

**Focal Adhesion Kinase Signaling Spatially Regulates Adhesion Dynamics in Fibroblasts.**

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**I dedicate this thesis to all my teachers.**

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## List of Abbreviations

ACF	Actin microtubule linker protein
ADF	Actin depolymerizing factor
ADP	Adenosie diphosphate
Arp	Actin related protein
ARPC	Actin related protein complex
ASAP	Arf GTPase activating protein
ATP	Adenosine triphosphate
Cas	Crk associated substrate
CAT	Collective amoeboid transition
Cdc42	cell division cycle GTP binding protein 42
CLASP	Cytoplasmic linker associated protein
CRD	Cystine rich domain
DAD	Dia autoregulatory domain
Dbl	Diffuse-B-cell lymphoma
DH	Dbl homology
Dia	Diaphanous
DID	Dia inhibitory domain
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchyma transition
Ena/Vasp	Enabled/Vasolidator-stimulated phosphoprotein
ERK	Extracellular regulated kinase
FAK	Focal adhesion kinase

FERM	erythrocyte band four.1-ezrin-radixin-moesin
FH	Formin homology
fMLP	formyl-methionyl leucine-phenylalanine
FRT	flp recombinase target
GAP	Guanine activating protein
GDI	Guanine dissociation inhibitor
GEF	Guanine exchange factor
GFP	Green fluorescent protein
GRAF	GTPase regulator associated with FAK
Grb	Growth factor receptor binding protein
GTP	Guanine triphosphate
HEK	Human embryonic kidney
HUVEC	Human umbilical endothelial cells
ILK	Integrin linked kinase
IRSp	Insulin receptor substrate protein
JAK-	Janus kinase
JMR	Juxtamembrane
LPA	Lysophosphatidic Acid
MAPK	Mitogen activated protein kinase
MEK	MAP kinase/ERK kinase
MHC	Myosin heavy chain
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MP	MAP kinase partner
NM	Non muscle myosin

PAK	p21 activated kinase
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PDZ	PSD95/dlg/ZO-1 domain
PH	Pleckstrin homology
PI3K	Phosphoinositol kinase 3
PIP2	Phosphoinositol 4,5 phosphate
PIP3	Phosphoinositol 3,4,5 phosphate
PKC	Protein kinase C
PTX	Pertussis toxin
PYK	Protein tyrosine kinase
Rac	Ras related protein
RACK	Receptor of activated protein kinase C
Raf	Ras activated factor
RBD	Rho binding domain
RFP	Red fluorescent protein
Rho	Ras homologous gene
RhoGEF	Rho guanine nucleotide exchange factor
RNAi	RNA interference
ROCK	Rho coil-coil associated kinase
SH2	Src homology 2
SH3	Src homology 3
Src	Sarcoma oncogene
WASP	Wiskot Aldrich syndrome protein





## Abstract

A key step in cell migration is the dynamic formation and disassembly of adhesions at the front and the concomitant movement and release of adhesions in the rear of the cell. In this thesis we present the evidence that the signals regulating adhesion dynamics in the rear of the cells differ from the signals that regulate adhesion dynamics in the front.

The first part of the work presented in this dissertation demonstrates that focal adhesion kinase (FAK) signaling to specific regulators of Rho GTPase controls adhesion movement and trailing edge retraction in fibroblasts. We present a model in which addition of Lysophosphatidic acid (LPA) induced the movement of adhesions and retraction of the trailing edge, thus mimicking tail retraction in migrating cells. FAK, guanine nucleotide exchange factors (GEFs) for Rho and the Rho effector, Rho Kinase II (ROCKII) are critical for the regulation of adhesion movement and trailing edge retraction. Down-regulation of FAK by small interfering RNAs (siRNAs) or small hairpin RNAs (shRNAs) blocked LPA induced adhesion movement and trailing edge retraction. This phenotype was rescued by ectopic expression of PDZ-RhoGEF or a Rho effector domain mutant that activates ROCK. The knockdown of PDZ-RhoGEF or ROCK II inhibited LPA-induced trailing edge retraction and adhesion movement. Moreover, over expressed PDZ-RhoGEF co-immunoprecipitated with FAK and localized to FAK containing adhesions. These studies support a model in which FAK and PDZ-RhoGEF co-operate to induce Rho/ROCKII-dependent adhesion movement and trailing edge retraction in response to LPA.

In the second part of this dissertation, we demonstrate the importance of a specific scaffold in targeting the MAP kinase pathway to adhesion structures found in the

areas of active protrusions (front). We show that the RACK1 (Receptor Activated Kinase C) scaffold protein specifically functions in integrin-mediated activation of the MAPK/ERK cascade and targets active ERK to adhesions present in the lamellipodia of protruding cells. We found that RACK1 associates with the core kinases of the ERK pathway, Raf, MEK and ERK, and that attenuation of RACK1 expression resulted in a decreased ERK activity in response to adhesion. RACK1 silencing also caused a reduction of active ERK in adhesions and decreased the rate of adhesion disassembly in the areas of active protrusions. Our data further indicate that FAK is an upstream activator of the RACK1/ERK pathway in the regulation of adhesion disassembly in the lamellipodia. We suggest that RACK1 tethers the ERK pathway to adhesions found in the lamellipodia and channels signals from upstream activation by integrins to downstream targets at these sites.

In summary, the data presented in Chapters 2 and 3 argue that signaling pathways regulating adhesion dynamics within the leading edge of migrating cell differ from the signals that regulate adhesion dynamics at the trailing edge of the cell. We propose that FAK is a major component in coupling the MAP kinase pathway adhesion disassembly in the leading edge and Rho pathway to adhesion movement at the trailing edge.

## CHAPTER 1

### Introduction

#### Why study cell migration?

The ability of cells to migrate is a fundamental biological phenomenon that is required for the persistence and survival of multicellular organisms (Horwitz and Webb, 2003). Cell migration starts from the moment of conception and continues throughout the life span of an organism. First, the fertilized egg has to migrate to the proper locale to prepare for the initial cell division. Second, in developing embryo primordial germ cells (precursors of gametes) migrate through the hindgut epithelium to the sites of gonad development (Laird et al., 2008). In adult organisms immune cells constantly survey tissues to look for potential pathogens (Cook and Bottomly, 2007). During injury (wound healing) stromal cells migrate to fill the injury site (Fuchs, 2007).

An alteration of the proper regulatory signals in migratory cells leads to pathological situations. In cancer, aberrant cell migration leads to the spread of cells to distant sites, resulting in dissemination of the cancer throughout the body (Wolf and Friedl, 2006). In muscular dystrophy, aberrant regulation of skeletal muscle stem cell migration leads to the failure of muscle repair following injury (Laird et al., 2008). Inflammation of arteries during atherosclerosis is induced in part by monocyte derived cells that fail to migrate out of the atherosclerotic lesions (Trogan et al., 2006). Therefore, understanding the biochemical and mechanical properties that regulate the migratory process is crucial for the development of therapeutics that may have useful applications in cancer progression, stem cell transplantation and inflammation associated with atherosclerosis.

In the introduction to the dissertation, I cover the fundamental issues that are critical for cell migration. Following the overview of cell migration, I will introduce selected extracellular matrix (ECM) components on which cells migrate. I will also review the ECM receptors expressed on the cell surface that are needed for cellular migration. Next, I will focus on the importance of cell adhesion to ECM and cytoskeletal network in cell migration. Further, I will review current knowledge about selected receptor mediated signal transduction pathways that have been implicated in the regulation of adhesions, cytoskeleton and cell migration. Finally, I will describe the plasticity of migratory behaviors that cells can adopt in disease like cancer and discuss the pro migratory signaling pathways that will be the main theme of this work.

### **Cell Migration an Overview**

Almost every aspect of life involves cell migration (Webb et al., 2005). During development, the blastocyst migrates as collective sheets to form three layers of the developing embryo (Anderson et al., 2000). Eventually cells within the developing embryo will migrate to form tissues and organs (Keller, 2005). In the developing cerebrum, neuronal stem cells migrate from the epithelial lining beneath the brain into the cerebrum, differentiate and extend axons to form synapses (Horwitz and Webb, 2003). Also the body plan of a vertebrate is entirely mediated by cell migration (Keller, 2006). For example, in the developing frog embryo the formation of a head and tail is achieved by polarized cell movement (Keller, 2002). These movements are known as convergence (to narrow the mediolateral aspect of the embryo) and extension (to elongate the anterior-posterior aspect of the embryo) of an embryo (Keller et al., 2000). Convergence/extension is achieved by increased mesoderm cell polarization followed by

medial and lateral protrusive activities within gastrula (Wallingford et al., 2000; Wilson and Keller, 1991). As a result, cells begin to migrate laterally in an up and down direction producing neurula and finally a tadpole (Keller, 2002).

Cell migration is fundamental not only in embryo development but also in maintaining adult homeostasis. For instance, epidermal cells of the skin are continuously renewed by virtue of dermal “newcomers” migration (Fuchs, 2007). Immune system homeostasis almost entirely relies on the migratory properties of cells (Ridley et al., 2003). Cells of the innate immune system migrate to survey tissues and organs during and post infection (Cook and Bottomly, 2007). Migration of cells of the acquired and humoral arm of the immune system is required for proper T and B cell maturation and their antigen dependent activation (Allen et al., 2007). Wound repair relies on cell migration. Following injury and platelet dependent fibrinogen production, fibroblasts first move into the wound to form new stromal cell layers and epithelial cells follow to complete wound closure (Watterson et al., 2007).

Cell migration is a cyclical process which starts with the ability of the cell to sense external signals and convert these signals into the mechanics of lamellipodia protrusion and trailing edge retraction necessary for cell translocation (Vicente-Manzanares et al., 2005). Migratory cells are coupled to the extracellular matrix via cells’ surface receptors localized within structures of close juxtaposition to the ECM (termed ECM adhesions). ECM adhesions connect the substrate on which the cell is migrating to the intracellular cytoskeleton. In the protruding lamellipodia, adhesions form and disassemble at relatively fast rates. In the trailing edge, adhesions tend to move inward and break during trailing edge retraction. In the following section, I will discuss the fundamental components of extracellular matrix that form the physical platform for cell migration.

## ECM Components

One of the most important cellular structures in biology are the sites of attachment of cells to basement membranes, attachment platforms rich in secreted extracellular matrix proteins. Basement membranes are present in the simplest multicellular sea creatures (for example, Coelenterates) as well as in the more complex and diverse higher mammals. The fundamental extracellular matrix proteins found in most basic basement membranes are type IV collagens and laminins (Hynes and Zhao, 2000). Both the fly and the worm have one pair of type IV collagen genes (Adams et al., 2000). In vertebrates there are three pairs of collagen IV genes that are arranged in an antiparallel (head to head) manner with a common promoter between them. Transcription of collagen IV in vertebrates results in production of heterotrimeric proteins containing two  $\alpha 1$  subunits and one  $\alpha 2$  subunit. Collagen IV molecules, through their triple alpha helical collagenous regions, interact with each other forming fibrils that play an essential role in the formation and maintenance of basement membranes (see Figure 1 A) (Kreis and Vale, 1999).

Laminins comprise another family of important extracellular matrix proteins. The laminins found in *Drosophila* and *C.elegans* are heterotrimers composed of one  $\alpha$ , one  $\beta$  or one  $\gamma$  subunit (Gotwals et al., 1994). In higher vertebrates the number of laminin subunits is far greater. In humans or mice there are five  $\alpha$ , three  $\beta$  and three  $\gamma$  subunits that make up sixteen different laminin heterotrimers (Aumailley et al., 2005). The sequence of the laminin  $\alpha$  subunit contains a number of tandem repeats of epidermal growth factor like domains, G like domains (sequences of approximately 200 residues that are repeated five times in the C-terminal globular region of the laminin  $\alpha$  subunit) and similarly N-like domains that are placed at the N terminus of the molecule (Figure

1B) (Colognato and Yurchenco, 2000). Similar to collagen IV, laminins play a central role in the formation and stability of basement membranes.

Fibronectin is an ECM protein that is not present in basement membranes but is essential for development. Fibronectin is a large glycoprotein composed of three different types of repeating modules, type I, type II and type III repeats (Figure 1C) (Pankov and Yamada, 2002; Potts and Campbell, 1996), (Hynes R.O, 1990). The type I repeat is composed of 45 amino acids and is found in the amino terminal and carboxy terminal regions of the protein (Bork et al., 1996). The amino terminal type I repeat facilitates interaction with other fibronectin molecules and collagen molecules and promotes formation of fibrils (Hohenester and Engel, 2002). Type II repeats are found within the N-terminal portion of the molecule and are positioned along with type I repeats within a collagen binding region of fibronectin that also contributes to fibril formation (Morla and Ruoslahti, 1992). Type III repeats make up the most of the molecule (Wierzbicka-Patynowski and Schwarzbauer, 2003). In contrast to type I and type II repeats, type III repeats are encoded by different exons within the fibronectin gene (Schwarzbauer et al., 1983). Alternative splicing of type III repeat exons can generate various fibronectin molecules that are expressed differentially during the development of the organism (Schwarzbauer et al., 1983). Type III repeats facilitate the interaction of fibronectin with other fibronectin molecules as well as cell surface receptors and heparins (Hynes and Yamada, 1982). Mice lacking the fibronectin gene die from defects in vascular development (Astrof et al., 2007). Thus, fibronectin along with collagen IV and laminin are required for development.

The role of ECM proteins is to provide attachment sites for cells expressing ECM receptors and provide a platform for cell migration. In the following section I will describe the variety of extracellular matrix receptors that engage different substrates.

## ECM Receptors

The integrins are major cellular receptors for ECM and are well conserved among the phyla (Hynes and Zhao, 2000). Integrins are heterodimeric receptors (comprised of an  $\alpha$  and  $\beta$  subunit) that link components of the ECM to the cell cytoskeleton (Carrell et al., 1985). The  $\alpha$  subunit noncovalently associates with  $\beta$  subunit to form a heterodimer (Gailit and Ruoslahti, 1988). The integrin  $\alpha$  and  $\beta$  subunits are transmembrane glycoproteins with short cytoplasmic tails (Hynes, 1992). The N terminus of the  $\alpha$  subunit contains the amino acid sequence (Asp-x-Asp-x-Asp-Gly-x-x-Asp) that supports divalent cation binding and is necessary for proper integrin function (Figure 2) (Kirchhofer et al., 1990). Common to all  $\beta$  subunits is a four fold repeat of a cysteine rich segment within the N terminal portion of the subunit. These repeats are thought to be important for ligand binding (Hynes, 1992). The carboxy terminal portions (cytoplasmic domains) of both subunits serve as docking sites for the cytoskeleton and cytoskeleton associated proteins (Liu et al., 2000b).

Integrins are found in organisms including sponges, corals, nematodes, echinoderms and mammals (Burke, 1999). The *Drosophila* genome encodes two  $\beta$  subunits ( $\beta$ PS and  $\beta$ v) and five  $\alpha$  subunits ( $\alpha$ PS1- $\alpha$ PS-5) (Gotwals et al., 1994). All five  $\alpha$  subunits pair to form complexes with  $\beta$ PS, it is not known if  $\beta$ v pairs with any of the  $\alpha$  subunits (Hynes and Zhao, 2000). The integrins,  $\alpha$ PS1 $\beta$ PS and  $\alpha$ PS2 $\beta$ PS are the receptors for laminin and collagen (Gotwals et al., 1994; Zavortink et al., 1993). In vertebrates there are eighteen  $\alpha$  subunits and eight  $\beta$  subunits and the combinatorial complexity yields twenty-two distinct integrins (Hynes, 1996). More than a half of vertebrate  $\alpha$  subunits contain an additional domain called the "I" domain (Hogg et al.,



1994). “I” domains are the major cation and ligand binding domains in integrins (Figure 2) (Nolte et al., 1999). I domains of  $\alpha_L$  integrins have been shown to bind to ICAM molecules (Luo et al., 2007). In vertebrates  $\alpha_{1-2}$  and  $\alpha_{6-7}$  subunits can pair with  $\beta_1$  subunit and yield receptors for collagen and laminin. Fibronectin is recognized by the following integrin dimers  $\alpha_{3-5}\beta_1$ ,  $\alpha_v\beta_6$ ,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_1$ ,  $\alpha_v\beta_5$  and  $\alpha_4\beta_7$  (Faull and Ginsberg, 1996). Genetic deletion of mouse integrin alpha subunits  $\alpha_{3,4,5,6,8}$  and  $\alpha_v$  is embryonic lethal (Georges-Labouesse et al., 1996; Kreidberg et al., 1996; Yang et al., 1993; Yang et al., 1995) (Hynes, 1996), whereas mice knocked out for  $\alpha_1$ ,  $\alpha_{IIb}$  and  $\alpha_E$  are viable and fertile (Gardner et al., 1996) (Hynes, 1996), suggesting some functional redundancy. However, targeted deletion of  $\alpha_7$  results in the development of muscular dystrophy (Mayer et al., 1997). Similarly, phenotypes due to deletion of mouse integrin  $\beta$  subunits differ. For instance, knock outs of  $\beta_1$  and  $\beta_4$  subunits are embryonic lethal (Fassler et al., 1995; van der Neut et al., 1996), whereas, knock outs of  $\beta_{2,3,5,6,7}$  are dispensable for development (Hynes, 1996).

Integrins are a major family of transmembrane receptors that translate extracellular cues into biochemical signals that regulate a variety of cellular processes (Schwartz and Ingber, 1994). The ability of integrins to regulate cell behavior is a result of the fact that integrins constitute an essential bridge between ECM and the cellular microfilament network, thus providing structure as well as sensors for the microenvironment. In the following section I will describe the nature of integrin substrate adhesions.

### **Integrin mediated adhesions.**

Integrin engagement with extracellular matrix components results in the formation of structurally distinct matrix adhesions (Horwitz et al., 1986). Early studies in cultured chicken fibroblasts revealed that matrix adhesions occur as elongated small regions along the ventral plasma membrane (Abercrombie and Dunn, 1975). These regions were termed focal adhesions and/or focal complexes (I will use the term adhesions throughout the thesis) (Burridge et al., 1988; Burridge et al., 1987). In the earlier experiments, it was recognized that adhesions were associated with the actin cytoskeleton and provided structural support for the attached cell (Burridge et al., 1987). In addition to the association of actin with adhesions, immunofluorescence experiments revealed the multi-protein nature of adhesions, containing transmembrane ECM receptors (integrins), lipid kinases/ phosphatases and non-enzymatic scaffold proteins (Zaidel-Bar et al., 2003). Because of the direct link to the cell cytoskeleton, adhesions are part of the cytoskeletal mechanics that drive cell migration. In the following chapter, I will focus on the role of cytoskeleton and selected associated proteins with the cytoskeleton in the regulation of cell migration.

### **Cytoskeleton and cell migration.**

Cellular migration is a mechanical process that involves the temporal and spatial interplay amongst components of the ECM, cellular adhesion and cytoskeleton. The initial event of polarized movement is the formation of a predominant membrane protrusion (lamellipodium or filopodia) which is driven by the actin cytoskeleton (Euteneuer and Schliwa, 1984; Pollard and Borisy, 2003; Verkhovsky et al., 1999). Actin

filaments are assembled from actin monomers (Drenckhahn and Pollard, 1986). Actin filaments within the protrusion are polarized in such a way that barbed ends (fast assembling) face the protrusion and pointed ends (slow assembling) face distally from the protrusion (Borisy and Svitkina, 2000). Organization of actin in the lamellipodium resembles a branching dendritic meshwork (Svitkina and Borisy, 1999), whereas in filopodia actin is organized in bundles (Schafer et al., 1998; Welch and Mullins, 2002). In lamellipodia, actin polymerization is catalyzed by components of the actin related protein complex Arp2 and Arp3 (Arp2/3). The Arp2/3 complex contains seven associated subunits that include Arp2, Arp 3 and ARPC1-5 (Machesky et al., 1994). Purified Arp2/3 can nucleate actin polymerization in vitro and cross-link (arrange) newly formed filaments into Y - branched arrays (Mullins et al., 1998). In lamellipodia, Arp2/3 induces the formation of new actin filaments that branch off already existing filaments (Pollard and Beltzner, 2002). In filopodia actin polymerization is also driven by Arp2/3-dependent mechanisms that promote actin filament barbed end assembly that pushes the membrane out (Miki et al., 1998). In addition filopodia can also be generated via Arp2/3 independent mechanisms, involving the formin -dependent barbed end polymerization and membrane extension (Pellegrin and Mellor, 2005).

The rate of actin polymerization in lamellipodia and filopodia is mainly controlled by actin-binding proteins that can directly promote or prevent polymerization of actin, disrupt existing actin filaments or stabilize actin filaments. For instance, profilin is an actin binding protein that can either promote or prevent actin polymerization (Kaiser et al., 1999). It promotes actin filament polymerization by targeting actin monomers to barbed ends of the filaments (Goldschmidt-Clermont et al., 1991). Profilin also prevents actin polymerization by decreasing the free monomer concentration available for spontaneous filament nucleation (Pollard and Borisy, 2003). Thymosin  $\beta$ 4 is an actin

binding protein that can regulate actin dynamics (Cassimeris et al., 1992). Contrary to profiling, thymosin  $\beta 4$  will not target actin monomers to barbed ends of polymerizing filaments (Goldschmidt-Clermont et al., 1992). Thus, it suppresses barbed end elongation by decreasing the pool of available actin monomers.

Another protein that can influence actin polymerization in the lamellipodium is the actin destabilizing factor ADF/cofilin (Ghosh et al., 2004). ADF/Cofilin is an actin severing molecule that cuts “old actin” filaments and creates free barbed ends for further polymerization, thus, it contributes to actin filament turnover (Andrianantoandro and Pollard, 2006). Studies in mammary adenocarcinoma cells revealed that lack of cofilin results in stabilization of actin filaments that induces persistence of lamellipodia (Sidani et al., 2007), supporting a role for cofilin in the turnover of pre-existing actin filaments.

Yet another mechanism for regulating actin dynamics within the lamellipodium is actin filament capping (Pollard and Borisy, 2003). Gelsolin is a protein that first cuts actin filaments and then caps one end of the filament (Kwiatkowski, 1999). This property of gelsolin has been attributed to its increased barbed end formation and stabilization of a polymerizing filament at the same time (Barkalow et al., 1996). Gelsolin null fibroblasts show increased actin bundling and decreased lamellipodia protrusions, probably due to decreased actin polymerization (Azuma et al., 1998).

In filopodial protrusions, another set of proteins has been found to regulate actin dynamics. Ena/Vasp proteins localize to the tips of barbed ends and antagonize capping and branching, thus promoting filament polymerization and stabilization (Krause et al., 2003). Mice lacking Ena/Vasp proteins are viable but show defects in brain development, and their neurons are severely depleted in filopodia (Kwiatkowski et al., 2007; Lebrand et al., 2004). Thus, Ena/Vasp plays a critical role in stabilization of polymerizing actin filaments that contribute to the formation of filopodia.

Another component of the cytoskeleton that regulates actin dynamics during cell migration is myosin (Vicente-Manzanares et al., 2007). The myosins are actin-based molecular motors that can pull and bundle actin filaments (Bresnick, 1999). The myosin II family includes skeletal, cardiac and smooth muscle myosin as well as non muscle myosin (NM) (Conti and Adelstein, 2008). Every myosin II molecule is a hexamer composed of Myosin Heavy Chain dimers (MHC) and two pairs of regulatory Myosin Light Chains (MLC). The MLCs are tightly bound to MHCs and play a role in stabilizing and regulating myosin structure (Amano et al., 1996a). The N-terminus of MHC is a globular head that contains ATPase activity and binds actin. The C-terminus of MHC is involved in myosin filament formation (Conti and Adelstein, 2008)(Figure 3). This arrangement of myosin allows for the actin filament to be translocated via the globular motor domain and to be maintained under tension via C-terminus association with other MHC molecules (cross-linking). NM II has been implicated in the mechanics of lamellipodial adhesion assembly, maturation and disassembly (Vicente-Manzanares et al., 2007) .

In addition to actin, microtubules have been implicated in regulation of migration in multiple cell types (Gundersen et al., 1998). Microtubules are comprised of tubulin dimers ( $\alpha$  and  $\beta$ ) that derive the energy for polymerization from the sustained binding of guanine tri-phosphates (GTP) to the  $\beta$  subunit (Howard and Hyman, 2003; Mitchison and Kirschner, 1984). Microtubule polymerization is favored when there is an abundance of GTP loaded dimers near the tips of microtubules (Howard and Hyman, 2003).

The regulation of microtubule stability is an important component of cell migration (Palazzo et al., 2004). In polarized fibroblasts, tips of stable (i.e., the rate of polymerization greatly exceeds the depolymerization rate) microtubules are predominantly found in protruding lamellipodia (Kaverina et al., 1999), as shown by total

internal reflection microscopy (TIR-FM). It has been proposed that the role of stable microtubule penetration into lamellipodia is to deliver lipid vesicles containing regulatory molecules (Lin et al., 2002).

Genetic attenuation of expression of microtubule tip binding proteins has been shown to perturb the stability of microtubules and the polarization of migratory fibroblasts. For instance, Cytoplasmic Linker Associated Protein -2 (CLASP-2) has been shown to be necessary for maintenance of stable microtubules in fibroblast lamellipodia (Drabek et al., 2006). Cells lacking CLASP-2 show multiple short lived lamellipodia and lose directional migration (Drabek et al., 2006). Another molecule that has been implicated in the regulation of microtubule dynamics within the lamellipodium is actin–microtubule linker protein ACF-7. Similar to CLASP-2 null fibroblasts, ACF-7 depleted cells have reduced levels of stable microtubules and fail to maintain directional migration (Kodama et al., 2003). In summary microtubules along with actin filaments are essential to drive polarized cell migration.

In previous paragraphs, I introduced the basic knowledge about components (substrate, adhesions and cytoskeleton, see Figure 4A) that contribute to the migratory process. Cell matrix adhesions along with the cytoskeleton are dynamic and undergo constant remodeling during attachment and cell migration. In the following paragraphs I will describe how adhesion dynamics are regulated during migration.

### **Dynamic Regulation of Adhesion**

Integrin-mediated adhesions are dynamic with defined lifespans (Webb et al., 2002). In the late 1970s, interference reflection microscopy was used to show that adhesions in chicken fibroblasts undergo dynamic changes during cell migration (Heath

and Dunn, 1978). From these studies, it was inferred that adhesion dynamics were regulated by the actomyosin network that directly connected to these adhesions. Twenty years later, researchers using the  $\beta 1$  integrin cytoplasmic domain fused to green fluorescent protein (GFP) observed that in stationary fibroblasts most of the integrin adhesions were highly mobile and tended to move along actin bundles towards the center of the cell. On the other hand, in actively moving cells, adhesions displayed polarized behavior. In the front of the cell adhesions were stationary with high frequency of disassembly whereas in the back of the cells, adhesions were elongated and moved along with the stress fibers towards the center of cell (Palecek et al., 1996; Smilenov et al., 1999). The movement of the elongated adhesions appear to result from the addition of adhesion molecules (e.g., vinculin, paxillin) at one end (assembly), and the removal of adhesion molecules at the other end (disassembly) of the adhesions – a process referred to as adhesion treadmilling (Digman et al., 2008) (Figure 4B). It is likely that changes in actomyosin contractility promote treadmilling of adhesions in the trailing edge (Digman et al., 2008).

Spatial difference in the adhesions dynamics (stationary adhesions vs treadmilling adhesions) is likely to involve regulation of myosins. In fibroblasts siRNA-dependent attenuation of non-muscle myosin IIA/B expression resulted in impaired lamellipodial and trailing edge adhesion dynamics (Vicente-Manzanares et al., 2007). Therefore, it is likely that signaling events that impact myosins are crucial in the spatial regulation of adhesion dynamics.

Microtubules have been shown to “target” adhesion sites and induce adhesion disassembly (Kaverina et al., 1999). Using TIRFM, researchers showed that tips of microtubules are able to target adhesion sites and induce their disassembly. The pharmacological disruption of microtubules results in adhesion growth, whereas the

removal of the inhibitor restores polymerization of microtubules and induces adhesion turnover (Ezratty et al., 2005). These observations suggested the hypothesis that protein trafficking associated with polymerizing microtubules could contribute to adhesion turnover. Caveolin have been shown to be delivered by microtubules to the plasma membrane (Mundy et al., 2002) and localized to adhesion sites (del Pozo et al., 2005). Interestingly, caveolin null fibroblasts have increased number of focal adhesions and their dynamics is impaired (Grande-Garcia et al., 2007). These data further suggested the hypothesis that microtubule-dependent delivery of caveolin is necessary for adhesion turnover. However, more experiments need to be done to fully explore this hypothesis.

Cellular proteases have been implicated in the regulation of adhesion dynamics. Early studies showed that inhibition of the calcium-dependent protease calpain reduces rates of cell migration, induces growth of adhesions and prevents trailing edge retraction (Huttenlocher et al., 1997). The likely mechanism for calpain-mediated regulation of adhesion is proteolytic degradation of adhesion components including FAK (Carragher et al., 2003) and talin (Franco et al., 2004).

In summary, substrate adhesions are dynamic entities that undergo constant remodeling during cell migration. The differences in their spatial behavior are most likely due to localized coupling of distinct signaling pathways. In the following paragraphs, I will introduce the initiation of signaling pathways from selected plasma membrane receptors that have been implicated in the control of adhesion dynamics and cell migration.



### **Adhesion Receptor Signaling.**

Abundant evidence exists indicating that integrin-mediated adhesions transmit signals from the outside of the cell to the inside as well as integrate signals generated by growth factor receptors (Schwartz, 2001). Evidence that integrin-mediated adhesions direct signals into the cells comes from experiments showing that integrin-dependent platelet aggregation induces tyrosine phosphorylation of several proteins in the range of 80 -130 kDa (Shattil and Brugge, 1991). In related experiments, adhesion of carcinoma cells or fibroblasts to fibronectin induced the tyrosine phosphorylation of similar sized proteins (Guan et al., 1991). Interestingly, the same set of proteins that were predominantly tyrosine phosphorylated in response to integrin mediated adhesion were found to be tyrosine phosphorylated in cells transformed with oncogenic Src (Sarcoma) kinase or in cells stimulated with soluble growth factors (Parsons and Parsons, 2004). These observations indicated that integrin-mediated adhesions induced intracellular signals in a similar fashion to already known signal transducers.

Another example of adhesion-mediated signaling is cytoplasmic alkalization (Hynes, 1992). In various cell types (e.g fibroblast, leukocytes, endothelial cells) integrin-mediated adhesion to fibronectin has been shown to induce changes in cytoplasmic pH that correlates with the ability of cells to spread on matrix proteins (Ingber et al., 1990). The mechanism of integrin control of intracellular pH involves microclustering and membrane immobilization of  $\alpha 5 \beta 1$  integrin and subsequent opening/activation of a sodium /hydrogen ( $\text{Na}/\text{H}^+$ ) antiporter (Schwartz et al., 1991). In some cases the activation of the  $\text{Na}/\text{H}^+$  antiporter is mediated by integrin-dependent augmentation of growth factor induced signaling to Protein Kinase C (Schwartz and Lechene, 1992). These

experiments showed that the pathways by which integrins transduce signals can synergize with growth factor receptor initiated pathways to regulate cellular alkalization.

The activation of integrins and transduction of adhesion-dependent signals involves many steps. First, prior to ligand binding to integrins, intermolecular interactions between cytoplasmic tails of  $\alpha$  and  $\beta$  subunits appear to be important in “priming” the affinity of integrin extracellular domains for the ligand (Hughes et al., 1996). The interaction between cytoplasmic tails of  $\alpha$  and  $\beta$  subunits depends on the conformational rearrangements mediated by the membrane spanning region of each integrin subunit (Du et al., 1993).

