REGULATION OF INTRACELLULAR TRAFFICKING OF LAMININ BINDING INTEGRINS IN PROSTATE CANCER

By

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DEDICATION

I dedicate this work to my parents - my first teachers.

And to everyone who has suffered cancer.
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ABSTRACT

Laminin binding integrins (α6β1 and α3β1) are persistently but differentially expressed throughout prostate cancer progression and metastasis. Prostate cancer primarily invades through laminin rich nerve for extracapsular escape during cancer metastasis. An intense expression of the pro-metastatic α6 integrin was observed during perineural invasion with a heterogeneous distribution of the integrin on the cancer cell membrane as well as intracellularly. Bone and soft tissue metastasis of human prostate cancer demonstrated a similar pattern where 75-80% of the cancers had significant intracellular staining. This was correlated with an mRNA overexpression of various intracellular trafficking regulators. Using a prostate cancer cell culture model of DU145 cells, the α6 integrin was found to be constitutively internalized in cancer cells at a rate of 3.25 min⁻¹, which was 3 fold greater than internalization rate of α3 integrin, classically considered a “non-circulating” receptor.

α6 and α3 integrins function coordinately to regulate cell migration during development, wound healing. Their orchestrated redistribution during these processes is well-known, but the mechanism remains elusive. Current study identifies intracellular trafficking of these integrins as a key mechanism of their coordination. Depletion of α3 integrin in prostate cancer cells significantly increased internalization of α6 integrin up to 1.7-fold and increased localization of α6 integrin at cell-cell membrane locations. There was a concomitant 1.8-fold increase in cell migration significantly dependent on α6 integrin. Depletion of α6 integrin expression however, had no effects on the
internalization of $\alpha_3$ integrin indicating that the identified coordination was unidirectional.

$\alpha_6$ integrin trafficking drives cancer invasion, but its selective regulators are unknown. Here, Rab11FIP5 was identified as a selective regulator of $\alpha_6$ integrin recycling to cell membrane. Interestingly, $\alpha_6$ integrin was found to be primarily recycled to the cell-cell membranes where it colocalized with Rab11 and Rab11FIP5. Depletion of Rab11FIP5 reduced such membrane expression of $\alpha_6$ integrin, inhibited cell-cell cohesion in 3D culture and significantly reduced cell migration. The localization of $\alpha_6$ and $\alpha_3$ integrin at these locations have been implicated in cell adhesion. Based on current study $\alpha_6$ recycling by Rab11FIP5 might be key to such function.

Another Rab11 effector protein Rab11FIP1 was identified as a regulator of both $\alpha_3$ and $\alpha_6$ integrin trafficking. Depletion of Rab11FIP1 reduced membrane expression of $\alpha_3$ integrin by significantly increasing its internalization and reducing the recycling. There was a major effect on $\alpha_6$ integrin internalization, which increased to an extent similar to that observed on $\alpha_3$ integrin depletion. Rab11FIP1 regulated $\alpha_6$ integrin recycling, in a pathway found to be independent of Rab11FIP5.

Taken together, current research defined Rab11FIPs as regulators of $\alpha_6$ and $\alpha_3$ integrins. A unidirectional coordination between $\alpha_6$ and $\alpha_3$ integrin was identified such that loss of $\alpha_3$ integrin, representative of high grade prostate cancer, amplifies integrin $\alpha_6$ integrin internalization and a resultant migratory phenotype.
I. Introduction

CANCER

Cancer is the second leading cause of death in both US and worldwide with an expected global burden of 21.7 million new cancer cases by 2030 (Torre et al., 2015). Prostate cancer is the most frequently diagnosed cancer in men and third leading cause of death in developed countries. Prostate cancer is slow growing indolent solid tumor that is manageable if it is locally contained. However, prostate cancer spread to distant sites (metastasis) is fatal, with only 29% survival (Siegel et al., 2017).

Current treatment for prostate cancer is dependent on accurate diagnosis of its metastatic potential which remains the biggest challenge in the field. Indolent disease with high metastasis risk calls for radical prostatectomy or radiation therapy. Whereas, locally advanced and metastatic cancer is principally treated with androgen deprivation therapy. Unfortunately, 70% of patients with metastatic prostate cancer become unresponsive to androgen deprivation therapy within 2-3 years and progress to more aggressive castration resistant prostate cancer leading to poor prognosis and survival of 16-18 months (Karantanos et al., 2013; Pienta and Bradley, 2006). Patients with low-risk of metastasis can instead be managed with active surveillance or minimally invasive strategies and avoid rigors of aggressive surgical treatment and related morbidity (Wilt et al., 2012). Hence, accurate diagnosis of metastasis potential is crucial to define individuals with propensity to develop incurable metastatic disease and prevent
overtreatment of low-risk patients. Moreover, pro-metastatic signatures need to be defined which can be clinically targeted to prevent aggressive metastatic spread and mortality.

Cancer metastasis is a complex multi-step process (Fig. 1.1) - first step being extracapsular escape of cancer cells from the primary tumor, followed by dissemination through the lymphatics and finally colonization at distant organs. Prostate cancer primarily invades via prostatic nerves and neurovascular bundles as a means of extracapsular escape during metastasis (Liebig et al., 2009; Marchesi et al., 2010; Sroka et al., 2010; Villers et al., 1989). This process is termed as Perineural invasion (PNI). PNI has emerged as an important pathologic feature of various other malignancies as well, like pancreatic, head and neck, colorectal and gastric cancer (Li et al., 2013; Liebig et al., 2009). PNI is a marker of poor prognosis and decreased patient survival (Harnden et al., 2007; Liebig et al., 2009). After escape from the prostate gland through nerves, prostate cancer cells primarily metastasize to bone. 90% of advanced prostate cancer metastases show skeletal metastasis causing severe bone pain in patients and significantly compromising the quality of life (Arya et al., 2006; Bubendorf et al., 2000). Metastasis to soft tissue, primarily to liver, lung and pleura and adrenal is less common, seen in 20% of patients.

Each step of the metastatic spread exploits the cellular migratory potential. Various cell surface proteins involved in cell adhesion, primarily the integrin receptors are dynamically utilized to modulate tumor cell interactions with surrounding microenvironment and dictate cell attachment and motility.
Figure. 1.1. The Metastasis cascade: First step is tumor invasion of basement membrane and escape of cells from cancer in situ. Second step is dissemination through the lymphatics which starts with tumor cells entering the blood vessels/lymphatics/nerves (intravasation). Final step is extravasation at distant organs and colonization. Cancer metastasis exploits dynamic changes in cell adhesion receptors like E-cadherin and integrins to modulate tumor cell motility and mediate tumor cell invasion (Guo and Giancotti, 2004).
Blocking integrin functions have been shown to significantly block cancer metastasis (Desgrosellier and Cheresh, 2010). Thus, understanding regulation of integrin mediated cell migration can reveal potential drug targets to block invasive spread of metastatic cancer.

**INTEGRINS**

Integrins are evolutionarily conserved cell surface receptors that interact with the surrounding extracellular matrix to regulate cell adhesion, migration and signaling pathways of cell proliferation, survival and differentiation (Hood and Cheresh, 2002; Hynes, 2002; Watt, 2002). Being transmembrane receptors, they physically connect intracellular cytoskeletal elements like actin and intermediate filaments with the extracellular matrix (ECM), and are important signal transducers between extracellular and intracellular environment (Fig. 1.2). Integrins can be activated upon ECM binding (outside-in signaling) or by binding of effectors to their cytoplasmic tails (inside-out signaling) (O'Toole et al., 1994; Shattil et al., 2010). Integrin activation is accompanied with major conformational switch from a bent V-shaped inactive form to an open extended active structure. Integrin-ligand binding initiates macromolecular assembly of intracellular effector proteins with actin at the cytoplasmic end to generate stable adhesions and tension for cellular spreading and migration (Vicente-Manzanares et al., 2009).
Figure 1.2. Bidirectional integrin signaling. Binding of integrins to their extracellular ligands changes the conformation of the integrin and leads to intracellular signals that control cell polarity, cytoskeletal structure, gene expression and cell survival and proliferation (Outside-in signaling). During 'inside–out' signalling, an intracellular activator, such as talin or kindlins, binds to the β-integrin tail, leading to conformational changes that result in increased affinity for extracellular ligands (integrin 'activation'). Inside–out signalling controls adhesion strength and enables sufficiently strong interactions between integrins and extracellular matrix (ECM) proteins to allow integrins to transmit the forces required for cell migration and ECM remodelling and assembly. Integrins also behave like traditional signalling receptors in transmitting information into cells by 'outside–in' signaling (Shattil et al., 2010).
Integrins are heterodimers of non-covalently linked alpha and beta subunits. In humans, 18 α and 8 β subunits are known to form 24 different receptors (Barczyk et al., 2010). They are broadly classified based on their extracellular ligand, each with distinct cellular functions (Fig. 1.3). Integrins heterodimerize before they are exported to the plasma membrane (Humphries, 2000). Cytoplasmic tails of the alpha integrins are highly divergent but contain the conserved GFFKR motif that is important for heterodimerization with beta subunit and hence membrane expression (Bauer et al., 1993; De Melker et al., 1997; De Melker and Sonnenberg, 1996; Kassner et al., 1994). 12 different alpha subunits pair with the β1 integrin but bind to different extracellular ligand and are functionally distinct. Targeted deletion of the β1 integrin gene in mice knocks out all αβ1 integrins leading to pre-implantation embryonic lethality (Fassler and Meyer, 1995; Stephens et al., 1995).

**LAMININ BINDING INTEGRINS (LBIs)**

Laminins are a major component of basement membrane that physically separates the epithelium, endothelium and nervous tissue from the surrounding connective tissue (Durbeej, 2010). Laminin binding integrins (LBI, α6β1 α6β4 and α3β1 heterodimers) represent a conserved class of integrins essential in various stages of development and maintenance of adult tissue. For simplicity, α6β1 and α6β4 integrins will be referred to as α6 integrin and α3β1 integrin as α3 integrin.
Figure 1.3. The integrin family. In vertebrates, 18 α and 8 β subunits are known to form 24 different heterodimeric integrins. They are broadly classified based on the extracellular matrix ligand (Barczyk et al., 2010).
Chapter I: Introduction

α6 integrin is essential for embryogenic processes like endoderm migration (Sutherland et al., 1993), kidney tubule formation (Sorokin et al., 1990), epidermal development and cerebral cortical and retinal lamination (De Arcangelis et al., 1999; Georges-Labouesse et al., 1996). α6 integrin is a stem cells receptor functionally important for maintenance of stem cell niches in the epithelium (Notta et al., 2011; Qian et al., 2006; Rowland et al., 2010). Human embryonic cell-derived progenitor expansion, migration and differentiation requires α6 integrin function (Ma et al., 2008). In adult humans, various mutations in α6β4 integrin subunits or its ligand laminin 5 lead to Junctional Epidermolysis Bullosa, a lethal genetic disorder characterized by severe blistering of the skin (Christiano and Uitto, 1996; Georges-Labouesse et al., 1996; Pulkkinen et al., 1997; van der Neut et al., 1996; Vidal et al., 1995).

Mutations in α3 integrin gene in human patients are associated with congenital nephrotic syndrome, interstitial lung and mild form of epidermolysis bullosa (Has et al., 2012; Nicolaou et al., 2012; Yalcin et al., 2015). Similarly, mice lacking α3 integrin develop skin blisters mainly due to basement membrane disorganization (DiPersio et al., 1997) but succumb to lung and kidney defects at later stages of development (De Arcangelis et al., 1999; Kreidberg et al., 1996). Double heterozygous mutant mice embryos for α6 and α3 (A6+/-/A3+/-) have additional defects not observed in single mutant animals like defective ectodermal ridge, limb abnormalities, neural tube closure, bilateral lung hypoplasia, urogenital tract abnormalities. These novel phenotypes demonstrated synergism between α6 and α3 integrins (De Arcangelis et al., 1999).
LBIs localize to the basolateral membrane of the epithelium where they form 2 different kinds of adhesion complexes—hemidesmosomes and focal adhesions. α6β4 integrin is a key component of hemidesmosome, a stable adhesion at the basal cell surface required for epithelial cell layer attachment to the basement membrane. The cytoplasmic tail of β4 integrin subunit is sufficient to allow assembly of a complex array of proteins and intermediate filaments to anchor the cells to the basement membrane via hemidesmosomes. LBIs in focal adhesions interact with intracellular actin filaments to form dynamic adhesion complexes important for cell spreading and migration.

The localization of LBIs at the lateral membrane locations of cell-cell adhesions have been well described (Underwood et al., 2009). However, their function at these locations remain elusive, although they have been implicated in cell-cell adhesion possibly via binding to ECM substrate between cells. Importance of α3β1 integrin in cell-cell adhesion has been described in various systems. α3β1 integrin was shown to associate with intercellular contact sites in lateral surfaces of basal keratinocytes (Carter et al., 1990; Symington et al., 1993). Similarly, blocking α3 integrin adhesion to ECM at basal surface induces cell-cell aggregation. The relocation of integrins from focal adhesions to cell-cell contacts result in detachment of cells from the basement membrane and increased cell-cell adhesion in the suprabasal cells (Carter et al., 1990). Cell clusters of cultured keratinocytes can be disrupted by blocking β1 integrin function without affecting cell-substrate adhesion (Larjava et al., 1990). Similarly, inhibition of α6 or β1 integrin with locally injected antibodies disrupt the cohesive nature of rostral migrator stream (Emsley, 2003). Thus, there is substantial evidence that integrins might
regulate cell-cell adhesion in epithelial cells which might be an important contributor to collective cell migration.

LBI localization at the cell-cell contact versus basal membrane is not only functionally but temporally coordinated. α3 integrin primarily localizes to cell-cell contact sites in early culture timepoints of keratinocytes, while its association with basal surface was a late event occurring after days of culture (Kaufmann et al., 1989). Similar temporally resolved localization of α6 integrin was observed in developing zebrafish epidermis with its distribution to cell-cell locations at early timepoints followed by its basal occurrence at later days of development (Sonawane et al., 2009).

α3 and α6 integrins coordinate cell migration in various stages of development including embryogenesis, development of epithelia/mesenchyma, wound healing and tissue regeneration in adults as well as in cancer invasion and metastasis (Huttenlocher and Horwitz, 2011). Laminin interaction with α6 integrin dictates directional migration of neuroblasts (Emsley, 2003). Cell migration is often accompanied with a major redistribution of integrins. During wound healing, the hemidesmosomes are disassembled and basal α6β4 integrin appears along the entire cell surface of the migrating epithelial sheet. α3 integrin is observed at the tip of the lamellipodia of migrating keratinocytes (Goldfinger et al., 1999) and is involved in the deposition of a provisional extracellular matrix (DiPersio et al., 1997), subsequently utilized by α6 integrin for collective epithelial migration (Margadant et al., 2009). In absence of α3 integrin, wound healing is faster with faster and persistent keratinocyte migration and cell spreading rescued by the α6 integrin (Margadant et al., 2009).
LBI IN CANCER

LBIs have been extensively studied for their role in tumorigenesis and metastasis. α3 and α6 integrins are expressed in various epithelial cancers in humans (Desgrosellier and Cheresh, 2010; Harryman et al., 2016; Ramovs et al., 2017; Stipp, 2010). They are the primary integrins detected in biopsy and prostatectomy specimens of primary prostate tumors, as well as in bone metastasis specimens (Schmelz et al., 2002), demonstrating a loss of the variety of integrin expression in prostate cancer as compared to normal glands (Cress et al., 1995). Previous work has shown a strong expression of α6 integrin during perineural invasion via laminin enriched nerves (Sroka et al., 2010). Although the majority of prostate cancers (80%) express either/both α3 or α6 integrins on the tumor cell surface, 26% express only integrin α6 (Schmelz et al., 2002). Additionally, surface α3 integrin expression is lost in advanced prostate cancer and positively correlates with high Gleason grade and the pathological stage of the cancer.

Expression of α6 integrin is an important determinant of tumor progression, reduced patient survival, and increased metastasis (Ports et al., 2009; Schmelz et al., 2002). α6 integrin is a marker of cancer stem cells or tumor initiating cells, known to be the most aggressive subset of tumor cells (Lathia et al., 2010; Lawson et al., 2007; Schmelz et al., 2005; Seguin et al., 2015). A tumor-specific functional variant of α6 integrin, α6p, is a key contributor to cancer metastasis (Demetriou and Cress, 2004; Ports et al., 2009). Functional blocking of this cleavage event curtails invasion and bone metastasis.
(Landowski et al., 2014; Ports et al., 2009). However, the role of α3 integrin in cancer progression remains less clear (Ramovs et al., 2017; Stipp, 2010). Several studies report α3 integrin is pro-metastatic (Mitchell et al., 2010; Zhou et al., 2014), while others have defined α3 integrin as a mediator of cell spreading and a negative regulator of metastasis (Owens and Watt, 2001; Varzavand et al., 2013).

**INTRACELLULAR TRAFFICKING OF INTEGRINS**

Membrane expression and localization of integrins is dynamically changed to coordinate cell migration (Caswell and Norman, 2008; Jones et al., 2006; Paul et al., 2015) (Fig. 1.4). The disassembly of stable cell adhesion complexes followed by an internalization of the components into the cells is first step to enable cell migration (Wehrle-Haller, 2012). The internalized integrins are returned back (recycled) to the cell surface to form fresh adhesions and forward sites of attachment to enable the forward movement of the cells. This process is strictly regulated through pathways of intracellular trafficking, blocking which significantly halts cell migration (Jones et al., 2006).
Figure 1.4: Integrin endocytosis and recycling back to plasma membrane is key to cell migration. Integrin molecules, bound to the ECM, become endocytosed into early endosomes (EE), transported to the recycling endosome (RE) and returned back to the cell surface to forward the cell migratory front (Ulrich and Heisenberg, 2009).
Both active and inactive integrins undergo intracellular trafficking (Arjonen et al., 2012) indicating trafficking signals are intrinsically present in the integrin subunits. Both the alpha and beta subunits of integrin contain endocytic sorting signals in their cytoplasmic tails (Caswell et al., 2009; Mai et al., 2011; Margadant et al., 2011). It is now well known that integrins sharing same β1 partners or even extracellular ligands can have distinct trafficking routes based on the alpha subunit (Bretscher, 1992) suggesting alpha subunit is key to such selectivity.

**INTRACELLULAR TRAFFICKING MACHINERY**

Intracellular trafficking is regulated by a machinery of membrane bound vesicular compartments, with distinct identity (Pfeffer, 2003). Each vesicular compartment is defined by its phosphoinositide composition and a family of small GTPases called RabGTPase (Campa and Hirsch, 2017). The Rabs have emerged at the root of metazoan evolution which marks the beginning of membrane bound compartmentalization of organelles (Klopper et al., 2012). There are 64 known RabGTPases known till date which are classified based on the functional pathway that they are involved (Stenmark, 2012). Each of these RabGTPase family members has characteristically distinct intracellular localization (Fig. 1.5).
Figure 1.5. Complex network of intracellular trafficking machinery in epithelial cells showing localization and function of RabGTPases. Each RabGTPase defines identity of the vesicular compartment and is responsible for distinct functions in terms of transport of selective cargo and directionality of movement (Stenmark, 2009)
Rabs control each step of vesicular transport which can be broadly classified into 5 steps (Zerial and McBride, 2001) (i) Establishment of vesicular identity by recruitment of cytoplasmic inactive GDP bound Rab to vesicular membrane (ii) cargo receptor selection and loading (iii) movement of vesicles on actin or microtubules via direct/ indirect interactions with motor proteins (iv) docking to target vesicle or plasma membrane (v) fusion with target membrane to mediate cargo transfer or exocytosis (Stenmark, 2009).

Rabs are regulated by a cycle of GTP bound active and GDP bound inactive state which involves major conformational changes, a process is catalyzed by GTP exchange factors (GEFs) and GTPase activating proteins (GAPs) respectively. Each Rab has a set of effector proteins most of which preferentially bind GTP bound active Rabs and provide selectivity and efficient execution of its function. Phosphorylation is central to recruitment of cytoplasmic inactive Rabs to the vesicular membrane. Additionally, Rabs can recruit appropriate kinases or phosphatases to modulate phosphoinositide composition of the vesicles. This coordination between the RabGTPase and phosphoinositide composition of membrane define vesicular identity and regulate spatiotemporal steps of trafficking (Jean and Kiger, 2012).

