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In The Footsteps Of Migrating Cancer Cells

by

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ABSTRACT

The capability of cells to migrate is vital for many physiological processes, including embryogenesis, tissue repair, and immune surveillance. However, cell migration becomes fatal when cancer cells acquire invasive properties and set out to conquer new metastatic niches in the body. In fact, metastasis is the major cause for cancer mortality. Thus, in order to selectively fight metastasising tumour cells, without harming migrating host cells, it is of great importance to understand the molecular machineries that orchestrate cell motility under different circumstances.

In the course of this thesis, we have unveiled conceptually new mechanisms that account for cancer cell migration in 2D and 3D matrix environments. We first discovered a novel function for p120RasGAP in the control of integrin recycling. Integrins are transmembrane receptors that crucially balance cell adhesion and migration. Their function is carefully regulated by ECM ligand-binding activity, but also by their targeted trafficking from and to the plasma membrane. We discovered a non-catalytic, competitive mechanism by which p120RasGAP replaces the endocytic Rab21 from the integrin cargo on early endosomes, which subsequently allows the $\alpha\beta$1-heterodimer to recycle back to the plasma membrane.
Besides the continuous traffic of integrins, also their localisation needs to be directed to dynamic sites of adhesion. We found that in metastasising lung cancer cells, the loss of intercellular contacts contributes to increased cell motility by the PKCε-dependent complex formation between the tight junction protein ZO-1 and α5β1-integrin. This complex localises to the leading edge, where it maintains the lamellipodium and supports local Rac1 activation. Thereby, the α5β1–ZO-1 duo promotes directionally persistent migration and may hence fuel the metastatic dissemination of various human tumour cells.
Finally, we shed light on the invasive switch induced by oncogenic c-Met signalling. The growth factor receptor is implicated in the progression of most carcinomas to metastatic disease. In this study, we identified novel c-Met effectors: RhoA, which promotes phenotypic alterations, and Hip1, which mediates c-Met–stimulated β1-integrin endocytosis. Moreover, we found that c-Met triggers tumour cell migration in 3D matrices with distinct invasion modes, depending on the mechanism of receptor activation.

Collectively, these findings provide new information on the complex molecular networks that drive cancer cell migration and invasion. As research progresses, increasing knowledge will help us to eventually design potent therapies to combat disseminating tumours.

Key words: Cell Migration, Integrin, Trafficking, c-Met, Metastasis, ZO-1

TABLE OF CONTENTS

ABBREVIATIONS

1D, 2D, 3D	One-Dimensional, Two-Dimensional, Three-Dimensional
AP2	Adaptor Protein 2
Arf6	ADP-Ribosylation Factor 6
Arg	Abl-Related Gene
Arp2/3	Actin-Related Protein 2/3
ATP	Adenosine Triphosphate
BIM I	Bisindolylmaleimide I
CAF	Cancer-Associated Fibroblast
CAT	Collective-Amoeboid Transition
CCP	Clathrin-Coated Pit
CME	Clathrin-Mediated Endocytosis
Col	Collagen
CSF-1	Colony-Stimulating Factor 1
Csk	C-terminal Src inhibitory Kinase
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DN	Dominant-Negative
ECM	Extracellular Matrix
EEA1	Early Endosome Antigen 1
EGF	Epidermal Growth Factor
EM	Electron Microscopy
EMT	Epithelial-Mesenchymal Transition
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERK	Extracellular signal-Regulated Kinase
ESCRT	Endosomal Sorting Complex Required for Transport
FA	Focal Adhesion
F-actin	Filamentous actin
FACS	Fluorescence-Activated Cell Sorting
FAK	Focal Adhesion Kinase
Fip2	Rab11 Family-Interacting Protein 2
FBS	Fetal Bovine Serum
FERM	Band Four-point-one, Ezrin, Radixin, and Moesin
Fig.	Figure
FN	Fibronectin
FP	Fluorescence Polarization
G-actin	Globular actin
GAP	GTPase-Activating Protein
GDI	Guanine nucleotide Dissociation Inhibitor
GDP	Guanosine Diphosphate
GEF	Guanine nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GSK	Glycogen Synthase Kinase
GST	Glutathione S-Transferase
GTP	Guanosine Triphosphate
HEK	Human Embryonic Kidney
HGF/SC	Hepatocyte Growth Factor / Scatter Factor

Hip1	Huntingtin-Interacting Protein 1
IF	Immunofluorescence
IFs	Intermediate Filaments
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
kDa	Kilodalton
mAb	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
MAT	Mesenchymal-Amoeboid Transition
MEF	Mouse Embryonic Fibroblast
MET	Mesenchymal-Epithelial Transition
MMP	Matrix Metalloproteinase
MVBs	Multivesicular Bodies
pAb	Polyclonal Antibody
PAK1	p21-Activated Kinase 1
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Pull-Down
PDGF	Platelet-Derived Growth Factor
PDZ	Post-synaptic density 95; Disc large tumour suppressor; Zonula occludens 1
PI, PtdIns	Phosphoinositide, Phosphatidylinositol
PKB / PKC	Protein Kinase B/C
PKD1	Protein Kinase D1
PLA	Proximity Ligation Assay
PNRC	Perinuclear Recycling Compartment
PTB	Phosphotyrosine-Binding
PTEN	Phosphatase and Tensin homologue
RCP	Rab-Coupling Protein
RFP	Red Fluorescent Protein
RNAi	RNA interference
ROCK	Rho Kinase
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcription PCR
shRNA	Short Hairpin RNA
siRNA	Small-interfering RNA
SCF	Stem Cell Factor
SSC	Squamous Cell Carcinoma
TAM	Tumour-Associated Macrophage
TCPTP	T-Cell Protein Tyrosine Phosphatase
Tet	Tetracycline
Tiam1	T lymphoma Invasion And Metastasis-inducing protein 1
TIMP	Tissue Inhibitor Metalloproteinase
TIFF	Telomerase-Immortalised Foreskin Fibroblast
TIRF	Total Internal Reflection Fluorescence
TGN	*trans*-Golgi Network
WASP	Wiskott-Aldrich Syndrome Protein
WB	Western Blot
WT	Wild-Type
ZO-1	Zonula Occludens 1

LIST OF ORIGINAL PUBLICATIONS

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

I **Competitive Binding of Rab21 and p120RasGAP to Integrins Regulates Receptor Traffic and Migration**
Mai A.*, Veltel S.*, Pellinen T., Padzik A., Coffey E., Marjomäki V., Ivaska J. (2011) *Journal of Cell Biology*

II **Oncogenic c-Met Signalling Enhances Integrin Trafficking and Stimulates Cell Invasion in Cooperation with RhoA and Hip1**
Mai A., Muharram G., Rantala J., Kermorgant S., Ivaska J. (manuscript)

III **PKCε Regulation of an α5-Integrin–ZO-1 Complex Controls Lamellae Formation in Migrating Cancer Cells**
Tuomi S., Mai A., Nevo J., Laine J.O., Vilkki V., Öhman T.J., Gahmberg C.G., Parker P.J., Ivaska J. (2009) *Science Signaling*

 In addition, unpublished data is presented in this thesis.

* Equal contribution

INTRODUCTION

Strategies for discovering and developing cancer therapies undergo constant change. While the focus was initially on mere killing of rapidly dividing cancer cells (with consequent severe side effects on rapidly dividing normal cells), current cancer research has evolved into aiming at a more selective targeting of cancer-unique traits. However, up to now the success rate for oncology drugs in clinical trials is one of the poorest - being more than three times lower than investigational compounds for cardiovascular diseases (Kamb *et al.*, 2007). The explanation for the high failure rate may be the dynamic genomic changes that drive tumourigenesis and the immensely adaptable nature of cancer cells. Moreover, the metastatic spread of tumour cells is largely hampering therapeutic interventions and accounts for the majority of cancer deaths (Jemal *et al.*, 2011).

Metastasis is a multistep process in which cancer cells acquire several attributes in order to disseminate from the primary tumour and establish new neoplastic lesions in distant organs. These encompass the gain of growth factor and attachment autonomy, as well as the ability to migrate and invade into the surrounding tissue (Bernards and Weinberg, 2002). Signals emanating from growth factor and integrin adhesion receptors regulate signalling pathways critical for cell adhesion, proliferation, survival, polarity, and migration, and are thus often derailed in metastasising cancer cells (Hanahan and Weinberg, 2011).

Growth factor receptors are transmembrane receptor tyrosine kinases (RTKs) that, following activation by their specific ligands, stimulate signalling cascades important for cell survival and proliferation. Signal termination is achieved by receptor dephosphorylation and/or internalisation followed by subsequent degradation (Birchmeier *et al.*, 2003). Moreover, endosomal signalling has been recognised to critically contribute to RTK function (Wang *et al.*, 2002).
Integrins, on the other hand, connect the cellular actin cytoskeleton to the extracellular matrix and transmit environmental cues into cellular responses, without possessing any intrinsic catalytic activity (Hynes, 2002). Integrin functionality is to a great extent controlled by their location. They not only undergo a continuous circuitry of endocytosis and recycling back to the plasma membrane, but are also selectively transported to sites of dynamic adhesion (Caswell *et al.*, 2009).

Therefore, efficient vesicular trafficking - selective internalisation, directed vesicle transit, and targeted recycling - is of tremendous importance for the kinetics and magnitude of signal transduction by these cell surface receptors. Small Rab GTPases orchestrate the endocytic machinery, and are hence implicated in aberrant integrin and RTK functionality (Mitra *et al.*, 2011). Moreover, emerging evidence highlights the significance of interactions between integrins and growth factor receptors, both during signalling and trafficking, and should be considered for future anti-cancer therapies (Soung *et al.*, 2010).

REVIEW OF THE LITERATURE

1. Cell Migration

Migration involves the transition of organisms (e.g. amoeba) from one location to another, but also the movement of single cells within complex, multicellular organisms. In mammals, the orchestration of cellular migration is especially central for the morphogenesis during embryonic development, but also in the adult individual crucial for the maintenance of tissue homeostasis, immune surveillance, and wound healing (Ridley *et al.*, 2003). Migration-orchestrating proteins are pivotal for the preservation of life and health, yet they also contribute to a number of pathological processes including chronic inflammatory diseases (such as rheumatoid arthritis and asthma) and tumour metastasis. Moreover, also impaired cell migration results in pathogenic alterations, for instance during brain and heart development, leading to epilepsy, mental retardation, or heart abnormalities (Horwitz and Webb, 2003).
Consequently, cell migration is a fundamental and dynamic process that underlies strict regulatory mechanisms orchestrated by an array of signalling molecules.

1.1 The Basic Migratory Cycle

Cell movement often arises from environmental, migration-promoting signals, both attractive and repulsive, and is classified as either random (-kinesis) or, more often, directed (-taxis). Cell motility can be initiated by small molecules (chemotaxis), gradients of substrate-bound chemoattractants (haptotaxis) or substrate rigidity (durotaxis), mechanical stimuli such as the loss of cell-cell contacts (mechanotaxis), or in response to an electric stimulus (electrotaxis) (Petrie *et al.*, 2009).
Depending on the cell type and the environment, the modes of migration can differ greatly. But generally, for a cell to move forward it has to undergo an integrated cycle of four basic steps: polarisation, formation of protrusions, adhesion, and retraction of the rear (Horwitz and Parsons, 1999).
As a first response to motogenic signals, cells polarise by establishing an asymmetric, well defined front and rear side. At the leading edge, plasma membrane extensions are stretched in the direction of migration. These protrusions are mainly driven by the remodelling of the actin cytoskeleton and stabilised by the formation of adhesive contacts with the underlying substrate. The sites of adhesion are predominantly mediated by integrins, a family of cell surface receptors that connect the actin cytoskeleton with the surrounding environment. Adhesive contacts mature, as the cell migrates, from nascent focal complexes at frontal protrusions to more complex focal adhesions underneath the cell body that provide the necessary traction forces for forward motion. The retraction of the trailing edge is finally realised by the coordinated disassembly of mature focal adhesions at the rear and the contraction of the actin cytoskeleton (Figure 1).

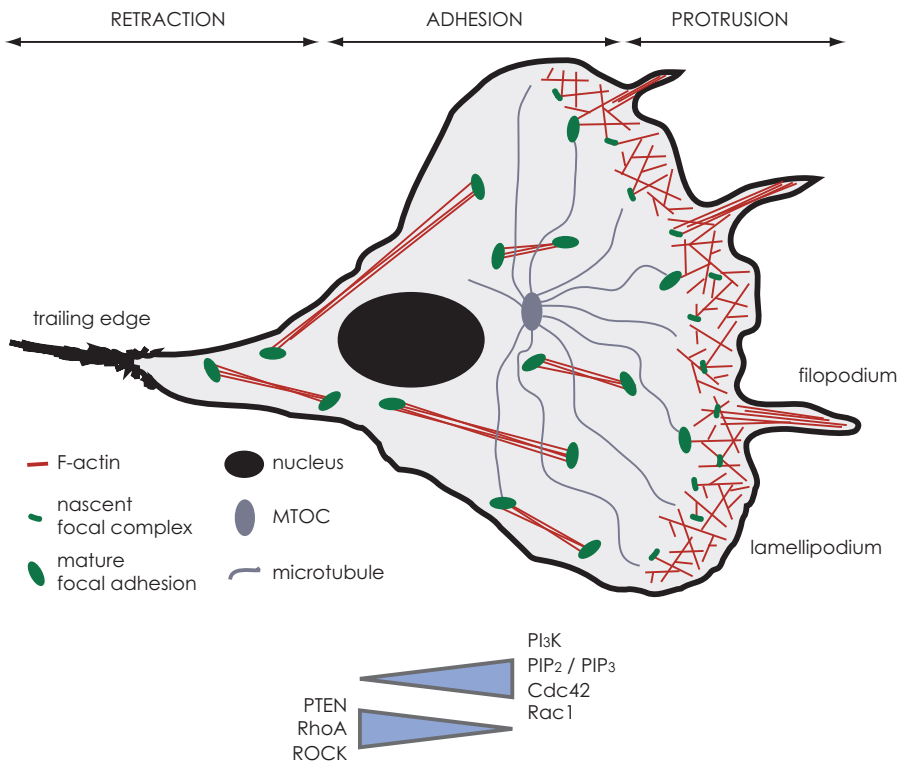

Figure 1: A Polarised Migrating Cell.
The prerequisite for directional migration is the development of a steady front-rear polarity. This depends largely on the asymmetric distribution of phosphoinositide phosphates (PIPs) and Cdc42. At the cell anterior, Cdc42 and Rac1 generate dynamic actin-based protrusions that sample the environment and guide the cell towards motogenic stimuli. Newly formed adhesion complexes create first adhesion sites with the underlying substrate and provide the necessary traction forces. These integrin-regulated structures mature into more complex focal adhesions, which orchestrate a wide range of cellular signalling cascades and jointly coordinate cell motility. In order to move forward, cells contract the actin cytoskeleton and retract the trailing edge - both RhoA-dependent processes.

1.2 A Cell's Skeleton: Actin, Microtubules, and Intermediate Filaments

How is the polarity in migrating cells established and retained?
This central event in the initiation of cell motility is realised by the serial recruitment of a range of interacting proteins to spatially and temporally restricted locations. All of these proteins regulate, in one way or the other, the remodelling of the actin cytoskeleton.

The cytoskeleton acts as a scaffold that supports cellular shape and tension, mediates cell-cell junctions, enables membrane organisation and intracellular transport, and thus essentially maintains normal cell function (Radulovic and Godovac-Zimmermann, 2011). The cytoskeleton of eukaryotic cells comprises three types of filamentous structures -

actin microfilaments, microtubules, and intermediate filaments - and their accessory proteins (Ward, 2011). These fibres are built from monomers, and their assembly and disassembly is tightly regulating their structure and function.

The family of intermediate filaments (IFs) is encoded by more than 60 genes, giving rise to a highly cell type-specific architectural component. Fibrous IFs assemble from monomeric IF-proteins that readily self-align in parallel to form coiled-coil dimers. These then instantly form anti-parallel tetramers, which further arrange longitudinally into extensive filaments with a diameter of about 10 nm (Herrmann *et al.*, 2007). Due to their anti-parallel association, IFs lack structural polarity and are more stable than micro-tubules and actin microfilaments. With these properties, IFs are the major contributor to a cell's topology and an important tool to functionally integrate all components of the cytoskeleton (Kim and Coulombe, 2007). Apart from their central contribution to the cytoarchitecture, the competencies of cytoplasmic IF-proteins have been extended to a growing number of other cellular functions. Vimentin, for example, has been shown to promote tumour cell migration (Vuoriluoto *et al.*, 2011) and to counterbalance pro-apoptotic stimuli by protecting ERK kinase from dephosphorylation and thus inactivation (Perlson *et al.*, 2006).

Microtubules build a more dynamic filamentous network with intrinsic polarity. They are composed of 13 proto-filaments - each assembled of α/β-tubulin heterodimers - that form a hollow cylindrical polymer with a diameter of 25 nm. The nucleation of micro-tubules starts in the microtubule organising centres (MTOC), typically close to the nucleus. Here, γ-tubulin creates a scaffolding platform for α- and β-tubulin monomers to assemble head-to-tail into proto-filaments with the slow growing (-)-end anchored in the MTOC and the faster growing (+)-end elongating towards the cell periphery. During polymerisation, both tubulin monomers are bound to GTP which stabilises them. But after assembly, only α-tubulin remains complexed with GTP, while β-tubulin rapidly hydrolyses GTP to GDP - resulting in the destabilisation of β-tubulin and its dissociation from the filament. Growing microtubules therefore constantly undergo cycles of growth and shrinkage at their (+)-ends, where β-tubulin is exposed. This so called 'dynamic instability' is regulated by a range of microtubule-associated proteins (MAPs) that bind and stabilise the growing (+)-ends and provide anchorage of microtubule filaments to the plasma membrane (Siegrist and Doe, 2007). In addition to MAPs, there is a set of motor proteins that use microtubules as tracks for their movement. These include the family of kinesins that move towards the (+)-end (Schaap *et al.*, 2011) and dynein that travels towards the microtubule (-)-end anchored in the MTOC (Bader and Vaughan, 2010). Generally, motor proteins use energy from ATP-hydrolysis to perform mechanical work by transporting cargo along microtubules. The great variety of motor proteins is thought to enable their cargo specificity and characteristic final destination within the cell (Ward, 2011).

The actin cytoskeleton forms a highly dynamic and widely branched network. There is only one class of motor proteins that uses actin filaments as their tracks: the diverse myosin family (Dantzig *et al.*, 2006). Generally, ATP-dependent myosins are classified as either conventional or unconventional, of which conventional myosins are found in the muscle, where they form filaments and participate in muscle contraction. Unconventional myosins are structurally different and perform a larger number of cellular functions, including intracellular trafficking - typically transporting cargo towards the elongating end of actin fibers.

Actin filaments (F-actin) are composed of globular actin subunits (G-actin) that assemble linearly into 6 nm wide, double helical polymers. Like microtubules, these filaments possess an intrinsic polarity with a so-called barbed (+)-end and a pointed (-)-end. Both, F- and G-actin, are bound to an Mg^{2+} ion and either ATP or ADP. During elongation, ATP-bound G-actin adds to the barbed (+)-end of the growing actin filament. ATP-hydrolysis, occurring during polymerisation, does not influence the rate of polymerisation, but has, however, a destabilising effect on the elongating actin filament. Once ATP is hydrolysed, F-actin is prone to collapse at the barbed end and to a lesser extent also at the pointed end. As a consequence, actin filaments simultaneously undergo elongation at one end and shrinkage that the other. This 'treadmilling' is only limited by the concentration of free monomers in the cytosol: The more subunits collide with the growing end, the faster the filament grows (Pollard and Borisy, 2003). Due to these kinetic properties, there is a certain concentration of G-actin at which the speeds of growth and shrinkage at both ends of an actin filament are equal. In this steady-state, the net length of a microfilament does not change.
In order to maintain a pool of G-actin monomers, allocated for sustaining the steady-state treadmilling as well as for availability if prompt actin growth is needed, there are two cooperating G-actin–binding proteins, which control the accessibility of free actin monomers in the cytosol. The first is thymosin-β4 (Tβ4) that binds and thereby sequesters G-actin by inhibiting its spontaneous *de novo* nucleation and elongation into F-actin. The other one is profilin, a Tβ4-competitor due to its higher actin-binding affinity. Also profilin inhibits F-actin nucleation, but unlike Tβ4, profilin catalyses the exchange of ADP to ATP and, moreover, allows the attachment of ATP-complexed G-actin to the barbed (+)-end. Jointly, profilin and Tβ4 control F-actin elongation and suppress spontaneous self-assembly (Pollard and Borisy, 2003).

1.3 The Actin Cytoskeleton under Continuous Reconstruction

As spontaneous self-assembly of monomeric actin is efficiently suppressed, new actin filaments arise predominantly from extension or severance of pre-existing filaments, but also from stimulated *de novo* nucleation. In any case, the assistance of actin filament-nucleating proteins is needed for actin polymerisation. Examples are the Arp2/3 complex, ADF/cofilin, and formins.
Arp2/3 is a complex comprising the actin-related proteins Arp2 and 3. This complex lacks intrinsic activity and is dependent on activation by nucleation-promoting factors such as the WASP family (e.g. N-WASP, WAVE) or cortactin. Once activated, Arp2/3 initiates F-actin elongation by binding to the pointed end of pre-existing filaments and stimulating their polymerisation towards the barbed end in a 70° angle to the existing filament. Similarly, Arp2/3 achieves branching of actin filaments by binding to the sides of F-actin and nucleating a new branch from there (Pollard and Borisy, 2003).
The elongation of F-actin is terminated by capping proteins such as capping protein (CP) and gelsolin. These proteins specifically bind the barbed (+)-end and inhibit both, its extension and shrinkage (Takeda *et al.*, 2010). In addition, the hydrolysis of ATP during actin polymerisation and the hence released γ-phosphate (γ-P_i) are responsible for the initiation of debranching. γ-Phosphate binds to ADF/cofilin, which in turn alters the actin filament structure depending on the local cofilin concentration. At low concentrations, ADF/cofilin causes the fragmentation of microfilaments. However, high amounts of cofilin favour F-actin polymerisation by increasing the number of free barbed (+)-ends and thus stimulating the nucleation of new actin filaments (Andrianantoandro and Pollard, 2006).

Taken together, although highlighting here only a few, there are a vast number of actin-associated proteins that cooperatively build, remodel, branch, and organise the highly versatile actin cytoskeleton.

1.4 Migration Devices: Lamellipodia and Filopodia

As mentioned earlier, cell locomotion is typically initiated by the formation of actin-rich protrusions in the direction of migration. These extensions are distinguished based on their shape as either lamellipodia or filopodia.
The lamellipodium is a broad but thin membrane protrusion, built from a highly branched network of short actin filaments. In contrast, filopodia (also called microspikes) are comprised of a tightly packed, parallel bundle of actin filaments, forming spike-like protrusions that extend from the lamellipodium (Vignjevic and Montagnac, 2008). While lamellipodia are thought to be the major engine for locomotion, filopodia are stable, sensory structures that probe the environment and guide the moving cell towards the migratory attractant (Figure 1).
Both itinerant organelles generate protrusions by creating a pushing force that spurs the plasma membrane forward. This force can be applied because the short and branched actin network in lamellipodia is less prone to bending compared to long, flexible filaments. The formation and maintenance of this highly dynamic, dendritic network is ensured by the cooperation of the Arp2/3 complex, which constantly initiates branching, and capping proteins, which limit the length of growing filaments (Huber *et al.*, 2008).
In contrast, the long and unbranched filaments in filopodia are maintained by elongation rather than by branched nucleation. This is ensured by nucleation-promoting factors of the Ena/VASP family. Enriched in filopodia tips, these proteins bind and tether barbed ends and prevent both capping and branching (Welch and Mullins, 2002). In order to also increase stiffness and avoid compression of filopodia, actin filaments are firmly bundled by a class of actin cross-linking proteins, such as filamin, α-actinin, or palladin (Dixon *et al.*, 2008).

1.5 The Conducting Family of Small Rho GTPases

Cell polarisation, in other words the establishment of a distinctive front-rear axis, is to a great extent regulated by the Rho family of small GTPases. Proteins of this class belong to the superfamily of Ras-related proteins and comprise approximately 20 members, including Rho (isoforms A, B, and C), Rac (isoforms 1, 2 and 3) and Cdc42 (Wherlock and Mellor, 2002). Most G-proteins of the Ras superfamily contain a C-terminal CAAX-motif (with C, cysteine; A, alanine; X, any amino acid), which is post-translational modified by prenyl transferases. The covalent attachment of prenyl groups, such as farnesyl or geranylgeranyl, dictates the localisation of this protein family. Once prenylated, cytosolic G-proteins are able to reversibly insert the hydrophobic anchor into lipid bilayers and to shuttle between the cytosol and membranes (Hoffman and Cerione, 2000).