Second, integrin ligation to extracellular matrix components (e.g. fibronectin) further induces conformational changes (activation of integrins) that are propagated to the transmembrane and cytoplasmic domain of integrins in an allosteric manner to induce  $\alpha\beta$  dimer formation (Liddington and Ginsberg, 2002).

Third, integrin oligomerization induces the recruitment of downstream effectors via interactions with the cytoplasmic tails of  $\alpha$  and  $\beta$  subunits (Brunton et al., 2004). The cytoplasmic tails of  $\beta$  subunits have been shown to mediate stable interactions with a greater number of partners than the cytoplasmic tails of  $\alpha$  subunits (Liu et al., 2000a). The  $\beta$  subunit cytoplasmic tail associates with an array of different proteins, including the actin binding proteins, talin, filamin,  $\alpha$ -actinin, and myosin (Horwitz et al., 1986; Jenkins et al., 1998; Otey et al., 1990; Pavalko et al., 1989), the scaffolding proteins, paxillin, Grb2, Rack 1 (Law et al., 1996; Liliental and Chang, 1998; Schaller et al., 1995) and the protein kinases, integrin linked kinase (ILK) and FAK (Hannigan et al., 1996) (Schaller et al., 1995).

Fourth, integrin oligomerization leads to activation of ILK and FAK. Integrin-initiated signaling and further signal propagation to integrin binding partners has been

shown to be important in many aspects of cell biology including cell migration. (Schwartz, 1997; Schwartz, 2001).

Integrin activation initiates signaling pathways that support membrane protrusions (Schwartz and Ginsberg, 2002). During cell migration, activated integrins are found predominantly at sites of lamellipodial protrusions (Kiosses et al., 2001). Integrins via their ability to initiate the formation of adhesions and localize signaling to the cytoskeleton are the main plasma membrane receptors that regulate protrusion formation and adhesion dynamics in polarized cell migration (Ridley et al., 2003). However, growth factor receptor activation has been shown to mediate pro - migratory signals. In the following paragraph, I will describe activation of selected soluble factor receptors that are important to consider when studying cell migration.

#### **Soluble Factor Receptor Activation.**

Cells plated on ECM components in the absence of soluble mitogenic factors initiate lamellipodia protrusions but fail to polarize and migrate (Schwartz and Ginsberg, 2002). These experiments indicate that soluble factors support polarization and cell migration. Among the vast array of cell surface soluble factor receptors, Lysophosphatidic Acid (LPA), and Platelet Derived Growth Factor (PDGF) receptors are relevant to this work. Therefore, I will focus my attention on these receptors.

LPA receptors belong to a family of Edg receptors. Edg receptors are G protein-coupled receptors. LPA can recognize four different Edg receptors (edg 1-4). Edg 1 receptor is the most widely expressed receptor in the body (Mills and Moolenaar, 2003). Fibroblasts isolated from Edg 1 receptor null mice showed decreased but not abrogated

migration in response to LPA, suggesting some redundancy among Edg receptors (Contos et al., 2000).

LPA binding to Edg receptors activates heterotrimeric guanine nucleotide binding regulatory proteins (G proteins) (Rozengurt, 2007). G proteins are composed of  $\alpha, \beta, \gamma$  subunits. G proteins are classified into Gs, Gi, Gq and  $G_{12/13}$  (Rozengurt, 2007). LPA has been shown to couple via Edg receptors to Gq, Gi and  $G_{12/13}$  (Mills and Moolenaar, 2003).

The activation of G proteins by agonist starts with binding of the cytoplasmic loop of an agonist receptor to a GDP-loaded  $\alpha$  subunit of the G protein. As a result agonist-bound receptor provokes the exchange of GDP for GTP within the G protein  $\alpha$  subunit. This reaction induces dissociation of the  $\alpha$  subunit from the  $\beta\gamma$  subunit and promotes both  $\alpha$  and  $\beta\gamma$ -dependent downstream interactions. The system returns to the native state by the hydrolysis of GTP by the  $\alpha$  subunit (Johnston and Siderovski, 2007).

There are two isoforms of PDGFR,  $\alpha$  and  $\beta$  (Ullrich and Schlessinger, 1990). Both isoforms are composed of extracellular ligand binding domains, transmembrane domains, juxtamembrane domains, protein tyrosine kinase domains and C terminal tail (Dibb et al., 2004). The ligand binding domain is composed of five immunoglobulin like repeats (Ullrich and Schlessinger, 1990). The transmembrane domain anchors the receptor within the plasma membrane, thereby connecting the extracellular environment with the internal compartment of the cell. The juxtamembrane (JMR) domain serves as an autoinhibitory domain in PDGFR kinase activation. Agonist binding promotes the kinase domain-catalyzed tyrosine phosphorylation on tyrosine residues within the receptor, thereby activating the PDGFR (Dibb et al., 2004).

Upon ligand binding to the extracellular domain, PDGFR dimerizes to form  $\alpha\alpha$ ,  $\beta\beta$  or  $\alpha\beta$  PDGFR dimers. Dimerization partially activates the kinase domain leading to

autophosphorylation of a critical tyrosine residue (Y-857) within the kinase domain. Following this partial activation, the JMR unlocks the catalytic activity of the receptor which promotes further phosphorylation of residues within the kinase domain, the JMR and the C terminal tail (Johnson et al., 1996). Tyrosine phosphorylation within these domains creates high affinity binding sites for tyrosine rich recognition sites containing (Src homology-2) proteins (Dibb et al., 2004)

Along with integrins, G proteins and tyrosine kinase receptors have been shown to coordinate polarized cell migration in various systems (Ridley et al., 2003). Their effect on migration is confined to the ability to regulate Ras homologous gene family of small GTPases (Rho GTPases) - the main drivers of cytoskeleton dynamics. In the following paragraph, I will focus on the activation of Rho-GTPases and their effect on the cytoskeleton.

### **Rho GTPase activation and the cytoskeleton**

Rho family GTPases belong to the low molecular weight (21 KD) protein family (small G proteins) of Ras-like GTPases (Ridley and Hall, 1992). Rho GTPases are ubiquitously expressed, and there are 22 members in the family. They act as molecular switches to control signal transduction pathways initiated by matrix and soluble growth factor receptors (Schwartz, 2004). The primary function of Rho GTPases is to regulate cytoskeleton dynamics (Bishop and Hall, 2000). Activation of Rho GTPases depends on the cycling between GDP- and GTP- bound state of the Rho molecule. When Rho is bound to GTP it is considered activated. On the other hand, when GDP is bound to Rho, the molecule is non-functional. These two nucleotide-bound states of Rho are tightly controlled by accessory proteins like guanine dissociation inhibitors (GDI), guanine

nucleotide exchange factors (GEF) and GTPase activating proteins (GAPs) (Rossman et al., 2005).

GDI binds the isoprenyl moiety of Rho and sequesters it from the plasma membrane, creating a cytoplasmic pool of Rho available for activation. GEFs have affinity for GDP-bound Rho and facilitate exchange of GDP to GTP, thereby activating Rho proteins. There are 80 known GEFs. Although, there is great structural diversity among various GEFs, one common theme is that most of them possess diffuse-B-cell lymphoma (Dbl) homology (DH) domains. The DH domain of GEFs is required for interaction with Rho and nucleotide exchange (Rossman et al., 2005). However, there are GEFs that do not have DH domains but instead utilize DOCK homology regions to activate Rho proteins (Brugnera et al., 2002; Meller et al., 2002) .

GAPs bind to GTP-loaded Rho, increase intrinsic enzymatic GTPase activity, and promote release of phosphate, thus, inactivating Rho. GDP-bound Rho is again sequestered by GDI from the plasma membrane to the cytosol, and the cycle repeats (Reuther et al., 2001).

Tissue culture studies using activated forms of Rho showed their involvement in the regulation of the actin cytoskeleton (Raftopoulou and Hall, 2004). For instance, the small G protein Rac, when overexpressed in fibroblastic cells, promotes formation of broad membrane protrusions. Rac-dependent induction of lamellipodial protrusions is ascribed to activation of the Arp2/3 complex that facilitates polymerization of actin within lamellipodia (Wells et al., 2004).

Another small G protein is Rho A. Overexpression of Rho A in fibroblasts promotes actin bundle and adhesion formation (Ridley and Hall, 1992). Rho A-mediated regulation of the actin cytoskeleton is mainly attributed to activation of Rho effectors including Rho-associated coil-coil kinase (ROCK) that regulates myosin-dependent actin

contractility and the formin diaphanous Dia-dependent actin polymerization (Bishop and Hall, 2000).

Still another member of the small G proteins is cell division cycle GTP binding protein 42 (Cdc42). Overexpression of Cdc42 in fibroblasts results in the predominant formation of filopodia (Ridley, 2001). Cdc42-dependent induction of filopodia is mainly mediated by its effector, Wiskot Aldrich Syndrome protein (WASP) (Welch and Mullins, 2002). However, recent studies indicated that filopodia could be formed in the absence of Cdc42, suggesting other compensatory mechanisms (Czuchra et al., 2005).

Coordination of cell protrusions with adhesion formation and disassembly, movement and trailing edge retraction during migration could be achieved by spatially localized signaling to small G proteins. One possible way to focus globally initiated small G protein signaling to a specific cellular region is the utilization of guanine exchange factors that are locally concentrated. Among the family of Dbp containing proteins there are GEFs that receive rather general signals from plasma membrane receptors and localize these signals to various cellular locales.

The group of Rho GEFs that link receptor-coupled heterotrimeric G protein signaling with Rho activation can localize to different subcellular locations (Banerjee and Wedegaertner, 2004). P115 RhoGEF and the PDZ domain-containing Rho GEF (PDZ-RhoGEF) operate via the regulator of G-protein signaling homology sequence (RGS) which binds to  $G\alpha_{12/13}$  and increases its nucleotide catalytic activity (Chikumi et al., 2004).  $G\alpha_{12/13}$  binding to the RGS domain of these GEFs promotes GEFs binding to Rho, nucleotide exchange activity within Rho and Rho activation (Rossman et al., 2005) (Figure 5). Although the ways of linking heterotrimeric G protein signaling to Rho activation are the same between these two GEFs, their subcellular localization suggests a differential focusing of the Rho A signal.

Over-expressed PDZ-Rho GEF but not p115RhoGEF is localized to the actin cytoskeleton in epithelial (Banerjee and Wedegaertner, 2004) and neutrophil cells (Wong et al., 2007). In neutrophils, PDZ-RhoGEF is mainly found within actin and myosin structures present in the trailing edge, suggesting that Rho signals to actomyosin within the trailing edge of migrating neutrophils (Wong et al., 2007). On the contrary, p115 RhoGEF is predominantly localized to the plasma membrane (Banerjee and Wedegaertner, 2004). When p115 RhoGEF is overexpressed in polarized neutrophils, it mainly localizes to the protruding front edge, suggesting Rho signals are localized to components within the membrane protrusions (Francis et al., 2006). It is clear from these experiments that signals imitated by the same set of G proteins can diverge to different cellular locations and this depends on specific GEF utilization.

GEF molecules can couple Rho signaling to distinct cellular locales, however, another set of Rho signaling proteins is required for Rho dependent cytoskeleton regulation. The immediate effectors of Rho signaling are the main bridges between Rho GTPase activation and cytoskeleton remodeling (Bishop and Hall, 2000). For instance, RhoA-mediated regulation of the actin cytoskeleton is ascribed to two effector molecules, ROCK and Dia (Schwartz, 2004).

ROCK belongs to the family of serine threonine kinases. The ROCK molecule contains a N-terminal kinase domain, the Rho binding domain (RBD) the pleckstrin homology domain (PH) and a cysteine-rich region (CRD) (Leung et al., 1995) (Figure 6). In the inactive state, the C-terminal RBD, the PH and the CRD of ROCK are folded over and bound to the N-terminal kinase domain (Figure 6). Binding of Rho to the RBD of ROCK induces the release of the kinase domain from the C-terminal domain, thus activating the kinase (Riento and Ridley, 2003) (Figure 6).



Two ROCK isoforms have been identified, ROCK I and ROCK II (Nakagawa et al., 1996). The amino acid sequences of both ROCKs have 65% overall identity, and within the kinase domain the identity is 92%, indicating ROCK's redundancy in substrate phosphorylation (Nakagawa et al., 1996). The isoforms differ in amino acid sequence within their PH domains, suggesting a possible source of specificity (Yoneda et al., 2005). ROCK I and ROCK II are ubiquitously expressed in tissues; however, the amount of protein expression varies among cell types. ROCK II seems to have a higher expression level in muscle cells and certain neuronal cells, which could indicate a predominant role in these types of cells (Leung et al., 1995; Nakagawa et al., 1996).

One of the hallmarks of both ROCK I and II activity is the phosphorylation of substrates that are linked to actin cytoskeleton contractility (Riento and Ridley, 2003). ROCK I and II negatively regulate myosin light chain phosphatase (MLCP) by phosphorylation at threonine 696 (Ito et al., 2004). Active MLCP negatively regulates myosin light chain activity by dephosphorylation (Kimura et al., 1996). In addition, ROCK I and ROCK II have been shown to directly phosphorylate serine 19 on myosin light chains, a main regulatory unit of non muscle myosin activity (Amano et al., 1996b). Pharmacological suppression of ROCK activity results in the loss of actin bundle formation, further supporting that the main function of ROCK is to regulate Rho-dependent actomyosin contractility (Yoneda et al., 2005).

Another RhoA effector implicated in the regulation of cytoskeleton dynamics is the filamentous actin nucleating protein diaphanous Dia (Bishop and Hall, 2000). There are two isoforms of mammalian diaphanous, mDia 1 and mDia 2. Similarly to ROCK I and II, mDia1 and mDia 2 are activated by Rho binding to RBD (Wallar et al., 2007). The role of mDia is confined to the Arp2/3 independent polymerization of actin and formation of filopodia (Pellegrin and Mellor, 2005) and, it was recently shown, to

mediate the formation of a subset of actin bundles (Hotulainen and Lappalainen, 2006) and stabilization of microtubules (Bartolini et al., 2008; Palazzo et al., 2001).

Rho GTPases are the main regulatory molecules of the actin cytoskeleton. Targeting Rho GTPase signaling to specific cellular compartments is achieved by selecting appropriate GEF and effector molecules that can activate the signals locally. In actively migrating cells, globally generated signals have to be directed to protrusions and trailing edges. In the following section I will discuss how globally generated signals co-ordinate cell migration.

### **Signaling and cell migration**

Cell migration is initiated when cell surface receptors recognize a stimulus that leads to the formation of a predominant lamellipodium in the direction of migration (Ridley et al., 2003). The abundance of receptors for soluble factors as well as integrins on the cell surface allows the cell to bind various ligands and transform the ligand receptor interaction into intracellular signals (Ridley et al., 2003). Studies in neutrophils have revealed that receptors that are coupled to heterotrimeric G proteins initiate intracellular signaling that drives polarized cell migration (Neptune and Bourne, 1997). Formyl-methionyl-leucine-phenylalanine (fMLP) binds to its receptor and activates the heterotrimeric protein  $G_i$  (Neptune and Bourne, 1997). The  $\beta\gamma$  subunits of  $G_i$  in neutrophils activate phosphatidylinositol kinase (PI3K) which in turn mediates localized production of phosphatidylinositol 3,4,5 phosphate (PIP3) and activation of Rac (Wang et al., 2002). Inhibition of heterotrimeric  $G_i$  activation with Pertussis toxin (PTX) results in the failure of neutrophils to form lamellipodia and polarize (Srinivasan et al., 2003). Therefore  $G_i$  activation is linked to lamellipodia protrusion by the local increase in PIP3

production and Rac dependent actin polymerization. While Gi is distributed evenly across the cell surface of neutrophils, PIP3 is detected only in lamellipodia but not the trailing edge. The localized presence of PIP3 phosphatase in the trailing edge is responsible for turning off the PIP3 signal. The polarized distribution of PIP3 phosphatase ensures the polarity following G coupled receptor activation (Iijima et al., 2002).

During fMLP stimulation, another set of heterotrimeric G proteins is activated. The fMLP-mediated activation of G12/13 leads to the induction of Rho activity, followed by activation of ROCK, and the subsequent activation of actomyosin contraction and trailing edge retraction (Xu et al., 2003). The forced expression of a dominant negative form of G 12/13, or the pharmacological inhibition of ROCK results in the failure of trailing edge retraction in neutrophils, indicating that the Rho pathway emanating from G12/13 is crucial to the control of trailing edge retraction during polarized migration (Xu et al., 2003). Rho A activation in the trailing edge of neutrophils is achieved by the coupling of PDZ-RhoGEF to actomyosin in the trailing edge (Wong et al., 2007). The experiments in neutrophils showed that signals initiated by heterotrimeric G proteins are spatially distributed by utilization of molecules that partition to the lamellipodium (PIP3) and the trailing edge (PDZ-RhoGEF).

In addition to receptors for soluble factors, the engagement of integrins with ECM has been shown to stimulate biochemical signals that support polarized protrusion formation (Ridley et al., 2003). Early studies indicated that cells plated on ECM components in the absence of growth factors extended protrusions in a similar fashion to adherent cells stimulated with growth factors (Schwartz, 2001). Subsequently it was shown that integrin -dependent protrusions correlated with tyrosine phosphorylation of the guanine nucleotide exchange factor Vav and activation of the small GTPases Rac and Cdc42 (del Pozo et al., 2003; Yron et al., 1999). Integrins activate protrusive signals by

stimulating localized translocation of activated Rac to specific membrane domains found in the regions of active protrusions (del Pozo et al., 2004). Targeting GTP-Rac to plasma membrane domains depends on adhesion-mediated regulation of specific membrane domains called lipid rafts. In cells that are detached, lipid rafts containing Rac are fully internalized into endosomal compartments. Following cell attachment, lipid rafts along with GTP-Rac are transported via microtubules and actin to the plasma membrane to support active protrusions (Balasubramanian et al., 2007). In support of this model, studies show that active integrins, active Rac and lipid rafts are predominantly localized to protruding lamellipodia during cell migration (Del Pozo et al., 2002; Kraynov et al., 2000). It is likely that localization of active Rac to lamellipodia protrusions is mediated by de novo integrin-dependent activation of a Rac GEF that is localized to membrane domains that are absent from the trailing edge.

In summary, signal transduction pathways that drive polarized migration are spatially restricted to specific cellular locales. The protrusion of the lamellipodia is driven by localized activation of Rac and Arp2/3-dependent actin polymerization. Trailing edge retraction is achieved by activation of Rho and actomyosin contractility. Any deviation from the proper signal localization can change the migratory behavior of normal cells and lead to the development of disease. In the following paragraphs I will describe the plasticity of migratory behaviors that are developed during cancer.

### **Cancer cell migration**

Cellular migration abnormalities have been attributed to pathological situations. Cancer metastasis is characterized by acquisition of new migratory proprieties that allow cells to disseminate to sites distant from the primary tumor. Tumor cells show great

diversity in migratory behavior (Wolf and Friedl, 2006). For instance, tumors of connective tissue such as fibrosarcomas (Wolf et al., 2003) exhibit increased fibroblast-like (mesenchymal) migration. Mesenchymal tumor migration relies on integrin-dependent adhesion dynamics and actomyosin contractility (Itoh et al., 1999). Tumors of the kidney (Wood, 1958), mammary gland (Farina et al., 1998), lymphomas, leukemia (Verschuere et al., 1991) and small cell carcinoma of the lung and prostate (Rintoul and Sethi, 2001) migrate distinctly from connective tissue tumors. They tend to glide over the substrate without establishing classical lamellipodia and trailing edges (amoeboid like migration). These tumors express low levels of  $\beta 1$  and  $\beta 3$  integrins and have a low degree of adhesion to the extracellular matrix (Kraus et al., 2002; Rintoul and Sethi, 2001). This property allows them to readily detach and migrate from the site of the primary tumor. A third type of migration, so-called chain migration (Friedl and Wolf, 2003) is exhibited by non-neoplastic neuronal crest cells (Jacques et al., 1998), myoblasts (El Fahime et al., 2000), melanomas (Friedl et al., 1997), mammary carcinomas and ovarian cancers (Pitts et al., 1991; Sood et al., 2001). Chain migration is characterized by one cell following another in a strand like fashion. These cells tend to align and move along ECM fibrils preserving their top and bottom cell-cell junctional contacts. It is likely that this form of migration depends on adhesion dynamics and actomyosin contractility (Wolf and Friedl, 2006). Another example of cancer cell migration is collective migration and invasion (Friedl and Wolf, 2003). This type of behavior is similar to the migration of cell sheets during three layer embryogenesis or mammary gland morphogenesis (Davidson and Keller, 1999). The primary characteristic of collective sheet migration is the preservation of strong cell-cell adhesions that allow formation of cortical actomyosin assembly around the group of cells (Hegerfeldt et al., 2002). A subset of highly protrusive cells is on the margins of the sheet. Cells using this form of movement appear

to use  $\beta 1$  integrins and adhesion/actomyosin-dependent tension to move the sheet on the basement membrane (Friedl et al., 1995). In addition, these cells secrete metalloproteases to degrade the ECM and move the sheet through the basement membrane (Nabeshima et al., 2000) (Figure 7).

Migratory tumor cells show vast plasticity in switching between types of migration modes. It often happens that tumor cells compensate the loss of one particular migratory characteristic by acquiring another one (Wolf and Friedl, 2006). The well established example of this phenomena is epithelial–mesenchymal transition (EMT) (Thiery, 2002). Epithelial tumor progression and differentiation results in transition from a collective tumor migration into a single cell disseminated migration in which the cells move in fibroblast-like fashion and tend to lose cell-cell junctions (Moll et al., 1993). Another example of tumor cell plasticity is mesenchymal amoeboid transition (AMT) (Wolf et al., 2003). This transition is characterized by cell morphology changes (fibroblastic-like shape to round and ecdysoid-like shape), a decrease in integrin expression and proteolytic activities.

The plasticity of the migratory properties of tumor cells is dictated in large part by changes applied to the environment in which these cells live. For instance, pharmacological therapies involving inhibition of integrins and metalloproteases results in changes in tumor cell migration from collective and/or mesenchymal like migration to amoeboid like migration (Hegerfeldt et al., 2002; Zucker et al., 2000). On the other hand, reports show that tumor cells treated with cytostatic drugs and irradiation can revert from amoeboid-like migration to a collective migration pattern (Kraus et al., 2002). These examples clearly indicate that changes in migratory behavior of tumor cells represent forms of evasive mechanisms from existing cancer therapy. Therefore, it is crucial to decipher the molecular mechanisms that regulate different types of migration adopted by

tumor cells during metastasis. One of the properties of highly metastatic tumors is hyperactivation of signaling pathways that regulate migration. In the following paragraphs, I will introduce selected protein kinases that have been implicated in the regulation of cell migration.

### **Src protein tyrosine kinase.**

In 1909, Peyton Rous working with avian sarcoma tumors noticed that cell free extracts from tumors could initiate tumor outgrowth in healthy animals. Later it was discovered that tumor cell free extracts contained virus particles that were responsible for tumor outgrowth (Martin, 2004). Characterization of the RNA genome of one transforming isolate, designated Rous Sarcoma Virus (RSV) showed that a single viral gene was responsible for the transforming activity of the virus (designated *v-Src*). Using DNA renaturation techniques, it was shown that this transforming gene was a mutated version of a normal avian Src gene (designated *c-Src*)(Cordell et al., 1978). In the late seventies, researchers inoculating rabbits with RSV raised the first antibodies to the protein product of *v-Src*, Src protein (Brugge and Erikson, 1977; Brugge et al., 1978). Subsequently it was recognized that the Src protein was a unique protein kinase, capable of phosphorylating tyrosine residues (Sefton et al., 1980).

The comparison of the amino acid sequences of multiple member of the tyrosine kinase family revealed several highly conserved domains, designated SH1, SH2 and SH3 for Src homology domains 1, 2 and 3. In the case of *c-Src*, a myristoylated N-terminal segment that is unique to Src is followed by the SH3 domains ( a domain that binds to proline-rich sequences), the SH2 domain (a recognition domain for phosphotyrosine containing sequence motifs), a linker region, the SH1 domain (the highly conserved

kinase domain) and a short C-terminal tail containing negative regulatory tyrosine residue 527 (Boggon and Eck, 2004). Crystallographic analysis of inactive c-Src revealed that the SH3 and SH2 domains turn inward over the kinase domain and lock the kinase domain in an inactive confirmation (Schindler et al., 1999) by virtue of an interaction between the c-terminal phosphotyrosine and the SH2 domain. Activation of c-Src is achieved by the release of the SH3 and SH2 domains from the kinase domain and by removal of the C-terminal phosphotyrosine. This event is initiated by protein phosphatase-dependent dephosphorylation of the negative regulatory phosphorylation on tyrosine 527 or high affinity ligand binding to the SH3 domain and release of intramolecular interactions within c-Src (Arias-Salgado et al., 2003). Following opening of the molecule, c-Src phosphorylates itself on tyrosine 416 within the kinase domain and achieves the complete activation state (Parsons and Parsons, 2004).

Activation of c-Src has been shown to take place downstream of substrate adhesion receptors (Shattil and Brugge, 1991). There are a series of experiments demonstrating that c-Src participates in adhesion dependent signaling (Playford and Schaller, 2004). First, Src has been found to interact directly with the  $\beta 3$  integrin subunit (Arias-Salgado et al., 2003) and adhesion components like FAK, paxillin and the scaffold protein p130 Cas (Parsons et al., 1994). Second, various forms of activated Src have been shown to localize to adhesion structures in fibroblastic cells (Yeo et al., 2006). Third, in Src-transformed cells, a adhesion-associated proteins showed increased tyrosine phosphorylation (Schaller et al., 1993). One of the proteins that showed increased phosphorylation in cells transformed with activated Src was FAK. In the following paragraphs I will describe proprieties of FAK as a main component of adhesion signaling.



### **FAK structure and regulation.**

FAK is a non-receptor tyrosine kinase of molecular weight 124 kDa (Guan et al., 1991; Hanks et al., 1992; Schaller et al., 1992). FAK is closely related to the protein tyrosine kinase PYK2. FAK and PYK2 share different degrees of sequence similarity within specific domains, with the highest similarity within the the kinase domains (60%). The amino terminal domain of FAK is termed the FERM domain (erythrocyte band four.1-ezrin-radixin-moesin), because of its sequence similarity to the domains found in JAK family kinases as well as, ezrin, radixin and moesin (Girault et al., 1999). FERM domains have been shown to interact directly with transmembrane receptors as well as phosphoinositides (Di Paolo and De Camilli, 2006; Garcia-Alvarez et al., 2003). The FERM domain of FAK is a three lobed protein-interaction domain (Lietha et al., 2007). Previous experiments demonstrated that this domain interacts with peptides derived from the cytoplasmic tails of  $\beta 1$  integrins (Schaller et al., 1995) and co-precipitates with the receptors for EGF and PDGF (Sieg et al., 2000). Using lipid protein overlay assays, it has been recently shown that the FERM domain of FAK interacts with phosphoinositide 4,5 phosphate (PIP2) (Cai et al., 2007). The FERM domain is connected to the central kinase domain via a 40 residue linker region containing a proline-rich domain that is positioned adjacent to tyrosine 397, which is the major autophosphorylation site within FAK. The C-terminal region of FAK is composed of 150 amino acids called the focal adhesion targeting domain (FAT). Located between the catalytic and FAT domain are two proline-rich sequences that mediate interactions with SH3-domain containing proteins (Playford and Schaller, 2004).

The crystal structure of autoinhibited FAK reveals that the FERM domain interacts with the kinase domain through the F-2 lobe of FERM and the C-lobe of the

kinase domain (Lietha et al., 2007). This intramolecular interaction creates a structure where the FERM domain blocks access to the active site cleft (where ATP binds) and to the kinase activation loop (required for transfer of the phosphate). Therefore, the proposed mode of FAK activation is release of the FERM domain from the kinase domain (Lietha et al., 2007) (Parsons et al., 2008) ( Figure 8). A likely hypothesis for FAK activation is that release of the FERM domain from the kinase domain is mediated by a high affinity ligand for the FERM domain.

FAK has been shown to be activated in response to integrins and soluble factor receptors. The mechanism of FAK activation by soluble factor receptors is poorly understood but it involves participation of Rho GTPases (Seufferlein and Rozengurt, 1994). On the other hand, integrin-dependent activation of FAK is well studied. Adhesion to a variety of ECM components induces tyrosine phosphorylation of FAK (Guan et al., 1991) predominantly on tyrosine 397. Current evidence is consistent with a model whereby the clustering of integrins on the cell surface via engagement with the ECM leads to the aggregation of the cytoplasmic tails of  $\beta$ -integrins ( $\beta$ 1,  $\beta$ 3 and  $\beta$ 5). This directly or indirectly leads to the recruitment of FAK, FAK activation (possibly through binding to the FERM domain of FAK, thus, relieving negative regulation) (Toutant et al., 2002) and outophosphorylation of tyrosine 397. Tyrosine phosphorylation of residue 397 creates a high affinity binding site for the SH2 domain of Src family kinases (Schaller et al., 1994) (Figure 8). The binding of Src to FAK leads to the activation of Src and downstream phosphorylation of adhesion substrates including paxillin and p130 Cas.

**FAK localization in cells.**

Immunostaining of chicken embryo fibroblasts with FAK-specific antibodies revealed that FAK was concentrated in the cell –ECM adhesion sites (Schaller et al., 1992), (Hanks et al., 1992). In addition, over-expression of FAK linked to GFP resulted in the localization of a large fraction of GFP-FAK to adhesion structures. However, FAK does not localize to adhesions that develop into  $\alpha 5$  integrin-containing fibrils (fibrillar adhesions) (Cukierman et al., 2001). Therefore, FAK localization into fibrillar adhesions is subjected to negative regulation most likely involving the usage of  $\beta$  integrin subunit subsets. In fibroblasts grown in a three-dimensional environment, FAK is localized to adhesions but fails to be tyrosine phosphorylated (Cukierman et al., 2001), indicating low integrin clustering at these sites (Cukierman et al., 2001). In microphages, FAK localizes with vinculin rich adhesions (Owen et al., 2007).

**FAK signaling.**

Phosphorylation of FAK in response to cell adhesion to the ECM leads to the formation of phosphotyrosine docking sites for several classes of signaling molecules. In addition to phosphotyrosine-mediated binding of proteins to FAK, the proline-rich domains found within the C-terminal region of FAK facilitate interaction with several other signaling proteins (Parsons, 2003). The most critical event in initiation of FAK downstream signaling is the clustering of integrins and integrin-dependent FAK tyrosine phosphorylation at position 397 (Schwartz, 2001). Phosphorylation of tyrosine 397 is crucial for the recruitment of Src to FAK and subsequent Src dependent tyrosine phosphorylation of FAK and FAK/Src-dependent phosphorylation of additional

substrates (Schaller et al., 1994; Schaller et al., 1995; Schaller and Parsons, 1995). Phosphorylation of Tyr397 also appears to be important for the recruitment of other SH2 domain containing proteins, including phosphoinositide 3 kinase (Akagi et al., 2002; Chen et al., 1996) and the adaptor growth factor receptor bound protein – 7 (Grb-7) (Han and Guan, 1999). The C-terminal domain of FAK has multiple protein-protein interaction sites. There are two proline-rich domains upstream of the FAT domain within the C-terminal region of FAK. The first proline-rich domain (Site I) provides the major binding sequence recognized by the SH3 domain of p130 Cas, a multifunctional adaptor protein (Harte et al., 1996). The second proline-rich domain (Site II) binds the SH3 domains of two regulators of small GTPases: GTPase regulator associated with FAK (GRAF) (Hildebrand et al., 1996) and a guanine activating protein for Arf1 and Arf6 GTPases (ASAP1) (Liu et al., 2002). The FAT domain of FAK has been shown to bind paxillin, talin (Chen et al., 1995) and the Rho guanine exchange factor (p190 RhoGEF) (Zhai et al., 2003). In addition, tyrosine 925 within the FAT domain has been shown to be a binding site for growth factor receptor bound protein (Grb2) (Schlaepfer et al., 1998) which in many systems has been shown to initiate extracellular regulated kinase signaling (ERK) (Kolch, 2005).

