Role of ICAP-1 in integrins' dynamic regulation, mechanosensing and contractility of osteoblast cells

Alexander Kyumurkov

Communauté
UNIVERSITÉ Grenoble Alpes

THESIS

To obtain the degree of

DOCTOR OF GRENOBLE ALPES UNIVERSITY

Speciality: Cell biology

Ministerial order: 25 may 2016

Defended by

Alexander KYUMURKOV

The PhD thesis is supervised by **Dr. Corinne ALBIGES-RIZO, CNRS** and
co-supervised by **Dr. Emmanuelle PLANUS, UGA,**
Prepared in **Institute for Advanced Biosciences,**
in **École Doctorale Chimie et Sciences du Vivant**

Role of ICAP-1 in integrins' dynamic regulation, mechanosensing and contractility of osteoblast cells.

Public PhD defense: **24 November 2017**,
in front of jury, composed of:

Prof. Franz BRUCKERT
Laboratoire des Matériaux et du Génie Physique, Member
Prof. Catherine PICART
Laboratoire des Matériaux et du Génie Physique,
Member, President of the jury
Dr. Amel METTOUCHI
Mediterranean Center for Molecular Medicine, Reviewer
Dr. Pere ROCA-CUSACHS
Institute for Bioengineering of Catalonia, Reviewer
Dr. Gregory GIANNONE
Institute for Interdisciplinary Neuroscience, Member

Communauté
UNIVERSITÉ Grenoble Alpes

THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ GRENOBLE ALPES

Spécialité : Biologie cellulaire

Arrêté ministériel : 25 mai 2016

Présentée par

Alexander KYUMURKOV

Thèse dirigée par **Dr. Corinne ALBIGES-RIZO, CNRS** et codirigée par **Dr. Emmanuelle PLANUS, UGA**

préparée au sein du **Laboratoire CRI IAB - Centre de Recherche Oncologie/Développement - Institutse for Advanced Biosciences**
dans **l'École Doctorale Chimie et Sciences du Vivant**

Rôle d'ICAP-1 dans la régulation de la mécanosensibilité et de la contractilité des cellules ostéoblastiques via la dynamique des intégrins

Thèse soutenue publiquement le **24 novembre 2017**, devant le jury composé de :

Dr. Corinne ALBIGES-RIZO
DR1, Institut pour l'Avancée des Biosciences, Directeur de these
Dr. Emmanuelle PLANUS
Maître de Conférences, Institut pour l'Avancée des Biosciences, CoDirecteur de these
Prof. Franz BRUCKERT
Professeur des Universités, Laboratoire des Matériaux et du Génie Physique, Examinateur
Prof. Catherine PICART
Professeur des Universités, Laboratoire des Matériaux et du Génie Physique, Examinateur, Président du jury
Dr. Amel METTOUCHI
Chargé de Recherche, Mediterranean Center for Molecular Medicine, Rapporteur
Dr. Pere ROCA-CUSACHS
Directeur de Recherche, Institute for Bioengineering of Catalonia, Rapporteur
Dr. Gregory GIANNONE
Directeur de Recherche, Institute for Interdisciplinary Neuroscience, Examinateur

'Brave are they who know everything
yet fear nothing!'

'Knowledge is power. Guard it well!'

'Heresy grows from idleness!'

-Imperial proverbs-

To my mother
À ma mère
На майка ми

Acknowledgements

As all paths come to an end my last adventure – my PhD has reached its inevitable end. Considerable amount of people have been by my side – some during the whole endeavor and some for a transient amount of time. I developed a particular relationship with all the people I have worked with in IAB (Institute for Advanced Bioscience), LIPhy (Laboratoire Interdisciplinaire de Physique) in Grenoble and the Institute for Interdisciplinary Neuroscience (IINS) in Bordeaux.

First and foremost, I would like to express my gratitude to the members of the honorable jury – **prof. Franz Brecket**, **prof. Catherine Picard**, **Dr. Amel Mettouchi**, **Dr. Gregory Giannone** and **Dr. Pere Roca-Cusachs**.

Thank you for agreeing to revise my work and come to Grenoble to hear my talk. I am sure that your scientific input will enrich my work and my culture. I hope you had good time reading and grading my work as I had good time working on it and writing it. I hope I can indulge you during the question time!

I would like to express my most honest gratitude to **Dr. Corinne Albigèz-Rizo** (the Boss!) for accommodating me in her well managed and open team. Together with **Dr. Emmanuelle Planus** (the co-Boss!) they seeded me as a young M2 student, watered me, manured me and eventually, for good or bad, the time has come to reap the harvest of my thesis. I hope my stay in the lab was as pleasant for them as it was for me. I shall leave with no regrets! Another special place of gratitude I owe to **Dr. Anne-Pascale Bouin** – we met in a challenging moment for both of us, but found the proper tune of each other and, one year later and half a dozen of unsuccessful attempts we were did publish together. Win-win. Dear **Dr. Eva Faurobert**… your witch laughter, combined with your constructive criticism and arguments will haunt my sleep for long time… Keep true! To exhaust the science staff - **Dr. Olivier Destaing** and **Dr. Cyril Boyault** – those two fabulous guys that was always next to me in everything – in biochemistry, in cell physiology, in the pub or at the running track.

= v =

This current eulogy would not be complete if I omit the backbone of team CAR – the technical staff. No DNA ligation, no Co-IP pulldown can oppose the queen of the molecular biology – **Christiane Oddou**. As wild as I am, very often I was very close to do something incredibly stupid or dangerous when our brave "Hygiene and security" responsible **Ingrid Bourrin Reynard** sat me down and explained to me very slowly and calmly how close I was to a disaster. I am sure I survived IAB because of her. The third and final pillar of success of the team CAR is **Sandra Manet-Dupe** who has the patience and the persistence to design a proper qPCR gene study from three different plates with no drama what so ever. I really admire the dedication and professionalism of these three ladies – my work would not be completed as such without the time and dedication of all of them.

Special thanks I owe to **Dr. Myriam Régent**, whom I have never met, but whose work I continued and build upon.

I cannot withhold my farewells to the two postdocs staying after me - **Dr. Sanela Vellino** and **Dr. Laure Forel**. Sanela, keep your energy high and your plans for the future even wilder. I know you will make it! Laure, keep being wild and never settle for mediocracy. You have the power in you!

Vacating my desk and my bench, I was feeling confident that I leave the lab to some dynamic and promising PhD students – the ambitious and spirited (!) **Adèle Kerjouan**, bright and lively **Mathilde Proponnet**, the strong and shy **Daphné Vannier** and always smiley and positive **Amaris Guevara**. I will miss you all.

During my Grenoble stay, I was able to create some very dear friendships – with the crazy wild American – **Kate Miroshnikova** (my Queen!) and the organized, fair and executive Teutonic maiden **Katharina Hennig**. From the beach to the forest, from downtown to the riverside you were my refuge from reality. The memory of you I shall cherish deeply forever.

I should mention the jolly gang from our neighboring LIPhy lab: **Tomas Andersen**, **Vanni Petrolli**, **Adel Djellouli** and **Tim Stoltmann**. Cheers to all of you, guys!

As I believe that the milestone of every success is proper family support I wish to include here also my dearest brother – **Boris**, for his exquisite humor and high spirit. In my darkest hours, I was always seeking his company. I also express my deepest thanks to my mother – **Violeta** for rising me honest and honorable and my father – **Petko** for showing me how to be a man.

Last but not least, I would like to thank the **ARC foundation** for granting me the funding to finish this project.

Concluding this part of my life, I can honestly say that my path would be darker if any of you was not there. Thank you all!

Abstract

Signal transduction from extracellular matrix originates at the membrane, where the clustering of adhesive receptors is a key step in adapting cellular force and transmitting a message. Both integrin classes β1 and β3 serve as a scaffold for numerous intracellular proteins and signaling pathway at the focal adhesions on fibronectin environment. In this molecular environment ICAP-1 has been identified as a specific partner of β1 integrin. First, I contributed to reveal how the monoubiquitylated form of ICAP-1 was involved in an elaborate signaling network responsible for maintaining cell tensional homeostasis, going from the dynamics of cell adhesion to the adaptation of contractile actomyosin machinery. We have then proposed that ICAP-1 monoubiquitylation by Smurf1 is a key event leading to a switch from ROCK2-mediated to MRCKa-mediated cell contractility. Moreover, ICAP-1 may function in β1 integrin dependent and -independent pathways to orchestrate both the chemo and mechanical regulation of cell migration on fibronectin environment. Therefore, we speculated on a more general role of ICAP-1 in cell adhesion and focal adhesion dynamics and the specifics objectives of my thesis was to investigate whether ICAP-1 can influence the behavior of integrins and consequently may affect cell function through regulation of cell contractility and force generation. For this purpose, I have worked with cellular environment where β1 integrin and/or ICAP-1 were absent by using four cell lines: WT osteoblast cells, β1 integrin KO osteoblast cells, ICAP-1 KO osteoblast cells and double KO β1 integrin/ICAP-1 osteoblast cells in order to monitor β3 integrin behavior. As expected, depletion of β1 integrin was associated with the loss of cell spreading and force generation according to traction force microscopy measurements. Surprisingly, the supplementary deletion of ICAP-1 leads to restoration of cell spreading and force generation which were dependent on enlarged β3 integrin FAs. β3 integrin-mediated forces were correlated with slow diffusion of β3 integrin within adhesion sites and slow turnover of β3 integrin containing focal adhesion. I addressed the question whether ICAP-1 might regulate β3 integrin endocytosis since ICAP-1 interacts with Nm23-H2, a nucleoside diphosphate kinase involved in dynamin-mediated endocytosis. I show that the deletion of either Nm23 or dynamin or clathrin in cells depleted in β1 integrin was able to mimic the combined loss of β1 integrin and ICAP-1 by restoring cell spreading, force generation and β3 integrin dynamics. My results propose that ICAP-1 might be involved in integrin dynamics and force generation by controlling integrin endocytosis through Nm23-dependent scission of endocytic clathrin coated pits.

Table of abbreviations

ABP Actin binding protein(s)

AFM Atomic force microscopy

AP2 Adaptor protein complex 2

CamKII Ca^{2+}/calmodulin–dependent protein kinase II

CCP Clatrin coated pits

Cdc42 Cell division control protein

CLIC Clathrin–independent carriers

ECM Extracellular matrix

ERK Extracellular signal–regulated protein kinase

FA Focal adhesions

FAK Focal adhesion kinase

FbN Fibronectin

FRAP Fluorescence recovery after photobleaching

HSPG Heparan sulphates

IAC Integrin adhesion complex

ICAP–1 Integrin cytoplasmic domain associated protein–1

IF Immunofluorescence

Krit–1 Krev interaction trapped–1

MLC Myosin light chain (aka RLC)

MLCK Myosin light chain kinase

MLCP Myosin light chain phosphatase

MRCK Myotonic dystrophy kinase-related CDC42-binding kinase

MMPs Matrix metalloprotease(s)

MT Microtubules

MYPT–1 Myosin phosphatase targeting protein, subunit of MLCP

PKC Protein kinase C

PM Plasma membrane

PTB Phosphotyrosine–binding domain

Rac1 Ras–related C3 botulinum toxin substrate 1

RLC Regulatory myosin chain (aka MLC)

ROCK Rho–associated kinase aka Rho kinase

SF(s) Stress fiber(s)

TF Traction forces

TFM Traction force microscopy

TIRF Total internal reflection fluorescence

VASP Vasodilatator stimulated phosphoprotein

WB Western blot

Table of content

V. Materials and methods

VI. Refferences

I.
Introduction

Chapter 1. Cell shape dictates cell function

How tissues and organs are formed and modified is still open question in the field of cell biology. Since the cell is the functional and structural unit of all the living tissues, changes and reorganizations at the cellular level impact the general tissue morphogenesis. The three main processes – cell division, cell growth and cell death, combined with the individual cell decisions (response to physical or chemical extracellular stimuli) put the basis of the tissue and organ morphogenesis.

1.1. Cell behavior and fate are result of coordination between physical and biological inputs

Cellular shape is the result of mechanical equilibrium between the forces exerted on the cell membrane by intracellular organelles (mainly cytoskeletal networks) and the outside environment (Ingber, 1993; Bereiter-Hahn, 2005).
Being so the physical reality of the cells is controlled by a number of biochemical processes. For better understanding of how the cell shape is controlled, a deeper understanding of the coordination between biochemical signals and cellular mechanical properties is required.
Cells in suspension present mostly round, oval shape, which is energetically favorable, without any visible polarization or special form. For example, non-activated platelets float in the blood stream as discoid particles and their shape is maintained by microtubules organized in ring structure (Diagouraga et al., 2014). Some cells or cell fragments as non-activated platelets retain that form for most of their existence. Majority of cell types though do organize their internal skeleton (cytoskeleton) in variety of modes in order to acquire desired shape and therefore regulate vital physiological processes like transport, replication, formation and maintaining of extracellular matrix and integrating the cells in the overall tissue.

Moreover different cell processes like cell division (cytokinesis), cell migration or contraction of a muscle fiber also require emerging of front-back polarity and generating anisotropic stress on the extracellular matrix (ECM) (Murrell et al., 2015). The summary balance of the external and internal forces is very closely regulated to ensure controlled cell shape changes, guaranteed by constant feedback between the cell mechanics and gene expression or protein activation.

1.2. How does the cell mechanics drive the cell shape

Cell shape and in general, the shape of objects is product of the mechanical forces forming them so the balance of the mechanical forces on the cell surface will be crucial in determining it. The internal forces on the cellular membrane are mainly due to the direct reorganization of three cytoskeletal networks – the microtubules, intermediate filaments and actin network. For example during the cytokinesis (Eggert et al., 2006), cell migration (Paluch et al., 2006; Lämmermann et al., 2008; Vicente-Manzanares et al., 2007), or apical contraction (Martin et al., 2009) the cell cytoskeletal networks do contract and therefore apply negative pressure on the cell membrane. On the other hand, the polymerization of actin drives membrane extension and formation of protrusions – lamellipodia and filopodia (Borisy and Svitkina, 2000). In addition, intracellular pressure can have osmotic origin or can be built via the contraction of the actin network (Figure 1.1) (Bereiter-Hahn, 2005; Mitchison et al., 2008; Sheetz et al., 2006).

The external forces applied on the cells are mainly from the cell adhesion on the cell microenvironment being cell – cell contacts or cell - extracellular matrix adhesion.

Forces applied on the cell surface can be categorized into three categories:

- actively generated within the cell – polymerization of actin or the contraction of actin network; opening of water or ion channels and ergo regulating the osmotic pressure inside the cell (De Vries et al., 2004);

- forces applied on the cell from outside – either by pressure from the other cells, either from the ECM fibers (Yusko and Asbury, 2014);
- forces generated at the plasma membrane (PM) itself – lipid segregation or recruitment of curvature inducing proteins, phospholipids or sugars (Lieber et al., 2013; Paszek et al., 2014).

Globally cells do have awareness about their shape and are able to control it directly. Examples of this mechanosensing feedback can be found in the organization of adhesion sites (focal adhesions) and cytoskeletal remodeling in

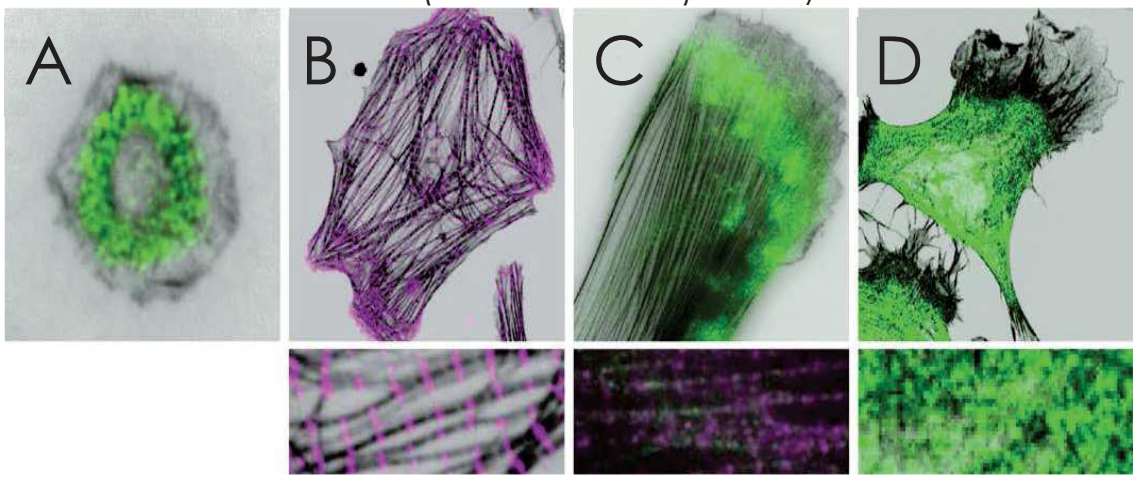


Figure 1.1. Different physical behaviors of the acto-myosin cytoskeleton in non-muscle and smooth muscle cells.
The molecular partnership between F-actin and non-muscle myosin II drive the generation of mechanical forces across various length scales in order to modulate cell shape, division and migration.
Several adherent cell types stained for actin, myosin II and a-actinin:
A. human platelets,
B. striated muscle from a rat heart,
C. smooth muscle from a human airway
D. mouse NIH 3T3 fibroblasts.
Different organization of the contractile cytoskeleton was shown in the magnified boxes.
Adapted from Murrell et al., 2015.

cultured cells. During mitosis eukaryotic cells actively respond to the deformations by recruiting heavily myosin II to counteract the change of the shape (Effler et al., 2006). Interphase fibroblasts also do reinforce their focal adhesions upon pulling (Riveline et al., 2001).

Multiple single factors do change according to the mechanical fate of the cell – the adhesion, the viscoelastic properties and cortical tension and they are tightly connected between them. Not surprising these mechanical properties are regulated by overlapping biochemical pathways forming self-regulating regulatory networks. As example, the proteins responsible for the actin turnover likely influence the cortical tension and also the viscoelastic properties in general (Janmey and McCulloch, 2007; Tabdanov et al., 2009).

1.3. Functional properties of ECM (Not just pretty fibrils!)

The ECM is more than just a passive network of ligands to support cell attachment: it contains different types of mechanical signals and it provides dimensionality. Here we are focusing on the contribution of the physical properties of the ECM environment on cellular mechanosensing (Hynes, 2009).

The extracellular matrix is present in all tissues and organs. It plays an essential role for the physical maintenance of the cellular components, enabling, in particular, the delimitation of the tissues and the individualization of the organs. Its role is not only structural; it intervenes in biochemical and biomechanical communication between cells, thus participating in differentiation, morphogenesis, homeostasis, etc. The extracellular matrix is essentially composed of water, fibrillar proteins and polysaccharides. However, its detailed composition and structure make it unique for each tissue. It is constantly developing: synthesized and remodeled by cells that are themselves influenced by the matrix. There is therefore a dialogue between the cells and their matrix. It has been presented a general view (Frantz et al., 2010) of the extracellular matrix in physiological condition and in pathological condition, as in the case of ovarian tumor (Figure 1.2) (Frantz et al.,

2010; Cho et al., 2015). The structure, composition, biological and mechanical properties as well as the balance of intrinsic and extrinsic forces are modified by tumorigenesis (Tilghman et al., 2010).

A large scale project, called "matrizome" has been recently introduced (Naba et al., 2012) where the in vivo and in silico ECM composition of normal and tumorous tissues have been catalogued to address various biological problems about ECM physiology and pathology.

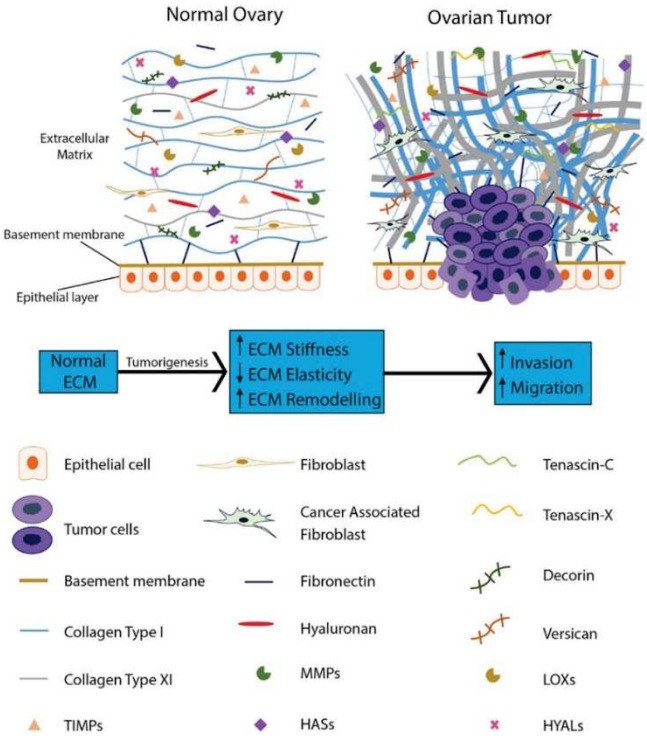


Figure 1.2. The ECM deregulation leads to ovarian tumorigenesis enhanced tumor progression
Normal ovarian ECM is composed mainly of highly organized clusters of collagen fibers with hyaluronic acid inserted between therefore regulating the dispersion of the collagen in the ECM (Cho et al., 2015). Some proteoglycans like decorin and versican are also present to secure pressure in the tissue. In epithelial ovarian cancer, the stromal fibroblasts are activated and the collagen is quickly remodeled into short, thick fibrils, randomly oriented into tracks and angles, tending toward perpendicular than parallel to the epithelial. Adapted from Cho et al., 2015.

1.4. Physical characteristics of the ECM

Another component of the cellular environment has long been ignored - the physical environment, which encompasses all the physical properties of the cellular and matrix environment, including the tissue rigidity. It seems clear, that the skin does not have the same rigidity or elasticity as the bone or the blood and lymphatic system. The rigidity of a number of tissues was measured (Cox and Erler,

2011) (Figure 1.3). There are 8 orders of magnitude between the softest solid tissue - the brain (10^2 Pa) and the stiffer tissue - the bone (10^9 Pa). Some variations in rigidity have long been used in clinical diagnostics to detect palpation of

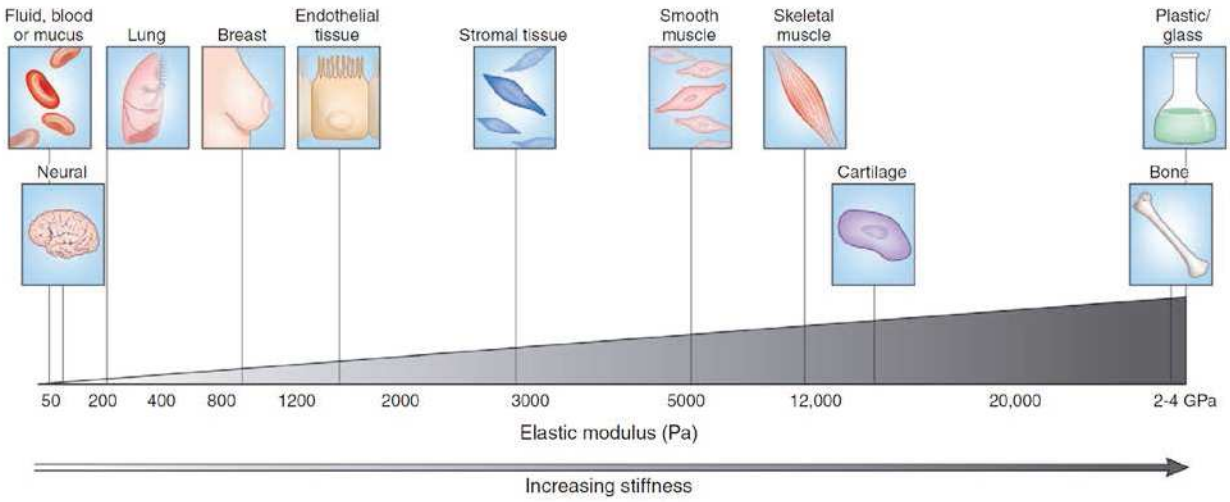


Figure 1.3. Variations in tissue stiffness. The biomechanical characteristics of different tissues in term of stiffness, measured in Pascals (Pa). It is evident that mechanically static tissues like brain or compliant like lungs exhibit low stiffness, while tissues, subjected to strong mechanical pressure like bone or skeletal muscles display stiffness several magnitudes higher. Adapted from Cox et al., 2011.

pathologies such as tumors. Other pathologies are associated with changes in the elastic properties of the tissues such as atherosclerosis, arthritis, osteoporosis, or fibrosis (sclerosis) of the heart, lungs, kidneys and liver (Ingber, 2003a). Finally, it has long been known that most cells from healthy tissue do not survive in suspension and require a solid support to grow in vitro. The lack of interaction between cells and the extracellular matrix causes apoptotic cell death called anoikis (Frisch and Francis, 1994). However, the engagement of integrins with matrix components in solution is not sufficient to inhibit this cell death. The survival signaling therefore depends on the physical properties of the matrix. Cellular growth independent of the support is one of the criteria for determining whether the cells are cancerous or not. Thus, the physics of the environment that influences the survival of the cells has to be considered.

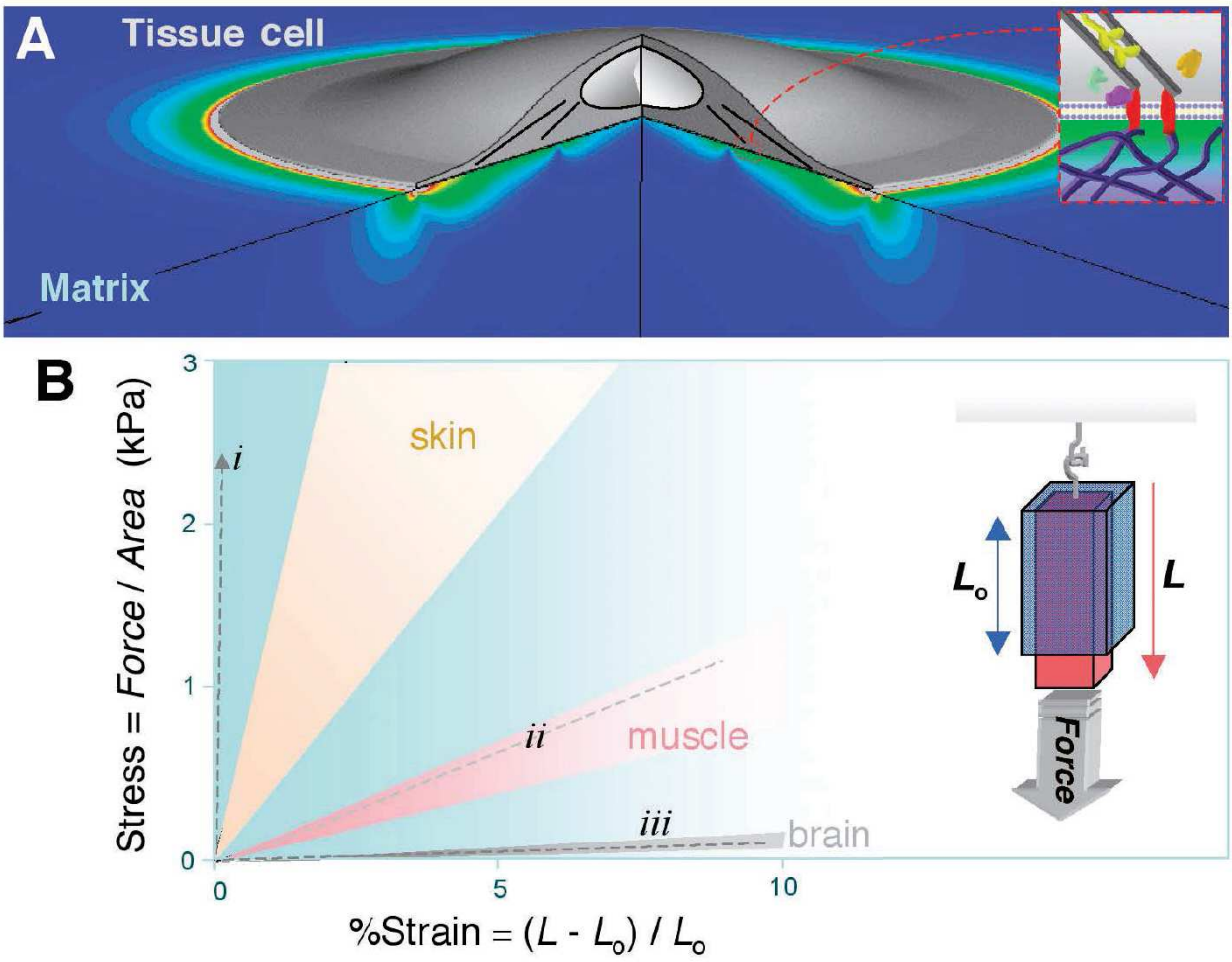


Figure 1.4. Substrate strain and tissue stiffness.
A. A in silico model of cell spread on soft matrix. The cell is modeled to be circular and to apply constant and sustained pulling pressure on the substrate from the edges to the nucleus (light grey).
B. Stress versus strain diagram for several soft tissues.
The range of slopes for these tissues is subjected to a small strain and gives the range of Young's elastic modulus, (E), for each tissue. Measurements are made on time scales of seconds to minutes and are in Pascal (Pa). The dashed lines (- - -) are those for (i) PLA, a common tissue-engineering polymer; (ii) artery-derived decellularized matrix; and (iii) matrigel. Adapted from Discher et al., 2005.

The physical environment of the cells is characterized not only by its mechanical properties. Topography and geometry also influence the cell behavior. The geometry of the environment matches the dimensional (2D vs 3D), the spatial organization of the components of the matrix, the orientation of protein fibers such as collagen, the tissue organization. It can be modeled by cell cultures in 3D or

pseudo-3D method using the method of the sandwich (Deroanne et al., 2001) or well (Ochsner et al., 2010), by the use of matrices organized by micropatterning (Thery and Bornens, 2006; Destaing et al., 2010) or structured matrices (Gardiner et al., 2015). The topography of the environment can be compared to the surface roughness that can be measured by atomic force microscopy by directly scanning the surface. Experiments done on the micro-pillars (Saez et al., 2005; Buguin et al., 2005) can address simultaneously the geometry (by spacing the pillars) or the topography of the surface (by regulating the height of the pillars and therefore their rigidity). The drawback of working on micro-pillars is that the adhesion surface is restricted and spaced artificially.

Stiffness is a measurement of the relationship between a force applied to a material and its deformation. Important parameters when measuring the stiffness are the Young's modulus (E) (also called modulus of longitudinal elasticity or traction) and Coulomb modulus (G) (also known as shear modulus or slipping) by the mode of application of force the material. For the Young's modulus, the force is applied perpendicularly to the surface of the material, whereas for the Coulomb module the force is applied parallel to this surface (Figure 1.4). These two modules are homogeneous on a constraint expressed in Pascals (Pa) corresponding to a force applied per unit area ($N/\mu m^2$); they are connected by the following equation: $E = 2G (1 + v)$ where v is the Poisson coefficient. For a material whose volume does not change under stress, this coefficient is a constant and is equal to 0.5. There is a simple relationship between E and G and measure the leads to measuring the other (Moore et al., 2010). The forces undergone by the cells may be due to flows of shearing fluids such as the blood circulating on the endothelial cells or the compression or tension of the tissues on the cells. They respond to their environment by applying opposing forces (Butcher et al., 2009). To study the effect of the rigidity due to extracellular matrix compression/tension forces on cell behavior, many natural or synthetic matrices have been developed (Ruprecht et al., 2017; Monge et al., 2015).

1.5. Some key components of the extracellular matrix

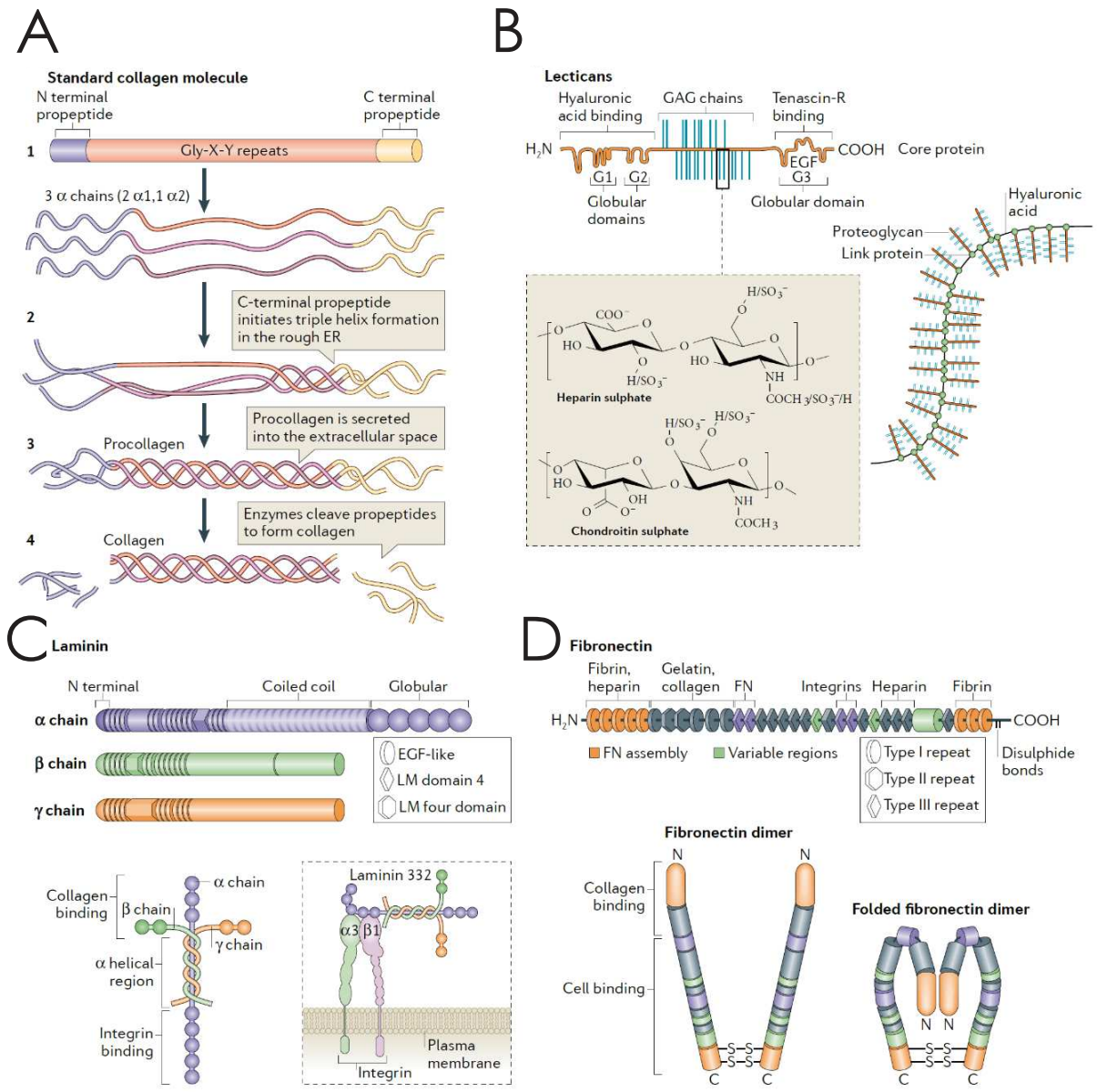
There are two major classes of macromolecules consisting of extracellular matrix: proteoglycans and fibrous proteins (Mouw et al., 2014) (Figure 1.5). Many growth factors do bind to matrix proteins and play a role in controlling cell proliferation and differentiation, sometimes synergistically with the matrix components(Hynes, 2009).

1.5.1. Proteoglycans

Proteoglycans are abundant components of the cell surface and the extracellular matrix; they regulate the distribution of extracellular signaling factors and modulate the signaling related to cell adhesion and motility. The proteoglycans consist of a protein core carrying long chains of linear disaccharides. Proteoglycans with heparan sulphates (HSPG) are grouped into three classes: transmembrane receptors, glypicons (membrane receptors) and secreted HSPGs (components of the extracellular matrix), including perlecan (Kirkpatrick and Selleck, 2007).

1.5.2. Laminins

Laminins are major components of the basal lamina, particular extracellular matrix defining epithelia and endothelia. They interact with numerous components of the matrix (collagens, glycoproteins like perlecan, etc.) They also form the mesh-like polymer by self-assembly of the basal lamina, much like collagen IV-shaped network. Laminins are hetero-trimeric glycoprotein formed by the combination of a chain α, a chain β and a chain γ. In mammals, there are 12 different heterodimers. The main laminin receptor are integrins β1 and β4, the dystroglycans and heparan sulphates (Colognato and Yurchenco, 2000).



1.5.3. Fibronectin

Fibronectin is produced by many cell types, and plays an important role in development, in angiogenesis, wound healing and bone physiology (Tang et al., 2004); its gene inactivation is lethal at the embryonic stage. It is a generally dimeric fibrillar protein whose subunits are covalently linked at their C-terminus by di-sulfide bridges. Each monomer is constituted by the repetition of three types of subdomains (Figure 1.6) which can be modified post-translationally in particular by glycosylations. There are at least 20 variants of human fibronectin, due to alternative splicing, particularly at the V domain (Figure 1.6). The solubility

properties of fibronectin and its interaction properties with its receptors depend on the presence or absence of the spliced domains. Fibronectin is abundantly present in a soluble form in the blood and in insoluble form in the extracellular matrix. The main roles of fibronectin have been attributed to the matrix and non-blood form. It is a ligand of many integrins. Among the sites of interaction between fibronectin and integrins include RGD (Arginine-Glycine-Aspartic acid) present in domain III 10 which is part of the cell binding domain (CBD): the FN fragment 8 - 10, and the V domain. The FN fragment 12-14 is the main heparin binding domain (HBD or Hep II) which serves Fibronectin also interacts with many other components of the extracellular matrix. Finally, fibronectin is organized by fibrils by the cells: it has self-assembly sites, but some of them will become available only following a conformational change induced by the traction of the cells (Zhong et al., 1998). This process is known as fibrillogenesis. These conformational changes of fibronectin can also lead to exhibition of cryptic sites that could link integrin under the influence of external force (Régent et al., 2011). This is why

Figure 1.5. The main macromolecular components of the extracellular matrix.
A. The standard fibrillar collagen molecule is characterized by amino- and carboxy-terminal propeptide sequences, which flank a series of Gly-X-Y repeats (where X and Y represent any amino acids but are frequently proline and hydroxyproline).
B. Lecticans have a main protein with binding domains for glycosaminoglycan (GAG) chains that has globular domains around it that interact with hyaluronic acid (at the N terminus) and tenascin R (at the carboxy terminus). Common GAGs are chondroitin sulphate and heparan sulphate, the chemical structures of which are shown.
C. Laminins are formed by the incorporation of α, β and γ chains into a cruciform, Y-shaped or rod-like structure.
D. The fibronectin molecule forms a dimer through disulphide bonds on its C terminus. The folded fibronectin molecule forms via ionic interactions between type III domains of neighboring molecules and is deformed by mechanical force to reveal cryptic binding sites for other fibronectin molecules and cell surface receptors when interacting with cells. Adapted from Mouw et al., 2014.

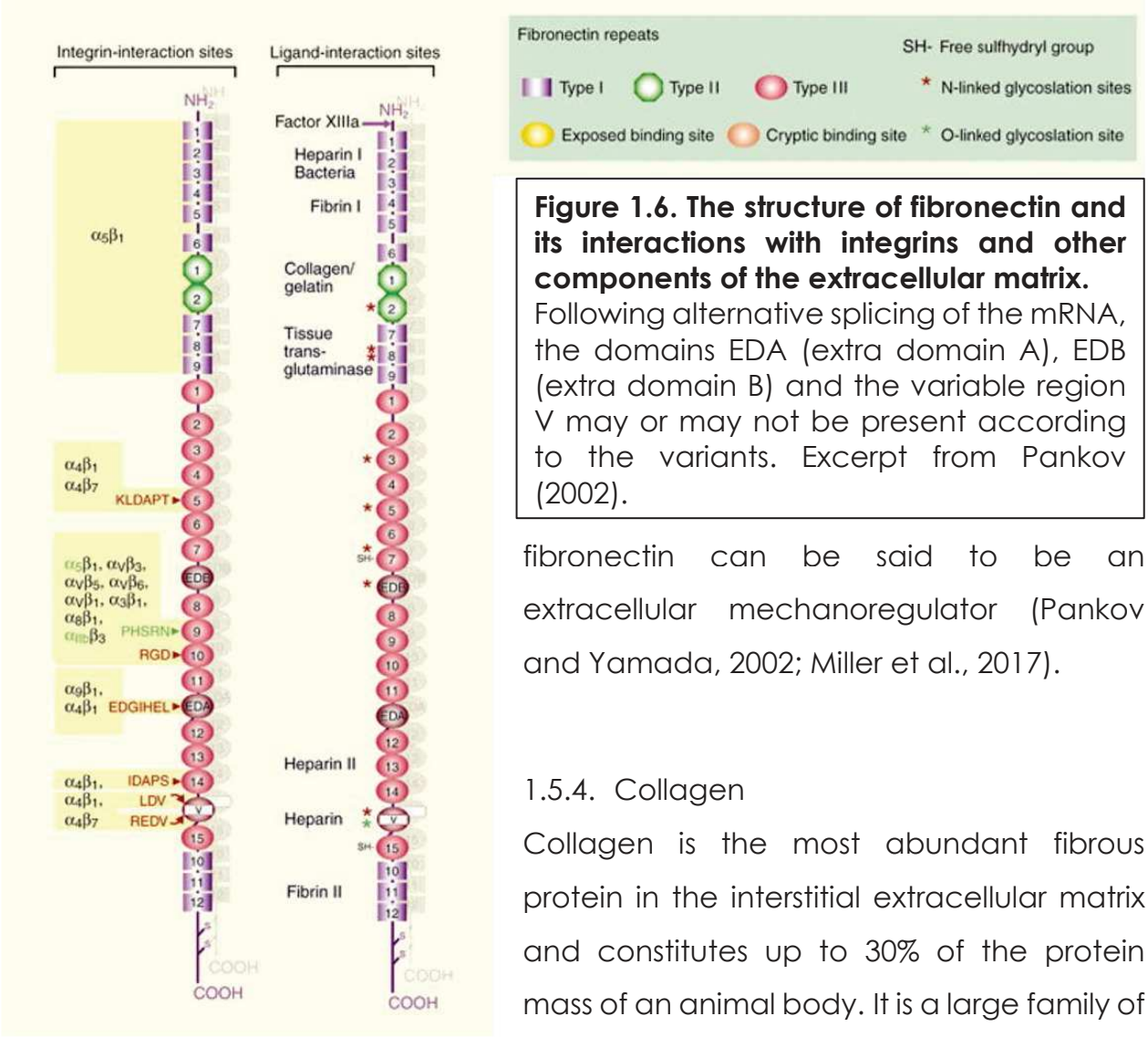


Figure 1.6. The structure of fibronectin and its interactions with integrins and other components of the extracellular matrix. Following alternative splicing of the mRNA, the domains EDA (extra domain A), EDB (extra domain B) and the variable region V may or may not be present according to the variants. Excerpt from Pankov (2002).

fibronectin can be said to be an extracellular mechanoregulator (Pankov and Yamada, 2002; Miller et al., 2017).

1.5.4. Collagen

Collagen is the most abundant fibrous protein in the interstitial extracellular matrix and constitutes up to 30% of the protein mass of an animal body. It is a large family of proteins possessing very different physical properties, conferring on each tissue its functional specificity. They play a very important role in resistance to tension in the tendons, cartilage and bones. With elastin, they ensure elasticity and cohesion of the skin. They also constitute the transparent matrix of the crystalline lens. A summary of the main classical knowledge about collagens has been published recently (Brinckmann, 2005). Collagens are trimeric proteins forming a straight super-helix. They can be organized in a sheet or cable by the cells, in particular by the fibroblasts (Mouw et al., 2014). Each polypeptide chain consists of a repeat of the tri-peptide Gxy (Glycine-xy) where x and y are frequently prolines and 4-hydroxyprolines. There are at least 28 kinds of collagen. The most common is

collagen type I. It forms fibrils and is present in tendons, ligaments, cornea, bones or skin. Type IV collagen forms a network and is specific to the basal lamina. Certain collagens possess an RGD (Arginine-Glycine-Aspartic Acid) or GFOGR (Glycine-Phenylalanine-Hydroxyproline-Glycine-Arginine) motif recognized by the integrins. Numerous post-translational modifications have been described. These influence the chemical properties of the collagens, participating in the bridging between themselves or with the other components of the extracellular matrix, which modulates the elastic properties of the collagens and consequently the physical properties of the matrix and the tissues.

1.5.5. Vitronectin

Vitronectin is a glycoprotein present in blood in monomeric form and in the extracellular matrix in multimeric form. Vitronectin is present in the connective tissues of many organs, the wall of blood vessels and lymph nodes. It is involved in many physiological and pathological processes such as homeostasis, angiogenesis, rheumatism and tumor invasion. The main function of vitronectin is to bind the inhibitor of plasminogen activator 1 (PAI-1) and to keep it in active conformation. Vitronectin is a ligand of the receptor uPAR (urokinase-like plasminogen activator receptor) and a ligand of integrins at its RGD motif (Madsen and Sidenius, 2008).

1.6. The extracellular matrix is a critical component of the metastatic niche

Cancer development and metastasis needs not only a localized niche to nourish the main tumor but also a metastatic niche to enable dissemination survival and colonization of distant tissues (Psaila and Lyden, 2009; Malanchi et al., 2012).
Tumor overgrowth naturally generates mechanical pressure on the adjacent tissues and therefore tumor compresses itself. These forces were speculated to

regulate the tumor growth. This mechanical pressure have been suggested to slow down the tumor evolution, but to trigger cell invasion and metastasis (Alessandri et al., 2013). That specific spot is composed of diverse ECM and sets of enzymes that reorganize it (matrix metalloproteases (MMPs), for example). The cancer cells in those niches cooperate with other local cell types such as bone marrow-derived cells, endothelial cells and fibroblasts. In Lewis lung carcinoma for example the production of fibronectin is increased in the places of future metastasis, which attracts BMDS cells through engagement of their α4β1 integrin (Kaplan et al., 2005). These cells also employ MMP9 to digest the basal membrane and like that, the ECM reorganizing happens in the early stages of metastasis to enable the circulating tumorigenic cells in the blood stream to colonize distant organs. Fibronectin is not the only ECM component to be engaged in building the metastatic niche. Another component, more predominant in stem cell niches is Tenascin C is secreted from breast cancer cells initially and sequentially by the stromal fibroblasts to ensure the survival and development of the lung metastasis (Oskarsson et al., 2011). Periostin (Malanchi et al., 2012), which plays important role in bone and teeth formation promotes the metastasis by recruiting WNT ligands and boosting the WNT pathways in cancer stem cells (Malanchi et al., 2012). Periostin secretion by fibro blasts is induced by invading tumor cells through TGFβ signaling and is needed to maintaining the stemness of the cancer stem cells. Curiously the endothelial tip cells forming the vascular sprouts secrete both TGFβ and periostin that induce the angiogenesis and micrometastatic formations (Ghajar et al., 2013). Considering the proteoglycanic ECM components LLC-conditioned growth medium contains versican – large chondroitin sulphate proteoglycan found to be upregulated in vast variety of human cancers. It activates macrophages via the Toll-like receptor 2 (TLR2) and induce the production of intereukin-6 (IL-6) and tumor necrosis factor (TNF) facilitating the formation of pro-inflammatory microenvironment that is conducive for metastatic growth (Kim et al., 2009).

Osteopontin, found in gliomas also preserve the stem cell properties and radioresistance through CD44 signaling (Pietras et al., 2014). More broad and systematic approach to point out the responses to ECM has also contributed to filling the library of the ECM molecules that promote metastatic behavior (Reticker-Flynn et al., 2012).

Taken together, these studies illustrate how the ECM modulates metastasis and implies that inhibiting the ECM niche may be therapeutically crucial in cancer treatment.

1.7. Cell can sense the physical environment and adapt its response (mechanoresponse)

The first technique was published in 1998 (Pelham and Wang, 1998) where biocompatible gel with controlled rigidity was used to show that that fibroblasts and epithelial cells can sense different rigidities of the substrate. Profound changes were found at several different levels in cell physiology - the morphology, lamellipodia's activity and migration were affected; the distribution of vinculin and the phosphorylation of tyrosines of several proteins are dependent on rigidity. Other teams were able to show that this sensitivity to the rigidity is for many cell types of different embryonic origins as endothelial cells (Deroanne et al., 2001; Yeung et al., 2005), neutrophils(Yeung et al., 2005) and smooth muscle cells (Engler et al., 2004), but this response is defected in transformed cancerous cells (Wang et al., 2013).

1.7.1. Cell growth and death

The cell cycle is controlled by the rigidity of the cellular microenvironment. The increase in the expression and function of cyclin D1 via FAK (Focal Adhesion Kinase)/Rac1 (Ras-related C3 botulinum toxin substrate 1) but not by ERK (Extracellular signal -regulated protein Kinase) (Klein et al., 2009) is increased

when the extracellular matrix rigidity is increased. Whereas the rate of cell death by apoptosis is decreased. This cellular sensitivity is lost with the transformation the cells into cancer (Wang et al., 2013). Within a monolayer of epithelial cells, regions where high physical stress is observed, correspond to regions where cell proliferation is increased. Inhibition of the physical tension induced by myosin (with blebistatin) or rupture of intercellular contacts release constraints and inhibit cell proliferation (Nelson et al., 2005).

1.7.2. Cell spreading through the organization of the actin cytoskeleton

Cell spreading is dependent on the stiffness of the extracellular environment and seems to be valid for most of the cell types. Study done on glioma cell lines (Tilghman et al., 2010) show that cancer cells also follow that general rule and the mechanoresponse is preserved even after transformation. The spreading area increases with stiffness as well as the number and size of adhesion structures such as focal adhesions. Actin filaments also adapt by forming thicker cables which are highly decorated with phosphor-myosin when the ECM stiffness is augmented. Figure 1.7 shows the type of response observed for fibroblasts or myoblasts, but also controls the organization of the actin cytoskeleton(Ochsner et al., 2010).

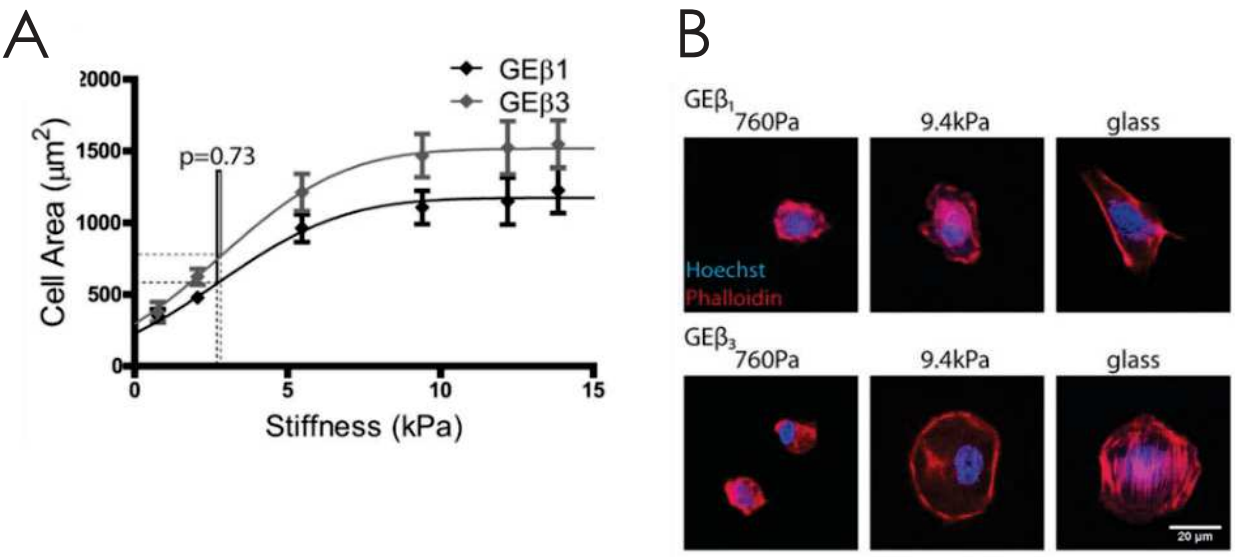


Figure 1.7. Cells respond to increased substrate stiffness by increased spreading.
A. Cellular spread area of mouse embryonic cells expressing β1 integrin (GEβ1) and or β3 integrin (GEβ3) cells over indicated rigidities. You can appreciate the increased surface area with the increased matrix stiffness.
B. Representative images of cells described in A. Notice the difference in actin organization and nuclear surface. Adapted from Balcioglu et al., 2015.

1.7.3. Cell migration

Cell migration is dependent on the physical environment. Indeed, the rigidity of the extracellular matrix modulates the speed of cell migration (Pelham and Wang,

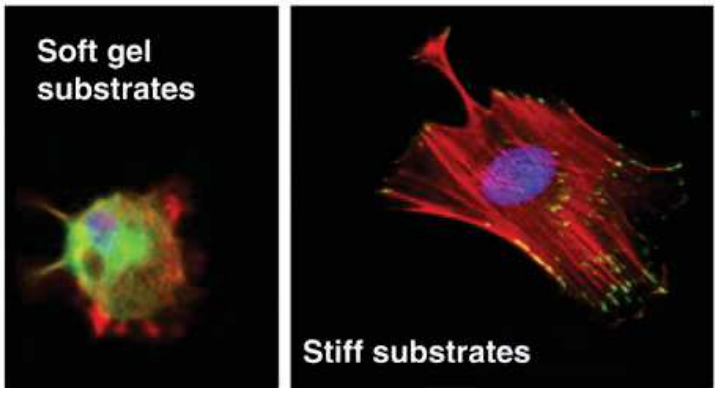


Figure 1.8. In vitro bioengineered substrates that approach soft (5 kPa) and stiff (40 kPa) tissue microenvironments (left) show that fibroblasts adhere more strongly to stiff substrates, create bigger focal adhesions and thicker actin-myosin stress fibers. Extracted from Discher et al 2009.

1998; Peyton and Putnam, 2005; Oakes et al., 2009; Ulrich et al., 2009; Stroka and Aranda-Espinoza, 2009). When plated on rigidity gradients, the cells migrate towards the stiffest substrates. In addition, cells do tend to have larger spreading area, more focal adhesions and thicker actomyosin filaments on rigid substrates. (Figure 1.8). The phenomenon of directed cellular migration towards the harder substrate is called "durotactics" in reference to the migration headed by a chemical factor gradient, called chemotaxis (Lo et al., 2000; Zaari et al., 2004). Cell migration also depends on the dimensionality of the environment: the migration in 1D fibroblasts (Doyle et al., 2009) resembles more the cellular migration observed in 3D fibrillar matrices than on 2D matrices. Unlike 2D migration, migration along 1D or 3D matrix fibers is independent of the density of the extracellular matrix and is faster.

1.7.4. Gene expression

Genes regulated by the mechanical properties of the environment are called mechanosensitive or mechanoresponsive genes. During development of the Drosophila for example the expression pattern of certain embryonic genes such as twist depends on the mechanical deformation of the epidermis (Desprat et al., 2008). When cancer cells – lung carcinoma and squamous cell carcinoma are cultured conventionally and in 3D pseudo-physiological conditions, several gene expression differences were established. In brief, cellular processes like cell-cell and cell-matrix adhesion immune cell response, tissue development is under the differential regulation of the physical 2D or 3D matrix based microenvironment. (Figure 1.9) (Zschenker et al., 2012; Le Beyec et al., 2007). Protein expression of differentiation factors like neurogenic p-NFH, myoblastic Myosin D and osteogenic BF a1 has different optimum depending on the rigidity of the environment in mesenchymal stem cells(Engler et al., 2006; Smith et al., 2017; Discher et al., 2017). Finally, the expression of many proteins of the extracellular matrix, signaling and cytoskeleton is regulated by extracellular mechanical forces and the forces generated by intracellular cells (Chiquet et al., 2009; Chan et al., 2010).

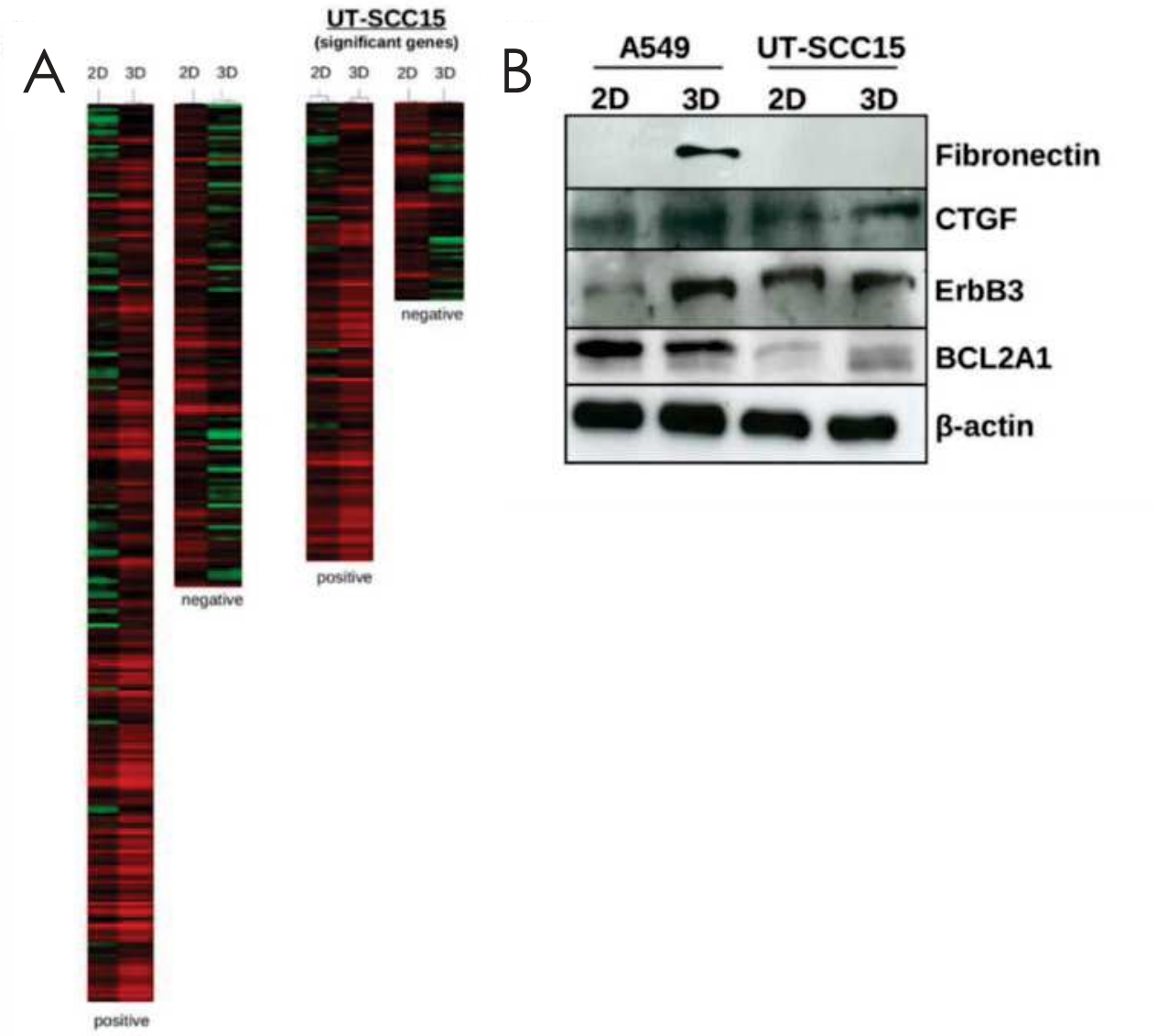


Figure 1.9. RNA ratios of differentially expressed genes and protein evidence of in 3D and 2D cell cultures of A549 and UT-SCC15 cells. (A) Hierarchical clusters of genes of 2D and 3D cell cultures at day 4 after plating. Red indicates overexpression, green - underexpression and black indicates average expression after Significance Analysis of Microarrays (SAM). ''Positive'' indicates genes upregulated in 3D versus 2D. ''Negative'' indicates genes downregulated in 3D versus 2D. (B) Western blot confirmation of several of the proteins identified in the microarray gene expression data. Fibronectin (240 kDa), CTGF (38 kDa), ErbB3 (180 kDa) and BCL2A1 (20 kDa) show important differences. β-actin served as loading control. Adapted from Zschenker et al., 2012.

1.7.5. Cell differentiation

Differentiation of stem cells is probably associated with the integrin-cytoskeletal-based feedback loop between mechanical and biochemical cues in a signaling network that determine their fates. Different mechanisms of matrix stiffness mechanotransduction may exist in three-dimensional environments, which are more physiologically relevant but still poorly studied (Lv et al., 2015) (Figure 1.10). The physical properties of the environment can be modeled by synthetic matrices or by cultures on monolayers of inactivated cells. It is possible to differentiate mesenchymal stem cells into neuronal, myoblastic or osteoblastic cells (Engler et al., 2006) by modulating the stiffness of the adhesion support without the addition of biochemical factors stimulating differentiation. The production of neuronal cells from mesenchymal stem cells is particularly unexpected since these cell types normally come from two different embryonic layers whose specification takes place at the time of gastrulation. The differentiation of mesenchymal stem cells is not only sensitive to the rigidity itself but also to the stiffness gradients of the extracellular medium (Tse and Engler, 2010). This regulation of differentiation by the physical properties of the cellular environment opens up perspectives in the field of biomaterials and regenerative medicine. Differentiation CDC cells (cardiosphere-derived cell) into mature cardiac cells can be controlled by the physical properties of a hydrogel. In addition, this gel is thermosensitive, biodegradable and compatible to myocardial injections (Li et al., 2011) and could be an important candidate for the contribution and the differentiation of cells to CDC after cardiac infarction. YAP (Yes-associated protein) and its partner TAZ are transcriptional activators that activate proliferation and are oscillating between the nucleus and the cytoplasm (Furukawa et al., 2017). YAP/TAZ were also identified as nuclear relays of mechanical cues from the ECM. Importantly YAP/TAZ are involved in the mechanical differentiation of mesenchymal stem cells. It can be concluded that

YAP/TAZ are sensors and mediators of mechanical cues instructed by the cellular microenvironment (Dupont et al., 2011).

