues following receptor internalization into endosomes. Following ligand-binding and internalization, receptors can undergo modifications that attract intracellular signalling molecules and can therefore continue to propagate signals (100). Early endosomes are considered to be a sorting station for internalized receptors, and are classified by their enrichment in Rab5 and EEA1 (early endosomal autoantigen 1) (100).

As previously described, clathrin-mediated endocytosis of TGFβ receptors targets their localization to the early endosome (63,97), which enhances TGFβ signalling. An elegant study performed by Runyan and colleagues illustrated that internalization of the TGFβ receptor complex is essential for maximal signal transduction (101). The authors illustrated that inhibition of clathrin-mediated endocytosis did not greatly prevent the ability of the receptor complex to phosphorylate Smad2; but inhibition of endocytosis *did* prevent nuclear translocation of Smad2 thus preventing TGFβ-dependent transcription (101). This paper suggested that there is a spatial component to TGFβ signalling.

One key player in the spatial control of TGFβ signalling is SARA (Smad anchor for receptor activation). SARA was first identified by Tsukazaki and

colleagues by screening a *X. laevis* expression library using Smad2 as a bait (102). SARA contains a FYVE domain (**F**ab1, **Y**OTB, **V**ac1, **E**EA1), a common motif in early endosomal proteins that has been shown to bind to phosphatidyl-inositol-3-phosphate (PtdIns3P) (103). Since phosphatidyl-inositol-3 (PI3) kinase activity has been implicated in vesicular trafficking, and FYVE domains can directly bind PtdIns3P, proteins containing FYVE domains have been suggested to mediate endosomal trafficking (103). SARA has also been shown to bind to Smad2 and Smad3 via their MH2 domains, and preferentially binds the unphosphorylated forms of the Smads (102). Furthermore, TGFβ receptors traffic into EEA1/SARA positive endosomes and disruption of SARA localization through the deletion of the FYVE domain perturbs Smad2/3 nuclear translocation (104). It has been proposed that SARA functions to link the TGFβ receptors with Smad2. Once Smad2 has been phosphorylated by the receptor complex, Smad2 dissociates from SARA and binds Smad4, translocating to the nucleus and initiating TGFβ-driven transcription (102).

The length of time which TGFβ receptors reside in the early endosome may also affect their signalling capacity. As previously mentioned, the early endosome is enriched in Rab5. Rab5 can control vesicular trafficking by promoting trafficking to the late endosome which is enriched in Rab7 (73). A Rab5 guanosine exchange factor (GEF), RIN1, has been shown to promote Smad signal transduction by activating Rab5 (105). Interestingly, SNAI1 a transcriptional target of TGFβ/Smad signalling, acts through a negative feedback

mechanism to decrease RIN1 expression and therefore decrease TGFβ signalling (105).

There is little information regarding the trafficking of TGFβ receptors following their endocytosis to the early endosome. It has previously been shown that TGFβ receptors can co-localize with Rab11, a recycling Rab protein (63). Furthermore, a dominant-negative version of Rab11 has been shown to impair the recycling of TGFβ receptors to the cell surface (97); however, the authors of this paper used a hybrid GMCSF-TβRII receptor composed of the extracellular domain of GMCSF and the cytoplasmic domain of TβRII, therefore studies using wild-type TβRII to assess TGFβ receptor recycling would further improve our understanding of TGFβ receptor recycling.

1.4.2 *The role of the caveolin-1 positive vesicle in TGFβ signalling*

Membrane raft endocytosis of TGFβ receptors results in receptors being targeted to the caveolin-1 positive vesicle. Indeed, Razani *et al.*, identified a caveolin-binding motif in the cytoplasmic tail of TβRI which mediates its interaction with the scaffolding domain of caveolin-1 (106). Unlike the early endosome, the caveolin-1 positive vesicle promotes association of Smad7, not Smad2, with the receptor complex (63). Smad7 belongs to the inhibitory Smads, or I-Smads, along with Smad6. Smad7 antagonizes the canonical TGFβ pathway and its expression is induced by TGFβ family ligands (107). The antagonistic role of Smad7 is mediated by two mechanisms. Firstly, Smad7 is able to interact with activated TβRI and therefore sterically inhibits the association of TβRI with

Smad2/3, preventing the subsequent activation of Smad2/3 and its association with Smad4 (108,109). Importantly, a mutant of Smad7 that is unable to bind to TβRI (Smad7 Δ408) loses its inhibitory activity of the TGFβ pathway, suggesting that the ability of Smad7 to bind to TβRI is critical in its antagonistic function (109). Secondly, Smad7 acts as an adaptor between the TGFβ receptor complex and a ubiquitin regulatory factor, Smurf2 (99). Smurf2 is an E3 ubiquitin ligase which is localized primarily in the nucleus (99). Upon TGFβ stimulation however, Smurf2 translocates to the cytoplasm and forms a stable interaction with Smad7 and the receptor complex (99). Smurf2 then ubiquitinates the receptor complex, targeting it for degradation *via* proteasomal and lysosomal pathways (99,110). Further supporting the role of membrane rafts in receptor degradation, an interesting study by Chen and colleagues found that increasing cholesterol concentrations can inhibit cell TGFβ responsiveness and promote receptor degradation. This suggests that cholesterol may shift TGFβ receptors into membrane rafts and subsequently caveolin-1-positive vesicles (111).

1.5 TGFβ Receptor Motifs Influencing Internalization

At the cell surface, TβRII and TβRI are generally found in a heteromeric complex consisting of two TβRII and two TβRI. It has been well established that these receptors undergo internalization and degradation, but the mechanisms directing this internalization are not clear. The internalization rate of the TGFβ receptors appears to vary depending on cell-type, with receptors being maximally internalized after 40 minutes of ligand-stimulation in Mv1Lu cells (112),

60 minutes after stimulation in HEK 293 cells (112), and less than 25% of receptors were internalized after 30 minutes in CR-26 mink lung cells (113). While other receptor systems, such as the EGFR and numerous GPCRs, display agonist-induced internalization (114,115), this is not the case for the TGFβ receptors. Mitchell and colleagues have shown that hybrid receptors consisting of the extracellular domain of GMCSFR and the intracellular domain of TβRII undergo similar rates of internalization and recycling both in the presence and absence of ligand (97). Similarly, it has been shown that wild-type full-length TβRII shows similar EEA1-endosomal enrichment (63) and partitioning into membrane rafts in the presence and absence of ligand (84).

While it does not appear that ligand binding plays a role in TGFβ receptor internalization, several studies have identified motifs in the cytoplasmic domains of both TβRII and TβRI that permit their association with components of endocytic machinery. Yao and colleagues illustrated that the cytoplasmic domains of both TβRII and TβRI can bind directly to the β2 subunit of AP-2 and clathrin (116). While TβRI has a slightly lower affinity for binding to AP-2 than TβRII, the presence of ligand does not affect the ability of either receptor to bind AP-2 or clathrin (116). The authors concluded that this interaction was essential for clathrin-mediated endocytosis of the receptors. TβRII has another consensus sequence in its cytoplasmic tail that links it to the clathrin-mediated endocytic machinery. Ehrlich *et al.* identified a di-leucine motif in the cytoplasmic tail of TβRII ($I_{218}I_{219}L_{220}$) which is essential for its clathrin-mediated internalization (117). Indeed, mutation of each of the residues in the di-leucine motif to alanines

prevented internalization of the receptors (117). Importantly, the constitutive internalization of TβRII via its di-leucine motif was also *independent* of ligand stimulation (117).

While studies have identified motifs that link TβRI and TβRII to components of the clathrin-mediated endocytic machinery, others have shown that the receptors can also associate with components of the membrane-raft endocytic machinery. As previously mentioned, Razani *et al.* identified a caveolin-binding motif in the cytoplasmic tail of TβRI which mediates its interaction with the scaffolding domain of caveolin-1 (106). Importantly, the interaction of TβRI with caveolin-1 has functional consequences as well. Caveolin-1 is a marker for the caveolin-1 positive vesicle, where receptors are targeted for degradation and sterically prevented from interacting with the R-Smads. In the study by Razani *et al.* it was shown that even when a constitutively active TβRI construct was used, co-expression of caveolin-1 decreased TGFβ-dependent transcription (106). Since the kinase activity of TβRI is necessary for propagating Smad signal transduction, the interaction of TβRI with caveolin-1 may act as a powerful negative regulator of TGFβ signalling.

1.5.1 *The role of receptor-interacting proteins on TGFβ endocytosis*

TβRII and TβRI can bind a number of proteins at the cell surface and many of these proteins have been shown to direct receptor endocytosis.

ADAM12 (a disintegrin and metalloprotease protein 12) is an example of a cell-surface TGFβ receptor interacting protein that enhances TGFβ signal transduction. ADAM12 is a proteoglycan with an extracellular metalloprotease domain and intracellular signalling domain (118). Using TβRII as bait, ADAM12 was identified as a novel binding partner for TβRII, directing the receptor to undergo clathrin-mediated endocytosis. ADAM12 traffics receptors into the early endosome, and in agreement with other studies, accumulation of receptors into the early endosome enhances TGFβ signal transduction (119). The authors illustrated this increase in TGFβ signal transduction by showing that ADAM12 increases Smad2 phosphorylation, Smad2-Smad4 association, as well as increased modulation of gene transcription (119).

There are also several cell-surface interacting proteins that direct TGFβ receptors into the degradative pathway. CD109 is a large glycophosphatidyl inositol (GPI)-linked protein which has been shown to bind TGFβ1 ligand and form a complex with TβRI, TβRII and TβRIII (120). As previously mentioned, GPI-linked proteins tend to accumulate in membrane rafts (77). Furthermore CD109 can also interact with caveolin-1 (121). An interesting paper by Bizet *et al.*, demonstrated that the association of CD109 with the TGFβ receptor complex increased the internalization of the receptors *via* caveolae and enhanced receptor degradation (121). In a follow-up paper, Bizet *et al.*, illustrated the importance of the subcellular localization of the receptors in terms of degradation, as CD109 enhanced the degradation of TβRI by Smad7/Smurf2 by

enhancing the co-localization of TβRI with Smurf2 (122).

The atypical protein kinase C (PKC) family represents another class of proteins that direct TGFβ internalization into the caveolin-1-positive vesicle. PKCζ and PKCι are members of the atypical protein kinase C family. These proteins are serine-threonine kinases, which unlike members of the classic or novel groups of PKC family do not require diacylglycerol for their activation (reviewed in (123)). Previously, Ozdamar *et al.*, observed that PKCζ interacts with TβRII through an association with Par6 to control EMT in breast cancer cells (124). Our lab has shown that the atypical PKCs direct TβRII into caveolin-1 positive vesicles, and treatment with either inhibitors to the atypical PKCs (such as GF109203X), or siRNA directed against the atypical PKCs, extends Smad2 phosphorylation and TβRII half-life (125). As the PKC family is primarily known for its role in GPCR endocytosis and trafficking (126), the identification of this family as controlling TGFβ receptor trafficking suggests that the PKCs may have a more general role in endocytosis than previously appreciated.

Finally, cytoplasmic proteins have also been shown to control the endocytosis of TGFβ receptors. βarrestin2 is a multi-functional scaffolding protein best known for its role in GPCR signalling. Upon agonist stimulation, GPCRs are phosphorylated by G protein receptor kinases (GRKs). Following GPCR phosphorylation, βarrestin2 binds to the phosphorylated receptor, promoting uncoupling of the receptor from the G protein and targeting the receptor for internalization (126). Interestingly, it has been shown that βarrestin2 is also able

to interact with TβRIII and mediate the endocytosis of TβRIII and TβRII (48). More specifically, the authors illustrated that TβRII phosphorylates TβRIII to recruit βarrestin2. They also showed that when βarrestin2 expression is decreased in HEK293 cells, TGFβ dependent apoptosis is increased (48), suggesting that βarrestin2 has a negative regulatory effect on TGFβ signalling. Since βarrestins have been shown to interact with components of the clathrin-mediated endocytic machinery, such as clathrin and AP-2 (reviewed in (127)), it would be expected that the interaction of βarrestin2 with TβRII and TβRIII would promote clathrin-mediated endocytosis, but the authors simply evaluated the internalization of the receptors, not their route. Furthermore, as clathrin-mediated endocytosis promotes TGFβ signalling (51,63), it would be of interest to assess the mechanism by which βarrestin2 mediates signal down-regulation, in particular in regards to trafficking.

1.6 TGFβ Biology

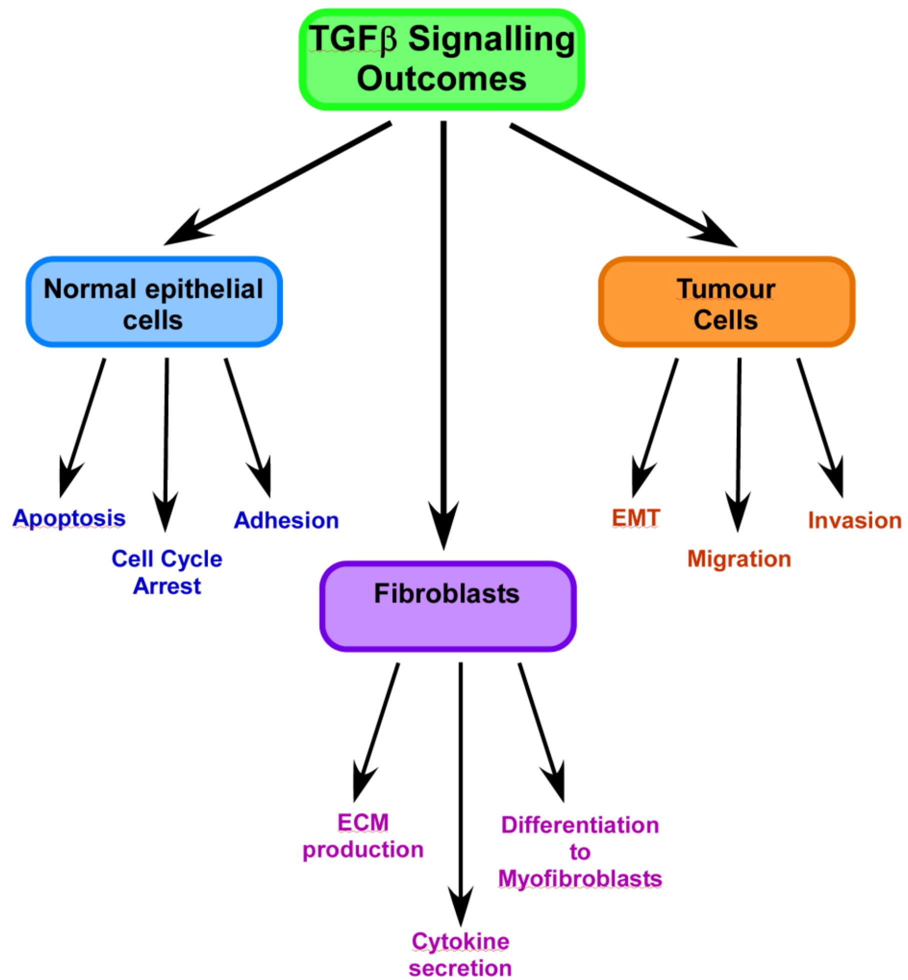
1.6.1 *TGFβ and Cell-cycle Arrest*

While virtually every cell in the body is responsive to TGFβ, its effects are context- and cell-dependent (Figure 1.6). TGFβ stimulates the growth of fibroblasts as well as their deposition of extracellular matrix proteins, such as fibronectin and collagen (128). However, in tissues such as the epithelium, mammary gland, endothelium and nervous system, TGFβ is growth inhibitory (129). Initial studies evaluating the growth-suppressive effects of TGFβ illustrated

Figure 1.6 Cell-type Dependent TGFβ Signalling Outcomes

While nearly all cells are responsive to TGFβ signalling, the outcome of TGFβ signalling is cell-type and context-dependent. In epithelial cells TGFβ is generally growth-inhibitory, while in fibroblasts it stimulates ECM deposition and cell differentiation. In tumour cells TGFβ transitions from being growth-inhibitory to stimulating migration and invasion.

Figure 1.6



that TGFβ prevented the phosphorylation of retinoblastoma protein (pRb) (130). Retinoblastoma protein has been proposed to function as a “gate-keeper” regulating the cell cycle. In its under-phosphorylated state, Rb protein arrests the cell in the G1 phase of the cell cycle, while in its phosphorylated state it allowsthe cell to undergo mitosis (131). The phosphorylation of pRb in the G1 phase is mediated by the cyclin-dependent kinases CDK4 and 6 (131). Later work illustrated that TGFβ inhibits cell-cycle progression by up-regulating the cyclin-dependent kinase inhibitors p15 and p21, therefore arresting cells by inhibiting the actions of CDK4 and CDK6 (129,132). TGFβ has also been shown to inhibit the expression of the growth-stimulatory transcription factor c-Myc through stimulating the formation of a complex consisting of Smad3, p107 and E2F4/5 (133). Therefore, TGFβ potently inhibits growth through two mechanisms in epithelial cells.

1.6.2 *TGFβ and Apoptosis*

Not only is TGFβ anti-tumourigenic through promoting cell-cycle arrest, but TGFβ has also been shown to induce apoptosis in a number of cells *via* several mechanisms. There are two principal apoptotic pathways: the extrinsic/death receptor pathway and the mitochondrial pathway (134). Activation of either pathway results in the cleavage of caspase-3, inducing DNA fragmentation, protein degradation, and the expression of ligands to stimulate phagocytic cells to engulf the apoptotic cell (134). The extrinsic/death receptor pathway is initiated by transmembrane receptors responding to extracellular stimuli, such as the Fas ligand/Fas receptor complex (134). The binding of ligand to the Fas receptor

induces the accumulation of intracellular adaptor proteins which eventually results in the formation of the DISC, or death-inducing signalling complex, which stimulates caspase cleavage (135). The intrinsic pathway is mediated by mitochondria responding to either apoptotic signals or to the absence of certain growth factors or signals (135). TGFβ has been shown to activate both apoptotic pathways. For example, TβRII is able to interact directly with Daxx, an intracellular component of the Fas-mediated apoptotic programme to activate JNK-mediated apoptosis (136). TGFβ can also activate apoptosis *via* the intrinsic pathway. Jang and colleagues illustrated that Smad signal transduction initiated by TGFβ induces the production of death-associated protein kinase, or DAP-kinase (137). DAP-kinase functions upstream of mitochondrial-induced apoptosis. It has been shown that a dominant-negative form of DAP-kinase blocks the ability of TGFβ to induce cytochrome C release from mitochondria (137).

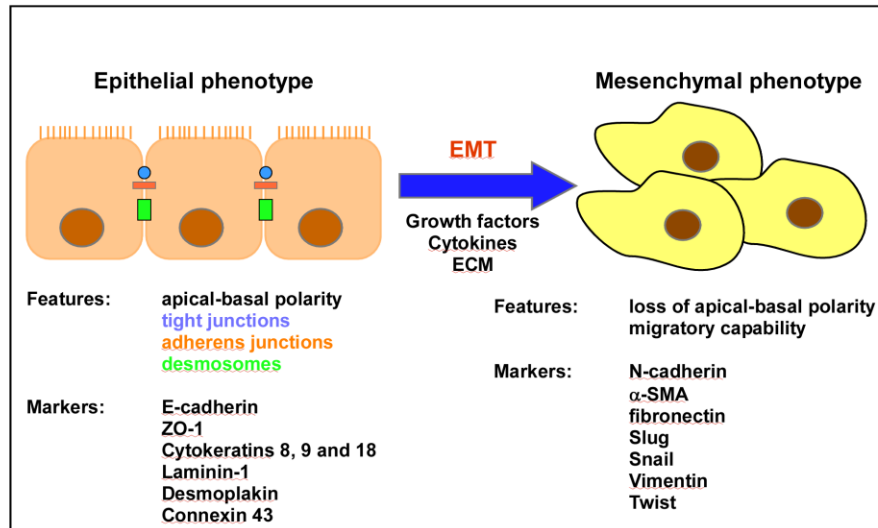
1.6.3 *TGFβ and EMT*

Another important and well-studied outcome of TGFβ signal transduction is EMT, a process whereby epithelial cells lose their apical-basal polarity and cell-cell junctions (such as tight junctions and adherens junctions) and gain a mesenchymal phenotype, increasing their ability to produce extracellular matrix proteins, migrate, and invade into other tissues (138) (Figure 1.7). The process of EMT is studied *in vitro* through the progressive loss of epithelial markers, such as E-cadherin, ZO-1, and cytokeratin; with the gain of mesenchymal markers such as N-cadherin, fibronectin, and vimentin (138).

Figure 1.7 The Epithelial-to-Mesenchymal Transition (EMT)

EMT can occur in both physiological conditions as well as pathologies such as cancer. During EMT epithelial cells lose their epithelial markers (such as E-cadherin and cytokeratins) as well as their apico-basal polarity and gain mesenchymal markers (such as N-cadherin and α-SMA) and a mesenchymal phenotype.

Figure 1.7



TGFβ was first shown to induce EMT in 1994 in a study by Miettinen and colleagues. They found that TGFβ induced mammary epithelial cells to gain a mesenchymal phenotype. This process is dependent on the type I TGFβ receptor, as the over-expression of a dominant-negative form of TβRI lacking the kinase domain prevented EMT (139). TGFβ mediates many of the phenotypic changes associated with EMT. One important event during EMT is the dissolution of tight junctions. A study by Ozdamar *et al.* showed that in epithelial cells, TGFβ treatment induces TβRII to phosphorylate Par6, thereby recruiting Smurf1, an ubiquitin ligase belonging to the same family as Smurf2, and targeting RhoA for degradation (124). The degradation of RhoA by Smurf1 begins the dissolution of tight junctions in epithelial cells (124). Another important step in EMT is the down-regulation of E-cadherin. E-cadherin is an epithelial cell-cell adhesion receptor and is important in regulating the epithelial phenotype (140). Loss of E-cadherin decreases epithelial cell junctions and also results in β-catenin being localized in the nucleus, activating the Wnt signalling pathway (140). TGFβ potently decreases E-cadherin levels through the induction of the transcription factors SNAI1 and SNAI2 via Smad3 (141). SNAI1 and SNAI2 repress E-cadherin transcription, therefore decreasing steady-state levels of E-cadherin (142,143). Furthermore, not only do cells decrease E-cadherin during EMT, but they also undergo a “cadherin switch” increasing the production of N-cadherin, or neuronal cadherin (144). N-cadherin up-regulation is also induced by TGFβ (145), and increased levels of N-cadherin are associated with an increase in cell motility, an important trait of mesenchymal cells (146). TGFβ also induces

the production of fibronectin, which contributes to cell-adhesion and motility (128).

EMT is essential in development for the generation of the three-layered body plan of the embryo consisting of endoderm, mesoderm, and ectoderm, which arises through gastrulation (140). Indeed, blocking EMT prevents development beyond the blastula stage (140). TGFβ is implicated in EMT during heart valve formation in the developing embryo. TGFβ3 expression is up-regulated by atrioventricular endothelial cells and works in concert with BMP-2 to initiate EMT in these cells (147).

While EMT is essential for body patterning and organogenesis in development, it has also been implicated in a number of pathologies including cancer. In order for a tumour cell to disseminate to distant sites, it must first detach from adjacent tumour cells, invade into the tissue, intravasate into the blood or lymphatic system, extravasate and grow at a distant site (148). In cancer, the ability of an epithelial-derived cancer cell to obtain mesenchymal characteristics permits the migration and invasion of that cell to distant sites (2). Indeed, a number of histological sections of cancers show cells undergoing EMT at the leading edge of the invasive front of the tumour (2). Interestingly, EMT has recently been shown to endow cancer cells with stem-cell like features, such as the expression of stem-cell markers and self-renewal (149,150). Therefore, EMT gives two advantages to a cancer cell. By giving cells the ability to migrate and invade, EMT promotes metastasis, but by promoting stem-cell features, EMT

also imparts on these cells the potential for self-renewal (149). Therefore, identifying the mechanisms whereby TGFβ induces and regulates EMT is an important area of research.

1.7 Smad-independent Signalling

While the activation of the classical Smad-mediated TGFβ pathway has been well established, TGFβ has also been shown to undergo cross-talk with several pathways including the PI3 kinase/Akt pathway, the Wnt pathway, the Notch pathway and the MAP kinase pathway (reviewed in (151)). For the purposes of this thesis I will focus on the cross-talk of TGFβ with the Wnt and MAPK pathways.

The WNT and TGFβ pathways are both implicated in important processes such as development, fibrosis, and cancer. Research by Labbe and colleagues illustrated that TGFβ has been shown to have a synergistic effect with Wnt signalling. For example, using a microarray approach, Labbe and colleagues found 78 novel genes up-regulated only by treatment with both TGFβ and Wnt3a and not each ligand independently, illustrating that the ligands are not simply having an additive effect (152). Importantly, a number of these TGFβ/Wnt3a target genes, such as CTGF, Inhba, and MMP14 are over-expressed in tumours from patients with familial adenomatous polyposis (152). Numerous cancers also have elevated Inhba, such as breast, lung, pancreatic and intestinal cancers (152). While the mechanism through which Wnt and TGFβ co-operatively signal

is not yet established, the co-operation of these two signalling pathways in tumourigenesis may have important implications in cancer progression.

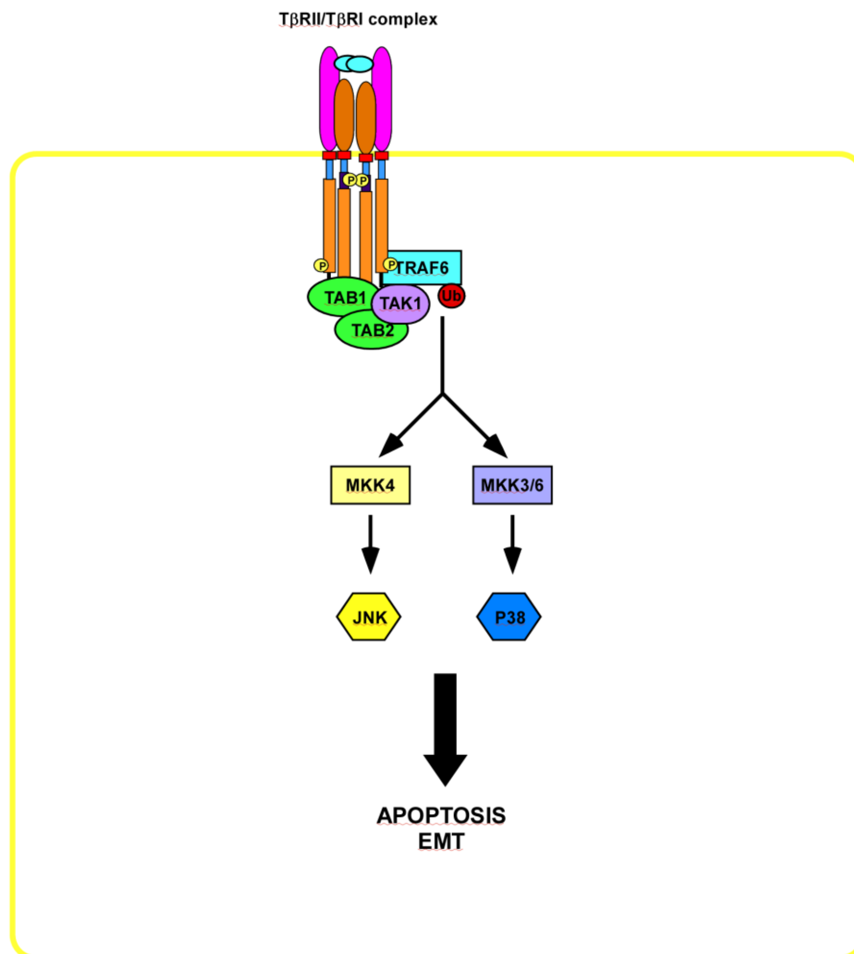
The ability of TGFβ to activate components of the MAP kinase pathway has long been observed, however, the exact mechanisms whereby it accesses this pathway *independently* of Smads have only been recently elucidated. The MAP kinase pathway consists of a series of phosphorylation events, ultimately resulting in the formation of a complex to activate transcription. There are three principle MAP kinase pathways: the ERK1/ERK2 pathway, the Jun N-terminal Kinase (JNK) pathway, and the p38 pathway. The MAP kinases are phosphorylated by the MAP kinase kinases (MAP2Ks), which in turn are activated by MAP kinase kinase kinases (MAP3Ks), which are activated in response to extracellular stimulation. ERK1/2 has been shown to be pro-tumourigenic, with high expression levels in many tumours, whereas JNK and p38 kinases are stress-induced pathways with more complex roles in cancer (153).

TGFβ can activate all three MAP kinase pathways. It has been shown that TGFβ activates Erk1/2 via TβRI. Briefly, TGFβ stimulation induces TβRI to directly phosphorylate ShcA on serine and tyrosine residues, though at a lower level than its phosphorylation of Smads (154). The phosphorylation of ShcA allows it to interact with Grb2, an adaptor protein which is constitutively associated with Sos (Son of sevenless) (155). Sos is a guanine nucleotide

Figure 1.8 Smad-independent activation of p38 and JNK by TGFβ

TGFβ stimulation can activate the p38 and JNK MAP kinase pathways. TGFβ stimulation facilitates Traf6 interaction with the receptor complex, where it is ubiquitinated. The ubiquitinated Traf6 then recruits TAK1 to activate p38 and JNK signalling.

Figure 1.8



exchange factor which promotes the exchange of GDP for GTP on Ras, thereby leading to the activation of Ras/ERK pathway (154,155).

TGFβ activates both p38 and JNK MAP kinase pathways *via* TAK1 (TGFβ activated kinase 1) (Figure 1.8). TAK1 is a MAP3K and was shown to be activated in response to TGFβ to induce MAPK-dependent transcription (156). A study by Yamashita and colleagues identified Traf6 as a functional link between the TGFβ receptors and TAK1 (157). Traf6 can interact directly with the TGFβ receptor complex and in response to TGFβ stimulation, is ubiquitinated which facilitates its interaction with TAK1 (157). The authors further illustrated that Traf6 is essential for TGFβ-mediated activation of the MAPK pathway, as siRNA mediated silencing of Traf6 abrogated the ability of TGFβ to increase p38 and JNK phosphorylation (157) (Fig. 1.8). The importance of the p38 pathway to TGFβ signalling has been highlighted by a study which illustrated that p38 is required for TGFβ-induced EMT as well as apoptosis (158). The p38 inhibitor, SB203580, blocked TGFβ induction of cleaved caspase as well as the loss of E-cadherin, but did not affect the ability of TGFβ to induce Smad2 phosphorylation; highlighting the fact that the p38 pathway is activated *independently* of Smad signalling (158). Therefore, when studying TGFβ signal transduction, it is important to assess both Smad-dependent and Smad-independent signalling pathways.

1.8 Purpose of study, hypothesis, aims

The TGFβ signalling pathway is crucial to both normal development and tissue homeostasis. The regulation of this pathway must be tightly controlled- this is evident in pathologies which show hyper-activation of the TGFβ pathway such as cancer and fibrosis. Indeed, TGFβ signalling is commonly dysregulated in cancer, and the ability of TGFβ to induce EMT is a crucial step in cancer progression and the dissemination of tumour cells to distant sites. Interestingly, the endocytic route of the TGFβ receptors directly influences their signalling outcome. Receptors internalized *via* clathrin-mediated endocytosis propagate TGFβ signalling, while internalization of TGFβ receptors via membrane rafts targets the receptors for degradation. Therefore, identifying proteins that direct TGFβ trafficking will directly impact TGFβ signal transduction. Therefore, **I hypothesize that protein interactions which alter TGFβ receptor endocytosis will have a direct effect on TGFβ signal transduction.** The **specific aims** of this study are:

Aim 1: Identify TβRII motifs that direct membrane raft partitioning.

Aim 2: Evaluate the role of TβRIII to TβRII/TβRI trafficking and signalling.

Aim 3: Assess the impact of β-arrestin2 on TGFβ receptor trafficking and signal transduction.

Aim 4: Study the role of TGFβ ligand sub-types on TβRII/TβRI trafficking and signalling in non-small cell lung cancer cells.

1.9 References

1. Wu, M. Y., and Hill, C. S. (2009) *Developmental cell* **16**(3), 329-343

2. Padua, D., and Massague, J. (2009) *Cell research* **19**(1), 89-102

3. Branton, M. H., and Kopp, J. B. (1999) *Microbes and infection / Institut Pasteur* **1**(15), 1349-1365

4. Schmierer, B., and Hill, C. S. (2007) *Nat Rev Mol Cell Biol* **8**(12), 970-982

5. Schlingensiepen, K. H., Schlingensiepen, R., Steinbrecher, A., Hau, P., Bogdahn, U., Fischer-Blass, B., and Jachimczak, P. (2006) *Cytokine & growth factor reviews* **17**(1-2), 129-139

6. Dunker, N., and Krieglstein, K. (2000) *European journal of biochemistry / FEBS* **267**(24), 6982-6988

7. Laverty, H. G., Wakefield, L. M., Occleston, N. L., O'Kane, S., and Ferguson, M. W. (2009) *Cytokine & growth factor reviews* **20**(4), 305-317

8. Brown, P. D., Wakefield, L. M., Levinson, A. D., and Sporn, M. B. (1990) *Growth factors (Chur, Switzerland)* **3**(1), 35-43

9. Lawrence, D. A. (2001) *Molecular and cellular biochemistry* **219**(1-2), 163-170

10. Sinha, S., Nevett, C., Shuttleworth, C. A., and Kielty, C. M. (1998) *Matrix Biol* **17**(8-9), 529-545

11. Miyazono, K., Olofsson, A., Colosetti, P., and Heldin, C. H. (1991) *The EMBO journal* **10**(5), 1091-1101

12. Sato, Y., and Rifkin, D. B. (1989) *The Journal of cell biology* **109**(1), 309-315

13. Yu, Q., and Stamenkovic, I. (2000) *Genes & development* **14**(2), 163-176

14. Pircher, R., Jullien, P., and Lawrence, D. A. (1986) *Biochemical and biophysical research communications* **136**(1), 30-37

15. Dallas, S. L., Zhao, S., Cramer, S. D., Chen, Z., Peehl, D. M., and Bonewald, L. F. (2005) *Journal of cellular physiology* **202**(2), 361-370

16. Annes, J. P., Rifkin, D. B., and Munger, J. S. (2002) *FEBS letters* **511**(1-3), 65-68

17. Shi, Y., and Massague, J. (2003) *Cell* **113**(6), 685-700

18. Derynck, R., and Feng, X. H. (1997) *Biochimica et biophysica acta* **1333**(2), F105-150

19. ten Dijke, P., Iwata, K. K., Goddard, C., Pieler, C., Canalis, E., McCarthy, T. L., and Centrella, M. (1990) *Molecular and cellular biology* **10**(9), 4473-4479

20. Brown, C. B., Boyer, A. S., Runyan, R. B., and Barnett, J. V. (1999) *Science (New York, N.Y* **283**(5410), 2080-2082

21. Shull, M. M., Ormsby, I., Kier, A. B., Pawlowski, S., Diebold, R. J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., and et al. (1992) *Nature* **359**(6397), 693-699

22. Sanford, L. P., Ormsby, I., Gittenberger-de Groot, A. C., Sariola, H., Friedman, R., Boivin, G. P., Cardell, E. L., and Doetschman, T. (1997) *Development (Cambridge, England)* **124**(13), 2659-2670

23. Proetzel, G., Pawlowski, S. A., Wiles, M. V., Yin, M., Boivin, G. P., Howles, P. N., Ding, J., Ferguson, M. W., and Doetschman, T. (1995) *Nature genetics* **11**(4), 409-414

24. Ferguson, M. W., and O'Kane, S. (2004) *Philosophical transactions of the Royal Society of London* **359**(1445), 839-850

25. Shah, M., Foreman, D. M., and Ferguson, M. W. (1995) *Journal of cell science* **108 (Pt 3)**, 985-1002

26. Occleston, N. L., Fairlamb, D., Hutchison, J., O'Kane, S., and Ferguson, M. W. (2009) *Expert opinion on investigational drugs* **18**(8), 1231-1239

27. Massague, J. (2008) *Cell* **134**(2), 215-230

28. Hau, P., Jachimczak, P., Schlingensiepen, R., Schulmeyer, F., Jauch, T., Steinbrecher, A., Brawanski, A., Proescholdt, M., Schlaier, J., Buchroithner, J., Pichler, J., Wurm, G., Mehdorn, M., Strege, R., Schuierer, G., Villarrubia, V., Fellner, F., Jansen, O., Straube, T., Nohria, V., Goldbrunner, M., Kunst, M., Schmaus, S., Stauder, G., Bogdahn, U., and Schlingensiepen, K. H. (2007) *Oligonucleotides* **17**(2), 201-212

29. Ghellal, A., Li, C., Hayes, M., Byrne, G., Bundred, N., and Kumar, S. (2000) *Anticancer research* **20**(6B), 4413-4418

30. van de Vijver, M. J., He, Y. D., van't Veer, L. J., Dai, H., Hart, A. A., Voskuil, D. W., Schreiber, G. J., Peterse, J. L., Roberts, C., Marton, M. J., Parrish, M., Atsma, D., Witteveen, A., Glas, A., Delahaye, L., van der Velde, T., Bartelink, H., Rodenhuis, S., Rutgers, E. T., Friend, S. H., and

Bernards, R. (2002) *The New England journal of medicine* **347**(25), 1999-2009

31. Massague, J., and Gomis, R. R. (2006) *FEBS letters* **580**(12), 2811-2820

32. Attisano, L., and Wrana, J. L. (1996) *Cytokine & growth factor reviews* **7**(4), 327-339

33. Henis, Y. I., Moustakas, A., Lin, H. Y., and Lodish, H. F. (1994) *The Journal of cell biology* **126**(1), 139-154

34. Lin, H. Y., Wang, X. F., Ng-Eaton, E., Weinberg, R. A., and Lodish, H. F. (1992) *Cell* **68**(4), 775-785

35. Lin, H. Y., Moustakas, A., Knaus, P., Wells, R. G., Henis, Y. I., and Lodish, H. F. (1995) *The Journal of biological chemistry* **270**(6), 2747-2754

36. Luo, K., Zhou, P., and Lodish, H. F. (1995) *Proceedings of the National Academy of Sciences of the United States of America* **92**(25), 11761-11765

37. Galliher, A. J., and Schiemann, W. P. (2007) *Cancer research* **67**(8), 3752-3758

38. Kang, J. S., Liu, C., and Derynck, R. (2009) *Trends in cell biology* **19**(8), 385-394

39. Bierie, B., and Moses, H. L. (2006) *Nature reviews* **6**(7), 506-520

40. Huse, M., Muir, T. W., Xu, L., Chen, Y. G., Kuriyan, J., and Massague, J. (2001) *Molecular cell* **8**(3), 671-682

41. Wieser, R., Wrana, J. L., and Massague, J. (1995) *The EMBO journal* **14**(10), 2199-2208

42. Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J., and Hill, C. S. (2002) *Molecular pharmacology* **62**(1), 65-74

43. Moren, A., Ichijo, H., and Miyazono, K. (1992) *Biochemical and biophysical research communications* **189**(1), 356-362

44. Gougos, A., and Letarte, M. (1990) *The Journal of biological chemistry* **265**(15), 8361-8364

45. Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W. S., and Massague, J. (1991) *Cell* **67**(4), 785-795

46. Guerrero-Esteo, M., Sanchez-Elsner, T., Letamendia, A., and Bernabeu, C. (2002) *The Journal of biological chemistry* **277**(32), 29197-29209

47. Yamashita, H., Ichijo, H., Grimsby, S., Moren, A., ten Dijke, P., and Miyazono, K. (1994) *The Journal of biological chemistry* **269**(3), 1995-2001

48. Chen, W., Kirkbride, K. C., How, T., Nelson, C. D., Mo, J., Frederick, J. P., Wang, X. F., Lefkowitz, R. J., and Blobe, G. C. (2003) *Science (New York, N.Y* **301**(5638), 1394-1397

49. Bernabeu, C., Lopez-Novoa, J. M., and Quintanilla, M. (2009) *Biochimica et biophysica acta* **1792**(10), 954-973

50. Wong, S. H., Hamel, L., Chevalier, S., and Philip, A. (2000) *European journal of biochemistry / FEBS* **267**(17), 5550-5560

51. McLean, S., and Di Guglielmo, G. M. *The Biochemical journal* **429**(1), 137-145

52. ten Dijke, P., Goumans, M. J., and Pardali, E. (2008) *Angiogenesis* **11**(1), 79-89

53. Compton, L. A., Potash, D. A., Brown, C. B., and Barnett, J. V. (2007) *Circulation research* **101**(8), 784-791

54. Lopez-Casillas, F., Wrana, J. L., and Massague, J. (1993) *Cell* **73**(7), 1435-1444

55. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) *Nature* **370**(6488), 341-347

56. Attisano, L., and Lee-Hoeflich, S. T. (2001) *Genome biology* **2**(8), REVIEWS3010

57. Chen, Y. G., Hata, A., Lo, R. S., Wotton, D., Shi, Y., Pavletich, N., and Massague, J. (1998) *Genes & development* **12**(14), 2144-2152

58. Macias-Silva, M., Abdollah, S., Hoodless, P. A., Pirone, R., Attisano, L., and Wrana, J. L. (1996) *Cell* **87**(7), 1215-1224

59. Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) *The EMBO journal* **16**(17), 5353-5362

60. Nakao, A., Roijer, E., Imamura, T., Souchelnytskyi, S., Stenman, G., Heldin, C. H., and ten Dijke, P. (1997) *The Journal of biological chemistry* **272**(5), 2896-2900

61. Xiao, Z., Latek, R., and Lodish, H. F. (2003) *Oncogene* **22**(7), 1057-1069

62. Doherty, G. J., and McMahon, H. T. (2009) *Annual review of biochemistry* **78**, 857-902

63. Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F., and Wrana, J. L. (2003) *Nature cell biology* **5**(5), 410-421

64. Marks, M. S., Woodruff, L., Ohno, H., and Bonifacino, J. S. (1996) *The Journal of cell biology* **135**(2), 341-354

65. Sorkin, A. (2004) *Current opinion in cell biology* **16**(4), 392-399

66. Praefcke, G. J., and McMahon, H. T. (2004) *Nat Rev Mol Cell Biol* **5**(2), 133-147

67. Chuang, E., Alegre, M. L., Duckett, C. S., Noel, P. J., Vander Heiden, M. G., and Thompson, C. B. (1997) *J Immunol* **159**(1), 144-151

