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THE INTEGRIN TAIL: A TALE OF CELL MOTILITY AND DIVISION

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”Imagination is more important than knowledge”

-Albert Einstein

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The Integrin Tail: A Tale of Cell Motility and Division

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ABSTRACT

Integrin transmembrane receptor functions are regulated by adaptor molecules binding to their α and β subunit intracellular domains, or tails, thus affecting integrin traffic and adhesion during e.g. cell motility. Interestingly, many cellular proteins function in both cell motility and cell division, thus raising the possibility that integrins might be involved in regulating the cell cycle. A thorough understanding of cell division is essential in cell biology and in human malignancies. It is well established that failures to complete cell cycle can give rise to genetically unstable cells with tumorigenic properties. Transformed cells promote the disruption of intercellular adhesions such as tight junctions, and this correlates with the onset of cell motility, invasion and unfavorable prognosis in cancer.

In this study, we analyzed integrin regulation, mediated by adaptor binding to the α subunit tail, during cell motility and cell division. We revealed a novel molecular mechanism by which Rab21, through association with the integrin α subunits, drives integrin endosomal traffic during mitotic phases. In addition, we found indications for this finding in vivo, as *RAB21* gene deletions were mapped in ovarian and prostate cancer samples. Importantly, the multinucleated phenotype of cultured ovarian cancer cells could be reverted by Rab21 overexpression. In this thesis work, we also show how the tight junction protein ZO-1 unexpectedly interacts with the α5 integrin cytoplasmic domain in the lamellipodia to promote cell motility and at the cleavage furrow to support separation of the daughter cells. The α5-ZO-1 complex formation was dependent on PKCε, which regulates ZO-1 phosphorylation and its subcellular localization. In addition, by an *in situ* detection method, we showed that a subset of metastatic human lung cancers expressed the α5β1-ZO-1 complex.

Taken together, we were able to identify new molecular pathways that regulate integrin functions in an α tail-mediated fashion. These findings firmly suggest that genetic alterations in integrin traffic may lead to progression of tumorigenesis as a result of failed cell division. Also, the interplay of integrins and ZO-1 in forming spatially regulated adhesive structures broadens our view of crosstalk between pathways and distinct adhesive structures that can be involved in cancer cell biology.

Keywords: Integrin, cell adhesion, tight junction, ZO-1, Rab21, cell motility, cell division

Saara Tuomi
Integriinin häntä -välitteinen soluliike ja solunjakautuminen syövässä

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TIIVISTELMÄ

Integriinit ovat solukalvon tarttumisreseptoreita, joiden toimintaa solun liikkumisen aikana säädellään niiden α/β-rakenteiden solunsisäisiin osiin, ”häntiin”, sitoutuvien signalointiproteiinien avulla. Monet signalointiproteiinit aktivoituvat samankaltaisesti sekä solujen liikkumisen että jakautumisen aikana. On siis mahdollista, että solujen liikkumiselle tärkeiden integriinien aktivoituminen on tärkeää myös solusyklin säätelyssä. Solunjakautumismekanismien tarkka ymmärtäminen on tärkeää sekä solubiologisesti että sairauksien hoitamisen kannalta. Solusyklin säätelyn virheet voivat johtaa geeniperimän epätasapainoon ja sitä kautta pahanlaatuisiin solumuutoksiin ja syövän eston kannalta tärkeiden solujen välisten liitosten (kuten tiivisliitos) hajoamiseen. Tämä vuorostaan edistää hajottavaa, invasiivista solujen liikkumista elimistössä ja siten syövän ennusteen huononemista.

Väitöskirjatutkimuksessani olen selvittänyt integriinien α-rakenteiden häntiin sitoutuvien molekyylien välittämiä signaaleja, jotka aktivoituvat syöpäsoluissa. Tutkimuksissamme osoitamme, että α-häntiin sitoutuva, integriinien solunsisäistä kuljetusta säätelevä Rab21 vaikuttaa solunjakautumisen onnistumiseen. DNA-analyysi osoitti myös *RAB21*-geenin häviämisen tietyistä munasarja- ja eturauhassyövistä. Soluviljelyolosuhteissa munasarjasyövän solujakautuminen oli häiriintynyt, jonka Rab21 pystyi normalisoimaan. Tässä työssä osoitamme myös, miten tavallisesti soluja suojeleva tekijä, tiivisliitosproteiini ZO-1, sitoutuu integriinin α5 häntään PKCε-kinaasin välittämänä ja avustaa pahanlaatuista syöpäsolujen liikkumista ja jakautumista elimistössä. Löydöksissämme etäpesäkkeisiin kuolleiden keuhkosyöpäpotilaiden syöpäsolut olivat saattaneet hyödyntää tätä ZO-1-avusteista liikkumismekanismia.

Tässä väitöskirjatyössä tarkasteltiin aiemmin tuntemattomia signalointireittejä, jotka välittyvät integriinin α-häntään sitoutuvien säätelytekijöiden avulla. Löydöstemme mukaan geeniperimän muutokset, jotka estävät integriinien solunsisäistä kuljetusta, voivat johtaa epäonnistuneen solujaon vaikutuksesta solun pahanlaatuisiin muutoksiin. Työssä tarkasteltiin myös ZO-1- proteiinin sitoutumista integriinin α5 häntään, jolla huomattiin olevan vaikutusta syöpäsolujen liikkumiseen sekä jakautumiseen. Yhdessä nämä tulokset lisäävät tietoutta syövässä tapahtuvista monimutkaisista muutoksista.

Avainsanat: Integriini, soluadheesio, tiivisliitos, ZO-1, Rab21, soluliike, solunjakautuminen

TABLE OF CONTENTS

ABBREVIATIONS

2D, 3D	Two-dimensional, three-dimensional
ADAM	A-disintegrin and metalloproteinase
Arp2/3	Actin-related protein 2/3
ATP	Adenosine triphosphate
BIM	Bisindolylmaleimide
CDK	Cyclin-dependent kinase
Col	Collagen
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ERM	Ezrin-radixin-moesin
EV1	Human echovirus 1
FA	Focal adhesion
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FN	Fibronectin
G-actin	Globular actin
GAP	GTPase activating protein
GEF	Guanosine nucleotide exchange factor
GFR	Growth factor receptor
GTP	Guanosine triphosphate
ILK	Integrin-linked kinase
kDa	Kilodalton
LN	Laminin
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MIDAS	Metal-ion dependent adhesion site
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
N-WASP	Neuronal Wiskott–Aldrich syndrome protein
pAb	Polyclonal antibody
Par3/6	Partitioning-defective-3/6
PCR	Polymerase chain reaction
PDZ	Post-synaptic density 95; Disc large tumor suppressor; Zonula occludens 1

PI, PtdIns	Phosphoinositide, phosphatidylinositol
PKB	Protein kinase B
PKC	Protein kinase C
PTB	Phosphotyrosine-binding
PTEN	Phosphatase and tensin homologue
ROCK	Rho kinase
RT-PCR	Reverse transcription PCR
SFK	Src family kinase
siRNA	Small-interfering RNA
TIRF	Total internal reflection fluorescence
VASP	Vasodilator-stimulated phosphoprotein
VN	Vitronectin
WASP	Wiskott-Aldrich syndrome protein
ZO-1/2	Zonula occludens 1/2
ZONAB	ZO-1 regulated nucleic acid binding protein

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-III:

I Teijo Pellinen*, **Saara Tuomi***, Antti Arjonen, Maija Wolf, Henrik Edgren, Hannelore Meyer, Robert Grosse, Thomas Kitzing, Juha K. Rantala, Olli Kallioniemi, Reinhard Fässler, Marko Kallio & Johanna Ivaska (2008). Integrin traffic regulated by Rab21 is necessary for cytokinesis. *Developmental Cell* **15**, 371-385. * Equal contribution

II **Saara Tuomi**, Anja Mai, Jonna Nevo, Tiina J. Öhman, Vesa Vilkki, Jukka Laine, Carl G. Gahmberg, Peter J. Parker & Johanna Ivaska (2009). A PKCε regulated α5 integrin-ZO-1 complex controls a migration switch in cancer cells. *Science Signaling* **2** (77), ra32.

III **Saara Tuomi**, Ylva Ivarsson, Pascale Zimmermann and Johanna Ivaska (2011). The regulation of the tight junction protein ZO-1 in cell division of epithelial cancer cells. Manuscript.

In addition, some unpublished data are presented in this thesis.

1. INTRODUCTION

Since the initial discovery in the 1980's of a cell membrane traversing molecule capable of binding the extracellular matrix (ECM) protein fibronectin and the intracellular actin filaments, integrin research has taken a huge leap ahead. To date, 24 different integrin heterodimers, arranged selectively from 18 α and 8 β subunits, are known and they display tissue specificity, prefer certain ECM ligands over others and transduce varying signals in the cells. In the integrin, the isoform-specific features are mediated largely by the heterogeneous intracellular domains, tails, of α and β subunits and more specifically by the proteins that bind to them and regulate actin-dependent processes inside the cell.

The role of integrins in supporting cell motility is well known and essential in all multicellular organisms, not only in cell sheet migration in development, but also throughout organism life, in wound repair and in immune surveillance. Activation of the cell motility machinery involves the disruption of intercellular adhesions. Critical, however, to the motile events like wound healing is the attenuation of the signaling and motility after wound closure or when the immune cell reaches the inflammated tissue. In cancer, integrin regulation is often derailed. For instance, cancer cells emanating from the primary tumor can exploit the integrin molecular machineries and thus disseminate to distant organs to form metastatic lesions without the attenuative control of the process (Ribatti 2007).

It is now widely understood that the major driving force of motility is the establishment of new adhesion sites and extension of the leading edge at the front of the cell, cell body contraction, and detachment of adhesions at the cell rear. All these steps require the spatio-temporally regulated assembly and disassembly of the acto-myosin based cytoskeletal structures via several family proteins to generate productive net forward movement (Ridley et al. 2003). During cell division, or cytokinesis, cells undergo notable changes in cell shape driven by the acto-myosin contractility and microtubule network. Adhesive structures in adherent non-motile cells are disassembled during the onset of mitosis and adherent cells undergoing mitosis in suspension have been shown to fail cytokinesis. Although a molecular outline of the sequential events involving alterations in the microtubule cytoskeleton, the formation of an actomyosin contractile ring and membrane traffic are known, many aspects of cytokinesis remain poorly characterized (Glotzer 2005).

Despite the knowledge of adhesion-dependency in cell division and that many motility-associated pathways are identical in executing cytokinesis, the role of integrins in this process has not been properly examined. In this thesis work, integrin regulation in cell division and cell motility was addressed. This study focused on investigating the integrin α subunit-mediated regulation and resulted in identification of two molecular pathways important for the successful cytokinesis and organized cell motility.

2. REVIEW OF THE LITERATURE

2.1. CELL DIVISION AND CELL MOTILITY ARE TIGHTLY REGULATED

2.1.1. Cell division

In an adult organism, cells divide in a continuous fashion and approximately 60 billion cells are generated (and lost) daily by tightly regulated events. Before the cell can start the duplication into two progeny cells, the synthesis of two sets of identical chromosomes and overproduction of cytoplasmic building blocks need to occur during interphase. The cell then enters mitosis, during which the chromosomes are separated; finally, the separation of the cytoplasmic material and the daughter cells takes place in cell division. The steps preceding cell division in mitosis, the prophase, prometaphase and metaphase, are tightly controlled in sequential "check-points" to detect and repair any damage that has occurred inside the cell before progression into the next phase in the division process. During these mitotic steps, the chromosomes in the nucleus condensate and are transported to the center of the cell to form and align at the metaphase plate. By this time, the nuclear membrane disrupts and the whole cell rounds up thereby losing adhesion strength to the surrounding matrix. Signaling from the remaining adhesive structures at this point is crucial, since losing cell attachment entirely in adherent cells may lead to adhesion-regulated programmed cell death called anoikis (Frisch, Screaton 2001) or aberrant cell division (Aszodi et al. 2003).

The multiple check-points exist to maintain homeostasis in tissues, such as controlling chromosome cohesion and tension during mitotic steps. For example, if the daughter cell chromosomes are incorrectly attached to microtubule (MT)-connecting kinetochores at the metaphase plate, the spindle check-point (SC) gives a "wait anaphase" signal until all attachments have been made correctly (Rieder et al. 1994). A mitotic check-point and chromosome passenger complex (CPC) protein Aurora B kinase has a role in correcting these impaired connections (Lampson et al. 2004) and inhibition of the Aurora B complex leads to defects in cytokinesis. The "wait anaphase" signal is produced and persists in the cell to inhibit the anaphase-promoting complex (APC), a mitotic ubiquitin ligase that promotes sister chromatid separation and exit from the mitotic steps (Rieder et al. 1995).

Mammalian cell cytokinesis, the division of the cytoplasmic material, is nowadays recognized as a two-step process executed by two functionally and mechanistically separate events (Rieder et al. 1994). It starts with the constriction of the cleavage furrow in anaphase followed by the physical separation of the two daughter cells by abscission in telophase. Cytokinesis requires at least 20 well established proteins to execute a successful daughter cell separation (Glotzer 2005). These include proteins of the central spindle, vesicle secretory pathway, cytoskeletal components, motor proteins and midbody-severing agents. Many additional proteins belonging to for example the family of intercellular adhesion molecules (Kojima et al. 2001) or tumor suppressors

(Daniels et al. 2004) have been identified in the cytokinetic structures, but their functions are not understood or necessarily required for animal cell division [for review see (Glotzer 2005)]. One example is the tumor suppressor BRCA2 which, when inhibited, impairs the cytokinetic progression in mammalian cells (Daniels et al. 2004).

2.1.1.2. The constriction of the cleavage furrow in anaphase

To separate the duplicated chromosomes, the cell forms a microtubule-containing machine called the central spindle, where the microtubule plus ends overlap and many mitotic regulators, like Aurora B or Polo-like kinase 1 (Plk1), concentrate (Figure 1). Plk-1 is a serine-threonine kinase involved in the regulation of cytokinesis and mitotic exit (Carmena et al. 1998, Descombes, Nigg 1998). It activates the early phases of cytokinesis by promoting the recruitment of the small guanosine triphosphatase (GTPase) RhoA GTP exchange factor Ect2 to the central spindle in anaphase (Petronczki et al. 2007, Santamaria et al. 2007). The recruitment of Ect2 activates RhoA, or Rho1, at the cell cortex which largely regulates the formation and correct positioning of the contractile ring that assembles around the cell equator and constricts inwards in anaphase (Bement, Benink & von Dassow 2005).

The contractile ring is a meshwork of filamentous actin (F-actin) and motor protein myosin II, which facilitates the movements of the actin fibres by providing the force for furrow ingression from ATP hydrolysis (De Lozanne, Spudich 1987). The myosin II motor activity depends on the myosin regulatory light chain (MRLC) phosphorylation (Komatsu, Hosoya 1996, Yamakita, Yamashiro & Matsumura 1994) which is regulated by RhoA and its effector proteins like Rho-kinase ROCK, citron kinase or the formin mDia (Bement, Benink & von Dassow 2005, Eda et al. 2001, Kosako et al. 2000). The actin nucleation at the furrow is likewise triggered by RhoA (Piekny, Werner & Glotzer 2005). Upstream of RhoA, MgcRacGAP, a GTPase-activating protein for RhoA, is required for the assembly of scaffold protein anillin and localized activation of myosin via Ect2 recruitment and Rho-dependent phosphorylation of MRLC. Anillin is an actin-, myosin-, and septin- binding scaffold protein and a substrate of the APC (Zhao, Fang 2005). Anillin localizes to the cleavage furrow in a Rho-dependent way and due to its potential membrane binding PH domain, has been proposed to link the contractile ring to the plasma membrane during cytokinesis (Hickson, O'Farrell 2008). In Drosophila, anillin depletion impairs the intercellular bridge and midbody stability leading to multinuclear cells (Echard et al. 2004). Furthermore, in Hela cells, anillin depletion impairs the accumulation of RhoA and active myosin at the furrow which leads to late stage cytokinesis defects (Zhao, Fang 2005). However, the data seems to be rather contradictory as to what is the temporal activity status of RhoA during cytokinesis. According to Yoshizaki and co-workers it seems to be the inactivation of RhoA, rather than activation of RhoA, that is important for cytokinesis (Yoshizaki et al. 2003, Yoshizaki et al. 2004).

The elongation of the MTs at the spindle is necessary for the separation, since severing the MT filaments in telophase prevents daughter cell separation and renders the separated nuclei to fall back together due to defects in actin-driven constriction (Straight et al. 2003). This and work by others suggests that the spindle MTs are

needed to keep the daughter cell nuclei separated until the constriction by actin-driven force is finished and also that these filamentous networks are interconnected (Gatti, Giansanti & Bonaccorsi 2000, Cimini et al. 1998).

2.1.1.3. Abscission in telophase: the midbody

During and after actomyosin constriction between the daughter cells, dynamic vesicle transport at the midzone occurs (Figure 1). The transport of endosomal vesicles via e.g. the GTPase Rab11-FIP3 complex is important for growing new plasma membrane at the furrow to complete the separation in abscission (Wilson et al. 2005). Since actin and its accessory proteins in the constriction site link to the inner plasma membrane (Wang 1985), they cannot seal the remaining gap in the intercellular bridge. Central to the abscission is the structurally rigid and invariantly sized midbody (sometimes called "the Flemming body" after its discoverer), which is mostly composed of disruption-resistant alpha and beta tubulins (Mullins, McIntosh 1982) along with several minor constituents, like CHO1, a mammalian kinesin-like motor protein of the MKLP1 subfamily. The CHO1 associates with the spindle midzone during anaphase and concentrates to a midbody matrix during cytokinesis. Inhibiting the function of CHO1 affected the formation of midbody matrix in dividing cells, caused the disorganization of midzone microtubules, and resulted in abortive cytokinesis implicating the functional role of midbody dynamics in telophase (Matuliene, Kuriyama 2002).

The midbody is the target site for membrane delivery and membrane fusion, the driving forces of abscission. In particular the unique midbody ring, located at the midpoint of the intercellular bridge, seems to be the key structural element that guides the events that lead to abscission (Pohl, Jentsch 2008). Membrane fusion proteins, like members of the SNARE family, are implicated in these processes as well as cytoplasmic Rab or Arf GTPase transporters and proteins of the vesicle secretion, the exocyst, complex (Low et al. 2003, Skop et al. 2001, Murthy, Schwarz 2004). Also the Rab11 effector FIP3 is needed in midbody abscission, as it binds to MgcRacGAP, which displaces Ect2 binding, and allows the disassembly of the contractile ring. Intriguingly, brefeldin A, which inhibits vesicle secretion by inhibiting the activation of the G-protein Arf, specifically inhibited the terminal stage of cytokinesis in *C. elegans* (Skop et al. 2001). Following closure of the intercellular bridge, the midbody disassembles in telophase and survives in one daughter cell often until the next division. Thus, in addition to its structural function, the midbody matrix may have an active role in the completion of cytoplasmic division by facilitating membrane fusion events. Yet a major gap remains in the scientific knowledge concerning this structure.

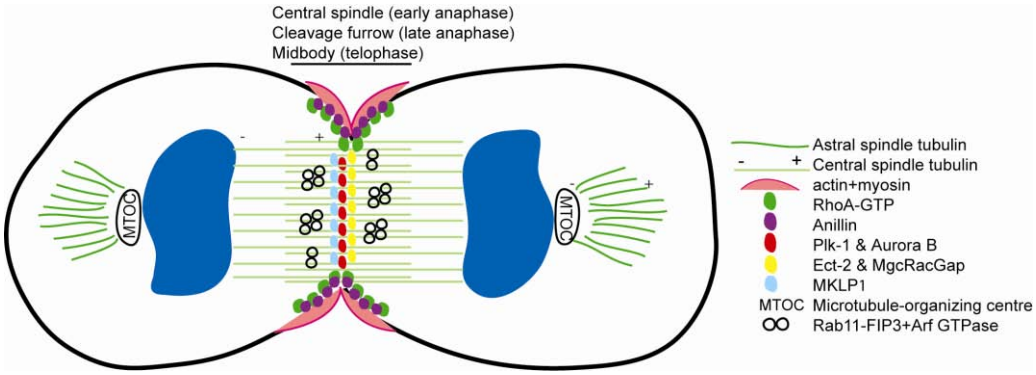

Figure 1. **Mammalian cell cytokinesis**. In early anaphase, microtubules at the central spindle elongate and transport the chromosomes (blue) to the nascent daughter cells. Central spindle proteins, like Plk-1, AurB, Ect2, MgcRacGAP concentrate at the spindle, where Plk-1 and MgcRacGAP are involved in recruiting Ect2 to the spindle. This in turn activates the GTPase RhoA at the cortex and decides the position of the acto-myosin contractile ring during furrowing of the cells. MgcRacGAP recruits the actin- and myosin- binding protein anillin to the midzone, which modulates the RhoA activity and myosin activation at the furrow. MKLP1 associates with the central spindle in anaphase and affects the stability of the midbody in telophase. Rab11 and its interacting protein FIP3 deliver membrane to the constriction site; FIP3 is also needed in the midbody abscission by binding to MgcRacGAP, which displaces Ect2 from MgcRacGAP thus allowing the contractile ring to disassemble.

2.1.2. Cell motility

During development, immunology or in pathological conditions like cancer, cell motility is essential in mediating the physiological response. These are the migration of cell cohorts in embryogenesis, immune cell penetration to the inflammated tissue or in cancer, the colonization of the secondary site to form metastatic lesions (Ridley et al. 2003, Friedl, Weigelin 2008). Cell motility is not always a function of only one cell, even though single cell migration is the best studied mode of cell motility. In fact, the co-migration of multiple cells in a cell sheet (collective migration) has been witnessed in xenograft mouse studies exploiting high-resolution multi-photon imaging techniques (Andresen et al. 2009). In this mode of motility, which varies to some extent e.g. during vascular sprouting, mammary gland morphogenesis or cancer cell invasion, the cells on the sheet edge lead the way slightly detached from the cells in the back and express motility-promoting proteins whereas the cells in the middle are connected via intercellular contacts and maintain the mass of cells by frequent cell division (Friedl, Gilmour 2009). These motile events require extensive re-shaping of the cells and modulation of adhesion structure turnover or strength in order to mold accordingly to the changing microenvironment.

2.1.2.1. Cell adhesion modulation

The modulation of the strength is especially crucial for some cell types due to their biological nature: immune cells that circulate in the bloodstream are virtually nonadherent until they detect an inflammatory tissue, upon which the cell adhesion machinery activates in subseconds to facilitate rapid adhesion to the tissue receptors (Grabovsky et al. 2000). In leukocytes, adhesion to endothelial cell surface receptors,

mediated by the β2 integrins, is essential for inflammatory response, as in the genetic disease LAD-1 (leukocyte adhesion deficiency-1) the integrin isoform is abnormally expressed causing severe bacterial infections (Bunting et al. 2002). Intermediate adhesion is the optimal for cell motility, since too strong adhesion prevents dynamic movement and disruption of adhesive structures, whereas too weak adhesion impairs motility and increases chances of anoikis, detachment induced cell death (Murphy-Ullrich 2001) (Figure 2). The strength of the adhesion is determined by the activity of hundreds of adaptor proteins and kinases that convey the message from the cytoskeletal structures containing actin, microtubules and intermediate filaments (Ridley et al. 2003).

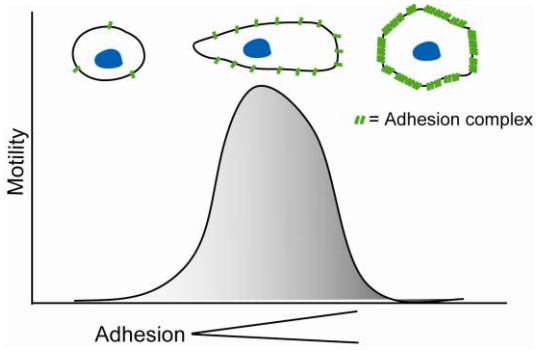

Figure 2. Adhesion strength correlates with motility. Moderate expression of adhesion complexes (green) on the cell surface favors optimal cell motility, as the balance between adhesion disassembly and the formation of new adhesions remains. Weak adhesion prevents motility and can lead to anoikis, whereas too strong adhesion disfavors dynamic turnover and is in fact detected mostly in non-motile cells.

During cellular reshaping, the actin cytoskeleton is thought to account for the major force, and one family of proteins is intimately related in regulating cell adhesion in shape changes, namely the Rho GTPases Rac, Cdc42 and Rho (Raftopoulou, Hall 2004). These proteins exist in GTP-bound active and GDP-bound inactive forms which can alternate in response to cellular demand. At the leading edge of the cell, actin is organized in parallel bundles in spike-like filopodia i.e. the cells sensors (regulated by Cdc42) and in a dense meshwork that forms ruffling lamellipodia. Rac generates a protrusive force through the localized polymerization of actin and initiative stimulation of integrin cell adhesion complexes (Raftopoulou, Hall 2004). In the cell body and at the cell trailing edge, Rho promotes acto-myosin contraction and deadhesion driven by filamentous actin stress fibers (Figure 3).

Other cytoskeletal constituents, like microtubules, contribute positively to cell adhesion complex turnover and motility in most cell types (Yoon, Shin & Mercurio 2005). However, unlike actin, microtubules can also inhibit cell protrusive activity in some cell types. Indeed, neutrophil motility is even increased in the absence of microtubules (Keller, Naef & Zimmermann 1984) whereas neuronal cells extend primarily via microtubulus-driven mobility (Etienne-Manneville 2004). Intermediate filaments (IFs) are a more diverse group of cytoskeletal components that participate in morphological reshaping and active mobilization of various types of cells (Ivaska et al.

2005, Hesse, Magin & Weber 2001). The best studied examples include the keratins in the keratinocytes and vimentin in mesenchymal and in malignant cells. One subtype of IFs, namely the lamins, locate in the nucleus to provide nucleoskeletal support (Helfand, Chang & Goldman 2004). The cytoskeletal components mediate signals from the plasma membrane adhesions to a variety of intracellular signalling molecules implicated in cell motility, like mitogen-activated protein kinase (MAPK) cascades, lipid kinases, phospholipases, Ser/Thr and Tyr kinases and scaffold proteins (Wiesner, Legate & Fassler 2005) (Figure 3).

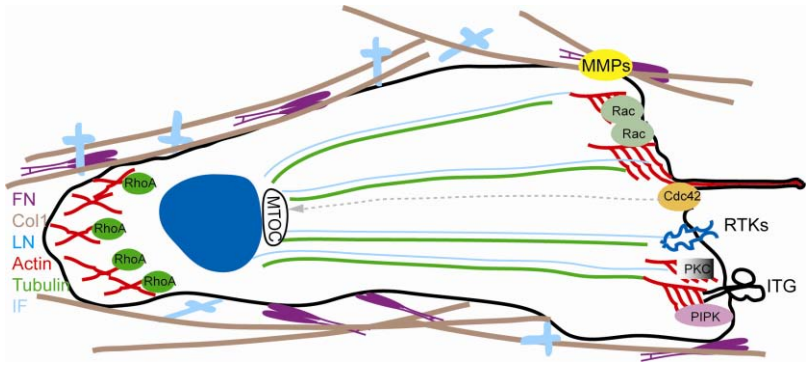

Figure 3. **Molecular events in motile cells**. During cell motility, Rac GTPase induces actin branching at the lamellipodia and stimulates integrin adhesion; Cdc42 triggers the formation of long actin-rich filopodia needed in matrix sensing and regulates the position of the MTOC, which is perpendicular to direction of migration. RhoA drives acto-myosin contractility at the trailing edge and detaches adhesions. Tubulin and intermediate filaments (IF) like vimentin promote cell motility by facilitating the transport of e.g. integrins to the plasma membrane. PIPK lipid kinases produce phospholipids at the leading edge membrane and for instance affect integrin-mediated adhesion during motility, and RTKs favor this process by responding to growth factors like HGF to dismantle intercellular adhesions and triggering cell scattering. MMPs degrade the ECM components fibronectin (FN), laminin (LN) and collagen (Col) during cell invasion through the matrix.