Rab11 is a major trafficking regulator in cells, crucial for cell survival (Welz et al., 2014). Rab11 defines the perinuclear recycling compartment (PNRC; also called apical recycling endosomes, ARE) (Ullrich et al., 1996) that forms a central hub of cellular trafficking to transport a variety of proteins and lipids from intracellular compartments to the plasma membrane. This includes recycling of transmembrane receptors like growth factor receptors, cell adhesion proteins like cadherins and integrins and channel proteins to
control plasma membrane composition as well as exocytosis of soluble ligands like neurotransmitters, immunoglobulins, matrix metalloproteases and growth factors like transferrin (Welz et al., 2014). The PNRC receives traffic from both apical and basolateral early endosomes, but efficiently sorts the apical and basolateral proteins by lateral segregation into subdomains of individual recycling endosomes (Thompson et al., 2007); thus has a key role in maintaining cellular polarity. Rab11 knockout mice are zygotic lethal, further confirming its importance in organism development and survival.

Rab11 is involved in each of the 4 key endocytic recycling steps (i) accepting cargo from early endosomes (ii) cargo sorting in PNRC (iii) movement of vesicles on actin or microtubules via direct/indirect interactions with motor proteins and (iv) docking and fusion with plasma membrane to mediate cargo transfer or exocytosis (Takahashi et al., 2012). A group of Rab11 effector proteins called Rab11 family interacting proteins (Rab11FIPs) regulate these temporally and spatially distinct steps of recycling (Horgan and McCaffrey, 2009). They localize to discrete subdomains in Rab11 recycling endosomal tubules and regulate recruitment of Rab11 to distinct recycling compartments (Baetz and Goldenring, 2013). Rab11FIPs enable the movement of Rab11 vesicles on cytoskeletal actin and microtubules by direct interaction with molecular motor proteins. Rab11FIPs are regulated by various kinases. Phosphorylation inhibitors or microtubule destabilizers increase localization of Rab11FIPs at plasma membrane.

The Rab11FIPs share a conserved C-terminal Rab11-binding domain. However, they are classified into two subfamilies with distinct functions owing to different N-terminal conserved motifs. Class I Rab11FIPs (Rab11FIP1, Rab11FIP2, Rab11FIP5) share a
homologous C2 domain well known to bind to membrane phosphoinositides. In fact, Rab11FIPs with intact C2 domain can move towards plasma membrane without binding to Rab11 but can’t transport the cargo receptor, suggesting the cargo loading requires Rab11. Class II Rab11FIPs contain a calcium binding EF hand motif and are crucial in intracellular transport during cell division, especially formation of cleavage furrow. Both these classes have been structurally resolved (Jagoe et al., 2006; Vetter et al., 2015). Rab11FIPs have both homo- and hetero-dimerizing capability (Wallace et al., 2002). Although each Rab11FIPs have distinct but not mutually exclusive functions, the functional importance of heterodimerization remains unclear.

The role of Rab11 and Rab11FIPs in trafficking of integrins is well known (Caswell et al., 2009); its deregulation being a key feature of cancer (Goldenring, 2013). Rab11FIPs are important candidates to provide specificity of trafficking to different integrin subunits, since each FIPs have specific though not necessarily mutually exclusive set of cargos. For example, integrin α5β1 is trafficked by Rab11FIP1 but not other class I FIPs FIP2 or FIP5 (Caswell et al., 2008), suggesting Rab11FIPs can provide specificity to recycling of specific integrin based on different alpha subunits.

Deregulated vesicular trafficking and receptor recycling represent emerging hallmarks of cancer (Goldenring, 2013; Mosesson et al., 2008). Genomic amplification, overexpression and deregulation of trafficking regulators RabGTPases, especially Rab11, contribute to initiation and progression of cancer (Mills et al., 2009; Mitra et al., 2011). Understanding trafficking regulation of known pro-metastatic factors can hence identify new locus as potential target to block cancer signaling and invasion.
DISSERTATION OBJECTIVES

Prostate cancer invades via laminin rich nerves and primarily metastasizes to bone. 80% of prostate cancer persistently but differentially express laminin binding α6 and/or α3 integrins throughout disease progression and metastasis. These integrins are known to function coordinately to regulate cell migration in various normal cellular processes especially during development, wound healing as well as cancer invasion and metastasis. Although the coordination of α3 and α6 function during these processes is well known, the molecular determinants of this regulation remain unclear. Integrin function in cell migration requires a dynamic assembly and disassembly of cell adhesion to extracellular environment strictly regulated by intracellular trafficking pathways. The objective of my dissertation was to identify regulators of intracellular trafficking of α6 and α3 integrins, and determine if a coordination exist between these integrins in prostate cancer.

Human prostate cancer tissue and patient derived xenograft models provided the clinical data to investigate trafficking regulators of these integrins. Quantitative techniques were employed to measure the trafficking kinetics of LBIs and revealed a unidirectional coordination between their internalization kinetics. Rab11FIP5 and Rab11FIP1 were discovered as regulators of intracellular trafficking of LBIs.

Phosphatase and tensin homolog (PTEN), a known regulator of membrane phosphoinositide composition and vesicular trafficking was tested as a potential regulator of LBI trafficking. This added to the clinical relevance of the study as PTEN
loss/mutation is an underlying mechanism of prostate cancer development. Finally, the function of LBIs in neurotropic motility of prostate cancer was investigated to understand the effect of nerve environment on perineural invasion of prostate cancer.
II. Internalization kinetics of Laminin Binding Integrins is Coordinated

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INTRODUCTION

The laminin binding integrins (α3 and α6 containing heterodimers; α3β1, α6β1, and α6β4) function coordinately during embryonic development (De Arcangelis et al., 1999; DiPersio et al., 1997) as well as in adult processes such as epithelial regeneration and wound healing (Longmate and Dipersio, 2014; Margadant et al., 2009). Mice lacking the α6 integrin die shortly after birth because of severe blistering of the skin and other epithelia (Georges-Labouesse et al., 1996), a defect that can only be partially compensated by α3 integrin (De Arcangelis et al., 1999; van der Neut et al., 1996). During development and wound healing, both integrins show an orchestrated redistribution of their cellular localization that affects their function (Shimizu et al., 2012). α3 integrin is observed at the tip of the lamellipodia of migrating keratinocytes (Goldfinger et al., 1999) and is involved in the deposition of a provisional extracellular matrix (DiPersio et al., 1997), subsequently utilized by α6 integrin for collective epithelial migration (Margadant et al., 2009). Thus, α3 integrin is important to establish and maintain basement membrane integrity and complements α6 function in forming stable adhesion of skin epidermis to the basement membrane (DiPersio et al., 1997).

In prostate cancer, the LBIs are differentially expressed. Majority of prostate cancers (80%) express the laminin binding α3 and α6 integrins on the tumor cell surface. Of these, 26% express only integrin α6 (Schmelz et al., 2002) and show a loss of α3 integrin. Interestingly, the loss of surface α3 integrin expression positively correlates with high Gleason grade and the pathological stage of the cancer (Schmelz et al., 2002).
Expression of α6 integrin is an important determinant of tumor progression, reduced patient survival, and increased metastasis (Ports et al., 2009). Previous work has shown a strong expression of α6 integrin during perineural invasion (Sroka et al., 2010) and bone metastasis in prostate cancer (Landowski et al., 2014). A tumor-specific functional variant, α6p, is a key contributor to cancer metastasis (Demetriou and Cress, 2004; Demetriou et al., 2008; Ports et al., 2009). However, the role of α3 integrin in cancer progression remains less clear (Stipp, 2010). Several studies report that α3 integrin is pro-metastatic (Mitchell et al., 2010; Zhou et al., 2014), while others have defined α3 integrin as a mediator of cell spreading and a negative regulator of metastasis (Owens and Watt, 2001; Varzavand et al., 2013). The coordination of α6 and α3 function on the cell surface may be an important factor to consider in evaluating α3 integrin function in cancer progression.

An important determinant of the cell surface expression and function of integrins is their intracellular trafficking. Cell surface integrins are continuously internalized to early endosomes and sorted through intracellular trafficking pathways to be either reexpressed back at the membrane via recycling or routed to the lysosome for degradation (Bridgewater et al., 2012; Ramsay et al., 2007). Internalization and recycling of α6 integrin is reported to be crucial for migration of neuronal cells during development (Strachan and Condic, 2004) and in hypoxia-induced breast cancer invasion (Yoon et al., 2005). Additionally, each alpha integrin is known to have a distinct cellular localization and internalization rates despite sharing the same β1 integrin partner (Bretscher, 1992).
Current chapter characterizes and compares the internalization kinetics of laminin binding integrins to determine if their trafficking is coordinated. Receptor internalization can be influenced by ligand binding, integrin activation state, integrin clustering, membrane micro domain location, cell type, pH, and temperature (De Franceschi et al., 2015). In order to minimize these variables, internalization rates were obtained using suspension cells as previously reported, to obtain “steady-state” or intrinsic trafficking characteristics of the receptor being studied. Quantitation of the kinetic parameters of the receptor(s) trafficking using flow cytometry and kinetic modeling (Wiley and Cunningham, 1982a) revealed that depletion of α3 integrin significantly increases α6 integrin internalization and a concomitant faster cell migration. Depletion of α3 integrin expression increased trafficking of α6β4 integrin to early endosomes and resulted in the redistribution of α6β4 integrin to the membrane at cell-cell locations.

**RESULTS**

*Internalization of α6 integrin containing heterodimers in prostate cancer cells:* Biotin labeled cell surface receptors in DU145 prostate cancer cell line were tested for internalization of α6 integrin containing heterodimers. Western blot analysis detected the internalized biotin labeled α6 integrin, the tumor variant α6p, and the partner subunits β1 and β4 within 10 minutes of incubation, reaching a maximum level at 30
Figure 2.1. Internalization of α6 integrin heterodimers in DU145 prostate cancer cells. Biotin-labeled cell surface proteins internalized for indicated time points were immunoprecipitated with α6 integrin-specific antibody (J1B5). Western blot analyses of cell lysates with streptavidin-HRP detected increasing internalization of α6, α6p, and their partner β1 and β4 integrin subunits with time.
minutes (Fig. 2.1). Each of the integrin subunits showed increased intracellular accumulation with time, indicating that DU145 cells accumulate internalized α6 and α6p containing β1 and β4 heterodimers.

Internalization rates of integrin subunits: Internalization kinetics were quantified for the laminin binding α3 and α6 integrins, their binding partner β1 integrin, vitronectin binding αv integrin, and an unrelated abundant transferrin receptor (TfR) in DU145 cells. The general schema is shown (Fig. 2.2A). The cell surface receptors were labeled with fluorophore conjugated antibodies at 4°C, and then allowed to internalize to measure receptor internalized in a single cycle of internalization. Labelling of surface receptors was observed as an increase in mean peak fluorescence (MPF) values as compared to unlabeled cells (Fig. 2.2B, left panel). With increased time of internalization, the labeled cell surface receptor accumulated inside the cells, demonstrated by a gradual increase in the MPF values of the intracellular label (Fig. 2.2B, right panel). The percent of internalized surface label was calculated from MPF value of internalized label as compared to total surface label and plotted for each timepoint up to 75 minutes (Fig. 2.2C).
Chapter II: LBI internalization is coordinated

Figure 2.2. Internalization kinetics of integrin subunits and transferrin receptor. (A) Schematic representation of the internalization assay. DU145 cells were surface labeled with fluorophore conjugated antibodies against integrin subunits α6 (A6-PE), α3 (A3-FITC), αv (Av-PE), β1 (B1-FITC), or transferrin receptor (TfR-FITC) at 4°C for 1 hr. followed by internalization at 37°C for different time intervals. Remaining surface label was removed and cells were fixed and analyzed by flow cytometry. (B) Flow histograms of receptor internalization. Left panel shows total surface levels of the indicated receptor and unlabeled cells (shaded) are shown as control. Right panel shows histogram profile of the labeled receptor internalized at representative time intervals showing increase in mean peak fluorescence with increase in time of internalization. (C) Internalization curve of the receptors. Percent label internalized (calculated as percent mean peak fluorescence of internalized label at a given time point versus total surface label) is plotted against time of internalization. First order curve is fitted using Kaleidagraph (R²>0.98). (D) Parameters of internalization kinetics: maximum intracellular accumulation (amplitude, b) and internalization rate constants (observed, k_{obs} and actual, k_{actual}) calculated using first order rate kinetics. Histograms and kinetic curves are representative of at least 3 independent experiments.
Significant α3 and α6 integrin internalization was observed within 10 minutes and approximately 40 to 60% of the label was internalized within one hour. α6 integrin was internalized at a rate 3.25-fold higher than α3 integrin ($k_{\text{actual}} = 3.25 \pm 0.16 \text{ min}^{-1}$ and $1.00 \pm 0.08 \text{ min}^{-1}$ respectively, Fig. 2.2D). Intracellular accumulation of α6 integrin was ~2-fold higher than α3 integrin (58.09±0.85, 30.46±0.67 percent of total surface integrin, respectively). αv integrin had a lower $k_{\text{actual}}$ than α6 integrin but a higher intracellular accumulation (64.92±2.94). Transferrin receptor (TfR) was internalized with a significantly higher rate ($k_{\text{actual}} = 15 \pm 4.60$), at least 4 times that of α6 integrin.

Internalization kinetics of β1 integrin (Fig. 2.2C) revealed a maximal internalization of 75%, while 45% of surface β1 integrin was already internalized at 0 min. This was not surprising, as caveolin mediated internalization of β1 integrin has been reported to occur at 4°C (Goldfinger et al., 1999; Teckchandani et al., 2009). Moreover, β1 integrin potentially has 11 alpha partners (α1-11), of which there is significant surface expression of α2, α3, α5, and α6 integrins in DU145 cells (Witkowski et al., 1993). Thus, the β1 subunit internalized at 4°C may reflect a cumulative internalization of various β1 integrin containing heterodimers. Interestingly, the other beta partner of α6 subunit, the β4 integrin, was completely internalized at 4°C at 0 min in these cells (Fig. 2.3). This suggests that internalization rates of α6 subunit measured under a physiologically relevant temperature of 37°C were due to the α6β1 heterodimer. This further underscores the differential regulation of α3 and α6 integrin internalization properties despite sharing the same β1 integrin partner.
Figure 2.3. **Internalization kinetic curve of β4 integrin.** DU145 cells were surface labeled with fluorophore conjugated antibody against β4 integrin subunit (B4-A660) at 4°C for 1 hour followed by internalization at 37°C for different time intervals. Remaining surface label was removed and cells were fixed and analyzed by flow cytometry. Percent label internalized was calculated and first order kinetic curve was fitted as previously described.
**Figure 2.4. α3 integrin expression in AR positive cell lines.** Total cell lysates of AR negative PCa cell line DU145 and AR positive PCa cell lines LNCaP, VCaP and 22Rv1 were immunoblotted with anti-α3 integrin antibody AB1920 and relative expression of the α3 integrin was quantified.
Silencing α3 integrin expression increases internalization of α6 integrin: Since both α3 and α6 integrin expression is preserved in the majority of human prostate cancers, experiments were designed to determine if their internalization is coordinated. The study was restricted to androgen receptor (AR) minus cell lines since AR positive cell lines (LNCaP, VCaP and 22Rv1) (van Bokhoven et al., 2003) were negative for α3 integrin expression (Fig. 2.4). Three other prostate cancer cell lines were tested (DU145, PC3, PC3B1). Since they are AR negative, they represent advanced castration resistant prostate cancer and express both α3 and α6 integrin. The expression of α3 or α6 integrin was silenced (siA3 or siA6 cells respectively) and the effect on the internalization kinetics of the non-targeted integrin was determined. Importantly, silencing expression of either integrin did not have a measurable effect on the total cellular expression of the non-targeted integrin as seen by immunoblot analysis (Fig. 2.5A).

Cell surface α6 integrin was labeled with PE-conjugated anti-α6 integrin antibody and a time-dependent increase in internalized label was observed. Representative flow profiles are shown for DU145 cells (Fig. 2.5B). In each of the three cell lines, DU145, PC3, PC3B1, the internalization of α6 integrin in siA3 cells was increased at each timepoint measured as compared to cells treated with non-targeting siRNA (siCON cells) (Fig. 2.5C).
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Figure 2.5. Internalization of integrin α6 after silencing integrin α3 expression. DU145, PC3 or PC3B1 cells were transfected with non-targeting siRNA (SiCON), siRNA against α3 integrin (siA3), or α6 integrin (siA6). (A) Immunoblot showing integrin α6 (AA6NT) and integrin α3 (AB1920) expression in untreated (WT), siCON, siA3, siA6 DU145 cells. (B) Internalization assay for integrin α6 and TfR were performed on siCON and siA3 cells. Flow histogram of total labeled integrin α6 at the surface and amount internalized at different time intervals in siCON and siA3 treated DU145 cells. (C) Internalization curve of α6 integrin (A6) in siCON, siA3, and siA6 cells and transferrin receptor (TfR) in siCON, siA3 treated DU145, PC3 and PC3B1 cells. Percent label internalized was calculated and first order kinetic curve was fitted as previously described in figure 1. (D) Maximum intracellular accumulation (amplitude, b) and internalization rate constants (observed, \( k_{obs} \) and actual, \( k_{actual} \)) calculated as per first order rate kinetics. Results represent 4 independent experiments. Statistical significance calculated for change in \( k_{actual} \) of siA3 versus siCON cells as per student’s t test, unpaired, *p<0.05, n=4.
Figure 2.6. Internalization kinetics of integrin α3 after silencing integrin α6 expression. Internalization assay for integrin α3 was performed on DU145 cells transfected with non-targeting siRNA (siCON), siRNA against α3 integrin (siA3), or α6 integrin (siA6). (A) Flow histogram of total labeled integrin α3 (A3) at the surface and amount internalized at different time intervals in siCON and siA6 treated DU145 cells. (B) Internalization curve of α3 integrin and transferrin receptor (TfR) in siCON, siA6, and siA3 treated DU145, PC3 and PC3B1 cells. (C) Maximum intracellular accumulation (amplitude, b) and internalization rate constants (observed, \( k_{\text{obs}} \) and actual, \( k_{\text{actual}} \)) calculated as per first order kinetics. Results represent 4 independent experiments.
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Kinetic rate constant analysis confirmed a statistically significant increase in $k_{\text{actual}}$ of α6 integrin internalization as compared to siCON cells. DU145 cells showed 1.47-fold increase in $k_{\text{actual}}$ (from 3.20 min$^{-1}$ to 4.72 min$^{-1}$), PC3 had a 1.71-fold increase (from 0.69 min$^{-1}$ to 1.18 min$^{-1}$) PC3B1 had a 1.44-fold increase (from 1.04 min$^{-1}$ to 1.47 min$^{-1}$) in siA3 cells versus siCON cells (Fig. 2.5D). The total intracellular accumulation, demonstrated by the amplitude of α6 integrin, was increased in each of the three cell lines (52.48 to 63.83, 11.35% increase in DU145; 34.61 to 42.10, 7.49% increase in PC3; and 40.32 to 52.36, 12.04% increase in PC3B1 cells). Depletion of α3 integrin expression in all three cell lines increased the α6 integrin internalization rate up to 1.71-fold, despite the differences in the intrinsic α6 integrin internalization rates (Fig. 2.5C, D).