One of the ways to activate the ERK pathway during integrin engagement is linking FAK to the canonical Ras pathway. The recruitment of Grb2 to FAK initiates the recruitment of the Ras GTPase exchange factor Son of Sevenless (SOS), which in turn activates Ras by catalyzing the exchange of GDP for GTP. The plasma membrane associated GTP-loaded Ras recruits its effector Ras activated factor (Raf), a serine threonine kinase. Membrane-associated Raf is activated by upstream kinases including Src (Hood et al., 2003) and PAK (King et al., 1998). Activated Raf phosphorylates and

activates MAP kinase and ERK kinase (Bourett et al.), which leads to ERK activation (Renshaw et al., 1996) (Schlaepfer et al., 1999).

Activation of Raf can be also independent of the Ras pathway. Protein kinase C alpha (PKC $\alpha$ ) can directly phosphorylate Raf and induce ERK activation distinct from canonical Ras pathway (Kolch et al., 1993). MEK and ERK are thought to be core components of the ERK pathway and are presented together by scaffold molecules for upstream activation by either integrins or growth factor receptors (Kolch, 2005).

The interaction of FAK with multiple binding partners opens many possibilities for integrin-dependent signal transduction pathways. The interaction of FAK with small GTPase regulating proteins and upstream ERK activators raises the possibility that FAK co-ordinates biological processes through the regulation of actin cytoskeleton.

#### **Genetic analysis of FAK function.**

The first insights into biological functions of FAK came from studies analyzing embryos from mice in which the FAK gene had been disrupted (Ilic et al., 1995). Homozygous mutant mouse embryos at day 8.5 exhibited poorly developed mesoderm with no notochord or somites. Whereas the heart and vasculature initially developed normally, their growth was subsequently retarded. The overall phenotype was lethal and similar to that observed in mice lacking fibronectin (George et al., 1993) or  $\beta 1$  integrin, suggesting that FAK contributes to fibronectin-integrin interactions supporting development. Isolation of fibroblast-like cells from mutant embryos revealed that FAK mutant cells were circular and formed larger adhesions and migrated slower when compared to control cells (Ilic et al., 1995). These studies concluded that FAK was essential for mesoderm development and that the developmental abnormalities observed

were a consequence of impaired cellular migration. However, due to the early developmental lethality of FAK mutant embryos, it was impossible to assess the role of FAK in different tissues.

The generation of mice with Cre-mediated targeted deletion of the FAK gene in differentiated tissues (Beggs et al., 2003) has allowed a more thorough analysis of the function of FAK in differentiated cells. Targeted deletion of FAK in the brain shows a role for FAK in the organization of the forebrain. Cortical lamination of basement membrane beneath the forebrain in tissues lacking FAK was disorganized. Disruption of FAK expression in the developing dorsal forebrain resulted in neuronal ectopias, manifested by misplacement of cells within the marginal zone of the forebrain. Cells often migrated into inappropriate compartments. Finally neurons from FAK-deleted brains exhibited increased dendrite branching and loss of dendrite orientation. Overall these studies showed that FAK is crucial for brain basal lamina organization and proper regulation of directional neuronal migration within the forebrain (Beggs et al., 2003).

The conditional deletion of FAK in osteoblasts showed that FAK-deficient bone cells were delayed in wound healing (Kim et al., 2007). In addition, the deposition of basal lamina and ECM mineralization was disrupted in FAK mutant osteoblasts. Overall, this study concluded that FAK is necessary for the organization of the ECM and proper cellular migration during bone injury.

Studies in mice where FAK was deleted in endothelial cells revealed that formation of vasculature is FAK dependent (Braren et al., 2006). Mice with targeted deletion of FAK in the endothelium exhibited irregular formation of vasculature resulting in hemorrhage and lethality. Endothelial cells isolated from FAK mutant mice showed increases in cell retraction, irregular migration and cell death during vascular formation. In another study, targeted deletion of FAK in cardiomyocytes revealed that FAK is

necessary for normal heart function (Hakim et al., 2007; Peng et al., 2006). FAK mutant mice died shortly after birth and the phenotype was traced to subaortic ventricular septal defects and abnormal alignment of the outflow track of the heart. In addition, the myocardium of FAK mutant mice exhibited disorganized myofibrils. Cardiomyocytes isolated from FAK mutant mice exhibited abnormal migration to chemotactic stimuli.

In a different study, the loss of FAK in the epidermis resulted in misregulation of the hair cycle, sebaceous gland hypoplasia and epidermal thinning (Essayem et al., 2006). Primary keratinocytes isolated from the epidermis of FAK mutant mice did not proliferate in culture and died within 48 hours, indicating that FAK is required for keratinocyte growth. However, in another study, FAK deficient keratinocytes were propagated on fibroblast feeder layers, with no defects in proliferation. In this study the major observed defect was abnormal keratinocyte migration (Schober et al., 2007).

In summary, studies using targeted disruption of the FAK gene have clearly shown that FAK deletion resulted in primary deficits in the organization of basal lamina in specific tissues. In addition, in all these studies, cellular migration was disturbed – cells migrated but not efficiently, often lacking direction. These results point to the role of FAK signaling in focal adhesion/cytoskeleton organization and dynamics. Proper regulation of adhesions and the cytoskeleton have been shown to influence ECM organization in tissue culture cells (Ilic et al., 2004; Yoneda et al., 2007). Regulation of focal adhesion dynamics in tissue culture cells has been previously shown to depend on FAK and its activity (Webb et al., 2004). In particular, FAK signaling to Rho and ERK was implicated as a major pathway regulating adhesion dynamics (Lim et al., 2008; Palazzo et al., 2004; Ren et al., 2000) (Webb et al., 2004). Interestingly, FAK expression and tyrosine phosphorylation are often correlated with the metastatic potential of many

tumor cells. In the following section, I will describe the current evidence that FAK initiated pathways in cancer cells could be a potential target for therapeutic interventions.

### **FAK and cancer progression.**

FAK was initially identified as a major tyrosine phosphorylated substrate in v-Src transformed chicken embryo fibroblasts (Schaller et al., 1992). It was subsequently demonstrated that Src directly interacted with FAK and phosphorylated FAK in vitro (Schaller et al., 1994). Later, it was shown that the human *fak* gene localized to chromosome 8 and was amplified in cell lines derived from metastatic tumors of head, neck and lung cancer (Agochiya et al., 1999). Moreover, FAK mRNA was increased in 49 neoplastic human tissue samples, including adenomatous tissues, invasive tumors and metastatic tumors of different origins (Weiner et al., 1993). In another study, the authors showed that levels of FAK expression were increased by 100% in colon cancer and 88% in breast cancer (Owens et al., 1995). In a variety of tumors, the overexpression of FAK was correlated with advanced disease and poor survival prognosis (Cance et al., 2000). FAK overexpression in ovarian cancer patients was linked to metastasis to the lymph nodes and distant organs (Sood et al., 2004). These findings suggested the possibility that FAK protein levels may be a prognostic marker of cancer disease progression (McLean et al., 2005).

The mechanism(s) that underlies FAK overexpression in tumors is not fully understood. Chromosomal polyploidy has been implicated in *fak* gene amplification in metastatic head, neck and lung cancer cell lines (Agochiya et al., 1999). Recent studies of the *fak* gene promoter indicate that FAK expression is regulated by the level of p53 tumor suppressor protein (Golubovskaya et al., 2007). Using chromatin immunoprecipitation



(Chipperfield and Pyle) assays it was demonstrated that p53 directly bound to the promoter region of the *fak* gene and that mutation of sites in p53 that mediate interactions with the *fak* promoter abolished the interaction. In addition, ectopic expression of p53 in cancer cells decreased expression of FAK. These studies indicate that transcriptional silencing of *fak* could involve regulation of p53 binding to the FAK promoter.

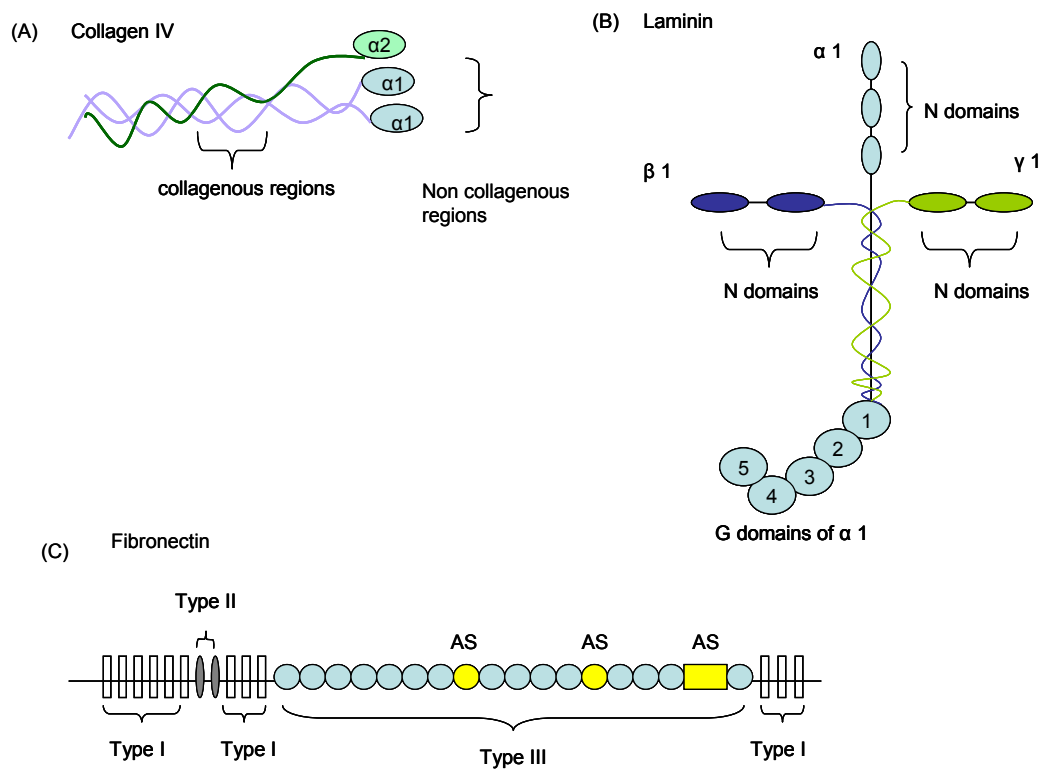
The FAK –Src signaling complex acts to recruit and phosphorylate a number of signaling proteins that are involved in the motile and invasive phenotype (Hsia et al., 2003; Schlaepfer et al., 1999). Increased tyrosine 397 phosphorylation of FAK has been reported to be associated with different tumors (Gabarra-Niecko et al., 2003). For instance, in ovarian and breast tissue, increased tyrosine phosphorylation of FAK was found in invasive tumors but not in benign tumors or normal epithelium (Sood et al., 2004).

Whereas there is little doubt that biochemical properties of FAK are changed in tumor tissues and cells, most of the evidence connecting FAK to tumorigenesis has been correlative. Today there are no data indicating that FAK mediates tumor formation or tumor progression. Ectopic over-expression of FAK in normal cells does not promote uncontrolled growth and transformation, a phenotype that is characteristic of Src overexpression. Nevertheless, the observations that FAK is overexpressed in multiple cancers and that its expression pattern correlates with poor clinical outcome indicate that FAK might be a useful therapeutic target.

## Figures

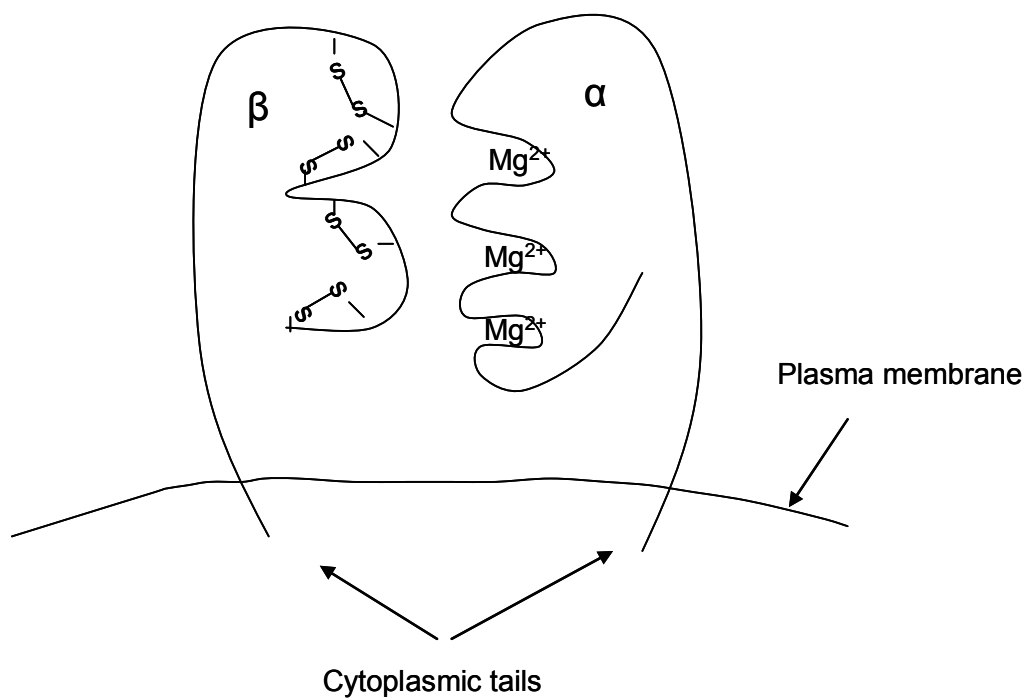
### Figure 1: Schematic representation of selected ECM proteins.

(A) Collagen trimer is composed of two  $\alpha 1$  and one  $\alpha 2$  subunits. Collagen subunits interact via  $\alpha$  helical collagenous regions. Non collagenous regions are positioned at the C-terminus of each subunit. (B) Laminin heterotrimer contains one  $\alpha$ , one  $\beta$ , and one  $\gamma$  subunit. Alpha subunit contains five G domains positioned at the C terminus. N domains are present at the N terminus of each subunit. (C) Fibronectin contains modules of type I, II and type III repeats. Alternative (AS) splicing of type III repeat exons can generate various fibronectin molecules (yellow).

**Figure 1**

**Figure 2. Schematic representation of  $\alpha$  and  $\beta$  subunits of integrins.**

Integrin,  $\alpha$  and  $\beta$  subunits are transmembrane glycoproteins with short cytoplasmic tails. The N terminus of the  $\alpha$  subunit contains the amino acid sequence (Asp-x-Asp-x-Asp-Gly-x-x-Asp) that supports divalent cation binding and is necessary for proper integrin function (I domain region). Common to all  $\beta$  subunits is a four fold repeat of a cysteine rich segment within the N terminal portion of the subunit. These repeats are thought to be important in ligand binding.

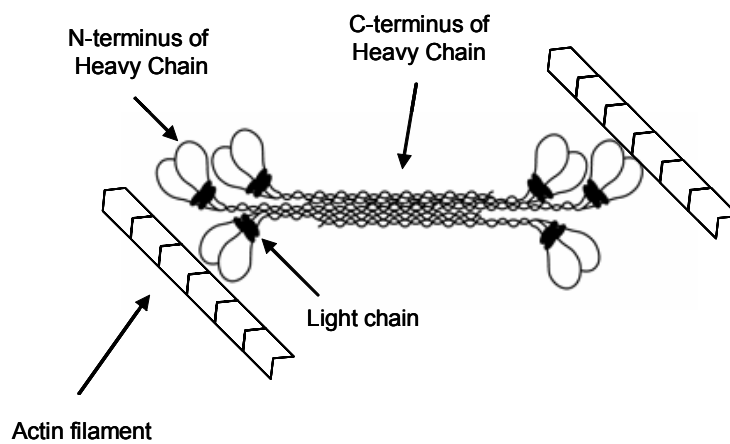
**Figure 2**

**Figure 3. Schematic representation of myosin molecule.**

Every myosin II molecule is a hexamer composed of Myosin Heavy Chain dimers (MHC) and two pairs of Myosin Light Chains (MLC). Myosin binds actin in a reversible manner and hydrolyzes ATP. The N-terminus of MHC is a globular head that contains ATPase activity and binds actin. The C-terminus of MHC is involved in myosin filament formation.

**Figure 3**

Myosin




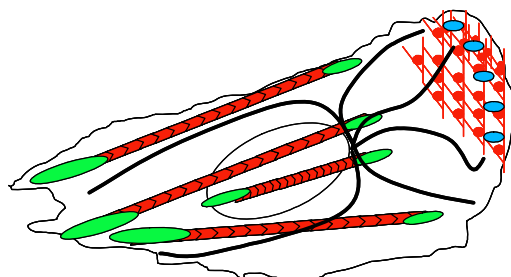


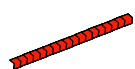

**Figure 4. Schematic representation of the cytoskeletal components during cell migration.**

(A) Cell migration is characterized by the formation of predominant a broad, protruding, leading edge and a narrow, trailing edge. Leading-edge protrusions are driven by Arp2/3-dependent actin polymerization and bending of the polymerized actin network. Protrusion is coupled to the formation of integrin–ECM adhesions, that can either disassemble or mature. Disassembly of some of the adhesions is facilitated by targeting of stable microtubules. Some of the formed adhesions will mature due to interaction with the actomyosin network. In the trailing-edge adhesions are elongated and tend to move inward during trailing edge retraction. (B) Adhesion movement (treadmilling) is characterized by simultaneous disassembly of one part of adhesion and assembly of another part of adhesion along the actomyosin cytoskeleton.

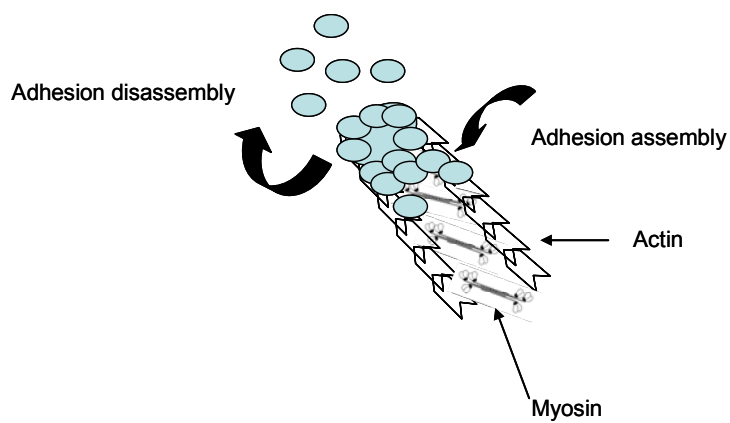


Figure 4

(A)

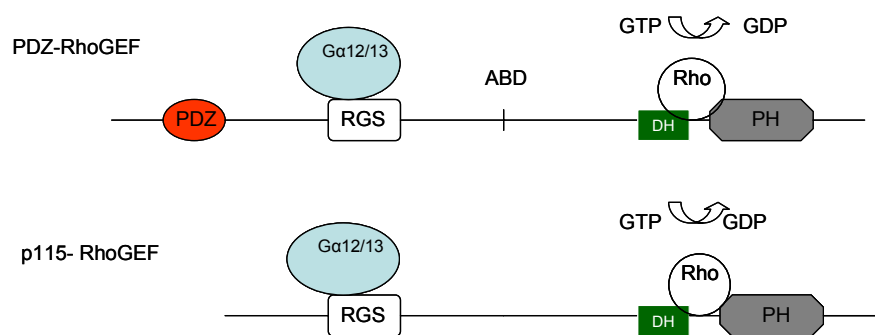
 Arp2/3 and actin at the leading edge nascent adhesions mature adhesions actomyosin bundles microtubules

(B)



**Figure 5. Domain composition of PDZ-RhoGEF and p115-RhoGEF.**

PDZ-RhoGEF and p115 RhoGEF belong to the RGS domain-containing GEFs that link heterotrimeric G protein signaling with Rho activation. p115 RhoGEF and PDZ-RhoGEF via the regulator of G-protein signaling homology sequence (RGS) bind to  $G\alpha_{12/13}$  and increase its nucleotide catalytic activity (Chikumi et al., 2004).  $G\alpha_{12/13}$  binding to the RGS domain of these GEFs promotes GEFs PH/DH domain binding to Rho, and Rho activation. In contrast to p115-RhoGEF, PDZ-Rho GEF has PDZ and actin binding (Horne et al.) domains.

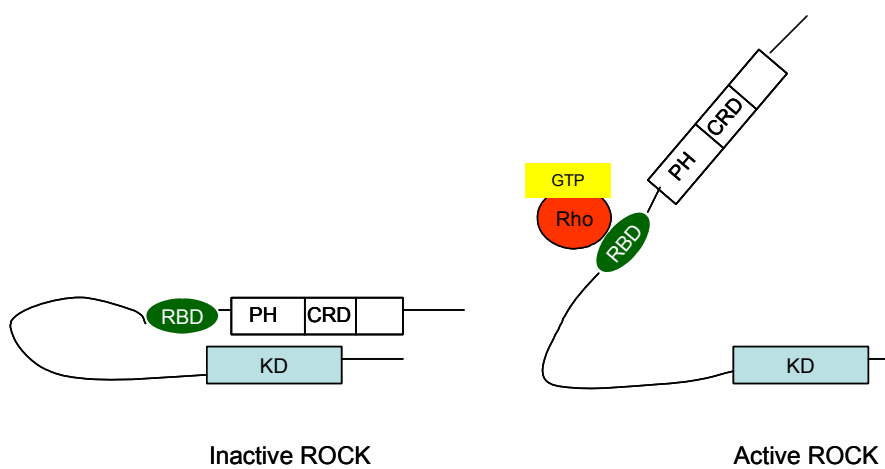
**Figure 5.**

**Figure 6. Schematic representation of Rho-induced activation of ROCK.**

ROCK belongs to the family of serine threonine kinases. The ROCK contains a N-terminal kinase domain, Rho binding domain (RBD,) pleckstrin homology domain (PH) and cystine rich region (CRD). In the inactive state, the C-terminal RBD, PH and CRD domains of ROCK are folded over and bound to the N-terminal kinase domain. Binding of Rho to the RBD of ROCK induces release of the kinase domain from the C-terminal interaction, thus activating the kinase.

**Figure 6**

Activation of ROCK



**Figure 7. Schematic representation of migratory proprieties that cancer cells can adopt.**

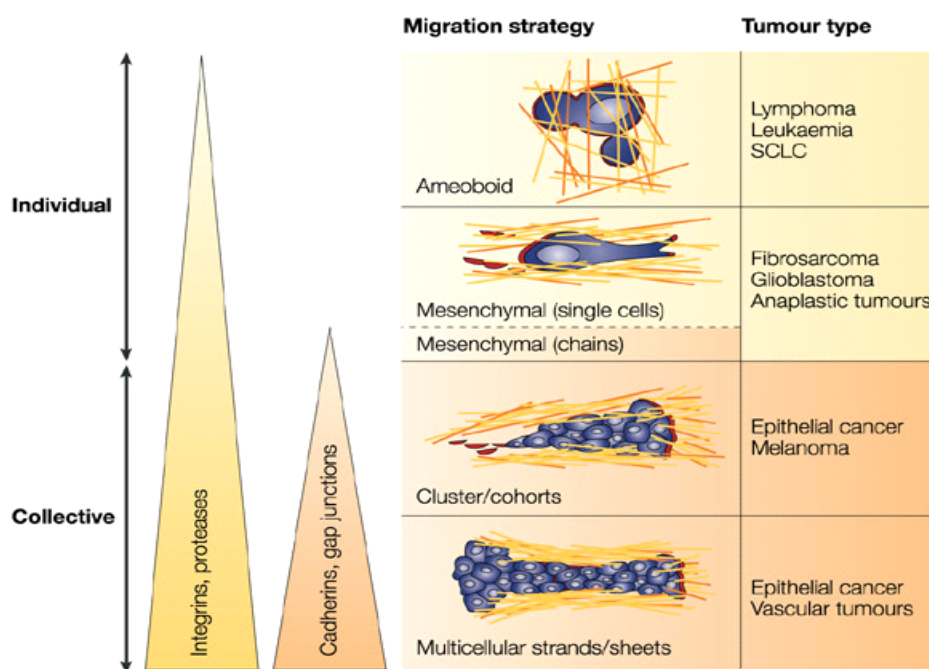
In amoeboid-like migration cells tend to glide over the substrate without establishing classical lamellipodia and trailing edges.

Mesenchymal tumor migration resembles fibroblast-like migration and relies on integrin-dependent adhesion dynamics and actomyosin contractility.

Chain migration is characterized by one cell following another in a strand-like fashion. These cells tend to align and move along ECM fibrils preserving their top and bottom cell-cell junctional contacts. It is likely that this form of migration depends on adhesion dynamics and actomyosin contractility.

In collective sheet migration cells preserve strong cell – cell adhesions that allow formation of cortical actomyosin assembly around the group of cells. On the margins of the sheet is a subset of highly protrusive cells. Cells using this form of movement appear to use  $\beta 1$  integrins and adhesion/actomyosin-dependent tension to move the sheet on the basal membrane. In addition these cells secrete metalloproteases to degrade matrix and move the sheet and invade through the basal membrane

Figure 7



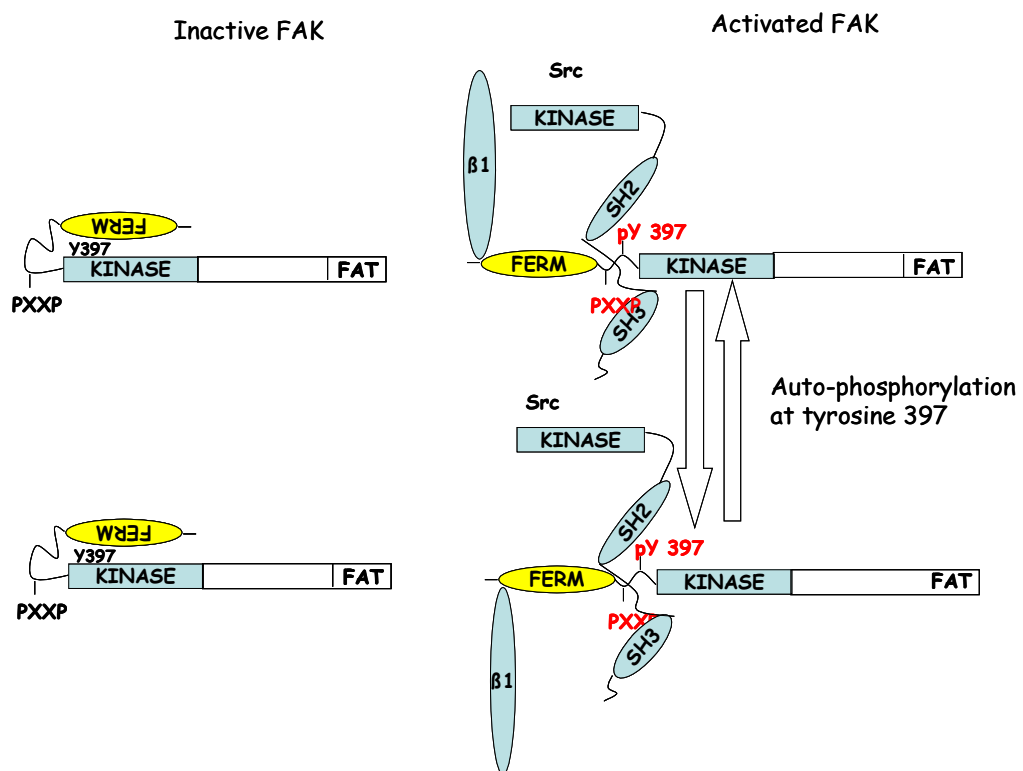
Adapted from :Peter Friedl & Katarina Wolf  
Nature Review Cancer 2003

**Figure 8. Integrin-dependent activation of FAK**

Engagement of integrins with ECM proteins results in recruitment of FAK to integrin  $\beta 1$  subunit cytoplasmic domains and release of the FERM domain from the kinase domain of FAK. FAK in its open confirmation is able to transphosphorylate itself at tyrosine 397. Phosphorylated tyrosine 397 provides a docking site for Src, which also interacts with the proline rich region of the FAK positioned upstream of tyrosine 397, initiating FAK/Src complex formation.



Figure 8



### **Dissertation focus.**

Cell migration is initiated by the formation of a leading edge lamellipodium and a distinct trailing edge. Lamellipodial substrate adhesions constantly form and disassemble whereas trailing edge adhesions tend to move inward and break during retraction. It is likely that signals regulating adhesion dynamics in the lamellipodia differ from the regulation of adhesions found in the trailing edge. In Chapter 2 and Chapter 3 of this thesis, I argue that the spatial coupling of distinct signaling pathways contributes to differential adhesion behavior found in migrating fibroblasts.

In the first part of this work I show that regulation of adhesions found within the trailing edge and concomitant trailing edge retraction in fibroblasts is regulated by growth factor-dependent regulation of FAK signaling to Rho. We chose a model in which adhesion dynamics and trailing edge retraction are promoted by activation of LPA receptors that induce Rho activation. Using this model, we discovered that FAK forms a complex with PDZ-RhoGEF. PDZ-RhoGEF localized to FAK-containing adhesions, and attenuation of either FAK or PDZ-RhoGEF resulted in the failure of LPA to induce adhesion dynamics and trailing edge retraction. Furthermore, we found that LPA-mediated adhesion dynamics and trailing edge retraction was also dependent on ROCK II signaling.

In the second part of this work which was performed in collaboration with Tomas Vomastek, I show that regulation of adhesions found in the lamellipodia depends on integrin/FAK-dependent RACK 1 targeting of the ERK pathway to de novo formed adhesions. We found that integrin-linked scaffold protein, RACK-1, channels ERK to adhesion sites for upstream activation and promotes adhesion disassembly and cell

migration. Attenuation of either RACK-1 or FAK interferes with active ERK localization to adhesions and inhibits adhesion disassembly in the lamellipodia.

In this dissertation I demonstrate that FAK signaling to Rho and the ERK pathway is critical for the regulation of different aspects of adhesion dynamics. Our work emphasizes that regulation of adhesion dynamics within distinct parts of a migrating cells depends on differential focusing of signals initiated by integrins and soluble factor receptors.

Plasticity of tumor cell migration is a result of aberrant regulation of the signals that control different migratory steps. Therefore, our work helps to understand the principles of cell migration which, when uncontrolled, could lead to tumor metastasis.

