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ACTIVATION INDEPENDENT FUNCTIONS OF COLLAGEN RECEPTOR INTEGRINS α1β1 AND α2β1

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To my Integrin family

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Activation independent functions of collagen receptor integrins α1β1 and α2β1
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ABSTRACT

Cell adhesion to extracellular matrix (ECM) molecules, such as collagen, is mediated by the integrin family of cell surface receptors. Integrins are also exploited by several viruses during cell entry. Integrins literally integrate the ECM to the cytoskeleton by binding ECM ligands with their large head domain, and by connecting to the cytoskeleton through various focal adhesion proteins that bind integrin tails. Integrin tails recruit over 200 focal adhesion proteins to mediate cellular signaling events to control adhesion dependent cell growth and cell movement. Aberrant integrin signaling can lead to uncontrolled cell growth, which in turn can induce cancer and metastases.

The affinity of integrins to their ligands, as well as interactions between integrin tails and focal adhesion proteins, are controlled by integrin activity and clustering. Integrins undergo conformational activation from a bent to primed/extended and finally to a fully active conformation. Integrin priming facilitates ligand binding, and ligand interactions further induce full activation through the separation of integrin legs, promoting protein recruitment to focal adhesions and integrin clustering. The bent integrin conformation has been considered nonfunctional, since the ligand binding domain faces the cell membrane and ligand interactions are thus mostly prevented. However, in this thesis work echovirus 1 was shown to interact specifically with the bent conformation of α2β1-integrins (I). Furthermore, it was shown that under flow conditions platelet α2β1-integrins can bind to collagen without receptor priming (III). The recruitment of focal adhesion proteins and the initiation of integrin signaling have been thought to require integrin conformational activation. Here, using echovirus 1 as a model system, I demonstrate that clustered α2β1-integrins can mediate Focal Adhesion Kinase signaling without conformational activation of the integrin (II).

Integrins are controlled not only by regulating their conformational activity and clustering but also by influencing their expression levels and the pool on the cell surface. A fourth level of regulation occurs at focal adhesions where integrins are connected to the cytoskeleton. In my thesis I show that during cell spreading the reduced recruitment of the cytoskeleton protein vimentin to focal adhesions induces the formation of lamellipodia independently of α1β1-integrin conformational activation (IV). I also show that β1-integrin-mediated cell adhesion can be prevented without affecting the activity or surface expression of the integrin (V).

In conclusion, this thesis emphasizes the importance of the *nonactivated* integrin conformation in regulating cell adhesion, and demonstrates that integrin mediated functions are regulated at several levels.

Keywords: integrins, cell adhesion, cytoskeleton, focal adhesions

Maria Lyydia Salmela

α1β1- ja α2β1-kollageenireseptori-integriinien aktivaatiosta riippumaton toiminta
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TIIVISTELMÄ

Integriinit ovat solukalvon reseptoreita, jotka säätelevät solujen tarttumista soluja ympäröivään soluväliaineeseen, kuten kollageeniin. Myös useat virukset käyttävät integriinejä sitoutuakseen solun pintaan. Integriinien kookas solun ulkopuolinen osa sitoutuu soluväliaineen molekyyleihin eli ligandeihin, ja integriinihäntien ympärille muodostuva adheesiokompleksi yhdistää integriinit solun tukirankaan. Adheesiokompleksin sadat signalointiproteiinit välittävät integriinien signalointia solukalvon puolin ja toisin ohjaten sekä solujen kasvua oikeanlaisessa ympäristössä että tarvittaessa solujen liikettä uuteen kasvuympäristöön. Integriinien muuttunut signalointi johtaa mm. solujen liikakasvuun ja syöpäkasvaimien muodostumiseen.

Integriinien toimintaa säädellään integriinien aktivaatiolla ja klusteroimisella, eli muuttamalla integriinien muotoa taipuneesta ojentuneeksi ja tuomalla integriinit lähelle toisiaan. Suuret ligandit sitoutuvat helpommin integriinien ojentuneeseen muotoon. Integriini–ligandi vuorovaikutuksen seurauksena tapahtuva integriinien klusteroituminen ja integriinihäntien erottuminen mahdollistavat adheesiokompleksien muodostumisen. Ligandien ei uskota sitoutuvan integriinien taipuneeseen muotoon, jossa sitoutumiskohta on painautuneena vasten solukalvoa ja siten useimpien suurten ligandien ulottumattomissa. Tästä syystä taipuneen muodon ei myöskään uskota osallistuvan solusignalointiin. Väitöstutkimukseni kuitenkin osoittaa ECHO-virus 1:n kykenevän sitoutumaan α2β1-integriinin taipuneeseen muotoon ja klusteroimaan integriinit (I). Lisäksi ECHO-virus 1:ä hyödyntäen havainnollistan, että myös taipuneessa muodossa olevat α2β1-integriinit voivat aktivoida solusignalointia aktivoimalla fokaaliadheesiokinaasia (II). Osoitamme myös verihiutaleiden sitoutuvan verisuonten kollageeniin ilman verihiutaleen pinnalla olevien α2β1-integriinien aktivaatiota (III).

Integriinien ilmentyminen sekä pitoisuus solukalvolla vaikuttavat niiden aktivaation ja klusteroitumisen lisäksi integriinien toimintaan. Integriinien toimintaa voidaan säädellä myös muuttamalla niiden yhteyttä solun tukirankaan. Työssäni näytän, miten solujen leviämistä voidaan lisätä irrottamalla solutukirangan vimentiini-säikeiden yhteys adheesiokomplekseihin integriinien aktivaatiosta riippumattomasti (IV). Lisäksi osoitan solujen leviämisen voivan estyä muuttamatta integriinien aktiivisuutta tai pitoisuutta solukalvolla (V). Tutkimukseni painottaa integriinien taipuneen muodon merkitystä soluissa ja korostaa aktivaation ylitse ulottuvaa monipuolisuutta integriinien toiminnassa.

Avainsanat: integriini, soluadheesio, solutukiranka, fokaaliadheesiot

CONTENTS

ABBREVIATIONS

Amino acids

Acidic		
D	Asp	Aspartate
E	Glu	Glutamate
Basic		
R	Arg	Arginine
H	His	Histidine
K	Lys	Lysine
Polar		
S	Ser	Serine
T	Thr	Threonine
N	Asn	Asparagine
Q	Gln	Glutamine
Y	Tyr	Tyrosine
C	Cys	Cysteine
Nonpolar		
G	Gly	Glycine
A	Ala	Alanine
I	Ile	Isoleucine
L	Leu	Leucine
M	Met	Methionine
F	Phe	Phenylalanine
W	Trp	Tryptophan
V	Val	Valine
P	Pro	Proline

ADMIDAS	adjacent to MIDAS
αI	alpha inserted domain
βI	beta inserted domain
DHPCC-9	1,10-Dihydropyrrolo[2,3-a]carbazole-3-carbaldehyde
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF(R)	epidermal growth factor (receptor)
EV1	echovirus 1
FA	focal adhesion
FAK	focal adhesion kinase
FAT	Focal adhesion targeting domain
FERM	band 4.1, ezrin, radixin, moiesin -domain
ICAM	intercellular adhesion molecule 1
ILK	integrin linked kinase
KD	kinase domain
LDV	leucine-aspartate-valine
LIMBS	ligand-associated metal binding site
MIDAS	metal ion-dependent adhesion site
PDGF(R)	platelet derived growth factor (receptor)
PI	phosphatidylinositol
PI3K	phosphoinositide 3-kinase
PIM	proto-oncogene serine/threonine-protein kinase
PIP_2	phosphatidylinositol (4,5)-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
PKC	protein kinase C
PSI	plexin-semaphorin-integrin
PTEN	phosphatase and tensin homolog
RGD	arginine-glycine-aspartate
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
TGF-β	transforming growth factor beta
TPA	12-O-tetradecanoylphorbol-13-acetate (PMA)
VCAM	vascular cell adhesion protein 1
vWF	von Willebrand factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to according to their Roman numerals (I-V)

I. Jokinen, J., White, D.J., **Salmela, M.**, Huhtala, M., Käpylä, J., Sipilä, K., Puranen, J.S., Nissinen, L., Kankaanpää, P., Marjomäki, V., Hyypiä, T., Johnson, M., Heino J. Molecular mechanisms of α2β1 integrin interaction with human echovirus 1. 2010. The EMBO Journal, vol 29, 196–208.

II. **Salmela, M.**, Jokinen, J., Tiitta, S., Rappu, P., Cheng, R.H., Heino, J. Integrin α2β1 in nonactivated conformation can induce focal adhesion kinase signaling. Manuscript.

III. Nissinen, L., Koivunen, J., Käpylä, J., **Salmela, M.**, Nieminen, J., Jokinen, J., Sipilä, K., Pihlavisto, M., Pentikäinen, O.T., Marjamäki, A., Heino, J. Novel α2β1 integrin inhibitors reveal that integrin binding to collagen under shear stress conditions does not require receptor pre-activation. 2012. Journal of Biological Chemistry, vol 287, 44694–44702.

IV. **Salmela, M.**, Rappu, P., Lilja, J., Niskanen, H., Taipalus, E., Jokinen, J., Heino, J. Tumor Promoter PMA enhances kindlin-2 and decreases vimentin recruitment into cell adhesion sites. 2016. International Journal of Biochemistry and Cell Biology, vol 78, 22–30.

V. Santio, N., **Salmela, M.**, Arola, H., Eerola, S.K., Heino, J., Rainio, E-M., Koskinen, P. The PIM1 kinase promotes prostate cancer cell migration and adhesion via multiple signaling pathways. 2016. Experimental Cell Research, vol 342, 113–124.

1 INTRODUCTION

In multicellular organisms, tissues are formed when different types of cells attach to the extracellular matrix (ECM) surrounding the cells. Cell adhesion to the ECM and other cells is strictly regulated by different cell adhesion receptors. Integrins are the most important ECM binding receptors in multicellular animals (Hynes, 2002). The binding of integrins to the ECM and counter receptors on adjacent cells establishes adhesion dependent cell signaling that drives essential cellular functions from differentiation to migration. Since integrins are key players in many normal cellular functions, abnormalities in their behavior can be connected to various diseases.

Integrin mediated cell adhesion is regulated at several levels. The first level of regulation is the control of the transcription of the genes coding for integrin subunits. A common feature of cancer cells, which have escaped from the environmental control, is altered integrin expression on the cell surface. The second level of integrin regulation is the recycling of integrins to and from the cell membrane without changing their overall expression. This is important during cell migration, when cells constantly form and break adhesions, and can also be linked to the malignant and metastatic behavior of cancer cells (De Franceschi et al., 2015). In addition, several viruses use integrin trafficking mechanisms to invade cells (Hussein et al., 2015). The third way of influencing the interaction between integrins and ECM ligands is to regulate the conformation of integrins from a bent-inactive to an extended-active molecule (Hynes, 2002). The regulation of integrin activity is very important for directing the timing and place of platelet and immune cell binding, and aberrant regulation can lead to e.g. thrombosis. The final mode of regulating integrin adhesion occurs at cell adhesion complexes in the cytoplasm. These protein complexes transmit integrin signaling from the ECM to the nucleus, thereby changing gene expression and determining the long term response to environmental cues. Adhesion complexes also connect integrins to the cytoskeleton and control the movements of cells (Legate et al., 2009).

This thesis focuses on the function and signaling of the collagen receptor integrins $\alpha2\beta1$ and $\alpha1\beta1$. The importance of the conformational activation of integrins for the binding of platelets to collagen and for the binding of echovirus 1 to its receptor, $\alpha2\beta1$-integrin, are studied. In addition, cancer related cell spreading and migration are studied focusing on the regulation of interactions between integrins, cell adhesion complexes and the cytoskeleton.

2 REVIEW OF THE LITERATURE

2.1 Integrins

Integrins are cell surface adhesion receptors that anchor cells to the surrounding extracellular matrix (ECM), as well as to other cells. Through integrins, cells can sense the properties of the pericellular environment, and respond to it (Hynes, 2002). By expressing 24 different integrin heterodimers with different ligand binding abilities, cells can respond to various stimuli coming from their surroundings, and adapt to different environments. While the composition of the ECM regulates cellular functions, the ECM is also constantly modified by the cells. Cell-ECM interactions create a unique environment in each tissue that rises from the diversity of the cells, integrins and ECM molecules involved (Frantz et al., 2010). The ECM is thus as important in regulating cellular functions as are soluble factors, such as growth factors and cytokines (Hynes, 2009). In addition to its composition, also the rigidity of the ECM plays a role in regulating cellular functions. Integrins bind their ligands with their large extracellular domains and *integrate* the extracellular matrix all the way to the cytoskeleton and the nucleus through their short intracellular tails. By forming a link from the ECM to the cytoskeleton, integrins participate in mechanotransduction and sense the rigidity of the matrix, too (DuFort et al., 2011). Through proteins that bind integrin tails, integrins mediate cellular signaling and control cell survival, proliferation and differentiation. Connections between integrins and the cytoskeleton dictate cell spreading and migration in response to mechanical forces. Due to their ability to regulate cell adhesion and behavior, integrins are involved in tissue morphogenesis, wound healing, and leukocyte and platelet binding (Figure 1) (Legate et al., 2009).

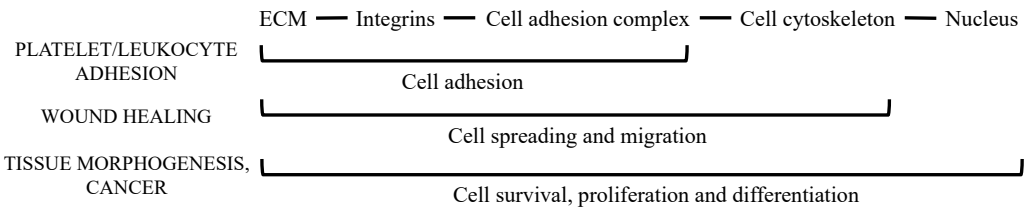

Figure 1. Integrin adhesions control the very basic functions of cells.

The interaction between an integrin and its ligand is strictly regulated and occurs through conformational changes in the integrin structure. Un-ligated integrins stay in their bent, inactive conformation until they are either primed for ligand binding from the inside of the cell, or interact with the ligand and the ligand induces their activation from the outside of the cell (Luo et al., 2007). Inside-out or outside-in activation leads to a firm integrin-ligand interaction and is followed by the recruitment of signaling proteins around the integrin tails. The recruited proteins form larger scaffolds called focal adhesions, where protein-protein interactions activate phosphorylation cascades that finally deliver the integrin dependent signals from the cell membrane to the nucleus (Legate et al., 2009). The details of integrin-ligand interactions, integrin conformational activation, protein binding to the tails of activated integrins, and the

formation of integrin signaling complexes, the focal adhesions, will be discussed in detail in the following chapters. Furthermore, integrin connections both with the ECM and the cytoskeleton enable mechanical sensing and transmission of forces through the integrins. These forces are important for cells to be able to spread and migrate. The role of integrins in mechanotransduction and cell migration will also be discussed.

2.1.1 Integrins recognize several ligands

The ECM is mainly composed of proteoglycans, which function as hydrating and buffering elements, and fibrous proteins such as collagen, fibronectin and laminin, which bring support and tensile strength to tissues (Frantz et al., 2010; Hynes, 2009). The ECM controls cell adhesion and proliferation through several cell adhesion molecules, of which the major receptors for the fibrous ECM proteins are integrins (Hynes, 2002). Integrins are heterodimers that consist of one α- and one β-subunit. In mammals, there are 18 different α-subunits and 8 different β-subunits that can form 24 different αβ-heterodimers (Figure 2). Integrins can be divided into four major groups according to their ligand binding preference: I) Collagen-binding integrins, II) LDV (leucine-aspartate-valine) -binding integrins, III) RGD (arginine-glycine-aspartate) -binding integrins, and IV) laminin binding integrins (Humphries et al., 2006).

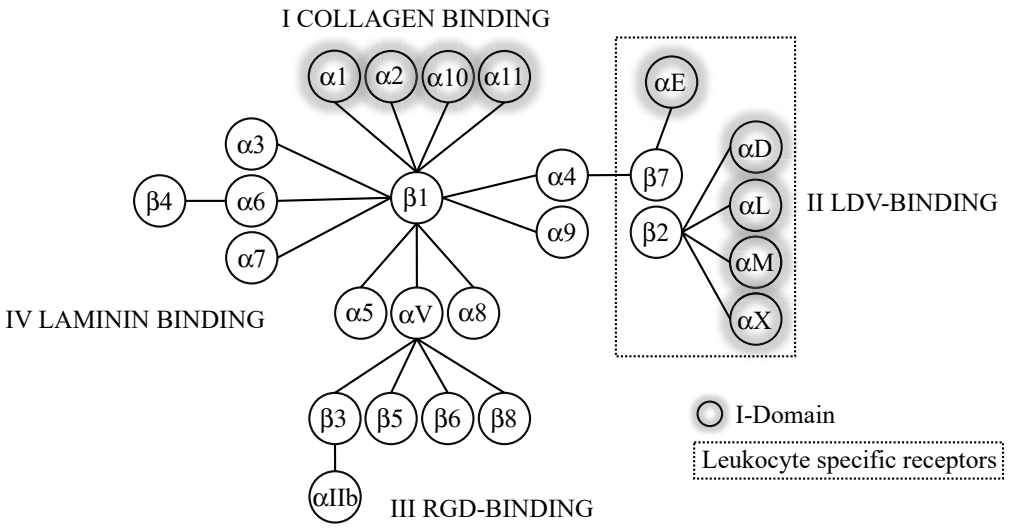

Figure 2. The integrin family. 24 integrin αβ-heterodimers are divided into four different groups based on their ligand binding specificities. Modified from Hynes, 2002.

The collagen binding integrins α1β1, α2β1, α10β1, and α11β1 belong to the group of I-domain containing integrins that have an extra ligand binding domain in their α-subunit. All of these integrins recognize the GFOGER motif in collagen triple helices (Heino 2014). Since collagen is the most abundant extracellular matrix protein in the human body, found in bone, cartilage, skin, and the basement membrane, these integrins have an essential role in maintaining tissue integrity. Collagen binding

integrins have other ligands too (see Table 1). α1-, α2- and α10- integrins also bind to laminins, and α2-integrin has several additional ligands.

The LDV-binding integrins include leukocyte specific αXβ2-, αMβ2-, αLβ2-, αDβ2- and αEβ7-integrins, as well as α4β1-, α4β7- and α9β1-integrins (Humphries et al., 2006). They recognize ligands that mediate cell-to-cell-contacts, such as ICAM, VCAM-1 and E-cadherin (Table 1). Like collagen binding integrins, αXβ2-, αMβ2-, αLβ2-, αDβ2- and αEβ7-integrins also possess the ligand binding I-domain in their α-subunit, whereas in α4β1-, α4β7- and α9β1-integrins the ligand binds to the interface between the α- and β-subunit. The integrin binding site in ligands that bind to the I-domain of leukocyte specific integrins is similar to the LDV-domain, which is why these integrins are grouped together (Humphries et al., 2006; Shimaoka et al., 2003).

Table 1. Integrin ligands (Humphries, Byron et al. 2006, Heino 2014)

Class	Heterodimer	Conventional ligands
I	α1β1	Collagen (IV, I, II, III, V, VI, IX, XVIII), laminin
I	α2β1	Collagen (I, II, III, V, IV, VIII, X, VII, IX, XVI, XXIII), laminin, osteopontin
I	α10β1	Collagen (I, II, III, IV, IV, VI, IX), laminin
I	α11β1	Collagen (I, II, IX)
II	αDβ2	ICAM, VCAM-1
II	αLβ2	ICAM
II	αMβ2	ICAM, fibrinogen
II	αXβ2	ICAM, fibrinogen, collagen
II	αEβ7	E-cadherin
II	α4β7	VCAM-1, osteopontin, fibronectin
II	α4β1	VCAM-1, osteopontin, fibronectin, thrombospondin
II	α9β1	VCAM-1, osteopontin, VEGF
III	α8β1	Fibronectin, osteopontin, vitronectin, LAP-TGF-β
III	α5β1	Fibronectin, osteopontin
III	αVβ1	Fibronectin, osteopontin, LAP-TGF-β
III	αVβ3	Fibronectin, osteopontin, vitronectin, LAP-TGF-β, thrombospondin, fibrinogen
III	αVβ5	Osteopontin, vitronectin, LAP-TGF-β
III	αVβ6	Fibronectin, osteopontin, LAP-TGF-β
III	αVβ8	Fibronectin, vitronectin, LAP-TGF-β
III	αIIbβ3	Fibronectin, osteopontin, vitronectin, fibrinogen
IV	α3β1	Laminin, thrombospondin
IV	α6β1	Laminin, VEGF
IV	α6β4	Laminin
IV	α7β1	Laminin

The RGD sequence was originally found in fibronectin (Pierschbacher and Ruoslahti, 1984) but is also the integrin binding site in several other proteins such as vitronectin, fibrinogen, vWF, laminin and thrombospondin (Ruoslahti, 1996). With such a wide set

of ligands, the RGD-binding integrins form the largest uniform group in the integrin family.

Laminins are one of the major components of the basement membrane, composing the lamina lucida part of basal lamina. Integrins α3β1, α6β1, α6β4, and α7β1 are classified as laminin binding integrins, but also the collagen binding integrins α1β1, α2β1 and α10β1 can bind to laminin. However, collagen binding integrins recognize a different binding site in the laminin structure. Laminin heterotrimers are composed of α-, β- and γ- chains, of which the α-chain is the binding site for integrins; laminin binding integrins bind the long arm at the C-terminal end, whereas collagen-binding integrins bind to the N-terminal short arm (Belkin and Stepp, 2000).

In addition to the extracellular matrix proteins of the host, several pathogens bind to integrins. Many viruses use integrins as their receptors on the cell surface and also exploit the internalization mechanisms of integrins to enter cells, where they can start replicating. The RGD-motif is a common motif in the protein capsid of viruses that can be recognized by integrins, mainly by αVβ3. Non-RGD binding integrins, however, can also act as receptors for viruses. A recent review by Hussein and colleagues (Hussein et al., 2015) summarizes the integrin binding viruses. Among these are HIV-1 and the Ebola virus, emphasizing the role of integrins in antiviral therapies.

2.1.2 Integrin structure

The integrin heterodimer is mostly extracellular with a large integrin head domain pointed towards the ECM, thus enabling interactions between the integrin and its ligands. Integrins can be divided into two groups based on the structure of their head domain: I-domain containing integrins and integrins without the I-domain (Figure 3). In I-domain integrins (left in the Figure 3), the ligand binding site is in the extra I-domain at the N-terminal end of the α-subunit. I-domain sequences are relatively similar, and the ligand binding mechanism is identical for all I-domain integrins. Integrins that do not have an I-domain bind their ligand with the interface between the β-I-domain and the β-propeller in the α-subunit. The β-I-like domain is also a regulatory domain in I-domain integrins. The metal ion-dependent adhesion site (MIDAS) in the α-I-domain and a similar site in the β-I-domain are the ligand binding sites. The 7th α-helix plays a central role in the conformational regulation of both the α- and β-I-domains (Arnaout et al., 2005).

The transmembrane domains in α- and β-subunit are single α-helixes. The tails can interact and modify the conformation of integrin by forming salt bridges with each other. The cytoplasmic tails of integrins are very short, the α-cytoplasmic tail (15-58 amino acid residues) being shorter than the β-cytoplasmic tail (46-64 amino acid residues), which is the major binding site for intracellular integrin binding partners. The β4-tail is an exception with its 1072 amino acid residues. The tails are important for the interactions between integrins and their intracellular binding partners. These interactions regulate the conformational activation of integrins (inside-out signaling) and integrin mediated (outside-in) signaling (Campbell and Humphries, 2011).

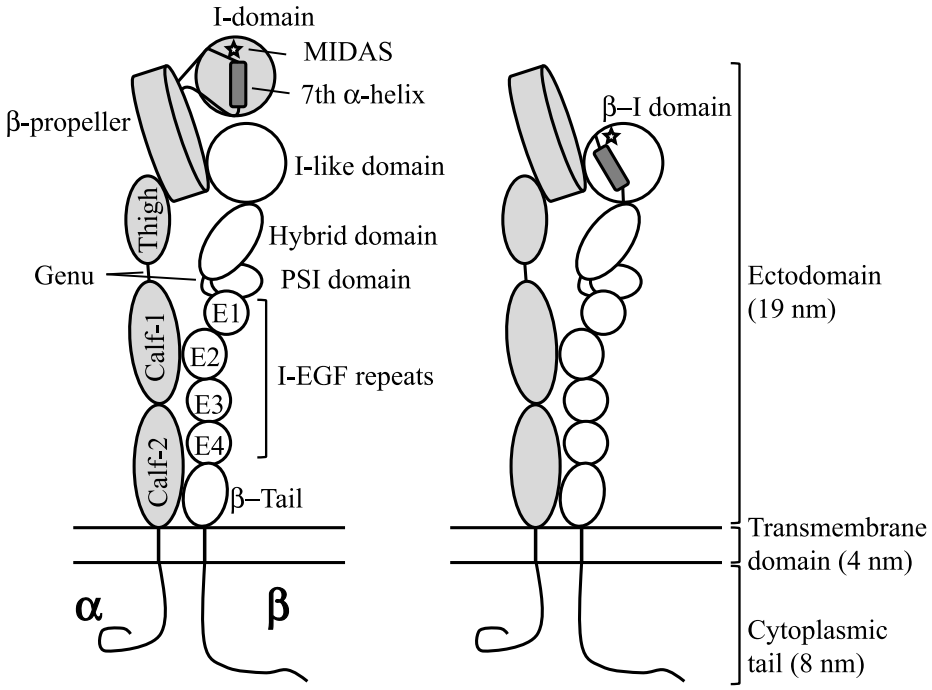

Figure 3. The structure of the I-domain containing integrins (left), and non-I-domain integrins (right). The α-subunit is composed of the immunoglobulin -like calf 1, calf 2 and thigh domains, with the genu, knee, in between to provide a site for integrin bending. The β-propeller is linked to the thigh in the α-subunit and the I-like domain in β-subunit. In I-domain integrins the β-propeller connects to the extra I-domain. The β-subunit is made up of 8 domains: the β-I-domain, hybrid domain, Plexin-semaphorin-integrin (PSI) domain, cysteine rich epidermal growth factor repeats (I-EGF), and the β-tail domain. The transmembrane domains of both subunits are single α-helices. The cytoplasmic tails of integrins are short and unfolded structures. The I-domain MIDAS and the 7th α-helix are important for ligand interactions. Adapted from Campbell and Humphries, 2011; Gahmberg et al., 2009.