*Silencing α6 integrin expression does not affect internalization rate of α3 integrin:* We next tested whether depletion of α6 integrin expression was a determining factor in α3 integrin internalization rates in the three cell lines DU145, PC3 and PC3B1. The silencing of α6 integrin (siA6) did not alter the total amount of α3 integrin expressed (Fig. 2.5A). The surface labeled α3 integrin internalized in a time dependent manner in siA6 or siCON cells as shown in representative flow cytometry profiles for the population of DU145 cells (Fig. 2.6A). Analysis of the MPF data obtained from the flow cytometry profiles generated the internalization curves (Fig. 2.6B). The internalization rate constant of α3 integrin was not significantly different in siCON and siA6 cells in any of the three cell lines. However, kinetic analysis predicted an increase in intracellular accumulation, represented by an increase in amplitude (31.27 to 42.11; 11% increase) of α3 integrin in siA6 cells in DU145 cells (Fig. 2.6C).
Figure 2.7. Actual internalization rate constant of laminin binding integrins on depletion of either subunit. Actual Internalization rate constant ($k_{\text{actual}}$) of $\alpha6$, $\alpha3$ or TfR internalization in DU145, PC3 and PC3B1 cells treated with siCON (grey bars), siA6 or siA3 treated cells (black bars). *p<0.05, n=4.
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Figure 2.8. Effect of ligand binding and functional blocking of integrin β1 on laminin receptor internalization. (A) Internalization curves for integrins α6 (A6) and α3 (A3) in untreated DU145 cells and cells treated with ligand mimetic peptide HYD1 and control peptide HYDS. (B) Internalization curves for integrins α6 and α3 in untreated and function blocking anti-β1 integrin antibody (AIIB2) treated DU145 cells.
An increase in amplitude was also observed in PC3 (63.77 to 66.67; 3% increase) and in PC3B1 (51.84 to 62.18; 10% increase) (Fig. 2.6C). We note that while the three cell lines have similar intrinsic internalization rate constants for α3 integrin (DU145, PC3, PC3B1 rates were 1.03, 1.20, 1.19, respectively) a statistically significant difference exists in the total intracellular accumulation of the α3 integrin in DU145, PC3 and PC3B1 cells (31.27, 63.77 and 51.84, respectively).

A comparison of the actual rate constant of internalization ($k_{actual}$) of the laminin binding integrins and whether depletion of either alpha subunit affects the internalization of the other alpha subunit was tested in the three cell lines (Fig. 2.7). Silencing α3 integrin expression led to a statistically significant increase in internalization of the α6 subunit by 1.44-1.71-fold, but did not affect the $k_{actual}$ of TfR internalization. The $k_{actual}$ of α3 internalization remained unchanged on silencing α6 integrin expression.

Since cells in suspension are used here to measure the intrinsic internalization rates, it is important to test the effect of ligand binding on integrin receptor internalization. We used a biologically active peptide called HYD1 that acts as ligand mimetic and blocks α3 and α6 adhesion without altering signaling (Sroka et al., 2006) HYD1 did not affect the internalization of either α3 or α6 integrin (Fig. 2.8A). Next, we tested an antibody AIIB2 that functionally blocks β1 integrin adhesion to laminin (Werb et al., 1989). Interestingly, the functional blocking of ligand binding using AIIB2 antibody treatment reduced internalization of α6 integrin but not α3 integrin (Fig. 2.8B).
Figure 2.9. Depletion of α3 integrin increased cell migration dependent on α6β1 integrin. DU145 cells were treated with non-targeting siRNA (siCON), siRNA targeting α3 integrin (siA3), and/or α6 integrin (siA6) and treated with a β1 integrin function blocking antibody AIIB2, followed by modified Boyden chamber cell migration assay for 6 hours. Statistical significance assessed by student’s unpaired t-test, (n=3, each experiment in triplicates), **p<0.005.
Silencing α3 integrin expression increased α6β1 integrin dependent cell migration: The increased α6 internalization on depletion of α3 integrin was tested for potential functional changes in cell motility. A modified Boyden chamber assay was employed where cells were plated on the top of the insert and allowed to migrate to the other side of the membrane coated with laminin. Silencing expression of α3 integrin (siA3) resulted in a marked increase in cell migration as compared to the untreated (siCON) cells (1.8 fold, **p<0.005 Fig. 2.9, black bars). The increased cell migration induced by silencing of α3 (siA3) was dependent upon α6 integrin since dual silencing of α6 and α3 integrin (siA3 + siA6), inhibited the increased in cell migration of α3 depleted cells (***p<0.005). Both the induced (siA3) and constitutive migration (siCON) was dependent on β1 integrin as shown by AIIB2 antibody treatment (Fig. 2.9).

Internalized α6, β4 and α3 integrin were targeted to early endosomes: The endocytic fate of the internalized integrins was determined by staining cells with markers for key endocytic vesicular compartments. α6 integrin co-localized with early endosome marker, early endosome antigen 1 (EEA1) in untreated DU145 cells (Fig. 2.10A, C). The co-localization increased significantly in cells depleted of α3 integrin expression (siA3) (Fig. 2.10B-D, Pearson’s Coefficient of Colocalization (Pr) increased from 0.28 to 0.36). Approximately 10% of EEA1 positive vesicles contained α6 integrin in untreated cells. Depletion of α3 integrin led to a 40% increase in α6 positive EEA1 vesicles (Fig. 2.10D). These early endosomes had specific domains positive for either EEA1 (green) or α6 integrin (red) or EEA1/ α6 integrin overlapped regions (yellow) (Fig. 2.10C).
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Figure 2.10. Distribution of α6 integrin in endosomal vesicles and redistribution on silencing α3 integrin expression. Surface integrin α6 was labeled with J1B5 in DU145 cells and allowed to internalize for 40 minutes in the presence of primaquine (a recycling inhibitor) to achieve maximum intracellular accumulation. Cells were fixed, permeabilized, and immunostained for markers of intracellular vesicular compartments. (A) Untreated DU145 cells, (B) siA3 treated cells. Integrin α6 (A6, red), early endosome antigen 1 (EEA1) positive early endosomes (green), Lamp1 vesicles (blue) and DAPI (gray) in merged image. Images acquired by deconvolution microscopy. (C) Magnified images of boxed sections are shown for untreated and siA3 treated DU145 cells. (D) Percent EEA1 or Lamp1 vesicles containing α6 integrin and mean Pearson coefficient of correlation of α6 integrin with EEA1 (Pr(α6/EEA1)) or with Lamp1(α6/Lamp1) are reported for untreated or siA3 treated cells based on 10 different field of view in 3 independent experiments (*p<0.05 **p<0.005). Bars, 10µm.
Figure 2.11. Distribution of α6β4 integrin in early endosomes on silencing α3 integrin expression. Surface α6 integrin was labeled with J1B5 and β4 integrin with ASC3 antibody in DU145 cells and allowed to internalize for 40 minutes in the presence of primaquine (a recycling inhibitor) to achieve maximum intracellular accumulation. Cells were fixed, permeabilized, and immunostained for markers of intracellular vesicular compartments. (A) Untreated DU145 cells, (B) siA3 treated cells. Integrin α6 (A6, red), early endosome antigen 1 (EEA1) positive early endosomes (green), integrin β4 (B4, blue) and DAPI (gray) in merged image. Images acquired by confocal microscopy. (C) Magnified images of boxed sections are shown for untreated and siA3 treated DU145 cells. (D) Percent EEA1 vesicles containing α6 or β4 integrin and mean Pearson coefficient of correlation of α6 integrin with EEA1 (Pr(α6/EEA1) or β4 with Lamp1(α6/EEA1) are reported for untreated or siA3 treated cells based on 10 different field of view in 3 independent experiments (*p<0.05 **p<0.005). Bars, 10µm.
The increase in α6 integrin in EEA1 vesicles was consistent with the increased intracellular accumulation of α6 integrin observed in the internalization assays (Fig. 2.5). There was no change in α6 integrin localization to lamp1 vesicles in untreated or siA3 cells (Fig. 2.10 A, B, D), indicating that the internalized integrin was not targeted for degradation.

Since α6 integrin can partner with either β1 or β4, we tested if the localization of the β4 integrin changed with respect to α6 integrin redistribution to cell-cell locations upon depletion of α3 integrin. In untreated DU145 cells, integrin α6 and β4 localized to EEA1 vesicles (Fig. 2.11A, C). The co-localization of the integrins with EEA1 increased significantly in cells depleted of α3 integrin expression (siA3) (Fig. 2.11B-D). Pearson’s Coefficient of Colocalization (Pr) increased from 0.20 to 0.35 for α6 integrin; 0.25 to 0.46 for β4 integrin (Fig. 2.11D). Both α6 and β4 integrin increased within EEA1 vesicles (14 % to 34% for α6 integrin and 22% to 38% for β4 integrin) in response to silencing of α3 integrin expression (siA3) (Fig. 2.11D). Similar to the results in Figure 6, early endosomes were detected that had specific domains positive for either EEA1 (green), α6 integrin (red), β4 integrin (blue) or EEA1/ α6/β4 integrin overlapped regions (white) (Fig. 2.11C). A discontinuous pattern of overlapping regions, as indicated by white pixels (Fig 2.11C, siA3, circles) was observed in α3 integrin silenced cells, suggesting distinct domains within the endosome. Integrin α3 silenced cells showed a marked redistribution of α6β4 integrin to cell-cell locations (Fig. 2.11B).
Figure 2.12. Distribution of α3 integrin in endosomal vesicles on silencing α6 integrin expression. P1B5 labeled surface integrin α3 in DU145 cells was allowed to internalize for 40 minutes in the presence of primaquine (a recycling inhibitor) to achieve maximum intracellular accumulation. Cells were fixed, permeabilized, and immunostained for markers of intracellular vesicular compartments. Untreated and siA6 treated DU145 cells stained for integrin α3 (A3, red) with early endosome antigen 1 (EEA1, green) in merged image (A) or Lamp1 (green) (B) and DAPI (blue). Magnified image of the boxed region are shown to demonstrate co-localization. Images acquired by confocal microscopy. (C) Percent EEA1 or Lamp1 vesicles containing α3 integrin and mean Pearson coefficient of correlation of α3 integrin with EEA1 (Pr(α3/EEA1)) or with Lamp1(α3/Lamp1) are reported for untreated or siA6 treated cells based on 10 different field of view in 3 independent experiments. Bars, 10µm.
Although α3 internalization rates were unaffected by the silenced expression of α6 integrin (Fig. 2.6), we tested whether the localization of α3 integrin was affected under these same conditions. Internalized integrin α3 showed significant co-localization with EEA1 in both untreated and α6 integrin silenced DU145 cells (siA6) (Fig. 2.12A, C). Pearson’s Coefficient of Co-localization (Pr) remained unchanged (0.48 for untreated and 0.52 for siα6 integrin) (Fig. 2.12C). Under both conditions, approximately 50% of EEA1 vesicles were positive for α3 integrin (Fig 2.12C) and α3 integrin was not significantly associated with lamp1 vesicles (Fig. 2.12B, C).

**DISCUSSION**

Internalization of cell surface integrins is a major regulator of their cell membrane expression and function and a prerequisite for integrin-mediated cancer cell migration (Desgrosellier and Cheresh, 2010). Here the internalization kinetics of laminin binding integrins are characterized using flow cytometry and a first-order kinetics model. The α6 integrin internalization rate was 3-fold higher as compared to α3 integrin, consistent with previous reports that the integrin alpha subunits have distinct internalization rates despite sharing the common integrin β1 partner (Bretscher, 1992; Winterwood et al., 2006). For comparative purposes, an unrelated integrin (αvβ3 had a slower internalization rate than α6 integrin (2.20 min⁻¹ versus 3.25 min⁻¹, respectively) whereas a growth-promoting receptor, the transferrin receptor, internalized with a rate of 15.08 min⁻¹. The data indicate that laminin binding integrins are internalized from the cell surface with significantly different rates from each other despite sharing the same β1
Chapter II: LBI internalization is coordinated

partner and the rates are distinct from non-laminin binding integrins and a growth-related receptor.

Interestingly, the internalization rate does not necessarily reflect intracellular accumulation since the αvβ3 integrin has a slower internalization rate compared to α6 integrin (2.2 min\(^{-1}\) versus 3.25min\(^{-1}\), respectively) but more internal accumulation compared to α6 integrin (65% versus 58%, respectively). Differences in accumulation likely reflect differences in recycling rate and adapter proteins for trafficking regulation. For example, Rab11FIP1 (RCP) machinery is used by fibronectin/vitronectin binding integrins (αvβ3 and α5β1) (Caswell et al., 2008). Following chapters explore which Rab11 adapter proteins regulate α6 versus α3 integrin trafficking and recycling.

The internalization kinetics measured for α6 subunit was a measure of the internalization of α6β1 heterodimer as the β4 integrin subunit was found to be completely internalized at 4°C. This reveals distinct internalization characteristics of the α6β1 and α6β4 integrins and indicates that the internalization of β4 integrin can occur at 4°C. Previous reports have characterized endocytosis at such lower temperatures to be a feature of caveolin mediated endocytosis (Ref). Future studies will be important to delineate the mechanism of the difference in temperature sensitive internalization of α6β4 and α6β1 heterodimers.

Integrins α3 and α6 work coordinately in normal cellular processes such as wound healing and epithelial development. In metastatic prostate cancer, distinct subtypes exist with differential expression of α3 and α6 integrins. High grade prostate carcinoma
shows selective loss of α3 integrin while preserving expression of α6 integrin (Schmelz et al., 2002). In model systems, α3 integrin expression can either prevent tumor progression or exacerbate it. In the current study, the presence of α3 integrin had a significant effect on internalization and cellular localization of α6 integrin. This could explain, in part, the previously discrepant reports since our results show that a coordination exists between α3 and α6 integrin. Here, depletion of α3 integrin led up to a 1.71-fold increase in internalization of α6 integrin. The increased internalization of α6 integrin by silencing α3 integrin was accompanied by an increased migration on laminin. The results were consistent with previous studies utilizing an α3 integrin null mouse, where wound healing is faster owing to rapid and persistent keratinocyte migration dependent on α6 integrin (Margadant et al., 2009). These results suggest a coordination of the laminin receptors may exist to influence cancer migration. Future studies will be important to determine which region of the α6 integrin is crucial for internalization in prostate cancer cells when α3 integrin is silenced. As specific regulators of α6 integrin internalization are found, it will be of interest to identify agonists and antagonists, as they may prove useful for blocking tumor metastasis via laminin lined structures. Silencing of α6 integrin expression did not alter the α3 integrin internalization rate, although an increased intracellular accumulation was observed. The data suggest a “unidirectional” regulation of internalization of laminin binding integrins. This is distinct from a previously reported reciprocal relationship between the recycling of fibronectin receptors α5β1 and αvβ3, where inhibition of either integrin promoted the recycling of the non-targeted integrin (Caswell et al., 2008).
The internalized α6 integrin was targeted to the early endosomes, which was markedly increased on depletion or functional blocking of α3 integrin. An interesting observation was that the endosomes had specific domains of the cargo integrin, as suggested by discontinuous overlap with EEA1 distribution (Figs 2.10C, 2.11C and 2.12A).

Integrin β4 was also found in the early endosomes containing α6 integrin. Importantly, α3 integrin depletion led to a redistribution of α6β4 to the plasma membrane at cell-cell contacts. This adds new interesting information that α6β4 integrin heterodimer localization is reminiscent of suprabasal distribution observed in the epidermis and results in enhanced tumorigenesis (Owens et al., 2003). This may possibly be due to the recycling of the internalized integrins from early endosomes to the cell-cell lateral membrane. In human keratinocytes, α6 integrin has been observed in vesicles close to the lateral membrane, as well as in intercellular spaces by electron microscopy (Poumay et al., 1993). Trafficking of α6 integrin to cell-cell lateral membrane has also been reported during epidermal development in zebrafish (Sonawane et al., 2009). Integrins at cell-cell membrane have been implicated in cell-cell adhesion (Chattopadhyay et al., 2003; Emsley, 2003) through the extracellular matrix present between cells (Behrendtsen et al., 1995). Future work will determine if α6β4 integrin may be important for cell-cell interaction and promote collective cell migration, a characteristic of human prostate cancer invasion and metastasis (Nagle and Cress, 2011).
III. Rab11FIP5 regulate Intracellular Trafficking of α6 integrin in aggressive prostate cancer

INTRODUCTION

Laminin binding integrin α6 is mainly restricted to the basolateral cell membrane in normal adult polarized tissue. (Carter et al., 1990; Sonnenberg et al., 1991). However, dynamic changes in its membrane expression and localization is observed during various normal cell processes associated with active cellular migration, like epithelia formation during development (Shimizu et al., 2012), rostral migratory stream migration during brain development (Belvindrah et al., 2007) or wound healing (Geuijen and Sonnenberg, 2002; Larjava et al., 1993; Margadant et al., 2009; Underwood et al., 2009). Similarly, in invasive cancer this phenomenon is amplified (Owens et al., 2003).

Intracellular trafficking of integrins is an important determinant of membrane expression and localization of integrins, and hence their function. Membrane integrins are continuously internalized, sorted through intracellular vesicular compartments and returned back to the cell surface by recycling (Bridgewater et al., 2012). The rate of recycling determines the flux of integrins on the membrane and is key to cell migration (Caswell and Norman, 2008). Once internalized, membrane receptors travel through a cascade of intracellular vesicular compartments defined by a family of small GTPases called Rab GTPases (Barr, 2013; Hutagalung and Novick, 2011; Mizuno-Yamasaki et al., 2012; Stenmark, 2012). Rab11 is one such major trafficking regulator that marks the perinuclear recycling compartment (PNRC; also called apical recycling endosomes, ARE) and is a central hub of cellular trafficking to transport a variety of proteins from intracellular compartments to the plasma membrane (Goldenring, 2015). The role of Rab11 in trafficking of integrins is well known (Caswell et al., 2009); its deregulation
being a key feature of cancer (Mosesson et al., 2008; Yoon et al., 2005). Integrin $\alpha_6\beta_4$ is known to be recycled by Rab11 in both normal and cancer cells. In breast cancer, Rab11 mediated $\alpha_6\beta_4$ recycling is associated with hypoxia induced cancer invasion (Yoon et al., 2005). Rab11 dependent $\alpha_6$ integrin recycling is important for rapid motility of cranial neural crest cell on laminin (Strachan and Condic, 2004).

Despite sharing the same beta partners or even extracellular ligands, integrins follow distinct temporal and spatial trafficking routes, selectivity determined by the alpha subunit (Bretscher, 1992; Caswell et al., 2009; De Franceschi et al., 2016; Mai et al., 2011). Most integrins, including the laminin binding integrins, share the central Rab11 recycling machinery and additional regulators are likely involved in providing selectivity to different integrins. A group of Rab11 effector proteins called Rab11 family interacting proteins ($\text{Rab11FIP}$s) are important candidates to provide such specificity as they regulate various temporally and spatially distinct steps of Rab11 dependent recycling (Baetz and Goldenring, 2013). Rab11FIPs are known to recycle a selective, though not necessarily exclusive, repertoire of cargo including integrins. (Horgan and McCaffrey, 2009). For example, $\alpha_5\beta_1$ integrin is trafficked by Rab11FIP1 but no other class I FIPs (FIP2 or FIP5) (Caswell et al., 2008), suggesting Rab11FIPs can provide specificity to recycling of specific integrin based on different alpha subunits. Rab11FIPs localize to discrete subdomains in Rab11 recycling endosomal tubules (Baetz and Goldenring, 2013) through a conserved C-terminal Rab11 binding domain (RBD) (Prekeris et al., 2001) (Prekeris et al., 2001). Class I Rab11FIPs (Rab11FIP1, Rab11FIP2, Rab11FIP5) contain the C2 domain that binds to membrane phosphoinositides required for docking
to membrane (Lindsay and McCaffrey, 2004). Class II Rab11FIPs (Rab11FIP3, Rab11FIP4) instead contain a calcium binding EF hand motif and are crucial in intracellular transport during cell division, especially formation of the cleavage furrow (Horgan and McCaffrey, 2009; Horgan et al., 2004). Rab11FIPs also provide directionality in vesicular movement and can be involved in both anterograde trafficking from early endosomes to Rab11 compartment as well as from Rab11 compartment to the plasma membrane (Grant and Donaldson, 2009; Schafer et al., 2016; Schonteich et al., 2008).