Ras-related proteins are regulated by switching between functional distinct GTP- and GDP-bound conformations (Figure 2). GTPases generally bind their effector molecules when complexed to GTP, but are inactive in the GDP-form. In order to switch between these two activation states, they require the help of regulatory proteins. GTPase-

activating proteins (GAPs) are inactivators that enhance the weak intrinsic GTPase activity of small G-proteins, thereby stimulating the hydrolysis of the bound GTP to GDP. Guanine nucleotide exchange factors (GEFs) activate GTPases by binding to the GDP-bound, inactive conformation and subsequently facilitating the release of GDP. Since the cellular concentration of GTP is about 10-fold higher than GDP, GTPases promptly bind GTP and are active again as soon as GDP is released (Itzen and Goody, 2011). Another level of regulation is achieved by multifunctional Guanine nucleotide dissociation inhibitors (GDIs), which block the release of bound nucleotides and inhibit GEF activity. More importantly, GDIs determine the location of small GTPases within the cell. They extract and bind to the membrane-inserted lipid moiety and retain the G-proteins soluble in the cytosol. Only GDI dissociation factors (GDFs) can then release the bound GTPases from GDIs (Geyer and Wittinghofer, 1997).

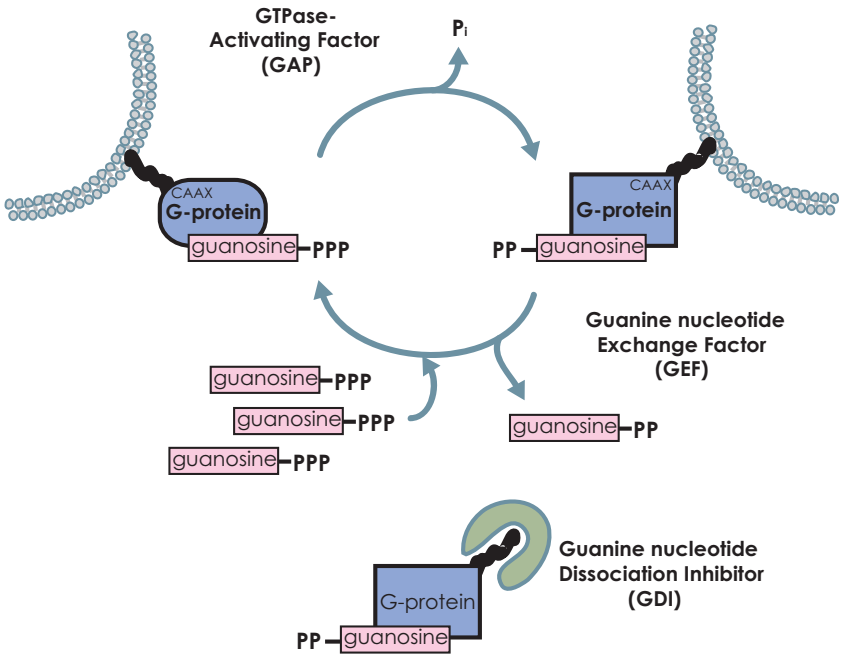

Figure 2: The Life Cycle of Small GTPases.
Small G-proteins are commonly bound to membranes via a hydrophobic lipid anchor, yet GDIs can retrieve and retain the GTPases in the cytoplasm. G-proteins are bound to either GTP or GDP. For conversion between the two conformations, GAPs and GEFs are required. When bound to GTP, GTPases generally recruit signalling molecules and exert their distinct functions.

Rho-related proteins conduct and orchestrate a multitude of cellular functions, including the organisation of the actin cytoskeleton. Rac1 activity is needed for the ruffling extension of lamellipodia; Cdc42 is associated with the formation of filopodia; and RhoA promotes focal adhesion maturation and regulates the arrangement of actin filaments

into stress fibres, which are crucial for the generation of traction forces during locomotion (Ridley, 2001). Due to their distinctive but cooperative functions, the timing and location of their activity is carefully balanced along the front-rear axis.

1.6 Cell Polarisation - How Cells Decide on a Direction

In response to external, migration-promoting signals, Cdc42 is activated at the plasma membrane, thereby initiating and determining intracellular asymmetry. The importance of this direction- sensing function of Cdc42 shows impressively in macrophages. The expression of a dominant-negative Cdc42 abolishes their ability to orientate towards a chemotactic signal, which results in random migration without any direction (Allen *et al.*, 1998).
Localised activation of Cdc42 is achieved by many different pathways, depending on the nature of the migratory stimulus (soluble chemokines, loss of cell-cell contacts and so on). Often, these signalling cues result in the establishment of a transient phosphoinositide phosphate (PIP) gradient with a high concentration of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ at the cell anterior. Phosphoinositides are generated by a network of lipid kinases, phosphatases, and phospholipase C (PLC), which orchestrate the interconversion of different phosphoinositide species (Bunney and Katan, 2010). $PI(3,4,5)P_3$ and $PI(3,4)P_2$ are commonly generated by PI3-kinase (PI3K) and degraded by the phosphatase PTEN. In response to chemotactic stimuli, the subcellular distribution of both enzymes is hence regulated reciprocally: PI3K is recruited to the plasma membrane at the site of the new leading edge, whereas PTEN translocates to the sides and the trailing edge (Devreotes and Janetopoulos, 2003). Positive feedback loops further amplify the accumulation of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ at the front of the cell (Figure 1). The hence originated gradient of phosphoinositides recruits PH-domain–containing effector proteins, which, either directly or indirectly, lead to the rapid and local activation of Cdc42 at the cell anterior (Horwitz and Webb, 2003).

Once activated, Cdc42 stimulates the complex formation of the atypical protein kinase C (aPKC) and the polarity proteins Par-3 and -6. This complex activates Rac1 and antagonises RhoA activity at the cell front by simultaneously activating Tiam1, a GEF for Rac1, and p190RhoGAP, which inactivates RhoA. In addition, the Par-3/Par-6/aPKC-complex is activated by Tiam1 in return and inactivated by the RhoA effector kinase ROCK (Hurd and Margolis, 2005). Localised Rac1 activity is further achieved by $PI(3,4,5)P_3$-sensitive GEFs (Welch *et al.*, 2003) and Cdc42 itself, which stimulates the Rac1-GEF βPIX via the kinase PAK1 (Cau and Hall, 2005). In brief, spatially restricted accumulation of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ as well as transient activation of Cdc42 determine the direction of migration and dictate the activation of Rac1 (and thus lamellipodium formation) at the cell front (Iden and Collard, 2008). At the same time, RhoA activity is antagonised as it counteracts the development of advancing extensions. This is achieved, for instance, by integrin-stimulated activation of the RhoA inhibitor p190RhoGAP. Ligated integrins promote Arg (Abl-related gene) kinase activity, which in turn phosphorylates p190RhoGAP. These phosphorylation sites serve as binding epitopes for the SH2-domains of p120RasGAP. The p190/p120-complex is consequently targeted to the plasma membrane, where p190RhoGAP locally suppresses RhoA activity (Bradley *et al.*, 2006).

In order to support the generation of a strongly branched lamellipodium at the leading edge, actin nucleation-promoting proteins of the WASP family are specifically activated at the cell front by PI(3,4,5)P3 and Rac1 (Suetsugu *et al.*, 2006). A high anterior concentration of ADF/cofilin cooperates with the Arp2/3 complex to promote the formation of a dendritic actin network (Carlier *et al.*, 1997). In addition, the Abl family of non-receptor tyrosine kinases (Abl and Arg) has been shown to reinforce actin dynamics by activating multiple signalling pathways, including the stimulation of the WASP family, cortactin, and the activation of the Rac1-specific GEF Sos-1 (Bradley and Koleske, 2009).

Besides extensive remodelling of the actin cytoskeleton, also the microtubule network is reoriented during cell migration. MT-associating proteins stabilise and anchor MTs at the leading edge. Also the MTOC itself is repositioned between the nucleus and the leading edge (Gomes *et al.*, 2005). By this, MTs radiate from the MTOC towards the motile front, maintain cellular polarity and deliver cargo vesicles in this dynamic region (Siegrist and Doe, 2007). In fibrillar one-dimensional (1D) and three-dimensional (3D) environments, however, cell motility features an anterior MT bundle and a posterior orientation of the MTOC (Doyle *et al.*, 2009).

1.7 Traction and Retraction: Finally, Movement!

Apart from establishing a propulsive front, in order to move forward, migrating cells have to translocate their cell body, too. This firstly requires the anchorage of frontal protrusions to the underlying substrate or adjacent cells. These sub-membrane sites of adhesion function as traction sites and are mainly mediated by transmembrane integrin receptors, which link the actin cytoskeleton to proteins of the surrounding extracellular matrix (ECM). Additionally, these integrin-rich adhesion plaques serve as mechanosensory devices, probing ECM rigidity and transmitting intracellular signals that alter cytoskeletal dynamics and adhesion size accordingly (Bershadsky *et al.*, 2003).

Cell-matrix adhesions assemble and mature gradually. The high Rac1 and Cdc42 activity in the lamellipodium encourages the formation of small, immature adhesions that stimulate Rac1 and Cdc42 activation in return. Rac1 and its effector kinase PAK, for instance, have been shown to recruit active integrins to the leading edge, thereby priming first adhesive contacts at the base of protruding lamellipodia (Kiosses *et al.*, 1999). The formation of nascent focal complexes starts with integrin aggregation and clustering induced by the binding to their specific ECM-ligands. Integrin engagement thus results in the recruitment of other adhesion components, either individually or in preformed complexes, such as the focal adhesion kinase (FAK) or the multi-domain signalling adapter proteins paxillin and tensins (Webb *et al.*, 2002).
As the anterior protrusions move on, focal complexes either disintegrate or mature into focal adhesions (FAs) of bigger size and more complex molecular composition. Maturation is induced by increased tensile forces and, in response, stronger RhoA- and myosin II-mediated contraction of actin stress fibers (Broussard *et al.*, 2008). The actin cross-linking protein α-actinin is especially enriched in focal adhesions, reinforcing the attachment of microfilaments to the membrane and stabilising mature adhesion sites (Webb *et al.*, 2002). FAs are further strengthened by the multi-adapter protein vinculin, which integrates most FA components and delays their disassembly (Humphries *et al.*, 2007).

Specialised cell-matrix adhesions evolve from FAs in a more soft and flexible ECM environment, with fibronectin being the major component. These so called fibrillar adhesions form upon dephosphorylation and subsequent dissociation of paxillin, and are typically rich in tensin and fibronectin-binding $\alpha5\beta1$-integrins. Their major function includes the formation of fibronectin fibrils underneath the cell body (Zaidel-Bar *et al.*, 2007).

Maturation of cell-matrix adhesions is antagonised by their turnover, both at the front, where new adhesion sites are inherently assembled and disassembled, as well as at the rear of the cell, where the trailing edge needs to be retracted (Webb *et al.*, 2002). The turnover of FAs is tightly accompanied by the trafficking of integrins that are continuously internalised at dissolving adhesions and recycled back to locations of newly forming adhesion sites (Caswell and Norman, 2006). Accordingly, loss of the FA-stabilising vinculin decreases integrin residency in FAs, and thus facilitates their disassembly (Humphries *et al.*, 2007). Similarly, also the FAK-induced dissociation of α-actinin weakens mature cell-matrix adhesions. Furthermore, activation of the protease calpain in response to high spatial calcium levels at the cell rear, leads to the degradation of FA components including integrins, FAK, and talin (Huttenlocher *et al.*, 1997). The disassembly of adhesions is also largely attributed to microtubules. Direct contact of MTs with FAs reduces compulsory tension at sites of adhesion and also enables the delivery of yet unknown signalling molecules conducting adhesion dissociation (Krylyshkina *et al.*, 2002).

In order to allow the translocation of the cell body, retraction of the trailing edge, and finally forward motion, several mechanisms converge to stimulate the myosin II-powered contraction of actomyosin bundles. For example, MT-associated RhoA GEFs are known to be released from microtubules at the cell posterior, where local RhoA activity, in conjunction with its effector kinase ROCK, supports actomyosin contraction (Broussard *et al.*, 2008). RhoA-stimulated actomyosin constriction thus causes pulling on mature FAs, generating the required traction forces for forward motion, and pulling on weaker, nascent adhesions in the trailing edge that hence rip off and are left behind as the cell moves on.

In summary, the locomotion of cells is precisely regulated by a lattice of scaffolding and signalling proteins, which jointly enable the induction and maintenance of cellular polarity, balance adhesion site maturation and turnover, and ultimately allow cell body translocation and rear retraction. Maintaining the equilibrium between adhesion and protrusion is thereby of particular importance as both too strong as well as too weak adhesion dynamics impede migration velocity (Gupton and Waterman-Storer, 2006). Small disturbances in this vital process can lead to pathological consequences, including the gain of invasive capabilities of malignant tumour cells.

1.8 Migration Modes: A Matter of Environmental Constraints

The migratory cycle described so far, portrays only the basic principles of cellular motility. However, cells adjust their migration mode according to specific environmental constraints, individual preferences, and functions.
A number of cells, including neural crest cells, fibroblasts or leukocytes, prefer to move as solitary entities. In contrast, epithelial or endothelial cells typically preserve strong

cell-cell contacts and migrate collectively as sheet-like cohorts (Farooqui and Fenteany, 2005). Moreover, some cells move relatively slowly, while others seem to rush forward. Fibroblasts migrate at low speed and exhibit distinctive steps of polarisation, protrusion, adhesion, and rear release during their locomotion. Fast moving cells, like leukocytes for example, display a less obvious separation between those motility stages. They seem to move effortlessly with blurred boundaries between adhesion and propulsion (Horwitz and Webb, 2003).

The differences between migration strategies become even more evident, when cell motility on planar, two-dimensional (2D) surfaces is compared to the movement of cells in a three-dimensional (3D) environment. In general, 2D substrates are more rigid and evenly distributed. Cells are forced into a dorsal-ventral polarity that allows adhesion only at the bottom side (Even-Ram and Yamada, 2005). Additionally, cells on 2D have been found to possess elevated Rac1 activity, consequently providing cells with multiple peripheral protrusions and thus leading to less directional persistent migration (Pankov *et al.*, 2005). In contrast, 3D environments are thought to better mirror *in vivo* conditions in tissues, where cells are entirely surrounded by their ECM-ligands. *In vivo*, cells encounter various complex environments with individual biophysical properties and thus diverse migratory challenges, including the interstitial fluid, the connective tissues or the highly impermeable basement membrane. Cellular migration strategies therefore depend to a great extent on the microenvironment (Poincloux *et al.*, 2011). But generally, the migration of cells is believed to be more directed, both *in vivo* and in the lap of artificial 3D surroundings (Even-Ram and Yamada, 2005).
When cells move within complex 3D environments, they face certain motional limitations. Owing to meshed networks of ECM components and depended on their elastic and porous properties, cells have to adapt their shape or employ strategies to remodel the ECM by local proteolysis. According to the matrix architecture, cells exploit different migration modes to move into and within the ECM (Friedl and Wolf, 2010). Roughly schematising, amoeboid and mesenchymal migration modes have been described (Figure 3).

Amoeboid migrating cells are typically roundish, lack a stable front-rear polarity and stress fibres, and exhibit only weak adhesive cell-matrix contacts, which rapidly turnover. In order to move forward, these cells employ RhoA-driven actomyosin contraction in conjunction with the dynamic reshaping of their actin cytoskeleton, which allows them to simply push and squeeze through pre-existing cavities in the matrix (Friedl *et al.*, 2001). Such highly adaptive, adhesion-independent movement is only limited by the size of the nucleus that cannot condense any further. However, pore diameters, greatly exceeding or falling short of the cell size, already slow down the migration speed (Friedl and Wolf, 2010).

Mesenchymal migration is characterised by increased attachment and cell-matrix interactions of elongated, spindle-like cells. They move in an integrin-dependent manner with Rac1-generated anterior protrusions. Unlike on 2D surfaces, these advancing extensions do not occur as sheet-like lamellipodia, but as thin cylindrical pseudopodia that extend in all three dimensions. Moreover, protease-driven degradation of the surrounding ECM generates the required migration tracks (Poincloux *et al.*, 2011).

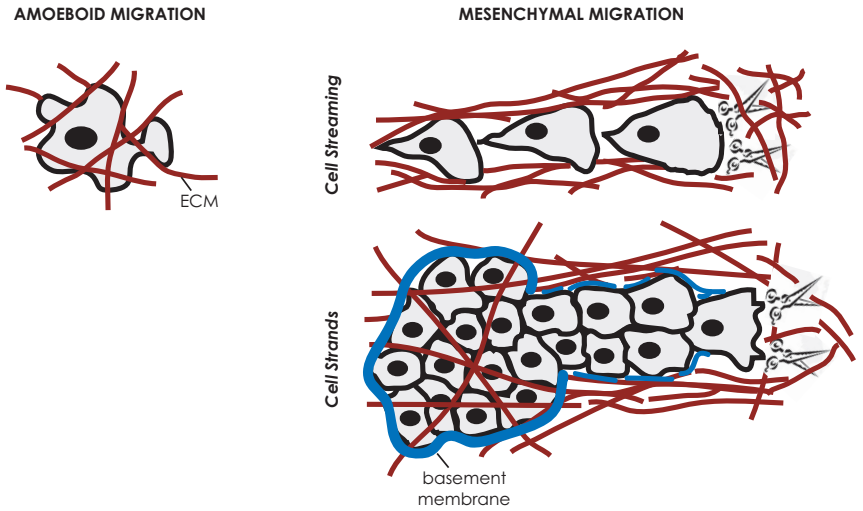

Figure 3: Distinct Migration Strategies.
In complex 3D environments, cells employ two different migration strategies: Amoeboid-migrating cells lack protease activity, and instead move forward by notable deformation of their cell body and actomyosin contraction. In contrast, mesenchymal-migrating cells are able to proteolytically degrade the surrounding matrix, thus creating migration tracks. In this mode, cells either preserve cell-cell contacts or not. In addition, cells are usually guided by a pioneering cell (of either epithelial or fibroblast origin), which expresses MMPs and deposits basement membrane components onto the advancing microtracks.

Cells exploiting the mesenchymal migration mode can move either individually or collectively (Figure 3). Individual cells migrate one after another along common trails. This so called 'cell streaming' is enabled by the vibrant formation of temporary intercellular connections, while tight cell-cell junctions are lacking. In contrast, if strong contacts between individual cells are retained, collective locomotion occurs. Collectively migrating, multicellular alliances foster a remarkable functional and structural organisation that extends across the entire cohort. Resembling individually moving cells, these collectives are governed by a front-rear asymmetry: Leading 'pioneer' cells at the tip fulfil exploratory tasks, expose highly dynamic pseudopodia, mediate integrin-dependent adhesion, and proteolytically degrade the surrounding ECM. In contrast, 'follower' cells at the rear of the assembly predominantly exert cohesion and retractile forces (Friedl and Wolf, 2010). Within the group there is a great deal of communication and division of tasks that can be genetically determined. During the embryonic development of zebrafish, for example, collectively migrating cell cohorts have been shown to display clear morphological and functional differences between 'leaders' and 'followers', which are attributed to distinctive gene expression profiles (Aman and Piotrowski, 2008). Division of tasks within a cell collective also shows in the concerted generation of migration tracks. Initial microtracks are formed by pioneering tip cells that employ cell surface-located matrix metalloproteinases (MMPs) to degrade the adjacent ECM. The path is then broadened by the joint proteolytic activity of the following cells, which cleave and re-align ECM fibres impeding the forward motion (Wolf *et al.*, 2007). In addition to proteolytic activity, tip cells pave the way and provide guidance for their accompanying

cells by secreting basement membrane components (laminins, perlecan, or type IV collagen) onto the newly formed microtracks (Friedl and Gilmour, 2009).

In conclusion, cells differ greatly in their migratory strategies, and it is the matrix architecture (both, on 2D and in 3D) that predominantly dictates the cellular migration mode. The switching between different migration modes is to a great extent regulated by the family of integrin cell surface receptors.

2. Integrin Adhesion Receptors

Integrins comprise a large family of transmembrane receptors with versatile functions. By integrating cell-matrix adhesion, intercellular interactions, and signal transduction, they regulate all cellular key functions: proliferation, differentiation, survival, angiogenesis, and cell motility. Generally speaking, integrins act as powerful sensors, which alter cellular functions in response to environmental cues.

2.1 A Multitasking Family

In vertebrates, integrins form a family of 24 specific α/β-heterodimers, composed from 18 α- and 8 β-subunits that non-covalently associate. Being transmembrane receptors, integrins employ their large extracellular domains (about 700 to 1000 residues) to bind components of the ECM, and interact with their short intracellular moieties (about 20 to 50 residues) with the actin cytoskeleton. The cytoplasmic tail is further crucial for integrin signalling properties as it interacts with an array of adapter and signalling molecules (Luo *et al.*, 2007). Each of the 24 heterodimers possesses characteristic ligand binding preferences and signalling properties. Based on the evolutionary relation of the α-subunits, four integrin subfamilies are distinguished: Firstly, the RGD-binding receptors ($\alpha5\beta1$, $\alpha v\beta3$, and $\alpha v\beta6$ being named as examples), which recognise and bind sequences rich in the amino acid triplet arginine-glycine-aspartic acid (RGD). ECM-components such as fibronectin or vitronectin contain RGD-motifs and are thus ligands for this integrin subfamily. Secondly, there is the subfamily of laminin-binding receptors ($\alpha3\beta1$, $\alpha6\beta1$, $\alpha7\beta1$, and $\alpha6\beta4$), which mediate adhesion to the basement membrane. However, the $\beta4$-subunit plays a quite unique role: Unlike other integrins, it possess a large cytoplasmic domain that harbours several phosphorylation sites and serves as an anchor for intermediate instead of actin filaments (Hynes, 2002). In addition to these two highly conserved subfamilies, there are also the collagen-binding integrins and, lastly, the more heterogeneous subfamily of residual integrins comprising $\alpha4\beta1$, $\alpha9\beta1$, and $\alpha4\beta7$, of which the $\beta7$-subunit is only expressed in leukocytes, where it recognises cell adhesion molecules on the vasculature (e.g. VCAM-1). Collagen-binding integrins are $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$, and $\alpha11\beta1$. A structural characteristic of this subgroup is the inserted I-domain (also called A-domain) in the α extracellular moiety that generates the ligand-binding site. This αI-domain is also found in α-subunits pairing with the leukocyte-specific $\beta2$-integrin, which is important for cell-cell adhesion during leukocyte migration across vascular and lymphatic endothelium (Johnson *et al.*, 2009).
Taken together, integrin cell surface receptors comprise a large family with diverse binding partners and thus cellular functions, albeit with varying significance in different tissues and cell types. But how are integrins activated and regulated?

2.2 Standing up Straight, Legs Apart: Integrin Activation

Unlike transmembrane receptors of the receptor tyrosine kinase (RTK) family, integrins do not possess any intrinsic catalytic activity. In order to mediate their functions, they rely on the recruitment of a lattice of adapter and signalling molecules that subsequently execute integrin-dependent cellular functions (Zaidel-Bar *et al.*, 2007). Nevertheless, integrins are far from being just 'passive' linking molecules! One of their most remarkable features is their ability for bidirectional signal propagation across the plasma membrane (Gahmberg *et al.*, 2009): Binding of their extracellular ligands induces conformational changes that are converted into intracellular signalling cascades and responses (the so called 'outside-in' signalling). In contrast, also intracellular proteins can either fuel or impede integrin activity, thereby altering receptor affinity towards ECM-ligands and thus fine-tuning integrin functions such as cell adhesion or migration ('inside-out' signalling).
When resting, integrins obtain a bent conformation with their N-terminal head-domains facing the plasma membrane and their two subunits being in close proximity. This closed, inactive configuration is stabilised by a salt bridge between the α- and β-cytoplasmic tails ('legs') in vicinity to the plasma mem-brane (Hughes *et al.*, 1996). Activation is achieved by either ligand binding to the extracellular domain or intracellular regulators that bind to the cytoplasmic integrin tail - both mechanisms leading to straightening and spatial separation of the 'legs'. With the cytoplasmic tails being apart, effector-binding epitopes are exposed and downstream signalling is enabled (Kim *et al.*, 2003).

2.3 Integrins and Their Little Helpers

The actin-binding protein talin has been recognised to actively participate in integrin ('inside-out') activation (Tadokoro *et al.*, 2003). In response to pro-migratory stimuli, but not exclusively, talin is recruited to the plasma membrane by RIAM, a Rap1 effector. This interaction exposes an integrin-binding site on the N-terminal tripartite FERM-(band 4.1, ezrin, radixin, and moesin) domain of talin, which contains a phosphotyrosine-binding (PTB)-domain. This PTB-domain is found in several integrin-accessory proteins, allowing the interaction with either of the two conserved NPxY-motifs (N, aspartic acid; P, proline; x, any amino acid; Y, tyrosine) on the integrin β-cytoplasmic tail (Calderwood *et al.*, 2002). A second hydrophobic interaction site of talin within integrin membrane-proximal residues additionally releases the inhibiting salt bridge between the 'legs', ultimately leading to integrin priming. This dual interaction with integrins is unique to talin and might explain why other PTB-containing proteins bind the β-NPxY-motif, but fail to activate integrins (Wegener *et al.*, 2007). However, talin has recently been found to not solely transmit integrin activity. Members of the kindlin protein family also contain FERM-domains, with which they bind integrin β-tails and activate the adhesion receptors in cooperation with talin (Moser *et al.*, 2008; Montanez *et al.*, 2008).

