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INTEGRIN TRAFFIC AND SIGNALLING – FROM PLASMA MEMBRANE TO ENDOSOMES

Jonna Alanko

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To my family
and in memory of my father

Jonna Alanko

Integrin traffic and signalling – from plasma membrane to endosomes

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ABSTRACT

Integrins are the main cell surface receptors by which cells adhere to the surrounding extracellular matrix (ECM). Cells regulate integrin-mediated adhesions by integrin endo/exocytic trafficking or by altering the integrin activation status. Integrin binding to ECM-components induces several intracellular signalling cascades, which regulate almost every aspect of cell behaviour from cell motility to survival, and dysregulation of integrin traffic or signalling is associated with cancer progression.

Upon detachment, normal cells undergo a specialised form of programmed cell death namely anoikis and the ECM-integrin -mediated activation of focal adhesion kinase (FAK) signalling at the cell surface has been considered critical for anoikis suppression. Integrins are also constantly endocytosed and recycled back to the plasma membrane, and so far the role of integrin traffic in cancer has been linked to increased adhesion site turnover and cell migration. However, different growth factor receptors are known to signal also from endosomes, but the ability of integrins to signal from endosomes has not been previously studied.

In this thesis, I demonstrate for the first time that integrins are signalling also from endosomes. In contrast to previous believes, integrin-induced focal adhesion kinase (FAK) signalling occurs also on endosomes, and the endosomal FAK signalling is critical for anoikis suppression and for cancer related processes such as anchorage-independent growth and metastasis. Moreover, we have set up a new integrin trafficking assay and demonstrate for the first time in a comprehensive manner that active and inactive integrins undergo distinct trafficking routes. Together these results open up new horizons in our understanding of integrins and highlight the fundamental connection between integrin traffic and signalling.

Keywords: Integrin traffic, integrin signalling, endosomal signalling, FAK, focal adhesion, Rab21, EEA1

Jonna Alanko

Integriinien liikenne ja signalointi – solukalvolta solunsisäisiin endosomeihin

Biokemian laitos, Turun yliopisto, Turun Biotekniikan keskus, Suomen teknologian tutkimuskeskus VTT, Biolääketieteen ja molekulaarisen luonnontieteen tohtoriohjelmat, Turun yliopisto, Suomi.

TIIVISTELMÄ

Integriinit ovat solujen tärkeimpiä solunpinnan tarttumisreseptoreita, joilla solut kiinnittyvät niitä ympäröivään soluväliaineeseen. Solut säätelevät integriini-välitteistä tarttumistaan integriinien kuljetuksella tai muuttamalla integriinien aktiivisuutta. Soluväliaineen aktivoima integriinien solunsisäinen signalointi säätelee lähes kaikkia solun normaaleja toimintoja, kuten solujen liikkumista ja selviytymistä, ja virheet joko integriinien kuljetuksen tai signaloinnin säätelyssä edesauttavat syövän syntymistä ja leviämistä.

Integriini-välitteinen tarttuminen on elintärkeä normaaleille soluille, koska ilman sitä solut kuolevat erityisen ohjelmoidun solukuoleman (anoikis) seurauksena. Soluväliaineen aiheuttama integriini-välitteinen FAK-signalointi estää anoikiksen, ja tämän on uskottu tapahtuvan ainoastaan solun pinnalta. Integriinejä kuitenkin kuljetetaan jatkuvasti solun sisään ja takaisin, mikä on tärkeää solujen liikkumiselle ja jakautumiselle. Syöpäsoluissa integriinien lisääntynyt kuljetus on yhdistetty syövän leviämiseen sekä etäpesäkkeiden muodostumisen lisääntyneeseen todennäköisyyteen. Erilaisten kasvutekijäreseptoreiden on jo pitkään tiedetty signaloivan myös solunsisäisistä vesikkeleistä, endosomeista, mutta integriinien kykyä signaloida endosomeista ei ole koskaan aikaisemmin tutkittu.

Tässä väitöskirjatyössä osoitan ensimmäistä kertaa, että integriinit signaloivat myös endosomeista. Vastoin aikaisempaa uskomusta, integriini-välitteinen FAK-signalointi tapahtuu endosomeista, mikä lisää alkuperäisestä kasvuympäristöstään irronneiden ja verenkiertoon kulkeutuneiden syöpäsolujen selviytymistä ja leviämistä. Lisäksi kehitimme uuden menetelmän integriini-liikenteen tutkimiseksi ja osoitamme ensimmäistä kertaa, että aktiiviset ja inaktiiviset integriinit kuljetetaan soluissa eri reittejä. Yhdessä tulokseni avaavat uusia näkökulmia integriinien toimintaan, ja korostavat integriinien liikenteen ja signaloinnin välistä vääjäämätöntä yhteyttä.

Avainsanat: Integriinien liikenne, integriinien signalointi, endosomaalinen signalointi, FAK, Rab21, EEA1

TABLE OF CONTENTS

ABBREVIATIONS

APPL 1/2	Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1/2
CCP	Clathrin-coated pits
CCV	Clathrin-coated vesicles
CIE	Clathrin-independent endocytosis
CLIC	Clathrin-independent carriers
CLIC3	Chloride Intracellular Channel Protein 3
CME	Clathrin-mediated endocytosis
CytD	Cytochalasin D
ECM	Extracellular matrix
EE	Early endosome
EEA1	Early endosome antigen-1
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FA	Focal adhesion
FAK	Focal adhesion kinase
FAT	Focal adhesion targeting domain
FERM	Band 4.1, ezrin, radixin, moesin -domain
FN	Fibronectin
IF	Immunofluorescence
IP	Immunoprecipitation
LE	Late endosome
NA	Nascent adhesion
PI	Phosphoinositide
PIP	Phophatidylinositol-phosphate
PM	Plasma membrane
PQ	Primaquine
Pyk2	Proline-rich tyrosine kinase-2
RCP	Rab-coupling protein
RGD	arginine-glycine-aspartic acid
RTK	Receptor tyrosine kinase
STED	Stimulated emission depletion

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 **Distinct recycling of active and inactive β1 integrins**
Arjonen A, Alanko J, Veltel S, Ivaska J. (2012) *Traffic*,13(4):610-25.

II **Integrin endosomal signalling suppresses anoikis**
Alanko J, Mai A, Jacquemet G, Schauer K, Kaukonen R, Saari M, Goud B, Ivaska J. (2015) *Nat Cell Biol*. 17(11):1412-21.

III **Endosomes: Emerging Platforms for Integrin-Mediated FAK Signalling**
Alanko J, Ivaska J. (2016) *Trends Cell Biol*. 26(6):391-398. (Opinion article)

1. INTRODUCTION

Multicellular organisms are extremely complex and fine-tuned systems composed of different tissues and organs. Cells in different tissues create their unique environment by secreting and remodelling extracellular matrix (ECM) components to form, together with other cells, all the structures that comprise a functional organism. The main cellular receptors for assembly, monitoring and binding the surrounding ECM are a family of transmembrane receptors called integrins (Hynes, 2002).

Every cell in each tissue type has its own place to maintain the homeostasis and the functionality of an organism. The correct location of cells is guaranteed largely by integrins: their intracellular signalling provides the basis for an elegant surveillance system called anoikis, ensuring that only cells in a right environment survive (Gilmore, 2005, Guadamillas et al, 2011, Paoli et al, 2013). On the other hand, the functionality of integrins themselves is regulated to a great extent by their subcellular location. Integrins undergo constant intracellular trafficking, which is essential for remodelling of cell adhesions and thus for cell migration, invasion and cancer metastasis. The intracellular trafficking of integrins, as with all transmembrane receptors, is achieved by vesicular transport in intracellular vesicles – the endosomes. As a result, the cytoplasm of a typical eukaryotic cell is highly populated with a variety of endosomes, each of them carrying specific cargo to specific destinations in a tightly orchestrated process. Given the fundamental role of integrins, not only in normal physiology but also in cancer progression, understanding the complex regulation of integrin traffic and signalling is crucial, and has been under intensive research over the last two decades.

In this thesis, the intracellular trafficking of integrins, mastered by small Rab GTPases, as well as the ECM-induced integrin signalling, crucial for cell survival and growth, will be discussed. Moreover, the concept of endosomal signalling in general will be introduced with a special focus on the unique features of endosomes which enable them to act as multifunctional signalling platforms, by which cellular signalling can be regulated, fine-tuned and amplified in a spatiotemporal manner.

2. REVIEW OF THE LITERATURE

2.1. CELLULAR MEMBRANES & PHOSPHOINOSITIDES

Cellular membranes define semi-permeable borders which separate cells from their environment, but also surround all the various intracellular compartments within a cell. These membranes are formed by a lipid bilayer, where proteins are inserted via transmembrane domains or post-translational modifications enabling membrane tethering. Cellular membranes have a key role in cellular signalling, as they not only enable receptors at the plasma membrane (PM) to communicate with the extracellular space, but also function to enrich the local concentration of intracellular signalling proteins, thereby facilitating protein-protein interactions, essential for signalling to occur and to be transmitted (van Meer et al, 2008).

In addition to these, cellular membranes bear specific lipids critical for binding of different signalling scaffolds. The lipid bilayer in mammalian cells is mainly composed of phospholipids, and among these, phosphatidylinositol and its phosphorylated derivatives, phosphoinositides (PIs) and phosphatidylinositol-phosphates (PIPs), form a small fraction of less than 10% (De Matteis & Godi, 2004). Irrespective of their low abundancy, these special lipids have been implicated in almost every aspect of cellular behaviour (Di Paolo & De Camilli, 2006). Phosphatidylinositol is composed of a d-*myo*-inositol-1-phosphate, which is linked to diacylglycerol via the phosphate group. The inositol ring, comprising six free hydroxyl groups, can be reversibly phosphorylated at positions 3, 4 and 5, thereby giving rise to seven distinct PIPs with specific subcellular locations. The most abundant PIP is PI(4,5)P2 (phosphatidylinositol-4,5-bisphosphate), which is mostly found from the PM, whereas PI(3)P (phosphatidylinositol-3-phosphate) for example is characteristic for early endosomes (De Matteis & Godi, 2004, Di Paolo & De Camilli, 2006) (Figure 1).

To produce and maintain different PIPs, cells have developed a large number of different organelle-specific kinases and phosphatases, which co-operatively produce PIPs either constitutively or in response to a stimulus, including integrin binding to the extracellular matrix (ECM). The main cellular function of PIPs is to specify membrane identity and to provide membrane-binding platforms for various signalling and adaptor proteins. By doing so, PIPs have a key role in multiple cellular functions, including cell signalling, adhesion and receptor trafficking (Di Paolo & De Camilli, 2006, Saarikangas et al, 2010).

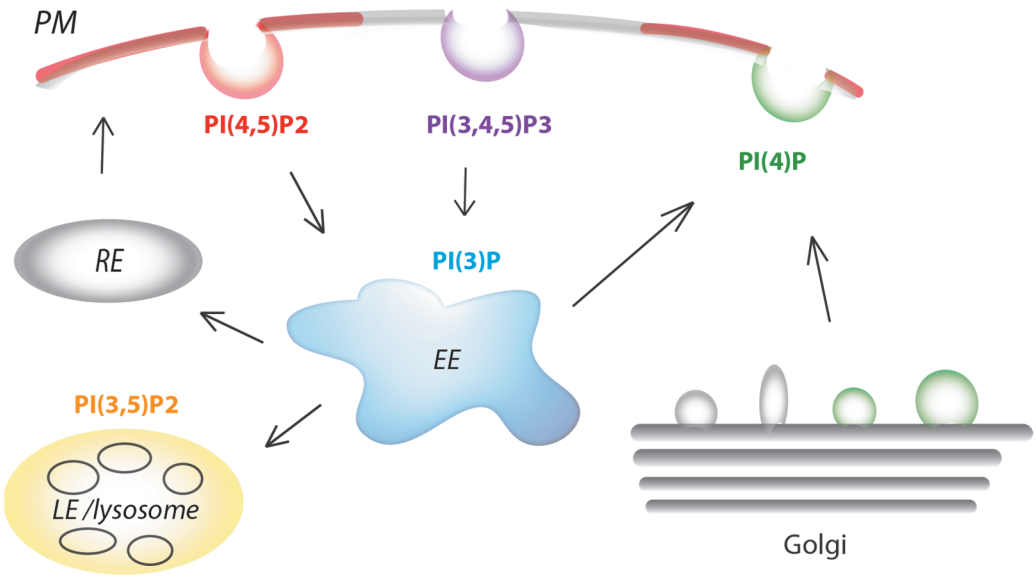

Figure 1. Subcellular distribution of phosphatidylinositol-phosphates. PI(4,5)P2 and PI(3,4,5)P3 are enriched at the plasma membrane (PM), PI(3)P is found almost exclusively from early endosomes (EE), PI(3,5)P2 is enriched in late endosomes (LE) / multivesicular bodies and lysosomes, whereas PI(4)P is enriched in at the Golgi complex. This figure illustrates a simplified picture, and overlap occurs between different PIPs in distinct subcellular compartments.

2.2. INTEGRINS

2.2.1. Integrin superfamily – general classification

Integrins are a family of heterodimeric transmembrane adhesion receptors mediating the bidirectional connection between cells and the surrounding extracellular matrix (ECM) (Hynes, 2002). These proteins were originally discovered as integral membrane proteins, therefore named as “integrins”, but the meaning was later strengthened by discovering their ability to physically connect - or integrate - the intracellular actin cytoskeleton to the ECM through various adaptor proteins (Hynes, 2002, Tamkun et al, 1986). In addition to their mechanical roles in anchoring cells, integrins transmit chemical signals into the cell, providing information on the cell’s location, adhesive state and surrounding matrix. Integrins have a fundamental role in biological processes such as embryonic development, tissue maintenance and repair, hemostasis

and immune response, among others (Harburger & Calderwood, 2009). As such, integrins are indispensable for the existence of metazoa, and dysregulation of integrin expression or function is implicated in a number of pathological conditions, including cancer (De Franceschi et al, 2015).

The heterodimerisation of 18 α-subunits and 8 β-subunits leads to the formation of at least 24 different integrin heterodimers with cell type-specific expression patterns and different, but overlapping, substrate specificity in all mammalian cells except for erythrocytes (Hynes, 2002). Each subunit is a type I transmembrane protein composed of a large extracellular domain, a transmembrane domain and, except for β4, a relatively short cytoplasmic tail domain of ~20-50 residues. The α- and β-subunits associate via non-covalent interactions and together form the heterodimeric integrin with a shape that resembles a large extracellular “head” and two membrane spanning “legs”. The head constitutes the main α/β interface, but also a putative salt bridge is formed between conserved membrane-proximal sequences in integrin α- and β-tails (Hynes, 2002). These cytoplasmic tails form the base for the assembly of large intracellular ECM-induced signalling complexes, including several actin-binding proteins, by which integrins link the extracellular matrix to the intracellular actin cytoskeleton (Campbell & Humphries, 2011).

The composition of extracellular matrix varies between different tissues, and integrins are traditionally classified into four different classes based on their ligand preference and cell type. These are (1) integrins that recognise the RGD (arginine-glycine-aspartic acid) tripeptide sequence in ligands such as fibronectin and vitronectin, (2) laminin-binding integrins, (3) collagen-binding integrins and (4) leukocyte-specific integrins, which bind cellular counter-receptors such as vascular cell adhesion molecule-1 (VCAM-1), thereby mediating cell-cell interactions between leukocytes and endothelial cells (Hynes, 2002). The most common integrin subunit is β1, which is ubiquitously expressed and forms half of the integrin heterodimers by pairing with 12 different α-subunits (Figure 2a). As a result, complete knockout of β1 in mice is embryonic lethal, while knockout of single α-subunits display predominantly milder phenotypes (Fassler & Meyer, 1995, Stephens et al, 1995).

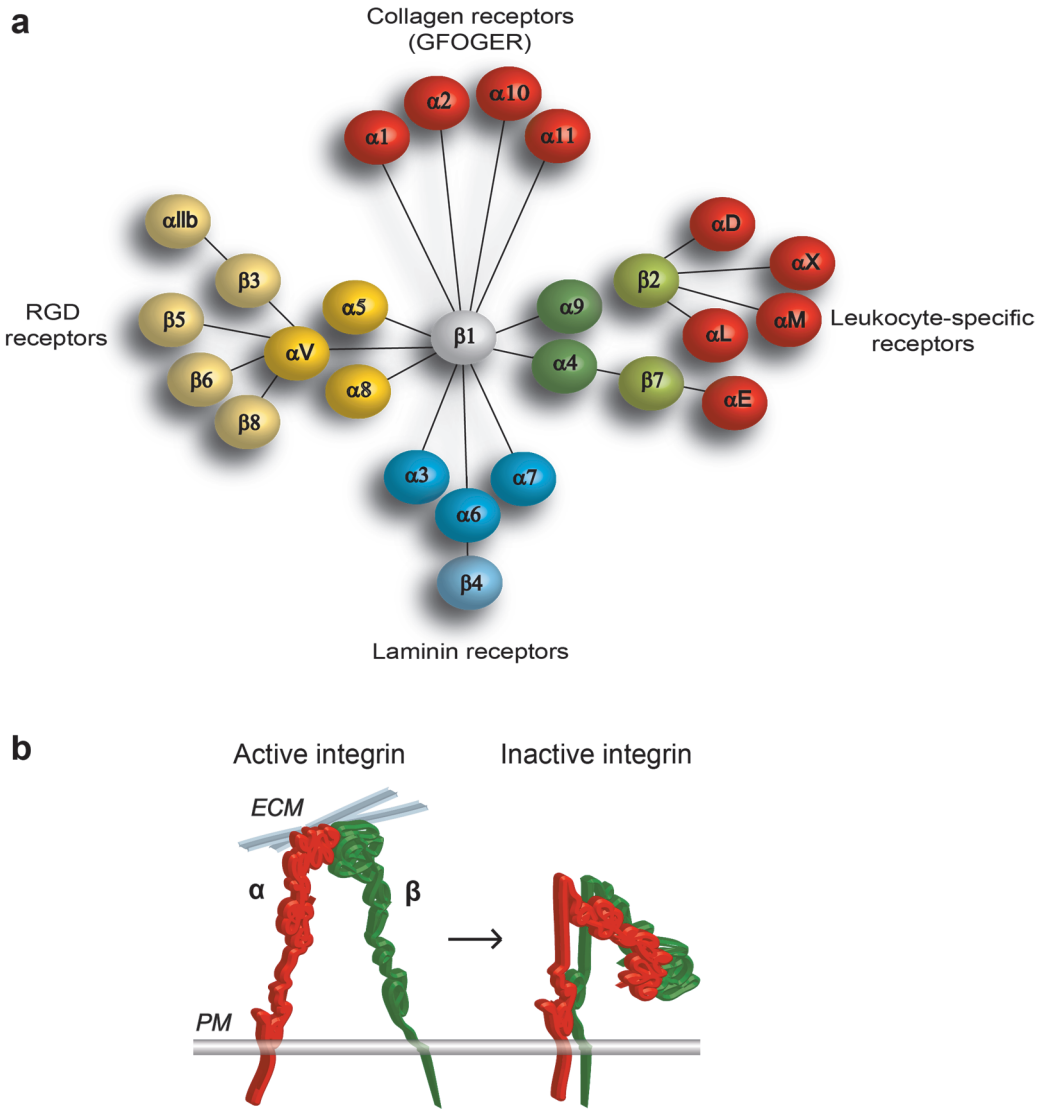

Figure 2. The integrin family. a) The integrin α- and β-subunits can form 24 different heterodimers, which can be divided into four main groups based on substrate recognition. The integrin α-subunits shown in red contain an αI-domain. **b)** Integrins can adopt an active, extended conformation or an inactive, bent conformation. (Hynes, 2002)

2.2.2. Integrin activation – outside-in & inside-out

Integrin receptors are unique in a sense that their affinity for a ligand can be modulated, and even from both sides of the PM. Most of the cell surface integrins exist in their inactive conformation, having low affinity for a ligand, but integrin activation can be triggered by integrin binding to an extracellular ligand or by specific integrin activators, which bind to integrin cytoplasmic tails (Campbell & Humphries, 2011, Hynes, 2002).