2.1.3. Molecular regulation of the cell cytoskeleton during cell division and cell motility

Cytoskeletal shape changes as well as targeted traffic and compartmentalization of cellular proteins, lipids and ions is needed to execute both cell division and motility in a regulated manner (Glotzer 2005). Polymerization of actin is a necessity for the animal and ameoboid life cycle: essentially all eukaryotes carry genes for actin, and nearly for all the genes for myosin motor proteins, which mediate force on the actin filaments (Adelstein, Conti 1975, Richards, Cavalier-Smith 2005). Being highly expressed in various cells, actin represents one of the most abundant proteins on earth. Therefore it is not a big surprise that actin is essential for the survival of most cells like are its regulatory proteins, that ultimately define the phenotype of the cytoskeletal structures. For example, actin filaments in the contractile ring often appear in parallel bundles, as opposed to the dendritic meshwork observed in the leading edge of a migrating cell. On the other hand, spatio-temporal signaling e.g. of small GTPase RhoA during telophase or cell invasion is essential for the contraction force needed during these events.

2.1.3.1. The acto-myosin force generation

The mechanism used by myosin to generate force and motility from adenosine triphosphate (ATP) hydrolysis was initially discovered in muscle cells (Geeves, Holmes 2005). It took two decades from the initial discovery, that actin and myosin were found to be expressed in other cell types as well (Hatano, Oosawa 1966, Adelman, Taylor 1969). Actin monomers (or G-actin, from Globular actin) line into long stable filaments with a helical arrangement spontaneously under physiological conditions (Pollard 2007). Since small actin oligomers are very unstable, polymerization starts off rather slowly but soon speeds up and forms a polar filament where all the subunits arrange into same direction and grow faster in the other end in a polarized fashion. In the growing end, actin binds ATP and at some point after filament assembly, it hydrolyzes the terminal phosphate and releases it. During cell division, the mechanochemical activity of myosin is latent until it interacts with actin filaments in the contractile ring, which then allows pinching off the two daughter cells in telophase (Straight et al. 2003). The RhoA-dependent myosin motor activity becomes important also in rear-end retraction in two-dimensional motility and in cell contraction during invasion in three-dimensional matrix (Raftopoulou, Hall 2004, Hooper, Gaggioli & Sahai 2010).

2.1.3.2. The actin regulatory proteins

Eukaryotic cells produce at least 100 actin regulatory proteins, the functions of which control actin polymerization, actin capping, severing, crosslinking and turnover [for review see (Pollard, Cooper 2009)]. The accessory proteins start up a new filament by forming it on the side of the existing filament, severing an existing one to make two new branches or growing it from actin monomers. The actin regulatory mechanisms are strikingly similar between amoebas, fungi and animals; most of the genes existed already 1 billion years ago before the different branches diverged on the phylogenetic tree (Richards, Cavalier-Smith 2005).

The organization of the filaments can be ascribed, at least in part, to the different nucleating complexes that generate these structures. The actin nucleating ARP2/3 (actin-related protein 2/3) complex, which is stimulated by WASP (Wiskott–Aldrich syndrome protein) family members, generates branched filaments and is critical for cell motility but not for the assembly of the contractile ring (Severson, Baillie & Bowerman 2002). Rather, formins, the second group of actin nucleating proteins, function on unbranched filaments and are essential for filopodia and contractile ring assembly in animal cells (DeWard, Alberts 2009). Formins cap the barbed end of the actin filament but allow filament growth (Kovar et al. 2003, Sagot, Klee & Pellman 2002). Together with profilin, the central region of formin induces ATP hydrolysis by actin and uses the released free energy to favor processive growth of actin filaments (Romero et al. 2004). In vivo, however, formins are autoinhibited because of intramolecular binding of the N and C termini. Active RhoA binds the N terminus and relieves this autoinhibition (Alberts 2001). Thus, spatially restricted RhoA activation could induce local activation of myosin activity and actin filament assembly. A protein called Spire represents the third class of actin nucleators that was discovered fairly recently in Drosophila and shown to induce the elongation of unbranched filaments (Quinlan et al.

2005). Interestingly, JMY, a p53 cofactor usually located in the nucleus, increased cell migration by combining Arp2/3-mediated actin branching and spire-mediated elongation of unbranched actin in neutrophil-like cells (Zuchero et al. 2009).

The filamentous actin bundles connecting the daughter cells need to be severed at the late stages of cytokinesis by actin-severing factors like gelsolin or cofilin/ADF (actin depolymerizing factor): in cofilin mutants the contractile ring contains an overabundance of actin filaments at late stages of cytokinesis (Maciver, Hussey 2002).

2.1.3.3. Regulation of the actin cytoskeleton by lipids

In actin-related motility and shape changes, membrane structures need to lead the way during the dynamic changes while connecting to the cytoskeleton via adaptor proteins. Membrane-bound lipids, like phosphatidyl inositol phosphates (PIPs) PI(4,5)P2 or the PI3K- synthesized PI(3,4,5)P3 are essential in regulating actin-dependent processes and they can re-structure the actin cytoskeleton in several ways. PI(4,5)P2 is generated by the activity of type I or type II phosphatidyl inositol phosphate kinase (PIPKI and PIPKII) isoforms α, β and γ, which utilize PI(4)P (phosphatidylinositol 4-phosphate) or PI(5)P (phosphatidylinositol 5-phosphate), respectively, as their substrates. Current evidence indicates that these kinases function and are regulated in an isoform-specific way (Emoto et al. 2005). PIPKIs are found at the plasma membrane and due to the localization, thought to account for the majority of PI(4,5)P2 synthesis; PIPKIIs are mainly localized to intracellular sites (Doughman, Firestone & Anderson 2003). Localized phospholipid, or PIP2, synthesis is also required for formation of apico-basal polarity in cyst structures by recruiting polarity proteins to apical surfaces (Martin-Belmonte et al. 2007).

2.1.3.3.1. PI(4,5)P2 in cell motility

The exact roles of PI(4,5)P2 during cell motility or cell division are not clear, even though its contribution to actin turnover in these processes is established. In black and white, high levels of PI(4,5)P2 are associated with actin polymerization, whereas low levels block assembly or promote actin severing activity. PI(4,5)P2 facilitates actin polymerization by 1) activating neuronal WASP -and Arp2/3 -mediated actin branching, 2) binding and impairing the activity of actin-severing proteins, such as gelsolin and cofilin/ADF and 3) uncapping actin filaments for the addition of new actin monomers (Sechi, Wehland 2000, Yin, Janmey 2003).

One of the reasons that make the exact lipid research difficult is the high abundance of PI(4,5)P2 on the membrane. Unstimulated neutrophils, for example, are decorated with 5 mM concentration of PI(4,5)P2 on the inner leaflet plasma membrane (Stephens, Hughes & Irvine 1991). These concentrations make it hard to assume that PI(4,5)P2 would use highly specific spatial control on the membrane. It is speculated by some researchers that due to its localization and actin-binding properties PI(4,5)P2 would act as a spatial organizer of actin branches in the growing lamellipodium, where the angle between the branches is thought to be 70° to attain optimal motility (Insall, Weiner 2001). In addition, PI(4,5)P2 induces a conformational change in actin-binding proteins such as vinculin, talin and ERM family proteins thereby anchoring the actin

cytoskeleton to the plasma membrane and affecting actin-driven motility (Sechi, Wehland 2000). One mechanism by which PI(4,5)P2 does this could be the co-transportation of PIPKIγ with talin to matrix adhesions where the kinase triggers local PI(4,5)P2 production (Ling et al. 2002).

2.1.3.3.2. PI(4,5)P2 in cell division

The regulation of PI(4,5)P2 is necessary for cleavage furrow ingression during cytokinesis (Janetopoulos, Devreotes 2006). The exact mechanism still remains unknown and current data are contradictory. Two rather recent studies demonstrate that PI(4,5)P2 increases by *de novo* synthesis at the cleavage furrow of mammalian cells transfected with PI(4,5)P2 -specific GFP-labelled PH domain from phospho-lipase C-delta (PLCδ) (Emoto et al. 2005, Field et al. 2005). Emoto and others also found that PIPKIβ, but not PIPKIγ, concentrated in the cleavage furrow of CHO (Chinese Hamster Ovary) cells and overexpression of a kinase-dead mutant of this isoform lead to multinucleated cell population. In addition, antibodies against PI(4,5)P2 as well as overexpression of PI(4,5)P2 -specific PH domain or synaptojanin (which degrades PI(4,5)P2), generated multinuclear cell population (Field et al. 2005). In these overexpression studies some cell types seemed to dissociate the F-actin from the plasma membrane without changing the total amount of actin, suggesting that one of the primary roles of PI(4,5)P2 is to promote cytoskeleton–membrane anchoring at the furrow (Logan, Mandato 2006). Any knowledge about possible linker proteins mediating this is however non-existing. In contrast, some studies (Brill et al. 2000) imply that it is the hydrolysis of $PI(4,5)P_2$ to its degradation products IP3 (inositol 1,4,5-triphosphate) and DAG (diacylglycerol) that is crucial for completion of cytokinesis, rather than activation of PI(4,5)P2. Phospholipids are involved also in regulating the spindle orientation during anchorage-dependent cell division. By silencing PTEN (a phosphatase that generates PIP2 from PIP3) or by depleting β1 integrin expression, PI3K activity was inhibited (and thus synthesis of PIP3), which resulted in failed localization of PIP3 at the cell cortex and compromised proper spindle orientation in metaphase (Toyoshima et al 2007).

2.1.3.4. Regulation by protein kinase C (PKC) isoforms

The PKC family of serine/threonine kinases affect cell motility, cell division, cell proliferation, growth or malignant transformation in an isoform-specific manner (Griner, Kazanietz 2007). The PKCs are divided into three functional subgroups according to their structure and modes of activation (Figure 4). The classical PKCs (α,β1,β2,γ) are activated by DAG or TPA/PMA (Tetradecanoylphorbol acetate/Phorbol myristate acetate), and calcium (Ca2+) binding. The novel PKCs (δ,ε,θ,η), however, do not require calcium for activation but solely depend on phospholipid binding. The atypical PKCs (ι,λ,ζ) , on the other hand, are triggered by ceramide and PIP3 binding.

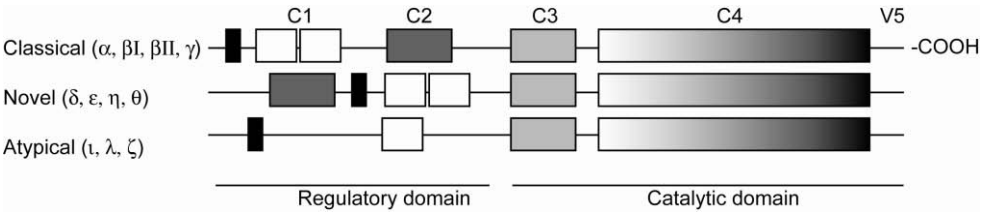

Figure 4. **Structure of the protein kinase C, PKC**. The classical PKCs are activated by C2 domain binding to calcium with concomitant C1 domain binding to DAG and phorbol esters. This alters the conformation of the kinase thus allowing substrate binding. The novel isoforms are incapable to bind calcium because of non-functinal C2 domain, but respond to DAG with higher affinity. The atypical PKCs lack the C2 domain altogether and have a non-conserved C1 domain, and thus are not activated by either calcium or DAG, but instead by ceramide and PIP3 binding to the C1 domain. The V5 region in the COOH-terminus is important in regulating the kinase activity of the PKCs.

The PKCs mature by the action of phosphoinositide-dependent kinase-1 (PDK1) phosphorylation in the activation loop (Le Good et al. 1998). This reveals the critical sites in the conserved COOH- terminal kinase domain (comprising of C3 and C4 domains where the ATP and substrate binding occurs), which leads to the autophosphorylation and stabilization of the kinase. The mature PKCs are recruited to the plasma membrane, where the response to stimulus differs between classical and novel PKCs. Because of the calcium-binding domain C2, the classical isoforms get rapidly recruited to the membrane, where DAG binds to C1 region. This induces massive reorganization in the kinase by releasing the pseudosubstrate domain and allowing substrate binding. The novel PKCs, which do not bind calcium, are targeted to the membrane less efficiently. However, since they bind DAG with higher affinity than the classical PKCs the signal transduction is being compensated over time [reviewed in (Steinberg 2008)].

The novel isoform PKCε is an oncogenic, actin nucleating protein that coordinately regulates changes in cell growth and shape. Cells constitutively expressing PKCε spontaneously acquire a polarized morphology and extend long cellular membrane protrusions. In the regulatory domain of PKCε, an actin binding motif induces the formation of invadopodial-like structures and metastasis of tumors grown in nude mice; removing this motif caused a dramatic reversion of tumor invasion (Tachado et al. 2002). PKCε has been demonstrated to be important in late phases of cell division (Saurin et al. 2008). The assembly of PKCε complex and its binding to the N-terminal alpha helix of 14-3-3 scaffold protein was crucial for the completion of cytokinesis. By inhibiting PKCε expression, the late stages of cell division were arrested, which associated with a delay in actomyosin ring dissociation and persistent RhoA activation at the abnormally positioned midbody (Saurin et al. 2008).

2.1.3.5. The interregulation of the microtubule and actin networks

Several reports from recent years imply that actin and microtubule networks are connected via regulation and act to rearrange and relocate the nuclear content during cell division and cellular or nuclear motility (Etienne-Manneville 2004, Zhen et al. 2002, Starr, Han 2003). In the anaphase of dividing cells the MTs form parallel bundles in between the daughter cells which interact with the cortical actin ring: if

either of these structures is disrupted, it prevents the proper organization of the other structure (Gatti, Giansanti & Bonaccorsi 2000) leading to defects in the late stages of cell division. This suggests strong co-operative interaction between these two structures during cytokinesis.

The interrelationship between tubulin and actin during cell motility can be appreciated in adhesion structure disassembly necessary for the organized movement of cells. Ezratty, Partridge and Gundersen (2005) showed that actin-rich adhesion disassembly and optimal motility involves microtubules and vesicle fission GTPase dynamin. Along the leading edge lamellipodium, which is devoid of any MT network in most cell types (Etienne-Manneville, Hall 2001), filamentous F-actin is nucleated at the inner plasma membrane (Wang 1985, Forscher, Smith 1988, Symons, Mitchison 1991). After actin nucleation, F-actin is transported rearward, away from the lamellipodium (Wang 1985). This retrograde actin flow in the leading edge induces rearward nuclear movement by pushing the nucleus or physically linking to the nuclear matrix (Gomes, Jani & Gundersen 2005, Zhen et al. 2002) and dictates the microtubule organizing centre (MTOC) positioning. The MTOC is known to adjust perpendicular to the direction of migration in a Cdc42-dependent manner (Etienne-Manneville, Hall 2001). Furthermore, nuclear positioning and migration are vital for normal mitotic and meiotic cell division, for the migration of pronuclei during fertilization, for normal brain development and during tumor cell invasion (Morris 2000) and the existing literature suggests that the two filament groups act to rearrange and relocate the nuclear content during these events.

2.1.4. Transformation arises out of multiple deregulated events

The cell needs to acquire at least 4-6 mutations to allow malignant transformation (Hahn, Weinberg 2002). The hallmarks of cancer (Hanahan, Weinberg 2000) entail the set of requirements that are to be met in a cell to allow malignant transformation and will be briefly discussed in the next chapter. In short, these acquired capabilities include self-sufficiency in growth signals, resistance to anti-growth signals and apoptosis, limitless replication potential, ability to form new blood vessels (angiogenesis) and sustained invasive potential. Despite the fact that the comprehensive view of tumorigenesis is far from being understood, the list of requirements has been extended and nowadays many of the individual steps in cancer progression are well understood.

2.1.4.1. The hallmarks of cancer

Genetic alterations and genomic instability characterize cancer development, but it is becoming increasingly clear that abnormalities in the epigenetic signature, known as the cellular memory that convey heritable information, affect the regulation of genes essential in neoplastic initiation and malignant progression (Feinberg, Ohlsson & Henikoff 2006). One of the most recognized forms of epigenetic regulation is the global methylation of bulk DNA and hypermethylation at promoter regions. Methylated DNA represents the transcriptionally repressed and the most abundant modified DNA in the cell. In cancer, many important genes for e.g. controlling cell-cycle progression, tissue development and tumor suppression are randomly methylated which prevents active gene transcription (Guo et al. 2008). Limitless replicative potential defines cancer cells,

since ordinary human cells can double only for 60-70 times before entering cell senescence or cell death. One of the most common explanations for this potential is the telomere maintenance by upregulating the enzyme telomerase that adds repeating units to the telomere DNA. In normal cells, the repeating units run out after fixed doubling times thus limiting the replication (Shay, Bacchetti 1997).

The mutations that affect the cells in e.g. the skin epithelium after UV radiation or in the gut epithelium after chemical or viral exposure, the body clears away by regulated cell death, apoptosis. Cancer cells have learned to evade the apoptosis machinery, which is one of the key characteristics of hyperproliferative cancer cells. For example, in a process called integrin-mediated death (IMD), unligated integrin on adherent cells recruit and activate caspase 8 resulting in apoptotic cell death (Stupack et al. 2001). In some cases, tumor cells get rid of caspase 8 expression to prevent cell death by IMD (Stupack et al. 2006). In addition, cancer cells can themselves over-produce anti-apoptotic factors, like Bcl-2 from the Bax family, to promote cell survival (Zhang et al. 1995). Figure 5.

Angiogenesis, the formation of new blood vessels, occurs during development and growth and during wound healing in adult tissues. During various malignancies, such as ischaemia, cardiovascular diseases and cancer, the regulation of angiogenesis is often altered to the disfavour of the patient (Carmeliet 2003). When tumors grow, they exploit the nutrients and oxygen of the neighbouring tissue until they reach a size of approximately 2 mm ∅ and need bigger supply. They start to induce the formation of their own vasculature by secretion of cytokines and growth factors necessary for vessel formation (Ribatti 2007) (Figure 5). Tumor vessels are structurally and biologically different from normal vessels, however: the leaky and fragile network allows easier intravasation of tumor cells, weakened blood flow and impaired drug delivery making them difficult therapeutical targets as well.

2.1.4.2. Control of cell division in cancer

During cell division, errors e.g. in chromosome delivery that compromise daughter cell integrity are detected in various check-point controls and if unrepairable, lead to programmed cell death to avoid unstable cells in the population. In cancer, the control mechanisms of cell division are compromised. Mutations in tumor-suppressor check-point proteins like *p53* or *Rb* (*Retinoblastoma protein*), cyclin D1 and $p16^{INK4a}$ are frequent in many cancers (Bachelder et al. 1999, Niehans et al. 1999) and cause defects in nuclear or cell division, like partial loss of spindle check-point (SC) function, improper chromosome attachment to kinetochores, cohesion defects between sister chromatids or imperfect separation of the daughter cells in telophase. These can all lead to division defects and ultimately to the generation of aneuploidy, deviation from the normal euploid chromosome number in a cell or in a cell population, which is a hallmark of tumorigenesis (Kops, Weaver & Cleveland 2005).

2.1.4.2.1. Aneuploidy

Solid tumors can be highly aneuploid, and in some tumors the aneuploid karyotype remains stable over time since the cells segregate their unusually multiple chromosomes fairly evenly in sequential cell divisions (Storchova, Pellman 2004).

Many of the aneuploid tumors, however, display high rates of chromosomal missegregation and chromosomal instability (CIN), making the tumor highly susceptible to phenotypic changes. The elevated level of chromosomal missegregations offer the aneuploid tumor cells an advantage to evolve in changing conditions. The disseminated cell population can have changed growth properties than their equivalent primary tumor (Kuukasjarvi et al. 1997) which poses a therapeutic challenge in cancer treatment. How tumor cells acquire and maintain the extra chromosomes during cell division is by far a mystery.

2.1.4.3. Epithelial-to mesenchymal transition

The regulated architecture of tissues and the epithelial layers with apical surfaces facing the lumen is critical for the normal organ function and homeostasis of the organism microenvironment. In an organized epithelium, such as the colon, all cells show apico-basal polarity. Epithelial-to-mesenchymal transition (EMT) and the reverse process, mesenchymal-to-epithelial transition (MET), are fundamentally important in a developing embryo or during wound healing (Hay 1995, Perez-Pomares, Munoz-Chapuli et al. 2002). Characteristic of EMT is the disruption of intercellular adhesions and activation of cell motility, which enables the dispersal of cells to the adjacent and distant tissues in developmental cell migration, for instance. During embryogenesis, several rounds of EMT and MET take place as several organs develop via various transient epithelial structures. For instance, the mesoderm needs EMTs for developing and differentiating into multiple cell types, and later in the development the mesoderm forms epithelial organs such as the kidney and ovary, via the action of METs (Mani et al. 2008).

Turning an epithelial cell into a mesenchymal cell requires alterations in morphology, cellular architecture, adhesion, and migration capacity. During developmental EMT, transcription of specific genes are turned on and turned off to mediate the migration of the mesodermal cells (Mani et al. 2008). Even though the complex process still remains elusive, many molecules, including transcription factors Twist, Snail and Slug, growth factors like the transforming growth factor-β (TGF-β), receptor tyrosine kinases like Met, Ras and other small GTPases, Src, vimentin, N-cadherin, fibronectin, β-catenin and integrins are known to influence the onset and progression of EMT (Figure 5). In fact, these molecules function primarily, either directly or indirectly, to downregulate the major epithelial regulator and marker E-cadherin (Guarino, Rubino & Ballabio 2007). Transformed cells have acquired the ability to harness the activation of the mesenchymal molecules and thereby invade the local tissue. Although there is an ongoing debate over whether cancer progression is truly an EMT process like during development, many of the molecular events are at least highly comparable (Lee et al. 2006).

2.1.4.3.1. Disruption of epithelial intercellular adhesions in cancer

The intercellular adhesions in between the epithelial cells control tissue integrity; down-regulation of the stronger E-cadherin adhesions and the concomitant production of weakly adhering N-cadherin at intercellular adherens junctions (AJs) loosens the epithelium and is the key switch in the EMT (Figure 5). The decreased E-cadherin expression correlates with the worse clinical outcome of cancer patients (Bringuier et al. 1993). In addition, E-

cadherin upregulation was shown with photoactivatable E-cadherin *in vivo* in mice tumors after treatment with the Src inhibitor dasatinib (Serrels et al. 2009).

In many diffuse, lobular tumors like the breast cancer or some carcinoma types of the gastrointestinal tract, the somatic loss of E-cadherin covers the whole tumor mass and correlates with the invasive, EMT gene expression pattern. Frequently, in solid tumors the loss of E-cadherin can be detected on the stroma-facing side of the tumor where individual cells invade the local tissue and colonize a secondary site. Interestingly, the colonization at this site can induce re-epithelialization and re-expression of E-cadherin as detected in many tumor types (Guarino, Rubino & Ballabio 2007). In addition, cell attachment to stromal fibronectin during branching morphogenesis of salivary glands induces local loss of E-cadherin in contacting epithelial cells, representing yet another mode of E-cadherin down-regulation (Sakai et al. 2003).

The mechanisms of E-cadherin down-regulation correlate largely with the tumor type: the promoter region of E-cadherin can be hypermethylated even in precancerous lesions of certain malignancies, whereas in diffuse infiltrating cancers the mutations are found in the genes of E-cadherin or α/β-catenins, the actin linker proteins of E-cadherin. At the invasive front of some solid tumors, E-cadherin machinery is shut down via β-catenin phosphorylation (Hirohashi, Kanai 2003). Several transcription factors repress E-cadherin (Slug, Snail, E12/E47, ZEB-1 and SIP-1) by direct binding to the proximal E-boxes of the E-cadherin promoter (Cano et al. 2000, Bolos et al. 2003, Perez-Moreno et al. 2001, Grooteclaes, Frisch 2000, Comijn et al. 2001). As the most apical structure between epithelial cells or endothelial cells, tight junctions (TJ) are recognized as the gatekeepers to control for the paracellular diffusion of ions and certain molecules. In addition to this role, TJs are found to regulate the cohesion of these epithelial monolayers and altered functions in TJ proteins can lead the way to invasive cellular behaviour, like E-cadherin down-regulation (Martin, Jiang 2009).

2.1.4.3.2. Cell invasion during metastasis

Metastasis, the dissemination of primary tumor cells to distant sites via local invasion, or tissue penetration, to the blood circulation (intravasation) and back to tissues (extravasation) to form secondary metastatic lesions around the body, is the primary cause of cancer deaths worldwide (Duffy, McGowan & Gallagher 2008). The activation of several transcription factors like Twist or FOXC2, have a profound role in metastasis progression, as their overexpression triggers EMT in vitro and inhibition prevents tumorigenesis in vivo (Yang et al. 2004). To this end, several theories are proposed and evolved in line with new emerging studies. However, none of the existing theories is sophisticated enough to explain all the twists and turns in the metastatic cascade (Li et al. 2009). The generally accepted model is the so-called clonal selection model, in which a pool of cells in the primary tumor acquires metastasis-promoting characteristics as the end result of minievolution which enables metastasis from the primary tumor (Klein 2008). This theory however is not waterproof, since metastatic signatures are detected already in primary tumors (van de Vijver et al. 2002) and in breast tumors, micrometastases exist already in the early evolution of cancer (Husemann et al. 2008). Instead, the co-operation between cancer

cells and the stromal components seems to be a common theme in complex metastatic cascade. For instance, it is proposed that tumor cells form a pre-metastatic niche by recruiting vascular endothelial growth factor receptor-1 (VEGFR1)-positive haematopoietic bone marrow cells to form a cluster to the site where the secondary tumor eventually forms. Indeed, tumor-derived growth-stimulatory factors at the future metastatic sites would invite fibroblasts to synthesize the extracellular protein fibronectin. This increased production of fibronectin would function to provide a docking site for the attachment of the bone marrow cells via integrin α4 –mediated adhesion and colonize the new site (Kaplan, Rafii & Lyden 2006). Figure 5.

