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Regulation of Smad anchor necessary for receptor activation (SARA) protein levels by the E3 ubiquitin-protein ligase Smurf2 and Smad7

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Physiology

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Abstract

The TGF β pathway, which regulates cell proliferation and differentiation, has also been shown to induce non-small cell lung cancer cell (NSCLC) migration and invasion. The TGF β pathway is initiated through the binding of TGF β to cell surface Ser/Thr kinase receptors. Activated receptors then phosphorylate intracellular signaling proteins, termed Smads, which translocate into the nucleus to regulate transcriptional responses. The protein Smad anchor for receptor activation (SARA) facilitates the phosphorylation of Smads and allows for efficient signal transduction. On the other hand, the inhibitory Smad, Smad7, recruits the E3 ubiquitin ligase, Smurf2, to catalyze the degradation of TGF β receptors. Since the signaling and degradation pathways target active receptor complexes, SARA and Smurf2-Smad7 may interact with common TGF β receptors. Therefore, the Smurf2-Smad7 complex may affect SARA steady state levels and influence TGF β signaling. I hypothesized that Smurf2-Smad7 induces SARA degradation through an ubiquitin-dependent pathway. I observed that SARA steady state levels decrease in the presence of Smurf2 and Smad7, and this is dependent on the HECT E3 ubiquitin ligase activity of Smurf2. In addition, I observed that SARA interacts with ubiquitinated proteins and is protected from degradation by the pharmacological inhibition of the proteasome. Finally, I assessed the functional outcome of reducing endogenous SARA levels. I observed that siRNA directed at SARA decreased both TGF β -dependent Smad2 phosphorylation as well as EMT. These data suggest that the interplay between SARA and Smurf2-Smad7 complexes can influence TGF β receptor signaling and may provide for a novel approach in targeting this pathway in NSCLC.

Keywords

TGF β , TGF β receptors, SARA, Smurf2, Smad7, early endosome, caveolin-1-positive early endosome, ubiquitination degradation, steady state levels

Co-Authorship Statement

All experiments were designed by S. Lee and J. Di Guglielmo, and completed by S. Lee.

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Abbreviations

Akt – AKR Thymoma / Protein Kinase B

ANOVA – Analysis of variance

ATF3 – Activating transcription factor 3

CaCl₂ – Calcium chloride

Cav-1 – Caveolin 1

CDK – Cyclin-dependent kinase

CO₂ – Carbon dioxide

Co-Smads – Common Smad, Smad4

ddH₂O – Double distilled water

DAPI – 4',6-Diamidino-2-Phenylindole

Daxx – Death-associated protein 6

DMEM – Dulbecco's Modified Eagle Medium

DNA – Deoxyribonucleic acid

E1 – Ubiquitin-activating enzyme

E2 – Ubiquitin-conjugating enzyme

E3 – Ubiquitin Ligase

E2F4/5-C/EBP β – E2F Transcriptional factors 4/5-Ccaat-enhancer-binding proteins

E-cad – E-cadherin

EDTA – Ethylenediaminetetraacetic acid

EEA-1 – Early Endosome Antigen 1

EMT – Epithelial to mesenchymal transition

ESCRT – Endosomal sorting complexes required for transport

FBS – Fetal bovine serum

F12K – Kaighn's Medium

FoxO – Forkhead box O

FYVE – Fab1, YOTB, Vac1 and EEA1

GS – Glycine/Serine

GTPase – Guanosine triphosphate hydrolase

HCl – Hydrogen chloride

HECT – homology to E6AP carboxyl terminus

HRP – Horseradish peroxidase

Hsc70 – Heat shock cognate 70 kDa protein 8

IB – Immunoblotting

Id1 – DNA-binding protein inhibitor 1

IF - Immunofluorescence

I-Smad – Inhibitory Smad, Smad 6 or Smad7

IP - Immunoprecipitation

JNK – c-Jun N-terminal kinases

KCl – Potassium chloride

LB – Lysogeny broth

Mad – Mothers against decapentaplegic

MAPK – Mitogen-activated protein kinase

MH1 – Mad Homology 1

MH2 – Mad Homology 2

MMP – Matrix metalloproteinase

NaCl – Sodium Chloride

N-cad – N-cadherin

NSCLC – Non small cell lung cancer

NTD – amino-terminal domain

p70S6K – Ribosomal protein S6 kinase beta-1

Par6 – Partitioning defective 6 homolog

PBS – Phosphate buffered saline

PI3K – Phosphoinositide 3-kinase

PI3P – phosphatidylinositol 3-phosphate

PMSF – Phenylmethane sulfonyl fluoride

PP2A – Protein phosphatase A

PS2 – Phospho-Smad2

Rab5 – Ras-related protein Rab-5A

RhoA – Ras homolog family member A

RNA – Ribonucleic acid

RNF11 – Ring-finger 11 protein

R-Smad – Receptor-regulated Smad, for e.g., Smad2 and Smad3

SARA – Smad anchor necessary for receptor activation

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA – Small interfering ribonucleic acid

Smurf1 – Smad ubiquitin related factor 1 / E3 ubiquitin-protein ligase SMURF1

Smurf2 – Smad ubiquitin related factor 2 / E3 ubiquitin-protein ligase SMURF2

T β RI – TGF β type I receptor

T β RII – TGF β type II receptor

T β RV – TGF β type V receptor

TBST – Tris-buffered saline supplemented with 0.05% Tween-20

TGF β – Transforming growth factor β

ZFYVE9 – zinc finger FYVE domain-containing protein 9 / also known as SARA

1 Introduction

1.1 Non small cell lung cancer

Lung cancer is the leading cause of cancer death in the world in that it is responsible to approximately 25% of all cancer mortality (Canada Cancer Society 2016; R. Siegel, Miller, and Jemal 2017). Over the past 30 years, the 5-year survival rate has not improved significantly and remains at 15% due to limiting treatment strategies and detection (R. Siegel, Miller, and Jemal 2017). There are two major forms of lung cancers: non small cell lung cancer (NSCLC) and small cell lung cancer (Herbst, Heymach, and Lippman 2008). NSCLC account for approximately 80-85% of all lung cancer cases (D'Addario et al. 2010). NSCLC can be divided into three major histological subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (Travis et al. 2004). Although the biological features are similar between the subtypes, their cell origin, location in the lung and pattern of growth are different suggesting that they are distinct diseases developing through different mechanisms (Pikor et al. 2013). Many human cancers, including lung cancer, often overexpress transforming growth factor β (TGF β), and this cytokine enhances the invasiveness and metastatic potential in late-stage tumors (Jeon and Jen 2010). Decreased or altered TGF β responsiveness and increased expression or activation of the TGF β ligand lead to cancer progression and metastasis (Roberts and Wakefield 2003). Therefore, TGF β induces cell proliferation, migration, invasion in NSCLC cells

(Kasai et al. 2005; Mooradian et al. 1992; Scagliotti, Masiero, and Pozzi 1995; Willis et al. 2005; Willis and Borok 2007).

1.2 Metastasis

Metastasis is the final stage of tumor progression and it accounts for up to 90% of deaths associated with tumors (Hanahan and Weinberg 2000). Metastasis consists of multiple process: cancer cells enter circulation, disseminate to capillary beds, enter by extravasation, adapt to the new microenvironment and grow into lethal colonies that invade blood vessels and enter the circulation to produce additional metastases in distal organs (Chambers, Groom, and Macdonald 2002; Fidler 2003; Gupta and Massagué 2006; Padua and Massagué 2009). The TGF β pathway has been identified as a key mediator of extravasation, microenvironment remodelling, homing, invasion, migration and survival and it impacts the ability of tumor cells, particularly by promoting EMT, to spread throughout the body (Gupta and Massagué 2006).

1.2.1 Epithelial to Mesenchymal Transition

TGF β enhances the migratory and invasive properties of cancer through epithelial-mesenchymal transition (EMT; Figure 1) (Padua and Massagué 2009). During EMT, epithelial cell characteristics are lost and an invasive and migratory mesenchymal phenotype is acquired. This allows the cells to leave the tissue parenchyma, undergo morphogenetic programs, generate new tissues during development or repair wounded ones, and to enter

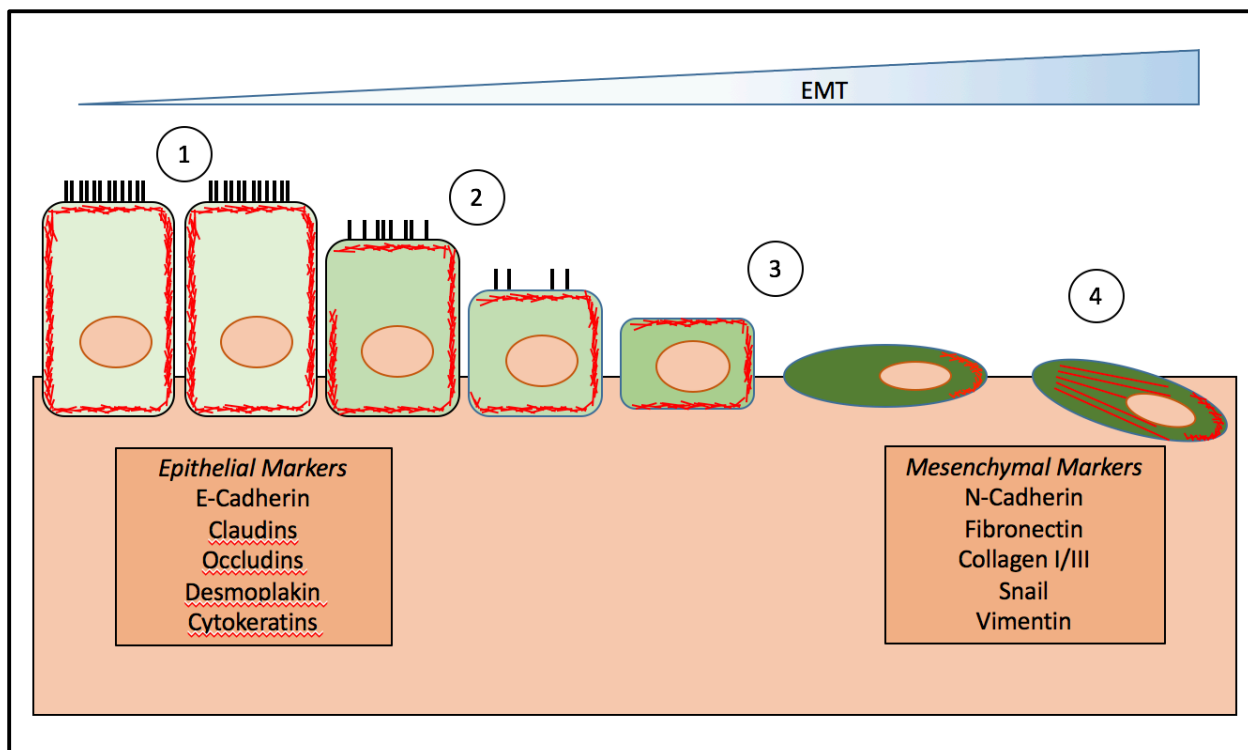


Figure 1. Epithelial to Mesenchymal Transition.

Epithelial to mesenchymal transition (EMT) is important during development and wound healing but can also be used by tumor cells to induce metastasis. There are four key steps during EMT: 1. Tight junction dissociation; 2. Adherent-junction and desmosome dissociation concomitant with the loss of apical-basal polarity; 3. Cytoskeleton reorganization (for e.g., cortical actin reorganizes into stress fibres; shown in red) and cell migration; and 4. Basement membrane degradation and invasion. EMT also involves a shift in expression of epithelial markers (for e.g., E-cadherin and ZO-1) to cells that express mesenchymal markers such as N-cadherin and vimentin and the transcription factors Snail and Slug.

the blood circulation during cancer metastasis (Gonzalez and Medici 2014; Thiery 2002; Valastyan and Weinberg 2011). EMT starts when intercellular junctions dissociate due to the downregulation of adhesion molecules, such as E-Cadherin, claudin, occludin, zona occludens-1, and desmoplakin, and epithelial cells lose their apical-basal polarity (Aroeira et al., 2013). Next, due to cytoskeletal reorganization, whereby cortical actin reorganizes to form stress fibres, cells adopt a front-back polarity and increase their migratory capacity (Kalluri and Neilson 2003; Thiery and Sleeman 2006). As EMT progresses, the later stages are characterized by cells acquiring the ability to degrade the basement membrane and invade the fibrotic stroma by upregulating their expression of matrix metalloproteinases (MMPs) (Aroeira et al. 2013). Upregulation of certain molecular markers during EMT such as, N-cadherin, vimentin and the transcription factors Snail and Slug, will indicate the later stage of EMT (Figure 1). EMT is a fundamental process in embryonic development and tissue repair; however, in human cancers, cytokines involved in the induction of EMT can be found in certain pathology sections and accounts for progression of diseases, including organ fibrosis and cancer (Gonzalez and Medici 2014; Padua and Massagué 2009; Thiery 2002).

1.3 Transforming Growth Factor β

The TGF β superfamily of cytokines regulate many biological responses such as cell growth, proliferation, differentiation and apoptosis (Asano et al. 2004; Izzi and Attisano 2004; Massagué 2012). Deregulation of the pathways initiated by these cytokines often leads to human disease such as cancer of the breast, colon, pancreas, and lungs (Carl Henrik Heldin, Vanlandewijck, and Moustakas 2012). TGF β superfamily members can

facilitate different cellular responses. TGF β induces all these responses through transmembrane receptor serine/threonine kinases and intracellular signaling cascades (Caestecker et al. 1998; Padgett 1999). During carcinogenesis, TGF β plays a dual role. It initially suppresses tumorigenesis through induction of growth arrest and promotion of apoptosis (C H Heldin, Miyazono, and ten Dijke 1997; Levy and Hill 2006). More specifically, TGF β can induce signaling at any stage in cell cycle but can only induce cell cycle arrest at G1 stage (Massague and Gomis 2006). Induction of G1 arrest is achieved by activation of various anti-proliferative responses such as the transcriptional upregulation of cyclin-dependent kinase (CDK) inhibitors p21 and p15 (Datto et al. 1995; Hannon and Beach 1994). In addition, TGF β causes transcriptional repression of c-Myc, a pro-growth transcriptional factor, and Id1, Id2 and Id3, which are inhibitors of differentiation (Alexandrow et al. 1995; Kang, Chen, and Massagué 2003; P. M. Siegel and Massagué 2003). Pro-apoptotic responses are also induced by TGF β through Phosphatidyl Inositol 3' kinase-protein kinase B (PI3K-Akt) pathway, where Akt binds and sequesters Smad3 from the transcriptional machinery (Conery et al. 2004; P. M. Siegel and Massagué 2003). However, due to oncogenic mutations in tumor cells, TGF β is repurposed to promote tumorigenesis by inducing EMT (Levy and Hill 2006). TGF β affects the cell-junction complexes and promotes EMT by Smad-dependent transcriptional events (Miettinen et al. 1994). E-cadherin, a cell-cell adhesion receptor, is the key target for repression during EMT (Miettinen et al. 1994; Oft et al. 1996). Snail and Slug, which are zinc-finger transcription factors are induced through Smad-mediated signaling through the TGF β pathway and repress the E-cadherin gene transcription (P. M. Siegel and Massagué 2003). In addition, the expression of N-cadherin, another EMT

marker, was observed to be significantly increased in peripheral areas of NSCLC tumors, which allows cells to infiltrate and destroy alveolar space (Funai et al. 2003; Watanabe et al. 2011). In later stages of cancer, tumor cells acquire the ability to evade growth suppression of TGF β and induce EMT, gaining a selective advantage for cancer cells to produce and activate TGF β to further promote carcinogenic process via fibroblast activation (Cantelli et al. 2017; Gupta and Massagué 2006; Kalluri 2016; Lee et al. 2006). TGF β also affects non-malignant cells during tumorigenesis by suppressing immune surveillance, promoting angiogenesis and recruiting inflammatory cells that secrete cytokines to act on the tumor cells (Carl Henrik Heldin, Vanlandewijck, and Moustakas 2012).