Integrins bind their ligands via the extracellular head domain, and ligand binding is dependent on divalent cations (Mg^{2+}, Mn^{2+} and Ca^{2+}). Half of the integrin α-subunits contain a conserved αI-domain, which is responsible for the ligand binding in these heterodimers. In the other half, ligands are bound to a crevice formed between α- and β-subunit in the integrin head (Hynes, 2002). Integrin activation and ligand binding involves huge conformational changes in integrin structure, leading to more straight and extended conformation with the legs and cytoplasmic tails of α/β-subunits moved apart from each other (Figure 2b). The separation of the cytoplasmic tails now allows the binding of multiple cytoplasmic proteins, including signalling proteins and adaptors, which further reinforce integrin activation. This so-called integrin “outside-in” signalling is counteracted by integrin inactivation, which leads to integrin bending, with the head pointing towards the PM and legs moving closer to each other, thereby reducing integrin affinity towards the ECM (Campbell & Humphries, 2011).

Intermediate to the two conformations, integrins can exist in a “primed” state, which resembles the active extended integrin, but having a low-affinity conformation in the β-subunit’s βI-domain in the absence of ligands (Askari et al, 2009). The primed state can be achieved by specific integrin activators, talin and kindlin, which bind to integrin β-tails, thereby leading to the separation of the transmembrane and cytoplasmic regions. This results in partial integrin activation from inside of the cell and is, for that reason, called integrin “inside-out” signalling. This type of activation occurs in situations where integrins are activated under stimuli received by other cell surface receptors, such as the receptors for cytokines, and therefore integrin inside-out signalling is particularly important for platelet activation (Luo et al, 2007, Moser et al, 2009).

The integrin β-tails comprise two well-defined motifs, a membrane-proximal NPxY and a membrane-distal NxxY, which serve as binding sites for talin and kindlins, respectively (Calderwood et al, 2003, Calderwood et al, 2013, Moser et al, 2009, Tadokoro et al, 2003). Both proteins bind integrins via their FERM (band 4.1, ezrin, radixin, and moesin) domains, and by binding to distinct sites in integrin tails, these two can cooperate to induce integrin activity. However,

the FERM-containing talin head-domain alone is sufficient to activate integrins in most cases (Calderwood et al, 1999, Moser et al, 2009), and talin plays a key role in mediating the mechanical link between integrin tails and the actin cytoskeleton.

Integrin activation has to be a tightly regulated process to avoid inappropriate cell adhesion and motility, and indeed, increased integrin activity is often associated with increased metastatic potential of cancer cells (Desgrosellier & Cheresh, 2010, Lee et al, 2013). In the cytoplasm, talin exists in an autoinhibited conformation, where the C-terminal talin rod blocks the integrin-binding site in the FERM-domain prior to talin activation at the PM. The rod region contains an additional binding site for integrin tails and multiple putative binding sites for other integrin-associated proteins such as vinculin. Binding of the talin head to integrin tails is thought to activate integrins by disrupting the salt bridge between the α- and β-subunits, which triggers the separation of integrin transmembrane and cytoplasmic domains. These conformational changes in the legs lead to further changes in the integrin extracellular domains, thereby increasing integrin affinity for ECM and promoting the assembly of integrin-mediated adhesions (Moser et al, 2009, Tadokoro et al, 2003).

The mechanism of selective talin targeting and activation at the PM is not completely understood, but it involves the locally produced PI(4,5)P2 at integrin adhesion sites (Martel et al, 2001). Moreover, activation of Rap1 at the PM has been shown to lead to recruitment of RIAM (Rap1-GTP-interacting adaptor molecule), which can bind and recruit talin to the PM (Han et al, 2006). In addition, talin can be phosphorylated at multiple sites, and interestingly, a recent study suggested that talin phosphorylation, but not talin expression as such, drives β1-integrin activation, thereby correlating with the potential of prostate cancer cell to metastasise in bone (Jin et al, 2015).

In contrast to talin and kindlin, proteins such as filamin (Kiema et al, 2006), ICAP1 (Bouvard et al, 2003) and sharpin (Rantala et al, 2011) can induce or sustain inhibition of integrin activity. Filamin and ICAP1 have been shown to interact with integrin β-tails and negatively regulate talin or kindlin recruitment (Bouvard et al, 2013). Also sharpin inhibits the interaction of talin and kindlin with integrin β-tails, although sharpin itself binds directly to integrin α-tails (Rantala et al, 2011).

2.2.3. Integrin-mediated adhesions

Upon integrin-ECM connection and ligand induced integrin clustering, proteins are recruited to the cytoplasmic leaflet of the PM to form complex multimolecular adhesion complexes, which link integrin tails to the actin cytoskeleton. This physical connection enables sensing and transmission of mechanical forces across the cell membrane, and allow integrins to regulate biochemical signals in response to chemical and mechanical environment (Evans & Calderwood, 2007, Hu & Luo, 2013). As the binding affinity of a single integrin heterodimer for its ECM-component is low, the formation of cell adhesions is achieved by integrin clustering, thereby increasing the overall binding avidity. Remarkably, even the first immature integrin-mediated adhesions on RGD-matrices are formed by clustering of approximately 50 αvβ3-integrins per adhesion site (Changede et al, 2015).

Integrin-ECM adhesions can be classified into different subtypes based on their morphology, protein composition and stability. The initial ECM-induced clustering of integrins at the PM generates short-living “nascent adhesions” (NA), which form in the absence of acto-myosin-dependent force (Choi et al, 2008, Choi et al, 2011). Although most of these have a lifetime of ~1 min, a subset of NAs are stabilised and progress to “focal contacts”, which can further mature into larger mechano-sensing “focal adhesions” (FA), the main sites for ECM-actin connection. Finally, fibronectin-bound α5β1-integrins can drive the formation of long and stable “fibrillar adhesions” together with adaptor protein tensin. Every maturation step involves the recruitment of various adaptor and signalling proteins to the vicinity of integrin tails, in a precise stoichiometric order, to drive the maturation and to strengthen the mechanical link between integrin and actin. The adhesion maturation occurs along the actin template that elongates centripetally from nascent adhesions towards the cell body, and therefore different adhesions display distinct subcellular locations in cells migrating on 2D. While nascent adhesions are seen in the leading lamellipodium, focal contacts localise close behind them and FAs are seen in the ends of actin bundles. The most stable fibrillar adhesions are formed by centripetal translocation of α5β1-integrins further towards the cell body (Valdembri & Serini, 2012).

The exact composition of integrin adhesion sites has been extensively researched over the past few years, and several studies have exploited mass spectrometric approaches to analyse the adhesion proteomics (Byron et al, 2012, Horton et al, 2015, Humphries et al, 2009). To date, over 2400 components have been identified to be involved in integrin-mediated adhesions (Horton et al, 2015), thereby giving an immediate indication of the complexity of the system. However, not all interactions are likely to occur in a given

situation and the formation of adhesion sites and the subsequent signalling is dependent not only on the cell type and integrin heterodimer, but also on the nature, organisation and stiffness of the ECM, the presence of co-signalling receptors and even the subcellular localisation of the integrin (Harburger & Calderwood, 2009).

Although the exact order of protein recruitments to assemble such complexes is not totally clear, what seem to be clear is that the first molecules recruited to nascent adhesions include FAK, paxillin, talin and kindlin, and the rest of the adhesion components join along the maturation (Case & Waterman, 2015). Some studies have described talin-mediated recruitment of FAK to adhesion sites, while others have demonstrated the opposite (Lawson et al, 2012). Moreover, integrin adhesions are highly dynamic, where components undergo rapid exchange with their cytoplasmic pools (Case & Waterman, 2015, Lele et al, 2008). Interestingly, while talin recruitment to nascent adhesions is inhibited in FAK-null cells, talin recruitment to mature adhesions remains unaltered, suggesting that the interplay between different adhesion components changes along the maturation (Lawson et al, 2012).

2.3. INTEGRIN SIGNALLING

2.3.1. Integrin signalling nexus

Integrin-mediated adhesions form the structural bases for integrin signalling, which eventually influences nearly every aspect of cell physiology. Integrins are often referred to as bidirectional signalling machines as they convey signals from the cell's exterior to the inside and *vice versa* (Hynes, 2002). The integrin cytoplasmic tails, despite of their small size, play the main role in integrin signalling - both in the "inside-out" and the "outside-in" signalling. As integrins do not possess any enzymatic activity of their own, the "outside-in" signalling is transmitted inside the cell by a large number of cytoskeletal and signalling proteins, which bind to integrin tails either directly or via each other (Harburger & Calderwood, 2009).

Integrin signalling is generated and transmitted via post-translational modifications. Upon ECM-integrin interaction, several kinases are recruited to adhesion sites to become activated and to trigger the phosphorylation and activation of the next signalling protein in the cascade. As evident from studies imaging cells with anti-phosphotyrosine antibodies or fluorescent phosphotyrosine-binding SH2 (Src homology 2) –domain, the adhesion sites are enriched with pY events (Ballestrem et al, 2006, Kirchner et al, 2003). This signalling from the plasma membrane to the cytoplasm and further to the

nucleus, eventually changes cell behaviour by modulating gene expression. Indeed, integrin-ECM interaction triggers a vast array of intracellular signalling events that ultimately determine cellular responses such as cell survival, proliferation, differentiation and motility, and provide a context for responding to other inputs, including those transmitted by growth factor receptors.

Given the multiple signalling cascades activated by integrins, together with 60 different proteins composing the consensus adhesome (Horton et al, 2015), it would be beyond the scope of this literature review to cover all of them. Hence, I will focus on describing only the most well understood components in the following chapters.

2.3.2. Focal adhesion kinase

Focal adhesion kinase (FAK, gene name PTK2) was among the first ones discovered to be phosphorylated as a part of integrin-mediated signalling, and at the same time, it is one of the first ones recruited to integrin-mediated adhesions (Kornberg et al, 1992, Schaller et al, 1992).

FAK is a ubiquitously expressed non-receptor tyrosine kinase with the unusual feature that its main activity is autophosphorylation. Although FAK has been reported to phosphorylate other adhesion components, including paxillin (Bellis et al, 1995, Schaller & Parsons, 1995), p130Cas (Schlaepfer et al, 1997) and PI3-kinase (Chen & Guan, 1994), it remains unclear whether the phosphorylation is directly executed by FAK. Therefore, rather than activating downstream pathways via phosphorylation, FAK functions as a phosphorylation-regulated signalling scaffold, which triggers the activation of multiple other signalling proteins by recruiting them directly or indirectly to the sites of integrin adhesions (Walkiewicz et al, 2015).

Structurally, FAK consists of a N-terminal FERM (band 4.1, ezrin, radixin, moesin) domain, a central kinase domain and a C-terminal focal adhesion targeting domain (FAT), separated by long linkers (about 50 and 220 residues, respectively) containing proline-rich domains (Walkiewicz et al, 2015) (Figure 3). The key step in FAK activation and signalling is FAK autophosphorylation at Tyr-397, which is located between the FERM and the kinase domains. Most of the cytoplasmic FAK exists in an autoinhibited conformation, in which the FERM domain is docked onto the kinase domain (Lietha et al, 2007), thereby sequestering the Y397 site as well as the phosphorylation sites in the FAK “activation loop”.

Upon ECM-integrin interaction, FAK is recruited to integrin cytoplasmic tails by interacting with paxillin via its FAT domain (Brown et al, 1996). In addition, integrin-induced local production of PI(4,5)P2 facilitates FAK recruitment to the PM via FAK-FERM – PI(4,5)P2 interaction. PI(4,5)P2 induces FAK targeting and clustering by interacting with a basic KAKTRLK -motif in the FERM domain, thereby releasing the inhibitory FERM-kinase interaction and allowing rapid FAK autophosphorylation at Y397 (Goni et al, 2014). This occurs presumably *in trans* by FAK dimerisation, which is thought to occur via FERM-FERM and FERM-FAT interactions upon local FAK enrichment (Brami-Cherrier et al, 2014, Walkiewicz et al, 2015).

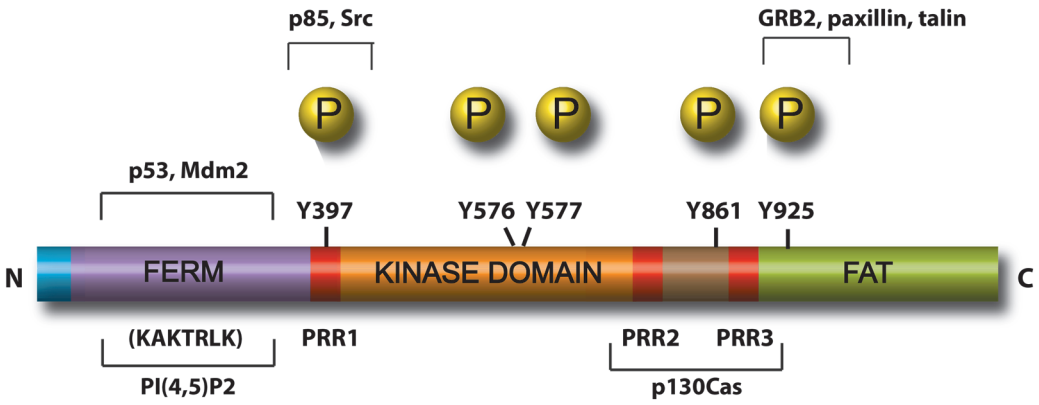

Figure 3. The structure of focal adhesion kinase. FAK contains a N-terminal FERM (band 4.1, ezrin, radixin, moesin) domain, a central kinase domain, a C-terminal focal adhesion kinase (FAT) domain and three proline-rich regions (PRR1-3). Presented are the FAK autophosphorylation site (tyrosine-397) and the main Src-mediated phosphorylation sites, as well as the FAK-binding proteins mentioned in the main text. Modified from (Mitra et al, 2005).

FAK phosphorylated at Y397 is considered activated and capable of interacting with a range of binding partners, some of which can further phosphorylate and activate FAK. In fact, more than 50 proteins have been reported to interact with FAK in different conditions (Walkiewicz et al, 2015), and FAK can be extensively phosphorylated having at least 25 putative phosphorylation sites (Grigera et al, 2005).

As one of the most upstream kinases in the integrin signalling network, FAK regulates several cellular functions. FAK is critical for the regulation of adhesion dynamics, as FAK-null cells display reduced FA turnover and decreased cell motility (Ezratty et al, 2005, Ilic et al, 1995). FAK promotes cell migration together with its downstream targets paxillin and p130Cas by signalling to Rho family GTPases Rac, Rho and Cdc42, whose coordinated action plays a key role in the regulation of cell polarity and contraction. In addition, FAK regulates cell survival and proliferation, as will be discussed in more detail in the following anoikis-chapter, and indeed, FAK is essential for embryonic development (Mitra & Schlaepfer, 2006, Walkiewicz et al, 2015).

In addition to these kinase-dependent functions, FAK has also kinase-independent scaffolding functions. For example in the nucleus, FAK FERM domain comprising a nuclear localisation signal, functions as an adaptor between p53 and an ubiquitin ligase Mdm2, thereby promoting p53 degradation and impairing the p53-mediated cell cycle arrest under stress conditions (Golubovskaya et al, 2004, Lim et al, 2008). Moreover, although FAK is one of the most classical integrin-induced signalling proteins, it also operates as a critical cross point in the co-operation of integrin and growth factor receptor signalling. Indeed, FAK is also activated upon growth factor stimulation, including epidermal growth factor (EGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) (Lee et al, 2015), and the HGF receptor Met has been shown to phosphorylate FAK directly (Chen & Chen, 2006).

Although FAK is recruited to the newly forming ECM-integrin sites, FAK-null fibroblasts are readily forming integrin-mediated adhesions and therefore FAK is clearly not essential for the adhesion formation (Ilic et al, 1995). Indeed, FAK has a close homologue, Pyk2 (proline-rich tyrosine kinase 2), which can compensate many FAK functions (Walkiewicz et al, 2015). Nevertheless, a recent study demonstrated that although inhibition of FAK or Src does not change integrin adhesion composition as such, inhibition of either of the two abolishes integrin-mediated signalling almost entirely (Horton et al, 2016), therefore highlighting that these two kinases are truly the key components of ECM-induced integrin signalling at the PM.

2.3.3. Signalling at focal adhesions

Focal adhesions are the sites for integrin-mediated link between the surrounding ECM and the intracellular actin cytoskeleton, therefore enabling mechano-sensing within FAs. In addition to anchoring cells, FAs transmit information about the state of the surrounding ECM into intracellular biochemical signalling pathways that control cell morphology, migration, differentiation and survival. Indeed, most of the known ECM-induced integrin signalling occurs at FAs, and these are the most well-studied integrin-mediated adhesion structures (Harburger & Calderwood, 2009, Legate et al, 2009).

One of the immediate changes arising from ECM-induced integrin activation is a local increase in PI(4,5)P2 concentration (Figure 4). PI(4,5)P2, generated by PIPKI (type I phosphatidylinositol phosphate kinase) –gamma at the PM, mediates the recruitment of several integrin-mediated adhesion components, including FAK, talin and vinculin to the sites of integrin tails (Legate et al, 2011). It remains unclear how the local activation of the PI(4,5)P2 producing enzymes occurs. Both talin and FAK have been shown bind and activate PIPKI-gamma, thereby leading to increased PI(4,5)P2 production (Di Paolo et al, 2002, Ling et al, 2002). However, since FAK recruitment and activation requires the FERM-PI(4,5)P2 interaction, talin is often considered to precede FAK. On the other hand, FAK is reported to mediate talin targeting to FAs (Lawson et al, 2012), and thus the exact order of FAK and talin recruitment is still a matter of debate.

Nevertheless, ECM-induced integrin activation leads to a rapid recruitment of FAK, and the subsequent autophosphorylation at Y397 creates a binding site for Src. FAK has been reported to bind integrin β-tails via the FERM-domain in vitro (Schaller et al, 1995), but based on the current view, integrin binding is indirect and FAK is recruited to integrin tails by binding to paxillin, and possibly talin, via its FAT-domain. Indeed, paxillin is one of the first proteins detected in nascent adhesions upon integrin-ECM interaction, but in contrast to talin and kindlin, paxillin binds to integrin α-tails (Legate et al, 2009, Liu et al, 1999). Src is a non-receptor tyrosine kinase, which binds FAK via its SH2-domain, but it can also associate with β3-integrins directly (Arias-Salgado et al, 2003).The interaction with FAK activates Src, which is then able to further phosphorylate FAK on at least six different tyrosine residues, including Y576 and Y577 in the activation loop, further stabilising the active FAK conformation (Calalb et al, 1995, Lietha et al, 2007). Together FAK and Src mediate almost all of the integrin induced signalling events (Horton et al, 2016) and co-operatively phosphorylate a wide range of target proteins, including paxillin and p130 Crk-associated substrate (p130Cas) (Schaller & Parsons, 1995, Schlaepfer et al, 1997). The binding of p130Cas to FAK leads to p130Cas phosphorylation,

which triggers downstream signalling leading to increased Rac activity and cell motility. Although the formation of FAK-Src complexes is well accepted, tumour cells lacking β1-integrin display defects in FAK but not in Src signalling, thus suggesting that in some cases FAK and Src can function independently (Berrier et al, 2008, White et al, 2004).

One of the main signalling pathways activated by FAK and Src is the Ras-MAPK (mitogen-activated protein kinase) –Erk1/2 (extracellular signal-regulated kinase 1/2) pathway, which regulates cell cycle progression and proliferation (Legate et al, 2009). Src-mediated phosphorylation of FAK at Y925 within the FAT domain creates a binding site for growth factor receptor bound protein 2 (GRB2), which is one of several adaptors mediating activation of this pathway by integrins (Schlaepfer et al, 1997).

