Talin: a Novel Inducible Antagonist of Transforming Growth Factor-beta 1 (TGF-β1) Signal Transduction

by

Shahrzad Rafiei

Department of Medicine, Division of Experimental Medicine

McGill University
Montréal, Québec, Canada

May, 2007

A thesis submitted to McGill University
in partial fulfillment of the requirements for the degree of
Master of Science (M.Sc.)

Your file Votre référence
ISBN: 978-0-494-38428-2
Our file Notre référence
ISBN: 978-0-494-38428-2

ABSTRACT

The survival of breast cancer patients declines when tumors are invasive and have an increased possibility of metastasizing to distal sites. Transforming Growth Factor-beta (TGF-β) suppresses breast cancer formation by preventing cell cycle progression in mammary epithelial cells. However, at late stage of mammary carcinogenesis, due to genetic and epigenetic alterations, TGF-β loses its cytostatic actions, and contributes to tumor invasion by promoting cell proliferation, Actin cytoskeletal reorganization, as well as Epithelial to Mesenchymal Transition (EMT). Despite the key role of TGF-β1 in tumor suppression as well as tumor progression, the molecular mechanisms underlying the conversion of TGF-β form an inhibitor of proliferation in mammary breast cancer cells to an inducer of their cell growth and EMT have not been fully elucidated. Thus, acquiring a basic knowledge on the mechanism of TGF-β regulating its target genes and its contribution to cancer progression may highlight new avenues for cancer therapy development. This prompted us to further investigate and identify TGF-β-inducible genes that may be involved in TGF-β biological responses during tumorigenesis.

In this thesis, we identified Talin as a novel TGF-β1 target gene that acts as an antagonist to inhibit TGF-β-mediated cell growth arrest and transcriptional activity in mammary cancer cell line, MCF-7. Searching for new partners of activated Smads, we found that TGF-β1 induces Talin translocation from cytosol to the plasma membrane where Talin physically interacts with the TGF-β1 signaling components, the Smads and the receptors. Furthermore, we observed that TGF-β1 stimulation leads to the formation of Actin stress fibers where Talin was detected at the end of these stress fibers. Taken all together, the obtained data show that TGF-β1 positively induced expression of Talin and suggests a role for Talin, which acts as a negative feedback loop to control TGF-β biological responses.

RÉSUMÉ

La durée de vie des patientes atteintes d'un cancer du sein est considérablement diminuée lorsque les tumeurs mammaires sont devenues invasives et développent des propriétés métastatiques. Le TGF-β1 (pour « Transforming Growth Factor beta-1) réprime la formation de cancer du sein en inhibant la prolifération des cellules épithéliales mammaires. Cependant, du fait d'altération génétiques ou épigénétiques, le TGF-β perd ses effets cytostatiques dans les phases tardives de la tumorigenèse mammaire. En effet, il participe dans ce cas à l'invasion des cellules tumorales en favorisant la prolifération cellulaire, la réorganisation de l'actine ainsi que la transition Epithelial-Mesenchymale (EMT). Bien que le rôle du TGF-β1 dans la suppression et la progression tumorale soit connu, les mécanismes moléculaires impliqués dans la transition du TGF-β1 en tant qu'inhibiteur de la prolifération des cellules cancéreuses mammaires à promoteur de leur croissance et de l'EMT reste peu caractérise. La compréhension du mécanisme par lequel le TGF-β régule ses gènes cibles, et sa contribution à la progression tumorale permettrait la mise en évidence de nouvelles approches pour le développement de traitements contre le cancer du sein. Nous avons identifié différents gènes induits par le TGF-β, potentiellement impliqués dans les effets biologiques du TGF-β1 durant la tumorogenèse.

Dans cette thèse, nous avons identifié le gène Talin comme une nouvelle cible du TGF- β1. Ce gène agit comme un antagoniste inhibant l'arrêt de prolifération induite par le TGF-β ainsi que ses activités transcriptionnelles dans la lignée cellulaire de cancer du sein MCF-7. En cherchant de nouveaux partenaires des protéines Smads activées, nous avons mis en évidence que le TGF-β1 induit la translocation de la protéine Talin depuis le cytosol jusqu'à la membrane plasmique. C'est à ce niveau que Talin interagit physiquement avec les acteurs de la signalisation du TGF-β1 : les récepteurs, ainsi que les protéines Smads. De plus, nous avons observé que la stimulation par le TGF- β1

entraîne la formation de fibre d'actine et la détection de la protéine Talin à l'extrémité de ces fibres. L'ensemble de ces résultats montre que le TGF-β1 induit l'expression de Talin et suggère que cette protéine intervient dans une boucle de régulation négative afin de contrôler les effets biologiques du TGF-β.

DEDICATION

This thesis is dedicated to my husband, Nima, whose deep devotion and boundless patience were the source and origin of my inspiration.

ACKNOWLEDGEMENTS

There are many people who I am thankful for their support during my research and learning experience. I am deeply indebted to my supervisor, Dr. Jean Jacques Lebrun from the Department of Medicine at McGill University, whose academic direction, guidance, stimulating suggestions, support, inspiration, and encouragement helped me throughout my research and the writing of my thesis. His constructive criticisms and mission for providing the only high-quality work have been of great values for my research work. I owe him lots of gratitude for having me shown this way of research.

I am grateful to the Department of Medicine, Division of Experimental Medicine, specially Marylin Linhares and Dominque Besso, who provided me with the answers to the administrative questions. Very special thanks to the Secretary of Hormones and Cancer Research Unit, France Pelletier for her helps with all the paperwork and administrative details. I also owe much thanks to the McGill University Health Center (MUHC) Research Institute for awarding me the MUHC Scholarship.

I extend my appreciation to the members of the examiner committee for accepting the examination of this thesis.

I would like to thank all my colleagues and friends from the Molecular Oncology Group (MOG) and Hormones and Cancer Research unit for their personal support during these two years. Dearest thanks goes to my lab-mates and friends (room H7-90), Shanhang Zhang, Hui Chen, Chantal de Guise, Melanié Roy, Loren Drevet, Johanna Mancini, Juliana Korah, Effie Cocolakis, Joanne Wing-Yee Ho, and Annie Lacerte for creating a unique learning environment and intellectual dynamic rarely experienced for which I feel privileged to have shared.

I am also greatly thankful to Lauren Drevet for helping me in the French translation of the Thesis abstract, and Dr. May Simaan for her helpful comments, references, and advice during the data analysis and thesis preparation. Merci beaucoup pour toutes les choses.

I am deeply thankful to my dear sister, Mehrzad, and the best parents in the world, Sharifeh and Behzad for their continuous love, tremendous support, and encouragement to pursue my goals, even if that meant an ocean's distance between us.

Saving for last, most heartfelt thanks goes to my soul mate, Nima for his loving insightful advice at key points along my path, his encouragement and understanding in pursuing this other love of my life. None of this could have been happening without all the support of my husband, Nima, who took this difficult road with me side by side throughout the years of my research and thesis work and being apart. I would not be able to start and accomplish this mission without you, my love.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER V: CONCLUSION ***Page***

LIST OF TABLES

LIST OF ABBREVIATIONS

ActRIIA:	Activin Receptor type IIA
ActRIIB:	Activin Receptor type II B
AKT:	Also known as PKB for Protein Kinase B
ALK5:	Activin Receptor-Like Kinase5
AMHRII:	Anti-Mullerian Hormone Receptor type II
AP1:	Activator Protein1
APC:	Anaphase Promoting Complex
ARC105:	Activator Recruited Co-factor 105kDa
ATF:	Activating Transcription Factor
BAMBI:	BMP and Activin Membrane-Bound InhibitorI
BMP:	Bone Morphogenetic Protein
BMPRII:	BMP Receptor type II
CBP:	CREB-Binding Protein
CDK:	Cyclin-Dependent Kinase
Co-Smad:	Common-partner Smads
cPML:	Cytoplamic form of ProtMyelocytic Leukemia protein
CREB:	cAMP-Response Element Binding
CRM1:	Chromosome Region Maintenance 1
C-TGF:	Connective-Tissue Growth Factor
DAPK:	Death Associated Protein Kinase
DAXX:	Fas Death domain-Associated protein
DNA:	Deoxyribo Nucleic Acid
DPC4:	Deleted in Pancreatic Carcinoma locus 4
E2F:	E2 promoter-binding Factor
ECM:	Extra Cellular Matrix
EMT:	Epithelial Mesenchymal Transition

ERK: Extracellular-signal Regulated Kinase

ERM: Ezerin, Radixin, Moesin

EVI1: Ecotropic Viral Integration site1

FA: Focal Adhesion

FAK: Focal Adhesion Kinase

FERM: Four point one Ezrin Radixin Moesin

FKBP12: FK506-Binding Protein 12kDa

FN Fibronectin

GAPDH: Glyceraldehyde-3 Phosphate DeHydrogenase

GDF: Growth and Differentiation Factor

GPCR: G-Protein Coupled Receptor

GRK2: G-coupled Receptor Kinase2

GS: Glycine Serine

HDAC: Histone Deacetylase

HMEC: Human Mammary Epithelial Cells

hTalin: human Talin

Idl: Inhibitor of differentiation 1

IFN-γ: Interferon-γ

IL11: InterLeukin11

IL2: InterLeukin2

I-Smad: Inhibitory-Smad

JAK: Janus Kinase

JNK: c-Jun N-terminal Kinase

LAP: Latency Associated Peptide

LLC: Large Latent Complex

LTBP: Latent TGF-β Binding Protein

MAPK: Mitogen-Activated Protein Kinase

MDCK: Madin-Darby Canine Kidney

MEC: Mammary Epithelial Cell

MH1: Mad-Homology 1

MH2: Mad-Homology 2

MIS:	Muellerian Inhibiting Substance
MKK:	MAPK Kinase Kinase
mRNA:	messenger RiboNucleic Acid
MSI:	Microsatellite Instability,
NCoR:	Nuclear Transcriptional CoRepressor
NES:	Nuclear Export Signal
NLS:	Nuclear Localization Signal
ORF:	Open Reading Frame
P/CAF:	p300/CBP-Associated Factor
PI3K:	PhosphatidylInositol 3-Kinase
PIP2:	PhosphatidylInositol biPhosphate
PIPKIγ:	type I gamma PhosphatidylInositol Phosphate Kinase
PP2A:	Protein Phosphatase 2A
PPM1A:	Protein Phosphatase 1A isoform 1
PTB:	Phospho Tyrosine Binding
PtdIns3P:	PhosphatidylInositol 3-Phosphate
Rb:	Retinoblastoma
RNA:	RiboNuclein Acid
R-Smad:	receptor-Regulated Smad protein
RTK:	Receptor Tyrosine Kinase
RUNX:	Runt-related transcription factor
SAD:	Smad Activation Domain
SARA:	Smad Anchor for Receptor Activation
SBE:	Smad Binding Element
SDS PAGE:	SDS PolyAcrylamide Gel Electrophoresis
SDS:	Sodium Dodecyl Sulfate
SHIP1:	SH2-doamin-containing Inositol-5′-Phosphatase1
SKI:	Sloan-Kettering Institute
SLC:	Small Latent Complex
Smad:	Contraction of *Drosophila* Mad and *C. elegans* Sma
SMURF:	Smad Ubiquitination Regulatory Factor

SnoN:	Ski-related novel gene N
STAT1:	Signal Transducer and Activator of Transcription 1
STRAP:	Serine Threonine kinase Receptor Associated Protein
TAK1:	TGF-β Activated Kinase1
TGF-β:	Transforming Growth Factor-beta
TGF-β1:	Transforming Growth Factor-beta1
TGF-β2:	Transforming Growth Factor-beta2
TGF-β3:	Transforming Growth Factor-beta3
TGIF:	TG-Interacting Factor
TIE:	TGF-β Inhibitory Element
TIEG1:	TGF-β-Inducible Early-response Gene
TLP:	TRAP-1-Like protein
TNFα:	Tumor Necrosis Factor α
TRAP1:	TGF-β Receptor Associated Protein 1
TRIP1:	TGF-β Receptor Interaction Protein-1
TβRI:	TGF-β Receptor type I
TβRII:	TGF-β Receptor type II
VEGF:	Vascular Endothelial Growth Factor

CHAPTER I: INTRODUCTION

Since individual cells evolved into multicellular organism and ultimately into complicated life forms, the intracellular communication systems and tight control over the movement, division, and differentiation among cells become essential to ensure the proper behavior in organism. This brings up one of the simple curiosities of life, that is, how the growth and development of a complete organism from a single cell are controlled. Part of the answer includes the involvement of growth factor receptors and their respective ligands which transduce signals across cell membrane and into the nucleus for the transcriptional readout. The molecular nature of these intracellular signals determines type of signals, pathways involved, subsequent regulatory interactions, and eventual activation or repression of transcriptional factor.

Among the secretory polypeptides, the Transforming Growth Factor-β (TGF-β) superfamily plays a major role in regulating cell growth and differentiation. TGF-β family members are expressed in most cell types. As a multi-potent cytokine, TGF-β plays a critical role in eukaryotic development and cell homeostasis through regulation of various cellular responses including cell growth, proliferation, differentiation, angiogenesis, immune suppression, Epithelial Mesenchymal Transition (EMT), cell migration, extracellular matrix production, as well as body organization (bone and sex organs) during embryonic development.

TGF-β exerts its biological effects and elicits multiple cellular responses by binding to its cell membrane receptor, a serine/threonine kinase designated types II (TβRII), which results in the recruitment of receptor type I (TβRI or ALK5). Upon formation of hetero-tetrameric receptor complexes at the membrane, the signal is propagated to the nucleus through Smad transcriptional complexes, a family of

transcription factors, to convert TGF-β signaling into gene activation or repression (Figure 1.1).

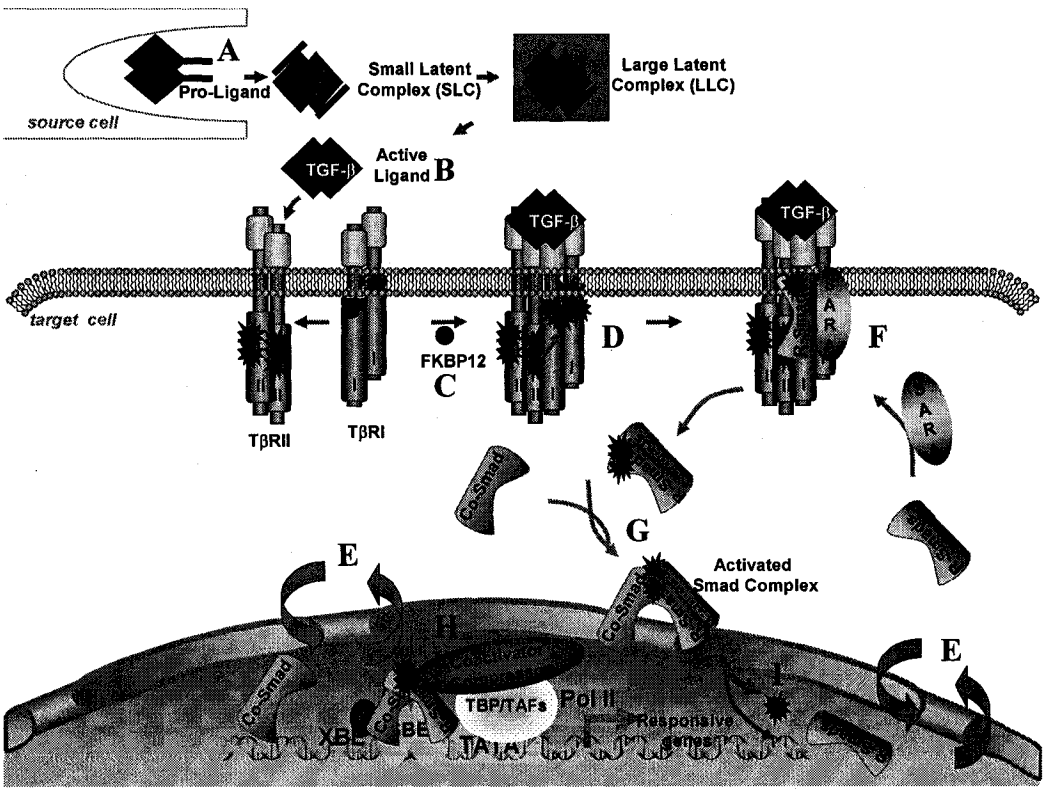


Figure 1.1: TGF-β-Smad signaling pathway. A) TGF-β is a dimeric ligand, which exists inactive in its latent form. **B)** Upon release of TGF-β, the ligand converts to its active form and binds to the TGF-β membrane receptor type II, which induces the recruitment of the receptor type I and formation of a hetero-tetrameric receptor complex. **C)** The inhibitory protein FKBP12, which enforces the inactive basal state by binding to the regulatory region (GS domain) of type I receptor is then released **D)** Upon the release of FKBP12, the type II receptor phosphorylates GS domain of type I receptor, which creates a docking site for receptor Regulated-Smad, Smad2/3 proteins. **E)** In the basal state, R-Smads and Co-Smad (Smad4) shuttle between nucleus and cytoplasm constantly. **F)** Anchor protein, SARA captures Smad2/3 and presents the R-Smad to the activated type I receptor, which will phosphorylate R-Smads at the C-terminus. **G)** The active R-Smads will be recognized by a basic motif in Smad4 resulting in the formation of Smads complex, **H)** which incorporates different DNA-binding cofactors and recruits either transcriptional coactivators or corepressors to regulate expression of several hundreds of target genes. **I)** Dephosphorylation of R-Smads will terminate this signaling cycle. Adapted from Massagué et al., (2005)

Despite the critical role of TGF-β signaling pathway in cellular homeostasis, aberrant TGF-β signaling (mutation or deletion of members of the signaling) or deregulation of TGF-β expression has been implicated in pathogenesis of many human diseases including carcinogenesis. On the basis of multiple evidences (Taya et al., 2003; Elliot and Blobe, 2005), TGF-β has a dual role in tumor pathogenesis. For instance, in the early stages of cancer development in tumor-derived mutations, TGF-β limits the growth of normal epithelial tumors that defines the role of TGF-β as a tumor suppressor. Whereas, in the later stages of cancer development despite of retaining TGF-β signaling components, the aggressively growing tumor cells become resistance to the TGF-β growth-suppressive effect due to the loss of the cytostatic gene responses. Moreover, cells become more invasive by undergoing Epithelial Mesenchymal Transitions (EMTs). In tumor-derived TGF-β (where TGF-β is actively secreted), cells that are in proximity to the growing tumors, such as immune cells are affected and the host-tumor immune response is suppressed. For the progressive growth and metastasis, TGF-β can also provide a proangiogenic environment through direct effect on endothelial cell growth, survival, and motility where the tumor cells metastasis to specific organs. As mentioned above, TGF-β signaling induction is involved in the regulation of gene expression, by which deregulation of this pathway may participate in cancer progression. Thus, understanding the molecular mechanism of TGF-β signaling pathway provides new insights and strategies for treatment of human cancer.

In this chapter, an overview followed by a literature review on TGF-β superfamily is presented. The attention is focused on the functional and structural features of the TGF-β signaling pathway; and the outcome of the regulation or defects in this signaling pathway in preventing or promoting of human tumorigenesis is discussed. Ultimately, a brief overview of previous studies is introduced to focus on Talin, a protein which has been demonstrated in this thesis for the first time to be positively regulated by TGF-β. The aims and the objectives of this thesis which are to characterize the role of Talin in TGF-β signaling pathway and its involvement in TGF-β biological responses in human breast cancer cell lines will be presented at the end of this chapter.

1.1 Overview of TGF-β Superfamily Members

Transforming Growth Factor-beta (TGF-β)s are members of a large superfamily of cytokines structurally related and characterized by six conserved cysteine residues that are encoded by 42 open reading frame (ORF)s in the human genome, nine in *Drosophila melanogaster* and six in the nematode *Caenorhabditis elegans* (Lander et al., 2001). The TGF-β superfamily members are regulated proteins that have been classified into two subfamilies (Figure 1.2): 1) the three mammalian TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3)/Activin/Nodal subfamily, and 2) the BMP (Bone Morphogenetic Protein)/GDF (Growth and Differentiation Factor)/MIS (Muellerian Inhibiting Substance) subfamily (Shi and Massagué, 2003). The TGF-β two subfamilies are not only defined by the sequence and structural homology but also by the particular signaling pathway that is activated through these ligands leading to different cellular responses.

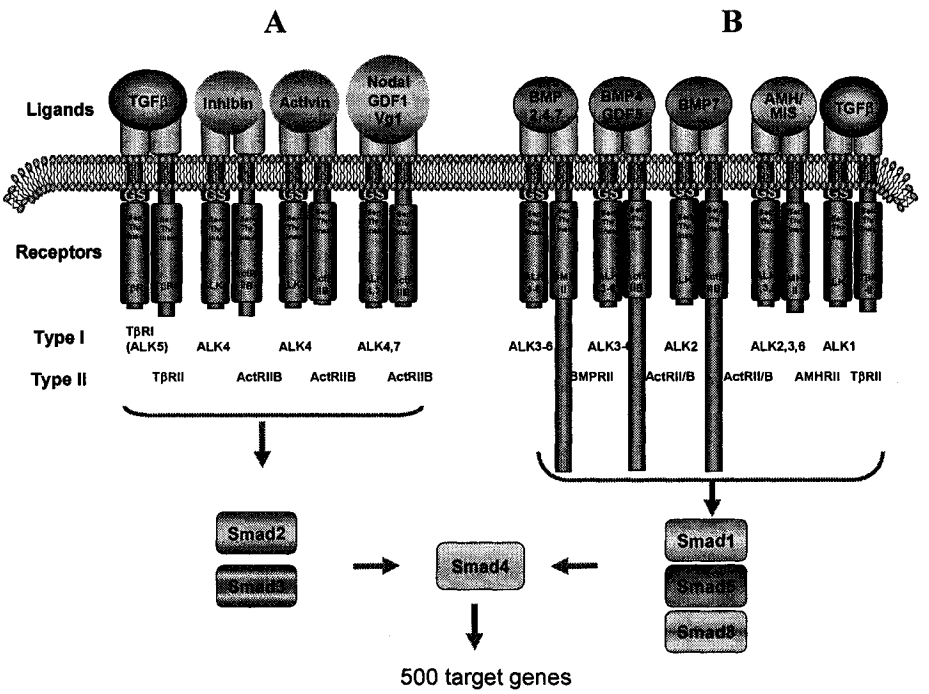


Figure 1.2: TGF-β two subfamilies. A schematic relationship of the ligands, the type I and II receptors, and the downstream R-Smad complexes illustrated for the two subfamilies: **A)** The TGF-β/Activin/Nodal subfamily, and **B)** BMP (Bone Morphogenetic Protein)/GDF (Growth and Differentiation Factor)/MIS (Muellerian Inhibiting Substance) subfamily. Adapted from Shi and Massagué, (2003)

1.2 Review of the Literature

1.2.1 TGF-β Discovery and Extracellular Regulation

TGF-β was initially described as an activity produced by retrovirally-transformed cells (Roberts et al., 1981). However, it is now obvious that the expression of TGF-β is not only seen in transformed cells, but also in many normal cells and tissues. There are three homologous TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) expressed in mammals, and each is encoded by a distinct gene expressing a set of structurally and functionally related homodimeric pro-proteins (pro-TGF-β) in a tissue-specific manner. TGF-β1 was the first member of the TGF-β superfamily that was discovered, purified, and isolated from human and porcine blood platelets (Assoian et al., 1983) and was found enriched in human placenta (Frolik et al., 1983) and bovine kidney (Roberts et al., 1983).

TGF-β exists as a latent secreted homodimer, which is required to be converted to its active state for eliciting cellular responses. Inside the cell in the trans-golgi, the mature dimeric TGF-β is proteolytically cleaved from the C terminus of its precursor protein by furin-type enzymes, and remains non-covalently bound to its pro-protein, the Latency Associated Peptide (LAP), forming the Small Latent Complex, SLC (Figure 1.1). Dissociation or activation of TGF-β from LAP is a critical regulatory event. Like any other growth factor, TGF-β has binding proteins that are involved in the regulation of its distribution and extracellular activation, thereby modulating its metabolic effects (Frolik et al., 1983). The Latent TGF-β Binding Proteins (LTBPs) are a family of extracellular glycoproteins also including fibrillin-1 and -2, the Extra-Cellular Matrix (ECM) proteins. Among the LTBP family members, the LTBP-1,-3,-4 are involved in the sequestering and binding to SLC via cysteine bonds to from a Large Latent Complex, LLC (Figure 1.1). This complex maintains the cytokine in an inactive state and prevents it form eliciting cellular responses. Once, LLC targets TGF-β availability to various extracellular structures and circulates it in the bloodstream, TGF-β is released and becomes active through proteolytic processing of the matrix-associated proteins. Only then, depending on the type and state of the cell, the active TGF-β ligands elicit different cellular processes

by binding to the extracellular domain of the TGF-β receptors and inducing specific signaling pathways (Annes et al., 2003; Rifkin et al., 2004).

1.2.2 TGF-β Signaling from the Cell Membrane to the Nucleus

1.2.2.1 TGF-β Signaling Receptors and Activation of TGF-β Receptor Complexes

The TGF-β superfamily members exert their biological effects through a conserved and well established signaling mechanism from the membrane receptors to the target genes in nucleus. The cell surface receptors that carry the TGF-β family signal into the cell are known as transmembrane serine/threonine kinases and are paired in different combinations as receptor complexes for different TGF-β family members. This serine/threonine kinase receptor complex consists of two distinct transmembrane proteins, known as the type I and type II receptors. TGF-β type I and II receptors are glycoproteins that have similar structural domains including, an extracellular N-terminal cysteine-rich ligand binding domain, a hydrophobic transmembrane domain, and a cytoplasmic C-terminal serine/threonine kinase domain. There is a conserved 30-amino regulatory segment rich in glycine and serine residues, termed the GS region that distinguishes type I receptor from type II. This GS region is named for the ^{185}TTSGSGSG^{192} sequence at its core and is located upstream of the serine/threonine kinase domain (Wieser et al., 1995). There are seven type I receptors also termed Activin receptor-Like Kinases (ALK1-7) and five type II receptors (ActRIIA, ActRIIB, BMPR-II, AMHRII and TβRII) encoded in human genome, dedicated to TGF-β signaling (Figure 1.2).

In the absence of ligand, TGF-β type I and type II receptors are present as homodimers in the plasma membrane. Studies using mutated forms of TGF-β type I and type II receptors have indicated the requirement of at least two TGF-β type I receptors in the signaling complex, and that the constitutively active transmembrane kinase activity of type II receptor is regulated through homodimerization (Dijkt et al., 2004). TGF-β signaling is initiated through activation of the ligand and formation of the type II receptor-ligand complex on the cell surface, where the ligand may undergo a

conformational change, exposing the binding epitope for the type I receptor (Shi and Massagué, 2003). Subsequently, receptor type I will be recruited and associated to the type II receptor, which in turn will be phosphorylated at the serine and threonine residues in the regulatory region (GS domain) by the type II receptor through its intrinsic kinase activity. In the absence of ligand, the GS region in type I receptor is hidden by FKBP12, an inhibitory protein which blocks the access to the phosphorylation sites and maintaining type I receptor inactive. While, upon TGF-β stimulation, FKBP12 is released and the cytoplasmic kinase domain of type I receptor becomes active and is capable to recognize its substrate, the receptor-Regulated Smad (R-Smad) proteins. In the basal state, R-Smads shuttle between nucleus and cytoplasm constantly. Anchor proteins capture R-Smads and present them to the activated type I receptor, which will phosphorylate R-Smads. Phosphorylation of R-Smads at two conserved serines at the C-terminal (SXS motif) produces a docking site for Smad4, the only Co-Smad, followed by Smad complex formations. Each of the R-Smads can then interact with a wide array of specific DNA binding proteins (Massagué et al., 2005; Attisano et al., 2002) to regulate transcriptional responses of TGF-β target genes (Figure 1.1).

1.2.2.2 TGF-β Accessory Receptors

Biochemical and genetic evidences have identified the existence of some accessory components of the TGF-β receptor complexes functioning as co-receptors including Betaglycan. This accessory receptor, also known as TGF-β type III, is a membrane anchored proteoglycan with a large extracellular region and a short cytoplasmic tail with no discernible kinase function identified to date (Lopez et al., 1991; Chifetz et al., 1992). However, it is involved in mediating ligand access, particularly TGF-β2 to the type II signaling receptors; since TGF-β2 has a low affinity for the type II receptor in contrast to TGF-β1 and TGF-β3 (Esparza et al., 2001; Lopez et al., 2001).

Betaglycan might also have additional functions. According to Chen and his colleagues (2003), TGF-β type II receptor can also phosphorylate the cytoplasmic part of Betaglycan, and as a result β-arrestin is recruited to the receptor which leads to

endocytosis of both Betaglycan and TβRII, and ultimately, down regulation of TGF-β signaling.

1.2.2.3 Internalization of Membrane Bound Receptors

Studies have suggested (Di Gugliemlo et al., 2003; Le Roy et al., 2005) that the internalization of membrane bound receptors including Receptor Tyrosine Kinases (RTKs), and G-Protein Coupled Receptors (GPCRs) occurs mostly through two pathways: 1) the lipid-raft (the caveolae to caveolin-positive vesicles) endocytotic pathway, and 2) the clathrin (the coated vesicles to early endosomes) endocytotic pathway. TGF-β receptor internalization also occurs through both of these endocytotic pathways.

The lipid-raft-mediated endocytosis promotes degradation of the receptor complex through association of the TGF-β receptors with the Smad7/SMURF2 complex that is then recruited to the caveolin-1-enriched lipid rafts leading to the ubiquitin-dependent degradation of the receptor. Besides trafficking in lipid-raft compartment, TGF-β receptor is also internalized into the early endosomes (clathrin endocytotic pathway). Despite the lipid-raft endocytotic pathway, the clathrin-mediated endocytosis plays a positive and crucial role in the TGF-β signal transduction through the TGF-β receptor-associated proteins. Among these proteins, SARA (Smad Anchor for Receptor Activation), which is abundant in endosomes, not only participates in internalization with the endocytic machinery in endosomes, but also links Smad2 to TGF-β type I receptor, stabilizes their interaction, which enhances ligand induced Smad phosphorylation, gene expression, and TGF-β signaling (Tsukazaki et al., 1998).

The involvement of this different intracellular pathway in regulation of the receptor signaling may be due to the composition of the heteromeric receptor complexes and the interaction of co-receptors, such as Betaglycan (Derynck et al., 2003). As mentioned before, the cytoplasmic part of Betaglycan is phosphorylated by TGF-β type II

receptor, leading to the recruitment of β-arrestin to the receptor and regulation of TGF-β receptor complexes internalization (Chen et al., 2003).