2.1.3 Ligand interaction with the integrin I-domain induces integrin outside-in activation

Integrins are thought to fluctuate between their inactive and active states. In the inactive, low affinity state, the I-domain is in its closed conformation, integrin is bent, and the ligand binding site is facing the cell membrane. Integrin priming extends the heterodimer to the intermediate affinity receptor. In the active state the I-domain is in its open conformation, the integrin is fully extended, and the integrin legs are separated (Figure 4). The conformation of the integrin, and thus activity, can be regulated by interactions between integrins and ligands (outside-in signaling) and between integrin tails and signaling proteins (inside-out signaling) (Hynes, 2002).

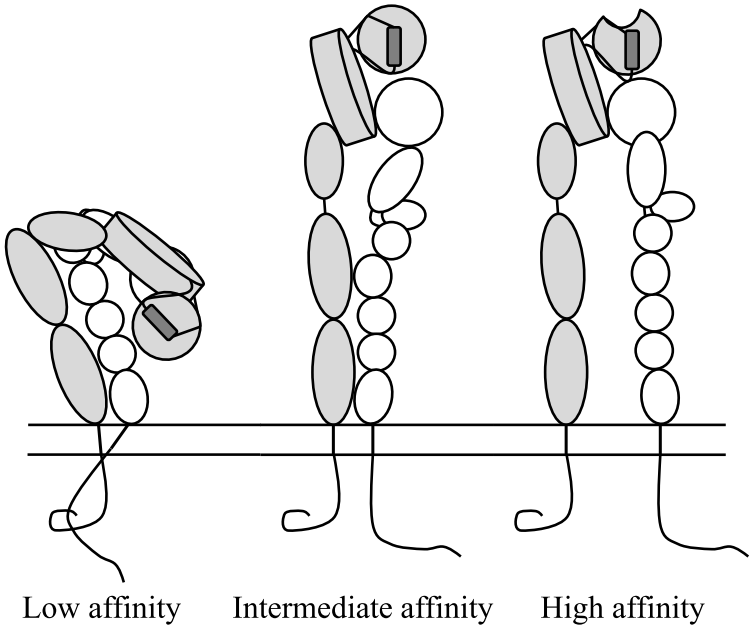

Figure 4. Integrin activity is regulated through conformational changes in structure. Adapted from Gahmberg et al., 2009; Hynes, 2002.

Ligand interactions with the I-domain

The integrin binding site in several ligands is in a loop structure that protrudes from the relatively large main molecule (Springer and Wang, 2004). A common feature of the integrin binding site is an acidic amino acid residue, such as glutamate or aspartate, which can interact with the MIDAS site in the I-domain. The interaction between the ligand and the ligand binding site has been extensively studied in the I-domain integrins α2, αL and αM. All of them show a similar mechanism in ligand binding and conformational opening of the I-domain (Liddington, 2014). A glutamate residue from the collagen triple helix or another acidic residue from different ligands changes the metal ion coordination in the MIDAS motif triggering large conformational changes in the I-domain. An α7-helix in the I-domain moves 10 Å downwards changing the interactions between the I-domain, the β-propeller and the β-I-domain. In all I-domain integrins, the last four residues of the α7-helix are conserved, and most importantly, they all possess an acidic glutamate as the second to last amino acid residue (310 in αL, E335 in α1, E336 in α2, and E320 in αM). This residue was proposed to function as an intrinsic ligand for similar MIDAS site in the β-I-domain (Alonso et al., 2002) and such an interaction was later confirmed (Yang et al., 2004). Although some integrins lack the ligand binding I-domain in their α-subunit, all integrins are activated in a very similar manner, because also the I-domain integrins need the β-I-domain for full activation. Integrins that do not have the I-domain in their α-subunit bind the ligand directly with their β-I-domain. The ligand interaction occurs through the β-MIDAS site and is assisted by Adjacent to MIDAS (ADMIDAS) and the ligand-

associated metal binding site (LIMBS) (Springer and Wang, 2004). Also in the β-MIDAS, the movement of the α7-helix triggers the opening of the β-I-domain, although here the α7-helix is rotated rather than shifted downwards (Liddington, 2014). In both types of integrins, those with and those without the α-I-domain, opening of the β-I-domain results in 40 to 60 degree swing of the β-hybrid domain in relation to the β-I-domain. As a result, the legs of the α-and β-subunits are separated and the integrin tails move further apart (Figure 4) (Arnaout et al., 2005; Liddington, 2014).

Integrin activation state can be modified by mutations

Mutations in the essential glutamates αL-E310 (Huth et al., 2000), α1-E335 (Lahti et al., 2011), α2-E336 (Connors et al., 2007) and αM-E320 (Alonso et al., 2002) abolish the collagen binding functions of these integrins. These mutations are thought to inactivate the integrins by preventing the interaction of the α7-helix with the β-I-domain and concomitantly prevent the conformational activation of the integrin. The balance in the integrin activation state can also be changed to favor the active conformation. Collagen binding integrins have an extra αC-helix at the collagen binding cleft that participates in ligand recognition (Emsley et al., 1997). At least the partial activation of integrins can be triggered by mutating the amino acid E318 in the α2-I-domain and E317 in α1-I-domain. The glutamate 318 in the α2-integrin α7-helix forms a salt bridge with arginine 288 in the αC-helix, holding the αC-helix in the "closed" conformation. An interaction with collagen and the following reorganization of metal coordination triggers the unwinding of the αC-helix (Emsley et al., 2000). After breaking the salt bridge by introducing the mutation E318W, the α2I-domain stays in the open conformation that is more easily accessible by collagen (Aquilina et al., 2002). The specificity of ligand binding is, however, somewhat lost (Tulla et al., 2008). A similar mutation in the α1I-domain, E317A, also induces the binding of the I-domain to collagen. However, relatively small structural rearrangements in the position of α7-helix were detected in the mutated α2- (E318W) and α1-(E317A) I-domains that were not bound to a ligand (Lahti et al., 2011; and our unpublished data), indicating that unwinding of the α-C-helix, rather than the shift in α7-helix position causes the increased activity in the mutated I-domains, and that ligand binding is still required for the full integrin activity.

2.1.4 Protein interactions with integrin tails induce integrin inside-out activation

Ligand binding to the integrin I-domain induces the conformational activation of the integrin by pushing the α- and β-legs apart, as was described in the previous chapter. Ligand induced integrin activation is termed outside-in activation, or outside-in signaling. However, also the interactions between proteins and integrin tails can affect the conformational activation of integrins. This would be referred to as inside-out activation. Inside-out activation also conformationally primes integrins for ligand binding, assisting especially in the binding of large ligands to the integrin ligand binding site, which can otherwise be buried in the cell membrane and be sterically unavailable (see Figure 4).

Transmembrane helices and tails keep integrin legs together

Although the transmembrane domains of integrins are short single helices, and the tails are short and unstructured, they have an important role in controlling the conformation of integrins. Interactions between α-and β-transmembrane domains and -tails are thought to keep the integrin legs together and these interactions must be broken to extend the conformation of the integrin. The salt bridge between the conserved α-tail arginine (R) and the conserved β-tail aspartate (D) were shown to be sufficient to hold the integrin in the resting state. The α-tail arginine is conserved in all α-subunits except α10, and the β-tail aspartate is conserved in all other subunits except β8. In addition to the R-D salt bridge, weak interactions keep the two transmembrane helices packed closely together, the α-tail straight perpendicular to the cell membrane, and the β-tail tilted (Ginsberg et al., 2005; Liddington, 2014). The conserved arginine in the α-tail is part of the α-tail hot spot sequence, GFFKR, where several α-tail binding proteins bind. If this area interacts with the β-tail, the binding site is hidden, preventing interactions. One of the β-tail protein binding hot spot areas, the HDR motif, contains the conserved aspartate participating in the formation of the salt bridge. This area is also buried in the membrane when the β-tail is tilted, and thus inaccessible for proteins (Figure 5)(Legate & Fassler, 2009).

Talin binding induces integrin priming

The salt bridge between the two tails can be broken when cytosolic proteins bind the integrin tails. The first, most studied, and also the most important such protein is talin. Talin is an integrin activating protein, which by binding to integrin β-tails initializes integrin inside-out signaling and primes integrins for ligand binding (Tadokoro et al., 2003). Talin can activate a single integrin molecule and increase its ligand binding affinity (Ye et al., 2013). It is assumed that both talin-1 and -2 activate all integrin heterodimers since the important binding domains are highly conserved in both isoforms (Anthis et al., 2009). The importance of talin in integrin activation has been shown with β3-integrins, the non-I-domain α5β1-integrin, and the I-domain-containing leukocyte αLβ2-integrin (Kim et al., 2003; Tadokoro et al., 2003), supporting that talin is important in cell adhesion to the ECM as well as for the adhesion of circulating blood cells (Theodosiou et al., 2016). Talin binds to a second hot spot area in the β-tails, the NPxY-membrane proximal domain that is not involved in salt bridge formation and tail-tail interactions. When talin binds to the β-tail NPxY-domain of the integrin, talin competes with a conserved α-tail arginine (R) for electrostatic interactions with the conserved aspartate (D) in the β-tail. This breaks the salt bridge that stabilizes the inactive conformation and allows integrin activation (Anthis et al., 2009). Talin must bind simultaneously to the β-transmembrane domain and to the inner plasma membrane to cause the "untilting" of the integrin transmembrane domain, separation of the α- and β-legs, and integrin activation (Anthis et al., 2009; Calderwood et al., 2013; Kim et al., 2012). The α-tail membrane distal domain (NxxY) outside the conserved GFFKR is also essential for talin mediated integrin activity (Liu et al., 2015a). See also Figure 5.

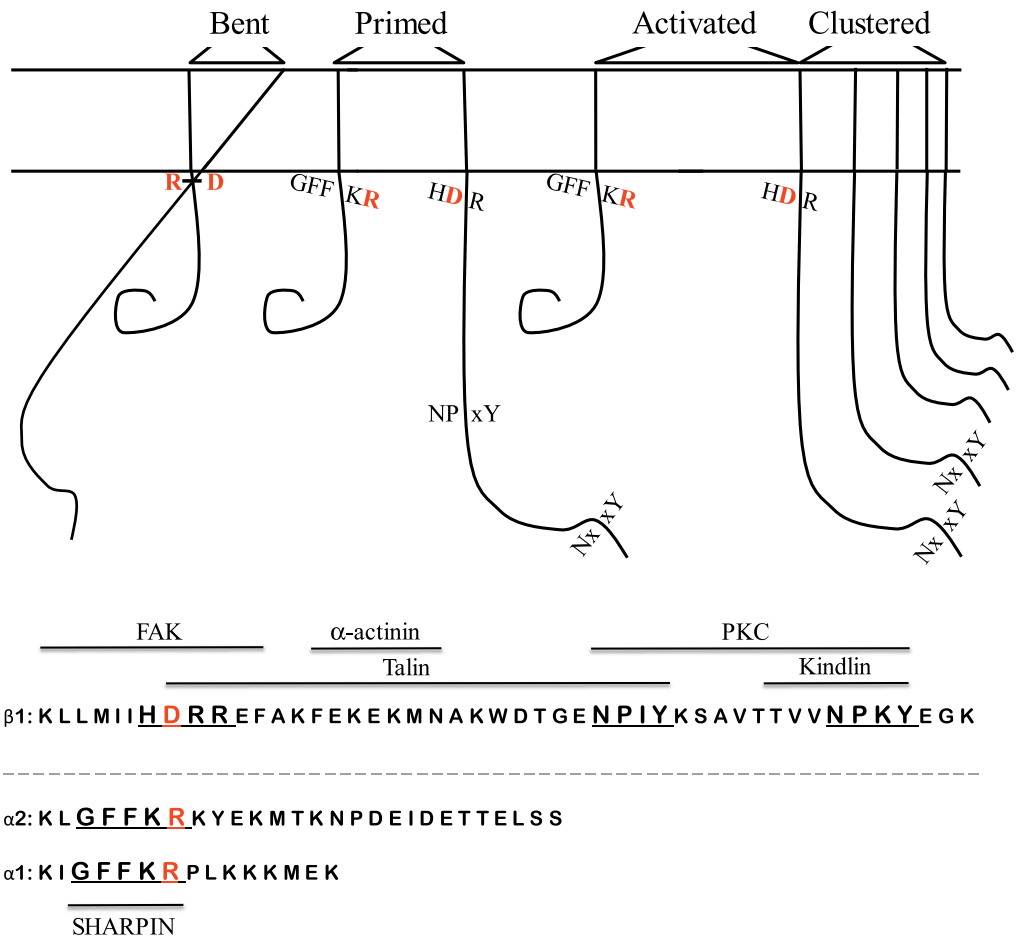

Figure 5. Protein interactions with integrin tails. The R-D salt bridge between the α- and β-tails is broken after talin binds to the integrin β-tail and the cell membrane. The β-HDR and α-GFFKR motifs are at the interface of the cell membrane and cytosol, half buried in the membrane. Protein access to these motifs is facilitated by the talin induced separation of integrin tails and by the untilting of the β-tail that exposes more amino acid residues for binding. The HDR-motif, membrane proximal NPxY-motif, and the membrane distal NxxY-motif in the β-subunits, and GFFKR-motif in the α-subunits are conserved in all integrin subtypes. These are the main protein binding sites in integrin tails.

Kindlins are integrin co-activators

The talin accompanying protein kindlin (kindlin-1, -2 and -3) is important for integrin inside-out and outside-in signaling. Several early papers showed that kindlins are important in activating integrins, similarly to talins. However, recent data suggests that kindlins are more important for integrin clustering. Kindlin-2 binds to the distal NxxY-motif that does not overlap with talin binding site (Montanez et al., 2008). Kindlin-2 was shown to be important for integrin activation in combination with talin (Montanez et al., 2008). Knocking out kindlin-2 prevents cell adhesion, the formation of focal

adhesions and the binding of antibodies that recognize the active integrin conformation (9EG7, WOW-1 and HUTS-4) (Askari et al., 2010; Montanez et al., 2008; Qu et al., 2011). A study with integrins attached on a nanodisc, an artificial lipid bilayer, showed that purified recombinant kindlin-3 could not activate monomeric integrins similarly to the talin head domain, but it could boost talin-mediated activation. Kindlin-3 also failed to induce the tilting of the integrin transmembrane domain the way talin did (Kim et al., 2012). In contrast, the binding of a multivalent ligand was markedly increased by the simultaneous overexpression of both talin and kindlin-1. In addition, silencing kindlin-2 prevented binding of a multivalent ligand, but not of a monovalent ligand. Kindlins could also promote the clustering of ligand bound integrins (Ye et al., 2013). These data show that kindlins are required for cell spreading and full talin-mediated integrin activation, but alone they probably cannot mediate the conformational activation of integrins.

Integrin inactivating proteins

Integrin tails are also recognized by inactivating proteins. Some of these proteins block the binding of the activating proteins talin and kindlin, some stabilize the bent conformation, and some reduce the amount of surface integrins by inducing trafficking. ICAP (integrin cytoplasmic domain associated protein 1), DOK1 (docking protein 1), DAB1 and DAB2 (disabled homolog 1 and 2), numb, and filamin are inhibitory proteins that compete with talin (Pouwels et al., 2012). Filamin also stabilizes the integrin α- and β-tail clasp and supports the inactive conformation (Liu et al., 2015b). PKD1 (protein kinase D1) and PKCα (protein kinase C alpha), on the other hand, regulate integrin activity by inducing receptor internalization (Pouwels et al., 2012). The recently found SHARPIN is an integrin inactivating protein that binds to the α-tail and blocks the binding of talin and kindlin, and might also stabilize the inactive conformation (Pouwels et al., 2012; Rantala et al., 2011a). Other α-tail binding inactivating proteins are Nischarin, CIB1 (calcium- and integrin-binding protein 1), MDGI (mammary-derived growth inhibitor), Rab21, P120RasGAP and GIPC1 (GAIP-interacting protein C terminus, member 1), but their functions are not well known (Pouwels et al., 2012).

Protein interactions with integrin α– and β–tails

Integrin β-tails are bound by several cytosolic proteins that either regulate the integrin activity (activate or inactivate), recruit other proteins to the proximity of integrin tails and function as scaffolding proteins, or activate other signaling molecules. Most proteins bind to hot spot areas that are conserved in all β-subunits (Figure 5) (Legate and Fassler, 2009). α-tail binding proteins are generally fewer in number. These proteins often recognize the GFFK sequence, which is the only conserved sequence in all α-tails. Since α-tails are less conserved than β–tails, α-tails can recruit proteins that regulate specific integrin functions (Morse et al., 2014). The collagen binding integrins α2 and α1, for example, mediate opposite signaling effects in cells (White et al., 2004). A subgroup of α-tails has a highly conserved Yxxϕ-sequence that regulates integrin endocytosis through the clathrin mediated

endocytic route (De Franceschi et al., 2016). Interestingly, the α2-tail has this sequence, whereas α1 does not.

As Figure 5 reveals, most integrin tail binding proteins bind to the same conserved sequences, or their binding sites overlap (Fagerholm et al., 2004; Liu et al., 2000; Morse et al., 2014; Pouwels et al., 2012). However, integrin signaling requires the co-operation of as many as 200 proteins. It is obvious that a single integrin cannot bind all of these proteins simultaneously. In addition, all of the proteins related to integrin signaling do not directly bind to integrin tails. Instead, when in clusters, several integrins can participate in interactions with cytosolic signaling proteins, and signaling proteins also bind and recruit other proteins. When viewed with a confocal microscope cells show densely packed integrin spots at the ends of actin stress fibers. These spots, focal adhesions, gather the integrin signaling proteins together and transmit integrin signals to cells.

2.1.5 Integrin clustering enhances ligand binding avidity

An interaction of an integrin with its ligand, the binding affinity, is relatively weak. However, the binding avidity is increased by recruiting several integrins close to each other to clusters where multiple weak interactions can mediate firm binding together. The clustering of integrins is not only important for stronger adhesion, but also for integrin signaling events (Legate et al., 2009). The threshold for integrin heterodimers to be able to form a signaling cluster (focal adhesions are discussed later) is four integrins at a 60 nm distance from each other (Schvartzman et al., 2011).

It is not clear whether integrin clustering is induced by a multivalent ligand, or if the binding and activation of one or a couple of integrins induce the recruitment of more integrins in a ligand independent manner. It is possible that, by inducing integrin priming and tail separation, inside-out signaling frees the tails for tail-tail interactions and enables microclustering (Iwamoto and Calderwood, 2015; Kim et al., 2004). Integrin binding to mobile RGD-particles showed that the initial binding triggers the lateral movement of integrins into clusters (Yu et al., 2011). Furthermore, proteins like kindlin have been shown to induce integrin clustering after ligand binding (Ye et al., 2013) so ligand multivalency is not necessary for promoting clustering. However, clustering by a multivalent ligand is nonetheless feasible, since according to molecular modelling, in type I collagen fibrils the α2β1-integrin binding sites can be close enough to cluster integrins. More importantly, according to the model, integrin tails (or proteins bound to them) can interact with each other (model by Santeri Puranen in Jokinen J, 2010, Annales Universitatis Turkuensis AI407). At least in two dimensional cell cultures, the formation of focal adhesions must be partly promoted by ligand independent integrin clustering and recruitment, since it is difficult to believe that ECM proteins in tissues and especially in cell culture would be precisely organized to induce the correct organization of adhesions. Probably clustering is induced both from the inside and the outside of the cells.

In addition to ligand induced clustering and clustering following integrin activation, integrin interactions with the ligand and cellular factors can be regulated by integrin compartmentalization at the membrane. Membrane order is higher around focal adhesions and partly dependent on integrin adhesion (Gaus et al., 2006). However, also inactive integrins localize close to these areas and their diffusion is restricted (Fiore et al., 2015). The cytoskeleton, especially the actin cortex right below the cell membrane, and the glycosyl-phosphatidylinositol-anchored proteins at the cell membrane, regulate the spacing of membrane proteins and also affect integrin mediated events (Köster and Mayor, 2016). Recently, another concept of regulated integrin rich areas have also arisen, namely the cell surface glycoproteins, which have been shown to regulate integrin clustering and ligand binding. These proteins are big, outweighing integrins in size and access to the ECM. In the presence of glycoproteins, integrins cannot reach and bind to ligands. However, glycoproteins can promote integrin clustering, ECM binding and the formation of focal adhesions by forming "kinetic traps" where pre-existing adhesion brings the ECM closer to cell surface and facilitates integrin binding (Paszek et al., 2014).

2.1.6 Echovirus 1 uses α2β1-integrin in the cell entry process

In addition to ECM proteins, integrins serve as cellular receptors for about 20 different viruses and also other pathogens. Integrins are present in every mammalian cell type, and can be internalized through several routes, making this receptor type useful for viruses (Hussein et al., 2015). Attachment to the cell surface is the first step in virus internalization. Several cell surface receptors can mediate virus binding and bring viruses close to the cell surface. Glycosaminoglycans and integrins are widely expressed and provide an easy target for viruses. After binding to their receptors, viruses can be internalized with the receptor by different pathways, such as clathrin-mediated and caveolar/lipid raft mediated endocytosis or micropinocytosis. These pinocytosis routes serve cells in nutrient uptake, and are constantly in use (Mercer et al., 2010). They are also involved in integrin trafficking during cell migration, focal adhesion turnover and matrix remodeling (De Franceschi et al., 2015).

The collagen receptor α2β1-integrin is the only cellular receptor for human echovirus 1 (EV1) (Bergelson et al., 1992). The EV1 binding site in the α2I-domain is different from the binding site of the ECM molecules (King et al., 1997). While collagen occupies the ligand binding MIDAS site, EV1 interacts with larger area of the I-domain and the MIDAS site is not involved in binding, although it faces the EV1 cleft. According to molecular modelling, collagen and EV1 should not be able to bind to the I-domain simultaneously because collagen peptide blocks the interaction between EV1 and the I-domain (Xing et al., 2004).

Several integrin binding viruses have an RGD peptide in their nucleo capsids that enables the binding to their integrin receptors in a mechanism similar to the binding of fibronectin (Hussein et al., 2015). However, the binding of EV1 and collagen to the α2I-domain differ remarkably. Whereas collagen binding to MIDAS requires a magnesium-ion to coordinate the binding, EV1 is not dependent on divalent cations

and can even bind to the I-domain in the presence of the chelating agent EDTA (ethylenediaminetetraacetic acid). The binding of all known integrin ligands is prevented by EDTA (Xie et al., 2004). Although EV1 specifically binds to α2-integrin, it is not dependent on the α-subunit during later events in virus entry, since EV1 could infect the cells even when the α2-tail was swapped or deleted (Bergelson et al., 1993). EV1 binding to α2-integrins triggers integrin clustering (Xing et al., 2004), that is followed by the internalization of the virus with the receptor in a caveolin-1 dependent manner (Marjomäki et al., 2002). Since the α2-tail is not important for virus entry, interactions between the β-tail and cytosolic proteins mediate the internalization. The α2-tail was recently shown to include a conserved Yxxϕ–motif that directs these integrins to clathrin-mediated endocytosis events (De Franceschi et al., 2016). It would be interesting to know how EV1 affects normal α2-integrin biology when triggering an alternative endocytic route. EV1 entry requires the activation of PKCα, Rac1 and Pak1 and occurs though a macropinocytosis like entry process (Karjalainen et al., 2008; Upla et al., 2004).

2.2 Integrin adhesions mediate integrin signaling and control cell attachment and movement

2.2.1 Integrin mediated cell adhesions

As described in the previous chapters, integrins can bind intracellular signaling molecules after the conformational activation of integrins and the following separation of the α- and β-tails. Integrin clustering leads to the formation of integrin dense areas, focal adhesions, where the proteins that are bound to integrins can also interact with each other and form scaffolds for other proteins to bind and interact.

Mature focal adhesions are preceded by nascent adhesions, that either disperse or develop into focal complexes and then into focal adhesions. Fibrillary adhesions develop on fibronectin surfaces and contribute to matrix remodeling. The principle of all of these structures is the same, to mediate integrin signaling after integrin ligand binding. The composition and the rate of protein recruitment, however, are different (Parsons et al., 2010). In addition, different integrin heterodimers and ECM molecules have been shown to recruit different components of adhesion proteins (Humphries et al., 2015). Adhesion sites are also the connection points for integrins and cytoskeletal proteins, such as actin. Classical images of cells show fibers of actin crossing the cell and ending in integrin rich bright dots at the edges of the cell (Figure 6). Although focal adhesions are clearly visible on cells cultured on 2D surfaces, the existence of similar structures in 3D cell cultures or tissues is still controversial. Some researchers have been able to show punctuate adhesions including integrins and other adhesion proteins, but the imaging of these structures and composition of artificial matrices complicates the research (Harunaga and Yamada, 2011).