Integrins promote cell survival also by activating the PI3K (phosphoinositide 3-kinase) -Akt signalling pathway. Akt (also known as protein kinase B, PKB) is a ubiquitously expressed serine/threonine kinase that drives integrin-mediated cell survival by regulating several targets both in the cytoplasm and in the nucleus (Legate et al, 2009). Active FAK can bind and phosphorylate the p85 regulatory subunit of PI3K at FAs (Chen & Guan, 1994), resulting in increased PI3K activity and local increase in PI(3,4,5)P3 levels. This leads to Akt recruitment to the PM, which allows PDK1 to access and phosphorylate Akt at the activation loop T308 residue. Akt is further phosphorylated at S473 by mTOR (Sarbassov et al, 2005) or by DNA-PK (Feng et al, 2004), which results in full activation of Akt (Hemmings & Restuccia, 2012).

As the principle adhesion receptors for ECM, integrins have a fundamental role in the processing and remodelling of ECM together with transmembrane type 1 matrix metalloproteinases (MT1-MMP) and secreted MMPs. FAK-Src signalling drives expression and secretion of MMPs, which is associated with metastatic potential of cancer cells (Hauck et al, 2002). Especially the membrane-bound collagenase MT1-MMP drives matrix degradation and is implicated strongly in invasion in collagen gels *in vitro* and metastasis *in vivo* (Poincloux et al, 2009, Sabeh et al, 2004). Interestingly, MT1-MMP also regulates the internalisation of FN-α5β1-integrin complexes (Shi & Sottile, 2011, Sottile & Chandler, 2005). Although ECM-remodelling has been mainly observed in invadopodia, which are specialised actin-rich structures important for 3D movement, the remodelling by MT1-MMPs occurs also in FAs in a FAK-p130Cas dependent manner (Stehbens et al, 2014, Wang & McNiven, 2012).

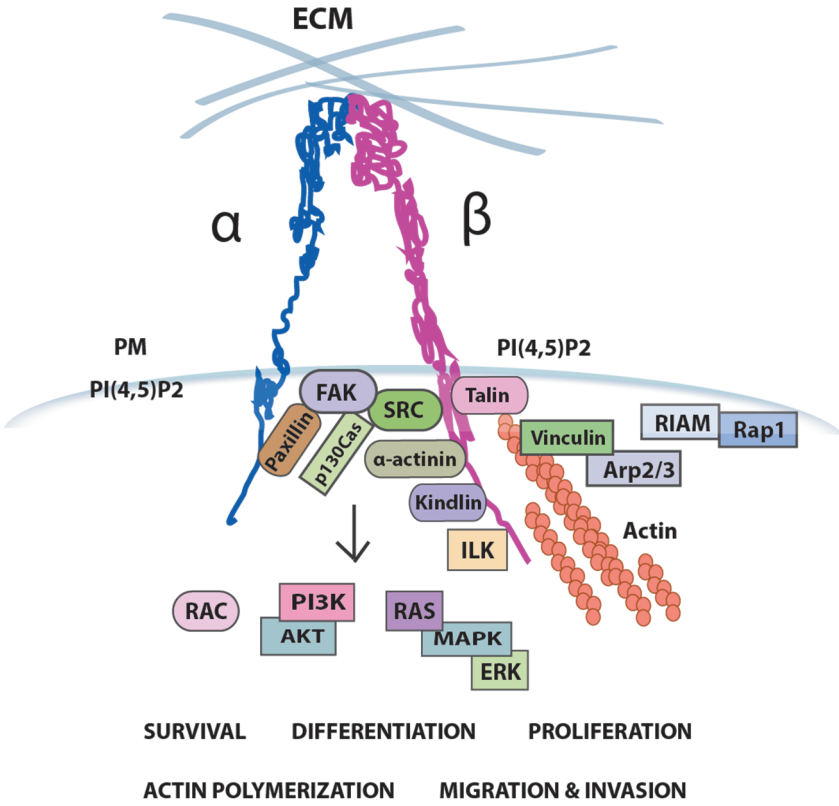

Figure 4. Integrin signalling at FAs. Integrin binding to the extracellular matrix (ECM) induces several intracellular signalling cascades, which ultimately regulate almost every aspect of cell behaviour, including cell survival, differentiation, proliferation and motility. Presented are the most classical integrin-associated signalling proteins described in the text.

Integrin tails are physically connected to actin filaments via multiple actin-binding proteins in FAs, most importantly by the force sensitive protein talin but also others such as α-actinin, filamin and vinculin (Legate et al, 2009). Talin binding to integrin β-tails is followed by force-dependent recruitment of vinculin, which does not bind integrins directly. Instead, vinculin binds to both talin and actin, thereby further reinforcing the mechanical integrin-actin linkage. In addition, vinculin interacts with other FA proteins including α-actinin, the Arp2/3 complex and paxillin (Atherton et al, 2015, Legate et al, 2009), and FAK-Src -mediated phosphorylation of paxillin has also been implicated in vinculin targeting to FAs (Pasapera et al, 2010). α-actinin acts as an actin cross-linker that binds directly to integrin β-tails. α-actinin has also been proposed to compete with talin for integrin-β3 binding after the initial talin-mediated integrin

activation (Roca-Cusachs et al, 2013). ILK (integrin-linked kinase) is a multidomain adaptor protein that associates with actin indirectly by forming a complex with PINCH and the actin- and paxillin-binding protein parvin. ILK interacts also with integrin-β tails (Hannigan et al, 1996), but at least in some cells the recruitment to integrins is dependent on kindlin, parvin or paxillin (Widmaier et al, 2012).

Actin polymerisation plays a key role in FAs, and the local actin polymerisation is driven by actin nucleating Arp2/3 complex together with its activator Wiskott-Aldrich syndrome protein (N-WASP) (Serrels et al, 2007, Tang et al, 2013). Arp2/3 is recruited to integrin adhesions by interacting with the FAK FERM-domain and vinculin, and the loss of Arp2/3 complex reduces FA assembly (Wu et al, 2012). The integrin-induced global regulation of actin dynamics is mediated by the Rho-GTPases Rac, Cdc42 and RhoA, which drive cytoskeletal rearrangements that allow cells to change their shape and initiate migration (Legate et al, 2009).

In addition to these, many other important integrin signalling proteins are found and more remain to be identified. Although a huge effort has been done to understand the molecular interactions involved in integrin signalling, how these signalling cascades are regulated spatially in the cytoplasm is still largely unclear. Nevertheless, recent advances in super-resolution imaging have allowed researchers to reveal the 3D-structure of FAs themselves (Case & Waterman, 2015, Kanchanawong et al, 2010).

2.3.4. Nanoscale organisation of focal adhesions

Based on three-dimensional super-resolution fluorescence microscopy, focal adhesions are assembled into conserved three-dimensional nano-architectures (Kanchanawong et al, 2010). In these structures, integrins and actin are vertically separated by ~40 nm focal adhesion complex, where integrin cytoplasmic tails, FAK and paxillin form the most membrane-proximal layer. The next layer contains vinculin and the most distant actin-regulatory layer contains actin as well as actin-associated proteins zyxin, VASP and α-actinin. As talin interacts both with integrin tails and actin, the integrin-binding head of talin localises together with paxillin and FAK near the PM, whereas the talin rod binds actin in the most outer layer. Vinculin is initially recruited near the PM, but is redistributed to localise with talin rod as the FA matures (Case & Waterman, 2015).

Although the overall 3D structure of FAs seems quite conserved, the proteins within it are highly dynamic and undergo constant turnover. Indeed, most FA

proteins, including FAK, paxillin, vinculin, α-actinin, talin, kindlin, zyxin, VASP and ILK, have been seen to undergo rapid exchange with their cytoplasmic pools (Case & Waterman, 2015, Lele et al, 2008). Moreover, a recent study suggested that proteins can enter and leave stable FAs as pre-assembled cytoplasmic building blocks enabling rapid, local assembly of adhesion sites. For example, FAK and paxillin from the same membrane-proximal layer (Kanchanawong et al, 2010) were suggested to diffuse together in the cytoplasm (Hoffmann et al, 2014). In addition, the effects of matrix organisation and stiffness on integrin signalling have become hot topics during the past years, as well as the question whether the signalling and adhesion formation are similar in 2D / 3D environments and *in vivo*. Despite possible mechanistic differences, focal adhesions, or at least focal contacts, are also seen in 3D matrices (Kubow & Horwitz, 2011), and nascent adhesions seem to form irrespective of the environment, even with a soluble ligand (Changede et al, 2015).

2.3.5. Anoikis

An ultimate evidence of the cell biological importance of accurate integrin-mediated adhesion is provided by anoikis. Anoikis is a form of programmed cell death induced upon loss of integrin-ECM interaction in normal adherent cells and thus it is triggered upon cell detachment or upon attachment to the wrong ECM. The word “anoikis” comes from Greek meaning “homelessness”, highlighting that cells forced to undergo anoikis have lost their correct place in tissue. Anoikis, as other forms of apoptosis, is crucial for development and proper tissue homeostasis in multicellular organisms, and as such, anoikis functions as a powerful surveillance system to prevent adhesion-independent cell growth and cell attachment to an inappropriate matrix. Anchorage-independent growth and epithelial-mesenchymal transition are features associated with anoikis suppression and cancer. Indeed, the ability of cancer cells to resist anoikis and to survive in the absence of anchorage is an established hallmark of cancer cells (Gilmore, 2005, Guadamillas et al, 2011, Paoli et al, 2013).

Cancer progression towards malignancy and dissemination to distant organs requires cells to overcome many obstacles. Cancer cells need to detach from the primary tumour, invade through the ECM, intravasate into the circulation, survive in blood and lymphatic vessels and finally to extravasate into secondary organs. Anoikis resistance of cancer cells plays a pleiotropic role during several steps in cancer progression, including the facilitation of anchorage-independent cell survival in the circulation. Therefore anoikis has emerged as an attractive pharmacological target for cancer therapies (Gilmore,

2005, Guadamillas et al, 2011, Paoli et al, 2013). Cancer cells can develop anoikis resistance by multiple mechanisms, including change in the expression pattern of different integrin heterodimers allowing them to grow in different environments, oncogene activation, overactivation of growth factor receptors or upregulation of key enzymes involved in integrin signalling, primarily FAK.

The ability of active FAK to protect cells from anoikis was reported already 20 years ago, shortly after the initial discovery of anoikis (Frisch et al, 1996). This effect was shown to be dependent on both the FAK kinase activity and phosphorylation at Y397. Moreover, active FAK rescued epithelial cells from anoikis onset and transformed normal cells by increasing their ability to grow anchorage-independently and to form tumours in mice (Frisch et al, 1996). This first study was performed using constitutively active transmembrane-anchored chimeric FAK, created by fusing the CD2 antigen ectodomain with full-length FAK (Chan et al, 1994). However, later several other studies have confirmed the critical role of FAK in anoikis suppression by other means such as siRNAs and antibodies against FAK (Hungerford et al, 1996, Ilic et al, 1998, Xu et al, 1996). The ability of FAK to suppress anoikis has also been shown to depend on FAK binding to paxillin, but this alone is not sufficient as mutation at Y925, which is a binding site for SH2 domain proteins such as Grb2 (Schlaepfer et al, 1994), also blocks the survival signalling (Zouq et al, 2009), thus indicating that FAK can suppress anoikis via multiple mechanisms.

Anoikis has been proposed to occur through both the intrinsic and extrinsic pathways. The intrinsic pathway involves mitochondrial permeabilisation, regulated by Bcl-2 family proteins, and the subsequent release of cytochrome-c. The extrinsic pathway is initiated by stimulation of cell surface death receptors, members of the TNF superfamily, leading to the assembly of a death-inducing signalling complex (DISC) and activation of caspase-8. Both pathways eventually lead to the activation of caspase-3, which initiates a downstream proteolytic cascade leading to DNA fragmentation and cell death (Gilmore, 2005, Paoli et al, 2013).

Although the precise mechanisms by which integrins promote cell survival and suppress anoikis remain to be fully elucidated, and seem to depend on cell type and on the signalling integrin, PI3K/Akt and Ras/Raf/MEK/Erk pathways constitute the most well-known signalling cascades promoting integrin-induced cell survival (Gilmore, 2005). Therefore, the importance of FAK and Src in integrin triggered survival signalling is intimately linked to their fundamental role in activating these downstream pathways. Akt and Erk signalling pathways promote cell survival by phosphorylating and thereby inhibiting the Bcl-2 family members, important for mitochondrial membrane permeability (Martin & Vuori, 2004). However, FAK and Src can contribute to cell survival also directly, as

Src can phosphorylate pro-CASP-8, thus preventing its maturation into functional caspase-8 (Cursi et al, 2006), whereas FAK can bind the death domain of receptor-interacting protein-1 (RIP1), thereby preventing the formation of DISC (Kurenova et al, 2004). In addition, FAK promotes cell survival by suppressing the tumour suppressor p53-mediated cell death (Ilic et al, 1998).

Different cell types are differently sensitive to anoikis, and for example epithelial and endothelial cells have been found to be more sensitive than fibroblasts, which are able to survive in the absence of ECM if growth factors are present (Gilmore, 2005). Indeed, the ability of integrins to crosstalk with growth factor receptors and activate some of the same signalling components is a well-known phenomenon, thus further increasing the complexity in signalling pathways regulating anoikis. Nevertheless, in both fibroblast and epithelial cells, anoikis is suppressed through activation of FAK and its downstream signalling (Ilic et al, 1998, Zouq et al, 2009).

2.3.6. FAK in cancer

The cellular features associated with tumour progression and metastases include increased cell migration and invasion, increased proliferation, angiogenesis and anchorage-independent cell survival. Considering the key role of FAK in all of these processes, it is not surprising that FAK is implicated also in cancers. Indeed, increased FAK expression and activity are seen in a number of human malignancies and increased FAK activation, as determined by antibodies recognizing the phosphorylated Y397, correlates with tumour progression and is often associated with poor clinical outcome (Golubovskaya et al, 2009, Lee et al, 2015, Zhao & Guan, 2009).

Interestingly though, unlike several classical oncogenes such as Ras and the tumour suppressor p53, FAK is rarely mutated, but instead overexpressed in a broad range of tumours. How this is achieved in cancer is not completely clear, but one possible mechanism is amplification of the gene encoding human FAK (PTK2), which is located at a chromosomal region that undergoes frequent aberrations in human cancers (Lee et al, 2015). In addition, FAK promoter activity is suppressed by p53, and thus cancer cells harbouring mutant p53 exhibit increased FAK expression (Golubovskaya et al, 2008). Moreover, increased intracellular pH commonly occurs in cancer, and recent studies have provided evidence linking FAK activity to cellular pH. The higher pH of cancer cells seem to decrease the FERM-kinase interaction in FAK, thus resulting in increased Y397 phosphorylation and FAK activity (Choi et al, 2013, Ritt et al, 2013).

FAK promotes malignancy by regulating a diverse array of cellular processes, but as already discussed, perhaps the most well-known cancer-promoting function of FAK is inhibition of anoikis. Active FAK is crucial for anoikis suppression and several studies have reported that inhibition of FAK activity and/or expression selectively prevents anchorage-independent cell survival without affecting proliferation or survival of adherent cells (Lee et al, 2015).

Owing to these, FAK has emerged as a potential target for cancer therapies, and in fact, several small molecule FAK inhibitors have been developed, which decrease tumour growth and metastasis in different preclinical models. Some of these have proceeded to Phase I or II clinical trials during the past few years, and at the moment, more than 10 clinical studies with different FAK inhibitors are ongoing or recruiting patients with different advanced cancers (Lee et al, 2015, Sulzmaier et al, 2014). Most of the FAK targeting drugs, and all of those in the clinical trials, are ATP-competitive FAK kinase inhibitors, which are designed to bind residues surrounding the ATP-binding pocket. However, the ATP pocket is similar in many different kinases, thereby increasing the potential for off-target effects. Moreover, these kinase inhibitors do not block the FAK kinase-independent scaffolding function in the nucleus, but could possible even enhance it, which might result in unpredictable outcomes in clinical trials (Sulzmaier et al, 2014). Another issue to consider in FAK-targeted therapies is Pyk2, which shares many of the FAK features and functions. Although Pyk2 is normally expressed only in specific cell types such as neurons, its expression can be increased upon loss of FAK due to knockout or specific inhibitors (Walkiewicz et al, 2015). For instance, FAK-inhibitor PF-573,228, which displays significantly greater selectivity for FAK over Pyk2, showed low anticancer effects possibly due to compensatory effects of Pyk2. However, the next-generation drug called Defactinib (VS-6063) currently in clinical trials is inhibiting both FAK and Pyk2, but whether other FAK targeting agents in clinical trials are inhibiting also Pyk2 is not clear (Lee et al. 2015).

Nevertheless, FAK inhibitors are generally well tolerated and have shown promising effects in phase I trials also as chemotherapy sensitisers in combination with cytotoxic drugs. However, since FAK is mastering multiple functions also in normal cells, designing a drug with selective and function-specific inhibitory effects would be more desirable.

2.4. INTEGRIN TRAFFIC

2.4.1. The general concept of integrin traffic

In addition to integrin activity, cells are able to regulate their integrin-mediated adhesions by integrin trafficking. Soon following the discovery and naming of these receptors as “integrins” (Tamkun et al, 1986), the first reports about their ability to traffic in cells emerged (Bretscher, 1992). Integrin traffic, comprising receptor endocytosis and recycling back to the PM, is critical for dynamic integrin functions at the PM and thus for processes such as cell migration, invasion and cytokinesis. In addition, integrins can co-traffic with other receptors, which also drives cancer invasion and metastasis (Caswell et al, 2008, Caswell et al, 2009, De Franceschi et al, 2015, Hognas et al, 2012, Muller et al, 2013, Paul et al, 2015).

Integrin traffic is an actively studied and increasingly complex area of research. Indeed, based on the current knowledge, integrin traffic is regulated on multiple levels and by a large and diverse array of proteins such that a specific integrin heterodimer can follow different trafficking routes in different cells and conditions (Bridgewater et al, 2012, Caswell et al, 2009, De Franceschi et al, 2015).

Integrins can be internalised by distinct entry portals, but irrespective of the mechanism, internalised integrins, as other cellular receptors, are generally first targeted to early endosomes (EE) for sorting to their final destinations. From here, receptors and/or their ligands can be taken to late endosomes (LE) and multivesicular bodies (MVB) to be degraded in lysosomes, or they can be recycled back to the PM via recycling endosomes (RE). Each step along the path is mediated by a different Rab GTPases.

2.4.2. Rab GTPases

Rabs (together with Arfs) are the master regulators of intracellular trafficking. Distinct Rabs have been implicated in almost every aspect of receptor trafficking, from cargo selection to vesicle formation, motility and docking. All this is accomplished by the ability of Rab-proteins to interact with various adaptors, tethering factors, kinases, phosphatases and motor proteins in a manner regulated by the nucleotide-loading status of the Rab. Not surprisingly, defects in different Rabs have been associated with various human diseases, including neurological disorders and cancer (Bhuin & Roy, 2014, Stenmark, 2009).

Rabs are small GTPases forming the largest branch of Ras superfamily, and they are found in all eukaryotes. The first mammalian Rab was identified from rat brain, thus leading to the term Rab as “Ras-like in rat brain” (Touchot et al, 1987), and since then, approximately 70 different Rab proteins have been identified in humans (Colicelli, 2004). Together with PIPs, Rabs are fundamental in specifying membrane identity and therefore different intracellular compartments can be defined by the presence of specific Rabs: EEs are enriched with Rab5 and Rab21, LEs are characterised by Rab7 and recycling endosomes by Rab4 and Rab11 (Figure 5). A comprehensive list of all Rabs can be found from (Bhuin & Roy, 2014).

