THE ROLE OF TYROSINE PHOSPHORYLATED CAVEOLIN-1 IN REGULATING FOCAL ADHESION DYNAMICS AND CANCER CELL MIGRATION

by

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ABSTRACT

Caveolin-1 (Cav1) is a conditional tumor suppressor whose expression is associated with a poor prognosis for many human cancer types. Cav1 is a Src kinase substrate phosphorylated on tyrosine-14 (pY14Cav1) that interacts with integrin and has been localized to focal adhesions (FAs). We undertook to study the role of pY14Cav1 in FA dynamics and tumor cell migration. Using FRAP analysis, we showed that pY14Cav1 phosphorylation and stabilization of FA kinase (FAK)-GFP in FAs occurs via a process that requires Rho/ROCK and Src signalling. In Cav1 expressing MDA-231 breast carcinoma cells, pY14Cav1 was enriched in purified pseudopodial fractions while in low Cav1-expressing MDA-435 tumor cells, transfected Cav1, but not the Y14F mutant of Cav1-mRFP, was recruited to actin-rich protrusions. In MDA-435 tumor cells, transfected Cav1 and the phosphomimetic Cav1 mutant, Cav1Y14D, were found in close proximity to FA-associated proteins. The recruitment of either Cav1 or Cav1Y14D to FAs was associated with increased cell migration. The positively charged Cav1Y14R mutant was not as closly associated with FA-associated proteins, and increased cell spreading and stress fiber formation. The spatial association of pY14Cav1 with FAs may therefore regulate FA signaling and dynamics.

The Mgat5 gene encodes the Golgi *N*-acetylglucosaminyltransferase-V that generates β1,6 branched *N*-glycans that bind galectin-3 at the cell surface forming a lattice domain. We found that the Mgat5/galectin lattice acts together with pY14Cav1 to stabilize FAK in FAs, thereby enhancing FA disassembly and de novo formation leading to the activation of directional tumor cell migration. Our data therefore argue that local interactions between the Mgat5/galectin lattice, Rho/ROCK and Src signalling and downstream phosphorylation of

pY14Cav1 at FA sites promote tumor cell migration by regulating local FA dynamics in protrusive domains of tumor cells.

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LIST OF ABBREVIATIONS

Bcl-2 – B-cell lymphoma/leukemia-2

Cav – Caveolin

CEM – Cholesterol-enriched membrane

CRD – Carbohydrate-recognition domain

CSD – Caveolin-1 scaffolding domain

Csk – C-terminal Src kinase

DRM – Detergent resistant membrane

ECM – Extracellular matrix

EGFR – Epidermal growth factor receptor

EMT – Epithelial mesenchymal transition

FA – Focal adhesion

FAK – FA kinase

FCS – Fluorescence correlation spectroscopy

FRAP – Fluorescence recovery after photobleaching

FRET – Fluorescence resonance energy transfer

FSM – Fluorescence speckle microscopy

Gal-3 – Galectin-3

GAP – GTPase activating factor

GDI – Guanine nucleotide dissociation factor

GDP – Guanine diphosphate

GEF – Guanine nucleotide exchange factor

GEM – Glycosphingolipid-enriched membrane

GFP – Green fluorescence protein

GlcNAc – *N*-acetylglucosamine

GlcNAc-TV – β1,6-*N*-acetylglucosaminyltransferase V

GM1 – Ganglioside monosialic acid 1

GPI – Glycosylphosphatidylinositol

Grb7 – Growth factor receptor-bound protein 7

GTP – Guanine triphosphate

HPV – Human papilloma virus

MMP – Matrix-metalloprotease

mRFP – Monomeric red fluorescence protein

MTOC – Microtubule organizing center

PTA – Podosome-type adhesion

PTEN – Phosphatase and tensin homolog

PTRF – Polymerase transcript release factor

pY14Cav1 – Tyrosine-14 phophorylated caveolin-1

PyMT - Polyoma middle T antigen

RGD – Arginine-Glycine-Aspartic Acid sequence

RNAi – Ribonucleic acid interference

ROCK – Rho kinase

SH2 – Src homology 2

shRNA – Short hairpin ribonucleic acid

siRNA – Small interfering ribonucleic acid

SW – Swainsonine

TGFβR – Transforming growth factor β receptor

TIRF – Total internal reflection fluorescence

TRAMP – Transgenic adenocarcinoma of mouse prostate

TTF-1 – Thyroid-specific transcription factor 1

UDP – Uridine diphosphate

VIP21 – Vesicular integral-membrane protein of 21 kDa

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For My Family

CHAPTER 1: INTRODUCTION

1.1 - TUMOR CELL MIGRATION AND ITS REGULATING FACTORS

1.1.1 – AN OVERVIEW OF TUMOR CELL MIGRATION

1.1.1.1 – The role of cell migration in tumor progression and metastasis

Abnormal cell migration and invasion is a characteristic of malignant cancer cells and represents one component of metastasis. The spread of cancer from its tissue of origin (primary site) and its subsequent growth in other organs (secondary sites) is the most fatal aspect of the disease (Sahai, 2005). At the time of cancer diagnosis approximately half of patients will have already developed metastasis (Hernandez-Alcoceba et al., 2000). The majority of cancer-related deaths are caused, not by the primary tumor itself, but by the subsequent development of metastases to distant organ sites. The ability to migrate away from their original site of origin and invade adjacent tissues, distinguishes cancerous tumors from their benign counterparts.

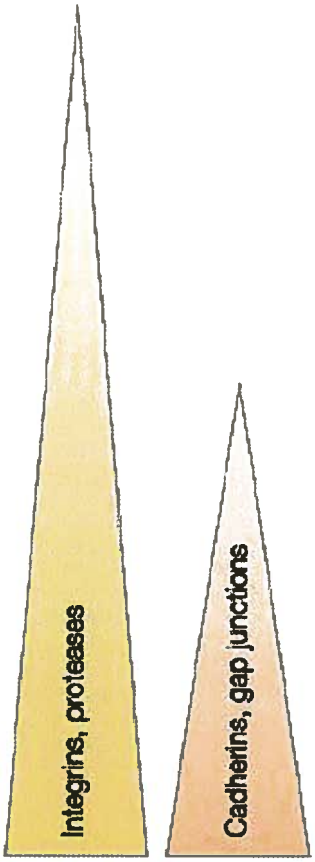
Many genes and proteins that are known to play essential roles during embryonic development are mutated or aberrantly expressed in cancerous cells. Microarray experiments have recently been reported to be able to distinguish which genes have an expression pattern that can be linked to the spread of cancer. This is done by analyzing clinical samples or comparing metastatic to non-metastatic cancer cell lines in experimental systems (Sahai, 2005). These abnormally expressed genes represent an ideal target for anti-motility therapies. Abnormal gene expression is further aided by other extrinsic factors that create a tumor micro-environment that is ideal for migration. For example, the production of matrix-

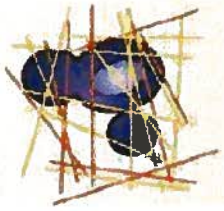
metalloproteases (MMPs), that are present in a majority of tumors, can degrade the extracellular matrix (ECM) that would otherwise inhibit cell migration (Grimshaw et al., 2004). Stromal cells have been shown to directly modulate cancer cell motility by producing growth factors in addition to these proteases (Grimshaw et al., 2004). Together, these factors allow tumor cells to successfully migrate into the ECM and infiltrate the lymphatics and vasculature systems, thereby allowing them to be transported to secondary sites elsewhere in the body.

1.1.1.2 – Tumor cell migration: the method and its modes

The current concept is that cellular migration is a multi-step, sequential system that may be referred to as haptokinetic migration that requires cellular polarization. The steps include the extension of protrusions at the front of the cell, formation of stable adhesions at the leading edge of the cell, reorientation of the microtubule organizing center (MTOC) and the Golgi toward the leading edge, translocation of the cell body in the direction of the movement, and focal adhesion (FA) release and retraction at the rear of the cell (Vicente-Manzanares et al., 2005). Many factors can influence each step of this system, and there have been vastly different modes of motility that have been characterized, specifically; mesenchymal (or fibroblast-like), amoeboid, and cell cluster migration (Friedl and Wolf, 2003) (Illustration 1). Both mesenchymal and amoeboid migrations are types of individual cell migration, whereas cell clustering refers to a collective cell migration strategy. The two modes of individual tumor cell movement are interchangeable and depend on environmental conditions. Not surprisingly, the ability of tumor cells to switch between different modes of motility may

Illustration 1: **The three different modes of tumor cell migration:** protease-independent amoeboid migration, protease-dependent mesenchymal migration, and protease/adherens junction-dependent collective cell migration (Adapted by permission from Macmillan Publishers Ltd: [NATURE REVIEWS. CANCER.] Friedl and Wolf, 2003).



Migration strategy		Tumour type
Ameoboid		Lymphoma Leukaemia SCLC
Mesenchymal		Fibrosarcoma Glioblastoma Anaplastic tumours
Cluster/cohorts		Epithelial cancer Melanoma

limit the effectiveness of single therapeutic agents aimed at reducing invasion (Wolf et al., 2003).

The first type of cell migration involves a switch from an epithelial to a mesenchymal phenotype. Epithelial cells normally form a monolayer that is tightly linked laterally by specialized junctions, including adherens junctions, desmosomes, tight junctions, and gap junctions. Additionally, these epithelial cells have an apical-basal polarity through their association with the basement membrane (Yang et al., 2004). This relationship with the basement membrane ensures that epithelial cells can only undergo lateral migration. Mesenchymal cells on the other hand rarely establish direct contact with other neighboring cells and exhibit a front-rear end polarity. They also have the ability to invade the underlying ECM as individual cells (Hay and Zuk, 1995). Transition between the two cell states was first identified in the early 1900s but was not recognized as a distinct cellular process, referred to as Epithelial-Mesenchymal Transition (EMT), until the 1980s (Greenburg and Hay, 1982). EMT is critical for tissue and organ formation during embryogenesis. In addition to its role in development, this process has a more threatening role in tumor progression (Thiery, 2002). Approximately 10-40% of carcinomas undergo EMT making it a very common element of the acquisition of cellular motility by tumor cells. For a long time EMT was not recognized as a potential mechanism for carcinoma progression as it could not be followed in human tumors. The great diversity of cellular organization present in human tumors makes it impossible to recognize EMT with certainty (Thiery, 2002).

The majority of solid tumors do not undergo EMT rather they employ an amoeboid method of migration. These cells have a rounded morphology and therefore do not have an obvious polarity. Proteases are also not relied upon as they are during mesenchymal migration (Sanz-Moreno et al., 2008) (Illustration 1). During amoeboid migration there is very active blebbing at the cell surface that is driven by actomyosin contractility (Wolf et al., 2003). This type of movement is similar to the invasion seen in tumor cells that have a distinct requirement for Rho/Rho kinase (ROCK) signalling (Sahai and Marshall, 2003). Recently, a Rho/ROCK/p38MAPK signalling pathway has been reported to stimulate both membrane blebbing and pseudopod formation (Jia et al., 2006). Imaging techniques have shown that amoeboid motility is up to 40 times faster, moving at a possible speed of 4μm/min, than mesenchymal migration (Friedl and Wolf, 2003).

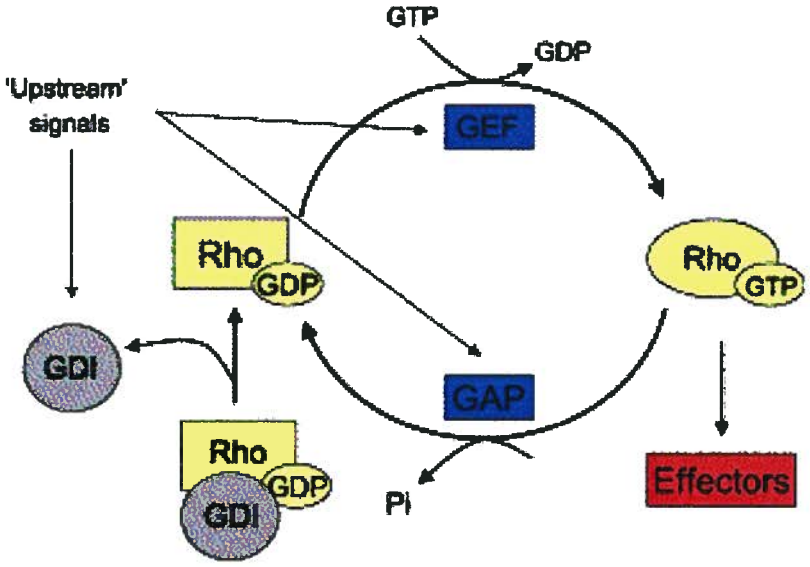
The final form of migration is referred to as collective cell migration. This involves the movement of whole clusters of invasive cells, and likely resembles the morphogenetic movement of epithelial sheets during development. Mechanistically, this form of migration is very similar to mesenchymal migration with the main exception being that lateral adherens junctions between cells are not severed (Sahai, 2005). Similar to mesenchymal migration, MMPs must be present to generate a path through the ECM for the cells to follow (Nabeshima et al., 2002) (Illustration 1). However, this migratory method is poorly understood due to the difficulty of modeling it in an *in vitro* system.

1.1.2 – CELLULAR MECHANISMS INVOLVED IN MIGRATION

1.1.2.1 – Actin dynamics

Optimal migration requires spatiotemporal feedback between actomyosin contraction, actin polymerization and repeated cycling between the formation and disassembly of adhesive complexes (focal adhesions) at the cell's leading edge (Goetz et al., 2008a; van der Flier and Sonnenberg, 2001). The polymerization of actin drives the protrusion of the cell membrane, cytoskeletal contraction and detachment at the rear (Lauffenburger and Horwitz, 1996). The most important intracellular regulatory elements with regards to migration are Rho, Rac and Cdc42; members of the Rho subfamily of the Ras family of GTP-binding proteins. They are all small GTPases that have control over the signal transduction pathways that mediate the distinct actin cytoskeleton changes required for cell migration (Jaffe and Hall, 2005; Joshi et al., 2008). These small GTPases function as molecular switches by cycling between an active, GTP-bound state and an inactive GDP-bound state. This cycling is tightly regulated by associated proteins, namely guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine dissociation inhibitors (GDIs). GEFs stimulate the exchange of bound GDP for GTP, leading to activation of the GTPases and binding of downstream effectors. Inactivation is promoted by GAPs and GDIs, that stimulate the intrinsic GTP hydrolysis rate of Rho-like proteins and inhibit the conversion of RhoGDP to RhoGTP, respectively (Van Aelst and D'Souza-Schorey, 1997) (Illustration 2). Actin polymerization, as well as the formation of nascent FAs, is stimulated by Rac and Cdc42 activation due to extracellular signals. As a result of external stimuli, Cdc42 and Rac activation lead to the formation of nascent FAs resulting in the development of actin-based pseudopodial protrusions referred to as filopodia and lamellipodia (Sander et al., 1999).

Illustration 2: **The regulatory cycle for the Rho family of GTPases:** A constant cycling between an active GTP-bound state and an inactive GDP-bound state is controlled by multiple factors, GEFs, GAPs and GDIs, and ultimately effects the migratory capabilities of a cell (Reprinted from Developmental Biology, Vol. 265, Issue 1, Raftopoulou and Hall, Cell migration: Rho GTPases lead the way, Pg. 25, 2004, with permission from Elsevier).


GTP
GDP
'Upstream' signals
GEF
Rho
GDP
Rho
GTP
GDI
GAP
Rho
GDP
GDI
Pi
Effectors

Subsequent Rho-activation, and the recruitment of its effector, ROCK, stabilizes mature FAs through an association with actin stress fibers and the actomyosin contractile machinery (Jaffe and Hall, 2005).

However, recent evidence has suggested that isoforms of Rho can regulate membrane protrusions, indicating that there may be a spatiotemporal dynamic associated with Rho GTPases (Jia et al., 2005; Jia et al., 2006; Pertz et al., 2006; Stuart et al., 2008). RhoC has been shown to play a role in tumor cell metastasis as opposed to acting as a FA stabilizing factor. RhoC signalling and its downstream activation of ROCK has been linked with an increase in tumor cell migration in colon and pancreatic carcinomas as well as A375 melanoma cells (Clark et al., 2000; Croft et al., 2004; Suwa et al., 1998). Activated Rho has also been identified at the leading edge of migrating metastatic breast carcinoma cells (Pertz et al., 2006; Stuart et al., 2008). Rho/ROCK signalling dynamics have been shown to play a key role in protease-independent, amoeboid tumor cell invasion (Sahai and Marshall, 2003; Wyckoff et al., 2006).

1.1.2.2 – Integrins

Integrins are transmembrane proteins involved in crosstalk between the ECM and the cytoskeleton of the cell. They aid in binding cells to various substrates of ECM, thus giving a cell the ability to generate contractile forces required for migration. They exist as a heterodimeric complex consisting of an α and β subunit. The 8 β subunits can assort with the 18 α subunits to create 24 distinct, non-redundant, integrins. The migration of tumor cells on specific substrates directly correlates with the combination and expression of distinct

integrins (Hynes, 2002; van der Flier and Sonnenberg, 2001). More specifically, integrins containing the α4-, α5-, α8-, αIIb-, or αV subunits bind to ECM components that contain the RGD (Arginine-Glycine-Aspartic Acid) sequence, namely fibronectin and vitronectin. Laminins and collagens also contain RGD sequences, but these are normally inaccessible. These ECM components are recognized by integrins containing the α3-, α6-, or α7-subunits (laminin-binding receptors) or the α1-, α2-, α10-, or α11-subunits (collagen-binding receptors) (van der Flier and Sonnenberg, 2001). Cells that have adhered to the substrate are reported to have constitutively activated integrins, although differences in the activation states have been seen at FAs within the same cell (Cruz et al., 1997). MMP1 is the preferred, non-matrix ligand for the α2-β1 integrin and can therefore aid in the migration of cells expressing this integrin (Dumin et al., 2001). Cell migration is a process that requires the precise regulation of integrin-mediated adhesion and its release (Lauffenburger and Horwitz, 1996).

Integrin clustering is controlled by remodeling of cytoskeletal elements and/or by multivalent ligands, resulting in an increase in their avidity and strong cell adhesion (van der Flier and Sonnenberg, 2001). The co-dependent role of actin-based pseudopodial protrusions and adhesion dynamics with regards to migration is clearly integral to cancer progression and metastasis.

1.1.2.3 – Cellular adhesions

Cell-ECM adhesions are essential for cell migration, tissue organization, and differentiation. The downstream signalling cascades that are initiated by the formation of these adhesions

regulate biological functions such as cell survival, cell proliferation, and tumorigenesis (Block et al., 2008). As stated previously, integrins are the key mediator of both cell-ECM attachments and the resulting signalling responses (Hynes, 2002). Upon binding the correct ligand, integrins induce reorganization of the actin cytoskeleton and associated proteins thereby causing the formation of cellular adhesions (Katz et al., 2000). These adhesion complexes show extraordinary molecular and structural diversity. Many distinct varieties of cell-ECM adhesions have been described, namely focal complexes, FAs (focal contacts), fibrillar adhesions, podosomes, and invadopodia (podosome-type adhesions) (Block et al., 2008).

Adhesive structures share many of the same proteins, although major structural differences have been observed. The first two forms of adhesive structures to be described are both podosome-type adhesions (PTA) and are referred to as podosomes and invadopodia. PTAs differ structurally in that they have a perpendicular cell-ECM adhesion arrangement and contain a ring of adhesive molecules centered on an actin column (Block et al., 2008). This contrasts the elongated structure and tangential orientation with the ECM found in the other three types of cellular adhesions. Depending on their structure and lifetime, PTAs are referred to as either podosomes or invadopodia. PTAs may be found in migratory and invasive cells (Block et al., 2008; Linder, 2007).

The idea of distinct types of ECM adhesions was recently confirmed in fibroblasts using digital microscopy by double-labeling for pairs of adhesion-associated molecules (Zamir et al., 1999). This study distinguished FAs from fibrillar adhesions by identifying differences

in their cytoskeletal association and in the composition of the submembrane plaques (Zamir et al., 1999). Another study on fibroblasts, using GFP-paxillin to analyse FAs and GFP-tensin to analyse fibrillar adhesions, has shown that both are highly dynamic structures with FAs primarily located at the cell periphery and fibrillar adhesions located centrally. Mature FAs can either disassemble under the approaching cell body during migration or they can form fibrillar adhesions that play critical roles in ECM modifications (Broussard et al., 2008). Fibrillar adhesions have the ability to reorganize a fibronectin substrate into fibrils, due to their association with α5-β1 fibronectin-binding integrin, thereby giving them a more elongated or beaded structure than FAs (Katz et al., 2000; Zamir et al., 2000). Nascent adhesion structures that form directly after the clustering of integrin, due to ECM binding and Rac activation, are referred to as focal complexes. They are small, 0.5- to 1-μm, dot-like contacts localized at the cell periphery. These structures are not connected to stress fibers although they have been shown to be linked to the actin network (Zamir and Geiger, 2001). These focal complexes mature, stabilize and develop into FAs (Nobes and Hall, 1995; Zamir and Geiger, 2001). It has been shown that the amount of phosphorylated focal adhesion kinase (FAK), an important protein involved in regulation of adhesion structure formation, is reduced in focal complexes compared to mature FAs (Zaidel-Bar et al., 2003).

