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β1 Integrin Regulation

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To my family

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β1 integrin regulation

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ABSTRACT

Integrins are heterodimeric adhesion receptors mediating adhesion to extracellular matrix proteins and to other cells. Integrins are important in embryonic development, structural integrity of connective tissue, blood thrombus formation, and immune defense system. Integrins are transmembrane proteins whose ligand binding capacity (activity) is regulated by large conformational changes. Extracellular ligand binding or intracellular effector binding to integrin cytoplasmic face regulate integrin activity. Integrins are thus able to mediate bi-directional signaling. Integrin function is also regulated by intracellular location. Integrins are constantly recycled from endocytic vesicles to plasma membrane, and this has been shown to be important for cell migration and invasion as well. Deregulation of integrin functionality can lead to deleterious illnesses, such as bleeding or inflammatory disorders. It is also evident that integrin deregulation is associated with cancer progression.

In this study, a novel β1 integrin associating protein, Rab21, was characterized. Rab21 binding to integrin cytoplasmic tail was shown to be important for β1 integrin endo- and exocytosis – intracellular trafficking. It was furher shown that this interaction has an important role in cell adhesion, migration, as well as in the final step of cell division, cytokinesis. This work showed that abrogation of Rab21 function or β1 integrin endocytic traffic, can lead to defects in cell division and results in formation of multinucleated cells. Multinucleation and especially tetraploidy can be a transient pathway to aneuploidy and tumorigenesis. This work characterized chromosomal deletions in *rab21* locus in ovarian and prostate cancer samples and showed that a cell line with *rab21* deletion also had impairment in cell division, which could be rescued by Rab21 re-expression. The work demonstrates an important role for Rab21 and β1 integrin traffic regulation in cell adhesion and division, and suggests a probable associaton with tumorigenesis.

In this study, β1 integrin activity regulation was also addressed. A novel cell array platform for genome-scale RNAi screenings was characterized here. More than 4500 genes were knocked-down in prostate cancer cells using siRNA-mediated silencing. The effects on β1 integrin activity were analyzed upon knock-downs. The screen identified more that 400 putative regulators of β1 integrin activity in prostate cancer. In conclusion, this work will help us to understand complex regulatory pathways involved in cancer cell adhesion and migration.

Key words: integrins, cell adhesion, cancer

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β1 integriinin säätely

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TIIVISTELMÄ

Integriinit ovat solukalvon adheesioproteiineja, jotka osallistuvat elimistön elintärkeisiin toimintoihin, kuten alkionkehitykseen, verenvuodon tyrehdyttämiseen sekä leukosyyttien kulkeutumiseen tulehtuneeseen kudoksen. Integriinit ovat inaktiivisia normaalissa tilassa, mutta aktivoituvat esimerkiksi soluväliaineen pilkistäessä verisuonten seinämän välistä tai tulehdusympäristössä missä mikrobi on päässyt tunkeutumaan kudokseen. Verisolujen ja immuunisolujen vajaatoimintaiset integriinit johtavat vakaviin sairauksiin, kuten verenvuototautiin (Glanzmann thrombasthenia) tai immunosupressiiviseen tilaan (leukocyte adhesion deficiencies, LAD I, III). Integriinien toiminnan säätelyn järkkymistä tapahtuu myös syöpäsoluilla, vaikkakin tätä ei ole tutkittu läheskään yhtä paljon kuin verisoluilla.

Tässä työssä tarkasteltiin β1 integriinien säätelyä syövässä, ja erityisesti rinta- ja eturauhassyövässä. Solukalvon reseptoriproteiineja, mukaan lukien integriinejä, säädellään myös niiden solunsisäisen liikenteen avulla. Integriinien kuljetus solukalvolta solunsisäisiin vesikkeleihin ja edelleen solun pinnalle uuteen kohtaan on osoitettu tärkeäksi solujen adheesion ja liikkumisen kannalta. Työssä löydettiin uusi β1 integriinien kuljetukseen osallistuva säätelyproteiini, Rab21. Tämä proteiini osoittautui merkittäväksi β1 integriinien välittämässä soluadheesiossa ja liikkumisessa. Näiden lisäksi huomattiin että Rab21 proteiinin välittämä integriinien solunsisäinen kuljetus on tärkeää myös solunjakautumisen viimeiselle vaiheelle, eli sytokineesille. Työssä Rab21:n tai integriinien kuljetuksen estäminen johti sytokineesihäiriön kautta monitumallisten solujen muodostumiseen, mitä pidetään yhtenä syövän syntymismekanismeista. Kromosomien DNA-analyysi osoittikin *rab21*-lokuksessa deleetiota muutamissa syöpänäytteissä. Työn tulokset viittaavat vahvasti siihen, että β1 integriinin solunsisäinen kuljetuksen säätely voi olla yhteydessä syövän muodostumiseen ja syöpäsolujen liikkumiseen elimistössä.

Lisäksi työssä tarkasteltiin integriinien aktiivisuuden säätelyä uudella soluarray-teknologialla, joka mahdollistaa tuhansien geenien hiljennyksen erillisissä reaktioissa samassa määrityksessä. Työssä hiljennettiin siRNA-molekyylien avulla yli 4500 geeniä viljellyissä eturauhassoluissa ja tarkasteltiin kunkin hiljennyksen vaikutus β1 integriinien aktiivisuuteen vasta-ainevärjäyksillä. Menetelmällä löydettiin yli 400 mahdollista aktiivisuuden säätelijää, joista validoitiin 100 geeniä kahdeksassa eri eturauhassolulinjassa. Tulokset avaavat uusia mahdollisuuksia syövän molekyylibiologisessa tutkimuksessa. Kaiken kaikkiaan työn tulokset auttavat ymmärtämään syöpäsolujen adheesio- ja liikkumismekanismeja.

Avainsanat: Integriinit, soluadheesio, syöpä

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ABBREVIATIONS

ADP	Adenosine diphosphate
CDS	Coding sequence
CHO	Chinese hamster ovary cell line
COL	Collagen
DAG	Diacyl glycerol
ER	Endoplasmic reticulum
FA	Focal adhesion
FN	Fibronectin
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanosine exchange factor
GTP	Guanosine triphosphate
ITAM	Immunoreceptor tyrosine activation motif
LAD	Leukocyte adhesion deficiency
LAM	Laminin
MVB	Multivesicular body
PNRE	perinuclear recycling endosome
RE	Recycling endosome
SH2	Src-homolgy domain
VIM	Vimentin
VN	Vitronectin
VWF	Von Willebrand factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred in the text by the Roman numerals I-III. The original communications have been reproduced with the permission of the copyright holders. Unpublished data is also included.

I Pellinen T, Arjonen A, Vuoriluoto K, Kallio K, Fransen JA, Ivaska J (2006). Small GTPase Rab21 regulates cell adhesion and controls endosomal traffic of beta1-integrins. *J Cell Biol*. 173:767-780.

II Pellinen T*, Tuomi S*, Arjonen A, Wolf M, Edgren H, Meyer H, Grosse R, Kitzing T, Rantala JK, Kallioniemi O, Fässler R, Kallio M, Ivaska J. (2008). Integrin trafficking regulated by Rab21 is necessary for cytokinesis. *Dev Cell*. 15:371-385.

III Juha Rantala*, Teijo Pellinen*, Rami Mäkelä, John Patrick Mpindi, Sami Kilpinen, Matthias Nees, Petri Saviranta, Johanna Ivaska, Olli Kallioniemi. Novel cell spot microarray method for genome-scale RNAi experiments and application for analysis of integrin β1 activation in prostate cancer cells. Submitted.

* Equal contribution

1. INTRODUCTION

The heterodimeric transmembrane integrins form important interactions with other cells, extracellular matrix proteins, and also with different soluble ligands. The combinations of 18 alpha and 8 beta subunits form 24 different heterodimers with additional splicing variants as well (Humphries 2000). Mouse knock-out studies have revealed embryonic lethality for β1, β8, α4, α5, and αV integrin knock-outs, but also severe defects in platelet aggregation (αIIb, β3, α2), leukocyte function (αL, αM, αE, β2, β7), and skin blistering or inflammation (α6, β4, α3, β6) (Hynes 2002).

Regulated adhesion and deadhesion of platelets and leukocytes are fundamental for human health as well. The pathological conditions of leukocyte adhesion deficiencies derive from the lack of functional β2 integrins (LAD-I), deficiency in β2 integrin activation (LAD-III) (Kuijpers, van de Vijver et al. 2008), or absence of functional selectin ligands (LAD-II), of which the selectin ligand, PSGL1, was shown recently to regulate β2 integrin activity as well (Zarbock, Abram et al. 2008). LAD-III syndrome influences also αIIbβ3 integrin inside-out activation leading to platelet aggregation defects and bleeding episodes (Pasvolsky, Feigelson et al. 2007; Kilic, Etzioni 2008). The importance of αIIbβ3 is well illustrated in the bleeding disorder Glanzmann thrombasthenia, where the integrin subunits contain mutations and platelets are unable to adhere properly or form proper platelet aggregates (Kato 1997).

Deregulation of integrin expression is associated with cancer progression. Collagen-binding α2 integrin is heavily downregulated in breast and prostate adenocarcinoma, but again upregulated in metastasis of the latter (Zutter, Krigman et al. 1993; Bonkhoff, Stein et al. 1993). In addition, α2β1 heterodimer seems to be highly expressed in prostate cancer stem cells (Collins, Berry et al. 2005; Mimeault, Batra 2007). A truncated isoform of platelet integrin αIIb is absent in normal prostate tissue, but is found in prostate adenocarcinoma with increased expression in metastatic foci in mouse metastasis model (Trikha, Cai et al. 1998; Trikha, Raso et al. 1998). Also β1 integrin, which will be the focus of this thesis, is consistently upregulated in prostate cancer, (Goel, Li et al. 2008).

The large-scale cancer-genome sequencing studies demonstrate that cell-matrix and cell-cell adhesion associated genes are among the highest scoring groups bearing somatic point-mutations or deletions/amplifications in protein coding sequences in breast and colorectal cancer (Sjoblom, Jones et al. 2006; Leary, Lin et al. 2008). Another genome-scale study shows that in 24 pancreatic cancer samples sequenced, 92% had a genetic alteration in one or more genes coding for "invasion" genes and 67% in integrin or integrin proximal genes, including integrins *ITGA4, ITGA9, ITGA11*, as well as an important integrin outside-in regulator, *ILK* (integrin-linked kinase) (Jones, Zhang et

al. 2008). These studies further demonstrate that cell adhesion and integrin function can have important roles in human carcinogenesis.

Expression levels of integrins are only one part of their story, and should only give direction of their existence or absence in certain tissue. Integrins' function lies in their elaborate regulation of activity, which is the ligand binding capability. For example, platelet αIIbβ3 and leukocyte αLβ2 (LFA-1) are in inactivated bent conformation as cells are circulating in blood. Upon vessel injury or pathogen attack, these integrins quickly extend and open up for ligand binding on endothelial surface and become arrested. If this and the subsequent relaxation are not finely regulated, bleeding, thrombosis, or inflammation can prevail.

Deregulation of integrin activity could also be important in the pathogenesis of cancer. In chronic myelogenous leukemia, BCR-ABL1 fusion oncoprotein is accounted for stronger β1 integrin-mediated cell adhesion to bone marrow stromal matrix proteins (Fierro, Taubenberger et al. 2008). In addition, BCR-ABL1 fusion results in aberrant adhesive properties and impaired leukocyte traffic due to constitutive inside-out activation of the β2 integrin LFA-1 (Chen, Malik et al. 2008). Crucial master regulators of integrin avidity (affinity plus clustering) are the small GTPase family members Rap1 and Rap2. The GTP-bound Raps are inactivated by GTPase activating proteins (GAPs), which hydrolyze GTP to GDP. Rap GAPs are strongly associated with cancer. *RAP1GAP* locus (1p36.1-p35) can be deleted in squamous cell carcinoma (Lefeuvre, Gunduz et al. 2008) or protein CDS point-mutated in breast carcinoma (Sjoblom, Jones et al. 2006). Knock-out mice of another Rap1/2 GAP, SPA-1, show diverse set of leukemia, and are strongly associated with invasion and metastasis (Minato, Hattori 2009). These studies suggest that constitutively active integrins could be associated with certain cancers. However, unlike in platelets and leukocytes, the regulators of integrin inside-out activation in cancer are still very poorly known.

Integrins are also regulated by their cellular localization. Cell motility and migration require dynamic assembly and disassembly of adhesion sites (Ridley, Schwartz et al. 2003). During the last few years, studies on intracellular integrin traffic have given important aspects on the mechanisms of cell migration (Caswell, Norman 2006). Recent studies also suggest that the endo-exocytic traffic of integrins could also play a role in cancer cell invasion (Caswell, Norman 2008).

The subject of this thesis is β1 integrin intracellular traffic and activity regulation. β1 integrin subunit forms dimers with α1, α2, α3, α4, α5, α6, αV, α7, α8, α9, α10, and α11 subunits. The ligands can be a diverse set of collagens, laminins, fibronectin, vitronectin, as well as VCAM-1 for α4β1. The first part of the work characterizes a novel association of β1 integrins with a small Rab GTPase, Rab21. This association is shown to be important for β1 integrin endo- and exocytosis. Perturbations of Rab21

function by dominant mutations or siRNA silencing resulted in cell adhesion and migration defects. In the second part of the thesis, Rab21-regulated β1-integrin traffic was investigated in cell division. Perturbing integrin traffic resulted in cytokinesis failure and multinucleation. In the last part, β1 integrin activity regulators in prostate cells were studied with a novel siRNA-based cell spot microarray screening. The screen identified in prostate cells several proteins that are known regulators of integrin activity in platelets and leukocytes. In addition, many new positive and negative regulators were found, and from these results possible pathways for integrin activity regulation in prostate were drawn.

2. REVIEW OF THE LITERATURE

2.1. Integrin traffic

Integrins are transmembrane proteins that can be endocytozed from plasma membrane to endosomal vesicles and recycled back to the plasma membrane. This endocytic integrin traffic enables cells to control adhesion in polarized manner during cell spreading or migration (Jones, Caswell et al. 2006). Endocytosis in general can be divided into 4 major pathways: clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin- and caveolin-independent endocytosis, and macropinocytosis (Mosesson, Mills et al. 2008).

Integrin internalization is mostly dependent on clathrin or caveolae (Table 1). The classical clathrin-mediated endocytosis transports cell-surface receptors to early endosomes and again to late endosomes or perinuclear recycling endosomes (PNRE), from where cargo can either be recycled back to the plasma membrane or targeted for lysosomal degradation. There are suggestions that early endosomes may work as converging points in caveolae- and clathrin-mediated endocytosis (Pelkmans, Burli et al. 2004). ADP-ribosylation factor GTPases (Arfs) are important in clathrin coat-complex formation and vesicular budding, whereas Rab family GTPases regulate transport and tethering/fusion of membrane vesicles (Casanova 2007; Zerial, McBride 2001). The most-studied of Rab GTPases is Rab5, which together with Rab21, Rab22, and Rab31 constitute the Rab5 subfamily, whose function is in the early endocytosis and for Rab31 also in anterograde traffic (Simpson, Jones 2005; Ng, Wang et al. 2007).

The regulation of integrin targeting to different endocytic compartments is poorly known, whereas regulators of integrin recycling have been well characterized. Both Arf6 and Rab11 contribute to β1 integrin recycling from the PNREs (Powelka, Sun et al. 2004). Recycling of α5β1 and αVβ3 through PNREs requires PI3K and AKT activity (Roberts, Woods et al. 2004). This "long-loop" recycling pathway is different form Rab4-assisted "short-loop" recycling pathway, which requires growth factor stimulation and protein kinase D (PKD) activity (Woods, White et al. 2004).

Recently, the endocytic recycling of integrins has gained a lot of attention in relation to its role in cancer cell metastasis, and especially in invasion (Caswell, Norman 2008; Ramsay, Marshall et al. 2007). Many studies show how perturbing integrin endocytic traffic leads to impaired cell adhesion, transwell migration, 2D cell motility, or reduced 3D matrigel invasion (see Tables 1 and 1b and Figure 1). Caswell and co-workers (2007) found an important role for Rab25 in the regulation of α5β1 integrin

recycling to pseudopodial projections in invasive ovarian cancer cells (Caswell, Spence et al. 2007). Rab25 is highly expressed in metastatic ovarian and breast cancer (Cheng, Lahad et al. 2004), where its cancer promoting functions could be associated with integrin regulation. Another group showed that the expression of αVβ6 and its positive regulator of endocytosis, HAX-1, correlate with more advanced invasive oral carcinoma (Ramsay, Keppler et al. 2007). Knocking-down HAX-1 decreased integrin endocytosis and was associated with reduced matrigel invasion of squamous cell carcinoma cells. These studies with the clinical evidence imply that integrin traffic could have a role in cancer metastasis, although this has not been shown by *in vivo* experimental analysis yet.

Cells adhering to 2D extracellular matrix (ECM) surfaces form specialized adhesion complexes, focal adhesions (FAs). These structures are composed of clustered ligand-bound integrins and endoplasmic complexes of proteins linking integrins to bundled actin cytoskeleton. FAs, although missing or different in 3D environment, are specialized platforms in extracellular force sensing and intracellular signal amplification, as several adaptor and effector proteins are bundled together (Broussard, Webb et al. 2008). Mutations in FA proteins can participate in formation of various pathologies, such as skin Kindler syndrome (FERMT1/Kindlin-1), lung cancer metastasis (paxillin), or chronic myelogenous leukemia (BCR-ABL1) (Lai-Cheong, Tanaka et al. 2008; Jagadeeswaran, Surawska et al. 2008; Weisberg, Sattler et al. 1997). FA turnover, the assembly and disassembly, is critical for cell motility (Broussard, Webb et al. 2008), and recent studies suggest that this turnover is coupled to integrin endo/exocytosis. Nichimura and Kaibuchi (2007) showed that a clathrin adaptor protein, NUMB, localizes to proximity of focal adhesions by binding to β1 and β3 integrin cytoplasmic tails and mediates integrin internalization from these structures, a process which is determined by a delicate balance of NUMB phosphorylation by atypical PKC (Nishimura, Kaibuchi 2007). Also, Ezratty and others (2005) showed that dynamin, which is implicated in β1 integrin endocytosis, modulates FA disassembly associated with actin dissociation and microtubule growth to FAs (Ezratty, Partridge et al. 2005). More recent still unpublished work form the same group shows that microtubule plus-ends direct clathrin-adaptor proteins, DAB1/2, to focal adhesions and mediate integrin endocytosis with FA disassembly (personal communication with Greg Gundersen).

It has been suggested that FA disassembly is associated with reorganization of the actin cytoskeleton, such that the link between integrins and actin is lost (Mitra, Hanson et al. 2005). Different cytoskeletal adaptor proteins, such as talin, filamin, paxillin, zyxin, and alpha-actinin form this link, with an important regulatory role of different FA protein phosphorylation, such as FAK and Src family kinases. This brings us to a model, where a very sensitive and delicate phosphorylation/dephosphorylation balance determines the loss of integrin-actin connection, growth of microtubules to the

structure with endocytic machinery proteins, and subsequent vesicular internalization. Future imaging improvements might enable us to see constant endo/exocytic traffic of integrins and focal adhesion proteins within the small subregions of the structure. There are probably tens of different proteins that might be involved in the regulation. However, it would be important to find out the major regulators and the stimulus that initiates the disassembly and how this is coupled to endocytic machinery. As the work of Gundersen and Kaibuchi suggest, integrin endocytosis from focal adhesions could be mediated by clathrin route. However, the molecular mechanisms that regulate integrin internalization outside focal adhesions are also of great interest.

Table 1. Regulators of integrin endocytosis.

Gene/molecule	Name	Description	Reference
CAV1	Caveolin 1	α5β1 integrin is important for fibronectin (Fn) endocytosis via CAV1-dependent pathway. However, α5β1 can be endocytozed without Fn. Could be important for ECM remodeling as Fn is degraded after endocytosis.	(Shi, Sottile 2008)
CD151	CD151	The CD151 cytoplasmic motif YRSL, which is bound by AP-2, is needed for CD151 vesicular localization and endocytosis. CD151 associates with α3-, α5-, and α6β1 integrins through extracellular domain and is co-endocytozed with these integrins. Mutation of YRSL motif impairs CD151 endocytosis toghether with its associated integrins, which is however a fraction of total integrins. YRSL mutation also decreased transwell migration to Fn or Lam1.	(Liu, He et al. 2007)
CDH13 (T-cadherin, T-CADH, H-CADH)	cadherin 13, H-cadherin (heart)	Over-expression of T-CADH in keratinocytes suppressed internalization of both β1 integrin and cholera toxin (CTX), a marker of caveolae-mediated endocytosis. T-CADH also reduced EGFR phosphorylation. T-CADH is considered as tumor suppressor.	(Mukoyama, Utani et al. 2007)
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	Constitutively active mutant form of the EGF receptor (EGFRvIII) in ovarian cancer cells led to reduction in integrin α2 surface expression, defects in cell spreading, and disruption of focal adhesions. EGFR and α2 integrin internalized to separate locations, α2 primarily to golgi and ER. Integrin endocytosis was caveolae-mediated.	(Ning, Zeineldin et al. 2005) (Ning, Buranda et al. 2007)
F11R (JAM-A)	F11 receptor, Junctional adhesion molecule A	Co-localizes with β1 integrin in vesicles in neutrophils and co-clusters with β1 upon integrin stimulation. JAM-A-deficient cells have impaired β1 integrin endocytosis leading to defects in uropod detachment and cell motility.	(Cera, Fabbri et al. 2009)

Gene/molecule	Name	Description	Reference
HAX1	HCLS1 associated protein X-1	HAX-1 associates with αvβ6 integrin in squamous cell carcinoma cell lines by directly interacting with the α6 cytoplasmic tail. Knock-down of HAX-1 impairs transwell migration against αvβ6 ligand, TGFb LAP, but not against other integrin ligands. Knock-down cells have defect in clathrin-mediated αvβ6 and transferrin internalization. Also knock-down of clathrin or expression of DN EPS15 decreased migration against LAP. HAX-1 also needed for VB6 invasion to matrigel.	(Ramsay, Keppler et al. 2007)
IQSEC1 (BRAG2); ARF6	IQ motif and Sec7 domain 1; ADP-ribosylation factor 6	Knock-down of Arf6 GEF, BRAG2, increased cell adhesion to Fn and enhanced spreading to Fn. BRAG2 knock-down cells had more surface β1-integrin, but ARF6 knock-down cells had less surface β1 integrin, implying that BRAG2 and Arf6 are needed for β1 integrin traffic, either endocytosis or recycling or both.	(Dunphy, Moravec et al. 2006)
L1CAM (L1)	L1 cell adhesion molecule	Antibody cross-linking of L1 results in L1 and α3β1 integrin co-clustering on cell surface and subsequent internalization to Rab5-positive endosomes. Clathrin coat assembly inhibition with modansyl cadaverine (MDC) or deletion of the AP2/clathrin binding motif (RSLE) from the L1 cytoplasmic domain inhibits L1/β1 endocytosis and transwell migration of HEK293 cells.	(Panicker, Buhusi et al. 2006)
LGALS3 (Gal-3)	Galectin-3	Gal-3 induces β1 integrin endocytosis with Gal-3, which is cholesterol-dependent and inhibited by lactose. This caveolae-dependent internalization is coupled to actin reorganization and increased spreading of breast carcinoma cells.	(Furtak, Hatcher et al. 2001)
NUMB, AP2A1 (AP-2), PRKCI (aPKC), PARD3 (PAR-3)	numb homolog (Drosophila); adaptor-related protein complex 2; alpha 1 subunit, protein kinase C, iota; par-3 partitioning defective 3 homolog (C. elegans)	The clathrin adaptor NUMB localizes to leading edge of cells around FAs and directly binds to β1 and β3 integrin cytoplasmic tails through its PTB domain. Knock-down of NUMB, AP-2, or clathrin inhibited β1/β3 endocytosis and cell migration. aPKC and PAR-3 bind to NUMB and regulate its polarized localization. Phosphorylation of NUMB by aPKC inhibits endocytosis, but phosphorylation/ dephospohorylation cycle is needed for endocytosis however.	(Nishimura, Kaibuchi 2007)
PRKCA (PKCa); PI3K; Dynamin	protein kinase C, alpha;	PKCα associates and partially co-localizes with active, ligand-bound β1 integrin in recycling endosomes and multivesicular bodies (MVBs) together with transferrin and Rab11 in MCF7 and MDA-MB-231 cells. Phorbol ester activation of PKCα results in increased endocytosis of β1 integrin, which is dynamin dependent. PKCα over-expression increases MCF7 transwell migration.	(Ng, Shima et al. 1999)

Gene/molecule	Name	Description	Reference
SERPINE1, (PAI-1)	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	Addition of PAI-1 to HT1080 cells initiates detachment from Vn, Fn, or collagen I. PAI-1 initiates LRP-1-dependent endocytosis of uPA-uPAR-αV-integrin complex, not free integrin.	(Czekay, Aertgeerts et al. 2003)
Sphingolipids and cholesterol		Glycosphingolipid C8-LacCer or cholesterol addition to fibroblasts induces β1 integrin activation, clustering and increases caveolae-mediated integrin endocytosis, leading to cell rounding and transient detachment. Endocytosis is preceded by Src activation, Cav-1 phosphorylation by Src, actin depolymerization, and RhoA dissociation from PM.	(Sharma, Brown et al. 2005)
TNF (TNFA)	tumor necrosis factor (TNF superfamily, member 2)	Exposure of TNFA to lung endothelial cells resulted in increased α5β1 integrin endocytosis and recycling. This could have a role in endothelial cell monolayer interaction with ECM and monolayer permeability.	(Gao, Curtis et al. 2000)

Table 1b. Regulators of integrin recycling.

Gene	Name	Description	Reference
ACAP1; CLTC	ArfGAP with coiled-coil, ankyrin repeat and PH domains 1; Clathrin heavy chain	ACAP1 directly interacts with clathrin and this is needed for β1 integrin and Glut4 recycling in adipocytes. Arf6 and GAP-activity are needed for the assembly of the complex.	(Li, Peters et al. 2007)
Arf6; Rab11	ADP-ribosylation factor 6; RAB11A, member RAS oncogene family	Dominant negative ARF6-T27N or Rab11-S25N efficently blocked serum-stimulated β1 integrin recycling from recycling compartments. Also a mutant Arf6 Q37E/S38I that inhibits actin rearrangements inhibited recycling. Recycling was important for 2D MDA-MB-231 cell migration.	(Powelka, Sun et al. 2004)
EHD1	EH-domain containing 1	Knock-down of EHD1 in Hela cells leads to β1 integrin and transferrin accumulation in recycling endosomes as a result of recycling defect (12G10 chase assay). Similar results using human fibroblasts or MEFs deficient with EHD1. EHD1-/- MEFs have increased focal adhesion formation and active β1, suggesting a general defect in endo- and exocytic traffic.	(Jovic, Naslavsky et al. 2007)

Gene	Name	Description	Reference
LGALS1 (Gal1)	lectin, galactoside-binding, soluble, 1 (Galectin-1)	Gal-1 knock-down leads to accumulation of β1 integrin, vimentin, and PKCe in perinuclear region in glioblastoma cells.	(Fortin, Le Mercier et al. 2008)
PI3K; AKT (PKB); and GSK3	PI3K; v-akt murine thymoma viral oncogene homolog; and glycogen synthase kinase 3	Chemical inhibitors of PI3K, expression of DN AKT, or knock-down of AKT1/2 inhibited both short-loop αvβ3 and long-loop αvβ3/α5β1 recycling to PM. GSK3 phosphorylation and inactivation by AKT was important for recycling and fibroblast spreading on vitronectin or fibronectin.	(Roberts, Woods et al. 2004)
PRKCE; VIM	Protein kinase C, epsilon and Vimentin	PKCε phosphorylates Vimentin in endocytosed vesicles. PKCe activity dissociates PKCε-Vim complex from endocytic vesicles. Expression of Vim mutant that cannot be phosphorylated by PKCε (S4,6,7,8,9A) resulted in vesicular PKCε and β1 integrin with defect in integrin recycling. PKC inhibitor BIM-1 impairs α2 integrin vesicular movement.	(Ivaska, Vuoriluoto et al. 2005)
PRKD1 (PKD1)	protein kinase D1 (PKC-μ)	PKD1 Ser-916 is critical for interaction with αvb3 and for Rab4-mediated short-loop recycling of this integrin. Inhibition of αvβ3 recycling by DN PKD1 S916A impairs fibroblast directional motility, by mechanism that involves increased α5β1 long-loop recycling and signaling to Rho-ROCK-Cofilin (random migration).	(White, Caswell et al. 2007)
PRKD1 (PKD1); RAB4	protein kinase D1 (PKC-μ)	PKD1 associates with c-terminal β3 integrin cytoplasmic tail. PDGF induces PKD1 activity, which is needed downstream of Rab4-mediated αvβ3 recycling.	(Woods, White et al. 2004b)
RAB11FIP1 (RCP)	RAB11 family interacting protein 1, RAB11 coupling protein	αvβ3 inhibition (cilengitide, RGD-peptides, ospteopontin) increases α5β1 recycling in ovarian cancer cell line, by mechanism that is dependent on RCP association with α5β1 and Rab11. This contributes to random migration with strong ruffles at cell front, but also 3D FN-matrigel invasion. EGFR is recycled with the same complex, increasing its signaling to AKT.	(Caswell, Chan et al. 2008)
RAB25 (RAB11C)	RAB25, member RAS oncogene family	Rab25 interacts directly with β1 integrin cytoplasmic tail and is important for ovarian cancer cell line A2780 or fibroblast invasion in Fn-matrigel. Ectopic expression increases migration persistence in cell-derived matrix with pseudopod morphology and actively recycling pool of α5β1 at the pseudopod tip.	(Caswell, Spence et al. 2007)

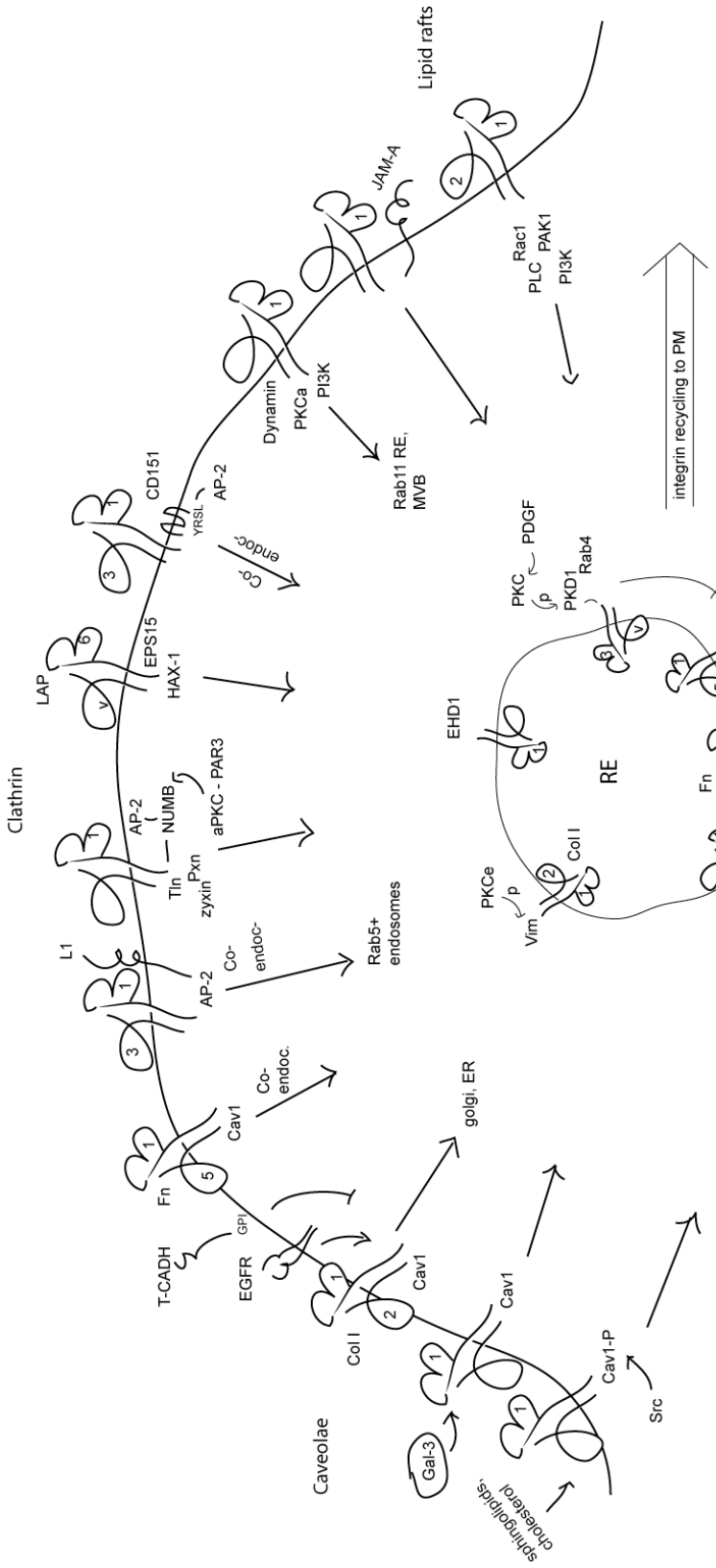
Clathrin
LAP
L1
AP-2
NUMB
aPKC - PAR3
Tln
Pxn
zyxin
HAX-1
EPS15
AP-2
Co-endoc.
Rab5+ endosomes
CD151
YRSL
AP-2
endoc.
Dynamin
PKCa
PI3K
Rab11 RE, MVB
JAM-A
Lipid rafts
Rac1
PLC
PAK1
PI3K
integrin recycling to PM
EHD1
PKCe
p
Vim
Col I
RE
PKC
PDGF
p
PKD1
Rab4
Rab11A
EGFR
RCP
Fn
Rab25
ACAP1
clathrin
TfR
AKT1
p
ARF6
Co-endoc.
Cav1
Fn
T-CADH
GPI
EGFR
golgi, ER
Col I
Cav1
Cav1
Caveolae
Gal-3
sphingolipids, cholesterol
Cav1-P
Src