1.2.2.4 The Intracellular Mediators of TGF-β Signaling

Smad Proteins

Smads are intracellular proteins downstream of distinct TGF-β family of serine/threonine kinase receptors playing a critical role in transmitting signals form the membrane to specific target genes in the nucleus. The prototypic members of the Smad family, SMA and MAD were first identified through genetic screens in worms: *Caenorhabditis elegans* (Das et al., 1999), and in flies: *Drosophila* (Raftery et al., 1999), respectively. The related proteins were consequently identified in mammals. Therefore the name "Smad" was created upon the similarity of human Smad1 sequence and function to the SMA protein in *C. elegans*, and the MAD protein in *Drosophila* (Liu et al., 1996). There are eight Smad proteins (Smad1-8) encoded in the human genome that are categorized into three distinct subclasses (Figure 1.3): Receptor-regulated Smads (R-Smads), Common-partner Smads (Co-Smads), and Inhibitory Smads (I-Smads), each of which plays a distinct role in the pathway.

Among the eight Smad proteins, there are five first identified substrates of the TGF-β family of type I receptors referred as Receptor-regulated Smads. Among R-Smads, Smad2 and Smad3 are substrates for the TGF-β, Activin, and Nodal receptors that transduce TGF-β-like signaling pathways; whereas Smad1, Smad5, and Smad8 are substrates for BMP and Anti-Muellerian (AMH) receptors that mediate signals characteristic of those initiated by BMPs (Figure 1.2). As suggested by Goumans and his colleagues (2003), these two signaling pathways have opposing effects and define the balance in endothelial cell migration and proliferation.

Smad4, also known as DPC4 (Deleted in Pancreatic Cancer-locus 4) belongs to the group of Co-Smad, which acts as a common partner for all R-Smads once phosphorylated.

Smad6 and Smad7 both are Inhibitory Smads (I-Smads) interfering with the Smad/Smad or Smad/receptor interactions, and thereby, terminating TGF-β signaling.

Structural Features of Smad Proteins

Smad proteins with around 500 amino acids in length contain two conserved globular domains, the amino-terminal Mad-Homology 1 (MH1) domain, and the carboxyl-terminal Mad-Homology 2 (MH2) domain flanking a proline-rich divergent middle linker region (Figure 1.3). The X-ray crystallographic analysis of individual Smad domains has provided insights into the important structural features of the MH1 and MH2 domains, including Smads interactions with other proteins as well as with DNA (Shi et al., 1998).

The MH1 domain (Figure 1.3) is conserved in all the R-Smads and the Co-Smad but not the I-Smads. With the exception of Smad2, the MH1 domain of Smads bind to a sequence-specific DNA via a β-hairpin structure and this binding is stabilized by a tightly bound zinc atom (Chai et al., 2003). However, Smad2 lacks this DNA-binding activity due to a 30 amino-acid insertion encoded by exon 3 which is thought to displace the β-hairpin loop and block DNA binding (Shi et al., 1998).

The flexible linker region, which is quite different between the subgroups, contains a PY motif that is recognized by SMURFs, the HECT-domain-containing E3 ubiquitin ligases (Figure 1.3). As one of the Smad-interacting proteins, SMURFs catalyze ubiquitin-mediated degradation of certain Smads and Smad-associated proteins, including the nuclear oncoprotein SnoN, and the TGF-β-receptor complex (Bonni et al., 2001; Kavsak et al., 2000; Ebisawa et al., 2001). In other words, Smads can function as adapters for the E3 ligases to induce degradation of Smad-associated proteins. Besides the PY motif, there are some phosphorylation sites for several protein kinases (Figure 1.4) allowing specific crosstalks with other signaling pathways including Mitogen-Activated Protein Kinases (MAPKs) that may prevent or activate the function of R-Smads (Zhang et al., 1999). Previously our lab identified another R-Smad-interacting protein, protein G-coupled Receptor Kinase 2 (GRK2), which also phosphorylates the linker region of the

R-Smads. The Phosphorylation of the R-Smads linker region by GRK2 was shown to inhibit phosphorylation of R-Smads carboxyl-terminal by the type I receptor kinase, thus preventing nuclear translocation of the Smads complex, leading to the inhibition of TGF-β-mediated target gene expression, cell growth inhibition and apoptosis (Ho et al., 2005)

The linker region in Smad4 contains a Nuclear Export Signal, NES (Pierreux et al., 2000) and a Smad Activation Domain, SAD (Qin et al., 1999) that overlaps the linker and MH2 regions (Figure 1.3). The SAD region in Smad4 is involved in transcriptional activation/repression through interactions with transcriptional activators/repressors (Qin et al., 1999; Massagué et al., 2005).

The highly conserved structure of C-terminal MH2 domain is a versatile protein interacting module, which provides specificity to the Smad function. For instance it mediates Smad activation through Smad-receptor association, Smads oligomerization through Smad-Smad interaction, and Smads nucleocytoplasmic shuttling through interacting with the nuclear pore complex (Xu et al., 2002). Smad mutational studies (Baker et al., 1996; Hata et al., 1997) suggest that there is an association between MH1 and MH2 domains in the inactive state. However, upon ligand stimulation and receptor activation, MH1 and MH2 dissociate from each other to be functionally active and be able to heterodimerize with Smad4 or other R-Smad partners. In the case of R-Smads there is a conserved C-terminal-Ser-X-Ser motif that becomes phosphorylated and activated by the phosphorylated type I receptors, and thus allowing hetero-oligomerization with Smad4 (Figure 1.3). A pocket lined with basic residues (basic pocket) has also been found in the MH2 domain of both R-Smads and Smad4 (Figure 1.3) that interacts with the phosphorylated region of the activated type I receptor in the case of R- Smads, and with the phosphorylated tail of R-Smads in the case of Smad4. According to the crystal structure analysis of the Smad2 (Wu et al., 2000), there is a set of hydrophobic patches (hydrophobic corridors) on the surface of MH2 domain (Figure 1.3), which serves as a binding site for several proteins including the DNA-binding cofactors, anchor proteins such as SARA to retain the R-Smads in the cytoplasm, and the nucleoporins for nucleocytoplasmic shuttling.

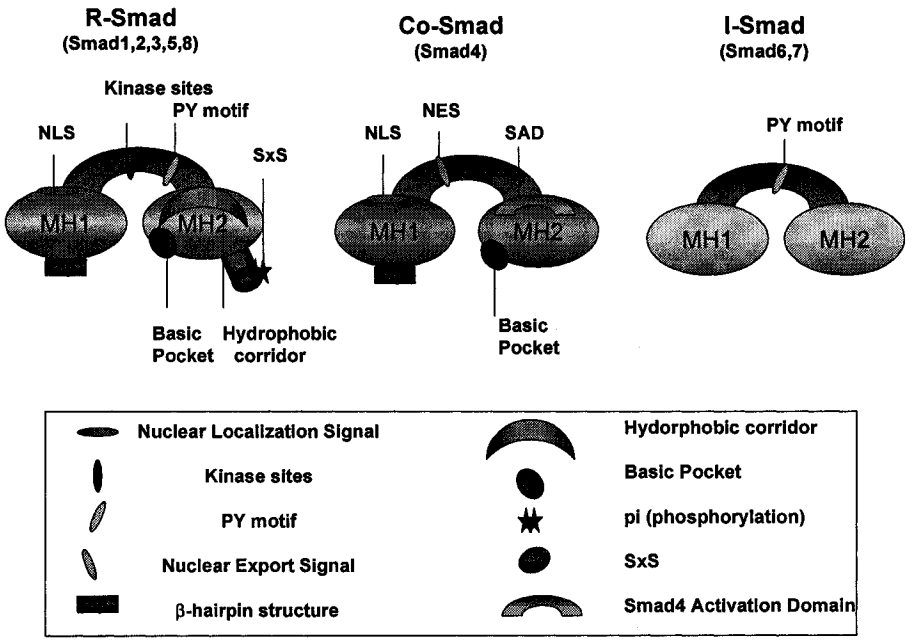


Figure 1.3: Schematic representation of Smad proteins and their structural elements. All Smad proteins consist of two conserved globular domains, the MH1 and MH2 domains which are linked to a variable linker region. Except for I-Smads, there is a β-hairpin structure in the MH1 domain which is essential for binding to DNA. The linker region in R-Smads and I-Smads contains a PY motif for recognition by the WW domains in SMURF ubiquitin ligases and a Nuclear Localization Site (NLS), while the linker region of Smad4 contains a Nuclear Export Signal (NES) domain important for shuttling; and a Smad4 Activation Domain (SAD) important for transcription. There is a basic pocket in the MH2 domain which is required for interaction of R-Smads with activated type I receptors in the case of R-Smads, and in the case of both the R-Smads and Smad4 is required for interaction with the pS–x–pS motif (red ball) of R-Smads. There is a set of hydrophobic patches or *hydrophobic corridor* on the surface of the MH2 domain of R-Smads which serves as a site for multiple interactions. Adapted from Massagué et al., (2005)

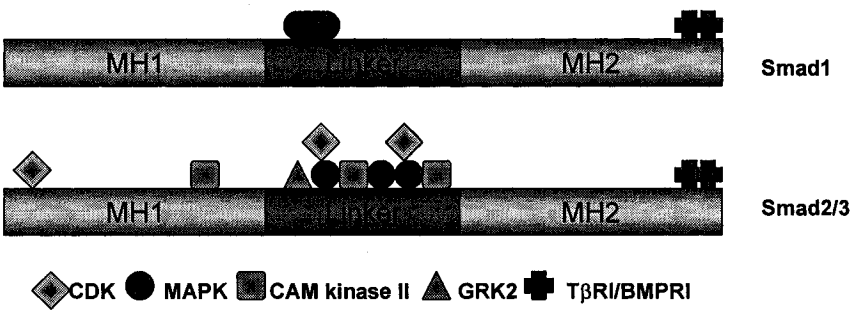


Figure 1.4: The position of phosphorylated residues in R-Smads. The linker region of R-Smads contains multiple phosphorylation sites for MAPKs, CDKs, GRK2, and other protein kinases which are indicated with different shapes. Adapted from Xu et al., (2006)

Smad Adaptors for Smad-Receptor Interaction

Studies based on genetic description, biochemical characterization, as well as structural features of Smad proteins, have provided an understanding of the molecular mechanism of how Smads are involved in transmitting TGF-β superfamily signals. These studies have also revealed essential signaling determinants that mediate the interaction of Smads with the receptors, transcriptional partners, and other associating proteins.

There are several adaptor proteins involved in facilitating Smad-receptor-interaction. Among them, SARA is the most well characterized functioning as Smad2/3 adaptor for cytosolic retention in the basal steady state (Tsukazaki et al., 1998). SARA is a multi-domain protein containing a FYVE-domain that mediates binding to phosphatidyl Inositol 3-phosphate (PtdIns3P) on the membrane (Itoch et al., 2002), being localized at plasma membrane, and being exclusively enriched in EEA1-containing early endosomes. SARA also contains an 80-amino-acid Smad-Binding Domain (SBD) that interacts with MH2 domain of Smad2 and Smad3 in the cytosol. Upon activation of receptor complex induced by TGF-β, SARA is required for the recruitment of Smad2/3 to the activated receptor complex at the plasma membrane. However, as SARA restricts Smad2/3 proteins to the plasma membrane as well as the early endosomes, this interaction may also appear to occur in early endosomes. Roy and his colleagues (2005) reported that SARA may recruit TGF-β receptor trafficking into the early endosome, leading to Smad2 phosphorylation. Therefore, the complex formation of receptors with SARA and Smad2/3 in the early endosomes may be important in TGF-β signaling.

Besides SARA, there are some other adaptor proteins that have also been suggested to facilitate Smad2/3-receptor interaction including Hgs (Miura et al., 2000), Disabled-2 (Hocevar et al., 2001), Dok-1 (Yamakawa et al., 2002), Axin (Furuhashi et al., 2001), ELF β-spectrin (Tang et al., 2003), and a cytoplasmic isoform of the ProMyelocytic Leukemia (cPML) protein (Lin et al., 2004).

Lin et al. (2004) have demonstrated the interaction of cPML with SARA, Smad2/3, and the TGF-β receptor and have suggested that this protein is critical for

phosphorylation of Smad2/3 and TGF-β signaling. Some proteins such as TRAP-1 (Wurthner et al., 2001) and TLP (Felici et al., 2003) have been reported to act as an adaptor through interacting with the receptor complex and mediating the formation of Smad2/3-Smad4 complexes.

In addition to adaptor proteins playing a role in Smad2/3-receptor interaction, cytoskeletal proteins are involved in the localization and signaling of Smads through interacting with R-Smads. For instance, TGF-β stimulation induces dissociation between unphosphorylated Smad2/Smad3 and microtubule filaments and enhancing Smad2/Smad3 phosphorylation and activation (Dong et al., 2000). Filamin, an Actin-binding protein that participates in the anchoring of membrane proteins to the Actin cytoskeletal protein is also involved in TGF-β signaling through interaction with Smads. It has been demonstrated that cells defective in Filamin expression have impaired TGF-β signaling and Smad2 phosphorylation (Sasaki et al., 2001).

Several other proteins may also interact with R-Smads acting as their adaptors or interacting partners. However, a detailed examination will be required before their involvement in TGF-β signaling can be predicted.

Phosphorylation and Activation of the Smad Proteins

As was mentioned previously, at the basal state, the inhibitory-FKBP12 protein interacts with Type I receptor at a conserved Leu-Pro motif adjacent to the GS domain and prevents phosphorylation, activation and internalization of type I receptor, and ultimately blocks R-Smads phosphorylation. However, upon stimulation of TGF-β ligand and formation of type II receptor-ligand complex on the cell surface, FKBP12 protein is released. As a result, the constitutively active transmembrane kinase domain of type II receptor phosphorylates the GS region of type I receptor which becomes active and exposes the GS region as a binding site for Smad2/3 (Huse et al., 2001). The activated cytoplasmic kinase domain of type I receptor then recognizes and phosphorylates the C-terminal SXS motif of R-Smads.

It has been reported that upon TGF-β stimulation, phosphorylation of Smad2/3 occurs within 15 to 30 minutes in cells being exposed to TGF-β. The steady-state of phospho-Smad levels will be maintained for several hours as long as TGF-β receptors remain active. Eventually, R-Smads will become dephosphorylated as a result of drop in TGF-β extracellular levels, negative feedback mechanisms, or inactivation of TGF-β receptors through internalization and degradation among others (Massagué et al., 2005). Thus, the activity of Smad signaling is tightly based on the receptor activation.

In addition to the C-terminal SSXS motif that is phosphorylated by type I receptors, R-Smads contain additional potential phosphorylation sites in the linker region (Figure 1.4) that are recognized by different cytoplasmic kinases including MAPK, which is involved in activation, and nuclear translocation of R-Smad; and GRK2 which inhibits R-Smads activation and nuclear translocation (Ho et al., 2005).

Phosphorylation of R-Smads is a key event in Smad activation and is involved in: 1) destabilizing R-Smads interaction with the receptor complex as well as SARA, and inducing the release of phosphorylated R-Smads from the complex presumably because of conformational changes; 2) allowing formation of R-Smads/Co-Smad heteromeric complex through providing a docking site for the basic surface pocket of the MH2 domain of Co-Smad of which Smad4 is the only member; and 3) exposing a nuclear import region on the Smad MH2 domain for the movement and accumulation of Smad complex in the nucleus (Attisano et al., 2002), where Smads can bind to DNA and transcription cofactors to regulate target gene expression.

Nuclear Localization of Smads

In the basal state, R-Smads are retained predominantly in the cytoplasm and Smad4 is located throughout the cell, shuttling between the cytoplasm and the nucleus (Watanabe et al., 2000). However, following TGF-β stimulation, the phosphorylated R-Smads are translocated into the nucleus via direct interaction of the hydrophobic corridor in the MH2 domain of R-Smads with the nuclear pore components, the Nup153 and Nup214 nucleoporins (Xu et al., 2002).

Upon receptor-mediated phosphorylation of R-Smads, Smad4 also becomes concentrated in the nucleus through heteromeric complex formation of R-Smad/Smad4. Moreover, the NES motif which is only found within the Smad4 linker region and is generally recognized by CRM1 (a nuclear export factor) will be masked and therefore blocking Smad4 nuclear export through CRM1 pathway (Xu et al., 2004).

Based on the over expression studies, it has been suggested that Smad3 and Smad4 can undergo nuclear import via an alternative mechanism, that is, importin-dependent pathway. Importin α and β are the nuclear translocation components, involved in transporting most cargo proteins to the nucleus. Importin α recognizes a Nuclear Localization Signal (NLS) motif in the cargo proteins and binds to importin β, which directly interacts with the nuclear pore components. The importin-β-importin-α-cargo complex then passes into the nucleus (Massagué et al., 2005). In case of Smad3, it has been demonstrated that importin β interacts with the nuclear NLS-like motif at N-terminal MH1 domain of Smad3 followed by translocation to the nucleus (Xiao et al., 2000). However, this importin-dependent pathway is considerably weaker and is not as efficient as the nucleoporins pathway (Xu et al., 2003).

As mentioned earlier, R-Smads contain additional potential phosphorylation sites in the linker region with multiple serine and threonine sites that are recognized by different cytoplasmic kinases including MAPK, which is involved in phosphorylation, activation, and nuclear translocation of R-Smad (Smad3). G1 Cyclin-Dependent Kinases, CDKs (Massagué et al., 2004) and ERK, another MAP kinase (kretzschmar et al., 1997), also mediate phosphorylation of the linker region in response to the ligand in a cell-context dependent manner. However, this phosphorylated site not only decreases Smad signaling activity (in both cases) but also attenuates nuclear accumulation (in the case of ERK).

In conclusion, Smad2, 3, and 4 directly interact with nucleoporins and undergo nuclear import. Nevertheless, in the case of Smad3 and 4, this process is mediated via nuclear translocation component, importin β.

Transcriptional Complexes of Smads

Upon receptor mediated R-Smad phosphorylation, a heterotrimeric complex comprising two phospho-R-Smad molecules and one Smad4 molecule is formed and translocated into the nucleus through nucleoporins pathway to activate or repress the expression of different TGF-β target genes. R-Smads (with the exception of Smad2) bind directly with a very low affinity to Smad-specific DNA-binding motifs in the promoters of target genes through their MH1 domain for transcriptional regulation.

The Smad specific DNA-binding motif, named Smad Binding Element (SBE), was originally defined as the 5′-GTCTAGAC-3′ sequence (Zawel et al., 1998) and later as 5′-GTCT-3′, or its complement 5′-AGAC-3′ sequence. Based on the crystal structure of the R-Smad MH1-SBE complex, Smads recognize the 5′-GTCT-3′ sequence through the β-hairpin in the MH1 domain (Shi et al., 1998). In addition to the canonical SBE motif, the Smad-DNA interaction can be also found in the GC-rich region in the promoters of *Vestigial* (Kim et al., 1997) and *Tinman* (Xu et al., 1998), which are required for the transcriptional repression. Furthermore, in the case of *Idl* promoter, SBE and GC-rich elements are both required for Smad complex (Smad1/Smad4 or Smad3/ Smad4) binding to DNA. Nevertheless, some TGF-β responsive regions such as TGF-β Inhibitory Element (TIE) in the *c-Myc* promoter lack a canonical SBE but contain a ''degenerate'' SBE which binds to Smad complex (Chen et al., 2002).

Since the affinity of Smad proteins for the SBE is too low (Shi et al., 1998), sufficient binding of the Smad complex for their cognate DNA binding sites and selectivity for specific target genes are achieved by incorporation of various sequence-specific transcription factors into the R-Smad/Smad4 complex at the promoters. These transcription factors are involved in the recruitment of a wide range of coactivators or corepressors for transcriptional activation or repression of target gene promoters.

Smad2, as compared to Smad3 can not bind directly to the specific DNA-binding motifs, owing to the presence of a 30 amino-acid insertion within the MH1 domain. Thus, the transcription of genes that are dependent on Smad2 requires the recruitment of

transcriptional factors such as FoxH1 (FAST-1), a Forkhead family member. FoxH1 was the first identified Smad-interaction transcription factor to permit the binding of the complex of Smad2/Smad4/FAST-1 to active-response element on the *Mix2* promoter region in response to Activin/Nodal-like signals in *Xenopus* (Chen et al., 1996; Chen et al., 1997).

Smad-Mediated Transcriptional Regulation of TGF-β Target Genes

Upon engagement of TGF-β signaling in the nucleus, the specificity of the Smad signaling in the regulation of transcriptional activity of TGF-β target genes is achieved through four levels: 1) *target gene specificity*: recognition of the target genes with the presence of the specific Smad cognate DNA sequence located in the proper orientation and distance on their promoter; 2) *cell type specificity*: expression of certain Smad DNA-binding protein partners in certain cell types for targeting a specific gene 3) *pathway specificity*: recognition of Smad2/Smad3 or Smad1/Smad5/Smad8 through the recruitment of proper DNA-binding protein partners; and finally 4) *specificity in transcriptional effect*: activation or repression of certain target genes through recruitment of specific cofactors (coactivators or corepressors).

The molecular mechanisms of transcriptional responses of the target genes through Smad complexes may either be direct or indirect. The direct mechanism is defined through association of Smads with specific protein partners such as FOX, HOX, RUNX, E2f, AP1, CREB/ATF, Zinc-finger and other families and their involvement in activation or repression of a gene promoter. Based on the nature of the Smad protein partners; coactivators such as p300/CBP, P/CAF and ARC105; or corepressors such as TG-Interacting Factor (TGIF), SKI, and SnoN are then recruited to a specific target gene promoter and assembled to the transcriptional complexes, which may be or not be restricted to a cell- type specific.

The indirect mechanism or ''self-enabled'' gene responses is defined by Smads interacting with the product of expression of certain genes induced through Smads enabling the expression of other Smad-dependent gene response (Figure 1.5). For

instance, in mammalian epithelial cells the Smad3-Smad4 complex binds to the ATF3 promoter and mediates expression of DNA-binding cofactor ATF3 (Kang et al., 2003). Following accumulation of ATF3, it can be recruited to the Id1 promoter as a partner of Smad3-Smad4 complex bound to the TGF-β responsive region to repress the expression of *Id1* gene (Figure 1.5).

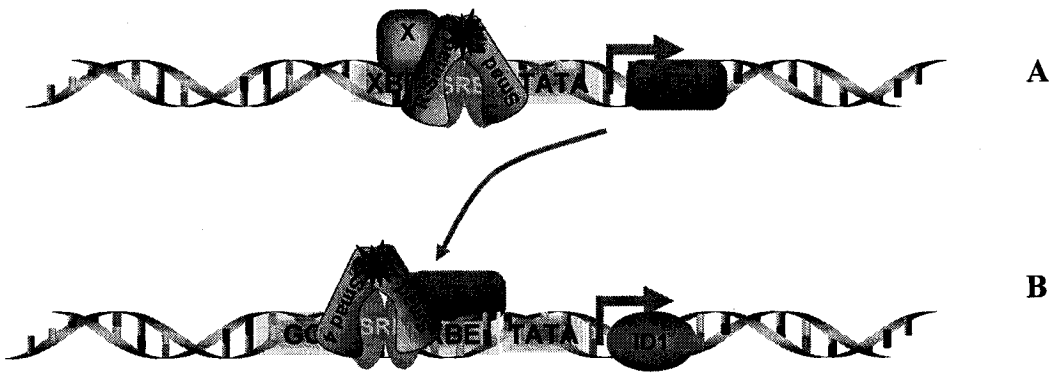


Figure 1.5: Self-enabling gene response: Induction of a repressor cofactor. A) Upon TGF-β stimulation, ATF3 expression which is a repressor cofactor is induced by the R-Smad/Smad4 complex binding to a specific DNA sequence in ATF3 promoter, in concert with the Smad partners, the cofactors. **B)** ATF3 expression becomes accumulated and then interacts with Smad3/Smad4 complex on the *Idl* promoter to mediate the repression of *Idl*. Adapted from Massagué et al., (2005)

1.2.3 Alternative TGF-β Signaling Pathways

Smads are known to be the crucial signal transducers in TGF-β signaling pathway that are involved in most actions of TGF-β family members. However, based on evidences regarding Smad4-deficient organisms and tumor cell lines, Smad4 is not necessary for all actions of TGF-β family members (Sirard et al., 1998). Therefore, signaling via R-Smads independently of Smad4 remains possible.

Upon binding of TGF-β ligands to their receptor complexes, several signaling pathways are activated that have some effects on physiological outcomes and regulation of TGF-β signaling. In addition, since the cells are almost exposed to several extracellular signals (mitogenic signals), TGF-β signaling can be propagated or modified by cross talking with other signaling cascades such as Ras pathway cooperatively as well as antagonistically during development and oncogenesis (Massagué et al., 2003). Moreover, activation of these signaling pathways following TGF-β treatment occurs in cell-context dependent manner and varies extensively in kinetics (Massagué et al., 2000). For example, receptor tyrosine kinases can modify TGF-β signals via activation of the Erk Mitogen-Activated Protein Kinase (MAPK) which phosphorylates R-Smads (Smads 1, 2, and 3) in the linker region. This phosphorylation occurs at low levels of TGF-β which regulates Smad activation through inhibiting ligand-induced nuclear localization of Smads stimulation and thereby the TGF-β antiproliferative response (kretzschmar et al., 1997). In contrast, at higher levels of TGF-β stimulation, phosphorylation of R-Smads in the linker region promotes accumulation of Smads in the nucleus, yet leads to alteration of other responses to Smads suggesting that ERK activation has other effects on Smad signaling. Wide range of studies supports that TGF-β superfamily and MAPK-coupled signals synergize rather than antagonize each another. Engel and his colleagues have demonstrated that mitogenic and stress signals activate JNK, another MAP kinases, which is involved in phosphorylation, activation, and nuclear translocation of Smad3 (Engel et al., 1999).

One molecular explanation of how TGF-β signaling pathway integrates with those of other growth factors can be through the interactions of Smads with the transcription factors that functions in other signaling cascades (Attisano et al., 2002). For instance in epithelial cells, p38 and JNK activity by agonists such as Tumor Necrosis Factor α (TNFα) induces *ATF3* expression, which represses *Id1* expression by TGF-β-activated Smads (Kang et al., 2003). Moreover, our lab demonstrated that activation of p38 MAPK pathway in response to Activin (a TGF-β family member) leads to phosphorylation of

ATF2 transcription factor which is required for cell growth inhibition of human breast cancer cells (Cocolakis et al., 2001).

Activation of PI3K-AKT pathway by mitogenic growth factors has also been reported to affect TGF-β signal transduction (Seoane et al., 2004). It has been shown that high levels of AKT activity phosphorylates FoxO (a Forkhead transcription factor) and blocks its localization into the nucleus to function as a partner of Smad3/Smad4 complexes (Brunet et al., 1999). Therefore, as a result of the activation of PI3K-AKT pathway and phosphorylation of FoxO, expression of p21Cip1 gene induced by TGF-β is attenuated leading to cell survival (Seoane et al., 2004).

TGF-β signaling can also be propagated independently of Smads through activation of Mitogen-Activated Protein Kinases (MAPKs); ERK1/2, p38, and JNK1/2; PI3-kinase/AKT; Protein Phosphatase2A (PP2A); and small Rho-like GTPases (RhoA, Rac and Cdc42). For instance, TGF-β activated kinase-1 (TAK1), a member of the MEKK family, links TGF-β receptors to the MAPK kinase kinase (MKK) activation biochemically and independently of Smads (Yamaguchi et al., 1995). It has also been reported that members of the Rho family of small GTPases are involved in the coupling of TGF-β receptors to JNK activation (Eagle et al., 1999). Nevertheless, the precise molecular mechanism of activation of these pathways by TGF-β receptors, the direct link between them and the TGF-β receptors, and their biological consequences remain unknown.

In addition to gene expression responses, the cross talks between TGF-β signaling pathway and other prominent signaling cascades are involved in the regulation of tumor biology as well. For instance, the Wnt pathway has been shown to cooperate with the TGF-β signaling pathway to regulate tumorigenesis (Nishita et al., 2000). Some other evidences have demonstrated that activation of small GTPases by TGF-β may play a part in mediating TGF-β-induced changes in cytoskeletal organization such as membrane ruffling, lamellipodia, stress-fiber formation, and epithelial-to-mesenchymal transdifferentiation (Edlund et al., 2002).

In summary, depending on the cell type and cell signaling context, several cytoplasmic kinases exert different or even opposite effects on both Smad-dependent and -independent TGF-β signaling pathway.

1.2.4 Negative-Regulation/Termination of TGF-β Signaling

TGF-β signaling is also modulated by negative regulators for the ligand access to the membrane receptors, which interferes with the ligand induced receptor complex formation; posttranslational modification of Smads (ubiquitination); recruitment of I-Smads, Smad phosphatases; transcriptional corepressors; or involvement of other signaling cascades, all leading to attenuation of TGF-β signal transduction.

1.2.4.1 Regulation of Ligands Access to the Signaling Receptors

The function and regulation of TGF-β is initiated with its secretion. Despite the numerous soluble proteins such as Latency-Associated Protein (LAP) which binds to TGF-β superfamily members and prevents their access to the membrane receptors, a group of membrane-anchored proteins such as Betaglycan has been identified as an enhancer of ligand-access to the TGF-β signaling receptors (Lopez et al., 1993). However, Betaglycan can also prevent Activin signaling by promoting Inhibin (an Activin antagonist) binding to the type II receptor and therefore, blocking Activin access to the type II receptor (Lebrun et al., 1997; Lewis et al., 2000).

1.2.4.2 Regulation of Membrane Receptors Activity

The crystal structure analysis of unphosphorylated TGF-β type I receptor has demonstrated that at basal state, a cytosolic inhibitory protein named Immunophilin FKBP12 binds to a region between unphosphorylated GS region and kinase domain of type I receptor (Huse et al., 1999). FKBP12 inhibits TGF-β signaling and prevents the leaky activation of ligand-independent phosphorylation of type I receptor by type II receptor or any other protein kinases (Chen et al., 1997) through blocking access to the

GS region phosphorylation site. The X-ray crystal structure of the FKBP12-TβRI cytoplasmic domain complex demonstrated that FKBP12 also stabilizes the inactive conformation of type I receptor kinase domain (Huse et al., 1999) and finally eliminating the binding site for R-Smad substrates.

BMP and Activin Membrane-Bound Inhibitor (BAMBI) is another negative regulator for membrane receptors with a similar extracellular domain sequence to TGF-β type I receptors (pseudoreceptor). This transmembrane protein interferes with the ligand induced receptor complex formation and activation of type I receptors by forming heterodimers with type I receptors (Onichtchouk et al., 1999).