68. Hopkins, C. R., Miller, K., and Beardmore, J. M. (1985) *J Cell Sci Suppl* **3**, 173-186

69. Stenmark, H. (2009) *Nat Rev Mol Cell Biol* **10**(8), 513-525

70. Barr, F., and Lambright, D. G. *Current opinion in cell biology* **22**(4), 461-470

71. McLauchlan, H., Newell, J., Morrice, N., Osborne, A., West, M., and Smythe, E. (1998) *Curr Biol* **8**(1), 34-45

72. Gorvel, J. P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991) *Cell* **64**(5), 915-925

73. Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005) *Cell* **122**(5), 735-749

74. Sonnichsen, B., De Renzis, S., Nielsen, E., Rietdorf, J., and Zerial, M. (2000) *The Journal of cell biology* **149**(4), 901-914

75. Simons, K., and Ikonen, E. (1997) *Nature* **387**(6633), 569-572

76. Staubach, S., and Hanisch, F. G. *Expert review of proteomics* **8**(2), 263-277

77. Simons, K., and Gerl, M. J. *Nat Rev Mol Cell Biol* **11**(10), 688-699

78. Brown, D. A. (2006) *Physiology (Bethesda, Md* **21**, 430-439

79. Plowman, S. J., Muncke, C., Parton, R. G., and Hancock, J. F. (2005) *Proceedings of the National Academy of Sciences of the United States of America* **102**(43), 15500-15505

80. Chichili, G. R., and Rodgers, W. (2007) *The Journal of biological chemistry* **282**(50), 36682-36691

81. Chichili, G. R., and Rodgers, W. (2009) *Cell Mol Life Sci* **66**(14), 2319-2328

82. Xu, X., Bittman, R., Duportail, G., Heissler, D., Vilcheze, C., and London, E. (2001) *The Journal of biological chemistry* **276**(36), 33540-33546

83. Lingwood, D., and Simons, K. (2007) *Nature protocols* **2**(9), 2159-2165

84. Luga, V., McLean, S., Le Roy, C., O'Connor-McCourt, M., Wrana, J. L., and Di Guglielmo, G. M. (2009) *The Biochemical journal* **421**(1), 119-131

85. McCabe, J. B., and Berthiaume, L. G. (2001) *Molecular biology of the cell* **12**(11), 3601-3617

86. Tang, Z., Scherer, P. E., Okamoto, T., Song, K., Chu, C., Kohtz, D. S., Nishimoto, I., Lodish, H. F., and Lisanti, M. P. (1996) *The Journal of biological chemistry* **271**(4), 2255-2261

87. Way, M., and Parton, R. G. (1995) *FEBS letters* **376**(1-2), 108-112

88. Razani, B., Wang, X. B., Engelman, J. A., Battista, M., Lagaud, G., Zhang, X. L., Kneitz, B., Hou, H., Jr., Christ, G. J., Edelmann, W., and Lisanti, M. P. (2002) *Molecular and cellular biology* **22**(7), 2329-2344

89. Parton, R. G., and Simons, K. (2007) *Nat Rev Mol Cell Biol* **8**(3), 185-194

90. Murata, M., Peranen, J., Schreiner, R., Wieland, F., Kurzchalia, T. V., and Simons, K. (1995) *Proceedings of the National Academy of Sciences of the United States of America* **92**(22), 10339-10343

91. Stahlhut, M., and van Deurs, B. (2000) *Molecular biology of the cell* **11**(1), 325-337

92. Bastiani, M., and Parton, R. G. *Journal of cell science* **123**(Pt 22), 3831-3836

93. Drab, M., Verkade, P., Elger, M., Kasper, M., Lohn, M., Lauterbach, B., Menne, J., Lindschau, C., Mende, F., Luft, F. C., Schedl, A., Haller, H., and Kurzchalia, T. V. (2001) *Science (New York, N.Y* **293**(5539), 2449-2452

94. Sotgia, F., Williams, T. M., Schubert, W., Medina, F., Minetti, C., Pestell, R. G., and Lisanti, M. P. (2006) *The American journal of pathology* **168**(1), 292-309

95. Williams, T. M., Medina, F., Badano, I., Hazan, R. B., Hutchinson, J., Muller, W. J., Chopra, N. G., Scherer, P. E., Pestell, R. G., and Lisanti, M. P. (2004) *The Journal of biological chemistry* **279**(49), 51630-51646

96. Lu, Z., Ghosh, S., Wang, Z., and Hunter, T. (2003) *Cancer cell* **4**(6), 499-515

97. Mitchell, H., Choudhury, A., Pagano, R. E., and Leof, E. B. (2004) *Molecular biology of the cell* **15**(9), 4166-4178

98. Ogunjimi, A. A., Briant, D. J., Pece-Barbara, N., Le Roy, C., Di Guglielmo, G. M., Kavsak, P., Rasmussen, R. K., Seet, B. T., Sicheri, F., and Wrana, J. L. (2005) *Molecular cell* **19**(3), 297-308

99. Kavsak, P., Rasmussen, R. K., Causing, C. G., Bonni, S., Zhu, H., Thomsen, G. H., and Wrana, J. L. (2000) *Molecular cell* **6**(6), 1365-1375

100. Platta, H. W., and Stenmark, H. *Current opinion in cell biology* **23**(4), 393-403

101. Runyan, C. E., Schnaper, H. W., and Poncelet, A. C. (2005) *The Journal of biological chemistry* **280**(9), 8300-8308

102. Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L., and Wrana, J. L. (1998) *Cell* **95**(6), 779-791

103. Lawe, D. C., Patki, V., Heller-Harrison, R., Lambright, D., and Corvera, S. (2000) *The Journal of biological chemistry* **275**(5), 3699-3705

104. Hayes, S., Chawla, A., and Corvera, S. (2002) *The Journal of cell biology* **158**(7), 1239-1249

105. Hu, H., Milstein, M., Bliss, J. M., Thai, M., Malhotra, G., Huynh, L. C., and Colicelli, J. (2008) *Molecular and cellular biology* **28**(5), 1573-1583

106. Razani, B., Zhang, X. L., Bitzer, M., von Gersdorff, G., Bottinger, E. P., and Lisanti, M. P. (2001) *The Journal of biological chemistry* **276**(9), 6727-6738

107. Yan, X., Liu, Z., and Chen, Y. (2009) *Acta biochimica et biophysica Sinica* **41**(4), 263-272

108. Nakao, A., Afrakhte, M., Moren, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N. E., Heldin, C. H., and ten Dijke, P. (1997) *Nature* **389**(6651), 631-635

109. Hayashi, H., Abdollah, S., Qiu, Y., Cai, J., Xu, Y. Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone, M. A., Jr., Wrana, J. L., and Falb, D. (1997) *Cell* **89**(7), 1165-1173

110. Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001) *The Journal of biological chemistry* **276**(16), 12477-12480

111. Chen, C. L., Liu, I. H., Fliesler, S. J., Han, X., Huang, S. S., and Huang, J. S. (2007) *Journal of cell science* **120**(Pt 20), 3509-3521

112. Zwaagstra, J. C., El-Alfy, M., and O'Connor-McCourt, M. D. (2001) *The Journal of biological chemistry* **276**(29), 27237-27245

113. Koli, K. M., and Arteaga, C. L. (1997) *The Journal of biological chemistry* **272**(10), 6423-6427

114. Dobrowolski, R., and De Robertis, E. M. *Nat Rev Mol Cell Biol* **13**(1), 53-60

115. Jean-Alphonse, F., and Hanyaloglu, A. C. *Molecular and cellular endocrinology* **331**(2), 205-214

116. Yao, D., Ehrlich, M., Henis, Y. I., and Leof, E. B. (2002) *Molecular biology of the cell* **13**(11), 4001-4012

117. Ehrlich, M., Shmuely, A., and Henis, Y. I. (2001) *Journal of cell science* **114**(Pt 9), 1777-1786

118. Kveiborg, M., Albrechtsen, R., Couchman, J. R., and Wewer, U. M. (2008) *The international journal of biochemistry & cell biology* **40**(9), 1685-1702

119. Atfi, A., Dumont, E., Colland, F., Bonnier, D., L'Helgoualc'h, A., Prunier, C., Ferrand, N., Clement, B., Wewer, U. M., and Theret, N. (2007) *The Journal of cell biology* **178**(2), 201-208

120. Finnson, K. W., Tam, B. Y., Liu, K., Marcoux, A., Lepage, P., Roy, S., Bizet, A. A., and Philip, A. (2006) *Faseb J* **20**(9), 1525-1527

121. Bizet, A. A., Liu, K., Tran-Khanh, N., Saksena, A., Vorstenbosch, J., Finnson, K. W., Buschmann, M. D., and Philip, A. *Biochimica et biophysica acta* **1813**(5), 742-753

122. Bizet, A. A., Tran-Khanh, N., Saksena, A., Liu, K., Buschmann, M. D., and Philip, A. *Journal of cellular biochemistry* **113**(1), 238-246

123. Alvi, F., Idkowiak-Baldys, J., Baldys, A., Raymond, J. R., and Hannun, Y. A. (2007) *Cell Mol Life Sci* **64**(3), 263-270

124. Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H. R., Zhang, Y., and Wrana, J. L. (2005) *Science (New York, N.Y* **307**(5715), 1603-1609

125. Gunaratne, A., Benchabane, H., and Di Guglielmo, G. M. *Cellular signalling* **24**(1), 119-130

126. Luttrell, L. M., and Lefkowitz, R. J. (2002) *Journal of cell science* **115**(Pt 3), 455-465

127. Ma, L., and Pei, G. (2007) *Journal of cell science* **120**(Pt 2), 213-218

128. Ignotz, R. A., and Massague, J. (1986) *The Journal of biological chemistry* **261**(9), 4337-4345

129. Siegel, P. M., and Massague, J. (2003) *Nature reviews* **3**(11), 807-821

130. Laiho, M., DeCaprio, J. A., Ludlow, J. W., Livingston, D. M., and Massague, J. (1990) *Cell* **62**(1), 175-185

131. Weinberg, R. A. (1995) *Cell* **81**(3), 323-330

132. Kang, Y., Chen, C. R., and Massague, J. (2003) *Molecular cell* **11**(4), 915-926

133. Chen, C. R., Kang, Y., Siegel, P. M., and Massague, J. (2002) *Cell* **110**(1), 19-32

134. Elmore, S. (2007) *Toxicologic pathology* **35**(4), 495-516

135. Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E. (1995) *The EMBO journal* **14**(22), 5579-5588

136. Perlman, R., Schiemann, W. P., Brooks, M. W., Lodish, H. F., and Weinberg, R. A. (2001) *Nature cell biology* **3**(8), 708-714

137. Jang, C. W., Chen, C. H., Chen, C. C., Chen, J. Y., Su, Y. H., and Chen, R. H. (2002) *Nature cell biology* **4**(1), 51-58

138. Kalluri, R., and Weinberg, R. A. (2009) *The Journal of clinical investigation* **119**(6), 1420-1428

139. Miettinen, P. J., Ebner, R., Lopez, A. R., and Derynck, R. (1994) *The Journal of cell biology* **127**(6 Pt 2), 2021-2036

140. Thiery, J. P., and Sleeman, J. P. (2006) *Nat Rev Mol Cell Biol* **7**(2), 131-142

141. Zavadil, J., Cermak, L., Soto-Nieves, N., and Bottinger, E. P. (2004) *The EMBO journal* **23**(5), 1155-1165

142. Batlle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J., and Garcia De Herreros, A. (2000) *Nature cell biology* **2**(2), 84-89

143. Bolos, V., Peinado, H., Perez-Moreno, M. A., Fraga, M. F., Esteller, M., and Cano, A. (2003) *Journal of cell science* **116**(Pt 3), 499-511

144. Wheelock, M. J., Shintani, Y., Maeda, M., Fukumoto, Y., and Johnson, K. R. (2008) *Journal of cell science* **121**(Pt 6), 727-735

145. Chimal-Monroy, J., and Diaz de Leon, L. (1999) *The International journal of developmental biology* **43**(1), 59-67

146. Nieman, M. T., Prudoff, R. S., Johnson, K. R., and Wheelock, M. J. (1999) *The Journal of cell biology* **147**(3), 631-644

147. Nakajima, Y., Yamagishi, T., Hokari, S., and Nakamura, H. (2000) *The Anatomical record* **258**(2), 119-127

148. Yilmaz, M., and Christofori, G. (2009) *Cancer metastasis reviews* **28**(1-2), 15-33

149. Scheel, C., and Weinberg, R. A. *International journal of cancer* **129**(10), 2310-2314

150. Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M., Campbell, L. L., Polyak, K., Brisken, C., Yang, J., and Weinberg, R. A. (2008) *Cell* **133**(4), 704-715

151. Guo, X., and Wang, X. F. (2009) *Cell research* **19**(1), 71-88

152. Labbe, E., Lock, L., Letamendia, A., Gorska, A. E., Gryfe, R., Gallinger, S., Moses, H. L., and Attisano, L. (2007) *Cancer research* **67**(1), 75-84

153. Sebolt-Leopold, J. S., and Herrera, R. (2004) *Nature reviews* **4**(12), 937-947

154. Lee, M. K., Pardoux, C., Hall, M. C., Lee, P. S., Warburton, D., Qing, J., Smith, S. M., and Derynck, R. (2007) *The EMBO journal* **26**(17), 3957-3967

155. Giubellino, A., Burke, T. R., Jr., and Bottaro, D. P. (2008) *Expert opinion on therapeutic targets* **12**(8), 1021-1033

156. Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995) *Science (New York, N.Y* **270**(5244), 2008-2011

157. Yamashita, M., Fatyol, K., Jin, C., Wang, X., Liu, Z., and Zhang, Y. E. (2008) *Molecular cell* **31**(6), 918-924

158. Yu, L., Hebert, M. C., and Zhang, Y. E. (2002) *The EMBO journal* **21**(14), 3749-3759

CHAPTER 2

THE EXTRACELLULAR DOMAIN OF THE TGFβ TYPE II RECEPTOR REGULATES MEMBRANE RAFT PARTITIONING

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2 Chapter 2

2.1 Chapter summary

TGFβ-dependent Smad signal transduction has been shown to be mediated by the endocytosis and trafficking of the TβRII/TβRI complex. Receptors internalized by clathrin-mediated internalization traffic to the early endosome and propagate Smad signaling, while those internalized by membrane-rafts traffic to the caveolin-1 positive vesicle and are targeted for degradation. However, the signal(s) which direct membrane raft partitioning of the signal complex are unknown. In this chapter, I evaluate structural motifs of TβRII which direct its partitioning and endocytosis. This chapter illustrates that the extracellular domain of TβRII increases its entry into membrane-raft fractions and that the glycosylation state of the cell as a whole, but not of TβRII itself, decreased entry of TβRII into membrane rafts. Importantly, I showed that a chimeric construct consisting of the extracellular domain of GMCSF and the intracellular domain of TβRII, does not greatly partition into membrane rafts, although the extracellular GMCSF is glycosylated similar to TβRII. My data from this chapter therefore suggests that a glycosylated protein interacts with the extracellular domain of TβRII to influence its partitioning.

2.2 Introduction

Endocytosis of cell surface proteins occurs *via* multiple pathways, two of which are clathrin-dependent and membrane raft-dependent, with the latter leading in part to entrance into caveolin-positive endosomes (1). Clathrin-coated vesicles, which form from the fission of plasma membrane clathrin-coated pits, carry receptors to the PtdIns3P-enriched early endosome, from which they can recycle back to the plasma membrane or continue into the late endosomal system for degradation (1). Membrane rafts are heterogeneous microdomains in the plasma membrane that act to compartmentalize cellular processes (2). They are enriched in cholesterol, sphingolipids, and glycolipids, and fractionate based on light buoyant density on sucrose gradient (3). Membrane raft/caveolar internalization of receptors occurs through small flask-shaped invaginations called caveolae (3). They are rich in caveolins, hairpin-like palmitoylated integral membrane proteins that bind cholesterol (3). Caveolae can act as signaling platforms from which receptors such as GPCRs, receptor tyrosine kinases, and steroid hormone receptors aggregate so as to facilitate downstream signaling events (4). Raft dependent internalization and caveolin-positive endosomes have also been described for the uptake of various viruses and toxins (4).

The transforming growth factor-β (TGFβ) superfamily regulates many cellular functions, including proliferation, differentiation, apoptosis, migration and extracellular matrix production (5). The signal transduction pathway initiated by cell surface TGFβ receptor complexes is dependent on ligand binding as well as receptor internalization and trafficking (1). Activation of the receptor is initiated

through ligand-induced heteromeric complex formation of type I (TβRI) and type II (TβRII) transmembrane Ser/Thr kinase receptors that target both Smad-dependent and independent signaling pathways (6). Smad activation by TGFβ involves the phosphorylation of the regulatory Smads, R-Smad2 and R-Smad3. TGFβ employs two internalization pathways one of which is clathrin-dependent and the other is clathrin-independent and membrane raft-dependent (rev. by (1)). Entrance into the early endosome is thought to promote Smad activation through Smad anchor for receptor activation (SARA) protein, which binds the receptors and recruits Smads to the membrane. The early endosome also contains hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), which co-operates with SARA to facilitate TGFβ signaling (7), and cytoplasmic promyelocytic leukemia protein (cPML), a scaffolding molecule that is necessary for TGFβ signaling (8).

TGFβ signaling is antagonized by the inhibitory Smad7, which interacts with TβRI and recruits the E3 ubiquitin ligase Smurf2, which directs ubiquitin-dependent degradation of the TGFβ receptors (9,10). Smad7 and Smurf2 complexes are localized to the caveolin-positive compartment and perturbation of membrane rafts increases signaling and reduces the rate of receptor degradation (11-13).

The partitioning of cellular transmembrane proteins into membrane rafts is complex and can be dependent on the extracellular domain, as in the case for the EGFR (14), the transmembrane domain as is the case for carboxypeptidase

E (15) or the intracellular domain as is the case for adenylyl cyclases (16). Recently, membrane rafts have been implicated in the endocytosis of other TGFβ superfamily members, namely bone morphogenetic receptor 1 and 2 (BMPRI, BRII) (17,18). While cell surface TGFβ receptors reside in both membrane raft and non-raft membrane domains (11-13,19) the determinant that controls partitioning is unknown. A short peptide sequence ($I_{218}I_{219}L_{220}$) on the cytoplasmic region of TβRII is the major signal for clathrin-mediated endocytosis (20) and the TGFβ receptors associate with both clathrin and AP-2 (21). However, the cytosolic TβRII domain also contains consensus sequences that were previously identified as caveolin scaffolding domains (22) and the major binding partner of TβRII, the type I TGFβ receptor, has also been shown to associate with caveolin-1 (13,23). On the extracellular surface of the plasma membrane Galectin 3, a β-galactose binding protein that contains a carbohydrate recognition domain (24), associates with receptors and is postulated to influence receptor internalization into EEA1 and caveolin-1 positive vesicles (25). Due to the numerous interacting partners on both facets of the plasma membrane, the primary determinants that partition receptors into membrane rafts remain unclear.

Using subcellular fractionation and mutants of TβRII I demonstrate that the extracellular domain of TβRII mediates receptor partitioning into membrane rafts. Consistent with these results, I illustrated that a GMCSF-TβRII hybrid receptor that replaces the native TβRII extracellular domain with that of the GMCSF

receptor, results in a chimeric receptor that is largely excluded from membrane rafts and caveolin-1-positive structures. Also, studies using tunicamycin perturbed receptor membrane raft partitioning. Importantly, I showed that this was not due to a disturbance of membrane raft formation, as treatment of cells with tunicamycin did not disrupt membrane rafts. Furthermore, the glycosylation of TβRII itself did not account for its differential membrane partitioning, suggesting that partitioning involves interactions with other cell surface glycoproteins. Taken together, my results indicate that the extracellular region of TβRII is necessary for receptor membrane raft partitioning.

2.3 Materials and Methods

2.3.1 *Reagents*

Polyclonal anti-HA, anti-Flag and anti-TβRII (Santa Cruz), monoclonal anti-HA (12CA5, Boehringer), monoclonal anti-EEA1 (Transduction Laboratories), monoclonal anti-Flag (Sigma), monoclonal anti-CD131 (Bioscience) and polyclonal anti-caveolin-1 (Transduction Laboratories) antibodies were used as *per* the manufacturers' instructions. Tunicamycin was purchased from Sigma-Aldrich. TGFβ1 ligand was purchased from Peprotech.

2.3.2 *Constructs*

Constructs encoding for the amino or carboxyl terminally hemagglutinin (HA) epitope-tagged wild-type TGFβ type II receptor (HA-TβRII or TβRII-HA; (26)), the intracellularly truncated receptor (HA-TβRII-ΔCyt; (27)) and the GMCSF-TβRII hybrid receptor (28), and the GMCSF2RB receptor (a.k.a. b_C;

(29)) were previously described. The full length carboxyl HA-tagged TβRII in pCMV5 was used as the template to construct a receptor that has the signal sequence and 13 amino acids of the extracellular domain (TβRII-ΔEX-HA) and the glycosylation mutant substituting asparagines 70, 94 and 154 of the TβRII to aspartic acid residues (TβRII-3ND-HA). All constructs were generated using a PCR-based mutagenesis approach and were validated by sequencing analysis.

2.3.3 *Cell Culture*

HEK 293T human kidney epithelial cells (American Type Culture Collection) were cultured in DME medium (Gibco) supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.3.4 *Transfection*

HEK 293T cells were transiently transfected using the calcium phosphate precipitation method as previously described (12). Briefly, HEK293T cells were plated at approximately 50% confluency in 6 well dishes. The following day cells were transfected with 1.0 μg of TβRII-HA, 1.5 μg of HA-TβRII-ΔCyt, 0.5 μg of TβRII-ΔEX-HA and/or 1.0 μg TβRI-flag as indicated in Figure 2.1.

2.3.5 *Preparation of Membrane Rafts/caveolin-enriched fractions*

The caveolin/raft-rich membrane fractions were isolated as previously described (12). Briefly, transfected cells grown to confluence in 100-mm dishes

were used to prepare the membrane fractions. All steps were carried out at 4°C. After two washes with cold 1× phosphate-buffer saline (PBS) cells were lysed with 0.5 M Na_2CO_3, pH 11.0, containing protease inhibitors. After scraping, the cell lysate was collected and homogenized three times for 10s bursts using a Polytron tissue grinder (Brinkmann Instruments). Homogenates were then sonicated three times for 20s with a Vibra Cell sonicator (Sonics Materials Inc.). The homogenates were adjusted to 40% sucrose and overlaid with 30% sucrose and 5% sucrose solutions. The samples were centrifuged at 200,000 x g_{av} for 16h at 4°C, using a Beckman SW41 rotor. Twelve 1-mL fractions were collected, and an aliquot of each fraction was eluted with Laemmli sample buffer, boiled, and subjected to SDS-PAGE followed by Western blot analysis.

2.3.6 *Immunoblotting*

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes by electrophoretic transfer. Blots were then blocked in 5% skim milk/TBS-5 for one hour. Following overnight incubation with primary antibody at 4˚C, blots were incubated with secondary antibody at room temperature for 45 minutes. Bound antibodies were then detected using SuperSignal chemiluminescence reagent (Pierce) and blots were imaged on a VersaDoc (11).

2.3.7 *Immunoprecipitation*

Transfected 293T cells were lysed (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 % Triton X-100, 1 mM PMSF, and cocktail protease inhibitors) and centrifuged at 15,000 × g_{av} at 4°C for 5 min. 50 μg aliquots of

supernatants were collected for analysis of total protein concentration. The remaining cell lysates were incubated with anti-Flag mAb followed by protein G sepharose incubation. The precipitates were washed 3 times, eluted with Laemmli sample buffer and subjected to SDS-PAGE and immunoblot analysis.

2.3.8 *Tunicamycin treatment*

A dose response curve of tunicamycin treatment on HEK293T cells was completed and a concentration of 2μg/mL was found to be optimal and did not induce cell death. HEK 293T cells were treated with 2 μg/mL tunicamycin in DMEM supplemented with 0.2% fetal bovine serum, 24h after transfection. After 20h of incubation with the antibiotic, cells were homogenized and fractionated by sucrose density ultracentrifugation as described above.

2.4 **Results**

2.4.1 *An extracellular truncation mutant of Tβ RII interacts with Tβ RI*

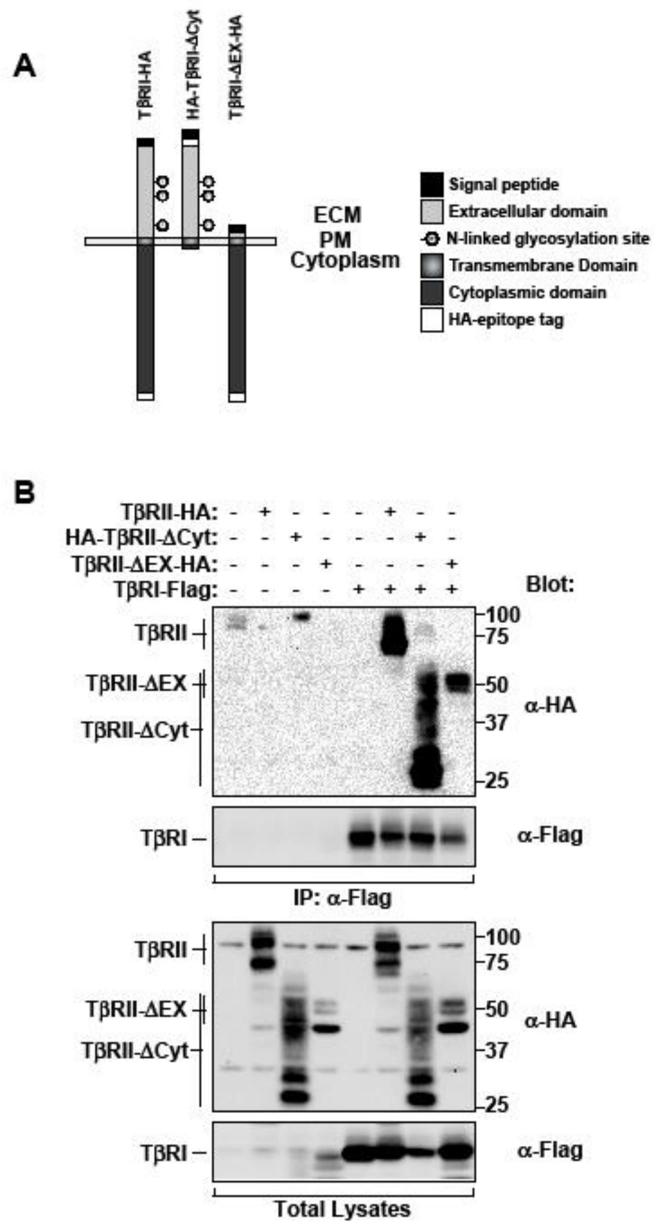
To understand if the partitioning of TGFβ receptors between raft and non-raft membrane domains is dependent on a molecular address in the receptor, we first explored the extracellular and intracellular domains of the TGFβ type II receptor (TβRII). A mutant containing only 13 amino acids of the extracellular domain (TβRII-ΔEX-HA) was generated and examined along with a truncated TβRII which contains only 10 amino acids of the intracellular domain (HA-TβRII-ΔCyt; (27); Figure 2.1A). The full length TβRII-HA and HA-TβRII-ΔCyt have been previously described and are both present at the cell surface. They also bind ligand and the TGFβ type I receptor (27). However, TβRII-ΔEX-HA had not been

Figure 2.1 Characterization of TβRII lacking the extracellular domain.

(**A**) TβRII mutants lacking the extracellular or the intracellular domain. Full length TβRII-HA and intracellularly truncated TβRII (HA-TβRII-ΔCyt) have been previously characterized (26,27). The extracellularly truncated receptor tagged at the carboxyl terminus (TβRII-ΔEX-HA) was generated for this study. The different domains of the receptors, the hemagglutinin tag (HA) as well as the plasma membrane (PM) and cytoplasm are indicated.

(**B**) TβRII-ΔEX-HA interacts with wild type TβRI. HEK 293T cells were transiently transfected with cDNA encoding the indicated receptors. Cell lysates were subjected to immunoprecipitation (IP) with mouse anti-Flag antibody followed by immunoblotting with rabbit anti-HA antibody (α-HA) to detect TβRI-associated type II receptor or rabbit anti-Flag (α-Flag) antibody to detect total TβRI expression as indicated (top panel). The relative expression of each construct was assessed by immunoblotting 50 μg of total cell lysates. The relative mobilities of the receptors are indicated (N=3).

Figure 2.1



characterized. Therefore, I sought to assess whether TβRII-ΔEX-HA is able to associate with the type I TGFβ receptor (TβRI). This occurs independently of ligand binding and is mediated by the cytoplasmic domains of the receptors (30). Indeed, immunoprecipitation of TβRI and immunoblotting for type II receptors revealed that TβRII-ΔEX-HA associated with TβRI (Figure 2.1B). It is noteworthy that both forms of the membrane-bound TβRII-ΔEX receptor, the 50 and 52 kDa forms, associate with the TβRI, whereas the 47 kDa cytosolic fragment of TβRII-ΔEX does not associate with membrane-localized TβRI. As positive controls, I assessed the interaction of TβRI with the wild type TβRII-HA as well as HA-TβRII-ΔCyt and I observed that they both associate with immunoprecipitated TβRI.

2.4.2 *TβRII-ΔEX-HA is largely excluded from membrane raft fractions*

Having found that the TβRII-ΔEX-HA associates with TβRI, the membrane raft partitioning of the three versions of TβRII was next compared (Figure 2.2). To assess membrane partitioning, cell homogenates were fractionated *via* sucrose density centrifugation as previously described (11-13,19). Briefly, cell homogenates were adjusted to 40% sucrose, overlaid with 30% and 5% sucrose cushions and centrifuged (Figure 2.2A; left panel). One mL fractions were then collected and immunoblotted with markers of either membrane rafts or early endosomes. Confirmation of the partitioning of raft from non-raft membranes is shown in Figure 2.2A (right panel) where fractions 4-6 on the sucrose gradient contained the majority of caveolin-1, a membrane raft resident protein (31) and

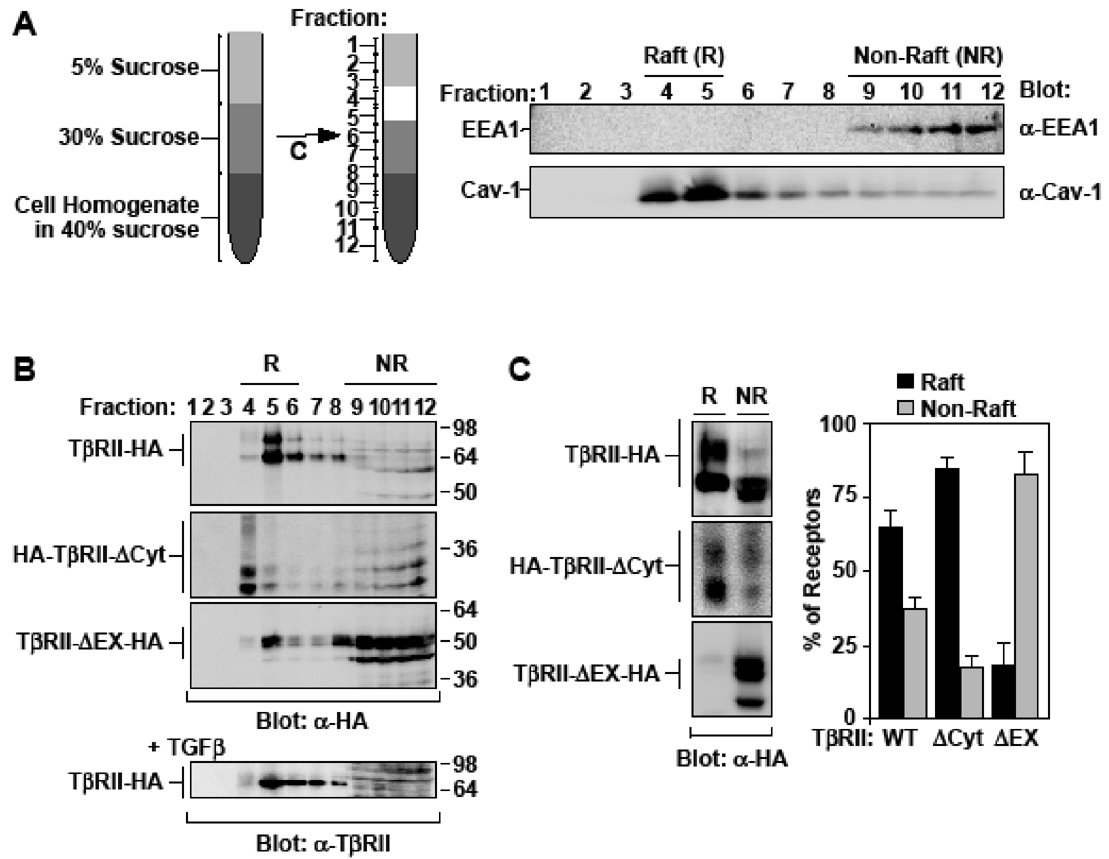
Figure 2.2 The TβRII extracellular domain is important for membrane raft partitioning.

(**A**) Subcellular fractionation of HEK 293T cells. Schematic representation of membrane raft isolation protocol (left panel). Cell homogenates were sonicated and adjusted to 40% sucrose, overlaid with 30% and then 5% sucrose cushions prior to ultracentrifugation (C). Twelve 1 mL fractions were then collected with membrane rafts (white region) usually present in fractions 4-6. Aliquots were immunoblotted for endogenous EEA1 (α-EEA1), or endogenous cav-1 (α-Cav-1), (right panel) (N=3).

(**B**) Membrane raft partitioning of full length TβRII and the truncation mutants. HEK 293T cells transiently transfected with cDNA containing the indicated receptor constructs were subjected to subcellular fractionation as described in panel A. All fractions were then immunoblotted with anti-HA antibody (α-HA). HEK 293T cells transiently transfected with wild-type TβRII cDNA were treated with 500 pMol TGFβ for 1 hour and subjected to subcellular fractionation (bottom panel). The fractions were then subjected to SDS-PAGE and immunoblotted with anti-TβRII antibody (N=3).

(**C**) Quantitation of wild-type and mutant forms of TβRII in membrane raft and non-raft fractions. Membrane raft (fractions 4-6) or non-raft (fractions 8-12) fractions from experiments described in (**B**) were pooled, adjusted to the same volume and immunoblotted with anti-HA (α-HA) antibody. The relative amount of TβRII receptors in membrane-raft and non-raft fractions were quantitated using QuantityOne software and graphed as a percentage of total receptors expressed. Each data point represents the mean of three experiments ± standard deviation (N=3).

Figure 2.2



fractions 8-12 contained the early endosomal auto-antigen 1 (EEA1), an early endosomal resident protein (32,33) . To analyze receptor partitioning TβRII-HA, HA-TβRII-ΔCyt or TβRII-ΔEX-HA was transiently expressed in HEK 293T cells and fractionated cell homogenates on sucrose gradients (Figure 2.2B). TβRII-HA was found to be present in both the raft and non-raft fractions and HA-TβRII-ΔCyt was also observed in raft and non-raft fractions (Figure 2.2B). In contrast, the TβRII-ΔEX-HA receptor was predominantly observed in the non-raft fractions. To assess whether ligand treatment affects TβRII membrane partitioning, I treated HEK 293T cells with 500 pM TGFβ one hour prior to membrane raft isolation. Following SDS-PAGE and immunoblotting, I observed that TGFβ treatment does not affect partitioning, as TβRII was still found primarily in raft fractions. To measure the relative amounts of receptors in the raft and non-raft fractions, raft fractions (fractions 4-6) or non-raft fractions (8-12) were pooled from experiments carried out as shown in Figure 2.2B, adjusted to the same volume and were subjected to quantitative immunoblotting (Figure 2.2C). It was observed that approximately 65% of TβRII was in rafts whereas 85% of TβRII-ΔCyt fractionated in rafts (Figure 2.2C; right panel). In marked contrast, only 20% of TβRII-ΔEX-HA was in the membrane raft fractions. These results indicate that the extracellular domain of TβRII mediates partitioning into membrane rafts, but the extracellular domain is not affecting partitioning via its ligand-binding capabilities.

2.4.3 *Perturbation of glycosylation alters membrane raft partitioning of TβRII*

Cells lacking proper glycosylation have defects in cytokine, and in particular TGFβ signaling (25). Therefore the pharmacological perturbation of cellular glycosylation was assessed to determine if this would alter TGFβ receptor partitioning in membrane raft domains (Figure 2.3). HEK293T cells transiently expressing wild-type TβRII were incubated with tunicamycin, an antibiotic that blocks the reaction of UDP-GlcNAc and Dol-P in the first step of glycoprotein synthesis and thus inhibits the synthesis of all N-linked glycoproteins (34). A reproducible decrease of receptors in the raft fractions and a concomitant increase in non-raft fractions in tunicamycin-treated cells was observed (Figure 2.3A). Importantly I illustrated that this shift was not due to perturbation of lipid rafts with tunicamycin treatment as shown in Figure 2.3B.

2.4.4 *The glycosylation status of TβRII does not alter its membrane raft partitioning*

These results suggest that the glycosylation state of TGFβ receptors and/or the proper glycosylation of other cell surface proteins regulate the membrane raft partitioning of receptors. To distinguish between these two possibilities, a construct of TβRII that contains mutations in all three putative N-linked glycosylation sites was generated (TβRII-3ND-HA; Figure 2.4A). Based on the molecular weight of the mutant compared to the wild-type receptor, it appears that the three putative sites are indeed glycosylation sites (Figure 2.4).The partitioning of TβRII-3ND-HA in membrane raft and non-raft fractions was next assessed. It was shown that this receptor co-fractionated with both membrane

Figure 2.3 Perturbation of glycosylation alters membrane raft partitioning of TβRII

(A) Quantitation of pharmacological inhibition of glycosylation on receptor partitioning. Membrane raft (fractions 4-6) or non-raft (fractions 8-12) fractions from experiments described in were pooled, adjusted to the same volume and immunoblotted with anti-HA (α-HA) antibody. The relative amount of TβRII receptors in membrane-raft and non-raft fractions were quantitated using QuantityOne software and graphed as a percentage of total receptors expressed. Each data point represents the mean of three experiments ± standard deviation.

(B) Evaluation of tunicamycin treatment on membrane raft formation. HEK 293T cells were grown in the presence or absence of 2 ug/mL tunicamycin for 20 hours. Cells were then subjected to sucrose-density ultracentrifugation and immunoblotted for early endosome autoantigen 1 (α-EEA1) or caveolin-1 (α-Cav-1) (N=3).

Figure 2.3

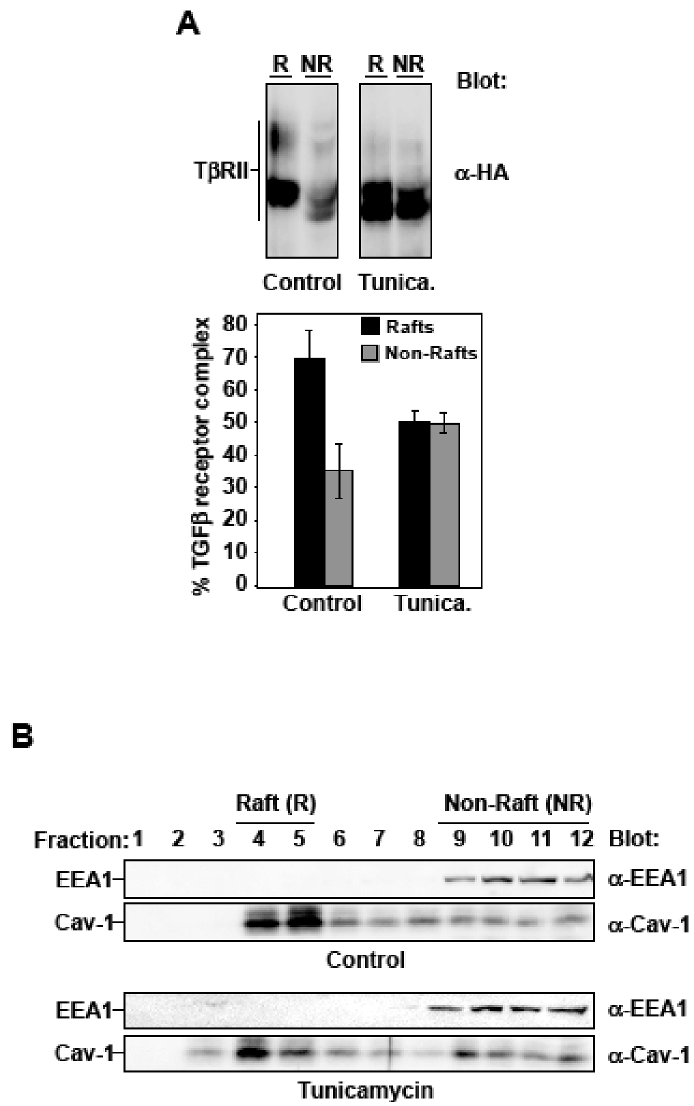
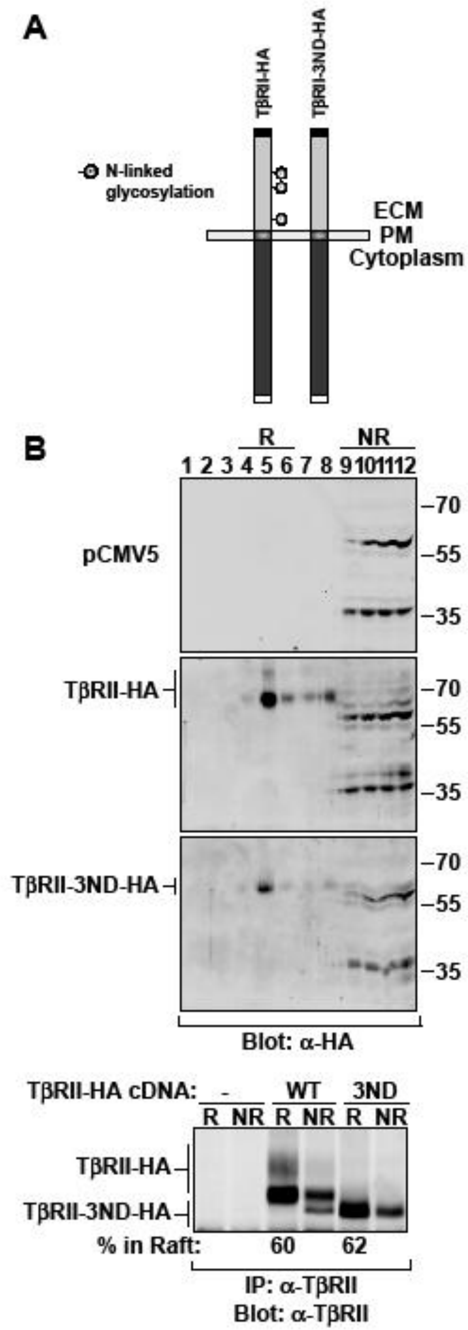


Figure 2.4. The TβRII glycosylation mutant partitions in membrane rafts.

