Mechanisms of action of CD109, a novel TGF-β co-receptor

by

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TGF-β rocks

(Massagué, 2007)

Abstract

Transforming Growth Factor β (TGF-β) is a multifunctional cytokine that plays a critical role in cell growth, differentiation and extracellular matrix deposition. Dysregulation of its pathway has been implicated in tissue fibrosis and cancer. TGF-β signals via the type I (TGFBR1) and type II (TGFBR2) receptor complex, which phosphorylates their intracellular substrates, SMAD2 and SMAD3. The phosphorylated SMAD2/3 then forms a complex with SMAD4 and regulates target gene expression. Alternatively to the SMAD2/3 (canonical) pathway, TGF-β also elicits signalling via non-canonical pathways, such as the ERK and p38 MAPK pathways.

TGF-β receptors internalize via the clathrin-coated pits route, which facilitates SMAD2/3 signalling, and via the caveolae route, which is associated with receptor degradation and with MAPK activation. Little is known regarding the factors regulating TGF-β receptor compartmentalization and turnover. Previously, CD109 was identified in our lab as a GPI-anchored protein that binds TGF-β and forms a heteromeric complex with the TGF-β receptors. The results presented here demonstrate that CD109 inhibits SMAD2/3-dependent signalling and responses, such as TGF-β-induced growth inhibition. Together, these results suggest that CD109 is a novel TGF-β co-receptor that negatively regulates TGF-β signalling.

I then explored the mechanism by which CD109 regulates TGF-β action. My results indicate that CD109 increases TGF-β receptor internalization via the caveolar pathway and enhances TGF-β receptor degradation by the E3 ubiquitin ligase Smurf2, leading to inhibition of TGF-β signalling.

Because TGF-β is a potent inducer of epithelial-mesenchymal transition (EMT), a process involved during cancer invasion and metastasis, I next investigated the role of CD109 in this process. CD109 inhibits TGF-β-induced EMT in both non-tumorigenic keratinocytes and squamous cell carcinoma cells via the SMAD and ERK and p38 MAPK pathways. Indeed,

CD109 modulates TGF-β-induced MAPK activation, in a caveolin-1 dependent manner. Collectively, these data suggest that CD109 exerts its function by promoting TGF-β receptor localization to caveolae, thereby accelerating their degradation and modulating TGF-β canonical and non-canonical signalling. Thus, CD109 may play a critical role in pathologies where TGF-β signalling is dysregulated, such as cancer progression.

Résumé

Le TGF-β (facteur de croissance transformant β) est une cytokine multifonctionnelle jouant un rôle important dans la croissance, la différentiation cellulaire et la déposition de la matrice extracellulaire. La dérégulation de la cascade de signalisation du TGF-β peut engendrer la fibrose des tissus ou des cancers. Le TGF-β transmet son signal grâce aux récepteurs de type I (TGFBR1) et de type II (TGFBR2), qui phosphorylent leurs substrats intracellulaires, SMAD2 et SMAD3. Les SMAD2/3 phosphorylées s'associent à SMAD4 et régulent l'expression de gènes cibles. En plus de la voie canonique de SMAD2/3, le TGF-β transmet aussi son signal par des voies non-canoniques, telle les voies des MAPKs p38 et ERK.

Les récepteurs du TGF-β sont internalisés dans des puits recouverts de clathrine (ce qui facilite la voie des SMAD2/3) et dans les cavéoles (ce qui entrainent la dégradation des récepteurs et l'activation des MAPKs). Peu de choses sont connues sur les facteurs régulant la compartimentalisation et la dégradation des récepteurs du TGF-β. CD109 a été identifié précédemment dans notre laboratoire en tant que protéine à ancre GPI capable de se lier au TGF-β et de former un complexe avec les récepteurs du TGF-β. Les résultats présentés ici démontrent que CD109 inhibe la voie des SMAD2/3 et leurs réponses associées, telles l'arrêt de la croissance cellulaire. Tout ceci suggère que CD109 est un nouveau corécepteur du TGF-β régulant de manière négative le signal du TGF-β.

J'ai ensuite exploré les mécanismes par lesquels CD109 régule l'action du TGF-β. Mes résultats indiquent que CD109 augmente l'internalisation dans les cavéoles des récepteurs du TGF-β et leur dégradation par l'E3-ubiquitine ligase Smurf2, conduisant ainsi à l'inhibition du signal du TGF-β.

Comme le TGF-β induit l'EMT (transition épithélium-mésenchyme), j'ai examiné le rôle de CD109 dans ce processus impliqué dans les métastases cancéreuses. CD109 inhibe

l'EMT dans les keratinocytes non tumorigéniques et dans les cellules de carcinomes squameux, via les voies des SMADs et des MAPKs p38 et ERK. CD109 régule l'activation des MAPKs, par un processus qui dépend de la cavéoline-1. L'ensemble de ces données suggèrent que CD109 exerce ses fonctions en facilitant la localisation des récepteurs dans les cavéoles, accélérant ainsi leur dégradation et modulant les voies canoniques et non canoniques du TGF-β. CD109 pourrait donc jouer un rôle crucial dans les pathologies où l'action du TGF-β est dérégulée, comme la progression des cancers.

Contributions to original knowledge

This thesis is assembled in accordance with the regulations given by the Faculty of Graduate Studies and Research, McGill University. It consists of an Abstract and its French translation (Résumé), and seven chapters entitled Chapter 1: Introduction; Chapter 2: Materials and Methods; Chapter 3: CD109 is a negative regulator of TGF-β signalling; Chapter 4: CD109 regulates TGF-β receptor internalization into caveolae; Chapter 5: CD109 regulates TGF-β receptor degradation; Chapter 6: CD109 regulates TGF-β-induced epithelial-mesenchymal transition (EMT) via SMAD and MAPK pathways; Chapter 7: Conclusions and Perspectives. A bibliography containing all the literature cited is provided at the end of the thesis.

Unless otherwise stated, I, Albane Bizet, under the close supervision of Dr. Anie Philip, oversaw experimental design and execution, data analysis and interpretation for the body of work presented here-in. The list of contributions to original knowledge I have made is presented below:

1) I have validated that CD109 is an inhibitor of SMAD2/3 signalling and TGF-β-induced growth inhibition.

2) I have demonstrated that CD109 can modulate TGF-β signalling independently of ligand sequestration.

3) I have demonstrated that CD109 associates with caveolin-1, and that this association is enhanced by TGF-β treatment and that CD109 co-localizes with the TGF-β receptors in caveolae.

4) I have demonstrated that CD109 enhances TGF-β receptor internalization via the caveolae and that CD109's effect on SMAD3 phosphorylation requires caveolin-1.

6) I have demonstrated that CD109 promotes TGF-β receptor degradation mediated by SMAD7/Smurf2 and the proteasome.

7) I have shown that CD109 enhances the association of the SMAD7/Smurf2 complex with the TGF-β receptors and promotes the co-localization of the TGF-β receptors with Smurf2.

8) I have demonstrated that CD109's effect on TGF-β-induced SMAD2/3 signalling requires SMAD7/Smurf2.

9) I have demonstrated that CD109 inhibits TGF-β-induced EMT, migration and Slug expression in HaCaT (non-tumorigenic) and A431 and SCC-13 (tumorigenic, epidermoid squamous cell carcinoma) cell lines.

10) I have demonstrated that CD109 modulates TGF-β-induced p38 and ERK MAPK in HaCaT and SCC cell lines.

11) I have demonstrated that CD109 regulates TGF-β-induced EMT, migration, Slug and fibronectin expression via SMAD and MAPK pathways.

Some of the data presented in this thesis are reported in the following articles:

- Finnson K.W., Tam B.Y.Y, Liu K., Marcoux A., Lepage P., Roy S., **Bizet A.A**. and Philip A., Identification of CD109 as part of the TGF-β receptor system in human keratinocytes. *FASEB J.* 2006 Jul;20(9):1525-7.
- **Bizet A.A.,** Liu K., Tran Khanh N., Saksena A., Vorstenbosch J., Finnson K. W., Buschmann M.D. and Philip A. The TGF-β co-receptor, CD109, promotes internalization and degradation of TGF-β receptors. *Accepted for publication in BBA-Molecular Cell Research*
- **Bizet A.A.,** Tran Khanh N., Saksena A., Liu K., Buschmann M.D. and Philip A. CD109-mediated degradation of the TGF-β receptors involves SMAD7 and Smurf2. *In preparation*
- **Bizet A.A.** and Philip A. CD109 inhibits TGF-β-induced Epithelial-Mesenchymal Transition via SMAD and MAPK pathways. *In preparation*

All the data presented in this thesis are the work of the author with the following exceptions:

1) The western blot of SMAD3 phosphorylation in HaCaT cells transfected with CD109 or its EV (Figure 3.1A) was performed by Ying Wang, a technician from Dr A. Philip's lab.

2) The sucrose gradient experiments (Fig 4.6A and B) and Internalization assay in HaCaT cells (Figure 4.3C-F and 4.5C) were realized by Kai Liu, a post-doctoral fellow in Dr A. Philip's lab. I have then done the densitometry, graphical representation and data analysis.

3) All confocal imaging was done in collaboration with Nicolas Tran-Khanh, a post-doctoral fellow from Dr M.D. Buschmann's laboratory (Ecole Polytechnique de Montréal).

4) The Western blot of SMAD3 phosphorylation in HaCaT cells transfected with CD109 (or EV), Dyn2K44A (or its control empty vector) (Fig 4.7A) and the co-immunoprecipitation of Smurf2/SMAD7, in the presence or absence of CD109 overexpression (Fig 5.3D) were done by Anshuman Saksena, a summer student working under my supervision.

5) HaCaT cells stably overexpressing CD109 or its EV were generated by Hahn Soe-Lin, a Master student from Dr A. Philip's lab.

6) CD109shRNA and scrambled shRNA constructs were made by Joshua Vorstenbosch, a PhD candidate from Dr A. Philip's lab. Joshua Vorstenbosch has also created the CD109 transgenic mice from which the primary mouse keratinocytes were isolated.

Abbreviations

α-2M	α2-macroglobulin
ActR2	Type II activin receptor
AF488	AlexaFluor488
AIP	Atrophin 1-interacting protein
ALK	Activin receptor-like kinase
α-SMA	α-Smooth muscle actin
AP1	Activator protein 1
AP2	Adaptor protein 2
APP	Amyloid precursor protein
ATF	Activating transcription factor
BAMBI	BMP and activin membrane-bound inhibitor
BMP	Bone Morphogenetic Proteins
BMPR	BMP receptor
BPE	Bovine Pituitary Extract
BRE	BMP-responsive element
BS^3	Bis(Sulfocsuccinimidyl)suberate
BSA	bovine serum albumine
bZIP	basic-leucine zipper
Cam Kinase II	Ca^{2+}-calmodulin-dependent protein kinase II
CBP	c-AMP-response element (CRE) binding protein
CD44, CD109...	Cluster of Differentiation 44, 109...
cdc42	Cell division control protein 42 homolog
CDK	Cyclin dependent kinase
CLIC	Clathrin-independent carrier
CMV	Cytomegalovirus
co-SMAD	Common-SMAD

cPML	Cytoplasmic promyelocytic leukemia protein
CRM1	Chromosome maintenance region 1
DAPI	4',6-diamidino-2-phenylindole
D-MEM	Dulbecco-modified Eagle's minimal essential medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EEA-1	Early endosomal antigen-1
EGF	Epidermal growth factor
EGF-CFC	Epidermal growth factor-Cripto, FRL-1, Criptic
EMT	Epithelial-mesenchymal transition
eNOS	Endothelial NO synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
EV	Empty vector
Fab	Fragment, antigen binding
FAM/USP9x	Fat facets in mammals/ubiquitin specific peptidase 9, X-linked
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GADD45β	Growth Arrest and DNA Damage 45β
GAG	Glycosaminoglycan
GDF	Growth Differentiation Factor
GEEC	GPI-anchored proteins enriched early endosomal compartment
GFP	Green fluorescent protein
GIPC	GAIP (Gα interacting protein)-interacting protein, C terminus
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinositol
Grab2	Growth factor receptor binding protein 2

GRK2	G protein-coupled receptor kinase 2
GSK3	Glycogen synthase kinase 3
GTP	Guanosine-5'-triphosphate
HECT	Homologous to E6AP COOH terminus
HEK 293	Human embryonic kidney 293
HGF	Hepatocyte growth factor
HHT1	Type I hereditary hemorrhagic telangiectasia
HRP	Horseradish peroxidase
ID1	Inhibitor of DNA binding 1
IF	Immunofluorescence
IFN	Interferon
IGFII/M6P	Insulin-like growth factor II/ mannose-6 phosphate
IgG	Immunoglobulin G
IKKα	Inhibitor of NF-κB subunit α
IL	Interleukin
IP	Immunoprecipitation
I-SMAD	Inhibitory SMAD
JaCoP	Just another co-localization Plug-in
JAK	Just another kinase (also known as Janus kinase)
JNK	c-Jun N-terminal kinase
kb	Kilobases
kDa	Kilodalton
LAP	Latency associated protein
LLC	Large latent complex
LRP-1	Lipoprotein receptor-related protein
LTBP	Latent TGF-β-binding proteins
MAD	Mother against Decapentaplegic
MAPK	Mitogen activated protein kinase

MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MEFs	Mouse embryonic fibroblasts
MEK	MAPK kinase
MEKK	MAPK kinase kinase
MET	Mesenchymal-epithelial transition
MH1 and 2	MAD-Homology 1 and 2
MIS/AMH	Müllerian inhibitory substance/anti-Müllerian hormone
MMP	Metalloproteinases
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of Rapamycin
Mv1Lu	Mink lung epithelial cell line
MβCD	Methyl-β-cyclodextrin
NANDOR	Non activating non-downregulating
NEDD4	Neuronal precursor cell-expressed developmentally downregulated 4
NES	Nuclear export sequence
NET1	Neuroepithelial cell-transforming gene 1 protein
NF-κB	Nuclear Factor-κB
NGS	Normal Goat serum
NMuMG	Non-transformed mouse mammary gland epithelial cell
O/N	Overnight
ONPG	2-Nitrophenyl-β-D-galactopyranoside
PAI-1	Plasminogen activator inhibitor-1
PAK	p21-Activated kinase
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDZ	Post synaptic density protein (PSD95), Drosophila disc large tumor
PI	Phosphatidylinositol

PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PIASγ	Protein inhibitor of activated STATγ
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol-3, 4, 5-triphosphate
PI-PL C or D	Phosphatidylinositol phospholipase C or D
PKCζ	Protein kinase Cζ
PMSF	Phenylmethylsulfonyl fluoride
PPM1A	Protein phosphatase 1A
PTB	Phosphotyrosine binding
PTEN	Phosphatase and tensin homolog
PTRF	Polymerase I and transcript release factor
RGM	Repulsive guidance molecule
RPTP-κ	Receptor type protein tyrosine phosphatase-κ
R-SMAD	Receptor-activated SMAD
SAD	SMAD4 activation domain
SARA	SMAD anchor for receptor activation
SBE	SMAD-binding element
SCC	Squamous cell carcinoma
SCF	Skp-1/Cul/F-box
SCP	Small C-terminal Domain Phosphatase
SDPR	Serum deprivation protein response
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard deviation of the mean
ShcA	Src homology 2 domain containing protein A
siRNA	Small interference ribonucleic acid
SLC	Small latent complex

Smurf	SMAD ubiquitination regulatory factor
SnoN	Ski-novel gene N
Sos	Son of Sevenless
STAT	Signal transducer and activator of transcription
STRAP	Serine-threonine kinase receptor-associated protein
TAB2	TAK-associated protein
TACE/ADAM-1	TNF-α converting enzyme/A Disintegrin And Metalloproteinase-1
TAK1	TGF-β activated kinase 1
TAZ	Transcriptional co-activator with PDZ-binding motif
TEF3	Transcriptional enhancer factor 3
TGFBR	TGF-β receptor
TGF-β	Transforming Growth Factor-β
Th17	T helper 17
TIE	TGF-β inhibitory element
TIF1γ	transcription intermediary factor 1γ
TMEPAI	Transmembrane TGF-β-Inducible Protein
TNF-α	Tumor Necrosis Factor-α
TPA	Tetradecanoyl phorbol acetate
TRAF	TNF Receptor Associated Factor
TSP1	Thrombospondin 1
uPAR	Urokinase receptor
VEGF	Vascular endothelial growth factor
WB	Western blot
YAP65	Yes-associated protein 65
ZEB	Zinc finger E-box binding homeobox 1
ZO-1	Zona occludens 1

Chapter 1: Introduction

1.1-TGF-β superfamily of ligands

1.1.1- TGF-β superfamily members and their functions

The Transforming Growth Factor-β (TGF-β) superfamily consists of more than 60 members in multicellular organisms. 42 sequences potentially coding TGF-β superfamily members have been identified in the human genome. To date, 29 members have been characterized in the human (Feng and Derynck, 2005). The TGF-β superfamily is composed of bone morphogenetic proteins (BMPs)/growth differentiation factors (GDFs), TGF-βs, activins, inhibins, nodal, lefty and the Müllerian inhibitory substance/anti-Müllerian hormone (MIS/AMH). They play fundamental roles during embryonic development and maintenance of homeostasis and mediate diverse cellular processes, such as proliferation, apoptosis, differentiation and regulation of immune response (Gordon and Blobe, 2008). TGF-β is a potent inhibitor of epithelial cell growth, a critical inducer of extracellular matrix (ECM) synthesis and it regulates immune and hematopoietic functions (Massagué and Chen, 2000). TGF-β also induces epithelial-mesenchymal transition (EMT), a process involved in development and cancer progression (Meulmeester and ten Dijke, 2011). Activins are particularly important for the control of reproductive axis functions (Harrison *et al.*, 2005). BMPs regulate haematopoiesis, neurogenesis and bone formation during the embryonic life and control bone mass in the adult (Gazzerro and Canalis, 2006). Dysregulations of TGF-β superfamily signalling pathways have been implicated in several human diseases, including cancer, impaired wound healing, developmental defects, auto-inflammatory diseases and neurodegenerative disorders (Gordon and Blobe, 2008).

1.1.2- TGF-β subtypes

TGF-β is composed of three different subtypes in mammals: TGF-β1, TGF-β2 and TGF-β3. They are encoded by different genes but share approximately 70% identity. The amino acid sequence of each (mature) subtype is highly conserved throughout evolution: TGF-β1 in human and mouse differs only by one amino acid. TGF-β1 is the most ubiquitous and abundant subtype, with TGF-β2 and TGF-β3 having a more restricted distribution. They have overlapping activity *in vitro* but distinct functions *in vivo*, as illustrated by the phenotype of *Tgf-β* null mice. *Tgf-β1* null mice die within 3 weeks of birth due to inflammation (Shull *et al.*, 1992). *Tgf-β2* null mice die in the perinatal period due to heart and pulmonary insufficiency (Sanford *et al.*, 1997), while *Tgf-β3* null mice die within 24hrs after birth and display a cleft palate (Proetzel *et al.*, 1995).

1.1.3- Synthesis and activation of latent TGF-β

TGF-β is transcribed as a pro-protein of 390 amino acids, consisting of a large pro-peptide and a C-terminal mature polypeptide (Gray and Mason, 1990). During its processing in the golgi apparatus, the pro-peptide is cleaved from the growth factor by pro-protein convertases, such as furin, as represented in Fig 1.1. The mature TGF-β, a homodimer of two 12.5kDa peptides joined by a disulfide bond, remains non-covalently attached to the homodimer of its pro-peptide (or latency associated protein, LAP). The association of the mature TGF-β with LAP renders TGF-β latent and this complex is referred as the small latent complex (SLC). The sequence of LAP is different for each TGF-β subtype and the LAPs are therefore termed LAP-β1, LAP-β2, and LAP-β3. In the secretory vesicles of most cell types, the SLC forms a covalent disulfide bond with glycoproteins of the latent TGF-β-binding proteins (LTBPs) family, thus forming the large latent complex (LLC) (Saharinen and Keski-Oja, 2000). After secretion, LTBP is covalently bound to components of the ECM, such as heparin and fibronectin, through the action of the extracellular tissue transglutaminase (Fig 1.1;(Kantola *et al.*, 2008)). LTBP thus

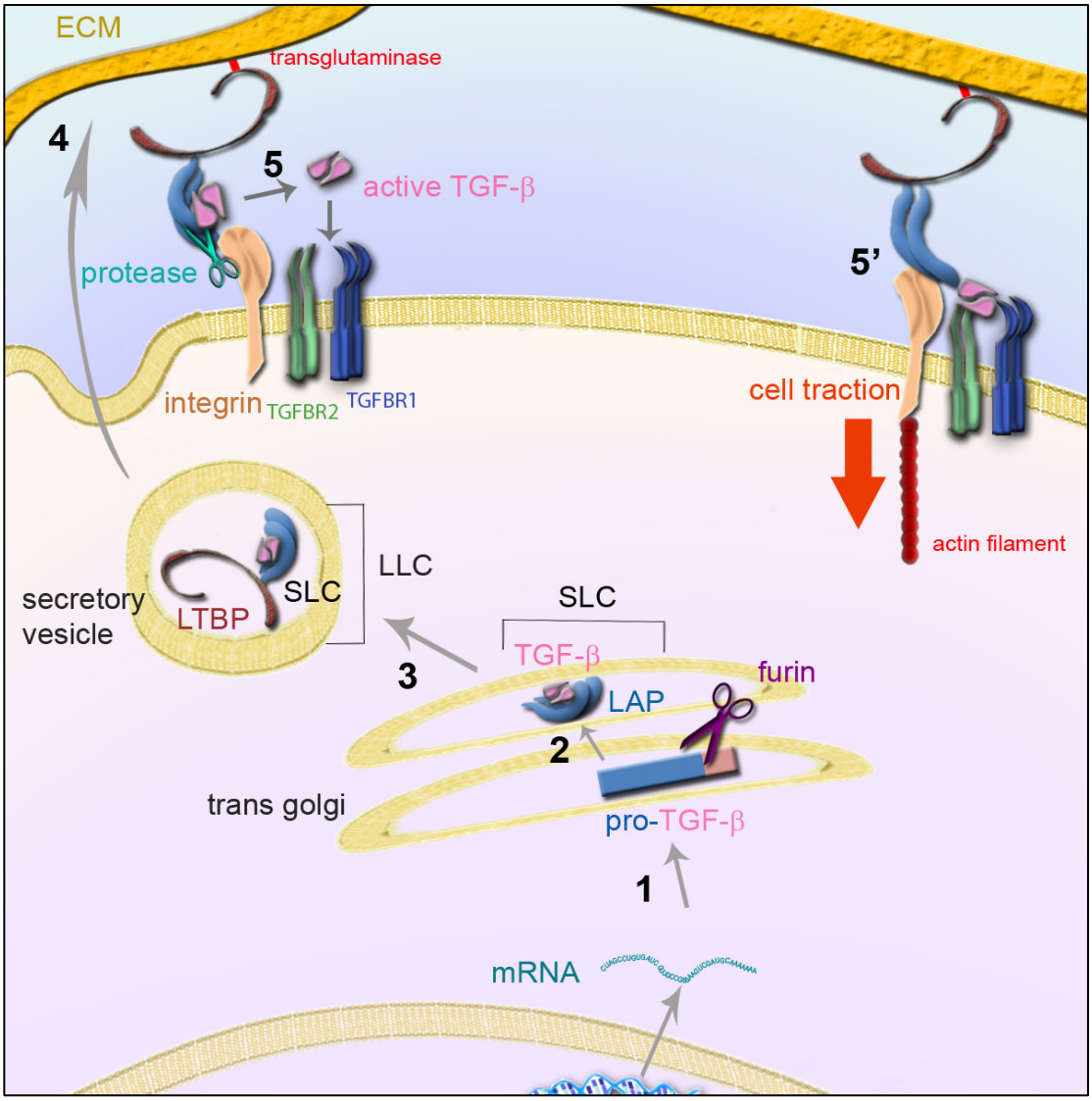


Figure 1.1: Schematic representation of TGF-β biosynthesis and activation.

1) TGF-β mRNA is transcribed as a pro-protein, containing a pro-peptide and the growth factor.

2) In the trans golgi, the pro-protein is cleaved by a proprotein convertase, such as furin. The pro-peptide (or Latent-associated-Protein, LAP) remains associated to TGF-β and dimerizes to form the small latent complex (SLC).

3) In secretory vesicles, SLC binds to LTBP, forming the large latent complex (LLC).

4) LLC is secreted and LTBP is covalently attached to the ECM via a transglutaminase link.

5) Integrin associates with both LAP and a protease, such as MMP, which cleaves LAP and release active TGF-β.

5') Alternatively, upon cell traction, integrin induces a conformational change in LAP, which activates TGF-β.

In addition, integrin clusters with TGF-β receptors.

determines the localization and distribution of TGF-β. The binding of latent TGF-β to matrix-associated proteins may play an important role in regulating TGF-β availability, by sequestering the ligand away from its receptor and by ensuring storage and control release of the ligand (Annes *et al.*, 2003).

As TGF-β is synthesized in excess, the activation of TGF-β is a limiting step controlling its bioavailability (Annes *et al.*, 2003). TGF-β activation is thus tightly regulated at a spatial and temporal level and in a subtype specific manner. Latent TGF-β can be activated by numerous processes, including heat, acid, reactive oxygen species, proteolysis, thrombospondin 1 (TSP-1) and/or integrin-mediated activation (Jenkins, 2008). Integrins αvβ5, αvβ6, αvβ8, and αvβ3 have been reported to bind TGF-β and participate in its activation in a subtype specific manner (Wipff and Hinz, 2008). Two main mechanisms, not mutually exclusive, have been proposed to explain how integrins activate latent TGF-β1: (1) Integrins αvβ3, αvβ5, αvβ6 and αvβ8 transmit cell traction forces that ultimately change the conformation of the latent TGF-β1 complex. This conformational change allows ligand activation and presentation to the receptor, but not necessarily release of free active TGF-β (Jenkins *et al.*, 2006). (2) integrins αvβ8 and αvβ3 could serve as docking proteins for latent TGF-β1 and for proteases, leading to enzymatic cleavage of the latent complex and release of active TGF-β1 (Wipff and Hinz, 2008). Moreover, integrins can recruit TGF-β receptors to locally activated TGF-β1 (Galliher and Schiemann, 2006; Scaffidi *et al.*, 2004). These mechanisms are illustrated in Fig 1.1.

Four different metalloproteinases (MMP) have been reported to activate TGF-β: MMP13 (or collagenase) (D'Angelo *et al.*, 2001), MMP2 and MMP9 (or gelatinase A and B) which use the integrin αvβ3 as a docking protein (Brooks *et al.*, 1996; Rolli *et al.*, 2003), and the transmembrane MMP14 (or MT1-MMP), which is responsible for TGF-β activation mediated by αvβ8 integrin (Mu *et al.*, 2002).

In addition, several serine proteases, including thrombin, plasmin, neutrophil elastase and mast cells chymase and tryptase, have been implicated in TGF-β activation in a cell-type specific manner (Jenkins, 2008). Thrombin can activate TGF-β by proteolytic cleavage (Taipale *et al.*, 1992). Moreover, thrombin, via the Protease Activated

Receptor-1 (PAR1), induces RhoA. In turn, RhoA promotes αvβ6 integrin activation of TGF-β (Jenkins *et al.*, 2006). Another protease, plasmin, cleaves ECM-associated-LTBP, at least *in vitro* (Jenkins, 2008). A second proteolytic cleavage release active TGF-β from the latent complex (Munger *et al.*, 1997). Plasmin is generated by proteolysis of plasminogen by the urokinase type plasminogen activator and the tissue type plasminogen activator. The plasminogen activator inhibitor-1 (PAI-1), a TGF-β target gene, inhibits plasmin production, thus forming a negative feedback loop (Jenkins, 2008).

Thrombospondin 1 (TSP-1), a glycoprotein secreted by activated platelet has been shown to activate latent TGF-β1 both *in vitro* and *in vivo* (Ahamed *et al.*, 2009). TSP-1 binds to plasminogen and facilitates its conversion into plasmin by the tissue type plasminogen activator (Jenkins, 2008). TSP-1 can also activate TGF-β independently of plasmin generation, by inducing a conformational change in LAP and exposing the receptor binding sites of the TGF-β (Schultz-Cherry *et al.*, 1994). The efficient activation of latent TGF-β by TSP-1 may require additional molecules, such as the αvβ3 integrin, that cluster all components at the cell surface (Harpel *et al.*, 2001; Yehualaeshet *et al.*, 1999). By all these mechanisms, TGF-β is activated in specific locations.

1.1.4- Structure of mature TGF-β

The structure of mature TGF-β family members is highly similar: they exist as dimers (homodimers in the case of the TGF-β1, 2 and 3) of 2 subunits of 12.5kDa and they all possess between six and nine conserved aligned cysteines that form a cysteine-knot. Four cysteine pairs are formed intramolecularly and the sixth cysteine (Cys 77) forms an intermolecular disulfide bond, resulting in dimer formation (Hinck *et al.*, 1996; Mittl *et al.*, 1996; Schlunegger and Grutter, 1992). The crystal structure of TGF-β subunit is often described as a hand: the wrist region is formed by a long helix (named H3), the knuckle region is located on the convex side formed by two anti-parallel β sheets, the fingers are formed by the four β strands and the thumb is made by the N-terminal sequence. The

dimeric interaction occurs between the H3 of one subunit and the β sheet of the other subunit and is stabilized by the intermolecular disulfide bond, thus giving the dimer a "butterfly" shape (Shi and Massagué, 2003).

1.2- Activation of TGF-β receptors

1.2.1- Structure of TGF-β receptors

The type I and type II TGF-β receptors (named TGFBR1 and TGFBR2) are structurally conserved serine/threonine kinases of 503 and 567 amino acids, respectively. They consist of a C-terminal intracellular domain with kinase activity, a single transmembrane domain and a short N-terminal extracellular ligand-binding domain subjected to N-glycosylation. They function as a heterotetramer formed by 2 type I and two 2 type II receptors, which bind to one TGF-β dimer (Feng and Derynck, 2005). The structure of the extracellular domain of TGF-β receptor is similar to a class of toxin and known as three-finger toxin fold (Greenwald *et al.*, 1999). The fold consist of three anti-parallel β sheets (β1-β2, β3-β4 and β5-β6), which all point in roughly in the same direction. This fold is dictated by four disulfide bonds formed by several conserved cysteines. The molecule has concave and convex interfaces arising mainly from the curvature of the first sheet (β1-β2) (Greenwald *et al.*, 1999).

1.2.2- Binding of TGF-β to its receptors

Upon TGF-β stimulation, the TGF-β receptor complex assembles sequentially, as illustrated in Fig 1.2. First, the ligand binds to the high affinity TGFBR2 receptor and induces the dimerization of TGFBR2. By imaging single molecule of green fluorescent protein (GFP)-labeled TGFBR2, Zhang et al. have demonstrated that the receptor exists as monomer at low expression level but that TGFBR2 dimerizes at high receptor concentration or following TGF-β addition (Zhang *et al.*, 2009). TGFBR2 does not undergo significant structural rearrangement upon binding and no contact is made

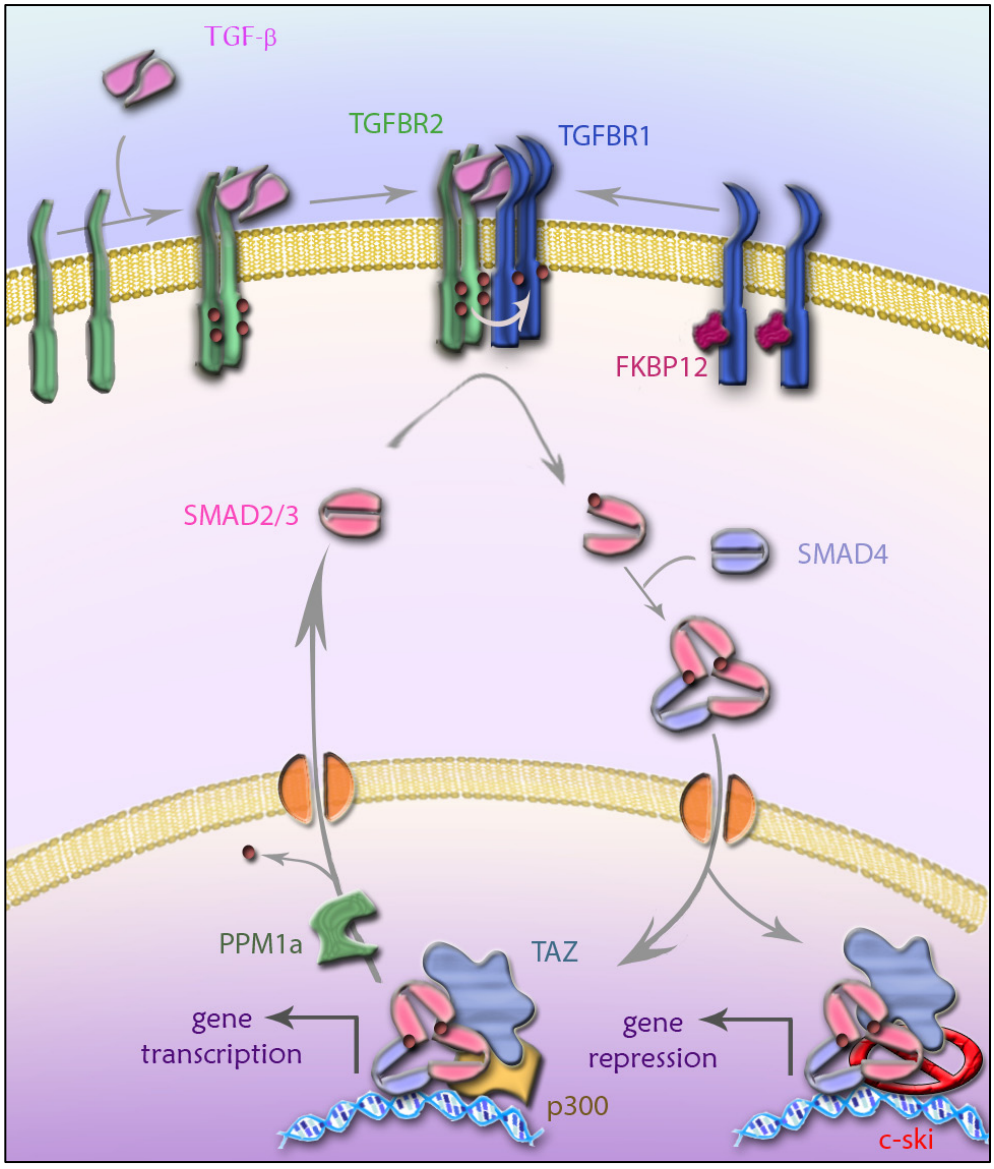


Figure 1.2: Schematic representation of the canonical TGF-β pathway. TGF-β induces dimerization of TGFBR2 and recruitment of TGFBR1. The constitutively active kinase of TGFBR2 phosphorylates TGFBR1 on its GS domain, leading to disruption of TGFBR1-FKBP12 interaction and activation of TGFBR1 kinase. TGFBR1 phosphorylates SMAD2/3 on its C-terminal SSXS motif. The phosphorylated SMAD2/3 forms a heteromeric complex with SMAD4. This complex accumulates in the nucleus, where it binds TAZ. SMAD2/3 associates with co-activators, such as p300 or co-repressors, such as c-ski to activate or repress gene transcription, respectively. The phosphatase PPM1A dephosphorylates SMAD2/3, leading to dissociation of SMAD4-SMAD2/3 complex. The SMADs are exported out of the nucleus, where they can be activated again by TGFBR1.

between the two TGF-β-bound TGFBR2 extracellular domains, as evidence by crystallography (Hart *et al.*, 2002).

After binding of TGF-β, the TGFBR2-TGF-β complex recruits the low affinity TGFBR1 (Fig 1.2). Similar to TGFBR2, TGFBR1 exists as a monomer at basal state and ligand addition induces its dimerization (Zhang *et al.*, 2010) and complex formation with TGFBR2. Surface Plasmon Resonance analysis using the TGF-β3 ligand confirms that the extracellular domain of TGFBR2 binds the ligand with high affinity (KD≈ 0.5μM). In contrast, TGFBR1 extracellular domain has low affinity for the ligand alone (KD≈ 188μM) but its affinity increases when the ligand is in complex with TGFBR2 (KD≈ 0.6μM), indicating cooperative assembly (Groppe *et al.*, 2008). Crystal structure of the ternary complex TGFBR2+ TGFβ3+ TGFBR1 reveals that the extracellular domain of TGFBR1 binds to both ligand and TGFBR2 extracellular domain (Groppe *et al.*, 2008). This result is in contrast to the structure of the BMP receptor complex, where no contact between the two receptor types is observed when the ligand is bound (Allendorph *et al.*, 2006). Moreover, BMP receptors interact with different regions of the ligand than TGF-β receptors (Groppe *et al.*, 2008). Three residues are critical for TGF-β3 binding to TGFBR2: TGF-β3-Val92 contacts TGFBR2-Ile53, TGF-β3-Arg25 binds to TGFBR2-Glu119 and TGF-β3-Arg94 interacts with TGFBR2 Asp32, Glu55 and Glu75. These three residues are unchanged in TGF-β1 but are substituted in TGF-β2 by Lys25, Ile92 and Lys94. The substitution in TGF-β2 is responsible for the lower affinity of this subtype for the TGFBR2 (about 1000 fold weaker) (De Crescenzo *et al.*, 2006).

1.2.3- Transphosphorylation of TGF-β type I receptor

TGFBR2 is a constitutively active kinase. Its full activity requires autophosphorylation at Ser213 and Ser409 (Luo and Lodish, 1997). Binding of TGF-β to the TGF-β receptor brings the constitutively active kinase of TGFBR2 at proximity to the GS (Gly-Ser) motif of TGFBR1. This GS motif is a 30 amino acids sequence located immediately upstream the kinase domain of TGFBR1 and is conserved among all type I receptor of the TGF-β superfamily. The transphosphorylation of multiple serine and threonine residues of the

GS motif by TGFBR2 is required for the downstream type I kinase to adopt a high activity state (Wieser *et al.*, 1995). The mutation T204D (a residue from the GS motif) renders the type I kinase constitutively active (Wieser *et al.*, 1995).

In the absence of ligand, the immunophilin FKBP12 binds to the GS region and prevents its inadvertent phosphorylation (Chen *et al.*, 1997). FKBP12, by pressing against the active center of TGFBR1 kinase, imposes a catalytically inactive conformation of the kinase (Huse *et al.*, 1999). The phosphorylation of the GS domain by TGFBR2 eliminates the binding site for FKBP12, while creating a new binding site for its substrates, the R-SMADs (Wrighton *et al.*, 2009). A model of the classical TGF-β pathway is shown in Fig1.2.

1.2.4- The possible combinations of TGF-β superfamily receptors

Ligands of the TGF-β superfamily bind to a specific combination of type I (also termed Activin receptor-like kinase) and type II receptors, which activate a particular group of R-SMADs, as illustrated in table 1 below:

	ligand	Type II receptor	Type I receptor	R-SMADs
Classical model	TGF-β	TGFBR2	ALK5 (TGFBR1)	SMAD2 SMAD3
	Activin Nodal	ACVR2 ACVR2B	ALK4 (ACVR1B) ALK7 (ACVR1C)	
	BMP	BMPR2 ACVR2	ALK1 ALK2 (ACVR1) ALK3 (BMPR1A) ALK6 (BMPR1B)	SMAD1 SMAD5 SMAD8
Alternative model in endothelial cells and chondrocytes	TGF-β	TGFBR2	ALK5 + ALK1	SMAD1 SMAD5
Alternative model in epithelial cells	TGF-β	TGFBR2	ALK5 + ALK2 ALK5 + ALK3	SMAD1 SMAD5

Table 1: Combinations of TGF-β superfamily ligands with their type I and type II receptors and the associated R-SMADs.

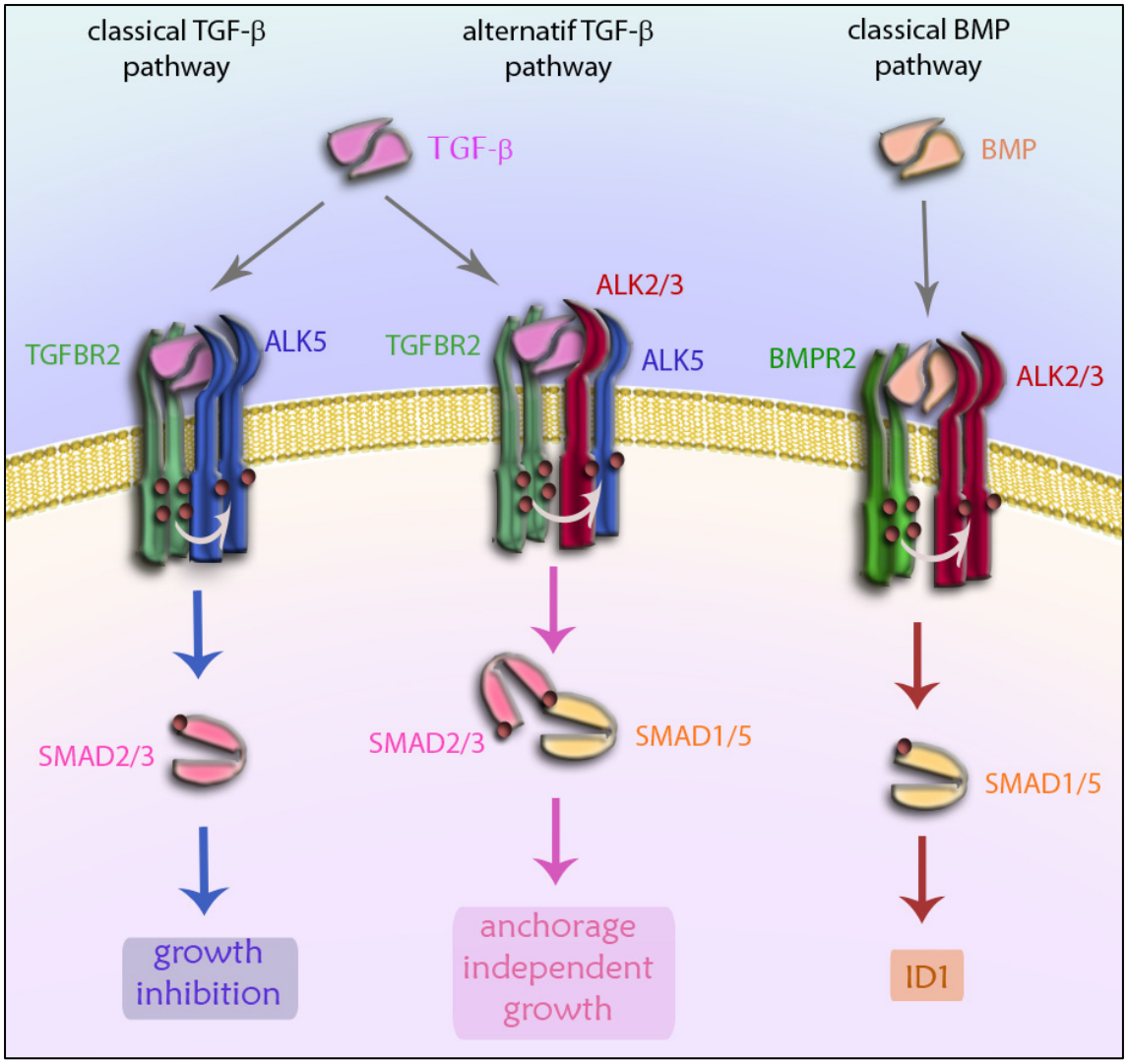


Figure 1.3: Schematic representation of the different combinations of TGF-β superfamily receptors occurring in epithelial cells.

In the classical model, TGF-β signals via TGFBR1 (also termed ALK5) and TGFBR2. Upon transphosphorylation of ALK5 by TGFBR2, ALK5 phosphorylates its substrates, SMAD2 and SMAD3. Activation of SMAD2/3 is involved in TGF-β-induced growth inhibition of epithelial cells.

BMP signals via the type I and type II BMP receptors, such as ALK2 or ALK3 and BMPR2. ALK2/3 phosphorylates its substrates SMAD1 and SMAD5, which induces the transcription of the inhibitor of DNA binding ID1.

Alternatively, TGF-β is thought to signal through a complex formed with both ALK5 and ALK2/3 in epithelial cells. This results in activation of SMAD1/5, formation of a mixed R-SMADs complex and in regulation of TGF-β-induced anchorage independent growth.

In the classical model, TGF-β signals via the type II receptor TGFBR2 and the type I receptor ALK5 (Feng and Derynck, 2005). The L45 loop of the type I receptor, located just downstream of the GS motif, defines which group of R-SMAD is recruited. The sequences of the L45 loop of ALK5 and ALK4 are identical and both bind to SMAD2 and 3, but they differ in four to seven amino acids from the L45 sequences of ALK1, ALK2, ALK3 and ALK6 that recruits SMAD1/5/8 (Feng and Derynck, 2005).

However, TGF-β is able to activate different receptor combinations in addition to the classical TGFBR2/ALK5 complex in a cell-specific manner and can therefore elicit different responses. In certain cell types, including endothelial cells and chondrocytes, TGF-β can signal via the classical TGFBR2+ ALK5 heteromeric complex to activate SMAD2 and 3, but also via a mixed TGFBR2+ALK5+ALK1 complex, and thereby activate SMAD1 and 5 (Finnson *et al.*, 2008; Goumans *et al.*, 2002; Goumans *et al.*, 2003). The ALK1-SMAD1/5 pathway opposes signalling via the ALK5-SMAD2/3 pathway (Finnson *et al.*, 2008; Goumans *et al.*, 2002). In addition, TGF-β can induce SMAD1/5 in several epithelial cells via ALK2 or ALK3, depending on the cell type. This activation of SMAD1/5 by TGF-β is not required for growth inhibition but is required for TGF-β induced anchorage-independent growth (Daly *et al.*, 2008). A schematic representation of the diverse combinations of TGF-β superfamily receptors occurring in epithelial cells is shown in Fig 1.3.

However, analysis of the crystal structure of the classical TGF-β receptor complex indicates that if a mixed complex composed of 2 TGFBR2+ ALK5+ALK1 (or ALK2 or ALK3) binds the TGF-β ligand, it may require a different set of interactions than in the classical complex and/or the presence of an adaptor protein (Groppe *et al.*, 2008). Moreover, another study has suggested that the L45 loop of ALK5 alone could mediate SMAD1/5 recruitment in tumorigenic but not in immortalized cells (Liu *et al.*, 2009b).

Furthermore, while TGFBR2 cannot bind to TGF-β2 on its own, some cells express a splice variant of TGFBR2, TGFBR2-B. TGFBR2-B contains a 26 amino acids insert after Ser31, replacing TGFBR2-Val32, a critical residue for interaction with TGF-β (De

Crescenzo *et al.*, 2006) and can thus interact with TGF-β2 in the presence of TGFBR1 (del Re *et al.*, 2004; Parker *et al.*, 2006; Rotzer *et al.*, 2001).

Together, these studies indicate that TGF-β elicits diverse responses via recruitment of different combinations of type I and type II receptors.

1.3- TGF-β signalling through the SMAD (canonical) pathway

1.3.1- Different classes of SMADs and their domains

SMADs were identified based on the homology with drosophila MAD (Mother against Decapentaplegic) protein and Caenorhabditis elegans Sma proteins. In mammals, there are 8 different SMADs, grouped into three different categories: (1) the R-SMAD (Receptor-activated SMAD), composed of SMAD2 and SMAD3, that are activated by ALK5, ALK4 and ALK7 and SMAD1, 5 and 8, activated by ALK1, 2, 3 and 6; (2) the common SMAD or co-SMAD, SMAD4, which forms a complex with the activated R-SMAD; and (3) the inhibitory SMADs or I-SMADs, SMAD6 and SMAD7, that inhibit TGF-β/BMP signalling.

SMAD2 and SMAD4 are essential for embryogenesis, as demonstrated by the phenotype of *Smad2-/-* and *Smad4 -/-* mice who die during embryonic development at day e8.5 and e6.5, respectively (Weinstein, 1998; Sirard, 1998). *Smad3 -/-* mice are viable but develop mucosal inflammation and inflammation-induced colon cancer (Zhu Y, 1998).

The R-SMADs and SMAD4 are proteins of about 500 amino acids (about 50kDa). They consist of two globular domains, the MAD-Homology (MH)1 and MH2 domains, joined by a less conserved linker region. This linker region includes a PY motif (in all SMADs except SMAD4 and SMAD8) that binds to WW domain containing proteins and several sites of regulatory phosphorylations (Attisano and Tuen Lee-Hoeflich, 2001; Massagué *et al.*, 2005).

The N-terminal MH1 domain contains binding sites for several transcription factors, including Jun, TFE3, Sp1 and Runx and mediates DNA binding via a β-hairpin structure (Attisano and Tuen Lee-Hoeflich, 2001). The β-hairpin is an 11 amino acid sequence

conserved in SMAD4 and all R-SMADs (but SMAD2) that forms hydrogen bonds with three base pairs of the SMAD-binding element (SBE) (Shi *et al.*, 1998). Although SMAD1 and SMAD3 share the same β-hairpin sequence, the structure of their DNA contact interface is different (BabuRajendran *et al.*, 2010). This may explain why SMAD1 and SMAD3 elicit different responses. In SMAD2, the insertion of 30 amino acids encoded by the exon 3 displaces the β-hairpin and prevents DNA binding. Thus, SMAD2 requires interaction with co-factor(s) in order to exert its transcriptional activity (Dennler *et al.*, 1999).

The C-terminal MH2 domain possesses a transcriptional activation domain. The MH2 domain of the R-SMADs contains a hydrophobic corridor responsible for binding to cytoplasmic retentions factors, to components of the nuclear pore complex and to DNA-binding cofactors. In SMAD4, a region overlapping the linker and the MH2 domains is involved in binding to transcriptional activators and repressors and is termed SAD (SMAD4 activation domain) (Attisano and Tuen Lee-Hoeflich, 2001; Massagué *et al.*, 2005).

Importantly, the MH2 domain of the R-SMADs contains a binding site for the activated TGFBR1, the L3 loop, and a SSXS motif located at the carboxyl terminus that is phosphorylated by TGFBR1. SMAD4 and the I-SMADs lack this SSXS motif (Attisano and Tuen Lee-Hoeflich, 2001; Massagué *et al.*, 2005).

1.3.2- Activation of R-SMADs

TGF-β binding to its receptors results in the direct phosphorylation of R-SMADs by the type I receptor, as illustrated in Fig 1.2. Upon TGF-β stimulation, the L45 loop of the activated TGFBR1 recognizes the L3 loop of an R-SMAD. As for the L45 loop, the specificity of the SMAD towards its receptor is determined by the L3 loop. Indeed, the L3 loop of SMAD2 and 3 are identical and binds to ALK5, ALK4 and ALK7, but they differ in two residues from the SMAD1/5/8 group (Feng and Derynck, 2005). A sequence

downstream of the L3 loop forms a basic/ positively charged pocket and stabilizes the interaction between the R-SMAD and TGFBR1 (Huse *et al.*, 2001).

The efficient recruitment of SMAD2/3 to the receptor requires SARA (SMAD anchor for receptor activation), a FYVE domain-containing protein. Via the interaction of its FYVE domain with the membrane lipid PI3P, SARA is localized at the plasma membrane and is enriched in early endosome (see section 1.6 below). SARA contains a SMAD-binding domain that associates with the hydrophobic corridor of SMAD2/3. Therefore, SARA facilitates SMAD2/3 localization to the membrane and enhances the interaction between SMAD2/3 and TGFBR1 (Tsukazaki *et al.*, 1998). Other proteins may also facilitate the interaction between TGFBR1 and the R-SMAD, such as the FYVE domain-containing protein Hgs, the β spectrin ELF, the clathrin adaptor Disabled-2 and Axin, a negative regulator of the Wnt signalling (Furuhashi *et al.*, 2001; Hocevar *et al.*, 2001; Miura *et al.*, 2000; Tang *et al.*, 2003).

The complex formation between TGFBR1 and R-SMADs results in phosphorylation of the last two serine residues of the SSXS motif. SMAD2/3 phosphorylation usually peaks 15-30 minutes after TGF-β addition and lasts several hours. The phosphorylation of SMAD2/3 destabilizes the interaction of SMAD2/3 with SARA, allowing dissociation of SMAD2/3 from the complex. The phosphorylated tail of R-SMAD interacts with the basic pocket of SMAD4, resulting in R-SMAD-SMAD4 complex formation (Feng and Derynck, 2005)(Fig 1.2).

In the current model, the R-SMADs and SMAD4 are thought to exist mainly as monomer in resting cells (Hill, 2009), although a previous study has shown that SMAD3 and SMAD4 may also exist as oligomers (Jayaraman and Massagué, 2000). SMAD4 possesses a unique H3/4 loop that prevents its oligomerization at basal state (Tada *et al.*, 1999). Similarly, SMAD2's MH1 domain interacts with its MH2 domain and prevents the formation of oligomers in resting cells (Hata *et al.*, 1997). Upon TGF-β treatment, the phosphorylation of the SSXS motif relieves the autoinhibitory interaction and promotes the association with SMAD4. Depending on factors present in the transcriptional complex, the activated R-SMAD and SMAD4 form heterodimers (1 SMAD4+ 1 R-SMAD)

or heterotrimers (2 identical R-SMADs + 1 SMAD4). Higher order assemblies and complexes containing SMAD2+ SMAD3+ SMAD4 have also been reported but their function is unknown (Jayaraman and Massagué, 2000). The oligomer formation is stabilized by extensive protein-protein interaction between the phosphorylated C-tail and the L3 loop of the other subunit (Chacko *et al.*, 2001). Thus, the unphosphorylated oligomers are likely to be unstable. Recently, it has been shown that the linker region of SMAD3 plays also a role in oligomerization in response to TGF-β (Vasilaki *et al.*, 2009). In most cases, SMAD4 preferentially participates to the complex formation due to specific residues on its MH2 domain, Asp493 and Arg378 that mediate complementary interaction with R-SMADs. However, in some instances, the inhibitor of NF-κB subunit α (IKKα) can replace SMAD4 and mediates some of the SMAD4-independent responses (Descargues *et al.*, 2008; He *et al.*, 2006). Notably, IKKα-SMAD2/3 mediates keratinocyte differentiation in a SMAD4-independent manner (Descargues *et al.*, 2008).