1.1.2.3.1 – Focal adhesions

FAs were initially described almost 40 years ago by electron and interference-reflection microscopy (Abercrombie et al., 1971). They are also the best known and characterized type of cellular adhesion. It was originally thought that FAs were artificial structures only present

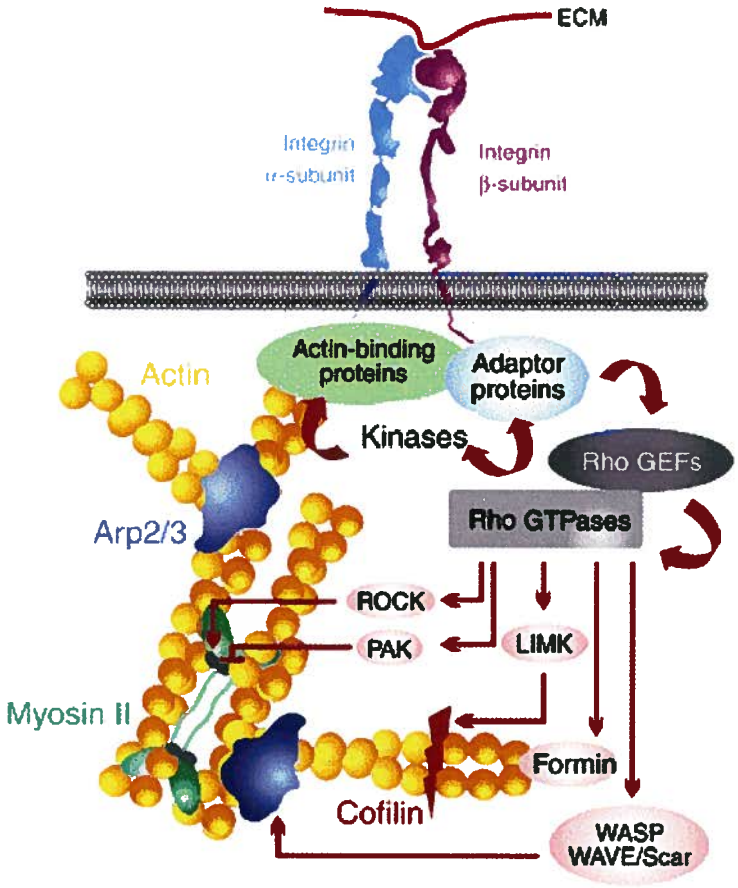
in cells cultured on rigid surfaces until they were identified *in vivo* at cell-ECM junctions (Cukierman et al., 2001; Fuchs et al., 1997).

FAs are 3-10μm in size and are found associated with actin stress fibers at the cell periphery. The formation, size and number of FAs are dependent on the arrangement of the actin cytoskeleton. Actin-binding proteins that are recruited to the cytoplasmic face of integrins provide both the scaffold and signalling platform from which a mature adhesion develops (Worth and Parsons, 2008) (Illustration 3). Inhibition of actin polymerization, using depolymerization agents such as latrunculin, causes the disassembly of mature FAs. Approximately 90 cellular molecules have been found to reside within FAs, with an additional 66 showing a transient interaction with them (Zaidel-Bar et al., 2007). Included in the multitude of molecules associated with FAs are anchoring and cytoskeletal proteins such as; talin, paxillin and vinculin. A number of signal transduction molecules including FAK, C-terminus Src kinase (Csk), protein kinase C, and others, are also present at FAs (Yamada and Miyamoto, 1995) (Illustration 3). Additionally, they contain relatively high levels of proteins with phosphorylated tyrosine residues (Katz et al., 2000). A vast majority of mature FAs are composed of either β1 or β3 integrins, with the dominant integrin being αV-β3 (Block et al., 2008; Zamir et al., 2000).

1.1.2.3.2 – Regulation of focal adhesion turnover

Interactions between proteins associated with FAs are under strict intracellular and extracellular control to tightly regulate the directionality and rate of cellular migration. A number of questions with regard to spatial recruitment, dynamic behavior, trafficking, and

Illustration 3: **Focal adhesion signalling pathways:** Integrin clustering and binding to the ECM induces the recruitment of different signalling elements. The major categories include; kinases, non-catalytic adaptor proteins, actin-binding proteins and tyrosine phosphorylated proteins. The majority of signalling requires Rho-GTPases which regulate actin polymerization and stability as well as actomyosin contractility (Reproduced with permission from The Company of Biologists: [Journal of Cell Science] Vicente-Manzanares et al., 2009).


ECM
Integrin
α-subunit
Integrin
β-subunit
Actin
Actin-binding proteins
Adaptor proteins
Kinases
Rho GEFs
Rho GTPases
Arp2/3
ROCK
PAK
LIMK
Myosin II
Formin
Cofilin
WASP
WAVE/Scar

binding partners of individual FA proteins are beginning to be addressed (Worth and Parsons, 2008). To understand FA dynamics a number of imaging techniques have been employed: Fluorescence recovery after photobleaching (FRAP) and fluorescence time-lapse imaging can be used to identify protein kinetics at FAs; total internal reflection fluorescence (TIRF) illuminates the molecular composition and dynamics at the site of the cell-ECM attachment; fluorescence resonance energy transfer (FRET) is used to identify direct protein-protein interactions; fluorescence correlation spectroscopy (FCS) can measure the specific protein concentration at a forming complex; and finally fluorescence speckle microscopy (FSM) is used to measure single molecule dynamics at FAs (Worth and Parsons, 2008). An understanding of FA dynamics in intact organisms is lacking and therefore three dimensional and *in vivo* imaging techniques have become available.

Upon formation, nascent adhesions can either turnover or mature. Integrins lack intrinsic catalytic activity and therefore to make this decision they require the recruitment of actin binding proteins as well as signalling molecules to trigger the generation of secondary intracellular signalling cascades (Illustration 3). Of particular importance is the presence of the non-receptor tyrosine kinase, FAK, which appears to be a key trigger in the turnover of adhesions (Broussard et al., 2008). Fibroblasts from FAK null mice were shown to have large FAs at the cell periphery (Ilic et al., 1995). Rises in calcium levels have been shown to induce adhesion disassembly by increasing the residency time of FAK at FAs (Giannone et al., 2004). FAK has also been shown to cause adhesion turnover by phosphorylating Src and p190RhoGAP which then decreases the activity of Rho and ROCK thereby shifting the Rho/Rac antagonistic interplay toward Rac activation and Rho inhibition. Increases in Rac

activation downregulate cytoskeletal tension at the leading edge resulting in FA turnover (Rottner et al., 1999). Interestingly, fibroblasts from Src null mice show a decreased rate of spreading and motility. It has been postulated that tyrosine-phosphorylated caveolin-1, phosphorylated by active c-Src, is located at adhesions along the leading edge where it couples with integrin and subsequently recruits FAK, resulting in stable turnover of FAs (Beardsley et al., 2005). Recently, the specificity of monoclonal antibodies raised against phosphorylated caveolin-1 has come into question, as they have been reported to recognize the tyrosine phosphorylated form of paxillin in immunofluorescence assays (Hill et al., 2007).

It is important to understand that interactions other than those occurring directly at the leading edge of the migrating cell also play a role in FA turnover. The rearrangement of FAs at the leading edge of the cell must be coupled with the correct adhesion dynamics at the rear of the cell for migration to be successful. Additionally, the contribution of mechanical forces to these processes cannot be overlooked. Under these forces, integrins remain associated with the substrate while the intracellular adhesive components, which are under tension, slide toward the cell body and eventually disperse (Crowley and Horwitz, 1995; Regen and Horwitz, 1992). Cytoskeletal contractions rupture weak mechanical interactions at the cell rear promoting the release of FAs (Webb et al., 2002)

The presence of over 150 FA associated molecules and the cytoskeletal forces that are affected by them, as well as the existence of a vast library of cell-types, make one precise definition of FA turnover difficult. Many explanations about how it occurs have been

suggested, however, the main requirements consistently include: the presence of FAK and Src, a cycling between active Rho and Rac, the coupling of adhesions with the actin cytoskeleton and its binding proteins, and lastly the dynamics of the cytoskeletal system.

1.2 – CELLULAR DOMAINS

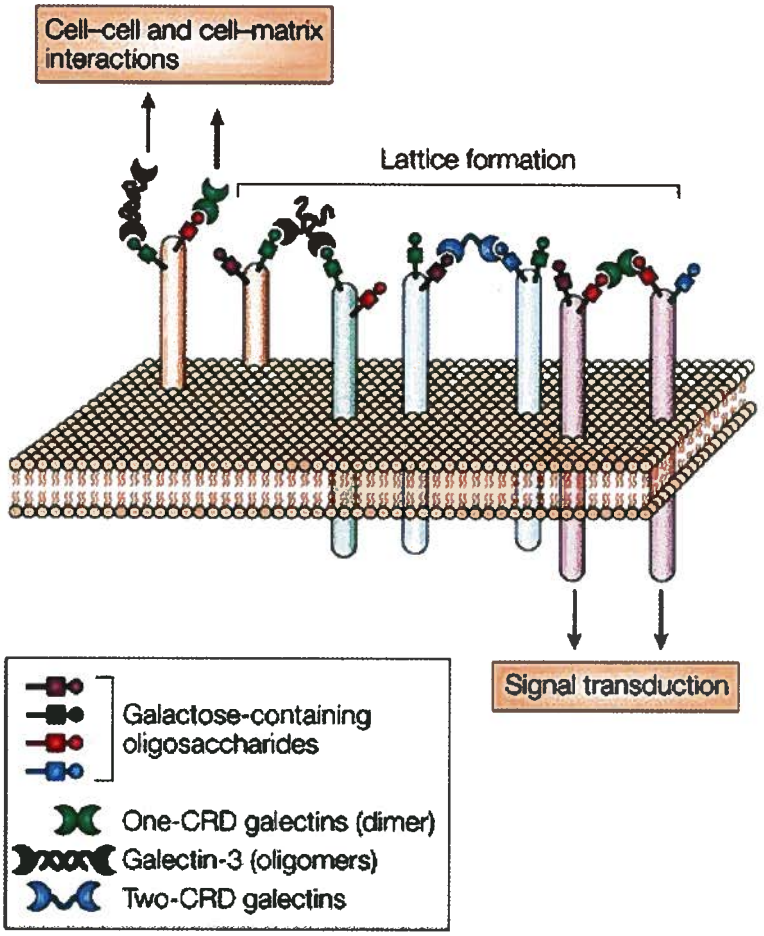
1.2.1 – THE MGAT5 / GALECTIN RELATIONSHIP

1.2.1.1 – Galectin family

Galectins are a large family of animal lectins which have a role in a variety of biological activities and recognize the carbohydrate, β-galactoside (Barondes et al., 1994). All galectins contain conserved carbohydrate-recognition domains (CRDs) of approximately 130 amino acids. These CRDs are the defining characteristic within the galectin family and are directly responsible for carbohydrate binding (Liu and Rabinovich, 2005). The ability of lectins to bind to specific glycoconjugates has proven to be a useful tool for studying changes in glycoprotein expression at the plasma membrane. To date, fifteen mammalian galectins have been identified, which can be subdivided into those that have one CRD and those that have two CRDs (Barondes et al., 1994) (Illustration 4). Galectins are found in the nucleus, cytoplasm, on the plasma membrane, and in the extracellular spaces. They have both an extracellular, by interacting with cell-surface and ECM glycoproteins and glycolipids, and intracellular function, by interacting with nuclear and cytoplasmic proteins (Liu and Rabinovich, 2005).

A structurally unique 31 kDa subfamily member is Galectin-3 (Gal-3), which has a single conserved CRD consisting of 130 amino acids, connected to a long N-terminal proline-, tyrosine- and glycine-rich domain (Ahmad et al., 2004; Barondes et al., 1994; Dumic et al., 2006; Liu and Rabinovich, 2005; Ramasamy et al., 2007) (Illustration 4). The C-terminal domain of Gal-3 is responsible for binding of the lectin to its specific carbohydrate

Illustration 4: **The galectin family of animal lectins and the formation of a galectin lattice:** Galectins can crosslink some glycoconjugates and trigger a cascade of transmembrane signalling events. They can potentially cause the clustering of multiple multivalent glycoconjugates, resulting in the formation of a lattice (Reprinted by permission from Macmillan Publishers Ltd: [NATURE REVIEWS. CANCER.] Liu and Rabinovich, 2005).


Cell–cell and cell–matrix interactions
Lattice formation
Signal transduction
Galactose-containing oligosaccharides
One-CRD galectins (dimer)
Galectin-3 (oligomers)
Two-CRD galectins

(Hsu et al., 1992; Mehul et al., 1995). The N-terminal domain of Gal-3 is required for the formation of oligomers, and ultimately, the plasma membrane galectin lattice microdomain, that is crucial for its cellular functions (Dumic et al., 2006; Nieminen et al., 2007) (Illustration 4). Upon proteolytic cleavage of the N-terminal domain the extracellular functions of Gal-3 are lost, presumably due to its inability to form oligomers (Nieminen et al., 2007). Gal-3 phosphorylation can occur at the N-terminal domain on the serine-6 and serine-12 residues (Gong et al., 1999). Phosphorylated Gal-3 can be found in both the nucleus and cytoplasm of mouse 3T3 fibroblast cells, whereas the non-phosphorylated form remains exclusively within the nucleus (Gong et al., 1999). Cell proliferation has been associated with an increased fraction of the phosphorylated form of Gal-3 (Gong et al., 1999).

1.2.1.2 – Mgat5 and protein glycosylation

Post-translational modification is critical for both the processing and the folding of proteins and their subsequent function at the cell membrane. A major class of modification is the addition of complex carbohydrates, or glycosylation that plays a critical role in signal transduction, cell-cell communication, and cell-ECM interactions. Glycosylated proteins exist in three classes: glycoproteins, proteoglycans, and glycosphingolipids (Ohyama, 2008).

Glycoproteins carry *O*-linked and *N*-linked glycans. *O*-linked glycans commonly have 1-3 sugar groups added to the hydroxyl group of serine, threonine, or hydroxylysine. *N*-glycans are more complex, with multiple sugar groups linked to the amide nitrogen of asparagine. These *O* or *N*-linked glycans can remain as a monosaccharide, but often have a complex

structure that is built up by stepwise addition of monosaccharides (Cooper et al., 1999). The assembly of an *N*-glycan chain is accomplished by a controlled sequence of glycosyltransferases beginning in the RER and continuing in the Golgi. *O*-glycans are synthesized solely by Golgi glycosyltransferases (Brockhausen et al., 1998).

The *Mgat5* gene encodes *N*-acetylglucosaminyltransferase V (GlcNAc-TV), a medial Golgi enzyme, that is required for the biosynthesis of β1,6GlcNAc-branched *N*-glycans. Mgat5 expression is regulated by the Ets transcription factors, that are activated by RAS-RAF-MAPK, a signalling pathway commonly active in cancer cells (Dennis et al., 1999). The addition of a sugar to a protein requires energy in the form of imported nucleotide mono/diphosphate sugar groups such as UDP-GlcNAc, one of nine sugar nucleotides used in glycosylation. The activity of Mgat5 depends on the presence of its UDP-GlcNAc substrate (Schachter, 1986). Mgat5 catalyses the transfer of GlcNAc from UDP-GlcNAc to the hydroxyl group on carbon-6 of the α1,6-linked mannose of *N*-glycans present on newly synthesized glycoproteins. This creates a poly-*N*-acetyllactosamine antennae extension on *N*-glycans. These *N*-acetyllactosamine antennae are the preferred ligand for Gal-3 (Dennis et al., 1999).

1.2.1.3 – The galectin lattice and its regulation of cell signalling

By binding their specific Mgat5-modified *N*-glycans on the plasma membrane, Gal-3 can oligomerize and help organize glycoprotein assemblies on the surface of the cell (Brewer et al., 2002; Partridge et al., 2004). This binding allows for the formation of a stable cell surface glycan-based domain, the Mgat5/galectin lattice (Brewer et al., 2002; Lajoie et al.,

2007) (Illustration 4). The strength of these interactions can be dynamically modulated by altering protein glycosylation or lectin expression (Collins and Paulson, 2004). Lattices have the ability to create homotypic or heterotypic glycoprotein complexes that can regulate a variety of cell functions, including proliferation, migration and apoptosis (Brewer et al., 2002) (Illustration 4). In particular, the Mgat5/galectin lattice has key roles in: organizing membrane domains, regulating cell surface signalling thresholds, and determining receptor residency time at the cell surface by inhibiting endocytosis (Garner and Baum, 2008). It has recently been demonstrated that the Mgat5/galectin lattice has the ability to limit the lateral diffusion of specific cell surface receptors, such as epidermal growth factor receptor (EGFR) (Lajoie et al., 2007; Lau et al., 2007; Partridge et al., 2004). In doing so, the Mgat5/galectin lattice sequesters EGFR away from negative cell surface caveolin-1 regulatory domains. By effectively preventing the recruitment of EGFR into stable inhibitory caveolin-1 plasma membrane domains, the Mgat5/galectin lattice allows for active EGFR signalling (Lajoie et al., 2007), like that seen in a variety of tumor types (Huang and Harari, 1999).

1.2.1.4 – Mgat5 / Galectin-3 and their relationship with cancer

Over-expression of galectin is often seen in cancerous cells and associated stromal cells (Liu and Rabinovich, 2005; van den Brule et al., 2004). Galectin levels have been shown to correlate with the aggressiveness of tumors and the acquisition of a metastatic phenotype in a variety of cancer types. Notably, Gal-3 inhibition, with the use of RNAi, has been linked with a suppression of cell transformation in diverse carcinomas (Takenaka et al., 2004; van den Brule et al., 2004). Monoclonal antibodies directed against Gal-3, labeling its extracellular domains, have been useful in confirming that cell surface Gal-3 expression is

increased in metastatic cells when compared to non-cancerous cell lines (Raz et al., 1986). Gal-3 expression shows a strong correlation with malignancy in colon, uterine, gastric, renal, melanoma, head and neck, and thyroid cancers. (Fukumori et al., 2007) The Golgi enzyme, Mgat5, has been shown to be upregulated in carcinomas (Partridge et al., 2004). As stated earlier, its expression initiates the production of poly-*N*-acetyllactosamine antennae on *N*-glycans, the high affinity ligand of Gal-3 (Partridge et al., 2004). The presence of increasing amounts of Mgat5 produced *N*-glycans is commonly associated with the malignant transformation of both murine and human cells while also showing a correlation with disease progression (Fernandes et al., 1991; Granovsky et al., 2000). Numerous glycoproteins, such as EGFR and transforming growth factor β receptor (TGFβR), have many *N*-glycan binding sites. Gal-3 has been shown to have a close relationship with these glycoproteins at the cell surface of Mgat5 expressing cell lines (Partridge et al., 2004). The association of EGFR and TGFβR with the Mgat5/galectin lattice prevents their endocytosis and retains them at the cell surface thereby enhancing their signalling capabilities and inducing cell survival and growth (Partridge et al., 2004). The addition of exogenous Gal-3 to Mgat5-positive cells results in the activation of FAK, phosphatidylinositol 3-kinase and the recruitment of α5-β1 integrin to fibrillar adhesions, all of which aid in the dynamic remodeling of microfilaments and substrate adhesions required for cell spreading and motility (Lagana et al., 2006).

Intracellular Gal-3 expression controls a variety of cellular functions that include apoptosis and T-cell growth. Upregulation of Gal-3, and its translocation to the nucleus, also occurs in proliferating cells, suggests that it has a function in normal cell growth (Yang et al., 1996). When T-cells are infected with human T-cell leukemia virus type 1, they show a high level of

Gal-3 expression (Yang et al., 1996). Gal-3 has also been shown to have a role as a suppressor of apoptosis by binding to the B-cell lymphoma/leukemia-2 protein (Bcl-2). Gal-3 binds Bcl-2 through its CRD and this interaction can be inhibited upon treatment with lactose, an inhibitory Gal-3 ligand (Reed, 1994; Yang et al., 1996). Gal-3 has been shown to be highly over-expressed in the nuclear compartment of rapidly proliferating human thyroid papillary carcinoma cell lines. In the nucleus Gal-3 acts as an up-regulator of thyroid-specific transcription factor 1 (TTF-1) transcriptional activity, whose expression contributes to the highly proliferative state of these cells (Paron et al., 2003).