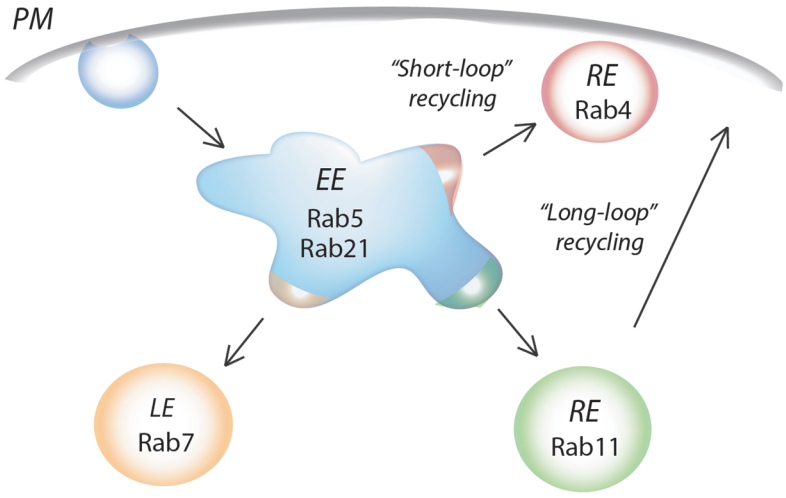

Figure 5. The main Rabs in integrin traffic. Rab21 and Rab5 guide integrins and other receptors to early endosomes (EE), from where integrins can be taken to Rab7-positive late endosomes (LE) and further to lysosomes for degradation. Alternatively, integrins can be recycled from EEs back to the plasma membrane (PM) via short, Rab4-mediated or via longer, Rab11-mediated recycling route.

As GTPases, Rabs can cycle between GTP (guanosine triphosphate) -bound “active” and GDP (guanosine diphosphate) -bound “inactive” state, and the switch between the two is spatiotemporally regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Rabs are inserted into membranes via one or two geranylgeranyl groups attached to the C-terminal cysteine residues of newly formed Rabs, and this is catalysed by

Rab geranylgeranyl transferases together with Rab escort proteins (REPs). Once attached to a correct membrane, Rab GEFs facilitate the removal of GDP, thereby stimulating the nucleotide exchange to GTP and activation of Rabs. This GTP-loading induces conformational changes in Rabs, allowing them to interact with multiple effector proteins required to fulfil their trafficking functions. GAPs catalyse the GTP-hydrolysis by Rabs converting them back to their inactive state, after which Rabs are extracted from membranes by guanine nucleotide dissociation inhibitors (GDIs), which bind and protect the hydrophobic prenylation. GDI bound Rabs are ready to be reinserted into new membranes to begin another cycle. The ability of Rabs to cycle between active and inactive state is critical for their function as endosome maturation and receptor trafficking can be inhibited by expression of constitutively active or inactive Rab mutants (Bhuin & Roy, 2014, Stenmark, 2009).

The endocytic carriers formed in the beginning of receptor internalisation at the PM first fuse with or mature into early endosomes (EE), from where the cargo can be taken to either degradation via Rab7-positive LEs or recycling via Rab4/11. The canonical EEs are marked by the presence of Rab5 and PI3P together with Rab5 effector early endosome antigen 1 (EEA1) (Zerial & McBride, 2001). EEA1 functions as endosomal scaffold, which binds specifically the GTP-loaded Rab5 and interacts with PI3P via its FYVE (zinc finger) -domain (Patki et al, 1997, Simonsen et al, 1998, Stenmark et al, 1996). EEA1 plays an important role in tethering and docking of endosomal vesicles before SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) -dependent membrane fusion (Christoforidis et al, 1999), and in fact, EEs undergo constant homotypic fusion.

Although most of the newly formed endocytic carriers fuse with EEA1-containing EEs, some have been seen to mature into those. APPL1 and 2 (adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1) form a subpopulation of EEA1-precursor endosomes, which are formed immediately after PM fission (Miaczynska et al, 2004). APPL1/2 binds directly to Rab5, but is replaced by EEA1 as APPL-endosomes move centripetally and PI3P is generated by phosphoinositide kinase Vps34, recruited to endosomes by Rab5 (Christoforidis et al, 1999). APPL1/2 and EEA1 are thought to compete for Rab5 binding with similar binding affinities, but the generation of PI3P on these endosomes favours EEA1 recruitment, thus functioning as a switch that triggers APPL to EEA1 conversion (Zoncu et al, 2009). Also Rab21, which mediates integrin endocytosis to EEs (Mai et al, 2011, Pellinen et al, 2006), can bind APPL1/2 in its GTP-loaded form, but the ability of Rab21 to interact with EEA1 has not been demonstrated (Mai et al, 2011, Zhu et al, 2007).

Although much is known about different steps in endosome transport, the detailed mechanism by which an endosome characterised with one Rab is turned into another remains strongly debated. Early endosomes are a morphologically heterogeneous population and their complexity is further enhanced by the presence of biochemically distinct membrane subdomains created by different Rabs together with their effector proteins (Zerial & McBride, 2001). Indeed, several studies have illustrated the presence of Rab5 and Rab7 simultaneously on the same endosome, but in distinct domains (Del Conte-Zerial et al, 2008, Rink et al, 2005, Vonderheit & Helenius, 2005). For the transition of Rab5 to Rab7 endosomes, two different models and evidence supporting each exist (Figure 6). In the first model, EEs and LEs are rather static and cargo is delivered between them in transport vesicles (the so called "vesicle transport" model). This is supported by the observation of Rab7-domains budding from Rab5-endosomes (Vonderheit & Helenius, 2005). In the second model, an endosome with one identity gradually matures into another one in a more dynamic fashion, and this is the model that seems to be more generally accepted. In fact, this "Rab conversion" model has been supported by several studies during the past few years, and Rab7 domains have been seen to grow progressively on the surface of Rab5 endosomes while the vesicles migrate from the cell periphery to the centre, ultimately replacing all Rab5 and converting the endosome into Rab7-positive late endosome (Del Conte-Zerial et al, 2008, Poteryaev et al, 2010, Rink et al, 2005). The Rab conversion is further supported by the finding that in macrophage-like *C.elegans* coelomocytes, the same protein complex that drives Rab5-GEF (RABX-5) detachment from EEs also mediates Rab7 recruitment possible through interaction with Rab7-GEF (Poteryaev et al, 2010).

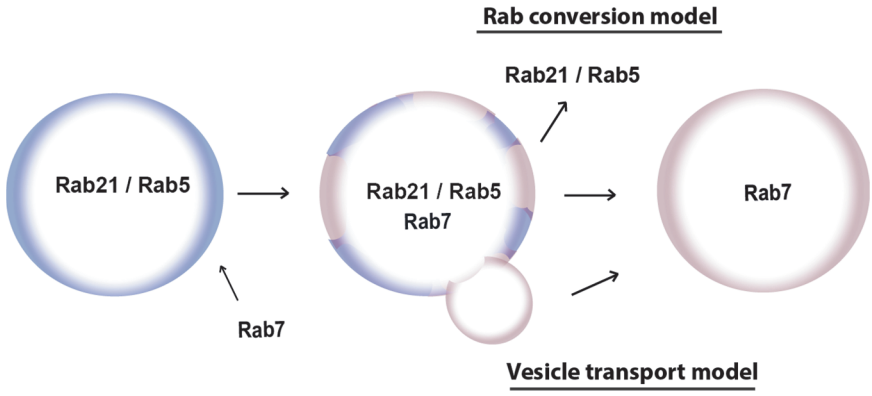

Figure 6. Endosome maturation. Two different models have been proposed for endosome maturation: the "Rab conversion model", where a set of Rab-proteins is gradually replaced by other Rabs, and the "vesicle transport model", where vesicles are budding from one endosomal compartment and fused with another.

2.4.3. Integrin endocytosis

Integrins, as other transmembrane receptors, can be internalised via different entry portals, which are commonly divided into two main categories: the clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE). In general, integrin endocytosis is a spatiotemporally regulated cascade of recruitment and dissociation of various endocytic adaptors and regulators interacting with integrin cytoplasmic tails, and the main GTPases mediating integrin endocytosis are Rab21 and Rab5. While Rab21 binds directly to integrin α-tails and thus plays a more integrin specific role, Rab5 is a more universal regulator of receptor endocytosis and has been shown to associate with various transmembrane receptors, including integrins (Mai et al, 2011, Pellinen et al, 2006, Zerial & McBride, 2001).

CME is the best characterised endocytosis route, and involves the recruitment of clathrin triskelia to the cytoplasmic leaflet of the PM, where they first assemble into flat lattices to form clathrin coated pits (CCP). These then further polymerise into hexagons and pentagons, which facilitates membrane curvature and drives the progressive invagination of the CCP together with various accessory proteins engaged at different stages of CCP-maturation. The final step in the formation of clathrin-coated vesicles (CCV) is the dynamin-mediated membrane scission, which releases the newly formed CCV from the PM. After this, clathrin is rapidly shed and ready to be used for the formation of another CCV (McMahon & Boucrot, 2011).

Clathrin is unable to associate with the PM or cargos directly, and thus additional adaptor proteins are needed for cargo selection and to physically link clathrin to the membrane. One of the key adaptors in CME is the AP2 complex, the second most abundant protein in CCPs after clathrin. AP2 interacts with PI(4,5)P2 at the PM (Honing et al, 2005) and binds cargo receptors via specific motifs, thereby critically regulating the cargo selection to be internalised via CCPs. AP2-binding motifs are found from many different classes of receptors, but were also recently found from a subset of integrins α-tails (De Franceschi et al, 2016). AP2 seems to prefer inactive integrins. This and the fact that the motif does not exist in all integrin α-tails, suggests a mechanism for potentially selective endocytosis of different integrin pools. AP-2, however, is not the sole clathrin adaptor in cells, and alternative adaptors Dab2 (disabled homologue 2) and Numb promote integrin endocytosis by interacting via their phosphotyrosine-binding (PTB) domains with the NPXY-motifs in β1-integrin tails (Calderwood et al, 2003, Nishimura & Kaibuchi, 2007, Teckchandani et al, 2009). How these different adaptors co-operate and determine cargo selection for internalisation is still largely an open question.

Clathrin-independent endocytosis (CIE) comprises a heterogeneous group of entry routes that all share a single common property - not being affected by clathrin inhibition. Pathways belonging to this group and utilised by integrins include caveolae-mediated endocytosis, clathrin-independent carriers (CLICs) and macropinocytosis (Bridgewater et al, 2012). However, compared to CME, the clathrin-independent routes are less well understood and especially the cargo selection is still more or less a mystery.

Caveolae are cholesterol- and sphingolipid-rich cup-shaped invaginations at the PM, decorated with caveolin from the cytoplasmic side, and the biogenesis of caveolae is dependent on cholesterol, caveolin and the recently identified cavins (Bastiani & Parton, 2010). As with CME, receptor endocytosis from these sites is dependent on dynamin, and indeed, the dynamin mediated abscission of vesicles budding from the PM is one of the most critical steps in both entry portals. Dynamin is a large GTPase, which wraps around the neck of newly budding vesicles and mediates membrane fission by GTP-hydrolysis in a process where also actin might play a role (Merrifield et al, 2002). The critical role of dynamin for efficient endocytosis is evident from widely used dominant-negative dynamin-2 mutants, defective in GTP binding and hydrolysis, such as the dynamin-2-K44A mutant (Dyn2-K44A). Dyn2-K44A blocks the formation of endocytic vesicles and results in accumulation of PM invaginations that are still in contact with the extracellular liquid, but fail to be closed and released from the PM (Damke et al, 1994, Damke et al, 2001). In addition to PM, dynamins have been found from other cellular locations and have been implicated for example in the recycling of transferrin receptor from recycling endosomes (van Dam & Stoorvogel, 2002).

More recently, in addition to these classical entry routes, integrins have been shown to be internalised via clathrin- and dynamin-independent carriers (CLICs), by macropinocytosis and Arf4 (ADP-ribosylation factor 4) –dependently in Rab25-expressing cells (Gu et al, 2011, Lakshminarayan et al, 2014, Rainero et al, 2015). CLICs represent a glycosphingolipid-dependent pathway by which β1-integrins are internalised after the binding of extracellular carbohydrate-binding protein Galectin-3 to the glycosylated extracellular domain of integrins (Lakshminarayan et al, 2014). Galectins have the ability to oligomerise into large lattices, and this is likely to drive integrin clustering at the PM and the following endocytosis. Integrins can also be internalised by macropinocytosis from PDGF (platelet derived growth factor) -induced massive circular dorsal ruffles, by which integrins can be efficiently transported to the leading edge of migrating cells (Gu et al, 2011). Arf4 (ADP-ribosylation factor 4)-dependent endocytosis of ligand-bound α5β1-integrins in Rab25-expressing cells represent another clathrin- and dynamin-independent entry route, but interestingly in contrast to others, Arf4 drives the internalisation of active

integrins from fibrillary adhesion directly to late endosomes/lysosomes without passing through EEA1-containing EEs (Rainero et al, 2015).

While some receptors are faithful for a single endocytosis route, integrins seem to be able to use alternative pathways, especially if the main route is blocked. As an example, β1-integrin is normally endocytosed via CME, as inhibition of clathrin or mutations in the clathrin adaptor –binding sequences (NXXY motifs, YYFF mutation) blocks integrin β1 endocytosis (Pellinen et al, 2008). Rab21 binds directly to integrins via the conserved membrane-proximal WKLGFFKR sequence found in most of the integrin α-tails and mediates integrin endocytosis to EEA1-containing EEs (Mai et al, 2011, Pellinen et al, 2008). However, overexpression of Rab21 can overcome the CME of β1-integrins and drive endocytosis by other, clathrin-independent means (Pellinen et al, 2008). Moreover, caveolin-1 has been implicated in α5β1-integrin endocytosis during fibronectin turnover (Shi & Sottile, 2008) and during the internalisation of virus- or antibody clustered α2β1 (Upla et al, 2004). Integrin endocytosis is also dependent on traction force, as rigid RGD matrix and high traction force promotes adhesion maturation and talin recruitment to integrin-β3 tails, whereas loss of force causes talin to be replaced by clathrin adaptors Dab2 and Numb, thereby resulting CME of RGD- integrin-β3 -clusters (Yu et al, 2015).

2.4.4. Focal adhesion turnover

Integrin endocytosis plays a critical role in FA turnover, and the tightly coordinated spatiotemporal regulation of cell adhesion dynamics is crucial for efficient cell migration. While the exact mechanism by which integrin-mediated adhesions are assembled is still not completely clear, the disassembly of FAs, or turnover, is even less well understood and the picture is far more fragmented. Nevertheless, some key players have been identified.

FAK-null fibroblasts display increased formation of integrin-mediated adhesions, suggesting a role for FAK in FA turnover (Ilic et al, 1995). Subsequently, the role of FAK as a central regulator of especially FA disassembly has been demonstrated in several studies. FAK phosphorylation of Y397 triggering recruitment of Src to FAs is critical for FA turnover and cell migration. In line with this, non-phosphorylatable FAK-Y397F shows longer residence time in FAs and reduced FA disassembly (Hamadi et al, 2005). However, as FA turnover is decreased also in Src-null fibroblast, Src-mediated phosphorylation of other sites in FAK may also play a role (Webb et al, 2004).

Another early discovery identified dynamic microtubules repeatedly targeting disassembling FAs (Kaverina et al, 1999). Although the mechanism by which microtubule targeting promotes FA turnover was, and still is, largely unknown, this phenomenon has been exploited to study FA disassembly. Indeed, the current knowledge of FA turnover is largely based on studies exploiting nocodazole, a microtubule-disrupting drug, which promotes stabilisation of FAs in adhesion cells. Nocodazole washout leads to microtubule regrowth and targeting to FAs, which induces a synchronised FA disassembly, thereby allowing analysis of FA disassembly separated from the assembly processes. These studies have further reinforced the requirement of FAK for FA turnover, as microtubule regrowth after nocodazole washout fails to induce FA disassembly in FAK-null fibroblasts (Ezratty et al, 2005, Kaverina et al, 1999).

Based on these studies, FA disassembly involves microtubule-induced internalisation of ECM-bound β1-integrins from FAs by a mechanism dependent on dynamin-2 and clathrin together with clathrin adaptors Dab2 (disabled-2) and ARH (autosomal recessive hypercholesterolemia) and/or AP2 (Chao & Kunz, 2009, Ezratty et al, 2005, Ezratty et al, 2009). During this process, FAK interacts with dynamin-2 and mediates its targeting around FAs (Ezratty et al, 2005). However, the targeting of these proteins to FAs is ultimately dependent on spatially restricted production of PI(4,5)P2 at the PM by type I phosphatidylinositol-phosphate-kinases (PIPKI) in response to integrin-ECM interaction. Knockdown of PI(4,5)P2 producing enzyme PIPKIβ blocks β1-integrin endocytosis and impairs FA turnover (Chao et al, 2010, Ling et al, 2002). In addition, microtubules are tethered to FAs via microtubule-associated CLASP proteins (cytoplasmic linker associated proteins 1 and 2), which are recruited to FAs independent of microtubules (Stehbens et al, 2014).

In addition to the above-mentioned players, several other proteins including Src, p130CAS, paxillin, Erk and MLCK (myosin light chain kinase) have been implicated in FA turnover by live-cell imaging of individual adhesion sites (Webb et al, 2004). Moreover, calcium-dependent protease Calpain-2 has been implicated in the regulation of FA turnover, presumably by cleaving some FA components and thereby triggering structural breakdown of FAs. As an example, calpain-mediated proteolysis of talin has been suggested to drive FA disassembly by mediating talin-dependent dissociation of paxillin, vinculin and zyxin from FAs (Chan et al, 2010, Franco et al, 2004).

Although the question of how microtubules induce FA turnover is still not clear, two possible mechanisms have been proposed. Microtubules have been speculated to activate dynamin to trigger adhesion disassembly or to mediate the delivery of some kind of relaxing signals to FAs, thereby leading to FA disassembly. In fact, kinesin-1 has been implicated in FA turnover, and

therefore microtubules could serve to deliver disassembly factors to FAs in a kinesin-dependent manner (Krylyshkina et al, 2002). However, the transport of such “relaxing factors” was postulated more than 15 years ago and the identity and/or existence of those remains still unknown (Kaverina et al, 1999).

Nevertheless, since FA disassembly involves microtubules and dynamin, the molecular mechanism of FA turnover is clearly distinct from the assembly process, and the need for dynamin in FA turnover suggests that the rate-limiting step involves endocytosis. In line with this, a recent study demonstrated Rab5 as a promoter of microtubule-induced FA disassembly, and Rab5 was shown to co-immunoprecipitate with β1-integrin, FAK, paxillin and vinculin. Moreover, a subpopulation of Rab5-positive endosomes was seen to localise to FAs prior to FA turnover, indicating the importance of integrin endocytosis for FA turnover (Mendoza et al, 2013).

2.4.5. Integrin recycling

Although fibronectin-engaged α5β1-integrins can be targeted to lysosomes for degradation (Dozynkiewicz et al, 2012, Lobert et al, 2010), the majority of endocytosed integrins are rapidly recycled back to the PM to provide the cell a fresh pool of integrins for new adhesions (Kharitidi et al, 2015). This can be rationalised by the fact that the half-life of β1-integrin is more than 20 hours, while one cycle of endocytosis/recycling occurs in less than 30 minutes (Bottcher et al, 2012). In line with this, integrin α5β1 has been shown to detach from FN in early endosomes at pH ~6, leading to FN targeting to lysosomes while allowing integrin resensitisation and recycling back to PM (Kharitidi et al, 2015).

As with the various endocytosis routes, integrins can be recycled via different pathways, and the choice is made based on the integrin heterodimer and the environmental cues (Bridgewater et al, 2012, De Franceschi et al, 2015). In general, integrins can be recycled from EEs via fast Rab4-mediated “short-loop” or via Rab11-dependent slower “long-loop” where receptors traffic through the perinuclear recycling compartments (PNRC) before returning to the PM (Caswell et al, 2009).

Several studies have reported different proteins promoting integrin recycling, but whether and how these proteins co-operate is unclear. Kindlin and sortin nexin 17 (SNX17) share the same binding site in the β1-integrin tail, and the switch from kindlin to SNX17 in EEs drives integrin recycling (Bottcher et al, 2012). A similar switch occurs with Rab21 and p120RasGAP (RASA1), which

bind the same site in integrin α-tails (Mai et al, 2011, Pellinen et al, 2006). Rab21 is replaced by p120RasGAP from α/β1-integrins in EEA1-containing EEs, thereby driving integrin progression into the recycling pathway and onwards to the PM. Moreover, recycling of α5-integrin is dependent on actin, Arp2/3 complex and the Arp2/3 nucleation promoting factor WASH (WASP and Scar homolog). WASH recruits Arp2/3 to Rab5 and Rab11 endosomes, thereby driving Arp2/3-dependent actin polymerisation on endosomes (Derivery et al, 2009, Duleh & Welch, 2010, Zech et al, 2011). Inhibition of WASH or Arp2/3 leads to α5 accumulation in EEs and subsequently to decreased number of FAs (Duleh & Welch, 2012). Indeed, although actin is dispensable for endocytosis in higher eukaryotes, it certainly has an important regulatory role (Kaksonen et al, 2006), and actin patches are frequently seen on endosomes (Derivery et al, 2009, Duleh & Welch, 2010).