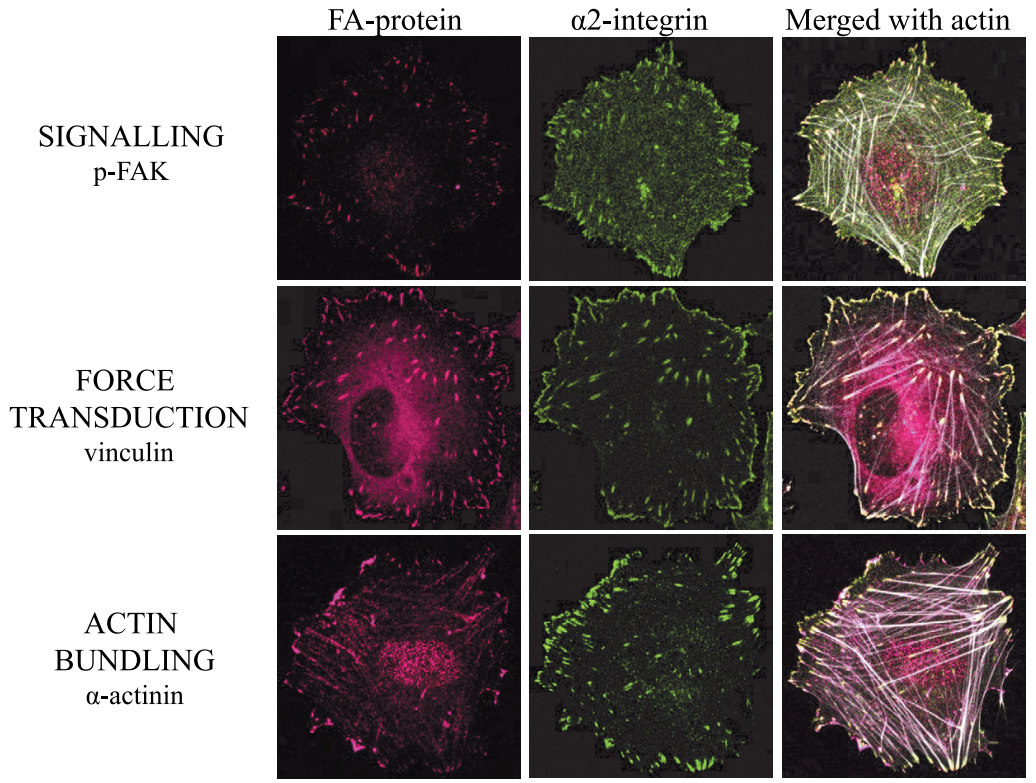

Figure 6. Focal adhesions. Integrins and signaling proteins such as FAK, and force transducing proteins such as vinculin, colocalize in focal adhesions. Actin bundles are connected to adhesions and stabilized by α-actinin. α-actinin can also be found in focal adhesions. Microscopy images by Maria Salmela.

The structure of focal adhesions

The integrin adhesome consists of around 150 focal adhesion proteins and almost 100 focal adhesion associated proteins (Winograd-Katz et al., 2014). These proteins serve as scaffolding proteins, they phosphorylate other recruited proteins, or they might be part of the cytoskeleton. The integrin adhesome project divides the proteins into 18 classes, of which the most abundant are 1) actin regulators (e.g. α-actinin, filamin), 2) adaptors (e.g. actin, kindlins, talin, vinculin, paxillin) and 3) adhesion receptors (e.g. integrins, syndecan) (Winograd-Katz et al., 2014). A very elegant model of the structure of focal adhesions was presented by the Waterman lab. They used super resolution imaging as a tool to measure the distance of each protein from the cell membrane and constructed a model of the layers of the focal adhesions (Kanchanawong et al., 2010). The first layer is the adhesion receptor integrins, the second is the signaling layer with molecules such as FAK and paxillin, the third layer is involved in force transduction and composed of vinculin and talin, the fourth layer is the actin regulatory layer with zyxin and VASP (vasodilator stimulated protein) and finally, the fifth layer is the actin bundles connected with α-actinin (Figure 7)(Liu et al., 2015c).

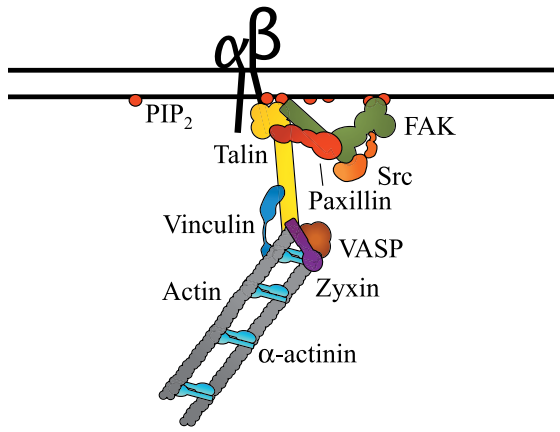

Figure 7. The structure of focal adhesion layers. Cell adhesion: Integrins. Signaling molecules: FAK, paxillin, Src, PIP_2. Force transduction layer: vinculin, talin. Actin regulatory layer: zyxin, VASP. Actin stabilization: α-actinin. Adapted from Kanchanawong et al., 2010.

Phosphatidylinositols PIP_2 and PIP_3

Phosphatidylinositols are involved in the spatiotemporal regulation of signaling at the cell membrane and thus are important in regulating the assembly of focal adhesions. The local concentrations of PIP_2 (phosphatidylinositol-4,5-biphosphate; $PI(4,5)P_2$) and the related molecule PIP_3 (phosphatidylinositol-3,4,5-triphosphate; $PI(3,4,5)P_3$) are increased after integrin mediated cell adhesion to the ECM. Integrin clustering induces the talin-dependent recruitment of Type I phosphatidylinositol phosphate kinase gamma (PIPKIγ) to focal adhesions, where it generates PIP_2 from the precursor PI (phosphatidylinositol) (Ling et al., 2002). PIP_2 and PIP_3 are required for maintaining cell polarity, e.g. during directional cell migration. They also regulate the polarized trafficking of signaling molecules (Thapa and Anderson, 2012). A local increase in PIP_2 recruits cytosolic proteins close to the membrane inducing their clustering and activation, thus regulating the assembly and disassembly of focal adhesions. PIP_2 has been shown to recruit talin and FAK (Goni et al., 2014; Legate et al., 2011), and both of these FA proteins also induce the synthesis of PIP_2 (Ling et al., 2002).

2.2.2 FAK orchestrates integrin adhesions and downstream signaling

The activation of Focal Adhesion Kinase 1 (FAK) is considered a hallmark for ECM mediated signaling, induced by integrin ligand binding, conformational activation and clustering. In addition to integrins, other receptors such as growth factor receptors, also activate FAK. FAK is an important regulator for both the assembly and disassembly of focal adhesions. FAK activation is required for the formation of focal adhesions and cell spreading, but prolonged FAK activity, on the other hand, induces the disassembly of focal adhesions (Mitra et al., 2005). A regulatory role similar to the one talin has in integrin inside-out signaling was also suggested for FAK (Hynes, 2002). Later reports, however, established a role for FAK both as a scaffolding protein that recruits other focal adhesion proteins and as a kinase that activates cellular signaling by

phosphorylating nearby proteins. Interestingly, a recent study suggests that FAK's scaffolding function is more important than its kinase function in the formation of focal adhesions and during cell spreading (Horton et al., 2016).

Interestingly, FAK signaling is not restricted to cell membranes, but it can continue in endosomes. Endosomes contain recycled integrins that can stay in their activated conformation and keep signaling through FAK, regulating cell anoikis (Alanko et al., 2015). FAK has a nuclear localization sequence and can thus be targeted to nucleus (Lim et al., 2008; Serrels et al., 2015). A role for FAK as a regulator of the transcription of inflammatory chemokines and cytokines was described recently (Serrels et al., 2015).

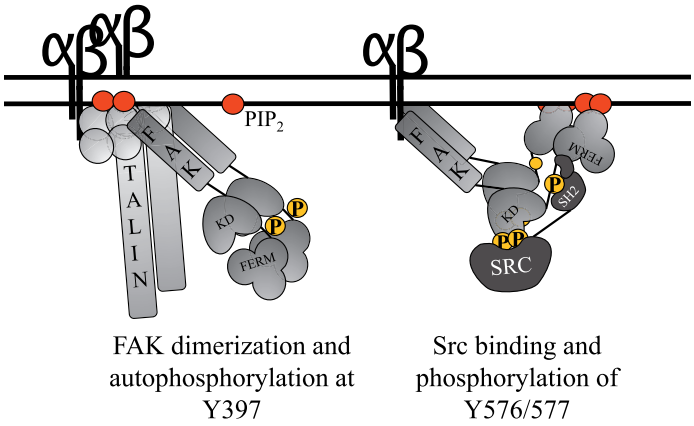

Figure 8. FAK activation. FAK autophosphorylation occurs through integrin mediated FAK dimerization, or clustering. Binding of the FAK FERM-domain to PIP_2 on the cell membrane enables Src to interact with pY397 and Src-mediated phosphorylation of Y576/Y577 sites. Adapted from Seong et al., 2013.

FAK activation requires dimerization and autophosphorylation

Dimerization and conformational alterations of the molecule are essential for FAK activation (Brami-Cherrier et al., 2014). FAK concentrations in cells are too low to promote spontaneous FAK dimerization, thus the recruitment of several FAK molecules to specific sites is required (Brami-Cherrier et al., 2014). Integrin clustering promotes the recruitment of FAK to focal adhesions, FAK dimerization and FAK activation. What brings FAK to close proximity of integrins is still being discussed. Researchers have suggested that FAK can directly bind to integrin β-tails (Schaller et al., 1995a), FAK is recruited by talin that is bound to clustered integrins (Frame et al., 2010), FAK FAT domain interacts first with paxillin (Brami-Cherrier et al., 2014), or local high concentrations of membrane bound phosphatidylinositol PIP_2 recruit FAK molecules to the cell membrane (Goni et al., 2014). All of these options initiate from integrin clustering, which leads to talin and paxillin recruitment and also to the increase in the local production of PIP_2 (Legate et al., 2009). Once recruited, FAK is activated *trans* by the autophosphorylation of tyrosine 397. For this phosphorylation to occur,

the inhibitory FERM domain must disconnect from the kinase domain (KD) to expose the loop where Y397 resides. It has been suggested that the FERM domain binds to PIP_2 at the cell surface opening the FAK structure (Seong et al., 2013). Once phosphorylated, Y397 provides a binding site for the Src kinase. Src induces the further phosphorylation of tyrosine residues 576 and 577, creating a binding site for other focal adhesion proteins (Figure 8). Fully activated FAK has a role as a kinase and a scaffolding protein (Arold, 2011; Frame et al., 2010).

2.2.3 Integrin induced downstream signaling pathways

Signaling induced by integrin adhesion regulates two main functions in cells: growth and movement. Cell survival, proliferation and differentiation, as well as cell spreading, migration and polarity, are dependent on integrin mediated adhesion and signaling (Figure 9; Kyle R. Legate et al., 2009).

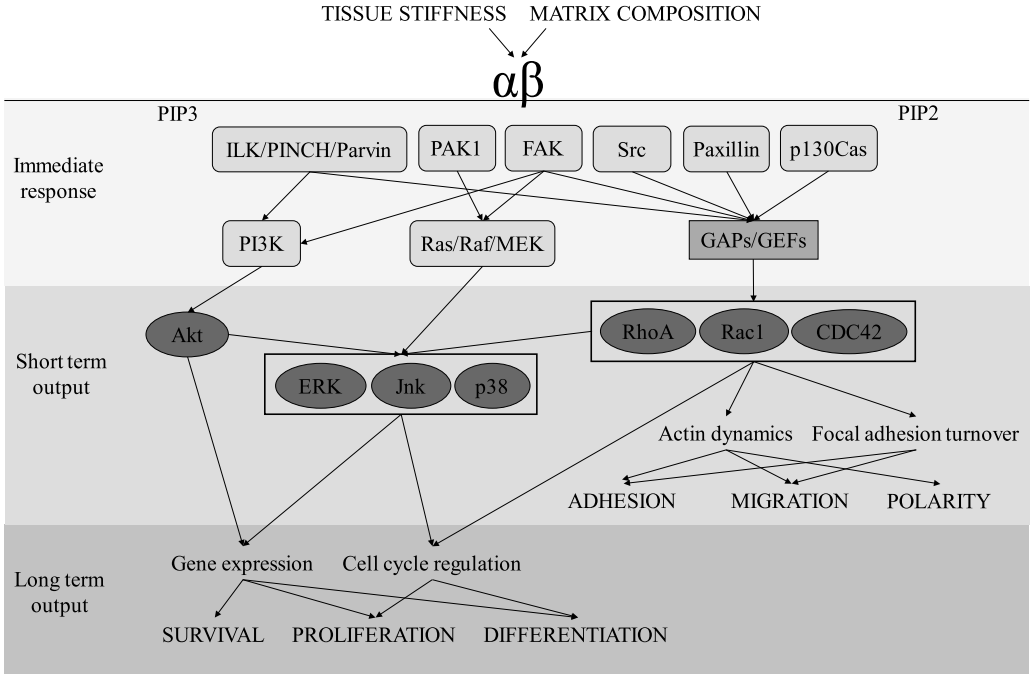

Figure 9. Integrin signaling. Adapted from Legate et al., 2009.

Integrin signaling maintains adhesion dependent growth

Integrins regulate cell proliferation and migration together with growth factor receptors. The importance of cell adhesion to cell growth is evident, since cells cannot proliferate in suspension, even with a supply of growth factors. On the other hand, integrins cannot induce cell proliferation without growth factors. In normal cells, the cell cycle is stopped between the G1 and S phase if cells are not adhered to the matrix. Cancer cells, however, have learned to escape from this regulation (Moreno-Layseca and Streuli, 2014). Integrins regulate cell proliferation through ILK, PI3K/Akt and

MAP-kinase pathways, and also Rho GTPases participate in these events (Moreno-Layseca and Streuli, 2014).

Integrin-linked-kinase, ILK, is an important regulatory protein in focal adhesions. ILK is a pseudokinase and unlike several of its fellow FA targeted kinases, it does not directly phosphorylate other proteins. Instead, ILK is a scaffold protein that forms a complex with Parvin and PINCH, and together they are recruited to focal adhesions, binding directly to the β-tails of integrins. The ILK-Parvin-PINCH-complex, the IPP complex, is important in linking the actin cytoskeleton and the ECM, probably through the direct binding of actin to parvin. Especially this complex is important in force transduction and the formation of fibronectin fibrils (Ghatak et al., 2013). ILK promotes adhesion dependent cell survival, and connects integrin signaling to growth factor receptor induced signaling through PINCH and Nck2. ILK has been shown to activate Akt signaling through PI3K (Hehlgans et al., 2007). The FAK–Src complex also recruits PI3K to focal adhesions and participates in Akt activation (Legate et al., 2009). Akt phosphorylates several targets, e.g. GSK3β and FOXP3, and regulates cell survival, growth, and proliferation (Faes and Dormond, 2015).

MAPKinases are mitogen activated kinases that respond to hormones, growth factors, and other cytokines as well as stress factors such as radiation, heat and mechanical stress. They are classified as p38 MAPKinases (α, β, γ, δ), extracellular signal-regulated kinases (ERK1, ERK2), and c-Jun NH_2-terminal kinases (JNK1, JNK2, JNK3) (Krishna and Narang, 2008). MAPKinases are activated through phosphorylation and respond mainly to receptor tyrosine kinases and G-protein coupled receptors. Integrin dependent adhesion affects the activity of MAPKinases through FAK/Src, PAK1, and RhoGTPase mediated Ras/Raf/MEK activation (Hehlgans et al., 2007; Juliano et al., 2004; Moreno-Layseca and Streuli, 2014). MAPKinases are activated by a chain of MAPKinase activating kinases (MAPKKs and MAPKKKs) and they further activate other kinases and eventually regulate the transcription of several genes (Krishna and Narang, 2008). ERK signaling is required for cell proliferation (Moreno-Layseca and Streuli, 2014). JNK mainly mediates stress responses together with p38 (Krishna and Narang, 2008). p38 MAPKinases participate in the regulating cell differentiation, and through the direct regulation of the cytoskeleton they promote cell migration and invasion (Cuenda and Rousseau, 2007).

Integrin signaling regulates cell movement

The FAK–Src complex is formed when integrins are clustered by interactions with the ECM and it functions upstream of several signaling pathways. During cell adhesion, several FA proteins such as talin, kindlin, vinculin, paxillin and α-actinin participate in integrin signaling too (Legate et al., 2009). The FAK–Src complex and other FA proteins such as paxillin and ILK activate Rho GTPases, small GTPases belonging to the Ras superfamily (Legate et al., 2009). Rac1, CDC42 and RhoA are the best characterized proteins in the Rho-family of GTPases and play an important role in regulating actin dynamics (Hall, 2012). Classically, the role of Rac1 is to induce the formation of lamellipodia, CDC42 induces filopodia protrusion, and RhoA regulates the maturation of focal adhesions and the formation of actin stress fibers (Hall, 2012).

GTPases are inactivated by GAPs (GTPase activating protein) that hydrolyze GTP to GDP, and activated by GEFs (guanine nucleotide exchange factor). In addition, the spontaneous activation of GTPases is prevented by GDIs (guanine nucleotide dissociation inhibitors). Rho GTPases are abundant in cells and their spatiotemporal control by GAPs and GEFs is crucial. In addition to a large number of proteins belonging to the Rho family (22) and the whole Ras-superfamily (160), the activation of GTPases is regulated by 70 GEFs and 70 GAPs, and over 100 targets have been identified only for Rho GTPases, clearly complicating the determination of the functions of a single family member (Hall, 2012). In general, the functions of Rho GTPases are so diverse that they most likely activate a large variety of downstream targets and their specificity is determined by the scaffolding functions of GAPs and GEFs instead of GTPases themselves (Hall, 2012). In focal adhesions, GAP and GEF activity and recruitment are regulated by the FAK–Src complex, targeting the functions of Rho GTPases to focal adhesions where they participate in the formation of focal adhesions and in actin polymerization and control directional cell migration (Tomar and Schlaepfer, 2009).

Signaling by collagen receptor integrins

Integrin α-tails have been shown to induce ligand/integrin specific signaling in cells, whereas the β-tails mediate common integrin signaling events described above. The collagen receptor integrins α1β1 and α2β1 serve as a good example of α-tail specificity. These integrins can bind to the same ligands, such as type 1 collagen, but induce specific signaling cascades and opposite outcomes in cells in an α-tail dependent manner (Heino, 2014). In response to cell adhesion to type 1 collagen, integrin α2β1 can activate p38 MAPKinase-α (Ivaska et al., 1999), whereas integrin α1β1 has been shown to activate the Ras-ERK pathway (Pozzi et al., 1998). These signaling events have opposite effects on collagen synthesis, since α2β1-integrin dependent p38-α activation leads to increased synthesis of type 1 collagen and also to upregulation of collagenases, whereas α1β1 induced ERK activation downregulates collagen synthesis (Langholz et al., 1995; Ravanti et al., 1999). Cell growth and movement are also differentially regulated by collagen receptors. α2-tail dependent p38 activation is required for EGF stimulated chemotactic cell migration on type 1 collagen (Klekotka et al., 2001). α1β1 on the other hand promotes cell proliferation through ERK (Pozzi et al., 1998). Interestingly, specific amino acids in the α2-tail, that are absent from the α1-tail, were shown to be necessary for directed cell migration. Mutation in the tyrosine (Y) or methionine (M) in the sequence YEKM in the α2-subunit abolished cell migration (Klekotka et al., 2001). Very recently some integrin α-subunits were shown to include YxxΦ-motifs that are required for integrin endocytosis and integrin mediated cell migration (De Franceschi et al., 2016). This motif corresponds to α2 YEKM, and is absent from α1, and could be responsible for the opposite signaling outcomes of these two receptors. Although other studies have showed that α1 activates ERK, and α2 does not, in the aforementioned study Klekotka and colleagues showed that lysine (K) and methionine (M) in the YEKM sequence are required for α2-mediated ERK activation and entry into the S-phase. These amino

acids are present in the α1-tail as well. Perhaps depending on the model system, α1 is usually responsible for ERK activation, but ligand bound α2 can either be endocytosed in a YxxΦ-dependent manner, and contribute to cell migration by activating p38, or α2 can stay at the focal adhesions where it contributes to ERK signaling and cell proliferation.

2.2.4 Integrins connect the cytoskeleton to the extracellular matrix and act as mechanosensors

Focal adhesions are not only signaling hubs but also the points where cells anchor to the ECM and integrate it to the cytoskeleton (Figure 10). Cells can use these anchor points to pull and push themselves during cell spreading and migration, and both create and sense mechanical tension.

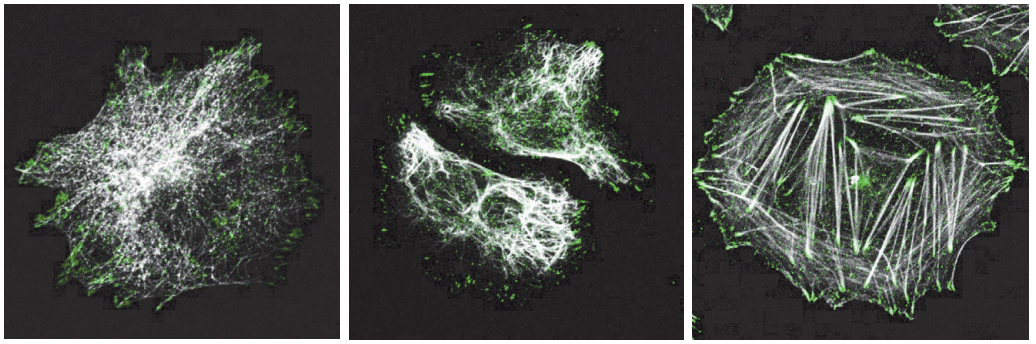

Figure 10. The cytoskeleton. The cytoskeleton consists of microtubules (left), intermediate filaments (middle) and the actin microfilaments (right). Cytoskeletal proteins are seen in white and the integrin focal adhesions in green. Microscopy images by Maria Salmela.

Cytoskeleton

The cytoskeleton consists of actin microfilaments, microtubules, intermediate filaments, and septins. These long protein bundles give cells their shape and strengthen their physical barrier. They also define the intracellular architecture and regulate the time and space of signaling events. The type III intermediate filament vimentin brings elasticity and stiffness to cells helping cells cope with different kinds of mechanical stress (Lowery et al., 2015). All of the components of the cytoskeleton participate in compartmentalizing intracellular organelles such as mitochondria, golgi and the nucleus. Microtubules also participate in organizing and separating chromosomes during cell division (Alfaro-Aco and Petry, 2015). Mechanosensing at the cell membrane can directly affect nuclear gene transcription through a link between integrins, focal adhesions, actin and intermediate filaments (Wang et al., 2009). In addition to the cell cortex, a filament rich layer of actin, septins and microtubules right beneath the plasma membrane restricts the diffusion of proteins and lipids at the cell membrane, thereby regulating cell signaling in space and time (Köster and Mayor, 2016). Especially septins have been shown to regulate cell signaling by forming local pools of PIP_2 (Bridges and Gladfelter, 2015). The cytoskeleton also directs cellular

trafficking and endocytosis. Actin participates in transferring cargo from the cell membrane to endosomes together with myosin motor proteins. Microtubules provide a track for kinesin motor proteins.

Actin, microtubules and intermediate filaments are in contact with integrin mediated focal adhesions (Figure 10), and of these cytoskeletal proteins actin has the most established role in regulating cell adhesion and migration. Actin fibers are built from g-actin monomers into fibrillary actin by different actin binding proteins, which also stabilize actin structures and link them to adhesions. Actin fibers can produce protrusive and contractile forces on cells. Protrusive forces are important during cell migration and in the formation of lamellipodia and filopodia, whereas contractile forces are important in mechanotransduction and the assembly of focal adhesions. Relatively stable stress fibers form different structures in cells, such as transverse arcs, and dorsal and ventral stress fibers. Dorsal stress fibers are situated at the leading edge of the cell and connect to focal adhesions at the one end and hold transverse arcs in position by binding to them with the other end. Transverse arcs form the lamella, right behind the protrusive lamellipodia, and contribute to cell migration. Ventral stress fibers produce a contractile force to assist in cell migration and cell retraction at the rear. Stress fibers are only produced when cells undergo mechanical stress, thus these structures are not present in all cells in tissues, but are important for example during wound healing (Tojkander et al., 2012).

Cells sense and adapt to mechanical forces through integrins, focal adhesions and the cytoskeleton

Cells are regulated by a cocktail of soluble chemical cues, such as growth factors and hormones, as well as by the various ECM proteins that bind to specific receptors embedded in the cell membrane. Different kinds of mechanical stimuli affect cell behavior, too. A most obvious mechanical disturbance in cells is created by muscle contraction and flowing liquids. Also interstitial fluid causes pressure to the tissues. Not just the composition of ECM, but also its stiffness that varies according to molecular components, affects cellular signaling (DuFort et al., 2011). Tension from cell-cell and cell-ECM contacts is sensed by a wide repertoire of receptors. Cell-ECM receptor integrins and cell-cell receptor cadherins are the most important sensors of mechanical stress, but also ion channels, receptor tyrosine kinases such as DDR and ephrin, and lipid rafts can sense forces independently or in collaboration with integrins and cadherins (Gasparski and Beningo, 2015). Even though a receptor does not sense mechanical stimuli directly, its function can be altered due to changes in the cytoskeleton and membrane organization, such as stretch-activated G-protein coupled receptors (Bukoreshtliev et al., 2013; DuFort et al., 2011). Mechanosensing has a fundamental role in regulating tissue formation. The differentiation of mesenchymal stem cells to specific lineages of neurons, osteoblasts and myoblasts, is regulated by the stiffness of the matrix. Cells respond to the matrix they are in contact with and adapt their stiffness to resemble that of the matrix (Engler et al., 2006). One study also demonstrated how a non-malignant cell line can become malignant when cultured on a stiff matrix resembling a tumor microenvironment (Paszek et al., 2005).

Mechanosensing relies on the conformational changes and elasticity of the molecules involved (DuFort et al., 2011). The ECM itself can be modified by stretching, and for example fibronectin contains integrin binding sites that are hidden without mechanical tension. ECM receptor integrins undergo conformational activation from the bent to extended conformation. Talin can also undergo structural changes, first to release autoinhibition and bind to integrins, and after that the simultaneous binding of talin to integrin tails and actin microfilaments straightens the talin molecule. More importantly, the pulling of the ECM and the actin cytoskeleton into opposite directions, leads to talin stretching (Figure 11). When stretched, new binding sites for actin and other proteins in talin are exposed. The recruitment of vinculin to vinculin binding sites in talin requires talin stretching. Vinculin can thus be recruited only when focal adhesions undergo mechanical stress (del Rio et al., 2009).