1.3.3- Nucleocytoplasmic shuttling of SMADs

The SMADs continuously shuttle between the cytoplasm and the nucleus, in the presence or absence of ligand. In the absence of TGF-β, SMAD4 localizes equally between the cytoplasm and the nucleus. In contrast, R-SMADs are found predominantly in the cytoplasm (Hill, 2009).

Upon TGF-β treatment, SMAD2/3-SMAD4 complexes slowly accumulate into the nucleus. The maximal nuclear localization signal is typically reached 45 minutes after ligand addition and lasts for 4 to 5hrs. The nuclear import of the SMADs is mediated by importin-dependent pathways or by direct interaction with nuclear pore components, depending on the SMADs and their oligomerization state (Chapnick and Liu, 2010; Chen *et al.*, 2005; Xu *et al.*, 2002). Phosphorylation of SMAD2/3 increases their interaction with importins and their nuclear import, as compared to unphosphorylated R-SMADs (Kurisaki *et al.*, 2001; Xiao *et al.*, 2000; Xu *et al.*, 2007).

Moreover, Varelas et al. have recently shown that TAZ is required for the nuclear accumulation of SMAD complexes in presence of TGF-β (Fig 1.2). In the nucleus, TAZ forms a complex with both the transcriptional machinery component Mediator and with SMAD2/3, but not with SMAD1/5/8 (Varelas *et al.*, 2008).

The export of SMADs complexes is achieved following dephosphorylation of the SSXS motif, leading to loss of affinity of the R-SMADs for the basic pocket of SMAD4 and dissociation of the complex. Because the association between SMAD4 and R-SMADs physically blocks the interaction between the Nuclear export sequence (NES) of SMAD4 (localized on its linker region) and CRM1/exportin1 (Chen *et al.*, 2005), dephosphorylation is a prerequisite for SMAD4 nuclear export. Once the complex is dissociated, SMAD2 and SMAD3 are exported via exportin 4 (Kurisaki *et al.*, 2006) or via RANBP3, a component of CRM1, which recognizes the unphosphorylated form of SMAD2/3 (Dai *et al.*, 2009). Recently, the nuclear phosphatase PPM1A has been shown to dephosphorylate R-SMAD, thereby increasing complex dissociation and nuclear export of the SMADs (Lin *et al.*, 2006) (Fig 1.2). The C-tail of SMAD1, but not SMAD2/3 can also be dephosphorylated by the nuclear phosphatases SCP1, 2 and 3 (Knockaert *et al.*, 2006). SMAD shuttling is thought to involve constant low level of R-SMAD dephosphorylation, resulting in export of monomeric SMADs to the cytoplasm. If the receptors are still active, the R-SMADs are re-phosphorylated and return to the nucleus. If the receptors are no longer active, the SMADs accumulate in the cytoplasm. Thus, nucleocytoplasmic shuttling guarantees that the concentration of active SMAD within the nucleus reflects the activity of the receptor complexes (Hill, 2009).

1.3.4- Regulation of gene expression by the SMAD complexes

In the nucleus, the SMADs activate or repress different set of genes, by interacting via their β-hairpin with the SMAD-binding element (SBE): 5'-GTCT-3' or its reverse complement 5'-AGAC-3' (Feng and Derynck, 2005; Massagué *et al.*, 2005). However, a single SBE is not sufficient to allow binding and transcriptional activity by the SMADs (Shi *et al.*, 1998). Although artificial concatemers of SBEs display a higher affinity for the

SMADs (Zawel *et al.*, 1998), natural promoters rarely contain SBE concatemers. Even when natural promoters contain four SBE, the SMADs still need additional cofactors to bind DNA efficiently (Massagué *et al.*, 2005). In addition, SMADs can bind to "degenerate" SBEs, such as the 5'-GGCTT-3' sequence found in the *c-MYC* promoter (called TGF-β inhibitory element (TIE)) (Chen *et al.*, 2002). Moreover, SMADs interact with GC rich sequences, as it is the case in the promoter of SMAD6. This sequence is termed BMP-responsive element (BRE) or SMAD1-binding element, but SMAD1/5/8 can also bind to SBEs (Massagué *et al.*, 2005).

SMAD complexes associate with a wide diversity of transcription factors, depending on the cell type and the activation states of other signalling pathways. The association with transcription factors can be specific for one subset of SMADs and thus may confer some specificity (Feng and Derynck, 2005; Massagué *et al.*, 2005).

The SMADs can bind to transcription factors of the forkhead family, such as FoxH1/FAST1, FoxO1, FoxO3 and FoxO4. Such interactions are involved in the activation of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ (Seoane *et al.*, 2004). Sp1, a Zn finger transcription factor is another partner of SMADs and the SMAD-Sp1 complex regulates the expression of $p15^{INK4b}$ and $p21^{Cip1}$ to mediate growth arrest (Feng and Derynck, 2005; Massagué *et al.*, 2005). In addition, SMADs cooperate with AP1 transcription factors to enhance c-jun expression. Furthermore, SMADs bind to c-jun, a basic-leucine zipper (bZIP) domain containing transcription factor, to regulate another set of genes (Feng and Derynck, 2005; Massagué *et al.*, 2005). SMADs also interact with other bZIP transcription factors, such as JunB, ATF2, ATF-3 and c-Fos (Feng and Derynck, 2005). The recruitment of ATF3 is particularly important for the repression of *ID1*. Moreover, SMAD can also cooperate with transcription factors of the RUNX family and with basic helix-loop-helix (bHLH) transcription factors, such as TFE3 (implicated in the regulation of PAI-1 and SMAD7 by SMAD3) (Hua *et al.*, 1998; Hua *et al.*, 2000), MyoD and myogenin, c-MYC and Max and with E2F family members to repress of *c-MYC* (Massagué *et al.*, 2005). This repression of *c-MYC* is involved in TGF-β-induced growth inhibitory response (Meulmeester and ten Dijke, 2011).

Importantly, SMADs recruit not only DNA-binding transcription factors, but also co-activators and co-repressors to modulate the amplitude of the transcriptional activation. For example, SMADs can bind to CBP/p300 and to the Mediator complex (Fig 1.2). These transcriptions co-activators then recruit the RNA polymerase II complex (Feng and Derynck, 2005). Moreover, p300 and CBP display histone actelytransferase activity that causes chromatin decompaction, facilitating DNA transcription. The binding of R-SMADs to CBP/p300 requires the phosphorylation of their C-terminus and their MH2 domain and additional interaction with the linker region (Wang *et al.*, 2005). The SAD domain of SMAD4 stabilizes the interaction between the R-SMAD and CBP/p300 (de Caestecker *et al.*, 2000).

SMADs can also interact with corepressor, such as c-Ski/SnoN, c-MYC and the homeobox transcription factor TGIF to repress gene transcription (Fig 1.2) (Feng and Derynck, 2005; Massagué *et al.*, 2005). c-ski and TGIF have been shown to recruit histone deacetylases. In addition, c-Ski mediates the dissociation of the R-SMAD-SMAD4 complex. By these mechanisms, c-ski negatively regulates TGF-β-induced growth inhibition: it inhibits the expression of $p15^{INK4B}$ and relieves *c-MYC* repression (Wu *et al.*, 2002). Finally, c-MYC associates with SMAD2/3 and Sp1 at the $p15^{INK4b}$ promoter and inhibits the transcriptional activity of Sp1-SMAD (Feng *et al.*, 2002). A fine balance between co-repressors and co-activators determine SMAD transcriptional responses. SMADs modulate a broad range of cellular responses, including cell cycle arrest, differentiation and ECM production, underscoring the need for a tight regulation of TGF-β signalling pathway.

1.4- Negative regulation of the TGF-β signalling pathway

TGF-β signalling is finely regulated, temporally and spatially, at different levels. There are several mechanisms to turn off TGF-β signalling, such as dephosphorylation (by PPM1A for example) and degradation of the activated components of the pathway. Some of

these mechanisms are controlled by negative regulators: the Inhibitory-SMADs (I-SMADs) (Itoh and ten Dijke, 2007).

1.4.1- Negative regulation by the Inhibitory SMADs

One of the major regulators of the TGF-β pathway is the 3rd class of SMADs: the Inhibitory-SMADs, SMAD6 and SMAD7. The importance of SMAD7 in regulating TGF-β signalling is illustrated by the severe phenotype of transgenic mice overexpressing *Smad7* in the epidermis (K5.*Smad7* mice). These mice exhibit defect in eyelid and cornea development, aberrant hair follicle formation and epithelial hyperplasia, resulting from increased proliferation and reduced apoptosis (He *et al.*, 2002a).

TGF-β superfamily ligands induce the expression of SMAD6 and SMAD7, thus creating a negative feedback loop. SMAD2/3-SMAD4 complexes mediate SMAD7 expression through interaction with a SBE on *SMAD7* promoter (Nagarajan *et al.*, 1999; Stopa *et al.*, 2000). In addition, inflammatory cytokines such as IL-1, IFN-γ and TNF-α as well as EGF, ultraviolet radiation, TPA and laminar shear stress can also induce the expression of SMAD6 and SMAD7 in a cell-type-dependent manner (Afrakhte *et al.*, 1998; Yan *et al.*, 2009b), conferring regulation of the TGF-β pathway by other signals.

Unlike the R-SMADs, the I-SMADs do not have an MH1 domain or a SSXS motif, but they possess an MH2 domain. The N domains of SMAD7 and SMAD6 have only 37% amino acids sequence identity. This difference might explain some of the divergent responses observed for SMAD6 and SMAD7: SMAD7 inhibits both TGF-β and BMP pathway whereas SMAD6 is more specific for the BMP pathway (Miyazono, 2008). Both SMAD6 and SMAD7 negatively regulate TGF-β/BMP signalling by diverse mechanisms. In addition, they can serve as scaffold proteins to promote non-canonical responses (see section 1.5).

Similar to the R-SMADs, the I-SMADs interact with the type I receptor via their L3 loop. Binding of SMAD7 or SMAD6 to the type I receptor does not result in phosphorylation of

the I-SMADs but blocks the access of the R-SMADs to the activated receptor (Hayashi *et al.*, 1997; Imamura *et al.*, 1997; Nakao *et al.*, 1997). Thus, the I-SMADs compete with R-SMADs for binding to the activated TGF-β receptors, resulting in a decrease in R-SMADs phosphorylation. The interaction between TGFBR1 and SMAD7 (but not SMAD6) can be stabilized by the WD-repeat protein STRAP and by Yes-associated protein (YAP)65 (Datta and Moses, 2000; Ferrigno *et al.*, 2002).

In addition to its ability to compete with R-SMADs for binding to type I receptors, SMAD6 has also been shown to interact with phospho-SMAD1 and therefore prevent the formation of SMAD1-SMAD4 complex (Hata *et al.*, 1998). Moreover, I-SMADs can interfere with R-SMADs transcriptional activity. For instance, SMAD7 interacts with SBE sequences via its MH2 domain and thus competes with the SMAD2+SMAD4+FoxH1 complex for DNA binding. As a result, TGF-β signalling is inhibited in the nucleus (Zhang *et al.*, 2007).

SMAD7 also promotes the dephosphorylation of TGFBR1, by recruiting GADD34, a subunit of the PP1 serine threonine phosphatase to the activated receptor (Shi *et al.*, 2004). This mechanism is thought to be facilitated by SARA, which binds and regulates PP1c subcellular localization and promotes the recruitment of SMAD7-GADD34 to PP1c (Shi *et al.*, 2004). Interestingly, in endothelial cells, TGF-β/ALK1 pathway upregulates SMAD7 and PP1α expression. SMAD7 recruits PP1α to ALK1, leading to ALK1 dephosphorylation and inhibition of SMAD1/5 signalling. In contrast, the ALK5 pathway is unable to do so (Valdimarsdottir *et al.*, 2006).

In addition to these above mentioned mechanisms, the I-SMADs promote degradation of the TGF-β receptor via the ubiquitin pathway by acting as an adaptor protein for E3 ubiquitin ligases, thus terminating TGF-β signalling (Ebisawa *et al.*, 2001; Kavsak *et al.*, 2000).

1.4.2- Regulation of TGF-β signalling by ubiquitination

1.4.2.1- The ubiquitin machinery

Proteins destined for degradation are commonly tagged by ubiquitin protein. This protein modification is realized by 3 different successive enzymes: E1, the ubiquitin activating enzyme; E2, the ubiquitin conjugating enzyme and E3, the ubiquitin protein ligase. The ubiquitin is first attached via a thioester bond to E1, in an ATP-dependent manner. Then, the activated ubiquitin is transferred to E2. There are two main classes of E3 ubiquitin ligases that mediate specificity towards the substrate: the RING and HECT domains E3. While the RING E3 transfers the ubiquitin moieties directly from the E2 to the substrate, the HECT E3 transfers the ubiquitin from the E2 to a Cysteine residue on their HECT domain and then to the substrate (Ravid and Hochstrasser, 2008).

Usually, polymers of ubiquitin on lys48 are attached to a protein, and this modification, called poly-ubiquitination, is recognized by the 26S proteasome. However, other ubiquitin modifications exist, such as Lys63 attachment and monoubiquitination. These modifications are thought to have other role than directing a protein for proteasomal degradation. For instance, they can be used as signal to target a protein for endocytosis (D'Azzo *et al.*, 2005).

1.4.2.2- Degradation of TGFBR1

<u>Degradation of TGFBR1 by SMAD7 and Smurf1/2</u>

Regulation of TGF-β receptor levels by ubiquitination and degradation ensure that proper amount of TGF-β receptor is expressed and can also lead to termination of the signalling. The SMAD ubiquitin related factor (Smurf)1 and Smurf2 are the first E3 ubiquitin ligases identified to target TGFBR1 for degradation (Ebisawa *et al.*, 2001; Kavsak *et al.*, 2000). They have redundant functions and while mice with individual knock-out of *Smurf1* or *Smurf2* are viable, double knock-out *Smuf1-/-*, *Smuf2-/-* mice die

around day e10.5 of gastrulation or neural tube defects (Narimatsu *et al.*, 2009). Smurf 1 and Smurf2 are expressed throughout early development and in most adult tissues. Smurf2 expression is induced by TGF-β treatment (via the PI3K/Akt pathway), thus creating a negative feedback loop (Ohashi *et al.*, 2005).

Smurf1 and Smurf2 contains a C-terminus HECT domain, an N-terminus C2 domain and in between two and three WW domains, respectively. The WW domain of Smurf interacts with the Pro-Pro-X-Tyr (PY) motif located on the linker region of SMAD7 (Ebisawa *et al.*, 2001; Kavsak *et al.*, 2000).

In resting cells, SMAD7 localizes principally in the nucleus. Co-expression of Smurf1/2 induces the translocation of the I-SMAD to the cytoplasm, through interaction of its NES located on its C-terminal domain with the nuclear export receptor CRM1 (Tajima *et al.*, 2003). Upon TGF-β stimulation, SMAD7 and Smurf2 are redistributed at the plasma membrane, with the C2 domain of Smurf2 acting in membrane targeting (Suzuki *et al.*, 2002). Once at the plasma membrane, SMAD7 interacts with the activated TGFBR1 and brings Smurf1 or Smurf2 at proximity to the receptor. The Smurfs then catalyze the poly-ubiquitination of SMAD7 and TGFBR1. The complex is then degraded by the proteasome and the lysosomal machinery, terminating TGF-β signalling (Ebisawa *et al.*, 2001; Kavsak *et al.*, 2000). The half-life of TGFBR2 (between 1hr and 2hr30) and the half-life of TGFBR1 (about 4-6hrs) decrease when the cells express SMAD7/Smurf2 (Chen, 2009).

In addition to facilitating TGFBR1 and its own ubiquitination, SMAD7 also enhances Smurf2 autoubiquitination and degradation (Ogunjimi *et al.*, 2005). Under basal conditions, the C2 domain of Smurf2 binds its HECT domain and occludes the catalytic cysteine, thereby inhibiting its own activity. SMAD7, by contacting the HECT domain of Smurf2, relieves this autoinhibition (Wiesner *et al.*, 2007). In addition, SMAD7, via a leucine rich motif on its N-terminal domain, recruits the E2 ubiquitin conjugating enzyme, UbcH7 to Smurf2 (Ogunjimi *et al.*, 2005). By these mechanisms, SMAD7 facilitates Smurf2 activity.

Regulation of SMAD7/Smurf1/2 activity

The activity of Smurf2 and SMAD7 is tightly regulated at several levels by a wide diversity of proteins and mechanisms: FKBP12 stabilizes the formation of ALK4, SMAD7/Smurf2 complex and enhances ALK4 ubiquitination (Yamaguchi *et al.*, 2006); AIMP4 stabilizes Smurf2 by preventing its autoubiquitination (Lee *et al.*, 2008); casein kinase-2 interacting protein-1 (CKIP-1) increases Smurf1, but not Smurf2 ubiquitin ligase activity (Lu *et al.*, 2008). On the other hand, Hsp90 prevent TGFBR1 from SMAD7/Smurf2-mediated degradation, thereby increasing TGF-β signalling (Wrighton *et al.*, 2008). SMAD7 levels are also controlled by the RING E3 ligase Arkadia (Liu *et al.*, 2006).

SMAD7 function can also be modulated by deubiquitination and by acetylation. The deubiquitinating enzyme UCH37 associates with SMAD7 and TGFBR1, counteracting Smurf2-mediated ubiquitination (Wicks *et al.*, 2005). The balance between ubiquitination and deubiquitination is likely to provide fine-tuning of TGF-β responses. In the nucleus, p300 acetylates lysine residues of SMAD7, protecting these lysine residues from ubiquitination by Smurf2 and stabilizing SMAD7 (Grönroos *et al.*, 2002). On the other hand, SMAD7 can also recruit histone deacetylases via its MH2 domain, which deacetylates SMAD7 and thus increases its ubiquitination and subsequent degradation (Simonsson *et al.*, 2005). Acetylation of SMAD7 may prevent its premature degradation by Smurfs in the nucleus, while deacetylation may help terminate the signal, by allowing ubiquitination of SMAD7. By these mechanisms, the levels of SMAD7 within the cell are tightly regulated.

Degradation of TGFBR1 via other E3 ubiquitin ligases

In addition to the Smurfs, SMAD7 also interacts with other HECT E3 ubiquitin ligases of the same family, such as WWP1/Tuil1 and NEDD4-2. These ubiquitin ligases have a distinct expression pattern from the Smurfs. WWP1/Tuil1 has four WW domains and interacts with all SMADs containing a PY motif (i.e. all SMADs but SMAD4 and SMAD8)

but does not ubiquitinate R-SMADs. Like Smurf1 and 2, WWP1/Tuil1 and NEDD4-2 bind to SMAD7 and induce its translocation out of the nucleus. Then, WWP1/Tuil1 and NEDD4-2 associate with TGFBR1 via SMAD7 and promote ubiquitin-mediated degradation of TGFBR1, leading to inhibition of TGF-β signalling, albeit with a lower efficiency than Smurf1 (Komuro *et al.*, 2004; Kuratomi *et al.*, 2005; Seo *et al.*, 2004).

1.4.2.3- Degradation of R-SMADs

Degradation of R-SMADs by the Smurfs

Smurf E3 ligases have been show to control the basal levels of some R-SMADs proteins, in order to avoid aberrant activation of the TGF-β signalling pathway. Smurf1 can mediate the proteasomal degradation of SMAD1 and SMAD5, in the absence of BMP signalling (Zhu *et al.*, 1999), while Smurf2 controls the degradation of SMAD1 and SMAD2 (Zhang *et al.*, 2001). Smurf2 cannot physically interact with SMAD4, due to the absence of PY motif on the linker region of SMAD4, and, although Smurf2 can bind to SMAD3, it is unable to ubiquitinate SMAD3 at least in some cell types (Zhang *et al.*, 2001). The interaction of Smurf2 and SMAD2 is increased by TGF-β treatment (Lin *et al.*, 2000), suggesting a role for the Smurfs in signal termination.

Phosphorylation of the linker region of SMAD1 has been shown to be a prerequisite for binding to Smurf1 (Fuentealba *et al.*, 2007; Sapkota *et al.*, 2007). Such phosphorylation of the linker region can be mediated by the MAPK and GSK3 (Fuentealba *et al.*, 2007; Sapkota *et al.*, 2007). SMAD1 and SMAD3 linker phosphorylations can also be mediated by the nuclear CDK8 and/or CDK9 following phosphorylation of SMAD's C-tail (Alarcón *et al.*, 2009). CDK8/9-dependent linker phosphorylations enhance on one hand the transcriptional activity of SMAD1 and SMAD3 and on the other hand, promotes SMAD1 binding to Smurf1 (Alarcón *et al.*, 2009) and SMAD2/3 interaction with the E3 ubiquitn ligase NEDD4L. SMAD2/3 is then targeted for degradation in the cytoplasm (Gao *et al.*, 2009), terminating TGF-β signalling.

Degradation of R-SMADs via other E3 ubiquitin ligases

Several other E3 ubiquitin ligases have been implicated in the regulation of SMADs steady-state levels as well as activated SMADs levels. The U-box-dependent E3 ubiquitin ligase CHIP downregulates SMAD1, SMAD3 and SMAD4 steady-state levels (Li *et al.*, 2004b; Xin *et al.*, 2005). Axin facilitates the phosphorylation of inactive SMAD3 by GSK3β, resulting in degradation of non-activated SMAD3 (Guo *et al.*, 2008). The E3 ubiquitin ligases NEDD4-2 and WWP1/Tuil1 promote the degradation of phosphorylated SMAD2 (Kuratomi *et al.*, 2005; Seo *et al.*, 2004) and phospho-SMAD3 degradation is mediated by the RING-H2 protein PRAJA and by Roc1, a component of the multisubunit RING E3 ligase Skp-1/Cul/F-box (SCF)$^{Fbw1a/\beta TRCP}$ complex (Fukuchi *et al.*, 2001; Saha *et al.*, 2005). Arkadia also induces the ubiquitination and degradation of phospho-SMAD2/3 but simultaneously enhances their transcriptional activity (Mavrakis *et al.*, 2007). Interestingly, a study has reported another role for ubiquitination of SMAD2. When SMAD2 is ubiquitinated by Itch/AIP4, SMAD2 is not targeted for degradation. Instead, this modification enhances SMAD2 phosphorylation by TGFBR1 and thus increases TGF-β signalling (Bai *et al.*, 2004).

1.4.2.4- Ubiquitination of SMAD4

Although SMAD4 does not have a PY motif, it has been proposed that Smurfs may ubiquitinate SMAD4, using the R-SMADs or the I-SMADs as adaptor proteins (Moren *et al.*, 2005). However, it is likely that other E3 ubiquitin ligases participate in the degradation of SMAD4. SMAD4 has been shown to interact with βTRCP and Skp2, two components of the SCF E3 ubiquitin complex, leading to ubiquitination and degradation of SMAD4 (Liang *et al.*, 2004; Wan *et al.*, 2004). Interestingly, some mutants of SMAD4 found in cancer bind Skp2 and βTRCP with higher affinity than SMAD4 wild-type, leading to increased degradation and to TGF-β unresponsiveness (Liang *et al.*, 2004).

SMAD4 function is controlled not only via polyubiquitination but also via monoubiquitination. In the nucleus, the monoubiquitin ligase Ectodermin/TIF1γ ubiquitinates SMAD4 in a ligand-dependent manner (Dupont *et al.*, 2009). The co-activator p300 enhances, while the co-repressor c-ski decreases SMAD4 mono-ubiquitination (Wang *et al.*, 2008). This modification disrupts the association between SMAD4 and the R-SMADs. The mono-ubiquitinated SMAD4 shuttles back to the cytoplasm, where it is deubiquitinated by FAM/USP9x (Dupont *et al.*, 2009). Similar to the cycle of phosphorylation and dephosphorylation of the R-SMADs, this cycle of monoubiquitination and deubiquitination may act as a sensor of TGF-β activity.

1.4.2.5- Control of TGF-β signalling by sumoylation

In addition to ubiquitination, components of the TGF-β signalling cascade can be sumoylated. Sumoylation is a protein modification involving the covalent attachment of SUMO, a ubiquitin-related polypeptide to a lysine residue by E1, E2 and E3 enzymes. Unlike ubiquitination, sumoylation does not target its substrate for degradation. SMAD4 is sumoylated by the E3 PIASγ (protein inhibitor of activated STATγ) and the E2 Ubc9. This modification can enhance SMAD4 stability and can increase or repress its transcriptional activity, depending on the promoter or on the recruitment of other proteins (Chang *et al.*, 2005; Lee *et al.*, 2003; Long *et al.*, 2004). In addition, SMAD3 can also be sumoylated by PIASγ, resulting in reduction of SMAD3 activity (Imoto *et al.*, 2003). Moreover, once activated, ALK5, but not the other ALKs, can be sumoylated. The sumoylation of ALK5 promotes its association with SMAD3, its subsequent phosphorylation and thus enhances TGF-β responses (Kang *et al.*, 2008).

1.5- TGF-β Signalling through non-canonical pathways

In addition to the canonical SMAD pathway, TGF-β is able to activate other signalling pathways, in a cell-type and cell-context dependent manner (Feng and Derynck, 2005).

Activation of these non canonical pathways broadens the diversity of TGF-β responses and can either synergize or antagonize SMAD signalling. The contribution of each of pathway in the regulation of a specific TGF-β response differs from cell type to cell type. For example, while TGF-β-induced growth arrest and PAI-1 expression are mediated via SMAD4 in HaCaT cells (Levy and Hill, 2005), they are mediated via SMAD4-independent pathways in embryonic fibroblasts (Sirard *et al.*, 2000).

1.5.1- TGF-β-induced MAPK pathways

The Mitogen Activated Protein Kinase (MAPK) pathways include the ERK, p38 and JNK pathways and consist of three sequentially acting kinases: the MAPK kinase kinases (MAPKKK or MEKK) are serine/threonine kinases that phosphorylate the MAPK Kinase (MAPKK or MEK). The MAPKK are dual specificity kinases and phosphorylate a MAPK on threonine and tyrosine residues. For instance, the MAPKKK Raf phosphorylates MEK 1 or 2, which in turn activates ERK1/2. Several MAPKKK participate in the JNK pathway, including TAK1, MEKK1-4 and MLK3. They activate MKK4 and MKK7, which phosphorylate JNK. MLK3, TAK1 or other MAPKKKs can phosphorylate MKK3 or MKK6 to activate p38 MAPK. Importantly, the function of these MAPKs highly depend on the duration, strength of the signal and on their location (Krishna and Narang, 2008).

1.5.1.1- TGF-β-induced ERK pathway

In many cell types, including epithelial cells and fibroblasts, a rapid activation of ERK occurs following TGF-β treatment. The kinetics of TGF-β-induced ERK phosphorylation depend on the cell types and on culture conditions (Zhang, 2009).

ERK is activated after tyrosine phosphorylation of TGF-β receptors (Lee *et al.*, 2007). Indeed, TGF-β receptors are evolutionary related to receptor tyrosine kinases (Huse *et al.*, 1999) and possess dual specificity kinase activity, although the tyrosine kinase activity is much lower than their serine/threonine activity (Lawler *et al.*, 1997). Upon TGF-β stimulation, TGFBR1 is subjected to tyrosine phosphorylation (either by itself or

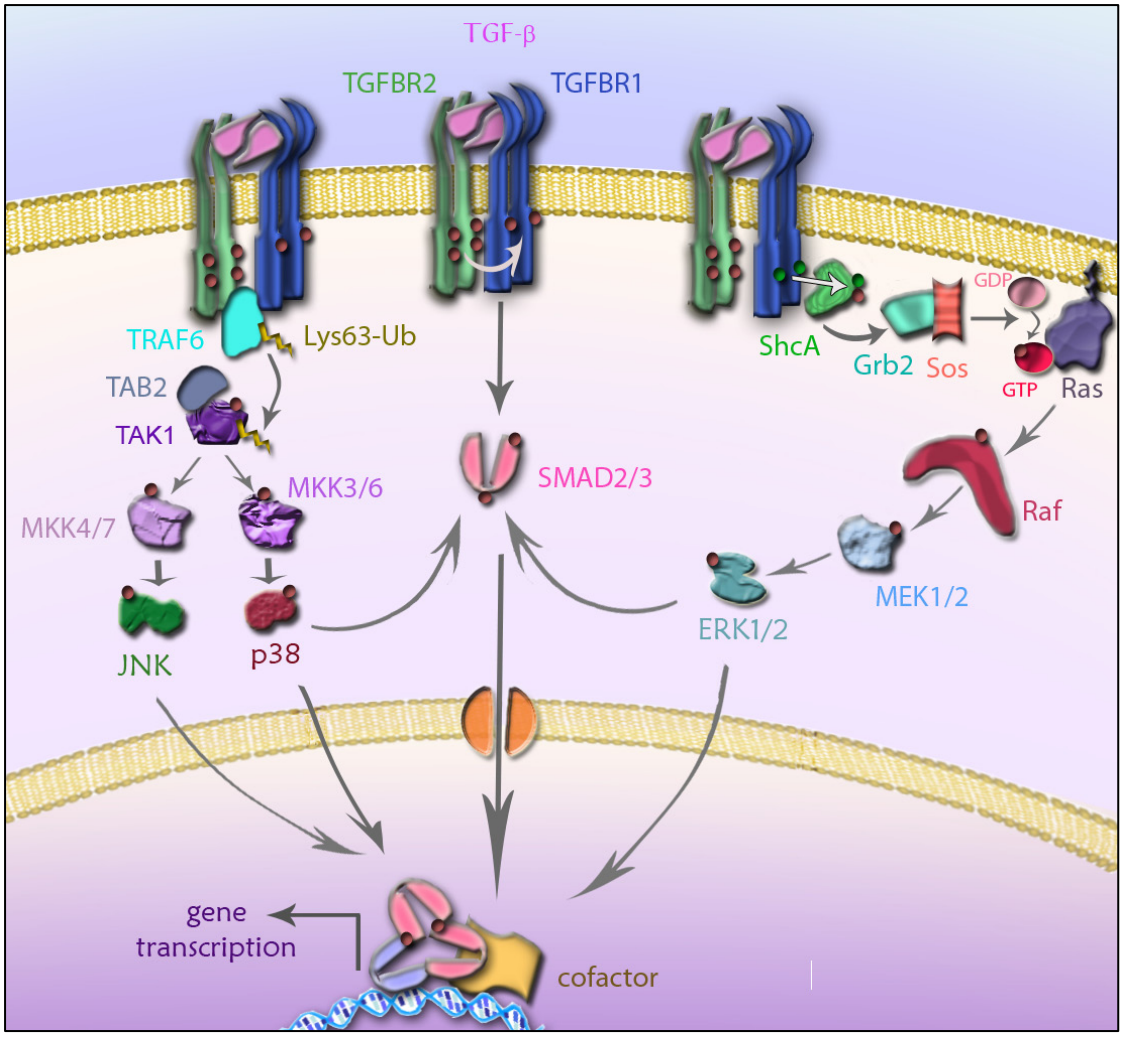


Figure 1.4: Schematic representation of TGF-β-induced MAPK pathways. TGF-β signals via the canonical SMAD2/3 pathway and via non-canonical pathways, such as the MAPK ERK1/2, JNK or p38 pathways, in a cell-specific manner. TGFBR2 and TGFBR1 are serine/threonine and tyrosine kinases. Tyrosine phosphorylated residues on TGFBR1 serves as docking sites for ShcA. Once phosphorylated, ShcA recruits Grb2 and Sos at the plasma membrane, where Sos catalyzes the exchange of Ras-GDP for Ras-GTP. Activated Ras-GTP phosphorylates Raf, which activates MEK1/2, the kinases upstream ERK1/2. TGFBR1 can also bind to the E3 Ubiquitin ligase TRAF6, which undergo auto-Lys63-ubiquitination. This modification is recognized by TAB2, an adaptor for TAK1. TAK1 is Lys63-ubiquitinated by TRAF6, which coincides with its phosphorylation. The activated TAK1 phosphorylates the MAPKK MKK4/7 and MKK3/6, which activates JNK and p38, respectively. The MAPKs can phosphorylate SMAD2/3 on its linker region, thus regulating its transcriptional activity and/or its cytoplasmic localization. In addition, MAPK activates transcription factors that synergize or antagonize with SMADs signalling.

by TGFBR2). TGFBR1 phosphorylated tyrosines serve as docking sites for the phosphotyrosine binding (PTB) domain of ShcA. TGFBR1 then phosphorylates directly ShcA on tyrosine and serine residues. The phosphorylated ShcA recruits the adaptor growth factor receptor binding protein 2 (Grb2) and Sos at the plasma membrane. Sos catalyzes the exchange of Ras-GDP to Ras-GTP, which activates Raf, leading to MEK and ERK activation (Lee *et al.*, 2007). A schematic representation of the TGF-β/ERK cascade is shown in Fig 1.4. TGF-β activation of ERK participates to the disassembly of cell adherens junctions and induction of cell motility and thus contributes to the epithelial-mesenchymal transition (EMT) in keratinocytes (Davies *et al.*, 2005; Zavadil *et al.*, 2001).

1.5.1.2- TGF-β-induced p38 and JNK pathways

The p38 and JNK MAPK pathways induce apoptosis in several cell systems, but have also been implicated in proliferation and differentiation (Boutros *et al.*, 2008). TGF-β rapidly activates JNK and/or p38 MAPK in several cell lines, in a SMAD-independent manner (Zhang, 2009). While the L45 loop of TGFBR1 is essential for SMAD2/3 interaction and activation, it is dispensable for JNK or p38 MAPK signalling (Itoh *et al.*, 2003; Yu *et al.*, 2002). However, TGFBR1 kinase activity has been shown to be necessary for p38 (and likely JNK) activation (Yu *et al.*, 2002).

Recently, it has been shown that the RING E3 Ubiquitin ligase TRAF6 binds to the TGF-β receptors. TGF-β stimulation triggers TRAF6 auto-Lys63-polyubiquitination, leading to the recruitment of the ubiquitin-binding protein TAB2 (TAK-associated protein 2) and TAK1. TAK1 is then Lys63-ubiquitinated by TRAF6. After that, TAK1 becomes phosphorylated and activated, recruits and induces the phosphorylation of MAPKK that activates downstream JNK/p38 pathways (Sorrentino *et al.*, 2008; Yamashita *et al.*, 2008). Fig 1.4 represents a schematic model of the TGF-β/p38/JNK pathway.

The adaptor SMAD7 was shown to act as a scaffold protein for TAK1, MKK3 and/or MKK6, thereby facilitating p38 activation (Edlund *et al.*, 2003). SMAD7 is also a scaffold

protein for p38, p53 and ATM, thus contributing to TGF-β-induced apoptosis (Zhang *et al.*, 2006). Therefore, it has been proposed that SMAD7 could facilitate the formation of TRAF6-TAK1-MAPK complex to facilitate apoptosis (Sorrentino *et al.*, 2008).

In addition to TAK1, other MAPKKKs have been implicated in TGF-β-induced JNK and p38, such as MEKK1 and MLK3 (Kim *et al.*, 2004; Zhang *et al.*, 2003). However, the mechanisms by which these MAPKKKs are activated remain obscure. Moreover, TGF-β stimulation leads to stabilization of Daxx, which binds to TGFBR2 and activates the JNK pathway, likely through ASK1 (Perlman *et al.*, 2001). β3integrin can facilitate TGFBR2 tyrosine phosphorylation by Src, thereby mediating TGF-β-induced p38 MAPK activation (Bhowmick *et al.*, 2001b; Galliher and Schiemann, 2006; Galliher and Schiemann, 2007).

In some cell types, TGF-β induces p38 after 1-2hrs of treatment. This delayed response, but not the early p38 activation, has been shown to be mediated by SMAD-dependent transcription of GADD45β. GADD45β activates MTK1/MEKK4, a MAPKKK upstream of the p38 pathway (Takekawa *et al.*, 2002). Similarly, a delayed response in JNK activation can be accounted for transcription of SMAD7 upon TGF-β treatment: SMAD7 can in turn induce prolonged JNK activation via an unknown mechanism (Mazars *et al.*, 2001).

1.5.2- TGF-β modulation of Rho-like GTPases

Several evidences indicate that Rho-like GTPases also contribute to TGF-β-induced cell cycle arrest and EMT. In epithelial cells, Par6, a protein that regulates cell polarity, interacts constitutively with TGFBR1 in the tight junctions. Following TGF-β stimulation, TGFBR2 associates with TGFBR1-Par6 and phosphorylates Par6, leading to the recruitment of Smurf1 at tight junctions. Smurf1 then induces the local degradation of RhoA, resulting in dissolution of the tight junctions (Ozdamar *et al.*, 2005).

In contrast to these studies, it was also shown that TGF-β rapidly activates RhoA in a SMAD-independent manner in keratinocytes (Bhowmick *et al.*, 2001a). A delayed activation of RhoA in response to TGF-β can also be observed and this delayed response

may be due to SMAD-induced transcription of NET1, a RhoA exchange factor (Shen *et al.*, 2001).

Moreover, TGF-β stimulation can lead to cdc42 activation, resulting in reorganization of actin filaments and membrane ruffling. This process involves SMAD7, which acts as a scaffold protein for cdc42 (Edlund *et al.*, 2004). In fibroblasts, cdc42, Rac1 and/or PI3K can activate p21-Activated kinase 2 (PAK2) in response to TGF-β, resulting in activation of mitogenic responses. In contrast, in most epithelial cells, PAK2 is not activated. This difference could explain why in fibroblasts, TGF-β stimulates cell proliferation whereas it inhibits cell growth in most epithelial cells (Wilkes *et al.*, 2005; Wilkes *et al.*, 2003; Wilkes *et al.*, 2009).

1.5.3- TGF-β-induced PI3K/AKT pathway

TGFBR2 associates indirectly with p85, the regulatory subunit of PI3K and upon ligand stimulation, TGFBR1 activates PI3K, independently of SMAD2/3 activation (Yi *et al.*, 2005). PI3K then generates PIP3 (phosphatidylinositol-3, 4, 5-triphosphate) from PIP2 (phosphatidylinositol 4,5-bisphosphate), which activates Akt.

A delayed activation of Akt can also be observed upon TGF-β treatment. TGF-β, in collaboration with the microRNA miR-192, induces the transcription of miR-216a and miR-217. These two miRNA target PTEN mRNA for degradation, releasing Akt inhibition by PTEN (Kato *et al.*, 2009).

TGF-β-induced Akt signalling has been shown to contribute to EMT. In the presence of TGF-β, Akt activates mTOR (mammalian target of Rapamycin), a key regulator of protein synthesis, which regulates cell size and invasion (Lamouille and Derynck, 2007). However, the Akt pathway antagonizes other TGF-β responses, such as apoptosis.

1.5.4- Cross-talks between TGF-β and other signalling pathways

The MAPK, the Rho-like GTPases and the PI3K/Akt pathways are activated not only by TGF-β but also in response to other signals and can modulate the TGF-β signalling pathway (Guo and Wang, 2009). In return, TGF-β interferes with other signalling pathways. The cross-talk between the different signalling pathways allows a high degree of regulation of cellular process in response to many signals. For example, the interplay between the EGF/Ras/ERK pathway and the TGF-β pathway play a central role during cancer progression. While TGF-β provokes growth arrest of "normal" epithelial cells, TGF-β treatment induces cell motility and invasiveness of Ras-transformed epithelial cells (Oft *et al.*, 1996). This suggests that Ras inhibits TGF-β-induced anti-proliferative responses, but cooperates with TGF-β to promote cell migration (Guo and Wang, 2009). Similarly, cross-talk between TGF-β and Wnt/β-catenin, Hedgehog or Notch pathways are essential during embryonic development and for the maintenance of homeostasis in the adult. Finally, a tight relationship between TGF-β and Interleukin (IL), Tumor Necrosis Factor (TNF)-α and Interferon-γ (IFN-γ) is critical for immune functions and inflammatory responses. For instance, TGF-β and IL-6 cooperate to induce the differentiation of Th17 cells, a cell type implicated in psoriasis initiation and progression (Kunz, 2009). Given the importance of these cross-talks, it is not surprising that a cell possesses multiple ways of integrating TGF-β signalling with other signals.

1.5.4.1- Cross regulation of cytokines production

TGF-β induces the production of numerous cytokines. Likewise, these cytokines can regulate TGF-β production. For example, the Ras/ERK pathway often induces TGF-β expression. Conversely, JNK antagonizes TGF-β signalling by inhibiting TGF-β1 autocrine expression. TGF-β and Wnt, Sonic Hedgehog or Notch reciprocally regulates their ligand production, a key step in the establishment of morphogenes gradients during embryonic

development. Additionally, TGF-β inhibits IL-2 production to suppress T cell proliferation (Guo and Wang, 2009).

1.5.4.2- Cross modulation of receptor activities

The integration of signalling from two different pathways can also occur via regulation of each other receptor. For example, ERK signalling activates the metalloprotease TACE/ADAM-1, which cleaves TGFBR1 and causes inhibition of TGF-β signalling (Liu *et al.*, 2009a). In keratinocytes, TGF-β induces the production of receptor type protein tyrosine phosphatase-κ (RPTP-κ). RPTP-κ dephosphorylates EGFR, thereby inhibiting EGFR signalling and proliferative response. This mechanism further enhances TGF-β anti-proliferative effect (Xu *et al.*, 2010).

1.5.4.3- Induction of I-SMADs by different pathways

Several cytokines also modulate I-SMAD expression as mentioned earlier. For instance, EGF, via the MAPK, IFN-γ, via JAK1/STAT1, TNF-α and IL-1β, via NF-κB, are all able to induce SMAD7 expression, thereby negatively regulating TGF-β signalling (Afrakhte *et al.*, 1998; Bitzer *et al.*, 2000; Ulloa *et al.*, 1999). Additionally, IL-7 inhibits TGF-β pro-fibrotic response by increasing SMAD7 expression in pulmonary fibroblasts (Huang *et al.*, 2002).

1.5.4.4- Regulation of SMAD activity by phosphorylation of its linker and MH1 domain via other pathways

SMAD function is tightly regulated in response to various signals, notably through phosphorylation of its linker region by many different kinases. Each kinase shows some preference for specific serine/threonine residues in the linker. Moreover, the outcome

of such linker phosphorylation varies greatly depending on the cell context. As mentioned above, phosphorylation of the linker domain of SMAD1 by the MAPK leads to SMAD1 degradation (Sapkota *et al.*, 2007). Phosphorylation of SMAD2/3 linker region by ERK can also impair their nuclear accumulation under some circumstances (Kretzschmar *et al.*, 1999; Sapkota *et al.*, 2007). In contrast, ERK-mediated phosphorylation of SMAD2 N-terminal increases SMAD2 stability and complex formation with SMAD4, thereby enhancing SMAD2 signalling (Funaba *et al.*, 2002).

Activation of Ras/ERK leads to the phosphorylation of SMAD4 linker region and can result in either an increase in its nuclear accumulation and transcriptional activity (Roelen *et al.*, 2003) or a blockage of SMAD4 nuclear accumulation and inhibition of TGF-β responses (Saha *et al.*, 2001), likely depending on the cell context. SMAD4 can also be phosphorylated by p38/JNK and these phosphorylations enhance its degradation by the SCF complex. SMAD4 cancer mutants which are more degraded than SMAD4-WT, display more phosphorylations by p38 and JNK (Liang *et al.*, 2004).

Moreover, p38 has been shown to phosphorylate SMAD2 and SMAD3 linker region, thereby increasing SMAD2/3 transcriptional activity (Kamaraju and Roberts, 2005). Similarly, JNK phosphorylates SMAD3 in response to TGF-β or HGF, and this positively or negatively affects SMAD activity depending on the promoter (Mori *et al.*, 2004).

Other kinases, such as PKC, a downstream effector of tyrosine kinase receptor, the GPCR inhibitor GRK2, Ca^{2+}-calmodulin-dependent protein kinase II (Cam kinase II), the nuclear CDK2, CDK4, CDK8 and CDK9 and GSK3 have been reported to phosphorylate SMAD2/3's MH1 and linker region, resulting in either enhancement of SMAD2/3 transcriptional activity or inhibition of TGF-β signalling (Alarcón *et al.*, 2009; Ho *et al.*, 2005; Matsuura *et al.*, 2004; Millet *et al.*, 2009; Wang *et al.*, 2009; Wicks *et al.*, 2000; Yakymovych *et al.*, 2001).

Within a cell, several kinases are likely to phosphorylate different sites on SMADs' linker in response to multiple signals. The resulting combination of sites that are phosphorylated may determine the effect on SMAD activity (Wrighton *et al.*, 2009). Moreover, the outcome may be different if the phosphorylation occurs in the cytoplasm

or in the nucleus. Finally, some phosphorylation needs priming kinases, such as phosphorylation by GSK3 which occurs after phosphorylation by MAPK (Fuentealba *et al.*, 2007). On the other hand, one phosphorylation may preclude others (Wrighton *et al.*, 2009). Thus, the net effect of all these kinases depend on the activity of the others kinases and on the balance with phosphatases, such as SCP1, 2, 3, which dephosphorylate SMADs' linker region (Sapkota *et al.*, 2006).

1.5.4.5-Cross-talk at the level of transcription factors

The MAPK and the other pathways can affect SMADs function, not only by phosphorylating its linker and MH1 domain, but also by phosphorylating several transcriptions factors that interact with SMADs. Notably, transcription factors from the Jun, Fos and ATF family, which form the AP-1 complex, are substrates for the MAPK, and can cooperate or interfere with SMAD transcriptional activity depending on the structure of the target gene promoter or the presence of other co-factors (Guo and Wang, 2009; Verrecchia *et al.*, 2001). For example, the SMAD3-SMAD4 complex and the AP-1 complex can each bind to two different elements of the c-jun promoter to induce its transcription in a synergistic manner (Wong *et al.*, 1999). This also illustrates the ability of SMAD signalling to regulate the transcription of its nuclear partners, adding another level of regulation to the process.

In addition, SMADs can bind to p53, β-catenin/Lef protein complex or to Notch transcription factor to regulate common target genes in a synergistic manner (Cordenonsi *et al.*, 2003; Zhang, 2009). Alternatively, Notch and Akt, in response to IGF, antagonize TGF-β signalling by sequestering the transcription co-factor from the SMADs (Masuda *et al.*, 2005; Seoane *et al.*, 2004). In addition, Akt phosphorylates the co-repressor Ski. This results in Ski degradation but relieves Ski-mediated repression of SMAD7. Thus, Akt has a dual effect on TGF-β signalling (Band *et al.*, 2009).

These numerous mechanisms integrate TGF-β signalling with other signalling pathways, allowing a cell to respond appropriately to simultaneous stimuli.

1.6- TGF-β receptor compartmentalization and internalization

1.6.1- Characteristics of the different modes of endocytosis

Endocytosis is a pivotal mechanism regulating cellular function, notably signal transduction. Different routes of endocytosis have been described and can be classified as non-lipid-raft, clathrin-dependent pathway and by opposition, lipid-raft, clathrin-independent pathways (Le Roy and Wrana, 2005; Puri *et al.*, 2001). The clathrin route is characterized by coated vesicles of 100-200 nm in diameter. The coat is formed after recognition of a transmembrane cargo molecule by an adaptor protein, such as AP2, Disabled 2, or β-arrestin and the subsequent assembly of clathrin-coated vesicle around the cargo (Benmerah and Lamaze, 2007). The vesicle is then pinched off the membrane by a mechanism involving the GTPase Dynamin. (There are 3 different dynamin genes: Dynamin2 is ubiquitously expressed and Dynamin1 and 3 have a much more restricted expression pattern). Dynamin multimers form a spiral around the membrane, constrict the neck of the vesicle and provoke the fission of the vesicle. The clathrin coated vesicle then loses its coat and the naked vesicle fuses with the early endosome (Doherty and McMahon, 2009).

Fusion between a compartment and a vesicle and trafficking of the vesicle along the cytoskeleton is controlled and specified by monomeric, membrane-bound Ras-like GTPases called Rab, which are specific to a cellular compartment. For example, Rab5 is located on the early endosome where it promotes the enrichment of this compartment in PI3P, thereby allowing the association of EEA-1 and other FYVE domain containing proteins, such as SARA, to the early endosome (Grosshans *et al.*, 2006). From the early endosome, cargos are sorted between several compartments. They can be directed to the trans-golgi network, to a rapid recycling/ Rab4 positive compartment, or to a slow recycling/ Rab8-Rab11-dependent route. Alternatively, the cargo can be sorted to the Rab7-Rab9-containing late endosome, a multivesicular body that send proteins for degradation into the lysosome (Grant and Donaldson, 2009).

The early endosomal compartment is not specific of the clathrin road but could also be the destination of other clathrin-independent routes. Most of the clathrin-independent pathways involve plasma membrane microdomains called lipid-raft. They are enriched in sphingolipids and cholesterol and are insoluble in non-ionic detergents, such as Triton X-100, a property used to isolate lipid-rafts (Thomas and Smart, 2008).

Among the non-clathrin pathways, the caveolar pathway is the best defined and is present in many, but not all cell types. Caveolae are characterized by flask or omega-shaped invaginations of 50-100nm diameter and generally by the presence of a member of the caveolin family. There are three members in this family and caveolin-1 has the widest distribution (Kiss and Botos, 2009). Caveolin are 18-22KDa proteins composed of a cytosolic N-terminal region involved in binding to the lipid-raft, to signalling molecules and in oligomerization, a middle region inserted into the inner leaflet of the membrane bilayer and a cytosolic C-terminus (Thomas and Smart, 2008). In addition to caveolin, SDPR (serum deprivation protein response) recruits PRTF/cavin to caveolar membrane and the two proteins induce and stabilize the membrane curvature (Hansen *et al.*, 2009). On the cell surface, caveolae appears to be relatively immobile structure, already pre-assembled. Receptor clustering or interaction of caveolae with ligands can cause the rapid internalization of caveolae (Kiss and Botos, 2009). Tyrosine phosphorylation of caveolin-1 stimulates the formation of vesicles and of long “caveolar tubes” (Orlichenko *et al.*, 2006). Dynamin then transiently associates with caveolae and induces the fission of the caveolar vesicle (Oh *et al.*, 1998). The caveolae vesicle then moves along the microtubules and forms the caveosome, a grape-like multicaveolar complex. The caveosome can serve as a way to escape lysosomal degradation for some viruses (Kiss and Botos, 2009). Conversely, caveosome can fuse with the early endosome, but their membrane domains do not seem to mix with each other (Pelkmans *et al.*, 2004).

Some of the non-clathrin, non-caveolar pathways are also dynamin dependent. This is the case of the IL-2 receptor endocytic route and the APP endocytosis in primary neurons (Kumari *et al.*, 2010). On the other hand, other endocytic processes do not require Dynamin function, such as the cdc42 (also called clathrin-independent carrier

(CLIC)/ GPI-anchored proteins enriched early endosomal compartment (GEECs) pathway) and the ARF6-dependent pathway. These pathways are the primary endocytic route for fluid phase markers and native GPI-anchored proteins (Sabharanjak *et al.*, 2002), but GPI-anchored proteins can take other routes than the CLIC/GEECs and ARF6 pathways, depending on their association with transmembrane proteins (Helms and Zurzolo, 2004; Lakhan *et al.*, 2009; Mayor and Pagano, 2007).

1.6.2- Internalization of TGF-β receptors

Internalization of TGF-β receptor is an important step in the control of TGF-β signalling. TGF-β receptors are internalized rather slowly, with a half-life of ≈15min and an endocytic rate (i.e. the amount of surface receptor internalized per minute) of 0.022 min^{-1}. This is 5-10 times slower that the internalization rate of the EGFR and 3-4 times slower than the endocytic rate of the growth hormone receptor (Anders *et al.*, 1997; Ehrlich *et al.*, 2001). TGF-β receptors have the particularity to undergo constitutive endocytosis and ligand addition does not change their endocytic rate (Chen, 2009; Ehrlich *et al.*, 2001; Mitchell *et al.*, 2004). Moreover, early studies have shown that TGF-β ligand is internalized along with its receptors and that the ligand is then degraded in lysosomes. During exposure of cells to TGF-β, the number of cell surface receptor remains constant, suggesting that after internalization, the receptors are recycled back at the cell surface or that the internalized receptors are degraded but that there is a large intracellular receptor storage pool (Massagué and Kelly, 1986). Evidences suggest that both recycling and intracellular storage pool contribute to this constant cell surface receptor level. Importantly, it was observed that ligand stimulation enhances TGF-β receptors degradation (Wells *et al.*, 1997).

1.6.2.1- TGF-β receptor internalization into the clathrin-coated pits

The endocytic routes through which the TGF-β receptors are internalized have been the subject of much controversy. Several studies implicate the clathrin-coated pathway as the major internalization route for the TGF-β receptors. First, it was shown that the endocytosis of the TGF-β receptor is greatly reduced after disruption of the clathrin lattice structure (Anders *et al.*, 1997; Ehrlich *et al.*, 2001). Similarly, clathrin siRNA blocks the internalization of chimeric receptors containing the cytoplasmic tails and transmembrane regions of the TGF-β receptors fused to the extracellular domains of the granulocyte macrophage colony-stimulating factor (GM-CSF). In contrast, blocking the lipid-raft pathway has no effect on chimeric receptor internalization (Mitchell *et al.*, 2004). Although this study suggests that TGF-β receptor can undergo clathrin-mediated endocytosis, it does not rule out the possibility that native TGF-β receptors are internalized via another route.