Here Rab11FIP5 is identified as a recycling regulator specific to α6 integrin that does not affect other integrins trafficked by Rab11 including the other laminin binding α3 integrin. In effect, offering a solution to the problem of how α3 and α6 integrins have different trafficking kinetics despite having the same β1 integrin subunit partner. Moreover, according to available literature Rab11FIP5 is not involved in trafficking of αv or α5 integrins (Caswell et al., 2008), thus making Rab11FIP5 specific to α6 integrin recycling. Depletion of Rab11FIP5 significantly reduced recycling of α6 integrin, a known pro-migratory integrin (Cruz-Monserrate and O’Connor, 2008; Ports et al., 2009; Sroka et al., 2010) and inhibited cell migration, adding a new way to interrupt α6 integrin function. In addition, Rab11FIP5 mRNA expression significantly correlated with intracellular α6 integrin staining in mouse xenografts of advanced human prostate cancer.
RESULTS

Cell membrane expression of α6β4 integrin requires Rab11FIP5: Cell membrane levels of integrin subunits α6, β4 and α3, non-integrin cell adhesion receptors CD44 and E-cadherin were measured using surface labelling of the receptors and flow cytometry analysis. The flow cytometry profile for α6 integrin showed that the mean peak fluorescence (MPF) of total cell population was markedly reduced in Rab11FIP5 silenced cells (siFIP5) as compared to untreated and non-targeting siRNA treated cell population (siCon) (Fig. 3.1A). In addition, the distribution of siFIP5 cells were overlapping with that of α6 integrin depleted cells (siα6) (Fig. 3.1A). Similar pattern was observed for β4 subunit, with lower surface levels in the total cell population of siFIP5 cells as compared to untreated or siCon cells. The membrane expression of β4 integrin was dramatically reduced on depletion of its heterodimeric partner α6 subunit (siα6 cells), as expected. The cell surface expression of the other laminin binding integrin, α3 integrin, the non- laminin binding cell surface receptor CD44 and cell-cell adhesion receptor E-cadherin remained unchanged in siFIP5 or siα6 cells (Fig. 3.1B). The MPF of surface integrin label was significantly reduced by ~50% for α6 integrin (**p<0.01, n=5) and ~70% for β4 integrin (**p<0.01, n=5) (Fig. 3.1B). The total protein expression as tested by immunoblotting for α6 and α3 integrins and Rab11 in lysates was not altered upon silencing Rab11FIP5 (Fig. 3.1C).
Figure 3.1. Membrane expression of α6β4 integrin is regulated by Rab11FIP5. DU145 cells were transfected with non-targeting siRNA (siCon), and siRNA against Rab11FIP5 (siFIP5) or α6 integrin (siα6) (A) Flow histograms of cell surface expression of laminin binding integrin subunits α6, β4 or α3 and unrelated adhesion receptors CD44 and E-cadherin using immunolabelling of receptors in fixed, non-permeabilized untreated (black), siCon (red), siFIP5 (blue) and siα6 (green) cells. (B) Relative mean peak fluorescence values of cell membrane expression of the receptors in untreated, siCon, siFIP5 and siα6 cells (n=5, **p<0.01). (C) Total cell lysate of untreated siCon cells and cells treated with two different siRNA against Rab11FIP5 (siFIP5 #1, #2) immunoblotted for Rab11FIP5, α6, α3, Rab11a and α-tubulin.
Figure 3.2. C2 domain of Rab11FIP5 is required for the membrane expression of α6 integrin. Rab11FIP5 mutants tagged with GFP were stably transfected into PC3N cell line- (i) GFP tagged full length Rab11FIP5 (ii) GFP tagged truncated Rab11FIP5 protein lacking C2 domain (∆C2FIP5-GFP) or (iii) a non-functional, Rab11 binding deficient, truncated Rab11FIP5 mutant tagged with GFP (I630E-∆C2FIP5-GFP) used as a vector control. (A) Schematic representation of the FIP5-GFP mutants used. (B) Total cell lysate of mutant FIP5-GFP transfected stable cell lines immunoblotted for Rab11FIP5 protein show expression of endogeneous, and overexpressed GFP tagged full length or truncated Rab11FIP5 protein. Total expression of α6 and α3 integrin subunits is shown. (C) Expression of FIP5-GFP mutant transfected PC3N cell lines imaged to show distinct cellular distribution of the FIP5-GFP mutant proteins (green)
The C2 domain of Rab11FIP5 is required for membrane expression of α6 integrin: Next, we assessed the functional domain of Rab11FIP5 that would be important for α6β1 integrin membrane expression using PC3N cells, a β4 null cell line. A comparison of cell surface expression of β4 integrin in PC3N cells with β4 integrin positive DU145 cells is shown in supplementary figure S.1. For this, we tested Rab11FIP5 mutants with defects in two key functionally conserved domains— the Rab11 binding domain (RBD) and the N-terminal, membrane phosphoinositide binding C2 domain. A schematic representation of the Rab11FIP5 mutants used is shown (Fig. 3.2A). The PC3N cells were stably transfected for either of the three mutant Rab11FIP5 proteins containing GFP: (i) a full length Rab11FIP5 (FIP5-GFP), (ii) a dominant negative truncated Rab11FIP5 protein lacking the C2 domain (∆C2 FIP5-GFP) and (iii) a Rab11 binding deficient ∆C2-FIP5 (I630E-∆C2 FIP5-GFP) yielding a non-functional Rab11FIP5 protein, each previously characterized (Junutula et al., 2004; Prekeris et al., 2000; Willenborg et al., 2011). The expression of mutant FIP5-GFP protein in respective mutant cell line is shown by immunoblotting (Fig. 3.2B). The endogenous Rab11FIP5 protein expression or the total expression of either α6 or α3 integrin was unaltered in each of the mutant cell lines. The cellular distribution of the FIP5-GFP mutants was imaged in fixed cells using deconvolution microscopy. Two different planes, one closer to the basal surface (Z=1) and other towards apical surface are shown (Fig. 3.2C). The full length FIP-GFP localized to the perinuclear region (white arrows) as well as cell edges (white triangles). The ∆C2
Figure 3.3. C2 domain of Rab11FIP5 is required for the membrane expression of α6 integrin. Rab11FIP5 mutants tagged with GFP were into PC3N cell line stably transfected with FIP5-GFP mutants (i) (A) Flow histograms of cell surface expression of α6 and α3 subunits in untransfected, and mutant FIP5-GFP transfected PC3N cells: GFP tagged full length Rab11FIP5 (FIP5-GFP, red), C2 domain lacking Rab11FIP5 (ΔC2FIP5-GFP, blue) and non-functional Rab11FIP5 containing vector (I630E-ΔC2FIP5-GFP, purple). (B) Relative mean peak fluorescence values of cell membrane expression of the integrin subunits in untreated and FIP5-GFP mutant transfected cells (n=4, *p<0.05).
FIP5-GFP mutant protein was localized tightly around the nucleus (white arrows) and was undetectable at the cell periphery. The non-functional Rab11FIP5, I630E-FIP5-GFP was distributed throughout the cell.

The FIP5-GFP mutant PC3N cells were tested for cell membrane expression of α6 and α3 integrins. Flow cytometry profiles show reduced surface α6 integrin expression in the ΔC2 FIP5-GFP mutant cell population (blue, Fig. 3.3A). The MPF of membrane α6 integrin was significantly reduced by ~25% in ΔC2-FIP5GFP as compared to untransfected, FIP5-GFP transfected or the non-functional I630E-ΔC2-FIP5GFP transfected PC3N cells (Fig. 3.3B). The flow cytometry profiles of membrane α3 integrin completely overlapped in each of the mutant cell lines (Fig. 3.3A) and MPF remained unchanged (Fig. 3.3B). These results demonstrate that the C2 domain of Rab11FIP5 selectively reduces membrane expression of α6 and not α3 integrin.

**Depletion of Rab11FIP5 increases intracellular accumulation of α6 integrin:** Since Rab11FIP5 affects the membrane expression of α6 integrin without affecting total cellular integrin levels, we tested if the internalization rate or total amount of integrin internalized was affected. Time courses of internalization of α6 integrin α3 integrin and transferrin receptor (TfR, CD71) in DU145 cells are shown (Fig. 3.4A). At each timepoint antibody labeled internalized surface receptors were plotted as a percent of total surface receptor and a first order kinetic model was fitted to obtain the internalization curve. The internalization curve for α6 integrin, α3 integrin and TfR were compared between untreated cells as control and siFIP5 cells (Fig. 3.4A). The percent α6 integrin
Figure 3.4. Depletion of Rab11-FIP5 expression increases intracellular accumulation of α6 integrin. (A) Internalization kinetic curve for α6, α3 or TfR in untreated DU145 and siFIP5 cells. Percent surface label internalized is plotted for different time points at 37°C and fitted with a first order kinetic curve ($R^2$>0.989). (B) Maximum intracellular accumulation and internalization rate constants (observed, $k_{\text{obs}}$ and actual, $k_{\text{actual}}$) calculated as per first order rate kinetics. Results represent 5 independent experiments. Statistical significance calculated for changes in label internalized at each timepoint and intracellular accumulation as per student’s t test, unpaired, *p<0.05, n=5.
internalized was not different between untreated and siFIP5 cells for the first 5 timepoints tested. But, there was a significant increase in total α6 integrin internalized in siFIP5 cells at 75 minutes, when the internalization reaches the maximum and a steady state (*p<0.05, n=5). There were no differences in the amount of internalized α3 integrin or TfR at any timepoint between untreated and Rab11FIP5 depleted cells (Fig. 3.4A). In one cycle of internalization in untreated cells, 46% of the total labeled membrane α6 integrin was internalized and accumulated intracellularly while in siFIP5 cells the amount of internalization was significantly greater reaching up to 58% (*p<0.05, n=5, Fig. 3.4B). The internalization rate was not altered in untreated and siFIP5 cells for either α6 (k_{actual} of 1.48 min^{-1}, 1.45 min^{-1} respectively) or α3 integrin (0.69 min^{-1}, 0.87 min^{-1}). The TfR tested as an unrelated transmembrane protein was unaltered for either internalization rate (10.37 min^{-1} for untreated, 9.66 min^{-1} for siFIP5) or intracellular accumulation (57.62% for untreated, 60.38% for siFIP5) in response to depletion of Rab11FIP5.

*Internalized α6 integrin accumulated in early endosomes and Rab11+ perinuclear recycling compartment (PNRC):* Next, we investigated which intracellular vesicular compartments contained the internalized α6 integrin in response to depletion of Rab11-FIP5 protein. To increase intracellular accumulation, live untreated control or siFIP5 DU145 cells were incubated with the anti-α6 integrin antibody, J1B5, for 40 mins. Cells were then fixed, permeabilized and stained with antibodies reactive to early endosome antigen 1 (EEA1) and Rab11a to visualize α6 integrin in early endosomes or in the perinuclear recycling compartment (PNRC), respectively.
Figure 3.5. Distribution of α6 integrin in early endosomes. Surface α6 integrin was labeled with J1B5 in DU145 cells and allowed to internalize for 40 minutes, fixed, permeabilized, and immunostained for the early endosome marker EEA1. (A) Untreated DU145 cells and (B) siFIP5 treated cells stained for α6 integrin (red), EEA1 (green) and DAPI (blue) in merged image. Images acquired by deconvolution microscopy and single Z-plane is shown. Bars, 20µm. (C) Magnified images of boxed sections for untreated and siFIP5 DU145 cells. White arrows indicate colocalization of α6 integrin in EEA1 vesicles. (D) Percent α6 integrin in EEA1 vesicles and mean Pearson coefficients of correlation of α6 integrin with EEA1 (Pr(α6/EEA1)) for untreated or siFIP5 cells are shown based on 10 different field of views in 3 independent experiments (*p<0.05).
In untreated control cells, α6 integrin was observed localized both at the cell membrane, primarily in cell-cell adhesion sites, and within the cytoplasm (Fig. 3.5A, red). The integrin localized to EEA1 positive endosomes in untreated cells (Fig. 3.5A, green). The siFIP5 cells showed reduced localization of α6 integrin at the cell-cell membrane locations and increased colocalization with EEA1 positive vesicles (Fig. 3.5B). The magnified cross sections show the colocalization of α6 integrin and EEA1 in untreated and siFIP5 cells marked by white arrows (Fig. 3.5C). In untreated cells, 16% of the integrin localized to early endosomes which increased up to 29% on Rab11FIP5 depletion. The Pearson’s coefficient for colocalization also increased significantly from 0.41 for untreated to 0.60 for siFIP5 cells (Fig.3.5D).

Since Rab11FIPs can be involved in receptor trafficking from early endosomes to Rab11a positive PNRC, we tested if absence of Rab11FIP5 interferes with this step in the trafficking of α6 integrin. Live cells that had up taken J1B5 antibody bound α6 integrin for 40 minutes were fixed, permeabilized and stained for Rab11a. In untreated cells, α6 integrin localized both at the cell membrane, primarily at cell-cell locations, and within the cytoplasm (Fig. 3.6A, red). Rab11a was distributed in vesicles close to the nucleus (Fig. 3.6A, green). The overlay of both images from untreated cells revealed that α6 integrin and Rab11a primarily colocalized at the cell-cell membrane locations in untreated cells. Depletion of Rab11FIP5 increased the intracellular localization of α6 integrin (Fig. 3.6B, red) and colocalization with the Rab11a marked PNRC (Fig. 3.6B, green). The magnified cross sections showed that the α6 integrin and Rab11a primarily colocalized at the cell-cell membrane locations in untreated cells (white triangles, Fig
Figure 3.6. Distribution of α6 integrin in Rab11a vesicles. Surface integrin α6 was labeled with J1B5 in DU145 cells and allowed to internalize for 40 minutes at RT, fixed, permeabilized, and immunostained with Rab11a. Cellular distribution of α6 integrin and Rab11a vesicles in (A) Untreated DU145 cells, (B) siFIP5 treated cells. Integrin α6 (red), Rab11a positive recycling vesicles (green) and DAPI (blue) in merged image. Images acquired by deconvolution microscopy and single Z-plane is shown. Bars, 20µm. (C) Magnified images of boxed sections. α6 integrin and Rab11a colocalize at cell-cell membrane locations (white triangles), mainly observed in untreated cells. Intracellular colocalization of α6 integrin and Rab11a (white arrows) is prominent in siFIP5 cells. (D) Percent α6 integrin in Rab11a vesicles Mean Pearson coefficients of correlation of α6 integrin with EEA1 (Pr(α6/EEA1)) or with Rab11a (Pr(α6/Rab11)) is reported for untreated or siFIP5 cells based on 10 different field of views in 3 independent experiments. (*p<0.05 **p<0.01).
Chapter III: Rab11-FIP5 regulates α6 integrin recycling

3.7C) whereas on depletion of siFIP5 the α6 integrin localized with intracellular Rab11a (white arrows, Fig. 3.6C). While 34% of the integrin colocalized with Rab11a vesicles in untreated cells, depletion of Rab11FIP5 led to a significant increase to up to 56% (Fig. 3.6D). This was accompanied with a significant increase in Pearson’s coefficient for colocalization from 0.46 for untreated to 0.62 for siFIP5 cells. Taken together, the data showed that depletion of Rab11FIP5 protein changes the distribution of α6 integrin from cell-cell membrane locations to intracellular early endosomes and Rab11a vesicles. The data showed that Rab11FIP5 loss did not affect trafficking of α6 integrin from early endosomes to Rab11a compartment, but suggest a role in downstream event such as recycling.

α6 integrin colocalizes with Rab11FIP5: Since Rab11FIP5 localizes to Rab11+ PNRC where the integrin accumulates on Rab11FIP5 depletion, we investigated the relative localization of Rab11FIP5 with α6 integrin. In untreated DU145 cells the Rab11FIP5 colocalized with α6 integrin primarily at cell-cell membrane locations (Fig. 3.7A), similar to Rab11a and α6 integrin colocalization seen earlier at these locations (Fig. 3.6A).

We also tested PC3N cell lines expressing FIP5-GFP mutants characterized earlier in figure 3.2. Since the Rab11FIP5 GFP mutants localized both at regions close to cell substratum attachment and towards the apical surface as seen in figure 3.2, both the planes were tested for localization of α6 integrin. The α6 integrin localization with FIP5-GFP was seen mainly towards the apical surface and not the region of cell attachment to the substratum.
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Figure 3.7. Rab11FIP5 colocalizes with α6 integrin. (A) Live DU145 cells or (B) FIP5-GFP mutant expressing PC3N cells were treated with antibody specific to α6 integrin for 40 minutes at RT, fixed permeabilized and immunostained with Rab11a. Images acquired by deconvolution microscopy. Single Z-plane is shown for DU145 and 2 Z-planes are shown for PC3NFIP5GFP mutants- Z1 (basal surface, closer to the cell substratum attachment region) and Z2 (towards apical side). Arrows show areas of colocalization in ΔC2-FIP5-GFP mutant expressing PC3N. Percent of α6 integrin in Rab11FIP5 vesicles is reported for FIP5-GFP and ΔC2-FIP5-GFP mutant, on 10 different field of views in 3 independent experiments. (*p<0.05).
It is interesting to note that PC3N cell lines grow as single cells and are devoid of epithelial like cell-cell membranes contacts and the α6 integrin that colocalized with Rab11FIP5 was not at the cell membrane but primarily adjacent to the nucleus. Interestingly, in C2 deficient FIP5-GFP expressing mutant PC3N cells (ΔC2-FIP5GFP) the α6 integrin accumulated with the intracellularly accumulated “punctae like” vesicular structures of ΔC2-FIP5GFP protein (white arrows, Fig. 3.7B). About 35% of α6 integrin colocalized with FIP5-GFP which increased up to 73% of the integrin colocalizing with ΔC2-FIP5GFP protein (Fig. 3.7B).

_Depletion of Rab11FIP5 reduces recycling of α6 integrin:_ The reduced cell surface levels and increased intracellular accumulation of α6 integrin in Rab11+ PNRC compartment on Rab11FIP5 depletion and with C2 domain deficient Rab11FIP5 suggested a possible defect in integrin recycling to the cell membrane. We measured the recycling kinetics of α3 and α6 integrins and the TfR using a flow cytometry based approach in DU145 cells treated with two different siRNA against Rab11FIP5 (siFIP5(#1) or siFIP5(#2)) and compared to the untreated cells (Fig. 3.8A). The recycling assay was performed at the lower temperature of 25°C to decrease the rate of recycling and improve the resolution of the rate kinetic curve. The rate kinetic curve for α6 integrin recycling at 25°C is compared to recycling at 37°C in Supplementary Figure S.2. The amount of recycled label was shown as a percent of the total amount of internalized receptor for different timepoints.
Figure 3.8. Depletion of Rab11-FIP5 expression reduces recycling of α6 integrin. DU145 cells were transfected with two different siRNAs against Rab11FIP5: siFIP5(#1) or siFIP5(#2) (A) Recycling kinetic curve for α6, α3 or TfR in untreated and siFIP5 DU145 cells. Percent label recycled to the cell surface at 25°C (calculated as percent mean peak fluorescence of recycled label at a given timepoint vs total label internalized) is plotted for different time intervals. First order kinetic curve is fitted using Kaleidagraph ($R^2$>0.98). (B) Maximum label recycled (%) and recycling rate constants (observed, $k_{obs}$, and actual, $k_{actual}$) calculated as per first order rate kinetics. Results represent 5 independent experiments. Statistical significance calculated for changes in label internalized at each timepoint and intracellular accumulation as per student’s t test, unpaired, *p<0.05 **p<0.01, n=5.
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The recycling curve was obtained by fitting a first order kinetic model. The recycling rate was decreased by 2.5-fold when the recycling assay was performed at 25°C instead of 37°C (2.06 min\(^{-1}\) and 4.91 min\(^{-1}\) respectively), and a better resolution of the kinetic curve was achieved. The total amount of labeled α6 integrin recycled at 25°C was comparable to 37°C.