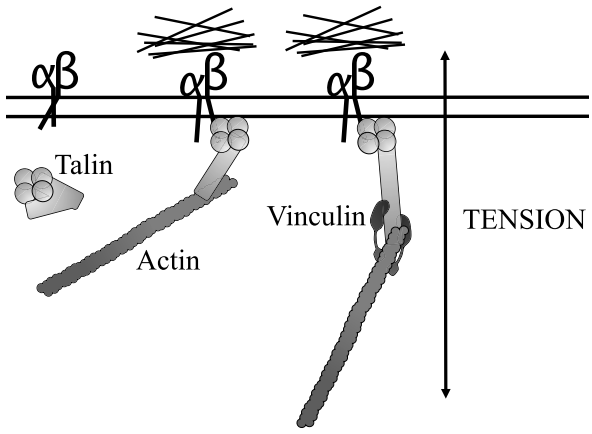

Figure 11. Mechanosensing through the talin-vinculin-actin complex. Talin stretching by integrins and actin microfilaments reveals several vinculin binding sites. Vinculin stabilizes the open conformation of talin and strengthens focal adhesions.

Focal adhesions grow in size and they are more stable when force is applied to them. Talin stretching and the following recruitment of vinculin are important for the maturation of adhesions, and thus focal adhesions fail to mature on soft surfaces where no tension is created (Haining et al., 2016). Tension to focal adhesions is also created during cell migration when cells grip onto the ECM and pull themselves forward. Vinculin plays an important role in regulating the retrograde flow of actin transverse arcs and in transmitting traction forces to the ECM (Atherton et al., 2016). Vinculin fluctuates between bound-active and unbound-autoinhibited forms in focal adhesions. The active form is stabilized by tension, and in contrast the release of vinculin occurs when there is less pulling from the actomyosin network (Atherton et al., 2016). This can provide a mechanism for how adhesions are reinforced at the leading edge where tension is greater and disassembled at the cell rear where the actomyosin network does not exert a similar force on the adhesions.

Mechanical tension can change integrin signaling through focal adhesions. For example the fibronectin receptor α5β1 can activate FAK (Y397) only on stiff surfaces

(Paszek et al., 2005; Seong et al., 2013), whereas the binding of α2β1-integrin to collagen can induce FAK activation even without a force (Seong et al., 2013). Force is not required for talin, FAK and paxillin recruitment (Yu et al., 2011), but to fibronectin stretching that has to take place to expose the integrin binding sites. Tumors are stiffer than normal tissues and have dramatic changes in the composition of the ECM around the tumor. Also integrin expression is altered leading to an abnormal interaction between cells and the ECM, followed by changes in cellular signaling (Paszek et al., 2005).

2.2.5 Integrins mediate cell migration

Cell migration can be regulated by different extracellular cues varying from chemical to electrical and mechanical stimuli. Chemotaxis is cell migration towards or away from chemical substances, such as growth factors, which are soluble. Haptotaxis on the other hand, is cell migration directed by bound substances that can be gradients of ECM molecules or other substances bound to the matrix. Chemotaxis and haptotaxis cannot be completely separated, since in tissues, several secreted molecules can become matrix bound, which then changes the availability of interacting matrix proteins. Also the electric field surrounding cells (electrotactic), shear stress (mechanotactic) and substrate rigidity (durotactic) can affect cell migration. In addition to the cues that direct the movement of individual cells, cells can also migrate collectively and receive migratory cues from other cells. Collective migration is important during tissue morphogenesis and wound healing, but also during cancer invasion and metastasis. During collective migration, both cell-cell interactions and cell-matrix interactions become important. In addition to integrins, adhesion receptors such as cadherins play an important role in connecting the cytoskeletons of neighboring cells (Haeger et al., 2015; Ricoult et al., 2015). In tissues, cells most likely need to react to all of these stimuli simultaneously. In most of the models for studying cell adhesion, mechanosensing and migration the situation is described in 2D, which *in vivo* applies only to the epithelial cells aligning the gut and veins, and to some extent to leukocytes circulating in our body. In 3D, and thus in most tissues, traction and force are differentially, but simultaneously, sensed from opposing sides of the cell (Doyle and Yamada, 2016). Signaling events are usually different in 2D cell cultures and in 3D models, such as cell spheres or collagen gels, even though the same cells and matrix components are used. Thus, the mechanical and molecular details we get from 2D models give us a general overview of cell adhesion and migration, but *in vivo* the molecular details can be different, and this must be taken into account in e.g. cancer treatment.

Cell migration is a collaboration between adhesion molecules, such as integrins, which control the attachment and detachment of cells, the cytoskeleton, which is assembled and disassembled to drive the movements of cells, and signaling molecules, such as Rho GTPases Rac1, CDC42 and RhoA, which orchestrate these events. Lipids in the cell membrane also play an important role in cell migration, especially the “handyman” PIP_2 that regulates the timing of events. When cells attach to the extracellular matrix, integrins play an important role in forming cell adhesions and in scanning the matrix. During migration, integrins are trafficked to the sites where new contacts with the

matrix are formed. Actin polymerization towards the attractant at the leading edge pushes the cell membrane forward and free integrins at the cell surface form new contacts with proteins in the matrix. Cells also need to be able to detach from the matrix during cell division and migration. At the cell rear, old contacts with the ECM are broken, integrins are internalized, and actin is retracted towards the leading edge with the trailing cell body (Fife et al., 2014).

Lamellipodia, the leading-edge actin network

Cells can proceed along 2D matrices by forming wide lamellipodia or spike-like filopodia. Actin polymerization drives the progress of lamellipodia, and this is activated by RhoA, through a formin mDia and ROCK (Hall, 2012). The formation of lamellipodia includes the branching of actin filaments at the protruding cell edge, and these branches are produced by the Arp2/3 complex and mediated by Rac signaling (Davidson and Wood, 2016; Hall, 2012). PIP_2 is involved in regulating the time and space of actin polymerization by recruiting WASP-proteins that then bind Arp2/3 proteins (Arjonen et al., 2011). During cell migration, a concentrated pool of PIP_2 is created by PI3K, which is localized to the leading edge. In contrast, at the cell rear, PTEN dephosphorylates PIP_2 back to PI (Wu et al., 2014). During the formation of lamellipodia, the intermediate filament vimentin is depolymerized at the sites where cells are protruding, to enable cell spreading. The depolymerisation of vimentin is a consequence of the Rac -mediated phosphorylation of vimentin at serine 38 (Helfand et al., 2011). At the leading edge of the lamellipodia, integrins attach to matrix molecules and induce the formation of small nascent adhesions. These transient adhesions are either disassembled or connected to the actin network at the border of the lamellipodia and lamella, where the exertion of force on the adhesions promotes the maturation of the adhesions into focal complexes and focal adhesions (Kuo, 2013).

Filopodia lead the way

Filopodia originate from lamellipodia structures at the leading edge, and probe the matrix for cues to direct cell migration. Filopodia, and in 3D matrices similar invadopodia, are also the drivers of cancer invasiveness, due to their ability to protrude into the surrounding matrix and enhance cell motility (Arjonen et al., 2011). The formation of filopodia is initiated by the activation of CDC42 and the Arp2/3 complex (Mattila and Lappalainen, 2008). In the filopodia protrusions, straight actin bundles are built by formins or the ENA/VASP complex and stabilized by actin binding proteins such as fascin and α-actinin (Köster and Mayor, 2016). Integrins and other filopodial components are cycled to the tips of forming filopodia along the actin filaments by myosin-X. Integrins are not required to initialize the formation of filopodia, but they stabilize the structure by attaching to the matrix (Arjonen et al., 2011). In addition to actin, other cytoskeletal proteins are also involved in the formation of filopodia. At the roots of filopodia, septins regulate the spacing of signaling events (Hu et al., 2012).

Focal adhesion turnover and integrin trafficking

Microtubules play an important role in regulating cell adhesion and migration by participating in the turnover of focal adhesions. Actin stress fibers are needed for the targeting of microtubules to focal adhesions, and if microtubules are disrupted, cells grow large focal adhesions and apply stronger contractility to their environment. As mentioned, integrin adhesions grow when force is applied, and disassemble with lower tension (Small et al., 2002). Microtubules attach to focal adhesions through CLASP proteins (Stehbens et al., 2014) and trigger the turnover of focal adhesions in a FAK and dynamin dependent manner (Ezratty et al., 2005). Microtubules have been suggested to traffic vesicles containing the metalloprotease MT1-MMP to adhesions, where this enzyme is released into the extracellular space and locally degrades the ECM and disrupts integrin mediated adhesions (Stehbens et al., 2014). From disintegrating adhesions, integrins are endocytosed in Rab5 positive endosomes and later recruited to Rab11 positive endocytic recycling compartments to be recruited back to the cell membrane. Active FAK is localized together with integrins and Rab11 in endocytic recycling compartments, where it recruits talin and maintains an active conformation of recycled integrins. A recent study claims that integrins are kept active during the recycling process, although they are not bound to ligands, and that their conformational inactivation dramatically reduces the reassembly of new adhesion sites. During the disassembly of focal adhesions FAK functions as a scaffolding protein and recruits dynamin, whereas during reassembly its kinase activity is required for the reformation of new adhesions. In addition to FAK, also the activity of Src family kinases is needed for trafficking the integrins back to the cell surface (Nader et al., 2016).

3 AIMS OF THE STUDY

Much of the work done with integrins has shown that these receptors need to be conformationally activated to be able to bind their ligands. However, most of the research was done on other than collagen binding integrins, which may in fact differ a lot in their binding and activation mechanism from collagen binding integrins. In the first paper of my thesis we showed that α2β1-integrins bind echovirus 1 without pre-activation of the receptor. Since integrin activation is known to lead to the formation of focal adhesion complexes that mediate integrin signaling, the next question was whether nonactivated integrins can mediate cellular signaling. Integrins mediate cell adhesion to the ECM and also direct the movements of cells. In the latter part of my thesis, I studied the impact of the integrin activation state on the initial binding of cells to ECM molecules, on the formation of cell adhesions and on the dynamics of cell spreading.

The specific aims in my thesis study were to determine:

1. Integrin ligand binding and signaling in the nonactivated conformation

2. The effect of the integrin activation state on cell adhesion and spreading

4 MATERIALS AND METHODS

The materials and methods used in the original publications are listed here, and are described in more detail in the publications.

Table 4.1. Methods

Method	Used in
BIAcore analysis	I
Binding site studies	III
Cell migration wound assay	V
Cell viability assay	V
Cellix: cell adhesion under flow*	III
Confocal microscopy: cells plated on cell culture plastic*	II, IV
Confocal microscopy: conventional*	I, II, IV, V
Covalent linking of EV1*	II
Echovirus 1 infection assay*	I, II
Flow cytometry*	I, II, III, IV,V
Image analysis: colocalization*	I, V
Image analysis: EV1 infected cells*	II
Image analysis: signal intensity on integrin rich areas	II
In vitro kinase assay	V
Integrin clustering with antibodies*	I
Isolation of cell adhesions*	IV
Live microscopy*	II, III, IV
Mass spectrometry	IV
Molecular modelling	I
Plate-and-wash assay: early cell adhesion*	I
Proteomics	IV
shRNA transfections: lentivirus system*	II
siRNA transfections*	II, IV, V
Solid phase binding assay	I
Western blot*	I, II, IV, V
xCelligence: real time cell adhesion measurement*	II, III, IV, V

Methods marked with * were carried out by MS

Table 4.2. Cell lines

Cell line	Type	Species	Culture media	Used in
SaOS-2	Primary osteogenic sarcoma	Human	DMEM	I, II, III, V
CHO	Ovary	Chinese hamster	alphaMEM	I, III, IV
HeLa	Epitheloid cervix carcinoma	Human	DMEM	II
PC-3	Prostate cancer bone metastasis	Human	RPMI	V
HEK-293T	Human embryonic kidney 293 cells + SV40 T-antigen	Human	DMEM	II

Table 4.3. Plasmids

Name	Backbone	Reference	Used in
α2	paWneo2	Ivaska *et al.,* 1999	I,II,III
α2E336A	pAWneo2	Jokinen *et al*., 2004	I,II,III
α2E318W	pcDNA3.1	(I)	I, III
α2E309A	pAWneo2	Connors *et al*., 2007	I
α2/α1tail	pAWneo2	Ivaska *et al.,* 1999	II
α2Y285F	pGEX-2T	Käpylä *et al*., 2007	III
α1	pcDNA3.1/Z-2	P. Ollikka (Biotie Therapies Corp)	IV
α1E317A	pcDNA3.1/Z-2	Lahti *et al*., 2011	IV
FoxP3	pFlag-CMV-2/ pIRES-puromycin/ pEGFP-C3	(V) Li *et al*., 2014 Held-Feindt *et al*., 2013	V
GSK3β	pEGFP-C3/ pGEX-6P-3/ pBJ5-HA	(V) Santio *et al.,* 2016 C. Beals (Stanford University, USA)	V
PIM1	pGEX-6P-3/ pTag-RFP-N/ pcDNA3.1./V5-HisC	Santio *et al*., 2016 Santio *et al*., 2016 Santio *et al*., 2016	V

Table 4.4. siRNAs and shRNAs

Target	Type	Target sequence 5'3'or type	Company	Used in
Hamster Kindlin-2/1	siRNA	AUUUCUCUGACCGAGUCUUtt	Ambion life technologies	IV
Hamster Kindlin-2/2	siRNA	CCAAAUGAGUCUGAUUGAAtt	Ambion life technologies	IV
Hamster Kindlin-2/3	siRNA	CGGUAACAUCACCAGAAAUtt	Ambion life technologies	IV
Hamster Kindlin-2/4	siRNA	AUGUAGCCCAAAUGAGUCUtt	Ambion life technologies	IV
Human FAK/1617	shRNA	TRCN000001617	Sigma	II
Human Talin-1/1	shRNA	TRCN0000299020	Sigma	II
Human Talin-1/2	shRNA	TRCN0000299022	Sigma	II
Human Talin-1/2,5	siRNA	HP GenomeWide siRNA	Qiagen	II
Human PIM-1	siRNA	GAUGGGACCCGAGUGUAUA	Dharmacon	V
Human PIM-2	siRNA	GUGGAGUUGUCCAUCGUGACA UU UGUCACGAUGGACAACUCCAC UU	Sigma-Aldrich	V
Human PIM-3	siRNA	GGCGUGCUUCUCUACGAUAUG UU CAUAUCGUAGAGAAGCACGCC UU	Sigma-Aldrich	V
Nontarget	siRNA	AllStars	Qiagen	IV
Nontarget	shRNA	SHC016	Sigma-Aldrich	II
Nontarget	siRNA	MISSION® siRNA Universal Negative Contol, SIC001	Sigma-Aldrich	V
Nontarget	siRNA	ON-TARGETplus	Dharmacon	V

Table 4.5. Antibodies

Antigen	Clone/ cat #	Dilution	Company	Appli-cation	Used in
β-actin	I-19/ Sc-1616	1:1000	Santa-Cruz Biotechnology	WB	I,II,IV,V
β-actin	13E5, #4970	1:1000	Cell Signaling Technology	WB	V
β-actin	D13K4803	1:20000	Sigma-Aldrich	WB	V
α-actinin	#3134S		Cell Signaing Technology	IF	II
AKT1/2/3	#9272	1:1000	Cell Signaling Technology	WB	V
AKT1/2/3 pS473	D9E, #9271	1:1000	Cell Signaling Technology	WB	V
EV1	Marjomäki *et al.,* 2002	1:10 000	(homemade)	IF	I,II
FAK	6110088	1:1000	BD Biosciences	WB	II
FAK	Clone 4.47	1:100	Millipore	IF	II
FAK pY397	3283S	1:1000	Cell signaling	WB	II
FAK pY397	44624G	1:100	Invitrogen	IF	II
FAK pY576/577	#3281P	1:1000	Cell signaling	WB	II
FAK pY925	#3284S	1:1000	Cell Signaling	WB	II
Flag	F1804	1:500	Sigma-Aldrich	WB	V
FoxP3	H-190, SC-28705	1:1000	Santa-Cruz Biotechnology	WB	V
GPVI	JAQ-1	8 μg/ml	Emfret Analytics	Bl	III
GSK3α/β pS21/9	#9331	1:3000	Cell Signaling Technology	WB	V
GSK3β	#9315	1:3000	Cell Signaling Technology	WB	V
HA	H3663	1:5000	Sigma-Aldrich	WB	V
Integrin-α2	12F1, BD555668	7.5 μg/ml	BD Pharmingen	FC	III, V
Integrin-α2	16B4		Serotec	IF	I
Integrin-α2	430903	1:50	R&D Systems	IF	II
Integrin-α2	P1H5	10 μg/ml	Santa Cruz Biotechnology	Bl	III
Integrin-β1 (active)	12G10, ab30394	7.5 μg/ml	Abcam	FC	V
Interin-β1	Mab13, BD552828	7.5 μg/ml	BD Pharmingen	FC	V
Kindlin-2	Ab74030	1:500	Abcam	WB	IV
P38 MAPK		1:1000	Cell Signaling Technology	WB	I
P38MAPK pT180/Y182	3D7 #9215L	1:1000	Cell Signaling Technology	WB	I

Antigen	Clone/ cat #	Dilution	Company	Appli-cation	Used in
p38MAPK pT180/Y182-Alexa488-conj.	28B10 #4551		Cell Signaling Technology	FC	I
Paxillin	610051	1:100 1:1000	BD Biosciences	IF WB	II
Paxillin pY118	2541S	1:1000	Cell Signaling Technology	WB	II
Phalloidin-alexa 633	A22284	1:30	Invitrogen	IF	II, IV
PIM1	#2907	1:1000	Cell Signaling Technology	WB	V
PIM1	12H8, SC-13513	1:500	Santa-Cruz Biotechnology	WB	V
PIM2	D1D2, #4730	1:1000	Cell Signaling Technology	WB	V
PIM3	D17C9, #4165	1:1000	Cell Signaling Technology	WB	V
PKCα	05-154	1:2000	Millipore	WB	I
PKCα pS657	06-822	1:1000	Millipore	WB	I
PTGS2	160116	1:2000	Cayman Chemical Company	WB	V
Src pY416	2101S	1:1000	Cell Signaling Technology	WB	II
Talin-1	MAB1676	1:100	Chemicon	IF	II
Type IV collagen	Ab52235		Abcam	IP	IV
Vimentin	VIM9/ V6389	1:400	Sigma	IF	IV
Vinculin	V9131	1:200	Sigma	IF	II, IV

WB: Western-blotting, IF: Immunofluorescence, FC: Flow cytometry, Bl: function blocking, IP: Immunoprecipitation

Table 4.6. Reagents

Reagent	Type	Conc.	Company	Used in
3,3´-dihexyloxacarbocyanine iodide	Fluorescent stain	1 μM	Invitrogen	III
BTT-3033	α2-integrin inhibitor	Varies	Biotie Therapies	III
BTT-3034	α2-integrin inhibitor	Varies	Biotie Therapies	III
CBL027	Integrin-α2 probe	50 μM	Koivunen *et al.*, 2011	III
Celecoxib	COX2 inhibitor	25 μM	Pfizer	V
DABI	Nuclear label	1:1000		II
DHPCC-9 (1,10-dihydropyrrol[2,3-a]carbazole-3-carbaldehyde)	PIM inhibitor	10 μM	Akué-Gédu *et al.,* 2009	V
D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone	anticoagulant	40 μM	Calbiochem	III
DRAQ5	Nuclear label	1:50	Cell Signaling	II
Fugene 6	Transfection reagent	*	Promega	II
Heparin	anticoagulant	7.5 U/ml	Leo Pharma	III
Indomethacin	COX1 inhibitor	50 μM	Sigma-Aldrich	V
NSC23766	Rac1 inhibitor	100 μM	Santa Cruz Biotechnology	II
Oligofectamine	Transfection reagent	*	Thermo Fischer Scientific	V
Phalloidin-Alexa 555/633 conjugated	Actin label	1:30	Invitrogen	II, IV
Safingol	PKC inhibitor	10 μM	Calbiochem	II
siLentFect lipid	Transfection reagent	*	Bio-Rad	II, IV
SYPRO Orange	Fluorescent dye		Invitrogen	III
TPA (PMA)	Phorbol ester	100 nM		I, III, IV
WST-1	Cell proliferation reagent	1:10	Roche Applied Science	I, III, (IV)

*According to the manufacturers' guide lines, the amount was optimized for each cell line.

Table 4.7. ECM proteins

Type	Details	Company	Used in
Collagen type I	HORM	Nycomed	III
Collagen type I	Bovine (PureCol)	Advanced BioMatrix	I, II, V
Collagen type I	Rat tail	BD Biosciences	III
Collagen type IV	Mouse Engelbreth-Holm-Swarm tumor	BD Biosciences	III, IV
convulxin	Snake venom	Coatech	III
Fibronectin	Fragment 40 kDa Fragment 120 kDa	Chemicon	III
Fibronectin	Human plasma	Chemicon International	I, II
Fibronectin	Human plasma	Sigma (F0895)	V
GFOGER	Collagen peptide	Auspep	I
Laminin-322		Chemicon	III
Poly-l-lysine	P9155	Sigma-Aldrich	II, V
vitronectin		Chemicon	III

5 RESULTS

5.1 Nonactivated integrins can bind to echovirus 1 (I)

α2β1 is the only cellular receptor for human echovirus 1 (EV1). EV1 exploits the integrin internalization pathways to infect cells, and thus provides a tool for studying the mechanisms of integrin ligand binding and internalization.

5.1.1 Integrin α2 can interact with EV1 in the nonactivated conformation

SaOS-WT cells cannot be infected with EV1, since they lack its receptor. However, when these cells are transfected with α2-integrin, they readily bind and internalize the virus. Surprisingly, transfecting the cells with the expected loss-of-function mutant of α2-integrin (E336A), did not block virus binding and entry (I, Fig. 6D). E336 is thought to act as an intrinsic ligand for the β-I-domain, causing the conformational changes in the integrin structure after ligand binding. A mutation in this essential acidic glutamate prevents the conformational activation of the I-domain integrins αL (E310; Huth et al., 2000), α1 (E335; Lahti et al., 2011), α2 (E336; Connors et al., 2007) and αM (E320; Alonso et al., 2002). Chinese hamster ovary cells (CHO), provide a clean system for studying specific integrin-ligand interactions since CHO cells lack all of the collagen receptor integrins. Studies in CHO cells confirmed that when cells interact with EV1, the loss-of-function mutation does not prevent cells from binding to the virus (I, Fig. 3C). In addition, expression of the gain-of-function mutant, E318W (Aquilina et al., 2002), reduced cell binding to EV1 (I, Fig. 2). The situation concerning collagen is quite the opposite. Cell binding to collagen was prevented when integrins were inactivated, and correspondingly increased when the receptor was pre-activated (I; Figs. 2,3C). Furthermore, addition of the Mg^{2+} and Ca^{2+} chelating agent EDTA, prevented the adhesion of CHO-α2 cells to collagen, but not to EV1 (I, Fig. S2A). EDTA is thought to promote the bent integrin conformation by removing the Ca^{2+} that supports the straightening of the integrin knee (Xie et al., 2004).

The same phenomenon was observed at the I-domain level. When the integrin α2-I-domain was allowed to bind to either a collagen peptide or EV1, the gain-of-function mutation that opens the αC-helix inhibited EV1 binding (I, Fig. 1C), but increased collagen binding (I, Fig. 1B). The wildtype I-domain, which stays in the closed conformation in the absence of a ligand, bound EV1 (Kd 0.8 ± 0.2 nM) even stronger than collagen peptide (Kd 39 ± 3.5 nM).

5.1.2 EV1 induces α2-integrin clustering, but not conformational activation

EV1 has 60 binding sites for integrins around the viral capsid, and according to a solid phase binding assay, one virus capsid can occupy up to 10 integrins simultaneously (I, Fig. 1E). α2-integrins are clustered around virus particles (I, Fig 6A), trafficked along actin fibers to bigger clusters and internalized (Upla et al., 2004). According to immunofluorescence studies of virus clustered integrins, an active β1-integrin

recognizing antibody did not colocalize with EV1 as readily as a conformation unspecific antibody for β1 (I, Fig. S3), indicating that virus binding does not require integrin activation, nor does it induce activation after binding.

Earlier studies have showed that ligand binding to an α2β1-integrin leads to the activation of the p38 MAPK pathway in a α2-subunit dependent manner (Ivaska et al., 1999). The activation of p38 MAPK requires the conformational activation of the α2-integrin, since the loss-of-function mutation E336A in the α2-subunit (I; Fig. 5B,C) as well as treatment with EDTA (I, Fig. S2B), prevent activation. Although both the binding of EV1 and an antibody against α2-integrins induce integrin clustering (I; Fig. 4A, 6A), only clustering by the α2-antibody could induce p38 phosphorylation (I, Fig. 6B). This suggests that antibody binding can induce the conformational activation of integrins, but EV1 cannot.

5.2 Nonactivated integrins can induce cell signaling (II)

EV1 binds to the closed I-domain of α2β1-integrins, without inducing the conformational activation of the integrins (I). Furthermore, EV1 binding triggers integrin clustering and EV1 internalization through a micropinocytosis-like mechanism (Karjalainen et al., 2008). Next, we asked, if nonactivated integrins can induce cellular signaling events. Is the signaling induced by integrins really depended on conformational activation, or is conformational activation only necessary for binding the ligand? If integrins can in some circumstances interact with a ligand in the nonactivated conformation, can they then also induce signaling?

5.2.1 Cells can spread on an EV1 coated surface using nonactivated integrins

As a model system for studying signaling induced by nonactivated integrins, we plated α2-integrin expressing cells on top of an EV1 layer (II, Fig. 1A). α2 is the only known receptor for EV1, thus all the contacts the cells can make with the environment, are expected to occur through this integrin. We confirmed the dependence on α2-integrin in the model when we plated wildtype SaOS cells, lacking α2, on a virus layer, and detected no binding (II, Fig. 1A). Furthermore, expressing the loss-of-function mutant, α2E336A, in SaOS cells rescued the binding to EV1 regardless of the inactive integrin conformation (II, Fig. 2B). We further treated SaOS-α2 and SaOS-α2E336A cells with 5 mM EDTA, and still saw binding to EV1, although EDTA completely blocks the ability of cells to bind to collagen (II, Fig. 2D). The amount of virus particles used for coating was estimated by the size (30 nm) and by the molecular weight of the virus. The goal was to have a uniform layer of particles, which would offer a maximal amount of binding sites for α2-integrins. This was to make sure that there were enough contact points for the formation of integrin mediated signaling complexes. These have been estimated to require 4 integrins at a 60 nm distance from one another (Schvartzman et al., 2011).

