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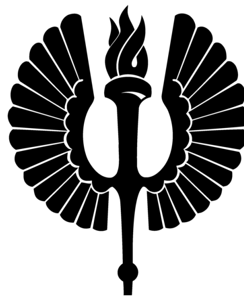
Integrins on the move

by

Antti Arjonen



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To Lenni and Kuutti

Antti Arjonen
Integrins on the move

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Annales Universitatis Turkuensis, Medica-Odontologica
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ABSTRACT

Cancer is a leading cause of death worldwide accounting for 13% of all deaths in 2005. The spread of cancer and formation of metastases is the major cause of mortality among cancer patients.

The spread of cancer is based on the cancer cell's ability to break away from the surrounding tissue and to migrate into new areas in the body. The ability of cells to bind its surroundings and to move is controlled by the mechanical cell surface adhesion receptors called the integrins. Integrins have a critical role in cell adhesion, cell motility and tissue homeostasis. By communicating with ECM, integrins transmit signals from the surrounding environment inside the cell and modulate the function of many important signalling pathways involved in cell survival, development, gene expression, proliferation, motility and cytoskeletal organization.

During cell migration integrin-matrix adhesions are formed in front of the cell while rear-adhesions are released during migration. Integrins are endocytosed from the plasma-membrane into the cytoplasm and partly recycled back to new adhesion sites in a process called integrin trafficking.

Also, the cell cytoskeleton and protrusions are important in cell migration. Finger-like actin protrusions called filopodia display an interesting cancer relevant cooperation with integrins that is required for cell migration.

The expression and function of integrins changes markedly as cells acquire carcinogenic properties. Changed integrin function is partly responsible for detachment of tumor cells from neighbouring cells and for providing enhanced invasive capabilities for tumor cells to disseminate. Similarly, the formation of filopodia is increased in cancer. High myosin-10 expression is related to poor outcome in breast cancer and increased cell migration. The proper function of myosin-10 induced filopodia needs association with β1 integrins.

The importance of integrin trafficking and filopodia formation is becoming increasingly more recognized in cancer. This thesis focusses on the role of integrins, integrin trafficking and myosin-10 induced filopodia cancer cell migration.

Keywords: Integrin, Filopodia, Endocytosis, Integrin trafficking, Myosin-10

Antti Arjonen
Integriinit liikkeessä

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TIIVISTELMÄ

Syöpä on yksi ihmisen yleisimmistä kuolinsyistä ja käsitti arviolta 13% kaikista kuolemantapauksista vuonna 2005. Syövän leviäminen ja etäpesäkkeiden muodostuminen on suurin kuolleisuuden aiheuttaja syöpäpotilailla.

Syövän leviäminen perustuu syöpäsolujen kykyyn irtautua ympäröivän elimistön kontrollista ja kykyyn tunkeutua muualle elimistöön. Solujen tarttumista ympäristöönsä ja liikkumista säätelevät solunpinnan tarttumisreseptorit, integriinit. Integriinit sitoutuvat solunulkoiseen väliaineeseen, ja välittävät viestejä solujen sisälle ylläpitäen elimistön normaalia tasapainoa. Integriinit säätelevät monien tärkeiden viestinvälitysketjujen toimintaa, ja osallistuvat solujen selviytymisen, solukehityksen, geeniekpression, solujakautumisen, liikkumisen ja solun tukirangan toimintaan.

Solujen liikkuessa uusia integriinien ja soluväliaineen välisiä tarttumakohtia muodostuu jatkuvasti solun etuosassa ja niitä puretaan solun takaosassa. Integriinit otetaan solun sisälle endosytoosin kautta ja kierrätetään vesikkeliliikennöinnissä takaisin solun pinnalle. Myös solujen tukirangalla ja solu-ulokkeilla on tärkeä merkitys solujen liikkumisessa. Sormimaiset aktiiniulokkeet, filopodiat, toimivat yhteistyössä integriinien kanssa solujen liikkumisen aikana ja niillä näyttäisi olevan tärkeä rooli myös syövän leviämisessä.

Integriinien säätely ja ilmentyminen muuttuvat merkittävästi kun terveestä solusta syntyy syöpäsolu. Muuttunut integriinien toiminta on osittain vastuussa syöpäsolujen irtautumisesta viereisistä soluistaan kudoksessa. Muuttunut integriinien toiminta ja liikennöinti edesauttavat syövän leviämistä. Myös filopodioiden ilmentyminen on lisääntynyt syövässä. Myosiini-10 aktiinimoottori on yksi filopodioita muodostavista proteiineista. Korkea myosiini-10 geenin ilmentyminen on yhteydessä huonoennusteiseen rintasyöpään, syövän leviämiseen ja lisääntyneeseen solujen liikkumiseen. Myosiini-10 proteiinin toiminta filopodioissa tarvitseen integriinien apua, jotta solujen liikkuminen olisi mahdollista. Tämä väitöskirja tutkii integriinien liikennöinnin ja myosiini-10 filopodioiden merkitystä syövän leviämisen kannalta.

Avainsanat: Integriini, filopodia, endosytoosi, reseptoriliikennöinti, myosiini-10

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ABBREVIATIONS

ARF6	ADP-ribosylation factor 6
Arp2/3 complex	Actin related proteins 2 and 3 complex
CavME	Caveolin-mediated endocytosis
CCV	Clathrin-coated vesicle
Cdc42	Cell division cycle 42
CLP	Calmodulin-like protein
CME	Clathrin-mediated endocytosis
DN	Dominant negative
DSHB	Developmental Studies Hybridoma Bank
ECM	Extracellular matrix
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EYFP	Enhanced yellow fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ESCRT	Endosomal sorting complex required for transport
FACS	Fluorescene-activated cell sorting
FERM	Band 4.1/ezrin/radixin/moesin
GPI	Glycosylphosphatidylinositol
IF	Immunofluorescence
IHC	Immunohistochemistry
IP	Immunoprecipitation
kD	Kilodalton
MTOC	Microtubule organizing center
MyTH4	Myosin tail homology 4 domain
PH	Pleckstrin homology
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PNRC	Perinuclear recycling compartment
PTB	Phosphotyrosine binding domain
RFP	Red fluorescent protein
Rluc	Renilla luciferase
RNA	Ribonucleic acid
siRNA	small interfering RNA
shRNA	small hairpin RNA
qRT-PCR	Quantitative real-time polymerase chain reaction
WASP	Wiskott-Aldrich syndrome proteins
WAVE	WASP family verprolin homologous
WB	Western blotting
wt	Wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals:

I **Small GTPase Rab21 regulates cell adhesion and controls endosomal traffic of β1-integrins**
Pellinen T, Arjonen A, Vuoriluoto K, Kallio K, Fransen JA, Ivaska J.
J Cell Biol. 2006

II **PtdIns(3,4,5)P_3 is a regulator of myosin-X localization and filopodia formation**
Plantard L*, Arjonen A*, Lock JG, Nurani G, Ivaska J, Strömblad S.
J Cell Sci. 2010

III **Distinct Recycling of Active and Inactive β1 Integrins**
Arjonen A, Alanko J, Veltel S, Ivaska J.
Traffic. 2012

IV **Myosin-10 promotes breast cancer invasion and metastasis downstream of mutant p53**
Arjonen A*, Kaukonen R*, Rouhi P, Högnäs G, Sihto H, Miller B, Bucher E, Cao Y, Sansom O, Joensuu H, Ivaska J
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* Equal contribution

1. INTRODUCTION

Cancer is a genetic disease that arises from dynamic changes in a human genome. The key features of cancer are altered patterns of gene function that eventually leads to cellular behaviour in which cells no longer are maintained in homeostasis with surrounding tissue. The hallmarks of cancer (Hanahan and Weinberg 2011) are changed patterns of cell or tumor-stroma behaviour nowadays well documented in cancer research. They include: sustainment of proliferative signals, protection from tumor suppressors, gaining replicative immortality, induction of angiogenesis, resistance of cell death, altered cellular metabolomics, avoiding immune destruction, tumor-pomoting inflammation, genomic instability and activation of invasion and metastasis - the hallmark discussed in this thesis. Cancer cells can proliferate without control giving rise to primary tumors, or they can invade surrounding tissues or even metastasize to different places in the body giving rise to secondary tumors (i.e metastasis).

Invasive and metastatic behavior of cancer is the main cause of death among all cancer patients. The basic life supporting functions of cancer patient's organs and the metabolic system collapse in the end, since the tumor cells, unable to fulfill the life supporting tasks of normal cells, have replaced the cells of the tissues in vital organs. The migration and invasion of cancer cells is directly implicated in spreading of cancer. Cell migration is a complex process involving transient signalling and spatio-temporal regulation of cell cytoskeleton, cellular adhesions and cell protrusions.

In this thesis the interplay of integrin function, integrin trafficking and regulation of filopodia is discussed. As a cell migrates new cell-ECM connections, i.e adhesion sites are, constantly formed and disassembled at the leading edge. This requires turnover and moving of integrins bilaterally and also via intracellular microtubule tracks – process called integrin endocytic trafficking. Making of new integrin adhesion sites also requires modulation of actin cytoskeleton. Filopodia function as antennae-like sensors probing for new integrin adhesions at the leading edge. Myosin-10 is a strong inducer of filopodia that needs integrin to fullfill its pro-migratory role. Myosin-10 and integrin cooperation is needed for cancer cell invasion.

The results in this thesis give novel mechanistical insigths how cancer cells employ integrin trafficking and make myosin-10 induced filopodia. Most importantly we are able to show myosin-10 is indeed needed for lung metastasis formation in mice and high myosin-10 expression correlates with poor survival among Finnish breast cancer patients.

2. REVIEW OF THE LITERATURE

2.1. CELL SURFACE IS THE MAIN INTERFACE IN LIFE

In order to understand the fundamental aspects of life and cell biology we must understand the structure and function of the cell surface and plasma-membrane. The plasma-membrane is an evolutionarily conserved lipid bilayer which as such has promoted and protected the development of life. The plasma-membrane has also been the prerequisite for development of all higher organisms and to be exact a platform for the key cellular processes discussed in this thesis. The plasma-membrane is a lipid bilayer composed of phospholipids, glycolipids and cholesterol. The phospholipids are polarized. The hydrophilic or polar (globular) heads are pointed towards the extracellular and intracellular environments whereas the hydrophopic sidechains form the inner parts of the bilayer (Alberts 1994). Several proteins are embedded in the lipid bilayer. Proteins can extend throughout the plasma-membrane (transmembrane proteins) or they can be peripheral only. Also, some of the proteins can move freely within the lipid bilayer whereas others are strictly organized as clusters or fixed to a certain location, kept in place by intracellular cytoskeletal elements.

Plasma-membranes are also known to contain microdomains with more highly organized and different lipid composition compared to the overall plasma-membrane. These patches at the plasma-membrane are also termed lipid rafts. Lipid rafts contain up to 5-fold more cholesterol, more sphingolipids and float freely in the plasma-membrane (Simons and Ikonen 1997). Lipid rafts serve as organization centers by sorting specific subtypes of membrane and assembling signalling proteins in close proximity (Janes et al. 2000). Lipid rafts especially associate with glycosylphosphatidylinositol (GPI) anchored proteins (Sangiorgio et al. 2004). Lipid rafts also have a central role in endocytic protein trafficking together with caveolins (Deckert et al. 1996) by mediating the endocytosis of GPI anchored proteins.

2.2. INTEGRINS

2.2.1 Integrin superfamily and structure

Integrins are a family of large transmembrane heterodimer cell surface adhesion receptors composed of 18 α and 8 β subunits. Integrins are expressed in all multicellular organisms and different cell types. Integrins regulate many pivotal cellular functions such as cell survival, motility, development and proliferation (Hynes 2002).

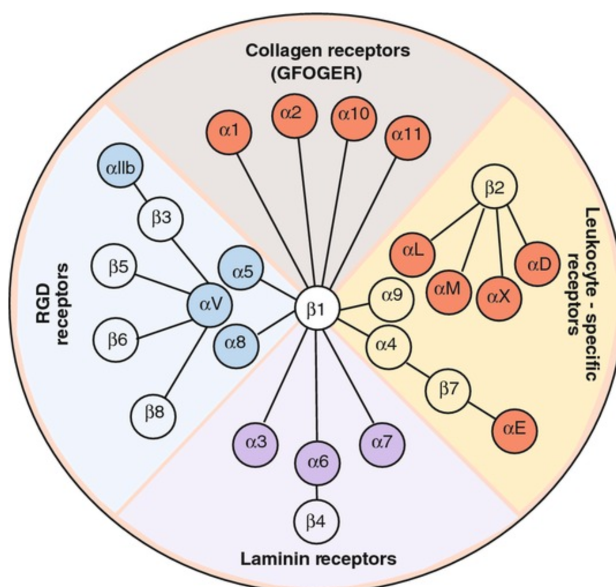

Figure 1. Different pairs of integrin heterodimers and their ligands. Adapted from (Barczyk et al. 2010).

The integrin subunits are non-covalently associated and form 24 different heterodimer pairs. Each heterodimer pair has a preference to bind specific extracellular matrix (ECM) proteins or cell surface ligands. Based on ligand specifity, integrin heterodimers can be classified to four main families: collagen, RGD (arginine-glycine-aspartic acid –motif), laminin and leukocyte-specific receptors (Barczyk et al. 2010).

Structurally, the largest part of the integrin heterodimer receptor is ligand binding globular extracellular domain. Both α and β subunits also have a single transmembrane domain and short cytoplasmic tail. Although short, the integrin tails are known to associate with many proteins and also with the cell cytoskeleton. Thus, integrins function as a linker between ECM and the cell body (Campbell and Humphries 2011).

2.2.2 Integrin activation and inactivation

The function of integrins is regulated by activation and inactivation of the receptor complex. Integrin activation involves a large conformational change mainly in the extracellular domain. Since integrins have only short cytoplasmic tails the signalling processes are carried out by intracellular tail binding proteins. Integrins can signal bidirectionally across the plasma-membrane. In the model of outside-in signalling the extracellular ligand binding results in activation of the receptor. The activation leads to conformational change, tail separation and allows cytoplasmic regulators bind the tails. Ligand binding also

clusters the integrins together to form multimeric complexes of several heterodimers. On the other hand, integrins can also be activated from inside of the cell. The inside-out signalling is triggered by an intracellular activator such as talin or kindlin that bind to the β tail. Talin binding results in a conformational change that increases the integrin affinity towards extracellular ligands (Shattil et al. 2010).

In the “switch-blade” model integrins change their conformation upon activation. Integrin receptors are either in a bent or extended conformation and the headpiece (ligand binding domain) is either closed or open (Takagi et al. 2002; Luo et al. 2007). Integrin receptor conformation fluxtuates between different conformation states that can be:

1) closed headpiece (bent). Low affinity.
2) closed headpiece (extended). Intermediate affinity. Also called primed.
3) open headpiece (extended). High affinity. Also called active or ligand-bound.

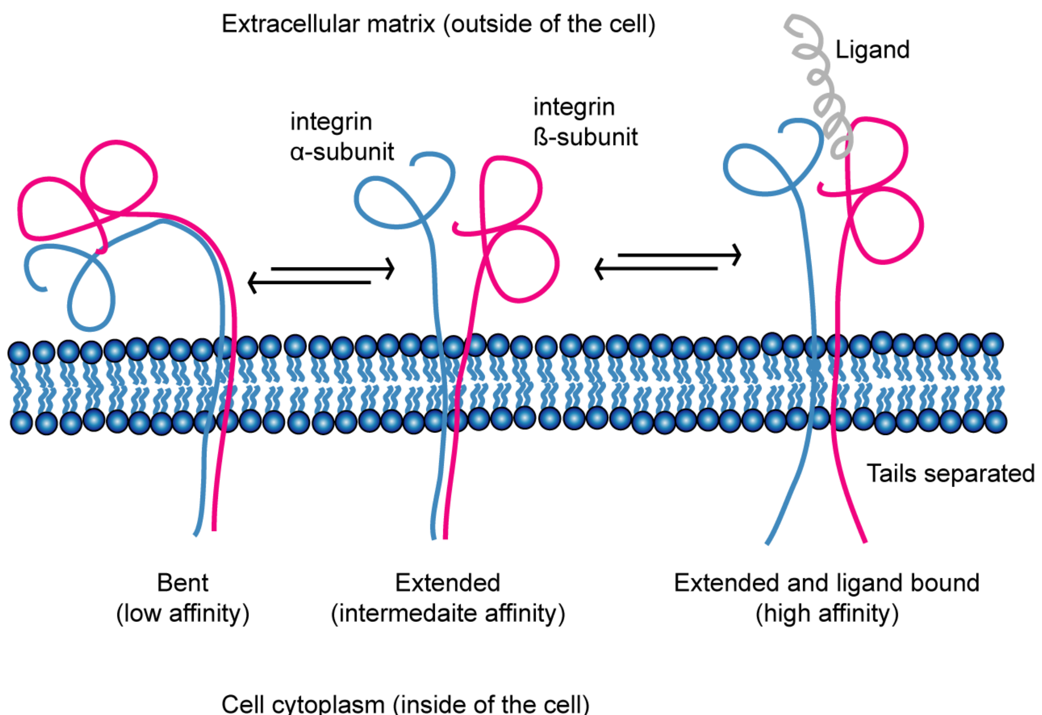

Figure 2. Artistic view of different integrin receptor conformations during activation

It is believed that in the bent conformation the ligand binding pocket is facing towards the plasma-membrane and there is no room for ligand binding. Integrin

extension would thus allow ligand binding to take place. In another controversial model of integrin activation called “deadbolt” also the bent conformation could bind the ligand (Xiong et al. 2003).