Using the optimized recycling assay at 25°C we found that the amount of α6 integrin that was recycled from cytoplasm to the cell surface was significantly reduced in the Rab11FIP5 depleted cells compared to untreated control cells at each timepoint tested. No differences were observed for α3 integrin or the TfR (Fig 3.8A). Depletion of Rab11FIP5 using two different siRNAs led to a significant ~2-fold reduction in the rate of recycling of α6 integrin from 2.06 min\(^{-1}\) to a \(k_{\text{actual}}\) of 1.22 min\(^{-1}\) for siFIP5 (#1) and 1.08 min\(^{-1}\) for siFIP5 (#2) (\(p<0.001, n=7\)) (Fig. 3.8B). The total amount of receptor recycled in one cycle was also significantly reduced from 36% in untreated to 29% in siFIP5(#1) and 30% in siFIP5(#2) cells (**\(p<0.01, n=7\)). This was consistent with the reduced membrane expression of α6 integrin in siFIP5 cells, as seen in figure 3.1. Only 17% of the internalized α3 integrin is recycled in DU145 cells at a rate of 1.33 min\(^{-1}\) which remains unaltered in siFIP5 cells (1.21 min\(^{-1}\) for siFIP5 (#1) and 1.38 min\(^{-1}\) for siFIP5 (#2); Fig. 3.8B).
Figure 3.9. α6 integrin recycled to cell-cell lateral membrane locations. Surface integrin α6 was labeled with J1B5 rat monoclonal antibody in adherent DU145 cell monolayer and allowed to internalize for 40 minutes at RT. Uninternalized label remaining at the cell membrane is blocked using Alexa 568 conjugated anti-rat secondary antibody. J1B5 antibody bound integrin protected inside the cells was allowed to recycle back and subsequently reacted with Alexa 488 conjugated anti-rat secondary antibody. (A) Total membrane and internalized intracellular α6 integrin in permeabilized cells shown as control. (B) Uninternalized α6 integrin (red) and recycled α6 integrin (green) at 0min, 10min and 40 minutes of recycling and 40min recycling with primaquine (PQ, recycling inhibitor) are shown in merged images with DAPI (blue) stained nucleus (top panel). Middle panel shows only the recycled α6 integrin label in grey. Bottom panel is magnified images of boxed sections marked for recycled integrin localized at cell-cell membrane locations (white arrows), uninternalized integrin at cell-cell locations (closed white triangles) and lamellipodia at cell front (open white triangles). Images acquired by deconvolution microscopy and single Z-plane is shown. Bars, 20µm (C) Bar graph showing average pixel intensity of recycled label plotted for different timepoints of recycling at RT (**p<0.01).
The rate of recycling of TfR is at least 5-fold faster than the integrins ($k_{\text{actual}}$ of 11.12 min$^{-1}$) and remained the same in either siFIP5(#1) ($k_{\text{actual}}$ of 10.20 min$^{-1}$) or siFIP5(#2) ($k_{\text{actual}}$ of 10.29 min$^{-1}$) cells. The total amount of TfR recycled also remained unchanged (21% untreated, 20% siFIP5 (#1) and 21% siFIP5 (#2); Fig. 3.8B). These results confirm the key and specific role of Rab11FIP5 for recycling of α6 integrin without affecting the other laminin binding α3 integrin or the TfR.

α6 integrin is recycled to the cell-cell membrane locations: The recycling assay was performed in adherent cell monolayer of DU145 cells and deconvolution microscopy used to determine the locations where recycled α6 integrin is targeted. In short, surface α6 integrin was immunolabeled with J1B5 followed by internalization for 75 minutes for maximum intracellular accumulation. Total membrane and internalized integrin labeled is shown (Fig. 3.9A, red). The uninternalized membrane integrin bound to J1B5 is labeled with a fluorescent a secondary antibody (Fig.3.9A, red) and appears in both cell-cell locations (closed white triangles) and lamellopodia (open white triangles). Intracellular J1B5 labeled α6 integrin was then allowed to recycle for different timepoints and the cell surface associated recycled integrin was stained with secondary antibody conjugated with a different fluorophore. Interestingly, within 10 mins the recycled α6 integrin (Fig. 3.9B, green) was detected at the cell-cell membrane locations (indicated by white arrows) and not in the lamellopodia. There was a significant increase in recycled label by 40 mins, primarily localizing to the cell-cell membrane locations and not lamellopodia. The recycled label disappeared on treatment with primaquine, a recycling inhibitor. The amount of label recycled was defined by average pixel intensity of the
recycled label and plotted as a bar graph for different timepoints (Fig. 3.9C). Levels of recycled α6 integrin increased significantly from 0 min (104 X10^3) to 10 mins (368 X10^3), 20 mins (689 X10^3) and 40 mins (774 X10^3) (**p<0.01, n=3).

Depletion of Rab11-FIP5 reduced cell-cell interactions in 2D and 3D cultures. DU145 cells grow as a cell monolayer with compact cell clusters and significant cell-cell adhesion when grown on 2D adherent surfaces. We found a significant change in the phenotype of Rab11FIP5 depleted cells, demonstrating a reduced cell-cell adhesion and a switch to a more “neuronal” type phenotype with long extending projections of cell membranes (Fig. S.3). About 20% of cells lose cell attachment to the adherent surface and become suspended in the growth media. The cells that lost adhesion when replated, adhered to the substratum again. This change in cellular phenotype of siFIP5 was found to be reversible after 14 days of culture when transiently silenced Rab11FIP5 protein is reexpressed.

α6 integrin has been implicated in cell-cell adhesion. Since silencing Rab11FIP5 resulted in loss of cell-cell contact and reduced α6 integrin at the cell surface, we hypothesized the collective cohesive structure formation might be affected.
Figure 3.10. siFIP5 affects cell-cell cohesion in 3D culture in Matrigel. DU145 cells grown on Matrigel form collective cohesive structures. Untreated control DU145 and siFIP5 cells were plated on Matrigel for 24 hours, fixed and immunostained for α6 integrin (red), Rab11a (green) and DAPI (blue). Note the remarkable decrease in the formation of the cohesive structures in siFIP5 cells. Images acquired by deconvolution microscopy and single Z-plane is shown. Bars, 100µm.
To test this, we employed a 3D culture model of DU145 prostate cancer cells. DU145 cells form cohesive networks when a 3D environment rich in basement membrane components is provided. DU145 cells plated on laminin rich Matrigel formed cohesive structures with substantial branching within 24 hours. These 3D networks of cancer cells were immunostained for α6 integrin and Rab11a (Fig. 3.10). The integrin was localized to areas of contact with the Matrigel as well as at areas of cell-cell contacts. Rab11a was also localized to locations of cell-cell contacts, in a pattern similar to that observed in DU145 cells grown in 2D adherent cultures. The formation of cohesive networks was significantly inhibited on depletion of Rab11FIP5 expression (Fig. 3.10).

**Depletion of Rab11FIP5 reduces cell migration:** Recycling of laminin binding integrins is a key requirement for cell migration on laminin (Strachan and Condic, 2004). α6 integrin is pro-migratory on laminin and blocking its recycling has been reported to reduce breast cancer cell migration (Yoon et al., 2005). Since Rab11FIP5 depleted cells had significant reduction of α6 integrin recycling, we hypothesized this would manifest in a cell phenotype of compromised motility. Migration of Rab11FIP5 depleted cells was tested using a modified Boyden chamber assay, with laminin coated underside and 10% FBS supplemented media as chemoattractant. Two different siRNAs against Rab11FIP5 had a similar effect on the migration of DU145 cells which was reduced by 62% in siFIP5(#1) and 48% in siFIP5(#2) treated cells as compared to the untreated cells (Fig. 3.11). The migration of these cells was confirmed to be integrin dependent, as blocking of β1 integrin function by the AIIB2 antibody significantly blocked cell migration by 81%.
Figure 3.11. Rab11FIP5 depletion reduces cancer cell migration. Untreated, AIB2 treated DU145 and siFIP5 cells were allowed to migrate in a modified Boyden chamber with 8μm pores in response to laminin enriched HaCaT conditioned media for 6 hours. (A) Percent untreated or siFIP5 cells migrated were counted in 50 field of view per sample (n=3, each experiment in triplicates) Statistical significance assessed by student’s unpaired t-test (**p<0.01, ***p<0.001).
Figure 3.12. Intracellular expression of α6 integrin prominent in advanced prostate cancer and correlated with Rab11FIP5. (A) Human prostate cancer reacted with antibody specific for α6 integrin (AA6NT). Both membrane and intracellular localization of α6 integrin was observed in perineural invasion, the characteristic feature of invasive prostate cancer (Ca). Nerve (N) and blood vessels (V) stained positive for α6 integrin as expected. (B) Established patient-derived xenografts (PDXs) obtained from 185 cases of advanced prostate cancer and metastases (121 bone metastases and 64 soft tissue metastases) were investigated for localization of α6 integrin by immunostaining with AA6NT. Intracellular versus the membrane staining intensity were scored for absence or presence of faint or intense staining of α6 integrin. (C) Genome wide gene expression analysis of subcutaneous PDX tissues were evaluated for genes correlated with cytoplasmic expression of integrin α6. Selected intracellular trafficking regulators with significant correlation to cytoplasmic α6 integrin are reported. Rab11FIP5 was identified as the top candidate.
**Intracellular expression of α6 integrin was prominent in prostate cancer and co-related with Rab11FIP5 mRNA expression:** Human prostate cancer tissue were stained by immunohistochemistry with the AA6NT antibody specific for α6 integrin to determine its cellular localization. Both membrane and intracellular localizations of α6 integrin were observed in prostate cancer cells that had invaded into the perineural space (Fig. 3.12A), characteristic of aggressive prostate cancer. In addition, nerve and endothelial cells stained positive for α6 integrin, as expected (Hogervorst et al., 1993). A tissue microarray of 185 metastatic sites (121 bone metastases and 64 soft tissue metastases including 35 lymph nodes, 19 livers and 10 other soft tissue sites) from 44 patients was stained for α6 integrin and revealed primarily cytoplasmic expression of α6 integrin in metastatic cancer cells. 76% of bone metastases cases showed positive cytoplasmic expression of the integrin and 26% revealed intense cytoplasmic staining. Similarly, 82% of soft tissue metastases were positive for cytoplasmic α6 integrin with intense staining in 38% of cases (Fig. 3.12B). The mRNA expression profile of patient-derived mouse xenografts (PDX) from the LuCaP series established from patients with castration-resistant metastatic prostate cancer (Nguyen et al., 2017) was evaluated for expression of genes encoding intracellular trafficking proteins. A LuCaP PDX tissue microarray was stained for α6 integrin and the association of genes encoding trafficking proteins with cytoplasmic α6 integrin staining was tested. Significantly correlated genes are reported (Fig. 3.12C). Interestingly, Rab11FIP5 was identified as a top hit with a positive correlation with increased cytoplasmic α6 integrin (Pearson coefficient, 0.535).
Figure 3.13. Rab11FIP5 is overexpressed in human prostate cancer. Human prostate cancer tissue immunostained with Rab11FIP5 (brown). Two different prostate tissue sections are shown each containing cancer (Ca) adjacent to the normal gland (N). Note the intense staining of Rab11FIP5 in the cancer. The bottom left panel is a representative of normal prostate tissue stained for Rab11FIP5, where a polarized apical staining is evident. In cancer (bottom right) the Rab11FIP5 is observed dispersed throughout the cytoplasm and the apical staining is markedly enhanced. A TMA of 45 human prostate tissue (containing 20 normal and 25 cancer tissue) were scored for Rab11FIP5 expression.
Rab11FIP5 protein was overexpressed in prostate cancer: Immunohistochemistry was performed on a TMA consisting of 20 normal prostate and 25 prostate cancer tissue to stain for Rab11FIP5 protein. Normal prostate tissue showed a cytoplasmic staining of Rab11FIP5 at apical locations in the luminal cells, as expected (Prekeris et al., 2000). In cancer, a higher intensity of Rab11FIP5 staining was observed. The apical localization was maintained in cancer, although diffuse cytoplasmic staining throughout the cancer cells was also demonstrated. The staining intensity was scored to reveal a statistically significant increase in Rab11FIP5 expression in prostate cancer (Fig. 3.13B).

DISCUSSION

Intracellular trafficking and subsequent recycling of integrins is an important determinant of their cell membrane expression and distribution and hence their function. Almost all integrins are known to employ the general Rab11 recycling machinery to be returned to the plasma membrane. Despite this, each integrin heterodimer is known to have distinct recycling characteristics (Das et al., 2017), suggesting that different Rab11 effectors might regulate the trafficking of individual integrins. Rab11FIPs are possible candidates to render such specificity as they are known to recycle a selective, although not necessarily exclusive, repertoire of cargo including integrins. Here, we report Rab11FIP5 as a recycling regulator specific for α6 integrin and is not involved in the recycling of the other laminin binding α3 integrin that also dimerizes with the β1 integrin subunit. Moreover, as per previous reports Rab11FIP5 is not involved in trafficking of other integrins like fibronectin binding α5β1 integrin or vitronectin receptor αvβ3, both being regulated by Rab11FIP1 (Caswell et al.,...
2008). Additionally, we found Rab11FIP5 did not affect membrane expression of other cell adhesion proteins like CD44, a non-integrin cell adhesion receptor-cadherin, the key cell-cell adhesion protein or the unrelated transferrin receptor.

The membrane expression of both α6 and β4 integrins was dependent on Rab11FIP5, raising an important question if it is an α6 or β4 dependent phenomenon. Since functional inhibition of Rab11FIP5 had similar effects on α6 integrin membrane expression in β4 null PC3 cell line, Rab11FIP5 clearly regulates α6 and not β4. This is in line with previous reports where the cytoplasmic tail of α6 integrin is sufficient to signal trafficking of a non-circulating CD8 receptor (Gaietta et al., 1994). This further emphasizes the role of alpha subunit in providing specificity of intracellular trafficking routes of integrins. Likewise, we found the LBIs have significantly different recycling kinetics. α6 integrin was recycled at a rate of 2.05 min⁻¹, 1.5 times greater than that of α3 integrin recycling rate of 1.33 min⁻¹.

In Rab11FIP5 depleted cells the internalization rate of α6 integrin was unaffected but the defect in recycling caused increased intracellular accumulation of α6 integrin. The resulting intracellularly accumulated integrins were found in both early endosomes (EE) and Rab11a endosomes, indicating that Rab11FIP5 is not involved in trafficking of α6 from EE to Rab11 but must be a regulator of trafficking of α6 integrin from Rab11 compartment to the plasma membrane.

Rab11FIP5 regulated membrane expression of α6 integrin was functionally dependent on the C2 domain of Rab11FIP5. The C2 domain is known to bind specific membrane
phosphoinositides like phosphatidylinositol 3,4,5 trisphosphate (PIP3) and phosphatidic acid (Lindsay and McCaffrey, 2004), and crucial for docking of cargo containing Rab11 vesicles to the plasma membrane (Lindsay and McCaffrey, 2004) before the Rab11 mediated exocytosis of vesicles at the targeted membrane (Takahashi et al., 2012). Interestingly, we observed α6 integrin was recycled to cell-cell membrane locations (Fig. 3.9). Additionally, there was a significant colocalization of α6 integrin with Rab11 and Rab11FIP5 at the cell-cell membrane locations (Fig. 3.7). Depletion of Rab11FIP5 significantly reduced such colocalization at the plasma membrane. If this was driven by specific membrane phosphoinositide composition of the targeted areas will be an interesting avenue of future research. Similar observations where α6 integrin is recycled to the cell-cell lateral membrane has been observed in model systems such as cultured human epidermal keratinocyte sheets (Poumay et al., 1993) and developing basal epidermis of zebrafish (Sonawane et al., 2009). Moreover, Rab11 is important for cell-cell communication during collective migration in border cell cluster migration in Drosophila (Ramel et al., 2013). Also, a loss of lateral β1 integrin staining was observed in Rab11c (also called Rab25) deficient mice (Nam et al., 2010), suggesting Rab11 might be important to maintain integrin levels at the cell-cell locations. The significance of targeting the recycled α6 integrin to the cell-cell locations in cancer is currently not understood. Integrins have been implicated to be involved in cell-cell adhesion by either direct interaction via intercellular ligand or indirectly by stabilization of E-cadherin mediated cell-cell adhesion complexes (Belvindrah et al., 2007; Hegerfeldt et al., 2002; Kaufmann et al., 1989; Larjava et al., 1993). The α6 integrin at lateral cell-cell membrane
locations can simply be a reservoir waiting to be recruited to cell-extracellular matrix adhesions at basal domains (Sonawane et al., 2009). Alternatively, α6 integrin might be of actual functional importance for cell-cell adhesion as has been published previously (Emsley, 2003).

Although depletion of Rab11FIP5 had no effect on membrane expression of E-cadherin, a cell-cell adhesion receptor, there was significant loss of cell-cell interaction A marked change in cellular phenotype was observed in 2D and the ability to form collective cohesive networks in 3D laminin rich environment was significantly compromised. It will be interesting for future study if the loss of cell-cell adhesion may be attributed to reduced integrin α6 at cell-cell locations or merely due to other possible downstream targets of other cell-cell adhesion molecules.

The prostate cancer cell migration on laminin enriched surfaces was significantly dependent on Rab11FIP5. We speculate that invasion via laminin enriched nerves and metastases to laminin rich bone might be dependent on α6 integrin recycling by Rab11FIP5. This mechanism of Rab11FIP5 dependent α6 recycling might be relevant in other systems that employ α6 dependent active cell migration e.g. epidermis movement during wound healing, rostral stream migration during nervous system development and epithelial layer development for embryogenesis.

Selective amplification of vesicular pathways involving specific integrins maybe important in cancer progression. The results of current study demonstrate that vesicular trafficking regulates amount of α6 integrin at the cell surface in prostate cancer.
Intracellular localization of α6 integrin was prominent in invasive prostate cancer as well as bone and visceral metastasis. The intracellular localization of the integrin might indicate active endocytosis of the integrin. We identified various intracellular trafficking regulatory proteins that significantly associated with the ratio of intracellular to cell surface localization of α6 integrin. Excitingly, Rab11FIP5 levels demonstrated the greatest associations with intracellular α6 integrin levels.

FIP5 in apical locations may provide a spatial advantage for recycling to the closer cell-cell lateral locations instead basolateral...

Recently, Rab11FIP5 was identified as marker of prognosis and poor outcome of ovarian cancer (Willis et al., 2016). Here, we identified Rab11FIP5 to be significantly overexpressed in human prostate cancer specimens as compared to normal prostate tissue. Based on current study, functional inhibition of Rab11FIP5 can provide specificity of targeting pro-metastatic α6 integrin. In future, it will be important to assess the clinical significance of Rab11FIP5 as a potential marker of aggressive prostate cancer.
IV. Rab11FIP1 regulates intracellular trafficking of α3 and α6 integrins
INTRODUCTION

α3 and α6 integrins function coordinately in various stages of development, wound healing and tissue regeneration in adults as well as in cancer invasion and metastasis (Huttenlocher and Horwitz, 2011). These processes are accompanied with dynamic changes in the membrane expression and cellular distribution of α6 and α3 integrins. During wound healing, following hemidesmosome disassembly, α6β4 integrin is released from basal membrane only to reappear along entire cell surface. α3 integrin on the other hand is observed at the tip of the lamellipodia where it functions to deposit a provisional matrix subsequently utilized by α6 integrin for collective epithelial migration. Although it is well established that the coordination of α3 and α6 expression during these processes is important the molecular determinants of this regulation remain unclear.

Intracellular trafficking is a key mechanism that cells employ to regulate membrane expression and distribution of cell surface receptors. Rab11 is a central regulator of intracellular trafficking. Rab11 effector proteins Rab11FIPs however traffic selective repertoire of cargo receptors. This is not only known to provide specificity but can be possible candidates to provide coordination between trafficking of certain receptors. In previous chapter we reported a coordination exists between α3 and α6 integrins in cancer, that significantly affects their trafficking, and cellular distribution and cell motility. Thus, understanding trafficking regulators of α3 integrin would be important to determine if modulating α3 integrin expression via trafficking regulation can coordinate α6 integrin trafficking.
Current chapter identifies another Rab11 effector protein of class I Rab11FIP family, Rab11FIP1 as a regulator of α3 integrin trafficking. Depletion of Rab11FIP1 led to a 41% decrease in membrane expression of α3 integrin accompanied with an increase in internalization and reduced recycling of α3 integrin. Rab11FIP1 depleted cells had major changes in α6 integrin trafficking demonstrated by a significant increase in its internalization, to the same magnitude as caused by α3 integrin depletion (chapter 2). Rab11FIP1 also regulated α6 integrin recycling, in a pathway independent of Rab11FIP5. Taken together, the data suggests that laminin binding integrins share a trafficking regulator Rab11FIP1, depletion of which phenocopies the effects of α3 integrin depletion.