There are several other receptor-interacting proteins with WW protein-protein interaction domain including TGF-β-Receptor Interaction Protein-1 (TRIP-1/TRAP-1), Protein Phosphatase2A (PP2A), and Serine Threonine kinase Receptor Associated Protein (STRAP) that could be involved in receptor regulation or signal propagation. For instance, STRAP interacts with both TGF-β type I and type II receptors in a ligand-independent manner and attenuates TGF-β signaling possibly by recruiting Smad7 (Datta et al., 1998).

1.2.4.3 Regulation of TGF-β Receptor Complex and R-Smads Level by the Ubiquitin Ligase, SMURF

Among several posttranslational modifications of Smads, ubiquitination is involved in termination of TGF-β signaling. One of Smads interacting proteins, Smad Ubiquitination Regulatory Factors (SMURFs) have been identified to ubiquitinate and degrade R-Smads. As mentioned previously, SMURFs are E3 ubiquitin ligases that contain a HECT-domain and a WW protein-protein interaction domain through which they recognize and interact with the PPXY motif found in the linker region of R-Smads and I-Smad. The interactions between SMURFs and R-Smads lead to their immediate ubiquitination and degradation. While, the interactions between SMURfs and Smad7 result in shuttling the Smad7/SMURFs complex to the cytoplasm and targeting SMURFs

to the TGF-β receptor complex for degradation leading to downregulation of TGF-β signaling (Kavsak et al., 2000).

In conclusion, SMURFs are involved not only in decreasing the steady-state levels of R-Smads and interfering with Smad-activated transcription, but also in the regulation of TGF-β membrane receptor complexes.

1.2.4.4 Feedback Regulation by Inhibitory Smads (I-Smads)

In contrast to R-Smads and Co-Smad carrying TGF-β signal from receptor into the nucleus, the I-Smads (Smad6, Smad7) are capable to attenuate ligand-induced Smad activation and gene expression and ultimately abrogate TGF-β family signaling. At the basal state, I-Smads reside in the nucleus and upon ligand stimulation the expressions of I-Smads are increased followed by their shuttling to the cytoplasm (Itoh et al., 1998), therefore, an auto-inhibitory feedback mechanism for ligand-induced signaling is achieved through elevated expression of the transcriptional regulators, I-Smads.

Inhibition of TGF-β signaling occurs through binding of the inhibitory Smad7 MH2 domains to the type I receptor (Heldin et al., 1997; Massagué et al., 1998), thus preventing R-Smads recruitment and activation (Figure 1.6). Whereas, inhibition of BMP signaling occurs through the inhibitory Smad6 which competes with Smad4 for binding to Smad1, and results in an inactive Smad1-Smad6 complex without interfering with the function of receptor (Massagué et al., 2000).

Besides TGF-β signaling pathway, other pathways such as interferon-γ (IFN-γ) is also involved in inducing Smad7 expression. IFN-γ increases Smad7 expression by Jak1 tyrosine kinase and STAT1 transcription factor therefore, inhibiting TGF-β-mediated Smad3 phosphorylation and signal transduction (Ulloa et al., 1999).

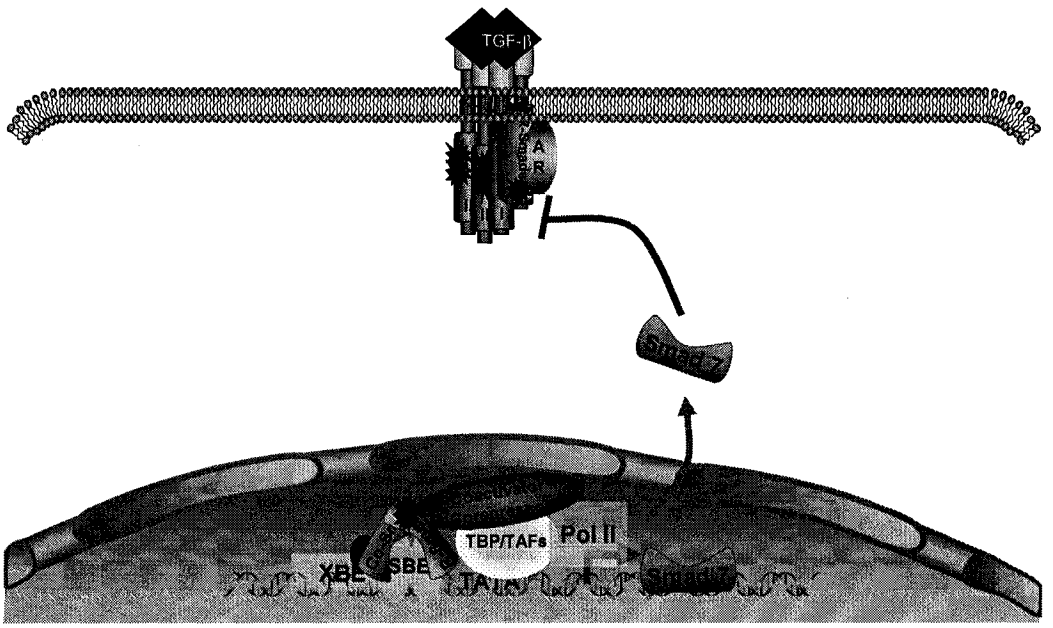


Figure 1.6: Auto-inhibitory feedback mechanism for TGF-β-induced signaling. Following TGF-β stimulation, the expression of the inhibitory Smad7 is increased and following translocation to the cytoplasm, it is capable to inhibit R-Smads phosphorylation and attenuate ligand-induced-Smad activation and gene expression.

1.2.4.5 Smad Clearance from the Nucleus by Activation of Smads Phosphatases

Lin and his colleagues uncovered PPM1A as Smad2 and Smad3 phosphatase through overexpression approach and screening for mammalian phosphatases that target R-Smads. They confirmed that PPM1A which is abundant in the nucleus may terminate TGF-β responses through dephosphorylation of Smad2 at the C-terminal SXS motif and facilitate nuclear export of Smad2 or Smad3. They also reported that high level of PPM1A inhibited the anti-proliferation function of TGF-β (Lin et al., 2006).

1.2.4.6 Transcriptional Corepressor, TG-Interacting Factor (TGIF), Sloan-Kettering Institute (c-Ski) or Ski-related novel gene N (SnoN)

Upon localization of Smad complex in to the nucleus, this complex may associate with transcription coactivators or alternatively with transcriptional corepressors. Depending on the nature of the Smad partners, the recruitment of corepressors such as

TGIF (Wotton et al., 1999), EVI1, SKI, or SnoN (Akiyoshi et al., 1999; Luo et al., 1996; Sun et al., 1999) to the Smad complex attenuates and antagonizes Smad-mediated transactivation of TGF-β signaling (Liu et al., 2001; Wang et al., 2000).

Wotton and his colleagues have reported that in response to TGF-β, TGIF interacts directly with Histone DeACetylases (HDACs) and recruits them to Smad2/Smad4 complex to inhibit nuclear function of Smads (Wotton et al., 1999). Lo and his colleagues demonstrated that in response to TGF-β, TGIF protein can be phosphorylated by MEK signaling (in Ras-MEK-MAPK pathway) therefore, resulting in the stabilization of TGIF protein and formation of Smad2/TGIF complex to repress target gene transcriptional activity (Lo et al., 2001).

Evi-1 corepressor inactivates TGF-β-responsive reporters and attenuates TGF-β-induced growth inhibition through interacting with Smad3 and preventing binding of Smad3/Smad4 complex to DNA (Kurokawa et al., 1998).

There are two mechanisms involved in repression of TGF-β signaling mediated by SKI and SnoN: 1) Recruitment of the nuclear transcriptional corepressor (N-CoR) and histone deacetylase and therefore, opposing the function of the histone acetyltransferase activity associated with the transcriptional coactivator, p300/CBP. 2) Competition with R-Smads for interaction with Smad4 and interfering with the formation of Smad4/R-Smads complexes (Kim et al., 2000). However, Upon TGF-β stimulation, the negative regulation on Smads mediated by SnoN is removed by ubiquitination and degradation either through recruitment of 1) E3 ubiquitin ligase, Anaphase Promoting Complex, APC (Stroschein et al., 2001), or 2) SMURF2 which is also bound to Smad2 (Ebisawa et al., 2001).

Taken all together, the activation or repression of TGF-β target genes is relied on the aspects that regulate Smads nuclear accumulation, post translational modification, and interaction with transcriptional cofactors.

1.2.5 TGF-β and Cancer

The complex process of tumor formation and progression requires a series of events to take place enabling cancer cells to acquire hallmarks such as: 1) resistance to growth inhibitory factors; 2) proliferation in the absence of exogenous growth factors; 3) escape from apoptosis; 4) angiogenesis induction; 5) immortality; 6) evasion form immune defense; and finally 7) invasion and metastasis (Hanahan et al., 2000). Signaling transduction pathways such as TGF-β cascades are involved in the regulation of each of these hallmarks to maintain tissue homeostasis whereas, alteration or disruption of these signaling pathways contribute to human tumorigenesis.

TGF-β maintains the cellular homeostasis through regulation of a diverse set of cellular processes, including cell growth, proliferation, differentiation, and apoptosis in various tissue or cell types such as epithelial, endothelium, stromal fibroblasts, and immune cells. Moreover, the balance between these cellular processes (rather than their simple presence or absence) is critical to many physiological processes which will determine the final output of TGF-β signaling. Breaking this balance is often associated with variety of diseases. There is enough evidence showing that deregulation in cell proliferation and cell death have been implicated in a variety of human diseases such as cancer. For instance, excess of apoptosis in immune cells results in immunodeficiency where as insufficient apoptosis may be observed in autoimmunity and human cancers (Gupta et al., 2000).

Although TGF-β is a potent growth inhibitor in epithelial tissues with cytostatic and differentiative effects preventing tumor emergence and progression, loss of these responses is a hallmark of cancer. Alterations in the TGF-β signaling cascade including mutations or deletions in the central components of TGF-β signaling pathway (the receptors and Smads) may disrupt the antiproliferative and apoptotic functions of TGF-β and contribute to tumor formation thus, supporting the tumor suppressive nature of TGF-β in human cancers.

While mutational inactivation of TGF-β signaling components increases cancer risk, high level of TGF-β secretions enhances the aggressiveness of several types of tumors. At later stages of carcinogenesis, while retaining functional TGF-β receptors and Smad activity, the aggressive human tumor cells that have lost their growth-inhibitory responsiveness to TGF-β and have become resistant to TGF-β mediated cytostasis or apoptosis may utilize TGF-β as a tumor progression factor. For instance, breast cancer cells often lose TGF-β cytostatic responses without inactivation of TGF-β signaling members (Jennings et al., 1998; Anbazhagan et al., 1999). In response to TGF-β stimulation, these tumor cells induce gene responses that promote tumor growth (Gold et al., 1999), cell migration, invasion (Derynck et al., 2001; Kang et al., 2005), evasion of immune surveillance (Gorelik et al., 2002; Thomas et al., 2005; Wojtowicz-Praga et al., 2003), and metastasis (Roberts et al., 2003; Siegel et al., 2003; Kang et al., 2005). Whereas, the tumor-derived TGF-β has the ability to affect the tumor cells as well as many other cell types including stromal fibroblasts, endothelial cells and immune cells that are in proximity to the growing tumor leading to invasion and metastasis to other tissues.

1.2.5.1 TGF-β Receptor and Smad Mutations in Cancer

The alterations in TGF-β signaling system such as mutations found in the TGF-β receptors or Smads disrupt the antiproliferative and apoptotic functions of TGF-β and promotes tumorigenesis validating the role of TGF-β signaling components as bona fide tumor suppressor (Massagué et al., 2000).

Mutational inactivation of TβRII is often found in most human colorectal and gastric carcinomas as the result of MicroSatellite Instability, MSI (Markowitz et al., 1995). In both sporadic and hereditary colon and gastric tumors, these mutations occur as a result of insertion or deletion of bases within TβRII extracellular domain generating truncated and inactivated forms of the receptor. It was also reported that 15% of MSI colon cancers containing missense mutations are mostly found within the kinase domain

of TβRII (Grady et al., 1999). Since the MSI tumor formation has not been found in the pancreas, liver, and breast cancers (Myeroff et al., 1995; Tomita et al., 1999; Furuta et al., 1999), the mutational inactivation found in TβRII are special for only certain cancers of specific tissue origins.

Mutational inactivation within TGF-β type I receptor are less frequently observed in ovarian (Wang et al., 2000), breast (Chen et al., 1998), pancreatic cancers (Goggins et al., 1998), as well as T-cell lymphomas (Schiemann et al., 1999). A variant of the TβRI gene (TβRI*6A) is carried by approximately 14% of the general population, which is involved in decreasing growth inhibition mediated by TGF-β (Kaklamani et al., 2003). Kaklamani and his colleagues have reported that the overall cancer risk is increased by 70 and 19% among TβRI*6A homozygotes and heterozygotes, respectively, suggesting that this variant of the TβRI gene (TβRI*6A) may become a target for cancer chemoprevention.

Among the Smads, Smad4 was first identified as a tumor suppressor gene that was homozygously deleted in 50% of pancreatic carcinoma (Hahn et al., 1996). Since then, Smad4 mutations were identified resulting in 10% of all colon cancers and 30% of metastatic colon cancers. The mutations found in Smad2 have been rarely found in colorectal and lung cancers (Eppert et al., 1996; Uchida et al., 1996). Moreover, no Smad3 mutation has yet been found in human cancer. However, homozygous deleted Smad3 mice develop metastatic colorectal cancer (Zhu et al., 1998).

1.2.6 Dual Role of TGF-β in Tumorigenesis

In the early stage of cancer, the tumor suppressor role of TGF-β is supported by cell growth inhibition in epithelial tissues with cytostatic and differentiative effects preventing tumor progression. Whereas, in the late stage of tumors, cells become resistant to growth inhibitory effect of TGF-β due to the loss of cytostatic gene responses and gain more aggressive phenotype such as: epithelial-mesenchymal transdifferentiation, invasion,

immunosuppression, angiogenesis, extravasation, and metastasis (Siegel et al., 2003; Elliott et al., 2005). However, the precise mechanism for the dichotomous function of TGF-β in human cancers remains elusive.

In conclusion, TGF-β a potent growth inhibitor in epithelial tissues, not only functions as a tumor suppressor at early stages of cancer, but also exerts tumorigenic effects and promotes cancer progression at later stages of carcinogenesis. Moreover, the loss of cytostatic, differentiative and antiproliferative effects of TGF-β in preventing tumor emergence as well as tumor progression are hallmark of cancer and are considered essential components of tumorigenesis.

1.2.7 Tumor Suppressor Role

1.2.7.1 Cell Cycle Arrest

Antiproliferative and mitogenic growth signals are involved in regulation of cellular proliferation by acting upon cell cycle regulators. The growth inhibitory effects of TGF-β as well as its ability to induce differentiation are also mediated through a program of cytostatic gene responses, which are barrier to tumor formation and progression (Massagué et al., 2006).

According to the initial studies and recent microarray analysis on transcriptional responses of TGF-β target genes in skin, lung, and mammary epithelial cells, a set of TGF-β gene responses have been found that are involved in cell cycle regulation and control the TGF-β cytostatic effect in a Smad-dependent manner (Massagué et al., 2006). Cyclin Dependent Kinase (CDK) inhibitors $p15^{INK4b}$ (Hannon et al., 1994), $p21^{CIP1}$ (Datto et al., 1995), and $p27^{Kip1}$ (Polyak et al., 1994), which are transcriptionally active and upregulated upon TGF-β stimulation, mediate TGF-β growth inhibition in late G1 phase at cell cycle arrest. These CDK inhibitors block cycling and prevent CDKs form phosphorylating the Retinoblastoma protein (Rb) allowing the hypophosphorylated form of Rb to bind and sequester the transcription factor E2F that mediates cyclins expressions necessary for S phase progression. At the same time, expression of *c-MYC* (Alexandrow

et al., 1995), a growth-promoting transcriptional factor, as well as *(ID)-1*, *-2*, and *-3* (which are nuclear factors involved in preventing cell differentiation) are repressed (Kang et al., 2003).

Since transcriptional activation or repression of genes in response to TGF-β are mediated by Smad-cofactors, $p21^{CIP1}$ and $p15^{INK4b}$ expressions are induced by activation of Smad-FoxO (Seoane et al., 2004) and Smad-FoxO-C/EBPβ transcriptional complexes (Gomis et al., 2006), respectively. While Smad-E2F4/5-C/EBPβ (Chen et al., 2002) and Smad-ATF3 complexes (Kang et al., 2003) repress expressions of *c-MYC* and *Id-1*, respectively. Interestingly, C/EBPβ not only induces $p15^{INK4b}$ expression but also represses *c-MYC* by Smads, and the induction of CDK inhibitors only occurs when *c-MYC* expression is declined.

Furthermore, there have been reports suggesting that the growth-inhibitory effect of TGF-β can be also mediated by the Smad-independent pathways including the MAPK (Hu et al., 1999) and the PP2A/p70S6 (Petritsch et al., 2000) kinase pathways.

1.2.7.2 Apoptosis

Besides regulation of cellular proliferation, TGF-β also controls cell number by induction of program cell death (apoptosis), which involves caspases activation (Inman et al., 2000) as well as the change in expression, localization, and activation of both pro- and anti-apoptotic members of the BCL2 family (Motyl et al., 1998).

Some TGF-β pro-apoptotic target genes have also been identified as components of the TGF-β cell-death network that are involved in the regulation of apoptosis in a cell and context dependent manner. For instance, increased expression of transcription factor, TGF-β-Inducible Early-response Gene1 (TIEG1) in various epithelial cell types (Tachibana et al., 1997), and Death–Associated Protein Kinase (DAPK) in hapatoma cells (Jang et al., 2002), inhibit proliferation and induce apoptosis.

Our lab has also demonstrated that in hematopoietic cells, TGF-β/Activin induces apoptosis through upregulation of SH2-doamin-containing Inositol-5-Phosphatase1 (SHIP1) expression, which inhibits the survival protein kinase AKT signaling in a Smad-dependent pathway (Valderrama-Carvajal et al., 2003).

Moreover, the Smad-independent pathways, including DAXX-mediated JNK activation, may also be involved in the regulation of programmed cell death by TGF-β (Perlman et al., 2001).

1.2.8 Tumor Promoter Role

1.2.8.1 Angiogenesis

The growth of solid tumors greater than 1 to 2 mm in diameter depends on the blood supply to provide the tumors required oxygen and nutrients through the formation of new blood vessels. Several evidences, including aberrant angiogenesis due to deletion of TGF-β1 (Dickson et al., 1995), TβRI (Larsson et al., 2001), and TβRII (Oshima et al., 1996) in mice have supported a pro-angiogenic role for TGF-β. The formation of new blood vessels and stimulation of angiogenesis mediated by TGF-β is accomplished through induction of Vascular Endothelial Growth Factor, VEGF (Yamamoto et al., 2001), which is a major stimulus in the promotion of angiogenesis among others.

1.2.8.2 Immunosuppression

Immune system is normally involved in recognizing cancer cells expressing tumor-specific antigen followed by their destruction. However, during tumorigenesis, most cancer cells are capable to escape this immunosurveillance. One of the major mechanism by which cancer cells escape an immune response is through producing and secreting TGF-β (Bodmer et al., 1989) known as a potent immunosuppressive cytokine (Kirkbride et al., 2003). The immunosuppressive effects of TGF-β are mediated through potent effects on Antigen Presenting Cells (APCs), and T cells (Brandes et al., 1991). With the lost of TGF-β cytostatic response in cancer cells, T lymphocytes become the

primary targets for TGF-β-mediated immune suppression during immune response. TGF-β which is produced by T cells prevents InterLeukin 2 (IL2) production and inhibits proliferation of T cells (Kehrl et al., 1986). It has also the ability to prevent naïve T cells from acquiring their effectors (cytotoxic or helper) functions and inhibits differentiation of T cells (Gorelik et al., 2002). TGF-β is also secreted by macrophages and is involved in preventing activation of tissue macrophages (Bogdan et al., 1992).

In summary, TGF-β plays a crucial role in the escape of tumor from host immunity and allowing the tumor to change the tumor microenvironment and host immune response.

1.2.8.3 Epithelial-Mesenchymal Transition (EMT)

Epithelial-Mesenchymal Transition (EMT) is a well-established biological process characterized by disassembly of cell-cell contacts, where epithelial cell layers lose their cellular polarity (epithelial phenotype) and manifest a flattened migratory phenotype (Thiery et al., 2002), which is usually accompanied by reorganization of the Actin cytoskeletal protein into stress fibers. This transition is critical for both embryonic development and wound healing. However, it also occurs during progression of benign tumors towards highly invasive malignancies including 24% to 45% of human breast cancers (Thiery et al., 2002; 2003, Zavadil et al., 2005).

The loss of cytoplasmic expression of E-cadherin protein, transcriptional repression of its mRNA, and increase in cell motility are the critical steps driving EMT (Thiery et al., 2002; 2003). Several autocrine factors and signal transduction pathways are contributed to EMT including TGF-β, which downregulates E-cadherin expression and therefore, enhances tumor cell invasiveness in a number of cancer models through both Smad-dependent (Zavadil et al., 2004), and Smad-independent signaling pathways including PAR6, PI3K-AKT, RHOA and p38 MAPK (Zavadil et al., 2005).

Based on phenotypic analysis in epithelial cells, EMT induced by TGF-β occurs in a coordinated temporal sequence of disassembly of cell junctions, cytoskeletal reorganization, loss of epithelial polarity, and remodeling of cell-matrix adhesions (Zavadil et al., 2001). However, the precise mechanism of TGF-β-induced EMT in vivo has not yet been clarified.

Since invasion and metastasis are the most lethal features of cancer and cause of cancer-related death, a thorough understanding of the molecular mechanisms underlying TGF-β mediating tumorigenesis through the cross-talk with other signaling pathways in different human cancers may shed new light in providing novel therapeutic modalities for the treatment of cancer.

1.2.9 Involvement of $\alpha_v\beta_3$ Integrin in Facilitating TGF-β-Mediated Induction of EMT in Mammary Epithelial Cells (MECs)

The organization of the epithelial cells within the tissue depends upon cell–cell adhesion as well as cell interactions with the extracellular matrix that underlies the epithelial units and makes up most of the organization of the stroma. Therefore, cell adhesion to the extracellular matrix is critical in many biological processes and is mediated by several different proteins that are associated with sites of cell adhesion to the Extra Cellular Matrix (ECM) such as ECM ligands, transmembrane adhesion receptors, adaptor proteins, cytoskeletal-binding proteins, and signaling proteins (Figure 1.7).

Cell adhesion to the ECM is primarily mediated by a class of heterodimeric transmembrane adhesion receptors called integrins, each composed of a αβ heterodimer. Integrins are a widely distributed family involved in the regulation of numerous biological processes including homeostasis, proliferation, differentiation, migration, tissue organization, immune response, wound healing, angiogenesis, embryogenesis, as well as tumorigenesis (Hynes et al., 1992).

These transmembrane receptors are clustered at focal adhesions and link the matrix with an intracellular structural scaffold, the cytoskeleton (Figure 1.7), as well as with signaling enzymes that direct cell survival, proliferation, differentiation, and migration. Therefore, integrin signaling affects cell adhesion, migration, differentiation, proliferation, and survival (Giancotti et al., 1997).

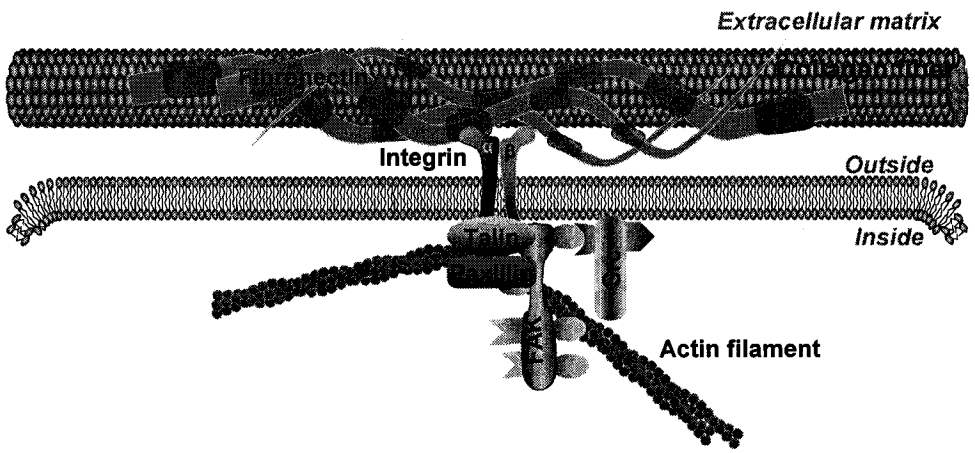


Figure 1.7: Proteins involved in linking the extracellular spaces to the inside the cell. Fibronectin, an ECM protein binds to integrin receptor, which are also linked to Actin cytoskeletal proteins at focal adhesion complexes consisting of Src, FAK, Talin, and Paxilin. Therefore, these transmembrane receptors, the integrins, link the matrix to an intracellular structural scaffold, the cytoskeleton. Adapted from Campbell, (2003)

TGF-β is a potent regulator of integrin-substrate interactions. It has been demonstrated that TGF-β upregulates the expression of several integrin subunits which obtain a more adhesive phenotype, as well as expression of ECM proteins (collagens, fibronectin [FN], and laminin) that are recognized by integrins (Ignotz and Massagué, 1986).

As mentioned previously, TGF-β suppresses tumor formation by inducing cell cycle arrest and apoptosis. However, during tumorigenesis TGF-β loses its cytostatic function (due to genetic and epigenetic changes) and acquires the ability to promote development of metastatic phenotypes. Unfortunately, the molecular mechanisms

underlying the conversion of TGF-β as a tumor suppressor to a tumor promoter are not well defined. However, according to Galliher and Schiemann (2006) this conversion may involve the signaling inputs from integrins. They have recently presented an approach for TGF-β mediated tumor suppression in progressing human breast cancers. They demonstrated that upon TGF-β stimulation in Mammary Epithelial Cells (MECs), $\beta_3$integrin expression increases which alters TGF-β signaling. They showed that the $\beta_3$integrin directly couples to the TGF-β signaling system by interacting physically with TβRII, promoting and Src-mediated TβRII tyrosine phosphorylation, enhancing cell proliferation and the ability of TGF-β to activate MAPKs and consequently, to induce EMT.

TGF-β contribution to tumor invasion, EMT, and cancer progression is also mediated by increasing the motility of tumor cells. Kloeker and et al. (2004) performed cDNA microarray analysis on a TGF-β-responsive cell line, Human Mammary Epithelial Cells (HMEC), to identify TGF-β-inducible genes and whether they are involved in TGF-β-mediated migration and EMT. They reported that Kindlerin, which has been implicated in human cancers and significantly overexpressed in lung and colon cancers (Weinstein et al., 2003) was found to be regulated by TGF-β at both mRNA and protein level. Kindlerin contains a putative FERM (Four point one Ezrin, Radixin, Moesin) domain identified in several proteins including Talin that connects the cytoplasmic domains of transmembrane proteins to the Actin cytoskeletal proteins (Bretscher et al., 2002).

Based on the homology of the FERM domain of Kindlerin with the integrin-binding residues found in Talin, they also demonstrated localization of kindlerin at sites of integrin-rich membrane-substratum adhesion (focal adhesions) and complex formation with the cytoplasmic domain of integrinβ. Since integrins have a critical role in mammalian cell adhesion and migration, based on siRNA studies performed by the same group, they were able to demonstrate the impact of kindlerin on cell spreading. They showed that in HACaT cells after 48 hours of exposure to TGF-β kindlerin levels increased; whereas, E-cadherin expression which is a marker for EMT declined. In

conclusion, they suggested that kindlerin may mediate TGF-β signaling in tumor progression via contributions to integrin-dependent cellular functions.

Since, all FERM domain containing proteins share homologous structural domains, they are therefore proposed to be regulated by similar mechanisms. Talin is known for its role in linking integrins to the Actin cytoskeletal proteins (Horwitz et al., 1986), as well as in integrin-mediated adhesion and developmental events. Mutant studies in *Drosophila* ovarian follicle cells have also uncovered a new role for Talin in regulating E-cadherin-mediated cell adhesion, that is, inhibition of DE-cadherin transcription through modulation of transcription mechanism independent of integrins (Becam et al., 2005). In this study, we found Talin, another FERM domain containing protein is positively regulated by TGF-β1 in breast cancer cell lines, which is involved in TGF-β biological responses. Therefore, to have a better understanding of Talin and its functional roles, a brief overview and a concise literature review on Talin are presented in the following section.

1.2.10 Talin

Talin is a member of the 'band 4.1' superfamily of membrane protein-cytoskeletal adaptor proteins (Rees et al., 1990; Pearson et al., 2000). This protein was initially identified as the first cytoplasmic protein binding to integrins (Horwitz et al., 1986) and linking them to the Actin cytoskeletal proteins (Jockusch et al., 1995; Critchley et al., 2000). Talin proteins are found in a wide variety of organisms, from slime molds (Kreitmeier et al., 1995) to human (Coiller et al., 1984) in different tissues including leukocytes, lung, placenta, small intestine, liver, kidney, spleen, thymus, colon, skeletal muscle, and heart (Ben-Yosef et al., 1999). Chen et al. (2000) also demonstrated that Talin is highly expressed in several prostate and breast cancer cell lines. Based on the obtained results in the present thesis, Talin is not only expressed highly in human breast cancer cells but also positively regulated by TGF-β1 (will be discussed in more details in Chapter-III).

The human Talin gene is mapped to chromosome 9p13 (Gilmore et al., 1995), extending over more than 23 kb consisting of 57 exons and 56 introns (Ben-Yosef et al., 1999). In mammals, there are two Talin genes, Tln1 (approx. 30 kb) and Tln2 (> 200 kb) encoding proteins containing 2541 amino acids with approximately 74% identity. However, Tln2 has a different size due to the presence of much larger introns and has a more restricted pattern of expression than the ubiquitous Tln1 (Monkley et al., 2001).

Regarding the distribution of Talin protein in various types of cells and tissues, immunocytochemical studies have demonstrated that Talin is localized in a variety of structures. For instance in platelets, it may be distributed from cytosol to membrane in response to thrombin activation (Table-1), which is associated with Talin phosphorylation on serine/threonine residues located on the Talin head domain (Bertagnolli et al., 1993; Critchley, 2004). However, after the discovery of the second Talin gene, Tln2 and its recognition by several antibodies it is difficult to interpret the reported data so far.