The conformational change of the extracellular domain upon integrin activation is followed by the separation of the α and β subunit cytoplasmic tails. The tails are kept together in close proximity by a salt bridge when the integrin is in the bent conformation. Following integrin activation, the tails become separated and the salt bridge is abolished. The separation of the tails is a key process in integrin function since it allows the binding of intracellular regulatory proteins to the integrin tails (Kim et al. 2003). The functional importance of tail separation is highlighted when the tails are forced together by a locked-together mutation (Askari et al. 2010). The locked together mutation prevented ligand binding and cell spreading.

The cytoplasmic domain of the integrin β-subunits contain two conserved NPXY (Amino acids: asparagine, proline, any, tyrosine) motifs which are recognized by phosphotyrosine binding (PTB) domain containing proteins (Calderwood et al. 2002). The two important cytoplasmic regulatory proteins talin (talin-1/2) and kindlin (kindlin-1/2/3) binds to the NPXY motif via the band 4.1/ezrin/radixin/moesin (FERM) domain. Talin binding is thought to be a key step in integrin activation and silencing talin inhibits the integrin activation (Tadokoro et al. 2003). While talin binds to the membrane proximal NPXY motif the kindlins bind to the membrane distal NPXY motif. Kindlins are thougth to regulate the integrin activation together with talin (Harburger et al. 2009). Integrin inactivation can be achieved by a competition for the same binding sites with the activators; talin and kindlin. At least filamin, dok1 and integrin-cytoplasmic-domain-associated protein 1 (ICAP1) have been shown to share an overlapping binding sites with the activators (ref). Recent studies have also identified novel integrin inactivators. SHARPIN was found to interact with the conserved GFFKR aminoacid sequence in the integrin α tail and to inhibit the recruitment of talin or kindlin to the β tail, leading to integrin inactivation (Rantala et al. 2011).

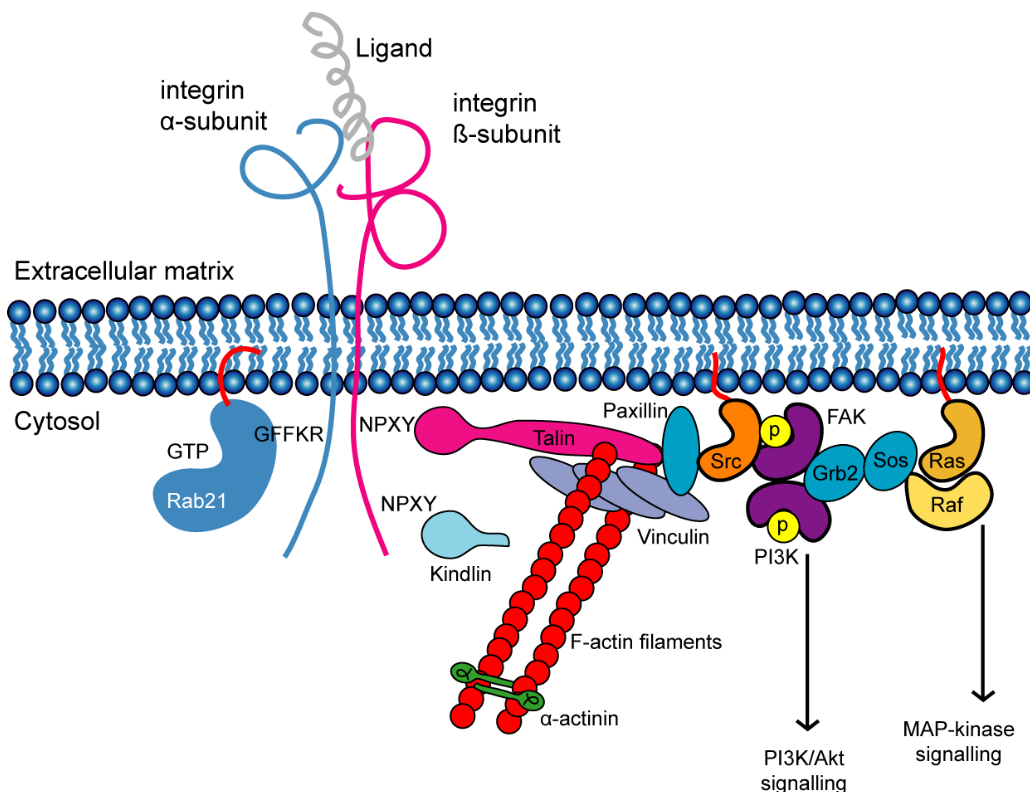

Figure 3. Integrins and the focal adhesion. Illustrated are the main components of the focal adhesion and the related signalling pathways triggered by integrin clustering. Note the connection to the actin cytoskeleton. Adapted from (Alberts B 1994).

2.2.3 Focal adhesions

Integrin containing adhesions are very dynamic. The mechanisms controlling adhesion formation are not well understood, but adhesion formation is known to require at least integrin activation and clustering. Adhesion formation presumably starts by focal complex formation. After the initial event where integrin-ECM contact has been established the adhesion sites are known to grow in size and to maturate from small dot-like focal contacts to large actin stress-fibre associated focal adhesions. During focal complex maturation more adhesion related structural component proteins, such as α-actinin, enter the adhesions. Some of these structural focal complex proteins, such as paxillin, are present at the adhesion from the beginning. Mature focal adhesions can even further evolve to fibronectin and tensin rich fibrillar adhesions (Pankov et al. 2000). Focal adhesions are large structural macromolecular assemblies containing about one hundred different adhesion related proteins. The function of focal adhesions is to connect cell's cytoskeleton to the extracellular matrix

but together with the anchoring function also mechanosensing and signalling functions have been descriped (Zamir and Geiger 2001; Wozniak et al. 2004).

2.2.4 Integrins and cell migration

Adhesive forces to other cells and to ECM maintain non-migrating cells at their positions. In order to move and spread within tissues, non-neoplastic cells and tumor cells can use different kind of migration strategies. Cancer cell migration is basically similar to migration of non-neoplastic cells. In short, cell migration is mechanistically a series of independent steps: First, polarization and elongation of the cell is needed to induce protrusions and actin polymerization in the direction of migration. Secondly, adhesions are formed which in turn preserve the polarity and actin polymerization at the leading edge. Thirdly, traction is generated throughout the cell body by cell contraction, and finally adhesions at cell's rear are disassembled and the trailing edge is retracted.

Upon initiation of migration the cells polarize and organize themselves into advancing and retracting portions. Polarization prior to the migration is essential, since front and rear portions have distinct signaling events, and these events create the actual "migration gradient" throughout the cell, which is the basis of cell movement. Leading edge formation marks the initiation of the polarization process and it starts in response to chemokine, growth factors or ECM molecules through cell surface receptors. Engagement of surface receptors at the plasma-membrane leads to localized activation of cell division cycle 42 (Cdc42) and Rac, which in turn decrease Rho activity and enhance PI3K activity to produce phospholipids. Localized production of lipids, such as PI(3,4)P2 and PI(3,4,5)P3 (shortly PIP3), enhances actin nucleation and polymerization and dictates the formation of the leading edge to this site. Phospholipids produced by PI3K at the leading edge are also principally responsible for the gradient formation towards the direction of migration throughout the cell (Ridley et al. 2003; Srinivasan et al. 2003).

In addition to intital polarizationthe maintenance of the polarized gradient is important to generate polarized and persistent cell migration. PTEN is a protein phosphatase which removes position 3-site phosphorylated residues from lipids and converts PIP3/PI(3,4)P2 back to their precursors PI(4,5)P2/PI4P respectively. In *Dictyostelium*, which is an amoeba and the best studied model organism in cell migration, PTEN further enhances and upkeeps the polarized gradient inside the cells by localizing to cell's edges and to the rear portion after initial leading edge formation. Thus, no PIP3/PI(3,4)P2 is present dominantly in polarized cells elsewhere, except in the leading edge (Funamoto et al. 2002). Similar exclusion of PTEN from leading edge is described in migration of mouse leukocytes, in which the pathway controlling localized F-actin formation at the leading edge is much more complex. Li et al. have suggested p21-activated kinase (PAK-1) dependent activation of Cdc42 is

needed for directional sensing and persistent migration (Li et al. 2003). Cdc42 also localizes the MTOC (microtubule organizing center) to the front of the nucleus oriented towards the direction of forward movement. As the name suggests, the MTOC mediates the formation of microtubule network. It may also contribute to polarized migration by organizing microtubule-mediated transport of vesicles and proteins to the leading edge providing new proteins from the Golgi to the advancing membrane and new protrusions (Rodriguez et al. 2003).

The local activation of Rac and Cdc42 induces actin nucleation at the leading edge together with Wiskott-Aldrich syndrome protein (WASP), WASP family verprolin homologous family protein (WAVE) and actin-related protein 2 and 3 (Arp2/3) complex (Ridley et al. 1992; Campellone and Welch 2010). This localized induction of actin polymerization is responsible for protrusion formation to the direction of migration. Formation of a protrusion also precedes adhesion formation and is essentially needed for the migration cycle to proceed. The formation of actin protrusion is mainly restricted to cell front as a result of PIP3 phosphatase PTEN. The protrusions are stabilized by integrins and other adhesion molecules, which link the dynamic actin cytoskeleton to ECM (Huttenlocher and Horwitz 2011).

A self-enhancing loop is established to support directional migration. New adhesions with ECM reinforce high Rac, PI3K and Cdc42 activity, which in turn stimulate actin polymerization and further prime new integrins to bind in a more sensitized manner to new ECM ligands (Ridley et al. 2003). This process of integrin priming or integrin activation to a high affinity state via the cytoplasmic tails of integrins is called integrin inside-out signaling (see chapter 2.2).

In order to complete the migration cascade, adhesions have to be disassembled. The mechanism controlling the adhesion disassembly is not well understood. Disassembly of adhesions takes place at the rear of the cell, and also in the front of the cells. In the front, adhesions are disassembled just behind the leading edge. Some focal contacts however are not disassembled and they mature into larger focal adhesions. The cell slides over the static adhesions as it migrates forward. Entry of α-actinin into focal contacts marks the maturation of focal contacts whereas targeting microtubules to focal contacts marks adhesion disassembly (Ridley et al. 2003; Small and Kaverina 2003). The disassembly of focal contacts is probably coordinated by a signaling cascade which includes the activation of focal adhesion kinase (FAK) and Src, and the accompanied formation of a so called adhesion signaling complex. Adhesion signaling complex formation mediates the localized activation of Rac and mitogen-activated protein kinase ERK which also marks the focal contact disassembly.

At the rear of the cell, similar FAK and Scr related disassembly cascades take place, but high tension in retracting fibres also seems to play an important role. Properly polarized and migrating cells usually have a distinct elongated tail. The tail is presumably formed because the leading edge moves forward and the tail is anchored to the matrix. The adhesions at the rear show strong interactions with the ECM, and the tension formed between front and the rear of a migrating cell is sufficient to break the molecular link between the integrins and the actin cytoskeleton. It has also been suggested that the tension in the rear can increase intracellular calcium levels via opening stretch-activated calcium channels in the plasma-membrane. The influx of extracellular calcium could further activate calcium dependent protease calpain, which cleaves many proteins present at focal contacts causing adhesions to disintegrate (Lee et al. 1999).

Strong anchoring of the tail to the matrix decreases the forward net migration but probably maintains the directional persistency of migration of a polarized cell (Ridley et al. 2003). Following the disassembly of adhesions, integrins are detached from their substrates and either internalized into the cell via endocytic vesicles or the link between cell cytoskeleton and integrin is cleaved and matrix bound integrin is left behind (Friedl and Wolf 2003).

Together with the actin polymerization that pushes lamellipodia forward the movement or force in cell migration is achieved via acto-myosin contraction. While integrins act as cells feet on the matrix, acto-myosin contraction is needed for contraction of the whole cell body. Contraction leads to forward gliding of the cell body and cell’s trailing tail. The force in contraction is generated via active myosin-II which binds to actin. Myosin-II motor proteins are able to walk along actin, and thus, stationary myosin-II proteins are pulling the actin fibres and generating the contraction. Myosin-II is activated by calcium and calmodulin dependent myosin light-chain kinase (MLCK). MLCK phosphorylates the light-chains of Myosin-II and activates it. The process is negatively regulated by MLC phosphatase, which dephosphorylates Myosin-II light-chains. MLC phosphatase is again phosphorylated and inhibited via RHO-associated serine/threonine kinase (ROCK) (Friedl and Wolf 2003). The cellular structures which are related in cell migration are illustrated in figure 4.

2.3. CELL CYTOSKELETON AND FILOPODIA

2.3.1 Cell cytoskeleton

The cell cytoskeleton is a network-like framework, cellular scaffolding, composed of different filamentous proteins. The main function of cell cytoskeleton is to maintain cell shape and to allow cell shape changes. The cytoskeleton is unique to eukaryotes. However, prokaryotes have been shown to have some kind of primitive cytoskeleton as well (de Souza 2012). The cytoskeleton is also needed for the organization of the internal compartments of the cell and to allow the movement of vesicles inside the cell body using the cell cytoskeleton as tracks of transportation in the process called protein trafficking. The cell cytoskeleton is mainly composed of three different filamentous protein networks: actin filaments, microtubules, and intermediate filaments. The functional importance of cell cytoskeleton is highlighted during cell migration and cell division; both of which require orchestrated cell shape changes.

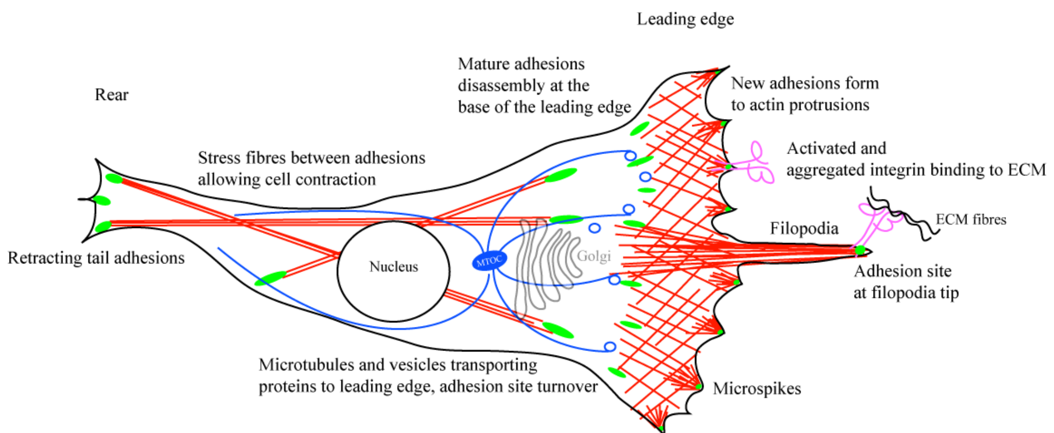

Figure 4. Schematic presentation of cell cytoskeleton. Actin cytoskeleton is in red. Integrin containing adhesion focal puncta and focal adhesions are in green. Microtubules are in blue originating from microtubule organizing center (MTOC). Integrin receptors are presented in here to be in focal puncta and at filopodia tips (magenta). Presented cell is polarized towards the direction of movement.