1.2.2 – LIPID RAFTS AND CAVEOLAE

1.2.2.1 – Lipid rafts

Lipid rafts are relatively stable membrane microdomains that are enriched in cholesterol and sphingolipids, and their formation is driven solely by lipid-lipid interactions. They are a heterogeneous group of domains that differ in their lipid and protein composition as well as their temporal stability. Additionally, they have been found to be insoluble in non-ionic detergents such as Triton X-100. These domains are therefore commonly referred to as CEMs (cholesterol-enriched membranes), GEMs (glycosphingolipid-enriched membranes), and DRMs (detergent resistant membranes) (Pike, 2004). The first identified raft domains were caveolae, that are found as solitary entities at the plasma membrane on various cell types (Palade, 1953) (Illustration 6). Caveolae were originally isolated by extracting cells with Triton X-100 and then floating the lysate on a sucrose gradient. Caveolin-1, GPI (glycosylphosphatidylinositol)-linked proteins and numerous proteins involved in cell signalling, were found associated with these buoyant DRM fractions. The presence of

signalling molecules in the DRM fraction led to the hypothesis that these domains are intimately involved in the process of signal transduction (Foster et al., 2003; von Haller et al., 2001). Lipid rafts have also been implicated in endocytic events and cholesterol trafficking (Ikonen and Parton, 2000; Nabi and Le, 2003). A non-detergent method has been employed to cleanly separate invaginated caveolae from DRMs rich in GPI-linked proteins. This resulted in a finding that suggested that DRMs are comprised of at least two types of domains; those that contain caveolin, which are referred to as caveolae, and those that lack caveolin, which are referred to as lipid rafts (Schnitzer et al., 1995). A formal definition of lipid rafts was recently adopted at the 2006 Keystone Symposium of Lipid Rafts and Cell Function: "Lipid rafts are small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions" (Pike, 2006).

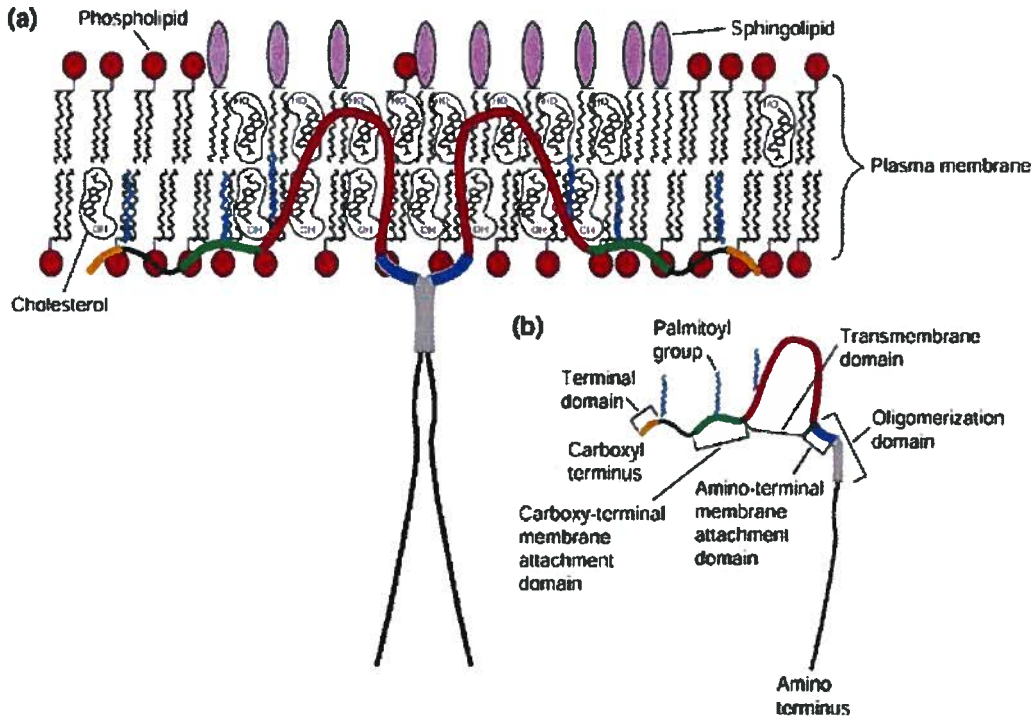
Lipid rafts have been implicated in a number of important cellular functions. Reports have shown that various transmembrane receptors, such as EGFR, are recruited to these domains. Partitioning of transmembrane receptors into raft domains results in a new micro-environment, where the phosphorylation state of the receptor can be modified by local kinases and phosphatases, thereby modulating downstream signalling (Mineo et al., 1999). The ability of rafts to diffuse throughout the membrane and coalesce with several other rafts can create what is known as a "signal transducing platform" that can amplify the receptor signalling cascade. By depleting cholesterol in the cell these amplified signalling platforms can be inhibited (Simons and Toomre, 2000). Another function associated with rafts is the

regulation of cellular migration. Asymmetric division of cellular components, including membrane microdomains, is an integral step in cell migration. The glycosphingolipid, ganglioside monosialic acid 1 (GM1), is commonly found in membrane raft domains. GM1 has been shown to be enriched in migrating MCF-7 breast carcinoma cells at their leading edge, while in leukocytes the accumulation of GM1 is at the trailing edge of the cell. This conflicting data suggests that lipid raft domain localization is cell-type specific (Gomez-Mouton et al., 2001; Manes et al., 1999).

1.2.2.2 – Caveolin family

Caveolin (Cav) sequences have been found in a wide range of vertebrates, including human, mouse, cow, and *Xenopus* (Tang et al., 1997; Williams and Lisanti, 2004b). The caveolin (Cav) gene family encodes for three different isoforms. The *Cav1* and *Cav2* genes are in close proximity on human chromosome 7q31.1, while *Cav3* is located on the 3p25 chromosome (Engelman et al., 1998c). They are predominantly expressed in plasma membrane domains, such as caveolae, due to three palmitoylation sites at the C-terminal region (cysteine-133, -143 and -156 on Cav1), but can also be found in the Golgi, the endoplasmic reticulum, at cytosolic locations, and in vesicles (Williams and Lisanti, 2004b). All three isoforms have both an N and C-terminus in the cytoplasm and a long membrane spanning hairpin-like domain (Dietzen et al., 1995; Monier et al., 1996; Williams and Lisanti, 2004b) (Illustration 5). Cav proteins exist as monomers in the Golgi apparatus but undergo homo-oligomerization upon being transported to the plasma membrane (Ren et al., 2004).

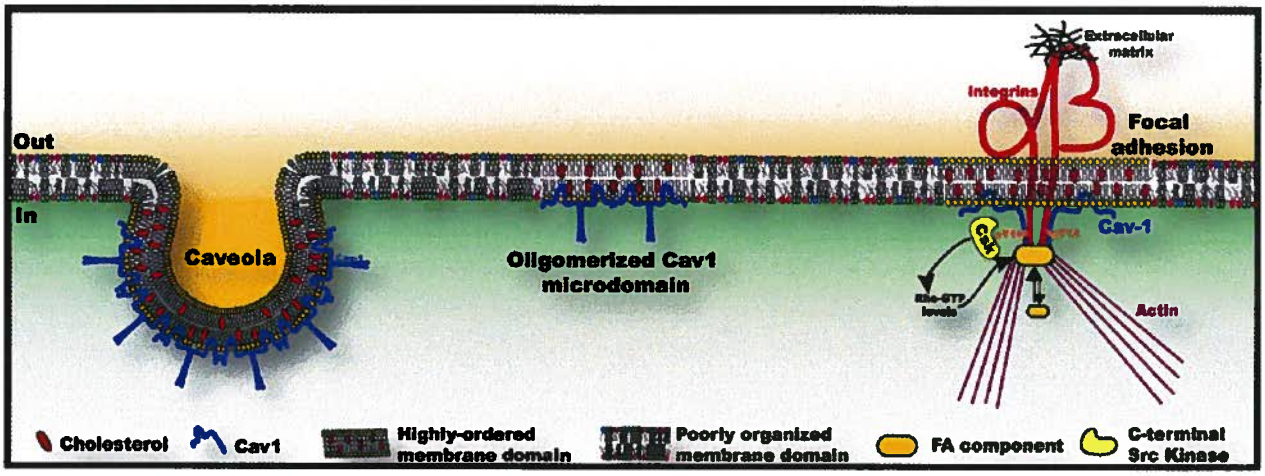
Illustration 5: **The primary structure and topology of Cav1:** (a) Two Cav1 monomers forming a homo-dimer (homo-oligomers containing 12-14 monomers are common) in the membrane. (b) The domains present in a Cav1 monomer (Reprinted by permission from BioMed Central Ltd. [GENOME BIOLOGY] Williams and Lisanti, 2004b).


(a)
Phospholipid
Sphingolipid
Plasma membrane
Cholesterol
(b)
Palmitoyl group
Transmembrane domain
Terminal domain
Oligomerization domain
Carboxyl terminus
Amino-terminal membrane attachment domain
Carboxy-terminal membrane attachment domain
Amino terminus

Caveolin-1 (Cav1 or VIP-21), the first identified isoform of Cav, is a 178 amino acid residue that exists in two separate sub-isoforms *in vivo*, α (contains residues 1-178) and β (contains residues 32-178) (Glenney, 1989). Cav1β has a 31 residue truncation at its amino terminus making it considerable shorter than its Cav1α counterpart (Scherer et al., 1995). The absence or presence of this truncation may be important in selectively targeting the two sub-isoforms of Cav1 to different cellular compartments (Li et al., 2001). Due to this truncation only Cav1α can be phosphorylated at its tyrosine-14 residue by active c-Src (Li et al., 1996). The Cav1 scaffolding domain (CSD) (residues 82-101) is the region where signalling molecules, such as the Src family tyrosine kinases, G-proteins, G-protein coupled receptors and growth factor receptors (ex. EGFR) can bind (Li et al., 1995; Zhang et al., 2007). The presence of a putative cholesterol binding domain imparts Cav1 with the ability to bind cholesterol at the plasma membrane (Murata et al., 1995). Cyclodextrin can prevent caveolae biogenesis by depleting cholesterol. Accelerated release of Cav1 from the Golgi complex has been shown to occur after the addition of cholesterol (Cheng et al., 2006). It has also experimentally been shown that the major sub-cellular location of Cav1 is at the plasma membrane. Cav1 is a relatively small protein being only 18-24 kDa, however its ability to form large oligomers comprised of 14-16 monomers, using its oligomerization domain (200-400 kDa), is the reason for its importance at the plasma membrane (Monier et al., 1995; Sargiacomo et al., 1995). The 41 residue long oligomerization domain directly juxtaposes the transmembrane domain of Cav1 (Sargiacomo et al., 1995). Palmitoylation of cysteine-133, -143 and -156 is essential to the formation of these large oligomers (Dietzen et al., 1995; Monier et al., 1996) (Illustration 5).

The plasma membrane localization of Cav1 oligomers is due in part to its involvement in the formation of caveolae, or omega shapped 50-100 nanometer wide invaginations of the plasma membrane (Kurzchalia and Parton, 1999; Williams and Lisanti, 2004b). Cav1 has been found to be the principal component of the caveolar protein coat, and is used as a marker for these domains, but is not solely capable of inducing caveolae formation (Breuza et al., 2002; Kurzchalia et al., 1992; Rothberg et al., 1992) (Illustration 6). It is believed that a threshold level of Cav1 is required in order for caveolar membrane domains to form (Fra et al., 1995). Polymerase transcript release factor (PTRF/Cavin) is located on the cytoplasmic face of caveolae where it triggers the deformation of the plasma membrane into flask-shapped invaginations (Hill et al., 2008). Animals lacking PTRF possess no morphologically detectable caveolae in any of the cell types (Liu et al., 2008). De novo formation of caveolae can be induced in Cav1 deficient cells, such as lymphocytes, by exogenously expressing Cav1 (Fra et al., 1995). These caveolar domains were identified by transmission electron microscopy greater than 50 years ago (Palade, 1953). Caveolae are anchored to the plasma membrane by the actin cytoskeleton and therefore represent a relatively immobile, highly stable plasma membrane structure (Thomsen et al., 2002). Caveolae have roles in many cellular processes including: endocytosis, transcytosis, cholesterol homeostasis, cell migration, cell polarity and calcium signalling, as well as a variety of other signalling events (Kurzchalia and Parton, 1999; Parton and Simons, 2007; Williams and Lisanti, 2005). The stability of caveolae within these specific plasma membrane domains is dependent upon the formation of complex Cav1 oligomers (Parton et al., 2006; Thomsen et al., 2002). Paralleling its essential role in caveolae formation, Cav1 is capable of forming functional membrane micro-domains completely independent of its ability to form caveolar domains

Illustration 6: **Cav1 and membrane organization:** Cav1 is required for the formation of caveolae, organized plasma membrane domains, and FA signalling platforms, with pY14Cav1 being specifically needed for the latter (With kind permission from Springer Science+Business Media: <Cancer Metastasis Review, Caveolin-1 in tumor progression: the good, the bad and the ugly, Volume 27, Copyright 2008, Page 718, Goetz J.G., Lajoie P., Wiseman S.M., Nabi I.R., Figure 3>).


Extracellular matrix
Integrins
Focal adhesion
Out
In
Caveola
Oligomerized Cav1 microdomain
Cav-1
Actin
Cholesterol
Cav1
Highly-ordered membrane domain
Poorly organized membrane domain
FA component
C-terminal Src Kinase

(Lajoie et al., 2007) (Illustration 6). Evidence has also suggested that Cav1 is important to β1 integrin-dependent fibronectin adhesion and FAK activation and that this might operate, at least in part, through the ability of Cav1 to regulate Src family kinase activity surrounding integrins. Cav1 therefore appears to be a general regulator of β1 integrin function (Wei et al., 1999) (Illustration 6).

The two other isoforms of Cav, Cav2 and Cav3, were identified after Cav1 and vary considerably in their cellular location and expression. Cav2 is commonly found co-expressed with Cav1 in many cell types and requires Cav1 for correct targeting to the plasma membrane. Cav3 has greater sequence similarity to Cav1 however its expression is muscle specific, being found in cardiac myocytes and skeletal muscle fibers (Williams and Lisanti, 2004a). Both Cav2 and Cav3 have the ability to form oligomers, although Cav2 requires the presence of Cav1 in order to form these high-molecular-weight complexes (Scherer et al., 1995; Tang et al., 1997). Even though Cav2 is commonly found associated with Cav1 and can form oligomers, its expression is not required for caveolae biogenesis (Lahtinen et al., 2003). Cav3 differs greatly from that of Cav1 or Cav2 in terms of tissue localization but its ability to form oligomers and its essential role in the formation of caveolae are similar to that of Cav1 (Galbiati et al., 2001).

1.2.2.3 – Caveolin-1 phosphorylation

The first major recognition of Cav1 as a tyrosine phosphorylated protein occurred in v-Src transformed chicken embryo fibroblasts (Glenney and Zokas, 1989). Another group found that if 3T3-L1 adipocytes were rapidly stimulated with insulin, Cav1 was put into a

transiently phosphorylated state (pY14Cav1). This phosphorylation event occurred indirectly through an endogenous Src family tyrosine kinase, rather than directly through the insulin receptor. The idea that a Src family tyrosine kinase was phosphorylating Cav1 is supported by the fact that a hetero-oligomeric complex consisting of Cav1 and active c-Src, as well as other Src family kinase members, can be purified together (Sargiacomo et al., 1993). Recent studies have shown that numerous other tyrosine kinases, including c-Abl and Fyn can phosphorylate Cav1 on its tyrosine-14 residue (Goetz et al., 2008b).

Caveolae, and its marker Cav1, have been shown to concentrate towards the rear of a polarized migrating cell, supporting their role in cellular locomotion. The importance of Cav1 and pY14Cav1 in establishing cell polarity during directional cell migration, through the coordinated signalling of Src kinases and Rho GTPases, has been shown in Cav1 knockdown experiments (Beardsley et al., 2005; Grande-Garcia and del Pozo, 2008). The loss of Cav1 polarity by targeted knockdown of the protein prevented cell polarization and impeded directional movement of endothelial cells. The ability of a cell to move requires there to be this asymmetrical division of proteins and cellular activities. It has been proposed that the distribution of Cav1 towards the rear of a migrating cell might prevent lamellipodial protrusions (Beardsley et al., 2005).

Src kinase mediated phosphorylation is the driving factor behind the redistribution of a small pool of Cav1 towards the leading edge of the cell (Parat et al., 2003). Transient transfection of exogenous Cav1-GFP constructs have shown that wild type Cav1-GFP and not non-phosphorylatable mutant, Cav1Y14F-GFP, can be localized in cell protrusions during three

dimensional migration (Grande-Garcia and del Pozo, 2008; Parat et al., 2003). This has also recently been shown in immortalized neurons (Lentini et al., 2008). The pY14Cav1 located at the leading edge of the cell may be recruiting signalling molecules required for the formation of FAs (Beardsley et al., 2005) (Illustration 6).

1.2.2.4 – Caveolin-1 and focal adhesions

As already discussed, recent studies have shown that Cav1 undergoes phosphorylation at its tyrosine-14 residue due to a number of stimuli, such as the presence of insulin and growth factors. During cell migration, pY14Cav1 located at FAs along the leading edge may be coupling integrin to non-receptor tyrosine kinases, such as Src, Fyn and FAK (Beardsley et al., 2005). This coupling is accomplished through the use of a docking site provided by pY14Cav1 that recruits SH2-domain containing proteins, such as Grb7 and Csk (Cao et al., 2004; Glenney and Zokas, 1989). Grb7 contains a phosphotyrosine-interacting region, as well as an SH2 domain, and therefore may act as a bridge connecting pY14Cav1 to other phosphorylated FA signalling molecules such as FAK (Kasus-Jacobi et al., 2000). pY14Cav1 is therefore a backbone working to connect the actin cytoskeleton, via integrins to the ECM, thereby creating a highly specialized signalling platform, FAs, at the leading edge of a migrating cell (Mettouchi et al., 2001). It has recently been shown that there is an association between EMT and FAK-mediated Cav1 expression (Bailey and Liu, 2008). Cav1 deficient fibroblasts show accelerated turnover of nascent FAs at the leading edge, indicating that Rho activation and the subsequent maturation of these structures may be inhibited (Vicente-Manzanares et al., 2005). These Cav1 deficient fibroblasts have enhanced protrusive characteristics throughout the perimeter of the whole cell, as opposed to just the

leading edge, indicating a loss of directional cell migration (Rottner et al., 1999). Additionally, they showed an increase in Rac activation which resulted in a markedly random pattern of migration (Grande-Garcia and del Pozo, 2008). Among other things, directional cellular migration is dependent upon the formation of leading edge FA signalling platforms that rely on the presence of Src-phosphorylated pY14Cav1 and active-Rho-GTPase for stability and maturation.

As previously discussed, the questionable reliability of mouse monoclonal anti-pY14Cav1 antibodies has hindered efforts to identify its function in FAs. Immunofluorescence experiments using this antibody indicating that pY14Cav1 is localized in FAs and therefore colocalize with FA proteins are no longer valid and must be reassessed. However, experiments using the anti-pY14Cav1 antibodies for Western blots or immunoprecipitations are still valid, as both phospho-paxillin and pY14Cav1 bands are present. Additionally, experiments utilizing Cav1Y14 mutants to identify the functional role of that amino acid are also valid. What remains to be determined is whether the phosphorylation of Cav1 regulates the caveolar localization of Cav1 (Hill et al., 2007).

1.2.2.5 – Caveolin-1 and its relationship with cancer

The role of Cav1 in the progression of cancer is controversial. The gene for *Cav1* maps onto a tumor suppressor locus that is commonly deleted in many types of human cancers including: prostate, breast, colorectal, renal, ovarian, and head and neck cancers. An inverse relationship has clearly been established between Cav1 expression and cellular transformation (Engelman et al., 1998b; Williams and Lisanti, 2005). It has been shown that

in transformed NIH-3T3 cells, Cav1 levels are significantly reduced (Koleske et al., 1995). Moreover, after depleting the level of Cav1 in NIH-3T3 cells using RNAi, what resulted was the induction of a transformed phenotype. This transformation was sufficient to form tumors in athymic (nude) mice and allow for cellular invasion into soft agar (Galbiati et al., 1998). Genetic support for the concept that Cav1 functions normally as a "transformation suppressor" gene has emerged with the study of Cav1 null mice. The Cav1 null mice formed more epidermally derived tumors in response to chemical carcinogenic treatment, which was preceded by epidermal hyperplasia due to cyclin D1 and Erk-1/2 upregulation (Williams and Lisanti, 2005). Cav1 expression by a highly metastatic Polyoma middle T antigen (PyMT) mammary carcinoma-derived cell line was found to be inversely related to the grade of the primary breast tumors, and subsequent upregulation of Cav1 was found to downregulate metastasis to distant organs (Sloan et al., 2004; Williams et al., 2004b). What they also found was that in less metastatic and non-metastatic breast cancer cell lines Cav1 expression was low, but not as low as the expression levels found in their highly metastastic counterparts (Sloan et al., 2004). It is evident that the loss of Cav1 results in cells obtaining a striking proliferative advantage, therefore suggesting that Cav1 may be acting as a tumor suppressor.