Figure 1. Integrin traffic. The most studied pathways for integrin internalization are caveolin- or clathrin-dependent. This can be due to better knowledge of these pathways and existing molecular tools to study these pathways. However, caveolae-mediated endocytosis internalize at least β1 integrins, α2β1 and α5β1, of which the latter can be endocytozed with or without its ligand, fibronectin (Fn) (Shi, Sottile 2008). Contrary to integrins, which are recycled back to the PM, Fn and other ECM proteins end up in lysosomes for degradation. Exogenously added sphingolipids, cholesterol, or carbohydrate-binding galectin-3 (Gal-3) induces caveolae-mediated β1 integrin endocytosis. Whereas Gal-3 is known to enhance integrin-mediated adhesion and integrin activity, sphingolipids or cholesterol addition round up and even detach fibroblasts from substratum (Sharma, Brown et al. 2005; Kahsai, Cui et al. 2008). Ning, Zeineldin, and others (2007) have shown that constitutively active EGFR induces α2β1 integrin endocytosis via caveolae, and that the two receptors traffic to distinct locations, α2β1 ending-up in golgi and ER. Interestingly, a GPI-anchored cadherin, T-CADH, suppresses EGFR phosphorylation and inhibits β1 integrin endocytosis through caveolae (Mukoyama, Utani et al., 2007). Clathrin-mediated endocytosis could be coupled to focal adhesion disassembly and the clathrin adaptor proteins AP-2 and NUMB could have both a structural and regulatory role in this process. AP-2 adaptor is required for co-endocytosis of α3β1–L1 adhesion protein and α3β1-CD151. The tetraspanin CD151 and L1 adhesion protein contain an YRLS-motif, where AP-2 binding is required for the endocytosis and integrin-mediated migration. Dissociation of CD151 from α3β1 with anti-CD151 antibodies leads to integrin inactivation and reduction in cell adhesion to laminin-10/11 (Nishiuchi, Sanzen et al. 2005). An AP-2-binding protein, NUMB, localizes to the vicinity of focal adhesions and is required together with AP-2 for β1 integrin internalization from these structures. The clathrin-mediated integrin internalization studies suggest a positive role of this process in cellular migration or invasion (see Table 1). Integrins traffic to different endosomal compartments, early and late endosomes, which are connected to lysosomes, multivesicular bodies, golgi, ER and exocysts. Recycling occurs from recycling endosomes (RE) or perinuclear recycling endosomes (PNRE). Here they are collectively called recycling endosomes. The most studied integrin recycling pathways rely on Rab family GTPases, Rab4, Rab11A, and Rab25 as well as Arf6 GTPase. The growth-factor-stimulated fast-recycling (short-loop) Rab4 pathway carries αvβ3 integrins, is dependent on protein kinase D1 (PKD1) kinase activity, and inhibits the slow-recycling (long-loop) Rab11 pathway, which transports α5β1 integrin (White, Caswell et al. 2007). The Rab4-mediated αvβ3 recycling reflects cells' ability to migrate persistently, whereas the Rab11 pathway enhances cell motility with impaired directionality. Interestingly, the random motility correlates with cells' ability to invade into fibronectin-containg matrigel (Caswell, Chan et al. 2008). If atypical PKCs (aPKC), PKCζ and PKCι, as well as the classical PKCα are implicated in integrin endocytosis, the novel PKCε regulates β1 integrin recycling. PKCε associates with β1 integrin positive endosomes and stimulates β1 integrin recycling by phosphorylating N-terminal serines in vimentin intermediate filament protein (Ivaska, Vuoriluoto et al. 2005). It would be interesting to study whether this correlates to vimentin's ability to induce epithelial-mesenchymal transition in cancer.

2.2. Cytokinesis

During cell division, vast morphological changes can be seen starting from rounding up in prometaphase, cell elongation during chromatid separation, ingression of plasma membrane during cytokinesis, and finally elongation and separation of daughter cells from each other (Glotzer 2001). These events are dependent on dynamics of the actin and microtubule cytoskeletons as well as membrane turnover. It is very interesting that the molecular machineries in cytokinesis resemble that of cell adhesion and migration. These include the family of Rho GTPases, its downstream effectors ROCK, myosin light chain kinase (MLCK or MYLK), cofilin etc., but also vesicular trafficking regulators such as Rab11, Arf6, and the exocyst complex (Ridley, Schwartz et al. 2003, Glotzer 2005; Pellinen, Ivaska 2006). There are studies showing that adherent cells require matrix adhesion for cytokinesis. For example chondrocytes with inactive β1 integrin have impaired cytokinesis, which is most probably due to defects in adhesion to collagen II and fibronectin in these cells (Aszodi, Hunziker et al. 2003). In addition, Reverte and others (2006) have shown that perturbation of β1 integrin activity by mutations lead to impaired microtubule spindle assembly in mitosis, which results in defective cytokinesis (Reverte, Benware et al. 2006). These results suggest a mechanistic link between cell division and cell adhesion.

The last step of cell division is the cytokinesis, where daughter cells are physically separated by ingression and cleavage of the membrane. In metazoan symmetric cell division, the localization of furrowing at the plasma membrane is determined by spindle pole-growing astral microtubules and central spindle microtubules (see Figure 2). The stability of microtubules in the central equatorial region allows centralspindlin proteins (MKLP1, CYK-4) to assemble to the central spindle (Odell, Foe 2008). This activates polo-like kinase 1 (PLK1), which activates RhoA through phosphorylation of Rho GEF, ECT2. RhoA action through its effectors (formins, ROCK, Citron kinase) is fundamental for furrow ingression as this pathway ultimately leads to myosin II activation and actin polymerization, which are needed for acto-myosin-driven contraction and formation of contractile actin ring (Piekny, Werner et al. 2005). This actin ring contraction bundles central spindle microtubules together and tightens them to form the midbody structure, where the final abscission occurs.

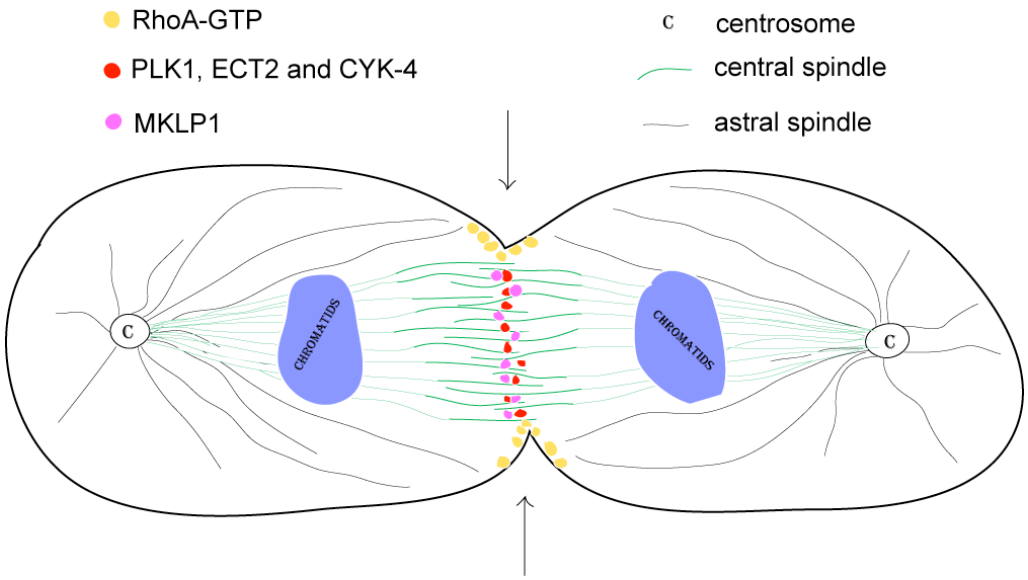

Figure 2. Metazoan symmetric cytokinesis. Separation of cytosol and sister chromatids occurs by ingression or invagination of plasma membrane (arrows). This equatorial midplane region (midzone) accumulates non-kinetochore antiparallel microtubules originating from centrosomes (central spindle). In addition to central spindle and kinetochore spindle, centrosomes direct the growth of astral spindle microtubules towards cortical plasma membrane, where they associate with actin (actin not shown). Stabilized astral and central microtubules decide the location of furrow formation by reqruiting RhoA activating protein ECT2 to the equatorial midplane (see text for details). RhoA effector, ROCK, is needed for myosin II activation and actin contraction, whereas another effector, mDia1 (formin) is needed for actin polymerization through profilin action (not shown). Together they enable the formation of contractile actin ring, which ingresses to form the midbody structure, where final abscission occurs.

During cytokinesis, a constant flow of membrane vesicles is transported to ingression and abscission sites. This apparently golgi- and endosome-derived material brings new membrane to the growing surface of ingressing plasma membrane (Goss, Toomre 2008; Albertson, Cao et al. 2008). For example Rab11 and its interacting protein FIP3 traffic via centrosome-associated spindle microtubules to cleavage furrow in endosomal vesicles (Wilson, Fielding et al. 2005). FIP3 (Nuf) modulates RhoA (Rho1) activity via RhoGEF2 in *Drosophila* and regulates actin polymerization in cleavage furrow (Cao, Albertson et al. 2008). Thus, it is clear that vesicle membrane flow in the furrow is not only for increased need in surface area, but also for effector functions in the process. FIP3 is also needed for the final midbody abscission, where it is recruited by binding to centralspindlin protein Cyk-4 (also called MgcRacGAP). This displaces ECT2 from binding to Cyk-4 allowing contractile ring to dissassemble (Simon, Schonteich et al. 2008). Interestingly, FIP3 and Rab11 bind to secretory exocyst complex, whose components also participate in the final abscission, but also in the ingression (Gromley, Yeaman et al. 2005; Cascone, Selimoglu et al. 2008). What exactly these endocytic or secretory vesicles are carrying as cargo and what their specific roles are in cytokinesis is still unknown.

Cytokinesis failure often leads to formation of a binucleate cell, with tetraploid progeny. Tetraploidy is considered to be a transient pathway to aneuploidy through subsequent erroneous chromosomal segregations. As aneuploidy feeds more and more random errors in cell division, changes gene expression signature, and accumulates DNA replication errors, its suggested role in tumorigenesis is mounting (Ganem, Storchova et al. 2007). Recently, Rancati and others created fast adaptive evolution in yeast by knocking *MYO1* gene (myosin II) and causing cytokinesis failure (Rancati, Pavelka et al. 2008). The failure resulted in formation of polyploidy, leading to aneuploidy, high variation in gene expression, and ultimately, selection for fittest cytokinesis phenotypes with specific changes in transcriptome. Similar evolution could also occur in tumorigenesis.

2.3. Integrin activity regulation

2.3.1. Introduction

Integrins are bidirectional signaling proteins. Extracellular ligand binding to integrin induces signal transduction across the plasma membrane in so-called outside-in activation. A stimulus from inside the cell can activate integrins to bind ligand, a process called inside-out activation. The current model is that integrins exist in equilibria of different conformations: 1) bent (inactive); 2) extended (primed); 3) extended and ligand-bound, where the different integrin conformations bear affinity differences to ligand as much as 10 000-fold (Luo, Carman et al. 2007). Ligand binding and tensile force favors the extended open conformation. Work on leukocytes and platelets suggests that rapid integrin activation occurs through inside-out activation (Alon, Ley 2008), whereas subsequent ligand binding and clustering (avidity) then stabilizes bond strength (Shattil, Newman 2004). During outside-in signaling integrins form connection to actin cytoskeleton through proteins that bind to conserved motifs in the cytoplasmic tails of β subunits. This further stabilizes bond strength, but also allows clustering of many cytoskeletal adaptor and effector proteins to regulate actin polymerization, cross-talk with receptor tyrosine kinases, and downstream signaling to MAP-kinases (Shattil, Newman 2004; Guo, Giancotti 2004; Cox, Natarajan et al. 2006).

The studies on regulation of integrin activity have been mainly carried out in platelets and leukocytes. This is due to the obvious bleeding or inflammatory pathological conditions associated with impaired integrin function in these cells (Glanzman thrombastenia, thrombosis, leukocyte adhesion deficiencies I, III). Blood cells also carry integrins in inactive conformation when circulating in blood, but in the site of vessel injury or inflammation, integrins need to be rapidly activated. Thus the nature of these cells makes them perfect for integrin activity studies, and will be discussed here in more detail.

2.3.2. Talin is the proximal integrin regulator

The final step in integrin activation is the binding of talin to the beta cytoplasmic tail (Tadokoro, Shattil et al. 2003; Petrich, Marchese et al. 2007; Nieswandt, Moser et al. 2007; Manevich, Grabovsky et al. 2007; Bouaouina, Lad et al. 2008). Talin interacts directly through its head FERM domain with NPXY (NPLY in β3) motif in beta cytoplasmic tails but also through its rod domain with the membrane proximal α-helix. A suggested mechanism for proximal integrin activation is that talin opens up the alpha and beta cytoplasmic tails by disrupting the salt bridge between membrane proximal α-helices of the cytoplasmic tails (Vinogradova, Velyvis et al. 2002; Rodius, Chaloin et al. 2008). This leads to conformational changes that increase the affinity of the extracellular domain to ligand (Luo, Carman et al. 2007). The FERM domain binding to NPLY is important for inside-out activation (Tadokoro, Shattil et al. 2003; Tanentzapf, Brown 2006; Zhang, Jiang et al. 2008), whereas the rod domain is essential for mediating interaction with the cytoskeleton. Intact talin is required for substrate adhesion and focal adhesion formation and actin polymerization in adhesion plaques (Zhang, Jiang et al. 2008). It is suggested that the rod domain may only bind to beta-tail when the integrin is activated and ligand-bound (Rodius, Chaloin et al. 2008; Tanentzapf, Brown 2006). Taken together, results suggest that talin is critical for bi-directional integrin signaling where the head FERM domain activates the integin conformational change and the rod domain further stabilizes the activated state by interacting with actin cytoskeleton and allowing force generation to substrate. The sustained adhesion and force generation is maintained by integrin outside-in signaling through proximal adaptor and effector proteins that will be discussed later.

2.3.3. Platelet integrin αIIbβ3 activation

Circulating platelets are activated when they form contacts with injured vessel wall exposing extracellular matrix proteins. Under physiological or high shear flow, the platelet glycoproteins GPVI and GPIb bind to collagen and collagen-associated von Willebrand factor (vWF) multimers, respectively, and tether platelets to the subendothelium (Andrews, Berndt 2008). This leads to inside-out activation of the platelet integrin αIIbβ3, allowing it to bind fibrinogen and vWF. During activation, platelets secrete alpha- and dense granules that contain platelet agonists ADP and thromboxane A2 (TXA2), which enhance αIIbβ3 activation through GPCR-signaling pathways and recruit new platelets (see Figure 3). Different *in vivo*-produced serine proteases, such as plasmin and thrombin cleave vessel wall proteins increasing the platelet coverage of the subendothelium (Komorowicz, McBane et al. 2002). Activated platelets twist the αIIbβ3 into high-affinity conformation allowing it to bind also soluble ligands in the bloodstream. Outside-in signaling of αIIbβ3 allows platelets to spread on fibrinogen and to form aggregates by platelet-platelet interactions through cross-linking fibrinogen multimers. Thrombus stability is maintained by correct cytoskeletal architecture, where outside-in signaling of αIIbβ3 plays a major role. Somatic mutations in αIIbβ3 may lead

to bleeding disorder, Glanzmann thrombasthenia, where platelets are unable to adhere properly or form proper platelet aggregates (Kato 1997). Another important platelet integrin is α2β1, which by binding to collagen I activates αIIbβ3 through pathways that are dependent on GPVI crosstalk with α2β1 (Nakamura, Kambayashi et al. 1999; Bernardi, Guidetti et al. 2006). Other evidence suggests that α2β1 integrin outside-in signaling in platelet activation can function also independently of GPVI (Inoue, Suzuki-Inoue et al. 2003).

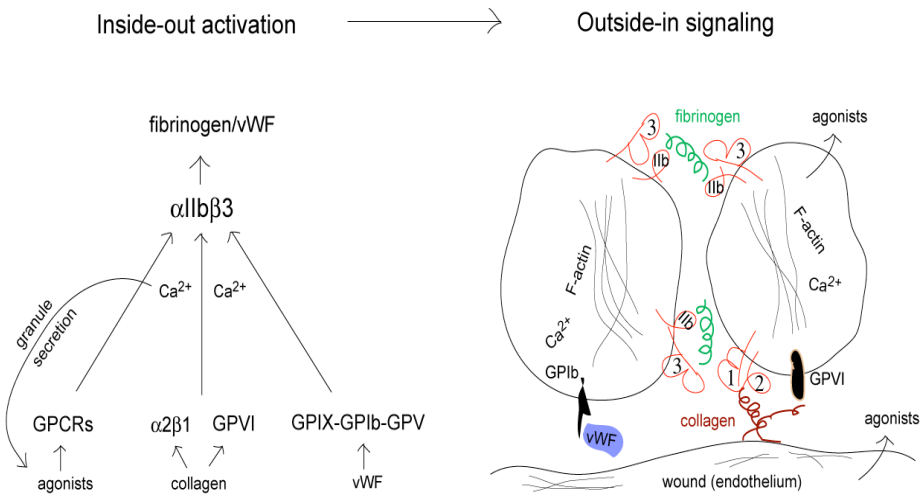

Figure 3. Platelet activation. Inside-out activation is triggered by platelet surface glycoprotein binding to endothelium-exposed collagen and/or von Willebrand Factor (vWF) (left). Cytoplasmic signaling pathways lead to conformational change of αIIbβ3 integrin that allows it to bind extracellular soluble fibrinogen and vWF. Cytoplasmic calcium flux activates alpha and dense granule secretion, which further activate platelet integrins. Ligand-bound αIIbβ3 signals to actin cytoskeleton and enhances calcium flux, which together strengthen and stabilize ligand binding allowing platelets to aggregate, contract, and form the blood clotting thrombus (outside-in signaling, right). Agonists secreted from platelets and endothelium contribute to sustained platelet activation.

2.3.3.1. Inside-out activation of αIIbβ3

2.3.3.1.1. A Complex of adaptor proteins recruit talin to activate αIIbβ3 integrin

Studies on CHO reconstitution model system demonstrate that αIIbβ3 integrin inside-out activation upon chemokine or phorbol ester stimulation is dependent on an "activation complex" consisting of PKCα, Rap1, RIAM, and talin (Han, Lim et al. 2006). Further studies have shown that knock-down of RIAM inhibits talin recruitment to αIIbβ3 integrin (Watanabe, Bodin et al. 2008). RIAM is a Rap1 effector protein, which binds to profilin and ENA/VASP proteins and is important in actin polymerization during integrin activation (Lafuente, van Puijenbroek et al. 2004). Two other adaptor proteins, ADAP (Fyb) and SKAP-55, play roles in recruiting RIAM and Rap1 to plasma membrane thus facilitating integrin activation in T-cells (Menasche, Kliche et al. 2007). In platelets,

ADAP forms a complex with another SKAP, SKAP-HOM. However, the knock-down of ADAP, but not SKAP-HOM, leads to αIIbβ3 integrin activation defects upon GPCR agonist or vWF-GPIb stimulation (Kasirer-Friede, Moran et al. 2007) (see Figure 4).

Phosphorylation of ADAP by Src family kinases (Fyn, Lyn) allows adaptor protein SLP-76 to bind ADAP through its SH2 domain (Musci, Motto et al. 1997). SLP-76 has been recognized as an important mediator of αIIbβ3 outside-in signaling, but also of collagen/GPVI-mediated inside-out activation of αIIbβ3 (Judd, Myung et al. 2000; Bezman, Lian et al. 2008). Mice deficient with SLP-76 show bleeding diathesis and decreased survival (Clements, Yang et al. 1998; Pivniouk, Tsitsikov et al. 1998), a moderate phenotype that could be seen as a result of an impairment of both the outside-in and inside-out signaling of αIIbβ3. In comparison, the knock-out of the Fcγ receptor (FcRγ), which is implicated in collagen and vWF-stimulated calcium signaling and integrin inside-out activation through Syk, shows only a platelet aggregation defect, but no bleeding or decreased survival (Poole, Gibbins et al. 1997). However, the knock-out mice of both Syk or its downstream player PLCγ2 have lethal bleeding diathesis (Cheng, Rowley et al. 1995) or severe internal bleeding (Wang, Feng et al. 2000), again probably due to their importance in both inside-out and outside-in αIIbβ3 integrin activation (see chapter outside-in signaling). Altogether, the above studies suggest that there is an important adaptor complex consisting of SLP-76 – ADAP – SKAP – RIAM, which is important in recruiting the inside-out activation players Rap1 and talin, but also in modifying the actin cytoskeleton for sustained adhesion through outside-in signaling of αIIbβ3 integrin.

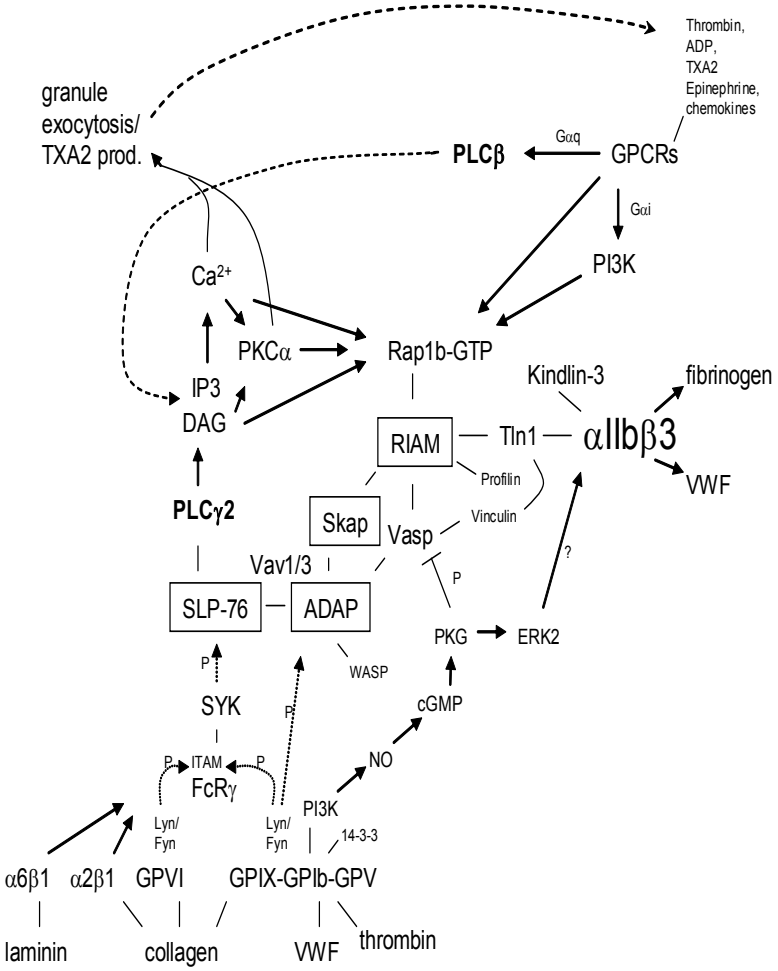

Figure 4. Inside-out activation of platelet integrin αIIbβ3. Platelets adhere to von Willebrand factor (VWF), collagen, and laminin exposed in injured vessel walls (bottom). Glycoprotein GPVI and a glycoprotein complex (GPIX-GPIb-GPV) initiate Src family-mediated phosphorylation directly to downstream adaptor proteins or to adaptors through Fc-receptor gamma (FcRγ) – Syk pathway. Spleen tyrosine kinase (Syk) is activated either by Src family phosphorylation or binding to phosphorylated immunoreceptor tyrosine activation motif (ITAM). Phosphorylated adaptor proteins (SLP-76 and ADAP) are recruited to αIIbβ3 integrin proximity by another adaptor protein RIAM, which is an effector for activated Rap1 and associates also with integrin activator, talin (Tln1). The adaptor proteins form the platform for effector proteins and actin cytoskeleton for integrin activation. Another ECM-stimulated pathway employs nitric oxide (NO), cGMP, and protein kinase G (PKG) cascade. This requires phophorylation of ERK2 kinase, but the final mechanism of integrin activation in this pathway is unclear (see text for details). PLC-gamma (PLCγ2) is important for second messenger generation (DAG, calcium). These messengers activate Rap1 either directly or through protein kinase C. Calcium and PKCs are also important in alpha- and dense granule secretion, which contain various GPCR agonists. GPCR-binding agonists activate neighboring platelets, but also self-activate in autocrine fashion. GPCRs signal to integrins through PLC-beta or PI3K-dependent pathways. Recently, Kindlin-3 binding to beta3 tail was shown critical for platelet integrin activation as well (Moser, Nieswandt et al. 2008).

2.3.3.1.2. PLCγ2 and PLCβ drive second messanger generation for platelet activation

PLCγ2 and PLCβ are lipid kinases generating calcium and DAG downstream of collagen/vWF and GPCR signaling, respectively (Suzuki-Inoue, Wilde et al. 2004; Mangin, Yuan et al. 2003; Offermanns, Toombs et al. 1997). The platelet agonists ADP, thrombin, and TXA2 signal through their cognate GPCRs to activate the G protein, Gαq leading to PLCβ activation, platelet aggregation, and granule release (Offermanns, Toombs et al. 1997). Platelet agonists may also activate Rap1b independent of PLCβ activity. This pathway requires Gαi-dependent activation of PI3K, especially the catalytic isoforms p110β and p110γ (Schoenwaelder, Ono et al. 2007) (see Figure 4).

Phospholipases catalyze the formation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) from inositol 4,5-bisphosphate (PIP2). DAG activates PKCα, and IP3 induces the mobilization of calcium through IP3 receptor channel (ITPR1) from the endoplasmic reticulum. In platelets, calcium is needed for the activation of Rap1b through the action of calcium and DAG-dependent Rap1 GEF, CalDAG-GEF1 (RASGRP1) (Crittenden, Bergmeier et al. 2004), but also for the activation of the calcium and DAG-dependent PKCα (Franke, Akkerman et al. 1997; Tabuchi, Yoshioka et al. 2003). A rare genetic CalDAG-GEF1 mutation causes a "leukocyte adhesion deficiency"-disease (LAD-III syndrome) with a clear abrogation of integrin inside-out activation in both leukocytes and platelets (Pasvolsky, Feigelson et al. 2007). Mice deficient with this protein show impaired platelet aggregation and prolonged bleeding times (Crittenden, Bergmeier et al. 2004). When Rap1b function is required for both GPCR-dependent and -independent integrin activation (Chrzanowska-Wodnicka, Smyth et al. 2005), CalDAG-GEF1 activity is required only for thrombin-stimulated GPCR (PAR4) - and Gαq-mediated activation pathway of integrin (Cifuni, Wagner et al. 2008). This pathway synergizes with ADP-stimulated Gαi pathway and ADP secretion-stimulating PKCα pathway.

Tec family kinases Tec and Btk can induce calcium mobilization by stimulating PLCγ2 (Quek, Bolen et al. 1998) or by store-mediated calcium entry (SMCE), which is independent of PLCγ2 function (Pasquet, Quek et al. 2000; Redondo, Rosado et al. 2005). Activation of Tec kinases in platelets may occur through activation of ITAM-bearing receptors GPVI-FcRγ (collagen) or Fc receptor FcγRII (antibody crosslinking) or by thrombin (Quek, Bolen et al. 1998; Redondo, Rosado et al. 2005; Oda, Ikeda et al. 2000). Tec kinases can be also directly activated by heterotrimeric G-proteins, Gαq and Gα12 (Jiang, Ma et al. 1998).

2.3.3.1.3. Nitric oxide – cGMP signaling; activator and inactivator?

The L-Arginine – NO – cGMP - pathway (see Figure 5) has been linked to inactivation of platelets (Radomski, Palmer et al. 1990; Chrzanowska-Wodnicka, Smyth et al. 2005). Platelets make nitric oxide (NO) and L-Citrulline from L-Arginine in a reaction catalyzed

by the enzyme nitric oxide synthase 3 (NOS3 or eNOS) (Radomsky et al., 1990). The reaction requires calcium as a cofactor, and it has been suggested that the increased calcium during platelet activation leads to suppression of aggregation depending on this particular pathway (Chrzanowska-Wodnicka, Smyth et al. 2005). NO activates guanylyl cyclase (GC) leading to the generation of cyclic guanosine monophosphate (cGMP) and subsequent activation of protein kinase G isoforms (PKGs), but also protein kinase A (PKA). Some time ago, however, vWF stimulated GPIb signaling was characterized as novel activator of L-Arg – NO – cGMP – PKG signaling leading to inside-out activation of αIIbβ3 integrin (Li, Xi et al. 2003). PKG deficient platelets showed reduced responses to vWF or to small amounts of thrombin and the knock-out mice had longer bleeding times. Li and others have further shown that cGMP-activated PKG leads to p38 and ERK2 phosphorylation, and that inhibition of p38 by dominant negative construct abolishes the integrin inside-out activation in a CHO reconstitution model (123 cells) (Li, Zhang et al. 2006). The same group also showed that the Src family kinase Lyn and its downstream PI3K-AKT1/2 activation are important for cGMP generation and PKG phosphorylation (Yin, Stojanovic et al. 2008; Yin, Liu et al. 2008) (see Figure 4). These results could imply that the NO – cGMP pathway has activating role in the early inside-out activation of αIIbβ3, but during the prolonged platelet activation and aggregation (outside-in signaling) the pathway gains inhibitory effect. The mechanism by which p38 or ERK2 induces integrin activation has not been studied.

An Ena/VASP family member VASP (vasodilator-stimulated phosphoprotein) could be the effector determining the positive or negative role of cGMP and cAMP in integrin activation. VASP is phosphorylated by PKG and PKA at sites Ser239 and Ser157, respectively. It directly binds actin and focal adhesion proteins and is implicated in the regulation of actin polymerization and bundle formation, cell adhesion, migration, and axon guidance (Krause, Dent et al. 2003). The phosphorylation of Ser157 of VASP correlates with αIIbβ3 inhibition measured by soluble fibrinogen binding and platelet aggregation (Horstrup, Jablonka et al. 1994). The Ser157 phosphorylation by PKA inhibits also agonist-induced shape change from discoidal low F-actin to spherical high F-actin (Jensen, Selheim et al. 2004). Also, as the phosphorylation of VASP Ser239 by PKG correlates with reduced Rap1b GTP loading in plateles, Rap1-signaling to integrin could be the ultimate target of NO-cGMP-mediated platelet inhibition (Danielewski, Schultess et al. 2005). This idea has been further studied by the work of Isenberg and coworkers, showing that Thrombospondin-1 (Tsp1) ligation to CD36 or CD47 receptors is able to block the inhibitory effect of NO-cGMP-PKG-VASP pathway in platelet adhesion and aggregation (Isenberg, Romeo et al. 2008) (Figure 5). They also showed that NO lowered the Rap1 activity and that addition of Tsp1 reversed this inhibition. I suggest that during inside-out integrin activation, unphosphorylated VASP could assist in forming the actin scaffold together with the adaptor proteins, RIAM and vinculin, to which it directly

binds. During sustained activation, phosphorylation of VASP by PKA or PKG could lead to inhibition of this actin scaffold, dissociation of the RIAM – Rap1b, and abrogation of integrin outside-in signaling. In leukocytes, PKA phosphorylates directly the α4 integrin cytoplasmic tail (Y991), which disrupts paxillin adaptor protein binding to it (Goldfinger, Han et al. 2003). This inhibits α4β1 (VLA-4) outside-in signaling and anchorage to actin during adhesion strengthening on VCAM-1 under shear flow (Alon, Feigelson et al. 2005). This means that PKA has distinct mechanisms of negative regulation of integrins in different cell types. However, this can be even more complex as cAMP can have a positive role in epithelial progenitor cell β1 and β2 integrin activation by stimulating the cAMP-activated Rap GEF, EPAC1 (Carmona, Chavakis et al. 2008).