2.3.2 Filamentous actin

Actin is the most abundant protein in the cells. Globular-actin (G-actin) is a small 43-kD monomeric protein that has the ability to self-polymerize into thin double-helical polymers of monomeric subunits called actin filaments or filamentous actin (F-actin). Several actin binding proteins bind to the different

ends of the actin filaments and regulate the turnover of the monomers in both ends. Adenosidetriphosphate (ATP) and adenosidediphosphate (ADP) nucleotides can bind to the actin monomers and the nucleotides regulate actin polymerization. ATP-actin has higher affinity for filament ends and thus is increasingly assembled into actin filaments. The actin filament functions also as an ATPase. Thus in the growing actin filament the nucleotide exchange takes place, and the other end (minus end) becomes enriched in ADP-actin. The nucleotide exchange creates the polarity of the actin filaments. The barbed end contains ATP-actin (fast growing end or plus-end) and has a higher turnover rate than the ADP-actin containing pointed end (slow growing minus end) (dos Remedios et al. 2003). The plus-ends are polarized towards the leading edge whereas the minus ends are directed away from the leading edge. The plus-end assembles and the minus end disassembles at steady state. Thus the fast growing plus-ends create the membrane forward pushing force in the lamellipodium that is needed for cell migration (Pollard et al. 2000). This ATP-dependent process is called actin treadmilling (Wang 1985; Pantaloni et al. 2001).

Actin nucleation and nucleation core formation is the initial step to create new actin filaments from free actin monomers since the kinetics for actin self-polymerization are unfavourable. The nucleation rate of free actin monomers is slow compared to the addition of free monomers to the existing actin filament. Arp2/3 is a seven-subunit complex and one of the identified nucleation factors. Arp2/3 complex can nucleate new actin filaments from the sides of pre-existing filaments (Amann and Pollard 2001). Subunits ARP2 and ARP3 share structural similarity with G-actin and they form the actin nucleation core by binding to the G-actin (Nurnberg et al. 2011). Arp2/3 complex nucleation activity is enhanced by nucleation promoting factors such as the Wiskott-Aldrich syndrome family proteins (WASP, N-WASP, WAVE, and WASH) (Higgs and Pollard 2000). Inactive and folded N-WASP becomes activated at the leading edge by binding to membrane bound PI(4,5)P2, Cdc42 and Grb2 (Pantaloni et al. 2001). The open conformation of N-WASP has an exposed C-terminal VCA (verprolin, central, acidic) domain that binds both G-actin and Arp2/3 complex in close proximity enhancing the actin nucleation activity of Arp2/3 complex (Marchand et al. 2001). After the actin nucleus is formed the speed of the addition of new monomers increases quickly at the plus-ends.

Another major actin nucleation promoting protein family is called the Formins. Formins are a class of proteins containing a conserved formin homology (FH2) domain. The FH2 domain can bind to actin filament plus-ends and nucleate actin polymerization. The major difference between ARP2/3 complex and formins is that Arp2/3 generates branched actin network whereas formins make straight actin filaments. FH2 domains can remain bound to the growing plus-end and thus also protect against capping proteins in order to maintain actin polymerization (Zigmond 2004).

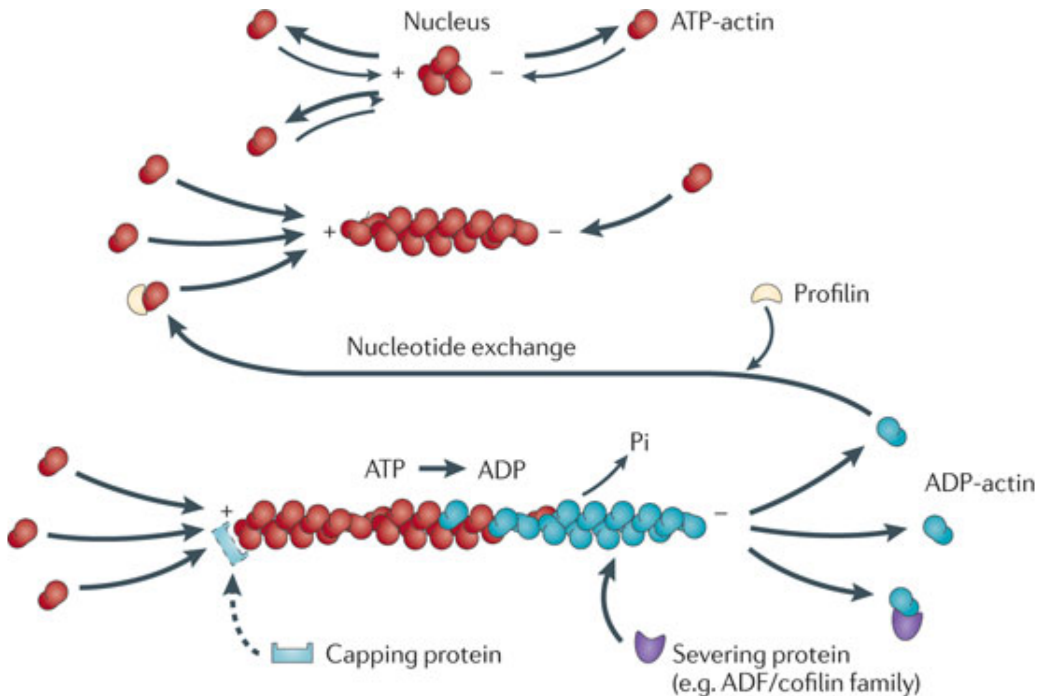

Figure 5. Actin polymerization and the regulators of actin treadmilling. Adapted from (Nurnberg et al. 2011).

Actin treadmilling is regulated by actin-depolymerizing factors (ADF/cofilin family proteins), profilin and capping proteins. The rate-limiting step in the F-actin treadmilling is the actin ATPase cycle. ADF/cofilin binds and accelerates the depolymerisation of the F-actin minus-ends by binding F-actin that has hydrolysed its bound ATP. This increases the pool of free ADF-ADP-G-actin. ADF/cofilin also severs actin filaments and creates new filament ends available for growing.

Another monomeric actin-binding protein profilin catalyzes the exchange of nucleotides (from ADP-G-actin to ATP-G-actin) increasing the pool of free monomeric-ATP-G-actin (Didry et al. 1998). Thus, ADF/cofilin together with profilin increases the grow-rate of the plus-ends of the actin-filaments and the cell motility (Carlier et al. 1997). ADF/cofilin and profilin have also been shown to localize in the leading edge of motile cells (Aizawa et al. 1995; Li et al. 2008).

Capping proteins, such as CapZ, bind to the plus-ends of actin filaments blocking the addition of new monomers and preventing the filament elongation

(Hug et al. 1995). Capping proteins increase the cell motility in synergy with Arp2/3 complex by increasing the rate of new short and branched filament nucleation (Cunningham et al. 1991; Akin and Mullins 2008). It has been proposed that by blocking most of the actin filament plus-ends the capping proteins increase the availability of free G-actin for Arp2/3 complex leading to increased new filament nucleation and actin based cell motility.

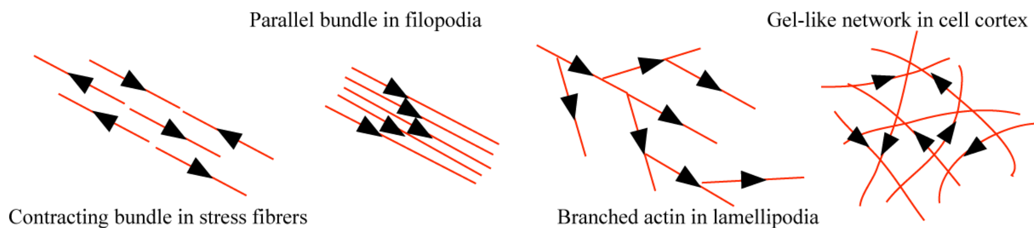

Figure 6. Different kind of F-actin structures. Adapted from (Alberts 1994).

2.3.3 Filopodia

Filopodia are short and dynamic, slender, finger-like, parallel and bundled actin containing protrusions originating from the dendritic actin network of the lamellipodia. Filopodia serve as cells “fingers” in order to probe the extracellular environment. Filopodia have fundamental and diverse biological functions (Mattila and Lappalainen 2008), which include:

1) Searching environmental cues
2) Precursors of cell adhesion sites
3) Growth cone guidance
4) Epithelial sheet closure
5) Wound healing
6) Embryonal development

Table 1. There are several proteins known to induce filopodia formation.

Protein	Type and function	Reference
Myosin-10	Unconventional myosin	(Berg et al. 2000)
Fascin	Actin crosslinker	(Vignjevic et al. 2006)
Arp2/3 complex	Actin nucleation factor	(Korobova and Svitkina 2008)
mDia2	Formin	(Yang et al. 2007)
Ena/VASP	Actin nucleation factor	(Lin et al. 2007)
IRSp53	Inverse BAR protein, deforms membranes	(Goh et al. 2012)
WAVE	Nucleation promoting factor	(Goh et al. 2012)

The difference between microspikes and filopodia is in the length of the protrusion. Microspikes are also embedded in the lamellipodia meshwork, but do not exeed the cell edge in the leading lamellipodia (Svitkina et al. 2003). Microspikes can also serve as precursors for filopodia initiation. Retraction fibres have similar kind of core structure to filopodia, but where filopodia are actively protruding in front of the cell, retraction fibres are left bound to the matrix as cell moves forward greating elongated thin protrusions behind the cells (Small 1988).

2.3.4 Filopodia formation

There are two models of filopodia initiation that rely on different kinds of actin nucleation mechanisms: convergent elongation model and tip nucleation model.

Arp2/3 complex plays a key role in the convergent elongation model. Arp2/3 complex nucleates branched actin filament network (such as seen in lamellipodia) and is activated by WAVE and N-WASP (Mullins et al. 1998). In contrast, the tip nucleation model relies on the function of formins that are capable of nucleating unbranched actin filaments and they also protect the plus-end from capping (Paul and Pollard 2009).

In the convergent elongation model the filopodia are formed from the lamellipodial actin-meshwork that is created by Arp2/3 complex. Some of the actin filament plus-ends are elongated by elongation factors such as Ena/VASP and formins resulting in parallel elongation of actin filaments and

convergence towards parallel actin filaments inside the branched actin meshwork. The filopodia become increasingly thicker with the help of actin cross-linking factors such as fascin that bundless the parallel filaments.

The tip nucleation model is much more straightforward. Clusters of formins come together and nucleate a group of actin filaments outward from the membrane and are cross-linked by fascin creating a filopodia. Arguments supporting the tip nucleation model are based on the fact that formin mDia2 upregulation induces and downregulation inhibits filopodia formation (Block et al. 2008). Likewise, filopodia can be formed without Arp2/3 complex or other nucleation promoting factors (Steffen et al. 2006).

An interesting and novel *in vitro* model also shows the flexibility of filopodia formation. Filopodia-like structures were able to self-form in the presence of negatively charged PI(4,5)P2 membranes and membrane curving I-BAR proteins. The membrane curvature recruited nucleation promoting factor N-WASP and Arp2/3 complex to the site of curvature (Lee et al. 2010).

2.3.5 The unconventional Myosin-10

Myosin-10 (*MYO10*) is a chromosome 5 located, actin-based motor protein and it belongs to the myosin superfamily. Myosin-10 belongs to the class of unconventional myosins and it is ubiquitously expressed in vertebrates, in a wide variety of different tissues and cells, but is not found in lower organisms such as *Drosophila* (Berg et al. 2000). The unconventional myosins are structurally different from the conventional (class II) muscle-contraction myosins. The unconventional myosins have unique tail domains with specific functions (Mooseker and Cheney 1995).

The cellular function of myosin-10 is to increase the number and length of filopodia and to undergo intrafilopodial (forward and rearward) movement along the actin fibers in filopodia (Berg and Cheney 2002).

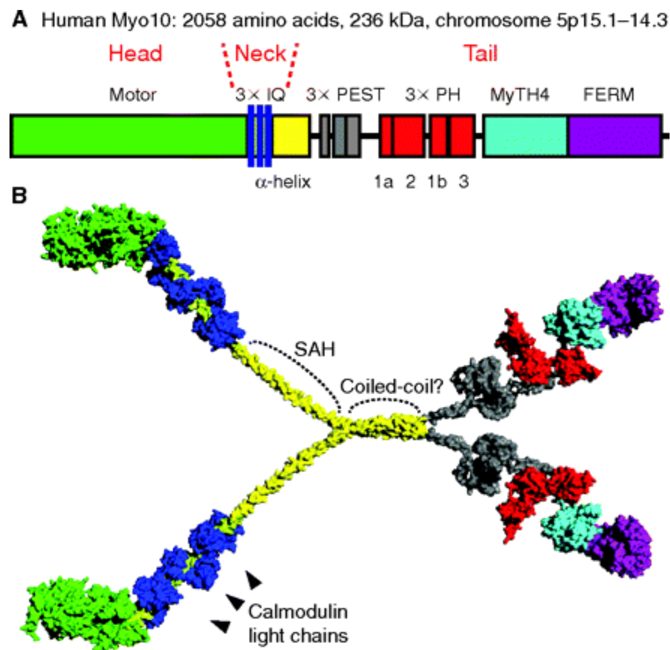

Figure 7. The structure of myosin-10. A) Different domains of myosin-10. B) Cartoon of myosin-10 dimer. Adapted from (Kerber and Cheney 2011)

The myosin-10 protein structure and domains are illustrated in figure 7. The functional domains are (starting from amino-terminus to the end):

1) Myosin motor domain. Involved in ATP-dependent force generation. Also known as the head domain.
2) Three IQ domains. Involved in calmodulin binding.
3) Coiled coil domain. Involved in dimerization of myosin-10.
4) PEST domain. Probably involved in proteolytic calpain-cleavage.
5) Three pleckstrin homology domains (PH-domains). Involved in phosphatidylinositol lipid binding.
6) Myosin tail homology 4 (MyTH4) -domain. Involved in microtubule binding.
7) Band 4.1/ezrin/radixin/moesin (FERM) domain. Involved in binding different cargo receptors (integrin, netrin)

The unique feature of Myosin-10 are the three PH-domains (Berg et al. 2000). PH-domains bind phosphatidyl inositol lipids. Based on the sequence analysis the PH2 and PH3 domains of myosin-10 have conserved residues suggesting binding of phosphatidylinositol lipids phosphorylated at the 3-position by phosphatidylinositol 3-kinase (PI3K). The PH1 domain is split in the middle by the insertion of PH2 domain (Isakoff et al. 1998). The role of the split PH1 domain is to enhance the function of the PH2 domain (Lu et al. 2011). The

binding of PI(3,4,5)P3 is probably involved in a conformational change in the myosin-10 structure. When PI(3,4,5)P3 is not bound, myosin-10 is in a folded autoinhibited conformation where the FERM and PH domains bind the head (motor) domain (Umeki et al. 2011). Upon PI(3,4,5)P3 binding the myosin-10 is dimerized and translocated to the membrane and induces filopodia formation (Plantard et al. 2010; Umeki et al. 2011).

Myosin-10 function is also regulated via Calmodulin-like protein (CLP) and it binds strongly to the three IQ motifs of myosin-10 (Rogers and Strehler 2001). Calmodulin-like protein increases Myosin-10 stability at the protein level and enhances Myosin-10 dependent filopodia formation and filopodia dependent migration (Bennett et al. 2007).

2.3.6 Myosin-10 and integrins

The tail of Myosin-10 has a structurally concerved FERM domain, that can bind to different cargo and to transport these cargo to filopodia tip. These cargos include: netrin receptors deleted in colorectal cancer (DCC) and neogenin, cell-cell adhesion receptor VE-cadherin, actin nucleation factor Mena/VASP and also β1 and β5 integrins (Tokuo and Ikebe 2004; Zhang et al. 2004; Zhu et al. 2007; Almagro et al. 2010). Myosin-10 FERM domain has been shown to directly bind β1, β3 and to β5 integrins (Zhang et al. 2004). The binding site has been mapped to the F2 and F3 domains of the FERM domain and to the membrane proximal NPXY motif of the β integrin cytoplasmic tails. Integrin dependent cellular functions such as cell adhesion are dependent in the link between myosin-10 and integrin. If myosin-10 is silenced the cell adhesion is inhibited (Hwang et al. 2009). Also if a mutant form of myosin-10 incabable of integrin binding (deletion of FERM F2 or F3 domain of full length myosin-10) is expressed, integrins are no longer localized to filopodia tips and filopodia are shorter (Zhang et al. 2004).

2.4. INTEGRIN TRAFFICKING

The function of cell surface receptors is regulated by two main mechanisms: by changing receptor activity or by downregulation of the receptor from cell surface (clearing the presence of the receptor from cell surface so it no longer can be activated by extracellular ligand). The downregulation of integrin adhesion receptors is necessary for: termination of the signalling of adhesion complexes at the plasma-membrane, disassembly of cell adhesions, integrin turnover and for re-sorting and re-assembly of receptors in other locations. Thus downregulation of integrins (or internalization/endocytosis) from the plasma-membrane is followed by sorting of integrins and the cargo in the endocytic machinery and by recycling of the non-degraded cargo back to the plasma-membrane. This process is called endocytic trafficking. The first evidence for integrins to participate in endocytic trafficking is from the year 1989 (Bretscher 1989). Focal adhesion disassembly and integrin endocytosis is particularly important and necessary for cell migration. If integrin endocytosis is inhibited, also cell migration is impaired (Broussard et al. 2008; Chao and Kunz 2009).