The existence of a dependent signaling mechanical links between the muscle and the skin in the nematode *Caenorhabditis elegans* has been also published (Zhang et al., 2011). This mechanotransduction is necessary also for the morphogenesis of the epithelium.

Thus, the physical environment of cells modulates many cellular processes. However, the molecular mechanisms that allow cells to sense physical

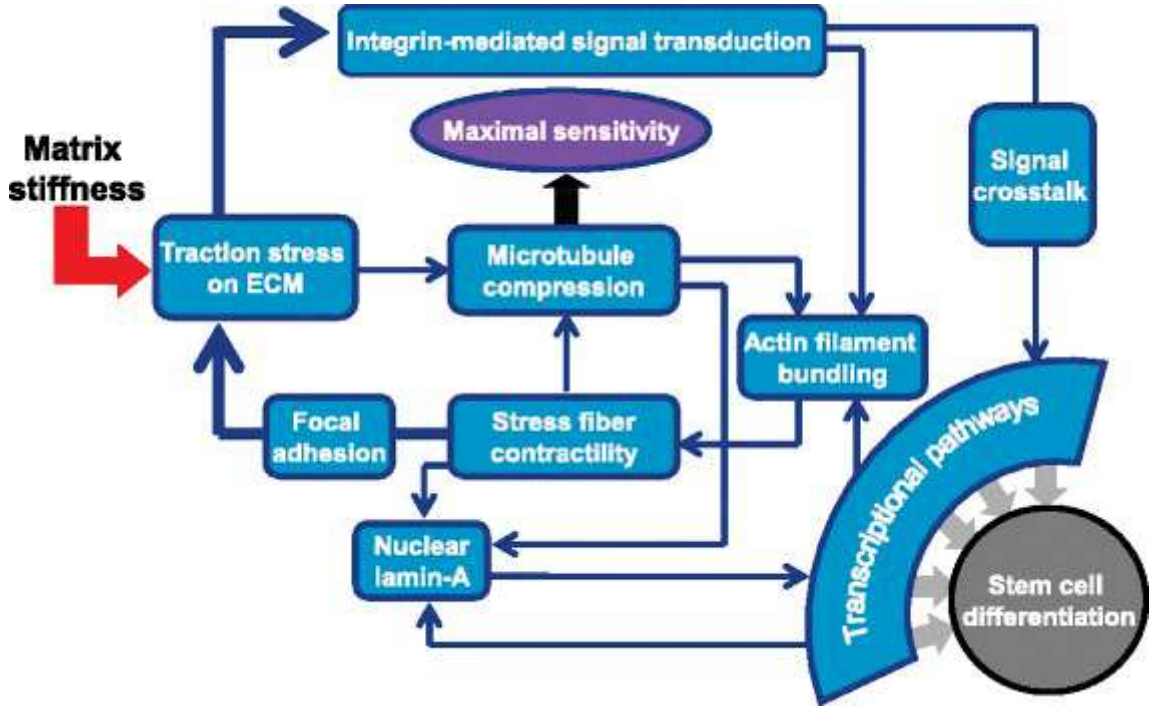


Figure 1.10. Mechanotransduction steps activated by matrix stiffness in stem cell differentiation

The original tension due to stress fiber contraction on their attachment spots on the ECM - FA is counteracted by the microtubules resistance. The cell balances the resultant force from the traction stress with activating integrin dependent signaling pathways that modulate actin polymerization and influences cell contractility. The initial stress is transmitted via the microtubules to the nucleus and is applied on lamin-A (form the family of the intermediate filaments) which activates transcriptional pathways that induces actin filament building. The cell is able to regulate its maximal mechanoresponse via cytoskeletal feedback loops. Adapted from Lv et al., 2015.

properties and respond to variations in this environment are not really known to this day. The molecular players in cell adhesion are a priori mechanical sensors as possible to form a link between the cell and its environment. It is therefore important to know the molecular actors of adhesion, their interactions and their involvement in adhesion, mechanosensitivity and cell migration. These topics will be discussed in details in the following introduction.

Chapter 2. Actin cytoskeleton as an internal mechanical element of the cell

2.1. Structure of actin microfibrils

Actin is a globular protein of about 44 kDa capable of self-assembly into double polarized helical filament (showing distinguished barbed end (+) and pointed (-) tip).

Figure 2.1 summarizes the dynamics of the actin filament. The equilibrium coefficients are different between the barbed end and the tip end, which explains the treadmilling motion of actin units within the filament in dynamic equilibrium. At the cellular level, the actin filaments can form very diverse intracellular structures such as a network of parallel filaments within filopodia or microvilli, a gel (highly branched and dynamic network) in the lamellipodia and the cell cortex or a cable antiparallel network, giving rise to different kind of stress fibers (SF). At the cellular level, all those structures are involved in the shape of the cell in the functioning of the internal machinery (including intracellular trafficking) and the regulation of cellular processes like migration.

To be so neatly organized, the dynamics of the F-actin are controlled by factors intrinsic to actin itself as the hydrolysis of ATP or arginylation of certain amino residues (Karakozova, 2006) or by extrinsic factors: regulatory actin binding proteins (ABP). These are grouped in two wide classes: proteins regulating the dynamics of actin filaments and proteins that regulate the organization of networks of filaments (Pollard, 2016; Blanchoin et al., 2014). All these proteins are themselves under the control of many diverse signaling pathways.

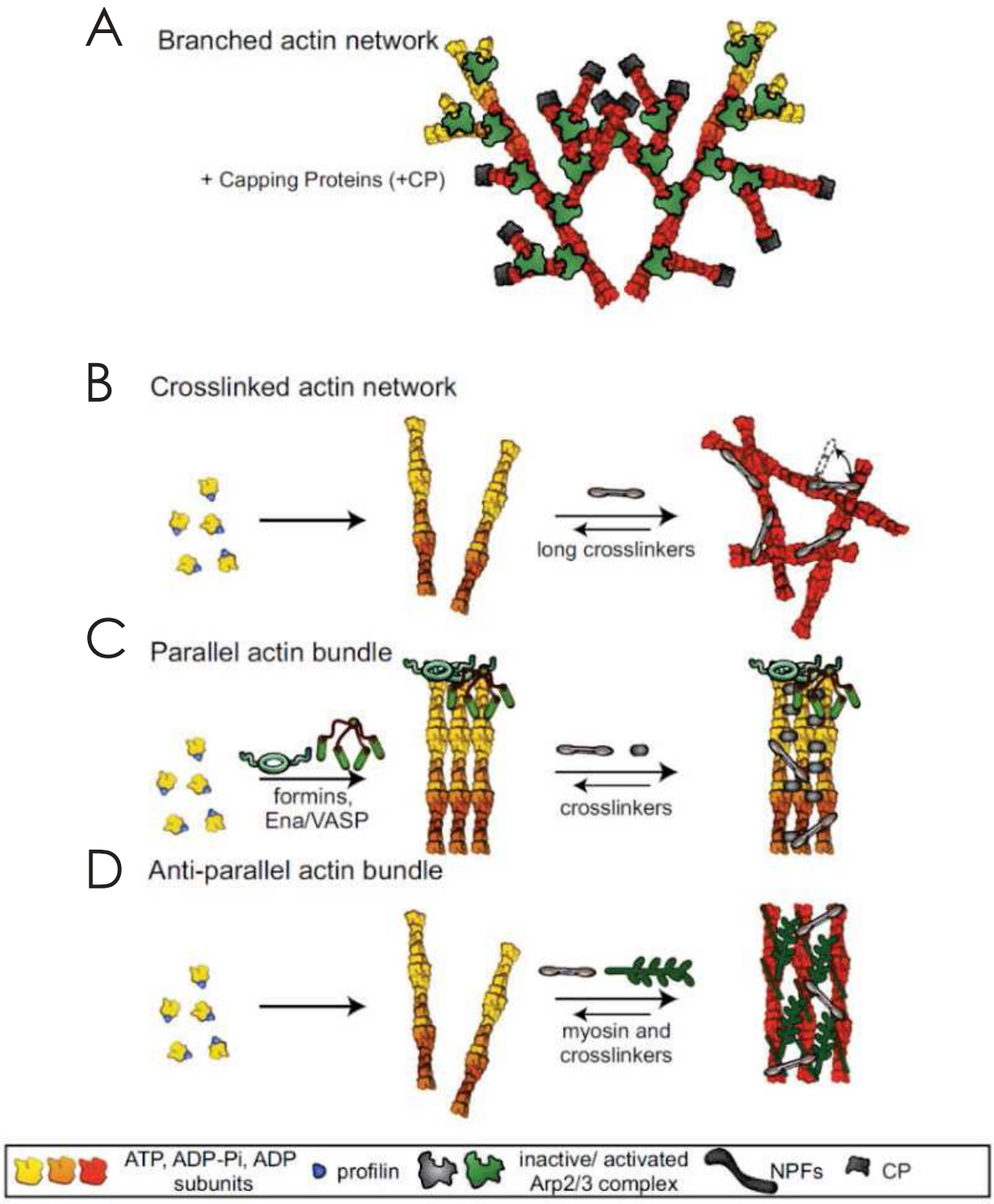

A
Branched actin network
+ Capping Proteins (+CP)
B
Crosslinked actin network
long crosslinkers
C
Parallel actin bundle
formins,
Ena/VASP
crosslinkers
D
Anti-parallel actin bundle
myosin and
crosslinkers
ATP, ADP-Pi, ADP
subunits
profilin
inactive/ activated
Arp2/3 complex
NPFs
CP

Figure 2.1. Dynamics of the different actin filaments
A. Branched actin network results from the activity of the Arp2/3 complex. Activated by nucleation promoting factors, the Arp2/3 complex forms a branch of actin from primer – already existing chain. In the presence of capping proteins, branches are shorter. This results in dense and rigid nets that can turn into a meshwork.
B. the long crosslinking proteins organize the linear actin filaments into nets. These connections act as rigid links and drive the global elasticity of the actin network according to their binding kinetics and concentration.
C. the short crosslinkers pack actin filaments more tightly into rigid fibers. They are controlled by formins or VASP systems and give rise actin fibers with parallel organization
D. molecular motors like myosin serve as dynamic connections between antiparallel filaments, that makes them effective contractile and spring units. Adapted from Blanchoin, et al., 2014.

2.2. Dynamics of actin microfibrils under the control of ABPs

Some proteins like the Arp2/3 complex promote the nucleation step of the filaments, which is highly energetically unfavorable. It facilitates formation of a branch on an already pre-existing filament. Other proteins regulate the actual growth of the filaments, their stability and/or disassembly. Examples are cap proteins that stabilize the ends of the filaments but also block their growth (tensin or gelsolin). Gelsolin is also able to cut the filaments. Members of the ADF/cofilin family bind the filaments of ADP-actin and promote dissociation of the actin units at the pointed end. Tropomyosins bind along the filaments and stabilize them by disadvantaging their spontaneous depolymerization and the cutting action of gelsolin or ADF / cofilin. They also regulate with troponins, interactions between F-actin and myosin in striated muscles. The dynamics of actin filaments of actin depends on the pool of unbounded G-actin, which is controlled by numerous proteins capable of binding to the monomers actin like profilin which promotes the exchange of nucleotide ADP by ATP and issue of ATP-actin monomers to the

barbed ends. Figure 2.2 summarizes nicely the complexity of the regulation of the actin cytoskeleton with the example of growing lamellipodium (Blanchoin et al., 2014; Pollard and Borisy, 2003; Pollard, 2016).

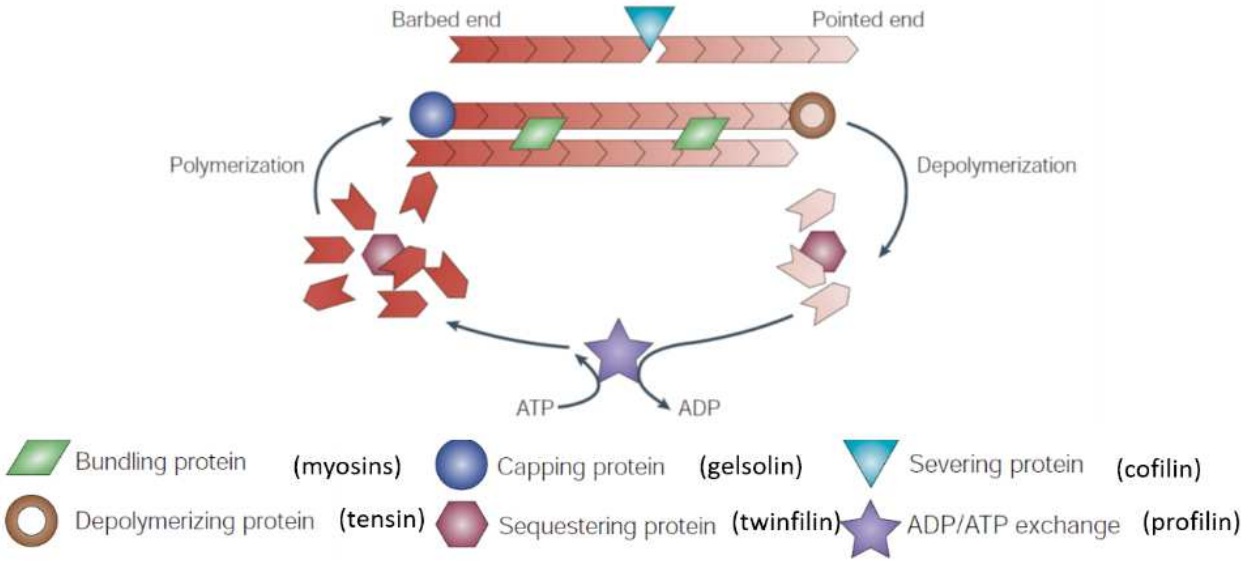


Figure 2.2. The F-actin filaments are formed by G-actin monomers until it reaches equilibrium. Filamentous (F)-actin is, asymmetric and the two extremities have different kinetics. Actin monomers assemble much more rapidly at the 'barbed end' compared to the 'pointed end'. When F-actin and G-actin are at equilibrium, the global critical concentration is intermediate between those of the two ends separately, therefore at this stage, there is a net loss of molecules at the pointed end and a net addition at the barbed end. The fiber seems in balance, which leads to treadmilling — an equal flow of actin subunits through the filament. G-actin binds either ATP or ADP. ATP monomers assemble faster than the ones, bound with ADP. After assembly on a treadmilling filament, ATP is hydrolysed to ADP this changes the filament conformation, forming less stable form at the pointed end, which depolymerizes. So, a treadmilling filament contains ATPbound subunits at the barbed end, but the actin monomers at the pointed end are ADP-bound. Many proteins bind to actin and influence its dynamics or activity. They are referred to as actin-binding proteins (ABPs). Among ABPs, some link actin filaments in a loose network (crosslinking proteins) or in a tight bundle (bundling proteins), or anchor filaments to the plasma membrane. Others bind to the barbed end of the filament and prevent further elongation (capping proteins), whereas some cause fragmentation of filaments (severing proteins) or might favor the depolymerization of pointed ends. ABPs also regulate the addition of monomers by sequestering them or favoring ADP/ATP exchange. Adapted from Revenu, et al., 2004.

2.3. Regulation of actin network by small GTPases

The small RhoGTPases (RhoA and Rac1) are central regulators of actin dynamics. They are capable to activate the Arp2/3 complex, ROCK family kinases and formins (mDia) (Mullins et al., 1998; Riento and Ridley, 2003). Traditionally Rac1 is described to operate in the leading edge of the migrating cell, where it acts on Arp2/3 complex for lamellipodia formation and RhoA is depicted in the cell rear, where it modulates actomyosin contractility via ROCK kinase family to retract the cell body (Ridley et al., 2003). This black and white view of Rho family proteins seems to be significantly challenged in 3D environment (Tomar and Schlaepfer, 2009; Yamazaki et al., 2009; Kim et al., 2015). Signaling from RhoA and Rac1 seems to occur in pseudo-oscillating fashion. Even more precisely it has been shown (Machacek et al., 2009) that RhoA activity is required in front of the Rac1 in the lamellipodium. In fibronectin rich environment cells presenting dominant RhoA activity at the leading edge tends to be more fast

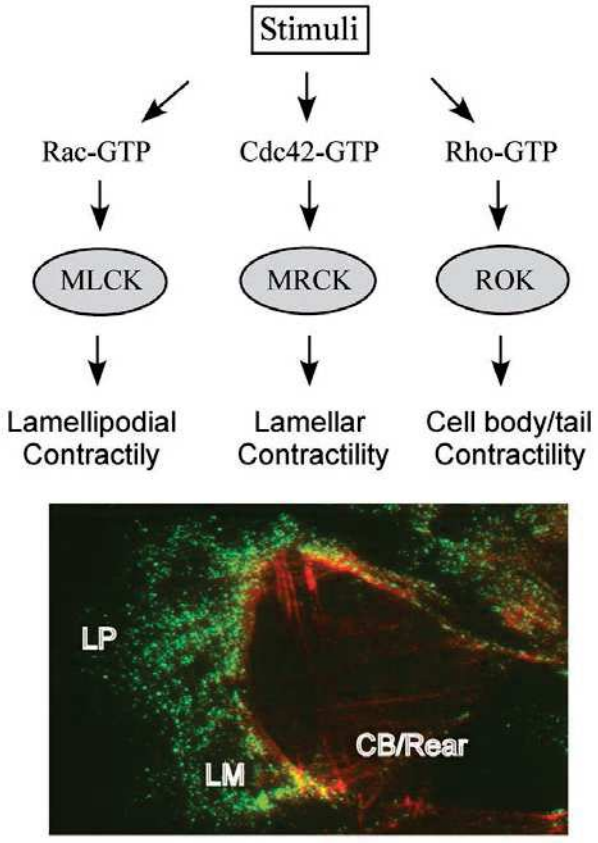


Figure 2.3. RhoGTPases display differential subcellular locations. Upper panel depicts a model for the specific activation of the different kinases, responsible for the phosphorylation of the MLC at various locations in the cell. In response to upstream signals, several kinases are activated and localized to different regions. The coordination of these signaling events is crucial for directional cell migration. Lower panel shows a typical front-rear location for Myosin 2A and 2B (in green) in a migrating U2OS cell. Actin is shown in red. LP – Lamellipodium, LM – lamella, CB.rear – cell body/rear part of the cell. Adapted from Tan et al., 2009.

in random 2D migration and to have increased invading capabilities in 3D tissue like microenvironment (Caswell et al., 2008; Muller et al., 2009; White et al., 2007; Jacquemet et al., 2013; Paul et al., 2015) (Figure 2.3).

2.4. Regulation of actin network by ABPs

There are clear distinguish between class of proteins allowing the alignment of filaments of actin cables in parallel or antiparallel fashion and that of the proteins allowing orthogonal (intersecting) bonds between filaments. All these proteins possess either multiple actin-binding domains or a single domain and then form multimers (Pollard, 2016). This is the case of a-actinin, which associates with an antiparallel dimer. It is involved in the formation of stress fibers. The spectral tetrameric complex is involved in the formation of the cortical actin gel network, particularly in the red blood cells.

2.5. Contractile actin cytoskeleton: actomyosin network

The growth in actin filaments is capable of generating forces, which can deform the semiliquid plasma membrane. In lamellipodia and filopodia, the development of membrane protrusions is due to the balance of forces on the membrane: the membrane resistance (which is constant) and the pushing forces from the growing of the cortical actin. That protrusion force is the balance between the polymerization of actin at the barbed ends, and the retrograde flow due to the action of myosins and treadmill movement of the actin. The actin filaments can push the membrane so that the actin network growth counteracts retrograde flow including through the network to the anchor extracellular matrix *through* integrins (the transmembrane receptors, that link the cell with the ECM fibers, see Chapter 1.5) and their cytoplasmic partners (Figure 2.4). This anchoring is called *clutch* and

involves talin (important component of the adhesion site) (Jiang et al., 2003; Giannone et al., 2009) (Chapter 3.5). Dendritic cells (Renkawitz et al., 2009) can migrate dependently or not on the integrins are able to compensate the increases in the retrograde flow and slip due to disengagement of integrins by an increase in the polymerization rate, keeping a speed of protrusion and a constant shape. That is not the case for fibroblasts migrating via integrin-dependent mechanism only.

The actin cytoskeleton builds the cell architecture, organization and cell neurites but the actin fibers are not alone in this network.

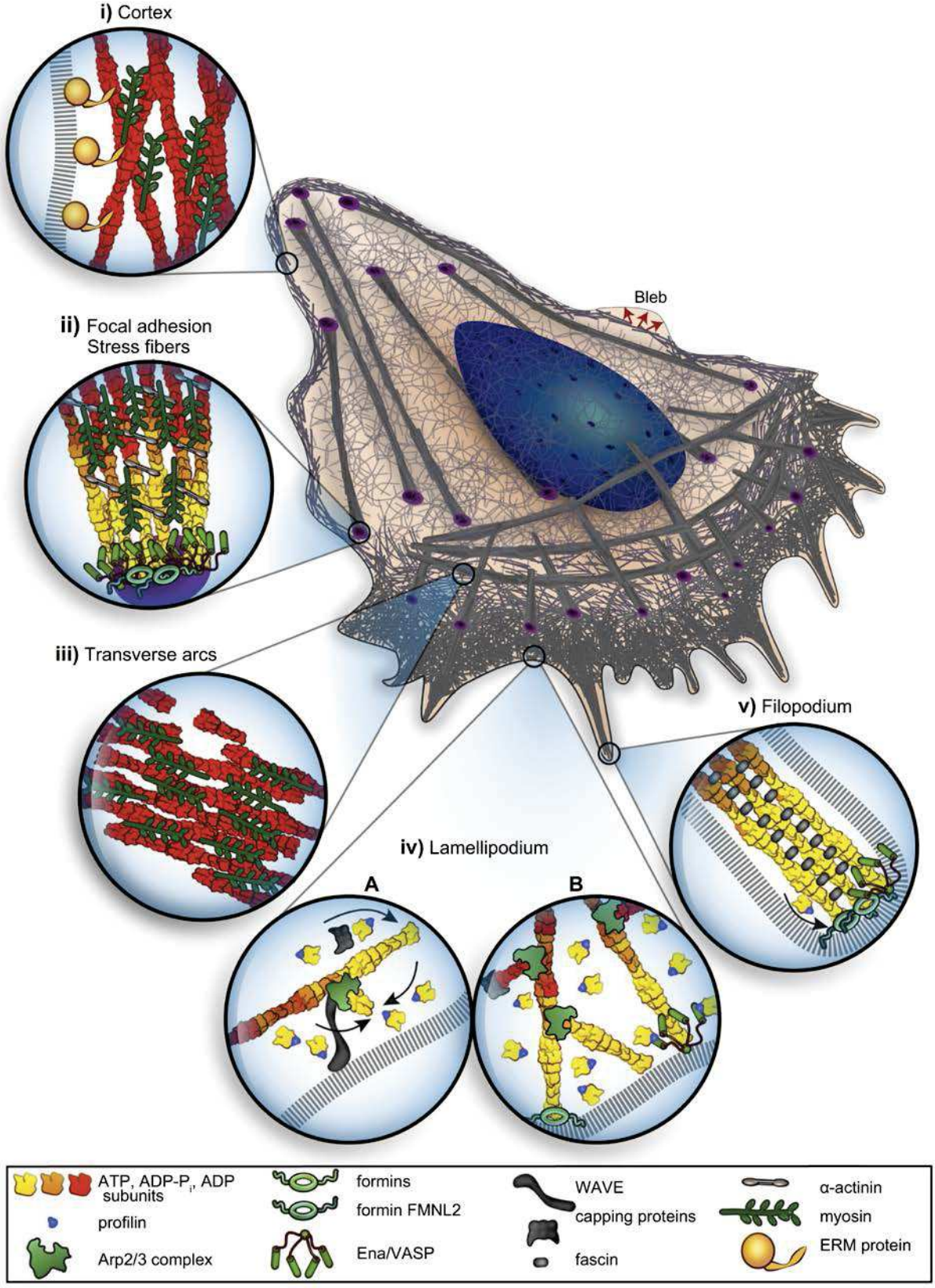
2.5.1. Cortical actin

The plasma membrane is closely related to the cellular cortex composed of an actin network, myosins superfamily proteins and membrane associated proteins. The myosin keeps the cortical actin under tension and applies hydrostatic pressure on the cytoplasm. Sometimes the plasma membrane detaches from the cortex and the cytoplasmic pressure causes a bulging of the membrane, forming a hemispherical bubble-like protrusion, called bleb. The assembly of a new actin cortex in the bubble and the activation of actin by myosin allows the retraction of this bubble. In cells that use amoeboid migration, there is a polarized formation of such bubbles in the direction of cellular movement, but the molecular mechanisms involved are still poorly understood (Charras and Paluch, 2008; Mulay et al., 2016).

2.5.2. Stress fibers – ventral (classical) SF, transverse fibers, and dorsal fibers

Stress fibers are contractile structures of actomyosin found in many non-muscular cell types. Numerous antiparallel actin filaments, cross-linked by bridges of a-actinin and by non-muscular myosins II, constitute them. The latter contract the filaments relative to one another continuously (and not discontinuously and induced like the muscular myosins) but not uniformly over the entire fiber.

I. Introduction



There are three categories of stress fibers (Lee and Kumar, 2016) (Figure 2.4): the ventral fibers, connected at each end to focal adhesion, the dorsal fibers connected at one end to a focal adhesion and on the other to the transverse fibers or arcs, which constitute the third category of fibers. Ventral fibers are responsible for changes in cell shape due to an increase in internal tension, such as the formation of the retraction tail in mesenchymal cell migration. During this cell migration, the transverse arcs are derived from the front edge towards the center of the cell, and the retrograde flow of actin is due in part to their contraction. This contractile force is transmitted to the substrate via the dorsal fibers (Hotulainen et al., 2006; Naumanen et al., 2008).

The formation of stress fibers increases with the rigidity of the extracellular matrix and the contractile state of the stress fibers is in equilibrium with the adhesion forces and the resistance to deformation of the cellular environment.

Figure 2.4. Actin organization in vivo.
Migrating cells have specific differentiated organization of the actin filaments in the different subcellular regions, responsible for distinct functions. The actin cortex is attached to the plasma membrane via ERM proteins and contracts via myosin motors.
One category of contractile fibers – the ventral stress fibers are parallel to the ventral PM and normally are organized along the direction of the movement. They connect with the PM via focal adhesions. They are also crosslinked and contracted by myosin motors Transverse arcs are also antiparallel and are found in the front part of the cell, just after the lamellipodium. They are also contractile, but are not connected to the focal adhesions. In the cell front the lamellipodium consists of quickly reorganized huge branched actin network; initiation of the branched network comes from activated Arp2/3 complex, attaching to an already existing filament and with the help of the proteins of WAVE family. Extension of the network comes through addition of profilin/actin complex (black arrows). Ena/VASP complex, the formin FMNL2 and capping proteins are antagonizing to control the elongation of the actin network by modulating the growing of the filament's barbed end (right zoom). While Ena/VASP and FMNL2 promotes the elongation of the network, the capping proteins stall it.
The sensory organels – filopods are packed with parallel actin filaments, also elongated by Ena/VASP and FMNL2 and crosslinked with fascin. Adapted from Blanchoin, et al., 2014.

2.5.3. Contractile actomyosin network

Myosins constitute a large family of molecular motor ATPases, capable of interacting with the actin filaments and generating tension between these filaments. Most of the myosins have three structural domains: a head that interacts with actin and hydrolyzes ATP to move along the filaments, an arm (or neck) on which light regulatory chains can attach, and a tail that allows interaction with other myosins to form filaments or with cargo molecules.

Based on phylogenetic criteria, 28 classes of myosin have been described in the animal kingdom (Hodge and Cope, 2000). The most ubiquitous class in non-muscular myosin class II.

Myosin II is composed of three pairs of peptides: two heavy chains of 230 kDa, two 20 kDa light chains regulating myosin activity (RLC or MLC) and two essential 17 kDa light chains that stabilize the structure of heavy chains (Figure 2.5A). The assembly of the myosins between them by the helical domain of the heavy chains allows the formation of the bipolar filaments of myosins. The non-muscular myosin II is evolutionary older and more diverse than the muscular myosins. The structures that these myosins form are more diverse, much less specialized than the sarcomeric repeats that are characteristic for the striated muscles. For NM decorated stress fibers is characteristic very variable frequency and amplitude of contraction which might stem from different affinity for actin crosslinker and other ABPs that can modulate the dynamics and restructuring of the actin network leading to increased turnover.

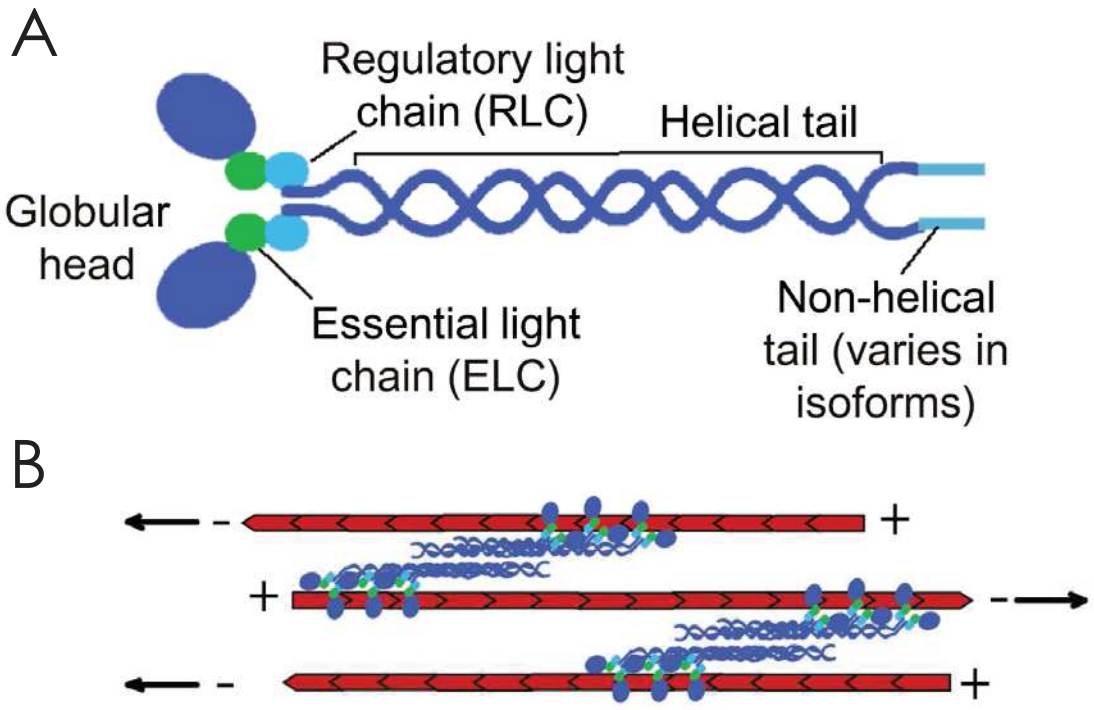


Figure 2.5. Myosin filament structure. Myosin is composed of two heavy chains, each consisting of a globular head and a tail, two essential light chains, and two regulatory light chains. The non-helical tail region varies in the three isoforms.
A. Schematic representation of the main components of the myosin complex.
B. Model of the contraction of acto-myosin fiber. Adopted from Lee et al., 2016.

2.5.4. Regulation by phosphorylation of MLC or MHC

The regulation of the formation of the myosin filaments and their ATPase activity depend on the phosphorylation of certain amine residues of the regulatory light chains and the heavy chains.

The regulation by phosphorylation of the light chains (MLC)

The phosphorylation of serine 19 increases the ATPase activity of myosin in the presence of actin by controlling the conformation of the myosin heads, but this phosphorylation does not affect myosin affinity for actin. Following phosphorylation of threonine 18 further enhances myosin's enzymatic activity.

Finally, it has been shown in vitro that phosphorylation of the light chain inhibits the intramolecular interaction of myosin and promotes both the formation of myosin bipolar filaments comprising between 14 and 20 molecules and the interaction of these filaments with the actin filaments. There are several kinases capable of phosphorylating the regulatory light chain (Figure 2.5B). The most well-known are MLCK (myosin light chain kinase) and ROCK (Rho-associated, coiled-coil containing protein kinase) which act on serine 19 and threonine 18. MLCK is activated by calmodulin-Ca2 + and is rather localized in the cellular periphery. ROCK is activated by the small RhoA GTPase protein; It can act not only on MLC but also on other substrates such as the myosin phosphatase (MLCP) subunit MYPT-1 (myosin phosphatase targeting protein); Finally, ROCK is located more centrally than active MLCK in cellular periphery (Totsukawa et al., 2000, 2004). MLC may also be phosphorylated by PKC (protein kinase C) at serine 1, 2 and threonine 9, which decreases the affinity of MLCK for MLC and thus decreases myosin activity.

The regulation by phosphorylation of the heavy chain

The phosphorylation of heavy chains favors the dissociation of myosin filaments or inhibits their formation in vitro. There are several C-terminal phosphorylation sites recognized by different kinases such as PKC or Casein kinase II (CK II) (Dulyaninova et al., 2005; Even-Faitelson and Ravid, 2006). These sites are different according to the isoform of the heavy chain. Phosphorylation can affect the subcellular localization of myosin IIA or the binding of the protein S100A4 (or MTS1), a protein known for its involvement in the metastatic invasion of cancer cells (Dulyaninova et al., 2005; Li and Bresnick, 2006).

The regulation of the activity of myosin by drugs

The ATPasie activity of myosin can be artificially blocked by blebbistatin. Its activity can also be indirectly controlled via inhibition of regulatory light chain

phosphorylation (MLC) either by inhibiting ROCK by Y27632 or by inhibiting MLCK by ML7.

Chapter 3. Cellular adhesions as actomyosin -dependent anchorages

Cellular adhesive machinery is responsible for cells to sense biochemical and physical properties of the microenvironment and to adapt cellular response through force transmission. It is therefore important to identify molecular actors to elucidate the organization and regulation of the macromolecular structures involved in cell adhesive machinery. Recent proteomic studies have shown that the adhesome consists of 232 molecules, including integrins, various actin regulators, adaptor proteins that link cytoskeletal structures to the cytoplasmic tails of integrins and multiple signaling molecules (Riveline et al., 2001; Zamir et al., 2000a; Byron et al., 2010; Danen et al., 2002; Schiller et al., 2013).

3.1. Integrins marshal cell adhesion and the subsequent signaling

Integrins are major receptors of cell adhesion to the extracellular matrix. They were discovered in the 1980s and the term "integrin" comes from the fact that this family of receivers plays a major role in the integration between the cytoskeleton inside the living cell and the extracellular matrix (Hynes, 2002).

The first gene of the integrin family was cloned in 1986 – the chicken β1 integrin (Tamkun et al., 1986). In three decades, more than 49,000 papers on the subject of the integrins have been published and they remain a vast subject of study.

Integrins form heterodimers composed of one of the 18 α- and one of 8 β subunits, that associate non-covalently with one another. Of the theoretically possible 144 associations, only 24 heterodimers are detected. The integrins can be grouped into subfamilies according to their composition: the subfamily of β1 integrin (11

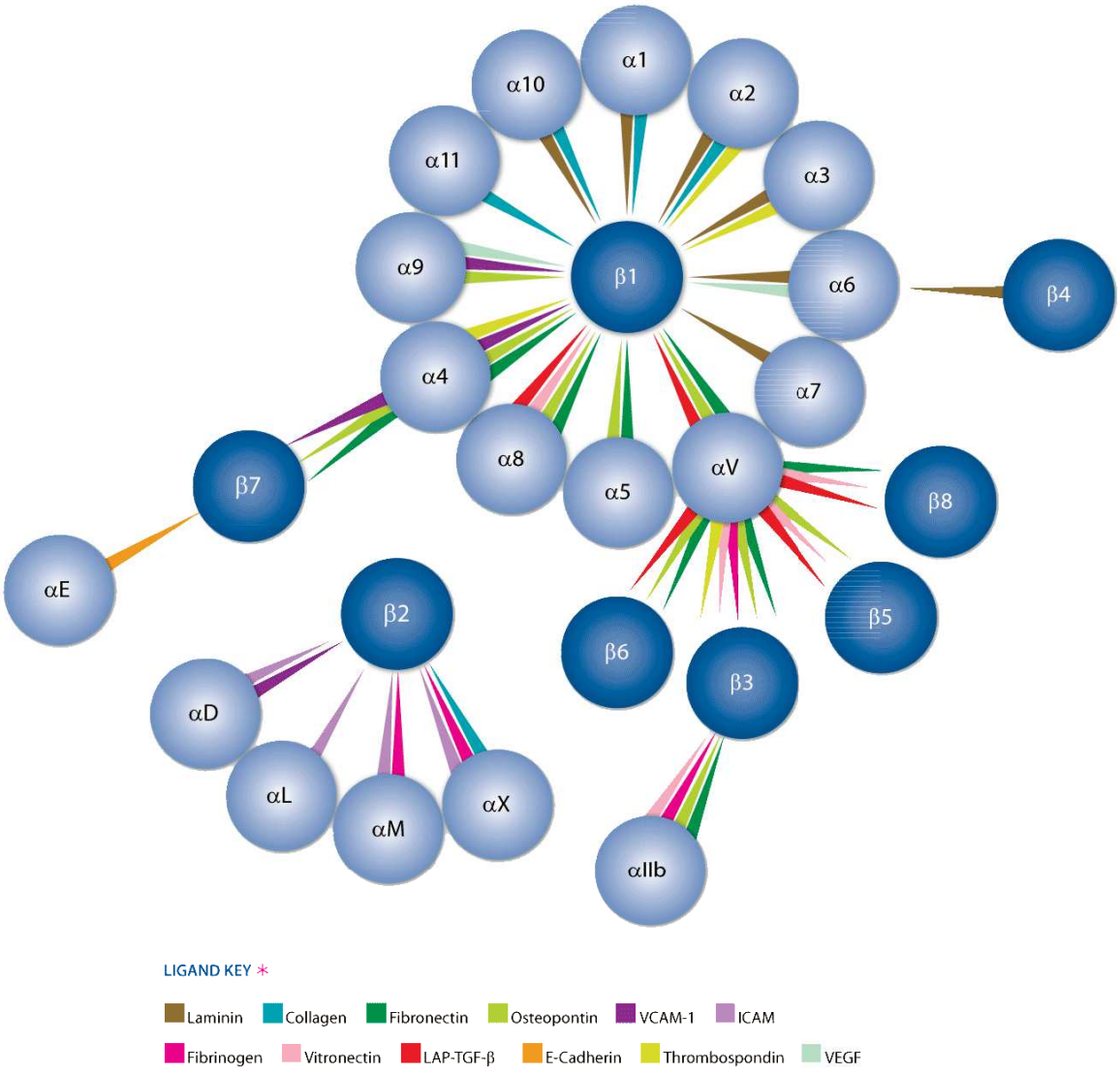


Figure 3.1. The integrin family contains 24 heterodimers in vertebrates. The principal matrix ligands with an RGD motif are fibronectin and vitronectin. The arrows, connecting a and β integrins represent the ECM ligand that this heterodimer will bind. Ref – R&D systems – a bio techie brand.

members), the subfamily of β2 integrins (4), of aV integrins (5), etc., or according to the nature of their matrix ligand (Figure 3.1): ligands with the RGD sequence are recognized by 8 integrins, mainly β1 and β3 integrins. These can also recognize the collagen RGD sequence and certain laminins when this sequence is exposed following denaturation or proteolytic cleavages of these ligands. Native collagen

is recognized via the GFOGER sequence by four integrin receptors, containing integrins β1. Their ability to interact depends also of the multi-molecular organization of collagen into fibrils. Some integrins can have several isoforms due to an alternative splicing, so this further increases the diversity of the integrin family. Not all subunits are expressed in all cells (Barczyk et al., 2010). The integrin repertoire of expression is dynamic, it changes during development and is highly modified in response to micro-environment conditions. Sometimes, the expression of a particular isoform is specific to a cell type or a state of differentiation. This integrins repertoire of attributes the cells both with a specificity of binding to the matrix and a signature of differentiation.

For examples, the cells expressing integrins β2 or β7 are part of the leukocyte lineage; integrin β1 isoform D is cell-specific to the skeletal muscle tissue. The expression of certain integrins may also depend on of the physical properties of the cellular environment (Elosegui-Artola et al., 2014). The integrin expression of β1 increases transiently when applying mechanical stress to chondrocyte progenitor cells and remains higher in stressed cells than in the control cells – where the pressure is applied. Contrarily the expression of integrin a5 is not modulated by the mechanical tension (Takahashi et al., 2003).

3.2. Physiological role

Integrins are involved in many processes from embryogenesis and during adult life and their deregulation or mutation may cause more or less severe pathologies. Although there is a redundancy between integrins for recognition of extracellular ligands, (Figure 3.2), they do not have the same affinity for a particular ligand, they are not all expressed in the same cells and they induce different signaling pathways, depending on their specific cytoplasmic partners.

The physiological importance can be underlined by the associated pathologies and by the effect of their gene inactivation in the mouse animal model. The 26

integrin genes have been inactivated separately, some of these inactivations are lethal at the embryonic and perinatal stages. For some genes, double inactivation was also achieved. So mice $a5^{-/-}$; $aV^{-/-}$ die 8 days after fertilization (E8), which corresponds to the gastrulation stage: the absence of the major fibronectin receptors blocks the formation of the anterior mesoderm, while the single inactivation are lethal later (E10, E12 - birth respectively). This shows that the compensation between integrins is partly possible. To better understand the importance of integrins whose gene inactivation is lethal in the embryonic stages,

Adaptor protein	Integrin to which adaptor binds	Reference
Structural adaptors		
α-actinin	β1, β2, β3	(Otey et al., 1993; Pavalko and LaRoche, 1993)
BP180	β4	(Koster et al., 2003; Schaapveld et al., 1998)
Filamin	β1, β2, β3, β7	(Calderwood et al., 2001; Kiema et al., 2006; Loo et al., 1998; Pfaff et al. 1998; Sharma et al., 1995; Travis et al., 2004; Zent et al., 2000)
Myosin	β1, β3, β5	(Jenkins et al., 1998; Sajid et al., 2000; Zhang et al., 2004)
Plectin	β4	(Geerts et al., 1999)
Skelemin	β1, β3	(Reddy et al., 1998)
Talin	β1, β2, β3, β5, β7	(Calderwood et al., 2003; Calderwood et al., 1999; Patil et al., 1999; Pfaff et al., 1998; Sampath et al., 1998)
Tensin	β1, β3, β5, β7	(Calderwood et al., 2003; McCleverty et al., 2007)
Scaffolding adaptors		
14-3-3	β1, β2, β3	(Fagerholm et al., 2005; Han et al., 2001)
β3 endonexin	β3	(Eigenthaler et al., 1997; Shattil et al., 1995)
CD98	β1, β3	(Zent et al., 2000)
Dab1	β1, β2, β3, β5, β7	(Calderwood et al., 2003)
Dab2	β3, β5	(Calderwood et al., 2003)
Dok1	β2, β3, β5, β7	(Calderwood et al., 2003)
Fhl2	β1, β2, β3, β6	(Wixler et al., 2000)
Fhl3	β1	(Samson et al., 2004)
Grb2	β3	(Blystone et al., 1996; Law et al., 1996)
IAP	β3	(Brown et al., 1990)
JAB1	β2	(Bianchi et al., 2000)
Kindlin 2	β1, β3	(Ma et al., 2008; Montanez et al., 2008)
Kindlin 3	β1, β3	(Moser et al., 2008)
Melusin	β1	(Brancaccio et al., 1999)
Numb	β3, β5	(Calderwood et al., 2003)
Paxillin	β1, β3	(Chen et al., 2000; Schaller et al., 1995)
Rack1	β1, β2, β5	(Liliental and Chang, 1998)
Shc	β3, β4	(Dans et al., 2001; Law et al., 1996)
TAP20	β5	(Tang et al., 1999)
WAIT1	β7	(Rietzler et al., 1998)
Catalytic adaptors		
Src	β3	(Arias-Salgado et al., 2003; Arias-Salgado et al., 2005)
Yes	β1, β2, β3	(Arias-Salgado et al., 2005)
Cytohesin 1	β2	(Kolanus et al., 1996)
Eps8	β1, β3, β5	(Calderwood et al., 2003)
ERK2	β6	(Ahmed et al., 2002)
FAK	β1, β2, β3, β5	(Chen et al., 2000; Eliceiri et al., 2002; Schaller et al., 1995)
Fyn	β3	(Arias-Salgado et al., 2005)
ILK	β1, β3	(Hannigan et al., 1996; Pasquet et al., 2002)
Lyn	β1, β2, β3	(Arias-Salgado et al., 2005)
PKD1	β1, β3	(Medeiros et al., 2005; Woods et al., 2004)
PP2A	β1	(Kim et al., 2004)
Shp2	β4	(Bertotti et al., 2006)
Other adaptors		
ICAP1a	β1	(Chang et al., 1997; Zhang and Hemler, 1999)
MIBP	β1	(Li et al., 1999)

Table 1. Adapter proteins that bind β-integrin cytoplasmic tail.

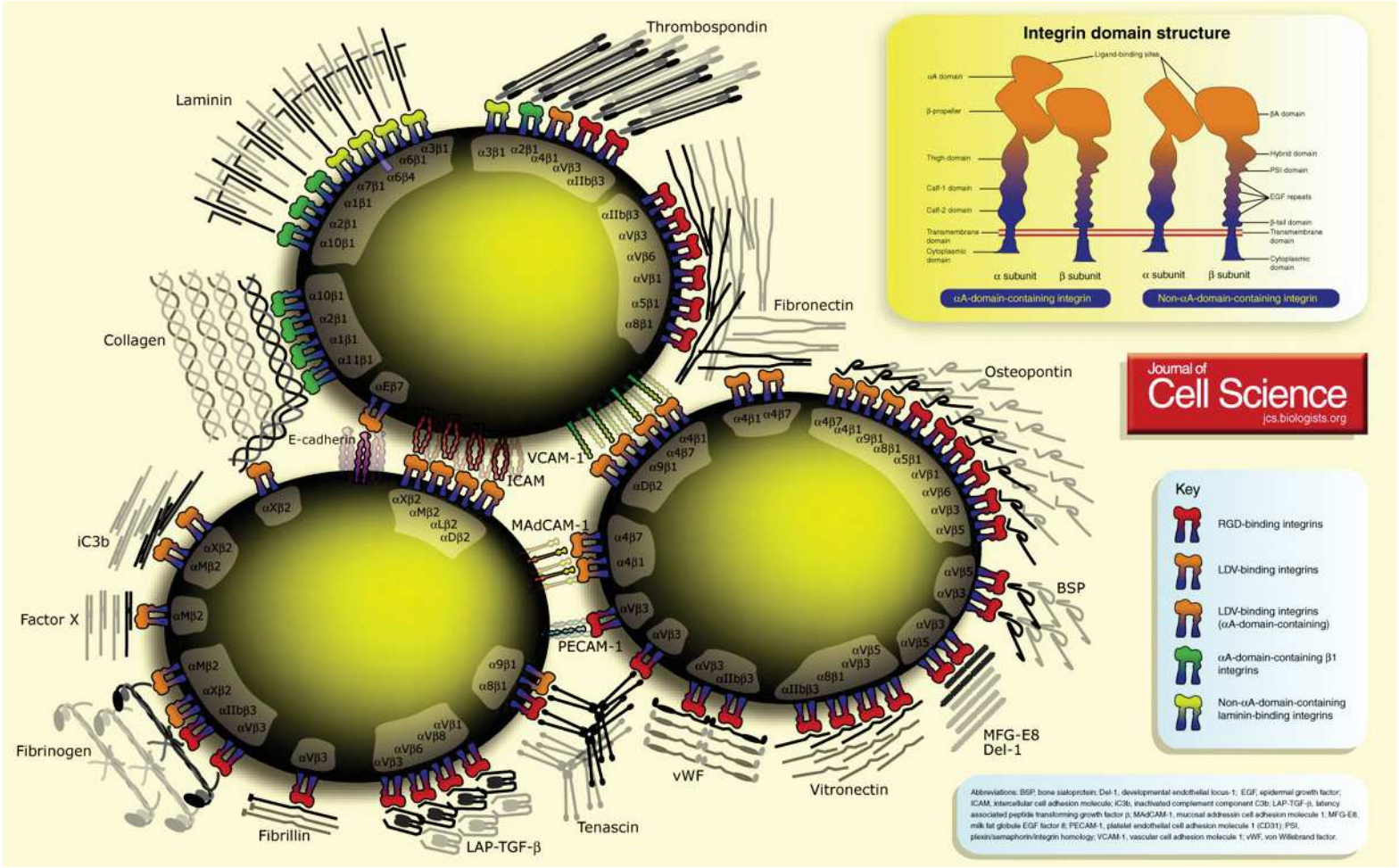


Figure 3.2. Integrins and their ligands. - BSP: bone sialic protein, Del: developmental endothelial locus, EGF: epidermal growth factor, ICAM: intercellular adhesion molecule, iC3b: inactivated complement C3b, LAP-TGF: latency associated peptide transforming growth factor, MAdCAM: mucosal addresin cell adhesion molecule, MFG-E8: milk fat globule EGF factor 8, PECAM: endothelial cell adhesion molecule platelet, PSI: plexin/semaphorin/integrin homology, VCAM: vascular cell adhesion molecule, vWF: von Willebrand factor. Extracted from Humphries et al. 2006.

organogenesis or adult physiology, conditional inactivation has sometimes been achieved. All these genetically modified animals are models for a number of human diseases such as *epidermolysis bullosa (*β4 or α6), muscular dystrophies (α2, α5, α7), osteoporosis (β3), leukocyte adhesion disability type I (LAD-I) (β2, β7 or αL), healing defects (α3, α9, αM or β3), cancers. Tumor progression is associated with overexpression or deregulation of certain integrins (Keely et al., 1998; Guo and Giancotti, 2004; Desgrosellier and Cheresh, 2010). As an example, integrin αVβ3 is associated with the formation of metastasis (Albelda et al., 1990); mutated integrin β1 causes squamous cell carcinomas of the tongue (SCC4) (Evans et al., 2003); integrins β1 seem important for metastasis and the loss of integrins β1 encourages spread of cancer cells in the lymph nodes (Kren et al., 2007); tumor angiogenesis is controlled by integrin α1 and α2; over-expression of integrin α5β1 increases the invasiveness of cells by increasing their internal contractility (Mierke et al., 2011).

3.3. The structure of the integrins

Integrins are transmembrane heterodimeric proteins. Each subunit has only one transmembrane domain. Integrin function depends on its activation state based on their conformational changes and state. The structure of several extracellular domains has been published. Most studied dimers are αVβ3 integrin with or without interaction with their ligand (Xiong, 2001; Xiong et al., 2002; Takagi et al., 2002), αIIbβ3 (Takagi et al., 2002) and α5β1 complexed to fibronectin (Takagi et al., 2003) or αxβ2 (Xie et al., 2010).

The extracellular domains of integrins have several conformations: a closed state where the interaction sites of integrins with their ligand is masked, an opened state where they are exposed and certainly many intermediate states (Xiong, 2001; Xiong et al., 2002; Hynes, 2002; Kinashi, 2005; Anthis and Campbell, 2011) (Figure 3.3).

At least three domains have been described to be involved in the interaction with the extracellular matrix ligands. Two of these sites are present on the a subunit whose site I/A which is present only on 9 of the 18 a subunits. The structure of the site I/A has been crystalized - it is involved in the coordination of bivalent cations (Mg^{2+}, Mn^{2+}) through its MIDAS motif (metal ion-dependent adhesion site) and is vital for binding certain ligands (Lee et al., 1995; Emsley et al., 2000). The last site is on the β chains. They contain a MIDAS site and an adjacent site (ADMIDAS) involved in inhibitory binding of Ca^{2+}. The exchange between Ca^{2+} ion with Mg^{2+} ion at this site causes a change in conformation of the integrin and activates it. Finally, integrin heterodimer binding to the extracellular matrix is due to the combination of two subunits, but the specificity of interaction between the integrin and the ligand is mainly due to the subunit a. The transmembrane domains are connected by a saline bridge which is broken after binding of certain intracellular proteins (talin) on the cytoplasmic domain of the β integrin, allowing the conformational change of the extracellular part to the open, active form and the intramembrane dissociation of the two subunits (Ye et al., 2010) (Figure 3.2). Except β4 integrin, the cytoplasmic tail of integrins is short (around fifty residues) and does not possess catalytic activity. They serve as platforms for numerous interactions, especially the β integrin, which have one or two NPxY (Asparagine-Proline-x-Tyrosine) interaction motifs to interact with PTB (phosphotyrosine binding) domains, contained in many proteins, which regulate the activation state of the integrins and the signaling pathways associated with them (Table 1).

3.4. Integrin associated complex

3.5.1. Extracellular ligands of integrins

A great diversity of ligands for integrin receptors and the greater or lesser specificity of a and β subunits have been largely documented including (Figure 3.3) matrix proteins (fibronectin, collagen, laminin, vitronectin ...), soluble ligands

(angiostatin, fibrinogen, prothrombin ...) (Humphries et al., 2006) and other membrane receptors presented by the neighboring cells (A disintegrin and metalloprotease molecule - ADAM, intercellular adhesion – ICAM, etc.). The specificity of ligand-integrin interaction and the stabilization of the interaction are based on the two integrin subunits that generally recognize short peptides of which one of the key residues is an acidic amino acid.

The binding of many integrins to fibronectin occurs at the level of the RGD motif carried by the domain III 10 of the fibronectin (Pankov and Yamada, 2002). There is an axillary site called PHSRN site (proline-histidine-serine-arginine-asparagine) at the domain III9 that is called synergistic site, since it promotes a better fixation of the integrin a5β1 to fibronectin (García et al., 2002; Friedland et al., 2009). This binding with the extracellular ligand causes a change in conformation and actually involves the integrins in the cellular signaling. This is called the outside-in signaling. Several proteomic and phosphorpoteomic studies have been done in the recent years (Schiller et al., 2013; Robertson et al., 2015; Humphries et al., 2009a) which shed more light on the integrin interactome and provided data for more comprehensive modeling of the FA.

3.5.2. The associated cytoplasmic proteins of integrins

Many proteins interact with the cytoplasmic tails of the integrins (Anthis and Campbell, 2011).

Integrins engaged with ECM recruit a large number of proteins that modulate the link to the actin cytoskeleton. An initial compilation based on published literature identified 156 components of the "adhesome", with 690 links (i.e. binding, activation, and inhibitory interactions) between the different components (Zaidel-Bar et al., 2007), though many more components within the adhesome have now been identified(Schiller et al., 2011a, 2013; Kuo et al., 2011; Ng et al., 2014). A more recent study combined the several proteomics datasets to create a "consensus-adhesome", which identified 60 components that represents the core components of IACs, present on all integrin ligand surfaces(Horton et al., 2015). Proteomic studies have given us insights into the complexities of IACs as signaling hubs. From the many FA proteins, nearly half of these are phosphorylated at adhesions (Robertson et al., 2015). On Figure 3.4 are presented typical MS analysis for isolated FA depending on their integrin content – only β1 integrin, only β3 integrin or both. There has been

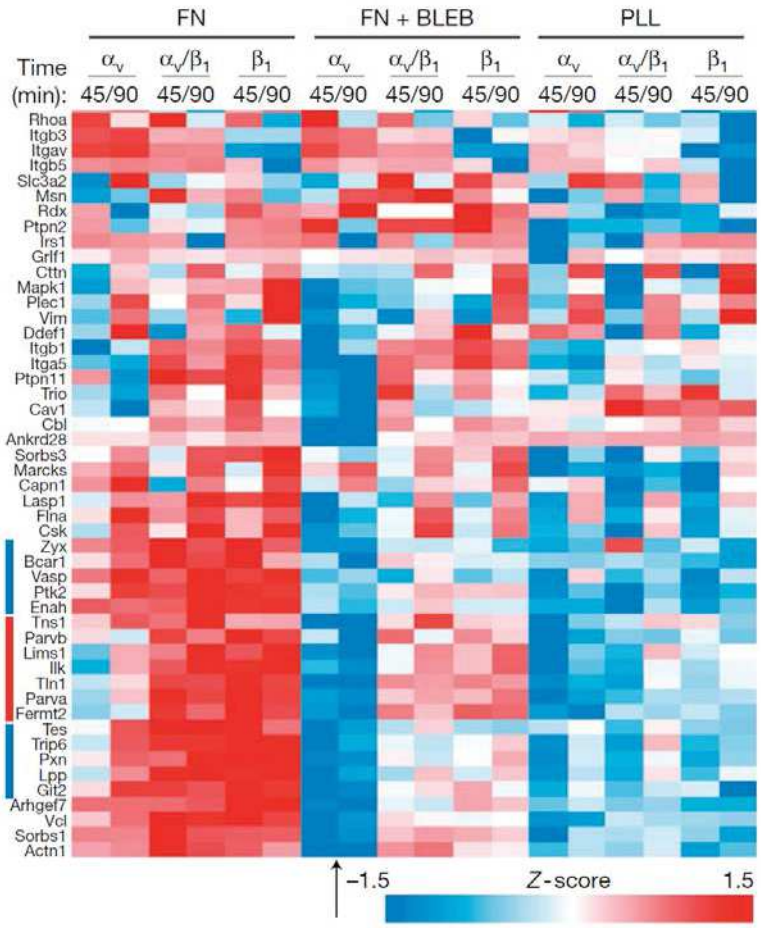


Figure 3.4. Focal-adhesion-enriched isolates analysed by MS before and after adding a contractile inhibitor blebbistatin.
The Z -scores of median MS intensities are color coded to indicate the relative protein abundance. A blebbistatin-non responcive cluster is marked with a red line and blebbistatin-sensitive clusters are marked with blue lines (on the left of the protein names). FN – Fibronectin, PLL – poly-L-lysin. Adapted from Schiller et al., 2012.

identified two subsets of phospho-proteins within IACs: some that are phosphorylated specifically

in response to integrin-ECM engagement, and others that are constitutively phosphorylated which are then recruited to IACs upon integrin-ECM engagement. Clearly, IACs are complex structures with a high degree of plasticity concerning their components. However, rather than being randomly organized, these proteins have a hierarchical structure.

Some proteins bind specifically to one or the other subunit (BP230, plectin, ICAP-1, etc.) while others bind to several subunits (a -actinin, talin, paxillin, etc.) (Figure 3.4). Some have a structural linking role between integrins and actin cytoskeleton or the cytoskeleton of the intermediate filaments while others have an intracellular signaling role with or without catalytic activity. Many studies have focused on the ability of some cytoplasmic partners to activate or inactivate integrins (inside-out) (Shattil et al., 2010). For example, talin is an important cytoplasmic adapter that activates integrin allowing its interaction with its extracellular ligand and with the actin cytoskeleton. ICAP-1, another cytoplasmic adapter, specifically maintains β1 integrin in its inactivated form by competing with kindlin, a co-activator of talin. The filamin A is another negative regulator, its action is in competition with migfiline. The phosphorylation of the cytoplasmic part of integrins modifies their interactions with their partners. For example, phosphorylation of integrins on certain serines and threonines by protein kinase C inhibits interaction with filamin but does not alter the interaction with the talin, whereas the phosphorylation of the tyrosine of the NPxY by Src inhibits the attachment of talin but promotes that of filamin and angiotensin (Kiema et al., 2006; McCleverty et al., 2007; Oxley et al., 2008; Takala et al., 2008) (Figure 3.5).

Finally, integrin signaling requires coordinated special and temporal assembly and disassembly of multiprotein complex that stems from the cytoplasmic tails of mainly β1 and β3 integrins.