(**A**) Schematic representation of wild type TβRII and the mutant in which the three N-linked glycosylation sites were mutated from asparagine (N) to aspartic acid (D) (TβRII-3ND-HA).

(**B**) Membrane raft partitioning of TβRII-HA and TβRII-3ND-HA. HEK 293T cells transiently transfected with control vector (pCMV5) or cDNA containing the indicated receptors were subjected to subcellular fractionation in order to separate cellular membrane raft and non-raft components. The fractions were immunoblotted with anti-HA antibody (α-HA; top panel). The membrane-raft and non-raft fractions were pooled, immunoprecipitated (IP) with anti-TβRII antibody and immunoblotted with anti-TβRII antibody (α-TβRII; bottom panel). The percentage of receptors in membrane rafts was quantitated using QuantityOne software and is indicated as % in the raft compartment (N=3).

Figure 2.4



raft and non-raft fractions in a similar fashion to the wild type TβRII (Figure 2.4B). Quantitation of pooled raft and non-raft fractions revealed that in both cases approximately 60% of the receptors were found in the raft fractions and 40% in the non-raft fractions (Figure 2.4B, bottom panel). Together, these results demonstrate that the glycosylation state of TβRII *per se* is not a determinant for membrane-raft partitioning, and suggest that partitioning may rely on the proper glycosylation of other cell surface proteins.

2.4.5 *GMCSF-TβRII does not partition with membrane rafts*

Having observed that the glycosylation state of the TβRII was not a factor in raft *vs*. non-raft partitioning, I therefore assessed whether a substitution of the extracellular domain of TβRII with another receptor that also contained 3 N-linked glycosylation sites would affect membrane raft partitioning. To do this I studied a hybrid GMCSF-TβRII receptor construct (28). This hybrid receptor contains the extracellular domain of the granulocyte/macrophage colony stimulating factor (GMCSF) 2B receptor, fused to the transmembrane and intracellular domains of the TβRII. Similar to the wild-type TβRII, it has three N-linked glycosylation sites but was reported to be excluded from caveolin-positive membrane domains structures ((35); Figure 2.5A). In contrast to the wild type TβRII, I found that the wild type GMCSF2BR was predominantly found to partition in non-raft fractions (Figure 2.5B and C). Interestingly, the GMCSF-TβRII chimeric receptor was also found to mostly partition with the non-raft fractions (Figure 2.5B and C). This supports the previous findings that the glycosylation state of the receptor does

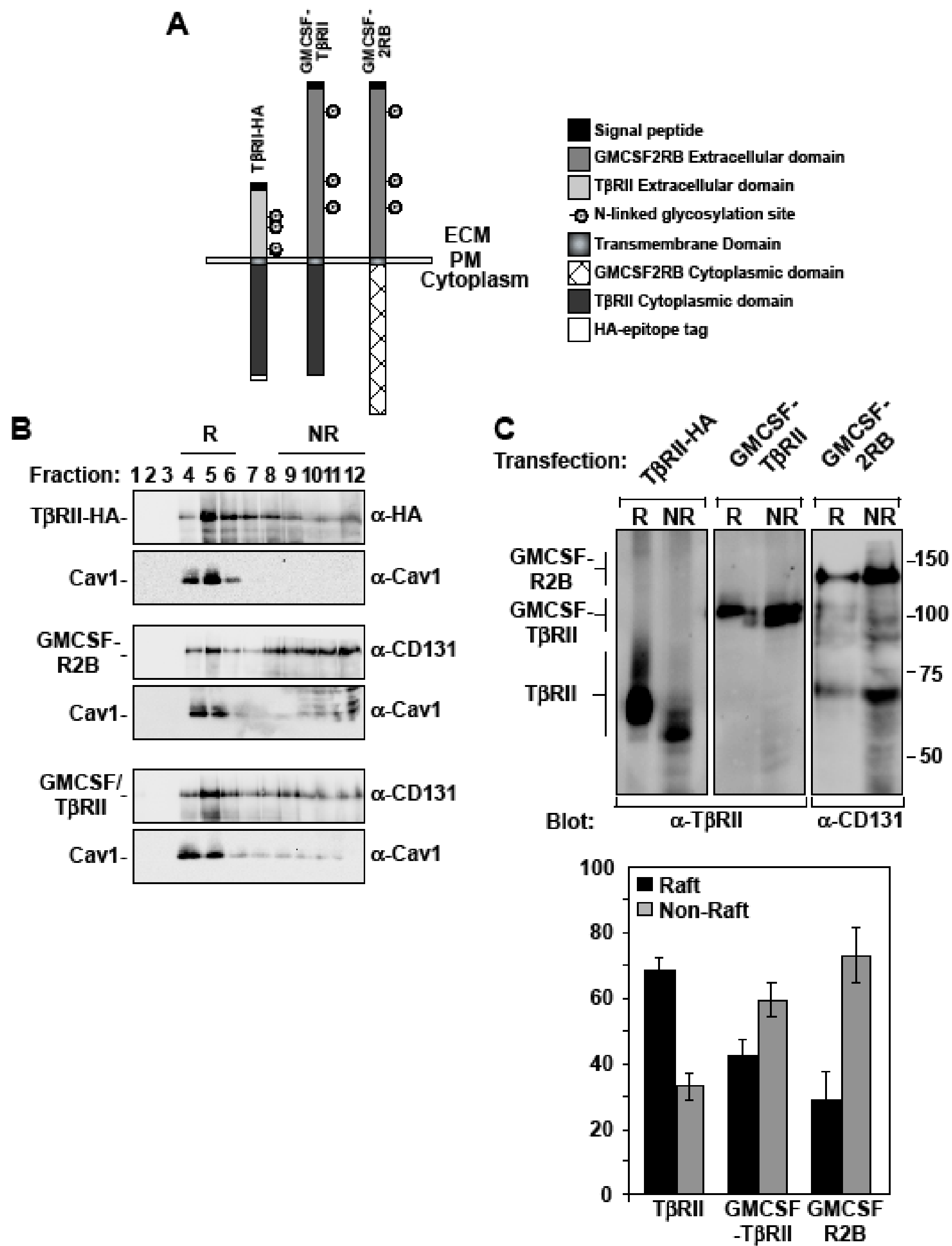
Figure 2.5 GMCSF-TβRII hybrid receptors partition predominantly in non-raft fractions.

(**A**) Schematic comparison of TβRII, the GMCSF-TβRII receptor hybrid, and GMCSF-2RB.

(**B**) HEK 293T cells transiently expressing the receptor constructs as indicated were subjected to subcellular fractionation to separate membrane raft and non-raft components. Fractions were then immunoblotted with anti-HA or anti-CD131 antibody as indicated. All fractions were also subjected to immunoblotting with caveolin-1 antibodies (α-Cav1) in order to indicate membrane rafts (N=3).

(C) Fractions from the experiments carried out as described in (B) were pooled into raft and non-raft fractions and adjusted to the same volume. Pooled membrane raft and non-raft fractions were then immunoblotted with anti-TβRII (α-TβRII) antibody or anti-CD131 (α-CD131) antibody (top panel), quantitated and graphed as a percentage of total receptor expressed (bottom panel). Each data point represents three experiments ± standard deviation.

Figure 2.5



not play a direct role in membrane raft partitioning and that the native extracellular domain of TβRII is essential for raft partitioning.

2.5 Discussion

Different transmembrane proteins have been shown to partition into membrane raft domains via their extracellular, intracellular or transmembrane regions (14-16). For TβRII it was unclear which domain is responsible because this receptor associates with proteins on both the extracellular and intracellular facet of the plasma membrane. On the outer aspect of the plasma membrane, Galectin 3 interacts with TβRII and maintains receptors at the cell surface (25) . The intracellular domain of the receptor associates with clathrin and AP2 (21), and the TβRII/TβRI complex associates with caveolin-1, a resident membrane raft protein (13,23).

To address this problem, the partitioning of TβRII lacking the majority of the extracellular or intracellular domains was assessed. It was found that a mutant TβRII that lacked the intracellular domain almost entirely partitioned with membrane rafts similarly to wild type TβRII, whereas the extracellularly truncated receptor was mostly excluded from rafts. These results indicate that while the extracellular domain of TβRII directs receptors into rafts, the intracellular domain directs them to non-raft domains. This was surprising because it was assumed that the intracellular domain, which contains a caveolin-1 binding motif, would play a larger role in membrane partitioning. If receptor partitioning is not

mediated by caveolin-1 binding, then the association may be important for other functions within the membrane raft compartment after receptor partitioning. Indeed, it was previously found in HEK 293T cells that the association of receptors with Smad7 and Smurf2 occurred in membrane rafts and the degradation of the receptor complex was enhanced when caveolin-1 protein was expressed (11). Furthermore, the chemical perturbation of membrane rafts induced an increase in TGFβ signal transduction. Therefore, the partitioning of receptor complexes may be important for receptor degradation and/or inhibition of signal transduction and this may be dependent on caveolin-1 association with receptors post membrane raft targeting.

The partitioning of receptors into membrane rafts also has important implications in receptor trafficking. The intracellular domain that has the AP2 and clathrin binding sites was shown to be important for clathrin-dependent internalization (20). Internalization from membrane rafts/caveolae leads to the formation of caveolin-1-positive vesicles. The balance between the two internalization pathways would therefore be an important mediator in receptor signaling and degradation.

Previous work showed that TGFβ receptors bound the cell surface N-glycan binding protein Galectin 3 (25). In that study, *Mgat5*$^{-/-}$ cells were found to contain more receptors in the EEA1-early endosomal compartment compared to wild type cells and the receptor half-life was prolonged (25). In this study, it was shown that mutation of all of the N-linked glycosylation sites of the full-length

receptor did not affect entrance into membrane rafts. Therefore, it was concluded that glycosylation of TβRII *per se* does not control trafficking but that the interaction of TβRII with other cell surface glycoproteins or glycoprotein binding proteins, are critical. This mode of raft/caveolae association is similar to that of the EGF receptor where the extracellular domain was found to be critical in the targeting of receptors to membrane rafts (14). In fact, there are several parallels between EGF receptor and TβRII raft partitioning: 1) they both bind cell surface Galectin-3, and 2) their partitioning is dependent on proper cellular glycosylation but not their own glycosylation. Moreover, studies have shown that the ubiquitination and trafficking of EGF receptors are membrane raft-dependent processes (36,37). It would therefore be of interest to assess if both of these receptors associate with common glycoproteins at the cell surface, which could direct them into membrane rafts.

Since the glycosylation state of the receptor was not responsible for the partitioning of receptors into membrane rafts and the scanning deletion mutants did not provide insight into the extracellular domain that regulate partitioning of receptors, I assessed if replacing the extracellular domain would influence raft partitioning. I therefore turned my attention to a chimeric receptor, GMCSF-TβRII, which has three N-linked glycosylation sites, as does wild-type TβRII, and upon endocytosis was shown to co-localize exclusively with clathrin-positive, but not caveolin-positive, structures (35). Based on previous data, I therefore considered that absence of the native extracellular domain of TβRII would interfere with normal raft partitioning and entrance into the caveolin-1

compartment. Indeed, I found that partitioning of the GMCSF-TβRII hybrid into rafts was substantially reduced compared to the wild type TβRII. Since the partitioning was not completely abolished, a question remains as to why there are still a proportion of the hybrid receptors observed in the raft fractions? Previously, it was shown that TβRI directly interacts with caveolin-1 via a consensus motif in the cytoplasmic tail of the receptor (23). Interestingly, TβRII contains putative caveolin-1 binding motifs, which may play a partial role in the partitioning of receptors into membrane rafts. It was estimated that this partitioning is approximately 20% based on the observations using the TβRII-ΔEX-HA construct (Figure 2.2). The wild type GMCSF receptors also partially partition into membrane rafts (Figure 2.5). Therefore the combination of a caveolin-1-binding motif and the small propensity of GMCSF receptors to partition into membrane rafts may account for the 40% of GMCSF-TβRII hybrid receptor membrane raft partitioning.

Receptors that are endocytosed via clathrin-mediated endocytosis enter the early endosome, access Smad 2/3, and propagate TGFβ signal transduction; however, receptors that are localized in membrane rafts are targeted for degradation by ubiquitination (1). While the downstream signal transduction of the TGFβ signalling pathway has been well characterized, the signal(s) directing receptors to either method of endocytosis is still unclear. Here, it is shown that the extracellular domain of the type II receptor is necessary for entrance into caveolin-1 positive vesicles. It is also shown that the glycosylation status of the

cell, but not of TβRII itself, affects the membrane partitioning of TβRII. This suggests that there may be interacting glycosylated protein(s) acting at the cell surface that direct the partitioning of the type II receptor.

Several articles have attempted to identify signals affecting TβRII partitioning at the cell surface. A study by Chen and colleagues illustrated that cells lacking heparin sulphate synthesis have significantly less TβRII found in membrane rafts (38). The authors postulated that since TβRIII has many proteoglycan attachments, it may be the signal that dictates TβRII internalization. TβRIII is the least well characterized of the TGFβ receptors, and its role in TGFβ signaling is only beginning to be understood. Several reports show that loss of TβRIII can potentiate TGFβ-dependent epithelial-to-mesenchymal transition. Gordon and colleagues have shown that TGFβ-dependent EMT in pancreatic cancer cells results in a loss of TβRIII expression (39). TβRIII loss also occurs in prostate cancer cells and non-small cell lung adenocarcinoma cells, with increased loss of TβRIII correlating with a more aggressive cancer phenotype (40, (40). However, a study by Criswell and colleagues showed that loss of TβRIII in breast cancer cells decreased TGFβ dependent invasion, migration, and signal transduction (41). Recently, an article evaluated the endocytosis of TβRIII and its membrane localization. The authors showed that while the receptor can internalize via both clathrin-dependent and independent mechanisms, inhibiting membrane raft internalization decreased TGFβ signal transduction (42).

All of these studies illustrate the fact that the role of TβRIII in TβRII endocytosis and signaling will be a fascinating area of study.

Another interesting line of investigation will be to assess the contribution of TGFβ type I receptors in the partitioning of receptor complexes. Huang and colleagues have observed that membrane raft and non-raft fractions contain altered ratios of the type I receptor to the type II receptor (38, 44, 45) . They further found that this ratio plays a role in clathrin *vs.* caveolar endocytosis of receptors and influences their signaling potential. Therefore, future studies identifying how the different TGFβ receptors, as well as other cell surface receptor-interacting proteins, regulate TGFβ signaling and trafficking will be of great interest.

2.6 References

1. Le Roy, C., and Wrana, J. L. (2005) *Nat Rev Mol Cell Biol* **6**(2), 112-126

2. Pike, L. J. (2006) *Journal of lipid research* **47**(7), 1597-1598

3. Parton, R. G., and Richards, A. A. (2003) *Traffic* **4**(11), 724-738

4. Patel, H. H., Murray, F., and Insel, P. A. (2008) *Annual review of pharmacology and toxicology* **48**, 359-391

5. Byfield, S. D., and Roberts, A. B. (2004) *Trends Cell Biol* **14**(3), 107-111

6. Attisano, L., and Wrana, J. L. (2002) *Science* **296**(5573), 1646-1647

7. Miura, S., Takeshita, T., Asao, H., Kimura, Y., Murata, K., Sasaki, Y., Hanai, J. I., Beppu, H., Tsukazaki, T., Wrana, J. L., Miyazono, K., and Sugamura, K. (2000) *Molecular and cellular biology* **20**(24), 9346-9355

8. Lin, H. K., Bergmann, S., and Pandolfi, P. P. (2004) *Nature* **431**(7005), 205-211

9. Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001) *The Journal of biological chemistry* **276**(16), 12477-12480

10. Kavsak, P., Rasmussen, R. K., Causing, C. G., Bonni, S., Zhu, H., Thomsen, G. H., and Wrana, J. L. (2000) *Mol Cell* **6**(6), 1365-1375

11. Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F., and Wrana, J. L. (2003) *Nat Cell Biol* **5**(5), 410-421

12. Ito, T., Williams, J. D., Fraser, D. J., and Phillips, A. O. (2004) *The Journal of biological chemistry* **279**(24), 25326-25332

13. Zhang, X. L., Topley, N., Ito, T., and Phillips, A. (2005) *The Journal of biological chemistry* **280**(13), 12239-12245

14. Yamabhai, M., and Anderson, R. G. (2002) *The Journal of biological chemistry* **277**(28), 24843-24846

15. Zhang, C. F., Dhanvantari, S., Lou, H., and Loh, Y. P. (2003) *Biochem J* **369**(Pt 3), 453-460

16. Crossthwaite, A. J., Seebacher, T., Masada, N., Ciruela, A., Dufraux, K., Schultz, J. E., and Cooper, D. M. (2005) *The Journal of biological chemistry* **280**(8), 6380-6391

17. Nohe, A., Keating, E., Underhill, T. M., Knaus, P., and Petersen, N. O. (2005) *Journal of cell science* **118**(Pt 3), 643-650

18. Hartung, A., Bitton-Worms, K., Rechtman, M. M., Wenzel, V., Boergermann, J. H., Hassel, S., Henis, Y. I., and Knaus, P. (2006) *Molecular and cellular biology* **26**(20), 7791-7805

19. Schwartz, E. A., Reaven, E., Topper, J. N., and Tsao, P. S. (2005) *Biochem J*

20. Ehrlich, M., Shmuely, A., and Henis, Y. I. (2001) *Journal of cell science* **114**(Pt 9), 1777-1786

21. Yao, D., Ehrlich, M., Henis, Y. I., and Leof, E. B. (2002) *Mol Biol Cell* **13**(11), 4001-4012

22. Couet, J., Li, S., Okamoto, T., Ikezu, T., and Lisanti, M. P. (1997) *The Journal of biological chemistry* **272**(10), 6525-6533

23. Razani, B., Zhang, X. L., Bitzer, M., von Gersdorff, G., Bottinger, E. P., and Lisanti, M. P. (2001) *The Journal of biological chemistry* **276**(9), 6727-6738

24. Liu, F. T., and Rabinovich, G. A. (2005) *Nat Rev Cancer* **5**(1), 29-41

25. Partridge, E. A., Le Roy, C., Di Guglielmo, G. M., Pawling, J., Cheung, P., Granovsky, M., Nabi, I. R., Wrana, J. L., and Dennis, J. W. (2004) *Science* **306**(5693), 120-124

26. Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F., and Massague, J. (1992) *Cell* **71**(6), 1003-1014

27. Wieser, R., Attisano, L., Wrana, J. L., and Massague, J. (1993) *Molecular and cellular biology* **13**(12), 7239-7247

28. Anders, R. A., and Leof, E. B. (1996) *The Journal of biological chemistry* **271**(36), 21758-21766

29. Zaks-Zilberman, M., Harrington, A. E., Ishino, T., and Chaiken, I. M. (2008) *The Journal of biological chemistry* **283**(19), 13398-13406

30. Feng, X. H., and Derynck, R. (1996) *The Journal of biological chemistry* **271**(22), 13123-13129

31. Kurzchalia, T. V., Dupree, P., and Monier, S. (1994) *FEBS Lett* **346**(1), 88-91

32. Patki, V., Virbasius, J., Lane, W. S., Toh, B. H., Shpetner, H. S., and Corvera, S. (1997) *Proc Natl Acad Sci U S A* **94**(14), 7326-7330

33. Stenmark, H., Aasland, R., Toh, B. H., and D'Arrigo, A. (1996) *The Journal of biological chemistry* **271**(39), 24048-24054

34. Lehle, L., and Tanner, W. (1976) *FEBS Lett* **72**(1), 167-170

35. Mitchell, H., Choudhury, A., Pagano, R. E., and Leof, E. B. (2004) *Mol Biol Cell* **15**(9), 4166-4178

36. Sigismund, S., Woelk, T., Puri, C., Maspero, E., Tacchetti, C., Transidico, P., Di Fiore, P. P., and Polo, S. (2005) *Proc Natl Acad Sci U S A* **102**(8), 2760-2765

37. Puri, C., Tosoni, D., Comai, R., Rabellino, A., Segat, D., Caneva, F., Luzzi, P., Di Fiore, P. P., and Tacchetti, C. (2005) *Mol Biol Cell* **16**(6), 2704-2718

38. Chen, C. L., Huang, S. S., and Huang, J. S. (2006) *The Journal of biological chemistry* **281**(17), 11506-11514

39. Gordon, K. J., Dong, M., Chislock, E. M., Fields, T. A., and Blobe, G. C. (2008) *Carcinogenesis* **29**(2), 252-262

40. Sharifi, N., Hurt, E. M., Kawasaki, B. T., and Farrar, W. L. (2007) *The Prostate* **67**(3), 301-311

41. Criswell, T. L., Dumont, N., Barnett, J. V., and Arteaga, C. L. (2008) *Cancer research* **68**(18), 7304-7312

42. Finger, E. C., Lee, N. Y., You, H. J., and Blobe, G. C. (2008) *The Journal of biological chemistry* **283**(50), 34808-34818

CHAPTER 3

TGFβ RECEPTOR TYPE III DIRECTS CLATHRIN-MEDIATED ENDOCYTOSIS OF TGF BETA RECEPTOR TYPES I AND II

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3 Chapter 3

3.1 Chapter Summary

Chapter 2 of my thesis illustrated that the extracellular domain of TβRII and the glycosylation status of the cell as a whole was important in directing membrane-raft partitioning of TβRII. This suggested that a glycosylated protein which could interact with TβRII at the cell surface may direct TβRII partitioning. In this chapter, I assessed a candidate protein, TβRIII, for its ability to direct internalization and trafficking of TβRII/TβRI complexes. Overall, I found that TβRIII increased non-membrane raft partitioning of TβRII/TβRI complexes and increased their trafficking to early endosomal compartments. Furthermore, the interaction of TβRIII with the receptor complex could occur both in the presence and absence of ligand. Finally, the interaction of TβRIII with TβRII/TβRI complexes increased the complex half-life as well as basal TGFβ signalling.

3.2 Introduction

The Transforming Growth Factor Beta (TGFβ) signalling pathway is necessary for the normal functioning of a variety of cells; its cell-type specific responses often mediate growth inhibition, extracellular matrix synthesis and cell migration. While this signalling pathway is crucial for normal development, it also plays a much more sinister role in a number of pathologies, including cancer (1). Somatic mutations of the TGFβ receptors, along with activation of potent growth-promoting oncogenes can override the tumour-suppressive effects of TGFβ (reviewed in (2)).

There are three principle receptor subtypes in the classical TGFβ pathway. TGFβ receptor I (TβRI) and TGFβ receptor II (TβRII) are structurally related glycoproteins with cytoplasmic serine/threonine kinase domains (reviewed in (3)). A third receptor, TGFβ receptor III (TβRIII) or betaglycan, is a large proteoglycan that is able to regulate TGFβ signal transduction by binding and presenting active TGFβ ligand to TβRII (1).

In the canonical TGFβ signalling pathway, the binding of ligand to TβRII causes TβRII to phosphorylate the TβRI receptor at serine-threonine residues in its GS domain (1). Activated TβRI then phosphorylates and activates Smad transcription factors. With the aid of specific nuclear localization signals, the Smad complex translocates to induce TGFβ-dependent transcriptional programmes (4). The signal transduction pathway of activated TGFβ receptors has been well characterized. However, the role of receptor interactions at the

plasma membrane and their effect on endocytosis and trafficking has yet to be fully evaluated.

Endocytosis of nutrients, growth factors and receptors is necessary for the normal functioning of a cell. When cell-surface receptors are activated, they are able to interact with cytosolic adaptor proteins that can promote polymerization of clathrin (reviewed in (5)). Clathrin from the cytosol is recruited to the plasma membrane and aggregates to form pits (6). These pits can then form vesicles for the transportation of nutrients and signals from the extracellular environment to the cell interior (7).

Clathrin-independent endocytosis through membrane rafts is also a common mechanism for the uptake of signals and nutrients from the extracellular environment. Membrane rafts are microdomains in the plasma membrane that are enriched in cholesterol and sphingolipids (8). Membrane rafts have been implicated in the endocytosis of a variety of receptors, including the group I metabotropic glutamate receptors (9), insulin-like growth factor receptor (10), and epidermal growth factor (reviewed in (11)). Similarly, membrane raft mediated endocytosis plays a crucial role in TGFβ signalling, specifically in regards to receptor turnover and degradation. TGFβ receptors endocytosed by clathrin-dependent endocytosis increase TGFβ signal transduction, while membrane raft endocytosis of receptors promotes receptor degradation (12). It has been shown that the extracellular domain of TβRII is necessary for entrance into membrane-raft domains (13). Furthermore the glycosylation state of the cell, though not of

TβRII itself, mediates TβRII endocytosis (13). This suggests that TβRII may interact with glycosylated protein(s) at the cell surface to direct receptor partitioning.

While many studies have evaluated the contribution of the type II and type I TGFβ receptors to Smad signalling, few have investigated the role of TβRIII. The type III TGFβ receptor is a highly glycosylated proteoglycan with a large extracellular domain (14, 15). Previously, TβRIII was thought to simply function in presentation of ligand to the type II receptor (16); however, several recent studies illustrate that TβRIII may play a crucial role in TGFβ-dependent cancer metastasis. Expression levels of TβRIII have been correlated with a number of cancers, including prostate cancer (17), ovarian cancer (18), granulosa tumours (19), and non-small cell lung adenocarcinomas (20). In some instances, TβRIII overexpression appears to contribute to cancer cell motility and invasion (21), while in other cases knockdown of TβRIII increases tumour cell metastasis (22). Furthermore, TβRIII endocytosis has been implicated in the activation of TGFβ signalling (23). These studies highlight the fact that TβRIII may play a crucial role in TGFβ signalling, particularly in cancer.

The objective of the present study is to evaluate the role of the type III TGFβ receptor in TGFβ receptor endocytosis and degradation. Using immunofluorescence microscopy and sucrose-density ultracentrifugation, I show that TβRIII directs TβRII and TβRI to undergo clathrin-mediated endocytosis. TβRIII also increases trafficking of TβRII into early endosomal compartments.

Furthermore, this re-directed trafficking increases TβRII/TβRI complex half-life and basal TGFβ signalling.

3.3 Materials and Methods

3.3.1 *Cell culture*

HEK (human embryonic kidney)-293T cells were maintained in DMEM (Dulbecco's modified Eagle's medium) (Gibco) supplemented with 10% fetal bovine serum. Mink Lung cells stably transfected with HA-tagged TβRII (Mink Lung HAT cells) were cultured in MEM supplemented with non-essential amino acids, 10% fetal bovine serum and 0.3% hygromycin. HepG2 cells were maintained in MEM supplemented with non-essential amino acids and 10% fetal bovine serum. All cells were maintained at 37°C in 5% CO_2.

3.3.2 *Constructs*

Constructs encoding the carboxy terminus hemagluttinin (HA) epitope tagged type II TGFβ receptor (TβRII-HA), the intracellularly truncated receptor (TβRII-Δcyt) and an extracellularly truncated receptor (TβRII-ΔEX) were previously described (13, 24). The GFP-tagged Rab5 wild-type (WT), constitutively active (Q79L) and dominant negative (S34N) constructs were used as previously described (25).

3.3.3 *Transfection*

HEK293T cells were transiently transfected using the calcium phosphate precipitation method as previously. Cells were plated at 50% confluency in 100mm dishes. The following day cells were transfected with 5 μg TβRII-HA, 5

μg of TβRI-flag, and 5 μg of myc-TβRIII. Mink lung HAT cells were transiently transfected using the polyethyleneimine (PEI) method.

3.3.4 *Isolation of caveolae/membrane-raft-enriched membrane fractions*

Membrane rafts were isolated as previously described (12, 13) . Briefly, transfected HEK293T cells were grown to confluence in 100-mm-diameter dishes. Cells were washed twice with cold 1X PBS and lysed in 0.5M Na_2CO_3, pH 11.0 containing protease inhibitors. After the cells were scraped, the cell lysate was homogenized in three 10 second bursts using a Polytron tissue homogenizer (Brinkmann Instruments). Cells were then sonicated three times for 20 seconds each with a Vibra Cell sonicator (Sonics and Materials). The homogenates were then adjusted to 40% sucrose, and overlaid with 30% sucrose and 5% sucrose solutions. The samples were centrifuged for 16h at 200,000 g_{av} at 4°C using a Beckman SW41 rotor. Following centrifugation, 12x1mL samples were collected and an aliquot of each sample was denatured with Laemmli sample prep buffer, boiled and subjected to SDS-PAGE followed by immunoblotting.

3.3.5 *Immunoblotting*

Proteins were separated by SDS-PAGE (10% gels) and transferred to nitrocellulose by electrophoretic transfer. Blots were incubated for 1 hr in 5% skim milk/TBST. After incubation with primary and secondary antibodies, bound antibodies were detected using SuperSignal chemiluminescence reagent (Pierce) and a VersaDoc imager (Biorad).

3.3.6 *Immunoprecipitation*

HEK293T cells transiently transfected with cDNA were lysed in TNTE (50mM Tris/HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 0.5% Triton X-100, 1mM PMSF and protease inhibitors) and centrifuged at 14000 g_{av} for 10 min. at 4°C. A protein assay was conducted on total cell lysates for analysis of protein concentration. The remaining cell lysates were then incubated with 1μg α-HA primary antibody for 16 hrs at 4°C followed by incubation with Protein G sepharose beads for 2 hrs at 4°C. The precipitates were washed three times, eluted with Laemmli sample prep buffer and subjected to SDS-PAGE (10% gels) and immunoblotting.

3.3.7 *Immunofluorescence/Receptor Internalization*

HAT Mv1Lu cells were plated on coverslips in 12 well dishes. Twenty-four hours post-transfection of myc-TβRIII (0.3 μg/well), Rab5-GFP (0.5 μg/well), Rab5-S34N-GFP (0.5 μg/well), Rab5-Q79L-GFP (0.5 μg/well) or Cav-1 GFP (0.5 μg/well) cDNA with polyethylenimine, cells were serum-starved and treated with 50 μM $ZnCl_2$ to induce HA-TβRII expression. The following day, cells were cooled to 4°C, and treated with α-HA antibody for 2 hours at 4°C to label receptors at the cell surface. Coverslips were then incubated with donkey anti-rabbit Cy3 antibodies. After labelling, cells were either permitted to internalize, by incubating at 37°C for 30 minutes or 1 hour, or were immediately fixed and permeabilized. Cells were incubated with α-EEA1 antibody, followed with donkey

α-mouse Cy5. All coverslips were then immunomounted and visualized using an IX81 inverted immunofluorescence microscope (Olympus, Canada).

3.3.8 *Affinity Labelling*

Transiently transfected HEK293T cells were labelled for 2 hrs with 250 pM [^{125}I] TGFβ1 ligand in 0.5% bovine serum albumin (BSA) in Krebs Ringers Hepes (KRH) at 4°C. Cells were cross-linked to ligand using disuccinimidyl suberate (DSS) as described previously (12). Cells were then either immediately lysed in 1XTNTE or incubated in media/10% FBS at 37°C for 2, 4 or 8 hrs prior to lysis. Samples were eluted with Laemmli sample prep buffer, and separated using SDS-PAGE (7.5% gels). Receptors were visualized using phosphorimaging (Molecular Dynamics).

3.3.9 *Luciferase Reporter Assay*

HepG2 cells were transiently transfected using the calcium phosphate precipitation method with an Activin Response element upstream of a luciferase construct (ARE-Lux), β-galactosidase, and FoxH1 alone (control) or with TβRI, TβRII and/or increasing concentrations of TβRIII. FoxH1 is a transcriptional co-activator necessary to induce maximal Smad-dependent transcription. Cells were serum-starved in 0.2% FBS/MEM for 4 hrs prior to treatment. Cells were incubated in the presence or absence of 100 pMol TGFβ1 for 16 hrs. Luciferase activity was normalized to β-galactosidase activity prior to analysis.

3.4 Results

3.4.1 *TβRIII is concentrated in non- raft membrane fractions*

Previous work has shown that the extracellular domain of the type II TGFβ receptor is necessary for membrane raft partitioning (13). Deletion of the extracellular domain decreases the endocytosis of TβRII receptors via membrane raft-dependent mechanisms (13). As both the type I and type III TGFβ receptors interact with the type II receptor, I first sought to identify the membrane localization of the three TGFβ receptor subtypes.

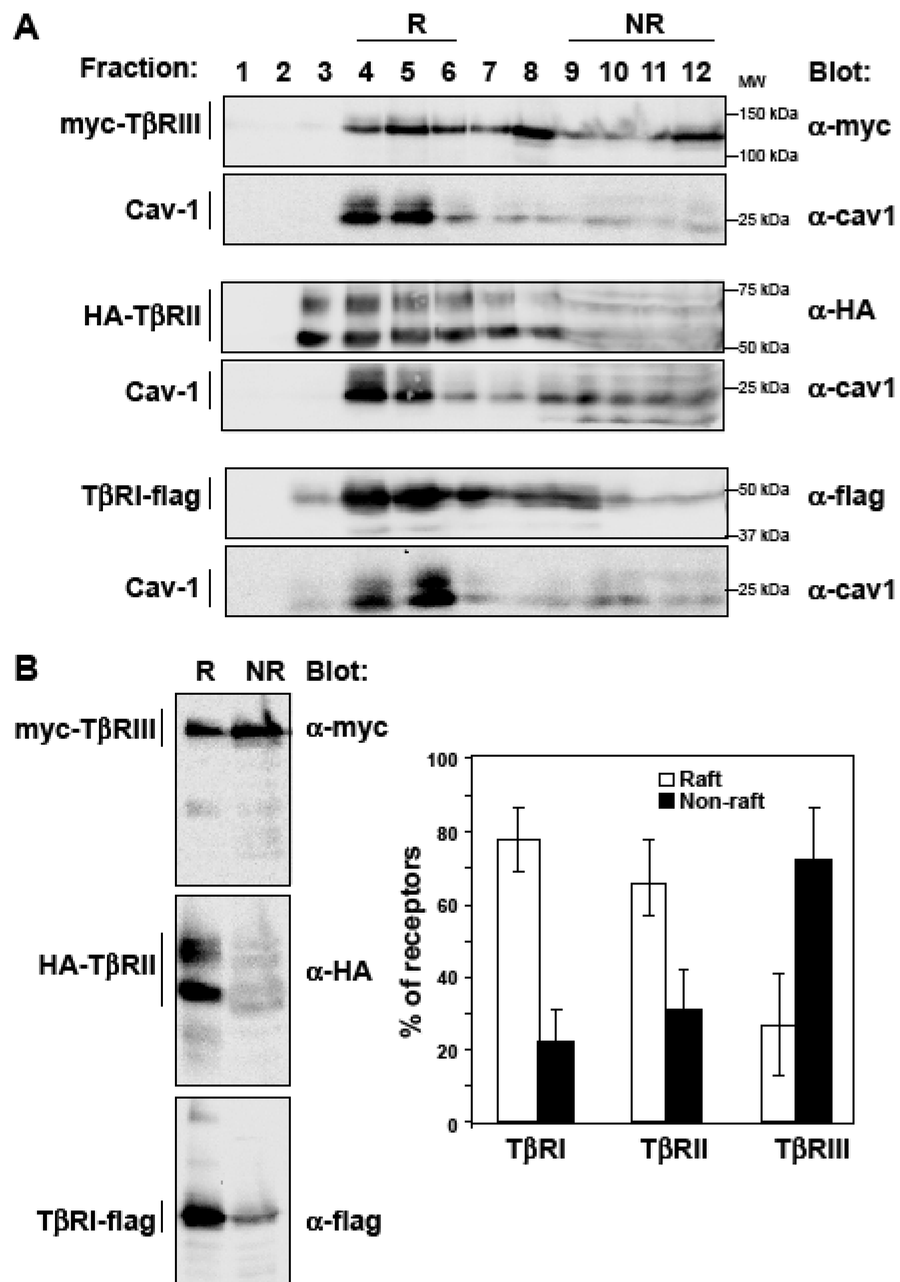
To evaluate the membrane localization of the TGFβ receptor subtypes, membrane raft fractions were isolated using sucrose-density ultracentrifugation as previously described (12,13). Briefly, HEK293T cells were transiently transfected with myc-TβRIII, TβRII-HA or TβRI-flag cDNA. Cells were lysed in 1 M Na_2CO_3 with protease inhibitors, homogenized and sonicated, then overlaid with a sucrose gradient. Following overnight high-speed ultracentrifugation, fractions were collected and subjected to SDS-PAGE. Immunoblotting for endogenous caveolin-1, a marker of membrane rafts, was performed to ensure membrane raft isolation. As shown in Figure 3.1A, membrane rafts were concentrated in fractions 4-6. Membrane raft and non-raft fractions were pooled, adjusted for the same volume, and subjected to SDS-PAGE as shown in Figure 3.1B. Interestingly, I observed that TβRII and TβRI largely partition in membrane raft fractions, with approximately 70% of type II receptors and 75% of type

Figure 3.1 Membrane partitioning of TGFβ receptors.

(A) HEK293T cells transiently expressing myc-TβRIII, HA-TβRII or TβRI-flag were subjected to sucrose-density ultracentrifugation as described in experimental procedures. Fractions 1-12 were then collected and immunoblotted using α-myc, α-HA, and α-flag antibodies as indicated. Fractions were also immunoblotted for endogenous caveolin-1, a marker for membrane rafts (N=3).

(B) Quantification of TGFβ receptor membrane partitioning. Fractions 4-6 (membrane raft) and 8-12 (non-raft) from each condition were pooled, adjusted to the same volume, and subjected to SDS-PAGE. Following immunoblotting with α-myc, α-HA or α-flag antibodies (left panel), receptors levels were quantified using QuantityOne software and graphed (right panel; N=3 ± SD).

Figure 3.1



receptors found in membrane raft fractions (Figure 3.1B). However, TβRIII is found more heavily concentrated in non-raft fractions with only 30% of receptors found in membrane raft fractions, and approximately 70% of receptors found in non-raft fractions (Figure 3.1B).

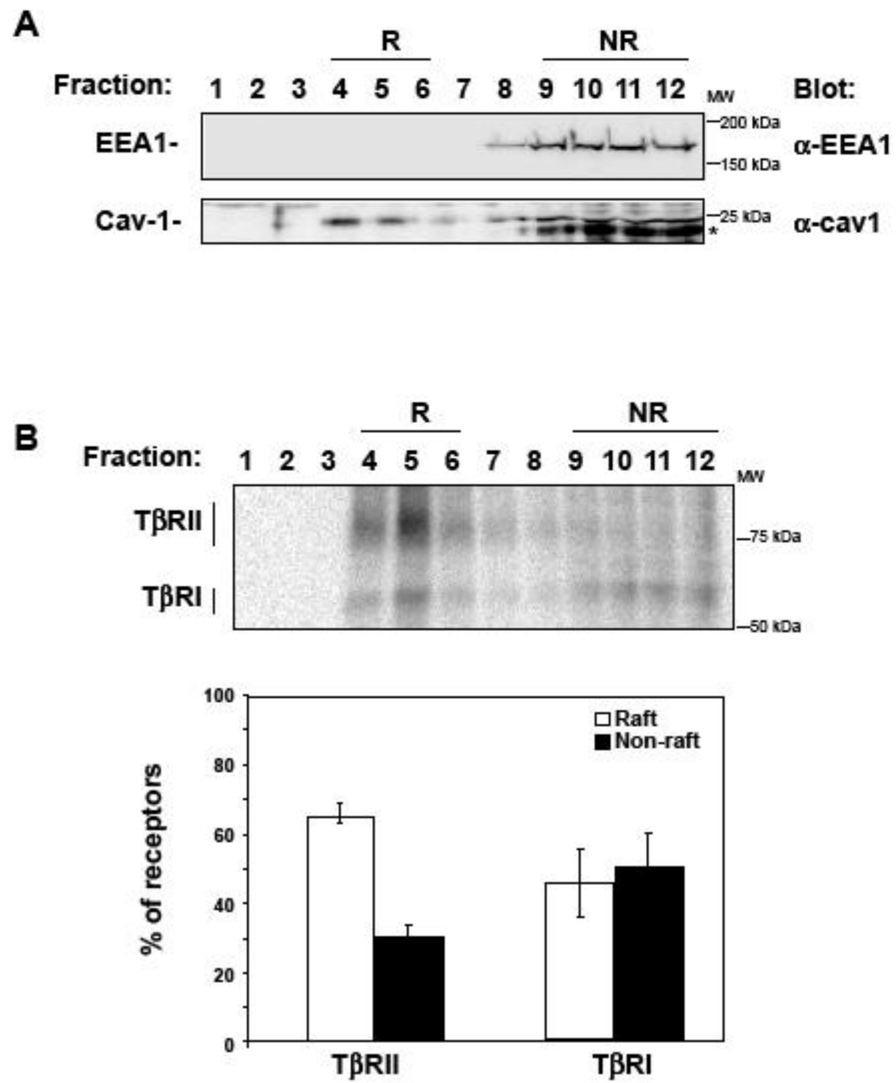
I also evaluated the partitioning of endogenous TβRII and TβRI in HepG2 cells by sucrose-density ultracentrifugation (Figure 3.2). I first assessed the membrane raft content of HepG2 cells by immunoblotting collected fractions for caveolin-1 (Figure 3.2A). To visualize endogenous receptors, I used [^{125}I] TGFβ1 ligand to label cell-surface receptors, subjected lysates to sucrose-density ultracentrifugation and performed autoradiography. Importantly, the radioactive ligand is cross-linked to the receptors, and and it has been shown that TGFβ ligand does not dissociate from the receptor complex at low pH (pH 2) (12). Similar to what has been previously shown for Mv1Lu cells (12), endogenous TβRII is found primarily concentrated in membrane raft fractions in HepG2 cells (Figure 3.2B), similar to my over-expression studies. TβRI is found in both raft and non-raft fractions, with slightly more receptors found in non-raft fractions (Figure 3.2B). While I show more TβRI in membrane raft fractions in our over-expression studies, (Figure 3.2B), this difference may be due to the relative levels of TβRI and TβRII in HepG2 cells.