1.4 The canonical TGF β pathway

TGF β signals through transmembrane TGF β type II and TGF β type I receptors (T β RII and T β RI, respectively), that contain intracellular serine and threonine (Ser/Thr) kinase domains (Macías-Silva et al. 1996). The canonical TGF β pathway is initiated through binding of TGF β ligand to T β RII (Massagué et al., 1994; Wrana et al., 1994). This recruits T β RI into a complex with T β RII, where T β RII then phosphorylates T β RI in its glycine/serine-rich (GS) domain (Zhang et al., 2001). This phosphorylation leads to an activation of a unique family of intracellular signaling proteins, termed Smads, which are related to *D. Melanogaster* Mothers Against Decapentaplegic (Derynck and Zhang 1996; Massagué 1996) Smads are highly conserved proteins across species (Sorrentino et al. 2012). They can be identified and classified into three groups based on their function and structure. Receptor-regulated Smads (R-Smads), such as Smad2 and Smad3, interact with

TGF β receptors directly, and are phosphorylated by the receptor complex (Graff, Bansal, and Melton 1996). Common Smads (Co-Smads) are responsible for forming heteromeric complexes by associating with R-Smads and further carry the signal to the nucleus. Smad4 is the only Co-Smad – it has a very similar structure to R-Smads but it does not get phosphorylated by TGF β receptors (Makkar et al. 2009); Finally, inhibitory Smads (I-Smads), such as Smad6 or Smad7, associate with TGF β receptors and act as pseudo substrate inhibitors as they cannot be phosphorylated by receptors. Their role is to inhibit the TGF β signaling mediated by R-Smads and Co-Smads (Hayashi et al. 1997; Nakao et al. 1997). All members of the family share structural domains such as Mad Homology (MH) 1 and MH2 domains in their the amino and carboxy-terminal regions, respectively (Baker and Harland 1996; Graff, Bansal, and Melton 1996). The MH1 domain contains a DNA binding region and contributes to its role in transcriptional activation (Shi et al. 1998). The MH2 domain mediates several interactions: 1. Between R-Smads and T β RI, 2. Between R-Smads and Co-Smads, 3. Between R-Smads and SARA, and 4. Between R-Smads and DNA binding factors (Hata et al. 1997; Macías-Silva et al. 1996) (Figure 2). Smads are proteins that function as intracellular signaling effectors in the TGF β pathway (Derynck, Zhang, and Feng 1998). The R-Smads, Smad2 and Smad3 are activated via C-terminal serine phosphorylation. Once activated, R-Smads associate with the Co-Smad, Smad4, and form a complex that translocates and accumulates in the nucleus to regulate transcriptional responses on many target genes (C H Heldin, Miyazono, and ten Dijke 1997; Miyazono 2000) (Figure 3). As mentioned before, upregulation of cell cycle

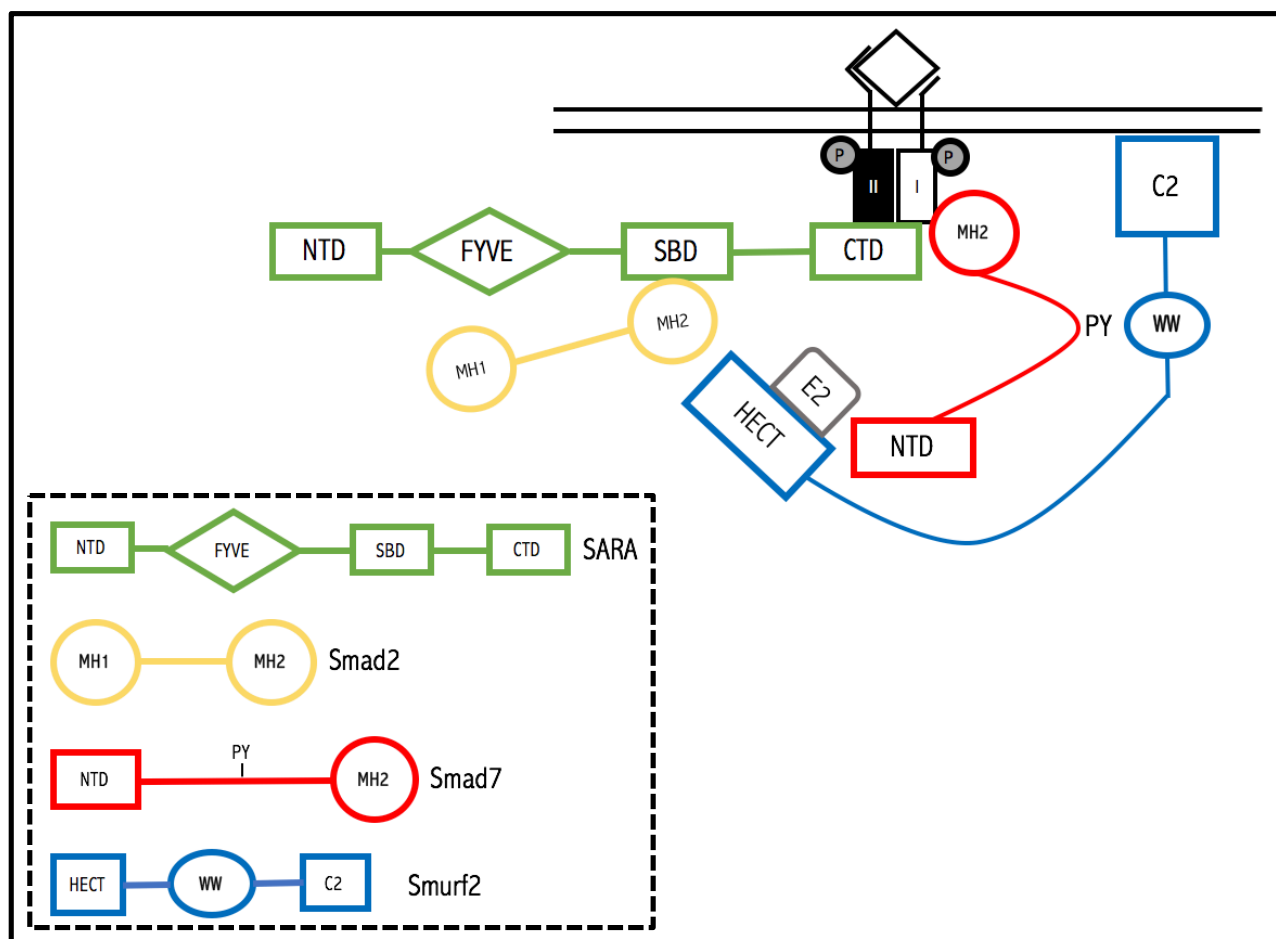


Figure 2. Protein domains in SARA, Smad2, Smad7 and Smurf2 that mediate protein-protein interactions.

SARA interacts with TGF β type II and type I receptors through the C-terminus domain. It contains a Smad binding domain where it interacts with R-Smads, such as Smad2 via the MH2 domain. Smad7 interacts with TGF β type I receptor via its MH2 domain. The PY motif of Smad7 is responsible for interaction with the WW domain of Smurf1 and the HECT domain of Smurf2. The NTD of Smad7 regulates the catalytic activity of the HECT domain by recruiting UbcH7 to the HECT domain.

NTD: Amino-Terminal Domain, FYVE: Fab1, YOTB, Vac1 and EEA1, SBD: Smad Binding Domain, CTD: Carboxyl-Terminal Domain, MH: Mad Homology, HECT: Homology to E6AP Carboxyl Terminus

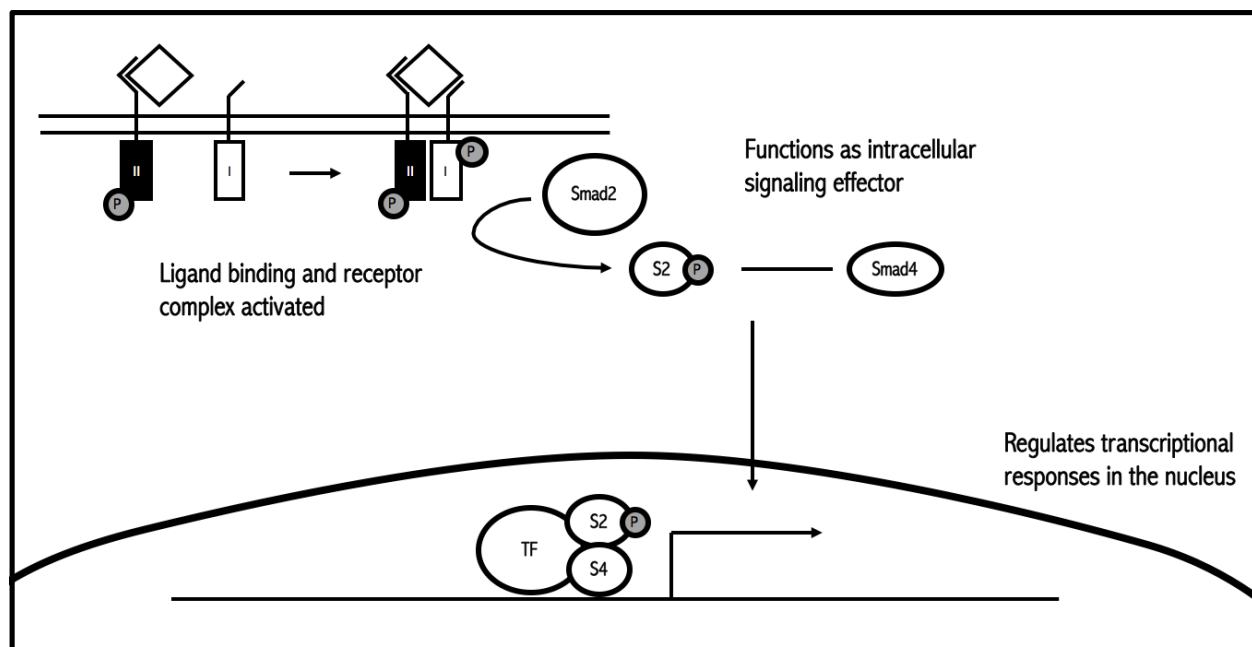


Figure 3. The canonical TGFβ pathway.

TGFβ signals through the transmembrane receptors, TGFβ type II receptor (TβRII) and TGFβ type I receptor (TβRI). The canonical TGFβ pathway is initiated through binding of TGFβ ligand to TβRII. This recruits TβRI into a complex with TβRII, where TβRII then phosphorylates TβRI in its GS domain. This phosphorylation leads to activation of TβRI and the phosphorylation of Receptor-regulated Smads (Smads), proteins that function as intracellular signaling effectors in the TGFβ pathway. Once phosphorylated, R-Smads associates with a common mediator-Smad (Co-Smad), such as Smad4, and form a complex that translocates and accumulates in the nucleus to regulate transcriptional responses.

S2: Smad2, S4: Smad4, TF: transcription factors

inhibitors is important in TGF β -dependent growth arrest. The formation of Smad complexes with FoxO, which belongs to the Forkhead transcription factor family, is necessary for the transcription of a myriad of genes (Wijchers, Burbach, and Smidt 2006). Also, transcriptional repression of c-Myc and Id1 are Smad-dependent (Chen, Kang, and Massague 2001; Gomis et al. 2006). The formation of Smad-E2F4/5-C/EBP β transcriptional complex is required for the repression of c-Myc and transcriptional repressor ATF3-Smad complex formation is necessary for the repression of Id1 (Chen, Kang, and Massague 2001; Kang, Chen, and Massagué 2003).

1.5 Non-canonical TGF β pathways

In addition to Smad-dependent pathways, there are non-Smad pathways that TGF β activates in order to achieve growth arrest, apoptosis and other cellular functions.

Griswold-Prenner, Kamibayashi, Maruoka, Mumby, & Derynck, 1998 showed that T β RI associate with the B α subunit of phosphatase A (PP2A) in epithelial cells, that leads to PP2A mediated dephosphorylation and inactivation of ribosome biogenesis and cell growth regulator, p70 S6K (Petritsch et al. 2000). Although the precise mechanism is unclear, many studies suggested that TGF β type V receptor (T β RV) induces inhibition of epithelial cell growth by forming a complex with T β RI upon TGF β treatment (S. S. Huang et al. 2003; Liu, Huang, and Huang 1997; Tseng, Huang, and Huang 2004). TGF β apoptosis can also be mediated through JNK and p38, which belong to the MAP kinase family. T β RII interacts with Daxx, pro-apoptotic adaptor protein and mediates JNK-dependent apoptosis (Hofmann et al. 2003; Perlman et al. 2001).

In addition to apoptosis, EMT also can be mediated through non-Smad pathways. Indeed, Par6, a tight junction protein, is phosphorylated by T β RII and regulates tight junction integrity via Smurf1, an E3 ubiquitin ligase, resulting in ubiquitination and degradation of RhoA GTPase and the dissolution of tight junctions (Ozdamar et al. 2005).

1.6 Smad Anchor necessary for Receptor Activation (SARA)

R-Smad expression, activation (by phosphorylation) and stability are regulated and facilitated by adaptor proteins such as Smad anchor necessary for receptor activation (SARA). SARA contains a Smad-binding domain (SBD) where it interacts with Smad2 on its MH2 domain (Wu et al. 2000), and the carboxyl-terminal region of SARA interacts with the TGF β receptor complex (Figure 2; Tsukazaki et al., 1998). SARA regulates the subcellular localization of R-Smads and acts as a recruitment factor that presents R-Smads to be phosphorylated by the receptors. When R-Smads are phosphorylated, they dissociate from SARA, associate with the Co-Smad and translocate to the nucleus (Qin et al. 1996; Tsukazaki et al. 1998). SARA is also a zinc finger FYVE (Fab1, YOTB, Vac1 and EEA1) domain-containing protein that binds to phosphatidylinositol 3-phosphate (PI3P), which localizes SARA to PI3P-containing membranes. Early endosomes are enriched with PI3P; hence, SARA is highly localized in endocytic compartment. Although, SARA co-localizes with Smad2 and TGF β receptors at the plasma membrane, it is thought to concentrate all of the signaling components on the early endosome, thereby suggesting a trafficking component to TGF β signaling (Di Guglielmo et al. 2003; Hayes, Chawla, and Corvera 2002). Due to the FYVE domain, SARA was shown to

contain a punctate cellular staining whereas a mutant form of SARA that lacks a FYVE domain remained cytosolic (Tsukazaki et al. 1998; Wu et al. 2000). In addition, disruption of localization of SARA from early endosomes (identified by the presence of early endosome antigen-1, EEA-1) inhibits the nuclear localization of Smad2 induced by TGF β (Hayes et al., 2002). Furthermore, endosomes play an important role as signaling centers, where they regulate the SARA-Smad2/3 complex as well as the assembly of specific TGF β -dependent multi-protein transducer complexes (Corallino et al., 2015). Therefore, receptor endocytosis and trafficking is required for optimal TGF β signaling; SARA facilitates the activation of R-Smads and allow efficient Smad phosphorylation (Itoh et al. 1998; Tsukazaki et al. 1998).

1.7 Smurf2 and Smad7

In addition to R-Smads and co-Smads, inhibitory Smads (I-Smads), such as Smad6 and Smad7, are important in the regulation of TGF β signaling pathways (Hayashi et al. 1997; Kavsak et al. 2000). I-Smads block TGF β signaling by competing against R-Smads for the association with T β RI or by targeting receptor complexes for ubiquitin-mediated degradation (Hayashi et al. 1997; Kavsak et al. 2000). Smad7 suppresses TGF β ligand dependent signaling by preventing phosphorylation of Smad2; hence, the Smad2-Smad4 complex cannot be formed and is unable to accumulate in the nucleus (Hayashi et al. 1997). It also stably binds to activated T β RI and cannot be phosphorylated since it does not contain the C-terminal serine residue motif (SSXS); thus, preventing the interaction between the receptor and Smad2 as a competitive inhibitor (Macías-Silva et al. 1996). Smad7 functions at an early step in the TGF β signaling pathway to inhibit the

phosphorylated Smad2 function, as opposed to a negative feedback loop towards Smad2-mediated responses (Hayashi et al. 1997). Indeed, it translocates out of the nucleus and targets the receptors on the membrane when the TGF β pathway is activated (Suzuki et al. 2002). I-Smads also recruit Smad related E3 ubiquitin ligases (Smurfs) to catalyze the degradation of receptor complexes via their HECT (homology to E6AP carboxyl terminus) domain (Hayashi et al. 1997; Kavsak et al. 2000; Nakao et al. 1997). In addition, Smurf2 associates with inactivated R-Smads, through the PPXY motif, and regulate ubiquitin-mediated auto-degradation (Izzi and Attisano 2004; Kavsak et al. 2000).

Cells utilize the ubiquitin proteasome pathway for degradation of many cellular proteins (Hershko and Ciechanover 1998). E1, ubiquitin-activating enzymes, E2, ubiquitin-conjugating enzymes and E3, ubiquitin-protein ligases, are responsible for regulating the multi-enzyme ubiquitination cascade (Hershko and Ciechanover 1998). E3 activity allows for specificity in which proteins are targeted for degradation by recruiting substrates to the ubiquitination machinery. Smad7 acts as an adaptor protein to recruit Smurf2 to the TGF β receptor complex (Kavsak et al. 2000). Smurf2 requires Smad7 for efficient interaction and to mediate the degradation of TGF β receptors whereas Smad7 associates with T β RI and binds directly to Smurf2 (Kavsak et al. 2000). Mutants that disrupt the binding of Smad7 to Smurf2 interfere with Smurf2 association with the receptor (Kavsak et al. 2000).

It is suggested that Smurf2 and Smad7 cooperation is important when Smad7 expression is low (Kavsak et al. 2000; Nakao et al. 1997). As TGF β signaling occurs, Smad7-Smurf2 may function to mediate rapid degradation of TGF β receptors as a negative

feedback loop; therefore, Smurf2 resets the Smad pathway by removing Smad7-bound receptor complex (Hanyu et al. 2001; Kavsak et al. 2000). When the TGF β receptor is not available as a target for ubiquitination, Smad7 functions to degrade Smurf2 via auto-ubiquitination to control the resting state levels of Smurf2 (Hanyu et al. 2001). It was previously reported that the amino-terminal domain (NTD) of Smad7, which is necessary for the regulation of catalytic activity of Smurf2 at the level of E2 recognition, and MH2 domain interact with each other – preventing MH2 to interact with TGF β receptors (Hanyu et al. 2001). The Smurf2 HECT domain interacts with the Smad7 NTD to liberate the MH2 domain, thereby promoting the interaction between TGF β receptors and Smad7 (Kavsak et al. 2000). Hence, Smurf2 and Smad7 are dependent on each other to maximally inhibit the activity of TGF β receptors (Kavsak et al. 2000).