2.4.1 Routes of integrin endocytosis

According to current knowledge there are five different routes for integrins to be endocytosed. The classification of these routes is based on the formation mechanism of the endocytic membrane invagination. Integrins have been shown to engage all of these four different routes of endocytosis (Figure 9). However, at present it is largely unknown how these different pathways of endocytosis are chosen spatio-temporally and what their specific roles are in cells.

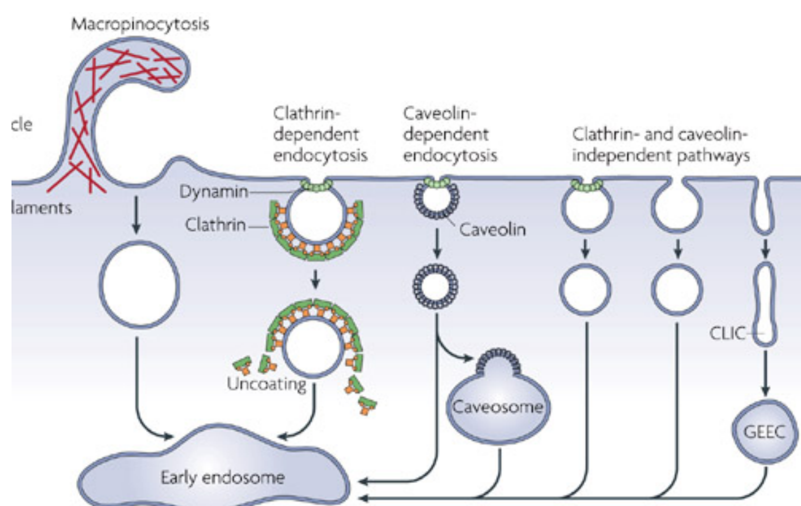

Figure 9. Pathways of endocytosis. CLIC: Clathrin- and dynamin-independent carriers. GEEC: GPI-anchored protein enriched early endosomal compartments. Figure adapted from (ref).

Clathrin dependent endocytosis (CDE)

The Clathrin-coated vesicles (CCVs) are important in selectively sorting different receptors at the plasma-membrane for endocytosis. Clathrin is a coat protein that forms a triskelion made of three heavy and three light chains. Several triskelia interact to form a polyhedral lattice (looking like a football made out of pentagon shaped pieces) that will deform and pull the plasma-membrane from inside (invagination). This clathrin dependent invagination is called a clathrin-coated pit (CCP). In order to form, first the CCP needs to nucleate. The key elements needed for CCP formation are EPS15, Intersectin and membrane bending F-Bar protein FCHO that all localise to PI(4,5)P2 lipid at the plasma-membrane. The nucleation complex will recruit AP-2 adaptor protein which initiates the clathrin-core build-up. AP-2 adaptor protein can recruit different receptors to the CCP by interacting with them directly or via other cargo specific adaptors (like Dab2 and Numb). The budding of the vesicle from the plasma-membrane is the latest step of the process and involves the function of another protein called dynamin. The clathrin coat is rapidly disassociated after the scission leading to uncoating of the newly formed vesicle (McMahon and Boucrot 2011).

Several studies show that clathrin adaptors regulate integrin endocytosis. Adaptor proteins Dab2 and Numb can directly bind to the NPXY motif in the β1 integrin tail via their phosphotyrosine-binding domains (PTB-domain) (Calderwood et al. 2003). Silencing of Dab2 or Numb inhibits the endocytosis of α1, α2, α3 and β1 integrins (Dab2) and β1 and β3 (Numb). Silencing of Dab2 and Numb also impair cell migration (Nishimura and Kaibuchi 2007; Teckchandani et al. 2009). Both adaptors, Dab2 and Numb, also localize to CCPs. Perturbation of either clathrin or dynamin function with dominant negative fusion constructs also effectively inhibits β1 integrin endocytosis (Arjonen et al. 2012).

β6 integrins are also endocytosed via CDE regulated by direct association between HS1-associated protein X-1 (HAX-1) and β6 tail. HAX-1 is an anti-apoptotic protein with many functions. The association between β6 and HAX-1 is not related to the NPXY motifs (Ramsay et al. 2007).

Small Rab GTPases and integrin endocytosis

Small Rab GTPases are Ras superfamily members and they regulate a multitude of aspects in membrane traffic dynamics. The function of Rabs is

regulated by a concerved mechanism called the GTPase cycle that switches the Rab-proteins on and off (Pfeffer and Aivazian 2004). Rabs are inactive in the guanosine diphosphate (GDP)-bound state and active in the guanosine triphosphate (GTP)-bound state. Rabs are present on the limiting membrane of the vesicles on the cytosolic side or in the cytosol where they are bound to the GDP-dissociation inhibitors (GDI). At the surface of membranes GDI-displacement factors (GDF) release GDI and allow the prenylated tails of Rabs to become embedded in the lipid bilayer. GTPase activating proteins (GAP) enhance the intrinsic GTPase activity of Rabs to hydrolyse the GTP to GDP, a process which turns Rab inactive and one phosphate group is released. GTP exchange factors (GEF) turn Rabs active by facilitating the exchange of GDP for GTP. GTPase and regulating proteins cycle is illustrated in figure 10.

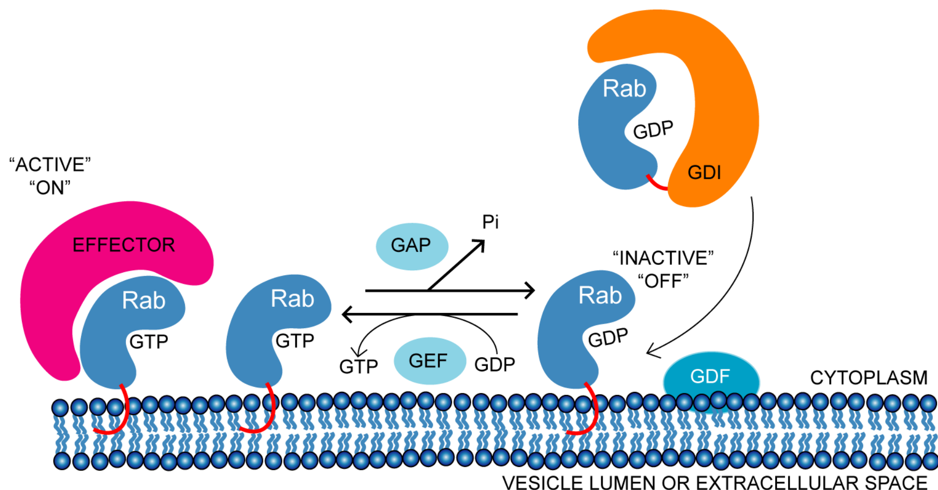

Figure 10. The GTPase cycle. Adapted from (Pfeffer and Aivazian 2004)

The best example of the role of Rabs in integrin endocytosis is the small GTPase Rab21. Rab21 is directly associated with the α2 integrin cytoplasmic tail in a GTPase dependent manner. Rab21 binds and positively regulates β1 integrin endocytosis and cell migration in its GTP-bound active form (Pellinen et al. 2006). Another Rab family member the small GTPase Rab25 binds to the β1 integrin cytoplasmic tail and regulates the migration of ovarian cancer cells on 3-dimensional ECM. However, the function of the Rab25 is to recycle α5β1 integrin back to the plasma-membrane in the protruding pseudopods (Caswell et al. 2007). Rabs are also involved when Neurophilin-1 (Nrp1), a vascular endotheliar growth factor coreceptor, regulates α5β1 integrin endocytosis and recycling Rab5 dependently in endothelial cells (Valdembri et al. 2009).

Focal adhesion (FA) disassembly has also been shown to require CME. During FA disassembly growing microtubule filaments are targeted to the focal adhesions after which the FA disassembles. The disassembly is dependent on

clathrin and its adaptors ARH and Dab2 and relies on endocytosis of β1 integrins. Also FAK and dynamin are needed and they interact with are present at the focal adhesions. Perturbation of FA disassembly also inhibits cell migration (Ezratty et al. 2005; Chao and Kunz 2009; Ezratty et al. 2009).

Clathrin independent endocytosis (CIE)

Caveolins are membrane integrated proteins and primary components of the 50-100 nm invaginations called caveolae. Caveolae are lipid membrane sub-domains with higher order including characteristics like detergent resistance (cold Triton X-100 resistance) and increased cholesterol and glycosphingolipid concentration. The structure, dimerization and interaction with membrane lipids are thought to bend the plasma-membrane in order to create the caveolar invaginations that are the basis for caveolin-mediated endocytosis. Caveolin-mediated endocytosis is functionally dependent on dynamin abscission (Mayor and Pagano 2007).

Many studies indicate that integrins are endocytosed independent of clathrin and via caveolin-mediated endocytosis (CavME). Integrins are found in detergent resistant membranes or so called lipid rafts (Fabbri et al. 2005). Silencing of caveolin-1 impairs fibronectin turnover and β1 integrin endocytosis (Shi and Sottile 2008). Clustered α2β1 integrin has also been shown to be endocytosed into caveosomes (Upla et al. 2004). Active β1 integrin has also been shown to use the caveolar endocytosis route on soft subrate in bone marrow mesenchymal stem cells (Du et al. 2011). Phosphorylated caveolin-1 is also present in focal adhesions and the internalisation of lipid rafts requires caveolin-1 and dynamin-2 and is regulated by integrin-dependent adhesion of cells to ECM (del Pozo et al. 2005).

Clathrin- and caveolin independent endocytosis routes

Clathrin independent carriers (CLICs) and glycosylphosphatidylinositol (GPI)-enriched early endosomal compartments (GEECs) are mediating endocytosis. CLIC and GEECs are cholesterol and actin dependent endocytosis routes (Chadda et al. 2007). CLICs have been shown to be a major endocytosis pathway. CLICs endocytose three times the volume compared to clathrin mediated endocytosis pathway in fibroblasts (Howes et al. 2010). CLICs are mostly responsible for the uptake of bulk membrane and fluid. However the proteomic profiling of CLIC identified several plasma-membrane receptors such as β1 integrin, CD44, CD109 and low-density lipoprotein receptor 1 (LRP1) and also several known regulators of CME such as Rab GTPases (Howes et al. 2010). CD44 is a cell surface glycoprotein and it is a receptor for hyaluronic acid (Thankamony and Knudson 2006). CD44 was found to be specific in CLIC endocytosis ruote. Using CD44 as a marker Howes et al.

showed CLIC pathway polarized towards the leading edge of migrating cells suggesting an important role in cell migration.

Circular dorsal ruffles and macropinocytosis

Another interesting clathrin and caveolin independent mechanism for integrin endocytosis is the growth factor stimulation dependent and actin dependent circular dorsal ruffle formation and the following massive macropinocytosis of integrin receptors (Gu et al. 2011). Gu et al. find that after growth factor stimuli integrins are macropinocytosed to the early endosomal trafficking route (EEA1, Rab5 and Rab4 positive) and recycled back to the ventral cell surface to form new focal adhesions in fibroblasts.

2.4.2 Integrin recycling pathways

After endocytosis, receptors and integrins are sorted to different endosomal compartments. The endocytic compartments regulate the sorting of the receptors and ligands in a sequential manner from one endocytic compartment to the next. Different endosomal compartments have their own separate functions and also the lipid composition of the compartments varies. Small RabGTPases are known to regulate the endosomal identity and function of endosomal compartments. By recruiting different effector proteins, motor proteins and kinases, the Rabs regulate the budding and fusion of the endosomes (Stenmark 2009). Different endosomal compartments are illustrated in figure 11.

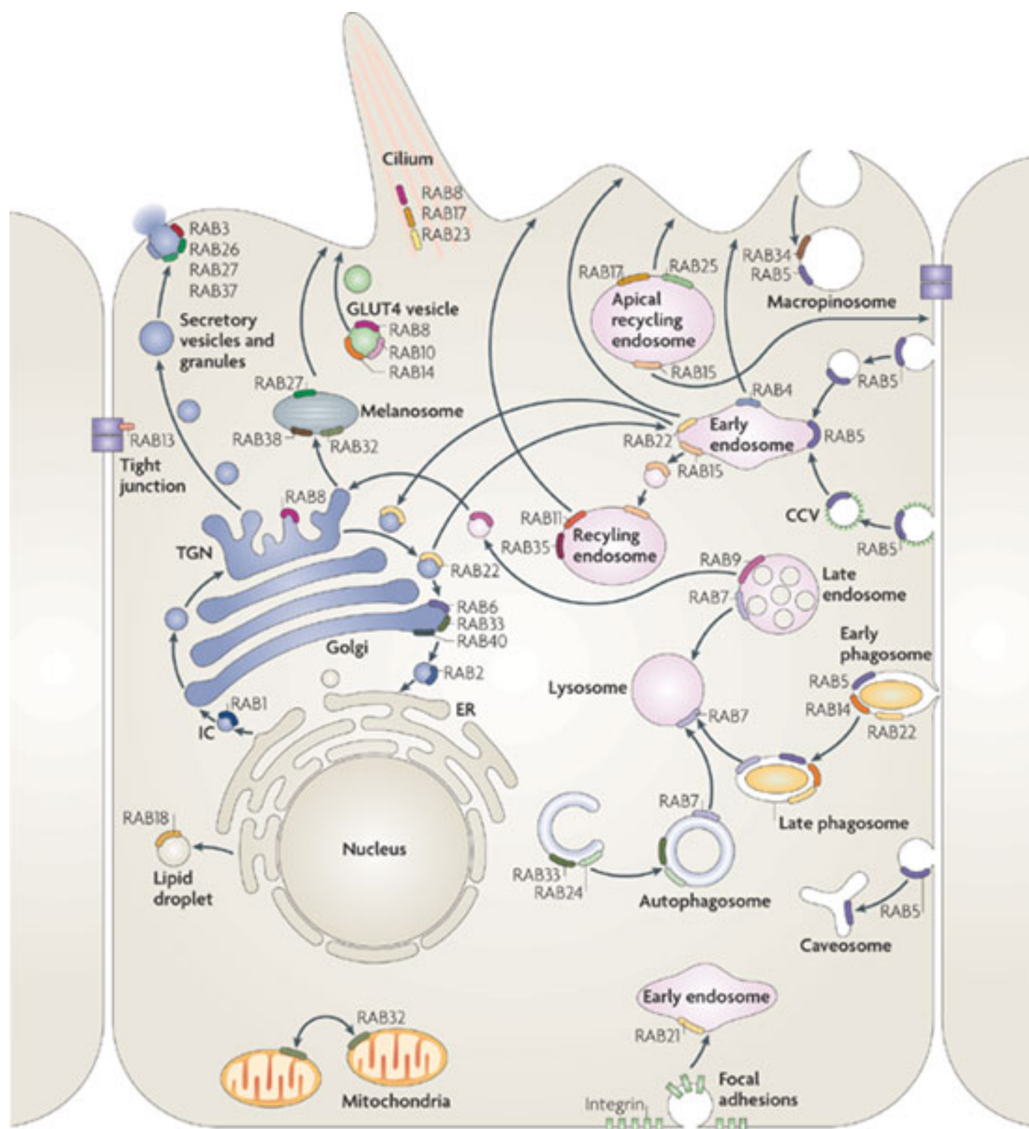

Figure 11. Endocytic trafficking in eukaryotic cells. Adapted from (Stenmark 2009).

After endocytosis, integrins destined for degradation are sorted through late endosomes to lysosomes. On the other hand integrins destined for recycling are sorted through recycling endosomes and transported back to the plasma-membrane. The half-life of integrins (degradation rate) is very long (halflife 18 hours) compared to the dynamics of integrin recycling (halflife approximately 30 minutes depending on the pathway) (Roberts et al. 2004; Lobert et al. 2010). The functional decision making step specifying recycling or degradation is poorly known and is discussed later. Sorting nexin 17 (SNX17) is a regulator

of protein trafficking. SNX17 has been shown bind the distal NPXY motif of β1 integrin cytoplasmic tail. SNX17 can retrieve β1 integrins from the degradative pathway by increasing β1 integrin recycling back to plasma-membrane (Steinberg et al. 2012).

Integrin recycling is a particularly important process during cell motility. ECM receptors, integrins and growth factor receptors are polarized in their localization in migrating cells. Recycling of integrins and growth factors towards the leading edge maintains the polarity and localized function of the receptors at the leading edge. Perturbation of the recycling pathways can compromise proper cell migration (Powelka et al. 2004; Woods et al. 2004).