Once activated, integrins bind their ECM-ligands and, in synergy with mechanical stress applied by the interactions with the rigid matrix, cluster to form nascent focal complexes (Ali *et al.*, 2011). This cooperative integrin stimulation leads to the activation of Src, a non-receptor tyrosine kinase. Src and its inhibitor Csk (C-terminal Src inhibitory kinase) are constitutively bound to the β-integrin tail. Receptor clustering results in the

dissociation of Csk and ultimately to activation of Src, which stimulates several downstream signalling pathways, reinforcing lamellipodia formation at the protruding front. Furthermore, Src promotes activation of the FA key regulatory protein FAK and phosphorylates the β-NPxY-motif itself, leading to the dissociation of talin, and hence to focal adhesion maturation (Arnaout *et al.*, 2007). FAK serves as an integrating adapter and signalling protein that promotes focal adhesion formation and turnover by recruiting multiple downstream targets, implicated in cell migration orchestration. FAK signalling is terminated by association of SOCS proteins (suppressors of cytokine signalling), initiating FAK polyubiquitination and subsequent degradation (Liu *et al.*, 2003). In addition, FAK is dephosphorylated by integrin-recruited and -activated protein tyrosine phosphatases such as Shp2 (von Wichert *et al.*, 2003) or PEST (Zheng and Lu, 2009). Src-mediated phosphorylation of the NPxY-motifs on the β-tail, as well as integrin phosphorylation by other specifically recruited kinases, generates characteristic binding epitopes for other integrin accessory proteins - ultimately leading to the assembly of signalling platforms at focal adhesion sites (Legate *et al.*, 2009). Moreover, phosphorylation of NPxY-motifs on β-cytodomains has been linked to the binding of Dok1, which has an inactivating effect on integrins (Anthis *et al.*, 2009).

In summary, integrins excel not only in their bidirectional signalling capabilities and their mechano-sensory function, they also integrate a multitude of vital signalling cascades. Each heterodimer thereby activates a characteristic set of targets (Wu *et al.*, 2008). Remarkably, ligand-bound integrins compete against each other, actively inhibiting their mutual activation and downstream signalling. Collagen-bound α2β1-integrin, for instance, restrains signalling of α5β1- and αvβ3-integrins via protein kinase A (PKA) activation. Conversely, PKCα recruitment by fibronectin-ligated α5β1 suppresses α2β1-integrin activation (Orr *et al.*, 2006). These properties, in conjunction with an extensive network of more than 150 accessory proteins (Zaidel-Bar *et al.*, 2007), make integrins powerful regulators, which are crucial for a vast number of cellular functions, including cell motility.

2.4 Seized by Wanderlust - Integrin Trafficking

Beyond regulation of ligand binding and activation at the cell surface, integrin activity is further controlled spatiotemporally: They travel steadily between the plasma membrane and various vesicular organelles in the cytosol.
The signalling of many cell surface receptors, including growth factor receptors, is terminated by internalisation into multivesicular bodies (MVBs), followed by degradation in lysosomes. For this purpose, cargo proteins are tagged by ubiquitination and sorted into degradation-destined MVBs by the ESCRT (endosomal sorting complex required for transport) machinery (Williams and Urbe, 2007). Also α5β1-integrin has been found to be subjected to this degradative pathway. Following fibronectin-binding, the ligated integrin is ubiquitinated at its α5-subunit, endocytosed into the cytosol together with FN, where it is recognised by ESCRT proteins and finally sorted to lysosomes (Lobert *et al.*, 2010). However, most integrin receptors evade this degradative fate and, instead, experience a constant flux of internalisation into endosomal compartments and recycling back to the plasma membrane. This selective endo/exocytic circuitry is to a great extent regulated by the large family of small Rab GTPases. Like other small G-proteins, Rabs switch between an active GTP- and an inactive GDP-bound conformation, controlled by

GEFs and GAPs (Figure 2). Rab GTPases are post-translational isoprenylated by covalent attachment of a geranylgeranyl group to a C-terminal cysteine residue. Owing to this hydrophobic lipid anchor, Rab proteins are reversely bound to membranes of specific cellular organelles. The targeting of Rabs to their designated membranes is achieved by GDIs that retrieve Rab GTPases from membranes, retain them in the cytosol and, on demand, recycle them back to their target membranes (Pfeffer, 1994).

Generally speaking, Rab proteins and their regulators coordinate intracellular trafficking by ensuring cargo specificity and supervising fusion organelle identity. Of the more than 60 Rab GTPases in humans, some have general functions, while others operate in a cell type-specific manner (Mitra *et al.*, 2011). However, a salient feature of all Rab proteins is their localisation to distinct organelles. Commonly, a certain Rab GTPase executes a particular step during vesicular traffic, while the cargo proceeds consecutively from one Rab-compartment to the next. Rab5, for example, localises specifically to the plasma membrane, caveosomes, and clathrin-coated vesicles, and stimulates their transport to early, sorting endosomes, where it tethers exclusively to the early endosome antigen 1 (EEA1). In contrast, Rab4 and Rab11 mediate recycling from early endosomal compartments, while Rab7 promotes maturation of vesicles into late endosomes and fusion with the lysosomes. Rab9, on the other hand, has been shown to guide late endosomes retrogradely to the *trans*-Golgi network (TGN). Despite this segregation of functions, some Rab proteins still localise to the same endosomal organelle, but compartmentalise the membrane into distinct microdomains. Late endosomes contain characteristic mosaic-like patterns for Rab7 and Rab9 (Stenmark, 2009). Similarly, Rab4, Rab5, and Rab11 colonise early and recycling endosomes in various, yet distinctive combinations (Sonnichsen *et al.*, 2000). Each of these organelles corresponds to a specific endosomal compartment, gradually sorting and handing cargo on through the trafficking machinery till the final destination (Zerial and McBride, 2001).

The guidance of integrins through the complex network of trafficking organelles is thoroughly controlled and greatly dependent on both the integrin heterodimer and cell type in question. Two main routes are used by integrins to enter the cell: clathrin-mediated endocytosis (CME) and clathrin-independent internalisation (Figure 4). CME is characterised by the cytoplasmic protein clathrin that is recruited to the plasma membrane, where it assembles into clathrin-coated pits (CCPs). Owing to a set of clathrin adapter proteins (AP2, Dab, Numb, and Hip1 being named representatively), these pits contain specifically selected cargo proteins. Sequential recruitment of clathrin co-factors then leads to the budding of pits, and subsequently the GTPase dynamin enables the pinching-off from the membrane. In the cytosol, clathrin-coated vesicles swiftly lose their clathrin coat and progress to early sorting endosomes (Ramsay *et al.*, 2007). Several cell surface molecules, including growth factor receptors, predominantly enter the cell via CCPs; whereas, for a long time, integrins have been believed not to favour this entry route. However, a growing number of integrins have recently been recognised to internalise clathrin-dependently, including $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 3$. $\beta 1$- and $\beta 3$-integrins directly interact with the clathrin adapter proteins disabled 2 (Dab2) and Numb, which recognise the cytoplasmic NPxY-motif in integrin β-tails by their PTB-domains (Caswell *et al.*, 2009). The $\beta 1$-integrin tail contains, moreover, two membrane-distal, conserved NxxY-motifs, which interact directly with the clathrin adaptor protein 2 (AP2). Integrin mutants carrying phenylalanine substitutions within these motifs ($\beta 1$YYFF) fail to internalise $\beta 1$-integrins through the clathrin-dependent endocytosis route (Pellinen *et al.*, 2008).

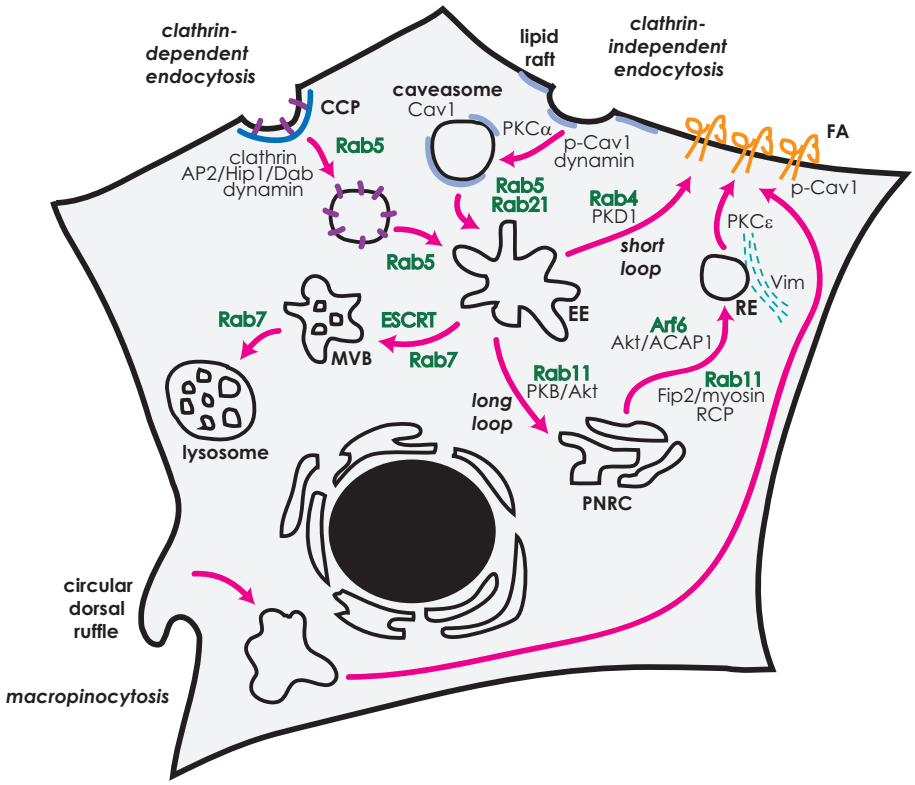

Figure 4: Integrin Trafficking Routes.
Integrins undergo a constant flux of endocytosis and recycling - lysosomal degradation occurs rarely. Endo/exocytic traffic is generally regulated by Rab GTPases that mediate distinct transport steps (see text for details). Integrins typically enter the cell by clathrin-mediated endocytosis or clathrin-independent, lipid raft-regulated caveolae. Following growth factor stimulation, rapid uptake of integrins arises also from macropinocytosis at circular dorsal ruffles. The return of integrins to the plasma membrane happens through the fast, Rab4-mediated or the slow, Rab11/Arf6-regulated route. The decision on the recycling path is made in the early, sorting endosome (EE) by a number of signalling proteins according to external stimuli. (CCP, clathrin-coated pits; Cav1; caveolin 1; FA, focal adhesion; PNRC, perinuclear recycling compartment; RE, recycling endosome; MVB, multivesicular bodies; Vim, vimentin)

Most integrins, however, have been found to internalise via clathrin-independent yet dynamin-dependent caveolar endocytosis. This route sprouts from cholesterol-rich lipid rafts on the plasma membrane, which enrich cargo proteins due to distinct biophysical and biochemical properties. Caveolae are small, uncoated invaginations within these lipid rafts. To date it is not fully understood how caveolae bud from the plasma membrane, albeit dynamin-dependent abscission is required. Furthermore, phosphorylated caveolin 1 has been found to stimulate caveolae internalisation. Phospho-caveolin 1 is usually retained by integrins at focal adhesion sites. Upon detachment or FA disassembly, phospho-caveolin 1 is released and targeted to caveolae, where it

induces their budding from the plasma membrane. Following re-attachment, integrins sequester phospho-caveolin 1 in FAs again and thereby impede their own internalisation at sites of adhesion (del Pozo *et al.*, 2005). After abscission, caveolae are first delivered to caveolin 1-containing endosomes, termed caveosomes, and then proceed from there to early and recycling endosomes (Nichols, 2003). α2β1-Integrin has been found to enrich in caveolae following antibody- or echovirus-1–induced integrin clustering. This process depends on PKCα activation and the α2-integrin subunit, as clustering of αv supported endocytosis into CCPs (Upla *et al.*, 2004). PKCα has been realised to be of general importance for the dynamin-dependent internalisation of β1-integrins (Ng, 1999). The kinase directly binds to the conserved NxxY-motifs in the β-tail of activated integrins and authorises their endocytosis into caveosomes (Parsons *et al.*, 2002).

Interestingly, some integrin heterodimers are promiscuous in their choice of an internalisation route. αvβ3-Integrin, for example, is optionally recruited to either caveolae or CCPs (Caswell *et al.*, 2009). Similarly, α5β1 generally enters the cell in an NxxY- and clathrin-dependent manner. However, if this entry route is blocked by either expression of mutant β1YYFF or the clathrin inhibitor monodansylcadaverine, α5β1-integrins are internalised Rab21-dependently (Pellinen *et al.*, 2008). Rab21, on the other hand, interacts with the conserved membrane-proximal GFFKR sequence that is common to most integrin α-subunits (Pellinen *et al.*, 2006). Resembling Rab5 (both GTPases belonging to the Rab5 subfamily), Rab21 critically regulates the endocytosis of several integrins, including α1β1, α2β1, and α5β1, and controls the movement of early endosomes along microtubules (Simpson *et al.*, 2004; Pellinen *et al.*, 2006). The general importance of Rab21-mediated integrin trafficking reflects in the finding that Rab21 not only critically regulates cell migration, but also controls cytokinesis, a late stage of mitosis, in which the two daughter cells divide. During cytokinesis, Rab21-vesicles target integrin cargo to and from the cleavage furrow, specifically guiding integrins to their sites of action (Pellinen *et al.*, 2008).

Another clathrin-independent internalisation mechanism, which has not been discussed thus far, is macropinocytosis emanating from circular dorsal ruffles. These structures are built from actin filaments and form temporarily at the dorsal plasma membrane in response to stimulation with growth factors, including EGF, PDGF, and HGF (Buccione *et al.*, 2004). Abscission of these large fluid-filled macropinosomes supposedly requires action of Rab5, dynamin, and cortactin. Integrins were found to rapidly traffic via this pathway following PDGF-stimulation (Gu *et al.*, 2011).

2.5 Back to the Future - Integrin Recycling

Irrespective of the internalisation route, cargo proteins enter the early, sorting endosome, where their final destiny is decided: transport to lysosomes for eventual degradation or recycling back to the plasma membrane for re-use (Ramsay *et al.*, 2007). Integrins are usually designated for the latter fate.

Receptor recycling occurs along two different routes: A Rab4-mediated, fast recycling path (the 'short-loop') that recycles cargo directly from the early endosome back to the plasma membrane; or a Rab11-controlled, slow recycling pathway (the 'long-loop')

that guides cargo through the perinuclear recycling compartment (PNRC) before returning it to the plasma membrane (Ramsay *et al.*, 2007). Which recycling route is to be chosen, is tightly orchestrated according to cellular and environmental cues (Figure 4).

Most integrins transit the PNRC and recycle through the Rab11-dependent long-loop, a process that requires PKB/Akt activation and consequent GSK-3 (glycogen synthase kinase) inactivation (Roberts *et al.*, 2004). Moreover, the return of β1-integrins to the plasma membrane is also dependent on PKCε-mediated phosphorylation of the IF-protein vimentin, which triggers the release of recycling vesicles from the tethering filaments (Ivaska *et al.*, 2005).

In cooperation with Rab11, also the ADP-ribosylation factor 6 (Arf6) has been found to regulate the slow recycling of integrins via the PNRC (Powelka *et al.*, 2004). Both GTPases exert their recycling function by recruiting a number of effector proteins. Rab11, for example, binds to the adapter protein Fip2 (Rab11 family-interacting protein 2) that bridges Rab11 and its cargo vesicle to the motor protein myosin Vb - hence allowing vesicular movement along actin filaments (Hales *et al.*, 2002). Also another member of the Fip family, the Rab-coupling protein (RCP), has been shown to be crucial for the recycling of β1-integrins (Caswell *et al.*, 2008). In contrast, Arf6 collaborates with ACAP1, which is not only an inactivating GAP for Arf6, but also its downstream effector. As a component of the coat complex, ACAP1 sorts the cargo protein at the recycling endosome. When phosphorylated by Akt, ACAP1 specifically binds to endosomal β1-integrin and thereby assigns it for recycling from the PNRC to the plasma membrane (Li *et al.*, 2005).

Another member of the Rab11 subfamily, thus promoting recycling from the PNRC, is Rab25. But unlike other Rab11 isoforms, Rab25 expression is restricted to cells of epithelial origin. Rab25 has been shown to directly bind to the cytoplasmic tail of β1-integrins and to direct them to the tips of invading pseudopodia. Interestingly, the migration-stimulating effect of Rab25 could only be observed in a 3D environment, but not on planar surfaces (Caswell *et al.*, 2007).

However, migrating cells are able to bypass the long recycling route. In order to rapidly supply nascent focal contacts with integrin adhesion receptors, and following PDGF-stimulation, αvβ3, but not α5β1, is diverted from the longer Rab11-route to the short Rab4-dependent path, triggering recycling directly from early endosomes (Roberts *et al.*, 2001). This selective redirecting of αvβ3 is achieved by direct association of the protein kinase D1 (PKD1) with the β3-cytoplasmic domain, which then enables the quick delivery of αvβ3 to nascent focal complexes at the advancing lamellipodium (Woods *et al.*, 2004). Moreover, if the short-loop recycling path is blocked by expression of a dominant-negative Rab4, αvβ3 still returns to the plasma membrane by taking the Rab11-route, but the integrin is no longer targeted solely to the cell front (Jones *et al.*, 2006).

2.6 Shifting Gears: Integrins in Cell Motility

Endocytic trafficking is essential for the generation and maintenance of cell polarity. The asymmetric distribution of migration-determining proteins, including integrins, arises from the targeted transport of vesicles into defined intracellular regions. Integrins are generally delivered to the advancing lamellipodium. This is achieved by increased internalisation and recycling rates at the cell front, while at the trailing edge trafficking is slowed down (Jones *et al.*, 2006). Moreover, also the lamellipodium itself plays a role in concentrating signalling molecules at the leading edge by simply restricting their lateral diffusion (Weisswange *et al.*, 2005).

The spatiotemporal regulation of endosomal trafficking not only restricts integrins to defined subcellular compartments, but also affects their downstream signalling and thus cellular responses. It has been shown that integrins mutually alter their itinerary routes and, as a result, impede their signalling. The best studied example is the differential recycling of αvβ3 and α5β1 that leads to a switch in the migration mode (White *et al.*, 2007). Cells move directionally persistently, when αvβ3 recycles rapidly by taking the PDGF-stimulated and Rab4-mediated short-loop. Being targeted to the leading edge, αvβ3 signalling supports local Rac1 activation, provides high levels of ADF/cofilin, and thus drives the extension of a single prominent lamellipodium (Danen, 2009). Besides provoking localised Rac1 activation, αvβ3 antagonises simultaneously the recycling of α5β1-integrin - possibly by sequestering RCP, a Rab11-adapter protein that specifically recycles β1-integrins (Caswell *et al.*, 2008). α5β1 also mediates cell adhesion to fibronectin, but unlike αvβ3 it inhibits cofilin and strongly activates RhoA signalling. Thus, cells that express only α5β1 migrate rapidly but randomly owing to multiple peripheral protrusions in various directions (Danen, 2009). Similarly, if αvβ3-integrin is inhibited, its α5β1-antagonising function is abrogated, resulting in increased recycling of this integrin and thus random cell motility (White *et al.*, 2007). Remarkably, this diminished persistent migration was only observed on 2D surfaces, whereas RCP-mediated recycling of α5β1-integrin increased the directed migration of cells into a fibronectin-rich 3D matrix (Caswell *et al.*, 2008).
In addition to striking differences of both RGD-binding receptors in their preference to activate either Rac1- or RhoA-signalling, αvβ3 and α5β1 further differ in the cell-matrix adhesions they support - with the first promoting static and the latter highly dynamic adhesions (Truong and Danen, 2009). Interestingly, it has been found that both integrins bind immobilised fibronectin equally well, but only α5β1 is additionally able to bind soluble fibronectin. It has been proposed that binding of this free fibronectin fraction (possibly secreted by the cell itself) competes and thus diminishes the interaction of α5β1 with the immobilised ligand in the ECM - hence making these cell-matrix adhesions more pliable and dynamic (Truong and Danen, 2009).
Taken together, integrins shift the gears in cell motility by adjusting distinct migration modes according to the environmental and cellular requirements. On 2D, β1-integrins generally support random migration, while β3-receptors promote directed motility.

It is further evident that an asymmetric activation of the opposing GTPases Rac1 and RhoA plays an essential role in the regulation of migration. But balancing these two is not only achieved by differential activation by integrins. It has been found that inactive Rac1 itself is retrieved by Rab5- and clathrin-dependent internalisation and guided to the early endosome, where Tiam1 (T lymphoma invasion and metastasis-inducing protein

1), a GEF protein, stimulates the exchange of GDP for GTP (Palamidessi *et al.*, 2008). Active Rac1 is subsequently targeted through the Arf6-mediated recycling route to the plasma membrane at the cell anterior. In addition to this CME-mediated mechanism, Rac1 can also rapidly localise to the protruding front via macropinosomes emanating from circular dorsal ruffles following growth factor stimulation. Also integrins are known to be recruited to these ruffles, internalised and instantly recycled to sites of newly forming adhesion complexes (Gu *et al.*, 2011).

In summary, integrin adhesion receptors comprise a large and diverse family exerting functions well beyond cell attachment regulation. With their unique ability for bidirectional signal transduction, integrins adapt cellular functions, such as survival and proliferation, according to environmental cues and simultaneously modify interactions with the environment, including adhesion and migration, corresponding to cellular needs. The ability to mutually interfere with their trafficking and signalling further adds diversity to integrin functions. And it is just this proficiency of integrins that makes them susceptible for exploitation in various pathologies, ranging from cardiovascular, dermatologic, and musculoskeletal disorders up to carcinogenesis (Moschos *et al.*, 2007; Wehrle-Haller and Imhof, 2003).

3. Cancer Cell Invasion and Metastasis

Integrins and associated signalling cascades are involved in nearly all steps of carcinogenesis, the transformation of a normal cell into a neoplastic cancer cell. During the progression into a malignant tumour, cells acquire gradually a set of distinctive and complementary capabilities - summarised by Hanahan and Weinberg as the hallmarks of cancer (Hanahan and Weinberg, 2011). These comprise: sustained and excessive proliferation; evasion of apoptosis, growth suppressors, and immunological destruction; induction of angiogenesis; gain of replicative immortality; alteration of the cellular energy metabolism; as well as invasion and metastasis - all of which are favoured by the inherent genomic instability of cancer cells and the occasional inadvertent support from immune cells during inflammatory defence reactions.

3.1 Sowing the Seeds of Cancer - Metastasis

The spread of cancer cells from the primary tumour to other, distant sites in the body is the most common lethal consequence of cancer. Difficulties in the systemic treatment and cure arise from the great biological heterogeneity of cancer cells in the primary tumour and in metastases, and also from responses of the host, which often are inadvertently tumour supporting (Fidler, 2003). Cancer cells constantly escape from the primary tumour, but seldom succeed to colonise to distant organs. The explanation for this is that the process of metastasis requires a cascade of discrete steps, all of which demand specific capabilities (Figure 5). Most cancer cells are insufficient in one or more of these steps, resulting in immediate failure to metastasise.

The relentless growth of a primary tumour demands an enormous supply with nutrients and oxygen as well as the removal of by-products. These needs can no longer be satisfied by simple diffusion if a tumour mass exceeds 1 to 2 mm in diameter. The resulting deficient oxygen supply (hypoxia) is most often the driving force for tumour

cells to initiate angiogenesis and lymph-angiogenesis in the surrounding host tissue and to establish an extensive network of capillaries (Lu and Kang, 2010). The metastatic cascade then begins with local invasion into the surrounding stroma and intravasation into lymphatic or blood vessels. In many cases, local lymph nodes are the primary target for metastatic colonisation (Psaila and Lyden, 2009). In the blood circulation, shear stress and the lack of attachment provide a hostile environment that is fatal for most cancer cells. The few that survive, escape from the vessels, transit the endothelium into the neighbouring tissue, and form small micrometastasis. Expansive proliferation of these lesions into macroscopic tumours, survival in the new, foreign microenvironment, neovascularisation, and evasion of immunological defence mechanisms, ultimately result in the settlement of a metastasis at a distant organ - being capable of seeding metastases itself (Fidler, 2003).