1.8 Two distinct TGF β endocytic pathways

In metazoan cells, endocytic membrane trafficking is an important process to ensure proper cell function, which includes delivering membrane components, receptor-associated ligands and solute molecules to intracellular destination; regulation of signal transduction and neurotransmission, and modulation of the composition of the plasma membrane (Arias, Siri, and Conde 2015; Conner and Schmid 2003; Mayor and Pagano 2007).

Cell surface proteins internalize from the plasma membrane via several pathways, including clathrin-mediated endocytosis, lipid-raft mediated endocytosis, micropinocytosis and macropinocytosis (Gruenberg 2001). TGF β receptors have been postulated to use two different internalization pathways: the clathrin-dependent pathway

and the caveolin-1-lipid raft-dependent pathway (Di Guglielmo et al. 2003).

Internalization into different endocytic compartments leads to Smad association with different proteins and distinct regulation of TGF β signaling. Differential composition of lipid bilayer in the two compartments is responsible for the separation of Smad signaling components in the two pathways (Di Guglielmo et al. 2003). In the clathrin-dependent pathway, SARA anchors Smad2 to the receptors on endosomal membrane and facilitates Smad2 phosphorylation (Penheiter et al. 2002; Roy and Wrana 2005; Sorkin and von Zastrow 2010). As mentioned before, SARA localization on the endosome is dependent on its interaction of FYVE domain with PI3P (Aasland 1996; Gaullier et al. 1998; Gaullier and Simonsen 1998; Hayakawa et al. 2004; Tsukazaki et al. 1998). However, Lu et al. (2002) has proposed that the clathrin-dependent pathway may not be essential for TGF β signaling by demonstrating an insignificant change in the TGF β signaling when the clathrin-pathway in HeLa cell was inhibited. Rather, Di Guglielmo et al. (2003) suggested that the function of the clathrin-dependent pathway is to sequester receptors away from the membrane rafts and caveolae, which can inhibit receptors by the binding of caveolin-1 to T β RI or through Smad7-Smurf2 ubiquitination (Razani, Zhang, Bitzer, Gersdorff, et al. 2001; Di Guglielmo et al. 2003)). In addition, potassium depletion, cytosol acidification or hypertonic buffer treatments dampened clathrin-dependent endocytosis and TGF β receptors were directed to caveolae at the plasma membrane or caveosomes in the cytoplasm (Di Guglielmo et al. 2003; Razani, Zhang, Bitzer, Von Gersdorff, et al. 2001; Zuo and Chen 2009). Therefore, endocytosis can occur independently of clathrin through membrane rafts (Henley et al. 1998; Sharma, Sabharanjak, and Mayor 2002). In the caveolin/ membrane raft-dependent pathway, the

cholesterol and sphingolipid-rich composition of membrane rafts preferentially associate with Smad7-Smurf2 complexes leading to degradation of the receptor complex (Di Guglielmo et al., 2003). When the expression of caveolin-1 was induced in HEK293T cells, TGF β receptors were down-regulated and this degradation was enhanced when Caveolin-1 was co-expressed with Smurf2 and Smad7 (Di Guglielmo et al. 2003). The two internalization pathways seem to have a delicate balance where disruption of one pathway may encourage receptor partitioning into the other pathway. When SARA is co-expressed with Smad7 and Smurf2, it stabilized receptor levels; however, SARA lacking its FYVE domain did not protect TGF β receptors from Smurf2-Smad7-mediated degradation (Di Guglielmo et al. 2003). Furthermore, Di Guglielmo et al. (2003) incubated Mv1Lu cells in media lacking potassium chloride in order to inhibit receptor endocytosis by blocking clathrin-coated pit formation. As a result, receptor-dependent Smad2 phosphorylation was suppressed. However, co-treatment with Nystatin, cholesterol-sequestering drug, restored phosphorylation by inhibiting caveolin-1-lipid dependent pathway (Di Guglielmo et al. 2003) (Figure 4).

Although the two internalization pathways are separately located at the plasma membrane, the two endocytic pathways may not be functionally separated post endocytosis. As clathrin-coated pits pinch off of the plasma membrane, vesicles lose their clathrin coats (Abrami et al, 2003; Massol et al., 2006; Rappoport et al., 2004). This presents the exposed uncovered lipid membranes to adjacent caveolar vesicles causing a partial fusion. Through the guidance of Rab5, caveolar and clathrin-coated vesicles move to the early endosome, forming the multifunctional caveolin-1-positive early endosomes

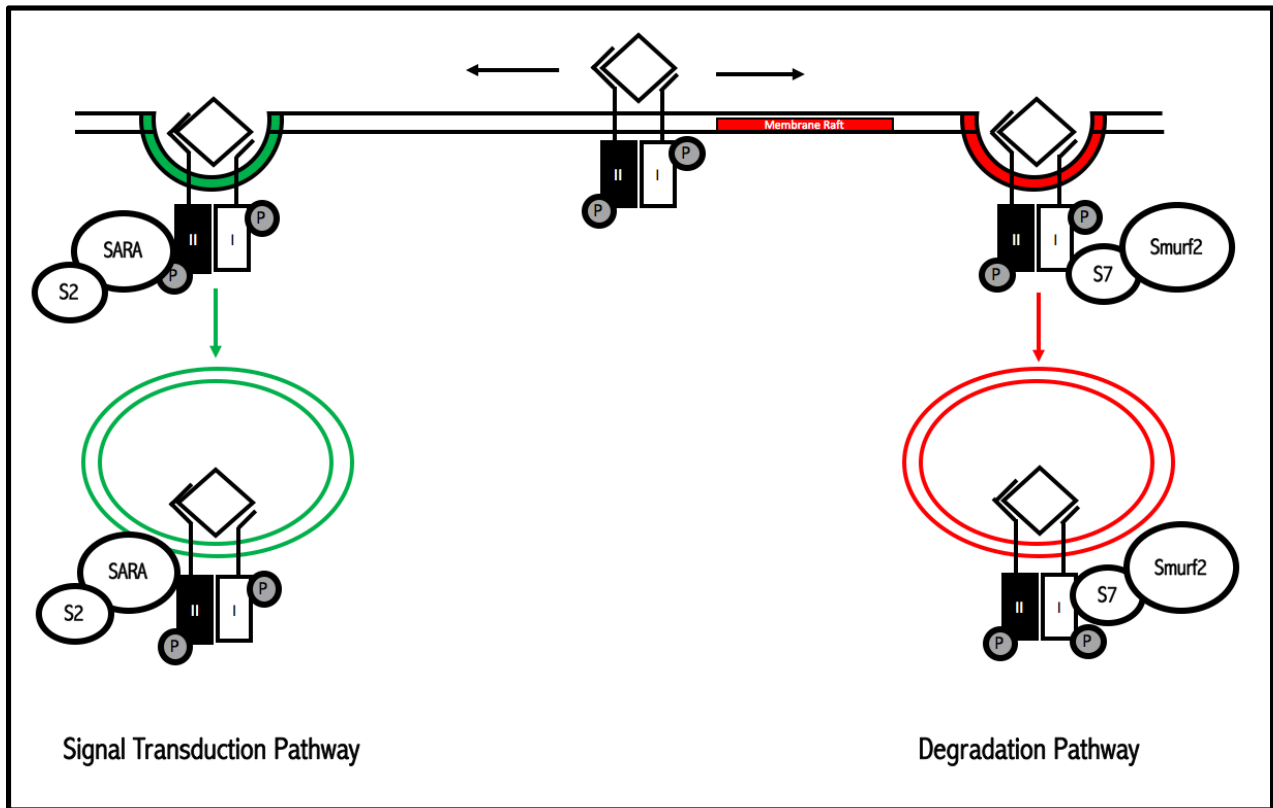


Figure 4. Two distinct internalization pathways for TGFβ receptors.

In the clathrin-dependent pathway, SARA anchors Smad2 to the receptors on the endosome and facilitates Smad activation. The function of clathrin-dependent pathway is to sequester receptors away from the rafts and caveolin, which can directly inhibit the receptors by binding of caveolin-1 to TβRI or through Smad7-Smurf2 ubiquitination. In the caveolin raft-dependent pathway, cholesterol and sphingolipid-rich composition of lipid rafts preferentially associate with Smad7-Smurf2 complexes leading to degradation of the receptor complex. The two internalization pathways seem to have a delicate balance where disruption of one pathway may push the receptors into the other pathway.

(He et al. 2015). These multifunctional endosomes have been proposed to contain EEA1, caveolin-1, Rab5, T β RI, SARA, and Smad7/Smurf2, thereby, allowing TGF β receptors to access different molecules to efficiently promote TGF β signaling and/or degradation (He et al. 2015).

1.9 Rationale and Hypothesis

Although the individual proteins involved in TGF β signaling have been studied, research in the interaction between the key proteins in receptor trafficking remains unclear. For e.g. the interaction between SARA and Smurf2 has not been assessed. SARA and Smurf2 are involved in distinct pathways that lead to different receptor signaling outcomes; however, whether they associate with each other by utilizing the same TGF β receptor complex is unknown. This allows the possibility of the interaction between the two proteins, directly or indirectly. He et al., (2015) observed a direct fusion of clathrin-coated and caveolin vesicles during TGF β receptor endocytic trafficking. Clathrin-coated vesicles and caveolin vesicles fused upon internalization and formed a multifunctional sorting compartment (He et al. 2015) (Figure 5). However, the study did not address the exact location or dynamics of each protein in the fused vesicle. Therefore, the Smurf2-Smad7 complex may affect SARA steady state levels and influence TGF β signaling.

Based on this rationale, I hypothesized that Smurf2 and Smad7 induce SARA degradation through a ubiquitin-dependent pathway.

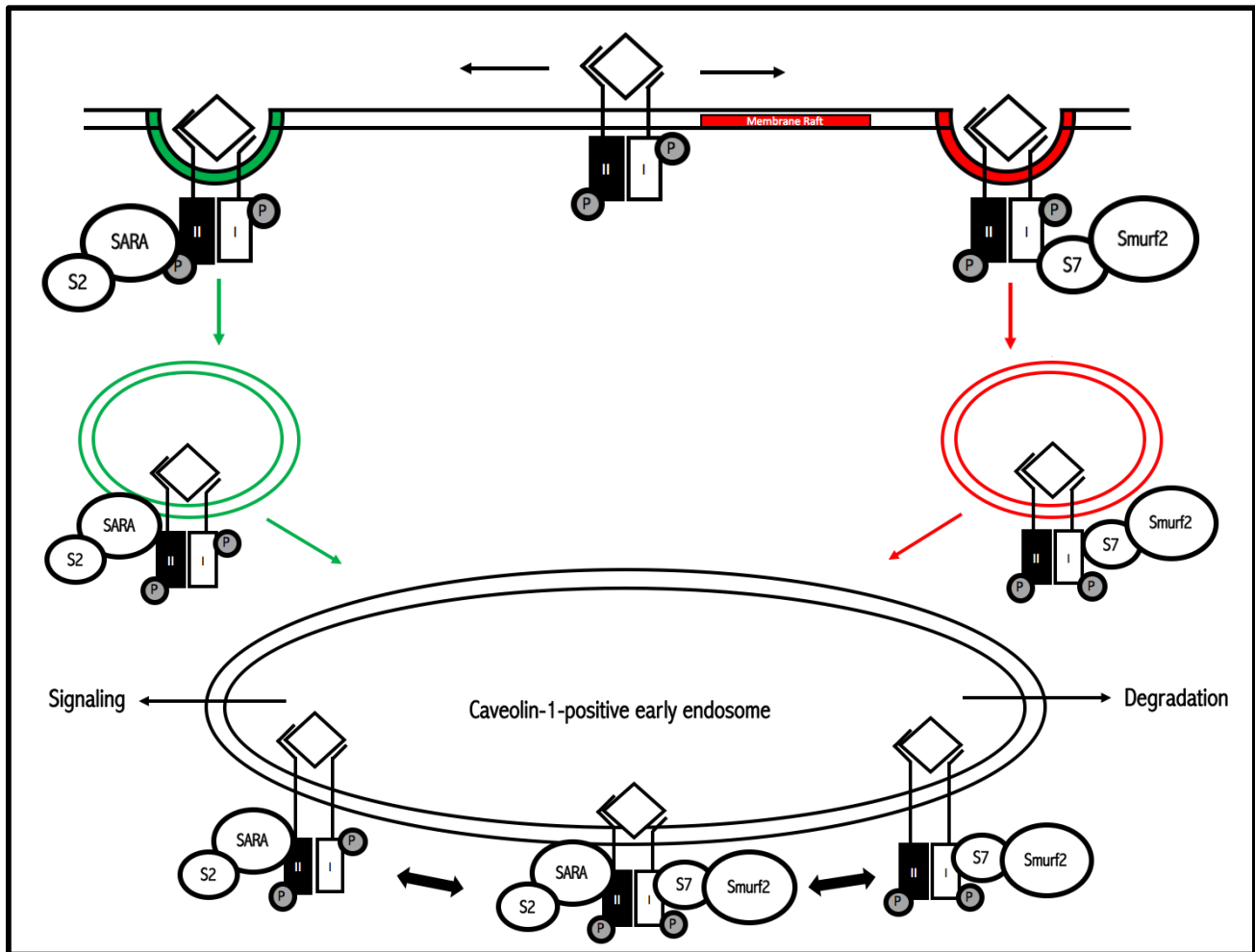


Figure 5. The caveolin-1 positive early endosome.

A direct fusion of clathrin-coated and caveolin vesicles during TGF β receptor endocytic trafficking was observed by He et al., 2015. Before the vesicles reach the early endosome, clathrin-coated vesicles and caveolin vesicles fused upon internalization and formed a multifunctional sorting device, caveolin-1-positive early endosomes, for TGF β receptors. It is in these caveolin-1 positive endosomes where it is postulated that the interaction between TGF β receptors and either signaling or degradation proteins occur. This introduces the possibility that proteins involved in TGF β signaling (for e.g., SARA) can interact and be influenced by degradation proteins (for e.g., Smurf2-Smad7) through the association with common receptors.

Based on the hypothesis state above, I attempted to address the following specific aims:

- 1) To determine whether SARA steady state levels are affected by Smurf2 and Smad7.
- 2) To determine whether Smurf2-Smad7-mediated degradation of SARA is dependent on an ubiquitin-degradation pathway.

2 Materials and Methods

2.1 Antibodies and Reagents

Primary antibodies were purchased from following vendors: anti- β -Actin (Sigma A2668), anti-Phospho-Smad2 (Cell Signaling, 3101), anti-Smad2/3 (BD, 610843), anti-SARA (Santa Cruz, sc-9135), anti-Flag (Sigma, F3165), anti-Smurf2 (Santa Cruz, sc-25511 and Santa Cruz, sc-393858), anti-Myc (Aves, ET-MY 100), anti-Smad7 (Santa Cruz, sc-7004 and Santa Cruz, sc-365846), anti-HA (Santa Cruz, sc-805), anti-EEA1 (BD, 610457), anti-Cav1 (Cell Signaling, 3238X), anti-E-cadherin (BD, 610182), anti-N-cadherin (BD, 610921), anti-Ubiquitin (Cell Signaling, 3936), HRP conjugated secondary goat-anti-rabbit (Thermo Scientific -31460), goat-anti-mouse (Thermo Scientific -31430) and donkey-anti-goat (Santa Cruz, sc-2020) were used for immunoblot analysis.

Fluorescently conjugated donkey α -mouse (Life Technologies, A21206), donkey α -rabbit (Life Technologies, A31572), and A555 conjugated Phalloidin (Invitrogen, A34055) were used for immunofluorescence studies. Protein G-Sepharose was purchased from GE Healthcare (17-0618-01).

siRNAs were purchased from following vendors: Silencer® Select Negative Control #1 siRNA (Ambion, 4390844), Silencer® Select siRNA to ZFYVE9 (Ambion, 4392420 ID: s17933), Silencer® Select siRNA to SMURF2 (Ambion, 4392420 ID: s34859), Silencer® Select siRNA to SMAD7 (Ambion, 4392420, ID: s8414), Smad7 siRNA (h) (Santa Cruz, sc-36508) and ON-TARGETplus SMARTpool for Human SMAD7

(Dharmacon™, L-020068-00). Shown in Table 1, is the list of antibodies and its dilutions used for immunoblotting and IF.

2.2 Cell Culture and Transfection

Human embryonic kidney cells 293 transformed with large T antigen (HEK293T) were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). A549 non-small cell lung cancer (NSCLC) cell lines were maintained in F12K with 10% FBS. Cells were kept in a tissue culture incubator at 37°C at 5% CO₂.