## CHAPTER 2

### FAK couples Rho Signaling to Adhesion Movement and Trailing Edge Retraction in Fibroblasts

#### Introduction

Fibroblast migration is a highly regulated process (Ridley et al., 2003). Protrusion of the leading edge and the establishment of new peripheral adhesions coupled with the maintenance of stable attachments at the cell rear create the typical asymmetrical profile of a migrating cell with a broad lamellipodia (front) and narrow long trailing edge (rear) (Regen and Horwitz, 1992). As the lamellipodium advances and trailing edge stretches the cell becomes elongated. Inward movement and subsequent release of adhesions at the rear lead to trailing edge retraction, cell body advance, and the cell becomes more rounded (symmetrical), and the cycle is repeated (Rid et al., 2005; Smilenov et al., 1999; Webb et al., 2005). Fibroblast movement requires spatial regulation of cell attachment to extra-cellular matrix (ECM). Adhesions are formed at sites of lamellipodial protrusion. Once formed, these adhesions must be strong enough to generate traction forces necessary to support translocation of the cell body. On the other hand, the trailing edge of the cells must be able to detach from the substrate to permit cell translocation (Regen and Horwitz, 1992). Detachment of the trailing edge requires continued myosin-driven cytoskeletal tension that results in movement and ultimate release (disassociation/degradation) of adhesions within the trailing edge (Palecek et al., 1998; Palecek et al., 1996; Rid et al., 2005; Smilenov et al., 1999).

LPA stimulation of several cell types induces tyrosine phosphorylation and activation of Focal Adhesion Kinase (FAK) (Bian et al., 2006; Luttrell et al., 1999; Seufferlein and Rozengurt, 1994). FAK is a non-receptor tyrosine kinase that is associated with adhesions (Hildebrand et al., 1993; Schaller et al., 1992) and sites of close contact between the cell and the extracellular matrix. FAK is activated in response to integrin engagement and its phosphorylation leads to the recruitment and activation of Src and the induction of several downstream signaling pathways (Parsons, 2003; Parsons et al., 1994). In addition FAK serves as a scaffold for the recruitment of the linker proteins, Cas (Burnham et al., 1996) and two GTPase activating proteins, ASAP (Liu et al., 2002) and GRAF (Hildebrand et al., 1996). Thus, FAK functions to recruit proteins that contribute to the regulation of adhesion signaling and promotes the dynamic remodeling of adhesions by triggering adhesion disassembly through a Src- and MEK-dependent cascade (Webb et al., 2004). Cells deficient for FAK exhibit defects in adhesion disassembly (Webb et al., 2004) and Rho regulation upon integrin (Ren et al., 2000) or LPA receptor stimulation (Palazzo et al., 2004)

In the present study, we investigate the role of LPA, FAK and PDZ-RhoGEF in the regulation of adhesion movement, trailing edge retraction and cell shape change. We observed that maintaining fibroblasts in the absence of serum results in stabilization of adhesions within the rear of the cell and significantly reduces trailing edge retraction resulting in an elongated cell phenotype. LPA but not PDGF was sufficient to restore trailing edge retraction and increase inward movement of adhesions, and to restore cell shape. Thus, the response of serum-deprived cells to LPA parallels events leading to tail retraction in a migrating cell. Using this paradigm, we provide evidence that LPA induces dynamic movement of adhesions present in the trailing edge and this process requires FAK, PDZ-RhoGEF and Rho/ROCK II function. This study provides evidence for the

spatial activation of an LPA receptor/FAK/ PDZ-Rho GEF signaling complex and suggests that modulation of adhesion movement within the trailing edges of fibroblasts is linked to Rho/ROCK II-dependent retraction.

## Materials and Methods

### Cell Culture, plasmids and siRNAs.

Rat2 and HEK293T cells were maintained in DMEM plus 10% fetal calf serum (GibcoBRL) at 37°C in 5% CO<sub>2</sub>. NIH3T3 cells were maintained in DMEM plus 10% calf serum (GibcoBRL), at 37°C in 5% CO<sub>2</sub>. For the FAK and PDZ-RhoGEF knockdown experiments, cells were transfected with siRNA oligonucleotides specific for rat FAK, GTRAP48 (PDZ-RhoGEF, Dharmacon pool) or with control siRNA oligonucleotides using calcium phosphate as previously described (Tilghman et al., 2005). FAK, ROCKI and ROCK II shRNA plasmids were obtained from Sigma (USA). For transient expression of myc-PDZ-RhoGEF, myc-PDZ-RhoGEF 1-585, myc-p115RhoGEF and GFP-FAK, Rat2 or HEK293T cells were plated in the presence of 10% serum in 100 mm<sup>2</sup> plastic culture dishes at densities 4x10<sup>5</sup> and 1.5x10<sup>6</sup> cells per dish respectively. The next day cells were transfected using Polyfect reagent (Qiagen) with 0.25 µg or 0.5 µg of the designated plasmid. Twenty four hours later cells were processed for experiments.

The Flp-In System (Invitrogen) was used to create stable cell lines expressing GFP-tagged or RFP-tagged proteins. Briefly, NIH3T3-LacZeo or RAT2-LacZeo cell lines with a flp recombinase target (FRT) site integrated in the genome were created. cDNAs encoding either Paxillin-GFP, GFP-FAK or  $\beta$ actin-mRFP fusion proteins were cloned into the *NheI* /*Bam*HI or *Hind*III/*Not*I sites of the pcDNA5/FRT vector. The host cells were then co-transfected with pOG44 plasmid encoding Flp recombinase and either pcDNA5/FRT-Paxillin-GFP, pcDNA5/FRT-GFP-FAK or pcDNA5/FRT- $\beta$ actin-mRFP, and recombinant cell lines were selected according to the manufacturer's instructions. Stable clones were analyzed for expression of paxillin-GFP, GFP-FAK and  $\beta$ actin-mRFP

by fluorescent microscopy and western blotting. Myc-tagged PDZ-RhoGEF, p115RhoGEF and (561-585) PDZ-RhoGEF-GFP plasmids were engineered as described elsewhere (Banerjee and Wedegaertner, 2004). Paxillin-GFP was a generous gift from Dr. Rick Horwitz, University of Virginia, and GFP-FAK was generated by cloning FAK from pCMV-myc -FAK vector (Xiong and Parsons, 1997) into pEGFP-C1 vector (Clontech, CA).  $\beta$ actin-mRFP was a generous gift from the laboratory of Frank Getler (MIT, Cambridge, MA). Rho effector domain mutants were generous gifts from Dr. Kodi Ravichandran, University of Virginia. FAK, ROCKI and ROCK II shRNA plasmids were obtained from Sigma (USA) shRNA library. Lentiviral packaging plasmids pMDG and pCMV $\delta$ R 8.2 were generous gifts from Dr. David Rekosh (University of Virginia). Lentiviral production was performed according to Sigma protocol. The organization of adhesions in cells knocked down for expression of FAK, PDZ-RhoGEF, ROCK I and ROCK II were assessed by immunostaining for FAK and paxillin.

#### **Serum starvation and LPA stimulation experiments.**

Serum deprivation is defined as removal of serum up to 6 hours. Serum starvation is defined as removal of serum for 12-16 hours. Rat 2 or NIH3T3 cells were plated overnight on fibronectin (1  $\mu$ g/ml) coated glass T dishes or plastic dishes in the presence of medium containing 10% serum. The following day, cells were provided medium containing serum, serum-free medium or serum free medium plus LPA (2  $\mu$ M).



### **Western Blotting.**

Cells were lysed in 200  $\mu$ l of RIPA buffer (50mM HEPES, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.1 M NaCl, 1 mM sodium orthovanadate, 0.1 M sodium pyrophosphate, 100 mM NaF and 1mM PMSF). Lysates were clarified by centrifugation at 13,000 rpm for 10 minutes. Clarified lysates were boiled in 1x sample buffer (0.04 M Tris-HCl pH 6.8, 1% SDS, 1%  $\beta$ -mercaptoethanol and 10% glycerol) for 10 minutes and resolved by SDS-PAGE. Proteins were transferred to Immobilon membrane (Whatman) and blocked with 5% BSA in PBS (140 mM NaCl, 0.27 mM KCL, 0.43 mM  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 0.14 mM  $\text{KH}_2\text{PO}_4$ , pH 7.3), 0.1% Tween 20, pH 7.2 for 1 hour at room temperature. The membranes were incubated overnight at 4°C with one of the following antibodies: anti-actin (Zymed, 1:5000 dilution), anti-FAK polyclonal antibody (UpstateBiochemicals, 1:1000 dilution), anti-pY397 FAK polyclonal antibody (Biosource, 1:1000 dilution), anti-MAPK monoclonal antibody (1:2000, Sigma), anti-phospho- MAPK polyclonal antibody (1:2000, gift from Dr. Michael J. Weber, University of Virginia), anti-GTRAP 48 (PDZRhoGEF) monoclonal antibody (1:1000, BD Transduction), anti-GFP polyclonal antibody (1:1000, Cell Signaling), anti-tubulin monoclonal antibody (1:5000, Sigma), anti-phospho tyrosine monoclonal antibody 4G10 (1:1000, UpstateBiochemicals) or anti-myc monoclonal antibody 9E10 (SantaCruz, 1:1000), polyclonal anti – ROCK II antibody (1:1000 , Upstate), monoclonal anti-ROCK I/II (1:1000, Upstate) or monoclonal anti-Paxillin antibody (1:1000, BD Transduction.) Membranes were subsequently probed with secondary antibodies linked to HRP (Amersham). Western blot membranes were developed using enhanced chemiluminescent substrate for detection of HRP (Amersham). Western blot results were prepared using AlphaEaseFC (FluorChem 8800) for Windows software.

**Immunoprecipitation experiments.**

Twenty four hours after transient transfection with indicated expression vectors cells were washed with ice cold PBS, and lysed in Immuno-Precipitation (IP) buffer (50 mM Tris-HCl, pH 7.6, 160 mM NaCl, 0.5 mM EDTA/EGTA, 1% Triton-X100, 10% Glycerol, 1 mM PMSF and 1 µg/ml Leupeptin). Cell extracts were clarified by centrifugation at 13,000 rpm and cell supernatants were applied to agarose beads (10% of total volume) conjugated with anti-myc 4A6 antibody (UpstateBiochemicals Inc). Immunoprecipitation was carried out for 2 hours at 4<sup>0</sup>C and immune complexes were washed 3 times with IP buffer and boiled in 1X sample buffer. Samples were processed for SDS-PAGE and western blotting as described above.

**Analysis of adhesion movement.**

Adhesion movements were visualized in serum deprived NIH3T3- or Rat2–Paxillin-GFP cells before and after the addition of 2 µM LPA using TIRF microscopy. The cells were maintained at 37° with a Biopetechs Delta T heated chamber system. Cells were imaged using a Nikon Eclipse TE2000-E inverted microscope equipped with TIRF illuminator, a 488 argon laser and a 60x DIC TIRF objective (NA 1.45) equipped with a Biopetechs objective heater. Images were acquired with a Retiga 1300i CCD camera and the QCapture Pro software (Q Imaging). Adhesion movement was quantified as described before (Smilenov et al., 1999); briefly the first frame of the movie was pseudo-colored green and the last frame was pseudo-colored red, and the two images were merged. Adhesions that contained both red and green and thus appeared yellow were scored as

poorly dynamic. The fraction of adhesions that moved was scored, determining the percentage of red adhesions relative to total adhesions in the first frame.

### **Phase microscopy.**

NIH3T3- $\beta$ actin-mRFP and Rat2 cells were serum deprived and filmed in the absence or presence of 2  $\mu$ M LPA using Zeiss Axiovert 135 TV microscope equipped with the CCD camera MTI 176.

### **Immunostaining of cells.**

Control, FAK or PDZ-RhoGEF siRNA-treated Rat2 fibroblasts were plated overnight on fibronectin-coated coverslips (1  $\mu$ g/ml, Sigma) in media containing 10% serum. Cells were either left untreated or serum starved overnight and then stimulated with, 2  $\mu$ M LPA with 0.1% carrier BSA or 10% FBS for indicated times. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS and blocked with 20% goat serum in PBS. The cells were washed 3 times with PBS and immunostained with the following antibodies: anti-tubulin B2-11 (Sigma, 1:5000 dilution) and anti-myc 9E10 (1:250, SantaCruz). To visualize actin cells were treated with AlexaFluor 488 conjugated phalloidin (Invitrogen, Inc). To visualize microtubules or myc-tagged GEF constructs cells were treated with secondary goat anti-mouse antibodies conjugated to AlexaFluor 594 (1:2000, Invitrogen, Inc). Fluorescent images were acquired using a Nikon Eclipse E600 upright fluorescence microscope equipped with a Hamamatsu ORCA CCD camera. Images were captured and analyzed with OpenLab (Improvision) software.

### **Analysis of trailing edge length and cell elongation.**

To measure trailing edge length, a line was drawn from the tip of the trailing edge towards the base of the nucleus. To measure cell length, a line was drawn from the center of the nucleus to the furthest visible microtubule tip. To measure cell width, a second line was drawn across the cell passing through the center of the nucleus at a 90° angle to the first line. To calculate the “elongation factor”, the length was divided by the width.

### **Analysis of lamellipodia protrusion.**

NIH3T3 cells stably expressing  $\beta$ actin-mRFP or Rat2 cells were plated in medium containing 10% serum on fibronectin-coated (1  $\mu$ g/ml) glass T dishes at the density of  $3 \times 10^4$  cells/dish. The next day, serum-containing medium was changed to serum free medium or serum free medium containing 2  $\mu$ M LPA. After 6 hours cells were filmed for a total of 15-20 min, collecting one image every 20 seconds using TIRF (NIH3T3) or DIC (Rat2) microscopy. Protrusion rates were calculated using Image J software (Wayne Rasband, National Institute of Health. USA).

### **Statistics.**

Statistical analysis was done using non-parametric, non assuming Gaussian distribution Mann-Whitney t-test using Prism software. The symbols denote the following statistical differences: \*\*\* p=0.001; \*\* p=0.01; \* p=0.05.

## Results

### **LPA stimulates trailing edge retraction and rear adhesion dynamics.**

NIH3T3 cells, cultured on fibronectin and deprived of serum for 6 hours, acquired a distinct morphology characterized by a protrusive lamellipodia and an elongated tail-like structure, which was approximately four times longer than control cells deprived of serum but then treated with LPA (Fig. 9). Interestingly, the rate of lamellipodia protrusion was the same in cells without serum and stimulated with LPA (Fig. 9). Cells serum starved overnight (12-16 hours) also displayed an elongated phenotype with significantly less protrusive activity (Fig. 10). Stimulation of serum starved NIH3T3 cells with 2  $\mu$ M LPA stimulated the retraction of the elongated tail and restored normal cell morphology whereas addition of 100 nM PDGF was much less effective at inducing normal morphology (Fig. 3). Time lapse imaging of serum starved cells stimulated with 2 $\mu$ M LPA revealed that the restoration of normal rounded morphology occurred with a  $t_{1/2}$  of 90 to 120 min (Fig. 11). To assess whether the LPA induced trailing edge retraction was reflected in changes in the dynamic behavior of adhesions, TIRF microscopy was used to monitor Paxillin-GFP labeled adhesions in cells serum starved over night and in LPA stimulated cells. As shown in Fig. 12, adhesions present in the rear of serum starved cells did not exhibit a change in either fluorescence intensity or position indicating that these adhesions were relatively stable. In contrast, LPA stimulation significantly increased the dynamics of the adhesions as measured by the inward movement of the adhesions over a 7 minute time period (Fig. 12). As previously reported the addition of LPA to serum starved cells induced a rapid increase in the tyrosine phosphorylation of FAK (Fig. 13A, B). These data indicate that LPA

signaling is sufficient to induce changes in the movement of mature adhesions found in the trailing edge and that the activation of FAK is concomitant with this process.

**The lack of FAK expression in fibroblasts results in an elongated phenotype in the presence of serum and failure to induce adhesion movement and trailing edge retraction.**

The knock down of FAK using either siRNAs designed to specifically target rat FAK (Tilghman et al., 2005) or shRNA RNA to specifically target mouse FAK resulted in an elongated cell morphology (Fig. 14A,B) when cells were grown in the presence of serum. To confirm that the observed elongation of the cells was due to the loss of FAK expression, a Rat2 cell line was created that constitutively expressed (at approximately 20% the level of endogenous FAK) GFP-tagged chicken FAK that was not targeted by the FAK siRNA (Fig. 15A). The morphology of cells expressing GFP-FAK was indistinguishable from control cells (Fig. 15B). While FAK siRNA treatment increased cell elongation in control Rat2 cells, the GFP-FAK expressing cells retained their more rounded morphology (Fig. 15B). FAK immunostaining revealed that in control cells FAK silencing resulted in the absence of endogenous FAK in adhesions (Fig. 15B). In contrast, FAK siRNA treatment of Rat2 cells expressing GFP-FAK did not decrease the expression of GFP-FAK or alter its localization to adhesions (Fig. 15B). Therefore, the changes in cellular morphology were a consequence of reduced FAK expression and correlated with the loss of FAK from cell matrix adhesions.

To examine whether FAK functions downstream of LPA to stimulate adhesion dynamics and trailing edge retraction, Rat2 cells that stably expressed paxillin-GFP (Rat2 Paxillin-GFP) were treated with control or FAK siRNA. Cells were serum starved for

twelve hours and stimulated with 2  $\mu$ M LPA and adhesion dynamics were measured using TIRF microscopy (Fig. 16). LPA readily induced dynamic movement of adhesions in control cells but failed to do so in FAK siRNA-treated cells (Fig. 16). This result is consistent with the failure of FAK siRNA treated cells to retract trailing edge in response to LPA. Consistent with these observations, addition of serum or LPA to serum starved control cells induced restoration of cell shape whereas serum starved FAK knockdown cells largely maintained their elongated morphology in response to either serum or LPA (Fig. 17). These observations are consistent with LPA controlling trailing edge retraction and changes in cell shape through a receptor pathway requiring FAK activation and downstream signaling.

**Over expression of specific Rho pathway components rescues FAK knock down phenotype.**

To determine the downstream effectors for FAK that contribute to adhesion movement and trailing edge retractions, we assessed the role of RhoGEFs and putative Rho effectors. The RhoGEF, PDZ-RhoGEF has been shown to be an important effector of the LPA receptor signaling pathway (Yamada et al., 2005). To investigate the role of this GEF, Rat2 cells treated with FAK siRNA to block FAK expression were transfected with myc-tagged PDZ-RhoGEF, PDZ-RhoGEF lacking Rho DH domain (PDZ-RhoGEF 1-585) and full length p115RhoGEF. The expression level of each of the Rho GEFs was similar (Fig. 18A) and the localization of GEFs was consistent with previously published data (Banerjee and Wedegaertner, 2004). Both PDZ-RhoGEF and PDZ-RhoGEF 1-585 but not p115RhoGEF localized to actin cytoskeleton (data not shown). As shown in Fig 18B the expression of wild type PDZ-RhoGEF but not PDZ-RhoGEF 1-585 or full length

p115RhoGEF reversed changes in cell shape induced by FAK siRNA treatment of Rat 2 cells. These data indicate that overexpressed full length PDZ-RhoGEF bypasses requirement for FAK in regulation of adhesion dynamics necessary for rear retraction and restoration of cell shape.

To assess which putative Rho effectors maybe important for the restoration of cell shape in FAK deficient NIH3T3 cells, we expressed two different active Rho effector domain mutants (Palazzo et al., 2001; Tosello-Tramont et al., 2003) in NIH3T3 cells with downregulated FAK expression (Fig.19A). As shown in Fig. 19B expression of the GFP-tagged Rho effector mutant that can activate both mDia and ROCK (RhoG14VF39V) efficiently rescued the elongated phenotype induced by FAK deficiency. In contrast, the negative control GFP-PDZ-RhoGEF (561-585) a non functional GEF with actin localization or the Rho effector mutant that activates only mDia (RhoG14VF39A) failed to rescue the elongated phenotype in FAK deficient cells (Fig. 19B). These data indicate that FAK and PDZ-RhoGEF cooperate to activate the Rho/ROCK pathway and induce trailing edge retraction.

#### **PDZ-RhoGEF-Rho-ROCK pathway is necessary for LPA induced trailing edge retraction and stimulation of adhesion dynamics**

We next sought to determine whether PDZ-RhoGEF forms a complex with FAK and localizes to FAK containing adhesions. Myc-PDZ-RhoGEF 1-585 or myc-p115RhoGEF was over expressed along with GFP-FAK in HEK293T cells. As shown in Fig.20A, GFP-FAK was readily detected in immune complexes with myc-PDZ-RhoGEF 1-585 but not in immune complexes with myc-p115RhoGEF. To test if FAK and PDZ-RhoGEF co-localized in adhesions we over expressed myc-tagged PDZ-RhoGEF, PDZ-RhoGEF 1-



585 and p115RhoGEF in Rat2 cells stably expressing GFP-FAK. We observed that myc-PDZ-RhoGEF, myc-PDZ-RhoGEF 1-585 but not myc-p115RhoGEF localized to FAK containing adhesions (Fig. 20B). To confirm the requirement for PDZ-RhoGEF in a signaling pathway that controls cell elongation downstream of the LPA receptor, Rat2 cells were treated with PDZ-RhoGEF siRNA to attenuate PDZ-RhoGEF expression (Fig. 21A). PDZ-RhoGEF siRNA treated cells exhibited elongated phenotype even in the presence of serum (Fig. 21B, phase images); a phenotype comparable to that observed in FAK siRNA treated cells. Stimulation of serum starved control and PDZ-RhoGEF siRNA treated cells with 2  $\mu$ M LPA revealed that the PDZ-RhoGEF siRNA treated cells remained elongated (Fig. 21C). TIRF microscopy was used to measure adhesion dynamics after LPA stimulation of serum starved control and PDZ-RhoGEF siRNA treated Rat2 -Paxillin-GFP cells (Fig. 22A). As shown in Fig. 22B LPA stimulation of control cells but not PDZ-RhoGEF siRNA treated cells accelerated adhesion dynamics. These data provide additional support for the role of PDZ-RhoGEF in a FAK-dependent pathway that contributes to the regulation of rear retraction and adhesion movement.

Since a Rho effector domain mutant that can not activate ROCK failed to rescue FAK knockdown phenotype we reasoned that shRNA mediated knockdown of ROCK I /II would prevent LPA from stimulating adhesion movement and trailing edge retraction in response to LPA. As shown in Fig. 23 A, shRNA mediated knockdown of ROCK II but not ROCK I expression induced cell elongation in the presence of serum. The phenotype of ROCKII knockdown cells however was distinct from that observed for FAK knockdown cells (shown in Figure 14AB) in that the cell bodies exhibit a somewhat flatter morphology. In contrast the knock down of ROCK I significantly impaired formation of mature adhesions and actin stress fibers (Figure 24 and data not shown) consistent with recent observations (Totsukawa et al., 2004; Yoneda et al., 2005). We

observed that LPA failed to induce adhesion movement in cells lacking either ROCK I or ROCK II (figure 24A,B) indicating that both ROCK I and II contribute to the regulation of adhesion mobility. As shown in Fig. 25 knockdown of ROCK II prevented the LPA-dependent stimulation of trailing edge retraction and concomitant decrease in cell elongation. These results support a model in which LPA activation of  $G_{12/13}$  leads to activation of FAK/PDZ-RhoGEF/Rho/ROCK II pathway that regulates the dynamics of mature adhesions leading to trailing edge retraction and concomitant decrease in cell elongation in serum starved cells treated with LPA.

### **Discussion.**

In this work we show that serum deprivation of rodent fibroblasts markedly reduced trailing edge retraction without having significant effect on lamellipodia protrusive activity at the leading edge. The perturbation in trailing edge retraction results in an elongated cell morphology, following over night serum starvation. Addition of LPA to serum starved cells stimulates rear retraction and decreases cell elongation and provides a model that parallels events within the trailing edge of a migrating fibroblast. Importantly, trailing edge retraction requires LPA induced stimulation of the dynamic movement of mature adhesions. Using this model, we have identified specific signaling components that contribute to trailing edge retraction in fibroblasts in response to LPA. LPA induced retraction of the trailing edge requires FAK. The siRNA mediated attenuation of FAK expression blocks the LPA induction of adhesion dynamics, trailing edge retraction and restoration of cell shape, suggesting that dynamic remodeling of adhesions is necessary for trailing edge retraction. LPA-induced changes in adhesion dynamics; trailing edge retraction and cell elongation also required PDZ-RhoGEF. The

siRNA mediated knockdown of PDZ-RhoGEF blocked LPA induced adhesion dynamics and prevented the restoration of cell shape. Furthermore, expression of PDZ-RhoGEF rescues FAK siRNA phenotype with respect to cell elongation (cells are less elongated). In addition PDZ-RhoGEF co-immunoprecipitated with FAK and co-localizes with FAK-containing adhesions suggesting that the activation of FAK and PDZ-RhoGEF are closely coupled. Finally, two lines of evidence suggest that one of the major targets of activated Rho in response to LPA is ROCKII. Expression of a Rho effector mutant that preferentially activates mDia failed to rescue the elongated phenotype of FAK knockdown cells; whereas a mutant that activates both mDia and ROCK pathways rescued the elongated phenotype of FAK siRNA treated cells. In addition the knockdown of ROCKII but not ROCK I induced a phenotype that parallels that observed in FAK-deficient cells with respect to cell elongation and retention of stress fibers and adhesions structures (Table 1). Our observations are consistent with a model in which LPA binding to its receptor stimulates activation of  $G_{12/13}$ , activation of FAK and the closely coupled activation of PDZ-RhoGEF, Rho and ROCKII, leading to spatial (trailing edge) stimulation of adhesion dynamics via localized increase in contractility, retraction of the cell rear and decrease in cell elongation (Figure 26).

There is abundant evidence that G protein-coupled receptors (GPCRs), such as those for LPA, activate Rho and Rho-dependent signaling pathways through the  $G_{12/13}$  family of heterotrimeric G proteins (Barr et al., 1997; Gohla et al., 1998; Majumdar et al., 1999; Offermanns et al., 1994). The RhoGEFs, p115RhoGEF, PDZRhoGEF and LARG (leukemia-associated RhoGEF) contain a regulator of G protein signaling (RGS) domain that binds activated  $G_{\alpha 12/13}$  resulting in RhoGEF activation (Wang et al., 2004). Thus, the RGS-RhoGEFs are thought to serve as effectors of activated  $\alpha 12/13$  and as molecular

bridges between the heterotrimeric G proteins and the Rho network. (Fukuhara et al., 1999; Reuther et al., 2001; Togashi et al., 2000; Zheng et al., 1995b).

A variety of studies show that activation of tyrosine kinases by GPCRs is required for the efficient stimulation of Rho and Rho signaling [for review see (Sah et al., 2000)]. Indeed, many GPCRs are potent activators of FAK, suggesting a close link between FAK activation and Rho signaling (Della Rocca et al., 1999). Furthermore, it has been reported that stimulation of  $G_{12/13}$  induces FAK dependent PDZ-RhoGEF tyrosine phosphorylation leading to enhanced activation of Rho (Chikumi et al., 2002). How FAK is participating in  $G_{12/13}$  signaling to Rho remains unclear. The observation that FAK forms a complex with PDZ-RhoGEF and PDZ-RhoGEF has been reported to oligomerize (Chikumi et al., 2004), leads us to speculate that LPA mediated activation of  $G_{12/13}$  may induce oligomerization of PDZ-RhoGEF and concomitant dimerization of FAK, leading to transphosphorylation and activation of FAK within such complexes. Alternatively, LPA stimulation of acidic phospholipids (PIP<sub>2</sub>) may activate FAK directly, as has been recently demonstrated by (Cai et al., 2007). We speculate that activated FAK may phosphorylate PDZ-RhoGEF stimulating GEF activity towards Rho and that this localized activation of Rho might lead to activation of effectors at or proximal to adhesion sites.

Previously it has been shown that LPA stimulation of serum-starved neuronal cells treated with ROCK inhibitor resulted in the failure of LPA to induce cell shape changes, rapid neurite retraction and cell rounding (Jalink et al., 1993; Kranenburg et al., 1999). However, this study did not delineate the contribution of ROCK I and ROCK II in the process of LPA induced neurite retraction. More relevant to the current study are the observations that knock down of ROCK II but not ROCK I yielded a cellular phenotype similar to that observed following FAK knock down with respect to cell shape,

augmented stress fibers and loss of dynamic adhesions (Yoneda et al., 2005). In addition, recent studies showed that Rho dependent breakdown of cell-cell adhesions requires ROCK II but not ROCK I (Samarin et al., 2007). Therefore, both studies point to a role for ROCK II in the induction of adhesion complex dynamics/breakdown. Recent evidence (Yoneda et al., 2005) indicates that ROCK I rather than ROCKII is important for stress fiber formation. ROCK II appears to act to regulate microfilament bundling at the adhesion site and is involved in phagocytosis of matrix-coated beads, a function not sensitive to ROCK I depletion.

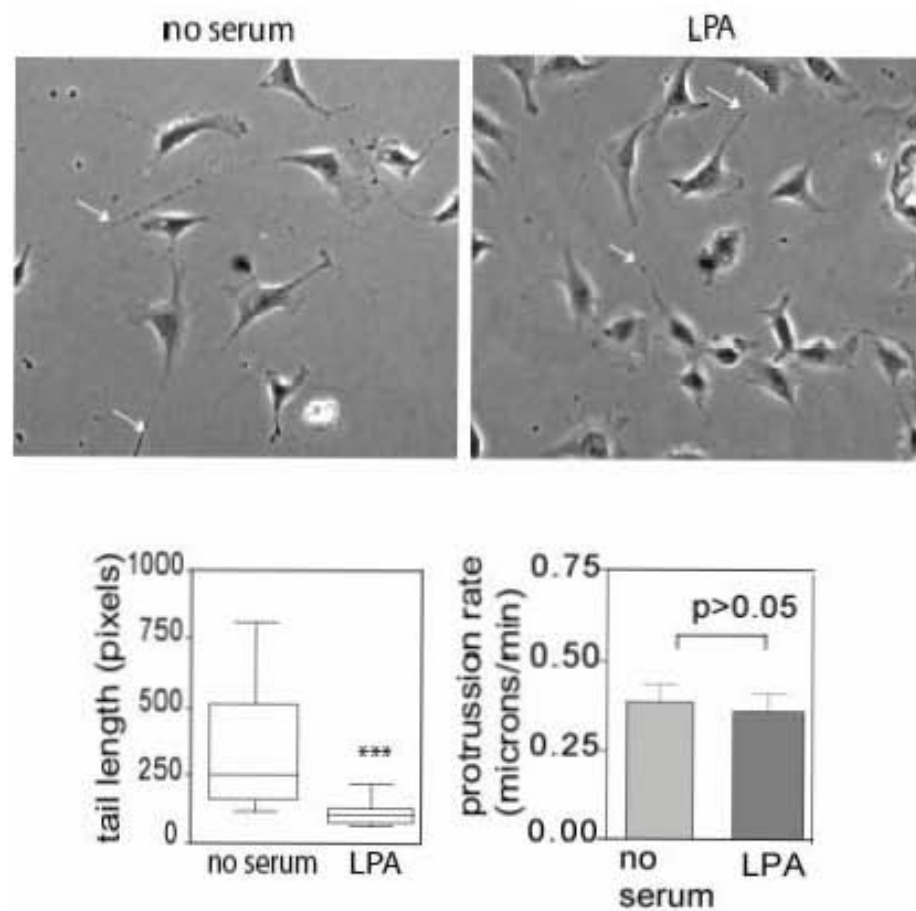
The effects of ROCK II on adhesion movement in trailing edge of fibroblasts could be attributed to induction of localized activation of myosin. We speculate that ROCKII-dependent tension at the trailing edge adhesions during LPA stimulation would contribute to the force that would promote adhesion inward movement and ultimate disassembly. In cells with attenuated FAK, PDZ-RhoGEF or ROCKII expression, adhesions remained stable even after LPA stimulation, indicating that this pathway is important in regulating localized changes in contractility. This observation would suggest that adhesion turnover requires localized modulation of myosin activity provided by the localized activation of the FAK-GEF-Rho-ROCKII pathway.