Table-1: Talin Distribution

- Cell-extracellular matrix junctions
  - Focal adhesion
  - Basal surface epithelial/endothelial
  - Myotendinous junctions
- Cell-cell junctions
  - Endothelial cell-cell junctions
  - T-cells at junctions with antigen presenting cells
  - Neuromuscular jucntions
- Other sites
  - Membrane ruffles
  - Golgi and endoplasmic reticulum
  - Platelets (translocates from cytosol to membrane in response to thrombin)

Table 1.1: Distribution of Talin in different cells and tissues. Adapted form Critchley, (2004)

1.2.10.1 Talin Domain Structure and Different Protein Binding Sites

Talin is a large (270 kDa) protein containing approximately 2541 amino acids which exists as a flexible, elongated (approx.60 nm) anti-parallel homodimer (Isenberg et al., 1998). As shown in Figure 1.8, Talin contains two protein domains, an N-terminal globular head region (residues 1-435) and a C-terminal flexible rod region (residues 2270-2541). There is a calpain proteolytic cleavage site between the Talin head and rod domains liberating the N-terminal head form the C-terminal Rod domain and another protease-sensitive region within the rod domain (Critchley, 2004).

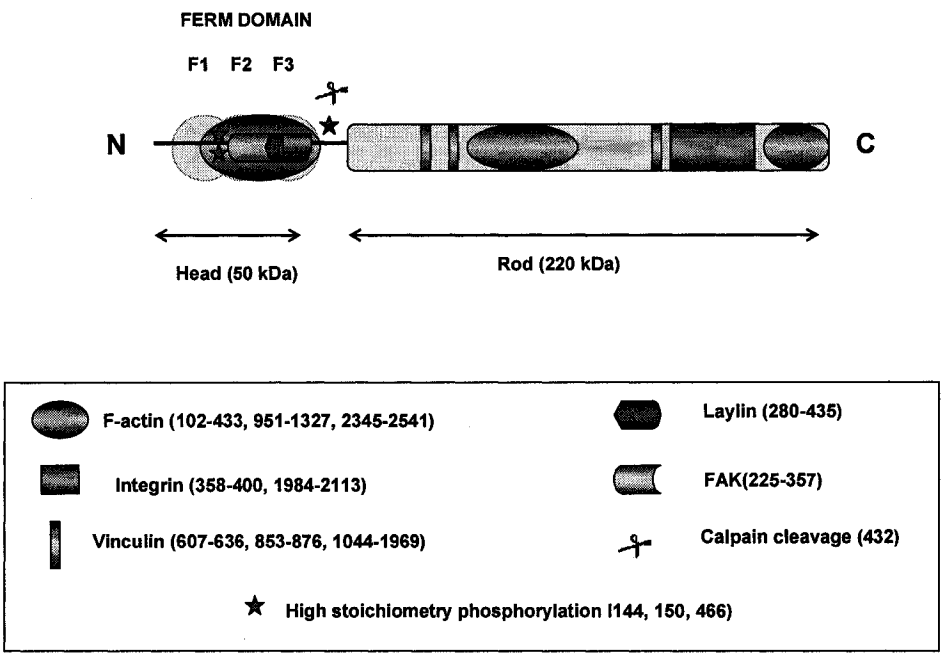


Figure 1.8: Talin domains. Protein domains of Talin include an N-terminal globular FERM domain (F1, F2, and F3) and a C-terminal flexible Rod domain. There is a calpain proteolytic cleavage site between these two domains. The binding sites for different proteins are shown in different shapes. The stars indicate sites with high possibility of phosphorylation. Adapted form Ratnikov et al., (2005)

The globular Talin head contains sequences similar to the FERM (band four-point-one, ezerin, radixin, moesin) domain found in the ERM (ezerin, radixin, moesin)

family of proteins that characterizes these proteins. FERM domain (residues 86-400) which consists of three subdomains F1, F2, and F3, is enriched in basic amino acids (Rees et al., 1990) with binding sites for β-integrin, layilin, Focal Adhesion Kinase (FAK), PIP kinase (type 1γ isoform of phosphoinositide 4,5-kinase), and Actin (Garcia et al., 2003; Borowsky et al., 1998; Ling et al., 2002; Hemmings et al., 1996).

F3, one of the subdomains of FERM domain, is the major binding site for Talin interacting with integrinβ subunit. This region has the same structural fold as Phospho Tyrosine Binding (PTB) domains, which interacts with the NPxY motif in the cytoplasmic tails of integrinβ subunit (Calderwood et al., 2002; Garcia-Alvarez et al., 2003). This interaction increases the affinity of integrin extracellular domains for ligands (inside-out signaling) through conformational changes in the integrin extracellular domain (Calderwood et al., 2004). Moreover, Talin head region contains binding sites for the type 1γ661 isoform of Phosphatidyl-inosital-4-phosphate 5-kinase, PIPK (Ling et al., 2002; Di paolo et al., 2002), and FAK (Borowsky et al., 1998), which are required for localization of Talin to the membrane and cell-ECM junctions (focal adhesions).

The Talin rod domain contains a conserved C-terminal tail with different binding sites including Actin-binding site (Hemmings et al., 1996), a second integrin-binding site (Xing et al., 2001; Tremuth et al., 2004), and several binding sites for vinculin, another cytoskeletal protein (Bass et al., 1999). It has been reported that this domain is also responsible for Talin homodimerization (Molony et al., 1987; Muguruma et al., 1995).

1.2.10.2 Talin Functional Roles

In addition to the key function of Talin in coupling the integrin family of cell adhesion molecules to the Actin cytoskeletal proteins (Critchley, 2004), Talin is also involved in integrin signaling. The integrin inside-out signaling occurs through binding of Talin FERM domain to the integrin cytoplasmic tails, which induces conformational changes in the integrin extracellular domains and therefore increases their affinity to its extracellular matrix ligands including fibronectin or laminin (Barsukov et al., 2003; de

Pereda et al., 2005; Di Paolo et al., 2002; Ling et al., 2002; Nayal et al., 2004). Whereas, binding of Talin FERM domain to FAK (a non-receptor tyrosine kinase) and layilin (a transmembrane protein) is involved in integrin outside-in signaling, modulating cell adhesion and motility (Borowsky et al., 1998; Bono et al., 2001).

Several evidences from different sources such as functional studies using antibody microinjection (Nuckolls et al., 1992) and antisense RNA (Albiges-Rizo et al., 1995) supported the essential role of Talin in integrin-mediated cell adhesion through confirming the involvement of Talin in focal adhesion assembly (cell-extracellular matrix junctions), actin stress fibers, and cell migration. Talin is also known for its role in a variety of integrin-mediated developmental events. Gene knockout studies (Cram et al., 2003, Brown et al., 2002; Monkley et al., 2000) where Talin (-/-) embryos were embryonic lethal at the gastrulation stage, have confirmed the pivotal role of Talin in embryogenesis development.

Interestingly, Becam et al. (2005) have reported that Talin has a second, integrin-independent function. They demonstrated that Talin downregulates DE-cadherin transcriptional expression in *Drosophila* ovarian follicle cells. This discovery placed Talin among the proteins found at cellular junctions that can also regulate gene expression. So far, the possible mechanism involved in Talin repressing DE-cadherin expression has not been defined. Tepass and Godt (2005) have suggested that since Talin has not been detected in the nucleus, perhaps it may recruit a transcriptional repressor to block DE-cadherin expression. Similar to the protein Zo-1 that sequesters ZONAB at the tight junction and suppresses proliferation in epithelial cells, Madin-Darby Canine Kidney (MDCK) cells (Balda et al., 2003). Therefore, it will be important to elucidate if Talin is essential in the regulation of E-cadherin gene expression in other tissues and organisms, and discover the signaling pathway and mechanism involved in this event. To accomplish this, a better understanding of how Talin becomes regulated and activated is required.

1.2.10.3 Talin Regulation and Activation

Since Talin may incorporate many signals, it would be interesting to know how Talin is activated. There are several potential mechanisms implicated in Talin regulation, including proteolytic cleavage by calpain separating the N-terminal-FERM domain from the C-terminal rod domain; Talin phosphorylation; or Talin interacting with inositol phospholipids, which will induce a conformational change in Talin enhancing its affinity for the β subunit of integrin cytoplasmic tail (Martel et al., 2001).

As illustrated in Figure 1.9, integrin signaling leads to tyrosine phosphorylation of type I gamma Phosphatidyl-Inositol Phosphate Kinase (PIPKIγ) by Src, which is potentially regulated by FAK. Phosphorylation of PIP kinase increases its affinity for binding to Talin and competing with integrin β subunit. Upon formation of PIPKIγ-Talin complex, not only PIPK is targeted to the focal adhesions but also increases its catalytic activity to produce PIP2 at focal adhesions. PIP2 is a key lipid messenger playing a pivotal role in regulating a variety of cellular signaling pathways. This membrane phospholipid promotes the recruitment of many cytosolic proteins to the plasma membrane for protein-protein interactions and for the modulation of protein activities through allosteric structural transitions. PIP2 production at FA also enhances integrin-Talin interaction which may then displace PIPKIγ from Talin leading to the reduction of PIP2 production pointing to a critical role of Talin in regulating the generation of PIP2 in integrin signaling complexes.

Since PIPKIγ661 and integrin share the same binding site on Talin F3 FERM subdomain (PTB domain), Ling and his colleagues (2003) demonstrated a model which was supported by mutational studies. They suggested that upon phosphorylation of PIPKIγ on Y644, Talin switches from binding to integrin to PIPKIγ661. Y644 forms a charge-charge interaction with Talin residues, K357 and R358, which are located close to Y644. Phosphorylation of integrin β subunit on Y788 results in the loss of integrin binding to Talin head domain due to the fact that this tyrosine-phosphorylated residue may be sterically hindered from interacting with K357, or R358 of Talin.

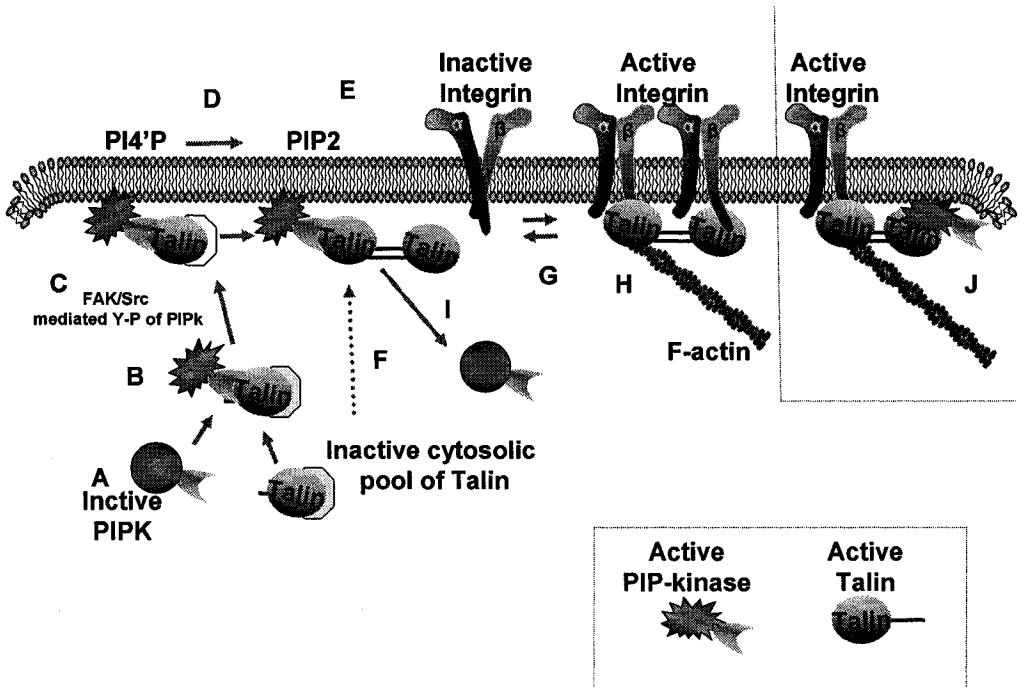


Figure 1.9: Activation of Talin and complex formation of integrin/Talin/actin. A) PIP kinase and Talin remain inactive in cytosol. **B)** Upon formation of Talin-PIP kinase complex, PIP kinase becomes active **C)** followed by the translocation of the complex to the plasma membrane where PIP kinase becomes further active through tyrosine phosphorylation of PIP kinase by Src and is exposed to its substrate PtdIns4P. This promotes **D)** conversion of PtdIns (4, 5) P3 to PtdIns (4, 5) P2; **E)** activation of the bound Talin at the plasma membrane where it exposes its integrin-binding site as well as dimerization sites; **F)** activation of the cytosolic pool of Talin; **G)** activation of integrin and dissociation of β-subunit from α-subunit by Talin; **H)** providing the link to the actin cytoskeletal protein; **I)** and finally displacement of PIP kinase to its inactive cytoplasmic pool, or **(J)** binding of PIP kinase to one of the two FERM domains in the Talin dimer. Adapted from Critchley, (2004)

In this thesis, Talin was explored to be positively regulated by TGF-β, translocated to the plasma membrane, co-localized at the end of Actin stress-fibers, and coupled to TGF-β signaling system. It was also demonstrated that Talin antagonizes TGF-β biological responses.

In summary, TGF-β as a member of a large superfamily of structurally related regulatory proteins regulates diverse cellular events including cell proliferation, differentiation and cell migration among others. Despite the fact that TGF-β is known as a potent growth inhibitory in epithelial cells, during tumorigenesis it functions as a tumor suppressor at early stages of carcinogenesis and as a stimulator of malignant progression at later stages. Under certain circumstances, one of these two roles of TGF-β may be dominant over the other. However, the mechanism underlying this switch is not well characterized. Thus, learning more about the TGF-β target genes and Smad interacting partners that may lead to cross talk with other signaling pathways is crucial.

1.2.11 Objectives and Hypothesis

TGF-β signaling cascade plays a crucial role in the maintenance of the cellular homeostasis in various tissues and organisms through regulation of different cellular physiological processes including proliferation, differentiation, and cell growth arrest among the others. Inappropriate regulation of TGF-β signaling pathway has been linked to many human pathogenesis including cancer. During tumorigenesis, TGF-β can either negatively regulate tumor development by inhibiting cell growth and proliferation or can positively promote cancer through enhancing EMT, angiogenesis and immune suppression. Although much is known about the mechanism of TGF-β signaling via knockout mouse models either for the ligands, TGF-β receptors, or downstream signaling proteins; the complex dual role of TGF-β during tumorigenesis remains elusive. Therefore, obtaining better knowledge of TGF-β signaling network, characterizing the novel Smad-interacting proteins and understanding the molecular mechanism by which aberrant TGF-β signaling may contribute to human cancer will reveal insights for improving targeting TGF-β signaling through the development of novel clinical treatments.

Kindlerin, a FERM domain-containing protein is positively regulated by TGF-β and is involved in TGF-β1-mediated migration and EMT in human mammary epithelial

cells (Kloeker et al., 2004). Similar to Kindlerin, Talin is also a FERM domain containing protein, which is highly expressed in breast cancer cell lines (Chen et al., 2000). Since FERM domain containing proteins share homologous structural domains, they are proposed to be regulated by similar mechanisms through physical interaction with the cytoplamic domains of transmembrane proteins or receptors (Bretscher et al., 2002). Youn Yi et al. (2002) demonstrated that TGF-β1 stimulation increased surface expression of integrin $\alpha_v\beta_3$ and induced EMT in human cervical squamous carcinoma cell line based on actin stress fiber formation, focal translocalization of Talin and integrin α_v subunit, as well as translocalization and down regulation of E-cadherin. Therefore, we hypothesized that Talin may also be a TGF-β target gene, which may be translocated to the plasma membrane following TGF-β1 stimulation and involved in TGF-β biological responses in mammary tumor cells. It has also been demonstrated that some Actin-binding proteins (Filamin) participating in the anchoring of membrane proteins for the actin cytoskeleal proteins are involved in TGF-β signaling through interaction with Smads (Sasaki et al., 2001). Since, Talin is a constituent of focal adhesions and an Actin-associated-integrin binding protein we hypothesized that Talin may also be involved in TGF-β signaling through interaction with Smads.

Briefly, the main goals of this thesis are to identify Talin as a downstream TGF-β target gene in human epithelial breast cancer cell lines and to elucidate the role of Talin in TGF-β signaling pathway and cellular responses.

1.2.12 Thesis Organization

In the earlier sections of this chapter (Chapter I) the introduction, a detailed review of the literature directly relevant to this research, motivation for this work and objectives were presented. In Chapter II, all the reagents as well as the experimental setup and procedures employed in this study are introduced. The experimental results are presented and discussed in Chapters III and IV. Finally, in Chapter V the contributions of this thesis are summarized, and some recommendations for extensions of this work are offered.

CHAPTER II: MATERIALS AND METHODS

2.1 Growth Factor, Antibodies, and Reagents

Human TGF-β1 #100-21R was purchased from Pepro Tech Inc. Mouse monoclonal [8D4] antibody against Talin (ab11188) was purchased form Abcam Inc. Rabbit polyclonal antibodies (pAb) against Smad2/3 (FL-425): sc-8332, TGFβ RI (V-22): sc-398, TGFβ RII (C-16): sc-220, SARA (H-300): sc-9135; and mouse monoclonal antibodies (mAb) against Smad4 (B-8) sc-7966 were all purchased form Santa Cruz Biotechnology Inc., CA. Rabbit polyclonal antibodies (pAb) against phospho-Smad3 [Sigma Chemical Co. (St. Louis, MO)]; mouse antibodies against β-Tubulin [BD Transduction Laboratories, Ontario, Canada]; and Polyclonal antibodies (pAb) against ERK1/2 [New England Biolabs, Pickering, Ontario, Canada] were used. Goat anti-mouse HorseRadish Peroxidase (HRP) and goat anti-rabbit HRP were supplied from Santa Cruz Biotechnology. Goat anti-mouse Rhodamine Red X was supplied from Jackson ImmunoResearch Laboratories (West Grove, PA). A- and G-Sepharose beads were purchased from Amersham Biosciences/GE Healthcare (Quebec, Canada). Oligonucleotides for human Talin primers and siRNA were purchased from Alpha DNA (Quebec, Canada) and Sigma respectively. LipofectAMINE 2000 reagent [Invitrogen (Ontario, Canada)]; and TRITC-conjugated phalloidin [Sigma Chemical Co., St. Louis, MO, USA)] have been utilized in this study.

2.2 Plasmid Constructs

The 3TPLuc reporter construct was a gift from Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, NY). This construct contains TGF-β/Activin-responsive elements of the plasminogen activator inhibitor 1 (PAI-1) and collagenase promoters which is widely used to assess TGF-β/Activin signaling requirements.

2.3 Cell Culture

Human mammary adenocarcinoma cell lines, MCF7 and MDA-MB-231 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) in the presence of 10% Fetal Bovine Serum (FBS), and 2 mM L-Glutamine. Cells were passed every 3rd day or at 80% confluency. They were detached by trypsinization, followed by washing with DMEM containing 10% FBS to neutralize the trypsin effect, counted with hematocytometer, and plated in the culture dish. Working cultures were incubated and maintained at 37°C in a humidified atmosphere of 95% air, and 5% CO_2.

2.4 Reverse-Transcription PCR (RT-PCR)

2.4.1 RNA Extraction

Cells were starved overnight and were then mock treated (control cells) or stimulated with 100 pM TGF-β1 in DMEM without serum media. The total RNA was extracted using Trizol reagents (Invitrogen) according to the manufacturer's protocol. Briefly, the cells were lysed directly in the culture dish by addition of Trisol solution. Cell lysates were then passed several times through a pipette to lyse the cells completely. RNA was extracted by the addition of Chloroform and was precipitated with isopropanol followed by washing with 70% ethanol (EtOH). The pellets were air dried, dissolved in diethylpyrocarbonate (DEPC) treated water, and incubated at 65°C for 15 minutes for complete dissolving. The total RNA extracts were quantified spectrophotometrically at a wavelength of 260 nm (Optimal density OD_{260nm} of 1 = 40 μg/ml of signal stranded RNA).

2.4.2 RT-PCR

cDNA synthesis was carried out using oligo-dT primers, 2 μg of total RNA, and Superscript First Strand Synthesis System for RT-PCR (invitrogen). Thermal cycling conditions were performed for 25 cycles of 94°C for 2 min, 94°C for 30 s, 57°C for 30 s, 72°C for 45 s. All PCR products were revealed by ethidium bromide staining of agarose gels. The oligonucleotide primer sequences used for PCR reactions were as follows:

hTalin-sense: 5′-GTCGCCAGGAAGATGTCATT-3′;
hTalin-antisense: 5′-CGCCAACCATCTTCTCTTTC-3′;
hGAPDH-sense: 5′-ACCACCATGGAGAAGGCTGG-3′;
hGAPDH-antisense: 5′-CTCAGTGTAGCCCAGGATGC-3′.

Densitometry analysis was performed for quantification of the mRNA induction by TGF-β1 normalized to GAPDH levels. $P<0.05$ compared with no TGF-β1 treatment.

2.5 siRNA Transfections

Silencing Talin expression in breast cancer cells was achieved by small interfering RNA (siRNA) with specific sequence for human Talin purchased from Sigma. Cells were plated in complete growth medium (DMEM containing 10% FBS) at 3×10^5 cells/ml density per well in 6-well dishes. The following day cells were washed with 1X Phosphate Buffer Saline (PBS) at pH 7.4 (8 g NaCl; 2 g KCl; 11.5 g Na_2HPO_4 H_2O; 2 g KH_2PO_4 per liter for 10X solution), and transfected or not with hTalin siRNA using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 120 nM of siRNA and 3 μl of Lipofectamine 2000 reagent were each diluted and mixed gently in the appropriate amount of Opti-MEM 1 Reduced Serum Medium without serum media (Invitrogen). After 5 minutes incubation at room temperature, the diluted Lipofectamine 2000 was added to the diluted siRNA, mixed and incubated for 15 minutes at room temperature to allow complex formation of siRNA/Lipofectamine 2000. The complete DMEM medium was replaced to Opti-MEM

medium followed by adding the siRNA/Lipofectamine 2000 complex to the cells and gently rocking the plate back and forth. After 4-6 hours incubation at 37°C in a 5% CO_2, the Opti-MEM 1 medium was replaced to DMEM containing 10% FBS and incubated for 24 hours. The following day, cells were trypsinized, split, plated into 6- or 12-well dishes, and allowed for recovery. Cells were then washed with PBS, cells were stimulated with 100 pM TGF-β1 in DMEM without serum media for the indicated periods of time. The control cells were mock treated in DMEM without serum media for the indicated periods of time as well. The expression levels of hTalin were monitored by Western blot and were maximally suppressed after 96 hours post-transfection. The sense siRNA sequence corresponds to the positions 6,043-6,063 relative to the Talin1 mRNA start codon. hTalin siRNA sequences were as follows:

hTalin-sense: 5′-AAUCGUGAGGGUACUGAAACU-3′;
hTalin-antisense: 5′-AGUUUCAGUACCCUCACGAUU-3′.

2.6 Cell Viability Assay (MTT Colorimetric Assay)

Cells were trypsinized and plated in 6-well dishes at 3×10^5 cells/ml density in complete medium (DMEM containing 10% FBS). The following day, cells were transfected or not with hTalin siRNA (described in siRNA transfections section) for 24 hours. Cells were then washed with PBS, trypsinized, and plated in triplicates in 96-well dishes, at 10×10^3 cells/100 ml density in DMEM containing 10% FBS. After allowing the cells to recover, cells were stimulated with 100 pM TGF-β1 in DMEM containing 2% FBS and incubated for 72 hours. The control cells were mock treated and incubated in DMEM containing 2% FBS for 72 hours as well. Cell viability was then assessed using the non-radioactive MTT cell growth assay for eukaryotic cells (Cell Titer 96. Promega G4000). Absorbance was measured at 570 nm with a reference wavelength at 450 nm, using a Bio-tek Microplate reader. Results are presented as mean ± standard deviation of 3 separate experiments.

2.7 Transfection and Reporter Assay

A day before transfection, cells were plated in 6-well plates at of 3×10^5 cells/ml density in complete medium (DMEM containing 10% FBS). The cells were co-transfected with expression plasmids encoding luciferase reporter construct 3TP-Luc (2 μg), β-galactosidase (pCMV-lacZ; 0.5 μg), in the presence or absence of hTalin siRNA (120 nM) using Lipofectamine 2000 (invitrogen). The following day, cells were trypsinized, split, and plated in 12 well dishes containing complete medium (DMEM containing 10% FBS). After allowing cells to recover, cells were starved overnight in DMEM with no serum and stimulated with 100 pM TGF-β1 for 18 hours. The control cells were mock treated and incubated for 18 hours as well. Cells were washed with PBS and lysed in 100 μl of lysis buffer (1% Triton X-100; 15 mM MgSO4; 4 mM EGTA; 1 mM dithiotheitol; 25 mM glycylglycine ph 7.8) on ice. The luciferase activity of each lysate was measured using 45 μl of cell lysate (EG & G Berthold Luminometer) and normalized to the relative values of β-galactosidase activity for transfection efficiency. Results are presented as mean ± standard deviation of 3 separate experiments.

2.8 Western Blot Analysis

Cells were stimulated with 100 pM TGF-β1or mock treated (control cells) and were then grown for the indicated periods of time in DMEM containing 2% FBS. Cells were washed with cold 1X PBS, lysed and harvested on ice in the lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 8, 0.1% SDS, 0.05% sodium deoxycholate) supplemented with protease inhibitors, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin. Total cell extracts were then separated on a 7.5% Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE), electrotransferred onto nitrocellulose at a constant current 0.15 mA for 1 hour in transfer buffer (25 mM Tris-base, 192 mM Glycine, 20% MeOH, 3.6% SDS). The membranes were blocked with TBS buffer (10 mM Tris at pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% dried nonfat milk for 1 hour and were then incubated with the indicated specific antibodies overnight at 4°C. All the antibodies were diluted in

antibody buffer (10mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20, 0.25% Gelatin, 0.05% NaAzide). Membranes were then washed twice for 10 minutes in TBST (50 mM Tris-Cl at pH 7.6, 200 mM NaCl, 0.05% Tween 20) and incubated with a proper secondary antibody conjugated to horseradish peroxidase (Santa Cruz; at a 1:10,000 diluted in blocking solution) for 1 hour at room temperature. Following incubation, membranes were washed four times for 15 minutes in TBST. Immunoreactivity was normalized by chemi-luminescence (Lumi-light Plus Western Blotting substrate, Roche) according to the manufacturer's instructions and revealed using an Alpha Innotech Fluorochem Imaging System (Pachard Canberra, Montreal, Quebec, Canada).

2.9 Co-Immunoprecipitation Analysis

Cell were starved overnight and the next day cells were stimulated with 100 pM TGF-β1 or mock treated (control cells) for the indicated periods of time as indicated in the figures. Cells were washed with 1X PBS and lysed in 1% NP-40 buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 8, 0.1% SDS, 0.05% sodium deoxycholate), supplemented with protease inhibitors 1 mM PhenylMethylSulphonyl Fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin. Lysates were incubated with 0.5 μg of rabbit anti-SMAD2/3 (Fl-425) antibody (Santa Cruz), rabbit anti-TGFβRI (V-22) antibody (Santa Cruz), rabbit anti-TGFβ RII (C-16), rabbit anti-SARA (H-300), or mouse monoclonal antibody against SMAD4 (B-8) for an overnight period. The following day, 40 μl of either a 50% protein A or protein G sepharose beads slurry solution (SantaCruz) was added for 2 hours at 4°C. Immunoprecipitates were then washed three times in the same RIPA buffer, eluted with 2% SDS loading buffer, boiled for 5 minutes, resolved by 7.5% SDS–PAGE, transferred onto nitrocellulose, and incubated with the indicated specific antibodies overnight at 4°C.

2.10 Confocal Immunofluorescene

MCF-7 cells were plated on 13 mm non-coated glass coverslips in complete medium. The next day, cells were stimulated with TGF-β1 or mock treated (control cells)

for the indicated periods of time in starvation medium and fixed with 4% paraformaldehyde in PBS at room temperature for 15 minutes. Fixed cells were permeabilized for 30 minutes with 0.2% Triton-X-100 and 2% BSA dissolved in PBS, washed three times for 5 minutes in PBS, and incubated for 1 hour with the primary antibody (mouse monoclonal anti-Talin) diluted in permeabilized buffer (1:400 dilution of mouse anti-Talin antibody). After three times washing in PBS for 5 minutes, cells were incubated for 1 hour with Goat anti-mouse Rhodamine Red X antibody and then co-stained with 1:500 dilution of TRITC-conjugated phalloidin (Sigma Chemical Co., St. Louis, MO, USA) for 30 minutes at 37°C, followed by three times washing in PBS for 5 minutes. Coverslips were mounted and observed using an LSM-510 Zeiss confocal microscopy.

2.11 Statistical Analysis

Results are expressed as mean ± standard deviation. Differences were assessed by one-way ANOVA or the unpaired *t* test. $P<0.05$ was considered significant.

CHAPTER III: RESULTS

3.1 TGF-β Upregulates Talin Gene Expression at mRNA Level in MCF-7 and MDA-MB-231 Human Mammary Adenocarcinoma Cell Lines

The role of TGF-β in tumor progression prompted us to further investigate and identify the potential TGF-β-inducible genes that may be involved in TGF-β biological events during tumorigenesis.

As discussed in the first chapter, Talin proteins are found in a wide variety of organisms, from slime molds to human. A survey of various human tissues by Northern blot showed Talin expression in leukocytes, lung, placenta, liver, kidney, spleen, thymus, colon, skeletal muscle, and heart (Ben-Yosef and Francomano, 1999). Chen et al. (2000) also demonstrated by Western-blot analysis that Talin is highly expressed in several prostate and breast cancer cell lines. To verify whether Talin expression itself is regulated by TGF-β1, we used two different human breast cancer cell lines MCF-7 and MDA-MB-231 that are highly responsive to TGF-β1. Moreover, these breast cancer cell lines display different malignant phenotypes ranging from highly to poorly tumorigenic and metastatic in the order of MDA-MB-231>MCF-7 (Xin et al. 2000). MCF-7 and MDA-MB-231 cells were first starved overnight and were then mock treated (control cells) or stimulated with TGF-β1 in starvation media. Total RNA was extracted and Reverse-Transcription Polymerase Chain Reactions (RT-PCRs) were performed (Figure 3.1, A and B) using primers specific to human Talin and the house-keeping gene, Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH). Following cDNA amplifications, densitometry analysis was performed for quantification of the mRNA induction by TGF-β1 normalized to GAPDH levels (Figure 3.1, C and D).

The resulting data revealed a slight increase in Talin gene expression following TGF-β1 treatment in both breast cancer cell lines, MCF-7 and MDA-MB-231, which led us further to evaluate the effect of TGF-β1 on the abundance of Talin protein.