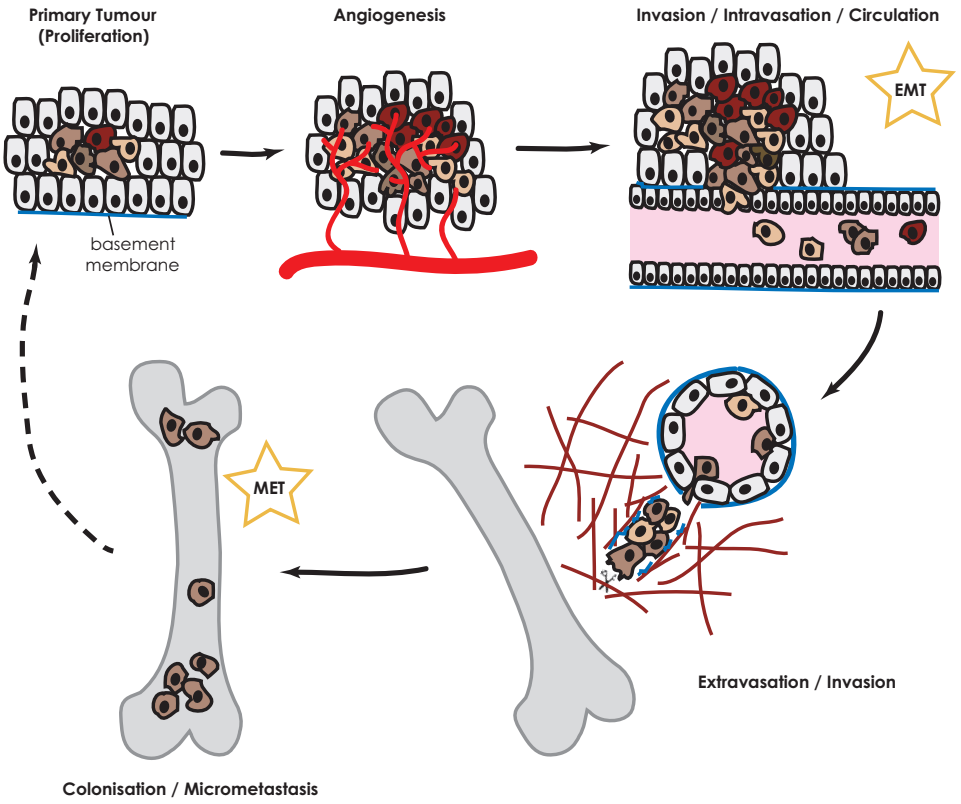

Figure 5: The Metastatic Cascade.
A metastasis-competent cancer cell must be a jack of many trades in order to successfully colonise a new site in the body. The metastatic process requires distinctive skills that enable the cancer cell to invade various tissue environments, to penetrate vessel walls, to endure the hostile milieu in the circulation, and, finally, to grow and survive in the new environment (see text for details). Thus, most cancer cells actually fail to metastasise. However, the few cells that possess these versatile requirements (usually a population of cells that complement each other's capabilities) are hard to eradicate and often resistant to conventional systemic therapies.

The success rate of planting a metastasis depends on the cells in the primary tumour - not all have the same potential to disseminate - and also on the new host environment. Certain organ sites (termed 'metastatic niches') have been realised to be especially permissive for metastatic colonisation. This has led to the so-called 'seed and soil' hypothesis, which encompasses two findings: Firstly, the primary tumour (the 'seed') consists of genetically and phenotypically heterogeneous cancer cell populations, which can complement the metastatic capabilities of one another. As different organs require unique survival and colonisation strategies, metastasis-triumphant cells are selected for specific survival-promoting attributes. Thus, distinct metastases may originate from the same primary tumour, yet from different cancer cell populations within the tumour. Consequently, metastases at distinct locations can differ biologically greatly from each other - one of the major obstacles in cancer treatment. And secondly, tumours also harbour host cells (such as fibroblasts or infiltrating leukocytes) that interact with the cancer cells and often in conjunction with the microenvironment ('the soil'), accidentally, support tumour growth, angiogenesis, and invasion - hence malignancy. This sinister cooperation between cancer cells and the host's microenvironment is especially significant during cancer cell invasion (Langley and Fidler, 2007). The impact of receptive metastatic niches is reflected in the common observation that many neoplasms are found to preferentially colonise to certain organ sites (Ben-Baruch, 2008).

3.2 Cutting the Cord - EMT

Cancer cells evolve a variety of strategies in order to break away from the primary tumour, invade into adjacent tissue, and resist apoptosis - and dysfunctional integrin signalling is involved in all of them.
Carcinoma cells (malignant tumours of epithelial origin) acquire invasive attributes by co-opting a regulatory mechanism normally employed during embryonic morphogenesis and wound healing: the epithelial-mesenchymal transition (EMT). During EMT, epithelial cells acquire a mesenchymal-like morphology by loosing their apical-basolateral polarity, disrupting cell-cell contacts, and, as a consequence, gain cell motility. In terms of malignant carcinoma cells, EMT induces complete or partial dedifferentiation resulting in cancer cells with highly dynamic and adaptive features - one of them being the acquirement of invasive capabilities (Thiery, 2002).

A number of transcription factors, including Slug, Snail, and Twist, orchestrate EMT during embryogenesis, and are known to be over-expressed in various tumours (Hanahan and Weinberg, 2011). Amongst other functions, these transcription factors suppress E-cadherin expression, and hence induce the weakening of adhesive bonds between cells. E-cadherin normally forms adherens junctions by mediating stable, homophilic interactions at the cell surface and connecting to the cortical actin cytoskeleton via α- and β-catenin. Some carcinoma cells express the neuronal (N-) cadherin in response to the loss of epithelial E-cadherin, but this adhesion molecule mediates only weak intercellular connections (Thiery, 2002). In addition to its cohesive role, E-cadherin also functions to represses cell proliferation ('contact-inhibition'). Loss of E-cadherin induces the translocation of the associated β-catenin to the nucleus, where it cooperates with transcription factors to regulate gene transcription and enable proliferation and survival even in the absence of cell-cell contacts (Brembeck *et al.*, 2006).

The integrity of functional epithelia is further supported by apically localised tight junctions, and loss of these structures can also induce EMT. Tight junctions are large multi-protein complexes that contain predominantly, but not exclusively, the transmembrane proteins occludin and claudins, as well as the cytosolic signalling and scaffolding protein zonula occludens 1 (ZO-1), which anchors membrane-integral components to the actin cytoskeleton (Reichert *et al.*, 2000). ZO-1 contains multiple protein-interaction domains, amongst others an N-terminal triplicate of PDZ- (Post-synaptic density 95; Disc large tumour suppressor; Zonula occludens 1) domains. PDZ-domains are superior signalling hubs that create functional protein complexes and determine their cellular localisation due to their multifaceted binding epitopes: They bind the C-terminus of several proteins, homo- or heterodimerise with other PDZ-containing proteins, interact with lipids in membranes, or bind to proline-rich internal peptide sequences that exhibit a kinked conformation (Nourry *et al.*, 2003). It is thus the PDZ-domains that target cytosolic ZO-1 to tight junctions. But also the actin-binding region has been recognised to affect ZO-1 location to the F-actin–rich tight junctions, and, interestingly, to likewise actin-rich, nascent adhesions at the leading edge of migrating cells (Fanning *et al.*, 2002).

In conclusion, the first step for dissemination from the primary tumour is commonly the weakening of intercellular contacts and the loss of apical-basolateral cell polarity. These and other invasive attributes are acquired by altered gene expression in the course of EMT. Conversely, at sites of metastatic colonisation, motile carcinoma cells can reverse EMT-induced morphology changes through the opposing mesenchymal-epithelial transition (MET). MET then results in the recovery of epithelial characteristics, which facilitates the integration of solitary cancer cells to establish a solid metastasis (Thiery, 2002).

3.3 Cancer Invasion as a Result of Usurpation and Adaptation

In practice, many cancer cells are not fully dedifferentiated and instead invade the adjacent stroma collectively as cell sheets, preserving intercellular junctions, coordinating cytoskeletal activities, and establishing a functional and structural polarity that extends across the entire cohort (Friedl and Gilmour, 2009). Collectively migrating cells are sterically more constrained than cells invading solitarily. Thus, the locomotion of multicellular collectives requires extensive modifications of the surrounding ECM. Integrin clustering and activation at the protruding front is known to attract surface-anchored MMPs and to recruit soluble proteases. $\alpha 2\beta 1$, for instance, directly binds and engages the cytoplasmic protease MMP1, and $\beta 1$- and $\beta 3$-integrins recruit MT1-MMP, both enzymes degrading collagen-matrices (Friedl and Wolf, 2003).
However, if ECM proteolysis is inhibited or integrin functions are impaired, instead of stopping their movement, cancer cells are able to adjust their mode of migration according to the new migratory challenges. Mesenchymal-like cells simply convert to amoeboid migrating cells that, independent of integrin engagement, squeeze through pre-existing gaps in the stroma. This compensatory process is termed mesenchymal-amoeboid transition (MAT) and is characterised by high Rac1 activity, while RhoA/ROCK-mediated signalling is diminished (Friedl and Wolf, 2003). Similarly, collectively migrating multicellular cohorts are able to individualise and continue invasion as single amoeboid migrating cells (collective-amoeboid transition, CAT). It has been shown that $\beta 1$-integrins are especially crucial for the maintenance of cell collectives, as blocking

α2β1, α3β1, and α5β1 resulted in the dissemination of single cells from primary melanoma explants (Hegerfeldt *et al.*, 2002).
In a nutshell, cancer cells are highly adaptive in their migration and invasion patterns and simply adjust to given environmental cues. The resulting diversity and flexibility of invasive migration modes certainly increases the difficulty to fight metastases.

Moreover, carcinoma cells that have not undergone EMT, and thus retain their epithelial attributes, such as squamous cell carcinoma (SCC) cells, are employing another strategy to remodel the surrounding matrix and invade: usurpation of accessory stromal fibroblasts. These fibroblasts not only release migration-promoting factors, but also actively pave the way for the following epithelial sheets by proteolytic ECM degradation (Gaggioli *et al.*, 2007; Hegerfeldt *et al.*, 2002). Remarkably, pioneering fibroblast and following carcinoma cells differently activate Rac1 and RhoA signalling pathways. While the fibroblast at the tip of the heterogeneous collective powers migration by generating traction forces through integrin-mediated RhoA activation and actomyosin contraction, SCCs need neither RhoA nor integrin engagement but Cdc42 activity to follow the pre-generated migration tracks.
But cancer-associated fibroblasts (CAFs) do much more than to just generate invasion paths for carcinoma cells. CAFs have been found to release chemokines and growth factors, including EGF, HGF, and TGFβ, and thereby to contribute to the growth and survival of solid tumours (Allen and Jones, 2011). A few CAF specimens have been reported to secrete angiogenesis-stimulating factors and thus to attract endothelial progenitor cells into the solid tumour (Orimo *et al.*, 2005). And CAFs are further involved in the generation of an altered ECM. This includes a modified matrix composition: most solid tumours possess a unique profile of ECM proteins, which derives from over-expression of e.g. fibronectin or tenascin-C, as well as from the expression of multiple, alternative splice variants, which are tumour-specific. The altered matrix composition is thought to provide additional integrin-binding sites, thereby promoting altered signalling and thus tumour growth and invasion (Allen and Jones, 2011). In addition to the remodelling of ECM patterns, CAFs also impact the mechanical properties of the matrix. A rigid surrounding has been shown to be favourable for tumour progression and dissemination. Reduction of ECM stiffness can even repress the malignant behaviour of breast epithelial cells (Paszek *et al.*, 2005). In order to generate a stiff matrix, some CAFs secrete lysyl oxidase, an enzyme that cross-links collagen and elastin fibres. The resulting tightened matrix reinforces β1-integrin clustering, enhances focal adhesion formation, and downstream signalling, which consequently results in profound and robust promotion of tumourigenesis (Levental *et al.*, 2009).
To date, the origin of CAFs is controversial. They may evolve from normal stromal fibroblasts in response to carcinoma cell-induced local stimulation or from cancer cells that have undergone EMT. It has even been reported that non-tumour associated fibroblasts, isolated from women with breast cancer, differ from those derived from women without breast cancer - pointing to an inherent (genetic or epigenetic) host predisposition for a pro-tumourigenic microenvironment (Allen and Jones, 2011). It is clear, however, that CAF properties differ from those of normal stromal fibroblasts. Immortalised but non-transformed prostate epithelial cells have been shown to acquire carcinogenic attributes when co-cultured with tumour-associated fibroblasts, but not in the presence of normal prostate fibroblasts. Interestingly, CAFs were not able to stimulate tumour development in non-immortalised benign prostate epithelium, indicating that some prior abnormality is conditional for a carcinogenic response to CAFs (Allen and Jones, 2011).

In addition to CAFs, also macrophages have been realised to be exploited by cancer cells. These so called tumour-associated macrophages (TAMs) are found in most solid tumours and, like CAFs, secrete tumour growth-, survival-, and invasion-promoting factors. TAMs presumably derive from activation by tumour-secreted inflammatory cytokines (Lewis and Pollard, 2006). Together with other inflammatory cells, TAMs are believed to contribute predominantly to the recruitment of endothelial progenitor cells and hence to tumour neovascularisation (Li Calzi *et al.*, 2010).

In conclusion, tumour cells are able to evolve a mere infinite number of strategies in order to capture and colonise new niches in the body. By adaptation to emerging growth and invasion challenges, as well as by exploitation of the host's microenvironment, metastasising cancer cells are notoriously hard to kill.

3.4 Invasion Devices: Invadopodia and Podosomes

Whenever malignant cells invade the stroma, they form specialised membrane protrusions dedicated for local ECM degradation - invadopodia. These structures are actin-enriched membrane extensions underneath the cell body, often in proximity to the nucleus. Invadopodia contain integrins, tyrosine kinases, and most importantly soluble and membrane-anchored MMPs (Ayala *et al.*, 2006). Invadopodia formation requires the small GTPase Arf6, as loss of Arf6 blocks both, invadopodia formation and carcinoma cell invasion, but has no impact on adhesive properties (Hashimoto *et al.*, 2004).
Podosomes, on the other hand, are dynamic and punctual structures, also rich in F-actin and located at the ventral site of the cell body. And like invadopodia, podosomes exhibit ECM proteolytic functions, but additionally fulfil adhesive tasks during migration. Podosomes resemble focal adhesions owing to their composition of vinculin, talin, and α-actinin, and additionally contain a number of actin remodelling proteins, such as gelsolin, Arp2/3, or N-WASP (Buccione *et al.*, 2004). Podosomes are formed by TAMs in response to the colony-stimulating factor 1 (CSF-1), a macrophage growth factor and chemoattractant secreted by carcinoma cells (Yamaguchi *et al.*, 2006). Notably, carcinoma cells and TAMs have been found to establish a paracrine CSF-1/EGF loop by which they mutually promote their joint invasion. Cancer cells secrete CSF-1 and express EGF-receptor (EGFR), while TAMs release EGF and expose the CSF-1 receptor. The subsequent formation of invadopodia and podosomes then synergistically promotes mammary tumour invasion (Wyckoff *et al.*, 2004).

3.5 Partners in Crime: Integrin and Growth Factor Receptors

Integrins and growth factor receptors exert a joint control on tumour survival, growth, and dissemination. The nature of their cross-talk is complex, and can be both, synergistic or mutually inhibitory (Alam *et al.*, 2007).
Integrins have been shown to trigger receptor tyrosine kinase (RTK) inactivation and even degradation. The T-cell protein tyrosine phosphatase TCPTP, for example, is recruited to the plasma membrane by the cytoplasmic domain of α1-integrin heterodimers. The hence activated phosphatase then dephosphorylates a number of growth factor receptors, including EGFR, PDGFRβ, and VEGFR2 (Mattila *et al.*, 2010). Moreover, integrins can also affect the rate of RTK degradation: Loss of cell attachment

resulted in immediate proteasomal degradation of the PDGFRβ, whereas integrin-mediated adhesion protected PDGFRβ from this catabolic fate (Baron and Schwartz, 2000).

But commonly, integrins and RTKs exert collaborative signalling functions. A prominent example for their synergistic action is the activation of the cytoplasmic kinase FAK, which serves as an integrative scaffold downstream of pro-migratory signals from both receptors. FAK is recruited by clustered integrins to sites of nascent focal complexes, where it is positioned close to its target effectors. Migration initiated by growth factor stimulation (by EGF and PDGF) requires concomitant association of FAK with RTKs and integrins, only then allowing the recruitment and activation of migration-promoting effectors such as the Src kinase (Sieg *et al.*, 2000). Another example is the laminin-binding integrin α6β4, which has been found to be crucial for the invasive growth of carcinoma cells following activation of EGFR or the c-Met receptor for the hepatocyte growth factor (HGF). Interestingly, α6β4 adhesion to laminin was not required, pointing to a non-adhesive role of this integrin as a RTK signalling-amplifier (Trusolino *et al.*, 2001).
Conversely, α2β1-integrin has been shown to play a more active and direct role in the activation of EGFR at sites of cell-cell contacts, where association of EGFR with α2β1 enables the activation of the RTK independent of EGF-stimulation but dependent on integrin-ligation (Yu *et al.*, 2000). Generally, integrins have been found to potentiate the signalling responses of RTK receptors for insulin, PDGF, EGF, and VEGF (Moro *et al.*, 2002).
The possible sinister cross-talk between integrins and growth factor receptors is especially evident in recent observations showing that integrin function-blocking agents, developed as potential anti-cancer drugs, might at low doses even stimulate tumour invasion and angiogenesis. Caswell and co-workers found that blocking of αvβ3-integrin by either the soluble ECM protein osteopontin or the αv-specific inhibitory peptide cilengitide abrogates its α5β1-antagonising function. Following αvβ3 inhibition, α5β1 strongly binds to EGFR and the recycling-promoting RCP adapter protein, which results in their concerted return to the plasma membrane. Thus, the consequences of αvβ3 inhibition are increased recycling of α5β1, which promotes carcinoma cell invasion in 3D, as well as rescue of EGFR from proteasomal degradation. Strikingly, EGFR signalling has been found to be amplified under these circumstances, leading to a strong activation of Akt/PKB, a known pro-invasive kinase (Caswell and Norman, 2008). Similarly, cilengitide-mediated blocking of αvβ3-integrin function was found to stimulate the recycling of VEGFR2 via the Rab4-mediated short-loop, instead of targeting the pro-angiogenic growth factor receptor to the degradative pathway. Thus as a consequence, tumour growth was supported by cilengitide *in vivo* owing to enhanced VEGF-stimulated angiogenesis (Reynolds *et al.*, 2009).

Taken together, the interactions between integrin and growth factor receptors are versatile, highly complex, and in some cases even adaptive. It has been suggested that integrins provide the 'cellular context' for the outcome of growth factor signalling - due to their cell type-specific expression and functionality, and owing to their ability to recruit different subsets of effector proteins in proximity to RTKs, thereby influencing growth factor fate and function (Alam *et al.*, 2007).

3.6 The Many Faces of c-Met

The hepatocyte growth factor HGF (also termed HGF/SF) and its receptor c-Met have been implicated in the progression of many solid tumours to metastatic disease. In fact, c-Met was even first identified as an oncogene in a human osteogenic sarcoma cell line (Gentile *et al.*, 2008). However, c-Met exerts also pivotal physiological functions during embryonic development and organ formation, as well as in adulthood for liver regeneration and wound healing. The physiologic roles of c-Met already imply its versatile biological functions: Activation of c-Met induces several signalling cascades that collectively drive a complex morphogenetic program termed as 'invasive growth'. This involves the loss of cell-cell contacts, scattering and gain of motility, induction of angiogenesis, escape from apoptosis, and the ability to proliferate and settle in a new environment. These c-Met–induced responses, which are required for normal cells during embryogenesis, are evidently also attractive attributes to hijack by metastasising carcinoma cells. It is thus no surprise that *MET* is one of the most frequently deregulated RTK oncogenes, and that constitutive c-Met activity is associated with poor patient prognosis (Knudsen and Vande Woude, 2008).

The HGF ligand is generally secreted by cells of mesenchymal origin, whereas the expression of c-Met is restricted to epithelial cells. The mature c-Met receptor is a tyrosine kinase, located at the plasma membrane and comprising a disulfide linked α/β-heterodimer. The short α-subunit is located entirely extracellularly, while the β-subunit encompasses a large extracellular region (which is, together with the α-subunit, involved in ligand-binding), a transmembrane domain, and an intracellular segment, which contains the juxtamembrane section, the kinase domain and a C-terminal, regulatory multifunctional docking site (Birchmeier *et al.*, 2003). HGF-binding induces c-Met homodimerisation and trans-phosphorylation of tyrosines within the catalytic domain (Y1230, Y1234 and Y1235). Phosphorylation of specific residues in the C-terminal tail, Y1349 and Y1356, then creates binding epitopes for a vast number of adapter and signalling proteins (Figure 6). Effectors bind c-Met either directly, such as Src or PI3K, or indirectly via its key scaffolding protein Gab1, which mediates most signalling events downstream of c-Met (Gentile *et al.*, 2008).

Termination of c-Met signalling is achieved by Cbl binding to Y1003 in the juxtamembrane domain. Cbl is an E3 ubiquitin ligase that initiates c-Met ubiquitination, internalisation via clathrin-coated vesicles, transit through the early sorting endosome, and eventual lysosomal degradation (Kermorgant and Parker, 2005). But notably, some c-Met–relayed signals even require receptor internalisation. For example, the activation of the MAP-kinase ERK1/2 by c-Met occurs only on early endosomes (Figure 6). From there, PKCε specifically targets active ERK1/2 to focal complexes at the plasma membrane, whereas PKCα promotes the transport of c-Met along microtubules to multivesicular bodies (MVBs), from where it is directed to the lysosomes (Kermorgant *et al.*, 2004). In addition, hepatic cells have been found to regulate c-Met activation not only by HGF-binding, but also by cell-cell contacts. Even in the presence of HGF, hepatocytes do not proliferate under confluent conditions - a process termed 'contact inhibition'. This is due to the c-Met–induced activation of the transmembrane protein tyrosine phosphatase LAR, which dephosphorylates c-Met and hence prohibits its mitogenic downstream signalling (Machide *et al.*, 2006).

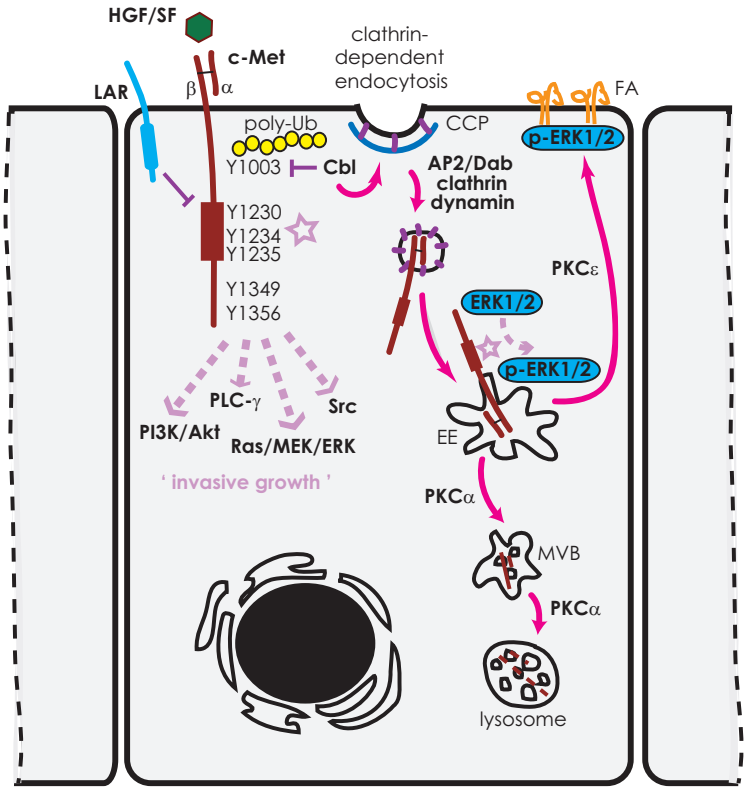

Figure 6: Versatile c-Met Signalling.
c-Met and its ligand HGF regulate a wide range of signalling cascades, stimulating cell proliferation, survival, angiogenesis, and motility (collectively termed as 'invasive growth'). C-Met signalling is vital during embryogenesis and wound healing processes, but owing to its versatile responses also exploited in many carcinoma cells. Following activation, c-Met is normally rapidly internalised and degraded. However, cancer cells have evolved a number of strategies to constitutively activate c-Met signalling (see text for details).

In order to avoid termination of signalling and to sustain continuous c-Met activity, carcinoma cells have evolved a number of mechanisms, which can be categorised in either 1) HGF-mediated receptor activation, 2) c-Met over-expression, or 3) gain-of-function mutations (Gentile *et al.*, 2008). The latter mechanism occurs more rarely, and tumours harbouring activating point-mutations are commonly characterised by a long latency. However, additional amplification of the mutant *MET* allele is often the second carcinogenic event that ultimately leads to tumour development (Graveel *et al.*, 2004). Examples of carcinomas with activating point-mutations in the c-Met kinase domain are lung, head and neck, and gastric cancer.
In contrast to gain-of-function mutations, the quantitative up-regulation of c-Met levels is the most frequent mechanism of oncogenic c-Met activation, and can be found in a large number of human tumours, for example in metastatic colorectal cancer and esophageal adenocarcinoma (Zou *et al.*, 2007). Over-expression of the receptor can occur as a

result of genomic amplification, mutations in the degradation-promoting juxtamembrane domain, or transcriptional mechanisms. Constitutive c-Met activity is then caused by increased ligand sensitivity or by HGF-independent receptor oligomerisation and reciprocal activation (Gentile *et al.*, 2008).
Ligand-mediated c-Met activation is also often found in human cancers and is associated with increased aggressiveness of the tumours (Stellrecht and Gandhi, 2009). Excessive HGF-stimulation by autocrine mechanisms, through simultaneous expression of c-Met and HGF, has been detected in many sarcomas and gliomas, but not in cancers of epithelial origin (Knudsen and Vande Woude, 2008). Paracrine c-Met activation is often characteristic for the frequently occurring host stroma stimulation of tumour angiogenesis, growth, and invasion (Bhowmick and Moses, 2005). Various cancer types, including prostate, stomach, skin, oral cavity, mammary gland, and colon cancer, secrete factors that enhance HGF expression in adjacent fibroblasts. Such carcinoma-derived HGF-inducing molecules are interleukin-1β (IL-1β), basic fibroblast growth factor (bFGF), PDGF, transforming growth factor-α, and prostaglandin E2. Fibroblast-emitted HGF, in turn, functions as a potent mitogenic, motogenic, and morphogenic factor that promotes carcinoma invasion and metastasis (Matsumoto *et al.*, 2008).