In support to a major role of the clathrin pathway for TGF-β receptor endocytosis, both TGFBR1 and TGFBR2 were found to directly interact with AP2 in a ligand-independent manner (Yao *et al.*, 2002). TGFBR2 major internalization signal is an isoleucine-leucine motif (amino acid 218 to 220), a typical consensus sequence for clathrin cargo (Ehrlich *et al.*, 2001). In contrast, the internalization signal of TGFBR1 is not a classical internalization sequence: it is a motif called the NANDOR (non activating non-downregulating) box (Garamszegi *et al.*, 2001). Internalization of TGF-β receptors through the clathrin-coated pits results in localization of the TGF-β receptors into the early endosomal compartment and then recycling of the TGF-β receptors (regardless of their activation state) via the Rab11-dependent pathway (“slow” recycling pathway)(Mitchell *et al.*, 2004), as represented in Fig 1.5. The clathrin associated protein Disabled 2 is essential for Rab11-dependent recycling (Penheiter *et al.*, 2010) and facilitates TGF-β signalling (Hocevar *et al.*, 2001).

The role of the endosomal compartment in TGF-β signalling is still a matter of debate. Because SARA localizes via its FYVE domain to PI3P enriched early endosome and facilitates SMAD2/3 binding to the receptor, it has been suggested that internalization of

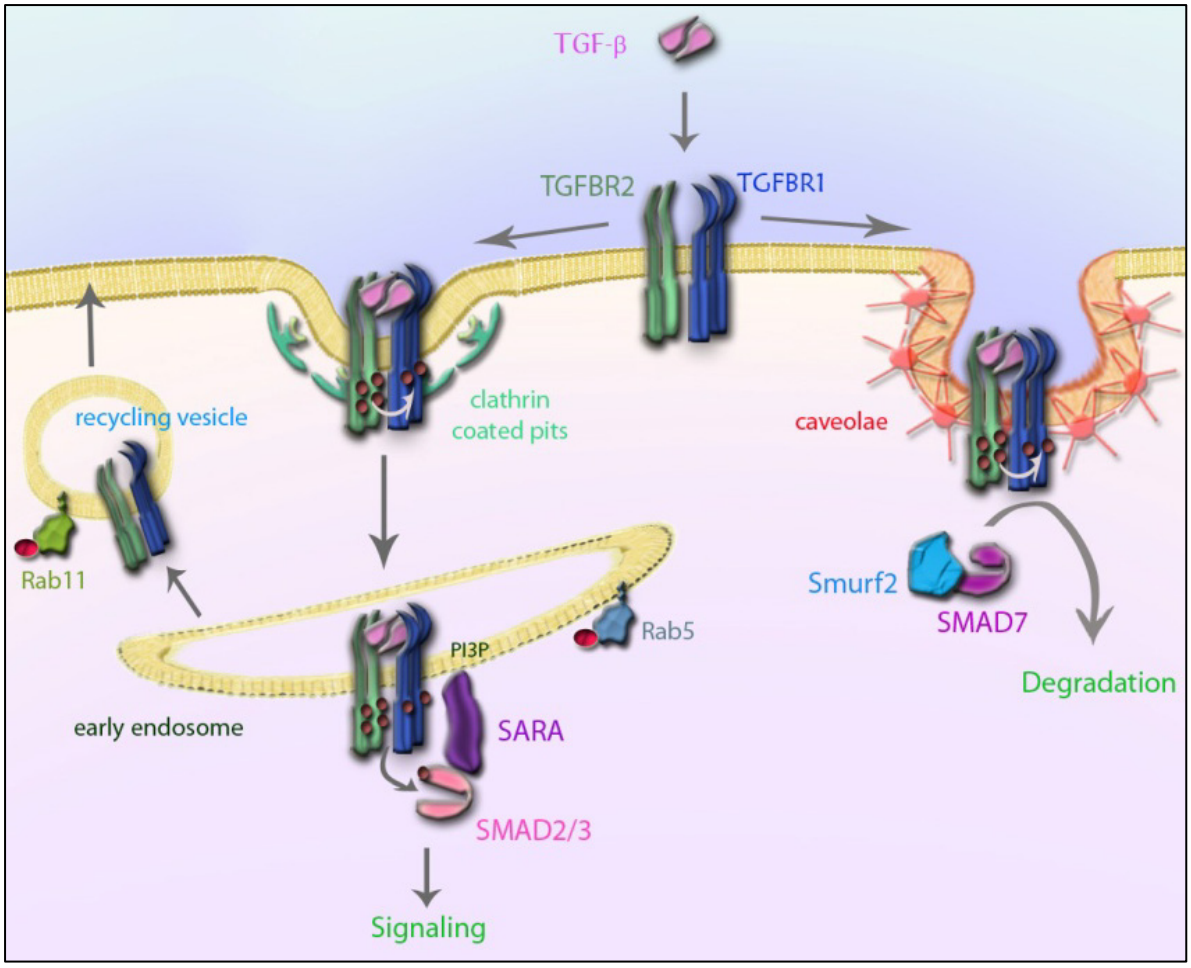


Figure 1.5: Schematic representation of TGF-β-receptor internalization. TGF-β receptors are internalized via two different routes. Internalization into clathrin-coated pits leads to localization of the receptors in Rab5-positive early endosome. The early endosomal membrane is enriched in PI3P, which binds to SARA FYVE domain. SARA is an adaptor for SMAD2/3 and facilitates TGF-β signalling. Thus, endocytosis of TGF-β receptor via the clathrin pathway is thought to promote TGF-β signalling. Moreover, the receptors are recycled back at the plasma membrane via Rab11-positive vesicles. On the other hand, TGF-β receptors can be internalized into caveolae vesicles, which are enriched in SMAD7/Smurf2 complex. SMAD7 serves as an adaptor protein for Smurf2, which ubiquitinates TGFBR1, resulting in lysosomal and proteasomal degradation of TGFBR1. Therefore, internalization of TGF-β receptors in caveolae decreases TGF-β signalling.

TGF-β receptor into the early endosome is essential for signalling (Di Guglielmo *et al.*, 2003) (Fig 1.5). In support with this notion, impairing SARA endosomal localization decreases SMAD2/3 nuclear accumulation and TGF-β transcriptional activity (Hayes *et al.*, 2002; Panopoulou *et al.*, 2002).

However, it has also been shown that the association of SARA and SMAD2 with the TGF-β receptors and SMAD2 phosphorylation occur at the plasma membrane, since these events are not inhibited by a dynamin dominant negative mutant. In contrast, downstream events, such as SMAD2/3 dissociation from SARA and SMAD2/3 transcriptional activity require vesicle formation (Penheiter *et al.*, 2002; Runyan *et al.*, 2005). Consistent with these studies, it was shown that dynamin mutant does not interfere with BMP-induced SMAD1/5 phosphorylation, but disrupting clathrin-coated pits formation decreases SMAD1/5 transcriptional activity (Hartung *et al.*, 2006). These studies suggest that although internalization may not be essential for the early events of TGF-β signalling, passage through the endosome is likely to be important for downstream signalling.

In contrast with all the above studies that suggest a critical role for receptor endocytosis in mediating TGF-β responses, it has been shown that blocking dynamin-dependent internalization or impairing SARA localization to the early endosome does not affect SMAD2/3 signalling (Lu *et al.*, 2002). Similarly, it has been reported that internalization of ALK4 was not necessary for the induction of activin target genes (Hagemann *et al.*, 2009; Zhou *et al.*, 2004). Together, this suggests that, in some instances, when trafficking is impaired, TGF-β/activin receptor can still find a way to elicit signalling.

Some of the differences observed after inhibition of the clathrin/endosomal-mediated internalization might be due to the presence or absence of proteins that regulate SARA function, such as the cytoplasmic promyelocytic leukemia protein (cPML) and the FYVE domain protein Hgs, which cooperates with SARA (Lin *et al.*, 2004; Miura *et al.*, 2000) and TMEPAI (Transmembrane TGF-β-Inducible Protein), which competes with SARA for binding to R-SMADs (Watanabe *et al.*, 2010).

1.6.2.2- TGF-β receptor internalization in caveolae

Several studies report that the TGF-β receptors can take an alternative road dependent on caveolae. Indeed, it has been shown that monodansylcadaverine, an inhibitor of clathrin invagination does not affect the internalization of TGF-β ligand (mediated by TGF-β receptors). Moreover, electron microscopy reveals the presence of TGF-β receptors into non-coated vesicles (Zwaagstra *et al.*, 2001). In addition, sucrose gradient fractionation experiments and confocal microscopy indicate that TGFBR1 and TGFBR2 localize to both caveolae positive lipid-raft and EEA-1 positive non-raft fraction (Di Guglielmo *et al.*, 2003; Razani *et al.*, 2001). TGFBR1 was shown to interact with caveolin-1 scaffolding domain and the interaction is enhanced by TGF-β stimulation (Razani *et al.*, 2001). However, ligand addition does not affect receptor partitioning between caveolar and non-caveolar compartment (Di Guglielmo *et al.*, 2003). The extracellular domain of TGFBR2 is required for its localization into the lipid-raft and this localization might be mediated by interaction between TGFBR2 with glycosylated protein(s)(Luga *et al.*, 2009). This may explain why, using GM-CSF/TGF-β chimeric receptors, Mitchell et al. could not detect TGF-β receptor in caveolae (Mitchell *et al.*, 2004).

Importantly, it has been demonstrated that, while clathrin-mediated internalization enhances SMAD2/3 signalling, internalization of the TGF-β receptor into caveolae leads to downregulation of TGF-β signalling (Di Guglielmo *et al.*, 2003). Caveolin-1 can inhibit TGFBR1 kinase activity, SMAD2 phosphorylation and subsequent signalling (Razani *et al.*, 2001). Furthermore, localization of TGF-β receptor into the caveolar/lipid-raft compartment facilitates TGF-β receptor degradation by SMAD7-Smurf2, which were shown to localize to lipid-raft/caveolae (Di Guglielmo *et al.*, 2003) (Fig 1.5). Blocking internalization via the caveolae enhances SMAD2/3 signalling, whereas blocking internalization via the clathrin-coated pits enhances receptor degradation, suggesting that impairing internalization through one route causes internalization through the alternative route (Di Guglielmo *et al.*, 2003). Similar to the TGF-β receptors, BMPR2 localizes to both clathrin and caveolar compartments. Inhibition of clathrin-mediated endocytosis decreases SMAD1/5 signalling, whereas inhibition of caveolae-mediated

endocytosis has no effect on SMAD1/5 signalling. However, caveolae seems to mediate BMP-induced non-SMAD signalling (Hartung *et al.*, 2006).

Growing evidence suggest that internalization of TGF-β receptor into the caveolae can lead to activation of non-canonical pathways. In HaCaT cells, endocytosis of the TGF-β receptor via the caveolae was shown to mediate TGF-β-induced ERK and p38 MAPK signalling, while reducing SMAD2/3 activation and had no effect on Akt signalling (Zuo and Chen, 2008). It is possible that SMAD7 is responsible for p38 activation in caveolae since SMAD7 localizes to caveolae (Di Guglielmo *et al.*, 2003) and can facilitate p38 activation (Edlund *et al.*, 2003). In addition, the preferential localization of H-Ras in the lipid-raft (Niv *et al.*, 2002) might explain that TGF-β induces ERK activation in lipid-raft. Additionally, in dermal fibroblasts, caveolin-1 can induce Akt/mTOR pathway to increase collagen type I expression (Kim *et al.*, 2008). Moreover, localization of TGF-β receptor into caveolae could negatively modulate eNOS activity in human endothelial cells (Schwartz *et al.*, 2005). Finally, it has been suggested that caveolin-1 could enhance TGF-β/ALK1 signalling, while inhibiting TGF-β/ALK5 responses in endothelial cells (Santibanez *et al.*, 2008).

1.6.2.3- Regulation of TGF-β receptor compartmentalization

TGF-β receptor trafficking is an elegant way to detect temporal changes in ligand concentration and differences in ratios of TGF-β subtypes. Any change in the internalization and recycling rates or in the ratio of constitutive to ligand-induced degradation rate is expected to impact on the strength and duration of the signal (Vilar *et al.*, 2006). Thus, molecules that regulates TGF-β receptor trafficking and compartmentalization are likely to play an important role in the control of TGF-β action.

To date, several molecules have been identified as regulator of TGF-β receptor trafficking, although the exact mechanism by which these molecules influence TGF-β receptor internalization is not always defined. One such molecule is FKBP12, which can abrogate TGF-β receptor endocytosis (Yao *et al.*, 2000). Hyaluronan promotes TGFBR1

compartmentalization into the lipid-raft and enhances the association of TGFBR1 with caveolin-1 and SMAD7, resulting in inhibition of TGF-β signalling (Ito *et al.*, 2004). Similarly, cholesterol addition increases the amount of TGF-β receptor in lipid-raft, resulting in decrease in TGF-β-signalling (Chen *et al.*, 2008). In contrast, deficiency in heparan sulfate synthesis decreases the amount of TGF-β receptor that localizes to caveolae and thus increases TGF-β signalling, suggesting that this modification affects glycoprotein(s) involved in regulation of TGF-β receptor compartmentalization (Chen *et al.*, 2006). In addition, the phosphatase Dullard, following dephosphorylation of ALK3, targets BMP receptors to caveolae and promotes their degradation (Satow *et al.*, 2006). Finally, Dapper2, an antagonist of the Wnt pathway, localize to late endosome, where it binds to TGF-β receptors and increases their lysosomal degradation (Su *et al.*, 2007; Zhang *et al.*, 2004). It is not clear, however, whether Dapper2 requires internalization via caveolae or via clathrin vesicles and whether SMAD7-Smurf2 are involved in the process.

On the other side, the metalloproteinase ADAM12 can prevent TGF-β receptor degradation by facilitating the relocalization of TGF-β receptor-SARA complex from the plasma membrane to the early endosome (Atfi *et al.*, 2007). Similarly, IL-6 promotes TGF-β receptor localization into the non-raft compartment, thereby decreasing receptor turnover and increasing TGF-β signalling (Zhang *et al.*, 2005b). Moreover, it has been shown that the β5 integrin subunit preferentially interacts with clathrin than caveolae and forms a complex with TGFBR1 in clathrin-coated pits. Thus, β5integrin may couple activation of latent TGF-β and signalling via the clathrin/endosomal pathway (Asano *et al.*, 2006).

In addition to these molecules, TGF-β co-receptors are also able to modulate TGF-β receptor internalization (see section 1.7 below).

1.7- TGF-β co-receptors

TGF-β signalling can be modulated at the receptor level by co-receptors. Co-receptors are cell-surface proteins that bind the ligand and regulate the binding to and signalling

of TGFBR1 and TGFBR2, but, unlike TGFBR1 and TGBR2, they have no evident signalling motifs. Some of these co-receptors have the particularity to bind preferentially to one subtype and thus confer subtype specificity for the cell expressing the co-receptor (Kirkbride *et al.*, 2005). TGF-β co-receptors play an important role in the formation of morphogene gradients, cell adhesion and in some instance, regulation of TGF-β receptor endocytosis (Mythreye and Blobe, 2009a).

1.7.1-Betaglycan

Betaglycan, also known as type III receptor, is the most abundant TGF-β binding proteins in many cell types, but its cellular distribution is more limited than TGFBR1 and TGFBR2: it is not expressed in some myoblasts, endothelial and hematopoietic cells (Cheifetz *et al.*, 1990; Ohta *et al.*, 1987; Segarini *et al.*, 1989). *Betaglycan* null mice die around day e13.5 from heart defects and apoptosis in the liver, suggesting an essential role of betaglycan during embryonic development (Stenvers *et al.*, 2003). Moreover, betaglycan expression is upregulated in non-Hodgkin's lymphomas and in B-cell chronic leukemia, while it is downregulated in neuroblastomas, prostate cancer and in a variety of carcinomas (Bernabeu *et al.*, 2009).

Betaglycan is a 200-300kDa proteoglycan that forms a complex with TGF-β receptors (Lopez-Casillas *et al.*, 1993) and, like TGFBR1 and TGFBR2, is capable of homodimerization (Henis *et al.*, 1994). It comports a short cytoplasmic tail containing a PDZ motif, a single transmembrane domain and a large extracellular domain heavily modified by heparan and chondroitin sulfate glycosaminoglycan (GAG) side chains (Bernabeu *et al.*, 2009).

Betaglycan can bind to all three TGF-β subtypes but has a higher affinity for TGF-β2 subtype. Betaglycan enhances TGF-β binding to TGFBR2, and this ligand presentation by betaglycan is particularly important for TGF-β2, since TGFBR2 does not bind TGF-β2 on its own. Thus, betaglycan is essential for TGF-β2 cellular response (Lopez-Casillas *et al.*, 1993). However, betaglycan can have either positive or negative effect on TGF-β signalling, likely depending on the cell context.

The dual effect of betaglycan on TGF-β signalling can be partly explained by the ability of the extracellular domain of betaglycan to be released from the cell surface following protease cleavage (Lopez-Casillas *et al.*, 1994; Velasco-Loyden *et al.*, 2004). The released/soluble betaglycan is able to sequester the ligand away from the TGF-β receptors (Fukushima *et al.*, 1993). In addition, the presence of large GAG chains on betaglycan may impair the association between TGFBR1 and TGFBR2, thus inhibiting TGF-β signalling, while shorter GAG chains results in increased TGF-β signalling by betaglycan (Eickelberg *et al.*, 2002). Thus, the presence of proteases that cleave betaglycan ectodomain and the amount of GAG modifications are likely to determine whether betaglycan acts as an antagonist or an agonist of TGF-β signalling.

The cytoplasmic domain of betaglycan has also an important role in regulating TGF-β signalling. Notably, the cytoplasmic tail of betaglycan has been shown to be phosphorylated by TGFBR2 (Blobe *et al.*, 2001). Once phosphorylated by TGFBR2, betaglycan, via its PDZ domain, recruits β-arrestin2 and internalizes with TGFBR2 (likely into clathrin-coated pits), in a ligand-independent manner (Chen *et al.*, 2003). Consistent with the above result, betaglycan can promote the localization of TGFBR1 and TGFBR2 to Rab5-positive early endosome and prevent SMAD7/Smurf2-mediated degradation of TGF-β receptor (McLean and Di Guglielmo, 2010). However, the effect of betaglycan on TGF-β receptor trafficking results in upregulation of TGF-β signalling only under basal conditions. In the presence of TGF-β, betaglycan downregulates TGF-β signalling and responses (Chen *et al.*, 2003; McLean and Di Guglielmo, 2010). To date, the mechanism leading to such a downregulation is not known. Localization of betaglycan into caveolae has also been reported and leads to its own degradation (Finger *et al.*, 2008).

Through its interaction with β-arrestin2, betaglycan can also constitutively activate cdc42, while downregulating NF-κB signalling, thereby reducing cell migration (Criswell and Arteaga, 2007; Mythreye and Blobe, 2009b; You *et al.*, 2009). Furthermore, in myofibroblasts, betaglycan, via its association with TGFBR2, is able to activate p38 MAPK (but not ERK or SMAD) in the absence of ligand and enhances fibronectin expression (Santander and Brandan, 2006).

Betaglycan is not only a co-receptor for TGF-β; it also binds BMP-2, BMP-4, BMP-7, and GDF-5 ligands and enhances the binding of these ligands to ALK3 and ALK6. Thus, betaglycan is also able to promote BMP signalling (Kirkbride *et al.*, 2008). In addition, betaglycan has been shown to promote distinct responses with ALK3 and ALK6 and control their trafficking differently. While betaglycan, via its interaction with β-arrestin2, enhances the internalization of ALK6, betaglycan maintains ALK3 at the cell surface, independently of β-arrestin2 (Lee *et al.*, 2009). Moreover, betaglycan is a co-receptor for inhibin. Betaglycan is thought to enhance inhibin affinity for ActR2, thus increasing the ability of inhibins to displace activin form ActR2 (Wiater *et al.*, 2009).

1.7.2- Endoglin

The TGF-β co-receptor endoglin (or CD105) is a 180kDa disulfide linked homodimeric glycoprotein that shares 70% homology with betaglycan. Like betaglycan, it possesses a short cytoplasmic domain that also contain a PDZ-binding motif, a single transmembrane domain and large extracellular domain with a zona pellucid domain involved in homodimerization and hetero-oligomerization with the TGF-β receptors. The expression of endoglin is more restricted than the one of betaglycan. Endoglin is expressed principally in endothelial cells (Cheifetz *et al.*, 1992), but also in hematopoietic and immune cells (Bühring *et al.*, 1991; Rokhlin *et al.*, 1995; Schmidt-Weber *et al.*, 2005; Zhang *et al.*, 1996), in chondrocytes (Parker *et al.*, 2003) and in basal keratinocytes (Quintanilla *et al.*). Endoglin haploinsufficiency causes type I hereditary hemorrhagic telangiectasia (HHT1), which is characterized by vascular dysplasia and hemorrhage (McAllister *et al.*, 1994). Moreover, like betaglycan, endoglin ectodomain can be cleaved and the resulting soluble endoglin is a marker for preeclampsia (Venkatesha *et al.*, 2006). In addition to its role in vascular diseases, endoglin plays also an important role during skin carcinogenesis. *Endoglin* heterozygous mice exhibit an increase in malignancy (Quintanilla *et al.*, 2003) and secretion of soluble endoglin is associated with progression of squamous cell carcinoma to a more invasive phenotype (Bernabeu *et al.*, 2009). However, in melanoma and Ewing sarcoma, endoglin is upregulated and

promotes tumor cell plasticity and invasion, likely by promoting BMP and focal adhesion kinase signalling (Pardali *et al.*, 2011).

Unlike betaglycan, endoglin is unable to bind TGF-β on its own. However, it does bind TGF-β1 and TGF-β3 (but not TGF-β2) with high affinity in the presence of TGFBR2 (Letamendia *et al.*, 1998). Moreover, endoglin can also bind activin-A, BMP-2 and BMP-7, through association with their corresponding receptor (Barbara *et al.*, 1999). Endoglin has also been shown to form a heteromeric complex with betaglycan, an association likely to be important in regulating TGF-β signalling (Wong *et al.*, 2000). Endoglin usually acts as an inhibitor of TGF-β responses, such as migration and growth inhibition in diverse cell lines (Letamendia *et al.*, 1998; Li *et al.*, 2000). Inhibition of these TGF-β responses by endoglin is thought to be mediated by endoglin's ability to regulate the balance between the TGF-β/ALK5 and the TGF-β/ALK1 pathway. In endothelial cells and in chondrocytes, endoglin interacts with ALK1 and enhances TGF-β/ALK1/SMAD1/5/8 signalling, while diminishing TGF-β/ALK5/SMAD2/3 signalling (Blanco *et al.*, 2005; Finnson *et al.*, 2010; Lebrin *et al.*, 2004).

Endoglin interacts with both type I and type II receptor in resting cells. TGFBR2, ALK1 and ALK5 phosphorylates endoglin cytoplasmic domain, resulting in modulation of endoglin function (Guerrero-Esteo *et al.*, 2002; Koleva *et al.*, 2006; Ray *et al.*, 2010). In return, endoglin affects the phosphorylation status of TGFBR1 and TGFBR2 to decrease TGF-β signalling and responses (Guerrero-Esteo *et al.*, 2002).

Via its cytoplasmic domain, endoglin associates with the adhesion proteins zyxin and ZRP-1 to reduce cell migration independently of TGF-β (Conley *et al.*, 2004; Sanz-Rodriguez *et al.*, 2004). Like betaglycan, endoglin can also interact with β-arrestin2, leading to endoglin internalization and inhibition of TGF-β-induced ERK signalling and endothelial cell migration (Lee and Blobe, 2007). However, the ability of endoglin to regulate TGF-β signalling receptor internalization has not been examined. In contrast, endoglin has been shown to localize to caveolae, where it forms a complex with eNOS (Toporsian *et al.*, 2005). Because caveolar localization has been shown to enhance TGF-

β/ALK1 signalling (Santibanez *et al.*, 2008), endoglin may modulate ALK1 localization in caveolae.

1.7.3- CD109

1.7.3.1- Identification of r150/CD109 as a novel TGF-β co-receptor

Previous results from our laboratory have unveiled the existence of a novel ≈150kDa TGF-β1 binding molecule on the cell surface of human keratinocytes, by using ^{125}I-TGF-β1 affinity labeling. This novel TGF-β1 binding molecule was called r150 and displays high affinity for TGF-β1, but lower affinities for TGF-β2 and TGF-β3 subtypes. Moreover, r150 forms a heteromeric complex with the TGFBR1 and TGFBR2 signalling receptors (Tam *et al.*, 1998). Further characterization showed that r150 is sensitive to PI-PLC treatment, suggesting that r150 is a GPI-anchored protein (Tam *et al.*, 1998). The released (soluble) r150 binds TGF-β1 and prevents TGF-β1 binding from the signalling receptors in human neonatal and in immortalized keratinocytes (HaCaT cells) (Tam *et al.*, 2001). In an attempt to uncovered r150 (and other GPI-anchored proteins) role in TGF-β signalling, mutant HaCaT cells deficient in GPI anchor synthesis (and therefore in r150) were created. These mutant cells exhibit enhanced TGF-β-induced SMAD2/3 phosphorylation and increased TGF-β signalling and responses, as compared to the parental HaCaT cells (Tam *et al.*, 2003). Together, these results suggest that r150 or other GPI-anchored proteins negatively regulate TGF-β signalling. More recently, r150 was cloned from keratinocytes by affinity purification on a TGF-β1 column and subsequent microsequencing (Finnson *et al.*, 2006). r150 cloning reveals that it represents CD109, a protein whose function was so far obscure. Later, the ability of CD109 to binds TGF-β1 and to form a heteromeric complex with the TGF-β receptors, in the presence and absence of ligand, was confirmed. Moreover, it was demonstrated that CD109 directly interacts with TGFBR1 (Finnson *et al.*, 2006), as illustrated in Fig 1.6A. In addition, preliminary results suggested that CD109 may inhibit TGF-β signalling.

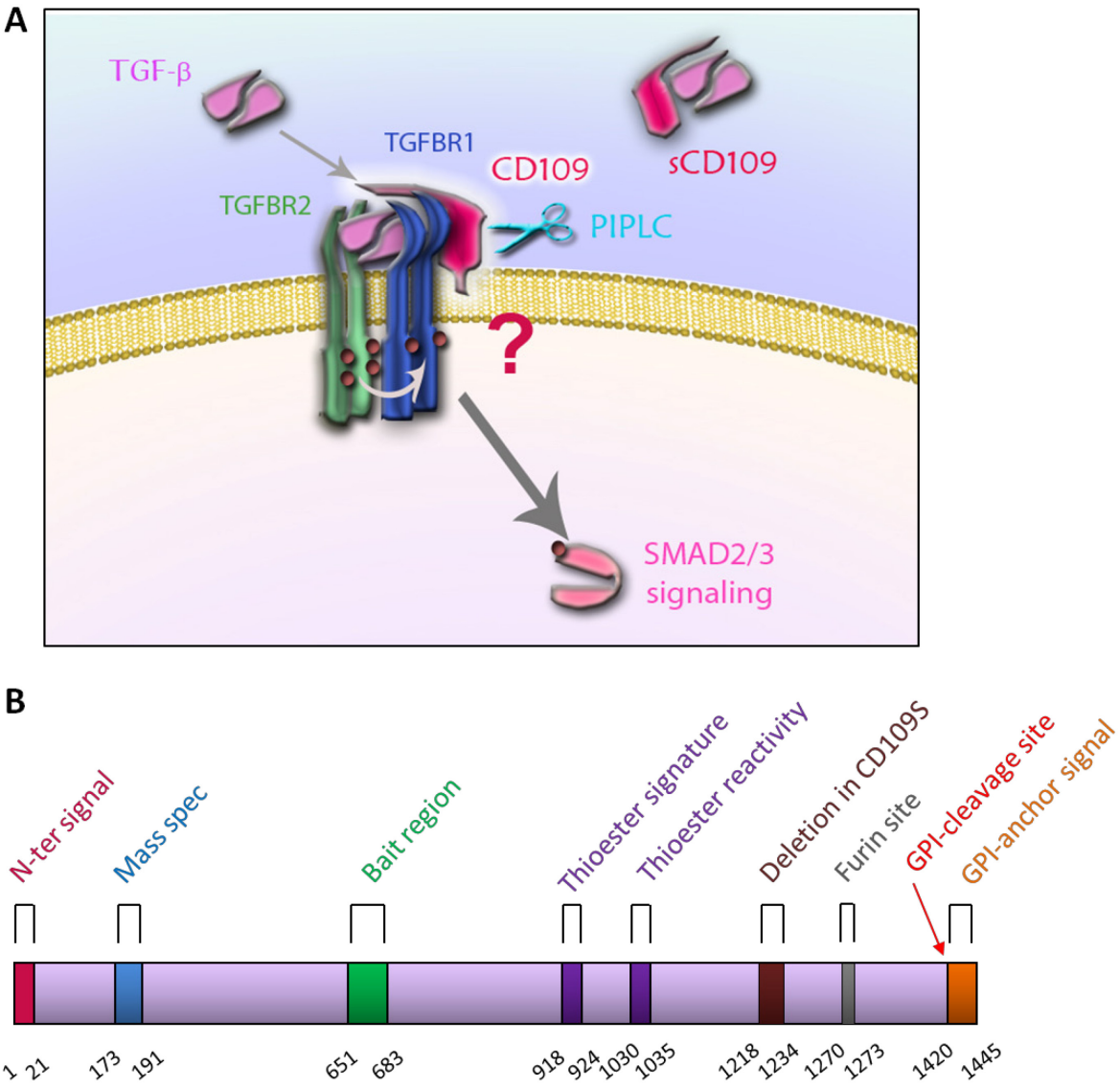


Figure 1.6: A. Schematic representation of CD109 function. CD109 is a GPI-anchored protein that can be cleaved by PI-PLC. Both soluble and membrane-anchored forms bind TGF-β1 subtype with high affinity. The soluble form can sequester TGF-β1 away from the signalling receptors. In addition, CD109 forms a heteromeric complex with TGF-β receptors and binds directly to TGFBR1. **B. Schematic representation of CD109 domains**. CD109 shares several features with members of the α2M/complement family, including a putative bait region, a thioester signature motif and thioester reactivity defining hexapeptide. CD109 is a GPI-anchored protein and as such, possesses an N-terminal signal sequence, a C-terminal GPI-anchor signal consensus sequence and a GPI-anchor cleavage site. The location of the microsequence obtained by mass spectrometry that allowed the identification of r150 as CD109, the sequence deleted in CD109S and the furin cleavage site are also indicated.

Collectively, these results indicate that CD109 is a novel TGF-β co-receptor and may thus modulate TGF-β signalling.

1.7.3.2- CD109: a member of the α2-macroglobulin/complement family

CD109 gene consists of 33 exons spanning a 128 kb region located on human chromosome section 6q13 and encodes a ≈1445 amino acid protein. Human CD109 possesses orthologs in mouse (the sequence identity is about 69%), rat, dog and cow (Solomon *et al.*, 2004). Different isoforms of CD109 results from alternative splicing (Solomon *et al.*, 2004). For instance, in the placenta, but not in keratinocytes, a shorter version of CD109 with 17 amino acid deleted at position 1218-1234 is found (and was termed CD109S) (Finnson *et al.*, 2006).

CD109 is a distant member (it has less than 30% amino acids sequence identity with other members of the family), and maybe the ancestor of the α2-macroglobulin (α2M)/complement family (Solomon *et al.*, 2004). This family consists notably in the C3, C4 and C5 complement system, that is activated during both acquired and innate immunity and that is involved in host defense and inflammation (Law and Dodds, 1997). α2M, another member of the (α2M)/complement family functions as a protease inhibitor, acts as a ligand for the low density lipoprotein receptor-related protein (LRP)-1 receptor and is a carrier for PDGF-BB and TGF-β1 in the plasma (O'Connor-McCourt and Wakefield, 1987). All members of the α2M family, including CD109, have evolved from a core of eight homologous macroglobulin domains (Janssen *et al.*, 2005) and contain a putative bait region involved in substrate specificity, a thioester signature sequence and a hexapeptide motif that defines the chemical reactivity of the thioester. The different motifs of CD109 are represented in Fig 1.6B. In the native molecule, the intrachain thioester bond is unreactive. Proteolytic cleavage of any of the target peptide bonds in the bait region of the molecule by specific activating enzymes (in the case of the complement proteins) or by a wide range of proteases (in the case of α2M) induces a major conformational change. The thioester becomes highly reactive toward

nucleophiles so that the protein becomes capable of covalently binding nearby molecules (Dodds and Law, 1998). By this mechanism, α2M entraps the reacting proteases within its hollow core structure. Moreover, the conformational change of α2M reveals binding sites for LRP-1 and enhances the affinity of α2M for PDGF-BB and TGF-β1 (Webb *et al.*, 1994). Under experimental conditions (by heating or chemical denaturation during SDS-PAGE samples preparation), the thioester may also undergo nucleophilic attack, resulting in autolytic cleavage of the protein (Sim and Sim, 1981). This autolytic cleavage can be prevented by pretreatment with methylamine. CD109 contains an intact thioester bond and the observed 150kDa band is actually the result of autolytic cleavage of the 180kDa band (Finnson *et al.*, 2006; Lin *et al.*, 2002). However, the function of this thioester bond remains to be determined. In addition, sequence alignment between CD109 and α2M indicates that the putative LRP-1 binding site in α2M is conserved in CD109 but so far, it is not known whether CD109 interacts with LRP-1 (Solomon *et al.*, 2004). Identification of the TGF-β1 binding site in CD109, based on similarity with the α2M sequence, is the object of another currently ongoing PhD thesis and of a patent application.

1.7.3.3- CD109: a GPI-anchored protein

Unlike most α2M/complement family members, CD109 possesses a GPI-anchor cleavage site and recognition sequence at the C-terminus. CD109 also contains 17 potential N-linked glycosylation sites and a 21 amino acid N-terminal signal peptide (Lin *et al.*, 2002) (Fig 1.6B). The N-terminal signal peptide allows translocation of CD109 into the endoplasmic reticulum (ER). In this compartment, the GPI-attachment signal sequence is recognized and cleaved. The “new” C-terminal amino-acid of the protein is then linked through a phosphodiester bond to a preassembled GPI anchor by the action of a GPI transamidase (Lakhan *et al.*, 2009). The GPI anchor consists of a phosphatidylinositol (PI) group attached to a carbohydrate moiety (trimannosyl-non-acetylated glucosamine) and is synthesized in the ER from PI through at least nine sequential reaction steps, involving

18 different enzymes. After attachment of the GPI precursor to the protein and further inositol deacylation in the ER, the GPI-anchored protein is transported into the golgi, where fatty acid remodeling and association with lipid-raft occur (Kinoshita *et al.*, 2008). The presence of GPI anchor allows CD109 (and other GPI-anchored proteins) to be released from the membrane by phospholipases. The phosphatidylinositol phospholipase C (PI-PLC) can hydrolyze the PI bond of GPI-anchor closest to the cell membrane, exposing a 1,2 cyclic monophosphate on the remaining part of the anchor, that is recognized by cross-reacting determinant(CRD) antibodies. Several evidences suggest the presence of endogenous PI-PLC activity in some cell types. For instance, using CRD antibody, it has been demonstrated that CD109/r150 is released by an endogenous PI-PLC in keratinocytes (Tam *et al.*, 2001). However, the molecular characterization of these enzymes in mammalian cells is still missing. In addition, PI-PLD is also able to cleave CD109 anchor (Lin *et al.*, 2002). Interestingly, normal human keratinocytes have very low levels of PI-PLD as opposed to mouse keratinocytes. Moreover, tumorigenic epidermoid squamous cell carcinoma A431 cells express a high level of GPI-PLD as compared to non-tumorigenic HaCaT cultured in the absence of serum, but serum addition upregulates PI-PLD expression in HaCaT cells (He *et al.*, 2002b). Thus, CD109 may be differentially shed in mouse versus human keratinocytes and in tumorigenic versus non-tumorigenic cells. Moreover, in certain cell types, a population of CD109 is resistant to PI-PLC but sensitive to PI-PLD, indicating that some of the CD109 GPI anchors are acylated on inositol (Lin *et al.*, 2002). This suggests that a proportion of CD109 may have distinct lipid solubility and may partition into distinct membrane domains. Moreover, it has been shown that T-cell CD109 is more soluble in non-ionic detergents than other GPI-anchor proteins and do not localize to Src-positive lipid-raft (Solomon *et al.*, 1998).

In contrast to previous studies that have demonstrated that CD109 is a single chain molecule (Smith *et al.*, 1995; Tam *et al.*, 2001), the 205Kda glycosylated form of CD109 have been shown to be cleaved in the golgi into a 180KDa form and a 25KDa form (that contain the GPI-anchor) by furin (Hagiwara *et al.*, 2010). The 180KDa and 25Kda forms of

CD109 remain associated at the cell surface and form a complex with TGFBR1. The cleavage by furin appears to be required for CD109 and TGFBR1 interaction. In addition, the authors found an important amount of the 180kDa form in the extracellular medium, suggesting that the 180KDa form can dissociate from the 25KDa membrane-anchored form. In contrast, only little amount of the 25KDa form (which may or may not remain associated with the 180KDa form) is detected in the medium, suggesting that secretion by cleavage of the GPI-anchor might not be the primary mechanism of CD109 shedding, at least in 293 cells (Hagiwara *et al.*, 2010). However, the mechanism regulating the dissociation of the 180KDa from the 25KDa form remains obscure. Moreover, preliminary results from our lab indicate that a significant portion of CD109 is not cleaved by furin.

1.7.3.4- Expression of CD109

CD109 was first characterized as a marker of hematopoietic stem cells (Furley *et al.*, 1986; Murray *et al.*, 1999). During hematopoietic differentiation, CD109 expression is restricted to myeloid precursors and then to megakaryoblastic progenitors and finally becomes undetectable on resting, mature blood cells. CD109 then reappears on activated T cells (CD4+ and CD8+) and activated platelets (Sutherland *et al.*, 1991). Interestingly, platelet CD109 carries the platelet-specific Gov antigen system, implicated in refractoriness to platelet transfusion (Smith *et al.*, 1995). A tyrosine at position 703 of CD109 defines the Gov a antigen, while a serine at this position defines the Gov b epitope (Schuh *et al.*, 2002). In addition, in platelet, CD109 is modified by glycosyltransferases so that platelet CD109 also carries the blood group A and B determinants (Kelton *et al.*, 1998).

More recently, a wider distribution of CD109 has been reported. For instance, CD109 was found on human keratinocytes (Finnson *et al.*, 2006), chondrocytes (Stevens *et al.*, 2008) and mouse and human testis (Hashimoto *et al.*, 2004). Importantly, CD109 expression is upregulated in many cancers, such as basal-like breast carcinoma (Hasegawa *et al.*, 2008), glioblastoma and in some adenocarcinoma and sarcoma, but its expression

remains low in neuroblastoma, small-cell lung carcinoma, leukemia and lymphoma cell lines (Hashimoto *et al.*, 2004). CD109 is also highly expressed in well differentiated squamous cell carcinomas (SCC) of various origins, as compared to normal epithelia (Hagiwara *et al.*, 2008; Hashimoto *et al.*, 2004; Sato *et al.*, 2007; Zhang *et al.*, 2005a). This upregulation of CD109 in SCC may be due to gene amplification (Järvinen *et al.*, 2008). However, CD109 expression levels decreases as the grade of differentiation of the SCC becomes poor (Hagiwara *et al.*, 2008). Moreover, CD109 cleavage by the protease mesotrypsin, which is upregulated in malignant breast cancer cells, appears to be important for malignant growth (Hockla *et al.*, 2009). While a study has found that CD109 transcripts level are increased in human malignant melanoma cell lines than in non-tumorigenic cells (Ohshima *et al.*), results from our lab suggest that CD109 mRNA expression is downregulated in the majority of melanoma analyzed (Finnson *et al.*, 2006). Such discrepancies between the two studies might be due to the low number of samples analyzed in both cases. In addition, *CD109* appears to be mutated in some colorectal cancers (Sjoblom *et al.*, 2006) and the *CD109* gene is differentially methylated (and thus silenced) during colorectal cancer progression: *CD109* gene becomes more methylated as the tumor stage advances. *CD109* gene methylation is also higher in proximal than in distal tumors, suggesting a role for CD109 during colon cancer metastasis (Mokarram *et al.*, 2009). Altogether, these studies indicate that CD109 is likely to play an essential role during cancer progression.

1.7.4- Other TGF-β superfamily "co-receptors": Cripto, RGM and BAMBI

The GPI-anchored protein Cripto is a member of the epidermal growth factor-Cripto, FRL-1, Criptic (EGF-CFC) family and function as a "co-receptor" for Nodal, activin and TGF-β. Cripto inhibits TGF-β signalling by competing with TGFBR1 for binding to the TGF-β1-TGFBR2 complex (Gray *et al.*, 2006) and may inhibit TGF-β tumor suppressive function during skin carcinogenesis (Shukla *et al.*, 2008). Cripto also antagonize activin signalling, by inhibiting ALK4 binding to activin and ActR2/2B (Gray *et al.*, 2003). In

addition, Cripto potentiates Nodal signalling, by promoting the interaction between Nodal and ALK4 and ALK7 (Yeo and Whitman, 2001). Cripto brings the precursor of Nodal (pre-Nodal) to the lipid-raft compartment and recruits proprotein convertases such as Furin and PACE4 to facilitate Nodal biosynthesis (Blanchet *et al.*, 2008a). Moreover, it directs Nodal at the limiting membrane of the endosome, bringing Nodal at proximity of SARA, SMAD2 and SMAD3 to facilitate signalling (Blanchet *et al.*, 2008b).

The GPI-anchored proteins of the repulsive guidance molecule family RGMa, RGMb (also known as DRAGON) and RGMc (or hemojuvelin) functions as co-receptors for BMP and, in their GPI-anchored form, enhance BMP signalling (Elena *et al.*, 2009). RGM proteins selectively bind BMP-2 and BMP-4 with high affinity (as compared to betaglycan), but do not bind TGF-β or BMP-7. RGM proteins are thought to allow BMP-2 and BMP-4 ligands, which normally signal via BMPR2 alone, to signal via ActR2A and thus, enhance BMP signalling. On the other hand, the soluble form of RGM protein sequesters BMP and inhibits BMP signalling (Babitt *et al.*, 2006; Babitt *et al.*, 2005; Samad *et al.*, 2005).

BAMBI (BMP and activin membrane-bound inhibitor) encodes a transmembrane protein with an extracellular domain that is closely related to TGF-βs type I receptors but lacks the intracellular serine/threonine kinase domain. BAMBI prevents the complex formation of type II receptors with most type I receptors (ALK3, ALK6, ALK1, ALK4 and ALK5), thereby inhibiting pan-TGF-β signalling (Onichtchouk *et al.*, 1999). Thus, BAMBI is considered as a pseudo-receptor. Recently, it has been shown that human BAMBI also inhibits TGF-β and BMP-mediated signalling not only by interfering with TGF-β receptor complex formation, but also by increasing the recruitment of SMAD7 to the type I receptor ALK5, thus impairing SMAD3 recruitment and phosphorylation by ALK5 (Yan *et al.*, 2009a). Interestingly, BAMBI also interacts with the Wnt receptor Frizzled and with its co-receptor LRP6 to increase Wnt/β-catenin signalling (Lin *et al.*, 2008).

1.8- Role of TGF-β in human skin diseases

1.8.1- Role of TGF-β in skin development and homeostasis

The skin, the largest organ of the human body, is composed of a stratified epithelium, the epidermis, separated from an underlying connective tissue, the dermis by a basement membrane. The epidermis and its appendages (such as hair follicles, sebaceous glands and nails) develop from the ectoderm during embryogenesis (Owens *et al.*, 2008). TGF-β2 has been shown to promote hair follicles development in mice, and, while deletion of individual R-SMAD in the epidermis does not impair skin development, deletion of SMAD4 results in loss of hair follicle, suggesting that the R-SMADs may have redundant function during skin development and differentiation (Owens *et al.*, 2008). Ectopic expression of SMAD7 in the epidermis leads to severe defects in epithelium and hair development, likely due to its ability to antagonize the R-SMADs and to promote β-catenin degradation by Smurf2 (He *et al.*, 2002a; Owens *et al.*, 2008). Together, these studies suggest that TGF-β and the SMADs play important roles during skin development via canonical and non-canonical pathways.

During epidermal differentiation, keratinocytes of the basal layer of the epidermis cease proliferating and enter suprabasal layers, where they undergo progressive differentiation. Eventually, the cells formed a cornified layer and slough off the skin (Li *et al.*, 2006). TGF-β is a major factor regulating skin homeostasis by modulating cell growth, apoptosis, differentiation, ECM production, migration, inflammation and angiogenesis (Massagué, 2008; White *et al.*, 2010). Given the critical role of TGF-β during skin development and homeostasis, it is not surprising that dysregulation of the TGF-β signalling pathway has been implicated in a variety of skin disorders including impaired wound healing (Bennett *et al.*, 2003; Cowin *et al.*, 2001), hypertrophic scarring (Armour *et al.*, 2007; Seifert and Mrowietz, 2009), psoriasis (Li *et al.*, 2004a), scleroderma (Leask and Abraham, 2004) and skin cancer, such as epidermoid squamous cell carcinoma (Li *et al.*, 2005).

1.8.2- Role of TGF-β signalling in skin squamous cell carcinomas

Squamous cell carcinoma (SCC) is the most common epithelial malignancy in the Human. SCCs usually occur in sun exposed skin and form islands of squamous epithelium in the interfollicular epidermis. The classification of SCCs is based on the degree of differentiation, determined by the degree of keratinization (Boukamp, 1999). A carcinoma can be characterized as either *in situ* (confined to the original site) or invasive, if the tumor has invaded underlying tissues. Skin SCCs metastase at a frequency of 3-10%, preferentially in the lymph nodes (Weinberg *et al.*, 2007).

Several animal models demonstrate a dual role for TGF-β during skin carcinogenesis. Transgenic mice overexpressing TGF-β1 in the epidermis and subjected to long-term chemical carcinogenesis treatment develop less and smaller tumors than their wild-type counterpart, indicating that TGF-β1 acts as a tumor suppressor during the early stage of the disease. In contrast, benign papillomas from TGF-β1 transgenic mice tend to metastasize more frequently than wild-type papillomas, indicating that TGF-β promotes tumor progression later on (Cui *et al.*, 1996; Weeks *et al.*, 2001).

1.8.2.1- TGF-β function as a tumor suppressor

TGF-β is a potent inhibitor of epithelial cell proliferation, inducing the expression of the CDK inhibitors $p15^{INK4b}$ and $p21^{Cip1}$ to cause G1/S arrest (Gomis *et al.*, 2006; Seoane *et al.*, 2004), while repressing the expression of the proto-oncogene c-MYC (Chen *et al.*, 2002). On the other hand, TGF-β promotes apoptosis by inducing specific transcription factors such as p53, in a p38 and SMAD-dependent manner (Heldin *et al.*, 2009) and induces epithelial differentiation by downregulating inhibitor of differentiation (ID 1, 2 and 3) (Kang *et al.*, 2003). TGF-β can thus limit pre-malignant growth (Padua and Massagué, 2009).

In order to progress into malignant and invasive tumors, cancer cells have to evade from TGF-β tumor suppressor activities. In many cancer, such as colon and pancreatic cancer,

this is achieved by inactivating mutation of TGF-β core signalling components. In human poorly differentiated SSC, SMAD2 and SMAD4 are often lost at the pretranscriptional level (Hoot *et al.*, 2008). Ablation of *Smad2* or *Smad4* gene in mouse epidermis results in increased proliferation of basal keratinocytes and spontaneous formation of malignant squamous cell carcinomas (Hoot *et al.*, 2008; Qiao *et al.*, 2005). However, deletion of *Tgfbr2* in the skin does not give rise to spontaneous formation of SCC, due to elevated apoptosis that compensates for the increase in cell proliferation (Guasch *et al.*, 2007). In contrast, deletion of *Tgfbr2* in combination with Ras activation, which are common events in human SCCs, leads to formation of aggressively invasive and metastatic squamous cell carcinomas in mice (Guasch *et al.*, 2007; Lu *et al.*, 2006; Wakefield and Stuelten, 2007). These mutations of TGF-β core signalling components are relatively rare in skin cancer. Other mechanisms, including the combined loss of $p15^{INK4b}$ expression and c-MYC repression (Chen *et al.*, 2001), result in the loss of the TGF-β cytostatic response (Bernabeu *et al.*, 2009; Padua and Massagué, 2009). Cancer cells that have evaded TGF-β-induced growth inhibitory response produce more TGF-β as a compensatory mechanism (Blobe *et al.*, 2000; Han *et al.*, 2005; White *et al.*, 2010). This overproduction of TGF-β further induces tumor invasion and metastases, by turning on TGF-β tumor promoting activities (Lu *et al.*, 2006; White *et al.*, 2010).

1.8.2.2- TGF-β function as a tumor promoter: critical role during EMT

TGF-β is able to promote angiogenesis, evasion of immunity, cell migration and invasion. Each of these processes favors tumor metastasis. TGF-β stimulates angiogenesis, which allows the tumor to receive nutriments and oxygen necessary for its growth and is essential for dissemination of tumor cells throughout the body (Padua and Massagué, 2009). TGF-β also recruits and activates T regulatory cells (Pyzik and Piccirillo, 2007) and suppresses the activity of dendritic cells and natural killer cells to escape the host immune system (Padua and Massagué, 2009).

TGF-β can also enhance the migratory and invasive properties of cancer cells, by inducing a process known as epithelial-mesenchymal transition (EMT). EMT is characterized by a series of events leading to the acquisition of a mesenchymal phenotype (Kalluri and Weinberg, 2009). One of the first events of EMT is the delocalization of tight junction proteins, such as ZO-1, claudin-1 and occludin, the dissolution of adherens junction complexes containing E-cadherin and β-catenin and the reorganization of the actin cytoskeleton into actin stress fibers (Miyazono, 2009). Then, the cell loses its apical-basal cell polarity, adopts a spindle-like morphology and starts to express mesenchymal markers, such as N-cadherin, vimentin, fibronectin and α-smooth muscle actin (α-SMA). Cells undergoing EMT show enhanced migratory and invasive capacity, increased production of ECM components and proteases and elevated resistance to apoptosis (Kalluri and Weinberg, 2009; Klymkowsky and Savagner, 2009; Miyazono, 2009).

The EMT program is activated during embryonic development and in adulthood during tissue repair, progression of fibrosis and cancer (Kalluri and Weinberg, 2009; Miyazono, 2009). In 1890, Santiago Ramon y Cajal described undifferentiated breast carcinomas as follows: “the epithelial islands are not surrounded by a basement membrane…We shall mention the fusiform, pear-like and star-like forms…the cells are not attached to each other….This explains their invasive ability, since free of intracellular cement, they can migrate through connective tissue”(Ramon y Cajal, 1890). This is likely the first description of the EMT phenomenon in cancer. Since then, evidence of an EMT process have been found in several human cancers, notably at their invasive front, a location rich in stromal TGF-β and other cytokines (Padua and Massagué, 2009). TGF-β cooperates with different signalling pathways, such as integrin, Notch, Wnt, TNF-α and EGF to induce EMT (Miyazono, 2009). Notably, TGF-β induces the expression of many transcriptions factors, including the zinc-finger factors Snail and Slug, δEF1/ZEB1, ZEB2, and Twist that control many of the aspect of EMT (Miyazono, 2009). TGF-β and these master regulators of EMT have also been shown to promote the expression of cell surface markers of “cancer stem cells”, the putative tumor-propagating cells (Mani *et*

al., 2008). The EMT process may not only enable cancer cell invasion and dissemination, but also enhances their self-renewal capability (Mani *et al.*, 2008).

Several evidences indicate that TGF-β promotes EMT via SMAD-dependent and independent mechanisms. In HaCaT cells, SMAD4 is required for TGF-β-induced cell cycle arrest and migration, but not for EMT (Levy and Hill, 2005). Zavadil and colleagues found that ERK is required for TGF-β-induced EMT-like phenotype in HaCaT cells (Zavadil *et al.*, 2001). In HaCaT cells, a more advanced EMT phenotype is seen after stimulation with both TGF-β1 and EGF (or oncogenic Ras) and involves SMAD3 and the MAPK (p38, JNK and ERK) pathways (Davies *et al.*, 2005). Similarly, in mammary epithelial cells, induction of Snail expression by the SMADs requires activation of the Ras-ERK MAPK pathway (Heldin *et al.*, 2009). Others pathways are also involved in TGF-β-mediated EMT, notably the Par6/Smurf1 pathway that promotes the dissolution of the tight junctions (Ozdamar *et al.*, 2005), as mentioned above.

Interestingly, SMAD2 and SMAD3 have opposite role during EMT: while *Smad3* null mice have unraveled an essential role for SMAD3 in TGF-β-induced EMT (Sato *et al.*, 2003), loss of SMAD2 in the skin confers enhanced EMT characteristics (Hoot *et al.*, 2008).

The notion of EMT during cancer metastasis implicates the additional concept of mesenchymal-epithelial transition (MET) to explain the formation of a secondary tumor with characteristics of the primary tumor (Kalluri and Weinberg, 2009). This implies that EMT is a transient and reversible phenomenon (Miyazono, 2009). Interestingly, it has been shown that long-term TGF-β exposure of mammary epithelial cells reversibly suppresses both SMAD2/3 anti-proliferative and p38 pro-apoptotic pathways and reduces survival and proliferative Akt and ERK pathways to a low but significant level, causing resistance to TGF-β-induced growth arrest and promoting EMT (Gal *et al.*, 2008). TGF-β may thus modulate its own signalling to facilitate switching from tumor suppression to tumor progression and withdrawal of TGF-β may lead to MET (Gal *et al.*, 2008).