Figure 3.2 Partitioning of endogenous TβRI and TβRII.

(A) HepG2 cells were subjected to sucrose density subcellular fractionation as previously described. Fractions were immunoblotted with a marker for the early endosome (α-EEA1) or a marker for membrane rafts (α-cav1) to ensure isolation of membrane raft fractions. A non-specific band is seen underneath the caveolin-1 protein band and is indicated (*) (N=3).

(B) HepG2 cells were affinity-labelled with $^{[125]}$I-TGFβ prior to sucrose density subcellular fractionation, as previously described. Following subcellular fractionation, lysates were subjected to SDS-PAGE. Receptor partitioning was visualized and quantified by phosphorimaging (Molecular dynamics). TβRI and TβRII partitioning into raft and non-raft fractions was quantified using QuantityOne software (N=3 ± SD).

Figure 3.2



3.4.2 *TβRIII forms a stable interaction with TβRII in the presence and absence of ligand and affects its membrane partitioning*

Having found that TβRIII was more concentrated in non-raft fractions, I sought to evaluate whether TβRIII could alter the partitioning of TβRII. I initially wanted to assess the ability of TβRIII to form a stable interaction with TβRII, as previous studies had simply suggested that the role of TβRIII was to present TGFβ ligand to the receptor. To address this question, I used a co-immunoprecipitation approach in HEK 293T cells transiently transfected with TβRII-HA, myc-TβRIII and TβRI-flag cDNA (Figure 3.3A). Following transfection, cells were serum-starved and then either treated with 500 pMol TGFβ for 1 hr or left untreated. Immunoprecipitation of cell lysates with α-HA pAb and subsequent immunoblotting with α-myc mAb revealed that TβRIII is able to form a stable interaction with TβRII in both the presence and absence of TGFβ (Figure 3.3A). The interaction of TβRIII with TβRII in the absence of ligand suggests that TβRIII may play a greater role in TGFβ signal transduction than simply ligand presentation.

Since TβRIII is found robustly in non-membrane raft fractions and can stably associate with TβRII, I speculated that the interaction of TβRIII with TβRII might increase the partitioning of TβRII in non-membrane raft fractions.

To evaluate the ability of TβRIII to differentially partition TβRII, HEK 293T cells transiently transfected with TβRII-HA and myc-TβRIII cDNA were subjected to sucrose-density ultracentrifugation as previously described. Figures 3.3B and

Figure 3.3. TβRIII stably interacts with TβRII and affects its membrane partitioning.

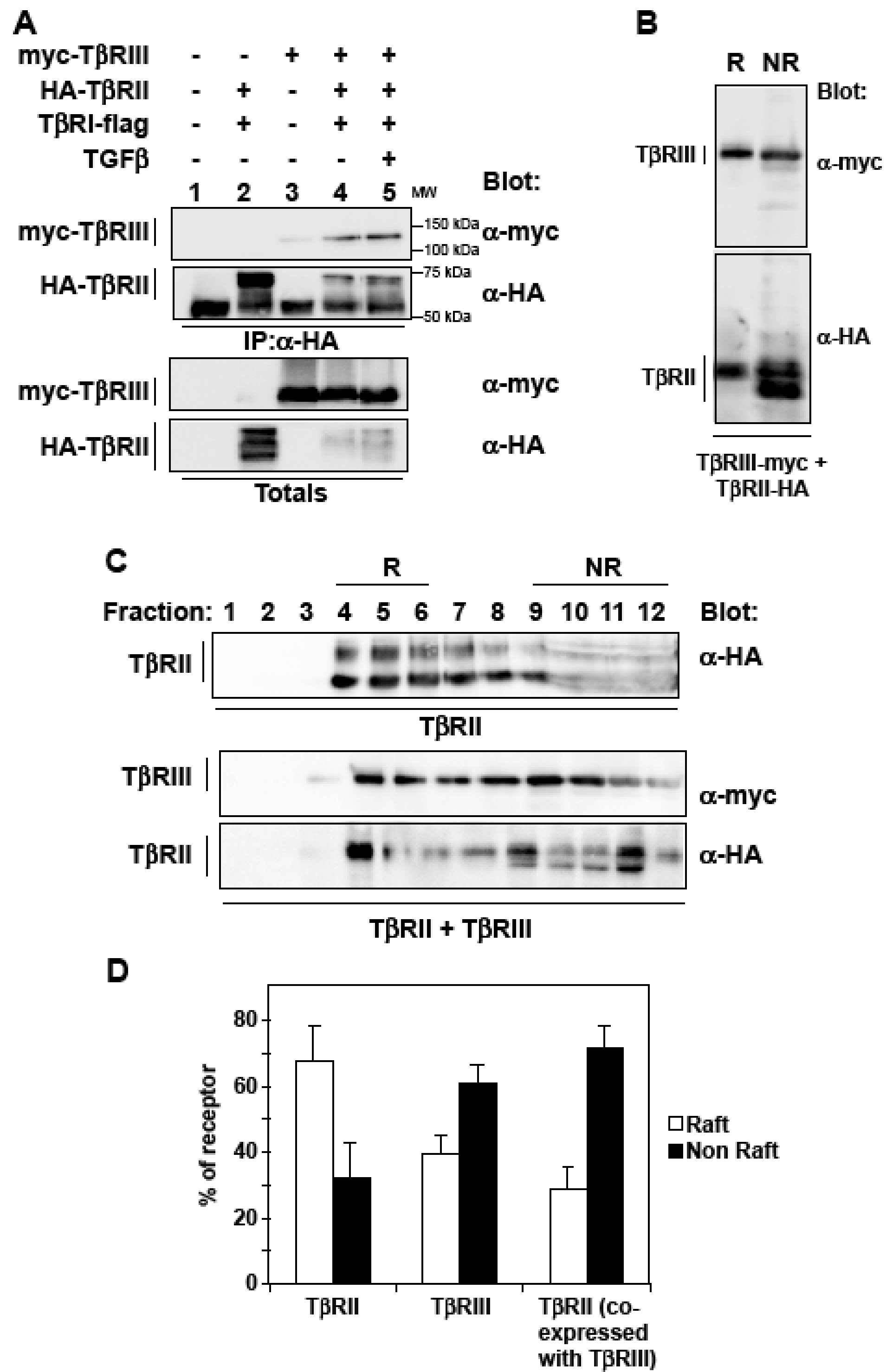
(A) HEK293T cells were transiently transfected with myc-TβRIII, TβRII-HA and/or TβRI-flag as indicated in the panel. Cells were then serum-starved and incubated in low-serum media in the presence or absence of 500 pMol TGFβ1 for 1 hr. Cell lysates were immunoprecipitated with α-HA antibody and subjected to SDS-PAGE and immunoblotting as indicated. The non-specific heavy chain of the immunoprecipitating antibody is indicated as IgG. (N=3)

(B) Fractions 4-6 (membrane raft; R) and 8-12 (non-raft; NR) were pooled, adjusted to the same volume, and subjected to SDS-PAGE and immunoblotting. (N=3)

(C) Membrane partitioning of TβRII in the presence and absence of TβRIII. HEK293T cells were transiently transfected with TβRII-HA or TβRII-HA + myc-TβRIII. Cells were then subjected to sucrose-density ultracentrifugation. Twelve 1mL fractions were collected and subjected to SDS-PAGE and immunoblotted as indicated. (N=3)

(D) Quantitation of membrane raft partitioning was then performed on the pooled fractions using QuantityOne software and graphed (N=3 ± SD).

Figure 3.3



3.3C illustrate that the interaction of TβRII with TβRIII increases the proportion of TβRII found in non-membrane raft fractions. Quantitation of membrane raft partitioning was performed by pooling raft (R) and non-raft (NR) fractions and performing SDS-PAGE (Figure 3.3B). Quantitative analysis of this differential partitioning was performed using Quantity One software. As shown in the graph in Figure 3.3D, upon co-expression with TβRIII, 72% of TGFβ type II receptors are found in non-membrane raft fractions, as opposed to the amount of TβRII found in non-membrane raft fractions in the absence of TβRIII (33%). This suggests that the association of TβRIII with TβRII increases the endocytosis of TβRII by clathrin-mediated mechanisms. As clathrin-dependent endocytosis promotes TGFβ signalling, TβRIII expression may increase downstream signalling events.

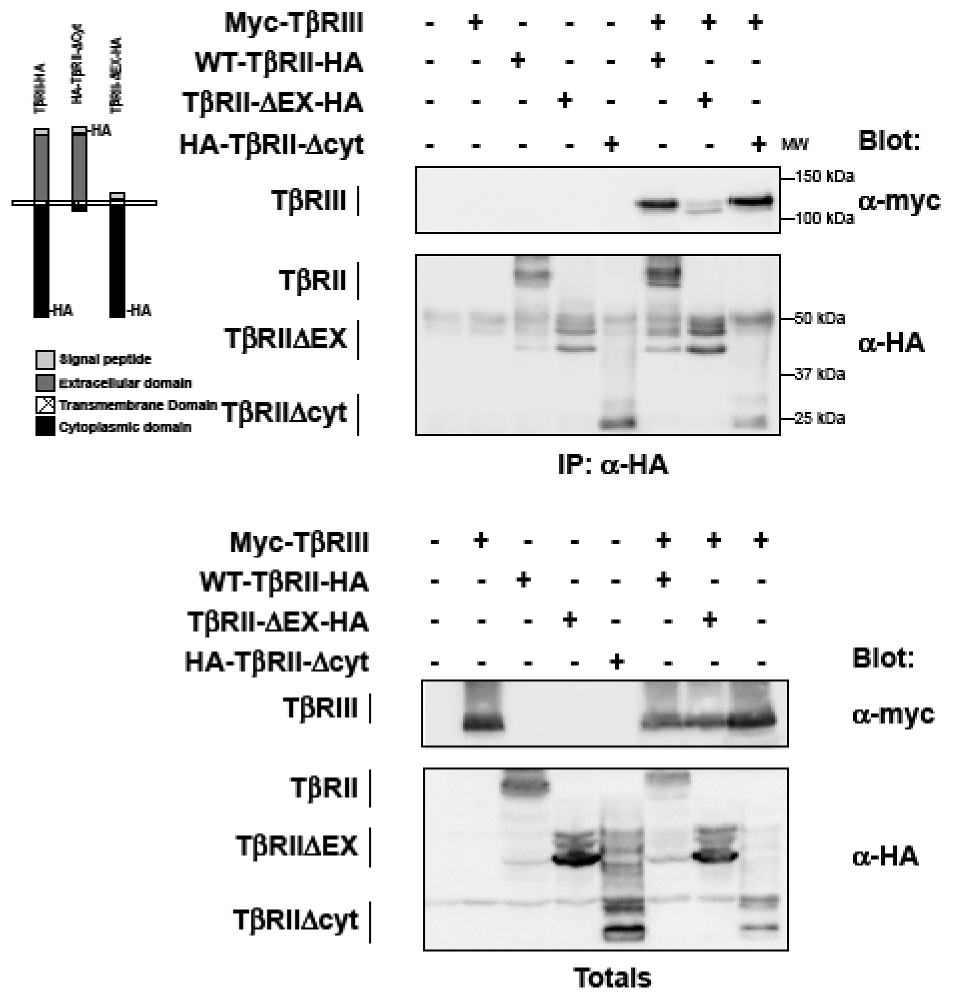
3.4.3 *TβRIII alters the endocytosis of the cytosolic truncation mutant of TβRII*

It has been previously shown that the cytosolic truncation mutant of TβRII, TβRIIΔcyt, is found nearly exclusively in membrane raft fractions (13). To assess whether TβRIII can also re-direct the partitioning of TβRIIΔcyt, I first performed a co-immunoprecipitation experiment to assess the interaction of TβRIII with TβRIIΔcyt and TβRIIΔEX, which lacks the extracellular domain (Figure 3.4). As TβRIII has a large, heavily glycosylated extracellular domain, I predicted that it primarily interacts with the extracellular domain of TβRII. I used α-HA primary antibodies to immunoprecipitate full-length TβRII and the truncation

Figure 3.4 TβRIII interacts with the extracellular domain of TβRII

HEK293T cells were transiently transfected with myc-TβRIII and full length TβRII-HA (WT), HA-TβRII lacking the intracellular domain (TβRII-Δcyt) or TβRII-HA lacking the extracellular domain (TβRII-ΔEX-HA). Cell lysates were immunoprecipitated with α-HA antibody and immunoblotted with α-myc or α-HA antibodies (top panel). Total cell lysates were also immunoblotted with α-myc or α-HA antibodies to indicate relative protein expression (bottom panel) (N=3).

Figure 3.4



mutants and to evaluate their association with the myc-TβRIII. I observed that while TβRIII can interact with both truncation mutants, it forms a more robust interaction with TβRIIΔcyt (Figure 3.4).

To address whether TβRIII can re-direct the partitioning of TβRIIΔcyt, I used sucrose-density ultracentrifugation to concentrate membrane rafts from HEK293T cells over-expressing TβRIIΔcyt, and TβRIIΔcyt in the presence of TβRIII (Figure 3.5). Interestingly, I found that similar to full-length TβRII, TβRIII also shifts TβRIIΔcyt from membrane rafts into non-membrane raft fractions (Figure 3.5). As an internal control, I also confirmed that TβRIII was able to alter the membrane partitioning of full-length TβRII (Figure 3.5). As TβRIII can re-direct the partitioning of TβRIIΔcyt, this illustrates that the ability of TβRIII to re-direct the partitioning of TβRII is not dependent on the intracellular domain of TβRII, which has binding sites for clathrin (26).

3.4.4 *TβRIII associates with TβRI in the absence of ligand and affects its partitioning*

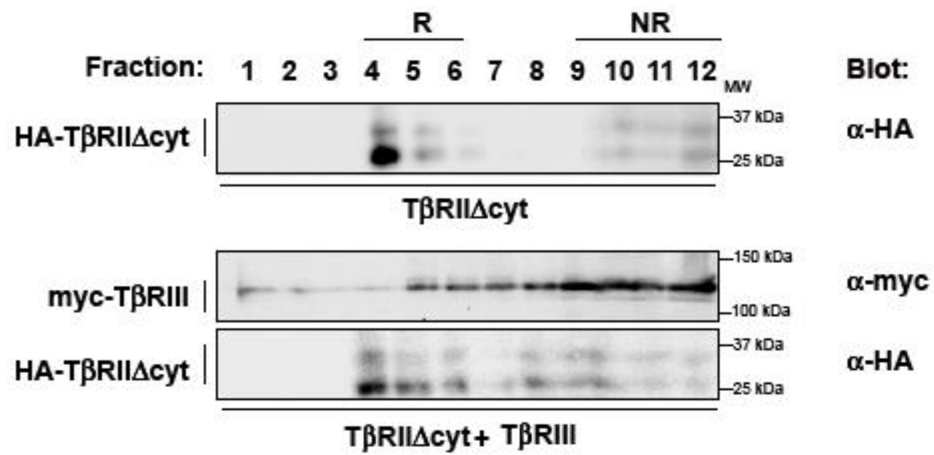
As TβRI plays a crucial role in the propagation of TGFβ signalling by phosphorylating downstream R-Smads, I also wanted to assess whether TβRIII may affect the partitioning of TβRI.

I first assessed whether TβRIII could interact with TβRI using a co-immunoprecipitation approach (Figure 3.6). I used α-flag antibodies to immunoprecipitate TβRIII, and similar to my results with TβRII, I found that TβRI

Figure 3.5 TβRIII moderately re-directs the membrane partitioning of a cytosolic truncation mutant of TβRII

HEK293T cells were transiently transfected with full length TβRII-HA, TβRII-HA lacking the intracellular domain (HA-TβRII-Δcyt) and/or myc-TβRIII and subjected to sucrose density subcellular fractionation and immunoblotted with α-HA or α -myc antibodies (N=3).

Figure 3.5



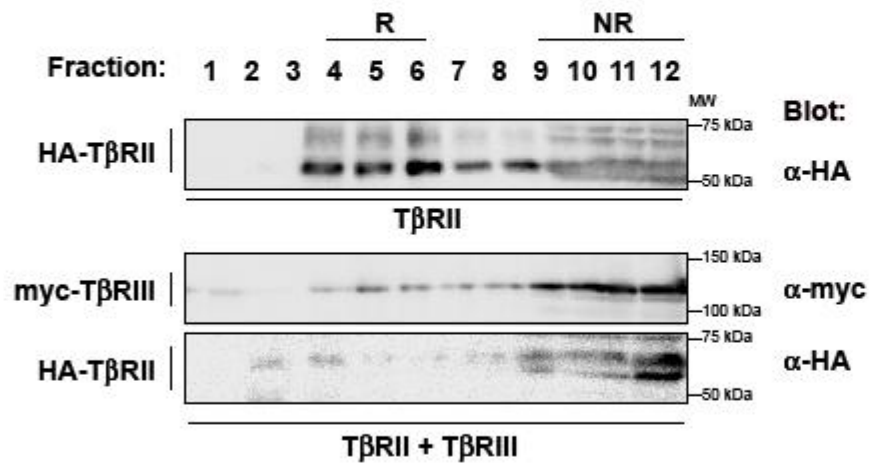


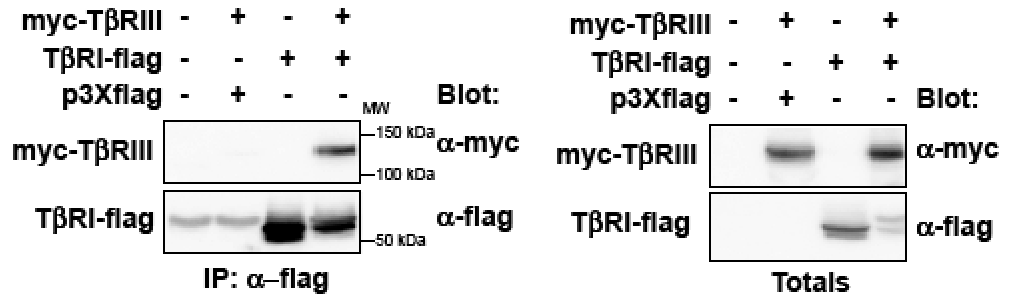
Figure 3.6. TβRIII interacts with TβRI in the absence of ligand and directs its membrane partitioning.

(A) HEK293T cells were transiently transfected with TβRI-flag, myc-TβRIII or p3xflag (control) as indicated. Cell lysates were immunoprecipitated with α-flag antibodies, processed for SDS-PAGE and immunoblotted with α-myc or α-flag antibodies (left panel). 50 μg of total cell lysates were also immunoblotted with α-myc or α-flag antibodies to indicate relative protein levels (right panel) (N=3).

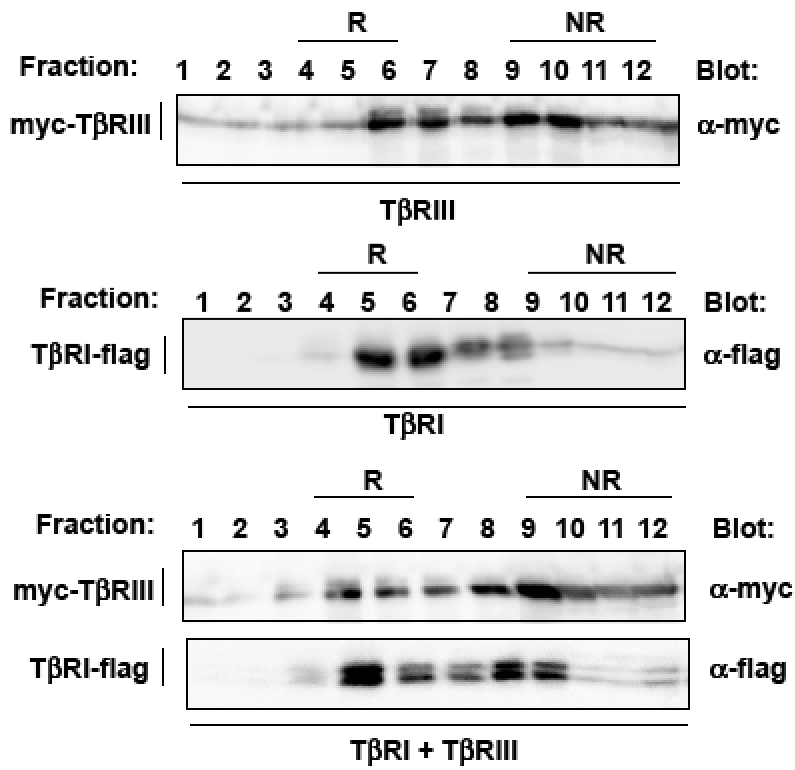
(B) HEK293T cells were transiently transfected with TβRI-flag, and/or myc-TβRIII and subjected to sucrose density subcellular fractionation as previously described. Fractions were then processed for SDS-PAGE and were immunoblotted with α-myc or α-flag antibodies (N=3).

Figure 3.6

A



B



can form a robust interaction with TβRIII in the absence of ligand (Figure 3.6A). Importantly, this interaction occurs in the absence of TβRII, as HEK293T cells express very little endogenous TGFβ receptors. To assess the membrane partitioning of TβRI, I again used sucrose-density ultracentrifugation to isolate membrane rafts. Similar to Figure 3.1A, TβRI is found predominantly in membrane raft fractions. To assess whether TβRIII can re-direct concentrated in membrane raft fractions (Figure 3.6B). Interestingly, and complementary to my findings with TβRII, TβRIII also shifts TβRI into non-raft fractions (Figure 3.6B). My results evaluating the partitioning of both TβRI and TβRII in the presence of TβRIII suggest that TβRIII is able to direct the partitioning of the TβRII/TβRI complex, but importantly, can interact with either receptor independently.

3.4.5 *TβRIII decreases entry of TβRII into caveolin-1-positive vesicles*

The intracellular trafficking of TGFβ receptors is also directly influenced by their endocytosis. When TGFβ receptors are endocytosed via membrane rafts/caveolae, the receptors enter into caveolin-1-positive vesicles, and the receptors are targeted for ubiquitination and degradation (12). Furthermore, no signal transduction occurs in caveolin-1-positive vesicles, as TβRI is blocked from interacting with Smad 2/3 by the inhibitory Smad, Smad7 (12). Thus, I predicted that since TβRIII shifted TβRII out of membrane raft fractions, less TβRII would also be found in caveolin-1 positive vesicles. To address this question, I used an immunofluorescence microscopy approach to visualize co-localization of TβRII and TβRIII with GFP-tagged caveolin-1. Mv1Lu HAT cells

stably over-express HA-TβRII under the control of a zinc-inducible promoter. Cells were transiently transfected with myc-TβRIII and GFP-tagged caveolin-1. The following day cells were cooled to 4˚C to prevent receptor internalization, and then were labeled with α-HA antibodies and Cy3 donkey anti-rabbit secondary antibodies. Receptors were then permitted to internalize by warming the cells to 37ºC. I then performed immunofluorescence microscopy to visualize TβRIII and caveolin-1. at 4˚C to label HA-tagged TβRII at the cell surface.

The top panel of Figure 3.7 shows that in the absence of TβRIII, a large fraction of TβRII co-localizes with caveolin-1. However, upon addition of TβRIII, less TβRII is found co-localized with caveolin-1 (Figure 3.7). Furthermore, very little co-localization between TβRIII and caveolin-1 was found. Not only do these results confirm my ultracentrifugation data, they also illustrate that TβRIII can direct TβRII out of the caveolin-1-positive vesicles, and therefore may also have a direct effect on TβRII half-life.

3.4.6 *TβRIII increases early-endosomal trafficking of TβRII*

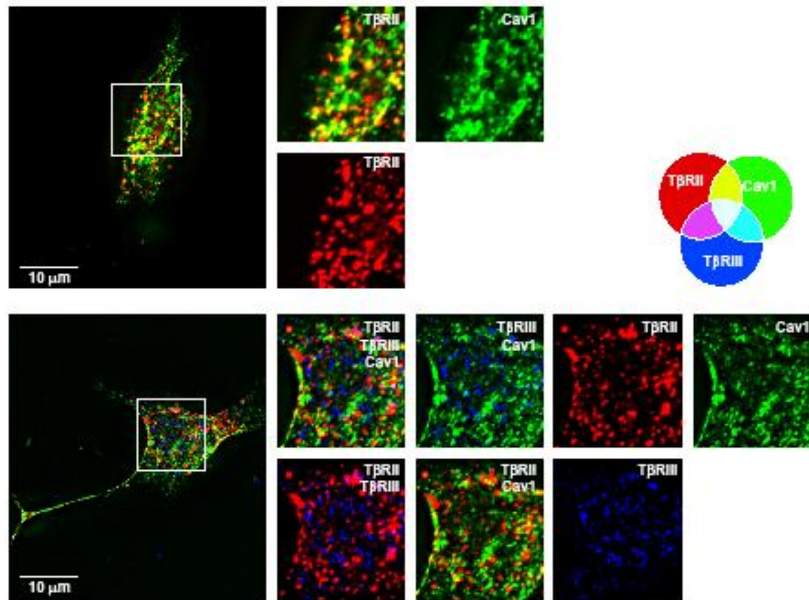
Having shown that TβRIII directs TβRII out of caveolar vesicles, I sought to evaluate whether TβRIII also increases TβRII entrance into the early endosome. It has been previously shown that receptors internalized via clathrin-coated pit mediated endocytosis traffic into early endosomes, where they can interact with Smad transcription factors to propagate TGFβ signalling (12).

Figure 3.7. TβRIII decreases TβRII localization into caveolin-1 positive vesicles

Top panel- Mv1Lu HAT cells stably expressing extracellularly HA-tagged TβRII were transiently transfected with caveolin-1 GFP. Tagged TβRII receptors were incubated with α-HA antibodies at 4ºC. Following incubation with Cy3-labelled secondary antibodies (red), the cells were incubated at 37ºC to allow receptor endocytosis. TβRII co-localizing with caveolin-1-GFP results in yellow staining (N=3).

Bottom panel- Mv1Lu HAT cells stably expressing extracellularly HA-tagged TβRII were transiently transfected with myc-TβRIII cDNA and caveolin-1 GFP. Tagged TβRII receptors were incubated with α-HA antibodies as described above. After receptor internalization, TβRIII was labelled with Cy5 secondary antibodies (blue) to assess receptor co-localization. TβRII co-localizing with caveolin-1 results in yellow staining, TβRII co-localizing with TβRIII results in magenta staining, and all three co-localizing results in white staining (N=3).

Figure 3.7



Therefore, if TβRIII can re-direct the intracellular trafficking of TβRII, then it may also have a direct effect on TGFβ signal transduction. To evaluate the ability of TβRIII to affect TβRII receptor trafficking I used an immunofluorescence microscopy approach to evaluate the co-localization of the receptors. As before, Mv1Lu HAT cells were transiently transfected with myc-TβRIII cDNA. Approximately 36 hrs post-transfection, receptors were labelled at the cell surface by cooling cells to 4°C and incubating with α-HA pAb. After labelling with α -rabbit Cy3, receptors were permitted to internalize by warming to 37°C. Cells were also labelled with α-myc primary antibody, followed by donkey α-mouse secondary antibody to visualize TβRIII. To evaluate early endosomal trafficking of receptors two markers for the early endosome, early endosomal antigen 1 (EEA1-FITC) (Figure 3.8A) and Rab5-GFP a GTPase involved in early endosomal sorting, (Figure 3.8B) were evaluated in terms of co-localization with receptors.

Using immunofluorescence microscopy, I found that in the absence of TβRIII, TβRII co-localized with EEA1; however, a substantial proportion of receptors did not localize with EEA1 (Figure 3.8A). One possibility is that receptors are internalized by both clathrin and non-clathrin mediated mechanisms. However, upon co-expression with TβRIII, more TβRII was found co-localized with EEA1, suggesting an increase in clathrin-mediated endocytosis or endosomal retention (Figure 3.8A). Co-localization experiments with Rab5 also showed that in the presence of TβRIII, it appears that more TβRII are found

Figure 3.8 TβRIII increases early-endosomal trafficking of TβRII

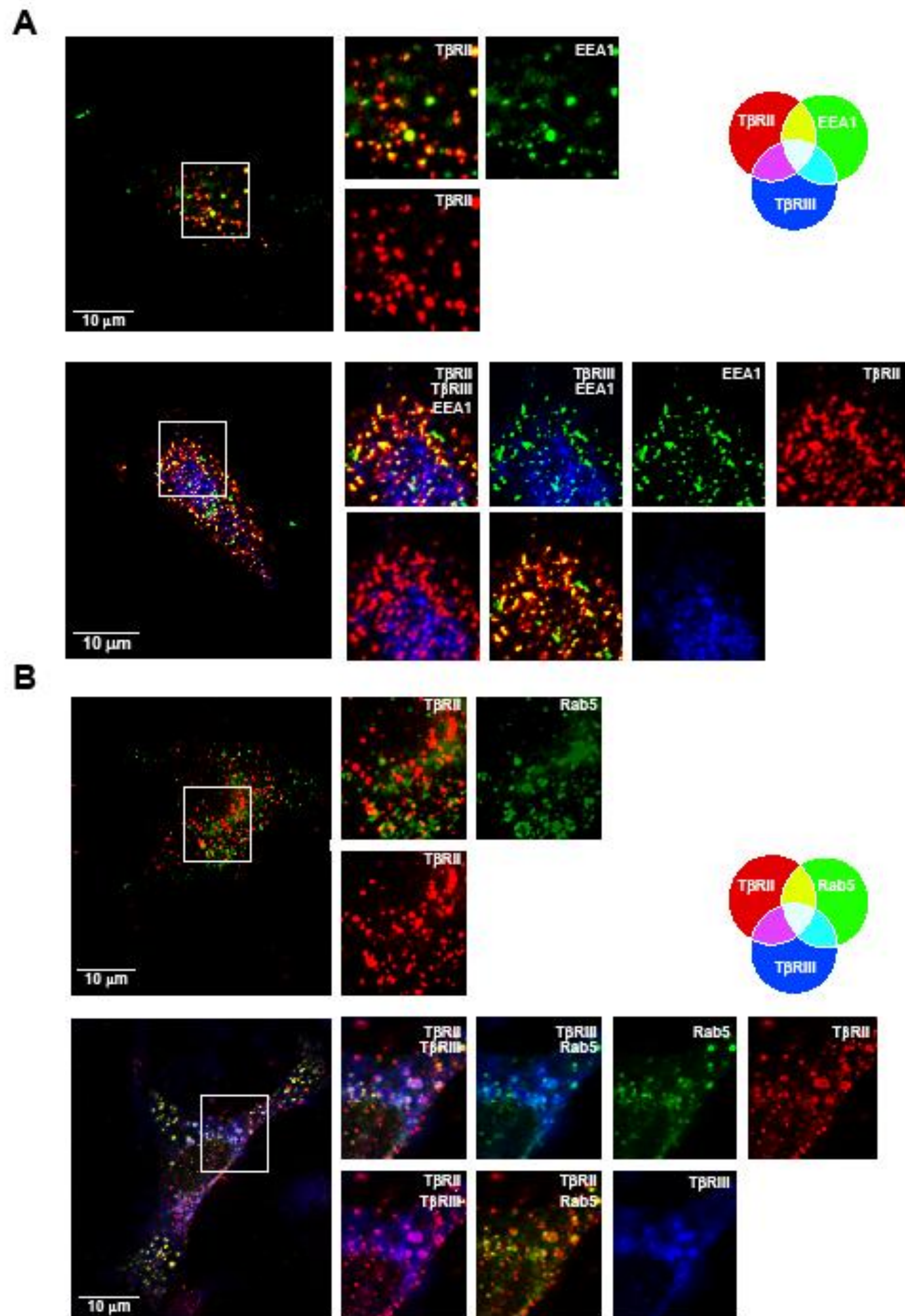
(A) Top panel- Mv1Lu HAT cells stably expressing extracellularly HA-tagged TβRII were incubated with α-HA antibodies at 4ºC. Following incubation with Cy3-labelled secondary antibodies (red), the cells were incubated at 37ºC for 1 hr to allow receptor endocytosis. After receptor internalization, the cells were immunostained with FITC-labelled α-EEA1 antibodies (green). TβRII co-localizing with early endosomes results in yellow staining and non-EEA1 localized TβRII is also found in the cytoplasm (red) (N=3).

Bottom panel- Mv1Lu HAT cells stably expressing extracellularly HA-tagged TβRII and transiently expressing myc-TβRIII were incubated with α-HA and α-Myc antibodies at 4ºC and processed for immunofluorescence microscopy as described in Figure 3.7 (N=3).

(B) Top panel- Mv1Lu HAT cells stably expressing extracellularly HA-tagged TβRII were transiently transfected with Rab5-GFP. Following incubation with Cy3-labelled secondary antibodies (red), the cells were incubated at 37ºC to allow endocytosis. TβRII co-localizing with Rab5-GFP results in yellow staining (N=3).

Bottom panel- Mv1Lu HAT cells expressing HA-tagged TβRII and myc-TβRIII were also assessed for their co-localization with Rab5-GFP. Cells were processed as before. TβRII co-localizing with Rab5-GFP results in yellow staining, TβRIII co-localizing with TβRII results in magenta staining, and all three co-localizing results in white staining (N=3). Bar= 10μm.

Figure 3.8



in early endosomes (Figure 3.8B). As Rab5 has been shown to cause endosomal enlargement, I also performed co-localization studies with GFP-labelled S34N (dominant-negative) or Q79L (constitutively-active) Rab5 mutants to assess whether the co-localization of the receptors with the Rab5-positive vesicle was simply due to endosomal enlargement (Figure 3.9). My results indicate that while Rab5 Q79L can cause endosomal enlargement, this does not appear to increase the localization of TβRII with the early-endosomal compartment (Figure 3.9). Overall, my study strongly suggests that the type III TGFβ receptor is able to direct the trafficking of TβRII.

3.4.7 *TβRIII extends the half-life of TβRII*

Efficient turnover of TGFβ receptors is essential for optimal TGFβ signal transduction, as TGFβ receptors and ligand are ubiquitously expressed. It has been previously shown that the intracellular compartmentalization of TGFβ receptors directs receptor degradation and recycling. Receptors in early endosomal compartments are recycled to the cell surface, whereas receptors localized in caveolin-1 positive compartments are targeted for ubiquitination and degradation (12). Therefore, having found that TβRIII re-directs trafficking of TβRII into early endosomal compartments, I predicted that TβRIII expression would also increase the half-life of TβRII. To assess this further, I used [^{125}I]-labelled TGFβ1 ligand to track cell-surface TGFβ receptor half-life in both the presence and absence of TβRIII. Briefly, HEK293T cells were transfected with TβRI, TβRII, TβRIII, Smurf2 and Smad7, as shown in Figure 3.10.

Figure 3.9 TβRII trafficking in the presence of S34N or Q79L Rab5.

(A) Top panel- Mv1Lu HAT cells stably expressing extracellularly HA-tagged TβRII and transiently expressing Rab5 S34N-GFP (Rab5-GDP) were incubated with α-HA antibodies at 4ºC. Following incubation with Cy3-labelled secondary antibodies (red), the cells were incubated at 37ºC for 1 hour to allow receptor endocytosis. TβRII co-localizing with Rab5-GDP results in yellow staining and non-Rab5-GDP localized TβRII is also found in the cytoplasm (red) (N=3).

Bottom panel- Mv1Lu HAT cells stably expressing extracellularly HA-tagged TβRII and transiently expressing myc-TβRIII and Rab5 S34N-GFP (Rab5-GDP) were incubated with α-HA and α-myc antibodies at 4ºC and processed for immunofluorescence microscopy as described above (N=3).

(B) Top panel- Mv1Lu HAT cells expressing HA-tagged TβRII were also assessed for their co-localization with transiently expressed Rab5 Q79L-GFP (Rab5-GTP), shown in yellow (N=3).

Bottom panel- Mv1Lu HAT cells expressing HA-tagged TβRII and myc-TβRIII were also assessed for their co-localization with Rab5 Q79L-GFP (Rab5-GTP). As before, receptors were labelled at the cell surface, internalized, then assessed for their co-localization. TβRII co-localizing with Rab5-GTP results in yellow staining, TβRIII co-localizing with TβRII results in magenta staining, and all three co-localizing results in white staining (N=3). Bar= 10μm.

Figure 3.9

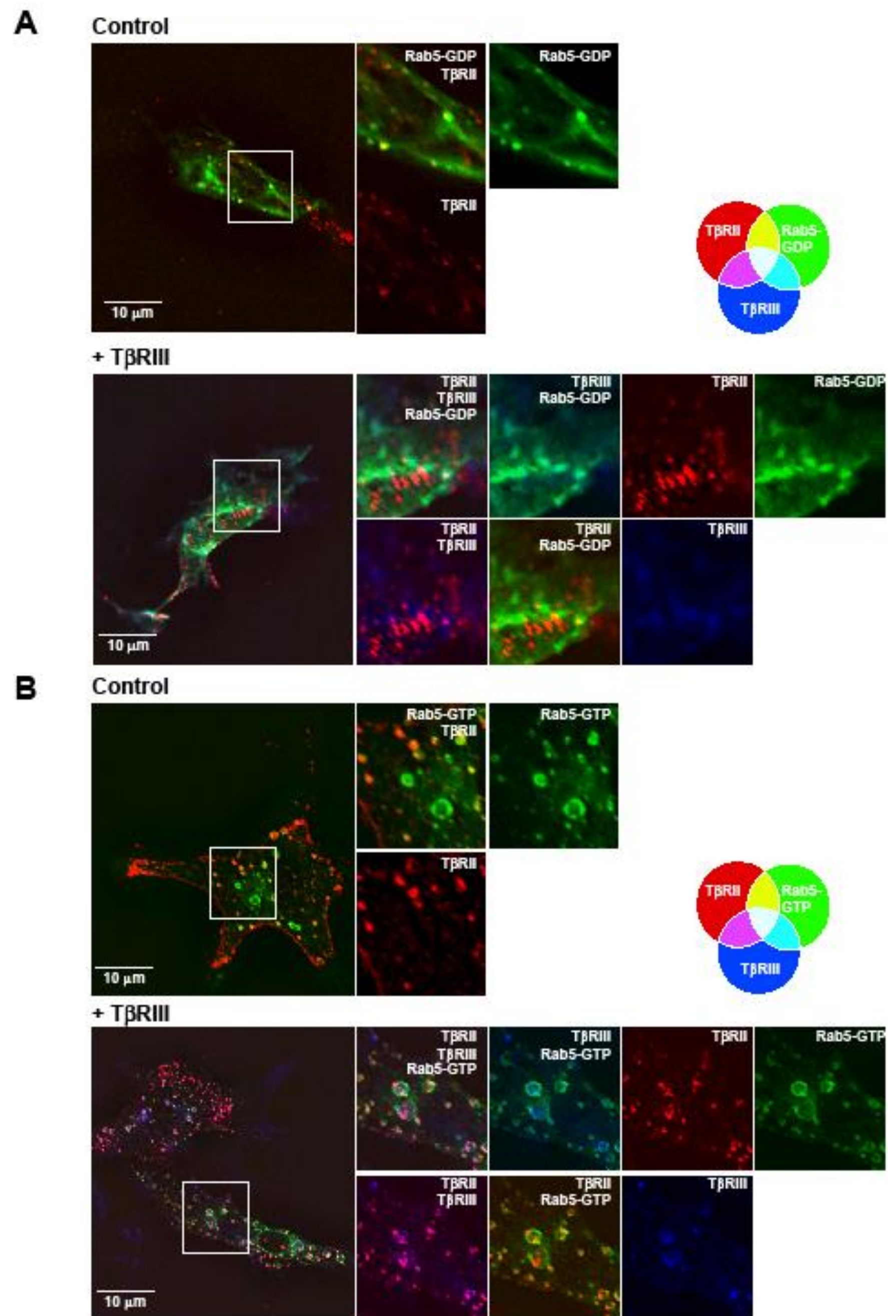
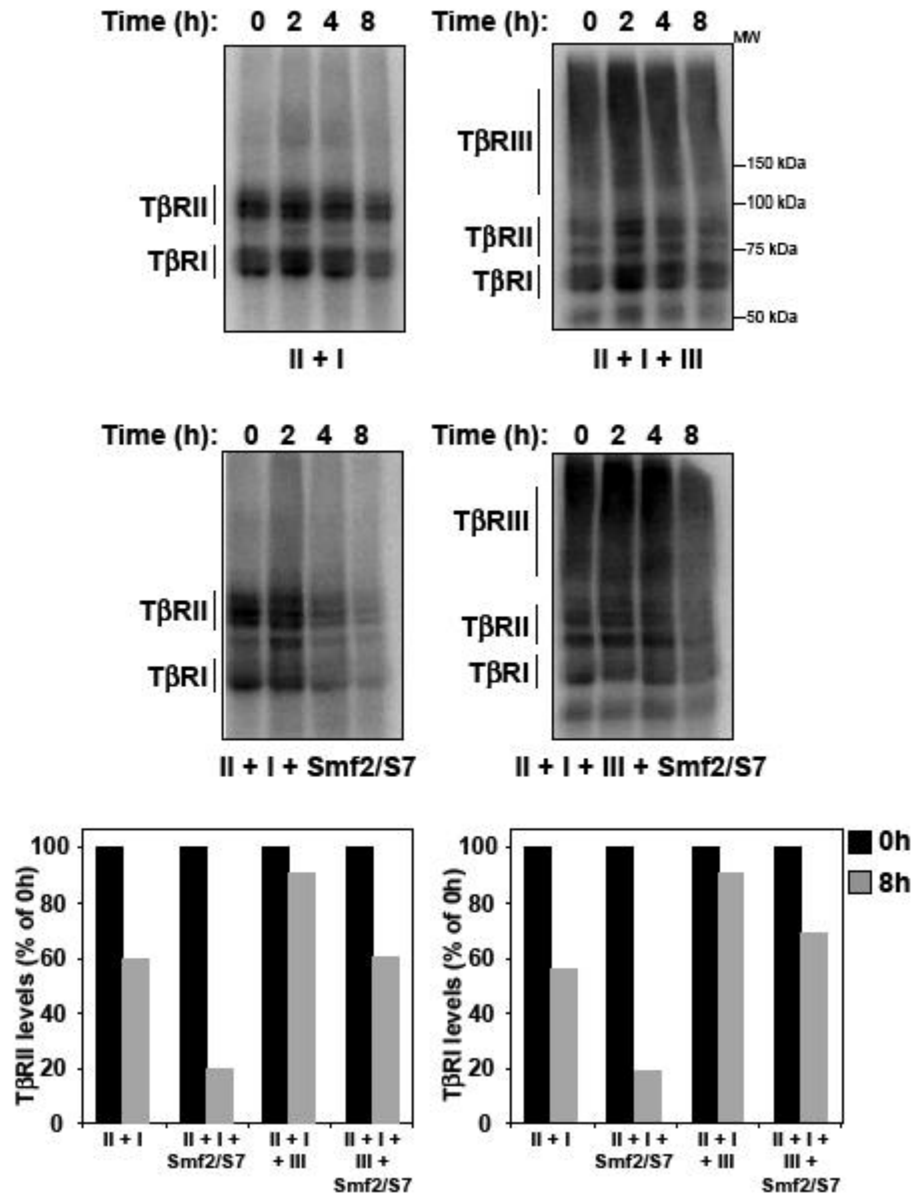


Figure 3.10 TβRIII expression reduces TβRII and TβRI complex degradation.