RESULTS

*Rab11FIP1 regulates membrane expression of α3 integrin:* Cell membrane levels of integrin subunits α6, α3 and the unrelated transferrin receptor (TfR) were measured in DU145 cells using surface labelling of the receptors and flow cytometry analysis. TfR was tested as a control as Rab11FIP1 is known to regulate its trafficking (Schafer et al., 2016). The mean peak fluorescence (MPF) of α6 and α3 integrins reduced when cells were silenced for Rab11FIP1 (siFIP1) expression compared to untreated control cell population. MPF of α3 integrin membrane expression was significantly reduced by 41% in siFIP1 cells (p<0.05, n=3). The MPF of surface integrin label for α6 integrin was reduced by 21%, but was not statistically significant. The cell surface expression of the TfR was significantly reduced by 49%, as expected.
Figure 4.1. Membrane expression of α3 integrin is regulated by Rab11FIP1. DU145 cells were transfected with siRNA against Rab11FIP1 (siFIP1) and surface expression of receptors were measured by flow cytometry. Relative mean peak fluorescence values of cell membrane expression of the α6, α3 integrins and transferrin receptor (TfR) in untreated and siFIP1 cells (n=3, *p<0.05).
Rab11FIP1 depletion increases internalization of α6 and α3 integrins: We tested if the reduced cell membrane expression of the receptors on depletion of Rab11FIP1 was a result of changes in their internalization. Using flow cytometry, we measured the amount of antibody labeled cell surface receptors internalized in one cycle of internalization in DU145 cells. The MPF of the internalized labeled receptors was plotted for different timepoints and fitted using a first order kinetic model to obtain the internalization curve. The internalization curve for α6, α3 integrins and TfR were compared between untreated control and siFIP1 treated cells (Fig.4.2A). For each timepoint measured there was a marked increase in percent α6 or α3 integrins internalized. In contrast, the amount of TfR internalized was reduced on siFIP1. The rate of α6 integrin internalization was increased by a significant measure of 1.8-fold in siFIP1 cells ($k_{\text{actual}}$ of 2.72 min$^{-1}$) compared to untreated control cells ($k_{\text{actual}}$ of 1.48 min$^{-1}$) (Fig. 4.2B). The internalization rate of α3 integrin significantly increased by 1.6-fold in siFIP1 cells when compared to untreated control cells ($k_{\text{actual}}$ of 2.38 min$^{-1}$, 1.45 min$^{-1}$ respectively) or α3 integrin (0.69 min$^{-1}$, 0.87 min$^{-1}$). The total amount of α6 integrin internalized was also markedly increased by 30% (from 46% in untreated to 77% in siFIP1 cells). Similarly, a 10% increase was observed for α3 integrin (46% in untreated to 77% in siFIP5 cells). The increase in intracellular accumulation of both the integrins on Rab11FIP1 depletion was statistically significant.
Figure 4.2 Depletion of Rab11FIP1 increases internalization of α6 and α3 integrins. Internalization kinetic curve for α6, α3 integrins and transferrin receptor (TfR) in untreated control DU145 and FIP1 silenced (siFIP1) cells. Labeled receptor internalized is plotted as a percent of total surface receptor labeled for different timepoints of internalization at 37°C. Internalization curve is obtained using a first order kinetic model ($R^2>0.989$). (B) Maximum intracellular accumulation and internalization rate constants (observed, $k_{obs}$ and actual, $k_{actual}$) calculated as per first order rate kinetics. Results represent 3 independent experiments. Statistical significance calculated for changes in rate constant and intracellular accumulation as per student’s t test, unpaired, *p<0.05, **p<0.01, n=3.
In contrast to the integrins, there was a significant 2.4-fold decrease in the internalization rate of TfR from 10.37 min$^{-1}$ in untreated control to 4.24 min$^{-1}$ in siFIP1 cells. This was accompanied by a significant 29% reduction in internal accumulation of the TfR. These results indicate that Rab11FIP1 negatively regulates internalization of the laminin binding integrins which is opposite to its role in TfR internalization.

*Depletion of Rab11FIP1 reduces recycling of $\alpha_6$ and $\alpha_3$ integrins:* Next, we tested if the increased intracellular accumulation of $\alpha_6$ and $\alpha_3$ integrins on Rab11FIP1 depletion may be a result of defective integrin recycling to the cell membrane. We measured the recycling kinetics of $\alpha_3$ and $\alpha_6$ integrins and the TfR using a flow cytometry in DU145 cells treated with two different siRNA against Rab11FIP1 (siFIP1(#1) or siFIP1(#2)) and compared to the untreated cells (Fig. 4.3A). Using the optimized flow cytometry based recycling assay at 25°C as described earlier, recycling kinetic curve was plotted for different time points and compared between untreated, siFIP1(#1) and siFIP1(#2) cells. For both the $\alpha_6$ and $\alpha_3$ integrins, the amount of integrin recycled to the cell surface was reduced on FIP1 depletion compared to untreated control cells at each time point tested. Recycling of TfR was also dramatically reduced.

Depletion of Rab11FIP1 using two different siRNAs led to a significant 1.6-1.7-fold reduction in the rate of $\alpha_6$ integrin recycling from 2.06 min$^{-1}$ to a $k_{\text{actual}}$ of 1.22 min$^{-1}$ for siFIP1 (#1) and 1.08 min$^{-1}$ for siFIP1 (#2) (p<0.05, n=3) (Fig. 4.3B). The total amount of $\alpha_6$ integrin recycled decreased from 34% in untreated cells to 30% in siFIP1(#1) and 32% in siFIP1(#2) cells (Fig. 4.3B).
Figure 4.3. Depletion of Rab11FIP1 expression reduces recycling of both α3 and α6 integrins. DU145 cells were transfected with two different siRNAs against Rab11FIP1: siFIP1(#1) or siFIP1(#2) (A) Recycling kinetic curve for α6, α3 or Tfr in untreated and siFIP1 DU145 cells. Percent label recycled to the cell surface at 25°C (calculated as percent mean peak fluorescence of recycled label at a given timepoint vs total label internalized) is plotted for different time intervals. First order kinetic curve is fitted using Kaleidagraph ($R^2>0.98$). (B) Maximum label recycled (%) and recycling rate constants (observed, $k_{obs}$ and actual, $k_{actual}$) calculated as per first order rate kinetics. Results represent 3 independent experiments. Statistical significance calculated for changes in label internalized at each timepoint and intracellular accumulation as per student’s t test, unpaired, *p<0.05, **p<0.01, n=3.
Similarly, rate of α3 integrin recycling was significantly reduced from 1.30 min⁻¹ in untreated cells to 0.76 min⁻¹ in siFIP1(#) and 0.80 min⁻¹ siFIP1 (#2) cells (Fig. 4.3B). The total amount of α3 integrin recycled in untreated cells was 17% and reduced to 15% in siFIP1(#1) and 12% in siFIP1(#2) cells (Fig. 4.3B). The rate of TfR recycling was remarkable diminished from 10.84 min⁻¹ for untreated cells to 2.94 min⁻¹ for siFIP1 cell. This was accompanied with a significantly reduced amount of total recycled TfR (25% untreated, 8% siFIP5 (#1)) (Fig.4.4B).

**Rab11FIP1 and Rab11FIP5 regulate α6 integrin recycling in independent pathways:** Rab11FIPs have the ability to heterodimerize (Wallace et al., 2002). Since, depletion of Rab11FIP1 reduces recycling of α6 integrin to a similar extent as depletion of Rab11FIP5, it was important to test if these Rab11FIPs regulate α6 recycling as a heterodimer. For this, we used DU145 cell lines silenced for either or both Rab11FIP1 and Rab11FIP5. The hypothesis was, if they function as a heterodimer, α6 integrin recycling would be equally affected whether one or both the Rab11FIPs are depleted. Otherwise an additive effect on decrease in recycling would indicate their independent role in α6 integrin recycling. Recycling assay was performed as described earlier and a rate kinetic curve for α6 and α3 integrins were determined for untreated cells, or cells depleted for Rab11FIP1 (siFIP1), Rab11FIP5 (siFIP5) or both (siFIP1/siFIP5) (Fig.4.4A).
Figure 4.4. α6 integrin is recycled by Rab11FIP5 and Rab11FIP1 in independent pathways. DU145 cells were transfected with either siRNAs against Rab11FIP5 (siFIP1), Rab11FIP5 (siFIP1) or both (siFIP5/siFIP1) (A) Recycling kinetic curve for α6, α3 or TfR in untreated, siFIP5, siFIP1 and siFIP5/siFIP1 DU145 cells. Percent label recycled to the cell surface at 25°C (calculated as percent mean peak fluorescence of recycled label at a given timepoint vs total label internalized) is plotted for different time intervals. First order kinetic curve is fitted using Kaleidagraph (R²>0.98). (B) Maximum label recycled (%) and recycling rate constants (observed, $k_{obs}$ and actual, $k_{actual}$) calculated as per first order rate kinetics. Results represent 3 independent experiments. Statistical significance calculated for changes in label internalized at each timepoint and intracellular accumulation as per student’s t test, unpaired, *p<0.05  **p<0.01, n=3.
Depletion of Rab11FIP1 or Rab11FIP5 decreased α6 integrin recycling to similar extents, reducing the rate constant \( (k_{\text{actual}}) \) of 2.06 min\(^{-1}\) in untreated control cells to a \( k_{\text{actual}} \) of 1.37 min\(^{-1}\) in siFIP5 and 1.32 min\(^{-1}\) in siFIP1 cells (Fig. 4.4B). Depletion of both Rab11FIP5 and Rab11FIP1 further decreased the rate of recycling to 1.01 min\(^{-1}\). The total amount of receptor recycled in one cycle was also significantly reduced from 34% in untreated to 28% in siFIP5, 28% in siFIP1 and 24% in siFIP5/siFIP1 cells. Thus, depletion of both the Rab11FIP proteins had an additive effect on reducing α6 integrin recycling.

The recycling curve for α3 integrin in siFIP1 overlapped with dual silenced siFIP5/siFIP1 cells. The recycling rate of α3 integrin was significantly reduced to a \( k_{\text{actual}} \) of 0.74 min\(^{-1}\) in siFIP1 and 0.68 min\(^{-1}\) in siFIP5/siFIP1 cells as compared to 1.07 min\(^{-1}\) in untreated control cells. There was no measurable difference between the rate of recycling (\( k_{\text{actual}} \) of 1.07 min\(^{-1}\) in untreated and 1.13 min\(^{-1}\) in siFIP1 cells) or the total amount of α3 integrin recycled (20.5%, 18.9%, respectively) in untreated and siFIP5 cells.

Taken together these data suggest that both Rab11FIP1 and Rab11FIP5 regulate recycling of α6 integrin, but in independent pathways. α3 integrin on the other hand is selectively regulated by Rab11FIP1 and not Rab11FIP5.

**DISCUSSION**

The function of α6 and α3 integrins are coordinated in various systems. We had earlier demonstrated a unidirectional coordination between the internalization of these integrins. Here, we identify a trafficking regulator Rab11FIP1 common to both α3 and α6 integrins. Rab11FIP1 was found to majorly regulate membrane expression of α3 integrin.
such that its depletion led to a 41% reduction of cell surface α3 integrin. This was a result of increased intracellular accumulation due to higher rates of internalization and significantly reduced recycling. Since the other class I C2 domain containing Rab11FIP, Rab11FIP5 was not involved in α3 integrin recycling, we suggest Rab11FIP1 as a regulator specific to α3 integrin.

Previous studies have reported Rab11FIP1 as a coordinator of the recycling of the fibronectin binding α5β1 integrin and vitronectin binding αvβ3 integrin, such that blocking αvβ3 adhesion resulted in increased interaction of Rab11FIP1 with α5 integrin and promote its recycling. We found the depletion of Rab11FIP1 and reduced membrane expression of α3 integrin and corresponded to a significantly higher rate of α6 integrin internalization to an extent similar to that observed in cells depleted of α3 integrin protein expression. It would be important to test in future, if the increased α6 integrin internalization was driven by Rab11FIP1 directly or through an indirect effect of reduced cell surface α3 integrin, which would indicate Rab11FIP1 as a coordinator of α6 and α3 integrin trafficking.

Rab11FIPs dimerize to bind to two different Rab11 molecules, a process critical for their function. Although homo and heterodimerizing ability of these Rab11FIPs have been identified based on cell free protein-protein interaction systems, possible function of the heterodimerization is not established in cell systems. Nevertheless, since both the Rab11FIP1 and Rab11FIP5 were found to significantly regulate recycling of α6 integrin, we tested if this could possibly require heterodimers of Rab11FIP5 and Rab11FIP1. However, we observed the dual silencing of both the Rab11FIPs had additional defects.
in recycling of α6 integrin, as compared to depletion of either Rab11FIPs. Thus, an additive function of these two Rab11FIPs in α6 integrin recycling was demonstrated, suggesting they recycle α6 integrin in independent pathways.
V. PTEN regulates α6 integrin trafficking
INTRODUCTION

PTEN is a tumor suppressor altered in various epithelial cancers (Chalhoub and Baker, 2009; Hollander et al., 2011; Li et al., 1997; Steck et al., 1997). PTEN is mutated or lost in at least 40% of prostate cancer and a predictor of aggressive disease (Hollander et al., 2011). PTEN mutation/deletion significantly associates with extracapsular extension and predicts prostate cancer recurrence (Troyer et al., 2015).

PTEN is a protein and lipid phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to phosphatidylinositol 4,5-bisphosphate (PIP2) to regulate the membrane phosphoinositide composition (Sun et al., 1999). Loss of PTEN activity leads to about 1.5-2 fold increase in PIP3 levels (Furumoto et al., 2006; Schubbert et al., 2016). PTEN localizes to apical membrane in polarized cells where it prevents PIP3 accumulation by converting it to PIP2 (Martin-Belmonte et al., 2007). Thus, PTEN is key in regulating cell polarity by restricting PIP3 to the basolateral surface (Gassama-Diagne et al., 2006). PTEN also regulates front-rear polarity in migrating cells by maintaining PIP2 at the trailing edge (Cain and Ridley, 2009) and allowing PIP3 at the leading edge of migrating cells (Karunarathne et al., 2013).

Coordination of phosphoinositides with RabGTPases is key to spatiotemporal regulation of vesicular trafficking and signaling pathways (Campa and Hirsch, 2017). Each vesicular compartment is defined by its phosphoinositide composition regulated by various kinases and phosphatases (Fig.5.2) (Shewan et al., 2011). Regulated phosphoinositide
Figure 5.1. Phosphoinositide composition of vesicular and cell membrane regulate intracellular trafficking. Subcellular distribution of Phosphoinositide species is shown. Phosphoinositides concentrate in cytosolic membranes, serving as discrete determinants of membrane identity. Interconversion of PIP3 (PtdIns(3,4,5)P_3) and PIP2 (PtdIns(4,5)P_2) by kinases and phosphatases are key to cellular polarity (Shewan et al., 2011).
interconversion is basis of appropriate vesicular delivery and fusion with destination membrane.

Membrane phosphoinositides form docking sites for C2 domain of Rab11FIPs (Lindsay and McCaffrey, 2004). Rab11FIPs have preferential binding affinity to certain phosphoinositides. Rab11FIP5 was reported to have higher affinity to PIP3 and phosphatidic acid as compared to PIP2 or other phosphoinositides. However, a different study reported that blocking kinase activity promotes translocation of C2 containing Rab11FIP5 to the plasma membrane. Thus, the function of Rab11FIP5 and its C2 domain might be regulated via a complex coordination of kinases and membrane phosphoinositides. Previous chapters identified Rab11FIP5 and its C2 domain as a crucial regulator of the recycling and membrane expression of α6 integrin. Work presented in this chapter was based on the hypothesis that changes in membrane PIP3 levels by PTEN may regulate trafficking of α6 integrin.

Current chapter explores if PTEN status of cancer can affect membrane expression and trafficking kinetics of α6 integrin. The results reported in this chapter provide preliminary support that PTEN status determines membrane expression of α6 integrin by regulating its recycling to the plasma membrane. Prostate cancer cell line DU145, which has heterozygous loss of one of the PTEN allele was used in this study. Moreover, a significant correlation was discovered between PTEN loss and membrane localization of α6 integrin in human prostate cancer tissue.
RESULTS

Loss of PTEN expression correlates with cell surface expression of α6 integrin in human prostate cancer tissue: Serial sections of human prostate cancer tissue were immunostained for PTEN and α6 integrin. PTEN negative cancer presented intense α6 integrin expression on the cell membrane that localized primarily to cell-cell membrane locations (Fig. 5.2). In contrast, PTEN positive cancer showed marked intracellular distribution of α6 integrin.

PTEN regulates cell surface expression of α6 integrin: Cell surface expression of α6 and α3 integrins were tested as a function of PTEN expression in prostate cancer cell line DU145, which has heterozygous deletion of one allele of PTEN allele. Flow cytometer histograms show a marked reduction of cell surface levels of α6 and β4 integrin subunits in cells silenced for PTEN expression (siPTEN) as compared to untreated or non-targeting siRNA treated cells (siCon), to an extent similar to α6 integrin silenced cells (siα6) (Fig. 5.3). Cell surface expression of the other LBI α3 integrin, and a non-integrin cell adhesion receptor CD44 remained unchanged in siPTEN, siCon, siα6 or untreated cells.
Figure 5.2. PTEN and α6 integrin distribution prostate cancer. Serial sections of human prostate cancer tissue were immunostained for PTEN and α6 integrin (n=12). Note the PTEN negative tumor shows distinct cell membrane expression of α6 integrin, primarily at the cell-cell locations, frequently observed in prostate cancer. PTEN positive cancer instead reveals a diffuse cytoplasmic distribution of α6 integrin.
Chapter V: PTEN regulates α6 integrin recycling

Figure 5.3. Membrane expression of α6β4 integrin is regulated by PTEN. DU145 cells were transfected with non-targeting siRNA (siCon), and siRNA against PTEN (siPTEN) or α6 integrin (sia6). Flow histograms of cell surface expression of laminin binding integrin subunits α6, β4 or α3 and unrelated adhesion receptor CD44 using immunolabelling of receptors in fixed, non-permeabilized untreated (black), siCon (red), siPTEN (purple) and sia6 (green) cells. Relative mean peak fluorescence values of cell membrane expression of the receptors in untreated, siCon, siPTEN and sia6 cells are shown (n=4, **p<0.01).
Silencing PTEN reduces recycling of α6 integrin: To test if the reduced cell membrane expression of α6 integrin in siPTEN cells was a result of defective trafficking, recycling kinetics of the integrin was determined in untreated and siPTEN DU145 cells. The amount of integrin label recycled at different timepoints was measured as a percent of total label internalized. Recycling curve was obtained by fitting a first order kinetic model as described earlier \((R^2 > 0.98)\). There was a significant reduction in α6 integrin recycling at each timepoint tested (Fig. 5.4A). The recycling curve for α3 integrin in siPTEN cells overlapped with untreated cells, without any changes in label recycled in any of the timepoints. The recycling rate for α6 integrin was significantly reduced by up to 2.45-fold from 1.45 min\(^{-1}\) in untreated cells to 1.0 min\(^{-1}\) in siPTEN cells (Fig. 5.4B). The total α6 integrin recycled was also reduced by ~9% (29.9%, 20.9% for untreated and siPTEN cells respectively). The rate of α3 integrin recycling (1.14 min\(^{-1}\), 1.04 min\(^{-1}\)) or the total integrin recycled (10.36%, 11.79%) was unchanged in untreated and siPTEN cells.
Figure 5.4. Depletion of PTEN expression reduces recycling of α6 integrin. DU145 cells were transfected with siRNA against PTEN (siPTEN) (A) Recycling kinetic curve for α6 and α3 integrins in untreated control and siPTEN DU145 cells. Percent label recycled to the cell surface at 25°C (calculated as percent mean peak fluorescence of recycled label at a given timepoint vs total label internalized) is plotted for different time intervals. First order kinetic curve is fitted using Kaleidagraph (R²>0.98). (B) Maximum label recycled (%) and recycling rate constants (observed, k_{obs} and actual, k_{actual}) calculated as per first order rate kinetics. Results represent 3 independent experiments. Statistical significance calculated for changes in label internalized at each timepoint and intracellular accumulation as per student’s t test, unpaired, *p<0.05  **p<0.01, n=3.
DISCUSSION

PTEN mutation or loss is a continuum of various epithelial cancers including prostate cancer. PTEN regulates phosphoinositide composition of both plasma membrane as well as intracellular vesicular membranes. Current chapter identifies PTEN expression as a regulator of cell surface expression and trafficking of α6 integrin.