Given the critical role of TGF-β during tissue homeostasis, inhibition of TGF-β signalling was predicted to affect the good and bad sides of TGF-β. However, it has been

demonstrated that long term blockage of TGF-β signalling using a soluble TGFBR2 does not significantly influence animal morbidity (Muraoka *et al.*, 2002; Yang *et al.*, 2002). Identification of TGF-β inhibitors may thus be of potential interest for the development of novel therapeutic strategies to treat disease with aberrant TGF-β signalling, such as cancer, fibrosis and impaired wound healing.

1.8.3- Role of TGF-β during wound healing

Wound healing is a well orchestrated phenomenon involving many cell types, including immune cells, endothelial cells, keratinocytes and fibroblasts and results from the coordination of various signalling pathways (Epstein *et al.*, 1999). Soon after injury, TGF-β1 is rapidly upregulated and secreted by macrophages, platelets and keratinocytes (Epstein *et al.*, 1999). This event is important for the initiation of the inflammatory response, as TGF-β1 acts as a chemoattractant to recruit additional inflammatory cells to the wound site and thus prevents infection (Barrientos *et al.*, 2008). Later on, TGF-β1 induces angiogenesis and enhances migration of keratinocytes and fibroblasts at the wound edge, by upregulating several MMPs (Lamar *et al.*, 2007; Madlener, 1998), cell migration-associated integrins (Gailit *et al.*, 1994) and by promoting the dissolution of cell-cell junction. Thus, TGF-β1 participates to the re-epithelialization of the wound. Then, TGF-β1 causes fibroblasts to differentiate into myofibroblasts, which stimulates wound contraction (Desmoulière *et al.*, 2005). Importantly, TGF-β is a potent inducer of ECM components, such as collagen and fibronectin and of proteases inhibitors, like PAI-1, thereby controlling the remodeling of ECM by fibroblasts (Schiller *et al.*, 2004). Although these ECM proteins provide strength and scaffolding to the healing wound, an overproduction of these proteins causes keloids and hypertrophic scarring (Barrientos *et al.*, 2008).

Unlike the other two TGF-β subtypes which promote scar formation, TGF-β3 appears to prevent scarring. Indeed, decreasing the TGF-β1/TGF-β3 ratio has been shown to improve scarring in animal models (Barrientos *et al.*, 2008; Ferguson and O'Kane, 2004).

On the other hand, a reduction of TGF-β1 expression is as deleterious and has been implicated in impaired wound healing, such as in diabetic foot ulcers and chronic venous ulcers (Cowin *et al.*, 2001; Jude *et al.*, 2002; Schmid *et al.*, 1993). Although exogenous administration of TGF-β1 is beneficial in animal models of impaired wound healing (Beck *et al.*, 1991; Beck *et al.*, 1993; Pierce *et al.*, 1989), application of TGF-β1 to human chronic ulcers has so far been discouraging (Bennett *et al.*, 2003). This might be due to inappropriate timing of administration or unavailability of the delivered factor. In our laboratory, we hypothesize that local manipulation of endogenous TGF-β action using regulatory molecules may provide an alternate approach to promote healing or reduce scarring. Thus, we have focused our research on characterizing such molecules.

1.9- Rationale and objectives for the current study

TGF-β is a multifunctional growth factor involved in many cellular processes, including cell growth, extracellular matrix production, cell migration and EMT. Dysregulation of its pathway have been implicated in several human diseases, notably in skin disorders such as impaired wound healing and hypertrophic scarring, fibrosis and squamous cell carcinomas. This underscores the need for a tight regulation of TGF-β signalling. TGF-β signals via the type I and type II receptors (TGFBR1 and TGFBR2) that transmit the signal intracellularly by phosphorylating SMAD2/3. Once phosphorylated, SMAD2/3 forms a complex with SMAD4 and accumulates into the nucleus, where it regulates gene transcription. In addition to this canonical pathway, TGF-β can also elicit signalling via the p38, ERK and JNK MAPK, via PI3K/Akt and via Rho-like GTPases, in a cell-specific manner. The TGF-β receptors have the particularity to internalize through two different routes. Endocytosis via the clathrin-coated pits results in SMAD2/3 signalling, whereas endocytosis into caveolae vesicles leads to MAPK signalling and receptor degradation, following recruitment by SMAD7 of the E3 Ubiquitin ligase Smurf2. Little is known regarding the factors regulating TGF-β receptors compartmentalization, degradation and MAPK activation.

Dr Philip's laboratory has previously identified a novel TGF-β co-receptor, CD109, in skin cells. CD109 is a GPI-anchored protein with high affinity for TGF-β1 subtype that forms a heteromeric complex with the TGF-β signalling receptors. Although previous results from our laboratory using GPI-mutant cells suggest that CD109 may be a negative regulator of TGF-β signalling, the full demonstration of CD109's function remained incomplete. In addition, CD109 was found to be mutated or dysregulated in many cancers, including in squamous cell carcinomas, suggesting that CD109 may play an important role during tissue homeostasis.

The objectives of this thesis were (1) to validate CD109's ability to inhibit TGF-β signalling and responses; (2) to investigate whether CD109 negatively regulates TGF-β signalling by regulating TGF-β receptor compartmentalization and degradation; and (3) to examine whether CD109 regulate the EMT process by modulating TGF-β-induced MAPK activation in the context of squamous cell carcinomas.

Chapter 2: Materials and methods

2.1- Cell culture

The human keratinocyte cell line HaCaT (Boukamp *et al.*, 1988) was kindly provided by P.Boukamp (Heidelberg, Germany), the HaCaT cells mutated in GPI anchor biosynthesis were previously generated in our lab (Tam *et al.*, 2003), human embryonic kidney (HEK) 293 cells, Mink Lung (Mv1Lu) cells, Cos-1 cells and A431 cells were purchased from the American Type Culture Collection. Cells were cultured in high glucose D-MEM (Gibco) supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and 50 μg/ml streptomycin (complete D-MEM) under standard conditions (37^{o}C in an atmosphere of 5% CO_2/air). TGF-β treatment was done in D-MEM containing antibiotics, but no FBS.

HaCaT clones stably expressing CD109 (clone CD3-3 and clone CD2-4) and the control cells (clone EV3-3 and clone EV3-4) were generated previously in our lab by transfection of CD109 cDNA in pDEST26 vector (which contains a neomycin resistance gene) or the empty vector. The cells were treated with 0.5mg/mL Geneticin until most cells died. The remaining colonies were isolated and cultured in complete D-MEM supplemented with 0.5mg/mL Geneticin.

A431 cells stably expressing CD109 or its EV (CD109-A431 and EV-A431) were generated by transfection of CD109 cDNA in pDEST26 vector or the empty vector. The transfected cells were selected and cultured in complete D-MEM supplemented with 0.5mg/mL Geneticin.

HaCaT cells stably expressing CD109 shRNA were generated by transfection of pENTR/H1/To plasmid (Invitrogen) in which CD109 shRNA was inserted by PCR using the following forward primer: 5'-CACCCCAGTAGAAATTTTAACCACGAATGGTTAAAATTTCTACTG-3' and reverse primer: 5'-AAAACCAGTAGAAATTTTAACCATTCGTGGTTAAAATTTCTACTG-3'. Alternatively, HaCaT cells were transfected with a scrambled shRNA containing plasmid. The transfected cells

were selected and cultured in complete D-MEM supplemented with 10 μg/ml Zeocin (Invitrogen).

Primary mouse keratinocytes from transgenic mice overexpressing CD109 in the epidermis (with human *CD109* under the control of the K14 promoter) or from wild-type control littermates were extracted from new born mice and cultured as described in (Pirrone *et al.*, 2005). Briefly, the limbs and the tail were removed (the tail was used for genotyping) and a longitudinal incision was made to detach the skin (dermis +epidermis) from the body. After several washes in PBS, the skin was laid down (dermis down) onto a sterile culture dish and allowed to dry for 5 to 10 min. Then, the skin was incubated with 0.25% Trypsin overnight at 4^{o}C. The next day, the epidermis was peeled off the dermis and minced into small pieces. The keratinocytes were then grown into collagen-coated dishes in N-medium (EMEM (Lonza) 0.06mM Ca2+, 10% chelexed FBS (to remove all calcium) mixed 1:1 with conditioned medium from mouse fibroblasts supplemented with 2ng/ml EGF, 10^{-10} M Cholera Toxin, 0.4 μg/ml Hydrocortisone, 0.75mM Aminoguanidine nitrate and penicillin/strepamycin, fungizone). The conditioned medium was collected every other day from primary mouse fibroblasts grown in EMEM 0.06mM Ca^{2+}, 10% FBS with antibiotics. TGF-β treatment was done in low serum medium (EMEM 0.06m Ca2+ mixed 1:1 with conditioned medium from mouse fibroblasts supplemented with penicillin/strepamycin and fungizone). The cells were cultured at 37^{o}C in an atmosphere of 5% CO_2/air. The absence of other cells in our culture was verified by staining the cells with K14, a marker of keratinocytes.

Mouse embryonic fibroblast (MEFs), a gift from A. Roberts (National Cancer Institute), were cultured in low glucose D-MEM supplemented with 10% FBS, 100U/ml penicillin and 50 μg/ml streptomycin.

Epidermal squamous cell carcinoma SCC-13 were purchased from the Harvard Skin Disease Research Center Cell Culture Core and culture in Keratinocyte Serum-free Media supplemented with 30 μg/ml Bovine Pituitary Extract (BPE), 0.1 ng/ml of recombinant EGF (Invitrogen) and 100U/ml penicillin and 50 μg/ml streptomycin. TGF-β treatment was done in Keratinocyte Serum-free Media without any supplement.

2.2- Iodination of TGF-β

Iodination of TGF-β1 (a gift from Genzyme) was performed using the chloramine T method as described previously (Philip and O'Connor-McCourt, 1991). Briefly, 2μg of carrier free TGF-β1 in 4mM HCl, 1M $NaPO_4$ solution was incubated with 1mCi ^{125}I-Na (purchased at Amersham then Perkin Elmer). The reaction was performed by 3 consecutives addition of Chloramine T (50 μg/ml in $NaPO_4$) at 0, 2min and 3.5 min. After 4.5 min, the reaction was stopped by the addition of 20μl 50mM Tyrosine, 200μl 60mM KI and 200μl urea (1.2g/ml in 1M HCl). The radiolabeled ligand was chromatographed on a PD10 column (GE healthcare) and twelve fractions were collected in 0.5ml of 0.1% BSA in 4mM HCl. The two fractions containing the highest radioactivity were pooled. Specific activity and concentration of the final preparation were calculated after determination of the percentage of the pre-chromatography reaction and of the final preparation that were precipitated by 10% Trichloroacetic acid. The biological activity was evaluated by labeling HaCaT cells in the presence or absence of increasing concentration of unlabeled TGF-β.

2.3- Affinity labeling

HaCaT or 293 cells were affinity labeled as described previously (Tam *et al.*, 2001). Briefly, cells were washed three times with ice-cold 0.1% BSA/D-PBS (containing Ca^{2+} and Mg^{2+}, pH 7.4) for 10 min per wash. The cells were incubated with 100pM ^{125}I-TGF-β1 for 3hrs at 4^{o}C, the ligand was then cross-linked to the receptors with 1mM Bis(Sulfocsuccinimidyl)suberate (BS^3) (Pierce) for 10min. The reaction was quenched by addition of 0.5M Glycine for 5min. After a quick wash with D-PBS, the cells were lysed in solubilisation buffer (20mM Tris pH 7.4, 1mM EDTA, 10% glycerol, 1% Triton X-100) containing protease inhibitors cocktail (Roche) and 2μM Phenylmethylsulfonyl fluoride (PMSF). Alternatively, cells were incubated at 37^{o}C for 0 to 8hrs prior to lysis in the presence or absence of 20μM MG132 (Sigma Aldrich) in binding medium (200mM Hepes-buffered D-MEM, pH 7.4, 0.2% BSA). Protein concentrations were determined

using a Lowry protein assay (BioRad). The lysates were diluted in 5X electrophoresis sample buffer (50% Glycerol, 0.25M Tris pH 6.8, 5% SDS, bromophenol blue) and boiled for 10 min. Samples were run on a 3%-11% gradient SDS-PAGE gel and analyzed by autoradiography. Densitometry analyses were performed using the ImageJ software.

2.4- Antibodies

Application: WB=Western blot; IF= Immunofluorescence; IP=Immunoprecipitation

All antibodies detect human proteins

Primary antibodies	**Company**	**Species**	**Application**	**Dilution**	**Diluents**	**Incubation (T^o, time)**
anti-actin (H-300)	Santa Cruz biotechnology	Rabbit polyclonal	WB	1/5000	5% milk	4°C, O/N
anti-β-actin (C4)	Santa Cruz biotechnology	Mouse monoclonal	WB	1/3000	5% milk	4°C, O/N
anti-caveolin-1 (2297)	BD biosciences	Mouse monoclonal	WB	1/2000	5% milk	4°C, O/N
			IP	2μg		4°C, O/N
anti-caveolin-1	BD biosciences	Rabbit polyclonal	WB	1/2000	5% milk	4°C, O/N
			IP	2μg		4°C, O/N
Cy3-conjugated anti-caveolin-1	Sigma Aldrich	Rabbit polyclonal	IF endogenous	1/100	1%BSA	37°C, 1hr
			IF overexpressed	1/500	1%BSA	37°C, 1hr
anti- CD109 (TEA 2/16)	BD biosciences	Mouse monoclonal	WB	1/2000	5% milk	4°C, O/N
			IF endogenous	1/200	1% NGS	37°C, 1hr
			IF overexpressed	1/500	1% NGS	37°C, 1hr
anti-CD109 (496901.11)	R&D systems	Mouse monoclonal	IP	2μg		4°C, O/N
anti-EEA-1	BD biosciences	Mouse monoclonal	WB	1/2000	5% milk	4°C, O/N

FITC-conjugated anti-E-cadherin	BD Biosciences	Mouse monoclonal	IF	1/500	1%NGS, 0.1%Triton X-100	37°C, 1hr
anti-Flag-M2	Sigma Aldrich	Mouse monoclonal	WB	1/2000	5% milk	4°C, O/N
anti-Flag	Sigma Aldrich	Rabbit	IF	1/250	1% BSA	37°C, 1hr
anti-fibronectin (cat#610406)	BD biosciences	Mouse monoclonal	WB	1/5000	5% milk	4°C, O/N
anti-HA (12CA5)	Abgent	Mouse monoclonal	WB	1/2000	5 % milk	4°C, O/N
anti-HA	Santa Cruz Biotechnology	Rabbit polyclonal	IP	2μg		4°C, O/N
AF488 conjugated anti-HA (16B12)	Molecular Probes	Mouse monoclonal	IF	1/200	1% BSA or 1% NGS	4°C, O/N
anti-Histone H1	Santa Cruz Biotechnology	Mouse monoclonal	IP	2μg		4°C, O/N
anti-myc (9E10)	ebioscience	Mouse monoclonal	WB	1/2000	5% milk	4°C, O/N
anti-p38 MAPK	Cell signalling Technology	Rabbit polyclonal	WB	1/2000	5% BSA	4°C, O/N
anti-phospho-p38 MAPK (Thr180/Tyr182)	Cell signalling Technology	Rabbit polyclonal	WB	1/2000	5% BSA	4°C, O/N
anti-p44/42 MAPK	Cell signalling Technology	Rabbit polyclonal	WB	1/3000	5% BSA	4°C, O/N
anti-phospho-p44/42 MAPK (Thr202/Tyr204)	Cell signalling Technology	Rabbit monoclonal	WB	1/3000	5% BSA	4°C, O/N
anti-PAI-1 (cat#612025)	BD bioscience	Mouse monoclonal	WB	1/3000	5% milk	4°C, O/N
anti-Slug (C19G7)	Cell signalling Technology	Rabbit monoclonal	WB	1/2000	5% BSA	4°C, O/N
anti-SMAD2 (L16D3)	Cell signalling Technology	Mouse monoclonal	WB	1/2000	5% milk	4°C, O/N

anti-phospho-SMAD2(Ser 465/467)	Cell signalling Technology	Rabbit polyclonal	WB	1/2000	5% milk	4°C, O/N
anti-SMAD2/3 (FL-425)	Santa Cruz biotechnology	Rabbit polyclonal	WB	1/2000	5% milk	4°C, O/N
anti-phospho-SMAD3 (Ser423/425)	Cell signalling Technology	Rabbit monoclonal	WB	1/2000	5% BSA	4°C, O/N
anti-TGFBR1	Cell signalling Technology	Rabbit polyclonal	WB	1/2000	5% BSA	4°C, O/N
Secondary Antibodies	**Compagny**	**Species**	**Application**	**Dilution**	**diluents**	**Incubation (T°, time)**
AF488 conjugated anti-mouse IgG	Molecular Probes	goat	IF	1/2000	1% NGS	RT, 1hr
Cy5-conjugated Fab fragment anti-mouse IgG	Jackson Immuno Research	goat	IF	1/1000	1% NGS	RT, 1hr
Cy3-conjugated anti-rabbit IgG	Sigma Aldrich	sheep	IF	1/1000	1% BSA	RT, 1hr
AF488 conjugated anti-FITC	Molecular probes	goat	IF	1/500	1% NGS, 0.1%Triton X-100	RT, 1hr
HRP-conjugated anti-mouse IgG	Cell Signalling Technology	N/A	WB	1/5000	Same as primary	RT, 1hr
HRP-conjugated anti-rabbit IgG	Cell Signalling Technology	N/A	WB	1/5000	Same as primary	RT, 1hr
Associated Reagents	**Compagny**	**Species**	**Application**	**Dilution**	**Diluents**	**Incubation (T°, time)**
AF647-streptavidin	Molecular Probes	N/A	IF	10μg/ml	binding medium	4°C, 1hr
Rhodamin-Phalloidin	Cytoskeleton	N/A	IF	98nM	1%NGS, 0.1%Triton X-100	RT, 30min
DAPI	Sigma	N/A	IF	1μg/ml	ddH_2O	RT, 10min

Table 2: List of antibodies used and their applications

2.5- Transient transfections and antisense delivery

HaCaT, 293 and Cos-1 cells were transiently transfected with different combinations of the following plasmids: CD109 or its empty vector (pCMVSport6), 3TP-lux reporter gene, (CAGA)12-lux reporter gene, CMV-β-galactosidase, caveolin-1 cDNA (gift from C. Hardin, University of Missouri), Dynamin2K44A (gift from S. Egan, University of Toronto), 6myc-SMAD7WT (gift from T.Imamura, The JFCR Cancer Institue, Tokyo), TGFBR2 and TGFBR1-WT cDNA or TGFBR1-T204D-HA cDNA, , HA-SMAD7-WT or ΔPY, Flag-Smurf2WT or Flag-Smurf2C716A (gifts from J. Wrana, University of Toronto), TGFBR1-WT-HA (gift from B. Kleuser, University of Berlin) or their corresponding empty vector using Superfect (Qiagen) according to the manufacturer's instruction. For antisense delivery, HaCaT cells were treated with CD109-specific antisense morpholino oligos: GCC-CTG-CAT-CTC-GAC-GGC-GTC-TGC-C, or with inverse control oligos (Gene Tools, Philomath, OR), according to the manufacturer's instructions. For knock-down experiments, HaCaT, A431, SCC-13 cells were transfected with CD109 siRNA (ID#129083), caveolin-1 siRNA (ID# 10297), SMAD7 siRNA # 1 (ID#17186), SMAD7 siRNA #2 (ID#17280) or a negative control siRNA (ID#4611) (all from Ambion) using lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation.

2.6- Co-immunoprecipitation

For immunoprecipitation of caveolin-1, 293 cells transfected with CD109 and caveolin-1 or their corresponding empty vectors were lysed in solubilisation buffer (20mM Tris pH 7.4, 1mM EDTA, 10% glycerol, 0.5% Triton X-100, 2μM PMSF and protease inhibitor cocktail (Roche)). Aliquots of the lysates were left non-immunoprecipitated in order to analyze the total level of transfected proteins (total lysates) while the remaining lysates were incubated with a mouse monoclonal anti-caveolin-1 antibody overnight at 4^{o}C. For immunoprecipitation of CD109, lysates from untransfected HaCaT cells (grown in 10 cm culture dishes) were incubated with mouse monoclonal anti-CD109 overnight at 4 ^{o}C, while a mouse monoclonal anti-histone H1 was used as control IgG. For

immunoprecipitation of caveolin-1 from untransfected HaCaT cells, HaCaT cells were serum starved for 2hrs prior addition of 25pM TGF-β1 for 30 min (in serum-free D-MEM). Cells lysates were prepared, protein concentration were determined and equal amount of protein were incubated with either a rabbit polyclonal caveolin-1 antibody or with an anti-HA antibody (used as a negative control) overnight at 4^{o}C. In all cases, immune complexes were incubated with Protein G Sepharose (Biovision) for 1hr at 4 ^{o}C and pulled down by centrifugation. Unbound proteins were removed by washing the beads three times with solubilisation buffer. Bound proteins were eluted in 1X electrophoresis sample buffer. Both total lysates and immunoprecipitated samples were run on a SDS-PAGE gel and analyzed by western blot using the indicated antibodies.

For immunoprecipitation of HA-TGFBR1, 293 cells were transfected with TGFBR1WT-HA or its control empty vector, TGFBR2, CD109 (or its empty vector), Flag-Smurf2 and 6myc-SMAD7 and treated with 100pM TGF-β1 for 90min. For immunoprecipitation of HA-SMAD7, 293 cells were transfected with TGFBR1, TGFBR2, CD109 (or its empty vector), Flag-Smurf2WT or C716A and HA-Smad7 (or its control empty vector) and treated with 100pM TGF-β1 for 90min. In both cases, the cells were lysed in 50mM Hepes pH 7.5, 150mM NaCl, 100mM NaF, 50mM Glycerophosphate, 5mM EDTA, 10% glycerol, 1% Igepal, 2μM PMSF and protease inhibitor cocktail (Roche) (1% Igepal buffer), immunoprecipitated by incubation with an anti-HA antibody overnight at 4^{o}C followed by incubation with protein G Sepharose (BioVision) for 1hr at 4 ^{o}C. The unbound proteins were removed by washing three times with 1% Igepal buffer, three times with 0.5% Igepal in PBS, three times with 0.1% Igepal in PBS and once with PBS. The bound proteins were eluted in 1X electrophoresis sample buffer. Both non-immunoprecipitated and immunoprecipitated samples were run on a SDS-PAGE gel and analyzed by western blot using the indicated antibodies.

2.7- Western blot

Cells, grown in 6-well plates, were left untreated or treated with TGF-β1 in serum-free medium for the indicated times and lysed in 1% Igepal buffer, supplemented with 60mM octyl-β-D-glucopyranoside (SigmaAldrich) to extract CD109. After determination of the protein concentration by Lowry protein assay (Biorad), cell lysates were diluted in 5X electrophoresis sample buffer. Samples were loaded on a SDS-PAGE gel and run at 100V. Proteins were then transferred to nitrocellulose membrane (Whatman) and the membrane was stained with Ponceau Red to verify equal loading of proteins. Non specific sites were blocked in 5% non-fat milk in TBST (Tris buffer saline, 1% Tween) for 1hr at Room Temperature and the membranes were incubated with the indicated antibodies overnight at 4^{o}C. The membranes were then washed three times in TBST, for 10 min per wash, incubated with the appropriate HRP-linked secondary antibody for 1hr at room temperature and washed three times in TBST. The membranes were subjected to chemiluminescence analysis (ECL, Amersham or Pierce). Membranes were stripped using 0.2M Glycine, pH 2.8 for 30 min and reprobe with anti-actin, anti-SMAD2/3 or anti-MAPK antibodies to demonstrate equal protein loading.

2.8- Plasma membrane preparation

Cell surface proteins from 293 cells transfected with TGFBR1, TGFBR2, CD109 or its empty vector were extracted using the Hook Cell Surface Protein Isolation kit (G-Biosciences, St-Louis, MO, USA) according to the manufacturer's instructions. Cell surface proteins were labeled with Hook-sulfo-NHS-SS-Biotin and then lysed. An aliquot was then analyzed by western blot for actin to make sure that similar amount of protein were used in the assay. Biotinylated proteins from the remaining lysates were isolated using streptavidin-agarose column, eluted and analyzed by western blot using the indicated antibodies.

2.9- Immunofluorescence

2.9.1- CD109 and caveolin-1 staining in HaCaT cells

Non transfected HaCaT cells grown in chamber slides until 60% confluency were washed twice with ice-cold PBS and fixed with 4% paraformaldehyde, pH 7.4 for 10 min at 37°C. After 3 washes with room temperature PBS, the cells were permeabilized with methanol for 2min at 4°C. After 3 more washes in PBS, non specific sites were blocked with 10% Normal Goat Serum (NGS) in PBS. Cells were then stained for CD109 using first an anti-CD109 antibody for 1hr at 37°C and second, an anti-mouse-AF488 for 1hr at room temperature. After a series of washes, cells were stained with an anti-caveolin-Cy3 for 1hr at 37°C. The cells were then immersed for 5 min in GOC anti-fade solution (117U/L of beef catalase, 1333U/ml of glucose oxidase and 1mg/ml of glucose) and mounted with Mowiol on slides with cover slips, as described previously (Blanc *et al.*, 2005). Images were acquired with a LSM 510 META Axioplan 2 confocal laser scanning microscope (Carl Zeiss Canada, Toronto, Ontario, Canada) and background was removed by deconvolution using Huygens Professional Software.

2.9.2- biotin-TGF-β1, CD109 and caveolin-1 staining in 293 cells

293 cells were grown in chamber slides coated with poly-L-lysine. 48hrs after transfection, 293 cells were incubated in binding medium (200mM Hepes-buffered D-MEM, pH 7.4, 0.2% BSA) for 1hr at 37°C to remove endogenous TGF-β, incubated with 250pM biotinylated-TGF-β (R&D System) for 2hrs at 4°C, washed 3 times with binding medium and incubated with 10μg/ml streptavidin-AF647 for 1hr at 4°C. Then, 293 cells were washed 3 times and incubated at 37°C in D-MEM, 10%FBS, for 30 min to allow receptor internalization. Alternatively, 293 cells were treated with 2.5mM Methyl-β-cyclodextrin (MβCD, Sigma), 25μg/ml Nystatin (Sigma) for 1hr prior to initiation of internalization or 100 μM Genistein (Sigma) for 4hrs prior to and during internalization. HEK 293 cells were then fixed, permeabilized and stained for CD109 and caveolin-1 as described in section 2.9.1, except that CD109 and Cy3-caveolin-1 antibodies were used

at 1:500. Images were acquired with a LSM 510 META Axioplan 2 confocal laser scanning microscope (Carl Zeiss Canada, Toronto, Ontario, Canada). Cells were manually counted to determine the percentage of caveolin-1 transfected cells that have incorporated biotin-TGF-β.

2.9.3- biotin-TGF-β1, TGFBR1-HA and caveolin-1 staining in 293 cells

293 cells were processed as before, except that they were stained for TGFBR1-HA instead of CD109. TGFBR1-HA staining was performed by incubating the cells with an AlexaFluor488 conjugated mouse anti-HA antibody (1:200 in 1% NGS) for 1hr at 37^{o}C.

2.9.4- CD109, TGFBR1-HA and caveolin-1 staining in 293 cells

293 cells were fixed and permeabilized as described in section 2.9.1. Non-specific sites were blocked with 10% NGS. Cells were stained for CD109 using an anti-CD109 antibody followed by Cy5-conjugated Fab fragment goat anti-mouse IgG (1:1000 in 1% NGS), to avoid cross-reaction with the AlexaFluor488 conjugated mouse anti-HA antibody used to detect TGFBR1-HA. Cells were then stained for caveolin-1 and TGFBR1-HA as described in the section 2.9.3.

2.9.5- biotin-TGF-β1, CD109 and Flag-Smurf2 staining in 293 cells

Biotin-TGF-β1 was detected using streptavidin-AF647, CD109 was detected using an anti-CD109 antibody followed by an AF488 conjugated anti-mouse antibody as described previously in section 2.9.2, whereas Flag-Smurf2 was detected using a rabbit anti-Flag antibody (1:250 in 1% BSA) for 1hr at 37^{o}C, followed by an Cy3 conjugated anti-rabbit antibody (1:1000 in 1% BSA) for 1hr at room temperature. Quantitative co-localization was performed using ImageJ software: background was removed by median filtering

(Landmann and Marbet, 2004) and the Pearson's correlation coefficient was calculated using the JaCoP plug-in (Bolte and Cordelieres, 2006).

2.9.6- CD109, Flag-Smurf2 and HA-SMAD7 staining in 293 cells

CD109 was detected using an anti-CD109 antibody followed by an Cy5-conjugated Fab fragment goat anti-mouse IgG, Flag-Smurf2 was detected using a rabbit anti-Flag antibody followed by an Cy3 conjugated anti-rabbit antibody and HA-SMAD7 was detected using an AF488 conjugated mouse anti-HA antibody, as described in the previous sections.

2.9.7- E-cadherin and F-actin staining in HaCaT, A431 and SCC-13 cells

HaCaT cells and A431 cells were treated with 0-100pM TGF-β1, in the presence or absence of various inhibitors for 36-40hrs. Cells were then washed twice with ice-cold PBS, fix with 4% paraformaldehyde, pH7.4 for 10 min at 37^{o}C and wash three times with PBS. Non-specific sites were blocked with 10%NGS, 0.1% Triton X-100 in PBS for 1hr at room temperature. E-cadherin was detected using a FITC-conjugated anti-E-cadherin antibody (1hr at 37^{o}C), followed by an AF488-conjugated anti-FITC antibody (1hr at room temperature) to amplify the signal. F-actin was detected using Rhodamin conjugated phalloidin and the nucleus was stained with DAPI. After immersion for 5 min in GOC anti-fading reagent, cells were mounted with Mowiol on slides with cover slips. Images were acquired with an Olympus BX60 fluorescent microscope coupled to a QColor3 camera (Olympus) using Image ProPlus 6.0 software.

2.10- Luciferase assay

The 3TP-lux construct contains three tetradecanoyl phorbol acetate (TPA)-responsive elements and TGF-β responsive elements from the PAI-1 promoter fused to the luciferase reporter gene (Wrana *et al.*, 1992). The $CAGA_{12}$-lux construct contains 12

CAGA repeats, which are binding sites for SMAD3, upstream of the luciferase gene (Dennler *et al.*, 1998). The pCMV-β-galactosidase was used to monitor transfection efficiency since the β-galactosidase gene is under the control of a constitutively active promoter. Cells were grown in 12-well plates and transfected as follow:

1) HaCaT cells and MEFs were co-transfected with CD109 or EV, 3TP-lux or $(CAGA)_{12}$-lux and β-galactosidase and treated with or without 100pM TGF-β1 for 16hrs,

2) 293 cells were co-transfected with 3TP-lux, β-galactosidase, TGFBR1-T204D (or its empty vector, pCMV5) and CD109 (or EV) and incubated overnight in the presence or absence of 10μg/ml TGF-β neutralizing antibody (1D11, Genzyme),

3) 293 cells were co-transfected with $(CAGA)_{12}$-lux, β–galactosidase, TGFBR1-T204D (or its empty vector pCMV5), CD109 (or its empty vector), Smurf2-WTor Smurf2-C716A.

Cells were lysed in 1% Triton, 15mM MgSO4, 4mM EGTA, 1mM DTT. To measure luciferase activity, cell lysates were mixed 10:1 with assay cocktail (30mM ATP, 0.1M KH_2PO_4, pH 7.8, 0.1M $MgCl_2$). Luciferin (80μg/ml in 0.1 M KH_2PO_4, pH7.8) was injected prior to reading in a luminometer. β-galactosidase activity was then measured to monitor for transfection efficiency. Briefly, 1.5mg/ml of 2-Nitrophenyl-β-D-galactopyranoside (ONPG) in β-galactosidase buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgCl_2$, 3.9μl/ml β-mercaptoethanol) was added 1:1 to the samples and incubated at 37^{o}C for 20-30 min. β-galactosidase activity was read on a spectrophotometer at λ=450nm. The graphs shown are expressed as luciferase activity values normalized to β-galactosidase activity.

2.11- Thymidine incorporation assay

1) HaCaT cells stably transfected with CD109 or its corresponding empty vector, 2) HaCaT cells treated with CD109 specific antisense morpholino oligos or inverse oligos, or 3) GPI mutant or its parental HaCaT cells were plated in triplicate in 24-well plates. The cells were then incubated with or without 50pM TGF-β1 in serum free medium for

24hrs. Cells were pulsed with 1μCi of ^{3}H-Thymidine for the final 6hrs of incubation. Cells were then washed three times with ice-cold PBS. Proteins were precipitated with 10% Trichloroacetic acid (American Chemicals LTD) for 20 min at 4^{o}C. After 2 washes, cells were solubilized in 1%SDS and scrapped. The incorporated radioactivity was counted on a beta-counter. The values are expressed as a percentage of untreated control cells.

2.12- Internalization assay

Internalization assay were performed as described in (Zwaagstra *et al.*, 2001) with some modifications. Briefly, HaCaT cells seeded in 12-well plates were washed twice with binding medium and incubated with or without 2.5mM MβCD in binding medium for 30 min at 4^{o}C. The cells were then treated with 100 pM ^{125}I-TGF-β1 (Perkin Elmer, Waltham, MA, USA) with or without 10 nM unlabeled TGF-β1 for 2 hr at 4^{o}C and transferred to 37^{o}C for the indicated time. After two washes with ice-cold D-PBS (containing Mg^{2+} and Ca^{2+}), 0.1% BSA, surface ligand was removed with 150 mM NaCl, 0.1% acetic acid, and 2M urea. Cells were then lysed with 1% Triton X-100 solubilization buffer in order to extract internalized ligand. The amount of surface and internalized ligand were quantified with a gamma counter. Specific cpm were determined by subtracting competed samples (with unlabeled TGF-β) from non-competed samples.

2.13- Sucrose gradient fractionation

Preparation of membrane fraction from HaCaT cells transfected with CD109 or empty vector and treated for 30 min without or with 25 pM of TGF-β1 was performed as described in (Ito *et al.*, 2004). Briefly, cells were mixed with sodium carbonate (500mM, pH 11) and homogenized using 10 strokes of a tight-fitting Dounce homogenizer followed by three 10 seconds bursts of a Polytron tissue grinder and by three 20 seconds bursts of sonication. The homogenized cell suspension was mixed with an equal volume of 90% sucrose (w:w) in MES-buffered saline (MBS: 25mM 2-(N-morpholino)ethanesulfonic acid, pH6.5, 0.15M NaCl), and a discontinuous

(45%/35%/4%) sucrose gradient was laid on top of the homogenized cell lysates. The resulting gradient was subjected to centrifugation (40,000rpm at 200,000g) using a Beckman SW-41 rotor under slow acceleration and deceleration at 16^{o}C for 16-20hrs. Twelve fractions were removed from the top of the tube and analyzed by western blot for EEA-1 and caveolin-1. The fractions containing EEA-1 or caveolin-1 were pooled and equal amounts of protein were analyzed by western blot for CD109 and TGFBR1.

2.14- *In vitro* wound healing (scratch) assay

Confluent monolayers of HaCaT or A431 cells were wounded by manually scrapping the cells with a 200μl pipette tip. Cells were washed twice with PBS and incubated with 100pM TGF-β1, in the presence or absence of 10μM U0126 (Cell signalling Technology), 1μM SB203580, 0.5μM SB431542 (both from Ascent) or Dimethyl sulfoxide (DMSO) in serum free medium for 24-48hrs. Pictures were taken at the same spot at 0hrs, 24hrs and 48hrs and "wound" area was measured using ImageJ software. The percentage of original wound area that was filled with keratinocytes was calculated.

2.15- Statistics

Numerical results are represented as means of n independent experiments ± SEM, according to the recommendations found in (Cumming *et al.*, 2007). A two-tailed Student t test was used to determine statistical significance between two groups. Comparisons within more than two groups were made by one-way analysis of variance. Multiple comparisons versus control (non-treated) group (fig 4.5B) or all pairwise multiple comparisons were made by Holm-Sidak test (post-hoc). A value of $p<0.05$ was considered significant.

Chapter 3: CD109 is a negative regulator of TGF-β signalling

3.1- Rationale

TGF-β is a multifunctional growth factor, which plays an important role in the maintenance of skin homeostasis and whose pathway is dysregulated in many skin diseases, including impaired wound healing, fibrosis and skin cancer. In order to understand TGF-β's action in the skin, previous work performed in Dr Philip's laboratory has aimed at characterizing TGF-β receptors and co-receptors profiles in skin cells by affinity labeling. From these experiments, a novel 150kDa TGF-β1 binding protein, called r150, was identified on the cell surface of HaCaT cells and early passage keratinocytes (Tam *et al.*, 1998). r150 is a GPI-anchored protein that can be released from the cell surface by an endogenous PIPLC (Tam *et al.*, 2001). Both soluble and membrane-anchored forms of r150 bind TGF-β1 with high affinity but have lower affinity for TGF-β2 and TGF-β3 subtypes. Moreover, r150 forms a heteromeric complex with the TGF-β signalling receptors (Tam *et al.*, 1998). Following studies have shown that human keratinocytes (HaCaT cells) defective in GPI-anchor biosynthesis display enhanced TGF-β responses, as evidenced by increased SMAD2 phosphorylation and increased TGF-β1-induced transcriptional activity (Tam *et al.*, 2003). These studies suggest that r150 (or other GPI-anchored proteins) may downregulate TGF-β signalling and responses in skin cells. Affinity purification and microsequencing of r150 revealed that r150 is CD109, a novel member of the α2-M/complement family (Finnson *et al.*, 2006), whose function has remained obscure. In addition, a novel isoform of CD109 was identified in the human placenta but not in keratinocytes (Finnson *et al.*, 2006). This placental isoform lacks 17 amino acids on position 1218 and was called CD109S. Preliminary results obtained with CD109S suggest that it may act as a negative regulator of TGF-β signalling. In addition, CD109 was shown to directly interact with TGFBR1 (Finnson *et al.*, 2006). Together, these findings imply that CD109 is a novel TGF-β co-receptor that inhibits TGF-β signalling.

In this chapter, I have further analyzed the role of CD109 (long version) in TGF-β canonical signalling and responses. In addition, I have investigated whether CD109 could exert its function independently of ligand sequestration. Together, these results linking CD109 function to regulation of TGF-β receptor activity suggest that CD109 plays a unique role in the regulation of TGF-β1 signalling in keratinocytes and are important for understanding the mechanisms underlying alterations of TGF-β action in various skin diseases.

3.2- Results

3.2.1- CD109 inhibits SMAD2/3 phosphorylation in various cell types

Because previous results in our laboratory have shown that CD109 binds TGF-β1 and forms a complex with the TGF-β signalling receptor, we investigated whether CD109 could modulate TGF-β signalling and responses. First, we analyzed the effect of gain and loss of function of CD109 on TGF-β1-induced phosphorylation of SMAD2 and SMAD3, one of the first events occurring after ligand binding to the TGF-β receptors. In human spontaneously immortalized keratinocytes (HaCaT cells), overexpression of CD109 results in a decrease of TGF-β-induced SMAD2 phosphorylation as compared to empty vector (EV) transfected cells, at all concentrations of TGF-β1 tested (fig 3.1 A). Total SMAD2 levels remained unchanged under these conditions. Similar results were obtained for SMAD3 phosphorylation in HaCaT cells (data not shown). Similarly, overexpression of CD109 in HEK293 cells leads to a marked decrease of TGF-β-induced SMAD3 phosphorylation, but does not affect the total amount of SMAD proteins (fig 3.1 B). Moreover, primary keratinocytes from transgenic mice overexpressing CD109 in the epidermis show a reduction in SMAD3 phosphorylation as compared to primary keratinocytes from wild-type littermates (fig 3.1 C). These results demonstrate that human CD109 conserves its ability to inhibit TGF-β-induced SMAD3 phosphorylation in murine cells, which is of importance for future studies of TGF-β responses using these

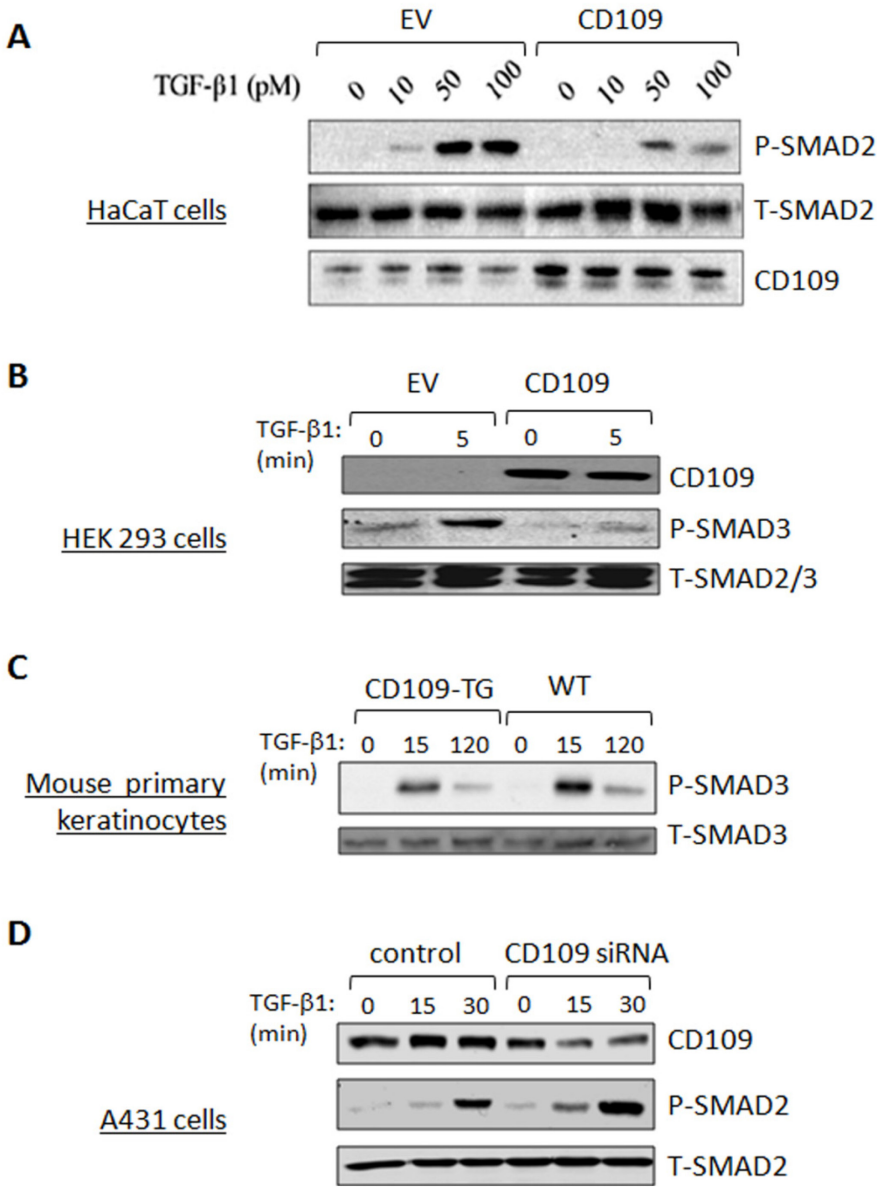


Figure 3.1: CD109 inhibits TGF-β-induced SMAD2/3 phosphorylation in a variety of cell types. A. HaCaT cells transfected with CD109 or its empty vector (EV) were treated with or without 10 pM TGF-β1 for 15 min or with 50 or 100 pM TGF-β1 for 60 min. **B.** HEK 293 cells transfected with CD109 or EV were treated with or without 250pM TGF-β1 for 5min. **C.** Mouse primary keratinocytes from transgenic mice overexpressing CD109 in the epidermis (CD109-TG) or from wild-type littermates (WT) and **D.** A431 cells transfected with CD109 siRNA or negative control siRNA were treated with 15pM TGF-β1 for the indicated times. In all cases, cell lysates were subjected to SDS-PAGE and analyzed by western blot using anti-phospho-SMAD2, anti-total SMAD2, anti-phospho-SMAD3, anti-total SMAD2/3 or anti-CD109 antibodies.

transgenic mice. Furthermore, in A431 cells, an epidermoid squamous cell carcinoma cell line, knock-down of CD109 expression using CD109-specific siRNA increases SMAD2 phosphorylation as compared to control siRNA transfected cells, indicating that endogenous CD109 can inhibit TGF-β-induced SMAD2 phosphorylation (fig 3.1 D). Altogether, these results indicate that CD109 inhibits SMAD2 and SMAD3 phosphorylation in numerous cell types.

3.2.2- CD109 inhibits TGF-β transcriptional activity

The effect of CD109 on TGF-β1-induced transcriptional activity was next investigated by using a TGF-β-responsive luciferase reporter construct, the 3TP-lux, which contains both PAI-1 and collagenase promoter elements upstream of the luciferase gene (Wrana *et al.*, 1992). Overexpression of CD109 in HaCaT cells significantly ($p<0.05$) reduces TGF-β-induced transcriptional activity when compared to empty vector (EV) transfected cells (fig 3.2 A). Similar results were obtained in mouse embryonic fibroblasts (MEFs) (fig 3.2 B), further validating that human CD109 can inhibit TGF-β signalling in mouse cells. Interestingly, the effect of CD109 can also be seen in the absence of TGF-β treatment (fig 3.2 A and B), suggesting that CD109 may also inhibit autocrine/ endogenously produced TGF-β.

I then looked more specifically to CD109's ability to regulate SMAD3 driven transcriptional responses, by using the $(CAGA)_{12}$-lux reporter construct, which contains 12 binding elements for SMAD3 upstream of the luciferase gene. Once again, overexpression of CD109 significantly reduces TGF-β1-transcriptional activity in both HaCaT cells and MEFs (fig 3.2 C and D). In addition, depletion of CD109 by stable transfection of a CD109 shRNA in HaCaT cells leads to an increase in SMAD3-dependent transcription, as compared to cells stably transfected with a scrambled shRNA (fig 3.2 E). These findings indicate that both endogenous and overexpressed CD109 are able to downregulate SMAD2/3 signalling.

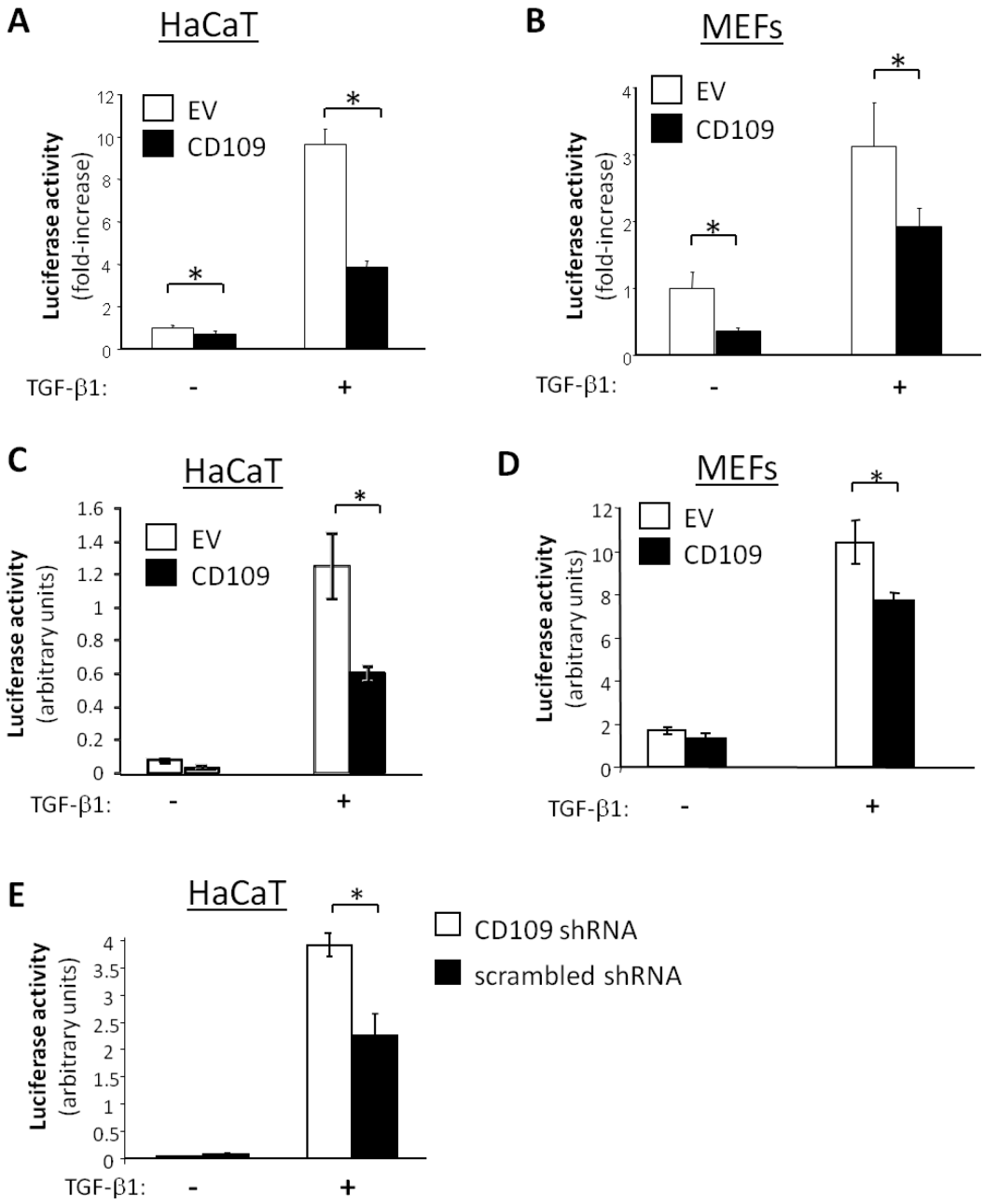


Figure 3.2: CD109 inhibits TGF-β1-induced transcriptional activity. A. HaCaT cells or **B.** MEFs were cotransfected with CD109 or EV along with 3TP-lux and β-galactosidase and treated with or without 100 pM TGF-β1 for 18 h. Cell lysates were analyzed for luciferase activity and expressed as fold-stimulation (normalized to β-galactosidase activity) relative to untreated cells . **C.** HaCaT cells or **D.** MEFs transfected with CD109 or EV or **E.** HaCaT cells stably transfected with CD109shRNA or scrambled shRNA were cotransfected with (CAGA)12-lux reporter and β-galactosidase and treated with or without 100 pM TGF-β1 for 18 h. Cell lysates were analyzed for luciferase activity and expressed as luciferase activity normalized to β-galactosidase activity. *p<0.05.

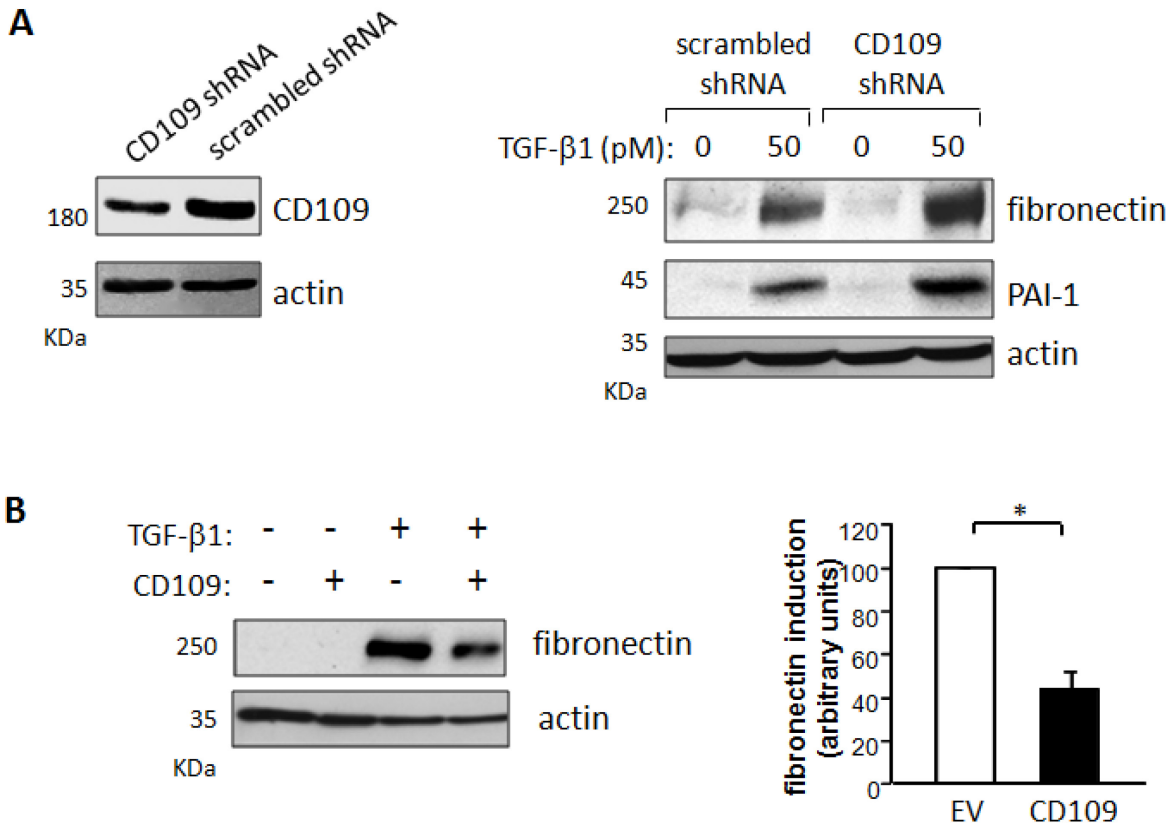


Figure 3.3: CD109 inhibits TGF-β-induced ECM production. **A.** *Left panel***:** Western blot showing that CD109 expression is decreased in CD109shRNA stably transfected cells, as compared to scrambled shRNA transfected cells. *Right panel*: HaCaT cells stably expressing CD109 shRNA or scrambled shRNA were treated with or without 50pM TGF-β1 for 16hrs. Cell lysates were analyzed for fibronectin and PAI-1 expression. The actin panel demonstrates equal protein loading. **B.** HaCaT cells overexpressing CD109 or its control EV were treated with 100pM TGF-β1 for 16hrs. Cell lysates were analyzed for fibronectin and actin expression. *Right panel*: Densitometry of n=3 independent experiments, ±SEM; *: P<0.05.