When SaOS-α2 cells spread on an EV1 coated surface, cells usually formed long filopodia-like protrusions (II, Fig. 1A), but the cell body stayed relatively immobile

(live-cell microscopy observations). A mass of α2-integrins at the tips of protrusions could represent adhesion sites, since no focal adhesion-like structures were formed (II; Fig. 1B). Actin fibers were thin and unorganized; no prominent stress fibers were observed in cells spreading on EV1 (II, Fig. S1A). Even after applying an additional force to the system by cross-linking EV1 covalently to a glass surface, we couldn't detect focal adhesions or actin stress fibers, although cells spread more (II, Fig. S1B). Thus the lack of tension required for the assembly of focal adhesions and the formation of actin stress fibers, was probably not caused by the substrate softness as such, but could instead be an outcome of abnormal protein recruitment to adhesion complexes.

5.2.2 Nonactivated integrins can induce the FAK pathway

We used the model system described in the previous section to study signaling pathways mediated by nonactivated integrins. FAK activation is the hallmark off integrin induced signaling and it was chosen to serve as a marker for integrin signaling also in our studies. When SaOS-α2 cells were plated on an EV1 coated surface, FAK phosphorylation increased gradually, in a similar manner to collagen induced FAK activation (II; Fig. 1C, S2). Importantly, activation was not seen on poly-l-lysine coated surfaces where cells attach independently of integrins (II, Fig. 1E). Both SaOS-α2 cells and cells harboring the inactivating mutant α2E336A induced FAK activation on an EV1 coated surface (II, Fig. 1C, 2C). The response in both cell lines, and more clearly so in α2E336A cells, often declined at 60 min time point (II; Fig. 1C, 2C). In addition, at longer time points (up to 120 min) the response to EV1 began declining after 60 min of cell spreading, whereas on Col I the activation seemed to reach the maximum intensity and remain there (II, Fig. S3). Thus EV1 induced FAK activation seemed to be transient. In addition to the nonactivated mutant being able to induce FAK activation, a treatment with EDTA did not abolish EV1 mediated FAK activation (II, Fig. 2E), strongly suggesting that integrins can indeed activate FAK without the conformational activation of integrins.

FAK activation by the phosphorylation of tyrosine 397 begins after the dimerization of the molecule and the release of the autoinhibition mediated by the FAK FERM and KD domains (Brami-Cherrier et al., 2014). As was described above, EV1 binding to the cell surface induces the clustering of α2-integrins, and we hypothesize that FAK can be recruited to integrin clusters and be activated most likely directly by the dimerization of FAK. After the binding of Src to Y397, additional sites of FAK, such as tyrosines 576/577, 861 and 925, are phosphorylated. This induces Src activation as well, and allows FAK interaction with other focal adhesion proteins and their phosphorylation and activation (Frame et al., 2010). When SaOS-α2 cells were plated on EV1, in addition to the phosphorylation of Y397, we could also observe an increase in the phosphorylation of tyrosines 567/577 (II, Fig. 4A), indicating that Src binds to FAK also in this case and causes the full activation of the FAK molecule. We also measured the activation of tyrosines 861 and 925, but managed to get proper antibody based readings only from a single experiment measuring Y925 (II, Fig. S5A). We also analyzed Src activation but only one experiment gave proper signal, although it did show gradual activation of Src (II, Fig. S5B). Instead, we observed paxillin

phosphorylation at tyrosine 118 in SaOS-α2 cells plated on EV1, indicating that FAK could bind and activate further downstream signaling proteins (II, Fig. 4B). More importantly, paxillin activation on EV1 was clearly transient compared to FAK activation on collagen (II, Fig. 4C).

5.2.3 FAK activation by nonactivated integrins is dependent on PKCα and independent of talin

The full activation of FAK by nonactivated integrins seemed to follow similar steps as by active integrins and lead to the activation of paxillin. However, on EV1 the phosphorylation of FAK and paxillin started to decline around 30-60 min after cell plating. Nonactivated integrins thus seemed to induce a transient activation of signaling and we wanted to know if there were critical differences between the adhesion complexes made of nonactivated and activated integrins. FAK can directly bind to β-tails (Schaller et al., 1995b), but it is more likely recruited to cell membranes by talin (Frame et al., 2010), paxillin (Brami-Cherrier et al., 2014) or membrane bound phosphatidylinositol PIP_2 (Goni et al., 2014). Several papers have shown that talin binding to integrin tails precedes the recruitment of FAK to focal adhesions. However, talin is an integrin activating protein, and here we were dealing with nonactivated integrins. When we looked at immunofluorescence images we saw that talin strongly colocalized with α2-integrin-rich areas on an EV1 coating (II, Fig. 3C). Similarly, loss-of-function α2-integrins in SaOS-cells (II; Fig. 3D,E) colocalized with talin. However, neither integrin nor talin was recruited to focal adhesions in SaOSα2E336A cells, even though these cells have other collagen binding integrins that can mediate cell adhesion. We silenced talin with talin-1 specific shRNA to see whether talin is also required for the FAK activation mediated by nonactivated integrins. Talin silencing was very efficient, almost 90 %, but it did not affect FAK activation (II, Fig. 3F). Even though talin seemed to be able to interact with nonactive integrins, we couldn't confirm a direct connection between talin and integrin tails, since talin was not required for the activation of FAK.

Since cells attached on EV1 did not form focal adhesions or actin stress fibers, we hypothesized that these adhesions lack tension. Interestingly, another focal adhesion protein, vinculin, was readily recruited to focal adhesions in both SaOS-α2 and SaOS-α2E336A cells on collagen, but did not colocalize with nonactivated integrins on an EV1 or collagen coated surface (II; Fig. 3C,D,E). The lack of vinculin colocalization with nonactivated integrins further suggested that these adhesions dot not mature similarly to the adhesions mediated by activated integrins.

PKCα is important for the EV1 life cycle and the inhibition of PKC activation by safingol inhibits virus entry into SaOS-α2 cells (II, Fig. S4A) (Pietiäinen et al., 2004; Upla et al., 2004). Furthermore, PKCα is activated when EV1 binds to α2-integrins (I, Fig. 6C). In our model system, safingol reduced the activation of FAK by nonactivated integrins (II, Fig. 3G), indicating that PKC activation is required for FAK activation. Also Rac1 plays a role in EV1 internalization through micropinocytosis (Karjalainen et al., 2008) and a Rac1 inhibitor completely prevents infection (II, Fig. S4B). However,

Rac1 activation seems to take place after FAK activation, since inhibiting Rac1 did not affect the activation of FAK on EV1 (II, Fig. 3H).

Together these results show that nonactivated α2-integrins can bind to EV1, and furthermore, cell adhesion mediated by nonactivated integrins can induce cell signaling. Nonactivated integrins do not, however, promote the formation of the mature focal adhesions that are associated with actin stress fibers and recruit talin and vinculin. Instead, the clustering of nonactivated integrins induces the transient activation of FAK and paxillin in a PKCα-dependent manner.

5.3 Nonactivated α2-integrins can bind to collagen I under flow (III)

The adhesion of platelets to injured vessel walls requires the interplay of integrins and other receptors. αIIbβ3-integrins mediate the binding of activated platelets to serum fibrinogen, and fibrinogen causes the aggregation of platelets (Sorrentino et al., 2015). α2β1-integrins together with GPVI, are collagen receptors on platelets. Collagen, unlike fibrinogen, is not present in the serum but becomes exposed only when there is a leak in the vessel wall due to wounding or an atherosclerotic plaque (Farndale et al., 2004). Small molecule inhibitors against α2-integrin were developed in collaboration with Biotie Therapies in the interest of preventing thrombosis in atherosclerosis patients. These inhibitors can also be used to study the mechanisms of ligand binding in integrins.

5.3.1 Integrin small molecule inhibitors BTT-3033 and BTT-3034 bind to different conformations of α2-integrin

Two small molecule inhibitors for α2-integrin, BTT-3033 and BTT3034, were found to selectively block α2-mediated cell adhesions to type I collagen (III, Fig. 1A-D). However, only BTT-3033 could effectively inhibit platelet binding to collagen I coated capillaries (III, Fig. 2A-D). The measurement was performed using a microfluidic system (Cellix) where human or mouse blood, or alternatively suspended cells, can be directed to pass through capillaries coated with e.g. collagen molecules. Platelet binding in this microfluidic system thus mimics their binding in blood vessels. When the binding of inhibitors to the α2-I-domain was studied more closely, gain-of-function activation of the I-domain was found to reduce the binding strength of BTT-3033 (III, Fig. 3B), indicating that BTT-3033 could not target the open I-domain conformation as effectively as the closed I-domain conformation.

5.3.2 BTT-3033 binds to the inactive integrin conformation and prevents platelet binding to collagen I under flow

To study the effect of conformational activation on the ability of α2-integrin to bind collagen under flow, we took advantage of CHO cells transfected with gain-of-function (E318W) and loss-of-function (E336A) α2 (III, Fig. 4A). The binding of CHO-α2 cells to collagen coated capillaries was shown to increase over time (III, Fig. 5A), and the binding could be blocked by an α2-integrin specific antibody (III, Fig. 5B).

Interestingly, the gain-of-function mutation E318W in the I-domain did not bring any advantage to cell binding to collagen under flow conditions (III, Fig. 5C) although it clearly increased cell binding under static conditions (I, Fig. 2). In addition, cells transfected with the α2-integrin loss-of-function mutation E336A could, under flow, bind to collagen as well as did cells expressing the wildtype integrin (III, Fig. 5C), although under static conditions the loss-of-function mutation clearly reduced binding (I, Fig. 3C). Finally, only BTT-3033 could prevent the binding of CHO-α2E336A cells to collagen (III, Fig. 6A), whereas BTT-3034 had no effect (III, Fig 6B). These results clearly suggest that the binding of platelets to collagen type I can be mediated by α2-integrin in its nonactivated conformation.

5.4 Tumor promoter TPA induces cell spreading and the formation of lamellipodia (IV)

When cells adhere to the ECM, integrins bind ECM molecules, become activated and cluster (Hynes, 2002). This enables the formation of focal adhesions through the recruitment of other proteins to bind to integrin tails and to form scaffolds. Integrin primers, talin and kindlins, play a role in the initial binding of integrins to the ECM by promoting the pre-activation of integrins. In the next series of events integrin mediated adhesions occupy FAK, paxillin and other kinases to induce signaling (Legate et al., 2009). The formation of focal adhesions has been studied by different approaches. We were interested in studying how the manipulation of integrin activity and ligand independent clustering prior to cell adhesion changes the recruitment of focal adhesion components. We manipulated integrin conformation with mutations, and additionally treated the cells with TPA, which has been shown to induce the ligand-independent clustering of integrins (Connors et al., 2007; Detmers et al., 1987) and increase the affinity of integrins for their ligand (Schürpf and Springer, 2011; Shimaoka et al., 2006).

5.4.1 Integrin pre-activation (E317A) induces cell adhesion, but does not change the appearance of cell adhesions or increase cell spreading

We have previously introduced mutations to the α1I-domain that mimic different integrin conformations (Lahti et al., 2011). α1E317A opens the I-domain αC-helix and increases collagen binding to the I-domain. The mutation α1E335A, on the other hand, lacks the important glutamate which, as an intrinsic ligand, pushes the α- and β-tails apart thus activating the integrins. In addition to these modifications, we have constructed a double mutant α1E335A/E317A that has an increased ability to bind to a ligand but simultaneously lacks the glutamate needed to fully activate the integrin. Cells expressing these three mutated integrins can spread on type IV collagen, although with different maximal spreading areas (Lahti et al., 2011). Interestingly, when we imaged the cells with confocal microscopy, the morphology of the double mutant α1E335A/E317A closely resembled that of the cells spreading on EV1, especially when cells were additionally treated with TPA to induce integrin clustering (Henri Niskanen, Pro gradu 2011). In both systems integrins can bind to a ligand and form clusters (α2-EV1, opened I-domain-Col IV), but they are not conformationally

activated (EV1 does not induce activation, E335A prevents activation). In addition, PKCα signaling is activated either by EV1 or TPA mediated stimulation. TPA activates PKC isoforms by mimicking a natural PKC activator diacylglycerol, which is produced in cells by the hydrolysis of PIP_2. TPA does not affect the turnover of phosphatidylinositols (Castagna et al., 1982), and thus does not perturb the association between the cell membrane and focal adhesion proteins.

We wanted to compare the composition of focal adhesions mediated by integrins locked in different conformational states and see how additional TPA mediated clustering and activation of integrins affects the formation of focal adhesions. We chose CHO cells lacking endogenous collagen receptors that were transfected with α1wt, α1E335A, α1E317A, and α1E335A/E317A. Cells were allowed to spread on type IV collagen for 1 hour, a time during which cells reach a maximal spreading area according to xCelligence studies (Lahti et al., 2011). We decided to isolate focal adhesions with a water spray based method developed by the Waterman lab (Kuo et al., 2011), and analyze isolated focal adhesions with mass spectrometry. At this stage we found that although the cells expressing α1E335A or α1E335A/E317A could spread on type IV collagen, they did not form adhesions strong enough to be collected and analyzed. Thus the adhesion complexes formed by conformationally nonactivated integrins must be analyzed using a different method, such as by fishing the cross-linked adhesion proteins with magnetic beads.

Interestingly, the pre-activation of α1-integrins by the E317A mutation did not affect the formation of focal adhesions as such, as could be seen by comparing the confocal microscopy images of CHO-α1 and CHO-α1E317A cells (IV, Fig. 2). In addition, when using a mixed population of CHO cells transfected with α1- and α1E317A-integrins we could not see notable differences in the speed of cell spreading (measured as cell index slope 0 to 15 min) or in the maximum spreading area either (measured as the maximum cell adhesion index)(Table 2). Similarly, the α2-integrin mediated cell spreading to collagen type I was not faster in cells expressing an integrin with the gain-of-function mutation (III, Fig. S3). In a plate-and-wash type of assay where the first 15 minutes of cell adhesion is measured, the gain-of-function α1 could mediate stronger binding to type IV collagen (Table 2), similarly to α2 binding to type I collagen (I, Fig. 2). These data suggest that in the case of collagen binding integrins, displacing the α-C helix and partial downward movement of the α7-helix, that opens the I-domain, improve integrin binding to collagen, but these changes do not dramatically affect the formation of cell adhesions or induce cell spreading.

Table 2. The effect of the gain-of-function mutation on adhesion and spreading in CHO-cells

Cell line	Cell spreading: Slope 0 to 15 min	Cell spreading: Max area	Cell adhesion: Adherent cells
CHO-α1	0.49±0.18	0.64±0.13	0.26±0.16
CHO-α1E317A	0.43±0.08	0.56±0.15	0.39±0.16
	n=4, p=0.31	n=4, p=0.16	n=4, p<0.001

5.4.2 TPA treatment immediately modifies the cytoskeleton and induces cell spreading

The introduction of the pre-activating mutant E317A to α1-integrin did not change the morphology of cells or cell adhesions as such (IV; Fig. 2A,B upper panels). In contrast, TPA had dramatic morphological effects on the cytoskeleton and cell adhesions (IV; Fig. 2A,B lower panels). In TPA treated cells, actin stress fibers were mainly absent. Instead actin framed the edges of the cells (IV; Fig. 2A,B), and also cell adhesions were rearranged to follow actin like a strand of pearls (IV; Fig. 2A,B). TPA has been shown to activate integrins (Shimaoka et al., 2006) and induce their clustering (Connors et al., 2007; Detmers et al., 1987). Here, however, integrins were already pre-activated suggesting that integrin activation is not the main mechanism for how TPA increases cell spreading and reorganizes the actin cytoskeleton.

Cell spreading was induced when cells were pre-treated with TPA for 5 to 10 minutes prior cell plating (IV, Fig. 3A-C). However, we wanted to see if TPA can affect cell spreading and cell adhesions also when spreading and adhesion formation had already started. We added TPA to cells prior to cell plating, and also 30 or 60 minutes after plating. TPA treated cells were followed up to 90 minutes with xCelligence, which measures real time cell adhesion, and in parallel fixed for microscopy. The addition of TPA increased the speed of cell spreading significantly, independent of the time when it was added (IV; Fig. 4A,B). A similar effect was seen in confocal microscopy images. A TPA treatment prior to plating or the addition of TPA after 30 or 60 minutes produced similar morphological changes in focal adhesions and the actin cytoskeleton (IV, Fig. 4C). Thus, TPA can instantly affect cell adhesions and the actin cytoskeleton connected to them regardless of the stability of adhesions at that point.

5.4.3 TPA affects the composition of α1-integrin mediated cell adhesions and significantly increases the amount of kindlin-2 and reduces the amount of vimentin

As was described earlier, we performed a mass spectrometry analysis to study the composition of focal adhesions in CHO-α1 and CHO-α1E317A cells, and looked at this data to gain a more precise idea of how focal adhesions are changed after the addition of TPA. Focal adhesion related proteins were selected by comparing the peptide list to a published integrin adhesome (Table S1, online version). The enrichment or reduction of each protein was compared to the amount of α1-integrin in the same sample (Table S2, online version).

The increased amount of kindlin-2 and the reduced amount of vimentin were the most significant changes in the composition of cell adhesions induced by TPA (IV, Fig. 5B). Kindlin-2 is an integrin activating protein that has been described to assist talin in integrin priming, but also to stabilize integrin clusters by crosslinking integrin tails (Ye et al., 2011). Both of these functions of kindlin-2 make it a strong candidate for mediating the effects of TPA, particularly the activation and clustering of integrins. A literature search also revealed that vimentin intermediate filaments are directly in contact with cell adhesions (Bershadsky et al., 1987; Gonzales et al., 2001).

Remarkably, the disconnection of vimentin has been reported to induce the formation of lamellipodia (Helfand et al., 2011). We decided to study the roles of kindlin-2 and vimentin in TPA induced cell spreading in more detail.

Cytoskeletal rearrangements were substantial in TPA treated cells. Actin microfilaments were pushed to the cell edges and vimentin filaments were retracted closer to the nucleus. Interestingly, we could detect that also the amount of plectin was reduced in focal adhesions after a TPA treatment, although this did not meet statistical significance (IV, Fig. 5B). Plectin is known as a connector protein for the cytoskeletal proteins actin, vimentin and tubulin. Plectin has been reported to link vimentin to focal adhesions and also to the actin cytoskeleton (Wiche et al., 2015). Recent work reported a role for vimentin in controlling lamellipodial actin arcs and the retrograde flow of actin, and for plectin as a mediator between vimentin and actin stress fibers (Jiu et al., 2015). Here, we did not study the role of plectin in more detail but hypothesized that it could play a role as a linker protein between vimentin and focal adhesions and also between vimentin and the actin cytoskeleton.

According to our proteomics analysis of TPA modified α1-integrin adhesions, the amount of calreticulin bound to α1-integrin was significantly reduced in three out of four experiments. An opposite observation has been made with α2-integrin in TPA treated Jurkat cells, where TPA increased the amount of calreticulin bound to the α2-subunit (Coppolino et al., 1995). Calreticulin has been reported to bind to the conserved area in the α-tail (Rojiani et al., 1991) and have a role in cell attachment to collagen (Coppolino et al., 1997). The interaction of calreticulin with integrin tails is thought to be transient, and the contacts are released as cell spreading proceeds (Coppolino and Dedhar, 1999). What makes calreticulin an interesting protein is its upregulation in several cancers. Calreticulin upregulation is connected to processes that induce cancer metastasis and invasion (Zamanian et al., 2013). Since these processes are induced in EMT, the role of calreticulin in the formation of lamellipodia would be interesting to study further. Here, however, the role of calreticulin in integrin adhesions was left for future studies.

A significant decrease in the recruitment of integrin α5 to adhesions after a TPA stimulation was also observed in three out of four experiments. α5-integrin is a fibronectin receptor and should not play a major role in cell adhesion to type IV collagen. However, it is possible that the binding of α1-integrin to type IV collagen induces the recruitment of other integrins to form adhesions in a ligand independent manner, and perhaps the recruitment is not limited to a single integrin subtype. The α1-tail is the shortest of all integrin α-tails and its ability to recruit focal adhesion proteins might be limited. By recruiting other integrin subtypes as well, the adhesion complexes could be more versatile in their functions. Why would the TPA treatment then reduce the relative number of α5-integrin? The question remains unanswered. One reason could be that this integrin stabilizes the connections with vimentin intermediate filaments. Also the trafficking of α5β1 during cell spreading and migration could explain why its numbers were reduced in adhesions. α5-integrin has been shown to be

trafficked to the cell rear during migration (Theisen et al., 2012) and it could thus be absent from protruding adhesions when TPA induces cell spreading.

5.4.4 Kindlin-2 is required for cell spreading, but kindlin-2 recruitment is not necessary for TPA effects

Kindlins are integrin activating proteins that have also been described to induce integrin clustering (Ye et al., 2011). Since TPA induces, independently of ligand binding, both integrin activation and clustering, recruiting kindlin-2 could be the mechanism for how TPA clusters and activates integrins through inside-out activation.

To study the importance of kindlin-2 in TPA induced cell adhesion, we designed siRNAs to silence kindlin-2 in CHO cells expressing pre-activated and wild type α1-integrins. Kindlin-2 silenced cells adhered to type IV collagen and initiated cell spreading (IV, Fig. 6B-E), but the formation of actin stress fibers and focal adhesions was prevented (IV; Fig. 6D,E). Kindlin-2 silenced cells were not dramatically smaller in size, but this might result from the small size of CHO cells in general. Integrin pre-activation by the mutation E317A did not rescue kindlin-2 silencing (IV, Fig. 6B-E). Although the pre-activation of integrin α1 does not induce the full activation of integrins, these results suggest that in this system the role of kindlin-2 is not to conformationally activate integrins to promote ligand binding. Instead, here kindlin-2 was required for full cell spreading, for the formation of focal adhesions and for the linking of actin stress fibers to these adhesions. Remarkably however, when we treated kindlin-2 silenced cells with TPA, we could no longer detect differences in cell morphology between kindlin-2 silenced cells and cells treated with non-targeting control siRNA (IV; Fig. 6D,E). The TPA treatment could completely overcome the effect of kindlin-2 silencing, so even though kindlin-2 mediates integrin clustering in TPA treated cells, its presence is not necessary. In another study, kindlin-2 silencing reduced cell spreading and adhesion formation as well. Kindlin-2 was found to recruit paxillin and induce FAK activation in focal adhesions. Moreover, FAK activation or overexpression as such, could rescue the defects in cell spreading caused by the absence of kindlin-2 (Theodosiou et al., 2016). It is possible that the TPA treatment can directly activate FAK, since TPA phosphorylates PKCα, which can act upstream of several protein kinases, and ultimately induce cell spreading without kindlin (or with reduced amount of kindlin present). We showed in the case of EV1 that PKCα activation was required for FAK activation (II, Fig. 3G).

5.4.5 TPA causes the retraction of vimentin filaments from cell adhesions

A mass spectrometric analysis of focal adhesions revealed that a TPA treatment decreases vimentin in focal adhesions (IV, Fig. 5B). In accordance with, confocal microscopy images showed that vimentin filaments were localized closer to the nucleus and did not reach focal adhesions similarly to untreated cells (IV; Fig. 1A,B). The TPA treatment seemed to induce the retraction of vimentin from cell adhesions. In addition, vimentin was clearly absent from strong lamellipodia-like cell protrusions. Earlier studies have suggested that vimentin filaments are connected to focal adhesions, and that the activation of integrins would disturb this connection (Kreis et

al., 2005). However, vimentin filaments were organized in a similar manner in cells expressing wildtype and pre-activated integrins, suggesting that integrin activation is not the main cause of vimentin retraction. Another recent study showed that the local dissociation of vimentin filaments at the cell edges strongly induces the formation of lamellipodia and cell spreading (Helfand et al., 2011). Our results support this study and show that vimentin is really dislocated from focal adhesions during cell spreading.

A study of human keratinocytes isolated from patients diagnosed with Kindlers syndrome, thus lacking kindlin-1, showed that kindlin-1 is required for the normal formation of lamellipodia and directed cell migration. More importantly, the activation of RhoGTPases Rac1 and RhoA, and to some extent CDC42, were reduced to 20% of the normal condition (Has et al., 2009). Also silencing of kindlin-2 has been shown to decrease Rac1 activation (Jung et al., 2011). Interestingly, Helfand and colleagues showed that Rho GTPase Rac1 directly phosphorylates vimentin causing its depolymerisation. Although the silencing of kindlin-2 could not prevent the TPA mediated effects, the recruitment of kindlin could be one of the steps in a pathway mediating TPA functions. TPA might also phosphorylate Rac1 directly, thus kindlin-2 would not be necessary for inducing cell spreading. TPA activated PKCα can be upstream of Rac1 as well as FAK (Kim et al., 2008). The inhibition of Rac1 or the activation of FAK with chemical inhibitors could clarify the mechanism of TPA mediated affects, and also the course of lamellipodia formation.

5.5 PIM kinases regulate integrin mediated cell adhesion (V)

PIM kinases, PIM1, PIM2 and PIM3, are serine/threonine kinases with overlapping functions. PIM kinases are overexpressed in several cancers, such as leukemia and prostate cancer, where they induce cell proliferation and prevent apoptosis (Santio et al., 2010). PIM overexpression has been shown to follow cancer treatments and promote cancer cell survival and cancer renewal (Zemskova et al., 2008). Thus PIM kinases are potential targets for cancer drugs, usually in combination with other drugs (Blanco-Aparicio and Carnero, 2013). PIM kinases are constantly in the active conformation, and their gene expression levels control their activity. A PIM kinase inhibitor DHPCC-9, which targets all PIM isoforms (1, 2 and 3), inhibits the migration of cancer cells and invasion without affecting cell viability or proliferation, or overall expression levels of PIM kinases. Similarly, the overexpression of any of the isoforms induces cell migration (Santio et al., 2010). Treating mice with a PIM-inhibitor prevents tumor growth and cancer metastasis to the lungs (Santio et al., 2015).