HEK293T cells were transfected at approximately 70% confluency using the calcium phosphate transfection method. Briefly, a mixture of water, cDNA constructs and 10% 2.5 M calcium chloride (CaCl₂) was mixed with 600 µl of 2X HEPES Buffered Saline (12 mM Dextrose, 50 mM HEPES, 10 mM KCl, 280 mM NaCl, 1.5 mM Na₂HPO₄•H₂O) and incubated for 20 minutes. Dropwise, the mixed transfection solution was added to the cells and incubated for 48 hours. Total of 3.5 µg of DNA constructs for 6-well dishes; total of 16.5 of µg of DNA constructs for 10 cm plates; pCMV5, an empty vector, was transfected to maintain the same concentration of DNA constructs throughout the experiment. For A549 cells, PolyJet™ DNA InVitro Transfection Reagent (FroggaBio) was used to transfect following the manufacturer's protocol. siRNA transfections were performed using Lipofectamine RNAi max (Invitrogen) according to the manufacturer's protocol.

Table 1. Antibodies and Dilutions

Antibody	Source	Catalogue No.	Dilution^a
β-actin	Sigma	A2668	1:1000 (IB)
Phospho-Smad2	Cell Signaling	3101	1:1000 (IB)
Smad2/3	BD	610843	1:1000 (IB)
SARA	Santa Cruz	sc-9135	1:1000 (IB); 1:100 (IF)
Flag	Sigma	F3165	1:1000 (IB) 1:100 (IF)
Myc	Aves	ET-MY 100	1:1000 (IB) 1:100 (IF)
Smurf2	Santa Cruz	sc-25511	1:1000 (IB); 1:100 (IF)
Smad7	Santa Cruz	sc-7004 sc-393858	1:1000 (IB); 1:100 (IF)
HA	Santa Cruz	sc-805	1:1000 (IB)
EEA1	BD	610457	1:100 (IF)
Cav1	Cell Signaling	3238X	1:100 (IF)
E-cadherin	BD	610182	1:1000 (IB); 1:100 (IF)
N-cadherin	BD	610921	1:1000 (IB); 1:100 (IF)
Ubiquitin	Cell Signaling	3936	1:1000 (IB)
HRP conjugated secondary goat-anti-rabbit	Thermo Scientific	31460	1:25000 (IB)
HRP conjugated secondary goat-anti-mouse	Thermo Scientific	31430	1:25000 (IB)

HRP conjugated donkey-anti-goat	Santa Cruz	sc-2020	1:25000 (IB)
Fluorescently conjugated donkey anti-mouse (488 nm)	Life Technologies	A21206	1:250 (IF)
Fluorescently conjugated donkey anti-rabbit (647 nm)	Life Technologies	A31572	1:250 (IF)
Fluorescently conjugated donkey anti-goat (555 nm)	Invitrogen	A21432	1:250 (IF)
A555 conjugated Phalloidin	Invitrogen	A34055	1:100 (IF)

- a. IF = Immunofluorescence
IB = Immunoblot

2.3 DH5 α transformation

cDNA constructs for flag-tagged SARA, myc-tagged Smurf2 WT and C716A, HA-tagged Smad7 and HA-tagged Ubiquitin were obtained from Di Guglielmo's lab. The constructs are in pCMV5 vector, antibiotic resistance to ampicillin. DH5 α transformation was utilized to amplify the cDNA constructs of interest. Firstly, 25 μ l DH5 α in tubes were thawed out on ice and added 1 μ l DNA of interest and incubated on ice for 15 minutes. Next, the cells were heat shocked in 42°C for 1 minute and back on ice for 2 minutes. Then, 500 μ l of lysogeny broth (LB) was added to the cells and placed in a shaker at 250 rpm at 37°C for 15 minutes. Next, the tube was centrifuged at 14000 rpm to obtain the pellet. Supernatant was removed and the pellet was re-suspended in 25 μ l of LB and the re-suspended cells were spread onto LB-agar plate with ampicillin (50 μ g/ml) and incubated overnight at 37°C. The following day, a colony was picked from the LB

plate and incubated in 150 mL of LB with 50 µg/ml of ampicillin overnight. To isolate plasmid DNA, PureYield™ Plasma Midiprep System (Promega) was used and assessed the purity of DNA using spectrophotometer, accepting the ratio of 1.8 at absorbance of 260 nm/280 nm.

2.4 Immunoblotting

Cells were lysed (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors, PMSF and Pepstatin A) and gently scraped using a scraper and collected in a 1.5 ml Eppendorf tube and centrifuged at 14,000 rpm at 4°C for 10 min. Twenty microliters of supernatants were collected for protein concentration assay which was determined using the Lowry method (Fisher). Remaining supernatant were recollected in another 1.5 ml Eppendorf tube and 8x Sample prep buffer (2.5 ml 1 M Tris-HCl, 0.5 ml of ddH₂O, 1.0g SDS, 0.8 ml 0.1% Bromophenol Blue, 4 ml 100% glycerol, 2 ml 14.3 β-mercaptoethanol, and adjust it to 10 ml with ddH₂O to make 4x stock) was added.

2.5 Immunoprecipitation

For immunoprecipitation, cell lysates were incubated with primary antibody overnight at 4°C. Next, the lysates were incubated with 10% slurry of protein G-Sepharose beads (Amersham) for an hour. The precipitates were collected and washed three times with wash buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl), and eluted with 2x sample prep buffer. Cell lysates were heated at 90°C for 5 minutes and then resolved by denaturing 10% polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, proteins were

transferred to nitrocellulose followed by blocking with with 5% skim milk diluted in Tris-buffered saline supplemented with 0.05% Tween-20 (TBST, 100 mM Tris-HCl pH 7.5, 0.9% NaCl) for 45 minutes at room temperature. Then, blots were incubated in primary antibody in TBST overnight at 4°C. The membranes were then washed three times for 10 minutes which was followed by 1-hour incubation with HRP conjugated secondary antibody. Again, the membranes were washed three times for 10 minutes. Proteins were then visualized using West Dura Super Signal ECL (Fisher) hand imaged on a VersaDoc Imaging System (BioRad). All results presented are a representative of 3-4 biological replicates. To quantify the protein levels in the immunoblots, equal sized rectangles were drawn around the protein bands of interest on scanned images using QuantityOne (BioRad). Background area and the pixel density of each protein bands were recorded; the densitometric value of each lane was obtained by subtracting the background values.

2.6 Immunofluorescence Microscopy

Glass cover slips were autoclaved to be sterilized. In order to plate HEK293T cells, glass cover slips were coated with Poly L Lysine (PLL). PLL was diluted in sterile PBS at 1:20 and each well was coated with 1 mL of diluted PLL. After 2-3 hours, the wells were rinsed with PBS and media and cells were plated onto a cover slip at approximately 60% confluency. After transfection with the cDNA constructs described in the figures, the cells were then fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were washed three times using 1X PBS and then permeabilized with 0.25% Triton X-100 in ddH₂O for 5 minutes at room temperature. Cells were again washed three times

using 1X PBS and then were blocked in 1X PBS containing 10% FBS for one hour at room temperature. Cells were incubated with the following antibodies at 4°C overnight with gentle rocking: anti-Smad2/3, anti-EEA1, anti-Cav1, anti-SARA, anti-Smurf2 and anti-Smad7. Next, cells were washed with 1X PBS three times and then were incubated with appropriate Cy-conjugated secondary antibodies for one hour at room temperature with gentle rocking, protected from the light. Cells were washed three times again with 1X PBS and incubated with 1 mg/ml DAPI (in ddH₂O) to visualize nucleus for 5 minutes protected from the light, with gentle rocking. Following a wash with 1X PBS, the glass cover slips were mounted onto microscope slides, using Immuno-mount mounting medium. Samples were dried overnight at room temperature in the dark before analysis. Finally, cells were visualized by immunofluorescence microscopy using an inverted IX81 Microscope (Olympus, Canada).

2.7 Phospho-Smad Signaling Assays

A549 cells were plated at approximately 70% confluency. Then, cells were incubated in serum-deprived F12K media (0.2% FBS) overnight. Then, cells were washed with sterile 1X PBS and were treated with either 0 pM, 10 pM, or 100 pM TGFβ in serum-deprived F12K media for 45 minutes. Cells were lysed and were processed for SDS-PAGE, and immunoblotted for Phospho-Smad2 and total Smad2 levels.

2.8 Epithelial to Mesenchymal Transition

A549 cells were plated at approximately 60% confluency. A549 cells were transfected with either control or ZFYVE9 (SARA) directed siRNA for 24 hours using PolyJet. Cells

were then serum starved using serum-deprived F12K media (0.2% FBS) overnight prior to TGF β treatment. Next, cells were washed with sterile 1X PBS and then incubated in serum free media containing 0 pM, 10 pM, or 100 pM TGF β for 0, 24 or 48 hours to induce EMT. Cells were then lysed and processed for SDS-PAGE to assess E-cadherin loss and increase of N-cadherin.

2.9 Statistical Analysis

One-way or Two-way ANOVA analysis followed by post-hoc Bonferroni's Tests were used to determine the significance of the results. Statistical analyses were performed using GraphPad Prism® Software 5.0 and p-values of < 0.05 were considered statistically significant. All experiments were conducted a minimum of 3 times.

3 Results

3.1 The effect on Smurf2-Smad7 on SARA steady state levels

SARA and Smad7-Smurf2 play key roles in two separate endocytic pathways. SARA acts as an anchor for Smad2 on the endosomal membrane and presents Smad2 to TGF β receptors during their endocytosis, which in turn stabilizes and facilitates R-Smad phosphorylation (Di Fiore & De Camilli, 2001; McPherson et al., 2001). The I-Smad, Smad7 in co-operation with Smurf2, facilitate the degradation of TGF β receptors (Kavsak et al. 2000). Smad7 acts as an adaptor protein to recruit the E3 ubiquitin ligase, Smurf2, which mediates the ubiquitination and degradation of TGF β receptor (Kavsak et al. 2000). Although the two pathways are separately located at the plasma membrane, after internalization, the two endocytic pathways are not functionally separated (Roy and Wrana 2005). He et al., 2015 showed that a partial fusion between clathrin coated vesicles and caveolar vesicles forming a multifunction endosome that contain many proteins involved in the TGF β pathway, including SARA, Smad7, and Smurf2. Since there is a potential interaction of SARA and Smurf2-Smad7 with common TGF β receptors, there is the possibility that SARA and Smurf2-Smad7 may affect each other's protein levels. In order to study this mechanistically, I first assessed the ectopic co-expression of SARA and Smurf2-Smad7 in HEK293T cells.

HEK293T cells, expressing flag-tagged SARA, myc-tagged Smurf2 or HA-tagged Smad7 separately (Figure 6) or in combination (Figure 7) were assessed by

immunofluorescence microscopy. When the proteins were expressed on their own, only the proteins of interest were observed in the analysis, indicating the specificity of the antibodies used (Figure 6). In the co-transfected cell analysis, $77\% \pm 19\%$ of the cells expressed all three proteins (Figure 7). Therefore, when multiple constructs were co-transfected into HEK293T cells, the majority of the cells overexpressed all three proteins of interest.

Having ascertained that all three proteins are co-expressed in the majority of cells, I next assessed if the expression of Smad7 and Smurf2 would have an effect on the steady state levels of expressed SARA. In order to do this, HEK293T cells expressing different combinations of flag-SARA, wild type (WT) myc-Smurf2, or a Smurf2 mutant that lacked a functional HECT E3 ubiquitin ligase domain (myc-Smurf2 C716A), as well as Smad7-HA were assessed by immunoblotting (Figure 8). When SARA was expressed alone or co-expressed with only Smurf2 WT, Smurf2 C716A mutant or Smad7, SARA steady state levels did not significantly change (Figure 8A, lane 6-8; Figure 8B).

However, in the presence of WT Smurf2 and Smad7, the steady state levels of SARA were greatly decreased (Figure 8A, lanes 2 vs. 11). Interestingly, in the presence of the E3 ligase mutant of Smurf2 (C716A) and Smad7, SARA steady state levels were affected to a lesser degree (Figure 8A, lanes 11 vs. 12). The steady state levels of SARA were quantitated to decrease significantly only in the presence of WT Smurf2 and Smad7 by $82\% \pm 13\%$ (Figure 8B). In addition, this decrease was dependent on the ubiquitin

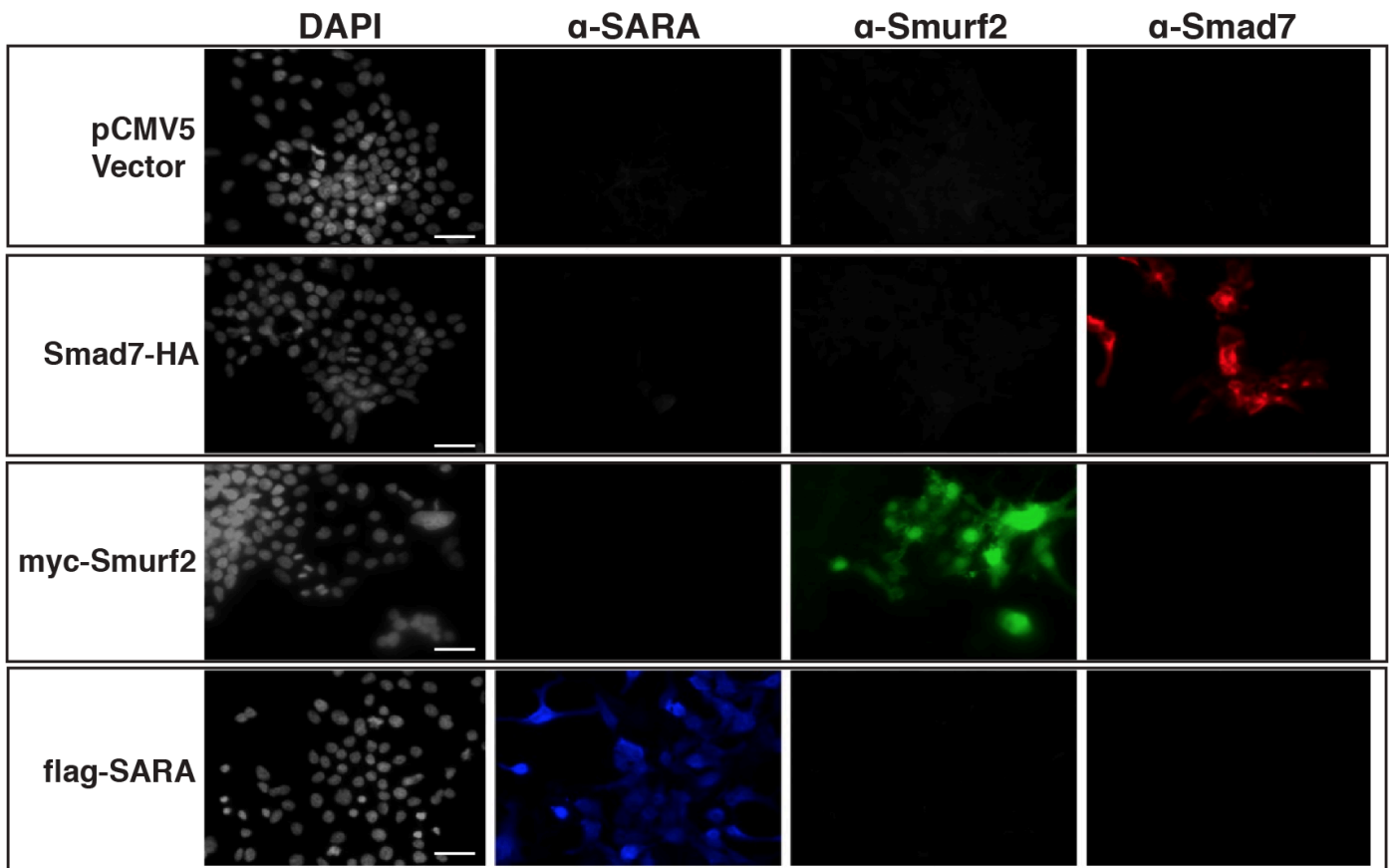


Figure 6. Exogenous expression of SARA, Smurf2 or Smad7 in HEK293T cells.