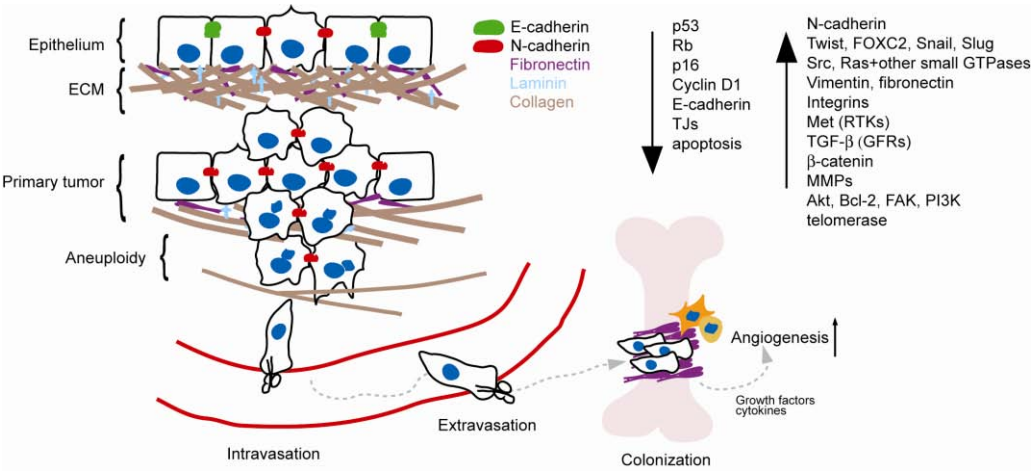

Figure 5. **Epithelial-to-mesenchymal transition (EMT).** In an intact epithelium, a cell can face multiple extracellular injuries like exposure to chemicals or carcinogens that can lead to transformation of the cell. Normal E-cadherin- guarded junctions are disorganized and replaced by N-cadherin that weakens the intercellular adhesions. Due to mutations in critical tumor suppressors like p53, Rb or apoptotic regulators, the transformed cell multiplies despite the chromosomal alterations, and grows into a primary tumor, or carcinoma in situ with characteristic aneuploid phenotype. Loss of tight junction (TJ) proteins and upregulation of several mesenchymal molecules (the arrows indicate frequently down-regulated or up-regulated molecules, respectively), like the intermediate filament (IF) vimentin, Twist and other transcription factors, ECM components and integrins contributes to EMT and invasion to vessels (intravasation) and from the vessels to tissues (extravasation) to colonize a secondary tumor at a distant site, like bone. At the secondary tumor, other cells like pericytes and bone marrow cells form a cluster where the tumor cells adhere. Angiogenic factors produced by the cells in the tumor mass start the formation of new vasculature.

2.2. THE INTEGRIN FAMILY OF CELL ADHESION RECEPTORS

2.2.1. Integrin structure and regulation of activity

The biology of integrin function resides in its membrane-spanning structure that allows cell adhesion to its immediate surroundings in extra-cellular matrix (ECM). Some lymphocyte or platelet integrins, namely αLβ2 and αIIbβ3, mediate adhesion to the surface of antigen-presenting and endothelial cells by binding to the ICAMs (inter-cellular adhesion molecule) induced by cytokines in inflammation (Shimaoka et al. 2003). Integrin was named after the early findings of its ability to link the ECM component fibronectin to the intracellular actin (Tamkun et al. 1986) and since then

several isoforms of this molecule have been discovered. Integrins heterodimerize in the endoplasmic reticulum (ER) by selective pairing between 18 α and 8 β subunits; there are currently 24 known distinct integrin receptors that bind various ECM ligands with different affinities (Luo, Carman & Springer 2007). Integrin subunits have large extracellular domains (approximately 800 amino acids), single transmembrane (TM) domains (approximately 20 amino acids), and short cytoplasmic tails, or "legs" (13 to 70 amino acids, except that of the β4 isoform). Figure 6.

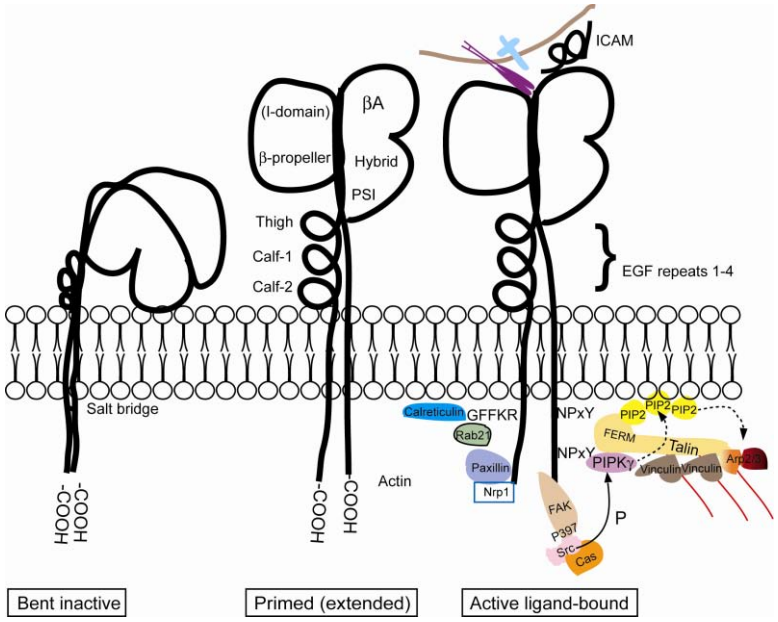

Figure 6. Integrin structure. Integrin extracellular domains bind to ligands in the ECM and receptors, like ICAMs, on other cells via the crevice between I-domains present in some α subunits or the A-domain in β subunits. The α subunit globular head bears a β-propeller and upper leg consists of Thigh and Calf 1/2 regions. The short transmembrane region links the cell exterior to the interior where the cytoplasmic tails are located. A conserved GFFKR sequence, where adhesion regulators like e.g. calreticulin or Rab21 bind, resides proximal to the plasma membrane. The COOH-terminus binds regulatory proteins like paxillin or Nrp1. The β subunit head region contains a hybrid region followed by PSI, plexin-semaphorin-integrin domain and Epidermal Growth Factor (EGF) -like repeats 1-4. The salt bridge between the cytoplasmic domains retains the integrin in the resting state. Talin binding to the β cytoplasmic domain NPXY motif disrupts the cytoplasmic interaction, and triggers the inside-out conformational change via PIPK, vinculin and actin-related protein 2/3. The COOH-terminus of integrin binds FAK and Src which leads to receptor activation. Activation is associated with slight shifting movement of the subunit 'stalk' regions relative to each other. The active integrin is presented as extended ligand-bound, whereas the inactive receptor adapts a bent conformation that can be primed (extended) to bind ligand.

With their globular extracellular domains, integrins bind to various ligands in the ECM in a sequence-specific manner. Different ECM ligands include macromolecular proteins like fibronectin (FN), vitronectin (VN) collagen (Col) and laminin (LN); in addition to these, integrins adhere to charged molecules or proteins in other cells in physiological conditions like blood clotting (Humphries, Byron & Humphries 2006). And of note, many viral pathogens in the cell exterior have learned to hijack integrins by mimicking the target sites in the ECM and thereby modulating and taking advantage of integrin biology (Jokinen et al. 2010). The ligand binding specification can be

categorized via the α subunits: fibronectin (α5, αv, αIIb and α8) and vitronectin (αIIb, αv and α8) integrins recognize the arginine-glycine-aspartic acid (RGD) motif while α4, α9, αE and α subunits pairing with the β2 subunits (αX, αM, αL and αD) recognize the functionally related leucine-aspartic acid-valine (LDV) motif in the ECM or ICAMs (Humphries, Byron & Humphries 2006, Ruoslahti, Pierschbacher 1987). The four collagen-specific (also laminin-binding) integrin receptors in vertebrates (α1, α2, α10, and α11) recognize the triple-helical sequence GFOGER in most collagen types, but their recognition ability in vivo is dependent on the fibrillar status (Knight et al. 2000, Barczyk, Carracedo & Gullberg 2010). Finally, the three β1 integrins (α3, α6, α7), plus α6β4, are highly selective laminin receptors and bind to different residues in laminin than the collagen-specific receptors. However, the exact molecular mechanisms of laminin binding are still unknown (Humphries, Byron & Humphries 2006). This specification is made according to the α subunits, the categorization could be elicited also according to the β subunits which evolved via a different evolutionary path (Johnson et al. 2009).

The α subunits can be further dissected to subcategories. Half of them contain an inserted "I" domain (or A domain because of its similar structure to the A-domains found in the protein von Willebrand factor) which presents the ligand-binding site in those integrins. Collagen-binding integrins carry this domain as well as integrins of the β2 family that mediate cell-cell adhesion. The αI domain possesses a conserved metal ion–dependent adhesion site (MIDAS), which binds divalent cations required for ligand binding by integrins. Integrins without an I domain bind ligands in a crevice between the αβ subunit interface, where a metal ion–occupied MIDAS within the A-domain in β subunit and the propeller domain of the α subunit interact (Moser et al. 2009b).

Integrins undergo global conformational changes that specify their activation state (Askari et al. 2010). Current view presents the inactive receptor in a bent conformation that can be primed (extended) for ligand binding and upon activation convert to a fully extended ligand-bound form (the 'switch-blade' model) (Takagi et al. 2002, Luo, Carman & Springer 2007) (Figure 6). Contradiction to this model brings the 'dead-bolt' model, according to which also bent conformation is able to bind ligand (Xiong et al. 2003, Arnaout, Mahalingam & Xiong 2005). The α and β subunit cytoplasmic tails, or legs, exhibit a functional interplay in the integrin activity regulation cascade. In fact, when integrins are in inactive form, a salt bridge is formed to connect and to keep the legs in close proximity (Czuchra et al. 2006). By preventing integrin leg separation with soluble tail-locked mutants of α5β1 integrin (Askari et al. 2010), ligand binding is inhibited simultaneously inhibiting cell spreading. In contrast, during activation, the integrin subunit legs and transmembrane regions are separated and the salt bridge severed to enable efficient signaling. The separation is elicited by ECM ligand binding or by intracellular regulatory proteins (Kim, Carman & Springer 2003). Important such regulatory proteins are e.g. talin and kindlin. They act by binding to the integrin β subunit conserved NPXY motifs via the FERM (4.1, ezrin, radexin, moesin) domain, and change the integrin from low-affinity (bent inactive) to high-affinity conformation (extended able to bind ligand). This alters the activation status of the integrin ('inside-out signaling') and is important in regulation of cell adhesion and cell motility in

physiological contexts like platelet activation in blood clotting (Harburger, Bouaouina & Calderwood 2009, Moser et al. 2009a,Wegener et al. 2007).

In response to integrin adhesion to the ECM ligands, cytoplasmic tails inside the cell separate in a process termed 'outside-in signalling' and recruit several adaptor and signalling proteins, such as focal adhesion kinase (FAK) and Src family kinases (SFKs). These and other proteins build up signalling platforms in the cell by connecting to the actin cytoskeleton and promoting integrin clustering to regulate a broad array of processes like cell survival, differentiation or proliferation (Figure 6). Because integrin receptors lack intrinsic enzymatic activity per se, these signaling proteins are needed to activate adhesion-dependent processes (Liu, Calderwood & Ginsberg 2000, Mitra, Hanson & Schlaepfer 2005). Integrin activation is especially intriguing in circulating blood cells as it demonstrates the fastest mode of integrin activation in response to extracellular stimulus. During tissue inflammation, chemokines are secreted on the vessel endothelium which triggers rapid lymphocyte adhesion to the vessel wall, via a subsecond inside-out activation of the integrin $\alpha 4\beta 1$ (Grabovsky et al. 2000). Integrin activity is important also during cell division, as perturbed integrin activity lead to impaired spindle microtubule assembly (Reverte et al. 2006) and defects in chondrocyte cytokinesis probably due to deregulated adhesion (Aszodi et al. 2003).

Despite of their small size, the integrin cytodomains are critical in mediating the specific functions of integrins (Hynes 2002). The α tails are highly heterogeneous except for the membrane proximal region that contains a conserved GFFKR motif in almost all of the subunits to which several adaptor proteins, like calreticulin, bind to modulate integrin adhesive properties (Coppolino et al. 1997) (Figure 6). In fact, many proteins that influence integrin functions recognize only a short amino acid sequence in the tail domains which is sufficient for exerting their functions (El Mourabit et al. 2002, Spicer et al. 2010). This is not an exceptional mode of regulation, also other cellular proteins i.e. neuropilin1 or cadherins are involved in cell adhesion regulation via cytoplasmic tails (Valdembri et al. 2009, Yap, Niessen & Gumbiner 1998). Ligand binding can be affected by a double proline- induced β-turn present in some of the α subunits (Filardo, Cheresh 1994, Cheng and Chang 1999).

In addition to functioning as the principle adhesion receptor mediating cell-matrix adhesion, integrins regulate e.g. matrix deposition and degradation during ECM remodelling by fibroblasts and offer signalling platforms to various other proteins in adhesion sites (Wiesner, Legate & Fassler 2005). The interplay with cell membrane receptor kinases is intriguing, especially from the therapeutical point of view. Activated integrin can transduce signalling cascades in multiple receptor tyrosine kinases, such as Met (Wang, Kobayashi & Bishop 1996), platelet-derived growth factor receptor [PDGFR; (Sundberg, Rubin 1996)], vascular derived growth factor receptor [VEGFR; (Wang, Zhang & Groopman 2001)] and epidermal growth factor receptor [EGFR; (Kuwada, Li 2000, Moro et al. 1998)]. Integrins are involved also in promoting cell survival by expressing anti-apoptotic factors (Zhang et al. 1995) and by activating extracellular signal-regulated kinases (ERKs) (Barberis et al. 2000) or

mitogen-activated protein kinases (MAPKs) (Schlaepfer et al. 1994), features that become essential during tumor progression.

2.2.2. Integrin adhesions

Anchoring, or adhering, junctions (adherens junctions, desmosomes, focal adhesions, and hemidesmosomes) are strong attachments that keep cells attached to each other or to the extracellular matrix. In addition, tight junctions which will be discussed later, and gap junctions, are involved in cell-cell adhesion in epithelia and endothelia. While adherens junctions and desmosomes use transmembrane cadherins to mediate cell-cell adhesion, focal adhesions and hemidesmosomes connect via integrins to the extracellular matrix (Figure 7). Inside the cell, adherens junctions and focal adhesions are bound to actin filaments, whereas desmosomes and hemidesmosomes link to intermediate filaments like keratin (Figures 7&9). Integrin linkage to the actin cytoskeleton is mediated by at least 50 cytoplasmic proteins recruited to the integrin cytoplasmic tails (Lo 2006).

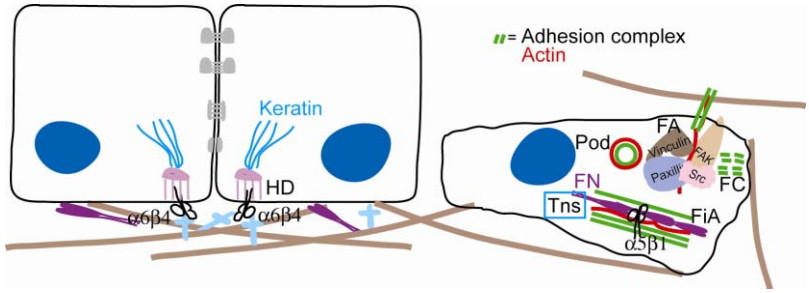

Figure 7. **Integrin adhesion types.** In stratified epithelium like the epidermis, integrin-mediated adhesion occurs via the hemidesmosomes (HD), where integrin isoform α6β4 forms the core of the laminin (cyan)-binding structure that links to keratin filaments inside the cells. In integrin-actin matrix adhesions, focal adhesions (FA), focal complexes (FC) and podosomes (Pod) share similar molecular constituents like vinculin and paxillin, but FAs have slower turnover and are bigger in size than the smaller FCs, whereas podosomes are formed in a circular structure. Fibrillar adhesion (FiAs) do not contain the common vinculin or paxillin, but instead parallel the actin fibres and underlying FN matrix with tensin (Tns), mastered by the integrin α5β1.

The integrin-actin matrix adhesions, or focal adhesions, can be subdivided further according to their characteristics, and are currently classified to focal adhesions, focal complexes, fibrillar adhesions and podosomes. The anatomy of the integrin-rich matrix adhesions is complex, since they vary in their expression, localization, protein composition, temporal regulation, size and so forth even if they all mediate the adhesion between cell and the ECM. Studies with fixed samples and live cell microscopy have been crucial in revealing the anatomy of matrix adhesions, since studies with isolated adhesions would be difficult due to their short half-lives.

2.2.2.1. Focal adhesions (FAs) and its variations

Focal complexes are small, punctate adhesions around 100 nm in size that usually reside in the area of membrane protrusion in migrating cells. They are highly dynamic and can mature into longer focal adhesions (around 1 μm in size) in a matter of

minutes. The central transducer of integrin signalling in these focal complexes is the focal adhesion kinase, FAK. Other common components include the kinase Src, integrin-binding protein paxillin and actin linker vinculin. The more static focal adhesions are frequently detected in resting or non-motile cells, and display a slower turnover rate. Focal adhesions are the sites of integrin extension (Askari et al. 2010). These bigger focal adhesions, or focal contacts, are often anchored to actin stress fibres, the concentration of which can be up to 1 mM in these sites (Abraham et al. 1999) (Figure 7).

A variation from focal adhesions is fibrillar adhesions, which are formed by many cell types exploiting integrin α5β1- mediated ligation to fibronectin. These adhesions reorganize the underlying fibronectin matrix to parallel intracellular actin stress fibres. Unlike focal adhesions, fibrillar adhesions are detected in tissues *in vivo* and do not contain typical adhesion proteins, such as vinculin or paxillin but are rich in tensin1, for instance (Zamir et al. 2000, McCleverty, Lin & Liddington 2007). Podosomes and invadopodia are dot-shaped matrix adhesions that are able to degrade the ECM components. Podosomes are also specialized structures in a way that they are expressed on the surface of osteoclasts and some hematopoietic cells such as neutrophils, and represent the only form of adhesion in those cells (Linder, Aepfelbacher 2003). Recently, however, podosome adhesions have been observed in some epithelial cell lines (Spinardi et al. 2004). In size and half-life they resemble focal complexes, but the adhesion complex structure differs dramatically from focal complexes: the adhesion proteins surrounding an actin core with actin regulators like cortactin, WASP or Arp2/3 are arranged in a circular form (Figure 7). Invadopodia, however, are mostly detected in cancer cells and lack a clear ring structure. One way to distinguish between these structures is monitoring the adhesion life-time and quantity, which is in 2-20 minutes for podosomes (20-100 per cell), but up to more than 1h for invadopodia (1-10 per cell) (Linder 2007).

2.2.2.2. Hemidesmosomes are specialized integrin-containing adhesions

Hemidesmosomes are specialized junctional cell-matrix adhesion structures found on the basal side of the epidermis and other stratified epithelium (Tidman, Eady 1984) (Figure 7). Hemidesmosomes connect the epithelial cells to the underlying extracellular matrix, which in the skin is the basement membrane, and link the cell to keratin filaments inside the cell. Defects in the adhesion mediated by hemidesmosomes can lead to different illnesses characteristic of epidermal detachment or blistering, such as in the case of junctional epidermolysis bullosa (JEB) (Borradori, Sonnenberg 1996, Tidman, Eady 1986). The integrin heterodimer α6β4 and the bullous pemphigoid antigen 180 (BP180) form the transmembrane core of the hemidesmosome structure and both have been suggested to bind the ligand laminin-5, the commonest component in the epidermal basement membrane (Niessen et al. 1994, Sonnenberg et al. 1991, Hopkinson, Riddelle & Jones 1992, Giudice, Emery & Diaz 1992, Jones et al. 1991). Mutations in either of the subunits of integrin α6β4 has been linked to some forms of JEB and mice null for α6 or β4 do not form any hemidesmosomes at all (Vidal et al. 1995, Pulkkinen et al. 1997, Dowling, Yu & Fuchs 1996, Georges-Labouesse et al.

1996, van der Neut et al. 1996). The β4 subunit is exceptional in a way that its cytoplasmic domain is 1000 amino acids long in comparison to the 13-70 residues long cytodomains of other β subunits. The long β4 cytoplasmic domain alone is sufficient for the recruitment and assembly of hemidesmosomal complex proteins, suggesting that it can induce ligand-independent hemidesmosome assembly (Nievers et al. 2000).

2.2.3. Integrin adhesion regulation via cytoplasmic tail binding proteins

As integrins provide a platform for several types of protein adhesion complexes, a recently introduced 'integrin adhesome', can consist of up to ~150 signalling proteins that interconnect in as many as ~700 ways and based on their common motifs form differential functional subnetworks in cell-matrix adhesions (Zaidel-Bar et al. 2007). Three types of proteins are recruited to cell-matrix adhesions: (1) integrin-binding proteins that give structural support (2) scaffolding proteins that provide additional adhesion protein binding sites and (3) enzymes (Table 1). The composition of these accessory proteins and for example the level of tyrosine phosphorylation can vary depending on whether the adhesions are formed in two- or three-dimensional matrix (Cukierman et al. 2001, Berrier, Yamada 2007). Furthermore, many of the regulatory proteins are indispensible, since studies with mice demonstrate embryonic lethality upon deletion of several critical components like talin (Monkley et al. 2000) or FAK (Furuta et al. 1995).

2.2.3.1. Talin, kindlin and actin-binding protein vinculin

Talin is a structural adaptor that exists in two isoforms, talin-1 and talin-2. Of these, talin-1 represents the ubiquitously expressed isotype that mediates the first steps in focal complex formation and integrin activation by binding to the conserved membrane-proximal NPxY motif in the integrin β subunit tails which affects the interactions between α and β tails (Calderwood et al. 1999). Another major integrin activator is the recently discovered protein kindlin and its family members, which bind to the membrane-distal conserved NPxY motifs to activate the integrins β1, β2 and β3 (Moser et al. 2009a, Moser et al. 2009b). Interestingly, other organisms express also talin homologues, even in the absence of integrin expression. In Dictyostelium discoideum, talin functions in actin-based motility and in myosin II- independent and adhesion-dependent cytokinesis (Hibi et al. 2004). Talin-1 is a large molecule composed of a C-terminal rod domain that facilitates actin binding and an N-terminal globular head domain which via its FERM domain engages and activates the integrin (Garcia-Alvarez et al. 2003). Integrin activation by talin includes a positive feed-back loop induced by local production of the lipid PI(4,5)P2. This production is thought to be mediated by PIPKγ, which is recruited to matrix adhesions in a complex with talin (Ling et al. 2002). PI(4,5)P2 binding to talin increases talin FERM domain binding to integrin β1 cytoplasmic tail (Martel et al. 2001). Also, the binding of PIPKγ and β1 integrin occurs at the same site on the talin head domain, and binding of these two is mutually exclusive. Both PI(4,5)P2 and talin bind to and activate the adhesion complex -localized actin-binding protein vinculin by relieving its autoinhibition. Vinculin could contribute to talin-induced bifurcated integrin signaling cascade by providing F-actin binders to the sites of focal complexes.

2.2.3.2. Paxillin

Paxillin is an important scaffolding adaptor in integrin-mediated cell-matrix adhesion as it provides additional docking sites for other proteins in adhesions. It does this via its repeating units, the N-terminal LD repeats and the C-terminal LIM domains. Paxillin can bind β1 integrin (Chen, Bailey & Fernandez-Valle 2000b, Schaller et al. 1995) but shows strongest affinity towards α4 integrin subunit (Liu et al. 1999). Indeed, paxillin binding to integrin α4 is regulated by phosphorylation on the integrin cytoplasmic tail. The PKA-mediated phosphorylation of S988 in integrin tail prevents paxillin binding and relocates the integrin to the leading edge to support cell motility, while the non-phosphorylated integrin binds to paxillin and they co-localize at the trailing edges of cells (Han et al. 2001, Goldfinger et al. 2003).

2.2.3.3. FAK, Src and other enzymes

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that gets activated upon integrin ligation and by other receptors and signals with great variety upon stimuli (Parsons 2003). FAK bears in its sequence a FERM homology domain which binds to β1 cytoplasmic tail upon integrin ligation (Schaller et al. 1995). The functional reason for the binding has not been discovered, as the integrin-binding region in FAK is not required for FA targeting (Shen, Schaller 1999). Upon integrin ligation, (inside the cell) FAK autophosphorylates at Tyr397, leading to formation of a high-affinity binding site for Src family kinases (SFKs) c-Src, Lyn, Fyn, c-Yes and Hck (Cobb et al. 1994, Schaller et al. 1995). The SFKs bind directly to the β3 tail, and some of them additionally to β1 and β2 tails (Arias-Salgado et al. 2003). The FAK/Src complex may in turn promote the tyrosine phosphorylation of the linker protein p130Cas which can recruit signalling adaptors like Crk and other substrates that regulate actin cytoskeleton. Adding to the co-regulatory roles of FA signalling proteins is the finding that Src phosphorylates the talin-binding sequence of PIPKγ, and increases the affinity to talin, in a FAK-dependent manner (Ling et al. 2002, Ling et al. 2003). The importance of FAK signalling to the various aspects of cell behaviour can be appreciated by the embryonic lethality of FAK null mice (Furuta et al. 1995). Several other kinases, including integrin-linked kinase (ILK) or PI3K, are important in focal adhesion dynamics. For instance, PI3K affects integrin-mediated motility in matrix adhesions by binding to the phospho-Tyr397 in FAK via its 85 kDa regulatory subunit (Chen et al. 1996). By generating a FAK mutant capable of binding Src but not PI3K (D395A), cell motility was decreased to an even greater extent than with chemical PI3K inhibitors wortmannin or LY294002 (Reiske et al. 1999).