α2-integrin is first downregulated in primary prostate tumors, but then upregulated in metastatic prostate cancer cells, and also in cancer stem cells (Collins et al., 2005). Prostate cancer often metastasizes to the bone, which is rich in the α2-integrin ligand, collagen type I. PIM kinases regulate the homing of bone marrow cells and the inhibition of PIM kinases efficiently prevented PC-3 cell metastasis to the lungs (Santio et al., 2015). Interestingly, the prostate cancer drug docetaxel was shown to increase the expression levels of PIM kinase in prostate cancer cells, such as DU145. The PIM kinase activity protected the cells from the cytotoxic events of the cancer drug (Zemskova et al., 2008). Data from our research group shows that DU145 cells

surviving the docetaxel treatment have an α2-integrin high subpopulation that could represent a cancer stem cell population (Elina Taipalus, Pro gradu 2015).

PIM kinases regulate the same targets and events in cells as α2β1 and other integrins, although the interplay between these two has not been reported. Taken together the earlier findings, there is plenty indirect evidence that PIM kinases regulate integrin dependent cell processes. Thus it was of interest to study whether PIM kinases can regulate α2-integrin functions in prostate cancer cells

5.5.1 Inhibition of PIM kinases prevents integrin mediated cell adhesion

Santio and colleagues found that the inhibition of PIM kinases remarkably reduced PC-3 cell migration in a wound closure assays (Santio et al., 2010). Due to the connection between PIM kinases and integrins, and since integrins are important mediators of cell migration, we decided to study the role of α2-integrins in PIM kinase regulated cell adhesion and migration. We saw that inhibiting PIM kinases with DHPCC-9 24 hours prior to cell plating, remarkably reduced α2-integrin mediated cell spreading on collagen type 1 (V, Fig. 7B). Since PIM kinases are transcription factors, we hypothesized that inhibiting PIM kinases could reduce the amount of surface integrins thus affecting cell adhesion. This was not, however, supported by the finding that also a 1 hour treatment with DHPCC-9 reduced cell spreading, and this time is too short to affect integrin expression (V, Fig. 7A). We confirmed with a flow cytometric analysis that there was no change in the surface expression of α2-integrins after a 1 or 24 hour pre-treatment with DHPCC-9 (V; Fig. S5,6). So, reduced integrin expression or induced integrin internalization were excluded as reasons for the reduction in cell spreading.

We were also interested to see, whether PIM kinases regulate α2-integrins specifically. We plated DHPCC-9 cells into fibronectin coated wells and followed whether spreading through fibronectin binding integrins is affected. As a control, we plated the cells onto poly-l-lysine, on which cells spread in an integrin independent manner. We saw a similar reduction in cell spreading to fibronectin, but no effect in cell spreading to poly-l-lysine. This indicates that PIM kinases affect specifically integrin mediated cell spreading, but they have an effect on several integrin subunits (V; Fig. 7A,B). With a flow cytometric analysis we also confirmed that surface levels of α5-integrins (not shown) or β1-integrins (V, Fig. S6) were not changed after treatment with an inhibitor.

Glycogen synthase kinase 3 beta (GSK3β) and FOXP3 were identified as targets for PIM kinases in this study. Both of these regulate the expression of prostaglandin-endoperoxide synthase 2 (PTGS2, COX-2), that is involved in β1-integrin mediated cell migration and invasion, and also influences integrin expression. Inducing the expression of *PTGS2* for 24 h has been shown to induce the expression of α2-integrin and α2-mediated cell spreading (Liu et al., 2010). Thus PTGS2 could be affected by the inhibition of PIM kinases and mediate the effect of PIM kinases to integrin mediated cell adhesion. We tested whether inhibiting PTGS2 with the selective

inhibitor celecoxib, affects integrin mediated cell adhesion. The 1 hour treatment with the inhibitor had no effect, and the 24 hour treatment affected also cell spreading on poly-l-lysine (V, Fig. S4). Thus the inhibition of PTGS2 is an unlikely outcome for the inhibition of PIM kinases, which affects integrins already after a 1 hour treatment.

5.5.2 PIM kinases do not regulate the conformational activity of integrins

Another way how PIM kinase inhibition could affect integrin mediated processes is the conformational inactivation of integrins. We treated cells with DHPCC-9 and analyzed the integrin activation state with flow cytometry using conformation specific antibodies (12G10: active β1, Mab13: total β1), and Mn^{2+} and EDTA as positive controls for activation and inactivation, respectively. We saw the effects of Mn^{2+} and EDTA on integrin activation, but no change in the conformation was seen after treatment with the PIM inhibitor DHPCC-9, indicating that PIM kinases do not regulate the conformational activation of α2-integrins (V, Fig. S6).

Since we could not observe changes in the surface levels or activation state of integrins, it is most likely that PIM kinases affect the adhesion complexes that bind integrin tails during cell attachment and migration. In the interest of time, the research was not carried on further, but it leaves interesting questions for future studies. Although we did not solve the molecular details, we demonstrated that PIM kinase signaling is connected to the integrin mediated regulation of cell adhesion and migration. We found that GSK-3β, an Akt target molecule that we have showed to be involved in α2-integrin signaling (Ivaska et al., 2002), is a PIM substrate. This brings PIM kinases close to the regulation of focal adhesions and future work will shed light on the details on how PIM kinases modify focal adhesions and the cytoskeleton during cell migration.

6 DISCUSSION AND GENERAL PERSPECTIVES

6.1 Nonactivated integrins can bind ligands and transmit cellular signaling

6.1.1 Nonactivated integrins as virus receptors

We showed that human EV1 binds to the bent conformation of α2β1-integrins while interacting with the closed ligand binding I-domain (I). Several integrin binding viruses have an RGD-motif in their protein capsid that mimics the natural ligand of integrins, and integrins bind these viruses in a similar mechanism as they bind ECM ligands. However, the situation could be different in the case of I-domain containing integrins and non-RGD-dependent viruses.

Non-RGD viruses bind to α1β1-, α2β1-, α4β1-, α6β1-, α9β1-, αxβ2- and α6β4-integrins (Hussein et al., 2015). Of these, only α1β1 and αxβ2 contain the I-domain, and possibly interact with viruses in a similar manner as α2β1. α1β1 is the only integrin type receptor for the Ross River virus, and according to current knowledge, it does not bind other viruses (Hussein et al., 2015). Binding of the Ross River virus to α1β1-integrin was enhanced in the presence of Mn^{2+} and an integrin activating antibody (9EG7), and blocked by EDTA, indicating that virus binding in this case requires receptor activation. The authors also suggested that part of the virus protein capsid mimics the extracellular matrix ligand, type IV collagen (La Linn et al., 2005). In addition to EV1, other α2β1-integrin binding viruses include human cytomegalovirus (HCMV), rotaviruses and simian virus 40 (Hussein et al., 2015). Interaction between HCMV and α2 might be difficult to decipher, since HCMV mainly interacts with cells through viral glycoproteins and heparin sulfate glycosaminoglycans on the cell surface, and in addition HCMV binds to EGFR, PDGFRα and BST2/Tetherin, and other integrins such as α6β1 and αvβ3 (Vanarsdall and Johnson, 2012). Rotaviruses use α2β1-integrins as key receptors during an infection. Several human rotavirus strains were shown to prefer the active integrin conformation in binding to integrins, but they did not seem to drive integrin activation since they did not induce p38 MAPKinase signaling. Furthermore, rotaviruses were dependent on different sites in the α2I-domain than were EV1 or collagen. Rotaviruses thus present a novel type of ligand binding to the integrin α2I-domain, but they still favor the active conformation (Fleming et al., 2011). For SV40, α2β1 is also the only currently known integrin type cellular receptor (Hussein et al., 2015). The conventional cell surface receptor for SV40 is glygosphingolipid GM1, and although α2 was reported to bind to SV40, and virus entry was found to be dependent on α2 (Stergiou et al., 2013) the exact mechanism for an SV40 infection is not known. It would, however, be interesting to see whether cells expressing an inactive form of the integrin, can be infected by SV40. Although EV1 has evolved to recognize an inactive integrin heterodimer and the closed I-domain, this doesn't seem to be a general phenomenon amongst viruses interacting with I-domain integrins.

Interestingly, there is another family of viruses, the pathogenic hantaviruses, that interacts with inactive rather than active integrins. Hantaviruses bind to αVβ3-integrins, but were found to interact with the PSI-domain instead of the ligand binding site in these integrins. The residues in PSI domain are available for the virus particularly when integrins are in the bent conformation. The virus can infect cells expressing only the loss-of-function αVβ3-integrin, but not cells expressing a gain-of-function mutant. Furthermore, by locking integrins into the bent conformation, the interaction between the virus and cell surface integrins disrupts normal cell functions, causing the severe symptoms of a hantavirus infection (Raymond et al., 2005).

Viruses have learnt to exploit host cell receptors in various ways. The two examples of viruses binding to inactive integrins using noncanonical ligand binding sites reminds researchers to pay attention to unusual binding mechanisms in virus-cell interactions.

6.1.2 Currently known interactions mediated by the inactive integrin conformation

Although the inactive integrin conformation has been described as a nonfunctional molecule, there are recent studies showing the importance of conformationally inactive integrins in regulating cellular functions.

A recent study demonstrates that the inactive α5-integrin conformation regulates fibronectin fibrillogenesis in the developing embryo. Fibronectin is synthesized on the tissue surface but not inside the tissue, and this spatial regulation of fibronectin fibrillogenesis was found to be regulated by the interaction of conformationally inactive α5-integrins on adjacent cells. Cadherin-2, which is mostly found in cell-cell contacts, stabilized the interaction between the two integrins. Furthermore, fibronectin was not necessary for the induction of the conformational activation of integrins, once the inhibition between adjacent integrins was released, indicating that integrins were mainly activated and clustered through the inside-out mechanism. If conformationally active integrins were present inside the tissue at cell-cell contacts, this led to aberrant fibronectin assembly (Jülich et al., 2015).

Also αvβ3-integrins can indirectly regulate matrix remodeling in their inactive conformation. Outside of focal adhesions, inactive αvβ3-integrins are bound in *cis* to Thy-1, a glycosphosphatidylinositol-anchored glycoprotein, and this interaction supports the tethering of inactive integrins to raft areas. Thy-1 also reduces the rate of integrin binding to fibronectin, and especially on soft surfaces inhibits integrin activation and fibronectin assembly, which would otherwise lead to increased ECM rigidity and changes in integrin signaling (Fiore et al., 2015). In lung cells, the loss of Thy-1 leads to stiffening of the matrix and the formation of fibrotic lung tissue (Hagood et al., 2005). However, in cancer associated fibroblasts, Thy-1 is often overexpressed, although also the cancer matrix is usually stiffer than the normal tissue (True et al., 2010). Also FAK and Src signaling are elevated in the absence of Thy-1 (Fiore et al., 2015), rather suggesting that Thy-1 should be anti-tumorigenic. An inactive αvβ3 in complex with Thy-1 recruits the Fyn kinase to membrane rafts, and preassembles signaling molecules required for the assembly of focal adhesions and

integrin signaling. Interestingly, the study proposes that Src can directly bind inactive integrins and that the clustering of integrins would enable clustering dependent Src activation (Fiore et al., 2015).

Although these two studies do not directly report cellular signaling mediated by the inactive conformation of integrins, they point out that the inactive conformation is also functional. This supports our perception of a nonactivated, rather than inactive, bent integrin conformation.

6.1.3 The benefit of the nonactivated integrin conformation

In an echovirus 1 infection

In the case of echovirus 1, its ability to interact with nonactivated α2β1-integrins can bring an advance in the competition for receptor binding. Natural ligands such as collagens readily accommodate activated receptors. According to our studies, echovirus 1 does not bind to ligand activated open I-domain (I). This would mean that echovirus 1 rather interacts with unoccupied α2-integrins. Echovirus 1 lacks the RGD-domain in its surface proteins that several other viruses use to bind to integrins. The ligand binding MIDAS site in the α-I-domain is too small for a large ligand, such as a virus to bind to, if the virus capsid lacks a protruding glutamate that can coordinate metal ions properly and induce the opening of the I-domain and the concomitant activation of the integrin conformation. Echovirus 1 interacts with a large interface of the I-domain (Xing et al., 2004), and this interaction does not trigger the movement of the α7-helix, which is required for the amino acid residue E336 to function as an intrinsic ligand for the β-I-domain.

EV1 might gain another advantage from binding to a nonactivated conformation of α2β1-integrins. Namely, EV1 enters the human body through the gastro-intestinal tract, which is lined with epithelial cells (Krieger et al., 2013). In the epithelial cells, most of the integrins are expressed at the basal surface, but small pools of α3β1- and α2β1-integrins are expressed also on the apical surface of the cells (Zuk and Matlin, 1996). On the apical surface, α2β1-integrins reside as a pool of bent integrins that do not have ligands such as collagen to bind to. The pool of nonactivated integrins on the apical surface is suggested to, through lack of signaling, participate in defining the apical-basal orientation of the epithelial cells (O'Brien et al., 2002). By binding to the bent α2, EV1 has a better chance of occupying a receptor and entering cells. In addition, nonactivated and active integrins are internalized through different routes that could also affect later events in virus entry and reproduction (Arjonen et al., 2012).

In platelet function

The role of αIIbβ3-integrin in platelet activation and aggregation has been studied extensively and is known in detail. During platelet activation in response to injury in the vessel wall, αIIbβ3-integrins are conformationally activated through inside-out signaling and they bind to serum fibrinogen and fibronectin. Several platelets aggregate

around fibrinogen and fibronectin molecules and form a clot sealing the leakage (Sorrentino et al., 2015). The activity of platelet integrins, similarly to leukocyte integrins, needs to be strictly regulated to prevent ligand binding under the wrong circumstances, since the excess binding of platelets leads to the formation of thrombi and can be lethal. This rule specifically applies to integrins such as αIIbβ3 whose ligands are constantly present in the blood serum. Another important integrin in platelets, α2β1, mainly binds to collagen. For α2β1, the major ligand collagen is not present in healthy vessels. Instead, collagen is exposed after injury in the vessel wall, or during the development of atherosclerosis (Farndale et al., 2004). This could be the reason why α2β1 can bind to collagen without pre-activation under flow conditions; it does not need to be conformationally regulated. α2β1 binding to collagen without conformational activation of the integrin might also enable quick binding to exposed ECM protein that can then start the platelet rolling and the platelet activation cascade. Some previous studies stated that α2β1 is important for the initial attachment of platelets, but not for the platelet activation (Schmaier and Kahn, 2009). Overall many studies tried to discover the activation mechanism for α2 in the platelets, but failed to find any, and thus falsely concluded that this integrin is unimportant for platelet activation. However, others have proposed that perhaps α2β1-integrins do not require activation to bind to collagen (Mazzucato et al., 2009).

Platelets adhere to fibrinogen only at low shear rates, but can bind e.g. vWF (Sorrentino et al., 2015) and collagens (Farndale et al., 2004) also at higher shear rates. To be able to properly interact with fibrinogen, platelets seem to need to decelerate first. This can take place through rolling, similarly to leukocytes (Sorrentino et al., 2015), and could be mediated by low-affinity interactions between conformationally nonactivated α2β1-integrins and collagen. Platelet activation requires α2β1-mediated Ca^{2+} influx (Mazzucato et al., 2009), and Src activation (Inoue et al., 2003), as well as PKCα signaling (Li et al., 2010). Platelet αIIbβ3 is activated through inside-out signaling by talin and kindlin-3 binding (Moser et al., 2008). Although we do not exclude the importance of α2β1-integrin activation in mediating stronger platelet binding to collagen and full platelet activation, the primary interaction with collagen without α2 activation could lead to the activation of PKCα, Src and FAK, as we show in original publication II. Furthermore, TPA has been widely used to activate platelets prior to ligand binding, and in addition to inducing PKCα signaling, we showed that TPA increased the recruitment of kindlin-2 to integrin tails as well as the reorganization of actin cytoskeleton (IV), both of which are important for platelet activation (Sorrentino et al., 2015). So there is a possibility that nonactivated α2-integrins could induce PKCα signaling and increase kindlin recruitment to activate both of the platelet integrins.

Recent research reported that platelets expressing activation-deficient β1-integrins showed reduced platelet activation. The mutation prevented kindlin binding to integrin tails, and thus was concluded to inactivate integrins (Petzold et al., 2013). The authors showed that lesions had reduced numbers of platelets expressing an activation-deficient β1. However, this gives information about the total amount of platelets recruited, not the interactions of a single platelet. They also did not study platelet binding under flow

conditions. The mutation does not necessarily prevent the primary α2 interaction with collagen even though it abolishes firm adhesions. The authors stated that the interaction between integrin and kindlin was required for inside-out, but not for outside-in signaling or the reorganization of the cytoskeleton during spreading. A kindlin-nonbinding mutant could also induce platelet spreading, FAK phosphorylation and platelet activation, although the final level of platelet activation was reduced. This supports the idea that platelets can initially interact with collagen without the pre-activation of α2β1-integrin, but that also α2β1-integrins contribute to the full activation of platelets and require conformational activation of the receptor for this.

The mechanism for activating platelets should be revisited with an open mind towards the possibility that α2β1 can initially interact with collagen, under flow conditions, without integrin pre-activation.

6.2 Integrin mediated cell adhesion and spreading can be controlled at several levels

6.2.1 Do β1-integrins require conformational activation?

β1, to bend or not to bend? Whether β1-integrins adopt the bent conformation at all, was a matter of discussion for years in the integrin field. The notion that β3-integrins in platelets and β2-integrins in leukocytes require strict regulation of their activity to avoid unwanted binding, was widely accepted. For these two classes of integrins three different conformations, namely the bent-closed, extended-closed and extended-open conformation (refer to Figure 4) were shown with different approaches (Takagi et al., 2002). In addition, the bent-closed and extended-closed conformations were shown to have a lower affinity to ligands than the extended-open conformation. Until very recently, the ability of β1-integrins to adopt the bent conformation had not been demonstrated. The Springer lab finally showed that also α5β1-integrin has three conformations with different ligand binding abilities (Su et al., 2016). In the case of EV1 binding to the α2I-domain it was clear that the closed I-domain conformation better interacts with the virus. This was evident based on molecular modelling and binding studies. Whether integrins adopted a bent conformation was, however, shown only indirectly and we are still lacking direct evidence, such as electron microscopy data, of the conformation of the virus bound integrin. The fact that β1-integrins had not been shown to adopt the bent conformation, and that they were even thought to be constantly activated/extended, compromised our claim that nonactivated integrins can induce cellular signaling. However, in light of the new evidence, we can assume that EV1-bound α2-integrins can actually adopt the bent conformation. Although EV1 binding does not induce the separation of the integrin tails and induce E336-dependent signaling, it cannot be excluded that EV1-bound integrins could still fluctuate between the closed-extended and closed-bent conformation. We don't know whether the clustering of integrins by EV1 supports either of these conformations, and whether fluctuation could affect the signaling events mediated by nonactivated integrins.

Integrins have been described to fluctuate freely between different conformations, although the closed-bent conformation could be favored in the absence of ligand binding in β2-and β3-integrins, whereas the bent conformation in α5β1 seemed to be less stable (Su et al., 2016). Also the study demonstrating the regulation of α5β1-integrin through trans interactions between the bent integrin conformation, suggested that without the stabilization of the bent conformation, these integrins rather spontaneously undertook the activated conformation (Jülich et al., 2015). Could it be that β1-integrins are allowed to fluctuate more freely than β2- and β3-integrins? Are these integrins constantly activated through inside-out signaling? Some proteins, such as sharpin and filamin, have been shown to inactivate integrins by binding to integrin tails and by supporting the bent conformation (Liu et al., 2015b; Rantala et al., 2011b). Filamin, which binds to the β3-tail, was thought to tilt the balance of the integrin activation state towards the bent conformation, since in the absence of filamin, integrins were found to be more active at the cell membrane (Liu et al., 2015b). The fact that α2β1-integrins did not require conformational pre-activation to bind to collagen under flow (III) could result from two facts: 1) these integrins are constantly in the active conformation and do not adopt the bent conformation 2) the I-domain is flexibly attached to the integrin head domain and can bind to its ligand without integrin extension. Studies with αXβ2-integrins have showed that the I-domain is surprisingly flexibly attached to the β-propeller (see Figure 12) (Xie et al., 2010).

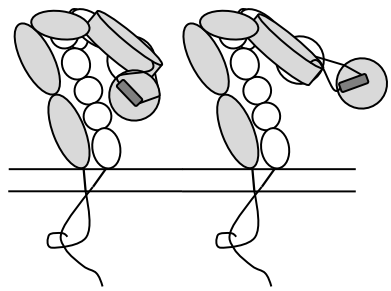

Figure 12. I-domain flexibility. The I-domain is flexibly bound to the β-propeller and could theoretically reach its ligand without integrin extension.

The flexibility of the I-domain is important for the ability of I-domain integrins to interact with bulky ligands (Xie et al., 2010). Furthermore, β1-integrins have now been shown to adopt the bent conformation. These facts would support the model where the α2-I-domain could bind to its ligand even though the integrins are in the bent-closed conformation. In addition, the αI-domains of collagen binding integrins bind their ligands relatively strongly also in the closed conformation, whereas the closed conformation of isolated leukocyte αI-domains bind their main ligands weakly (Lahti et al., 2013). However, in leukocytes, αLβ2-integrins that do not normally contribute to rolling adhesion were found to do so if the closed-extended integrin was induced. Closed-extended integrins increased cell rolling, but prevented adhesion, which still requires open-extended integrins. The closed-bent integrins didn't participate into rolling, and the mutation αLE310A completely prevented rolling adhesion. This was, however, restored by using an α/β-I-like allosteric agonist that bound to the β2

interface and induced the opening of the I-like-domain in the β-subunit and stabilized the extended conformation, but prevented the opening of the α-I-domain (Salas et al., 2004). It cannot be excluded that *in viv*o, during rolling adhesion, leukocyte integrins could also mediate low-affinity binding in the closed-extended conformation, but since this is not normally observed, β2-integrins might predominantly reside in the bent conformation. If α2β1-integrins can mediate platelet rolling adhesion in the bent conformation, there must be an explanation why αLβ2-integrins cannot do the same. Perhaps the closed I-domain affinity to its ligand is critically lower in leukocyte integrins preventing the bent-closed interaction with the ligand. Otherwise, the speculations would incline towards the assumption that β1-integrins undergo fluctuations more freely and interact with ligands in the extended-closed conformation without the additional pre-activation of the receptors.

6.2.2 Cell spreading and migration after established cell adhesion is controlled through the focal adhesion–cell cytoskeleton axis

The closed I-domain of collagen binding integrins binds to collagen relatively strongly. Despite this, the loss-of-function mutation in the α-I-domain that interrupts the cross-talk between the α- and β-subunits, inhibits cell adhesion and spreading under static conditions as has been shown with α2E336A binding to collagen I (I) and α1E335A binding to collagen IV (Lahti et al., 2011). However, cell adhesion is not completely prevented, although the strength of adhesion is compromised. The gain-of-function mutation in the αI-domain improves integrin binding to collagen both at the I-domain level (binding studies) and at the cellular level (plate-and-wash assays), as has been shown with α2E318W binding to collagen I (I, III) and α1E317A binding to collagen IV (IV, (Lahti et al., 2011)). However, cell spreading was not induced by the activating mutation α2E318W (III) or α1E317A (IV), indicating that even though integrin binding to a substrate is enhanced, cell spreading is no longer controlled by the activation state of the integrin head domain. TPA has been described to induce the activation of integrins, as well as cell spreading mediated by integrins. We could detect TPA induced cell spreading even when integrins were preactivated with the gain-of-function mutation. Furthermore, cell spreading was induced at the point where cells without treatment had already reached the maximum spreading area (IV). This suggests that cell spreading is not controlled by integrin activity, but rather by the connection between integrins and cytoskeleton, and that TPA can disturb this connection. Silencing of kindlin-2 reduced cell adhesion, and the formation of focal adhesions and actin stress fibers, even in cells expressing gain-of-function integrins (IV). This means that to establish primary cell adhesion and connections with the cytoskeleton, integrin activation and binding to a ligand is not enough, but that integrin-tail interactions with signaling molecules are required as well. In addition, the role of kindlin in adhesion stabilization rather than in integrin activation was emphasized.

The study with PIM kinase inhibitors showed that β1-integrin dependent cell spreading was reduced without affecting the surface expression levels or the conformational activity of β1-integrins. Even though we didn't explore the mechanism in more detail, we can hypothesize that PIM kinase inhibitors change the dynamics of either focal

adhesions or the cytoskeleton (V). PIM kinases have a role in cell adhesion and spreading, and it has been shown that PIM overexpression in tumors induces cell migration. Both TPA and the PIM kinase inhibitor seem to modify integrin mediated cell adhesion by affecting later events in integrin-ligand interactions and the formation of focal adhesions, not by directly modifying the binding affinity of the integrin ligand or integrin recycling. The effects of TPA were explained by modifications in the connections between the cytoskeleton and focal adhesions, since the recruitment of the intermediate filament vimentin was reduced. PIM kinases very likely have targets at focal adhesions or their mediators, such as GSK3β that was shown to be a substrate for PIM kinases (V). In summary, the conformational activation of integrins is not always the main facilitator in integrin mediated cell adhesion and spreading (Figure 13). Especially after cell adhesion is established, the focal adhesion–cell cytoskeleton linkage plays an important role in inducing cell migration and spreading.