Cav1 has also been shown to have a relationship with a variety of oncogenes, some of which include, but are not limited to, c-Myc, v-Abl, Bcr-Abl, and Neu/ErbB2 (Engelman et al., 1998a; Koleske et al., 1995; Park et al., 2001; Timme et al., 2000). Through transcriptional regulation, these oncoproteins are able to downregulate Cav1 expression levels. Interestingly, viral oncoproteins, such as human papilloma virus (HPV), are also able to indirectly affect Cav1 levels by inactivating the well characterized tumor suppressor gene,

p53. p53 has been shown to be a positive regulator of both Cav1 transcription and translation. Additionally, Cav1 levels have been shown to be reduced, *in vitro*, in p53-deficient fibroblasts and *in vivo*, in p53 null mice (Williams et al., 2004a; Williams and Lisanti, 2005). It has been suggested that Cav1 itself may be directly regulating the level of PTEN (a phosphatase possessing tumor suppressor functions) since PTEN has been found to colocalize in caveolae and form hetero-complexes with Cav1. PTEN levels have been found to be simultaneously downregulated in response to Cav1 downregulation in follicular thyroid neoplasms (Aldred et al., 2003; Caselli et al., 2002).

In direct contrast to its tumor suppressing capabilities, Cav1 expression has been correlated with a poor prognosis in a number of human tumor types (Yang et al., 1998). Interestingly, the expression of Cav1 may vary depending on the stage of cancer progression (Bender et al., 2000). Numerous studies have reported that Cav1 upregulation correlates with tumor progression; a good example is illustrated by the Cav1 promotion of tumor progression in an autochthonous mouse model of prostate cancer. Cav1 null mice were bred with TRAMP (transgenic adenocarcinoma of mouse prostate) mice, and TRAMP-Cav1 null mice exhibited low metastatic ability and reduced prostate tumor burden (Williams et al., 2005). Recent data has also correlated Cav1 expression with tumor progression and a poor prognosis for both esophageal squamous cell carcinomas and human breast cancer (Ando et al., 2007).

With so many conflicting studies it remains to be determined whether Cav1 is indeed a tumor suppressor and/or an oncogene. It has been suggested that in the early stages of transformation and tumor growth decreased Cav1 expression may be necessary, while

metastasis and drug resistance require higher levels of Cav1 expression (Bender et al., 2000). Recent work has also shown that Cav1 may be acting as a conditional tumor suppressor that may be overridden by the expression of the Mgat5/galectin lattice (Lajoie et al., 2007).

1.3 – THESIS HYPOTHESIS

In part 1 of chapter 3 of my thesis I will be displaying my contribution to the work done in Joshi et al., 2008. We hypothesized that pY14Cav1 expression is correlated with stable FA turnover leading to increased tumor cell migration and is being regulated in a Rho/ROCK- and Src-dependent manner. We therefore postulate that Cav1 is a critical effector of Rho/ROCK- and Src-dependent tumor cell migration and invasion that acts by regulating FA dynamics.

In part 2 of chapter 3, we hypothesize that mutating the tyrosine-14 (Y14F, Y14D, Y14R) residue responsible for recruitment of pY14Cav1 to FAs might effect migration and invasion in tumor cells. Additionally, we will study the phenotypes seen with the Cav1Y14 mutant cell lines.

In part 3 of chapter 3 of my thesis I will be displaying my contribution to the work done in Goetz et al., 2008a, as well as a continuation of that work. Earlier it was demonstrated that pY14Cav1 was a regulating factor in FA dynamics. Interestingly, this pY14Cav1 regulation was reliant upon the presence of the Mgat5/galectin lattice. We therefore postulate that FA dynamics and ultimately tumor progression require concerted regulation by Gal-3 and pY14Cav1.

My thesis therefore investigates the interactions between, and the role of, the Mgat5/galectin lattice, Rho/ROCK and Src signalling and downstream phosphorylation of pY14Cav1 at FA

sites in promoting tumor cell migration through the regulation of FA dynamics in protrusive domains of cancer cells.

CHAPTER 2: MATERIALS AND METHODS

Antibodies and reagents

Swainsonine, β-lactose, sucrose, mouse anti-β-actin antibodies, PP2, Y27632, crystal violet and paraformaldehyde (PFA) were purchased from Sigma-Aldrich (St. Louis, Missouri, U.S.A.). The Gal-3 anti-mouse monoclonal IgG and anti-myc primary antibodies were purchased from Santa Cruz Biotechnology Incorporated (Santa Cruz, California, U.S.A.). Mouse anti-vinculin antibodies were purchased from Millipore (Billerica, Massachusetts, U.S.A). HRP-conjugated mouse secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania, U.S.A.). Geneticin, blasticidine, Lipofectamine 2000 reagent, phalloidin and secondary antibodies conjugated to Alexa 488, 568, or 647 were bought from Invitrogen (Burlington, Ontario, Canada). Triton X-100 and Tween were both purchased from Fisher Scientific (Ottawa, Ontario, Canada). Effectene transfection reagent, ECL Western Blotting Detection Reagent and CelVol (Gelvatol) were purchased from Qiagen (Mississauga, Ontario, Canada), Amersham (Pittsburgh, Pennsylvania, U.S.A.), and Celanese Ltd. (Dallas, Texas, U.S.A.), respectively.

Cell culture

MDA-435, MDA-231, PC3, DU145, HCT116, and HT29 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 medium (Invitrogen) supplemented with 1% L-glutamine, 1% penicillin/streptomycin, 1% Hepes pH buffer (Invitrogen), and 10% fetal bovine serum (FBS) (Medicorp, Montreal, Quebec, Canada). Stable MDA-435 cell mutants (Cav1-myc-mRFP, Cav1Y14F-myc-mRFP, Cav1Y14D-myc-mRFP, Cav1Y14R-myc-mRFP, and myc-DsRed) were selected for using RPMI 1640

medium supplemented with geneticin. Stable MDA-231 cell mutants (Cav1-shRNA) were selected for using RPMI 1640 medium supplemented with blasticidine. Mgat5 WT, Mgat5 22.9, Mgat5 22.9-Rescue, Mgat5 22.10, and Mgat5 22.10-Rescue cells (provided by Dr. Jim Dennis, Mount Sinai Hospital, Toronto, Ontario, Canada) were cultured in high glucose DMEM (Invitrogen) supplemented with 1% L-glutamine, 1% penicillin/streptomycin, 1% vitamins (Invitrogen), and 10% FBS. Newly thawed cells were passaged at least twice before they were used in experiments. Cells were maintained at 37°C in an incubator containing 5% CO^2/air. All cell lines were frozen in a mixture of 10% DMSO (Fisher Scientific), and 90% FBS before being stored in a -80°C freezer overnight and then ultimately in liquid nitrogen (Praxair, Danbury, Connecticut, U.S.A.).

Plasmid and small interfering RNA transfections

Over-expression transfections were carried out using an Effectene tranfection reagent and left to incubate with FAK-GFP (Dr. Mike Gold's Lab, Vancouver, B.C., Canada), Paxillin-GFP (Dr. Konstantin G. Birukov, Chicago, Illinois, U.S.A.), Cav1-myc-mRFP, Cav1Y14F-myc-mRFP, Cav1Y14D-myc-mRFP, Cav1Y14R-myc-mRFP or β-actin-GFP for 24 hours before analysis was done.

siRNA transfections were carried out using Lipofectamine 2000 reagent and left to incubate for 48 hours prior to analysis. Validated human c-Src (SMARTpool reagent M-003175-01-0005) and ROCK1 small interfering RNAs (siRNA; SMARTpool reagent M-003536-01-0005), as well as control siRNAs and siGLO Red (D-001630-02-05), were purchased from Dharmacon (Chicago, Illinois, U.S.A.). For Gal-3 knockdown in all cell lines, both mouse

and human, the cells were transfected with a pool of Gal-3 siRNA oligonucleotides (custom synthesized) or control siRNA (si*CONTROL* nontargeting siRNA no. 1; Dharmacon) using Lipofectamine 2000 transfection reagent following a specific protocol (Henderson et al., 2006).

Stable cell line generation using various Cav1-mRFP and Cav1 shRNA constructs

MDA-231 Cav1 shRNA cells were a kind gift from Dr. Michael Cox, Vancouver, British Columbia, Canada. Briefly, the Cav1 shRNA lentiviral construct was prepared using the following core sequence (5'-CTGTTCCCATCCGGGAACAGGGCAACAT-3') which was cloned into pSHAG-1 plasmid (provided by Dr. G. Hannon, Cold Spring Harbor, New York, U.S.A.). Subsequently, the shRNA cassette was transferred into pLenti6/BLOCK-iT^{TM}-DEST (Invitrogen) that carries a blasticidin selection marker. Lentiviral vector from conditioned media of HEK293T cells transfected with Cav1 shRNA-pLenti6/BLOCK-iT^{TM}-DEST was used as a source of virus particle to infect MDA-231 cells. A pooled population of blasticidin-selected MDA-231 cells exhibiting maximal Cav1 suppression was used for subsequent experiments (Kojic et al., 2007).

The human Cav1-myc-mRFP was cloned into mammalian expressing pRFP-N1 placed under a CMV promoter. To prepare phosphomimetic Cav1Y14D-mRFP, Cav1Y14D cDNA was PCR amplified from GFP-Cav1Y14D (provided by Rich Minshall, University of Illinois at Chicago, Chicago, Illinois, U.S.A.) using Cav1-BamHI-Rev (5' GGG GAT CCC TAT TTC TTT CTG CA AGT TGA TGC GGA C 3'), Cav1-HindIII-For (5' GGA AGC TTA GCA TGT CTG GGG GCA AAT AC 3') primers. Amplified product and pRFP-N1 vector were

restriction digested with HindIII–BamHI, gel purified, cloned, and then verified by sequencing. Tyrosine (Y)14 in human Cav1-mRFP was mutated to either phenylalanine (F) or arginine (R) by a PCR-based overlapping extension technique with the altered codon indicated in bold (Forward primers: 5' G GGA ATT CTA GCA TGT CTG GGG GCA AAT ACG TAG ACT CGG AGG GAC ATC TC**T TCA** ML 3', 5' AAG CTT AGC ATG TCT GGG GGC AAA TAC GTA GAC TCG GAG GGA CAT C**TC C**GC ACC 3'; Reverse primer: 5' GGG ATC CCC AGA TCC TCT TCT GAG ATG AG 3') (Goetz et al., 2008a).

In order to prepare the various MDA-435 stable cell lines were seeded at approximately 50% confluency and allowed to grow for 24 hours prior to transfection. The next day these cells were transiently transfected with, Cav1-myc-mRFP, Cav1Y14F-myc-mRFP, Cav1Y14D-myc-mRFP, or Cav1Y14R-myc-mRFP plasmids using Effectene transfection reagent following the protocol supplied with the reagent. Two days post-transfection the cells were washed with phosphate buffered saline (PBS) and supplied with fresh medium supplemented with geneticin (400μg/ml) and allowed to grow for 24 hours. Initially, every 24 hours for two cycles, dead cells were removed, washed with PBS and fresh medium supplemented with geneticin was added. Subsequently, cells were washed and supplied with fresh medium containing geneticin every 72 hours for a period of two weeks. Emerged pulled populations, stably expressing the various Cav1 mutants or wild type cells, were verified by imaging with an FV1000 Olympus (Markham, Ontario, Canada) confocal microscope at 568 nm wavelength, which was further validated by performing a western blot using an anti-myc antibody.

Immunofluorescence labeling

MDA-435 stable cells were plated on glass coverslips and allowed to grow to 80% confluency before being washed with PBS three times and then fixed for 15 minutes with 3% PFA and permeabilized for 10 minutes with 0.1% Triton X-100 in PBS supplemented with 1M $CaCl_2$ and 1M $MgCl_2$ (PBS-CM) (Sigma-Aldrich). A blocking solution containing PBS-CM + 1% bovine serum albumen (BSA) (Fisher Scientific) was then added to cells for 15 minutes. The cells were then incubated with mouse anti-vinculin primary antibody for 30 minutes, rinsed three times with blocking solution, and then further incubated with phalloidin conjugated Alexa-488 along with the appropriate secondary antibody for an additional 30 minutes. The cells were rinsed three times for 10 minutes with blocking solution. Coverslips were mounted in CelVol and either stored at 4°C or imaged with a 100x planapochromatic objective on an FV1000 Olympus confocal microscope.

Fluorescence Recovery After Photobleaching (FRAP)

MDA-231, MDA-231 Cav1 shRNA, MDA-435 (including all MDA-435 transiently transfected with Cav1 or a Cav1 mutant), PC3, DU145, HCT116, HT29, Mgat5 WT, Mgat5 22.9, Mgat5 22.9-Rescue, Mgat5 22.10, and Mgat5 22.10-Rescue cells were plated at low density in eight chamber slides (IBIDI, Munich, Germany), transiently transfected 24 hours later with FAK-GFP and then left to incubate at 37°C for an additional 24 hours. FRAP was done at 37°C using a 60x planapochromatic objective on an FV1000 Olympus confocal microscope. Images were taken in four second intervals, for 25 intervals in total on each individual FA. Two pre-bleaching images were obtained followed by a bleached image (using a 405-wavelength SIM scanner). The degree of recovery was determined by the

remaining four second, post-bleaching intervals where a plateau was reached. The data was then normalized to the pre-bleach intensity and the mobile and immobile fractions for each condition were obtained. Each FRAP experiment was repeated three times and the graphs represent the average of these experiments. When MDA-435 cells transiently transfected with Cav1-mutant plasmids were compared, the cells were co-transfected with FAK-GFP. The mRFP was used to identify positively transfected cells, and the FAK-GFP was then bleached and analyzed. PP2 (10μmol/L) and Y27632 (20μmol/L) treatments were applied 30 minutes prior to FRAP experiments. Prism Graphpad 4 (La Jolla, California, U.S.A.) graphing software was used to calculate the mobile fraction for each experiment and Microsoft Excel 2007 was used to determine the significance.

Live cell movies

MDA-435 and DU145 cells were plated at low density in eight chamber slides and transiently co-transfected 24 hours later with β-actin-GFP and either Cav1-mRFP or Cav1Y14F-mRFP using Effectene transfection reagent. After 24 hours images were taken of cells under each condition every 30 seconds for 30 minutes at 37°C using a 60x planapochromatic objective on an FV1000 Olympus confocal microscope. Kymographs were created using ImagePro (Media Cybernetics, Bethesda, Maryland, U.S.A.) software by drawing a line perpendicular to a lamellipodial region. MDA-435 stable cell lines were prepared in the same way as stated above with FAK-GFP being transiently transfected into the cells 24 hours prior to imaging.

Masks and FA tracking

Live cell 30 minutes movies using all MDA-435 stable cell lines were made at 37°C using a 60x planapochromatic objective on an FV1000 Olympus confocal microscope as stated in the previous section. The generated movies were then imported into Slidebook Version 4.2 software supplied by Intelligent Imaging Innovations (I^3, Denver, Colorado, U.S.A.). To create a mask of FAK-GFP at 488 nm wavelength the dynamic range must be segmented. On a 4095 grey level scale, the low grey level must be brought up to 1500 to narrow the dynamic range. The mask is then made more functional by eliminating objects (individual FAs) smaller than 10 pixels, objects with a path length less than three, and objects that move more than 10 pixels. FAs are then tracked using the center of area to measure the total displacement over time. Microsoft Office Excel 2007 is used to compare the total displacement for each cell line.

Migration assay

Stable MDA-435 cell mutants (Cav1-myc-mRFP, Cav1Y14F-myc-mRFP, Cav1 Y14D-myc-mRFP, Cav1Y14R-myc-mRFP), MDA-435, MDA-231 and stable MDA-231 Cav1 shRNA cells were plated and 24 hours later Gal-3 was knocked down using Gal-3 siRNA and Lipofectamine 2000 transfection reagent. After a 48 hour incubation the cells were trypsonized using 0.05% Trypsin-EDTA (Invitrogen), counted, and 30,000 cells were transferred to 8.0μm pore size inserts (Falcon, Mississauga, Ontario, Canada) in 2% serum containing medium and placed in wells of uncoated 24-well plates (Falcon) with 10% serum containing medium. Non-migrating cells were removed after 24 hours, from the top of the filter using a cotton swab. The migrating cells at the bottom of the well were fixed using

PFA and stained with 5% crystal violet and then counted. Control-siRNA was used for each cell line to show that the results were due to the knockdown of Gal-3 and not an effect of the transfection reagent.

Western blots

Cells were allowed to grow to 80% confluency in complete media supplemented with the appropriate selection drug prior to lysate preparation. Subsequently, cells were washed one time in cold PBS, and then harvested in M2 lysis buffer (20 mM Tris-HCl, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA (Fisher Scientific)) containing freshly added 2 mM DTT, 0.5 mM PMSF, 1 mM sodium vanadate, 2.5 mM sodium fluoride, and 1 μM leupeptin (Sigma-Aldrich). Cells suspended in M2 lysis buffer were rotated for 30 minutes at 4°C and then centrifuged at 13000 rpm for 10 minutes at 4°C. Supernatant was collected and stored at -80°C. The proteins were separated on a 12% SDS-PAGE gel and electroblotted for 2 hours onto a nitrocellulose membrane (Amersham) by semi-dry technique and subjected to western blot probing. Galectin-3 anti-mouse monoclonal IgG or other indicated primary antibodies of interest were incubated overnight at 4°C in PBS. The next day the western blots were washed in PBS + 0.2% Tween (PBS-T) and incubated with horseradish peroxidase-conjugated donkey anti-mouse IgG or the otherwise indicated secondary antibodies were incubated for 1 hour at room temperature. After washing with PBS-T three times, a signal was detected using ECL Western Blotting Detection Reagent and imaging film (Clonex Corporation, Markham, Ontario, Canada).

Cell spreading assay

MDA-435 and MDA-435 stable cell lines were plated in 96-well plates at a density of 1,000 cells/well in serum containing medium for 48 hours. The cells were fixed for 15 min with 3% PFA at 20°C, and then permeabilized in 0.1% Triton X-100 and blocked in PBS plus 1% BSA for 30 minutes. Phalloidin secondary antibodies conjugated to Alexa 568, and Hoechst were added at 1/500 and 1/100, respectively, in PBS plus 1% BSA and incubated for 30 minutes at 20°C. After washing one time, the plates were scanned using an ArrayScan automated fluorescence microscope (Cellomics Inc., Pittsburgh, Pennsylvania, U.S.A.). The difference in cell spreading was determined on 5 fields/well for all of the conditions. The average area was then calculated and graphed using a Microsoft Excel 2007 spreadsheet. The graphs represent a minimum of 4 experiments.

TIRF imaging

Stable MDA-435 cell mutants (Cav1-myc-mRFP, Cav1Y14F-myc-mRFP, Cav1Y14D-myc-mRFP, Cav1Y14R-myc-mRFP) were plated at low density in eight chamber slides, transiently transfected 24 hours later with FAK-GFP or Paxillin-GFP and then left to incubate at 37°C for an additional 24 hours. Cells were then imaged using a Plan-Fluar 100x/1.45 oil objective on a Zeiss Axiovert 200M microscope with a Photometrics QuantEM 512SC camera. For TIRF movies, images were taken every 30 seconds for the appropriate period of time. Analysis of the captured data was done using Slidebook Version 4.2 software.

CHAPTER 3: RESULTS

3.1 – Phosphorylated caveolin-1 regulates Rho/ROCK-dependent FA dynamics and tumor cell migration and invasion (Joshi et al., 2008).

pY14Cav1 is expressed in select metastatic cell lines (Joshi et al., 2008).