In addition to Rab21, another Rab directly binding to integrin tails is Rab25, a member of Rab11 subfamily. Unlike the ubiquitously expresses Rab11, Rab25 expression is restricted to cells of epithelial origin and has been implicated in aggressive cancers (Cheng et al, 2004). Rab25 binds directly to β1-integrin tails (Caswell et al, 2007) and guides the ligand-bound active α5β1-integrins to LEs/lysosomes for degradation, but intriguingly, the lysosomal degradation can be circumvented by Chloride Intracellular Channel Protein 3 (CLIC3). CLIC3 is often upregulated in Rab25-expressing cancer cells, where it can drive α5β1-recycling from LEs/lysosomes while still in their active conformation (Dozynkiewicz et al, 2012). However, it is important to keep in mind that these two Rabs discussed here are exceptions among Rabs, as no other Rab has so far been demonstrated to bind integrins directly.

In addition to the previously mentioned Arf4 implicated in a distinct integrin endocytosis route (Rainero et al, 2015), another Arf family member Arf6 has been implicated in integrin traffic. Surprisingly though, Arf6 regulates both integrin endocytosis and recycling, and the ultimate decision seems to depend at least partly on the associated Arf GAP: ACAP1 promotes β1-integrin recycling from tubular Rab11-containing recycling compartments, while ARAP2 mediates β1-integrin endocytosis to APPL1-containing endosomes (Chen et al, 2014, Li et al, 2005).

2.4.6. Trafficking for cell migration

Cell migration plays a fundamental role in the development and maintenance of multicellular organisms, and is essential for wound healing, for proper tissue formation during embryogenesis and for leukocytes to get into the sites of inflammation. However, cell migration and invasion become fatal when cancer

cells start to move from primary tumour to conquer other sites in the body (Caswell & Norman, 2008, Franz et al, 2002, Paul et al, 2015).

Directed cell migration is enabled by cellular sensing of local gradients and can be triggered by multiple cues, including soluble factors such as cytokines and growth factors (chemotaxis) or gradients in the extracellular matrix stiffness (durotaxis). Migrating cells are polarised and the driving force required for cell movement is provided by the actin cytoskeleton, which pushes the PM forward in the leading edge of cells and mediates the retraction of the trailing edge together with myosins (Franz et al, 2002, Paul et al, 2015). Integrins regulate cell migration not only by providing the mechanical link between ECM and the actin cytoskeleton to apply the necessary traction forces, but also by modulating the activity of Rho GTPases to drive local actin polymerisation. Different integrin heterodimers can activate different Rho GTPases, with strong impact on the mode of migration. Indeed, αvβ3-integrin supports the activation of Rac1, thus driving lamellipodia formation and slow persistent migration, while α5β1 promotes activation of RhoA-ROCK signalling cascade thereby driving rapid random cell migration (Danen et al, 2005). Inhibition of Rab4-mediated αvb3 recycling leads to increased Rab11-dependent recycling of α5β1 and thus to a switch from persistent to random cell migration (Caswell & Norman, 2008, White et al, 2007).

Initially, it was assumed that in migrating cells integrins are endocytosed from the rear of the cell and transported to the front to provide new adhesion sites. Although this type of long-range traffic does occur in some special conditions (Lawson & Maxfield, 1995, Pellinen et al, 2008), it is now evident that integrin endocytosis and recycling occurs at both ends of migrating cells. The local integrin endo/exocytic traffic in the front of migrating cells promotes FA turnover and actin dynamics to drive membrane protrusions needed for migration and invasion (Caswell et al, 2009). Rab25 promotes cell invasion in 3D fibronectin-rich matrices by targeting and retaining inactive α5β1-integrins at the tips of pseudopodia in front of cells, while ligand-bound α5β1 is targeted to LEs/lysosomes towards the cell body (Caswell et al, 2007, Dozynkiewicz et al, 2012). On the other hand, CLIC3-mediated recycling of active α5β1 from these degradative compartments back to the PM promotes cell invasion and cancer metastasis by activating Src signalling and driving forward movement of the rear of the cell (Dozynkiewicz et al, 2012, Paul et al, 2015).

Integrin traffic regulates cell migration also by controlling the traffic of other receptors. Rab-coupling protein, RCP, is a class I Rab11 family interacting protein (also called Rab11-FIP1), which plays a key role in co-trafficking of integrin and EGFR (Caswell et al, 2008). In cells expressing both αvβ3 and α5β1, RCP is associated with αvβ3, but inhibition of αvβ3 or mutant p53 leads

to displacement of RCP from β3 to interact with α5β1.This increases α5β1 recycling and fibronectin-dependent cell migration and invasion. Interestingly, RCP drives invasion by promoting a complex formation between α5β1 and EGFR, which is required for efficient EGFR recycling and EGF-induced Akt signalling, without changes in cell adhesion (Caswell et al, 2008, Muller et al, 2009).

In addition, a recent study proposed that the key route for inactive (α5) β1-integrins involves retrograde trafficking via the Golgi. Inactive integrins are transported to the leading edge of migrating cells via retrograde trafficking from the PM to the trans-Golgi network, from where integrins can be recycled back to the PM (Shafaq-Zadah et al, 2016) in a polarised manner. This route might be important in situations where cells need to migrate in a highly polarised and persistent manner, such as during the gonad development in *C.elegans* (Shafaq-Zadah et al, 2016).

2.5. ENDOSOMAL SIGNALLING

2.5.1. Endosomes as signalling platforms

Our understanding of endocytosis has evolved remarkable during the past two decades. Endosomes are easy to depict as intracellular transport wagons, necessary to move receptors and other plasma membrane components to different localisations within a cell, but in addition to this, endocytosis is a critical regulator of receptor signalling. Indeed, endocytosis has long been recognised as a means to terminate ligand-induced receptor signalling at the PM and to downregulate the PM availability of the receptor by targeting them for lysosomal degradation. However, based on a mounting body of evidence from studies with different receptor tyrosine kinases (RTKs), this is only a part of the whole truth. In fact, in addition to their fundamental role in cell logistics, endosomes are remarkable signalling devices, allowing a spatiotemporal regulation of signalling, and are ultimately linked with almost every aspects of cellular signalling (excellently reviewed in (Sigismund et al, 2012)).

The first compelling evidence for endosomal signalling came from pioneering work exploiting a dominant negative dynamin mutant (Dyn2-K44A) to study the signalling of EGFR (Vieira et al, 1996). By blocking EGFR endocytosis with mutant dynamin, the activation of signalling pathways downstream of EGFR was compromised, demonstrating the requirement of efficient endocytosis for full EGFR signalling. Indeed, several signalling receptors, including integrins, are frequently detected ligand-bound in endosomal compartments, thereby

enabling some receptors to signal persistently post-endocytosis. Endosomes are known to bear several unique features enabling them to fulfil multiple roles in the regulation of signal propagation and duration in several signalling pathways (Alanko et al, 2015, Sigismund et al, 2012).

2.5.2. Different roles of endocytosis in receptor signalling

Endocytosis can regulate signalling by terminating the receptor signalling emanating from the PM, and extreme examples of defects in this are seen in different human cancers (Sigismund et al, 2012). For example, hypoxia-inducible factor HIF1α regulates the transcription of certain Rab5 effectors, thereby inhibiting Rab5-endosome fusion and prolonging the endosomal residence of active EGFR. This leads to sustained endosomal signalling of EGFR, and HIF1α overexpression is seen in various human cancers correlating with tumour progression and poor prognosis (Wang et al, 2009, Zhong et al, 1999).

However, endocytosis can modulate receptor signalling also by many other ways, and endosomes can for example sustain, amplify or fine-tune the signals, transmit the signals over long distances beyond the limits of passive diffusion or even lead to a completely distinct signalling outcome (Alanko et al, 2015). One of the most fascinating examples of endocytosis-dependent signalling outcomes arises from the tumour necrosis factor receptor 1 (TNFR1). Once activated by TNF at the PM, this receptor mediates anti-apoptotic signals by recruiting RIP-1 and TRAF-2 and by activating the nuclear factor kappa B (NF-kappaB) transcription factor (Schneider-Brachert et al, 2004). However, internalisation of TNFR1 leads to the recruitment of TRADD, FADD and caspase-8 to form the “death-inducing signalling complex” (DISC) thus ultimately promoting apoptosis (Schneider-Brachert et al, 2004, Schutze et al, 2008). Therefore, the same receptor is able to produce signals to either promote or to inhibit cell death, and the final outcome appears to be solely dependent on the subcellular localisation of the receptor.

Endosomes can also fine-tune signalling based on the ligand-receptor concentration, which is the case with EGFR. EGFR is preferentially endocytosed via CME if the ligand (EGF) concentration is low, allowing prolonged endosomal signalling of EGFR and recycling back to PM. However, high EGF concentration induces additional CIE of the EGFR leading to receptor degradation (Sigismund et al, 2008).

Endosomes can generate distinct signals in response to different endocytic routes or different ligands. In addition to the previously mentioned EGFR, TGFβ can undergo both CME and CIE. Activated TGFβ entering the cells via CME is targeted to SARA-containing, signalling-active endosomes, while TGFβ trafficking through the NCE route is targeted for degradation (Di Guglielmo et al, 2003). Moreover, different ligands can also lead receptors to distinct trafficking fates. EGF and TGFα are both ligands for EGFR, and both ligands are readily internalised with the receptor. However, the interaction with EGF is less sensitive to lower endosomal pH and thus EGFR is actively signalling in endosomes until lysosomal degradation. In contrast, TGFα is detached from EGFR early along the internalisation, which leads to recycling of EGFR back to the PM, promptly ready for new ligands (Sigismund et al, 2012).

Perhaps the most common and natural way for endocytosis to influence receptor signalling is by prolonging and sustaining the signalling emanating from the PM. Several examples from RTKs and G-protein coupled receptors exist, and to mention few, endocytosis is required for insulin receptor mediated Erk1/2 signalling (Ceresa et al, 1998) and for angiotensin II mediated Akt signalling (Nazarewicz et al, 2011). Endocytosis can also sustain signalling by protecting it from PM protein tyrosine phosphatases, although some phosphatases, such as PTEN is recruited directly to PI3P-positive early endosomes (Naguib et al, 2015). As an example of a signalling protector role, endocytosis of active VEGF-receptor protects the receptor-mediated PLC-gamma/MAPK signalling by sequestering VEGFR away from PM phosphatases (Lanahan et al, 2010).

2.5.3. Unique features of endosomes

So what makes endosomes good for signalling? Described below are some of the unique features that make endosomes ideal signalling platforms including size, composition, pH and mobility (Figure 7).

2.5.3.1. Confined size & curvature

Endosomes are a highly heterogeneous population of membrane-surrounded compartments varying in size typically between 50-200 nm. Nevertheless, the space in endosomes is limited and this has been proposed to facilitate receptor-mediated signalling in endosomes mainly by two ways (Sigismund et al, 2012): (1) by forcing the ligands to stay in close proximity to the receptors due to the confined volume and (2) by restricting different signalling

components close to each other due to the limited size of the endosomal membrane. Another unique feature of endosomes that likely contributes to the ligand-receptor interaction and signalling is the high membrane curvature of endosomes compared to the flat PM. In endosomes, the curved membrane forces the cytoplasmic domains of two neighbouring receptors further apart and at the same time directs the former extracellular domains of receptors to point in the centre of the endosome. Therefore, especially in case of receptors, such as integrins, where multiple receptors are able to bind the same ECM-component, the high curvature of endosomes could reinforce the overall integrin-ligand interaction.

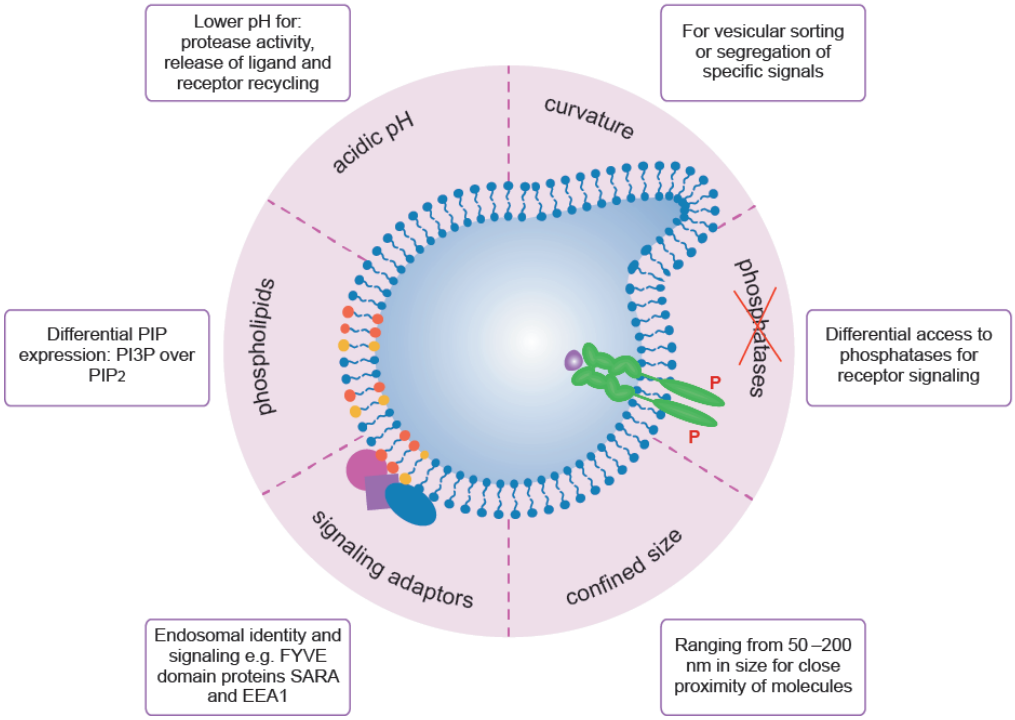

Figure 7. The unique features of endosomes. Endosomes bear several features making them ideal for signalling purposes. These include specific PIPs, acidic pH, high curvature compared to PM, endosome specific components and adaptors, such as the FYVE-domain containing proteins, confined size and directional movement, which may facilitate receptor escape from PM phosphatases. Adapted from (Alanko et al, 2015),

The curvature is also recognises by BAR (Bin/Amphiphysin/RVS) domain containing proteins, such as APPL1/2, which enables the assembly of unique signalling platforms on endosomes (Miaczynska et al, 2004). BAR domains are dimeric crescent-shaped modules that sense, bind and support nanoscopic membrane curvature by interacting with negatively charged membrane lipids, such as PIPs (Peter et al, 2004)

2.5.3.2. Endosome specific components

PIPs, together with Rabs, are the key components establishing and defining organelle identity and by doing so, they also functions as fundaments for endosome specific signalling complexes.

The main PIPs at the PM are PI(4,5)P_2 and PI(3,4,5)P_3, which are sequentially converted during endocytosis by a cooperation of different kinases and phosphatases into PI(3)P, the main PIP in early endosomes. However, endosome maturation and PIP turnover are highly dynamic processes, where endosomes are constantly modified and fused with each other, and thus a single endosome is likely to have different PIPs at any given time. Indeed, some PI(3,4,5)P_3 has been detected on early endosomes, albeit most likely in distinct domains than PI3P (Naguib et al, 2015). Nevertheless, PI3P in early endosomes is recognised by a wide range of proteins, mainly via FYVE and PX domain mediated interactions. One of these FYVE-domain containing proteins is EEA1 (Lawe et al, 2000), which is required for angiotensin II (AngII) induced Akt signalling in vascular smooth muscle cells, but is dispensable for AngII-induced Erk signalling (Nazarewicz et al, 2011). In contrast, APPL1/2, which is detached from Rab5-endosomes upon PI3P generation, has been shown to mediate EGFR-induced MAPK but not Akt signalling, therefore highlighting that different endosomes can propagate distinct signalling (Miaczynska et al, 2004, Zoncu et al, 2009).

A third subpopulation of early endosomes is comprised of yet another FYVE-domain containing protein called SARA (Smad anchor for receptor activation) (Itoh et al, 2002, Tsukazaki et al, 1998). SARA was first demonstrated in transforming growth factor β (TGFβ) signalling, where it was shown to recruit SMAD2 to the endosomal TGFβ receptor thus facilitating SMAD2 phosphorylation and translocation into the nucleus (Tsukazaki et al, 1998). Later, SARA endosomes have been implicated also in the NOTCH signalling and in the division of intestinal stem cells in Drosophila (Coumailleau et al, 2009, Montagne & Gonzalez-Gaitan, 2014).

2.5.3.3. Acidic pH

The luminal pH of endosomes drops along the internalisation, and while EEs are weakly acidic with a pH 6.8 - 5.9, pH in LEs varies between 6.0 – 4.0 (Maxfield & Yamashiro, 1987). Decreasing pH certainly influences the ability of receptor to interact with their ligands, and thus affect the ligand induced signalling. In the case of integrins, ECM-components have been reported to detach from integrins in late endosomes, after which integrins can be recycled back to the PM, while the ligands are left behind for degradation in lysosomes (Kharitidi et al, 2015). In some cases, however, decreasing pH can be required for signal propagation as demonstrated with the NOTCH receptor. NOTCH plays a key role in modulating cell fate decisions during development, and NOTCH receptor is cleaved at the PM leading to endocytosis of the transmembrane and cytoplasmic domains. In endosomes, the receptor is further cleaved by gamma-secretase, a protease only functional at low pH, and this is required for the nuclear transport of NOTCH to activate transcription (Vaccari et al, 2008).

2.5.3.4. Directional movement

One of the biggest advantage of signalling endosomes is their microtubule- (and actin to some extent) -based directional motility, which enables signal transmission over the limits of passive diffusion. The intracellular organisation of endosomes is mediated by the cellular microtubule network, and endosomes can be moved along microtubules with the help of microtubule associated motor proteins, dyneins and kinesins. Dynein drives endosome movement to the (-) end while kinesins transport their cargos to the (+) end of microtubules (to the PM). These motor proteins are coupled to endosomes via specific Rabs and members of the sortin nexin (SNX) family (Hunt & Stephens, 2011).

The reason for the great benefit of microtubule based trafficking is that the interior of a cell can be a highly crowded place, as elegantly demonstrated by a structurally detailed molecular model of *E.coli*'s cytoplasm (McGuffee & Elcock, 2010). Due to the viscous and ultimately crowded environment, the canonical free random diffusion of phosphorylated signalling proteins seems inefficient and slow, and thus directional transport vehicles would be more cost-effective method to take information from the plasma membrane to the cell interior. Fast and secured transport of signals is especially important in neurons, where the distance between the signal originating and the receiving site can be up to a meter. Indeed, in nerve terminals, the nerve growth factor (NGF) binds to its receptor TrkA and activates the TrkA-mediated Erk1/2 and PI3K/Akt signalling and the subsequent clathrin-mediated endocytosis of the NGF-TrkA complex.

The long distance signalling in a limited time is accomplished with sustained endosomal signalling, and the actively signalling NGF-TrkA in endosomes is transported along microtubules to the neuronal body and to the nucleus to ultimately promote cell survival (Grimes et al, 1996, Howe & Mobley, 2005).