HEK293T cells were transiently transfected with TβRII-HA (II), TβRI-flag (I) and myc-TβRIII (III) in the presence or absence of Smurf2/Smad7 (Smf2/S7). Cells were then incubated with $^{[125]}$I-TGFβ , cross-linked and incubated at 37˚C for 2, 4 or 8 hrs. Following cell lysis and SDS-PAGE, receptors levels were visualized and quantified by phosphorimaging (Molecular dynamics). Relative receptor levels of TβRII (left) and TβRI (right) were compared to the amount of receptor measured at time zero and graphed (bottom panel; N=3). Shown is a representative graph of receptor levels.

Figure 3.10



Smurf2 and Smad7 were transiently transfected to promote receptor degradation, as in the absence of Smurf2 and Smad7 TGFβ receptors over-expressed in HEK293T cells have a prolonged half-life (Figure 3.10, top panel). Post-transfection, cells were labelled with [^{125}I] TGFβ1 at 37˚C. The ligand was then cross-linked to receptors and the cells were warmed to 37ºC to promote receptor internalization. Cells were lysed at 0, 2, 4 and 8 hrs of internalization, subjected to SDS-PAGE and visualized using autoradiography.

The top panels of Figure 3.10 illustrate that in the absence of Smurf2 and Smad7, TβRII has a prolonged half-life both in the presence and absence of TβRIII. However, upon addition of Smurf2 and Smad7, which promote receptor ubiquitination and degradation, TβRIII greatly extends the half-life of both TβRI and TβRII. These results confirm my receptor trafficking studies and imply that TβRIII can have direct effects on TGFβ signal transduction by altering the TβRII/TβRI complex half-life.

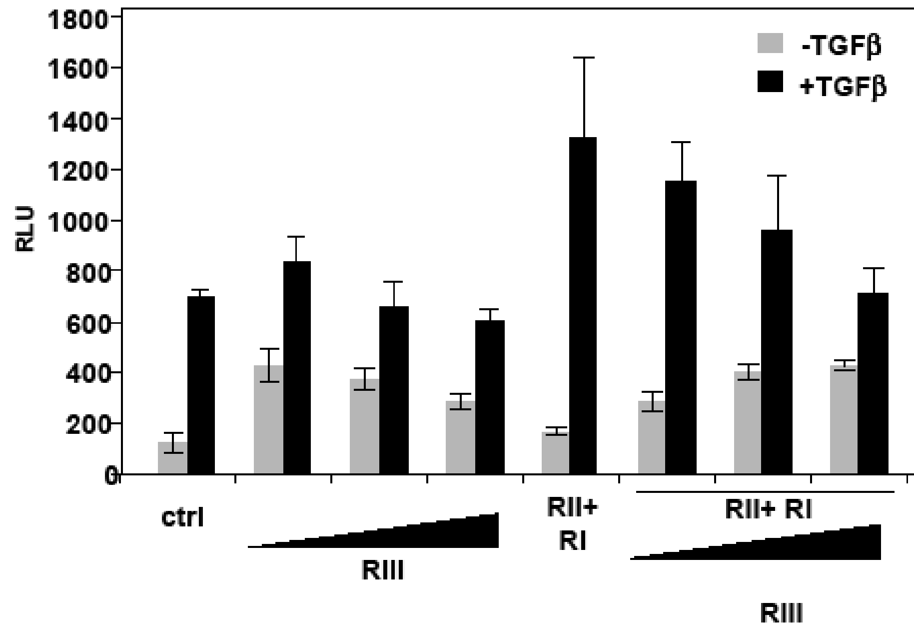
3.4.8 *TβRIII enhances basal TGFβ signalling*

As it has been previously shown that clathrin-mediated endocytosis enhances TGFβ signal transduction (12), I sought to identify whether TβRIII expression could affect TGFβ signalling (Figure 3.11). To address this question, I used a TGFβ-responsive promoter upstream of a luciferase construct to quantitatively assess the role of TβRIII expression on signalling. HepG2 cells have previously been shown to be TGFβ-responsive and are amenable to calcium chloride transfection and were therefore used as my model system to

Figure 3.11 TβRIII expression increases basal TGFβ signalling

HepG2 cells were transfected with ARE-lux, β-gal and FoxH1 alone (control) or together with TβRII/I (RII+RI) and/or increasing concentrations of TβRIII (RIII). Transfected cells were incubated in the absence (gray bars) or presence (black bars) of 100 pM TGFβ. Luciferase activity was normalized to β-galactosidase activity and is plotted as the mean ± SD of triplicates from a representative experiment (N=3).

Figure 3.11



assess TGFβ-depdendent transcription (12). HepG2 cells were transiently transfected with ARE-Lux, β-galactosidase, FoxH1 (a transcriptional co-activator) and/or TβRII and TβRI, or increasing concentrations of TβRIII (Figure 3.11). Interestingly, it seems that TβRIII expression may increase Smad-dependent signalling in the absence of TGFβ (Figure 3.11). This result was surprising, as TβRIII is best known for its role in ligand presentation. I hypothesize that the ability of TβRIII to enhance basal TGFβ signalling is due to its enhanced clathrin-mediated endocytosis of the TβRII/TβRI complex. Indeed, it has been shown that TGFβ receptors can signal at a basal level in the absence of ligand (27). In contrast to this result, it appears that increasing levels of TβRIII cDNA transfection decreases TGFβ signalling in the presence of ligand (Figure 3.11). I hypothesize that this may be accounted for the ability of over-expressed TβRIII to pre-load the early endosomes with receptors. This would result in a loss of TβRII/TβRI from the cell surface, and therefore may explain why luciferase activity is decreased in the presence of increasing concentrations of TβRIII. Future studies to evaluate the role of TβRIII to mediate expression of validated TGFβ-responsive genes, such as PAI-1 and Smad7 should be performed to confirm these results.

In conclusion, analysis of both membrane fractionation and receptor trafficking illustrate that TβRIII promotes clathrin-mediated endocytosis of both TβRII and TβRI and directs TβRII into the early endosome. TβRIII expression therefore has functional consequences on TGFβ signal transduction, as it

extends receptor half-life by re-directing the TβRII/TβRI complex out of the degradative membrane raft pathway and enhances basal TGFβ signalling.

3.5 Discussion

The mechanism of endocytosis at the cell membrane can have immediate downstream effects in signal transduction. In the canonical TGFβ signalling pathway, clathrin-mediated endocytosis increases TGFβ signalling through enabling the association of the receptors with SARA (Smad anchor for receptor activation) in the early endosome. SARA is able to mediate the interaction of the TGFβ receptors with Smad proteins, which are the downstream effectors of TGFβ signal transduction (12). Membrane raft/caveolar endocytosis however, decreases TGFβ signalling through promoting the degradation of the receptors (12). In membrane rafts, Smurf2 (Smad ubiquitination regulatory factor 2) associates with the receptors, promotes the ubiquitination and degradation of the TGFβ receptors, and prevents the association of Smad proteins (reviewed in (7)).

Therefore, since the endocytic mechanism of TGFβ receptors can have such profound effects on TGFβ signalling, and a number of pathologies including metastatic cancers and fibrotic diseases show aberrant TGFβ signalling, an in-depth study evaluating the mechanism through which TGFβ receptors are directed to endocytose is warranted.

To address the issue of endocytic partitioning in TGFβ signalling, I first attempted to evaluate the contribution of TGFβ receptor subtypes to the raft and

non-raft partitioning of TβRII. Interestingly, using co-immunoprecipitation studies, I showed that TβRIII, the least characterized TGFβ receptor, is able to associate with both TβRII and TβRI even in the absence of ligand. I confirmed these results with sucrose-density ultracentrifugation, which quantitatively illustrated that the association of TβRIII with TβRII and TβRI can greatly shift the partitioning of TβRII and TβRI into non-membrane raft/clathrin fractions. In support of my subcellular fractionation studies, I also show that TβRIII directs TβRII into the early endosome and out of the degradative pathway using immunofluorescence microscopy. Using [^{125}I] labelled TGFβ1 to track TβRII and TβRI half-life, I show that TβRIII can have a direct effect on the signalling capacity of the TβRII/TβRI complex, as its association can extend the half-life of TβRII/TβRI. Finally, I illustrate that TβRIII increases basal TGFβ signalling, but decreases signalling in the presence of ligand.

While my study evaluates the contribution of TβRIII to TGFβ receptor trafficking, other studies have also attempted to evaluate factors that affect TGFβ receptor half-life. Koli and Arteaga illustrated that binding of TGFβ to TβRII can shorten its half-life (28). Interestingly, it has also been recently shown that inhibiting clathrin-mediated endocytosis of TβRII extends receptor half-life and promotes TGFβ signalling (29). In this study, the authors illustrated that inhibitors of clathrin-mediated endocytosis prevent internalization of TGFβ receptors, but allow the association of SARA and TβRI at the cell surface; this then promotes and extends TGFβ signalling (29).

Another study has attempted to evaluate the contribution of TβRIII to TβRII membrane partitioning. The authors concluded that TβRIII was endocytosed via membrane rafts in Cos7 and HepG2 cell lines (23). Furthermore, they reported that membrane-raft associated TβRIII regulates phosphorylation of Smad2 and p38 (23). While I show differing results in this report, in that TβRIII is primarily found in non-membrane raft fractions, and is able to differentially partition the TβRII/TβRI complex, this result may be due to the cell types used in both studies. Differences in membrane raft content between HepG2 and HEK 293T cells may account for some of the discrepancies observed. This study nonetheless illustrates that while TβRIII endocytosis can affect TGFβ signalling, there may be other interacting protein partners that influence the effect of TβRIII. Indeed, a recent review highlights several RhoGTPases which can have a modulating effect on TGFβ endocytosis and signal transduction (30).

The importance of the effect of TβRIII on TGFβ signal transduction is only beginning to be explored. This TGFβ receptor has recently drawn attention due to its aberrant expression in several cancers. Indeed, TβRIII overexpression is found in seminomas (31) and knockdown of TβRIII decreases invasiveness and motility of breast cancer cells (21). However, it has also been shown that loss of TβRIII can promote metastasis and invasiveness in a number of cancers, including non-small cell lung cancer (20), pancreatic cancer (22) and prostate cancer (17,32). Our finding that TβRIII promotes basal TGFβ signalling but

decreases ligand-dependent signalling may help explain some of the duality of its function in cancer. Perhaps the levels of TβRIII may play a role- a total loss of TβRIII may increase TGFβ signalling by preventing ligand sequestration from the TβRII/TβRI complex and thus may promote the metastatic effects of TGFβ signalling. While the opposing effects of TβRIII warrant further investigation of this pathway, my studies suggest that TβRIII expression may play a critical role in control of TGFβ signal transduction.

Overall, in this study I have shown that TβRIII directs TβRII and TβRI to undergo clathrin-mediated endocytosis. This altered endocytosis directs TβRII into early endosomal pathways, extends TβRII and TβRI half-life and enhances basal TGFβ signalling. As TβRIII is aberrantly expressed in a number of pathologies, my study suggests that TβRIII may mediate TGFβ signal transduction by altering TGFβ receptor endocytosis.

3.6 References

1. Siegel, P. M., and Massague, J. (2003) *Nat Rev Cancer* **3**(11), 807-821

2. Blobe, G. C., Schiemann, W. P., and Lodish, H. F. (2000) *N Engl J Med* **342**(18), 1350-1358

3. Shi, Y., and Massague, J. (2003) *Cell* **113**(6), 685-700

4. Kang, Y., Chen, C. R., and Massague, J. (2003) *Mol Cell* **11**(4), 915-926

5. Rappoport, J. Z. (2008) *Biochem J* **412**(3), 415-423

6. Traub, L. M. (2003) *The Journal of cell biology* **163**(2), 203-208

7. Le Roy, C., and Wrana, J. L. (2005) *Nat Rev Mol Cell Biol* **6**(2), 112-126

8. Simons, K., and Toomre, D. (2000) *Nat Rev Mol Cell Biol* **1**(1), 31-39

9. Francesconi, A., Kumari, R., and Zukin, R. S. (2009) *J Neurosci* **29**(11), 3590-3602

10. Salani, B., Briatore, L., Contini, P., Passalacqua, M., Melloni, E., Paggi, A., Cordera, R., and Maggi, D. (2009) *Biochem Biophys Res Commun* **380**(3), 489-492

11. Patra, S. K. (2008) *Biochim Biophys Acta* **1785**(2), 182-206

12. Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F., and Wrana, J. L. (2003) *Nat Cell Biol* **5**(5), 410-421

13. Luga, V., McLean, S., Le Roy, C., O'Connor-McCourt, M., Wrana, J. L., and Di Guglielmo, G. M. (2009) *Biochem J* **421**(1), 119-131

14. Wang, X. F., Lin, H. Y., Ng-Eaton, E., Downward, J., Lodish, H. F., and Weinberg, R. A. (1991) *Cell* **67**(4), 797-805

15. Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W. S., and Massague, J. (1991) *Cell* **67**(4), 785-795

16. Derynck, R., and Feng, X. H. (1997) *Biochim Biophys Acta* **1333**(2), F105-150

17. Turley, R. S., Finger, E. C., Hempel, N., How, T., Fields, T. A., and Blobe, G. C. (2007) *Cancer Res* **67**(3), 1090-1098

18. Hempel, N., How, T., Dong, M., Murphy, S. K., Fields, T. A., and Blobe, G. C. (2007) *Cancer Res* **67**(11), 5231-5238

19. Bilandzic, M., Chu, S., Farnworth, P. G., Harrison, C., Nicholls, P., Wang, Y., Escalona, R. M., Fuller, P. J., Findlay, J. K., and Stenvers, K. L. (2009) *Mol Endocrinol* **23**(4), 539-548

20. Finger, E. C., Turley, R. S., Dong, M., How, T., Fields, T. A., and Blobe, G. C. (2008) *Carcinogenesis* **29**(3), 528-535

21. Criswell, T. L., Dumont, N., Barnett, J. V., and Arteaga, C. L. (2008) *Cancer Res* **68**(18), 7304-7312

22. Gordon, K. J., Dong, M., Chislock, E. M., Fields, T. A., and Blobe, G. C. (2008) *Carcinogenesis* **29**(2), 252-262

23. Finger, E. C., Lee, N. Y., You, H. J., and Blobe, G. C. (2008) *The Journal of biological chemistry* **283**(50), 34808-34818

24. Wieser, R., Attisano, L., Wrana, J. L., and Massague, J. (1993) *Mol Cell Biol* **13**(12), 7239-7247

25. Hunyady, L., Baukal, A. J., Gaborik, Z., Olivares-Reyes, J. A., Bor, M., Szaszak, M., Lodge, R., Catt, K. J., and Balla, T. (2002) *The Journal of cell biology* **157**(7), 1211-1222

26. Ehrlich, M., Shmuely, A., and Henis, Y. I. (2001) *J Cell Sci* **114**(Pt 9), 1777-1786

27. Kang, J. S., Liu, C., and Derynck, R. (2009) *Trends in cell biology* **19**(8), 385-394

28. Koli, K. M., and Arteaga, C. L. (1997) *The Journal of biological chemistry* **272**(10), 6423-6427

29. Chen, C. L., Hou, W. H., Liu, I. H., Hsiao, G., Huang, S. S., and Huang, J. S. (2009) *J Cell Sci* **122**(Pt 11), 1863-1871

30. Kardassis, D., Murphy, C., Fotsis, T., Moustakas, A., and Stournaras, C. (2009) *Febs J* **276**(11), 2947-2965

31. Dias, V., Rajpert-De Meyts, E., McLachlan, R., and Loveland, K. (2009) *Reproduction*

32. Sharifi, N., Lechleider, R. J., and Farrar, W. L. (2007) *J Mol Endocrinol* **39**(5), 329-332

CHAPTER 4

βARRESTIN2 INTERACTS WITH TβRII TO REGULATE SMAD-DEPENDENT AND SMAD-INDEPENDENT SIGNAL TRANSDUCTION

A version of this chapter has been submitted to Cellular Signalling, manuscript # CLS-D-12-00204.

4 Chapter 4

4.1 Chapter Summary

In chapter 3 I assessed the ability of TβRIII, a heavily-glycosylated cell-surface protein, to mediate receptor partitioning of TβRII/TβRI complexes. Reports have shown that TβRIII interacts with βarrestin2 to mediate its internalization with TβRII. Therefore, in this chapter I assessed the ability of βarrestin2 to influence TGFβ receptor trafficking and signal transduction. Interestingly, I found that βarrestin2 can interact with TβRII in the absence of TβRIII. Furthermore, βarrestin2 traffics to the early endosome with TβRII where it increases the association of TβRII with SARA. Depletion of endogenous βarrestin2 increased levels of TβRII at the cell-surface and also induced hyper-phosphorylation of p38. Increased phosphorylation of p38 correlated with an increased sensitivity of cells to cell death both in the presence and absence of TGFβ.

4.2 Introduction

The Transforming Growth Factor Beta (TGFβ) signalling pathway is a cell and context-dependent pathway that is under intense study due to its complex roles in cancer and fibrotic diseases. In the classical TGFβ pathway, TGFβ ligands stimulate the formation of a heteromeric serine/threonine kinase complex of TGFβ receptor I (TβRI) and TGFβ receptor II (TβRII). Ligand-binding to TβRII activates its kinase domain, promoting phosphorylation of TβRI on its GS domain (reviewed in (1)). Activated TβRI then induces a Smad signalling cascade by phosphorylating Smad2/3, which allow them to interact with the Co-Smad, Smad4, and translocate to the nucleus to activate cell-type specific transcriptional programmes (1).

While endocytosis of plasma membrane receptors is classically thought to function to downregulate signalling, it has been shown in the TGFβ pathway that the endocytic route of the receptors can play a direct role in signalling outcome. TβRII/TβRI complexes internalized via clathrin-coated pits enter the early endosome, where SARA (Smad Anchor for Receptor Activation) enhances Smad-dependent signal transduction by recruiting Smad 2/3 and facilitating their phosphorylation by TβRI (2,3). Receptor complexes internalized by clathrin-independent, caveolin-positive vesicles however, are sterically prevented from signalling by the inhibitory Smad7 and targeted for degradation by Smurf2, an E3 ubiquitin ligase (2). Indeed, several studies have shown that trafficking of the TGFβ receptors inside the cell has important signalling consequences. For

example, it has been shown that Dab2 is necessary for sorting of TBRII from the early endosome into the late endosome (5). Furthermore, the regulation of trafficking to late endosomes plays a role in Smad signal transduction, as a dominant-negative Rab5 causes constitutive, ligand-independent Smad signalling and nuclear translocation (5).

While the importance of trafficking in regards to signal transduction is well-appreciated in the TGFβ pathway, the specific signal(s) directing receptors to be internalized and trafficked via either pathway is not well-understood. Previously I have shown that the type III TGFβ receptor (TβRIII, or betaglycan) which was thought to simply present ligand to TβRII, can direct TβRII/TβRI complexes into the early endosome (3). As others have shown that TβRIII can interact with βarrestin2 (7), and βarrestin2 is known to have an important role in GPCR internalization (4), I sought to evaluate the contribution of βarrestin2 to TβRII internalization, trafficking and signal transduction.

The relatively simplified view of βarrestin2 solely acting to internalize GPCRs has been challenged by a myriad of experiments that implicate it as a pleiotropic scaffolding protein. While it is true that βarrestin2 can interact with components of the endocytic machinery, including clathrin (5) and AP-2 (6), it can also act as a scaffolding protein and binds to a variety of proteins in the cytoplasm including Src, cofilin, Akt and MAP kinases (reviewed in (7)). Indeed, βarrestin2 has been implicated in diverse processes such as Ras-independent cytoskeletal re-arrangement (12), ERK signal transduction (8), activation of beta-

catenin via endothelin receptors (14) and activation of epidermal growth factor receptors (9). Based on this plurality of βarrestin2 function, and the fact that TβRIII can bind to βarrestin2 (7), I sought to further evaluate the role of βarrestin2 in TGFβ signal transduction. In this chapter I show that βarrestin2 interacts with TβRII and that decreased βarrestin2 levels cause an increase in TβRII cell-surface levels, and the phosphorylation of Smad2. Interestingly, decreased βarrestin2 levels do not increase Smad-dependent transcription, as decreased βarrestin2 decreases the production of luciferase under the control of a Smad-responsive promoter. I also assessed a Smad-independent pathway, the p38 pathway, and found that the increased phosphorylation of p38 through decreased βarrestin2 levels functionally results in an increase in TGFβ-dependent and -independent apoptosis.

4.3 Materials and Methods

4.3.1 *Antibodies and reagents*

Commercially available antibodies were used as per manufacturers' instructions from the following sources: α-HA (Santa Cruz-Y11-SC-805), α -myc (Santa Cruz sc-40), α-flag (Sigma F3165), α-EEA1 (BD Trans Labs-610457), α-GFP (Clontech- 632381), α-PSmad2 (Millipore- AB3849), α-βarrestin2 (Abcam-AB54790), α-actin (Sigma-A2668), α-Smad2/3 (BD- 610843), α-PSmad3 (Cell Signaling- 3101), α-pp38 (Cell Signaling-92115), α-p38 (Cell Signaling-92125), α-PARPc (Cell Signaling- 9541). HRP-conjugated goat α-mouse (Thermo Scientific- 31430), and goat α-rabbit (Thermo Scientific-31460) were used for

western blotting. Fluorescently labelled donkey α-rabbit Cy3 (Jackson- 711-165-152) and donkey α-mouse AlexaFluor 647 (Invitrogen- A21236) were used for visualization of immunofluorescence experiments. The constructs encoding TβRII-HA, myc-TβRIII, βarr2-flag, βarr2-GFP and SARA-flag were used as previously described (3,10,11). Stealth siRNA (negative control, 634978; βarrestin2 siRNA1, ARRB2VHS40600; or βarrestin2 siRNA2, ARRB2VHS40604) and Lipofectamine RNAiMax were purchased from Invitrogen. TGFβ1 was purchased from Peprotech. Hoechst 33342 was a generous gift from Dr. S. Cregan (Robarts Research Institute, Western University).

4.3.2 *Cell Culture*

HEK 293T human embryonic kidney cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Mink lung cells stably expressing a carboxy terminus HA tagged TβRII construct (HAT Mv1Lu) were maintained in Modified Eagle's medium (MEM) supplemented with 10% FBS, non-essential amino acids and 0.3% hygromycin. A549 non-small cell lung adenocarcinoma cells (American Type Culture Collection) were maintained in F12K media supplemented with 10% FBS. H1299 non-small cell lung adenocarcinoma cells (ATCC) were maintained in RPMI media supplemented with 10% FBS. HepG2 hepatocellular carcinoma cells were maintained in Modified Eagle's medium (MEM) supplemented with 10% FBS and non-essential amino acids. All cells were kept in a humidified atmosphere at 37°C and 5% CO_2.

4.3.3 *Transfection*

HEK 293T and HepG2 cells were transiently transfected using the calcium phosphate method. HAT Mv1Lu cells were transfected using polyethylenimine.

4.3.4 *Immunoprecipitation*

Transfected HEK 293T cells were lysed (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, and cocktail protease inhibitors) and centrifuged at 14,000 × g_{av} at 4°C for 10 min. Aliquots of supernatants were collected for analysis of total protein concentration. The remaining cell lysates were incubated with antibody followed with protein G sepharose incubation. The precipitates were washed 3 times, eluted with Laemmli sample buffer and subjected to SDS-PAGE and immunoblot analysis.

4.3.5 *Immunoblotting*

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes by electrophoretic transfer. Following blotting with primary and secondary antibodies, bound antibodies were detected using SuperSignal chemiluminescence reagent (Pierce).

4.3.6 *Isolation of Caveolae/membrane-raft enriched membrane fractions*

The caveolin/raft-rich membrane fractions were isolated as previously described (12). Briefly, transfected HEK 293T cells grown to confluence in 100-mm dishes were used to prepare the membrane fractions. All steps were carried out on ice. After two washes with cold phosphate-buffer saline (PBS), cells were lysed with 0.5 M Na_2CO_3, pH 11.0, containing protease inhibitors. After scraping,

the cell lysate was collected and homogenized three times for 10 s by using a Polytron tissue grinder (Brinkmann Instruments). Homogenates were then sonicated three times for 20 s with a Vibra Cell sonicator (Sonics Materials Inc.). The homogenates were adjusted to 40% sucrose and overlaid with 30% sucrose and 5% sucrose solutions. The samples were centrifuged at 200,000 x g_{av} for 16 h at 4°C, using a Beckman SW41 rotor. Twelve 1-mL fractions were collected, and an aliquot of each fraction was eluted with Laemmli sample buffer, boiled, and subjected to SDS-PAGE followed by Western blot analysis.

4.3.7 *Immunofluorescence/Receptor Internalization*

HAT Mv1Lu cells were plated on coverslips in 12 well dishes. Twenty-four hours post-transfection of βarrestin2-GFP cDNA with polyethylenimine, cells were serum-starved and treated with 50 μM $ZnCl_2$ to induce HA-TβRII expression as previously described (3,10). The following day, cells were cooled to 4°C, and treated with α-HA antibody for 2 hours at 4°C to label receptors at the cell surface. Coverslips were then incubated with donkey anti-rabbit Cy3 antibodies. After labelling, cells were either permitted to internalize, by incubating at 37°C for 30 minutes or 1 hour, or were immediately fixed and permeabilized. Cells were incubated with α-EEA1 antibody, followed with donkey α-mouse Cy5. All coverslips were then immunomounted and visualized using an IX81 inverted immunofluorescence microscope (Olympus, Canada).

4.3.8 *siRNA-mediated Knockdown of βarrestin2 in A549 and H1299 cells*

Endogenous levels of βarrestin2 protein were decreased by Stealth siRNA (Invitrogen). At approximately 50% confluency, cells were transfected with siRNA (negative control, βarrestin2 siRNA1, or βarrestin2 siRNA2) using Lipofectamine siRNA Max as per manufacturers' instructions. Forty-eight hours following transfection, cells were assayed for silencing by SDS-PAGE and immunoblotting with α-βarrestin2 antibodies and α- actin antibodies.

4.3.9 *TGFβ Receptor Binding Assay*

A549 cells were transiently transfected with βarrestin2 siRNA as previously described. Approximately 48 hours following transfection, cells were placed on ice and then labelled with 250 pM [^{125}-I] labelled TGFβ ligand. Cells were incubated with ligand for 2 hours at 4°C and then receptors were cross-linked using 10 mg/mL DSS in DMSO for 15 minutes. Cells were then either lysed in 1X TNTE (time 0) or were placed in 10% FBS in DMEM and incubated at 37°C for 2, 4, or 8 hours prior to lysis. Lysates were then subjected to SDS-PAGE and visualized using phosphorimaging.

4.3.10 *Phospho-Smad and phospho-p38 Time Course*

A549 and H1299 cells were transiently transfected with control or βarrestin2 siRNA as described above. Prior to TGFβ treatment, cells were serum-starved overnight in 0.2% FBS in F12K or 0.2% FBS in RPMI media. The following day cells were treated with 250 pM TGFβ ligand for 30 minutes. Cells were then washed with PBS, and were either lysed or incubated at 37°C for an

additional 1 or 4 hours. Lysates were subjected to SDS-PAGE and were immunoblotted with α-phospho-Smad2, α-phospho-Smad3, α-phospho-p38, α-p38 or α-Smad2/3 antibodies.

4.3.11 *siRNA-mediated Knockdown of βarrestin2 in HepG2 cells*

Endogenous levels of βarrestin2 were decreased by Stealth siRNA (Invitrogen) using a reverse-transfection method as per manufacturers' instructions. Approximately 72 hours following transfection, cells were assayed for knockdown by SDS-PAGE and immunoblotting.

4.3.12 *Luciferase reporter assay*

HepG2 cells were transiently transfected using the calcium phosphate precipitation method with ARE (activin-response element)-Lux (luciferase), β-galactosidase, βarrestin2 cDNA and FoxH1 reporter plasmids. Cells were serum-starved in 0.2% FBS/MEM/NEAA for 4 hours prior to treatment. Cells were then incubated in the presence or absence of 250 pM of TGFβ for 16 hours. Luciferase activity was normalized to *β*-galactosidase activity.

4.3.13 *cleaved-PARP assay*

A549 cells were transiently transfected with control or βarrestin2 siRNA as previously described. Sixteen hours following transfection, cells were serum-starved in 0.2% FBS/F12K media for 4 hours. Cells were then incubated in the presence or absence of 250 pM TGFβ for 48 hours. Cells were then lysed, and subjected to SDS-PAGE and immunobloted with α-cleaved-PARP antibodies.

4.3.14 *Hoechst Cell Death assay*

A549 cells were transiently transfected with control or βarrestin2 siRNA as previously described. Sixteen hours following transfection, cells were serum-starved in 0.2% FBS/F12K media for 4 hours. Cells were then incubated in the presence or absence of 250 pM TGFβ for 48 hours. Following the 48 hour incubation, cells were treated with cell-permeant Hoechst (Hoechst 33342) for 30 minutes at 37˚C. Apoptotic and non-apoptotic cells were then visualized using an IX71 inverted immunofluorescence microscope (Olympus, Canada). The number of apoptotic *vs.* non-apoptotic cells were then counted and plotted as percentage of apoptotic cells per condition. Statistical analysis was performed using GraphPad Prism® 5.0 software.

4.3.15 *Statistical analysis*

One-way ANOVA analyses followed by post-hoc Bonferroni test were used to evaluate the significance of the results. Statistical analyses were performed using GraphPad Prism ® 5.0 software and p values of <0.05 were considered statistically significant.

4.4 **Results**

4.4.1 *TβRII binds βarrestin2 in the absence of TβRIII*

TβRII functions primarily as a serine/threonine kinase capable of both autophosphorylation and TGFβ signal propagation through the trans-phosphorylation of TβRI (reviewed in (13)). As βarrestin2 has been shown to bind

TβRIII following its phosphorylation by TβRII (14), I sought to assess whether TβRII may also be able to bind βarrestin2.

To address this question I used an immunoprecipitation approach using HEK293T cells, as they express very few endogenous TGFβ receptors (10). HEK293T cells were transiently transfected with combinations of βarr2-Flag, TβRII-HA, and myc-TβRIII cDNA. Cells were then lysed and immunoprecipitated with α-Flag antibodies and subjected to SDS-PAGE and immunoblotting (Figure 4.1). I observed that TβRII interacts with βarrestin2 both in the presence and absence of TβRIII (Figure 4.1, lanes 4 and 8). As a positive control, I evaluated the ability of βarrestin2 to interact with TβRIII. Indeed, I was able to immunoprecipitate TβRIII with βarrestin2 (Figure 4.1, lane 6). Since TβRII is essential for TGFβ signal transduction, my finding that TβRII can interact with βarrestin2 when they are over-expressed in HEK 293T cells suggests that βarrestin2 may have an important role in TβRII signal transduction and receptor internalization.

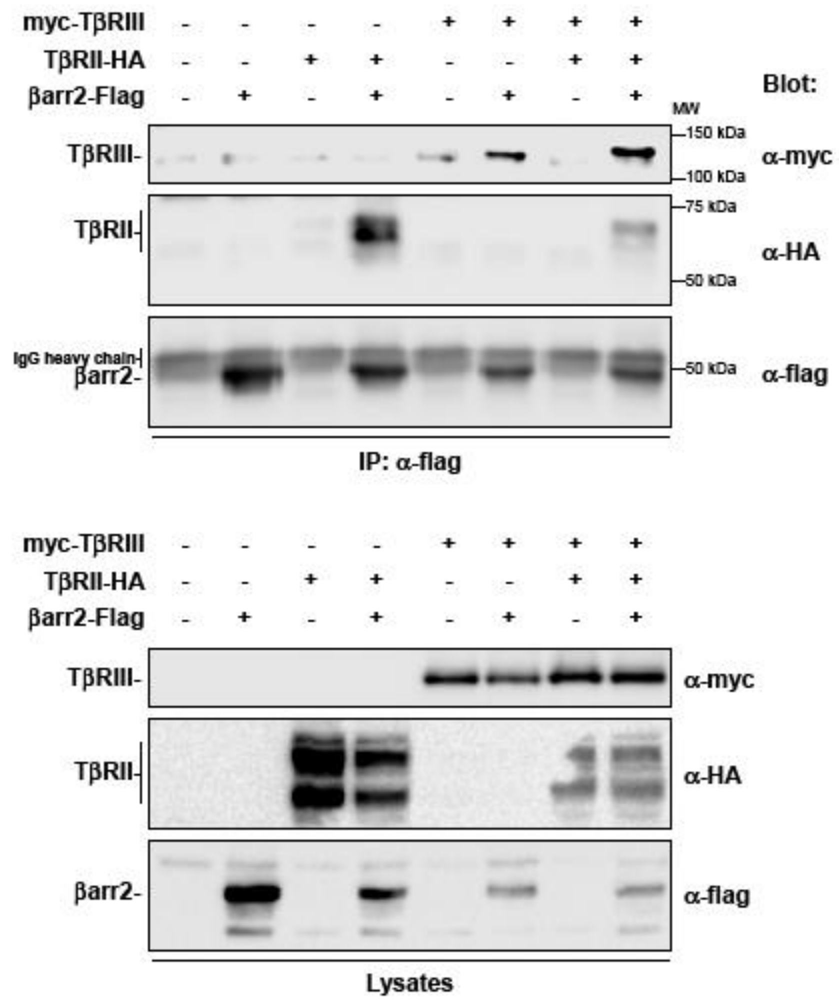
4.4.2 *βarrestin2 localizes to early endosomal compartments with TβRII*

The intracellular trafficking of the TGFβ receptor complex can have significant implications in TGFβ signal transduction, as TGFβ receptors that traffic to the early endosome have enhanced TGFβ signalling capacity (2). Having shown that βarrestin2 can interact with TβRII, I next assessed the intracellular

Figure 4.1 TβRII interacts with βarrestin2 in the absence of TβRIII

HEK 293T cells were transiently transfected using the calcium phosphate precipitation method to express Flag-tagged βarrestin2, HA-tagged TβRII and/or myc-tagged TβRIII, as indicated. Cells were lysed, immunoprecipitated with 1 μg α-Flag antibodies and subjected to SDS-PAGE and immunoblotting as indicated. Fifty μg of lysates was used to assess total expression. The top panel shows immunoprecipitated proteins, while the bottom panel illustrates total protein expression in cell lysates. (N=3).

Figure 4.1



trafficking of TβRII/βarrestin2 complexes. In order to address this question, I used a receptor-chase approach. Briefly, Mv1Lu cells stably expressing TβRII were cooled to 4˚C to halt receptor internalization, and receptors were labelled at the cell surface. Cells were then warmed to 37˚C to permit receptor internalization for 30 minutes or 1 hour, and then the cells were fixed, permeabilized, and processed for immunofluorescence microscopy. Early endosomal compartments were visualized by using antibody against early endosomal autoantigen 1 (EEA1). As I have previously shown, at time 0 all TβRII is found at the cell surface and does not co-localize with EEA1 (3) (Figure 4.2, top panel). Following 30 minutes of internalization, TβRII began to cluster and partially co-localize with EEA1, and it appears that further co-localization with EEA1 occurring at 60 minutes (Figure 4.2, middle panel). Interestingly, it appears that βarrestin2 co-localizes with TβRII at the early endosome at 30 minutes (Figure 4.2, middle panel). Strikingly, following 60 minutes of internalization, TβRII was observed to co-localize with EEA1 and βarrestin2 in large vesicles (Figure 4.2, bottom panel).

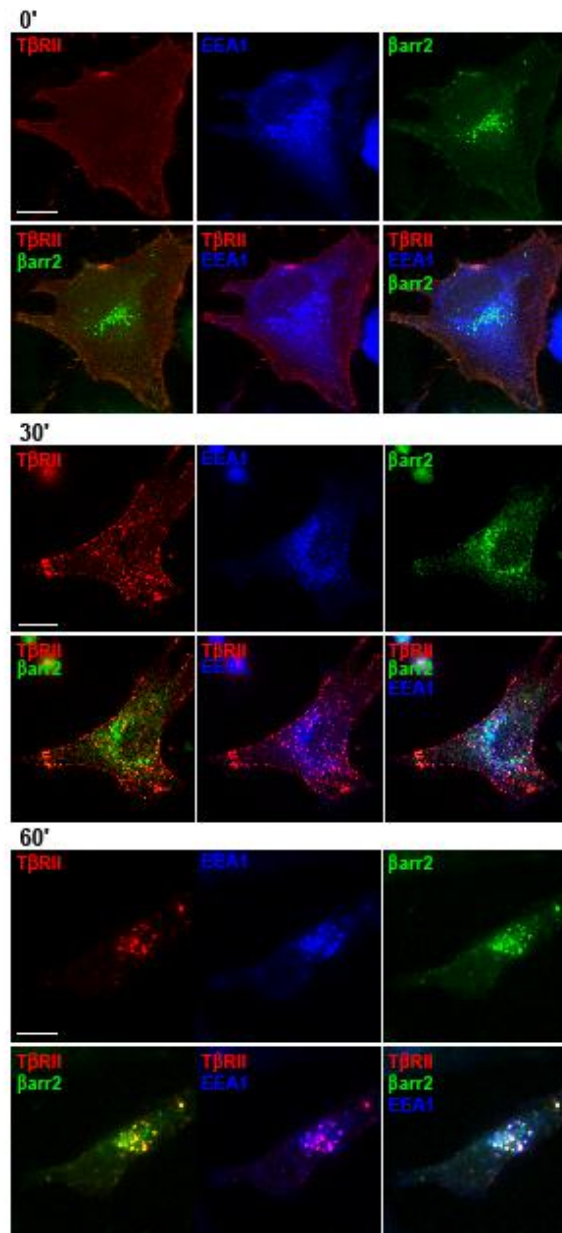
4.4.3 *βarrestin2 does not alter the membrane raft partitioning of TβRII*

The intracellular trafficking of TGFβ receptors is dependent upon their internalization route; TGFβ receptors gain access to the early endosome following clathrin-mediated internalization. Having found that TβRII can directly interact with βarrestin2, and since βarrestin2 has been shown to directly interact

Figure 4.2. βarrestin2 localizes to the early endosome with TβRII

Mv1Lu cells stably transfected with HA-tagged TβRII were transiently transfected with βarr2-GFP cDNA. To assess receptor internalization and trafficking, cells were incubated at 4˚C to prevent receptor internalization, and cell-surface receptors were labeled with α-HA antibody. Following incubation with fluorescently labeled secondary antibodies, cells were either immediately fixed and permeabilized (time 0, top panel), or were warmed to 37˚C and permitted to internalize for 30 minutes (middle panel) or 60 minutes (bottom panel). Cells were incubated with α-EEA1 antibodies followed by Cy5 secondary antibody to visualize early endosomes (N=5). To analyze receptor co-localization, 5-10 cells per condition per experiment were evaluated. Shown are representative cells from each condition. Bar= 10 μm

Figure 4.2



with components of the clathrin-coated pit machinery (6,15) I sought to establish whether βarrestin2 could enhance clathrin-mediated internalization of TβRII. To evaluate this question, I performed sucrose-density ultracentrifugation of HEK 293T cells as previously described (3,12). I found TβRII to be primarily in membrane raft fractions, TβRIII in both membrane raft and non-raft fractions, and βarrestin2 solely enriched in non-membrane raft fractions (Figure 4.3). The co-expression of βarrestin2 with TβRII did not shift its partitioning into non-membrane raft fractions (Figure 4.3). Since I have previously shown that TβRIII is able to increase clathrin-mediated endocytosis of TβRII (3), I next sought to establish whether the interaction of TβRIII and βarrestin2 with TβRII could further drive its non-membrane raft partitioning. Co-expression of TβRII and TβRIII shifts the partitioning of TβRII into non-membrane raft fractions, as I have previously shown (3). However, the addition of βarrestin2 did not further increase the partitioning of TβRII into non-membrane raft fractions (Figure 4.3).