Table 1. Adaptor proteins that bind to integrin β cytoplasmic domains

Adaptor protein	**Integrin to which adaptor binds**	**Reference**
Structural adaptors		
Alpha-actinin	β1, β2	(Otey et al. 1993, Pavalko, LaRoche 1993)
BP180	β4	(Nievers et al. 2000)
Filamin	β1, β2, β3, β7	(Calderwood et al. 2001, Kiema et al. 2006, Loo, Kanner & Aruffo 1998, Pfaff et al. 1998, Sharma, Ezzell & Arnaout 1995, Travis et al. 2004, Zent et al. 2000)
Platelet myosin (β3) Myosin-X (β1)	β1, β3	(Jenkins et al. 1998, Zhang et al. 2004)
Plectin	β4	(Geerts et al. 1999)
Skelemin	β1, β3	(Reddy et al. 1998)
Talin	β1, β2, β3, β5, β7	(Calderwood et al. 2003, Pfaff et al. 1998, Calderwood et al. 1999, Patil et al. 1999, Sampath, Gallagher & Pavalko 1998)
Tensin isoforms	β1, β3, β5, β7	(Calderwood et al. 2003, McCleverty, Lin & Liddington 2007)
Scaffolding adaptors		
14-3-3 αβ, δζ	β1, β2, β3	(Fagerholm et al. 2005, Han, Rodriguez & Guan 2001)
β3 endonexin	β3	(Eigenthaler et al. 1997, Shattil et al. 1995)
CD98	β1, β3	(Zent et al. 2000)
Dab1	β1, β2, β3, β5, β7	(Calderwood et al. 2003)
Dab2	β3, β5	(Calderwood et al. 2003)
Dok1	β2, β3, β5, β7	(Calderwood et al. 2003)
Fhl2	β1, β2, β3, β6	(Wixler et al. 2000)
Fhl3	β1	(Samson et al. 2004)
Grb2	β3	(Law, Nannizzi-Alaimo & Phillips 1996)
IAP	β3	(Brown et al. 1990)
JAB1	β2	(Bianchi et al. 2000)
Kindlin 1,-2	β1, β3	(Harburger, Bouaouina & Calderwood 2009)
Kindlin 3	β1,β2, β3	(Moser et al. 2008, Moser et al. 2009a)
Melusin	β1	(Brancaccio et al. 1999)
Numb	β3, β5	(Calderwood et al. 2003)
Paxillin	β1, β3	(Chen, Bailey & Fernandez-Valle 2000a, Schaller et al. 1995)
Rack1	β1, β2, β5	(Liliental, Chang 1998)
Shc	β3, β4	(Law, Nannizzi-Alaimo & Phillips 1996, Dans et al. 2001)
TAP20	β5	(Tang, Gao & Ware 1999)
WAIT1	β7	(Rietzler et al. 1998)
Catalytic adaptors		
Src	β3	(Arias-Salgado et al. 2003)
Yes	β1, β2, β3	(Arias-Salgado et al. 2005)
Cytohesin 1	β2	(Kolanus et al. 1996)
Eps8	β1, β3, β5	(Calderwood et al. 2003)
ERK2	β6	(Ahmed et al. 2002)
FAK	β1, β2, β3, β5	(Chen, Bailey & Fernandez-Valle 2000b, Eliceiri et al. 2002, Schaller et al. 1995)
Fyn	β3	(Arias-Salgado et al. 2005)
ILK	β1, β3	(Hannigan et al. 1996, Pasquet, Noury & Nurden 2002)

Lyn	β1, β2, β3	(Arias-Salgado et al. 2005)
PKD1	β1, β3	(Medeiros et al. 2005, Woods et al. 2004)
PP2A	β1	(Kim et al. 2004)
Shp2	β4	(Bertotti, Comoglio & Trusolino 2006)
Other adaptors		
ICAP1	β1	(Calderwood et al. 2003)
MIBP	β1	(Li, Mayne & Wu 1999)

Modified from (Legate, Fassler 2009)

2.2.3.3.1. The disassembly of FAs

Adhesion break-down is important in the dynamic regulation of cell motility. Talin degradation is a critical event for the turnover of the adhesions (Franco et al. 2004) as might be expected by its fundamental role in the assembly process. The proteolysis of talin occurs by calpain (calcium-dependent ubiquitous proteases) cleavage, which is a common mode of regulation of various FA-related proteins (Franco, Perrin & Huttenlocher 2004) and integrin β tails (Du et al. 1995, Pfaff, Du & Ginsberg 1999). The disassembly of focal adhesions, as proposed in (Ezratty, Partridge & Gundersen 2005), involves the targeting of microtubules to the adhesion sites. In this study, the authors showed that the disruption of FAs requires the vesicle scission- associated GTPase dynamin and if inhibited, cell migration is prevented possibly due to sustained FA stability. Interestingly, FAK was required to recruit dynamin to adhesion sites and upon adhesion break-down, the level of FAK autophosphorylation at Y397 was decreased. According to this model, FA disassembly is more than just the reversal of its assembly process.

2.2.3.4. Other proteins regulating integrins via cytoplasmic recruitment

Most of the known integrin-binding adaptors recognize the intracellular tail of the β subunit whereas scarce knowledge exists of the α subunit binding proteins. Recently, several new molecules have been identified to specifically bind the α subunit cytodomain and modulate cell adhesive properties (Table 2, negative adhesion regulation is indicated with bold text). Some of them exhibit high isoform specificity, while others bind to the conserved sequences in the α subunits. These include proteins of unclear functions (Ramsay et al. 2007), from cytoplasmic compartments (Alahari, Lee & Juliano 2000), phosphatases (Mattila et al. 2005), fatty-acid binding proteins (Nevo 2010) and PDZ adaptor proteins (El Mourabit et al. 2002).

Table 2. Adhesion regulators binding to the α subunit tails

TCPTP	α1	(Mattila et al. 2005)
MDGI	α1, α2, α10, α11,α5	(Nevo et al. 2010)
GIPC-1	α5,α6	(El Mourabit et al. 2002)
Calreticulin	GFFKR of α isoforms	(Rojiani et al. 1991)
Paxillin	α4, α9	(Liu et al. 1999, Young et al. 2001)
Nischarin	α5	(Alahari, Lee & Juliano 2000)
Rab21	GFFKR of α isoforms	(Pellinen et al. 2006)
ICAM-1	αL	(Shimaoka et al. 2003)
CIB1	αIIβ	(Leisner et al. 2007)
Caveolin-1	transmembrane region of α isoforms	(Wary et al. 1998)
DRAL/FHL2	α3A, α3B and α7A	(Wixler et al. 2000)

2.2.3.4.1. The PKCs in integrin-mediated functions

The PKCs regulate a multitude of functions of different integrin heterodimers. For instance, the αvβ3-driven melanoma cell invasion is boosted by the expression of PKCα and PKCδ together with the activation of Src (Putnam et al. 2009). Parsons et al. (2002) report that the phorbol ester-induced cell polarization and directional motility in breast carcinoma cells is determined by a short motif in the PKCα V3 hinge domain which also promoted a direct association between PKC and β1 integrin. Efficient binding of β1 integrin to PKC requires the presence of both NPXY motifs in the integrin cytoplasmic domains. A cell-permeant inhibitor which prevented the PKC binding to β1 integrin, was shown to block both PKC-driven and epidermal growth factor (EGF)-induced chemotaxis. PKCε, on the other hand, has been shown to control the traffic of β1-integrin to the plasma membrane thereby promoting cell migration (Ivaska et al. 2002) and was later demonstrated to phosphorylate vimentin in integrin containing intracellular vesicles to regulate exit from this compartment to the plasma membrane (Ivaska et al. 2005).

2.2.4. Integrin endocytosis, recycling and invasion

It is not long ago, when the endocytic process was described solely to function in the downregulation of growth factor receptor signalling. Nowadays, however, the picture of how we conceive the endocytosis- related processes, is crystallizing and it is known that e.g. integrin- mediated cell motility exploits this mechanism (Caswell, Norman 2008). During dramatic cell shape changes, the balance between plasma membrane surface area and associated molecules is maintained by the actions of endo- and exocytic machineries. On the plasma membrane, the traversing and associated proteins are taken inside the cell via endocytosis and targeted to intracellular compartments or recycled back to the plasma membrane, which is important in controlling cell adhesion in a polarized way during cell motility (Pellinen et al. 2006, Caswell, Norman 2008). The regulated intake and degradation of ECM proteins is also important as impaired removal can lead to disease states like fibrosis, arthritis or cancer (Holmbeck et al. 1999, Mosesson, Mills & Yarden 2008).

Integrin endocytosis, or internalization, occurs mostly via clathrin- or caveolin-mediated routes. Other internalization pathways include the clathrin- and caveolin-independent endocytosis and macropinocytosis, but the data linking these to integrin internalization remains scant (Doherty, McMahon 2009). One such indication was shown with the leukocyte integrin αLβ2 (LFA-1) that internalised through a clathrin-independent cholesterol-sensitive pathway during cell motility (Caswell, Norman 2008). In clathrin-mediated endocytosis, the clathrin triskelion and its adaptor proteins form a coat complex on the membrane surrounding the cell-surface receptors, such as integrins, to facilitate membrane invagination and endocytotic vesicle formation. The vesicles pinching off the plasma membrane are decorated with molecules specialized in cargo transport. Once in the cell, the cargo is transported to early endosomes (EEs) and to perinuclear recycling endosomes (PNREs) or late endosomes (LEs), from where it is shuffled back to the plasma membrane or to lysosomal degradation, respectively. One family of molecules implicated in this delivery is the Rab GTPases whose function is

to regulate the endosomal traffic of proteins with the help of many accessory proteins like EEA-1 (early endosome antigen-1) or Rabenosyn-5, that mediate sorting and vesicle fusion events in the endocytic process (Mishra et al. 2010).

The Rab GTPases constitute a functionally rich family of guanosine triphosphate hydrolases that shuffle between active (GTP-loaded) and inactive (GDP-loaded) conformations with the help of guanosine exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Pellinen et al. 2006). The most-studied of Rab GTPases is Rab5, which together with Rab21, Rab22 and Rab31 comprise the Rab5 subfamily with implications in the early endocytosis (Simpson et al. 2004, Simpson, Jones 2005). Recent data from our lab demonstrates the essential role of Rab21 in endocytosis and cell motility of β1-integrins (Pellinen et al. 2006). Indeed, the regulation of integrin function was shown to act via cytoplasmic association between Rab21 and the conserved GFFKR sequence of integrin α subunit cytoplasmic tails. More specifically, point mutation analysis revealed that the conserved residues arginine (R1161) and lysine (K1162), which is involved in forming the α -helical structure of the α tail, were critical for the association. Expression of GTP-locked active Rab21 in MDA-MB-231 cells resulted in accumulation of intracellular β1 integrin in intracellular vesicles whereas the GDP-locked version left the integrin in focal adhesion structures and these mutations both affected the motility of the cells. This study was the first to show that Rab GTPases regulate integrin traffic and motility via associating with the integrin α subunit and that the integrin localization phenotype is dependent on the activity status of the GTPase.

Rab11 GTPase is an important regulator of endocytic membrane traffic, the functions of which are mediated by the Rab11-family interacting proteins (FIPs). Based on the primary structure, the alternatively spliced FIPs have been sub-classified into two groups – class I FIPs [FIP2, RCP (Rab coupling protein) and Rip11 (Rab11-interacting protein)] and class II FIPs (FIP3 and FIP4) (Horgan, McCaffrey 2009). RCP, for instance, regulates protein sorting into tubular endosomes (Peden et al. 2004). Rab11, together with FIP3, supports endosomal recycling to the cleavage furrow during cell division and contributes to β1 integrin recycling from the PNREs to the membrane, via a "long-loop" pathway which differs from the Rab4- regulated "short-loop" recycling pathway from EEs to the plasma membrane or Rab5/7- regulated lysosomal targeting via EEs and LEs (Wilson et al. 2005, Ullrich et al. 1996, Powelka et al. 2004, Daro et al. 1996).

Endocytosis of integrins is needed during matrix remodelling (Shi, Sottile 2008). Recently it was shown that the plasma membrane glycoprotein Nrp1 complexed with GIPC1 (GAIP-interacting protein C-1) can specifically induce the endocytosis of active form of integrin α5 in endothelial cells; this process was occurring at the sites of abundant fibronectin deposition indicating a link between endocytosis and matrix remodelling (Valdembri et al. 2009). During tumour progression, cancer-associated fibroblasts (CAFs) promote the dissemination of cancer cells in the body. Hooper, Gaggioli & Sahai (2010) describe in their work how Rab21 contributes to this by releasing integrin α5β1, abundant in fibroblasts, from the Rab5 positive endosomes to the plasma membrane to mediate extensive matrix remodeling. The Rab21- mediated recycling of integrin α5β1 promoted actomyosin contractility thereby increasing the

invasive potential of the cells.The endocytic machinery is often deregulated in cancer by oncogenic alterations or genetic interference (Mosesson, Mills & Yarden 2008). Mutant form of the tumor suppressor p53 enhanced the endocytic recycling of integrin α5β1 and epidermal growth factor receptor which depended on Rab-coupling protein (RCP), thereby boosting cell invasion and metastasis (Muller et al. 2009). It was shown that the mutant p53 functions by binding to and inhibiting the function of p63, a p53 family tumor suppressor. This is intriguing, since scientists have been puzzled for a long time over the putative functions of this mutant form which is frequently overexpressed in many human tumors (Selivanova, Wiman 2007). Integrin αvβ6 is frequently upregulated in epithelial cancers and has been shown to internalize via a clathrin-dependent mechanism when it binds to a cytoplasmic protein HAX-1. Disruption of this interaction inhibits invasion to the matrigel (Ramsay et al. 2007), linking integrin endocytosis directly to invasive migration. Another Rab family member, Rab25, promotes integrin α5β1 recycling and 3D invasion in ovarian carcinoma by binding to β1 subunit in a GTPase- dependent manner (Caswell et al. 2007). Figure 8.

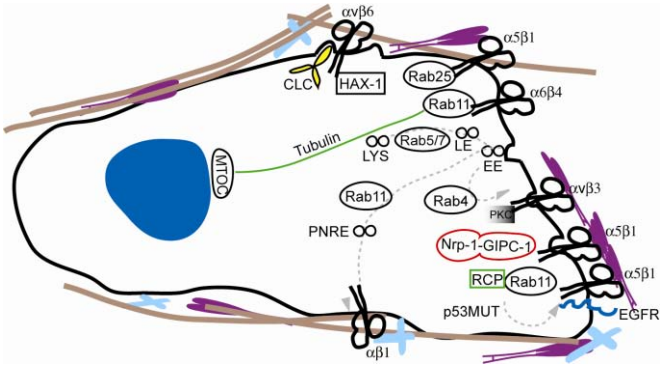

Figure 8. Integrin endocytosis, recycling and invasion. Rab11 contributes to β1 integrin recycling to the plasma membrane from the PNREs following integrin endocytosis through EEs (early endosome) to PNRE (perinuclear recycling endosome) via a "long-loop" pathway. This differs from the Rab4- regulated "short-loop" recycling pathway from EEs to the plasma membrane. Integrins can also be targeted to lysosomes (LYS) via EEs and LEs (late endosomes) by the action of Rab5/7 isoforms. PKCs control the traffic and release of β1-integrin to the plasma membrane thereby promoting cell migration. Integrin αvβ6 internalized via a clathrin (CLC)-dependent mechanism when bound to a cytoplasmic protein HAX-1, and Nrp1/GIPC1 complex specifically induced the endocytosis of active form of integrin α5 in endothelial cells. Microtubule-regulated Rab11-mediated trafficking of integrin α6β4 to the plasma membrane can increase invasive potential of cells in hypoxia. Mutant p53 (p53MUT) drives the Rab11-RCP-dependent recycling of integrin α5β1 and EGFR thereby boosting cell invasion, and Rab25 is upregulated in ovarian carcinoma and promotes α5β1 recycling, invasion and cancer progression.

2.2.5. Integrins in cancer

Integrin- activated signalling pathways are frequently exploited by cancer cells. For instance, FAK signalling which promotes survival mechanisms in cells is upregulated in a multitude of tumor cells and cancers (Mitra, Hanson & Schlaepfer 2005, Slack-Davis et al. 2007). In line with this, several integrin isoforms show increased expression in malignant tissues in comparison to their healthy counterparts. Some integrin subunits are ubiquitously expressed throughout the body, while other subunits are expressed in a tissue- or stage-restricted manner (Humphries, Byron & Humphries

2006). For example, in the adult epithelia, the expression of subunits like αvβ3, α5β1 or αvβ6 is barely detectable but can be skyhigh in some epithelial-derived tumors like repeated reviews indicate (Desgrosellier, Cheresh 2010). In non-small cell lung cancers (NSCLC), the lymph node status is one of the most important prognostic factors. However, about 40% of the node-negative patients die of cancer recurrences, suggesting a systemic cancer spread at the time of tumor removal (Mountain 1997). The high expression level of integrin α5β1 in lung cancers has been associated with tumor suppressor as well as tumor promoter functions depending on the tumor stage (Adachi et al. 2000). Indeed, the authors in this study demonstrated that the advanced node-negative tumor grade correlated with high integrin α5β1 and poor prognosis where the integrin was speculated to serve as a marker for micrometastases. In lower grade tumors, on the other hand, the integrin upregulation was inversely correlated with the prognosis.

Sometimes it is difficult to determine whether the observed upregulation is merely a functional outcome of a changed tumor microenvironment rather than the ultimate cause of malignancy. ECM-tumor rigidity is critical for the prognosis, since integrins like α5β1 can sense the stiffness of the ECM and signal thereby to the disfavour of the patient (Paszek et al. 2005). More specifically, Levental et al. (2009) stated later that breast tumorigenesis was driven by collagen crosslinking-induced ECM stiffening and increased focal adhesions and PI3 kinase (PI3K) activity, thereby promoting cell invasion to the tissue. Additionally, other alterations in the tumor environment affect integrin functions. Hypoxia, the prolonged decrease of oxygen levels, which often develops inside larger tumors, stabilized the microtubule network thereby facilitating the Rab11-mediated trafficking of integrin α6β4 to the plasma membrane and resulting in increased invasive potential of the cells (Yoon, Shin & Mercurio 2005).

In addition to having modulated functions in cancer cells, integrins are important for the host cellular response mediated by various cell types during tumor progression (Desgrosellier, Cheresh 2010). Endothelial cells lining the cancerous leaky blood vessels express integrin αvβ3, in contrast to the healthy quiescent counterpart (Brooks, Clark & Cheresh 1994). It is possible that this observed upregulation serves to recruit and adhere to the large body of matrix proteins deposited in the tumor microenvironment. Underlining the endothelium, perivascular cells such as smooth muscle cells or pericytes promote the vessel maturation. Pericytes connect to the endothelium via integrin α4β1, which stabilizes the vessel (Garmy-Susini et al. 2005). In tumors, this stability is compromised leaving the vessels leaky (Baluk, Hashizume & McDonald 2005). In the case of non-small cell lung carcinoma, stromal fibroblasts, via expressing the integrin α11β1 trigger the release of insulin-like growth factor, thereby increasing tumor growth and highlighting the importance of the stromal components to the progression of cancer (Zhu et al. 2007). Multiple cell types emanate from the bone marrow and circulate throughout the body in immunosurveillance functions. Upon encountering a tumor, immune cells like macrophages help in tumor suppression via an integrin αvβ3 –dependent manner, since macrophages from *Itgb3* $^{-/-}$ mice failed to infiltrate the tumor (Taverna et al. 2004). Although integrins as such are not oncogenic, some of them have been reported to cooperate with oncogenes or receptor tyrosine

kinases to promote tumorigenesis. In a study by Guo et al. (2006) integrin α6β4 was shown to boost the function of the oncogenic receptor tyrosine kinase ErBB2 to enhance breast tumor onset and invasion. Additionally, the oncogenic fusion protein BCR-ABL1 in blood cells promoted aberrant integrin adhesion due to constitutive inside-out activation (Chen et al. 2008) thereby contributing to the invasive potential of the cells (Fierro et al. 2008).

2.2.5.1. Integrins in targeted cancer therapy

Because of their wide expression in cancer progression- associated cell types and for their cooperative role with growth factor receptors, integrins are appealing as therapeutical targets. In addition, integrin expression is often highly upregulated in tumor cells and can be thus easily targeted by integrin antagonists, like antibodies or small molecules. Integrin αvβ3 is frequently observed upregulated in several different malignancies and therapeutics against this isoform have been developed: one such example is cilengitide, an inhibitor of αvβ3 and αvβ5, but not of αIIbβ3. Cilengitide is currently being tested in Phase II trials in patients with lung and prostate cancer and for glioblastoma patients, Phase II and Phase III tests are currently taking place. So far, the results have been encouraging in a way that late-stage glioblastoma patients' survival has increased while maintaining the side effects minimal (Desgrosellier, Cheresh 2010). However, it should be noted that the benefit for other cancers has been minimal and instead, nanomolar concentrations of cilengitide have been shown to increase the tumor size of certain cancers (Reynolds et al. 2009). In addition to being a target in drug therapy, integrin expression signature can be used as biomarker when the efficacy of certain anti-angiogenic or anti-tumor drugs is assessed, like is the case with the isoform αvβ3 (Winter et al. 2003, Haubner et al. 2005).

2.3. THE TIGHT JUNCTIONS MEDIATE INTERCELLULAR ADHESION

In between the cells of endothelia and over a hundred varieties of different epithelia in the body, intercellular adhesion complexes throughout the cell orthogonal axis participate in dividing the cell into two plasma membrane domains, the apical and basal, and in the sealing of the cell monolayer facing the vessel or tissue lumen (Le Gall et al. 1995). Starting from the basal side, hemidesmosomes and desmosomes (in stratified epithelium) mediate cell-matrix or cell-cell adhesion, respectively, and linkage to the keratin filaments; higher up in the cells are cylinder-like gap junctions built from the transmembrane protein connexins, which permit the free passage of ions and small molecules (up to a molecular weight of about 1000 daltons) between the cells; reaching the sub-apical membrane are cadherin and catenin- rich adherens junctions (AJs) as well as desmosomes and as most apical adhesion structures towards the lumen, tight junctions control the lateral diffusion of macromolecules (Claude, Goodenough 1973) or the blood-brain barrier (BBB) in the brain (Harhaj, Antonetti 2004) (Figure 9).

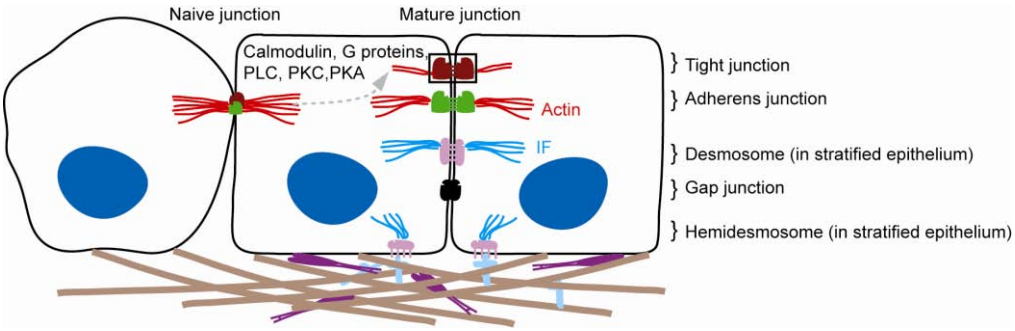

Figure 9. Intercellular adhesion types. Naïve junctions containing TJ and AJ components mature into TJs via the action of calmodulin, G proteins, PLC, PKA and PKC and to AJs via E-cadherin-mediated mechanism. Mature junctions contain tight and adherens junctions that link to actin cytoskeleton, whereas desmosomes and hemidesmosomes connect to the intermediate filament (IF) network between cells or between cell and the matrix, respectively, in the epidermis and other stratified epithelia. Gap junctions mediate ion flow between cells via connexin proteins. Detailed TJ structure is depicted in Figure 10.

For long, tight junctions were considered as simple compartmental barriers, but recent evidence has revealed, that they are actively participating in cell proliferation and gene expression via bi-directional signaling at the intercellular adhesion sites, executed via several identified protein complexes (Balda et al. 1991). One interesting field of research is the role of TJs in the transit of ions and molecules through junctions between the epithelial or endothelial cells, called the paracellular route. Whereas lipophilic drugs are able to cross the epithelial or endothelial cell monolayer via the transcellular pathway, i.e. through the cells, hydrophilic drugs enter the tissue via the paracellular route. The delivery of hydrophilic drugs is enhanced via peptide-, siRNA-, or toxin-mediated manipulation of the TJ structures to open the paracellular route (Gonzalez-Mariscal, Hernandez & Vega 2008).

2.3.1. Signaling at tight junctions

Tight junctions are involved in two types of signal-transduction processes: signals from inside the cell towards forming tight junctions regulate their assembly, whereas TJ -derived signals towards cell interior affect gene expression, cell proliferation and differentiation (Matter, Balda 2003). Signaling to the tight junctions affects initiation and maturation of naive junctions and thereby affects the integrity of the junctional structure in the epithelial monolayer (Gonzalez-Mariscal, Chavez de Ramirez & Cereijido 1985). In Madin-Darby-Canine-Kidney (MDCK) cells, which form a polarized epithelial layer when grown in cell culture conditions (Gumbiner, Simons 1986), the calcium-dependent, E-cadherin-mediated cell-cell adhesion functions as the initial signal for the assembly of intercellular adhesions, which induces the formation of naive junctions containing TJ and AJ components (Figure 9). These then mature further to separate junctions. The maturation of tight junctions is affected by several proteins, including G proteins, calmodulin, PLC, protein kinase A and C family isoforms (Balda et al. 1991). The functions of these proteins on TJs might be opposite depending on the experiment and cellular context. For instance, inhibition of cPKC/nPKCs can either block the assembly or disassembly, suggesting that transient

activity, rather than persistent activation, is required for restructuring the dynamical tight junctions.

2.3.2. The multidomain tight junction protein Zonula occludens-1 (ZO-1)

Tight junctions are multiprotein complexes containing at least forty different proteins (Gonzalez-Mariscal et al. 2003) composed of integral membrane proteins that associate with cytoplasmic plaque proteins (Figure 10). The former mediate cell–cell adhesion, while the latter function as a bridge between the TJ and the actin cytoskeleton (Gonzalez-Mariscal, Tapia & Chamorro 2008). Of the integral proteins, occludin, junctional adhesion molecules (JAMs) and claudins with the 20 family members constitute the major strand builders of the tight junctions. Cytoplasmic proteins at TJs include cingulin, AF-6 (or afadin), MAGI-1,-2 and -3, 7H6, Pals and many others. The peripheral cytoplasmic proteins, the homologous Zonula Occludens (ZO) –1, -2 and -3 represent a diverse group of scaffold proteins linking the cell-cell adhesions to the cytoskeleton: these globular proteins belong to the membrane-associated guanylate kinase homologs (MAGUKs) (Fanning et al. 2007).

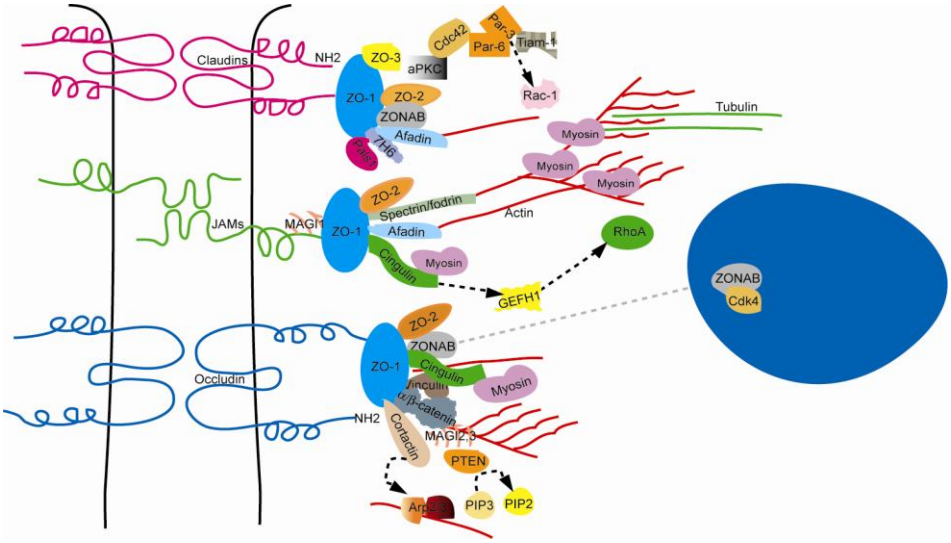

Figure 10. The tight junction complex. At the TJ, claudins, JAMs and occludin traverse the plasma membrane and connect to several cytoplasmic TJ adaptors. ZO-1 binds directly to the COOH- termini of the transmembrane proteins and to other ZO proteins, cingulin, catenins and afadin. Additionally, ZO-1 forms plaque structures with other actin(myosin) –linking proteins like spectrin/fodrin, cortactin (that regulates actin polymerization via Arp2/3) and polarity proteins like Par3 that control TJ assembly through PAR-6/aPKC-independent mechanism by regulating Rac1 activation via TIAM1 to formulate F-actin/myosin binding and cell adhesion. TJ formation (JAMs/Cingulin complex) contributes to the down-regulation of RhoA activation and actin-based motility in high-density epithelial cells by inhibiting GEFH1 (Guanine Nucleotide Exchange Factor-H1). ZO1 also regulates proliferation and interacts with the Y-box transcription factor ZONAB, a protein that is required for normal proliferation and can shuttle to nucleus to regulate e.g. Cdk4. Modified from www.sabiosciences.com/images/EpithelialTightJunction.