The fast delivery also ensures that the message remains complete and that information is not lost or changed on the way. Endosomes can protect phosphorylated signalling proteins from cytoplasmic phosphatases by faster delivery, as proposed in another mathematical model (Howe, 2005). Transcription factor STAT3 is phosphorylated and activated at the PM by different receptors after which STAT3 needs to translocate into the nucleus. In the model by Howe, the random diffusion combined with probabilistic modelling of dephosphorylation kinetics was compared to directed microtubule-based endosome movement from the PM to the nucleus. Based on this model, the endosome-mediated transport of phosphorylated signalling proteins is more cost-effective and less information is lost due to dephosphorylation events compared to random diffusion if the distance exceeds 200 nm, which is easily the case in normal cells. However, it is important to note that this is not necessary true with all receptors and signalling pathways, and in some cases the signalling intensity might be high enough to reach the required threshold even with simple diffusion. Nevertheless, endosomal trafficking via directional microtubules is a faster, and a more sophisticated, route to the nucleus than a random walk.

As a conclusion, in light of these examples and numerous others not described here, endosomal signalling seems to be more a rule than an exception in the world of receptor-mediated signalling. Moreover, although the examples discussed here highlight the fundamental role of endocytosis in regulating cellular signalling, signalling can also regulate endocytosis on multiple levels. For example, activation of different RTKs as well as activation and clustering of integrins by ECM induces clathrin nucleation at the PM, thereby driving endocytosis (McMahon & Boucrot, 2011).

3. AIMS OF THE STUDY

Integrins are fundamental for the existence of metazoans as ECM-mediated integrin signalling regulates almost every aspect of cell behaviour, from cell survival and anchorage-independent growth to cell migration and invasion – all features critically driving cancer progression. Indeed, cancer metastasis is the main cause of cancer-related deaths due to the lack of efficient therapies, and for this, if for no other reason, understanding the regulation of integrins in cells, their trafficking and signalling, is critically important.

Although integrin traffic has been under intense research during the last decades, and both active and inactive integrins are known to undergo constant endo/exocytic trafficking, how integrin activity and traffic are exactly connected has remained incompletely understood. Individual studies have exploited different methods in different cell lines to look at specific integrin heterodimers, but these pieces of information have been difficult to interpret. Therefore a systematic approach to investigate the connection between integrin traffic and activity was needed.

Moreover, although much information has been gained on endosomal signalling since its original discovery (Grimes et al, 1996, Vieira et al, 1996) and a wide range of RTKs are known to signal also from endosomes, surprisingly, the ability of integrins to propagate signals from endosomal compartments had not been previously investigated.

The specific aims in this work were:

- To set up a new antibody-based assay to study the traffic of integrins in different activation states
- To compare the trafficking routes exploited by active and inactive β1-integrins
- To investigate whether integrins are signalling from endosomes
- To analyse the biological significance of integrin-endosomal signalling

4. MATERIALS AND METHODS

More detailed descriptions of the methods and reagents are available in the original publications (I-II).

Experimental procedures

Method	Used in
Cell culture	I,II
DNA and siRNA transfection	I,II
Antibody-based trafficking assay	I
Biotin-based trafficking assay	I
Biotin-based assay with ELISA-based detection	I
Immunofluorescence microscopy and image analysis	I,II
Spinning-disk confocal microscope	I,II
Fluorescence microscope	I
Stimulated emission depletion (STED) microscope	II
Immunoprecipitation (IP)	I
Flow cytometry	I,II
Western blotting	I,II
Subcellular fractionation	II
In situ proximity ligation assay (PLA)	II
Activation of integrin signalling by replating	II
Immunofluorescence staining	I,II
Integrin activation with microbeads	II
Recombinant protein interaction with endosomal fraction	II
Anoikis assay	II
Anchorage-independent growth assay	II
Micropatterns and probabilistic density maps	II
Extravasation assay in mice	II
Human phospho-RTK array	II
Mass spectrometry and data analysis	II
Statistical analysis	I,II

DNA constructs

DNA construct	Description	Used in
pEGFP-C1	Clontech	I,II
GFP-Dyn2-K44A	Dominant negative dynamin-2 (Altschuler et al. 1998)	I,II
GFP-Eps15-EH29	Dominant negative Eps15 (Benmerah et al. 1999)	I
GFP-caveolin-1	Dominant negative caveolin-1 (Pelkmans et al. 2001)	I
EGFP-Arf6	Wildtype Arf6	I
EGFP-Rab4a	Wildtype Rab4a	I
EGFP-Rab4a-S22N	Dominant negative (Nagelkerken et al. 2000)	I
EGFP-Rab5	Wildtype Rab5	I
EGFP-Rab7	Wildtype Rab7	I
EGFP-Rab11	Wildtype Rab11	I
EGFP-Rab21	Wildtype Rab21 (Pellinen et al. 2006)	I,II
EGFP-Rab25	Wildtype Rab25	I
pET15b-FN III (7-10)	Fibronectin fragment (Takahashi et al. 2007)	I,II
GFP-Rab5-Q79L	Constitutively active Rab5	II
pIRES-GFP-α2-WT	Wildtype α2-integrin (Pellinen et al. 2008)	II
pIRES-GFP-α2-AA	Rab21-binding deficient α2-integrin (K1160A, R1161A) (Pellinen et al. 2008)	II
GFP-EEA1	Addgene plasmid 42307 (Lawe et al. 2005)	II
GFP-FAK	Wildtype FAK (Ilic et al. 1998)	II
GFP-FAK-FERM (1-402)	FAK's FERM -domain (Schlaepfer et al. 2007)	II
GFP-FAK-FAT	FAK's FAT-domain (Ilic et al. 1998)	II

siRNAs

Name or target	Description or cat. no and supplier	Used in
Allstars	Negative control siRNA,1027281, Qiagen	II
EEA1	5'-ATGGATAACATGACCTTGGAA-3'	II
EEA1 (smart pool)	5'-AGCCGCTATATTAGACTTGGA-3' 5'-AAGCTAAGTTGCATTCCGAAA-3' 5'-CCCGGCACAGAATGTGAGTTA-3'	II
β1-integrin	5'-CCCGACATCATCCCAATTGTA-3' 5'CTGGTCCATGTCTAGCGTCAA-3'	II
Rab21	5'-AAGGCATCATTCTTAACAAAG-3' (3'-AlexaFluor555)	II
APPL	5'-CAGGACAATCTCGGCCACCGA-3'	II

Cell lines

Cell line	Description	Used in
MDA-MB-231	Human breast adenocarcinoma	I,II
PC-3	Human prostate adenocarcinoma	I
NCI-H460	Human non-small cell lung carcinoma	I,II
TIFF	Human telomerase-immortalised foreskin fibroblasts	II
CHO	Chinese hamster ovary cells	II
Fak-/- MEF	FAK-null mouse embryonic fibroblasts (Schlaepfer et al. 2007)	II
Fak+/+ MEF	Wildtype mouse embryonic fibroblasts (Schlaepfer et al. 2007)	II

Antibodies

Antibody	Species	Description and supplier	Application	Used in
β1-integrin	Mouse	K20, Beckman Coultier	IF, ELISA	I,II
β1-integrin	Mouse	12G10, Abcam	IF	I,II
β1-integrin	Rat	mAb13, BD Biosciences	IF	I
β1-integrin	Mouse	4B4, Beckman Coultier	IF	I
β1-integrin	Rat	9EG7, BD Pharmingen	IF	I,II
β1-integrin	Mouse	Huts-21, BD Pharmingen	IF	I
β1-integrin	Mouse	P1H5, Santa Cruz	IF	I
β1-integrin	Mouse	mab1998, Millipore	IF	I
anti-Alexa Fluor 488	Rabbit	Quenching antibody, Molecular Probes	IF	I
Biotin		(HRP)-conjugated, # 7075, Cell Signaling Tech	WB, ELISA	I
β1-integrin	Mouse	MAB2252, Millipore	WB	I
β1-integrin	Mouse	610468, BD Transduc.Lab	WB	I
α2-integrin	Rabbit	AB1936, Millipore	WB	II
AF-488/555/647	Various	Alexa Fluor secondary antibodies, Life Tech.	IF	I,II
DyLight 680/800	Various	Secondary antibodies for Odyssey, Thermo Sci.	WB	I,II
GST	Rabbit	A5800, Invitrogen	WB, IF	II
pFAK-Y397	Rabbit	44-624G, Life Technologies	WB, IF	II
GFP	Rabbit	A11122, Life Technologies	WB	II
FAK	Mouse	610088, BD Biosciences	WB	II
EEA1	Mouse	610457, BD Biosciences	WB, IF	II
EEA1	Rabbit	07-292, Upstate	WB	II
APPL	Rabbit	ab59592, Abcam	WB	II
pAkt-S347	Rabbit	9271S, Cell Signaling Tech	WB	II
pErk1/2-T202/Y204	Rabbit	4370, Cell Signaling Tech	WB	II
pSrc-Y416	Rabbit	2101S, Cell Signaling Tech	WB	II

Fibronectin	Rabbit	F3648, Sigma	IF	II
β-actin	Mouse	A1978, Sigma	WB	II
α-tubulin	Mouse	12g10, Hybridoma bank	WB	II
* Rab21	Rabbit	Affinity purified from serum	WB	II
Paxillin	Mouse	612405, BD Biosciences	WB	II
Vimentin	Mouse	sc-6260, Santa Cruz	WB	II
Talin	Mouse	T3287, Sigma	WB	II
Vinculin	Mouse	V9131, Sigma-Aldrich	WB	II
Mega-520	Rabbit	Secondary antibody for STED, Sigma	IF	II
Atto-647	Mouse	Secondary antibodies for STED, Sigma	IF	II

* Affinity purified from rabbit antisera using Affi-Gel10 (Biorad). Rabbit polyclonal antisera for Rab21 was raised by Innovagen (Sweden) using the whole purified Rab21 as an antigen.

Reagents

Compound	**Supplier**	**Used in**
OptiMem	Invitrogen	I,II
AF488-protein/antibody labelling kit	Molecular probes	I
HiPerfect	Qiagen	I,II
Lipofectamine 2000	Life Technologies	I,II
EZ-link cleavable sulfo-NHS-SS-biotin	Thermo Scientific	I
Hanks' balanced salt solution	Sigma	I
HyQTase	HyClone	II
Odyssey blocking buffer	Fisher	I,II
Phalloidin-Atto647N	Fluka	II
CellTrace green/far-red	Molecular Probes	II
Dynasore monohydrate	Sigma	II
Dimethylsulphoxide (DMSO)	Sigma	II
Collagen type I solution	Sigma	II
Dyngo4a	Abcam	II

PF-562271 (FAK inhibitor)	Selleckchem	II
PF-573228 (FAK inhibitor)	Selleckchem	II
Kinetic Caspase-3/7 Apoptosis Assay Kit (Nucview)	Essen Bioscience	II
LY294002 (PI3K-inhibitor)	Life Technologies	II
AF-568-conjugated transferrin	Life Technologies	I,II
Fibronectin (bovine plasma)	Merck	I,II
Polybead Microspheres 6.00 µm	Polysciences	II
FAK inhibitor 14	R&D Systems	II
Human Phospho-RTK Array	R&D Systems	II
Active (pY397) recombinant FAK	ProQinase	II
Non-activated recombinant FAK	ProQinase	II

5. RESULTS & DISCUSSION

5.1. INTEGRIN TRAFFIC IS REGULATED BY INTEGRIN ACTIVITY (I)

5.1.1. Active and inactive β1-integrins display distinct subcellular localisation (I)

To gain a general view of integrin traffic, we chose to concentrate on integrin-β1, the most common integrin subunit in mammals (Hynes et al. 2002). Several monoclonal antibodies have been raised against conformation-dependent epitopes in β1-integrins, which detect specifically active or inactive integrins (Byron et al, 2009). By using several such antibodies with fixed NCI-H460 lung cancer cells on 2D, it was evident that active and inactive β1 display distinct subcellular localisation. Focusing on the middle section of these cells with confocal microscope, the active β1 was mainly detected inside the cell, in dot-like structures possibly in endosomes, whereas the inactive β1 was found almost exclusive at the cell surface (I, Fig.1B). Moreover, while active integrin was localised in the interface between cells and the supporting surface at the bottom of the cells, inactive β1 was seen in the non-adhered membrane protrusions at the cell edges (I, Fig.1C). The predominant PM localisation of inactive integrin was further confirmed by measuring the levels of active and inactive β1 with flow cytometry in three different cell lines (NCI-H460, MDA-MB-231 & PC-3). Indeed, most of the cell surface integrins were inactive (mab13, 4B4), whereas only ~20% of integrins were active (12G10, 9EG7) compared to inactive integrins (I, Fig.1A), which is in line with a previous report (Tiwari et al, 2011).

These observed differences in cell surface localisation are easy to rationalise, as in 2D environment integrin-mediated adhesion can only form at the bottom of the cells, where integrins are mostly active. In the middle and further above, cells have nothing to adhere to and thus cell surface integrins are mostly inactive. In 3D-environment the distribution is likely to be more even and probably a bigger portion of cell surface integrins are in their active conformation. However, the 2D-situation highlights the difference between the intracellular localisation of active and inactive β1, as although most of the β1-integrins were inactive, hardly any inactive β1 could be detected inside the cells.

5.1.2. The trafficking of active and inactive β1 follow distinct kinetics (I)

The observed difference in the subcellular localisation of active and inactive β1 suggests that the two are trafficking differently. The trafficking of plasma membrane receptors have been traditionally studied with a biotinylation-based assay, where the amount of endocytosis or recycling is detected with Western blotting. However, this method does not allow visualisation of the different integrin conformations, and therefore we set up a new antibody-based trafficking assay, which is outlined in (I, Fig.3A). In brief, cell surface integrins are labelled with fluorescent Alexa Fluor (AF) –conjugated antibodies and following internalisation the remaining signal at the cell surface is depleted with specific anti-AF quenching antibody. The amount of endocytosis or recycling is detected with a fluorescent plate reader or microscope and can be quantified by measuring the total signal intensity. The assay was validated by using an antibody against the total β1-integrin pool and by comparing the levels of endocytosis and recycling to those gained with the classical biotin-assay (I, Fig.3B,C, Fig.S2A).

The use of primary and secondary antibodies together in live cells could potentially lead to small scale integrin clustering, thereby triggering increased integrin endocytosis (Upla et al, 2004). However, the kinetics of β1 endocytosis were virtually the same with antibody-based assay as with the classical biotin-assay, where this type of clustering does not occur. Moreover, similar results have been gained with immunoglobulin molecules and monovalent Fab-fragments against integrin-β1, indicating that the potential clustering of integrins does not play a significant role (Powelka et al, 2004). Integrin traffic occurs in a time-dependent manner, and the results obtained with the two different methods were in line with each other, although slightly less recycling was detected with the antibody-based method after 30min. However, the kinetics were nearly identical, indicating that the method can be used to study integrin traffic.

By using the antibody-based assay, we were able to compare the levels of endocytosis and recycling of active and inactive β1-integrins. In all of the three cell lines, around half of the active β1 was endocytosed already after 10 minutes, and all of them after 30 min in two different cell lines, while less than 50% of inactive integrin was seen internalised even after 30min (I, Fig.4A). These are in line with the observed differences in the subcellular localisation of active and inactive β1 at steady-state. The total β1 showed similar results with inactive β1, further supporting the finding that most integrins at the cell surface are inactive. However, these measurements represent the net-endocytosis, as the amount of internalised integrins is balanced by recycling. In fact, when cells were treated with Primaquine (PQ), an anti-malaria drug that blocks endosomal

recycling of integrins (I, Fig.3C, (Somasundaram et al, 1995, van Weert et al, 2000), the localisation of inactive β1 was dramatically changed. In contrast to its normal PM staining, the inactive β1 now emerged in large, EEA1-positive endosomes together with active β1 (I, Fig.7A,B,D & S6). Although subtle changes in the subcellular localisation of active β1 could also be detected, unlike the inactive β1, the amount of intracellular active β1 was not significantly changed (I, Fig.7A, C). Together these results indicate that the steady-state localisation of inactive β1 is greatly influenced by its recycling, and therefore the fast recycling of inactive β1 could explain the low net-endocytosis seen compared to active β1.

Cells are constantly sensing and monitoring the surrounding ECM, and thus cells need to have an adequate pool of inactive integrins constantly at the PM ready to be activated. The slower recycling of active β1 compared to inactive β1 is easy to rationalise by the recent observation that active integrins need to detach from their ligands prior to recycling (Kharitidi et al, 2015). In contrast, inactive β1 does not require similar resensitisation, but can be readily transported back to the PM to form new adhesions. Efficient integrin traffic is especially important for cell motility, and vesicular transportation of integrins to the correct sites is likely to be faster compared to PM diffusion. Moreover, ECM-binding induces integrin clustering and the transport of this type of multiprotein complex is likely to be slower compared to inactive integrins, which do not form similar clusters.

5.1.3. Active and inactive β1 traffic via different routes (I)

Given the significant difference in the sensitivity of active and inactive β1 to PQ-treatment, we hypothesised that the two might be trafficking via different routes. The most classical routes for integrin endocytosis are dynamin-2 and clathrin or caveolin dependent (Bridgewater et al, 2012, De Franceschi et al, 2015). However, when these endocytic routes were inhibited, no significant difference was detected between active and inactive β1. The endocytosis of both active and inactive β1 was inhibited in cells expressing a dominant-negative dynamin-2 (Dyn2-K44A, (Damke et al, 1994, Damke et al, 2001) or Esp15-mutant, which inhibits clathrin-mediated endocytosis (CME) by blocking AP2-clathrin complex formation (Benmerah et al, 1999). In contrast, the N-terminally EGFP-tagged caveolin-1, which has been shown to function as dominant-negative inhibitor of SV40-virus uptake (Pelkmans et al, 2001) had no effect in either of the two (I, Fig. 6A,B). In line with this, antibody-staining of clathrin light chain showed more colocalisation with both integrin conformations compared to caveolin-1 (I, Fig.S5B,C). Integrin-β1 has been reported to undergo CME also previously (Pellinen et al, 2008), although examples of

caveolin-dependent entry route also exist. For instance, virus- or antibody-clustered α2β1 has been shown to associate with caveolin-1 (Upla et al, 2004), and likewise, α5β1 has been reported to undergo caveolin-dependent endocytosis during fibronectin assembly (Shi & Sottile, 2008). As we only used one approach to block caveolin-dependent endocytosis, we cannot fully exclude the possibility that a fraction of β1-integrins would undergo this route. Nevertheless, both active and inactive β1 displayed similar effects in all of the endocytosis modulation experiments, therefore suggesting that the endocytosis routes utilised by the two are shared.

However, it is important to note, that although the internalisation of both active and inactive β1 is dependent on clathrin-mediated endocytosis, the two may not be internalised from the same clathrin coated pits. Indeed, as we did not co-image active and inactive β1 in live cells, it is not clear whether the two are endocytosed together or from distinct sites at the PM. AP-2 was recently reported to promote predominantly the endocytosis of inactive integrins by binding to a subset of integrin α-tails carrying a conserved YxxΦ motif (De Franceschi et al, 2016), whereas integrin endocytosis from FAs, where integrins are presumably in their active conformation, is dependent on other clathrin adaptors ARH (autosomal recessive hypercholesteremia) and Dab2 (Disabled-2) (Ezratty et al, 2009). Together, these results suggest that the internalisation of different integrin pools might be mediated by distinct clathrin adaptors, but it remains to be discovered whether they are jointly internalised in the same endocytic vesicles.

In addition to the shared clathrin-dependency, both active and inactive β1 co-localised with EGFP-tagged Rab5, Rab21, Rab4 and Rab11 after 30 min internalisation in MDA-MB-231 cells (I. Fig.5A-C). Interestingly though, only active β1 was detected in EGFP-Rab7-positive endosomes, which is in line with reports demonstrating that active integrins are transported to LEs to be degraded or resensitised prior to their recycling back to the PM (Kharitidi et al, 2015, Lobert et al, 2010). However, although both conformations were seen to localise to fast recycling Rab4-endosomes, only the traffic of inactive β1 was affected by expression of dominant-negative Rab4 mutant (Rab4a-S22N) (I, Fig.7E). Specifically, the amount of internalised inactive β1 was increased, suggesting that inactive β1 is transported back to the PM via a Rab4-dependent fast recycling loop, explaining why in a steady-state situation the inactive β1 is mainly localised at the cell surface. In contrast, a recent study showed that inactive β1-integrins are re-secreted from the Golgi apparatus instead of trafficking via Rab4-endosomes (Shafaq-Zadah et al, 2016). However, the retrograde transport is likely to be exploited mostly in situations, where cells need to migrate in a highly polarised and persistent manner, and inactive integrins are transported longer distances, from the rear of the cell to the front. Corroborating this, the retrograde transport appears to be important

especially during wound healing and gonad development in *C.elegans* (Shafaq-Zadah et al, 2016).