Several different recycling routes have been described to facilitate integrin traffic back to the plasma-membrane. These include the Rab4-dependent “short-loop”, Rab11-dependent “long-loop”, stimulation-dependent ARF6 and tubular actin-dependent recycling pathways (Roberts et al. 2001; Powelka et al. 2004; Roberts et al. 2004; Fang et al. 2010).

αvβ3 and α5β1 integrins are first endocytosed through early endosomes and Rab4 positive endosomes to the perinuclear Rab11 positive recycling compartment ref. The perinuclear recycling compartment is “deeper” inside the cell and integrins take a longer time to reach this compartment. Thus, the nomenclature of long and short loop of recycling. Functional Rab11 is required for the recycling of integrins from the perinuclear recycling compartment (PNRC) back to the plasma-membrane. However, upon growth factor stimulus, faster Rab4 dependent recycling of αvβ3 integrin bypassing the PNRC is triggered (Roberts et al. 2001). Another example of stimulation-dependent recycling is from Powelka et al. 2004. β1 integrins were shown rapidly recycle back to plasma-membrane (5 min timepoint) after growth factor stimuli. The recycling of β1 integrins was dependent on functional Rab11 and ADP-ribosylation factor 6 (ARF6). Also, blocking F-actin polymerization by drug treatment inhibited the recycling (Powelka et al. 2004). Another example of F-actin involvement in β1 recycling comes from experiments with Supervillin (an F-actin and Myosin II binding protein). Supervillin knockdown and inhibition of F-actin polymerization inhibited Rab4-dependent fast integrin β1 recycling but did not effect the β1 endocytosis (Fang et al. 2010). The results for fast Rab4-dependent recycling are also supported by the observation that inactive integrin preferentially recycles via the fast Rab4-pathway and is F-actin dependent (Arjonen et al. 2012).

2.4.3 Integrins in lysosomal pathway

Cargo bound integrins have also been described in the lysosomal pathway. A fraction of fibronectin bound integrins are ubiquitinated and sorted for lysosomal degradation (Lobert et al. 2010). Ubiquitin is a small "tagging protein" used in eukaryotes in many regulatory purposes. Ubiquitin can be added to integrin α5 tail lysines by a ubiquitin ligase and thus integrins can be marked for degradation. Lobert et al. 2010 demonstrated that ligand bound α5β1 integrins were sorted via multivesicular endosomes for degradation in a process involving the function of endosomal sorting complex required for transport (ESCRT) protein. Also, a higher degree of colocalization has been detected with endocytosed and active ligand-bound β1 integrins compared to the endocytosed inactive β1 integrins in late endosomes and lysosomes (Arjonen et al. 2012; Dozynkiewicz et al. 2012) indicating that ligand-bound integrins are *en route* for degradation. However, the results by Dozynkiewicz et al. indicate that active integrins can also be recycled back to plasma-membrane from the lysosomes – an intriguing observation discussed later in *results and discussion*.

2.4.4 Integrin trafficking and polarized cell migration

Efficient and directed cell migration is dependent on polarization of key plasma-membrane proteins and cell surface receptors towards the direction of movement. The polarization of proteins needs to be maintained constantly during the cell migration. In epithelial sheets, tight junction proteins physically prevent the diffusion of proteins and maintain dorsal-apical polarization (Balda and Matter 1998). However, migrating cells are lacking obvious physical diffusion barriers and the limited diffusion (and rear-front polarity) is maintained by other methods. Limited diffusion of plasma-membrane proteins is seen for example in axon hillock (a part of neuron cell body that connects axon) but the mechanism is not completely understood (Kobayashi et al. 1992). Actin polymerization at the leading edge has been proposed to upkeep the lipid diffusion barrier and polarity (Weisswange et al. 2005). Weisswange et al. have studied how the filament plus-ends could interact and stabilize the positions of certain proteins and thus limit the diffusion of lipids at the leading edge. Inhibition of actin polymerization by a drug Cytochalasin D leads to disruption of actin filaments and the diffusion barrier (Weisswange et al. 2005).

Another mechanism to maintain the polarization is endo-exocytic trafficking of proteins towards the leading edge. Integrins are known to be polarized in migrating cells and to undergo dynamic endocytic trafficking (Bretscher 1989). There are many examples how integrin trafficking and especially recycling of integrins towards the leading edge is related to cell migration. While the severing activity of cofilin and the actin nucleating activity of Arp2/3 complex generates the membrane forward pushing forces the stabilization of the leading

lamellipodia is mediated via ECM binding integrins (Ballestrem et al. 2001; DesMarais et al. 2004). Disruption of endocytic trafficking by blocking the clathrin coated bit formation makes cells to lose their polarized actin cytoskeleton (Altankov and Grinnell 1993). Altankov et al. also showed integrin distribution was altered and much less integrin was seen in focal adhesions when compared to properly polarized cells in clathrin blocked cells.

A good example of integrin polarization in migrating cells was shown by Tuomi et al. 2009. Resting non-small cell lung cancer cells have epithelial-like phenotype with established cell-cell contacts and show non-polarized distribution of α5β1 integrins. However, when cells were plated sparsely, they become polarized and migratory, and α5β1 integrin was extensively localized to the leading edge (Tuomi et al. 2009).

Also αvβ3 integrins are polarized during cell migration (Roberts et al. 2001). αvβ3 is known to be recycled Rab4 GTPase and PDGF stimulation dependently. Expression of dominant negative GDP-bound Rab4 blocks the αvβ3 recycling and results loss of the polarized αvβ3 distribution (Roberts et al. 2001). The αvβ3 integrin was later shown to associate with protein kinase D 1 (PKD1) in vesicular compartments during inhibition of integrin recycling (Primaquine treatment). Moreover the kinase activity of PKD1 was needed in the recycling of αvβ3 integrin to focal adhesions at the leading edge. Expression of kinase inactive form of PKD1 or suppression of PKD1 by RNA interference reduced cell migration probably because αvβ3 recycling was blocked (Prigozhina and Waterman-Storer 2004; Woods et al. 2004).

While β3 integrin recycling seems to be dependent on Rab4 GTPase, the β1 integrin recycling has been shown to be dependent on Rab11 GTPase and ARF6 function (Powelka et al. 2004). Dominant negative (DN, GDP-bound) forms of ARF6 and Rab11 GTPases (but not DN-Rab4 GTPase) were shown to inhibit β1 integrin recycling. DN-ARF6 also inhibited cell migration towards collagen I gradient suggesting recycling of collagen-binding β1 integrins (α1/2/10/11 paired with β1) is needed for proper cell migration.

Integrin β1 recycling has also shown to be regulated by Rab25 (Rab11c) (Caswell et al. 2007). Rab25 binds directly to the β1 integrin cytoplasmic tail. Rab25 was shown to regulate the recycling of β1 integrin in pseudopodia tips that were extended towards the direction of cell migration. Photoactivation of α5 (pair of β1) integrin in a Rab25-positive endosome showed integrin recycling back to the plasma-membrane. Rab25 and α5β1 integrin vesicles also localized to the pseudopods extending to the direction of migration. Overexpression of Rab25 increased cell invasion in 3D most likely by promoting α5β1 integrin recycling in the pseudopod tips.

The recycling of $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrin can support two different kinds of cell migration phenotypes. Integrin $\alpha v\beta 3$ is supporting directional and more persistent cell migration whereas $\alpha 5\beta 1$ integrin is related to rapid and random cell migration (Danen et al. 2005). Adhesion of cells via $\alpha v\beta 3$ integrin activates the actin severing protein cofilin resulting a single lamellipodia. On the other hand the activity of $\alpha 5\beta 1$ integrin phosphorylated and inactivated cofilin leading to improper polarization and more random cell migration (Danen et al. 2005). The mediator of $\alpha 5\beta 1$ integrin functions on actin cytoskeleton was shown to involve increased RhoA and Rho-associated protein kinase (ROCK) activity whereas Rac was activated downstream of $\alpha v\beta 3$ integrin. The $\alpha 5\beta 1$ integrin recycling and the related rapid/random migration were enhanced when $\alpha v\beta 3$ integrin recycling (or $\alpha v\beta 3$ integrin ligand binding) was blocked. This suggests that the activity and the recycling of $\alpha v\beta 3$ integrin are suppressing the $\alpha 5\beta 1$ integrin recycling (White et al. 2007).

The recycling of integrins can also modulate growth factor receptor signalling at the leading edge by changing the kinetics of growth factor receptor trafficking. RCP and $\alpha 5\beta 1$ integrin can make a complex with EGFR1 and increase EGFR1 recycling and EGFR1 downstream signalling (Caswell et al. 2008). EGFR1 further activates PI3-kinase and its downstream promigratory kinases protein kinase B and Akt. The enhanced levels of PI(3,4,5)P3 produced by PI3-kinase activation have been shown to directly increase actin polymerization and cell migration (Keely et al. 1997; Niggli 2000).

2.5. FILOPODIA AND INTEGRINS IN CANCER

2.5.1 Integrins in cancer

Cancer spreading needs cells which have became more metastatic and migratory. Metastasis is a multistep process involving interplay of many signalling pathways. Integrins contribute to tumor cell survival, migration and invasion and to proliferation (Desgrosellier and Cheresh 2010). One of the key changes cancer cells develop to gain a more migratory phenotype is to change their affinity and avidity to the ECM. Numerous studies have reported changed integrin expression in malignant cells (Hood and Cheresh 2002).

Table 2. Higher (up) or lower (down) integrin expression in metastatic tumor cells compared to the primary tumor cells (Mizejewski 1999; Desgrosellier and Cheresh 2010):

Cancer type	α1β1	α2β1	α3β1	α6β1	α5β1	αVβ3	α4β1
Breast	up	down			up	up	
Skin		up	up	up		down	
Ovary						up	up
Colon		down			up	up	
Kidney							
Lung		down			up		
Melanoma	up	up		up	up	up	
Prostate						up	
Pancreatic						up	
Cervical						up	
Glioblastoma						up	

The changes in integrin subtype expression is not only limited to the cell-ECM relationship but also regulates several other aspects leading to a more metastatic phenotype.

Changes in integrin expression related to metastasis are:

1) Degradation of basement membrane: Integrins have been reported to directly activate or indirectly modulate the function of matrix degrading matrix metalloproteinases (MMP) (Morozevich et al. 2009; Jiao et al. 2012). Also MMPs have been show to inactivate integrins to promote migration (Kryczka et al. 2012).

2) Deposition of new matrix and remodelling of ECM: Osteopontin (OPN) is a metastasis-promoting protein secreted by tumor cells and cells in tumor stroma. Integrins can bind to OPN which can enhance cell survival and cell migration (Anborgh et al. 2010).

3) Cytoskeletal organization and force generation: Following integrin activation and FAK signalling in focal contacts, small G-proteins like Cdc42 and RAC are activated leading to cytoskeletal changes and to epithelial-mesenchymal transition (EMT). Cdc42 activation induces filopodia formation and RAC promotes membrane ruffling and migration in integrin-dependent migration (Keely et al. 1997; Filipenko et al. 2005).

4) Survival: Unligated integrins signal cells to undergo apoptosis, a process called anoikis (Matter and Ruoslahti 2001). This prevents anchorage-independent growth if cells become unattached from the ECM. In order to survive during metastasis, integrins need to feed the cells with pro-survival signals. Changes in integrin expression can either kill the apoptotic switch or enhance pro-survival signals when cells have metastasized to “wrong environment” (Eliceiri et al. 2002).

5) Integrin crosstalk with growth factors and cytokines: Growth factor stimulation of cancer cells can promote invasion by activating integrins resulting in integrin mediated cell migration (Klemke et al. 1994; Ricono et al. 2009).

2.5.2 Filopodia in cancer

Although the role of the cell cytoskeleton and filopodia is implicated in cell migration, little is known about the role of filopodia in cancer. The existence of filopodia in 3D tissues is under debate, but the invasive structures in human cancers (membrane blebs, invadopodia and pseudopodia) closely resemble the filopodia in their proteomal composition (Nurnberg et al. 2011). Among filopodia inducing genes, fascin1 is one of the best characterised in cancer. Fascin1 is an actin bundling protein, and has been associated with invasive migration *in vitro* in 3D environments (Li et al. 2010). Fascin-1 is also reported to be upregulated in colorectal cancer and to localize at the invasive front of tumors (Vignjevic et al. 2007). Fascin1 is also a target gene for the Wnt/β-catenin-TCF signalling pathway which is upregulated in many cancers. In line with this, high fascin expression has been shown to correlate with EMT and loss of E-cadherin (epithelial cadherin, a marker for healthy epithelial tissues) (Zou et al. 2010). Fascin is associated also with poor clinical outcome and is upregulated in many metastatic epithelial cancers (Machesky and Li 2010).

Another group of filopodia inducing genes, formins, have also been implicated in cancer (Nurnberg et al. 2011). Diaphanous homolog 1 (Diaph1) regulates breast cancer cell invasion (Lizarraga et al. 2009) and Formin-like 2 (FMNL2) shows increased expression levels metastatic colorectal tumors. (Zhu et al. 2008). FMNL2 and FMNL3 suppression has also been shown to inhibit amoeboid-like (non-adherent and round cells) cell invasion in vitro (Kitzing et al. 2010; Vega et al. 2011).

A very interesting link between filopodia-expression in breast cancer was reported recently. In silico analysis of human breast cancers and filopodia-inducing genes was performed on the clinically evaluated Swedish breast cancer patients (Miller et al. 2005; Arjonen et al. 2011). The higher mRNA expression of many filopodia inducing genes correlated positively with the poor-outcome basal-like breast cancers when compared to luminal or normal-like breast cancers (Arjonen et al. 2011). Basal-like breast cancers are triple negative, i.e estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2) negative, and usually have a poorer outcome when compared to luminal i.e hormone-receptor positive breast cancers. The role of filopodia in breast cancer is further discussed in results and discussion.

3. AIMS OF THE STUDY

The general aim of this thesis work is to study cell adhesion and migration in cancer. More specifically, the function of integrin adhesion receptors, integrin trafficking and filopodia induced by myosin-10 has been studied.

With respect to integrin function, little is known about the regulators of integrin trafficking. Furthermore, the relationship between the active and inactive conformations of integrin and integrin endocytosis is unclear.

With respect to filopodia formation, myosin-10 is a known inducer of filopodia, but the regulation of myosin-10 function is unclear. Also the role of filopodia in cell migration has been accepted, but there is no clear evidence demonstrating a role for myosin-10 and filopodia in cancer progression.

The specific aims of this study were:

- To identify novel regulators of β1 integrin endocytosis
- To study the endocytosis of active and inactive β1 integrins
- To study the regulation of myosin-10 dependent filopodia formation
- To understand the relationship between myosin-10 dependent filopodia formation and cancer cell invasion

4. MATERIALS AND METHODS

More detailed description of the methods and reagents can be found in the original publications (I-IV).