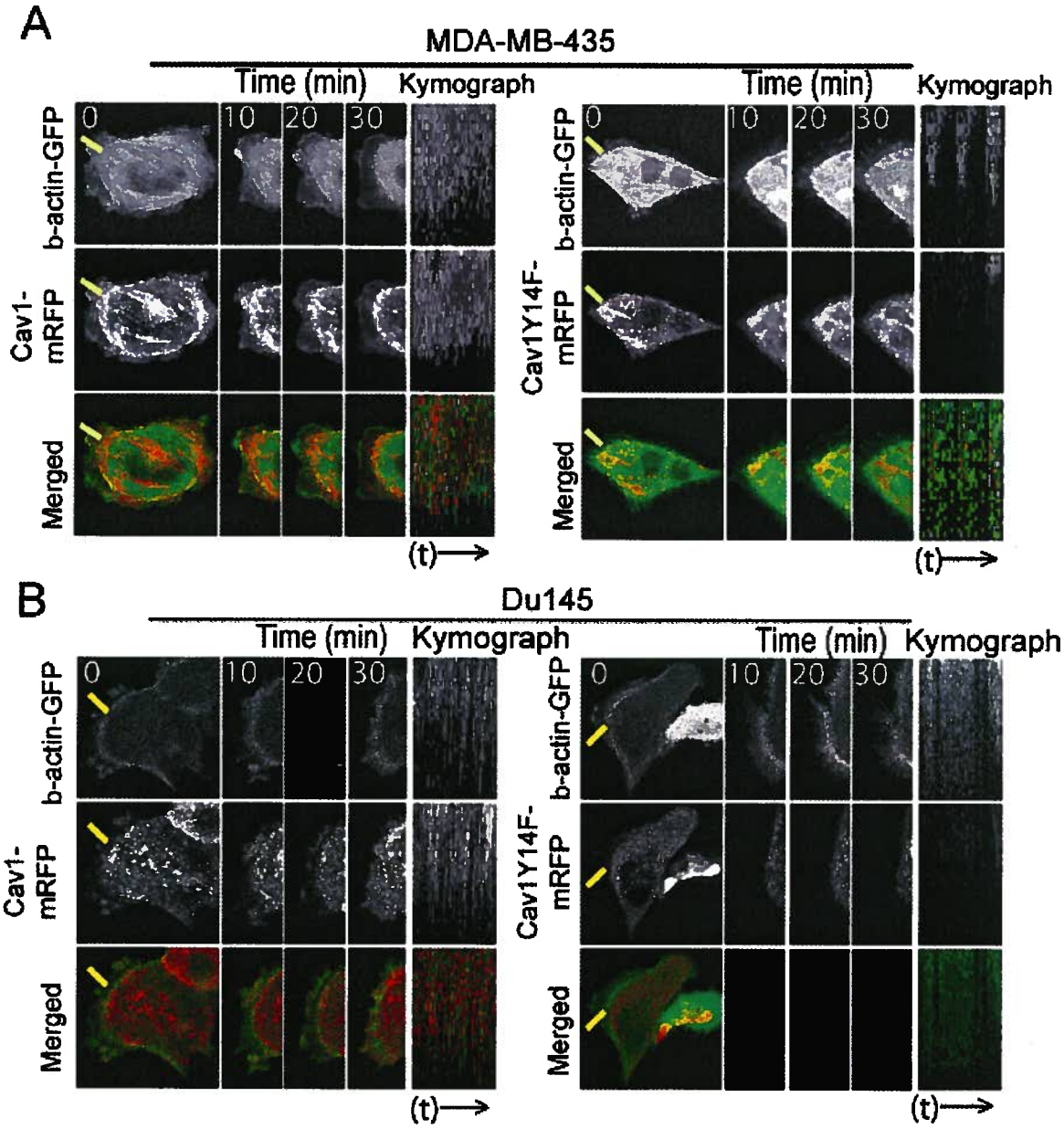
To understand the relationship between pY14Cav1 expression and FA dynamics and migration I wanted to compare cell lines showing differential expression of both Cav1 and pY14Cav1. Numerous cell lines have previously been profiled for both Cav1 and pY14Cav1 (Joshi et al., 2008). To ensure that all results are robust, I chose cell lines derived from human breast (MDA-231, MDA-435), prostate (PC3, DU145) and colon (HT29, HCT116) tumor types.

The expression of Caveolin-1/2 (Cav1/2) and pY14Cav1 has been profiled in MDA-231, MDA-435, DU145, PC3, HCT116, and HT29 cell lines. The metastatic MDA-231, PC3, DU145, and HCT116 cell lines were found to have significantly increased Cav1/2 expression relative to the other cell lines (Joshi et al., 2008). pY14Cav1 levels were selectively detected in MDA-231, HCT116, and PC3 cells, where it was found to correlate with either elevated Cav1 levels and/or elevated expression of phosphorylated Src (Joshi et al., 2008). pY14Cav1 levels have also been shown to be increased in the previously characterized MSV-MDCK-INV canine cell line relative to non-invasive MDCK epithelial cells (Jia et al., 2005; Joshi et al., 2008).

Purification of pseudopodial domains, by harvesting them after they have projected through 1 μm filter pores, determined that both Cav1/2 and pY14Cav1 were localized to tumor cell protrusions (Joshi et al., 2008). The psuedopodial fraction of MSV-MDCK-INV and MDA-231 cells was shown to be significantly enriched in both pY14Cav1 and pSrc relative to the cell body. Additionally, Cav1/2 has also been found in the pseudopodial fraction, localizing caveolin to tumor cell protrusions (Joshi et al., 2008, by permission).

By immunofluorescence, Cav1/2 distribution in MSV-MDCK-INV, HCT116, PC3, and MDA-231 cells did not exhibit a polarized distribution and extended to peripheral cellular regions. To verify the distribution of Cav1 to protrusive cellular domains, we cotransfected the MDA-435 cell line that expresses reduced levels of endogenous Cav1 (Figure 1A) and few caveolae (Kojic et al., 2007), as well as DU145 cells that express Cav1 but not pY14Cav1 (Figure 1B), with Cav1-mRFP or Cav1Y14F-mRFP and β-actin-GFP. Time lapse imaging shows clearly that Cav1-mRFP is present in protrusive lamellipodial domains defined by β-actin-GFP expression (Figure 1A and B). In contrast, mutant Cav1Y14F-mRFP is excluded from the β-actin-GFP-rich leading edge of moving cells (Figure 1A and B). Kymograph analysis showed that wild-type (WT) Cav1, but not Cav1Y14F, was expressed throughout the lamellipodial region. These data suggest that pY14Cav1 is targeted to tumor cell protrusions (Joshi et al., 2008, by permission).

Figure 1: **Tyrosine phosphorylated Cav1 is localized to tumor cell protrusions:** MDA-435 (A) and DU145 (B) cells were cotransfected with β-actin-GFP and either Cav1-mRFP or Cav1Y14F-mRFP and imaged every 30 s for 30 min. Representative images taken every 10 min are shown, and merged images show β-actin-GFP in green and Cav1-mRFP and Cav1Y14F-mRFP in red. Kymograph analysis of a line drawn perpendicular to the lamellipodial region (left) is shown from 0 to 30 min. Whereas Cav1-mRFP extends to protrusive domains, the Cav1Y14F-mRFP mutant is excluded from protrusive lamellipodial domains (Joshi et al., 2008, by permission).


A
MDA-MB-435
Time (min)
Kymograph
Time (min)
Kymograph
b-actin-GFP
Cav1-mRFP
Cav1Y14F-mRFP
Merged
0
10
20
30
(t)
B
Du145
Time (min)
Kymograph
Time (min)
Kymograph
b-actin-GFP
Cav1-mRFP
Cav1Y14F-mRFP
Merged
0
10
20
30
(t)

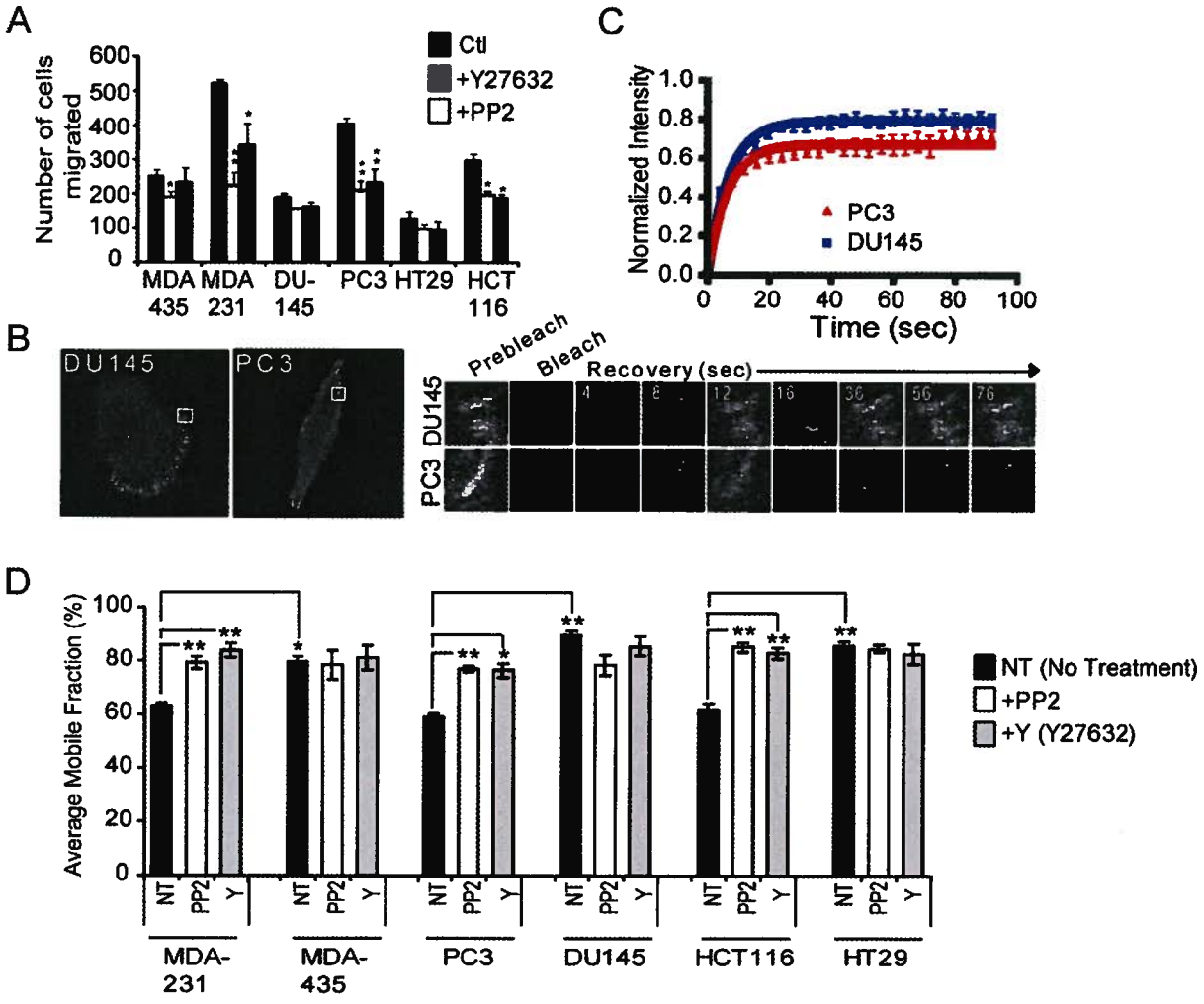
Increased FA dynamics in pY14Cav1-expressing tumor cell lines is Src-dependent and ROCK-dependent (Joshi et al., 2008).

The mechanism behind pY14Cav1-mediated increases in FA dynamics required further investigation. Src kinase has a close association with Cav1, in that Cav1 is phosphorylated by active c-Src on its tyrosine-14 residue (Sargiacomo et al., 1993). Rho/ROCK signalling is enriched in cell protrusions and ruffles at the leading edge of a cell and plays a major role in the formation of actin networks in these pseudopodial domains (Jia et al., 2006). Rho/ROCK signalling has also been associated with increased tumor cell invasivity via a nonproteolytic, amoeboid mode of tumor cell migration (Sahai and Marshall, 2003). The presence of pY14Cav1 in protrusions (Figure 1A and B) indicates that it might be an effector of Rho/ROCK-dependent tumor cell migration.

Using a Boyden chamber assay, we detected increased migration of pY14Cav1 expressing MDA-231, PC3, and HCT116 cells relative to MDA-435, DU145, and HT29 cells that do not express elevated pY14Cav1 levels (Figure 2A). The migration of pY14Cav1-expressing MDA-231, PC3, and HCT116 cells, but not of MDA-435, DU145, and HT29 tumor cells, was sensitive to ROCK (Y27632) and Src (PP2) inhibition (Figure 2A) (Joshi et al., 2008, by permission).

We observed that pY14Cav1 stabilizes FAK exchange in FAs leading to enhanced FA turnover (Goetz et al., 2008a). The presence of pY14Cav1 in cellular protrusions led us to investigate FA dynamics in these cancer cell lines. Cells were transiently transfected with

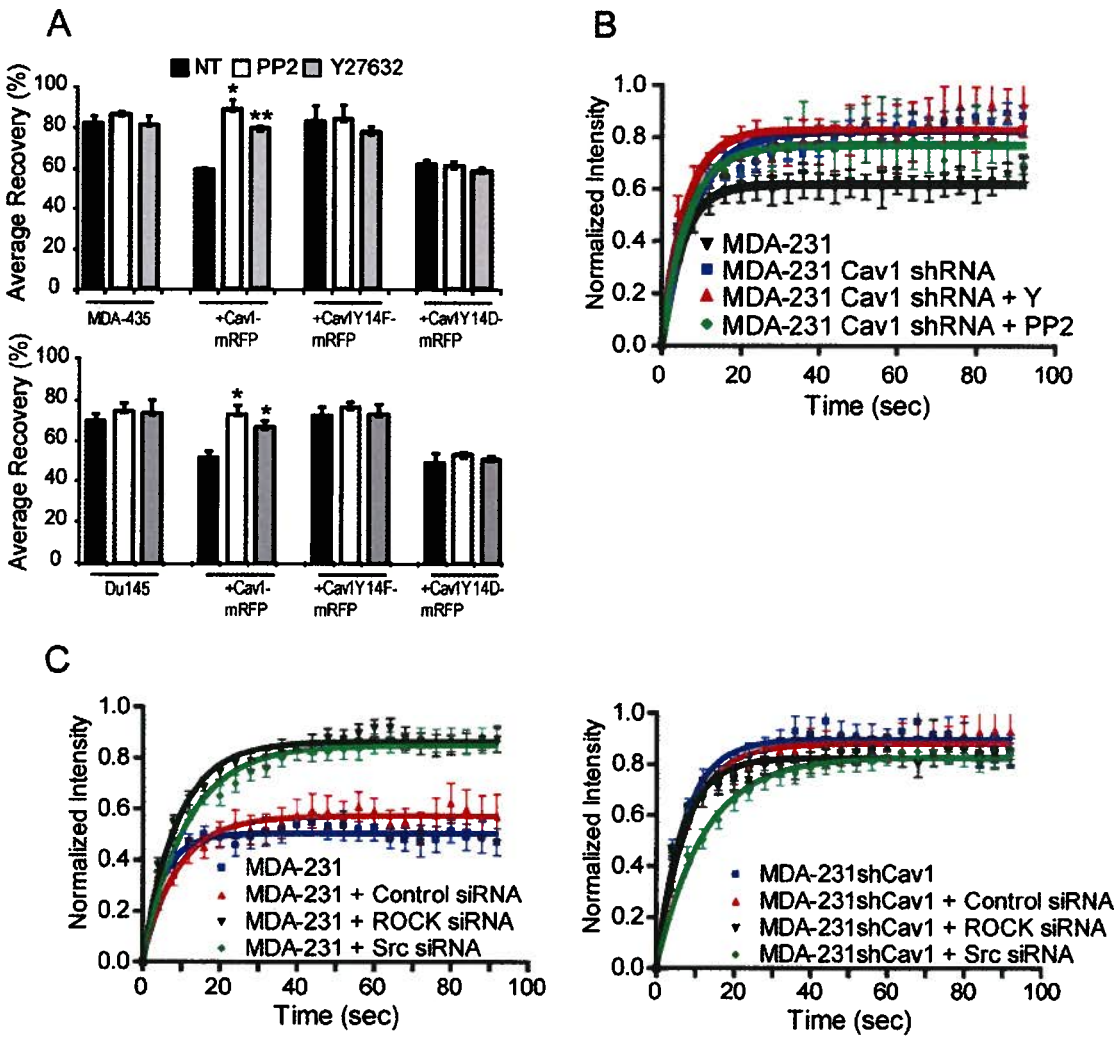
Figure 2: **Src and ROCK dependence of tumor cell migration and FAK stabilization is associated with pY14Cav1 expression:** (A) Boyden chamber transwell migration assay of MDA-435, MDA-231, DU145, PC3, HT29, and HCT116 cells was performed in the presence of Src inhibitor PP2 (10 μmol/L) or ROCK inhibitor Y27632 (20 μmol/L), as indicated (n = 3; FSE). *, $P < 0.05$; **, $P < 0.001$ relative to untreated cells. (B-D) MDA-231, MDA-435, PC3, DU145, HCT116, and HT29 cells were transiently transfected with FAK-GFP and subjected to FRAP of FAK-GFP in peripheral FAs. (B) Representative images of DU145 and PC3 cells with FAs selected for photobleaching in boxes. Right, prebleach, bleach, and recovery images of the selected FA. (C) Representative graphs show average percentage intensity (n = 3; FSE) in the bleached zone of FAK-GFP during recovery for PC3 and DU145 cells. (D) MDA-231, MDA-435, PC3, DU145, HCT116, and HT29 cells were transiently transfected with FAK-GFP and treated for 30 min with the Src inhibitor PP2 (10 μmol/L) or ROCK inhibitor Y27632 (20 μmol/L) before FRAP analysis of FAK-GFP in peripheral FAs. Graphs show average mobile fraction (n = 3; FSE) in the bleached zone of FAK-GFP during recovery. See Table 1 for calculation of the rate of recovery and immobile fraction (n = 3). *, $P < 0.05$; **, $P < 0.001$ (Joshi et al., 2008, by permission).


A
Number of cells migrated
600
500
400
300
200
100
0
Ctl
+Y27632
+PP2
MDA 435
MDA 231
DU-145
PC3
HT29
HCT 116
C
Normalized Intensity
1.0
0.8
0.6
0.4
0.2
0.0
PC3
DU145
0
20
40
60
80
100
Time (sec)
B
DU145
PC3
Prebleach
Bleach
Recovery (sec)
4
8
12
16
36
56
76
D
Average Mobile Fraction (%)
100
80
60
40
20
0
NT
PP2
Y
MDA-231
MDA-435
PC3
DU145
HCT116
HT29
NT (No Treatment)
+PP2
+Y (Y27632)

FAK-GFP, and FRAP of FAK-GFP in FAs was determined as previously described (Giannone et al., 2004; Goetz et al., 2008a; Hamadi et al., 2005). Integrin-mediated adhesion sites show molecular and functional heterogeneity so that only peripheral FAs were photobleached, and the rate of fluorescence recovery in the bleached region followed over time (Figure 2B and C). Increased FAK exchange between FA and cytosolic pools, reflected in the higher mobile fraction, was consistently detected in pY14Cav1-negative MDA-435, DU145, and HT29 cells compared with pY14Cav1-positive MDA-231, PC3, and HCT116 tumor cell lines (Figure 2D; Table 1). Treatment of cells with the ROCK inhibitor Y27632 and the Src inhibitor PP2, under conditions that reduce Cav1 tyrosine phosphorylation, selectively affect FAK exchange in pY14Cav1 expressing MDA-231, PC3, and HCT116 tumor cell lines. Y27632 and PP2 did not affect FAK exchange in MDA-435, DU145, and HT29 tumor cells that do not express elevated levels of pY14Cav1 (Figure 2D; Table 1) (Joshi et al., 2008, by permission).

Transfection of MDA-435 cells with Cav1-mRFP, but not Cav1Y14F-mRFP, results in FAK stabilization in FAs (Goetz et al., 2008a). Similarly, transfection of either MDA-435 or DU145 cells with Cav1-mRFP or the phosphomimetic Cav1Y14D-mRFP, but not Cav1Y14F-mRFP, showed increased FAK stabilization in FAs (Figure 3A). Importantly, whereas Cav1 expression rendered FAK exchange sensitive to PP2 and Y27632, in cells expressing the phosphomimetic Cav1Y14D, FAK-GFP stabilization in FAs was no longer sensitive to Src or ROCK inhibition (Figure 3A; Table 1). This provides direct evidence of a role for tyrosine phosphorylation of Cav1 in Src-dependent and ROCK-dependent regulation of FAK stabilization in FAs (Joshi et al., 2008, by permission).

Figure 3: **pY14Cav1 expression determines the Src and ROCK dependence of FAK stabilization in FAs:** (A) MDA-435 and DU145 cells were transiently transfected with FAK-GFP and either Cav1-mRFP, Cav1Y14F-mRFP, or Cav1Y14D-mRFP and treated for 30 min with the Src inhibitor PP2 (10 μmol/L) or ROCK inhibitor Y27632 (20 μmol/L) before FRAP analysis of FAK-GFP in peripheral FAs. Graph shows average mobile fraction (n = 3; FSE) in the bleached zone of FAK-GFP during recovery (n = 3). *, $P < 0.05$; **, $P < 0.001$. (B) MDA-231 and Cav1 shRNA MDA-231 cells were transiently transfected with FAK-GFP and subjected to FRAP analysis of FAK-GFP in peripheral FAs. Cav1 shRNA MDA-231 cells were also treated for 30 min with the Src family kinase inhibitor PP2 (10 μmol/L) or Rho kinase inhibitor Y27632 (20 μmol/L) before FRAP analysis. (C) MDA-231 (left) and Cav1 shRNA MDA-231 (right) cells were transfected with control, ROCK, and Src siRNA together with siGlo and after 24h with FAK-GFP and subjected to FRAP analysis of FAK-GFP in peripheral FAs of siGlo-positive cells. Graph shows average mobile fraction (n = 3; FSE) in the bleached zone of FAK-GFP during recovery. See Table 1 for calculation of the rate of recovery and immobile fraction (n = 3). *, $P < 0.05$; **, $P < 0.001$ (Joshi et al., 2008, by permission).