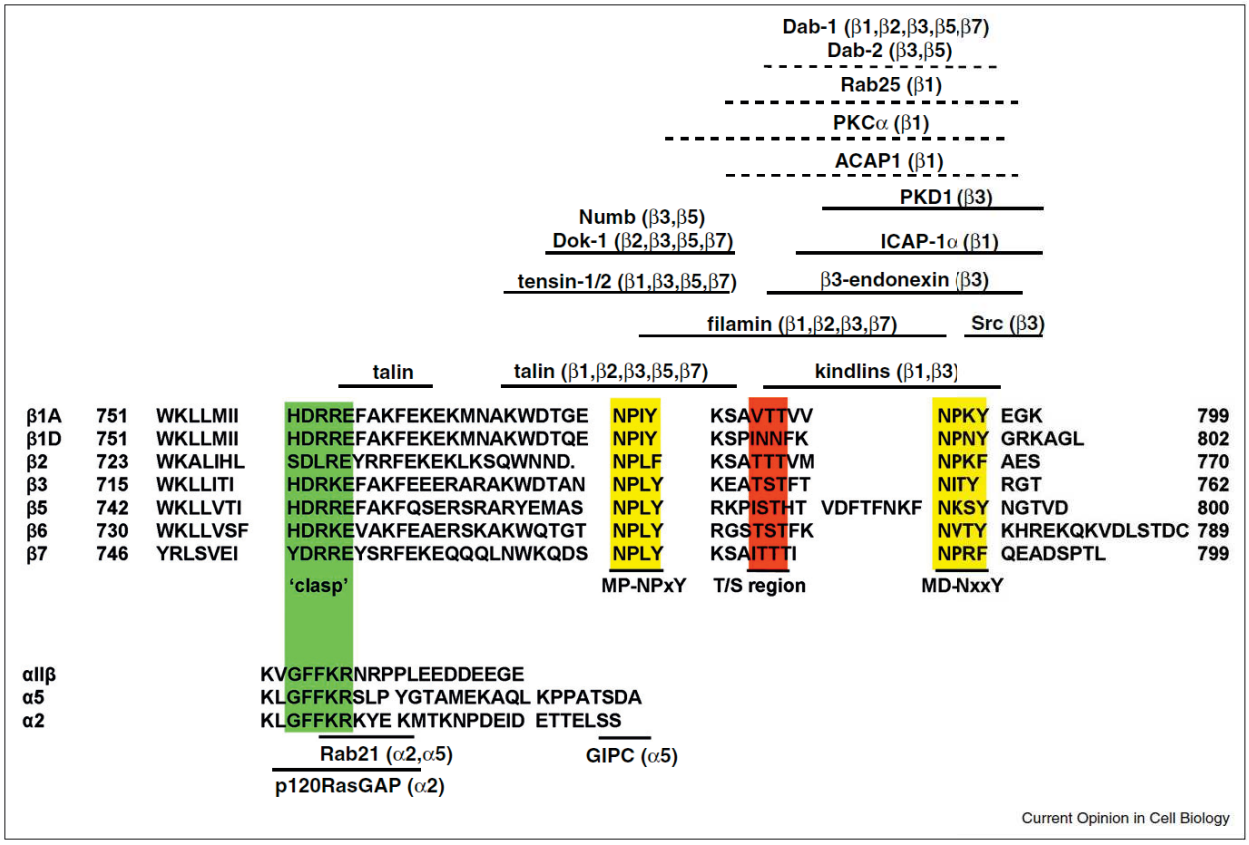


Figure 3.5. Direct integrin cytoplasmic tail partners. Interactions between integrin cytoplasmic tails and intracellular regulators modulates integrin activity and downstream signaling. This is a short summary of the direct integrin interactors, their sites of interaction on a- or β-tails, and their functional role by color. Conserved tail residues are displayed in uppercase; highly conserved residues are bolded. Extracted from Margadant et al., 2011.

3.5. Focal adhesion architecture

3.5.1. Nanoscale organization

The flawless distribution of the FA components is crucial for its proper function. Considering that the FA are relatively densely packed any change of the players inside or their ratio can lead to different cellular response. Using elegant multicolor

super-resolution microscopy, that question have been addressed (Shroff et al., 2007). This study revealed that even though some adhesion protein might appear co-localizing via conventional fluorescence microscopy they are indeed specially differentiated as distinct nanoclusters. Recently similar data were obtained for integrins β1 and β3 – both residing in the FA, but forming separate homoclusters at nanoscale with their own separate dynamics (Rossier et al., 2012). 3D iPALM super-resolution study showed that vertical organization of FA is also highly regulated and structured (Kanchanawong et al., 2010). The authors describe a layer of 40 nm between the short integrin tails and the actin filaments. That layer contains number of signaling proteins like FAK and paxillin, intermediate layer containing talin and vinculin and the upper, closer to the F-actin layer, accommodating zyxin and a-actinin.

That spatial segregation of protein interaction can mean that their activity is also temporarily regulated due to dynamic nature of the FA and depending on its maturity. This unorthodox spatial segregation of particular proteins and therefore protein interactions into distinct nano-clusters can prove to be efficient way to regulate spatially but also temporally protein activity. With the maturation of the FA can be expected that these interaction will also evolve s since FA maturation is associated for instance with increased interactions of a-actinin in competition with talin for β-integrin tails (Roca-Cusachs et al., 2013). Importantly the mechanical cues transmitted via the adhesion structures could also regulate the in time and space the probability of specific protein interaction as force can distort some mechano-responsive proteins and expose cryptic binding sites as is the case for talin (Liu et al., 2015) (Figure 3.6).

3.5.2. Dynamics at nanoscale

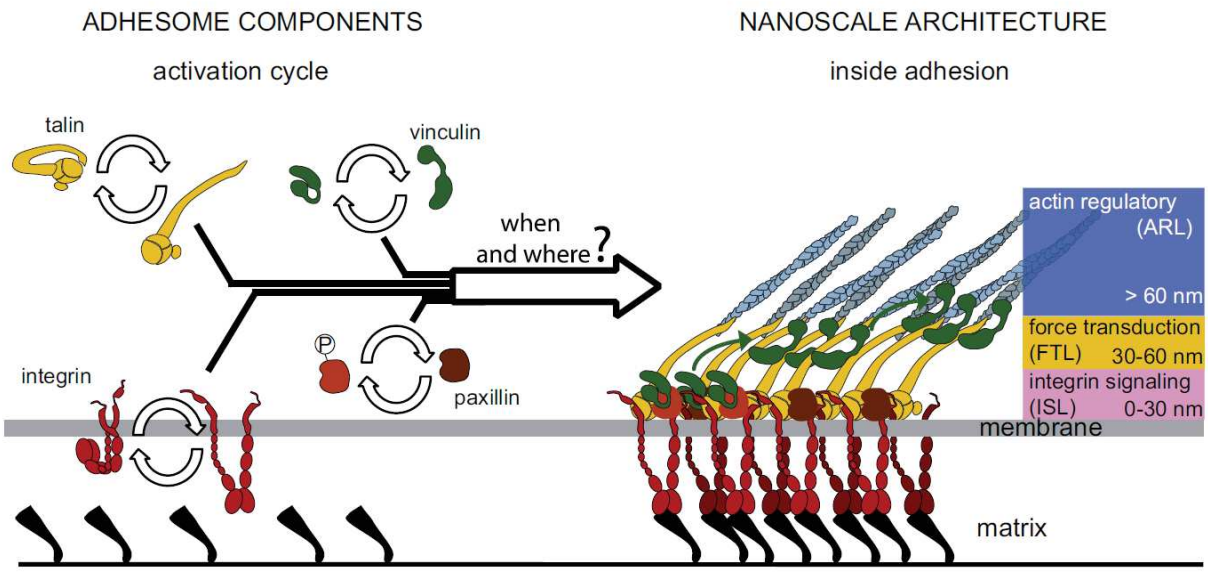


Figure 3.6. Nanoscale organization of FA. FA structure have been exposed by recent high-resolution microscopy techniques as 3D layers, containing specific proteins with specialized functions. The first layer – the integrin signaling layer (in pink) holds the integrin cytoplasmic tails and their immediate partners – FAK and paxillin. The intermediate force transduction layer (yellow) contains the mechanosensitive proteins talin and vinculin, and the upper actin regulatory level (in blue) is composed from a-actinin and zyxin. In addition, the proteins of the adhesome undergo cycles of activation and inactivation via different manners (conformation, phosphorylation, mechanical stretching, etc.) that can be responsible for their location or the recruitment inside FA. Adapted from Rossier et al., 2016.

Crystallographic data indicates that integrins can adopt different conformation (Zhu et al., 2008b) (Chapter 3.1). The shift between different states regulates the binding (and detachment) of different integrin partners. Low resolution dynamics of FA and more specifically integrins have been performed using FRAP (Ballestrem et al., 2001a), but it stays only dynamical measurements on large ensemble of molecules with low spatial resolution. FRAP data reveal the so-called immobile fraction – proteins that do not recover their fluorescence in time. Inside that pool is hidden the real dynamics of single molecules interacting inside the borders of FA. Using single particle tracking (SPT) (Wiseman et al., 2004; Brown et al., 2006;

Bachir et al., 2014). This is special kind of fluorescent microscopy that detects macromolecular assemblies inside FA that form fluorophores clusters – speckles. The mobility of these spots can be measured in various conditions. In the case of membranous receptor like integrins several studies have been carried out monitoring their interaction with ECM ligands (Rossier et al., 2012; Cairo et al., 2006; Rossier and Giannone, 2016). The ability to follow single (or very few) integrins allows for registering the transition between their activation states. It has been reported (Shibata et al., 2012; Leduc et al., 2013; Rossier et al., 2012) that integrins are capable to enter freely FA by diffusion and perform several cycles of activation (immobilization) and deactivation (free diffusion) before exiting FA. These studies show that most integrins inside FA are not always attached to actin cytoskeleton.

Chapter 4. Cell adhesion dynamics

Cell adhesion of a single cell is a very dynamic process resulting from the coordination between adhesion sites (focal adhesions) and cytoskeleton as illustrated by cell spreading and cell migration. Cell migration and cell spreading are driven by integrin-mediated focal adhesions (FAs), protein assemblies that couple contractile actomyosin bundles to the plasma membrane, transmit force generated by the cytoskeleton to the ECM, and convert the mechanical properties of the microenvironment into biochemical signals, a process called mechanotransduction. The establishment, maintenance and dynamics of these structures during the steps of cell spreading or cell migration are highly regulated (Cavalcanti-Adam et al., 2007). There are three basic components contributing to force on the ECM at an FA: (1) myosin II, which produces force on (2) actin filaments, which act as a conduit of the force to (3) FA proteins and integrins, which comprise the linkage between actin and the ECM through the plasma membrane. Dynamic changes in assembly/disassembly, activity, or protein–protein interactions within any of these three components could be responsible for mediating the fluctuations in force transmission seen in FA (Plotnikov et al., 2012).

4.1. The stages of cell spreading

The spreading of cells on hard substrate is a complex model for understanding the mechanisms associated with cell adhesion dynamics, which are crucial for cell migration and force balance. The spreading of cultured fibroblasts on rigid surfaces proceeds in two distinct stages: radial spreading followed by cell polarization important for cell migration. Cells drastically reorganize their cytoskeleton during this extremely dynamic process. The main visible feature is the increased surface area of contact between the cells and the extracellular matrix

that is accompanied by a significant flattening of the cell body and an increase of the total surface of the plasma membrane by exocytosis(Gauthier et al., 2009). There are two modes of cell spreading: an isotropic and anisotropic way(Dubin-Thaler et al., 2004). The anisotropic mode shows greater fluctuations in the transient and stochastic membrane edges and an increase in the contact

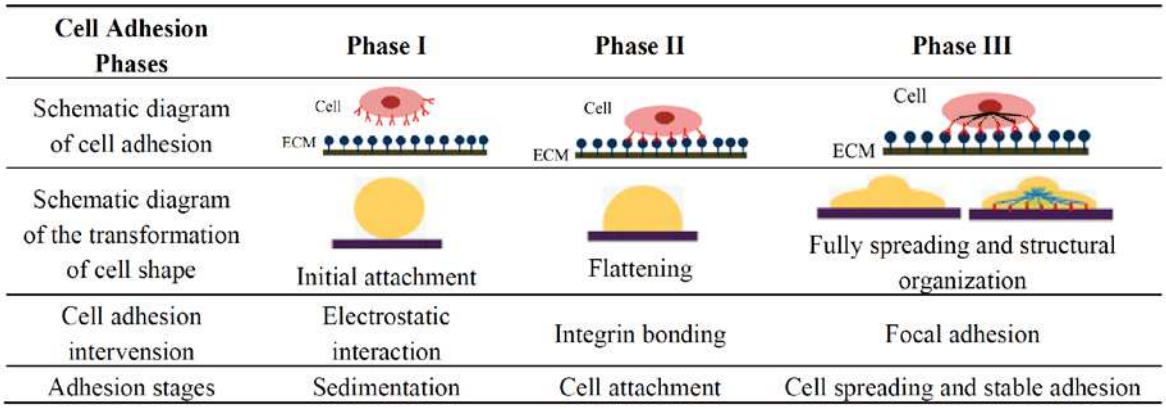


Figure 4.1. Characterizing of the adhesion stages. The spreading surface increases (Phase I) following the inhibition of the internal contractility (Phase II) allowing the protrusions to grow (Phase III), then the cell stabilizes its shape by strengthening the contacts and the tensile forces with the substrate. While the talin do not seem to be necessary for the first two phases of spreading, it is essential for the tensioning of the cells. Adapted from Khalil et al., 2015.

area, which is less rapid than in the isotropic mode. For the isotropic mode, the analysis of the spreading surface over time helped to identify three phases(Doebereiner et al., 2004) (Figure 4.1).

4.1.1. Initial phase

During the initial phase, the cell comes into contact with the matrix. Binding of integrins to the active matrix activates Rac1 pathway and decreases the cortical contractility *via* a decrease in the ROCK pathway. This promotes cell spread that allows new interactions between the matrix and other integrin molecules.

4.1.2. The intermediate phase

The intermediate phase of rapid expansion is linked to the formation of large lamellipodes where the polymerization of the actin filaments is important and allows the protrusion of the plasma membrane.

4.1.3. During the stabilization phase

During the stabilization phase, the cell develops specific adhesion with the matrix and testing the rigidity of the microenvironment via cytoskeletal contraction cycles at the maturing adhesions (Giannone et al., 2004, 2007). The establishment of cellular polarity depends on the spatial coordination between the microtubule organization and the contractility of the actomyosin network.

4.2. Cell migration

Cell migration is a phenomenon involved in many physiological processes (embryogenesis, inflammatory response, wound healing) and pathological (cancer, arthrosis, atherosclerosis, osteoporosis). Cell migration is the dynamic and complex result of interactions between the organization and the contractile state of the cytoskeleton, the dynamics of adhesions between cells and the extracellular matrix, vesicular traffic and cellular polarity. This requires integration and temporal and spatial coordination of many subcellular processes. In the case of collective migration, the dynamics of the intercellular junctions are added to this complexity. In this introduction only the migration of isolated cells will be addressed.

In the wide range of the observed cellular forms, two main types of isolated migration have been described: mesenchymal migration of keratocytes and fibroblasts and amiboid migration of neutrophils with reference to the cellular movements of amoebae, the unicellular protozoa.

4.2.1. Mesenchymal migration

Mesenchymal migration was mainly described on observations of cells evolving in a 2D environment but is now also much studied in 3D matrices, due to the involvement of fibroblasts as lead cells in the collective migration of metastatic cells. The migratory cycle was divided into four stages: protrusion, adhesion, traction and retraction. Initially, the polymerization of the dense actin network pushes the plasma membrane forward, thus forming a thin lamellipode oriented in the direction of migration. In a second step, the protrusion adheres to the extracellular matrix by forming nascent adhesions at the front of the lamellipod. Some of them will mature in focal adhesions and will be translocated to the rear base of the cell. Stress fibers are organized from these focal adhesions and connect them to the focal adhesions of the back of the cell. Thirdly, the contraction of these stress fibers reinforces the adhesions at the front, weakens those at the back and causes the cell body to move forward. Finally, the adhesions at the back are detached allowing the back membrane to retract (Figure 4.2 and 4.3) (Gupton and Waterman-Storer, 2006; Mogilner and Keren, 2009; Friedl and Wolf, 2003; Parri and Chiarugi, 2010; Parsons et al., 2010; Scales and Parsons, 2011). At the lamellipodia located at the front of the cell, protrusion-retraction cycles are observed. During these cycles, myosin pulls the lamellipodial actin network backwards, causing a retraction of the leading edge and initiation of new adhesion sites. The network of actin, condensed by myosin, is detached from the front. The polymerization of a new actin network pushes the membrane forward creating a new protrusion. The introduction of a new myosin cluster at the migration front initiates a new retraction-protrusion cycle (Giannone et al., 2007). This migration is slow (0.1 to 1 µm/min) and is characterized both by significant

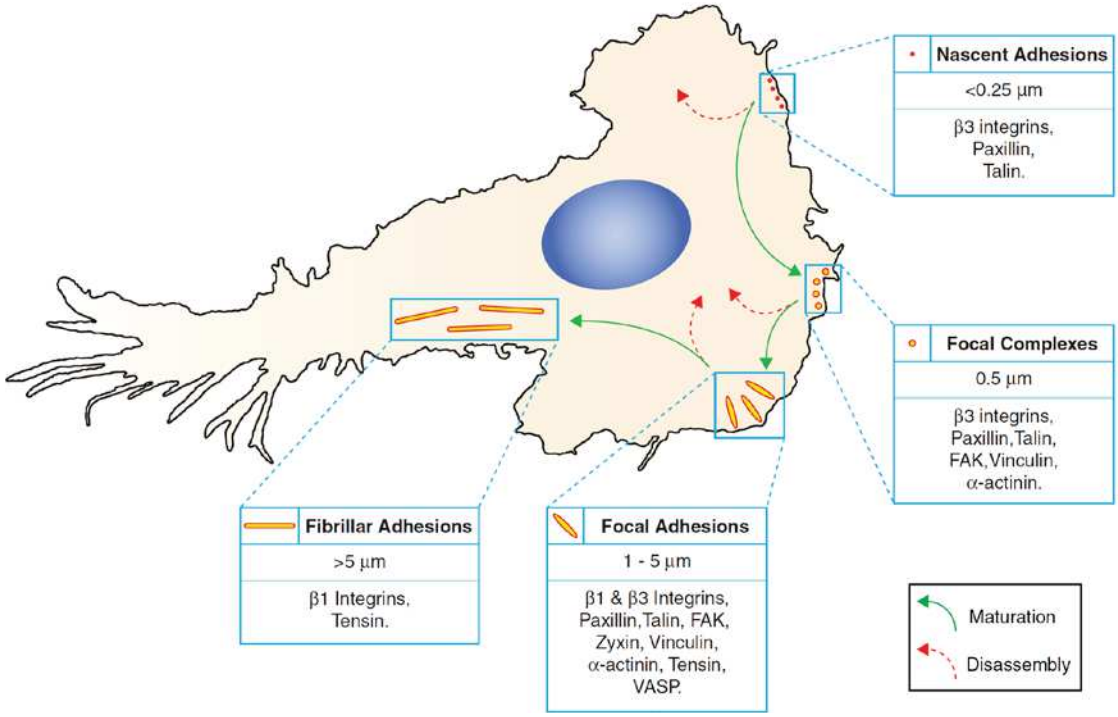


Figure 4.2. Typical representation of cell-matrix adhesions during mesenchymal cell migration. Outlined are the nascent adhesions, the focal complexes, focal adhesions and the fibrillary adhesions and their evolution – assembly in green arrows and disassembly in red arrows. Also, size and known partners are represented. Adapted from Scales and Parsons, 2011.

antero-posterior cell polarization and by a strong dependence on the dynamics of cell-extracellular matrix adhesions, in particular the family of focal adhesions required for the development of traction forces (Friedl and Wolf, 2003). The adhesion dynamics are controlled by the balance between the Rho-GTPase RhoA - of the lamellum at the back of the cell that promotes the maturation of focal adhesions and stress fibers - and Rho-GTPase Rac1 - in the lamellipodia, which promotes the dynamics of nascent adhesions and the polymerization of actin in a dense lattice (Figure 4.2). Moreover, a recent more detailed study of the spatio-temporal localization of the activity of RhoA, Rac1 and Cdc42 proteins at the front edge of the cell allowed us to show another spatial-temporal coordination between these small GTPase proteins, Initiation or stabilization of

protrusions (Machacek et al., 2009). There is therefore a spatial control of the activation of RhoA and Rac1 to allow directional migration (Parri and Chiarugi, 2010).

4.2.2. Amoiboid migration

Amoiboid migration is mainly studied in 3D. It is less dependent on cell adhesions-extracellular matrix involving integrins than mesenchymal migration and is independent of matrix metalloproteases. Amoiboid migration is poorly directional, but can reach high displacement rates (0.1-20 µm / min) (Friedl and Wolf, 2003). On the other hand, it is highly dependent on the ROCK contractility pathway (Figure 4.3) and is characterized by propulsion movements. Two modes of amoeboid migrations are distinguished: the pseudopodial protrusive mode and the so-called blebby contractile mode due to the presence of membrane

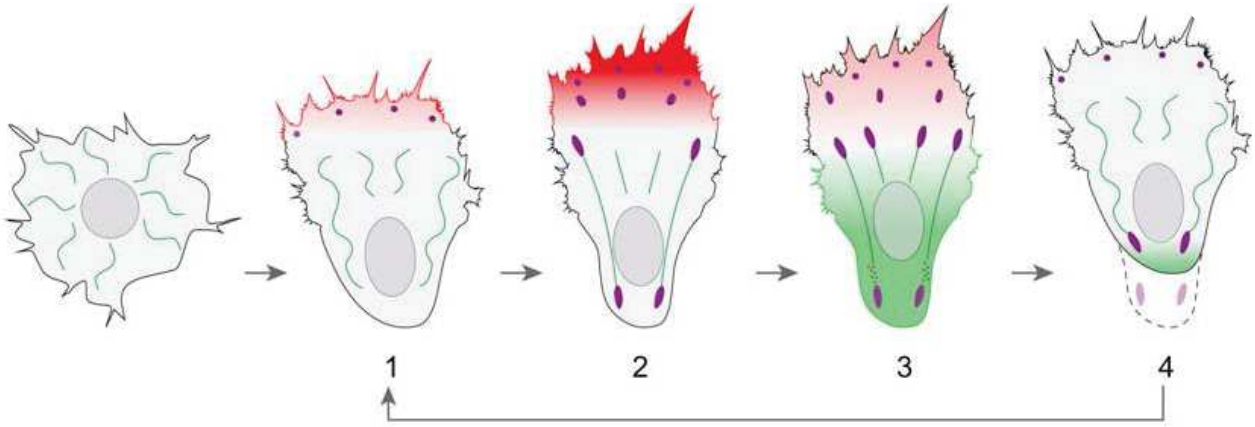


Figure 4.3. Acto-adhesive events during cell migration in culture. Obvious are actin polymerizing events (red), substrate adhesive events (focal adhesions in purple) and the myosin II dependent events (contraction of the cytoskeleton in green). Cells moving on 2D surfaces undergo repeated steps of: (1) extension of the leading edge and formation of immature cell-substrate adhesions; (2) maturation of cell-substrate adhesions; (3) forward translocation of the cell body; and (4) disassembly of focal adhesions coupled to retraction of the rear edge. Adapted from Reig et al., 2014.

bubbles (bleb). There are also models of amoeboid migration in two-dimensional cell environments (Renkawitz et al., 2009). There is a balance between the

adhesive forces due to the interaction between the cell and its support, protrusive forces due to actin polymerization, and internal contractile forces due to actomyosic activity (Lämmermann et al., 2008). Depending on the balance between these types of forces, amoeboid migration will be more or less protrusive or contractile (Renkawitz et al., 2009).

4.3. Growth and maturation of cell adhesion: membership structures

Focal adhesions are mainly observed *in vitro* on cells grown in 2D media. Their existence *in vivo* or 3D *in vitro* is debated, but recent papers (Kubow E. Kristopher et al., 2011; Fraley et al., 2010) show that that these adhesions do exist and can be visualized in cells in 3D matrices. All focal type adhesions have a connection to the actin cytoskeleton, which is, oriented parallel to the membrane in contact with the substrate. The earliest adhesions are called nascent adhesions (sometimes referred as focal complexes). They will dissociate or mature into focal complexes. During cell spreading, some of these complexes will fuse and mature into larger sized adhesions called focal adhesions that can develop into fibrillar adhesions (Figure 4.4).

In migrating (mesenchymal) cells, nascent adhesions form at the front of the lamellipodia-like protrusions. They can either disassemble or elongate at the

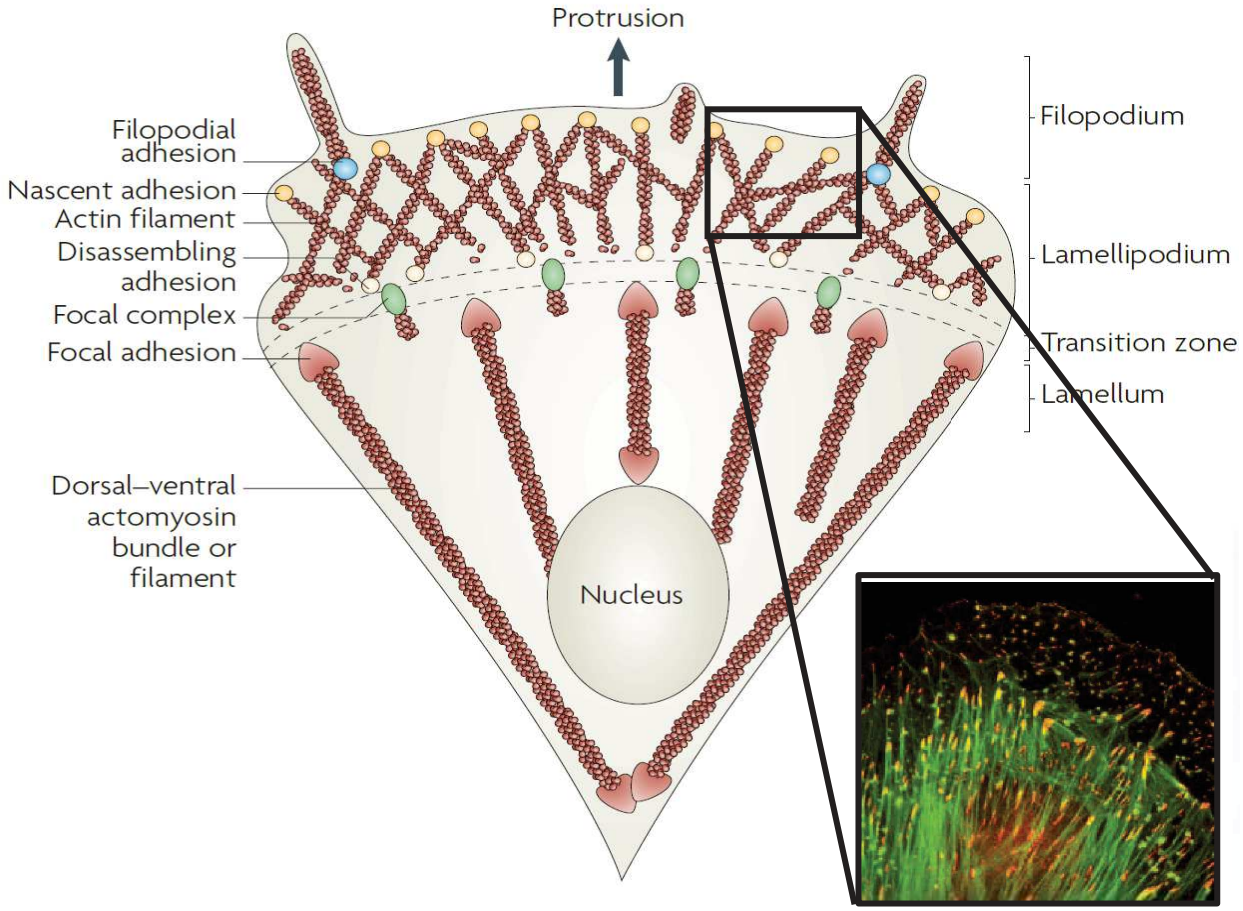


Figure 4.4. Structural elements of the adhesion of a migrating cell.
Adhesion is closely related with the protrusions of the leading edge - filopodia and lamellipodia. The nascent adhesions initially form in the lamellipodia (although some
adhesions may also be associated with filopodia) and the rate of nascent adhesion assembly correlates with the rate of cell protrusion. Nascent adhesions either disassemble or stabilize and elongate at the convergence of the lamellipodia and lamella. The maturation to focal complexes and focal adhesions is accompanied by the bundling and cross-bridging of actin filaments, and actomyosin-induced contractility reinforces and stabilizes adhesion formation and increases adhesion size. Adapted from Parsons et al., 2010 and Gimona et al., 2005.

transition between the posterior border of the lamellipodia and the lamella, giving rise to the focal complexes and then subsequently the fibrillar adhesions. This maturation of the adhesions is associated with a modification of the organization of the actin network in fibers. The introduction of myosin II on the actin fibers

induces a local increase in contractility, which stabilizes the adhesions and promotes their growth (Figure 4.4).

4.3.1. Nascent adhesions

Nascent adhesions are complex emerging on the edge of lamellipodia are mainly visible by TIRF microscopy. They are small (<1 µm) and dynamic (life span of about 60 seconds) and are formed independently of the activity of myosin II, but in combination with actomyosin fibers. A portion of them can mature in focal complexes (Choi et al., 2008) which are slightly larger (1-2 µm), less dynamic (few minutes) and present at the interface between the lamellipodia and lamella. Their formation is stimulated by Rho-GTPases Rac1 and Cdc42. Their molecular composition is not very well characterized, especially because the distinction between nascent adhesion, focal complex and focal adhesion is not always obvious: the notion of continuum is increasingly preferable to the existence of

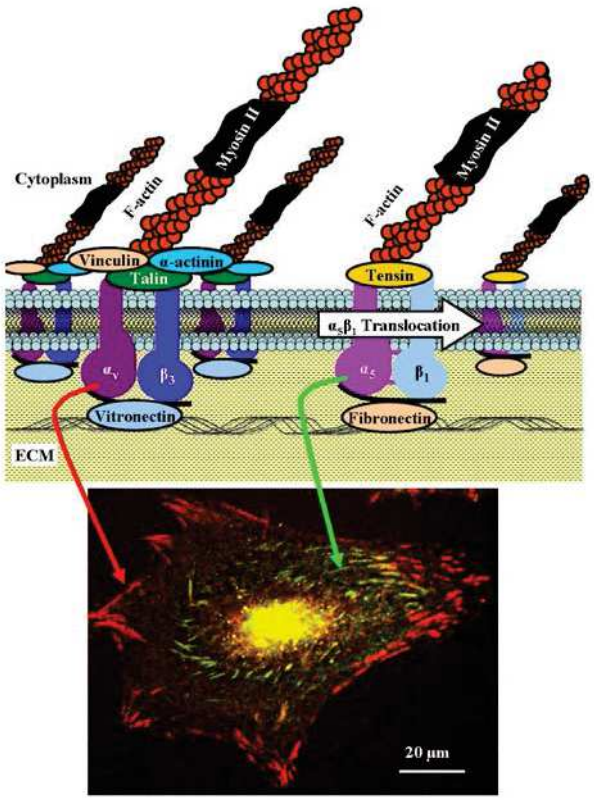


Figure 4.5. Organization of focal and fibrillar adhesions.
Focal adhesions are elongated spindle-like structures located at the periphery of the cell that connect bundles of actin stress fibers (F-actin) via many proteins, including avβ3 integrins and structural proteins like a-actinin, vinculin and talin. Fibrillar adhesions are more centrally located and contain extracellular fibronectin, a5β1 integrins and tensin. The translocation of fibrillar adhesions is highly directional, starting centripetally from the cell periphery towards the center. Double immunostaining for aV (red) and a5 (green) in cells attached to fibronectin revealed the segregation between focal and fibrillar adhesion. Adapted from Marquis et al., 2009.

distinct classes of adhesions (Parsons et al., 2010). These adhesions contain, in addition to integrins, a number of structural proteins: talin, paxillin, vinculin and probably kindlins (Figure 4.5). FAK and Src signaling proteins are also present, they occur in particular in the regulation of assembly and disassembly of these adhesions *via* activity of adapter proteins such as paxillin, ERK or MLCK (Webb et al., 2004). The VASP proteins (vasodilatator stimulated phosphoprotein) and Arp2/3, promoting actin polymerization, are recruited by vinculin and FAK. Arp2/3 also promotes the branching of actin therefore aiding the propagation of the lamellipodia. Local stimulation of the polymerization and the branching of actin promotes the assembly of nascent adhesions (Choi et al., 2008) and indirectly promote the formation of complex by grouping probably focal integrins located in the vicinity of newly formed adhesions (Geiger and Bershadsky, 2001). The nascent adhesions are not sensitive to agents depolymerizing actin, which is why they are supposed to be independent of mechanical forces. For focal complexes, this insensitivity to tension forces is questioning due to the presence of mechanosensitive proteins such as vinculin, and RPTPα (Receptor Protein-Tyrosine Phosphatase α), p130CAS, etc.

4.3.2. Focal adhesions

Focal adhesions are best characterized as incipient adhesions or focal complexes. Discovered by electron microscopy (Abercrombie et al., 1971) on adherent cells cuts *in vitro*, these adhesions have also been observed *in vivo* (Lo et al., 1997). Focal adhesions are elongated structures about 2 µm wide and 3 to 10 µm long, extended by the stress fibers of actomyosin and have a long life (between 30 and 90 minutes). Their recruitment is stimulated by the Rho-GTPase RhoA *via* its action on ROCK and mDia. ROCK activates the light chain of myosin II both by direct phosphorylation and indirectly by inhibiting its MLCP phosphatase *via* phosphorylation the MYPT-1 subunit. This allows the formation of bipolar antiparallel filaments of myosin II and their interaction with the actin

filaments to assemble into contractile stress fibers. All this increases the internal tension. That mechanical tension will promote the clustering of the integrins, the formation of the focal adhesions and their maturation. Many proteins take part in scaffolding the focal adhesions others are transiently associated. More than 180 proteins and 690 interactions have been described. All these adhesions associated proteins is called "adhesome" (Zaidel-Bar et al., 2007; Zaidel-Bar and Geiger, 2010; Schiller et al., 2011a). These proteins have been grouped into 17 classes according to their biological activity. There are structural proteins (talin, paxillin, vinculin, the a-actinin, zyxin, etc.), Tyrosine kinase signaling proteins - FAK, Src, PYK2 *(Proline-rich tyrosine kinase 2)*, serin-treonin kinases - ILK (Integrin like kinase), PKC), regulators of small GTPases - p190RhoGAP, p190RhoGEF, tyrosine phosphatase, regulators of actin polymerization. Most important proteins are shown in Figure 4.5. The composition of adhesions depends on the type of integrins involved in connection with the matrix (Worth et al., 2010), but also the contractile state of the cell (Kuo et al., 2011; Schiller et al., 2011a). A recent proteomic study identified 905 proteins in adhesions (Humphries et al., 2009b). For 459 of them, their abundance in adhesions varies with the inhibition of myosin II. 73% of these proteins are depleted in adhesions when myosin is inhibited, among them are regulators of RhoA activity, proteins activating the formation of stress fibers and proteins involved in disassembling adhesions. In contrast, other proteins (27%) are enriched in adhesions when myosin is inhibited; they are essentially the proteins of the nascent adhesions such as the regulators of the activity of Rac1. It is unlikely that all these proteins are directly dependent on the activity of myosin, these changes in the proteome of the adhesions should rather be the result of a molecular cascade initiated by mechanosensitive proteins. The transmembrane proteoglycans, containing extracellular heparan sulfates, are co-receptors of cell adhesion. They participate in the regulation of focal adhesions and sometimes act in synergy with the integrins. Similar is the case for some growth receptors that work in cooperation with integrins (Fourel et al., 2016). Specifically

β3 integrin is required to interact with BMP receptors in order to control Smad signaling and mechanical stress homeostasis (Fourel et al., 2016).
Disassembly of focal adhesions resulting from both FAK signaling, Src *via* phosphorylartion of paxillin, ERK and MLCK (Webb et al., 2004), the proteolytic cleavage induced by calpain, the targeting microtubules and endocytosis (Dubash et al., 2009). Inhibition of contractility in stress fibers results in rapid disassembly of focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996), whereas disassembly of microtubules induces their formation (Enomoto, 1996).

4.3.3. Fibrillar adhesions

Fibrillar adhesions are observed only on fibronectin ECM. They are thinner and longer than the focal adhesions, they are also more central position in the cell and not perform like focal adhesions. They are dragged by centripetal translocation of integrin *a*5β1 along contractile stress fibers (Zamir et al., 1999, 2000b; Pankov et al., 2000). This translocation is highly dependent on the intracellular contractility generated by myosin II and ROCK signaling. Meanwhile, fibronectin molecules bound to integrins are stretched, revealing cryptic sites allowing their assembly into fibrils parallel to fibronectin stress fibers and fibrillar adhesions (Pankov et al., 2000). This process is called fibrillogenesis. Fibrillar adhesions are depleted of talin and paxillin, but enriched in a-actinin or tensin (Figure 4.5).

4.4. Integrin endocytosis

Integrins (both a and β chains) are internalized by different ways like by macropinocytosis, clathrin-dependent endocytosis and clathrin-independent endocytosis, which includes endocytosis mediated by caveolae and clathrin-independent carriers (CLICs) (Bridgewater et al., 2012). The integrin heterodimers can be endocytosed independently on their attachment to the ligand. There is

clear data that the internalization and the turnover of the integrin dimers is important step for regulation the cell migration in both 2D and 3D matrices (Caswell and Norman, 2008; Valdembri and Serini, 2012; Webb et al., 2002).

4.4.1. Clathrin dependent endocytosis

A classical pathway for clathrin dependent integrin endocytosis is presented at Figure 4.6A. The inactive heterodimer links with adapter proteins like Dab2, forms clathrin coated pit and is internalized. Interestingly if cells do not sense enough physical resistance from the ECM β3 integrin clusters quickly recruit Dab2 and attract the endocytic machinery (Yu et al., 2015).

FA turnover can be facilitated by the microtubules (and more specifically kinesin 15). Figure 4.6B illustrates the delivery of the necessary MMP to cut the ECM links and free the bonded integrins and the molecular players that carry out the integrin endocytosis, including dynamin 2 (Dyn2) (Ezratty et al., 2005; Stehbens et al., 2014). Dyn2 polymerize around the budding vesicle and cut it from the PM (McMahon and Boucrot, 2011). Several adaptors like Dab2, Eps8 and Numb are

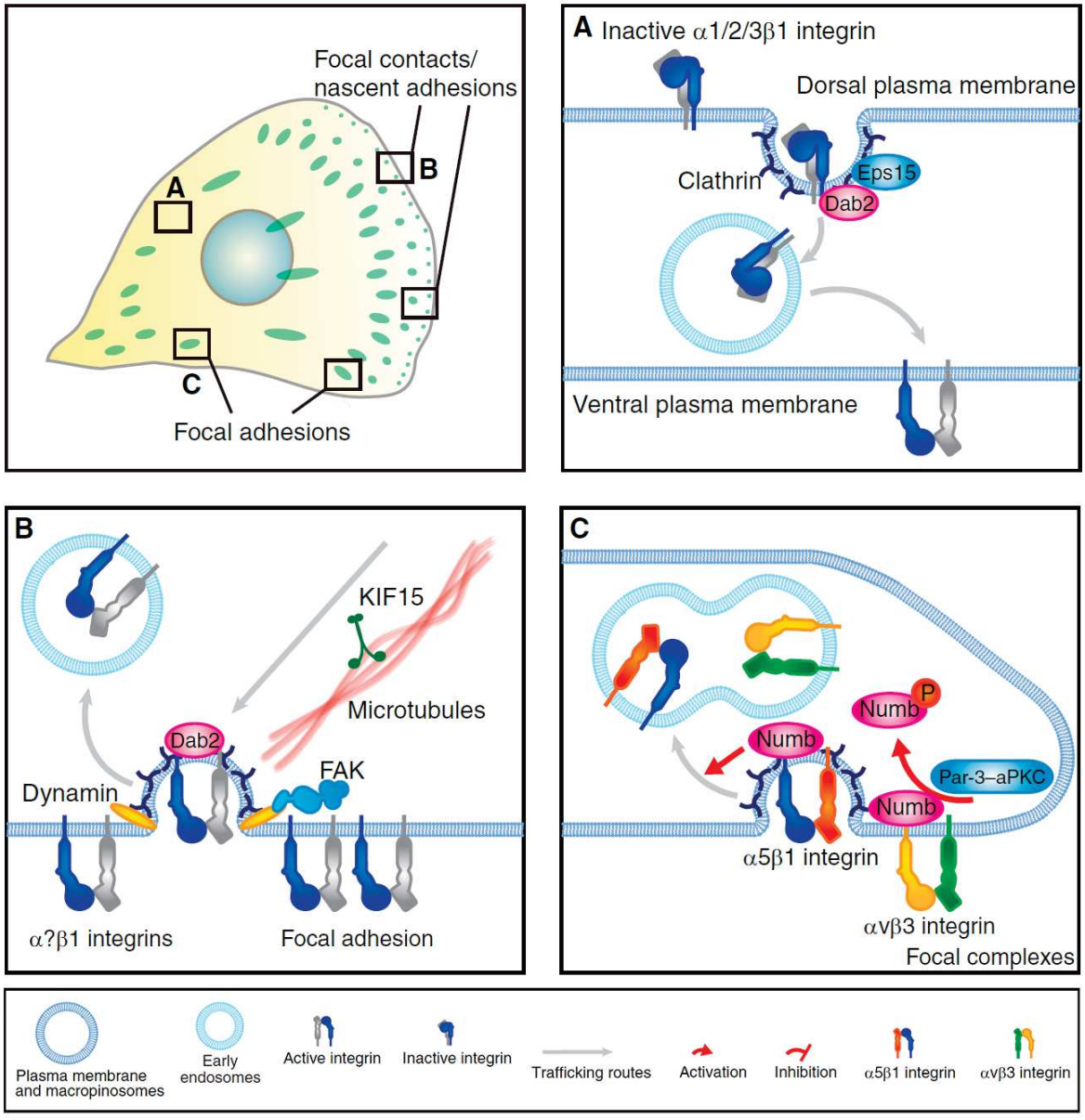


Figure 4.6. Clatrin dependent endocytosis and adhesion turnover.
A. Clatrin dependent endocytosis of β1 integrin via the adapter protein Dab2. B. Microtubule dependent adhesion disassembly leads to deliver of Dab2 at the vicinity of the FA.
C. The cycle of phosphorylation of the adapter protein Numb regulates endocytosis of β1 and β3 integrins at the leading edge of the cell. Adapted from Paul et al., 2015.

involved in the regulation of clathrin dependent endocytosis that interact directly via their phosphotyrosine-binding (PTB) domains with conserved NPxY/NxxY motifs

within β-subunit cytoplasmic tails (Calderwood et al., 2003) Figure 4.6C. Numb is of particular interest since it binds β1 and β3 integrin at the PM. Together with the adapter protein Ap2 regulate the turnover of β1 and β3 integrin at the leading edge to facilitate the cell migration (Nishimura and Kaibuchi, 2007). Even more the clathrin-dependent endocytosis relies on ARF GTPases: the Arf6 GAP ARAP2 (ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2) is necessary for β1 integrin internalization and disassembly of the FA (Chen et al., 2014).

4.4.2. Clathrin-independent ways of integrin endocytosis

The biogenesis of clathrin-independent carriers (CLICs) at the PM requires s glycosphingolipids, the N-glycan-binding protein galectin-3 and GRAF1 (GTPase regulator associated with FAK 1) (Lakshminarayan et al., 2014; Doherty et al., 2011). Figure 4.7A shows that GARF1 binds phosphorylated form of FAK and is reported to localizes to podosome-like adhesions in HeLa and is necessary for cell migration (Doherty et al., 2011). Caveolae are specific membrane domains, rich in cholesterol and sphingolipids that are important both for clathrin-independent and clathrin-dependent transport of integrins (Figure 4.7B). Integrin endocytosis via caveolae has been demonstrated (Upla et al., 2004; Shi and Sottile, 2008a; Du et al., 2011).

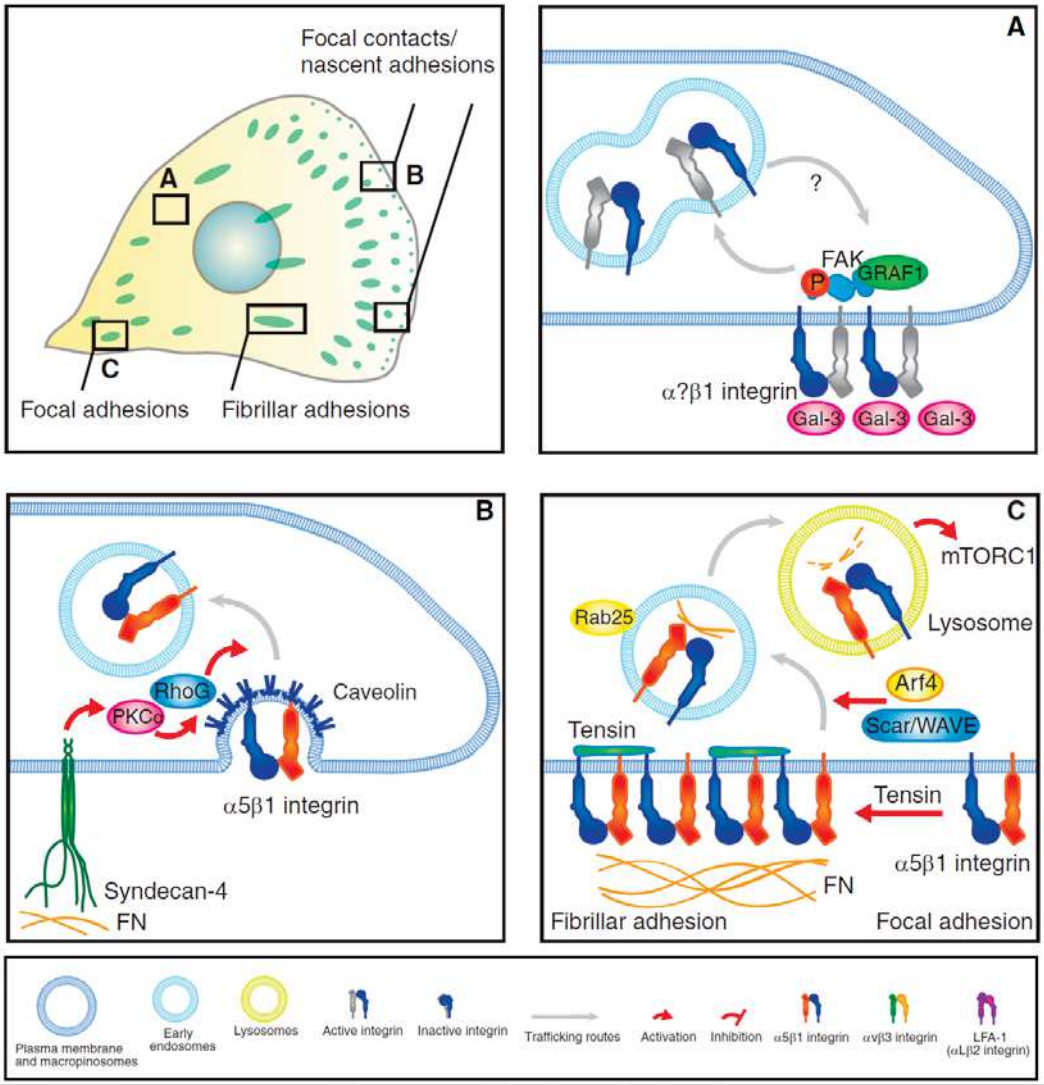


Figure 4.7. Clatrin independent endocytosis and adhesion turnover.
A. CLICs require transmembrane galectin 3 and intracellular GRAF1 to form complex with pFAK in order to facilitate endocytosis of β1 integrin.
B. Syndecan-4 (and ECM receptor, that binds among other ligands FbN). It activates PKCa and RhoG and induces the internalization of β1 integrin in the caveolae.
C. A peculiar method for internalizing a5β1 integrin from fibrillar adhesions does not require Dyn2 or clathrin, but Arf4 and SCAR/WAVE. The charged integrins are transported to the lysosomes where they activate mTOR pathway. Adapted from Paul et al., 2015.

Chapter 5. Mechanosensing

Cells feel many chemical and physical parameters of the environment and they respond. They adapt their adhesive structures to external constraints such as rigidity or density of the extracellular matrix (Albiges-Rizo et al., 2009). The forces exerted on the cells by outside factors (external or internal) are felt at adhesion structures regardless of their origin (Riveline et al., 2001). The stretching of adherent cells on elastic substrates while as the activation of myosin causes an increase in the size of focal adhesions and activation of FAK (Hamasaki et al., 1995). The focal adhesions are mechano-sensitive (Riveline et al., 2001; Collin et al., 2008). Maturation and growth of focal adhesions require strengthening of the cell/matrix bond in response to the forces exerted by the extracellular microenvironment. This capacity depends on the integrin β1(Friedland et al., 2009), talin(Zhang et al., 2008) and also involves ROCK, who activates myosin, and mDia (formin) that promotes the nucleation of actin and elongation of a parallel network of actin filaments (Chrzanowska-Wodnicka and Burridge, 1996; Rottner et al., 1999). Myosin exerts a force of 5,2 nN/μm^2 on focal adhesions (Schwarz et al., 2002) which exerts a force of 5,5 nN/μm^2 on the substrate (Schwarz et al., 2002). The activation of myosin and the increased cellular contractility changes the protein composition of adhesions (Kuo et al., 2011).

Cellular sensitivity to extracellular stiffness depends on at least two important parameters: the dynamics of adhesion sites and cytoskeletal tension (Fereol et al., 2009), which themselves depend on the components of the physical link between the extracellular matrix and intracellular cytoskeleton (Schwartz, 2010).

Cellular Adaptation to the biophysical properties of the microenvironment requires at adhesion sites the presence of molecular factors capable of adhering, applying pressure and transforming a mechanical signal into an intracellular biochemical signal: this phenomenon is called mechanotransduction.

I. Introduction

Although cells possess several force sensing devices (such as force sensitive channels (Kobayashi and Sokabe, 2010), and cell–cell adhesions (Huveneers and de Rooij, 2013), due to space constraints we focus here on mechanotransduction events at integrin-associated complexes. These directly connect the ECM with the actin cytoskeleton and are one of the major contributors to mechanotransduction. We will discuss protein dynamics of integrin associated complex in relation to their function in mechanosensing and −signaling.

Four key parameters determine the range of rigidity that the cell can sense via the integrins: the binding force between the integrins and the matrix, the traction force of the cells, the speed of this retraction and the sensitivity of the mechanosensors (Moore et al., 2010). In addition, five types of successive noncovalent interactions are required for the development of forces at the level of the cellular binding with the extracellular matrix: the interaction between myosin and actin, the polymerization of monomeric actin into filaments, the interaction of these filaments with actin binding proteins, the interaction of these proteins with integrins, and finally the interaction of integrins with the extracellular matrix (Moore et al., 2010). The forces of interactions between the actin binding proteins or the integrins are still poorly known.

5.1. Integrins as mechanoreceptors

Complex conformational changes of the integrins control both their affinity for ECM proteins and their association with cytoskeletal partners. In the formation of adhesive contacts, integrins follow a mechanical cycle(Puklin-Faucher and Sheetz, 2009): they bind ECM and cytoskeletal filaments, transforming mechanical forces into intracellular biochemical signal, cluster together and form growing adhesion where the strength of resistance increases and in time, they detach and are being subsequently recycled. Experimental data suggest that the conformation of integrins a5β1 can be modulated by external forces.

The conformation of fibronectin-related integrin a5β1 is initially loose; it corresponds to the pre-activated state of the integrin and is force independent. The exertion of mechanical forces modifies this conformation to and extended and fully active form where integrin binding to fibronectin is enhanced by the presence of an additional interaction between integrin and fibronectin at the synergistic site(Friedland et al., 2009). This bond, reinforced and stabilized by the force, is called "catch bond"(Dembo et al., 1988) and is characteristic for a5β1. The bindings destabilized by mechanical tension are called "slip bonds" and are employed by integrins aVβ3. However, functional differences exist between the different bindings: the slip bond is less resistant to forces than that catch bond, but it does initiates signal transduction(Roca-Cusachs et al., 2009).

5.2. Mechanosensors associated with integrins

There are five mechanisms (Moore et al., 2010) that are responsible for cellular mechanosensitivity via integrins: the formation of physical attachment bonds between integrins and their ligands and between actin and myosin, the opening of transmembrane mechanosensitive channels, regulation the exposure of phosphorylation sites and exposure of binding sites of certain enzymes. Among these mechanisms, several are due to changes in protein conformation that are mechanosensitive, which positively or negatively regulate the activity of proteins.

I. Introduction

The mechanosensitive channels are transmembrane ion channels capable of converting a mechanical force into an electrochemical signal. They are mostly described in sensory mechanisms as hearing (Chalfie, 2009). However, mechanical stress generated by integrin-bound beads on the surface of endothelial cells causes calcium to enter in a few seconds (Matthews et al., 2006), so there are mechanosensitive ion channels coupled to integrins or activated by the mechanical strain on the integrins.

The activity of several kinases depends on the extracellular rigidity (Table 2) (Paszek et al., 2005).

Adhesion parameter	Soft matrix	Stiff matrix
Adhesion	+++	+++
Adhesion strength	+++	+++
Adhesion size	++	++++++
β1 integrin	+++	+++
Activated β1 integrin	+++	+++
Talin	+++	+/-
Lck & Lck^{pY505}	+++++	+/-
Lynz & Lyn^{pY507}	+++++	-
SrcpY416	+++++	+
Src	+	+++
FAK^{pY861}	+++	+++
FAK^{pY397}	-	+++
Vinculin	-	+++
Actin stress fibers	+	+++
ERK activation	+	+++

Table 2. The extracellular stiffness influences the behavior of epithelial cells (Paszek et al., 2005).

The most well-known is FAK (Focal Adhesion Kinase) protein kinase, whose activity increases with mechanical forces (Wang et al., 2001; Michael et al., 2009). The kinases of the Src kinase family are quickly (300 ms) activated by the RPTP protein in response to the application of extracellular forces by fibronectin beads (von Wichert et al., 2003). However, RPTPa is not directly activated by mechanical forces (Moore et al., 2010). Some proteins change their conformation in response to their mechanical stretching. These changes allow the exposure of so-called cryptic sites which may be phosphorylation sites, as is the case for the Cas protein family(Sawada et al., 2006), or sites of interactions. The interaction of paxillin, FAK protein, p130Cas with the cytoskeleton increases with tension (Sawada and Sheetz, 2002). The binding of vinculin to talin is dependent

on stretching of the latter (del Rio et al., 2009). This stretching leads to the exposure of specific binding sites for vinculin.

Recently the binding between talin and vinculin was clarified using an innovative approach (Hu et al., 2016) - monitoring the *in situ* dynamics of the talin dimer stretch it was shown that that optimal vinculin and vinculin head binding occurred when talin was stretched to 180 nm. Also, multiple vinculins bound within a single second in narrowly localized regions of the talin rod during stretching. As a conclusion, talin stretches as an antiparallel dimer and that activates vinculin

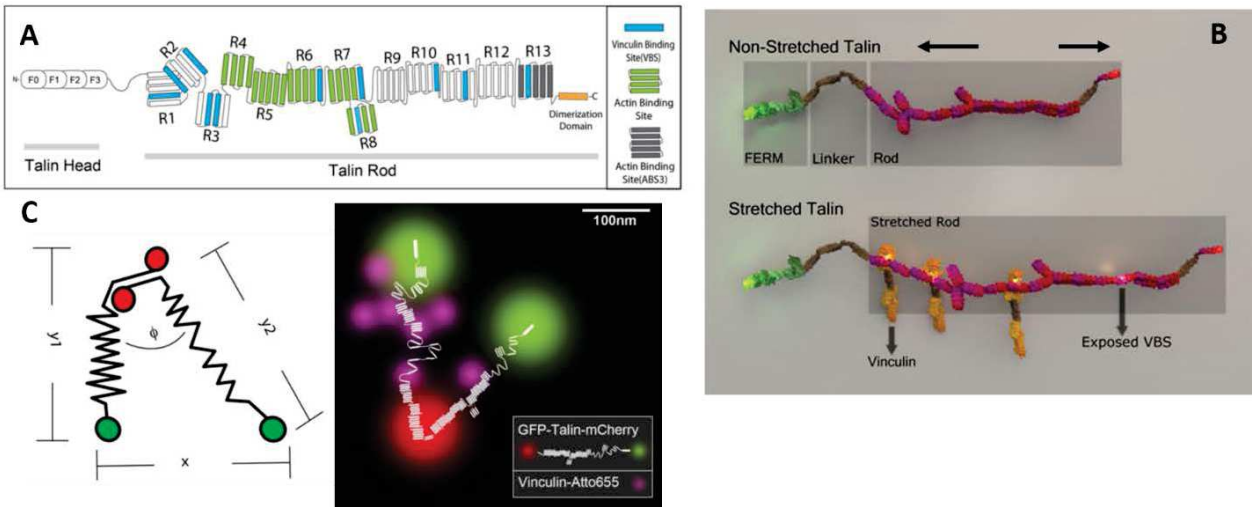


Figure 5.1. Interaction between talin and vinculin is facilitated if talin is stretched mechanically.
A. Talin head domain contains a FERM domain (50 kDa), followed by a flexible "neck" (10 kDa), which is followed by the head domain to its C-terminal rod domain (220 kDa). In blue are presented the vinculin binding parts (see legend).
B. Under the action of force in the direction of the black arrows, the tail of the talin begins to stretch. When the vinculin binding sites (VBS) are exposed to the vinculin, the latter binds to the talin.
C. Hu et al., 2016 proposed that talin do forms antiparallel dimers that activate the cooperative recruitment of vinculin. Extracted from Hu et al., 2016

binding in a cooperative manner, consistent with the stabilization of folded talin by other binding proteins (Figure 5.1).

I. Introduction

Several years ago (Grashoff et al., 2010) it has been created a biosensor of the mechanical tension applied in the adhesions: it is built on the base of vinculin, where a stretchable protein motif was surrounded by two fluorescent proteins and it has been inserted between the head and the tail of the vinculin (Figure 5.2). Vinculin binds talin and thus indirectly integrins and the extracellular matrix with its head and the contractile actin cytoskeleton with its tail. In this position the sensor is able to feel the changes in tension between the inside and the outside of the cell. These force variations are measurable via the level of energy transfer between the two fluorescent proteins (FRET) (Doyle and Yamada, 2010).

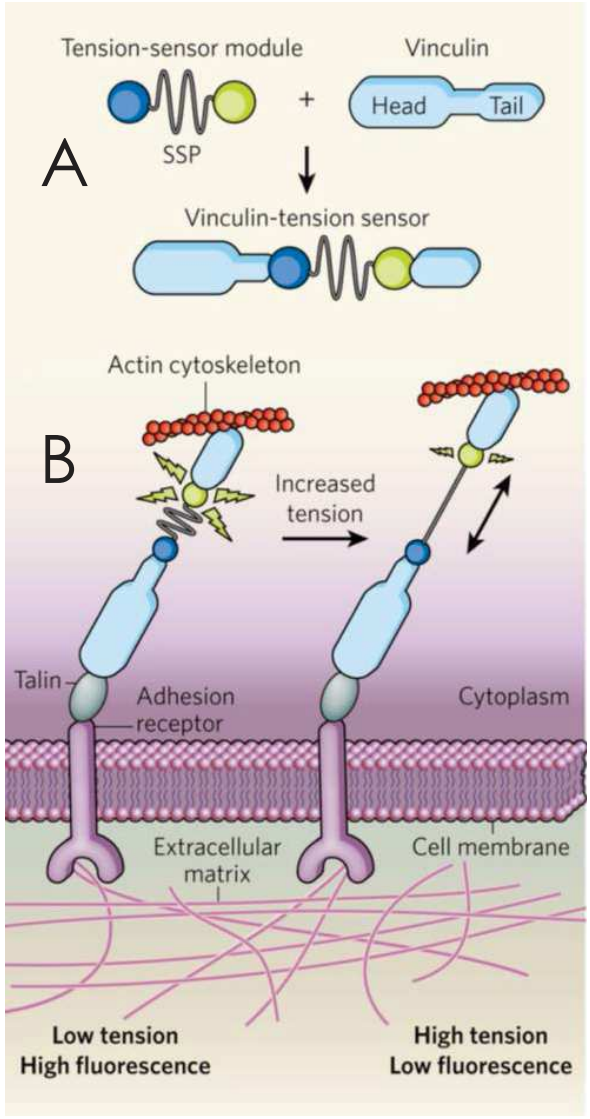


Figure 5.2. Force biosensor.
A. An elastic fragment of an SSP spider silk protein surrounded by two fluorescent proteins was introduced into the vinculin.
B. An increase in the tension in the biosensor causes a stretching of the SSP module and therefore a remoteness of the fluorescent proteins and a reduction of the energy transfer (FRET). Adopted from Doyle and Yamada, 2010.

5.3. Mechanotransduction at a distance

Within the focal adhesions, the integrins are linked to the actin cytoskeleton. Actin network is also connected to nuclear structural molecules (lamines), chromatin and the DNA. Mechanical forces applied to the cell surface at the integrins not

only activate signaling pathways from the membrane but also cause structural rearrangements in the cytoplasm and nucleus (Maniotis et al., 1997). Mechanical forces applied to the cell surface can act at a distance and can be converted into a chemical-mechanical signal directly into the nucleus in addition to the cytoplasmic mechanotransduction initiated at the plasma membrane by the integrin-associated mechanosensors. This structural connection linked to the possibility of propagating forces over long distances (at the cell scale) is called tensegrity (Ingber, 2003b; a). Mechanical linkage allows mechanical propagation of the signal faster than the propagation of a chemical signal (Figure 5.3A). Numerous proteins are involved in the continuity of the physical bond between the extracellular matrix and the nucleus, in particular the focal adhesion components, the actin cytoskeleton and the nesprine/SUN/lamin network present at the nuclear envelope (figure 5.3B). The deformation of the nuclear envelope induced by a force applied to the surface of the cell stimulates an entry of calcium through nuclear ion channels, which induces the expression of certain genes. The identity of these mechanosensitive nuclear channels is still unknown, but these are certainly related to the mechanosensitive cytoskeleton and to the structural proteins of the nucleus(Wang et al., 2009). Different mechanisms for converting the mechanical signal received by the nucleus into a biochemical signal are possible. The opening of tension-sensitive nuclear pores can modify the transcriptional state of the nucleus. The deformation of the nuclear envelope can reflect on the chromatin and make it more or less accessible to the transcription factors; this deformation can also cause the double helix of DNA to be opened via attachment to the nuclear matrix; the deformation of intra-nuclear structural

molecules allows the recruitment of factors involved in transcriptional regulation or chromatin modification(Wang et al., 2009).