Experimental procedures

Method	Used in
Cell culture	(I-IV)
Yeast two-hybrid and yeast mating	(I)
DNA cloning	(I,II)
Site-directed mutagenesis	(I,II)
Immunofluorescence microscopy	(I-IV)
Immunoelectron microscopy	(I)
Immunoprecipitation (IP)	(I,II)
Western blotting (WB)	(I-IV)
ELISA-based detection	(III)
Live-cell microscopy	(I,II,IV)
Cell adhesion assay	(I,IV)
Cell migration assay	(I,IV)
Cell invasion assay	(IV)
Cell spreading assay	(IV)
Statistical analysis	(I-IV)
Sucrose gradient fractionations	(I)
DNA and siRNA transfection	(I-IV)
Immunohistochemistry and clinical studies	(IV)
In silico data analysis	(IV)
Antibody-based integrin internalization assay	(III)
Antibody-based integrin recycling assay	(III)
Biotin-IP-based integrin internalization assay	(I,III)
Biotin-IP-based integrin recycling assay	(I,III)
Protein purification	(II)
Lipid pull-down assay	(II)
Cold triton fractionation	(II)
Image analysis	(I-IV)
Flow cytometry	(III)
Quantitative real-time polymerase chain reaction (RT-PCR)	(IV)
Zebrafish invasion assay	(IV)
Mouse lung extravasation assay	(IV)

DNA constructs

DNA construct	**Description**	**Used in**
EGFP-mRAB21	Small GTPase Rab21	(I)
EGFP-mRAB21GTP	Small GTPase Rab21 GTP-locked (Q76L)	(I)
EGFP-mRAB21GDP	Small GTPase Rab21 GDP-locked (T31N)	(I)
EGFP-mRAB21CCSS	Small GTPase Rab21 prenylation motif mutant CC218/219SS	(I)
EGFP-mRAb21 C-term	Small GTPase Rab21 C-terminal deletion after E144	(I)
Rluc-mRAB21	As above, with Renilla luciferase tag	(I)
Rluc-mRAB21GTP	As above, with Renilla luciferase tag	(I)
Rluc-mRAB21GDP	As above, with Renilla luciferase tag	(I)
Rluc-mRAB21CCSS	As above, with Renilla luciferase tag	(I)
Rluc-mRAb21 C-term	As above, with Renilla luciferase tag	(I)
EGFP-α2 integrin	A2 integrin	(I)
EGFP- α2A integrin	A2 integrin, K1160A	(I)
EGFP- α2AA integrin	A2 integrin KR1160/1161AA	(I)
EGFP-Rab4	Small GTPase Rab4	(II,III)
EGFP-Rab4-S22N	Small GTPase Rab4 GDP-locked (S22N)	(II,III)
EGFP-Rab5a	Small GTPase Rab5A	(I)
EGFP-Rab7	Small GTPase Rab7	(I)
EYFP-Rab9	Small GTPase Rab9	(I)
EGFP-Rab11	Small GTPase Rab11	(I)
EYFP-mTalin1	Talin-1	(I)
Myo10-PH1	PH1-domain only, P1170-G1378	(II)
Myo10-PH2	PH2-domain only, E1206-A1304	(II)
Myo10-PH3	PH3-domain only, E1386-D1491	(II)
Myo10-tail	Myo10 tail only, R1160-R2052	(II)
mCherry-Myo10	Full length (FL) Myo10	(II)
Myo10-PH1pm	Full length Myo10, point mutation at K1179A	(II)
Myo10-PH2pm	Full length Myo10, point mutation at KK1215/1216AA	(II)
Myo10-PH3pm	Full length Myo10, point mutation at K1395A	(II)
mCherry-Myo10 delPH	Full length Myo10 with all PH-domains deleted	(II)

DNA construct	**Description**	**Used in**
mCherry-Myo10Btk	Full length Myo10 with PH2 domain swapped to Btk PH-domain	(II)
mCherry-Myo10PLCδ1	Full length Myo10 with PH2 domain swapped to PLCδ1 PH-domain	(II)
mCherry-Myo10TAPP1	Full length Myo10 with PH2 domain swapped to TAPP1 PH-domain	(II)
EGFP-C1	Clontech cloning vector	(I-IV)
EGFP-DynaminK44A	Dominant negative Dynamin	(III)
EGFP-Eps15 EH29	Dominant negative Eps15	(III)
EGFP-Caveolin-1	Dominant negative Caveolin-1	(III)
EGFP-ARF6	ADP-ribosylation factor 6	(III)
EGFP-Rab25	Small GTPase Rab25	(III)
EGFP-Myo10	Full length Myosin-10	(IV)
EGFP-Myo10 delFERM	FERM-domain deleted	(IV)
p53-R273H	Mutant p53	(IV)

siRNAs and shRNAs

Name or target	**Description or sequence (sense)**	**Used in**
Rab21	ggcaucauucuuaacaaagtt	(I)
Rab21 #2	ggucaagagagauuccaugtt	(I)
Scramble control	Ambion's negative control, scramble sequence	(I)
Allstars negative	Qiagen's negative control, mix of three non-effective siRNAs	(IV)
Scramble shRNA	gatcccgcgaatcctacaagcgcgcttgatatccg gcgcgctttgtaggattcgtttttccaaa	(I)
Rab21 shRNA	gatccggtcaagagagagettccatgttcaagag acatggaatctctcttgacctga	(I)

Cell lines

All cell lines used in this thesis are of human origin, exept COS-7 and CHO.

Cell line	Description	Used in
NCI-H460	Non-small lung cancer cells	(III)
MDA-MB-231	Breast adenocarsinoma cells	(I,III,IV))
MCF10A	Nontumorigenic breast epithelial cells	(IV)
MCF7	Luminal-like breast cancer cells	(IV)
HCT116-p53 (-/-)	Colorectal cancer cells, *TP53* null	(IV)
COS-7	Immortalized monkey kidney cells	(II)
HeLa	Cervical cancer cells	(I,II)

HEK-293T	Embryonic kidney cells	(I)
CHO	Chinese hamster ovary cells	(I)
HT1080	Fibrosarcoma cells	(I)
PC-3	Prostate cancer cells	(I,III)
U87-MG	Glioblastoma cells	(IV)
Saos-2	Osteosarcoma cells	(I)
MAE	Mouse aortic endothelial	(II)

Antibodies

Antigen	**Species**	**Description**	**Used in**
EEA1	Rabbit	Santa Cruz	(I)
EGFR	Mouse	151-IgG, DHSB, Iowa	(I)
RFP	Rabbit	PM005, MBL	(II)
β-Tubulin	Mouse	12G10, DHSB, Iowa	(II,IV)
β-Actin	Mouse	JLA20, DHSB, Iowa	(II,IV)
LAMP1	Goat	Santa Cruz	(II)
MBP-Probe	Rabbit	N17, Santa Cruz	(II)
EGFP	Mouse	Clontech	(II)
AKT	Rabbit	Cell Signaling	(II)
p-AKT	Rabbit	Ser473, Cell signaling	(II)
Myosin-10	Rabbit	2243.00.02 SDIX	(II)
Myosin-10	Rabbit	845-944, Strategic Diagnostics	(IV)
Rab7	Mouse	Rab7-117, Abcam	(II)
IgG	Rabbit	Sigma	(II)
Rab21	Rabbit	Ab sera, Innovagen	(I)
Rab21	Rabbit	Opdam et al 2000	(I)
Rab5a	Rabbit	Santa Cruz	(I)
EGFP	Rabbit	Rabbit	(I)
Rab7	Rabbit	Santa Cruz	(I)
Rab11	Rabbit	Santa Cruz	(I)
Caveolin-1	Rabbit	Santa Cruz	(I)
AF488	Rabbit	A-11094, Molecular Probes	(III)
α1-integrin	Mouse	AB1937, Chemicon	(I)
α2-integrin	Mouse	MCA2025, Serotec	(I)
α2-integrin	Rabbit	AB1934, Chemicon	(I)
α2-integrin	Mouse	P1H5, Santa Cruz	(III)
α2-integrin	Mouse	mAb1998, Millipore	(III)
α5-integrin	Mouse	BIIG2, DHSB, Iowa	(I)
α6-integrin	Mouse	MAB699	(I)
β1-integrin	Mouse	HUTS-21, BD Biosciences	(I,III)
β1-integrin	Mouse	K20, Beckman Coultier	(III)
β1-integrin	Mouse	12G10, Abcam	(III,IV)
β1-integrin	Rat	mAb13, BD Biosciences	(III,IV)

β1-integrin	Mouse	4B4, Beckman Coultier	(III)
β1-integrin	Rat	9EG7, BD Pharmingen	(I,III,IV)
β1-integrin	Mouse	P5D2, DHSB, Iowa	(I)
β1-integrin	Mouse	P4G11, DHSB, Iowa	(I)
β1-integrin	Mouse	MAB2252, Chemicon	(I,IV)
β1-integrin	Mouse	AIIB2, DHSB, Iowa	(I)
Talin1	Mouse	T3287, Sigma	(IV)
p53	Mouse	DO-7, Santa Cruz	(IV)

Reagents and chemicals

Compound	**Supplier**	**Used in**
HiPerfect	Qiagen	(I-IV)
Lipofectamine 2000	Invitrogen	(I-IV)
OptiMem	Invitrogen	(I-IV)
DAPI	Sigma-Aldrich	(I-IV)
Phalloidin-AF 488/561/647	Molecular Probes	(I-IV)
Fibronectin	Calbiochem	(I)
Collagen	Sigma-Aldrich	(I)
Laminin	Sigma-Aldrich	(I)
Vitronectin	Sigma-Aldrich	(I)
Mowiol	Calbiochem	(I-IV)

5. RESULTS AND DISCUSSION

5.1. Small GTPase Rab21 and β1-integrin trafficking (I)

Illustrations of integrins and the focal adhesion complex usually depict the integrin heterodimer with a β-tail that has many interaction partners, but with a short α-tail that lacks binding partners (Harburger and Calderwood 2009). To challenge this unbalanced view of identified α and β-tail binders we screened the embryonic cDNA library (mouse) by yeast-two-hydrid using the integrin α2-integrin intracellular tail as bait. One of the novel hits that was found was the small GTPase Rab21. Rab proteins regulate intracellular vesicle trafficking and are known to participate in endocytosis and exocytosis. Rab21 has been previously found to be involved in the regulation of the endocytic pathway (Simpson et al. 2004). However this was the first time a direct interaction of Rabs with their endocytic cargo was charactericed.

To further verify the interaction we used co-immunoprecipitation in human cells and found that Rab21 was associated with many α-tails and β1 integrin (I). Most of the integrin α-tails have a conserved GFFKR amino acid sequence, and indeed point mutations within this sequence abolished the interaction with Rab21. In particular, mutagenesis of the conserved positively charged arginine (R1161A) reduced the binding of Rab21 to α2-integrin. We also found the Rab5 (a close homolog to Rab21) but not Rab7 or Rab11a associated with β1 integrin. Later Rab25 (Rab11c, but not Rab11a/b) was shown to associate with β1 integrin (Caswell et al. 2007).

Overexpression of EGFP-Rab21 wt increased the formation of large vesicles in human MDA-MB-231 cells with integrin and ECM matrix staining in the vesicle lumen. Live-cell imaging demonstrated that smaller Rab21 vesicles both fused with and budded from larger Rab21 vesicles. . Overexpression of Rab5 also promoted formation of integrin positive vesicles and overlap of EEA1 staining. Rab5 is a known regulator of early endosome function (Gorvel et al. 1991). Interestingly, EEA1 staining overlapped with Rab21 in a limited fashion. Transferrin endocytosis experiments (a classical model for clathrin mediated endocytosis) showed that transferrin colocalized well with Rab5 whereas Rab21 only showed partial colocalization with transferrin. These results suggest the Rab21 endocytosis pathway is not a component of the classical clathrin-mediated Rab5 to early endosome route.

The GTP-locked Rab21 mutant (defective in GTP-hydrolysis, Rab21 Q76L) localized to tubulovesicular structures whereas the GDP-locked Rab21 mutant (GTP-binding deficient, Rab21 T31N) was mostly seen in membrane ruffles. The expression of GDP-locked Rab21 induced formation of large integrin containing focal adhesions whereas the GTP-locked Rab21 accumulated with integrins to large cytosolic vacuoles. If the Rab21 prenylation motif was

mutated (loss of membrane association, Rab21 CC218/219SS, later Rab21-CCSS) the Rab21 vesicles were lost and integrin became localized to focal adhesions,as with Rab21-GDP expression. Prenylation tail deficient Rab21 also associated with β1 integrin to a lesser extent. Total internal reflection fluorescence microscopy (TIRFM) revealed Rab21 vesicles (wt) moved back and forth in the close proximity to, but without fusion with, the plasma-membrane. GDP-locked Rab21 showed diffuse non-vesicular staining at the plasma-membrane in TIRFM. The localization of Rab21 seems to be regulated by the GTPase cycle, and the proper GDP/GTP switch is needed for vesicle formation. The GTPase cycle of Rab21 also regulates integrin subcellular localization indicating that Rab21 is needed for integrin trafficking. However, overexpresson of Rab21 changes the subcellular balance between different Rab GTPases. Thus, the overexpression of one particular Rab could have a 'dominant' effect, re-routing all cargo through the pathway controlled by this Rab, creating an artefact. Similar effects were seen in (III), where the distribution of active/inactive integrin β1 confomers was studied by overexpression of different Rabs, and onlyonly minor differences between the treatments were observed. In the future, a better way to study the role of Rabs in integrin trafficking would be silencing rather than overexpression. However, there is the problem of redundancy: for example, if Rab21 is silenced, the balance of integrin trafficking could be shifted towards its close homolog Rab5.

The effect of the Rab GTPase cycle on the association with integrin was also studied. The GTP-locked Rab21 showed increased integrin association while the GDP-locked Rab21 decreased the association. A similar observation was made by Caswell et al. Rab25-GTP associated with β1 integrin, whereas GDP-loaded Rab25 did not. In our experiments the GTP-state of Rab21 also affects the subcellular localization of Rab21, and thus the mislocalization of Rab21 could explain the decreased association between β1 integrin and Rab21. However, the Rab25-β1 interaction study was performed *in vitro* and is therefore free of subcellular localization effects. Later, Mai et al. showed using fluorescence polarization assay *in vitro* that the Rab21-GTP/GDP both bind equally well the conserved α2 tail (Mai et al. 2011). These results suggest Rab21 interaction with integrins is nucleotide independent and localization of Rab21 is regulated by the Rab21-GTPase cycle is important for the integrin association.

The altered localization of endogenous integrins upon Rab21 overexpression indicated that Rab21 could regulate integrin trafficking. We found the overexpression of Rab5 and Rab21, but not Rab11a, increased integrin endocytosis. In line with this, the silencing of Rab21 reduced β1 integrin endocytosis. Expression of the GDP-locked Rab21 inhibited integrin endocytosis when compared to Rab21 wild-type (wt). Also, the expression of the GTP-locked Rab21 inhibited integrin endocytosis in short timepoint but accumulated more integrins overtime increasing the total level of endocytosed

integrin. The accumulation of integrins in endocytosis experiment is in line with the observation that the overexpression of Rab21-GTP showed enlarged integrin vacuoles in cell body. This could also be an effect of inhibited integrin recycling.

There is an interesting correlation between expression of Rab21-GDP increasing the number of integrin-positive focal adhesions and inhibition of integrin endocytosis. If we think that focal adhesion disassembly is regulated by integrin endocytosis (Ezratty et al. 2005; Chao and Kunz 2009; Ezratty et al. 2009), it would follow that the perturbation of integrin endocytosis by Rab21 that cannot associate with integrin (Rab21-GDP) or is mislocalized (Rab21-CCSS) could induce enlarged focal adhesions. In line with this we see increase in cell surface β1 integrin expression when Rab21-GDP is expressed when compared against Rab21-GTP or wild type in FACS.

Rab21 mediated integrin trafficking is also functionally important in cell adhesion and migration. Overexpression of Rab21 and Rab5 increased cell adhesion to the α2 integrin substrate collagen I, whereas Rab7, Rab11a and Rab9 did not. Cell migration was also increased by Rab21 overexpression in wound healing experiment. The increased in Rab21 mediated cell adhesion was dependent on α2-Rab21 association since the coexpression of α2 integrin mutant that was deficient in Rab21 binding (KR1160/1161AA) did not increase the cell adhesion. Conversely, silencing of endogenous Rab21 decreased cell adhesion and cell migration.

More recently Rab21 has been also associated with EGFR endocytosis (Yang et al. 2012). Overexpression of Rab21 increased EGFR endocytosis to Rab21 positive endosomes leading to increased lysosomal degradation of EGFR and thus decreasing the basal MAPK-ERK signalling pathway activity. Rab21 also associated with EGFR in immunoprecipitation experiments. Interestingly it also has been shown by Caswell et al. (2008) that integrin α5β1 is crosslinked to EGFR1 by their cytoplasmic tails being bridged by Rab-coupling-protein (RCP or Rab11-FIP1). RCP promotes the recycling of α5β1-RCP-EGRF1 complex back to the plasma-membrane leading to enhanced Akt and MAPK-ERK pathway signalling (Caswell et al. 2008; Muller et al. 2009). Integrins are known to associate with many growth factor receptors physically at the plasma-membrane (Ivaska and Heino 2010) and the effect of Rab21 regulating EGFR1 endocytosis could be co-endocytosis together with integrins. These results show nicely how integrin trafficking coordinates growth factor receptor signalling at the plasma-memrane. In general these pbservations suggest that endocytosis suppresses,but recycling enhances, intracellular signalling pathways.

	Integrin association	Intracellular vesicles	Integrins in FA	Integrin endocytosis	Cell adhesion	Cell migration
Rab21 wt	+	++	-	+	+	+
Rab21 GTP	++	++	-	- (later t. +)	0	0
Rab21 GDP	-	-	++	-	0	0
Rab21 CCSS	-	-	++	nd	nd	nd
Rab21 silencing	nd	nd	nd	-	-	-

Table 3. Effects of Rab21 in the regulation of integrin function. Not determined (nd). Negative (-). Positive(+). No effect (0).

5.2. Active and inactive β1-integrin trafficking (III)

In has been known integrins undergo endocytic traffic for over fifteen years and someof the key regulatory components and adaptors of the integrin trafficking have been identified (Caswell et al. 2009). However, the relationship between integrin activation and integrin trafficking has been incompeletely studied. There are studies with results related to the active and inactive integrin endocytosis (Powelka et al. 2004; Teckchandani et al. 2009; Jokinen et al. 2010) but systematic approaches have been missing.