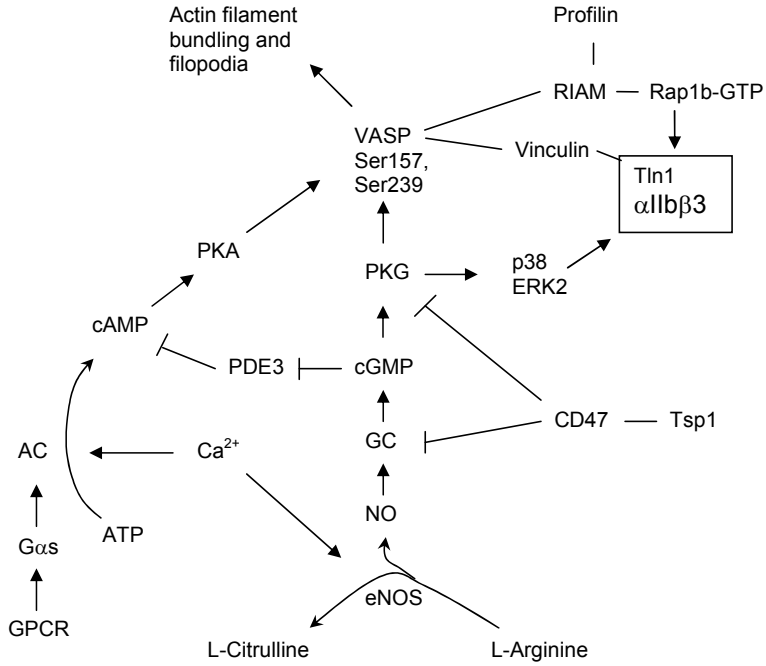

Figure 5. NO – cGMP pathway in platelet integrin activation. Nitric oxide synthase (eNOS) catalyzes the formation of nitric oxide (NO) from L-Arginine. Guanylate cyclase (GC) drives the formation of cGMP, which activates protein kinase G (PKG). cGMP also elevates cAMP levels by inhibiting the enzyme responsible for cAMP degradation (PDE3) (Sheth and Colman, 1995). The inhibitory effect of cGMP and cAMP in platelet function is mediated by VASP, as in VASP-deficient platelets cGMP and cAMP are unable to inhibit aggregation (Aszodi et al., 1999). The phosphorylation of VASP at Ser157 and Ser239 correlates to reduced Rap1b activation, αIIbβ3 activation, and platelet aggregation. VASP binds directly to actin and proximal integrin adaptors, and its Ser157 phosphorylation negatively affects its positive function in actin filament bundling and filopodia formation (Pula et al., 2006). NO – cGMP – PKG signaling may also activate αIIbβ3 through p38 and ERK2 MAP-kinases, and cAMP can be a potent activator of Rap1 in other cell types (see text). Altogether, NO signaling is very complex, probably due to rapid flux of the various second messangers involved in the pathway, which I suggest leads to intimate balance of phosphorylation/ dephosphorylation of VASP with dynamic effects on actin turnover.

2.3.3.1.4. ERK2 (MAPK1) and integrin activation in platelets

ERK2 seems to be a critical mediator of platelet integrin αIIbβ3 activation. Von Willebrand factor (vWF)-initiated NO-cGMP-PKG pathway requires ERK2 and p38 phosphorylation for the early inside-out activation of the integrin. Also the collagen stimulated GPVI-FcRγ signaling results in thromboxane 2 (TXA2) formation and ADP secretion, which both activate ERK2 in Galpha(q)- and Galpha(i) (P2Y12)-dependent manner, respectively (Roger, Pawlowski et al. 2004). Thrombin acts synergistically with TXA2 to increase ADP and activate ERK2 through the P2Y12 Galpha(i) signaling (Falker, Lange et al. 2004). In addition, ERK2 activation itself is important for the production of TXA2 (Garcia, Quinton et al. 2005), which further enhances integrin activation through the GPCR-mediated signaling. αIIbβ3 integrin also promotes ERK2 activation by outside-in signaling through Rho signaling pathway (Mazharian, Roger et al. 2007). In this study integrin outside-in activation was analyzed by looking at cell spreading on fibrinogen after addition of PAR4 agonist peptide (PAR4-AP). ERK2 activation was dependent on PAR4-AP-induced ADP signaling and spreading to fibrinogen and resulted in myosin light chain (MLC) phosphorylation, whereas p38 phosporylation, which was integrin independent but ADP dependent, resulted in actin polymerization. Another group has showed that MLC binding to tyrosine phosphorylated αIIbβ3 is important for blood clot retraction and stabilization of thrombus downstream of ephrin receptors (Prevost, Woulfe et al. 2005). However, they did not look at the possible function of ERK2 in this model. Thus, ERK2 activation can have roles in both the early wave integrin activation and late sustaining of fibrinogen binding, which is important for platelet aggregation and thrombus formation.

2.3.3.2. Outside-in signaling of αIIbβ3

Thrombus formation requires stabile platelet – platelet association, where αIIbβ3 integrins crosslink adjacent cells through fibrinogen binding. Integrin – fibrinogen interaction leads to changes in actin cytoskeleton and tyrosine phosphorylation of multiple proteins, which together sustain the activation state of the integrin and allow even tighter cell - cell association and clot retraction. Obviously, similar molecular requirements as in inside-out activation are required for sustaining of integrin activity and signaling to downstream proteins. As talin cross-links integrins to the actin cytoskeleton and enables force generation and spreading of cells to substrate, it must be crucial for the outside-in signaling as well. Zhang and others (2008) recently showed that talin1/talin2 deficient fibroblasts have deficiencies in sustained spreading and adhesion, although the initial spreading was not affected. The knock-out cells were unable to form focal adhesions with activated FAK, and F-actin was diffuse in the cytoplasm. However, the Src family kinase phosphorylation was not compromised, and inhibition of Src by PP2 abrogated the initial fibronectin – integrin-mediated spreading. This work nicely illustrated the importance of talin in sustaining the outside-in signaling of integrins to the actin cytoskeleton and to

focal adhesion – associated proteins, but also highlighted the role of Src family kinases in the early spreading.

2.3.3.2.1. Src family kinases

Src family kinases (SFKs) (c-Src, Fyn, Lyn, c-Yes, and Hck) bind directly to β3-integrin cytoplasmic tails, whereas Lyn, c-Yes, and Hck also bind to β1 and β2 tails (Arias-Salgado, Lizano et al. 2003). Mice platelets deficient in multiple Src-family kinases fail to spread on fibrinogen and show impaired general tyrosine phosphorylation, whereas c-Src-only-deficient platelets spread normally (Arias-Salgado, Lizano et al. 2003). Inhibition of SFKs by chemical inhibitors or truncation of β3-tail after tyrosine-759, where C-Src binds, or addition of RGT-peptide that mimics the C-terminus of β3-tail, impairs integrin outside-in signaling, but not inside-out signaling (Obergfell, Eto et al. 2002; Xi, Bodnar et al. 2003; Su, Mi et al. 2008). C-Src is constantly associating with αIIbβ3 in circulating platelets and becomes rapidly activated upon fibrinogen binding (Obergfell, Eto et al. 2002). In Obergfell's model, c-src tyrosine kinase (Csk) also associates with αIIbβ3 and keeps c-Src inactivated by phosphorylating the inhibitory tyrosine-530. Fibrinogen binding to integrin releases the inhibitory Csk and c-Src is activated by the phosphorylation of its activation loop tyrosine-419. More recent work has demonstrated a role for PTP-1B (PTPN1) in the dephosphorylation of tyrosine-530 of c-Src in platelets (Arias-Salgado, Haj et al. 2005). As Src family kinases have a prominent role in integrin outside-in signaling and will be discussed more below, the activation mechanism of Src family kinases is depicted in Figure 6.

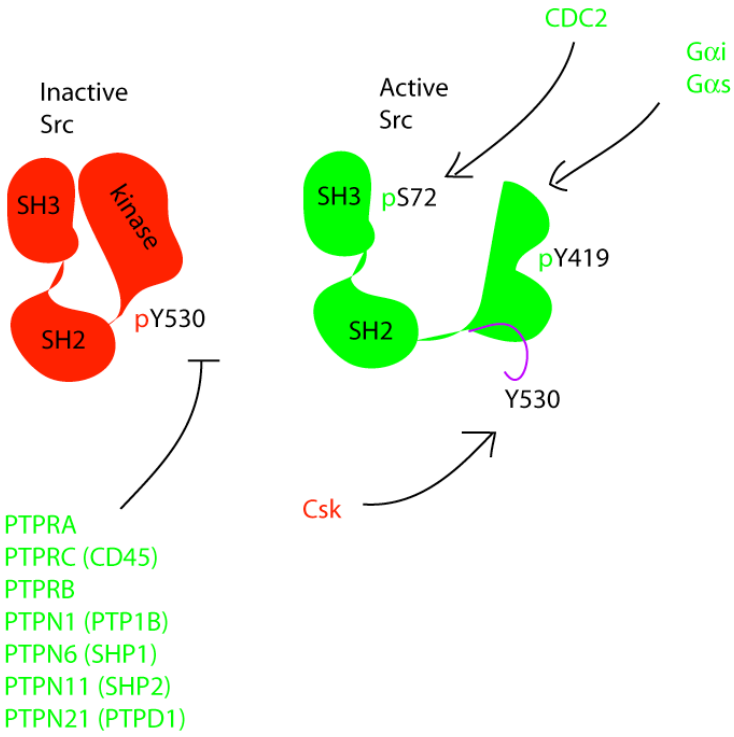

Figure 6. Mechanisms of Src family kinase activation. Src is in inactive conformation when the C-terminal inhibitory loop tyrosine Y530 is phophorylated. Several tyrosine phosphatases can release this inhibition by dephosphorylating the pY530 (Bjorge, Jakymiw et al. 2000). C-Src tyrosine kinase (Csk) is a negative regulator of Src family kinases. Autophosphorylation at Y419 increases Src access to substrate. Other activators include CDC2 (CDK1) (Roskoski 2005) and the G-proteins downstream of GPCRs. G-proteins, Gαs and Gαi bind to the catalytic domain and change the conformation of Src, leading to increased accessibility of the active site to substrates (Ma, Huang et al. 2000).

2.3.3.2.2. Signaling through Syk, SLP-76, and VAV1/3

Syk (spleen tyrosine kinase) is a kinase directly binding to C-terminal unphosphorylated β3 cytoplasmic tail, upon which Syk is activated (Woodside, Obergfell et al. 2001). It also becomes associated with αIIbβ3-integrin after platelet binding to fibrinogen, and is important in phosphorylating a Rac-GEF Vav1/3, and the adaptor protein SLP-76 (Obergfell, Eto et al. 2002). SLP-76 is important for αIIbβ3-integrin outside-in signaling as its depletion in mice cause fetal hemorrhage and the platelets show impaired spreading and much lower overall tyrosine phosphorylation (Judd, Myung et al. 2000). Syk activation upon integrin ligation leads to formation of a complex consisting of SLP-76, an adaptor Nck, and a Rac effector, PAK1, which enhance cell spreading and lamellipodia formation in a CHO reconstitution model (Obergfell, Judd et al. 2001). SLP-76 associates with ADAP constitutively, but their phosphorylation and association with VASP is increased upon integrin binding to fibrinogen in platelets (Obergfell, Judd et al. 2001). Both Syk and SFKs phosphorylate Vav1 and Vav3, which have redundant

roles in platelets and are important in PLCγ2 activation and spreading on fibrinogen independently of Rac activation (Pearce, McCarty et al. 2007). Same authors also showed that spreading and lamellae formation in the absence of Vav1/3 can be induced by thrombin-stimulated GPCR-signaling through PLCβ and Rac activation. As in inside-out activation of αIIbβ3, calcium mobilization through the action of PLCγ2 is needed for platelet spreading on fibrinogen and also for clot retraction (Wonerow, Pearce et al. 2003; Suzuki-Inoue, Hughes et al. 2007). Recently, an ITAM-bearing Fc receptor, FcγIIRa, was found to be involved in αIIbβ3 outside-in signaling leading to PLCγ2 phosphorylation (Boylan, Gao et al. 2008). This pathway requires SFK-mediated phosphorylation of the ITAM-motif and a subsequent association of Syk with ITAM. Altogether this means that Syk, SLP-76, Vav1/3, and PLCγ2 are all involved in both inside-out – and outside-in signaling of αIIbβ3.

2.3.3.2.3. PKCs

Platelet agonist-stimulated αIIbβ3 inside-out activation is regulated by calcium and DAG-dependent (PKCα, CalDAG-GEF) or calcium-independent pathways (Gαi) (Cifuni, Wagner et al. 2008). PKCs are known to stimulate dense granule release containing ADP and TXA2 important for second wave integrin activation, and it has been shown that PKCα is upstream of Rap1 in αIIbβ3 activation (Han, Lim et al. 2006; Cifuni, Wagner et al. 2008). However, it was suggested by Giuliano and coworkers that PKCs are required for bidirectional αIIbβ3 signaling, where PKC activity is needed for the initial integrin activation, but also for maintaining the stationary platelet adhesion on vWF downstream of αIIbβ3 (Giuliano, Nesbitt et al. 2003). More recent work of the Shattil's group has shown that PKCβ is activated by fibrinogen binding to αIIbβ3 by 9-fold and that the PKCβ-deficient platelets have impaired spreading on fibrinogen (Buensuceso, Obergfell et al. 2005). They also showed that PKCβ associates with the β3 cytoplasmic tail through the receptor for activated C kinase 1 (RACK1). Similar role in outside-in signaling was also found for PKCθ (theta), which associates constitutively with αIIbβ3, but is phosphorylated only after fibrinogen binding (Soriani, Moran et al. 2006). PKCθ phosphorylation downstream of αIIbβ3 correlated with integrin association with Syk and BTK, but also with phosphorylation of WASP, which is a positive regulator of Arp2/3-mediated actin polymerization.

2.3.3.2.4. Tyro3, CD40L, or Ephrin-B1 signaling leads to β3 integrin phosphorylation

Angelillo-Scherrer and others found a platelet dysfunction in mice lacking a Tyro3-family ligand, Growth association specific 6 (Gas6) (Angelillo-Scherrer, de Frutos et al. 2001). Platelets deficient with this protein protected mice against artificially induced-thrombosis, but had normal bleeding times in tail cut experiments. Similarily, deficiency in any of the Tyro3-family receptor tyrosine kinases (Tyro3, Axl, or Mer) or delivery of soluble extracellular domain of Axl, protected mice against thrombosis (Angelillo-

Scherrer, Burnier et al. 2005). They also showed that β3 integrin cytoplasmic tail phosphorylation is induced by Gas6 and is dependent on PI3K – AKT signaling. More recently, the redundancy of the three receptors was suggested by single, double, and triple knock-out models (Wang, Chen et al. 2007). Tail bleeding transections, platelet aggregation studies, and assays on dense granule secretion (ATP release) verified the importance of this RTK-family in hemostasis.

CD40L is a platelet- and leukocyte-derived ligand for a tumor necrosis family receptor CD40 that plays a positive role on inflammation and thrombus formation by activating circulating leukocytes and platelets (Andre, Nannizzi-Alaimo et al. 2002). Furthermore, platelet surface-expressed CD40L mediates interaction with endothelial CD40 inducing endothelial cells to secrete chemokines and to express adhesion molecules for leukocyte arrest (Henn, Slupsky et al. 1998). Interestingly, André and coworkers (2002) showed that recombinant soluble CD40L binds directly via its KGD motif to purified αIIbβ3 and to activated platelets in β3 integrin-dependent manner. Mice deficient with CD40L had an impairment of thrombus stabilization, whereas mice lacking CD40 were normal. Further studies showed that CD40L ligation to αIIbβ3 increases β3-tail phosphorylation (Y759) and induces soluble fibrinogen binding (Prasad, Andre et al. 2003). An interesting finding in this study was that platelets on CD40L-coated surfaces aggregated and showed β3-tail phosphorylation much more that platelets on fibrinogen. The above findings suggest a stimulatory role for CD40L in outside-in signaling of αIIbβ3 integrin in platelets. Despite of the stimulatory role of CD40L in platelets and leukocytes, its use as a growth inhibitory molecule in several neoplastic tumors shows promising results (Tong, Stone 2003).

Ephrins are either GPI-anchored or transmembrane ligands for Eph receptor tyrosine kinases that mediate bidirectional cell-cell signaling, but also modulate integrin adhesive function in a negative or positive manner (Nakamoto, Kain et al. 2004). For example EphA2 associates with FAK and upon Ephrin-A1 binding induces inactive conformation of β1 integrin and dephosphorylation of FAK by SHP2 phosphatase (Miao, Burnett et al. 2000). Conversely, transmembrane ephrin-B1 and –B2 may stimulate integrin adhesion and outside-in signaling (Huynh-Do, Stein et al. 1999; Meyer, Hafner et al. 2005). In platelets, ephrin-B1 was shown to enhance soluble fibrinogen binding and aggregation upon thrombin or TXA2 agonist stimulation (Prevost, Woulfe et al. 2004). In another study by the same group, addition of GST-ephrin-B1, which oligomerizes through GST, substantially increased Tyr-773 phosphorylation of the β3-integrin tail in a Src-dependent manner (Prevost, Woulfe et al. 2005). The phoshorylation also required the presence of soluble fibrinogen. The monomeric His-ephrin-B1 alone was not able to induce phophorylation, but was able to do so in the presence of His-Epha4 and ADP. The Epha4-ephrin-B1 interaction was shown to stimulate phosphorylation of Y773 and Y785 and binding of myosin to β3-tail supporting clot retraction. Taken the results

together, they suggested a mechanism where ephrin-B1 – Epha4 interaction leads to Src association with Epha4 and subsequent phosphorylation of β3 integrin tail (Y773 and Y785), where myosin can bind and promote clot retraction.

2.3.3.2.5. Tetraspanins in integrin activation in platelets and cancer

Tetraspanins are membrane spanning small proteins that associate with several integrins and form tetraspanin microdomains, which at least in platelets are distinct from lipid raft microdomains (Israels, McMillan-Ward 2007). Platelets express at least five different tetraspanins – CD9, CD151, TSPAN9, TSSC6, and CD63, of which CD9, CD151, TSSC6, and CD63 are known to associate with αIIbβ3, and TSPAN9 with α6β1 in platelets (Israels, McMillan-Ward et al. 2001; Lau, Wee et al. 2004; Goschnick, Lau et al. 2006; Protty, Watkins et al. 2009). CD151 and TSSC6 (TSPAN32) have clear positive effects on platelet spreading and aggregation mediated by αIIbβ3 outside-in signaling (Lau, Wee et al. 2004; Goschnick, Lau et al. 2006). CD9 might have a positive role also in inside-out activation of αIIbβ3, as anti-CD9 mAbs stimulate soluble fibrinogen binding as well as platelet secretion and aggregation (Wu, Peng et al. 1999).

CD9 has been extensively studied in various cancer cell lines and clinical tumour samples. Kotha and others have shown that ectopic expression of CD9 in CHO cells leads to enhanced β1 integrin activation upon RGDS peptide addition and also enhances cell migration depending on α5β1 integrin and PI3K-AKT signaling (Kotha, Longhurst et al. 2008). In prostate cancer cells (PC-3M-LN4), CD9 over-expression was shown to stimulate *in vitro* invasiveness, but had no effect on lymph node metastasis in a mouse model (Zvieriev, Wang et al. 2005). Although CD9 can potentiate adhesion and migration in *in vitro* experiments, its expression is largely diminished or can be even absent in metastatic tumors, such as in multiple myeloma, colon cancer, cervical cancer, small cell lung cancer, prostate cancer, and breast cancer (De Bruyne, Bos et al. 2008; Cajot, Sordat et al. 1997; Sauer, Windisch et al. 2003; Saito, Tachibana et al. 2006; Wang, Begin et al. 2007; Sauer, Kurzeder et al. 2003). For example Cajot and coworkers showed that in colon cancer, cells derived from primary tumor samples had higher CD9 expression and much greater migration potential compared to cell samples from metastatic regions (Cajot, Sordat et al. 1997). In multiple myeloma, CD9 is lost from plasma cells derived from active disease bone marrow samples due to epigenetic silencing, raising a question whether this could be a general mechanism of CD9 downregulation during cancer progression (De Bruyne, Bos et al. 2008). Interestingly, Hori and others have shown that CD9 expression increase is observed in severe vessel invasion, active lymph node metastasis, and in advanced stage of gastric cancer (Hori, Yano et al. 2004). The above examples imply that the relationship of CD9 and integrin activity regulation is complex and that integrins are probably not the only functional targets of CD9. Furthermore, CD9 is known to complex with different integrins including αIIbβ3, α5β1 and α3β1, and it

could be that the CD9-integrin complex is different depending on cell type and also the effect on integrin activity either positive or negative in different environment. A good example of this is the work of Sauer and others (2003), which showed that CD9 is lost in most invasive cervical carcinomas, but can be highly re-expressed in certain "hot-spots" of transendothelial migration during lymph node metastasis.

2.3.4. Integrin activation in leukocyte arrest and migration

2.3.4.1. Chemokines trigger integrin activation during leukocyte rolling

Upon pathogen attack, tissue injury, or antigen encounter, various chemoattractants are produced and released from activated cells. This leads to selectin and integrin-mediated leukocyte rolling and arrest to vascular endothelial surfaces, integrin-mediated leukocyte spreading on endothelium, extravasation from lumen to the tissue, and chemotactic migration towards the attractant (Springer 1994) (Figure 7). Circulating leukocytes arrest to inflamed vascular endothelial surfaces by β2 integrins – LFA-1 (αLβ2) and Mac-1 (αMβ2) as well as by β1 integrin VLA-4 (α4β1) or by another α4 integrin, α4β7. Polymorphonuclear neutrophils are more dependent on LFA-1 and Mac-1 mediated arrest, whereas monocytes use primarily VLA-4. Leukocyte adhesion deficiency type I reflects the importance of β2 integrins in adaptive immunity, as in this genetic disease, β2 integrins are not properly expressed with a consequence of severe bacterial infections (Bunting, Harris et al. 2002). The arrest becomes possible during rolling of leukocyte on P- and E-selectin glycoproteins on endothelial membrane, but also by L-selectin expressed on leukocyte membrane. This rolling enables the association of leukocyte chemokine receptors (GPCRs) with endothelium-bound chemokines, leading to very fast and local inside-out activation of leukocyte integrins. The neutrophil arrest on endothelium is dependent on LFA-1 conformational change from intermediate to high affinity state (Green, Schaff et al. 2006). Similarly to platelets agonists, endothelium-derived chemokines operate through GPCR - Gαi signaling to activate integrins. Recently it has become apparent that also the P- and E-selectin ligand PSGL-1 on neutrophil membrane can partially activate integrins by a pathway that is independent of Gαi signaling (Urzainqui et al., 2002), but signals through ITAM-motif containing receptors FcRγ and DAP12 (TYROBP) as well as Syk kinase (Zarbock, Abram et al. 2008). This integrin activation pathway interestingly resembles that of platelet integrin initiated by GPIb or GPVI glycoproteins contacting with ECM proteins that signal also via FcRγ-ITAM-Syk pathway. Syk, which can bind directly to αIIbβ3 and is involved in outside-in signaling, associates also with LFA-1 and is critical for neutrophil adhesion and migration to inflamed tissue (Schymeinsky, Then et al. 2007).

The signaling from chemokine recpeptors to leukocyte integrins through Gαi also resembles that of platelet agonist-mediated integrin activation. Gαi2-null mice have 50% reduction in neutrophil recruitment to sites of inflammation, and *in vitro* assays

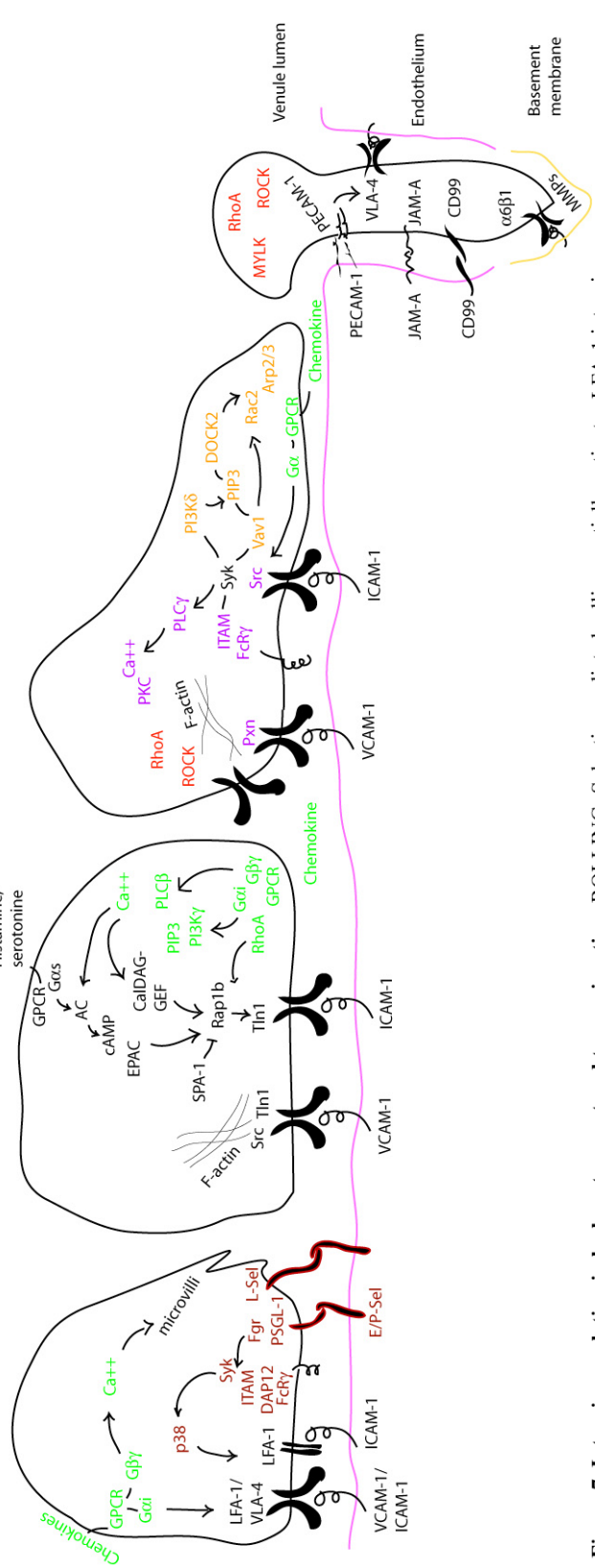

Figure 7. Integrin regulation in leukocyte arrest and transmigration. ROLLING: Selectin-mediated rolling partially activates LFA-1 integrins through ITAM-bearing receptors, DAP12 or FcRg, which are phosphorylated by Src-family Fgr and can then bind Syk. The partial integrin activation (extension) by selectins and also chemokines prime integrin to bind ligand. This slows down rolling speed. ARREST: Endothelium-immobilized chemokines and other soluble chemoattractants activate integrins by inside-out signals that are mainly second-messangers, such as calcium, cAMP, PIP2, and PIP3. Rap1-assisted Talin1 (Tln1) binding to β2- and β1-cytoplasmic tails opens up integrin heterodimer. POST-ARREST and SPREADING: Chemokines keep activating integrins, but now integrin bonds are strengthened by integrin outside-in signaling and a connection to actin cytoskeleton to resist shear flow. During spreading, Src-family kinases and Syk downstream of integrins activate multiple kinases and GTPases needed for F-actin stabilization at cell body and rear, but also for actin-driven membrane flow at leading edge. RhoA and ROCK-mediated contraction liberates adhesions at the rear, thus enabling cell body movement. DIAPEDESIS: Different adhesion molecules at cell-cell junctions form homophilic interactions between leukocyte and endothelium. PECAM-1 and CD99 amplify VLA-4 and LFA-1 adhesions and contribute also to increase α6β1 integrin expression needed for efficient binding and invasion to basement membrane. Also matrix-metlloprotease (MMP) exression is induced during transmigration for example by Wnt/Fzd/b-catenin canonical pathway (See text for references).

show impaired neutrophil arrest on P-selectin, ICAM-1, CXCL1-coated flow chambers (Zarbock, Lowell et al. 2007). Similarly, the Gαi downstream effector, PI3Kγ (p110γ) has important role in neutrophil arrest, especially in the early adhesion strengthening, which is abrogated in PI3Kγ-null neutrophils (Smith, Deem et al. 2006). In effector T cells, PI3Kγ is important for migratory phenotype (Thomas, Mitchell et al. 2008; Martin, Schwartz et al. 2008). PLCβ, which is an effector of Gαq and Gβγ, is also important for certain leukocytes, since inhibition of PLC in monocytes leads to impaired arrest on VCAM-1 surfaces due to low affinity VLA-4 (Hyduk, Chan et al. 2007). However, PLCβ does not seem to have important role in T-cell migration to inflamed tissue, since the double knock-out (β2 and β3) has no overt effects (Lin, Wang 2000).

2.3.4.2. Integrin outside-in signaling strengthens adhesions

During integrin bond strengthening and cell spreading, the outside-in signaling from ligand-bound open integrin headpiece to cytoplasm is mediated by Src family kinases. Lck-deficient lymphocytes show impaired adhesion to VCAM-1 or fibronectin with reduced affinities, but also reduced chemotactic migration in primary T cells and natural killer cells (Feigelson, Grabovsky et al. 2001; Inngjerdingen, Torgersen et al. 2002). Triple-negative Src (lyn–/–hck–/–fgr–/–) or PP2-inhibited neutrophils have impaired adherence and arrest to ICAM-1 or HUVEC monolayer, and also show almost total block in HUVEC transmigration (Sarantos, Zhang et al. 2008). As IL-8 is able to induce ICAM-1-Alexa488 binding in triple KO cells, Src family kinases are not involved in the inside-out-, but outside-in signal transmission. Another Src-family kinase, Hck, plays a role in thrombohemorrhagic vasculitis. LPA- and TNFα-induced vasculitis is dependent on Mac-1 integrin binding to complement C3 on vessel walls, and subsequent Hck and Syk-mediated elastase secretion, as deficiency of any of these components prevents the disease-causing hemorrhage and thrombosis (Hirahashi, Mekala et al. 2006). The Syk kinase downstream of Src family kinases is also important for outside-in signaling. Syk is activated either by Src family kinase phosphorylation or binding of Src-phosphorylated immune tyrosine activation motif (ITAM) present in integrin-associating transmembrane proteins, such as FcRγ, Dap12, and CD3 proteins (Tsang, Giannetti et al. 2008). Syk-null neutrophils have a defect in adhesion and a spreading to cremaster muscle venules with reduced extravasation to inflamed tissue or infiltration to wound in a mouse wound healing model (Schymeinsky, Sindrilaru et al. 2006; Schymeinsky, Then et al. 2007). Interestingly, double knock-out of FcRγ and Dap12 reduces the Syk phosphorylation and activation and concomitant adhesion and spreading to β2-integrin ligands (Mocsai, Abram et al. 2006). This means that immunoreceptor ITAM-based signaling is important in integrin inside-out activation, but also downstream of integrins after ligand binding. This is very similar to platelets, where FcRγ mediates GPVI and GPIb-induced inside-out activation and FcγIIRa is important downstream of αIIbβ3. Mutation of tyrosine residues in Syk (Y348F or Y323F), which recruit downstream players Vav1/3 and

PI3Kdelta, respectively, leads to impaired directed migration by formation of excessive lamellae with no proper leading edge polarization (Schymeinsky, Sindrilaru et al. 2006; Schymeinsky, Then et al. 2007).