Finally, the mechanosensitivity of the cells is a global phenomenon, at the level of the adhesions and at the whole cell level. This sensitivity relies on the intracellular mechanical integrity due to the cytoskeletons and the mechanical continuity with the ECM thanks to the connection of the actin cytoskeleton to the extracellular matrix via integrins and their cytoplasmic partners.

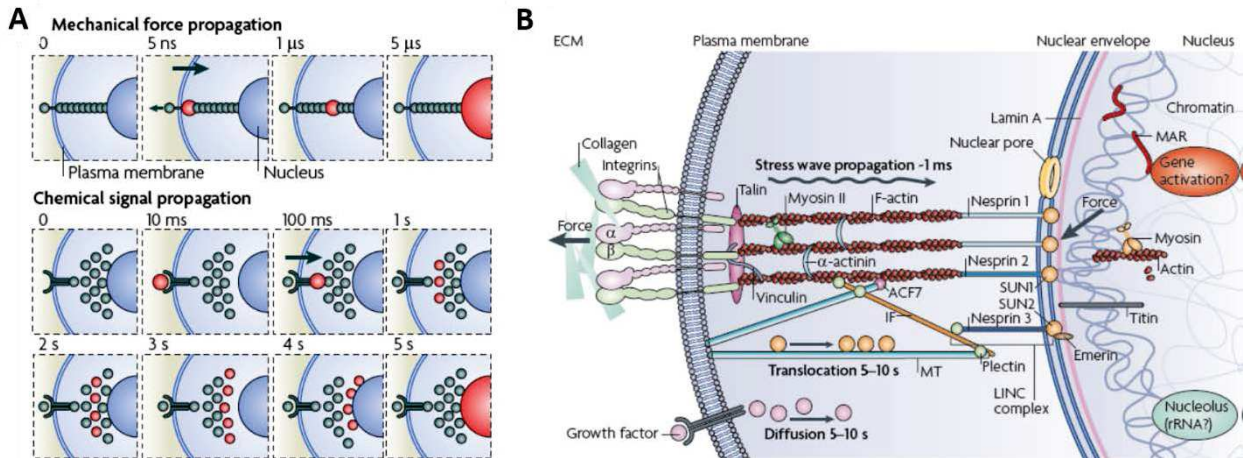


Figure 5.3. Mechanical transduction at a distance.
A. Comparison of the propagation of a mechanical or chemical signal. A force applied to the integrin-bound cytoskeleton reaches the nucleus in less than 5 µs (top). The mechanical signal transmitted to the cytoskeleton is symbolized by the red dot to the nucleus. A growth factor-type chemical signal is received by tyrosine kinase receptors and propagates into the cytoplasm by a secondary (lower) messenger activation cascade. The signal reaches the nucleus in 5 s. The arrows indicate the direction of the applied force (top) or direction of the propagation signal through the membrane (bottom).
B. The molecular connection between the extracellular matrix and the nucleus involves on the one hand the integrins and the focal adhesions and on the other hand the nesprins linked to the SUN proteins inserted in the internal nuclear membrane themselves connected to chromatin and laminins responsible for the structure of the nucleus and finally the actin cytoskeleton which physically connect the two previous macromolecular structures. MT: microtubules, IF intermediate filaments. Adapted from Wang et al. (2009).

Chapter 6. ICAP-1 as a regulator of the cellular mechanoresponse

The ICAP-1 protein (integrin cytoplasmic domain associated protein-1) has been identified by screening of the cytoplasmic partners of the integrin subunit β1A by the two-hybrid technique (Chang et al., 1997). This interaction was confirmed by several other labs (Zhang and Hemler, 1999a; Degani et al., 2002; Ng et al., 2014; Morse et al., 2014). It is a small protein of 200 amino acids (21 kDa). It is formed by two protein domains: a serine and threonine rich domain and a phosphotyrosine binding domain (PTB), which is able to interact with β1 integrin (Figure 6.1).

ICAP-1 is a phosphoprotein within eukaryotic cells (Zhang and Hemler, 1999a). The phosphorylation sites include, PKA, PKC, and CamKII consensus phosphorylation motifs, all located in the N-terminal half of the protein. The T38D mutant of ICAP-1 that mimics the phosphorylated form of the protein strongly increases its

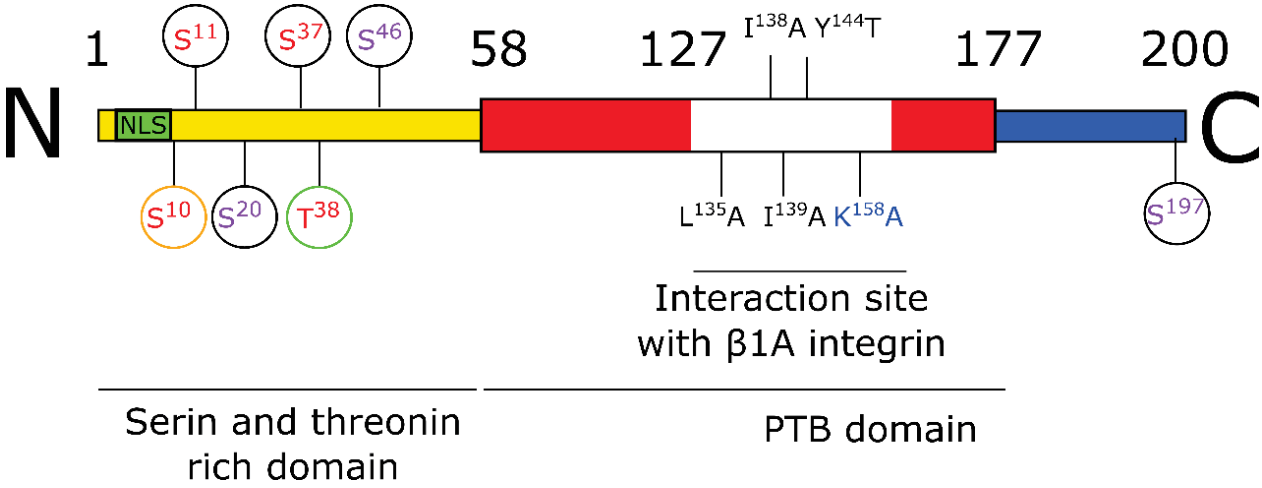


Figure 6.1 ICAP-1. The serine and threonine rich domain form a nuclear localization signal (NLS) and numerous consensus phosphorylation sites (circled): in red the sites recognized by ROCK (RxxS/T or RxS/T), in violet the protein Kinase C (S/TxK/R), orange protein kinase A (RxS/T or RR/KxS/T), green CamKII (I/LxRxxS/T). The mutations indicated in the binding domain with the cytoplasmic part of integrin β1 are known to block this protein interaction.

interaction with β1 integrin (Morse et al., 2014) and impairs CHO cell spreading on

fibronectin, suggesting that ICAP-1 behaves as a negative regulator of β1 integrin-mediated cell adhesion under the control of protein phosphorylation (Bouvard and Block, 1998). ICAP-1 interacts specifically with the C-terminal NPXY motif of the β1 integrin cytoplasmic domain. Alanine scanning mutagenesis of the cytoplasmic tale of β1 integrin reveals that Val(787), Val(790), and (792)NPKY(795) are critical for ICAP-1 binding. The NPXY motif is a known binding site for phosphotyrosine binding (PTB) domain proteins and, computational modeling reveals that amino acids 58– 200 can fold into a PTB motif (Domain et al., 2002) .

The specificity of interaction with the various integrin subunits was analyzed in double yeast hybrids and by in vitro interaction assays (Chang et al., 1997; Zhang and Hemler, 1999a; Degani et al., 2002). ICAP-1 does not bind to the subunits β1B, 1D, 2, 3 nor to the a2, 3, 4, 5, 6, V, L subunits. Mutagenesis implicated the integrin β1 tail NPKY motif and a preceding VTTV sequence as important for ICAP1 binding, and molecular modeling suggested the interaction happens via a classical PTB-domain ligand interaction (Domain et al., 2002). Consistent with published mutagenic analyses (Domain et al., 2002), more recent structural studies (Liu et al., 2013) show that the interaction is mediated by integrin β1 residues S783I to N792I (SAVTTVVN). This interaction interface is highly conserved and broadly hydrophobic, with V787I and V790I packing against hydrophobic patches. Surprisingly, although the β1 integrin N792PxY motif binds ICAP1 and mutagenesis confirms the importance of Y795I(Domain et al., 2002), Y795I binding to the ICAP1 PTB domain is not observed in the crystal structure.

The binding of ICAP-1 to subunit β1 is specific of the isoform A at the second site NPxY^{795}. Valines at position -5 and -8 by tyrosine are essential for interaction (Domain et al., 2002). From 8 β integrin chains, only β1A contains the pattern VxxVxNPxY which explains its molecular specificity. However this specificity was questioned (Degani et al., 2002) by showing a two hybrid interaction between ICAP-1 and the β5 integrin tail, contrary to the preceding groups. This interaction does not seem to have been confirmed to date.

6.1. ICAP-1: a cell adhesion regulator via the β1 integrin

The ICAP-1 protein is a negative regulator of the cellular spreading that engages β1 integrin. This has been shown by overexpression (Bouvard et al., 2003; Degani et al., 2002) and by depletion (Bouvard et al., 2007) of the ICAP-1 protein on ECM which can be recognized by β1 integrin such as fibronectin, laminin or collagen. The interaction of ICAP-1 with β1 integrin negatively affects the integrin's affinity for its ligand (Bouvard et al., 2007; Millon-Frémillon et al., 2008). During the initial stages of spreading, ICAP-1 is localized at the cellular periphery at the level of the lamellipodia with integrins β1 (Fournier et al., 2002a). Despite the direct interaction between ICAP-1 and integrins β1A, ICAP-1 has never been visualized in focal adhesions (Bouvard et al., 2003) even though the deletion of ICAP-1 leads to the redistribution of focal adhesion all over the ventral face of the osteoblastic, fibroblastic and endothelial cells. The increase of β1 integrin– dependent cell motility on fibronectin upon ICAP-1 overexpression (Chang et al., 1997; Domain et al., 2002; Zhang and Hemler, 1999a), the increase of collective migration (Zhang and Hemler, 1999a; Alvarez et al., 2008) and cell rounding up after overexpression of a phosphomimetic mutant of ICAP-1 at the CaMKII site (Brunner et al., 2011b; Millon-Frémillon et al., 2013) suggest that ICAP-1 might regulate β1 -integrin function. Biochemical studies and FRAP analysis have highlighted the impact of ICAP-1 on cell adhesion dynamics through its ability to slow down focal adhesion assembly by competing with the co-activator of talin called kindlin (Bouvard et al., 2003; Millon-Frémillon et al., 2008; Brunner et al., 2011b). Unexpectedly, the ICAP-1-dependent decrease in integrin affinity allows cell sensing of matrix surface density suggesting that ICAP-1 might be involved in mechanotransduction process. Later on, the lab has demonstrated the importance of ICAP-1 in inhibiting ROCK1-mediated cell contractility via the regulation of β1 integrin affinity (Faurobert and Albiges-Rizo, 2010). Altogether, these results clearly demonstrated

that a switch between high and low affinity integrin states is required to control cell contractility and to drive an integrated cell response that is appropriate for the ECM environment.

6.2. ICAP-1: a regulator of cell proliferation through regulation of β1 integrin.

Over-expression of ICAP-1 stimulates cell proliferation whereas its depletion slows down the cell growth (Henri-Noel Fournier, Sandra Dupe-Manet, Daniel Bouvard et al., 2005; Bouvard et al., 2007). The protein ICAP-1 has a functional nuclear localization signal (KKNH[9]) and is targeted to the nucleus after the first steps of the adhesion. The double substitution of lysines in alanines abolishes its nuclear localizing signal. This nuclear location depends on the adhesion engagement of the β1 integrins and stimulates proliferation via activation of the c-myc gene (Henri-Noel Fournier, Sandra Dupe-Manet, Daniel Bouvard et al., 2005). It has been shown that activation is related to the interaction between ICAP-1 and the protein Nm23-H2 (Henri-Noel Fournier, Sandra Dupe-Manet, Daniel Bouvard et al., 2005).

6.3. ICAP-1: a regulator of cell homeostasis and tissue integrity

The PTB domain allows ICAP-1 to interact with the NPxY motifs. However, these are present in many proteins. Thus, proteins with PTB domains can have several partners with NPxY motifs as well as proteins with NPxY motifs may have multiple partners with PTB domains. These motifs are, of course, present in the cytoplasmic domains of integrins (Brunner et al., 2011b; Faurobert and Albiges-Rizo, 2010; Ren et al., 1999) (Figure 3.3). At present, about ten proteins have been described as partners for ICAP-1 (Table 3).

Partner	Method of identification	Recognized motif	Reference
ECM receptor			
Integrin β1A	Two hybrid technique, pull down, co-immunoprecipitation	NPxY	Chang et al. (1997); Zhang et Hemler (1999); Degani et al. (2002)
Integrin β5	Two hybrid technique	NPxY	Zhang et al. (2002)
LDL receptors			
LRP-1	Two hybrid technique, pull down	NPxY?	Gotthardt et al. (2000)
ApoER2	Two hybrid technique	NPxY?	Gotthardt et al. (2000)
Megaline	Two hybrid technique, pull down	NPxY?	Gotthardt et al. (2000)
Signaling proteins			
Rac1	Pull down	?	Degani et al. (2002)
Cdc42	Pull down	?	Degani et al. (2002)
ROCK	Two hybrid technique, co-immunoprecipitation, FRET	?	Stroeken et al. (2006)
Others			
Krit-1/CCM1	Two hybrid technique, pull down, co-immunoprecipitation	NPxY	Zhang et al. (2001) ; Zawistowski et al. (2002)
Nm23-H2	Two hybrid technique, pull down, ELISA	?	Fournier et al. (2002)

Table 3. Proteins, reported to interact with ICAP-1.

6.3.1. ICAP-1 regulates cell adhesion independently on β1 integrins

Nm23 proteins are proteins kinases that catalyze the phosphorylation of nucleotide diphosphates (NDP) from nucleotide triphosphates (NTP), allowing a transfer of energy from the ATP to the GTP, that are involved in many cellular processes. The interaction between ICAP-1 and Nm23-H2 was shown by the our laboratory and later confirmed (Miyamoto et al., 2009). The complex ICAP-1-Nm23-H2 co-locates with β1 integrins in early stages of cell spreading. This localization of Nm23-H2 by ICAP-1 at the integrins suggests that Nm23-H2 might modulate signaling induced by Rho-GTPases activated by integrin engagement during cell adhesion (Fournier et al., 2003). It has been reported that ICAP-1 interacts with Rac1 and Cdc42 but not with RhoA (Degani et al., 2002). ICAP-1 inhibits the activation of these Rho-GTPases in the early stages of cell spread, but

expression of a constitutively active mutant of Cdc42 lifts the inhibition of ICAP-1-mediated spreading, indicating that ICAP-1 works upstream of the small protein G. The dissociation of the GDP with Cdc42 is reduced in the presence of ICAP-1 while dissociation of Cdc42 with the plasma membrane is increased by ICAP-1. This indicates that ICAP-1 would be a GDI (Guanine nucleotide Dissociation Inhibitor) for Cdc42. This function could not be shown for Rac1, indicating that regulation of Rac1 by ICAP-1 would be indirect. However, these results are surprising because the structure of ICAP-1 does not resemble the immunoglobulin domain of the conventional Rho-GDIs; these results also remain to be confirmed.
ROCK is a kinase involved in cellular contractility. It interacts with ICAP-1 (Stroeken et al., 2006) and the complex locates at the membrane edges with β1 integrins. ICAP-1 might recruit ROCK near β1 integrins, thus locating the activity of ROCK to the membrane. Inhibition of ROCK or depletion of ICAP-1 diminishes the collective cell migration (Alvarez et al., 2008). However, the biological function of ROCK-ICAP-1 complex remains little understood. This interaction suggests though that ICAP-1 could intervene in the control of intracellular contractility. As Nm23 proteins are also involved in endocytosis of receptors, it could not be excluded the involvement of ICAP-1 in integrin trafficking.

6.3.2. ICAP-1 takes part in osteoblast differentiation and angiogenesis

Krit-1 (Krev interaction trapped-1) / CCM1 is a protein with a FERM domain (band 4.1, ezrin, radixin, moesin) initially described as a partner of GTPase Rap1, also called Krev1 (Serebriiskii et al., 1997). Krit-1 maintains cell-cell junction integrity in the endothelium of cerebral vessels (Béraud-Dufour et al., 2007; Glading et al., 2007) and is associated with Congenital cerebrovascular disease CCM (Cerebral cavernous malformation) causing epilepsy and cerebral hemorrhages due to fragility of the endothelia of these vessels. Two other genes called CCM2 and CCM3 are associated with this disease and it has been shown in vitro that the three CCM proteins can associate to form a ternary complex (Hilder et al., 2007).

The interaction between Krit-1 and ICAP-1 was shown multiple times (Zhang et al., 2001; Zawistowski et al., 2002). Krit-1 has an NPxY motif and the interaction with ICAP-1 is carried out at the level of the PTB, Krit-1 is therefore in competition of the β1 integrins for ICAP-1 binding (Zhang et al., 2001). The binding of ICAP-1 to Krit-1 activates the latter by breaking of intramolecular interactions and unveils the FERM domain, an interactive platform with other proteins and allowing its localization to the cytoplasmic membrane (Béraud-Dufour et al., 2007). Loss of interaction between Krit-1 and ICAP-1 leads to the degradation by the Krit-1 as well as ICAP-1 by protesomal degradation (Béraud-Dufour et al., 2007). The binding of CCM2 to the
Krit-1-ICAP-1 complex inhibits translocation of the complex in the nucleus. Finally ICAP-1/CCM complex is at the crossroad between integrin-mediated FAs and cadherin mediated adhesion junctions(Faurobert and Albiges-Rizo, 2010).

6.4. The physiological Importance of ICAP-1

The ICAP-1 protein is expressed in all organs except the liver but its level of expression varies according to the tissues and cell types (Zhang and Hemler, 1999a). Although, to date, no genetic disease is associated with its loss or its increase in function due to mutations or genetic deletion of the icap-1 gene, the physiological importance of ICAP-1 can be emphasized and analyzed by the effect of its gene inactivation in mice (Bouvard et al., 2007). Unlike inactivation of its main partner, β1 integrin, inactivation of icap-1 is not lethal at the embryonic stage. At birth and in adulthood, ICAP-1-deficient mice are smaller than wild mice and have several phenotypes: neurological disorders, bone defects (Bouvard et al., 2007), fertility defects and vascular defects (Faurobert et al., 2013). The severity of this the last phenotype depends on the genetic background.
Bone defects are best described in the literature: deficient mice in ICAP-1 show growth retardation and retardation of bone mineralization, a craniofacial

malformation and a lack of ossification of the calvaria due to decreased proliferation and abnormal cell differentiation (Bouvard et al., 2007). In vitro, the ICAP-1$^{-/-}$ osteoblasts show defects in adhesion, cell migration, compaction and organization of fibronectin matrix (Bouin et al., 2017b) required for bone mineralization, explaining in part the bone phenotype observed in vivo (Bouvard et al., 2007; Millon-Frémillon et al., 2008; Brunner et al., 2011b). Osteoblasts expressing a mutated β1 integrin, that no longer interacting with ICAP-1 have the same defects as ICAP-1$^{-/-}$ osteoblasts[6,9]. This suggests that bone defects observed in vivo are due to the interaction between ICAP-1 and the β1 integrin.

II.
Scientific context and general aim of the study

Scientific context and general aim of the study

Cells perceive their environment by sensing the chemical and physical properties of the extracellular matrix as a result of the organization on its surface of adhesive machinery which is a proper molecular platform organized around the mechanoreceptors called Integrins. Cells are thus both able to adapt to the topography, the chemical composition and the elasticity of its environment, but they are also able to modify it (degradation, fibrillogenesis).

Usually cells held in place by direct bonding with a complex network of extracellular macromolecules secreted by the cell and also with neighboring cells. Cell adhesion assumes the existence of a physical interface consisting of a hierarchical multi-protein complex. Through this molecular complex, the cell may develop a mechanical action on the environment (the fibrillogenesis is exemplary) and in return, receive information from the environment in the form of stresses and strains transmitted until the nucleus. Adhesion is thus a key to controlling cell proliferation, but also cell migration and cell differentiation. These interactions between the cell and its environment are due to specific transmembrane proteins (first **integrins**) playing the role of **adhesion molecules** and who are known to be also sensitive to the environment, as mechanoreceptors. Integrins are described as biphasic transmembrane receptors that connect the intracellular polymers (actin filaments) to the extracellular polymers (the fibrillar extracellular matrix components). Depending on the mechanical properties of the substrate, and in particular its rigidity, adhesive interfaces are established with specific protein architecture, allowing a cell behavior adapted to this environment. On a substrate such as stiffness exceeds tens of kPa, cells establish stable adhesions with significant size (several μm^2) called

focal adhesions. This cellular adaptation is thinly regulated thanks to the molecular dynamics of the adhesion structures.

Integrin **β1** and **β3** are observed and co-localized in focal adhesions. Integrin β1 and β3 have specific mechanical roles and are associated with different signaling pathways. If β1 is widely associated with traction forces generated at the cell/substrate interface, β3 is associated with the transmission and traduction of the mechanical signal into a biochemical signal: the mechanotransduction (Roca-Cusachs et al., 2009). These distinct mechanical roles could explain their co-localization and/or their segregation in focal adhesion sites (Zamir et al., 2000a), but today a little is known about their regulation.

The comprehension of this regulation participates to the general understanding of the mechanism at the origin of the cell sensitivity to the mechanical properties of their environment. This field is the subject of intense multidisciplinary research in which Corinne Albigès-Rizo team participates actively. These searches require both the knowledge and skills for the molecular manipulation of tools for cell engineering, measurement techniques of local forces across the cell and measurements of molecular complex dynamic. A unique property of integrins is their tuneable conformation since they can switch from close to open conformation leading respectively to integrins activation or inactivation (Shattil et al., 2010). Some adapters control the activation of integrin which corresponds to a conformational switch from a low to high affinity state for extracellular ligand. For example, whereas talin is important to activate integrin and to connect actin filaments, **ICAP-1 specifically maintains β1 integrin in its inactivated form**. The laboratory has shown that ICAP-1 enables the cell to sense

extracellular ligand density and adapt its adhesive and migratory responses (Millon-Frémillon et al., 2008).

It has been also demonstrated considerable differences in the organization and dynamics of β3 and β1 integrins within focal adhesions. While β3 integrin was stationary within focal adhesions, a significant proportion of immobilized β1 integrins displayed retrograde translocation similar to that of F-actin. Such differences would allow the spatial compartmentalization of specific integrins and differential transduction of mechanical force from the actin cytoskeleton to the extracellular matrix, and *vice versa*. Overall, the data were consistent with the notion that, within focal adhesions, β3 and β1 integrins exhibit distinct biomechanical and mechanoresponsive properties.

Indeed, if a potential crosstalk between β1 and β3 integrin in term of spatial organization exists then the dynamics of one integrin should be influenced by modulation in activity of the other one. Here we chose to characterize the cell/substrate interface from a structural and rheological point of view through the regulation of adhesive interfaces involving β1 and β3 integrins and ICAP-1 as a particular partner. To determine β3 integrins' behavior in focal adhesion according to β1 integrin incidence, we have generated ICAP-1$^{-/-}$ osteoblasts mutants to achieve up-regulated conformation of β1 integrins, β1$^{-/-}$ osteoblasts mutants to delete β1 completely and osteoblasts deficient in both β1 integrin and ICAP-1 (displaying in that case only β3 integrins). The specifics objectives of my thesis were to investigate whether β1 integrin can influence the behavior of β3 integrin and consequently may affect cell function through regulation of cell contractility and force generation in regards to ICAP-1 context.

II. Scientific context and general aim of the study

III.
Article and results

Chapter 7. Article: ICAP-1 monoubiquitylation coordinates matrix density and rigidity sensing for cell migration through ROCK2–MRCKa balance

7.1. Specific scientific context:

The cellular conversion from a non-tumorigenic state to a metastatic one is of critical interest in cancer cell biology, as most deaths from cancer occur due to metastasis (Pollard and Borisy, 2003). This metastatic conversion is one of the hallmarks of cancer (Hanahan and Weinberg, 2011). It is highly regulated and defined as a multistep process including cell plasticity, a dysregulation of cell adhesion, degradation of the extracellular matrix (ECM), acquirement of a motile phenotype, vascular infiltration, exit and colonization to a new organ site, dormancy, and re-activation. In addition to genetic factors, environmental factors control this conversion. Indeed cell sample in space and time the heterogeneity in the composition, topography and stiffness of their extracellular matrix (ECM) through integrin-mediated adhesive machinery to adapt their migratory behavior and to invade surrounding tissues (Wirtz et al., 2011) (Pollard and Borisy, 2003). As physical links between ECM and cellular actin cytoskeleton, integrins are membrane mechanoreceptors crucial for force transmission and signal transduction to adapt cell behavior. Actin cytoskeleton generates appropriated traction forces under the control of kinases which activate the motor myosin (Bustelo et al., 2007). In response to different micro-environmental stimuli, cells build diverse contractile networks of actin filaments and myosin motors to orchestrate cell shape changes and optimize cell migration. Cell contractility of the actomyosin has to be tightly regulated in space and time by different biochemical pathways involving the activities of several kinases (ROCK1,

ROCK2, MRCK, DMPK, CRIK.) responsible for myosin phosphorylation. Furthermore, actomyosin networks display complex dynamics, such as flows and pulses, which participate in spatial and temporal distribution of myosin and evolution of forces during cell adaptation to the microenvironment for cell migration optimization. Whether cell contractility relies on the balance between different kinase activities to control the spatiotemporal activity of myosin or elongation and organization of actin filament need to be investigated. How activities of different kinases are controlled by ECM properties and whether some molecular switches allow interconnection between two types of contractility to adapt the mechanical behavior of motile cells are not understood.

7.2. Specific objectives of the study

ICAP-1, a negative regulator of β1 integrin enables the cell to sense ECM density to adapt its adhesive and migratory responses (Millon-Frémillon et al., 2008) and to control fibronectin (FbN) remodeling (Brunner et al., 2011a; Faurobert et al., 2013). ICAP-1 specifically binds to the cytoplasmic tail of β1 integrin maintaining the integrin in its inactivated form by competing with the two activators named Kindlin and talin (Montanez et al., 2008; Ye et al., 2014; Brunner et al., 2011a; Millon-Frémillon et al., 2008). ICAP-1 also binds to ROCK1 (Stroeken et al., 2006). Thanks to these interactions, ICAP-1 may be a good candidate for regulating myosin-based contractility and cellular response to ECM stiffness. Tunable post-translational modifications may control ICAP-1 functions enabling the cell to adapt its migratory response. Our laboratory has already shown that the N terminal domain of ICAP-1 contains multiple phosphorylation consensus sites. The calcium and calmodulin-dependent serine/threonine protein kinase of type II (CaMKII) is an important regulator of ICAP-1 for controlling focal adhesion dynamics (Millon-Frémillon et al., 2013, 2008). As ubiquitination is emerging as

important for cell migration dynamics and cell contractility (Sahai et al., 2007; Su et al., 2013; Wang et al., 2003; Schaefer et al., 2012; Carvallo et al., 2010), we addressed whether ubiquitination may control ICAP-1 functions enabling the cell to adapt its migratory response.

7.3. Conclusion

Our results show that ICAP-1 is monoubiquitinated by SMAD ubiquitin regulatory factor 1 (Smurf1) and that Smurf1 is a node to control focal adhesion dynamics and cell contractility. This monoubiquitination impairs ICAP-1 binding to β1 integrin and is involved in ECM density and rigidity sensing as well as in coordination of the dynamics of adhesion sites and contractile machinery. ICAP-1 monoubiquitination plays an important role in the responses of migrating cells to mechanical inputs in a β1 integrin independent manner by promoting the switch from a ROCK-mediated to a MRCK-mediated contractility pathway.

7.4. Contributions to the article

-cellular culture, SiRNA experiments, TFM, Western blot of P-Myosin, statistical analysis, Critical assistance in the preparation of the manuscript.

III. Article and results

© 2017. Published by The Company of Biologists Ltd | Journal of Cell Science (2017) 130, 1195 doi:10.1242/jcs.202218

CORRECTION

Correction: ICAP-1 monoubiquitylation coordinates matrix density and rigidity sensing for cell migration through ROCK2–MRCKα balance

Anne-Pascale Bouin, Alexander Kyumurkov, Myriam Régent-Kloeckner, Anne-Sophie Ribba, Eva Faurobert, Henri-Noël Fournier, Ingrid Bourrin-Reynard, Sandra Manet-Dupé, Christiane Oddou, Martial Balland, Emmanuelle Planus and Corinne Albiges-Rizo

There was an error published in *J. Cell Sci.* **130**, 626-636.

The name of the author Alexander Kyumurkov was incorrect in the original author list. The correct spelling is given above.

The authors apologise to the readers for any confusion that this error might have caused.

© 2017. Published by The Company of Biologists Ltd | Journal of Cell Science (2017) 130, 626-636 doi:10.1242/jcs.200139

RESEARCH ARTICLE

ICAP-1 monoubiquitylation coordinates matrix density and rigidity sensing for cell migration through ROCK2–MRCKα balance

Anne-Pascale Bouin[1,2,3], Alexander Kyurmurkov[1,2,3], Myriam Régent-Kloeckner[1,2,3,*], Anne-Sophie Ribba[1,2,3], Eva Faurobert[1,2,3], Henri-Noël Fournier[1,2,3], Ingrid Bourrin-Reynard[1,2,3], Sandra Manet-Dupé[1,2,3], Christiane Oddou[1,2,3], Martial Balland[3,4], Emmanuelle Planus[1,2,3] and Corinne Albiges-Rizo[1,2,3,‡]

ABSTRACT

Cell migration is a complex process requiring density and rigidity sensing of the microenvironment to adapt cell migratory speed through focal adhesion and actin cytoskeleton regulation. ICAP-1 (also known as ITGB1BP1), a β1 integrin partner, is essential for ensuring integrin activation cycle and focal adhesion formation. We show that ICAP-1 is monoubiquitylated by Smurf1, preventing ICAP-1 binding to β1 integrin. The non-ubiquitylatable form of ICAP-1 modifies β1 integrin focal adhesion organization and interferes with fibronectin density sensing. ICAP-1 is also required for adapting cell migration in response to substrate stiffness in a β1-integrin-independent manner. ICAP-1 monoubiquitylation regulates rigidity sensing by increasing MRCKα (also known as CDC42BPA)-dependent cell contractility through myosin phosphorylation independently of substrate rigidity. We provide evidence that ICAP-1 monoubiquitylation helps in switching from ROCK2-mediated to MRCKα-mediated cell contractility. ICAP-1 monoubiquitylation serves as a molecular switch to coordinate extracellular matrix density and rigidity sensing thus acting as a crucial modulator of cell migration and mechanosensing.

KEY WORDS: Cell migration, Rigidity sensing, ICAP-1, Integrin, Monoubiquitylation, Cell contractility

INTRODUCTION

Motile cells continuously sample in space and time the heterogeneity in the composition and stiffness of their extracellular matrix (ECM) through integrin-mediated focal adhesions (FAs) (Moore et al., 2010). As a mechanical link between ECM and actin stress fibers, integrins are crucial for force transmission and signal transduction (Moore et al., 2010). FA assembly, growth and maintenance depend on actomyosin traction forces, which adapt to the substrate elasticity (Burridge and Wittchen, 2013). In spite of alternative pathways involving MRCK (which has two isoforms, MRCKα and MRCKβ, also known as CDC42BPA and CDC42BPB, respectively), MLCK (also known as MYLK) or mDia (Burridge and Wittchen, 2013; Chen et al., 2014; Jégou et al., 2013; Totsukawa et al., 2004), a key event is the modulation of cellular contractility through myosin-based contractility and ROCK (which has two isoforms, ROCK1 and ROCK2) activity. However, signaling pathways underlying FA-mediated rigidity sensing and the mechano-response are not fully understood.

ICAP-1 (also known as ITGB1BP1), a negative regulator of β1 integrin, enables the cell to sense ECM density to adapt its adhesive and migratory responses (Millon-Frémillon et al., 2008) and to control fibronectin (FN) remodeling (Brunner et al., 2011; Faurobert et al., 2013). ICAP-1 specifically binds to the cytoplasmic tail of β1 integrin, maintaining the integrin in its inactivated form by competing with the two activators named Kindlin and talin (Brunner et al., 2011; Millon-Frémillon et al., 2008; Montanez et al., 2008; Ye et al., 2014). ICAP-1 also binds to ROCK1 (Peter et al., 2006). Thanks to these interactions, ICAP-1 may be a good candidate for regulating myosin-based contractility and cellular response to ECM stiffness. Tunable post-translational modifications may control ICAP-1 functions enabling the cell to adapt its migratory response. As ubiquitylation is emerging as important for cell migration dynamics and cell contractility (Carvallo et al., 2010; Sahai et al., 2007; Schaefer et al., 2012; Su et al., 2013; Wang et al., 2003), we addressed whether ubiquitylation may control ICAP-1 functions, enabling the cell to adapt its migratory response. Here, we show that ICAP-1 is monoubiquitylated by SMAD ubiquityl regulatory factor 1 (Smurf1). This monoubiquitylation impairs ICAP-1 binding to β1 integrin and is involved in ECM density and rigidity sensing as well as in coordination of the dynamics of adhesion sites and contractile machinery. ICAP-1 monoubiquitylation plays an important role in the responses of migrating cells to mechanical inputs in a β1 integrin-independent manner by promoting the switch from a ROCK2-mediated to an MRCKα-mediated contractility pathway.

RESULTS

ICAP-1 is monoubiquitylated by Smurf1 at the β1 integrin-binding site

To investigate ICAP-1 ubiquitylation, we performed nickel-bead pulldown experiments on Chinese Hamster Ovary (CHO) cells transfected with ICAP-1 either in the presence or absence of co-transfection with His-tagged ubiquitin. The proteasome inhibitor MG132 was added to prevent proteasomal degradation of any ubiquitylated ICAP-1. When expressed alone, ICAP-1 appeared on a western blot an apparent molecular mass that was slightly greater than 20 kDa, whereas co-transfection with His-tagged ubiquitin and pulldown on nickel beads resulted in isolation of ICAP-1 with higher molecular mass forms, with a band above 35 kDa (Fig. 1A), showing that ICAP-1 is indeed ubiquitylated. This band above 35 kDa most likely corresponds to ICAP-1 monoubiquitylation. HA-tagged ubiquitin (HA–Ubi) was also coexpressed with ICAP-1 fused to Flag and our results show that ICAP-1–Flag can be recognized by both anti-Flag and anti-HA antibodies after immunoprecipitation with anti-Flag antibodies (Fig. 1B),

[1]INSERM U1209, Grenoble F-38042, France. [2]Université Grenoble Alpes, Institute for Advanced Biosciences, Grenoble 38042, France. [3]CNRS UMR 5309, Grenoble F-38042, France. [4]Laboratoire Interdisciplinaire de Physique, UMR CNRS, Grenoble 5588, France.
*Present address: Cergy Pontoise University, France

‡Author for correspondence (Corinne.albiges-rizo@ujf-grenoble.fr)

H.-N.F., 0000-0002-3918-7330; C.A., 0000-0002-6333-4150

Received 28 November 2016; Accepted 8 December 2016

Journal of Cell Science (2017) 130, 626-636 doi:10.1242/jcs.200139

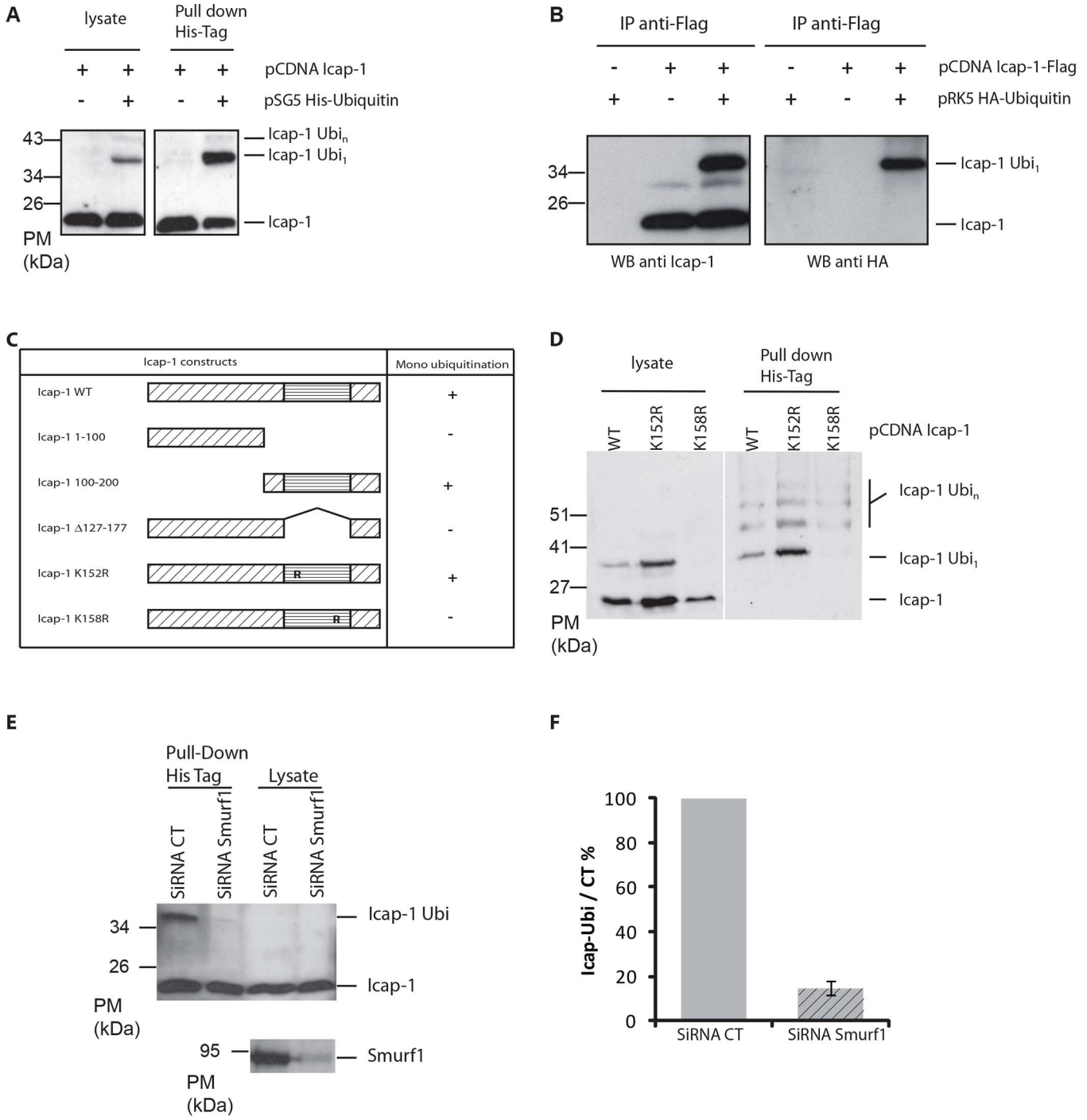


Fig. 1. The Smurf1 ubiquitin ligase is responsible for ICAP-1 monoubiquitylation. (A) ICAP-1 was overexpressed in CHO cells with or without His-tagged ubiquitin. After pulldown on TALON resin, the ubiquitylated proteins were analyzed by western blotting with the anti-ICAP-1 antibody. ICAP-1 was monoubiquitylated (Icap-1 Ubi$_1$) and weakly polyubiquitylated (Icap-1 Ubi$_n$). (B) ICAP-1–Flag immunoprecipitated by anti-Flag antibody can be recognized by anti-HA antibodies (as assessed by western blotting) after co-transfection with HA–Ubi and ICAP-1–Flag in CHO cells. The results are representative of more than three independent experiments. (C) Different ICAP-1 constructs were used to identify the ubiquitylated lysine residue. The horizontal-striped box corresponds to the β1 integrin-binding site. (D) ICAP-1 WT, ICAP-1 K152R or ICAP-1 K158R were overexpressed in CHO cells with His-tagged ubiquitin. His-tagged pulldown assays show that only the ICAP-1 K158R mutant was not monoubiquitylated. The results are representative of three independent experiments. (E) HeLa cells with or without Smurf1 knockdown were co-transfected with ICAP-1 and His-tagged ubiquitin. After pulldown on TALON resin, the ubiquitylated proteins were analyzed by western blotting with the anti-ICAP-1 antibody. Non-ubiquitylated ICAP-1 was used to ensure equivalent ICAP-1 levels in both lysates. CT, control. (F) Quantification of the level of ubiquitylated ICAP-1 in Smurf1-silenced HeLa cells. Error bars represent s.e.m. (n=3). PM, position of molecular mass makers.

confirming that ICAP-1 can be ubiquitylated. Furthermore, to identify which lysine residue is monoubiquitylated, we analyzed whether truncated forms of ICAP-1 could be monoubiquitylated (Fig. 1C). We determined that the monoubiquitylation site was located in the binding domain for β1 integrin. The point mutation of either one of the two lysine residues present in this domain identified lysine K158 as the site of monoubiquitylation, as its replacement with arginine led to the absence of the 35 kDa band

Journal of Cell Science (2017) 130, 626-636 doi:10.1242/jcs.200139

(Fig. 1D) without changing the ICAP-1 polyubiquitylation states (Fig. 1D). The non-ubiquitylable K158R mutant was even less stable than wild-type (WT) ICAP-1, suggesting that the monoubiquitylated form of ICAP-1 is not targeted for proteasomal degradation but rather may have a signaling function (Fig. S1A,B). Because Smurf1 catalyzes the ubiquitylation of the integrin activator talin (Huang et al., 2009), we hypothesized that Smurf1 could be responsible for ICAP-1 monoubiquitylation. To test this hypothesis, Smurf1 was silenced by small interfering RNA (siRNA); there was a high efficiency in reducing Smurf1 transcript and protein levels without affecting ICAP-1 expression (Fig. 1E). ICAP1 monoubiquitylation was blocked when Smurf1 was knocked down, suggesting that Smurf1 is necessary for promoting ICAP-1 monoubiquitylation (Fig. 1E,F). A pulldown assay shows that purified recombinant Smurf1–GST is able to bind to exogenously expressed ICAP-1 in CHO cells, in contrast to the null interaction with GST alone (50-fold less) or with the weak binding to GST fused to Smurf2 (10-fold less) (Fig. S1C). Smurf2 had been chosen as a control because overlapping but distinct substrate and regulator specificity has been observed between Smurf1 and Smurf2 (Lu et al., 2008, 2011). The co-immunoprecipitation between Smurf1–Myc and ICAP-1–Flag expressed in CHO cells confirms that Smurf1 and ICAP-1 belong to the same complex (Fig. S1D). A direct interaction between Smurf1 and ICAP-1 was demonstrated by an ELISA assay using purified recombinant GST–Smurf1 and purified recombinant ICAP-1–His (Fig. S1E). Taken together, our results indicate that Smurf1 is responsible for ICAP-1 monoubiquitylation.

The monoubiquitylation of ICAP-1 prevents binding to β1 integrin and regulates β1 integrin-dependent adhesion

According to structure predictions and crystallographic data (Chang et al., 2002; Liu et al., 2013), the monoubiquitylation site is located in the β1 integrin-binding domain of ICAP-1 facing the isoleucine residue important for the binding to β1 integrin (Fig. 2A). As this monoubiquitylation could interfere with the interaction between ICAP-1 and β1 integrin, we used two classical methods to produce an ubiquitylated form of a protein (Torrino et al., 2011; Visvikis et al., 2008), first by co-transfecting ICAP-1 with His-tagged ubiquitin and second by creating a chimera made of ubiquitin fused to the C-terminal tail of ICAP-1 (ICAP-1–Ubi) (Fig. 2B). We tested the ability of WT, non-ubiquitylatable (K158R) and monoubiquitylated ICAP-1 (endogenous ubiquitylation or chimera) to interact with the cytoplasmic domain of either β1 integrin or β3 integrin fused with GST or with GST alone by pulldown assay (Fig. S2A) or by ELISA assay (Fig. 2C). As previously reported (Millon-Frémillon et al., 2008), we confirmed that ICAP-1 specifically interacts with the cytoplasmic domain of β1 integrin (Fig. 2C; Fig. S2A). Furthermore, the non-ubiquitylated K158R mutant retained the ability to interact with the cytoplasmic domain of β1 integrin, whereas both ubiquitylated forms of ICAP-1 (His-tagged and chimeric) lost the capacity to interact with the cytoplasmic domain of β1 integrin (Fig. 2C; Fig. S2A). These results show that ICAP-1 monoubiquitylation prevents the interaction of ICAP-1 with β1 integrin.

Next, we investigated whether the monoubiquitylation of ICAP-1 could affect FA organization by rescuing ICAP-1-deficient osteoblast cells with a similar stable expression of WT ICAP-1, non-ubiquitylatable ICAP-1 K158R and of the chimeric ubiquitylated form. All osteoblast cell lines were able to spread onto FN and develop FAs containing β1 integrins, as revealed by 9EG7 antibody staining for activated β1 integrin (Fig. 2D). Like ICAP-1-deficient cells, cells expressing the ubiquitylated form of ICAP-1 displayed more numerous β1 integrin-containing FAs compared with cells expressing the WT form (Fig. 2D–F) because of the inability of the monoubiquitylated ICAP-1 to inhibit β1 integrin. Conversely, cells expressing the non-ubiquitylatable ICAP-1 K158R mutant displayed fewer, smaller and more-punctate adhesion sites (Fig. 2D–F) compared with those of WT ICAP-1, likely due to its ability to interact with β1 integrin and thus inhibit the assembly of larger FAs (Bouvard et al., 2007; Millon-Frémillon et al., 2008).

As Smurf1 is responsible for ICAP-1 monoubiquitylation, we investigated whether the formation of β1 integrin-containing FAs was dependent on Smurf1 activity. As expected, the deletion of Smurf1 led to a decrease in the number and area of β1 integrin-containing FA (Fig. S2B,C,D) phenocopying the non-ubiquitylatable ICAP-1 K158R phenotype (Fig. 2D–F). Conversely, the ubiquitylated ICAP-1 was able to bypass the destructive effect of Smurf1 deletion on β1 integrin-containing FAs (Fig. S2B,C,D). Thus, Smurf-1-mediated ICAP-1 monoubiquitylation plays a crucial role in the organization of β1 integrin-containing FA by preventing or disrupting the ICAP-1–β1-integrin interaction.

ICAP-1 monoubiquitylation is a signal coordinating FN density sensing with rigidity sensing

We wondered whether ICAP-1 monoubiquitylation was involved in FN density and rigidity sensing. To test an effect on FN density sensing, single-cell tracking of sparse cells was performed to monitor the migration speed of ICAP-1-deficient osteoblast or mouse embryonic fibroblast (MEF) cells expressing WT ICAP-1, K158R ICAP-1 or ICAP-1–Ubi in the presence of increasing concentrations of FN. As expected (Discher et al., 2005; Engler et al., 2006; Raab et al., 2012), WT ICAP-1-expressing osteoblasts (Fig. 3A; Movies 1,2) or MEFs (Fig. S3A) displayed faster migration rates with increasing FN density. While the migratory speed of the cells expressing the ubiquitylated ICAP-1 form depended on ECM density, like ICAP-1 null cells, the cells expressing the non-ubiquitylatable K158R mutant maintained the same migration speed whatever the density of FN coating (Fig. 3A; Fig. S3A, Movies 3,4). Moreover, the ability to adapt their migration response to ECM density was lost in cells treated with siRNA against Smurf1 but was rescued in cells co-expressing the monoubiquitylated ICAP-1 showing that the Smurf1-dependent monoubiquitylation of ICAP-1 is necessary for cells to sense and respond to FN density (Fig. S3B).

To explore the possibility that the inability of the K158R mutant to adapt its migration speed to FN density could be due to a greater capacity to lock β1 integrin in its inactivated form than with WT ICAP-1, we analyzed the response of cells treated with β1 integrin-blocking antibodies to increasing FN density. We showed that these cells were unable to sense the density of FN or adapt their migratory behavior (Fig. 3B), confirming the requirement for β1 integrin activation for the adaptation of the cell migration rate to the FN density. Additionally, cells co-expressing a β1 integrin mutant that lacks ICAP-1 binding (β1 V787T) with the ICAP-1 K158R mutant or in the context of silenced Smurf1 were still able to adapt their migration speed to the FN density (Fig. 3B; Fig. S3C). Therefore, the unresponsiveness of cells to the FN density is most likely due to the inhibitory interaction between the non-ubiquitylatable ICAP-1 and β1 integrin. Overall, ICAP-1 monoubiquitylation by Smurf1 is required to release ICAP-1 inhibitory effect on β1 integrin in order to permit the adaptation of cell migration to ECM density.

Journal of Cell Science (2017) 130, 626-636 doi:10.1242/jcs.200139

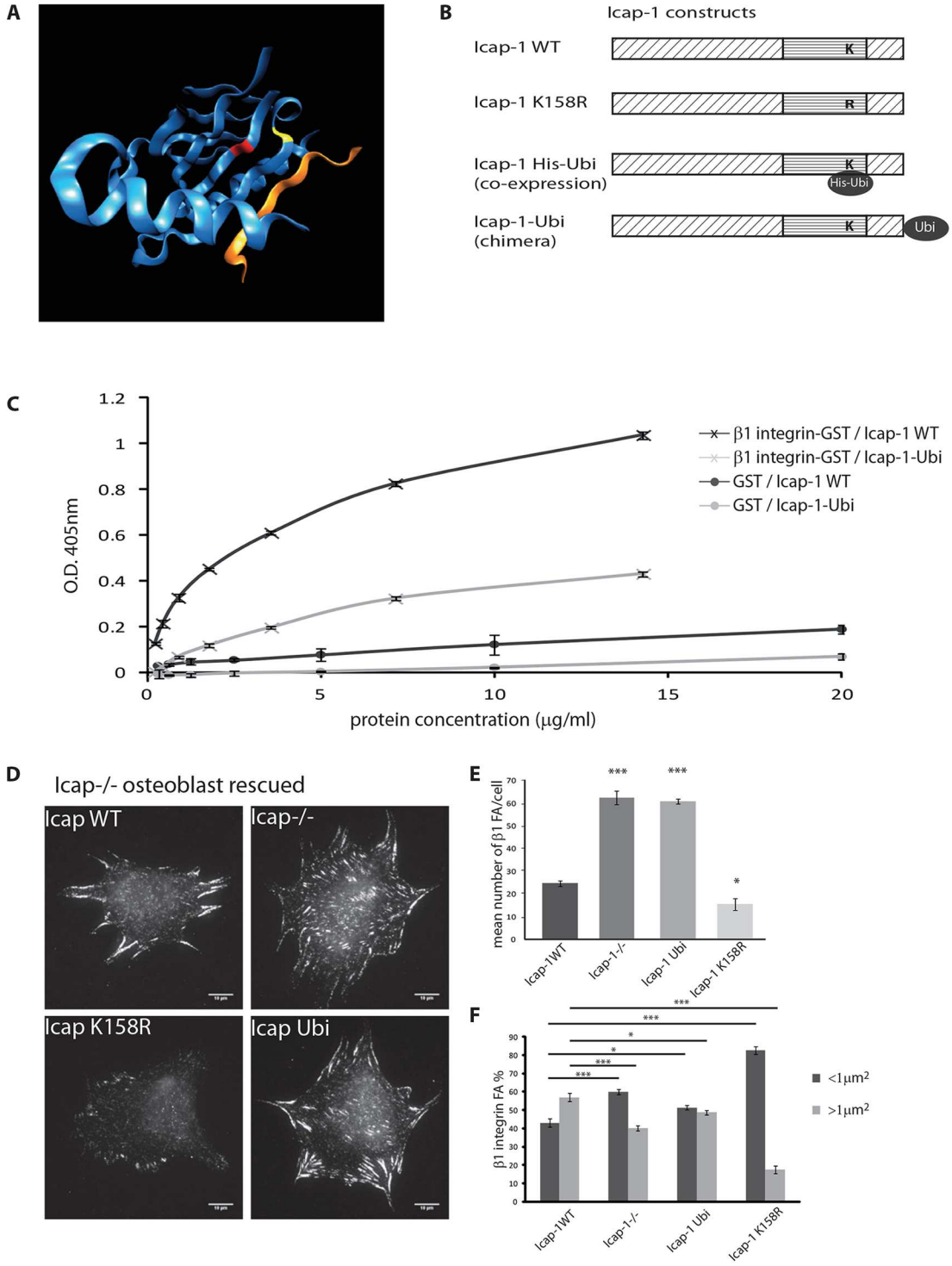


Fig. 2. Ubiquitylated ICAP-1 does not interact with the β1 integrin cytoplasmic tail and disorganizes FA distribution. (A) Recently published structure of ICAP-1 interacting with the β1 integrin cytoplasmic tail (PDB 4DX9) (Liu et al., 2013). Blue, ICAP-1 protein with I139 represented in yellow and K158 represented in red. Orange, β1 integrin cytoplasmic tail. This image was made with VMD, NAMD, BioCoRE, JMV and other software support (these software packages are developed with NIH support by the Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign). (B) ICAP-1 constructs used for the study. K158R is the non-ubiquitylatable form. ICAP-1–His–Ubi results from the overexpression of ICAP-1 and His-tagged ubiquitin proteins in CHO cells. ICAP-1–Ubi is a chimeric form with ubiquitin fused at the C-terminus of ICAP-1 to mimic constitutive monoubiquitylation. (C) Interaction between recombinant ICAP-1–His or ICAP-1–Ubi–His and recombinant GST or the GST–β1 integrin cytoplasmic domain as determined by an ELISA assay. The results are representatives of three independent experiments. (D) β1 integrin staining in ICAP-1-null osteoblasts or ICAP-1-null cells rescued with ICAP-1 WT, non-ubiquitylatable ICAP-1 or the ICAP-1 ubiquitin chimera spread on FN for 2.5 h. The cells expressing the non-ubiquitylatable form (K158R) display smaller β1 integrin FAs compared with the cells expressing ICAP-1 WT. Scale bars: 10 μm. (E) Quantification of the β1 integrin focal adhesion number and (F) distribution of the β1 integrin focal adhesion areas. Analyses were performed on 30–40 cells from two independent experiments. Error bars indicate s.e.m. **P*<0.05, ****P*<0.0005 (one-way ANOVA and Tukey's HSD test).

Journal of Cell Science

Journal of Cell Science (2017) 130, 626-636 doi:10.1242/jcs.200139

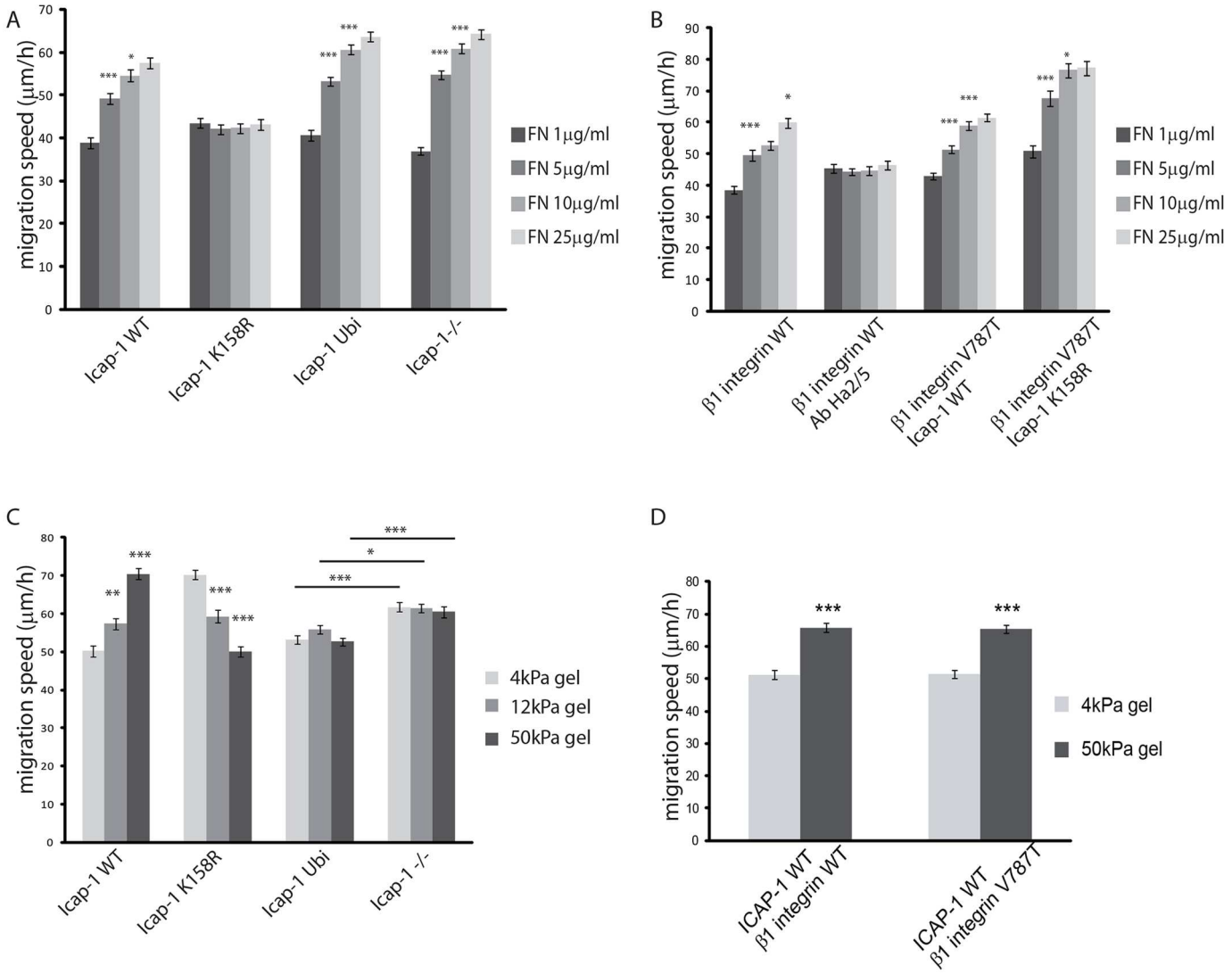


Fig. 3. ICAP-1 ubiquitylation controls FN density and rigidity sensing. Osteoblasts were spread on increasing concentrations of FN and migration was monitored for 5 h using time-lapse microscopy. Cell velocity was determined by individually tracking 150–200 cells from three independent experiments. (A) Cells expressing ICAP-1-WT, the ICAP-1 ubiquitin chimera or cells deficient in ICAP-1 adapted their migratory speed according to the FN density, whereas the cells expressing the ICAP-1 K158R mutant maintained the same speed regardless of the FN density. (B) Similar to the cells expressing ICAP-1 K158R, cells treated with a blocking anti-β1 integrin antibody (Ab Ha2/5) were unable to adapt their migration speed to the FN density. β1 integrin-null cells expressing the β1 integrin mutant that lacks ICAP-1 binding (V787T) were not affected by K158R ICAP-1 expression. (C,D) Osteoblast cells were spread on FN-coated PAA gels of different rigidities. Cell migration was monitored for 5 h using time-lapse microscopy. The cell velocity was determined by individually tracking 150–300 cells in three independent experiments. Similar to in ICAP-1-deficient cells, ICAP-1–Ubi cells did not change their velocity according to gel rigidity whereas WT cells moved more quickly in stiffer gels (C). β1 integrin-null cells expressing the β1 integrin mutant that lacks ICAP-1 binding (V787T) responded to gel rigidity similarly to control cells (D) indicating that the interaction between β1 integrin and ICAP-1 is not necessary to adapt cell migration to substrate stiffness. Error bars indicate the mean±s.e.m. **P*<0.05, ***P*<0.005, ****P*<0.0005 (one-way ANOVA and Tukey's HSD test).

We next evaluated the effects of ICAP-1 monoubiquitylation on the ECM rigidity sensitivity. Osteoblast cells (Fig. 3C) or MEF cells (Fig. S3D) infected with ICAP-1 WT, ICAP-1 K158R and ICAP-1–Ubi were plated onto FN-conjugated elastomeric polyacrylamide (PAA) gels with increasing Young's modulus (*E*) and monitored for cell migration. As expected, the WT ICAP-1 cells moved more quickly on stiffer gels than they did on softer gels (40% increase on the stiffer substrate) (Fig. 3C; Fig. S3D, Movies 5,6). Cells expressing ICAP-1 K158R still responded to the increase in matrix rigidity, whereas cells expressing the monoubiquitylated ICAP-1 displayed a constant migration velocity that was independent of the stiffness of the substrate, like ICAP-1-deficient cells (Fig. 3C; Fig. S3D, Movies 7,8). However, the migration speed of ICAP-1$^{-/-}$ cells was slightly but significantly higher as compared to that of ICAP-1–Ubi cells. This suggests that ICAP-1 monoubiquitylation also controlled the capacity of cells to adapt their velocity to ECM rigidity. As monoubiquitylation prevents ICAP-1 and β1 integrin interaction, we then investigated whether rigidity sensing was dependent on ICAP-1 and β1 integrin interaction. Cells expressing the β1 integrin V787T mutant that are unable to interact with ICAP-1 still adapt their velocity in response to the external rigidity (Fig. 3D) whereas ICAP-1 deficiency led to insensitiveness to substrate stiffness (Fig. 3C). Thus, the presence of ICAP-1 is required even though ICAP-1 interaction with β1 integrin is dispensable for rigidity sensing. Monoubiquitylation of ICAP-1 is a signal that allows the sensing of matrix density and rigidity by

Journal of Cell Science (2017) 130, 626-636 doi:10.1242/jcs.200139

decoupling the inhibitory role of ICAP-1 on β1 integrin from an unexpected role that is independent of its interaction with β1 integrin.