HEK293T cells were transiently transfected with cDNA encoding flag-SARA, myc-Smurf2 or Smad7-HA. The cells were then fixed, permeabilized and immunostained with anti-SARA rabbit (blue), anti-Smurf2 mouse (green) or anti-Smad7 goat (red) antibodies, conjugated with donkey-anti-rabbit, donkey-anti-mouse and donkey-anti-goat secondary antibodies respectively. The nuclei were visualized using DAPI stain and are shown in white. Shown here are representative immunofluorescence images taken with an Olympus IX81 microscope at 40x magnification (n=3, bar = 30 μ m)

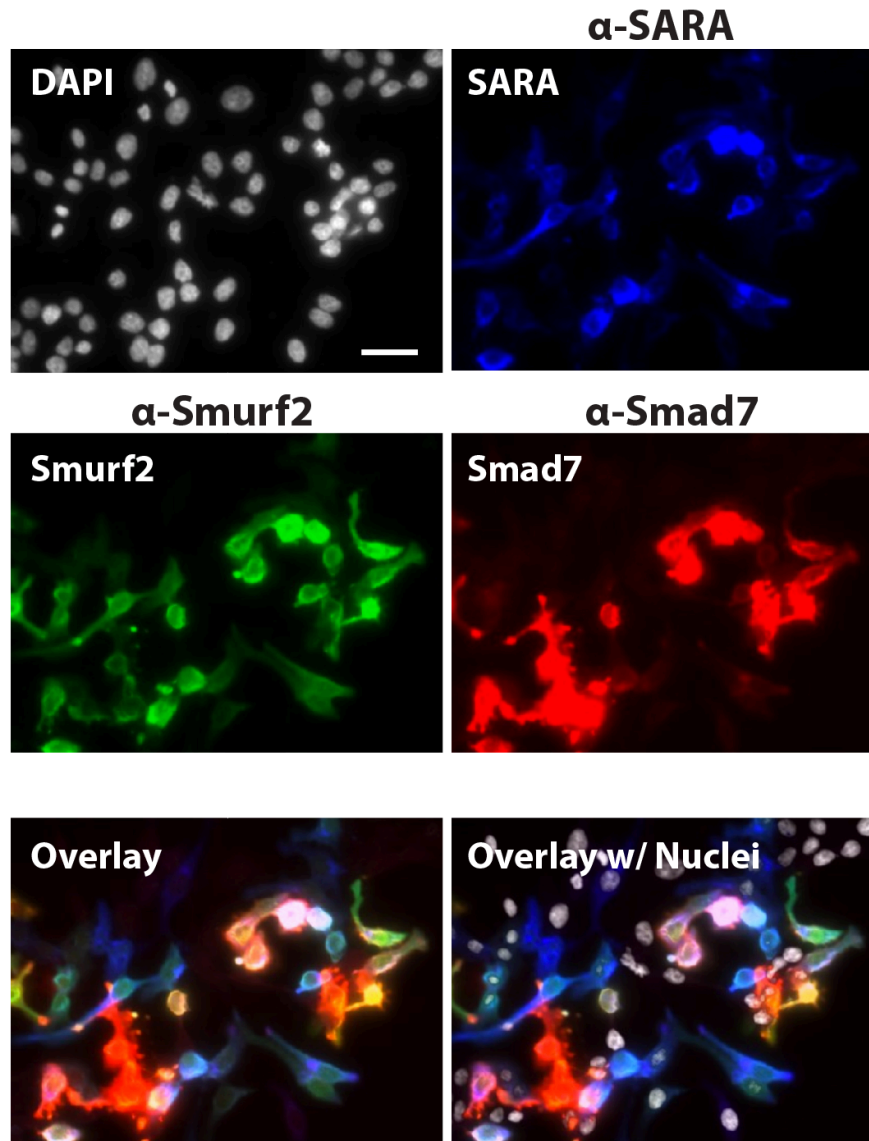


Figure 7. Co-expression of SARA, Smurf2 and Smad7.

HEK293T cells were transiently transfected with cDNA encoding flag-SARA, myc-Smurf2 and Smad7-HA. The cells were then fixed, permeabilized and immunostained with anti-SARA rabbit (blue), anti-Smurf2 mouse (green) or anti-Smad7 goat (red) antibodies, conjugated with donkey-anti-rabbit, donkey-anti-mouse and donkey-anti-goat secondary antibodies respectively. Transfected cells were counted at ten different fields of views. Shown here are representative immunofluorescence images taken with an Olympus IX81 microscope at 40x magnification. Bar = 30 μ m

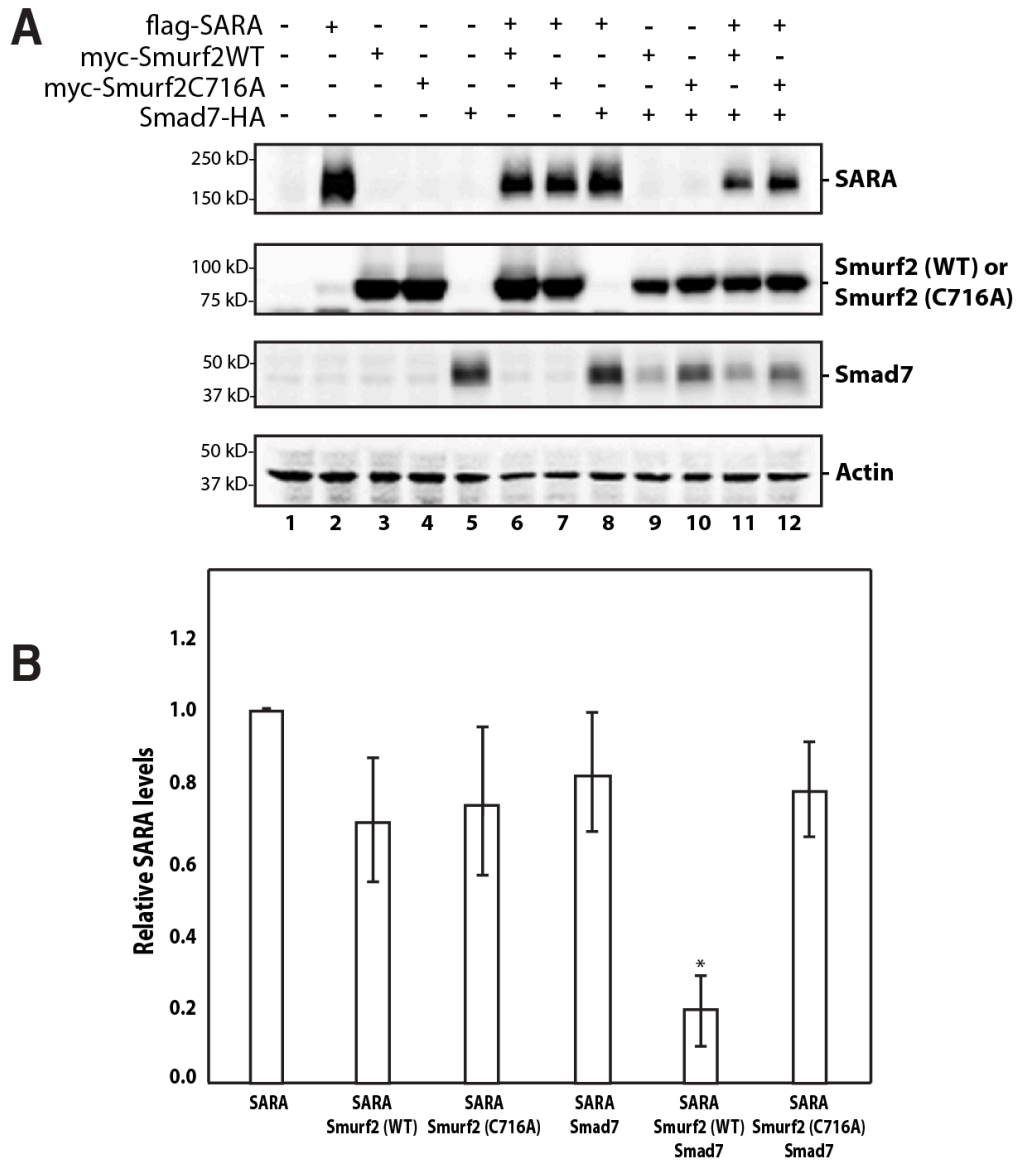


Figure 8. Effect of Smurf2/Smad7 on SARA steady state levels.

A) HEK293T cells were transfected with cDNA constructs encoding flag-tagged SARA, myc-tagged WT Smurf2, or Smurf2 containing a HECT domain mutation (C716A) or HA-tagged Smad7 as indicated at the top of the panel. Cell lysates were then subjected to SDS-PAGE followed by immunoblotting with anti-SARA, anti-Smurf2 or anti-Smad7 antibodies. The lysates were also immunoblotted with anti- β actin antibodies (equal protein loading control). The relative migration of each overexpressed protein, compared to the molecular mass markers (shown on the left) is indicated.

B. Three separate experiments were carried out as described in panel A and the relative levels of expressed SARA were quantitated using QuantityOne software (BioRad) and expressed as the mean \pm SD (n=3). The asterisk (*) indicates $p < 0.05$ relative to the level of SARA when SARA is transfected alone (One-way ANOVA).

ligase activity of Smurf2. When SARA was co-expressed with Smad7 and Smurf2 C716A, a mutant of Smurf2 where the ligase activity is inactive, the SARA steady state levels were significantly higher than SARA steady state levels in the presence of WT and Smad7 (Figure 8B).

Taken together, our results suggest that Smurf2 WT and Smad7 function cooperatively to decrease the steady state levels of SARA and that the ligase activity of Smurf2 may play an important role in this process. Having observed that Smurf2 WT and Smad7 influence the steady state levels of over-expressed SARA levels, I next assessed if this could be observed endogenously.

3.2 Relative levels of SARA, Smurf2, Smad7 when SARA or Smurf2 are silenced

In order to determine whether Smurf2 influences the steady state levels of SARA endogenously, I utilized siRNA to silence proteins of interest. A549 cells were transfected with siRNAs via Lipofectamine[®] RNAiMAX and assessed by immunoblotting. Increasing concentrations (0-37.5 nM) of SARA siRNA or Smurf2 siRNA were utilized to silence SARA and Smurf2 respectively (Figure 9). When SARA was silenced, the endogenous steady state levels of Smurf2 and Smad7 did not change. Also, endogenous steady state levels of SARA and Smad7 did not change when Smurf2 was silenced. Together, our results indicate that silencing SARA does not affect the endogenous level of Smurf2 and Smad7; more interestingly, silencing Smurf2 alone does not influence the endogenous level of SARA. This suggests that in terms of Smurf2-dependent effect on SARA, silencing Smurf2 alone does not affect SARA. Smad7 is the

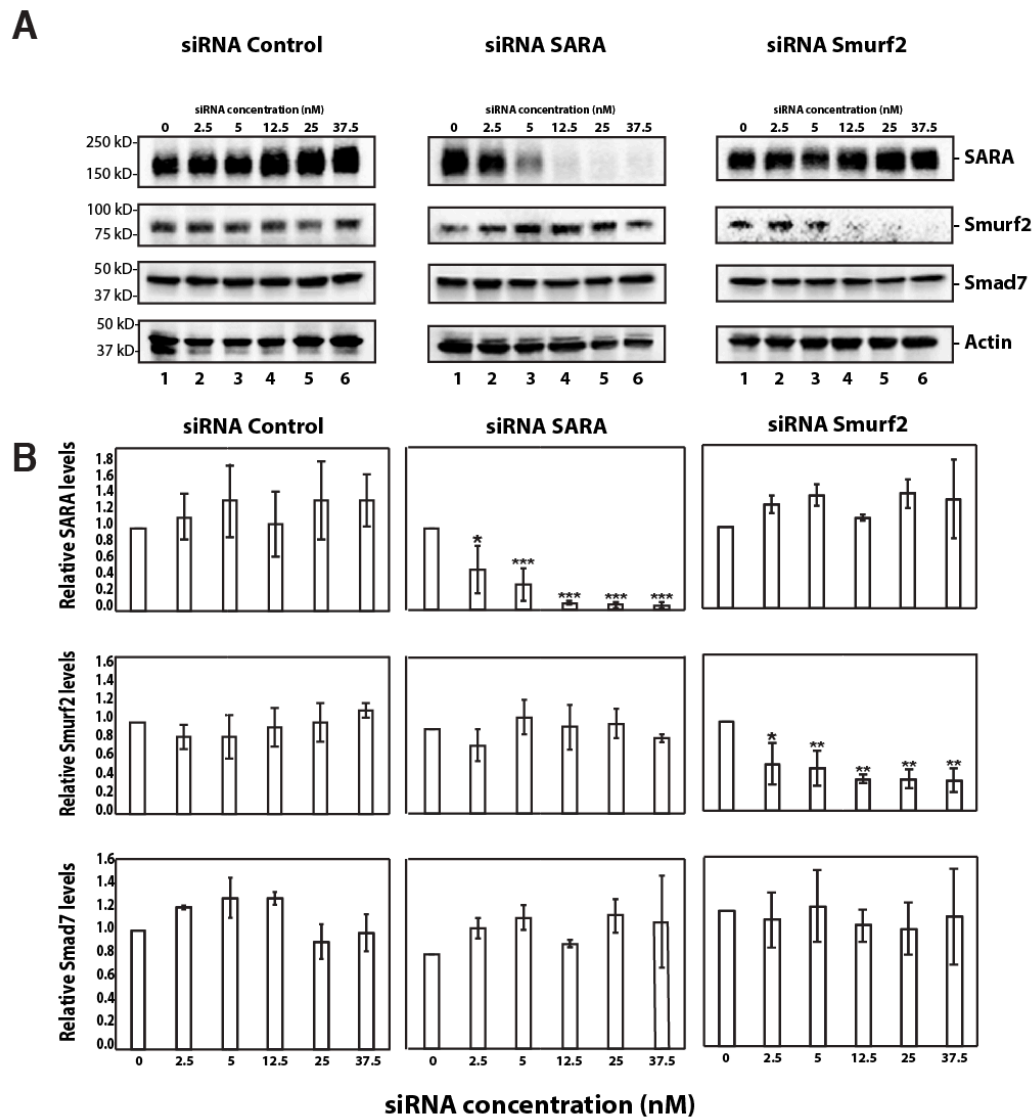


Figure 9. Relative levels of SARA, Smurf2 and Smad7 when SARA, Smurf2 are silenced.

A) A549 cells were transfected with a scrambled (control) siRNA or siRNA targeting SARA or Smurf2. Cell lysates were then subjected to SDS-PAGE followed by immunoblotting with anti-SARA, anti-Smurf2, anti-Smad7 antibodies. The lysates were also immunoblotted with anti- β -antibodies (equal protein loading control). The relative migration of each protein, compared to the molecular mass markers (shown on the left) is indicated.

B) Three separate experiments were carried out as described in panel A and the relative levels of endogenous SARA, Smurf2 and Smad7 were quantitated using QuantityOne software (BioRad) and expressed as the mean \pm SD (n=3) and asterisk (*) indicates $p < 0.05$, ** indicate $p < 0.01$, and *** indicate $p < 0.001$ relative to the level of SARA, Smurf2 and Smad in the control (One-way ANOVA).

adaptor protein that acts as the bridge for Smurf2 to associate with TGF β receptor. It is possible that silencing of Smad7 in combination with Smurf2 is necessary to alter the endogenous steady state levels of SARA. Unfortunately, in the analysis, I was unable to silence Smad7, despite using different siRNAs from various companies (including Ambion, 4392420, ID: s8414; Santa Cruz, sc-36508 and Dharmacon™, L-020068-00; data not shown). Future studies using different approaches may be necessary to address this limitation (please see discussion for details).

Having ascertained that overexpressed Smurf2 and Smad7 reduce the steady state levels of SARA, I next wanted to determine whether SARA and Smurf2 could physically interact with each other.

3.3 Immunoprecipitation of Smurf2 and associated proteins

Based on the overexpression studies described above, Smurf2 and Smad7 cooperatively decrease the steady state levels of SARA (Figure 8). It is possible that the three proteins can associate directly with each other, or through a common bridge such as the TGF β receptor complex. In order to address if SARA and Smurf2-Smad7 form a complex, I carried out co-immunoprecipitation analysis. Briefly, HEK293T cells expressing flag-SARA, myc-Smurf2 WT or myc-Smurf2 C716A and Smad7 were immunoprecipitated with α -myc antibodies to immunoprecipitate for Smurf2 and immunoblotted for Smurf2, SARA and Smad7 (Figure 10). I observed that Smad7 co-precipitated with both the WT and C716A mutant of Smurf2 (Figure 10, Lanes 3-6); however, SARA was not present in any of the immunoprecipitations. These results suggest that SARA and Smurf2 do not form a stable interaction. Although Smurf2 and SARA do not form a stable interaction,

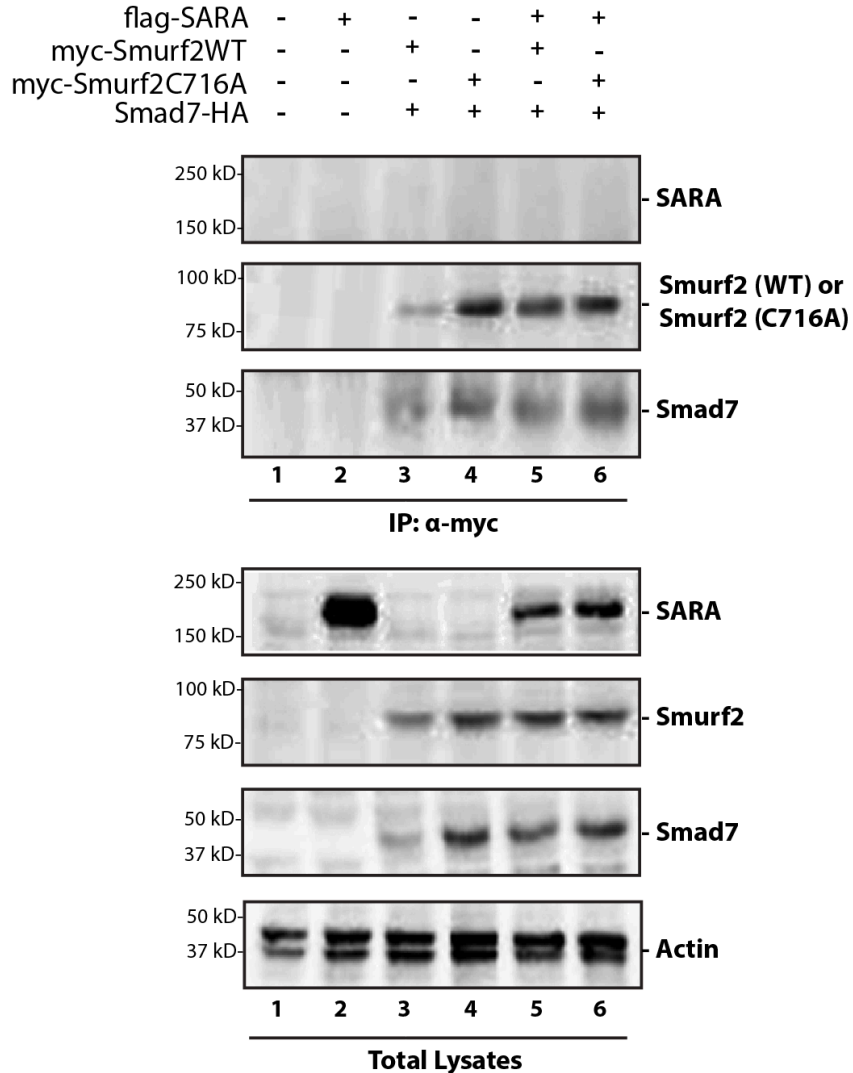


Figure 10. Smurf2 co-immunoprecipitates with Smad7 but not SARA.