Interestingly, it has recently been appreciated that oncogenic c-Met signalling requires vivid trafficking from the plasma membrane to endosomes (Joffre *et al.*, 2011). Tumour-associated activating mutations in c-Met failed to promote *in vivo* tumourigenesis and metastasis whenever receptor endocytosis was blocked. Thus, the oncogenic potential of c-Met arises not only from its sustained activity, but also from its localisation and signalling on endosomes. Moreover, mice harbouring different point-mutations in the *MET* gene locus have been found to develop mutant-specific tumours (Graveel *et al.*, 2004). This formation of characteristic neoplasms arises supposedly from individual, tumor-dependent requirements that are enabled by distinctively affected downstream signalling of the respective mutant c-Met.

Even though deregulated c-Met signalling supports multiple stages of tumour progression, the *de novo* formation or initiation of cancer by c-Met alone is slow and occurs only after a long latency period (Knudsen and Vande Woude, 2008). Instead, dysfunctional c-Met signalling is implicated in the progression of various cancers to disseminating and invading tumours. As it is just metastasis that is the most fearsome aspect of cancer, much effort has been made to develop a variety of therapeutic inhibitors against aberrant c-Met signalling - each with the common goal to retain malignant tumours 'static', in both growth and spreading, and hence support surgical removal of the tumour and allow disease-free survival of the patient (Eder *et al.*, 2009).
Currently, HGF-neutralising antibodies, c-Met–targeting siRNAs, dominant-negative proteins, small molecule c-Met tyrosine kinase inhibitors (such as SU11274), and microRNAs that target c-Met mRNA (Tan *et al.*, 2011) are being investigated as possible therapeutic tools against sustained c-Met activation (Stellrecht and Gandhi, 2009). Owing to the very diverse effects of oncogenic c-Met, it has also become increasingly evident that optimised patient selection for clinical trials, together with the development of diagnostic biomarkers, are invaluable for the effective therapeutic targeting of aberrant c-Met signalling (Cecchi *et al.*, 2010).

AIMS OF THE STUDY

Cancer becomes life-threatening when malignant cells acquire the ability to escape the primary tumour and to penetrate and invade the surrounding tissue in order to colonise distant sites in the body. The ominous gain of invasive capabilities provides the potential to be therapeutically targeted, yet cell migration is also essential during many physiological processes, such as tissue homeostasis and wound healing. For the development of cancer-selective drugs in the future, it is thus of great importance to broaden our basic understanding of the complex molecular machineries that orchestrate cell migration under diverse circumstances and in different cell types.
Integrin cell surface receptors are engaged in the perception and integration of endogenous and environmental cues. The functional importance of integrin trafficking to and from the plasma membrane is known for some time, but yet the detailed molecular mechanisms remain ambiguous. How are endocytosis and recycling routes coordinated? What targets integrins to the leading edge of a cell? How does integrin trafficking contribute to directed movement? How do growth factor receptors account for cancer cell migration and invasion? Even more complexity arises, when cell motility is studied in a 3D environment. Are the cellular mechanisms underlying cancer cell invasion *in vivo* distinct from the experimental results obtained on tissue culture plates?
The aim of this study was to explore migratory mechanisms in 2D and 3D in more detail and to find new regulatory pathways that might eventually be exploited in targeted cancer therapy.

Specific aims were:

- ✓ To identify new regulatory proteins, which cooperate with Rab21 on the control of integrin trafficking and cell migration.
- ✓ To elucidate the mechanisms underlying the breakdown of intercellular junctions and resultant increased cancer cell motility.
- ✓ To investigate signalling pathways that account for the c-Met–induced invasive phenotype in a cancer-mimicking 3D cell model.

MATERIAL AND METHODS

A more detailed description of the methods and reagents can be found in the original publications (I-III).

Method	Used in
PKC Kinase Assay	III
In situ Proximity Ligation Assay	III
Immunohistochemistry	III
Phosphoprotein Extraction	III
ELISA Binding Assay	III
Adhesion, Migration and Invasion Assays	I, II, III
Scratch-Wound Assay	I
3D Cell Culture and Time-Lapse Microscopy	II
Cell Proliferation Assay	III
Rac and Rho Activation Assay	I, II, III
Flow Cytometry (FACS)	I, II, III
Cell Culture	I, II, III
Immunoprecipitation	I, II, III
Western Blot Analysis	I, II, III
Biotinylation-based Trafficking Assay	I, II, III
Antibody-based Trafficking Assay	I
Pull-Down Assay	I
Immunofluorescence Microscopy	I, II, III
Immunoelectron Microscopy	I
Surface Plasmon Resonance Protein Interaction Assay	I
Fluorescence Polarization Protein Interaction Assay	I
Protein Expression and Purification	I, III
GAP Assay	I
Subcellular Density Fractionation	I
Statistical Analysis	I, II, III

DNA constructs

The following constructs were generated:
Used in I: pEGFP-C2-Rab21, pEGFP-C2-p120RasGAP and its kinase-dead mutant (R789A) and ΔGAP construct, pGEX-p120RasGAP full length and its GAP-domain alone, pGEX-Rab21, pGEX-APPL1
Used in III: siRNA-resistant variants of pEGFP-N3-α5 and pEGFP-C1-ZO-1; pGFP-N-ZO-1, pGFP-C-ZO-1, pLNCX-Flag-ZO-1WT, pLNCX-3Flag-ZO-1PDZ1; pGFP-PKCεWT and pGFP-PKCαWT and their kinase-dead mutants

Expression vectors for the following constructs were generous gifts:
Used in I: pEYFP-N1-RFP and pRFP-N1-232 constructs from A.J. Koleske, Yale University, New Haven, CT, USA; pGEX-4T1 p120RasGAP from A. Wittinghofer, MPI of Molecular Physiology, Düsseldorf, Germany; pET15b-APPL1 from X.C. Zhang, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA

siRNAs

Target	Sequence (sense) or name	Supplier	Used in
ZO-1	AAAACAGTCACTCCAGCATAC	Qiagen	III
ZO-1	AAGATATTGTTCGGTCCAATC	Qiagen	III
α5-integrin	ATCCTTAATGGCTCAGACAT	Qiagen	III
PKCε	GAUCGAGCUGGCUGUCUUUTT	Qiagen	III
PKCε	AAAGACAGCCAGCUCGAUCTT	Qiagen	III
Scr	Allstars negative control	Qiagen	III
Scr	Scramble control	Ambion	I
Rab21	GGCAUCAUUCUUAACAAAGTT	Ambion	I
Rab21	GGUCAAGAGAGAUUCCAUGTT	Ambion	I
p120RasGAP	GGAAGAAGAUCCACAUGAATT	Qiagen	I
p120RasGAP	GCUCCCAUAUACCAUUAAATT	Qiagen	I
Hip1	GGAACUUGCCACAAGCCAATT	Qiagen	II
Hip1	GGCUUAGGAUCGACAAGAATT	Qiagen	II
RhoA	CGGAAUGAUGAGCACACAATT	Qiagen	II
RhoA	AGCUAGACGUGGGAAGAAATT	Qiagen	II

Peptides

TAT-conjugated α5-peptides as well as biotin-conjugated integrin-peptides were custom synthesised by GeneCust, EDANS-labelled peptides by GenScript.

Name	Sequence	Used in
TAT- α5 WT	PPATSDA	III
TAT-α5 ADA	PPATADA	III
TAT-α5 PPAA	AAATSDA	III
β1 cyt	WKLLMIIHDRREFAKFEKEKMNAKW DTGENPIYKSAVTTVVNPKYEGK	I
α1 cyt	WKIGFFKRPLKKKMEKRPLKKKMEK	I
α2 cyt	WKLGFFKRKYEKM	I
α2 12AA	AALGFFKRKYEKM	I
α2 34AA	WKAAFFKRKYEKM	I
α2 56AA	WKLGAAKRKYEKM	I
α2 78AA	WKLGFFAAKYEKM	I

Cell Lines

Cell line	Origin	Used in
NCI-H460	human non-small cell lung cancer	III
MDA-MB-231	human breast cancer	I, III
KF28 and KFr13	human ovarian cancer	I
MEF	murine embryonic fibroblasts	I, III
TIFF	human telomerase immortalised foreskin fibroblasts	I
T-REx293–GFP-cMet	Tet-inducible, human embryonic kidney	II
MKN-45 and GTL-16	human gastric cancer (*MET*-amplified)	II

Antibodies

Antigen	Description	Used in
α5-integrin	MCA1949 and Mab1999, Chemicon	III
α5-integrin	MCA1187, Serotec	III
α4-integrin	Mab16983, Chemicon	III
αV-integrin	L230, Alexis Chemicals	III
ZO-1	mouse mAb, Zymed	III
ZO-1	rabbit pAb, Zymed	III
FAK	goat pAb, Santa Cruz	III
FLAG	mouse mAb, Sigma-Aldrich	III
Vinculin	mouse mAb, Sigma-Aldrich	III
PKCε	rabbit pAb, Upstate	III
EEA1	rabbit pAb, Upstate	I
Rab11	rabbit pAb, Invitrogen	I
Rab21	mouse mAb, Santa Cruz	I
Rab21	mouse mAb, Abnova	I
β1-integrin	mouse mAb P5D2, Hybridoma Bank	I, III
β1-integrin	mouse mAb P4G11, Hybridoma Bank	I
β1-integrin	mouse mAb AIIB2, Hybridoma Bank	I
β1-integrin	mouse mAb 12G10, Abcam	I
β1-integrin	mouse mAb 9EG7, BD Pharmingen	I
β1-integrin	mouse mAb Mab13, BD Pharmingen	I, II
β1-integrin	mouse mAb Mab2252, Chemicon	I, III
β1-integrin	mouse mAb K20, Beckman coulter	I, II
β4-integrin	mouse mAb Mab2060, Chemicon	II
p120RasGAP	rabbit pAb, BD Biosciences	I
p120RasGAP	mouse mAb, BD Biosciences	I
p190RhoGAP	rabbit pAb, BD Biosciences	I
RhoA	mouse mAb, Cell Signalling	I, II
RFP	rabbit pAb, MBL	I
GST	rabbit pAb, Invitrogen	I, III
GFP	rabbit pAb, Invitrogen	I, II, III

Antigen	Description	Used in
Biotin	goat HRP-linked Ab, Cell Signalling	I, II, III
c-Met	goat pAb, R&D Systems	II
phospho-Met Y1234/5	rabbit mAb, Cell Signalling	II
Vimentin	mouse mAb, Santa Cruz	II
α-Tubulin	mouse mAb, Santa Cruz	I, II, III
HIP1	rabbit pAb AB9880, Chemicon	II
Fibronectin	HFN7.1, Hybridoma Bank	II

Reagents

Reagent	Application	Supplier	Used in
Glutathione-sepharose beads	Protein expression and Pull-down analysis	GE Healthcare	I, III
Thrombin protease	Protein expression		I
PreScission protease	Protein expression		I
Lipofectamine 2000	Transfections	Invitrogen	I, II, III
HiPerfect	Transfections	Qiagen	III
Collagen I	Cell culture dish coating		I
Fibronectin	Cell culture dish coating		III
Growth factor reduced Matrigel	3D Cell culture	BD Biosciences	II
Calphostin C	PKC inhibitor		III
Y-27632	ROCK inhibitor	Sigma	II
CT04 (C3)	Rho inhibitor	Cytoskeleton	II
SU11274	c-Met kinase inhibitor	Calbiochem	II
Tetracycline	Protein expression		II
HGF	Activation of c-Met		II
ATP	PKC kinase assay		III
GTP and [32P-γ]GTP	GAP assay		I
^{32}P	Phosphoprotein extraction	Amersham Biosciences	III
DAPI	Immunofluorescence		I, II, III
Mowiol	Immunofluorescence	Calbiochem	I, II, III
Vectashield mounting medium	Immunofluorescence	Vector Labs	I, II, III
Dabco	Immunofluorescence	Sigma	I, II, III
EZ-Link Sulfo-NHS-SS-Biotin	Biotinylation-based trafficking assay	Pierce	I, III
MesNa	Biotinylation-based trafficking assay	Fluka	I, III
Iodoacetamide (IAA)	Biotinylation-based trafficking assay	Sigma	I, III
Streptavidin-sepharose beads	Pull-down analysis	GE Healthcare	I
Protein G-sepharose beads	Immunoprecipitation	Amersham Biosciences	I, III

RESULTS

1. Balanced Integrin Trafficking - A Prerequisite for Controlled Cell Migration and Invasion (I, II, III)

The findings of the publications included in this thesis collectively highlight the general importance of balanced and targeted integrin trafficking for proper cell migration and invasion. It has become evident that not only integrin endocytosis enables cell migration, but also their targeted redistribution to the plasma membrane is essential at the advancing cell front, where integrins are engaged again (Powelka *et al.*, 2004). Moreover, cell migration is also a balancing act between cell attachment and detachment, as too much as well as too little adhesion is not favourable for forward motion (Strachan and Condic, 2004).

1.1 p120RasGAP Controls Integrin Recycling (I)

In our previous work, we have identified the small GTPase Rab21 as a regulator of integrin endocytosis (Pellinen *et al.*, 2006). With the aim of identifying regulatory proteins that are implicated in Rab21-driven integrin trafficking, p120RasGAP aroused our interest. p120RasGAP (subsequently abbreviated as p120) is a well-known GAP protein for Ras, but has also been suggested as a negative regulator for Rab5, a close homologue of Rab21 (Liu and Li, 1998).
In order to test p120 implication in integrin endo/exocytic transport pathways, we performed a number of biochemical and microscopic trafficking assays. First, we followed the itinerary fate of cell surface-labelled β1-integrins in MDA-MB-231 breast cancer cells using a biotinylation-based method. While silencing of p120 had no significant effect on integrin endocytosis (I, Fig.1A), siRNA-induced loss of p120 readily diminished the recycling rate of β1-integrins compared to control-silenced cells (I, Fig.1B). Similarly, when labelling surface integrins with an anti-β1 antibody and following their endo/exocytic traffic, integrins were equally well internalised in both control and p120-silenced cells under serum-free conditions (I, Fig.1C). However, serum-stimulation triggered the redistribution of endocytosed integrins back to the plasma membrane only in control cells, whereas p120-siRNA transfected cells showed a reduced ability to recycle integrins. Third, we confirmed these findings by electron microscopy (EM), where integrins at the plasma membrane were labelled with a gold-conjugated antibody. Also with this assay, we observed a reduced ability of p120-silenced cells to return integrins to the plasma membrane. Instead, labelled β1-integrins accumulated in cytoplasmic vesicles (I, Fig.1D).

In line with our previous finding that Rab21 regulates integrin trafficking, we repeated the antibody-based trafficking assay with superficially labelled β1-integrins and consecutive internalisation and recycling steps, but additionally co-stained for endogenous Rab21. In the absence of p120, there was a strong co-localisation of Rab21 and β1-integrins on endosomes, both after the internalisation and the recycling step (I, Fig.7A). In contrast, control cells showed in both cases only limited overlap between Rab21 and integrins, suggesting that integrins did not remain for long in Rab21-positive endosomes. In compliance with these results, we detected also in an EM-based assay a strong

cytoplasmic co-localisation of surface-derived integrins with Rab21 in p120-silenced cells (I, Fig.7C). In control cells, 10 nm-gold–labelled integrins were found predominantly at the plasma membrane, distant from Rab21 5 nm-gold labels found on vesicular structures. In order to identify the endosomal compartment, where in the absence of p120 integrins accumulate with Rab21, we stained p120-silenced cells for different endosomal markers. As a result, we found that, in the absence of p120, integrins got trapped in EEA1-positive early sorting endosomes, whereas there was only limited overlap with Rab11 or RCP, two markers of recycling endosomes (I, Fig.7B).

In summary, these results implicate the requirement of p120 in the recycling of Rab21-internalised β1-integrins. If p120 is lost, integrins accumulate in early endosomes instead of redistributing to the cell surface.

1.2 Integrin Recycling Regulates Cell Migration (I)

As derailed integrin recycling has been correlated with changes in cellular migration modes (White *et al.*, 2007; Caswell and Norman, 2008), we aimed to determine the impact of p120 on cell motility. We therefore silenced p120 in a number of cell lines, tracked their movement with time-lapse microscopy, and then evaluated their migration speed and persistence. MDA-MB-231 breast cancer cells, when transfected with p120-siRNA, migrated with greater speed (I, Fig.S2A) and also closed a wound faster than control cells (I, Fig.2A). Importantly, the stimulated migratory behaviour of MDA-MB-231 cells was specifically attributed to the loss of p120, as re-expression of GFP-tagged wild-type (WT) p120 reversed the phenotype (I, Fig.2A). Likewise, loss of p120 also stimulated the migration of human telomerase-immortalised foreskin fibroblasts (TIFFs) and mouse embryonic fibroblasts (MEFs) into a wound (I, Fig.S2B, C). The more rapid wound closure was a result of both increased cell migration speed and persistence. The observed enhanced cellular motility is likely to be due to aberrant integrin recycling in p120-silenced cells, as no alterations regarding β1-integrin cell surface expression or activation status could be observed compared to control cells (I, Fig.S3).

Remarkably, the stimulation of cell motility following loss of p120 was only observed in cells expressing Rab21. We made this observation using two related ovarian cancer cell lines: KF28 and KFr13. In contrast to the parental KF28 cells, KFr13 cells harbour a chromosomal deletion in the *RAB21* gene locus, thus lacking Rab21 but not β1-integrin, Rab5, or p120 (I, Fig.S4A). The absence of Rab21 in KFr13 cells resulted in the failure of β1-integrin endocytosis (I, Fig.S4B) and a mere ventral plasma membrane distribution of β1-heterodimers (I, Fig.2C). In contrast, parental KF28 cells internalised β1-integrins normally and showed a typical distribution of the integrin, both at the cell surface and in endosomes in the cytosol. Moreover, silencing of p120 resulted in enhanced persistent migration only of KF28 cells, whereas loss of p120 had no impact on KFr13 cell motility (I, Fig.2D).

In brief, p120 appears to regulate cell migration by enabling the redistribution of Rab21-internalised β1-integrins back to the plasma membrane. Derailed integrin recycling, due to the loss of p120, results in increased cell motility in a number of different cell lines.

1.3 Directed Migration Requires the Targeted Transport of Integrins and ZO-1 to the Leading Cell Edge (III)

Apart from balanced trafficking, integrins also need to be specifically targeted to the advancing lamellipodium at the cell front. Furthermore, the generated leading edge needs to be maintained and stabilised in order to allow directionally persistent cell migration. With the aim to investigate how integrins are delivered to the emerging leading edge and how the cell front in turn is stabilised, we used the invasive non-small cell lung cancer cell line NCI-H460.

In functional epithelia, integrins mediate cell-matrix contacts on the basolateral side of the polarised cell layer. Accordingly, when NCI-H460 cells were resting in a confluent monolayer, $\alpha5\beta1$-integrins resided in intercellular contact sites (III, Fig.1A). However, following a migratory stimulus by applying a scratch wound to the monolayer, NCI-H460 cells polarised towards the wound edge. Simultaneously, $\alpha5\beta1$-integrin, but not $\alpha2\beta1$, was redistributed to the advancing lamellipodium (III, Fig.1A; Fig.S1A, B). This specific recruitment of $\alpha5\beta1$ to the cell front was required for the breakdown of cell-cell contacts, the initiation of cell motility, and also for invasion into a 3D fibronectin-matrix, as blocking $\alpha5\beta1$ function with an antibody or siRNA inhibited all these functions (III, Fig.S2A; Fig. 1B, C).

The integrity of functional epithelia is further supported by apically localised tight junctions, with the signalling and scaffolding protein ZO-1 being one of their major components. Since it has been shown that ZO-1 not only localises to tight junctions, but also to nascent adhesion sites at the leading edge of migrating cells (Fanning *et al.*, 2002), we aimed to investigate the localisation of $\alpha5\beta1$-integrin and ZO-1 in NCI-H460 cells. Indeed, in immotile cells, there was no co-localisation of the two proteins (III, Fig.2A, B). Both proteins were found at intercellular junctions, but ZO-1 was restricted to the apical site and $\alpha5\beta1$ to the basolateral surface. However, in sub-confluent actively migrating cells, this compartmentalisation was lost, and ZO-1 and $\alpha5\beta1$ strongly co-localised at the advancing lamellae as assessed by IF (III, Fig.2A, B) and a proximity ligation assay (PLA; III, Fig.3C, D). Correspondingly, we could co-immunoprecipitate $\alpha5\beta1$ and ZO-1 only from cell lysates that derived from sub-confluent migrating cells, but not from a confluent cell monolayer (III, Fig.3A). The concomitant recruitment of ZO-1 and $\alpha5\beta1$-integrin to the leading edge was integrin-specific, as we did not detect a ZO-1 association with $\alpha2\beta1$ (III, Fig.3B). However, we observed $\alpha5\beta1$–ZO-1 co-localisation also in MDA-MB-231 breast cancer cells, indicating a cell type-independent mechanism (III, Fig. S4A).

In order to understand the role of ZO-1 at the lamellipodium, we analysed the effect of ZO-1 loss on the migration of NCI-H460 cells. We found that ZO-1–silencing significantly reduced cell migration towards a chemotactic stimulus in a Transwell assay (III, Fig.2C) and abolished overall directionally persistent migration (III, Fig.D, E). In line with its role in directional cell motility, ZO-1 loss in NCI-H460 cells also resulted in the impaired formation of a prominent lamellipodium. Instead, the cells exhibited multiple protrusions in several directions compared to control cells that were clearly polarised (III, Fig.2F). The deficiency in lamellipodia formation was truly attributed to the loss of ZO-1, as re-expression of GFP-tagged ZO-1 rescued the defect (III, Fig.2G). Furthermore, the induction of multiple protrusions in ZO-1–depleted cells was accompanied by elevated

Rac1 activity, which is consistent with the observed increased random cell migration (III, Fig.S5). However, the loss of ZO-1 and hence the deficient formation of a stable lamellipodia had no effect on the recruitment of α5β1-integrin to the cellular protrusions (III, Fig.2F). Silencing of α5-integrin, on the other hand, inhibited the formation of a ZO-1–positive lamellipodium, and re-expression of GFP-α5 reconstituted the defect (III, Fig. 5B).

Taken together, these data assigned a new role for the tight junction protein ZO-1 in the regulation of persistent cell migration. Following a migration-promoting stimulus, α5β1-integrin and ZO-1 are specifically targeted to the emerging cell front. ZO-1 is not important for α5β1 redistribution to the leading edge *per se*, but instead contributes to lamellipodium stabilisation and hence directionally persistent cell migration. Direct interaction with α5β1-integrin thereby anchors ZO-1 at the cell front. Interestingly, the α5β1–ZO-1 complex formation in motile carcinoma cells found *in vitro* has also clinical relevance in metastasising lung carcinomas *in vivo*. We analysed 48 lung cancer patient samples and found α5β1–ZO-1 complex formation exclusively in metastatic cancer specimens, but not in samples of primary carcinomas that had not spread (III, Fig.S7). Thus, the interaction of α5β1-integrins with ZO-1 at the leading edge of motile cancer cells may be a critical switch that allows cancer cell dissemination and invasion.

1.4 Oncogenic c-Met Signalling Enhances Integrin Trafficking and Stimulates Cell Invasion in 3D Matrigel (II)

Deregulated activation of the growth factor receptor c-Met has been implicated in the progression of a stationary primary tumour to a disseminating and metastasising cancer. In order to study the molecular changes that facilitate cancer cell invasion, we utilised an epithelial cell model, in which we mimicked oncogenic c-Met signalling caused by either c-Met over-expression or excessive HGF-mediated activation.