The monoubiquitylation of ICAP-1 increases cell contractility

As rigidity sensing is associated with cell contractility, we sought to determine whether the monoubiquitylated form of ICAP-1 might interfere with cell contractility. First, as a contractility marker, we analyzed the phosphorylation state of myosin light chain (pMLC) by western blotting lysates from WT, and ICAP-1–Ubi and ICAP-1-deficient cells plated onto FN-coated plastic or elastomeric PAA gels with a Young's modulus (E) of 4 or 50 kPa (Fig. S4A). As expected, the level of pMLC in total cell lysates of cells expressing ICAP-1 WT increased with the substrate rigidity. ICAP-1-deficient cells displayed the same behavior as ICAP-1 WT cells. In contrast, cells expressing the monoubiquitylated ICAP-1 showed a constant level of pMLC independently of the rigidity of the substrate. This loss of pMLC regulation is correlated with the inability of ICAP-1–Ubi cells to adapt their velocity to ECM rigidity (Fig. 3C). In addition, an increase of pMLC staining along the stress fibers in ICAP-1–Ubi cells was noted (Fig. 4A). To investigate whether the monoubiquitylated ICAP-1 is involved in the genesis and modulation of forces applied to the substratum, traction force microscopy (TFM) was used. Traction forces generated by the cells were twice as high in ICAP-1–Ubi cells as compared to the WT cells and ICAP-1-deficient cells (Fig. 4B). Therefore, the monoubiquitylation of ICAP-1 increases cell contractility by forcing the phosphorylation of myosin independently of the substrate rigidity.

The monoubiquitylation of ICAP-1 drives MRCKα-mediated cell contractility

Cell contractility relies on the balance between ROCK, MLCK and mDia activities to control elongation and organization of actin filament (Burridge and Wittchen, 2013). To explore the contractility pathways potentially affected by ICAP-1–Ubi, a pharmacological approach was used by testing ROCK, MLCK and mDia inhibitors (Y27632, ML7 and SmifH2, respectively) on the migration of osteoblasts adhered to 4 kPa gels coated with 5 µg/ml of FN. Like WT cells, ICAP-1–Ubi cells migrated slower upon MLCK and mDia inhibition (Fig. S4B). As previously described (Totsukawa et al., 2000), WT cells migrate faster upon ROCK inhibition. In contrast, ICAP-1–Ubi cells were insensitive to Y27632 treatment since no change in migratory speed response was observed as compared with the WT cells (Fig. S4B). This insensitivity to ROCK inhibition in ICAP-1 Ubi cells is not due to the loss of the interaction between ICAP-1–Ubi and β1 integrin since cells expressing the V787T mutant of β1 integrin, which is unable to interact with ICAP-1, are still sensitive to ROCK inhibition (Fig. S4C). Thus, ICAP-1–Ubi cell migration is independent of ROCK-controlled contractility, suggesting an alternative contractile pathway for ICAP-1–Ubi cells.

Besides regulating ROCK1 (Peter et al., 2006), ICAP-1 has been shown to inhibit Cdc42 and Rac1 (Degani et al., 2002), which are involved in the regulation of MRCK. Therefore, we sought to assess whether ICAP-1 could regulate MRCK-dependent cell contractility (Leung et al., 1998). To test this hypothesis, we used a siRNA strategy to knockdown ROCK1, ROCK2, MRCKα and MRCKβ (Fig. 4C,D). The WT ICAP-1 cells moved more quickly on stiffer gels than they did on softer gels whatever the siRNA used except in conditions of ROCK2 deletion suggesting that WT cells adapt their migratory behavior through a ROCK2-dependent contractility and this behavior is independent of ROCK1, MRCKα and MRCKβ (Fig. 4C). In contrast, only MRCKα silencing in ICAP-1–Ubi cells led to an increase in the cell migration speed when rigidity of the substrate was increased (Fig. 4D). Thus, the cell contractility mode imposed by ICAP-1 monoubiquitylation is dependent on MRCKα and is independent of ROCK1, ROCK2 and MRCKβ. To confirm the involvement of MRCKα in the monoubiquitylated ICAP-1-dependent phosphorylation of myosin, we tested the effect of siRNA against MRCKα or ROCK2 on the decoration of stress fibers by T18/S19 phosphorylated MLC (ppMLC) (Fig. 4E). Whereas the siRNA against ROCK2 decreased the level of ppMLC in WT cells, the depletion of MRCKα significantly reduced the level of ppMLC in cells infected with ICAP-1–Ubi. Thus, ICAP-1 monoubiquitylation favors the phosphorylation of myosin II that is dependent on the activity of MRCKα whereas ROCK2 activity is responsible for the phosphorylation of myosin II in WT cells. Taken together, these results show that ICAP-1 monoubiquitylation allows the switch from ROCK2-mediated to MRCKα-mediated cell contractility.

DISCUSSION

Our data show that monoubiquitylation of ICAP-1, a protein that associates with integrin cytoplasmic domains, by Smurf1 is involved in regulating the balance between adhesion and contractility. ICAP-1 monoubiquitylation inhibits its binding to β1 integrin, subsequently regulating the number and organization of β1 integrin-containing FAs. ICAP-1 and its monoubiquitylated form may be crucial mediators involved in the balance between ROCK2 and MRCKα activities in order to adapt cell contractility to the variability of ECM stiffness. Our results show that these two functions of ICAP-1 are integrated by the cell to sense both matrix density and rigidity.

Smurf1 as a node to control focal adhesion dynamics and cell contractility

In addition to its ability to ubiquitylate talin (Huang et al., 2009), Smurf1 was a good candidate for ICAP-1 monoubiquitylation because Smurf1 associates with the cerebral cavernous malformations (CCM) complex (Crose et al., 2009), which interacts with ICAP-1 (Hilder et al., 2007). Smurf1 also possesses an NPxY motif that might be able to interact with ICAP-1 phosphotyrosine-binding (PTB) domain. Smurf1 is also involved in cell polarity and cell migration (Sahai et al., 2007; Wang et al., 2003). We demonstrated that the monoubiquitylation of ICAP-1 by Smurf1 is not involved in ICAP-1 degradation via the proteasome, but rather, regulates the assembly and organization of FAs by modulating the ICAP-1–β1-integrin interaction. The ICAP-1–β1-integrin interface is likely disrupted upon ICAP-1 monoubiquitylation since K158 is in close vicinity to the I138 residue known to be important for the β1 integrin interaction (Chang et al., 2002; Liu et al., 2013).

In addition to their canonical roles in cell growth and differentiation mediated through TGF signaling (Zhu et al., 1999), accumulating evidence indicates that Smurfs play key roles in regulating cell adhesion and migration. Smurf1 is localized in lamellipodia and filopodia, with a fraction of Smurf1 in FAs (Huang et al., 2009; Wang et al., 2003). Smurf1 ubiquitylates molecules involved in both cell adhesion and contractility. Smurf1 controls talin head degradation, and subsequently adhesion stability and cell migration (Huang et al., 2009). RhoA ubiquitylation by Smurf1 causes its degradation at the leading edge of migrating cells and promotes lamellipodium formation (Sahai et al., 2007; Wang et al.,

Journal of Cell Science (2017) 130, 626-636 doi:10.1242/jcs.200139

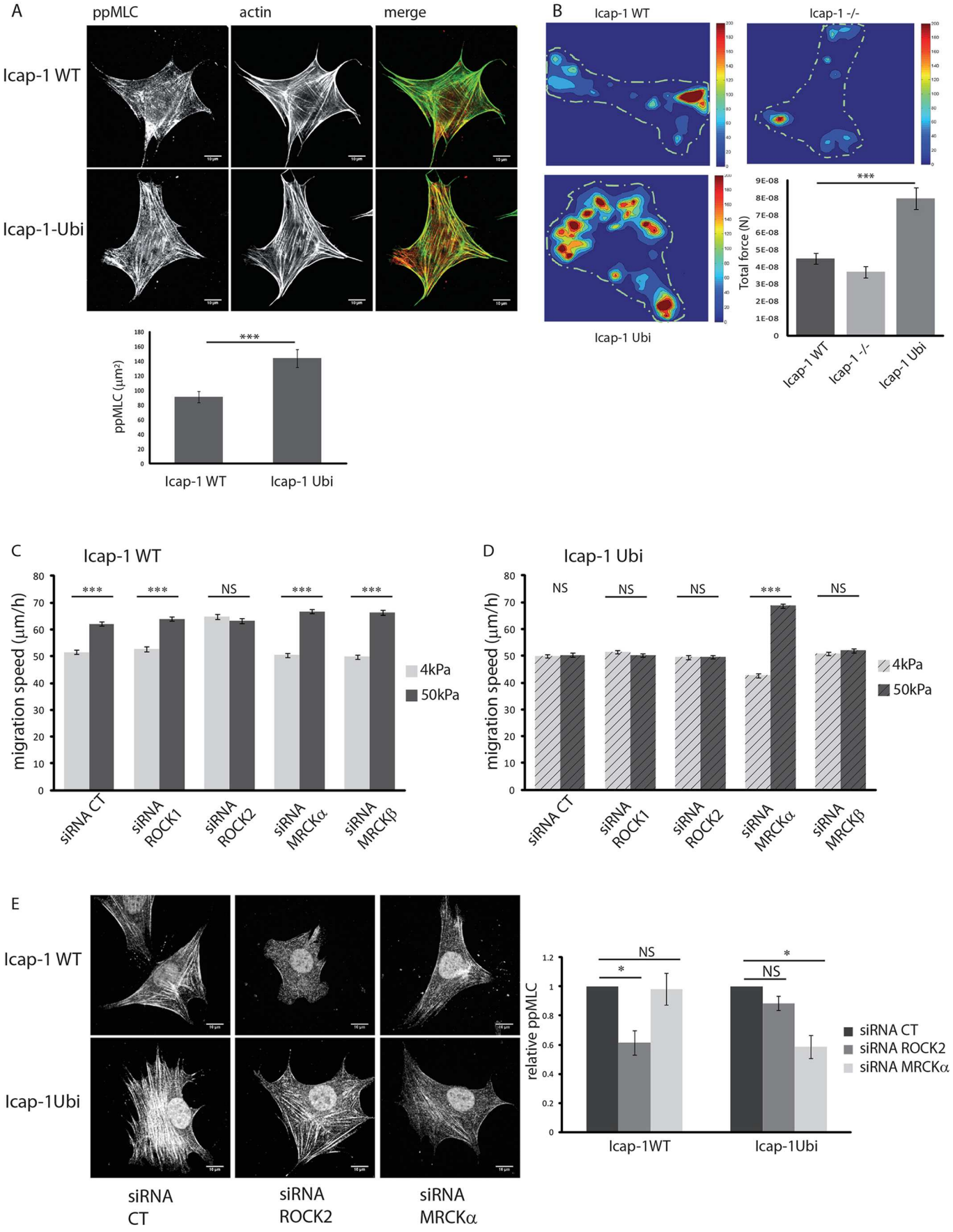


Fig. 4. See next page for legend.

Journal of Cell Science

Journal of Cell Science (2017) 130, 626-636 doi:10.1242/jcs.200139

Fig. 4. The monoubiquitylation of ICAP-1 drives an MRCKα-mediated cell contractility. (A) Immunostaining of ppMLC and actin (phalloidin) in WT cells and ICAP-1–Ubi osteoblast cells. Note the increase of ppMLC along the stress fibers as seen from the quantification of 80 cells from three independent experiments. (B) Representative traction force maps obtained by TFM in ICAP-1 WT, ICAP-1-deficient and ICAP-1–Ubi osteoblast cells (images). TFM experiments showed an increase of the force applied on the substrate in ICAP-1–Ubi cells as compared to ICAP-1 WT and ICAP-1-deficient cells (n=78 from three independent experiments) (graph). Error bars indicate the mean±s.e.m. ***P<0.0005 (one-way ANOVA and Tukey's HSD test). (C) Osteoblasts were spread on FN-coated PAA gels of different rigidities. Cell migration was monitored for 5 h using time-lapse microscopy. Cell velocity was determined by individually tracking 200–300 cells in three independent experiments. Monitoring of WT cells migration after treatment with scrambled siRNA (siRNA CT) or siRNA against ROCK1, ROCK2, MRCKα or MRCKβ on 4 or 50 kPa gels. Note that WT cells are sensitive to ROCK2 siRNA treatment. (D) Monitoring of ICAP-1–Ubi osteoblast cells migration after treatment with scrambled siRNA (siRNA CT) or with siRNA against ROCK1, ROCK2, MRCKα or MRCKβ on 4 or 50 kPa gels. Note that ICAP-1–Ubi cells are sensitive to MRCKα siRNA treatment. (E) Immunostaining of ppMLC in WT osteoblast cells and ICAP-1–Ubi osteoblast cells after treatment with siRNA against ROCK2 or MRCKα (left panel). The right-hand panel shows a quantification of ppMLC staining. Note the decrease of ppMLC staining along the stress fibers after siRNA against ROCK2 for the WT cells whereas the decrease of ppMLC is observed after treatment with siRNA against MRCKα for ICAP-1–Ubi cells (n>80). Error bars indicate the mean±s.e.m. *P<0.05; ***P<0.0005; NS, not significant (one-way ANOVA and Tukey's HSD test). Scale bars: 10 µm.

2003). Our data demonstrate that Smurf1 is a node controlling both FA dynamics and cell contractility through a common target, ICAP-1. ICAP-1 monoubiquitylation not only regulates the number and organization of β1 integrin-containing FAs but also inhibits ROCK signaling and promotes the MRCK signaling pathway. Therefore, we add another piece of evidence showing that the RhoA–ROCK pathway is inhibited by Smurf1, and we demonstrate for the first time that Smurf1 controls a switch from a ROCK-dependent to a MRCK-dependent cell contractility.

The monoubiquitylation of ICAP-1 as a switch from ROCK2-mediated to MRCKα-mediated contractility

In addition to its role in the β1 integrin activation cycle (Millon-Frémillon et al., 2008), ICAP-1 interferes with small GTPase signaling and cell contractility by putting a cap on RhoA activation (Faurobert et al., 2013) and inhibiting Rac1 and Cdc42 (Degani et al., 2002). So far, how ICAP-1 can regulate both RhoA–ROCK signaling and the Cdc42 and Rac1 pathway was unclear. It has been described that a cooperation between RhoA–ROCK and Cdc42 or Rac1–MRCK signaling can control cell contractility cell polarity, morphology and morphogenesis (Gally et al., 2009; Unbekandt and Olson, 2014; Wilkinson et al., 2005). Their respective contribution might depend on ECM rigidity. ICAP-1, independently of its interaction with β1 integrin, could act as a sensor of ECM rigidity differently modulating the activity of each enzyme depending on the substrate stiffness. It could act by playing on the level of activation of RhoA, Rac1 or Cdc42 and by directly modulating the activity of ROCK2 and MRCKα. Thus, we propose that ICAP-1 monoubiquitylation by Smurf1 is a key event leading to a switch from ROCK2-mediated to MRCKα-mediated cell contractility. ICAP-1 and its monoubiquitylated form regulate ROCK2- and MRCKα-dependent MLC phosphorylation independently of interaction with β1 integrin. This is in line with previous studies, which do not attribute a major role of β1 integrin to ECM rigidity sensing (Jiang et al., 2006). Taken together, our results show that ICAP-1 contributes to an elaborate signaling network responsible for maintaining cell tensional homeostasis, going from the dynamics of cell adhesion to the adaptation of contractile actomyosin machinery. ICAP-1 may function in β1 integrin-dependent and -independent pathways to orchestrate both the chemo and mechanical regulation of cell migration. These two pathways might regulate distinct signaling cascades through a switch operated by Smurf1 to adapt the cellular migratory response (Fig. 5). ICAP-1 is essential in rigidity sensing and its monoubiquitylation might be crucial for the adaptation of cells to a local variation of ECM stiffness in tissues or a change of ECM composition during development or in pathological situations. ICAP-1 monoubiquitylation would allow the cell to adapt its the contractility depending on substrate stiffness by controlling the balance between ROCK2-and MRCKα-mediated cell contractility. In future studies, it will be important to identify the factors that are regulated by ICAP-1 independently of its interaction with β1 integrin in order to develop a more complete understanding of the functions of ICAP-1 in mechanosensing.

MATERIALS AND METHODS

Plasmid construction

The plasmids pCMVFlag-Smurf1 WT, pGEX4T1-Smurf1 WT, pGEX4T1-Smurf2 WT, pRK5-Myc-Smurf1 and pRK5-HA-Ubiquitin-WT were obtained from Addgene (Cambridge, MA; numbers 11752, 13502, 13504, 13676 and 17608). pGEX4T1 plasmids containing the β1 or β3 integrin cytoplasmic domain, as well as pCLMFG retroviral vectors containing WT β1 integrin or the V787T mutant, have been previously described (Brunner et al., 2011). The pSG5-ubiquitin-His vector was a kind gift from Saadi Khochbin (U823 INSERM-UJF, Grenoble, France). The full-length cDNA of WT human ICAP-1 was subcloned into the *Eco*RI and *Bam*HI sites of the pBabe-puro retroviral vector (pBabe-ICAP-1 WT). The K158R substitution was introduced into the ICAP-1 cDNA via site-directed mutagenesis (pBabe-ICAP-1 K158R). The Myc tag was inserted at the 3′ end of the ICAP-1 or ubiquitin cDNA using PCR. The Myc-tagged ICAP-1 cDNA was subcloned between the *Bam*HI and *Eco*RI sites of the pcDNA3.1 expression vector and mutated to generate the K158R mutant.

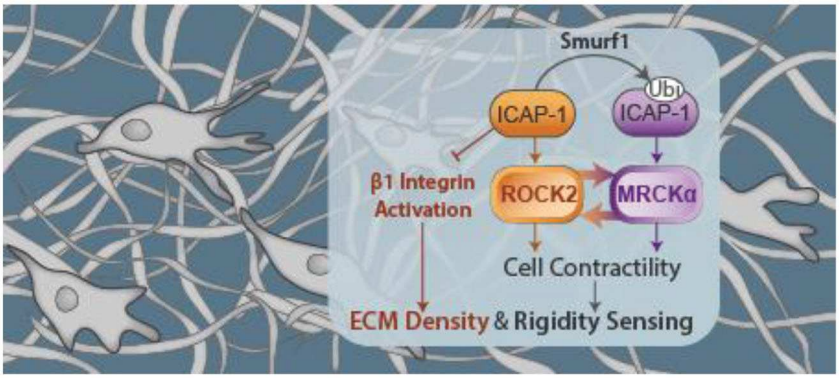


Fig. 5. A ROCK2–MRCKα switch operated through the monoubiquitylation of ICAP-1 by Smurf1 to adapt the cellular migratory response. Smurf1 is able to monoubiquitylate ICAP-1. The monoubiquitylation of ICAP-1 by Smurf1 is required to release inhibitory effect of ICAP-1 on β1 integrin, thereby facilitating the activation–deactivation cycle of β1 integrin important for ECM density sensing and adaptive cell migration responses. The monoubiquitylation of ICAP-1 allows the switch from ROCK2-mediated to MRCKα-mediated cell contractility to control ECM rigidity sensing.

Journal of Cell Science (2017) 130, 626-636 doi:10.1242/jcs.200139

The cDNA of Myc-tagged ubiquitin was amplified and inserted at the 3′ end of the ICAP-1 cDNA, between the *Eco*RI and *Xho*I sites of the pcDNA3.1 vector (pcDNA3.1-ICAP-1-myc, pcDNA3.1-ICAP-1 K158R-myc and pcDNA3.1-ICAP-1-Ubi-myc). The ICAP-1-Ubi-myc cDNA was subcloned into the pBabe-puro, between the *Bam*HI and *Sal*I sites (pBabe-ICAP-1-Ubi-myc).

Cell culture, transfection and antibodies

Immortalized osteoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Life Technologies, Cergy Pontoise, France), CHO cells and HeLa cells were grown in αMEM (PAA) at 37°C in a humidified, 5% CO_2 chamber. All media are supplemented with 10% fetal calf serum (FCS; Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin. Immortalized osteoblasts from *icap-1*$^{-/-}$*; Itgb1* $^{flox/flox}$ mice were generated as previously described (Bouvard et al., 2007). These cells were treated with or without adenoCre viruses obtained from the gene transfer vector core (University of Iowa) to generate β1 integrin-null cells. The ICAP-1-null cells were incubated with or without retroviral particles to obtain rescued cells expressing ICAP-1 WT, ICAP-1 K158R or the ICAP-1–Ubi chimera. The cells were selected with 1 mg/ml puromycin to produce cell populations with heterogeneous ICAP-1 expression levels. β1 integrin-null cells that had already been rescued with ICAP-1 were again infected with retrovirus to obtain double-rescued cells expressing ICAP-1 (WT or mutant) and WT β1 integrin or the V787T mutant. For all experiments, cells were trypsinized and washed in PBS before plating in DMEM containing 4% FN-free FCS for 3 h. Osteoblasts (90×10^4 cells) were transfected with 25 pmol siRNA and 6 µl Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. The cells were used 2 days after transfection. SMARTpool siRNA (Dharmacon Research Inc., Lafayette, LA) was used against appropriate proteins, along with the control siRNA sequence 5′-AGGUAGUGUAAUCGCCUUG-3′. HeLa cells were transfected with control or Smurf1 siRNA SMARTpool siRNA (Dharmacon Research Inc.) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions; two rounds of transfection were performed. ICAP-1 and His-tagged ubiquitin were overexpressed using Fugene (BD Biosciences, Le Pont de Claix, France) according to the manufacturer's instructions. CHO cells were transfected with ExGen (EUROMEDEX, Souffelweyersheim, France) following the manufacturer's instructions using pcDNA3.1-ICAP-1-myc, pcDNA3.1-ICAP-1K158R-myc or pcDNA3.1-ICAP-1-Ubi-myc. CHO cells were cotransfected with pcDNA3.1-ICAP-1-myc or pcDNA3.1-ICAP-1 K158R-myc and pSG5-ubiquitin-His. After 24 h, the transfected cells were incubated with the proteasome inhibitor MG132 (20 µM) for 4 h. The antibodies used in this study were the following: rat anti-β1 integrin 9EG7 (1:100; BD Biosciences, 553715), donkey anti-rabbit-IgG conjugated to HRP (1:12,000; Jackson ImmunoResearch, UK, 711-036-152), goat anti-rat-IgG conjugated to Alexa Fluor 488 (1:1000; Invitrogen, A-11006), mouse anti-actin (1:1000; Sigma-Aldrich, Saint Quentin Fallavier, France, A2066), mouse anti-Smurf1 (1:1000; Santa Cruz Biotechnology, Heidelberg, Germany, Sc-100616) rabbit anti-T18/S19 MLC [1:1000 (western blotting) or 1:100 (immunofluoresence); Cell Signaling Technology, Leiden, The Netherlands], and rabbit anti-ICAP-1 (1:1000; Millon-Frémillon et al., 2008).

Purification of His-tagged ubiquitylated proteins

Transfected CHO cells were lysed in phosphate-buffered saline (PBS) containing 10% glycerol, 0.3% NP40, 5 mM NEM, 10 mM NaF, phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich), and a protease inhibitor cocktail (cOmplete, EDTA-free, Roche, Meylan, France). After centrifugation (15,000 ***g*** for 20 min), the supernatants were incubated with Talon Metal Affinity resin (Clontech, Saint Germain en Laye, France) for 2 h. After three washes, the proteins were eluted in Laemmli buffer and analyzed by western blotting (3% of the total lysate is used for the input track).

Pulldown assays

GST–Smurf1 and GST–Smurf2 were expressed in *E. coli* (BL21 DE3 RIL) as previously described (Wang et al., 2006). Transfected CHO cells were lysed in buffer containing 50 mM Tris-HCl pH 7.7, 150 MG132, protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3. The supernatants were incubated for 3 h with GST–Smurf1-, GST–Smurf2- or GST-coupled glutathione–Sepharose beads. After five washes in lysis buffer, the samples were eluted in Laemmli buffer and analyzed by western blotting (3% of the total lysate is used for the input track). GST–β1-integrin and GST–β3-integrin were expressed in *E. coli* (BL21 DE3 RIL), and pulldown experiments with supernatants from transfected CHO cells were performed as previously described (Brunner et al., 2011).

ICAP-1 protein lifetime measurement

Transfected CHO cells were incubated with 100 µg/ml cycloheximide (Sigma-Aldrich) with or without 20 µM MG132. Cells were lysed in RIPA buffer at the indicated times, and the protein concentration was measured using the BCA assay. Total proteins (20 µg) were separated by SDS-PAGE and immunoblotted as below.

Flag immunoprecipitation

Transfected CHO cells were lysed in lysis buffer (50 mM NaCl, 10 mM Pipes, 150 mM sucrose, 50 mM NaF, 40 mM $Na_4P_2O_7{\cdot}10H_2O$, 1 mM Na_3VO_4, pH 6.8, 0.5% Triton X-100, 0.1% sodium deoxycholate, and protease inhibitor cocktail). The supernatants were incubated for 1 h with anti-Flag M2 magnetic beads (Sigma-Aldrich). After four washes with lysis buffer, the samples were eluted in lysis buffer containing 100 µg/ml Flag peptide (Sigma-Aldrich) and analyzed by western blotting (3% of the total lysate is used for the input track).

ELISA assay

The interaction between recombinant ICAP-1 and ICAP-1–Ubi was analyzed using a solid-phase assay. Briefly, a 96-well tray (MaxiSorp, Nunc) was coated with either ICAP-1-His or ICAP-1–Ubi–His (40 µg/ml) for 16 h at 4°C and blocked with 3% BSA in PBS for 1 h at room temperature. Increasing concentrations of GST, the GST–β1-integrin cytoplasmic domain or GST–Smurf1 were added for 1 h. After three washes in PBS with 0.1% Tween20, detection of bound proteins was performed by using the antibodies directed against β1 integrin cytoplasmic domain or Smurf1. Nonspecific binding to BSA-coated wells was subtracted from the results as background.

pMLC western blot analysis

Cells were plated on plastic or on PAA gels with controlled rigidities of 50 kPa or 4 kPa (Cell Guidance System, Cambridge, UK) coated with 1 µg/cm^2 (5 µg/ml) of FN. The next day cells were lysed in Laemmli buffer and analyzed by western blotting. Immunoblots were visualized using the ECL system (Biorad) and Chemidoc imaging system (Biorad).

Traction force microscopy

The PAA substrates were prepared on two-well LabTek slides (Thermo Fischer Scientific, Ulm, Germany) using 8% acrylamide mixed with appropriate percentage of bis-acrylamide and 10 mM HEPES (pH 8.5) gels. After two Sulfo-SANPAH (Thermo Fischer Scientific, Ulm, Germany) activations, the gels were coated with 5 µg/ml FN (1 µg/cm²) at 4°C overnight. We used a concentration of 0.15% of bis-acrylamide to create gels with controlled rigidities of 5 kPa. Cells were plated at an approximate density of 2×10^4 cells per cm^2 for 3-4 h and images were acquired on an iMIC Andromeda (FEI, Gräfelfing, Germany) microscope at 40x magnification. Force calculations were performed as previously described (Tseng et al., 2011).

Random migration analysis

Cells were plated on a 12-well plate containing a PAA substrate (Cell Guidance System) or on an 8-well LabTek slide coated with various FN concentrations at an approximate density of 1.2×10^5 per cm^2 for 3 h in CO_2-independent DMEM containing 4% FN-free FCS. The cells were maintained at 37°C and imaged on an inverted microscope (Zeiss Axiovert 200) equipped with a motorized stage, cooled CCD camera (CoolSnap HQ2, Roper Scientific) and a 10× objective (EC Plan-Neofluar) for live-cell imaging for 5 h at a frequency of 1 image every 4 min. Inhibitors were added as indicated to the medium 10 min prior to the initiation of image acquisition and maintained throughout the migration assay at a final

concentration of 10 µM for Y27632 (Calbiochem), 5 µM for ML7 (Calbiochem) and 2 µM for SmifH2 (Calbiochem). Cell velocity was obtained using the manual tracking plug-in in ImageJ software. A total of 150–300 cells were analyzed from at least five different locations in each experiment, and results were collected from three independent experiments.

Immunofluorescence

Cells were plated at an approximate density of 2×10^4 cells per cm^2 for 2.5 h in 24-well plates on slides coated with 0.6 µg/cm^2 (2 µg/ml) or 1.5 µg/cm^2 (5 µg/ml) of FN in DMEM containing 5% FN-depleted serum; the cells were then fixed and immunostained as previously described (Millon-Fremillon et al., 2008). For the focal adhesion analysis, images were acquired on an Axio Imager (Zeiss) microscope at with a 63× objective. We analyzed the β1 integrin staining of 30–40 cells from two independent experiments using a thresholding method and the particle analyzer in ImageJ. Particles larger than 0.5 µm^2 were analyzed. Internal focal adhesions are defined as a FA that was more than 3 µm distal to the plasma membrane. For the ppMLC-decorated stress fibers, images were acquired on an iMIC Andromeda (FEI) microscope at with a 40× objective. We analyzed the phosphorylation of Thr18 and/or Ser19 on the light myosin chain in 90–100 cells from three independent experiments by using the 'Unsharp mask' and the particle analyzer plug-in in ImageJ software. Objects bigger than 0.5 µm^2 were analyzed.

Statistical tests

All data sets were analyzed with R (http://www.R-project.org/). We used an ANOVA-2 analysis and Tukey's HSD post-hoc test when necessary. Results are mean±s.e.m. Significance is indicated with asterisks (*P<0.05, **P<0.005, ***P<0.0005).

Acknowledgements

We thank Daniel Bouvard for osteoblast production, the Institut Albert Bonniot Cell Imaging facility, and Jacques Mazzega and Alexei Grichine for their assistance with the microscopy studies. We are indebted to O. Destaing and Richard Demetz for scientific discussions, Rachel Ramchurn for manuscript reading, to Saadi Khochbin for biological tools.

Competing interests

The authors declare no competing or financial interests.

Author contributions

C.A.-R., A.-P.B., and E.P. designed and analyzed the experiments. A.-P.B., M.R.-K., A.-S.R., A.K., E.F., H.-N.F., I.B.-R., S.M.-D., C.O. and M.B. helped with the experimental design and the procedures, performed the experiments, and analyzed the data. C.A.-R., A.P.-B. and E.P. wrote the manuscript. All of the authors provided detailed comments. C.A.-R. initiated the project.

Funding

This work was funded by Fondation ARC pour la Recherche sur le Cancer and Institut National Du Cancer (INCA). The C.A.-R. team is supported by la Ligue Contre le Cancer (Equipe labellisée Ligue 2014). M.R.-K., A.K., and H.-N.F. were supported by Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche fellowships.

Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.200139.supplemental

References

Bouvard, D., Aszodi, A., Kostka, G., Block, M. R., Albigès-Rizo, C. and Fässler, R. (2007). Defective osteoblast function in ICAP-1-deficient mice. *Development* **134**, 2615-2625.

Brunner, M., Millon-Frémillon, A., Chevalier, G., Nakchbandi, I. A., Mosher, D., Block, M. R., Albigès-Rizo, C. and Bouvard, D. (2011). Osteoblast mineralization requires {beta}1 integrin/ICAP-1-dependent fibronectin deposition. *J. Cell Biol.* **194**, 307-322.

Burridge, K. and Wittchen, E. S. (2013). The tension mounts: stress fibers as force-generating mechanotransducers. *J. Cell Biol.* **200**, 9-19.

Carvallo, L., Muñoz, R., Bustos, F., Escobedo, N., Carrasco, H., Olivares, G. and Larraín, J. (2010). Non-canonical Wnt signaling induces ubiquitination and degradation of Syndecan4. *J. Biol. Chem.* **285**, 29546-29555.

Chang, D. D., Hoang, B. Q., Liu, J. and Springer, T. A. (2002). Molecular basis for interaction between Icap1 alpha PTB domain and beta 1 integrin. *J. Biol. Chem.* **277**, 8140-8145.

Chen, C., Tao, T., Wen, C., He, W.-Q., Qiao, Y.-N., Gao, Y.-Q., Chen, X., Wang, P., Chen, C.-P., Zhao, W. et al. (2014). myosin light chain kinase (MLCK) regulates cell migration in a myosin regulatory light chain phosphorylation-independent mechanism. *J. Biol. Chem.* **289**, 28478-28488.

Crose, L. E. S., Hilder, T. L., Sciaky, N. and Johnson, G. L. (2009). Cerebral cavernous malformation 2 protein promotes Smad ubiquitin regulatory factor 1-mediated RhoA degradation in endothelial cells. *J. Biol. Chem.* **284**, 13301-13305.

Degani, S., Balzac, F., Brancaccio, M., Guazzone, S., Retta, S. F., Silengo, L., Eva, A. and Tarone, G. (2002). The integrin cytoplasmic domain-associated protein ICAP-1 binds and regulates Rho family GTPases during cell spreading. *J. Cell Biol.* **156**, 377-387.

Discher, D. E., Janmey, P. and Wang, Y. L. (2005). Tissue cells feel and respond to the stiffness of their substrate. *Science* **310**, 1139-1143.

Engler, A. J., Sen, S., Sweeney, H. L. and Discher, D. E. (2006). Matrix elasticity directs stem cell lineage specification. *Cell* **126**, 677-689.

Faurobert, E., Rome, C., Lisowska, J., Manet-Dupé, S., Boulday, G., Malbouyres, M., Balland, M., Bouin, A.-P., Kéramidas, M., Bouvard, D. et al. (2013). CCM1-ICAP-1 complex controls β1 integrin-dependent endothelial contractility and fibronectin remodeling. *J. Cell Biol.* **202**, 545-561.

Gally, C., Wissler, F., Zahreddine, H., Quintin, S., Landmann, F. and Labouesse, M. (2009). Myosin II regulation during C. elegans embryonic elongation: LET-502/ROCK, MRCK-1 and PAK-1, three kinases with different roles. *Development* **136**, 3109-3119.

Hilder, T. L., Malone, M. H., Bencharit, S., Colicelli, J., Haystead, T. A., Johnson, G. L. and Wu, C. C. (2007). Proteomic identification of the cerebral cavernous malformation signaling complex. *J. Proteome Res.* **6**, 4343-4355.

Huang, C., Rajfur, Z., Yousefi, N., Chen, Z., Jacobson, K. and Ginsberg, M. H. (2009). Talin phosphorylation by Cdk5 regulates Smurf1-mediated talin head ubiquitylation and cell migration. *Nat. Cell Biol.* **11**, 624-630.

Jégou, A., Carlier, M.-F. and Romet-Lemonne, G. (2013). Formin mDia1 senses and generates mechanical forces on actin filaments. *Nat. Commun.* **4**, 1883.

Jiang, G., Huang, A. H., Cai, Y., Tanase, M. and Sheetz, M. P. (2006). Rigidity sensing at the leading edge through alphavbeta3 integrins and RPTPalpha. *Biophys. J.* **90**, 1804-1809.

Leung, T., Chen, X.-Q., Tan, I., Manser, E. and Lim, L. (1998). Myotonic dystrophy kinase-related Cdc42-binding kinase acts as a Cdc42 effector in promoting cytoskeletal reorganization. *Mol. Cell. Biol.* **18**, 130-140.

Liu, W., Draheim, K. M., Zhang, R., Calderwood, D. A. and Boggon, T. J. (2013). Mechanism for KRIT1 release of ICAP1-mediated suppression of integrin activation. *Mol. Cell* **49**, 719-729.

Lu, K., Yin, X., Weng, T., Xi, S., Li, L., Xing, G., Cheng, X., Yang, X., Zhang, L. and He, F. (2008). Targeting WW domains linker of HECT-type ubiquitin ligase Smurf1 for activation by CKIP-1. *Nat. Cell Biol.* **10**, 994-1002.

Lu, K., Li, P., Zhang, M., Xing, G., Li, X., Zhou, W., Bartlam, M., Zhang, L., Rao, Z. and He, F. (2011). Pivotal role of the C2 domain of the Smurf1 ubiquitin ligase in substrate selection. *J. Biol. Chem.* **286**, 16861-16870.

Millon-Frémillon, A., Bouvard, D., Grichine, A., Manet-Dupé, S., Block, M. R. and Albiges-Rizo, C. (2008). Cell adaptive response to extracellular matrix density is controlled by ICAP-1-dependent beta1-integrin affinity. *J. Cell Biol.* **180**, 427-441.

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

Moore, S. W., Roca-Cusachs, P. and Sheetz, M. P. (2010). Stretchy proteins on stretchy substrates: the important elements of integrin-mediated rigidity sensing. *Dev. Cell* **19**, 194-206.

Peter, J. M. S., Belén, A., Jacco van, R., Yvonne, M. W., Dirk, G., Kees, J., Ed, R., Stroeken, P. J., Alvarez, B., Van Rheenen, J. et al. (2006). Integrin cytoplasmic domain-associated protein-1 (ICAP-1) interacts with the ROCK-I kinase at the plasma membrane. *J. Cell. Physiol.* **208**, 620-628.

Raab, M., Swift, J., Dingal, P. C. D. P., Shah, P., Shin, J.-W. and Discher, D. E. (2012). Crawling from soft to stiff matrix polarizes the cytoskeleton and phosphoregulates myosin-II heavy chain. *J. Cell Biol.* **199**, 669-683.

Sahai, E., Garcia-Medina, R., Pouysségur, J. and Vial, E. (2007). Smurf1 regulates tumor cell plasticity and motility through degradation of RhoA leading to localized inhibition of contractility. *J. Cell Biol.* **176**, 35-42.

Schaefer, A., Nethe, M. and Hordijk, P. L. (2012). Ubiquitin links to cytoskeletal dynamics, cell adhesion and migration. *Biochem. J.* **442**, 13-25.

Su, Y.-T., Gao, C., Liu, Y., Guo, S., Wang, A., Wang, B., Erdjument-Bromage, H., Miyagi, M., Tempst, P. and Kao, H.-Y. (2013). Monoubiquitination of filamin B regulates vascular endothelial growth factor-mediated trafficking of histone deacetylase 7. *Mol. Cell. Biol.* **33**, 1546-1560.

Torrino, S., Visvikis, O., Doye, A., Boyer, L., Stefani, C., Munro, P., Bertoglio, J., Gacon, G., Mettouchi, A. and Lemichez, E. (2011). The E3 ubiquitin-ligase HACE1 catalyzes the ubiquitylation of active Rac1. *Dev. Cell* **21**, 959-965.

Totsukawa, G., Yamakita, Y., Yamashiro, S., Hartshorne, D. J., Sasaki, Y. and Matsumura, F. (2000). Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J. Cell Biol.* **150**, 797-806.

Totsukawa, G., Wu, Y., Sasaki, Y., Hartshorne, D. J., Yamakita, Y., Yamashiro, S. and Matsumura, F. (2004). Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts. *J. Cell Biol.* **164**, 427-439.

Tseng, Q., Wang, I., Duchemin-Pelletier, E., Azioune, A., Carpi, N., Gao, J., Filhol, O., Piel, M., Théry, M. and Balland, M. (2011). A new micropatterning method of soft substrates reveals that different tumorigenic signals can promote or reduce cell contraction levels. *Lab. Chip* **11**, 2231-2240.

Unbekandt, M. and Olson, M. F. (2014). The actin-myosin regulatory MRCK kinases: regulation, biological functions and associations with human cancer. *J. Mol. Med.* **92**, 217-225.

Visvikis, O., Lorès, P., Boyer, L., Chardin, P., Lemichez, E. and Gacon, G. (2008). Activated Rac1, but not the tumorigenic variant Rac1b, is ubiquitinated on Lys 147 through a JNK-regulated process. *FEBS J.* **275**, 386-396.

Wang, H.-R., Zhang, Y., Ozdamar, B., Ogunjimi, A. A., Alexandrova, E., Thomsen, G. H. and Wrana, J. L. (2003). Regulation of cell polarity and protrusion formation by targeting RhoA for degradation. *Science* **302**, 1775-1779.

Wang, H.-R., Ogunjimi, A. A., Zhang, Y., Ozdamar, B., Bose, R. and Wrana, J. L. (2006). Degradation of RhoA by Smurf1 ubiquitin ligase. *Methods Enzymol.* **406**, 437-447.

Wilkinson, S., Paterson, H. F. and Marshall, C. J. (2005). Cdc42-MRCK and Rho-ROCK signalling cooperate in myosin phosphorylation and cell invasion. *Nat. Cell Biol.* **7**, 255-261.

Ye, F., Snider, A. K. and Ginsberg, M. H. (2014). Talin and kindlin: the one-two punch in integrin activation. *Front. Med.* **8**, 6-16.

Zhu, H., Kavsak, P., Abdollah, S., Wrana, J. L. and Thomsen, G. H. (1999). A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature* **400**, 687-693.

Supplemental information:

Supplemental information includes 4 supplemental figures and 8 supplemental movies.

Supplemental figures:

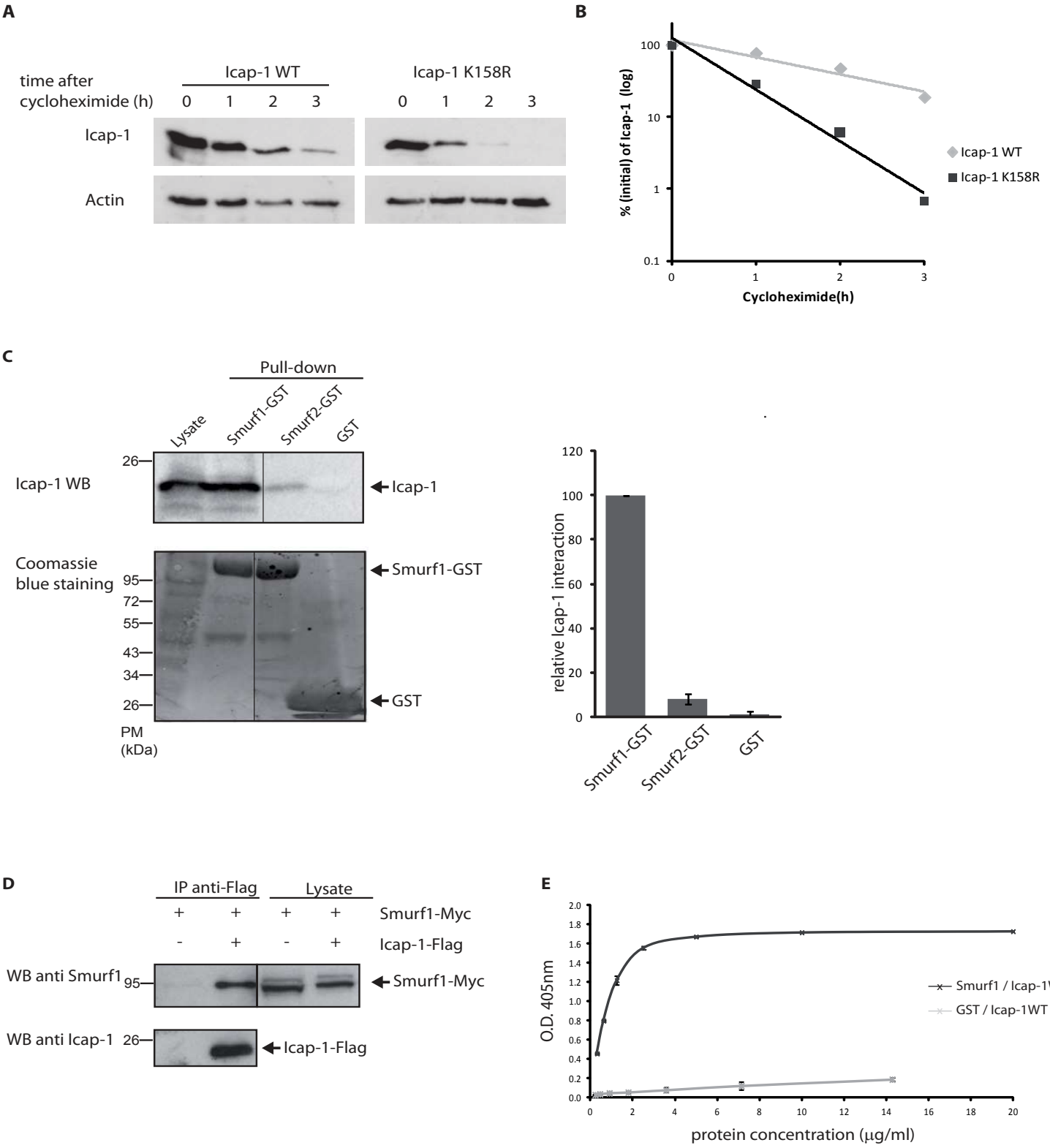


Fig. S1: Monoubiquitination of ICAP-1 by Smurf1 does not lead to ICAP-1 degradation.

A. Cycloheximide was added at t=0 to block protein synthesis. The ICAP-1 protein content in the total lysates was visualized at the indicated times by Western blotting. The results are representative of three independent experiments. B. Quantification of ICAP-1 WT or K158R mutant protein levels over a time-course after the inhibition of protein synthesis. The results are the mean of three independent experiments. C. CHO lysates overexpressing ICAP-1 were incubated with immobilized recombinant Smurf1-GST, Smurf2-GST or GST protein as a control. Interacting protein was analyzed by Western blotting with the anti-ICAP-1 antibody (left panel) and quantified (right panel). The GST protein quantities were controlled using Coomassie blue staining. The results are the mean of two independent experiments. D. Smurf1-myc and ICAP-1-Flag are co-expressed in CHO cells and coimmunoprecipitated with anti-Flag antibodies before blotting against either with anti-Smurf1 or anti-ICAP-1 antibodies. E. Elisa assay showing the direct interaction between Smurf1 and ICAP-1 by using purified recombinant GST-Smurf1 and purified recombinant ICAP-1-His.

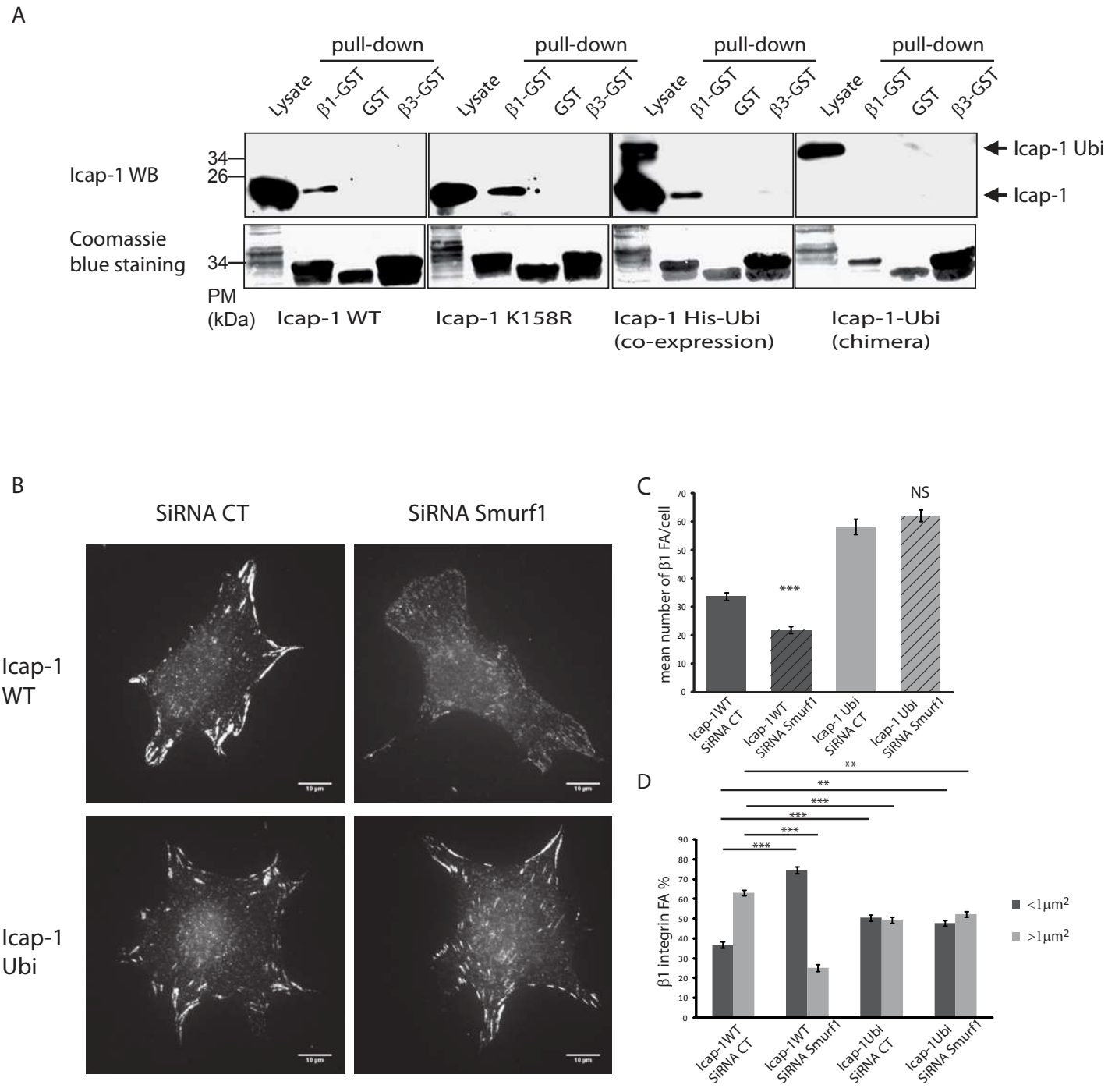
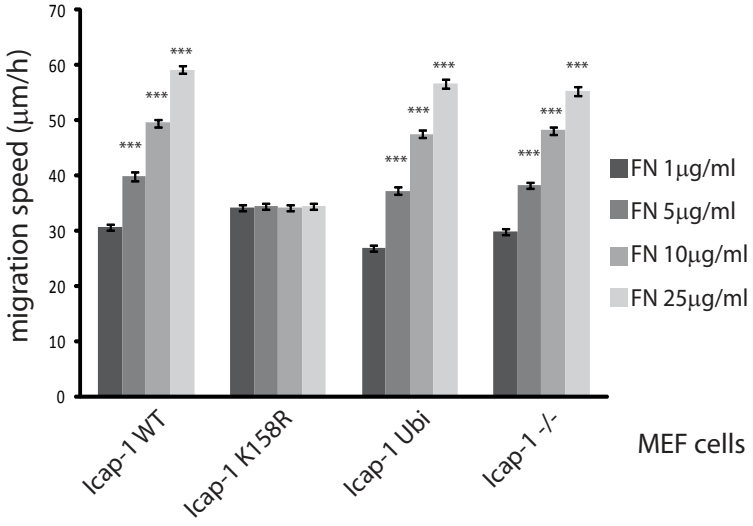


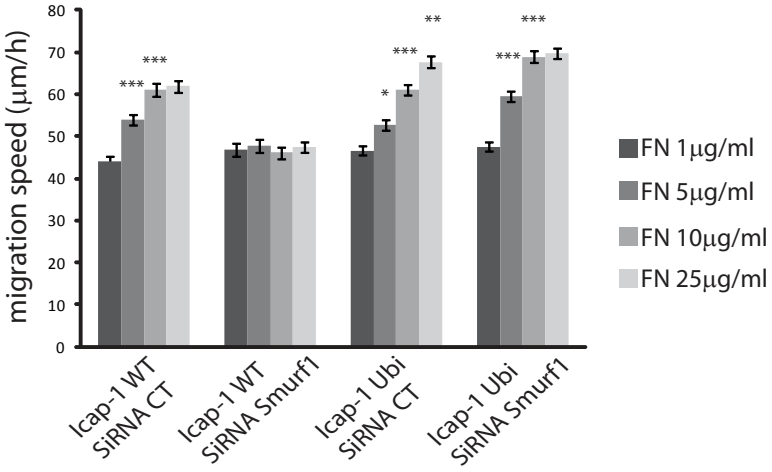
Fig. S2: The deletion of Smurf1 leads to focal adhesion disorganization like ICAP-1 K158R. A. CHO lysates overexpressing the different ICAP-1 constructs were incubated with immobilized recombinant β1-integrin-tail-GST, β3-integrin-tail-GST or GST protein as a control. The interacting proteins were analyzed by Western blotting with the anti-ICAP-1 antibody. The GST protein quantities were controlled using Coomassie blue staining. The results are representatives of three independent experiments. B. β1 integrin staining of ICAP-1-null osteoblasts rescued with ICAP-1 WT or the ICAP-1 ubiquitin chimera treated with

control or Smurf1 siRNA and spread on FN for 2.5 h. Similar to the cells expressing ICAP-1 K158R, the cells expressing ICAP-1 WT that were treated with Smurf1 siRNA displayed fewer and smaller β1 focal adhesions than the cells treated with control siRNA. C. Quantification of the β1 integrin focal adhesion number. D. Distribution of the β1 integrin focal adhesion areas. Analyses were performed on 30-40 cells from two independent experiments. Error bars indicate SEM. *p< 0.05, **p<0.005, ***p<0.0005.

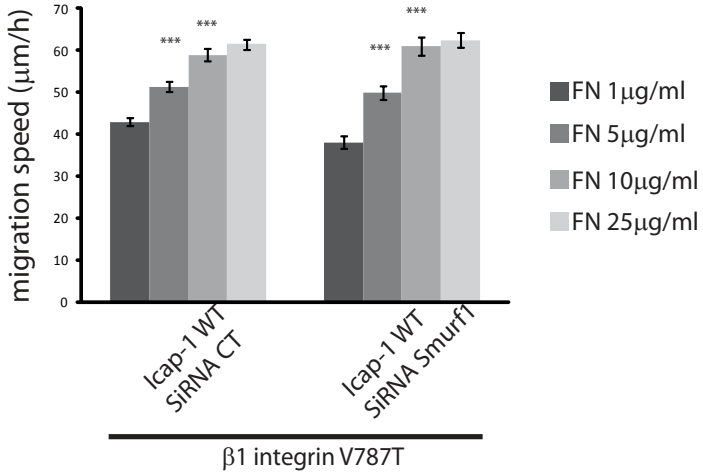
A


migration speed (µm/h)
FN 1µg/ml
FN 5µg/ml
FN 10µg/ml
FN 25µg/ml
Icap-1 WT
Icap-1 K158R
Icap-1 Ubi
Icap-1 -/-
MEF cells

B


migration speed (µm/h)
FN 1µg/ml
FN 5µg/ml
FN 10µg/ml
FN 25µg/ml
Icap-1 WT SiRNA CT
Icap-1 WT SiRNA Smurf1
Icap-1 Ubi SiRNA CT
Icap-1 Ubi SiRNA Smurf1

C


migration speed (µm/h)
FN 1µg/ml
FN 5µg/ml
FN 10µg/ml
FN 25µg/ml
Icap-1 WT SiRNA CT
Icap-1 WT SiRNA Smurf1
β1 integrin V787T

D

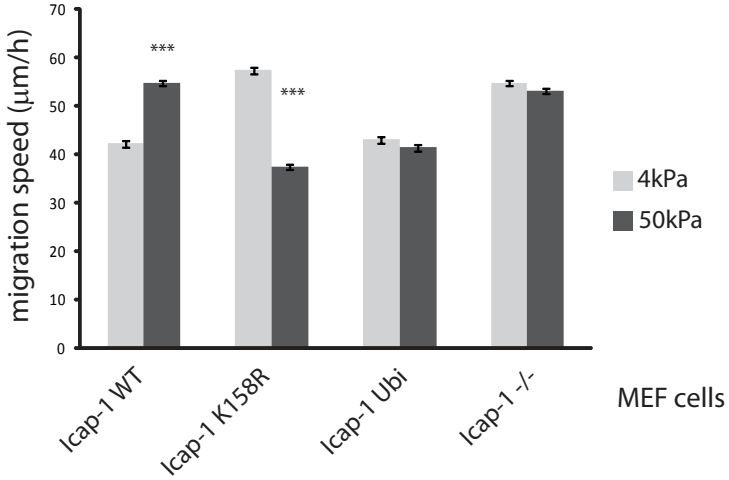

migration speed (µm/h)
4kPa
50kPa
Icap-1 WT
Icap-1 K158R
Icap-1 Ubi
Icap-1 -/-
MEF cells

Fig. S3: The deletion of Smurf1 leads to the unresponsiveness of cells to the FN density like ICAP-1 K158R. A. Fibronectin density sensing assay in MEF cells. MEF cells were spread on increasing concentrations of FN and migration was monitored for 5 h using time-lapse microscopy. Cell velocity was determined by individually tracking 150-200 cells from three independent experiments. The cells expressing ICAP-1 WT or the ICAP-1 ubiquitin chimera or cells deficient in ICAP-1 adapted their migratory speed according to the FN density, whereas the cells expressing the ICAP-1 K158R mutant maintained the same speed regardless of the FN density. B. ICAP-1 WT-expressing osteoblast cells that were treated with Smurf1 siRNA were unable to adapt their migratory speed to increasing FN density. This defect was rescued by the ICAP-1 ubiquitin chimera. C. β1 integrin-null osteoblast cells expressing the β1 integrin mutant that lacks ICAP-1 binding (V787T) were not affected by Smurf1 siRNA treatment. D. Rigidity sensing assay in MEF cells. Error bars indicate the mean +/- SEM. *p< 0.05, **p<0.005, ***p<0.0005.

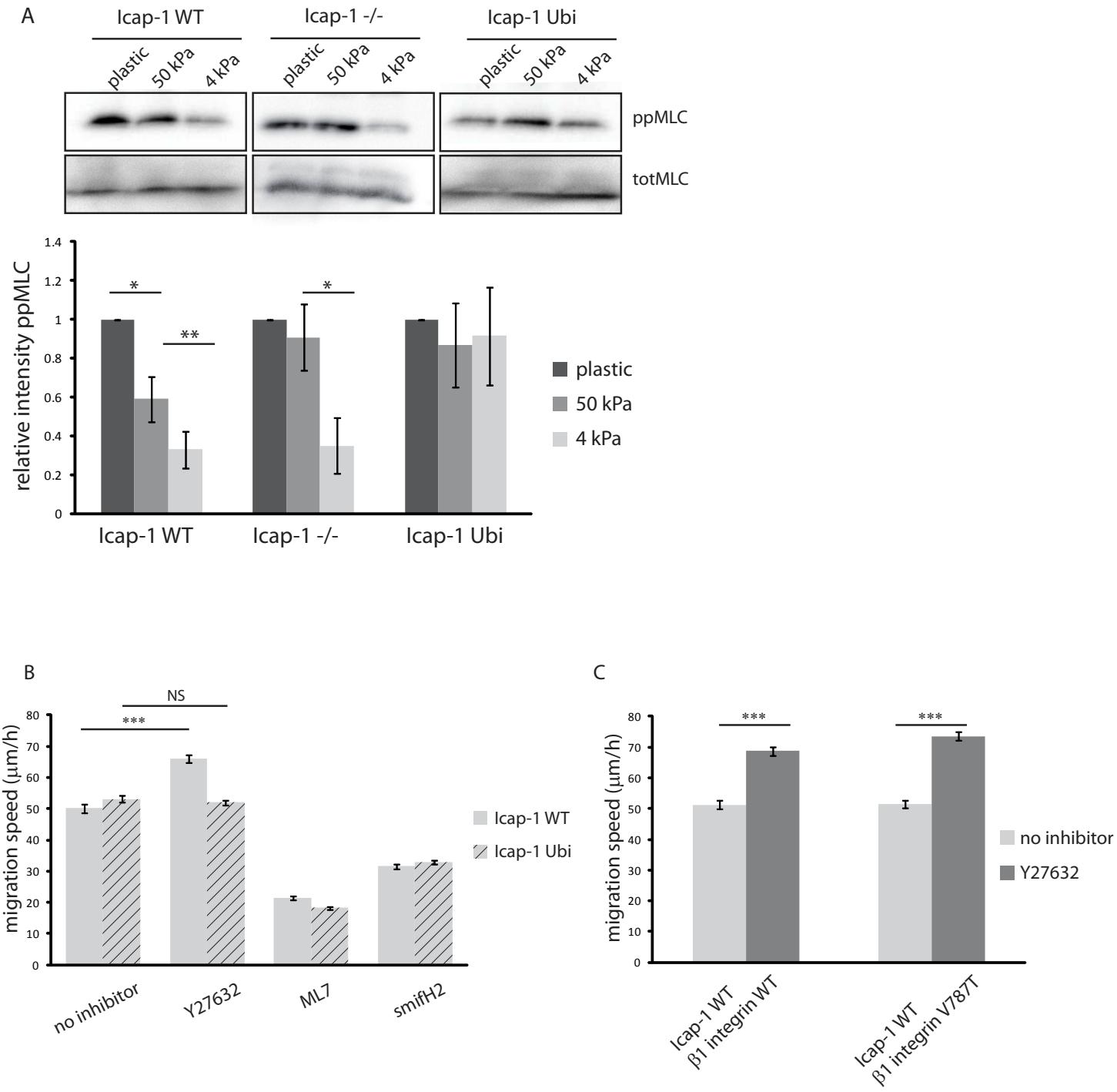


Fig. S4: Identification of contractile pathway in ICAP-1 Ubi osteoblast cells. A. The level of P-myosin is evaluated by western blot in ICAP-1 WT, ICAP-1 deficient and ICAP-1 Ubi cells (upper panel). Note the constant level of P-myosin in ICAP-1 Ubi cells whatever the substrate rigidity after the quantification of the western blot (bottom panel). B. Osteoblasts were spread on FN-coated PAA gels of different rigidities. Cell migration was monitored for 5 h using time-lapse microscopy. Cell velocity was determined by individually tracking of 200-300 cells in three independent experiments to test the effect of inhibitors on WT Osteoblasts cells and osteoblasts transfected with ICAP-1 Ubi cells on 4kPa gels (Y27632: ROCK

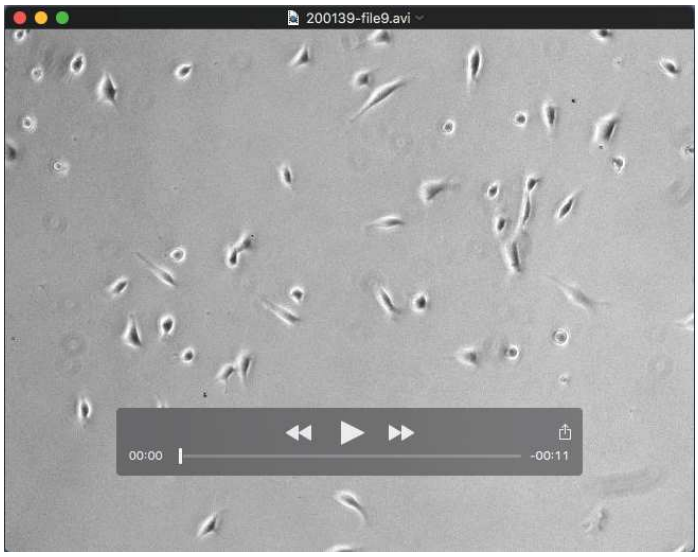
inhibitor, 10 μM, ML7: MLCK inhibitor, 5 μM, SmifH2: mDia inhibitor 2 μM). Note that cells expressing ICAP-1 Ubi are insensitive to Y27632. C. β1 integrin-null cells expressing the β1 integrin mutant that lacks ICAP-1 binding (V787T) on 4kPa gels responded to Y27632 treatment in a similar manner to that of the control WT osteoblast cells.