A
NT
PP2
Y27632
Average Recovery (%)
MDA-435
+Cav1-mRFP
+Cav1Y14F-mRFP
+Cav1Y14D-mRFP
Du145
B
Normalized Intensity
MDA-231
MDA-231 Cav1 shRNA
MDA-231 Cav1 shRNA + Y
MDA-231 Cav1 shRNA + PP2
Time (sec)
C
MDA-231
MDA-231 + Control siRNA
MDA-231 + ROCK siRNA
MDA-231 + Src siRNA
MDA-231shCav1
MDA-231shCav1 + Control siRNA
MDA-231shCav1 + ROCK siRNA
MDA-231shCav1 + Src siRNA

A stable lentiviral-infected Cav1 shRNA MDA-231 cell line expresses reduced Cav1 levels (Kojic et al., 2007) and shows an increased FAK-GFP mobile fraction in FAs that is no longer sensitive to PP2 and Y27632 treatment (Figure 3B; Table 1). Furthermore, both ROCK and Src siRNA increased the rate of FAK exchange in MDA-231 cells, but not FAK exchange in Cav1 shRNA MDA-231 cells (Figure 3C; Table 1). pY14Cav1 expression is therefore associated with ROCK-sensitive and Src-sensitive FA dynamics and tumor cell migration (Joshi et al., 2008, by permission).

Conversely, stable transfection of Cav1-mRFP but not CavY14F-mRFP in MDA-435 cells was associated with increased stabilization of FAK in FAs. Stable DsRed transfection controls did not show a significant difference in FAK exchange compared to Cav1Y14F-mRFP transfectants but did show a significant increase in FAK mobile fraction relative to Cav1-mRFP stable transfectants (Figure 4; Table 1) (Joshi et al., 2008, by permission).

Figure 4: **Cav1 expression stabilizes FA dynamics:** Stably transfected MDA-435 cell lines expressing Cav1-mRFP, Cav1Y14F-mRFP or DsRed cells were transiently transfected with FAK-GFP and subjected to FRAP analysis of FAK-GFP in peripheral FAs. See Table 1 for calculation of the rate of recovery and immobile fraction (Joshi et al., 2008, by permission).

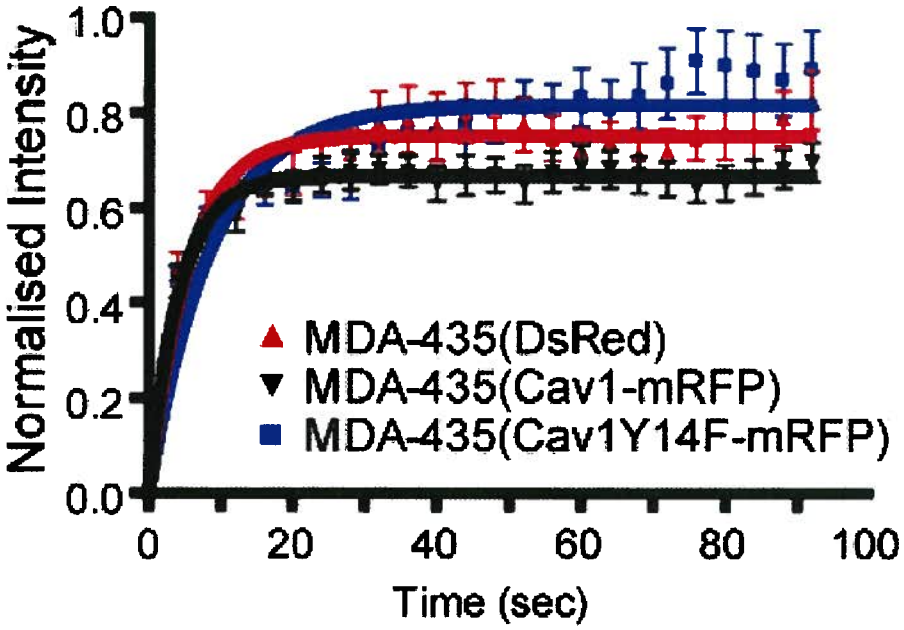

Normalised Intensity
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Time (sec)
MDA-435(DsRed)
MDA-435(Cav1-mRFP)
MDA-435(Cav1Y14F-mRFP)

Table 1: **Average mobile fraction of FAK-GFP in FAs as determined by FRAP** (Joshi et al., 2008, by permission).

CELL LINES	Treatment	n	Average mobile fraction %
BREAST			
MDA-231	NT	12	63.0 ± 1.3
	+PP2	3	79.3 ± 2.2[b]
	+Y27632	3	83.9 ± 2.8[b]
MDA-435	NT	9	79.6 ± 1.9[a]
	+PP2	3	78.4 ± 5.5
	+Y27632	3	81.1 ± 4.7
Stable Transfectants			
MDA-231 shCav1	NT	3	84.0 ± 3.6[a]
	+PP2	3	82.5 ± 1.3[a]
	+Y27632	3	85.5 ± 0.8[b]
MDA-435(DsRed)		3	75.2 ± 2.3[c]
MDA-435(Cav1-mRFP)		3	66.8 ± 2.2
MDA-435(Cav1Y14F-mRFP)		3	82.9 ± 3.2[c]
PROSTATE			
PC3	NT	9	59.4 ± 1.1
	+PP2	3	76.9 ± 3.0[e]
	+Y27632	3	76.4 ± 2.6[d]
DU145	NT	9	89.7 ± 1.4[e]
	+PP2	3	78.6 ± 3.9
	+Y27632	3	85.5 ± 3.6
COLON			
HCT116	NT	9	62.0 ± 2.3
	+PP2	3	85.2 ± 1.6[f]
	+Y27632	3	82.9 ± 2.1[f]
HT29	NT	9	85.8 ± 1.4[f]
	+PP2	3	84.7 ± 1.4
	+Y27632	3	82.8 ± 3.8

Data are presented as means ± SEM.
NT = No treatment
[a]$p < 0.05$ compared with MDA-231.
[b]$p < 0.005$ compared with MDA-231.
[c]$p < 0.05$ compared with Cav1-mRFP.
[d]$p < 0.05$ compared with PC3.
[e]$p < 0.005$ compared with PC3.
[f]$p < 0.005$ compared with HCT116.

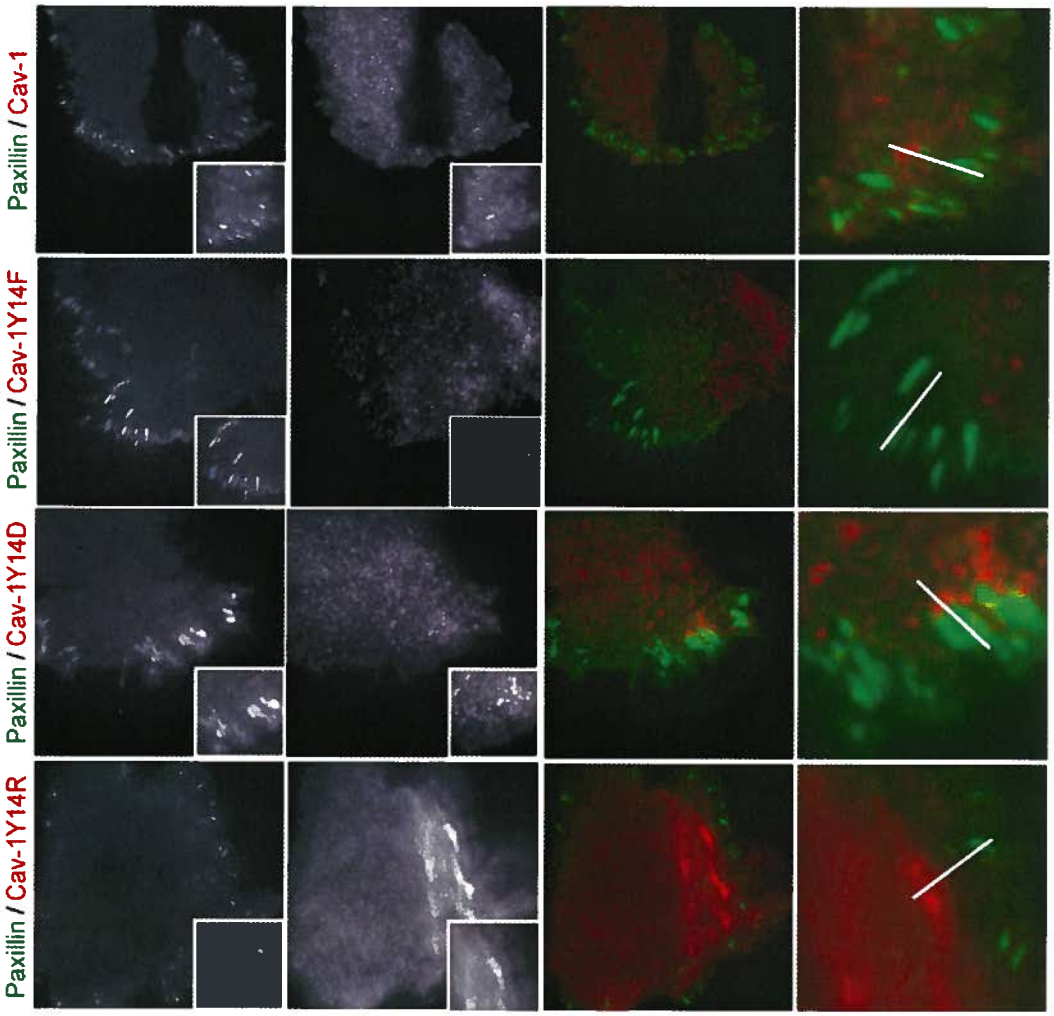
3.2 – The phosphorylation and resulting protein conformational changes are required for the recruitment of Cav1 to the leading edge

Cell surface interaction between Cav1 and focal adhesion proteins is phosphorylation-dependent.

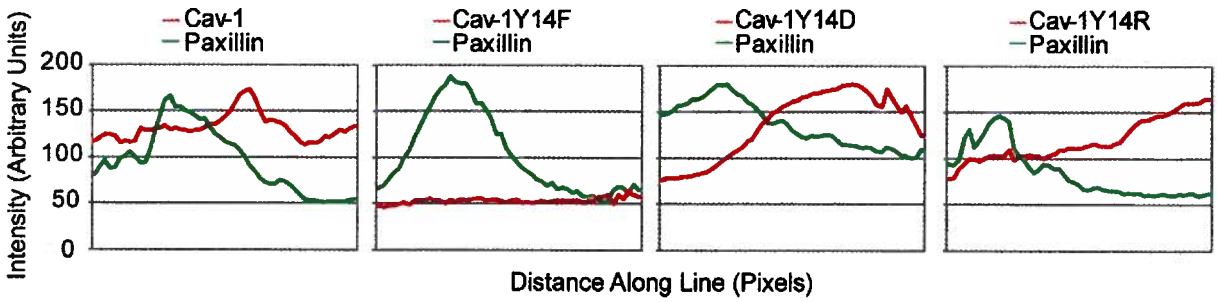
The human MDA-435 cancer cell line expresses a low level of Cav1 and has very few caveolae (Kojic et al., 2007). The transient overexpression of Cav1-mRFP, but not the non-phosphorylatable mutant Cav1Y14F-mRFP, in MDA-435 cells promotes FA stabilization (Goetz et al., 2008a; Joshi et al., 2008). An analysis of the cell-substrate interacting region was carried out by means of total internal reflection fluorescence (TIRF) microscopy. In MDA-435 cells stably expressing either Cav1-mRFP or the mutant phosphomimetic, Cav1Y14D-mRFP, paxillin-GFP localizes directly in front of concentrated pools of both of these mRFP-tagged Cav1 constructs at the leading edge of the cell (Figure 5A). The inability of mutant Cav1Y14F-mRFP to be phosphorylated prevents its recruitment to the leading edge of the cell and therefore does not bring it into close proximity with FAs and FA-associated proteins, such as paxillin (Figure 5A). The positively charged arginine (R) residue, which replaces the negatively charged phosphotyrosine in the MDA-435 stably expressing mutant Cav1Y14R-mRFP, prevents its concentration directly behind the FA-associated protein, paxillin-GFP, but does not polarize it at the rear of the cell as the Cav1Y14F-mRFP mutant does (Figure 5A). There is no direct interaction with paxillin or any Cav1 construct at the cell surface, but phosphorylation does localize Cav1 into close proximity with FAs, indicating that it may play a role in FA dynamics.

Figure 5: **The close proximity of Cav1 to FAs at the cell surface is phosphorylation-dependent:** (A) Representative TIRF images of paxillin-GFP (green) and either stably expressing mRFP-tagged Cav1, Cav1Y14F, Cav1Y14D, or Cav1Y14R (red) in MDA-435 cells. White lines highlight an expression profile analysis through a focal adhesion for each cell type. (B) Graphs show expression profile analysis of a line drawn through a focal adhesion. Curves represent the relative intensities of transiently transfected paxillin (green) and either MDA-435 cells stably expressing mRFP-tagged Cav1, Cav1Y14F, Cav1Y14D, or Cav1Y14R (red) for each pixel along the line.

A


Paxillin / Cav-1
Paxillin / Cav-1Y14F
Paxillin / Cav-1Y14D
Paxillin / Cav-1Y14R

B

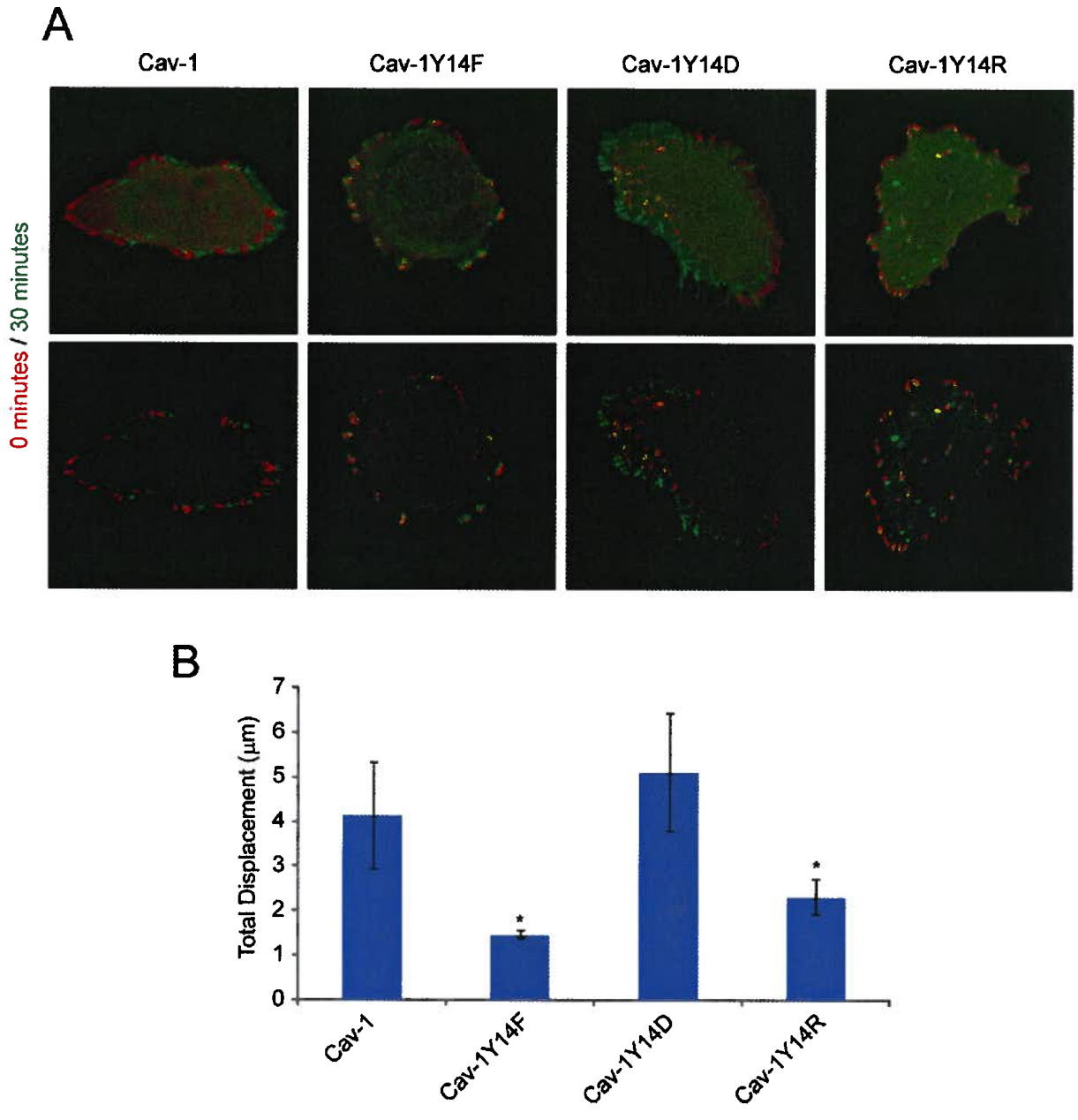

Cav-1
Paxillin
Cav-1Y14F
Paxillin
Cav-1Y14D
Paxillin
Cav-1Y14R
Paxillin
Intensity (Arbitrary Units)
200
150
100
50
0
Distance Along Line (Pixels)

Using TIRF images of MDA-435 cells stably expressing mRFP-tagged Cav1, Cav1Y14F, Cav1Y14D or Cav1Y14R, an expression profile line can be drawn through focal adhesion regions of each cell. The curves on the graphs (Figure 5B) represent the average relative intensities of paxillin-GFP and the Cav1-mRFP constructs on each pixel along the profile expression line. Both Cav1-mRFP and Cav1Y14D-mRFP intensities begin to increase directly after paxillin-GFP intensity levels off (Figure 5B). Cav1Y14R-mRFP expression, although not found directly behind paxillin-GFP expressing regions, were found towards the front of the cell. Increases in intensity of Cav1Y14F-mRFP were not found at regions adjacent to paxillin-GFP expression (Figure 5B).

The pY14Cav1 regulation of migration in MDA-435 human cancer cells is charge dependent.

Over-expression of Cav1-mRFP, as opposed to the mutant Cav1Y14F-mRFP, in MDA-435 cells stabilizes FA dynamics thereby increasing the rate of cell migration (Goetz et al., 2008a). pY14Cav1 reduces the availability of FAK within FAs for dynamic exchange with the cytosol, promoting membrane order within FAs (Gaus et al., 2006; Goetz et al., 2008a). To determine the degree of migration in the phosphomimetic, Cav1Y14D-mRFP, and the positively charged, Cav1Y14R-mRFP MDA-435 stable cell lines, time-lapse images were acquired every 30 seconds for 30 minutes (Figure 6A). Cav1Y14D-mRFP transfected cells underwent disassembly of FAs at the trailing edge of the cell, as did the Cav1-mRFP transfected cells. The protrusive leading edge of Cav1Y14D-mRFP transfected cells also exhibited increased formation of nascent FAs. In contrast, both Cav1Y14F-mRFP and Cav1Y14R-mRFP transfected cells showed limited stable turnover of FAs throughout the

Figure 6: **Tumor cell migration requires Cav1 phosphorylation:** MDA-435 cells stably expressing Cav1-mRFP, Cav1Y14F-mRFP, Cav1Y14D-mRFP, or Cav1Y14R-mRFP were transfected with FAK-GFP and imaged every 30 seconds for 30 minutes. (A) Overlay of FAK-GFP images at time 0 (red) and 30 minutes (green) shows FA displacement over time. (B) Masks were generated using FAK-GFP at the 488 nm wavelength. Masks were made functional by narrowing the dynamic range and eliminating objects smaller than 10 pixels, objects with a path length less than three frames and objects that move more than 10 pixels. Individual focal adhesions were tracked using center of area and averages were calculated. (B) Bar graph represents the average total displacement (μm) for each condition. *, $P < 0.05$; **, $P < 0.001$ relative to MDA-435 Cav1Y14D-mRFP stable cell line.