2.3.4.3. Integrins and leukocyte transmigration

Prior to transmigration, leukocytes laterally migrate on the endothelial apical surface to cell-cell junctions, and this movement is dependent on functional LFA-1 and Mac-1 on monocytes and ICAM-1 and ICAM-2 on endothelium (Huang, Larbi et al. 2006). Neutrophils, however use mainly LFA-1/ICAM-1 interaction for the arrest, but Mac-1/ICAM-1 interaction during lateral intraluminal crawling (Phillipson, Heit et al. 2006). The squeezing through endothelial junctions (paracellular diapedesis) is dependent on various adhesion molecules – β2 integrin associations with ICAM-1 and ICAM-2, homophilic interactions by JAM-A, CD99, and PECAM-1 expressed on both leukocytes and endothelium (Nourshargh, Krombach et al. 2006; Lou, Alcaide et al. 2007). Interestingly, during diapedesis VLA-4 is interacting with a secreted protein acidic and rich in cysteine (SPARC), whose KO impairs *in vitro* leukocyte transmigration and *in vivo* migration to inflamed peritoneum (Kelly, Allport et al. 2007). Leukocyte transmigration has been mainly studied with neutrophils and monocytes, but recently T lymphocytes were found to use Wnt/Fzd/β-catenin canonical signaling for induction of MMP expression needed for transmigration through EC and basement membrane *in vitro* and *in vivo* (Wu, Crampton et al. 2007).

Of these different adhesion molecules, at least PECAM-1 (CD31) is regulating integrin function. The immune tyrosine inhibition motif (ITIM) bearing PECAM-1 upon oligomerization induces β1- and β2-mediated T cell adhesion and spreading to VCAM-1 and ICAM-1 (Tanaka, Albelda et al. 1992). In a CHO expression model, antibody-assisted oligomerization of PECAM-1 induces α5β1-mediated adhesion and spreading to fibronectin (Zhao, Newman 2001). Also PECAM-1 homophilic interaction on transmigrating neutrophils upregulates α6β1, which is important for invasion through perivascular basement membrane (Dangerfield, Larbi et al. 2002). Importantly, PECAM-1 deficient platelets show impaired spreading on fibrinogen with a defect in clot retraction due to failure in outside-in αIIbβ3 signaling (Wee, Jackson 2005). These studies together with examples of CD99 regulating integrin adhesive properties (Hahn, Kim et al. 1997; Kasinrerk, Tokrasinwit et al. 2000; Bernard, Raimondi et al. 2000), it can be suggested that leukocyte integrins are critical during endothelial transmigration and basement membrane invasion, but they need to work together with different adhesion molecules.

2.3.4.4. Integrin α4β1 (VLA-4) has unique properties

VLA-4-mediated rolling and adhesion to VCAM-1 is important for monocytic cell lineage and T-cell arrest on endothelial surfaces (Ley, Laudanna et al. 2007). VLA-4 is

also important in spreading and transmigration of leukocytes across the inflammation activated endothelium. Multiple myeloma cells utilize this integrin in trafficking to bone marrow stroma, and also melanoma cells in transmigration across endothelium (Sanz-Rodriguez, Ruiz-Velasco et al. 1999; Sanz-Rodriguez, Hidalgo et al. 2001; Klemke, Weschenfelder et al. 2007). α4β1-integrins are unique as they have a subset of semi-activated or extended state conformations that can spontaneously bind to VCAM-1 in the absence of selectin or chemokine activation, and this characteristic could be mediated by leukocyte Src-family kinase, Lck (Feigelson, Grabovsky et al. 2001). According to Chigaev and others, the extension or unbending accounts for increased ligand capture efficiency, and this population can be increased by chemokines (Chigaev, Waller et al. 2007). The correct spatiotemporal regulation of VLA-4 affinity during transmigration is critical, as rendering the integrin constitutively active by arginine substitution with alanine in the conserved α4 GFFKR tail, immobilizes cells due to aberrant trailing tail detachment (Imai, Park et al. 2008).

In addition to its unique heterogeneous affinity states in resting leukocytes, α4β1 is also differently regulated in its inside-out and outside-in signaling. Rap1 GTPase, which is known to positively regulate at least αIIbβ3, αLβ2 and other β1 integrins, is not playing a role in α4β1 activation in T-cells (Ghandour, Cullere et al. 2007). Similarly, as shown by the same authors, the Rap1 GEF, CalDAG-GEF1, whose nonfunctionality is the primary cause of type III leukocyte adhesion deficiency (LADIII), does not mediate VCAM-1 binding in T-cells. Another interesting feature of α4 integrin is that paxillin adaptor protein binds directly to its cytoplasmic tail and mediates important outside-in signaling by stabilizing linkage to actin and polarizing Rac GTPase to leading edge during leukocyte migration (Hyduk, Oh et al. 2004; Manevich, Grabovsky et al. 2007; Rose 2006). Mice bearing a mutation in α4 tail (Y991A) that disrupts paxillin binding have defective mononuclear leukocyte recruitment to sites of inflammation (Feral, Rose et al. 2006). Paxillin binding to non-phosphorylated Y991 of α4 tail is important for talin1 association and anchorage to actin during adhesion strengthening on VCAM-1 under shear flow (Alon, Feigelson et al. 2005). However, during migration, paxillin is a negative regulator of Rac activation by recruiting an Arf-GAP protein that decreases the active Rac (Nishiya, Kiosses et al. 2005). Paxillin binds to high affinity ligand-bound non-phosphorylated integrin at the sides and posterior of the cell enabling PKA-mediated α4 tail phosphorylation at the leading edge during directed cell migration (Goldfinger, Han et al. 2003; Lim, Han et al. 2007). Thus, paxillin has a dual role in leukocyte trafficking – Firstly to mediate adhesion strengthening during arrest and secondly, to promote proper polarization for cell migration. It could be that Rac-assisted lamellae formation does not favor too stabile integrin bond formation, and this is probably why PKA activity dislocates paxillin from α4 tail.

The stabilization of integrin bonds is mediated by other GTPases – The Rho family GTPases. Inhibition of Rho GTPases by C3 transferase was shown by Laudanna and others (1996) to block formyl peptide or IL-8 induced adhesion of reconstituted B-cells to purified VCAM-1. Stimulation by agonists was also shown to increase the cellular levels of GTP-loaded RhoA. In a recent and elegant study of Pasvolsky, Grabovsky et al. (2008), RhoA was shown to be critical for LFA-1 extension prior to ligand binding. However, this was only restricted to CXCL-12 (SDF-1) induced activation, but not to induction with another CXCR4 ligand, CXCL-9, which surprisingly showed only outside-in LFA-1 activation independent of RhoA. Whether the same applies to VLA-4 was however not studied. Also, it would be interesting to see whether the constitutive suppression of LFA-1 activation by RhoH is also amenable to VLA-4 function, as it has been shown that RhoH by inhibiting NFKB, Rac1, Cdc42, and RhoA signaling, suppresses LFA-1 activation in resting T-cells (Cherry, Li et al. 2004).

Table 2. Regulators of integrins in leukocyte arrest and migration. Negative regulators are with red color.

Gene	Integrin	Cells	Function	Reference
CBLB (CBL-B); YWHAB (14-3-3b)	LFA-1	bone marrow-derived mononuclear phagocytes (BMDMs)	Phagocytes deficient with CBL-B had increased adhesion to endothelium in vitro and peritoneal recruitment in vivo, which was dependent on LFA-1 activity upregulation. Cbl-b deficiency resulted in increased phosphorylation of T758 beta2-tail of LFA-1 and enhanced association of 14-3-3b protein with the beta2-chain.	(Choi, Orlova et al. 2008)
CCL21	LFA-1	Naive T cells, plt/plt mice lack CCL21 in peripheral lymph node HEVs	plt/plt mice (lack CCL21) have defect in T cell homing to PLN. plt/plt T cells roll but don't arrest in HEVs. Intracutaneus CCL21 addition to plt mice rescued phenotype, which was LFA-1 dependent.	(Stein, Rot et al. 2000)
CCL21; CCL25; CCL28; CXCL12	α4β7	Lymphocytes	Immobilized chemokines induced arrest on co-immobilized MAdCAM-1. Potential role for these chemokines in the arrest of lymphocytes on postcapillary venules in the gut.	(Miles, Liaskou et al. 2008)
CD44	VLA-4	Murine T cells	CD44 associates with active VLA-4 through its tail. CD44-hyaluronan interaction promotes VLA-4-VCAM-1 interaction and firm arrest in laminar flow *in vitro* assay. Activated lymph node T cells recruited to inflammatory peritoneal cavities, but CD44 w/o tail or CD44-/- cells did not.	(Nandi, Estess et al. 2004)
CD81; PRKCB (PKCb)	VLA-4, VLA-5	Primary murine B splenocytes from wt or CD81-/- mice and monocytes (U937), effect not seen in T cells	CD81 KO cells had decreased rapid sub-second adhesion strengthening to VCAM-1 or FN, but no change in sVCAM-binding or clustering. Outside-in avidity enhancment. DAG-like phorbol ester PMA increased VLA-4 avidity (clustering), but not if CD81 was depleted or PKCb inhibited.	(Feigelson, Grabovsky et al. 2003)
CD9; CD151	VLA-4, LFA-1	Peripheral blood lymphocytes (PBLs)	Knock-down of CD9/CD151 or addition of CD9-large extracellular loop (LEL)-glutathione S-transferase (GST) peptides prevented lymphocyte transendothelial migration and increased lymphocyte detachment under shear flow. Tetraspanins associate with adhesion molecules and regulate their function.	(Barreiro, Yanez-Mo et al. 2005)

Gene	Integrin	Cells	Function	Reference
CSK	MAC-1	Granulocytes	CSK-deficient granulocytes showed hyperadhesiveness to serum, which was β2 integrin dependent. Cells also bound more soluble fibrinogen, but also MAC-1 expression higher. CSK-/- cells had 5-fold higher HCK kinase activity, higher paxillin and SYK phosphorylation, implying stronger outside-in signaling.	(Thomas, Schmedt et al. 2004)
CX3CL1 (FKN)	VLA-4	T-cells, human intestinal microvascular endothelial cells (HIMECs)	TNFa, IFNG, or leukocyte adhesion increased FKN surface expression by HIMECs. IBD mucosa contained significantly more CX3CR1+ leukocytes than control mucosa. FKN increased adhesion to VCAM-1 and HIMEC transmigration. FKN activates β1 integrins measured by 12G10 mAb.	(Sans, Danese et al. 2007)
CXCL12 (SDF-1a)	VLA-4	periferal blood lymphocytes (PBLs)	Immobilized CXCL12 induced firm adhesion to VCAM-1 in laminar flow chamber. LDV peptide-based small molecule that preferentially binds high-affinity VLA-4 reduced PBL firm adhesion to VCAM-1 by 90% and increased rolling. VLA-4 high activity is induced.	(DiVietro, Brown et al. 2007)
CXCL13	LFA-1, a4b7	B cells	CXCL13 induces adhesion to ICAM-1 and MAdCAM-1 under static and flow conditions. CXCL13 null mice have decreased B cell adherence to HEVs of mesenteric lymph nodes and payer's patches. Superfusion of CXCL13 rescues adherence. CXCL13 activates Rap1GTP, and if Rap effector RAPL is depleted, partial failure in ICAM-1 adherence.	(Kanemitsu, Ebisuno et al. 2005)
CXCL9; CXCR3	LFA-1	Lymphocytes	CXCL9 triggers robust Gi-mediated activation of LFA-1 adhesiveness to both low and medium densities of ICAM-1 under shear flow conditions, but not changes in LFA-1 conformation. RhoA 23/40-independent outside-in activation, post-ligand stabilization. PMA works similar manner, but independent of RhoA.	(Pasvolsky, Grabovsky et al. 2008)
DAP12 (TYROBP); FcRg (FCER1G); SYK	LFA-1, Mac-1	Neutrophils and macrophages from wt or KO mice	Double KO (DAP12, FcRg) neutrophils have decreased adhesion and spreading on fibrinogen or poly-RGD, and defective degranulation and oxidative burst. Only partial migration defects agains chemokine gradient. Tyr phosphorylation of Vav, Pyk2, Erk, p38, SYK was impaired upon integrin ligation. The ITAM-binding SYK SH2-domain is critical for integrin outside-in signaling.	(Mocsai, Abram et al. 2006)
DOCK2	LFA-1, VLA-4	Pr. Splenocytes or T lymphocytes from WT mice or DOCK-/- mice	DOCK-/- cells had impared motility on purified ICAM-1 or VCAM-1 and on endothelial basal layer. No effect on affinity or adhesion strengthening.	(Shulman, Pasvolsky et al. 2006)
DOCK2	LFA-1, VLA-4, a4b7	B cells	DOCK-/- B cells had impaired static adhesion to ICAM-1, VCAM-1, and MADCAM-1. IVM demonstrated impaired adhesion to PLN and PP venules. T cells had no defects. DOCK2 important for T and B cell migration however.	(Nombela-Arrieta, Lacalle et al. 2004)
DOCK2	ND	Neutrophils	fMLP and PMA induced Rac1 and Rac2 GTP-loading, but 70% less if DOCK2-/- neutrophils used. C5a- or fMLP-induced chemotaxis was >95% inhibited in DOCK2-/- neutrophils. Also defects in superoxide production. Null cells migrate slower in 2D and have lower persistence. DOCK2 associates with PIP3 and stabilizes it to leading edge.	(Kunisaki, Nishikimi et al. 2006)

Gene	Integrin	Cells	Function	Reference
DRD2; DRD3	VLA-4, VLA-5	T cells from peripheral blood of healthy donors.	Dopamine or agonist -hydroxy-DPAT (DPAT) induced T cell adhesion to FN. Dopamine antagonist or VLA-4/5 mAb can inhibit this.	(Levite, Chowers et al. 2001)
EPAC (RapGEF)	VLA-4 or VLA-5 and LFA-1	Endothelial progenitor cells (EPCs)	EPAC activator 8-pCPT-2'-O-Me-cAMP increased cell adhesion to HUVECs and FN. Also migration increased. Also increased polarization of ITGB1/B2 and CD44 to front and back in suspension. β1 affinity increased, when measured with HUTS21 mAb FACS. EPC homing to ischemic limbs increased.	(Carmona, Chavakis et al. 2008)
EPAC1 (RapGEF3)	VLA-4	U937 monocytes and human periferal blood monocytes (PBMs)	8CPT-2Me-cAMP activated Rap1 and promoted cell adhesion to fibronectin and endothelial cells. Epac1 activation resulted in a rapid and significant increase of activated β1 on the cell surface followed by their down-modulation (12G10 FACS). They also noted that serotonin activates Rap1, through cAMP dependent way.	(Lorenowicz, van Gils et al. 2006)
Formyl peptide (FP, fMLP); C5a; PAF; CXCL12	VLA-4	Monocytes U937 and human blood monocytes	Treatment of U937-FPR cells with either FP or SDF-1 resulted in sVCAM-1/Fc binding at 30 s. Immobilized chemokines induced arrest on VCAM-1, and was blocked by soluble VCAM. FP or CXCL12 increased HUTS-21 mAb binding. CytD inhibited rolling and arrest, but not high affinity integrin.	(Chan, Hyduk et al. 2001)
GNAI2 (Gai2)	LFA-1	Neutrophils	CXCL1 or LTB4-induced arrest was decreased in GNAI2-/- cells in flow chamber coated with P-sel/ICAM-1/chemokine. Also in vivo cremaster muscle venule recruitment defect in GNAI2-/- mice. In peritonitits or lung inflammation model, GNAI2-/- hematopoietic cells had decreased recruitment.	(Zarbock, Deem et al. 2007)
GNAS (Gαs); HRH2; ADRB2		Lymphoma U937, pr. monocytes	Decreased VLA-4 ligand binding (LDV-FITC) after Gαi stimulation and decreased aggregation. Gαs - cAMP is inhibitory.	(Chigaev, Waller et al. 2008)
HCK, FGR, VAV1, RAC2, PAK	ND	Neutrophils	Chemotactic peptide formyl-methionyl-leucyl-phenilalanine (fMLP) induced F-actin polymerization and cell migration through 1μm pores, but not if Hck/Fgr-/- neutrophils used. Also VAV1, PAK phosphorylation decreased in null cells with no effects on PI3K or PLC activities.	(Fumagalli, Zhang et al. 2007)
HCK; FGR	LFA-1	Neutrophils	Rapid arrest or adhesion of Hck/Fgr-/- neutrophils to co-immobilized ICAM1/P-Sel/chemokine was unaffected. Also, chemokine stimulation did not show integrin affinity defects in KO cells measured with soluble ICAM-1. However, neutrophil spreading defect on β2 substrates. Also, defect in in vivo arrest to inflamed muscle venules of >60 micrometer in diameter. Outside-in signaling.	(Giagulli, Ottoboni et al. 2006)
HCK; FGR; LYN	LFA-1	Polymorphonu-clear leukocytes (neutrophils)	Src inhibitor PP2 or KO cells (lyn–/–hck–/–fgr–/–) had decreased adhesion, arrest, and transmigration on HUVECs due to impaired outside-in signaling or LFA-1. Src inhibition decreases sICAM-1 binding to cells, but IL-8 can overcome this, suggesting that Src is not involved in inside-out activation, but outside-in activation.	(Sarantos, Zhang et al. 2008)

Gene	Integrin	Cells	Function	Reference
HCK; SYK; C3; ELA	Mac-1	Neutrophils	Thrombohemorrhagic vasculitis was induced by TNFa and LPS. Mac-1 binding to C3 activated HCK, SYK and elastase release leading to pathological thrombosis. Mac-1 null mice were normal.	(Hirahashi, Mekala et al. 2006)
HRAS	LFA-1	Jurkat T-cells	Constitutively active HRAS (D12) inhibits CXCL12-induced soluble ligand binding. CA or DN HRAS (D12/N17) abrogate LFA-1 avidity, not VLA-4.	(Weber, Ostermann et al. 2001)
IFNG (IFNγ); STAT1	ND	Monocytes	IFNG inhibited migration in chemotactic chamber assay in response to CCL2 gradient. This was dependent on STAT1. Inhibition correlated with decreased phosphorylation of PYK2, JNK, and PAK, as well as lower GTP-loading of CDC42 and RAC.	(Hu, Hu et al. 2008)
IL-6	VLA-4	macrophages, primary monocytes	IL-6 increased adhesion to fibronectin, migration on fibronectin and HUVEC transmigration. Correlated well with increased β1 integrin activation measured with 9EG7 mAb FACS.	(Clahsen, Schaper 2008)
IL-6; IL-11; PTPN11 (SHP2)	VLA-4	primary T-cells	IL-6 increased transwell migration when coated with fibronectin. IL-6 increased β1 integrin activation measured with 9EG7 mAb FACS. IL-6 independent of Gα, since PTX had no effect. IL-6 induction was dependent on gp130-dependent activation of SHP2 (PTPN11), but not STAT activation.	(Weissenbach, Clahsen et al. 2004)
IL-8, ERK1/2 (MAPK3)	LFA-1, Mac-1	Neutrophils	ICAM-1 expressed on endothelial L cell monolayer induced rolling and arrest under flow only upon IL-8 induction. ERK1/2 inhibitor decreased this efficiently. IL-8 effects dependent on Gαi.	(Simon, Hu et al. 2000)
LAD (SH2D2A, TSAd)	ND	T cells	CXCL12 induced T cell transwell migration that was inhibited by LAD knock-down. LAD interacts with Gβ upon agonist stimulation. Lad associated with the tyrosine kinases LCK and Zap-70 upon chemokine stimulation. Chemokine induced PYK2 and paxillin phosphorylation, which was absent in LAD knock-down cells.	(Park, Park et al. 2007)
LCK	VLA-4, VLA-5	T-cells	LCK-deficient cells show impaired adhesion to VCAM-1 and FN, and lower affinity for ligand as measured by high-affinity b1 recognizing antibodies, 15/7, HUTS4, HUTS21, 9EG7, but also VLA-4 binding LDV-peptide or soluble VCAM-Ig.	(Feigelson, Grabovsky et al. 2001)
LCK; CSK; CXCL12		Human naive T-cells and NK cells, Jurkat cells	LCK-deficient cells show decreased chemotaxis upon CXCL12 induction. Rescued by expression of LCK, but not if CSK is cotransfected. CXCL12 induced LCK phosphorylation. From inside-out activation to outside-in signaling.	(Inngjerdingen, Torgersen et al. 2002)
LTB4; LTB4R (BLT1)	LFA-1	Cytotoxic T effector CD8+ cells wt or LTB4R-/-	LTB4 superfusion to cremaster muscle venules increased T cell accumulation due to arrest and integrin activation. In acute peritonitis, LTB4R was required for efficient T cell recruitment. Also in IVM of cremaster muscle venules, T cell sticking was dependent on LTB4R expression. Also in vitro chemotaxis was affected.	(Goodarzi, Goodarzi et al. 2003)
LYN	LFA-1	primary CD34+ stem/progenitor cells, and Mo7e, HL-60, and Nalm-6 myeloid cell lines	Lyn knock-down (KD) decreased directed 2D chemotactic migration against CXCL12 gradient. KD increased attachment to stromal cells and to purified ICAM-1, not VCAM-1. Lyn kinase inhibits the affinity of LFA-1 to ICAM-1. Also fMLP induces LYN activity.	(Nakata, Tomkowicz et al. 2006)

Gene	Integrin	Cells	Function	Reference
MIF; CXCR2 (IL8RB)	VLA-4, LFA-1	Monocytes and lymphocytes	MIF mediates mononuclear cell arrest to endothelium through CXCR2, CD47, CXCR4 and VLA-4. Increases chemotaxis. Stimulates also lymphocyte arrest on ICAM-1 by LFA-1 conformational change, as measured with mAb 327C. CXCR2 genetic deletion decreased leukocyte recruitment to MIF-induced peritonitis. Also, high fat-diet apoe-/- mice had decreased atherosclerosis if MIF was blocked with antibodies.	(Bernhagen, Krohn et al. 2007)
MYLK (MLCK1), PYK2 (PTK2B, FAK2)	LFA-1	Neutrophils	Ex vivo LPS-induced lung injury model, where MYLK-null neutrophils had decreased recruitment. Adhesion and transmigration in vitro abolished on endothelial layer. In vivo transalveolar migration impaired. Impaired soluble ICAM-1-Fc ligand binding. C-SRC, PYK2 phosphorylation decreased 80/60%. MYLK interacts with PYK2 and phosphorylates it. MYLK null neutrophils migrate faster but not directly on 2D.	(Xu, Gao et al. 2008)
PI3K; CXCL12; CCL19; CCL21	LFA-1	Lymphocytes	Any one of these chemokines increased adhesion to ICAM-1 and HEV Payer's patches. Adhesion was Gα and PI3K dependent when ICAM-1 at low densities. PI3K needed for clustering not affinity regulation as measured with soluble 125I-ICAM-1.	(Constantin, Majeed et al. 2000)
PIK3CD (PI3Kdelta, p110d)	VLA-4	periferal blood lymphocytes (PBLs) and THP-1 cells	p110d inhibitor IC87114 was efficient in blocking adhesion and spreading on VCAM-1, but also in β1 integrin affinity upregulation. No effects on ICAM-1. RAC1 and CDC42 activity upregulation involved. (See SYK)	(Ferreira, Isaacs et al. 2006)
PIK3CG (PI3Kg), p110-gamma; CXCL1; CXCR2	LFA-1	Leukocytes	PI3Kgamma null mice showed an 80% decrease in CXCL1-induced leukocyte adhesion in venules of the exteriorized mouse cremaster muscle. Defect in integrin bond stabilization. In vitro chamber flow adhesion to P-selectin/ICAM-1/ CXCL1 substrate was abrogated.	(Smith, Deem et al. 2006)
PIP5K1C (PIP5KC)	LFA-1	T lymphocytes	PIP5KC is a downstream effector of both RHOA and RAC1 and is activated by phospatidic acid (PLD1 product). It binds directly talin1. Knock-down decreased static and flow adhesion to ICAM-1, showed a defect in the ability to bind soluble ICAM-1, and prevented binding of high-affinity LFA-1 reproter mAb327C. No effects on binding of LFA-1 extension reporter KIM127. Regulates the final conformational trasnsition of LFA-1. RHOA, RAC1 and PLD1 contribute to PIP2 levels.	(Bolomini-Vittori, Montresor et al. 2009)
PLC; CALM; FP; CXCL12; CCL5	VLA-4	U937 monocytes and human periferal blood monocytes (PBMs)	Inhibition of PLC or calmodulin, not PI3K or PKC, decreased VLA-4 affinity to ligand (LDV-FITC, sVCAM-1) upon chemokine induction. Also decrease in arrest to HAEC monolayer.	(Hyduk, Chan et al. 2007)
PLD1	LFA-1	T lymphocytes	PLD1 is RHOA and RAC1 effector. Inhibition of RHOA or RAC1 consistently prevented PLD1 activity triggering by CXCL12. N-butanol, the scavenger of PLD1 product phosphatidic acid, inhibited T lymphocyte static adhesion to ICAM-1, tethering under flow, and extended or high-affinity LFA-1. Tat-P1-PLD1 peptide, which blocks RHOA-PLD1 interaction, also inhibited adhesion and LFA-1 affinity upregulation.	(Bolomini-Vittori, Montresor et al. 2009)

Gene	Integrin	Cells	Function	Reference
PRKCZ (PKCzeta, PKCz); CCL21	LFA-1	Lymphocytes	PKCz regulates lateral mobility, not affinity by CCL21 stimulation. CCL21 induced a consistent and rapid increase of PKCz kinase activity. Important for adhesion to low density ICAM-1.	(Giagulli, Scarpini et al. 2004)
PSGL1; SYK; TNFα; CXCL1/ CXCR2	LFA-1	Neutrophils	Blocking LFA-1 not Mac-1 increased neutrophil rolling velocities on E-selectin/ICAM-1 surface. E-selectin is required for LFA-1 activity in the absence of chemokines. SYK is required for E-sel and P-sel integrin activation. Intermediate LFA-1 is needed for slow rolling but high affinity LFA-1 for arrest. SYK is needed for slow rolling, TNFa and CXCR2 for arrest.	(Zarbock, Lowell et al. 2007)
PSGL1; SYK; FGR; DAP12 (TYROBP); FcRγ	LFA-1	Neutrophils	E-selectin–mediated phosphorylation of SYK and slow rolling was abolished in neutrophils from fgr–/– mice. Neutrophil recruitment into the inflamed peritoneal cavity was suppressed in DAP12–/– Fcrγ–/– mice.	(Zarbock, Abram et al. 2008)
PXN (Paxillin)	VLA-4	Jurkat T-cells, human PBLs	PXN KD decreased adhesion to low/medium not high concentration VCAM-1 under shear flow but not static. No affinity changes when measured with VCAM-Fc or 15/7 anti-LIBS. Outside-in signaling mediated by PXN increases shear resistance.	(Manevich, Grabovsky et al. 2007)
RAC1; CDC42	LFA-1	T lymphocytes	Tat–Rac1-N17(DN) inhibited CXCL12-triggered static lymphocyte adhesion to ICAM-1 or lowered amounts of lymphocytes arresting to ICAM-1 under flow for <1s, <3s, <10s, suggesting a role for inside-out and outside-in activation. Rac1 inhibition or siRNA knock-down prevented CXCL12-induced LFA-1 extended or high-affinity conformations (KIM127, 327C) in primary T lymphocytes. In contrast Tat–Cdc42-V12(CA) or Tat–Cdc42-L61(CA) decreased all these parameters.	(Bolomini-Vittori, Montresor et al. 2009)
RAC1; WAVE2; RAP1; RAPL; CCL25; CXCR9	VLA-4	CD4+CD8+ and CD4+CD8– T-cells	Knock-down of any one of these genes decreased CCL25-CCR9-induced adhesion to FN or VCAM-1. RIAM KD had no effect. Thymocyte RAC1-WAVE2 and RAP1-RAPL pathways for inside-out signaling and stimulated adhesion.	(Parmo-Cabanas, Garcia-Bernal et al. 2007)
RAP1; CalDAG-GEF1; PLC; SPA-1 (SIPA1); RAP1GAP	LFA-1	Primary CD3+ human T cells	CXCL12 or PMA induced Rap1 GTP-loading. PMA-induced adhesion to ICAM-1, not VCAM-1 or FN, was decreased if SPA-1 or RAP1GAP expressed. SPA-1 decreased active conformation of LFA-1 as measured with KIM127 mAb. CalDAG-GEF1 KD decreased static and flow adhesion to ICAM-1. PLC inhibitor (U73122) or calcium chelator BAPTA decreased adhesion to both substrates.	(Ghandour, Cullere et al. 2007)
RAP1; PLC; Ca2+	VLA-4	Eosinophils	Inhibition of PLC (U73122) or Ca2+ depletion (ETGA) abolished cell adhesion to VCAM-1. Also decrease in attachment to HUVECs under flow by U73122, which was specific to VLA-4. Correlates with RAP1 activity.	(Ulfman, Kamp et al. 2008)
RAP1; SPA1 (SIPA1); CCL21; CXCL4 (PF4)	LFA-1, VLA-4	Lymphocytes	Chemokines increased adhesion to ICAM-1 and VCAM-1 and MBEC4- transmigration only under shear flow. RAP1 was activated within seconds and this was inhibited by RAP1 GAP, SPA1 expression. Dominant active Rap1V12 induced adhesion, transmigration, and polarization of lymphocytes.	(Shimonaka, Katagiri et al. 2003)

Gene	Integrin	Cells	Function	Reference
RAP1B	VLA-4	B cells	In vitro adhesion to VCAM-1 and chemotactic migration was impaired in RAP1B-null B cells. Also defect in B cell homing to lymph nodes. CXCL12-induced PYK2 phosphorylation decreased in RAP1B-/- cells.	(Chen, Yu et al. 2008)
RAPL (RASSF5)	LFA-1, VLA-4	Lymphocytes, thymocytes, dendritic cells	Adhesion of RAPL-null T and B cells to ICAM-1 or VCAM-4 was reduced to 75-80% upon CCL21 or CXCL12 induction. Transmigration through endothelial cells also impaired. RAPL-null T cells failed to become polarized and form LFA-1 patches by CCL21. Null mice have defective lymphocyte homing to lymphoid tissues. Impaired DC adhesion, migration, and traffic to lymph nodes.	(Katagiri, Ohnishi et al. 2004)
RASGRP1 (CalDAG-GEF1)	LFA-1	Neutrophils and primary T cells from LADIII patients	LADIII neutrophils or primary T cells defective in arrest on TNFa-stimulated endothelium. CXCL12-induced in vitro arrest on immobilized ICAM-1 and chemokines failed. Extension/high affinity LFA-1 failure. Also partial defects in VLA-4 arrest.	(Pasvolsky, Feigelson et al. 2007)
RHOA, ROCK	beta2	Monocytes	C3 RHOA inhibitor inhibited monocyte transwell migration through HUVEC layer. No effects on adhesion or spreading. Tail retraction defect by C3 or RBD of Rhotekin on serum or HUVECs. Constitutively active ROCK rescued C3 inhibition. C3 mislocalizes beta2 to trailing tail.	(Worthylake, Lemoine et al. 2001)
RHOA; CCL21; CCR7; CCR10	LFA-1	Lymphocytes	23–40 and 92–119 P1-RHOA peptide mimetics prevented rapid adhesion to ICAM-1 induced by CCL21. RHOA important for LFA-1 inside-out activation and lateral mobility. RhoA inhibition in lymphocytes decreased arrest to PP-HEVs.	(Giagulli, Scarpini et al. 2004)
RHOA; CXCL12; CXCR4	LFA-1	Lymphocytes	RHOA inhibitory peptide, P1–23/40, when treated with lymphocytes, decreased rapid and stable adhesion to PP-HEV in vivo. Rho-23/40- interfering peptide (P1–23/40) eliminated all CXCL12-triggered LFA-1 adhesion in vitro. CXCL12 induces extended LFA-1.	(Pasvolsky, Grabovsky et al. 2008)
SELE; p38	LFA-1, Mac-1	Neutrophils	SELE and ICAM-1 expressed on endothelial L cell monolayer induced rolling and arrest under flow w/o chemokine. p38 inhibitor decreased this greatly. Independent of Gαi.	(Simon, Hu et al. 2000)
ST3GAL4; CXCL1, CXCL8 (IL8), CXCR2 (IL8RB),	LFA-1	Neutrophils	CXCL1 or CXCL8 injection to inflamed mirovessels in ST3GAL4-null mice had marked leukocyte adhesion deficiency. Also, decreased leukocyte arrest on P-selectin/ICAM-1/CXCL1-immobilized chamber or transendothelial migration in vitro. Also in vivo extravasation on TNFa-inflamed cremaster muscle venules inhibited. Sialylation critical for chemokine interaction with its receptor.	(Frommhold, Ludwig et al. 2008)
SYK	LFA-1	Leukocytes	SYK-/- mice had decreased leukocyte adhesion efficiency to cremaster muscle venules. When stimulated with fMLP, no difference in the early point, but superfusion more that 5min decreased adhesion. Also spreading defect on venules. Neutrophils tested in vitro also. Outside-in signaling.	(Frommhold, Mannigel et al. 2007)

Gene	Integrin	Cells	Function	Reference
SYK	LFA-1	Neutrophil-like HL-60 cells and monocytic THP-1 cells	Inhibition or KD of SYK resulted in massive lamellae formation, polarization defect and directional migration defect. Polarized localization of SYK was dependent on β2 integrin and fibrinogen.	(Schymeinsky, Then et al. 2005)
SYK, VAV1/3	LFA-1	Neutrophils	In inflamed cremaster muscle, SYK-/- neutrophils revealed a defect in adhesion and migration, with decreased extravasation to inflamed tissue. Mutation in VAV1/3 binding site (Y348F) caused excessive lamellae formation and migration defects.	(Schymeinsky, Sindrilaru et al. 2006)
SYK; PIK3CD (PI3Kdelta)	ND	PMNs	SYK-/- neutrophils transfected with SYK Y323F, which cannot bind p110δ, showed impaired lamellae polarization and directed migration. Also Syk-/- neutrophils incabable of infiltrating to wound in mouse healing assay. p110d is downstream of SYK.	(Schymeinsky, Then et al. 2007)
TLN1	VLA-4	Jurkat T-cells, human PBLs	Knock-down decreased adhesion to all concentrations of VCAM-1. This correlated with VLA-4 affinity decrease measured with soluble VCAM-1/Fc-beads or 15/7 anti-LIBS. TLN1 is responsible for CXCL12 induced affinity upregulation, but is not involved in shear resistance (affinity maturation).	(Manevich, Grabovsky et al. 2007)
TLR2; CD14	MAC-1	Monocytes	*P. gingivalis* fimbriae binding to macrophages leads to inside-out activation of MAC-1, but not if CD14 or TLR2 is knocked-out. CD14-TLR4 axis is important for adhesion and endothelial transmigration, where Rac and PI3K downstream.	(Harokopakis, Albzreh et al. 2006)
VAV1/3	MAC-1	Neutrophils	VAV1/3 are phosphorylated in neutrophils adhering to complement fragment C3bi through αMβ2. VAV1/3-null cells have decreased adhesion strength and spreading to C3bi. VAV1/3-null cells arrest in response to fMLP but exhibit significantly reduced sustained adhesion in vivo. Defects in AKT, PAK, PYK2, PXN, MLC phosphorylations, not SFKs, when plated on C3bi, but no changes if cells in suspension and CXCL12-induced. VAV1/3 is downstream of integrin activation.	(Gakidis, Cullere et al. 2004)

2.3.5. T-cell receptor signaling to integrins LFA-1 and VLA-4

A beautiful example of integrin regulation is the immunological synapse (IS) that is formed between antigen presenting cell (APC) and T cell. As IS matures, antigen-bound T cell receptor (TCR) is accumulated in the central region of the conjugate and integrins (LFA-1 and VLA-4) are accumulated at the periphery of the complex, thus forming the central and peripheral supramolecular activation clusters (cSMAC, pSMAC), respectively (Monks, Freiberg et al. 1998). Formation of mature IS requires integrin-mediated adhesion together with cytoskeletal rearrangements (Wulfing, Davis 1998). TCR binding to antigen stimulates integrin adhesiveness (Dustin, Springer 1989) and together these receptors mediate important signals for T cell to activate transcription, proliferate, and differentiate (Van Seventer, Shimizu et al. 1990). The convergence between TCR and integrin signaling is at least at the level of transcriptional regulation,

as both stimulate MAPK signaling to activate transcription (Wang, Shibuya et al. 2008; Perez, Mitchell et al. 2003). However, the convergence is already at the level of receptor complex formation, as TCR and integrins share many of the same molecular constituents downstream of them. The adaptor complexes and effector proteins important for integrin activation downstream of TCR are also important for sustaining integrin adhesion and linking integrins to the cytoskeleton. Src family kinases, Syk (Zap-70), PLCγ, Vav1/3, as well as the adaptors SLP-76 and ADAP are needed for platelet integrin αIIbβ3 outside-in signaling. Similarly, SLP-76 and ADAP are phosphorylated and activated by integrin ligation during IS formation in T cells (Nguyen, Sylvain et al. 2008; Suzuki, Yamasaki et al. 2007) and Vav1 is activated downstream of LFA-1 activation (Sanchez-Martin, Sanchez-Sanchez et al. 2004).