We took the advantage of monoclonal antibodies recoqnising the different conformations (active or inactive) of β1 integrins (Byron et al. 2009) and used them to study integrin endocytosis and recycling. We observed that the subcellular localization of active and inactive β1 integrins was different. Active β1 integrin was mostly intracellular whereas the inactive β1 integrin was at the plasma-membrane. This initial observation is against the well accepted idea whereby the ligand bound, active integrins are mostly localized to focal adhesions in fibroblasts (Askari et al. 2010). However, the cells used in our study (epithelial breast adenocarsinoma MDA-MB-231 cells) do not display large focal adhesion-like structures in large scale. MDA-MB-231 cells instead show large and dynamic lamellipodial-like structures and ruffling edges.

The observation of active integrin being mostly intracellular would suggest the active integrin is endocytosed more compared to the inactive conformation. This is in line with growth factor receptor endocytosis, where the ligand binds the receptor which is in turn dimerized, phosphorylated and endocytosed (e.g c-MET). We tested this hypothesis further by incubating cells with antibodies against the active and inactive conformations (12G10 and mAb13 respectively) and saw a higher rate of active integrin endocytosis compared to the inactive. The integrin α5β1 ligand, fibronectin (FN) fragment (FNIII(7-10)) (Cutler and Garcia 2003), also endocytosed together with the active β1 integrin antibody to the same endosomal compartments whereas inactive did not. This shows endogenous, active, ligand-bound β1 integrins are endocytosed to the same endocytic compartments as the antibodies against the active conformation, an important observation used to validate the use of antibodies to study integrin endocytosis.

To further evaluate the use of antibodies in the study of integrin endocytosis we developed an antibody-based integrin endocytosis and recycling assay. The assay is outlined in original publication (III) figure 2. We compared our assay against the gold-standard cell surface biotinylation and immunoprecipitation based integrin trafficking method (Bretscher 1989; Roberts et al. 2001) and were able to measure similar kinetics of integrin endocytosis and recycling with both assays. This is an important result since it shows both assays probably measure the same phenomena although they are

quite different. Monoclonal β1 integrin antibodies bind only to the β1 integrin receptors whereas the cell-surface biotinylation labels all the amines exposed on cell-surface (integrins, growth factor receptors etc.). In the antibody based method we can distinguish the active and inactive confomers whereas with the biotinylation method it is not possible (denaturing conditions during gel electrophoresis). Denatuting conditions could also be avoided using Enzyme-Linked Immunosorbent Assay (ELISA) in detection.

On the other hand the biotin-tag is very small compared to the size of monoclonal antibody and thus biotin would not interfere with the natural function of integrins and could be more suitable for the experiments. We also tested the downstream signalling effects of both methods and found no significant upregulation of integrin dependent signalling pathways (phospho-FAK or phospho-ERK1/2) when activating 12G10 β1 integrin antibody or cell surface biotinylation was used. Nevertheless, we suggest both assays would be used side-by-side to study integrin trafficking since the overall number of integrin trafficking experiments (especially with antibody based method) is still quite limited in the literature.

Using the antibody-based endocytosis assay we measured endocytosis of both active and inactive conformations. Active β1 integrins were endocytosed to a greater extent than inactive β1. We also studied the endocytosis pathways undertaken by the two different confomers using antibody chase and overexpression of EGFP-tagged small Rab GTPases known to function in their own distinct pathways. The endocytosis pathways of both confomers overlapped to a large extent, except only the active β1 integrin was seen to colocalize with Rab7 positive vesicles. This indicates that the endocytic pathways of both confomers follow similar routes in the beginning but are later separated and only ligand-bound integrins traffic to the late endosomes. This is in line with Lobert et al. 2010, who showed that fibronectin bound α5β1 integrins travel to late endosomes/lysosomes (Lobert et al. 2010). Similarly, Dozynkiewicz et al. 2012 observed that active α5β1 integrin and fibronectin route to lysosomes (Dozynkiewicz et al. 2012).

The endocytosis mechanisms of both confomers were also similar since the perturbation of CME using dominant negative mutants of EPS15 mutant (EH29) and dynamin-2 (K44A) mutant blocked the endocytosis of active and inactive β1 integrins. Dominant negative caveolin-1 did not effect endocytosis of either conformer although it has been published that caveolin-1 also regulates active β1 integrin endocytosis (Shi and Sottile 2008). Both confomers also colocalised with dynamin and clathrin but not with caveolin. In fact, clathrin was seen to colocalize at the front of the cells together with active and inactive β1 integrin whereas caveolin localized to the rear of the cell. This polarized distribution of endocytic trafficking has been noted also before. In a study

showing thatcaveolar endocytosis usually takes place at the rear of migrating cells(Fletcher and Rappoport 2010).

Since the endocytosis of integrins was measured with no growth factor stimulus in our experiments, it is always balanced with integrin recycling (both endocytosis and recycling take place simultaneously at a given time at steady state). We tested whether the observed slower net-endocytosis rate of inactive β1 integrins would be balanced by constant recycling. Inhibition of integrin recycling with anti-malaria drug Primaquine increased the net endocytosis rate of inactive β1 integrin whereas only a small increase was seen on active β1 integrin. The mechanism of Primaquine to block recycling is related to the neutralisation of the endosomal pH (van Weert et al. 2000). In line with this, the inactive integrin also showed marked change in the subcellular localization after the Primaquine treatment. In steady state, the inactive integrin was mostly at the plasma-membrane whereas after Primaquine treatment the inactive integrin became notably localized into endosomes. Active integrin on the other hand was already vesicular at steady state and showed only a small increase in the endosomal pool after Primaquine treatment. These results suggest that inactive integrins move very rapidly and constantly through the endosomal recycling pathway. If the recycling is blocked, they accumulate into the endosomes. If Primaquine is washed away by changing the cell media, the localization of inactive integrin is restored to plasma-membrane.

The inactive β1 integrin containing endosomes induced by the blocking of the recycling localized close to the plasma-membrane and colocalized with early endosome antigen 1 (EEA1). Also, the colocalization between the two confomers increased significantly after the recycling was blocked. These results tell us the recycling of inactive β1 integrin separates the trafficking of the two integrin conformations. In line with this, expression of dominant negative EGFP-Rab4a-S22N (GTP binding deficient, fast integrin recycling pathway blocked) also increased the pool of inactive β1 integrin but had little effect on active β1 integrin. The use of dominant negative Rab4a was a good and more specific control in comparison to Primaquine to block integrin recycling. The effects of Primaquine could perturbate the vesicle recycling pathway in large scale.

Although we could show the recycling dependent separation of the two β1 integrin conformer pools the mechanism is still unclear. One possibility is that the tails of the active and inactive β1 integrins are recoqnised by regulators of integrin recycling and the pools are separated by these regulators. One example of this is the function of p120RasGAP (RASA1) displacing the rab21 bound on the integrin α tail (overlapping binding sites) and enhancing integrin recycling back to the plasma-membrane (Mai et al. 2011). Whether RASA1 would function in the Rab4a dependent recycling pathway is still unclear. Also, it is not known does RASA1 prefer binding to the active or inactive integrins.

We also noticed the recycling of inactive β1 integrin back to the plasma-membrane is dependent on actin polymerization. The inactive β1 integrin endosomes induced by blocking the integrin recycling with the Primaquine are positive for F-actin. When the Primaquine is washed away and inactive integrins are able to recycle back to the plasma-membrane. If however the polymerization of filamentous actin is inhibited by drug Cytochalasin D, the inactive integrin stays in the endosomal compartment. The F-actin dependent inactive integrin recycling endosomes were also positive for ARF6 which has been linked to actin-positive protrusions and to integrin recycling before (Radhakrishna and Donaldson 1997; Powelka et al. 2004). These results support the role of F-actin in inactive integrin recycling.

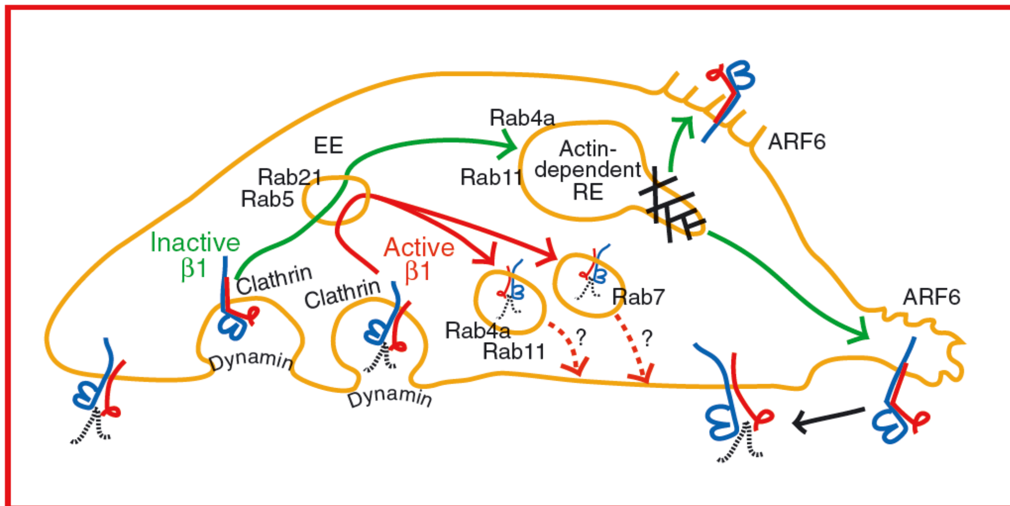

Figure 13. Model of active and inactive β1 integrin recycling in cancer cells. Both confomers are endocytosed clathrin and dynamin dependently to Rab5/Rab21 positive early endosomes. Fast actin and Rab4a-dependent recycling of inactive β1 integrin is seen to ARF6 positive membrane ruffles. Active β1 integrin accumulates in Rab7 positive endosomes. The recycling of active β1 integrin back to plasma-membrane is plausible but requires further investigation. Adapted from (Arjonen et al. 2012).

Our results would suggest that active β1 integrin is not efficiently recycled, at least compared to the levels of inactive integrin recycling. There are however studies showing active integrin is recycled to the plasma-membrane from lysosomes. Dozynkiewicz et al. 2012 use photoactivation of constitutively active EGFP-α5 integrin and an antibody (9EG7) to show active β1 integrin is recycled back to the plasma-membrane in its active conformation. This poses interesting questions: does integrins need to change their conformation in order to be recycled? Or are the observed recycled active integrins just forced to be recycled regardless of the conformation. The antibody-labelled integrins

are locked to the active conformation and thus the antibody-integrin complex would show lower recycling rates. Also the constitutively active α5 followed in the recycling experiments is unnatural since it probably cannot change its conformation. Another interesting unanswered question is related to the ligand of the active integrin. Is the ligand separation from integrins a sufficient signal to recycle the receptor back to plasma-membrane?

5.3. PI(3,4,5)P3 binding regulates the function of Myosin-10 (II)

Phosphatidylinositol phosphates (PIPs) are signaling lipids recruiting different proteins to the cell membranes to fullfill their tasks in cell growth and migration. PIPs interact with proteins containing the conserved pleckstrin homology domain (PH domain). There are three different signalling PIPs in cells depending on the site of phosphorylation (Figure 12). For example, PI3K targets and phosphorylates the third position in the aromatic carbon-ring generating PI(3)P. Depending on the exact PH-domain amino acid structure, PH domain containing proteins are either more specific in binding to PI(4,5)P2 or to PI(3,4,5)P3 (Park et al. 2008). The function PI(3,4,5)P3 is well documented in the cell migration. Upon stimulation, PI(3,4,5)P3 becomes transiently localized to the leading edge (cell polarization, the first step in the cell migration cascade) where it supports localized actin polymerization (Insall and Weiner 2001).

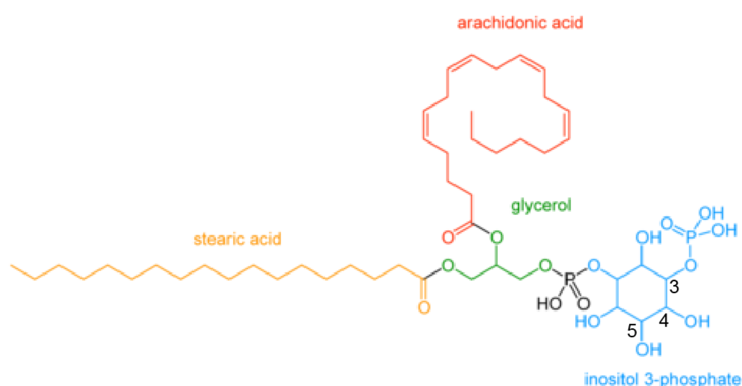

Figure 12. The strurcture of PI(3)P and the different phosphorylation positions on the aromatic ring.

Myosin-10 is unique among the myosin motors since it contains three PH domains (Berg et al. 2000) (see figure 7). EGFP-Myosin-10 expression induces filopodia formation and it localises as bright puncta to filopodia tips. The tips of filopodia are mostly attached to the ECM at the substratum level (plane of adhesion) but also dorsal puncta are seen to some extent. Deletion of all three myosin-10 PH domains changed the localization of EGFP-myosin-10 drastically, showing cytoplasmic puncta in the cell body. This indicates that the PH domains of myosin-10 are needed for proper localization of myosin-10 and suggests that PIP binding could recruit myosin-10 to plasma-membrane. Similar results were observed by using the PI3K inhibitors LY294002 and Wortmannin. Full length myosin-10 was very rapidly localized to cytoplasmic puncta after the production of PIP3 was pertubated. The filopodial localization

was also transiently restored after inhibitor washout indicating constant and dynamic regulation of myosin-10 localization in cells.

The functional relationship between PI3K and myosin-10 has also been noted before during macrophage phagocytosis (a method through which leukocytes engulf and eat bacteria by endocytosis of large particles). Phagocytosis requires actin polymerization, actin protrusions and PIP3 production by PI3K. Myosin-10 was shown to be translocated to phagocytic cups. However if a PI3K inhibitor was used myosin-10 no longer colocalised with the phagocytic cups and related F-actin (Cox et al. 2002). Also microinjection of an antiboby against myosin-10 inhibited the phagocytosis. These results indicate myosin-10 has biological importance during phagocytosis in a PI3K-dependent manner. Myosin-10 could support phagocytic cup formation by enhancing protrusion formation to engulf the bacterium.

Myosin-10 has been suggested to prefer PI(3,4,5)P3 binding over PI(4,5)P2 by *in silico* analysis of the myosin-10 PH domain amino acids (Park et al. 2008). In our lipid pull-down experiments we confirmed the myosin-10 binds PIP3 with high specifity. The binding site was mapped to the second PH domain of myosin-10 (called PH2 for simplicity) whereas the other PH domains (PH1 or PH3) did not bind to any lipids. The results were confirmed *in vitro* using point mutations in the PH domains. The EGFP-myosin-10-PH2pm (point mutated PH2 domain KK1215/6AA) showed a cytoplasmic punctate phenotype whereas point mutations to PH domain 1 or 3 conserved residues showed no relocalization.

All three myosin-10 PH domains are tandemly organized. However the PH2 domain is inserted in the middle of PH1 domain for unknown reason (from N to C-terminus: PH1a-PH2-PH1b-PH3). The split PH domain structure is uncommon among other PH domain containing proteins. However the split PH domain structure is conserved in the myosin-10 sequence among different species indicating functional importance (Lu et al. 2011). The split PH domain structure was further studied by Lu et al.. They where able to verify our results and confirm that the PH2 domain of myosin-10 is important in PIP3 binding specificity (Lu et al. 2011), and further showed that the PH1 domain inserts on both sides of PH2 domain increase the membrane binding avidity (binding strength of multiple single affinities) of the tandem PH1a-PH2-PH1b.

The biological relevance of myosin-10 PIP3 binding is seen during filopodia formation. Expression of the PH2 mutant unable to bind PIP3 showed fewer filopodia, whereas the PH1 or PH3 mutants showed expression of filopodia similar to the wild type full length myosin-10. In line with this the use of PI3K inhibitors reduced filopodia formation. Interestingly, when the full length myosin-10 PH2 domain was swapped with PH domains from other PH domain containing proteins using Bruton's tyrosine kinase (Btk), Phospholipase C

gamma (PLCδ1) and tandem PH-domain-containing protein 1 (TAPP1) which bind to PI(3,4,5)P3, PI(4,5)P2 and PI(3,4)P2 respectively, only Btk and PLCδ1 PH domains (PI(3,4,5)P3 and PI(4,5)P2 binding) supported myosin-10 localization to filopodia tips. This shows that the localization of myosin-10 to the plasma-membrane is needed for filopodia formation since both PIP3 and PI(4,5)P2 have been described to be enriched at the plasma-membrane (Heo et al. 2006) whereas the PI(3,5)P2 is an endolysosome specific lipid (Dong et al. 2010).