HEK293T cells were transfected with cDNA constructs encoding flag-tagged SARA, myc-tagged Smurf2 WT or HECT domain mutant (C716A) or HA-tagged Smad7 as indicated at the top of the panel. Cell lysates were immunoprecipitated (IP) with α -myc to precipitate myc-tagged Smurf2 and associated proteins (top panel). The immunoprecipitates and remaining cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-SARA, anti-Smurf2 or anti-Smad7 antibodies. The lysates were also immunoblotted with anti-beta actin antibodies (equal protein loading control). The relative migration of each overexpressed protein, compared to the molecular mass markers (shown on the left) is indicated.

our previous results suggest that Smurf2 WT and Smad7 decrease SARA in an ubiquitin ligase activity-dependent manner. Therefore, I next turned my attention towards assessing the involvement of ubiquitination and the proteasome on the steady state levels of SARA.

3.4 Effect of MG132 on SARA steady state levels

Smurf2 is an E3 ubiquitin ligase that catalyzes the degradation of receptor complexes. The work shown above demonstrates that when the ligase activity of Smurf2 is disabled, SARA steady state levels do not decrease. In order to determine whether the decrease of SARA steady state levels is dependent on the proteasome, I pharmacologically targeted the proteasomal degradation pathway using MG132. HEK293T cells expressing flag-tagged SARA, myc-tagged Smurf2 or HA-tagged Smad7 were treated with MG132 (10 μ M) for 6 hours and were assessed by immunoblotting (Figure 11). In the presence of Smurf2 WT and Smad7, the steady state levels of SARA significantly decreased by 82% \pm 13% as observed above. As expected, the E3 ubiquitin ligase mutant of Smurf2 increased the steady state levels of SARA compared to the levels in the presence of Smurf2 WT and Smad7 (Figure 11B). A similar rescue effect was seen when the cells were treated with MG132. When the proteasome was inhibited, SARA steady state levels did not decrease even in the presence of Smurf2 WT and Smad7 (Figure 11A, Lanes 5 vs 7). As expected, Smurf2 C716A and Smad7 in the presence of MG132 did not alter the steady state levels of SARA. Based on the results above, the decrease of SARA steady state levels is dependent on the ubiquitin ligase activity of Smurf2. Therefore, the results suggest that SARA follows an ubiquitin degradation pathway. Thus far, the literature has only described Smurf2-Smad7 affecting the degradation of TGF β receptors

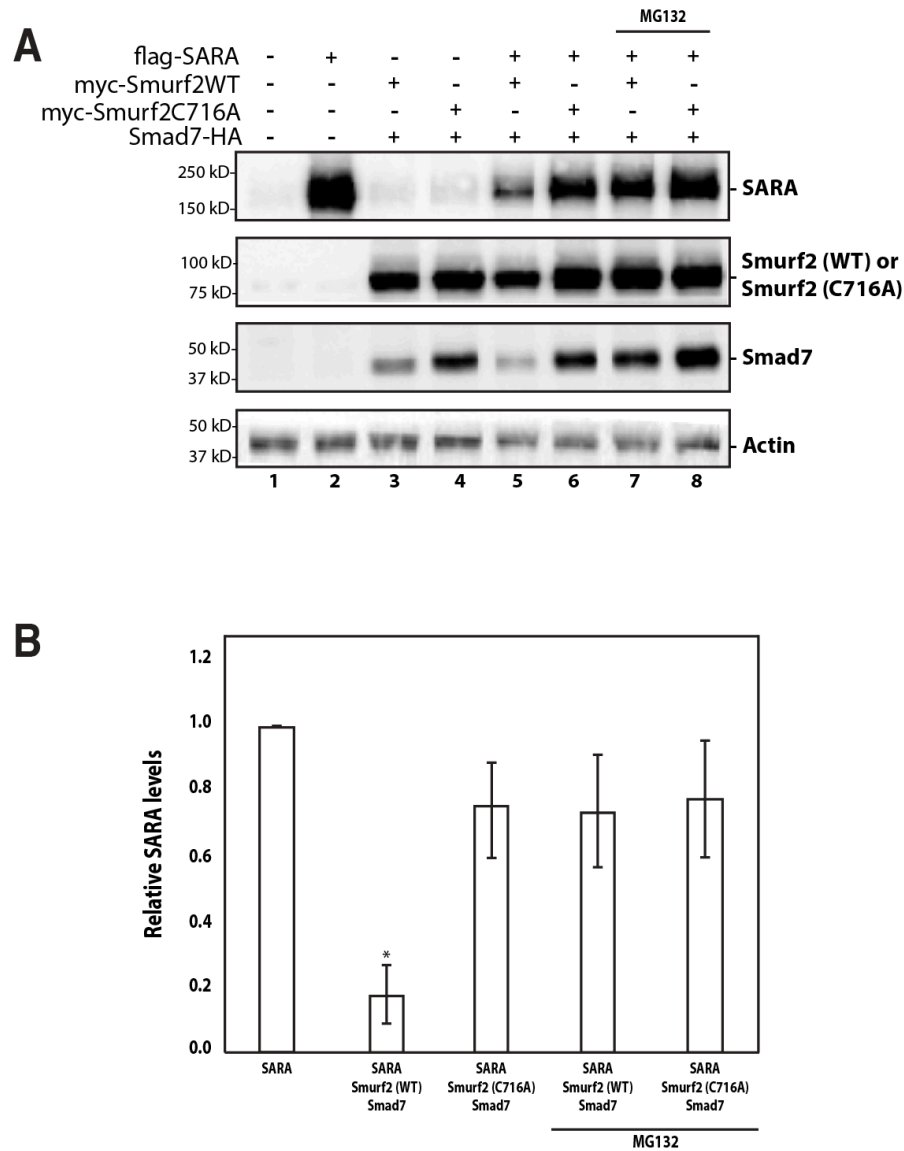


Figure 11. Proteasome inhibition protects SARA from Smurf2/Smad7-mediated degradation.

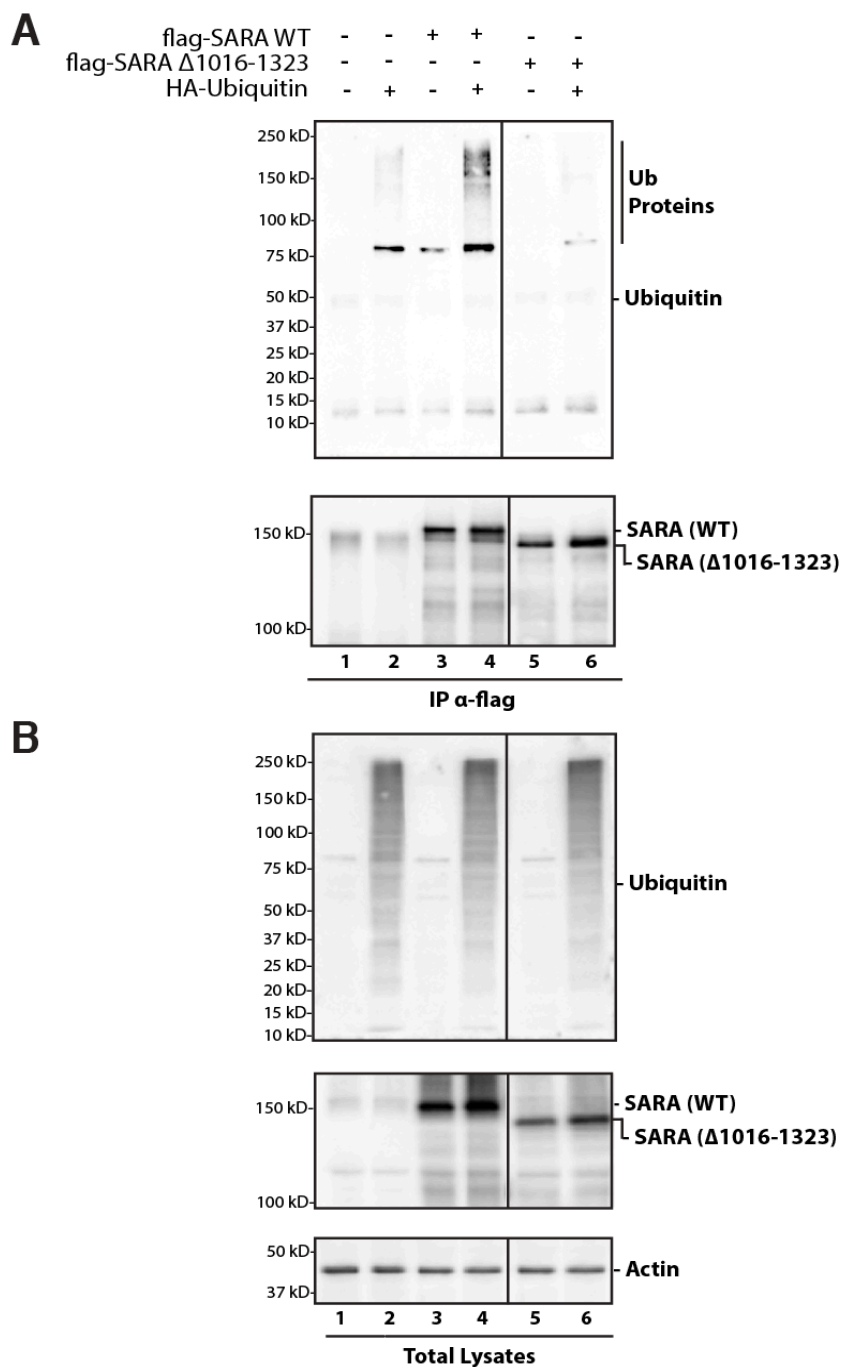
A. HEK293T cells were transfected with cDNA constructs encoding flag-tagged SARA, myc-tagged Smurf2 and/or HA-tagged Smad7 as indicated at the top of the panel. Cells were treated with MG132 (10 μ M) for 6 hours. Cell lysates were then subjected to SDS-PAGE followed by immunoblotting with anti-SARA, anti-Smurf2 or anti-Smad7 antibodies. The lysates were also immunoblotted with anti- β actin antibodies (equal protein loading control). The relative migration of each overexpressed protein, compared to the molecular mass markers (shown on the left) is indicated.

B. Three separate experiments were carried out as described in panel A and the relative levels of expressed SARA were quantitated using QuantityOne software (BioRad) and expressed as the mean \pm SD (n=3). The asterisk (*) indicates $p < 0.05$ relative to the level of SARA when SARA is transfected alone (One-way ANOVA).

(Kavsak et al. 2000). It is therefore possible that by interacting with ubiquitinated receptors, SARA is itself targeted for degradation. In order to assess this possibility, I utilized a SARA construct (SARA Δ 1016-1323) that lacks the C-terminal domain, which is responsible for interacting with TGF β receptors (Tsukazaki et al. 1998).

3.5 SARA and Ubiquitin

In order to determine whether SARA is involved in the ubiquitin-degradation pathway, I wanted to first observe whether SARA is ubiquitinated, or if it interacts with ubiquitinated proteins. HEK293T cells, expressing flag-tagged WT or Δ 1016-1323 SARA and HA-tagged Ubiquitin were immunoprecipitated with α -flag to immunoprecipitate SARA and immunoblotted for SARA and Ubiquitin (Figure 12). I observed a characteristic high molecular weight ubiquitinated protein species in the lanes containing WT SARA, indicating that either SARA is ubiquitinated or it interacts with ubiquitinated proteins (Figure 12A, Lane 4). These results showed that SARA is in close proximity to ubiquitin and suggests two possibilities: 1) SARA may be acting as an ubiquitin sensor and associates with ubiquitinated proteins, or 2) SARA itself is ubiquitinated. SARA may be interacting with proteins, such as TGF β receptor I and II, which are ubiquitinated by Smurf2 and targeted for degradation. To assess this possibility, I used a C-terminal deletion of SARA that no longer interacts with TGF β receptors (Tsukazaki et al. 1998). Indeed, this mutant of SARA (Δ 1016-1323 mutant) did not immunoprecipitate with ubiquitin (Figure 12, Lane 6). Taken together, these results suggest that the interaction of SARA with TGF β receptors may be necessary for SARA to bind to ubiquitinated proteins.



3.6 Effect of Smurf2-Smad7 on SARA Δ 1016-1323 steady state levels

Above, I have shown that a decrease in SARA steady state levels is dependent on the presence of Smurf2 and Smad7 (Figures 8 and 11) and that the presence of TGF β receptor interacting domain of SARA is necessary for the immunoprecipitation of SARA and ubiquitin (Figure 12). Therefore, I next assessed whether the TGF β receptor interaction domain of SARA is necessary for the Smurf2-Smad7-dependent decrease in SARA steady state levels. HEK293T cells expressing flag-tagged SARA, flag-tagged SARA Δ 1016-1323, myc-tagged Smurf2 WT and HA-tagged Smad7 were assessed by immunoblotting. Consistent with the results above, SARA steady state levels significantly decreased in the presence of Smurf2 WT and Smad7. However, when the carboxy terminal domain of SARA is deleted, the steady state levels are no longer affected by the ubiquitin ligase activity of Smurf2 (Figure 13). Our result suggests that in order for Smurf2 and Smad7 to regulate SARA steady state levels in the TGF β signaling pathway, the interaction with the TGF β receptor complex is necessary.

3.7 Effects of silencing SARA on TGF β -dependent signal transduction and EMT

Thus far, the results have shown that SARA steady state levels are regulated by Smurf2 and Smad7. In order to determine the consequences of modulating SARA levels in the TGF β signaling pathway, I first assessed the effect of reduced SARA levels on Smad2

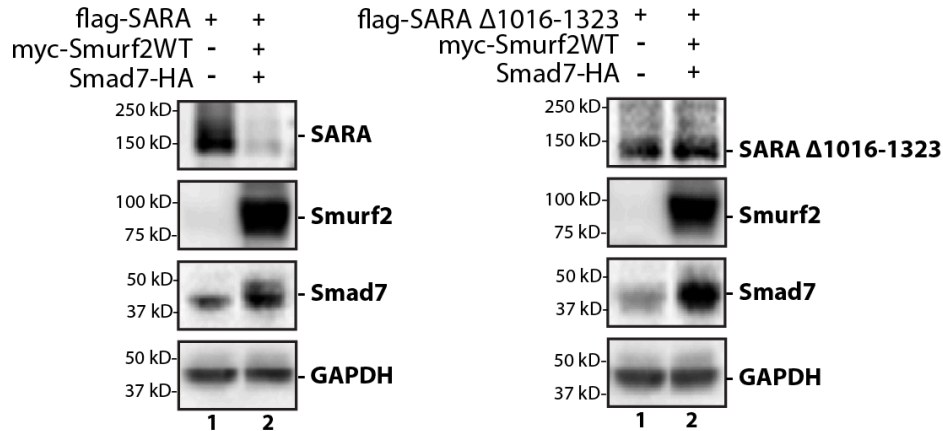
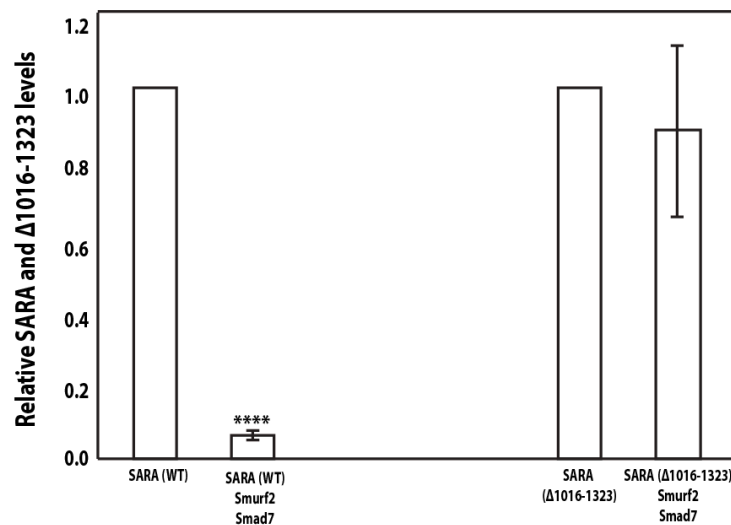
A**B**

Figure 13. Effect of Smurf2/Smad7 on SARA Δ 1016-1323 steady state levels.