Movie S1: Migration of WT osteoblast cells on 1 μg/ml FN

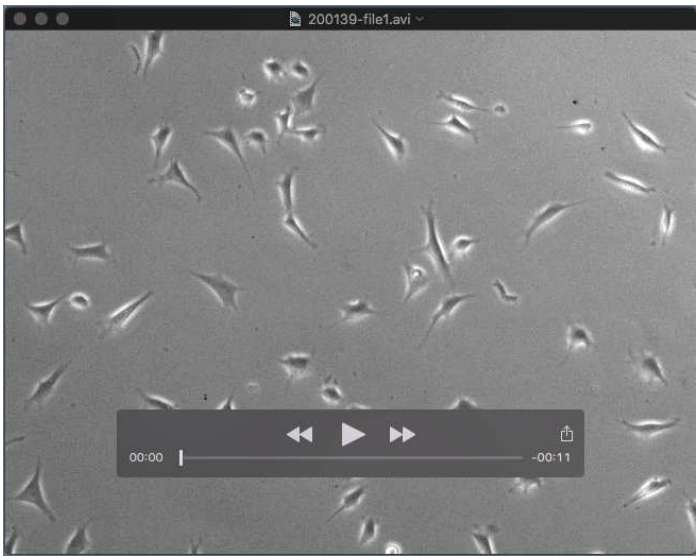
Movie S2: Migration of WT osteoblast cells on 25 μg/ml FN

Movie S3: Migration of ICAP-1 K158R osteoblast cells on 1 μg/ml FN

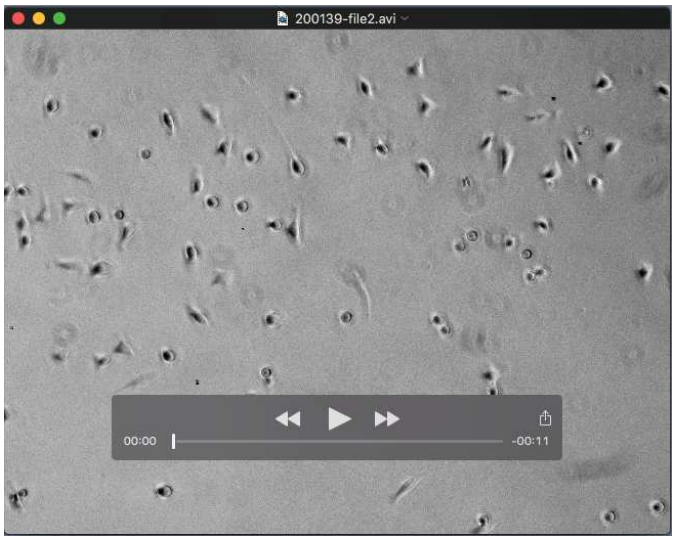
Movie S4: Migration of ICAP-1 K158R osteoblast cells on 25 μg/ml FN



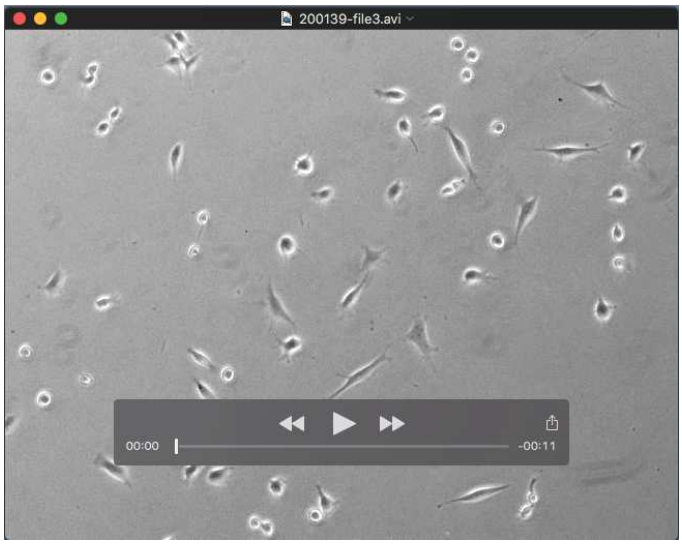
Movie S5: Migration of WT osteoblast cells on 4 kPa gel coated with 5 μg/ml FN



Movie S6: Migration of WT osteoblast cells on 50 kPa gel coated with 5 μg/ml FN



Movie S7: Migration of ICAP-1 Ubi osteoblast cells on 4 kPa gel coated with 5 μg/ml FN



Movie S8: Migration of ICAP-1 Ubi osteoblast cells on 50 kPa gel coated with 5 μg/ml FN

Chapter 8. ICAP-1 is involved in integrin dynamics and force generation by controlling integrin endocytosis through Nm23-dependent clathrin coated pits.

8.1. Specific scientific context

Signal transduction from extracellular matrix (ECM) originates at the membrane, where the clustering of adhesive receptors is a key step in adapting cellular force and transmitting a message (Cebecauer et al., 2010; Groves and Kuriyan, 2010; Salaita et al., 2010). By following this rule, adhesive receptors, most notably the integrins help cells for perceiving their microenvironment by sensing chemical, physical and mechanical cues of ECM through adhesive machinery and actomyosin-based contractility (Albiges-Rizo et al., 2009; Engler et al., 2006). Actomyosin-mediated contractility is a highly conserved mechanism for generating mechanical stress in animal cells and underlies cell shape, cell migration, cell differentiation and morphogenesis (Murrell et al., 2015). Cell adhesion mediated by fibronectin-binding integrins leads to the formation of nascent adhesions that eventually mature into large focal adhesions connected to actin stress fibers and then eventually convert into central or fibrillar adhesions (Geiger et al., 2001). Most of cells exploring fibronectin-based microenvironments engage a5β1 and avβ3 class integrins to orchestrate membrane protrusions, cell contractility and cell migration. The regulation of integrin function can be achieved on several levels, including ligand engagement and binding of intracellular adaptors. These intracellular adaptors are able to control their clustering state and their activation switch crucial for modulating integrin–ligand binding affinity and for serving as nucleation points for the assembly of larger signaling and structural scaffolds (Legate and Fässler, 2009). For example, talin is

one of the adaptor important for building actin-bound mechanosensitive complexes (Klapholz and Brown, 2017; Shattil et al., 2010). In turn, mechanical force is known to be an important factor in integrin activation and can contribute to both outside-in and inside-out signaling (Alon and Dustin, 2007; Zhu et al., 2008a). Different studies uncovered striking differences in the ability of a5β1- and avβ3-class integrins to adjust adhesion lifetime and strength to mechanical forces. Whereas a5β1 is known to adhere efficiently to fibronectin and generate cellular traction force, av-class integrin-mediated adhesion is strongly reinforced by myosin II, and with time the av-class integrins accumulate in adhesion areas exposed to the highest traction forces (Kuo et al., 2011; Schiller et al., 2013, 2011b). These finding are reminiscent of single protein tracking experiments of β1- and β3-integrins, which showed that β3-integrins are immobilized in large focal adhesions, whilst β1-integrins are more mobile (Rossier et al., 2012). Interestingly, avβ3-integrin has been shown to be needed for cell stiffening on force application, which might be consistent with a role in structural reinforcement of the adhesion (Roca-Cusachs et al., 2009). Reinforcement of focal adhesion and cell contractility is likely coupled with the inhibition of focal-adhesion dissolution process. Focal adhesion disassembly involves microtubule targeting, enhanced integrin endocytosis, calpain-mediated cleavage of talin, and loss of tension following Rho kinase inhibition (Wehrle-Haller, 2012). Together, these observations suggest that a5β1 integrin and avβ3 integrin are subjected to functional specifications for adjusting adhesion lifetime and strength to mechanical tension. These studies also predict that the collaborative work between integrin heterodimers is more complex than previously thought. It is still unclear whether and how the two Fibronectin-binding integrin classes signal to each other to orchestrate assembly or disassembly of adhesion sites or to strengthen adhesion or to adapt force to Fibronectin-based environment. The signaling pathways controlling the reciprocity between avβ3 and a5β1 need to be explored.

ICAP-1 was first identified as a specific partner of β1 integrin (Zhang and Hemler, 1999b; Degani et al., 2002). ICAP-1 encompasses a phosphotyrosine binding domain (PTB domain) which interacts specifically with the cytoplasmic tail of β1 integrin at the membrane-distal NPXY motif. It was subsequently found to be a partner of Nm23-H2, the human metastatic suppressor (Fournier et al., 2002b). ICAP-1, Nm23-H2 and β1 integrin are colocalized at the leading edges of cells during the early stages of spreading suggesting a transitory role of this complex in adhesion site dynamics (Fournier et al., 2002b). However, the biological relevance of ICAP-1/Nm23-H2 interaction in cell adhesion field is still unknown. The N terminal domain of ICAP-1 contains multiple phosphorylation consensus sites. The calcium and calmodulin-dependent serine/threonine protein kinase of type II (CaMKII) is an important regulator of ICAP-1 for controlling focal adhesion dynamics (Millon-Frémillon et al., 2013, 2008). Mechanistically, we previously reported that ICAP-1 limits both talin and kindlin interaction with β1 integrin and thereby restraining focal adhesion assembly. ICAP-1 direct interaction with the β1 integrin tail and the modulation of β1 integrin affinity state are required for down regulating focal adhesion assembly. We have also shown that ICAP-1 is involved in cell mechanoresponse and cell differentiation in a β1 integrin dependent manner (Brunner et al., 2011b; Faurobert et al., 2013; Bouvard et al., 2007; Renz et al., 2015; Millon-Frémillon et al., 2008). However, as ICAP-1 is also able to adapt cell migration in response to substrate stiffness in a β1-integrin-independent manner (Bouin et al., 2017b), we speculated on a more general role of ICAP-1 in cell adhesion and focal adhesion dynamics. The involvement of ICAP-1 in cell contractility and fibronectin fibrillogenesis makes it an attractive candidate for adapting cell migration (or behavior) by playing a role in force regulation.

8.2. Specific objectives of this study

To address this question, we have established cellular environment where ICAP-1 and its favorite β1 integrin were absent by using four cell lines: WT osteoblast, β1 integrin KO osteoblast cells, ICAP-1 KO osteoblast cells and double KO β1/ICAP-1 osteoblast cells in order to monitor β3 integrin behavior. Unexpectedly, our data show that β3 integrins are able to generate forces in cells depleted in β1 integrin and ICAP-1. Our results suggest that ICAP-1 is involved in β3 integrin dynamics and force generation by controlling β3 integrin endocytosis through Nm23-dependent scission of endocytic clathrin coated pits.

8.3. Results

8.3.1. β3 integrin is able to generate traction force in absence of β1 integrin and ICAP-1

We addressed the question about β1 integrin specificity and the role of its negative regulator called ICAP-1 on β3 integrin cell adhesive behaviors by using four osteoblast cell lines: *β1 integrin$^{+/+}$-icap-1$^{+/+}$, β1 integrin$^{+/+}$-icap-1$^{-/-}$, β1 integrin$^{-/-}$-icap-1$^{+/+}$ and β1 integrin$^{-/-}$-icap-1$^{-/-}$* cells. The deletion of β1 integrin or ICAP-1 was confirmed by western blot (Fig. 8.3.1A). Based on western blot analysis (Fig. 8.3.1A), qPCR analysis (Fig. 8.3.1B) and FACS analysis (Fig. 8.3.1C), we checked that the deletion of β1 integrin or ICAP-1 do not affect β3 integrin expression and its cell surface expression.

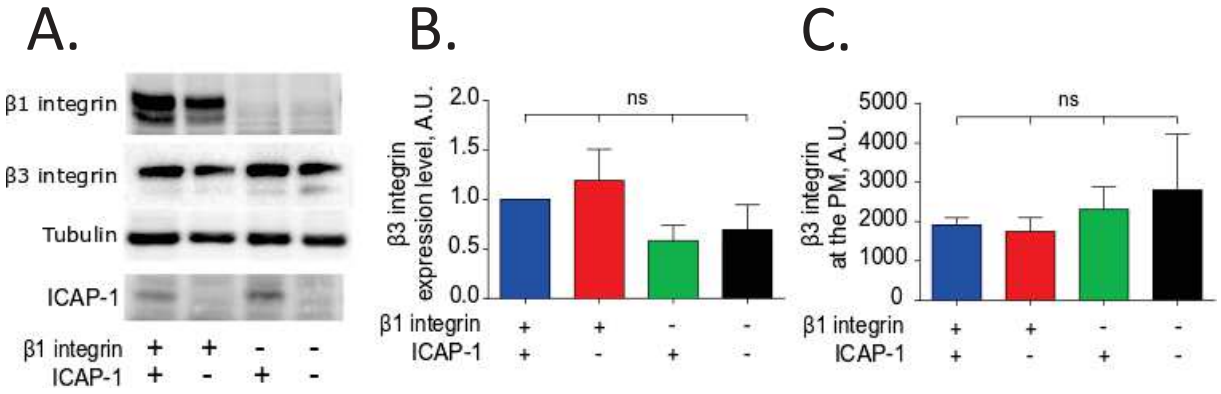


Figure 8.3.1. ICAP-1 KO osteoblasts are able to exert traction force on fibronectin substrate in the absence of β1 integrin.
A-C Western blot of total cell lysate confirmed the deletion of β1 integrin or ICAP-1 (A). Based on western blot analysis (B), qPCR analysis (C) and FACS analysis (D), we checked that the deletion of β1 integrin or ICAP-1 do not affect β3 integrin expression and its cell surface expression. Error bars represent standard deviation of at least 20 cells/experiment. ns adjusted p.value > 0.05. Experiment was done three independent times.

To understand to what extent individual integrin classes β1 and β3 integrins bound to the same ECM ligand use specific pathways to sense and exert force, we designed a quantitative traction force microscopy of osteoblast mutants seeded on fibronectin (FbN) coated hydrogels with a Young's modulus (E) of 5 kPa. First,

our results reveal that osteoblasts deficient in β1 integrin (*β1 integrin$^{-/-}$-icap-1$^{+/+}$*) are defective in force generation (Fig. 8.3.1D-E) as it has been already described in the literature (Danen et al., 2002; Schiller et al., 2013). The total cell force measured on β1 integrin KO cells decreased by more than 50% as compared to wild-type cells (Fig. 8.3.1E).

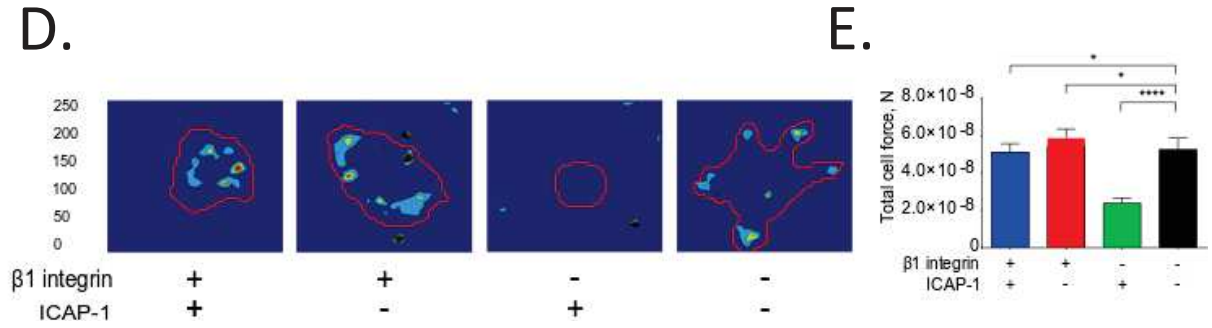


Figure 8.3.1. Osteoblasts are able to exert traction force on fibronectin substrate in the absence of β1 integrin.
D-E. Representative traction forces maps and quantification (E) of the total force applied (N) on fibronectin-coated polyacrylamide gel with a defined rigidity of 5 kPa. β1 integrin KO cells exert less force than the other osteoblasts mutants. The additional deletion of ICAP-1 led to generation of TFs revealing a novel pathway independent of β1 integrins to generate TFs on fibronectin. Error bars represent standard deviation of at least 20 cells/experiment. **** adjusted p.value ≤ 0.0001, * adjusted p.value ≤ 0.05.

Secondly and unexpectedly, the additional loss of ICAP-1 in osteoblasts cells deficient for β1 integrin was able to rescue the developed contractile energy (Fig. 8.3.1D-E) which is supported by the drastic increase of P-myosin light chain (pp-MLC) as judged by the western blot using double phospho sites specific antibody against myosin light chain (Fig. 8.3.1F).

The rescue of traction force in *β1 integrin$^{-/-}$-icap-1$^{-/-}$* cells was associated with a rescue of cell spreading and restoration of thick stress fibers highly decorated with P-myosin light chain as compared to *β1 integrin$^{-/-}$* cells which display limited spreading and poorly spread actin cytoskeleton (Fig. 8.3.1G-H). These results indicate that traction strength on deformable FbN substrate is not strictly correlated with the presence of β1 integrins and is likely related with another integrin regulated by the presence of ICAP-1.

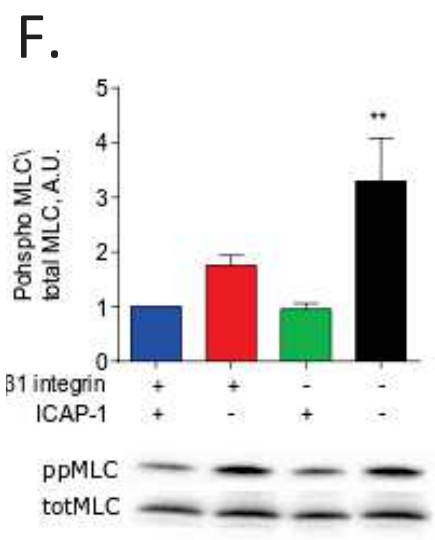


Figure 8.3.1. Osteoblasts are able to exert traction force on fibronectin substrate in the absence of β1 integrin.
F. The level of the double phosphorylation (T18/S19) of the MLC was assessed via Western Blot against the total level of MLC of cell lysates of cells spread for 4 hours on fibronectin covered glass. The increased TFs in the β1$^{-/-}$/ICAP-1$^{-/-}$ cells correlated with an elevated levels of double phosphorylated myosin light chain (MLC or RLC). Error bars represent standard deviation. ** adjusted p. value ≤ 0.01. Experiment was done three independent times.

As FbN- coated surface mediates RGD binding to a5β1 and avβ3 integrins (Leiss et al., 2008) which exert both specific and redundant functions (Ballestrem et al., 2001b; Danen et al., 2002), we investigated whether the increase of traction forces in *β1 integrin$^{-/-}$-icap-1$^{-/-}$* cells was dependent on β3 engagement. Silencing the expression of β3 integrins via RNA interference lead to a dramatic decrease in cell spreading (Fig. 8.3.1I-J) and ppMLC staining (Fig. 8.3.1I-J). On line with this, siRNA against β3 integrin treatment abolished the high traction forces generated by *β1 integrin$^{-/-}$-icap-1$^{-/-}$* cells compared to control (Fig. 8.3.1K-L). It is noteworthy that siRNA β3 integrin treatment leads to the rounding up of the β1 integrin null (Fig. 8.3.1I) suggesting that their modest spreading is dependent on the expression of

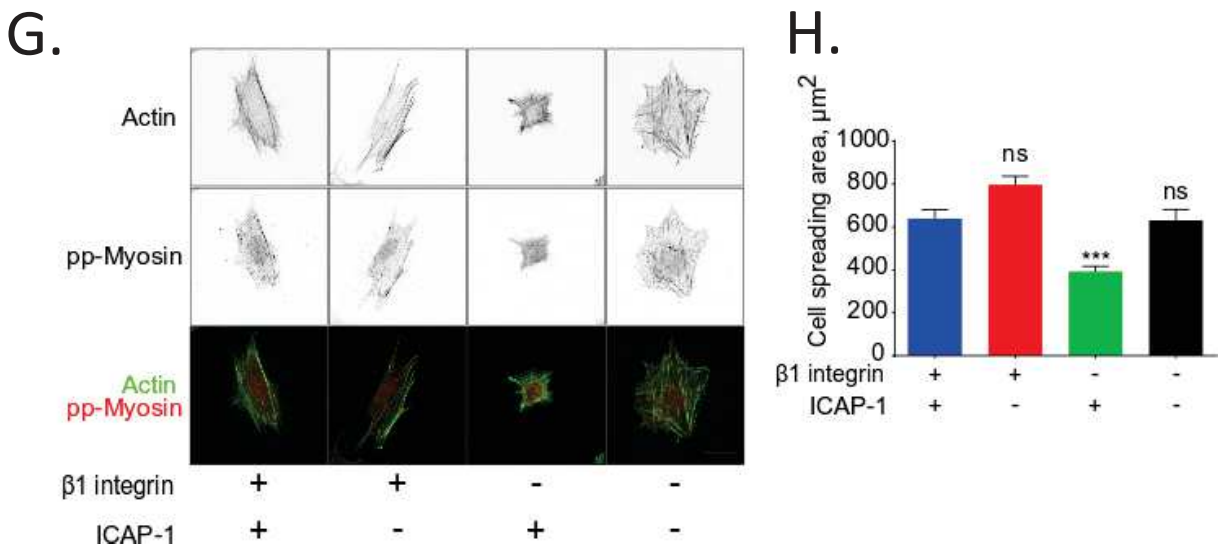
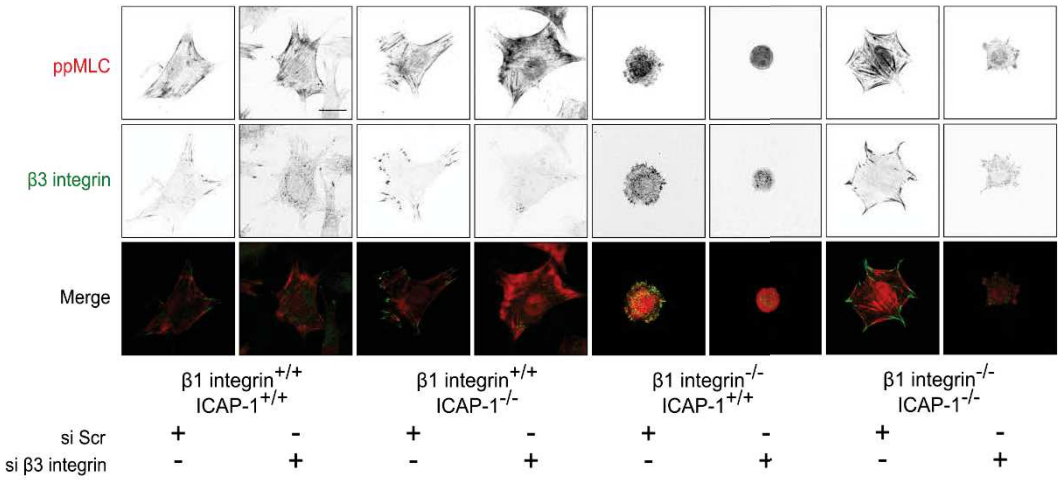


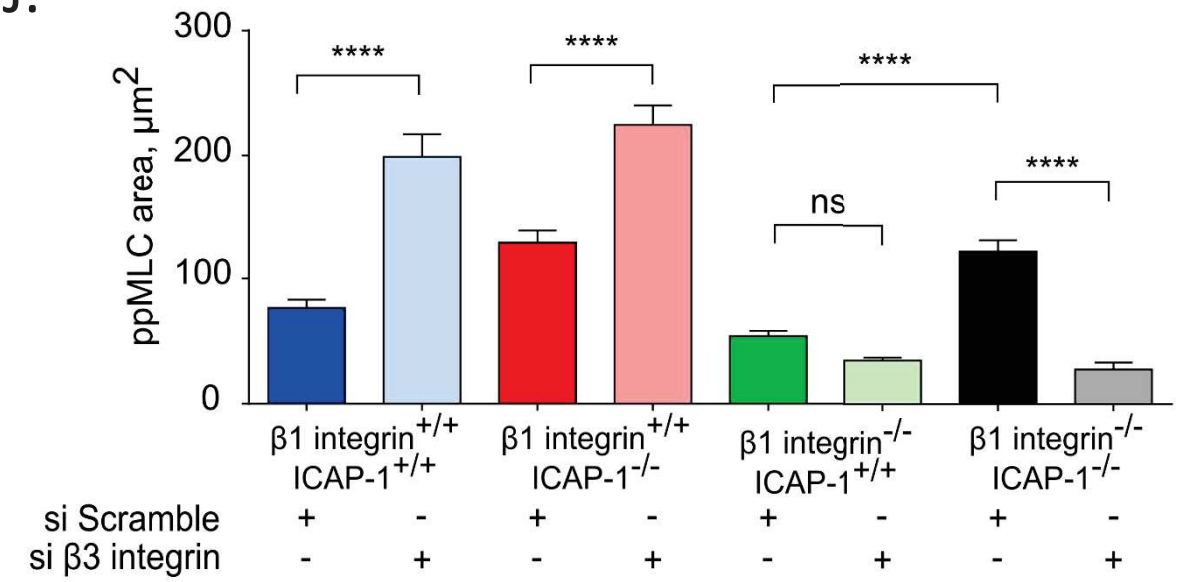
Figure 8.3.1. Osteoblasts are able to exert traction force on fibronectin substrate in the absence of β1 integrin.
G-H. Immunofluorescence staining of the ppMLC (red) and actin-F (phalloidin, blue) in the four osteoblasts mutants showed that deletion of ICAP-1 alone does not change the organization of acto-myosin CSK but increases slightly the intensity and the thickness of the fibers (see quantification of the ppMLC area in panel H). Deletion of β1 integrin leads to drastic decrease of the cell area and disorganization and decrease of thickness and number of the ppMLC decorated stress fibers. Error bars represent standard deviation of at least 20 cells/experiment. ** adjusted p.value ≤ 0.01. Experiment was done three independent times.

β3 integrins (Fig. 8.3.1I-J). Moreover, it is important to notice that β3 integrins silencing increases by more than twice the myosin light chain phosphorylation along the actin stress fibers for both *β1 integrin*$^{+/+-/-}$*-icap-1*$^{+/+}$ cells and *β1 integrin*$^{-/-}$ *-icap-1*$^{-/-}$ cell lines, suggesting that β3 integrins down regulate myosin II reinforcement dependent on β1 integrin when cells are spread on FbN stiff environment (Milloud et al., 2017) (Fig. 8.3.1I). Altogether these results identify not only the cross talk between β1 and β3 integrin to regulate cell spreading and force generation but also ICAP-1 as a molecular link to regulate this cross-talk. Finally, our results suggest a new regulatory role for ICAP-1 in the actomyosin contractility loading on β3 integrins.

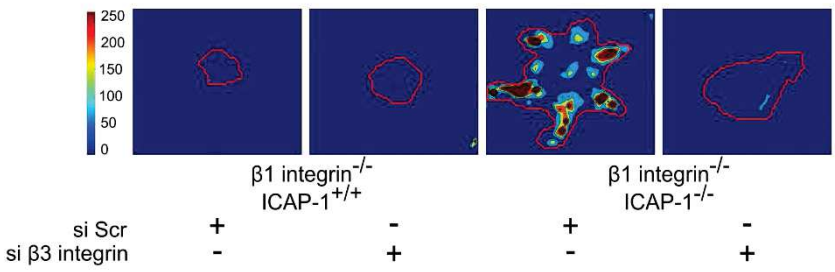
I.


ppMLC
β3 integrin
Merge
β1 integrin+/+ ICAP-1+/+
β1 integrin+/+ ICAP-1-/-
β1 integrin-/- ICAP-1+/+
β1 integrin-/- ICAP-1-/-
si Scr
si β3 integrin

J.


ppMLC area, µm2
300
200
100
0
****
****
****
****
ns
β1 integrin+/+ ICAP-1+/+
β1 integrin+/+ ICAP-1-/-
β1 integrin-/- ICAP-1+/+
β1 integrin-/- ICAP-1-/-
si Scramble
si β3 integrin

K.


250
200
150
100
50
0
β1 integrin-/- ICAP-1+/+
β1 integrin-/- ICAP-1-/-
si Scr
si β3 integrin

L.

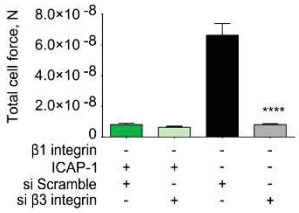

Total cell force, N
8.0×10-8
6.0×10-8
4.0×10-8
2.0×10-8
0
****
β1 integrin
ICAP-1
si Scramble
si β3 integrin

Figure 8.3.1. Osteoblasts are able to exert traction force on fibronectin substrate in the absence of β1 integrin.
I-J. Osteoblasts cells were transfected with β3 integrin siRNA for 48h and then let spread in on Fbn coated glass for 24 hours. After PFA fixation, stainings of ppMLC (red) and β3 integrin (green) were performed and analysed by fluorescent microscopy. Silencing the expression of β3 integrin *via* siRNA increased the stress fibers even more in the β1 positive cells (WT and ICAP-1 KO). In the $\beta1^{-/-}$/ICAP-$1^{-/-}$ cell line removing β3 integrin lead complete abolishment of the ppMLC decorated SF (stress fibers) and shrinkage of the cell area. Scale bar represents 20 µm. Quantification of the ppMLC area from the spinning disk images. Customized particle analysis script from ImageJ was used after application of Unsharpen mask and Despecle filters. The error bars represent standard deviations. **** adjusted p.value ≤ 0.0001. Experiments were done 3 times with at least 20 cells per condition.
K-L. TFM analysis on beta1 deficient cells shows that the additional silencing of β3 integrin in the $\beta1^{-/-}$/ICAP-$1^{-/-}$ cells decimates the TFs, confirming that generation of strong cellular contractility is dependent on β3 integrin in absence of β1 integrin and ICAP-1. Quantification of the total forces from TFs maps. Error bars represent standard deviation of at least 20 cells/experiment. ** adjusted p.value ≤ 0.01. Experiment was done three independent times.

8.3.2. β3 integrin dependent traction forces are associated with redistribution, lower membrane mobility and higher life time of large β3 integrin focal adhesions.

Next, we investigated whether the loss of β1 integrins and ICAP-1 and the associated contractile behavior might be related to a change in β3 integrin focal adhesions (FAs) organization and dynamics. All four osteoblast cells lines used here, were able to develop β3 integrin FAs on FbN coated surface as revealed by immunostaining or expression of β3 integrin-GFP (Fig. 8.3.2A). Nevertheless, the KO of β1 integrins reduced noticeably the cell spreading without affecting the adhesion area occupied by β3 integrins FAs (Fig. 8.3.2A-B and Fig. 8.3.2G) meaning that β3 integrins alone in FbN mediated adhesions fails to support the typical osteoblasts spreading.

Remarkably, the additional depletion of ICAP-1 in β1 integrin deficient cells (by gene KO or RNA silencing (Fig. 8.3.3A-B)) promotes significantly and considerably the cell spreading and the size of β3 integrins FAs (+/- 80 μm^2/cell: twice as more than the other mutants) (Fig. 8.3.2A-B and Fig. 11.3B).

Our results reveal a crucial role for ICAP-1 in controlling the β3 integrin clustering and the distribution of β3 integrins containing FAs in absence of β1 integrins. We hypothesized then a possible link between β3 integrins exchange rates and the presence of ICAP-1. To explore this hypothesis, we compared the dynamic of β3 integrins exchange rate in large peripheral focal adhesions link to actin stress fibers. We performed Fluorescent Recovery After Photobleaching (FRAP) using Total Internal Reflection Fluorescent microscopy (TIRF) experiments on the four cell lines transfected with β3 integrin- eGFP (Fig. 8.3.2C-F). It appeared that neither the loss of β1 integrins nor the one of ICAP-1 alone have any significant effect on the β3 integrin-eGFP exchange rate in FAs. However, the oversized β3 integrin FAs observed in *β1 integrin$^{-/-}$-icap-1$^{-/-}$* cells correlates with a threefold slower β3-EGFP integrin exchange rate (Fig. 8.3.2C) and with an increase of β3- integrin-eGFP FAs life time (Fig. 8.3.2D-E) which correlate with the development of stress fibers highly

decorated with P-myosin light chain. In the context of β1 null cell mutants, these data hint that the loss of ICAP-1 strongly affects the behavior of β3 integrins. These findings reveal that β3 integrin clustering and dynamics is under the control of ICAP-1 in conditions where β1 integrin is absent.

Figure 8.3.2. Size and dynamic of β3 integrin FAs are dependent on β1 integrins and ICAP-1

A. Indirect immunofluorescence surface staining of β3 integrins (Luc.A5 antibody, red) was carried on GFP-osteoblast cells spread for 4 hours cells on fibronectin coated coverslips. β1 integrin positive cell lines ($\beta1^{+/+}$/ICAP-$1^{+/+}$ and $\beta1^{+/+}$/ICAP-$1^{-/-}$) show classical distribution of β3 FAs - peripheral and elongated towards the SFs. β1 null cell line display small and punctuated FAs around the peripheral rim and multiple punctuated FAs at the ventral cell spread face. The $\beta1^{-/-}$/ICAP-$1^{-/-}$ exhibits both peripheral elongated FAs, and ventral elongated FAs, suggesting different activity states of β3 integrins. Scale bar represents 20 µm.

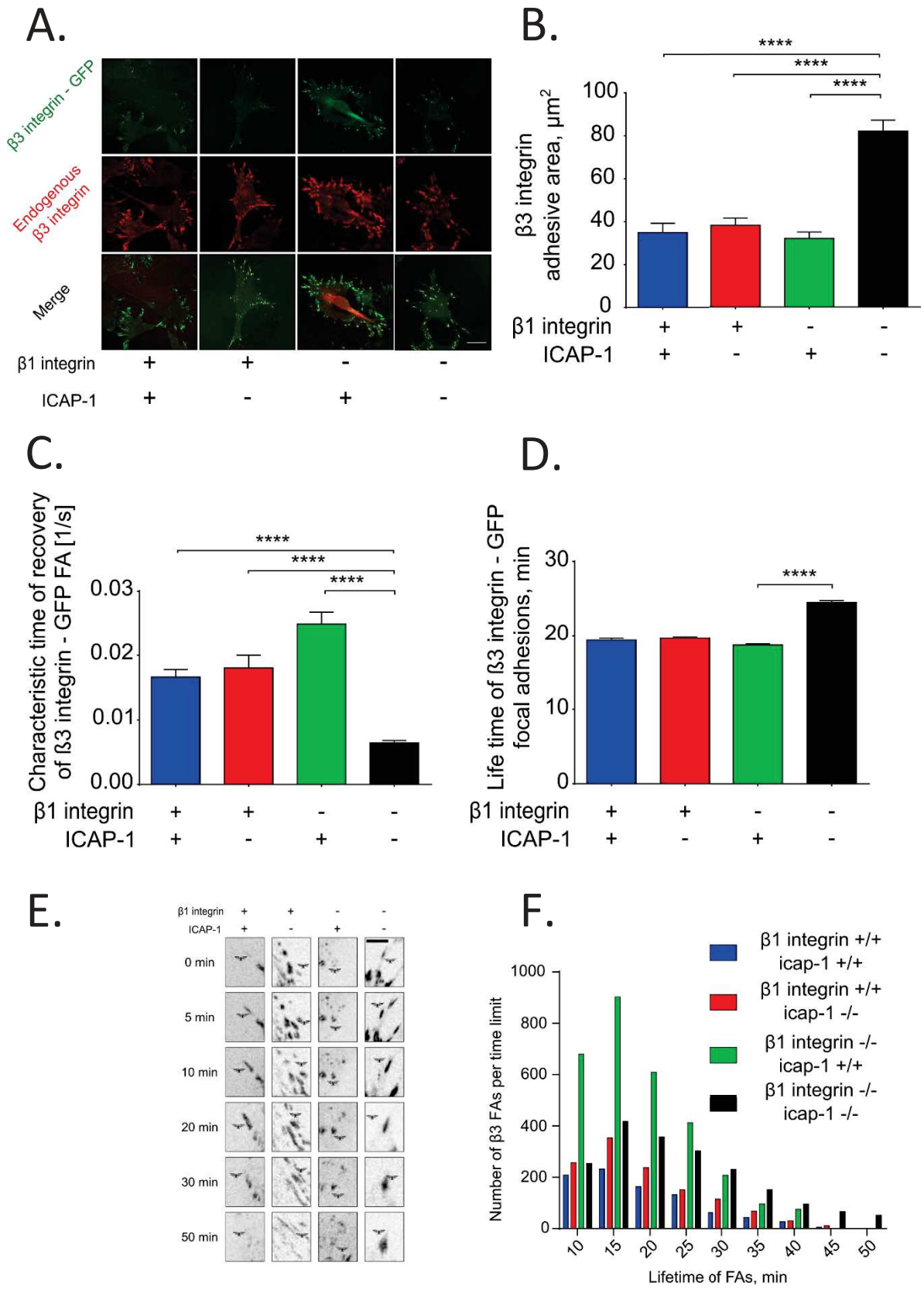
B. Quantification of the total focal adhesion area of β3 integrin FAs. Loss of β1 integrins decreases slightly the total β3 integrin adhesive area, while subsequent deletion of ICAP-1 drives massive leap of β3 integrin stained adhesive area. **** adjusted p.value ≤ 0.0001. Experiments were done 3 times with at least 20 cells per condition.

C. FRAP analysis on the GFP-β3 integrin FAs reveal that the β3 integrin mobility at the plasma membrane is halted in the absence of ICAP-1 and β1 KO. 5 FAs per cell were bleached and their recovery were monitored for 5 min. At least 10 cells were analyzed every experiment. Error bars represent standard deviation of at least 20 cells/experiment. **** adjusted p.value ≤ 0.0001. The experiment was performed in triplicate.

D. TIRF lifetime analysis on the GFP-β3 integrin FAs. The lifetime of the GFP-β3 integrin containing focal adhesions is increased in β1 integrin$^{-/-}$/ICAP-$1^{-/-}$ cells. Spinning disk videos of GFP-β3 integrin were taken for the duration of 2 hours. The adhesion lifetime was analyzed by Focal Adhesion Analysis Server (FAAS) (Berginski and Gomez, 2013) and verified visually. **** adjusted p.value ≤ 0.0001. The experiment was performed in triplicate.

E. Representative time series of the lifetime of β3 integrin – GFP focal adhesions of the four osteoblastic cell lines. Eagles point out FAs in the $\beta1^{-/-}$/ICAP-$1^{-/-}$ cells with typical slow down disassembly lifetime. Bar represents 5 µm.

F. Frequency distribution of the FAs lifetimes. The $\beta1^{-/-}$/ICAP-$1^{-/-}$ cells shows higher number of FA in the late time points.


A.
β3 integrin - GFP
Endogenous β3 integrin
Merge
β1 integrin + + - -
ICAP-1 + - + -
B.
β3 integrin adhesive area, μm²
100
80
60
40
20
0
****
****
****
β1 integrin + + - -
ICAP-1 + - + -
C.
Characteristic time of recovery of ß3 integrin - GFP FA [1/s]
0.03
0.02
0.01
0.00
****
****
****
β1 integrin + + - -
ICAP-1 + - + -
D.
Life time of ß3 integrin - GFP focal adhesions, min
30
20
10
0
****
β1 integrin + + - -
ICAP-1 + - + -
E.
β1 integrin + + - -
ICAP-1 + - + -
0 min
5 min
10 min
20 min
30 min
50 min
F.
Number of β3 FAs per time limit
1000
800
600
400
200
0
10 15 20 25 30 35 40 45 50
Lifetime of FAs, min
β1 integrin +/+ icap-1 +/+
β1 integrin +/+ icap-1 -/-
β1 integrin -/- icap-1 +/+
β1 integrin -/- icap-1 -/-

8.3.3. β3 integrin dependent traction forces are associated with formation of tensin dependent fibrillar adhesion

We addressed the question whether the increase of β3 integrin clustering and force generation in condition where β1 integrin and ICAP-1 are depleted, are associated with the ability to form fibrillary adhesions known to be formed under tension. We have already described the ability of *β1 integrin$^{+/+}$-icap-1$^{-/-}$* cells to develop more central β1 integrin containing focal adhesions as compared to *β1 integrin$^{+/+}$- icap-1$^{+/+}$* cells (Millon-Frémillon et al., 2008; Faurobert et al., 2013). At longer times of spreading, *beta1 integrin$^{+/+}$- icap-1$^{+/+}$ and beta1 integrin$^{+/+}$-icap-1$^{-/-}$* cells were able to form elongated fibrillar adhesions (Fig. 8.3.3A). Those elongated adhesions were enriched in active β1 integrins stained by 9EG7 antibody specific for activated β1 integrin chain which co-localized with P-myosin light chain decorating actin stress fibers (Fig. 8.3.3A). As expected, β1 integrin$^{-/-}$ cells did not display any elongated fibrillar adhesions whereas the additional loss of ICAP-1 lead to fibrillar adhesions colocalizing with myosin II stress fibers and enriched in β3 integrins but devoid of β1 integrins (Fig. 8.3.3B). In fibroblast like cells, it is well described that, integrins translocate centripetally from focal adhesions to mature, centrally located, elongated matrix contacts termed fibrillar adhesions, which are enriched in active β1 integrins and tensin (Pankov et al., 2000; Zamir et al., 2000b). To explore where tensin resides in cells lacking β1 integrins to undergo effective fibrillar adhesions, we investigate tensin co-localization in regard to expression of ICAP-1. Because of antibody limitations, we were unable to study endogenous tensin in mouse osteoblasts, therefore we transiently expressed GFP-tensin-1 in osteoblasts cell lines. Based on TIRF microscopy and analysis by plot profiles of GFP-tensin-1/β3 integrin, tensin-containing β3 integrins adhesion were localized at the periphery of *beta1 integrin$^{+/+}$-icap-1$^{+/+}$ and beta1 integrin$^{+/+}$- icap-1$^{-/-}$* cells (Fig. 8.3.3C-D). Many elongated adhesions of GFP-tensin were observable in the cell center but were not necessarily associated with β3 integrins (Fig. 8.3.3D). The tensin association must be related to β1 integrins (data not shown). For β1$^{-/-}$

integrin cells (*β1 integrin$^{-/-}$-icap-1$^{+/+}$ and β1 integrin$^{-/-}$-icap-1$^{-/-}$* cells), tensin regionalization was dependent on the presence of ICAP-1. Indeed, tensin can be associated with β3 integrins at the cell periphery but no co-localization was observed with β3 in cell center of *β1 integrin$^{-/-}$-icap-1$^{+/+}$* cells. The additional removal of ICAP-1 promotes the co-localization of tensin with β3 integrins at the rear of the cell and with elongated tensin fibrillar adhesions centrally positioned on the ventral cell surface (Fig. 8.3.3C-D). Moreover, we noted that the general distribution of β3 integrin containing FAs was affected by the lack of β1 integrins and not by the lack of ICAP-1. Indeed, in the case of *β1 integrin$^{+/+}$* cell lines, β3 integrins FAs localize at the periphery of spread cells as contrary to *β1 integrin$^{-/-}$* cell lines where β3 integrin FAs were rapidly redistributed between the cell periphery and randomly over the ventral cell face (Fig. 8.3.3E). Together, these results show that the distribution of β3 integrin FAs is regulated by the occurrence of β1 integrins. However, the additional lack of ICAP-1 promotes the formation of bulky β3 integrin FAs and β3 enriched fibrillar adhesions associated to tensin.

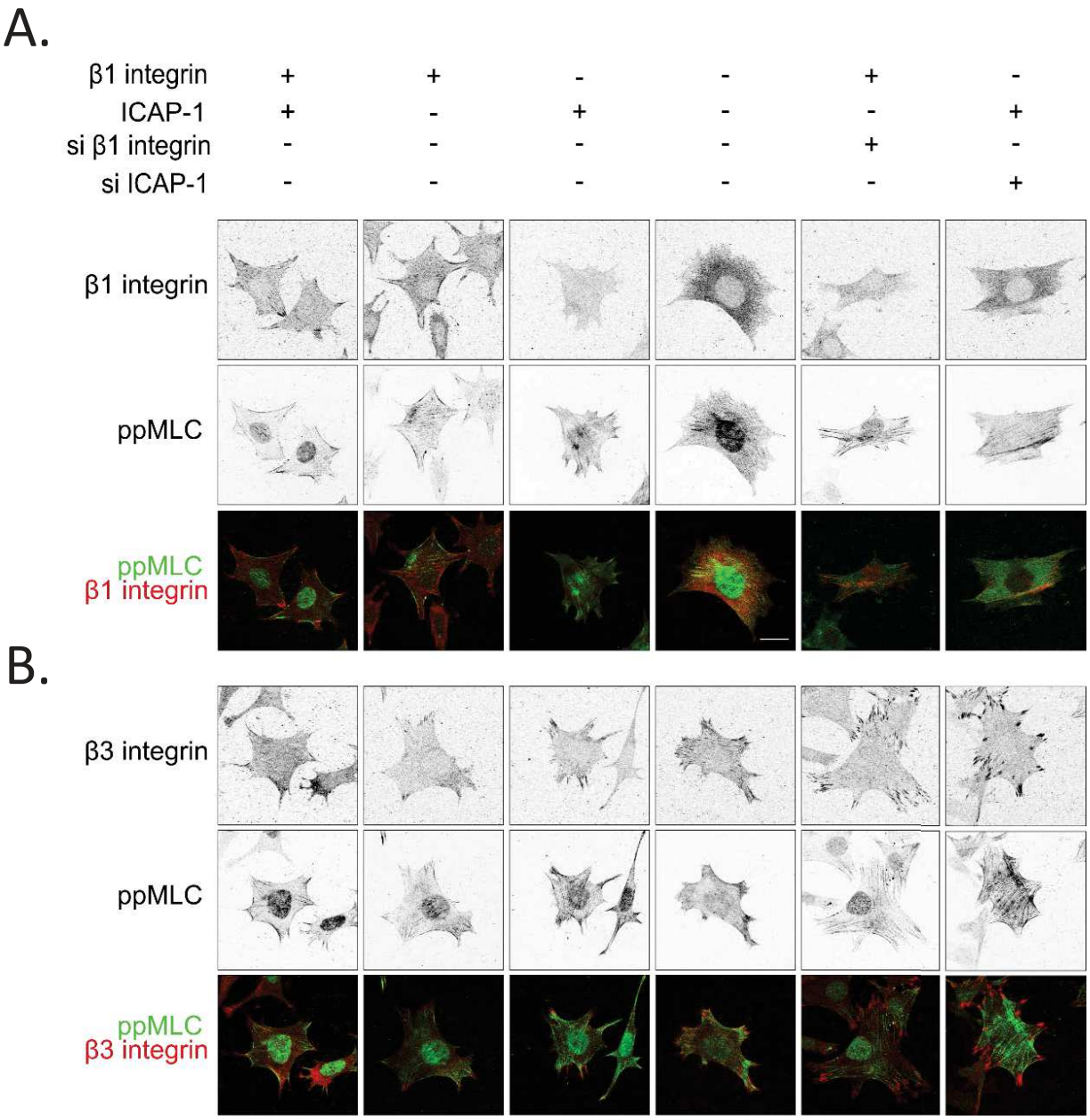

A.
β1 integrin + + - - + -
ICAP-1 + - + - - +
si β1 integrin - - - - + -
si ICAP-1 - - - - - +
β1 integrin
ppMLC
ppMLC
β1 integrin
B.
β3 integrin
ppMLC
ppMLC
β3 integrin

Figure 8.3.3. Restoration of traction forces in the β1$^{-/-}$/ICAP-1$^{-/-}$ is associated with the recruitment of tensin 1 along the fibrillar β3 integrin adhesions.
A-B. Indirect immunofluorescence surface staining of β1 integrins (9EG7, red) or β3 integrins (Luc.A5, red) and ppMLC (T18/S19, green) of osteoblastes mutants spread on Fbn coated glass. β1+/+ mutants express elongated activated β1integrins adhesion from the periphery to the ventral face co-localized with myosin stress fibers and β3 FAs to the cell periphery at the end of myosin stress fibers; β1-/- cells express β3 FAs to the cell periphery to the ventral face co-localized with slight myosin stress fibers, no β1 staining. β1$^{-/-}$/ICAP-1$^{-/-}$ cells express large β3 FAs to the cell periphery at the end of myosin stress fibers and elongated β3 integrins adhesion from the periphery to the ventral face co-localized with thick myosin stress fibers, no β1 staining. Note that siRNA-β1 integrin reverts the phenotype β1$^{+/+}$/ICAP-1$^{+/+}$ to a β1$^{-/-}$/ICAP-1$^{+/+}$ phenotype and siRNA-ICAP-1 reverts the phenotype β1$^{-/-}$/ICAP-1$^{+/+}$ to a β1$^{-/-}$/ICAP-1$^{-/-}$ phenotype.

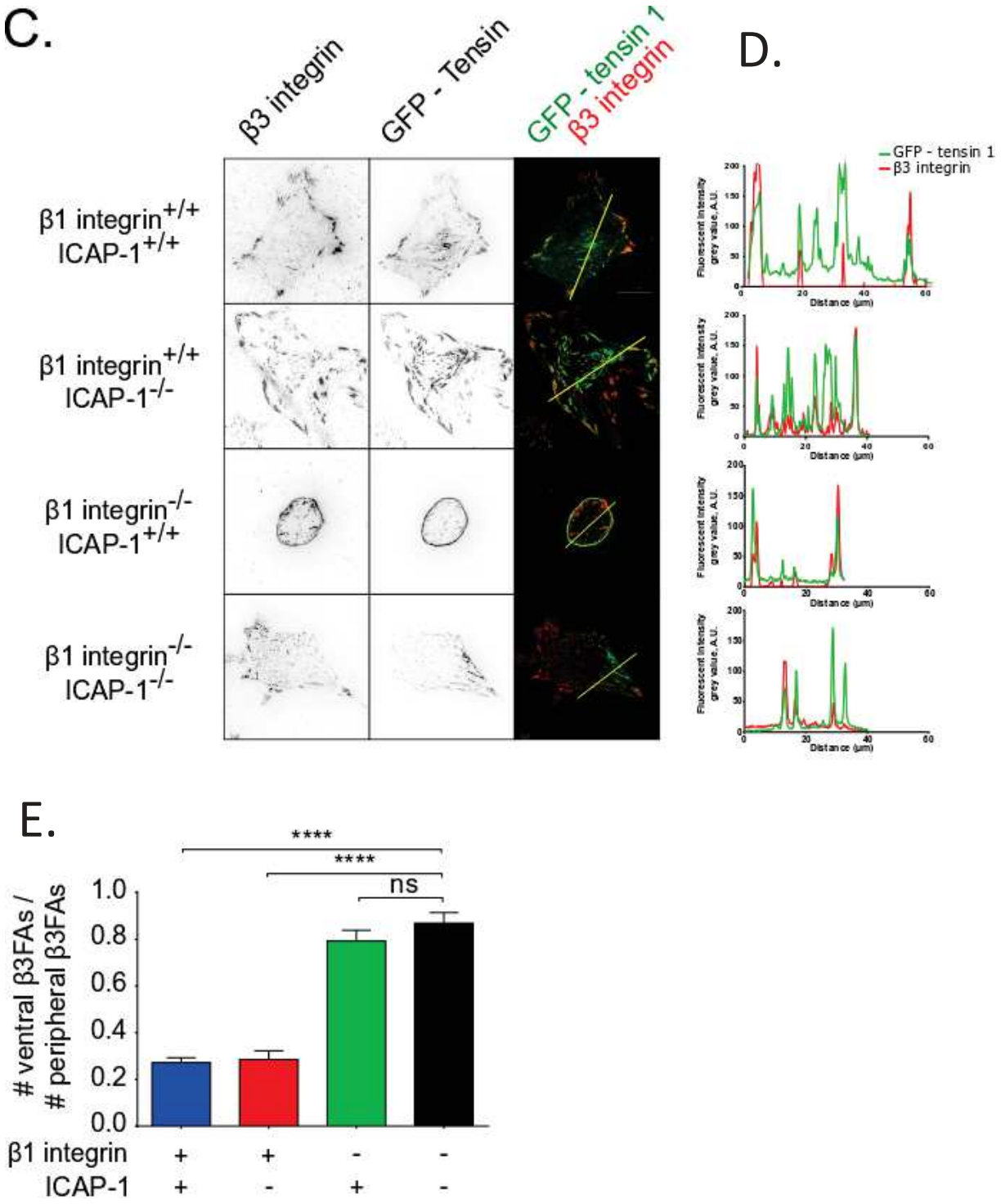

C.
β3 integrin
GFP - Tensin
GFP - tensin 1
β3 integrin
β1 integrin+/+
ICAP-1+/+
β1 integrin+/+
ICAP-1-/-
β1 integrin-/-
ICAP-1+/+
β1 integrin-/-
ICAP-1-/-
D.
GFP - tensin 1
β3 integrin
Fluorescent intensity grey value, A.U.
Distance (µm)
E.
ventral β3FAs / # peripheral β3FAs
1.0
0.8
0.6
0.4
0.2
0.0
****
****
ns
β1 integrin + + - -
ICAP-1 + - + -

Figure 8.3.3. Restoration of traction forces in the β1$^{-/-}$/ICAP-1$^{-/-}$ genotype leads to recruiting tensin 1 along the fibrillar β3 integrin adhesions.
C-D. representative TIRF microscopy images and plot profiles of osteoblasts mutants expressing GFP-tensin-1 spread on Fbn and stained for β3 integrins (Luc.A5, green). Intensities profiles of the GFP-tensin and β3 integrin were obtained across the yellow lines in each corresponding image. localization of GFP-tensin1 shows typical retardation in respect to β3 integrin containing focal adhesions in β1$^{+/+}$ cell lines. Plot profiles show that the co-localization of tensin and β3 integrins covered a larger cell area. Conversely, in β1 deficient cells co-localization of tensin and β3 integrins is stuck at the cell periphery, the additional loss of ICAP-1 redistributes the co-localization of tensin and β3 integrins all over the ventral cell area. Bar scale represents 20 µm.
E. Quantitative ratio between the unusual ventral β3 FAs vs the more classical peripheral. It is obvious that β1 KO cells have excess of ventral FAs, but with different morphology and probably function. **** adjusted p.value ≤ 0.0001. Experiments were done 3 times with at least 20 cells per condition.

8.3.4. Loss of ICAP-1 promotes β3 integrin fibrillar adhesion which are associated with FbN fibrillogenesis.

Our results suggest an unexpected role of ICAP-1 on β3 integrins clustering and maturation into fibrillar adhesion associated with an actomyosin contractility. We addressed the question of the cellular functionality of this β3 integrin clustering by analyzing the FbN fibrillogenesis process, known to be dependent on the actomyosin contractility (Wu et al., 1995). Indeed, FbN matrix assembly results from the coordinated engagement of a5β1 and aVβ3. In this process the application of actomyosin-dependent tensile forces and the translocation of a5β1 to form fibrillar adhesions are essential to regulate FbN fibrillogenesis.

To assess the regulation of the actomyosin tensile forces loading on β1 and/or β3 integrins by ICAP-1, fibrillogenesis assays were performed in the four osteoblast cell lines. Osteoblasts cell lines were cultured on uncoated cover slides in the absence of serum for 12h to 16h hours, to give them time to synthesize and organized their own FbN fibrils. The organization of the FbN fibrils is dependent on the secreted endogenous FbN. In condition where endogenous FbN is reduced, using specific FbN RNAi only few cells were competent to adhere and spread (Fig. 8.3.4F), confirming that the FbN fibrillogenesis process observed *in vitro* over uncoated substrate is largely dependent on secretion of FbN by osteoblasts cells.

In these conditions we observed that the area and the length of the *de novo* FbN fibrils were slightly increased with the deletion of ICAP-1 as compared to *β1 integrin*$^{+/+}$*-icap-1*$^{+/+}$ (Fig. 8.3.4.A-C-D-E). On the other hand, the lack of β1 integrins drastically impaired the formation of dense meshwork of FbN fibrils as already described (Brunner et al., 2011b). As assumed, the additional loss of ICAP-1 allowed

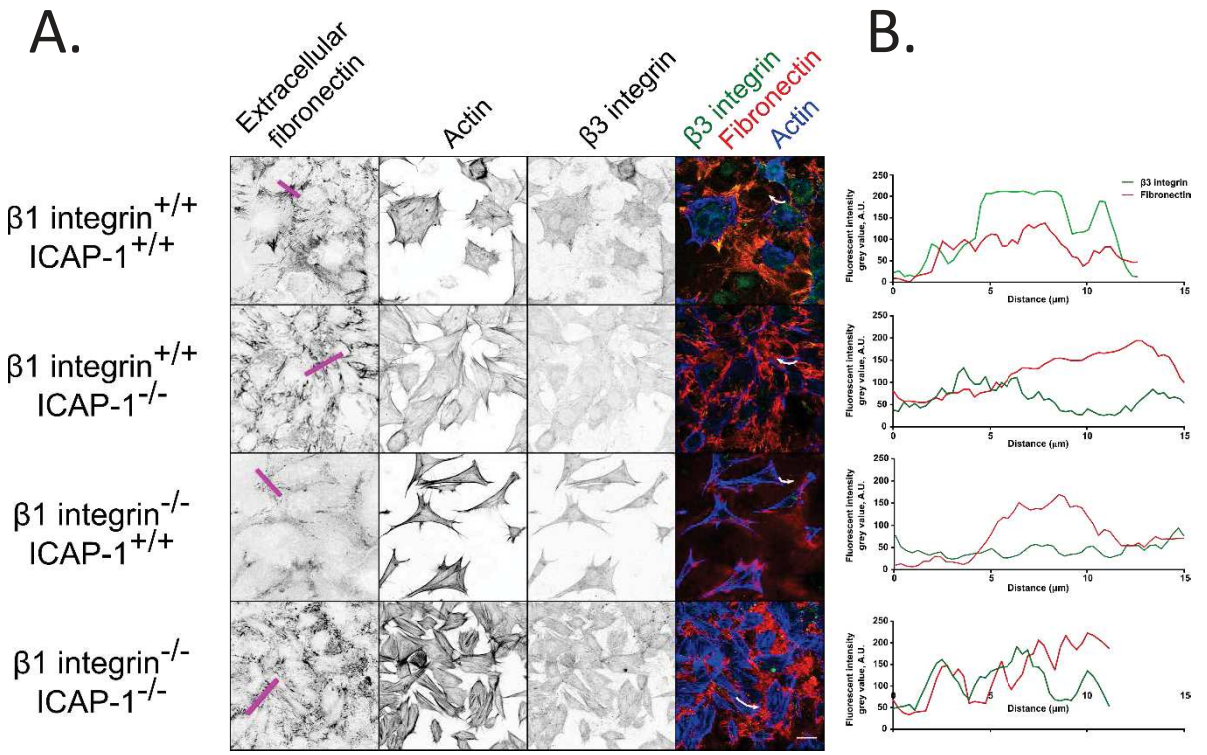


Figure 8.3.4. Loss of ICAP-1 promotes β3 integrin fibrillar adhesions which are associated with FbN fibrillogenesis.
A. Osteoblasts cells were spread in serum free medium on glass for 24 hours. Indirect immunofluorescence was performed visualizing the extracellular fibronectin (cellular fibronectin antibody, red), F-actin (phalloidin, blue) and β3 integrins (Luc.A5 antibody, green) and cell culture were analyzed by fluorescent microscopy. Not surprisingly, the $\beta1^{+/+}$ cell lines orchestrate FbN fibrillogenesis events and $\beta1^{-/-}$ cells demonstrated very poor organization of synthetized fibronectin. β1 $integrin^{-/-}$/$ICAP\text{-}1^{-/-}$ on the other side showed significant amount of FbN fibrillogenesis. Bar scale represents 20 µm.
B. Representative plot intensities profiles of β3 integrin (green) and FbN fibrils (red) were obtained across curves (whit arrow) in each corresponding cell lines. Co-localization of FbN fibrils and β3 integrin adhesions covered a long distance in β1+/+ cells and in the double KO. Conversely, in β1 deficient cells co-localization of FbN fibrils and β3 integrins only on a short distance. see text for more explanation.

the formation and organization of short but thick and numerous FbN fibrils (Fig. 8.3.4.A-4C-4D-4E). The plot profiles of fluorescent fibrils intensity confirm thinner and less dense FbN fibrils in *β1 integrin$^{-/-}$-icap-1$^{+/+}$* cells as compared to *β1 integrin$^{+/+}$-icap-1$^{+/+}$* and to the double KO *β1 integrin$^{-/-}$-icap-1$^{-/-}$* (Fig. 8.3.4.D, plot profiles trail the red lines). Despite their better spreading induced by the deficiency of serum

in the cell culture medium, $\beta1^{-/-}$ integrin cells were not able to organize well the FbN into fibrils as compared to the double KO cells (Fig. 8.3.4.A). It is important to note that the level of FbN fibrillogenesis is largely dependent on cell culture confluence. Nevertheless, even at high confluence only some short and thin FbN fibrils (< 5 µm) could be seen (data not show).