3.2.3- CD109 inhibits TGF-β-induced ECM production

TGF-β is one of the most powerful inducer of ECM, and dysregulation of TGF-β signalling has been implicated in many fibrotic disorders, including scleroderma and hypertrophic scarring (Leask and Abraham, 2004). Thus, I looked at CD109's ability to regulate TGF-β-induced ECM by analyzing fibronectin and PAI-1 production. In HaCaT cells, TGF-β1 treatment causes a marked increase in fibronectin and PAI-1 expression levels, as expected (Fig 3.3A and B). Importantly, knock-down of CD109 expression using shRNA further enhances TGF-β-induced PAI-1 and fibronectin expression (Fig 3.3A), suggesting that endogenous CD109 negatively regulates TGF-β-induced ECM production. Consistent with this result, overexpression of CD109 in HaCaT cells decreases fibronectin expression following TGF-β stimulation, as compared to EV transfected cells (Fig 3.3B). This result is in agreement with previous results obtained in our lab (Finnson *et al.*, 2006) and together, they indicate that CD109 inhibits the TGF-β-induced expression of components of the ECM.

3.2.4- CD109 reduces TGF-β-induced growth inhibition

Because TGF-β is a potent growth inhibitor of epithelial cells, including keratinocytes (Siegel and Massagué, 2003), I investigated whether CD109 could regulate TGF-β antiproliferative effect, by measuring ^{3}H-thymidine incorporation. HaCaT cells stably transfected with CD109 display a significant ($p<0.05$) reduction of TGF-β1-induced inhibition of thymidine incorporation as compared to empty vector transfected cells (demonstrated by an increase in cpm) (fig 3.4A). Similarly, transfection with CD109 antisense oligonucleotides results in a significant ($p<0.05$) increase in TGF-β1-induced growth inhibition as compared to cells transfected with control inverse oligonucleotides (fig 3.4B). This result indicates that endogenous CD109 is also able to negatively regulate TGF-β-induced growth inhibition, and that the effect observed in fig 3.4A is not an artifact due to overexpression. Furthermore, HaCaT cells defective in GPI anchor biosynthesis (GPI M) also shows an increase in TGF-β1-induced growth inhibition as

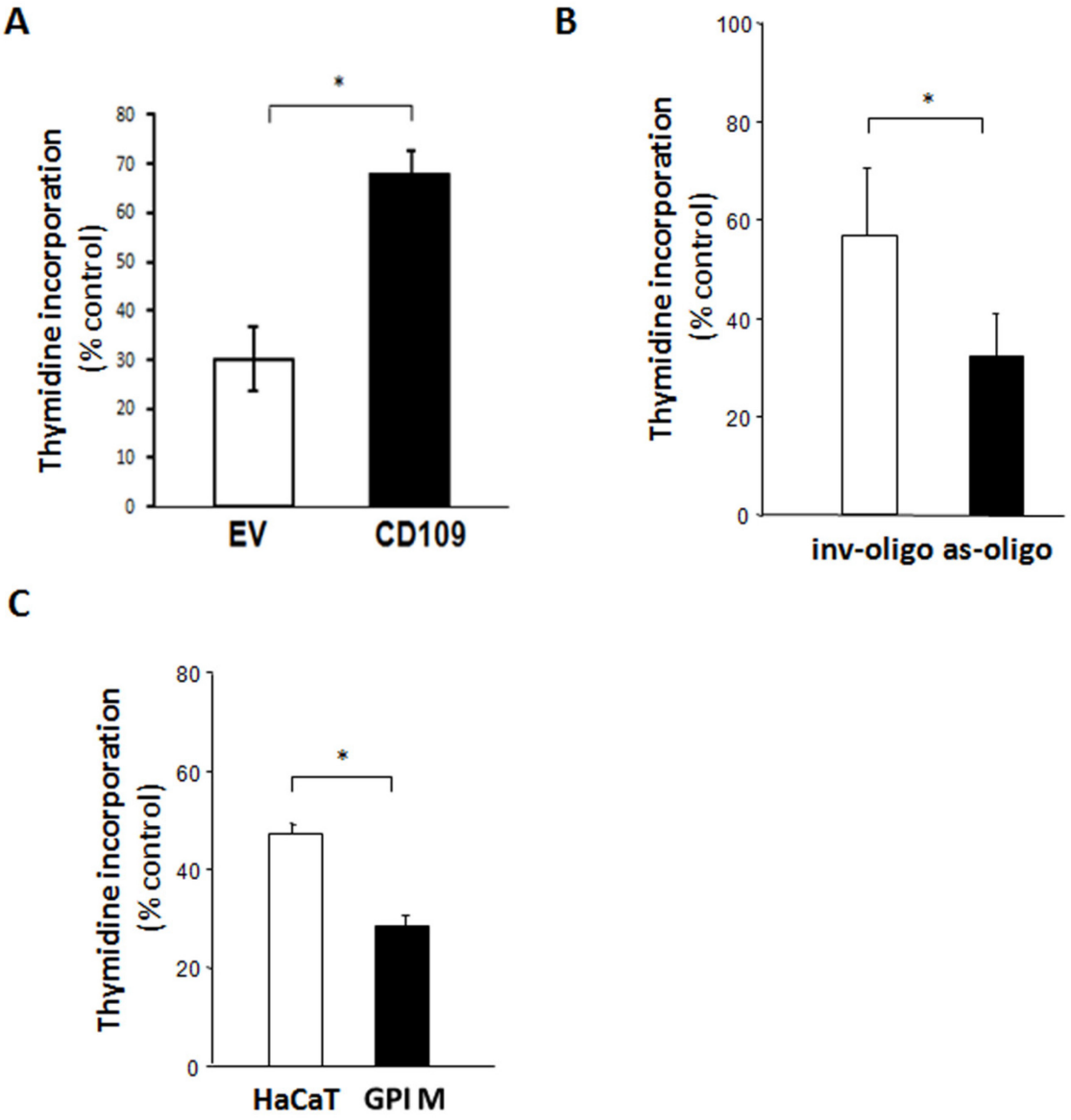


Figure 3.4: CD109 inhibits TGF-β-induced growth inhibition. **A.** HaCaT cells stably overexpressing CD109 or its EV, **B.** HaCaT cells transfected with CD109-specific antisense morpholino (as-oligo) or inverse control oligos (inv-oligo) and **C.** HaCaT cells defective in GPI-anchor synthesis (GPI M) or parental HaCaT cells were incubated with or without 50 pM TGF-β1 for 24h and ^{3}H-thymidine incorporation was determined as described in Materials and Methods and expressed as a percentage of untreated cells. *: $p < 0.05$.

compared to parental HaCaT cells (fig 3.4C). Collectively, these results suggest that CD109 negatively regulates TGF-β-induced growth inhibition in human keratinocytes.

3.2.5- CD109 can inhibit TGF-β transcriptional activity independently of ligand sequestration

Because previous studies from our laboratory have shown that soluble CD109 can bind TGF-β1 on its own (Tam *et al.*, 2001), it is possible that CD109 acts solely by sequestering the ligand away from the signalling receptors. However, because CD109 directly binds to TGFBR1 (Finnson *et al.*, 2006), CD109 may also directly modulate TGF-β receptors activity. To determine the mechanism of action of CD109, the effect of CD109 on the transcriptional activity induced by a constitutively active TGFBR1-(T204D) was analyzed in the absence of ligand, using a 3TP-lux assay (fig 3.5). The assay was performed in 293 cells, because they can be efficiently transfected and have very low amount of endogenous TGF-β receptors (Itoh *et al.*, 2003). As expected, transfection of TGFBR1-T204D in 293 cells results in an increase in luciferase activity, as compared to cells transfected with a control vector. Importantly, CD109 co-transfection leads to a significant ($p < 0.05$) decrease in this TGFBR1-T204D-induced transcriptional activity, as compared to EV co-transfection (fig 3.5). To make sure that the effect of CD109 is not due to autocrine TGF-β, 293 cells were treated with a pan TGF-β neutralizing antibody (1D11). In the presence of 1D11, CD109 is still able to inhibit TGFBR1-T204D activity (fig 3.5). These results indicate that CD109 can exert its inhibitory effect on TGF-β signalling independently of ligand sequestration.

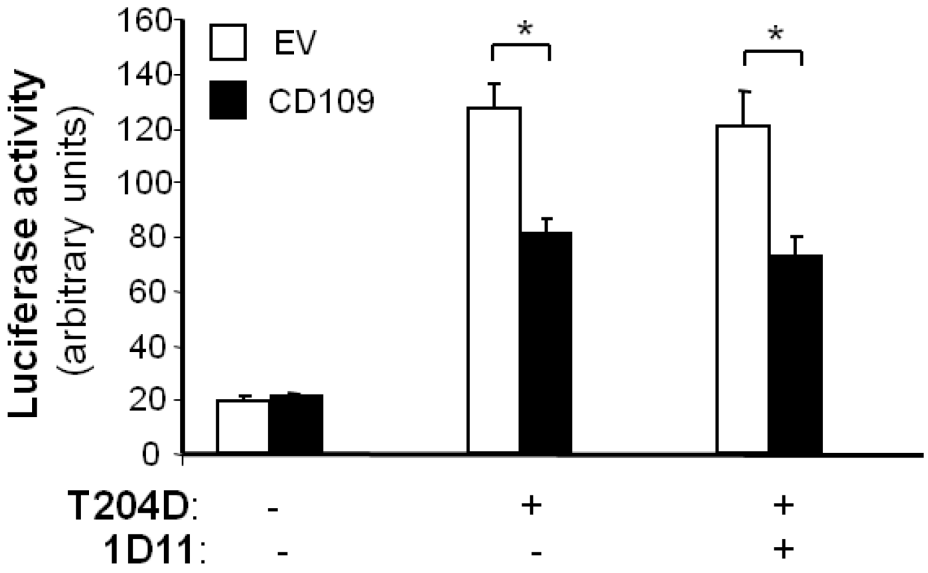


Figure 3.5: CD109 inhibits TGF-β receptor signaling independently of the TGF-β ligand. HEK 293 cells were co-transfected with 3TP-lux, β-galactosidase, TGFBR1-T204D (or its empty vector) and CD109 (or its empty vector, EV). The cells were then incubated overnight in the presence or absence of a pan TGF-β neutralizing antibody (1D11, 10 μg/ml). Cell lysates were analyzed for luciferase and β-galactosidase activities and expressed as luciferase activity normalized to β-galactosidase activity. The result is representative of three independent experiments. *$p<0.05$.

3.3- Discussion

TGF-β is multifunctional cytokine, involved in many cellular processes and whose action is impaired in several human diseases. This underscores the need for a tight regulation of TGF-β signalling pathway. TGF-β signalling can be regulated at many levels, including at the receptor level, by molecules called co-receptors. Our group has previously reported that keratinocytes defective in GPI-anchor biosynthesis display increase TGF-β responses, suggesting that (a) GPI-anchor protein(s) may regulate TGF-β signalling (Tam *et al.*, 2003). In addition, a GPI-anchored protein able to bind TGF-β1 was identified on the cell surface of human keratinocytes (Tam *et al.*, 1998; Tam *et al.*, 2001). Molecular cloning of this protein reveals that it represents CD109 (Finnson *et al.*, 2006), a member of the α2M/complement family, whose function remained obscure. CD109 forms a heteromeric complex with TGF-β signalling receptors and binds directly (*in vitro*) to TGFBR1 (Finnson *et al.*, 2006). Here, I have demonstrated that CD109 acts as an inhibitor of TGF-β signalling and responses in a variety of cell types and that it can regulate TGF-β signalling independently of ligand binding. Together, these findings suggest that CD109 is a novel TGF-β co-receptor, which is able to negatively regulate TGF-β signalling and responses.

Results shown in this chapter indicate that CD109 is able to inhibit TGF-β-induced SMAD2/3 phosphorylation, SMAD3 transcriptional activity and TGF-β-mediated ECM production and growth inhibition in all cell lines analyzed. These results suggest that CD109 is able to negatively regulate TGF-β signalling and to inhibit TGF-β-induced responses *in vitro*. Moreover, our results indicate that its ability to antagonize TGF-β signalling is conserved between cell types and species. My *in vitro* results showing that a human CD109 is able to inhibit TGF-β signalling in mouse cells set the stage for further studies of transgenic mice overexpressing human CD109 in the epidermis (Vorstenbosch *et al.*, 2011). Despite the fact that posttranscriptional modifications (such as glycosylation) may differ between murine and human cells, CD109 retains its ability to

bind TGF-β1 (data not shown) and to decrease TGF-β-induced SMAD2/3 phosphorylation and transcriptional activities in mouse cells.

Interestingly, molecular cloning of CD109 has revealed the occurrence of two different forms of CD109 in the placenta: a long version (termed CD109) and a shorter version (called CD109S), which is missing 17 amino acids at position 1218 (Fig 1.6B and (Finnson *et al.*, 2006)). The observed deletion in CD109S is likely the result of alternative splicing since the 51bp sequence encoding the 17 missing amino acids in *CD109* are flanked by consensus RNA splicing sites. In contrast to placenta, HaCaT cells only express the long version of CD109, suggesting that alternative splicing of CD109 mRNA occurs in a tissue-specific manner (Finnson *et al.*, 2006). Preliminary results from our laboratory were performed using CD109S in HaCaT cells and show that CD109S inhibits TGF-β-induced SMAD3 transcriptional activities and growth inhibition (Finnson *et al.*, 2006). In this thesis, all results presented use CD109 long form and suggest that, like CD109S, CD109 is able to inhibit TGF-β signalling and responses.

The ability of CD109 to reduce TGF-β-induced growth inhibition in HaCaT cells suggest that CD109 may play an important role in skin homeostasis, maintaining a fine balance between proliferation and differentiation. Moreover, CD109 may have a potential role during tumor formation. Dysregulation of CD109 levels has been observed in many cancers, including in squamous cell carcinoma originating from keratinocytes (Hagiwara *et al.*, 2008; Hashimoto *et al.*, 2004). An upregulation of CD109 in these cells may lead to an increase of proliferation, causing tumor growth. The role of CD109 as a negative regulator of TGF-β signalling and responses and its possible implication during cancer initiation or progression emphasize the potential importance of this novel TGF-β co-receptor. Thus, understanding the mechanism of action of CD109 is crucial to the development of novel therapeutic strategies involving CD109.

Potential mechanisms by which CD109 may inhibit TGF-β signalling include decreasing ligand availability to the signalling receptors and/or modulating receptor activity. It has been previously demonstrated that the GPI-anchored protein r150/CD109 can be released from the cell surface (either via PI-PLC or by furin cleavage) (Hagiwara *et al.*, 2010; Tam *et al.*, 2001). The released/ soluble CD109 retains its ability to bind TGF-β1 and reduces TGF-β1 binding to signalling receptors (Tam *et al.*, 2001). However, the release occurs only in small amounts in the absence of exogenously added PI-PLC. Thus, ligand sequestration may not be the primary mechanism of action of CD109. Indeed, previous results showing that CD109 directly binds to TGFBR1 (Finnson *et al.*, 2006) and my result demonstrating that CD109 can inhibit transcriptional activity induced by a constitutively active form of type I TGF-β receptor in the absence of TGF-β ligand strongly suggest that CD109 is able to modulate TGF-β receptor activity independently of ligand sequestration. Delineation of the precise molecular mechanism(s) by which CD109 acts as a negative regulator of TGF-β signalling is the object of the following chapters.

Chapter 4: CD109 regulates TGF-β receptor internalization into caveolae

4.1- Rationale

We previously identified a novel TGF-β co-receptor, CD109, that negatively regulates TGF-β signalling and responses in a variety of cell types ((Finnson *et al.*, 2006), Chapter 3). Moreover recent reports have shown that CD109 expression is deregulated in basal-like breast carcinoma (Hasegawa *et al.*, 2007), squamous cell carcinoma (Hagiwara *et al.*, 2008; Hashimoto *et al.*, 2004; Sato *et al.*, 2007; Zhang *et al.*, 2005a) and melanoma (Finnson *et al.*, 2006) and that CD109 is mutated in colorectal cancer (Sjoblom *et al.*, 2006). Despite its important role in TGF-β signalling and its potential significance in cancer progression, its mechanism of action is not yet understood. CD109 is a 180 kDa glycosylphosphatidylinositol (GPI)-anchored protein that can be released from the cell surface by PI-PLC treatment. Both soluble and membrane-anchored CD109 binds the TGF-β1 subtype with high affinity. However, my previous results indicate that CD109 is able to inhibit TGF-β signalling independently of ligand sequestration (Fig 3.5). This suggests that, by forming a heteromeric complex with the TGF-β signalling receptors, CD109 may modulate TGF-β receptor activity.

Interestingly, it has been shown that TGF-β receptors are internalized via both clathrin- and caveolae-dependent pathways (Di Guglielmo *et al.*, 2003). Internalization of the TGF-β receptors via the clathrin-coated pits has been linked with signalling via SMAD2/3 and receptor recycling (Hayes *et al.*, 2002; Itoh *et al.*, 2002; Mitchell *et al.*, 2004; Runyan *et al.*, 2005). In contrast, TGF-β receptor localization in caveolae is associated with downregulation of SMAD2/3 signalling and receptor degradation following ubiquitination by the E3-ubiquitin ligase Smurf2 (Di Guglielmo *et al.*, 2003; Razani *et al.*, 2001). Because CD109 is a negative regulator of TGF-β signalling, I have investigated whether CD109 exerts this effect by modulating caveolae-mediated internalization of the TGF-β receptors.

4.2- Results

4.2.1- CD109 associates with caveolin-1 at endogenous concentration

I sought to determine whether CD109 exerts its negative effects on TGF-β signalling by modulating TGF-β receptor localization into the caveolae, a process shown to be associated with downregulation. The association between CD109, a GPI-anchored protein, with caveolin-1, a major component of the caveolae was first examined by co-immunoprecipitation and immunofluorescence studies. Co-immunoprecipitation experiments reveal that CD109 associates with caveolin-1 in 293 cells co-transfected with both CD109 and caveolin-1 (fig 4.1A). In the absence of caveolin-1 transfection, (i.e. when caveolin-1 expression is undetectable (Wharton *et al.*, 2005)), CD109 is not co-immunoprecipitated (fig 4.1A), demonstrating the specificity of the association.

In untransfected HaCaT cells, caveolin-1 is co-immunoprecipitated by an anti-CD109 antibody (fig 4.1B). Similarly, low amount of CD109 is co-immunoprecipitated by an anti-caveolin-1 antibody (fig 4.1C), confirming that the interaction between CD109 and caveolin-1 is not an artifact of overexpression and that it occurs at endogenous concentrations of caveolin-1 and CD109 (fig 4.1B and C). Importantly, the association between CD109 and caveolin-1 is greatly enhanced after TGF-β treatment (fig 4.1C).

Immunofluorescence microscopy of untransfected HaCaT cells (fig 4.2A) shows that caveolin-1 (*in red*) displays a punctate staining pattern at the plasma membrane, with some punctate staining in the cytoplasm, which may represent neo-synthesized or internalized caveolin-1. Endogenous CD109 (*blue*) localizes mainly at the plasma membrane in both non-caveolar and caveolin-1 positive compartments. The co-localization of CD109 with caveolin-1 (in *purple,* fig 4.2A) suggests that CD109 is able to associate with caveolin-1 in the same cellular compartment, supporting the results obtained in fig 4.1.

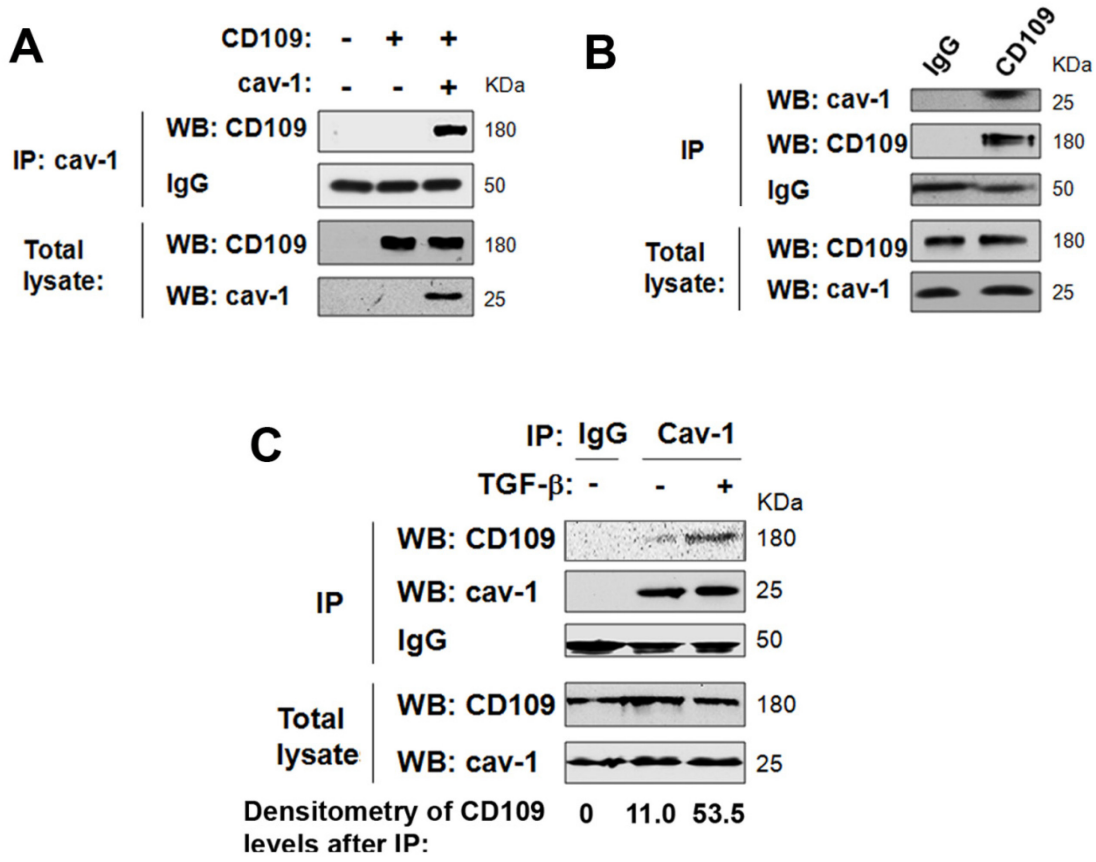


Figure 4.1: CD109 associates with caveolin-1. **A.** Cell lysates from 293 cells transfected with CD109 and caveolin-1 or their corresponding empty vector were immunoprecipitated with an anti-caveolin-1 (cav-1) antibody followed by western blot with an anti-CD109 antibody. The IgG band demonstrates equal loading. **B.** Cell lysates from non-transfected HaCaT cells were immunoprecipitated with an anti-CD109 antibody or with an anti-histone antibody (used as control IgG), followed by western blot for CD109 and caveolin-1. **C.** Non-transfected HaCaT cells were treated with or without 25pM TGF-β1 for 30 min. Cell lysates were then subjected to immunoprecipitation with an anti-caveolin-1 antibody or with an anti-HA antibody (used as control IgG), followed by western blot for CD109 and caveolin-1. Densitometry of CD109 (in the immunoprecipitates) is indicated.

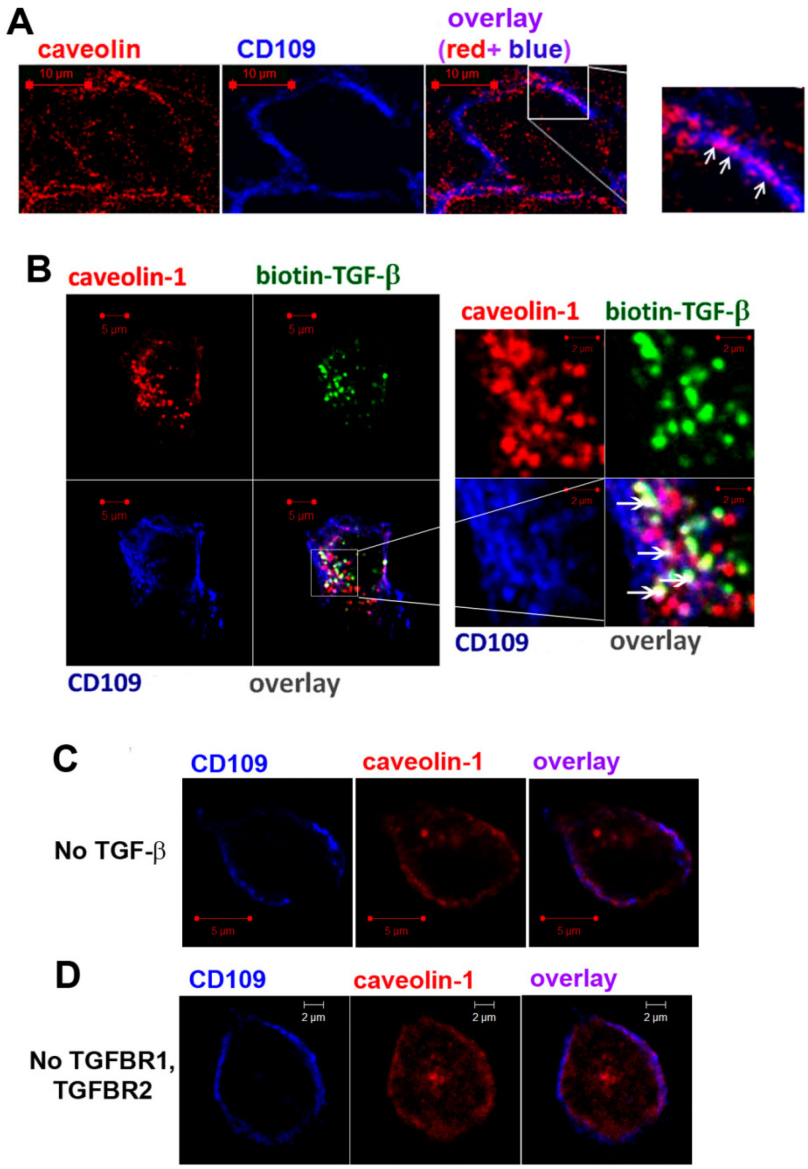


Figure 4.2: CD109 co-localizes with the TGF-β receptors in caveolae. A. HaCaT cells were immunostained for endogenous caveolin-1 (*red*) and endogenous CD109 (*blue*). Co-localization of CD109 with caveolin-1 appears in purple. **B.** 293 cells transfected with CD109, caveolin-1, TGFBR1/TGFBR2 were stained with biotin-TGF-β1/streptavidin (*green*) and incubated for 30 min at 37^{o}C to allow receptor internalization. After fixation, cells were stained for CD109 (*blue*) and caveolin (*red*) and analyzed by confocal microscopy. Triple co-localization of caveolin-1, CD109, and biotin-TGF-β1 appears in white. A representative cell of n= 5 independent experiments is shown. **C.** 293 cells transfected as in (B) were left untreated and stained for CD109 (*blue*) and caveolin-1 (*red*). Co-localization of CD109 with caveolin-1 appears in purple. A representative cell of n= 3 experiments is shown. **D**. 293 cells transfected with CD109, and caveolin-1 but not with TGFBR1/TGFBR2 were stained as in (C).

4.2.2- CD109 and TGF-β receptors co-localize in caveolae

Previous results from Dr Philip's laboratory have shown that CD109 binds directly to TGFBR1 (Finnson *et al.*, 2006). Thus, I sought to determine whether TGF-β receptors co-localize with CD109 in caveolae. Because the endogenous levels of TGF-β receptors in HaCaT cells are too low to allow detection of the internalized ligand, 293 cells were transfected with TGFBR1/TGFBR2, and caveolin-1 and labeled with biotin-TGF-β, followed by streptavidin-AF647 treatment as described in Chapter 2. After 30 min at 37^{o}C to allow receptor and ligand internalization, the cells were immunostained for CD109 and caveolin-1 (fig 4.2B). Importantly, a significant amount of triple co-localization (*visualized in white*) is observed between the internalized biotin-TGF-β (*in green*), CD109 (*in blue*), and caveolin-1 (*in red*) (fig 4.2B). The proportion of internalized biotin-TGF-β that is not associated with CD109 and caveolin-1 (fig 4.2B) may represent molecules that are internalized via the clathrin pathway (associated with SMAD signalling) or that are in recycling compartments. In the absence of TGF-β treatment, CD109 localizes mainly at the plasma membrane, where it partially co-localizes with caveolin-1 (fig 4.2C), as observed in untransfected HaCaT cells (fig 4.2A). Thus, it appears that CD109 is localized at the cell surface in the absence of TGF-β treatment and that it becomes internalized following TGF-β stimulation.

Interestingly, in the absence of TGF-β receptor transfection, low levels of co-localization between CD109 and caveolin-1 could be observed (fig 4.2D). It is possible that the interaction between the GPI-anchored CD109 and the integral membrane protein caveolin-1 is mediated via endogenous TGF-β receptors in that case, although the contribution of other transmembrane proteins or lipid molecules cannot be ruled out.

The notion that the biotin-TGF-β staining is specific for biotin-TGF-β bound to TGFBR1/TGFBR2 complex and not to other TGF-β binding molecules under our detection settings is supported by the following results: (1) when TGFBR1/TGFBR2 are not transfected, no staining is observed for biotin-TGF-β, even in the presence of CD109 (fig 4.3A); (2) when biotin-TGF-β but not AF647-strepatvidin treatment is omitted, no signal is observed (fig 4.3B), confirming the specificity of the staining; (3) biotin-TGF-β

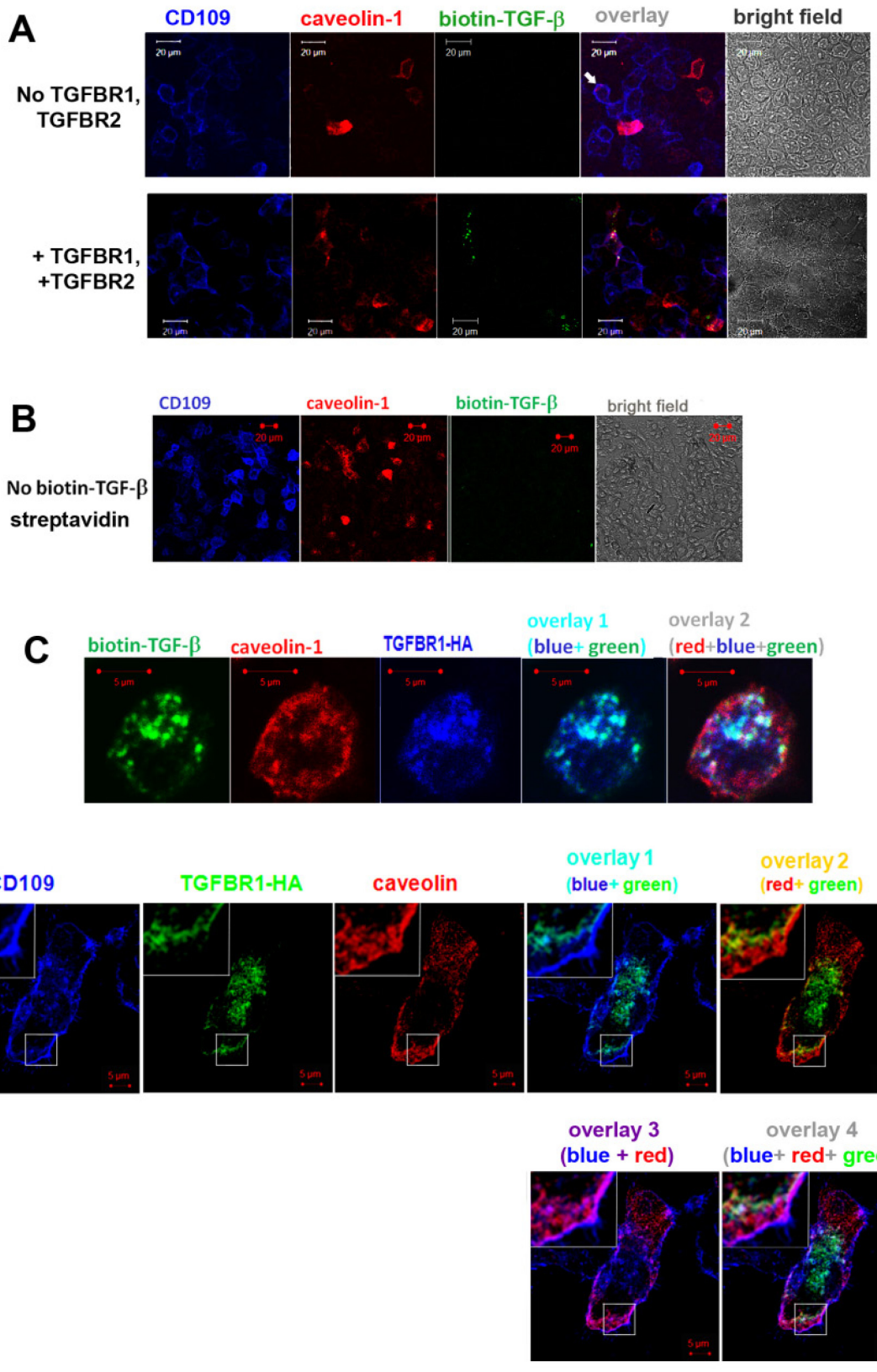

A
CD109
caveolin-1
biotin-TGF-β
overlay
bright field
No TGFBR1, TGFBR2
+ TGFBR1, +TGFBR2
B
CD109
caveolin-1
biotin-TGF-β
bright field
No biotin-TGF-β streptavidin
C
biotin-TGF-β
caveolin-1
TGFBR1-HA
overlay 1 (blue+ green)
overlay 2 (red+blue+green)
D
CD109
TGFBR1-HA
caveolin
overlay 1 (blue+ green)
overlay 2 (red+ green)
overlay 3 (blue + red)
overlay 4 (blue+ red+ green)

Figure 4.3: Streptavidin-AF647 staining is specific for biotin-TGF-β and represents TGF-β bound to the TGF-β receptors. **A.** 293 cells transfected with CD109, caveolin-1 and TGFBR1/TGFBR2 or their corresponding empty vector were incubated with biotin-TGF-β1 and streptavidin-AF647 (*green*) and placed at 37^{o}C for 30 min. After fixation, cells were immunostained for CD109 (*blue*) and caveolin-1 (*red*) and analyzed by confocal microscopy. No stain for biotin-TGF-β1 is detected in the absence of TGF-β receptors transfection. A white arrow points at a cell where co-localization between CD109 and caveolin-1 can be observed in the absence of TGF-β receptor transfection. **B.** 293 cells transfected with CD109, caveolin-1 and TGFBR1/TGFBR2 were incubated without biotin-TGF-β1 but with streptavidin-AF647 (*green channel*) and placed at 37^{o}C for 30 min. After fixation, cells were immunostained for CD109 (*blue*) and caveolin-1 (*red*) and analyzed by confocal microscopy. As expected, no stain for biotin-TGF-β1 is detected. **C.** 293 cells transfected with TGFBR1-HA, TGFBR2, and caveolin-1 were stained with biotin-TGF-β1/streptavidin (*green*) and incubated for 30 min at 37^{o}C. After fixation, cells were stained for TGFBR1-HA (*blue*) and caveolin-1 (*red*) and analyzed by confocal microscopy. The co-localization of biotin-TGF-β1 with TGFBR1-HA appears in turquoise (overlay 1). Triple co-localization of biotin-TGF-β1, TGFBR1 and caveolin-1 appears in white (*overlay 2*) and indicate that biotin-TGF-β1 follow the same internalization route as TGFBR1. **D.** 293 cells transfected with CD109, TGFBR1-HA, TGFBR2 and caveolin-1 were treated with unlabeled TGF-β1 for 30 min at 37^{o}C and immunostained for CD109 (*blue*), caveolin (*red*) and TGFBR1-HA (*green*). The co-localization of CD109 and TGFBR1 appears in turquoise (overlay 1), the co-localization of TGFBR1 and caveolin appears in yellow (overlay 2) while the co-localization of CD109 and caveolin-1 appears in purple (overlay 3). Triple co-localization appears in white (overlay 4) and suggests that CD109 and TGFBR1 form a complex in caveolae.

co-localizes with HA-tagged TGFBR1 (fig 4.3C). This suggests that TGF-β follows the same internalization route as TGFBR1, as reported by others (Di Guglielmo *et al.*, 2003; Zwaagstra *et al.*, 2001); (4) HA-TGFBR1 co-localizes with CD109 in caveolae vesicles and at the plasma membrane (fig 4.3D). Together, these data indicate that biotin-TGF-β can be used to assess TGF-β receptor localization and suggest that CD109 is able to form a complex with TGF-β-bound TGF-β receptors (activated receptors) in caveolae.

4.2.3- CD109 enhances binding of TGF-β to TGF-β receptors and TGF-β-bound receptor internalization

The effect of CD109 on TGF-β-bound receptor internalization was then investigated. For this, 293 cells were transfected with TGFBR1/TGFBR2, caveolin-1 and CD109 or its control empty vector (EV), treated with biotin-TGF-β/streptavidin and incubated for 30 min at 37^{o}C to allow receptor internalization as described above. CD109 overexpression significantly ($p<0.05$) increases the number of cells that have internalized biotin-TGF-β (i.e. TGF-β-bound receptors) as compared to empty vector (EV) transfection in 293 cells (fig 4.4A and B). After normalization for transfection efficiency (as determined by caveolin-1 expression), the result indicate that 64% of caveolin-1 positive cells internalize biotin-TGF-β in the presence of CD109, whereas only 27% incorporate biotin-TGF-β in EV transfected cells (fig 4.4B), suggesting that CD109 increases the internalization of TGF-β-bound receptors. The amount of internalized biotin-TGF-β reflects the levels of receptors that underwent endocytosis, and also any recycling and/or degradation that may have occurred during that time period. Notably, CD109 transfection does not alter the levels of TGF-β receptors or caveolin-1, as compared to EV transfection (fig 4.5B and data not shown), suggesting that CD109 only affects the amount of internalized TGF-β-bound receptors.

Next, the effect of CD109 on the internalization of ^{125}I-TGF-β was examined in HaCaT cells at endogenous TGF-β receptor concentration. The ^{125}I-TGF-β internalization assay has been shown to reliably reflect receptor internalization (Guimond *et al.*, 2002; Zwaagstra *et al.*, 2001). At early time points (15 and 30 min), CD109 enhances ^{125}I-TGF-β

internalization (fig 4.4C). The increase of ^{125}I-TGF-β internalization in CD109 overexpressing cells is followed by a decline after 30 min whereas a decline occurs only after 60 min in EV transfected cells (fig 4.4C). The decline in internalized ^{125}I-TGF-β has been reported previously and might be due to ligand depletion from the extracellular milieu and/or degradation (Zwaagstra *et al.*, 2001). Importantly, these results suggest that CD109 enhances ligand-bound receptor internalization.

Interestingly, CD109 overexpression is associated not only with an increase of ^{125}I-TGF-β internalization, but also with an increase of surface-bound ^{125}I-TGF-β at 30min (fig 4.4D). The ratio Internalized/ Surface-bound ^{125}I-TGF-β (I/S) is higher in CD109 transfected cells, as compared to EV cells at both 15 and 30 min (fig 4.4E), suggesting that CD109 may accelerate TGF-β-bound receptor endocytosis. Moreover, knocking down CD109 expression using CD109 siRNA leads to a decrease in both internalized and surface-bound ^{125}I-TGF-β at 30 min (fig 4.4F). These results indicate that endogenous CD109 increases surface binding and internalization of TGF-β.

To determine whether the increase in TGF-β binding to the cell surface is due to an increase in TGF-β binding to the TGF-β receptors and not solely due to an increase in TGF-β binding to CD109, I have performed affinity labeling of cell surface receptors at 4^{o}C followed by SDS-PAGE analysis. In HaCaT cells and 293 cells, ^{125}I-TGF-β is bound to a 50kDa band and a 70 kDa band representing TGFBR1 and TGFBR2; CD109 is detected as two bands: a 180kDa band and a 150kDa band resulting from autolytic cleavage, as previously observed (Finnson *et al.*, 2006). Importantly, CD109 siRNA transfection in HaCaT cells decreases the amount of cell surface ^{125}I-TGF-β-bound receptors (fig 4.5A), but does not affect the total levels of TGFBR1, as demonstrated by western blot of the same lysate used for affinity labeling (fig 4.5A). Similarly, CD109 overexpression in 293 cells increases the amount of cell surface ^{125}I-TGF-β-bound receptors without affecting the total amount of TGFBR1, as shown by western blot (fig 4.5B). Because the amount of TGF-β receptor present at the cell surface represents only a small proportion of the total amount of TGF-β receptor, I then verified that overexpression of CD109 does not alter the cell-surface level of TGF-β receptors (fig 4.5B). These results indicate that

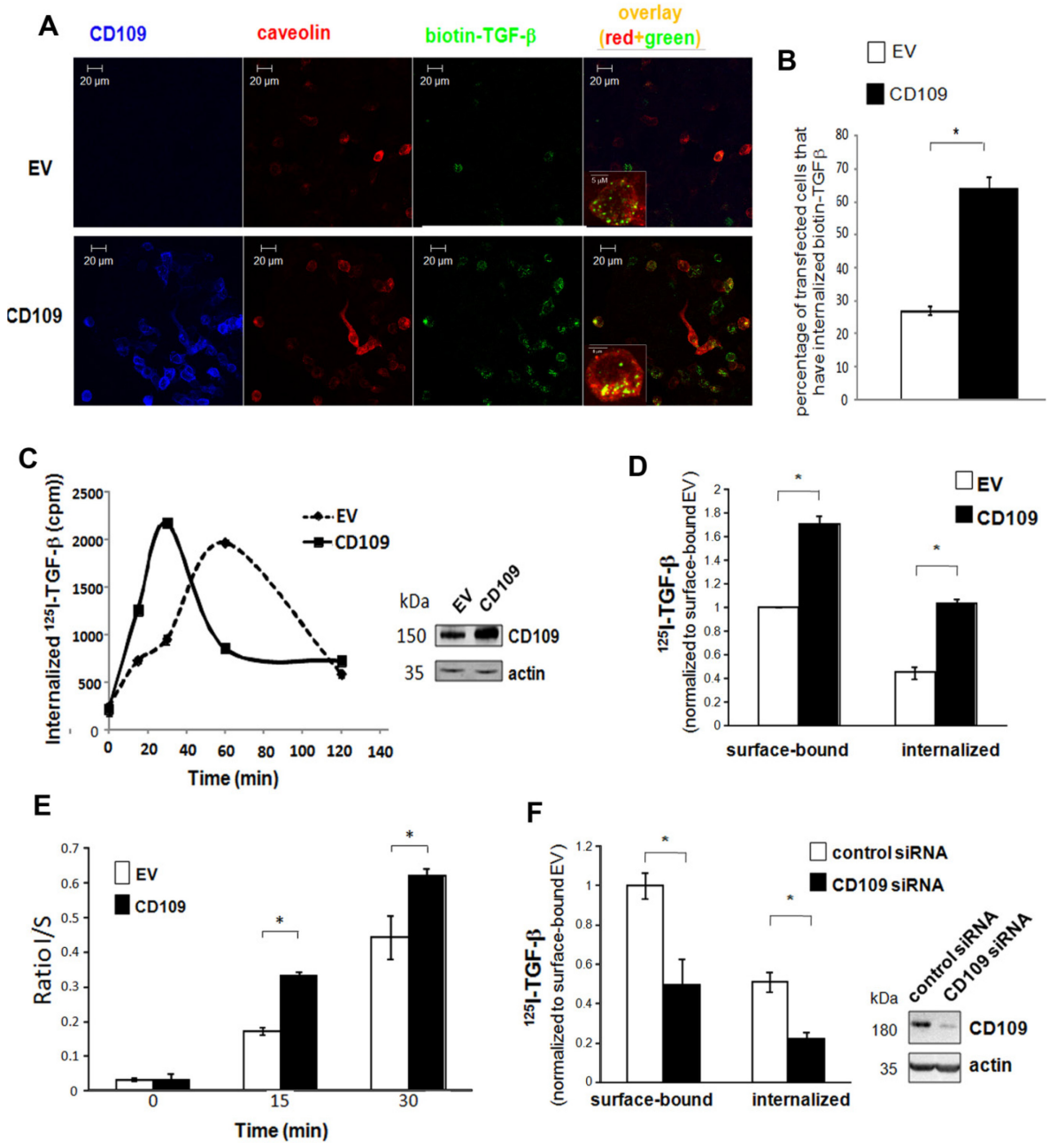

A
CD109
caveolin
biotin-TGF-β
overlay
(red+green)
EV
CD109
20 μm
B
EV
CD109
percentage of transfected cells that have internalized biotin-TGFβ
C
Internalized 125I-TGF-β (cpm))
EV
CD109
Time (min)
kDa
150
CD109
35
actin
D
125I-TGF-β
(normalized to surface-bound EV)
surface-bound
internalized
E
Ratio I/S
Time (min)
F
control siRNA
CD109 siRNA
180
CD109
35
actin

Figure 4.4: CD109 promotes TGF-β internalization. ***A-B.*** *Immunofluorescence*: 293 cells were stained with biotin-TGF-β1/streptavidin (*green*) and incubated for 30 min at 37^{o}C to allow receptor internalization. Cells were fixed and immunostained for CD109 (*blue*) and caveolin (*red*) and analyzed by confocal microscopy. **A.** Comparison of populations of CD109 and control EV transfected cells. A representative cell showing internalization of biotin-TGF-β1 is enlarged in the left corner of the overlay. **B.** Percentage of transfected (caveolin-1 positive) cells that have internalized biotin-TGF-β1 (mean of n=5 independent experiments ± SEM, *: p< 0.05).***C-F***. *^{125}I-TGF-β internalization assay*: **C.** *Left panel*: HaCaT cells transfected with CD109 or EV were treated with 100 pM ^{125}I-TGF-β1 and incubated at 37°C for the indicated times. The graph shows the amount of internalized ^{125}I-TGF-β1 (expressed as specific cpm). *Right panel*: western blot showing CD109 overexpression in HaCaT cells. **D.** Amount of surface-bound and internalized ^{125}I-TGF-β1 after 30 min incubation at 37^{o}C in HaCaT cells transfected with CD109 or EV (normalized to surface-bound EV). **E.** Ratio of internalized / surface-bound ^{125}I-TGF-β1 (I/S) in HaCaT cells transfected with CD109 or EV. **F.** *Left panel*: Amount of surface-bound and internalized ^{125}I-TGF-β1 after 30 min incubation at 37^{o}C in HaCaT cells transfected with CD109 siRNA or control siRNA (normalized to surface-bound control siRNA). *Right panel*: Western blot showing CD109 siRNA efficacy. All graphs show the mean of n≥3 independent experiments, ± SEM, *: p<0.05.

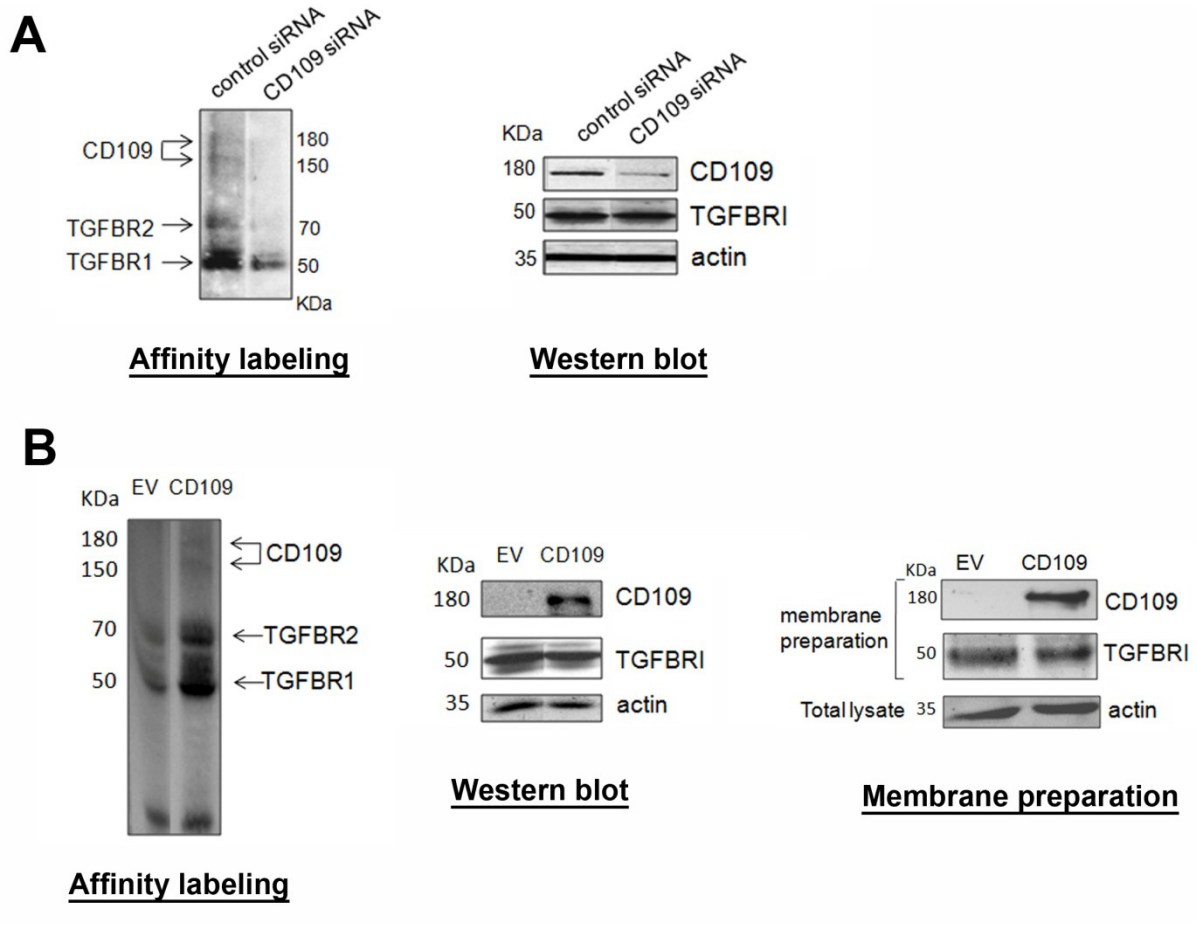


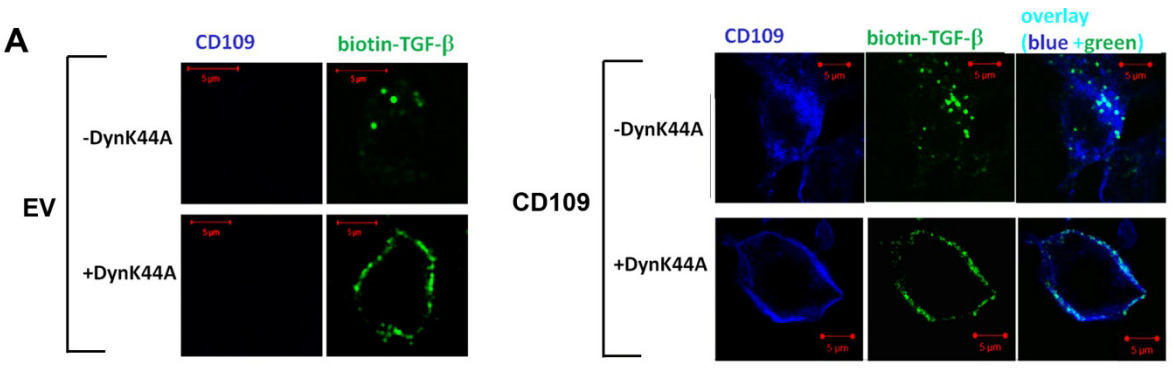
Figure 4.5: CD109 enhances TGF-β binding to the TGF-β receptors without altering the total or cell surface receptor levels. **A.** HaCaT cells transfected with control or CD109 siRNA were affinity labeled with 100pM ^{125}I-TGF-β1. *Left panel*: The amount of cell surface receptors bound to TGF-β1 is visualized by autoradiography. *Right panel*: The same lysates were analyzed for TGFBR1 by western blot. **B.** 293 cells transfected with TGFBR1, TGFBR2 and CD109 or EV were affinity labeled with 100pM ^{125}I-TGF-β1. *Left panel*: autoradiography showing the amount of cell surface TGF-β-bound receptors. *Middle panel*: The same lysates were analyzed for TGFBR1 by western blot. *Right panel*: Cell surface proteins from 293 cells transfected with TGFBR1/TGFBR2, CD109 or EV were labeled with Hook-sulfo-NHS-SS-Biotin, pulled down with streptavidin and analyzed by western blot with CD109 and TGFBR1 antibodies. Same amount of proteins were used in the experiment as demonstrated by the actin blot. All results are representative of n=3 independent experiments and all lanes from a panel come from the same blot.