2.3.2.1. ZO-1 structure

ZO-1 is a large 225 kD phosphoprotein that exists in two isoforms, α- isoform which has restricted tissue expression e.g. in the dynamic TJs of endothelia and α+ isoform which

is expressed in all epithelial cells expressing less dynamic TJ strands and naive AJs (Balda, Anderson 1993). The structure is characterized by a core complex of protein-binding domains, including a guanylate kinase homology (GUK) domain and a Src homology 3 (SH3) domain intersected by unique (U) regions U5 and U6, COOH-terminal actin binding region (ABR) and three N-terminal postsynaptic density protein-95, disk large, zonula occludens-1 (PDZ) domains intersected by unique regions U1-4 (Fanning et al. 2007) (Figure 11). The function of the different modules in ZO-1 (and in all MAGUKs) is to bind various protein ligands in order to assemble multiprotein complexes. Crystallographic studies have illuminated the interconnection of some of these domains (Lye et al. 2010). The SH3 and GUK domains form a functional core by intramolecular interactions to collectively influence protein-protein interactions with other domains, and this core formation is coordinated by the U6 region (Fanning et al. 2007). Indeed, deletion of U6 region in ZO-1 was shown to displace its normal junctional localization thereby affecting the subcellular distribution of other TJ components.

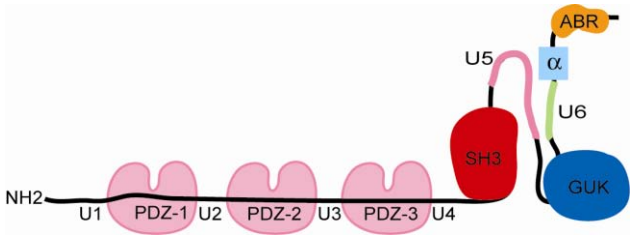

Figure 11. The ZO-1 structure. At the NH2-terminus, the three PDZ domains are flanked by unique regions 1-4; the middle part and COOH-terminus containing the SH3 and GUK domains interact and are flanked by unique regions 5 and -6 followed by the α isoform motif. In the COOH-terminus, the actin-binding region (ABR) mediates direct linkage to actin cytoskeleton.

PDZ domains are small, ~80-90 residue modules that bind to the 4-5 C-terminal peptide residues of various transmembrane proteins. They often homo- or heterodimerize with other PDZ domains and multimers to organize protein signaling complexes at specific cellular locations (Nourry, Grant & Borg 2003). The second PDZ domain of ZO-1 links to other ZO-1 molecules or occludin (Utepbergenov, Fanning & Anderson 2006). The crystal structures have revealed that the PDZ domains comprise of six β strands (βA to βF) and two α-helices, αA and αB, tightly organized into a globular structure. Moreover, peptide binding of the ligand takes place in an elongated surface groove as an antiparallel β strand interacts with the βB strand and the αB helix (Nourry, Grant & Borg 2003). They are vaguely reminiscent of those of PH/PTB domains in that six antiparallel β strands and an internal helix are arranged in a configuration similar to that in the PH/PTB domains (Balla 2005).

2.3.2.1.1. The biology of PDZ domain binding to short peptide ligands

PDZ domains are classified into three classes on the basis of the peptide sequences they prefer to bind (Songyang et al. 1997, Jelen et al. 2003, Nourry, Grant & Borg 2003). These always have a hydrophobic (valine, leucine or isoleucine) C-terminal residue, and a serine or threonine (Class I), a hydrophobic (tyrosine, phenylalanine or valine; Class II) or an acidic (Class III) residue at the –2 position. There are several basic

residues in some PDZ domains (many of which are conserved in some, but not all, PDZ domains) that form a basic pocket analogous to the inositide-binding site of PH domains relative to the peptide-binding site. In fact, phospholipid binding to syntenin PDZ domain has been shown to control its membrane localization (Zimmermann et al. 2002). In ZO-1 and ZO-2 PDZ2 domain, phospholipid binding occurs via identified residues in the binding groove, and binding controls e.g. the subnuclear localization of ZO-2 (Meerschaert et al. 2009).

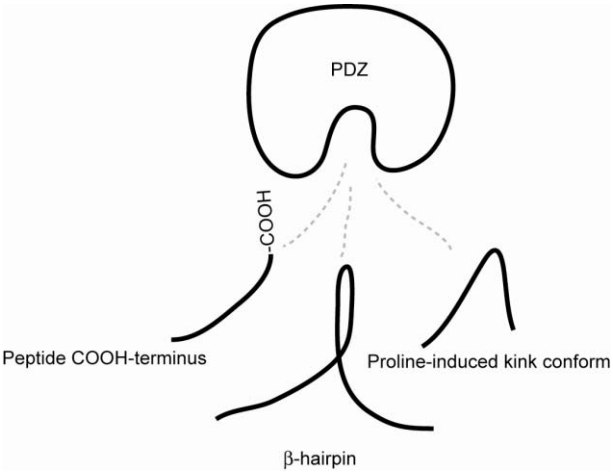

Figure 12. The ligand binding of PDZ domains. Ligand, or peptide, binding takes place in an elongated surface groove of the PDZ domain. In addition to the conventional protein COOH-terminus, binding of internal sequences in target proteins in the presence of kink conformation, induced by sharp β-hairpin structures or proline- induced turns in the peptide, can occur.

The interaction of PDZ domains with their COOH-terminal peptide ligands are conventionally thought to represent the only mode of binding. However, evidence exists to show that the modes of interaction can extend to include binding of internal sequences in target proteins in the presence of kink conformation, which still allows binding to the deep groove, in the target peptide (Hillier et al. 1999) (Figure 12). Such conformations could be induced by sharp β-hairpin structures or the amino acid proline- induced turns in the peptide, since an internal proline causes about 25° kink in a helical peptide (Cheng and Chang, 1999). Proline is unique among the 20 naturally occurring amino acids in its lack of an amide proton to provide a hydrogen bond donor. As a result, it is found more frequently in sharp turns than helical or β-strand structures (Cheng and Chang, 1999).

2.3.2.1.2. The actin-binding region (ABR) of ZO-1

The C-terminus of ZO-1 facilitates direct binding to the actin cytoskeleton, via a domain called the actin-binding region (ABR). This region is required to localize ZO-1 to an actin-rich pool at the free edge of cells before initiation of cell-cell contact (Fanning, Ma & Anderson 2002) and with actin is thought to place ZO-1 at the junctions in the intercellular junctions. Data indicating against this idea, however, is the finding that ZO-1 expression construct lacking the ABR still localizes to the

junctions in confluent MDCK cells (Fanning, Ma & Anderson 2002). However, accumulation of constructs lacking the ABR was markedly reduced at tight junctions in confluent cells, suggesting that the ABR does play a role in the localization of ZO-1 at junctions, probably by contributing to the stability of ZO-1. The stabilization of ZO-1 at the junctions is dependent also on myosin light chain kinase (MLCK), as inhibition of this protein prevented the exchange of ZO-1 and lead to enhanced accumulation and barrier function in the cells (Yu et al. 2010). Moreover, the ABR was shown to control this stabilization, since ZO-1 lacking the ABR was not stabilized in the presence of MLCK inhibitor.

2.3.2.2. ZO-1 function at intercellular adhesions

ZO-1 mediates the maturation of naive AJs to linear AJs (Yamazaki et al. 2008) and to TJs which requires Rac1 activation and myosin II recruitment to the naive AJs (Ikenouchi et al. 2007). In detail, the peripheral TJ protein afadin, through its two proline-rich regions, interacts with the SH3 domain of ZO-1 before the formation of TJs. Depletion of afadin impaired the maturation of both AJs and TJs in MDCK cells, whereas knockdown of ZO-1 impaired the formation of TJs, but not AJs. During and after the formation of TJs, ZO-1 dissociated from afadin and associated with JAM-A (Ooshio et al. 2010). These studies suggest that ZO-1 is not necessary for the initial formation of AJs but instead for the maturation into TJs, which includes polymerization of TJ-specific transmembrane strands, like JAM-A or claudins, the most abundant transmembrane proteins building the TJ structure. Claudins bind to ZO-1 PDZ1 domain via the COOH- terminal sequence, which is important for initiating the polymerization of claudins at the correct site claudins in the cell-cell junctions (Umeda et al. 2006).

2.3.2.2.1. Regulation of gene expression

Tight junctions and ZO-1 participate directly in the control of gene expression in epithelial cells (Balda, Matter 2000). In proliferating cells, a Y-box transcription factor ZONAB (ZO-1-associated nucleic acid-binding protein) localizes to the nucleus and binds to sequences of specific promoters containing an inverted CCAAT box (Figure 10). In (Balda, Matter 2000), ZONAB was demonstrated to bind to the SH3 domain of ZO-1 at TJs, where ZONAB and ZO-1 functionally interacted to regulate the receptor tyrosine kinase ErbB-2 (erythroblastic leukemia viral oncogene homolog-2) promoter in a cell density-dependent manner with consequences on paracellular permeability. The authors showed that in confluent cells, ZO-1 expression and that of ErbB-2 at the TJs was high with concomitant low expression of ZONAB at the TJs. Subconfluent cells, on the other hand, expressed low levels of ZO-1 and high nuclear ZONAB, which decreased ErbB-2 expression. ZONAB also localized at TJs of proliferating cells, but was not detectable in the nucleus of nonproliferating high density cells, suggesting that accumulation of ZONAB in the nucleus may be required for efficient proliferation (Balda, Matter 2000).

2.3.2.2.2. Endocytosis of tight junction proteins

When intercellular adhesions disrupt e.g. during the onset of cell motility, plasma membrane proteins reorganize and internalize into cellular compartments. The membrane-traversing tight junction protein occludin is endocytosed via caveolin-1 when induced with TNF thus increasing the MLCK- dependent paracellular permeability and diarrhea phenotype in mouse intestine epithelia (Marchiando et al. 2010). During cell division, integral tight junction proteins occludin and claudin-1 concentrate to the midbody whereas cytoplasmic ZO-1 and ZO-2 were shown to localize beneath the midbody, and the authors suggest that the transmembrane and cytoplasmic proteins are regulated in a different manner to reach these locations (Kojima et al. 2001). The internalization routes of ZO-1 are not well established, but some indications exist to show that it would occur via a clathrin-and caveolin-independent mechanism (Khandelwal, Ruiz & Apodaca 2010). The authors showed that during bladder voiding, recycling of membrane components and internalization of ZO-1 occurred in a β1 integrin- stimulated manner which depended on the vesicle scission GTPase dynamin, actin and RhoA activity (Figure 13).

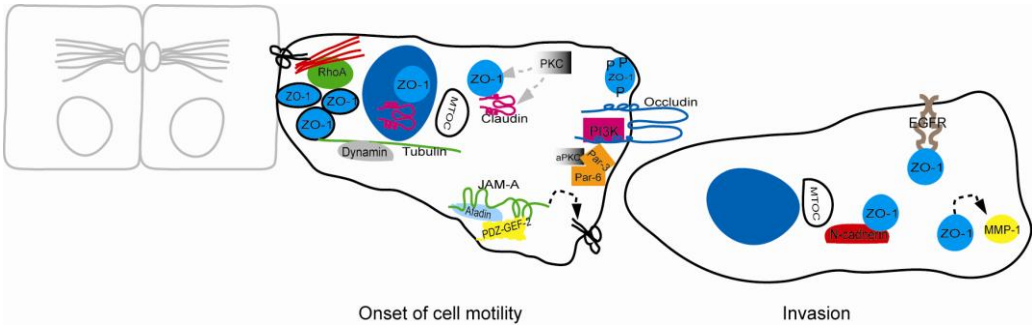

Figure 13. ZO-1 and other TJ proteins in cell motility and in invasion. At the onset of cell motility, the membrane-traversing tight junction protein occludin is endocytosed via caveolin-1-dependent manner and trafficked to the leading edge of the cell to regulate Par3-Par6-PATJ complex and PI3K- regulated actin motility. Internalization of ZO-1 can occur in a β1 integrin- stimulated manner which depends on the GTPase dynamin, actin and RhoA activity and ZO-1 can be found at the leading edge of fibroblasts heavily phosphorylated. Claudin and ZO-1 can be shuttled to nucleus upon cell motility via their nuclear localization signals; their subcellular localization is affected by PKCs. JAM-A, together with Afadin and PDZ-GEF-2, affects β1 integrin surface levels and cell motility. During melanoma or colorectal cancer cell invasion, ZO-1 associates with N-cadherin or EGFR, respectively, to promote invasion; ZO-1 can affect tumorigenesis also by triggering MMP-1 promoter by activating the β-catenin/Tcf/Lef transcriptional activity.

2.3.2.3. ZO-1 localization in motile cells

Epithelial cells control adhesion to the basement membrane and extracellular matrix to maintain an intact barrier that can reseal quickly in response to injury. Disruption of the epithelial barrier stimulates a fraction of cells to extend protrusions into the wound space, which can lead to intercellular junction disruption and release of proteins from their scaffolds to the cytoplasm, nucleus or to the leading edge. Retargeting these proteins to the migrating edge helps polarize migrating cells in the direction of movement via reorientation of the Golgi, MTOC and the microtubule cytoskeleton along the axis of migration (Brennan et al. 2010).

ZO-1 (and ZO-2 and ZO-3) contains several nuclear localization and nuclear export signals (NLS and NES, respectively) (Islas et al. 2002). In fact, ZO-1 has been reported to shuttle to nucleus upon cell stress or if cells are plated sparsely in cell culture conditions (Islas et al. 2002, Gottardi et al. 1996). It is known that the nuclear expression of ZO-1 can inhibit proliferation (Traweger et al. 2003). Also other TJ proteins, like the transmembrane protein claudin-1, travel to the cytoplasm or nucleus (French et al. 2009). It has been suggested that the translocation of claudin-1 into the nucleus may require ZO-1 or ZO-2, possibly as shuttles for claudin-1 (Dhawan et al. 2005). Also, ZO-1 has been reported to locate to the fibroblast lamellipodium, heavily phosphorylated, together with actin during wound healing process (Taliana et al. 2005) (Figure 13).

2.3.2.3.1. Other TJ proteins in cell motility

In addition to its functions as a TJ barrier integrity regulator, JAM-A was recently discovered to regulate integrin β1 expression levels by binding to afadin and PDZ-GEF-2 (that activates Rap-1) and thereby contribute positively to cell motility (Severson et al. 2009). Some known members of the cell polarity regulators, like the Par (partitioning defective)-Tiam1 complex, mediate apico-basal gradient during intercellular adhesion as well as front-rear polarization of motile cells thereby regulating local molecular composition or persistent migration (Pegtel et al. 2007). The translocation of these proteins, like the polarity complex Par3 (partitioning defective 3)-aPKC-Par6 (partitioning defective 6) to TJs is regulated by transmembrane junction proteins which triggers TJ formation to separate apical and basal domains (Joberty et al. 2000, Lin et al. 2000). Atypical PKC-Par3 proteins, regulated by PATJ (Pals1-associated tight junction) have been reported to be recruited to the leading edge of migrating cells and regulate the polarization of MTOC during wound healing (Shin, Wang & Margolis 2007). In MDCK cells, occludin knockdown disrupted accumulation of aPKC-Par3 and PATJ at the leading edge, and led to a disorganized microtubule network and misorientation of the MTOC during cell motility (Du et al. 2010). In addition, depletion of occludin abrogated the recruitment and activation of PI3K at the leading edge, which resulted in abrogation of actin protrusions and cell migration (Figure 13).

2.3.2.4. ZO-1 regulation via phosphorylation

2.3.2.4.1. PKCs

According to published literature and database search (Olsen et al. 2006, Gnad et al. 2007), ZO-1 contains over 30 phosphorylatable amino acids in its sequence, most of which (~30) are serine or threonine residues. The roles of PKCs have been frequently documented in ZO-1 function modulation and TJ restructuring events (Harhaj, Antonetti 2004). The PKCs phosphorylate serine and threonine residues in conserved sequences of conform R/K1-3_X2-0 _S/T-X2-0_R/K1-3 (Steinberg 2008) and putative PKC sites exist in ZO-1. The downregulation of ZO-1 was demonstrated by introduction of exogenous VEGF, which resulted in tyrosine phosphorylation and degradation of ZO-1. ZO-1 phosphorylation was reverted by chemical inhibition of

MAPK or PKC in nephritic cells (Leung et al. 2005). Tyrosine phosphorylation of ZO-1 has been linked to its down-regulation also in cancer (Kaihara et al. 2003). The PKC isoforms α and β dictated the subcellular localization of ZO-1 and paracellular permeability in colonocytes (Chen, Pothoulakis & LaMont 2002). In addition, the subcellular translocation of claudin-1 involved direct phosphorylation by PKC and PKA (Dhawan et al. 2005). Claudin-4- dependent TJ integrity was compromised in ovarian cancer cells by PKCε phosphorylation on two residues (D'Souza, Indig & Morin 2007) and occludin phosphorylation by PKCη on threonine residues had a crucial role in the assembly and/or maintenance of TJs in Caco-2 and MDCK cell monolayers (Suzuki et al. 2009). Together these data indicate the general contribution of PKC isoforms to the regulation of TJ proteins.

2.3.2.5. ZO-1 in cancer

ZO-1 can mediate pro- or antitumorigenic functions depending on the cellular context. ZO-1 expression level can change dramatically between different tumor stages in colorectal cancers (Kaihara et al. 2003). At the apical cell borders of normal colorectal epithelium, the luminal side of which has tubular gland structures, ZO-1 was expressed normally at the intercellular junctions. Tubular gland structures of colorectal cancer have been demonstrated to undergo dedifferentiation at the primary site, with the gland structures reforming in liver metastases. The authors showed that in contrast with the normal epithelium, the ZO-1 expression level was frequently reduced in primary CRC but was re-expressed again in liver-metastasized cancers. In addition, in primary colorectal tumors, ZO-1 associated with EGFR irrespective of the phosphorylation status of EGFR with concomitant high tyrosine phosphorylation of ZO-1. In liver-metastatic cancers, however, ZO-1 tyrosine phosphorylation was absent. The authors suggested that tyrosine phosphorylation of ZO-1 leads to its functional down-regulation and dedifferentiation of the glands in colorectal cancers, and these phenomena contribute to liver metastases. ZO-1 loss has been linked to poor prognosis in breast cancer (Hoover, Liao & Bryant 1998), with significantly reduced levels of TJ-associated ZO-1 in patients with metastatic disease compared to those remaining disease-free (Martin et al. 2004). In contrast, when expressed in melanoma, ZO-1 associates with the mesenchymal protein N-cadherin thus promoting cell invasion and adhesion (Smalley et al. 2005). ZO-1 can affect tumorigenesis also by activating migration-promoting proteins: ZO-1 was shown to induce MMP-1 promoter by activating the β-catenin/Tcf/Lef transcriptional activity (Polette et al. 2005) (Figure 13). Together, these data imply the important, albeit context-dependent role of ZO-1 in cancer progression.

3. AIMS OF THE STUDY

Shape changes are characteristic to both cell division and motility and require dynamic alterations of the cell cytoskeleton, vesicular compartments and the plasma membrane. On the plasma membrane, adhesive structures need to disassemble and rebuild again to facilitate and support protrusive activity, invasion through the matrix or cell rounding in mitosis. Loss of adhesion leads to cytokinesis defects in normal adherent cells. Integrins are cell surface receptors consisting of α and β subunits that are important regulators of cell motility. However, the exact role of integrins as adhesion mediators during cell division is enigmatic and unexplored.

Integrin adhesive functions in motile events are modulated by cytoplasmic adaptor proteins binding to the integrin cytoplasmic domain, or tail. Several such regulators are known, most of them acting via binding to the β subunit. In fact, compared to the β subunits, very little is known about the integrin function regulation via the diverse α subunits.

In the tissue, integrins are involved in mediating the adhesion between the cell and the immediate surrounding matrix. In intercellular adhesions that connect e.g. the epithelial cells in tissues, typically different types of molecules are involved. In the classical view, proteins from different adhesive structures act locally in a limited fashion by interacting with proteins only from the same compartment. Recently, however, some examples of cross-reactivity between different types of adhesions have been reported and the strict concept of adhesion protein dynamics has started to loosen. The general aim of this study was to identify new integrin tail binding proteins regulating integrin adhesion and functions in cell motility and, possibly, in cell division.

The specific aims of this study were:

- To analyze Rab21-mediated integrin function during cell division and integrin targeting to the cleavage furrow
- Identifying integrin α5 cytoplasmic tail binding proteins that regulate its subcellular localization during lung cancer cell motility
- To study the co-localization and co-operation between integrin α5 and tight junction protein ZO-1 in cell motility
- To explore the function of ZO-1 in cytokinetic structures

4. MATERIALS AND METHODS

More detailed information on methods and reagents is available in the original publications (I-III). * = generated for these studies.

DNA constructs and peptides

pEGFP-Rab21 and variants (I); DsRedm-Rab21* (I); pIRES2-a2-EGFP and variants *(I); siRNA-resistant α5WT and α5PPAA* (II,III); ZO-1-GFP (II, III); ZO-1-GFPrescue* (II); ZO-1WT-Flag and mutants ZO-1S168A-Flag*, ZO-1S168D-Flag* (II,III); ZO-1PDZ1-3-Flag (II); ZO-1-N-GFP, ZO-1-C-GFP, GFP-PKCεwt, the GFP-PKCεwt kinase-dead mutant, GFP-PKCαwt and GFP-PKCα(T497A) (II); GFP-α5 (I); mCherry-α5 (III); GFP-RBD (I,III); GFP-PH-PLCδ (III); GFP-ZO-1K253A* (III). GST-ZO-1PDZ1-3 (II,III); TAT-α5SDA (II,III); TAT-α5ADA, TAT-α5PPAA (II).

SiRNAs

- SCRamble Qiagen's AllStars Neg. Control siRNA (1027281)
- Rab21 5'AAGGCATCATTCTTAACAAAG-3'
- Clathrin heavy chain predesigned SMARTpool duplexes, Dharmacon (4 duplexes)
- FIP3 5'-AAGGCAGTGAGGCGGAGCTGT-3' [from (Wilson et al. 2005)]
- α5 integrin 5'ATCCTTAATGGCTCAGACAT-3' (validated siRNA from Qiagen)
- ZO-1a 5'AAAACAGTCACTCCAGCATAC-3'
- ZO-1b 5'AAGATATTGTTCGGTCCAATC-3' (from (McNeil, Capaldo & Macara 2006))
- PKCε a 5'-GAUCGAGCUGGCUGUCUUUTT-3'
- PKCε b 5'-AAAGACAGCCAGCUCGAUCTT-3' [from (Kermorgant, Zicha & Parker 2004)]

Cell lines

Cell line	Description	Used in
NCI-H460	Non-small lung cancer cell	(I,II,III)
MDA-MB-231	human breast cancer cell	(II,III)
GD25β1 and mutant variants	murine fibroblasts	(I)
MEF-PKCε-/- and MEF-PKCεRE	mouse embryonic fibroblasts	(II)
KF28 and KFr13	human ovarian cancer cells +cisplatin-resistant variant	(I)
COS-7	immortalized monkey cells	(III)
Hela	human cervical cancer	(I)
HEK-293T	human embryonic kidney	(I)
CHO	Chinese hamster ovary	(I)

Antibodies

Target epitope	Description	Application	Used in
Integrin α5	mouse (Serotec MCA 1187)	Immunocytochemistry	(II,III)
Integrin α5	mouse MAB1999, Chemicon	IF	(II)
ZO-1	mouse mAb, Zymed	IF	(II,III)
ZO-1	rabbit pAb, Zymed	IF, WB	(II,III)
Occludin	rabbit, Zymed	IF	(III)
α-tubulin	rat 6160-100, Abcam	IF	(I,III)
Rab21	rabbit pAb sera, Innovagen	IF	(I)
β1-integrin	mouse mAb 12G10, Chemicon	IF, FACS	(III)
β1-integrin	rat mAb 9EG7, Amersham	IF	(I)
β1-integrin	mouse P5D2, Developmental Studies Hybridoma Bank, Iowa	IF	(I)
β3-integrin	M109-3MBL, MBL	IF	(I)
Plk1	mouse 14209-50, Abcam	IF	(I)
GFP	rabbit pAb, InVitrogen	WB, IP	(II)
Vinculin	mouse mAb, Sigma-Aldrich	IF	(II)
FLAG	mouse mAb M2, Sigma-Aldrich	IF	(II,III)
Integrin α5	rabbit 1949, Chemicon	IF	(I,II)
Clathrin	rabbit ab21679, Abcam	IF	(I)
β1-integrin	mouse MCA2025, Chemicon	IF, FACS	(I, II)
α4-integrin	mouse MAB16983,Chemicon	FACS	(II)
FAK	goat sc-557-G, Santa Cruz Biotechnology	IF	(II)
αV -integrin	L230, Alexis Chemicals	FACS	(II)
β1-integrin	MAB2252, BD Transduction Laboratories	WB	(II)
PKCε	rabbit antisera	WB	(II)
Incenp	rabbit 36453, Abcam	IF	(I)
Aurora B	rabbit 2254, Abcam	IF	(I)

Reagents and compounds

Reagent	**Application**	**Used in**
HiPerfect (Qiagen)	Transfection of siRNAs	(I,II,III)
Lipofectamine 2000 (Invitrogen)	Transfection of DNA	(I,II,III)
OptiMem (Dulbecco's)	Transfection solution	(I,II,III)
Calphostin C	Chemical inhibition of PKC	(II,III)
GFP-RBD	Detection of active Rho	(I,III)
DAPI	Nuclear stain	(I,II,III)
TPA/PMA	Chemical activation of PKC	(II,III)
ATP-γ-32P	Kinase activity assay	(II,III)
Phalloidin-488/561/647	Staining filamentous actin	(I,II,III)
Fibronectin (FN)	Matrix coating of dishes	(I,II,III)
Collagen	Matrix coating of dishes	(I,II)
Laminin	Matrix coating of dishes	(I)
Vitronectin	Matrix coating of dishes	(I)
Wst-1	Proliferation reagent	(I)
Vectashield	Anti-fading agent, Vector labs	(I,II,III)

Experimental procedures

Method	**Used in**
Cell culture	(I,II,III)
Yeast two-hybrid	(I)
DNA cloning	(I,II)
Site-directed mutagenesis	(I,II,III)
Immunofluorescence microscopy	(I,II,III)
Immunoprecipitation (IP) and Western blotting (WB)	(I,II,III)
Live-cell microscopy	(I,II,III)
Adhesion assay	(II)
Taqman PCR	(I)
Migration and invasion assay	(II)
Statistical analysis	(I,II,III)
In vitro kinase assay	(III)
DNA and siRNA transfection	(I,II,III)
Phosphoprotein extraction (Qiagen)	(II)
ELISA binding assay	(II)
Immunohistochemistry	(II)
In situ proximity ligation	(II)
PKC kinase assay	(II)
Rac activity assay	(II)
Integrin internalization assay	(II)

5. RESULTS

5.1. Rab21 mediated integrin traffic and adhesion at the cleavage furrow (I)

Many of the molecular requirements for successful cell motility are identical to those functioning during cell division (Glotzer 2005). Integrins have not been seriously looked at in the context of cell division, despite their important role in cell motility and the knowledge regarding adhesion-dependent cytokinesis in adherent cells. The recent work from our laboratory describing the GTPase Rab21 in integrin traffic and adhesion by binding to the integrin α tail prompted us to look at the Rab21-mediated integrin profile during the events of cell division. First, to gain insight on integrin localization in dividing cells, cells in different phases of mitosis were examined. During prometaphase and metaphase, β1 integrin localized diffusely in the cell (I: Figure 1A, B). Upon furrow ingression in early telophase, integrins started to accumulate at the cleavage site and confocal imaging revealed the β1 integrin in close proximity to the underlying matrix (I: Figure 1C-1E) suggesting that integrins might work at the furrow as mechanical anchors or in cell-matrix signal transduction during cell division. To evaluate whether integrin traffic during cell division is needed, the role of Rab21 was analysed. The expression of the GFP-fused GTP-locked mutant of Rab21 caused severe cytokinesis defects in several cell lines when monitored with immunofluorescence or live-cell analysis (I; Figure 2A, B, C, S1A, B, S4A). The observed defects were not due to impaired central spindle assembly (I: Figure 2D, S2A-C) that could lead to similar cytokinesis defects (Glotzer 2005) but rather indicated that the Rab21GTP- induced defect occurs at a later stage after normal spindle formation.