Most of the integrin activation studies in IS formation focus on cell adhesion to purified integrin ligands or to APC, as well as integrin localization and clustering during conjugate formation showing that the effect is downstream of TCR, but disregard whether it is also downstream of integrin ligation (see Table 3). The discrimination between inside-out and outside-in integrin signaling during IS formation is not so straightforward, though. If adhesion defect can be rescued by PMA, it only means that activation of PKC, which can be upstream or downstream of integrin, can rescue the defect. It is also misleading to say that the effect is upstream of integrin, if exogenous addition of integrin activating antibody or manganese can rescue adhesion or IS formation. The use of manganese and activating antibodies (ligand mimetics) in excess stabilize the active conformation and rule out the slightest possibility for integrin to be in inactive conformation and at the same time superactivate all the remaining integrins that are not affected from within. The above-mentioned assays are wrongly interpreted in too many studies. Integrin affinity to its ligand can be analyzed by measuring soluble ligand binding to cell surface. If for example TCR activation by mAb cross-linking (anti-CD3, anti-CD8) induces soluble ICAM-1 or VCAM-1 binding or integrin clustering, but fails to do so after knock-down of the studied gene, the gene regulates inside-out activation of integrin. Failure in sustained adhesion or spreading on integrin ligand, can be regarded as a defect in outside-in signaling. Additionally, antibodies mAbKIM127 and mAb327C detect extended or high affinity conformations of LFA-1, respectively, and can be used for verifying whether the effect is on affinity or clustering. A monovalent Fab should be used for affinity measurements as whole antibody induces clustering. Studies of integrin activation during IS formation are listed in Table 3 with short descriptions. Most of the proteins involved in IS formation are the same as discussed earlier in platelet activation and leukocyte arrest. Figure 8 puts together the proteins that have been considered as important integrin inside-out regulators downstream of TCR stimulation.

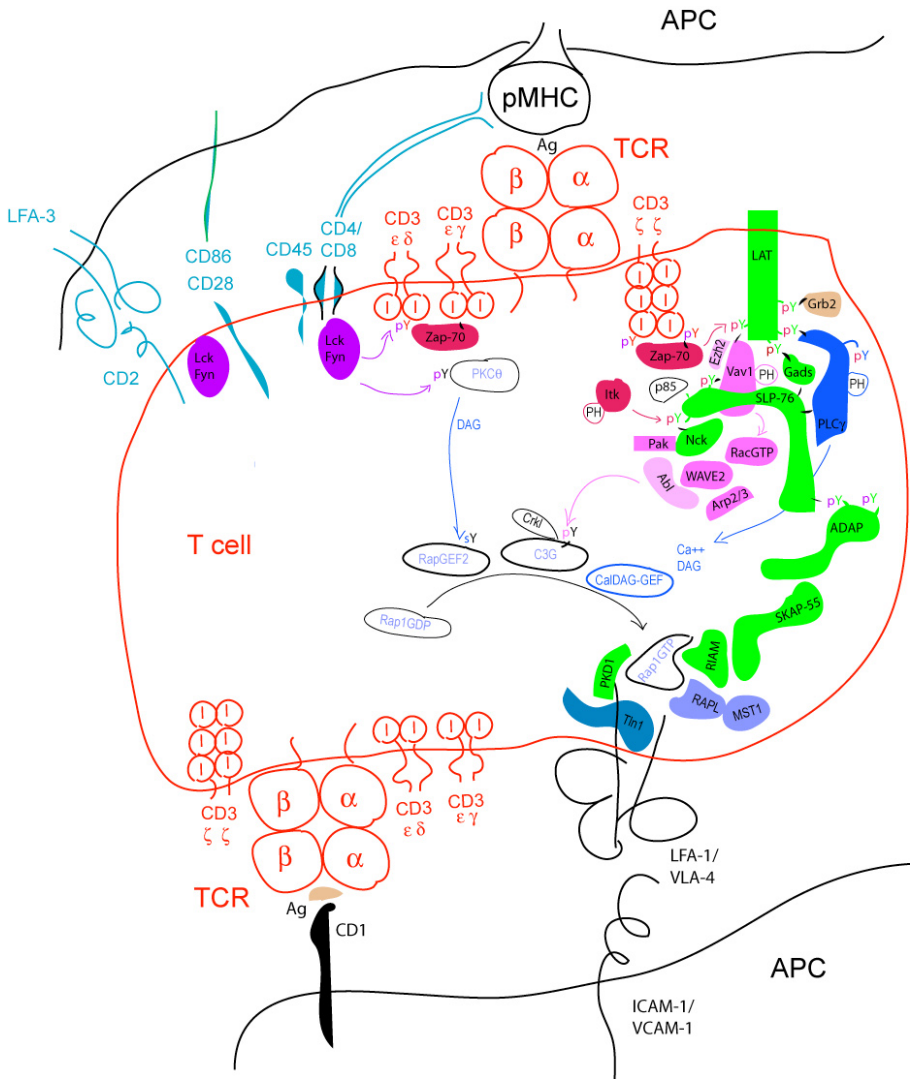

Figure 8. TCR signaling to integrins in immunological synapse (IS). Stable conjugate formation between antigen presenting cell (APC) and T cell requires integrin-mediated adhesion and signaling, which is activated by TCR signaling within a supramolecular activation complex (SMAC). Co-stimulatory receptors recruit and activate Src kinases (Lck, Fyn), which phosphorylate ITAMs (I) in TCR. Phosphorylated ITAMs and Src kinases recruite and activate Zap-70 (Syk in platelets), which together with TEC kinase, Itk, phosphorylate proximal adaptors (LAT, SLP-76) and effectors (Vav1/3, PLCγ1), leading to actin polymerization and calcium/ DAG flux. Adaptor complex, ADAP - SKAP-55 - RIAM recruits activated GTP-loaded Rap1 and integrin binding cytoskeletal protein Talin1 (Tln1) to activate integrins (LFA-1, VLA-4). PKCs, and especially PKCtheta, act upstream of Rap1 by activating Rap GEFs. Also Abl, which is recruited by WAVE2, is able to phosphorylate and activate a Rap GEF (C3G). Protein kinase D1 (PKD1), which is an effector kinase of PKCs and associates with C-terminal β1 integrin tail, is needed for the recruitment of activated Rap1 to IS. Another Rap1 effector, RAPL, and its interactor, MST1 (STK4), are involved in LFA-1 traffic and clustering to IS. Thus, Rap1 GTPase is the central player in integrin activation regulation in the formation of SMAC, and especially at the level of integrin recruitment and clustering (see Table 3 for references).

Table 3. Regulators of TCR and BCR signaling to integrins.

Gene	Study description	Reference
ADAP; SKAP-55	SKAP-55-/- T cells show defects in β1 and β2 integrin adhesion, LFA-1 clustering, production of the cytokines IL-2 and IFNg, and proliferation. SKAP-55 is the effector of ADAP-SKAP-55 complex. TCR/CD3 microcluster localization at the IS reduced in -/- cells. Subset of peripheral T cells has no defects.	(Wang, Liu et al. 2007)
ADAP-SKAP55-RIAM-Rap1	SKAP55/RIAM complex is essential for TCR-mediated adhesion and for efficient conjugate formation between T cells and antigen-presenting cells. RIAM is needed for Rap1 localization to IS and integrin activation.	(Menasche, Kliche et al. 2007)
αPIX (ARHGEF6); PAK2	αPIX-/- T cells form impaired IS with B cells loaded with OVA II peptide. Pak phosphorylation down and LFA-1 clustering to IS weaker upon TCR-stimulation. However, no differences in Rac or Cdc42 GTP loading, but GIT1 expression is lower.	(Missy, Hu et al. 2008)
CD2	CD2 physically interacts with both Lck and Fyn inside lipid rafts. CD2 can mediate the interaction between the two kinases and the consequent boost in kinase activity in lipid rafts.	(Nunes, Castro et al. 2008)
CD2; CD28; CD3; CD7	Antibody cross-linking increases soluble FN binding, HUTS-21 (active b1 recognizing ab) binding to Jurkat and peripheral T cells. Also adhesion to immobilized FN is increased.	(Woods, Cabanas et al. 2000)
CD2; CD3	CD2- or CD3-ligation leads to LFA-1-mediated increase in T cell adhesion. CD3-triggered adhesion is transient, whereas CD2 causes persistent stimulation. Both trigger PKC activation as does PMA.	(van Kooyk, van de Wiel-van Kemenade et al. 1989)
CD28; LCK; PKCtheta	Anti-CD3 triggers Lck-driven membrane localization of PKCtheta. CD28 needed for proper PKCtheta localization in the cSMAC and LFA-1 localization in the pSMAC for sustained T cell activation (>4hours).	(Huang, Lo et al. 2002)
CTLA-4	Anti-CTLA-4 increases cell adhesion to immobilized ICAM-1 and is additive to anti-CD3. Anti-CD3 increases LFA-1 clustering and anti-CTLA-4 is additive. Anti-CD3 fails to induce LFA-1 clustering and adhesion, when CTLA-4 depleted. CTLA-4 ligation activates Rap1 and is additive to CD3-ligation. Rap1-N17 can block CTLA-4 increased adhesion and Rap1-V12 can mimic CTLA-4 in its cooperation with anti-CD3.	(Schneider, Valk et al. 2005)
CTLA-4	Anti-CTLA-4 (CTLA-4 ligation) increases T cell motility on ICAM-1. CD3 ligation decreases cell motility or arrests cells on ICAM-1-coated plates. Coligation with CD3 and CTLA-4 reverses the arrest or speed reduction. CTLA-4 positive CD4+ cells fail to stop upon peptide challenge in cervical lymph nodes followed by two photon microscopy. More transient contacts with APCs in vitro if CTLA-4 positive cells.	(Schneider, Downey et al. 2006)
DNAM1 (CD226)	DNAM1 associates with LFA-1. Mutant (Y-F322) CD226 transferred into naive CD4+ helper T cells (Ths) inhibits interleukin (IL)-12-independent Th1 development initiated by CD3 and LFA-1 ligations. Proliferation induced by LFA-1 costimulatory signal is suppressed in mutant (Y-F322) CD226-transduced naive CD4+ and CD8+ T cells in the absence of IL-2.	(Shibuya, Shirakawa et al. 2003)
ITK/RLK	ITK/RLK-deficient cells or Itk-deficient cells showed decreased adhesion to ICAM-2 upon CD3 ligation, but PMA stimulus was intact. TCR-stimulated Tyr phosphorylation of Vav1 and ADAP, but not Pyk2, is intact in Tec kinase-deficient cells. LFA-1, Tln1, Vav1, PKCtheta, Pyk2, and actin fail to polarize in T cells towards anti-TCR beads. Vav1-/- cells have same phenotype, suggesting that Tec kinases are needed for correct Vav1 localization and integrin clustering.	(Finkelstein, Shimizu et al. 2005)

Gene	Study description	Reference
PKCtheta; RapGEF2 (PDZGEF1); Rap1	Direct phosphorylation of RapGEF2 at Ser960 by PKCtheta regulates Rap1 activation and LFA-1 adhesiveness to ICAM-1. In OT-II TCR-transgenic CD4+ T cells, clustering of LFA-1 after Ag activation was impaired in the absence of PKCtheta.	(Letschka, Kollmann et al. 2008)
PKD1 (PRKD1)	Expression of PKD1 lacking PH-domain blocks CD3- or PMA-stimulated adhesion of Jurkat cells to FN. CD3/PMA stimulation increases PKD1 or PH mutant localization to PM β1 integrins, but integrin clustering defective if PH mutant used. KD of PKD1 decreases adhesion to FN. PMA stimulates association of PKD1 and Rap1, and CA Rap1 associates constitutively. CD3/PMA induces Rap1 GTP loading but not if PKD1 PH-mutant expressed. Complex formation of Rap1-PKD1-ITGB1 upon stimulation. PH domain not needed for PKD1-ITGB1 association. Rap1 activation requires ITGB1 expression and its c-terminus.	(Medeiros, Dickey et al. 2005)
PLCG1; CalDAG-GEF; RAP1	TCR cross-linking triggered persistent Rap1 activation, and SDF-1 (CXCL12) activated Rap1 transiently. PLC inhibitor, U73122 abrogated Rap1 activation triggered by both the TCR and SDF-1 (CXCL12). PLCg1-deficient Jurkat T cells showed a marked reduction of TCR-triggered Rap1 activation and adhesion to intercellular adhesion molecule-1 (ICAM-1) mediated by LFA-1	(Katagiri, Shimonaka et al. 2004)
RAP1	Anti-CD3, phorbol-dibutyrate, or ionomycin activate Rap1A in T cells. V12Rap1A thymocytes from transgenic mice (CD2 promoter) have increased binding to FN (VLA-4), 130kD FN (VLA-5), and to ICAM-1 (LFA-1). MgCl2 and EGTA increase cell adhesion to ICAM-1 or binding of soluble ICAM-1, suggesting that V12Rap1A does not modulate affinity, but clustering (avidity), which is also shown by confocal.	(Sebzda, Bracke et al. 2002)
RAP1	IgG+ A20 B cell lymphoma or A20 B cells spread on immobilized anti-IgG or anti-LFA-1, but not to anti-CD40 or anti-FcgRII. 10-μM CytD or LatA almost totally blocked this and PP2 about 70-80%. RapGAP2 expression or Rap1N17 blocked spreading on these antibodies. Plating cells on antibodies activates Rap1. Rap activation is critical for formation of LFA-1-containing pSMACs and for LFA-1 to enhance Ag accumulation at the IS. Particulate BCR ligands induce the formation of F-Actin-rich cups via Rap activation. BCR signaling to ERK and AKT is compromised when Rap1GAPII expressed (Ig-bead adhesion stimulation).	(Lin, Freeman et al. 2008)
RAPL; MST1 (STK4)	RAPL binds directly and activates MST1 kinase activity. MST1 can activate LFA-1 adhesion, which requires the kinase activity and C-terminal regulatory region of MST1. KD impairs adhesion and LFA-1 polarization. KD also impairs IS formation and adhesion upon CCL21 or TCR ligation. No affinity changes.	(Katagiri, Imamura et al. 2006)
SKAP55	KD reduced LFA-1 clustering around CD3-TCR and reduced T cell-APC conjugation (50%) even with intact CD3-TCR capping. SKAP-55 needed for LFA-1 inside-out activation upon TCR stimulus. SKAP-HOM (SKAP-55R) can't rescue the defect.	(Jo, Wang et al. 2005)
SKAP55	SKAP-55 expression induces T cell adhesion to FN and ICAM-1 and increases LFA-1 clustering at IS. Needs SH3-domain, which binds ADAP, for these effects. T cell - APC conjugation leads to SKAP-55 localization to lipid rafts.	(Wang, Moon et al. 2003)
SLP76 - ADAP	ADAP binding to the SLP-76 SH2 domain is needed for TCR-induced integrin adhesion to FN or ICAM-1 and T cell–APC conjugation. PMA rescues the defect. ADAP binding to VASP has no role in conjugate formation.	(Wang, McCann et al. 2004)
SLP76 - GADS	SLP-76 peptide that binds Gads is used to inhibit interaction. Peptide inhibits partly Jurkat adhesion to FN and ICAM-1 upon TCR-stimulation (OKT3 ab) or PMA-stimulation and manganese restored the defect (not outside-in signaling). No effects on conjugate formation however. Maybe not as important as SLP-76 - ADAP - SKAP55 interaction.	(Jordan, Maltzman et al. 2007)

Gene	Study description	Reference
SRC family; PI3K (p110δ); VAV1/2; RAC2; RAP1	LFA-1 pSMAC formation is needed for efficient antigen accumulation. Src-family kinases critical for BCR-induced IS-formation. Rac2-deficient B cells exhibit lower amounts of Rap1-GTP and severe actin polymerization defects. BCR stimulation leads to activation of Rac2 via Src family kinases, PI3K, Vav1/2. Rac2-/- cells have decreased binding to ICAM-1, cell adhesion to ICAM-1 bilayers, and deficiency in Ag clustering. CA Rac2 rescues mutant BCR B cell adhesion to ICAM-1.	(Arana, Vehlow et al. 2008)
Src kinases; ADAP; SLP76; JNK	LFA-1 ligation induces actin cloud formation and requires ADAP, SLP-76, and JNK, but not ZAP-70 or TCR. Outside-in signaling of LFA-1 forms actin cloud at the center of T cell - APC interface, and this lowers the threshold for T cell activation. JNK is activated upon adhesion to anti-CD3 or anti-LFA-1 measured by phospho-Jun. JNK inhibitor also abrogates IS formation (CD3 clustering)	(Suzuki, Yamasaki et al. 2007)
TLN1 (Talin1)	Decreased adhesion and spreading to ICAM-1-Fc in siRNA-treated cells upon CD3 crosslinking or PMA. See that cells don't adhere to ICAM-1 without any stimulation. Tln1 KD causes reduced LFA-1 affinity in ctrl and PMA-stimulated cells (measured by antibody), but no differences when affinity stimulated from outside by manganese. However, adhesion defect is not rescued by manganese, because also LFA-1 clustering and polarization defect on anti-CD3 beads. Talin1 is required for T cell-APC conjugation (superantigen-bearing Daudi B cells).	(Simonson, Franco et al. 2006)
VAV1	Whereas basal adhesion is same, anti-CD3 stimulation increases adhesion to FN, COL4, Lam, Vn, or ICAM-1, but not if Vav1-null cells used. PMA or Mg2+ rescued defect. Null cells have defective conjugation with APCs, because defect both in TCR and LFA-1 clustering. WASP-null cells have TCR cluster defect but not LFA-1 cluster defect. PYK2 not phosphorylated upon TCR- or LFA-1-stimulation in VAV1-null cells. Inside-out- and outside-in activation.	(Krawczyk, Oliveira-dos-Santos et al. 2002)
WAVE2; ABL1; CRKL; C3G; RAP1	TCR-stimulation (anti-CD3) increases T cell binding to FN and ICAM, but not if WAVE2 KD cells. Anti-CD3 increases Rap1 GTP-loading, not if WAVE2 depleted (Vinc. siRNA no effect). siRNA of Crkl or C3G (RapGEF) inhibit CD3-stimulated Rap1 activity and adhesion to FN/ICAM-1. Crkl and C3G needed for β1 localization to IS upon superantigen (SEE)-pulsed Raji B cell encounter and β1 activation upon PMA or a-CD3. Abl1 regulates TCR-mediated activation of Rap1 and integrin affinity maturation, where WAVE2 is needed for Abl localization. No effects on Zap70 or PLCg1 phophorylation.	(Nolz, Nacusi et al. 2008)
WAVE2; ARP2/3; Vinculin (Vinc); Tln1	TCR-stimulation leads to WAVE2-Arp2/3-Vinc-Tln1 complex formation at the IS. KD of WAVE2 or Vinc inhibits activation-dependent induction of high-affinity integrin binding to VCAM-1.	(Nolz, Medeiros et al. 2007)
ZAP70; LAT	CD3/TCR-mediated increases in β1 integrin adhesion and activation of PI3K are abrogated in Zap-70-null Jurkat cells. Substitution Y315F of Zap-70 abrogates kinase activity, phosphorylation of LAT cytoplasmic tail and adhesion as well as PI3K induction. Also LAT-deficient Jurkat cells have decreased β1-mediated adhesion.	(Goda, Quale et al. 2004)

2.3.6. Summary of integrin activity regulation in platelets and leukocytes

As seen above, platelets and leukocytes share common regulatory pathways for integrin inside-out activation as well as outside-in signaling to cytoskeleton. Figure 9 puts the essential together and shows the schematics from inside-out activation to outside-in signaling.

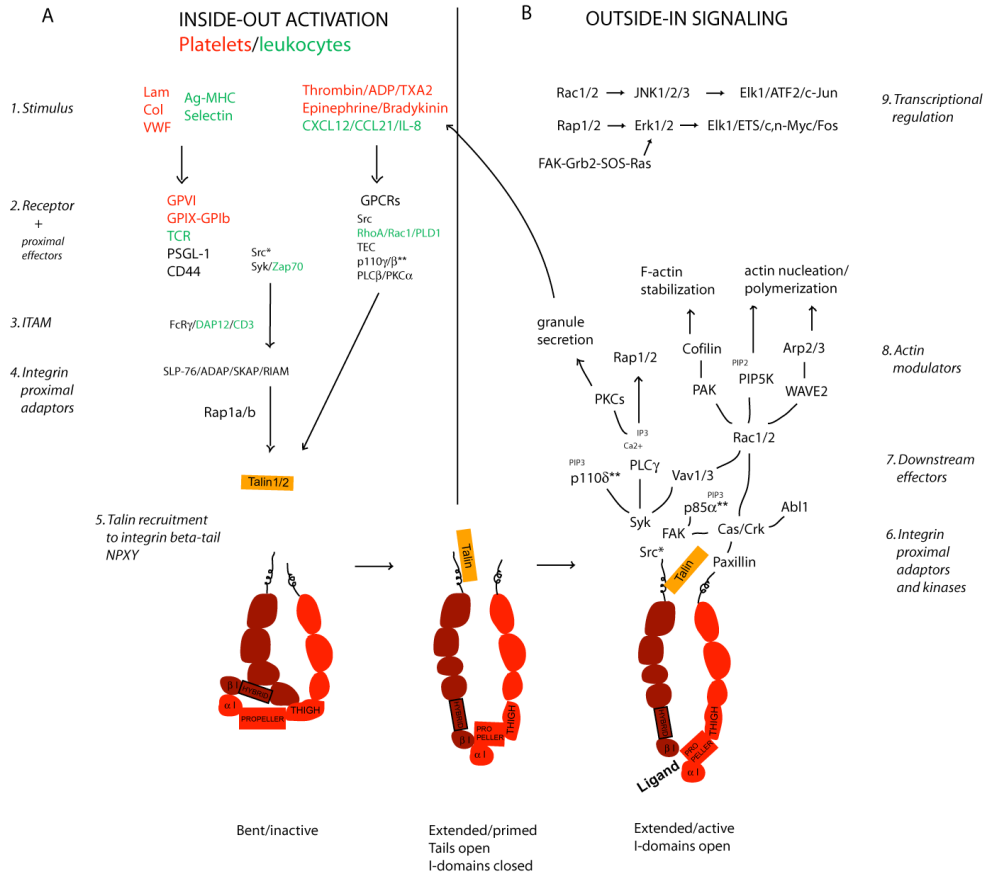

Figure 9. Common pathways in integrin activity regulation and signaling in platelets and leukocytes. A. Inside-out activation. The stimulus for integrin activation initiates from the exposed ECM proteins in endothelial walls for platelets. Selectin-mediated rolling and signaling as well as antigen encounter lead to leukocyte integrin activation. Different GPCR agonists sustain platelet integrin αIIbβ3 activity, whereas in leukocytes different chemokines are essential for early integrin inside-out activation during leukocyte arrest on endothelial wall. The essential players downstream of ECM receptors and T cell receptor (TCR) are the Src family kinases and Spleen tyrosine kinase (Syk) or Zap70 in leukocytes. Src family kinases either directly activate Syk/Zap70 or by phosphorylating tyrosines in ITAM-motifs, where Syk/Zap70 then bind and become activated. The integrin proximal adaptor complex SLP-76/ADAP/SKAP/RIAM is formed upon Syk/Zap70 and TEC kinase family (TEC, Btk, Itk, Rlk) mediated phosphorylation. This adaptor complex then recruits Rap1a/b GTPase and talin1/2, of which the former is known to regulate integrin clustering and the latter integrin affinity by directly binding to conserved NPXY motif in beta-tails of integrin heterodimers. Talin binding to beta-tail disturbs salt-bridge interaction between alpha and beta tails and leads to a conformational change in the extracellular part (red) by swing-out of the beta-integrin hybrid domain (boxed). Now, the extended integrin heterodimer can more readily bind to its ligand, and is thus activated from within the cell. Here the example is leukocyte integrin LFA-1, where ligand binds to the headpiece alpha I domain. αIIbβ3 does not contain alpha I domain, and the ligand binding is mediated by the beta-propeller of the alpha-integrin and the I-like domain of beta-integrin **B. Outside-in signaling**. Ligand binding to integrin stabilizes integrin extended conformation with the headpiece I domains open. Integrins signal to cytoskeleton and to transcriptional regulation through various adaptor and effector proteins, of which only some are drawn here. The details of signaling to cytoskeleton and references can be found in the text. For integrin regulated transcriptional regulation, readers are suggested to read Giancotti, Ruoslahti (1999) and Aplin, Stewart et al. (2001). * Src = Src family kinases (Fyn, Lyn, Lck, Hck, Fgr). ** Subunits of PI3K. The integrin conformational change cartoon was modified from Alon and Ley (2008).

2.4. Integrin regulation in cancer

Deregulation of integrin expression is often associated with cancer progression. Collagen-binding α2 integrin is heavily downregulated in breast and prostate adenocarcinoma, but again upregulated in metastasis of the latter (Zutter, Krigman et al. 1993; Bonkhoff, Stein et al. 1993). In addition, α2β1 heterodimer seems to be highly expressed in prostate cancer stem cells (Collins, Berry et al. 2005; Mimeault, Batra 2007). A truncated isoform of platelet integrin αIIb is absent in normal prostate tissue, but is found in prostate adenocarcinoma with increased expression in metastatic foci (Trikha, Cai et al. 1998; Trikha, Raso et al. 1998). Also β1 integrin is consistently upregulated in prostate cancer, (Goel, Li et al. 2008). The importance of β1 integrin has been studied with mouse models. Targeted β1 integrin deletion in mouse breast cancer model (MMTV/PyV MT) or in pancreatic cancer model (Rip1Tag2), results in significantly reduced primary tumour growth in both cancers, but also reduces metastasis of the latter (White, Kurpios et al. 2004; Kren et al., 2007).

Whereas the primary tumour invasion might occur even in the absence of integrin adhesion (Friedl, Wolf 2003), the arrest to endothelium and subsequent extravasation is dependent on this (Lammermann, Bader 2008). Humphries, Olden and others (1986) have shown that systemic injection of melanoma cells can result in pulmonary metastasis, but co-injection of integrin ligand-mimetic peptide, GRGDS, dramatically inhibits this. Thus, it is the extravasation step of metastasis, where integrin function and activity regulation could play important roles. This is evident for example in chronic myelogenous leukemia, where BCR-ABL1 fusion oncoprotein results in aberrant adhesive properties and impaired leukocyte traffic due to constitutive inside-out activation of the β2 integrin LFA-1 (Chen, Malik et al. 2008). Also, strong β1 integrin-mediated cell adhesion to bone marrow stromal matrix proteins induced by BCR-ABL1, accounts for part of the malignant phenotype (Fierro, Taubenberger et al. 2008). In a xenograft model of leptomeningeal leukemia, an adherent subpopulation of a cell line with constitutively active β1, β2, and β3 integrins (L1210-A), when injected to blood stream, shows much higher metastatic potential and lower survival as compared to the parent cell line (L1210-S) (Brandsma, Reijneveld et al. 2002). Also human melanoma cell lines with high metastatic potential (MV3, BLM), but not those of low metastatic potential (IF6, 530), adhere and migrate on VLA-4 integrin ligand VCAM-1 without chemokine stimulation due to constitutively active VLA-4 (Klemke, Weschenfelder et al. 2007). These studies suggest that constitutively active or dysregulated integrins could be associated with certain cancer cell extravasation and apparently stronger cancer cell – endothelial cell interactions could contribute to this. However, it is also suggested that neutrophils and platelets could help circulating cancer cells to arrest on endothelium. For example, Liang and Dong (2008) have shown that association of melanoma cancer cells with polymorphonuclear neutrophils (PMNs) promotes transmigration through

endotehelium in high shear conditions, whereas direct integrin VLA-4 /VCAM-1 interaction is sufficient in low shear conditions. Felding-Habermann, O'Toole and others (2001) showed that breast cancer cells with constitutively active αVβ3 from metastatic xenograft tumours adhere to collagen-activated platelets and become co-arrested in laminar flow conditions *in vitro*. They also showed that expression of constitutively active β3 (D723R) in β3-null breast cancer cells, but not that of β3 WT, leads to platelet interaction and high vitronectin haptotactic migration *in vitro*, as well as strongly increased pulmonary metastasis *in vivo*. Earlier, Filardo, Brooks et al. (1995) have demonstrated an important role for talin-binding NPLY-motif in β3-tail in melanoma cell migration *in vitro* and in pulmonary metastasis in chick embryo *in vivo* assay.