In order to imitate the quantitative up-regulation of c-Met, we exploited a recently developed epithelial cell model (Kermorgant, manuscript) with a tetracycline (Tet)-inducible expression system for GFP-tagged c-Met. When induced with Tet, these human embryonic kidney (HEK) 293 cells (subsequent abbreviated as HEK-Met cells) showed sustained activation of c-Met (II, Fig.1A), similarly to tumours that carry an amplification of the *MET* locus (Zou *et al.*, 2007). Interestingly, following Tet-induction, these HEK-Met cells underwent a peculiar morphology change on planar surfaces. They rounded up (II, Fig.1B) and acquired the ability to survive anchorage-independently, which is one of the hallmarks of cancer cells. These c-Met–induced morphological changes were accompanied by the loss of β1-integrins from the cell surface owing to an enhanced endocytosis rate (II, Fig.1C, D).
Notably, when embedding HEK-Met cells into a more physiological 3D environment, into 50 % Matrigel, they eagerly formed multicellular spheric clusters (II, Fig.2A; movie S1). However, upon Tet-induction and hence cancer-like c-Met over-expression, HEK-Met cells lost the spheroid phenotype instantly and instead converted into a loose assembly of individual round cells with amoeboid characteristics (II, Fig.2A; movie S2). Nevertheless, the cells remained alive, as even after 72 hours of Tet-induction the cells were moving on the spot and proliferating actively (II, movie S3). Moreover, the induction of these morphological changes was prevented in the presence of the c-Met kinase

inhibitor SU11274, indicating that c-Met catalytic activity provoked this so-called 'oncogenic rounding' (II, Fig.2A). Importantly, when embedding carcinoma cells originating from human gastric tumours with *MET* amplification into Matrigel, these cells also adopted an amoeboid phenotype similar to our Tet-induced HEK-Met cells (II, Fig.2D).
In order to characterise the nature of the oncogenic morphology changes in more detail, we fixed and stained HEK-Met cells in 3D culture in the absence or presence of Tet and stained for β1-integrin, actin, and vimentin (II, Fig.2B). Without induction, HEK-Met cells formed dense spheric clusters. Interestingly, the IF-protein vimentin was found to be expressed only by cells in the outer layer of the spheroid, but not by cells inside the structure. After Tet-induction, vimentin was present in all cells of the fragmented spheroid. Moreover, in line with the earlier findings on 2D, we noticed also in these 3D immuno-stainings that integrins obtained a predominantly cytosolic distribution following Tet-induction compared to non-stimulated cells (II, Fig.2C). It has been shown that the formation of spheroids in 3D depends on both cell-cell and cell-matrix interactions (Casey *et al.*, 2001). Thus, the abrogation of the spheroids by c-Met activation may partially be attributed to the loss of integrins from the cell surface.

We also observed c-Met–induced invasive alterations in WT HEK293 cells, which were stimulated with exogenous HGF. With these conditions, we mimicked paracrine c-Met activation typically found in many CAF-stimulated carcinoma cells (Knudsen and Vande Woude, 2008). When embedding WT HEK293 cells into 50 % Matrigel, they formed multicellular spheroids just like the HEK-Met cells (II, Fig.3A). However, following stimulation with excessive HGF, HEK293 cells did not round up. Instead, the cells adopted a mesenchymal phenotype, started to disseminate from the spheric cell cluster and progressively invaded as sheet-like cohorts into the matrix (II, Fig.3A; movie S4). In the presence of the c-Met kinase inhibitor, HEK293 cells significantly lost their ability to disseminate from the spheroid, indicating that the gain of invasive capabilities was owing to c-Met signalling (II, Fig.3B). Consistently, in an *in vitro* invasion assay, HEK293 cells migrated through 3D Matrigel towards a gradient of HGF, but remained largely non-invasive in the absence of HGF-stimulation (II, Fig.3C). In addition, akin to Tet-induced HEK-Met cells, also WT HEK293 cells lost β1-integrins from the cell surface following stimulation with HGF (II, Fig.3D).

In summary, we have developed an *in vitro* 3D cell model that allows the investigation of morphological and migratory changes induced by sustained c-Met activation. Moreover, we found that the effects of oncogenic c-Met signalling vary depending on the receptor-activating mechanism: While the over-expression of c-Met initiated an amoeboid phenotype, HGF-mediated receptor stimulation triggered a mesenchymal morphology with distinct cellular invasion characteristics. However, enhanced integrin trafficking, at least partially, accounted for the c-Met–induced invasive switch - irrespective of the mechanism of c-Met activation.

2. Novel Protein-Protein Interactions and Functions in the Control of Cell Migration (I, III)

In the course of our research on integrin traffic and cell migration, we found conceptually new mechanisms, which regulate the function and interaction of integrin-associated proteins.

2.1 An Unexpected Non-GAP Function for p120RasGAP in the Regulation of Vesicular Trafficking (I)

When we first found a link between Rab21-controlled endocytosis and p120-stimulated recycling of β1-integrins, we were assuming a negative regulatory function of p120 as a GAP for Rab21. However, after performing an *in vitro* GTP-hydrolysis assays with recombinant proteins and γ-[^{32}P]GTP, we found that the purified GAP-domain of p120 was not able to increase the intrinsic GTP-hydrolysis rate of Rab21 (I, Fig.3A). In contrast, p120 GAP-domain did significantly stimulate the GTP-hydrolysis of H-Ras, an established substrate of p120.
Furthermore, we were able to rescue the integrin recycling defect (I, Fig.6A) as well as the migration-promoting phenotype (I, Fig.6C, D) in p120-silenced MDA-MB-231 cells by the re-expression of both, GFP-tagged WT p120 as well as the catalytically inactive R789A mutant form of p120.
Together, these results unexpectedly indicated to us that p120 GAP-activity is not needed for the regulation of neither integrin recycling nor cell migration.

2.2 p120RasGAP Binds Integrins Directly via its GAP-domain and Competes with Rab21 (I)

Although the catalytic activity of p120 is not needed, the protein itself is nevertheless crucial for appropriate integrin trafficking and cell migration. So how does it function then? To address this question, we first performed pull-down experiments with GST-tagged p120 constructs comprising either the full-length protein, the GAP-deficient R789A mutant, or the p120 GAP-domain alone. Interestingly, all three constructs were sufficient to pull-down β1-integrins from cell lysates, indicating that the GAP-domain itself interacts with the integrin (I, Fig.4B). Using purified GST-tagged p120 protein and biotinylated peptides corresponding to the α1-, α2-, or β1-integrin cytodomain in a pull-down assay (I, Fig.4C) as well as with a surface plasmon resonance-based (Biacore™) method (I, Fig.S5C), we found that p120 GAP-domain directly binds to the α1- and α2-subunit of integrins, but not to β1.
Furthermore, we analysed the interaction between p120 and the α-subunit of integrins in more detail using a fluorescence polarization (FP)-based assay. And also with this method, we detected a direct interaction of the α2-tail peptide with the p120 GAP-domain, as well as with the full-length WT or mutant p120 protein (I, Fig.4D). Moreover, we determined the equilibrium dissociation constants (K_d) with a FP-based titration experiment, and found that the GAP-domain alone displayed a similar binding affinity to the integrin α2-tail as the full-length p120 protein (I, Fig.4E). In line with this, we found that p120-constructs lacking the GAP-domain but retaining all classical protein-protein interaction domains (such as PH-, SH2-, and SH3-domains) failed to rescue the integrin recycling defect (I, Fig.6A) as well as the migration-promoting phenotype (I, Fig.6C, D) in p120-silenced MDA-MB-231 cells.

Similar to p120, we also demonstrated that Rab21 interacts directly and exclusively with the α-subunit of β1-integrin heterodimers (I, Fig.4B, C). Interestingly, this interaction was not dependent on the nucleotide status of the GTPase, as Rab21 loaded with either GDP or the non-hydrolysable GTP-analogue GppNHp had the same binding affinity towards the α2-cytodomain (I, Fig.3D).

Having found that p120 and Rab21 bind to both, the α1- and the α2- cytoplasmic tails, we suspected their integrin-binding epitope to be within the conserved membrane-proximal sequence that most α-subunits have in common: WKLGFFKR (Hynes, 2002). Moreover, the arginine R1161 within this sequence has also earlier been shown to be important for Rab21 function on integrin traffic (Pellinen *et al.*, 2006). In order to confine the integrin binding epitope for p120 and Rab21, we performed a FP-based titration experiment using integrin peptides with alanine-mutations within the WKLGFFKR sequence (I, Fig.5A). The determination of the corresponding K_d-values revealed that alanine substitutions within the residues WK and FF equally strong reduced the binding affinities of both, Rab21 and p120 (I, Fig.5B). The KR motif seemed to be important, too, but to a lesser extent. In other words, under these *in vitro* conditions, p120 and Rab21 possess overlapping binding sites on the α-cytoplasmic tail of integrins.
This finding led us to perform another FP-based experiment: We first determined that p120 and Rab21 do not form a ternary complex with the α2-tail peptide (I, Fig.5C). This could be excluded because addition of Rab21 to a pre-formed, saturated p120–α2 complex did not yield a further increase of the binding signal. In addition, Rab21 was not able to replace p120 from the integrin peptide, as the binding signal did not drop either. Conversely, when p120 was added to a complex of Rab21 and α2-peptide, the binding signal further increased (I, Fig.5C). Since the final value was identical to the one obtained for α2-peptide and p120 alone, and because we also already excluded the formation of a ternary complex, we concluded that p120 was able to replace Rab21 from the integrin peptide.

The physiological relevance of our competitive hypothesis in cells was supported by the findings that p120-constructs, specifically lacking the GAP-domain, were not able to rescue neither the integrin recycling deficiency (I, Fig.6A) nor the migration-stimulating effect in p120-silenced MDA-MB-231 cells (I, Fig.6C, D). In contrast, the catalytically inactive R789A p120 mutant was able to fully reverse the traffic and migration alterations induced by the loss of p120 (I, Fig.6).

In conclusion, our data support a model in which Rab21-binding to the membrane-proximal sequence of the α-subunit enables β1-integrin endocytosis and transport to the early sorting endosome. There, due to its high cytosolic concentration and its slightly higher affinity, p120 is able to replace Rab21 from the integrin tail, thus allowing integrin transit through the recycling compartments back to the plasma membrane.
Balanced integrin traffic is a prerequisite for controlled cellular migration. However, in the absence of p120, β1-integrin heterodimers are still endocytosed, yet they cannot leave from the early endosome. The consequent derailed integrin trafficking resulted in stimulated cell migration in a number of cell lines. Conversely, if Rab21 is lost, β1-integrins fail to be internalised, and hence lack of p120 has no effect on β1-integrin trafficking-dependent cell migration (I, Fig.8).

2.3 A Non-canonical PDZ-Binding Motif in the α5-Integrin Cytodomain Mediates the Direct Interaction with ZO-1 (III)

The joint function of α5β1-integrin and ZO-1 at the leading edge of migrating cells in the stabilisation of a prominent lamellipodium, prompted us to analyse the nature of this protein-protein interaction in more detail.

ZO-1 contains three N-terminal PDZ-domains that crucially regulate ZO-1 function and location. In order to test whether ZO-1 binds α5β1 via its PDZ- or other binding domains, we used GFP-tagged constructs comprising only the N-terminal or C-terminal region of ZO-1 in immunoprecipitations. The specific co-IP of the integrin with solely the N-terminal region of ZO-1 indicated that the interaction occurs through the PDZ-domains (III, Fig.4A). This assumption was affirmed by the finding that expression of the C-terminal ZO-1 construct, unlike the ZO-1 N-terminus, was not able to support cell migration (III, Fig.4C, D). In contrast, a GFP-tagged ZO-1 construct comprising only the three PDZ-domains was sufficient to co-localise with α5β1-integrin at the leading edge (III, Fig.4E).

We next sought to determine the binding epitope of the ZO-1 PDZ-domains on the integrin. Is this interaction even direct? We addressed these questions with an ELISA assay using a GST-tagged construct comprising the three ZO-1 PDZ-domains and a peptide containing the seven C-terminal residues of the α5-cytodomain (PPATSDA). We narrowed our search of the integrin-binding epitope to only the α5-subunit as we have observed earlier that other β1-integrin heterodimers, such as α2β1, did not co-localise with ZO-1 at the leading edge, indicating that it is not the β1-subunit that mediates the interaction (III, Fig.3B). Furthermore, we choose only the seven last integrin residues, because PDZ-domains classically bind to the C-terminus of interacting proteins (Nourry *et al.*, 2003). And indeed, with the ELISA assay we found that the α5-tail peptide was able to directly bind to the ZO-1 PDZ-construct (III, Fig.5A).
It has been shown earlier that α5- and α6-cytoplasmic tails directly bind to the PDZ-domain of a protein named TIP-2 through their C-terminal SDA-sequence (El Mourabit *et al.*, 2002), and that this interaction is abolished by mutating the serine within this sequence to alanine (ADA). Accordingly, we repeated the ELISA assay with a mutant α5-peptide carrying this serine to alanine substitution. But we did not detect any decrease in the interaction with the ZO-1 PDZ-construct, indicating that other than the canonical residues are important for the interaction (III, Fig.5A).
Since PDZ binding motifs are highly variable and bind also to internal peptide sequences that exhibit a kinked conformation, we decided to mutate the two proline residues within the α5-tail peptide, as they potentially display such a kinked conformation. And indeed the proline-mutant α5-peptide was no longer able to bind to the ZO-1 PDZ-domains in the ELISA assay (III, Fig.5A). When transfecting α5-depleted NCI-H460 cells with a GFP-tagged mutant α5-integrin, in which the two prolines had been substituted with alanines, the cells localised the mutant integrin to the cell surface and normally adhered to fibronectin (III, Fig.S6). However, expression of this mutant did not rescue the lamellipodia defect and instead it stimulated the formation of multiple protrusions (III, Fig.5B, C). This phenotype was also reflected in the migratory behaviour of α5 proline-mutant–expressing α5-silenced cells. While control or WT α5-integrin–rescued cells migrated predominantly in a directionally persistent manner with a single lamellipodium, proline-mutant α5-expressing cells migrated more randomly with multiple protrusions in several directions (III, Fig.5D; Fig.S6C; movie S5).

Taken together, our data revealed that the PDZ-domains of ZO-1 directly bind to a non-canonical peptide sequence at the C-terminus of the integrin α5-subunit that contains two crucial proline residues. Mutations within these proline residues abolish the α5β1–ZO-1 complex formation and hence result in derailed cell migration.

2.4 PKCε Phosphorylation of ZO-1 Regulates the Spatiotemporal Complex Formation with α5β1-Integrin (III)

The next question to answer was about the stimulus that triggers the spatiotemporally restricted α5β1–ZO-1 complex formation at the leading cell edge, but not at intercellular contact sites. In order to localise to the advancing lamellipodium, ZO-1 has to be released from its function in cell-cell contacts. Several PKC isoforms have been implicated in the disassembly of tight junctions (Avila-Flores *et al.*, 2001), and ZO-1 phosphorylation following RTK activation is known to influence its localisation and function (Harhaj and Antonetti, 2004). We therefore first tested whether the phosphorylation status of integrin or ZO-1 differs between immotile, confluent and actively migrating, sub-confluent cells. While α5β1 was not phosphorylated in either situation (III, Fig.S8A), we found more phosphorylated ZO-1 in cell lysates derived from sub-confluent compared to confluent cells, indicating that ZO-1 is specifically phosphorylated in migrating cells (III, Fig.6A). Furthermore, the PKC inhibitor Calphostin C prevented ZO-1 re-localisation to the cell front (III, Fig.S8C, D), pointing to a role of a PKC isoform for ZO-1 phosphorylation - just which isoform?

To address this question, we transfected NCI-H460 cells with either dominant-negative (DN) or WT PKCα and PKCε. Both isoforms were expressed by the NCI-H460 cells and have been linked to cell migration before (Ivaska *et al.*, 2005). While no morphological alterations were observed for the PKCα forms, expression of DN PKCε readily abolished lamellae formation (III, Fig.6B). Similar results were obtained with a PKCε-specific siRNA (III, Fig.6C). Moreover, the loss of PKCε resulted in a preferentially nuclear localisation of ZO-1 (III, Fig.6C). To confirm our results, we analysed the localisation of ZO-1 and α5β1 in PKCε-null MEFs, which were either un-transfected or reconstituted with PKCε. In the absence of PKCε, α5β1 and ZO-1 rarely co-localised in membrane ruffles, quite contrary to the strong co-localisation observed in PKCε reconstituted MEFs (III, Fig.6D). In addition, we found more ZO-1 co-precipitating with α5β1 from PKCε-null MEFs that had been reconstituted with PKCε compared to those that had not (III, Fig.7A). In line with this, PKCε activation was significantly higher in scratch-wounded compared to confluent NCI-H460 cells (III, Fig.6E, F). Interestingly, increased PKCε activity in motile cells had no effect on the endocytosis rate of β1-integrins (III, Fig.S9). However, the translocation of α5β1 to the cell surface was increased in wounded cells (III, Fig.6G). Notably, PKCε has previously been implicated in the recycling of β1-integrins (Ivaska *et al.*, 2002). Thus, PKCε might contribute to the α5β1–ZO-1 complex formation by enhancing the delivery of integrins to the cell surface at the leading edge.

Moreover, we directly implicated PKCε in the phosphorylation of ZO-1, as loss of PKCε in motile cells resulted in a reduced level of phosphorylated ZO-1 (III, Fig.7B). ZO-1 contains a putative PKC phosphorylation site (RxxS^{168}xR) between the first and second PDZ-domain. Therefore, we mutated the crucial serine residue to either alanine (inactive mutant) or aspartic acid (phospho-mimetic mutant). As assumed, the S168A mutation abolished the translocation of ZO-1 to the advancing lamellipodium in motile cells, while having no impact on the ZO-1 location to cell-cell contacts in confluent cells (III, Fig.7C). In contrast, the phospho-mimetic S168D mutation in ZO-1 resulted in the induction of protrusions even in confluent, immotile cells (III, Fig.7C). Consistent with the requirement of PKCε for α5β1–ZO-1 complex formation, expression of the S168A ZO-1 mutant failed to bind to α5β1-integrin in motile cells, whereas the phospho-mimetic S168D mutant form interacted with the integrin (III, Fig.7D). In addition, expression of S168A

mutant ZO-1 in PKCε-null MEFs retained mutant ZO-1 at intercellular contact sites irrespective of PKCε re-expression (III, Fig.S10). Conversely, WT ZO-1 localised to anterior cell protrusions when PKCε was reconstituted and resided in cell-cell contacts only in the absence of PKCε (III, Fig.S10).

In summary, following migration-promoting stimuli, PKCε is activated. Elevated PKCε activity on the one hand supports the recycling of endocytosed β1-integrins to the cell surface and on the other hand enables ZO-1 phosphorylation. Phosphorylated ZO-1 consequently translocates from cell-cell contacts to the leading edge, where it binds to α5β1-integrin. The spatiotemporally controlled α5β1–ZO-1 complex formation is crucial for the maintenance of a pronounced lamellipodium and thereby contributes to directionally persistent cell migration. In carcinomas, the α5β1–ZO-1 complex might hence contribute to the transition from a polarised, immotile epithelial cell into an invasive carcinoma cell.

3. Sustained c-Met Activation Induces Cancer Cell Invasion in Cooperation with RhoA and Hip1 (II)

After having found a feasible cell model that allows the investigation of c-Met–induced pro-invasive changes in a 3D environment, we sought to identify the effectors of oncogenically activated c-Met. For this purpose, we performed an RNAi-based high-throughput cell spot microarray (Rantala *et al.*, 2011) with the Tet-inducible HEK-Met cells. With this technique, we identified a number of siRNAs that prevented the oncogenic rounding despite the over-expression of c-Met (II, Fig.4). Among the best hits were 14 that have been linked with RhoA signalling, including RhoA itself, and Hip1, which we became interested in as it is a known clathrin-adapter protein implicated in endocytosis and tumourigenesis (Hyun and Ross, 2004).

3.1 RhoA, a New Key Player Downstream of Oncogenic c-Met

RhoA expression levels are known to correlate with malignancy progression in breast and testicular germ-cell tumours (Sahai and Marshall, 2002). Likewise, the activation of the Rho-dependent kinase ROCK has been shown to induce tumour cell dissemination (Croft *et al.*, 2004). However, to the best of our knowledge, to date RhoA has not been shown to function as a transducer for c-Met–stimulated cancer cell invasion. In support of our finding, it has recently been discovered that c-Met over-expression correlates with the over-expression of RhoA in invasive NSCL carcinomas, yet the molecular mechanisms remain unclear (Gumustekin *et al.*, 2011).

In order to verify our finding that RhoA is crucial for the morphology changes provoked by sustained c-Met activity, we used HEK-Met cells and inhibited RhoA signalling either directly with the Rho-specific inhibitor C3 or by inhibiting its effector kinase ROCK in the absence or presence of Tet. As a result, we confirmed that the inhibition of RhoA and ROCK prevented the c-Met–induced oncogenic rounding on 2D (II, Fig.5A). In addition, RhoA was significantly activated following Tet-induction as assessed by a biochemical Rho-activation assay (II, Fig.5B). Moreover, the disruption of spheroids in 3D Matrigel by induction of c-Met over-expression was specifically prevented by the inhibition of ROCK

activity (II, Fig.5C). Consistently, HEK-Met cells that were depleted of RhoA due to siRNA-silencing failed to respond to Tet-induction and maintained their spheric phenotype in 3D Matrigel (II, Fig.5D). Notably, when expressing a constitutively active RhoA mutant (RhoA-QL) in HEK-Met cells, they failed to form multicellular spheroids in Matrigel, whereas the expression of dominant negative RhoA (RhoA-DN) had no effect on the spontaneous formation of spheric cell clusters (II, Fig.5E). Together, our data supported the observation that oncogenic c-Met over-expression causes the induction of an amoeboid phenotype, which is strongly RhoA/ROCK-dependent (Gadea *et al.*, 2008; Friedl *et al.*, 2001).

Interestingly, impeding RhoA signalling by the use of a ROCK inhibitor also prevented the HGF-stimulated mesenchymal-type invasion of WT HEK293 cells into Matrigel (II, Fig.5F). In brief, our results thus point to a key role of RhoA as an effector of oncogenic c-Met. RhoA activation is needed for both, morphological changes and stimulated cell invasion induced by sustained c-Met activity.

3.2 Hip1 Mediates c-Met–Stimulated Integrin Endocytosis

We then sought to determine the role of Hip1 for the c-Met–mediated invasive switch in cancer cells. As Hip1 is important for clathrin-mediated endocytosis, we first checked the effect of Hip1-silencing on the cell surface level of β1-integrins. In HEK-Met cells, the loss of Hip1 prevented the clearance of integrins from the plasma membrane following Tet-induction (II, Fig.6A) and likewise impeded the Tet-stimulated endocytosis rate of integrins (II, Fig.6C). Consistently, WT HEK293 cells that had been silenced for Hip1 failed to reduce integrin surface levels upon HGF-stimulation (II, Fig.6B).

In the 3D environment, Hip1-silenced WT HEK293 cells normally assembled into spheroids, but stimulation with HGF did no longer induce the collective dissemination of cells from the spheric cluster (II, Fig.6D). We thus speculated that Hip1 critically regulates integrin-dependent cell migration downstream of activated c-Met. In order to test this hypothesis, we stimulated HEK293 cells with exogenous HGF and followed their invasion into Matrigel in the presence or absence of the β1-integrin function-blocking antibody Mab13 (II, Fig.6F). In fact, also the function-blocking antibody prevented the dissemination of WT HEK293 cells from the spheroid, just as observed for Hip1-depleted cells. Together with the finding that also the broad-spectrum MMP inhibitor III prevented the mesenchymal-type invasion of HGF-stimulated HEK293 cells (II, Fig.6F), our results support the observation that ligand-mediated c-Met stimulation induces an integrin- and MMP-dependent mesenchymal invasion mode. C-Met–recruited Hip1 thereby regulates the surface availability of β1-integrins and thus contributes to the stimulated invasive behaviour of the cells.

In contrast to the HGF-induced mesenchymal phenotype, when embedding Hip1-silenced HEK-Met cells into Matrigel, c-Met over-expression by Tet-induction still resulted in the rapid fragmentation of spheroids into individual round cells (II, Fig.6G). This could be prevented by the concomitant presence of the c-Met kinase inhibitor or the ROCK inhibitor (II, Fig.6G). Likewise, neither Mab13 nor MMP inhibitor III did prevent the Tet-induced amoeboid-type morphology changes (II, Fig.S3). These findings are consistent with the suggested amoeboid phenotype, which is triggered by c-Met over-expression.

Taken together, our results assign a new role for Hip1 in the endocytosis of β1-integrins following cancer-like sustained c-Met activation (irrespective of the mechanism of activation). Hip1 crucially regulated the integrin-dependent mesenchymal migration of HGF-stimulated HEK293 cells, but was dispensable for the amoeboid phenotype induced by c-Met over-expression.

DISCUSSION

Following migratory impulses, integrin trafficking is often altered. Conversely, derailment of integrin traffic results in distorted cell migration. In the publications included in this thesis, we have found new mechanisms that haven broadened our understanding about these two interdependent cellular processes. However, still not all questions are answered, and more riddles are yet to be solved.

1. Differential Roles of p120RasGAP during Cell Migration

In the first publication included in this thesis we have identified a novel function for p120 in the regulation of cell migration, which is independent of its catalytic activity but nevertheless dependent on its GAP-domain (Figure 7). Moreover, the migration-repressing function of p120 described by us does not involve the established interaction with p190RhoGAP and the concomitant inhibition of RhoA activity.

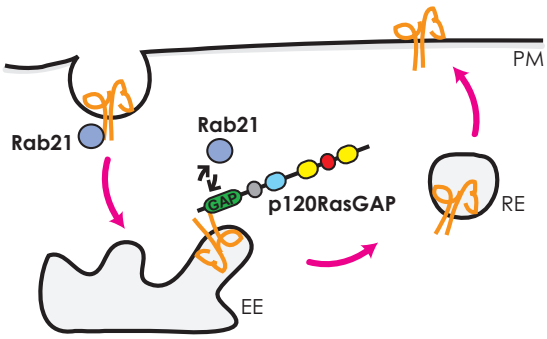

Figure 7: Joint Regulation of β1-Integrin Trafficking by Rab21 and p120.
Rab21 mediates the endocytosis of β1-integrins and their transport to the early endosome (EE). P120 then replaces Rab21 on the integrin α-cytodomain and thereby permits the recycling of integrins back to the plasma membrane (PM).