A) HEK293T cells were transfected with cDNA constructs encoding flag-tagged SARA, flag-tagged SARA mutant Δ 1016-1323, myc-tagged Smurf2 and/or HA-tagged Smad7 as indicated at the top of the panel. Cell lysates were then subjected to SDS-PAGE followed by immunoblotting with anti-flag, anti-Smurf2 or anti-HA antibodies. The lysates were also immunoblotted with anti-GAPDH (equal protein loading control). The relative migration of each overexpressed protein, compared to the molecular mass markers (shown on the left) is indicated.

B) Three separate experiments were carried out as described in panel A and the relative levels of expressed SARA were quantitated using QuantityOne software (BioRad) and expressed as the mean \pm SD (n=3). The asterisk (****) indicates $p < 0.0001$ relative to the level of SARA when SARA is transfected alone (One-way ANOVA).

phosphorylation. Briefly, A549 cells were transfected with control siRNA or siRNA targeting SARA and TGF β signal transduction was assessed by immunoblotting for phospho-Smad2 (Figure 14). After 45 minutes, even at a relatively low concentration of TGF β (10 pM), Smad2 phosphorylation was observed in cells expressing SARA (Figure 14A, Lane 2). However, the phospho-Smad2 levels were significantly reduced in SARA siRNA targeted cells (Figure 14A, Lanes 5 and 6). The result suggests that in the absence of SARA, the phosphorylation of Smad2 is dampened, presumably because Smad2 is no longer tethered to the membrane where it can interact with TGF β receptors.

A downstream effect of TGF β -dependent Smad2 phosphorylation is the induction of transcriptional activity and the shift in expression of different cadherin proteins involved in EMT. In order to assess the effect of reduced SARA (and Smad2 phosphorylation) on TGF β -dependent EMT, A549 cells were transfected with control siRNA or siRNA targeting SARA and incubated with TGF β for 24 or 48 hours to induce EMT. To assess that EMT was occurring, immunoblotting for the loss of E-cadherin (epithelial cell marker) and increase in N-cadherin (mesenchymal cell marker) was carried out (Figure 15). I observed that when cells were treated with control siRNA, there was time-dependent reduction in E-cadherin levels and a concomitant increase in N-cadherin levels (Figure 15) These Cadherin changes were significant and reproducible (Figure 15B).

Consistent with my observations that SARA silencing reduced Smad2 phosphorylation, I also observed a blunted effect in the cadherin shift (Figure 15A and B).

Taken together, my results suggest that SARA can be targeted for degradation by Smurf2 and Smad7 and a reduction in SARA levels will affect the robustness of TGF β -dependent signaling and processes involved during EMT.

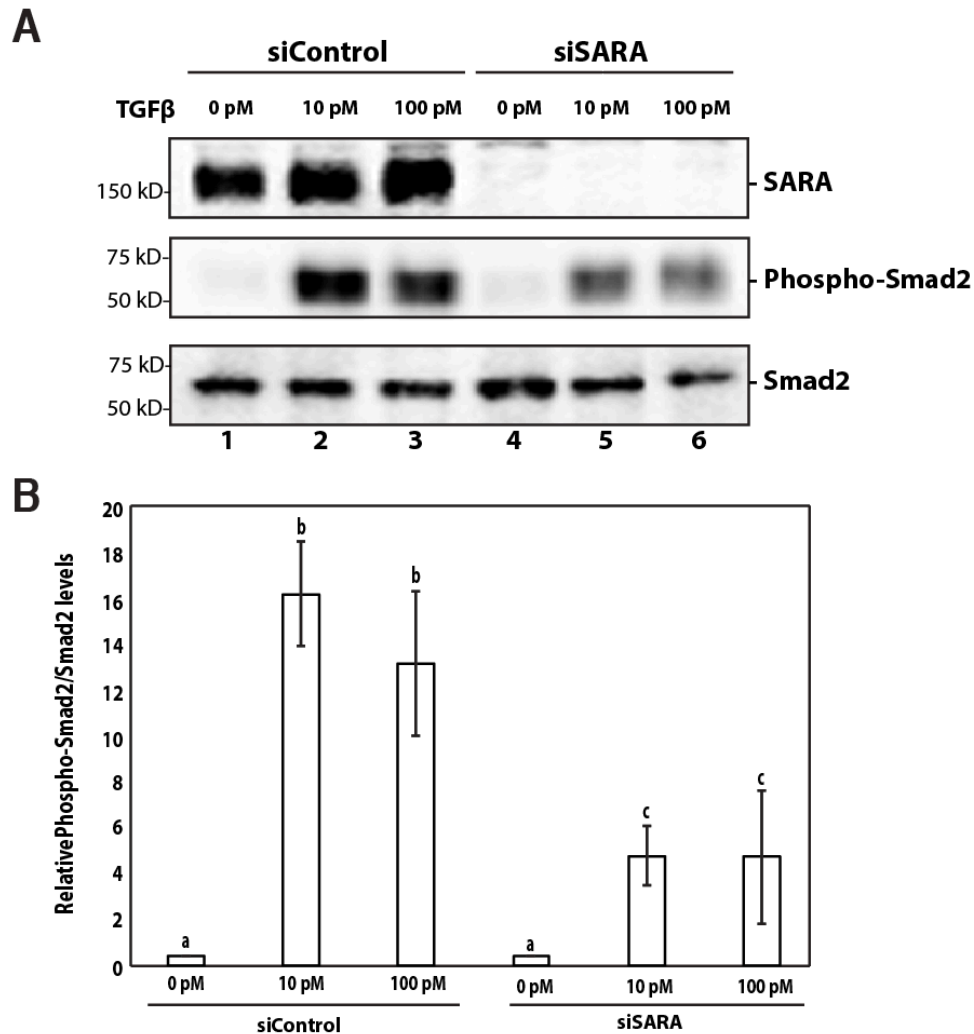


Figure 14. TGFβ signaling in the presence or absence of SARA.

A) A549 cells were transfected with a scrambled (control) siRNA or siRNA targeting SARA. Cells were then cultured in the indicated treatment concentration of TGFβ for 45 minutes. Cell lysates were then subjected to SDS-PAGE followed by immunoblotting with anti SARA and anti-phospho-Smad2. The lysates were also immunoblotted with anti-Smad2 antibody (equal protein loading control). The relative migration of each protein, compared to the molecular mass markers (shown on the left) is indicated.

B) Three separate experiments were carried out as described in panel A and the relative levels of endogenous pS2 and Smad2 were quantitated using QuantityOne software (BioRad) and expressed as the mean ± SD (n=3) and different letters denote significant differences (p<0.05, One-way ANOVA).

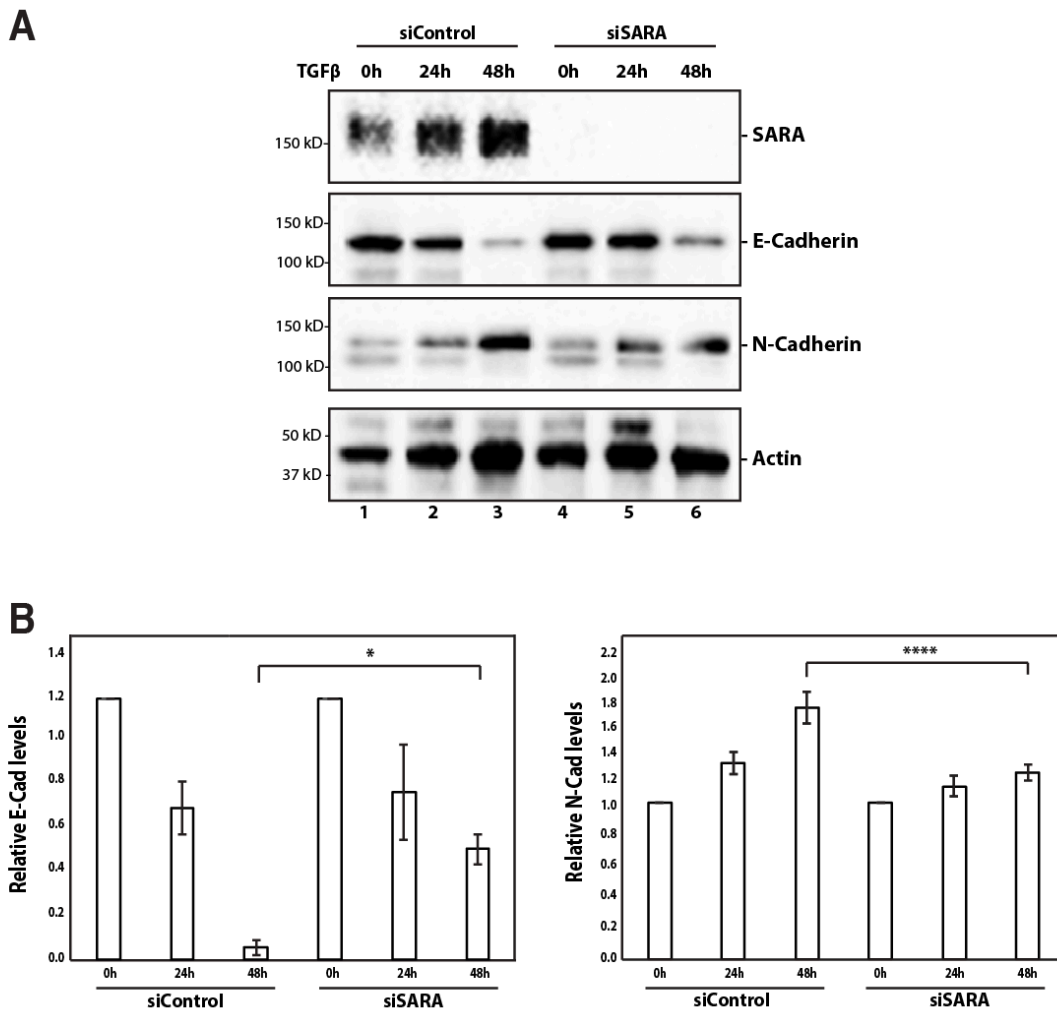


Figure 15. TGFβ-dependent EMT in the absence of SARA.

A) A549 cells were transfected with a scrambled (control) siRNA or siRNA targeting SARA. Cells were then cultured in the indicated treatment concentration of TGFβ for 0, 24, and 48 hours. Cell lysates were then subjected to SDS-PAGE followed by immunoblotting with anti-E-cadherin and anti-N-cadherin antibodies. The lysates were also western blotted with anti-β actin antibodies (equal protein loading control). The relative migration of each protein, compared to the molecular mass markers (shown on the left) is indicated.

B) Three separate experiments were carried out as described in panel A and the relative levels of endogenous E-cadherin and N-cadherin were quantitated using QuantityOne software (BioRad) and expressed as the mean ± SD (n=3) and asterisk (*) indicates $p < 0.05$, **** indicates $p < 0.0001$ (One-way ANOVA).

4 Discussion

4.1 Summary and general discussion

In the TGF β pathway, receptors have been postulated to internalize through both the clathrin-dependent and caveolin raft-dependent pathway in mammalian cells (Di Guglielmo et al. 2003; Roy and Wrana 2005). In the clathrin-dependent pathway, SARA anchors Smad2 to the receptors and facilitates Smad activation (Di Guglielmo et al. 2003; Penheiter et al. 2002; Roy and Wrana 2005; Sorkin and von Zastrow 2010). In the caveolin raft-dependent pathway, cholesterol and sphingolipid-rich composition of lipid rafts preferentially associate with Smad7-Smurf2 complexes leading to degradation of the receptor complex (Di Guglielmo et al. 2003). Although the individual proteins involved in TGF β signaling have been studied, research in the interaction between the key proteins in receptor trafficking remain unclear. Although the two internalization pathways are thought to be distinct, the endocytic pathways may not be functionally separated. Indeed, clathrin-coated and caveolin-coated vesicles have been postulated to fuse upon internalization and formed a multifunctional sorting compartment, where SARA, the TGF β receptor complex, Smad7 and Smurf2 reside (He et al. 2015). Although both SARA and Smurf2 interact with activated TGF β receptor complexes, it was still unknown whether SARA and Smurf2 associate with a common receptor complex. In this scenario, SARA and Smurf2-Smad7 may influence the ability of SARA to facilitate TGF β signaling by modulating its steady state levels. Indeed, my research shows a new potential mechanism whereby SARA degradation is regulated by Smurf2 and Smad7 in the TGF β pathway.

4.2 Role of Smurf2 and Smad7 on SARA steady state levels

SARA plays a crucial role in stabilizing and facilitating an efficient Smad signaling (Itoh et al. 1998; Tsukazaki et al. 1998). Consistent with previous literature, my data showed that in the absence of SARA, phosphorylation of Smad2 decreases significantly, presumably because SARA can no longer act as the anchor for Smad2 to efficiently associate with TGF β receptor; thereby reducing the signaling efficiency of Smad2 (Hayes, Chawla, and Corvera 2002; Tsukazaki et al. 1998). Tsukazaki et al., 1998 utilized different mutants of SARA to show that the disturbance in the ability of SARA to associate with endosomes (via FYVE domain mutants) or Smad2 (via SBD domain mutants) can mislocalize Smad2 and blunt TGF β signaling. Hayes et al., 2002 showed that by inhibiting clathrin-dependent receptor internalization via potassium depletion or transfecting a dominant negative dynamin, the stimulation of Smad2 nuclear translocation and transcriptional activation was greatly impaired. TGF β receptors accumulate in the early endosome, where SARA is localized, during the signaling process and plays an important role in sustaining the stimulation of Smad2 signaling (Di Guglielmo et al. 2003; Hayes, Chawla, and Corvera 2002). In addition, my data shows that when SARA is absent in NSCLC cells, TGF β signaling and the EMT-dependent cadherin shift (E-cadherin shift to N-cadherin) is decreased.

My results have also shown that in the presence of Smurf2 and Smad7, SARA steady state levels decrease. Previous literature has shown that Smurf2 and Smad7 cooperation is important for maximal inhibition of the activity of the TGF β receptors (Kavsak et al.

2000). Our work further shows the importance of the cooperation between Smurf2 and Smad7 in controlling the turnover of SARA steady state levels mechanistically in the TGF β signaling pathway. Smurf2 alone did not decrease steady state levels of SARA, whereas in combination with Smad7, it was capable to do so in an ubiquitin-dependent manner. Smad7 acts as the adaptor protein so that Smurf2 may interact with TGF β receptors (Kavsak et al. 2000). Therefore, this suggests that Smurf2 could influence the steady state levels of SARA at the level of TGF β receptor complexes. Interestingly, ubiquitin ligase activity of Smurf2 plays an important role in the decrease of SARA steady state levels. When Smurf2 loses the ability to ubiquitinate target proteins, SARA steady state levels do not decrease. In addition, pharmacologically inhibiting the proteasome, one of the end points of the degradation pathway, protects SARA steady state levels, suggesting that SARA follows an ubiquitin-dependent degradation pathway initiated by Smurf2 and Smad7.

The interaction of SARA with the TGF β receptor complex is necessary in the maintenance of the steady state levels by Smurf2 and Smad7. Indeed, my results show that a SARA mutant that cannot associate with the receptor complex is also not affected by Smurf2 and Smad7. In addition, SARA does not associate with ubiquitin if SARA cannot interact with the receptors. Smurf2 is an E3 ubiquitin-protein ligase that target TGF β receptors for ubiquitin-mediated degradation (Hayashi et al. 1997; Izzi and Attisano 2004; Kavsak et al. 2000; Nakao et al. 1997). Based on our work, there is a possibility that SARA may be another target for ubiquitination by the Smurf2-Smad7 complex. This suggests that there are three possibilities whereby SARA is degraded in response to Smurf2-Smad7 association with TGF β receptors. The first is that TGF β

receptors are ubiquitinated by Smurf2 while SARA is associating with the receptor and SARA passively follows TGF β receptors, leading to degradation of both receptors and associated SARA. The second scenario is where SARA acts as an ubiquitin-sensor protein, binding to TGF β receptors that are ubiquitinated by Smurf2. Finally, it is also possible that SARA is a target of Smurf2 and gets ubiquitinated for degradation (Figure 16).

In support of scenario 1, previous work by Di Guglielmo et al., 2003 showed that SARA and Smurf2-Smad7 regulate the fate of TGF β receptors. Smurf2-Smad7 reduces the half life of receptors significantly by targeting the receptors in the raft compartment for degradation (Di Guglielmo et al. 2003). However, they observed that in the presence of SARA, the receptors are mostly localized in the early endosome stabilizing the receptors and inhibiting the degradation pathway. Furthermore, they also showed that mutants of SARA that could neither associate with the endosomal membrane, nor interact with the receptor complex could protect TGF β receptors from degradation. Furthermore, He et al., 2015 showed that a portion of clathrin-coated vesicles and caveolar vesicles fuse after internalization into cells and create a multi-sorting vesicle called caveolar-1-positive early endosome. Based on these studies and my work, SARA may interact with the receptors that are targeted for degradation within the multi-sorting compartment and follow for Smurf/Smad7-dependent degradation.