Human prostate cancer tissue with a loss of PTEN expression demonstrated an intense staining of α6 integrin at the cell membrane, specifically at cell-cell locations. PTEN positive cancer on the other hand were positive for α6 integrin expression which showed reduced membrane and prominent diffuse intracellular staining.

However, studies on the prostate cancer cell line DU145 revealed that siRNA mediated PTEN depletion significantly reduces the membrane expression of both α6 and β4 integrin subunits. One of the many plausible reasons for these contrasting results is the interplay between kinase signaling cascades. It has been shown that alterations in PI3K/PTEN status can modulate alternative kinase cascades such as ERK signaling (Lee et al., 2008). The PTEN/PI3K may regulate vesicular transfer by not only controlling the phosphoinositide composition as well as activate various effector proteins downstream of the kinase signaling. Hence, the contribution of compensatory kinase signaling pathways need to be assessed in the regulation of α6 integrin.

The reduced membrane expression of α6 integrin was attributed to a defective recycling in absence of PTEN. The fate of the intracellularly accumulated α6 integrin remains to be determined. PTEN can dephosphorylate RabGTPases and hence depending on their
target RabGTPase the PTEN status can dictate the trafficking route of the cargo receptor. For example, PTEN loss have been earlier described to increase the internalization of E-cadherin routing it to the lysosomal degradation pathway (Shinde and Maddika, 2016). Thus, determining the vesicular compartment where the α6 integrin resides due to defective recycling can provide key information to PTEN dependent regulation of α6 integrin recycling.

Despite a significant effect on the trafficking of α6 and β4 integrins, depletion of PTEN did not affect the membrane expression or recycling rate of the other laminin binding α3 integrin. Additionally, there was no effect on a non-laminin cell surface adhesion receptor CD44. This suggests a selective regulation of α6β4 integrin by PTEN. Since previous chapters have established that the regulation of membrane expression and trafficking kinetics of α6 integrin is significantly different than α3 integrin even though their trafficking is coordinated. It will be important to test if PTEN mediated dephosphorylation may provide selective advantage to trafficking regulators selective for α6 integrin like Rab11FIP5. Future research will be important to determine if PTEN dependent regulation of α6 integrin is dependent on Rab11FIP5 and the selective functional affinity of its C2 domain to PIP3. Additionally, it would be interesting to test if α6 recycling to the cell-cell locations may be driven by distinct phosphoinositide composition, possibly PIP3 enriched domains at those locations.
Chapter IV: α6 integrin in Perineural Invasion

VI. α6 integrin in Perineural Invasion

PMID: 26239765

INTRODUCTION

Prostate cancer is a neurotropic cancer that invades through prostatic nerves and neurovascular bundles as a means of extracapsular escape during metastasis (Fig. 6.1) (Liebig et al., 2009; Marchesi et al., 2010; Sroka et al., 2010; Villers et al., 1989). Various other epithelial cancers including pancreatic, colorectal and head and neck cancer similarly employ the complex neuroanatomy of highly innervated organs for primary tumor cell escape (Marchesi et al., 2010). This process is known as perineural invasion (PNI). PNI is clinically a factor of metastasis and poor patient outcome both in terms of recurrence after surgical tumor resurrection and decreased patient survival (Harnden et al., 2007; Liebig et al., 2009; Zareba et al., 2017). About 85% of prostate cancer cases demonstrate PNI. The peripheral zone of the prostate gland known to be significantly innervated is the predominant site for prostate tumor development (Chang et al., 1998; Powell et al., 2005). Despite the well-known clinical significance of PNI in development and metastasis of epithelial cancers, the mechanisms are poorly understood.

Perineural invasion is classically defined as “cancer in, around, and through the nerve”. This includes invasion of any of the three layers of nerve- epineurium, perineurium or endoneurium. Tumor cells undergoing PNI invade along the perineural space or into the endoneurium (Sroka et al., 2010) and disrupt and damage nerve axons. The endoneurium contains Schwann cells that secrete various factors like neurotrophic factor pleiotrophin and myelin-associated glycoprotein (MAG) that promote cancer.
Figure 6.1 Anatomy of prostatic nervous system. The prostatic nervous system penetrates into the peripheral zone of the prostate gland where most prostate tumors develop. Different layers of the nerve sheath include the epineurium, perineurium and endoneurium from outside in, respectively. Perineural and endoneural invasion of prostate tumor cells along the nerve route is used for extracapsular escape. Inset: α6 integrin (brown) is expressed on human prostate tumor cells during tumor invasion on prostatic nerves. Note the absence of cancer cell (CA) invasion along vessels (V) when compared to significant invasion of the nerve (N). Integrin α6 is expressed around vessels as expected (Sroka et al., 2010).
invasion (Swanson et al., 2007; Yao et al., 2013). Thus, a complex interaction between factors secreted from nerve cells and tumor entails during PNI. The two kinds of Schwann cells-myelinating and non-myelinating, may secrete specific factors to modulate tumor cell invasion.

The Peripheral nervous system is rich in laminin (Yu et al., 2007). Engagement of laminin through integrin receptors, specifically α6β1 and α6β4 integrins, is a requirement for Schwann cell regulated myelination of axons during development of peripheral nervous system (Dubovy et al., 1999; Niessen et al., 1994; Terpe et al., 1994; Yu et al., 2007). Expression of LBIs may provide tumor cells an advantage for attachment to laminin enriched nerve and they use it to escape from the gland. Our group had earlier identified laminin receptor α6 integrin to be significantly expressed in PNI (Sroka et al., 2010). Interestingly, the prominent laminin chains expressed in neoplastic lesions and PNI comprise the ligands of α6 integrin, the laminin α5, α4, and γ1 chains. The expression of α6 integrin is maintained throughout prostate cancer progression and metastasis to laminin rich bone. A functional post-translational cleavage of the extracellular ligand binding domain of α6 integrin is crucial for this pro-metastatic function of α6.

Here the α6 integrin is tested for its potential role in perineural invasion of human prostate cancer. A cell culture model is employed, where neurotropic factors released by Schwann cells are tested for effect on prostate and pancreatic cancer cell invasion. Tumor cell migration was increased up to 2-fold when conditioned medium from cultured myelinated Schwann cells was used. This was found to be dependent on the
post-translational functional cleavage of extracellular ligand binding domain of α6 integrin on the cell surface, a phenomenon previously shown to promote cancer invasion and metastasis to bone. Prostate cancer presenting PNI revealed collectively invading cancer with heterogenous cell population surrounding or invading nerves. Two different phenotypes in terms of cellular distribution of α6 integrin- both strong membrane and intracellular expression was identified with latter being pronounced in prostate cancer cells that had invaded into the endoneural space.

RESULTS

α6 integrin expression in PNI: Human prostate cancer tissue were stained with neural specific marker PGP 9.5 and α6 integrin (Fig. 6.2, red and brown respectively). Both perineural invasion and endoneural invasion was observed and representative images are presented. Both perineural and endoneural invasion had significant expression of α6 integrin. In PNI the tumor cells can be seen surrounding an intact nerve sheath containing schwann cells and are separated from the nerve by the perineurium (Fig. 6.2A). There is heterogeneity in the localization of the integrin, as both membrane (white triangles) and intracellular expression (asterisk) of the integrin is observed in PNI. In endoneural invasion, the tumor can be seen invaded into the perineural space with interspersed nerve cells. Intense expression of α6 integrin is observed in endoneural invasion as well (Fig. 6.2B). Cytoplasmic staining of α6 integrin was pronounced in endoneural invasion. Figure 6.2C shows both perineural and endoneural invasion of the cancer cells with heterogenous distribution of α6 integrin.
Figure 6.2. α6 integrin expression in endoneural and perineural invasion in human prostate cancer. Human prostate cancer tissue was stained for the α6 integrin (brown) and a neural specific marker PGP 9.5 (red). Cancer cell invasion of perineurium (P), endoneurium (E) of nerves (N) demonstrate both membrane (white triangle) and intracellular/cytoplasmic diffuse staining (*). (A) Perineural invasion. (B) Endoneural invasion of cancer, shows cancer cells in dispersed nerve (C) Both endoneural and perineural invasion of cancer can be seen.
Chapter IV: α6 integrin in Perineural Invasion

*S16 Schwann cell conditioned media increases cancer cell invasion dependent on α6 integrin:* Next, we tested the effect of nerve cells, the Schwann cells, on invasion of cancer cells and if it was dependent on α6 integrin. Cell conditioned media from two different types of immortalized Schwann cells, myelinating (S16) and non-myelinating (S16Y) derived from neonatal mice (Hai et al., 2002; Toda et al., 1994) were tested for effects on cancer cell invasion. Two prostate cancer cell lines- DU145 (Fig. 6.3A) and PC3 (Fig. 6.3B) and a pancreatic cancer cell line CFPAC (Fig. 6.3C) were tested. Each cell line demonstrated a 1.6-2 fold increase in invasion when treated with conditioned media from myelinating cells (S16) as compared to FBS treatment. While conditioned media from non-myelinating S16Y cells had an opposite effect and reduced cell invasion by up to a half (Fig. 6.3).

Blocking pro-metastatic functional cleavage of α6 integrin by J8H antibody significantly reduced the S16 induced cell invasion in DU145, PC3 and CFPAC. The cancer cell invasion was dependent on β1 integrin function, as blocking cell attachment to laminin by antibody AIIB2 treatment completely abrogated the S16 induced cell invasion in all cell lines tested (Fig. 6.3).

*Schwann cell conditioned media alter α6 integrin cleavage:* Since S16 conditioned media induced cell invasion was dependent on functional cleavage of α6 integrin, we tested if S16 conditioned media alters α6 integrin cleavage and production of α6p. Each of the cell lines that increased cell invasion on treatment with S16 conditioned media were tested. An additional normal immortalized prostate cell line RWPE-1 was also tested.
Figure 6.3. S16 conditioned media increased tumor cell invasion dependent on α6β1 integrin. Invasion of (A) DU145, (B) PC3, and (C) CFPAC1 cells were analyzed using a modified Boyden chamber invasion assay with laminin 111. The fold-increase in invasion was determined for cancer cells invading towards FBS (grey), S16 (black bars), or S16Y (gray bars) conditioned media. Both untreated cells and cells treated with function blocking antibody against α6 integrin (J8H) or β1 integrin (AIIB2) were tested for invasion under the effects of FBS and S16 conditioned media. Results shown are a mean of 3 independent experiments. Statistical significance is denoted *p<0.05, **p<0.005, ***p<0.0001.
Figure 6.4. Suppression of α6p production in tumor cells by S16Y cell (non-myelinating phenotype) conditioned media. (A) DU145, PC3, CFPAC1 tumor, and normal prostate (RWPE-1) cells were treated with DMEM control media (C), or S16 and S16Y conditioned media for 24 h. α6 integrin and α6p were immunoprecipitated using the J1B5 antibody and detected by immunoblot. (B) Quantitative analysis of the immunoblot experiments to determine the ratio of area density of α6p to α6 as shown. The results are representative of three independent experiments and the asterisk denotes a significant difference (*p<0.05)
Each cell line was treated with S16 and S16Y conditioned media for 24 hours, followed by cell lysis and immunoprecipitation for α6 integrin and western blot analysis. S16 conditioned media treatment increased α6p production. Interestingly, S16Y blocked the α6 integrin cleavage as seen by a reduction/loss of the α6p band in each of the cell lines (Fig.6.4A). The ratio of cleaved to full length α6 integrin increases on S16 conditioned media treatment and is significantly reduced in S16Y conditioned media treated cells (Fig.6.4B).

**DISCUSSION**

Various epithelial cancers including prostate, pancreatic, colorectal, head and neck cancer employ the complex neuroanatomy of highly innervated organs for primary tumor cell escape. The peripheral nervous system is rich in laminin and an overexpression of α6β1 integrin has been observed in human prostate cancer cases with PNI. Current chapter reports a heterogeneous expression of integrin α6 in human prostate cancer during perineural and endoneural invasion. Intense expression of α6 integrin was observed in both cell membrane and intracellular staining pattern in prostate cancer surrounding the nerves. Interestingly, endoneural invasion demonstrated prominent intracellular expression of the integrin. The heterogeneous expression of α6 integrin in PNI suggests dynamic regulation of membrane expression of α6 integrin, possibly to attach and invade the laminin rich niche of the perineurium. Furthermore, similar heterogeneity in membrane and intracellular staining pattern of α6 integrin observed in metastatic prostate cancer, as reported in chapter 3, had correlated with increased expression of various proteins that regulate intracellular trafficking (Fig.
3.12). Such dynamic patterns of membrane and intracellular expression may indicate a possible role of intracellular trafficking in PNI, which needs to be tested in future.

A reciprocal signaling between the nerve cells and invading tumor has been identified as a promoter of PNI. Tumor invasion into the nerve cause considerable damage to nerve cells, leading to secretion of a myriad of chemoattractive and adhesive factors by nerve cells. Myelinating Schwann cells secrete a variety of neurotrpins, cytokines and extracellular laminin during nerve regeneration (Campana, 2007; Jessen and Mirsky, 2005; Stoll and Muller, 1999). Here, we report that the S16 cells, which exhibit characteristics of myelinating Schwann cells, secrete factors that promote cancer cell invasion of prostate and pancreatic cancer cell lines (DU145, PC3 and CFPAC) on laminin. This invasive phenotype was promoted by factors specific to myelinating Schwann cells, as the non-myelinating Schwann cells S16Y, had the opposite effect on cancer invasion leading to a significant reduction of the cell invasion.

Specifically, the Schwann cell secreted factors altered the production of the pro-metastatic variant of α6 integrin, α6p, produced by cleavage of the extracellular ligand binding domain on the cancer cell surface. The myelinating Schwann cells increased the production of α6p. Using the function blocking antibody J8H which blocks this α6p production, had curbed the S16 induced cell invasion. J8H treatment has previously shown to delay the onset of bone metastasis of prostate cancer (Ports et al., 2009). A variety of factors secreted by S16 may promote α6p production. Components of uPA/uPAR axis are promising candidates, since it is known to regulate α6 integrin cleavage (Pawar et al., 2007; Ports et al., 2009). S16Y on the other hand significantly
blocked the production of α6p and reduced cell invasion. In summary, the results in current chapter demonstrate the importance of α6 integrin function and its cleavage in perineural invasion of prostate cancer. The Schwann cells present in the endoneurium dictate invasive capability of cancer cells by secreting tropic factors that modulate α6 integrin function.
Laminin binding integrins (LBIs) are persistently but differentially expressed throughout prostate cancer progression and metastasis. Being a neurotropic cancer, LBI expression in prostate cancer cells must provide advantage to attach and migrate on laminin enriched nerves. Additionally, 90% of prostate cancer metastasizes to bone, again a laminin rich environment that may provide attachment cues and facilitate subsequent homing of the cancer cells. Intense expression of $\alpha_6$ integrin was demonstrated during perineural invasion of prostate cancer, the primary route of gland escape. Interestingly, human prostate cancer tissue of PNI and bone and visceral metastasis presented with a heterogeneous pattern of $\alpha_6$ integrin localization such that both cell-cell membrane and intracellular distribution was observed. The cytoplasmic expression became pronounced when cancer cells had invaded into the endoneural space. These observations suggest that changes in integrin distribution might be key to invasion during cancer progression.

Cytoplasmic expression of $\alpha_6$ integrin was found to associate with overexpression of various proteins that regulate intracellular vesicular trafficking. Cell surface integrins are known to continuously internalize and sorted through intracellular trafficking pathways before they are returned back to the cell membrane. This is crucial to enable cell migration as release of integrins after disassembly of focal adhesions is facilitated by endocytosis, a process important for detachment of cell-rear. These integrins are then available to be returned back to the membrane to forward sites of cell adhesion. We
found that both \( \alpha_6 \) and \( \alpha_3 \) integrins undergo these processes of internalization and recycling but with significantly different rate kinetics.

The LBIs function coordinately during embryonic development and adult processes such as wound healing and epithelial regeneration. Their orchestrated redistribution in cells during cell migration is well-known, but the mechanism is not clearly understood. Current study identifies the intracellular trafficking of \( \alpha_6 \) and \( \alpha_3 \) integrins as a key mechanism of coordination of their function and cellular distribution. \( \alpha_3 \) integrin negatively regulated the internalization of \( \alpha_6 \) integrin. Depletion of \( \alpha_3 \) integrin redistributed the internalized \( \alpha_6 \) integrin to the cell-cell location, reminiscent of suprabasal localization of \( \alpha_6 \) integrin in epidermis and a common phenomenon demonstrated in Prostatic intraepithelial neoplasia (PIN) lesions of human prostate cancer. The relative localization of \( \alpha_6 \) and \( \alpha_3 \) integrin in human prostate cancer specimens will be interesting to study in future. Based on the observations reported in this dissertation, a loss of \( \alpha_3 \) integrin, known to occur in 90% of advanced prostate cancer, would promote such localization of \( \alpha_6 \) integrin at cell-cell locations and promote a more invasive phenotype.

\( \alpha_6 \) integrin recycling is known to drive cell migration and cancer invasion. The important pro-metastatic role of \( \alpha_6 \) integrin with a distinct pattern of intracellular integrin expression drove a need to understand the regulation of \( \alpha_6 \) integrin trafficking in cancer. Current research successfully identified trafficking regulators selective to \( \alpha_6 \) integrin. Upon investigation of patient derived xenografts, various trafficking regulators potentially involved in \( \alpha_6 \) integrin trafficking were identified. Intracellular trafficking
machinery is strictly governed by a family of small GTPases RabGTPases and their effectors. Rab11 is a central hub of trafficking, recycling a myriad of proteins. A family of Rab11 effector proteins, Rab11FIPs mediate various steps of Rab11 regulated trafficking, including sorting of cargo, inter-vesicular transfer and docking and fusion of the recycled vesicles to plasma membrane. Here, Rab11FIP5 was discovered as a selective regulator of α6 integrin recycling from cytoplasm to the plasma membrane. Interestingly, it did not affect trafficking of the other laminin binding α3 integrin. Also, previous studies did not find any role of Rab11FIP5 in trafficking of fibronectin binding α5β1 integrin or the vitronectin binding αvβ3 integrin, indicating Rab11FIP5 selectively regulates α6 integrin trafficking.

Another Rab11 effector protein Rab11FIP1 was found to regulate trafficking of both α6 and α3 integrin. Rab11FIP1 depletion reduced membrane expression of α3 integrin by increasing its internalization and reducing its recycling. Interestingly there was a major effect on α6 integrin internalization, which increased to a similar extent when α3 integrin expression was depleted. These results demonstrated that FIP1 traffics both the LBIs, and resulting in reduced membrane α3 integrin and same functional effects as loss of α3 integrin expression. Rab11FIP1 hence might be a possible coordinator of the trafficking of the two LBIs which would be important to study in future.

Study of α6 integrin recycling characteristics revealed an interesting phenomenon of recycled integrin being targeted to the cell-cell membrane locations in cancer cells. The presence of both LBIs at cell-cell locations is well established, but the function remains elusive. Although LBIs have been implicated in cell-cell adhesion in various systems,
possibly via extracellular matrix in inter-cellular space. We found the α6 integrin was primarily recycled to these locations instead to lamellipodia in the cell front. Such pattern of α6 integrin distribution is common in PIN lesions and aggressive prostate cancer cells where the basal α6 integrin is found to appear along the entire tumor cell surface, including the cell-cell locations. The significance of such localization of α6 integrin which is actively replenished by recycling must underline a major function of the α6 integrin at these locations and will be an interesting avenue of future research.