Rab21 belongs to the Rab5 superfamily of small GTPases that share several effector proteins and thus mediate some overlapping functions (Simpson,J.C. 2005). To get more insight on the specific role of Rab21, the integrin recruitment to the furrow of NCI-H460 cells was analyzed in the absence of Rab21. In Rab21siRNA treated cells the integrin localization at the furrow was lost (I: Figure 3C). The observation of integrin at the opposing poles of the daughter cells implies that Rab21 could mediate traffic also there. This was studied further by monitoring the temporal activity of these proteins with live-cell imaging of Rab21 and integrin α5. The two proteins co-localized in (trafficking) vesicles in cells undergoing furrow ingression (I: Figure 3E). The co-localization at the furrow increased later in telophase and continued at the opposing poles at the end of the division where daughter cells increase adhesion to the substratum and pull away from each other. The β1 integrins can traffic via other Rab proteins as well, and the Rab11-interacting proteins FIP3 has been implicated in integrin traffic to the cleavage furrow (Wilson et al. 2005). To test if this route is active also in cells where Rab21 directs integrin traffic, we silenced FIP3 and monitored β1 integrin localization in these cells. FIP3 silencing caused telophase arrest and accumulation of β1 integrins in perinuclear regions of cells (I: S3A, B). Also, in contrast to the Rab21-silenced cells, integrins were detected at the furrow of FIP3-

silenced cells (I: S3A, arrow). These results would indicate that Rab21 and Rab11-FIP3 pathways both regulate β1 integrins during cell division, but that the mechanisms differ probably by local and temporal regulation of the effector proteins.

Rab21 associates with the conserved membrane proximal GFFKR sequence found in almost all α subunits of integrin heterodimers (Hynes 2002). Mutations to the critical residues in the conserved sequence in the collagen-binding integrin α2, the highly conserved R1161 to alanine (α2AA) or the α- helical structure forming K1162 to alanine mutation (α2P), abolishes association with the Rab21 (I: Figure 4A and (Pellinen et al. 2006). The association drives integrin traffic and facilitates cell motility (Pellinen et al. 2006). We hypothesized that interaction of Rab21 and integrin α2 may be needed during cell separation, and expressed these mutants in CHO cells (which lack endogenous collagen-binding integrins, including α2). Indeed, cells expressing Rab21-binding deficient collagen-binding integrins failed to undergo cytokinesis even if they adhered normally to their substrate collagen (I: Figure 4B, C). However, expressing α2WT or an α2 mutant (α2ARA) still capable of Rab21 association, executed normal cell division. Expectedly, the traffic of these mutants correlated with the immunodetection studies. While the α2ARA was efficiently internalized from the cell surface, α2AA failed to follow endo/exocytic routes in the cell (I: Figure 4D) which was observed also in confocal studies showing the lack of vesicles in the presence of α2AA mutant (I: Figure 6D). Adhesion and cell division in cells expressing the mutants was normal when they were plated on the matrix fibronectin and thus could adhere via functional endogenous fibronectin-binding integrins. Taken together, these data imply that Rab21 association with the integrin α subunit conserved sequence is needed for the cell division which depends on Rab21-mediated traffic.

The integrin β1 subunit tail harbours two conserved NPxY motifs, found also in other cell surface receptors, that bind to clathrin adaptor proteins in endocytosis (Ohno et al. 1995). We speculated that clathrin-mediated endocytosis of integrins might be functional during cell division. We investigated the effects of this NPxY mutation (Y783, Y795 mutated into phenylalanines) in β1-null mouse embryonic fibroblast cells reconstituted with wild-type β1 (GD25β1WT) or β1YYFF (GD25β1YYFF) (Wennerberg et al. 2000). Mutation to the conserved tyrosines altered integrin localization to pronounced focal adhesions and interfered with internalization of integrin (Figures 5A), but not transferrin (I: Figure S4B). In contrast, the cells reconstituted with GD25β1WT integrin was found intracellularly and efficiently internalized from the cell surface. The GD25 cells are from β1 null differentiated embryonic stem cells and could have developed phenotypes irrelevant to integrin function. Thus, we used two separate MEF cell lines, isolated from β1 null embryos that carry this mutation in the germline. In these cells, the YYFF mutation failed similarly to endocytose the integrin (I: Figure 5B). To evaluate the effect of integrin endocytosis to cell division in these cells, β1wt MEFs or β1YYFF MEFs were plated on β1–specific matrix laminin and scored for nuclear status. Interestingly, the cells which adhered via β1YYFF integrin, failed in cytokinesis which resulted in cell population of bi- and multinucleated cells (I: Figure 5C, D, 6A). It is shown that in the

presence of a non-functional integrin, MEFs upregulate the integrin isoform β3 (Danen, Sonnenberg 2003). Indeed, in GD25 cells on fibronectin, β1WT was recruited to the cleavage furrow while in GD25β1YYFF cells, the β3 was upregulated. Moreover, overexpression of Rab21, but not Rab7 which is unable to associate with integrins, in the cells expressing the YYFF integrin rescued the cytokinesis defect and integrin internalization (Figure 6A, B). However, Rab21 seemed to induce integrin internalization by a clathrin-independent mechanism, as the clathrin inhibitor monodansyl-cadaverin did not inhibit β1YYFF endocytosis but did prevent β1WT internalization (I: Figure S5A, B).

The fact that altered chromosomal quantity, aneuploidy, can drive tumorigenesis, is not a new idea but instead was recognized nearly a century ago (Boveri, 1914). Now it is becoming clear that aneuploidy, originating from defects in the precise control of cell cycle can cause tumorigenic properties to cells (Fujiwara et al. 2005). So we asked what is the relevance of Rab21-regulated integrin traffic *in vivo*? If the cytokinesis defect is only observed when integrin function on its specific matrix is inhibited, what can be the biological relevance? We performed a CGH (comparative genomic array) analysis in two ovarian cancer cell lines (KF28 and KFr13) and a prostate cancer tumor. Deletions in *RAB21* gene locus on chromosome 12 were found in the KFr13 cells and in the prostate tumor (I: Figures 7A, S6). Intriguingly, cell culturing displayed differences in the ploidy of the two cell lines. The parental cell line that expressed Rab21 was mononuclear, whereas the variant, and the parental cell line upon Rab21 silencing, showed an increased trend towards multinuclearity (I: Figure 7C, D). Importantly, the multinucleated phenotype could be reversed by the exogenous expression of Rab21 into the KFr13 cells (I: Figure 7D). This result and other data strongly favors the idea that abrogations in integrin traffic and the consequent defects in executing cell division could link to the real clinical samples and thus present one mechanism of how multinucleation can arise in tumorigenesis.

5.2. The integrin α5 –ZO-1 complex formation (II, III)

In an intact epithelial cell monolayer, adjacent cells are held together by intercellular adhesions while the monolayer is firmly adhering to the underlying basement membrane via cell–matrix adhesion. This homeostatic structure is disrupted in carcinogenesis whereby epithelial intercellular adhesions weaken and cell-matrix adhesions containing integrins are altered. Tight junctions are epithelial junctions, which reside on the apical face of the monolayer and whose integrity is often lost in cancer. One of the first identified tight junction protein, ZO-1, protects intercellular integrity by binding to transmembrane proteins and linking the structure to actin cytoskeleton. Expectedly, the function of ZO-1 is altered in carcinogenesis but by mechanisms that are not understood. In epithelial lung carcinoma, integrin α5 expression increases (Adachi et al. 2000). The frequency of its role in supporting cancer cell motility is established; the possible mechanisms, however, remain elusive. We investigated α5 regulation in lung carcinoma cell line NCI-H460. We detected a dramatic change in subcellular localization of the integrin α5 from the intercellular adhesions to the leading edge of the cells upon cell motility (II: Figure 1A). This

correlated with increased α5 surface expression in motile cells and could not be detected with all α isoforms, like α2 (Figure S1B). The localization correlated with cell motility and invasion. Whereas SCRsiRNA treated cells invaded through the three-dimensional fibronectin-Matrigel matrix, inhibition of α5 with siRNA or antibody significantly decreased this (Figures 1B, C) which is likely a result of lost integrin-rich lamellipodia (Figure S2C). Also, wound closure and motility in 2D was prevented in α5/β1 antibody treated cells (Figures S1B, S2A) but not when treated with α2 antibody. These results confirm an active contribution of integrin α5 in cell motility that was not due to altered integrin α5 expression or proliferative activity (Figure S1A, C).

The altered subcellular localization of integrin α5 suggested the involvement of associated molecules that could regulate this movement from intercellular sites to the leading edge. Integrin α5 (and α6) COOH-terminus contain sequence (T)SDA which can be bound by class I PDZ binding motifs (Nourry, Grant & Borg 2003), and in fact, the TIP2/GIPC protein recognizes this sequence via its PDZ-binding domain (El Mourabit et al. 2002). This study encouraged us to look at the most common proteins that contain PDZ domains and reside in intercellular junctions. One such candidate was a tight junction protein ZO-1, which contains three PDZ domains (Fanning, Ma & Anderson 2002), via which it binds to short PDZ binding motifs in COOH-terminal ends of various proteins (Songyang et al. 1997), and is able to leave its junctional localization upon cell stress or in sparse cell culture conditions (Islas et al. 2002, Gottardi et al. 1996). In immunolocalization studies, ZO-1 was detected in the lamellipodia upon scratch-wounding or in subconfluent cells and co-localized with integrin at the free edges of elongated cells (II: Figure 2A, lower panel and 2B, right panel). In contrast, in confluent cells which displayed intense ZO-1 staining restricted to the subapical part of lateral membranes, the integrin was detected in basolateral membranes when monitored with confocal microscope (II: Figure 2A, upper panel and 2B, left panel). Of note, co-localization in lamellipodia was detected also in MDA-MB-231 breast cancer cells, demonstrating that the co-existence of these proteins could be relevant also in other cells (II: Figure S4A).

To characterize further whether association between the integrin and ZO-1 exists and whether it could be regulated by cell confluency, NCI-H460 cells were seeded in plates and allowed to grow until confluent or subconfluent and exposed to integrin immunoprecipitation. Co-precipitation with ZO-1 was detected in subconfluent cells, whereas confluent cells containing junction-associated ZO-1 did not support integrin-ZO-1 association (II: Figure 3A). Also, an in situ antibody-based detection method (proximity ligation assay, or PLA) which reveals close proximity (10-100 nm) between two proteins by fluorescent signal was used to assess the local interaction between integrin and ZO-1 (Soderberg et al. 2008). As expected, the PLA signal was detected only in the lamellipodia of cells at the wound edge, whereas in intercellular junctions no signal between integrin and ZO-1 antibodies was seen (II: Figure 3C, D). This implies that integrin α5 and ZO-1 associate preferably in the lamellipodia whereas in

cells connected with intercellular junctions, ZO-1 and integrin are involved in separate types of adhesions.

Several integrin tail binding proteins have been characterized (see tables I and II in the Literature Review). However, most of the studies have assessed these molecules regarding the β subunit tails, whereas research into the functional contribution of the α subunit has been more limited. Regulatory proteins, like Rab21 or calreticulin, associate with the conserved motif GFFKR found in almost all integrin α subunits. Therefore, we wanted to assess the specificity of the interaction between ZO-1 and α5 integrin. According to flow cytometry analysis, the integrin α2 subunit was expressed in NCI-H460 cells similarly to α5 (II: Figures S1A, S2B, S8E) and thus its association with ZO-1 was tested. Co-immunoprecipitation was detected between ZO-1 and α5 integrin, but not with α2 subunit indicating that binding is most likely not mediated via the conserved sequences in α subunits but rather in an subunit-specific way (II: Figure 3B).

ZO-1 sequence entails various protein-protein interaction domains, and one of its functions is to anchor the transmembrane molecules to the intercellular junctions via its NH2- terminal PDZ domains and link via the COOH-terminal proline-rich region to the actin cytoskeleton and signaling pathways (Fanning, Ma & Anderson 2002). To identify which region of ZO-1 mediates the binding to integrin α5, expression constructs comprising of either the COOH-terminal (ZO-1-C-GFP) or NH2-terminal (ZO-1-N-GFP) parts of ZO-1 were used in a co-immunoprecipitation and localization study. According to the data, the NH2-terminus of ZO-1, but not the COOH-terminus or GFP alone, is capable of binding to integrin α5 (II: Figure 4A) and which seemed to be refined to the lamellipodia (II: Figure 4B). Furthermore, expression of the NH2-terminal ZO-1 supported NCI-H460 cell motility, whereas cells expressing the COOH-terminal ZO-1 construct displayed non-motile behaviour in time-lapse imaging (II: Figure 4C, D). ZO-1 has been shown to relocalize from the tight junctions to the cytoplasm during EMT and expression of PDZ domains of ZO-1 is sufficient to induce EMT like changes in MDCK cells (Polette et al. 2005, Reichert, Muller & Hunziker 2000). In NCI-H460 cells, the expression of a construct consisting of only the three NH2-terminal PDZ domains (ZO-1PDZ1-3Flag) induced and exhibited lamellipodial localization together with integrin α5 (II: Figure 4E), thus indicating an actin-independent movement of ZO-1 to the leading edge together with integrin α5, which has implications in cell motile behavior.

Upon silencing of ZO-1, integrin α5 was nevertheless able to localize in protrusions at the front of the cells (II: Figure 2F). However, in comparison to the broad integrin – containing lamellipodia of SCRsiRNA cells, the ZO-1siRNA cells failed to form these kind of structures but instead displayed multiple smaller protrusions containing integrin α5, together with observed retractions in between the cells (II: Figure 2G). This phenotype correlated with decreased motile activity, since ZO-1siRNA treated cells portrayed decreased migration in 3D Transwell assays and decreased persistent motility (and increased velocity) on 2D matrix surfaces over time (II: Figures 2C-E). Importantly, the loss of broad lamellipodia in ZO-1siRNA cells could be rescued with

exogenous siRNA-resistant GFP-ZO-1 (II: Figure 2G). So it is possible that transport of integrins to the leading edge occurs independently of ZO-1, but that ZO-1 is needed in modulation of adhesions to promote cell motility. This theory would be supported by the altered adhesion complex phenotype (II: Figure S4B) and increased Rac activity in ZO-1siRNA cells (Figure S5). These data are in line with the knowledge of increased Rac1 activity regulating transient protrusions and inhibiting directional cell motility (Pankov et al. 2005).

5.2.1. Direct interaction of integrin α5 and ZO-1

To assess whether the binding between integrin and ZO-1 is direct, an ELISA assay that measures interaction between GST-ZO-1PDZ1-3 and integrin α5 cytoplasmic tail sequence fused to TAT-peptide, was performed. Indeed, the peptide comprising of seven COOH-terminal residues of integrin α5 bound the bacterially produced GST-ZO-1PDZ1-3, and not the GST alone (II: Figure 5A). When the critical serine in the integrin α5 and α6 tails was mutated to alanine, the binding of TIP2/GIPC PDZ domain to integrin was abolished (El Mourabit et al. 2002). Thus, we speculated that the serine^{1047}, situated in the putative PDZ-binding motif, might regulate the binding of GST-ZO-1-PDZ1-3 to the integrin α5. Mutating that serine had, however, no effect on the binding (II: Figure 5A). Thus, the interaction observed had to be mediated by some of the remaining residues in the peptide. Internal sequences, in the context of hairpins or β-turns, in PDZ-binding proteins have been shown to interact with the PDZ peptide binding groove, and sometimes even competing with the linear ends of peptides (Hillier et al. 1999). In the integrin α5 tail, two sequential prolines could introduce a kink conformation and thus serve as a structure that can be recognized by the PDZ domain. To evaluate this possibility, we used a peptide where the two prolines were changed to alanines and measured for interaction in a similar ELISA binding assay. The alanine substitution in the integrin tail decreased the interaction with the ZO-1PDZ1-3 as much as ~50 % when compared to wild-type integrin tail peptide (II: Figure 5A). This suggests that the two prolines in the integrin tail serves to introduce binding sites to scaffold domains like PDZ. The contribution of this binding site was assessed additionally in vivo. NCI-H460 cells were silenced with integrin α5siRNA, and transfected with integrin α5 rescue constructs containing either the wild-type sequence (α5WT rescue) or the double proline-to-alanine mutation (α5PPAA rescue). Whereas the wild-type α5 construct rescued the lamellipodial phenotype that was absent in α5-silenced cells, the mutant was unable to rebuild prominent lamellipodia but instead was observed in smaller protrusions throughout the cell surface (II: Figure 5B, C). The defect was however not due to altered adhesive properties of the cells (II: Figure S6A, B). The abrogated phenotype was evident also in live cell recordings of α5siRNA- silenced and rescued cells. The wild-type promoted lamellipodial protrusion in the direction of movement, whereas the mutant cell displayed random motility with numeral smaller and less stable protrusions (II: Figure S6C, 5D).

The fact that we saw the complex formation primarily in cells with motile phenotype, led us to ask the question of the possible existence of integrin α5-ZO-1 complex in vivo. By using the same PLA *in situ* detection method as in *in vitro* assays (II: Figure

3C) we stained 48 paraffin-embedded lung carcinomas with antibodies against α5 and ZO-1. Out of these, 3 samples with metastasis signature, stained positive for the complex whereas non-metastatic carcinomas were negative for α5-ZO-1 association (II: Figure S7). The positive signal was detected only in a minute fraction of cells in the tumor mass, which could relate to the in vivo demonstration of the metastatic process, where only a small number of cells detach from the tumor to facilitate dissemination in the body (Sahai 2007).

In addition to the observed subcellular change in localization during cell motility, integrin α5 was detected also in the cleavage furrow of dividing NCI-H460 cells, co-localizing with ZO-1 (III: Figure 1C). Given the results of Rab21-regulated integrin β1 recruitment to the cleavage furrow (I: Figure 1A-E), we wanted to investigate the role of integrin α5 specifically mediated by ZO-1 during cell division. During constriction in anaphase, integrin α5 ablated cells expressing α5WT rescue underwent normal separation with characteristic tubulin-driven midbody formation in telophase (III: Figure 1B). The α5siRNA treated cells expressing the α5PPAA rescue construct, however, failed to execute the symmetrical actin-driven constriction in elongated anaphase cells (III: Figure 1B). In addition, live cell imaging of α5siRNA cells revealed defects in daughter cell separation, as tripolar division or cell fusion was frequently detected, resulting in one or two daughter cells with abnormal midbody positioning as detected with tubulin and Plk-1 antibodies (III: Figure 1A). To characterize the temporal regulation of these proteins during cell division, COS-7 cells were co-transfected with GFP-ZO-1 and mCherry-a5 and the movement followed over time with time-lapse microscopy. The live cell analysis demonstrated that integrin α5 moves in vesicle-like structures towards the cleavage furrow in anaphase. ZO-1, however, moves on the membrane and concentrates inbetween the daughter cells and forms a midbody-like structure that finally adapts an intercellular adhesion-like form (III: Figure 1D). These results would suggest that integrin α5 and ZO-1 reach the cleavage furrow of NCI-H460 cells independently and that the interaction is essential for normal separation in NCI-H460 cells.

5.3. The regulation of integrin α5 and ZO-1 by PKCε (II, III)

Integrin α4 cytoplasmic tail is phosphorylated by PKA at S988 which prevents binding of paxillin and instead favors its translocation to the leading edge to favour cell motility (Han et al. 2001). This suggested to us the possibility that ZO-1 binding to the integrin tail in subconfluent cells might be regulated by integrin α5 tail phosphorylation. To evaluate this, radioactive phosphate incorporation to NCI-H460 cells extracted from confluent or subconfluent conditions was done in the presence of phosphatase inhibitors. The data showed, however, no incorporation, indicating that α5 integrin isoform is not phosphorylated (II: Figure S8A) and thus is not the mediator of subcellular complex formation. ZO-1 localization at the junctions is controlled by growth factor- and cytokine- mediated phosphorylation at the intercellular junctions (Harhaj, Antonetti 2004). To test whether the translocation of ZO-1 to the leading edge requires phosphorylation, the junction-associated or lamellipodial ZO-1 was compared in confluent and subconfluent cells, respectively, by using an affinity column that collects

all phosphoproteins from the eluates. We could see more phosphorylated ZO-1 in subconfluent cells, with lamellipodial phenotype whereas total cellular ZO-1 or FAK phosphorylation were not markedly altered (II: Figure 6A). Frequently, the subcellular localization of ZO-1 is regulated by some member of the PKC kinase isoforms (Avila-Flores et al. 2001). Indeed, the PKCε was seen with ZO-1 in the lamellipodia of NCI-H460 cells whereas the co-localization with PKCα was absent (II: Figure 6B). In fact, expression of kinase activity deficient PKCε induced massive outgrowth of long protrusions which mislocalized the ZO-1 (II: Figure 6B, upper panel). Similar defect was observed in NCI-H460 cells treated with PKCεSiRNA, where the subcellular localization of both α5 and ZO-1 changed dramatically (II: Figure 6C). Upon PKCε silencing, which was 70-100% in all experiments (II: Figure S3C; III: Figure 5F), the integrin α5 was observed diffusely in the cytoplasm and ZO-1 additionally in the nucleus whereas in SCRsiRNA cells the localization was in the leading edge lamellipodia (II: Figure 6C). To investigate the contribution of PKCε to the subcellular localization of α5 and ZO-1 in more detail, we used mouse embryonic fibroblasts (MEFs) null for PKCε or reconstituted with full-length PKCε for immunodetection (Ivaska et al. 2002). In the presence of PKCε, α5 and ZO-1 were detected in membrane ruffles, which was not seen in PKCε null cells. In addition, the MEFs null for PKCε displayed a similar nuclear ZO-1 to that observed in PKCεSiRNA treated cells (II: Figure 6D). These data implicates PKCε in control of the subcellularly restricted α5-ZO-1 complex formation.

Traffic of β1 integrins can be regulated by PKCε during cell motility (Ivaska et al. 2002). Since PKCε seemed to be essential in regulating the lamellipodial α5-ZO-1 recruitment, we went ahead to investigate if PKCε kinase activity controls this. In scratch-wounded NCI-H460 cells, compared to confluent intact cells, activation of PKCε was substantially increased, with coinciding integrin α5 surface upregulation (II: Figures 6E-G). This, however, does not occur via changes in integrin turnover from the membrane (II: Figure S9) or adhesion to the matrix, as cells treated with SCRsiRNA, ZO-1siRNA or PKCεSiRNA adhered equally well to fibronectin matrix (II: Figure S8E). This indicates that kinase activity of PKCε regulates integrin α5 release to the cell surface and/or affects α5-ZO-1 complex formation locally during cell motility. This is supported by the finding that the α5 integrin-ZO-1 complex was detected in PKCεRE but not PKCε null cells (II: Figure 7A). Additionally, with chemical inhibition by Calphostin C, which prevents PKC activation by DAG, the lamellipodial formation was inhibited. This altered the localization of both integrin α5 and ZO-1, and the molecules were prevented from reaching the leading edge (II: Figure S8C, D).

Given the results of PKC-mediated control of α5-ZO-1 interaction, we hypothesized that the α5-ZO-1 complex formation is regulated by PKCε phosphorylation. Indeed, in phosphoaffinity column, the presence of phosphorylated ZO-1 was decreased by 50 % in cells silenced or null of PKCε compared to SCRsiRNA or PKCεRE cells, suggesting a PKCε-mediated ZO-1 phosphorylation in subconfluent cells (II: Figure 7B). In line with this, in an in vitro assay, PKCε was found to directly phosphorylate the substrate GST-ZO-1-PDZ1-3 (III: Figure 5C). This implicates PKCε in direct control of ZO-1. Indeed, upon knock-down of PKCε in NCI-H460 cells, we identified similar aberrant

midbody phenotype in cells undergoing cell division as we did with ZO-1siRNA (III: Figure 5D). PKCε activity, however, has been linked to successful cytokinesis already by others and it is shown that inhibiting PKCε or its association with 14-3-3 protein leads to aberrant cytokinesis with similar defects in sustained daughter cell connection and aberrant midbody formation (Saurin et al. 2008). In live cell imaging of PKCεSiRNA cells, the daughter cells underwent aberrant division and resulted in one progeny cell due to one daughter cell dying or fusing with the other cell whereas in SCRsiRNA cells the division was completed in less 30 minutes (III: Figure 5). This suggests that PKCε might work upstream of ZO-1 in regulating cytokinesis, but due to its involvement with other events regulating cytokinesis, like association with 14-3-3, might display more severe defects than ZO-1 silencing alone.

5.4. RhoA targeting in Rab21siRNA and ZO-1siRNA cells (I, III)

RhoA is well-known for its role in regulating the acto-myosin constriction in anaphase by concentrating and activating at the site of the furrow (Bement, Benink & von Dassow 2005, Yoshizaki et al. 2003) and defects in executing the final steps in telophase have been linked to abnormal RhoA activity (Piekny, Werner & Glotzer 2005, Saurin et al. 2008). Also, integrins are known to regulate RhoA via adhesion-dependent intracellular signalling. To investigate if the observed integrin localization at the furrow coincides with that of the active RhoA, we used an affinity-based GFP-tagged probe that is based on the Rho-binding domain of Rhotekin and that recognizes GTP-loaded active RhoA *in situ* (Berdeaux et al. 2004). In control siRNA treated NCI-H460 cells, active RhoA was detected in the narrow zone in the ingressing furrow and actin localized symmetrically around the active RhoA (I: Figure 3D; III: Figure 2D and quantification in E). In Rab21-silenced cells, where integrin did not localize to the cleavage furrow anymore, the active RhoA localization was lost (I: Figure 3D). In ZO-1siRNA cells, where integrin was seen in retractions, both actin and RhoA appeared diffuse around the middle of the cells probably due to the loss of organized midbody structure (III: Figure 2C and quantitation in D). Thus, based on these results it is possible that the loss of integrin targeting to the furrow upon Rab21 silencing abrogates RhoA (or its effectors') activity. Additionally, ZO-1 may, directly or indirectly, affect the midbody targeting of RhoA thereby affecting cell division.