1.1 The Promiscuous Role of p120RasGAP during Cell Migration

P120 is a multifunctional protein that exerts various cellular functions. The GAP protein has first been identified as a negative regulator of Ras signalling downstream of numerous growth factor receptors (Marshall *et al.*, 1989). However, depending on the microenvironment, p120 can function up- or downstream of Ras and also Ras-independently. P120 exerts survival, pro- or anti-proliferative functions, and also critically regulates cell migration (Pamonsinlapatham *et al.*, 2009).

We found that down-regulation of p120 by RNAi stimulated the directional motility of a number of cell types. In cells that already migrated intrinsically persistent, for example in MDA-MB-231 cells and in MEFs, the lack of p120 increased only the speed of migration (I, Fig.2A; Fig.S2C). In contrast, in cells that displayed more random motility (e.g. KF28 cells or TIFFs), silencing p120 resulted in the strong induction of directionally persistent motility (I, Fig.2D; Fig.S2B). Moreover, the migration-promoting effect was dependent on the knockdown efficiency of the respective p120-siRNA. As seen in figure S2C (I) for the MEFs, siRNA 6 reduced p120 levels not quite as efficiently as siRNA 3. Consequently,

also the stimulating effect on the migration of cells transfected with this siRNA was more modest compared to siRNA 3.

However, in contrast to our findings, earlier work by Kulkarni and colleagues reported that MEFs, isolated from p120 null mice, failed to polarise and migrate towards a wound, yet stimulation with PDGF rescued the migration defect (Kulkarni *et al.*, 2000). They therefore hypothesised that p120 critically regulates cell migration in a Ras-dependent manner. But PDGFR stimulation has been found to promote cell migration also Ras-independently by activating Rac1 at the leading cell edge and via PDGFR endocytosis (Kawada *et al.*, 2009). More recently, King and co-workers found also β1-integrins to be important for the chemotactic migration of fibroblasts towards PDGF. Following stimulation, PDGFR prompted the activation of β1-integrins at the leading edge. Active β1-integrin, in turn, stabilised and activated Cdc42 and N-WASP, two proteins that permit directed migration of polarised cells (King *et al.*, 2011). In other words, the PDGF-stimulated rescue of the migration-deficiency of p120 null MEFs observed by Kulkarni *et al.* may be attributed to other, Ras-independent mechanisms.
Nevertheless, the p120 knockout MEFs used for their study did not migrate much under serum-free conditions, which is contradictory to the migration-promoting effect we observed following siRNA-mediated loss of p120. This discrepancy may be attributed to the different silencing efficiencies obtained by either siRNA-transfection (about 80-90% reduction) or complete loss of the protein in cells derived from knockout animals. Owing to the early lack of p120, these MEFs may have evolved other, p120-independent strategies to compensate for the loss. In line with this hypothesis are our observations in the ovarian cancer cell line KF28 and its Rab21-deficient variant KFr13 (I, Fig.2C, D). KFr13 cells lack Rab21 due to a chromosomal deletion. Despite their inability to traffic β1-integrins, KFr13 cells were able to migrate actively, in striking contrast to MDA-MB-231 cells that have been silenced for Rab21 only temporary using shRNA, and whose migration was strongly impaired (Pellinen *et al.*, 2006). Thus, KFr13 cells have presumably acquired the capability to use for their migration other integrins, whose trafficking is not dependent on Rab21 (and thus p120). Similar compensatory mechanisms may have evolved in p120 null MEFs.
It would be interesting to see whether the re-expression of Rab21 in KFr13 cells would make their migration susceptible for p120 intervention. Similarly, would loss of p120 still affect the migration of KF28 cells, if they were silenced for Rab21? Presumably, those cells would not migrate (as seen for Rab21-silenced MDA-MB-231 cells) and would not respond to the knockdown of p120, because they are not able to traffic β1-integrins and also have not had enough time to evolve other migratory mechanisms. Alternatively, different cell types may simply have different potential to compensate for p120 deficits.
Another indication that p120 regulates cell migration independent of Ras inactivation is the work by Pamonsinlapatham and colleagues. They showed that p120 knockdown in cells expressing constitutively active, oncogenic K-Ras^{V12} resulted in increased cell migration (Pamonsinlapatham *et al.*, 2008). Of the cells that we have used, also MDA-MB-231 cells harbour an oncogenic Ras mutation, but none of the other cell lines. However, the finding of Pamonsinlapatham *et al.* already points to a migration-regulating function for p120 that does not rely on Ras inactivation.

In the study of Kulkarni *et al.*, also the re-expression of p120 rescued the migration defect of p120 null MEFs in a concentration-dependent manner (Kulkarni *et al.*, 2000). This was accompanied by the complex formation between p120 and p190RhoGAP (subsequently abbreviated as p190). It has been known for a while that following EGF-

stimulation, p120 and p190 interact, and that this interaction critically regulates actin cytoskeleton reorganisation (Chang *et al.*, 1995). Later, Hu and Settleman found that receptor tyrosine kinase activation leads to Src-mediated phosphorylation of two tyrosine residues in the p190 protein (Y1105 and Y1087), which then serve as dual binding epitopes for the two SH2-domains of p120 (Hu and Settleman, 1997). The importance of the p120–p190 complex for cell spreading and polarisation has been shown by Bradley *et al.* (2006). Moreover, they reported that integrin-mediated adhesion results in the activation of the non-receptor tyrosine kinase family Abl, which includes Abl and Arg. Like Src, also active Arg kinase phosphorylates p190 and thus promotes the complex formation with p120. Bradley and colleagues showed that p120 association with p190 was critical for the redistribution of the complex to the cell periphery, where p190 transiently inactivates RhoA (Bradley *et al.*, 2006). Expression of a RFP-tagged p120 fragment comprising its SH2- and SH3-domains (RFP-232) impeded the p120–p190 interaction and thus prevented p190 re-localisation to and activity at the plasma membrane. Notably, the findings of this study were obtained during initial cell attachment and spreading on fibronectin.
We have been using the same dominant-negative RFP-232 construct in order to elucidate a possible contribution of the p120–p190 complex for cellular migration. However, we did not observe any significant differences in the motility of either RFP or RFP-232 expressing cells (I, Fig.S5B). In addition, we also did not detect changes in the cellular level of active RhoA following p120 knockdown (I, Fig.S5A), indicating that the p120–p190 interaction and subsequent RhoA inhibition do not regulate cell migration *per se*. Instead, this complex appears to be important for efficient cell spreading, the formation of initial cellular protrusions, and the sampling of the environment (Arthur *et al.*, 2002). This hypothesis is supported by the work of Tomar and co-workers, who reported that integrin-activated FAK targets the p120–p190 complex to the leading cell protrusions, where it maintains cell polarity (Tomar *et al.*, 2009). Indeed, RhoA activity has been found to undergo a dynamic tri-step regulation: Integrin-signalling initially promotes RhoA inactivation, which locally abrogates RhoA-mediated contractility and prevents the maturation of nascent adhesion complexes. This allows the formation of dynamic cellular protrusions that probe the environment. Subsequently, RhoA activity is elevated again, triggering stress fibre formation and focal adhesion maturation, which leads to the establishment of a prominent protrusion in the direction of migration. Finally, RhoA activity returns to a basal level, which is important for the maintenance of cell polarity (Arthur and Burridge, 2001). Interestingly, it has been shown that these patterns of RhoA activity at membrane protrusions depend on extracellular cues and the migration-initiating stimulus. In other words, the spatiotemporal regulation of RhoA activity in growth factor-stimulated cells is not the same as in randomly migrating cells or in cells that move towards an induced wound (Pertz *et al.*, 2006).

In conclusion, these findings, in conjunction with our results, allocate a possible dual function to p120 during the regulation of cell migration: First, p120 is necessary for the supply of p190 to the plasma membrane, where RhoA is locally and transiently inactivated. Thus, in complex with p190, p120 enables cell spreading on extracellular matrices and the establishment of a polarised cell. Second, p120 is required on early endosomes in the cytosol, where it allows the recycling of β1-integrins during cell migration. It may be possible that these two cooperative p120 functions occur consecutively (after p190 dephosphorylation, p120 may be released and return to the cytosol, where it then regulates integrin recycling) or by different pools of p120.

Alternatively, it has been suggested that EGFR, p120, Ras, and Annexin A6 jointly internalise through clathrin-coated vesicles (Vila de Muga *et al.*, 2009). It is thus possible that p120 is targeted to early endosomes via EGFR co-endocytosis. However, it is conceivable that the requirement for p120 to facilitate cell spreading at the plasma membrane is transient, and that after the cell has spread and orientated towards the migratory stimulus, p120 predominantly regulates integrin trafficking. This might explain why we did not observe any basal changes in RhoA activity in p120-silenced cells.

1.2 Impaired Integrin Recycling: Pro- or Anti-Migratory?

In the first paper we also studied the role of integrin trafficking during cell migration. We found that derailed recycling of integrins, owing to the loss of p120, has a stimulatory effect on the migration of a number of cell types.
It has been known for some time that integrin trafficking, and recycling in particular, critically regulates cell migration (Jones *et al.*, 2006; Petrie *et al.*, 2009). However, impaired recycling has often been linked with diminished cell motility (Ivaska *et al.*, 2002; Powelka *et al.*, 2004; Mammoto *et al.*, 1999). Other studies have shown that it is not mere inhibition of general recycling pathways that alters cellular motility, but rather the mutual influence of integrin-specific trafficking routes that contributes to the respective migration mode (White *et al.*, 2007; Caswell and Norman, 2008). Moreover, the cellular context has been demonstrated to determine whether increased integrin recycling supports or compromises cell migration. For example, in breast and ovarian cancer cells, the increased expression of Rab25 has been found to promote β1-integrin recycling and consequently the invasion of these tumour cells into a 3D matrix (Caswell *et al.*, 2007). By contrast, in colon cancer, the loss of Rab25 has been correlated with poorer patient survival (Goldenring and Nam, 2011). Rab25-deficient mice showed elevated levels of intracellular β1-integrin in the cells of the villus, which was associated with increased susceptibility to colon carcinogenesis. It has thus been proposed that both over-expression as well as loss of Rab25 result in imbalances in the surface delivery of integrins or associated proteins (e.g. EGFR), and hence contribute to impaired cell migration (Goldenring and Nam, 2011).

We have found that p120 operates on early sorting endosomes (I, Fig.7B), where it substitutes Rab21 from the integrin α-subunit. This replacement is necessary to allow the integrin to proceed to the PNRC and via recycling endosomes back to plasma membrane. It remains a topic for further investigation how p120 itself is released from the integrin cytodomain and where this release occurs. Does p120 withdrawal occur already on early endosomes? Does RCP, known to be critical for the recycling of β1-integrins, pursue the redistribution of integrins from the PNRC onwards - maybe even by releasing p120? Or does p120 accompany the itinerary integrin all the way back to the plasma membrane? The latter hypothesis may be subverted by the only limited co-localisation we observed between integrins and p120 under steady-state conditions (I, Fig.5D). This implies that the encounter between p120 and integrins is rather brief, and that p120 is presumably released on-site.

As the loss of p120 resulted in strong accumulation of integrins in Rab21-positive vesicles (I, Fig.7A, C), we hypothesise that p120 functions as a universal regulator for the recycling of the majority of β1-integrins. This hypothesis is supported by the integrin-binding site of p120, which lies within the WKLGFFKR sequence that is common to most

α-subunits (I, Fig.5B). Even though we determined the p120-binding epitope only on the α2-cytodomain in more detail, it is conceivable that the same residues of other integrins also mediate the interaction with p120. Thus, p120 may indeed have a role as a master-regulator for integrin recycling. Upon the loss of p120, this extensive recycling mechanism is impaired, but other recycling pathways may then account for the targeted delivery of integrins to the plasma membrane. For example, it is possible that in the absence of p120, integrins bypass the commonly used long recycling route and, instead, exit the early endosome and return to the plasma membrane via the short, Rab4- and PKD1-dependent recycling loop. This may be feasible only for certain integrins, but not the majority of adhesion receptors. Integrin αvβ3, for example, is able to rapidly recycle through the short-loop pathway (Woods *et al.*, 2004). Moreover, this integrin has been associated with increased directional persistent migration (Danen *et al.*, 2005).
Alternatively, instead of circumventing the long recycling route, it is also possible that following p120 loss, other collateral recycling pathways come to the fore, which were otherwise masked by the potent effect of p120. The hence reduced, but possibly more targeted recycling of integrins would allow for the formation of a pronounced protrusion and thus support directed migration. In order to confirm that neither the Ras-inactivating catalytic activity of p120 nor its N-terminal classical protein-protein interaction domains account for the regulation of integrin recycling and cell migration, one could express the p120 GAP-domain alone and test whether it can rescue the defects in p120-silenced cells.

In general, a stringent balance between integrin internalisation and recycling seems to be of importance during cell migration. Moreover, the consequences of impaired trafficking have been shown to not only depend on the cell type, but also to a great extent on the microenvironment. Strachan and Condic (2004) demonstrated that cells preferentially recycled those integrins, which were mediating their motility. Moreover, they proposed that efficient migration not only relies on substrate composition, but also on the ratio of integrin cell surface level to ligand availability. The dependency of efficient cell motility on integrin recycling was more pronounced in cells cultured on high ligand concentrations. By contrast, in the event of low ligand concentrations, the block of recycling had less impact on the migration speed and was instead more dependent on integrin activation/inactivation (Strachan and Condic, 2004). Therefore, when studying the impact of integrin trafficking on the motility of cells, it seems important to bear in mind that substrate composition and density, as well as cell type and integrin heterodimer characteristics influence the significance of individual trafficking routes.

1.3 How Do Rab21 and p120RasGAP Cooperate?

The mechanism by which p120 elicits β1-integrin progression through the endosomal trafficking machinery implies the replacement of bound Rab21 from the integrin α-cytodomain on early endosomes. Both proteins bind integrin heterodimers on membrane proximal residues, which are conserved in most α-subunits (I, Fig.5B). However, the binding affinities towards the integrin differ: *In vitro*, p120 possess a slightly higher affinity than Rab21, which might *in vivo,* due to its elevated concentration in the cytosol, account for the competitive binding ability of p120 to the integrin (I, Fig.5). This conceptual new mechanism arises many interesting questions for further investigation.
First, can p120 also replace other Rab proteins, possibly Rab5, from integrins? If p120 would indeed be capable to also replace Rab5, then this competitive binding would be a

novel, general mechanism how early endosomal Rab proteins are released and the cargo is handed on. However, to date it has not yet been shown that Rab5 even binds directly to integrins. Thus, the competitive mechanism demonstrated by us may be unique for the Rab21-driven endocytosis route. It would be interesting, however, to test whether recycling Rab GTPases (such as Rab7, Rab11, or Rab25) are in turn able to (directly or indirectly) replace p120 from the integrin receptor. Rab25 has been shown to bind to the β1-subunit (Caswell *et al.*, 2007), but it could still be possible that integrin-bound p120 would be released due to conformational changes induced by the Rab25-binding or due to steric hindrance.

Another question to address is what happens to the integrin cargo when p120 is bound? Does the interaction influence its downstream signalling or activation state? And conversely, does integrin-binding influence the catalytic activity of p120? The latter is feasible to imagine, as p120 interacts with the integrin via its GAP-domain. Further analysis will reveal whether p120 GAP activity is elevated or decreased. Interestingly, the PH-domain of p120 has been proposed to bind to the GAP-domain and thereby to competitively prevent Ras-binding and inactivation (Drugan *et al.*, 2000). May the p120–integrin binding therefore prevent Ras inactivation as well, or may the interaction even release inhibitory contacts and stimulate Ras GTP-hydrolysis? Moreover, it will be exciting to test in a FP-based competition assay, whether Ras-binding to p120 influences its interaction with the integrin α-cytodomain and hence its competition with Rab21. Furthermore, since it is the GAP-domain of p120 that binds to the integrin α-subunit, the classical N-terminally located protein-protein interaction domains may be accessible for binding of other proteins. For example, RCP, a Rab11 adapter protein, has been found to link EGFR to α5β1-integrins and to regulate their joint recycling from the PNRC to the plasma membrane (Caswell *et al.*, 2008). What if p120 would be able to concomitantly bind integrins and activated RTKs (or other cargo proteins) and thereby prevent their sorting to lysosomal degradation and instead retarget them to the recycling pathway?

Finally, the regulatory proteins that control Rab21 activity still remain to be determined. What are the GEFs and GAPs that regulate Rab21-dependent integrin trafficking and cell migration? We found that Rab21 binds integrins in an unexpected nucleotide-independent manner (I, Fig.3D). However, previous work indicated that Rab21 function on integrin trafficking still depends on its nucleotide-bound conformation (Pellinen *et al.*, 2006). When expressing a GDP-locked Rab21 mutant, integrins failed to internalise. Conversely, when expressing a constitutively active GTP-mutant, integrins accumulated inside the cell. Therefore, GEF and GAP proteins are certainly required to regulate Rab21 function. However, Rab GTPases are known to recruit a number of effector proteins that are then responsible for vesicle budding and fusion (Stenmark, 2009). The competitive replacement by p120 might hence not be needed for the transport of Rab21 vesicles, but rather to release the early endosomal Rab and to allow late endosomal Rab proteins to take over.

In summary, we found a new function for p120 in the regulation of integrin recycling and cell migration. On early sorting endosomes, p120 binds to the α-subunit of integrins via its GAP-domain and thereby replaces the bound endocytic protein Rab21. This competitive mechanism allows for the transfer of the integrin cargo through the recycling compartment back to the plasma membrane. P120 has hence a critical role in the careful balance of integrin endocytosis and recycling during cell migration.

2. Novel Regulators of c-Met–Induced Cancer Cell Invasion

We established an *in vitro* 3D cell model for the investigation of oncogenic c-Met activation in a more physiological surrounding than the commonly used monolayer cell culture. We therefore embedded epithelial HEK293 cells in 50 % Matrigel, which is a reconstituted basement membrane matrix, especially rich in collagens, laminin, fibronectin, and proteoglycans (Shaw *et al.*, 2004), and investigated the cellular behaviour following oncogenic c-Met activation by either HGF-stimulation or c-Met over-expression. With this cell model, we identified new regulatory mechanisms implicated in c-Met–induced cancer progression (Figure 8). HGF-mediated stimulation of c-Met was found to induce collective cell invasion with a mesenchymal phenotype. These cells relied on integrin engagement and proteolytic ECM remodelling. In contrast, c-Met over-expression stimulated an amoeboid invasion mode, which required RhoA/ROCK activation.

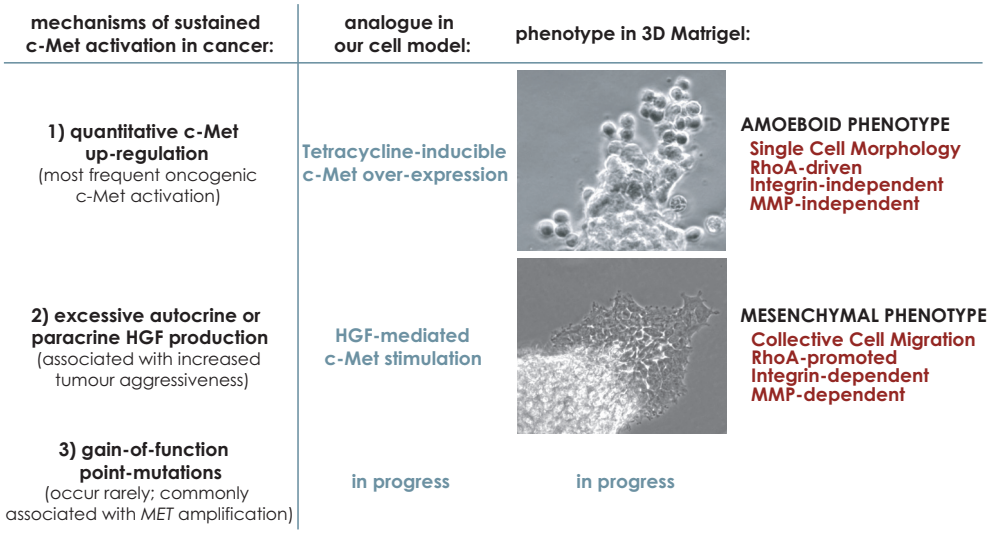

Figure 8: c-Met Effects on Cancer Cell Invasion.
Oncogenic c-Met signalling triggers the invasion of numerous tumour cells. However, the c-Met–promoted migration strategy depends on the activating stimulus: HGF-mediated receptor activation results in the collective invasion of cells with mesenchymal characteristics. In contrast, c-Met over-expression contributes to an amoeboid phenotype.

2.1 A 3D Cell Model to Study Oncogenic c-Met Signalling

Unexpectedly, the cellular responses to sustained c-Met activity differed depending on the mechanism of receptor activation (Figure 8).
Exogenous HGF-stimulation of WT HEK293 cells induced the dissemination of these cells from the spheric cell cluster and their collective invasion into the surrounding matrix, while retaining intercellular contacts. HGF/c-Met signalling has been shown to activate matrix-degrading MMPs during the mobilisation of haematopoietic cells (Tesio *et al.*, 2011). We showed that also the mesenchymal invasion mode of HGF-stimulated

HEK293 cells is dependent on the proteolytic activity of MMPs, while the Tet-induced amoeboid phenotype is not. With additional experiments, we aim to test whether MMPs are indeed distinctively activated or expressed following c-Met activation by either ligand-stimulation or receptor over-expression.

In striking contrast to the HGF-triggerd mesenchymal phenotype, the Tet-induced over-expression of c-Met caused an amoeboid cell morphology, which resulted in the disruption of the spheroids into loose assemblies of individual cells with only weak intercellular junctions. However, no apparent induction of amoeboid-type cell invasion was observed, as the cells stayed on the spot and moved only marginally (II, movie S3). But as the amoeboid invasion mode does not rely on proteolytic ECM remodelling, it depends to a great extent on the architecture of the matrix (Friedl and Wolf, 2010). Matrigel constitutes a stiffer and more sterically constrained matrix than, for instance, a 3D collagen environment. Moreover, the average pore size in 50 % Matrigel is about 2 µm and thus much smaller than the cellular dimensions (Zaman *et al.*, 2006). Therefore, over-expression of c-Met may induce the switch towards amoeboid invasion, yet under the given experimental conditions, the cells were unable to move forward due to steric hindrance. It is therefore the next step to analyse the motility of Tet-induced HEK-Met cells in lower Matrigel concentrations as well as in a collagen environment. Moreover, it would be interesting to co-culture HEK-Met cells with MMP-expressing fibroblasts in 50 % Matrigel, and to see whether the ECM-remodelling ability of the fibroblasts would then enable the HEK-Met cells to migrate within Matrigel in a c-Met–dependent manner.

We also found vimentin expression to be up-regulated following c-Met over-expression. Vimentin is an IF-protein that is commonly restricted to cells of mesenchymal origin, but has been found to be important for cancer cell migration (Vuoriluoto *et al.*, 2011) and to be up-regulated in metastatic carcinoma cells (Mendez *et al.*, 2010). Therefore, vimentin has become an established marker for cancer cells undergoing EMT, and its expression correlates with tumour progression (Ivaska, 2011). It has recently been reported that Matrigel is able to induce vimentin expression (Dal Vechio *et al.*, 2011), and thus it remains a topic for further investigation, whether the specific vimentin pattern in spheroids seen by us is due to the Matrigel environment or not. However, vimentin has been shown to be sufficient to provoke cell motility and cell type-depending morphological changes (Mendez *et al.*, 2010). The observed c-Met–stimulated expression of vimentin may thus contribute to the suggested amoeboid invasion mode.
Interestingly, it has been reported that also the MT-destabilising protein stathmin is over-expressed in metastatic sarcomas (Belletti *et al.*, 2008). If stathmin was protected from inactivation by ECM-contact, the cells obtained a round morphology and gained invasive potential by adopting the amoeboid migration mode. In relation to this finding, we speculate that elevated c-Met levels may either directly increase MT-dynamics or indirectly support stathmin activity by initiating weaker cell-matrix interactions. Stable MTs can be determined by staining for post-translational modifications, such as acetylation (Belletti *et al.*, 2008). We therefore aim to examine the level of acetylated/stabilised MTs and stathmin in HEK-Met cells with and without Tet-induction.