4.4.4 *Loss of βarrestin2 increases steady-state levels of cell-surface TβRII*

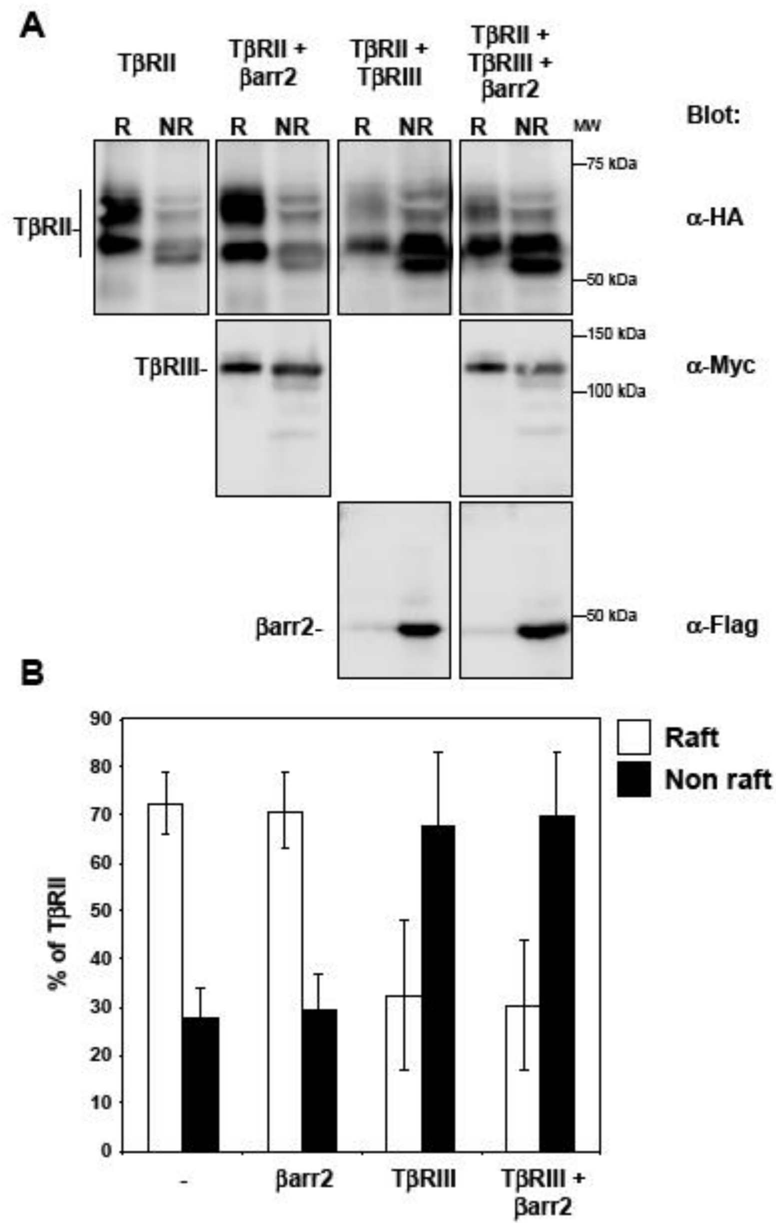
The path of TβRII trafficking directly influences TβRII recycling and/or turnover. Receptors trafficked to the early endosome promote signal propagation; while receptors trafficked to the caveolin-1 positive vesicles are targeted for degradation. As I have shown that βarrestin2 traffics to the early endosome with

Figure 4.3. βarrestin2 does not alter the membrane raft partitioning of TβRII

(A) Lipid raft partitioning of TβRII upon co-expression with TβRIII, βarrestin2 both individually and together. HEK 293T cells transiently expressing the indicated constructs were subjected to sucrose-density ultracentrifugation as described in methods. Samples were then pooled into raft (R) and non-raft (NR) fractions and subjected to SDS-PAGE and immunoblotting with the antibodies indicated. (N=3)

(B) Using Quantity One software, partitioning of TβRII into either raft or non-raft fractions was measured and calculated as a % of total TβRII levels for all conditions. The graph shown is the mean ± SD. (N=3)

Figure 4.3



TβRII, I wanted to assess the role of βarrestin2 in a second way, and evaluate its role in the internalization of cell-surface TGFβ receptor complexes. To assess the role of βarrestin2 in internalization, I again used siRNA to decrease βarrestin2 protein levels.

Following siRNA transfection, cells were treated at 4˚C with 250 pM [^{125}I-TGFβ] to label cell-surface receptors. Following cross-linking, cells were either immediately lysed, or warmed to 37˚C and incubated for 2, 4, or 8 hours. Lysates were subjected to SDS-PAGE and then developed by phosphorimaging. I observed that decreasing βarrestin2 levels had little effect on TGFβ receptor half-life (Figure 4.4A). Surprisingly, decreasing protein levels of βarrestin2 induced increased TβRII cell-surface levels at time zero, which were sustained at 2 hours. To quantify my results, phosphorimaging analysis was performed. Figure 4B illustrates TβRII levels as a percentage of TβRII levels in the negative control condition at time 0. Quantification revealed that the levels of TβRII were approximately 1.5 fold higher at time 0 in the βarrestin2 siRNA conditions compared to the siRNA control (Figure 4.4A and 4.4B).

4.4.5 *Effects of βarrestin2 siRNA on Smad2 phosphorylation levels*

Having discovered that decreased βarrestin2 expression increased TβRII cell-surface levels, I was interested to assess the effect of decreased βarrestin2 levels on Smad signal transduction. TβRII initially propagates the Smad signalling cascade by phosphorylating TβRI, which can then propagate signal

Figure 4.4. Decreased βarrestin2 protein expression increases TβRII levels at the cell surface.

(A) A549 cells transiently transfected with control siRNA (Ctrl siRNA) or two different siRNA to βarrestin2 (βarr2 siRNA1 or βarr2 siRNA2) were assessed for TβRII half-life. Following labelling with ^{125}I-TGFβ1 at 4˚C for 2 hours, cells were cross-linked and immediately lysed, or were warmed to 37˚C and were permitted to internalize for 2, 4 or 8 hours. Lysates were subjected to SDS-PAGE and visualized using phosphorimaging (left panel) (N=4). Total lysates not labelled with ^{125}I-TGFβ1 were also subjected to SDS-PAGE and immunoblotted to ensure efficient βarrestin2 silencing (right panel).

(B) Receptor levels were quantitated from experiments carried out as described in Panel A and graphed as receptor levels (% of control siRNA at time zero) *vs.* time for each condition (mean ± SD, N=4)

Figure 4.4

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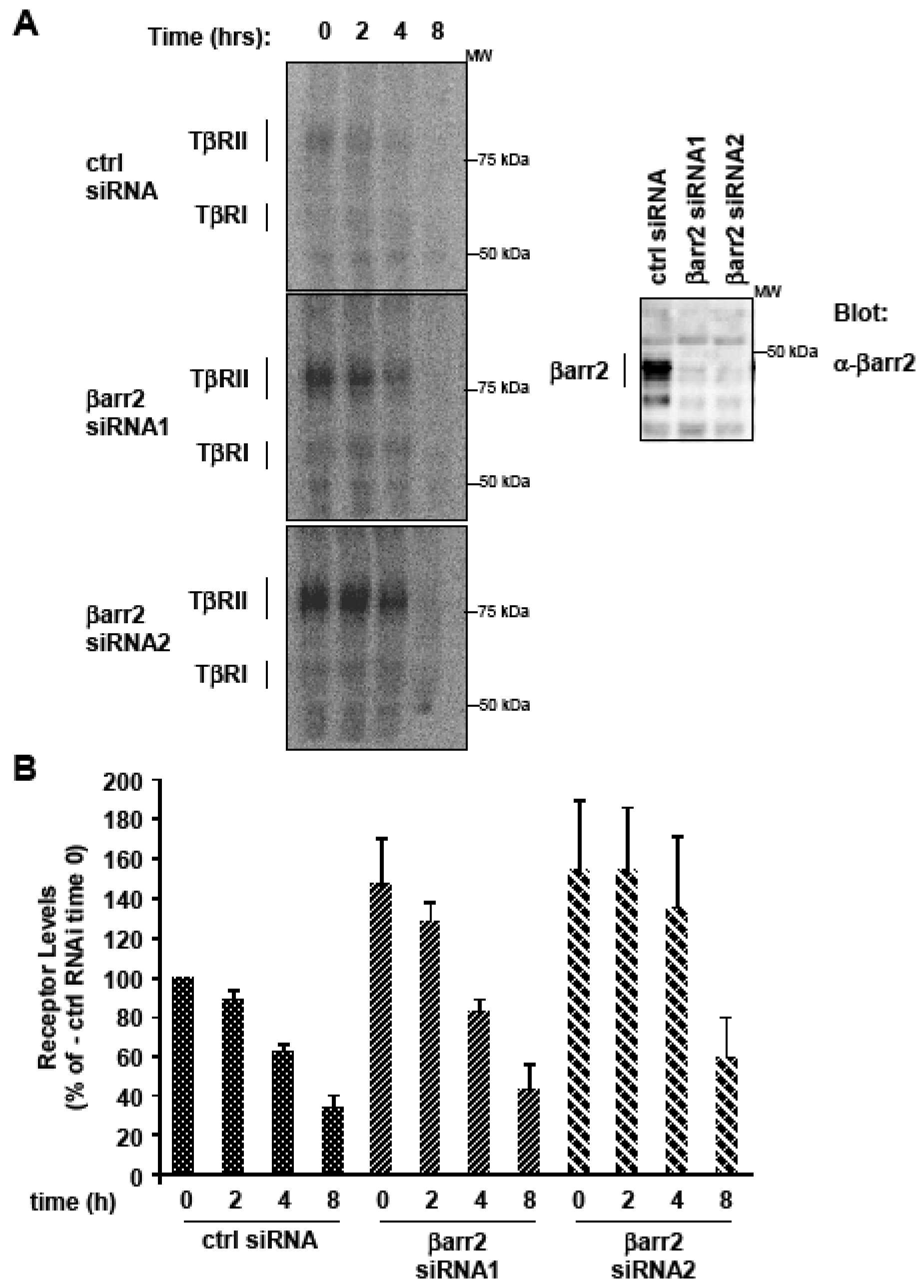


transduction by phosphorylating Smad2 (reviewed in (1)). As it is necessary for TβRII to bind TGFβ ligand in order to activate TβRI, I predicted that increased cell-surface levels of TβRII should increase Smad 2 phosphorylation, as a greater number of receptors would be exposed to ligand.

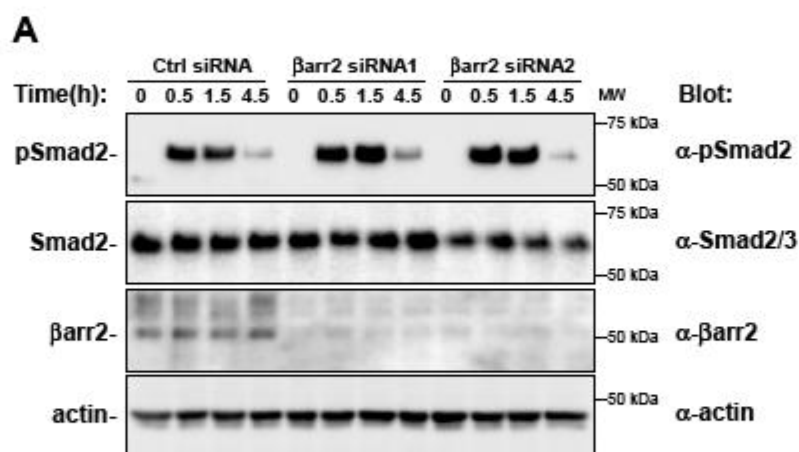
To assess the role of βarrestin2 in Smad signalling, I performed a phospho-Smad signalling assay. Non-small cell lung adenocarcinoma A549 cells were chosen for the signal transduction studies, as these cells have negligible TβRIII levels (16), and any results I observed would therefore be TβRIII-independent. In A549 cells transfected with control siRNA I observed that 250 pM TGFβ induced robust Smad2 phosphorylation within 30 min of ligand treatment which then gradually decreased after 1.5 hours and 4.5 hours. In cells transfected with siRNA constructs towards βarrestin2, the phospho-Smad2 levels appeared similar to those transfected with negative siRNA control (Figure 4.5A). I confirmed these results in another non-small cell lung cancer cell line, H1299 cells, which also showed little difference in phospho-Smad2 levels between βarrestin2 siRNA treated cells and control (Figure 4.6). To ensure that I was accurately assessing the levels of phospho-Smad2 in each instance, I quantified the levels of phospho-Smad2 using Quantity One software and densitometric analysis (Figure 4.5B). Indeed, quantitative analysis supported my observation that the silencing of βarrestin2 increased TGFβ-dependent Smad2 phosphorylation.

Figure 4.5 Effects of decreased βarrestin2 protein expression on Smad2 phosphorylation

(**A**) A549 cells transiently transfected with control siRNA (Ctrl siRNA) or two different siRNA to βarrestin2 (βarr2 siRNA1 or βarr2 siRNA2) were assessed for TGFβ-dependent Smad2 phosphorylation. Serum-deprived cells were either immediately lysed or treated with 250 pM TGFβ1 for 30 minutes. Cells were then washed in PBS and further incubated for 1 or 4 hours or were lysed. All lysates were subjected to SDS-PAGE and immunoblotted for phospho-Smad 2, Smad 2, βarrestin2 and actin levels (N=3).

(**B**) Using Quantity One software analysis, phospho-Smad2 levels as a ratio of total Smad2 levels were plotted for all conditions. The graph shown represents the mean ±SD *vs.* time (N=3).

Figure 4.5



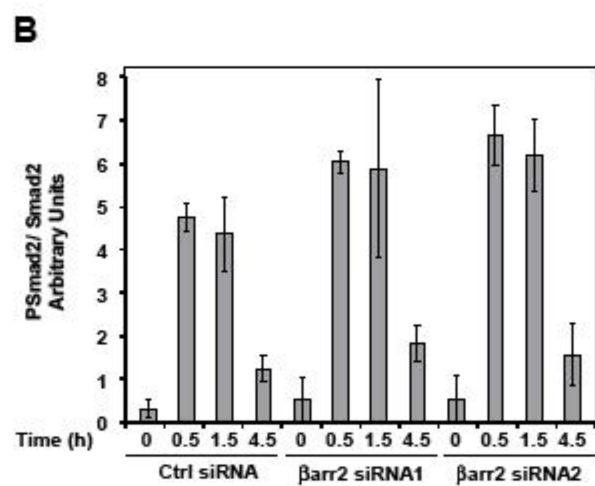
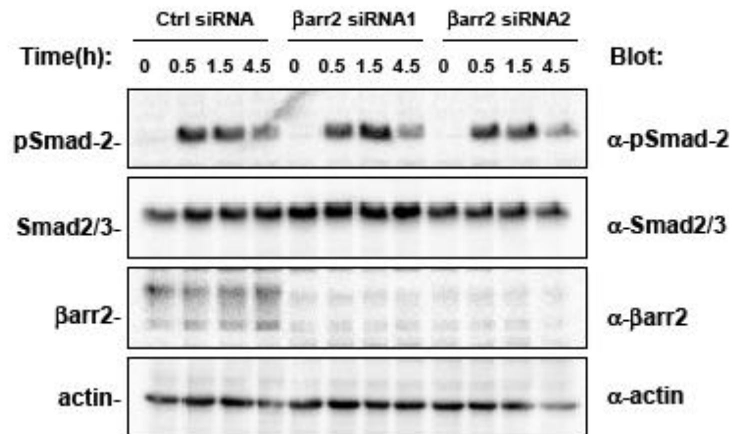


Figure 4.6 Effects of decreased βarrestin2 protein expression on PSmad2 levels in H1299 cells

H1299 cells transiently transfected with non-specific control siRNA (Ctrl siRNA) or two different siRNA to βarrestin2 (βarr2 siRNA1 or βarr2 siRNA2) were assessed for TGFβ-dependent Smad2 phosphorylation. Serum-deprived cells were either immediately lysed or treated with 250 pM TGFβ1 for 30 minutes. Cells were then washed in PBS and further incubated for 1 or 4 hours or were lysed. All lysates were subjected to SDS-PAGE and immunoblotted for phospho-Smad 2, Smad 2, βarrestin2 and actin levels (N=3).

Figure 4.6



4.4.6 *Transcription of a Smad-dependent luciferase construct in response to decreased βarrestin2 protein expression*

In the canonical TGFβ signalling pathway, following phosphorylation of Smad2 by TβRI, phospho-Smad2 translocates to the nucleus with Smad4 to activate TGFβ-dependent signal transduction. My results thus far illustrated that decreasing βarrestin2 protein levels increased Smad2 phosphorylation. I was therefore interested to assess the effects of βarrestin2 expression on TGFβ-dependent transcription. To address this question I performed ARE-Lux luciferase analysis (Figure 4.7). I was surprised to find that loss of βarrestin2 decreased TGFβ-dependent transcription (Figure 4.7A). While siRNA1 had a modest dampening effect on luciferase production, siRNA2 showed greater than two-fold decreases in luciferase production relative to negative control (Figure 4.7A).

As a complementary approach, I sought to evaluate the effect of increasing levels of βarrestin2 on TGFβ-dependent transcription. As in Figure 4.7A, HepG2 cells were transiently transfected with ARE-lux construct, as well as FoxH1 and β-galactosidase. Furthermore, cells were transfected with increasing amounts of βarrestin2 as indicated (Figure 4.7C). The specificity of my experiment was assessed using the inhibitory Smad, Smad7, which decreases transcriptional responses and causes decreased luciferase production in this assay. I observed that increasing concentrations of βarrestin2 enhanced TGFβ-dependent luciferase transcription (Figure 4.7C). As a control, we also transfected increasing amounts of βarrestin1 cDNA and found that βarrestin1

Figure 4.7 Decreased βarrestin2 protein levels decreases TGFβ-dependent transcription

(**A**) HepG2 cells were transiently transfected with control siRNA (Ctrl) or two different siRNA to βarrestin2 (siRNA1 or siRNA2). The following day, cells were transfected with cDNA encoding ARE-lux, FoxH1, and β-galactosidase. To induce ARE-lux activation, transfected cells were incubated in the absence or presence of 250 pM TGFβ overnight. The graph is representative of the mean of triplicates (± SD) from one representative experiment. (N=4)

(**B**) HepG2 cells were transiently transfected with control siRNA (Ctrl) or two different siRNA to βarrestin2 (siRNA1 or siRNA2) as described in Panel A. Forty eight hours post-transfection, cells were lysed, subjected to SDS-PAGE and immunoblotted to assess βarrestin2 protein expression.

(**C**) HepG2 cells were transiently transfected with cDNA encoding ARE-lux, FoxH1, and β-galactosidase and increasing amounts of βarrestin2 cDNA or βarrestin1 cDNA (as indicated). Smad7 was also transfected in condition 2 to assess the robustness of the system. Transfected cells were incubated in the absence or presence of 250 pM TGFβ. Luciferase activity was normalized to β-galactosidase activity and is represented as the mean ± SD of triplicates from one representative experiment (N=3).

Figure 4.7

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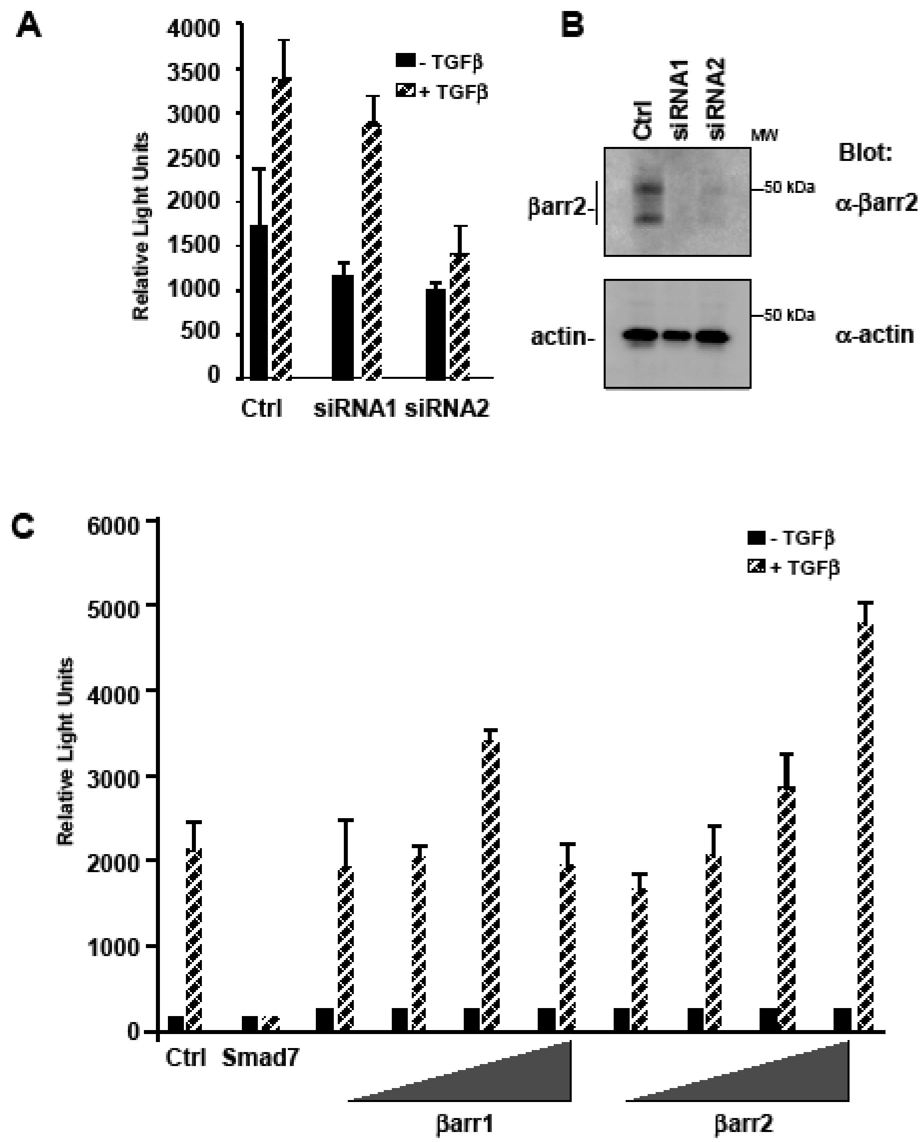


did not greatly increase TGFβ-dependent transcription (Figure 4.7C). This result supported my finding in Figure 4.7A that βarrestin2 mediates TGFβ transcriptional responses.

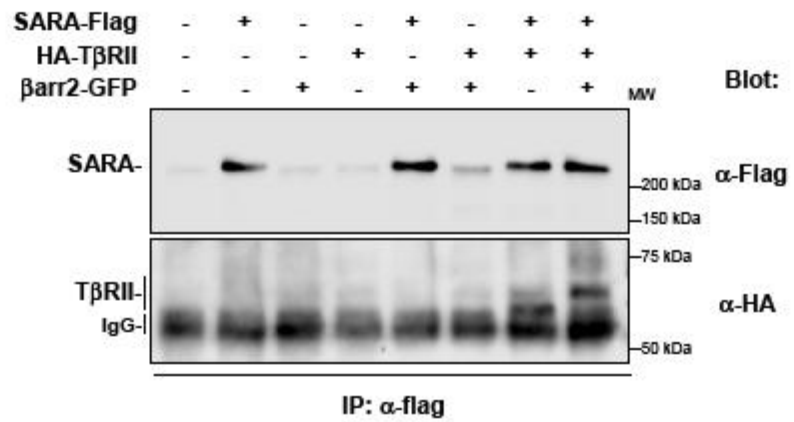
4.4.7 *βarrestin2 expression increases SARA-TβRII association*

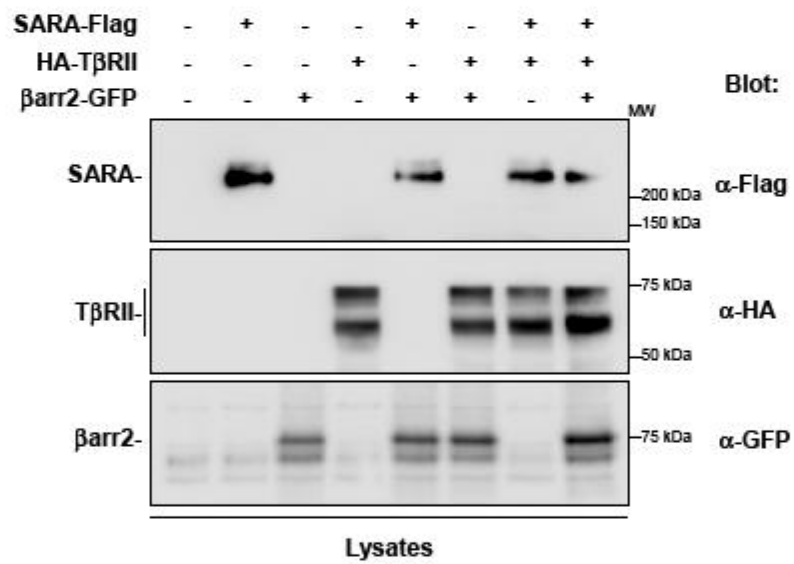
My results from figures 4.5 and 4.7 showed paradoxical effects. Although loss of βarrestin2 had minimal effects on Smad2-phosphorylation, loss of βarrestin2 decreased Smad-dependent transcription. To confirm this result, we showed that increasing the amount of βarrestin2 increased TGFβ-dependent luciferase production. Runyan et *al*. illustrated that Smad2 phosphorylation can occur independently of receptor internalization, but receptor internalization is necessary for TGFβ-dependent transcription. Since SARA is enriched in the early endosome, and it has been shown that TGFβ receptor localization to the early endosome propagates TGFβ signal transduction (10), I decided to assess whether βarrestin2 modulated the interaction of TβRII with SARA. Using a co-immunoprecipitation approach in HEK293T cells, I observed that over-expression of βarrestin2 increased the association of TβRII with SARA (Figure 4.8). Since access to the early endosome facilitates TGFβ signalling, the ability of TβRII to associate with components of the early endosome may therefore be a regulatory mechanism in TGFβ signal transduction. Thus, my finding that βarrestin2 increases SARA-TβRII association and Smad-dependent transcription suggests

Figure 4.8 βarrestin2 increases the interaction of SARA with TβRII

HEK293T cells were transiently transfected with Flag-tagged SARA, GFP-tagged βarrestin2 and/or HA-tagged TβRII, as indicated. Approximately 36 hours post-transfection, cells were lysed and immunoprecipitated with α-Flag antibodies. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting. The top panel shows immunoprecipitated proteins, while the bottom panel shows total protein expression levels (N=3).

Figure 4.8





that βarrestin2 may enhance Smad-dependent TGFβ signal transduction.

4.4.8 *siRNA directed to βarrestin2 enhances p38 phosphorylation*

TGFβ can also activate signal cascades that are independent of the canonical Smad pathway. As I had discovered that loss of βarrestin2 protein expression caused increased phosphorylation of Smad2 but decreased Smad-dependent transcription, I wanted to assess the effect of decreasing βarrestin2 levels on TGFβ-dependent, Smad-independent signalling pathways.

Several groups have shown that TGFβ can activate the MAPK pathway through MKK3/6 (22,23). Importantly, the induction of p38 and JNK phosphorylation by TGFβ is *independent* of the Smad pathway and is mediated by TRAF6 and TAK1 (17,18).

To evaluate the role of βarrestin2 on Smad-independent pathways, I silenced endogenous βarrestin2 levels in A549 cells using siRNA, then treated the cells with 250 pM TGFβ1 for 30 minutes (Figure 4.9). I observed that phosphorylated p38 increased robustly at 1.5 hours, and is sustained at 4.5 hours (Figure 4.9A). Similar to my observations with phosphorylated Smad2, I found that loss of βarrestin2 increases levels of phosphorylated p38, with a statistically significant difference between negative control and βarrestin2 knockdown occurring at 4.5 hours (Figure 4.9B). I also assessed levels of phosphorylated p38 in H1299 cells (Figure 4.10), and found that loss of βarrestin2 levels also increased phosphorylated p38 levels, similar to my results with A549 cells.

Figure 4.9 Decreased βarrestin2 expression increases p38 phosphorylation

(**A**) A549 cells transiently transfected with non-specific control siRNA (Ctrl siRNA) or two different siRNA to βarrestin2 (βarr2 siRNA1 or βarr2 siRNA2) were assessed for TGFβ-dependent p38 phosphorylation. Serum-deprived cells were either immediately lysed or treated with 250 pM TGFβ1 for 30 minutes. Cells were then washed in PBS and further incubated for 1 or 4 hours or were lysed. All lysates were subjected to SDS-PAGE and immunoblotted for phospho-p38, total p38, βarrestin2 and actin levels (N=3).

(**B**) Using Quantity One software, phospho-p38 levels as a ratio of total p38 levels were plotted for all conditions. The graph shown represents the mean ±SD vs. time (N=3). *$p<0.05$

Figure 4.9

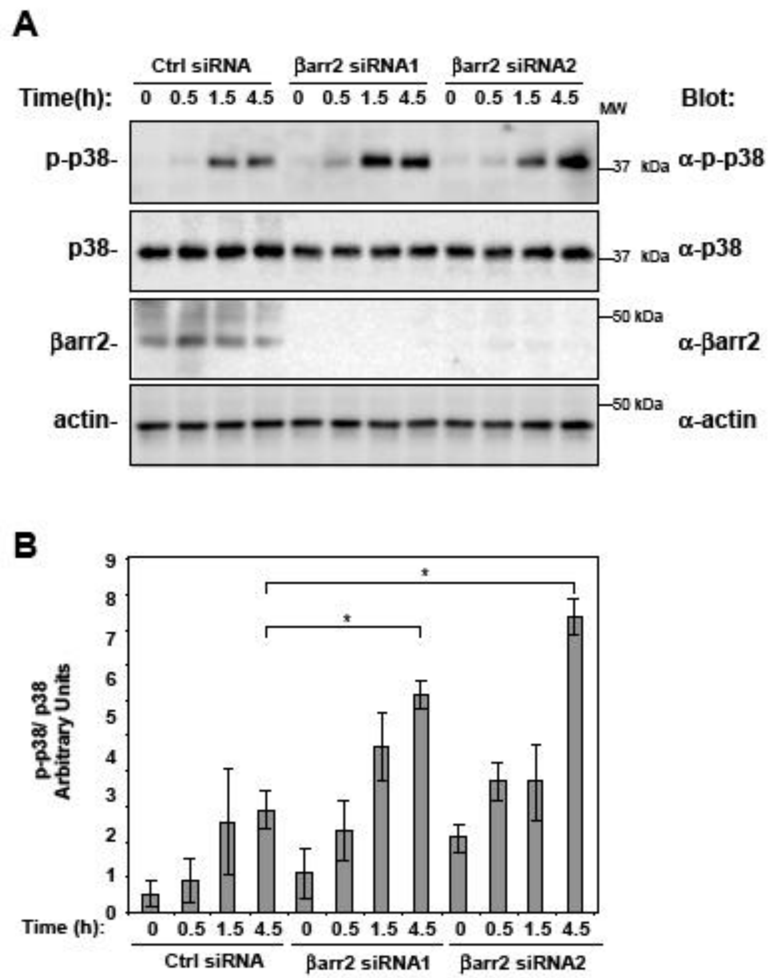
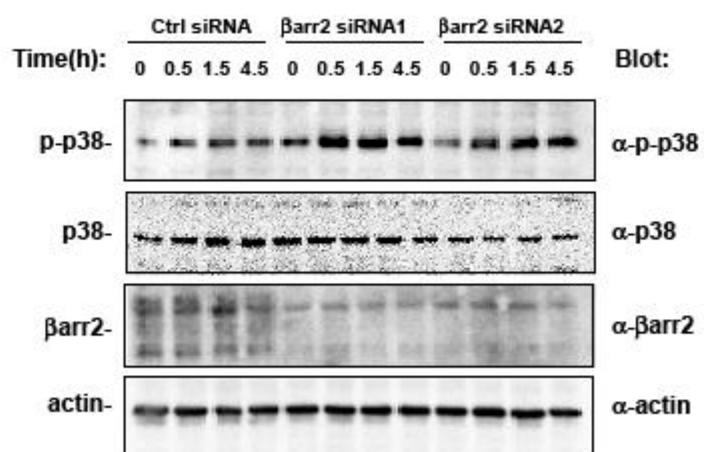


Figure 4.10 Decreased βarrestin2 expression increases p38 phosphorylation in H1299 cells

H1299 cells transiently transfected with non-specific control siRNA (Ctrl siRNA) or two different siRNA to βarrestin2 (βarr2 siRNA1 or βarr2 siRNA2) were assessed for TGFβ-dependent p38 phosphorylation. Serum-deprived cells were either immediately lysed or treated with 250 pM TGFβ1 for 30 minutes. Cells were then washed in PBS and further incubated for 1 or 4 hours or were lysed. All lysates were subjected to SDS-PAGE and immunoblotted for phospho-p38, total p38, βarrestin2 and actin levels (N=3).

Figure 4.10



4.4.9 *siRNA directed to βarrestin2 predisposes cells to apoptosis and increases TGFβ-dependent apoptosis*

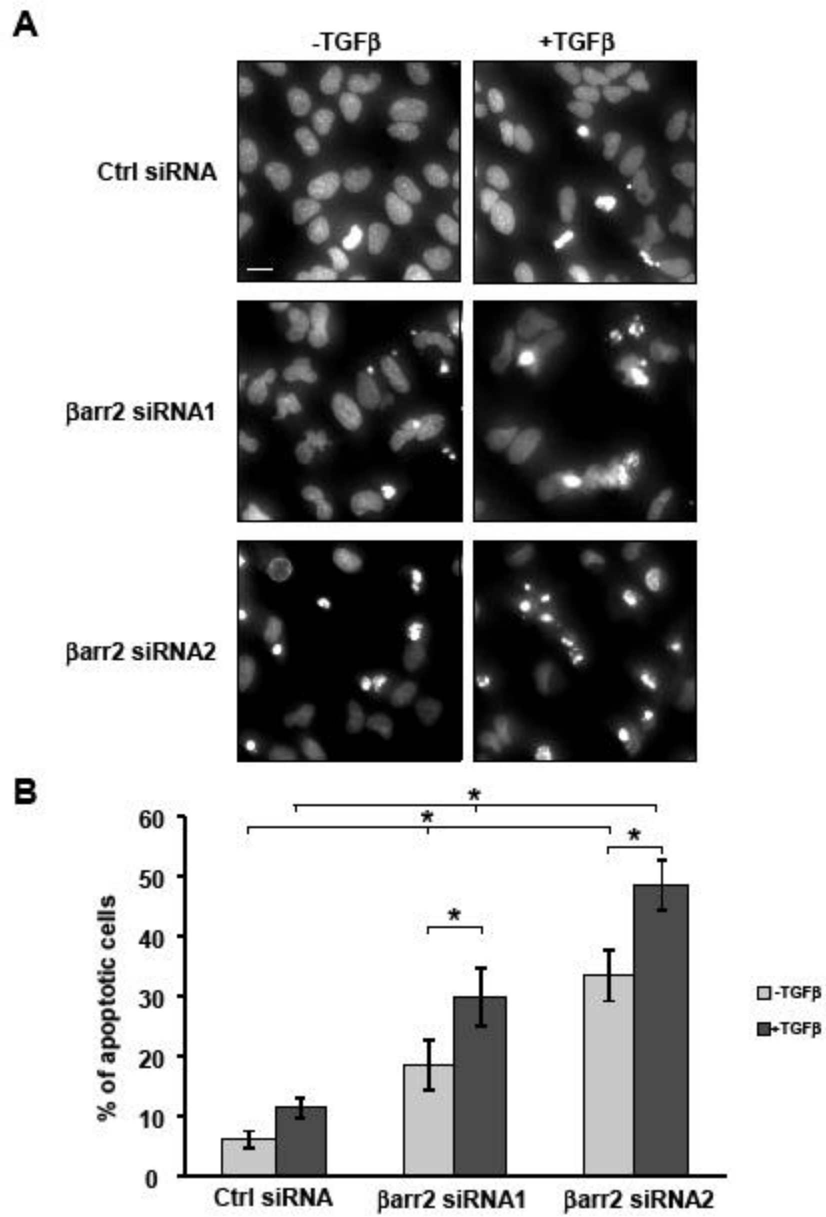
Having found that decreasing βarrestin2 expression increased levels of phosphorylated p38, I wanted to assess whether this increase in phosphorylation would have functional outcomes for the cell; or, similar to my results with phosphorylated Smad2, would not result in increased signal transduction. The p38 MAPK pathway is well-known as a stress-activated pathway, and its activation has been shown to induce apoptosis (19). Furthermore, Yu and colleagues have shown that p38 is necessary for TGFβ-induced apoptosis (20). Therefore, I decided to assess TGFβ-induced apoptosis in A549 cells as a functional read-out for phosphorylated p38. A549 cells were plated in 12-well dishes and transfected with siRNA against βarrestin2, as previously described. The day following transfection, cells were serum-starved in 0.2% FBS/F12K for 4 hours, then were either left in low-serum media or were treated with 250 pM TGFβ for 48 hours. Following treatment, cells were incubated with Hoechst 33342 prior to imaging on an inverted IX-71 immunofluorescence microscope. Figure 4.11 illustrates the outcome of the apoptosis assays. In the negative control siRNA treated cells, cells that were not stimulated with TGFβ demonstrated little apoptosis (approximately 7% of cells) (Figure 4.11A, 4.11B), while those treated with TGFβ had a moderate increase in apoptosis (approximately 12% of cells) which did not reach statistical significance (Figure 4.11).

Figure 4.11 Decreased βarrestin2 protein levels increase cell death

(A) A549 cells were transiently transfected with ctrl siRNA or two different siRNA to βarrestin2 (βarr2 siRNA1 or βarr2 siRNA2). Following transfection cells were serum-starved and either treated with 250 pM of TGFβ1 for 48 hours or left untreated. Cells were then incubated with Hoechst 33342 and imaged on an inverted IX-71 immunofluorescence microscope. Panel A illustrates representative fields of view for the different conditions. (N=4). Bar= 10 µm.

(B) Quantification of apoptosis assay. Nine fields of view over four separate experiments (>100 cells/condition/experiment) were quantified by dividing apoptotic cells by total number of cells per field of view. The graph illustrates the percentage of apoptotic cells. One-way ANOVA followed by Bonferroni correction statistical analysis was performed. (*) indicate a statistically significant difference between the indicated conditions ($p<0.05$).

Figure 4.11



Interestingly, in the absence of TGFβ stimulation, both βarrestin2 siRNA conditions had a statistically significant increase in apoptotic cells compared to control (Figure 4.11B). Furthermore, both βarrestin2 siRNA conditions showed a statistically significant increase of apoptosis with TGFβ treatment, with approximately a 10% increase in total apoptosis (Figure 4.11B). This figure illustrates that decreasing βarrestin2 expression sensitizes cells to cell death.

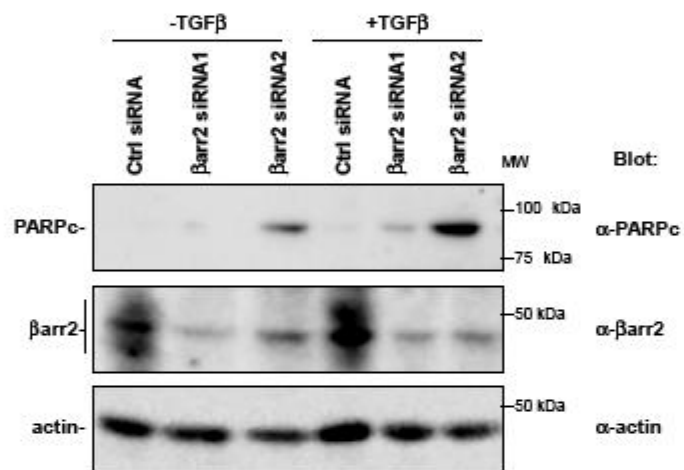
As the Hoechst apoptosis assay only allowed me to qualitatively assess dead cells, I wanted to ensure the cell death that I had found was indeed apoptosis and not necrosis. In order to answer this question, I performed a western blot to evaluate levels of the protein cleaved PARP. Poly(ADP-ribosylation) is a post-translational modification that is commonly implicated in DNA repair (21). The process of poly(ADP-ribosylation) is regulated in part by PARP, poly(ADP-ribosylation) polymerase (21). During the intermediate phase of apoptosis, PARP is activated but is later cleaved by a number of proteases, with the best-known being caspase-3 (22). The cleavage of PARP inactivates its activity, and apoptosis continues to progress.

To assess levels of cleaved-PARP in my cells, I performed western blot analysis and immunoblotting (Figure 4.12). Cells were transfected with βarrestin2 siRNA, then 16 hours post-transfection, cells were serum-starved for 4 hours and then either treated with 500 pM TGFβ for 48 hours or left untreated. Similar to my results using the Hoechst assay, I found that decreasing βarrestin2 levels increased the amount of cleaved-PARP, in the absence of TGFβ, particularly in the case of siRNA2 (see Figure 4.12, βarr2 siRNA2). However, similar to the

Figure 4.12 Decreased βarrestin2 levels increase cleaved-PARP

A549 cells were transiently transfected with ctrl siRNA or two different siRNA to βarrestin2 (βarr2 siRNA1 or βarr2 siRNA2). Following transfection, cells were serum-starved and either treated with 500 pM of TGFβ1 for 48 hours or left untreated. Cells were then lysed and subjected to SDS-PAGE and immunoblotting for cleaved-PARP as well as βarrestin2 and actin (N=3).

Figure 4.12



Hoechst experiments I saw an inductive effect of TGFβ in enhancing levels of cleaved-PARP (Figure 4.12).

Taken together, my results indicate a role for βarrestin2 in modulating TGFβ signalling pathways.

4.5 Discussion

While receptor complexes were previously thought to signal solely at the cell surface, a number of studies have shown that the localization of receptor complexes in different subcellular compartments can directly affect signal transduction. For example, it has recently been shown that the trafficking of the VEGF receptor, VEGFR-2, is dependent on its interaction with NRP-1, which enhances VEGFR-2 signalling and entrance into a recycling pathway (23). Similarly, Purvanov and colleagues found that the early endosomal GTPase Rab5 can directly interact with Go to activate the planar-cell-polarity pathway of Frizzled signalling (24). In the TGFβ signalling pathway, SARA, an early endosomal protein, enhances the ability of TβRI to phosphorylate Smads by bringing them into close proximity with one another (3, 27).

In this chapter, I have attempted to elucidate the role of βarrestin2 in TGFβ signal transduction. βarrestin2 has greater than twenty known binding partners (7), and is an important scaffolding protein in GPCR signalling. Chen *et al.*, originally reported that βarrestin2 functions to promote the internalization of TβRIII/TβRII complexes following the phosphorylation of TβRIII by TβRII (7). However, I have shown that the role of βarrestin2 in TGFβ signal transduction is

not merely limited to its interactions with TβRIII. Rather, I have shown that βarrestin2 can interact with TβRII in the absence of TβRIII. This interaction has significant implications in TGFβ signal transduction, as A549 cells, which have been shown to have very little endogenous TβRIII (16), show significant differences in TGFβ signal transduction through both canonical and non-canonical pathways. My results indicate that the loss of βarrestin2 promotes increased phosphorylation of both Smad2 and p38. However, the phosphorylation of Smad2 does not translate into functional signalling, as loss of βarrestin2 decreases TGFβ-dependent luciferase production. In their manuscript, Chen *et al* suggested that βarrestin2 increased TGFβ receptor endocytosis and loss of βarrestin2 increased TGFβ signalling (14). In this chapter, I have shown that loss of βarrestin2 increases the phosphorylation and activity of the p38 pathway.

While it may seem contradictory that loss of βarrestin2 increases phosphorylated Smad2 levels but decreases TGFβ-dependent transcription, an elegant study by Runyan *et al.* supports my findings. In their report, Runyan and colleagues evaluated the role of internalization in TGFβ-dependent Smad signal transduction. Using human kidney mesangial cells, the authors illustrated that inhibition of internalization only slightly affected levels of phosphorylated Smad2, and Smad2-SARA complexes (25). However, the authors also showed that inhibition of endocytosis greatly decreased Smad2 nuclear localization as well as Smad2-dependent transcriptional activation (25). Indeed in my study, I found

increased cell-surface levels of TβRII in the absence of βarrestin2. Since the inhibition of internalization does not affect the ability of Smad2 to be phosphorylated (25), it follows then that loss of βarrestin2 should not decrease Smad2 phosphorylation. However, given our finding that βarrestin2 increases the association of TβRII with SARA, it is plausible that while loss of βarrestin2 does not affect Smad2 phosphorylation, it may traffic with the receptor complex to the early endosome and enhance SARA-TβRII association.