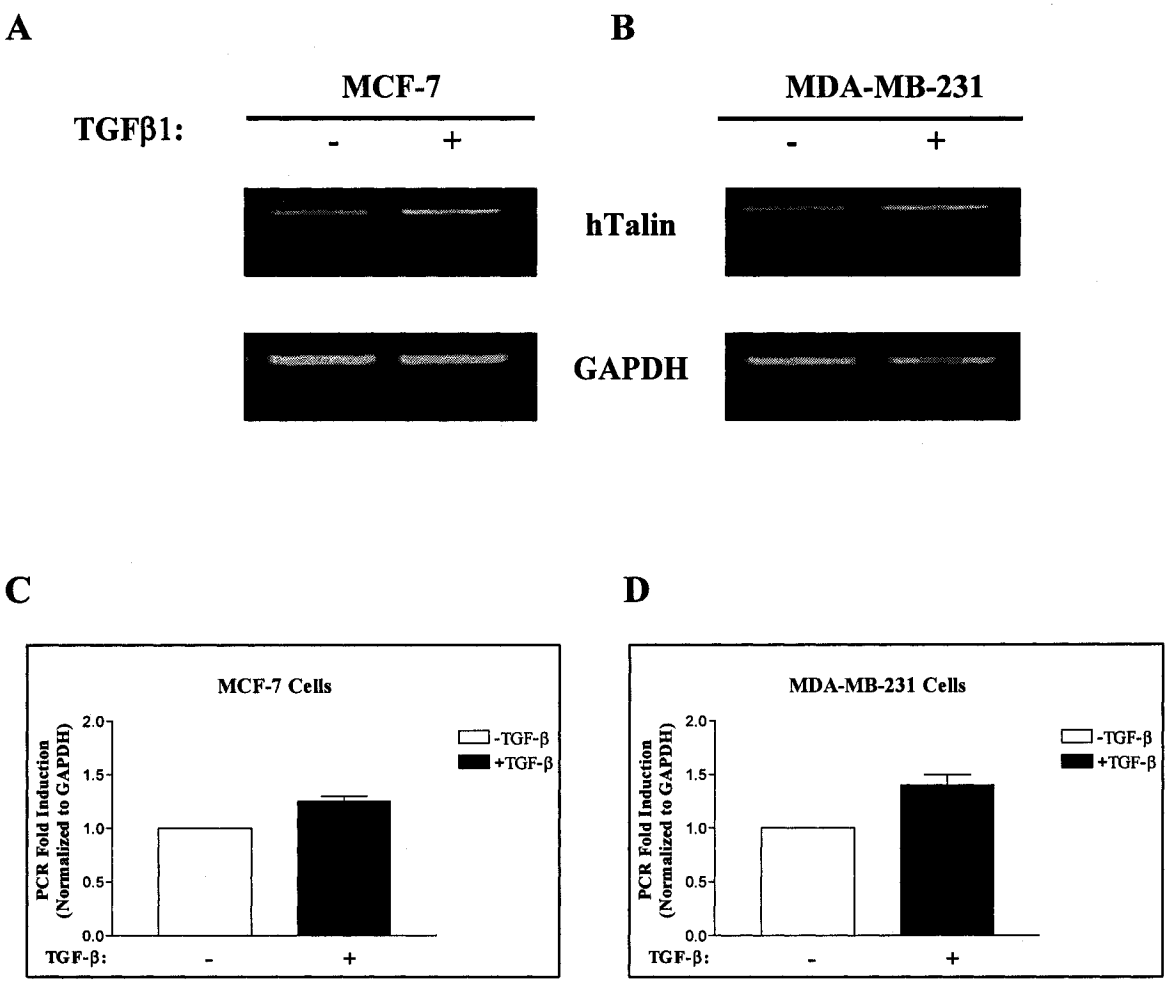


Figure 3.1: TGF-β upregulates Talin mRNA expression in human breast cancer cell lines. (A, B): MCF-7 and MDA-MB-231 cells were starved overnight. The cells were then stimulated with TGF-β1 for 8 h or were mock treated (control cells) in starvation medium, followed by the total RNA extraction. Reverse transcription reactions were performed using oligo-dT. cDNAs were amplified for 20 cycles using specific oligonucleotide sequences to hTalin and GAPDH which served as an internal control. **(C, D):** Densitometry analysis was performed for quantification of the mRNA induction by TGF-β1 normalized to GAPDH levels. $P<0.05$ compared with no TGF-β treatment.

3.2 TGF-β Increases Talin Expression in MCF-7 and MDA-MB-231 Human Mammary Adenocarcinoma Cell Lines

In order to verify the induction of Talin protein expression by TGF-β1, Western blot analysis was performed on MCF-7 and MDA-MB-231 whole cell extracts, which were first mock-treated (control cells) or stimulated with TGF-β1 in DMEM without serum for 24 hours. Cell lysates were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were transferred to a nitrocellulose membrane for immunoblotting using an antibody against human Talin (hTalin). Despite an slight increase in Talin expression at mRNA levels following TGF-β1 treatment, TGF-β1 enhanced significantly the levels of Talin protein (shown by arrow) in both MCF-7 and MDA-MB-231 cell lines (Figure 3.2, A and B, upper panel). The membranes were stripped and re-probed with β-Tubulin antibody to normalize for equal protein levels (lower panel). Collectively, based on the obtained data from RT-PCR and Western blot analysis, for the first time, TGF-β1 has been identified as a positive regulator of human Talin expression in breast cancer cell lines. Due to an increase in Talin expression level following TGF-β1 stimulation, we speculated that Talin might act as a downstream mediator of TGF-β1-induced cellular responses in breast cancer cell lines. Thus, we next investigated contribution of Talin in TGF-β signal transduction.

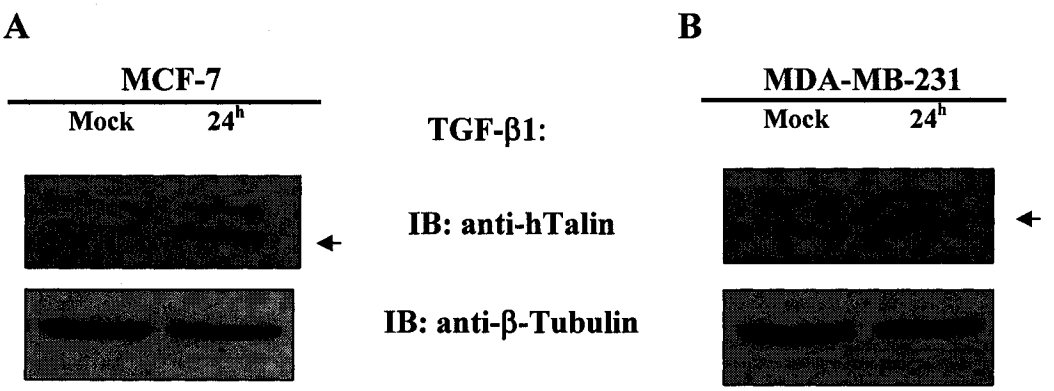


Figure 3.2: TGF-β induces upregulation of Talin protein in breast cancer cell lines. (A and B) MCF-7 and MDA-MB-231 cells were first mock treated (control cells) or stimulated with TGF-β in starvation medium for 24 hours. The whole cell lysates were then analyzed by Western blot using a specific monoclonal antibody against hTalin (upper panel). Equal loadings were confirmed by re-probing the membrane with β-Tubulin antibody (lower panel).

3.3 Talin Is Subcellulary Translocated from Cytosol to the Plasma Membrane and Co-localized to the Ends of Actin Stress Fibers in Response to TGF-β1 in MCF-7 Cells

The capacity of TGF-β inducing Epithelial-Mesenchymal Transition (EMT) contributes to invasive transition of tumors at later stages of carcinogenesis. Youn Yi et al. (2002) demonstrated that TGF-β1 stimulation increased surface expression of integrin $\alpha_v\beta_3$ and induced EMT in human cervical squamous carcinoma cell line based on actin stress fiber formation, focal translocalization of Talin and integrin α_v subunit, as well as translocalization and down regulation of E-cadherin.

Similar to integrin $\alpha_v\beta_3$, in this study Talin was shown to be positively regulated by TGF-β1. Moreover, as tabulated in Table 1.1, immunocytochemical studies have demonstrated that Talin protein is localized in a variety of structures in different types of cells and tissues, for instance in platelets, it may be distributed from cytosol to membrane in response to thrombin activation.

Since Talin as a constituent of focal adhesions and an Actin-associated-integrin binding protein establishes a pivotal role in bi-directional integrin signaling; in order to gain insights into the role of Talin in TGF-β1 mediated cytoskeletal reorganization we speculate that in response to TGF-β1, Talin is translocalized to the plasma membrane and co-localized with Actin cytoskeletal protein, which may function as one of the downstream mediators of TGF-β-initiated EMT in breast cancer cell lines.

To determine the subcellular localization of the Talin protein and Actin cytoskeletal reorganization induced by TGF-β1, MCF-7 cells were plated on non-coated glass coverslips and were mock treated (control cells) or stimulated with TGF-β at the indicated periods of time. The cells were then fixed and incubated with specific primary mouse monoclonal antibody against Talin. Immunofluorescence staining was performed using goat anti-mouse Rhodamine Red X secondary antibody to detect localization of

endogenous Talin which was co-stained with fluorescent TRITC-conjugated phalloidin to visualize Actin. Based on immunstaining analysis under confocal microscopy, visualization of filamentous Actin by fluorescent phalloidin in green and Talin in red demonstrated that after 1 hour of TGF-β1 treatment, Actin cytoskeletal proteins were reorganized from a network of filaments delineating each cell colony (Figure 3.3, A), to the formation of short and thin Actin stress fibers (Figure 3.3, B, C and D). Simultaneously, localization of a fraction of Talin to the ends of Actin stress fibers became apparent following 1 hour stimulation with TGF-β1 (Figure 3.3, D).

In conclusion, TGF-β1 induced the reorganization of the Actin cytoskeleton from circumferential ring-like structure into Actin stress-fiber-like projections in MCF-7 cells. Moreover, localization of Talin to plasma membrane was noticed after exposure to TGF-β1 for short period of time. Interestingly, Talin was also detected at the end of Actin stress-fibers in a ligand dependent manner, suggesting that Talin may be involved in TGF-β1-mediated cytoskeletal reorganization. However, since Talin upregulation induced by TGF-β1 occurred after 24 h ligand stimulation, we speculate that Talin may be a consequence rather than a causing event of TGF-β1-induced cytoskeletal reorganizations.

A: MOCK

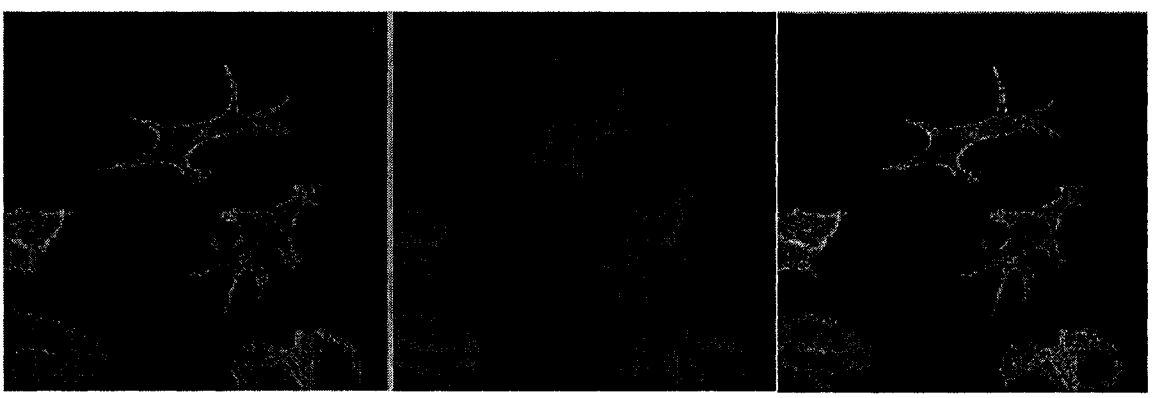
Actin **Talin** **Overlay**

B: TGF-β1–15′

Actin Talin Overlay

C: TGF-β1–30′

Actin Talin Overlay



D: TGF-β1–60′

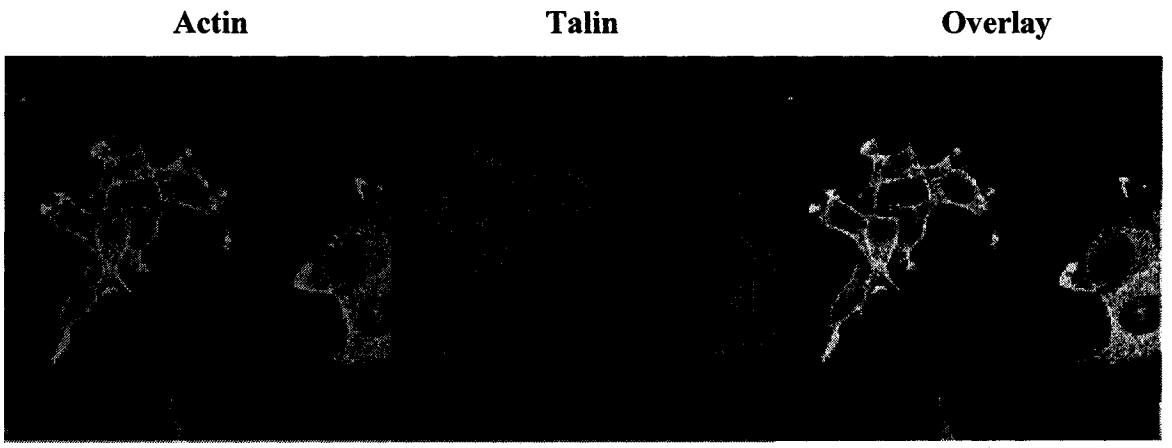


Figure 3.3: TGF-β effect on Talin subcellular localization. (A-D): MCF-7 cells were plated on non-coated glass coverslips and were mock treated (control cells) or stimulated with TGF-β1 at the indicated periods of time in starvation medium. Cells were then fixed, followed by incubation with specific primary mouse monoclonal antibody against Talin. The endogenous Talin proteins were labeled with goat anti-mouse Rhodamine Red X secondary antibody and co-stained with TRITC-conjugated phalloidin to visualize Actin.

3.4 Talin Knock Down in MCF-7 and MDA-MB-231 Cells

To determine the function of Talin in TGF-β signal transduction, small interfering RNA (siRNA) approach was first employed to inhibit Talin expression in human breast cancer cell lines. For this, cells were transfected or not (mock) with different concentrations of siRNA duplexes specific to human Talin. After 72 hours posttransfection of the siRNA, as shown by Western blot analysis (Figure 3.4), Talin expressions were significantly reduced in lysates prepared form MCF-7 and MDA-MB-231 cells transfected with different concentrations of Talin siRNA (60, 120 and 200 nM) as compared with the lysates prepared from the cells that were not transfected with Talin siRNA (Mock). However, the efficiency of Talin knockdown by siRNAs appears to work more efficiently in MCF-7 (A) vs. MDA-MB-231 (B) cell lines. To demonstrate equal loading, the membranes were stripped and probed using β-Tubulin antibody.

Since Talin expression was successfully diminished via siRNA duplexes specific for Talin in all indicated concentrations, to assess the function of Talin in TGF-β1 signal transduction, we employed Talin siRNA (120 nM) approach in the following sections.

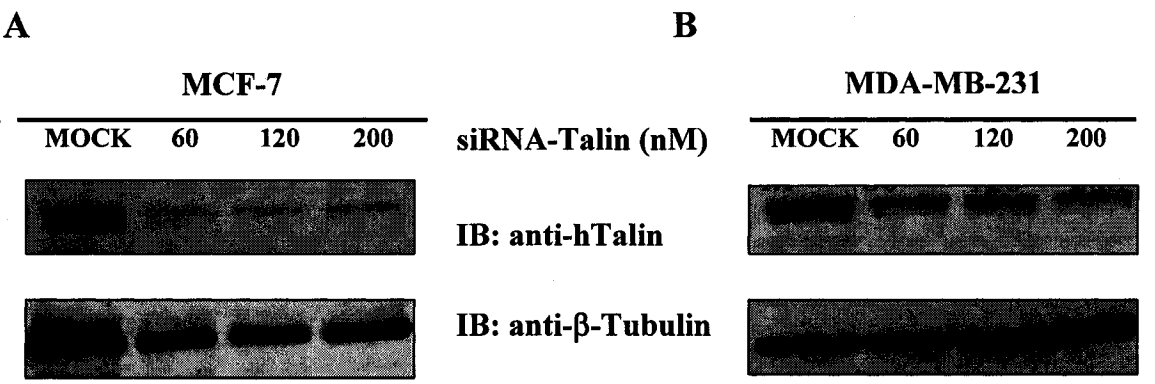


Figure 3.4: Talin knock down. (A and B): MCF-7 cells were transfected or not (mock) with specific siRNA duplexes targeted against human Talin using lipofectamine 2000. Western blot analysis was performed on MCF-7 and MDA-MB-231 cell lysates from mock and Talin siRNA transfected cells. The membranes were probed with Talin antibody (upper panel). To demonstrate equivalent protein levels, β-Tubulin antibody was used (lower panel).

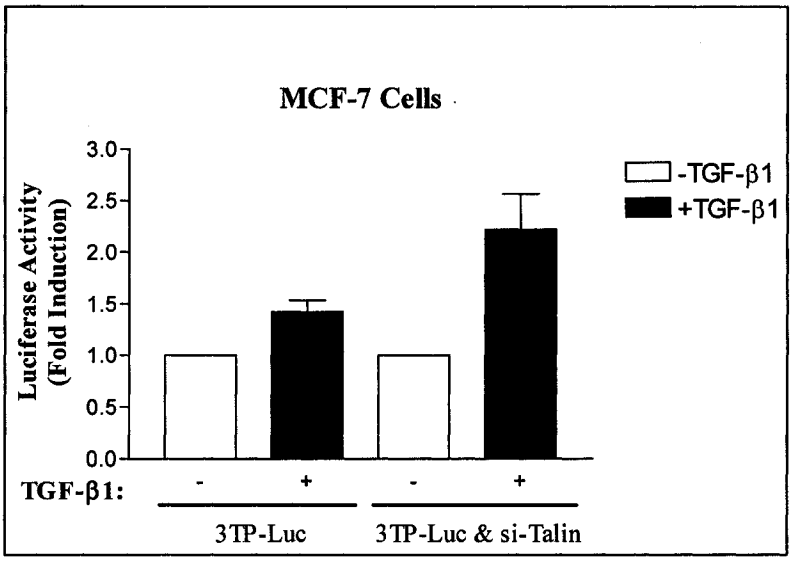
3.5 The Inhibitory Effect of Talin on TGF-β1-Mediated 3TP-Luc Promoter Activity in MCF-7, Breast Cancer Cell Line

To determine the role of Talin in TGF-β-mediated gene expression, the activity of TGF-β-responsive gene promoter (3TP-luc) was assessed in breast cancer cell lines. MCF-7 and MDA-MB-231 cells were first co-transfected with or without Talin siRNA along with 3TP-luc reporter construct fused to the luciferase gene and β-galactosidase expression plasmids. After 48 hours posttransfection cells were mock treated or stimulated with TGF-β1 for 18 hours before the luciferase activity was assessed.

As shown in Figure 3.5 (A), 3TP-luc luciferase activity was significantly induced in response to TGF-β1 stimulation in MCF-7 cells. However, when Talin expression was inhibited using Talin siRNA, the TGF-β1 effect on 3TP-luc luciferase activity was significantly enhanced; suggesting that Talin may play an inhibitory role downstream of TGF-β1 in MCF-7 cells (Figure 3.5, A). Interestingly, the TGF-β1-mediated an increase in 3TP-Luc luciferase activity observed in MDA-MB-231 cells was not further increased when Talin siRNA was used (Figure 3.5, B); suggesting that Talin may mediate its inhibitory effect on TGF-β1 signaling pathway only at early stage of cancer and not when the tumor cells become more invasive at late stage of carcingogenesis. Moreover, this difference may also reflect the slight difference in the efficiency of Talin siRNA in these cell lines.

Having established that TGF-β1 treatment induces an upregulation of Talin protein levels in breast cancer cell lines, and inhibition of Talin enhances TGF-β1-mediated gene expression significantly in MCF-7 cells; the effect of Talin on TGF-β1-induced cell growth inhibition was next examined.

A



B

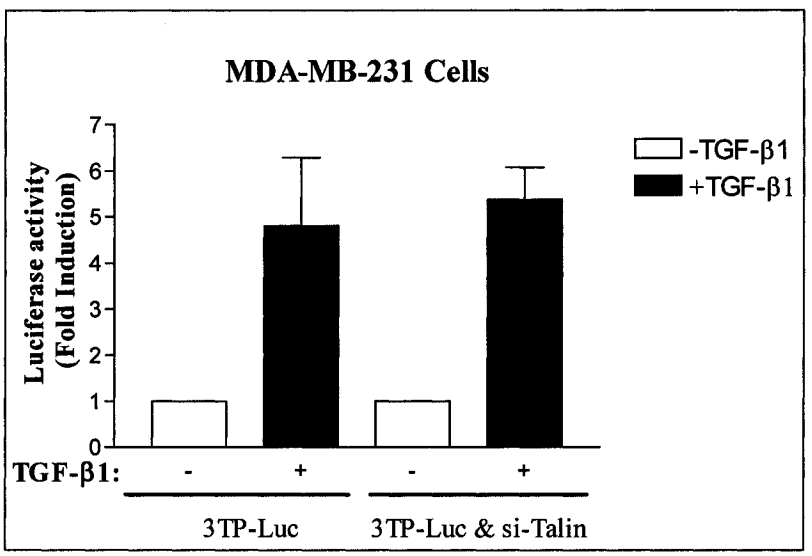


Figure 3.5: Talin inhibits TGF-β-mediated gene expression in MCF-7 cells. MCF-7 **(A)** and MDA-MB-231 **(B)** cells were co-transfected with 2 μg 3TP-luc reporter construct (as a control), 0.5 μg β-galactosidase expression plasmid, and cotransfected or not with 120 nM of specific Talin siRNA. After 48 hours posttransfection, cells were mock treated or stimulated with TGF-β1 for 18 hours. The TGF-β1 response was measured by luciferase assay and the activities were normalized to the relative β-galactosidase activities. Results represent means and standard deviations of three independent experiments. $P<0.05$ compared with no TGF-β treatment (Mock).

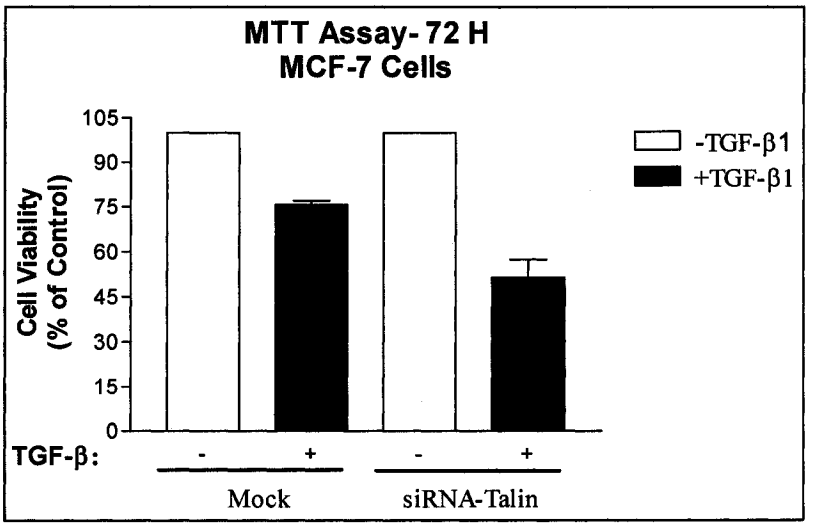
3.6 Talin Inhibits TGF-β1-Induced Cell Growth Arrest in MCF-7 Cells

TGF-β is known to induce cell growth arrest in a variety of cell lines including epithelial, endothelial, lymphoid, and hematopoietic cells (Burgess et al., 1996, Burrows et al., 1995). To evaluate Talin role in TGF-β-induced cell growth inhibition, MCF-7 and MDA-MB-231 cells were first transfected or not with siRNA duplexes specific to Talin to diminish Talin expression. After 24 hours posttransfection, the cells were mock treated or stimulated with TGF-β1 for 72 hours and the cell viability was assessed by MTT assay.

As shown in Figure 3.6 (A), TGF-β1 stimulation of the MCF-7 cells resulted in a 25% cell growth inhibition. However, when Talin expression was inhibited using Talin siRNA, TGF-β1 significantly enhanced the cell growth inhibition to 56%, suggesting that Talin may play an inhibitory role in cell growth arrest downstream of TGF-β1 in MCF-7 cells (Figure 3.6, A). In contrast, since MDA-MB-231 cells have high capacity of invasion and have lost their sensitivity to TGF-β1 mediated cell growth inhibition, cell viability was not affected in response to TGF-β1 as compared to the mock (Figure 3.6, B). Moreover, when Talin expression was diminished using siRNA approach, cell viability in response to TGF-β1 stimulation insignificantly decreased (only by 10%); suggesting that once again, Talin may mediate its inhibitory effect on TGF-β1-mediated cell growth arrest only at early stage of cancer. In addition, the different efficiency of Talin siRNA in these cell lines may also reflect this difference in the inhibitory effect of Talin on TGF-β1 biological response in MCF-7 and MDA-MB-231 cell lines.

Taken all together, these findings strongly indicate that elevated levels of Talin expression in MCF-7 cells induced by TGF-β1 may contribute to Talin's antagonistic effect upon TGF-β-induced growth inhibition and 3TP-Luc promoter activity at early stage of cancer in MCF-7 cells.

A



B

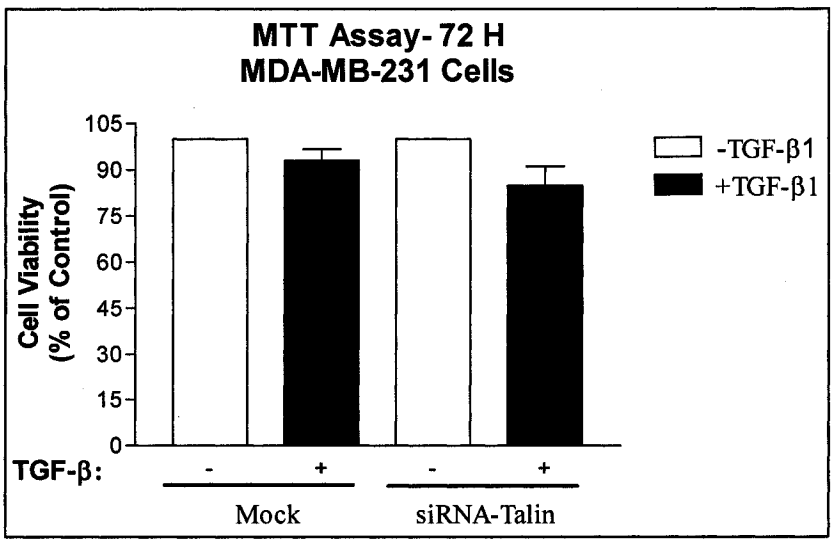


Figure 3.6: Talin inhibits TGF-β-induced cell growth arrest in MCF-7 cells. MCF-7 **(A)** and MDA-MB-231 **(B)** cells were mock transfected, or transfected with specific siRNA sequence to Talin. Cells were mock treated or stimulated with TGF-β1 for 72 hours before cell growth was assessed by cell viability colorimetric (MTT) assay. Values are representative of three independent experiments performed in triplicate, and are expressed in arbitrary units. $P<0.05$ compared with no TGF-β1 treatment.

3.7 Talin Knock Down Has no Effect on TGF-β1-Induced R-Smad Phosphorylation in MCF-7 Cells

Similar to Talin, Filamin is also an Actin-binding protein that participates in the anchoring of membrane proteins to the Actin cytoskeletal protein and is also involved in TGF-β signaling. It has been demonstrated that cells defective in Filamin expression have impaired TGF-β signaling and Smad2 phosphorylation (Sasaki et al., 2001). To explain the potential mechanism for Talin preventing TGF-β1-induced cell growth arrest and gene expression, we hypothesized that the antagonistic effect of Talin on TGF-β biological events may be due to Talin having an effect on Smad3 activation. Thus, we next examined Smad3 phosphorylation induced by TGF-β1 in the absence of Talin. For this, MCF-7 cells were transfected or not with Talin siRNA followed by TGF-β1 treatment for the indicated short period of time. Western blot analysis was performed on the total cell lysates using a specific antibody recognizing C-terminal phosphorylated serine residues of Smad3 and the membrane was stripped and re-probed using ERK1/2 antibody. As shown in Figure 3.7 (upper panel), TGF-β1-induced Smad3 phosphorylation was not affected in the absence of Talin; suggesting that antagonistic effect of Talin on TGF-β biological events is not due to Talin having an effect on Smad3 activation.

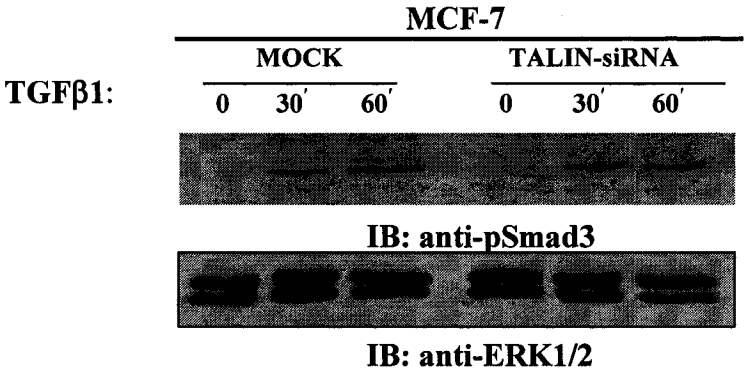


Figure 3.7: Talin has no effect on Smad3 phosphorylation induced by TGF-β1. MCF-7 cells were transfected or not with Talin siRNA duplexes (120 nM). A day after transfection cells were trypsinized and divided into three. Cells were starved overnight prior to the stimulation with TGF-β1 for the indicated periods of time. Protein phosphorylation level was then monitored by Immunoblotting using antibodies against anti-phospho-Smad3 (upper panel).The membrane was stripped and reprobed with anti-ERK1/2 antibody (lower panel) to verify the equal protein levels.