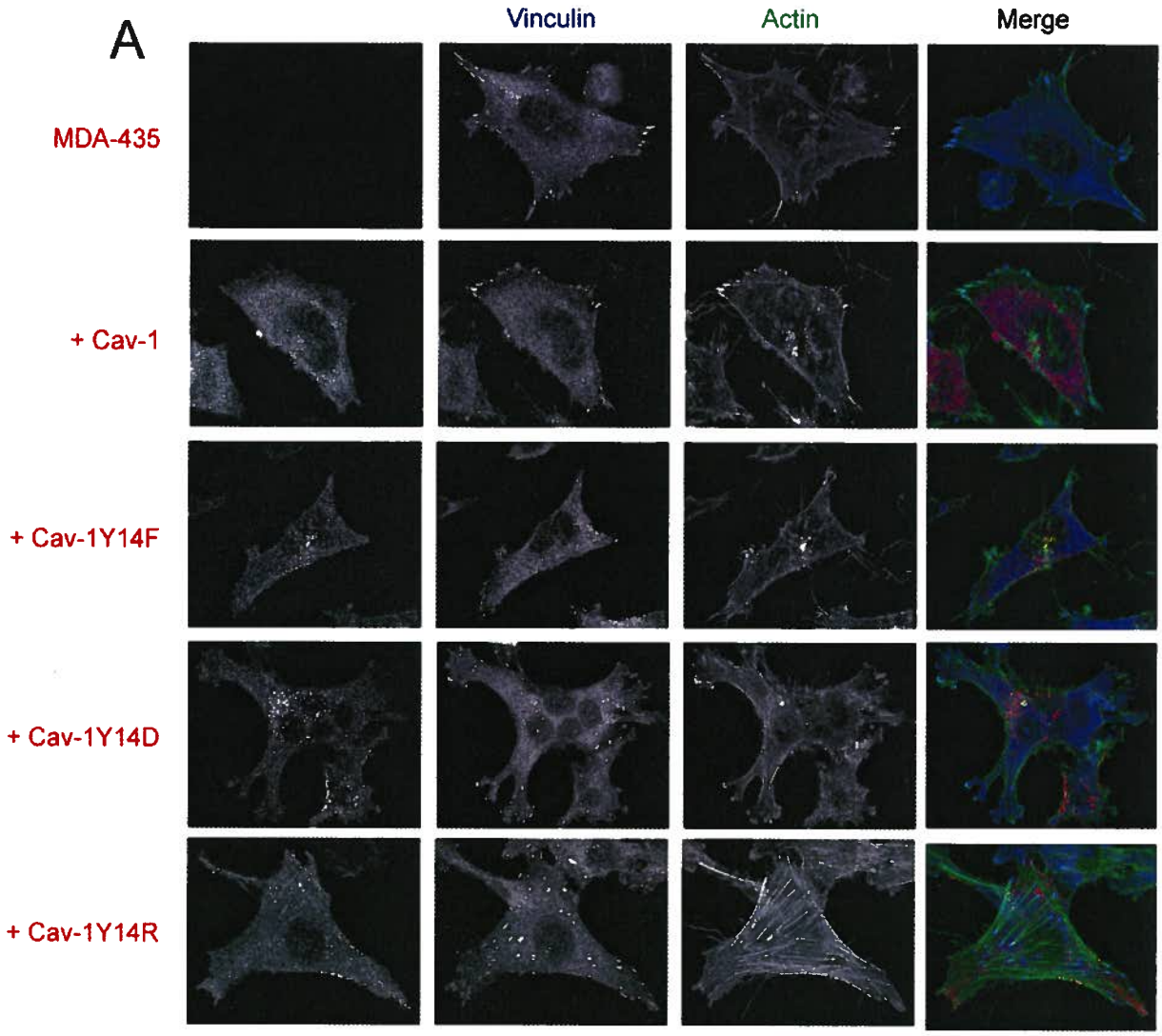

A
Cav-1
Cav-1Y14F
Cav-1Y14D
Cav-1Y14R
0 minutes / 30 minutes
B
Total Displacement (μm)
0
1
2
3
4
5
6
7
*
*
Cav-1
Cav-1Y14F
Cav-1Y14D
Cav-1Y14R

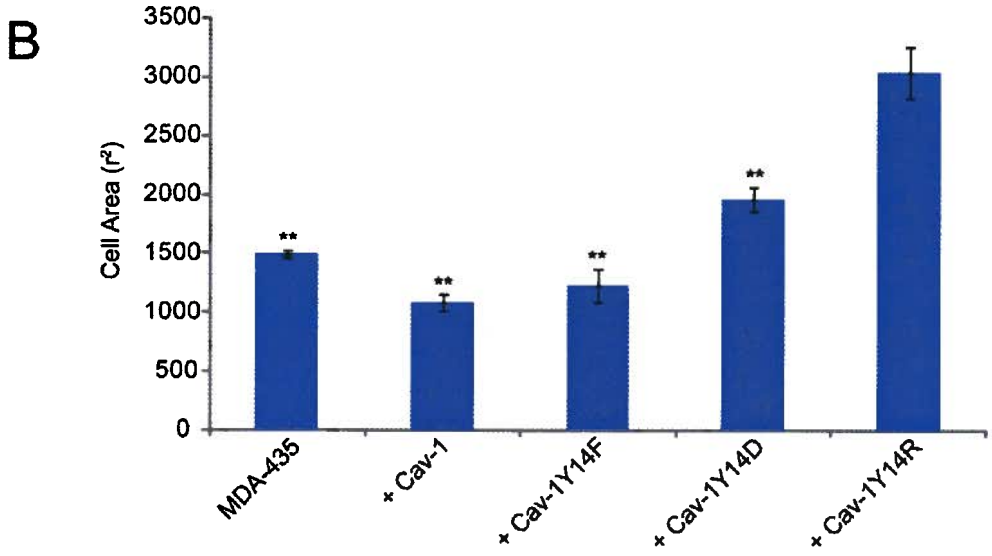
cell (Figure 6A). FA tracking, through the generation of a mask of FAK-GFP, revealed that FAs in both Cav1Y14D-mRFP and Cav1-mRFP transfected cells migrated significantly more from a point of origin than FAs in Cav1Y14F-mRFP and Cav1Y14R-mRFP transfected cells which remained static (Figure 6B).

Increased stress fiber formation and cell spreading occurs in positively charged Cav1 Y14R-mRFP transfected MDA-435 cells.

The activation of Rho, and the subsequent recruitment of its effector, ROCK, leads to the stabilization of mature FAs due to an association with actin stress fibers (Jaffe and Hall, 2005). Immunofluorescence analysis using phalloidin (actin-labelling) revealed that Cav1Y14R-mRFP stably transfected MDA-435 cells show increased actin stress fiber formation (Figure 7A). Labelling of anti-vinculin, a FA associated protein, clearly indicates a close interaction between FAs and actin stress fibers only in the Cav1Y14R-mRFP transfected cells. Additionally, vinculin labeled FAs can be found both peripherally and centrally in the Cav1Y14R-mRFP transfected cells, in contrast to the other stable cell lines where vinculin is only found peripherally (Figure 7A). Scan array analysis of cell spreading in MDA-435 cells, and the MDA-435 mRFP-tagged stable cell lines; Cav1, Cav1Y14F, Cav1Y14D, and Cav1Y14R, corresponded with actin stress fiber formation, where significant increases in spreading occurred in Cav1Y14R-mRFP transfected cells (Figure 7B). This suggests that the positively charged arginine residue might be activating Rho, causing actin stress fiber formation and cell spreading thereby decreasing cell migration.

Figure 7: **Stress fiber formation and cell spreading is increased in MDA-435 Cav1 Y14R stable cells:** (A) MDA-435, MDA-435 mRFP-tagged Cav1, Cav1Y14F, Cav1Y14D, and Cav1Y14R stable cell lines plated on glass coverslips and immunofluorescently labeled with anti-vinculin (blue), and Alexa488-phalloidin (green) antibodies. (B) Scan array determination of cell spreading in MDA-435, MDA-435 mRFP-tagged Cav1, Cav1Y14F, Cav1Y14D, and Cav1Y14R stable cell lines was completed 48 hours after plating. Graph shows average spreading area (r^2) (n = 4; FSE). *, $P < 0.05$; **, $P < 0.001$ relative to MDA-435 Cav1Y14R-mRFP stable cell line.


A
Vinculin
Actin
Merge
MDA-435
+ Cav-1
+ Cav-1Y14F
+ Cav-1Y14D
+ Cav-1Y14R


B
Cell Area (r²)
3500
3000
2500
2000
1500
1000
500
0
**
**
**
**
MDA-435
+ Cav-1
+ Cav-1Y14F
+ Cav-1Y14D
+ Cav-1Y14R

3.3 – Concerted regulation of FA dynamics by galectin-3 and tyrosine-phosporylated caveolin-1 (Goetz et al., 2008a).

Reduced FAK exchange requires expression of both the Mgat5/Gal-3 lattice and pY14Cav1 (Goetz et al., 2008a).

Expression of the *Mgat5* gene as well as its β1,6GlcNAc-branched N-glycans in human breast and colon carcinomas correlate with a poor prognosis and decreased survival time (Fernandes et al., 1991). Its expression has also been shown to directly cause tumor progression and metastasis in mice (Dennis et al., 2002).

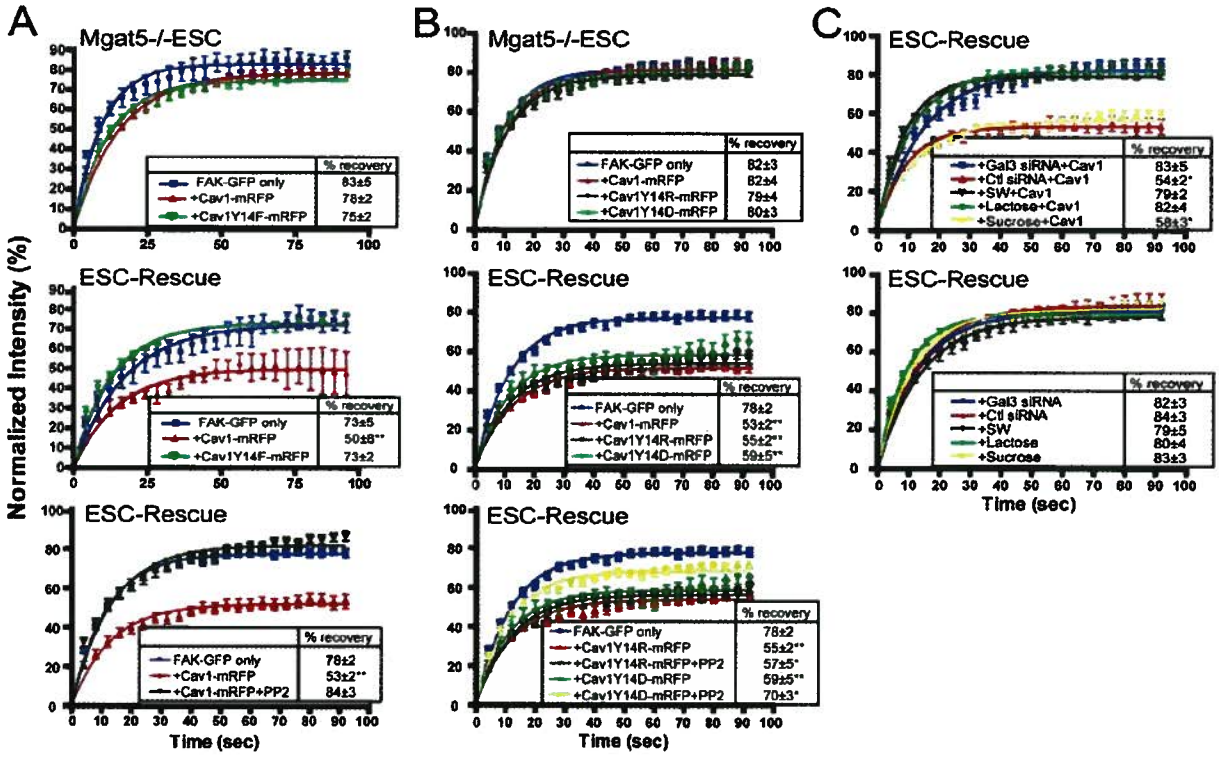
It had previously been identified in the lab that Mgat5/Gal-3 expression directly affected FA dynamics, just as pY14Cav1 expression did in the human tumor cell lines that I tested. I therefore wanted to determine whether Mgat5/Gal-3 and pY14Cav1 were working concertedly to regulate FA dyanamics and cell migration.

The Mgat5 cell lines ($Mgat5^{+/+}$, $Mgat5^{-/-ESC}$ and ESC-Rescue) were generated from MMTV-PyMT murine mammory tumors (Granovsky et al., 2000). $Mgat5^{-/-}$ mice displayed delayed tumor development and significantly lower lung metastases compared to the $Mgat5^{+/+}$ mice (Granovsky et al., 2000). The tumors found in MMTV-PyMT $Mgat5^{-/-}$ mice were small ($<2cm^3$), but a minority showed accelerated growth, indicating that they may have escaped the effects of an Mgat5 deficiency. The $Mgat5^{-/-ESC}$ cell line was generated from these larger, rapid growing tumors found in MMTV-PyMT $Mgat5^{-/-}$ mice (Granovsky et al.,

2000). By stably infecting Mgat5$^{-/-ESC}$ cells with an Mgat5 expression vector, we generated ESC-Rescue cells that have restored ß1,6GlcNAc-branched N-glycan expression.

Both Mgat5$^{-/-ESC}$ and ESC-Rescue cells lack Cav1 expression, additionally, the latter has had Mgat5 expression rescued and can form an Mgat5/galectin lattice. To determine whether reduced FAK exchange requires the presence of both the Mgat5/galectin lattice and pY14Cav1, we cotransfected Mgat5$^{-/-ESC}$ and ESC-Rescue cells with FAK-GFP and either Cav1-mRFP or non-phosphorylatable, dominant negative Cav1Y14F-mRFP and performed FRAP analysis of FAK-GFP. Cav1-mRFP, but not Cav1Y14F-mRFP, stabilized FAK in FAs of ESC-Rescue cells (Fig. 8A). As for Mgat5$^{-/-}$ cells, neither Cav1-mRFP nor Cav1Y14F-mRFP affected FAK exchange in FAs of Mgat5$^{-/-ESC}$ cells (Figure 8A). To assess whether Src-dependent phosphorylation of Cav1 was required for stabilization of FAK exchange in FAs, ESC-Rescue cells cotransfected with FAK-GFP and Cav1-mRFP were treated with PP2, a well-characterized inhibitor of Src family kinases. PP2 completely prevented Cav1-mRFP stabilization of FAK-GFP exchange in ESC-Rescue cells (Figure 8A). PP2 did not affect FAK-GFP exchange in ESC-Rescue cells transfected with FAK-GFP alone. To determine whether tyrosine phosphorylation of Cav1 is required for Cav1 regulation of FAK exchange in FAs, we introduced a phosphomimetic glutamate (D) residue or a positively charged arginine (R) residue in place of Y14 on Cav1. Expression of Cav1Y14D-mRFP or Cav1Y14R-mRFP did not affect FAK exchange in Mgat5$^{-/-ESC}$ cells, but both stabilized FAK-GFP in FAs of ESC-Rescue cells to a similar extent as Cav1-mRFP (Figure 8B). PP2 only partially, but significantly, enhanced FAK-GFP stabilization in Cav1Y14D-mRFP transfected cells and did not affect FAK-GFP stabilization in Cav1Y14R-

Figure 8: **pY14Cav1 regulates FAK exchange in Mgat5-expressing cells:** FRAP analysis was performed on $Mgat5^{-/-ESC}$ and ESC-Rescue cells transfected with FAK-GFP and either Cav1-mRFP or Cav1Y14F-mRFP (A) or with Cav1-mRFP, Cav1Y14R-mRFP, or Cav1Y14D-mRFP (B) and treated (or not) for 1 h with PP2, as indicated. (C) ESC-Rescue cells were either treated with 20 mM lactose, 20 mM sucrose, or 1 μg/ml SW, or transfected with Gal-3, control (CTL), or Cav1 siRNA for 48 h and then transfected with FAK-GFP (top) or cotransfected with FAK-GFP and Cav1-mRFP (bottom). FRAP analysis of FAK-GFP in FAs was then performed. Percent intensity (±SEM) in the bleached zone of FAK-GFP during recovery and quantification of percentage of recovery (box) are shown ($n = 3$; ± SEM; *, $P < 0.05$; **, $P < 0.01$) (Adapted from Goetz et al., 2008a, by permission).


A
Mgat5-/-ESC
% recovery
FAK-GFP only 83±6
+Cav1-mRFP 78±2
+Cav1Y14F-mRFP 75±2
ESC-Rescue
% recovery
FAK-GFP only 73±5
+Cav1-mRFP 50±8**
+Cav1Y14F-mRFP 73±2
ESC-Rescue
% recovery
FAK-GFP only 78±2
+Cav1-mRFP 53±2**
+Cav1-mRFP+PP2 84±3
Normalized Intensity (%)
Time (sec)
B
Mgat5-/-ESC
% recovery
FAK-GFP only 82±3
+Cav1-mRFP 82±4
+Cav1Y14R-mRFP 79±4
+Cav1Y14D-mRFP 80±3
ESC-Rescue
% recovery
FAK-GFP only 78±2
+Cav1-mRFP 53±2**
+Cav1Y14R-mRFP 55±2**
+Cav1Y14D-mRFP 59±5**
ESC-Rescue
% recovery
FAK-GFP only 78±2
+Cav1Y14R-mRFP 55±2**
+Cav1Y14R-mRFP+PP2 57±5*
+Cav1Y14D-mRFP 59±5**
+Cav1Y14D-mRFP+PP2 70±3*
Time (sec)
C
ESC-Rescue
% recovery
+Gal3 siRNA+Cav1 83±5
+Ctl siRNA+Cav1 54±2*
+SW+Cav1 79±2
+Lactose+Cav1 82±4
+Sucrose+Cav1 58±3*
ESC-Rescue
% recovery
+Gal3 siRNA 82±3
+Ctl siRNA 84±3
+SW 79±5
+Lactose 80±4
+Sucrose 83±3
Time (sec)

mRFP transfected cells (Figure 8B). The inability of Src inhibition to completely prevent Cav1Y14D-mRFP or Cav1Y14R-mRFP stabilization of FAK-GFP in FAs is indicative of a role for Src-dependent phosphorylation of Cav1Y14 in FA turnover (Goetz et al., 2008a, by permission).

To verify that pY14Cav1 regulation of FAK stabilization in FAs in ESC-Rescue cells was indeed due to the presence of the galectin lattice, we disrupted the galectin lattice with lactose competition of galectin binding, swainsonine (SW) inhibition of *N*-glycan terminal processing, and Gal-3 siRNA (Figure 8C). These treatments did not affect FAK recovery in FAs of ESC-Rescue cells; however, lactose, SW, and Gal-3 siRNA, which disrupt cell surface Gal-3 binding but not sucrose or control siRNA, all prevented Cav1-dependent stabilization of FAK in FAs (Goetz et al., 2008a, by permission).

We have therefore shown that an interdependence of the Mgat5/galectin lattice and pY14Cav1 on FA dynamics exists in murine mammary tumor cells. The novel finding that both Cav1Y14D and Y14R affect FA dynamics independently of Src, indicates that pY14Cav1 recruitment to FAs regulates the order and structure of these cellular domains.

Cav1 phosphoryation and Gal-3 expression regulate cancer cell migration.

FA dynamics tightly regulate the directionality and rate of cellular migration (Worth and Parsons, 2008). Upon identification of a concerted role for pY14Cav1 and the Mgat5/galectin lattice in regulating FA dynamics, I wanted to determine whether this relationship had an effect on human tumor cell migration.

Using a Boyden chamber assay I detected increased migration of pY14Cav1 expressing cells (MDA-231, and Cav1, Cav1Y14D, Cav1Y14R MDA-435 stable cells) relative to cell lines that do not express pY14Cav1 (MDA-435, MDA-231 shCav1 and Cav1Y14F MDA-435 stable cells) (Figure 9). Gal-3 siRNA treatment negatively affected cell migration in MDA-231 and Cav1, Cav1Y14D, Cav1Y14R MDA-435 stable cells, (Figure 9) indicating that both Cav1 phosphorylation and Gal-3 expression play a concerted role in human tumor cell migration.

Figure 9: **Gal-3 and pY14Cav1 regulate cancer cell migration:** Boyden chamber transwell migration assay of MDA-231, MDA-231 shCav1, MDA-435, MDA-435 mRFP-tagged Cav1, Cav1Y14F, Cav1Y14D and Cav1Y14R was performed 48 hours after transfection of Ctl siRNA, or Gal-3 siRNA as indicated (n = 3; FSE). *, $P < 0.05$; **, $P < 0.001$ relative to untransfected cells.