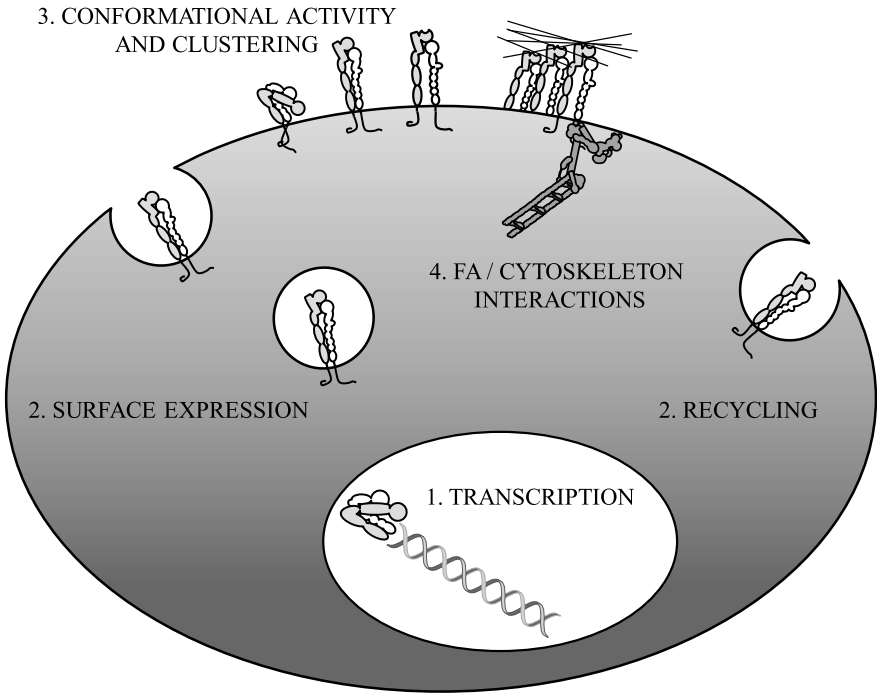

Figure 13. Integrin regulation occurs at different levels. The importance of integrin transcriptional regulation (1) is emphasized in cancer where integrins are often up- or down regulated. Integrin availability at the cell surface (2) critically affects cell adhesion through integrins. In addition, integrin recycling (2) plays important role during cell migration. Integrin conformational activation and receptor clustering are usually required for ligand interaction (3), and activation is strictly regulated in most integrins. Integrin connection to focal adhesions (FA) and cell cytoskeleton (4) regulates cell migration.

6.3 Aspects of integrin based therapy

Since aberrant integrin function is related to several pathological conditions, such as cancer invasiveness and metastasis, thrombosis, excessive bleeding, and defects in the

immune system, integrins are targets for drug development. Several integrin targeting blocking antibodies, peptides and small molecules against stroke, inflammatory bowel diseases, cancer, allergy and rheumatoid arthritis are under development (Hehlgans et al., 2007). αIIbβ3, α4β1 and α4β7 targeting molecules are currently in clinical use (Ley et al., 2016).

FAK in cancer

In 2D cell cultures, FAK promotes cell migration and EMT, and it is required for integrin mediated signaling. FAK signaling is not required for normal morphogenesis in mammary tissue, but increased FAK signaling is connected to tumor growth in the breast. FAK overexpression did not increase cancer cell proliferation, but it was shown to remarkably protect cancer cells from anoikis. Importantly, in normal mammary gland acini, FAK was phosphorylated only in cells in contact with the ECM, but FAK overexpression induced FAK phosphorylation throughout the acini (Walker et al., 2016). FAK is not mutated in cancer, but its transcription and signaling are elevated in several cancers, which makes this molecule a potent target in cancer treatments. To develop effective drugs, the details of FAK activation mechanism must be known. Most importantly, the relative importance of FAKs scaffolding function and its kinase function in cancer cells must be determined (Frame et al., 2010). Several FAK inhibitors are being developed to treat cancer and have reached clinical trials (Sulzmaier et al., 2014). Since integrin transcription is also altered in several cancers and, depending on the cancer type and integrin subunit, either up or downregulated, the link between altered integrin expression, elevated FAK signaling levels, and escape from the environmental control is very interesting. Overexpression of integrins could lead to a situation where integrin recruitment to membrane rafts and integrin interactions with tail-binding proteins are changed, supporting ligand independent integrin clustering. If the number of integrins at the cell membrane exceeds the threshold value, integrins could spontaneously activate FAK signaling in an ECM independent manner, since the nonactivated conformation, as is shown in this thesis, can induce FAK phosphorylation.

α2β1-integrin inhibitors in treating thrombosis vs. inflammation

The importance of β1-integrins in preventing thrombosis in atherosclerotic patients has been highlighted (Petzold et al., 2013). We report here a small molecule inhibitor that prevents platelet binding to collagen under flow conditions (III). BTT-3035 is a small molecule inhibitor that closely resembles BTT-3033. A study, where BTT-3035 was tested against platelet binding using blood from healthy donors revealed that α2β1-integrin expression levels in platelets affected the effectivity of the inhibitor. The binding was significantly reduced in platelets that expressed higher amounts of α2. The dimorphism C/T807 in the *ITGA2* gene causes differences in the expression levels of α2-integrin, and higher expression is related to an increased risk of stroke (Nissinen et al., 2016). Thus patients in the risk group can benefit from these inhibitors. Antibodies that target αIIbβ3 and prevent platelet binding are in clinical use. Although the platelet restricted expression of αIIbβ3 supports the targeting of this platelet receptor, an

adverse effect of several antibodies is increased bleeding. Since α2β1 KO mice do not have increased bleeding or any other major phenotype (Mercurio, 2002), α2β1-integrin inhibitors could presumably be better tolerated and have less side effects, being advantageous especially for patients with $\alpha2^{high}$ platelets.

A follow-up study with the small molecule integrin inhibitors BTT-3033 and BTT-3034 showed that in inflammation, the drug that recognizes the active conformation of integrins (BTT-3034) plays a role in reducing inflammation (Nissinen et al., 2015). In platelets, integrin-α2 most likely plays a role in platelet rolling and deceleration, and also later in establishing firm adhesion to collagen through activated α2-integrins. In leukocytes, α2 doesn't necessarily play a role in cell rolling, but is involved in cell invasion to collagenous tissues. Collagen degradation requires integrin activation and thus an inhibitor that prevents firm ligand binding is important under these circumstances. The possibility to target specific α2-integrin conformations increases the potency of these small molecule inhibitors as drugs.

Potential use of α2β1-integrin inhibitors in prostate cancer treatment

Both PIM kinase and α2-integrin help prostate cancer cells survive and become resistant towards a docetaxel drug treatment. They also potentially induce cancer cell migration, metastasis and homing to the bone marrow. PIM kinase inhibitors are thus potential cancer drugs to be used in combination with other drugs, especially to target the drug-resistant stem cell-like population of cancer cells. Small molecule inhibitors against α2 are potential anti-thrombotic and anti-inflammation drugs, but their use in preventing cancer cell homing to the bone marrow in an integrin dependent manner is also an interesting area of research.

7 CONCLUSIONS

Integrins have an essential role in regulating cell movement and cell growth. The importance of integrins is emphasized in malignancies and diverse diseases where normal cell behavior is changed due to dysfunctional integrin control. This thesis work demonstrates, through the following findings, that collagen receptor integrins have activation independent functions in cells:

I. α2β1-integrins can mediate ligand binding in the nonactivated conformation

II. The clustering of nonactivated α2β1-integrins can induce cellular signaling by activating FAK

III. Integrin mediated cell spreading and migration can be regulated by affecting the connection between integrins, focal adhesions and the cytoskeleton, without affecting the conformational activation or the cell surface expression levels of α1β1- and α2β1-integrins

The results emphasize the diversity of the ways integrin mediated functions can be regulated. Although the conformational regulation of an integrin's affinity towards its ligand is important for engaging integrins and initiating cell adhesion, it does not seem to play a central role after cells have adhered and start to migrate. The fact that α2β1-integrins can bind ligands in the nonactivated conformation also points out that the 24 different integrins are most likely regulated in different manners. The collagen binding integrins with their β1-tail and the I-domain in the α-subunit are much less studied both structurally and functionally than α5β1-integrin without the I-domain, or I-domain and β2-containing leukocyte integrins, or non-I-domain β3-integrins, whose function related structures have been elucidated. Collagen integrins form a small subgroup of 4 integrins with a unique ligand binding domain–tail combination. Based on the results presented in this thesis, we suggest that at least one of these integrins can be functional in the nonactivated conformation. The same conclusion does not apply to all integrins, which must meet different criteria in the regulation of their function. When studying the structure-function relationship of integrin subgroups, we should pay attention not to generalize the results to apply to all integrins.

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9 REFERENCES

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

Alfaro-Aco, R., and Petry, S. (2015). Building the microtubule cytoskeleton piece by piece. J. Biol. Chem. *290*, 17154–17162.

Alonso, J.L., Essafi, M., Xiong, J.-P., Stehle, T., and Arnaout, M.A. (2002). Does the Integrin alphaA Domain Act as a Ligand for its betaA Domain? Curr. Biol. *12*, R340–R342.

Anthis, N.J., Wegener, K.L., Ye, F., Kim, C., Goult, B.T., Lowe, E.D., Vakonakis, I., Bate, N., Critchley, D.R., Ginsberg, M.H., et al. (2009). The structure of an integrin/talin complex reveals the basis of inside-out signal transduction. EMBO J. *28*, 3623 LP-3632.

Aquilina, A., Korda, M., Bergelson, J.M., Humphries, M.J., Farndale, R.W., and Tuckwell, D. (2002). A novel gain-of-function mutation of the integrin alpha2 VWFA domain. Eur. J. Biochem. *269*, 1136–1144.

Arjonen, A., Kaukonen, R., and Ivaska, J. (2011). Filopodia and adhesion in cancer cell motility. Cell Adhes. Migr. *5*.

Arjonen, A., Alanko, J., Veltel, S., and Ivaska, J. (2012). Distinct Recycling of Active and Inactive beta1 Integrins. Traffic *13*, 610–625.

Arnaout, M.A., Mahalingam, B., and Xiong, J.-P. (2005). Integrin Structure, Allostery, and Bidirectional Signaling. Annu. Rev. Cell Dev. Biol. *21*, 381–410.

Arold, S.T. (2011). How focal adhesion kinase achieves regulation by linking ligand binding, localization and action. Curr. Opin. Struct. Biol. *21*, 808–813.

Askari, J.A., Tynan, C.J., Webb, S.E.D., Martin-Fernandez, M.L., Ballestrem, C., and Humphries, M.J. (2010). Focal adhesions are sites of integrin extension. J. Cell Biol. *188*, 891–903.

Atherton, P., Stutchbury, B., Jethwa, D., and Ballestrem, C. (2016). Mechanosensitive components of integrin adhesions: Role of vinculin. Exp. Cell Res. *343*, 21–27.

Belkin, A.M., and Stepp, M.A. (2000). Integrins as receptors for laminins. Microsc. Res. Tech. *51*, 280–301.

Bergelson, J.M., Shepley, M.P., Chan, B.M., Hemler, M.E., and Finberg, R.W. (1992). Identification of the integrin VLA-2 as a receptor for echovirus 1. Science *255*, 1718–1720.

Bergelson, J.M., Chan, B.M.C., Finberg, R.W., and Hemler, M.E. (1993). The integrin VLA-2 binds echovirus 1 and extracellular matrix ligands by different mechanisms. J. Clin. Invest. *92*, 232–239.

Bershadsky, a D., Tint, I.S., and Svitkina, T.M. (1987). Association of intermediate filaments with vinculin-containing adhesion plaques of fibroblasts. Cell Motil. Cytoskeleton *8*, 274–283.

Blanco-Aparicio, C., and Carnero, A. (2013). Pim kinases in cancer: Diagnostic, prognostic and treatment opportunities. Biochem. Pharmacol. *85*, 629–643.

Brami-Cherrier, K., Gervasi, N., Arsenieva, D., Walkiewicz, K., Boutterin, M.C., Ortega, A., Leonard, P.G., Seantier, B., Gasmi, L., Bouceba, T., et al. (2014). FAK dimerization controls its kinase-dependent functions at focal adhesions. EMBO J. *33*, 356–370.

Bridges, A.A., and Gladfelter, A.S. (2015). Septin form and function at the cell cortex. J. Biol. Chem. *290*, 17173–17180.

Bukoreshtliev, N. V., Haase, K., and Pelling, A.E. (2013). Mechanical cues in cellular signalling and communication. Cell Tissue Res. *352*, 77–94.

Calderwood, D. a, Campbell, I.D., and Critchley, D.R. (2013). Talins and kindlins: partners in integrin-mediated adhesion. Nat. Rev. Mol. Cell Biol. *14*, 503–517.

Campbell, I.D., and Humphries, M.J. (2011). Integrin Structure , Activation , and Interactions. 1–15.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. *257*, 7847–7851.

Collins, A.T., Berry, P.A., Hyde, C., Stower, M.J., and Maitland, N.J. (2005). Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res. *65*, 10946–10951.

Connors, W.L., Jokinen, J., White, D.J., Puranen, J.S., Kankaanpaa, P., Upla, P., Tulla, M., Johnson, M.S., and Heino, J. (2007). Two Synergistic Activation Mechanisms of alpha2beta1 Integrin-mediated Collagen Binding. J. Biol. Chem. *282*, 14675–14683.

Coppolino, M.G., and Dedhar, S. (1999). Ligand-specific, transient interaction between integrins and calreticulin during cell adhesion to extracellular

matrix proteins is dependent upon phosphorylation/dephosphorylation events. Biochem. J. *340 (Pt 1*, 41–50.

Coppolino, M., Leung-Hagesteijn, C., Dedhar, S., and Wilkins, J. (1995). Inducible Interaction of Integrin alpha2beta1 with calreticulin: Dependence on the activation state of the integrin. J. Biol. Chem. *270*, 23132–23138.

Coppolino, M.G., Woodside, M.J., Demaurex, N., Grinstein, S., St-Arnaud, R., and Dedhar, S. (1997). Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion. Nature *386*, 843–847.

Cuenda, A., and Rousseau, S. (2007). p38 MAP-kinases pathway regulation, function and role in human diseases. Biochim. Biophys. Acta *1773*, 1358–1375.

Davidson, A.J., and Wood, W. (2016). Unravelling the Actin Cytoskeleton: A New Competitive Edge? Trends Cell Biol.

Detmers, P. a., Wright, S.D., Olsen, E., Kimball, B., and Cohn, Z. a. (1987). Aggregation of complement receptors on human neutrophils in the absence of ligand. J. Cell Biol. *105*, 1137–1145.

Doyle, A.D., and Yamada, K.M. (2016). Mechanosensing via cell-matrix adhesions in 3D microenvironments. Exp. Cell Res. *343*, 60–66.

DuFort, C.C., Paszek, M.J., and Weaver, V.M. (2011). Balancing forces: architectural control of mechanotransduction. Nat. Rev. Mol. Cell Biol. *12*, 308–319.

Emsley, J., King, S.L., Bergelson, J.M., and Liddington, R.C. (1997). Crystal structure of the I domain from integrin alpha2beta1. J. Biol. Chem. *272*, 28512–28517.

Emsley, J., Knight, C.G., Farndale, R.W., Barnes, M.J., and Liddington, R.C. (2000). Structural basis of collagen recognition by integrin alpha2beta1. Cell *101*, 47–56.

Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E. (2006). Matrix Elasticity Directs Stem Cell Lineage Specification. Cell *126*, 677–689.

Ezratty, E.J., Partridge, M.A., and Gundersen, G.G. (2005). Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. Nat. Cell Biol. *7*, 581–590.

Faes, S., and Dormond, O. (2015). PI3K and AKT: Unfaithful partners in cancer. Int. J. Mol. Sci. *16*, 21138–21152.

Fagerholm, S.C., Hilden, T.J., and Gahmberg, C.G. (2004). P marks the spot: Site-specific integrin phosphorylation regulates molecular interactions. Trends Biochem. Sci. *29*, 504–512.

Farndale, R.W., Sixma, J.J., Barnes, M.J., and De Groot, P.G. (2004). The role of collagen in thrombosis and hemostasis. J. Thromb. Haemost. *2*, 561–573.

Fife, C.M., McCarroll, J.A., and Kavallaris, M. (2014). Movers and shakers: Cell cytoskeleton in cancer metastasis. Br. J. Pharmacol. *171*, 5507–5523.

Fiore, V.F., Strane, P.W., Bryksin, A. V., White, E.S., Hagood, J.S., and Barker, T.H. (2015). Conformational coupling of integrin and Thy-1 regulates Fyn priming and fibroblast mechanotransduction. J. Cell Biol. *211*, 173–190.

Fleming, F.E., Graham, K.L., Takada, Y., and Coulson, B.S. (2011). Determinants of the specificity of rotavirus interactions with the alpha2beta1 integrin. J. Biol. Chem. *286*, 6165–6174.

Frame, M.C., Patel, H., Serrels, B., Lietha, D., and Eck, M.J. (2010). The FERM domain: organizing the structure and function of FAK. Nat. Rev. Mol. Cell Biol. *11*, 802–814.

De Franceschi, N., Hamidi, H., Alanko, J., Sahgal, P., and Ivaska, J. (2015). Integrin traffic - the update. J. Cell Sci. *128*, 839–852.

De Franceschi, N., Arjonen, A., Elkhatib, N., Denessiouk, K., Wrobel, A.G., Wilson, T.A., Pouwels, J., Montagnac, G., Owen, D.J., and Ivaska, J. (2016). Selective integrin endocytosis is driven by interactions between the integrin alpha-chain and AP2. Nat. Struct. Mol. Biol. 1–10.

Frantz, C., Stewart, K.M., and Weaver, V.M. (2010). The extracellular matrix at a glance. J. Cell Sci. *123*, 4195–4200.

Gahmberg, C.G., Fagerholm, S.C., Nurmi, S.M., Chavakis, T., Marchesan, S., and Grönholm, M. (2009). Regulation of integrin activity and signalling. Biochim. Biophys. Acta - Gen. Subj. *1790*, 431–444.

Gasparski, A.N., and Beningo, K.A. (2015). Mechanoreception at the cell membrane: More than the integrins. Arch. Biochem. Biophys. *586*, 20–26.

Gaus, K., Le Lay, S., Balasubramanian, N., and Schwartz, M.A. (2006). Integrin-mediated adhesion regulates membrane order. J. Cell Biol. *174*, 725–734.

Ghatak, S., Morgner, J., and Wickström, S. a (2013). ILK: a pseudokinase with a unique function in the integrin-actin linkage. Biochem. Soc. Trans. *41*, 995–1001.

Ginsberg, M.H., Partridge, A., and Shattil, S.J. (2005). Integrin regulation. Curr. Opin. Cell Biol. *17*, 509–516.

Goni, G.M., Epifano, C., Boskovic, J., Camacho-Artacho, M., Zhou, J., Bronowska, A., Martin, M.T., Eck, M.J., Kremer, L., Grater, F., et al. (2014). Phosphatidylinositol 4,5-bisphosphate triggers activation of focal adhesion kinase by inducing clustering and conformational changes. Proc. Natl. Acad. Sci. *111*, E3177–E3186.

Gonzales, M., Weksler, B., Tsuruta, D., Goldman, R.D., Yoon, K.J., Hopkinson, S.B., Flitney, F.W., and Jones, J.C. (2001). Structure and function of a vimentin-associated matrix adhesion in endothelial cells. Mol. Biol. Cell *12*, 85–100.

Haeger, A., Wolf, K., Zegers, M.M., and Friedl, P. (2015). Collective cell migration: Guidance principles and hierarchies. Trends Cell Biol. *25*, 556–566.

Hagood, J.S., Prabhakaran, P., Kumbla, P., Salazar, L., MacEwen, M.W., Barker, T.H., Ortiz, L.A., Schoeb, T., Siegal, G.P., Alexander, C.B., et al. (2005). Loss of fibroblast Thy-1 expression correlates with lung fibrogenesis. Am. J. Pathol. *167*, 365–379.

Haining, A.W.M., Lieberthal, T.J., and del Río Hernández, A. (2016). Talin: a mechanosensitive molecule in health and disease. FASEB J. *30*, 2073–2085.

Hall, A. (2012). Rho family GTPases. Biochem. Soc. Trans. *40*, 1378–1382.

Harunaga, J.S., and Yamada, K.M. (2011). Cell-matrix adhesions in 3D. Matrix Biol. *30*, 363–368.

Has, C., Herz, C., Zimina, E., Qu, H.-Y., He, Y., Zhang, Z.-G., Wen, T.-T., Gache, Y., Aumailley, M., and Bruckner-Tuderman, L. (2009). Kindlin-1 Is required for RhoGTPase-mediated lamellipodia formation in keratinocytes. Am. J. Pathol. *175*, 1442–1452.

Hehlgans, S., Haase, M., and Cordes, N. (2007). Signalling via integrins: Implications for cell survival and anticancer strategies. Biochim. Biophys. Acta - Rev. Cancer *1775*, 163–180.

Heino, J. (2014). Cellular signaling by collagen-binding integrins. Adv. Exp. Med. Biol. *819*, 143–155.

Helfand, B.T., Mendez, M.G., Murthy, S.N.P., Shumaker, D.K., Grin, B., Mahammad, S., Aebi, U., Wedig, T., Wu, Y.I., Hahn, K.M., et al. (2011). Vimentin organization modulates the formation of lamellipodia. Mol. Biol. Cell *22*, 1274–1289.

Horton, E.R., Humphries, J.D., Stutchbury, B., Jacquemet, G., Ballestrem, C., Barry, S.T., and Humphries, M.J. (2016). Modulation of FAK and Src adhesion signaling occurs independently of adhesion complex composition. J. Cell Biol. *212*, 349–364.

Hu, J., Bai, X., Bowen, J.R., Dolat, L., Korobova, F., Yu, W., Baas, P.W., Svitkina, T., Gallo, G., and Spiliotis, E.T. (2012). Septin-driven coordination of actin and microtubule remodeling regulates the collateral branching of axons. Curr. Biol. *22*, 1109–1115.

Humphries, J.D., Byron, A., and Humphries, M.J. (2006). Integrin ligands at a glance. J. Cell Sci. *119*, 3901–3903.

Humphries, J.D., Paul, N.R., Humphries, M.J., and Morgan, M.R. (2015). Emerging properties of adhesion complexes: What are they and what do they do? Trends Cell Biol. *25*, 388–397.

Hussein, H.A.M., Walker, L.R., Abdel-Raouf, U.M., Desouky, S.A., Montasser, A.K.M., and Akula, S.M. (2015). Beyond RGD: virus interactions with integrins. Arch. Virol. *160*, 2669–2681.

Huth, J.R., Olejniczak, E.T., Mendoza, R., Liang, H., Harris, E.A.S., Lupher, M.L., Wilson, A.E., Fesik, S.W., and Staunton, D.E. (2000). NMR and mutagenesis evidence for an I domain allosteric site that regulates lymphocyte function-associated antigen 1 ligand binding. Proc. Natl. Acad. Sci. *97*, 5231–5236.

Hynes, R.O. (2002). Integrins: Bidirectional, allosteric signaling machines. Cell *110*, 673–687.

Hynes, R.O. (2009). The extracellular matrix: not just pretty fibrils. Science *326*, 1216–1219.

Inoue, O., Suzuki-Inoue, K., Dean, W.L., Frampton, J., and Watson, S.P. (2003). Integrin alpha2beta1 mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLCgamma2. J. Cell Biol. *160*, 769–780.

Ivaska, J., Reunanen, H., Westermarck, J., Koivisto, L., Kähäri, V.M., and Heino, J. (1999). Integrin alpha2beta1 mediates isoform-specific activation of p38 and upregulation of collagen gene transcription by a mechanism involving the alpha2 cytoplasmic tail. J. Cell Biol. *147*, 401–416.

Ivaska, J., Nissinen, L., Immonen, N., Eriksson, J.E., Kähäri, V.M., and Heino, J. (2002). Integrin alpha2beta1 promotes activation of protein phosphatase 2A and dephosphorylation of Akt and glycogen synthase kinase 3 beta. Mol. Cell. Biol. *22*, 1352–1359.

Iwamoto, D. V, and Calderwood, D.A. (2015). Regulation of integrin-mediated adhesions. Curr. Opin. Cell Biol. *36*, 41–47.

Jiu, Y., Lehtimäki, J., Tojkander, S., Cheng, F., Jäälinoja, H., Liu, X., Varjosalo, M., Eriksson, J.E., and Lappalainen, P. (2015). Bidirectional Interplay between Vimentin Intermediate Filaments and Contractile Actin Stress Fibers. Cell Rep. *11*, 1511–1518.

Juliano, R.L., Reddig, P., Alahari, S., Edin, M., Howe, a, and Aplin, a (2004). Integrin regulation of cell signalling and motility. Biochem. Soc. Trans. *32*, 443–446.

Jung, G.-Y., Park, Y.-J., and Han, J.-S. (2011). Mediation of Rac1 activation by kindlin-2: an essential function in osteoblast adhesion, spreading, and proliferation. J. Cell. Biochem. *112*, 2541–2548.

Jülich, D., Cobb, G., Melo, A.M., McMillen, P., Lawton, A.K., Mochrie, S.G.J., Rhoades, E., and Holley, S.A. (2015). Cross-Scale Integrin Regulation Organizes ECM and Tissue Topology. Dev. Cell *34*, 33–44.

Kanchanawong, P., Shtengel, G., Pasapera, A.M., Ramko, E.B., Davidson, M.W., Hess, H.F., and Waterman, C.M. (2010). Nanoscale architecture of integrin-based cell adhesions. Nature *468*, 580–584.

Karjalainen, M., Kakkonen, E., Upla, P., Paloranta, H., Kankaanpää, P., Liberali, P., Renkema, G.H., Hyypiä, T., Heino, J., and Marjomäki, V. (2008). A Raft-derived, Pak1-regulated entry participates in alpha2beta1 integrin-dependent sorting to caveosomes. Mol. Biol. Cell *19*, 2857–2869.

Kim, C., Ye, F., Hu, X., and Ginsberg, M.H. (2012). Talin activates integrins by altering the topology of the β transmembrane domain. J. Cell Biol. *197*, 605–611.

Kim, M., Carman, C. V, and Springer, T. a (2003). Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. Science *301*, 1720–1725.

Kim, M., Carman, C. V, Yang, W., Salas, A., and Springer, T.A. (2004). The primacy of affinity over clustering in regulation of adhesiveness of the integrin alphaLbeta2. J. Cell Biol. *167*, 1241–1253.