3.8 Talin Physically Associates with Smad3, Smad4, TβRI, and SARA; and Dissociates from TβRII in a TGF-β1-Dependent Manner in MCF-7 Cells

Smad-dependent signaling initiated by TGF-β superfamily members can be modulated by a variety of interacting proteins. It has been demonstrated that some Actin-binding proteins (Filamin) participating in the anchoring of membrane proteins to the actin cytoskeletal protein are involved in TGF-β signaling through interaction with Smads (Sasaki et al., 2001). As a plasma membrane protein, Talin is thought to participate in many different signaling pathways through interaction with plasma membrane proteins as well as different transmembrane receptors such as integrins. In this study, it is speculated that one potential mechanism by which Talin modulates TGF-β biological events may be through formation of possible immunocomplexes with TGF-β signaling components such as the receptors and the Smads.

To determine if Talin binds to R-Smads and whether this interaction is mediated by TGF-β1stimulation, co-immunoprecipitation experiments were performed using anti-Smad2/3 antibody in the cell lysates prepared form MCF-7 cell lines, which were mock treated (control cells) or stimulated with TGF-β1 for the indicated periods of time (Figure 3.8, A). The proteins in the complex were then revealed by immunoblot analysis using a mouse monoclonal antibody directed against Talin to recognize the endogenous protein. As illustrated in Figure 3.8, A (first panel), TGF-β1 stimulation induced the formation of an endogenous Talin/Smad2/3 complexes after 1 hour, suggesting that Talin may act as R-Smad interacting partner upon ligand stimulation. Moreover, Since Talin does not antagonize TGF-β1-mediated endogenous Smad2/3 phosphorylation (Figure 3.5) but inhibits TGF-β1 transcriptional activity (Figure 3.6, A), it is expected that Talin interacting with Smad2/3 leads to a reduction in TGF-β1-induced Smad2/3/Smad4 heterocomplex. Therefore, the membrane was stripped and re-blotted against Smad4 antibody. As shown in Figure 3.8, A, (second panel), on the contrary to our expectation, no reduction was observed in the formation of Smad2/3/Smad4 heterocomplex.

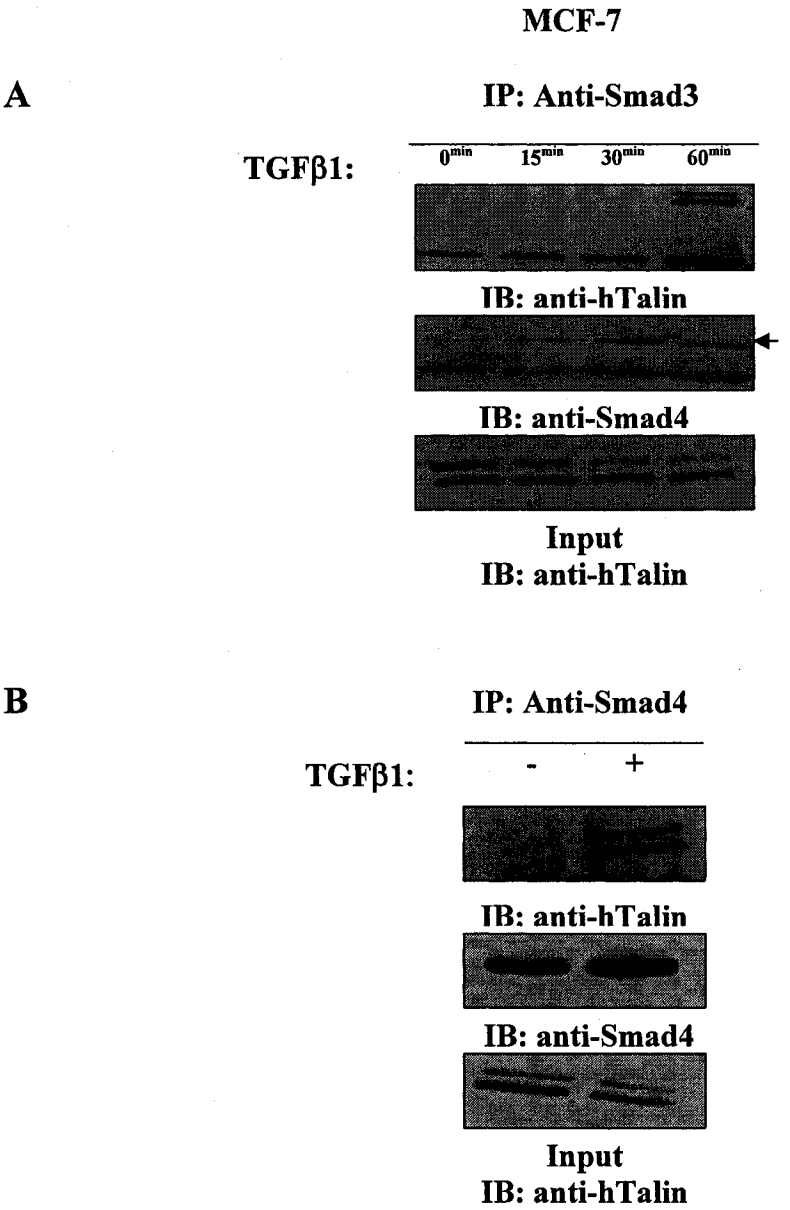
To further support this result, immunocomplex formation between Talin and Smad4 was then examined. For this, the cell lysates prepared form MCF-7 cell lines were mock treated or stimulated with TGF-β1 for the 1 hour and were immunoprecipitated using anti-Smad4 antibody (Figure 3.8, B). The proteins in the complex were then revealed by immunoblot analysis using Talin antibody. We found that Talin also interacts with Smad4 in response to TGF-β1, suggesting that Talin may act as an adaptor protein for Smads which does not prevent formation of Smad2/3/Smad4 heterocomplex in response to ligand stimulation. Based on these results, Talin (similar to Filamin) interacts with Smads which may be the mechanism of how Talin has an effect on TGF-β biological responses in MCF-7 cells.

We next evaluated the complex formation between Talin and SARA (membrane-associated Smad Anchor for Receptor Activation) using anti-SARA antibody based on the following reasons: 1) Talin is translocated from cytosol to plasma membrane upon TGF-β1 stimulation (Figure 3.3, D), 2) Talin interacts with Smads (Figure 3.8, A), and 3) R-Smads are localized to the plasma membrane by SARA to be recognized by TGF-β type I transmembrane receptor. As shown in Figure 3.8 (C) upon ligand stimulation, Talin strongly interacted with SARA. It would be interesting to determine if Talin interacts with Smad2/3 through SARA and forms a heterocomplex of Talin/SARA/Smad2/3.

As an anchor protein, since SARA presents Smad2/3 to TGF-β type I receptor, the affinity of Talin for the TGF-β1 receptors was next evaluated using anti-TβRI and TβRII antibodies. As shown in Figure 3.8 (D and E), the immunocomplexes results for Talin and TGF-β1 receptors indicate that at basal state, Talin is constantly associated with TβRII. However, in the presence of the ligand, Talin loses its affinity for TβRII and associates with TRβI.

Collectively, in MCF-7 cells, following TGF-β1 stimulation, Talin association with Smads, SARA, and TβRI may be one potential mechanism involved in Talin

antagonistic effect in TGF-β1 mediated gene transcriptional activity and cell growth arrest. Moreover, these associations can not be related to the TGF-β1-induced increase in Talin expression which occurs at later time point. The MCF-7 immunocomplexes data prompted us to further examine the complex formation of TGF-β1 signaling system in MDA-MB-231 cells. Since Talin mediates its inhibitory effect on TGF-β1 signaling pathway at early stage and not at later stage of tumorigenesis (possibly through interaction with Smads, SARA, and TβRI), we hypothesized that Talin association with Smads, SARA, and TGF-β type I receptor will be attenuated in MDA-MB-231 cells following ligand stimulation.



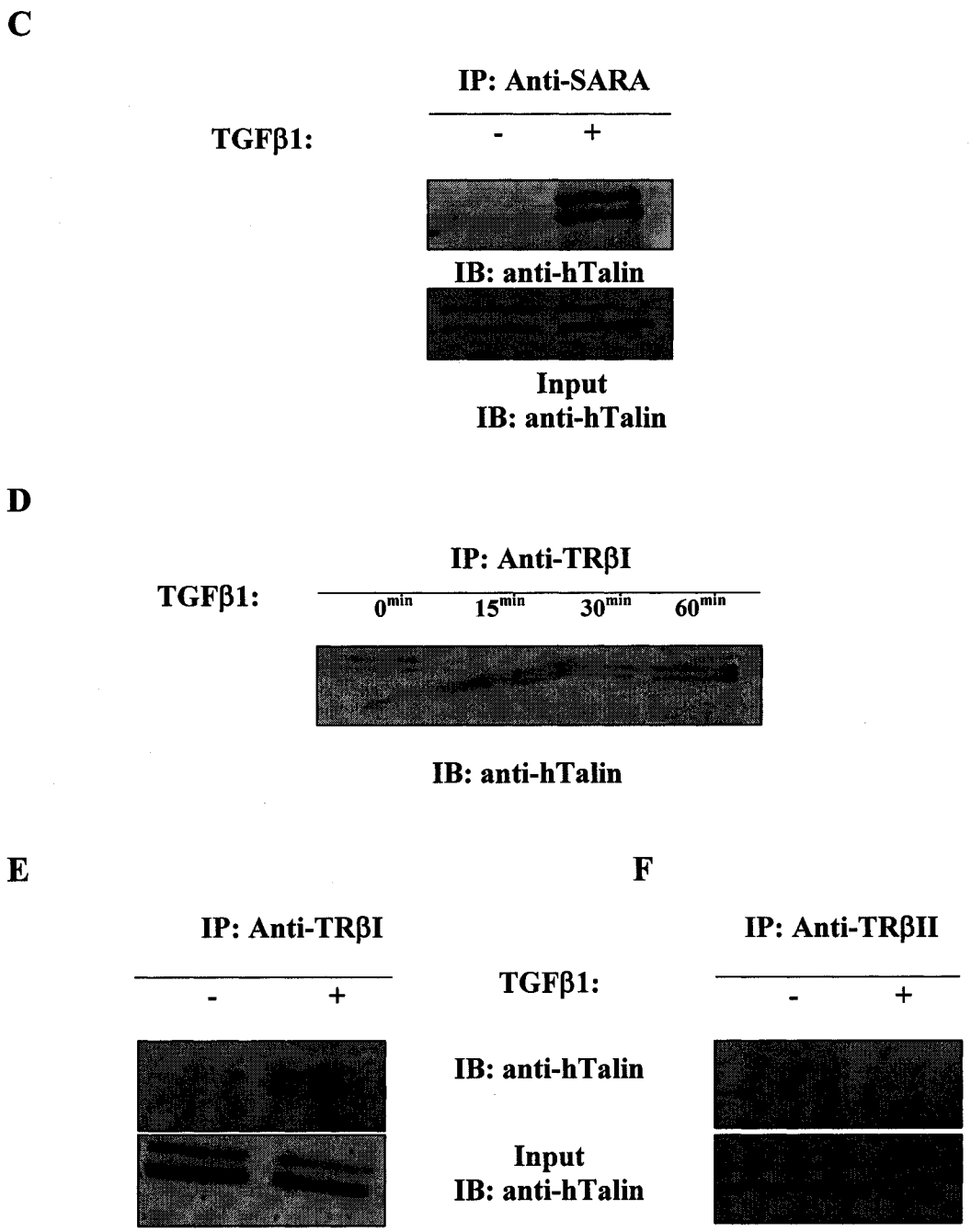


Figure 3.8: TGF-β1 induces complex formation between Talin and Smad3, Smad4, TβRI, and SARA; and attenuates the formation Talin/TβRII complex in MCF-7 cells. (A-F): MCF-7 cell were mock treated and stimulated with TGF-β1 for the indicated periods of time and the cell lysates were immunoprecipitated with anti-Smad3, -Smad4, -SARA, -TβRI, and -TβRII antibodies. The immunocomplex formations were analyzed by immunoblotting using anti-hTalin antibody. Immunoblotting using antibody against Smad4 was used as a positive control for Smad3 immunoprecipitation. The total cell lysates, representing 10% of the immunoprecipitating inputs were analyzed by immunoblotting, using antibodies against hTalin.

3.9 Talin Physically Associates with TβRII, and Dissociates from Smad3, SARA in a TGF-β-Dependent Manner in MDA-MB-231 Cells

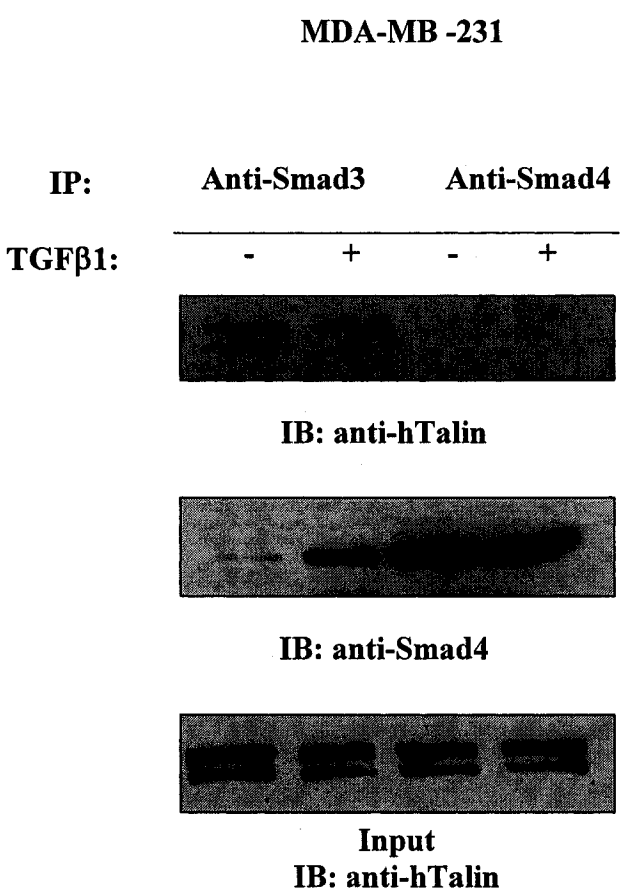
In previous section, we speculated that one potential mechanism by which Talin modulates TGF-β1 biological responses in MCF-7 may be through formation of possible immunocomplexes with TGF-β1 signaling components such as the receptors and the Smads. Based on the immunoprecipitation experiments performed in MCF-7 cells, Talin was shown to have high affinity for Smad2/3, Smad4, SARA, and TβRI after 1 hour exposure to TGF-β1, whereas Talin affinity for TβRII was lost. We speculated that these interactions would not occur in MDA-MB-231 cells, which may explain why Talin has no affect in TGF-β1 biological responses in this invasive cell line.

To determine the formation of possible immunocomplexes between Talin and TGF-β1 signaling components in MDA-MB-231 cells following TGF-β1 stimulation, co-immunoprecipitation experiments were performed using anti-Smad2/3, -Smad4, -TβRI, -TβRII and -SARA antibodies in the cell lysates prepared form MDA-MB-231 cell lines stimulated with TGF-β1 for the indicated periods of time or mock treated (Figure 3.9, A-D). The proteins in the complex were revealed by Immunoblot analysis using a mouse monoclonal antibody directed against Talin. In contrast to MCF-7, the immunocomplexes results in MDA-MB-231 cells revealed that in the absence of the ligand, Talin is in complexes with Smad3 and SARA (Figure 3.8, A-C), and upon TGF-β1 treatment for 1 hour, Talin dissociates from Smad3, and SARA and physically interacts with TβRII (Figure 3.9, D). Interestingly, no immunocomplex formation was observed between Talin and Smad4 (Figure 3.9, B), and in the case of TRβI, Talin was weakly bound to TRβI in a ligand independent manner (Figure 3.9, C).

These differences in immunocomplex formations in these two cell lines might reflect the stage of carcinogenesis these cell lines are at or the activity of other signals, in particular integrin signaling. As discussed in the review of literature, Galliher and Schiemann (2006) have recently shown that β3integrin alters TGF-β signaling in

Mammary Epithelial Cells (MECs) by interacting physically with the TβRII mediating TβRII tyrosine phosphorylation by Src, thereby enhancing TGF-β-mediated invasion and EMT. Since, Talin is an integrin-interacting partner involved in integrin cellular responses, we speculate that in highly invasive MDA-MB-231 cells formation of Talin-TβRII complex upon TGF-β stimulation may involve integrin signaling, enhancing TGF-β1 mediated invasion and EMT, which require further investigations.

A



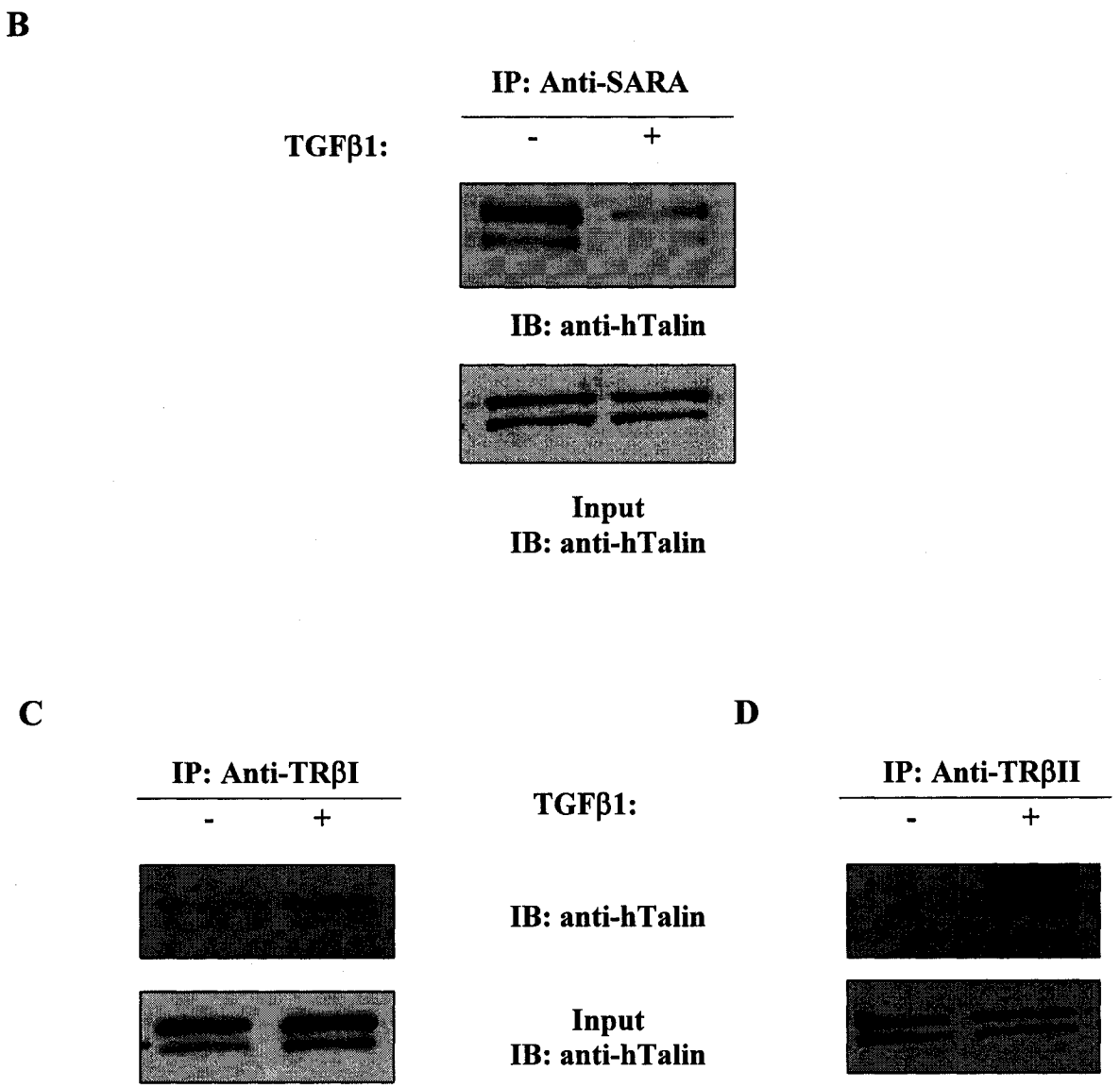


Figure 3.9: TGF-β induces hTalin/TβRII complex formation; attenuates Talin/Smad3, and Talin/SARA complex formations; and has no effect on Talin/TβRI complex formation in MDA-MB-231 cells. (A-D): MDA-MB-231 cells were mock treated or stimulated with TGF-β1 for the indicated periods of time. The cell lysates were immunoprecipitated with anti-Smad3, -Smad4, -TβRI, -TβRII, and -SARA. The immunocomplexes were analyzed by immunoblot using anti-hTalin antibody. Cell lysates representing 10% of the immunoprecipitating inputs were analyzed by immunobloting using antibodies against hTalin.

In summary, in the present study, we found Talin as a novel TGF-β1 target gene which is positively regulated both at mRNA and protein levels in presence of the ligand. Moreover, we showed that at short time exposure to TGF-β1, Actin structure was reorganized; Talin was translocated to the plasma membrane and colocalized at the end of Actin stress fibers. Interestingly, we also found that at early stage of cancer, Talin acts as an antagonist inhibiting TGF-β1-mediated cell growth arrest and transcriptional activity. To explain the potential mechanism involved in antagonistic effect of Talin on Smad-dependent signaling initiated by TGF-β, we showed: 1) Talin has no effect on Smad3 activation by using siRNA approach. 2) Talin physically interacts with or dissociate from TGF-β1 signaling components, raising the possibility that Talin may be involved in the modulation of TGF-β-Smad-dependent signaling through physical interactions with TGF-β1 signaling system which requires further investigation.

CHAPTER IV: DISCUSSION

TGF-β superfamily members are known as potent growth inhibitors in epithelial cells. At early stages of carcinogenesis, TGF-β1 is known to act as a tumor suppressor (Pierce at al., 1995). However, as tumor cells acquire several genetic alterations they may be rather insensitive to TGF-βs in regard to growth inhibition. There are several evidences showing that TGF-β contributes to malignant progression (Arteaga at al. 1993) which is attributed to its effect on EMT that promotes cell migration via Actin cytoskeletal reorganization (Boland et al. 1996). The molecular mechanisms underlying this conversion of TGF-β function require further research. Since TGF-β elicits its most biological effects by activation or inhibition of a Smad-dependent transcriptional program, identification of TGF-β target genes that function downstream of TGF-β signaling and have an impact on TGF-β physiological effects are likely to be critical.

In the present study, we identified Talin as a novel TGF-β1 target gene, which is positively regulated in response to the ligand, and its contribution to TGF-β cellular responses in mammary cancer cell lines has also been investigated.

4.1 Regulation of Talin Expression by TGF-β1

Several pieces of circumstantial evidences prompted us to determine the physiological relevance of Talin in TGF-β mediated cellular responses in mammalian cancer cells, which are as followings: 1) Talin protein is found in a wide variety of organisms including various human tissues (Ben-Yosef and Francomano, 1999; Chen et al., 2000), and is highly expressed in human breast cancer cell lines (Chen et al., 2000); 2) TGF-β1-induced EMT at subcellular levels in human cervical squamous carcinoma cell line was based on several criteria including actin stress fiber formation, focal

translocalization of Talin, as well as translocalization and down regulation of E-cadherin (Youn Yi et al., 2002); 3) Kindlerin protein, which is a FERM domain containing protein similar to Talin is positively regulated by TGF-β1 and is involved in TGF-β1-mediated migration and EMT in human mammary epithelial cells (Kloeker et al., 2004); and 4) since FERM domain containing proteins share homologous structural domains, they are therefore proposed to be regulated by similar mechanisms through physical interaction with the cytoplasmic domains of transmembrane proteins or receptors and therefore, connecting them to Actin cytoskeletal protein (Bretscher et al., 2002).

Based on these rationales, we first assessed the regulatory effect of TGF-β1 on Talin mRNA and protein expressions in mammary cancer cell lines, MCF-7 and MDA-MB-231. We characterized Talin as a novel TGF-β1 target gene in breast cancer cell lines by RT-PCR and Western blot analysis, and similar to Kindlerin, Talin protein expression is positively induced by TGF-β1 at later time points. However, further investigation is required to determine if the enhanced transcription of the Talin gene occurs through a direct transcriptional regulatory mechanism or a secondary response to TGF-β1 stimulation by using the protein synthesis inhibitor, cycloheximide.

4.2 The Net Effect of Talin on TGF-β Physiological Cell Responses

As discussed in review of the literature, TGF-β/Smad signaling has been recognized as a prometastatic pathway contributing to tumor invasion and cancer progression by inducing EMT and promoting cell migration via Actin cytoskeletal reorganization in mammary tumors (Siegel et al, 2003; Boland et al., 1996; Bakin et al., 2000; Bhowmick et al., 2001). Some key proteins involved in tumor invasion have been identified as targets in the TGF-β pathway, such as Snail and Kindlerin, which are positively regulated by TGF-β and are known to mediate EMT (Romano and Runyan, 2000; Kloeker et al., 2004). Recently, Galliher and Schiemann (2006) demonstrated that TGF-β stimulation induced $\alpha_v\beta_3$ integrin expression in a manner that coincided with EMT through down regulation of E-cadherin in Mammary Epithelial Cells (MECs).

Since, Talin is an integrin-interacting protein partner, which provides the initial connections between integrins and the Actin cytoskeletal protein and establishes a pivotal role for these connections in bi-directional integrin signaling; we investigated the subcellular translocalization of Talin in response to TGF-β1 stimulation. As tabulated in Table 1.1, Talin protein is localized in a variety of structures in different types of cells and tissues. For instance in platelets, it may be distributed from cytosol to membrane in response to thrombin activation.

In this study, the confocal immunstaining analysis in MCF-7 cells showed that Talin is translocalized to the plasma membrane after exposure to TGF-β1 for a short period of time. In addition, we also investigated other characteristic features of EMT including Actin cytoskeletal reorganization. It was observed that upon TGF-β1 stimulation, Actin structure is reorganized from circumferential ring-like into stress-fiber-like projections, where Talin is co-localized at the end of these actin stress fibers in a ligand dependent manner. This suggests that Talin may be involved in TGF-β1-mediated cytoskeletal reorganization. However, since Talin upregulation induced by TGF-β1 occurred after 24 hours ligand stimulation, we speculate that Talin may be a consequence rather than a causing effect of TGF-β1-induced cytoskeletal reorganizations. Based on the obtained data, to explore if Talin has a role in TGF-β1-mediated EMT or cell invasion, further criteria such as changes in cell morphology leading to the loss of cell-cell contact and instability of E-cadherin on the cell surface are required to be investigated.

Since TGF-β1 is known to play a major role in cell growth arrest and transcriptional activity, Talin contribution to the regulation of these cellular responses was next investigated. To assess the function of Talin, small interfering RNA (siRNA) was employed to knock down Talin expression in the cells. It was observed that following TGF-β exposure, Talin is able to enhance a marked proliferative response and block transcriptional activity in MCF-7 but not in MDA-MB-231 cells.

Taken all together, these findings indicate that elevated levels of Talin expression induced by TGF-β1 may contribute to the antagonistic effect of Talin on TGF-β-induced

growth inhibition and 3TP-Luc promoter activity at early stage of cancer in MCF-7 cells. We suggest that the different effects of Talin on these breast cancer cell lines not only reflect the different stages of carcinogenesis these cell lines are at, but may also reflect the different efficiency of Talin siRNA in these cell lines.

4.3 The Potential Mechanism Involved in Antagonistic Effect of Talin on TGF-β Biological Events

Studies on specific TGF-β target genes have identified numerous non-Smad proteins that interact with Smads to cooperate with the Smad and regulate expression of TGF-β target genes. Filamin, an Actin-binding protein that participates in the anchoring of membrane proteins for the Actin cytoskeleton is also involved in TGF-β signaling through interaction with Smads. It has been demonstrated that cells defective in filamin expression have impaired TGF-β signaling and Smad2 phosphorylation (Sasaki et al., 2001).

Since Talin is an Actin-binding protein and in this study was shown to be involved in TGF-β biological responses (Figure 3.5, 3.6), we next investigated the phosphorylation state of Smad2/3 upon TGF-β1 stimulation in the absence of Talin to find out if Talin is involved in modulation of Smad2/3 activation. As is illustrated in Figure 3.7, the involvement of Talin in TGF-β biological events is not related to Smad2/3 activation.

Galliher and Schiemann (2006) showed that β_3 integrin interacted physically with the TGF-β receptor type II (TβR-II), thereby enhancing TGF-β-mediated gene transcription, blocking cell growth arrest, and increasing TGF-β mediated invasion and EMT. Since Talin is an integrin interacting partner involved in regulation of integrin signaling, and its expression is increased following TGF-β1 treatment, it was hypothesized that Talin may also regulate TGF-β signaling in part via interacting with TGF-β signaling components. Co-immunoprecipitation studies in MCF-7 cells suggest that at basal state, Talin is bound to TGF-β receptor type II, and upon ligand binding

Talin dissociates from receptor type II and associates with receptor type I, Smad2/3, and Smad4. Moreover, a physical interaction between Talin and SARA protein is also observed following exposure to TGF-β1. On the contrary, in MDA-MB-231 cells in the absence of ligand, Talin is shown to be bound to Smad3, SARA, and in a ligand independent manner to TGF-β type I receptor. However, following exposure to TGF-β1, the activated TGF-β receptor type I terminates or reduces all these interactions, and Talin interacts with TGF-β type II receptor. In addition, in contrast to MCF-7 cells, Talin has no affinity for Smad4 in MDA-MB-231 cells in the presence or absence of the ligand. These differences in MCF-7 and MDA-MB-231 cells may reflect the stage of carcinogenesis these cell lines are at, or may be due to the activity of other signals, in particular integrin signaling. Thus, to further elucidate the different effects of Talin on these cell lines, it is required to investigate the differences between these cells in terms of integrin signaling, or any other signaling pathways that might contribute the differences observed in TGF-β signaling with respect to Talin.

To conclude, it can be speculated that at early stage of cancer, due to the physical interactions between Talin and TGF-β receptor type I and Smads following ligand stimulation in MCF-7 cells, a co-repressor may be recruited to the Smad2/3/Smad4 complex which will bind to Smad3 and blocks the transcriptional activity of cell cycle dependent kinase inhibitors and therefore enhancing cell proliferation. Besides, since Talin is not found in nucleus (Tepass and Godt, 2005), it is expected that Talin dissociates from the Smads complex and will be released in cytosol following nuclear tranlocalization of Smads complex. Comparing to MCF-7 cells, since these interactions between Talin and TGF-β signaling components are different in MDA-MB-231 cells, it is suggested that upon ligand stimulation, Talin association with TGF-β type II receptor may be the reason Talin has no antagonistic effect on cell growth arrest or transcriptional activity induced by TGF-β1 and may have other potential effects on TGF-β signal transduction such as EMT or cell migration.