The analysis of intensities profiles of fibronectin/β3 integrin (Fig. 8.3.4.B) of $\beta1^{+/+}$ cell lines reveal the co-localization of β3 integrins and FbN fibrils followed by their mismatch, suggesting that the late elongation of the FbN fibrils is supported by another integrin (Fig. 8.3.4.B). Indeed, the fibrils elongation to the cell center is related to β1 integrins in the two cell lines containing $\beta1^{+/+}$ integrin (Fig. 8.3.4.G). Besides, in the case of the double KO of β1 integrin/ICAP-1, the intensities profiles show the co-localization of β3 integrin all along FbN fibrils and validate the mechanical involvement of the β3 integrins fibrillar adhesions in the assembly of the FbN fibrils (Fig. 8.3.4.B). By following the FbN fibrils and β1 integrins all over the cell shape with optical sections of confocal images we confirm the congruence of β1 integrins with FbN fibrils from the basal to dorsal face in $\beta1^{+/+}$ integrin cell lines (Fig. 8.3.4.G). On the other hand, following FbN fibrils and β3 integrins reveal that FBN fibrils on dorsal face can also be accompanied by β3 integrins but it occurs only in conditions where β1 integrin is present. (Fig. 8.3.4.G-H). Thus, even though β3 integrins containing FAs are oversized and elongated as fibrillar adhesion in the case of the double KO of β1 integrin/ICAP-1, they lake the facility to move to the dorsal face. These results suggest that the loss of ICAP-1 confers to β3 integrins the capabilities to build up an efficient contractile apparatus to remodel the fibronectin matrix at the basal face of the 2D spread cells but impaired in 3D matrix organization. According to our results, it may exist two distinct types of fibronexus in our osteoblasts cell lines: (i) a ventral substrate-adhesive nexus consisting of fibronectin and β3 integrins containing adhesions and (ii) dorsal association matrix fibers consisting of FbN fibrils and β1 integrins adhesions with or without β3 integrins. In order to observe the dynamic of FbN remodeling process in the four

osteoblasts cell lines, we took advantage of the biochemical properties of FbN and its high affinity for gelatin (Engvall and Ruoslahti, 1977). We used conjugated gelatin with Fluo Alexa 563 to localize cell surface fibronectin fibrils in live osteoblasts in cultures (Hsieh et al., 1980). To do so, osteoblast cell mutants were seeded in serum freemedium on uncoated cover slide chamber for overnight time long then the synthesized endogenous fibronectin hidden on the surface of the cells was probe using fluorescent gelatin and live time imaging records were performed (Fig. 8.3.4.I). As expected, we observed a fibrillogenesis extended and organized in FbN fibrils in *β1 integrin*$^{+/+}$ cell lines as well as in β1 integrin null cells deficient in ICAP-1. In contrary but as well as expected, the β1 integrin 1 KO cells do not exhibit FBN fibrils on their surface. However, it was observed numerous endocytosis vesicles full of fluorescent gelatin revealing in this mutant a strong internalization process of matrix. Thus, β3 integrin FAs dependent traction forces promoted by the loss of ICAP-1 are efficient and functional enough to remodel the fibronectin matrix. Furthermore, it would appear that this phenotype is associated with a defect of endocytosis of β3 integrins binding FbN at the cell membrane.

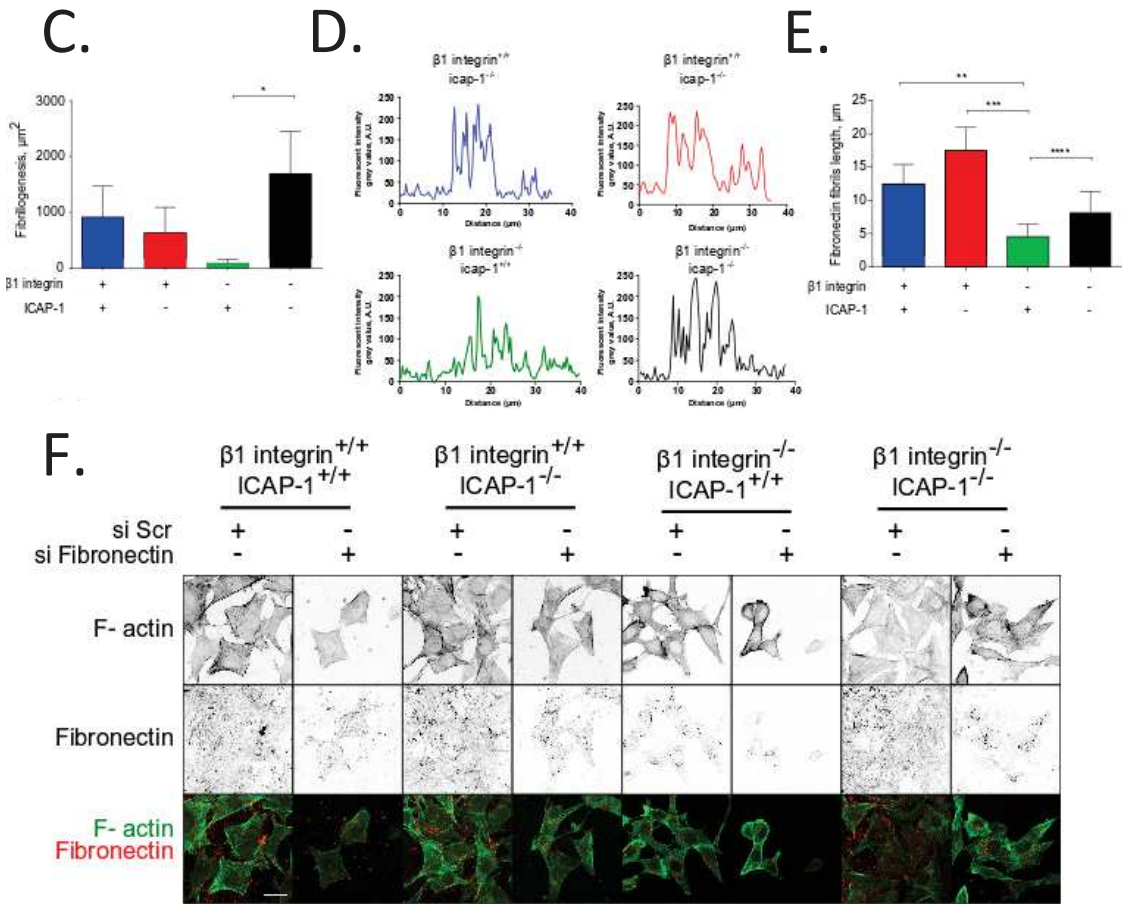


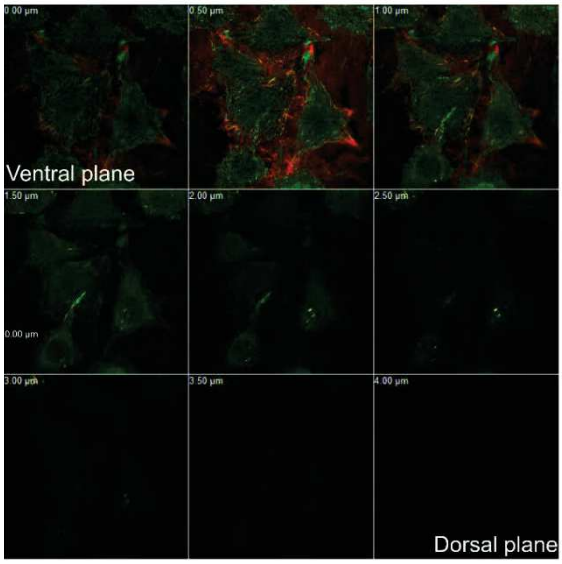
Figure 8.3.4. Loss of ICAP-1 promotes β3 integrin fibrillar adhesions which are associated with FbN fibrillogenesis.

C-E. Thresholded images of deposited and organized in the process of fibrillogenesis were processed and quantified. (C) quantification of FbN fibrils area. (D) Intensity profiles of FbN fibrils were obtained across the red lines in each corresponding cell culture. The intensity of the fluorescence reveals the density of FbN in fibrils and the picks area the thicker of the fibrils. The loss of ICAP-1 in β1-/- cells increases both the density and the thicker of FbN fibrils. (E) The FbN fibrils length quantification reveals that the loss of ICAP-1 increases the length compare to the respective cell lines with or without β1-/- integrin.

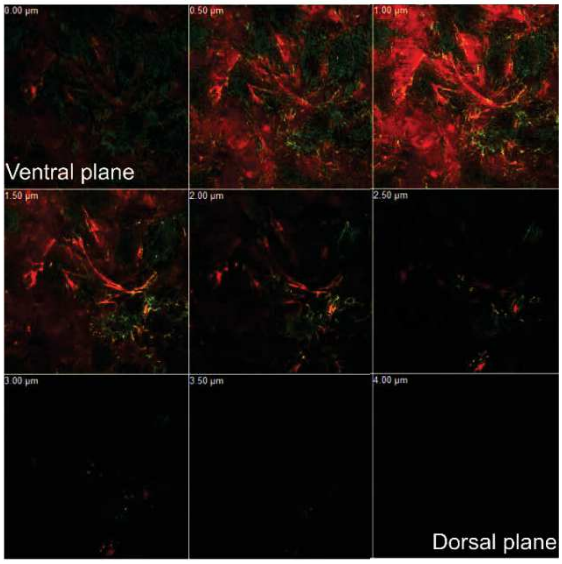
F. Osteoblasts cells were transfected with fibronectin or scramble siRNA for 48h and then let spread in serum free medium on uncoated glass for 24 hours. After PFA fixation, staining of F-actin (phalloidin, green) and extracellular fibronectin (gelatin-Alexa 563, red) were performed and analyzed by fluorescent microscopy. Not surprisingly, fibronectin siRNA drastically impairs FbN fibrillogenesis in all osteoblasts mutants. All four osteoblast cell lines require endogenous FbN to spread and organize their actin cytoskeleton since disruption of FbN expression diminishes severely the spreading area. Bar scale represents 20 µm.

G.

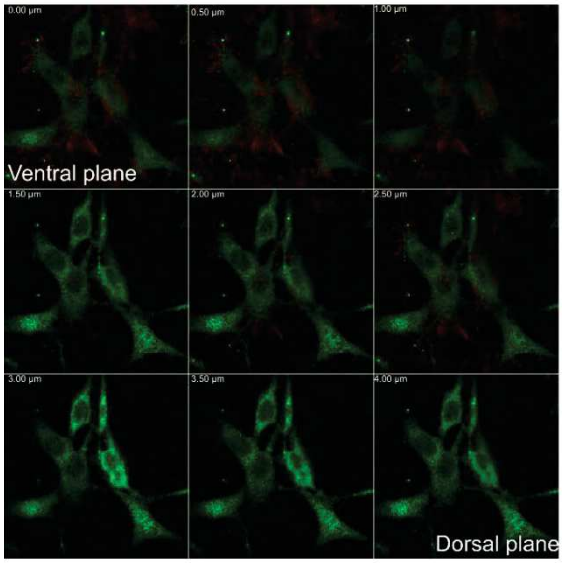
β1 integrin$^{+/+}$
ICAP-1$^{+/+}$



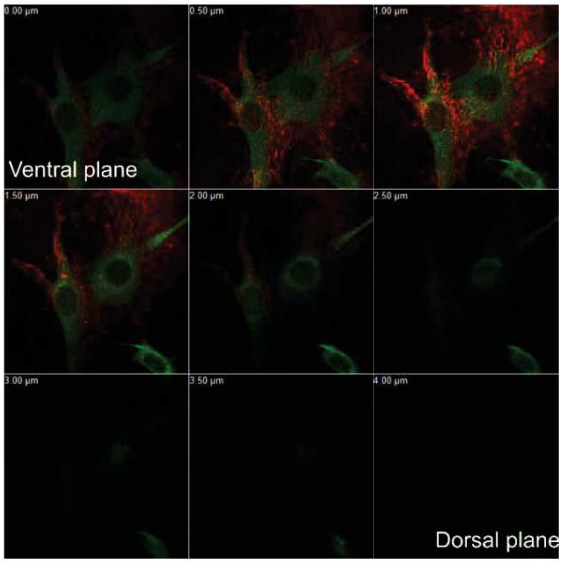
β1 integrin$^{+/+}$
ICAP-1$^{-/-}$



β1 integrin$^{-/-}$
ICAP-1$^{+/+}$



β1 integrin$^{-/-}$
ICAP-1$^{-/-}$



β1 integrin
Extracellular
fibronectin

H.

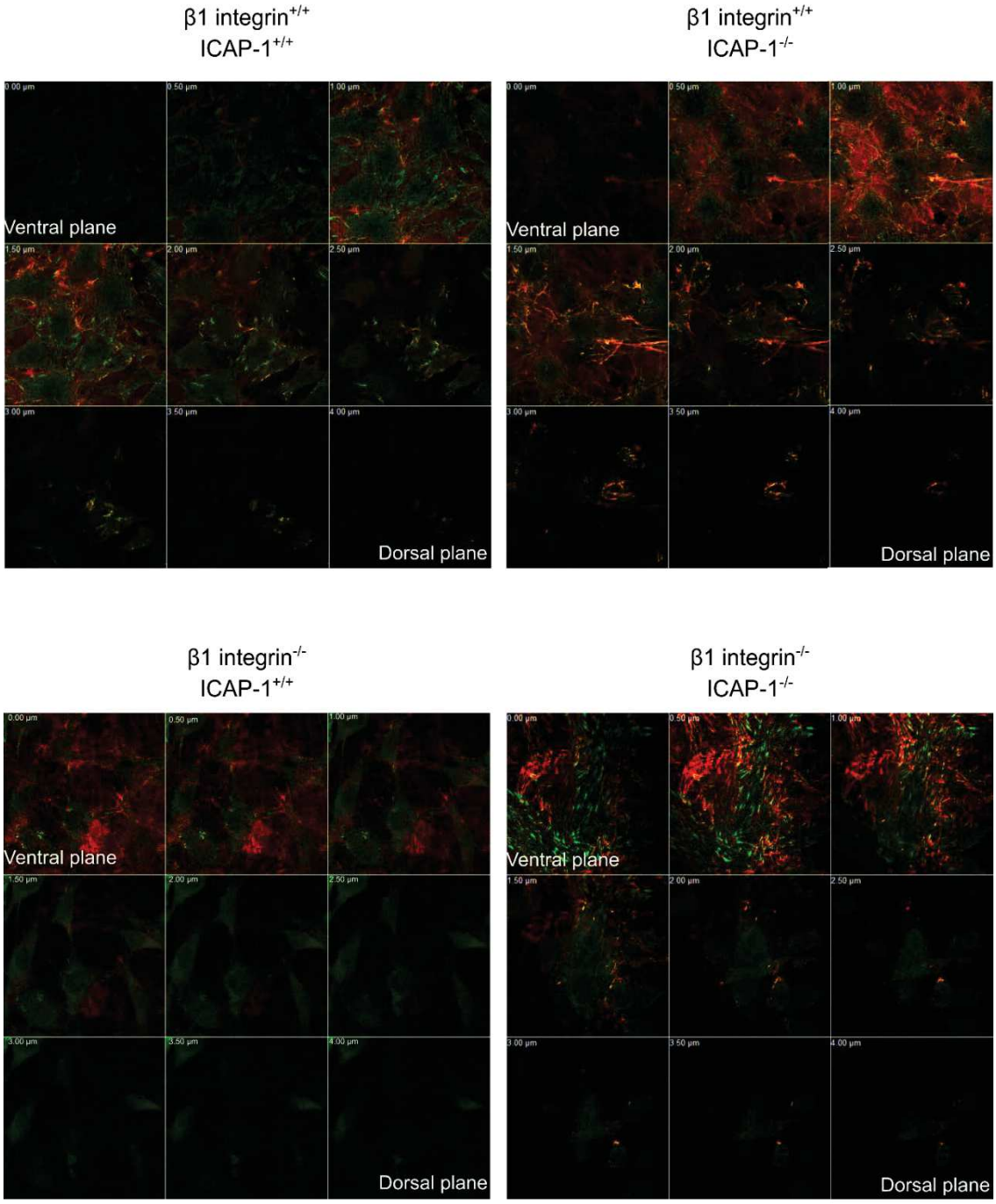

β1 integrin+/+
ICAP-1+/+
β1 integrin+/+
ICAP-1-/-
β1 integrin-/-
ICAP-1+/+
β1 integrin-/-
ICAP-1-/-
Ventral plane
Dorsal plane
β3 integrin
Extracellular
fibronectin

Figure 8.3.4. Loss of ICAP-1 promotes β3 integrin fibrillar adhesions which are associated with FbN fibrillogenesis.
G-H. Optical sections of osteoblasts cell cultures undergoing fibrillogenesis process. After PFA fixation and staining, confocal galleries images from the ventral to the dorsal sides were generated every 1µm, to reveal double fluorescence and co-localization of FbN fibrils (red) with β1 or β3 integrin adhesions (green, G and H respectively). Note that for β1 integrin deficient cells β3 integrin adhesions associated with FbN fibrils could not move to the cell dorsal side and they are keep on the ventral side. Indeed, β1 deficient cells were not able to pull FbN fibrils over the cell roofs, even though $\beta1^{-/-}$/ICAP-$1^{-/-}$ has no troubles in organizing ventral FbN fibrils. Bar scale represents 20 µm.

I.

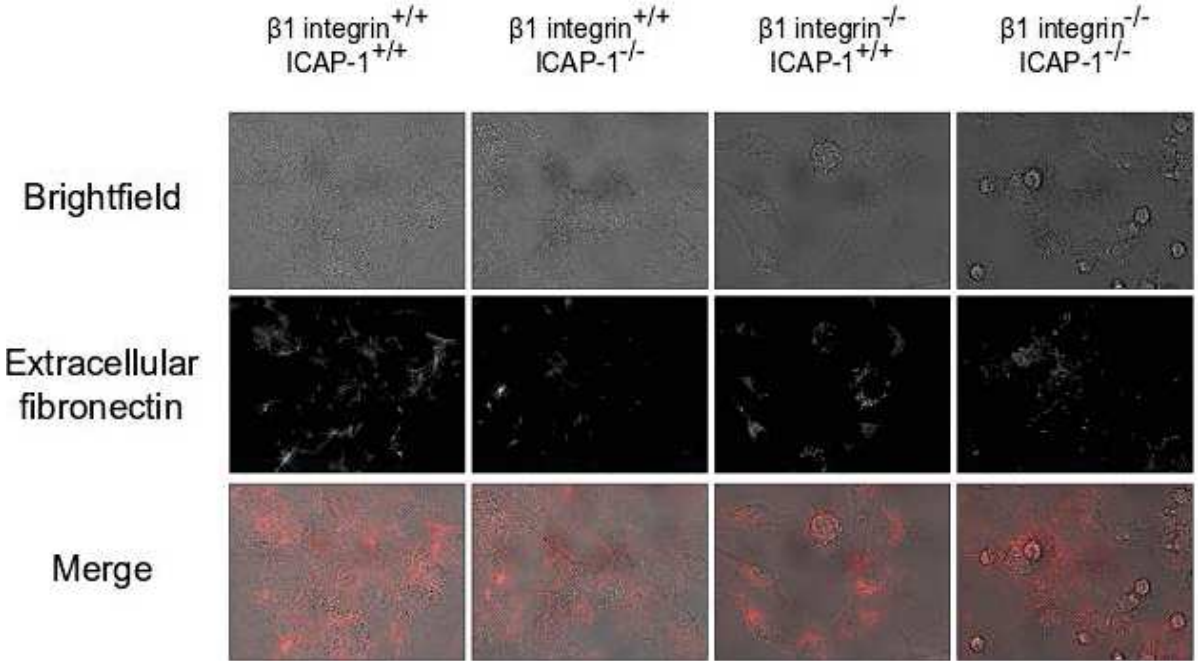


Figure 8.3.4. Loss of ICAP-1 promotes β3 integrin fibrillar adhesions which are associated with FbN fibrillogenesis.
I. Representative images of live time imaging records. Osteoblast cell mutants were seeded in serum free medium on uncoated cover slide chamber for overnight. Endogenous fibronectin hidden on the surface of the cells was probe using fluorescent Alexa 563 gelatin and washout after 30 min incubation. Extended and organized FbN fibrils were observed for *beta1 integrin*$^{+/+}$ and for beta 1 integrin$^{-/-}$/ ICAP-1$^{-/-}$. In contrary beta 1 KO cells do not exhibit FbN fibrils on their surface but numerous endocytosis vesicles full of fluorescent. Bar scale represents 20 µm.

8.3.5. The β3 integrin dependent contractility in cells devoid of β1 integrin and ICAP-1 is associated with a defect of β3 integrin endocytosis

As endocytic membrane traffic regulates bioavailability of cell-surface molecules and therefore the intensity and/or specificity of receptor-initiated signals (Ceresa and Schmid, 2000; Scita and Di Fiore, 2010), we hypothesize that the decrease of β3 integrin turnover observed in β1/ICAP-1 double KO might be linked to a defect in β3 integrin endocytosis. For this, cells were plated onto glass surfaces coated with fibronectin and confocal microscopy was used to determine the uptake of

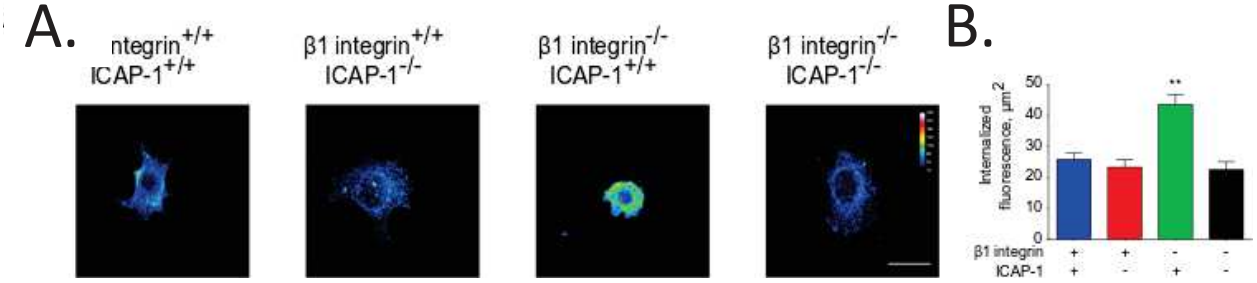


Figure 8.3.5.1. The β3 integrin dependent contractility is associated with the defect of β3 integrin endocytosis
A. The β3 integrin uptake was measured using β3 integrin specific antibody (LucA.5). Representative confocal images of osteoblast cells plated on fibronectin coated glass and stained for FITC conjugated β3 integrin specific antibody (LucA.5). Shown are x-axis and Z-axis after 3D reconstruction profile. The images were taken after 20 min incubation at 37°C followed by acid wash. Scale bar is 20 µm.
B. Quantification of the proportion of endocytosed β3 integrin receptors based on antibody staining (n=20 cells pooled from three independent experiments, mean ± SEM) after 3D reconstruction of z-stacks. Note higher β3 integrin endocytosis in the case of *β1 integrin$^{-/-}$-icap-1$^{+/+}$* cell line and a rescue of β3 integrin endocytosis rate in *β1 integrin$^{-/-}$-icap-1$^{-/-}$* cell line.
** adjusted p.value ≤ 0.01. Experiment was done three independent times.

anti-integrin β3 antibodies (LucA.5 conjugated with FITC). Fluorescent antibodies were transported into cytoplasmic vesicles that were visible above the plane of cell surface in the case of WT osteoblasts (Fig. 8.3.5.1A and 8.3.5.1B).

The number of LucA5-positive intracellular vesicles was significantly reduced in the case of the double deletion β1integrin/ICAP-1 compared to β1 null cell line, suggesting a defect in β3 integrin endocytosis. As surface β3 integrins are

constitutively endocytosed through clathrin-mediated routes (Arjonen et al., 2012; Yu et al., 2015; Ezratty et al., 2009), we addressed the question whether the deletion of clathrin or dynamin might mimic the loss of ICAP-1 in cells depleted in β1 integrin. First alterations of clathrin and dynamin expression in WT cells lead to an increase of cellular spreading which is correlated with an increase of β3 integrin focal adhesion area and P-myosin light chain intensity (Fig. 8.3.5.2). Whereas β1 integrin deficient cells displayed a decrease in their spreading surface (Fig. 8.3.1G) and a reduction in area of β3 integrin containing focal adhesion (Fig. 8.3.2B) as compared to the WT cells, the deletion of clathrin (Fig. 8.3.5.2A-D) or dynamin (Fig. 8.3.5.2A-D) in β1 integrin deficient cells restore their ability to spread, to form bigger β3 integrin focal adhesion and to generate actin stress fibers highly decorated with P-myosin light chain. Importantly, the deletion of either clathrin or dynamin did not change the phenotype of cells depleted in ICAP-1 or in cells devoid of both ICAP-1 and β1 integrin in term of cell spreading, size of β3 integrin focal adhesions and P-myosin staining. Firstly, our results demonstrate that impairment of endocytosis modifies β3 integrin signaling through an increase of β3 integrin clustering which is correlated with an increase of cell spreading and reorganization of actomyosin cytoskeleton. Secondly our data show that cells devoid of ICAP-1 are not sensitive to the alteration of clathrin-based traffic machinery suggesting a potential role of ICAP-1 in β3 integrin endocytosis.

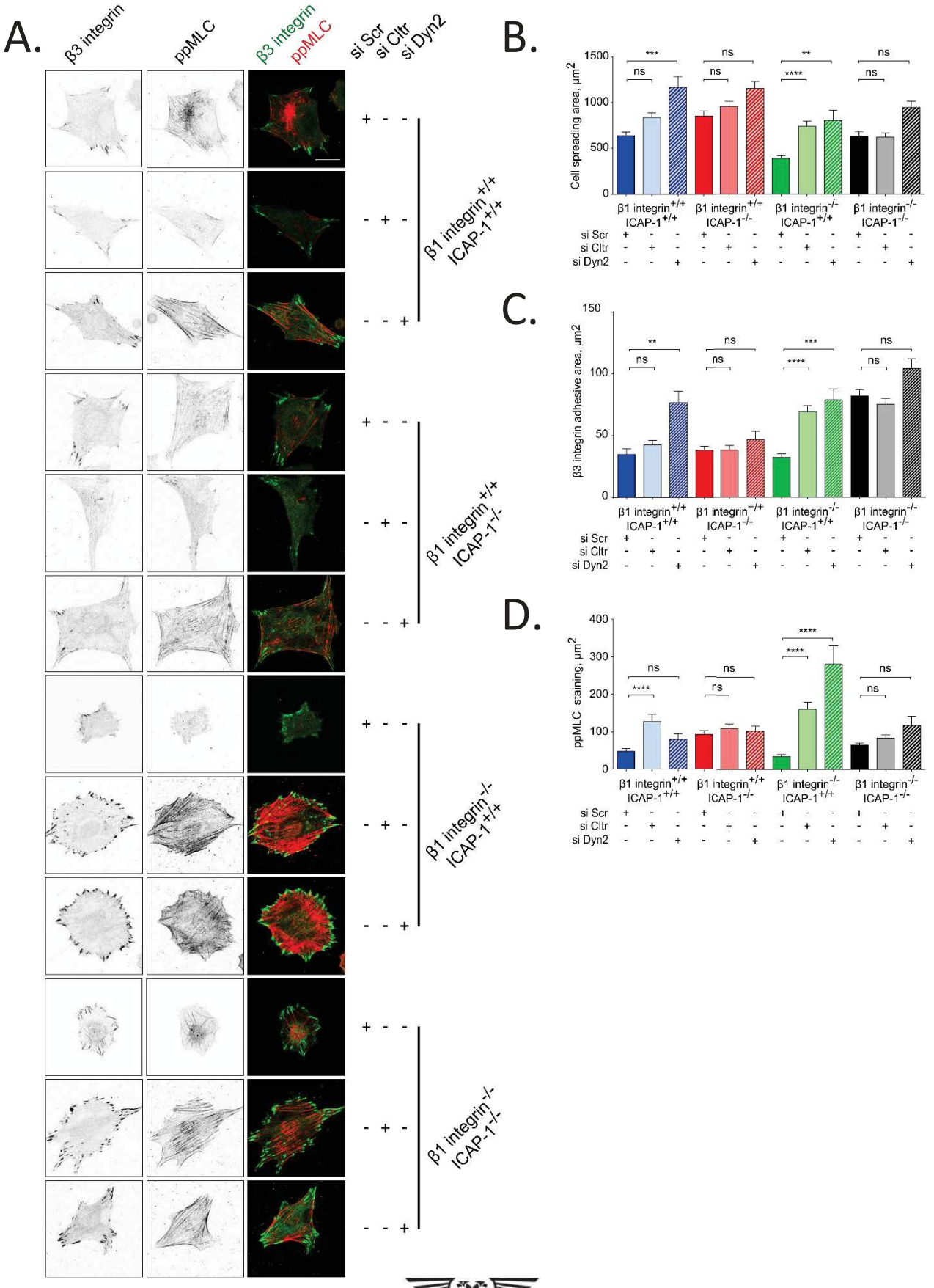

A.
β3 integrin
ppMLC
β3 integrin
ppMLC
si Scr
si Cltr
si Dyn2
β1 integrin+/+ ICAP-1+/+
β1 integrin+/+ ICAP-1-/-
β1 integrin-/- ICAP-1+/+
β1 integrin-/- ICAP-1-/-
B.
Cell spreading area, µm2
C.
β3 integrin adhesive area, µm2
D.
ppMLC staining, µm2

Figure 8.3.5.2. Clathrin and dynamin inhibition in β1 KO cells restores cellular β3 integrin mediated spreading and acto-myosin cytoskeleton mimicking the phenotype of *β1 integrin$^{-/-}$-icap-1$^{-/-}$* cells

A. Spinning disk representative micrographs of the four osteoblast cell lines treated or not with clathrin or dynamin siRNAs. Inhibition of clatrin and dynamin in *β1 integrin$^{-/-}$-icap-1$^{+/+}$* cell line allows the rescue of cell spreading through β3 integrin mediated focal adhesion and development of acto-myosin cytoskeleton (see green bars in quantification graph below). Note the lack of effect of SiRNA in cell lines depleted in ICAP-1 (*β1 integrin$^{+/+}$-icap-1$^{-/-}$* in red bars and *β1 integrin$^{-/-}$-icap-1$^{-/-}$* in black bars). Scale bar is 20 µm.

B. Quantification of cell spreading area before and after treatment with clathrin SiRNA and dynamin siRNA.

C. Quantification of area of β3 integrin containing adhesion sites area before and after treatment with clathrin SiRNA and dynamin siRNA.

D. Quantification of P-myosin staining area before and after treatment with clathrin SiRNA and dynamin siRNA.

ns adjusted p.value > 0.05; * - p.value ≤ 0.05; ** p.value ≤ 0.01; *** p.value ≤ 0.001; **** p.value ≤ 0.0001.

8.3.6. ICAP-1 partner, Nm23-H1/2, is involved in the β3 integrin dependent contractility and β3 integrin dynamics

Next, we investigated the mechanism by which ICAP-1 might affect β3 integrin endocytosis. We have previously shown the interaction between ICAP-1 and the Nucleoside Diphosphate Kinase called Nm23-H2 (Fournier et al., 2002b). Genetic and functional studies have demonstrated the ability of Nm23-H2 to fuel dynamin to drive clathrin dependent endocytosis (Boissan et al., 2014; Krishnan et al., 2001; Dammai et al., 2003; Nallamothu et al., 2008). Based on these findings, we addressed the question whether Nm23-H2 might impact on β3 integrin dynamics and consequently on cell spreading and cell contractility. For this purpose, we analyzed adhesive behavior of cells in conditions where Nm23-H2 was deleted. As Nm23-H2 forms a complex with Nm23-H1 to be recruited to clathrin-coated pits by their physical interaction with dynamin (Boissan et al., 2014), both Nm23-H1 and Nm23-H2 were knocked down using specific RNAi since they form a complex. The deletion of Nm23 rescued the ability of β1 integrin depleted cells to spread onto fibronectin (Fig. 8.3.6A, B). The restoration of cell spreading is associated with an increase of β3 integrin clustering as demonstrated by the increase of the area of β3 integrin containing focal adhesion (Fig. 8.3.6C, E) and reorganization of actomyosin cytoskeleton as confirmed by the increase of P-myosin staining (Fig. 8.3.6D). More importantly, the deletion of Nm23 leads to a decrease of β3 integrin turnover as judged by FRAP experiments performed onto β1 integrin depleted cells (Fig. 8.3.6C). The deletion of Nm23 is inefficient in the case of cells already depleted in ICAP-1. The effect of Nm23 down regulation is significantly detected in WT cells as judged by the increase of cell spreading and the reorganization of actomyosin network whereas the turnover of β3 integrin and the area of β3 integrin containing focal adhesion are not changed. The presence of β1 integrin might protect β3 integrin from the deletion of Nm23. Finally, our results show that ICAP-1 and Nm23 are both implicated in cell spreading and actomyosin

reorganization likely through a fine tuning of β3 integrin endocytosis which impacts on β3 integrin turnover and size of associated focal adhesion.

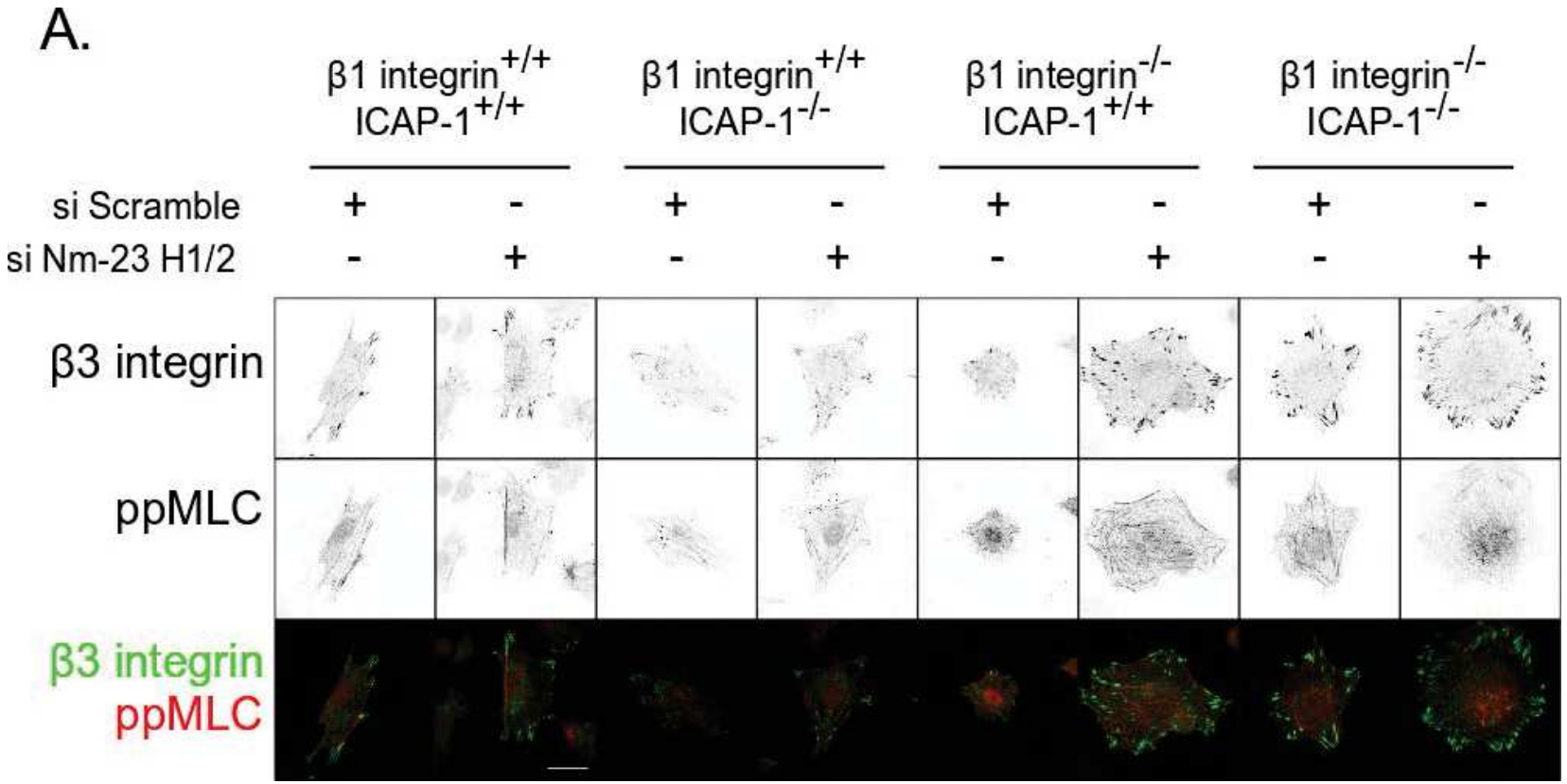


Figure 8.3.6. Nm23-H1/2 (partner of ICAP-1, CCP complex and dynamin) is involved in the β3 integrin dependent contractility and dynamics

A. Spinning disk representative micrographs of the four osteoblast cell lines before and after silencing of Nm-23-H1/2. Silencing of Nm-23-H1/2 in *β1 integrin$^{-/-}$-icap-1$^{+/+}$* cell line is able to rescue of cell spreading through β3 integrin mediated focal adhesion and development of acto-myosin cytoskeleton likely through β3 integrin trapping at the cell membrane (see green bars in quantification graph below). No effect of SiRNA Nm-23-H1/2 in cell lines depleted in ICAP-1 (*β1 integrin$^{+/+}$-icap-1$^{-/-}$* in red bars and *β1 integrin$^{-/-}$-icap-1$^{-/-}$* in black bars). Scale bar is 20 µm.

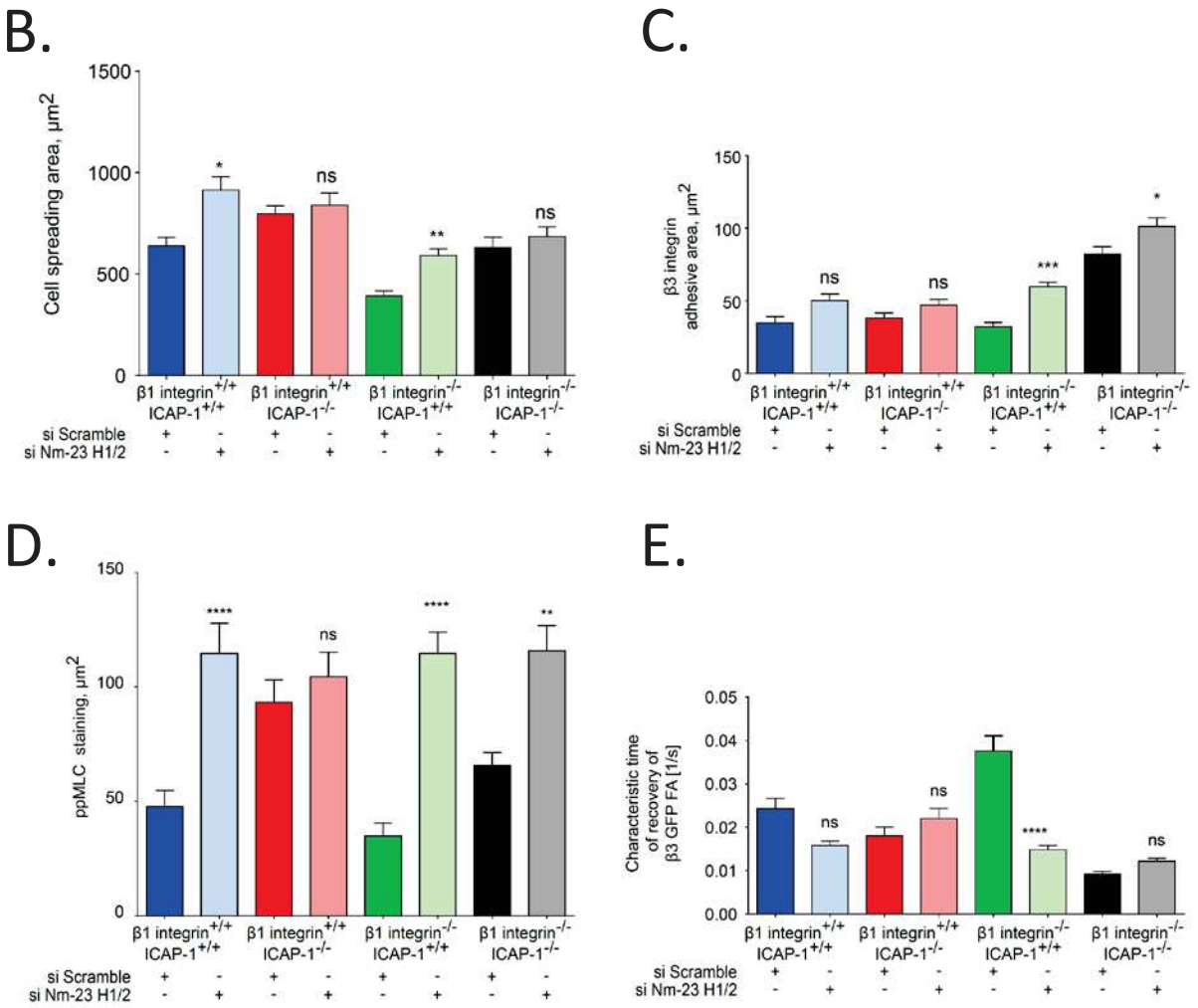


Figure 8.3.6. Nm23-H1/2 (partner of ICAP-1, CCP complex and dynamin) is involved in the β3 integrin dependent contractility and dynamics

B. Quantification of cell spreading area before and after silencing of Nm-23-H1/2.

C. Quantification of area of β3 integrin containing adhesion sites area before and after silencing of Nm-23-H1/2.

D. Quantification of P-myosin staining area before and after silencing of Nm-23-H1/2.

E. TIRF/FRAP analysis shows that deletion of Nm-23-H1/2 complex impedes the turnover of β3 integrin – GFP at the plasma membrane in *β1 integrin$^{-/-}$-icap-1$^{+/+}$* cell line. 50 FA (5 FA per cell) were bleached for each experiment and their recovery was monitored for 5 min. Error bars represent standard deviation of at least 10 cells/experiment. **** adjusted p.value ≤ 0.0001. The experiment was performed in triplicate. ns adjusted p.value > 0.05; * - p.value ≤ 0.05; ** p.value ≤ 0.01; *** p.value ≤ 0.001; **** p.value ≤ 0.0001.

IV.
Discussion, conclusion and perspectives

8.4. Discussion

8.4.1. Force development and mechanical remodeling of ECM through β3 integrins are regulated by ICAP-1

Our results show that the deletion of ICAP-1 restores cellular spreading of β1 integrin KO cells which are known to be round and devoid of traction forces. In these conditions, the cellular spreading is due to the increase of β3 integrin clustering which is associated with the impressive development of the actomyosin cytoskeleton related to high contractile events on the FbN substrate and the development of FBN fibrils. Our data demonstrate that ICAP-1 modulates the organization of cell matrix adhesion and actomyosin contractility loading on β3 integrins independently of β1 integrin signaling.

It has been previously proposed that the progression from newly formed nascent adhesions to mature focal adhesions and fibronectin–matrix remodeled fibrillar adhesions can be conceptualized as a myosin II-dependent maturation process from small cell–matrix adhesions with high turnover rate to progressively larger and enduring adhesion sites (focal adhesion and fibrillar adhesion) stabilized by the connection to the cytoskeleton (Schiller et al., 2011b). Here, our data show that the loss of ICAP-1 decreases drastically the turnover of β3 integrins in the focal adhesions and one of the major consequence was to increase the actomyosin pattern of the spread cells increasing the associated contractile energy at the adhesions sites with the fibronectin substrate. In the lab, we have previously demonstrated that in endothelial cells the destabilization of ICAP-1 through the loss of its partner CCM1/2 complex resulted in an increase of β1 activation and led to increase RhoA-dependent contractility. The resulting abnormal distribution of forces led to aberrant extracellular matrix remodeling around lesions of CCM1- and CCM2-deficient mice (Faurobert et al., 2013). Here, for osteoblasts which spread over fibronectin substrate, the effect of the loss of ICAP-1 is not actually significant on cell contractility and on the associated extracellular remodeling

even though we can observe a tendency to slightly increase actomyosin contractility with an increase in the associated fibrillogenesis. Effect of the loss of ICAP-1 becomes major when β3 integrins are no longer associated with the expression of β1 signaling. As focal adhesions always contain combinations of several integrins it is unknown whether to what extent individual integrin classes bound to the same ECM ligand (*e.g.* a5β1 and avβ3 on FbN), use special pathways to sense and exert force. In 2013, Shiller et al (Schiller et al., 2013) identified a functional synergy between a5β1 and av-class integrins signaling hubs leading to feedback amplification of myosin II activity. In this study, by comparing β1 integrin KO cells et β1 integrin/ICAP-1 double KO cells, we were authorized to reveal the influence of ICAP-1 over β3 integrins by pointing out the restoration of spreading and traction force in β1 integrin null cells. One can then ask the question of why the cells in the absence of β1 integrins and in the presence of ICAP-1 express a poor spreading and contractile phenotype on FBN substrate. This can be considered by the view that for β1 integrin KO cells, ICAP-1 does not anymore interact with β1A integrins cytoplasmic domain and released from this interaction would no longer be titrated by β1 integrins. Thus, liberated ICAP-1 could interact and inhibit other partners such as Rac1 or Cdc-42 that could lead to the down regulation of activity of lamellipodia/filopodia motilities and to a poor cell spreading (Degani et al., 2002). As described before, a5β1 and avβ3 integrins bind to FBN. However, a5β1 but not avβ3 supports high levels of RhoA activity at later stages of cell adhesion, which are associated with recruitment of tensin into fibrillar adhesions and FBN fibrillogenesis (Danen et al., 2002). Expression of an activated mutant of RhoA stimulates avβ3-mediated fibrillogenesis as the loss of ICAP-1 in osteoblasts β1 integrin KO mutant does. It would be easy then to formulate the link between the loss of ICAP1 and the activation of RhoA. Unlike Rac1 and Cdc-42, no direct link was established between ICAP-1 and RhoA. However, the plausible global GDI activity of ICAP-1 is controversial and is not supported by solid structural data, with the sequence and conformation of ICAP

being relatively far-off from those of the classic Rho GDI. On the other hand, ICAP-1 and ROCK (the Rho kinase) were co-immunoprecipitated from C2C12 cells and also identified by using the yeast two-hybrid assay (Stroeken et al., 2006; Alvarez et al., 2008). Thus, ICAP-1 might interact with ROCK and regulate the stiffening of the intracellular environment. We have recently shown that ICAP-1 and its monoubiquitylated form regulate ROCK2- and MRCKa-dependent myosin phosphorylation independently of interaction with β1 integrins (Bouin et al., 2017b). Indeed, our results propose a novel role of ICAP-1 on β3 integrins clustering, β3 dynamics and on the associated actomyosin contractile pathway. In this issue, ICAP-1 might negatively control contractile actomyosin cytoskeleton organization and would no longer allow the maturation of adhesion sites into FAs and then fibrillar adhesions. We cannot rule out the fact that the increase of contractile actomyosin cytoskeleton can be also lead to a defect in membrane tension and consequently in endocytic process. A recent study corroborates with these later findings by demonstrating the sensitivity of β3 integrins regarding force generation. Indeed the loss of physical forces on ligand-bound integrins can switch β3 activation from classical focal adhesion formation to a pathway of clathrin-mediated endocytosis of β3 clusters (Yu et al., 2015). Finally, our results identify ICAP-1 as a novel factor contributing to β3 integrin regulation through an elaborate signaling network responsible for maintaining cell tensional homeostasis independently of its interaction with β1 integrins.

8.4.2. ICAP-1 as an adaptor protein in integrin endocytic process to regulate β1 and β3 integrin cooperativity

Integrin internalization occurs through clathrin-dependent and clathrin independent mechanisms and many integrins can follow more than one route into the cell to control adhesion turnover, cell migration, morphogenesis and cancer metastasis (Caswell et al., 2009; Shi and Sottile, 2008b; Mellman and Yarden, 2013; Yu et al., 2015). Clathrin-dependent trafficking of integrins has

proven to be essential for adhesion disassembly and this process is governed by the intracellular domains of both α and β subunits (Margadant et al., 2011; De Franceschi et al., 2016; Ezratty et al., 2009; Arjonen et al., 2012). Nevertheless, the existence of specific regulatory pathways that would trigger preferential internalization of one integrin heterodimer over another is still matter of debate.

Our study demonstrated that β3 integrin dynamics is affected by the simultaneous loss of β1 integrin and ICAP-1. This decrease of β3 integrin dynamic in absence of ICAP-1 and β1 integrin is correlated with the alteration of β3 integrin trafficking. Our data show also that the additional loss of clathrin or Nm23-H2 in cells depleted in β1 integrin mimics the loss of ICAP-1. Moreover, the loss of Nm23-H2 or clathrin does not allow restoration of β3 integrin mediated cell spreading in cells depleted in ICAP-1. These results suggest that ICAP-1, Nm23-H2 and clathrin might work together to control β3 integrin endocytosis. On line with this, Clathrin/AP2 mediated endocytosis is associated with adhesion disassembly and clathrin coated pits (CCP) are enriched at adhesive contacts by colocalizing with integrins (Ezratty et al., 2009). However in addition to the essential core components of cargo, AP-2 adaptor and clathrin, many other endocytic accessory proteins or co-adaptors associate with CCP and aid in cargo selection, in the efficiency of cargo enrichment at the CCP, and in execution of subsequent membrane deformation, fission, uncoating and endosomal fusion events (Yap and Winckler, 2015). Some prominent examples are PTB domain proteins named Dab2 and Numb known to associate with conserved NPXY motifs shared by all the cytoplasmic domains of β-integrin subunits and to be involved in integrin trafficking by interacting with endocytic machinery like AP2 and clathrin or endocytic accessory proteins (Eps15). Dab2 and Numb accumulate at or near focal adhesions shortly before their disassembly (Chao and Kunz, 2009; Teckchandani et al., 2009; Nishimura and Kaibuchi, 2007; Ezratty et al., 2009). Like Numb and Dab2, ICAP-1 belongs to the PTB domain protein family. Whereas ICAP-1 interacts with the distal NPXY motif of β1 integrin, Dab2 binds the same distal

NPXY motif of β1, β2, β3, β5 and β7 integrins and Numb associates with the proximal NPXY of β3 and β5 integrins. The involvement of ICAP-1 in the endocytic machinery is supported by its direct interaction with nm23-H2 (Fournier et al., 2002), which is a nucleoside diphosphate kinases (NDPKs) involved in dynamin-mediated endocytosis (Boissan et al., 2014). Nm23-H2 is complexed with AP2 (Zala and Boissan 2017) and is important for scission of endocytic clathrin coated pits (Boissan et al., 2014). Many proteomic based studies have identified nm23 as partner of both β1 and β3 integrin associated complexes (Kuo et al., 2011; Schiller et al., 2011b, 2013). It is tempting to think that ICAP-1 might be involved in the membrane localization of nm23-H2 as already described (Fournier et al., 2002b), in the activity of nm23-H2 or in the physical proximity between nm23-H2 and integrin. Whereas we have shown that ICAP-1 is involved in β3 integrin endocytosis, we cannot exclude the implication of ICAP-1 in β1 integrin endocytosis. Preliminary experiments have confirmed a defect in β1 integrin endocytosis in ICAP-1 depleted cell highlighting a general role of ICAP-1 in integrin endocytosis (data not shown). As ICAP-1 is not observed in focal adhesion, we can suppose that ICAP-1 would play its role in integrin endocytosis outside focal adhesions. This hypothesis is supported by high resolution imaging of ICAP-1 showing its ability to localize outside focal adhesion site and to conserve its membrane localization even in cells depleted in β1 integrin (Collaboration with Giannone lab).

Despite these advances, it remains unclear how the individual NPXY motifs present in the cytoplasmic domain of integrins regulate trafficking of different integrin and through which PTB domain proteins this is effectuated. The physiological relevance of having many adaptors or co-adaptors like ICAP-1, Numb or Dab2 in the same cell might respond to integrin specificity and physical properties of the microenvironment. The mechanical state of a cell is a master regulator of its endocytic clathrin coat dynamics (Ferguson et al., 2017). Tension on the membrane can hinder this process as it increases the energy cost of curvature formation (Sheetz, 2001). As an illustration, Dab2 is not found at β3 integrin-

mediated focal adhesion sites formed on RGD-glass suggesting different adaptors depending on the stiffness of the microenvironment. Indeed, it has been shown that the development of actomyosin contractility inhibits Dab2 binding to β3 integrin, and the loss of cell–matrix force development is one of the key mechanisms activating Dab2 binding to β3 integrin clusters resulting in endocytosis of b3 integrins in soft conditions (Yu et al., 2015). Assuming a possible interaction between β3 integrin and a modified form of ICAP-1, ICAP-1 might take over on Dab2 in stiffer microenvironment as ICAP-1 and Dab2 are sharing the same distal NPXY motif to interact with integrin. As far as β1 integrin endocytosis is concerned, Dab2 and clathrin are not found to activated β1 integrin clusters (Yu et al., 2015) indicating that the turnover of β1 integrin may be regulated by a different pathway involving eventually ICAP-1 in stiffer microenvironment.

8.5. Conclusion and perspectives

Our data demonstrate that ICAP-1 regulates β3 integrins and therefore it influences the organization of cell matrix adhesion and actomyosin contractility independently of β1 integrin signaling.

Finally, because migratory and adhesive behavior is mediated by β1 and β3 integrin cooperation (Schiller et al., 2013), the regulation of their respective intracellular trafficking in a coordinated manner is likely essential for rapidly and efficiently adapting the responsiveness of migratory cells to extracellular guidance cues. Originally identified as specific for β1 integrin, ICAP-1 might coordinate β1 and β3 integrin endocytosis to adapt integrin dynamics and force generation in a context dependent manner, supporting the idea that endocytic process is a good way to tune integrin cooperativity.

Several questions still remain unanswered though. Are β3 integrins recruiting other partners at the level of FAs as a consequence of their slow turnover? It is not difficult to imagine that the molecular environment of β3 integrin will change if its mobility is delayed and it will modulate its signaling at the FA level. A feasible

approach to identify the different β3 integrin partners in different cellular environment is subcloning it in BioID2 vector – an engineered biotin ligase that is able to biotinylate neighboring partners and subsequently analyze the biotinylated proteins by quantitative mass spectrometry. Also, results from variation in the length between β3 integrin and the biotin ligase (to discriminate between close and more distant partners) could shed more light on the protein content of the β3 FAs in an ICAP-1 context of β1 integrin null cells. Since FA turnover and microtubule (MT) dynamics has been described (Stehbens and Wittmann, 2012). Even more since the MT are main ways to deliver endocytosis related protein as dynamin (Chao and Kunz, 2009; Engler et al., 2007; Ezratty et al., 2009) and/or are implicated in Rho-ROCK contractility pathway by delivering GEF-H1 (Heck et al., 2012). It seems feasible to hypothesize that the MT dynamics would influence the turnover and the actomyosin contractility loading on β3 integrins at the cell membrane. ICAP-1 can influence that process directly at MT level, since as we show, it is important for the proper function of Nm-23 complex and Nm23 is reported to interact with directly with the MT (Ikeda, 2010). The interaction between ICAP-1 and Nm-23-H2 has been published before (Fournier et al., 2002a), but direct interaction between β3 integrin and Nm-23-H2 are still not solid despite several proteomic studies (Bharadwaj et al., 2017; Humphries et al., 2009b; Alanko et al., 2015. Integrin Endosomal Signalling Suppresses Anoikis -, 2015). Proximity ligation assays (*in situ* PLA) – is a powerful technology which allows direct detection of endogenous protein interactions and modifications with high specificity and will efficient to establish if indeed β3 integrin/Nm-23-H2 form a complex at the plasma membrane and whether ICAP-1 behavior could influence the integrins turn over.

In collaboration with Giannone's laboratory, using super-resolution microscopy and single particle tracking we have recently shown that β1 and β3 integrins move and function as distinct homotypic nanoclusters within focal adhesion demonstrating the nanoscale dynamics of integrins within focal adhesion (Rossier

et al., 2012). This nano-organization and clear segregation of β integrins seems to be a universal phenomenon since we have also shown these patterns in other adhesion structures such as invadosome (Destaing et al., 2010). My working hypothesis here, should be based on my current results identifying ICAP-1 as molecular basis of the cross-talk between β1 and β3 integrins. The attracting hypothesis is that the dynamic of β3 integrins within focal adhesion might be regulated by ICAP-1 at the cell membrane nearby to control development of local forces and to drive associated signaling necessary for cell adhesion, migration and invasion.

V.
Materials and methods

9. Materials and methods

9.1. Antibodies and chemicals

Human plasma fibronectin was purchased from Sigma-Aldrich. All siRNA (ON-TARGETplus) were obtained from ThermoFisher. Anti β3 integrin antibody was purchased from Emfret (Clone LucA.5, #M030-0, for the variant, conjugated with FITC - # M031-1), the double phosphorylated (T18/S19) myosin light chain antibody was obtained from Cell signaling (#3674), the unmodified myosin light chain antibody and tubulin were purchased from Sigma-Aldrich, #M4401 and #T4026 respectively. The fibronectin antibody was bought from Milipore (#AB2033). The transferrin antibody was purchased from Abcam (#ab82411).
The HRP conjugated antibodies were obtained from Jackson ImmunoResearch - $F(ab')_2$ Anti-Rabbit HRP (#711-036-152) or Anti-Mouse IgG, Light Chain HRP (#115-035-174).
The fluorescent secondary antibodies conjugated with AlexaFluor 488 (#A-11063), AlexaFluor 546 (#A-11003) or AlexaFluor 633 (#A-21053) were obtained from ThermoFisher Scientific. Phalloïdin, coupled with Atto 647 was also purchased from ThermoFisher scientific (#A22287).

9.2. Cell culture

Immortalized osteoblasts from *icap*-$1^{+/+}$; *β1 integrin*$^{flox/flox}$ and *icap*-$1^{-/-}$; *β1* integrin$^{flox/flox}$ mice were generated as described previously (Bouvard et al., 2007). These cells were infected or not by adenoCre viruses from gene transfer vector core (University of Iowa) in order to obtain *β1* integrin-null cells. Clonal linages of cells were maintained in culture in DMEM (Life technologies #31966-021) supplemented with 10 % FBS (Dominique Dutcher, #S1810-500), 100 U/mL penicillin

and 100 µg/mL streptomycin (PAN Biotech #P06-07100) at 37°C in a 5% CO_2-humidified chamber. For all experiments, cells were washed by PBS (Dominique Dutcher, #L0615-500), detached using trypsin (Dominique Dutcher, #L0615-500) and treated with 1mg/mL trypsin inhibitor (Sigma-Aldrich, #T6522). Cells were then plated in DMEM containing 10 % FBS for 4h and then the appropriate analysis was carried out. Where needed a serum free medium OptiMEM was used (Life Technologies, #51985-026) as substitute.

9.3. Production of β3-GFP expressing osteoblast cell lines

The four osteoblast clones - *icap*-1$^{+/+}$; *icap*-1$^{-/-}$; *β1* integrin$^{floxed/floxed}$; *β1 integrin*$^{floxed/floxed}$ and *icap-1*$^{-/-}$ were infected using lentiviral infection system from Invitrogen with pLenti – murine β3-GFP vector.

9.4. Western blotting

Cells were plated on 50% confluence and left to spread overnight. The next day, the dishes were washed twice with ice cold PBS and lysed in cold RIPA buffer, supplemented with 1x cOmplete protease inhibitors, 5 mM NaF and 2 mM Na-orthovanadate. After protein quantification via Pierce™ BCA Protein Assay (ThermoFisher Scientific, #23227), the samplers were mixed with Laemmli sample buffer (0.4% SDS, 20% glycerol, 120 mM Tris-Cl (pH 6.8) and 0.02% (w/v) bromophenol blue) and loaded on electrophoretic PAA gels. Following the standard wet blotting protocol, the nitrocellulose membranes (Amersham, #10600003) the membranes were probed with the appropriate primary antibodies, diluted in 5 % BSA in TTBS and incubated overnight. The membrane was subsequently incubated with the appropriate secondary antibodies, also diluted in 5 % BSA in TTBS for one hour and then developed using Clarity ECL kit

(Biorad, #170-5061) and recorded with ChemiDoc Imaging System and analyzed with ImageLab software.

9.5. Traction force microscopy

The poly-acrylamide hydrogels with defined rigidity of 5 kPa and containing fluorescent microbeads (Life technologies, #F8783) were cast in 2 well LabTeks (ThermoFisher, #154461), coated with BindSilane (Sigma-Aldrich, #GE17-1330-01) and covered with coverslip, coated with Sigmacote (Sigma-Aldrich, #SL2). After the polymerization of the polyacrylamide the wells were flooded with water and the coverslips were detached gently. For the functionalization a protocol from Przybyla et al., 2015 was used. Briefly, solution of tetramethacrylate, N6 and Irgacure was deposed on the gels and baked under UV light (312 nm) for 5 min. Then 5 µg/ml fibronectin was deposed on the gels and incubated at 4°C overnight.