The rescue of myosin-10 localization to filopodia tips by a PI(4,5)P2 binding PH domain is also interesting from the filopodia formation point of view. It has been shown that filopodia-like structures can be formed *de novo* in the presence of PI(4,5)P2, membrane bending F-BAR proteins and actin nucleation factors Arp2/3 complex (Lee et al. 2010). What if myosin-10 cooperates with these proteins in the filopodia formation? Cooperation with other proteins to make myosin-10 dependent filopodia is plausible since myosin-10 does not have any functional domains to induce actin polymerization itself. On the other hand silencing of myosin-10 in mouse fibroblasts has been shown to reduce the endogenous microspikes seen in lamellipodia (Tokuo et al. 2007). The observed overall reduction in filopodia formation detected with F-actin staining indicates a more general role for myosin-10 in filopodia formation although myosin-10 is not necessary for filopodia formation.

One important aspect of the myosin-10 filopodia inducing function has been suggested to be related to the dimerization of myosin-10. The Myosin-10 protein structure also contains a coiled-coil domain. Coiled-coils are structural motifs where two or more α-helices are wound together like strands of rope, and they usually mediate dimerization of multimeric proteins. It has been suggested that the myosin-10 coiled-coil would make a dimer (Berg et al. 2000) but it has been never verified experimentally. The dimerization has also been suggested to be the mechanism responsiple for filopodia formation in the actin fiber convergence model (Tokuo et al. 2007). We observed that the EGFP-myosin-10-PH2 mutant did not completely abolish the myosin-10 filopodial localization. One explanation for the effect could be that the endogenous myosin-10 (with fully operational PH domain and PIP3 binding) could make a dimer with the exogenously expressed PH2 mutant and partially rescue the mislocalization. Using co-immunoprecipitation between overexpressed EGFP-myosin-10 and mCherry-myosin-10 we indeed saw that myosin-10 was dimerized. EGFP-myosin-10 was associated with mcherry-myosin-10. The presence of the PH domain mutation (overexpression of EGFP-myosin-10 and mcherry-myosin-10 with PH domain mutations in both) abolishing the PIP3 binding did not affect the dimerization in our experiments. Umeki et al. 2011 have also studied the structure and dimer formation of myosin-10. In conflict with our results they show that the binding of PIP3 to myosin-10 would induce dimer formation (Umeki et al. 2011). Our mutants

were unable to bind PIP3 but still dimerized. Umeki et al. concluded myosin-10 would be monomeric and the PH and FERM domain of myosin-10 would fold on itself and bind intramolecularly the head (motor domain) of myosin-10 forming an autoinhibited conformation. PIP3 binding would open up this conformation and allow myosin-10 to dimerize, localize to plasma-membrane and make filopodia. Our results with myosin-10 dimerization are based on co-immunoprecipitation only whereas the results by Umeki et al. have structural visualisation of the myosin-10 protein folding using electron microscopy. The results (monomeric myosin-10 in the absence of PIP3) by electron microscopy are could be more accurate since immunoprecipitation of overexpressed constructs from cell lysates can be prone to false positive results. Also mechanistically when looking at the big picture of myosin-10 function and activation, the monomeric autoinhibited folding would make sense. Another FERM domain containing protein involved in integrin binding is focal adhesion kinase (FAK). FAK also adobts and autoinhibited conformation where FERM domain binds the kinase domain intramolecularly (Lietha et al. 2007). FAK autoihnibition is regulated by phosphorylation of Tyr397. Myosin-10 function could also be regulated by phosphorylation together with the PIP3 binding to myosin-10. Indeed several databases show multiple detected phosphorylation sites on myosin-10 at the region of FERM domain and close to the PH domains (Phosida, Scansite). The phosphorylation of myosin-10 could also explain the difference in our results compared to Umeki et al. In our model (cells in vitro) myosin-10 could be phosphorylated whereas in the structural studies the other modificators and cofactors are missing. I would speculate the phosphorylation of myosin-10 could open up the myosin-10 conformation allowing its dimerization and making it active.

We further characterised the myosin-10 cytoplasmic puncta seen PI3K inhibitor treated cells expressing wild type EGFP-myosin-10. We found the puncta were moving similarly as vesicles in live cell imaging experiments. Also the microtubule (MT) depolymerizing drug nocodazole pertubed the movement of myosin-10 positive vesicles confirming they are vesicles moving along MT tracks. The same observation was made with EGFP-myosin-10-PH2 mutants. These myosin-10 vesicles were positive for the small GTPase late endosomal marker Rab7 and lysosomal-associated membrane protein 1 (LAMP1, a lysosomal marker). Markers for early endosomes or recycling endosomes were negative (Rab5, Rab21, Rab4a and Rab11). This was an unexpected observation since myosin-10 has not been described to localize to the late endosomal pathway before. Also, we found the Rab7/myosin-10 vesicles moved in close proximity to the plasma-membrane, seen by evanescence wave illumination in TIRF microscopy (100nm above the cell's plane of adhesion). Myosin-10 and Rab7 have been descriped in phagosome formation. Also, Rab7 vesicles and late endosomes are known to fuse with phagosomes. Thus, Rab7 vesicles could transport myosin-10 to the site of phagosome formation (Harrison et al. 2003).

5.4. Myosin-10 promotes breast cancer invasion (IV)

The role of myosin-10 in cell migration has been firmly established (Pi et al. 2007; Hwang et al. 2009). However the role of myosin-10 in cancer has not been studied before. We observed by analysing published *in silico* data that among Swedish and Norwegian breast cancer patients the high myosin-10 expression was associated with the worst outcome basal-like breast cancer subtype (triple negative) (Sorlie et al. 2001; Miller et al. 2005).

Also, the expression of myosin-10 on protein level was high in basal-like breast cancer cell line (MDA-MB-231) but not in luminal-like or epithelial-like cell lines (MCF7 and MCF10A respectively). We confirmed the inhibitory effect of myosin-10 silencing on cell migration and adhesion in the MDA-MB-231 breast cancer cell line. The effect was also seen in filopodia formation and breast cancer cell invasion *in vitro*. These experiments imply the role of myosin-10 in breast cancer invasion is important, since silencing the myosin-10 gene alone is enough to show effects in four important invasion related biological aspects: cell adhesion, filopodia formation, cell migration and cell invasion into matrigel.

We next continued to study the myosin-10 related breast cancer cell (MDA-MB-231 cells) invasion using *in vivo* models. We used Zebrafish *Danio Rerio* tumor invasion model where myosin-10 shRNA expressing cells were injected into the the perivitelline cavity of living Zebrafish embryos. The invasion of cells was monitored four days after injection. Myosin-10 silenced cells invaded significantly less in the trunk and tail region of the Zebrafish. Similar results were obtained in mice. Myosin-10 silenced breast cancer cells were injected to the tail vein of mice and the invasion to mouse lungs was studied. Myosin-10 silenced cells extravasated less to the lungs of mice in this *in vivo* tumor invasion model. This is the first time that myosin-10 has been shown to be important in cancer cell invasion.

The localization of integrins to filopodia tips has been shown to require binding of myosin-10 FERM domain to the integrin β tail (Zhang et al. 2004). If the myosin-10 FERM domain is mutated, or if the FERM domain binding site in the β1 tail (W775A) is mutated, the filopodia are shorter. In our experiments we saw similar effects using integrin β1 function blocking antibodies (mAb13). Inhibition of integrin β1 binding to the matrix abolished attachment of the filopodia tip to the matrix, and resulted in shorter filopodia. These unanchored filopodia were shorter and kinked, indicating thatfilopodia formation was not hampered but instead the anchoring or stabilization of myosin-10 induced filopodia was inhibited. Timelapse microscopy confirms this observation showing EGFP-myosin-10 transports myosin-10 to the filopodia tips but at given timepoints integrins stay at the filopodia tips (keeping the filopodia elongated and attached to the matrix) whereas myosin-10 moves back and fourth. The binding of myosin-10 to the integrins was also needed for cancer

cell invasion. The cells overexpressing EGFP-myosin-10-delF2 (FERM domain with the subdomain 2 deleted) invaded matrigel plugs less than cells overexpressing wild type myosin-10.

Based on the encouraging results that silencing myosin-10 *in vivo* inhibited cell invasion, we next studied the protein-level expression of myosin-10 in breast cancer samples of Finnish patients. The high myosin-10 expression correlated with lower survival in the *in silico* mRNA datasets (Sorlie et al. 2001; Miller et al. 2005). We saw similar correlation in our tissue microarray dataset of over 1300 patient samples. High myosin-10 immunohistochemistry staining correlated positively with lower survival. Among the tumor samples the myosin-10 staining was higher at the invasive edges of the tumors compared to the middle regions of the tumor. The association of high myosin-10 staining and low survival was even stronger and more significant with the subpopulation of patients with lymph-node positive (spread) tumors (580 patients). The tissue microarray data supports the *in silico* observations that myosin-10 expression is high in cancer.

We also noticed the high myosin-10 expression correlated with the clinical status of mutated TP53 oncogene. TP53 is a tumor suppressor gene that regulates the genome integrity by sensing the mutations in the DNA. If mutations are sensed by TP53 it can induce apoptosis or growth arrest and active many DNA repair genes. Mutations in the TP53 gene are associated with more then half of human tumors and thus the growth arrest or apoptosis related guardian functions are lost. In the *in silico* data, and in the TMA staining, high myosin-10 levels correlated with high p53 levels. In the breast cancer cell lines, the basal-like MDA-MB-231 cells, known to express mutant p53, had high myosin-10 expression whereas the wild-type p53 cell lines MCF7 and MCF10A had low myosin-10 expression. To our surprise, the exogenous expression of mutant p53 (p53 R273H, one of the hot-spot p53 mutations very common in many cancers) increased the protein levels of myosin-10 in p53 null background (knock-out colon cancer p53 -/- cells). In line with this silencing, the mutated p53 gene in MDA-MB-231 cells resulted in the loss of myosin-10 expression. These results suggest the expression of myosin-10 is regulated by mutant p53.

Mutant p53 has been shown to gain functional properties in many ways in cancer. Although wild type p53 is a cancer suppressor, the mutations found in the p53 gene in cancer are mostly in the DNA binding domain of p53. Thus, these mutations abrogate the DNA binding and function of the wild type p53 and promote novel gain-of-function properties (Freed-Pastor and Prives 2012). The best studies gain-of-function mechanisms of the mutant p53 include: inhibition of the function of p63/p73 transcription factor by a direct association, binding to and enhancing the function of other transcription factors, binding of mutant p53 to promoter regions of other genes resulting transcriptional

activation and binding of mutant p53 to proteins that are not transcription factors.

Our preliminary results show mutant p53 could bind to the promoter region of the transcription factor early growth response 1 (EGR1), which has been shown to regulate myosin-10 transcription (Weisz et al. 2004; Cermak et al. 2010). Via this mechanism mutant p53 could first activate EGR1 transcription by binding to its promoter region, and promote EGR1 expression and thus myosin-10 transcription.

There are also other studies showing that integrin dependent cancer cell migration is enhanced by mutant p53. Mutant p53 has been shown to increase the recycling of EGFR/integrin receptor complex and to maintain the activation of EGFR/integrin signalling pathways via repressing p63 function (Muller et al. 2009). The recycling of the hepatocyte growth factor receptor c-MET was also shown to be enhanced by mutant p53 and to promote cell invasion (Muller et al. 2013). Mutant p53 could promote myosin-10 dependent invasion is via PI3K. The enhanced EGFR/integrin signalling could result in activation of PI3-kinase. The activation of PI3-kinase could thus lead to enhanced production of PI(3,4,5)P3 and myosin-10 translocation to filopodia tips and induction of pro-invasive filopodia.

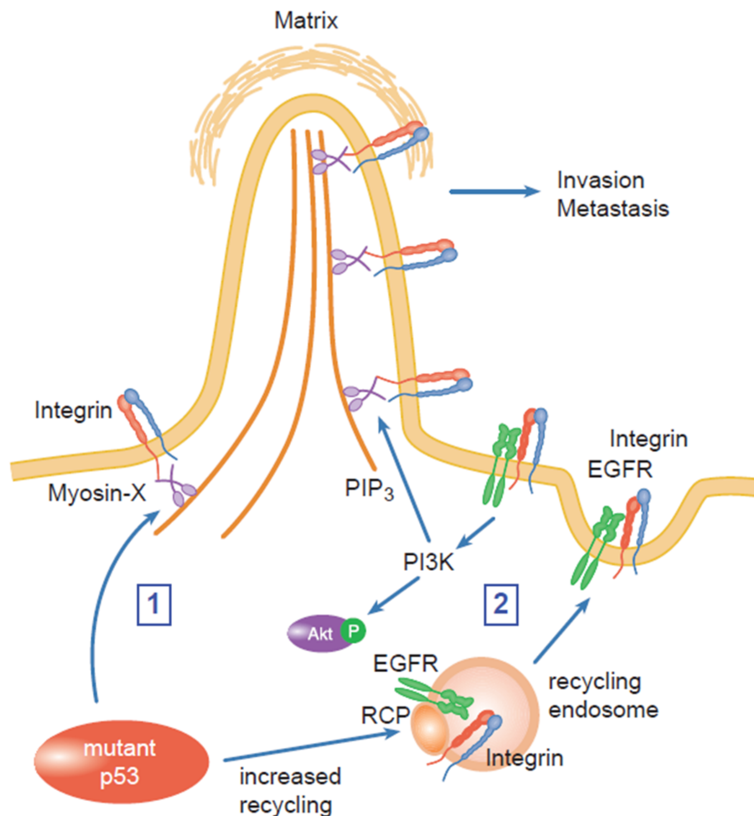

Figure 14. Mechanism of myosin-10 dependent filopodia induction leading to increased invasion. 1) Mutant p53 binds to the promoter of EGR1 and EGR1 is a transcription factor for myosin-10 leading to increased myosin-10 levels. 2) Mutant p53 increases the recycling of EGFR/integrin complex and enhances the PI3K signalling. Increased production of PIP3 supports myosin-10 mediated filopodia formation. Adapted from (Arjonen, Kaukonen et al. 2013, in revision).

6. CONCLUSIONS

The aim of this thesis was to understand how integrin trafficking and filopodia function could regulate cell migration and how these prosesses could affect cancer spreading.

The concept of integrin trafficking and its role in cell migration is a relatively new topic in the field of integrin research. We showed that the small GTPase Rab21 binds directly to the α2 integrin cytoplasmic tail and regulates the subcellular localization of integrins, and that Rab21 (and Rab5) is a positive regulator of integrin endocytosis. The regulation of integrin endocytosis had direct implications on cancer cell adhesion and migration both of which are very relevant biological functions in cancer spreading. The results also further highlight the importance of receptor trafficking in cancer, an emerging hallmark of cancer spreading, and provide new targets for cancer therapy.

We also raised an important discussion about the relationship between the integrin conformation and the regulation of integrin endocytic trafficking. By directly comparing active and inactive integrin β1 trafficking we noticed inactive β 1 integrin was rapidly recycled back to the plasma-membrane in an actin dependent manner, whereas the active ligand-bound β 1 integrin was retained in the lysosomal pathway. These results are mechanistically important in order to understand how the ligands of integrins (outside activators) and the cytoplasmic tail binding partners (inside activators and inactivators) regulate the integrin trafficking and participate in cell migration.

Actin-rich cellular protrusions, filopodia, are also very important in cell migration during development and growth cone guidance serving as antennae probing the matrix. Myosin-10 is an intriguing motor since it can induce filopodia and transport integrins to the tips of filopodia. We studied how myosin-10 filopodia formation is regulated and found that the PI3-kinase product PI(3,4,5)P3's binding to pleckstrin homology domain 2 of myosin-10 is an essential process for the filopodia formation in cancer cells.

The role of myosin-10 dependent filopodia in cancer spreading has not been studied before. In this thesis we show myosin-10 is pro-invasive and is involved in cancer spreading in an integrin-dependent fashion. High myosin-10 levels are associated with poor survival among Finnish, Swedish and Norwegian breast cancer patients. At the level of the cancer cell, inhibition of myosin-10 function inhibits filopodia formation and affects filopodia dependent cell adhesion, migration and invasion. The *in vivo* models of metastasis formation (zebrafish invasion, mouse lung extravasation and orthotopic mouse lung metastasis) show that RNAi mediated inhibition of myosin-10 decreases

the number or metastasis formed by human breast cancer cells. We also found that the mechanism of myosin-10 upregulation is related to the widely studied oncogene TP53 (mutated in half of cancers). Mutant TP53 binds to and activates the promoter region of EGR1 which in turn activates myosin-10 transcription. Targeting this TP53-EGR1-myo10 axis could be a novel treatment to brake down breast cancer invasion. Furthermore, targeting filopodia formation in general would represent a novel way to treat cancer.

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