Collectively, in this study for the first time, a novel role for Talin in modulating TGF-β biological events through interacting with TGF-β transmembrane serine/threonine

kinase receptors and Smads mediated TGF-β signal transduction was established. To have a better understanding of the mechanism, further investigations are required such as the incorporation of genomic and proteomic approaches combined with mouse genetic manipulation, which will provide better understanding of the antagonistic effects of Talin on cellular responses to TGF-β in maintaining cell and tissue homeostasis.

CHAPTER V: CONCLUSION

5.1 Conclusion

In this thesis, Talin has been identified as a novel TGF-β target gene, antagonizing TGF-β1 biological effects at early stage of mammary carcinogenesis. However, since Talin upregulation induced by TGF-β1 occurred after 24 h, we speculate that this antagonistic effect of Talin may be a consequence rather than a causing event.

To obtain a better understanding of how Talin negatively modulates TGF-β/Smad signal transduction, the following model is proposed as is schematically illustrated in Figures 5.1-3. At the basal state, Talin has been shown to be associated with TGF-β receptor type II in MCF-7 cells (Figure 5.1).

However, upon ligand binding (Figure 5.2) and activation of TGF-β type I receptor (A), Talin loses its affinity for TβRII and physically interacts with TGF-β receptor type I, Smad3, and SARA followed by the recruitment of Smad3-SARA complex (B). On the other hand, TGF-β1 induces Talin subcellular translocalization, where it can interact with plasma membrane proteins, SARA and R-Smads functioning as an adaptor protein. Upon Smad2/3 activation, SARA is dissociated (C) and Talin/Smad3 complex becomes free to bind to Smad4 (D). Since Talin is not detected in the nucleus, it is speculated that Talin dissociates from the Smads complex and is released in the cytosol (E). We hypothesize that the antagonistic effect of Talin on TGF-β1-mediated cell growth arrest and transcriptional activity is expected to occur via recruitment of a repressor to Smad4/Smad2/3 complex. Thus, upon release of Talin, a repressor is recruited to the Smads complex followed by translocation to the nucleus (F). The Smads complex is then bound to the Smad Binding Element and through recruitment of a

corepressor (G) blocks transcriptional activity of TGF-β target genes involved in cell growth arrest (H).

Taken all together, as illustrated in Figure 5.3, Talin is an inducible antagonist of TGF-β signal transduction, mediating cell proliferation and inhibiting gene expression.

Figure 5.1: Physical interactions of Talin with TGF-β signaling components in the absence of TGF-β in MCF-7 cells

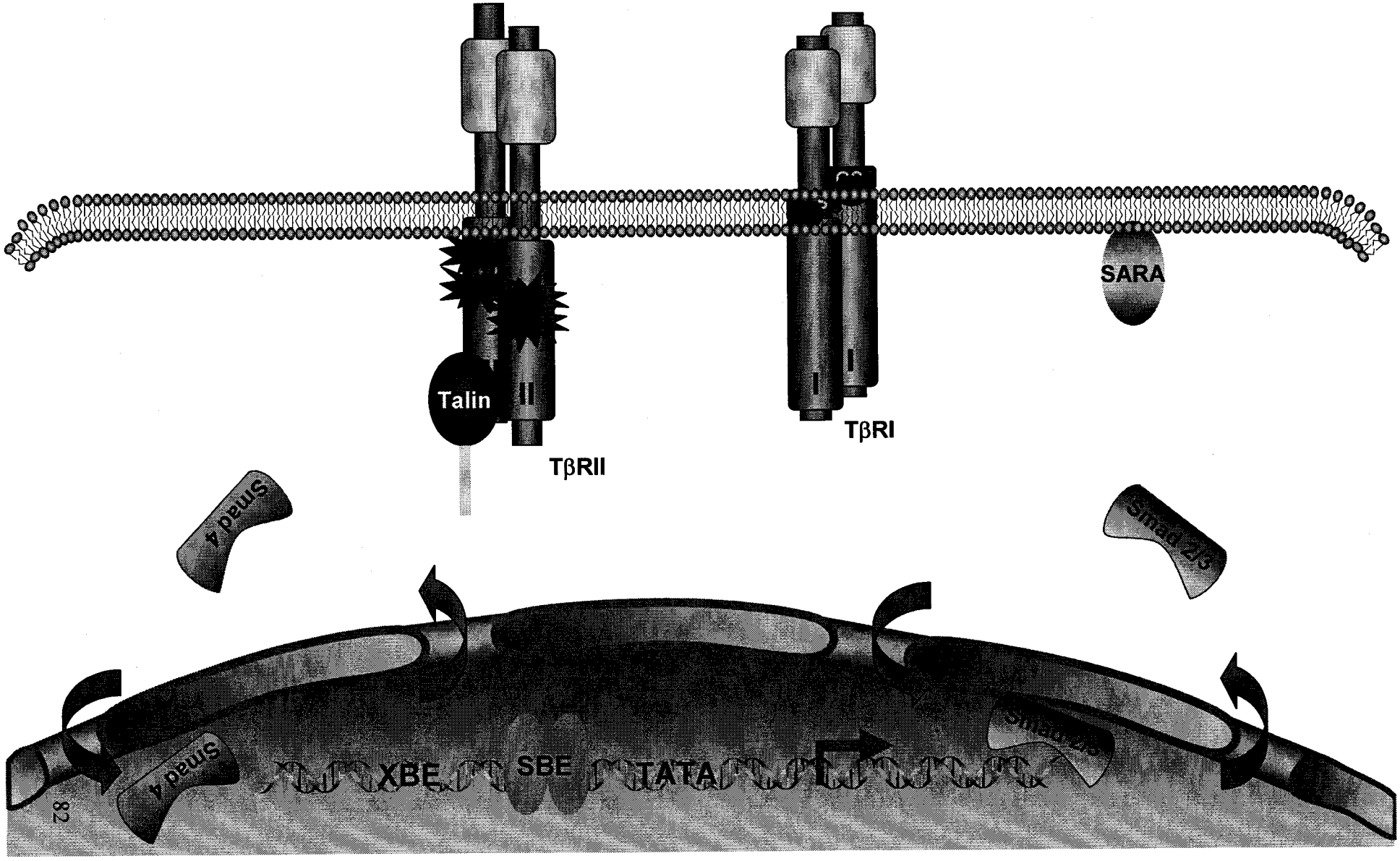


Figure 5.2: Physical interactions of Talin with TGF-β signaling components in the presence of TGF-β in MCF-7 cells

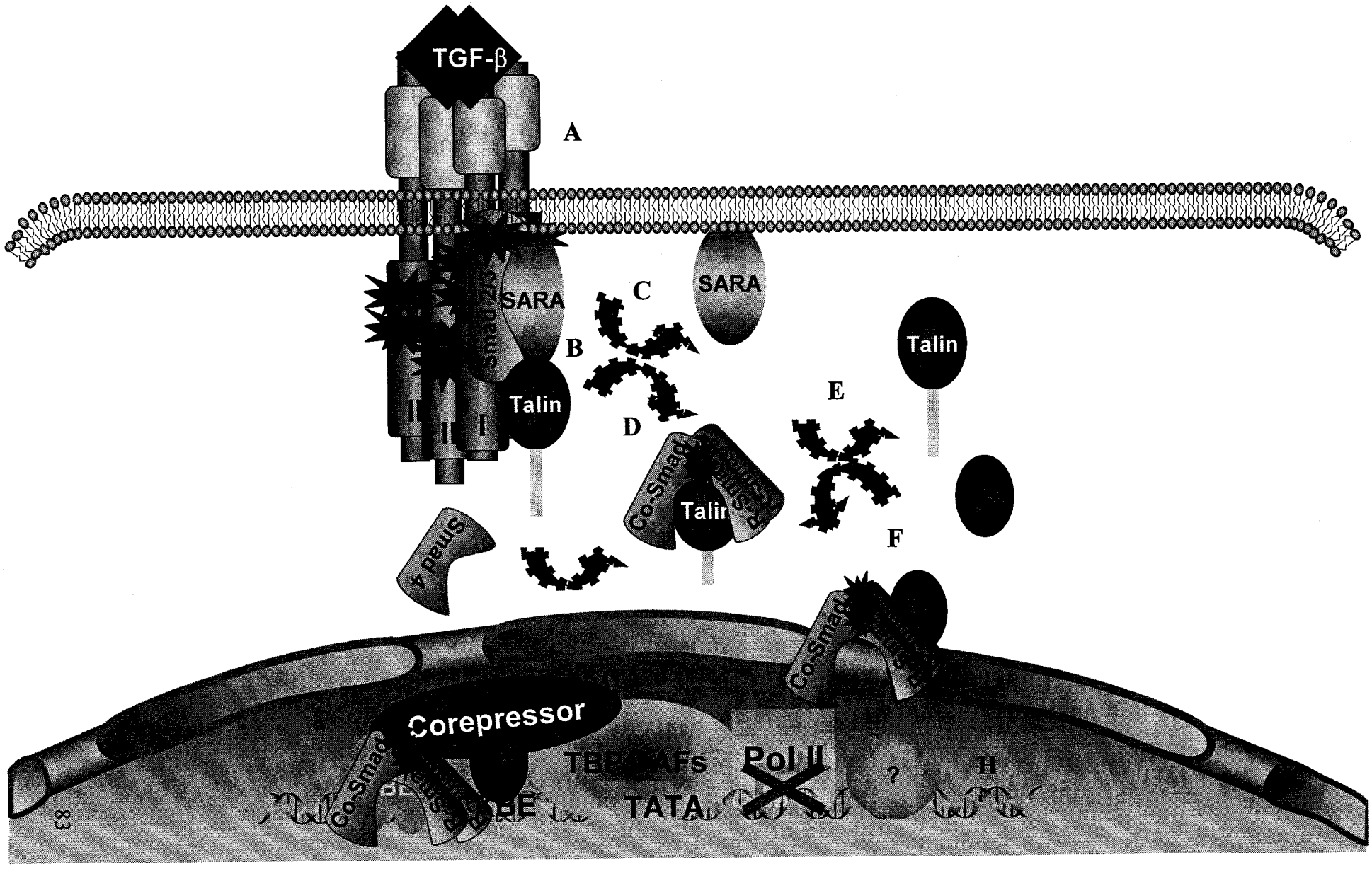
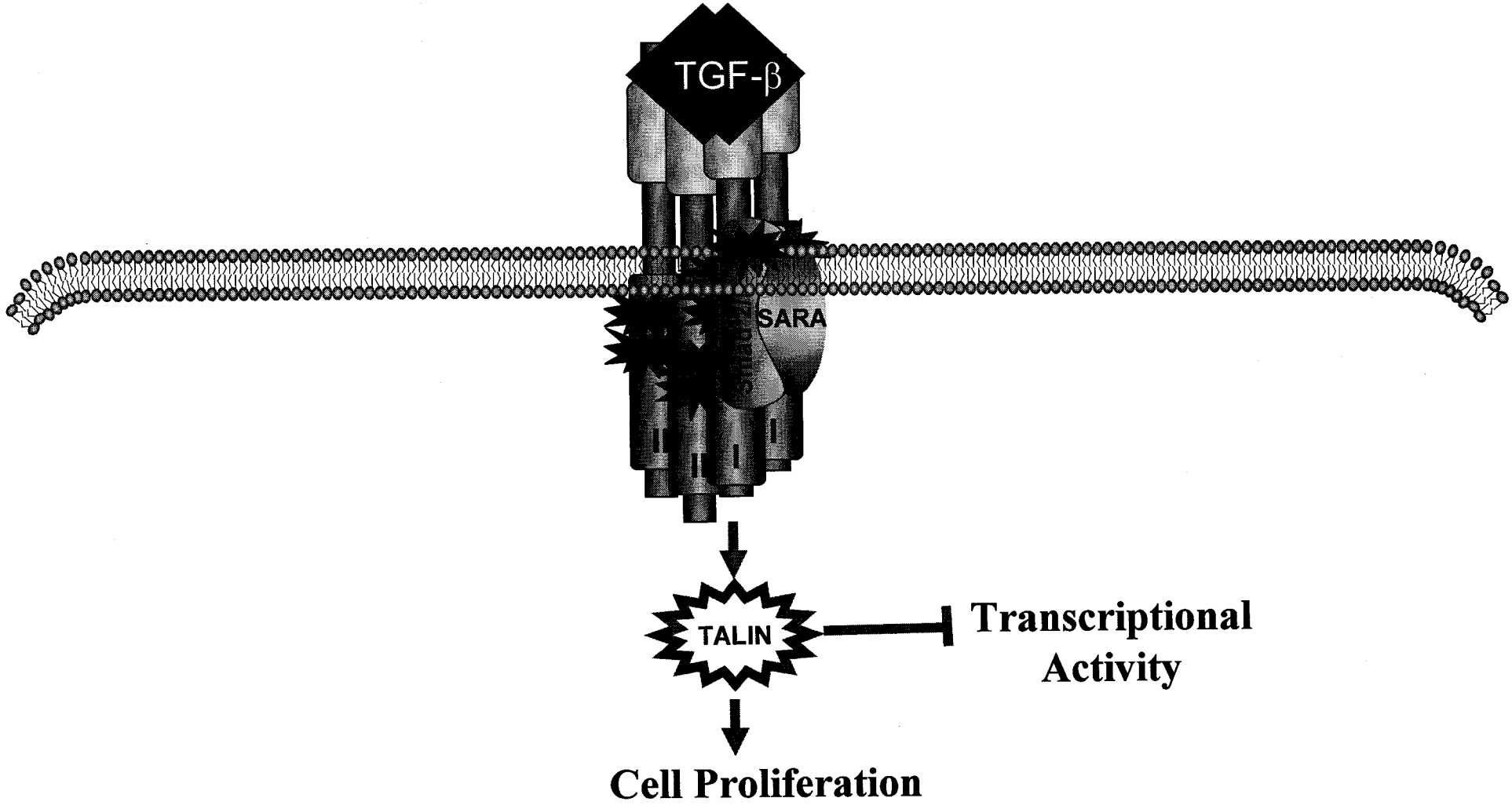


Figure 5.3: Antagonistic effects of Talin in TGF-β signal transduction in MCF-7 cells



5.2 Future Remarks

Based on the obtained data the future extensions of this study can be summarized as followings:

Talin was shown to be positively regulated by TGF-β1. To reveal if this regulation is through a direct or a secondary response to TGF-β1, the transcriptional regulatory mechanism of Talin gene should be investigated by using the protein synthesis inhibitor, cycloheximide.

As discussed in Chapter I, Talin head region contains binding sites for the type 1γ661 isoform of Phosphatidyl-inosital-4-phosphate 5-kinase, PIPK (Ling et al., 2002; Di paolo et al., 2002), and FAK (Borowsky et al., 1998), which are required for localization of Talin to the membrane and cell-ECM junctions (focal adhesions). Therefore, the possibility of the involvement of FAK, and PI3-K in TGF-β signaling pathway leading to TGF-β1-induced Talin translocalization and Actin cytoskeletal reorganization should be examined.

Kloeker and his colleagues (2004) illustrated that Kindlerin, a FERM domain containing protein (similar to Talin) is positively regulated by TGF-β, which is known to mediate EMT in human mammary epithelial cells. Therefore, to find out if Talin is involved in TGF-β-mediated EMT, further criteria such as changes in cell morphology leading to the loss of cell-cell contact and instability of E-cadherin on the cell surface are required to be investigated in terms of Talin.

Moreover, Becam et al. (2005) have reported that Talin has a second, integrin-independent function. They demonstrated that Talin downregulates DE-cadherin transcriptional expression in *Drosophila* ovarian follicle cells. To explore if Talin has any effect on E-cadherin expression in MCF-7 and MDA-MB-231 breast cancer cell lines, hTalin siRNA technique should be employed.

To better understand the potential mechanism of Talin antagonistic effect on TGF-β-induced cell growth arrest, the regulation of cyclin-dependent kinase inhibitors p15INK4B, p21CIP1WAF1, and p27 and other cell cycle regulatory genes induced by TGF-β1 should be investigated in terms of Talin.

We have illustrated that Talin physically interacts with Smads and TGF-β receptors in response to TGF-β1 in breast cancer cell lines. Thus, the identification of the required domains involved in these interactions, and if these interactions lead to cross talk with other signaling pathways may provide a new path for future studies.

As mentioned before, Talin is an integrin-interacting partner involved in integrin cellular responses. In this thesis it was shown that Talin is an inducible antagonist of TGF-β signal transduction at early stage of tumorigenesis. We speculate that in highly invasive MDA-MB-231 cells formation of Talin-TβRII complex upon TGF-β stimulation may involve integrin signaling, enhancing TGF-β1 mediated invasion and EMT. Therefore, further investigations are required in terms of integrin signaling, or any other signaling pathways that might contribute to the differences observed in TGF-β signaling in MCF-7 and MDA-MB-231 cells with respect to Talin.

Studies have shown that phosphorylation of the FERM family of proteins regulates their interactions with transmembrane proteins (Tsukita and Yonemura, 1999). There are several evidences showing that Talin as a FERM containing protein is also phosphorylated on different residues (Qwarnstrom et al., 1991 ; Tidball and Spencer, 1993 ; Turner et al., 1989 ; Watters et al., 1996 ; Beckerle et al., 1989). For example, it has been illustrated that Talin is an in vitro substrate of Protein Kinase C (PKC) and is phosphorylated on both serine and threonine residues in blood platelets (Litchfield and Ball, 1990 ; Litchfield and Ball, 1986 ; Murata et al., 1995). Given the number of protein partners that Talin interacts with, Talin phosphorylation could control when and where these interactions occur (Ratnikov et al. 2005). In this thesis it has been shown that in response to TGF-β1, Talin physically interacts with TβRI in MCF-7 cells and TβRII in MDA-MB-231 cells. Thus, to point to the possibility that Talin phosphorylation could be

of importance for regulating its biological functions and its antagonistic effects in TGF-β signal transduction, it is important to investigate if these serine/threonine kinase receptors or any other kinases are involved in phosphorylation of Talin in response to TGF-β1.

REFERENCES:

Akiyoshi, S., Inoue, H., Hanai, J., Kusanagi, K., Nemoto, N., Miyazono, K. and Kawabata, M. (1999). c-Ski acts as a transcriptional co-repressor in transforming growth factor-beta signaling through interaction with smads. *J Biol Chem* **274**, 35269-77.

Albiges-Rizo, C., Frachet, P. and Block, M. R. (1995). Down regulation of talin alters cell adhesion and the processing of the alpha 5 beta 1 integrin. *J Cell Sci* **108 (Pt 10)**, 3317-29.

Alexandrow, M. G. and Moses, H. L. (1995). Transforming growth factor beta and cell cycle regulation. *Cancer Res* **55**, 1452-7.

Anbazhagan, R., Fujii, H. and Gabrielson, E. (1999). Microsatellite instability is uncommon in breast cancer. *Clin Cancer Res* **5**, 839-44.

Annes, J. P., Munger, J. S. and Rifkin, D. B. (2003). Making sense of latent TGFbeta activation. *J Cell Sci* **116**, 217-24.

Arteaga, C. L., Hurd, S. D., Winnier, A. R., Johnson, M. D., Fendly, B. M., Forbes, J. T. (1993) Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* **92**, 2569-76.

Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M. and Sporn, M. B. (1983). Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. *J Biol Chem* **258**, 7155-60.

Attisano, L. and Wrana, J. L. (2002). Signal transduction by the TGF-beta superfamily. *Science* **296**, 1646-7.

Baker, J. C. and Harland, R. M. (1996). A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. *Genes Dev* **10**, 1880-9.

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

Balda, M. S. and Matter, K. (2003). Epithelial cell adhesion and the regulation of gene expression. *Trends Cell Biol* **13**, 310-8.

Barsukov, I. L., Prescot, A., Bate, N., Patel, B., Floyd, D. N., Bhanji, N., Bagshaw, C. R., Letinic, K., Di Paolo, G., De Camilli, P. et al. (2003). Phosphatidylinositol phosphate kinase type 1gamma and beta1-integrin cytoplasmic domain bind to the same region in the talin FERM domain. *J Biol Chem* **278**, 31202-9.

Bass, M. D., Smith, B. J., Prigent, S. A. and Critchley, D. R. (1999). Talin contains three similar vinculin-binding sites predicted to form an amphipathic helix. *Biochem J* **341 (Pt 2)**, 257-63.

Becam, I. E., Tanentzapf, G., Lepesant, J. A., Brown, N. H. and Huynh, J. R. (2005). Integrin-independent repression of cadherin transcription by talin during axis formation in Drosophila. *Nat Cell Biol* **7**, 510-6.

Beckerle, M. C., Miller, D. E., Bertagnolli, M. E. and Locke, S. J. (1989). Activation-dependent redistribution of the adhesion plaque protein, talin, in intact human platelets. *J. Cell Biol.* **109**, 3333-3346.

Ben-Yosef, T. and Francomano, C. A. (1999). Characterization of the human talin (TLN) gene: genomic structure, chromosomal localization, and expression pattern. *Genomics* **62**, 316-9.

Bertagnolli, M. E., Locke, S. J., Hensler, M. E., Bray, P. F. and Beckerle, M. C. (1993). Talin distribution and phosphorylation in thrombin-activated platelets. *J Cell Sci* **106 (Pt 4)**, 1189-99.

Bhowmick, N. A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C. A., Engel, M. E., Arteaga, C. L. and Moses, H. L. (2001). Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* **12**, 27-36.

Bodmer, S., Strommer, K., Frei, K., Siepl, C., de Tribolet, N., Heid, I. and Fontana, A. (1989). Immunosuppression and transforming growth factor-beta in glioblastoma. Preferential production of transforming growth factor-beta 2. *J Immunol* **143**, 3222-9.

Bogdan, C., Paik, J., Vodovotz, Y. and Nathan, C. (1992). Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10. *J Biol Chem* **267**, 23301-8.

Boland, S., Boisvieux-Ulrich, E., Houcine, O., Baeza-Squiban, A., Pouchelet, M., Schoevaert, D., Marano, F. (1996). TGF beta 1 promotes actin cytoskeleton reorganization and migratory phenotype in epithelial tracheal cells in primary culture. *J Cell Sci* **109 (Pt 9)**, 2207-19.

Bonni, S., Wang, H. R., Causing, C. G., Kavsak, P., Stroschein, S. L., Luo, K. and Wrana, J. L. (2001). TGF-beta induces assembly of a Smad2-Smurf2 ubiquitin ligase complex that targets SnoN for degradation. *Nat Cell Biol* **3**, 587-95.

Bono, P., Rubin, K., Higgins, J. M. and Hynes, R. O. (2001). Layilin, a novel integral membrane protein, is a hyaluronan receptor. *Mol Biol Cell* **12**, 891-900.

Borowsky, M. L. and Hynes, R. O. (1998). Layilin, a novel talin-binding transmembrane protein homologous with C-type lectins, is localized in membrane ruffles. *J Cell Biol* **143**, 429-42.

Brandes, M. E., Wakefield, L. M. and Wahl, S. M. (1991). Modulation of monocyte type I transforming growth factor-beta receptors by inflammatory stimuli. *J Biol Chem* **266**, 19697-703.

Bretscher, A., Edwards, K. and Fehon, R. G. (2002). ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol* **3**, 586-99.

Brown, N. H., Gregory, S. L., Rickoll, W. L., Fessler, L. I., Prout, M., White, R. A. and Fristrom, J. W. (2002). Talin is essential for integrin function in Drosophila. *Dev Cell* **3**, 569-79.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J. and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-68.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G. and Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu Rev Cell Biol* **4**, 487-525.

Burgess, J. R., Shepherd, J. J., Parameswaran, V., Hoffman, L., and Greenaway, T. M. (1996). Prolactinomas in a large kindred with multiple endocrine neoplasia type 1: clinical features and inheritance pattern. *J Clin Endocrinol Metab.* **81**, 841-5

Burrows, F. J., Derbyshire, E. J., Tazzari, P. L., Amlot, P., Gazdar, A. F., King, S. W., Letarte, M., Vitetta, E. S., and Thorpe, P. E. (1995). Up-regulation of endoglin on vascular endothelial cells in human solid tumors: implications for diagnosis and therapy. *Clin Cancer Res* **1**, 1623-34.

Calderwood, D. A., Tai, V., Di Paolo, G., De Camilli, P. and Ginsberg, M. H. (2004). Competition for talin results in trans-dominant inhibition of integrin activation. *J Biol Chem* **279**, 28889-95.

Calderwood, D. A., Yan, B., de Pereda, J. M., Alvarez, B. G., Fujioka, Y., Liddington, R. C. and Ginsberg, M. H. (2002). The phosphotyrosine binding-like domain of talin activates integrins. *J Biol Chem* **277**, 21749-58.

Campbell, I. D. (2003) Modular proteins at the cell surface. *Biochemical Society Transactions* **31**, 1107-1114.

Cheifetz, S., Bellon, T., Cales, C., Vera, S., Bernabeu, C., Massagué, J. and Letarte, M. (1992). Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. *J Biol Chem* **267**, 19027-30.

Chen, C. R., Kang, Y., Siegel, P. M. and Massagué, J. (2002). E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. *Cell* **110**, 19-32.

Chen, H., Ishii, A., Wong, W. K., Chen, L. B., and Lo, S. H. (2000). Molecular characterization of human tensin. *Biochem J* **351(Pt 2)**, 403-411.

Chen, T., Carter, D., Garrigue-Antar, L. and Reiss, M. (1998). Transforming growth factor beta type I receptor kinase mutant associated with metastatic breast cancer. *Cancer Res* **58**, 4805-10.

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). Beta-arrestin 2 mediates endocytosis of type III TGF-beta receptor and down-regulation of its signaling. *Science* **301**, 1394-7.

Chen, X., Rubock, M. J. and Whitman, M. (1996). A transcriptional partner for MAD proteins in TGF-beta signaling. *Nature* **383**, 691-6.

Chen, Y. G., Liu, F. and Massagué, J. (1997). Mechanism of TGFbeta receptor inhibition by FKBP12. *Embo J* **16**, 3866-76.

Cocolakis, E., Lemay, S., Ali, S. and Lebrun, J. J. (2001). The p38 MAPK pathway is required for cell growth inhibition of human breast cancer cells in response to activin. *J Biol Chem* **276**, 18430-6.

Collier, N. C. and Wang, K. (1982). Human platelet P235: a high Mr protein which restricts the length of actin filaments. *FEBS Lett* **143**, 205-10.

Cram, E. J., Clark, S. G. and Schwarzbauer, J. E. (2003). Talin loss-of-function uncovers roles in cell contractility and migration in C. elegans. *J Cell Sci* **116**, 3871-8.

Critchley, D. R. (2000). Focal adhesions - the cytoskeletal connection. *Curr Opin Cell Biol* **12**, 133-9.

Critchley, D. R. (2004). Cytoskeletal proteins talin and vinculin in integrin-mediated adhesion. *Biochem Soc Trans* **32**, 831-6.

Das, P., Maduzia, L. L. and Padgett, R. W. (1999). Genetic approaches to TGFbeta signaling pathways. *Cytokine Growth Factor Rev* **10**, 179-86.

Datta, P. K., Chytil, A., Gorska, A. E. and Moses, H. L. (1998). Identification of STRAP, a novel WD domain protein in transforming growth factor-beta signaling. *J Biol Chem* **273**, 34671-4.

Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y. and Wang, X. F. (1995). Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci* **92**, 5545-9.

de Pereda, J. M., Wegener, K. L., Santelli, E., Bate, N., Ginsberg, M. H., Critchley, D. R., Campbell, I. D. and Liddington, R. C. (2005). Structural basis for phosphatidylinositol phosphate kinase type Igamma binding to talin at focal adhesions. *J Biol Chem* **280**, 8381-6.

Derynck, R., Akhurst, R. J. and Balmain, A. (2001). TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* **29**, 117-29.

Derynck, R. and Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signaling. *Nature* **425**, 577-84.

Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F. and Wrana, J. L. (2003). Distinct endocytic pathways regulate TGF-beta receptor signaling and turnover. *Nat Cell Biol* **5**, 410-21.

Di Paolo, G., Pellegrini, L., Letinic, K., Cestra, G., Zoncu, R., Voronov, S., Chang, S., Guo, J., Wenk, M. R. and De Camilli, P. (2002). Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 gamma by the FERM domain of talin. *Nature* **420**, 85-9.

Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S. and Akhurst, R. J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* **121**, 1845-54.

Dong, C., Li, Z., Alvarez, R., Jr., Feng, X. H. and Goldschmidt-Clermont, P. J. (2000). Microtubule binding to Smads may regulate TGF beta activity. *Mol Cell* **5**, 27-34.

Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T. and Miyazono, K. (2001). Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. *J Biol Chem* **276**, 12477-80.

Edlund, S., Landstrom, M., Heldin, C. H., and Aspenstrom, P. (2002). Transforming growth factor-beta-induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA. *Mol Biol Cell* **13**, 902-14.

Elliott, R. L. and Blobe, G. C. (2005). Role of transforming growth factor Beta in human cancer. *J Clin Oncol* **23**, 2078-93.

Engel, M. E., McDonnell, M. A., Law, B. K. and Moses, H. L. (1999). Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J Biol Chem* **274**, 37413-20.

Eppert, K., Scherer, S. W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L. C., Bapat, B., Gallinger, S., Andrulis, I. L. et al. (1996). MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* **86**, 543-52.

Esparza-Lopez, J., Montiel, J. L., Vilchis-Landeros, M. M., Okadome, T., Miyazono, K. and Lopez-Casillas, F. (2001). Ligand binding and functional properties of betaglycan, a co-receptor of the transforming growth factor-beta superfamily. Specialized binding regions for transforming growth factor-beta and inhibin A. *J Biol Chem* **276**, 14588-96.

Felici, A., Wurthner, J. U., Parks, W. T., Giam, L. R., Reiss, M., Karpova, T. S., McNally, J. G. and Roberts, A. B. (2003). TLP, a novel modulator of TGF-beta signaling, has opposite effects on Smad2- and Smad3-dependent signaling. *Embo J* **22**, 4465-77.

Frolik, C. A., Dart, L. L., Meyers, C. A., Smith, D. M. and Sporn, M. B. (1983). Purification and initial characterization of a type beta transforming growth factor from human placenta. *Proc Natl Acad Sci* **80**, 3676-80.

Furuhashi, M., Yagi, K., Yamamoto, H., Furukawa, Y., Shimada, S., Nakamura, Y., Kikuchi, A., Miyazono, K. and Kato, M. (2001). Axin facilitates Smad3 activation in the transforming growth factor beta signaling pathway. *Mol Cell Biol* **21**, 5132-41.