5.5. ZO-1 binding to PI(4,5)P2 (III)

Integrin adhesion turnover is required for cell motile events and is executed by a number of molecules, such as the FAK-Src complex, ERK (extracellular regulated kinase), MLCK, microtubules and dynamin (Webb et al. 2004, Ezratty, Partridge & Gundersen 2005). Furthermore, integrin adhesion disassembly has been shown to involve clathrin-dependent active integrin endocytosis, and it may require also localized actions of lipid kinases (Chao et al. 2010). The active integrin-rich retractions in TIRF imaging and similar structures observed with live cell imaging of dividing ZO-1siRNA cells indicated failed adhesion disassembly during cell division. Interestingly, the integrin appeared to reach the furrow (epifluorescence) but failed to adhere to the substratum (TIRF) in the absence of ZO-1 (III: Figure 3A, B). Also, ZO-1 depleted

cells displayed loss of cell symmetry and vesicular targeting associated with sustained daughter cell contact and delocalized midbody formation in telophase (III: Figure 2A-D). These results are indicative of abnormal actin cytoskeletal activity that could stem from remaining integrin signaling in the retractions and could involve alterations in lipid kinase activities. By inhibiting the lipid kinase PIPKβ or dynamin-2-FAK interaction, both critical steps in adhesion break-down, formation of the clathrin complex was prevented (Chao et al. 2010). ZO-1 (and ZO-2) PDZ2 domain binds to phosphoinositide lipid PI(4,5)P2, that is synthesized by type I and type II PIPKs (Meerschaert et al. 2009). Whereas the PI(4,5)P2 binding to ZO-2 PDZ2 domain seems to regulate the localization of ZO-2 to nuclear speckles, the functional outcome of lipid binding to ZO-1 is unknown. Therefore, PIP2 localization was studied in SCRsiRNA or ZO-1siRNA treated NCI-H460 cells transfected with a PIP2-detecting (binding) PH-domain of PLCδ and assessed for localization during cell division. In SCRsiRNA treated cells, GFP-PH-PLCδ accumulated in the furrow (III: Figure 4A). In contrast, ZO-1 depleted cells displayed PIP2-rich retractions at the cell periphery, indicative of lipid involvement in ZO-1siRNA induced defects in adhesion disassembly. We hypothesized that ZO-1 silencing induced retractions might involve alterations in lipid dynamics. Indeed, we were able to demonstrate binding between PI(4,5)P2 and GST-ZO-1PDZ1-3 in BIACORE experiments (III: Figure 4B). Interestingly, the interaction between PI(4,5)P2 and GST-ZO-1PDZ1-3 was prevented following preincubation of the GST-ZO-1PDZ1-3 with integrin α5 tail peptide (III: Figure 4B). This suggests that when integrin α5 is present, ZO-1 is unable to interact with the lipid PI(4,5)P2.

The retractions observed during cell division could be related to the phenotype observed in sparsely seeded NCI-H460 cells depleted of ZO-1, in which tail-like retractions containing integrin α5 were left at the rear of the cells upon migration (II: Figure 2F). In addition, these data might be related to the observations of increased surface expression of integrin isoforms β1, active β1, inactive β1 and α5 in ZO-1siRNA cells compared to SCRsiRNA cells (III: Figure 3C) and thus indicate a role for ZO-1 in integrin adhesion turnover. Similar kind of phenotype was detected in cells treated with chemical PKC inhibitor Calphostin C, as the cleavage furrow was rich in integrin α5-positive retractions with concomitant loss of ZO-1 from the furrow (III: Figure 4A). These data could mean that ZO-1, possibly via upstream regulation by PKCε, regulates integrin adhesion turnover. The effects can be visualized as tail-like phenotype during cell motility, frequently observed in cells with derailed kinase/phosphatase activity (Lawson, Maxfield 1995), or as active integrin-containing adhesion remnants around the furrow of dividing cells indicative of deregulated adhesion disassembly.

The specific residues mediating PIP2-ZO-1 binding are identified and lysine-to-alanine mutation to K253 abolishes the binding completely (Meerschaert et al. 2009). The inability of lipid binding to ZO-1 in the presence of integrin α5 could mean that both PIP2 and integrin bind to the same site in the PDZ2 domain of ZO-1. To assess this, we performed lysine-to-alanine mutagenesis to K253 in the GFP-ZO-1 expression construct. In immunodetection of GFP-ZO-1K253A and endogenous integrin α5, the

co-localization of these proteins appeared even more pronounced (III: Figure 4C) than with endogenous proteins in these same cells (II: Figure 2A). More specifically, both proteins were able to be recruited to the leading edge in motile cells, were frequently detected in perinuclear compartments and displayed prominent expression and strong co-localization at the opposing poles of separating daughter cells (III: Figure 4C). These data thus votes against a mutual binding site for PIP2 and integrin but suggests that the inability of ZO-1 to bind PIP2 increases its interaction with integrin α5. However, this speculation is based on the co-localization data merely, since we have not verified the binding between GFP-ZO-1K253A and integrin α5 with proper assays.

5.6. ZO-1 S168A phosphorylation (II, III)

According to a phosphorylation site database PHOSIDA, ZO-1 contains a putative PKC target sequence (RXXS^{168}XR) in between the first and the second PDZ domain (Gnad et al. 2007). To assess the functionality of this site to ZO-1 subcellular localization, we mutated this serine to alanine (loss-of-function) and aspartic acid (phosphomimic) in Flag-tagged ZO-1 expression construct (ZO-1S168A-Flag and ZO-1S168D-Flag, respectively). Whereas the wild-type ZO-1WT-Flag localized to intercellular punctae in confluent cells and to the free edges in sparse cells, the ZO-1S168A-Flag failed to localize to the leading edge in subconfluent cells (II: Figure 7C). The phosphomimic ZO-1S168D-Flag was, however, recruited to the leading edge, in a broad lamellipodia. In confluent monolayer, the ZO-1S168D-Flag could not form intercellular adhesions that were observed upon ZO-1WT-Flag or ZO-1S168A-Flag expression but instead localized in protrusions even in confluent cells (II: Figure 7C). Similar results were obtained also in untransfected and reconstituted MEF cells, where the expression of ZO-1WT-Flag was seen in membrane ruffles in the presence of PKCε and in the absence of PKCε, ZO-1WT-Flag was mainly found at the intercellular adhesions (II: Figure S10). In contrast, the expression of ZO-1S168A-Flag was observed at the intercellular contacts, irrespective of the PKCε expression (II: Figure S10). In analogy with the finding of the PKCε requirement for α5-ZO-1 complex formation (II: Figure 7A), the phosphorylation was required for α5-ZO-1 interaction, as the PLA signal between α5 and ZO-1 was reduced in the NCI-H460 cells expressing the ZO-1S168A-Flag (II: Figure 7D). This data means that phosphorylation at S168 regulates ZO-1 subcellular localization, directly or indirectly via PKCε. Given the role of ZO-1 in cleavage furrow constriction, we wanted to test if this was dependent on phosphorylation at S168. Expression of ZO-1WT-Flag was mainly detected slightly co-localizing with tubulin at the midbody and with integrin α5 at the opposing poles of daughter cells (III: Figure 4G). The alanine mutant, however, failed to co-localize at the midbody structures, which were aberrant in the cells expressing ZO-1S168A-Flag (III: Figure 4G) and reminiscent of those observed in ZO-1siRNA cells (III: Figure 2B). The phosphomimic ZO-1S168D-Flag localization did not markedly differ from the wild-type ZO-1 expressing cells (III: Figure 4G). This suggests that the phosphorylation of ZO-1 at S168 regulates its subcellular localization which is required during cell division.

5.7. ZONAB localization and regulation in NCI-H460 cells

ZO-1 is involved in gene regulation by associating with a transcription factor ZONAB (ZO-1-associated nucleic acid binding protein) that shuttles between tight junctions and the nucleus (Balda, Garrett & Matter 2003). The prevailing view is that during cell confluency, ZONAB is sequestered by ZO-1 to the intercellular junctions and this prevents ZONAB-associated cell proliferation by Cdk-1 or Cdk-4 in MDCK cells. In motile cells, on the other hand, ZONAB localizes to the nucleus and promotes cell proliferation (Balda, Garrett & Matter 2003). Evidence suggests that some cancer and normal epithelial cells do not proliferate during migration but do so when sitting on laminin-rich basement membrane (when they are polarized and confluent).

Due to the important role of ZO-1 in mediating cell motility and division, we investigated its interaction partner ZONAB in these processes. In NCI-H460 cells, contradictory to the prevailing model, ZONAB localization in the nucleus was highest during cell confluency, whereas in cells with lamellipodial localization of ZO-1, ZONAB was detected, besides the nucleus, also in the cytoplasm (Figure 14). This suggests that in cancer cells, where proliferative control is frequently altered, the regulation of ZONAB subcellular localization might differ from the studies done in non-transformed epithelial cell line.

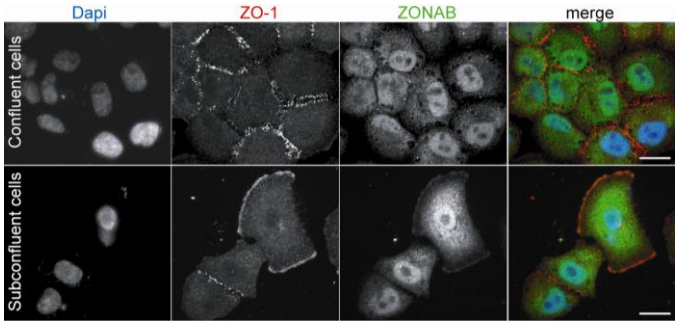

Figure 14. ZONAB localization in NCI-H460 cells. In confluent cells, ZONAB (green) localizes to the nucleus (DAPI, blue) and ZO-1 (red) to tight junctions. During subconfluency, when ZO-1 relocates to the lamellipodia, ZONAB is detected in the cytoplasm and in the nucleus. Scale bar, 10 μm.

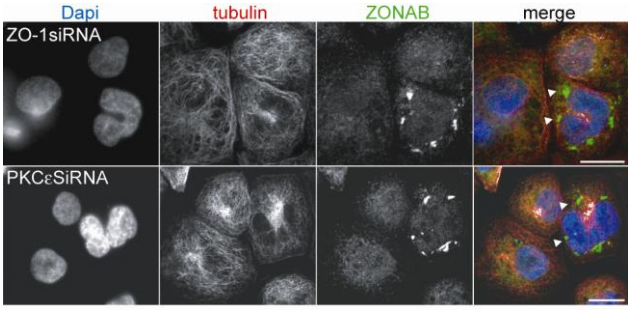

Figure 15. ZONAB localization in ZO-1 and PKCε silenced NCI-H460 cells. NCI-H460 cells were treated with ZO-1 or PKCε siRNA and stained for ZONAB (green) and tubulin (red) localization or DAPI to detect the nucleus. In cells with abnormal ploidy, ZONAB was detected in perinuclear compartments (arrowheads) around the nuclei. Scale bar, 10 μm.

ZO-1, as well as PKCε depletion from the NCI-H460 cells affected the execution of cell division (III). To establish whether these findings were associated with differences in ZONAB localization, we investigated ZONAB in the silenced cells. Interestingly, in cells depleted of ZO-1 or PKCε, the localization of ZONAB was diffuse throughout the cells. In multinuclear cells, ZONAB was always detected in perinuclear compartments surrounding the nuclei (Figure 15, arrowheads). This indicates that in NCI-H460 cells following ZO-1 or PKCε depletion, ZONAB loses its distinct localization in the nucleus and additionally is localized in perinuclear compartments in cells with abnormal ploidy. This most likely could affect the proliferative status of the cells and thus should be investigated in more detail.

6. DISCUSSION

6.1. The regulation of integrin adhesion during cell division (I, III)

We have demonstrated that during cell division, integrins traffic to the cleavage furrow where they exert mechanistic-adhesive functions required for successful daughter cell separation. These results offer mechanistic insight to the findings where loss of adhesion to the substratum in adherent cells leads to multinucleate cell population due to defects in cell division (Ben-Ze'ev, Raz 1981) or how in chondrocytes, β1 integrin regulates the entry into S phase, and, cytokinesis (Aszodi et al. 2003). The data in the first study suggests that Rab21-mediated integrin transport during cell division requires association between Rab21 and a conserved GFFKR sequence in the cytoplasmic tails of integrin α-chains (Pellinen et al. 2006). Perturbating this by Rab21 silencing, GTPase-defective Rab21 expression, inhibiting Rab21-integrin association and endocytosis, all lead to late stage cytokinesis defect and generation of multinucleate cell population in several cell lines. Additionally, loss of integrin targeting at the cleavage furrow by ZO-1 depletion similarly abrogates proper cell separation underscoring the existence of multi-level integrin regulation.

What is the function of integrin targeting to the furrow? The results from TIRF imaging confirm that active β1 integrin at the cleavage furrow mediates close proximity to the underlying surface. In addition to the physical anchoring of the cleavage furrow the results would implicate a signalling function for integrins. Integrin signalling is known to regulate RhoA (Hall 2005). The active integrin-rich retractions observed in ZO-1siRNA cells could abrogate actin-related processes in telophase. Our observations of the aberrantly activated/targeted RhoA at the furrow in Rab21- and ZO-1- silenced cells could be a result of various factors. First, in Rab21siRNA cells where active RhoA was largely absent from the furrow, furrow ingression proceeded initially. This could be due to incomplete Rab21 silencing. If Rab21 silencing resulted in complete inhibition of RhoA, furrow ingression should be totally prevented which was not the case. Also, since RhoA is regulated by other factors than integrin signalling, it is logical to assume that the effect is partial. The ZO-1 silencing abrogated the midbody formation as such and the RhoA targeting/activity in these abnormal structures was difficult to decipher exactly. The aberrant structure still seemed to contain active RhoA but the localization was diffuse. This could mean that ZO-1 does not directly affect RhoA activity but rather influences cell morphology and symmetry which may then indirectly affect RhoA activation and localization. This could be investigated further by measuring activity of RhoA in pull-down assays from synchronized cells treated with ZO-1 or Rab21 siRNA.

In Rab21 and ZO-1 silenced cells, the impaired midbody structure could stem from 1) direct effect on vesicular traffic to the midbody matrix which is known to depend on the action of other Rabs, Arfs and SNARE vesicular components (Skop et al. 2001, Murthy, Schwarz 2004) 2) additional role in regulating acto-myosin contractility during furrowing, since Rab21 is implicated in acto-myosin force generation (Hooper, Gaggioli & Sahai 2010) and ZO-1 binds to actin and regulates myosin-2 recruitment at

intercellular junctions (Yamazaki et al. 2008) 3) alterations in microtubule elongation in anaphase that interfere with the actin dynamics, as the interdependence of these filamentous networks is established (Straight et al. 2003) 4) integrin-mediated deregulation of other small Rho GTPases, namely Cdc42 and Rac at the furrow (Yoshizaki et al. 2003, Etienne-Manneville, Hall 2001, Hall 2005). Cdc42 is essential in maintaining cell polarity by positioning the MTOCs and is detected in the midbody and in intracellular vesicles symmetrically at the opposing poles of daughter cells. RhoA and Rac, on the other hand, are detected at the plasma membrane and Rac activity increases at the daughter cell poles towards telophase as detected with FRET-probes (Yoshizaki et al. 2003). It is possible that integrin deregulation affects actin dynamics and cell polarity via Cdc42- mediated fashion, which could explain the asymmetrical vesicular distribution and microtubule emanation from the MTOC in ZO-1siRNA cells. The altered signalling at the furrow/midbody should be studied further with photoactivatable probes of the GTPases or biochemical assays.

We monitored regulators of other vesicular compartments as well, especially Rab11 and its effector protein FIP3, which are reported to be active during cell division (Wilson et al. 2005). We saw also the requirement for FIP3 in NCI-H460 cell division. In FIP3-silenced cells, the β1 was able to traffic to the cleavage furrow, but was observed trapped in perinuclear compartments in later stages. In addition, the actin filamentous network was altered in FIP3-silenced cells. These defects might affect the overall cell adhesion and lead to impaired anchoring to the substratum at the daughter poles. Huge vesicular structures containing GFP-Rab21 were observed in cells silenced of FIP3. This could reflect the imbalance between continuous endocytosis (Rab21) and inhibited recycling (FIP3-siRNA). Altogether, this would mean that overlapping vesicular complexes exist to mediate integrin adhesion required in separate steps during cell division.

The generation of chromosomal instability and aneuploidy following defective cell division are causal in carcinogenesis (Fujiwara et al. 2005). We show here that deregulated integrin traffic can lead to aberrant cell division and altered ploidy in a cell population. What is the clinical relevance of these findings? To answer this, comparative genomic hybridization (CGH) analysis was done in two ovarian cancers and a prostate tumor sample. Genetic ablation of *RAB21* gene and the subsequent loss of mRNA expression were found in ovarian cancer cell line KFr13 with alterations in integrin traffic. Interestingly, a similar chromosomal deletion was found in a prostate cancer sample. These clinical findings indicate that in a subset of cancers, *RAB21* deletion could lead to deregulated integrin traffic and aneuploidy and thus serve as a marker of a multinucleate cancer cell phenotype.

The contribution of integrin function to cell cycle control can be further discussed. Importantly, follow-up studies (unpublished) elucidating the long-term effects of aberrant integrin function in cell division have been conducted in our laboratory by using non-transformed mouse embryonic fibroblasts reconstituted with β1WT or β1YYFF integrin. When these cells are plated on laminin (β1- specific matrix) and undergo several rounds of cell division, the β1YYFF cells with cytokinesis defects

generate an aneuploid cell population whereas the β1WT cells maintain normal euploidy in the population. The aneuploid cells are characterized by EMT-related gene upregulation and malignant properties such as anchorage independence and formation of tumors in mice. This thus brings molecular level data on how abrogations in integrin traffic can lead to the generation of aneuploidy and tumorigenesis. Altered integrin function is implicated in other diseases as well and for instance, can lead to severe infectious conditions (Bunting et al. 2002).

6.2. The integrin α5 –ZO-1 interaction (II, III)

Both integrin α5 upregulation and the loss of ZO-1 from tight junctions to the cytoplasm or nucleus are linked to tumorigenesis, but the regulation behind these events is unknown. According to our findings, ZO-1, a protein thought to mainly guard the integrity of epithelial sheets, serves an essential function in the leading edge lamellipodium. The Jekyll and Hyde –function presented here indicates that loss of ZO-1 from intercellular junctions, a frequent phenomenon in cancer *in vivo*, can lead to its active participation in supporting invasive structures in motile cells rather than e.g. to its degradation. The main function of ZO-1 in some cells types could actually be to boost cell motility as its expression is not restricted to cells which form TJs or have polarized membrane domains at confluency (eg. fibroblasts). One mechanism how ZO-1 might regulate cell motility is the adhesion site turnover. The vinculin-positive adhesions in ZO-1siRNA cells were smaller in size and concentrated on the cell edges whereas in SCRsiRNA cells they were larger and extended under the cell body. These might correlate with the increased velocity and reduced persistence, since alterations in Rac activity, which is known to modulate lamellipodial protrusions (Pankov et al. 2005), were detected in ZO-1 siRNA cells. In detail, we describe a direct interaction between ZO-1PDZ domain and a non-canonical internal PDZ binding motif in integrin α5 tail and show that this interaction promotes cancer cell motility. Although PDZ domain binding to the COOH-termini of integrin α5 and α6 cytoplasmic domains has been reported (El Mourabit et al. 2002) our study represents the first demonstration of an interaction between tight junction proteins and internal sequence in integrins. The exact binding mode between the PDZ domain binding groove and integrin COOH-terminus can only be speculated, however.

Internal sequences in PDZ-binding proteins have been shown to efficiently target to the binding groove, and sometimes even competing with the linear peptides (Hillier et al. 1999). Furthermore, β turns in the context of double prolines have been reported in integrin α tails with implications in cell adhesion regulation (Filardo, Cheresh 1994). The tail domain of integrin αV(β3) was shown to produce a kink conformation by PPQEE sequence and affect ligand binding. The double proline in the integrin α5 tail (PPATSDA-cooh) situates fairly close to the C-terminal end, and citing the critic at cell-cell junction-focused conference, it remains "questionable if the site can really be termed 'internal' ". It is possible, that the prolines do serve a binding site by physical means, but the actual binding site is still the more conventional TSDA at the distal integrin tail end. The abolished binding between the mutant integrin peptide (AAATSDA) and GST-ZO-1PDZ1-3 could also reflect differences in binding affinities

between the wild-type and the mutant peptide that cannot be detected with the ELISA method used but instead would require e.g. BIACORE experiments with calculated affinities. The mutation most probably linearizes the tail peptide and brings the α tail closer to the β tail which could sterically inhibit the binding due to adaptor binding to the β tail. Alternatively, the mutation could prevent the binding of other proteins to the tail that enhances the binding of ZO-1. However, the ELISA assay was done in the presence of only the α tail and the GST-ZO-1PDZ1-3 peptide which indicates that the abolished interaction is due to the loss of conformation-dependent binding site (Figure 16). In order to further clarify the exact requirements for this interaction, ELISA assay with integrin α6 peptide would probably be useful, since it contains the same COOH-terminal TSDA amino acids, but does not harbour a double proline in its immediate sequence, as does integrin α5. In addition, α6 subunit was detected at the intercellular junctions and to some extent at the leading edge in NCI-H460 cells together with the α5 subunit thus suggesting a similar localization phenotype as the integrin α5.

The significance of the double proline mutation was further verified with cell biological experiments. The α5siRNA-treated cells with α5PPAA rescue displayed cell morphology without prominent lamellipodia. Here, the mutation could involve alterations in recruitment of other cytoplasmic proteins, but this was not tested. Expectedly, the mutation affected the cell motile characteristics. While the WTα5 rescue construct promoted lamellipodial protrusion in the direction movement, the α5PPAA cells displayed random motility with numeral smaller and less stable protrusions, reminiscent of those seen in ZO-1-depleted cells. The presence of multiple smaller protrusions correlated with increased velocity and Rac activity in ZO-1siRNA cells. The Rac activity from the α5PPAA rescued cells should be similarly measured.

The double proline mutation was non-functional also during furrow ingression. In cells depleted of endogenous integrin α5, the α5PPAA expressing cells displayed abrogated or absent constriction in anaphase with a phenotype that was more severe than with the α5 siRNA treatment only. This indicates that the complex formation at the time of cytokinesis is required for furrow ingression in these cells. However, we have not formally shown that the complex is formed in cytokinetic structures. To do this, NCI-H460 cells in mitosis should be enriched in the population by arrest techniques like thymidin block or nocodazole treatment and analyze complex formation with co-IP or PLA in those cells.

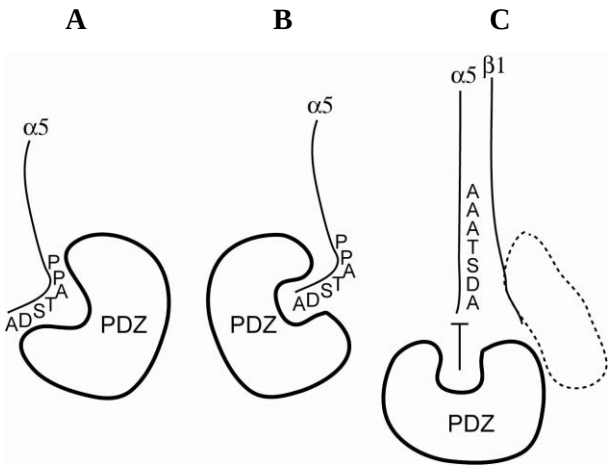

Figure 16. Models for integrin α5 tail binding to ZO-1 PDZ domain. Integrin binding to the PDZ domain binding groove requires the prolines for interaction: in (A), the binding occurs via the non-canonical way whereas in (B) the PDZ domain recognizes the three COOH-terminal residues that represent a classical PDZ- binding motif. In (C), the proline-to-alanine mutations prevent kink conformation in the integrin tail. This could sterically inhibit PDZ domain binding due to adaptor binding to the β subunit.

We wanted to investigate the temporal regulation of α5 and ZO-1 at the furrow. The GFP-tag on ZO-1 did not interfere with the association with mCherry-tagged integrin α5 (not shown) and the movements of GFP-ZO-1 and mCherry-α5 were assessed in COS-7 cells due to the relatively easy co-transfection achievable in these cells. This revealed the differential paths of α5 and ZO-1 to the furrow. ZO-1 stays at the plasma membrane throughout the mitotic phases (Reinsch, Karsenti 1994). In COS-7 cells, ZO-1 moved along the membrane steadily to the furrow where it was seen in two focal clusters, closely resembling the appearance of the midbody, and later in a belt-like localization between the nascent daughter cells. Integrin α5, however, moved in a dynamic fashion and more randomly around the cell, concentrating at the furrow during ingression and moving to the opposing poles later in telophase. Only limited co-localization of the two proteins was seen at the furrow as compared to the immunodetection of α5 and ZO-1 in NCI-H460 cells. However, any other experiments in COS-7 cells were not done, and in these cells regulation of the division process might differ.

The fact that similar translocation of integrin α5-ZO-1 complex to the leading edge was observed in other cell lines, like the breast cancer line MDA-MB-231 or the mouse cell lines indicates, that the function of this complex might be more general. The expression of several integrin heterodimers such as αvβ3, αvβ6 and α5β1 is very low or undetectable in adult epithelia, but frequently gets highly upregulated in several tumours as in the case of integrin α5β1 in non-small cell lung carcinomas (Adachi et al. 2000) or melanomas (Danen et al. 1994). Interestingly, the expression profiles of integrin α5β1 and ZO-1 were highly correlative in tumors of patients with metastatic melanoma (unpublished data). This is interesting since overexpression of both integrin

α5β1 and ZO-1 (via interacting with N-cadherin) has been shown to correlate with bad prognosis in melanoma patients (Danen et al. 1994, Smalley et al. 2005). In the epidermis, ZO-1 is involved in the terminal differentiation (Morita et al. 1998) and integrin α5 mediates the epidermal migration during wound healing by responding to the keratinocyte growth factor (KGF) release (Koria, Andreadis 2007). It could be that during melanoma progression, terminal differentiation and normal ZO-1 function are impaired and instead ZO-1 associates with the "bad" N-cadherin. This could promote invasion, possibly via KGF release- mediated integrin α5 motility. Whether these two proteins act co-operatively to promote cell motility and invasion as they do in lung cancer cells, would be relevant to study further in established melanoma cell lines like M16.