In my study I also assessed a Smad-independent pathway, the p38 pathway, and my results showed that loss of βarrestin2 greatly increases p38 phosphorylation in the presence of TGFβ. Since one possibility is that greater numbers of TGFβ receptors are found at the cell surface in the absence of βarrestin2, this would mean that more receptors would be exposed to ligand and therefore activated for signalling. Since the activation of the p38 pathway by TGFβ is Smad-independent (17, 18); the internalization of the receptor complex does not appear to be necessary for p38 signal transduction. Indeed, I showed that increased levels of phosphorylated p38 had a functional outcome in the cell, as loss of βarrestin2 increased apoptosis, a well-established signalling event downstream of p38 signal transduction.

The trafficking of TGFβ receptors plays a crucial role in signal transduction, not only biochemically but in disease states as well. Recently, Park and colleagues showed that a TβRII mutant found in oral squamous cell carcinoma exhibits delayed internalization and promotes cancer cell migration

and invasion (26). Similarly, in scleroderma, a fibrotic disease which exhibits elevated TGFβ signalling, patients exhibit decreased levels of caveolin-1, a key component of membrane raft dependent internalization (32). These studies suggest that internalization and trafficking of TGFβ receptors can have significant implications in disease states. Therefore proteins like βarrestin2, which alter TGFβ trafficking can have significant affects on signal transduction and should be studied in detail to evaluate their effects in disease states such as cancer and fibrosis.

4.6 References

1. Shi, Y., and Massague, J. (2003) *Cell* **113**(6), 685-700

2. Lin, X., Liang, M., and Feng, X. H. (2000) *The Journal of biological chemistry* **275**(47), 36818-36822

3. McLean, S., and Di Guglielmo, G. M. *The Biochemical journal* **429**(1), 137-145

4. Marchese, A., Paing, M. M., Temple, B. R., and Trejo, J. (2008) *Annual review of pharmacology and toxicology* **48**, 601-629

5. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* **383**(6599), 447-450

6. Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G. (2000) *The Journal of biological chemistry* **275**(30), 23120-23126

7. DeFea, K. A. *Cellular signalling* **23**(4), 621-629

8. Lin, F. T., Daaka, Y., and Lefkowitz, R. J. (1998) *The Journal of biological chemistry* **273**(48), 31640-31643

9. Zajac, M., Law, J., Cvetkovic, D. D., Pampillo, M., McColl, L., Pape, C., Di Guglielmo, G. M., Postovit, L. M., Babwah, A. V., and Bhattacharya, M. *PloS one* **6**(6), e21599

10. Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F., and Wrana, J. L. (2003) *Nature cell biology* **5**(5), 410-421

11. Pampillo, M., Camuso, N., Taylor, J. E., Szereszewski, J. M., Ahow, M. R., Zajac, M., Millar, R. P., Bhattacharya, M., and Babwah, A. V. (2009) *Molecular endocrinology (Baltimore, Md* **23**(12), 2060-2074

12. Luga, V., McLean, S., Le Roy, C., O'Connor-McCourt, M., Wrana, J. L., and Di Guglielmo, G. M. (2009) *The Biochemical journal* **421**(1), 119-131

13. Derynck, R., and Feng, X. H. (1997) *Biochimica et biophysica acta* **1333**(2), F105-150

14. Chen, W., Kirkbride, K. C., How, T., Nelson, C. D., Mo, J., Frederick, J. P., Wang, X. F., Lefkowitz, R. J., and Blobe, G. C. (2003) *Science (New York, N.Y* **301**(5638), 1394-1397

15. Goodman, O. B., Jr., Krupnick, J. G., Gurevich, V. V., Benovic, J. L., and Keen, J. H. (1997) *The Journal of biological chemistry* **272**(23), 15017-15022

16. Finger, E. C., Turley, R. S., Dong, M., How, T., Fields, T. A., and Blobe, G. C. (2008) *Carcinogenesis* **29**(3), 528-535

17. Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995) *Science (New York, N.Y* **270**(5244), 2008-2011

18. Yamashita, M., Fatyol, K., Jin, C., Wang, X., Liu, Z., and Zhang, Y. E. (2008) *Molecular cell* **31**(6), 918-924

19. Wagner, E. F., and Nebreda, A. R. (2009) *Nature reviews* **9**(8), 537-549

20. Yu, L., Hebert, M. C., and Zhang, Y. E. (2002) *The EMBO journal* **21**(14), 3749-3759

21. Soldani, C., and Scovassi, A. I. (2002) *Apoptosis* **7**(4), 321-328

22. Decker, P., and Muller, S. (2002) *Current pharmaceutical biotechnology* **3**(3), 275-283

23. Ballmer-Hofer, K., Andersson, A. E., Ratcliffe, L. E., and Berger, P. *Blood* **118**(3), 816-826

24. Purvanov, V., Koval, A., and Katanaev, V. L. *Science signaling* **3**(136), ra65

25. Runyan, C. E., Schnaper, H. W., and Poncelet, A. C. (2005) *The Journal of biological chemistry* **280**(9), 8300-8308

26. Park, I., Son, H. K., Che, Z. M., and Kim, J. *Cancer letters*

CHAPTER 5

TGFβ3 IS A LESS POTENT INDUCER OF TGFβ SIGNALING THAN TGFβ1 IN NON-SMALL CELL LUNG CANCER CELLS

5 Chapter 5

5.1 Chapter summary

In Chapters 3 and 4 of my thesis, I assessed the ability of proteins which interact with TβRII, namely TβRIII and βarrestin2, to influence trafficking and signaling of the receptor complex. In this chapter I have evaluated the role of TGFβ ligand types 1 and 3, which can bind all three TGFβ receptors, in their ability to affect TGFβ trafficking and signaling. I show that overall TGFβ3 is much less potent than TGFβ1 at propagating TGFβ signalling. While I initially hypothesized that this would be due to alterations in endocytosis and trafficking similar to my other chapters, I found that both TGFβ ligands induced similar membrane raft partitioning and trafficking of the TGFβ receptor complex. However, I found that TGFβ3 induced a different binding ratio of TβRII/TβRI cell-surface complexes than TGFβ1. Therefore the level of receptor engagement at the cell surface may differ between the two ligands and may be able to account for the observed differences in signal transduction.

5.2 Introduction

The TGFβ superfamily consists of structurally and functionally related cytokines that are released into the extracellular matrix as inactive precursors (1). The TGFβ superfamily has two distinct subfamilies: the TGFβ/Activin/Nodal subfamily and the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF)/ Muellerian inhibiting substance (MIS) subfamily (2). The TGFβ/Activin/Nodal subfamily binds to serine-threonine kinase receptors at the cell surface which results in signal propagation utilizing Smads 2 and 3 (3). Similarly, signal transduction by the BMP/GDF/MIS subfamily is also propagated by serine-threonine kinase receptors, but their activation results in signal transduction utilizing Smads 1, 3, 5 and 8 (2). While signal transduction by the TGFβ superfamily has been implicated in normal development, such as dorsal/ventral patterning and angiogenesis, the TGFβ superfamily, in particular the canonical TGFβ pathway has been implicated in pathologies such as cancer and fibrosis (3).

There are three TGFβ ligands which share significant sequence homology and have relatively specific, non-overlapping functions *in vivo*: TGFβ1, TGFβ2 and TGFβ3 (4). For example, while *Tgfb1*$^{-/-}$ mice and *Tgfb2*$^{-/-}$ mice both generally die during development, *Tgfb1*$^{-/-}$ mice have significant vasculogenic defects (5), whereas *Tgfb2*$^{-/-}$ mice have cardiovascular, skeletal and pulmonary issues (6). Interestingly, *Tgfb3*$^{-/-}$ mice survive gestation but die shortly after birth due to an inability to suckle caused by cleft palate (7). Although TGFβ ligands

have different functional roles, they are all secreted as inactive, homodimeric proproteins that must be cleaved by TGFβ activating molecules, such as matrix metalloproteinase 2, thrombospondin-1 and plasmin (8-10) . Active TGFβ is a homodimer stabilized by disulfide bridges and hydrophobic interactions (11). Once TGFβ has been activated, it is able to elicit downstream transcriptional events through binding and activating TGFβ receptors.

To propagate TGFβ signaling, ligand is presented to the TβRII with the aid of TβRIII. The binding of ligand to TβRII causes TβRII to transphosphorylate the TβRI at serine-threonine residues in its GS domain (12). Phosphorylated TβRI recruits the receptor-regulated Smads, or R-Smads, and phosphorylates, and thereby activates them. Once the R-Smads have been phosphorylated, they are able to recruit the Co-Smad, Smad4, to form a heteromeric complex. With the aid of specific nuclear localization signals, the heteromeric Smad complex is able to translocate to the nucleus and interact with transcriptional co-activators and co-repressors in order to induce cell-specific transcriptional programs (11).

Despite activating the same signal transduction pathway, the TGFβ ligands have vastly different effects in the wound microenvironment. For example, TGFβ1 signalling in fibroblasts promotes ECM production and myofibroblast differentiation, resulting in a scar following wound-resolution (13). Similarly, inhibition of either TGFβ1 or TGFβ2 with neutralizing antibodies improves wound resolution and scar appearance in adult rodent wounds (14,15). However, application of exogenous TGFβ3 results in scar-free wound-resolution

in the same model system (16). This suggests that the different TGFβ ligands do not have the same signalling effect in the wound microenvironment.

The vast majority of studies on TGFβ in cancer have focussed on TGFβ1 (4). TGFβ1 has a dual role in cancer: in early stages of tumourigenesis, TGFβ1 is growth-inhibitory and induces cell cycle arrest (17). However, in advanced cancers, TGFβ1 promotes epithelial-to-mesenchymal transition of tumour cells and promotes migration and invasion (17). While all three TGFβ ligands are elevated in various tumours, the role of TGFβ3 has simply been assumed to be the same as TGFβ1 without subsequent in-depth biochemical studies to support this notion (4). Given the opposite roles of TGFβ1 and TGFβ3 in the wound microenvironment and in development, it is unlikely that these two ligands have the same signalling outcome in the tumour microenvironment and cancer cells. Therefore in this chapter I have attempted to evaluate the mechanism of how structurally related TGFβ ligands activate receptor signaling cascades and downstream cellular events.

5.3 Materials and Methods

5.3.1 *Cell culture*

HEK293T cells were maintained in DMEM (Dulbecco's modified Eagle's medium) (Gibco) supplemented with 10% fetal bovine serum. A549 cells were maintained in F12K media (ATCC) supplemented with 10% fetal bovine serum. Mink Lung cells stably transfected with HA-tagged TβRII (Mink Lung HAT cells)

were cultured in MEM supplemented with non-essential amino acids, 10% fetal bovine serum and 0.3% hygromycin. All cells were maintained at 37°C in 5% CO_2.

5.3.2 *Constructs*

The pCMV5 cDNA construct encoding the carboxy terminus hemagluttinin (HA) epitope tagged type II TGFβ receptor (TβRII-HA) was used as previously described (18).

5.3.3 *Transfection*

HEK293T cells were transiently transfected using the calcium phosphate precipitation method; 5μg of TβRII-HA was transfected per 100mm dish (19,20).

5.3.4 *Isolation of caveolae/membrane-raft-enriched membrane fractions-*

Membrane rafts were isolated as previously described (18-20). Briefly, transfected HEK293T cells were grown to confluence in 100-mm-diameter dishes. Cells were washed twice with cold 1X PBS and lysed in 0.5 M Na_2CO_3, pH 11.0 containing protease inhibitors. After the cells were scraped, the cell lysate was homogenized in three 10 second bursts using a Polytron tissue homogenizer (Brinkmann Instruments). Cells were then sonicated three times for 20 seconds each with a Vibra Cell sonicator (Sonics and Materials). The homogenates were then adjusted to 40% sucrose, and overlaid with 30% sucrose and 5% sucrose solutions. The samples were centrifuged for 16h at 200,000 g_{av} at 4°C using a Beckman SW41 rotor. Following centrifugation, 12x1mL samples were collected and an aliquot of each sample was denatured

with Laemmli sample prep buffer, boiled and subjected to SDS-PAGE followed by immunoblotting.

5.3.5 *Immunofluorescence Microscopy*

Mv1Lu HAT cells were plated on coverslips in a 12 well plate and transfected with the indicated constructs. Cells were then incubated with biotinylated-TGFβ1 or biotinylated-TGFβ3 (Peprotech) in 0.5% BSA in KRH for 2hrs at 4°C. Cells were washed three times with buffer, and then incubated with Cy3-labelled streptavidin for 1 hr at 4°C. Cells were fixed, permeabilized and incubated with anti-EEA1 primary and secondary antibodies as described previously (18,19). Images were obtained using an Olympus Ix81 inverted microscope using InVivo® software.

5.3.6 *Immunoblotting*

Proteins were separated by SDS-PAGE (10% gels) and transferred to nitrocellulose by electrophoretic transfer. Blots were incubated for 1 hr in 5% skim milk/TBST. After incubation with primary and secondary antibodies, bound antibodies were detected using SuperSignal chemiluminescence reagent (Pierce) and a VersaDoc imager (Biorad).

5.3.7 *Epithelial to mesenchymal cell marker analysis*

A549 cells were incubated with low-serum containing control medium or low-serum media containing increasing concentrations of TGFβ1 or TGFβ3 for 48 hours. Cells were then lysed and immunoblotted with α-E-cadherin (epithelial cell marker) or α-N-cadherin (mesenchymal cell marker) as described above.

5.3.8 *Affinity labelling*

A549 cells were labelled for 2 hrs with 250 pM [^{125}I] TGFβ1 or 250 pM [^{125}I] TGFβ3 ligand (Peprotech) in 0.5% BSA in KRH at 4°C. Cells were cross-linked to ligand using DSS as described previously (19). Cells were then either immediately lysed in 1XTNTE or incubated in media with 10% FBS at 37°C for 2, 4 or 8 hrs prior to lysis. Samples were eluted with Laemmli sample prep buffer, and separated using SDS-PAGE (7.5% gels). Receptors were visualized using phosphorimaging (Molecular Dynamics).

5.4 **Results**

5.4.1 *TGFβ3 is less potent at inducing Smad2 phosphorylation than TGFβ1*

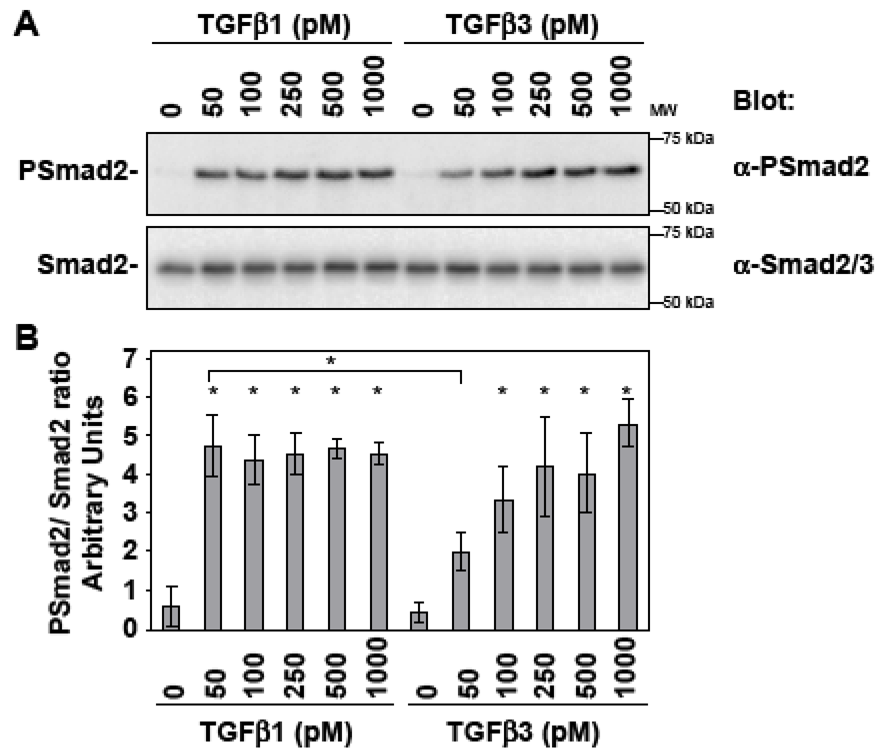
As it has previously been shown that TGFβ1 and TGFβ3 exert vastly different outcomes in the wound microenvironment (21), I wanted to assess the ability of these ligands to transmit signals in cancer cells, as many of the same growth factors elevated in the wound microenvironment are also elevated in the tumor microenvironment. A549 non-small cell lung adenocarcinoma cells were used as a model to begin to characterize the role of these ligands in cancer cell signaling. A549 cells were serum-starved overnight prior to a one hour treatment with TGFβ1 or TGFβ3 ligand, ranging in concentration from 50 pM to 1 nM prior to lysis and immunoblotting with anti-phosphoSmad2 antibodies (Figure 5.1A). I observed that TGFβ1 induced the phosphorylation of Smad2 maximally at 50 pM. This is consistent with our previous results using Mv1Lu cells (20). TGFβ3 was also observed to stimulate a similar amount of Smad2 phosphorylation, however

Figure 5.1. TGFβ3 is less potent than TGFβ1 in inducing Smad2 phosphorylation.

(**A**) A549 cells were serum-starved and treated with increasing concentrations of TGFβ1 or TGFβ3 for 1 hour. Following lysis, cells were subjected to SDS-PAGE and immunoblotting for phosphorylated Smad2 (PSmad2) or Smad 2 (N=3).

(**B**) Three separate experiments as described in Panel A were carried out and the amounts of PSmad2 and Smad2 were quantified using QuantityOne software and plotted as the ratio of PSmad2/Smad2. The mean (Arbitrary Units) ± SD is shown. One-way ANOVA followed by a Tukey's Multiple Comparison test was conducted to evaluate statistical significance. * indicates a statistical significance of $p<0.05$.

Figure 5.1



at a concentration of 250 pM (Figure 5.1B). This five-fold difference in the stimulation of Smad2 phosphorylation was next functionally tested in the ability of TGFβ1 and TGFβ3 to induce differences in protein expression of the EMT markers E-cadherin and N-cadherin.

5.4.2 *TGFβ1 is more potent at altering steady-state cellular EMT markers than TGFβ3*

In order for epithelial-based cancers to metastasize, they first must release their cell-cell contacts, change their cytoskeletal arrangement, and acquire a motile phenotype (22). Loss of E-cadherin, an adhesion molecule, is one of the first indications of epithelial-to-mesenchymal transition. As TGFβ1 has been shown to induce loss of E-cadherin (reviewed in (22)), I wanted to assess whether TGFβ3 also shared this capability. A549 cells were serum-starved overnight and then treated with increasing concentrations of TGFβ1 or TGFβ3 for 48 hours (Figure 5.2). I observed that TGFβ1 stimulated a loss of steady state E-cadherin levels in A549 cells that was maximal at 250 pM (Figure 5.2B). Interestingly, TGFβ3 did not induce a pronounced decrease in the steady-state levels of E-cadherin, compared to TGFβ1 (Figure 5.2A). Indeed, I observed a 10-fold difference in the abilities of TGFβ3 or TGFβ1 to decrease the steady-state levels of E-cadherin by 50% (Figure 5.2B; 100 pM for TGFβ1 *vs.* 1 nM for TGFβ3).

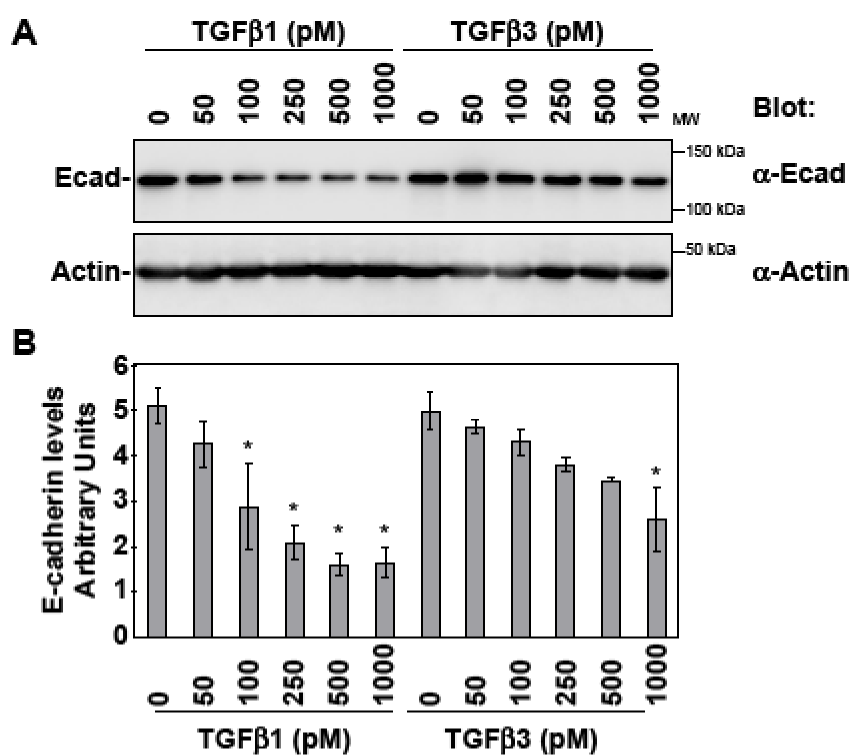
While loss of E-cadherin occurs frequently with the progression of cancer, upregulation of N-cadherin, a neuronal cadherin, has also been shown to occur during EMT (23). Increased levels of N-cadherin are correlated with tumor

Figure 5.2. TGFβ1 is more potent than TGFβ3 at reducing E-cadherin levels

(**A**) A549 cells were serum-starved and treated with increasing concentrations of TGFβ1 or TGFβ3 for 48 hours. Following lysis, cells were subjected to SDS-PAGE and immunoblotting for E-cadherin (Ecad) or Actin (N=3).

(**B**) Three separate experiments as described in Panel A were carried out and the level of E-cadherin was quantitated using QuantityOne software. The means (Arbitrary Units) ± SD are shown. Statistical significance was evaluated using a One-Way ANOVA test followed by a Bonferroni correction (* $p<0.05$).

Figure 5.2



metastasis (23). Therefore I wanted to assess the effect of TGFβ3 on steady-state levels of N-cadherin. Again, A549 cells were serum-starved then treated with increasing concentrations of either TGFβ1 or TGFβ3 for 48 hours (Figure 5.3). I observed that TGFβ3 is less potent than TGFβ1 in increasing N-cadherin steady-state levels (Figure 5.3A). While TGFβ1 induces more N-cadherin protein at concentrations as low as 100 pM, TGFβ3 required higher concentrations to induce only very minor increases in N-cadherin levels (Figure 5.3B). These differences in steady state EMT markers may be a global effect that these two TGFβ sub-types have on cells.

5.4.3 *TGFβ1 and TGFβ3 ligand treatment do not alter TGFβ receptor membrane partitioning*

As I have shown that TGFβ1 and TGFβ3 have strikingly different signal transduction capabilities I hypothesized that these differences in signaling could be accounted for by receptor membrane partitioning, similar to my other data chapters. To address this question, HEK293T cells were transiently transfected with HA-TβRII cDNA. Cells were serum-starved 16 hours prior to membrane raft preparation, and were then treated with either 250 pM TGFβ1, 250 pM TGFβ3, 0.2% FBS or 10% FBS (as indicated) for 30 minutes. Cells were subjected to sucrose-density ultracentrifugation and immunoblotting with anti-HA antibodies to identify tagged TGFβ receptors. Figure 5.4 illustrates that in the presence and absence of ligand, the TGFβ receptors were observed primarily in membrane raft fractions. Therefore, it appeared that ligand treatment of specific TGFβ isoforms did not directly affect membrane partitioning of receptors.

Figure 5.3. TGFβ1 induces greater N-cadherin steady-state levels than TGFβ3

(**A**) A549 cells were serum-starved and treated with increasing concentrations of TGFβ1 or TGFβ3 for 48 hours. Following lysis, cells were subjected to SDS-PAGE and immunoblotting for N-cadherin (Ncad) or Actin (N=3).

(**B**) Three separate experiments as described in Panel A were carried out and the level of N-cadherin was quantitated using QuantityOne software. The means (Arbitrary Units) ± SD are shown. One-way ANOVA followed by a Tukey's Multiple Comparison test was conducted to evaluate statistical significance. * indicates a statistical significance of $p<0.05$, whereas ** indicates a statistical significance of $p<0.001$.

Figure 5.3

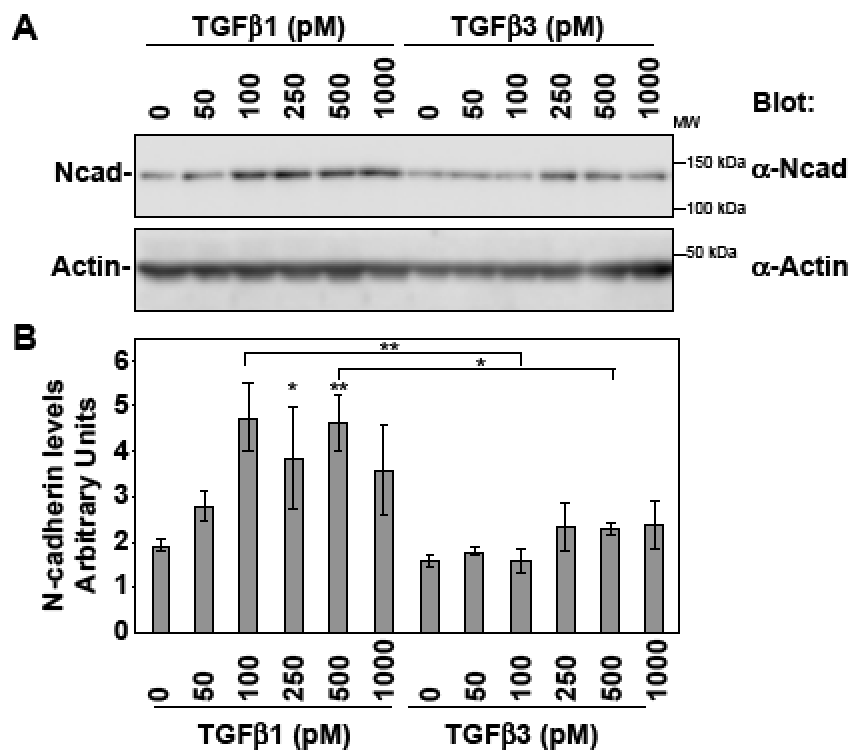
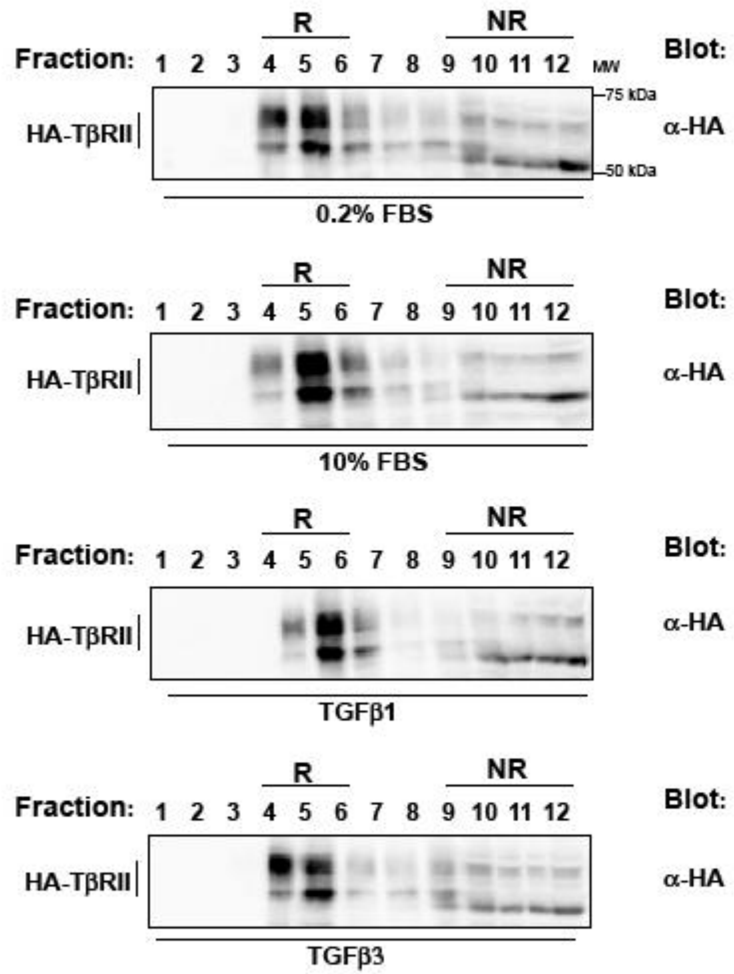


Figure 5.4. Ligand treatment does not influence TGFβ receptor partitioning

HEK 293T cells were transiently transfected with TβRII-HA cDNA. Cells were serum-starved for 16 hours prior to a 30 minute treatment with 250 pM ligand or FBS (as indicated). The cells were then lysed and processed for ultracentrifugation as previously described (18). The collected fractions containing membrane rafts (R) and non-raft (NR) were immunoblotted with anti-HA antibodies to visualize the partitioning of expressed TβRII (N=3).

Figure 5.4



5.4.4 *TGFβ1 or TGFβ3 treatment do not differ in their trafficking of TβRII to the early endosome*

Although TGFβ1 and TGFβ3 do not alter TGFβ receptor complex membrane partitioning, I wanted to assess the trafficking of receptors following ligand treatment. It has been shown that the greater the amount of time spent in the early endosome, the greater the Smad signalling potential (20,24,25). Therefore, I hypothesized that perhaps the differences in signaling could be accounted for by receptor trafficking to the early endosome. Using biotinylated-TGFβ1 or TGFβ3, HAT Mv1Lu cells stably expressing TβRII were labelled at the cell surface at 4˚C. Receptors were then incubated with streptavidin-Cy3, and permitted to internalize for 10, 20, or 60 minutes (Figure 5.5). Using EEA1 (early endosomal autoantigen-1) as a marker for the early endosome, I found that both ligands co-localized in EEA1-positive compartments at 20 minutes, and significant co-localization occurred at 60 minutes.

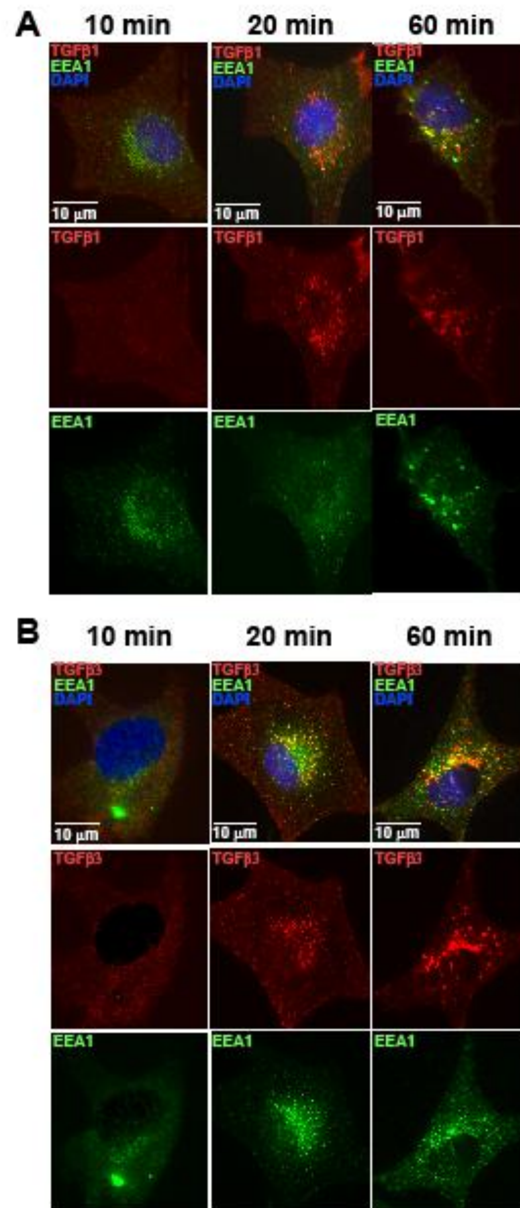
5.4.5 *TGFβ3 promotes a different binding ratio of TβRII/TβRI complexes than TGFβ1*

While it has been previously found that TGFβ3 binds to TβRII dimers with a greater affinity than TGFβ1 (26), I wanted to ensure that our ligands bound similarly in our experiments. To address this question, TGFβ1 and TGFβ3 were labelled with ^{125}I and performed receptor binding studies. Briefly, Mv1Lu cells were labelled with saturating doses of ^{125}I-TGFβ isoforms, for 2 hours at 4˚C. The ligands were cross-linked, and lysates were subjected to SDS-PAGE.

Figure 5.5 Cells treated with TGFβ1 or TGFβ3 exhibit similar trafficking of TβRII

Mv1Lu cells stably over-expressing HA-TβRII were labelled at 4˚C for 2 hours with biotinylated TGFβ1 (**A**) or TGFβ3 (**B**). Following incubation of Cy3-labelled streptavidin at 4˚C, cells were incubated for 10, 20 or 60 minutes (as indicated) at 37˚C to permit receptor internalization. Standard immunofluorescence staining was used to visualize EEA1, a marker for the early endosome, and nuclei (DAPI staining). Cells were assessed for receptor complex co-localization with the early endosome, which results in a yellow overlay (N=4). Bar= 10 μm

Figure 5.5



I observed that both TGFβ1 and TGFβ3 associated with TβRII (Figure 5.6A). However, it appears that the amount of TβRI recruited to the TGFβ/TβRII complex was lower for TGFβ3 than TGFβ1 (Figure 5.6B). As the kinase activity of TβRI is responsible for initiating the Smad signaling cascade, and TβRI must be activated by TβRII following ligand binding to initiate the cascade, TβRII/TβRI ratios may play a crucial role in determining signaling potential. Therefore, if TGFβ3 induces less TβRI to be bound to TβRII, this may result in fewer activated TβRI to propagate Smad signal transduction.

5.5 Discussion

In the canonical TGFβ signaling pathway, the association of TGFβ to the type II receptor (TβRII) is the first step in activation of the signaling cascade (27,28). This is followed by the recruitment of the type I receptor (TβRI) to the TβRII/TGFβ complex and the transphosphorylation of TβRI in its GS domain by TβRII. The now active TβRI initiates downstream R-Smad (Smad2/3) phosphorylation leading to the formation of a complex between R-Smads with the common Smad, Smad4, and their nuclear import to affect transcription (2).

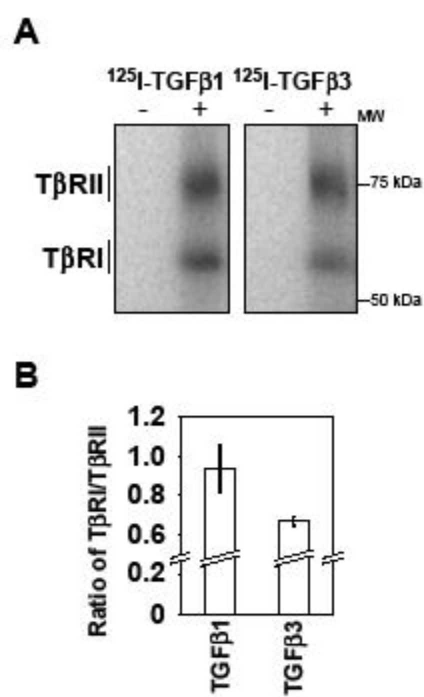
In this chapter, I have demonstrated that the degree of recruitment of TβRI to the TβRII/TGFβ complex is dependent on isoform-specific TGFβ ligands. I observed that TGFβ1 is more effective in recruiting TβRI to the complex than TGFβ3. This has greater implications to signal transduction and I observed a muted response to ligand-dependent activation of the Smad signaling pathway

Figure 5.6. TGFβ isoform specific receptor complex formation

(**A**) Mv1Lu cells were labelled at 4˚C for 2 hours with ^{125}I-TGFβ1 or ^{125}I-TGFβ3, cross-linked, subjected to SDS-PAGE and visualized by phosphorimaging (N=3).

(**B**) Ratios of TβRI and TβRII were determined using QuantityOne software. The graph illustrates the amount of TβRI associated with TβRII. (mean ± SD). (N=3)

Figure 5.6



and muted changes of EMT markers when cells were incubated with TGFβ3 compared to TGFβ1. As previously mentioned, during adult wound healing, TGFβ1 is found at very high levels, and promotes myofibroblast differentiation, extracellular matrix production, and fibroblast chemotaxis (29). In contrast, TGFβ3 promotes scar-free healing (29). Therefore, my results may account for some of the differences seen in wound healing because even though TGFβ ligands can activate the same receptors to propagate signal transduction, the level of isoform specific receptor engagement will influence transcriptional outcome in TGFβ signaling. To confirm these results, it may be of interest to assess the ability of TGFβ3 to induce differentiation of fibroblasts to myofibroblasts.

Though studies have shown that TGFβ3 promotes scar-free healing (29), few studies have assessed the ability of TGFβ3 to induce myofibroblast differentiation. Interestingly, a study by Waddington and colleagues showed that increasing the bioavailability of TGFβ3 may increase its efficacy in promoting scar-free wound-healing. In this study, the authors created a mutant TGFβ3 which did not bind latent TGFβ binding protein (LTBP), thereby increasing its bioavailability, as LTBP has been shown to sequester TGFβ ligand in the ECM (30). The authors used a lentiviral-based delivery system to deliver this construct *in vivo* and illustrated that it decreased scar tissue markers in a mouse wounding model (30). Therefore, modulating the bioavailability of TGFβ3 in the ECM may provide an interesting therapeutic target for scar tissue formation. Since my

results also show differences between TGFβ1 and TGFβ3 signalling ability in cancer cells, it may be interesting to assess whether modulating the bioavailability of TGFβ3 in the tumour microenvironment through a similar system could mediate tumourigenicity in an *in vivo* model.

An intriguing avenue of study to complement my work on isoform-specific TGFβ signal transduction would be to assess the binding capacity of TGFβ1 and TGFβ3 to the TGFβ receptor complex. It has been reported that TGFβ3 has the greatest ability to bind TβRII (4). Therefore it would be interesting to assess whether TGFβ1 and TGFβ3 could compete for binding of TβRII. If TGFβ3 is able to displace TGFβ1 from TβRII, and since my results show that TGFβ3 induces less signal transduction in cancer cells, increasing the amount of TGFβ3 may be able to competitively displace TGFβ1 and therefore decrease the detrimental effects of TGFβ1 signalling in cancer cells.

TGFβ ligands normally dimerize to associate and cluster receptors effectively. A recent study substituted one of the dimerized TGFβ3 with TGFβ3 WD (31). The TGFβ3 WD that was designed by the authors is a heteromeric TGFβ ligand composed of one wild-type TGFβ3 and one TGFβ3 in which Arg25 and Arg94 were substituted with glutamate, and Tyr90 was substituted with alanine (31), residues shown to be important for ligand binding. These residues are also missing in TGFβ2. This TGFβ3 wild-type/ TGFβ3 WD dimer was found to associate with TβRII and recruited the TβRI with affinities similar to wild-type TGFβ3, but with one-half the stoichiometry (31). TGFβ3 WD was further shown

to retain approximately half the signaling activity of TGFβ3 in three TGFβ assays such as Smad3 phosphorylation and growth inhibition. The authors also provided evidence that the two TβRI/TβRII heterodimers bind and signal in an independently of one another. Taken together, these results and my observations suggest that TGFβ1 and TGFβ3 have different receptor clustering potential. Future work comparing the amino acid sequences of the TGFβ1 *vs.* TGFβ3 ligands, which are >76% identical, may show specific regions of the ligands that are responsible for receptor binding and engagement.

Another interesting consequence of my study was the observation that TβRII membrane partitioning and trafficking is not influenced by isoform-specific ligand binding. This is consistent with previous results which illustrated that the trafficking of TβRII is not dependent on ligand association (20). Indeed, the recruitment of TβRI to the constitutively trafficking TβRII may be the limiting factor in receptor signaling potential, where TβRI must access the early endosome to phosphorylate Smad2 and initiate the signal transduction cascade. Further studies on the effect of ligand specific trafficking of TβRI may further elucidate this theory.

Since the mechanism of TGFβ receptor endocytosis has a profound effect on TGFβ signaling, and a number of pathologies show aberrant TGFβ signaling, an in-depth study evaluating the mechanism through which TGFβ receptors are directed to internalize is warranted.