## Figures and Tables

### **Figure 9. Serum starvation of NIH3T3 cells results in the failure of trailing edge retraction.**

Length of the trailing edge and protrusion rates in NIH3T3- $\beta$ actin-mRFP cells in the absence of serum and in the absence of serum plus 2  $\mu$ M LPA. For trailing edge measurement, cells were filmed for 6 hours immediately after serum removal with images acquired at 5 minutes intervals, using phase contrast microscopy. Images acquired at the end of the sixth hour are shown and quantified. The data represents the analysis of 60 cells from two different experiments. Images acquired at the end of 6<sup>th</sup> hour are shown. For protrusion of the lamellipodia, cells were starved for 6 hrs and at the end of 6th hour, images of  $\beta$ -actin-mRFP in protruding lamellipodia were obtained for 20 minutes at 20 second intervals. The data represent analysis of 6 protruding lamellipodia per experimental condition.

Figure 9

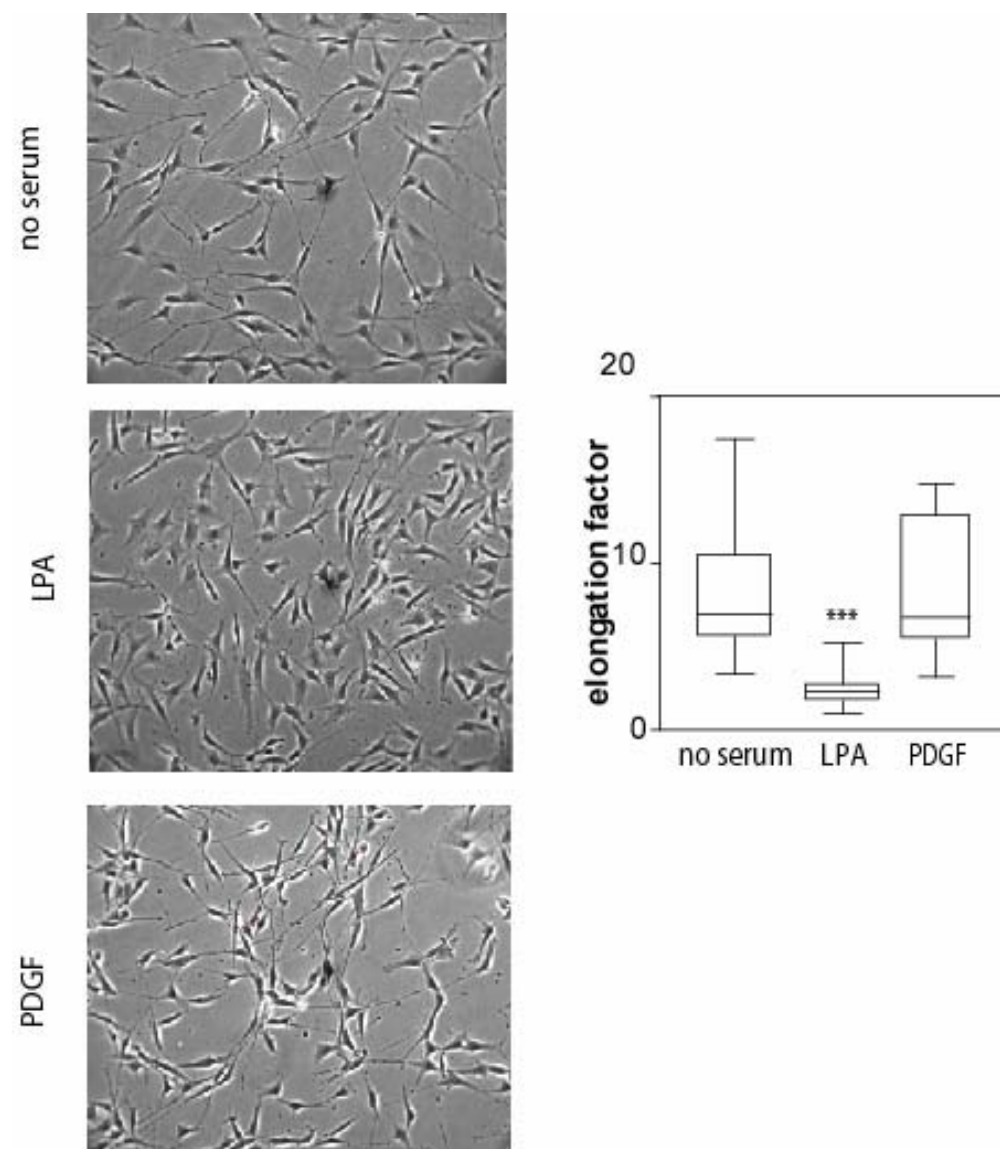


**Figure 10. Failure of NIH3T3 cells to retract trailing edges results in cell shape changes that depend on LPA but not PDGF.**

Phase contrast images and quantification of cell elongation of serum starved cells, serum starved cells treated with 2  $\mu$ M LPA or 100 nM PDGF. The data represent the analysis of 50-72 cells per condition from two different experiments.



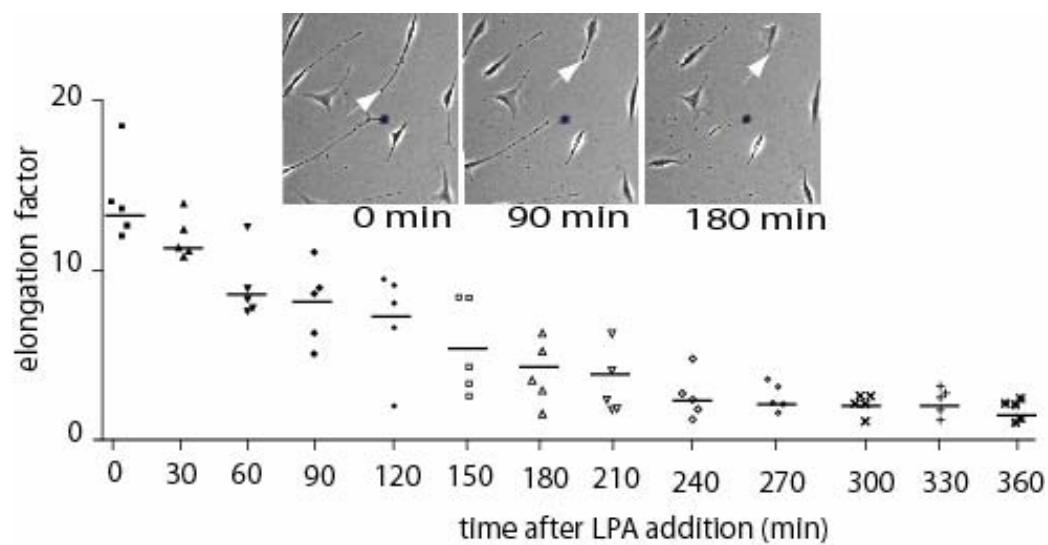
Figure 10



**Figure 11. LPA induces trailing edge retraction in serum deprived NIH3T3 cells**

Kinetics of changes in cell elongation upon LPA stimulation of serum starved fibroblasts.

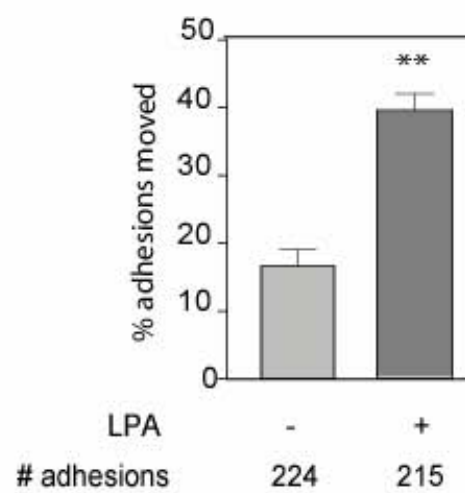
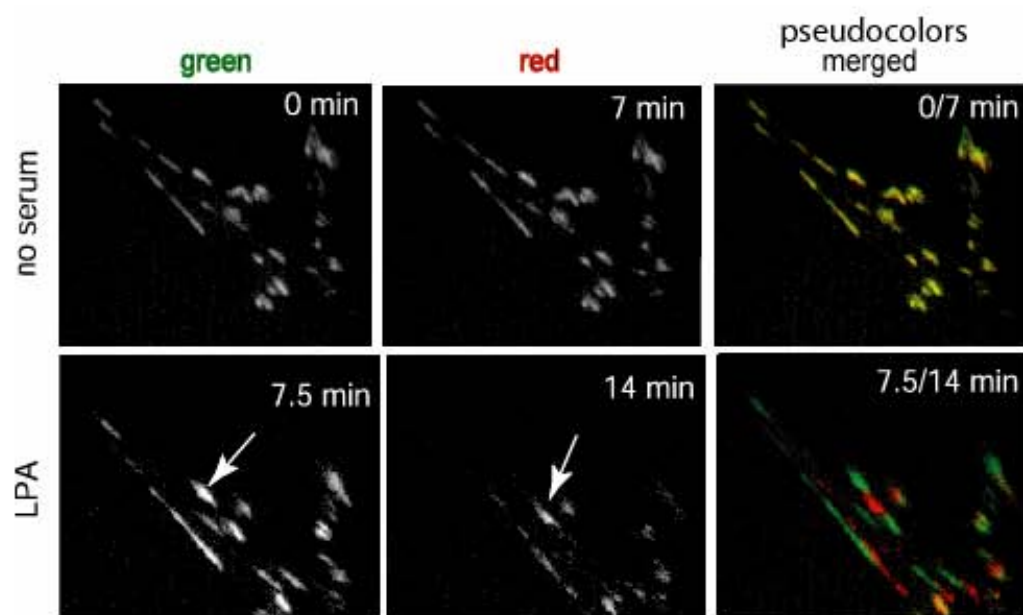
Figure 11



**Figure 12. LPA stimulates movement of adhesions found in the trailing edge of NIH3T3 cells.**

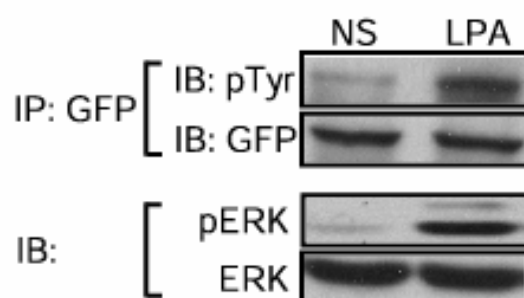
NIH3T3 cells stably expressing Paxillin GFP were plated in the presence of serum containing medium (10%) on dishes coated with 1  $\mu$ g/ml fibronectin. The following day cells were placed in serum free medium for 12 hours and subsequently stimulated with 2  $\mu$ M LPA and the movement of adhesions was analyzed using TIRF microscopy as described in Materials and Methods. Cells were filmed for 7 minutes prior to addition of LPA and 7 minutes after addition of LPA (arrows indicate representative adhesions). Bar graphs represent quantification of adhesion movement induced by LPA in NIH3T3 cells expressing Paxillin-GFP.

Figure 12



**Figure 13. LPA stimulation of serum starved NIH3T3 and Rat 2 cells induces activation of Focal Adhesion Kinase.**

(A) Western blot analysis of FAK activation by LPA in Rat 2 cells stably expressing GFP-FAK. Cells were stimulated with LPA for 5 minutes and GFP-FAK phosphorylation was assessed using anti-pTyr antibody as described in Materials and Methods. The increase in phosphorylation of ERK was determined by immunoblotting using antibodies against phosphorylated and total ERK (B) LPA activation of endogenous FAK in Rat 2 cells. Cells were stimulated with LPA for 5 minutes and western blots were probed with anti-pTyr397 or anti-FAK antibody as described in Materials and Methods

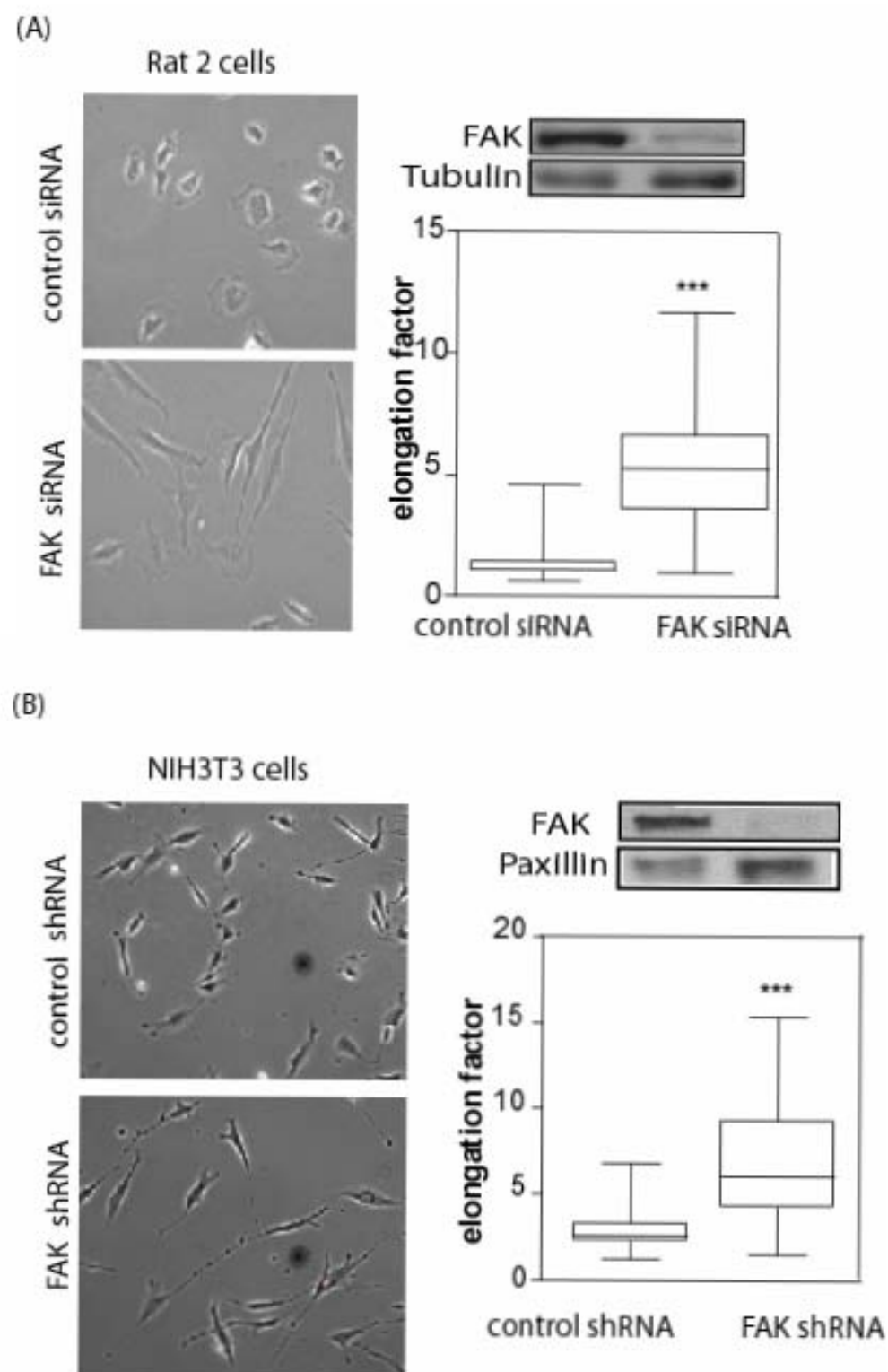
**Figure 13****(A)****(B)**

**Figure 14. Knockdown of FAK expression induces cell elongation in the presence of 10% serum.**

**(A,B)** Phase contrast images, western blot analysis of FAK protein expression and quantification of cell elongation in control and FAK siRNA treated Rat2 cells and FAK shRNA treated NIH3T3 cells. Rat2 cells were treated with either siRNAs specific for FAK **(A)** or NIH 3T3 cells were stably transfected with shRNA specific for FAK **(B)** as described in Materials and Methods. The extent of knockdown was determined by western blotting using FAK specific monoclonal antibodies and for the loading controls tubulin or paxillin specific antibodies.



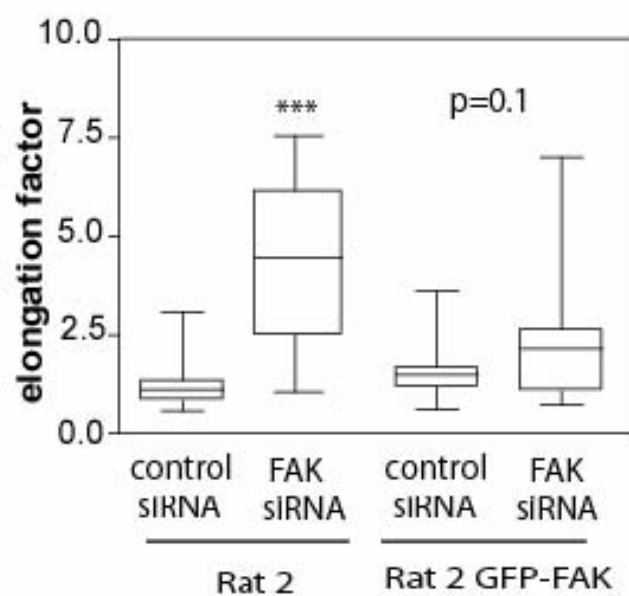
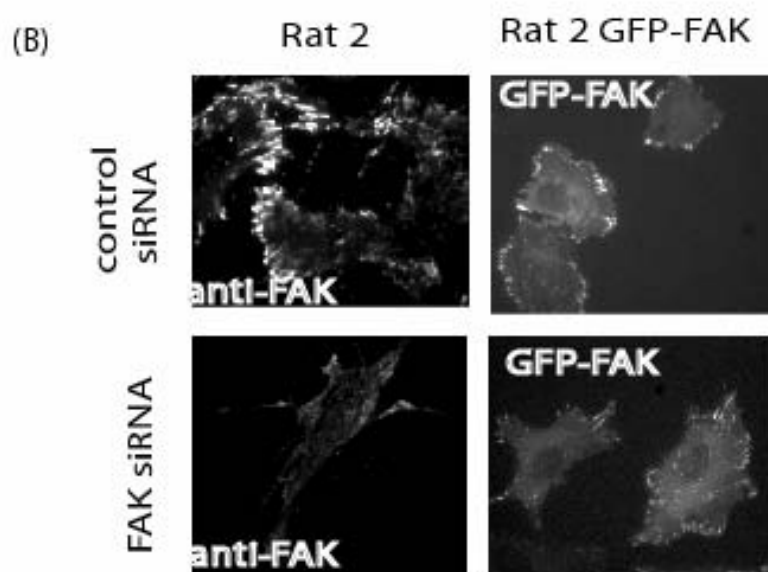
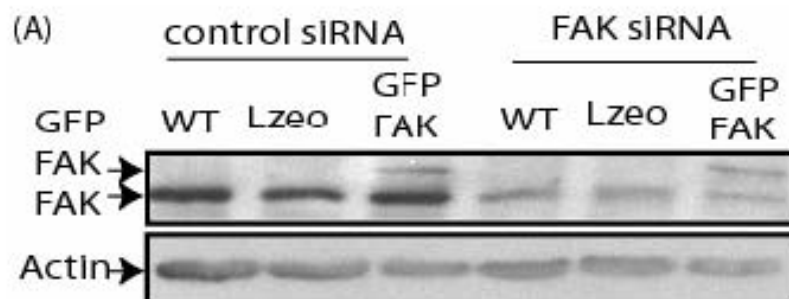
Figure 14



**Figure 15. Re-expression of FAK in cells treated with FAK siRNA rescues the defect in cell elongation.**

(A) Western blot analysis of FAK expression in wild type Rat 2 cells, Rat2 cells expressing LacZeo and Rat2 cells expressing GFP-FAK. (B) GFP-FAK expression decreases cell elongation resulting from FAK siRNA treatment. LacZeo expressing cells and GFP-FAK expressing cells were treated with control or FAK siRNA and stained with anti FAK(LacZeo) or left unstained to visualize GFP (GFP-FAK). The images illustrate the reduced FAK staining in LacZeo expressing Rat2 cells after FAK siRNA treatment but not control siRNA treatment (left). FAK siRNA treatment of GFP-FAK expressing cells did not alter GFP-FAK localization to adhesions (right panel). Control and FAK siRNA treated cells were immunostained with anti-tubulin (not shown) and the shape of individual cells was assessed by measuring the Elongation Factor.

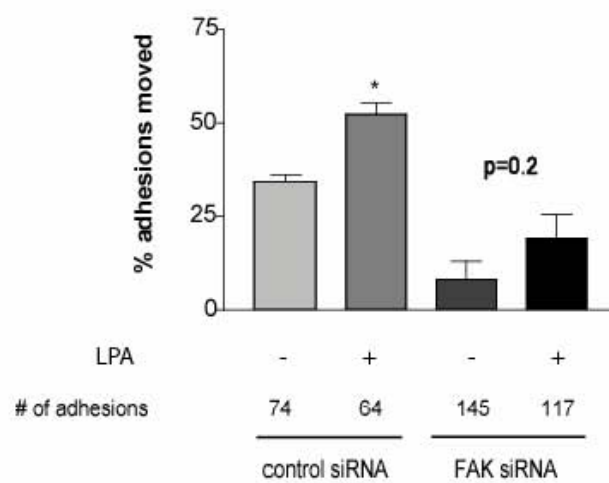
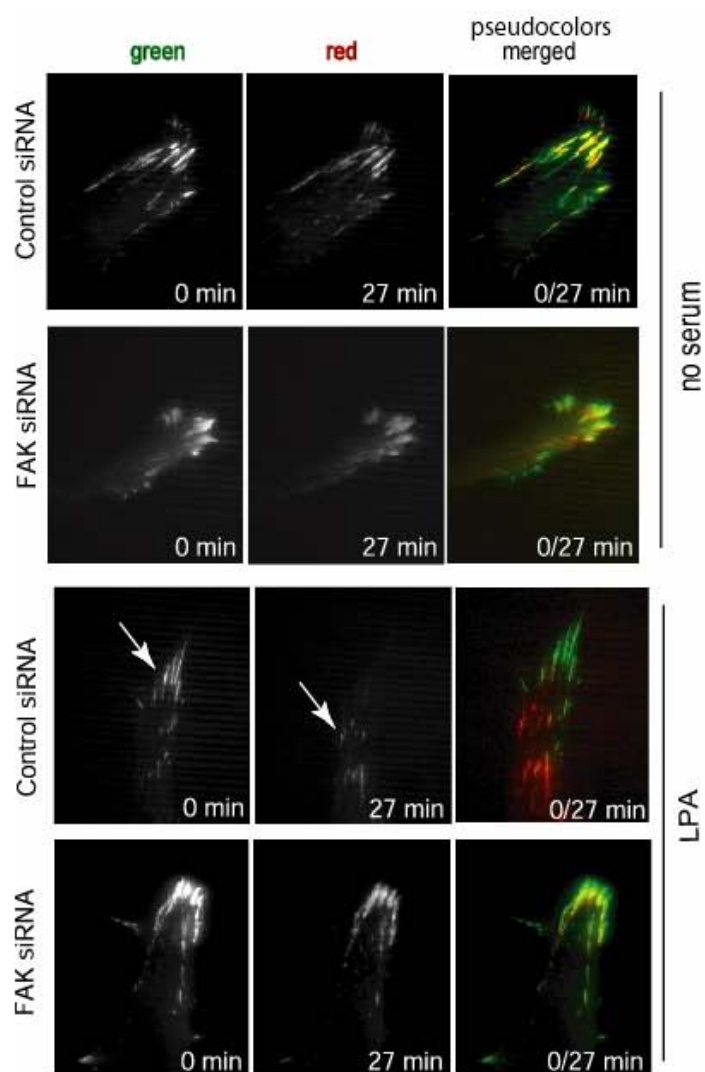
Figure 15



**Figure 16. LPA-induced adhesion movement is FAK-dependent**

Analysis of adhesion movement in response to 2  $\mu$ M LPA. Control or FAK siRNA treated Rat2 cells stably expressing Paxillin-GFP were plated on fibronectin coated 1 $\mu$ g/ml glass T dishes in the presence of 10 % serum containing medium. The following day, cells were serum starved for 12 hours and stimulated with 2  $\mu$ M LPA. Adhesion movement was analyzed using TIRF microscopy as described in Fig. 3. Cells were filmed for 27 minutes prior to the addition of LPA and 27 minutes after the addition of LPA. Bar graphs represent quantification of dynamic adhesions that was assessed as described in Fig. 5 (arrows indicate representative adhesions). The experiment was performed 3 times with 6 to 8 cells analyzed per experimental condition.

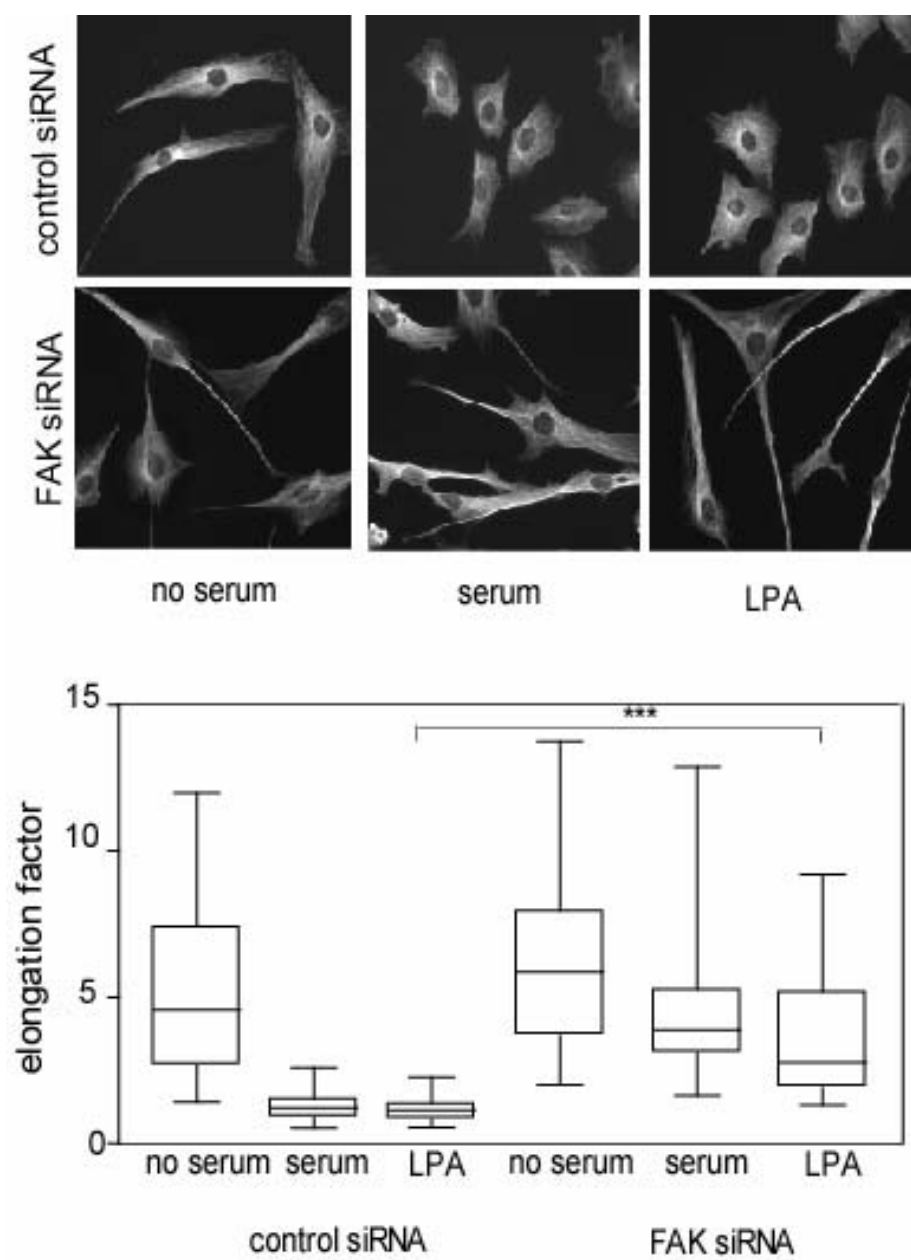
Figure 16



**Figure 17. FAK is required for LPA-mediated induction of trailing edge retraction and the restoration of normal morphology.**

Representative images of  $\alpha$  tubulin immunostained control and FAK siRNA treated cells that were either serum starved or serum starved and stimulated with 10% serum or 2  $\mu$ M LPA are shown. The graphs show quantification of the elongation factor for siRNA treated cells following treatment of over night serum starved cells with serum or LPA for 7 hours. The experiment was repeated two times and 50 to 60 cells were analyzed per condition.

Figure 17

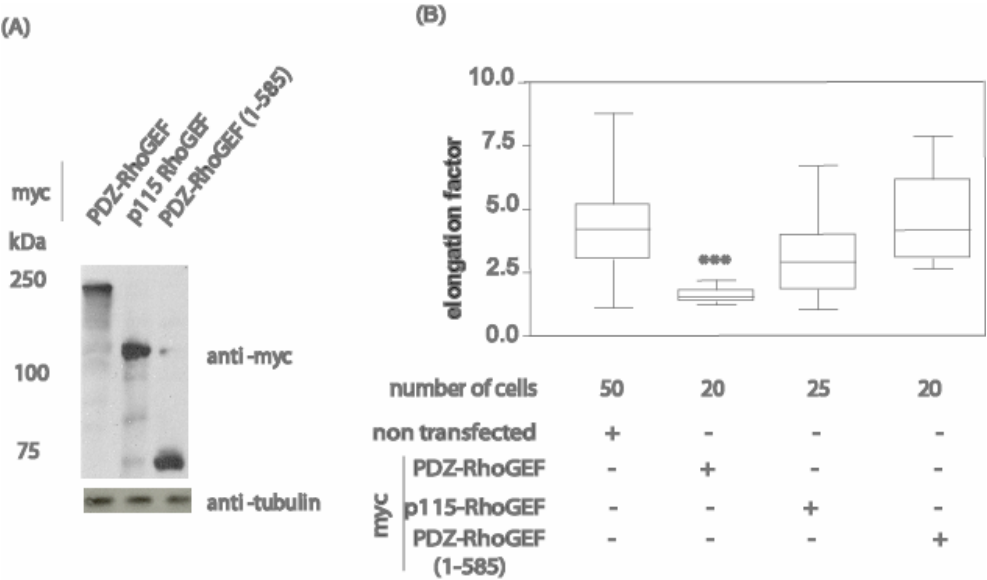


**Figure 18. Ectopic expression of PDZ-RhoGEF rescues the elongation induced by knockdown of FAK.**

(A) NIH3T3 cells were transiently transfected with PDZ-RhoGEF, p115RhoGEF and PDZ-RhoGEF 1-585, as described in Materials and Methods. Twenty four hours after transfection, lysates were prepared and blotted with anti myc antibody (B) Quantification of cell elongation in FAK siRNA treated cells expressing different Rho GEF molecules.