Upon ligand binding, integrin cytoplasmic tails form connection to actin cytoskeleton through adaptor proteins, such as talin, paxillin, and vinculin (Geiger, Bershadsky et al. 2001). These adaptors also recruit different kinases important for integrin outside-in signalling, such as focal adhesion kinase (FAK), Src family kinases (c-Src, Fyn, Lyn, Lck), Abelson tyrosine kinase (Abl), and spleen tyrosine kinase (Syk). The role of integrin outside-in signalling in platelets is to strengthen cell–cell adhesion through fibrinogen cross-linking for aggregate formation, and in leukocytes to strengthen post-arrest adhesion to endothelium before leukocyte extravasation. The molecular mechanisms and contribution of different focal adhesion proteins in platelet and leukocyte integrin activation are well characterized (Shattil, Newman 2004; Guo, Giancotti 2004; Cox, Natarajan et al. 2006; Alon, Ley 2008). However, in spite of the well acknowledged roles of FAK, Src family kinases, and Abl in cancer promotion, their role in the process of cancer cell extravasation has not been studied (Mitra, Schlaepfer 2006; Sirvent, Benistant and Roche 2008). It is very probable that the constitutive nature of integrin activity and adhesion is partly maintained in cancer cells by these crucial integrin regulators, whose aberrant function is then reflected to aberrant adhesive interactions with other cells and extracellular matrix.

3. AIMS OF THE STUDY

Cell migration is a complicated process involving simultaneus adhesion and deadhesion in different locations of a motile cell. As integrins are pivotal molecules mediating adhesion to extracellular proteins and other cells, the regulation of integrin function must also be important for cell motility. Cell migration requires fast changes in integrin locations and function, and cannot be regulated solely by transcriptional control of integrin expression. Recent findings in cell biology research demonstrate that cell surface receptors, including integrins and G-protein coupled receptors, are not internalized only for degradation, but also for recycling back to the plasma membrane. And it is this endocytic trafficking that could determine the polarization and localized activation of integrins in migrating cells. In spite of elegant studies on integrin recycling mechanisms, the endocytic routes and pathways are still very much unknown. **The first aim of the studies was to identify important regulators of β1 integrin endocytosis during cell migration and secondly to broaden this to dividing cells as well.**

Another level on integrin regulation lies in its conformation. Integrins are able to change their conformation and ligand-binding capacity in subsecond time-scale. Proteins from within the cells, by binding to cytoplasmic tails of integrins, can mediate conformational changes through transmembrane domain all the way to ligand-binding head domain, which then increases binding capacity (affinity) for the ligand. **The third goal of the study was to identify proteins regulating β1 integrin conformational changes or stability of ligand binding in prostate cancer cells**.

4. MATERIALS AND METHODS

DNA constructs

If constructed in-house**, bold** character.

pEGFP-Rab21 and pRluc-Rab21, and their variants (**I**, II); DsRedm-Rab21 (**II**); pEGFP-Rab5A (I) (Gomez et al., 2003); pYGFP-Rab7 (I) (Wilcke et al., 2000); pEGFP-Rab9 (I) Barbero et al., 2002); pEGFP-Rab11 (I) Lebrand et al., 2002); pAWNEOα2 (I), (Ivaska et al., 1999); pEGFP-α2 and variants (**I**); pIRES2-α2-EGFP and variants (**II**); pGBKT7-α2 and variants (**I**); pGADT7-Rab21 (95-222) (**I**); pSilencer Rab21-shRNA (I).

siRNAs

Rab21 siRNAs (see different target sequences in I) (I, II)
Clathrin heavy chain siRNA (Predesigned SMART POOL from Dharmacon) (II)
FIP3 siRNA (Target: AAGGCAGTGAGGCGGAGCTGT)
Scramble siRNA (Qiagen AllStar, 1027281) (I, II, III)
Scramble siRNA (Ambion Silencer negative control) (I, III)
siRNAs in CSMA, see original publication III Materials and Methods

Cell lines

Cell line	Species/origin	Used in
MDA-MB-231	human breast carcinoma	(I, II)
HT-1080	human fibrosarcoma	(I)
Hela	human cervical cancer	(I)
Saos-2	human bone osteosarcoma	(I)
Saos-2 with ITGA2	human bone osteosarcoma modified	(I)
CHO	hamster ovary	(I, II)
HEK-293T	human embryonic kidney	(I, III)
PC3	human prostate cancer	(I)
GD25b1 and variants	murine fibroblasts	(II)
MEFs	murine embryonic fibroblasts	(II)
VCaP	human prostate cancer	(III)
KFr13 and KF28	human ovarian cancer	
NCI-H460	human lung cancer	(II)

Antibodies

Target	Description	Used in
EEA1	rabbit pAb Santa Cruz	(I)
Rab5A	rabbit pAb Santa Cruz	(I)
Rab7	rabbit pAb Santa Cruz	(I)
Rab11	rabbit pAb Santa Cruz	(I)
Caveolin-1	rabbit pAb Santa Cruz	(I)
Rab21	rabbit pAb (Opdam et al., 2000)	(I)
Rab21	rabbit pAb generated in (II)	(II)
β1-integrin	mouse mAb P5D2 hybridoma bank	(I, II)
β1-integrin	mouse mAb AIIB2 hybridoma bank	(I)
β1-integrin	mouse mAb Mab1997, Chemicon	(I, II)
β1-integrin	mouse mAb HUTS-21, Amersham	(I)

Target	Description	Used in
β1-integrin	mouse mAb 12G10, Chemicon	(I, III)
β1-integrin	rat mAb 9EG7, Amersham	(II, III)
β1-integrin	mouse mAb Mab2252, Chemicon	(I)
β1-integrin	mouse mAb P4G11, hybridoma bank	(I)
α5-integrin	mouse mAb Mab1949, Chemicon	(I, II)
α5-integrin	mouse mAb BIIG2, hybridoma bank	(I)
α2-integrin	rabbit pAb AB1936, Chemicon	(I, II)
α2-integrin	mouse mAb MCA2025, Millipore	(I, II)
α1-integrin	rabbit pAb AB1934, Chamicon	
α1-integrin	mouse mAb Mab1973, Chamicon	(I)
α6-integrin	rabbit pAb MAB699, Chemicon	(I)
β3-integrin	mouse mAb M109-3MBL, MBL	(II)
EGFR	mouse mAb, 151-IgG, hybridoma bank	(I)
Collagen I	mouse mAb RAHC11, Imtek	(I)
alpha-tubulin	mouse mAb 6160-100, Abcam	(II)
Biotin-HRP	Cell Signaling Technologies	(II)
Plk1	14209-50, Abcam	(II)
clathrin	ab21679, Abcam	(II)
GFP	rabbit pAB, InVitrogen	(I)

Reagents and compounds

Reagent	Application	Used in
Phalloidin-488/561/647	Filamentous actin staining, InVitrogen	(II, III)
DAPI	Nuclei staining	(I, II, III)
Lipofectamine 2000	Transfection, InVitrogen	(I, II, III)
HiPerfect	Transfection, Qiagen	(II)
Fibronectin	Cell dish coating	(I, II)
Collagen	Cell dish coating	(I, II)
Laminin	Cell dish coating	(I, II)
Vitronectin	Cell dish coating	
Affi-Gel 10	pAb purification, Biorad	(II)
WST-1	Cell proliferation, Roche	(II)
NHS-SS-Biotin	Cell surface protein labeling, Pierce	(I, II)
GFP-RBD	Detection of active Rho	(II)
Coelenterazine	Renilla luciferase substrate, Nanolight	(I)

Methodology

Method	Used in
Cell culture	(I, II, III)
DNA cloning	(I, II)
Yeast two-hybrid	(II)
Cell array siRNA screening	(III)
Immunoprecipitations and Western blotting	(I, II)
Integrin internalization and recycling	(I, II)
Immunoelectron microscopy	(I)
Immunofluorescence microscopy	(I, II, III)
Live-cell microscopy	(I, II)
Adhesion and migration assays	(I, II)
Matrigel invasion assays	
RT-PCR	(II, III)
Sucrose gradient fractionation	(II)
Statistical analysis	(I, II, III)

5. RESULTS

5.1. Rab21 regulates β1 integrin traffic

5.1.1. Rab21 associates with αβ1 integrins in α-tail dependent manner (I)

Many proteins are known to bind integrin beta-cytoplasmic tails, but only few to alpha-tails. NMR studies however show that alpha and beta-tails associate weakly with each other, and integrin inside-out activation disrupts this interaction. This could mean that alpha-tails have also important function in integrin functional regulation. To study what proteins might bind to integrin alpha-tails, alpha2 integrin cytoplasmic tail was used as bait in yeast two-hybrid screening.

Yeast two-hybrid screen of mouse embryonic cDNA library with α2-integrin cytoplasmic tail identified C-terminal Rab21 (residues 95-222) as a "hit" in several yeast clones. Further yeast rematings and biochemical immunoprecipitation assays identified α-integrin membrane proximal conserved motif GFFKR important for the association (I: Fig 1D, E). Several point mutations in the tail confirmed arginine (R) as critical amino acid in the motif for association. Immunoprecipitations were done in CHO cells expressing different cytoplasmic variants of α2 integrin. CHO cells don't express endogenous α2 integrin and thus are suitable for ectopic expression of this integrin or its mutant variant.

Immunoprecipitation studies included a novel assay for protein association by using a luciferase tag. Rab21 WT or mutants were expressed as fusion proteins with *Renilla luciferase* (Rluc) in different cell lines. Immunoprecipitations with anti-α2 or anti-β1 integrin antibodies enriched Rab21 luciferase activity, whereas control precipitations did not (I: Fig 1b, C; Table S1). Best association was seen with a Rab21 mutant unable to dissociate GTP, whereas a GDP-locked Rab21 mutant or Rab21 prenylation mutant had lower association with β1 integrins (I: Fig 1B, C). This would imply that the association favors membrane localization (prenylation) and endosomal vesicles where Rab5 family effectors are working in a GTP-dependent manner (Zerial, McBride 2001). Rab21 belongs to the Rab5 family, and the downstream effectors of Rab5 are well characterized (Christoforidis, Zerial 2000). Endogenous Rab21 was also shown to associate with β1 integrins in MDA-MB-231 cells (I: Fig 1F).

5.1.2. Rab21 affects β1 integrin localization and intracellular traffic (I, II)

The close relative to Rab21, Rab5 is required for early endosome fusion and motility of early endosomes on microtubules (Nielsen, Christoforidis et al. 2000; Nielsen, Severin et al. 1999). To study the localization of Rab21 and its effects on β1 integrin, Rab21 was expressed as fusion with EGFP in MDA-MB-231 cells and β1 integrin was stained

with different antibodies. Confocal microscopy analysis revealed that Rab21 expression dramatically influenced β1 integrin localization in MDA-MB-231 cells (I: Fig 2A, B). In Rab21-expressed cells, active β1 integrin was enriched in intracellular vesicles. Expression of different mutant variants of Rab21 revealed that it's GTP-locking resulted in strong intracellular accumulation of β1 integrins, whereas GDP-locked mutant or prenylation mutant resulted in focal adhesion localization of β1 integrins (I: Fig 2D, E, F, G, H, I). These results implied that Rab21 could possibly regulate integrin traffic between plasma membrane and endocytic vesicles. In addition, as the Rab21-positive vesicles contained both active β1 integrin and its ligand collagen I, the results could suggest that β1 integrins and collagen are co-endocytozed (I: Fig 2L).

Cell biotinylation in +4 °C is practical for labeling cell surface proteins, as endocytosis is non-existent in this temperature. Transferrin labeled cells on > +20 to 22 celcius allows endocytosis to occur. By using this method, Rab21 was found to associate only with the internalized population of β1 integrins (I: Fig 3A), suggesting that Rab21 is not involved in reqruitment of β1 integrins for endocytosis, or regulating integrin clustering or lateral mobility in the plasma membrane, which are often prerequisites for receptor endocytosis. However, Rab21 overexpression increased the early 10 minute internalization and subsequent recycling of β1 integrin, whereas Rab21GDP inhibited endocytosis and Rab21GTP increased intracellular accumulation of β1 integrins, confirming that Rab21 does regulate β1 integrin endocytic traffic (I: 3B). Similarly, knocking-down Rab21 expression by shRNA silencing resulted in reduced β1 integrin endocytosis (I: 3C). However, Rab21 expression did not affect transferrin receptor endocytosis; which is classical marker of clathrin-mediated endocytosis (I: Fig S2A) (Hirst, Robinson 1998).

As Rab21 associates with β1 integrins through alpha-subunit tail GFFKR-dependently, a mutant version with point mutations was tested in CHO cell endocytosis. α2AA mutant integrin (GFFAA) when expressed in CHO cells was not internalized, whereas another mutant, a2ARA (GFFARA), which retains integrin association, was nicely endocytosed (II: Fig 4D, E).

β1 integrin cytoplasmic domain contains two NPXY motifs, which bind to clathrin adaptor proteins and are required for clathrin-mediated endocytosis of other cell surface receptors, such as transferrin receptor, IGF2R, and LRP1 (Ohno, Stewart et al. 1995; Owen, Evans 1998) To study in more detail the β1 integrin endocytosis, β1 integrin deficient fibroblasts expressing WT β1 integrin or mutant β1 integrin, where the two NPXY tyrosines were substituted with phenylalanines (β1 YYFF), were used. Expression of this mutant β1 integrin blocked its endocytosis and also resulted in β1 integrin accumulation into focal adhesions (II: 5A). A reduction of β1 integrin endocytosis was also seen in another cell line cloned from embryos carrying an YYFF mutation in germline (II: 5B). In addition, β1 integrin endocytosis was also reduced in GD25 WT cells upon inhibition

of clathrin coat formation with monodansyl cadaverine (MDC) (II Fig S5). This suggests that in these fibroblast cell lines, β1 integrin endocytosis occurs mostly through clathrin-dependent mechanism. However, over-expression of Rab21 in clathrin-inhibited cells (YYFF and MDC) rescued the endocytosis defect, verifying that Rab21 can induce a non-clathrin endocytosis of β1 integrins.

What could be the mechanism by which Rab21 positively affects integrin endocytosis, if it does not associate with the plasma membrane β1? Easiest explanation would be that Rab21 is generally regulating some endocytosis pathway, which would be other than clathrin-mediated. Immunofluorescent stainings with different endosomal markers demonstrate that β1 integrins partly colocalize with early endosomal marker EEA1 (I: Fig 1 G), which is the convergence point for clathrin and caveolae-mediated pathways. β1 integrin was also accumulated in GFP-Rab5 or GFP-Rab21 positive vesicles with patches of caveolin lining the membrane. Immunogold labeling and electron microscopy revealed that some GFP-Rab21-positive large vesicles had a physical appearance of multivesicular bodies (MVBs) with Lamp1 co-localization (I: Fig S1, not shown). MVBs have been earlier demonstrated to associate with β1 integrin recycling (Ng, Shima et al., 1999). The above results would suggest that Rab5 and Rab21 target β1 integrins via early endosomes through pathways dependent on caveolin endocytosis or caveosome maturation, and that at least Rab21 could target β1 integrins to MVBs, but this remains to be studied. A study of Hagiwara and others (2009) would support the caveolin-pathway, as they show that caveolin-1 directly interacts with Rab5 and stimulates its GTP-loading and cholera toxin B (ChTB) endocytosis.

Endogenous Rab21 was also seen associating with F-actin, and depolymerization of F-actin with cytochalasin impaired GFP-Rab21-positive vesicle motility at the cell periphery (not shown). This could mean that Rab21 affects integrin endocytosis by affecting the actin cytoskeleton, which is accumulated in integrin adhesion contacts. Rab5 is known to regulate the actin cytoskeleton dynamics in processes forming circular or peripheral ruffles by activation of PI3K and Rac GTPases (Lanzetti, Palamidessi et al. 2004; Palamidessi, Frittoli et al. 2008). These ruffles are transient (5 to 20 min) structures, and are implicated both in cell migration and macropinocytosis (Buccione, Orth et al. 2004). Whether Rab5 and Rab21 regulate β1 integrin endocytosis through pathways dependent on macropinocytosis would be of great interest to study.

5.1.3. Rab21 influences cell adhesion and migration (I)

Several studies have shown a positive link between intracellular integrin traffic and cell spreading or migration (see Table 1 Litterature Review). As Rab21 regulates both the endocytosis and recycling of β1 integrins, it is very probable also that it positively affects cell adhesion turnover and migration. Indeed, Rab21 and Rab5 expression in PC-3 and Rab21 expression MDA-MB-231 cells increased cells adhering to collagen I during

30 min incubation (I: Fig 6A, B). Hovever, CHO cells with mutant α2AA integrins, were unable to increase their adhesion upon Rab21 expression (I: Fig 6E), suggesting that the effect of Rab21 on adhesion is dependent on the association and stimulation of integrin endo-exocytic traffic. Similarly, siRNA-mediated partial knock-down of Rab21 in MDA-MB-231 cells decreased early cell adhesion to collagen (I: Fig6 D).

Cell migration requires cell spreading at the leading edge and adhesion disassembly further back in the cell, and it has been suggested that during cell migration integrins would be endocytosed from the rear and transported through the cell to the leading edge (Bretscher 1989). Live-cell Rab21-vesicle tracking during active phase of cell spreading demonstrated that integrins could be trafficked at the leading edge (I: Fig 7A), rather than disassembling only at the cell rear. To study whether Rab21 is needed for cell motility on 2 dimensional surfaces, mutant Rab21-expresssing or Rab21-deficient cells were used in scratch wound assays. Perturbation of Rab21 function by these means demonstrated that Rab21 is important for cell migration, but is not absolutely necessary (I: Fig 7B, C).

5.1.4. Rab21 regulates β1 integrin traffic in cytokinesis (II)

There are clear similarities in the molecular requirments in cytokinesis and in cell adhesion/migration (Glotzer 2005; Ridley, Schwartz et al. 2003). It is also known that adherent cells often fail to undergo cytokinesis in suspension (Pugacheva, Roegiers et al. 2006; Thullberg, Gad et al. 2007). These facts prompted us to look at possible function of Rab21 and β1 integrins in cell division. Perturbation of Rab21 normal function by expressing a GTP-locked Rab21 mutant lead to a cytokinesis failure and subsequent binucleation in NCI-H460 and GD25b1 cells (II: Fig 2A, B, C, S1A, B). Similarly, knocking-down Rab21 in NCI-H460 cells resulted in bi- or multinucleate cell formation (II: Fig 3A). During cytokinesis, Rab21-silenced cells had β1 integrin distributed all over the plasma membrane, whereas in control cells it was nicely accumulated to the cleavage furrow (II: Fig 3C), suggesting that β1 integrin traffic could be important during cytokinesis as well.

To look at the possible role of Rab21 association with αβ1 integrin in cytokinesis, CHO cell lines with wild type or mutant α2 integrins were used. α2 integrin mutant (α2AA), which is unable to associate with Rab21, caused a cytokinesis defect and binucleation in CHO expression model, wich nicely correlated with impaired α2β1 integrin endocytosis and absence of vesicular appearance in the cleavage furrow (II: Fig 4).

During the progression of cytokinesis, daughter cells separate by pulling themselves away from each other. Also Rab21 and β1 integrins traffic in a co-localized manner from the cleavage furrow to the opposing ends of newly forming daughter cells (II: Fig 3E). Antibody chasing experiments further verified that Rab21 silencing results in decreased β1 integrin traffic at the cleavage furrow during ingression (II: Fig 3F). However,

even more pronounced defect in traffic was seen during the stage when newly forming daughter cells should start to elongate away from each other (II: Fig 3F, lower panel). As perturbation of β1 integrin traffic leads to regression of cytokinesis during this late stage of plasma membrane ingression (II: Fig 2C, D), our studies suggest that β1 integrins are especially needed during the conversion where daughter cells start to elongate and pull each other apart.

As it was already shown, mutations in β1 integrin cytoplasmic tail two NPXY motifs (YYFF) blocks integrin internalization in GD25 cells and MEFs. Interestingly, when these cells were cultured on laminin (β1 specific), cells formed bi- and multinucleate phenotypes (II: Fig 5 C, D, 6A). To see whether these defects were associated with β1 integrin traffic defect, Rab21 was expressed in GD25β1 (YYFF) cells and phenotypes were counted again to see whether Rab21 can rescue the defects. Indeed, Rab21 was able to rescue the normal mononucleate phenotype by rescuing β1 integrin endocytosis (II: 6A, B).

Cytokinesis failure often leads to tetraploidy and subsequent aneuploidy by aberrant chromosomal rearrangements in cell divisions, and thus may facilitate tumorigenesis (Fujiwara, Bandi et al. 2005). Analysis of CGH data in different cancer cells revealed a deletion of *rab21* locus in one ovarian cancer cell line, KFr13, and in one prostate cancer sample (II: 7A, S6). These Rab21-deficient KFr13 cells showed multinucleate phenotype and later have been shown to be defective in β1 integrin endocytosis as well (II: Fig 7B, C, and not shown). This multinucleation was essentially due to a defect in Rab21 function, as re-expression of Rab21 in these cells rescued the normal mononucleate phenotype (II: Fig 7D). These results suggest that deregulated β1 integrin traffic due to non-functional Rab21 could be a driving force in tumorigenesis. Further studies should be addressed to look for possible somatic mutations in *rab21* or other *rab5*-family genes in different cancer specimens and cell lines. Also changes in Rab5/21 regulator activities, such as in GEFs and GAPs in different cell lines, could be addressed.

5.2. β1 integrin activity regulation in prostate cells

5.2.1. Novel siRNA-based cell spot microarray screen (CSMA)

The fast development of RNAi tools is providing researchers with high-throughput screening possibilities to identify genes important in diseases such as cancer. The current silencing methods rely on microtiter-plate assays, where siRNAs are transferred to cells with so-called “reverse-transfection” protocol. Basically, cells are mixed with siRNA, liposome-based transfection reagent, and medium. Due to high reagent demand, the microtiter-plate set-up often requires mixing of different siRNAs into pools, from where “hit” wells are chosen for secondary screens with individual siRNAs per well. For the

assay read-out, most published genome-scale studies have relied on either simple bulk detection reagents (Martin, Jones et al. 2007) or model cell lines expressing reporter gene constructs (Lin, David et al. 2008). Only recent ground breaking genome-scale experiments from large screening consortia have managed to push applications towards more functional assay approaches including the use of wound healing assays as readout (Simpson, Selfors et al. 2008).

Cell microarray technologies have the great potential to miniaturize assay size and provide the means to multiply experiment repeats and thus yield statistical power. In 2001, Ziauddin and Sabatini demonstrated the use of the first cell microarray, with cells growing as confluent carpet, and cDNA constructs transfected on pre-defined areas from top of the cells (Ziauddin, Sabatini. 2001). This system was later adapted for the use of synthetic siRNAs by Mousses, Caplen and others (2003). The pitfalls of this kind of assay set up are that cells are in direct contact with each other, creating the possibility of reagent or cell mixing between the neighboring transfection areas. Since these pioneering studies, cell microarray technology has not gained wider use in the scientific community, with exception of a few "proof-of-principle" studies about the feasibility of the system.

In this thesis, the final part of the work consists of a new cell microarray technology with an application of a screen for β1 integrin activity regulation. The system is based on siRNA transfection from pre-defined spots, where cells adhere and become transcriptionally silenced (see Figure 10). The spots consist of mix of Matrigel, liposome reagent, and siRNA, which all can be robotically spotted to pre-defined places on plastic plate. Cells adhere strongly to hardened Matrigel, "eat" the siRNA, and become silenced within few days. The advantage of this setup is that cells grow on separate spots and genes are silenced with no spot-to-spot mixing or reagent diffusion. The assay requires an optimized washing step after cell adherence to spots to minimize cells adhering to plastic between spots.

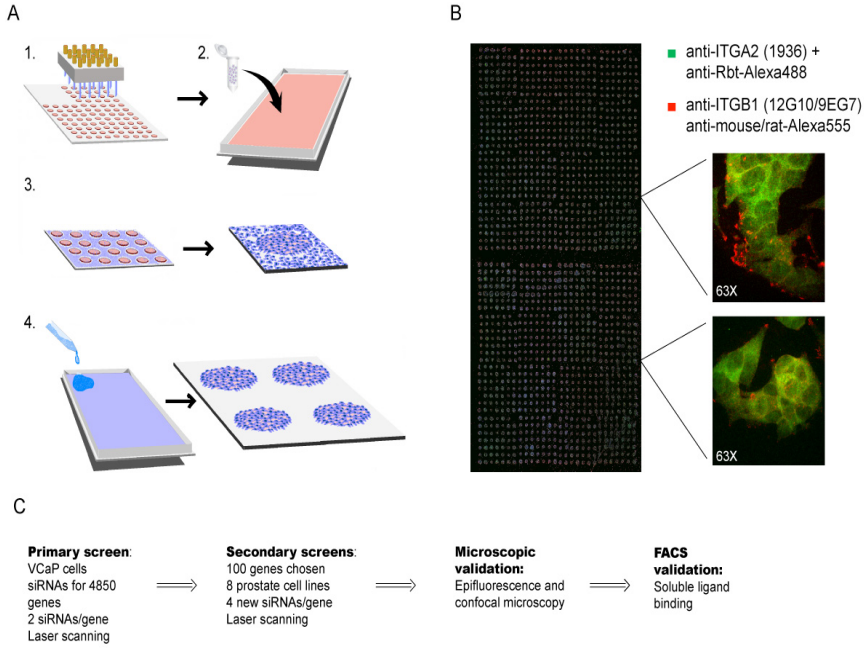

Figure 10. Cell spot microarray protocol and assay setup for active β1 integrin screening. **A**. (1) Transfection reagent and siRNA are spotted with Matrigel-containing mixture to defined areas on assay plate. One spot contains one siRNA. (2) Cells are added on top of the spots with high confluency. (3) Cells adhere more rapidly to Matrigel spots, but loosely to plastic as well. (4) Assay plate is washed with medium to wash away unattached and loosely attached cells. Cells spread and grow on spots and take in liposome-complexed siRNAs from the spots. **B**. After two days of incubation, cells are fixed, permeabilized and stained with antibodies. Staining intensities are measured with confocal laser scanner (left). On the right are shown epifluorescent microscope images of individual spots. For analyzing β1 integrin activity, active ligand-bound β1 integrin (ITGB1) with two different conformation-specific antibodies were used. Both 12G10 and 9EG7 are so called anti-CLIBS (cation- and ligand-influenced binding site) monoclonal antibodies that recognize only the ligand-bound active β1 integrin (Bazzoni, Ma et al. 1998). 12G10 and 9EG7 stainings were normalized with anti-α2 integrin staining (Ab1936). Also filamentous actin was stained with phalloidin from the same assay plates (not shown). **C**. The strategy consisted of primary screen with one cell line, secondry screen with 100 chosen genes and 8 different cell lines, microscopic validations, and soluble ligand binding assay.

5.2.2. Assay validation

To validate the method, GFP-expressing HEK293 cells were silenced on array spots containing different concentrations of siRNA against GFP. Optimized 835-μM concentration of siRNA in spots was sufficient to silence GFP in these cells during two days incubation (III: Fig 1C). The GFP knock-down efficiency as measured with laser scanner was about 75% with a p-value of $7x10^{-7}$ (n=150). The method was also validated with siRNAs against α2 and β1 integrins. Both knock-downs were analyzed with confocal microscopy directly from the immunostained spots. The results demonstrate that both integrins are nicely silenced (III: Fig 1D). As a control in these

experiments, siRNAs against PTK2 (FAK) were used. Its knock-down is known to result in accumulation of small immature focal adhesions, which was clearly visible in VCaP cell line as well.

5.2.3. Primary screen

VCaP cell line was used for studying the effects of 4850 different gene knock-downs. VCaP cell line is thought to be a good model for studying prostate cancer. It has been harvested from a vertebral metastatic lesion, and it contains many of the characteristics of clinical prostate carcinoma, including expression of PSA, PAP, and AR (Korenchuk, Lehr et al. 2001). It also bears the TMPPRSS2-ERG promoter fusion with androgen-responsive promoter driving expression of an ETS transcription factor, ERG, which has been associated with cancer cell invasion (Tomlins, Rhodes et al. 2005; Tomlins, Laxman et al. 2008).

The results from primary screens were plotted according to staining intensities of active β1 integrin (III: Fig 2A). The z-score plots demonstrate that a relatively small population of gene knock-downs showed statistically significant down- or upregulation of integrin activity (about 100 up and 100 down in both experiments). The primary screen VCaP results with 50 selected gene knock-downs resulting in β1 integrin inactivation are shown in Table 4, and selected 50 genes increasing β1 integrin actitivy in Table 5.

5.2.4. Secondary β1 integrin screen

A possible problem with siRNA screening is that one siRNA targeting to a specific gene has silencing effect against another gene (off-target effect). To overcome this problem, four new siRNAs were used for silencing again these selected 100 genes in VCaPs, but also in seven other prostate cell lines (III: Fig S1). In the secondary screens, stainings were done only with the other β1 integrin antibody, 12G10. However, experiments were done now twice with every cell line, yielding a total of 8 knock-downs per gene per cell line. In total this makes 64 knock-downs per gene. Comparison of the primary screen results with that of secondary screens demonstrated that about 85% of the knock-downs showed same direction in integrin activity regulation (III: Fig 2C). However, more differences was seen when different cell lines were compared with each other (III: Fig 2D). This was of course anticipated, as these cell lines can be very different from each other. PC3 and ALVA31 are androgen insensitive, whereas VCaP, RWPE1, MDA-PCA2B, and 22RV1 are androgen sensitive cell lines. The heat map clusters cell lines and genes according to the color pattern, implying that PC3 and ALVA31 would have much more similar responses to knock-downs compared to PC3 and VCaP for example. The replicate experiment with each cell line, however demonstrated that the assay is very repetitive and accurate, as heat-map clusters all the same cell line experiments

Table 4. Selected 50 knock-downs causing β1 integrin inactivation in primary screens.