5.6 References

1. Feng, X. H., and Derynck, R. (2005) *Annual review of cell and developmental biology* **21**, 659-693

2. Massague, J. (2008) *Cell* **134**(2), 215-230

3. Wu, M. Y., and Hill, C. S. (2009) *Developmental cell* **16**(3), 329-343

4. Laverty, H. G., Wakefield, L. M., Occleston, N. L., O'Kane, S., and Ferguson, M. W. (2009) *Cytokine & growth factor reviews* **20**(4), 305-317

5. Shull, M. M., Ormsby, I., Kier, A. B., Pawlowski, S., Diebold, R. J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., and et al. (1992) *Nature* **359**(6397), 693-699

6. Sanford, L. P., Ormsby, I., Gittenberger-de Groot, A. C., Sariola, H., Friedman, R., Boivin, G. P., Cardell, E. L., and Doetschman, T. (1997) *Development (Cambridge, England)* **124**(13), 2659-2670

7. Proetzel, G., Pawlowski, S. A., Wiles, M. V., Yin, M., Boivin, G. P., Howles, P. N., Ding, J., Ferguson, M. W., and Doetschman, T. (1995) *Nature genetics* **11**(4), 409-414

8. Annes, J. P., Munger, J. S., and Rifkin, D. B. (2003) *Journal of cell science* **116**(Pt 2), 217-224

9. Sato, Y., and Rifkin, D. B. (1989) *The Journal of cell biology* **109**(1), 309-315

10. Yu, Q., and Stamenkovic, I. (2000) *Genes & development* **14**(2), 163-176

11. Shi, Y., and Massague, J. (2003) *Cell* **113**(6), 685-700

12. Mehra, A., and Wrana, J. L. (2002) *Biochemistry and cell biology = Biochimie et biologie cellulaire* **80**(5), 605-622

13. Biernacka, A., Dobaczewski, M., and Frangogiannis, N. G. *Growth factors (Chur, Switzerland)* **29**(5), 196-202

14. Shah, M., Foreman, D. M., and Ferguson, M. W. (1992) *Lancet* **339**(8787), 213-214

15. Shah, M., Foreman, D. M., and Ferguson, M. W. (1994) *Journal of cell science* **107 (Pt 5)**, 1137-1157

16. Shah, M., Foreman, D. M., and Ferguson, M. W. (1995) *Journal of cell science* **108 (Pt 3)**, 985-1002

17. Akhurst, R. J., and Derynck, R. (2001) *Trends in cell biology* **11**(11), S44-51

18. Luga, V., McLean, S., Le Roy, C., O'Connor-McCourt, M., Wrana, J. L., and Di Guglielmo, G. M. (2009) *The Biochemical journal* **421**(1), 119-131

19. McLean, S., and Di Guglielmo, G. M. *The Biochemical journal* **429**(1), 137-145

20. Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F., and Wrana, J. L. (2003) *Nature cell biology* **5**(5), 410-421

21. Occleston, N. L., Fairlamb, D., Hutchison, J., O'Kane, S., and Ferguson, M. W. (2009) *Expert opinion on investigational drugs* **18**(8), 1231-1239

22. Bierie, B., and Moses, H. L. (2006) *Nature reviews* **6**(7), 506-520

23. Hazan, R. B., Qiao, R., Keren, R., Badano, I., and Suyama, K. (2004) *Annals of the New York Academy of Sciences* **1014**, 155-163

24. Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L., and Wrana, J. L. (1998) *Cell* **95**(6), 779-791

25. Itoh, F., Divecha, N., Brocks, L., Oomen, L., Janssen, H., Calafat, J., Itoh, S., and Dijke Pt, P. (2002) *Genes Cells* **7**(3), 321-331

26. ten Dijke, P., Iwata, K. K., Goddard, C., Pieler, C., Canalis, E., McCarthy, T. L., and Centrella, M. (1990) *Molecular and cellular biology* **10**(9), 4473-4479

27. Attisano, L., Wrana, J. L., Lopez-Casillas, F., and Massague, J. (1994) *Biochimica et biophysica acta* **1222**(1), 71-80

28. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) *Nature* **370**(6488), 341-347

29. Ferguson, M. W., and O'Kane, S. (2004) *Philosophical transactions of the Royal Society of London* **359**(1445), 839-850

30. Waddington, S. N., Crossley, R., Sheard, V., Howe, S. J., Buckley, S. M., Coughlan, L., Gilham, D. E., Hawkins, R. E., and McKay, T. R. *Mol Ther* **18**(12), 2104-2111

31. Huang, T., David, L., Mendoza, V., Yang, Y., Villarreal, M., De, K., Sun, L., Fang, X., Lopez-Casillas, F., Wrana, J. L., and Hinck, A. P. *The EMBO journal* **30**(7), 1263-1276

CHAPTER 6

GENERAL DISCUSSION

6 Chapter 6

6.1 Summary and General Discussion

The TGFβ signalling pathway is a cell-type and context-dependent pathway which has pleiotropic effects. While it was initially thought that TGFβ signal transduction simply occurred from the cell surface and was mediated by the Smad family of transcription factors, it is now understood that TGFβ receptor internalization and trafficking play key roles in regulating signalling outcome. It has been shown that receptors internalized by clathrin-mediated endocytosis traffic to the early endosome where they can interact with SARA to propagate Smad-mediated transcription (1-3). However, receptors internalized by membrane raft-dependent, clathrin-independent mechanisms traffic to the caveolin-1 positive vesicle, are prevented from signal propagation and are targeted for degradation. While the role of endocytosis and trafficking in this pathway has now been established, the signal(s) directing the TGFβ receptors to internalize *via* clathrin-dependent endocytosis or clathrin-*independent* endocytosis were not well understood. The overall purpose of my study was to evaluate factors directing TGFβ receptor internalization. I evaluated the role of domains of TβRII, the interaction of TβRII with TβRIII, the interaction of TβRII with βarrestin2, and the role of TGFβ ligand isoforms on TGFβ receptor trafficking and subsequent signal transduction. Therefore, in this body of work I have assessed both intracellular and extracellular factors which direct TGFβ receptor trafficking and have direct effects on signalling outcome.

6.1.1 *The extracellular domain of TβRII directs entry into membrane-raft fractions*

My work, presented in chapter 2, complemented a study I carried out with Valbona Luga to identify domains of TβRII which may direct receptor internalization. We used truncation mutants of TβRII and evaluated their ability to partition into membrane raft fractions. Previously very little work had been done on identifying motifs involved in TβRII internalization. A study performed by Ehrlich and colleagues had identified a di-leucine motif in the cytoplasmic domain of TβRII which regulated clathrin-mediated endocytosis (4); but no studies had attempted to evaluate the role of the extracellular domain in regulating TβRII internalization. In chapter 2, I showed that both the extracellular and intracellular truncation mutants of TβRII were able to interact with TβRI. This was an important parameter to assess as it has been shown that TβRII and TβRI form heterocomplexes at the cell surface (5) and it has been suggested that the binding ratio of TβRII:TβRI may also affect endocytic trafficking of the receptors (6). Upon analysis of TβRII receptor partitioning, we found that the extracellular truncation mutant of TβRII had a marked decrease in membrane-raft internalization. As TβRII has several extracellular glycosylation sites (7) we assessed whether the glycosylation status of TβRII affected its ability to partition into membrane rafts. We found that the glycosylation status of TβRII itself did not affect its membrane raft partitioning, but the glycosylation status of the cell as a whole did affect the partitioning of TβRII. Importantly, this was not due to a disruption in the ability of the cell to produce membrane rafts, as I illustrated that

treatment with nystatin did not disrupt membrane raft formation. Finally, I illustrated using a chimeric receptor composed of the GMCSF receptor extracellular domain and the intracellular domain of TβRII that it is specifically the extracellular domain of TβRII, and not a similarly glycosylated receptor, which directs entry of TβRII into membrane rafts.

6.1.2 *TβRIII increases clathrin-mediated endocytosis of TβRII/TβRI complexes and basal TGFβ signalling*

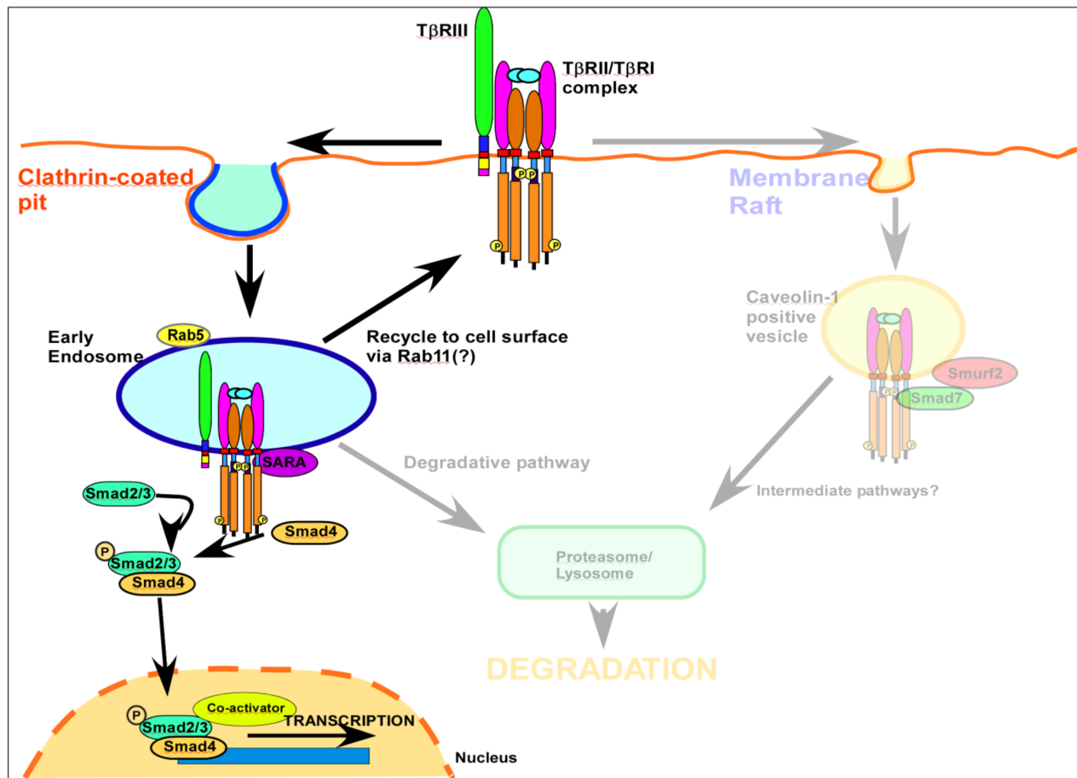
As shown in Chapter 2 that the extracellular domain of TβRII and the glycosylation status of the cell as a whole affected membrane raft partitioning of TβRII, I decided to assess the role of TβRIII in directing TβRII/TβRI internalization and trafficking. Previously the role of TβRIII in TGFβ signal transduction had been thought to be simply involved in ligand-presentation to TβRII. Since TβRIII has a large, heavily glycosylated extracellular domain (7) and it has been shown to interact with TβRII, I thought it could potentially be the signal regulating TβRII trafficking based on my work from Chapter 2.

In Chapter 3, I demonstrated that TβRIII was able to interact with both TβRII and TβRI in the absence of ligand. Furthermore, unlike TβRII and TβRI which were found primarily localized in membrane-raft fractions, TβRIII was found enriched in non-membrane raft fractions. Interestingly, its association with TβRII and TβRI re-directed both receptor complexes into non-membrane raft fractions and subsequently into the early endosome (Figure 6.1). This altered

Figure 6.1 TβRIII increases clathrin-mediated endocytosis of TβRII/TβRI complexes

In Chapter 3 of my thesis, I evaluated the ability of TβRIII to direct internalization of TβRII/TβRI complexes. My work illustrated that TβRIII could direct the TβRII/TβRI complex to increase its non-membrane raft partitioning, traffic to the early endosome, and increase its half-life and basal signalling potential.

Figure 6.1



internalization had effects on signal transduction as well, as the association of TβRIII with the TβRII/TβRI receptor complex increased their half-life and basal TGFβ signal transduction. As mentioned, while the role of TβRIII had previously been thought to be in ligand-presentation, I showed in Chapter 3, that TβRIII could alter the trafficking of the receptor complex thus supporting my model, that this altered receptor trafficking has direct implications in TGFβ signalling potential.

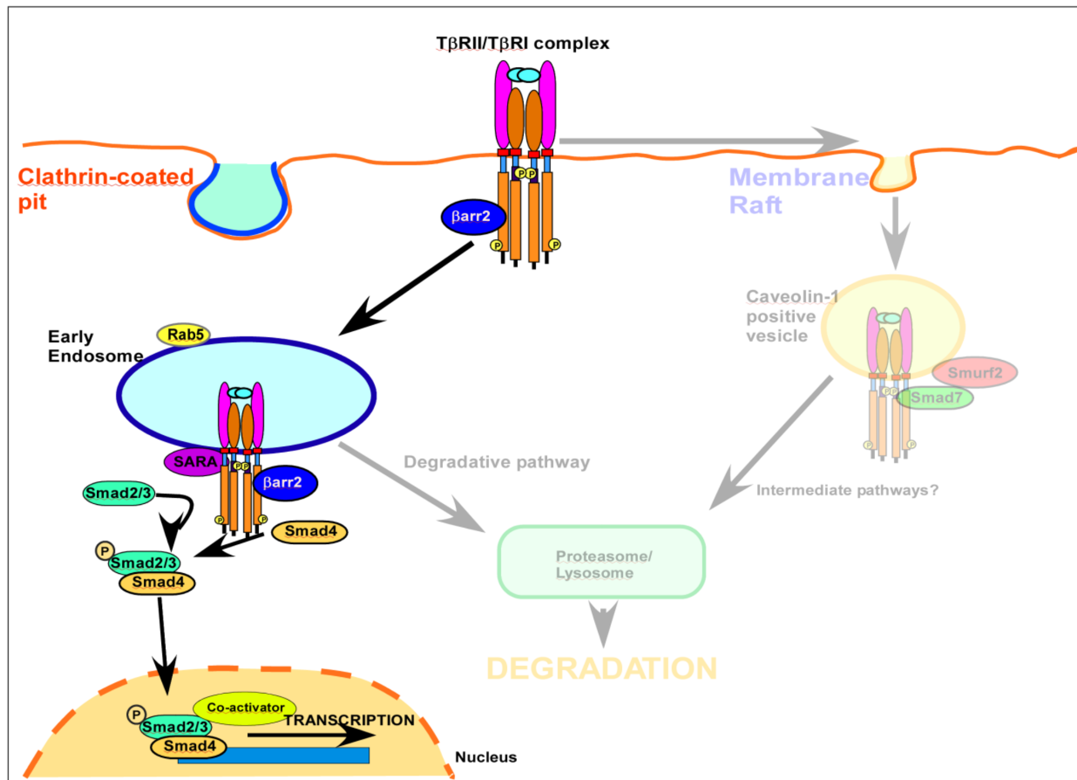
6.1.3 *βarrestin2 interacts with TβRII to mediate Smad-dependent and Smad-independent signal transduction*

I was interested in studying the role of βarrestin2 in TGFβ signal transduction, as Chen and colleagues had found a novel role for βarrestin2 in mediating TβRII/TβRIII endocytosis through binding TβRIII (8). Since they showed that βarrestin2 promoted endocytosis of TβRII/TβRIII, I was interested in assessing whether this interaction directed membrane trafficking of the receptors. As βarrestin2 bound to a phosphorylated threonine residue on TβRIII, and TβRII has multiple phosphorylated residues on its intracellular domain (7), I wanted to test the possibility that βarrestin2 could bind TβRII in the absence of TβRIII. In Chapter 4, I illustrated that TβRII and βarrestin2 could interact in the absence of TβRIII (Figure 6.2). Furthermore, this interaction had direct consequences on signalling. I used a cell line which has been shown to have very little endogenous TβRIII (A549 cells) (9), and illustrated that loss of βarrestin2 protein expression increased phosphorylation of both Smad2 and p38. Interestingly, the increased

Figure 6.2 βarrestin2 interacts with TβRII to increase early endosomal trafficking of TβRII and enhance Smad-dependent signal transduction

In Chapter 4 of my thesis I evaluated the ability of βarrestin2 to affect TGFβ signalling. My work illustrated that βarrestin engages TβRII and traffics with TβRII to the early endosome. Here, its presence increases TβRII-SARA interaction and Smad-dependent transcription.

Figure 6.2



phosphorylation of Smad2 did not translate into increased Smad signal transduction as βarrestin2 increased TβRII trafficking to the early endosome and mediated the interaction of TβRII with SARA. However, increased phosphorylation of p38 in the absence of βarrestin2 predisposed cells to apoptosis both in the presence and absence of TGFβ.

Therefore, in Chapter 4 I illustrated that βarrestin2 associates with TβRII and may direct Smad-dependent and Smad-independent responses to TGFβ stimulation.

6.1.4 *TGFβ3 is a less potent inducer of TGFβ signalling than TGFβ1 in non-small cell lung cancer cells*

During my preparation for my comprehensive exam, I noticed that the majority of work done on TGFβ in cancer had been done with TGFβ1 ligand and very few research articles had evaluated the role of TGFβ2 or TGFβ3 in cancer. Furthermore, it was generally assumed that the role of TGFβ2 and TGFβ3 would be the same as TGFβ1 in TGFβ signalling in cancer. Interestingly, in the wound microenvironment, TGFβ3 has opposite effects to TGFβ1 and TGFβ2 and promotes scar-free wound resolution, whereas TGFβ1 and TGFβ2 both cause scar formation (10). Since many of the same cellular players are present in the wound microenvironment and the tumour microenvironment, I predicted that TGFβ3 would have different effects than TGFβ1 in TGFβ signal transduction in cancer cells.

In Chapter 5 I evaluated the signalling ability of TGFβ1 and TGFβ3 in A549 cells. In this chapter, I illustrated that TGFβ1 was a much more potent inducer of TGFβ signal transduction than TGFβ3 and increased both Smad2 phosphorylation and EMT to a greater extent than TGFβ3. Initially, I predicted that this difference in signal transduction would be due to an alteration of TGFβ receptor partitioning and membrane trafficking, as was the case for my other studies. However, I found that both ligand treatments induced similar membrane-raft partitioning and trafficking of the receptor complex. Interestingly I did observe differences in cell-surface receptor complex formation with TGFβ3 treatment compared to TGFβ1 treatment (Figure 6.3). I found that TGFβ3 induced fewer TβRI bound to TβRII than TGFβ1 treatment. The generally accepted model of TGFβ complex formation is that at the cell surface TβRII and TβRI form a hetero-oligomeric complex composed of two TβRII and two TβRI (11). As TβRI phosphorylates the downstream Smad transcription factors, having less TβRI associated with TβRII may cause less activated TβRI available for activating the Smad pathway.

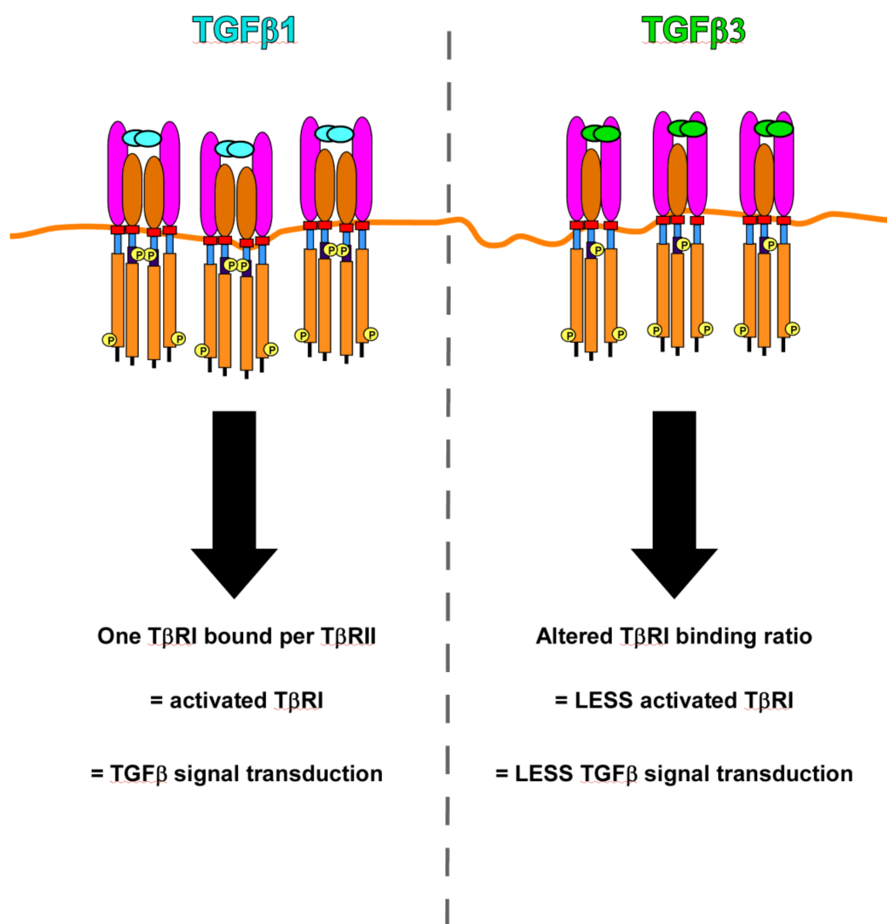
6.2 Limitations and Future Studies

It is important to note that all of the studies performed in this thesis used immortalized cultured cell lines and purified ligands. The majority of my work is heavily mechanism-based and assesses TGFβ signal transduction pathways. Using cell culture models that have been established for TGFβ signalling assays allowed me to carry out my work in a simplified and well-characterized system.

Figure 6.3 TGFβ ligands cause altered TGFβ receptor complex formation

In Chapter 5 of my thesis I evaluated the role of TGFβ1 and TGFβ3 to induce Smad signal transduction in a non-small cell lung cancer cell line. I found that TGFβ3 induces less Smad-signal transduction than TGFβ1 and alters cell-surface TGFβ receptor complex formation.

Figure 6.3



Furthermore, using these established cell lines where receptor levels and TGFβ signalling capacity had been established allowed me to manipulate different protein-interactions to assess the effects on trafficking and signal transduction.

In all of my data chapters I used HEK293T cells to assess TGFβ receptor interactions and membrane raft partitioning. These cells are an ideal model system for this type of work, as HEK293T cells do not express high levels of endogenous TGFβ receptors and also have a high level of membrane raft content (1). This allowed me to assess receptor interactions in over-expression assays using tagged receptors for ease of detection. However, there are some issues regarding relying solely on over-expression studies to assess membrane raft partitioning. It is possible that the over-expression of a construct, such as TβRIII may alter its distribution in the plasma membrane relative to endogenous levels of receptors. Ultimately, it would have been ideal to assess levels of endogenous TβRIII in mediating the membrane partitioning of TβRII/TβRI complexes. Unfortunately, there are few established antibodies available for the detection of endogenous TGFβ receptors. Therefore, when possible, I attempted to evaluate endogenous TGFβ receptors using ^{125}I-TGFβ ligand to bind receptors, as this method is highly sensitive and can allow for detection of receptors even in cell lines with low receptor levels. I used this method to detect endogenous levels of receptors in HepG2 cells in membrane raft partitioning

experiments in Chapter 3, and this method should be used to confirm membrane raft partitioning results in future studies.

To confirm my membrane raft isolation experiments, I also used a well-established cell line, Mv1Lu HAT cells, to evaluate receptor trafficking using immunofluorescence microscopy. This cell line is ideal for evaluating TβRII trafficking, as it is stably transfected with a zinc-inducible HA-tagged TβRII construct. These cells are also amenable to PEI transfection, which allowed me to evaluate the effects of TβRIII and βarrestin2 on TβRII trafficking in Chapters 3 and 4, respectively. While it would be ideal to assess the ability of endogenous receptors to traffic into different endosomal compartments, we do not currently have a sufficiently sensitive immunofluorescence approach to answer this question.

Finally, to evaluate Smad-dependent transcription, I employed the use of luciferase assays under the control of a Smad-responsive promoter in HepG2 cells in Chapters 3 and 4. HepG2 cells have been established for luciferase assays and are easily transfected using the calcium-phosphate precipitation method. Therefore, this approach allowed me to evaluate the role of different combinations of receptors and their interacting protein(s) on TGFβ-dependent transcription. When possible, I confirmed these results with signalling assays in non-small cell lung cancer cells, such as A549 and H1299 cells, which allowed me to assess the role of endogenous proteins on Smad-dependent and independent signal transduction.

Chapter 2 of this thesis provided evidence that the extracellular domain of TβRII regulated the entrance of TβRII into membrane raft fractions. Furthermore, it was shown that the glycosylation state of the cell as a whole, but not TβRII itself, was involved in regulating its membrane partitioning. This suggests that there are cell-surface glycosylated TβRII-interacting protein(s) which may direct the entrance of TβRII into membrane rafts. I evaluated one of these candidate proteins, TβRIII, in Chapter 3 of this thesis, but actually found that TβRIII increased clathrin-dependent internalization of TβRII, and not clathrin-independent internalization, as was suggested in Chapter 2. This leads to the notion that there are likely other proteins directing TβRII into membrane raft fractions. One such protein is CD109, which is a glycoprotein with 17 potential N-linked glycosylation sites (12) and has been shown to enhance internalization of TGFβ receptors into the caveolin-1 positive vesicle (13). Future studies to identify other glycosylated cell-surface proteins directing TGFβ receptor endocytosis may have important implications in TGFβ signal capacity, and could potentially be performed by isolating membrane raft fractions, performing co-immunoprecipitations to purify TβRII and subjecting the immunoprecipitated proteins to mass spectroscopy to identify novel proteins.

Though I initially thought that TβRIII would promote clathin-independent internalization of TβRII/TβRI complexes, the finding that TβRIII promotes clathrin-dependent internalization of receptors has important implications in TGFβ receptor biology. TβRIII has been shown to be dysregulated in a number of

cancers (14-19), and the effect of its altered expression is similar to that of TGFβ signalling as a whole: at times, loss of TβRIII inhibits cancer progression and metastasis, while at other times it appears to increase tumorigenicity. While initially it could be thought that these discrepancies in the role of TβRIII may be due to its role in ligand-presentation, my work suggests that the level of available TβRIII may affect TGFβ signal transduction through increasing the trafficking of the receptors to the early endosome and extending their half-life. Therefore, it would be interesting to evaluate the effect of knockdown of TβRIII in cancer cell lines at different stages of tumorigenicity and evaluate the trafficking and receptor half-life of TβRII.

Chapter 4 of my thesis evaluated the role of βarrestin2 in modulating TGFβ signal transduction. While it had previously been shown that βarrestin2 could interact with TβRIII, my work illustrating that βarrestin2 can interact with TβRII and affect Smad-dependent and Smad-independent signalling pathways suggests that it has a greater role than simply receptor internalization, as initially thought (8). I found it particularly interesting that βarrestin2 caused such a marked increased in p38 phosphorylation resulting in apoptosis. It has been shown that Smad-independent pathways can have important effects in cancer progression such as through mediating EMT, cell survival and apoptosis (reviewed in (20)).Therefore, it would be interesting to see if loss of βarrestin2 could drive apoptosis in numerous cancer cell lines resistant to the growth-inhibitory effects of TGFβ. Furthermore, it would be fascinating to evaluate the

role of βarrestin2 in mediating metastasis of tumors *in vivo*. If loss of βarrestin2 was also able to induce apoptosis *in vivo,* this may represent a novel mechanism to disrupt TGFβ-dependent metastasis and tumor outgrowth.

Finally, in Chapter 5 of my thesis I attempted to assess the differences between TGFβ1 and TGFβ3 in TGFβ signalling in a non-small cell lung cancer cell line, A549 cells. Overall, I showed that TGFβ3 was much less potent than TGFβ1 in inducing TGFβ-dependent signalling, especially in terms of EMT. Since EMT is a necessary step for epithelial-based tumors to escape the primary tumor site and metastasize, future studies should evaluate the role of TGFβ3 in cancer cell migration and invasion. Furthermore, it would be interesting to assess mechanistically how TGFβ3 induce less potent signalling even though both TGFβ1 and TGFβ3 bind to the same cell-surface receptors. Initial work in Chapter 5 of this thesis suggests that these ligands may induce different signalling capacities through TGFβ receptor complex formation at the cell surface. However, as these ligands share 76% amino acid identity in their active forms (21), it may be important to identify differences in the amino acid sequence which could explain their differential ability to induce receptor complex formation. One important way in which these ligands differ is in terms of their post-translational modifications. Upon comparison of the sequences of TGFβ1 and TGFβ3, it appears that TGFβ3 has a putative glycosylation site that is not found in TGFβ1. Therefore, manipulating the glycosylation state of TGFβ3 may affect its ability to bind to TβRII and may change receptor complex formation. Overall,

the differences in the ability of TGFβ1 and TGFβ3 to induce signal transduction represent an exciting field of study applicable to numerous processes in addition to cancer, such as development and fibrotic conditions.

6.3 Context of Findings in the Field of TGFβ Signalling Regulation

In this thesis I focus on the mechanisms whereby protein interactions with the TGFβ receptor complex can alter both membrane trafficking and signal transduction. However, there are many other mechanisms which regulate TGFβ signal transduction.

For example, as suggested in the introduction, the bioavailability of TGFβ ligand in the extracellular matrix may greatly regulate TGFβ signalling. The latent TGFβ complex is composed of homodimeric TGFβ, latency associated peptides, and the latent TGFβ binding protein (LTBP). Importantly, the LTBP is important for sequestering the TGFβ ligand complex in the extracellular matrix and TGFβ in its latent form is unable to bind TGFβ receptors (22). Therefore, the ECM acts as a reservoir for latent TGFβ; however, TGFβ activation can occur in several ways. For example, TGFβ can be activated *in vivo* in low pH environments, such as in lacunae surrounding osteoclasts (23, 24). Furthermore, a number of proteases such as plasmin and thrombospondin can cleave latent TGFβ at the cell surface and at wound-healing sites, respectively (22). Interestingly, TβRIII may regulate the bioavailability of active TGFβ through its ectodomain shedding. Soluble TβRIII can be found naturally in serum and the

ECM and occurs as a protease cleavage of its extracellular, TGFβ-binding domain (25). It has been shown that soluble TβRIII can bind and inhibit all three TGFβ ligands with relatively high affinity, but is best at binding and inhibiting the actions of TGFβ2 (25). The protease responsible for producing the soluble form of TβRIII is still currently unknown (25). In chapter 3 of this thesis I examined the role of TβRIII in regulating the trafficking and signalling of the TβRII/TβRI complex. In figure 3.11 I show that TβRIII may increase the basal signalling of the TGFβ receptor complex, but decreases Smad signalling in the presence of TGFβ1 ligand. One possible explanation for this finding is that the over-expressed form of TβRIII may be shed from the cell surface and therefore acting to sequester TGFβ ligand from the TGFβ receptor complex. Once the protease which is responsible for inducing the ectodomain shedding of TβRIII is discovered, it will be possible to evaluate the potential for TβRIII in negatively regulating TGFβ signalling.

In many different receptor families, regulating the level of cell-surface receptors is an important mechanism for regulating signal transduction. Indeed, in the TGFβ receptor family, this is also the case. For example, it has been shown that regulating the levels of endoglin can affect the ability of breast cancer cells to perform angiogenesis (26). Li and colleagues illustrated that decreasing the levels of endoglin in human vascular endothelial cells (HUVECs) inhibited *in vitro* angiogenesis and mAb to endoglin in a mouse model of breast cancer induced regression of breast cancer (26). Furthermore, the cell surface levels of

TGFβ receptors can also be affected by glycosylation. It is thought that glycosylated receptors act as a network at the cell surface and delay internalization (27). It was shown that Mgat5, a Golgi enzyme involved in N-glycan processing, sensitized cytokine receptors such as EGFR and TGFβ receptors by keeping them at the cell surface (27). Indeed, loss of Mgat5 decreased cytokine signalling and this effect could be rescued by treating with inhibitors of endocytosis (27). In chapter 4 of this thesis, I found that loss of βarrestin2 expression appeared to increase steady-state levels of TβRII at the cell-surface but did not appear to affect the half-life of the receptor. This increase in levels of receptor at the cell-surface may allow for the formation of more signalling complexes; however since receptor internalization is necessary for Smad signal transduction, this may preferentially activate Smad-independent pathways, such as the p38 pathway.

The main focus of this thesis was the role of receptor endocytosis on signal transduction. While the model that I propose in this thesis suggests that clathrin-mediated endocytosis enhances TGFβ signalling and membrane-raft/caveolae-dependent endocytosis decreases TGFβ signalling, it is possible that membrane-rafts could function to compartmentalize other non-Smad signalling pathways. Zuo and Chen published an interesting article in which they illustrated that the membrane-raft localization of the TGFβ receptor complex was important for the activation of ERK and p38, but not Smad2/3 by TGFβ (28). Indeed, they illustrated that depleting cholesterol, which is an integral component

of membrane rafts, decreased TGFβ activation of ERK and p38 and also decreased the migration of HaCaT cells in response to TGFβ (28). Further supporting a role for caveolae in non-Smad signal transduction is a paper by Meyer *et al.* that showed that caveolae formation in hepatocytes was necessary for non-Smad mediated activation of the Akt pathway (29). In chapter 4 of this thesis, I found that decreasing levels of βarrestin2 enhanced TGFβ-induced phosphorylation of p38. The mechanism of this enhanced phosphorylation could occur by several means. First of all, as I suggested in chapter 4, loss of βarrestin2 also enhanced the levels of TβRII at the cell surface which may increase the ability of the receptor complex to activate non-Smad signalling pathways. However, another possibility is that loss of βarrestin2 may shift endogenous TβRII into membrane raft fractions, which can then activate p38 signalling as suggested by Zuo and Chen (28). While I found that over-expressing βarrestin2 did not further shift TβRII into non-membrane raft fractions, it is essential to evaluate endogenous receptors and βarrestin2 to completely discount the role of βarrestin2 in membrane raft partitioning.

6.4 Significance of Findings and Conclusion

In this thesis I provide evidence that TGFβ receptor internalization and trafficking can have direct effects on TGFβ-dependent signalling. I have identified regions of TβRII that are important in mediating its trafficking, as well as both cytoplasmic and extracellular interacting-proteins which direct TGFβ receptor

trafficking. Furthermore, I have illustrated that the roles of TGFβ trafficking in mediating signalling output are not limited to Smad-dependent pathways, as the Smad-independent p38 pathway is also affected by changes in TGFβ receptor trafficking.

The TGFβ signalling pathway is important in normal physiological conditions, such as development and cell differentiation, and pathological conditions such as cancer and fibrosis. As I have demonstrated that the trafficking of the receptors, and proteins which direct this trafficking, have implications in TGFβ signal transduction, my work has implications in a number of fields. There is also support in the literature that perturbations in TGFβ receptor trafficking can influence disease states such as cancer and fibrosis. For example, it has recently been shown that a mutation in TβRII which disrupts its endocytosis promotes cancer cell migration and invasion in an oral squamous cell carcinoma (30). Similarly, in fibrotic conditions, such as systemic sclerosis which also show enhanced TGFβ signalling, perturbations in TGFβ endocytosis have also been demonstrated. Patients with systemic sclerosis have decreased caveolin-1 expression in their lungs and studies have shown that decreased caveolin-1 expression increases collagen production (31). Therefore, identifying factors which promote and control TGFβ endocytosis will have significant effects in disease states in which TGFβ signal transduction is deregulated.

6.5 References

1. Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F., and Wrana, J. L. (2003) *Nature cell biology* **5**(5), 410-421

2. Runyan, C. E., Schnaper, H. W., and Poncelet, A. C. (2005) *The Journal of biological chemistry* **280**(9), 8300-8308

3. Mitchell, H., Choudhury, A., Pagano, R. E., and Leof, E. B. (2004) *Molecular biology of the cell* **15**(9), 4166-4178

4. Ehrlich, M., Shmuely, A., and Henis, Y. I. (2001) *Journal of cell science* **114**(Pt 9), 1777-1786

5. Moustakas, A., Lin, H. Y., Henis, Y. I., Plamondon, J., O'Connor-McCourt, M. D., and Lodish, H. F. (1993) *The Journal of biological chemistry* **268**(30), 22215-22218

6. Huang, S. S., and Huang, J. S. (2005) *Journal of cellular biochemistry* **96**(3), 447-462

7. Derynck, R., and Feng, X. H. (1997) *Biochimica et biophysica acta* **1333**(2), F105-150

8. Chen, W., Kirkbride, K. C., How, T., Nelson, C. D., Mo, J., Frederick, J. P., Wang, X. F., Lefkowitz, R. J., and Blobe, G. C. (2003) *Science (New York, N.Y* **301**(5638), 1394-1397

9. Finger, E. C., Turley, R. S., Dong, M., How, T., Fields, T. A., and Blobe, G. C. (2008) *Carcinogenesis* **29**(3), 528-535

10. Occleston, N. L., Laverty, H. G., O'Kane, S., and Ferguson, M. W. (2008) *Journal of biomaterials science* **19**(8), 1047-1063

11. Siegel, P. M., and Massague, J. (2003) *Nature reviews* **3**(11), 807-821

12. Hagiwara, S., Murakumo, Y., Mii, S., Shigetomi, T., Yamamoto, N., Furue, H., Ueda, M., and Takahashi, M. *Oncogene* **29**(15), 2181-2191

13. Bizet, A. A., Liu, K., Tran-Khanh, N., Saksena, A., Vorstenbosch, J., Finnson, K. W., Buschmann, M. D., and Philip, A. *Biochimica et biophysica acta* **1813**(5), 742-753

14. Gatza, C. E., Holtzhausen, A., Kirkbride, K. C., Morton, A., Gatza, M. L., Datto, M. B., and Blobe, G. C. *Neoplasia (New York, N.Y* **13**(8), 758-770

15. Kim, J. H., Yu, S. J., Park, B. L., Cheong, H. S., Pasaje, C. F., Bae, J. S., Lee, H. S., Shin, H. D., and Kim, Y. J. *Digestive diseases (Basel, Switzerland)* **29**(3), 278-283

16. Lambert, K. E., Huang, H., Mythreye, K., and Blobe, G. C. *Molecular biology of the cell* **22**(9), 1463-1472

17. Zakrzewski, P. K., Mokrosinski, J., Cygankiewicz, A. I., Semczuk, A., Rechberger, T., Skomra, D., and Krajewska, W. M. *Cancer investigation* **29**(2), 137-144

18. Bilandzic, M., Chu, S., Farnworth, P. G., Harrison, C., Nicholls, P., Wang, Y., Escalona, R. M., Fuller, P. J., Findlay, J. K., and Stenvers, K. L. (2009) *Molecular endocrinology (Baltimore, Md* **23**(4), 539-548

19. Criswell, T. L., Dumont, N., Barnett, J. V., and Arteaga, C. L. (2008) *Cancer research* **68**(18), 7304-7312

20. Zhang, Y. E. (2009) *Cell research* **19**(1), 128-139

21. Laverty, H. G., Wakefield, L. M., Occleston, N. L., O'Kane, S., and Ferguson, M. W. (2009) *Cytokine & growth factor reviews* **20**(4), 305-317

22. Hyytiainen, M., Penttinen, C., and Keski-Oja, J. (2004) *Critical reviews in clinical laboratory sciences* **41**(3), 233-264

23. Salo, J., Lehenkari, P., Mulari, M., Metsikko, K., and Vaananen, H. K. (1997) *Science (New York, N.Y* **276**(5310), 270-273

24. Oursler, M. J. (1994) *J Bone Miner Res* **9**(4), 443-452

25. Vilchis-Landeros, M. M., Montiel, J. L., Mendoza, V., Mendoza-Hernandez, G., and Lopez-Casillas, F. (2001) *The Biochemical journal* **355**(Pt 1), 215-222

26. Li, C., Guo, B., Bernabeu, C., and Kumar, S. (2001) *Microscopy research and technique* **52**(4), 437-449

27. Partridge, E. A., Le Roy, C., Di Guglielmo, G. M., Pawling, J., Cheung, P., Granovsky, M., Nabi, I. R., Wrana, J. L., and Dennis, J. W. (2004) *Science (New York, N.Y* **306**(5693), 120-124

28. Zuo, W., and Chen, Y. G. (2009) *Molecular biology of the cell* **20**(3), 1020-1029

29. Meyer, C., Godoy, P., Bachmann, A., Liu, Y., Barzan, D., Ilkavets, I., Maier, P., Herskind, C., Hengstler, J. G., and Dooley, S. *Journal of hepatology* **55**(2), 369-378

30. Park, I., Son, H. K., Che, Z. M., and Kim, J. *Cancer letters* **315**(2), 161-169

31. Del Galdo, F., Lisanti, M. P., and Jimenez, S. A. (2008) *Current opinion in rheumatology* **20**(6), 713-719

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Sarah McLean, Gianni M. Di Guglielmo. TGFβ (transforming growth factor β) receptor type III directs clathrin-mediated endocytosis of TGFβ receptor types I and II. Biochem. J. (2010). 429: 137-145.

Valbona Luga, **Sarah McLean**, Christine Le Roy, Maureen O'Connor-McCourt, Jeff L Wrana, Gianni M Di Guglielmo. The extracellular domain of the TGFβ type II receptor regulates membrane raft partitioning. Biochem. J. (2009) 421: 119-131.

Anita Woods*, Daphne Pala*, Laura Kennedy, **Sarah McLean**, Jason Rockel, Guoyan Wang, Andrew Leask, Frank Beier. Osteoarthritis Cartilage (2009) 3: 406-413.

Shaoqiong Chen, **Sarah McLean**, David E. Carter, Andrew Leask. The gene expression profile induced by Wnt 3a in NIH 3T3 fibroblasts. J. Cell Commun. Signal. (2007) 1: 175-183.

Abstracts:

Sarah McLean, John Di Guglielmo. TGFβ3 is a less potent inducer of TGFβ signalling than TGFβ1 in non-small cell lung cancer cells. Poster Presentation. American Association for Cancer Research 103rd Annual Meeting. April 2nd, 2012, Chicago, Illinois, USA.

Sarah McLean, John Di Guglielmo. Research in Scleroderma: From Understanding to Developing Therapies. Invited speaker. Scleroderma Society of Ontario London Support Group Meeting, October 2011.

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Sarah McLean, John Di Guglielmo. Perspectives on Cellular Research in Scleroderma. Invited speaker. Scleroderma Society of Ontario London Support Group Meeting, May 2010.

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Sarah McLean, John Di Guglielmo. TβRIII directs clathrin-mediated endocytosis of TGFβ type I and II receptors. Poster Presentation. American Society for Cell Biology Meeting, San Diego, California. December 2009.

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Sarah McLean, Adrian Gunaratne, Moshmi Bhattacharya, John Di Guglielmo. β-arrestin2 directs clathrin-mediated endocytosis of TGFβ receptors. Poster Presentation. Margaret Moffatt Research Day, University of Western Ontario, March 2009.

Sarah McLean, Adrian Gunaratne, Moshmi Bhattacharya, John Di Guglielmo. β-arrestin2 directs clathrin-mediated endocytosis of TGFβ receptors. Poster Presentation. American Society for Cell Biology Meeting, San Francisco, California, December 15th, 2008.

Sarah McLean, Adrian Gunaratne, Moshmi Bhattacharya, John Di Guglielmo. β-arrestin2 directs clathrin-mediated endocytosis of TGFβ receptors. Poster Presentation. Stevenson Research Day, University of Western Ontario, November 2008.

Sarah McLean, Valbona Luga, Christine Le Roy, Jeffrey L. Wrana, Moshmi Bhattacharya, John Di Guglielmo. 2008. The Extracellular Domain of the TGFβ type II receptor is necessary for lipid raft partitioning. Poster Presentation. Margaret Moffatt Research Day, University of Western Ontario, March 2008.

Sarah McLean, Jeff Cowger, David E. Carter, Shaoqiong Chen, Andrew Leask. 2007. Wnt Signalling in fibroblasts: a new target for anti-fibrotic therapy in scleroderma? Poster Presentation. Canadian Scleroderma Researcher's Group