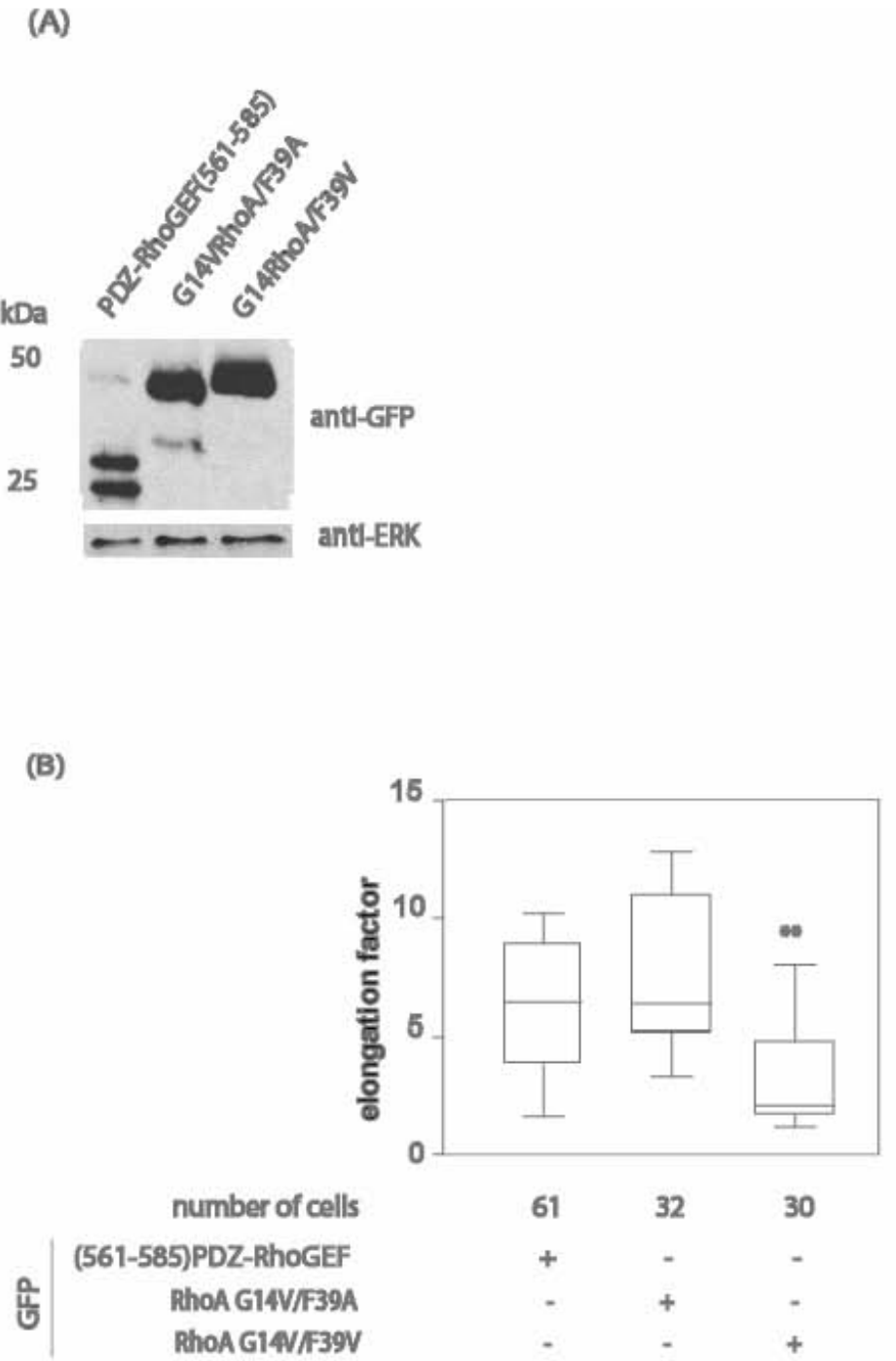
Figure 18



**Figure 19. Ectopic expression of Rho effector domain mutant that activates ROCK but not mDia rescues the elongation induced by knockdown of FAK expression.**

**(A)** FAK shRNA treated cells were transiently transfected with control, non-functional (561-585) PDZ-RhoGEF-GFP or G14VRho/F39A-GFP or G14VRho/F39V-GFP molecules as described in Materials and Methods. Twenty four hours after transfection, lysates were prepared and blotted with anti GFP antibody. **(B)** Quantification of cell elongation in FAK shRNA treated cells expressing different Rho effector domain mutant molecules.

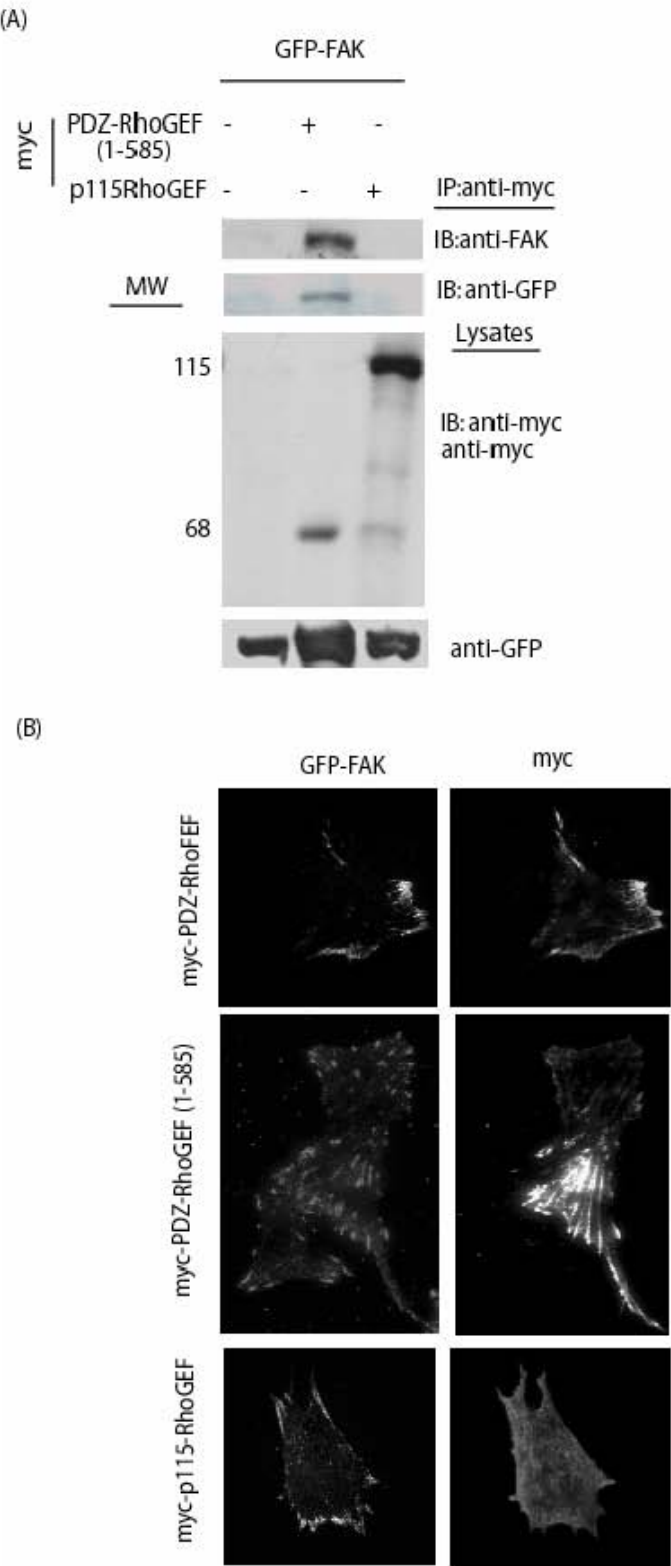
Figure 19



**Figure 20. GFP-FAK co-immunoprecipitates and localizes with co-expressed myc-tagged PDZ-RhoGEF and myc-tagged PDZ-RhoGEF 1-585.**

**(A)** HEK293T cells were transfected with with 0.5  $\mu$ g GFP-FAK and myc-PDZ-RhoGEF 1-585 or myc-p115RhoGEF. Twenty four hours post transfection cells were processed for anti-myc immunoprecipitation, SDS PAGE and western blotting with anti GFP and anti FAK antibodies as described in Materials and Methods. **(B)** Rat2 cells stably expressing GFP-FAK were transiently transfected with myc-PDZ-RhoGEF, myc-PDZ-RhoGEF 1-585 or myc-p115RhoGEF. Twenty four hours after transfection cells were detached and seeded on fibronectin coated (1  $\mu$ g/ml) glass T dishes. Twenty four hours later, cells were fixed and immunostained with anti-myc antibody as described in Materials and Methods and visualized using TIRF microscopy.

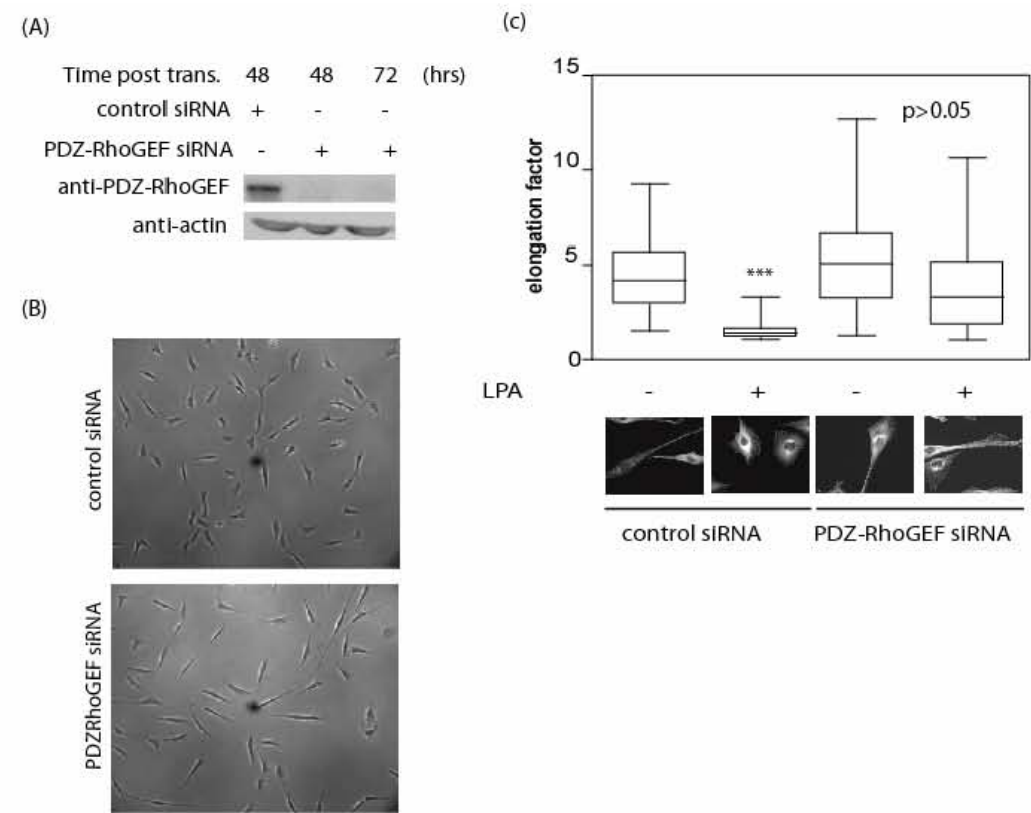
Figure 20



**Figure 21 PDZ-RhoGEF is required for LPA stimulated trailing edge retraction.**

(A) Western blot analysis of endogenous PDZ-RhoGEF (GTRAP48) protein expression and phase contrast images of control and PDZ-RhoGEF siRNA treated Rat2 cells. (B) fluorescent images and cell elongation quantification of control and PDZ-RhoGEF siRNA treated cells. Control or PDZ-RhoGEF siRNA treated cells were plated for 24 hours on fibronectin (1  $\mu$ g) coated microscope coverslips in the presence of 10% serum. Next day, cells were serum starved for 12-16 hours. Following starvation, cells were stimulated with 2  $\mu$ M LPA for 7 hours. Cells were fixed and stained with anti  $\alpha$ -tubulin antibody as described in Materials and Methods.

Figure 21

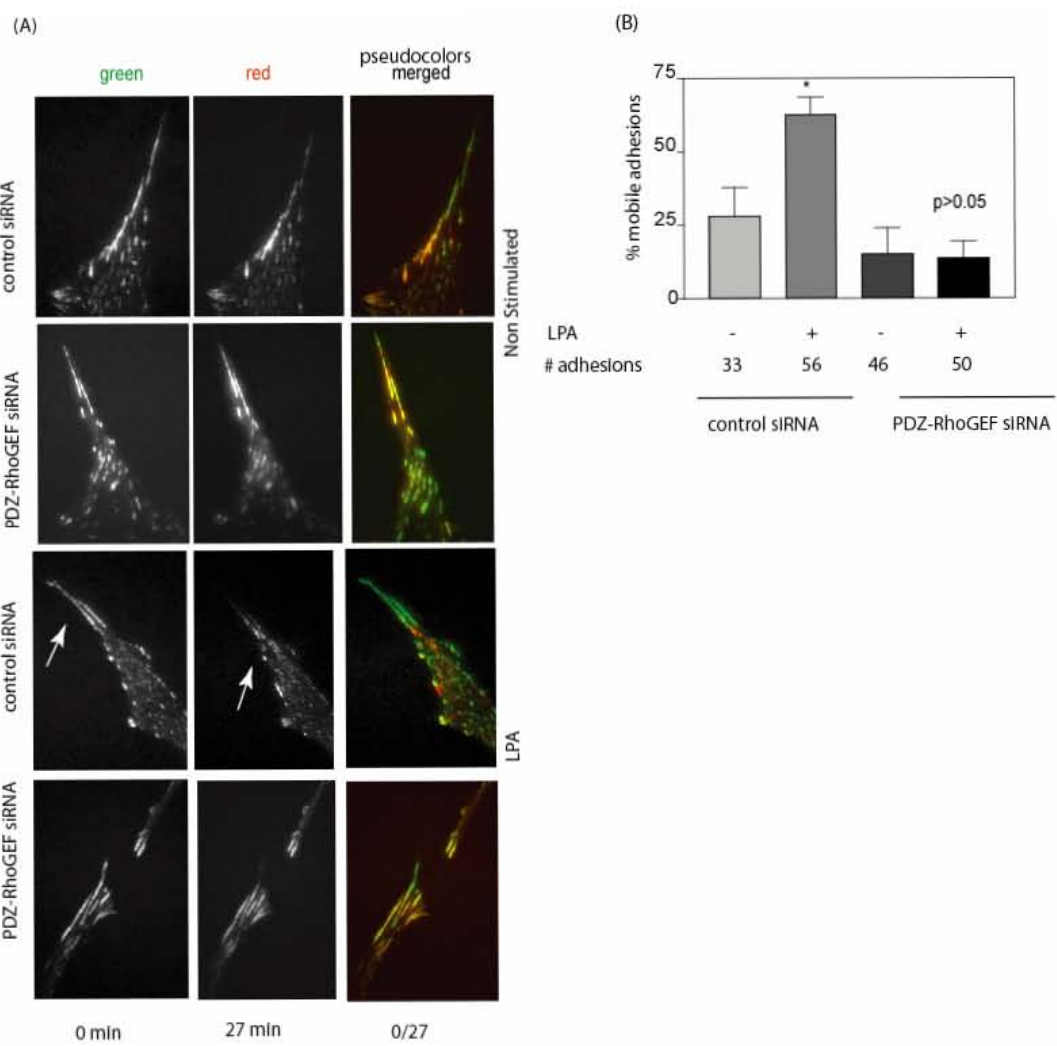


**Figure 22. PDZ-RhoGEF is required for LPA induction of adhesion movement.**

**(A)** Analysis of adhesion movement in response to LPA. Control or PDZ-RhoGEF siRNA treated Rat2 cells stably expressing Paxillin-GFP were plated on fibronectin coated 1  $\mu$ g/ml glass T dishes in the presence of 10 % serum containing medium and the following day cells were serum starved for 12-16 hours and then stimulated with 2  $\mu$ M LPA. Adhesion dynamic was analyzed using TIRF microscopy as described in Fig. 3. Cells were filmed for 21 minutes prior to the addition of 2  $\mu$ M LPA and 27 minutes after the addition of 2  $\mu$ M LPA. **(B)** Quantification of dynamic adhesions was assessed as described in Fig. 2 (arrows indicate representative adhesions). The experiment was performed 2 times with 5 to 7 cells analyzed per condition.



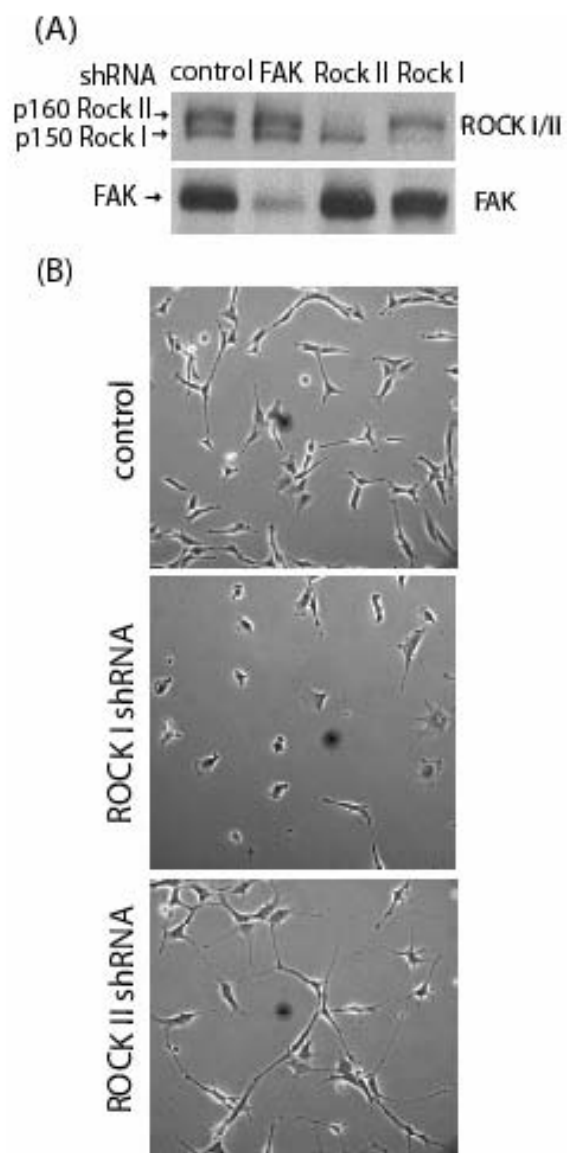
Figure 22



**Figure 23. shRNA mediated attenuation of ROCK II but not ROCK I induces phenotype similar to FAK and PDZ-RhoGEF knock down.**

Western blot analysis of ROCK I/II protein expression in control, FAK, ROCK I and ROCK II shRNA-treated NIH3T3 cells (top panel) and phase contrast images of control, ROCK I and ROCK II shRNA treated cells (bottom panel)

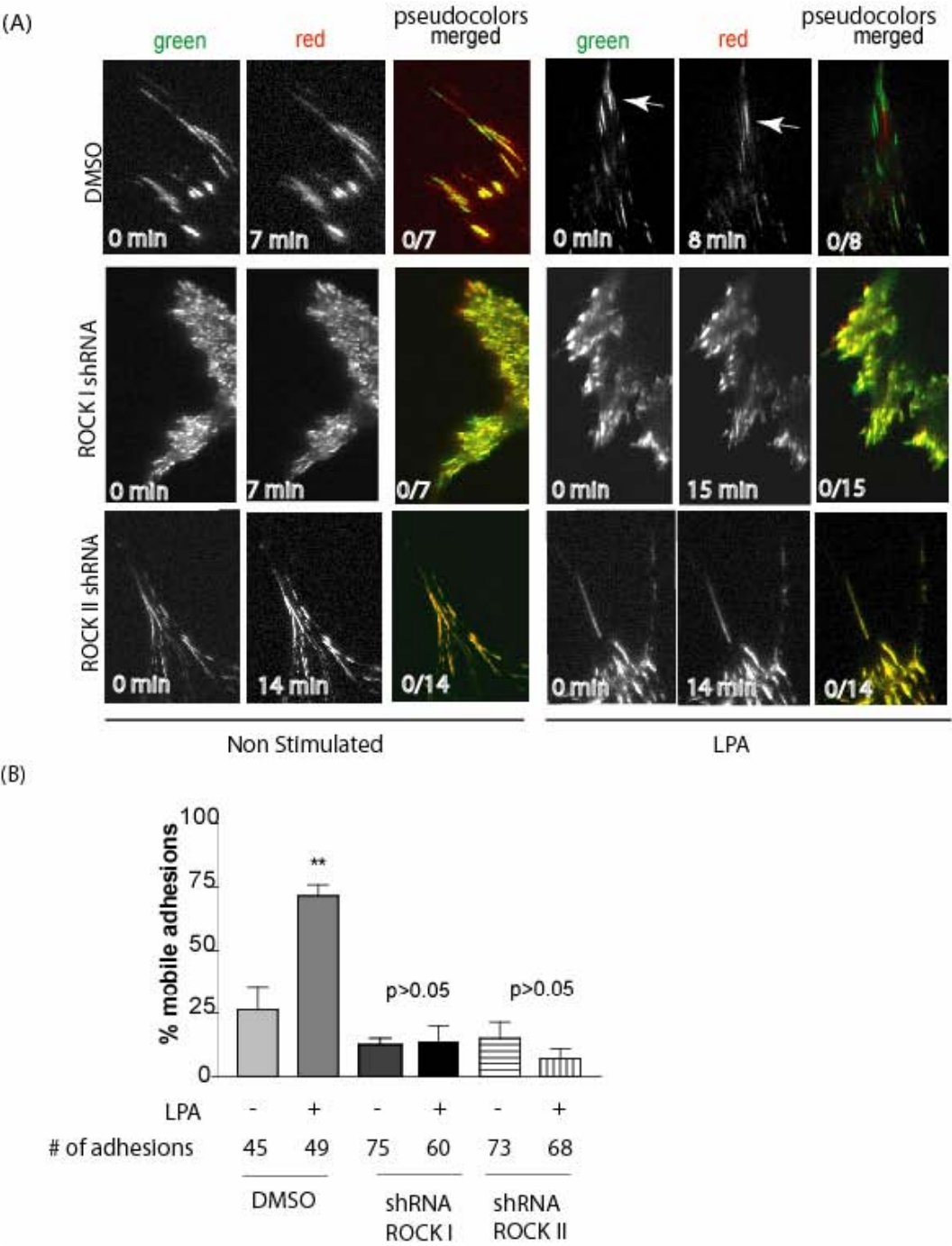
Figure 23



**Figure 24. LPA-induced adhesion movement requires both ROCK isoforms.**

(A) Control ROCK I and ROCK II shRNA treated cells were serum starved for 12 hours. Following the starvation, cells were stimulated with 2  $\mu$ M LPA, and the adhesion movement was visualized by TIRF microscopy. (B) Quantification of adhesion movement (as described in Materials and Methods) in serum starved cells and serum starved cells stimulated with LPA (arrows indicate representative mobile adhesions). The experiment was performed 2 times with 6-8 cells analyzed per experimental condition.

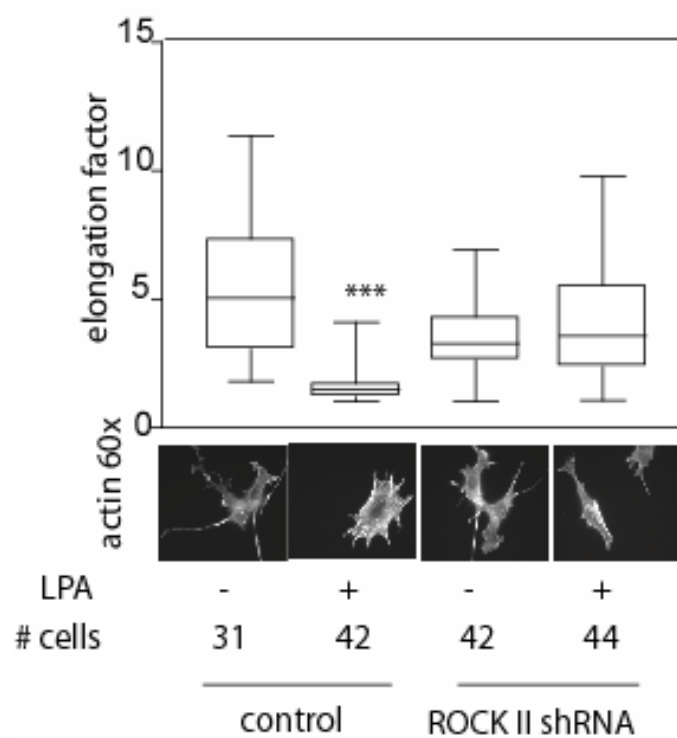
Figure 24



**Figure 25. ROCK II is required for LPA induced trailing edge retraction.**

Control and ROCK II shRNA treated cells were serum starved for 12 hours. Following the starvation, cells were stimulated with 2  $\mu$ M LPA for 7 hours. Quantification of cellular elongation in LPA stimulated control and ROCK II shRNA treated cells. The experiment was performed 2 times.

Figure 25



**Table 1. Summary of Trailing edge and adhesion movement studies in control, FAK , PDZ-RhoGEF , ROCK I and ROCK II siRNA or shRNA treated cells.**

sh RNA	Average elongation factor	LPA induced adhesions movement	LPA induced trailing edge retraction
control	1.2	YES	YES
FAK	5.2	NO	NO
PDZ RhoGEF	4.8	NO	NO
ROCK I	1.3	NO	
ROCK II	5.1	NO	NO

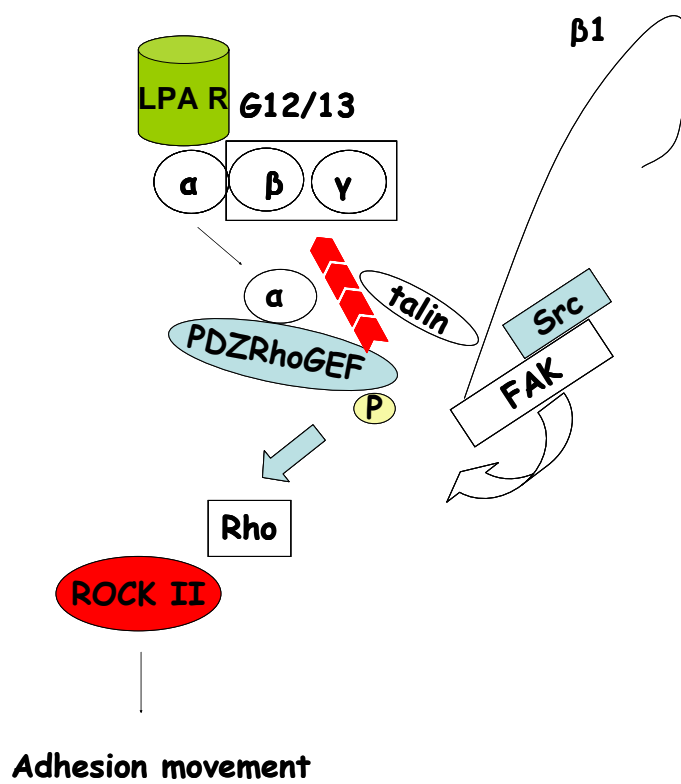
ROCK I shRNA treated NIH3T3 cells are small and do not show any distinct trailing edges.



**Figure 26. Current model of FAK-dependent regulation of adhesion movement and trailing edge retraction.**

Activation of LPA receptor results in the activation of the  $\alpha$  subunit of G-proteins. PDZ-RhoGEF that is present on actin filaments via its RGS domain binds to GTP loaded  $\alpha$  subunit and catalyzes GTP hydrolysis. FAK/Src, via  $\beta 1$  integrin and talin tethered to actin, phosphorylates PDZ-RhoGEF and leads to activation of Rho and Rho effector ROCK II. ROCK II via unknown mechanisms controls adhesion movement and trailing edge retraction.

Figure 26



## CHAPTER 3

### **RACK 1 Organizes FAK-induced ERK Activation and Localization to Adhesions**

#### **Introduction**

ERK signaling converts extracellular stimuli into a variety of specific intracellular biological responses such as differentiation, cell movement, cell division, apoptosis and oncogenic transformation. Extracellular stimuli, including growth factors, hormones and adhesion to ECM proteins, activate Raf, which phosphorylates and activates MEK, which in turn phosphorylates and activates ERK (Chang et al., 2001) (Pearson et al., 2001; Rubinfeld and Seger, 2005). Following activation active ERK localizes to different subcellular compartments including the nucleus, the plasma membrane, endosomes, Golgi apparatus and adhesions (Fincham et al., 2000; Kolch, 2005; Mor and Philips, 2006). At these various locations, ERK can phosphorylate a specific constellation of diverse substrates, leading to changes in gene expression, intracellular trafficking, and cell movement appropriate for the cell and stimulus.

Activated ERK localizes to adhesion structures that through integrins link extracellular matrix proteins to the actin cytoskeleton and anchor the cell to the substratum. Integrin engagement is sufficient to activate ERK, and in cells spreading on fibronectin active ERK localizes to newly formed adhesions (Chen et al., 1994; Fincham et al., 2000). However, the functional significance of active ERK in adhesions has not been well understood.

One mechanism by which ERK signaling can be directed to specific locations and functions is by scaffold or anchor proteins. These non-enzymatic entities associate

with and enhance functional interaction of the components of the ERK pathway and can regulate the amplitude, timing and location of signals (Kolch, 2005; Morrison and Davis, 2003; Schaeffer and Weber, 1999).

In this work we show that the scaffold protein RACK1 links adhesion signaling to the ERK pathway activation. Furthermore, we demonstrate that RACK1 is required for efficient ERK activation in response to adhesion, but is not necessary for stimulation by growth factor. We also show that RACK1 targets active ERK to a specific intracellular location, adhesion structures found in the protruding areas of the cell, and at this location ERK promotes adhesion disassembly. In addition, we show evidence that FAK is also necessary for ERK activation at adhesion sites. Our data are consistent with the model in which RACK 1 targets the ERK pathway components to adhesions for integrin- and FAK –dependent activation.

## **Materials and Methods.**

### **Cell lines and transfections.**

NIH3T3 cells were maintained in Dulbecco's modified Eagle's media (DMEM; Gibco BRL) supplemented with 10% calf serum (Gibco BRL). CCL39, RAT2 and REF52 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco BRL). CCL39 cells were transfected using LipofectAMINE (Gibco BRL) according to manufacturers' instructions. FlipIn system (Invitrogen) was used for generation of NIH3T3 cell line stably expressing GFP-Paxillin. Briefly, NIH3T3 cells were transfected with pFRT/LacZeo vector and a Zeomycin resistant clone (NIH3T3-LacZeo) was isolated. NIH3T3-LacZeo cells were then co-transfected with pOG44 and GFP-Paxillin-pcDNA5-FRT constructs, and a Hygromycin resistant cell population (NIH3T3-GFP-Paxillin) was established. NIH3T3-GFP-Paxillin cells have been shown to be GFP-Paxillin positive by western blotting and TIR fluorescence microscopy (data not shown).

### **Immunoprecipitations and Immunoblotting.**

Cells were lysed 24 hours post-transfection in FLAG lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM EDTA, 0.5 mM EGTA, pH 7.3 supplemented with 50 mM NaF, 5 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 0.2 mM  $\text{Na}_3\text{VO}_4$  and protease inhibitors). Clarified extracts were incubated for 2 hours with M2 anti-FLAG affinity resin (Sigma) at 4°C. Immunoblots were carried out with the following antibodies: anti-FLAG M2 (Sigma) and anti-HA 12CA5 (Babcock). Secondary goat anti-rabbit and anti-mouse antibodies coupled to horseradish peroxidase and enhanced chemiluminescence (ECL) reagents (Amersham Biosciences) were used for protein detection.