Another major finding of current research was the regulation of pro-invasive recycling of α6 integrin by PTEN. PTEN is lost/mutated in 40% prostate cancer and other epithelial carcinomas. PTEN was tested as a prime candidate owing to its role in regulating membrane phosphoinositides. Vesicular transport heavily relies on changing phosphoinositide composition of vesicular and cell membranes. Moreover, the C2 domain of Rab11FIP5 was earlier reported to have a higher affinity to PIP3 as compared to PIP2 or other phosphoinositides. We hypothesized PTEN driven changes in the PIP3 levels in plasma membrane might regulate α6 integrin membrane expression which was earlier found to be dependent on the C2 domain of Rab11FIP5.

In summary, current research defined Rab11FIPs as regulators of α6 and α3 integrins. A unidirectional coordination between α6 and α3 integrin was identified such that loss of α3 integrin, representative of high grade prostate cancer, amplifies integrin α6 integrin internalization and a resultant migratory phenotype. Based on current study it is evident that cancer cells can employ changes in intracellular trafficking pathways to trigger preferential internalization or recycling of one integrin over another. This might provide
an efficient mechanism to quickly respond to respond efficiently to changing extracellular environment during cell migration. The differential expression and trafficking of the LBIs and heterogeneous distribution of α6 integrin in prostate cancer cells observed during perineural invasion and metastasis might be a mechanism to dynamically regulate cell surface adhesion to the laminin rich niche for deployment through nerve and homing in bone for successful metastasis. Based on current study Rab11FIPs are strong candidates to execute this complex LBI interregulation during prostate cancer progression.
VIII. Material and Methods

Cell lines

All cell lines were obtained from ATCC unless otherwise mentioned and cultured in appropriate culture media as mentioned namely, Dulbecco’s Modified Eagle’s Medium (DMEM), Iscove’s Modified Dulbecco’s medium (IMDM) or Roswell Park Memorial Institute (RPMI) 1640 medium from Invitrogen (Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) from HyClone Laboratories (Novato, CA) and grown at 37 °C in a 5% CO₂ humidified incubator. Trypsin-EDTA (Gibco, NY) or non-enzymatic Cellstripper (CellGro, Manassas, VA) were used to disassociate adherent cells into suspension.

Human prostate cancer cell line DU145 originated from brain metastasis, PC3 from bone metastasis, were grown in IMDM with the standard 10% FBS supplement and conditions mentioned above. PC3N cell line, an N-cadherin expressing variant of bone metastatic human prostate carcinoma cells PC3 (Tran et al., 1999) and PC3B1 prostate cancer cells isolated from the bone marrow of SCID mice that had been injected six weeks previously with the PC3 cell line as described earlier (Ports et al., 2009) were maintained in IMDM. LnCaP a prostate cancer cell line obtained from lymph node metastasis was grown in RPMI 1640 supplemented with 10% FBS. VCaP, a vertebral metastasis and RWPE-1 an immortalized cell line transformed using HPV were grown in DMEM. CFPAC a pancreatic cancer cell line used was grown in IMDM. The two nerve cell lines S16 and S16Y were
grown in DMEM in poly-lysine coated tissue culture dishes and conditioned media was harvested after 24 hours. HaCaT cells were grown in DMEM and the conditioned media enriched in laminin-511 and laminin-332 (Sroka et al., 2008) was harvested.

Transfection and generation of stable FIP-GFP mutants: GFP tagged mutant Rab11FIP5 constructs characterized earlier (Prekeris et al., 2000) were a kind gift from Dr. Rytis Prekeris (University of Colorado, Denver, CO). They were transfected into PC3N cell line using lipofectamine 2000 (Invitrogen, Carslbad) as per manufacturer’s protocol and stable clones were selected using G418 and sorted using the FACSaria III (BD Biosciences, San Jose, CA).

Antibodies

Antibodies for flow cytometry are listed based on the experiments. Membrane expression analysis by flow cytometry - rat phycoerythrin (PE) conjugated GoH3 (eBioscience, San Diego, CA) against α6 integrin, eFluor 660 conjugated 439-9B(eBioscience, San Diego, CA) against β4 integrin, rabbit AB1920 (Chemicon, Temecula, CA), against α3 integrin, mouse CD44 antibody (Cell Signaling Technologies, CST, Danvers, MA), rabbit E-cadherin (CST, Danvers, MA), anti-rabbit AlexaFluor 488 (A488) or AlexaFluor 633 (A633) conjugated secondary antibodies and anti-mouse A633 conjugated secondary antibody (Invitrogen, Carlsbad, CA).
Internalization/recycling assay by flow cytometry- Phycoerythrin (PE) conjugated GoH3 (eBioscience, San Diego, CA) and A488 conjugated conjugated GoH3 against α6 integrin were used in internalization and recycling assay respectively. Fluorescein-conjugated IA3 (FAB1345, R&D Systems, Minneapolis, MN) against α3 integrin or FITC conjugated anti-transferrin receptor CD71 (TfR) antibody (eBiosciences) were used for flow cytometry based recycling assay. FITC conjugated TS2/16 (eBiosciences) against-β1 integrin and Alexa Fluor 660 conjugated anti-β4 antibody (clone 439-9B, eBiosciences) used for internalization assay and FITC conjugated anti-β4 antibody (Abcam) for recycling assay.

Fluorophore quenching antibodies required for recycling assay were anti-A488, anti-Fluorescein (Invitrogen, Carlsbad, CA) and anti-FITC mouse monoclonal antibody (Abcam, Cambridge, MA).

Antibodies used for immunofluorescence staining include anti-α6 integrin J1B5 rat monoclonal antibody, anti-EEA1 (Early Endosome Antigen-1) polyclonal rabbit antibody (CST #2411, Danvers, MA), and anti-Rab11a rabbit polyclonal antibody (CST #2413, Danvers, MA), anti-rat Alexa 546, anti-rabbit Alexa 488 conjugated secondary antibodies (Invitrogen, Carlsbad, CA) or anti-rabbit DyLight 650 conjugated secondary antibody (BD Biosciences, San Jose, CA).

Antibodies used for western blotting were anti-α6 integrin antibody AA6NT (Ports et al., 2009), anti-α3 integrin rabbit AB1920 antibody (Chemicon, Temecula, CA), anti-Rab11a (CST, Danvers, MA ), anti-Rab11FIP5 rabbit polyclonal (Novus Biologicals, Littleton, Colorado) and horseradish peroxidase—conjugated secondary antibodies (Jackson
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Immuno-Research Laboratories, Inc., West Grove, PA). Anti-β1 integrin antibody AlIB2 (Developmental Studies Hybridoma Bank, University of IOWA, Iowa City, Iowa) was used for blocking adhesion function of the integrin.

METHODS

Silencing expression by siRNA: Cells (30% confluent) were treated with 25nM Dhharmacon siRNA (Thermo Scientific, Lafayette, CO) specific for protein of interest or with the non-targeting siRNA (SiGENOME Control Pool Non-Targeting #2) using DharmaFECT transfection reagent for 72 hours.

Biotin internalization assay: DU145 cells were harvested using Cellstripper, washed with PBS, and treated with EZ-link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) for 20 minutes at room temperature to biotinylate cell surface proteins. After two washes with PBS, cells were incubated in IMDM containing 10% FBS at 37°C for 0 to 60 minutes for internalization of cell surface biotinylated proteins. Cell were immediately chilled on ice and remaining surface biotin was removed using PBS containing 40 mM glutathione. Cells were lysed in RIPA buffer containing protease inhibitors (Roche) and immunoprecipitated using 1:1000 dilution of J1B5 antibody.

Flow cytometry of membrane expression: DU145 or FIP5-GFP mutant PC3N cells were obtained with Cellstripper (Corning, Manassas, VA) and fixed in 2% formaldehyde. After blocking in 2% BSA/PBS, cells were incubated with receptor specific primary antibodies listed earlier for 30 minutes followed by three PBS washes and 20 minutes incubation
with respective secondary antibodies. Unbound secondary antibodies were washed off and fluorescence of labeled cell surface receptors was measured using BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA).

*Flow cytometry internalization assay:* DU145 cells were obtained with Cellstripper and washed with cold PBS (Phosphate buffer saline) containing 0.2% BSA. Cell surface receptors were labeled with fluorophore-conjugated antibodies specific for integrin subunits or transferrin receptor at 4°C for 45 minutes. Unbound antibody was removed with cold PBS. Cells were incubated in media supplemented with 10% FBS at 37°C for internalization, followed by stopping the reaction at 4°C. Antibody remaining at the cell surface was removed using cold acid solution (0.5M NaCl, 0.2M CH₃COOH) for 5 minutes followed by PBS wash. Cells were fixed in PBS containing 1% formaldehyde and analyzed using a FACScan (BD Biosciences, San Jose, CA). Internalized label for each time point was calculated as a percent of the full label mean peak fluorescence (MPF) value. At least three independent experiments were performed for each receptor. A first-order kinetics model was created using KaleidaGraph (Synergy Software) and provided a good fit for the receptor internalization kinetics ($R^2$>0.98) according to the following formula,

$$y = a + b \left[1 - \exp (-k_{obs} t)\right],$$

where, $y$ is the amount of receptor internalized at time $t$ (in min), $b$ is the maximum intracellular accumulation of the receptor, and $k_{obs}$ is the observed first-order rate constant (min⁻¹) (Wiley and Cunningham, 1982b). Actual rate constant $k_{actual} = b \times k_{obs}$
was calculated as a measure of net internalization rate at the steady state of maximum internal accumulation. All values are reported as mean±standard error.

**Flow cytometry recycling assay:** Cell surface integrins or TfR were labeled with fluorophore-conjugated antibodies at 37°C in 10% FBS supplemented media for 60mins for maximum internalization. Fluorescence of uninternalized cell surface antibodies was quenched by incubating with respective anti-fluorescent quenching antibodies (listed above) at 4°C for 30 minutes. Intracellularly protected fluorescent antibody labeled receptors were allowed to recycle back to the cell surface at 25°C. The recycled label was also quenched same as earlier. Cells were fixed and analyzed using BD Accuri C6 flow cytometer as above. The amount of recycled label was measured as a loss of intracellular signal and plotted as a percent of total internalized intracellular label. A first-order kinetics model \( y = a + b \left[ 1 - \exp\left(-k_{\text{obs}}t\right)\right] \) was fitted \( R^2>0.98 \) using KaleidaGraph (Synergy Software) to obtain the recycling curve where, \( y \) is the amount of receptor recycled at time \( t \) (in min), \( b \) is the total amount of receptor recycled, and \( k_{\text{obs}} \) is the observed first-order rate constant \( (\text{min}^{-1}) \) (Wiley and Cunningham, 1982a). Actual recycling rate constant \( k_{\text{actual}} = b \cdot k_{\text{obs}} \) was calculated as a measure of net recycling rate at the steady state. All values are reported as mean±standard error.

**Immunofluorescence staining:** DU145 cells grown to 70% confluence on glass coverslips were incubated with anti-α6 integrin antibody diluted to 1:100 IMDM+10% FBS media for 40 minutes. The cells were fixed using PBS containing 2% formaldehyde and permeabilized with PBS containing 0.2% Triton X-100. After blocking with 2% BSA for 20 minutes at room temperature, cells were incubated with primary antibodies against
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endocytic markers EEA1 (1:100) and Rab11a (1:100) for 30 minutes followed by incubation with anti-rabbit A488 or anti-rabbit DyLight 650 conjugated secondary antibodies for 20 minutes. Coverslips were mounted on slides using Prolong diamond antifade (Invitrogen, Carlsbad, CA). Images were acquired using specimens were imaged using a DeltaVision Core system (GE Healthcare Bio-Sciences, Pittsburgh, United States of America) equipped with an Olympus IX71 microscope, a 60X objective (NA 1.20), and a cooled charge-coupled device camera (CoolSNAP HQ2; Photometrics). Single plane images were acquired and deconvolved with softWoRx v1.2 software (Applied Science). Images were analyzed using ImageJ plugin Just Another Colocalization Plugin (JACoP) to measure Pearson coefficient of co-localization (Pr) and percent colocalization.

Immunofluorescence imaging based recycling assay: Adherent cell monolayer of DU145 cells grown on glass coverslips were reacted with anti-α6 integrin rat antibody (J1B5) at room temperature (RT) for 60 mins to allow surface labelling and internalization of antibody bound integrin. Uninternalized α6 integrin at the cell surface were blocked by incubating with anti-rat A564 fluorophore conjugated antibody at 4°C for 30 minutes. Cells were then incubated at RT for 0, 10, 20 and 40 minutes (with or without 0.5 mM primaquine (Invitrogen, Carlsbad, CA), a recycling inhibitor used for control) to allow recycling of internalized J1B5 labeled integrin for different time intervals. Cells were fixed with 2% formaldehyde and recycled integrin was stained with anti-rat A488 fluorophore conjugated antibody. Coverslips were mounted on slides using Prolong diamond antifade (Invitrogen, Carlsbad, CA) and imaged and deconvolved using the 40X objective (NA 1.20) of the DeltaVision Core system as explained in previous section.
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Average pixel intensity of recycled label was measured using ImageJ and reported standardized as per number of cells.

Cell migration assay: Cell migration was assessed using a modified Boyden chamber assay. Cell culture inserts (BD Biosciences, San Jose, CA, USA) of pore size 8 μm were coated on the underside with laminin enriched 50 μL HaCaT conditioned media overnight at 4°C. Approximately 15,000 cells (siRNA treated) in 200 μL IMDM were plated into the upper chamber of each insert. Function blocking anti-β1 integrin antibody AIIB2 (1 mg/ml; 1:100) was added to cells prior to plating. Inserts were placed into wells containing 600 μL IMDM plus 10% FBS in a 24-well tissue culture plate and incubated for 6 hours at 37°C in a humidified incubator. Cells on the underside of the insert were fixed/permeabilized in methanol/acetone and stained with 4', 6-diamidino-2-phenylindole (DAPI) (1μg/mL) (Sigma Chemical Co., St. Louis, MO, USA) for nuclei detection. The cells were imaged in fifty sections of each insert using the 20X objective of above mentioned DeltaVision Core system. The average number of cells / field of view was counted based on DAPI stained nucleus using “Analyze particles” plugin of ImageJ.

Cultrex invasion assay: The Cultrex laminin invasion assay (Trevigen Inc., Gaithersburg, MD) was performed as recommended by the manufacturer. Briefly, DU145, PC3, and CFPAC1 cells were plated in 10 cm plates and cultivated until 80% confluent and then serum starved overnight. The upper chambers of the Cultrex plates were coated with 50 μL of 0.2x mouse laminin 111 (5x laminin solution diluted in 1x coating buffer supplied by Trevigen) at 37°C overnight. The cells were harvested and 50 μL of cell suspension
was added to each well. The J8H and AllB2 blocking antibodies (1 mg/ml; 1:100) were added to the cells prior to plating. The access port was used to add 150 µL of control or conditioned medium to each well. The cells were then incubated for 24 h in a 37 °C CO₂ incubator. The following day, the top and bottom chambers were aspirated and washed twice, and invaded cells were detected using calcein-AM containing cell dissociation buffer and an immunofluorescence plate reader at 485/520 nm per the manufacturer’s instructions.

*Human prostate tissue microarray and Immunohistochemistry:* Tissue microarray (TMA) of patient cancers were obtained from the Prostate Cancer Donor Rapid Autopsy Program at the University of Washington (UW) from consented subjects (Roudier et al., 2003). Samples from bone and grossly visible metastatic disease in lymph nodes, liver, lung and other sites were collected in a systematic and consistent fashion on every case. A TMA (UWTMA21) was constructed from 44 patients with 185 metastatic sites of which 121 are bone metastases and 64 are soft tissue metastases (Zhang et al., 2011). The soft tissue metastatic sites included 35 lymph nodes, 19 livers, and 10 other soft tissue sites. Each metastatic site was represented by 2 cores. The immunohistochemical staining was performed as described. Briefly, tissues were retrieved using Na/EDTA buffer pH 8.0 and immunohistochemistry performed using the AA6NT antibody (1:700) on the Discovery XT Automated Immunostainer (Ventana Medical Systems, Inc., Tucson, AZ). Membrane and cytoplasmic staining were scored separately by a pathologist (BSK).

*Immunoblotting:* Cell lysates were prepared by harvesting the cells into PBS (phosphate buffer solution, pH 7.4) and centrifuging the cells (2500 rpm for 5 min at 4°C). The pellet
was resuspended in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris, 150 nmol/L NaCl, 1% Triton X-100, 0.10% sodium dodecyl sulfate, and 1% deoxycholate) with complete mini protease inhibitor cocktail (Roche, Indianapolis, IN). The lysates were incubated on ice for 30 min and centrifuged (14,000 rpm for 5 min at 4 °C). Protein quantification was performed using the Bradford Assay (Bio-Rad Laboratories, Hercules, CA). Samples were loaded onto 10% or 4-12% Pre-cast SDS-PAGE Bis-Tris gels (Life Technologies) and electrophoresis was performed at 160V using 1X MOPS running buffer (Life Technologies).

After electrophoresis of the proteins, the SDS-PAGE gel was transferred onto a MeOH activated Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) using a transfer cassette of the Novex Mini cell system, at 35 volts for 2 hours. The membranes were blocked in 10mL Tris-buffered saline with 1% (v/v) Tween (TBST) containing 5% (w/v) powdered milk for 1 hour at room temperature and probed for protein of interest by incubating with primary antibody at 4°C overnight. Membranes were washed with TBST 3 times and HRP-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) was added at 1:10,000 dilution. in 10mL of 5%milk/TBST and incubated for 1 hour. Secondary antibodies were washed off using TBST. The proteins were then visualized using chemiluminescence (Western Lightning Plus ECL Western Blotting Detection System, Perkin Elmer). And a western blot imaging system (Syngene, Frederick, MD).

Statistical analysis: Student’s t test for unpaired samples was used throughout. For internalization/recycling assays, statistical significance is tested for each timepoint as
well as for the calculated rate kinetic parameters using student’s t-test for unpaired samples assuming unequal variance. A P-value lower than 0.05 was considered statistically significant.
**Figure S1. PC3N cells are negative for β4 integrin expression.** Du145 and PC3 cells were labeled with an antibody against β4 integrin to and cell surface levels of β4 integrin was measured using flow cytometry. Flow histograms of β4 surface levels in unlabeled PC3N cells (black), PC3N cells labeled with a non specific fluorophore conjugated secondary antibody (2° Ab only, brown) and DU145 (red) and PC3N (blue) cells labeled with antibody against β4 integrin. The mean peak fluorescence values for each condition is reported.
Figure S.2 α6 integrin recycling at 25°C versus 37°C. Recycling assay performed for α6 integrin in DU145 cells at 37°C or 25°C. Recycling kinetic curve measured and plotted as described earlier and protocols. First order kinetic curve is fitted using Kaleidagraph ($R^2>0.98$).
Figure S.3. Depletion of Rab11FIP5 expression reduces recycling of β4 integrin. Recycling assay was performed for β4 integrin in untreated and siFIP5 (#1) treated DU145 cells. Percent label recycled to the cell surface at 25°C (calculated as percent mean peak fluorescence of recycled label at a given timepoint vs total label internalized) is plotted for different time intervals. First order kinetic curve is fitted using Kaleidagraph ($R^2>0.98$). Maximum label recycled (%) and recycling rate constants (observed, $k_{obs}$ and actual, $k_{actual}$) calculated as per first order rate kinetics. Results represent 3 independent experiments. Statistical significance calculated for changes in label internalized at each timepoint and intracellular accumulation as per student’s t test, unpaired, **p<0.01, n=3
Figure S.4. Phenotype of DU145 silenced for Rab11FIP5 expression. Untreated and siFIP5 treated DU145 cells show significantly different morphologies. siFIP5 cells lose the epithelial phenotype observed in wild type DU145 cells. About 20% of cells loose cell adhesion to substrate and are found suspended in the media. The cell media was centrifuged and the cells in suspension were replated and allowed to grow for 72 hours (bottom left). The cells in suspension reattached. siFIP5 cells grown for a long period of time of 14 day reverse their phenotype and start resembling the wild type DU145 cells.
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