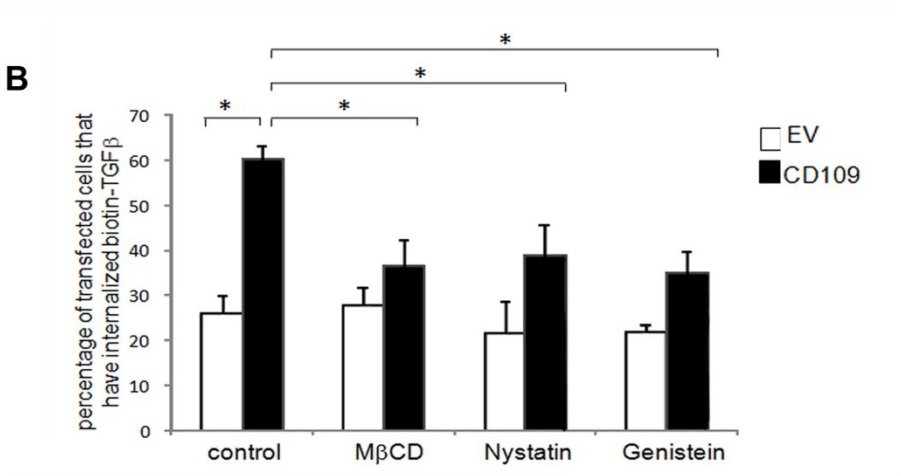
CD109 is able to enhance TGF-β binding to the receptors and together with previous findings from Dr Philip's lab demonstrating that CD109 forms a complex with the TGF-β receptors (Finnson *et al.*, 2006), suggest that such association is an important step to CD109's ability to increase TGF-β binding to this multimeric TGF-β receptor complex and enhances the internalization of this TGF-β bound-complex.

4.2.4- CD109 enhances TGF-β receptor internalization via the caveolar pathway

Next, I assessed if the CD109-induced increase in TGF-β receptor internalization could be mediated by the caveolar compartment. Internalization of biotin-TGF-β in EV transfected 293 cells is prevented by co-transfection of the Dynamin2-K44A mutant (Dyn2K44A) (fig 4.6A), which blocks endocytosis via both clathrin and caveolar pathways (Puri *et al.*, 2001) and is consistent with previous reports demonstrating that TGF-β receptors are internalized via clathrin and/or caveolae pathways (Di Guglielmo *et al.*, 2003; Luga *et al.*, 2009). More importantly, internalization of biotin-TGF-β in CD109 transfected 293 cells is also blocked by co-transfection of the Dynamin2-K44A mutant (Dyn2K44A) (fig 4.6A). In the presence of Dyn2K44A, the biotin-TGF-β staining appears at the plasma membrane in 81 (± 5.2) % of the cells and co-localizes with CD109 staining, whereas the biotin-TGF-β staining is found at the plasma membrane in only 5.1 (± 4.6)% of the cells in the absence of Dyn2K44A (fig 4.6A). This indicates that CD109 promotes dynamin-dependent internalization of the TGF-β-bound receptor.

Because CD109 is an inhibitor of TGF-β signalling (Chapter 3 and (Finnson *et al.*, 2006)), I looked more specifically at CD109's ability to increase internalization via caveolae, a route that has been shown to lead to receptor degradation and signalling downregulation. Pre-treatment of 293 cells with three different inhibitors of lipid-raft/caveolar-dependent internalization: MβCD, Nystatin and Genistein (Mitchell *et al.*, 2004; Puri *et al.*, 2001)), significantly ($p<0.05$) reduces the CD109-induced increase in biotin-TGF-β internalization (fig 4.6B).


A
CD109
biotin-TGF-β
overlay
(blue +green)
EV
-DynK44A
+DynK44A
CD109
-DynK44A
+DynK44A
5 μm


B
percentage of transfected cells that have internalized biotin-TGFβ
EV
CD109
control
MβCD
Nystatin
Genistein
*

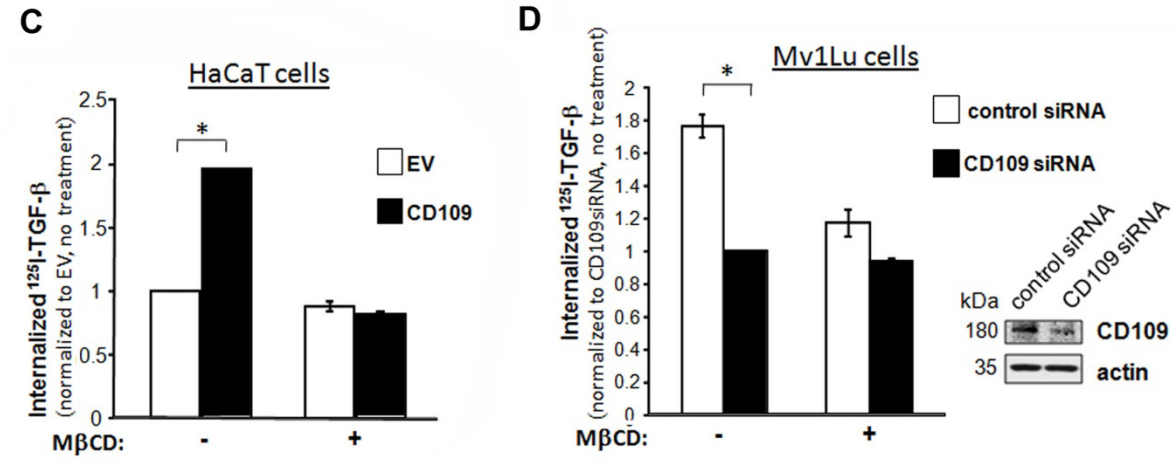

C
HaCaT cells
Internalized 125I-TGF-β
(normalized to EV, no treatment)
EV
CD109
MβCD:
-
+
D
Mv1Lu cells
Internalized 125I-TGF-β
(normalized to CD109siRNA, no treatment)
control siRNA
CD109 siRNA
MβCD:
kDa
control siRNA
CD109 siRNA
180
CD109
35
actin

Figure 4.6: CD109 enhances TGF-β internalization via the caveolar pathway. ***A-B.*** *Immunofluorescence*: **A.** 293 cells transfected with TGFBR1/TGFBR2, CD109 (or its EV) and Dyn2K44A (or its control vector) were treated with biotin-TGF-β1/streptavidin (*green*), incubated for 30 min at 37^{o}C and stained for CD109 (*blue*). Co-localization of CD109 and biotin-TGF-β1 appears in turquoise (overlay). Representative cells are shown. **B.** 293 cells transfected with CD109 or EV, TGFBR1/TGFBR2 and caveolin-1 were treated with MβCD, Nystatin or Genistein prior initiation of internalization. The cells were stained for biotin-TGF-β1/streptavidin and caveolin-1. The graph shows the percentage of transfected (caveolin-1 positive) cells that have internalized biotin-TGF-β1. ***C-D.*** *^{125}I-TGF-β internalization assay*: **C.** HaCaT cells transfected with CD109 or EV were treated with or without MβCD prior to incubation with ^{125}I-TGF-β1. The graph shows the amounts of internalized ^{125}I-TGF-β1 after 30 min incubation at 37^{o}C (normalized to EV, no treatment). **D.** *Left panel*: Mv1Lu cells transfected with CD109 siRNA or control siRNA were treated with or without MβCD prior to incubation with ^{125}I-TGF-β1. The graph shows the amounts of internalized ^{125}I-TGF-β1 after 30 min incubation at 37^{o}C (normalized to CD109 siRNA, no treatment). *Right panel*: western blot showing CD109 siRNA efficacy in Mv1Lu cells. All graphs show the mean of n≥ 3 independent experiments, ± SEM; *: p< 0.05.

Moreover, an alternative approach using ^{125}I-TGF-β to monitor endogenous TGF-β receptor internalization in HaCaT cells shows that pre-treatment with MβCD blocks CD109's effect on ^{125}I-TGF-β internalization in HaCaT cells (fig 4.6C). The slight (and non-significant) decrease observed after MβCD treatment in EV transfected cells suggests that the internalization of TGF-β via the caveolar pathway at basal conditions is modest in HaCaT cells. This is not consistent with the report showing that about 50% of TGF-β receptors localized in caveolae in Mv1Lu cells (Di Guglielmo *et al.*, 2003). To determine whether the discrepancy is due to cell-type specific differences, the effect of CD109 on caveolae-mediated internalization of ^{125}I-TGF-β was examined in Mv1Lu cells. In this cell type, CD109 siRNA decreases ^{125}I-TGF-β internalization as expected, in the absence of MβCD (fig 4.6D). Importantly, treatment of MβCD leads to a reduction of ^{125}I-TGF-β internalization in control siRNA but not in CD109 siRNA transfected cells, indicating that, in Mv1Lu cells, a large proportion of TGF-β is internalized via the caveolae and that endogenous CD109 mediates this effect. In addition, these results support the argument that the amount of TGF-β internalizing via the caveolae varies between cell-types. Also, the difference observed in control siRNA Mv1Lu or EV HaCaT cells in presence of MβCD is due to cell type specificity because no difference was observed between EV and control siRNA transfection in the absence of MβCD (data not shown). Together, these results suggest that CD109 increases the proportion of TGF-β-bound receptors that internalize via the caveolar pathway.

4.2.5- CD109 promotes TGFBR1 compartmentalization into the caveolae in a ligand-dependent manner

TGF-β receptors have been shown to traffic independently of ligand (Di Guglielmo *et al.*, 2003; Mitchell *et al.*, 2004). Thus, we determined whether the effect of CD109 on TGFBR1 trafficking occurs in a ligand-independent manner. By sucrose gradient centrifugation, the raft fractions (fractions 5 and 6) were separated from the non-raft fractions (fractions 8 to 12). The purity of the raft and the non-raft fraction was

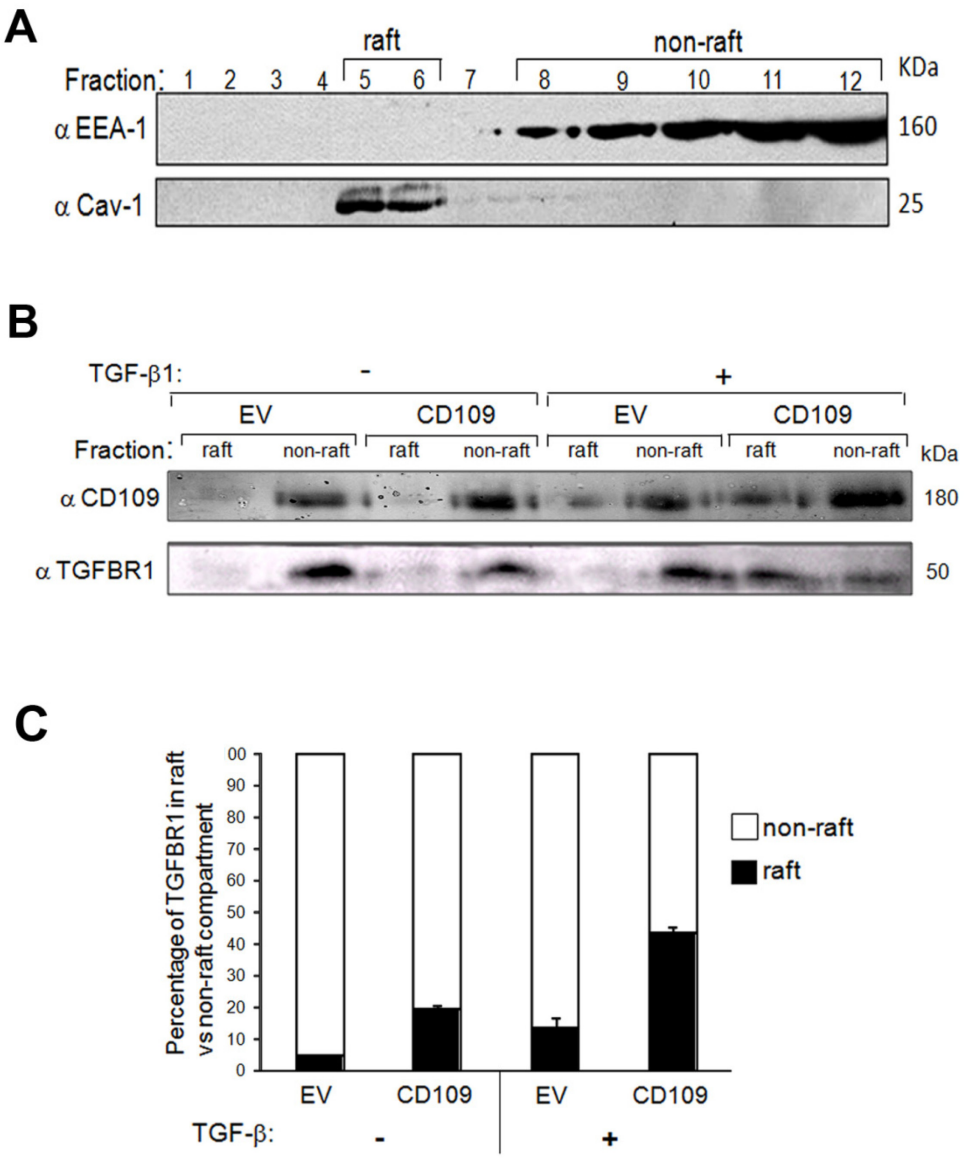


Figure 4.7: CD109 promotes TGFBR1 entry into the caveolar compartment in the presence of TGF-β. A. Lysates from HaCaT cells transfected with CD109 or EV and treated without (-) or with (+) 25 pM TGF-β1 for 30 min were subjected to sucrose gradient centrifugation. The resulting fractions were analyzed by western blot for EEA1 and caveolin-1. **B.** The pooled raft (fractions 5-6) and pooled non-raft (fractions 8-12) fractions were analyzed by western blot for CD109 and endogenous TGFBR1. A representative western blot is shown. **C.** Densitometric analysis of TGFBR1 levels of n=2 experiments.

verified using the early endosome antigen-1 (EEA-1) and caveolin-1, two markers conventionally used for the non-raft/endosomal and the raft/caveolar compartment, respectively (Di Guglielmo *et al.*, 2003; Ito *et al.*, 2004) (fig 4.7A). Analysis of TGFBR1 and CD109 localization in the pooled raft and pooled non-raft fractions was performed on the same gel by western blot (fig 4.7B). In the absence of exogenous TGF-β, TGFBR1 is present predominantly in the non-raft fraction in both CD109 and EV transfected cells. In contrast, TGF-β treatment leads to a marked increase in the amount of TGFBR1 in the raft fraction of CD109 transfected cells as compared to EV transfected cells (fig 4.7B and C). In addition, TGF-β treatment of EV transfected cells results in a modest increase in the amount of TGFBR1 in the raft fraction (fig 4.7B and C), presumably due to the presence of endogenous CD109. The low amount of TGFBR1 detected in the raft fraction in HaCaT cells is consistent with the above results in this cell type (fig 4.6C) showing that only a small amount of TGF-β-bound receptors is internalized via the lipid-raft/caveolar pathway in EV transfected HaCaT cells. Under basal conditions, CD109 localizes mainly in the non-raft compartment, but is also present in the lipid-raft compartment (visualized on a more exposed film), which agrees with the immunofluorescence data (fig 4.2A). The effect of TGF-β on the pattern of CD109 localization is similar to the one observed for TGFBR1: TGF-β treatment increases the localization of CD109 in the raft compartment, which is consistent with my co-immunoprecipitation data (fig 4.1C). Together, these results suggest that CD109 promotes TGFBR1 entry into the lipid-raft and that this effect is enhanced by TGF-β treatment.

4.2.6- Inhibition of TGF-β signalling by CD109 involves caveolae-mediated internalization

The results presented so far show that CD109 promotes TGF-β receptor internalization via the caveolae. I next investigated if this step is involved in mediating CD109's inhibitory effect on TGF-β signalling. First, to determine whether internalization is required for CD109's ability to reduce SMAD3 phosphorylation, HaCaT cells were

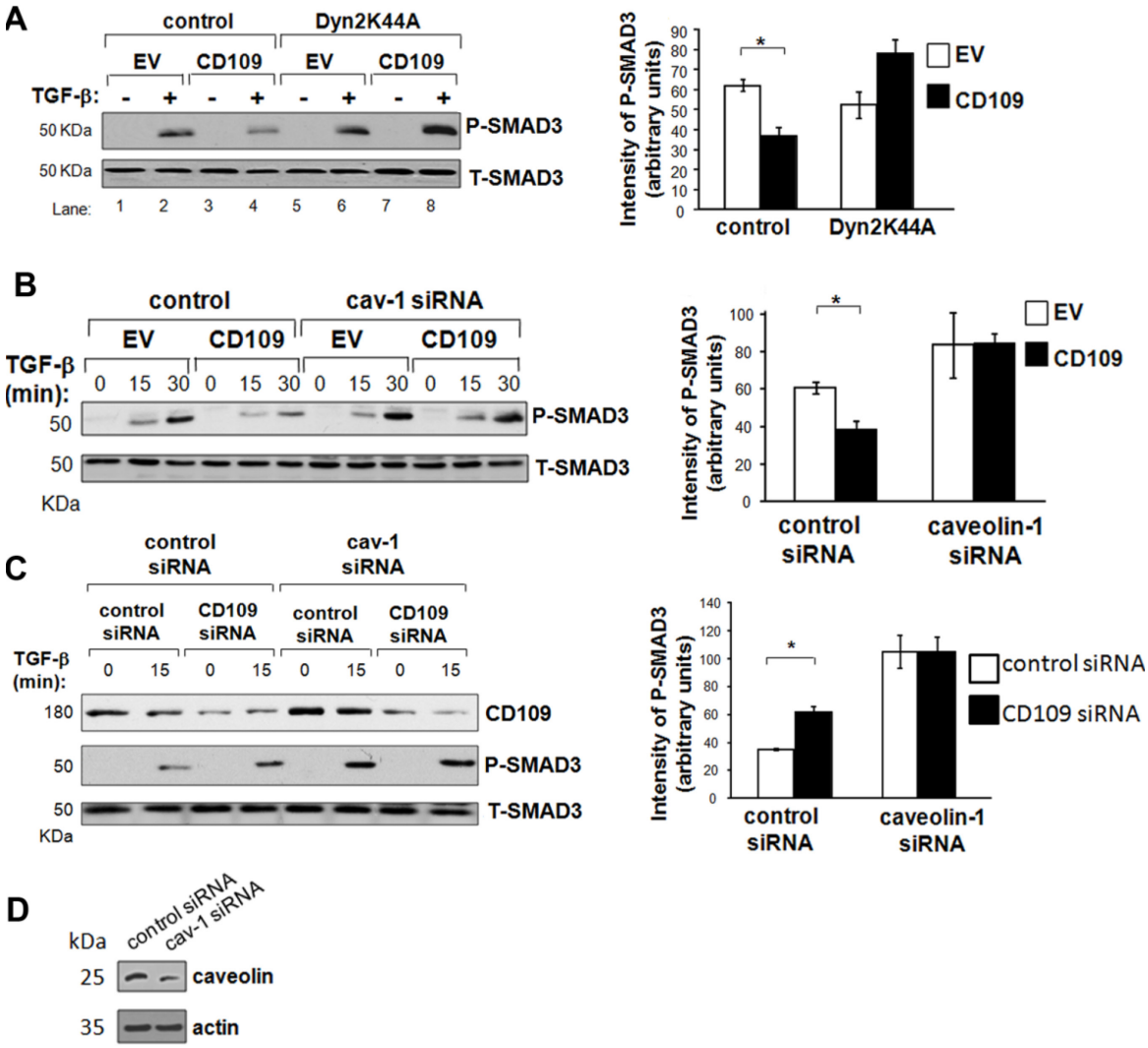


Figure 4.8: CD109's inhibition of TGF-β signaling involves internalization via the caveolar pathway. ***A-D.*** *Western Blot*: **A.** HaCaT cells transfected with CD109, Dyn2K44A or their corresponding empty vectors (EV, control), were treated for 30 min with 15pM TGF-β1. Cell lysates were analyzed by western blot for phospho-SMAD3 and total SMAD3. *Right panel*: Densitometry of P-SMAD3 (mean of n=5 independent experiments ± SEM, *p<0.05). **B.** HaCaT cells transfected with CD109, caveolin-1 siRNA or their corresponding controls were treated with 15pM TGF-β1. Cell lysates were analyzed by western blot as in (A). *Right panel*: Densitometry of P-SMAD3 after 30 min TGF-β1 treatment (mean of n=4 independent experiments ± SEM, *p<0.05). **C.** HaCaT cells transfected with CD109 siRNA, caveolin-1 siRNA or control siRNA were treated with 15pM TGF-β1 for 15 min. Cell lysates were analyzed by western blot as in (A). *Right panel*: Densitometry of P-SMAD3 (mean of n=4 independent experiments ± SEM, *p<0.05).**D.** Western blot showing the efficiency of caveolin-1 siRNA in HaCaT cells.

transfected with CD109 (or EV) and Dyn2K44A (or its control vector) (fig 4.8A). Overexpression of CD109 inhibits TGF-β-induced SMAD3 phosphorylation (fig 4.8A, lane 4 vs. 2), as shown in Chapter 3. The CD109's inhibitory effect on SMAD3 phosphorylation is abrogated by the presence of Dyn2K44A (fig 4.8A, lane 8 vs. 6), suggesting that CD109's ability to downregulate TGF-β signalling involves an internalization step. In the presence of Dyn2K44A, high levels of SMAD3 phosphorylation are still observed (fig 4.8A, lane 6 vs. 2), consistent with the notion that SMAD3 can be phosphorylated at the plasma membrane, prior to receptor internalization (Lu *et al.*, 2002; Runyan *et al.*, 2005). Importantly, CD109's inhibition of SMAD3 phosphorylation is also blocked by co-transfection of caveolin-1 siRNA (fig 4.8B), indicating that CD109' effects on TGF-β signalling involve the caveolar pathway. Similarly, knocking down CD109 expression using siRNA results in an increase in TGF-β-induced SMAD3 phosphorylation, and when caveolin-1 siRNA is co-transfected, CD109 has no effect (fig 4.8C). This result suggests that downregulation of TGF-β signalling by endogenous CD109 involves internalization via the caveolar pathway.

4.3- Discussion

Our group has previously identified CD109, a GPI-anchored protein, as a novel TGF-β co-receptor (Finnson *et al.*, 2006). CD109 has high affinity for TGF-β1, forms a complex with the TGF-β signalling receptors and negatively regulates TGF-β signalling *in vitro* (Chapter 3 and (Finnson *et al.*, 2006)). Mutation of CD109 or deregulation of its expression has been reported in many cancers (Hagiwara *et al.*, 2008; Hasegawa *et al.*, 2007; Hasegawa *et al.*, 2008; Hashimoto *et al.*, 2004; Sato *et al.*, 2007; Sjoblom *et al.*, 2006; Zhang *et al.*, 2005a). Because of CD109's potential relevance as a regulator of TGF-β action *in vivo*, I examined the mechanism by which it regulates TGF-β signalling. Here, I show that CD109 increases TGF-β binding to its receptors and promotes TGF-β receptor compartmentalization and internalization into caveolae. By this mechanism, CD109

downregulates SMAD3 signalling. In addition, I have shown that ligand addition may regulate CD109 effect on TGF-β receptor localization to lipid-raft/caveolar compartment.

Caveolae serve as platforms for signalling molecule assembly, endocytosis of receptors, and modulation of signalling (Parton and Simons, 2007). They are a subset of lipid-raft characterized by the insertion of caveolin-1 in the membrane bilayer and are enriched in GPI-anchored proteins. Here, I report that caveolin-1 associates with CD109, suggesting that CD109 can localize to caveolae. Interestingly, immunofluorescence and sucrose gradient analyses reveal that CD109 is present in both caveolar and non-caveolar compartments. Whether the non-caveolar compartment represents a storage area for CD109 or whether CD109 has other roles in that compartment remains to be determined.

The ability of CD109 to enhance the binding of TGF-β to its receptors on one hand and to inhibit TGF-β signalling on the other suggests that CD109 may direct the ligand and its receptors into a compartment where they cannot signal. The results showing that inhibitors of caveolae-mediated endocytosis block CD109's effect on TGF-β internalization suggest that CD109 facilitates TGF-β-bound receptor internalization via the caveolae, a compartment associated with TGF-β signalling downregulation and receptor degradation (Di Guglielmo *et al.*, 2003; Razani *et al.*, 2001). Accumulation of receptors in caveolae has been shown to trigger the rapid internalization via caveolae (Kiss and Botos, 2009). Thus, CD109 may facilitate receptor clustering in the presence of ligand to promote their internalization via the caveolar route. Together, this study demonstrates that activated TGF-β receptors can internalize via the caveolae and that this event is regulated by CD109. Thus, an alteration in CD109 concentration, as seen in many cancers, may lead to a change in the proportion of TGF-β receptor internalizing via caveolae, resulting in aberrant TGF-β signalling and action.

Several molecules have been implicated in the regulation of TGF-β receptor compartmentalization. Hyaluronan has been shown to modulate TGF-β receptor partitioning to caveolae (Ito *et al.*, 2004). The TGF-β co-receptors betaglycan and its homologue endoglin have been shown to facilitate TGF-β receptor endocytosis through their interaction with β-arrestin2, a component of the clathrin-coated pits (Chen *et al.*, 2003; Lee and Blobe, 2007). However, there is a discrepancy in the results reported, since internalization via the clathrin pathway leads to inhibition of TGF-β signalling in one study (Chen *et al.*, 2003), and delay in receptor degradation in another study (McLean and Di Guglielmo, 2010). The proportion of TGF-β receptors that internalize via the caveolae may vary depending on the cell type (possibly due to a difference in the levels of caveolin, receptors and/or co-receptors), as observed in the current study, and may explain the discrepancy. Here, we demonstrate that, in contrast to other TGF-β co-receptors, CD109 may regulate TGF-β receptor compartmentalization in a ligand-dependent manner.

Although the events downstream of TGF-β receptor internalization are ligand-dependent (Anders *et al.*, 1997; Kavsak *et al.*, 2000), studies on TGF-β receptor endocytosis have been unable to demonstrate an effect of ligand on TGF-β receptor compartmentalization or trafficking (Di Guglielmo *et al.*, 2003; Dore *et al.*, 2001). The results presented in this chapter reveal that ligand may play a role in CD109's effect on TGF-β receptor compartmentalization. Sucrose gradient fractionation experiments show that CD109 promotes localization of TGFBR1 into the caveolae, in the presence of TGF-β. Moreover, CD109 associates more efficiently with caveolin-1 after TGF-β stimulation. These findings are in line with the results obtained for several receptors, such as insulin, β2adrenergic and EGF receptors, which localize into caveolae in a ligand-dependent manner (Rybin *et al.*, 2000; Sigismund *et al.*, 2005; Vainio *et al.*, 2002). Although the molecular mechanisms involved in TGF-β receptor internalization are less well understood than those of the receptors above, this study provides evidence that ligand can modulate TGF-β receptor localization into caveolae in the presence of CD109.

An important finding in the present chapter is that CD109 inhibits SMAD3 phosphorylation in a caveolin-dependent manner. One potential explanation is that CD109, by promoting TGF-β receptor endocytosis into the caveolae, sequesters the receptors away from SMAD2/3. CD109 may thus inhibit TGF-β signalling. In addition, the caveolar compartment has also been shown to be associated with TGF-β receptor degradation. In the next chapter, I investigate whether CD109 could also negatively regulate TGF-β responses by promoting TGF-β receptor degradation.

Chapter 5: CD109 regulates TGF-β receptor degradation

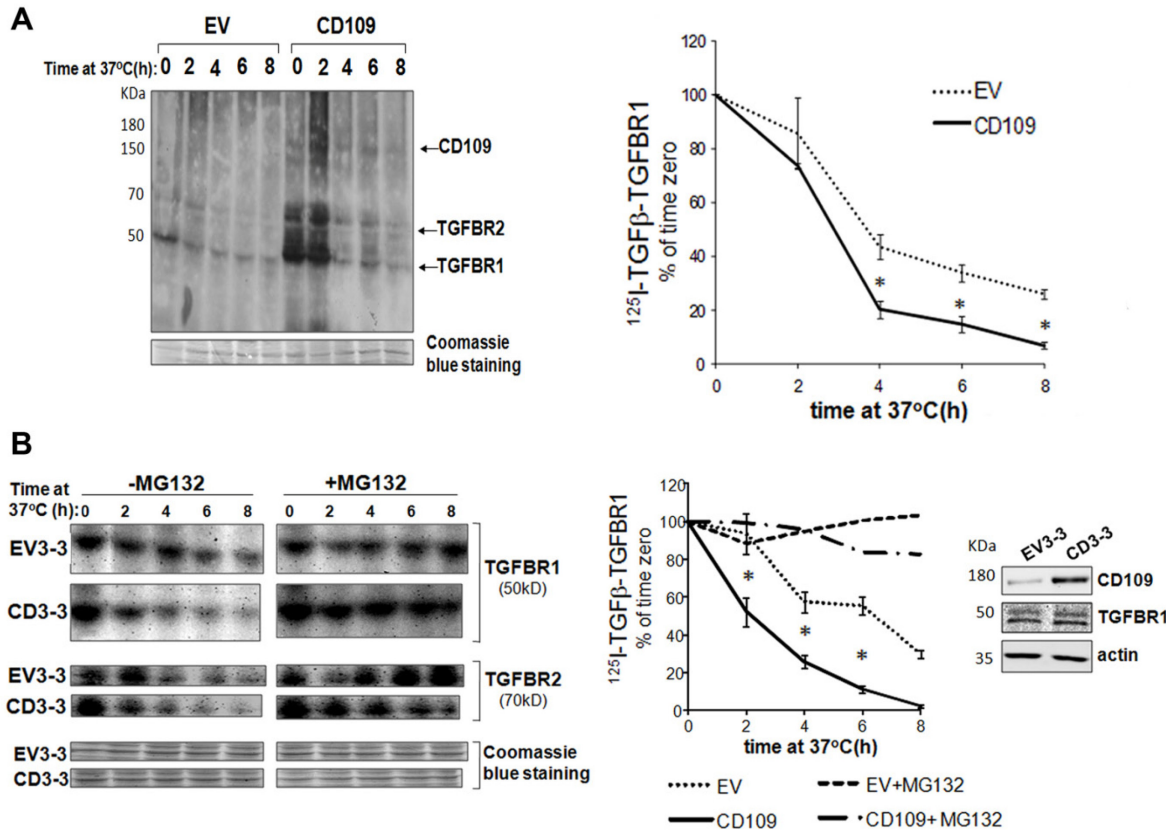
5.1- Rationale

TGF-β is implicated in many cellular processes, and dysregulation of its pathway occurs in several human diseases. TGF-β signaling is tightly regulated, notably at its receptor level. TGF-β receptors internalization via caveolae is associated with a decrease in SMAD2/3 phosphorylation and also with receptor degradation (Di Guglielmo *et al.*, 2003). SMAD7 recruits the E3 ubiquitin ligase Smurf2 to the activated TGFBR1 in caveolae, resulting in ubiquitination followed by receptor degradation and termination of signalling (Di Guglielmo *et al.*, 2003; Ebisawa *et al.*, 2001; Kavsak *et al.*, 2000).

While in most resting cells SMAD7 localizes in the nucleus, upon TGF-β stimulation or after Smurf2 overexpression, SMAD7 is exported out of the nucleus and the Smurf2/SMAD7 complex interacts with the activated TGFBR1 at the plasma membrane (Suzuki *et al.*, 2002). This localization of the SMAD7/Smurf2 complex at the plasma membrane is essential for SMAD7 inhibitory function. Thus, molecules that modulate SMAD7/Smurf2 localization could be used as potential target to regulate TGF-β signalling.

Previous results from Dr Philip's lab have previously identified a novel co-receptor, CD109, a GPI-anchored protein that negatively regulates TGF-β signalling (Chapter 3, (Finnson *et al.*, 2006)). In the previous chapter, I showed that CD109 downregulates TGF-β signalling by promoting TGF-β receptor localization into the raft/caveolae compartment. Since the caveolar compartment is associated with TGF-β receptor degradation by SMAD7/Smurf2, in this chapter, I investigate whether CD109 could modulate SMAD7/Smurf2-mediated degradation of the TGF-β receptors and whether CD109 could regulate the localization and the association of SMAD7/Smurf2 with TGFBR1.

5.2- Results


A
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Time at 37°C(h): 0 2 4 6 8 0 2 4 6 8
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EV
CD109
time at 37°C(h)
B
Time at 37°C (h):
-MG132
+MG132
EV3-3
CD3-3
TGFBR1 (50kD)
TGFBR2 (70kD)
Coomassie blue staining
EV
EV+MG132
CD109
CD109+ MG132
KDa
EV3-3
CD3-3
CD109
TGFBR1
actin
C
Time at 37°C (h): 0 4 6 8
EV 1-6
CD2-4
←TGFBR1 (50kDa)
Coomassie staining
D
DMSO
MG132
Chloroquine
CD109siRNA: - + - + - +
CD109
TGFBR1
actin
TGFBR1 levels (normalized to control siRNA + DMSO)
control siRNA
CD109 siRNA
DMSO
MG132
chloroquine

Figure 5.1: CD109 accelerates TGFBR1 proteasomal degradation. ***A-B*** *Affinity Labeling*: **A.** 293 cells transfected with CD109 or EV and TGFBR1/TGFBR2 were affinity labeled at 4^{o}C with ^{125}I-TGF-β1, which was then covalently cross-linked to the receptors, and incubated at 37^{o}C for 0 to 8hrs to allow receptor internalization. *Left panel*: the labeled receptors were visualized by autoradiography. Coomassie blue staining shows equal protein loading. *Right panel*: the amount of labeled TGFBR1 was quantified by densitometry (mean of n=4 independent experiments ± SEM, * difference between EV and CD109 groups is statistically significant: $p<0.05$). **B.** HaCaT cells stably transfected with CD109 (clone CD3-3) or EV (clone EV3-3) were affinity labeled as in (A), in the presence or absence of 20 μM MG132. *Left panel*: labeled TGFBR1 and TGFBR2 were visualized by autoradiography. Coomassie blue staining shows equal protein loading. *Middle panel*: the amount of labeled TGFBR1 was quantified by densitometry (mean of n=3 independent experiments ± SEM, *: $p<0.05$). *Right panel*: Western blot showing CD109 overexpression in CD3-3 clone, compared to EV3-3 clone. **C.** HaCaT cells stably transfected with CD109 (clone CD2-4) or EV (clone EV1-6) were affinity labeled as in (A). Labeled TGFBR1 was visualized by autoradiography. Coomassie blue staining shows equal protein loading. **D.** *Western Blot*: HaCaT cells transfected with control or CD109 siRNA were treated with 100pM TGF-β1 for 16hrs, in the presence or absence of MG132 or chloroquine. Cell lysates were analyzed by western blot using the indicated antibodies (*left panel*). *Lower panel*: Densitometry of TGFBR1 (mean of n=3 independent experiments, ± SEM, *: $p<0.05$).

5.2.1- CD109 accelerates TGF-β receptor degradation

First, I tested if CD109 modulates TGF-β receptor degradation, by monitoring the levels of affinity labeled receptors (fig 5.1A). Consistent with my previous findings, CD109 overexpression increases the binding of ^{125}I-TGF-β1 to its receptors, compared to EV transfection (see time 0). TGFBR1, TGFBR2 and CD109 levels decrease over time, suggesting that the CD109-TGF-β-bound-receptor complex is being degraded (fig 5.1A). Quantification of TGFBR1 levels by densitometry (expressed as a percentage of time 0) reveals that CD109 accelerates degradation of TGF-β-bound receptors (fig 5.1A, right panel, $p< 0.05$). In addition, stable transfection of CD109 in HaCaT cells (clone CD3-3 and CD2-4) accelerates the degradation of endogenous TGFBR1 and TGFBR2, as compared to stable transfection of EV (clone EV3-3 and EV1-6) (fig 5.1B and C, $P< 0.05$). Furthermore, the increased rate of degradation is prevented by MG132, a proteasome inhibitor, indicating that CD109 enhances proteasomal degradation of TGF-β receptors (fig 5.1B). Moreover, transfection of CD109 siRNA in TGF-β-treated HaCaT cells significantly ($p<0.05$) increases TGFBR1 levels, as compared to control siRNA transfection and this effect of CD109 can be blocked by addition of MG132, but not chloroquine, an inhibitor of lysosomal degradation (fig 5.1D). These results indicate that endogenous CD109 facilitates proteasomal degradation of TGF-β receptors and confirms that the previous results (fig 5.1A-C) were not an artifact due to CD109 overexpression.

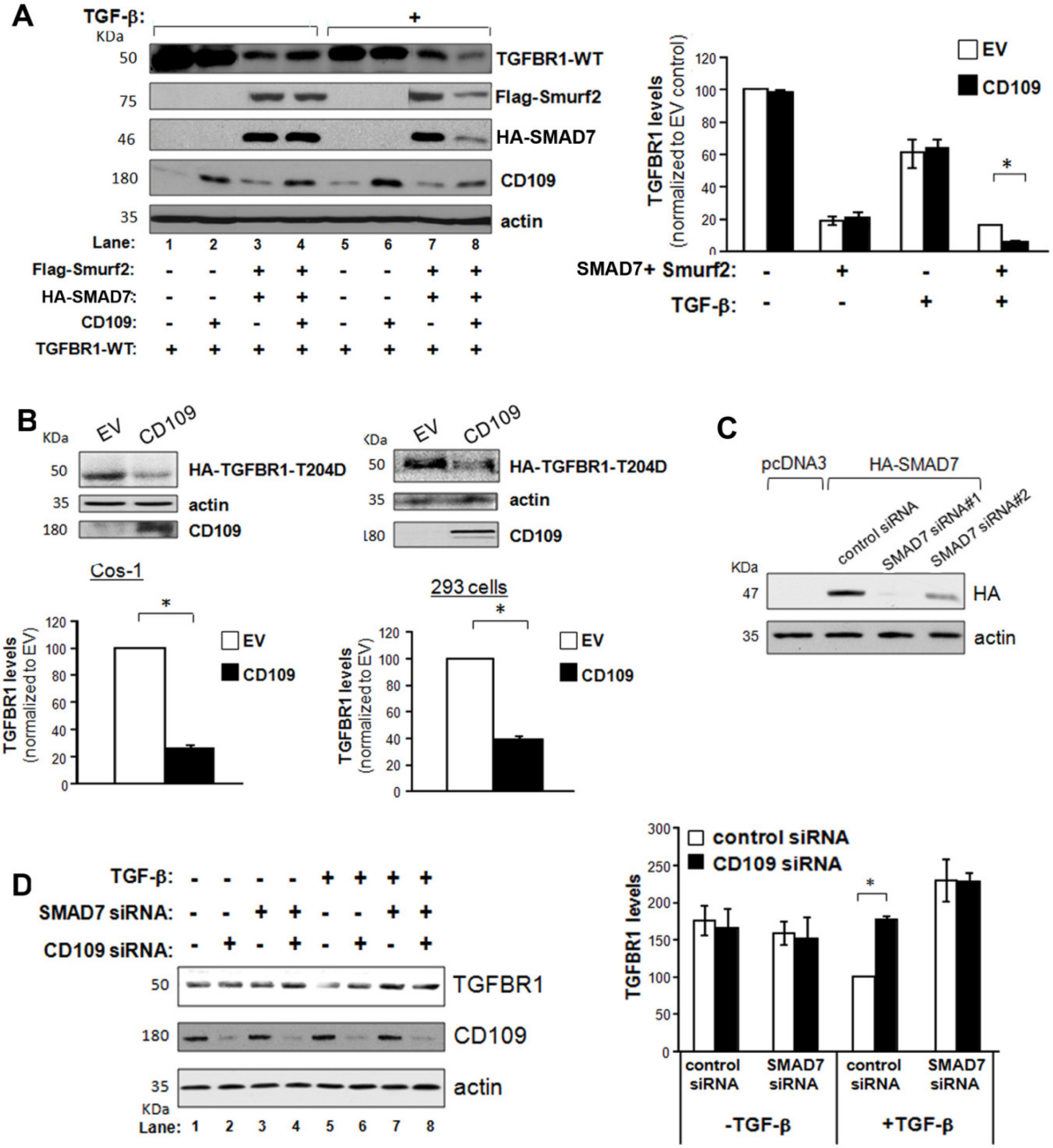
5.2.2- CD109 facilitates SMAD7/Smurf2-mediated degradation of TGFBR1 in a TGF-β-dependent manner

In the previous chapter, we have seen that CD109 promotes localization of TGF-β receptor in the caveolar compartment in a ligand-dependent manner. Therefore, I investigated whether CD109's effect on TGFBR1 degradation is ligand-dependent, as for CD109's effect on TGFBR1 caveolar localization. Moreover, since the E3 ubiquitin ligase

Smurf2 and its adaptor SMAD7 have been shown to promote TGF-β receptor degradation in caveolae, I also looked whether CD109's effect on TGFBR1 is mediated by SMAD7/Smurf2. Overexpression of Smurf2 and SMAD7 in COS-1 cells leads to a decrease in TGFBR1 levels, consistent with their known ability to induce TGFBR1 degradation (fig 5.2A, lane 3&4 vs. lane 1&2 and lane 7&8 vs. 5&6). Importantly, in the presence of TGF-β, CD109 overexpression further decreases the level of TGFBR1 when SMAD7 and Smurf2 were co-transfected (fig 5.2A, lane 8 vs. 7), suggesting that CD109 acts synergistically with SMAD7 and Smurf2 to mediate TGFBR1 degradation. However, in the absence of TGF-β, CD109 has no effect on TGFBR1 degradation (fig 5.2A, lane 4 vs. 3). Interestingly, in the presence of TGF-β, CD109 overexpression also decreases SMAD7 and Smurf2 levels. This observation is consistent with previous reports demonstrating that both SMAD7 and TGFBR1 are ubiquitinated by Smurf2 and that Smurf2 can mediate its own auto-ubiquitination (Ogunjimi *et al.*, 2005). More importantly, this result suggests that CD109 can promote the degradation of SMAD7/Smurf2/TGFBR1 complex.

I further examined the ability of CD109 to promote the degradation of an activated TGFBR1 in different cell lines. CD109 overexpression significantly decreases the level of a constitutively active TGFBR1 (TGFBR1-T204D) in the presence of SMAD7 and Smurf2 in both COS-1 and 293 cells (fig 5.2B), indicating that CD109 enhances degradation of activated TGFBR1.

In order to study the role of endogenous SMAD7 in CD109-mediated degradation of TGFBR1, I first validated the efficiency of SMAD7 siRNAs in knocking down SMAD7-HA expression in transfected 293 cells (fig 5.2C), as an anti-SMAD7 antibody only detected non-specific bands in HaCaT cells. I next investigated the role of endogenous CD109 in SMAD7/Smurf2-mediated degradation of TGFBR1 in HaCaT cells. TGFBR1 levels decrease after TGF-β addition (fig 5.2D, lane 1 vs. 5), but this decrease is not observed when CD109 expression is knock-downed (fig 5.2D, lane 5 vs. 6), suggesting that endogenous CD109 promotes TGFBR1 degradation, in a ligand-dependent manner. Moreover, CD109 has no effect on TGFBR1 levels when SMAD7 expression is knocked-down (fig 5.2D, lane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KDa
50
75
46
180
35
TGFBR1-WT
Flag-Smurf2
HA-SMAD7
CD109
actin
Lane: 1 2 3 4 5 6 7 8
Flag-Smurf2: - - + + - - + +
HA-SMAD7: - - + + - - + +
CD109: - + - + - + - +
TGFBR1-WT: + + + + + + + +
TGFBR1 levels (normalized to EV control)
EV
CD109
SMAD7+ Smurf2: - + - +
TGF-β: - - + +
B
KDa
EV
CD109
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35
180
HA-TGFBR1-T204D
actin
CD109
Cos-1
293 cells
TGFBR1 levels (normalized to EV)
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pcDNA3
HA-SMAD7
control siRNA
SMAD7 siRNA#1
SMAD7 siRNA#2
KDa
47
35
HA
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TGF-β: - - - - + + + +
SMAD7 siRNA: - - + + - - + +
CD109 siRNA: - + - + - + - +
50
180
35
TGFBR1
CD109
actin
KDa
Lane: 1 2 3 4 5 6 7 8
TGFBR1 levels
control siRNA
CD109 siRNA
control siRNA
SMAD7 siRNA
control siRNA
SMAD7 siRNA
-TGF-β
+TGF-β

Figure 5.2: CD109 increases SMAD7/Smurf2-mediated TGFBR1 degradation. **A.** Cell lysates from COS-1 cells co-transfected with the indicated cDNA and treated with or without 100 pM TGF-β1 for 90 min were analyzed by western blot using anti-TGFBR1, anti-HA, anti-Flag and anti-CD109 antibodies. *Right panel:* Densitometry of TGFBR1. **B.** Lysates from COS-1 cells and 293 cells co-transfected with HA-TGFBR1-T204D, HA-SMAD7, Flag-Smurf2 and CD109 or EV were analyzed by western blot using anti-HA, anti-CD109 and anti-actin antibodies. *Bottom panel*: Densitometry of TGFBR1. **C.** 293 cells transfected with HA-SMAD7 or its control empty vector, pcDNA3, were co-transfected with control siRNA, SMAD7 siRNA#1 or SMAD7 siRNA#2. Cell lysates were analyzed by western blot for SMAD7 expression using an anti-HA antibody. The actin blot shows equal protein loading. **D.** Western blot of lysates from HaCaT cells transfected with CD109 siRNA, SMAD7 siRNA#1 or negative control siRNA and treated with 100pM TGF-β1 for 16hrs. *Right panel*: Densitometry of TGFBR1. All experiments are representative of n=3 independent experiments,
*: $p<0.05$.

7 vs. 8 and fig 5.2), suggesting that CD109's ability to promote TGFBR1 degradation requires SMAD7 expression.

5.2.3- CD109 promotes the association of SMAD7/ Smurf2 with TGFBR1

Next, I studied the possibility that CD109 promotes TGFBR1 degradation by altering SMAD7/Smurf2 recruitment to the TGF-β receptors. In 293 cells, co-immunoprecipitation experiments reveal that the association between TGFBR1 and SMAD7 is enhanced when CD109 is overexpressed, as compared to EV transfected cells (fig 5.3A). This result indicates that CD109 may promote or stabilize the interaction between SMAD7 and TGFBR1. Moreover, both SMAD7 and CD109 are co-immunoprecipitated with TGFBR1, suggesting that CD109 may form a complex with TGFBR1 and SMAD7. Consistent with the above result, CD109 is able to associate with SMAD7 as shown by co-immunoprecipitation of CD109 with SMAD7 (fig 5.3B). In addition, a strong co-localization signal was found between CD109 (visualized *in blue*), SMAD7 (*in green*) and Smurf2 (*in red*), as demonstrated by immunofluorescence and confocal microscopy (fig 5.3C), suggesting that a complex between CD109, SMAD7 and Smurf2 is formed. Furthermore, CD109 overexpression increases the association between SMAD7 and Smurf2-WT (visualized on a high exposure film) or Smurf2-C716A (visualized on a lower exposed film), as compared to EV transfection (fig 5.3D). Collectively, these results suggest that CD109 enhances TGFBR1 degradation by promoting and/or stabilizing the association of SMAD7/Smurf2 with the TGF-β receptors.

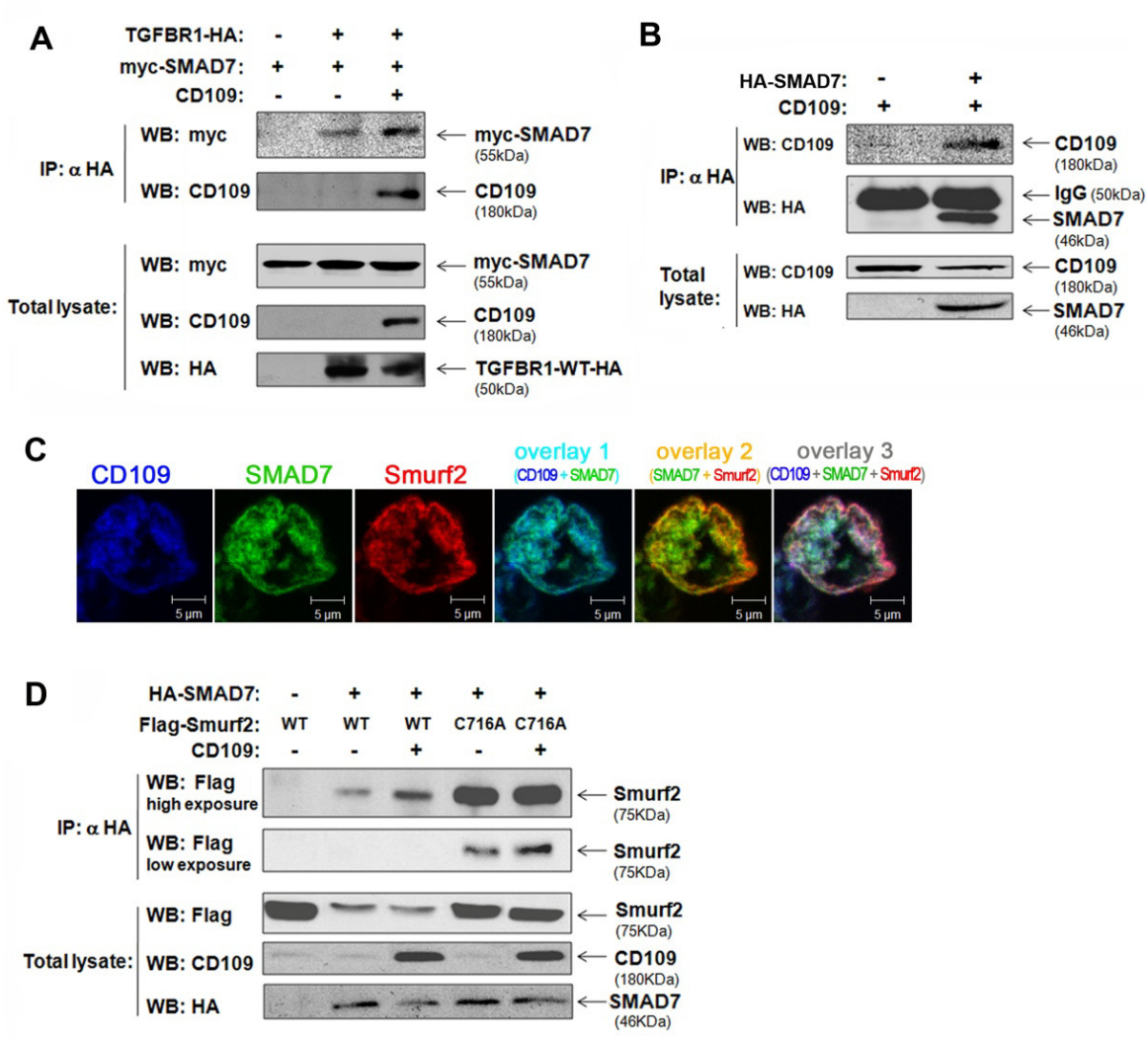


Figure 5.3: CD109 promotes the association of SMAD7/Smurf2 with TGFBR1. ***A, B and D.*** *Co-Immunoprecipitation*: Lysates from 293 cells transfected with TGFBR1/TGFBR2 and the indicated cDNAs and treated with TGF-β1 for 90 min were subjected to immunoprecipitation with an anti-HA antibody followed by western blot using the indicated antibodies. 6myc-SMAD7 run at the expected size of 55kDa (6myc tag: 10kDa + SMAD7: 46 KDa). ***C.*** *Immunofluorescence*: Transfected 293 cells were stained for CD109 (*blue*), Flag (to detect Flag-Smurf2-C716A, *in red*) and HA (to detect HA-SMAD7, *in green*) and analyzed by confocal microscopy. Co-localization of CD109 and SMAD7 appears in turquoise (overlay 1), while co-localization of SMAD7 and Smurf2 appears in yellow (overlay 2). Triple co-localization appears in white (overlay 3). A representative cell is shown.