PRIMARY SCREEN β1 INACTIVATION			Experiment 1		Experiment 2		Experiments 1+2	
GENE	NAME	siRNA ID	z-score 12G10	z-score Phalloidin	z-score 9EG7	z-score Phalloidin	Mean 12G10 + 9EG7	Mean Phalloidin
AKT3	v-akt murine thymoma viral oncogene homolog 3	Q006903	-1.209	-1.507	-2.279	-2.025	-1.744	-1.766
ALDH4A1	aldehyde dehydrogenase 4 family, member a1	Q020861	-1.630	-0.330	-2.529	-1.642	-2.079	-0.986
AP1S1	adaptor-related protein complex 1, sigma 1 subunit	Q018565	-1.880	-1.361	-2.138	-1.913	-2.009	-1.637
ATF2	activating transcription factor 2	Q018635	-2.563	-1.730	-1.565	-1.381	-2.064	-1.555
BAI2	brain-specific angiogenesis inhibitor 2	Q023838	-2.076	-1.607	-1.811	-1.508	-1.944	-1.558
BDKRB1	bradykinin receptor b1	Q023841	-1.563	-1.062	-1.691	-1.390	-1.627	-1.226
CACNA1I	calcium channel, voltage-dependent, alpha 1i subunit	Q020957	-2.497	-0.459	-1.750	-1.193	-2.123	-0.826
CAPN2	calpain 2, (m/ii) large subunit	Q018409	-2.898	-2.139	-2.283	-1.878	-2.590	-2.009
CCNE2	cyclin e2	Q021007	-2.164	-0.444	-1.581	-0.870	-1.872	-0.657
CD14	cd14 antigen	Q018471	-3.037	-1.846	-2.176	-2.027	-2.607	-1.937
CD1A	cd1a antigen	Q018453	-3.591	-1.768	-2.004	-1.790	-2.797	-1.779
CD1C	cd1c antigen, c polypeptide	Q018455	-2.615	-1.874	-2.142	-1.881	-2.378	-1.878
CD3D	cd3d antigen, delta polypeptide (tit3 complex)	Q018459	-3.336	-1.677	-2.234	-1.633	-2.785	-1.655
CD8B	cd8 antigen, beta polypeptide 1 (p37)	Q018469	-2.953	-1.914	-2.727	-1.885	-2.840	-1.899
CD81	CD81 molecule	Q018505	-2.947	-0.329	-0.238	-0.057	-0.193	-1.593
CD9	cd9 antigen (p24)	Q018470	-1.511	-1.120	-2.500	-1.321	-2.006	-1.221
COL9A1	collagen, type ix, alpha 1	Q018603	-2.114	-1.540	-1.521	-1.348	-1.818	-1.444
DHRS4	dehydrogenase/reductase (sdr family) member 4	Q021518	-0.617	-0.831	-2.217	-1.625	-1.417	-1.228
EDG5	endothelial differentiation, g-protein-coupled receptor, 5	Q006012	-2.475	-1.967	-2.065	-1.502	-2.270	-1.734
ELK1	elk1, member of ets oncogene family	Q018840	-2.221	-1.394	-1.170	-1.045	-1.696	-1.220
EXO1	exonuclease 1	Q021009	-2.664	-0.563	-1.875	-1.123	-2.269	-0.843
EZH2	enhancer of zeste homolog 2 (drosophila)	Q018894	-2.960	-1.795	-2.221	-1.329	-2.590	-1.562
FAT	fat tumor suppressor homolog 1 (drosophila)	Q018921	-2.075	-1.949	-0.867	-0.514	-1.471	-1.231
FAT2	fat tumor suppressor homolog 2 (drosophila)	Q018922	-0.334	-0.295	-2.158	-0.950	-1.246	-0.623
FKBP5	fk506 binding protein 5	Q018957	-0.441	-0.663	-2.446	-2.109	-1.444	-1.386
FZD5	frizzled homolog 5 (drosophila)	Q024048	-2.581	-1.676	-0.736	-0.562	-1.658	-1.119
GJA1	gap junction protein, alpha 1, 43kda (connexin 43)	Q019066	-2.309	-1.573	-1.864	-1.684	-2.087	-1.629
GPR143	g protein-coupled receptor 143	Q023944	-1.933	-1.380	-2.587	-2.478	-2.260	-1.929
GPX4	glutathione peroxidase 4 (phospholipid hydroperoxidase)	Q019129	-2.127	-1.098	-1.389	-1.097	-1.758	-1.098
HDAC4	histone deacetylase 4	Q021193	-1.646	-1.312	-2.040	-1.520	-1.843	-1.416
HUWE1	hect, uba and wwe domain containing 1	Q021287	-2.503	-0.329	-1.971	-0.935	-2.237	-0.632
IFNA10	interferon, alpha 10	Q019291	-2.081	-1.816	-2.060	-2.297	-2.071	-2.056
IRS2	insulin receptor substrate 2	Q020862	-1.611	-0.247	-2.538	-1.699	-2.075	-0.973
JAK2	janus kinase 2 (a protein tyrosine kinase)	Q024691	-2.943	-3.424	1.103	0.152	-0.920	-1.636
LCK	lymphocyte-specific protein tyrosine kinase	Q006620	-1.806	-2.062	-2.716	-1.950	-2.261	-2.006
NCAM1	neural cell adhesion molecule 1	Q019738	-2.191	-1.177	-0.939	-1.449	-1.565	-1.313
PAK3	p21 (cdkn1a)-activated kinase 3	Q024737	-2.545	-1.727	-1.986	-1.722	-2.266	-1.725
PFTK1	pftaire protein kinase 1	Q006680	-3.952	-4.068	-2.026	-1.606	-2.989	-2.837
PIK3R4	phosphoinositide-3-kinase, regulatory subunit 4, p150	Q007003	-2.210	-3.837	-1.250	-1.094	-1.730	-2.466
PLCL3	phospholipase c-like 3	Q021660	-2.103	-0.577	-0.422	-0.555	-1.263	-0.566
PRKAG2	protein kinase, amp-activated, gamma 2 non-catalytic subunit	Q025087	-2.403	-1.549	-1.739	-1.064	-2.071	-1.306
PRKG2	protein kinase, cgmp-dependent, type ii	Q024812	-2.110	-1.597	-2.162	-1.599	-2.136	-1.598
PTP4A3	protein tyrosine phosphatase type iva, member 3	Q024457	-2.807	-0.464	-1.549	-0.820	-2.178	-0.642
PTPN11	protein tyrosine phosphatase, non-receptor type 11	Q024402	-1.215	0.207	-5.364	-4.503	-3.290	-2.148
RICS	rho gtpase-activating protein	Q021188	-1.260	-0.961	-2.112	-1.381	-1.686	-1.171
RIPK4	receptor-interacting serine-threonine kinase 4	Q025099	-2.631	-2.005	-1.854	-1.620	-2.242	-1.812
TEC	tec protein tyrosine kinase	Q006797	-2.081	-1.500	-0.252	0.266	-1.167	-0.617
TNFSF11	tumor necrosis factor (ligand) superfamily, member 11	Q020843	-2.064	-0.355	-2.050	-1.096	-2.057	-0.726
USP6NL	usp6 n-terminal like	Q021181	0.040	-0.109	-2.017	-1.241	-0.988	-0.675
WISP3	wnt1 inducible signaling pathway protein 3	Q020933	-2.179	-0.422	-1.824	-1.178	-2.001	-0.800

next to each other (III: Fig S2). The secondary screen heat-maps also show that gene knock-downs clustering to either extreme up or down in the figure, show same direction of integrin regulation in the cell lines. Whether these “master regulators” are specific to prostate cells or are also similar in other cell types as well, will be of future study interest.

Table 5. Selected 50 knock-downs causing β1 integrin activation in primary screens.

PRIMARY SCREEN β1 ACTIVATION			Experiment 1		Experiment 2		Experiments 1+2	
GENE	NAME	siRNA ID	z-score 12G10	z-score Phalloidin	z-score 9EG7	z-score Phalloidin	Mean 12G10+9EG7	Mean Phalloidin
AKAP14	a kinase (prka) anchor protein 14	Q025214	2.982	2.313	1.805	2.681	2.497	2.394
AKT1	v-akt murine thymoma viral oncogene homolog 1	Q024551	0.013	-0.166	2.298	1.227	0.530	1.156
ATR	ataxia telangiectasia and rad3 related	Q006480	1.731	-0.491	12.631	5.722	2.616	7.181
ATRN	attractin	Q020810	0.958	-0.269	2.389	1.064	0.397	1.673
AVPR1A	arginine vasopressin receptor 1a	Q005758	2.914	-0.169	1.761	0.767	0.299	2.337
CD2	cd2 antigen (p50), sheep red blood cell receptor	Q018458	2.823	0.380	2.241	1.331	0.856	2.532
CDC25B	cell division cycle 25b	Q024334	3.900	1.634	4.113	2.256	1.945	4.007
CDK5R1	cyclin-dependent kinase 5, regulatory subunit 1 (p35)	Q006855	2.052	2.385	2.474	2.398	2.391	2.263
CDKN3	cyclin-dependent kinase inhibitor 3 (cdk2-associated dual specificity phosphatase)	Q024336	2.592	1.982	9.304	8.808	5.395	5.948
CXCL2	chemokine (c-x-c motif) ligand 2	Q019159	2.014	1.447	0.439	0.349	0.898	1.227
DEDD	death effector domain containing	Q021021	2.981	0.697	3.198	1.528	1.113	3.090
EPHA4	EPH receptor A4	Q006562	3.394	3.616	1.421	2.089	2.852	2.407
EPS15	epidermal growth factor receptor pathway substrate 15	Q018866	-0.019	-0.677	5.097	5.056	2.189	2.539
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1	Q018868	2.053	1.004	0.788	0.415	0.709	1.421
ESRRA	estrogen-related receptor alpha	Q018878	-0.669	-0.351	2.273	1.686	0.667	0.802
FBN2	fibrillin 2 (congenital contractural arachnodactyly)	Q018925	1.444	1.910	3.799	4.913	3.411	2.622
FMO3	flavin containing monooxygenase 3	Q018968	-0.155	-0.417	3.854	3.727	1.655	1.850
FUS	fusion (involved in t(12;16) in malignant liposarcoma)	Q018998	1.687	-0.162	4.515	1.893	0.866	3.101
GABBR1	gamma-aminobutyric acid (gaba) receptor, rho 1	Q005823	1.920	3.059	1.423	2.338	2.698	1.672
GAMT	guanidinoacetate n-methyltransferase	Q019031	2.078	1.912	0.961	0.582	1.247	1.520
GAST	gastrin	Q018997	0.241	0.545	2.336	2.396	1.471	1.288
GDF10	growth differentiation factor 10	Q019049	3.228	2.445	-0.207	0.023	1.234	1.511
GPR39	g protein-coupled receptor 39	Q023940	0.844	0.048	2.158	0.575	0.311	1.501
GPR88	g protein-coupled receptor 88	Q006094	0.937	1.891	3.157	4.898	3.395	2.047
GRM6	glutamate receptor, metabotropic 6	Q005874	5.238	5.082	1.446	1.510	3.296	3.342
HIP1	huntingtin interacting protein 1	Q019217	2.973	-1.631	-0.918	-0.812	-1.221	1.027
IFNA1	interferon, alpha 1	Q019284	-0.705	-0.443	3.954	3.191	1.374	1.624
IL2RB	interleukin 2 receptor, beta	Q019326	0.818	0.363	3.634	2.566	1.464	2.226
INPP1	inositol polyphosphate-1-phosphatase	Q006270	4.402	2.942	2.775	3.957	3.450	3.589
KLK4	kallikrein 4 (prostase, enamel matrix, prostate)	Q021163	-1.997	-0.327	3.144	4.744	2.208	0.574
KNTC1	kinetochore associated 1	Q021184	2.896	0.094	4.796	1.439	0.767	3.846
MAST2	microtubule associated serine/threonine kinase 2	Q025037	2.779	1.474	0.481	1.781	1.627	1.630
MASTL	microtubule associated serine/threonine kinase-like	Q007103	10.751	7.703	-0.328	-0.818	3.443	5.211
MMP8	matrix metallopeptidase 8 (neutrophil collagenase)	Q019661	2.132	0.526	2.590	2.812	1.669	2.361
MSX1	msh homeobox homolog 1 (drosophila)	Q019693	4.203	3.541	9.319	8.711	6.126	6.761
MUSK	muscle, skeletal, receptor tyrosine kinase	Q006642	-1.634	-2.410	5.295	1.476	-0.467	1.830
MYCL1	v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	Q019718	2.779	0.423	-1.881	-0.593	-0.085	0.449
NCBP2	nuclear cap binding protein subunit 2, 20kda	Q021643	1.990	8.812	2.127	2.186	5.499	2.059
NCOR1	nuclear receptor co-repressor 1	Q021158	4.569	0.973	0.809	0.766	0.869	2.689
NR5A2	nuclear receptor subfamily 5, group a, member 2	Q018990	2.574	1.578	-0.400	-0.945	0.316	1.087
OR10H1	olfactory receptor, family 10, subfamily h, member 1	Q006060	8.216	-1.782	1.938	0.513	-0.634	5.077
OR7A5	olfactory receptor, family 7, subfamily a, member 5	Q006062	2.916	-0.688	0.278	-0.137	-0.412	1.597
PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	Q024750	2.442	2.274	0.593	1.408	1.841	1.517
PTPRB	protein tyrosine phosphatase, receptor type, b	Q024409	1.148	1.928	2.114	1.474	1.701	1.631
RFPL2	ret finger protein-like 2	Q021477	2.758	0.269	2.186	1.776	1.022	2.472
SARM1	sterile alpha and tir motif containing 1	Q021682	2.165	0.530	1.136	0.823	0.676	1.651
SLC25A44	solute carrier family 25, member 44	Q021174	2.782	1.068	2.802	1.314	1.191	2.792
STX6	syntaxin 6	Q021335	2.342	0.200	4.885	2.075	1.138	3.613
USP13	ubiquitin specific peptidase 13 (isopeptidase t-3)	Q020973	0.707	0.145	2.808	3.778	1.961	1.758
YWHAG	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	Q020693	1.718	1.233	3.575	4.846	3.039	2.647

5.2.5. Hit validation with soluble ligand binding and confocal microscopy

In addition to the use of conformation-specific antibodies, integrin ligand-binding capacity and activity can be probed with soluble ligand binding (see Table 2 in Litterature review). The antibodies we have used, 12G10 and 9EG7, are anti-CLIBS antibodies, which mean that these antibodies are able to bind to β1 integrin only when a stabilizing

cation (Mg^{2+}) and a ligand are already bound to the integrin (Bazzoni, Ma et al. 1998). Thus, anti-CLIBS binding tells mostly about integrin-ligand stability in a given situation, which is largely dependent on intracellular connection to the actin cytoskeleton and its stabilizing adaptor proteins, such as talin, FAK, and paxillin, for example. Soluble ligand binding, on the contrary, could tell about three different things that are difficult to distinguish from each other. Firstly, soluble ligand binding capacity could reflect the pre-existing conformation of integrins, where rapid ligand binding would mean that cells have integrin conformation equilibrium more towards the extended or primed version. It could also mean that integrin conformational change form the inactive bent to primed extended conformation upon ligand binding is fast. Thirdly, directly after ligand binding, intracellular adaptor proteins and cytoskeleton must be reorganized in order to strengthen and sustain the ligand binding (affinity maturation). This might involve also integrin clustering, which is also largely dependent on actin cytoskeleton dynamics. To see whether different knock-downs could also affect β1 integrin ligand binding capacity, α5β1 integrin ligand binding (fibronectin-Alexa555) was studied with VCaP adherent cells in microtiter-well assay format. Labeled fibronectin was incubated with cells for 30 minutes in 37 °C, allowing also outside-in signaling to occur. When these results were compared to those of the 12G10 stainings in secondary screen array, a very high correlation was marked (III: Fig 3A). The mechanisms, by which ligand binding is higher or lower in different experiments, will be interesting to follow up in the future. Rapid cell adhesion to immobilized ligands with time-scale of seconds or even sub-seconds could give insights into the affinity regulation. This type of adhesion could be measured for example with atomic force microscopy.

People usually believe what they see. This is why numerical values from laser scannings were also made visible to eye by using confocal fluorescence microscopy. Spinning disc confocal system was used for imaging several different spots from screening plates. Confocal microscopy gives high resolution three-dimensional images, but also enables pixel intensity measurements from the images. Images taken from VCaP cell line showed remarkable changes in β1 integrin activity upon gene knock-downs (III: Fig 4). The images also demonstrated that ligand-bound active β1 integrin was often found in addition to its normal basal localization, in cell-cell contacts and sometimes clustered in strong patches (siGRM6 and MAST2). VCaP cells grow on top of each other and β1 integrin can be seen often enriched in between these cells. However, when β1 integrin activity is very low due to knock-down of certain gene, cells are unable to grow so much on top of each other. This could mean that VCaPs are producing extracellular matrix proteins themselves and adhere or cluster to each other through integrins and ECM cross-linking.

From secondary screens, three "master regulator" genes were chosen for confocal studies in different cell lines (III: Fig 5). Of these genes, COL9A1 and CD9 knock-downs

inactivated integrin, except that in ALVA31 cell line COL9A1 siRNA had a slightly activating function, as was also seen in the heat-map (III: Fig 2D). For these gene knock-downs, also invasion assays were carried out in ALVA31 cells. ALVA31 was chosen for its relatively high invasive capacity in previous assays (personal communication with Ville Härmä). The matrigel invasion assays showed that CD9 knock-down almost totally blocked ALVA31 cell invasion, whereas MASTL and COL9A1 increased it (III: Fig 5F). Further studies showed that MASTL knock-down resulted in collective growth to the matrigel rather than invasion. In addition, the high "hills" generated upon knock-down were hollow inside, suggesting some sort of lumen formation, which is characteristic to luminal prostate tissue. More studies on invasion will be required to see how β1 integrin activity regulation correlates with invasive or lumen formation capacity of prostate cells.

6. DISCUSSION

6.1. Rab21 and β1 integrin traffic

The results in the thesis suggest that Rab21 is important for β1 integrin endocytic traffic. Perturbation of this traffic has consequences on cell adhesion, migration, and cytokinesis. The exact pathway of β1 integrin internalisation remains still to be solved. Our initial studies suggest however that Rab21 drives β1 integrin endocytosis to a non-clathrin-dependent pathway, and that caveolae or caveosomes could be associated instead. The presence of GFP-Rab21 in multivesicular bodies (MVBs) and examples of previous studies (Ng, Shima et al. 1999) suggest that β1 integrins could also be targeted there. MVBs are normally linked to transmembrane protein degradation upon monoubiquitination. However, a recycling role for this compartment has been suggested at least for MHC class-II proteins and some lipid raft-associated proteins (de Gassart, Géminard et al. 2004). Figure 11 depicts the schematics of Rab21-mediated β1 integrin traffic.

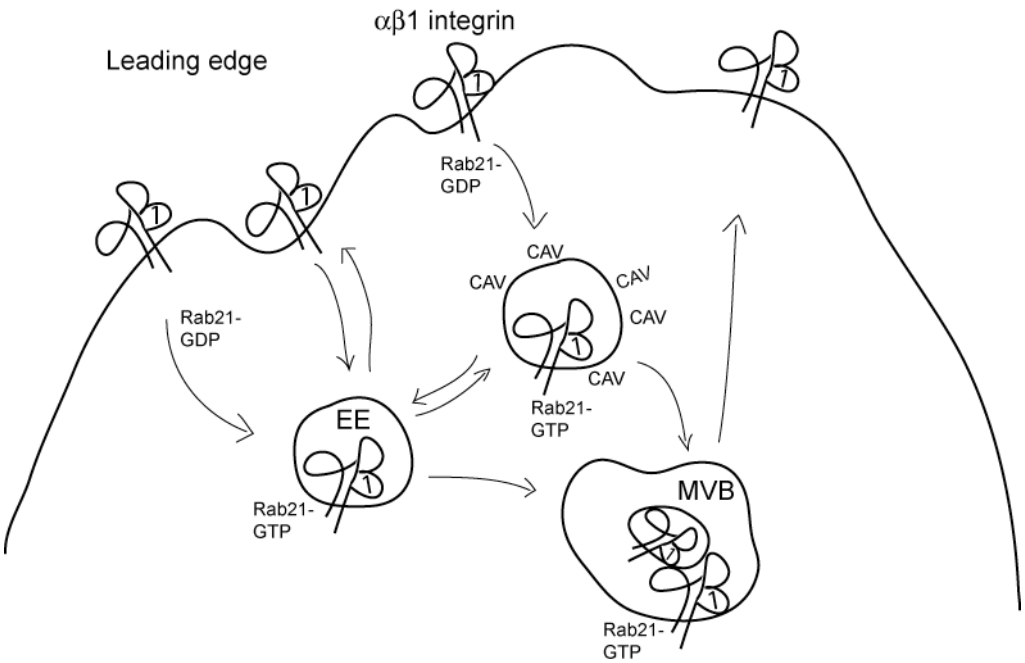

Figure 11. Rab21 controls β1 integrin traffic during cell spreading and migration. Rab21 targets β1 integrins to early endosomes and caveolin-positive vesicles. This requires exchange of GDP to GTP in Rab21. Rab21 is also present in multivesicular bodies (MVBs), where β1 integrins are also known to localize. The molecular architecture of different endocytic vesicles is constantly changing thus making it difficult to define exact locations of integrins. However, Rab21 stimulates also β1 integrin recycling back to the plasma membrane. This endo- and exocytic traffic seems to be essentially important in the leading edge of migrating or spreading cells.

Rab21 is a Rab5-family GTPase, and Rab5A function has been linked to migration and cell-motility activation. Palmidessi, Frittoli and others (2008) showed that Rab5-dependent general endocytosis is needed for Rac1 activation in early endosomes by a Rac GEF, Tiam1, upon growth factor stimulation. They also showed that Rab5 expression-induced Rac recycling between plasma membrane and endocytic vesicles enhace actin dynamics, membrane ruffling, as well as cell motility in 2D. Finally, Rab5 expression was also shown to increase β1 integrin-positive protrusive structures and cell invasion in 3D Matrigel and collagen, as well as modulate cell morphology plasticity. Rab5 and its guanine nucleotide exchange factor, Rin1, are also known to regulate receptor tyrosine kinase (RTK) internalization by a mechanism where growth factor-activated Ras binding to Rin1 activates its GEF-activity to Rab5 (Barbieri, Kong et al. 2003; Tall, Barbieri et al. 2001). In a very elegant study, Jékely, Sung and others (2005) showed how *Drosophila* Rin1/2/3 homolog, Sprint, by inducing RTK endocytosis in correct place, controls the polarization of RTK signaling to actively internalizing pool and thus is important for polarized oocyte border cell migration *in vivo*. Also an E3 ubiquitin kinase, Cbl, and its binding to intracellular tyrosine-phosphorylated RTK tail together with Sprint, was required for efficient RTK endocytosis and polarized signaling from these structures. These above studies raise an important question; whether cell polarity guidance cues, such as those of RTK activating growth factors, could induce Rab5-mediated localized RTK endocytosis and signaling together with integrin endo/exocytosis. In other words, is localized RTK activation inducing also localized integrin endocytic traffic in polarized fashion for polarized cell motility? Similarily this question could also be extended to other guidance cues, cytokines and chemokines, as their receptor internalization and subsequent resensitization can also be regulated by Rab5 family GTPases (Seachrist J and Ferguson S 2003). It is also known that angiotensin receptor, AT1AR (AGTR1), upon agonist-binding, can activate Rab5-GTP loading and receptor endocytosis involving direct binding of Rab5 to the receptor (Seachrist et al. JBC 277 2002).

Given the important well-established roles of integrin-RTK cross-talk in Ras-signaling, cell proliferation, and anchorage-independent growth, as well as GPCR signaling in integrin activity regulation, it would not be surprising to find coupling of endocytosis between these molecules as well. Indeed, α2β1 integrin endocytosis is stimulated upon EGF addition or expression of constitutively active EGFR in ovarian cancer cells (Ning, Buranda et al. 2006). Also, in cutaneous squamous cell carcinoma (SCC), inhibition of EGFR by inhibitors or siRNA-mediated knock-down of EGFR, both reduce caveolin-mediated internalization of β1 integrin, whereas activation by EGF results in increase in its endocytosis (Mukoyama, Utani et al. 2007). A study from White, Caswell and Norman (2007) suggest that αvβ3 integrin recycling from endocytic vesicles back to plasma membrane is stimulated by growth factors and requires Rab4 and phosphorylation and kinase activity of protein kinase D1 (PKD1). They also show that this fast short-loop

recycling is important for persistent cell migration in scratch wound assays, whereas the Rab11-dependent long-loop α5β1 integrin recycling supports more random migration involving ROCK – phospho-cofilin signaling. Furthermore, co-regulated recycling of EGFR and α5β1 integrin from endocytic compartments to plasma membrane plays an important role in A2780 ovarian cancer cell invasion to fibronectin-containing Matrigel (Caswell, Chan et al. 2008). Here, a Rab11 effector protein, FIP1 (Rab-coupling protein, RCP) was found to be important in bridging α5β1 integrin and EGFR1 association for polarized recycling into protrusive F-actin structures in invasive cells. Interestingly, stimulation of α5β1 and EGFR1 recycling by inhibition of αvβ3 integrin function also increased the autophosphorylation of EGFR1 by EGF. These studies do suggest an important mechanistic link between integrin endocytic traffic and RTK phosphorylation and signaling.

Do these integrin traffic regulators have any clinical significance in cancer? The Rab coupling protein (RCP) is found in an amplicon (8p11-12) in breast cancer, and a Rab11-family member, Rab25 (Rab11C), is associated in metastatic breast and ovarian cancer and amplified in about half of these cancers (Cheng, Lahad et al. 2004; Wang, Goswami et al. 2004). Figure 12 shows Affymetrix analysis of Rab25 expression in 64 different cell lines and validates that Rab25 can be overexpressed in breast and ovarian cancer, but also in prostate cancer. Furthermore, hypoxic conditions, which are characteristic of many solid tumours, stimulate Rab11-dependent α6β4 integrin recycling and invasive migration by mechanisms that involves AKT-GSK3β driven stabilization of microtubules (Yoon, Shin et al. 2005). Also Rab5A can be overexpressed in metastatic human lung adenocarcinoma, whereas we have found deletion of *rab21*-locus in ovarian cancer cell line and in clinical prostate cancer specimen (Yu, Hui-Chen et al. 1999; II: 7A, S6), suggesting that enhanced integrin traffic could support metastasis, whereas its deregulation could promote carcinogenesis by other means, such as by cell division defect, which is discussed next.

In addition to focusing on integrin traffic in cell migration, this thesis work also addressed the question of whether integrin traffic plays a role in cell division as well. The conclusions were following: 1) β1 Integrin endocytic traffic regulated by Rab21 association with integrin alpha cytoplasmic tail, is important for adherent cell cytokinesis. 2) β1 integrin traffic is enriched in the furrow region during cytokinesis, which probably promotes proper actin constriction during ingression by mechanism that involves integrin-mediated adhesion in midzone region. 3) Perturbation of β1 integrin traffic also impairs later step integrin vesicle movement away from the furrow towards opposing poles, resulting in nascent daughter cell elongation defect. 4) Finally, these defects lead to regression of daughter cell separation and formation of bi- or multinucleated cell of nascent daughter cells or cell detachment and apoptosis. Figure 13 shows schematics of β1 integrin traffic during cytokinesis. The results of this work imply that deregulation of normal β1 integrin

Figure 12. Affymetrix expression analysis of Rab25, RCP, and Rab21 in 64 different cell lines. High expression of Rab25 can be seen in breast (red) and ovarian cancer cells (orange), but also in some prostatic cells as well (green). RCP seems to be well expressed in almost all the cell lines except in glioma cell lines. Lower Rab21 expression can be readily seen in KFr13 ovarian cell lines (bold), which have a heterozygous deletion in the *rab21-locus*, whereas breast cancer cell line, ZR751 has a heterozygous amplification of *rab21* (not shown).

traffic might have consequences on carcinogenesis as well. Especially, the formation of multiploidy and subsequent aneuploidy as a result of erraneus cytokinesis could be a driving force for genome instability, a hallmark of cancer (Ganem, Storchova et al. 2007).

Figure 13. Model for β1 integrin traffic in cytokinesis. **A**. Normal cytokinesis. β1 integrins localize to plasma membrane and endocytic vesicles (green). During furrow ingression (5 min), β1 integrins move in dynamic fashion at the equatorial midzone concentrating more to the basal site of the cell. As cytokinesis proceeds, actin-myosin-based constriction bundles spindle microtubules allowing the midbody structure to form (black). Now, β1 integrins start to move and polarize more to the direction of doughter cell elongation, again at the basal site of the cells. As the endocytic traffic of integrins is needed for the formation of new adhesion sites during cell spreading or migration, it is conceivable that inhibition of the traffic would impair this phase of cytokinesis. **B**. Inhibition of β1 integrin traffic impairs cytokinesis. Knock-down of Rab21, inhibits the endocytic motility of β1 integrins at the cleavage furrow, but also decreases the amount of endocytozed integrin vesicles (5 min). During progression of cytokinesis, doughter cells are unable to elongate and separate from each other, leading to furrow regression and formation of binucleated cell. Many cells also detach from the substratum during this stage and undergo apoptosis.

What are the mechanisms by which perturbation of Rab21 lead to cytokinesis defect? As Rab21 knock-down impairs also the proper formation of midbody structure, it could have an additional role during the acto-myosin constriction. We have not seen defects in spindle microtubule assembly and its molecular constitutents, suggesting that something goes wrong with the actin machinery. Indeed, we see inhibition of Rho activity during

telophase upon Rab21 silencing (II: Fig 3D). This must however be partial, as furrowing should not initiate at all in the absence of Rho activity. The mechanism of how Rab21 silencing stops telophase before final midbody formation is still difficult to decipher. It is also possible that the defects resulting from Rab21 silencing during the furrow ingression are independent of β1 integrin function. The same family GTPase, Rab5 (Rab5 family), is known to activate Rac GTPase in early endosomes, which is important for actin dynamics (Palamidessi, Frittoli et al. 2008). Regulated actin dynamics is essential for furrow constriction during cytokinesis as well. To answer these questions, we should use the α2 or α5-integrin construct with a GFFKR-mutation, where Rab21 is unable to associate, and then follow integrin traffic in these cells.

The results demonstrate that Rab21-mediated β1 integrin traffic is necessary for successful cytokinesis, at least in adherent cells. This raises a question, whether abrogation or deregulation of normal integrin traffic could result in tetraploidy, subsequent aneuploidy, and carcinogenesis. Accordingly, CGH-analysis of gene copy number revealed *rab21*-deletions in ovarian and prostate cancer specimens suggesting that this could hold true. Further unpublished studies in our lab has shown that perturbation of β1 integrin traffic in MEFs with repeated selection for survivors, created aneuploidy and highly invasive transformed phenotypes with major changes in gene expression profiles. Similar observations have been done recently by Rancati, Pavelka and others (2008), where they created adaptive evolution with yeast by perturbing cytokinesis and selecting for survivors. Here they showed that tetraploidy preceded aneuploidy and the survivors had specific gene expression changes. They also suggested that cancer drugs inhibiting cell division/cytokinesis could lead to undesired selection for aneuploid phenotypes with drug resistance. Thus, inhibition of cell division machinery as cancer targeting therapy could promote rather than suppress cancer progression in certain cases.

6.2. β1 integrin activity regulation in prostate cancer

6.2.1. Conserved hits in VCaP prostate cancer cell line

Important integrin regulators in platelets and leukocytes showed up as hits in VCaP screen. Due to the screening setup, which is 2 days silencing during sustained matrigel adhesion; the hits probably reflect more outside-in signaling of β1 integrins. However, as it can be seen from the literature review, integrin inside-out and outside-in regulators overlap significantly. The best characterized integrin regulators, Src family kinases and spleen tyrosine kinase Syk, do appear as strong hits in the screen (see table 6). Syk can be activated by Src family kinases, but also by immunoreceptor tyrosine activation motifs (ITAMs), which are present for example in CD3 subunits of TCR. As the T cell receptor co-stimulatory CD8B and the TCR subunits CD3D, CD3Z score high in the

list, it is tempting to speculate that ITAM-based signaling would also be stimulatory for prostate cancer integrins. Table 6 shows the results of genes that are well characterized regulators for platelet and leukocyte integrins, and Figure 14 depicts the platform for β1 integrin activity regulation in VCaPs.

Table 6. Well characterized integrin regulators show up in VCaPs. Shown are the z-scores of antibody intensities with two siRNAs per target. Each value represents the median of two siRNA knock-downs per target. Hck, Fyn, and Lck are Src family kinases. Genes with bold character were chosen for secondary screens and were validated in VCaP cell line and were tested in other prostate cell lines as well.