### **Cell stimulation.**

To determine ERK activation status in response to EGF REF52 cells were cultured in 10% FBS and either left untreated or serum deprived for 4 hours and stimulated with EGF in DMEM for 10 minutes. To determine ERK activation status in response to adhesion REF52 cells were cultured in 10% FBS, detached by trypsin, and trypsin was stopped by addition of trypsin inhibitor. Cells were pelleted, resuspended in DMEM, kept in suspension for 90 minutes and plated on dishes precoated with fibronectin (10µg/ml) for 15 min. Cells were lysed in RIPA lysis buffer and processed as above with the following antibodies: anti-ERK2 B3B9, anti-FAK (Upstate), anti-ppERK, anti-RACK1 and anti-Paxillin (Signal Transduction Laboratories), and anti-FAKpY397. Western blot membranes were developed using ECL (Amersham).

### **siRNA gene silencing assay.**

A double stranded siRNA targeting the 21-nt sequence AAGGTGTGGAATCTGGCTAAC conserved between the mouse and rat RACK1 genes was synthesized by Qiagen and the Non-Specific Control Duplexes-XIII was obtained from Dharmacon Research. The siRNA was transfected into REF52 and RAT2 cells using the calcium phosphate protocol and 48-72 hours post-transfection cell lysates were probed as indicated. Mock control cells received transfection reagent only. NIH3T3 cells were transfected with Lipofectamine2000 (Invitrogen). A second RACK1 siRNA (GCTAAAGACCAACCACATTTT) was utilized as well and generated identical biological results to those shown in this paper. FAK siRNA oligonucleotide has been described previously (Tilghman et al., 2005).

### **Immunostaining.**

Cells were washed twice with PBS, fixed in 1% paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 4 mM MgSO<sub>4</sub>, pH6.9) supplemented with 0.2 mM vanadate and 50 mM  $\beta$ -glycerophosphate for 20 min. and extracted with 1% CHAPS in PHEM buffer for 5 min. Cells were blocked in 20% normal goat serum (NGS, Invitrogen) in MBST (50 mM MOPS, 150 mM NaCl, 0.05% Tween 20, pH7.4) for 1 h, and incubated with rabbit polyclonal anti-ppERK antibody diluted in 5% NGS in MBST for 1 h. Cells were washed with MBST three times and subsequently treated with secondary goat anti-rabbit antibody conjugated to biotin (Vector laboratories), diluted in 5% NGS in MBST for 1 h followed by incubation with Texas Red-avidin (Vector Laboratories). Paxillin was detected with mouse monoclonal anti-Paxillin antibody and visualized with goat anti mouse antibody conjugated to either FITC (Jackson Laboratories) or Cy5 (Molecular Probes). The following primary antibodies were used: rabbit polyclonal anti-ppERK (diluted 1:50-300; Cell Signaling) and mouse monoclonal anti Paxillin clone 5H11 (1:1000, Upstate). Fluorescent images were acquired using a Nikon Eclipse E600 upright fluorescent microscope equipped with a Hamamatsu ORCA CCD camera. Images were captured and analyzed with OpenLab (Improvision) software.

OpenLab (Improvision) software was used to measure the length of adhesions. For each experimental condition the length (in pixels) of approximately 200 adhesions from 10 cells was measured. Statistical analysis (Mann-Withnay non-paired (not assuming Gaussian distribution) t-test) was done using Prism software (GraphPad Software, Inc).

**TIRF microscopy and adhesion disassembly quantification.**

To record dynamics of adhesions, NIH3T3-GFP-Paxillin stable cell line was grown on Biopetechs T-Dishes (Fisher Scientific) to confluency. To induce cell migration the wound was made and 5 hrs later cells images were acquired every 3 min. Cells were visualized using a Nikon Eclipse TE2000-E inverted microscope equipped with a TIRF illuminator, a 488 argon laser and a 60xDIC TIRF objective (NA 1.45) equipped with a Biopetechs objective heater. Images were acquired with a Retiga 1300i CCD camera and the QCapture Pro software (Q Imaging). To quantify the rates of adhesion assembly and disassembly OpenLab (Improvision) software was used to measure fluorescent intensity of individual adhesions. Changes in fluorescent intensity of adhesion were plotted in a semilogarithmic plot as a function of time, and the constant rate of adhesion disassembly was calculated from the slope as described before. Plotting of individual adhesion disassembly rates and statistical analysis (Mann-Whitney non paired t-test) were done using Prism software (GraphPad Software).

**Cell migration assay.**

The bottom sides of 24 well plate cell culture inserts (8 $\mu$ m pores; Biocoat Becton Dickinson) were precoated with fibronectin (1.0  $\mu$ g/ml) overnight at 4<sup>0</sup>C. To determine cell migration 1.10<sup>5</sup> REF52 cells were plated on the top of the insert in serum free media and allowed to migrate for 6 hours. Nonmigrating cells were wiped from the top by cotton swab. Cells were washed with PBS, fixed in 3% paraformaldehyde and stained with crystal violet (0.1 % in 20% EtOH). Cell migration was determined by counting the cells at the bottom of five fields.



## **Results.**

### **Identification of RACK1 as a MAPK/ERK pathway scaffold.**

To determine whether RACK1 associates with components of the MAP/ERK Kinase pathway in living cells, we co-expressed RACK1 together with individual components of the ERK pathway and examined their association. RACK1 associated with Raf-1 (Fig. 27A), the ERKs (Fig. 27B) and MEKs (Fig. 27C), supporting the concept that RACK1 can serve a scaffolding function for the ERK pathway.

### **RACK1 regulates adhesion-induced ERK activation**

RACK1 has been reported to interact with integrin  $\beta$  subunits and with the protein kinase Src (Chang et al., 1998). Integrins and Src play important roles in ERK activation during cell adhesion (Chen et al., 1994; Pullikuth et al., 2005; Schlaepfer et al., 1997). We therefore hypothesized that RACK1 may also be important for ERK activation during adhesion. Silencing of RACK1 expression by small interfering RNA (siRNA) in REF52 fibroblasts suppressed ERK activation in cells plated on fibronectin (Fig.28A). RACK1 function was specific to adhesion-induced ERK activation, as RACK1 silencing did not inhibit ERK activation in response to EGF (Fig. 28A). These data are consistent with the hypothesis that scaffold RACK1 localizes MAPK/ERK pathway components for integrin mediated activation. Clearly, growth factor mediated activation of MAPK/ERK pathway must depend on a distinct scaffold from RACK 1.

**RACK1 is required for active ERK localization to adhesions**

Next, we sought to explore the consequences of ERK and RACK1 signaling in response to adhesion. Since RACK1 depletion inhibited ERK activation mediated by integrin engagement to the extracellular matrix, we investigated whether RACK1 controlled the localization of active ERK to adhesions. Control or RACK1-depleted REF52 cells were plated on fibronectin-coated coverslips for 15 min. in serum-free media, fixed, permeabilized under conditions that retain adhesions and immunostained with antibody recognizing diphosphorylated, active ERK and antibody recognizing the adhesion marker paxillin. Consistent with previous reports (Fincham et al., 2000; Slack-Davis et al., 2003b) we found that control cells spreading on fibronectin formed peripheral adhesions and a fraction of the cellular pool of active ERK localized to these adhesions (Fig. 28B). RACK1 silencing did not inhibit cell spreading on fibronectin and assembly of adhesions. In fact, RACK1-depleted cells formed visibly more adhesions than control cells (discussed below). Importantly, RACK1 silencing resulted in the absence of active ERK in adhesions (Fig. 28B).

This finding suggests that RACK1 is essential for localization of active ERK to adhesions and that the existing cellular pool of active ERK can not be stably targeted to adhesions in the absence of RACK1. RACK1 silencing also reduced the level of active ERK in adhesions of adherent cells migrating into a wound (Fig. 29) further supporting the idea that active ERK is targeted to newly formed adhesions in a RACK1-dependent process.

**RACK1 promotes adhesion disassembly and cell motility**

In REF52 cells adhering to fibronectin, RACK1 silencing resulted in a noticeable change in adhesion architecture, particularly with respect to adhesion distribution. The cells with silenced RACK1 contained more adhesions, and these adhesions became significantly longer in adherent cells (see Fig. 28B). These observations suggested that regulation of adhesion lifetime could be affected by attenuation of RACK1. Previously published observations suggested that ERK activity is necessary for proper adhesion turnover, particularly adhesion disassembly (Webb et al., 2004). Since ERK presumably needs to act at these adhesions, we hypothesized that RACK1 silencing would specifically alter disassembly of adhesions. To determine whether RACK1 affects the dynamic behavior of adhesions in real time we established a NIH3T3 cell line stably expressing GFP-paxillin and measured the dynamics of adhesions in a wound-healing assay. Adhesions containing GFP-paxillin were recorded using time-lapse total internal reflection fluorescence (TIRF) microscopy, because TIRF microscopy selectively excites fluorophores close to the solid surface (within  $\sim 100$  nm), thus minimizing the background fluorescence of cytoplasmic GFP-paxillin. The wound-healing assay was chosen because cells migrating into the wound display a uniform direction of migration and the adhesions are very dynamic, rapidly appearing and disappearing at the front of the migrating cell. In a wounded NIH3T3 monolayer active ERK localized to dynamic adhesions at the leading edge of the cells, and RACK1 silencing almost completely abolished the appearance of active ERK at the adhesions in the cells migrating into the wound (Fig. 29).

Both control cells and cells with silenced RACK1 migrating into the wound were capable of forming new adhesions at the front of the cell. However, in RACK1-depleted

cells adhesions at the front of the cell persisted for a longer time than adhesions in control cells (Fig. 30A). The kinetics of adhesion disassembly showed that in the control cells adhesions were disassembled in approximately 10 min (Fig.30B). By contrast, the disassembly of adhesions in cells with silenced RACK1 was greatly delayed and this defect in adhesion disassembly resulted in enhanced adhesion growth and elongation (Fig. 30A,B). Analysis of the assembly and disassembly rates of dynamic adhesions at the front of the cell showed that RACK1 silencing reduced the disassembly rate by more than 50% (Fig. 30B) but had no effect on the rate of adhesion assembly (Fig. 30C). These data suggest that RACK1 is a functional component of the ERK signaling pathway that regulates adhesion disassembly.

Since the disassembly of adhesions is an important step in the regulation of cell motility (Webb et al., 2004) we determined whether RACK1 regulates cell motility. In REF52 cells RACK1 was depleted by siRNA, and cell migration toward fibronectin was measured in a Boyden chamber assay. Consistent with the reduced adhesion disassembly rate, we found that RACK1 silencing resulted in significantly reduced migration toward fibronectin (Fig. 30D). The reduced rate of adhesion disassembly was also reflected in an increase of adhesion length in cells migrating into a wound (see Fig. 30B). Quantification of the adhesion length showed that in cells plated on fibronectin in the presence of serum, RACK1 knockdown resulted in approximately a two fold increase in adhesion length (Fig. 31A).

**FAK, RACK1 and ERK are components of a signaling pathway that regulates active ERK localization to adhesions and adhesion dynamics.**

RACK1 has been shown previously to associate with Src and regulate its activity (Chang et al., 1998). In addition, Src phosphorylates several adhesion proteins and regulates adhesion disassembly (Parsons, 2003). This raises the question whether the defects in adhesion dynamics seen in RACK1-knockdown cells are a consequence of impaired Src signaling. We thus determined whether pharmacological inhibition of ERK by the MEK inhibitor UO126 affects adhesion length. Similarly to RACK1 knockdown in RAT2 cells ERK inhibition resulted in a significant increase of adhesion length (Fig. 31B). In addition, we observed an increase in adhesion length in cells with silenced ERKs (data not shown). These data are consistent with the previous finding that ERK pharmacological inhibition reduces the rate of adhesion disassembly (Webb et al., 2004).

Our data suggested that integrin engagement is necessary for active ERK localization to adhesions. The activation of Focal Adhesion Kinase (FAK) is a central step in integrin signaling and it has been proposed that both FAK and ERK signaling are linked to adhesion disassembly. These findings indicated that FAK and RACK1 may be components of a signaling pathway that regulates adhesion dynamics through ERK. If FAK regulates adhesion disassembly through ERK it should also regulate presence of active ERK in adhesions. To test this we depleted the FAK protein level by siRNA and determined the localization of active ERK. FAK silencing efficiently decreased the total level of FAK protein (Fig. 32A). Importantly, in spreading cells grown in the presence of serum, FAK silencing completely abrogated the appearance of active ERK in adhesion structures (Fig. 32B). In addition, similarly to RACK1 knockdown we observed that FAK depletion by siRNA resulted in adhesion elongation at the front edge of cells migrating

into the wound (data not shown). Our data are consistent with the model in which RACK1 functions in targeting MAPK/ERK pathway to the integrin/FAK pathway for activation to control adhesion dynamics and cell migration.

### **Discussion.**

In summary, the data presented here show that RACK1 acts as a scaffold for the ERK pathway core kinases and localizes these enzymes to the integrin/FAK adhesion signaling pathway and controls the distribution of active ERK to de novo formed adhesion structures, where it can act on substrates essential for adhesion disassembly and motility. Although RACK1 selectively facilitates ERK activation in response to cellular adhesion, it is dispensable for EGF-mediated ERK activation.

RACK1 associates with multiple components of the ERK pathway including ERK1/2, MEK1/2, and Raf. In addition, RACK1 has been reported to associate with components of the adhesion signaling pathway, including integrin  $\beta$ -subunits and the Src protein kinase (Chang et al., 1998; Liliental and Chang, 1998), and we show here that it functionally cooperates with FAK. Integrin engagement to ECM potently activates Src (Sandilands et al., 2004), and genetic evidence suggests that Src is an upstream component in integrin mediated ERK activation. In Src deficient cells ERK activation is inhibited in response to adhesion, but the Src family protein kinases are dispensable for ERK activation by the soluble growth factor PDGF (Klinghoffer et al., 1999) (Schlaepfer et al., 1997). Similarly to Src, depletion of RACK1 inhibits ERK activation in response to integrin engagement but not in response to EGF. Thus, the functional and binding characteristics of RACK1 are consistent with its functioning as a scaffold in integrin-

mediated activation of the ERK pathway and suggest that RACK1 regulates ERK activation by its ability to localize ERK pathway components to adhesions.

Integrin engagement during adhesion not only is sufficient to induce ERK activation but also is required to target active ERK to adhesions. This is shown by the fact that integrin-independent cell adhesion to polylysine neither activates ERK nor targets active ERK to adhesions. Moreover, in cells spreading on fibronectin or in cells with a polarized migratory phenotype, active ERK intensively associates with the presumed sites of acute integrin engagement to extracellular matrix and *de novo* adhesion formation (Chen et al., 1994; Fincham et al., 2000). Taken together, these data suggest that in migrating cells integrin attachment to the substratum coordinately induces both new adhesion assembly and ERK activation that is spatially linked to these newly formed adhesions. We observed that RACK1 regulates ERK activation specifically in response to integrin engagement to extracellular matrix and that depletion of RACK1 results in the absence of active ERK in adhesions. Importantly, the bulk cellular pool of active ERK is not able to compensate for the absence of active ERK in adhesions when RACK1 is depleted. RACK1 has been reported to localize to adhesions. Our data show that RACK1 regulates adhesion disassembly. Thus, RACK1 may promote ERK pathway activation at the sites of integrin engagement and at the sites of *de novo* adhesion formation (areas of active protrusions). At adhesion sites ERK signaling facilitates adhesion disassembly consistent with the previous finding that ERK signaling regulates this process. (Webb et al., 2004)

Integrin engagement leads to FAK activation and active FAK localization to *de novo* formed adhesions. FAK is a central transducer of integrin signaling as it activates numerous pathways including ERK. Previous reports suggested that FAK plays an important role in regulation of adhesion dynamics. It has been shown that FAK null cells

display an increase in adhesion size and number, probably as a consequence of reduced adhesion disassembly (Ilic et al., 1995; Webb et al., 2004). FAK regulates adhesion turnover at least partly through the ERK pathway because the reduced adhesion disassembly in FAK null cells can be rescued by expression of constitutively active MEK. These data suggest that the ERK pathway acts downstream of FAK to regulate adhesion disassembly.

Since we show that active ERK localization to adhesions is necessary for proper adhesion disassembly these data suggest that FAK may regulate activation of an ERK pool at newly formed adhesions. Indeed, in actively protruding cells we observed that FAK silencing resulted in the absence of active ERK in adhesions. In addition similarly to RACK1 knockdown and ERK pharmacological inhibition, adhesions in FAK silenced cells were significantly longer, suggesting that FAK silencing inhibited adhesion disassembly. Collectively, these data suggest that the tools for adhesion disassembly in protruding areas of the cell are generated coordinately in space and time with the assembly of adhesions, through the ability of RACK1 to bind integrins and the MAP kinase enzymes and to locate these signaling components proximal to the upstream activator FAK.

Expression of constitutively active MEK rescues adhesion disassembly in FAK null cells and, conversely, MEK inhibition decreases the adhesion disassembly rate (Webb et al., 2004). In addition, we observed that MEK inhibition, RACK1 and FAK knockdown result in a similar phenotype, i.e. increase in adhesion length in actively spreading cells. These data suggest that RACK1 regulates adhesion dynamics through the ERK pathway, specifically coupling the ERK pathway to the upstream activator (FAK), consistent with a RACK1 scaffolding function.



The molecular mechanism(s) by which ERK regulates adhesion disassembly remains to be elucidated. One possibility is that active ERK promotes disassembly of adhesions via suppression of integrin affinity for ECM (Hughes et al., 1997). Myosin light chain kinase (MLCK) is a potential downstream target, as it has been shown to be phosphorylated by ERK and to regulate adhesion disassembly (Klemke et al., 1997). ERK may also activate proteases such as calpain to induce calpain-mediated proteolysis of adhesion components and thus regulate adhesion disassembly (Carragher et al., 2003; Franco et al., 2004; Glading et al., 2004). In addition, ERK phosphorylates paxillin on serine residue 83 which may modulate the association of specific adhesion molecules (Ishibe et al., 2004). In summary, we propose that RACK1 serves as a scaffold protein that organizes integrin- and FAK- induced ERK activation and localization to adhesions. In this way, a pool of active ERK is created that is spatially and temporally restricted to the site of integrin engagement and adhesion formation and is separated from bulk cellular ERK signaling, providing a specific channel for activation and regulation of the MAP Kinase pathway. This localized ERK activation, which is generated coordinately with integrin-induced adhesion assembly, represents a negative regulatory loop which promotes adhesion disassembly.

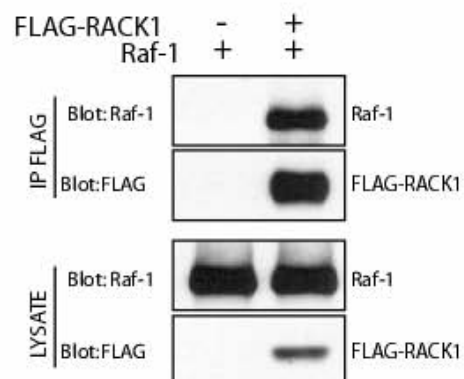
**Figures****Figure 27. RACK1 associates with components of ERK pathway.**

**(A)** Co-immunoprecipitation of Raf-1 with RACK1. CCL39 cells were transfected with either control vector or FLAG-RACK1 and untagged Raf-1. RACK1 precipitation and immunodetection were performed as in Fig. 1A with the exception that Raf-1 antibody was used to detect Raf-1. **(B)** Co-immunoprecipitation of RACK1 with ERK1 and ERK2. CCL39 cells were transfected with control vector, FLAG-ERK1 or FLAG-ERK2 constructs and HA-RACK1. ERK precipitation and immunodetection were performed as in Fig. 1A and RACK1 was detected with HA antibody. **(C)** Co-immunoprecipitation of RACK1 with MEK1 and MEK2. CCL39 cells were transfected with either control vector, FLAG-MEK1 or FLAG-MEK2 constructs and HA-RACK1. MEK precipitation and immunodetection were performed as in Fig. 1B. Upper band in MEK blot represents FLAG antibody heavy chain)

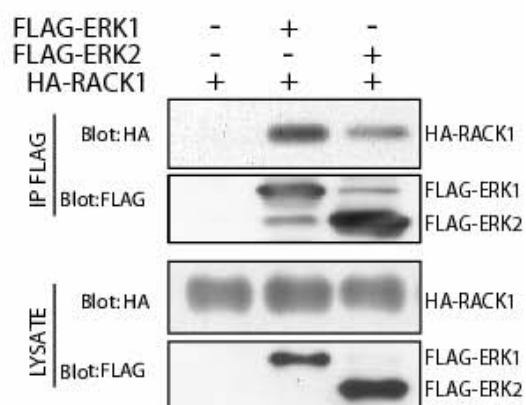
**Experiment performed by Tomas Vomastek**

Figure 27.

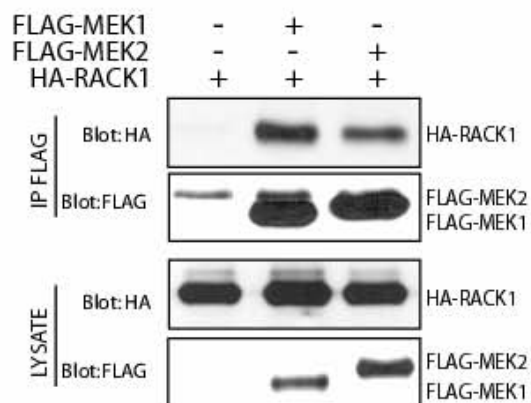
A.



B.



C.

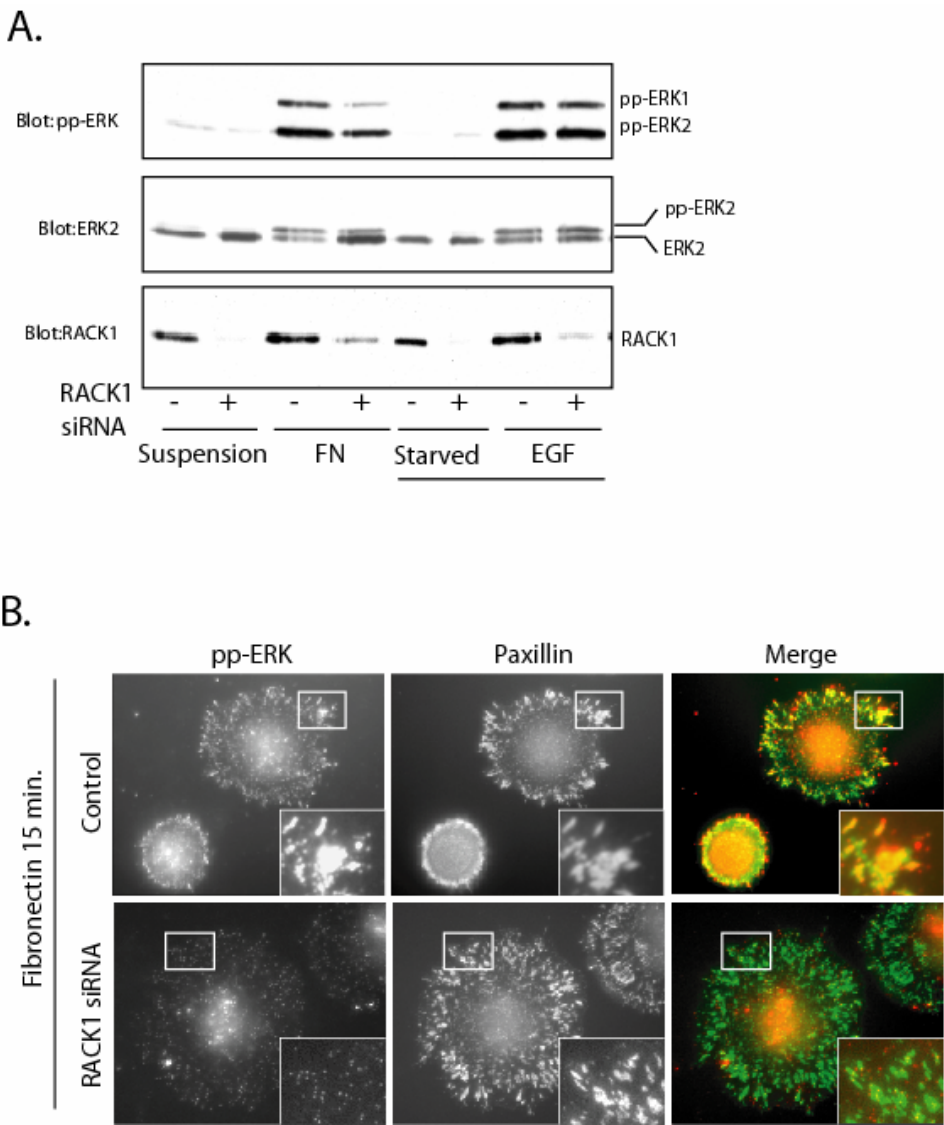


**Figure 28. Suppression of RACK1 interferes with ERK activation in response to adhesion.**

**(A)** Downregulation of endogenous RACK1 protein inhibits ERK activation in response to adhesion. REF52 cells were transfected with siRNA for 48 hours, suspended in serum-free medium for 90 minutes and replated on fibronectin coated dishes for 15 minutes. Cell lysates were probed with antibody recognizing doubly phosphorylated active form of ERK and with antibody directed against ERK2 and RACK1 to confirm equal loading of proteins and knockdown efficiency, respectively. Depletion of RACK1 protein by siRNA targeting different regions of RACK1 mRNA also inhibited ERK activation in response to adhesion to fibronectin confirming the siRNA specificity (data not shown). **(B)** RACK1 silencing inhibits active ERK localization to substrate adhesions in response to fibronectin. REF52 cells were transfected with siRNA for 48 hours, suspended and replated on fibronectin for 15 minutes. Cells were co-stained with antibodies recognizing active form of ERK (red) and paxillin (green). The inset shows a higher magnification of boxed area. Cell lysates were probed with antibody directed against RACK1 and ERK2 to confirm knockdown efficiency and equal loading of proteins, respectively (right panel)

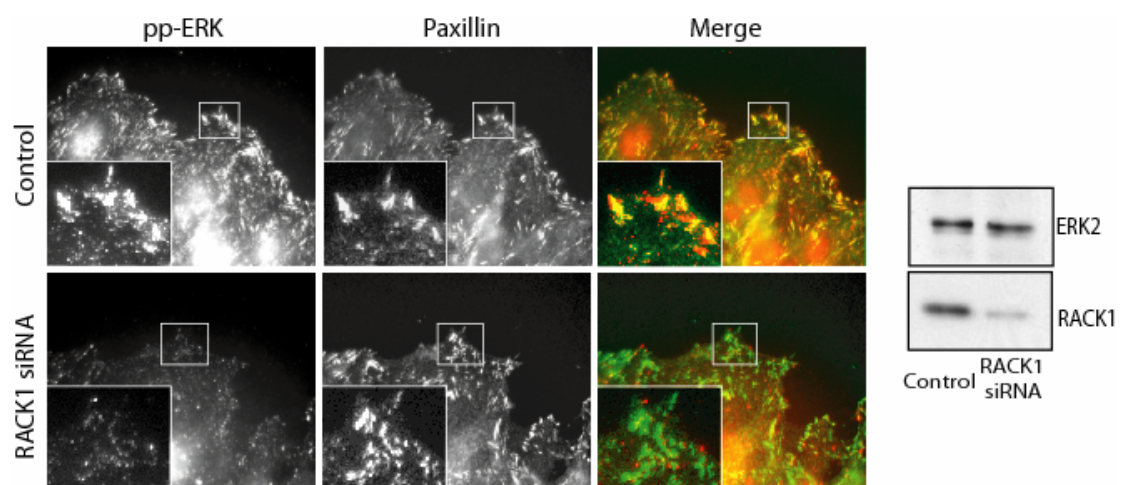
**Experiment performed by Tomas Vomastek (A) and Marcin Iwanicki (B)**

Figure 28



**Figure 29. RACK 1 regulates the localization of active ERK to adhesions in wounded cell monolayers.**

NIH3T3 cells were transfected with siRNA and 48 hours after the transfection wound was made in confluent monolayer of cells in the presence of 10% serum. 5 hours after the wound cells were co-stained with antibodies recognizing the active form of ERK (red) and paxillin (green). The inset shows a higher magnification of boxed area. Western blot shows levels of RACK 1 expression in this experiment. **Experiment performed by Marcin Iwanicki.**

**Figure 29**

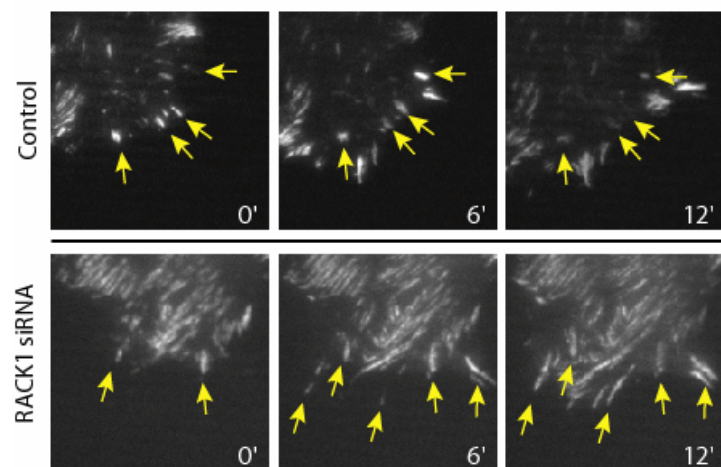
**Figure 30. RACK1 regulates adhesion disassembly and cell motility.**

**(A)** Series of images from time-lapse TIRF microscopy recording the dynamics of GFP-paxillin adhesions in control (upper panel) and RACK1 knockdown (lower panel) cells. Arrows indicate examples of dynamic adhesions at the protrusive region of the cell where both assembly and disassembly were seen. NIH3T3 cells stably expressing GFP-paxillin were transfected and wounded as described in Fig. 22. GFP-paxillin containing adhesions were imaged 5 hrs after wounding by time-lapse TIRF microscopy by collecting images every 3 min. **(B)** Rate of adhesions disassembly. Rate constants of adhesion disassembly were determined from values obtained from 37 adhesions in 4 cells (control cells) or 30 adhesions in 5 cells (RACK1 siRNA treated cells). Each point represents one adhesion analyzed. Only adhesions that displayed an initial increase in fluorescent intensity (i.e. adhesion assembly) were included in the analysis. **(C)** Rate of adhesion assembly. Rate constants of adhesion assembly were determined from values obtained from 16 adhesions in 4 cells (control cells) or 15 adhesions in 5 cells (RACK1 siRNA treated cells). **(D)** RACK1 depletion inhibits cell migration toward fibronectin. REF52 cells were transfected with siRNA for 48 hours, and suspended, and cell migration toward the fibronectin was measured in triplicates in a Boyden chamber assay. The data represent the average  $\pm$  standard deviation. Cell lysates were probed with antibody directed against RACK1 and ERK2 to confirm knockdown efficiency and equal loading of proteins, respectively (right panel). Depletion of RACK1 protein by siRNA targeting different regions of RACK1 mRNA also inhibited cell migration toward fibronectin (data not shown). **Experiment performed by Marcin Iwanicki (Rodriguez-Frade et al.) and Tomas Vomastek (D)**

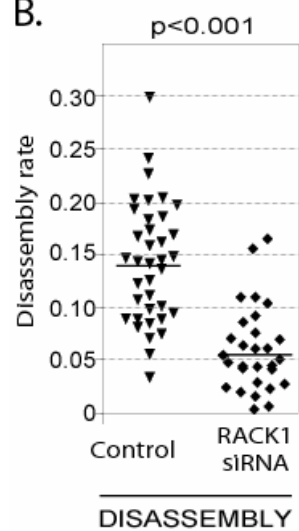


Figure 30

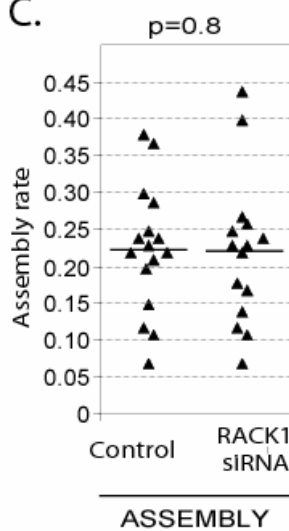
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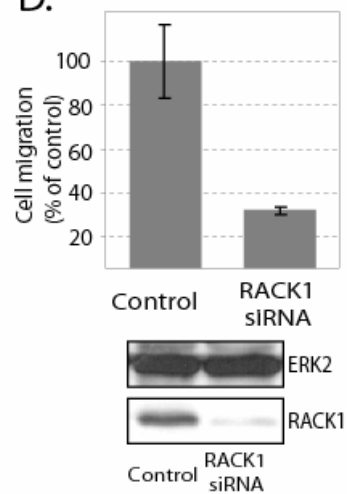
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C.