Depending on the cellular context, ZO-1 expression can be pro- or antitumorigenic. In breast cancer cell line BT549, expression of ZO-1 or only the NH2-terminal fragment containing the PDZ domains, promotes invasiveness by upregulating MT-MMP1 and the β-catenin/TCF/LEF pathway (Polette et al. 2005) which is frequently deregulated in cancers (Esufali, Bapat 2004). This was associated with cytoplasmic localization of ZO-1. We observed ZO-1 and the PDZ1-3 construct, or in MDA-MB-231 breast cancer cells, ZO-2 (where ZO-2 was more abundant than ZO-1) in the leading edge while the PDZ1-3 of ZO-2 was detected in the nucleus of MDA-MB-231 cells (not shown). The activation of the β-catenin/TCF/LEF pathway and EMT-like changes were observed also in MDCK cells expressing the ZO-1 PDZ1-3 domain construct (Reichert, Muller & Hunziker 2000). We did not look at any down-stream effects in our assays. The activation of the β-catenin/TCF/LEF pathway could be tested in scratch wound assay, where upregulation of integrin α5 and PKCε was observed and also upon ZO-1 silencing when cell motility was decreased. Interestingly, Rac1 GTPase activity positively regulates β-catenin pathway and enables cell proliferation by targeting the *Cyclin D1* (Esufali, Bapat 2004). According to our data, ZO-1 depletion increases Rac1 activity and alters, similarly to PKCε, ZONAB localization. *Cyclin D1* is also a target of ZONAB (Sourisseau et al. 2006). Upon PKCε silencing the cells were bigger in size which indicates derailed proliferation. Also, the cells had lost their characteristic lamellipodial ZO-1 which was instead expressed in the nucleus where it is reported to downregulate transcription. With for instance luciferase transcriptional assays, we could evaluate the promoter activity of *Cyclin D1* and thus study whether PKCε or ZO-1, through ZONAB, could interfere with proliferation via regulation of *Cyclin D1*.

To evaluate the clinical relevance of the α5-ZO-1 complex, we used an established in situ detection method combined with paraffin-embedded tissue microarray (TMA) samples to detect this complex in vivo. In three patient samples that had metastasized, we could detect a PLA signal (and thus interaction between ZO-1 and α5) in a subfraction of cells. This could relate to the findings in *in vivo* imaging of tumor cell invasion in mice, where only a subpopulation of cells display motile characteristics whereas the rest of the tumor cells maintain intercellular adhesion or undergo cell proliferation (Sahai 2007). The identity of the cells displaying PLA signal is, however, not known. Even though most of the TMA samples are extracted from cancerous

lesions, it could be that we detected PLA signal in pericytes, stromal fibroblasts or in inflammatory cells. In theory, we should also detect PLA signal of ZO-1-α5 interaction additionally in dividing cells, whereas during intercellular adhesion, which represents most of the tumor mass, no signal should be detected. Possibly with more optimized antibody pairs and different sample preparation (frozen sections) the detection level of the complex could be higher and visible in dividing cells as well. The observed α5β1-ZO-1 complex in metastatic tumors makes the study clinically relevant. A small molecule inhibitor targeting the "bad" α5 integrin-ZO-1 complex in certain cancer types might block cell motility and thus could interfere with cancer progression in vivo in a way that our data suggests.

In general, our data implies the importance of integrin adhesion regulation via effector proteins in cell division and motility. How to then exploit these results in therapeutical/diagnostic purposes when the same pathway seems to favour or disfavour malignant properties in the cell? For a fact we do not know that the pathways are similar. Several effector proteins are known to regulate the Rab GTPases and this could vary locally and depending on membrane concentration. Interphase cells are normally spread out facing the underlying surface with a flat morphology and invariantly sized protrusions projecting from the cell. In a dividing cell, on the other hand, the plasma membrane and cytoplasm are more condensed at the furrow area. This might affect Rab21 effector concentration and localization due to 1) altered actin filament structure (branched actin via Arp2/3 versus non-branched actin via formin) 2) tubulin modification such as acetylation or 3) prenylation of Rab21 on the membrane and thus involvement in pathway regulation. Another aspect is the tumor stage. Depending on the stage of tumorigenesis, upregulation of certain integrins can predict prognosis in either way (Adachi et al. 2000). Likewise the expression of the effector proteins could be subject to alterations in a stage-dependent manner.

6.3. The regulation of the integrin-ZO-1 complex by PKCε (II, III)

PKC regulation of tight junction modulation and integrin traffic is demonstrated in several studies (Ivaska et al. 2002, Avila-Flores et al. 2001). ZO-1 NH2-terminus contains a putative PKC phosphorylation target site (RXXS^{168}XR) and we found this to regulate, via direct or indirect PKCε phosphorylation, ZO-1 subcellular localization during cell-cell adhesion break-down and onset of motility. Mutating this site between the first and second PDZ domain to alanine inhibited lamellipodial recruitment and prevented normal cell separation. We do not know how ZO-1 reaches the plasma membrane or when and where in the cell it gets phosphorylated at S^{168}. ZO-1 is known to dimerize via the second PDZ domain (Utepbergenov, Fanning & Anderson 2006), which might regulate its scaffolding functions and/or recruitment to the leading edge lamellipodia. It is possible that the S^{168}A mutation abrogates the homodimerization of ZO-1 or its association with another adaptor molecule thereby inhibiting its recruitment to the lamellipodia and interaction with integrin α5. As an example are the ubiquitous 14-3-3 proteins that recognize preferentially e.g. phosphorylated mode I binding motifs RSXpS/TXP (where X denotes any residue and "p" phosphorylated residue) in their target proteins, such as PKCε, and regulate thereby protein localization and

conformation (Dougherty, Morrison 2004). Hypothetically, 14-3-3 could recognize phosphorylated ZO-1 S^{168}, situated in a sequence RSLpS^{168}PR and thus regulate ZO-1 subcellular localization. The 14-3-3 isoforms can alter the conformation of their target proteins significantly (Yaffe 2002) and therefore 14-3-3 binding to ZO-1 might alter its globular conformation. The possible involvement of such adaptor proteins binding to ZO-1S^{168} should be investigated by pull-down, yeast-two-hybrid or mass spectrometry experiments. The ZO-1S^{168} was mainly detected in the lamellipodia and thus motile cells, whereas the alanine mutant ZO-1S^{168A} still retained normal localization at the junctions and mediated adhesion to adjacent cells. This could mean that in the clinical samples integrin α5 is complexed with phosphorylated ZO-1^{S168} and thus the "bad" form of ZO-1. An antibody which would specifically recognize this pro-migratory form of ZO-1 could be used to assess the universality of this finding and possibly in diagnostic purposes in the future. In dividing cells, the alanine mutant ZO-1S^{168A} failed to execute proper cell division. These experiments were not done in ZO-1 null cells, and thus may explain the less dramatic phenotype to that observed with ZO-1siRNA cells, possibly due to the presence of endogenous expression.

PKCε phosphorylates vimentin tethered to integrin-containing vesicles thereby enabling the release of integrins to the plasma membrane to support cell motility (Ivaska et al. 2005). We saw increased PKCε activity and α5 surface expression upon wounding the confluent NCI-H460 monolayer (II: Figure 6). This could mean that, upon onset of cell motility, activation of PKCε in the cells would increase integrin α5 release to the cell surface where it meets ZO-1 for interaction. It is also possible that α5 and ZO-1 meet already in vesicles, since ZO-1 positive vesicles were observed in NCI-H460 cells expressing GFP-ZO-1 (not shown) and are reported in the endocytosis of apical proteins in bladder cells (Khandelwal, Ruiz & Apodaca 2010). These theories would be interesting to test in live cells by using chemical inhibitors, like Calphostin C or BIM, or siRNA against PKCε and monitor the traffic of FP-tagged α5 and ZO-1 to the leading edge or to the cleavage furrow over time.

ZO-1 localization in the nucleus has been reported in conditions where the cell faces stressful conditions, in sparsely seeded cells or upon exposure to nicotine (Hawkins et al. 2004). The observed loss of ZO-1 and α5 from the lamellipodia upon PKCε silencing could be due to secondary effect, since PKCε inhibition might contribute, via actin-binding properties, to the general abrogation of motility-promoting structures and thus indirectly affect ZO-1 localization. In PKCε silenced cells, ZO-1 was seen repeatedly in the nucleus, where it is shown to negatively regulate cell proliferation (Traweger et al. 2003). Does the nuclear ZO-1 affect proliferation upon loss of PKCε expression in MEF cells or in NCI-H460 cells? The NCI-H460 cells were fewer and bigger in size following PKCε depletion, which could be a sign of defected G1/S transition. However, in PKCε null MEFs, the cells on a culture dish seemed identical in size and quantity, indicating that in these cells the PKCε/ZO-1 pathway does not represent such an important role as in NCI-H460 cells. Alternatively, it could be that in NCI-H460 cancer cells the cell-cycle regulation is more prone to defects induced by PKCε silencing than the mouse epithelial cell line. The possible effect on proliferation

should be tested with WST-1 or comparable reagent which measures proliferative activity of cells. The localization of the integrin α5 and ZO-1 varied in PKCε null or PKCεRE cells which might reflect differences between cell types and the lack of cell-cell polarization in fibroblasts.

PKCε and specifically its regulatory domain are needed in neurite outgrowth during neuronal differentiation (Zeidman et al. 1999). In addition, overexpression of PKCε induces nerve growth factor- stimulated phosphorylation of MAPKs and neurite outgrowth (Hundle et al. 1995). In our observations, the overexpression of kinase-dead mutant of PKCε or siRNA against PKCε prevented ZO-1 lamellipodial localization and induced long protrusions in the NCI-H460 cells that very much exhibited neuron cell-like phenotype. Thus, the effects of PKCε expression in these experimental setups are totally contrasting and might reflect the cell context differences. Indeed, the phosphorylation of MAPK in NCI-H460 cells would be intriguing to study in the presence of SCRSiRNA and PKCε siRNA, the GFP-tagged PKCε WT or kinase-dead overexpression constructs. Additionally, establishing upstream inducers of the PKCε activity and the following α5-ZO-1 complex formation would be important. We have been studying cell motility -related events in an artificially induced context, as scratch wounding does not occur inside tissues in vivo. Stimulation of PKCε activity and the following downstream effects by growth factors, such as NGF, HGF (KGF in melanoma cells) or the like could be studied. Additionally, ischemia or inflammation related epithelial cell damage models could be considered as study platforms.

PKCε and α5 upregulation as well as disruption of intercellular adhesions are all events linked to tumorigenesis. Where would this "bad" pathway function in vivo? Interestingly, PKCε is abnormally highly expressed in non-small cell lung cancers where it protects the cells against the drug etoposide and doxorubicin (Ding et al. 2002). In addition, the overexpression of this isoform is linked to cisplatin resistance in some ovarian cancer cell lines (Basu, Weixel 1995). Whether PKCε would regulate the integrin α5-ZO-1 complex in these cell types, would be interesting to test e.g. by using the ovarian cancer model KF28 and its cisplatin-resistant derivative KfR13. The protective role of some PKC isoforms upon harmful cigarette smoke exposure has been demonstrated (Park et al. 2008). The PKCε response to cigarette smoke- derived carcinogens would be nice to test in NCI-H460 lung cancer cells, as nicotine has been shown to cause ZO-1 relocalization to the nucleus of endothelial and certain mouse epithelial cells and to increase metastatic potential (Hawkins et al. 2004, Davis 2009). This would further characterize the findings of integrin α5-ZO-1 complex in the context of lung cancers.

6.4. The role of ZO-1 during cell division (III)

It is proposed that intercellular junctions are maintained throughout the course of cell division (Baker, Garrod 1993). We demonstrate however, that ZO-1 would lead an active role in mediating cell separation that is somehow linked to integrin adhesion regulation. What could be the function of ZO-1 at the furrow? ZO-1 is characterized by several protein-protein interaction domains by which it associates with other tight junction proteins (Umeda et al. 2006), transmembrane receptors and actin (Fanning,

Ma & Anderson 2002). Thus it could be involved in scaffolding several independent events during cell division. Active integrin remnant adhesions were seen projecting from ZO-1 depleted cells but were not seen mediating adhesion to the substratum. However, the integrin seemed to be targeted to the furrow area indicating a functional delivery. This suggests that ZO-1 function is in situating the delivered integrin firmly to the bottom of the furrow and the disassembly of integrin adhesions during cell shape changes in mitosis. Thus it also suggests that different cellular pools of integrin are regulated by different pathways, which partly explains the differences in cells silenced of Rab21 or ZO-1.

The deregulated integrin adhesion could directly contribute to filamentous forces on the cells (Hall 2005). Actin regulates the MTOC (Etienne-Manneville, Hall 2001) which could explain why cells depleted of ZO-1 display defects in the orientation of nuclei during last steps of cell division. Interestingly, knocking down a motor protein kinesin CHO1 induces a similar phenotype and has been suggested to slide the antiparallel microtubules *in vitro* (Matuliene, Kuriyama 2002). This could suggest that ZO-1 affects, either directly or indirectly, the filamentous structures that are crucial during cell division. The integrin-rich retractions observed in the ZO-1siRNA cells undergoing division might be due to loss of ZO-1-directed myosin recruitment to the adhesion sites since MLCK is required for disassembly of integrin adhesions (Webb et al. 2004). Additionally, Yamazaki and others (2008) show that ZO-1 and ZO-2 recruit myosin-2 to the newly forming cell-cell junctions. Since myosin-2 is the known actin-driving motor in cellular motility, it is intriguing to think that the cytokinesis defect would occur because of weak myosin-2 recruitment and hence, inadequate actin forces, to the cleavage furrow. ZO-1 silenced cells should be studied in the presence of a live actin probe which could reveal the temporal occurrence of the defect in filament assembly. Considering that the filamentous actin structures would project in all dimensions during cell division, analysis in 3D environment would likely be of use, since signalling from the underlying basement membrane would imagine to contribute to the division process. Interestingly, in monolayers of polarized epithelial cells such as MDCK cells, the dividing cells are encapsulated by the neighbouring cells and the forming midbody ends up very close to TJs. It could be that midbodies serve as platforms for junctional assembly and ZO-1 is critical for this due to its role in maturation of naive junctions into AJs and TJs (Ikenouchi et al. 2007). Loss of ZO-1 at junctions would lead to incomplete intercellular adhesion with malignant implications. The contribution of the neighbouring cells to the adhesive need for cytokinesis would be important to study further in 3D. Most of the experiments carried out in this study focused on individual cells where the adhesion via cleavage furrow may become far more important than in cells which are surrounded by other cells and thus with increased adhesive surfaces.

After constriction, the remaining cytoplasmic bridge is severed to complete the cytokinesis (Gromley et al. 2005). Interestingly, ZO-1 silencing displayed midbody remnants indicative of falsely severed connection between the cells. PKCε knock-out cells and knock-out cells reconstituted with 14-3-3- binding deficient PKCε exhibited a

similar cytokinetic phenotype with persistent bundled tubulin in the midbody (as in cells depleted of ZO-1) (Saurin et al. 2008). The authors reasoned that this observed delay in cytokinesis occurred prior to actomyosin ring dissociation, since the rounded daughter cells remained attached to each other for a substantially longer period when compared to wild type cells. The abnormal telophase observed in ZO-1siRNA cells could also result from impaired function of vesicular SNAREs (Low et al. 2003) and Rabs (Skop et al. 2001) needed in abscission, since according to the unpublished results in our laboratory, ZO proteins are able to associate with e.g. Rab21 (in cells). These data and the results from our PKCε knock-down studies implicates PKCε and ZO-1 in a common signalling pathway that is activated during cell division. It would be interesting to test if the ZO-1 silencing induced defects could be partly rescued by PKCε overexpression.

6.5. The ZONAB localization phenotype in NCI-H460 cells

The regulatory mechanism of ZO-1-ZONAB complex on gene expression and cell proliferation has been proposed (Matter, Balda 2003). As shown by Balda and co-workers, the cooperative function of these proteins is regulated by cell density: during high cell confluency, the expression of ZO-1 at tight junctions increases (Balda, Matter 2000). This leads to low ZONAB expression and its recruitment to tight junctions with subsequent inhibition of cell proliferation. Conversely, as the cells are seeded in low density, the expression of ZO-1 decreases leading to high nuclear ZONAB expression and cell proliferation. In contrast, according to our observations, the nuclear enrichment of ZONAB was evident in NCI-H460 cells during high cell density, whereas junctional ZONAB was hardly detectable. ZO-1 expression in tight junctions was nevertheless high. In subconfluent cells, where ZO-1 is upregulated in the leading edge lamellipodia to promote cell motility, ZONAB was distributed evenly in the cytoplasm concomitantly with some nuclear localization. This controversy in the findings could reflect the differences in the origin of the cell lines or reflect the differences between transformed and normal epithelial cells. Interestingly, in our knock-down studies, the cellular distribution of ZONAB was altered upon ZO-1 or PKCε knock-down. More specifically, in depleted cells that were multinuclear indicative of defects in cell division, ZONAB was localized in perinuclear compartments, whereas in mononuclear cells the localization was cytoplasmic and diffuse. However, the nature of these compartments is not known and should be characterized, for instance, by endosomal markers. This suggests that the traffic of ZONAB is altered by ZO-1/PKCε, possibly cell-cycle dependently. Also, the current model of the localization of ZONAB might be inadequate, since it is based on merely MDCK cells and should be extended with studies from several cell lines, including cancer cell lines that are known to display altered proliferative phenotypes.

6.6. PIP2 association with ZO-1 (III)

Phospholipid PI(4,5)P2, or PIP2, binding to ZO-1 PDZ2 domain is established to occur via specific residues in the PDZ (Meerschaert et al. 2009). Our results likewise demonstrate the binding of PIP2 to ZO-1. Furthermore, we show that integrin α5 tail

peptide abolishes ZO-1 binding to PIP2. This would favour the idea of a mutual binding site of integrin and PIP2 in ZO-1. However, mutation to the lysine K253 in ZO-1 PDZ2 domain, which abolishes PIP2 binding to ZO-1 and ZO-2 PDZ domain (Meerschaert et al. 2009), had no effect on the co-localization of integrin α5 and K253A-ZO1 suggesting that this does not hold true. However, we did not confirm that these two proteins would still interact. Given that they would not bind, we would expect to see phenotypes such as those with α5PPAA construct, small protrusions projecting from the cells or reduced motility. The association should be verified with co-IP studies before proceeding with other functional studies. Considering that α5 binding to ZO-1 inhibited PIP2 binding (that most likely is not because of mutual binding site), brings out the possibility that integrin binding to ZO-1 occurs via conformational changes in ZO-1. It is possible that in the furrow, PIP2 competes with α5 tail in binding to ZO-1PDZ. This should be assessed in Biacore experiments by immobilizing biotin-α5 peptide to the sensor chip and check whether ZO1-PDZ1-3 binds to α5 in the presence of PIP2.

Phospholipids (specifically PIP2) and actin have been shown to contribute to the formation of clathrin-coated pits (CCPs), together with the major components clathrin and adaptor protein AP-2 (Boucrot et al. 2006). In CCPs, integrin is internalized from the cell surface and sorted to endosomal compartments. Lipid kinase activity might very well play a role in disassembling integrin α5 adhesions, since inhibiting PI3K with LY294002 caused integrin α5 accumulation to large focal adhesions and prevented lamellipodial localization in NCI-H460 cells (not shown). ZO-1 depletion increased the surface expression of integrin subunits α5 and β1 and failed to disassemble active β1 integrin containing retractive adhesions. This and the fact that similar kind of retractions were observed also in the motile NCI-H460 cells upon ZO-1 knock-down, could suggest that ZO-1 affects integrin uptake from the cell surface and thereby regulates integrin-mediated adhesion during cell motility and division. Another interesting point is that depletion of AP-2, the clathrin adaptor protein, in NCI-H460 and MDA-MB-231 cells induced similar tail-like morphology and changes in the surface expression profile of integrin subunits α5 and β1, although somewhat more pronounced than ZO-1 depletion (not shown). The localization of integrin in AP-2 silenced cells was not studied with TIRF microscopy. However, this implicates that the internalization route taken by the integrins could go via AP-2-assisted clathrin route in these cells. Does ZO-1 link to this hypothesis in any way? It would certainly be appealing to think so, since 1) ZO-1 goes to the membrane periphery and is known to bind actin, an important player in CCP assembly 2) ZO-1 binds PIP2, another important CCP assembly factor, but only when it is not binding to integrin α5 tail, and 3) ZO-1 binds those integrins, which are suggestively internalized via clathrin (or AP-2) in our system. If ZO-1 would be involved in clathrin-mediated integrin endocytosis, could it play a role during cell division as well? Studies regarding ZO-1 endocytosis are virtually non-existent, except for the recent paper describing ZO-1 in β1 integrin - regulated, clathrin-independent endocytosis (Khandelwal, Ruiz & Apodaca 2010). It would be worthwhile to study whether clathrin-mediated endocytosis occurs at the cleavage furrow and if this would be affected by ZO-1 depletion. A way to get an idea

would be to co-transfect SCRsiRNA or ZO-1siRNA-treated NCI-H460 cells with fluorescently tagged clathrin light chain and integrin α5 and monitor vesicle movement in the furrow of dividing cells.

Talin binding to PIPKγ and associated PIP2 production occurs during integrin activation (Ling et al. 2003). ZO-1 might mediate also the linkage between actin and integrin in adhesions, where PIP2 would be involved in membrane linkage and possibly integrin adhesion site activation/inactivation by binding to talin. Additionally, PLC-mediated hydrolysis of PIP2 and the downstream activation of Ca2+/CaM and PKC influences acto-myosin contractility by activating MLCK, leading to phosphorylation of myosin regulatory light chain (Iwasaki et al. 2001). ZO-1 depletion could also partially inhibit PIP2 hydrolysis at the furrow that could cause telophase defects. ZO-1 might also regulate the generation of polarized PIP-domains in motile/dividing cells. PTEN is a phosphatase that generates PIP2 from PIP3 and counteracts PI3K activity in polarized and motile epithelial cells. In addition, PTEN contains a PDZ-binding motif (Valiente et al. 2005) that could hypothetically interact with ZO-1 PDZ domains to locally regulate phospholipid levels.

Regulation of cell adhesion is not important only for maintaining tissue integrity and polarity but also for the organized movement and structured division of cells. Our results provide mechanisms for how these complex processes in certain types of cells may be regulated. In an intact epithelium, the loss of *RAB21* gene e.g. via somatic mutation could result in aberrant integrin traffic and in generation of an aneuploid cancer cell population that feeds subsequent malignant properties in progeny cells. The genetic loss of *RAB21* could be used as a prognostic marker of such aneuploid cancers. We demonstrated that upstream regulation of ZO-1 by phosphorylation (possibly via PKCε) is crucial for proper cell division (Figure 17). Abnormal ZO-1 function could compromise epithelial integrity through incomplete cell separation/junctional maturation, thus emphasizing the interdependency of successful cell division and intercellular adhesion. Our data may additionally help understand the pathogenesis of some lung epithelial cancers, where altered expression of TJ markers (such as ZO-1), integrin α5 or PKCε are previously reported but not linked together. This study shows that in some lung cancers, PKCε may locally activate the integrin α5 –ZO-1 complex thus facilitating cell motility. Therefore, our data could offer mechanisms for clinical interventions by e.g. targeting the integrin α5–ZO-1 complex formation in certain types of cancers.

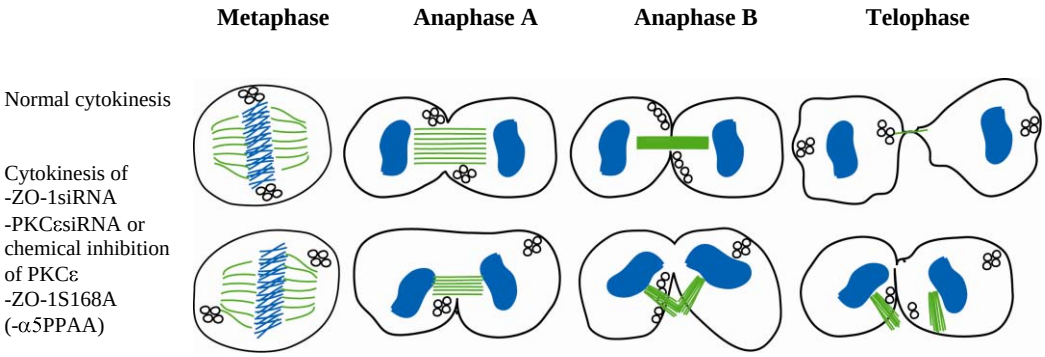

Figure 17. Model of the asymmetrical cell division in ZO-1siRNA cells. At the central spindle, i.e. the mid axis of the dividing cells, microtubules (green) elongate and move the separated chromosomes (blue) towards the opposing poles of the forming daughter cells. This triggers the actin-driven constriction of the plasma membrane at the central spindle or cleavage furrow, which resembles the tightening of a purse string and results in two individual cells with identical cytoplasmic and genomic content. During normal cytokinesis, cell separation, starting from metaphase and ending in telophase with two daughter cells, is completed in ~30 minutes. In anaphase, cell elongation driven by microtubules separates the chromosomes and vesicular structures (**oo**) that form symmetrically around the ingressing furrow. In late anaphase (B) microtubules condensate and form a midbody connecting the daughter cells. In telophase, exocyst vesicles accumulate in the other cell and locally severe the midbody which becomes ingested by the other cell only. At the plasma membrane of the new daughter cells, adhesion to the substratum increases and empowers the final separation. In ZO-1-depleted cells, the vesicular structures localize to random positions in metaphase cell and throughout the division. The cell ingresses asymmetrically in early anaphase (A) and the nuclei have lost their positioning. The midbody formation occurs but shifts away from the intercellular bridge and is detected as a loose floating structure. Daughter cells with midbody remnants remain attached for a substantially long time. Similar defects were observed in cells depleted of PKCε or treated with chemical PKC inhibitors.

7. SUMMARY AND CONCLUSIONS

Constantly fluctuating cellular events, cell motility and cell division, all require remarkable flexibility from the cell in respect of cell shape. The plasma membrane must coordinate the intake and re-organization of surface proteins while holding the cell tightly on the underlying extracellular matrix. Cells need to adhere, anchor, to their environment and integrins help the cells in this process. Previous studies strongly imply the need for integrin-mediated adhesion in mitosis. However, until this study, the role of integrins in regulating the events during cell division, was unknown. Integrins are themselves being regulated by an ever growing set of molecules. Most of them work by associating with the cytoplasmic tails of integrins. Importantly, whereas most studies discuss the regulation via integrin β subunit tail, we present here evidence for α tail –mediated regulation, as direct interaction of ZO-1 to integrin α5 tail regulated integrin functions in cell motility and constriction of daughter cells in cytokinesis.

Integrin biology extends far. Ultimately, this study and these results underscore the important point that there are no established rules or restrictions on how the proteins and pathways can come together in the cell. Firstly, this study shows that integrins, originally named after their ability to integrate the cell interior (actin) to the exterior (fibronectin) by Richard Hynes lab in the 80's and implicated in cell adhesion and motility, unexpectedly serve to assist the cellular division. Secondly, our findings challenge the view where tight junction proteins and integrins mediate physically and functionally separate types of cellular adhesions by demonstrating direct interaction of ZO-1 with integrin α5 with functional outcomes in cell motility and division. It is not a big surprise that, even if taking into account the multitude of cellular molecules and interactions known to date, the complex cell has made some energy-saving wise decisions by using certain multi-modal molecules in several situations. The identity and tasks of these molecules are important to the knowledge of the scientist and they may help understand the contradiction and context-dependency of varying results. So, finally, in this study, we have provided knowledge of such molecular decisions the cell has made and can make in various cellular contexts. This knowledge might become useful in designing personalized medicine. For example, targeting integrins in tumors with antagonists due to massive overexpression could be relevant in metastatic cancers, whereas in aneuploid tumors, integrin activation by agonists might help in cell adhesion and thus in executing successful dell division.

8. ACKNOWLEDGEMENTS

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