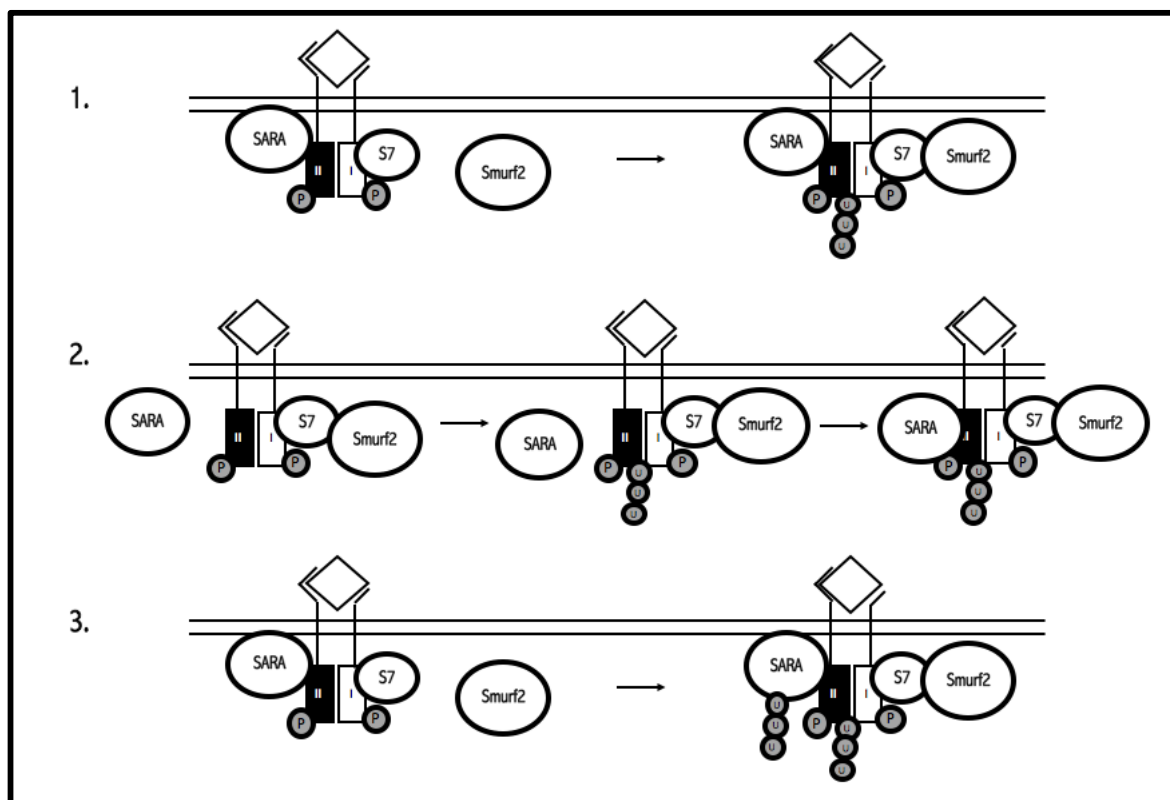


Figure 16. Potential scenarios for Smurf2/Smad7 dependent reduction to steady state SARA levels.

1. Smurf2 will ubiquitinate TGFβ receptors. Associated SARA with the receptors will passively follow leading to degradation of both proteins.
2. Smurf2 will ubiquitinate TGFβ receptors. SARA acts as an ubiquitin-sensor protein and associates with the ubiquitinated TGFβ receptors for degradation.
3. SARA is also ubiquitinated with TGFβ receptors and are both targeted for degradation.

To discuss more specifically about how proteins are ubiquitinated, there are three types of enzymes that play a key role: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) (Connor and Seth 2004). First, E1 activates ubiquitin and transfers it to E2. Then, ubiquitin is transferred to the target protein either by an E2 or with E3 (Connor and Seth 2004). There are different classifications of E3 ligase, such as HECT- and RING- (Connor and Seth 2004; Joazeiro and Weissman 2000). HECT- ligases, such as Smurf2, are ubiquitinated by E2s and afterwards, transfer the ubiquitin to the target protein. RING-type E3 ligases have two types: 1) ones that are able to cause ubiquitination on its own; and 2) those that act as in a multi-subunit E3 complex (Connor and Seth 2004; Joazeiro and Weissman 2000). One example of RING-type E3 ligase that acts in a multi-subunit is the ring-finger 11 (RNF11) protein. Subramaniam et al., 2003 and Connor & Seth, 2004 identified that RNF11 protein contains the PY motif, like Smad2 and Smad3, and interacts with the WW domain of Smurf2, and contains ring finger domain for the purpose of protein-protein interactions including E2s.

Subramaniam et al., 2003 further discussed the requirement of RNF11 for the ligase activity of Smurf2. They identified that RNF11 interacts with an E2 called UbcH5 enzymes through the ring finger domain and interacts with Smurf2 through the WW domain. By bringing the E2 and E3 subunits together, ubiquitin moieties are transferred from UbcH5 to Smurf2. However, when Smurf2 is associated with RNF11, the PY motif of RNF11 interferes with the binding of Smurf2 and Smad7, inhibiting the degradation of TGF β receptor (Subramaniam et al. 2003). In addition, Kostaras et al., 2012 showed that RNF11 is a SARA-interacting protein localized in the early endosome and there is a high possibility that both proteins are structurally and functionally associated with endosomal

sorting complexes required for transport -0 (ESCRT-0), which are protein complexes that generate multi-vesicular bodies via ubiquitinated proteins/receptors and deliver to lysosome for degradation (Wollert and Hurley 2010). Therefore, according to scenario 2 (Figure 16), SARA may be a key protein that is associated with the ESCRT system, specifically with ESCRT-0 complex that regulates degradation through lysosome. We propose that SARA may target the receptors to two degradation pathways depending on its protein-protein interaction. The first is the association with RNF11-Smurf2 complex in the early endosome, or in the multi-functional vesicle that He et al., 2015 proposed, will target TGF β receptors to lysosomal degradation pathway. Alternatively, association of SARA with TGF β receptors targeted by Smurf2-Smad7 will follow the receptors to proteasomal degradation pathway. Indeed, Kavsak et al., 2000 have demonstrated that endogenous TGF β receptors are degraded through both the proteasome and lysosome pathway. There may be a possible balance that cells are trying to maintain through the use of the four proteins RNF11, SARA, Smurf2, and Smad7, regulating the fate of TGF β receptors.

Finally, in scenario 3 (Figure 16), although our result shows that SARA immunoprecipitates with ubiquitin, Kostaras et al. (2012) suggested that SARA lacks a ubiquitin interacting motif; therefore, it is very unlikely for this scenario to happen.

Based on the previous literature and my work, the association of Smurf2, SARA and RNF11 may be the deciding complex that controls the fate of TGF β receptors. I propose that when TGF β signaling is activated, SARA acts as the facilitator and stabilizer for efficient signaling. After Smad7 translocates from the nucleus to the cytoplasm, it acts as an adaptor protein for Smurf2 to associate with the receptors and degrade the receptors

through proteasomal degradation pathway. When the receptors are turned over, SARA follows along and can be degraded as well. At some point in the process where the TGF β receptors need to be delivered to lysosome instead, RNF11 may associate with Smurf2 to interfere with the association of Smurf2 with Smad7. RNF11 may transfer the ubiquitin moieties to Smurf2 and along with SARA, the complex may associate with ESCRT system to target TGF β receptors for lysosomal degradation pathway. However, this needs more investigation and will be an important question to answer to further reveal the regulation of TGF β receptors by SARA and Smurf2.

4.3 Limitations and Future Directions

Although I have investigated and uncovered a novel regulatory mechanism in the regulation of TGF β signaling potential via the interaction of signaling (SARA) and degradative (Smurf2-Smad7) proteins, there is still much work to be done to fully understand this mechanism. I utilized siRNA for Smurf2 and SARA to observe the changes in the endogenous steady state levels. The result showed that silencing Smad7 in combination with Smurf2 may be necessary for the increase of SARA steady state levels. I attempted to address this by utilizing three different siRNA for Smad7, each from different company (Ambion, 4392420, ID: s8414; Santa Cruz, sc-36508; Dharmacon™, L-020068-00). However, I was not successful in silencing Smad7 in A549 cells (data not shown). Therefore, it would be important to potentially utilize different techniques, such as CRISPR/Cas9 gene editing, to manipulate the levels of Smad7 in A549 cells to determine whether SARA will be influenced.

When I attempted to investigate the association between SARA and Smurf2-Smad7, I did not observe these proteins to co-immunoprecipitate. Although there is the possibility that SARA and Smurf2 are not in close proximity, my work has shown that Smurf2 plays a crucial role in regulating the steady state levels of SARA via an ubiquitination degradation pathway. Therefore, it is more likely that the Smurf2 and SARA interaction is very transient and does not withstand co-immunoprecipitation. Whether SARA associates with the ESCRT system or follows the ubiquitin-tagged receptors for degradation, Smurf2-Smad7 is important in regulating SARA steady state levels through the ubiquitination-degradation pathway. Interestingly, however, SARA did not immunoprecipitate with Smurf2. He et al., 2015 showed that there is a fusion of the two vesicles upon internalization forming a multifunctional vesicle that contains proteins involved in the TGF β pathway, such as TGF β receptors, SARA, Smurf2 and Smad7. The fusion of these vesicles may be very transient for the proteins to form stable complexes. In addition, there is the possibility that Smurf2 and SARA may interact with the TGF β receptor at completely different time point in the multifunctional vesicle; it would be an important study to follow up. To determine whether Smurf2 and SARA are in close proximity throughout the TGF β pathway, techniques, such as the Proximity Ligation assay (PLA). In this technique, fixed cells are probed with antibodies conjugated to oligonucleotides. If two antibodies are in close proximity (less than 40 nm), the probes can be annealed and following an in situ DNA synthesis (using fluorescent probes), the signal is amplified and observed via immunofluorescence microscopy (Gustafsdottir et al. 2005).

Lastly, we showed that Smurf2 alone cannot decrease the steady state levels of SARA; however, with enough over-expression of Smurf2, we believe that Smurf2 can associate with the endogenous Smad7. Therefore, we suggest a titration experiment with increasing concentration of Smurf2 transfection to determine the levels of Smurf2 required to interact with endogenous Smad7 to affect SARA steady state levels. Although TGF β signaling and its endocytic pathways have been investigated, there are still many questions that need to be answered. Previous studies and my work have shown how important SARA and Smurf2 are in the regulation of TGF β signaling (Di Guglielmo et al. 2003; F. Huang and Chen 2012; Itman et al. 2011; Moustakas, Souchelnytskyi, and Heldin 2001; Runyan et al. 2009). It will be very important to answer how SARA and Smurf2 are interacting with RNF11 and Smad7. As suggested, TGF β receptors may have different end points in the degradation pathway depending on which combinations of proteins associate with the receptors. Investigating further into the relationship between these proteins will provide many answers.

My work has only shown a small glimpse of how SARA, TGF β receptors and Smurf2-Smad7 may possibly interact. There are still many questions that have to be answered to fully understand the way cells control TGF β receptors in the pathway. Besides the mechanistic aspect of TGF β signaling, more studies can be done on the functional outcomes of the signaling pathway, such as EMT. There are many EMT markers that can be analyzed; therefore, by silencing SARA, a future study can determine the effect it has on the levels of all the EMT markers as well as how it modulates the actin cytoskeleton by assessing stress fibre formation. In addition, TGF β -dependent invasion and migration can be studied using techniques such as transwell migration and scratch assays. Knocking

down SARA and Smurf2-Smad7 may have severe negative consequences in growth, as Tojo, Takebe, Takahashi, Tanaka, & Alk, 2012 observed that Smad7-deficient mice showed growth retardation with reduced viability. Therefore, it would be interesting to utilize the knockdown technique on adult mice and observe the influence of SARA knockdown has on metastasis. SARA is a potential target to reduce the rate at which tumor cells metastasize and will be an interesting subject for future investigation.

4.4 Significance

The TGF β pathway enhances the migratory and invasive properties of cancer through EMT (Padua and Massagué 2009). Furthermore, EMT can be found in certain pathology sections and accounts for progression of diseases, including organ fibrosis and cancer (Gonzalez and Medici 2014; Padua and Massagué 2009; Thiery 2002). The final stage of tumor progression is metastasis and is responsible for 90% of deaths associated with tumors (Hanahan and Weinberg 2000). Our research proposes that the cooperative effect of Smurf2 and Smad7 on TGF β receptors can influence a major key protein involved in TGF β signaling, SARA. Smurf2-Smad7 decrease the steady state levels of SARA through the ubiquitination degradation pathway. As TGF β signaling progresses, cells activate the negative feedback by recruiting Smurf2 to associate with the receptors using the Smad7 as an adaptor protein (Hayashi et al. 1997). Our study proposes that as the receptors are being transported for degradation, SARA follows the receptor to be degraded. As the signaling progresses, the receptors turnover potentially through a raft-dependent degradation and SARA follows the receptors for degradation; hence, decreasing the availability for cells to utilize SARA in the signaling pathway to stabilize

and facilitate Smad2 signaling. Therefore, this will lead to a decreased phosphorylation activation of Smad2 and affecting the functional outcome of TGF β signaling such as EMT. SARA may be a promising target in cancer cells; by controlling the steady state levels of SARA through Smurf2-Smad7, TGF β signaling may be suppressed, dampening the process of EMT and finally, leading to decreased metastasis.

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SAM SANGHYUN LEE

Education & Awards

Education

Masters of Science in Physiology, University of Western Ontario, London ON, 2014-2017

Bachelor of Medical Science, Honour Specialization in Physiology, University of Western Ontario, London ON, 2010-2014

Awards

Global Opportunities Award 2012
Western Scholarship of Excellence 2011

Publication & Presentation

Publication

Characterization of Smad Anchor Receptor necessary for Activation (SARA) in TGF β receptor signaling (In preparation)

Cytoprotective effects of crude polysaccharide fraction of Panax quinquefolius against cyclophosphamide toxicity in The 11th International Symposium of Ginseng, October 27-30, 2014. Page 214-224

Presentations

Lee, S. and Di Guglielmo, G. Characterization of Smad Anchor Receptor necessary for Activation (SARA) in TGF β receptor signaling.

At: WCRG 3rd Biennial International Cancer Research Conference in Windsor, 2016
James A.F. Stevenson Distinguished Lecture and Research Day at UWO, 2016
American Association for Cancer Research, New Orleans in New Orleans, 2016
London Health Research Day in London, 2016
Charles W. Cowdey Distinguished Lecture and Research Day at UWO, 2015

Research Experience

Graduate Researcher at UWO from 2014-2017

- Carried out research with minimal supervision and oversaw the completion from the beginning to the end
- Designed a cancer cell metastasis research project and discovered a novel characteristic
- Collected quantitative data utilizing densitometry western blot
- Imaged cancer cells using immunofluorescent microscopy to determine the effect of drugs
- Analyzed the collected data using statistical analytic program such as GraphPad Prism
- Prepared quantitative data into a manuscript for a thesis and a presentable form of posters and PowerPoint presentations

Undergraduate Honours Research at UWO from 2013-2014

- Worked in collaboration with two undergraduate colleagues to successfully complete the honours research project
- Organized and coordinated a schedule to distribute daily work-load evenly and to ensure the studies are done efficiently
- Designed a novel study about the protective effect of ginseng on rheumatoid arthritis
- Collected qualitative & quantitative data: scoring and observing the behavior of mice and harvesting the organs to measure certain protein levels
- Follow-up studies were done to determine the long-term effect of ginseng on the mice
- Assisted my supervisor in writing the grant proposal based on the project that we created

Work Experience

Research Assistant at UWO from 2011-2013

- Worked in collaboration with a Ph.D. research associate and successfully published an article in the 11th International Symposium of Ginseng in 2014
- Utilizing mice model, we studied the protective effect of ginseng against an adverse cytotoxic effect of chemotherapeutic drug
- Conducted scientific literature searches, and organized a literature database

Teaching Assistant at UWO from 2014-2016

- Worked in a Physiology Laboratory course with 10 new students every unit (4 units in total)
- Responsible for coordinating and creating presentations to teach the techniques and theory behind what the students were learning
- Collaboratively worked with other TAs to create a student-focused learning environment, encouraging a hands-on learning experience in the physiology lab

Surgical Preparation Unit at University Hospital from 2012-2013

- Assisted SPU staffs and nurses with the daily running of the clinic in the OR waiting room
- Collected patients from the waiting room area and guided them to the appropriate preparation area and assigned beds
- Organized patient registration papers and recorded patient arrival time, bed # assignment on log sheet and sort list

General Medical Team in Costa Rica, 2012

- Through International Service Learning, travelled to San Jose, Costa Rica, to provide health care service to underdeveloped communities that have limited access to hospitals
- Worked as a team of 18 members to provide the highest quality of health care with utmost respect and professionalism
- Assisted the doctors with patient interviews, taking vital signs and physical examination
- Trained for basic pharmacology and assisted with pharmacy at the clinic