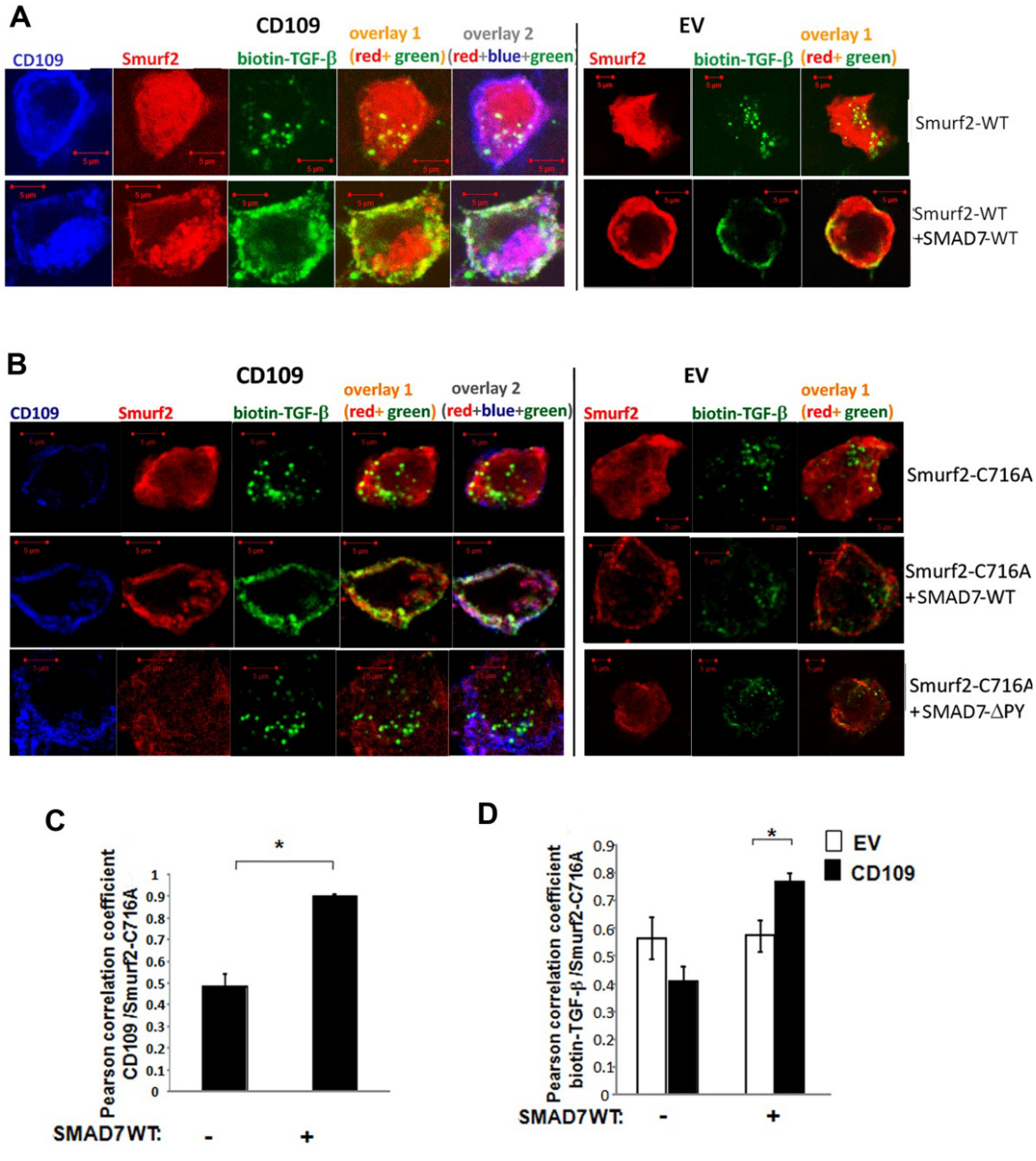

A
CD109
overlay 1
overlay 2
CD109
Smurf2
biotin-TGF-β
(red+ green)
(red+blue+green)
EV
Smurf2
biotin-TGF-β
overlay 1
(red+ green)
Smurf2-WT
Smurf2-WT
+SMAD7-WT
B
CD109
overlay 1
overlay 2
CD109
Smurf2
biotin-TGF-β
(red+ green)
(red+blue+green)
EV
Smurf2
biotin-TGF-β
overlay 1
(red+ green)
Smurf2-C716A
Smurf2-C716A
+SMAD7-WT
Smurf2-C716A
+SMAD7-ΔPY
C
Pearson correlation coefficient
CD109 /Smurf2-C716A
*
SMAD7WT:
-
+
D
Pearson correlation coefficient
biotin-TGF-β /Smurf2-C716A
*
EV
CD109
SMAD7WT:
-
+

Figure 5.4: CD109 promotes co-localization of Smurf2 with TGF-β receptors, in a SMAD7-dependent manner. A. 293 cells transfected with TGFBR1/TGFBR2, CD109 or its empty vector (EV), Flag-Smurf2-WT and HA-SMAD7-WT or its empty vector, were incubated with biotin-TGF-β1, AF647-streptavidin (*in green*) and placed at 37°C for 30min. After fixation, cells were stained for CD109 (*blue*) and Flag-Smurf2 (*red*) and analyzed by confocal microscopy. Co-localization of biotin-TGF-β1 and Flag-Smurf2-WT appears in yellow (overlay 1). Triple co-localization of Flag-Smurf2-WT, CD109 and biotin-TGF-β1 appears in white, while co-localization of CD109 and Flag-Smurf2-WT appears in purple (overlay 2). **B.** 293 cells transfected with CD109, TGFBR1/TGFBR2, Flag-Smurf2C716A, HA-SMAD7WT or HA-SMAD7-ΔPY or their corresponding empty vectors were treated and stained as in (A). The co-localization of biotin-TGF-β1 and Flag-Smurf2-C716A appears in yellow (overlay 1). The co-localization of CD109 and Flag-Smurf2-C716A appears in purple and triple co-localization of TGF-β1, Smurf2 and CD109 appears in white (overlay 2). Representative cells from n=3 independent experiments are shown. **C-D.** Quantitative analysis (using Pearson correlation coefficient) of the co-localization between CD109 and Flag-Smurf2-C716A (**C**) and between biotin-TGF-β1 and Flag-Smurf2-C716A (**D**), (mean ± SEM, *p<0.05).

5.2.4- CD109 increases co-localization Smurf2 with the TGF-β receptors, in a SMAD7-dependent manner

Because localization of SMAD7/Smurf2 to the receptors is a critical event regulating their cellular function, I next examine the ability of CD109 to affect the co-localization of Smurf2 with the TGF-β receptors in the presence or absence of SMAD7. The use of biotin-TGF-β followed by streptavidin treatment was previously validated to detect TGF-β-bound to TGFBR1/TGFBR2 in 293 cells (fig 4.3). Thus, I used this system to analyze the co-localization of CD109 (*blue*), biotin-TGF-β (*green*) and Smurf2 (*red*), in the presence or absence of SMAD7, by confocal microscopy (fig 5.4A and B). Because Smurf2-WT induces TGF-β receptor degradation, quantitative co-localization analyses were conducted with Smurf2-C716A, a mutant unable to ubiquitinate the receptors (Kavsak *et al.*, 2000). However, I did not notice any major differences between Smurf2-WT and Smurf2-C716A transfected cells in the cellular localization of Smurf2, CD109 or biotin-TGF-β (fig 5.4A versus 5.4B). As reported previously (Kavsak *et al.*, 2000; Suzuki *et al.*, 2002), transfection of SMAD7-WT induces a relocalization of Smurf2-C716A mainly at the plasma membrane (and in vesicles) where it co-localizes with biotin-TGF-β (i.e. activated receptors) (fig 5.4B, middle panel). Interestingly, overexpression of SMAD7-WT increases the co-localization of Smurf2-C716A with CD109 (fig 5.4B and C), suggesting that CD109 forms a complex with Smurf2, in the presence of SMAD7. More importantly, in the presence but not in the absence of SMAD7-WT, CD109 overexpression enhances the co-localization of Smurf2-C716A with biotin-TGF-β (fig 5.4B and D). In contrast, in EV transfected cells, or in the absence of SMAD7, biotin-TGF-β is found mainly in cytoplasmic vesicles (fig 5.4B, top and middle panel). These results are in agreement with the co-immunoprecipitation data and further suggest that CD109 promotes the association between SMAD7/Smurf2 and the activated TGF-β receptor that occurs at the plasma membrane. Interestingly, in the presence of SMAD7-ΔPY, a mutant unable to bind Smurf2, biotin-TGF-β is located in cytoplasmic vesicles in both EV and CD109 transfected cells (fig 5.4B, bottom panel), indicating that CD109's ability to enhance Smurf2/TGF-β receptor co-localization requires the interaction of SMAD7 with Smurf2.

5.2.5- Inhibition of TGF-β signalling by CD109 involves SMAD7 and Smurf2 ubiquitin ligase activity

The results presented so far show that CD109 promotes SMAD7/Smurf2-dependent degradation of TGF-β receptors. Thus, I investigated if this mechanism is involved in mediating CD109's inhibitory effect on TGF-β signalling and responses. First, I examined whether CD109's inhibitory effect on the transcriptional activity induced by a constitutively active TGFBR1 (TGFBR1-T204D) requires Smurf2 ubiquitin ligase activity using the SMAD3-responsive $(CAGA)_{12}$-lux reporter construct in 293 cells. CD109 inhibits TGFBR1-T204D-induced signalling in control (pcDNA3 co-transfected) and in the presence of Smurf2-WT, but not in the presence of Smurf2-C716A (fig 5.5A). The decreased transcriptional activity in Smurf2-WT (compared to pcDNA3 transfected cells) is likely due to an increase in TGFBR1 degradation, while the increased transcriptional activity in Smurf2-C716A (compared to pcDNA3 transfected cells) may be due to absence of TGFBR1 degradation (endogenous TGFBR1 and transfected TGFBR1-T204D).

TGF-β signalling is a potent inducer of extracellular matrix protein, such as fibronectin and PAI-1, and dysregulation of its pathway has been observed in many fibrotic disorders. Thus, I examined whether Smurf2 ubiquitin ligase activity is required for CD109 inhibitory effect on TGF-β-induced fibronectin synthesis in HaCaT cells. CD109 knock-down increases TGF-β-induced fibronectin expression in control siRNA transfected cells (fig 5.5B, lane 3 vs. 4) and in Smurf2WT (fig 5.5B, lane 5 vs. 6) but not Smurf2-C716A transfected cells (fig 5.5B, lane 7 vs. 8), indicating that endogenous CD109 dampens TGF-β responses by a mechanism that involves Smurf2 activity. In the absence of TGF-b, fibronectin was not detected in control transfected cells (fig 5.5B, lane 1 &2) and in Smurf2-WT and Smurf2-C716A transfected cells (data not shown).

In addition, transfection of CD109 siRNA leads to an increase in PAI-1 levels (fig 5.5C, lane 3 vs. 4) in control cells, but not in SMAD7 siRNA transfected cells (fig 5.5C, lane 7 vs. 8 and lane 11 vs. 12). This result suggests that CD109's inhibition of TGF-β responses requires SMAD7 expression. Altogether, my results indicate that CD109 negatively regulates TGF-β action by enhancing SMAD7/Smurf2-mediated TGFBR1 degradation.

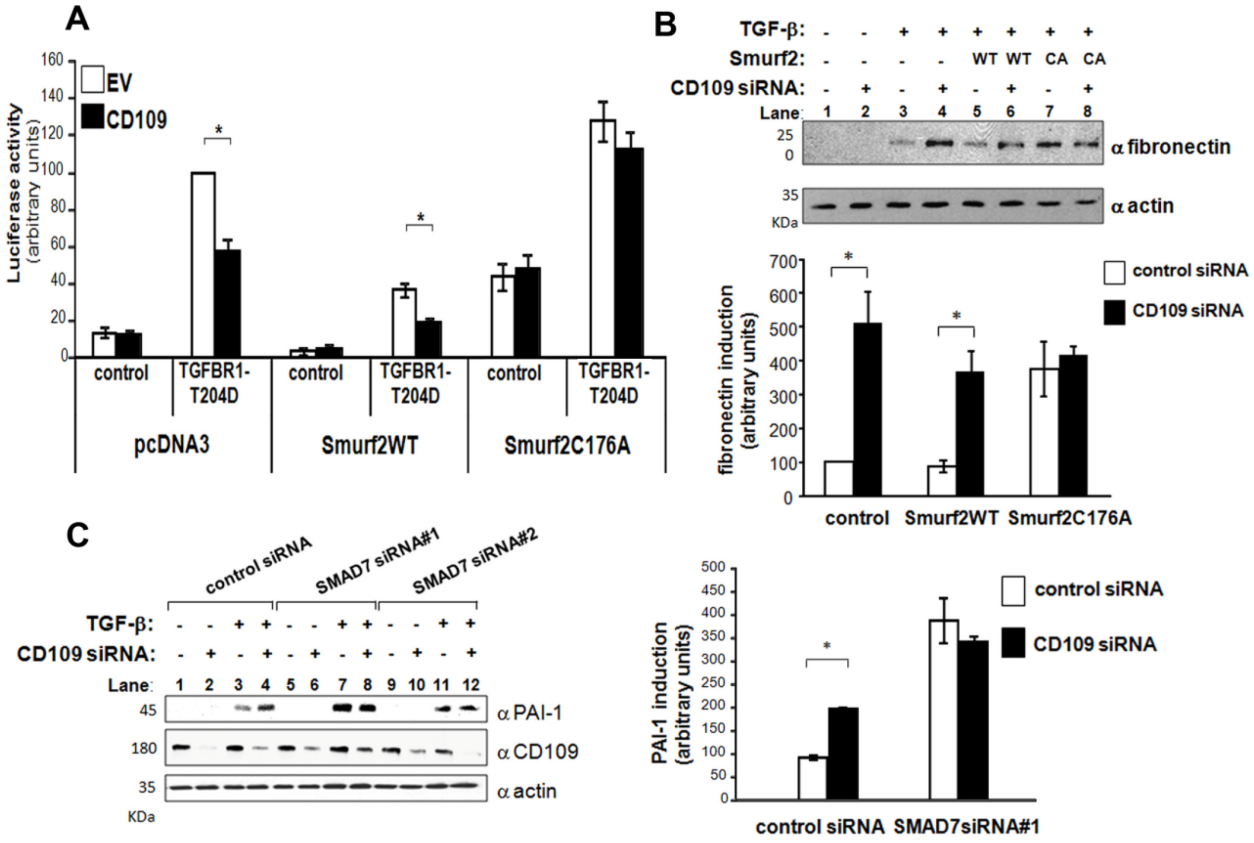


Figure 5.5: CD109's inhibition of TGF-β signalling involves SMAD7 and Smurf2 ubiquitin ligase activity. A. Cell lysates from 293 cells transfected with CD109, TGFBR1-T204D, Smurf2-WT or Smurf2-C716A or their empty vectors, $(CAGA)_{12}$-lux and β-galactosidase were analyzed for luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase and results were expressed as percentage of EV+TGFBR1-T204D control (mean of n=4 independent experiments ± SEM, * p< 0.05). **B.** Western blot of fibronectin and actin in HaCaT cells transfected with CD109 siRNA or negative control siRNA, Smurf2-WT or Smurf2-C716A and treated for 16hrs with or without 100pM TGF-β1. *Bottom panel*: Densitometry for fibronectin of n=3 independent experiments. **C.** *Left panel*: Western blot for PAI-1, CD109 and actin in HaCaT cells transfected with CD109 siRNA, SMAD7 siRNAs or negative control siRNA and treated with 100pM TGF-β1 for 16hrs. *Right panel*: Densitometry of n=3 independent experiments, * p<0.05.

5.3- Discussion

The novel TGF-β co-receptor, CD109, is a GPI-anchored protein with high affinity for the TGF-β1 subtype which forms a complex with the TGF-β signalling receptors and inhibits TGF-β signalling *in vitro* (Chapter 3 and (Finnson *et al.*, 2006)). Mutation of CD109 or deregulation of its expression occurs in many cancers (Hagiwara *et al.*, 2008; Hasegawa *et al.*, 2007; Hasegawa *et al.*, 2008; Hashimoto *et al.*, 2004; Sato *et al.*, 2007; Sjoblom *et al.*, 2006; Zhang *et al.*, 2005a), underscoring its potential relevance as a regulator of TGF-β action *in vivo.* Thus, I examined the mechanism by which it regulates TGF-β signalling. In Chapter 4, I showed that CD109 downregulates TGF-β signalling by promoting TGF-β receptor localization into the raft/caveolae compartment. Consistent with a previous report demonstrating that the caveolar compartment is associated with degradation of the TGF-β receptors by SMAD7/Smurf2 (Di Guglielmo *et al.*, 2003), I show in this chapter that CD109 inhibits TGF-β responses by enhancing TGF-β receptor degradation. My results indicate that CD109 promotes SMAD7/Smurf2-mediated degradation of TGF-β receptors. I have also shown that CD109 increases the association of SMAD7/Smurf2 with the activated TGF-β receptors and that CD109's inhibitory effect on TGF-β signalling and responses involve SMAD7 expression and Smurf2 ubiquitin ligase activity. Taken together, these results imply that CD109 negatively regulates TGF-β signalling by promoting and/or stabilizing that association of SMAD7/Smurf2 to the activated receptors, thereby enhancing their degradation.

TGF-β receptors have been shown to be degraded by both lysosomal and proteasomal machineries following receptor ubiquitination (Kavsak *et al.*, 2000; Kowanetz *et al.*, 2008). Although lysosomal degradation has been shown to be facilitated by Dapper 2 (Su *et al.*, 2007), limited data are available on factors controlling TGFBR1 proteasomal degradation. My results showing that CD109 enhancement of TGFBR1 degradation is blocked by the proteasome inhibitor MG132 but not by the lysosome inhibitor chloroquine, suggest that CD109 may regulate TGFBR1 proteasomal degradation.

In Chapter 4, I showed that CD109 facilitates the caveolar localization of TGFBR1, in a TGF-β-dependent manner. Here, I provide evidence that CD109 promotes TGFBR1 degradation also in a ligand-dependent manner. Thus, these findings reconcile TGF-β receptor compartmentalization/ trafficking with ligand-induced receptor degradation. Moreover, my results showing that CD109 decreases the levels of TGFBR1 in the presence, but not in the absence of ligand and that CD109 reduces the level of a constitutively active TGFBR1 suggest that CD109 acts preferentially on activated TGFBR1. One possible explanation is that CD109 preferentially form a complex with SMAD7 /Smurf2 and TGFBR1 when the later displays an active conformation.

Consistent with the notion that the caveolae mediates TGF-β-receptor degradation due to the presence of SMAD7 and Smurf2 in this compartment (Di Guglielmo *et al.*, 2003), I have demonstrated that CD109 enhances TGF-β receptor degradation induced by SMAD7/Smurf2. Moreover, I observe that CD109 co-localizes with Smurf2 and SMAD7, which agrees with the finding that both CD109 (fig 4.2) and SMAD7 (Di Guglielmo *et al.*, 2003) localize to caveolae. Together, these results suggest that CD109 promotes the localization of TGFBR1 to the caveolae, thereby facilitating the recruitment of SMAD7/Smurf2 to the activated receptor and its degradation.

The localization of the SMAD7/Smurf2 complex is essential for their function. Thus, CD109, by regulating the localization of SMAD7/Smurf2 to the plasma membrane (and to the TGF-β-bound receptors), may act as an important modulator of SMAD7 and Smurf2 function. By this mechanism, the localization of CD109 may control where the degradation of TGFBR1 occurs, thereby regulating specific cellular function. Thus, it is likely that an aberrant localization of CD109 may lead to mis-degradation of TGFBR1, resulting in uncontrolled TGF-β signalling.

TGF-β has been shown to induce SMAD7 expression (Nakao *et al.*, 1997), which acts in a negative feedback loop by recruiting the E3 ubiquitin ligase Smurf2 to the activated receptor, leading to TGF-β receptor degradation and termination of signalling (Kavsak *et*

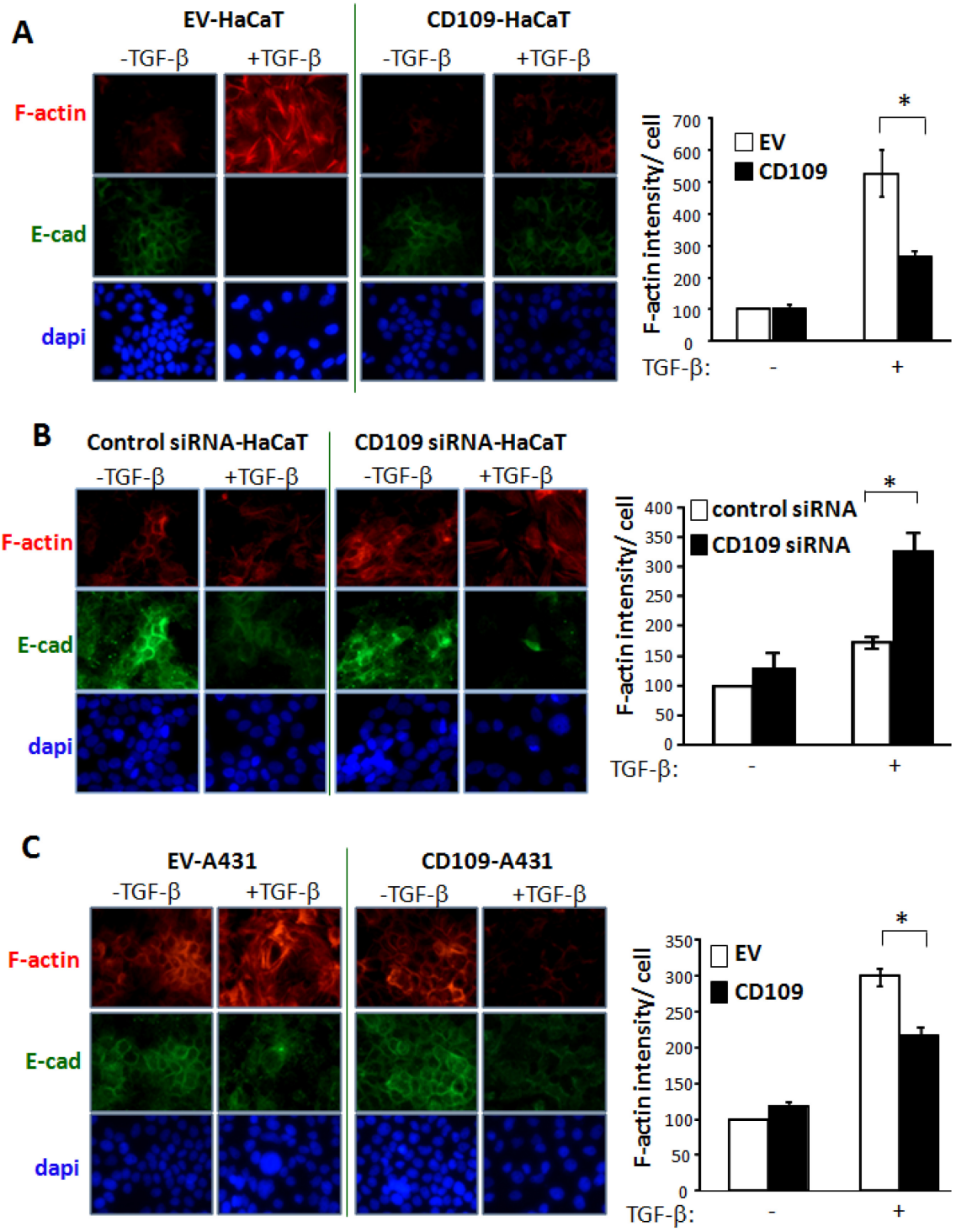
al., 2000). My findings that CD109 enhances SMAD7/Smurf2 recruitment to the TGF-β receptors and that CD109 enhances SMAD7/Smurf2-mediated degradation of the activated TGFBR1 suggest that CD109 may participate in the SMAD7 negative feedback loop to decrease the strength and duration of TGF-β signalling. Furthermore, the observation that CD109's inhibition of fibronectin and PAI-1 expression involves Smurf2 activity and SMAD7 expression indicates that CD109 can downregulate TGF-β responses by promoting TGF-β receptor degradation. Together, these results imply that CD109 has a critical role in TGF-β signal termination. CD109 may thus dampen TGF-β responses in order to avoid a deleterious effect of excess TGF-β that often leads to many human diseases such cancer metastasis and tissue fibrosis. Because an alteration in CD109 level can result in abnormal TGF-β activity, CD109 may represent a novel therapeutic target for treatment of diseases in which TGF-β is known to play a pathophysiological role.

Chapter 6: CD109 regulates TGF-β-induced epithelial-mesenchymal transition (EMT) via SMAD and MAPK pathways

6.1- Rationale

TGF-β is a potent growth inhibitor for epithelial cells and thus, acts as a tumor suppressor during the early stage of cancer. Paradoxically, TGF-β is upregulated in many malignant human tumors including squamous cell carcinomas (SCCs) (Akhurst and Derynck, 2001; Li *et al.*, 2006; Padua and Massagué, 2009). The high TGF-β level at late stages of cancer is thought to promote tumor progression, invasion and metastasis by inducing epithelial-mesenchymal transition (EMT). EMT is a process by which a polarized epithelial cell acquires a mesenchymal phenotype, characterized by an increased migratory capacity, increased production of extracellular matrix components, reorganization of the cytoskeleton and loss of the cell-cell adhesion molecule, E-cadherin (Kalluri and Weinberg, 2009).

TGF-β mediates its cellular effects by signalling through the SMAD2/3 pathway (Feng and Derynck, 2005; Schmierer and Hill, 2007). In addition to this canonical pathway, TGF-β also activates non-canonical pathways in a cell context dependent manner. For instance, TGF-β is able to modulate the ERK, p38 or JNK MAPK pathways (Feng and Derynck, 2005; Moustakas and Heldin, 2005; Zhang, 2009). The MAPK rapidly activates nuclear transcription factors to regulate numerous cellular processes, including differentiation and migration. Several studies have indicated that both SMAD and MAPK pathways contribute to TGF-β-induced EMT in keratinocytes (Zavadil, 2001, Davies, 2005). Interestingly, localization of TGF-β receptors to the lipid-raft/caveolae has been shown to be critical for TGF-β-induced MAPK activation and TGF-β-induced EMT (Zuo and Chen, 2008).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EV
CD109
TGF-β:
-
+
*
B
Control siRNA-HaCaT
CD109 siRNA-HaCaT
-TGF-β
+TGF-β
-TGF-β
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-
+
*
C
EV-A431
CD109-A431
-TGF-β
+TGF-β
-TGF-β
+TGF-β
F-actin
E-cad
dapi
F-actin intensity/ cell
EV
CD109
TGF-β:
-
+
*

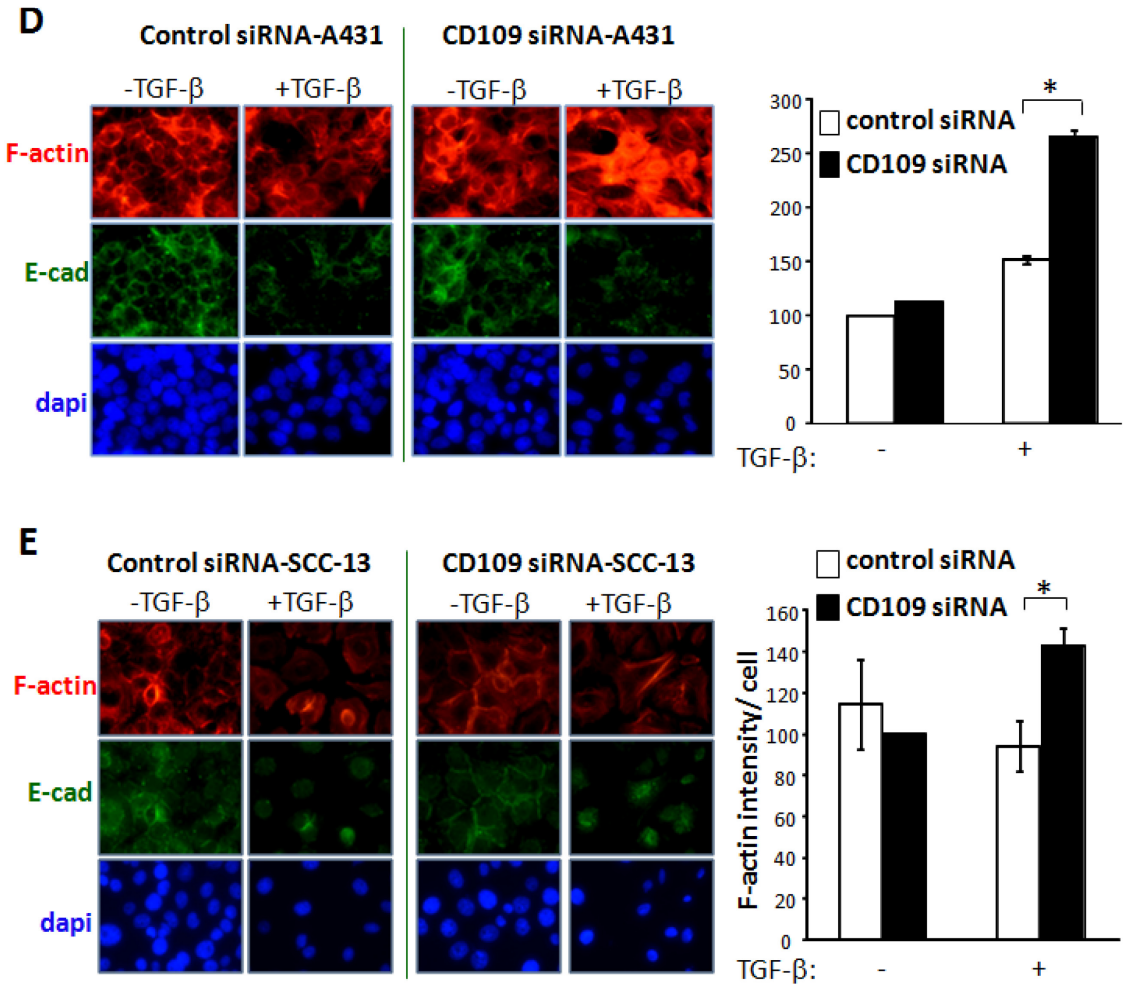


Figure 6.1: CD109 inhibits TGF-β-induced EMT. A. HaCaT stably transfected with CD109 or EV were treated with 100pM TGF-β1 for 48hrs, fixed and stained with Rhodamin-Phalloidin (stain for F-actin, *in red*), E-cadherin (*in green*) and DAPI (*blue*). *Left:* representative panels of cells are shown. *Right panel*: Densitometry of actin staining (mean of n= 4 experiments ± SEM; *: p<0.05) **B.** HaCaT cells transfected with control or CD109 siRNA were treated with 100pM TGF-β1 for 36hrs and stained as in (A). *Left:* representative panels of cells are shown. *Right panel*: Densitometry of actin staining (mean of n= 5 experiments ± SEM; *: p<0.05). **C.** A431 stably transfected with CD109 or EV were treated and stained as in (A). *Left:* representative panels of cells are shown. *Right panel*: Densitometry of actin staining (mean of n= 3 experiments ± SEM; *: p<0.05). **D.** A431 transfected with CD109 siRNA or a control siRNA were treated with 50pM TGF-β1 for 48hrs and stained as in (A). *Left:* representative panels of cells are shown. *Right panel*: Densitometry of actin staining (mean of n= 3 experiments ± SEM; *: p<0.05). **E.** SCC-13 transfected with CD109 siRNA or a control siRNA were treated and stained as in (C). *Left:* representative panels of cells are shown. *Right panel*: Densitometry of actin staining (mean of n= 3 experiments ± SEM; *: p<0.05).

Results presented in Chapter 4 show that CD109 regulates TGF-β receptor localization in caveolae. Because this compartment is associated with MAPK activation, it is of importance to examine whether CD109 regulates TGF-β-induced non-canonical pathways, in particular p38 and ERK MAPK pathways. Interestingly, CD109 expression has been shown to be elevated in premalignant lesional and well-differentiated SCCs as compared to normal epithelia, and its expression level decreases as the grade of differentiation becomes poor in SCCs (Hagiwara *et al.*, 2008). This study suggests that the reduction of CD109 levels may contribute to SCC progression. Thus, in this chapter, I have investigated whether CD109 regulates TGF-β-induced EMT in keratinocytes and in epidermoid SCCs cells and whether such regulation involves the SMAD and MAPK pathways.

6.2- Results

6.2.1- CD109 inhibits TGF-β-induced EMT in HaCaT and SCC cell lines

Because TGF-β is a potent inducer of EMT, a process involved in tumor invasion, I investigated the role of the TGF-β co-receptor CD109 in regulating TGF-β-induced EMT, by first looking at two different markers: the appearance of actin stress fibers and the disappearance of membrane E-cadherin. HaCaT cells stably overexpressing CD109 show a marked decrease in actin stress fibers formation upon TGF-β1 treatment (the actin filament appears to be more cortical) as compared to EV transfected cells (fig 6.1A). Quantification of F-actin staining demonstrates that the effect of CD109 on TGF-β-induced actin stress fibers appearance is statistically significant ($p<0.05$). In adition, HaCaT cells overexpressing CD109 also display a decrease in TGF-β-induced loss of membrane E-cadherin, as compared to EV transfected cells (fig 6.1A). Because E-cadherin is delocalized from the cell membrane to the cytoplasm upon TGF-β treatment, quantification of E-cadherin levels was not a good indicator of the EMT process.

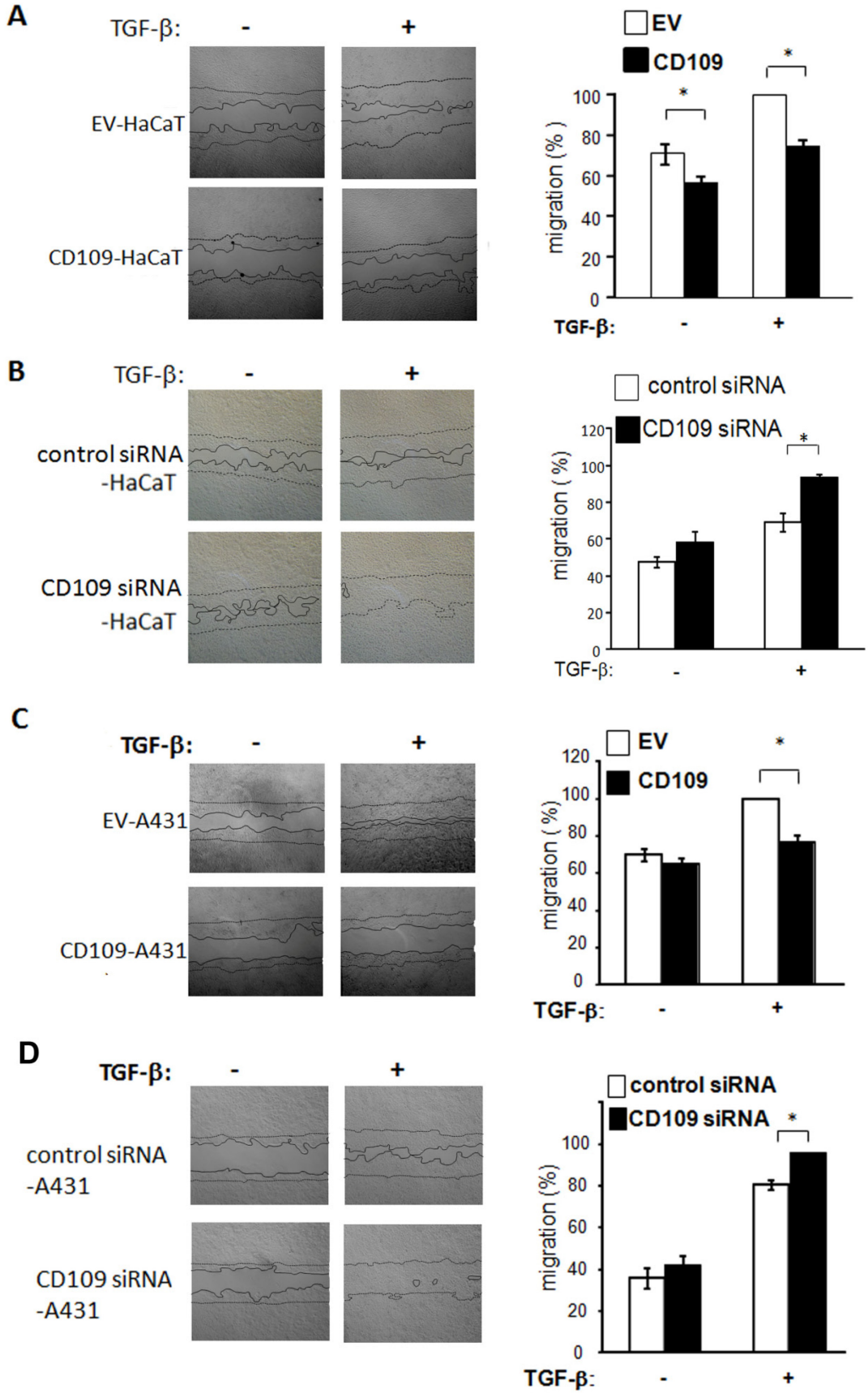
Together, these results suggest that, in human non-tumorigenic keratinocytes, CD109, when overexpressed, can inhibit some of the characteristics of TGF-β-induced EMT.

Similarly, knock-down of CD109 expression in HaCaT cells results in a significant ($p<0.05$) increase of actin stress fibers appereance and an increase of membrane E-cadherin delocalization upon TGF-b treatment, as compared to control siRNA transfected cells (fig 6.1B). This result indicates that endogenous CD109 reduces TGF-β-induced EMT.

I next examined whether CD109 inhibits TGF-β-induced EMT in tumorigenic cell lines, A431 and SCC-13, both of which are derived from epidermal squamous cell carcinomas. In the presence of 100pM TGF-β1, A431 cells stably overexpressing CD109 display less actin stress fibers and reduced relocalization of E-cadherin compared to EV transfected cells (fig 6.1C). In the absence of TGF-β, F-actin is cortical and E-cadherin localized at the plasma membrane in both EV and CD109 transfected cells (fig 6.1C). These results suggest that exogenous CD109 prevents TGF-β-induced EMT in SCC cells. I then determined whether endogenous CD109 inhibits TGF-β-induced EMT by using CD109-specific siRNA. Transfection of CD109 siRNA in A431 and SCC-13 cells results in more actin stress fibers ($p<0.05$) and a more cytoplasmic localization of E-cadherin than in control siRNA transfected cells, after 50pM TGF-β treatment (fig 6.1D and E), suggesting that endogenous CD109 is able to inhibit TGF-β-induced EMT.

6.2.2- CD109 inhibits cell migration in HaCaT and SCC cell lines

Cells undergoing EMT display enhanced migratory capacity (Kalluri and Weinberg, 2009). Thus, *in vitro* wound healing (scratch) assays were performed to evaluate the role of CD109 in regulating TGF-β-induced cell migration (Liang *et al.*, 2007). Results shown in fig 6.2 demonstrate that, in HaCaT and A431 cells, TGF-β treatment decreases the wounded area 24hrs after scratching, as compared to untreated cells, which is consistent with TGF-β's ability to promote cell migration (Muraoka *et al.*, 2002; Padua and Massagué, 2009). Importantly, HaCaT cells stably overexpressing CD109 display a less migratory phenotype, in the absence and in the presence of TGF-β, as compared to


A
TGF-β:
-
+
EV-HaCaT
CD109-HaCaT
EV
CD109
migration (%)
100
80
60
40
20
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TGF-β:
-
+
B
TGF-β:
-
+
control siRNA
-HaCaT
CD109 siRNA
-HaCaT
control siRNA
CD109 siRNA
migration (%)
120
100
80
60
40
20
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TGF-β:
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TGF-β:
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EV-A431
CD109-A431
EV
CD109
migration (%)
120
100
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control siRNA
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CD109 siRNA
-A431
control siRNA
CD109 siRNA
migration (%)
100
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TGF-β:
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Figure 6.2: CD109 inhibits cell migration. **A.** HaCaT cells stably transfected with CD109 or EV were grown until confluency and scratched with a pipette tip. The cells were treated with or without 100pM TGF-β1 for 24hrs. **B.**HaCaT cells transfected with control or CD109 siRNA were treated and scratched as in (A). **C.** A431 cells stably transfected with CD109 or EV were treated and scratched as in (**A**). **D.** A431 cells transfected with CD109 siRNA or control siRNA were treated and scratched as in (**A**). Pictures of the wounded area were taken at 0 and 24hrs and the percentage of the original wound area filled with keratinocytes were calculated. The graphs shows the mean of $n \geq 3$ independent experiments ±SEM; *: $p<0.05$

▪▪▪▪▪▪ : wound margin at 0hr, ——— : wound margin at 24hr.

EV transfected cells (fig 6.2A). This result confirms previous observation from our laboratory showing that CD109S, the shorter, placental version of CD109, inhibits migration of HaCaT cells (Finnson *et al.*, 2006). The effect of CD109 on cell migration in the absence of TGF-β treatment might be due to inhibition of autocrine TGF-β signalling. The reduction in wound gap closure observed in CD109 overexpressing cells is not the result of reduced cell growth, since CD109 enhances cell proliferation (see Chapter 3 and (Finnson *et al.*, 2006)).

In addition, knock-down of CD109 expression enhances TGF-β-induced "wound" closure, as compared to control siRNA transfected cells (fig 6.2B), indicating that endogenous CD109 is able to inhibit TGF-β-induced migration.

Moreover, CD109 overexpression inhibits TGF-β-induced migration of A431 cells (fig 6.2C) while knock-down of CD109 increases migration (fig 6.2D). Together, these results suggest that endogenous and exogenous CD109 can decrease the migratory rate of SCC cells in response to TGF-β.

6.2.3. CD109 inhibits TGF-β-induced ECM production in SCC cell lines

Because another hallmark of cells undergoing EMT is their increased production of ECM components (Kalluri and Weinberg, 2009), such as fibronectin, I evaluated CD109's ability to inhibit TGF-β-induced fibronectin expression in SCC cells. Overexpression of CD109 leads to a reduction in fibronectin production in A431 cells (fig 6.3A). Similarly, knock-down of CD109 in SCC-13 cells enhances TGF-β-induced fibronectin expression (fig 6.3B). These results are in agreement with our data obtained in HaCaT cells (fig 3.3, fig 6.6A and (Finnson *et al.*, 2006)) and demonstrate that CD109 is able to inhibit TGF-β-induced ECM production in epidermoid SCC cells.

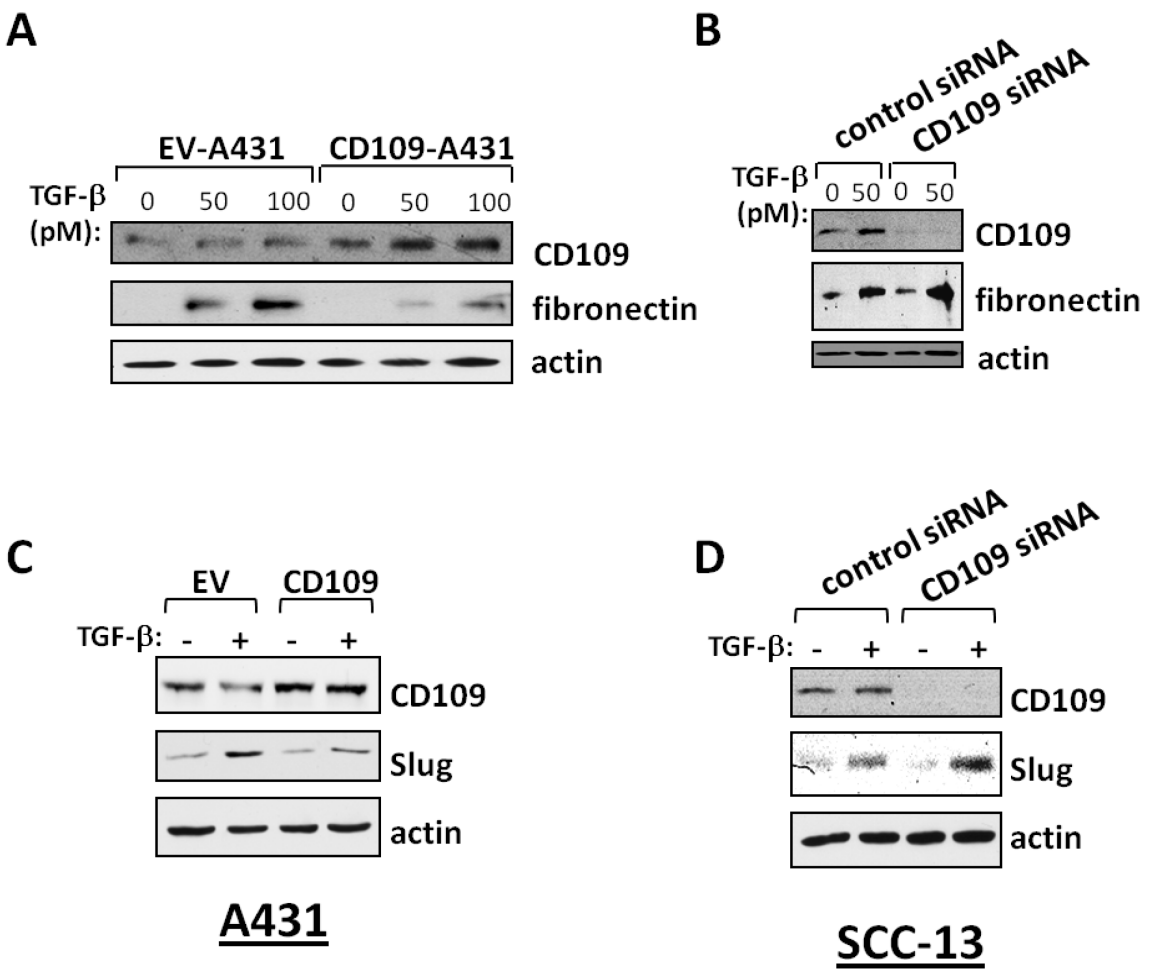

Figure 6.3: CD109 inhibits TGF-β-induced fibronectin and Slug expression in SCC cell lines. **A.** A431 cells stably transfected with CD109 or EV were treated with 0-100pM TGF-β1 for 16hrs. **B.** SCC-13 transfected with control or CD109 siRNA were treated with 50pM TGF-β1 for 5hrs. **A-B.** Cell lysates were analyzed by western blot for fibronectin expression. The actin blot demonstrates equal protein loading. **C.** A431 cells stably transfected with CD109 or EV were treated with or without 100pM TGF-β1 for 3 hrs; **D.** SCC-13 cells transfected with CD109 siRNA or a control siRNA were treated with 100pM TGF-β1 for 2hrs. **C-D.** Cell lysates were analyzed by western for Slug expression. The actin panel demonstrates equal protein loading.

6.2.4- CD109 inhibits TGF-β-induced Slug expression in SCC cell lines

Slug, a member of the Zn finger transcription factor family, initiates EMT and acts as master regulator of the EMT process (Savagner *et al.*, 1997). Slug has been shown to be a target gene of TGF-β in many cell types, including in keratinocytes (Herfs *et al.*, 2008). Thus, CD109's effect on TGF-β-induced Slug expression was examined in SCC cells. Overexpression of CD109 in A431 cells reduces the level of Slug expression, as compared to EV transfection (fig 6.3C). Similarly, CD109 siRNA transfection in SCC-13 cells increases TGF-β-induced Slug expression (fig 6.3D). Collectively, these results imply that endogenous and exogenous CD109 can reduce TGF-β-induced Slug expression in SCC cells, and this may contribute to inhibition of TGF-β-induced EMT by CD109.

6.2.5- CD109's effects on EMT involve both SMAD and MAPK pathways

Several studies indicate an important role for both SMAD and p38 and ERK MAPK pathways in TGF-β-induced EMT (Davies *et al.*, 2005; Zavadil *et al.*, 2001; Zuo and Chen, 2008). Therefore, I investigated whether CD109's ability to inhibit TGF-β-induced EMT involves the SMAD or the MAPK pathway. For this purpose, I used three different inhibitors: SB431542 is an inhibitor of the type I receptors ALK4, ALK5 and ALK7 that was shown to inhibit the SMAD pathway, but not other pathways (Inman *et al.*, 2002); U0126 is an inhibitor of MEK 1/2 and therefore of the ERK1/2 pathway, while SB203580 is an inhibitor of p38 MAPK.

First of all, the effective concentration and specificity of each inhibitor were evaluated (fig 6.4 and data not shown). Treatment of HaCaT cells with 0.5 μM SB431542 prevents TGF-β-induced SMAD2 phosphorylation, but does not affect ERK nor p38 phosphorylation. 1 μM of SB203580 prevents TGF-β-induced p38 autophosphorylation without affecting ERK or SMAD2 phosphorylation (fig 6.4). However, higher concentrations of SB203580 and SB431542 had non-specific effects (data not shown).

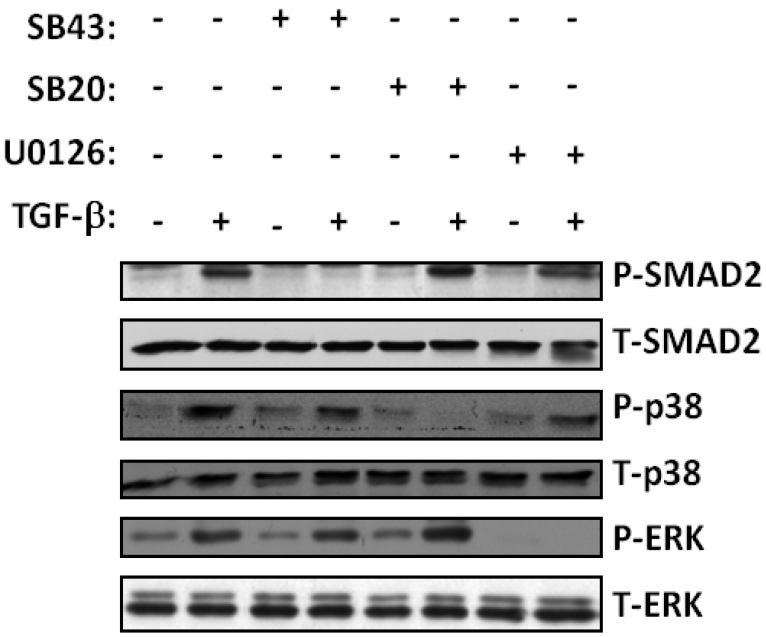

Figure 6.4: Specificity of SB431542, U0126 and SB203580 inhibitors. HaCaT cells were pre-treated for 1hr with 0.5μM SB431542 (SMAD inhibitor), 1μM SB203580 (p38 inhibitor) and/or 10μM U0126 (ERK inhibitor) and treated with 15pM TGF-β1 for 15 min. Cell lysates were analyzed for phospho-SMAD2, phospho-p38 and phospho-ERK levels by western blot. The membranes were then stripped and reprobed for total levels of the above proteins.

Finally, 10μM of U0126 inhibits ERK activation but not p38 or SMAD2 phosphorylation (fig 6.4). These results show that each inhibitor specifically blocks each respective pathway at the concentration used.

Using these concentrations of inhibitors, I examined the involvement of each pathway in CD109's ability to inhibit EMT. As expected, CD109 siRNA transfected HaCaT cells display more actin stress fibers and less membrane E-cadherin than control siRNA transfected cells (fig 6.5). Treatment with SB431542, U0126 and SB203580 had no effect in the absence of TGF-β (data not shown). Importantly, SB431542, U0126 or SB203580 treatment in the presence of TGF-β reduces (but does not abrogate) the effect mediated by CD109 siRNA transfection on actin stress fibers appearance and relocalization of E-cadherin. These findings suggest that, when CD109 levels are reduced, TGF-β induces EMT at least via the SMAD2/3, ERK and p38 pathways. In control siRNA transfected cells, only SB431542 prevents the appearance of actin stress fibers, suggesting that the SMAD2/3 pathway is the principal pathway mediating EMT in these cells. Interestingly, the combination of SB431542+ U0126, SB431542+ SB203580 or SB203580+ U0126 totally abrogates the appearance of actin stress fibers and the relocalization of membrane E-cadherin: the phenotype of the cells treated with two different inhibitors resembles the one of cells untreated with TGF-β, in both CD109 siRNA and control siRNA transfected cells, suggesting that these pathways may cooperate to promote TGF-β-induced EMT. Together, these results suggest that, when CD109 expression is reduced, TGF-β mediates its effect on EMT via both SMAD and MAPK pathways.

In addition, knock-down of CD109 increases cell migration, in the presence of TGF-β (fig 6.6). This increase is partially reversed by SB431542 and to a lesser extend by U0126 and SB203580 treatment (fig 6.6). The combination of inhibitors (SB431542+ U0126, SB431542+ SB203580 or SB203580 +U0126) totally blocks the effect of CD109 siRNA on TGF-β-induced migration (fig 6.6), suggesting once again that the MAPK pathways cross-talk with the SMAD pathway to regulate cell migration.

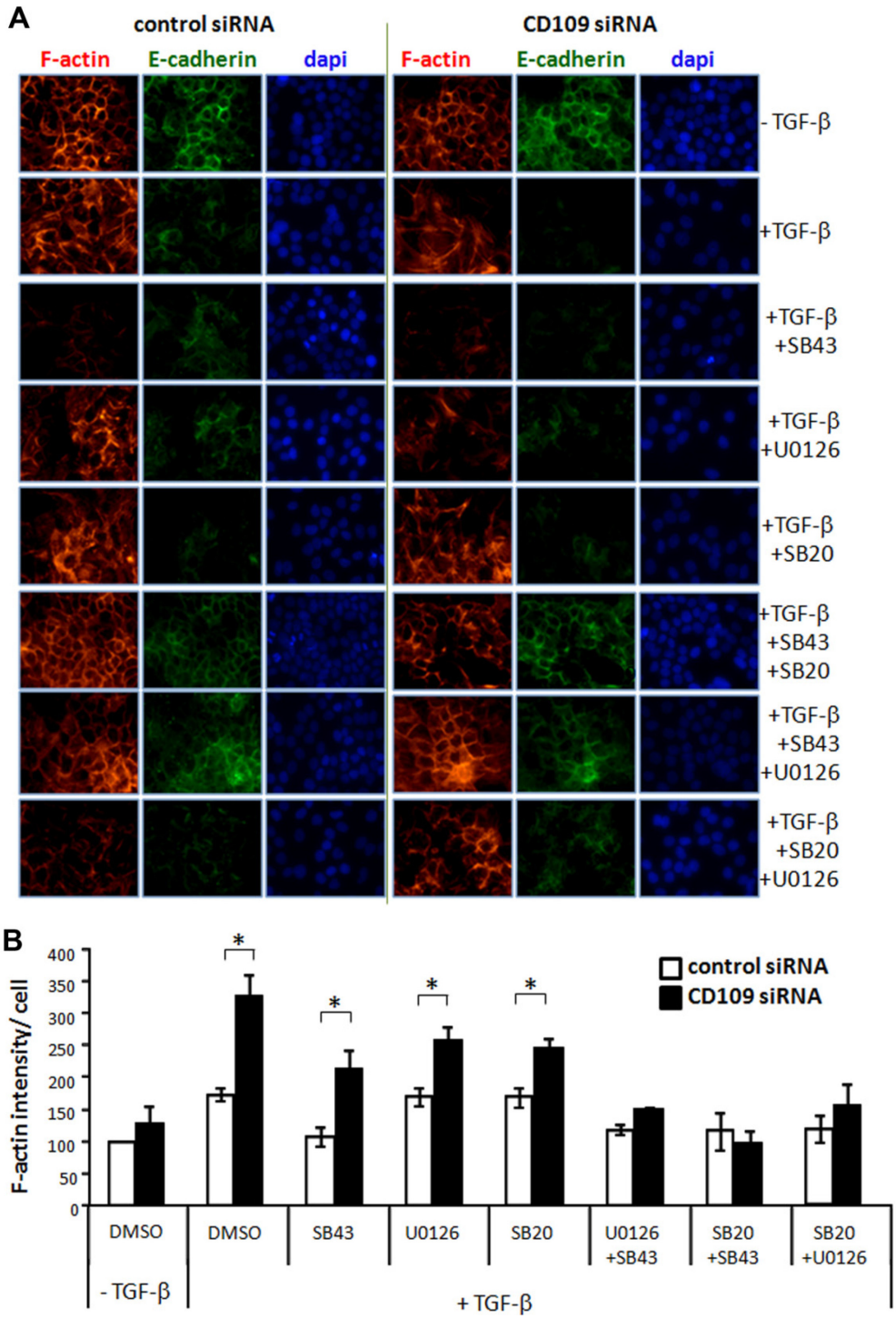

Figure 6.5: CD109 inhibits EMT via the SMAD and MAPK pathways. HaCaT cells transfected with control or CD109 siRNA were treated for 36 hrs with 100 pM TGF-β1, in the presence or absence of 0.5μM SB431542, 1μM SB203580 and/or 10μM U0126. Cells were then fixed and stained for F-actin, E-cadherin and with Dapi. **A**. Representative panels of cells are shown. **B**. Densitometry of F-actin staining (mean of n=3 independent experiments ± SEM; *P<0.05).