GENE	NAME	12G10	9EG7	Phalloidin 1	Phalloidin 2
CD8B	CD8b molecule	-2.436	-2.347	-2.173	-1.788
CD3D	CD3d molecule, delta	-1.871	-0.930	-1.366	-0.668
CD3Z	CD3z molecule, zeta	-0.456	-0.853	-0.770	-0.579
HCK	hemopoietic cell kinase	-1.031	-1.333	-1.100	-1.175
FYN	FYN oncogene related to SRC	-1.125	-1.045	-0.905	-1.077
LCK	lymphocyte-specific protein tyrosine kinase	-1.264	-1.094	-1.383	-0.901
SYK	spleen tyrosine kinase	-1.275	-0.639	-1.206	-1.218
TEC	tec protein tyrosine kinase	-1.916	-1.360	-1.633	-1.038
NCK1	NCK adaptor protein 1	-1.687	-0.690	-0.999	-0.749
PIK3CG	PI3K, catalytic, gamma, p110g	-2.007	-1.023	-2.789	-0.738
PIK3R4	PI3K, regulatory, p150	-1.934	-1.818	-1.266	-1.241
AKT3	v-akt murine thymoma viral oncogene 3	-1.267	-1.740	-1.398	-1.628
ABL1	c-abl oncogene 1, receptor tyrosine kinase	-4.299	0.348	-4.926	-1.178
PLCG1	phospholipase c, gamma 1	-1.030	-0.704	-0.541	-0.653
PAK3	p21 protein (Cdc42/Rac)-activated kinase 3	-1.665	-1.148	-1.243	-0.688

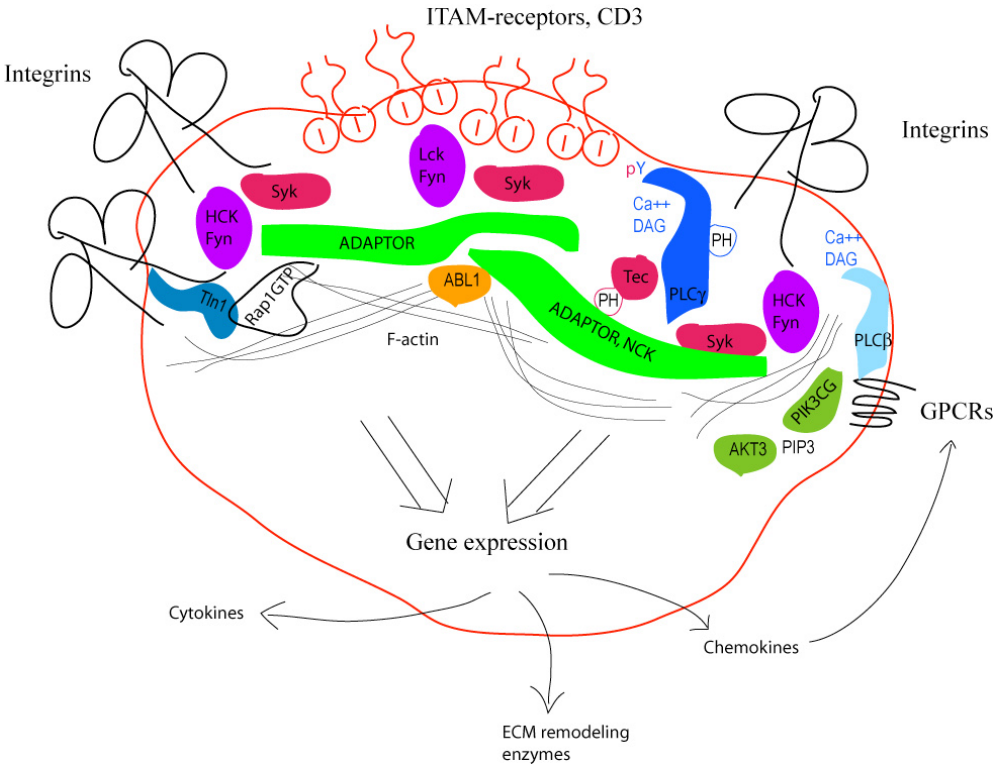

Figure 14. The platform for positive integrin activity regulation in VCaP prostate cancer cell line. Src family kinases Hck, Fyn, Lck phosphorylate Syk and ITAM-receptors, as well as proximal integrin adaptors. Downstream of Syk are PLC-gamma and TEC-kinase, the former important in calcium and DAG release, and the latter in NCK phosphorylation. Adaptors, such as NCK, enable the recruitment of various effector proteins (ABL1) important in integrin activity regulation and in actin cytoskeleton modulation (Bubeck Wardenburg et al., 1998). In addition to generation of calcium and DAG via PLC-beta, GPCRs signal to integrins through PI3-kinases, and especially through the isoform p110-gamma (PIK3CG). Calcium and DAG activate PKCs, which are important for Rap1/2 GTPase activation. Rap1/2 is needed for activation of integrins by recruiting the final integrin activator, Talin (Tln). Probably due to redundancy, the screen did not show up PKCs, talin, or Rap GTPases in the screen. Talin has two isoforms (TLN1 and TLN2) and it's known that integrin inactivation in certain cell lines requires the depletion of both isoforms (Zhang, Jiang et al. 2008). Integrin signaling is known to stimulate MAP-kinase activation and result in gene transcription modulation. The model suggested here also relates to platelet and leukocyte function, as upon activation, both are known to secrete various chemokines and small molecules to attract other platelets and leukocytes. In platelets, the secretion of alpha- and dense granules, which is mostly calcium-, PKC-, or MAPK-dependent, contributes to the so called "second wave integrin activation", where GPCRs mediate stimulatory signals to integrins to sustain adhesion, aggregation, and thrombus formation. I suggest that a similar mechanism could function in VCaPs as well.

6.2.2. GPCR signaling to β1 integrins in prostate cancer

Many gene hits in the screen are involved in GPCR signaling (Table 7). The signaling pathways initiating from GPCRs activate integrins and has been studied intensively in

leykocytes and platelets. GPCR functions in leukocytes to attract cells, arrest them by integrin activation, and promote chemotactic migration and extravasation to tissue. In platelets, GPCRs are activated in the second wave of integrin activation by thrombin, ADP, thomboxane, and different hormones to sustain adhesion and aggregation. The same activation pathways could be seen in cancer to promote invasive chemotactic migration from primary tissue towards endothelium, arrest cancer cells to endothelium in a distant site, transmigrate through endothelium and basement membrane to peripheral tissue, and finally induce aggregation and anchorage-independent growth. In one patient, different subpopulations of transformed cells could mediate different actions during metastasis progression and this certainly also reflects to the nature of different cancer cell lines in our study. For example, VCaPs are non-motile and are more likely to function as platelets, whereas PC-3 cells migrate and invade a bit more like lymphocytes do. The types of GPCRs and their ligands expressed in cells could have a role in this. Figure 15 shows some GPCR hits in VCaPs and possible downstream pathways for integrin activation.

Table 7. Examples of GPCRs and ligands from VCaP primary integrin screen. Shown are the z-scores of antibody intensities with two siRNAs per target. Each value represents the median of the two siRNA knock-downs. Grey-colored genes are ones that have already been published to affect integrin activity (Tables 2 and 3), and genes with bold-character were validated in our secondary screens. Note that CCL19 and CCL21 are ligands for CCR10 and CXCL12 is a ligand for CXCR4. The cholinergic nicotinic receptors form heterocomplexes with each other, which could mean that knocking-down any one gene of the complex, would give the effect.

GENE	NAME	12G10	9EG7	Phalloidin 1	Phalloidin 2
ADORA1	adenosine A1 receptor	-1.079	-1.213	-1.030	-1.195
BAI2	brain-specific angiogenesis inhibitor 2	-1.413	-1.476	-1.381	-1.164
BDKRB1	bradykinin receptor B1	-1.563	-1.691	-1.062	-1.390
CCL19	chemokine ligand 19	-0.606	-1.501	-0.440	-1.749
CCL21	chemokine ligand 21	-0.046	-1.658	0.121	-1.868
CCR10	chemokine receptor 10	-1.486	-0.391	-1.110	-0.366
CHRM3	cholinergic receptor, muscarinic 3	-0.817	-1.371	-1.165	-1.460
CHRNA2	cholinergic receptor, nicotinic, alpha 2	-1.191	-1.198	-1.152	-1.064
CHRNB2	cholinergic receptor, nicotinic, beta 2	-0.877	-1.107	-1.260	-1.314
CHRNB4	cholinergic receptor, nicotinic, beta 4	-1.045	-0.933	-1.271	-0.999
CXCL12	chemokine ligand 12 (SDF-1a)	-0.716	-0.933	-0.930	-1.606
CXCR4	chemokine receptor 4	-1.533	-0.938	-1.547	-1.038
DRD1	dopamine receptor D1	-1.568	-1.083	-0.826	-1.188
DRD3	dopamine receptor D3	-1.335	-0.943	-1.251	-1.003
EDG5	sphingosine-1-phosphate receptor 2	-1.883	-0.581	-1.297	-0.873
EDG7	lysophosphatidic acid receptor 3	-1.158	-1.531	-1.132	-1.365
EDNRA	endothelin receptor type A	-1.923	-0.739	-1.588	-1.163
EDNRB	endothelin receptor type B	-1.524	-0.885	-1.051	-1.306
FPR1	formyl peptide receptor 1	-0.856	-1.614	-0.762	-2.371
FZD5	frizzled homolog 5	-2.031	-0.541	-1.368	-0.385
GPR109B	G protein-coupled receptor 109B	-1.123	-1.522	-1.393	-1.919
GPR143	G protein-coupled receptor 143	-1.266	-1.414	-1.169	-1.044
GPR75	G protein-coupled receptor 75	-1.863	-1.689	-1.414	-1.547

GPR83	G protein-coupled receptor 83	-1.635	-1.009	-0.901	-0.651
GRIA2	glutamate receptor, ionotropic, AMPA 2	-1.576	-0.765	-1.393	-0.590
HRH2	histamine receptor H2	-0.699	-1.533	-0.790	-1.404
LTB4R	leukotriene B4 receptor	-1.013	-1.527	-0.517	-1.464
OPN3	opsin 3	-1.665	-2.290	-1.525	-1.781
OR1A2	olfactory receptor, 1A2	-1.343	-1.295	-1.646	-1.198
OR2M4	olfactory receptor, 2M4	-0.929	-1.418	-0.666	-0.878
OR2W1	olfactory receptor, 2W1	-1.188	-1.968	-0.574	-1.845
OR7C2	olfactory receptor, 7C2	-2.034	-0.441	-1.482	-0.927
OR8B8	olfactory receptor, 8B8	-1.563	-0.885	-1.430	-1.169
OR8G2	olfactory receptor, 8G2	-1.792	-0.615	-1.109	-0.586
P2RY14	purinergic receptor P2Y, 14	-1.078	-0.769	-1.439	-0.870
P2RY2	purinergic receptor P2Y, G-protein coupled, 2	-1.195	-0.360	-0.762	-0.428
P2RY5	purinergic receptor P2Y, G-protein coupled, 5	-1.177	-1.456	-1.140	-1.519
TACR2	tachykinin receptor 2	-1.770	-0.962	-0.920	-0.827
TACR3	tachykinin receptor 3	-1.652	-0.720	-1.370	-0.654
TSHR	thyroid-stimulating hormone receptor	-1.512	-1.576	-1.299	-1.509

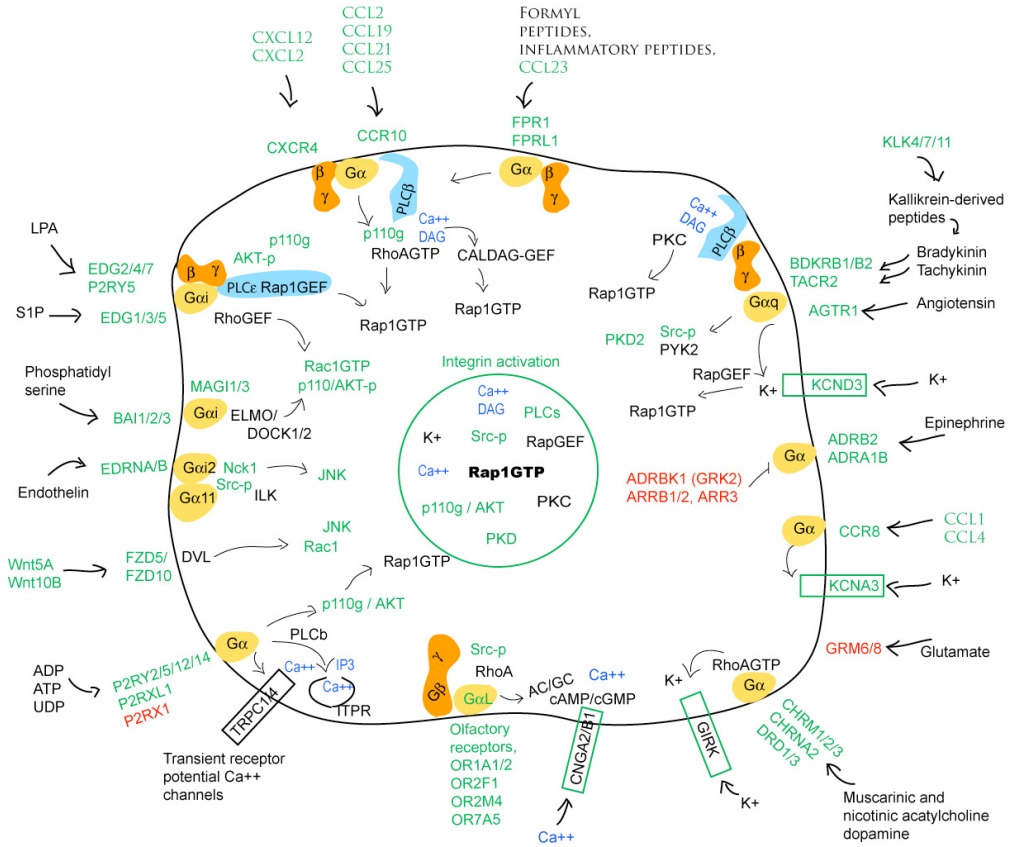

Figure 15. GPCR signaling to β1 integrins in VCaPs. The genes with green color are positive regulators of β1 integrin activity, as their knock-downs caused inactivation of the integrin in the screens. Red colored genes showed opposite effect in the screens. Chemokines, hormones, neuropeptides, lipids, and different small molecules bind to GPCRs and activate downstream signaling via G-proteins, Gα and Gβ/γ. The pathways downstream involve PLCβ, PI3-kinase subunits p110β/γ, Rho GTPases, MAP-kinase JNK, and Rap1/2 GTPases.

The classical leukocyte integrin activation is initiated by different chemokines, such as CXCL12 (SDF-1a), CCL19 (SLC), and formyl peptides. Knock-down of chemokines or their cognate receptors decreases β1 activity in VCaPs as well. Knock-down of the critical GPCR effectors, the PI3K subtype p110γ (PIK3CG), also results in inactivation of β1 integrin (zmed= -1.51). The other main pathway is the Galphaq-coupled PLCβ activation that is needed for calcium and DAG second messenger production and subsequent activation of Rap1/2. PLCβ did not appear in the VCaP hit list, but its homologues PLCD1 (zmed= -1.11) and PLCL3 (12G10 med= -0.93) did. Interesting PLC isoform is PLCε, which binds to Gβγ, is activated by Rap1/2 and RhoA, but also works as a Rap1 GEF to activate Rap1 upon stimulation with lysophosphatic acid (LPA), sphingosine-1-phosphate (S1P) or thrombin (Citro, Malik et al. 2007). LPA may also stimulate a RhoGEF, FLJ00018 to activate Rac1 GTPase (Ueda, Nagae et al. 2008). In PC3 prostate cancer cell line, LPA stimulates matrigel invasion through activation of RhoA and NF-kappaB activity (Hwang, Hodge et al. 2006). Another known GPCR to activate Rac is the phosphatidyl serine receptor BAI1 (Brain-specific angiogenesis inhibitor-1), which acts at least through ELMO/DOCK Rac-GEF-complex (Park, Tosello-Trampont et al. 2007). Endothelin receptor A (EDNRA) induces JNK activation and cell migration through the adaptor protein NCK1, but is also mediating invasive phenotype via integrin-linked kinase (ILK), which could link endothelin recptors directly to integrin signaling (Miyamoto, Yamauchi et al. 2004; Rosano, Spinella et al. 2006). The purinergic recptors P2RYs that bind to adenosine or uridinine nucleotides, activate the classical PI3K (p110γ) and PLC pathways, but also increase calcium influx by activating purinergic calcium channels, such as P2RXL1 (zmed= -1.03), or transient-receptor potential calcium channels (TRPs) (Tolhurst, Vial et al. 2005).

It is very surprising to find such many olfactory receptors (ORs) in the integrin activity screen. ORs couple to GalphaL, which is an olfactory bulb-specific G-protein. Its knock-down also shows some response in VCaPs (zmed= -0.54). Interestingly, Regnauld and coworkers have shown the expression of olfactory specific GalphaL (Gaolf) in epithelial cancer cell lines, including a prostate cancer cell line LnCaP (Regnauld, Nguyen et al. 2002). They showed that GalphaL was important in cellular invasion in transformed cells, but it also induced neuroendocrine differentiation of LnCaPs. ORs activate adenylate/guanylate cyclase-dependent (AC/GC) cyclic-nucleotide-gated channels (CNGs), of which the isoform CNGA2 (zmed= -0.68) is olfactory-specific (Kaupp, Seifert 2002). CNGs form heterotetrameric complexes, such that CNGA2 associates with CNGB1 (zmed= -1.10). Given the large repertoire of olfactory receptors in the screen hits and also their downstream players, olfactory receptors, whatever their ligands might be, could be interesting drug targets in prostate cancer. OR inhibition might cause severe defects in wine tasting sensibility, though.

Potassium channels have been recently identified as novel regulators of cell adhesion and migration. For example, α9β1 integrin can activate potassium inward rectifier channel Kir4.2/KCNJ15 (zmed= -1.39), whose knock-down decreases cell migration persistence and causes irregular lamellae formation (deHart, Jin et al. 2008). Another family channel, the delayed rectifier Kv2.1/KCNB1 potassium channel, interacts with FAK and enhances its phosphorylation at Y397 or Y576/577 upon fibronectin-integrin ligation and knock-down of this channel leads to impaired directional cell migration (Wei, Wei et al. 2008). Many GPCRs form complexes with G protein-gated inwardly rectifying K+ (GIRK) channels (Kir3s) and either activate or inhibit their function (Lei, Jones et al. 2003). The dopamine and acetylcholine receptors function in adhesion and migration modulation could be a result of GIRK channel regulation (Levite, Chowers et al. 2001; Varker, Williams 2002). Levite and coworkers have shown that exogenous addition of potassium to T-cells activates β1 integrin, even in the absence of chemokines (Levite, Cahalon et al. 2000). They also showed that the activation of β1 integrin with exogenous potassium or CCL4 (MIP-1b) can be blocked with inhibitors of Kv.3.1 channel/KCNA3 (zmed= -0.73), suggesting that influx of potassium is important for β1 integrin activation in T-cells. Potapova and others have recently shown that stimulation of angiotensin receptor I (AGTR1), by binding to Rap1GEF, leads to activation of Rap1, and that this is greatly boosted by co-expression of Kv.3.4 potassium channel KCND3 (zmed= -1.42), which also associates with AGTR1 (Potapova, Cohen et al. 2007). Accordingly, AGTR1 stimulation leads to inside-out activation of β1 integrin in cardiac fibroblasts (Stawowy, Margeta et al. 2005). Given the above facts, it is not surprising to see potassium channels as positive regulators of β1 integrin activity in VCaPs (KCND3, KCNJ15, KCNA3), but interestingly also a few as negative regulators (KCNQ4, KCNC3).

Finally, the importance of GPCR signaling in β1 integrin activation can be validated by looking at negative regulators of GPCR signaling. Galpha GAP (ADRBK1/GRK2 zmed= +2.19) and arrestins (ARRB1/2 (+0.53) and ARR3 (+1.95)) knock-downs increase β1 integrin activation in the screens. Very high β1 activation can be seen also by knocking-down type III glutamate receptors GRM6/8 (+2.59/+0.93), whose functions are not well known.

6.2.3. T cell receptor signaling to β1 integrin in VCaPs

The contribution of CD8 and CD3 receptors in integrin activation in VCaPs suggests that T cell receptor (TCR) signaling could intensify β1 integrin ligand binding and possibly integrin signaling. Indeed, T cell receptor gamma-subunit is expressed in epithelia of prostate cancer as alternatively spliced isoform (TARP) (Essand, Vasmatzis et al. 1999; Wolfgang, Essand et al. 2000). Many TCR downstream players implicated in leukocyte integrin activation (LFA-1 and VLA-4 (α4β1) also show up as prominent hits in VCaPs (see Figure 16).

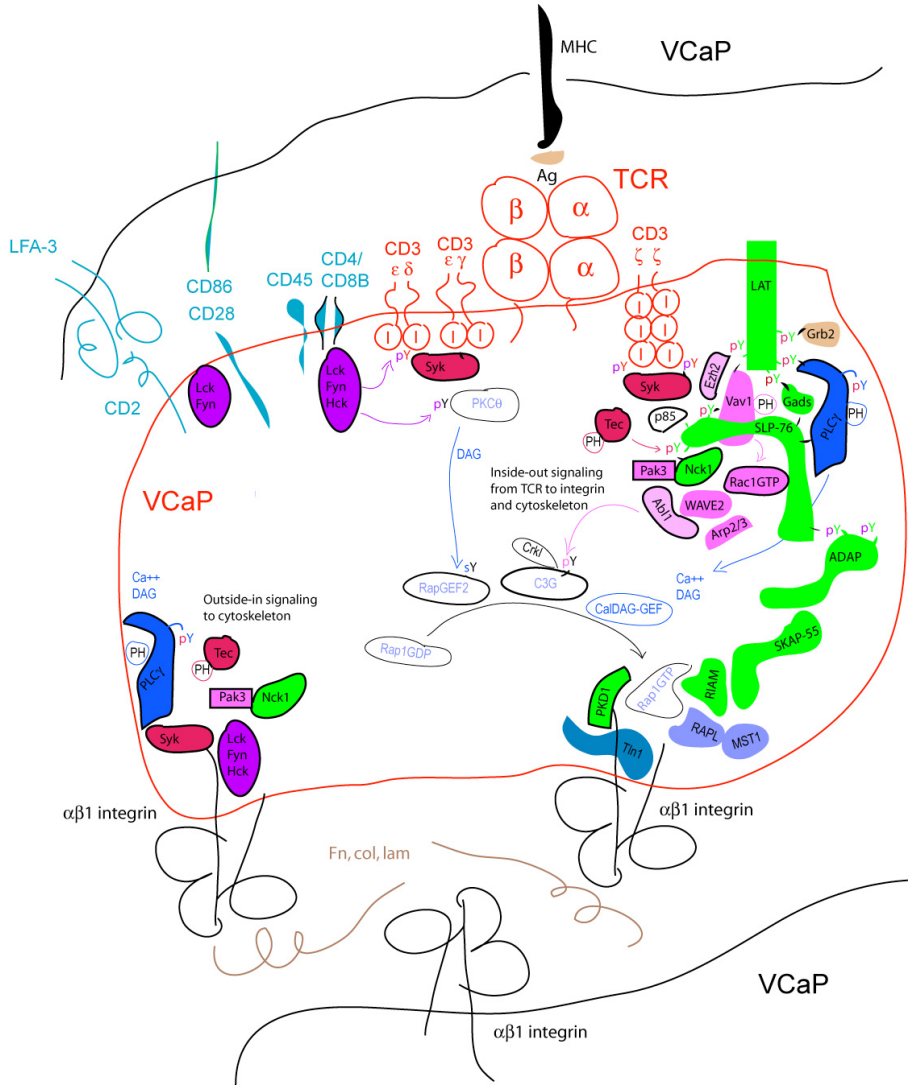

Figure 16. TCR signaling to β1 integrins in VCaPs. Hits found in the screens are marked with black borders around the genes. Src family kinases (Lck, Fyn, Hck) are recruited to TCRs by co-stimulatory proteins, such as CD4/8 and CD28. Src family kinases activate Syk by phosphorylation, but also by phosphorylating tyrosines in ITAM-motifs, where Syk binds and is activated. Lck also phosphorylates PKCtheta, which is known to activate Rap1 via RapGEF2. CD3 cross-linking or antigen stimulation recruits SLP76 and NCK adaptor proteins to TCR. NCK contributes to actin remodeling and polymerization during synapse formation by recruiting WASP protein (Barda-Saad, Braiman et al. 2005), but can also activate WAVE1 (Eden, Rohatgi et al. 2002). WAVE1 is important for reqruiting ABL1 kinase, which can activate a Rap1-GEF, C3G (Nolz, Nacusi et al. 2008). PAK (PAK1 in T cells) binding to NCK increases PAK kinase activity, cellular motility, and focal adhesion formation in endothelial cells (Bokoch, Wang et al. 1996; Master, Jones et al. 2001; Stoletov, Ratcliffe et al. 2001). A T cell TEC-kinase, Itk, contributes to VAV1 mobilization and activation in T cells, but TEC-family kinases are also required for calcium mobilization via PLCγ upon antigen stimulation (Finkelstein, Shimizu et al. 2005; Takesono, Finkelstein et al. 2002). Finally, the very proximal integrin activators consist of Rap1GTP, its effectors RIAM and RAPL, protein kinase D (PKD1), and talin (Tln1). Src family kinases and SYK are also important for integrin outside-in signaling.

One interesting feature of VCaP cell line is that is forms aggregates and grows on top of each other. Active β1 integrin staining demonstrates that there is a large population of active, ligand-bound β1 integrin in cell-cell contacts, sometimes as strong patches. I suggest that this integrin-mediated cell aggregation could be similar to platelet or leukocyte aggregation. It is also tempting to think that VCaP cancer cells could form "immunological synapses" with each other, displaying antigens as "antigen-presenting cells" and recognize them as "T cells". CD1A is a lipid-antigen-presenting molecule on T cell surface, but was found to be a major hit in the VCaP integrin screen and was further validated to be a positive regulator of β1 integrins in all 8 tested prostate cell lines. It would be also interesting to find out whether prostate cells express MHC class molecules, and whether they have a contribution to integrin activity. The above-mentioned ideas rise up a question, whether prostate cancer cells could have adopted a mechanism for immune escape by forming "immunological synapses" by themselves and thus preventing efficient immune cell encounter. Accordingly, from the screening results we should try to find proteins, which are important for cancer cell integrin activation, but unnecessary or even inhibitory for immune cells. Targeting these proteins in cancer would suppress cancer growth/metastasis, and at the same time stimulate immune cells to kill cancer cells.

6.2.4. Outside-in β1 integrin signaling in VCaPs

Outside-in integrin signaling stabilizes adhesions by anchoring integrins to the actin cytoskeleton. Thus, proteins implicated in actin polymerization and turnover would be expected to have consequences to integrin ligand-binding ability as well. It is also difficult to know whether a knock-down that results in integrin inactivation is due to direct effect on integrin conformation or due to indirect effect by cytoskeleton disruption. The cross-talk between integrins and the actin cytoskeleton is probably also very different during static adhesion, cell spreading, or during cell migration. For example in the leading edge of a cell, a constant retrograde flow of actomyosin is required for efficient polarization and migration (Tan, Yong et al. 2008), whereas further back, the cell body has to form more stabile adhesions, such as focal or fibrillar adhesions, for cell to be able to pull the trailing tail of the cell. Figure 17 shows a model for β1 integrin outside-in signaling to cytoskeleton and also to possible gene transcriptional regulation in VCaP cell line.

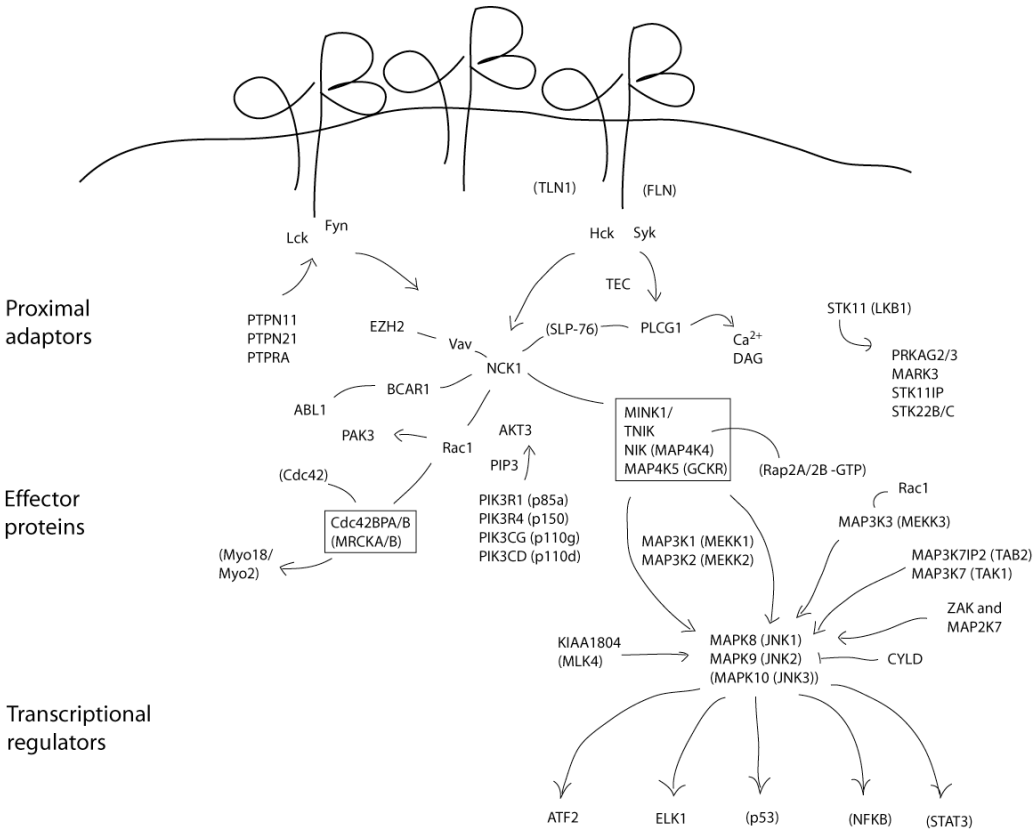

Figure 17. Possible pathways for β1 integrin outside-in signaling in VCaPs. In the figure, all genes except ones with brackets were found as positive regulators of β1 integrin in the screens. The additional genes with brackets are added to help in understanding the pathways. Integrin signaling to cytoskeleton is critical for adhesion maturation and strengthening. Integrin beta1-tail-interacting proteins talin (TLN1) and filamin (FLN) associate with the actin cytoskeleton. Src family kinases (Lck, Fyn, Hck) together with Syk also bind integrins and phosphorylate cytoskeleton-linked adaptor proteins, such as NCK1 and SLP-76. Src kinase activation involves dephosphorylation of the inhibitory loop tyrosine-530 by phosphatases. Adaptors recruit downstream effector proteins that can be kinases (PAK3, AKT3, ABL1, TEC, TNIK), GTPases (Rac1, Cdc42, Rap1/2), or lipid modifying enzymes (PLCG1) all important in cytoskeletal dynamics and/or cell polarization. These effectors also modulate focal adhesion proteins in strengthening or weakening the link to cytoskeleton (assembly/dissassembly). Additionally, the effector proteins signal through MAP-kinases to activate transcription of several proteins, such as cytokines, chemokines, and ECM-proteins important in adhesion regulation. Interestingly, many effector proteins signal to JNK MAP-kinase. The boxed proteins contain a CN-domain (Citron homology domain), and these proteins have been shown to function as effectors for Rac1/Cdc42 and Rap2 GTPases, but also to bind directly β1A integrin cytoplasmic tail (NIK). The CN-domain bearing proteins belong to STE20-familiy of serine-threonine kinases, whose member STK4 (MST1) shows effector functions for Rap1-RAPL in integrin clustering and polarization upon chemokine or TCR ligation. An interesting STE-kinase could be also STK11 (LKB1), which binds directly to Cdc42 and PAK and is important for motile cell leading edge polarization (Zhang, Schafer-Hales et al. 2008). Also its downstream substrates show up in the screen. Another STE-kinase with CH-domain (MRCKA) was recently shown to regulate actomyosin retrograde flow in the leading edge of migrating cells (Tan, Yong et al. 2008).

7. SUMMARY AND CONCLUSIONS

Since the finding of first integrins as fibronectin receptors during 80's, the integrin field has taken a huge step forward. 18 different alpha-subunits and 8 beta subunits are known to form at least 24 different heterodimers, which can bind to multitude of ligands starting from extracellular matrix collagen to soluble blood fibrinogen. The very recent findings in integrin field have revealed mechanisms of conformational activation and elegant intracellular trafficking pathways in functional regulation of integrins. This work was also implemented to study regulatory mechanisms of integrins, and especially the β1 integrins. Firstly, the work illustrates how a cytoplasmic small GTPase, Rab21, associates with β1 integrins to regulate its intracellular trafficking to different locations in cells. This interaction was found to be important for cell adhesion to extracellular matrix protein collagen and migration on this as well. Furthermore, the studies demonstrated a new link between β1 integrin endocytosis and cell division. Perturbation of β1 integrin traffic by Rab21 silencing or mutations in β1 integrin cytoplasmic tail abrogated normal cytokinesis. The results demonstrate that deregulated β1 integrin endocytic traffic could be associated with tumorigenesis and cell migration during cancer progression. However, *in vivo* work in mice should be implemented in order to study more the effects of impaired integrin traffic in invasion or metastasis for example.

Integrin activity regulation is very well characterized at the structural level. Also many cytoskeletal adaptor and effector proteins are known to bind directly or indirectly to integrin cytoplasmic tails to regulate inside-out activation or outside-in signaling of integrins. The regulatory proteins have been characterized in blood cells. However, very little is known about integrin activation pathways in cancer. This work characterized and validated 100 novel β1 integrin regulators in prostate cells. Results will give great possibilities to look at specific pathways and nodes in integrin regulation and will enable a large amount of new mechanistic studies to implement. Interesting findings included characterization of T cell specific pathways in prostate cancer cell integrin activity regulation. This could reflect cancer cells' adaptivity to arrest or invade into endothelium during metastasis progression. Future *in vitro* and *in vivo* studies will be carried out to look at these possibilities.

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