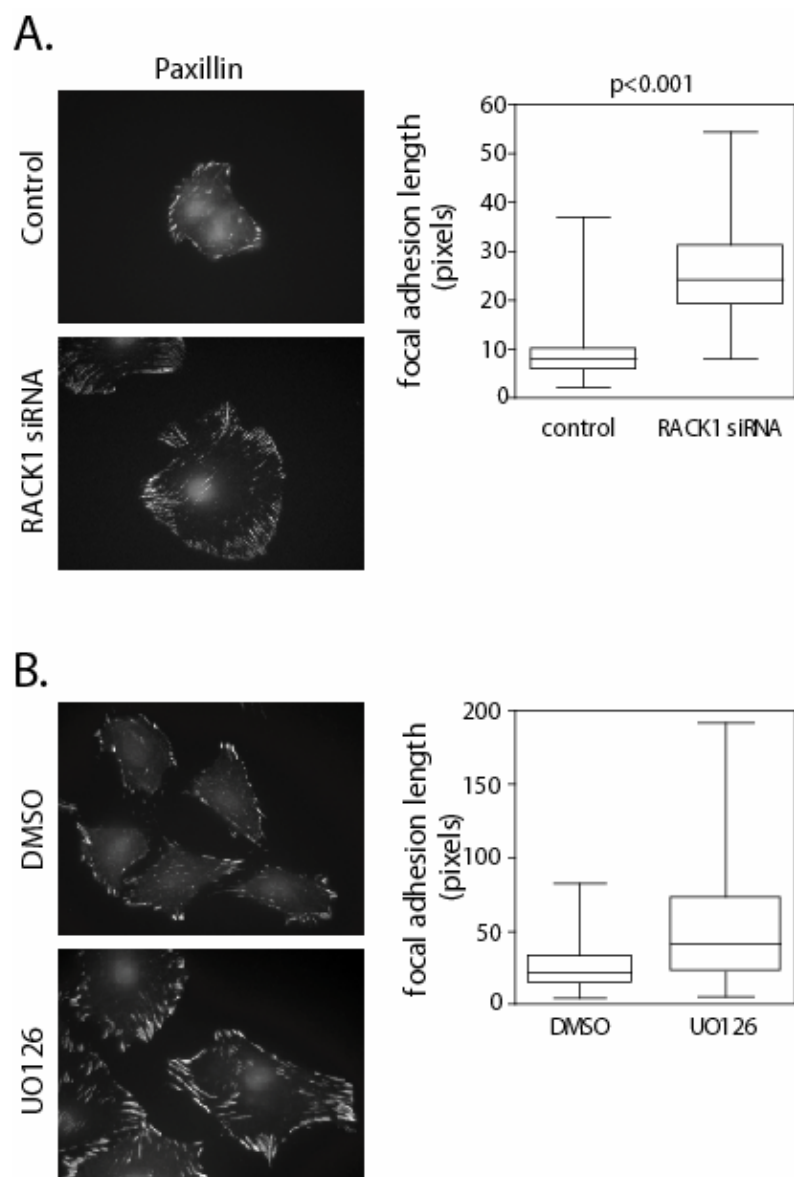
D.



**Figure 31 RACK1 and ERK regulate adhesion length.**

**(A)** RACK1 silencing increases adhesion length. RAT2 cells were transfected with siRNA, and 48 hours after the transfection cells were plated on fibronectin (1 $\mu$ g/ml) coated coverslips in the presence of 10% serum. 4 hours after the plating cells were stained with antibody recognizing paxillin. Right panel shows the quantification of adhesion length in control and RACK1 siRNA transfected cells (determined from values obtained from ~200 adhesions in 10 cells). Only peripheral adhesions were included in the analysis. Depletion of RACK1 protein by siRNA targeting different regions of RACK1 mRNA also increased the length of the adhesions (data not shown). **(B)** MEK inhibition induces increase in adhesion length. RAT2 cells were plated on fibronectin (1 $\mu$ g/ml) coated coverslips and treated with the MEK inhibitor UO126 (10mM) for 48 hrs. Cell staining and quantification of adhesion length were done as in A. **Experiment performed by Marcin Iwanicki (A) and Tomas Vomastek (B).**

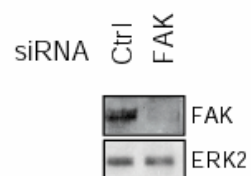
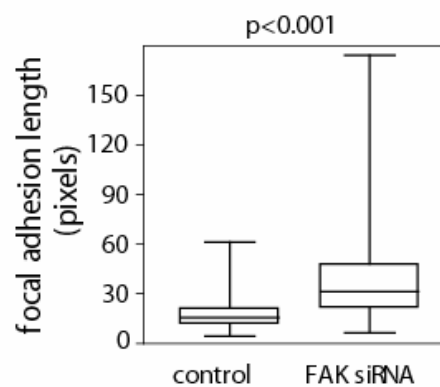
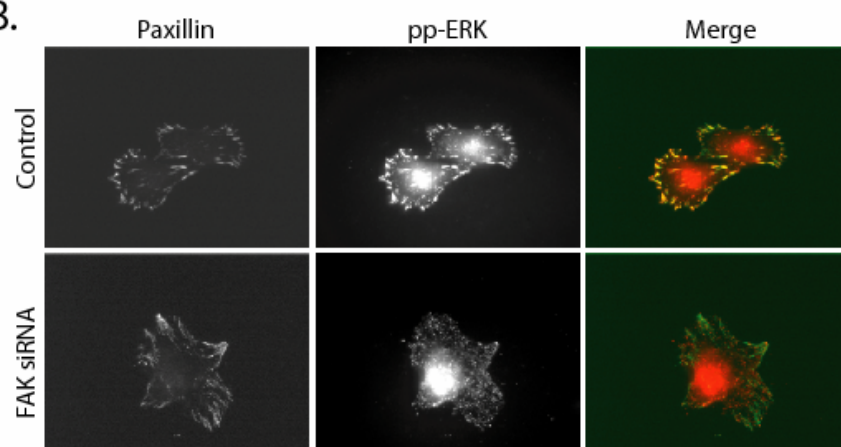
Figure 31



**Figure 32. FAK regulates active ERK localization to adhesions and adhesion length .**

**(A)** Efficiency of FAK knockdown. RAT2 cells were transfected with FAK siRNA for 48 hours, suspended in medium with 10% serum and replated on fibronectin-coated dishes for 4 hours. Cell lysates were probed with antibodies recognizing FAK, active FAK (FAKpY397), ERK and active ERK. **(B)** FAK silencing inhibits active ERK localization to adhesions. RAT2 cells were transfected with FAK siRNA for 48 hours, suspended in medium with 10% serum and replated on fibronectin coated coverslips. 4 hours after the plating cells were co-stained with antibodies recognizing the active form of ERK (red) and paxillin (green). Graph represents quantification of adhesion length in control and FAK siRNA treated cells.

**Experiment performed by Tomas Vomastek.**

**Figure 32****A.****B.**

## CHAPTER 4

### Summary and possible models

Cell migration is a biological process required for development and adult tissue homeostasis. The mechanical events that characterize cell migration include polarized protrusion of the lamellipodium and distinct trailing edge retraction. Cells that migrate form and disassemble adhesion structures within the protruding lamellipodia and mature, move and break adhesions within the trailing edges. The goal of this research was to identify the signaling pathways that control dynamic behavior of adhesions found in the rear and the front of polarized fibroblasts.

In chapter 2 of this dissertation, we described experiments that showed that FAK, through the involvement of PDZ domain-containing guanine nucleotide exchange factor, couples Rho and ROCK II signaling at mature adhesions to control adhesion movement (treadmilling) and trailing edge retraction. In chapter 3 of this dissertation, we identified the requirements for the scaffold protein RACK 1 in localizing the ERK pathway components to integrin/FAK signaling to control adhesion disassembly in the protruding lamellipodia of fibroblasts.

In the first and second part of this discussion, I will summarize the data and present major conclusions drawn thus far. In the third part of the discussion, I will combine conclusions from part 1 and part 2 and present a model for signal-dependent spatial regulation of adhesion dynamics during cell migration.

### **Role of FAK in fibroblast trailing edge retraction and adhesion movement.**

First insights into the role of FAK in cell migration came from the studies using fibroblasts that were derived from embryos in which the FAK gene had been disrupted. FAK-null fibroblasts plated on fibronectin-coated dishes showed two-fold reduced migration as compared to control cells. In addition, in steady state growth conditions, FAK null fibroblasts had enlarged adhesions (Ilic et al., 1995). These data suggested that the events that regulate adhesion life cycle could be directly linked to the migratory process and FAK was part of this process. Later, two studies showed that FAK null fibroblasts did not properly regulate Rho activity in response to fibronectin adhesion (Ren et al., 2000) and LPA stimulation (Palazzo et al., 2004). These studies indicate that FAK participates in RhoA mediated regulation of adhesion and cytoskeleton dynamics. Recent studies showed that the FAK–Src complex phosphorylated certain Rho GEFs and promoted Rho activation (Chikumi et al., 2002; Zhai et al., 2003) further arguing for a role for FAK in Rho regulation.

In chapter 2 we provide the first evidence that FAK signaling to a specific adhesion/actin associated Rho guanine nucleotide exchange factor regulates Rho GTPase-dependent dynamics of mature adhesions found in trailing edges of polarized fibroblasts. The siRNA mediated attenuation of FAK expression in fibroblastic cells resulted in stabilization of trailing edges and overall cellular elongation, suggesting defects in trailing edge retraction. Similar phenotypes were observed in serum starved fibroblasts, suggesting that trailing edge retraction is driven by a factor present in the serum. In serum-starved fibroblasts, activation of LPA receptors that are coupled to heterotrimeric G proteins but not activation of tyrosine kinase receptors (e.g PDGFR) resulted in rapid trailing edge retraction and restoration of normal cell morphology. These

data indicate that trailing edge retraction was regulated by events downstream of heterotrimeric G proteins. LPA-induced trailing edge retraction was accompanied by the activation of FAK and rapid inward movement of GFP-paxillin- or GFP-FAK- containing adhesions found in the trailing edge of fibroblasts. Furthermore, siRNA-mediated attenuation of FAK expression in fibroblasts blocked LPA receptor-mediated trailing edge retraction and inward movement of GFP-paxillin-containing adhesions. These data indicate that FAK is a key component, regulating trailing edge retraction and adhesion movement in response to activated G proteins.

It is well established that activation of LPA receptors (G protein-coupled receptors) increases tyrosine phosphorylation of FAK (Luttrell et al., 1997) and induces Rho mediated cytoskeletal changes (Ridley and Hall, 1992). LPA-mediated activation of Rho is very well understood (Moolenaar et al., 2004). Binding of LPA to LPA receptors induces association of LPA receptors with heterotrimeric G proteins (G12, G13). The alpha subunit of the heterotrimer binds GTP and upon interaction with the RGS domain-containing Rho GEF becomes activated. Following this interaction, Rho GEF binds via its DH domain to Rho GTPase and facilitates exchange of GDP to GTP (Fukuhara et al., 1999; Reuther et al., 2001; Togashi et al., 2000; Zheng et al., 1995a). As shown in Fig. 20, we find that FAK forms a complex and co-localizes with the RGS domain-containing Rho GEF (PDZ-RhoGEF). In addition, ectopic expression of PDZ-RhoGEF but not PDZ-RhoGEF lacking the DH domain or p115RhoGEF (a close family member that does not localize to adhesions) rescued trailing edge retraction in cells where FAK was attenuated. These data argue that this downstream effector of G12/G13 can rescue trailing edge retraction in the absence of FAK.

Consistent with these results, we observed that siRNA-mediated inhibition of PDZ-RhoGEF in fibroblasts resulted in the phenotype that was similar to that observed in



FAK siRNA treated cells. PDZ-RhoGEF siRNA treated cells exhibited an elongated phenotype in the presence of 10% serum, indicating defects in trailing edge retraction. Indeed, LPA stimulation of adhesion dynamics or trailing edge retraction in PDZ-RhoGEF siRNA treated cells was significantly decreased. These observations are in agreement with the previous published results showing that the FAK/Src complex phosphorylates PDZ-RhoGEF and promotes Rho activation in response to G12/G13 agonists (Chikumi et al., 2002).

FAK has been shown to regulate Rho activity through promoting phosphorylation of a different RhoGEF, p190-RhoGEF (Zhai et al., 2003). In contrast to PDZ-RhoGEF, p190RhoGEF does not have an RGS domain and it has not been shown to be downstream effector of heterotrimeric G proteins (Rossman et al., 2005). However, similar to PDZ-RhoGEF, p190 RhoGEF localizes to adhesions and siRNA-mediated attenuation of p190 RhoGEF in fibroblasts induces cell elongation, suggesting a defect in trailing edge retraction (these data were published concurrently with our data) (Lim et al., 2008). Both observations are consistent with the model in which FAK-dependent localized activation of Rho induces adhesion inward movement that is concomitant with trailing edge retraction.

Fibroblast trailing edge retraction depends on actomyosin regulation (Riento and Ridley, 2003; Vicente-Manzanares et al., 2007). ROCK has been shown to directly regulate actomyosin contractility through modulation of myosin light chain phosphorylation (Yoneda et al., 2005). In our experiments, restoration of trailing edge retraction in FAK siRNA-treated cells depended on Rho-mediated activation of ROCK I/II but not the other Rho A effectors, mDia I /II. Consistent with these data, we showed that siRNA-mediated attenuation of ROCK II but not ROCK I resulted in a similar phenotype as FAK or PDZ-RhoGEF knock-down cells. ROCK II- but not ROCK I-

depleted cells became elongated, with prominent mature adhesions in the trailing edge. LPA-induced trailing edge retraction and adhesion movement are dependent on ROCKII. Interestingly, cells in which expression level of ROCK I was decreased did not become elongated and had very small adhesions, a phenotype opposite to FAK, PDZ-RhoGEF and ROCK II knock-down cells. This observation is consistent with the requirement for ROCK I to maintain actomyosin contractility and adhesion stability.

These data indicate that ROCK I and ROCK II are not redundant and contribute differently to trailing edge retraction and adhesion dynamics in fibroblasts. Recently published studies support the differential roles of ROCK I and ROCK II in the regulation of actomyosin and adhesion (Samarin et al., 2007; Yoneda et al., 2005; Yoneda et al., 2007). In these studies, ROCK II attenuation did not alter myosin activation as evidenced by MLC phosphorylation, actomyosin or adhesion integrity, the events sensitive to ROCK I depletion. Our studies also showed that ROCK II, FAK or PDZ-RhoGEF knock-down cells did not exhibit alterations in LPA-induced MLC phosphorylation (data not shown). In preliminary experiments, shRNA-mediated attenuation of FAK, ROCK II and PDZ-RhoGEF, increased adhesion size, whereas attenuation of ROCK I decreased adhesion size, indicating that ROCK I regulates adhesion dynamics distinctly from FAK, PDZ-RhoGEF and ROCK II. One possibility is that ROCK I directly regulates via increased actomyosin contractility adhesion assembly and maturation. ROCK I signaling to the actomyosin may be triggered by the activation of the LPA receptor and downstream signals independently of FAK, PDZ-RhoGEF and ROCK II. FAK, PDZ-RhoGEF and ROCK II however, may play a role in promoting adhesion disassembly. Therefore, lack of adhesion movement (e.g., treadmilling) in the absence of FAK, PDZ-RhoGEF or ROCK II may reflect the failure to stimulate the turnover of components within the adhesion. Since adhesions remain under tension from the ROCK I pathway,

addition of new molecules to the adhesions could take place and be independent of the FAK/PDZ-RhoGEF/ROCK II pathway. In support of this hypothesis, we have observed in preliminary experiments that adhesions increase in length in cells lacking the expression of FAK, PDZ-RhoGEF or ROCK II.

The mechanisms that regulate adhesion treadmilling are poorly understood. It is clear that adhesion treadmilling is contractility-dependent adhesion assembly at the one end of the adhesion and disassembly at the other end of the adhesions. We speculate that the LPA/FAK-dependent RhoA and ROCK II activation contributes to adhesion disassembly and that LPA/RhoA/ROCK I activation increase actomyosin contractility and promotes adhesion assembly. Therefore, the ROCK I and the ROCK II pathways are both necessary for adhesion treadmilling.

**Role of RACK 1 in the regulation of FAK /ERK-dependent adhesion disassembly in the lamellipodia of migrating cells.**

A key event in directional migration is the formation of protrusive lamellipodia in the direction of cell migration. In the lamellipodia of polarized fibroblasts, adhesion dynamics are tightly controlled by temporal and spatial organization of signaling modules within adhesions. In Chapter 3 of this thesis, we presented evidence that the integrin-linked scaffold protein RACK 1 associates with core components of the MAP kinase pathway and targets this pathway to adhesions for activation.

A plethora of receptor agonists will activate the MAP kinase pathway (Kolch, 2005). The mechanism by which ERK is coupled to different receptors depends on scaffold or anchoring proteins (Kolch, 2005). These non-enzymatic molecules channel the ERK pathway to upstream activators. For instance, MAP Kinase Partner 1 (MP1)

associates with MEK and ERK and present this pathway to the upstream activator Rac/cdc42-PAK pathway (Pullikuth et al., 2005). MP1, by virtue of its ability to interact with MEK 1 and PAK, regulates phosphorylation of serine residue 298 of MEK 1 and primes MEK for activation by Raf (Pullikuth et al., 2005).

Consistent with these results, we observed that another scaffold protein RACK 1 tethers components of the MAP kinase pathway (Raf, MEK and ERK) and presents this pathway to upstream activators. A role for PAK in the activation of this pathway is indicated by our observation that in the absence of RACK 1, integrin-dependent activation of Raf by PAK (data not shown) and subsequent ERK activation was decreased. These results lead us to propose that RACK 1 is a scaffold that links integrin engagement with PAK/Raf-dependent MAP kinase activation. RACK 1 binds MP1 a scaffold protein for MEK, ERK and PAK, thus, it is possible that in the absence of RACK 1, PAK is never recruited to the complex (Pullikuth et al., 2005) and this results in the failure to activate the ERK pathway (Figure 33).

Integrin engagement results in the formation of distinct adhesion structures. Activated ERK has been shown previously to be part of adhesions in cells spreading on fibronectin (Slack-Davis et al., 2003a). Consistent with these data, we observed that attenuation of RACK 1 expression by siRNA altered localization of active ERK in adhesions. In cells with actively protruding lamellipodia, RACK 1 was required for activated ERK localization to de novo formed adhesions. Our data are consistent with a model in which RACK 1 targets components of MAP kinase signaling to the integrin pathway.

One of the properties of MAP kinase signaling is the ability to regulate adhesion disassembly and cell migration (Webb et al., 2004). Cells treated with MEK inhibitor migrate poorly and have low rates of adhesion disassembly. Consistent with these data,

we observed that siRNA attenuation of RACK1 expression impaired cell migration and adhesion disassembly in the protruding areas of the cell. A reduced rate of adhesion disassembly during wound-induced migration was also reflected in an increase of adhesion length, suggesting reduced turnover of these adhesions. Our data argue that coupling of the ERK pathway to newly assembled adhesions within lamellipodia provides a negative feedback loop that promotes adhesion disassembly.

The disassembly rates of adhesions in the lamellipodia depend on FAK signaling. Forced expression of activated MEK in FAK null cells partially rescues adhesion disassembly rates in cells with protruding lamellipodias (Webb et al., 2004). Furthermore, FAK has been shown to regulate integrin-dependent activation of MAPK during acute integrin engagement (Slack-Davis et al., 2003a). Consistent with these data, we observed that FAK was necessary for active ERK localization to adhesions in the presence of RACK 1. Furthermore, similar to RACK 1-depleted cells or MEK inhibitor-treated cells, FAK siRNA treated cells showed elongated focal adhesions. These results agree with the model in which RACK1 organizes MAP kinase core components and recruits it to adhesions for the activation.

The biochemical mechanisms by which ERK contributes to regulation of adhesion disassembly remain to be elucidated. Studies focusing on identification of new substrates for ERK during acute integrin engagement are likely to shed some light. Nevertheless, ERK has been shown to phosphorylate substrates implicated in the regulation of adhesions (Klemke et al., 1997), (Ishibe et al., 2004) (Carragher et al., 2003; Franco et al., 2004) and to inhibit activation of integrins (Hughes et al., 1997), which most likely leads to weakening of adhesions and adhesion disassembly. However, in all these examples, the requirement for ERK specific phosphorylation in the regulation of adhesion dynamics is yet to be determined.

**FAK couples two distinct signaling pathways to spatially regulate adhesion dynamics.**

Protrusions at the front of the cell and trailing edge retraction are tightly coupled to the regulation of substrate adhesions found in the front and back of migrating fibroblasts (Vicente-Manzanares et al., 2007). In the protruding areas, adhesions are stationary but disassemble at relatively fast rates. On the other hand, during trailing edge retraction, adhesions move in the inward direction but disassemble slower than front adhesions (Palecek et al., 1998; Smilenov et al., 1999). The work presented in this thesis argues that the spatial dichotomy of adhesion behavior is regulated by two distinct signaling pathways with a common initiator. Based on our studies we suggest the following model.

During lamellipodial adhesion formation, integrin activation leads to adhesion-localized activation of the ERK pathway. In this model, integrin-dependent activation of ERK relies on two events: First, the integrin-linked scaffold RACK1 recruits MAP kinase core components to the adhesion. Second, activation of FAK results in the downstream activation of MAP kinase components via one or more pathways (Figure 33). Therefore, FAK-dependent activation of MAP kinase at de novo formed adhesion sites primes these adhesions for disassembly. FAK-mediated activation of the ERK pathway may occur via different routes. First, phosphorylation of FAK by Src at tyrosine 925 may initiate the Grb2/Ras pathway leading to activation of the Raf kinase. Second, integrin-mediated activation of Rac may lead to the activation of PAK, promoting the phosphorylation of either Raf at serine 338 (King et al., 1998) and/or MEK at serine 298 (Slack-Davis et al., 2003b). Either pathway would lead to the subsequent activation of the ERK pathway. This model also predicts that failure to either activate FAK or localize RACK 1 would

result in the attenuation of integrin-dependent activation of Raf or MEK respectively and decrease adhesion disassembly rates (Webb et al., 2004). Therefore, localized events that negatively regulate FAK activity or prevent the localization of RACK 1 would fail to positively regulate ERK activation at newly formed adhesions and allow the adhesions to persist for longer times.

More persistent (mature) adhesions are predominantly found in the trailing edge of polarized fibroblasts (Rid et al., 2005). In contrast to de novo formed stationary front adhesions, these adhesions appear to be elongated and mobile (Smilenov et al., 1999). Interestingly, activated ERK staining in these adhesions seems to be less prominent than in leading edge adhesions (our unpublished observations). Thus we argue the possibility that signals controlling mobility/treadmilling of the adhesions found in the trailing edge differ from the signals controlling leading edge adhesion disassembly.

To test the model presented in this thesis, there is a need for further experiments addressing the issue of contribution of the Rho pathway to the regulation of disassembly rates of leading edge adhesions. We do not know if PDZ-RhoGEF and/or ROCK II contribute to leading edge adhesion disassembly and localization of active ERK to these adhesions. We speculate that the localization of active ERK and rates of leading edge adhesion disassembly should not depend on PDZ-RhoGEF or ROCK II. However, one possible outcome is that the absence of PDZ-RhoGEF or ROCK II may not alter integrin-mediated ERK activation or active ERK localization to adhesion structures but still inhibits leading edge adhesion disassembly. If this result is observed, it would indicate that there is an ERK-independent but Rho-dependent mechanism to regulate adhesion disassembly at the front, or that both pathways are necessary.

Our preliminary experiments indicate that pharmacological inhibition of MEK did not prevent LPA from stimulating adhesion movement in serum starved cells. These

preliminary observations argue that activation of ERK may not contribute significantly to controlling mature adhesion mobility. RhoA activation has been shown to occur at the leading edge of migrating fibroblasts (Pertz et al., 2006). In addition, RhoA effector mDia2 has been implicated in the regulation of adhesion dynamics in the lamellipodia of epithelial cells (Gupton et al., 2007). These data indicated that the activation of the RhoA/mDia2 pathway in the lamellipodia couples to leading edge adhesion turn-over. It is possible that PDZ-RhoGEF activates the RhoA/mDia2 pathway in the lamellipodia, thus participating in the regulation of leading edge adhesion turnover.

The work presented in this thesis places Focal Adhesion Kinase as a central initiator of different signaling pathways that spatially regulate adhesion dynamics in polarized fibroblasts. FAK has been shown to be involved in the regulation of cell migration (Parsons et al., 2008). Interestingly cancer cell types that exhibit fibroblastic migration show increased activation of FAK (McLean et al., 2005). Therefore it is reasonable to think that interference with FAK signaling to the ERK or the Rho pathway could modulate migration of selected cancer cells.

Migration of fibrosarcoma cells (HT1080) in vivo is characterized by formation of front protrusions and retraction of the trailing edge, a phenotype very similar to fibroblast migration in tissue culture conditions (Peter Friedl, Cell migration in Invasion and Inflammation, Keystone 2008). Interestingly, HT1080 cells show increased phosphorylation of FAK and FAK activation-dependent matrigel invasion (Park et al., 2005). Hyper-phosphorylation of FAK in HT1080 cells correlates with sustained Rho activation (Mukai et al., 2002). Furthermore, HT1080 fibrosarcoma cells possess oncogenic mutant alleles of N-Ras (Gupta et al., 2001) that constitutively activate the MAP kinase pathway. Inhibition of the MAP kinase pathway with a MEK inhibitor stops matrigel invasion of HT1080 cell, suggesting the involvement of the ERK in migration of



these cells (our observations). Thus, it is attractive to hypothesize that changes in FAK-dependent regulation of ERK and Rho pathways at adhesion structures regulate the metastatic potential of HT1080 cells. The use of advanced imaging techniques combined with genetic approaches will be necessary to address the issues of the in vivo relevance of FAK signaling to MAP kinase and Rho in the regulation of cell migration.

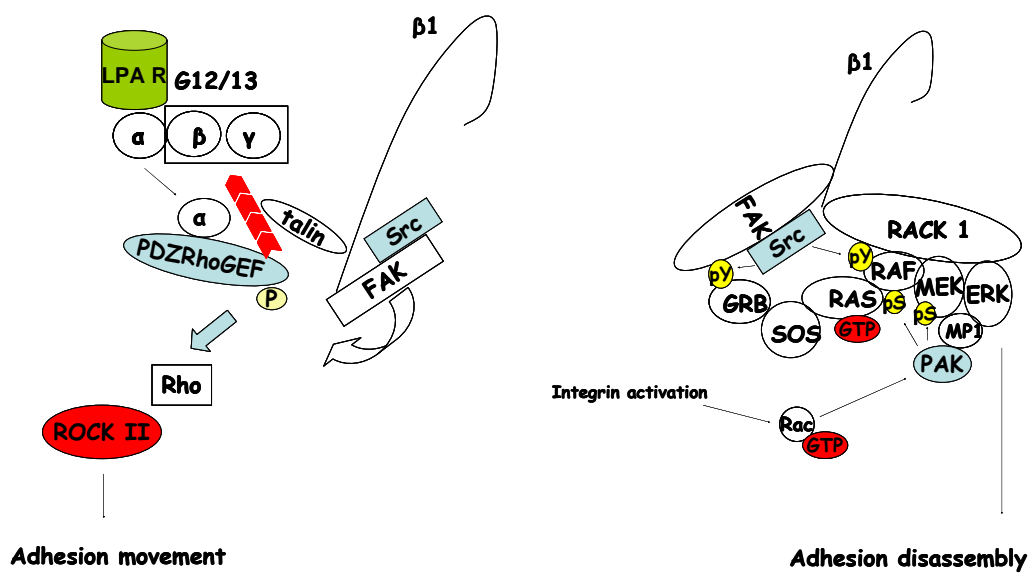
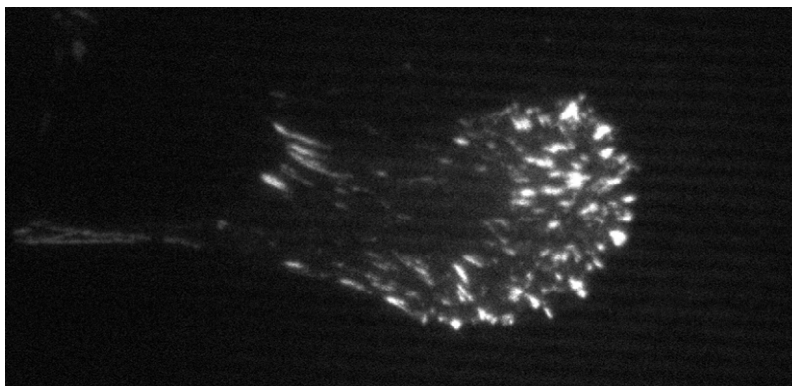
## Figures

**Figure 33. Proposed model of how FAK could spatially regulate adhesion dynamics in the lamellipodia and trailing edge of migrating cells.**

In the lamellipodia, integrin engagement with substrate induces recruitment of FAK to cytoplasmic portion of  $\beta 1$  integrin subunits. FAK phosphorylates itself at tyrosine 397 and recruits Src to form a bi-partite complex. In turn Src phosphorylates FAK at tyrosine 925 which serves as a docking site for the adaptor protein Grb2. Grb2, by recruitment of SOS initiates Ras signaling to activate the ERK pathway. Alternatively, Src can phosphorylate Raf at tyrosine 340/341 and initiate Raf dependent activation of the ERK pathway. In addition to Src, MP1/PAK complex is activated by integrin-dependent Rac activation. Activated PAK phosphorylates Raf at serine 338 and MEK and serine 298 and initiates the ERK pathway. Independently of FAK, the MAP kinase scaffold RACK1 is recruited to integrins and recruits the ERK (Raf, MEK, ERK) pathway to FAK for activation. In turn active ERK via downstream unknown molecules initiates adhesion disassembly.

In the trailing edge PDZ-RhoGEF that is present on actin filaments links LPA receptor activation with adhesion dynamics. PDZ-RhoGEF, via its RGS domain, binds to the GTP-loaded  $\alpha$  subunit of heterotrimeric G proteins and subsequently Rho A. The FAK/Src complex phosphorylates PDZ-RhoGEF and leads to PDZ-RhoGEF-mediated activation of Rho. Rho binds to ROCK II and activates it. ROCK II, via unknown mechanisms controls adhesion movement and trailing edge retraction.

Figure 33.



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