While neither active nor inactive β1 were observed in EGFP-Rab25-positive endosomes even after 2 hours, inactive β1 showed increased accumulation in Arf6-positive compartments (I, Fig.S4A,B & 5C). Inactive β1 showed also significantly more co-localisation with Arf6 compared to active β1, especially in membrane protrusions and in the tips of EGFP-Arf6-positive dorsal cell surface microspikes (I, Fig. 9A-D). Arf6 is often implicated in Rab11-dependent "long-loop" recycling (Powelka et al, 2004), whereas based on our observation, recycling of inactive β1 is mediated by Arf6 and Rab4, thus implicating Arf6 in the "short-loop" recycling. However, in studies addressing 'long-loop' recycling, integrin activity was not addressed, and fast integrin recycling has been associated with Arf6 also previously (Fang et al, 2010). Moreover, although the recycling of inactive β1 was observed to be dependent on Rab4 and it localised to Arf6-positive compartments, we cannot exclude that the two would be separate pathways, and inactive β1 was also detected in Rab11-endosomes. The active β1 localised less to Arf6-positive compartments most likely due to its targeting to Rab7-positive late endosomes. Arf6 is also known to mediate distinct trafficking steps determined at least partly by the associating Arf-GAP. Arf6 has been reported to drive integrin recycling from Rab11-positive compartments when associated with ACAP1, whereas another Arf6-GAP, ARAP2, drives integrin endocytosis through APPL-endosomes (Chen et al, 2014). Therefore, it is possible that Arf6 operates in addition to Rab11 also with Rab4 by associating with another, yet unidentified GAP-protein.

Arf6 localises to the PM and regulates β1-integrin recycling together with actin (Al-Awar et al, 2000, Fang et al, 2010, Powelka et al, 2004, Radhakrishna & Donaldson, 1997). In line with those data, the PQ-induced enlarged endosomes comprising the inactive β1 were found to overlap with actin staining (I, Fig.8A), and actin was further shown to be required for the recycling of inactive β1 by inhibiting actin polymerisation with Cytochalasin D (CytD) during PQ washout. In contrast, no change was detected in the intracellular levels of active β1 upon CytD treatment (I, Fig.8B,C), suggesting that although the entry routes of active and inactive β1 are identical, their recycling is different. However, as the recycling of active β1 is relatively slow compared to inactive β1, Rab4 as well as actin might play a role also in the recycling of active β1 at later time-points. Nevertheless, the difference in the recycling rate is evident.

Taken together, the above results indicate that while both active and inactive β1 are trafficking via Rab5/Rab21/ EEA1 positive early endosomes, a significant proportion of active β1 is targeted to Rab7-positive LEs, whereas the inactive β1 is recycled rapidly in a F-actin- and Rab4-dependent manner to Arf6-positive protrusions at the PM (Figure 8). Therefore in the steady state situation, most of the inactive β1 is seen on the cell surface, whereas the slowly recycling active β1 is more intracellular. The mechanisms by which active and inactive β1 are distinguished in cells is not clear, but different possibilities are discussed later (Chapter 5.6.).

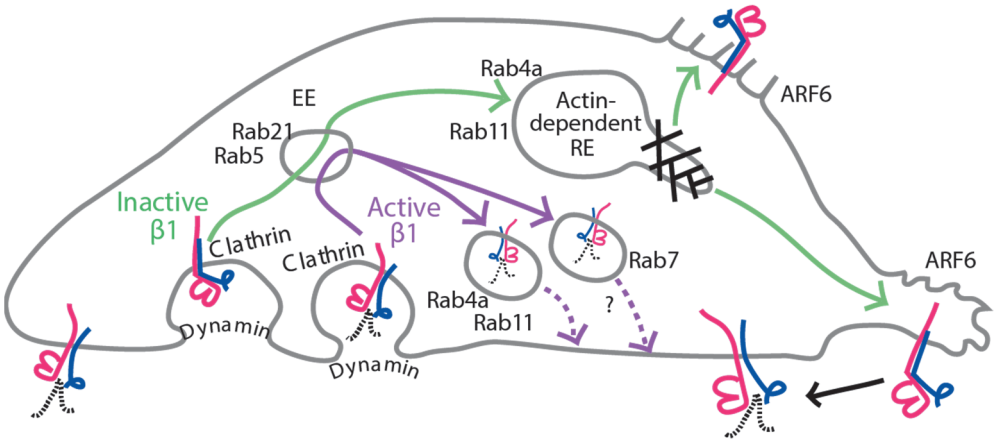

Figure 8. Active and inactive β1 recycle via different routes. Although both active and inactive β1 are endocytosed in clathrin- and dynamin-dependent manner to Rab21/Rab5-endosomes, recycling of inactive β1 integrin is faster compared to active β1. Inactive β1 is recycled to Arf6-positive protrusions at the PM in a Rab4- and actin-dependent manner, whereas a significant portion of active β1 is taken to Rab7-positive late endosomes. Adapted from (Arjonen et al, 2012).

5.2. ACTIVE β1 IS ENDOCYTOSED TOGETHER WITH ITS LIGAND (I, II)

The activation of integrins can be primed by binding to an extracellular ligand, and the talin-mediated mechanical connection between integrin tails and the actin cytoskeleton has been proposed to be required for full integrin activation (Legate et al, 2009, Tadokoro et al, 2003). For this reason, the ability of integrins to stay active in endosomes, where similar actin-mediated forces are unlikely to be exerted, has been under discussion. Nevertheless, several

independent studies have reported active integrins in endosomes together with their ligands (Ng et al, 1999, Pellinen et al, 2006) and fibronectin-bound α5β1-integrins are targeted to multivesicular endosomes/lysosomes to be degraded (Lobert et al, 2010) or recycled in their active conformation in Rab25 and CLIC3 expressing cancer cells (Dozynkiewicz et al, 2012). In line with these, we detected active β1 colocalising with labelled fibronectin fragment (FN(7-10)III) in endosomal structures in MDA-MB-231 cells (I, Fig. 4B) and with fibronectin staining in constitutively active GFP-Rab5 (GFP-Rab5-CA/ Q79L) -positive endosomes in TIFFs (human telomerase-immortalised foreskin fibroblasts) plated on fibronectin (II, Fig. 2A top panel). Surprisingly though, in addition to endosomal actin (I, Fig. 8A & II, Fig.6E), also talin was detected in the integrin-containing endosomal fraction in subcellular fractionations of MEFs (mouse embryonic fibroblast, II, Fig.2B). Therefore, all the main protein components implicated in integrin activity at the PM are also present in endosomes, where endosomal talin may function to sustain integrin activity. Whether some local tension occurs also on endosomes or whether integrins can stay active in endosomes without tension, especially when the complex has once been fully activated at the PM, remains to be discovered. Nevertheless, integrins can clearly be in their active conformation also in endosomes.

5.3. INTEGRINS SIGNAL FROM ENDOSOMES (II, III)

5.3.1. Integrin endocytosis is required for full ECM-induced integrin signalling (II)

Several RTKs are known to signal from early endosomes (Alanko et al, 2015, Sigismund et al, 2012, Vieira et al, 1996), and given the ability of integrins to co-traffic with their ECM-ligands to these compartments, we investigated whether integrin endocytosis would be needed for its signalling. To induce integrin signalling, cells were plated on integrin ligands, collagen or fibronectin for 0-45 min, which triggers time-dependent activation of the classical integrin-induced signalling proteins, FAK, Akt, Erk and Src (II, Fig.S2A). As integrins and activated RTKs are known to activate some of the same signalling pathways (Ivaska & Heino, 2011), to detect specifically the integrin-mediated signalling, cells were kept under serum starvation prior to experiments. Under these conditions, RTKs were not significantly activated (II, Fig.S2B). When integrin endocytosis was inhibited with a dynamin inhibitor (dynasore), giving around 50% reduction in the endocytosis of active β1 with the used concentration (80 μM) (II, Fig.S3A, B), ECM-induced integrin signalling was significantly reduced, as determined by immunoblotting against pFAK-Y397, pErk1/2, pAkt-S473 and pSrc-Y416 (II, Fig.3A-D & S4A-D). Whereas pAkt, pErk and pSrc displayed cell line and/or ligand-dependent effects, the level of

pFAK-Y397 was significantly decreased in all conditions. Indeed, the reduced pFAK-Y397 was observed in two different carcinoma cell lines (NCI-H460 and MDA-MB-231) and in immortalised fibroblasts (TIFF) both on fibronectin and on collagen, therefore indicating that the effect is not cancer cell- or ligand/integrin heterodimer-specific.

Dynamin-2 has been implicated not only in receptor endocytosis, but also in the recycling of transferrin receptor (van Dam & Stoorvogel, 2002), and therefore the reduced integrin signalling observed under dynasore-treatment could be due to reduced integrin recycling and PM signalling. However, when the levels of cell surface β1 were measured with flow cytometry, dynasore-treatment slightly increased the integrin-β1 surface levels, in line with the reduced endocytosis (II, Fig.S4G). On the other hand, the dynasore-treated cells were slightly less spread than the control cells especially after 45 min plating, and therefore the reduced signalling could be due to the fact that these cells are not able to form the same amount of integrin-mediated adhesions (II, Fig.S5A,B). This possibility was excluded by inducing integrin signalling in suspension with collagen-coated beads (II, Fig.S5B) or by plating the cells on small round micropatterns forcing the cells into a same adhesion area (II, Fig.4F). In both cases, similar decreases in the pFAK-Y397 levels (around 50%) were observed following dynasore treatment. Integrin endocytosis-dependent FAK activation was further confirmed with another dynamin inhibitor Dyngo4a (II, Fig.S4E) and with Dyn2K44A expression (II, Fig.S4F). As dynamin-2 has a general role in receptor endocytosis, integrin endocytosis was inhibited also more specifically by Rab21-silencing (II, Fig.3F). Rab21-silencing gave a similar reduction in pFAK-Y397 levels compared to dynamin inhibition, thus indicating that integrin endocytosis is required for full integrin signalling. Moreover, the level of pFAK-Y397 was significantly reduced also in cells expressing Rab21-binding-deficient α2-integrin (II, Fig.3G) and accordingly, Rab21 or Rab5-CA overexpression increased the pFAK-Y397 levels (II, Fig.S6A, B). Taken together, these results indicate for the first time that Rab21-mediated integrin endocytosis is required for full integrin-induced FAK signalling.

Integrin traffic has been linked to cellular signalling also previously. Integrin co-trafficking with EGFR stimulates EGF-induced Akt signalling (Caswell et al, 2008), Akt activity has been shown to promote integrin recycling (Roberts et al, 2004) and endocytosis of active, ligand-bound integrin enables TIAM-mediated Rac1 activation on endosomes (Sandri et al, 2012). Moreover, in line with our results, overexpression of wild-type Rab5 has been reported to increase pFAK-Y397 levels in hepatocellular carcinoma cell lines (Geng et al, 2015). Thus, although cross-talk between integrin traffic and cellular signalling pathways is well established, until our study, direct evidence for endocytosis-dependent integrin-induced pFAK signalling was missing.

5.3.2. Integrin-mediated FAK signalling occurs on endosomes (II)

FAK is a well-known component of focal adhesions, and although FAK is known to translocate also to the nucleus, it is commonly considered to localise to integrin-mediated adhesions at the PM (Lim et al, 2008, Mitra et al, 2005). However, when we studied the localisation of active β1 and pFAK-Y397 in fixed cells on crossbow-shaped fibronectin-coated micropatterns, giving the cells a controlled polarised shape, we noticed that the two localised not only in FA resembling structures at the cell edge, but also inside the cells (I, Fig.1A & S1D). The intracellular staining of both active β1 and pFAK-Y397 was even more prominent in 3D-probabilistic density maps created from the stainings. When cells were transfected with GFP-Rab21 or GFP-Rab5-CA to generate larger endosomes, pFAK-Y397 was seen to localise with active and total β1-integrin to these endosomes (II, Fig.1C & S1E). The localisation of pFAK-Y397 together with active β1 in Rab5-CA endosomes was further confirmed using super-resolution STED imaging, where the two were seen to colocalise (II, Fig.1D). Moreover, pFAK-Y397 and active β1 localised together with endogenous EEA1-staining (II, Fig. 1B), indicating that the endosomal localisation of FAK is not caused by the overexpression of Rabs. The localisation with EEA1 is in line with the fact that Rab21 mediates integrin endocytosis to EEA1-positive endosomes (Mai et al, 2011, Pellinen et al, 2006).

Our data suggest that integrin signalling occurs also on endosomes, and the endosomal localisation of FAK was further verified with subcellular fractionation of FAK-null MEFs reconstituted with GFP-FAK expression. The fractionation assays indicated that β1-integrin, Rab21 and EEA1 are present in the endosomal fraction together with GFP-FAK (II, Fig.2B). In contrast, paxillin was completely absent and only very low levels of vinculin could be detected (II, Fig.2B). Thus, the integrin proximal components on the endosomes are at least partially distinct from the focal adhesion components. In line with the absence of paxillin, FAK-FAT domain alone was not recruited to the endosomal fraction (II, Fig.4E). In contrast, the FAK-FERM domain alone was sufficient to localise to integrin-containing endosomes. This suggests that FAK endosomal targeting is driven by a mechanism distinct from FAK targeting to focal adhesions. Moreover, the integrin endosomal localisation is not dependent on FAK, as β1-integrin localises to endosomal fraction also in FAK-null MEFs (II, Fig.4A).

5.3.3. The endosomal signalling nexus is distinct from canonical FAs (II, III)

The above results indicate that integrin signalling is dependent on endocytosis, and could be explained by the following scenarios: the integrin signalling originating from the PM might be sustained on endosomes, for example by transporting integrins away from PM phosphatases. Alternatively, more signalling proteins might be recruited to endosomes, thereby amplifying the signal. A third option is that the canonical signalling complexes at FAs are replaced by another, endosome-specific signalling nexus, which could be called as integrin “endoadhesomes”. To date, the subcellular location of FAK-targeting phosphatases is unclear, but to investigate whether integrin-induced FAK signalling could be amplified on endosomes, we analysed the binding of activated (pY397) and non-activated recombinant FAK proteins to isolated integrin-containing endosomes derived from FAK-/- MEFs. Surprisingly, both active and non-active FAK were able to associate with the isolated endosomal fraction (II, Fig.4B), and interestingly, the endosome-associated FAK was even activated in the presence of ATP (II, Fig.4D). Moreover, the association of active FAK with endosomes was at least partly dependent on β1-integrin (II, Fig.4C), suggesting the possibility that more FAK could be recruited and activated also after integrin endocytosis.

EEA1 and APPL1 have previously been implicated in growth factor-induced endosomal signalling (Miaczynska et al, 2004, Nazarewicz et al, 2011). Similarly, EEA1 silencing significantly decreased ECM-induced FAK signalling, whereas no difference was observed with APPL1-silencing (II, Fig.6A,C). EEA1 is known to associate with endosomes by binding to PI(3)P (Lawe et al, 2000), and accordingly, inhibition of PI(3)P-producing enzyme (PI3K) decreased pFAK-Y397 levels to the same extent as dynasore (II, Fig.6D), without changing the total levels of cellular EEA1 (II, Fig.6E). Importantly, as silencing of neither EEA1 nor APPL1 affected the endocytosis of active β1 (II, Fig.6B), irrespective of EEA1-dependent FAK signalling, these results suggest that EEA1 functions as an endosomal signalling platform needed for integrin-mediated signalling. Therefore this, together with the lack of paxillin and vinculin in the integrin/FAK/talin-containing endosomal fraction, favours the existence of a distinct endosomal signalling complex formed after endocytosis.

To further investigate the components responsible for integrin-mediated endosomal signalling, we performed mass spectrometric analysis of the endosomal fraction together with PM and cytoplasmic fractions (II, Fig.5A-D). In addition to integrins and FAK, several known FAK and integrin-interacting proteins were identified from endosomes, most importantly the integrin ligands collagen and fibronectin. Moreover, while many classical FA components were

found in both the PM fraction and the endosomes, including different kinases and phosphatases, actin-binding proteins and GTPase-regulating proteins, some FA proteins were not detected in endosomes, such as paxillin. On the other hand, some proteins not identified in the PM fraction were detected on endosomes, including ARFGEF2 (also known as GEF-H1), a microtubule-associated Rho GEF (Ren et al, 1998), thus supporting the existence of a unique endosomal signalling nexus. Some of the most interesting proteins identified from endosomes are illustrated in III, Fig. 2. In general, only a minority of the detected proteins were seen enriched in endosomes compared to other fractions, which could indicate that the components required for integrin endosomal signalling play a role also in other cellular compartments. Moreover, although several endosomal proteins were identified in the endosomal fraction, the technique used in this study for subcellular fractionation is basic, and thus we cannot exclude the possibility that the endosomal fraction might also contain proteins from other compartments. To identify the endosomal proteins more specifically, other techniques, such as sucrose gradient fractionation, immunoprecipitation of endosomes or proximity-based biotinylation assay would be required in future studies.

5.3.4. The endosomal FAK signalling supports anchorage-independent cell survival (II)

As one of the most upstream kinases in ECM-induced integrin signalling, FAK is implicated in almost every aspect of integrin-mediated cellular functions from cell proliferation to motility and survival. Given its well-known role in anoikis suppression (Frisch et al, 1996), to highlight the significance of endosomal pFAK-Y397 we chose to concentrate on anoikis. In line with the reduced pFAK-Y397 levels upon inhibition of integrin endocytosis, the number of TIFFs going through anoikis after 5h in suspension was significantly increased with dynasore or Dyngo4a treatment, or with Rab21- or EEA1-silencing. In contrast, no significant difference was observed in adherent cells or in APPL1-silenced cells (II, Fig.7A,C,D & S7A,B). Moreover, no difference was detected in FAK-/- MEFs following dynasore treatment, indicating that the increased anoikis sensitivity is dependent on FAK, and cannot be compensated by increased expression of FAK homologue Pyk2 (II, Fig.7B). Cancer cells are less sensitive to anoikis, but the anchorage-independent cell survival of MDA-MB-231 cells was also significantly reduced by FAK inhibition (II, Fig.S8A) and by EEA1- or Rab21-silencing (II, Fig.7E). Accordingly, when EEA1- or Rab21-silenced cells were co-injected with control cells into the tail veins of mice in extravasation assays, both silenced cell populations displayed significantly reduced metastasis to lungs compared to controls (II, Fig.7F,G), presumably due to reduced survival in the vasculature. Therefore, reduced integrin endosomal

FAK signalling sensitises cancer cells to anoikis and also impairs their metastatic potential *in vivo*.

It is, however, important to note that EEA1 is implicated also in the signalling of other receptors (Alanko et al, 2015, Nazarewicz et al, 2011), and Rab21-mediated integrin endocytosis is likely to influence also the intravasation of cells from the circulation (Pellinen et al, 2006). Identification of the exact mechanism of FAK recruitment to endosomes would potentially allow specific inhibition of endosomal FAK signalling, thereby enabling more precise investigation of its significance in cancer metastasis. The detailed mechanism of FAK endosomal recruitment is still unknown, as will be discussed in the following chapter, and the most efficient targeting strategy will naturally depend on that. However, in the light of our observation of EEA1 being critical for FAK signalling, one possibility to target specifically the endosomal FAK signalling would be to develop an EEA1-binding FAK-inhibitor.