Kim, Y., Lee, Y.S., Choe, J., Lee, H., Kim, Y.M., and Jeoung, D. (2008). CD44-epidermal growth factor receptor interaction mediates hyaluronic acid-promoted cell motility by activating protein kinase C signaling involving Akt, Rac1, Phox, reactive oxygen species, focal adhesion kinase, and MMP-2. J. Biol. Chem. *283*, 22513–22528.

King, S.L., Kamata, T., Cunningham, J.A., Emsley, J., Liddington, R.C., Takada, Y., and Bergelson, J.M. (1997). Echovirus 1 interaction with the human very late antigen-2 (integrin alpha2beta1) I domain: Identification of two independent virus contact sites distinct from the metal ion-dependent adhesion site. J. Biol. Chem. *272*, 28518–28522.

Klekotka, P.A., Santoro, S.A., Wang, H., and Zutter, M.M. (2001). Specific Residues within the alpha2 Integrin Subunit Cytoplasmic Domain Regulate Migration and Cell Cycle Progression via Distinct MAPK Pathways. J. Biol. Chem. *276*, 32353–32361.

Kreis, S., Schönfeld, H.-J., Melchior, C., Steiner, B., and Kieffer, N. (2005). The intermediate filament protein vimentin binds specifically to a recombinant integrin alpha2/beta1 cytoplasmic tail complex and co-localizes with native alpha2/beta1 in endothelial cell focal adhesions. Exp. Cell Res. *305*, 110–121.

Krieger, S.E., Kim, C., Zhang, L., Marjomaki, V., and Bergelson, J.M. (2013). Echovirus 1 entry into polarized Caco-2 cells depends on dynamin, cholesterol, and cellular factors associated with macropinocytosis. J. Virol. *87*, 8884–8895.

Krishna, M., and Narang, H. (2008). The complexity of mitogen-activated protein kinases (MAPKs) made simple. Cell. Mol. Life Sci. *65*, 3525–3544.

Kuo, J.C. (2013). Mechanotransduction at focal adhesions: Integrating cytoskeletal mechanics in migrating cells. J. Cell. Mol. Med. *17*, 704–712.

Kuo, J.C., Han, X., Yates, J.R., and Waterman, C.M. (2011). Isolation of focal adhesion proteins for biochemical and proteomic analysis. Methods Mol. Biol. *757*, 297–323.

Köster, D. V., and Mayor, S. (2016). Cortical actin and the plasma membrane: Inextricably intertwined. Curr. Opin. Cell Biol. *38*, 81–89.

Lahti, M., Bligt, E., Niskanen, H., Parkash, V., Brandt, A.M., Jokinen, J., Patrikainen, P., Kapyla, J., Heino, J., and Salminen, T.A. (2011). Structure of collagen receptor integrin alpha(1)I domain carrying the activating mutation E317A. J Biol Chem *286*, 43343–43351.

Lahti, M., Heino, J., and Käpylä, J. (2013). Leukocyte integrins alphaLbeta2, alphaMbeta2 and alphaXbeta2 as collagen receptors - Receptor activation and recognition of GFOGER motif. Int. J. Biochem. Cell Biol. *45*, 1204–1211.

Langholz, O., Röckel, D., Mauch, C., Kozlowska, E., Bank, I., Krieg, T., and Eckes, B. (1995). Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by alpha1beta1 and alpha2beta1integrins. J. Cell Biol. *131*, 1903–1915.

Legate, K.R., and Fassler, R. (2009). Mechanisms that regulate adaptor binding to -integrin cytoplasmic tails. J. Cell Sci. *122*, 187–198.

Legate, K.R., Wickström, S.A., and Fässler, R. (2009). Genetic and cell biological analysis of integrin outside-in signaling. Genes Dev. *23*, 397–418.

Legate, K.R., Takahashi, S., Bonakdar, N., Fabry, B., Boettiger, D., Zent, R., and Fässler, R. (2011).

Integrin adhesion and force coupling are independently regulated by localized PtdIns(4,5)2 synthesis. EMBO J. *30*, 4539–4553.

Ley, K., Rivera-Nieves, J., Sandborn, W.J., and Shattil, S. (2016). Integrin-based therapeutics: biological basis, clinical use and new drugs. Nat Rev Drug Discov *15*, 173–183.

Li, Z., Delaney, M.K., O'Brien, K.A., and Du, X. (2010). Signaling during platelet adhesion and activation. Arterioscler. Thromb. Vasc. Biol. *30*, 2341–2349.

Liddington, R.C. (2014). I Domain Integrins (Springer Netherlands).

Lim, S.-T., Chen, X.L., Lim, Y., Hanson, D.A., Vo, T.-T., Howerton, K., Larocque, N., Fisher, S.J., Schlaepfer, D.D., and Ilic, D. (2008). Nuclear FAK Promotes Cell Proliferation and Survival through FERM-Enhanced p53 Degradation. Mol. Cell *29*, 9–22.

Ling, K., Doughman, R.L., Firestone, A.J., Bunce, M.W., and Anderson, R. a (2002). Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions. Nature *420*, 89–93.

La Linn, M., Eble, J.A., Lübken, C., Slade, R.W., Heino, J., Davies, J., and Suhrbier, A. (2005). An arthritogenic alphavirus uses the alpha1beta1 integrin collagen receptor. Virology *336*, 229–239.

Liu, J., Wang, Z., Thinn, A., Ma, Y., and Zhu, J. (2015a). The dual structural roles of the membrane distal region of the -integrin cytoplasmic tail during integrin inside-out activation. J. Cell Sci. *128*, 1718–1731.

Liu, J., Das, M., Yang, J., Ithychanda, S.S., Yakubenko, V.P., Plow, E.F., and Qin, J. (2015b). Structural mechanism of integrin inactivation by filamin. Nat Struct Mol Biol *22*, 383–389.

Liu, J., Wang, Y., Goh, W.I., Goh, H., Baird, M. a., Ruehland, S., Teo, S., Bate, N., Critchley, D.R., Davidson, M.W., et al. (2015c). Talin determines the nanoscale architecture of focal adhesions. Proc. Natl. Acad. Sci. 201512025.

Liu, J.-F., Fong, Y.-C., Chang, C.-S., Huang, C.-Y., Chen, H.-T., Yang, W.-H., Hsu, C.-J., Jeng, L.-B., Chen, C.-Y., and Tang, C.-H. (2010). Cyclooxygenase-2 enhances alpha2beta1 integrin expression and cell migration via EP1 dependent signaling pathway in human chondrosarcoma cells. Mol. Cancer *9*, 43.

Liu, S., Calderwood, D. a, and Ginsberg, M.H. (2000). Integrin cytoplasmic domain-binding proteins. J. Cell Sci. *113 (Pt 2*, 3563–3571.

Lowery, J., Kuczmarski, E.R., Herrmann, H., and Goldman, R.D. (2015). Intermediate Filaments Play a Pivotal Role in Regulating Cell Architecture and Function. J. Biol. Chem. *290*, 17145–17153.

Luo, B.-H., Carman, C. V, and Springer, T. a (2007). Structural basis of integrin regulation and signaling. Annu. Rev. Immunol. *25*, 619–647.

Marjomäki, V., Pietiäinen, V., Matilainen, H., Upla, P., Ivaska, J., Nissinen, L., Reunanen, H., Huttunen, P., Hyypiä, T., and Heino, J. (2002). Internalization of echovirus 1 in caveolae. J. Virol. *76*, 1856–1865.

Mattila, P.K., and Lappalainen, P. (2008). Filopodia: molecular architecture and cellular functions. Nat. Rev. Mol. Cell Biol. *9*, 446–454.

Mazzucato, M., Cozzi, M.R., Battiston, M., Jandrot-Perrus, M., Mongiat, M., Marchese, P., Kunicki, T.J., Ruggeri, Z.M., and De Marco, L. (2009). Distinct spatio-temporal Ca2+ signaling elicited by integrin alpha2beta1 and glycoprotein VI under flow. Blood *114*, 2793–2801.

Mercer, J., Schelhaas, M., and Helenius, A. (2010). Virus Entry by Endocytosis. Annu. Rev. Biochem. *79*, 803–833.

Mercurio, A.M. (2002). Lessons from the alpha2 Integrin Knockout Mouse. Am. J. Pathol. *161*, 3–6.

Mitra, S.K., Hanson, D.A., and Schlaepfer, D.D. (2005). Focal adhesion kinase: in command and control of cell motility. Nat. Rev. Mol. Cell Biol. *6*, 56–68.

Montanez, E., Ussar, S., Schifferer, M., Bosl, M., Zent, R., Moser, M., and Fassler, R. (2008). Kindlin-2 controls bidirectional signaling of integrins. Genes Dev. *22*, 1325–1330.

Moreno-Layseca, P., and Streuli, C.H. (2014). Signalling pathways linking integrins with cell cycle progression. Matrix Biol. *34*, 144–153.

Morse, E.M., Brahme, N.N., and Calderwood, D.A. (2014). Integrin Cytoplasmic Tail Interactions.

Moser, M., Nieswandt, B., Ussar, S., Pozgajova, M., and Fässler, R. (2008). Kindlin-3 is essential for integrin activation and platelet aggregation. Nat. Med. *14*, 325–330.

Nader, G.P.F., Ezratty, E.J., and Gundersen, G.G. (2016). FAK, talin and PIPKIgamma regulate endocytosed integrin activation to polarize focal adhesion assembly. Nat. Cell Biol. *18*, 491–503.

Nissinen, L., Ojala, M., Langen, B., Dost, R., Pihlavisto, M., Käpylä, J., Marjamäki, A., and Heino, J. (2015). Sulfonamide inhibitors of alpha2beta1 integrin reveal the essential role of collagen receptors in in vivo models of inflammation. Pharmacol. Res. Perspect. *3*, e00146.

Nissinen, L., Rappu, P., Ollikka, P., Nieminen, J., Marjamäki, A., and Heino, J. (2016). Platelet response to a small molecule inhibitor of alpha2beta1 integrin is associated with ITGA2 C807T dimorphism. Platelets *27*, 378–380.

O'Brien, L.E., Zegers, M.M.P., and Mostov, K.E. (2002). Opinion: Building epithelial architecture: insights from three-dimensional culture models. Nat. Rev. Mol. Cell Biol. *3*, 531–537.

Parsons, J.T., Horwitz, A.R., and Schwartz, M.A. (2010). Cell adhesion: integrating cytoskeletal dynamics and cellular tension. Nat Rev Mol Cell Biol *11*, 633–643.

Paszek, M.J., Zahir, N., Johnson, K.R., Lakins, J.N., Rozenberg, G.I., Gefen, A., Reinhart-King, C.A., Margulies, S.S., Dembo, M., Boettiger, D., et al. (2005). Tensional homeostasis and the malignant phenotype. Cancer Cell *8*, 241–254.

Paszek, M.J., DuFort, C.C., Rossier, O., Bainer, R., Mouw, J.K., Godula, K., Hudak, J.E., Lakins, J.N., Wijekoon, A.C., Cassereau, L., et al. (2014). The cancer glycocalyx mechanically primes integrin-mediated growth and survival. Nature *511*, 319–325.

Petzold, T., Ruppert, R., Pandey, D., Barocke, V., Meyer, H., Lorenz, M., Zhang, L., Siess, W., Massberg, S., and Moser, M. (2013). B1 Integrin-Mediated Signals Are Required for Platelet Granule Secretion and Hemostasis in Mouse. Blood *122*, 2723–2731.

Pierschbacher, M.D., and Ruoslahti, E. (1984). Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature *309*, 30–33.

Pietiäinen, V., Marjomäki, V., Upla, P., Pelkmans, L., Helenius, A., and Hyypiä, T. (2004). Echovirus 1 endocytosis into caveosomes requires lipid rafts, dynamin II, and signaling events. Mol. Biol. Cell *15*, 4911–4925.

Pouwels, J., Nevo, J., Pellinen, T., Ylanne, J., and Ivaska, J. (2012). Negative regulators of integrin activity. J. Cell Sci. *125*, 3271–3280.

Pozzi, A., Wary, K.K., Giancotti, F.G., and Gardner, H.A. (1998). Integrin alpha1beta1 mediates a unique collagen-dependent proliferation pathway in vivo. J. Cell Biol. *142*, 587–594.

Qu, H., Tu, Y., Shi, X., Larjava, H., Saleem, M. a, Shattil, S.J., Fukuda, K., Qin, J., Kretzler, M., and Wu, C. (2011). Kindlin-2 regulates podocyte adhesion and fibronectin matrix deposition through interactions with phosphoinositides and integrins. J. Cell Sci. *124*, 879–891.

Rantala, J.K., Pouwels, J., Pellinen, T., Veltel, S., Laasola, P., Mattila, E., Potter, C.S., Duffy, T., Sundberg, J.P., Kallioniemi, O., et al. (2011a). SHARPIN is an endogenous inhibitor of beta1-integrin activation. Nat. Cell Biol. *13*, 1315–1324.

Rantala, J.K., Pouwels, J., Pellinen, T., Veltel, S., Laasola, P., Mattila, E., Potter, C.S., Duffy, T., Sundberg, J.P., Kallioniemi, O., et al. (2011b). SHARPIN is an endogenous inhibitor of beta1-integrin activation. Nat. Cell Biol. *13*, 1315–1324.

Ravanti, L., Heino, J., López-Otín, C., and Kähäri, V.M. (1999). Induction of collagenase-3 (MMP-13) expression in human skin fibroblasts by three-dimensional collagen is mediated by p38 mitogen-activated protein kinase. J. Biol. Chem. *274*, 2446–2455.

Raymond, T., Gorbunova, E., Gavrilovskaya, I.N., and Mackow, E.R. (2005). Pathogenic hantaviruses bind plexin-semaphorin-integrin domains present at the apex of inactive, bent alphaVbeta3 integrin conformers. Proc. Natl. Acad. Sci. U. S. A. *102*, 1163–1168.

Ricoult, S.G., Kennedy, T.E., and Juncker, D. (2015). Substrate-bound protein gradients to study haptotaxis. Front. Bioeng. Biotechnol. *3*, 40.

del Rio, A., Perez-Jimenez, R., Liu, R., Roca-Cusachs, P., Fernandez, J.M., and Sheetz, M.P. (2009). Stretching Single Talin Rod Molecules Activates Vinculin Binding. Science (80-.). *323*, 638 LP-641.

Rojiani, M. V, Finlay, B.B., Gray, V., and Dedhar, S. (1991). In vitro interaction of a polypeptide homologous to human Ro/SS-A antigen (calreticulin) with a highly conserved amino acid sequence in the cytoplasmic domain of integrin alpha subunits. Biochemistry *30*, 9859–9866.

Ruoslahti, E. (1996). RGD and other recognition sequences for integrins. Annu. Rev. Cell Dev. Biol. *12*, 697–715.

Salas, A., Shimaoka, M., Kogan, A.N., Harwood, C., Von Andrian, U.H., and Springer, T.A. (2004). Rolling adhesion through an extended conformation of integrin alphaLbeta2 and relation to alpha I and beta I-like domain interaction. Immunity *20*, 393–406.

Santio, N.M., Vahakoski, R.L., Rainio, E.-M., Sandholm, J. a, Virtanen, S.S., Prudhomme, M., Anizon, F., Moreau, P., and Koskinen, P.J. (2010). Pim-selective inhibitor DHPCC-9 reveals Pim kinases as potent stimulators of cancer cell migration and invasion. Mol. Cancer *9*, 279.

Santio, N.M., Eerola, S.K., Paatero, I., Yli-Kauhaluoma, J., Anizon, F., Moreau, P., Tuomela, J., Härkönen, P., and Koskinen, P.J. (2015). Pim kinases promote migration and metastatic growth of prostate cancer xenografts. PLoS One *10*.

Schaller, M.D., Otey, C.A., Hildebrand, J.D., and Parsons, J.T. (1995a). Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. J. Cell Biol. *130*, 1181–1187.

Schmaier, A. a, and Kahn, M.L. (2009). Platelet integrin signaling: wherefore art thou? Blood *114*, 2571–2572.

Schvartzman, M., Palma, M., Sable, J., Abramson, J., Hu, X., Sheetz, M.P., and Wind, S.J. (2011). Nanolithographic control of the spatial organization of cellular adhesion receptors at the single-molecule level. Nano Lett. *11*, 1306–1312.

Schürpf, T., and Springer, T.A. (2011). Regulation of integrin affinity on cell surfaces. EMBO J. *30*, 4712–4727.

Seong, J., Tajik, A., Sun, J., Guan, J., Humphries, M.J., Craig, S.E., and Shekaran, A. (2013). Distinct biophysical mechanisms of focal adhesion kinase mechanoactivation by different extracellular matrix proteins. Proc Natl Acad Sci U S A *110*, 19372–19377.

Serrels, A., Lund, T., Serrels, B., Anderton, S.M., Nibbs, R.J.B., Frame, M.C., Byron, A., Mcpherson, R.C., Von Kriegsheim, A., Gó Mez-Cuadrado, L., et al. (2015). Nuclear FAK Controls Chemokine Transcription, Tregs, and Evasion of Anti-tumor Immunity. Cell *163*, 160–173.

Shimaoka, M., Xiao, T., Liu, J.-H., Yang, Y., Dong, Y., Jun, C.-D., McCormack, A., Zhang, R., Joachimiak, A., Takagi, J., et al. (2003). Structures of the αL I Domain and Its Complex with ICAM-1 Reveal a Shape-Shifting Pathway for Integrin Regulation. Cell *112*, 99–111.

Shimaoka, M., Kim, M., Cohen, E.H., Yang, W., Astrof, N., Peer, D., Salas, A., Ferrand, A., and Springer, T.A. (2006). reveals chemokine-induced affinity up-regulation in lymphocytes. *103*, 13991–13996.

Small, J.V., Geiger, B., Kaverina, I., and Bershadsky, A. (2002). How do microtubules guide migrating cells? Nat. Rev. Mol. Cell Biol. *3*, 957–964.

Sorrentino, S., Studt, J.-D.D., Medalia, O., Tanuj Sapra, K., and Sapra, K.T. (2015). Roll, adhere, spread and contract: structural mechanics of platelet function. Eur. J. Cell Biol. *94*, 129–138.

Springer, T.A., and Wang, J.-H. (2004). The Three-Dimensional Structure of Integrins and their Ligands, and Conformational Regulation of Cell Adhesion. Adv. Protein Chem. *68*, 29–63.

Stehbens, S.J., Paszek, M., Pemble, H., Ettinger, A., Gierke, S., and Wittmann, T. (2014). CLASPs link focal-adhesion-associated microtubule capture to localized exocytosis and adhesion site turnover. Nat. Cell Biol. *16*, 561–573.

Stergiou, L., Bauer, M., Mair, W., Bausch-Fluck, D., Drayman, N., Wollscheid, B., Oppenheim, A., and Pelkmans, L. (2013). Integrin-Mediated Signaling Induced by Simian Virus 40 Leads to Transient Uncoupling of Cortical Actin and the Plasma Membrane. PLoS One *8*.

Su, Y., Xia, W., Li, J., Walz, T., Humphries, M.J., Vestweber, D., Cabañas, C., Lu, C., and Springer, T.A. (2016). Relating conformation to function in integrin alpha5beta1. Proc. Natl. Acad. Sci. U. S. A.

Sulzmaier, F.J., Jean, C., and Schlaepfer, D.D. (2014). FAK in cancer: mechanistic findings and clinical applications. Nat. Rev. Cancer *14*, 598–610.

Tadokoro, S., Shattil, S.J., Eto, K., Tai, V., Liddington, R.C., de Pereda, J.M., Ginsberg, M.H., and Calderwood, D. a (2003). Talin binding to integrin beta tails: a final common step in integrin activation. Science (80-.). *302*, 103–106.

Takagi, J., Petre, B.M., Walz, T., and Springer, T.A. (2002). Global conformational earrangements in integrin extracellular domains in outside-in and inside-out signaling. Cell *110*, 599–611.

Thapa, N., and Anderson, R.A. (2012). PIP2 signaling, an integrator of cell polarity and vesicle trafficking in directionally migrating cells. Cell Adhes. Migr. *6*, 409–412.

Theisen, U., Straube, E., and Straube, A. (2012). Directional Persistence of Migrating Cells Requires Kif1C-Mediated Stabilization of Trailing Adhesions. Dev. Cell *23*, 1153–1166.

Theodosiou, M., Widmaier, M., Böttcher, R.T., Rognoni, E., Veelders, M., Bharadwaj, M., Lambacher, A., Austen, K., Müller, D.J., Zent, R., et al. (2016). Kindlin-2 cooperates with talin to activate integrins and induces cell spreading by directly binding paxillin. Elife *5*.

Tojkander, S., Gateva, G., and Lappalainen, P. (2012). Actin stress fibers - assembly, dynamics and biological roles. J. Cell Sci. *125*, 1855–1864.

Tomar, A., and Schlaepfer, D.D. (2009). Focal adhesion kinase: switching between GAPs and GEFs in the regulation of cell motility. Curr. Opin. Cell Biol. *21*, 676–683.

True, L.D., Zhang, H., Ye, M., Huang, C.-Y., Nelson, P.S., von Haller, P.D., Tjoelker, L.W., Kim, J.-S., Qian, W.-J., Smith, R.D., et al. (2010). CD90/THY1 is overexpressed in prostate cancer-associated fibroblasts and could serve as a cancer biomarker. Mod. Pathol. *23*, 1346–1356.

Tulla, M., Lahti, M., Puranen, J.S., Brandt, A.-M., Käpylä, J., Domogatskaya, A., Salminen, T. a, Tryggvason, K., Johnson, M.S., and Heino, J. (2008). Effects of conformational activation of

integrin alpha1I and alpha2I domains on selective recognition of laminin and collagen subtypes. Exp. Cell Res. *314*, 1734–1743.

Upla, P., Marjomäki, V., Kankaanpää, P., Ivaska, J., Hyypiä, T., Van Der Goot, F.G., and Heino, J. (2004). Clustering induces a lateral redistribution of alpha2beta1 integrin from membrane rafts to caveolae and subsequent protein kinase C-dependent internalization. Mol. Biol. Cell *15*, 625–636.

Vanarsdall, A.L., and Johnson, D.C. (2012). Human cytomegalovirus entry into cells. Curr. Opin. Virol. *2*, 37–42.

Walker, S., Foster, F., Wood, A., Owens, T., Brennan, K., Streuli, C.H., and Gilmore, A.P. (2016). Oncogenic activation of FAK drives apoptosis suppression in a 3D-culture model of breast cancer initiation. Oncotarget; Vol 7, No 43.

Wang, N., Tytell, J.D., and Ingber, D.E. (2009). Mechanotransduction at a distance: mechanically coupling the extracellular matrix with the nucleus. Nat Rev Mol Cell Biol *10*, 75–82.

White, D.J., Puranen, S., Johnson, M.S., and Heino, J. (2004). The collagen receptor subfamily of the integrins. Int. J. Biochem. Cell Biol. *36*, 1405–1410.

Wiche, G., Osmanagic-Myers, S., and Castañón, M.J. (2015). Networking and anchoring through plectin: A key to IF functionality and mechanotransduction. Curr. Opin. Cell Biol. *32*, 21–29.

Winograd-Katz, S.E., Fässler, R., Geiger, B., and Legate, K.R. (2014). The integrin adhesome: from genes and proteins to human disease. Nat. Rev. Mol. Cell Biol. *15*, 273–288.

Wu, C.Y., Lin, M.W., Wu, D.C., Huang, Y.B., Huang, H.T., and Chen, C.L. (2014). The role of phosphoinositide-regulated actin reorganization in chemotaxis and cell migration. Br. J. Pharmacol. *171*, 5541–5554.

Xie, C., Shimaoka, M., Xiao, T., Schwab, P., Klickstein, L.B., and Springer, T. a (2004). The integrin alpha-subunit leg extends at a Ca2+-dependent epitope in the thigh/genu interface upon activation. Proc. Natl. Acad. Sci. U. S. A. *101*, 15422–15427.

Xie, C., Zhu, J., Chen, X., Mi, L., Nishida, N., and Springer, T. a (2010). Structure of an integrin with an alphaI domain, complement receptor type 4. EMBO J. *29*, 666–679.

Xing, L., Huhtala, M., Pietiäinen, V., Käpylä, J., Vuorinen, K., Marjomäki, V., Heino, J., Johnson, M.S., Hyypiä, T., and Cheng, R.H. (2004). Structural and functional analysis of integrin alpha2I domain interaction with echovirus 1. J. Biol. Chem. *279*, 11632–11638.

Yang, W., Shimaoka, M., Salas, A., Takagi, J., and Springer, T.A. (2004). Intersubunit signal transmission in integrins by a receptor-like interaction with a pull spring. Proc. Natl. Acad. Sci. U. S. A. *101*, 2906–2911.

Ye, F., Kim, C., and Ginsberg, M.H. (2011). Molecular mechanism of inside-out integrin regulation. J. Thromb. Haemost. *9 Suppl 1*, 20–25.

Ye, F., Petrich, B.G., Anekal, P., Lefort, C.T., Kasirer-Friede, A., Shattil, S.J., Ruppert, R., Moser, M., Fässler, R., and Ginsberg, M.H. (2013). The mechanism of kindlin-mediated activation of integrin alphaIIbbeta3. Curr. Biol. *23*, 2288–2295.

Yu, C., Law, J.B.K., Suryana, M., Low, H.Y., and Sheetz, M.P. (2011). Early integrin binding to Arg-Gly-Asp peptide activates actin polymerization and contractile movement that stimulates outward translocation. Proc. Natl. Acad. Sci. U. S. A. *108*, 20585–20590.

Zamanian, M., Veerakumarasivam, A., Abdullah, S., and Rosli, R. (2013). Calreticulin and cancer. Pathol. Oncol. Res. *19*, 149–154.

Zemskova, M., Sahakian, E., Bashkirova, S., and Lilly, M. (2008). The PIM1 kinase is a critical component of a survival pathway activated by docetaxel and promotes survival of docetaxel-treated prostate cancer cells. J. Biol. Chem. *283*, 20635–20644.

Zuk, A., and Matlin, K.S. (1996). Apical beta1 integrin in polarized MDCK cells mediates tubulocyst formation in response to type I collagen overlay. J. Cell Sci. *109 (Pt 7*, 1875–1889.