**
# of Migrating Cells
800
700
600
500
400
300
200
100
**
**
**
**
MDA-231
+ Ctl-siRNA
+ Gal-3-siRNA
MDA-231 shCav-1
+ Ctl-siRNA
+ Gal-3-siRNA
MDA-435
+ Ctl-siRNA
+ Gal-siRNA
MDA-435 Cav-1
+ Ctl-siRNA
+ Gal-3-siRNA
MDA-435 Cav-1Y14F
+ Ctl-siRNA
+ Gal-3-siRNA
MDA-435 Cav-1Y14D
+ Ctl-siRNA
+ Gal-3-siRNA
MDA-435 Cav-1Y14R
+ Ctl-siRNA
+ Gal-3-siRNA

CHAPTER 4: DISCUSSION

Cav1 and its regulatory role on FA dynamics

The location of the human *Cav1* gene was first detected using fluorescence in situ hybridization on the q31.1 region of chromosome 7 at the D7S522 locus (Engelman et al., 1998b). In human breast, prostate, colorectal, head and neck, renal, and ovarian cancers, this locus is commonly found deleted (Engelman et al., 1998b). This suggests a possible role for Cav1 as a tumor suppressor. Additionally, a sporadic mutation of the *Cav1* gene at codon 132 (P132L) has been detected in 16% of human breast cancer cases (Hayashi et al., 2001). The role of Cav1 as a negative regulator of transformation has clearly been demonstrated in a variety of models. However, Cav1 is a protein with numerous functions, and our observations, in addition to those in current literature, focus on the varied contribution of Cav1 to tumor biology, but nevertheless suggest that Cav1 expression does play an important role in cancer progression and metastasis (Joshi et al., 2008). Cav1 has long been associated with poor prognosis and poor patient survival in prostate cancer (Yang et al., 1999). It has also been found to be overexpressed in invasive compared to benign and *in situ* breast tumors, is associated with the basal-like phenotype in sporadic and hereditary breast cancer, and is associated with poor patient outcome (Savage et al., 2007; Van den Eynden et al., 2006; Yang et al., 1998).

Using the fluorescent probe Laurdan that labels ordered membrane domains, the FA membrane has been shown to be highly ordered, more so than caveolae and cholesterol-dependent raft domains (Gaus et al., 2006). Stabilized FA domains are a result of pY14Cav1

recruitment to the leading edge of the cell promoting the recruitment of FA-associated proteins, such as FAK, paxillin and Src, that effect FA turnover (Wei et al., 1999).

Exploring the localization of pY14Cav1 without using the cross-reactive antibody

Recent studies have clearly displayed the cross-reactivity of the anti-pY14Cav1 antibody with phospho-paxillin, bringing into question the result stating that pY14Cav1 is present in pseudopodial domains at the leading edge of migrating cells (Hill et al., 2007).

To reconfirm previous results that were found to be inaccurate, I explored the questions that arose due to the cross-reactivity of the anti-pY14Cav1 antibody by transfecting human cell lines, that under normal conditions express low levels of both Cav1 and pY14Cav1, with wild-type Cav1 or a non-phosphorylatable Cav1Y14F mutant. The presence of wild-type Cav1 and absence of mutant Cav1Y14F in protrusive domains indicates the importance of tyrosine-14 phosphorylation with regards to Cav1 recruitment to the leading edge and thus tumor cell migration. In support of our findings, $Cav1^{-/-}$ mouse embryonic fibroblasts have been shown to contain fewer newly formed adhesions in cellular protrusions resulting in defects in polarized spreading and ultimately reduced mobility (Grande-Garcia et al., 2007)

Additionally, my FRAP experiments have conclusively shown that pY14Cav1 expression and localization plays a critical role in regulating FAK molecular dynamics. FRAP analysis of tumor cell lines with differential pY14Cav1 expression, or cells exogenously overexpressing either mutant Cav1Y14F or wild-type Cav1, showed that Cav1 promotes FA turnover, thereby implicating pY14Cav1 expression in tumor cell migration. We therefore

confirmed that the stability in FAs necessary for substrate adherence of cellular protrusions and directional motility is in part reliant upon pY14Cav1 expression.

Cav1 requires integrin binding and activation for its Src-dependent phosphorylation, it then subsequently becomes part of a signalling mechanism that helps integrins to indirectly drive cell migration (Goetz et al., 2008a; Li et al., 1996). The less than 1% of Cav1 that is phosphorylated is essential for membrane organization at FAs (del Pozo et al., 2005; Gaus et al., 2006). What has remained elusive is the temporal and spatial relationship of pY14Cav1 with FAs, and FA-associated proteins.

Here I have demonstrated using TIRF imaging on MDA-435 cells, a spatial relationship between wild type Cav1, or the phosphomimetic Cav1Y14D and the FA-associated protein, paxillin. In doing so I have further explored the controversies brought about by the inaccuracies of the anti-pY14Cav1 antibody. While a direct co-localization does not occur at the cell-substrate interacting region beween these Cav1 constructs and paxillin, a close association appears to be present as mRFP-tagged Cav1 and Cav1Y14D localize directly posterior to paxillin-GFP. The absence of Cav1Y14F-mRFP from FA domains indicates that Y14 phosphorylation is essential for this spatial association. Interestingly, the positively charged Cav1Y14R does not concentrate at the the rear of the cell, as does Cav1Y14F, nor does it show a close association with paxillin, as is the case for the traditionally phosphorylated forms of Cav1. The conformational changes associated with a phosphorylation event seem to be recruiting Cav1Y14R away from the rear of the cell.

Although, it cannot be ruled out that the absence of a negative charge, in the form of arginine (R), may be preventing its interaction with FAs and their associated proteins.

Interdependence of pY14Cav1 and the Mgat5/galectin lattice on FAs and migration

FA precursor formation is generated by the lateral association of integrins into clusters that is a direct result of a combination of various extracellular and intracellular stimuli including ligand binding, integrin activation, and actin polymerization (DeMali et al., 2003; Ginsberg et al., 2005). Gal-3 expression, pentamerization, and subsequent binding to its preferred ligand, *N*-glycans, causes the formation of Mgat5/galectin lattice domains (Hirabayashi et al., 2002). The affinity of Gal-3 to *N*-glycans is proportional to the GlcNAc branching found on glycoproteins (Hirabayashi et al., 2002). We have shown that the formation of an Mgat5/galectin lattice defines the ability of pY14Cav1 to regulate FA dynamics at the leading edge of the cell.

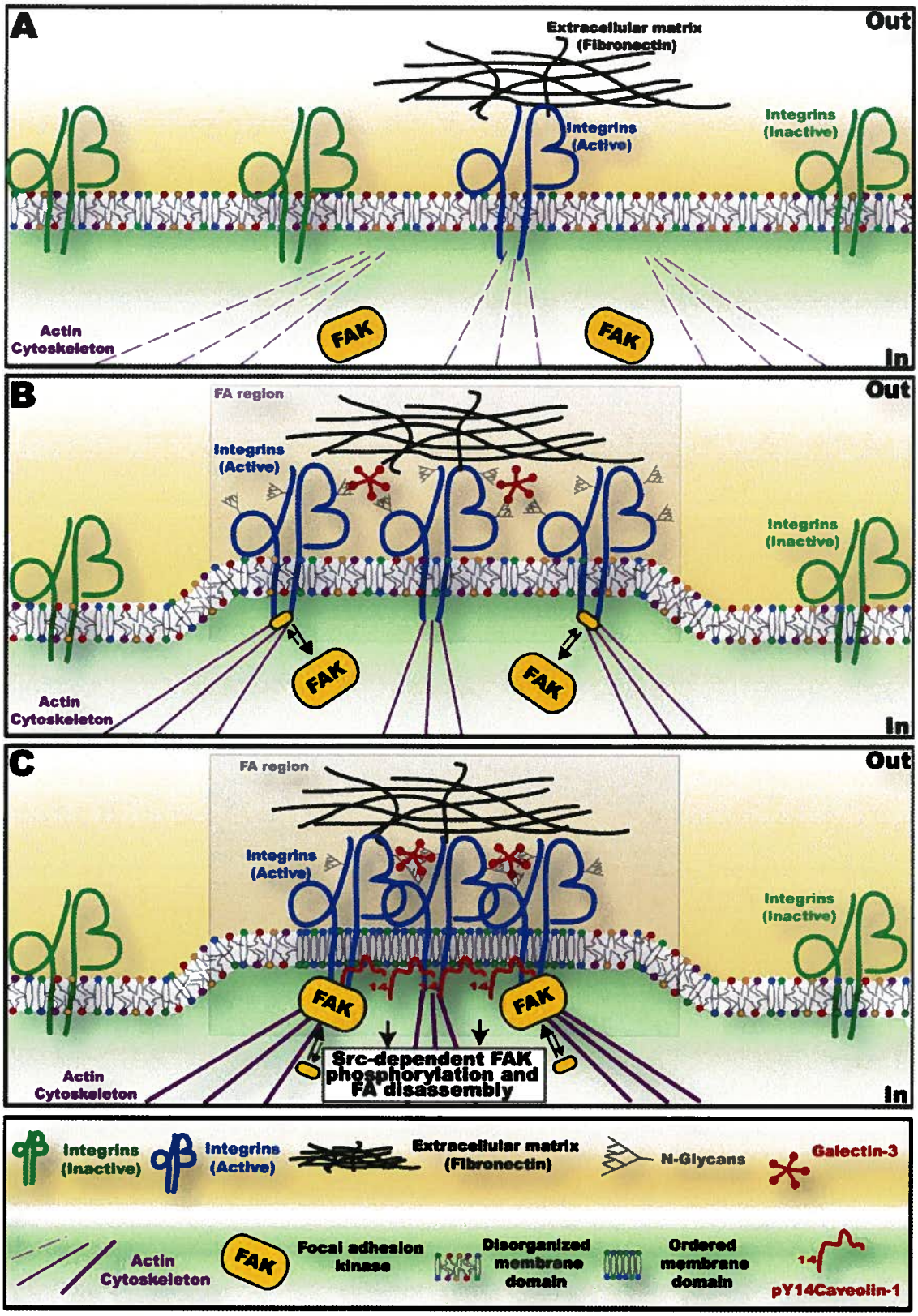
Clustering of Mgat5-modified *N*-glycans at the cell surface is Gal-3-dependent and leads to integrin activation and the FAKY397 phosphorylation necessary for the assembly and disassembly of FAs (Lagana et al., 2006). FAKY397 is the high affinity binding site for the SH2 domain found on Src kinase that is therefore recruited and further phosphorylates FAK and other FA associated proteins (Mitra and Schlaepfer, 2006). As previously stated, a phosphorylation induced increase in activation at FAs results in the recruitment of adaptor proteins, such as paxillin, that regulate FA dynamics and tumor cell migration (Giannone et al., 2004; Hamadi et al., 2005; Owen et al., 2007; Webb et al., 2002). By inhibiting lattice formation, either chemically or by Gal-3 knockdown, I was able to destabilize FAs and

increase the mobile fraction of FAK in pY14Cav1 expressing cells. Here I have identified that the Mgat5/galectin lattice regulates FA dynamics and that this activity is dependent upon the expression and tyrosine-14 phosphorylation of Cav1. Therefore, Gal-3 and pY14Cav1 work in concert to stabilize FAK and decrease its mobile fraction in FAs. Gal-3-mediated integrin activation acts through pY14Cav1 to induce FA membrane organization and stabilization thereby restricting exchange of FAK and other FA components while enabling FA turnover (Goetz et al., 2008a).

The binding of Gal-3 to Mgat5-modified *N*-glycans induces fibronectin fibrillogenesis and cell motility via activation of α5-β1 integrin (Lagana et al., 2006). Mgat5 knockdown has clearly been shown to disrupt formation of the Mgat5/Gal-3 lattice and attenuate EGF-induced FAK dephosphorylation and activate Shp2 and inhibit tumor cell motility and invasiveness (Guo et al., 2007). My results using Gal-3 siRNA knockdown show that the degree of migration in pY14Cav1 expressing human tumor cell lines is significantly decreased as opposed to cell lines that express low levels or have deficiencies of pY14Cav1. By preventing the formation of the Mgat5/Gal-3 lattice, recruitment of integrins to nascent FAs at the leading edge is inhibited leading to decreases in pY14Cav1-dependent signalling and stability. It is possible that these inhibitory effects decrease both adhesion turnover and ultimately cell migration.

Our data therefore supports a model in which Gal-3 binding promotes integrin clustering, pY14Cav1 recruitment and formation of stable focal contacts leading to an increase in directional cell migration (Goetz et al., 2008a) (Illustration 7). This is an example of outside-

Illustration 7: **The model of FA regulation by both Gal-3 and pY14Cav1:** (A) The inability to from an Mgat5/galectin lattice in Mgat5$^{-/-}$ cells prevents FA formation and membrane organization. (B) ESC-Rescue cells, where Mgat5 expression has been restored, show integrin clustering and activation due to Gal-3 binding but are unable to stabilize FAK in FAs. (C) pY14Cav1 expression results in the formation of ordered membrane domains resulting in the stabilization of FA-associated proteins, such as FAK. The stable association of phospho-FAK with FAs leads to their disassembly and turnover. The need for lattice formation and Cav1 phosphorylation at FA domains indicates the importance of both Gal-3 and pY14Cav1 with respect to FA dynamics and tumor cell migration (Goetz et al., 2008a, by permission).


A
Out
Extracellular matrix
(Fibronectin)
Integrins
(Active)
Integrins
(Inactive)
Actin
Cytoskeleton
FAK
FAK
In
B
FA region
Out
Integrins
(Active)
Integrins
(Inactive)
FAK
FAK
Actin
Cytoskeleton
In
C
FA region
Out
Integrins
(Active)
Integrins
(inactive)
FAK
FAK
Src-dependent FAK
phosphorylation and
FA disassembly
Actin
Cytoskeleton
In
Integrins
(Inactive)
Integrins
(Active)
Extracellular matrix
(Fibronectin)
N-Glycans
Galectin-3
Actin
Cytoskeleton
FAK
Focal adhesion
kinase
Disorganized
membrane
domain
Ordered
membrane
domain
pY14Caveolin-1

in signalling, such that the interdependence of FA turnover on both extracellular Gal-3 and intracellular pY14Cav1 regulates integrin activation and cell adhesion (Luo et al., 2007). This indicates that Gal-3 and pY14Cav1 are required, but not necessarily sufficient, for the stabilization of FA components and promotion of FA signalling, disassembly, and translocation. Both Gal-3 and Cav1 have numerous functions and a multitude of binding proteins. Therefore, Gal-3 stimulation of pY14Cav1 may not be limited to FAs, and it is also possible that Gal-3 and pY14Cav1 play multiple roles in FA turnover and maturation.

The relationship between Src/ROCK and pY14Cav1 and its effects on FA dynamics and migration

The formation of a FAK/Src signalling complex, after integrin clustering, causes a phosphorylation induced increase in activation in FAs. Adaptor proteins such as, but not limited to, p130Cas, paxillin, and Crk are then recruited and promote FA dynamics and tumor cell motility (Mitra and Schlaepfer, 2006). The generation of a docking site on pY14Cav1 in FAs allows it to bind SH2-domain containing proteins, such as Grb7 (Lee et al., 2000), and Csk causing the down-regulation of Src activity through phosphorylation (Cao et al., 2002). RhoA/ROCK signalling promotes migration by limiting integrin activity and membrane protrusions to the leading edge and prevents the formation of multiple competing lamellipodia (Worthylake and Burridge, 2003). In inflammatory breast cancer, Cav1 and Cav2 expression correlate with RhoC expression (Van den Eynden et al., 2006). Additionally, both activated Rho (Worthylake and Burridge, 2003) and pY14Cav1 can be found localized to tumor cell protrusions (Parat et al., 2003). The Y14-dependent enrichment of pY14Cav1 in protrusions indicates that an interaction with ROCK must occur within

protrusive domains of tumor cells. By regulating the activation state of Src, which in turn regulates signalling by Rho GTPases, Cav1 controls cell polarity required for directional tumor cell migration (Grande-Garcia et al., 2007). The ability of both Src and Rho/ROCK signalling to promote Cav1 tyrosine phosphorylation in the tumor cell lines studied here is indicative of a feedback loop that maintains local Rho activation and pY14Cav1 expression in tumor cell protrusions.

FA organization is significantly disrupted in $Cav1^{-/-}$ mouse embryonic fibroblasts and is more efficiently restored upon transfection with Cav1 wild-type than in cells transfected with the non-phosphorylatable Cav1Y14F mutant (Goetz et al., 2008a). I found that by inhibiting Src or ROCK activity with PP2 or Y27632, respectively, there was a decrease in FA stability and an increase in FAK turnover in cells expressing wild-type Cav1. Cav1Y14F mutant expressing cells were not sensitive to Src or ROCK inhibition. Additionally, there appears to be a reduced sensitivity to Src and ROCK inhibition of FAK stabilization in FAs by Cav1Y14D compared with wild-type Cav1. This suggests that pY14Cav1 recruitment to FAs regulates the order and structure of these cellular domains. The ability of a non-traditional Cav1Y14R mutant, where the negatively charged phosphotyrosine is being substituted for a positively charged arginine residue, to functionally mimic pY14Cav1 in a Src-independent manner indicates that conformational changes due to Y14 phosphorylation may be involved in pY14Cav1 function (Goetz et al., 2008a). I demonstrated in both murine and human tumor cell lines that pY14Cav1 reduces the availability of FAK within FAs for dynamic exchange causing a decrease in FAK turnover. This is consistent with the ability of pY14Cav1 to promote membrane order within FAs (Gaus et al., 2006). Reducing FAK exchange gives the

cell more opportunity to recruit effectors that lead to FA disassembly and turnover, ultimately promoting cell migration (Giannone et al., 2004; Hamadi et al., 2005). Regulation of membrane order within FAs by pY14Cav1 can therefore be considered a determinant of the dynamics and stabilization of FA components, including FAK (Gaus et al., 2006).

Our results suggest that Cav1 is a critical effector of Rho/ROCK- and Src-dependent tumor cell migration and invasion through regulation of FA dynamics. MDA-231 cells, shown here to present elevated pY14Cav1, Rho activation (Joshi et al., 2008), and Rho/ROCK dependent migration, exhibit a protease-dependent, mesenchymal mode of migration (Wolf et al., 2007). Regulation of tumor cell invasion by Rho/ROCK signalling may therefore involve pY14Cav1-dependent FA turnover in protrusive domains of tumor cells invading via a mesenchymal mode of migration in addition to matrix deformation and proteolysis-independent cell invasion (Sahai and Marshall, 2003; Wyckoff et al., 2006). Rho/ROCK signalling has also been shown to regulate mRNA translocation to tumor cell protrusions, the invasive response to invadopodia formation and hypoxic conditions (Cardone et al., 2005; Jia et al., 2005; Stuart et al., 2008; Vishnubhotla et al., 2007). This suggests that Rho/ROCK signalling might be playing a variety of important roles with regards to tumor cell migration and invasion. The critical role described here for pY14Cav1, as a regulator of Src- and ROCK-dependent tumor cell migration and invasion, describes a mechanistic explanation for the close association between Cav1 expression and poor survival and distant metastasis in various human tumors. The functional interaction between Rho/ROCK signalling and pY14Cav1-mediated FAK exchange defines a novel interaction between these metastasis-related processes.

The Cav1Y14R phenotype

Actin stress fibers are both lengthy and complex structures that have been found to disassemble in response to the *Clostridium botulinum* C3 toxin for which the Rho family of small GTPases is the major cellular target (Chardin et al., 1989). By microinjecting RhoA, the frontrunner with regards to actin stress fiber formation, into fibroblasts, an extensive network of stress fibers are rapidly formed (Paterson et al., 1990). ROCK, a downstream effector activated by Rho signalling has been shown to phosphorylate multiple targets involved in stress fiber formation, thereby identifying it as a main player in actomyosin contractility (Pellegrin and Mellor, 2007). It was evident throughout our research, and as later quantified by scan array analysis, that stable overexpression of Cav1Y14R in MDA-435 cells lead to an increase in cell spreading. As expected, Cav1Y14R overexpressing cells also displayed an elaborate network of actin stress fibers. The fibers showed a close interaction with the FA marker vinculin. This indicates that the positively charged arginine residue might be activating the Rho/ROCK signalling pathway leading to cell stabilization and spreading.

Several studies have reported that stress fibers are more prominent in static, non-migratory cells, indicating that they prevent cell migration (Burridge, 1981). Time lapse imaging of our MDA-435 stable cell lines overexpressing mRFP-tagged Cav1, Cav1Y14F, Cav1Y14D and Cav1Y14R, lead to multiple conclusions. Phosphorylation of Cav1 either transiently (Cav1-mRFP) or consistently (Cav1Y14D-mRFP), was required for correct disassembly/assembly of FAs. Secondly, the probable activation of the Rho/ROCK signalling pathway in

Cav1Y14R-mRFP overexpressing cells prevents cell migration and limits FA dynamics seemingly due to the formation of elaborate actin stress fiber networks.

Conclusion

Our results and others observations have found conflicting evidence that would suggest that Cav1 upregulation correlates with metastatic potential and that it is reliant upon both Src and Rho kinases. However, the phosphorylation of Cav1, induced by both upstream Src and Rho kinase signalling is alone not enough to drive FA turnover and cell migration. The expression and formation of an Mgat5/galectin lattice, and subsequent integrin activation, recruit pY14Cav1 to the protrusive tumor cell domains leading to FA disassembly and directional tumor cell migration. By clarifying the regulatory roles of both integrin and Rho/Src kinase signalling pathways with regards to pY14Cav1-dependent tumor cell migration, we have gained some insight into the relationship between Cav1 expression and reduced survival in individuals diagnosed with cancer.

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