Furuta, K., Misao, S., Takahashi, K., Tagaya, T., Fukuzawa, Y., Ishikawa, T., Yoshioka, K. and Kakumu, S. (1999). Gene mutation of transforming growth factor beta1 type II receptor in hepatocellular carcinoma. *Int J Cancer* **81**, 851-3.

Galliher, A. J. and Schiemann, W. P. (2006). Beta3 integrin and Src facilitate transforming growth factor-beta mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. *Breast Cancer Res* **8**, R42.

Garcia-Alvarez, B., de Pereda, J. M., Calderwood, D. A., Ulmer, T. S., Critchley, D., Campbell, I. D., Ginsberg, M. H. and Liddington, R. C. (2003). Structural determinants of integrin recognition by talin. *Mol Cell* **11**, 49-58.

Giancotti, F. G. (1997). Integrin signaling: specificity and control of cell survival and cell cycle progression. *Curr Opin Cell Biol* **9**, 691-700.

Gilmore, A. P., Ohanian, V., Spurr, N. K. and Critchley, D. R. (1995). Localisation of the human gene encoding the cytoskeletal protein talin to chromosome 9p. *Hum Genet* **96**, 221-4.

Goggins, M., Shekher, M., Turnacioglu, K., Yeo, C. J., Hruban, R. H. and Kern, S. E. (1998). Genetic alterations of the transforming growth factor beta receptor genes in pancreatic and biliary adenocarcinomas. *Cancer Res* **58**, 5329-32.

Gold, L. I. (1999). The role for transforming growth factor-beta (TGF-beta) in human cancer. *Crit Rev Oncog* **10**, 303-60.

Gomis, R. R., Alarcon, C., Nadal, C., Van Poznak, C. and Massagué, J. (2006). C/EBPbeta at the core of the TGFbeta cytostatic response and its evasion in metastatic breast cancer cells. *Cancer Cell* **10**, 203-14.

Gorelik, L. and Flavell, R. A. (2002). Transforming growth factor-beta in T-cell biology. *Nat Rev Immunol* **2**, 46-53.

Goumans, M. J., Valdimarsdottir, G., Itoh, S., Lebrin, F., Larsson, J., Mummery, C., Karlsson, S. and ten Dijke, P. (2003). Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. *Mol Cell* **12**, 817-28.

Grady, W. M., Myeroff, L. L., Swinler, S. E., Rajput, A., Thiagalingam, S., Lutterbaugh, J. D., Neumann, A., Brattain, M. G., Chang, J., Kim, S. J. et al. (1999). Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res* **59**, 320-4.

Gupta, S. (2000). Molecular steps of cell suicide: an insight into immune senescence. *J Clin Immunol* **20**, 229-39.

Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H. et al. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* **271**, 350-3.

Hanahan, D. and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* **100**, 57-70.

Hannon, G. J. and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* **371**, 257-61.

Hata, A., Lo, R. S., Wotton, D., Lagna, G. and Massagué, J. (1997). Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. *Nature* **388**, 82-7.

Heldin, C. H., Miyazono, K. and ten Dijke, P. (1997). TGF-beta signaling from cell membrane to nucleus through SMAD proteins. *Nature* **390**, 465-71.

Hemmings, L., Rees, D. J., Ohanian, V., Bolton, S. J., Gilmore, A. P., Patel, B., Priddle, H., Trevithick, J. E., Hynes, R. O. and Critchley, D. R. (1996). Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. *J Cell Sci* **109 (Pt 11)**, 2715-26.

Ho, J., Cocolakis, E., Dumas, V. M., Posner, B. I., Laporte, S. A. and Lebrun, J. J. (2005). The G protein-coupled receptor kinase-2 is a TGFbeta-inducible antagonist of TGFbeta signal transduction. *Embo J* **24**, 3247-58.

Hocevar, B. A., Smine, A., Xu, X. X. and Howe, P. H. (2001). The adaptor molecule Disabled-2 links the transforming growth factor beta receptors to the Smad pathway. *Embo J* **20**, 2789-801.

Horwitz, A., Duggan, K., Buck, C., Beckerle, M. C. and Burridge, K. (1986). Interaction of plasma membrane fibronectin receptor with talin-a transmembrane linkage. *Nature* **320**, 531-3.

Hu, P. P., Shen, X., Huang, D., Liu, Y., Counter, C. and Wang, X. F. (1999). The MEK pathway is required for stimulation of p21(WAF1/CIP1) by transforming growth factor-beta. *J Biol Chem* **274**, 35381-7.

Huse, M., Chen, Y. G., Massagué, J. and Kuriyan, J. (1999). Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. *Cell* **96**, 425-36.

Huse, M., Muir, T. W., Xu, L., Chen, Y. G., Kuriyan, J. and Massagué, J. (2001). The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. *Mol Cell* **8**, 671-82.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.

Ignotz, R. A. and Massagué, J. (1986). Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem* **261**, 4337-45.

Ignotz, R. A. and Massagué, J. (1987). Cell adhesion protein receptors as targets for transforming growth factor-beta action. *Cell* **51**, 189-97.

Inman, G. J. and Allday, M. J. (2000). Apoptosis induced by TGF-beta 1 in Burkitt's lymphoma cells is caspase 8 dependent but is death receptor independent. *J Immunol* **165**, 2500-10.

Isenberg, G. and Goldmann, W. H. (1998). Peptide-specific antibodies localize the major lipid binding sites of talin dimers to oppositely arranged N-terminal 47 kDa subdomains. *FEBS Lett* **426**, 165-70.

Itoh, F., Divecha, N., Brocks, L., Oomen, L., Janssen, H., Calafat, J., Itoh, S. and Dijke Pt, P. (2002). The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF-beta/Smad signaling. *Genes Cells* **7**, 321-31.

Itoh, S., Landstrom, M., Hermansson, A., Itoh, F., Heldin, C. H., Heldin, N. E. and ten Dijke, P. (1998). Transforming growth factor beta1 induces nuclear export of inhibitory Smad7. *J Biol Chem* **273**, 29195-201.

Jang, C. W., Chen, C. H., Chen, C. C., Chen, J. Y., Su, Y. H. and Chen, R. H. (2002). TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. *Nat Cell Biol* **4**, 51-8.

Jennings, M. T. and Pietenpol, J. A. (1998). The role of transforming growth factor beta in glioma progression. *J Neurooncol* **36**, 123-40.

Jockusch, B. M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G. and Winkler, J. (1995). The molecular architecture of focal adhesions. *Annu Rev Cell Dev Biol* **11**, 379-416.

Kaklamani, V. G., Hou, N., Bian, Y., Reich, J., Offit, K., Michel, L. S., Rubinstein, W. S., Rademaker, A. and Pasche, B. (2003). TGFBR1*6A and cancer risk: a meta-analysis of seven case-control studies. *J Clin Oncol* **21**, 3236-43.

Kang, Y., Chen, C. R. and Massagué, J. (2003). A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol Cell* **11**, 915-26.

Kang, Y., He, W., Tulley, S., Gupta, G. P., Serganova, I., Chen, C. R., Manova-Todorova, K., Blasberg, R., Gerald, W. L. and Massagué, J. (2005). Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. *Proc Natl Acad Sci* **102**, 13909-14.

Kavsak, P., Rasmussen, R. K., Causing, C. G., Bonni, S., Zhu, H., Thomsen, G. H. and Wrana, J. L. (2000). Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* **6**, 1365-75.

Kehrl, J. H., Wakefield, L. M., Roberts, A. B., Jakowlew, S., Alvarez-Mon, M., Derynck, R., Sporn, M. B. and Fauci, A. S. (1986). Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med* **163**, 1037-50.

Kim, J., Johnson, K., Chen, H. J., Carroll, S. and Laughon, A. (1997). Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. *Nature* **388**, 304-8.

Kim, R. H., Wang, D., Tsang, M., Martin, J., Huff, C., de Caestecker, M. P., Parks, W. T., Meng, X., Lechleider, R. J., Wang, T. et al. (2000). A novel smad nuclear interacting protein, SNIP1, suppresses p300-dependent TGF-beta signal transduction. *Genes Dev* **14**, 1605-16.

Kirkbride, K. C. and Blobe, G. C. (2003). Inhibiting the TGF-beta signaling pathway as a means of cancer immunotherapy. *Expert Opin Biol Ther* **3**, 251-61.

Kloeker, S., Major, M. B., Calderwood, D. A., Ginsberg, M. H., Jones, D. A. and Beckerle, M. C. (2004). The Kindler syndrome protein is regulated by transforming growth factor-beta and involved in integrin-mediated adhesion. *J Biol Chem* **279**, 6824-33.

Kreitmeier, M., Gerisch, G., Heizer, C. and Muller-Taubenberger, A. (1995). A talin homologue of Dictyostelium rapidly assembles at the leading edge of cells in response to chemoattractant. *J Cell Biol* **129**, 179-88.

Kretzschmar, M., Doody, J. and Massagué, J. (1997). Opposing BMP and EGF signaling pathways converge on the TGF-beta family mediator Smad1. *Nature* **389**, 618-22.

Kurokawa, M., Mitani, K., Irie, K., Matsuyama, T., Takahashi, T., Chiba, S., Yazaki, Y., Matsumoto, K. and Hirai, H. (1998). The oncoprotein Evi-1 represses TGF-beta signaling by inhibiting Smad3. *Nature* **394**, 92-6.

Lander, E. S. Linton, L. M. Birren, B. Nusbaum, C. Zody, M. C. Baldwin, J. Devon, K. Dewar, K. Doyle, M. FitzHugh, W. et al. (2001). Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921.

Larsson, J., Goumans, M. J., Sjostrand, L. J., van Rooijen, M. A., Ward, D., Leveen, P., Xu, X., ten Dijke, P., Mummery, C. L. and Karlsson, S. (2001). Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. *Embo J* **20**, 1663-73.

Le Roy, C. and Wrana, J. L. (2005). Clathrin- and non-clathrin-mediated endocytic regulation of cell signaling. *Nat Rev Mol Cell Biol* **6**, 112-26.

Lebrun, J. J. and Vale, W. W. (1997). Activin and inhibin have antagonistic effects on ligand-dependent heteromerization of the type I and type II activin receptors and human erythroid differentiation. *Mol Cell Biol* **17**, 1682-91.

Lewis, K. A., Gray, P. C., Blount, A. L., MacConell, L. A., Wiater, E., Bilezikjian, L. M. and Vale, W. (2000). Betaglycan binds inhibin and can mediate functional antagonism of activin signaling. *Nature* **404**, 411-4.

Lin, H. K., Bergmann, S. and Pandolfi, P. P. (2004). Cytoplasmic PML function in TGF-beta signaling. *Nature* **431**, 205-11.

Lin, X., Duan, X., Liang, Y. Y., Su, Y., Wrighton, K. H., Long, J., Hi, M., Davis, C. M., Wang, J., Brunicardi, F. C., Shi, Y., Chen, Y. G., Meng, A., and Feng, X. H. (2006). PPM1A functions as a Smad phosphatase to terminate TGFbeta signaling. *Cell* **125**, 915-28.

Ling, K., Doughman, R. L., Firestone, A. J., Bunce, M. W. and Anderson, R. A. (2002). Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions. *Nature* **420**, 89-93.

Ling, K., Doughman, R. L., Iyer, V. V., Firestone, A. J., Bairstow, S. F., Mosher, D. F., Schaller, M. D. and Anderson, R. A. (2003). Tyrosine phosphorylation of type Igamma phosphatidylinositol phosphate kinase by Src regulates an integrin-talin switch. *J Cell Biol* **163**, 1339-49.

Litchfield, D. W. and Ball, E. H. (1986). Phosphorylation of the cytoskeletal protein talin by protein kinase C. *Biochem. Biophys. Res Commun.* **134**, 1276-1283.

Litchfield, D. W. and Ball, E. H. (1990). Phosphorylation of high molecular weight proteins in platelets treated with 12-O-tetradecanoylphorbol-13-acetate. *Biochem. Int.* **20**, 615-621.

Liu, F., Hata, A., Baker, J. C., Doody, J., Carcamo, J., Harland, R. M. and Massagué, J. (1996). A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature* **381**, 620-3.

Liu, X., Sun, Y., Constantinescu, S. N., Karam, E., Weinberg, R. A. and Lodish, H. F. (1997). Transforming growth factor beta-induced phosphorylation of Smad3 is required for growth inhibition and transcriptional induction in epithelial cells. *Proc Natl Acad Sci* **94**, 10669-74.

Liu, X., Sun, Y., Weinberg, R. A. and Lodish, H. F. (2001). Ski/Sno and TGF-beta signaling. *Cytokine Growth Factor Rev* **12**, 1-8.

Lo, R. S., Wotton, D. and Massagué, J. (2001). Epidermal growth factor signaling via Ras controls the Smad transcriptional co-repressor TGIF. *Embo J* **20**, 128-36.

Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W. S. and Massagué, J. (1991). Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. *Cell* **67**, 785-95.

Lopez-Casillas, F., Wrana, J. L. and Massagué, J. (1993). Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* **73**, 1435-44.

Luo, K. and Lodish, H. F. (1996). Signaling by chimeric erythropoietin-TGF-beta receptors: homodimerization of the cytoplasmic domain of the type I TGF-beta receptor and heterodimerization with the type II receptor are both required for intracellular signal transduction. *Embo J* **15**, 4485-96.

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B. et al. (1995). Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* **268**, 1336-8.

Martel, V., Racaud-Sultan, C., Dupe, S., Marie, C., Paulhe, F., Galmiche, A., Block, M. R. and Albiges-Rizo, C. (2001). Conformation, localization, and integrin binding of talin depend on its interaction with phosphoinositides. *J Biol Chem* **276**, 21217-27.

Massagué, J. (1998). TGF-beta signal transduction. *Annu Rev Biochem* **67**, 753-91.

Massagué, J. (2000). How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* **1**, 169-78.

Massagué, J. (2003). Integration of Smad and MAPK pathways: a link and a linker revisited. *Genes Dev* **17**, 2993-7.

Massagué, J. (2004). G1 cell-cycle control and cancer. *Nature* **432**, 298-306.

Massagué, J. and Gomis, R. R. (2006). The logic of TGFbeta signaling. *FEBS Lett* **580**, 2811-20.

Massagué, J., Seoane, J. and Wotton, D. (2005). Smad transcription factors. *Genes Dev* **19**, 2783-810.

Miura, S., Takeshita, T., Asao, H., Kimura, Y., Murata, K., Sasaki, Y., Hanai, J. I., Beppu, H., Tsukazaki, T., Wrana, J. L. et al. (2000). Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. *Mol Cell Biol* **20**, 9346-55.

Molony, L., McCaslin, D., Abernethy, J., Paschal, B. and Burridge, K. (1987). Properties of talin from chicken gizzard smooth muscle. *J Biol Chem* **262**, 7790-5.

Monkley, S. J., Pritchard, C. A. and Critchley, D. R. (2001). Analysis of the mammalian talin2 gene TLN2. *Biochem Biophys Res Commun* **286**, 880-5.

Monkley, S. J., Zhou, X. H., Kinston, S. J., Giblett, S. M., Hemmings, L., Priddle, H., Brown, J. E., Pritchard, C. A., Critchley, D. R. and Fassler, R. (2000). Disruption of the talin gene arrests mouse development at the gastrulation stage. *Dev Dyn* **219**, 560-74.

Motyl, T., Grzelkowska, K., Zimowska, W., Skierski, J., Wareski, P., Ploszaj, T. and Trzeciak, L. (1998). Expression of bcl-2 and bax in TGF-beta 1-induced apoptosis of L1210 leukemic cells. *Eur J Cell Biol* **75**, 367-74.

Muguruma, M., Nishimuta, S., Tomisaka, Y., Ito, T. and Matsumura, S. (1995). Organization of the functional domains in membrane cytoskeletal protein talin. *J Biochem (Tokyo)* **117**, 1036-42.

Murata, K., Sakon, M., Kambayashi, J., Okuyama, M., Hase, T. and Mori, T. (1995). Platelet talin is phosphorylated by calyculin A. *J. Cell Biochem.* **57**, 120-126.

Myeroff, L. L., Parsons, R., Kim, S. J., Hedrick, L., Cho, K. R., Orth, K., Mathis, M., Kinzler, K. W., Lutterbaugh, J., Park, K. et al. (1995). A transforming growth factor beta receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. *Cancer Res* **55**, 5545-7.

Nayal, A., Webb, D. J. and Horwitz, A. F. (2004). Talin: an emerging focal point of adhesion dynamics. *Curr Opin Cell Biol* **16**, 94-8.

Nishita, M., Hashimoto, M. K., Ogata, S., Laurent, M. N., Ueno, N., Shibuya, H. and Cho, K. W. (2000). Interaction between Wnt and TGF-beta signaling pathways during formation of Spemann's organizer. *Nature* **403**, 781-5.

Nuckolls, G. H., Romer, L. H. and Burridge, K. (1992). Microinjection of antibodies against talin inhibits the spreading and migration of fibroblasts. *J Cell Sci* **102 (Pt 4)**, 753-62.

Onichtchouk, D., Chen, Y. G., Dosch, R., Gawantka, V., Delius, H., Massagué, J. and Niehrs, C. (1999). Silencing of TGF-beta signaling by the pseudoreceptor BAMBI. *Nature* **401**, 480-5.

Oshima, M., Oshima, H. and Taketo, M. M. (1996). TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev Biol* **179**, 297-302.

Pearson, M. A., Reczek, D., Bretscher, A. and Karplus, P. A. (2000). Structure of the ERM protein moesin reveals the FERM domain fold masked by an extended actin binding tail domain. *Cell* **101**, 259-70.

Perlman, R., Schiemann, W. P., Brooks, M. W., Lodish, H. F. and Weinberg, R. A. (2001). TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. *Nat Cell Biol* **3**, 708-14.

Petritsch, C., Beug, H., Balmain, A. and Oft, M. (2000). TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest. *Genes Dev* **14**, 3093-101.

Pierreux, C. E., Nicolas, F. J. and Hill, C. S. (2000). Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus. *Mol Cell Biol* **20**, 9041-54.

Pierce, D. F. Jr., Gorska, A. E., Chytil, A., Meise, K. S., Page, D. L., Coffey, R. J. Jr, Moses, H. L. (1995). Mammary tumor suppression by transforming growth factor beta 1 transgene expression. *Proc Natl Acad Sci* **92**, 4254-8.

Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massagué, J., Roberts, J. M. and Koff, A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev* **8**, 9-22.

Qin, B., Lam, S. S. and Lin, K. (1999). Crystal structure of a transcriptionally active Smad4 fragment. *Structure* **7**, 1493-503.

Qwarnstrom, E. E., MacFarlane, S. A., Page, R. C. and Dower, S. K. (1991). Interleukin 1 beta induces rapid phosphorylation and redistribution of talin: a possible mechanism for modulation of fibroblast focal adhesion. *Proc. Natl. Acad. Sci.* **88**, 1232-1236.

Raftery, L. A. and Sutherland, D. J. (1999). TGF-beta family signal transduction in Drosophila development: from Mad to Smads. *Dev Biol* **210**, 251-68.

Ratnikov, B., Ptak, C., Han, J., Shabanowitz, J., Hunt, D. F., and. Ginsberg, M. H. (2005). Talin phosphorylation sites mapped by mass spectrometry *Journal of Cell Science* **118**, 4921-4923.

Rees, D. J., Ades, S. E., Singer, S. J. and Hynes, R. O. (1990). Sequence and domain structure of talin. *Nature* **347**, 685-9.

Rifkin, D. B. (2005). Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. *J Biol Chem* **280**, 7409-12.

Roberts, A. B., Anzano, M. A., Meyers, C. A., Wideman, J., Blacher, R., Pan, Y. C., Stein, S., Lehrman, S. R., Smith, J. M., Lamb, L. C. et al. (1983). Purification and properties of a type beta transforming growth factor from bovine kidney. *Biochemistry* **22**, 5692-8.

Roberts, A. B., Lamb, L. C., Newton, D. L., Sporn, M. B., De Larco, J. E. and Todaro, G. J. (1980). Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. *Proc Natl Acad Sci* **77**, 3494-8.

Roberts, A. B. and Wakefield, L. M. (2003). The two faces of transforming growth factor beta in carcinogenesis. *Proc Natl Acad Sci* **100**, 8621-3.

Romano, L. A., Runyan, R. B. (2000). Slug is an essential target of TGFbeta2 signaling in the developing chicken heart. *Dev Biol.* **223**, 91-102.

Sasaki, A., Masuda, Y., Ohta, Y., Ikeda, K. and Watanabe, K. (2001). Filamin associates with Smads and regulates transforming growth factor-beta signaling. *J Biol Chem* **276**, 17871-7.

Schiemann, W. P., Pfeifer, W. M., Levi, E., Kadin, M. E. and Lodish, H. F. (1999). A deletion in the gene for transforming growth factor beta type I receptor abolishes growth regulation by transforming growth factor beta in a cutaneous T-cell lymphoma. *Blood* **94**, 2854-61.

Seoane, J., Le, H. V., Shen, L., Anderson, S. A. and Massagué, J. (2004). Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* **117**, 211-23.

Shi, Y. and Massagué, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.

Shi, Y., Wang, Y. F., Jayaraman, L., Yang, H., Massagué, J. and Pavletich, N. P. (1998). Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. *Cell* **94**, 585-94.

Siegel, P. M. and Massagué, J. (2003). Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* **3**, 807-21.

Sirard, C., de la Pompa, J. L., Elia, A., Itie, A., Mirtsos, C., Cheung, A., Hahn, S., Wakeham, A., Schwartz, L., Kern, S. E. et al. (1998). The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev* **12**, 107-19.

Stroschein, S. L., Bonni, S., Wrana, J. L. and Luo, K. (2001). Smad3 recruits the anaphase-promoting complex for ubiquitination and degradation of SnoN. *Genes Dev* **15**, 2822-36.

Sun, Y., Liu, X., Ng-Eaton, E., Lodish, H. F. and Weinberg, R. A. (1999). SnoN and Ski protooncoproteins are rapidly degraded in response to transforming growth factor beta signaling. *Proc Natl Acad Sci* **96**, 12442-7.

Tachibana, I., Imoto, M., Adjei, P. N., Gores, G. J., Subramaniam, M., Spelsberg, T. C. and Urrutia, R. (1997). Overexpression of the TGFbeta-regulated zinc finger encoding gene, TIEG, induces apoptosis in pancreatic epithelial cells. *J Clin Invest* **99**, 2365-74.

Tang, Y., Katuri, V., Dillner, A., Mishra, B., Deng, C. X. and Mishra, L. (2003). Disruption of transforming growth factor-beta signaling in ELF beta-spectrin-deficient mice. *Science* **299**, 574-7.

ten Dijke, P. and Hill, C. S. (2004). New insights into TGF-beta-Smad signaling. *Trends Biochem Sci* **29**, 265-73.

Tepass, U. and Godt, D. (2005). Talin's second persona. *Nat Cell Biol* **7**, 443-4.

Thiery, J. P. (2002). Epithelial-mesenchymal transitions in tumor progression. *Nat Rev Cancer* **2**, 442-54.

Thiery, J. P. (2003). Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* **15**, 740-6.

Thomas, D. A. and Massagué, J. (2005). TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* **8**, 369-80.

Tidball, J. G. and Spencer, M. J. (1993). PDGF stimulation induces phosphorylation of talin and cytoskeletal reorganization in skeletal muscle. *J. Cell Biol.* **123**, 627-635.

Tomita, S., Deguchi, S., Miyaguni, T., Muto, Y., Tamamoto, T. and Toda, T. (1999). Analyses of microsatellite instability and the transforming growth factor-beta receptor type II gene mutation in sporadic breast cancer and their correlation with clinicopathological features. *Breast Cancer Res Treat* **53**, 33-9.

Tremuth, L., Kreis, S., Melchior, C., Hoebeke, J., Ronde, P., Plancon, S., Takeda, K. and Kieffer, N. (2004). A fluorescence cell biology approach to map the second integrin-binding site of talin to a 130-amino acid sequence within the rod domain. *J Biol Chem* **279**, 22258-66.

Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L. and Wrana, J. L. (1998). SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* **95**, 779-91.

Tsukita, S. and Yonemura, S. (1999). Cortical actin organization: lessons from ERM (ezrin/radixin/moesin) proteins. *J. Biol. Chem.* **274**, 34507-34510.

Turner, C. E., Pavalko, F. M. and Burridge, K. (1989). The role of phosphorylation and limited proteolytic cleavage of talin and vinculin in the disruption of focal adhesion integrity. *J. Biol. Chem.* **264**, 11938-11944.

Uchida, K., Nagatake, M., Osada, H., Yatabe, Y., Kondo, M., Mitsudomi, T., Masuda, A., Takahashi, T. and Takahashi, T. (1996). Somatic in vivo alterations of the JV18-1 gene at 18q21 in human lung cancers. *Cancer Res* **56**, 5583-5.

Ulloa, L., Doody, J. and Massagué, J. (1999). Inhibition of transforming growth factor-beta/SMAD signaling by the interferon-gamma/STAT pathway. *Nature* **397**, 710-3.

Valderrama-Carvajal, H., Cocolakis, E., Lacerte, A., Lee, E. H., Krystal, G., Ali, S. and Lebrun, J. J. (2002). Activin/TGF-beta induce apoptosis through Smad-dependent expression of the lipid phosphatase SHIP. *Nat Cell Biol* **4**, 963-9.

Wakefield, L., M, Roberts, A., B. (2002). TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* **12**, 22-9.

Wang, W., Mariani, F. V., Harland, R. M. and Luo, K. (2000). Ski represses bone morphogenic protein signaling in Xenopus and mammalian cells. *Proc Natl Acad Sci* **97**, 14394-9.

Watanabe, M., Masuyama, N., Fukuda, M. and Nishida, E. (2000). Regulation of intracellular dynamics of Smad4 by its leucine-rich nuclear export signal. *EMBO Rep* **1**, 176-82.

Watters, D., Garrone, B., Gobert, G., Williams, S., Gardiner, R. and Lavin, M. (1996). Bistratene A causes phosphorylation of talin and redistribution of actin microfilaments in fibroblasts: possible role for PKC-delta. *Exp. Cell Res.* **229**, 327-335.

Weinstein, E. J., Bourner, M., Head, R., Zakeri, H., Bauer, C. and Mazzarella, R. (2003). URP1: a member of a novel family of PH and FERM domain-containing membrane-associated proteins is significantly over-expressed in lung and colon carcinomas. *Biochim Biophys Acta* **1637**, 207-16.

Wieser, R., Wrana, J. L. and Massagué, J. (1995). GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. *Embo J* **14**, 2199-208.

Wojtowicz-Praga, S. (2003). Reversal of tumor-induced immunosuppression by TGF-beta inhibitors. *Invest New Drugs* **21**, 21-32.

Wotton, D., Lo, R. S., Lee, S. and Massagué, J. (1999). A Smad transcriptional corepressor. *Cell* **97**, 29-39.

Wu, G., Chen, Y. G., Ozdamar, B., Gyuricza, C. A., Chong, P. A., Wrana, J. L., Massagué, J. and Shi, Y. (2000). Structural basis of Smad2 recognition by the Smad anchor for receptor activation. *Science* **287**, 92-7.

Wurthner, J. U., Frank, D. B., Felici, A., Green, H. M., Cao, Z., Schneider, M. D., McNally, J. G., Lechleider, R. J. and Roberts, A. B. (2001). Transforming growth factor-beta receptor-associated protein 1 is a Smad4 chaperone. *J Biol Chem* **276**, 19495-502.

Xiao, Z., Liu, X., Henis, Y. I. and Lodish, H. F. (2000). A distinct nuclear localization signal in the N terminus of Smad 3 determines its ligand-induced nuclear translocation. *Proc Natl Acad Sci* **97**, 7853-8.

Xin, H., Stephans, J. C., Duan, X., Harrowe, G., Kim, E., Grieshammer, U., Kingsley, C. and Giese, K. (2000). Identification of a novel aspartic-like protease differentially expressed in human breast cancer cell lines. *Biochim. Biophys. Acta* **1501**, 125-137

Xing, B., Jedsadayanmata, A. and Lam, S. C. (2001). Localization of an integrin binding site to the C terminus of talin. *J Biol Chem* **276**, 44373-8.

Xu, L., Alarcon, C., Col, S. and Massagué, J. (2003). Distinct domain utilization by Smad3 and Smad4 for nucleoporin interaction and nuclear import. *J Biol Chem* **278**, 42569-77.

Xu, L., Kang, Y., Col, S. and Massagué, J. (2002). Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus. *Mol Cell* **10**, 271-82.

Xu, L. and Massagué, J. (2004). Nucleocytoplasmic shuttling of signal transducers. *Nat Rev Mol Cell Biol* **5**, 209-19.

Xu, X., Yin, Z., Hudson, J. B., Ferguson, E. L. and Frasch, M. (1998). Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the Drosophila mesoderm. *Genes Dev* **12**, 2354-70.

Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E. and Matsumoto, K. (1995). Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. *Science* **270**, 2008-11.

Yamakawa, N., Tsuchida, K. and Sugino, H. (2002). The rasGAP-binding protein, Dok-1, mediates activin signaling via serine/threonine kinase receptors. *Embo J* **21**, 1684-94.

Yamamoto, T., Kozawa, O., Tanabe, K., Akamatsu, S., Matsuno, H., Dohi, S. and Uematsu, T. (2001). Involvement of p38 MAP kinase in TGF-beta-stimulated VEGF synthesis in aortic smooth muscle cells. *J Cell Biochem* **82**, 591-8.

Youn Yi, j., Chung Hur, K., Lee, E., Jin, Y., J., Arteaga, C., L., Son, Y., S. (2002). TGFb1-mediated epithelial to mesenchymal transition is accompanied by invasion in the SiHa cell line. *European Journal of Cell biology* **81**, 457-468.

Zavadil, J., Bitzer, M., Liang, D., Yang, Y. C., Massimi, A., Kneitz, S., Piek, E. and Bottinger, E. P. (2001). Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proc Natl Acad Sci* **98**, 6686-91.

Zavadil, J. and Bottinger, E. P. (2005). TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene* **24**, 5764-74.

Zavadil, J., Cermak, L., Soto-Nieves, N. and Bottinger, E. P. (2004). Integration of TGF-beta/Smad and Jagged1/Notch signaling in epithelial-to-mesenchymal transition. *Embo J* **23**, 1155-65.

Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B. and Kern, S. E. (1998). Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol Cell* **1**, 611-7.

Zhang, Y. and Derynck, R. (1999). Regulation of Smad signaling by protein associations and signaling crosstalk. *Trends Cell Biol* **9**, 274-9.

Zhu, Y., Richardson, J. A., Parada, L. F. and Graff, J. M. (1998). Smad3 mutant mice develop metastatic colorectal cancer. *Cell* **94**, 703-14.