The clear differences in the cellular responses following either c-Met over-expression or HGF-stimulation raise the question for the underlying mechanism. We studied oncogenic c-Met signalling using the same cellular background and the same 3D microenvironment, thus the two cell models differed only in the mechanism of c-Met activation. Sustained c-Met stimulation induced cell invasion in both models, yet with distinct

migration characteristics. These different invasion modes may result from differences in the duration or magnitude of signal strength. Tet-induced over-expression of c-Met might possibly result in a longer or more potent activation of c-Met downstream signalling compared to the stimulation with exogenous HGF, where concomitant receptor degradation may restrict c-Met–dependent signalling (Lemmon and Schlessinger, 2010). In future experiments we thus plan to examine c-Met activity and protein level in WT HEK293 cells over a longer incubation time with HGF.
Alternatively, the different cellular responses to sustained c-Met activity may not derive from the signal magnitude. Is it instead possible that the respective c-Met–induced downstream signalling pathways rely on the activating stimulus? This merits further investigation. One could, for instance, envisage that active c-Met recruits a certain set of core effectors, yet accessory pathways may possibly be activated distinctively - depending on the mechanism of c-Met activation. It has already been shown in mice that c-Met–activating point-mutations cause the development of mutant-specific tumours (Graveel *et al.*, 2004). This discovery already points to mutation-unique effector recruitment by oncogenic c-Met. Similar effector selectivity may thus also arise from receptor activation by either HGF-stimulation or c-Met over-expression. In addition, the potential c-Met bias for certain downstream pathways may result from differential cellular locations of the activated receptor. It is already known that c-Met trafficking (Joffre *et al.*, 2011) as well as its signalling from early endosomes (Kermorgant and Parker, 2005) greatly contributes to receptor function and specificity. Moreover, highly invasive MDA-MB-231 breast cancer cells have been found to localise a C-terminal c-Met–fragment to the nucleus, where it contributed to the invasiveness of the cells by a yet unknown mechanism. In contrast, low invasive MCF-7 breast cancer cells showed no nuclear localisation of c-Met (Matteucci *et al.*, 2009). Although the mechanism remains elusive, these observations still underscore the significance of c-Met localisation for its effects on cellular behaviour. Future experiments will thus be aimed at investigating differences in c-Met localisation and signalling following either receptor over-expression or HGF-stimulation.
In line with our discovery that c-Met effects on cell invasion rely on the mechanism of activation is the finding that c-Met can be activated in a HGF-dependent and -independent manner but via distinct mechanisms (Hui *et al.*, 2009). While ligand-independent, integrin-mediated activation of c-Met required Src and FAK activation, the stimulation of c-Met by exogenous HGF did not rely on Src. It is thus conceivable that not only the activating mechanism may differ, but also the c-Met downstream signalling.

In conclusion, we found that oncogenic c-Met activity results in differential cellular responses depending on the mechanism of receptor activation. HGF-mediated c-Met stimulation contributed to mesenchymal-type collective cell invasion, whereas c-Met over-expression may induce single cell migration with amoeboid characteristics. Our established 3D model will provide the possibility to examine in more detail the possible differences in the downstream signalling pathways that may arise from distinct c-Met–activating stimuli. Moreover, with our 3D cell model we mimic the two major mechanisms that account for oncogenic c-Met activation in numerous human tumours. Our *in vitro* model is therefore a suitable and powerful tool to easily test new pharmacological agents targeting sustained c-Met signalling, while considering the distinct therapeutic challenges that may arise from oncogenic c-Met activity caused by either excessive HGF-stimulation or receptor over-expression.

2.2 RhoA, Handyman for c-Met–Mediated Cancer Cell Invasion

In order to identify new regulatory proteins that accomplish oncogenic c-Met effects, we used the Tet-inducible HEK-Met cells, that undergo easily detectable morphological changes following sustained c-Met activation, and performed a high-throughput RNAi-based cell spot microarray (Rantala *et al.*, 2011). The most persuasive signalling cascade that was found to be crucial for the induction of the oncogenic rounding was the RhoA pathway. In fact, we found RhoA to be activated following Tet-induced c-Met over-expression, and inhibition of RhoA, its effector kinase ROCK, as well as loss of RhoA by RNAi blocked the c-Met–induced morphological changes. In addition, also the myosin light chain (MLC) was found to be increasingly phosphorylated (II, Fig.1A), a finding which is consistent with RhoA/ROCK-stimulated cell contractility.
RhoA activation following c-Met over-expression is in line with the proposed induction of an amoeboid invasion mode. Amoeboid migrating cells are characteristically roundish and exploit a propulsive RhoA-driven pushing migration mode (Friedl *et al.*, 2001). Moreover, RhoA-stimulated contractility has been found to contribute to the disruption of cell-cell contacts (Zhong *et al.*, 1997), and this may allow single cell migration out of the spheroid. In support of our results is also the recent finding that in human non-small cell lung (NSCL) carcinomas, c-Met over-expression correlates with RhoA and TIMP over-expression in invasive specimens (Gumustekin *et al.*, 2011). TIMP (Tissue Inhibitor Metalloproteinase) is a MMP-antagonist that prevents ECM proteolysis, and together with the earlier report that the NSCL carcinoma cell line H1299 preferentially migrates in the amoeboid mode (Carragher *et al.*, 2006), these findings support our discovery that c-Met over-expression initiates a RhoA-dependent, but MMP-independent amoeboid invasion mode (Figure 8).

Unexpectedly, RhoA/ROCK signalling was also crucial for the HGF-induced mesenchymal-type collective invasion of WT HEK293 cells. In other words, although HGF-stimulated HEK293 cells employ a different migration strategy than HEK-Met cells, they still require RhoA activity for their invasion. This finding seems contradictory to the prevailing view that RhoA/ROCK signalling is dispensable for the mesenchymal migration mode (Pankova *et al.*, 2010). However, extensive research on cell motility in 3D matrices has led to the discovery of an increasing number of migration strategies, and the differences in the invasive behaviour of various cell types can possibly not be explained with only two classical migration modes (Mierke *et al.*, 2008). Consistently, Poincloux and colleagues recently described the motility of MDA-MB-231 breast cancer cells in Matrigel. The cells combined characteristics from both the mesenchymal and the amoeboid migration mode (Poincloux *et al.*, 2011). In addition, ROCK-dependent local matrix remodelling and deformation has also been shown to critically contribute to the migration of fibroblasts in 3D culture (Kim *et al.*, 2006). Thus, mesenchymal cells seem to employ various strategies to locally remodel the microenvironment - one of them being RhoA activation.
In addition, it is also conceivable that increased RhoA/ROCK activity has an impact on the cellular distribution of c-Met and thus on its specific downstream signalling. The spatial organisation and responsiveness of EGFR has been reported to be also dependent on the organisation of the actin cytoskeleton (Chung *et al.*, 2010). Thus, RhoA/ROCK-mediated actin reorganisation may possibly affect also c-Met distribution and thereby enable the effector selectivity needed for the HGF-triggered mesenchymal migration mode. It remains a topic for further investigation whether the magnitude or duration of RhoA activation following c-Met over-expression is stronger than upon HGF-

stimulation and whether this putative difference accounts for the distinct morphological and migratory phenotypes in our cell models.
Alternatively, the critical balance between RhoA and Rac1 activation, which controls the plasticity of tumour cell invasion, may be differentially shifted in our two cell models. The amoeboid migration mode requires high levels of active RhoA, but Rac1 activity needs to be low at the same time, as Rac1 counteracts RhoA-mediated actomyosin contractility (Sanz-Moreno *et al.*, 2008). In addition, it has been found that Src promotes the mesenchymal migration of cancer cells, but is dispensable for the amoeboid-type motility (Carragher *et al.*, 2006). More recently, it has been discovered that Src phosphorylates ROCK II on tyrosine Y722, thereby rendering ROCK inert to RhoA activation (Lee *et al.*, 2010). Together, these findings allow the hypothesis that sustained c-Met activity results in RhoA activation, irrespective whether the receptor was activated by ligand-stimulation or over-expression. However, Src may be specifically and selectively activated upon HGF-mediated c-Met–stimulation and thus inhibit the amoeboid invasion mode by inhibiting ROCK signalling. The consequent higher Rac1 activity in HGF-stimulated HEK293 cells may then promote the observed mesenchymal invasion mode. In order to test this hypothesis, we will analyse and compare the Rac1 activation status in our two cell models.

In summary, our data indicate that RhoA is a key effector of sustained c-Met signalling, independent of the receptor-activating mechanism. Thus, RhoA/ROCK signalling contributes to the broad migration-promoting effect of oncogenic c-Met, yet other accessory signalling pathways may then account for the different modes of migration.

2.3 The Enigmatic Role of Hip1 in Tumourigenesis and Metastasis

We found that sustained c-Met activation by either receptor over-expression or HGF stimulation resulted in decreased β1-integrin cell surface levels. We hence speculated that c-Met–initiated alteration of integrin trafficking is responsible for the onset of migration following oncogenic c-Met activation.
Having this hypothesis in mind, the endocytic protein Huntingtin-interacting protein 1 (Hip1) caught our attention in the hit list from our screen for novel c-Met effectors (II, Fig.4). Hip1 is an accessory protein in the clathrin-mediated endocytosis pathway, yet to date its actual function is still unclear (Gottfried *et al.*, 2009). In neurons derived from Hip1 null mice, AMPA receptor failed to internalise upon ligand stimulation (Metzler *et al.*, 2003). And also Gottfried and colleagues (2009) assigned a role for Hip1 during the early internalisation step in the abscission of clathrin-coated pits. In contrast, other studies have found that Hip1 over-expression in HEK293T cells prolonged EGFR activity following EGF-stimulation by blocking receptor targeting from the early endosomes to the lysosomal degradative pathway (Hyun and Ross, 2004). Recent work by the same group reported that Hip1 also retards the degradation of the SCF-activated receptor tyrosine kinase c-Kit in Merkel cell carcinomas of the skin (Ames *et al.*, 2011). Moreover, owing to a correlation between Hip1 over-expression and concomitant elevated RTK activity in a number of carcinomas, they proposed that the Hip1 protein level may be used as a predictive marker for the presence and progression of some tumours to aggressive disease (Rao *et al.*, 2003; Bradley *et al.*, 2005; Ames *et al.*, 2011).
Because of the proposed functions of Hip1, we assumed that it may be recruited for the c-Met–stimulated integrin endocytosis. Moreover, as RTKs preferentially enter the cell via the clathrin-mediated route, Hip1 might even be involved in the co-endocytosis of c-

Met and integrins. In line with this, we detected a strong co-localisation between the growth factor receptor and β1-integrins on endosomes following c-Met activation (II, Fig.1E). In accordance with the observations by Ross and colleagues (Ames *et al.*, 2011), one could also imagine that Hip1 protects activated c-Met receptor from degradation by targeting c-Met and integrins to the recycling pathway. But this hypothesis awaits further investigation. To date we do not yet know whether Hip1-silencing impedes c-Met internalisation or degradation.
However, we found that in the absence of Hip1, both WT HEK293 and HEK-Met cells failed to induce β1-integrin internalisation following oncogenic c-Met activation. Therefore, we propose a new function for the endocytic protein Hip1 in the internalisation of β1-integrins downstream of activated c-Met. Consistently, a correlation between increased c-Met/HGF signalling and decreased α4β1-integrin (VLA-4) cell surface levels was recently discovered in B-chronic lymphocytic leukemia (Eksioglu-Demiralp *et al.*, 2011). Although in this study the researchers focused only on the consequent downstream signalling effects, it is conceivable that the elevated c-Met signalling had caused the diminished α4β1 surface levels via Hip1-mediated endocytosis.

We also found that the loss of Hip1 prevented the HGF-stimulated mesenchymal invasion mode, which is known to depend on integrin engagement (Friedl and Gilmour, 2009). Integrin-mediated adhesion is required whenever high traction forces are needed, for example during the crossing of the basement membrane (Renkawitz and Sixt, 2010). Therefore, the loss of Hip1 appears to impair the integrin-dependent mesenchymal invasion of WT HEK293 cells through Matrigel. This hypothesis is supported by our finding that also the presence of a β1-integrin function-blocking antibody prohibited HGF-stimulated cell invasion into the matrix.
Alternatively, it is also possible that the loss of Hip1 impairs the trafficking of the c-Met receptor itself. Thus, although c-Met is activated by HGF, it fails to promote oncogenic, pro-invasive signals from endosomes. The importance of the endosomal localisation for transforming c-Met signalling has recently been reported (Joffre *et al.*, 2011). We therefore aim to examine possible alterations in the distribution of c-Met following Hip1 loss.

In contrast to the mesenchymal invasion mode, amoeboid-type cell motility, triggered by c-Met over-expression, depends on RhoA/ROCK signalling, but integrin engagement is thought to be dispensable (Friedl and Wolf, 2003). Consistently, we found that the presence of the β1 function-blocking antibody Mab13 had no impact on the initiation of the amoeboid phenotype in 3D Matrigel upon Tet-induction. Moreover, Hip1-silencing in HEK-Met cells did not prevent the induction of the amoeboid-like morphological changes following c-Met over-expression, while in the concomitant presence of either c-Met or ROCK inhibitor the fragmentation of the spheroids was inhibited. This indicated to us that Hip1-altered integrin trafficking does not play a role for the amoeboid-type invasion of cells in 3D Matrigel. However, under the conditions of our cell spot microarray screen, on planar cell culture plates, the loss of Hip1 did prevent the rounding of Tet-induced HEK-Met cells. The explanation for this seeming discrepancy is that amoeboid migration does not depend on integrin engagement in a 3D environment. However, on 2D surfaces, cell motility is only governed by a careful balance between adhesion and traction, and cell movement slows down if the adhesive strength is either too high or too low (Zaman *et al.*, 2006). In other words, on 2D cell culture plates, cells rely only on adhesion and traction, and thus altered integrin trafficking by Hip1 has an impact on the morphology of the cells. Conversely, in 3D, cells are also affected by the matrix architec-

ture and stiffness, and because amoeboid motility does not depend on integrin engagement, Hip1-silencing has no apparent effect on the phenotype of HEK-Met cells in 3D Matrigel. However, although Hip1-mediated integrin trafficking has no influence on the amoeboid morphology, it may still influence the signalling events that account for this invasion mode. Further research will reveal whether Hip1 additionally contributes to the trafficking of c-Met and whether it allows collaborative signalling of integrins and c-Met (on endosomes?).

3. The Role of the α5β1–ZO-1 Complex during Cell Migration

In the third publication, we identified a new function for the tight junction protein ZO-1 in the regulation of cell migration (Figure 9). Following a migration-promoting stimulus, elevated PKCε activity initiated the redistribution of ZO-1 from cell-cell contacts to the leading protrusion, where it, in cooperation with α5β1-integrin, maintained cell polarity and stimulated directionally persistent migration. The ominous role of the α5β1–ZO-1 interaction for tumour invasion *in vivo* is underlined by the finding that the complex was exclusively present in patient samples from metastasising lung carcinoma specimens.

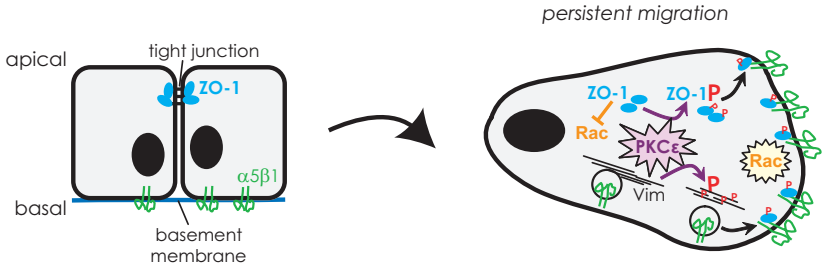

Figure 9: ZO-1 and α5β1, a Migration-Promoting Duo.
In immotile epithelial cells, ZO-1 is a component of tight junctions and α5β1-integrins mediate cell attachment to the basement membrane. However, following migration-triggering impulses, high PKCε levels result in α5β1–ZO-1 complex formation and facilitate integrin recycling from trapping vimentin (Vim) filaments. The α5β1–ZO-1 duo restricts Rac1 activation to the leading edge, thereby supporting cell polarity and directed migration.

3.1 What Guides ZO-1 to the Leading Edge?

We demonstrated with a biochemical ELISA assay *in vitro* that the integrin α5-subunit binds directly to the ZO-1 PDZ-domains via a non-canonical internal peptide sequence at its C-terminus (III, Fig.5A). We do not yet know which of the three PDZ-domains is crucial for the interaction. But more importantly, we found that in cells, ZO-1 required PKCε-dependent phosphorylation on specific residues located between the first and the second PDZ-domain in order to interact with α5β1 at the lamellipodium (III, Fig.7). This finding provides a first indication of the mechanism that guides ZO-1 to the leading edge of the cell. We have found that integrin α5β1 was necessary to anchor ZO-1 at the lamellipodium, but PKCε-dependent phosphorylation of the tight junction protein was needed to target ZO-1 to α5β1 at the advancing protrusion. It is hence conceivable that

PKCε creates binding epitopes, which recruit other cellular effector proteins that then guide ZO-1 from tight junctions to the lamellipodium in proximity to α5β1. The importance of ZO-1 phosphorylation for its cellular localisation has been proposed before (Taliana *et al.*, 2005), yet the mechanism is still not known. Alternatively, it may be possible that ZO-1 phosphorylation is needed only for its release from tight junctions, yet other accessory proteins guide ZO-1 to the leading edge. It has recently been demonstrated that the Cdc42-activated MRCKβ kinase binds ZO-1 and directs the complex to the leading edge (Huo *et al.*, 2011). However, also in this study the molecular basis for the ZO-1 redistribution has not been elucidated.

3.2 How Does the α5β1–ZO-1 Duo Maintain the Lamellipodium?

Another question to be answered concerns the mechanism of how the α5β1–ZO-1 complex promotes directed cell migration. Local Rac1 activation at the lamellipodium is required, but how is that achieved by the α5β1–ZO-1 interaction? One possible mechanism involves the above mentioned work by Huo *et al.* (2011). They demonstrated that ZO-1 was required to anchor the Cdc42-MRCKβ complex at the leading lamellae. One could thus envisage that ZO-1 guides and ties this complex to α5β1-integrin. Moreover, Cdc42 has an established role for cell polarisation and local Rac1 activation (Iden and Collard, 2008). Thus, by the connective function of ZO-1, a multi-protein complex involving α5β1, Cdc42, and MRCKβ could allow for Rac1 activation at the advancing lamellipodium and consequently account for directed cell migration. In contrast, if ZO-1 is absent, the Cdc42-MRCKβ complex is no longer distributed to the cell front, and instead Rac1 activation is stimulated throughout the cell. This would cause the formation of multiple protrusions and result in random motility (Pankov *et al.*, 2005). In addition, it may also be possible that non-phosphorylated ZO-1, which is not engaged in tight junctions, recruits a Rac1-specific GAP protein. This putative Rac1-inhibitory interaction may be lost when ZO-1 is phosphorylated and thus complexed with Cdc42, MRCKβ, and α5β1-integrin - or when ZO-1 is depleted by RNAi.

An alternative mechanism of how the α5β1–ZO-1 duo may promote spatially restricted Rac1 activation comprises the Par-Tiam1 complex. The Par polarity complex has been found to target the Rac1-specific GEF Tiam1 to the leading edge and by subsequent local Rac1 activation to promote directed cell migration (Pegtel *et al.*, 2007). Interestingly, the Par complex is crucial for the assembly of tight junctions, and its clustering with junctional adhesion molecules (JAMs) mediates the association with ZO-1 (Ebnet *et al.*, 2003). Phosphorylation-induced disassembly of cell-cell contacts (Lee and Daar, 2009) may hence cause the joint redistribution of the Par complex and ZO-1, accompanied by Tiam1, to α5β1-integrin at the leading edge.

Yet another hypothesis involves the proteoglycan syndecan-4. This protein generally suppresses Rac1 activation, except at the leading cell edge, where its binding to fibronectin promotes PKCα-dependent Rac1 activation (Bass *et al.*, 2007). But what mechanism activates syndecan-4 specifically at the lamellipodium? We propose that a cooperative interaction between syndecan-4 and α5β1-integrin, possibly mediated by ZO-1, could contribute to the local Rac1 activation. In the event of lost ZO-1, matrix engagement at the plasma membrane could still lead to the activation of syndecan-4 and thus to high levels of active Rac1, but this would no longer be restricted to the leading cell edge and could, instead, occur at all sites of matrix engagement.

But whatever the molecular mechanism for the spatially restricted Rac1 activation by the α5β1–ZO-1 complex is, it remains also unclear how this is restricted to the plasma membrane at the leading edge. This process might possibly be controlled by the localisation of α5β1-integrin. Targeted recycling (e.g., resulting from elevated PKCε activity) as well as enrichment of the integrin in specific lipid domains at the lamellipodium (Weisswange *et al.*, 2005) may cooperatively contribute to α5β1 concentration at the cell front. Moreover, the second PDZ-domain of ZO-1 has been demonstrated to bind to $PI(4,5)P_2$, $PI(3,4,5)P_3$, and $PI(3,4)P_2$ with low affinity (Meerschaert *et al.*, 2009). The latter two phosphoinositides are specifically generated at the leading edge, and albeit the affinity of the PDZ-domain may not be strong enough to recruit ZO-1 to the plasma membrane, it may still have a modulating signalling or targeting role.

3.3 Possible Role for ZO-1 in the Nucleus

We also found a peculiar localisation of ZO-1 in the nucleus whenever PKCε activity was impaired (II, Fig.6). ZO-1 has been acknowledged to be an itinerary protein with localisation-dependent functions (Polette *et al.*, 2007). In stationary cells, ZO-1 is an important structural component of tight junctions. In addition, it sequesters the transcription factor ZONAB at intercellular contact sites, hence diminishing ZONAB nuclear levels and its ability to promote epithelial cell proliferation (Matter and Balda, 2007). The redistribution of ZO-1 to the nucleus has been described controversially, but there are studies reporting a nuclear localisation of ZO-1 in migrating cells, where ZO-1 has been found to induce vimentin and MMP expression and to stimulate the invasiveness of carcinoma cells (Polette *et al.*, 2007). Recent work by Remue and colleagues (2010) reported an association of the EMT-inducing transcription factor TAZ with the first PDZ-domain of ZO-1 and ZO-2. However, nuclear co-localisation with the transcription factor was predominantly observed for ZO-2, whereas ZO-1 interacted with TAZ mainly in the cytosol and only occasionally also in the nucleus (Remue *et al.*, 2010). However, cell type-related differences were observed, too. A recent publication reported a nuclear localisation of ZO-1 specifically for non-transformed cells (Belgiovine *et al.*, 2011).

Taken together, there is indeed controversial data on the nuclear localisation of ZO-1. Thus, cell type, differentiation state, and migratory circumstances may critically influence ZO-1 function in the nucleus. Our observation that ZO-1 shuttled to the nucleus in migrating cells following PKCε depletion suggests that PKCε may serve as a guardian of ZO-1 localisation and function. If the tight junction protein is released from cell-cell contact sites, PKCε phosphorylation labels ZO-1 for its α5β1-dependent migration-promoting function at the leading cell edge - possibly by the recruitment of accessory shuttle proteins. In contrast, in the absence of PKCε, ZO-1 is released from tight junctions, but redistributed to the nucleus, where it may influence EMT-inducing gene regulation in cooperation with certain transcription factors. In other words, ZO-1 may possibly contribute to the invasiveness of carcinoma cells directly or indirectly, depending on the presence or activity of PKCε, which itself depends on cellular and environmental cues.

SUMMARY AND CONCLUSION

At the beginning of this study, many questions were waiting to be answered. So we set out to explore novel regulatory mechanisms that urge malignant cells to roam. Cell migration is a highly complex process, which is carefully orchestrated in time and space, and which furthermore possesses great context- and cell type-dependency. In the course of this study, we succeeded to elucidate new, unconventional and unexpected signalling concepts, which broaden our basic knowledge of cell migration and invasion, and which may potentially help to discover novel therapeutic strategies in the future.

First, we identified a non-catalytic function of the Ras GAP protein p120 for the trafficking of integrins. By replacing the early endosomal Rab21 from the α-cytodomain of β1-integrins, p120 enables integrins to recycle back to the plasma membrane. The binding occurs via the GAP-domain of p120 and not through the canonical protein-protein interaction domains at its N-terminus. In addition, we showed that also Rab21 binds to the integrin tail in an unusual nucleotide-independent manner. Any intervention in this competitive mechanism results in imbalanced integrin trafficking and thus contributes to altered cell migration. We found that the loss of the tumour suppressor protein p120, as it occurs in many cancer types, may promote tumourigenesis not only by abolished control of mitogenic Ras signalling, but also by boosting the motility of cancer cells.
Second, we shed light on migratory strategies of cells in a 3D matrix environment. We hypothesise that the same growth factor receptor, c-Met, induces distinct modes of cell motility following different mechanisms of activation. Most solid carcinomas have evolved a number of ligand-dependent and -independent tactics to avoid termination of c-Met signalling. For future therapeutic intervention it might thus be important to consider that paracrine/autocrine HGF-mediated c-Met stimulation promotes collective cell invasion with mesenchymal characteristics, whereas c-Met over-expression triggers amoeboid-type single cell migration. Both migration modes differ in their executing molecular machineries and may hence require differential treatment.
Third, we discovered a molecular collaboration between two functionally divergent adhesion-associated molecules: integrins and the tight junction protein ZO-1. Following the breakdown of cell-cell contacts, these two proteins form a PKCε-dependent complex, which possesses migration-promoting capabilities. The α5β1–ZO-1 interaction may thus be fatal, when cancer cells lose their intercellular junctions in the course of EMT and set out to conquer new metastatic niches.

Together these findings add another sand grain of information to the vast beach of knowledge about cancer cell biology. However, with persistence and perseverance we may eventually accumulate a sufficient understanding in order to catch up with tumour cells - instead of chasing the footsteps of migrating cancer cells.

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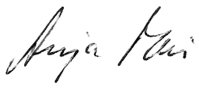

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