Although increased FAK expression and activity are often associated with highly malignant cancers, how FAK activity is sustained in metastasising cancer cells upon loss of adhesion has remained unclear. The ability of FAK to bind and become activated on endosomes could provide a potential explanation. FAK dimerisation, induced by local FAK enrichment, has been proposed to drive FAK autophosphorylation and activation (Brami-Cherrier et al, 2014). Although previously the local enrichment has been observed only in FAs, the endosomal membranes comprising integrin and talin might facilitate similar FAK enrichment and activation. In this respect, the activation of recombinant FAK on isolated endosomes could be argued to result from high FAK concentration, thereby facilitating rather abnormal FAK activation. However, as increased FAK levels are also commonly found in advanced human cancers, the endosomal activation could also explain increased FAK activity *in vivo*. In addition, increased integrin activity and traffic have been implicated in cancer metastasis (De Franceschi et al, 2015, Lee et al, 2015), and β1-integrins are endocytosed in PC-3 cells also in suspension (I, Fig.S1B), which could further promote higher FAK activity in anoikis-resistant cancer cells. Moreover, FAK activity has been seen to promote anchorage-independent survival of murine ovarian carcinoma cells independently of Src activity (Ward et al, 2013), which is in line with our observation of endocytosis-independent Src signalling (II, Fig.3B-D).

5.4. HOW IS FAK RECRUITED TO ENDOSOMES? (II, III)

Although paxillin is considered as the main FAK-recruiting protein in FAs, also talin has been reported to interact with the FAK-FAT domain (Chen et al, 1995). Moreover, talin functions as a mechanosensor that can be stretched and is able to bind different proteins depending on the applied force (Yan et al, 2015). For instance, talin has been shown to bind vinculin only upon mechanical stretching of the talin rod (del Rio et al, 2009). Since endosomes are unlikely to be exposed to the same extent of mechanical tension as FAs, this would be in line with our observation that vinculin is only weakly detected in the endosomal fraction. Although the FAK-FAT domain alone was unable to bind isolated endosomes, the endosomal talin might still facilitate FAK recruitment to these compartments. On the other hand, the FAK-FERM domain has also been reported to bind directly to integrin β-tails *in vitro* (Schaller et al, 1995), which could provide another mechanism for endosome association. This would be in line with the fact that targeting of pFAK-Y397 to the endosomal fraction is at least partly dependent on β1-integrin. The remaining binding could be due to a compensatory increase in β3-expression (II, Fig.S4I). However, the ability of β3-integrins to signalling from endosomes remains to be investigated. The binding of non-activated FAK might be more dependent on integrins, but also this remains to be tested. Another possible mechanism by which FAK could associate with endosomes is the endosomal actin and the actin-nucleating Arp2/3-complex, as FAK has been shown to interact with Arp3 via its FERM domain (Serrels et al, 2007). FAK is known to interact also with different growth factor-activated RTKs, and although FAK activation was clearly dependent on integrin endocytosis in the absence of growth factors and RTK activation, we cannot exclude that those would play a role.

At FAs, the FAK-FERM domain binds PI(4,5)P2, but it is also able to bind PI(3,4,5)P3 (Goni et al, 2014), which has been detected in minor quantities in early endosomes (Naguib et al, 2015). This raises the possibility that FAK targeting to endosomes is mediated by PIPs. In fact, our unexpected finding of endosomal pFAK-Y397 and talin was further confirmed by another group soon after our study (Nader et al, 2016), and interestingly in this study, also PIPKI-gamma was detected on the same endosomes. The endosomal FAK was suggested to phosphorylate and activate PIPKI-gamma, thus driving the production of PI(4,5)P2 on endosomal membranes. This would suggest that FAK might also be recruited to endosomes via PI(4,5)P2, in a similar manner to FAK-PM targeting. Indeed, perhaps both FERM-PIP interactions and FAT-talin interactions are needed to target the full-length FAK to endosomal integrin tails. This could be tested by investigating whether FAK lacking the FERM-domain is still able to localise to endosomes. Moreover, as active β1 and pFAK-Y397 were seen in Rab11 endosomes (Nader et al, 2016), this suggests that the integrin-endosomal FAK signalling is sustained beyond the observed

Rab5/Rab21/EEA1-positive early endosomes, possibly as far as the PM (Nader et al, 2016). Finally, the question of how and when FAK is inactivated provides an interesting avenue for future research.

5.5. HOW IS INTEGRIN ACTIVITY SUSTAINED IN ENDOSOMES? (I, II)

Another interesting question is how integrins are maintained in their active conformation in endosomes and whether this requires integrins to be constantly bound to their ligands in order to signal. Several studies, including ours, have reported integrins to localise in endosomes together with their ECM-ligands (Lobert et al, 2010, Ng et al, 1999, Pellinen et al, 2006), but endosomes are known to have gradually decreasing pH along the way from EEs (pH ~6) to LEs and further to lysosomes (pH < 5.5) (Kharitidi et al, 2015). How is the acidic pH in endosomes affecting the integrin-ligand interaction, or in other words, is it even possible for integrins to bind their ligands at pH 6 or below? The studies in Rab25-expressing cells, where integrins are seen to recycle from LEs/lysosomes while still in their active state strongly support this idea (Dozynkiewicz et al, 2012). In the case of αvβ3, the pH 6.0 has been shown to even increase its affinity for RGD-ligand (Paradise et al, 2011). The lower pH was proposed to open the αvβ3 headpiece, thus leading to fully activated extended integrin conformation. However, based on a recent study on α5β1, almost 70% of integrin-bound FN is lost at pH 6.1, thereby allowing most of the integrins to be resensitised and recycled back to PM, while the rest of the integrin-FN complexes continues to degradation (Kharitidi et al, 2015). Moreover, FN binding to integrin α5β1 was shown to trigger integrin ubiquitination and internalisation together with FN, but dissociation of FN due to endosome acidification promoted deubiquitination and recycling (Kharitidi et al, 2015). In line with this, the integrin-containing endosomes analysed in our study did indeed contain a variety of ubiquitin-related proteins together with fibronectin and collagen. These seemingly contradictory results could be explained by different pH sensitivity in case of different integrin-ligand complexes. Another possibility is that although integrin affinity for ligands decreases along pH, it may not prevent continued rebinding of ligands in endosomes with restricted space and integrin heads pointing towards the lumen. On the other hand, as integrins can clearly be still in their active state while recycled (I), perhaps once the fully active integrin has been established together with talin and others also present in endosomes, the ligand binding is no longer required for integrin activity and signalling. This intriguing possibility of integrins to signal in the absence of ligand remains to be fully investigated and one approach would be to test whether PQ treatment increases endosomal FAK signalling.

5.6. HOW ARE ACTIVE AND INACTIVE INTEGRINS DISTINGUISHED IN CELLS? (I, II)

Cells need to be able to distinguish active and inactive integrins in order to direct them to distinct trafficking routes. How this is accomplished is still unknown, but the distinction has to occur based on the components associated with integrin cytoplasmic tails. Indeed, the only difference between active and inactive integrins is in their conformation, and the short cytoplasmic integrin tails with different interactors are the only cues for the intracellular transportation machinery. Moreover, as both active and inactive β1 are internalised to same early endosomes, the distinction is likely still occurring also in these compartments. As active integrin and talin are both present in endosomes, the difference in actin-binding could play a role, but also several proteins regulating integrin traffic and activity are known to share the same binding sites in integrin tails. Therefore, the interplay between these proteins could determine the trafficking fate of different integrin pools. However, it is important to note that ligand-induced integrin clustering gathers multiple integrin tails to the same adhesion complex. Therefore, it is possible that different proteins sharing the same binding site may not always have to compete with each other, but might bind separate integrin tails in a same integrin cluster.

For instance, SNX17 (sorting nexin-17) shares the same binding site in integrin β-tails with the integrin activator kindlin (Moser et al, 2009). SNX17 protects integrins from lysosomal degradation by driving integrin recycling from EEA1-positive endosomes (Steinberg et al, 2012). GGA3 (ADP-ribosylation factor-binding protein) is required for the endosomal localisation of SNX17 and for SNX17- and Arf6-dependent integrin recycling (Ratcliffe et al, 2016). As mainly inactive β1 was detected in Arf6-positive compartments (I), the GGA3-SNX17 -complex could selectively drive the recycling of inactive integrins. This would be consistent with a potential competition between SNX17 and kindlin.

Another example is provided by Rab21, which shares a partially overlapping binding site in integrin α-tails with the integrin inhibitor sharpin (Mai et al, 2011, Pellinen et al, 2006). This would suggest that Rab21 drives selectively the endocytosis of active integrins, but based on our observation also the inactive β1 localises to Rab21-endosomes. Perhaps sharpin-binding is needed only at the PM and sharpin is replaced by Rab21 upon internalisation, thereby allowing the endocytosis of both active and inactive integrins. In this case, other components would be required to mark the integrin as inactive in order to direct it to the correct traffic route.

The endosomal PIPs could also play a role in targeting different integrin pools to distinct trafficking routes. Although the prevailing view is that PI(4,5)P2 at the PM is converted into PI(3)P along endosome maturation (De Matteis & Godi, 2004), PI(4,5)P2 was recently observed also in integrin/FAK/talin-containing Rab11-endosomes (Nader et al, 2016). Moreover, the endosomal PI(4,5)P2 was shown to be required for endosomal talin recruitment and thus for sustaining integrin activity and signalling in endosomes. Based on our study also PI(3)P-interacting EEA1 is required for endosomal FAK signalling and inhibition of PI3K decreases the levels of pFAK-Y397, suggesting that the conversion into PI(3)P is needed for integrin endosomal signalling. Since endosomal membranes are known to comprise distinct subdomains, different integrin pools might localise to distinct membrane domains even in the same endosomes. Active integrins could thus be sustained in endosomal PI(4,5)P2-domains, where FAK-mediated activation of PIPKI-gamma2 is needed for talin recruitment and for subsequent integrin activity (Nader et al, 2016). In this case, PI(3)P and the associating EEA1, known to mediate homotypic fusion between newly formed early endosomes, could be needed in general for integrin traffic to the correct endosomal location. Moreover, an adequate size of EEA1-positive endosomes could be necessary to trigger fission processes driving receptor trafficking to different destinations. SNX17 drives integrin recycling by interacting with endosomal PI(3)P, and thus inactive integrins could localise specifically to PI(3)P-positive subdomains, thereby allowing SNX17-mediated recycling of inactive integrins. However, SNX17 has been shown to promote also the recycling of active β1 (Steinberg et al, 2012), but whether p120RasGAP-mediated integrin recycling is selective for different integrin pools is yet unknown.

A simple explanation for the distinct recycling of active and inactive β1 would be that the endosomal signalling complex formed in the tails of active integrin causes steric hindrance making the tails of active integrin less available to proteins mediating integrin recycling, such as p120RasGAP and SNX17 (Mai et al, 2011, Steinberg et al, 2012). This would enable inactive β1 to recycle faster and permit active integrins to signal longer before returning to the PM.

5.7. FUTURE PERSPECTIVES (I, II, III)

Integrin traffic has been extensively studied during the past 15 years, but due to the multiple and interconnected trafficking routes and the variety in integrin heterodimers, the findings from different studies have been difficult to interpret. We here systematically analysed the trafficking fate of β1-integrin in its active and inactive conformation. To get a full and inclusive understanding of integrin traffic in cells, a high throughput approach using labels against different heterodimers in their different activation states together with different endocytic markers, time-points and stimuli could be instrumental to clear this long-lasting condundrum. The antibody-based trafficking assay developed in this study would allow this, as it is designed to be used with multiwell plate reader in a high-throughput system, where imaging and quantification are automated, thereby enabling simultaneous analysis of multiple receptors under various conditions. Indeed, as AP-2 for instance was recently discovered to couple only with a subset of integrin-α tails (De Franceschi et al, 2016), it will be interesting to see how different α-subunits contribute to β1-integrin traffic.

We studied the trafficking routes exploited by integrins by overexpression of different Rabs, an approach that has been widely used in the trafficking field. However, overexpression of Rab proteins may unbalance the trafficking network and therefore affect the trafficking kinetics of integrins in different compartments. Although this is still unlikely to drive integrin traffic to compartments that would not otherwise be used, the increased use of the CRISPR/Cas system to tag endogenous Rab proteins with fluorescent proteins shall provide further insights in the trafficking kinetics in the future.

Although much information has been gained from signalling endosomes since their original discovery (Grimes et al, 1996, Vieira et al, 1996), as highlighted in this study, this is likely to be only a small part of the whole truth. In general, studying endosomal signalling is challenging as endocytosis, recycling and endosome maturation are extremely dynamic processes, different endosomes fuse with each other and the repertoire of associated proteins is constantly changing. Even more challenging is the study of signalling proteins that are activated by endosomal kinases, but are themselves only transiently in contact with endosomes. This seems to be the case for example with Erk1/2 (II, Fig.S4H). So far the main techniques available to study endosomal signalling consist of those used in this study, and although all these techniques are definitely useful and help us to broaden our knowledge about this intriguing topic, new methods are in need to facilitate research in this field. Indeed, with the current knowledge and tools, it is not possible to inhibit specifically the endosomal FAK signalling and thus define its specific impact in different cellular functions. Therefore, the identification of the exact mechanism of FAK

recruitment to endosomes will be important, as well as system biological approaches together with advanced high-resolution live cell imaging to fully understand the complicated and multilaterally regulated network of integrin traffic and signalling.

We demonstrated with different methods that integrin-induced FAK signalling occurs also on endosomes, but several important questions still remain. For instance, FAK has been shown to have FERM-dependent functions in the nucleus, but how FAK is translocated there is currently unknown. Interestingly, EGFR was recently shown to localise in nuclear envelope-associated endosomes, derived from early endosomes, which can deliver PM proteins to the nucleus by fusing with the nuclear envelope (Chaumet et al, 2015). Indeed, the ability of integrin-containing endosomes to function as transport wagons to take pFAK-Y397 to the nucleus would be a fascinating model, although just bringing pFAK-Y397 closer to the nucleus might be sufficient. Moreover, although we observed that FAK can associate with and become activated on isolated endosomes, some pFAK-Y397 might still co-endocytose with integrins from the PM. Whether integrin signalling is sustained and amplified in endosomes or whether a completely new signalling nexus is assembled on endosomes remains to be fully investigated. Other important outstanding questions include whether all integrin heterodimers are able to signal from endosomes, how other FAK phosphorylation sites contribute to endosomal signalling, and what signalling pathways and cellular processes are dependent on integrin endosomal signalling, other than suppression of anoikis. Finally, it remains to be elucidated how long endosomal integrin signalling is sustained, how and when it is terminated, and importantly, how these factors contribute to integrin recycling.

Resistance to anoikis and anchorage-independent growth are among the many hallmarks of cancer cells, and the endosomal FAK signalling is clearly playing a role in these processes. Several FAK inhibitors already exist and some of those are currently in clinical trials in Phase I/II. However, these drugs do not block the kinase-independent functions of FAK in the nucleus and FAK has multiple roles also in healthy cells. For these reasons, function or rather location-specific inhibition of FAK would be needed, and discovering the mechanism underlying the endosomal targeting of FAK could provide better options for future drug development.

6. CONCLUSIONS

Remarkably, already 10 years ago, a review from our group stated that "Integrin trafficking and signaling are probably therefore tightly coupled. Indeed, signaling might also occur in endocytic vesicles bearing integrins and be different from that at the classical adhesions." (Pellinen & Ivaska, 2006). Although this thesis work started years later and the statement was mainly forgotten, this insightful idea has now been confirmed.

The publications in this thesis work highlight the indispensable connection between integrin traffic and signalling, and demonstrate for the first time how integrin endocytosis critically regulates integrin signalling and, *vice versa*, how integrin signalling ultimately determines its trafficking fate (Figure 9).

In this thesis, we have developed a new antibody-based trafficking assay to study the connection between integrin traffic and activity in a comprehensive manner. With this method, we show that although both active and inactive β1-integrins share the same endocytic route, the recycling kinetics and pathways are distinct. The same assay can be used to investigate the trafficking of any antibody-detectable receptors and is therefore likely to benefit also research fields other than integrins.

Importantly, we show for the first time that integrin endocytosis is needed for full ECM-induced integrin signalling, and integrin-mediated endosomal FAK signalling contributes to cancer-related processes such as anchorage-independent survival and metastasis. Together these results open up new horizons in the field of integrin signalling and reveal integrin endocytosis as a novel mechanism hijacked by cancer cells to support their survival. In the future, unravelling the mechanism of endosomal FAK activation may uncover novel therapeutic targets in metastasising cancer cells.

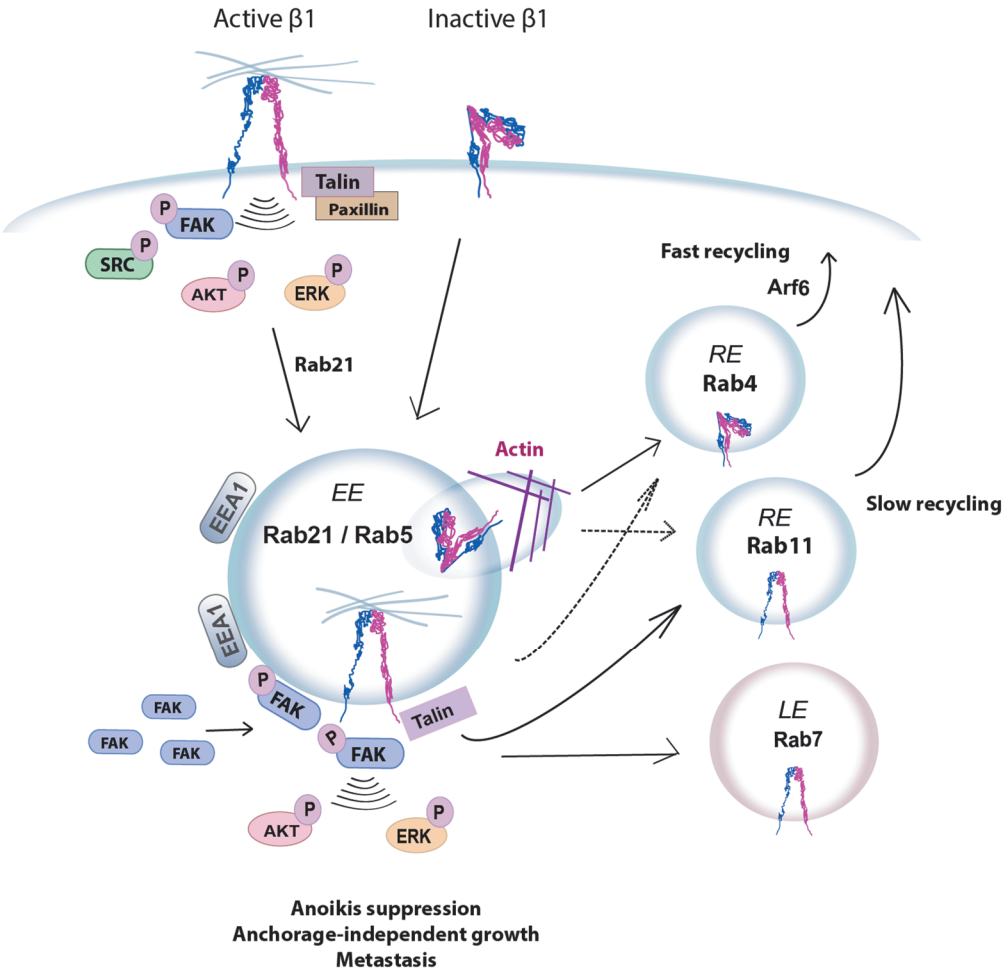

Figure 9. Summary of the results presented in this thesis. Both active and inactive β1 are endocytosed clathrin-dependently into Rab5/Rab21/EEA1-positive early endosomes, where active β1 together with its ligand remains actively signalling. The ECM-induced endosomal FAK signalling is critical for anoikis suppression and for anchorage-independent growth and metastasis of cancer cells. A major fraction of active β1 continues to Rab7-positive late endosomes, whereas the inactive β1 undergoes a rapid actin- and Rab4- dependent recycling to Arf6-positive protrusions at the PM.

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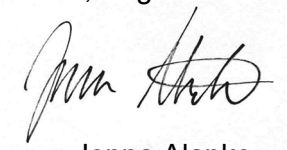

Jonna Alanko

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