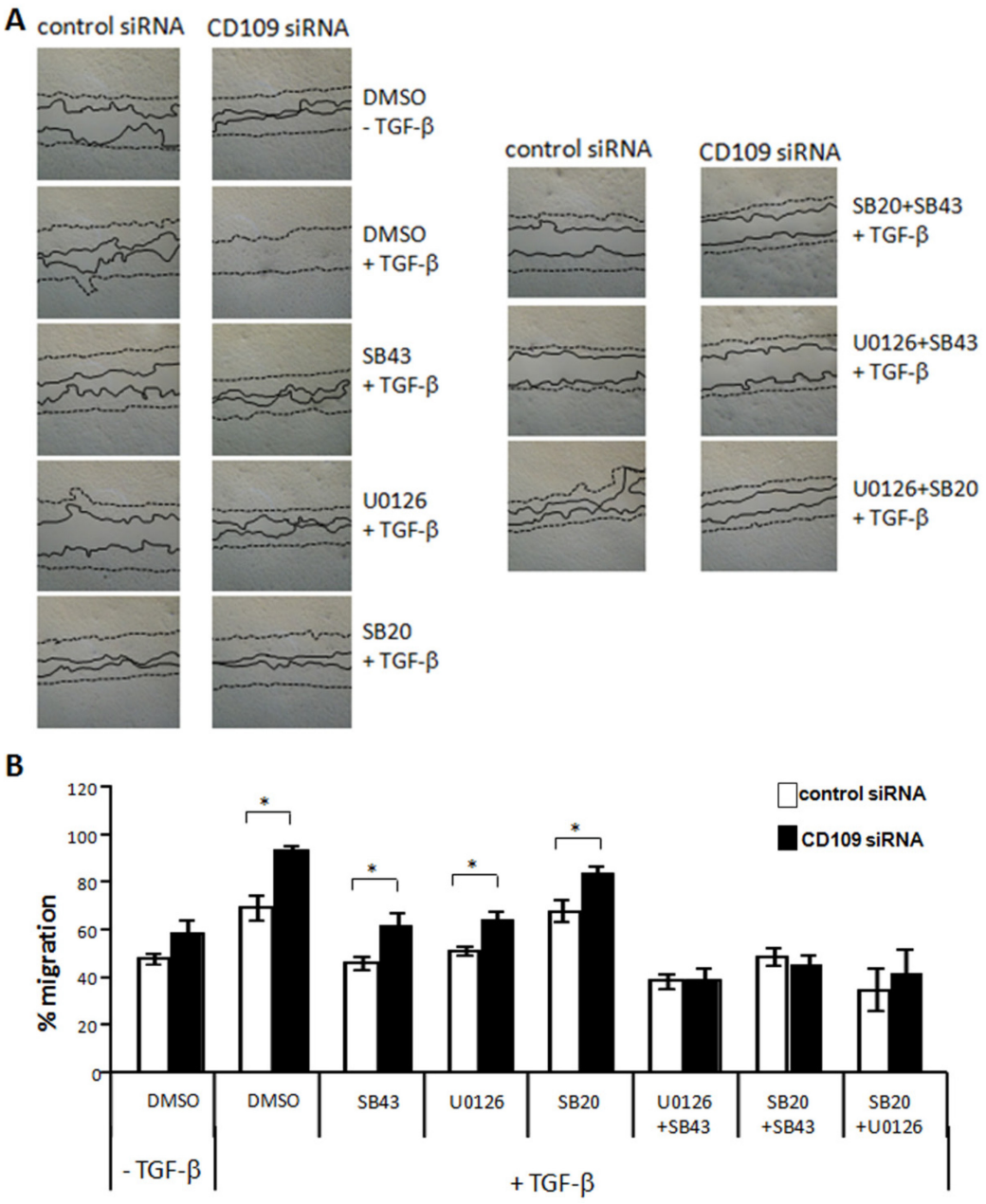

Figure 6.6: CD109 regulates migration via the SMAD and MAPK pathways. A. Scratch assay in HaCaT cells transfected with CD109 siRNA or control siRNA and treated with 100pM TGF-β1, 0.5 μM SB431542, 1μM SB203580 or 10μM U0126 for 36hrs.
····· : wound margin at 0hr, ——— : wound margin at 36hrs. **B.** The graph shows the percentage of migration (compared to wound at 0hr), *: $p<0.05$, n=5 independent experiments.

Similar results were obtained in the absence of TGF-β treatment (data not shown), suggesting that the same pathways may be involved in the regulation of autocrine TGF-β signaling by CD109. Collectively, these results suggest that CD109 negatively regulates cell migration by modulation of the SMAD and MAPK pathways.

Next, the contribution of the SMAD and MAPK pathways in CD109's effect on fibronectin expression was analyzed. Knock-down of CD109 using CD109 siRNA in HaCaT cells dramatically increases TGF-β-induced expression (fig 6.7A). Fibronectin production is totally abrogated by SB431542 treatment, suggesting that the SMAD pathway is essential for fibronectin expression. In these cells with CD109 knock-downed, SB203580 and U0126 also decrease fibronectin production, suggesting that p38 and ERK also participate although to a much lesser extent than the SMAD pathway (fig 6.7A).

Finally, I investigated the involvement of SMAD and MAPK pathways on CD109's regulation of TGF-β-induced Slug expression. As in SCC cell lines, transfection of CD109 siRNA in HaCaT cells leads to an increase in TGF-β-induced Slug expression (Fig 6.7B). This increase is abrogated after inhibition of SMAD and ERK pathway, using SB431542 and U0126, respectively, suggesting that SMAD2/3 and ERK1/2 contribute to the elevation of Slug expression mediated by CD109 siRNA (Fig 6.7B). Surprisingly, SB203580 treatment increases Slug expression in the absence (data not shown) and in the presence of TGF-β, in HaCaT cells transfected with control and CD109 siRNA (fig 6.7B), suggesting that p38 may inhibit Slug expression in HaCaT cells.

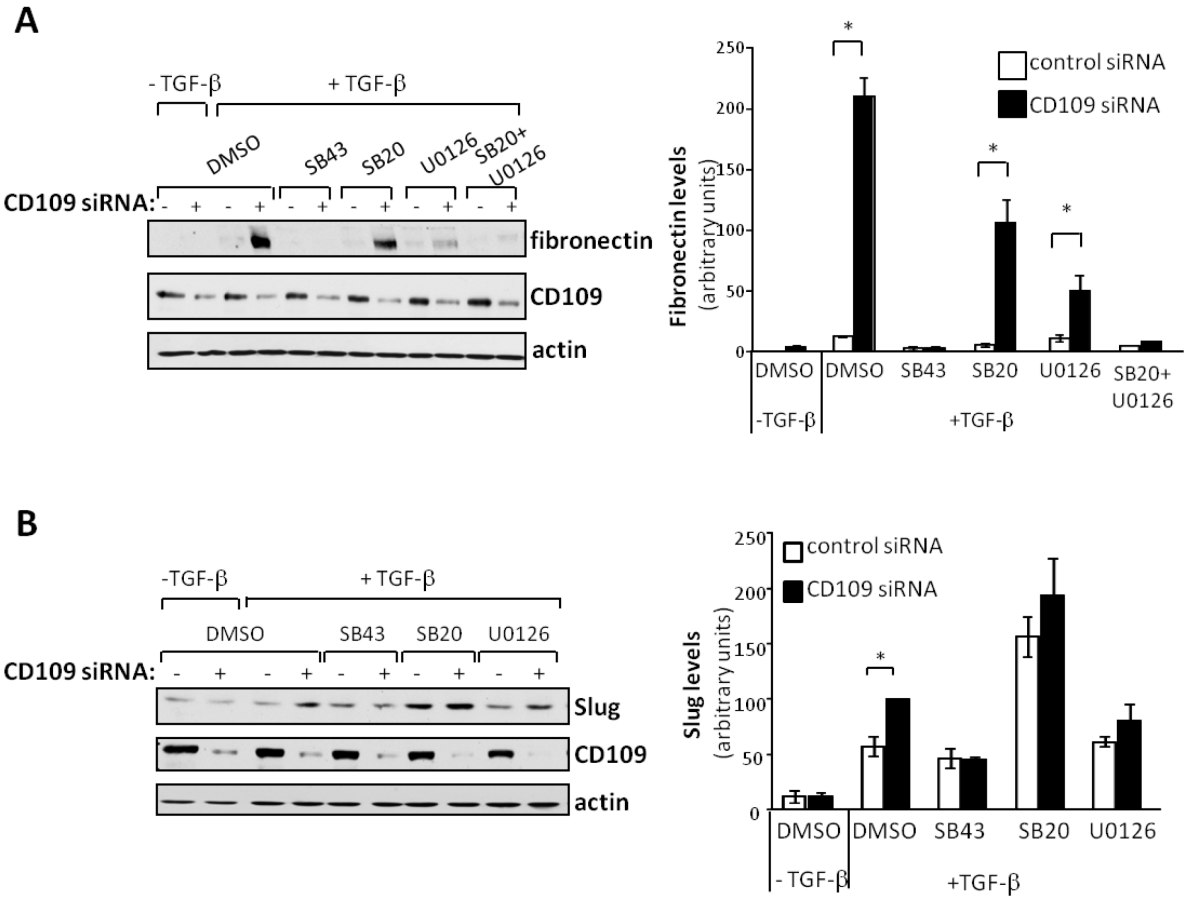

Figure 6.7: CD109 inhibits fibronectin and Slug expression via the SMAD and MAPK pathways. A. HaCaT cells transfected with control or CD109 siRNA were treated for 16hrs with or without 100pM TGF-β1, in the presence or absence of 0.5 μM SB431542, 1μM SB203580 or 10μM U0126. Cell lysates were analyzed by western blot using the indicated antibodies. *Right panel*: Densitometry of fibronectin (mean of n=3 independent experiments, ± SEM; *: $p<0.05$).**B.** HaCaT cells transfected with control or CD109 siRNA were treated with 100pM TGF-β1 for 4hrs, in the presence of absence of SB431542, SB203580 and/or U0126 and analyzed by western blot for Slug expression. *Right panel*: Densitometry of n=4 independent experiments, ± SEM; *: $p<0.05$.

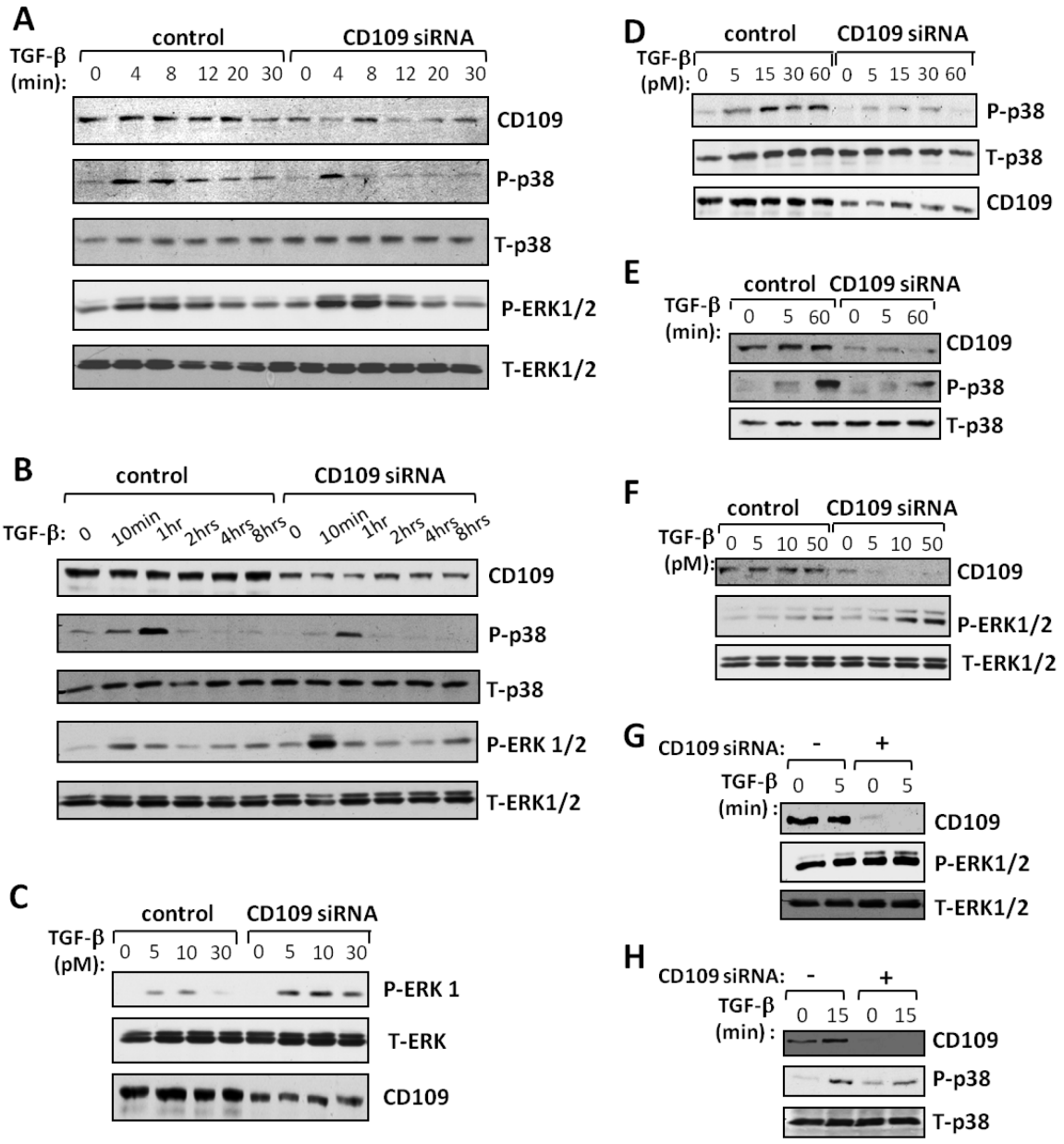

Figure 6.8: CD109 modulates TGF-β-induced p38 and ERK1/2 phosphorylation. **A-B.** HaCaT cells transfected with CD109 siRNA or control siRNA were treated with 15pM TGF-β1 for the indicated time. **C-D.** HaCaT cells transfected with CD109 siRNA or control siRNA were treated with 0-60pM TGF-β1 for 10 min. **E-F.** A431 cells transfected with control or CD109 siRNA were treated with 15pM TGF-β1 for the indicated times (**E**) or with 0-50pM TGF-β for 10 min (**F**). **G-H.** SCC-13 transfected with control or CD109 siRNA were treated with 15pM TGF-β1. All cell lysates were analyzed by western blot for phospho-ERK1/2, total ERK1/2, phospho-p38, total p38 or CD109.

6.2.6- CD109 modulates TGF-β-induced MAPK phosphorylation

The results presented so far suggest that CD109 could not only negatively regulate SMAD2/3 signalling in keratinocytes, but also could modulate non-canonical pathways, notably p38 and ERK1/2 MAPK pathways. Thus, CD109's ability to regulate TGF-β-induced p38 and ERK1/2 phosphorylation was investigated. Treatment of HaCaT cells with 15pM of TGF-β1 results in rapid phosphorylation of p38 and ERK1/2 (fig 6.8A and B). The rapid activation of p38 and ERK upon TGF-β treatment suggests that it occurs independently of the SMAD. Importantly, knock-down of CD109 using siRNA (or shRNA, data not shown) leads to a decrease in TGF-β-induced p38 phosphorylation and an increase in TGF-β-induced ERK1/2 phosphorylation, especially at early (4min to 1hr) time points (fig 6.8 A and B). In addition, examination of TGF-β dose response demonstrates that CD109 knock-down increases ERK phosphorylation (fig 6.8C) and decreases p38 phosphorylation (fig 6.8D) at all doses of TGF-β tested, as compared to control siRNA transfection. No change in the levels of total ERK and total p38 were observed. Together, these results suggest that endogenous CD109 is able to promote TGF-β-induced p38 activation while inhibiting ERK activation in HaCaT cells.

Next, I studied CD109's ability to modulate TGF-β-induced MAPK pathways in SCC cell lines. Similar to what was observed in HaCaT cells, CD109 siRNA transfection reduces TGF-β-induced p38 phosphorylation and enhances TGF-β-induced ERK phosphorylation in both A431 (fig 6.8 E and F) and SCC-13 cells (fig 6.8 G and H), as compared to control siRNA transfection. Collectively, these results suggest that CD109 inhibits the TGF-β-induced SMAD2/3 (Chapter 3) and ERK1/2 pathways, but promotes TGF-β-induced p38 MAPK pathway in human keratinocytes and in SCC cell lines.

6.2.7- CD109's effects on TGF-β-induced MAPK activation involve the caveolae

In Chapter 4, I have shown that CD109 promotes the localization of the TGF-β receptors to the caveolae. Because localization of the TGF-β receptors to the caveolar

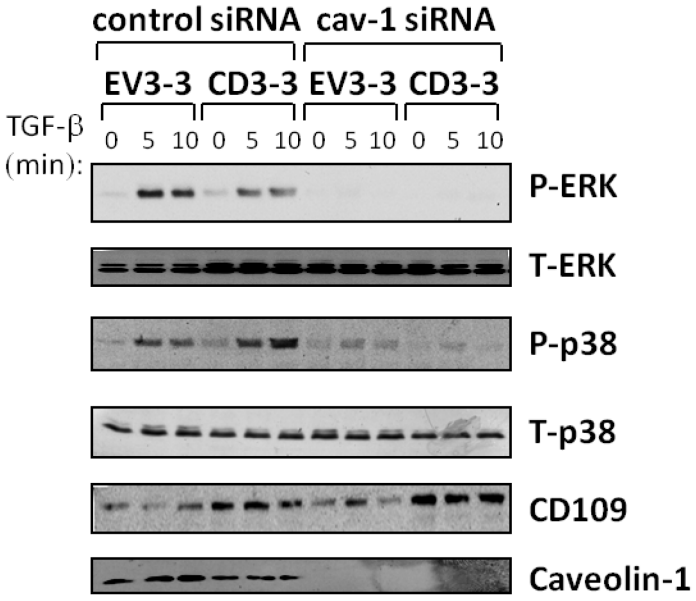

Figure 6.9: CD109's effects on TGF-β-induced MAPK activation involve the caveolae. HaCaT cells stably transfected with CD109 (clone CD3-3) or its empty vector (clone EV3-3) were transiently transfected with caveolin-1 siRNA or negative control siRNA. The cells were then treated with or without 15pM TGF-β1 for 5-10 min. Cell lysates were analyzed by Western blot with the indicated antibodies.

compartment has been implicated in MAPK activation (Zuo and Chen, 2008), I investigated whether CD109's ability to regulate TGF-β-induced p38 and ERK phosphorylation requires the caveolar pathway. Consistent with the above results, overexpression of CD109 in HaCaT cells leads to a reduction in TGF-β-induced ERK phosphorylation and an increase in TGF-β-induced p38 phosphorylation, as compared to EV transfected cells (fig 6.9). Importantly, the effect of CD109 on ERK and p38 activation is abrogated by caveolin-1 siRNA transfection (fig 6.9), suggesting that CD109, by promoting TGF-β receptor localization to the caveolae, is able to regulate TGF-β-induced MAPK activation.

6.3- Discussion

CD109 is a GPI-anchored protein whose expression is deregulated in many cancers, including in squamous cell carcinomas (SCC) (Finnson *et al.*, 2006; Hagiwara *et al.*, 2008; Hasegawa *et al.*, 2007; Hasegawa *et al.*, 2008; Hashimoto *et al.*, 2004; Sato *et al.*, 2007; Zhang *et al.*, 2005a). Our group has previously shown that CD109 is a novel TGF-β co-receptor that binds to TGF-β1 with high affinity, forms a heteromeric complex with the TGF-β signalling receptors and negatively regulates SMAD2/3 pathway (Finnson *et al.*, 2006). In addition, I showed that CD109 promotes the localization of TGF-β receptors in caveolae and enhances their degradation (Chapter 4 and 5). Because TGF-β is a potent inducer of EMT, a program leading to the escape of cancer cells from solid tumors to form metastasis (Miyazono, 2009; Padua and Massagué, 2009), I sought to determine whether CD109 affects TGF-β-induced EMT in SCCs-derived cell lines. In this study, I demonstrated that CD109 negatively regulates several key features of EMT: in the presence of TGF-β, CD109 inhibits the appearance of actin stress fibers, the relocalization of cell-cell adhesion molecule E-cadherin and cell migration. Moreover, CD109 inhibits TGF-β-induced ECM production, as evidenced by lower fibronectin expression and decreases TGF-β-induced expression of the transcription factor and initiator of EMT, Slug, in both HaCaT and SCC derived cell lines. Using specific inhibitors, I

also showed that CD109 mediates its diverse effects on TGF-β-induced EMT via both the SMAD and p38 and ERK1/2 MAPK pathways. Finally, my results indicate that CD109 is able to increase TGF-β-induced p38 phosphorylation, while reducing TGF-β-induced ERK1/2 activation in HaCaT and SCC-derived cells, probably by targeting the receptor localization to the caveolae. I conclude that CD109 regulates both TGF-β canonical and non-canonical signalling pathways to inhibit TGF-β-induced EMT.

My results suggest that, in keratinocytes, inhibition of TGF-β-induced EMT by CD109 involves ERK1/2, p38 MAPK and SMAD pathways in human keratinocytes. Interestingly, the contribution of each pathway differs for the cellular responses analyzed in the current study. For instance, SMAD inhibition totally abrogates the effect of CD109 on TGF-β-induced fibronectin expression but only partially blocks cell migration and actin stress fibers formation. Also, slug expression is mediated by SMAD and ERK but is inhibited by p38. My findings that SMADs, ERK and to a lesser extent p38 pathways, contribute to TGF-β-induced actin stress fiber appearance, E-cadherin relocalization, cell migration and fibronectin expression in HaCaT is consistent with previous studies showing that SMADs and MAPKs mediate several features of the EMT in HaCaT cells (Zavadil *et al.*, 2001; Zuo and Chen, 2008). However, other studies have demonstrated that the contribution of each pathway in mediating TGF-β-induced EMT differs between cell types. For example, in NMuMG cells, p38 is required but ERK is not involved in TGF-β-mediated EMT (Bakin *et al.*, 2002), p38 is essential for TGF-β-induced fibronectin expression in fetal lung fibroblasts (Kucich *et al.*, 2000) and Slug expression is mediated by ERK but not by SMAD or p38 in lens epithelial cells (Choi *et al.*, 2007). Altogether, these findings further emphasize that the control of EMT by TGF-β involves different signalling pathways in a cell-type dependent manner and that CD109 differentially regulates these pathways to inhibit TGF-β-induced EMT.

Here, I have provided evidence that CD109 reduces TGF-β-induced SMAD and ERK1/2 phosphorylation, but increases p38 phosphorylation levels. CD109-mediated reduction

of ERK1/2 activation levels and CD109's ability to inhibit EMT are consistent with ERK's involvement in cytoskeleton remodelling, disassembly of cell adherens junction and cell motility (Boutros *et al.*, 2008; Krishna and Narang, 2008; Pullikuth and Catling, 2007). The function of ERK and p38 MAPK highly depends on the duration, strength of the signal and on their location (Boutros *et al.*, 2008; Krishna and Narang, 2008). Consistent with previous results shown in Chapter 4 demonstrating that CD109 promotes the localization of TGF-β receptors into lipid-raft/caveolae and that TGF-β-induced MAPK activation occurs in lipid-raft (Zuo and Chen, 2008), fig 6.9 suggests that CD109's activation of p38 and downregulation of ERK may occur in caveolae. It is thus possible that the function of these MAPKs in caveolae differs from their function in other cell compartments, as they may not interact with the same partners. The precise mechanisms by which CD109 modulates TGF-β-induced MAPK activation in caveolae remain to be determined.

My results suggest that p38 activation decreases Slug expression, thus CD109, by increasing TGF-β-induced p38 activation may further decrease Slug expression. While inhibition of p38 pathway increases Slug expression, it is not associated with a reduction of E-cadherin expression, as evidence by immunofluorescence. One possible explanation is that p38 pathway inhibits Slug expression, but differentially affects other transcription factors, such as Snail and Twist, whose function are known to overlap with Slug's (Moreno-Bueno *et al.*, 2006). Interestingly, inhibition of the p38 pathway has only a minor effect on actin stress fibers appearance, E-cadherin relocalization, cell migration and fibronectin expression. This suggests that the increased activation of p38 by CD109 is not directly involved in CD109's inhibition of EMT. In contrast, the effect of CD109 was totally abrogated when SB203580 was used in combination with SMAD or ERK inhibitor, indicating the existence of a cross-talk between the different pathways. Indeed, MAPK can phosphorylate the SMAD on their linker region to regulate their transcriptional activity. MAPK can also activate transcription factors that synergize or antagonize SMADs function (Guo and Wang, 2009; Javelaud and Mauviel, 2005). Thus, CD109 may

activate p38 MAPK pathway, which may decrease Slug expression, and may modulate SMAD activity in order to downregulate TGF-β-induced EMT.

Results from this chapter indicate that CD109 inhibits several features of EMT induced by TGF-β. The ability of TGF-β to mediate an EMT-like phenotype (or partial EMT) in HaCaT cells has been reported previously (Lee *et al.*, 2004; Zavadil *et al.*, 2001). However, it has been shown that, in addition to TGF-β treatment, Ras activation is required in order to obtain a more mesenchymal phenotype (Davies *et al.*, 2005), indicating that synergy between different pathways is necessary for the cells to undergo a full EMT during cancer progression. Although in the presence of TGF-β alone, HaCaT cells do not undergo a full EMT but rather a partial EMT, any changes in cell migration are likely to be of importance during cancer invasion and metastasis (Akhurst, 2008). Thus, the finding that manipulation of CD109 expression levels leads to changes in migration of SCC cells may be of critical importance in cancer progression. In agreement with this notion, it has recently been reported that CD109 expression is elevated in premalignant lesional and well-differentiated SCCs as compared to normal epithelia, and that its expression level decreases as cells become poorly differentiated (Hagiwara *et al.*, 2008). Based on my results showing that CD109 reduces TGF-β-induced growth inhibition in HaCaT cells (Finnson *et al.*, 2006) and that CD109 inhibits TGF-β-induced EMT in HaCaT and SCCs cells, a potential role for CD109 in cancer progression is proposed as depicted in fig 6.10. The upregulation of CD109 may contribute to tumor progression by inhibiting TGF-β-induced growth arrest during the early stage of the disease, while the loss of CD109 expression at later stage of the disease may relieve the inhibition of TGF-β-mediated EMT, thus promoting tumor invasion (fig 6.10). CD109 may thus represent a potential molecular target for cancer therapy.

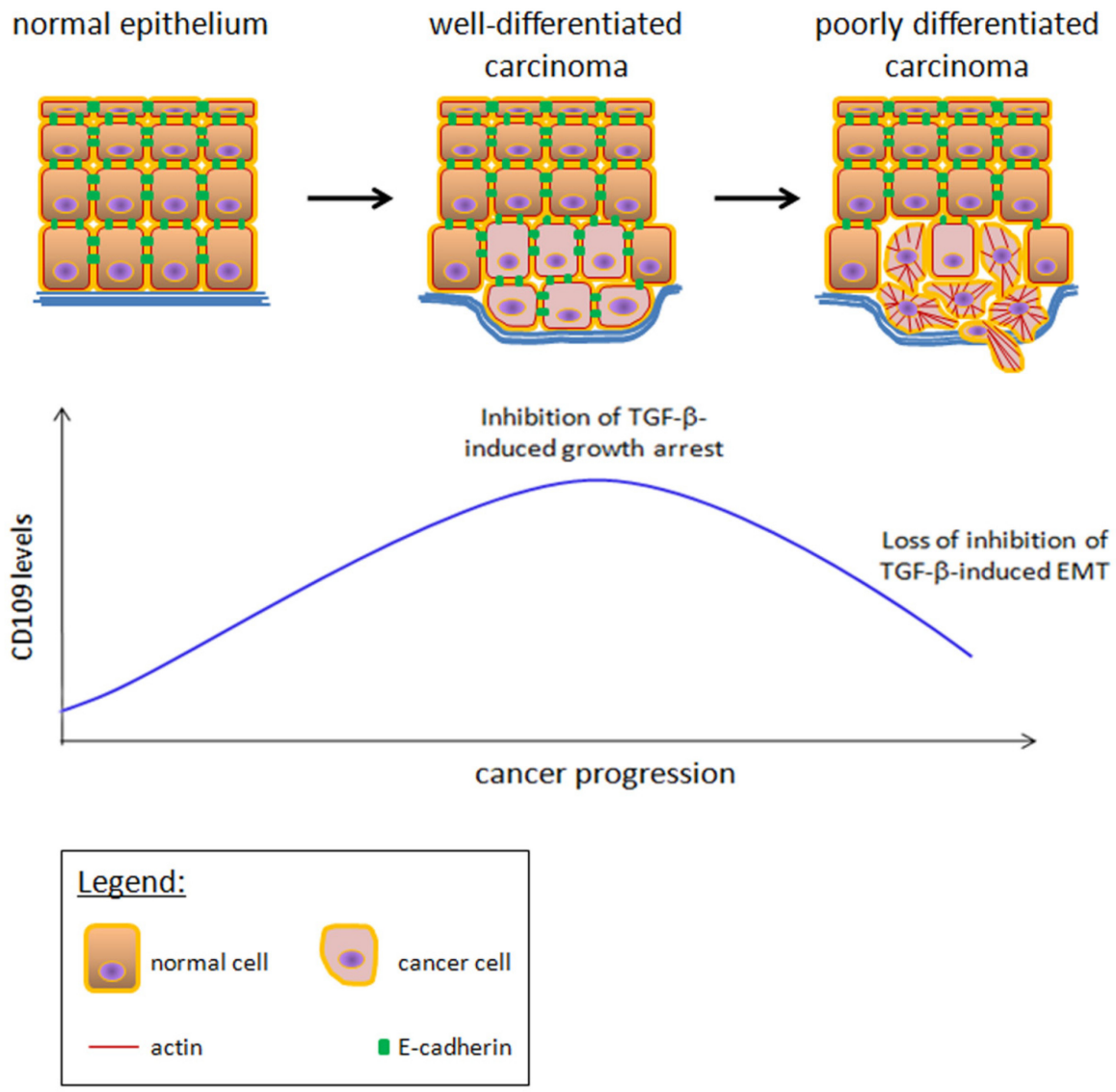

Figure 6.10: Schematic model of CD109's potential role during cancer progression. CD109 expression is increased in well-differentiated SCC and may contribute to tumor growth by inhibiting TGF-β-induced growth arrest. Because CD109 also inhibits TGF-β-induced EMT (characterized by the apparition of stress fibers, loss of junctional E-cadherin), the decrease of CD109 expression in poorly differentiated SCC may participate in tumor invasion and metastasis.

Chapter 7: Conclusions and Perspectives

TGF-β is a multifunctional growth factor that controls many cellular processes, such as cell proliferation, migration, ECM production and EMT. In the skin, TGF-β plays a critical role in the maintenance of skin homeostasis and dysregulation of its pathways has been implicated in many skin disorders, including squamous cell carcinomas, impaired wound healing and hypertrophic scaring, psoriasis and scleroderma (Kunz, 2009; Leask, 2010; Li *et al.*, 2004a; Seifert and Mrowietz, 2009). Our group hypothesized that manipulation of TGF-β action represents a novel strategy for the treatment of these clinical problems. This prompted us to characterize molecules that could regulate TGF-β action in skin cells and led to the identification of a novel TGF-β binding protein, CD109. CD109 is a 180 kDa GPI-anchored protein and a distant member of the α2M/complement family. Our group has previously shown that CD109 could be released from the cell surface by PI-PLC treatment and that both soluble and membrane anchored-form could bind TGF-β1 subtype with high affinity, but shows lower affinity toward the other TGF-β subtypes. Moreover, we have demonstrated that CD109 binds directly to TGFBR1 *in vitro* and forms a heteromeric complex with TGF-β signalling receptors. In addition, preliminary results using CD109S, a shorter, placental version of CD109 indicate that CD109S is able to inhibit TGF-β signalling and responses in keratinocytes, suggesting that CD109 may be a novel TGF-β co-receptor. In this thesis, I have further characterized the role of CD109 in skin cells and studied its mechanism of action. More specifically, I have shown that CD109, like CD109S, is able to inhibit TGF-β-induced SMAD2/3 phosphorylation and transcriptional activities and negatively regulates TGF-β-induced growth inhibition. These results strongly suggest that CD109 is an antagonist of TGF-β action. Although it has been previously shown that released/soluble CD109 can sequester TGF-β1 away from the signalling receptor, my results indicate that CD109 is also able to inhibit TGF-β receptor activity independently of ligand sequestration. This prompted me to investigate whether CD109 could direct TGF-β receptor into caveolae, a compartment associated

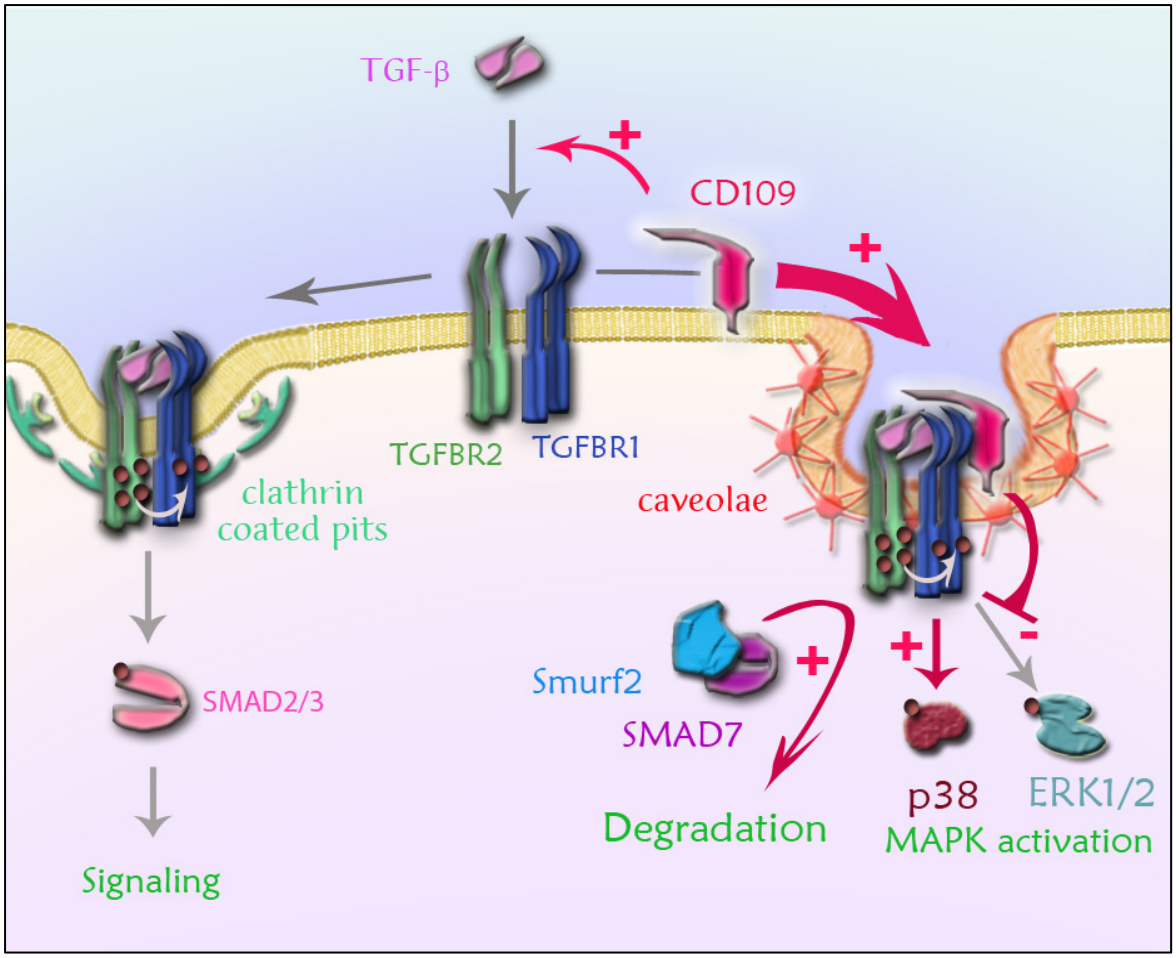

Figure 7.1: Schematic representation of the potential mechanism by which CD109 negatively regulates TGF-β responses. The TGF-β receptors internalized via two different pathways. Internalization via the clathrin-coated pits leads to SMAD2/3 signalling, while internalization via the caveolae is associated with TGF-β receptor degradation and MAPK activation. CD109 increases TGF-β binding to TGF-β receptors and promotes TGF-β receptor localization to the caveolae. Thus, CD109 facilitates TGF-β receptor endocytosis via the caveolar pathway; enhances proteasomal degradation of TGFBR1 by SMAD7/Smurf2 and/or modulates TGF-β-induced ERK and p38 action. These effects of CD109 ultimately lead to downregulation of TGF-β signalling and responses, such as EMT.

with TGF-β signalling downregulation and with TGF-β receptor degradation. I have shown that CD109 associates with caveolin-1, the major component of caveolae and localizes with the TGF-β receptors in lipid-raft/caveolae, in the presence of TGF-β. Although CD109 increases TGF-β binding to TGFBR1 and TGFBR2, CD109 enhances internalization of TGF-β receptor via the caveolar pathway, leading to inhibition of SMAD2/3 phosphorylation. Moreover, I have shown that CD109 accelerates TGF-β receptor degradation, in the presence, but not in the absence of TGF-β. CD109 increases the association and the co-localization of the E3 ubiquitin ligase Smurf2 and the adaptor SMAD7 with the activated TGFBR1. Together, my results suggest that CD109 increases TGF-β binding to TGF-β receptor and promotes TGF-β receptor localization to the caveolae, thereby facilitating TGF-β receptor endocytosis via the caveolar pathway and/ or enhancing SMAD7/Smurf2-mediated proteasomal degradation of TGFBR1. By these mechanisms, CD109 can downregulate TGF-β signalling (fig 7). Because the caveolar compartment has also been implicated in TGF-β-induced MAPK activation, which is important during EMT, I then looked at CD109's effects on TGF-β-induced p38 and ERK activation and at CD109's ability to regulate EMT in the context of squamous cell carcinomas (SCC). My results suggest that CD109 enhances TGF-β-induced p38 phosphorylation but decreases ERK activation, by a mechanism that involves caveolin-1 (fig 7). Importantly, CD109 inhibits TGF-β-induced EMT via both the SMAD and MAPK pathways in keratinocytes. This suggests that CD109 may play a role during cancer progression.

My results showing that CD109 negatively regulates TGF-β signalling at the receptor level together with previous results from our lab demonstrating that CD109 associates with TGF-β signalling receptors and binds to TGF-β1 define CD109 as a novel TGF-β co-receptor alongside betaglycan, endoglin and cripto. TGF-β co-receptors play an important role in regulating subtypes-specific TGF-β signalling and responses. In addition, TGF-β co-receptor such as betaglycan and CD109 are able to control compartmentalization, internalization and degradation of TGF-β signalling receptors. Finally, co-receptors have been shown to modulate SMAD2/3 pathway and also non-

canonical pathways in a cell-types specific manner. Thus, the relative proportion of TGF-β co-receptor may determine: 1) the intensity of subtype-specific TGF-β responses; 2) which internalization route will be used by the TGF-β signalling receptors; 3) the canonical and non-canonical pathways that will be activated in a given cell. So far, most studies have focused on the role of one type of co-receptor on TGF-β responses. However, previous results from our lab indicate that CD109 associates with betaglycan in keratinocytes (Finnson *et al.*, 2006). It would thus be interesting to take into account the role of all co-receptors present on a cell and to study their functional interaction. It is possible that a co-receptor affects the function and the internalization of another co-receptor.

Despite CD109's ability to enhance the binding of TGF-β to its receptors, I have shown that CD109 inhibits SMAD2/3 signalling and responses. This inhibition is likely mediated by CD109's capacity to direct TGF-β receptors into caveolae and to promote their degradation. The ability of TGF-β receptors to internalize into the caveolae was called into question in a previous study by Mitchell et al (Mitchell *et al.*, 2004). However, the above study used chimeric receptors in which the cytoplasmic/ transmembrane domains of the TGF-β receptors were fused to the ligand-binding domain of the GM-CSF receptors (Mitchell *et al.*, 2004). Under such conditions, TGF-β binding by CD109 (Tam *et al.*, 1998) and CD109 interaction with TGF-β receptor's extracellular domain (Finnson *et al.*, 2006) would not occur and may account for the inability to demonstrate a significant role of the caveolar pathway for TGF-β receptor endocytosis in Mitchell et al. study. Supporting this notion, the extracellular domain of TGFBR2 has been shown to be essential for TGF-β receptor localization in caveolae (Luga *et al.*, 2009). In addition, it has been suggested that an unknown glycosylated protein associates with TGFBR2 extracellular domain to direct TGF-β receptor compartmentalization to lipid-raft domains (Luga *et al.*, 2009). It would be of interest to determine whether CD109 binding to TGF-β, its interaction with TGF-β receptor and/or its glycosylation status are critical for CD109's effect on TGFBR1 caveolar localization. These could be studied by first delineating the domain(s) of interaction between CD109 and TGF-β1 and between

CD109 and TGFBR1, and then by creating mutants of CD109 unable to bind to TGF-β or to the TGF-β receptors. The importance of glycosylation could also be analyzed by treating the cells with tunicamycin (Luga *et al.*, 2009) and/or by mutating CD109 glycosylation sites. These approaches should provide us with further insights on the mechanism by which CD109 promotes TGF-β receptor localization to caveolae.

The localization of CD109 in lipid-raft/caveolae may depend on the cell type and on the composition of its GPI anchor. Indeed, it has been proposed that in some cell types, CD109 may partition into distinct membrane domains due to acylation of its GPI-anchor (Lin *et al.*, 2002). It would thus be of interested to study whether modifications of CD109 GPI-anchor affect its function on TGF-β signalling, internalization and degradation of TGF-β receptor. Such modification may affect CD109 localization to caveolae and association with caveolin-1. I have shown that CD109 associates with caveolin-1 (fig 4.1) and with SMAD7 (fig 5.3). These associations are likely indirect since CD109 is a cell-surface GPI-anchored protein while caveolin-1 is an integral membrane protein that never reaches the extracellular milieu (Kiss and Botos, 2009) and SMAD7 is a cytosolic protein. Thus, another area of investigation would be to determine which molecules mediate these associations. It is highly possible that TGFBR1 links CD109 with caveolin-1 and with SMAD7, as TGFBR1 has been shown to interact directly with CD109 (Finnson *et al.*, 2006), caveolin-1 (Razani *et al.*, 2001) and SMAD7 (Hayashi *et al.*, 1997). In order to prove that TGFBR1 mediates the association between CD109 and caveolin-1 and CD109 and SMAD7, co-immunoprecipitation should be performed in the presence or absence of TGFBR1 siRNA. Because I have also shown that TGF-β enhances the association between CD109 and caveolin-1, we can expect that this association will be enhanced after overexpression of a constitutively active TGFBR1, but not of a kinase dead mutant. Similarly, because my results show that receptor activation is important for TGF-β receptor localization to caveolae and also for degradation, overexpression of a kinase dead TGFBR1 mutant is likely to decrease the association between CD109 and SMAD7.

Although I have shown that CD109's effect on p38 and ERK activation involves caveolin-1, the exact mechanisms by which CD109 modulate these MAPKs remain to be elucidated. Despite CD109's ability to promote TGF-β receptor localization to caveolae, a compartment associated with ERK activation, CD109 decreases ERK phosphorylation. This suggests that CD109 may either inhibit tyrosine phosphorylation of TGFBR1 or may inhibit the recruitment of the ShcA-Raf complex (Lee *et al.*, 2007). Because SMAD7 has been shown to reside in caveolae and to mediate p38 activation (Di Guglielmo *et al.*, 2003; Edlund *et al.*, 2003), SMAD7 may play a role in CD109's ability to enhance TGF-β-induced p38 phosphorylation. CD109 may facilitate the formation of the TRAF6-SMAD7 complex in caveolae, thus enhancing p38 activation. While CD109 increases p38 phosphorylation in keratinocytes, preliminary results indicate that CD109 inhibits p38 in HEK 293 cells, suggesting that the mechanism by which CD109 modulates TGF-β-induced MAPK activation is cell-type specific. Thus, CD109 may elicit different responses in different cell types. It would also be interesting to investigate whether CD109 can modulate other TGF-β non-canonical pathways in order to broaden its cellular action. Future work could be to look whether CD109 modulates Rho-like GTPase activities, since I have noticed, while performing immunofluorescence and confocal microscopy, that CD109 localizes to region that resembles membrane ruffles. Finally, it is of interest to determine whether CD109, like endoglin, can regulate the balance between the classical TGF-β-induced SMAD2/3 signalling and the TGF-β-induced SMAD1/5 pathway.

CD109 is a GPI-anchored protein that can be released from the cell surface either via endogenous PI-PLC, PI-PLD (Tam *et al.*, 1998; Tam *et al.*, 2001) or after furin cleavage (Hagiwara *et al.*, 2010). The furin cleaved form of CD109 has been shown to remain associated with the TGF-β receptor (Hagiwara *et al.*, 2010). Thus, it is possible that the released/soluble form of CD109 is able to promote TGF-β receptor internalization via the caveolae and degradation. Our preliminary results reveal that addition of recombinant (and soluble) CD109 decreases TGFBR1 levels (Litvinov *et al.*, 2011), suggesting that released CD109 may associate with the TGF-β receptors to target them for degradation.

We can thus wonder whether the release of CD109 is an important step for CD109 action and whether the membrane-bound and soluble CD109 have the same potency in regulating TGF-β signalling. Interestingly, our preliminary data also suggest that the release of CD109 might be increased in psoriasis (Litvinov *et al.*, 2011). It is therefore important to identify factors that control the release of CD109. A previous study has demonstrated that TGF-β enhances the release of PI moiety in chondrocytes, suggesting that TGF-β induces a PI-PLC-like activity (Vivien *et al.*, 1993). This could be of potential importance in psoriatic keratinocytes, where elevated levels of TGF-β1 are associated with the degree of the disease (Flisiak *et al.*, 2002). Such increase in TGF-β1 may cause the release of GPI-anchored protein, including CD109. CD109 may then decrease TGF-β bioavailability and TGF-β receptor levels, resulting in the keratinocytes hyperproliferation characteristic of psoriasis.

While in psoriasis, the release of CD109 may lead to downregulation of TGF-β receptor levels and inhibition of TGF-β-induced growth arrest, in well-differentiated SCCs, upregulation of CD109 expression levels has been suggested to be responsible for the lack of TGF-β responsiveness (Hagiwara *et al.*, 2008). However, the mechanism by which such an upregulation of CD109 causes aberrant TGF-β signalling in SCCs remains unknown. Thus, it would be of interest to determine whether upregulation of CD109 enhances receptor localization to caveolae and degradation of the TGF-β receptor in SCCs. Moreover, because CD109 is upregulated in well-differentiated SCCs but becomes downregulated in poorly differentiated SCCs, understanding how CD109 expression is (de)regulated is also of importance. Although the upregulation of CD109 in well-differentiated SCCs is likely the result of gene amplification (Järvinen *et al.*, 2008), the mechanism leading to CD109 decrease in poorly differentiated SCCs is unknown. This decrease of CD109 expression may occur at the protein, at the mRNA or at the gene level. For example, it is possible that the degree of methylation of *CD109* affects its expression during SCCs, as observed in colorectal cancer (Mokarram *et al.*, 2009).

Further insight on the mechanisms leading to CD109 deregulation in SCCs may also be gained by cloning CD109's promoter and mapping its functional features.

My results show that decreasing CD109 expression using siRNA enhances TGF-β-induced EMT in SCC-derived cell lines. It is therefore important to validate the role of CD109 in SCC metastasis *in vivo*. For this purpose, SCC cells stably transfected with CD109 shRNA or its empty vector control could be injected subcutaneously into athymic nude mice as described in (Galer *et al.*, 2010). The presence of metastasis in lymph nodes, lung and skin could then be examined 3 weeks after injection. We expect that mice injected with CD109shRNA transfected cells develop more metastasis than control mice. Alternatively, nude mice could be injected with SCC cells and 7 days after injection (a period during which tumor develop), the mice could be treated with recombinant CD109 protein to prevent the appearance of metastasis.

The effect of CD109 in TGF-β-induced EMT may have its importance not only during cancer progression, but also in the development of fibrosis. Indeed, it has been proposed that a significant proportion of fibroblasts may be generated via EMT, causing excess of ECM production and destruction of the epithelium, a characteristic of advanced fibrosis (Guarino *et al.*, 2009). CD109 may thus improve fibrosis by inhibiting TGF-β-induced EMT. Consistent with this notion, recent results from our laboratory indicate that mice overexpressing CD109 in the epidermis display better collagen organization and decreased dermal thickness following bleomycin injection, a potent inducer of fibrosis (Al-Ajmi *et al.*, 2011). In addition, CD109 inhibits SMAD2/3 phosphorylation and reduces collagen type I and fibronectin expression in response to bleomycin treatment (Al-Ajmi *et al.*, 2011), supporting our *in vitro* data. Although CD109 is able to inhibit TGF-β signalling and ECM production in both an *in vivo* model of fibrosis (Al-Ajmi *et al.*, 2011) and in fibroblasts from sclerodermic patients (Man *et al.*, 2011), CD109 is upregulated in sclerodermic fibroblasts and keratinocytes. The increase of CD109 in the context of scleroderma may represent an adaptative mechanism to inhibit

the excessive production of ECM (Man *et al.*, 2011). Interestingly, preliminary results suggest that while CD109 downregulates TGF-β receptor levels in normal fibroblasts, CD109-mediated degradation of TGF-β receptor may be impaired in sclerodermic fibroblasts. Caveolin-1 has been shown to be downregulated in sclerodermic fibroblasts (Del Galdo *et al.*, 2008; Qian and Ueno, 2010) and may explain CD109's inability to mediate TGF-β receptor degradation in these cells. In addition, a micro-array analysis has revealed that *caveolin-1* null fibroblasts overexpressed CD109, as compared to wild-type fibroblast (Pavlides *et al.*, 2010). This could represent a compensatory mechanism to avoid an excess of TGF-β signalling. However, as the level of caveolin-1 becomes too low, CD109 becomes unable to decrease TGF-β receptor levels. This results in an increase of TGFBR1 levels, which has been shown to be associated with scleroderma fibrosis (Pannu *et al.*, 2006).

In addition to the role of CD109 during bleomycin-induced fibrosis *in vivo*, studies of transgenic mice overexpressing CD109 in basal keratinocytes have provided us with critical insight on the role of CD109 during wound healing. Notably, CD109 potently inhibits scarring and inflammation in both incisional and excisional wound models but does not affect normal physiology (Vorstenbosch *et al.*, 2011). As I have observed *in vitro*, my colleague has demonstrated that CD109 promotes keratinocytes proliferation *in vivo* during wound healing (Vorstenbosch *et al.*, 2011). Another study from our laboratory has demonstrated that CD109 transgenic mice display decreased ECM production and decreased dermal thickness in their hypoxic wounds as compared to hypoxic wounds of wild-type animals and to non-hypoxic control wounds (Winocour, 2010). This further emphasizes the ability of CD109 to downregulate ECM production (likely via inhibition of TGF-β signalling) in the skin. The next logical step would be the creation of knock-out mice to better understand the overall biology of CD109, notably its function during development and immune homeostasis. Using the cre-lox technology, we could also study the loss of function of CD109 in a particular tissue.

In summary, I have demonstrated that the novel TGF-β co-receptor CD109 negatively regulates TGF-β signalling and responses, by promoting TGF-β receptor internalization into caveolae and by facilitating TGF-β receptor degradation. In addition, I have shown that CD109 inhibits TGF-β-induced EMT via the SMAD and MAPK pathways. Together, these results may provide a basis for the development of novel therapeutic strategies based on CD109 properties to prevent or treat diseases where TGF-β is known to play an important pathophysiological role, such as impaired wound healing, fibrosis, psoriasis and cancer progression.

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