Cells were allowed to adhere and spread 4 hour in DMEM containing 10% FBS and then placed in 4% FBS. Just before the acquisition, the membrane was stained with red fluorescent membrane marker PKH26 (Sigma-Aldrich, # PKH26GL).

Images were taken using spinning disk microscope, equipped with heating chamber, CO_2 installation, using 40x magnification oil objective. A fluorescent image of the beads with the cell spread on and fluorescent image of the cell membrane was obtained. Then the culture medium was replaced with pure solution of trypsin and after verification that cells were completely detached the second image of the fluorescent beads were taken. Isolated cells were randomly chosen for each experimental condition.

Force calculations were performed as previously described (Tseng et al., 2011) Briefly the displacement fields describing the deformation of the PA substrate are determined from the analysis of fluorescent beads images before and after removal of the adhering cells with trypsin treatment. The displacement field is

obtained by a two-step method consisting of particle image velocimetry followed by individual bead tracking (Butler et al., 2002; Sabass et al., 2008). A special procedure is used to evaluate displacements in the area of the adhesive pattern where gel deformation is expected to be largest. Depending on the pattern shape, traction forces may be strongly localized leading to large displacements in very small areas. In this case, failure to correctly track a few beads in such areas would significantly alter the calculated force magnitude. Therefore, the pattern area is divided into smaller windows that are allowed to overlap, before applying the cross-correlation and tracking analysis. Reducing the size of the windows makes it possible to retrieve larger displacements with cross-correlation and, using overlapped windows, we can avoid missing beads close to the windows boundaries. All image processing and analysis were performed using Matlab (Gao and Kilfoil, 2009).54 To calculate cell-induced traction stress from displacement data, we have used the Fourier-transform traction cytometry (FTTC) method (Sabass et al., 2008). We kept the regularization parameter at small values ($\lambda < \sim 10^{-9}$) in order to maintain the best spatial resolution, which is estimated to be about 5 mm in our case.

9.6. Plasmids and DNA constructions

The GFP – tensin 1 construction was cloned in the lab. Briefly, the chicken variant of tensin 1 was subcloned in pEGFP-C2 expression vector.

For the detection of the expression levels of β3 integrin, we used the following qPCR primers - 5' – AGC AAC GTC CTC CAG CTC ATT G and 3' – TTG AGG GTG GCA TTG AAG C.

9.7. Focal adhesion lifetime analysis

Cells stably expressing β3-GFP were spread in 2 well LabTeks and left to spread for 4h. Spinning disk videos were taken for the length of 2h with 1 min frequency. The lifetime analysis was performed with Focal Adhesion Analysis Server (Berginski and Gomez, 2013).

9.8. Immunofluorescence

Cells were plated at an approximate density of 6 x 10^4 per cm^2 for 4h, fixed with 4% PFA, permeabilized with 0.3% Triton X-100 and blocked with 10% goat-serum in PBS then with appropriate primary antibodies and after rinsing, with appropriate Alexa-Fluor-conjugated secondary antibody and phalloïdin. Finally, the coverslips were mounted in Mowiol/DAPI solution.

9.9. siRNA treatments

Cells were plated in six well plate at low density – 6x10^3 cells per cm^2 and left to spread overnight. The next day they were transfected with the appropriate siRNA using RNAiMAX system (ThermoFisher Scientific). The medium was changed the next day and a second hit with the same siRNA was performed. The transfected cells were used in 24 hours after.

All used siRNA were purchased from Dharmacon as follows: siRNA against β3 integrin – L-040746-01-0005; siRNA against dynamin 3 - L-044919-02, siRNA against clatrin – L-063954-00, siRNA against caveolin – L-058415-00, siRNA against Nm23-H1 – L-040142-00 and siRNA against Nm23-H2 - L-040143-00; for all experiments we used an non targeting siRNA as control – D-001810-10-20.

9.10. FRAP analysis

Cells stably expressing β3-GFP were spread in 2 well LabTeks and left to spread for 4h. FRAP videos were taken using multimodal microscope for photo manipulations equipped with TIRF 63x objective. The analysis was carried out using build in FRAP analysis module in the FEI offline analysis.

9.11. Image analysis and statistical tests

For ppMLC staining or surface analysis, we measured the necessary signal using a thresholding method with manuel correction when needed. More than 30 cells were measured in each condition that allowed us to do a non-parametric Kruskal-Wallis test (non-parametric) followed by Wilcoxon test with a Bonferroni correction when KW tests were significant (using GraphPad); experiments were done at least 3 times.

For focal adhesion analysis, we measured the β3 staining signal of at least 20 cells using a manual threshold and the particle analyser of ImageJ software. Particles over 1 μm^2 were analysed. The number of focal adhesion per cell and the total adhesive area per cell were analysed by a Kruskal-Wallis test (non-parametric) followed by Wilcoxon test with a Bonferroni correction when KW tests were significant; the mean area of focal adhesions was analyzed by an anova-2 analysis and TukeyHSD post-hoc tests (using GraphPad). Experiments were done 3 times.

9.12. Fibronectin fibrillogenesis

100,000 cells were plated on lab-Tek glass slide 4 chambers and allowed to adhere for 24 h in serum free medium made of OptiMEM. Then cells were fixed with 4% PFA. Cells were stained with anti-fibronectin, anti-beta1 (9EG7), anti-beta3

(LucA.5), phalloidin or DAPI. The length of individual fibers was determined manually in ImageJ. To assess fibronectin coverage, the images were processed with fast Fourier transform bandpass filters to visualize all fibers, and the amount of fibronectin was measured by thresholding using Fiji (Schindelin et al., 2012).
For live-cell imaging, 70,000 cells were plated on cover glass slide 4 chambers and allowed to adhere for 24 h in serum free medium made of Opti-MEM. Then, Alexa 568 Gelatin (diluted 1/20 in PBS) were added to cell culture for 30 min. Labelling gelatin with Alexa 568 dye was described somewhere else (Sharma et al., 2013). Unbound gelatin was washout and replace by OptiMEM serum free medium, live time imaging records were performed.

9.13. Labeling Gelatin with Alexa 546 Dye

Bio-Gel P-30 powder was diluted in PBS and loaded onto glass column. After the colon was drained of the PBS, a mixture of 0.2% gelatin solution and 0.2 M sodium bicarbonate solution was prepared. The Alexa 546 dye was diluted in DMSO and added to the gelatin solution and rotated at RT for 1 h. The dye–gelatin solution was deposited on the top of the column. As the dye–gelatin solution reaches the bottom of the column, the dye labeled gelatin was collected into eppendorf tubes stored at 4 °C for up to 2 months.

9.14. Fluorescent integrin antibody uptake assays

Cells were spread for 4h on FbN coated LabTek slides in DMEM, supplemented with 10% FBS and 1% P/S. When the cells were fully spread the fluorescent antibody (5 µg/ml) was added and the cells were incubated for 20 min at 37°C. At the end of the incubation period the cells were acid washed (0.2 M glycin, 0.15M NaCl, pH 3) for 3 min, fixed in 4% PFA, permeabilized and co-staining for transferrin was

performed to confirm that intracellular vesicles, marked by fluorescent β3 integrin antibody are due to endocytic process.

9.15. Fluorescence-activated cell sorting

Cells were gently detached with trypsin, then treated with trypsin inhibitor (#T0256). Then, they were placed in round-bottom 96 well plate and blocked with 1% BSA in PBS for 30 min at 37°C. Then cells were incubated with the appropriate antibodies and secondary antibodies as control diluted in PBS/1%BSA for 30 min on ice. After subsequent incubation with secondary antibodies cells were fixed in 4% PFA for 10 min and surface staining was detected with BD Accuri C6 flow cytometer and analyzed with the provided software.

VI. References

Chapter 10. References

Abercrombie, M., J.E.M. Heaysman, and S.M. Pegrum. 1971. The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading lamella. *Exp. Cell Res.* 67:359–367. doi:10.1016/0014-4827(71)90420-4.

Alanko et al., 2015. Integrin Endosomal Signalling Suppresses Anoikis -. 2015.

Albelda, S.M., S.A. Mette, D.E. Elder, R.M. Stewart, L. Damjanovich, M. Herlyn, and C.A. Buck. 1990. Integrin Distribution in Malignant Melanoma: Association of the β3 Subunit with Tumor Progression. *Cancer Res.* 50:6757–6764.

Albiges-Rizo, C., O. Destaing, B. Fourcade, E. Planus, and M.R. Block. 2009. Actin machinery and mechanosensitivity in invadopodia, podosomes and focal adhesions. *J. Cell Sci.* 122:3037–3049. doi:10.1242/jcs.052704.

Alessandri, K., B.R. Sarangi, V. V. Gurchenkov, B. Sinha, T.R. Kiessling, L. Fetler, F. Rico, S. Scheuring, C. Lamaze, A. Simon, S. Geraldo, D. Vignjevic, H. Domejean, L. Rolland, A. Funfak, J. Bibette, N. Bremond, and P. Nassoy. 2013. Cellular capsules as a tool for multicellular spheroid production and for investigating the mechanics of tumor progression in vitro. *Proc. Natl. Acad. Sci.* 110:14843–14848. doi:10.1073/pnas.1309482110.

Alon, R., and M.L. Dustin. 2007. Force as a facilitator of integrin conformational changes during leukocyte arrest on blood vessels and antigen-presenting cells. *Immunity*. 26:17–27. doi:10.1016/j.immuni.2007.01.002.

Alvarez, B., P.J.M. Stroeken, M.J. Edel, and E. Roos. 2008. Integrin Cytoplasmic domain-Associated Protein-1 (ICAP-1) promotes migration of myoblasts and affects focal adhesions. *J. Cell. Physiol.* 214:474–482. doi:10.1002/jcp.21215.

Anthis, N.J., and I.D. Campbell. 2011. The tail of integrin activation. *Trends Biochem. Sci.* 36:191–198. doi:10.1016/j.tibs.2010.11.002.

Arjonen, A., J. Alanko, S. Veltel, and J. Ivaska. 2012. Distinct recycling of active and inactive β1 integrins. *Traffic*. 13:610–25. doi:10.1111/j.1600-0854.2012.01327.x.

Bachir, A.I., J. Zareno, K. Moissoglu, E.F. Plow, E. Gratton, and A.R. Horwitz. 2014. Integrin-associated complexes form hierarchically with variable stoichiometry in nascent adhesions. *Curr. Biol.* 24:1845–1853. doi:10.1016/j.cub.2014.07.011.

Ballestrem, C., B. Hinz, B.A. Imhof, and B. Wehrle-Haller. 2001a. Marching at the front and dragging behind: Differential αVβ3-integrin turnover regulates focal adhesion behavior. *J. Cell Biol.* 155:1319–1332. doi:10.1083/jcb.200107107.

Ballestrem, C., B. Hinz, B.A. Imhof, and B. Wehrle-Haller. 2001b. Marching at the

front and dragging behind: differential alphaVbeta3-integrin turnover regulates focal adhesion behavior. *J. Cell Biol.* 155:1319–32. doi:10.1083/jcb.200107107.

Barczyk, M., S. Carracedo, and D. Gullberg. 2010. Integrins. *Cell Tissue Res.* 339:269–280. doi:10.1007/s00441-009-0834-6.

Béraud-Dufour, S., R. Gautier, C. Albiges-Rizo, P. Chardin, and E. Faurobert. 2007. Krit 1 interactions with microtubules and membranes are regulated by Rap1 and integrin cytoplasmic domain associated protein-1. *FEBS J.* 274:5518–5532. doi:10.1111/j.1742-4658.2007.06068.x.

Bereiter-Hahn, J. 2005. Mechanics of crawling cells. *Med. Eng. Phys.* 27:743–753. doi:10.1016/j.medengphy.2005.04.021.

Berginski, M.E., and S.M. Gomez. 2013. The Focal Adhesion Analysis Server: a web tool for analyzing focal adhesion dynamics. *F1000Research*. doi:10.12688/f1000research.2-68.v1.

Le Beyec, J., R. Xu, S.-Y. Lee, C.M. Nelson, A. Rizki, J. Alcaraz, and M.J. Bissell. 2007. Cell shape regulates global histone acetylation in human mammary epithelial cells. *Exp. Cell Res.* 313:3066–3075. doi:10.1016/j.yexcr.2007.04.022.

Bharadwaj, M., N. Strohmeyer, G.P. Colo, J. Helenius, N. Beerenwinkel, H.B. Schiller, R. Fässler, and D.J. Müller. 2017. aV-class integrins exert dual roles on a5β1 integrins to strengthen adhesion to fibronectin. *Nat. Commun.* 8:14348. doi:10.1038/ncomms14348.

Blanchoin, L., R. Boujemaa-Paterski, C. Sykes, and J. Plastino. 2014. Actin dynamics, architecture, and mechanics in cell motility. *Physiol. Rev.* 94:235–63. doi:10.1152/physrev.00018.2013.

Boissan, M., G. Montagnac, Q. Shen, L. Griparic, J. Guitton, M. Romao, N. Sauvonnet, T. Lagache, I. Lascu, G. Raposo, C. Desbourdes, U. Schlattner, M.-L. Lacombe, S. Polo, A.M. van der Bliek, A. Roux, and P. Chavrier. 2014. Nucleoside diphosphate kinases fuel dynamin superfamily proteins with GTP for membrane remodeling. *Science (80-.).* 344:1510–1515. doi:10.1126/science.1253768.

Borisy, G.G., and T.M. Svitkina. 2000. Actin machinery: Pushing the envelope. *Curr. Opin. Cell Biol.* 12:104–112. doi:10.1016/S0955-0674(99)00063-0.

Bouin, A.-P., A. Kyumurkov, M. Régent-Kloeckner, A.-S. Ribba, E. Faurobert, H.-N. Fournier, I. Bourrin-Reynard, S. Manet-Dupé, C. Oddou, M. Balland, E. Planus, and C. Albiges-Rizo. 2017a. Correction: ICAP-1 monoubiquitylation coordinates matrix density and rigidity sensing for cell migration through ROCK2–MRCKa balance. *J. Cell Sci.* 130:1195–1195. doi:10.1242/jcs.202218.

Bouin, A.-P., A. Kyurmurkov, M. Régent-Kloeckner, A.-S. Ribba, E. Faurobert, H.-N.

Fournier, I. Bourrin-Reynard, S. Manet-Dupé, C. Oddou, M. Balland, E. Planus, and C. Albiges-Rizo. 2017b. ICAP-1 monoubiquitylation coordinates matrix density and rigidity sensing for cell migration through ROCK2–MRCKa balance. *J. Cell Sci.* 130:626–636. doi:10.1242/jcs.200139.

Bouvard, D., A. Aszodi, G. Kostka, M.R. Block, C. Albigès-Rizo, and R. Fässler. 2007. Defective osteoblast function in ICAP-1-deficient mice. *Development*. 134:2615–25. doi:10.1242/dev.000877.

Bouvard, D., and M.R. Block. 1998. Calcium/calmodulin-dependent protein kinase II controls integrin alpha5beta1-mediated cell adhesion through the integrin cytoplasmic domain associated protein-1alpha. *Biochem Biophys Res Commun*. 252:46–50. doi:S0006-291X(98)99592-4 [pii]\r10.1006/bbrc.1998.9592.

Bouvard, D., L. Vignoud, S. Dupe-Manet, N. Abed, H.N. Fournier, C. Vincent-Monegat, S. Francesco Retta, R. Faessler, and M.R. Block. 2003. Disruption of focal adhesions by integrin cytoplasmic domain-associated protein-1a *J. Biol. Chem.* 278:6567–6574. doi:10.1074/jbc.M211258200.

Bridgewater, R.E., J.C. Norman, and P.T. Caswell. 2012. Integrin trafficking at a glance. *J. Cell Sci.* 125:3695–3701. doi:10.1242/jcs.095810.

Brinckmann, J. 2005. Collagens at a glance. *Top. Curr. Chem.* 247:1–6. doi:10.1007/b103817.

Brown, C.M., B. Hebert, D.L. Kolin, J. Zareno, L. Whitmore, A.R. Horwitz, and P.W. Wiseman. 2006. Probing the integrin-actin linkage using high-resolution protein velocity mapping. *J. Cell Sci.* 119:5204–5214. doi:10.1242/jcs.03321.

Brunner, M., A. Millon-Fremillon, G. Chevalier, I.A. Nakchbandi, D. Mosher, M.R. Block, C. Albiges-Rizo, and D. Bouvard. 2011a. Osteoblast mineralization requires {beta}1 integrin/ICAP-1-dependent fibronectin deposition. *J Cell Biol*. 194:307–322. doi:10.1083/jcb.201007108.

Brunner, M., A. Millon-Frémillon, G. Chevalier, I. a. Nakchbandi, D. Mosher, M.R. Block, C. Albigès-Rizo, and D. Bouvard. 2011b. Osteoblast mineralization requires β1 integrin/ICAP-1-dependent fibronectin deposition. *J. Cell Biol.* 194:307–322. doi:10.1083/jcb.201007108.

Buguin, A., P. Chavrier, B. Ladoux, O. du Roure, A. Saez, and P. Silberzan. 2005. [An array of microfabricated pillars to study cell migration]. *Med Sci*. 21:765–767. doi:10.1051/medsci/2005218-9765.

Bustelo, X.R., V. Sauzeau, and I.M. Berenjeno. 2007. GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *BioEssays*. 29:356–370. doi:10.1002/bies.20558.

Butcher, D.T., T. Alliston, and V.M. Weaver. 2009. A tense situation: forcing tumour

progression. *Nat. Rev. Cancer*. 9:108–122. doi:10.1038/nrc2544.

Butler, J.P., I.M. Tolic-Norrelykke, B. Fabry, and J.J. Fredberg. 2002. Traction fields, moments, and strain energy that cells exert on their surroundings. *AJP Cell Physiol.* 282:C595–C605. doi:10.1152/ajpcell.00270.2001.

Byron, A., M.R. Morgan, and M.J. Humphries. 2010. Adhesion signalling complexes. *Curr. Biol.* 20. doi:10.1016/j.cub.2010.10.059.

Cairo, C.W., R. Mirchev, and D.E. Golan. 2006. Cytoskeletal Regulation Couples LFA-1 Conformational Changes to Receptor Lateral Mobility and Clustering. *Immunity*. 25:297–308. doi:10.1016/j.immuni.2006.06.012.

Calderwood, D.A., Y. Fujioka, J.M. Pereda, B. GarcA-a-Alvarez, Nakamoto, T, B. Margolis, C.J. McGlade, R.C. Liddington, and M.H. Ginsberg. 2003. Integrin beta cytoplasmic domain interactions with phosphotyrosine- binding domains: a structural prototype for diversity in integrin signaling. *Proc. Natl. Acad. Sci. U. S. A.* 100:2272–2277.

Carvallo, L., R. Muñoz, F. Bustos, N. Escobedo, H. Carrasco, G. Olivares, and J. Larraín. 2010. Non-canonical Wnt signaling induces ubiquitination and degradation of Syndecan4. *J. Biol. Chem.* 285:29546–55. doi:10.1074/jbc.M110.155812.

Caswell, P., and J. Norman. 2008. Endocytic transport of integrins during cell migration and invasion. *Trends Cell Biol.* 18:257–263. doi:10.1016/j.tcb.2008.03.004.

Caswell, P.T., M. Chan, A.J. Lindsay, M.W. McCaffrey, D. Boettiger, and J.C. Norman. 2008. Rab-coupling protein coordinates recycling of α5β1 integrin and EGFR1 to promote cell migration in 3D microenvironments. *J. Cell Biol.* 183:143–155. doi:10.1083/jcb.200804140.

Caswell, P.T., S. Vadrevu, and J.C. Norman. 2009. Integrins: masters and slaves of endocytic transport. *Nat. Rev. Mol. Cell Biol.* 10:843–853. doi:10.1038/nrm2799.

Cavalcanti-Adam, E.A., T. Volberg, A. Micoulet, H. Kessler, B. Geiger, and J.P. Spatz. 2007. Cell spreading and focal adhesion dynamics are regulated by spacing of integrin ligands. *Biophys. J.* 92:2964–74. doi:10.1529/biophysj.106.089730.

Cebecauer, M., M. Spitaler, A. Sergé, and A.I. Magee. 2010. Signalling complexes and clusters: functional advantages and methodological hurdles. *J. Cell Sci.* 123:309 LP-320.

Ceresa, B.P., and S.L. Schmid. 2000. Regulation of signal transduction by endocytosis. *Curr. Opin. Cell Biol.* 12:204–10.

Chalfie, M. 2009. Neurosensory mechanotransduction. *Nat. Rev. Mol. Cell Biol.*

10:44–52. doi:10.1038/nrm2595.

Chan, M.W.C., B. Hinz, and C.A. McCulloch. 2010. Mechanical Induction of Gene Expression in Connective Tissue Cells. 98. 179-205 pp.

Chang, D.D., C. Wong, H. Smith, and J. Liu. 1997. ICAP-1, a novel β1 integrin cytoplasmic domain-associated protein, binds to a conserved and functionally important NPXY sequence motif of β1 integrin. *J. Cell Biol.* 138:1149–1157. doi:10.1083/jcb.138.5.1149.

Chao, W.-T., and J. Kunz. 2009. Focal adhesion disassembly requires clathrin-dependent endocytosis of integrins. *FEBS Lett.* 583:1337–43. doi:10.1016/j.febslet.2009.03.037.

Charras, G., and E. Paluch. 2008. Blebs lead the way: how to migrate without lamellipodia. *Nat. Rev. Mol. Cell Biol.* 9:730–736. doi:10.1038/nrm2453.

Chen, P.W., R. Luo, X. Jian, and P.A. Randazzo. 2014. The Arf6 GTPase-activating proteins ARAP2 and ACAP1 define distinct endosomal compartments that regulate integrin α5βS1 traffic. *J. Biol. Chem.* 289:30237–30248. doi:10.1074/jbc.M114.596155.

Chiquet, M., L. Gelman, R. Lutz, and S. Maier. 2009. From mechanotransduction to extracellular matrix gene expression in fibroblasts. *Biochim. Biophys. Acta - Mol. Cell Res.* 1793:911–920. doi:10.1016/j.bbamcr.2009.01.012.

Cho, A., V.M. Howell, and E.K. Colvin. 2015. The Extracellular Matrix in Epithelial Ovarian Cancer – A Piece of a Puzzle. *Front. Oncol.* 5:1–16. doi:10.3389/fonc.2015.00245.

Choi, C.K., M. Vicente-Manzanares, J. Zareno, L.A. Whitmore, A. Mogilner, and A.R. Horwitz. 2008. Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nat. Cell Biol.* 10:1039–50. doi:10.1038/ncb1763.

Chrzanowska-Wodnicka, M., and K. Burridge. 1996. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J. Cell Biol.* 133:1403–1415. doi:10.1083/jcb.133.6.1403.

Collin, O., S. Na, F. Chowdhury, M. Hong, M.E. Shin, F. Wang, and N. Wang. 2008. Self-Organized Podosomes Are Dynamic Mechanosensors. *Curr. Biol.* 18:1288–1294. doi:10.1016/j.cub.2008.07.046.

Colognato, H., and P.D. Yurchenco. 2000. Form and function: The laminin family of heterotrimers. *Dev. Dyn.* 218:213–234. doi:10.1002/(SICI)1097-0177(200006)218:2<213::AID-DVDY1>3.0.CO;2-R.

Cox, T.R., and J.T. Erler. 2011. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. *Dis. Model. Mech.* 4:165–

78. doi:10.1242/dmm.004077.

Dammai, V., B. Adryan, K.R. Lavenburg, and T. Hsu. 2003. Drosophila awd, the homolog of human nm23, regulates FGF receptor levels and functions synergistically with shi/dynamin during tracheal development. *Genes Dev.* 17:2812–2824. doi:10.1101/gad.1096903.

Danen, E.H.J., P. Sonneveld, C. Brakebusch, R. Fässler, and A. Sonnenberg. 2002. The fibronectin-binding integrins a5β1 and avβ3 differentially modulate RhoA-GTP loading, organization of cell matrix adhesions, and fibronectin fibrillogenesis. *J. Cell Biol.* 159:1071–1086. doi:10.1083/jcb.200205014.

Degani, S., F. Balzac, M. Brancaccio, S. Guazzone, S.F. Retta, L. Silengo, A. Eva, and G. Tarone. 2002. The integrin cytoplasmic domain-associated protein ICAP-1 binds and regulates Rho family GTPases during cell spreading. *J. Cell Biol.* 156:377–387. doi:10.1083/jcb.200108030.

Dembo, M., D.C. Torney, K. Saxman, and D. Hammer. 1988. The reaction-limited kinetics of membrane-to-surface adhesion and detachment. *Proc. R. Soc. London. Ser. B.* 234:55–83. doi:10.1098/rspb.1988.0038.

Deroanne, C.F., C.M. Lapiere, and B. V. Nusgens. 2001. In vitro tubulogenesis of endothelial cells by relaxation of the coupling extracellular matrix-cytoskeleton. *Cardiovasc. Res.* 49:647–658. doi:10.1016/S0008-6363(00)00233-9.

Desgrosellier, S.J., and A.D. Cheresh. 2010. Integrins in cancer: biological implications and therapeutic opportunities. *Nat. Rev. Cancer.* 10:9–22. doi:10.1038/nrc2748.

Desprat, N., W. Supatto, P.A. Pouille, E. Beaurepaire, and E. Farge. 2008. Tissue Deformation Modulates Twist Expression to Determine Anterior Midgut Differentiation in Drosophila Embryos. *Dev. Cell.* 15:470–477. doi:10.1016/j.devcel.2008.07.009.

Destaing, O., E. Planus, D. Bouvard, C. Oddou, C. Badowski, V. Bossy, A. Raducanu, B. Fourcade, C. Albiges-Rizo, and M.R. Block. 2010. β1A Integrin Is a Master Regulator of Invadosome Organization and Function. *Mol. Biol. Cell.* 21:4108–4119. doi:10.1091/mbc.E10-07-0580.

Diagouraga, B., A. Grichine, A. Fertin, J. Wang, S. Khochbin, and K. Sadoul. 2014. Motor-driven marginal band coiling promotes cell shape change during platelet activation. *J. Cell Biol.* 204:177–185. doi:10.1083/jcb.201306085.

Discher, D.E., L. Smith, S. Cho, M. Colasurdo, A.J. García, and S. Safran. 2017. Matrix Mechanosensing: From Scaling Concepts in 'Omics Data to Mechanisms in the Nucleus, Regeneration, and Cancer. *Annu. Rev. Biophys.* 46:295–315. doi:10.1146/annurev-biophys.

Doebereiner, H.G., B.J. Dubin-Thaler, G. Giannone, H.S. Xenias, M.P. Sheetz, H.G. Dobereiner, and H.-G. Döbereiner. 2004. Dynamic Phase Transitions in Cell Spreading. *Phys. Rev. Lett.* 93:108105. doi:10.1103/PhysRevLett.93.108105.

Doherty, G.J., M.K. Ahlund, M.T. Howes, B. Moren, R.G. Parton, H.T. McMahon, and R. Lundmark. 2011. The endocytic protein GRAF1 is directed to cell-matrix adhesion sites and regulates cell spreading. *Mol. Biol. Cell*. 22:4380–4389. doi:10.1091/mbc.E10-12-0936.

Domain, P.T.B., D.D. Chang, B.Q. Hoang, J. Liu, and T. a Springer. 2002. Molecular basis for interaction between Icap1 alpha PTB domain and beta 1 integrin. *J. Biol. Chem.* 277:8140–5. doi:10.1074/jbc.M109031200.

Doyle, A.D., F.W. Wang, K. Matsumoto, and K.M. Yamada. 2009. One-dimensional topography underlies three-dimensional fibrillar cell migration. SUPP MAT. *J. Cell Biol.* 184:481–90. doi:10.1083/jcb.200810041.

Doyle, A.D., and K.M. Yamada. 2010. Sensing tension. *Nature*. 466:5–6. doi:10.1126/scisignal.2004355.

Du, J., X. Chen, X. Liang, G. Zhang, J. Xu, L. He, Q. Zhan, X.-Q. Feng, S. Chien, and C. Yang. 2011. Integrin activation and internalization on soft ECM as a mechanism of induction of stem cell differentiation by ECM elasticity. *Proc. Natl. Acad. Sci.* 108:9466–9471. doi:10.1073/pnas.1106467108.

Dubash, A.D., M.M. Menold, T. Samson, E. Boulter, R. García-Mata, R. Doughman, and K. Burridge. 2009. Chapter 1 Focal Adhesions: New Angles on an Old Structure. *Int. Rev. Cell Mol. Biol.* 277:1–65. doi:10.1016/S1937-6448(09)77001-7.

Dubin-Thaler, B.J., G. Giannone, H.-G. Döbereiner, and M.P. Sheetz. 2004. Nanometer Analysis of Cell Spreading on Matrix-Coated Surfaces Reveals Two Distinct Cell States and STEPs. *Biophys. J.* 86:1794–1806. doi:10.1016/S0006-3495(04)74246-0.

Dulyaninova, N.G., V.N. Malashkevich, S.C. Almo, and A.R. Bresnick. 2005. Regulation of myosin-IIA assembly and Mts1 binding by heavy chain phosphorylation. *Biochemistry*. 44:6867–6876. doi:10.1021/bi0500776.

Dupont, S., L. Morsut, M. Aragona, E. Enzo, S. Giulitti, M. Cordenonsi, F. Zanconato, J. Le Digabel, M. Forcato, S. Bicciato, N. Elvassore, and S. Piccolo. 2011. Role of YAP/TAZ in mechanotransduction. *Nature*. 474:179–183. doi:10.1038/nature10137.

Effler, J.C., Y.S. Kee, J.M. Berk, M.N. Tran, P.A. Iglesias, and D.N. Robinson. 2006. Mitosis-Specific Mechanosensing and Contractile-Protein Redistribution Control Cell Shape. *Curr. Biol.* 16:1962–1967. doi:10.1016/j.cub.2006.08.027.

Eggert, U.S., T.J. Mitchison, and C.M. Field. 2006. Animal cytokinesis: from parts list to mechanisms. *Annu. Rev. Biochem.* 75:543–566.

doi:10.1146/annurev.biochem.74.082803.133425.

Elosegui-Artola, A., E. Bazellières, M.D. Allen, I. Andreu, R. Oria, R. Sunyer, J.J. Gomm, J.F. Marshall, J.L. Jones, X. Trepat, and P. Roca-Cusachs. 2014. Rigidity sensing and adaptation through regulation of integrin types. *Nat. Mater.* 13:631–7. doi:10.1038/nmat3960.

Emsley, J., C.G. Knight, R.W. Farndale, M.J. Barnes, and R.C. Liddington. 2000. Structural basis of collagen recognition by integrin alpha2beta1. *Cell*. 101:47–56. doi:10.1016/S0092-8674(00)80622-4.

Engler, A., L. Bacakova, C. Newman, A. Hategan, M. Griffin, and D. Discher. 2004. Substrate compliance versus ligand density in cell on gel response. *Biophys. J.* 86(1):617–628. doi:10.1016/S0006-3495(04)74140-5.

Engler, A.J., F. Rehfeldt, S. Sen, and D.E. Discher. 2007. Microtissue Elasticity: Measurements by Atomic Force Microscopy and Its Influence on Cell Differentiation. *Methods Cell Biol.* 83:521–545. doi:10.1016/S0091-679X(07)83022-6.

Engler, A.J., S. Sen, H.L. Sweeney, and D.E. Discher. 2006. Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell*. 126:677–689. doi:10.1016/j.cell.2006.06.044.

Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. cancer*. 20:1–5.

Enomoto, T. 1996. Microtubule disruption induces the formation of actin stress fibers and focal adhesions in cultured cells: possible involvement of the rho signal cascade. *Cell Struct. Funct.* 21:317–326. doi:10.1247/csf.21.317.

Evans, R.D., V.C. Perkins, A. Henry, P.E. Stephens, M.K. Robinson, and F.M. Watt. 2003. A tumor-associated β1 integrin mutation that abrogates epithelial differentiation control. *J. Cell Biol.* 160:589–596. doi:10.1083/jcb.200209016.

Even-Faitelson, L., and D. Ravid. 2006. PAK1 and aPKCζ Regulate Myosin II-B Phosphorylation: A Novel Signaling Pathway Regulating Filament Assembly. *Mol. Biol. Cell*. 17:2869–2881. doi:10.1091/mbc.E05.

Ezratty, E.J., C. Bertaux, E.E. Marcantonio, and G.G. Gundersen. 2009. Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. *J. Cell Biol.* 187:733–47. doi:10.1083/jcb.200904054.

Ezratty, E.J., M. a Partridge, and G.G. Gundersen. 2005. Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat. Cell Biol.* 7:581–590. doi:10.1038/ncb1262.

Fereol, S., R. Fodil, V.M. Laurent, M. Balland, B. Louis, G. Pelle, S. Henon, E. Planus, and D. Isabey. 2009. Prestress and adhesion site dynamics control cell

sensitivity to extracellular stiffness. *Biophys. J.* 96:2009–2022. doi:10.1016/j.bpj.2008.10.072.

Faurobert, E., and C. Albiges-Rizo. 2010. Recent insights into cerebral cavernous malformations: A complex jigsaw puzzle under construction. *FEBS J.* 277:1084–1096. doi:10.1111/j.1742-4658.2009.07537.x.

Faurobert, E., C. Rome, J. Lisowska, S. Manet-Dupé, G. Boulday, M. Malbouyres, M. Balland, A.-P. Bouin, M. Kéramidas, D. Bouvard, J.-L. Coll, F. Ruggiero, E. Tournier-Lasserve, and C. Albiges-Rizo. 2013. CCM1-ICAP-1 complex controls β1 integrin-dependent endothelial contractility and fibronectin remodeling. *J. Cell Biol.* 202:545–61. doi:10.1083/jcb.201303044.

Ferguson, J.P., S.D. Huber, N.M. Willy, E. Aygün, S. Goker, T. Atabey, and C. Kural. 2017. Mechanoregulation of clathrin-mediated endocytosis. *J. Cell Sci.* jcs.205930. doi:10.1242/jcs.205930.

Fourel, L., A. Valat, E. Faurobert, R. Guillot, I. Bourrin-Reynard, K. Ren, L. Lafanechère, E. Planus, C. Picart, and C. Albiges-Rizo. 2016. β3 integrin-mediated spreading induced by matrix-bound BMP-2 controls Smad signaling in a stiffness-independent manner. *J. Cell Biol.* 212:693–706. doi:10.1083/jcb.201508018.

Fournier, H.N., C. Albiges-Rizo, and M.R. Block. 2003. New insights into Nm23 control of cell adhesion and migration. *J Bioenerg Biomembr*. 35:81–87.

Fournier, H.N., S. Dupé-Manet, D. Bouvard, M.L. Lacombe, C. Marie, M.R. Block, and C. Albiges-Rizo. 2002a. Integrin cytoplasmic domain-associated protein 1a (ICAP-1a) interacts directly with the metastasis suppressor nm23-H2, and both proteins are targeted to newly formed cell adhesion sites upon integrin engagement. *J. Biol. Chem.* 277:20895–20902. doi:10.1074/jbc.M200200200.

Fournier, H.N., S. Dupé-Manet, D. Bouvard, M.L. Lacombe, C. Marie, M.R. Block, and C. Albiges-Rizo. 2002b. Integrin cytoplasmic domain-associated protein 1a (ICAP-1a) interacts directly with the metastasis suppressor nm23-H2, and both proteins are targeted to newly formed cell adhesion sites upon integrin engagement. *J. Biol. Chem.* 277:20895–20902. doi:10.1074/jbc.M200200200.

Fraley, S.I., Y. Feng, R. Krishnamurthy, D. Kim, A. Celedon, G.D. Longmore, D. Wirtz, and S. Louis. 2010. A distinctive role for focal adhesion proteins in three-dimensional cell motility. *Nat. Cell Biol.* 12:598–604. doi:10.1038/ncb2062.A.

De Franceschi, N., A. Arjonen, N. Elkhatib, K. Denessiouk, A.G. Wrobel, T.A. Wilson, J. Pouwels, G. Montagnac, D.J. Owen, and J. Ivaska. 2016. Selective integrin endocytosis is driven by interactions between the integrin a-chain and AP2. *Nat. Struct. Mol. Biol.* 23:172–179. doi:10.1038/nsmb.3161.

Frantz, C., K.M. Stewart, and V.M. Weaver. 2010. The extracellular matrix at a

glance. *J. Cell Sci.* 123:4195–4200. doi:10.1242/jcs.023820.

Friedl, P., and K. Wolf. 2003. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat. Rev. Cancer*. 3:362–74. doi:10.1038/nrc1075.

Friedland, J.C., M.H. Lee, and D. Boettiger. 2009. Mechanically activated integrin switch controls alpha5beta1 function. *Science*. 323:642–644. doi:10.1126/science.1168441.

Frisch, S.M., and H. Francis. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* 124:619–626. doi:10.1083/jcb.124.4.619.

Furukawa, K.T., K. Yamashita, N. Sakurai, and S. Ohno. 2017. The Epithelial Circumferential Actin Belt Regulates YAP/TAZ through Nucleocytoplasmic Shuttling of Merlin. *Cell Rep.* 20:1435–1447. doi:10.1016/j.celrep.2017.07.032.

Gao, Y., and M.L. Kilfoil. 2009. Accurate detection and complete tracking of large populations of features in three dimensions. *Opt. Express*. 17:4685. doi:10.1364/OE.17.004685.

García, A.J., J.E. Schwarzbauer, and D. Boettiger. 2002. Distinct activation states of a5β1 integrin show differential binding to RGD and synergy domains of fibronectin. *Biochemistry*. 41:9063–9069. doi:10.1021/bi025752f.

Gardiner, B.S., K.K.L. Wong, G.R. Joldes, A.J. Rich, C.W. Tan, A.W. Burgess, and D.W. Smith. 2015. Discrete Element Framework for Modelling Extracellular Matrix, Deformable Cells and Subcellular Components. *PLoS Comput. Biol.* 11. doi:10.1371/journal.pcbi.1004544.

Gauthier, N.C., O.M. Rossier, A. Mathur, J.C. Hone, and M.P. Sheetz. 2009. Plasma Membrane Area Increases with Spread Area by Exocytosis of a GPI-anchord Protein Compartment. *Mol. Biol. Cell*. 20:3261–3272. doi:10.1091/mbc.E09.

Geiger, B., and A. Bershadsky. 2001. Assembly and mechanosensory function of focal contacts. *Curr. Opin. Cell Biol.* 13:584–592. doi:10.1016/S0955-0674(00)00255-6.

Geiger, B., A. Bershadsky, R. Pankov, and K.M. Yamada. 2001. Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. *Nat. Rev. Mol. Cell Biol.* 2:793–805. doi:10.1038/35099066.

Ghajar, C.M., H. Peinado, H. Mori, I.R. Matei, K.J. Evason, H. Brazier, D. Almeida, A. Koller, K.A. Hajjar, D.Y.R. Stainier, E.I. Chen, D. Lyden, and M.J. Bissell. 2013. The perivascular niche regulates breast tumour dormancy. *Nat. Cell Biol.* 15:807–817. doi:10.1038/ncb2767.

Giannone, G., B.J. Dubin-Thaler, H.G. Döbereiner, N. Kieffer, A.R. Bresnick, and M.P. Sheetz. 2004. Periodic lamellipodial contractions correlate with rearward actin waves. *Cell*. 116:431–443. doi:10.1016/S0092-8674(04)00058-3.

Giannone, G., B.J. Dubin-Thaler, O. Rossier, Y. Cai, O. Chaga, G. Jiang, W. Beaver, H.G. Döbereiner, Y. Freund, G. Borisy, and M.P. Sheetz. 2007. Lamellipodial Actin Mechanically Links Myosin Activity with Adhesion-Site Formation. *Cell*. 128:561–575. doi:10.1016/j.cell.2006.12.039.

Giannone, G., R.M. Mege, and O. Thoumine. 2009. Multi-level molecular clutches in motile cell processes. *Trends Cell Biol.* 19:475–486. doi:10.1016/j.tcb.2009.07.001.

Glading, A., J. Han, R.A. Stockton, and M.H. Ginsberg. 2007. KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell-cell junctions. *J. Cell Biol.* 179:247–254. doi:10.1083/jcb.200705175.

Grashoff, C., B.D. Hoffman, M.D. Brenner, R. Zhou, M. Parsons, M.T. Yang, M.A. McLean, S.G. Sligar, C.S. Chen, T. Ha, and M.A. Schwartz. 2010. Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature*. 466:263–6. doi:10.1038/nature09198.

Groves, J.T., and J. Kuriyan. 2010. Molecular mechanisms in signal transduction at the membrane. *Nat. Struct. Mol. Biol.* 17:659–65.

Guo, W., and F.G. Giancotti. 2004. Integrin signalling during tumour progression. *Nat. Rev. Mol. Cell Biol.* 5:816–826. doi:10.1038/nrm1490.

Gupton, S.L., and C.M. Waterman-Storer. 2006. Spatiotemporal Feedback between Actomyosin and Focal-Adhesion Systems Optimizes Rapid Cell Migration. *Cell*. 125:1361–1374. doi:10.1016/j.cell.2006.05.029.

Hamasaki, K., T. Mimura, H. Furuya, N. Morino, T. Yamazaki, I. Komuro, Y. Yazaki, and Y. Nojima. 1995. Stretching mesangial cells stimulates tyrosine phosphorylation of focal adhesion kinase pp125FAK. *Biochem. Biophys. Res. Commun.* 212:544–549. doi:10.1006/bbrc.1995.2004.

Hanahan, D., and R.A. Weinberg. 2011. Hallmarks of cancer: The next generation. *Cell*. 144:646–674. doi:10.1016/j.cell.2011.02.013.

Heck, J.N., S.M. Ponik, M.G. Garcia-Mendoza, C.A. Pehlke, D.R. Inman, K.W. Eliceiri, and P.J. Keely. 2012. Microtubules regulate GEF-H1 in response to extracellular matrix stiffness. *Mol. Biol. Cell*. 23:2583–2592. doi:10.1091/mbc.E11-10-0876.

Henri-Noel Fournier, Sandra Dupe-Manet, Daniel Bouvard, F.L., S.F.R. Simona Degani, Marc R. Block, and C. Albiges-Rizo. 2005. Nuclear Translocation of Integrin Cytoplasmic Domainassociated Protein 1 Stimulates Cellular Proliferation. *Mol. Biol. Cell*. 16:1–13. doi:10.1091/mbc.E04.

Hilder, T.L., M.H. Malone, S. Bencharit, J. Colicelli, T.A. Haystead, G.L. Johnson, and C.C. Wu. 2007. Proteomic identification of the cerebral cavernous malformation signaling complex. *J. Proteome Res.* 6:4343–4355. doi:10.1021/pr0704276.

VI. References

Hodge, T., and M.J.T. V. Cope. 2000. A myosin family tree. *J. Cell Sci.* 113:3353–3354.

Horton, E.R., A. Byron, J.A. Askari, D.H.J. Ng, A. Millon-Frémillon, J. Robertson, E.J. Koper, N.R. Paul, S. Warwood, D. Knight, J.D. Humphries, and M.J. Humphries. 2015. Definition of a consensus integrin adhesome and its dynamics during adhesion complex assembly and disassembly. *Nat. Cell Biol.* 17:1577–1587. doi:10.1038/ncb3257.

Hotulainen, P., P. Lappalainen, X. Wang, P. Hotulainen, P. Lappalainen, T. Pollard, J.-Q. Wu, D. Dean, A. Napolitano, J. Youssef, J. Morgan, D. Dean, A. Rago, J. Morgan, W. Legant, A. Pathak, M. Yang, V. Deshpande, R. McMeeking, C. Chen, T. Boudou, S. Thomas, J. Overdevest, M. Nitz, P. Williams, C. Owens, M. Sanchez-Carbayo, H. Frierson, M. Schwartz, D. Theodorescu, L. Castella, L. Buscemi, C. Godbout, J.-J. Meister, B. Hinz, D. Discher, P. Janmey, Y.-L. Wang, A. Saez, A. Buguin, P. Silberzan, B. Ladoux, M. Ghibaudo, A. Saez, L. Trichet, A. Xayaphoummine, J. Browaeys, P. Silberzan, A. Buguin, B. Ladoux, D. Mitrossilis, J. Fouchard, A. Guiroy, N. Desprat, N. Rodriguez, B. Fabry, A. Asnacios, A. Zemel, F. Rehfeldt, A. Brown, D. Discher, S. Safran, V. Deshpande, R. McMeeking, A. Evans, D. Nickerson, N. Smith, P. Hunter, V. Barocas, R. Tranquillo, V. Barocas, R. Tranquillo, G. Zahalak, J. Wagenseil, T. Wakatsuki, E. Elson, M. Marchetti, J. Joanny, S. Ramaswamy, T. Liverpool, J. Prost, M. Rao, R. Simha, J. Prost, F. Jülicher, J.-F. Joanny, A. Hill, S. Pellegrin, H. Mellor, Y. Sawada, M. Tamada, B. Dubin-Thaler, O. Cherniavskaya, R. Sakai, S. Tanaka, M. Sheetz, E. Puklin-Faucher, M. Sheetz, et al. 2006. Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. *J. Cell Biol.* 173:383–94. doi:10.1083/jcb.200511093.

Hsieh, P., R. Segal, and L.B. Chen. 1980. Studies of fibronectin matrices in living cells with fluoresceinated gelatin. *J. Cell Biol.* 87:14–22.

Hu, X., C. Jing, X. Xu, N. Nakazawa, V.W. Cornish, F.M. Margadant, and M.P. Sheetz. 2016. Cooperative vinculin binding to talin mapped by time-resolved super resolution microscopy. *Nano Lett.* 16:4062–4068. doi:10.1021/acs.nanolett.6b00650.

Humphries, J.D., A. Byron, M.D. Bass, S.E. Craig, J.W. Pinney, D. Knight, and M.J. Humphries. 2009a. Proteomic analysis of integrin-associated complexes identifies RCC2 as a dual regulator of Rac1 and Arf6. *Sci. Signal.* 2:ra51. doi:10.1126/scisignal.2000396.

Humphries, J.D., A. Byron, M.D. Bass, S.E. Craig, J.W. Pinney, D. Knight, M.J. Humphries, W. John, D. Knight, M.J. Humphries, J.W. Pinney, D. Knight, and M.J. Humphries. 2009b. Proteomic analysis of integrin-associated complexes identifies RCC2 as a dual regulator of Rac1 and Arf6. *Sci. Signal.* 2:ra51. doi:10.1126/scisignal.2000396.

Humphries, J.D., A. Byron, and M.J. Humphries. 2006. Integrin ligands at a glance. *J. Cell Sci.* 119:3901–3903. doi:10.1242/jcs.03098.

Huveneers, S., and J. de Rooij. 2013. Mechanosensitive systems at the cadherin-F-actin interface. *J. Cell Sci.* 126:403–413. doi:10.1242/jcs.109447.

Hynes, R.O. 2002. Integrins: Bidirectional, allosteric signaling machines. *Cell*. 110:673–687. doi:10.1016/S0092-8674(02)00971-6.

Hynes, R.O. 2009. The extracellular matrix: not just pretty fibrils. *Science*. 326:1216–1219. doi:10.1126/science.1176009.

Ikeda, T. 2010. NDP kinase 7 is a conserved microtubule-binding protein preferentially expressed in ciliated cells. *Cell Struct. Funct.* 35:23–30.

Ingber, D.E. 1993. The riddle of morphogenesis: a question of solution chemistry or molecular cell engineering? *Cell*. 75:1249–52. doi:0092-8674(93)90612-T [pii].

Ingber, D.E. 2003a. Tensegrity II. How structural networks influence cellular information processing networks. *J. Cell Sci.* 116:1397–408. doi:10.1242/jcs.00360.

Ingber, D.E. 2003b. Tensegrity I. Cell structure and hierarchical systems biology. *J. Cell Sci.* 116:1157–1173. doi:10.1242/jcs.00359.

Jacquemet, G., D.M. Green, R.E. Bridgewater, A. von Kriegsheim, M.J. Humphries, J.C. Norman, and P.T. Caswell. 2013. Rcp-driven a5β1 recycling suppresses rac and promotes rhoa activity via the racgap1-iqgap1 complex. *J. Cell Biol.* 202:917–935. doi:10.1083/jcb.201302041.

Janmey, P.A., and C.A. McCulloch. 2007. Cell mechanics: integrating cell responses to mechanical stimuli. *Annu. Rev. Biomed. Eng.* 9:1–34. doi:10.1146/annurev.bioeng.9.060906.151927.

Jiang, G., G. Giannone, D.R. Critchley, E. Fukumoto, and M.P. Sheetz. 2003. Two-piconewton slip bond between fibronectin and the cytoskeleton depends on talin. *Nature*. 424:334–337. doi:10.1038/nature01805.

Kanchanawong, P., G. Shtengel, A.M. Pasapera, E.B. Ramko, M.W. Davidson, H.F. Hess, and C.M. Waterman. 2010. Nanoscale architecture of integrin-based cell adhesions. *Nature*. 468:580–584. doi:10.1038/nature09621.

Kaplan, R.N., R.D. Riba, S. Zacharoulis, A.H. Bramley, L. Vincent, C. Costa, D.D. MacDonald, D.K. Jin, K. Shido, S.A. Kerns, Z. Zhu, D. Hicklin, Y. Wu, J.L. Port, N. Altorki, E.R. Port, D. Ruggero, S. V. Shmelkov, K.K. Jensen, S. Rafii, and D. Lyden. 2005. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature*. 438:820–827. doi:10.1038/nature04186.

Karakozova, M. 2006. Arginylation of -Actin Regulates Actin Cytoskeleton and Cell Motility. *Science (80-.).* 313:192–196. doi:10.1126/science.1129344.

VI. References

Keely, P., L. Parise, R. Juliano, E.A. Clark, J.S. Brugge, D.A. Lauffenburger, A.F. Horwitz, K. Burridge, M. Chrzanowska-Wodnicka, C. Rosales, et al., M.A. Schwartz, M.D. Schaller, M.H. Ginsberg, T.R. Polte, S.K. Hanks, A. Richardson, J.T. Parsons, L.V. Owens, et al., Q. Chen, et al., Q. Chen, et al., D.D. Schlaepfer, et al., X. Zhu, R.K. Assoian, K.K. Wary, et al., T.H. Lin, et al., D.D. Schlaepfer, M.A. Broome, T. Hunter, E.A. Clark, R.O. Hynes, M.E. Bottazzi, R.K. Assoian, J.E. Meredith, M.A. Schwartz, S. Miyamoto, et al., T.H. Lin, et al., B.L. Ziober, C.S. Lin, R.H. Kramer, T. Tennenbaum, et al., J.F. Marshall, I.R. Hart, R.L. Juliano, J.A. Varner, J.A. Varner, D.A. Cheresh, G. Radeva, et al., W. Kolanus, et al., U.P. Naik, P.M. Patel, L.V. Parise, H. Kashiwagi, et al., J.J. Meredith, et al., M. Fornaro, et al., D. Sheppard, R.B. Dixit, et al., J. Nip, et al., M. Friedlander, et al., F. Mainiero, et al., A.S. Clarke, et al., S.Y. Kim, et al., C. Chao, et al., R.L. Klemke, et al., C. Schreiner, et al., F.G. Giancotti, E. Ruoslahti, J.A. Varner, D.A. Emerson, R.L. Juliano, V. O'Brien, S.M. Frisch, R.L. Juliano, Z.H. Zhang, et al., et al. 1998. Integrins and GTPases in tumour cell growth, motility and invasion. *Trends Cell Biol.* 8:101–6. doi:10.1016/S0962-8924(97)01219-1.

Kiema, T., Y. Lad, P. Jiang, C.L. Oxley, M. Baldassarre, K.L. Wegener, I.D. Campbell, J. Ylänne, and D.A. Calderwood. 2006. The molecular basis of filamin binding to integrins and competition with talin. *Mol. Cell*. 21:337–347. doi:10.1016/j.molcel.2006.01.011.

Kim, S., H. Takahashi, W.-W. Lin, P. Descargues, S. Grivennikov, Y. Kim, J.-L. Luo, and M. Karin. 2009. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature*. 457:102–106. doi:10.1038/nature07623.

Kim, T.H., N. Monsefi, J.H. Song, A. Von Kriegsheim, D. Vandamme, O. Pertz, B.N. Kholodenko, W. Kolch, and K.H. Cho. 2015. Network-based identification of feedback modules that control RhoA activity and cell migration. *J. Mol. Cell Biol.* 7:242–252. doi:10.1093/jmcb/mjv017.

Kinashi, T. 2005. Intracellular signalling controlling integrin activation in lymphocytes. *Nat. Rev. Immunol.* 5:546–559. doi:10.1038/nri1646.

Kirkpatrick, C. a, and S.B. Selleck. 2007. Heparan sulfate proteoglycans at a glance. *J. Cell Sci.* 120:1829–1832. doi:10.1242/jcs.03432.

Klapholz, B., and N.H. Brown. 2017. Talin - the master of integrin adhesions. *J. Cell Sci.* 130:2435–2446. doi:10.1242/jcs.190991.

Klein, E.A., L. Yin, D. Kothapalli, P. Castagnino, F.J. Byfield, T. Xu, I. Levental, E. Hawthorne, P.A. Janmey, and R.K. Assoian. 2009. Cell-Cycle Control by Physiological Matrix Elasticity and In Vivo Tissue Stiffening. *Curr. Biol.* 19:1511–1518. doi:10.1016/j.cub.2009.07.069.

Kobayashi, T., and M. Sokabe. 2010. Sensing substrate rigidity by mechanosensitive

ion channels with stress fibers and focal adhesions. *Curr. Opin. Cell Biol.* 22:669–676. doi:10.1016/j.ceb.2010.08.023.

Kren, A., V. Baeriswyl, F. Lehembre, C. Wunderlin, K. Strittmatter, H. Antoniadis, R. Fässler, U. Cavallaro, and G. Christofori. 2007. Increased tumor cell dissemination and cellular senescence in the absence of beta1-integrin function. *EMBO J.* 26:2832–42. doi:10.1038/sj.emboj.7601738.

Krishnan, K.S., R. Rikhy, S. Rao, M. Shivalkar, M. Mosko, R. Narayanan, P. Etter, P.S. Estes, and M. Ramaswami. 2001. Nucleoside diphosphate kinase, a source of GTP, is required for dynamin-dependent synaptic vesicle recycling. *Neuron*. 30:197–210.

Kubow E. Kristopher, H.A.R., K.E. Kubow, A.R. Horwitz, and H.A.R. Kubow E. Kristopher. 2011. Reducing background fluorescence reveals adhesion in 3D matrices. *Nat Cell Biol*. 13:3–7. doi:10.1038/ncb0111-3.Reducing.

Kuo, J.-C., X. Han, C.-T. Hsiao, J.R. Yates, and C.M. Waterman. 2011. Analysis of the myosin-II-responsive focal adhesion proteome reveals a role for β-Pix in negative regulation of focal adhesion maturation. *Nat. Cell Biol.* 13:383–393. doi:10.1038/ncb2216.

Lakshminarayan, R., C. Wunder, U. Becken, M.T. Howes, C. Benzing, S. Arumugam, S. Sales, N. Ariotti, V. Chambon, C. Lamaze, D. Loew, A. Shevchenko, K. Gaus, R.G. Parton, and L. Johannes. 2014. Galectin-3 drives glycosphingolipid-dependent biogenesis of clathrin-independent carriers. *Nat. Cell Biol.* 16:595–606. doi:10.1038/ncb2970.

Lämmermann, T., B.L. Bader, S.J. Monkley, T. Worbs, R. Wedlich-Söldner, K. Hirsch, M. Keller, R. Förster, D.R. Critchley, R. Fässler, and M. Sixt. 2008. Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature*. 453:51–5. doi:10.1038/nature06887.

Leduc, C., S. Si, J. Gautier, M. Soto-Ribeiro, B. Wehrle-Haller, A. Gautreau, G. Giannone, L. Cognet, and B. Lounis. 2013. A highly specific gold nanoprobe for live-cell single-molecule imaging. *Nano Lett.* 13:1489–1494. doi:10.1021/nl304561g.

Lee, J.O., P. Rieu, M. a Arnaout, and R. Liddington. 1995. Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD11b/CD18). *Cell*. 80:631–638. doi:10.1016/0092-8674(95)90517-0.

Lee, S., and S. Kumar. 2016. Actomyosin stress fiber mechanosensing in 2D and 3D. *F1000Research*. 5:2261. doi:10.12688/f1000research.8800.1.

Legate, K.R., and R. Fässler. 2009. Mechanisms that regulate adaptor binding to beta-integrin cytoplasmic tails. *J. Cell Sci.* 122:187–98. doi:10.1242/jcs.041624.

Leiss, M., K. Beckmann, A. Girós, M. Costell, and R. Fässler. 2008. The role of integrin

binding sites in fibronectin matrix assembly in vivo. *Curr. Opin. Cell Biol.* 20:502–507. doi:10.1016/j.ceb.2008.06.001.

Li, Z., X. Guo, S. Matsushita, and J. Guan. 2011. Differentiation of cardiosphere-derived cells into a mature cardiac lineage using biodegradable poly(N-isopropylacrylamide) hydrogels. *Biomaterials*. 32:3220–3232. doi:10.1016/j.biomaterials.2011.01.050.

Li, Z.H., and A.R. Bresnick. 2006. The S100A4 metastasis factor regulates cellular motility via a direct interaction with myosin-IIA. *Cancer Res.* 66:5173–5180. doi:10.1158/0008-5472.CAN-05-3087.

Lieber, A.D., S. Yehudai-Resheff, E.L. Barnhart, J.A. Theriot, and K. Keren. 2013. Membrane tension in rapidly moving cells is determined by cytoskeletal forces. *Curr. Biol.* 23:1409–1417. doi:10.1016/j.cub.2013.05.063.

Liu, J., Y. Wang, W.I. Goh, H. Goh, M.A. Baird, S. Ruehland, S. Teo, N. Bate, D.R. Critchley, M.W. Davidson, and P. Kanchanawong. 2015. Talin determines the nanoscale architecture of focal adhesions. *Proc. Natl. Acad. Sci.* 112:E4864–E4873. doi:10.1073/pnas.1512025112.

Liu, W., K.M. Draheim, R. Zhang, D.A. Calderwood, and T.J. Boggon. 2013. Mechanism for KRIT1 Release of ICAP1-Mediated Suppression of Integrin Activation. *Mol. Cell*. 49:719–729. doi:10.1016/j.molcel.2012.12.005.

Lo, C.M., H.B. Wang, M. Dembo, and Y.L. Wang. 2000. Cell movement is guided by the rigidity of the substrate. *Biophys. J.* 79:144–152. doi:10.1016/S0006-3495(00)76279-5.

Lo, S.H., Q.C. Yu, L. Degenstein, L.B. Chen, and E. Fuchs. 1997. Progressive kidney degeneration in mice lacking tensin. *J. Cell Biol.* 136:1349–1361. doi:10.1083/jcb.136.6.1349.

Lv, H., L. Li, M. Sun, Y. Zhang, L. Chen, Y. Rong, and Y. Li. 2015. Mechanism of regulation of stem cell differentiation by matrix stiffness. *Stem Cell Res. Ther.* 6:103. doi:10.1186/s13287-015-0083-4.

Machacek, M., L. Hodgson, C. Welch, H. Elliott, O. Pertz, P. Nalbant, A. Abell, G.L. Johnson, K.M. Hahn, and G. Danuser. 2009. Coordination of Rho GTPase activities during cell protrusion. *Nature*. 461:99–103. doi:10.1038/nature08242.

Madsen, C.D., and N. Sidenius. 2008. The interaction between urokinase receptor and vitronectin in cell adhesion and signalling. *Eur. J. Cell Biol.* 87:617–629. doi:10.1016/j.ejcb.2008.02.003.

Malanchi, I., A. Santamaria-Martinez, E. Susanto, H. Peng, H.-A. Lehr, J.-F. Delaloye, and J. Huelsken. 2012. Abstract SY28-02: Interactions between cancer stem cells and their niche govern metastatic colonization. *Cancer Res.* 72:SY28-02-SY28-02. doi:10.1158/1538-7445.AM2012-SY28-02.

Maniotis, A.J., C.S. Chen, and D.E. Ingber. 1997. Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. *Proc. Natl. Acad. Sci. U. S. A.* 94:849–54. doi:10.1073/pnas.94.3.849.

Margadant, C., H.N. Monsuur, J.C. Norman, and A. Sonnenberg. 2011. Mechanisms of integrin activation and trafficking. *Curr. Opin. Cell Biol.* 23:607–14. doi:10.1016/j.ceb.2011.08.005.

Martin, A.C., M. Kaschube, and E.F. Wieschaus. 2009. Pulsed contractions of an actin-myosin network drive apical constriction. *Nature*. 457:495–9. doi:10.1038/nature07522.

Matthews, B.D., D.R. Overby, R. Mannix, and D.E. Ingber. 2006. Cellular adaptation to mechanical stress: role of integrins, Rho, cytoskeletal tension and mechanosensitive ion channels. *J. Cell Sci.* 119:508–18. doi:10.1242/jcs.02760.

McCleverty, C.J., D.C. Lin, and R.C. Liddington. 2007. Structure of the PTB domain of tensin1 and a model for its recruitment to fibrillar adhesions. *Protein Sci.* 16:1223–9. doi:10.1110/ps.072798707.

McMahon, H.T., and E. Boucrot. 2011. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nat Rev Mol Cell Biol*. 12:517–533. doi:10.1038/nrm3151.

Mellman, I., and Y. Yarden. 2013. Endocytosis and cancer. *Cold Spring Harb. Perspect. Biol.* 5:a016949. doi:10.1101/cshperspect.a016949.

Michael, K.E., D.W. Dumbauld, K.L. Burns, S.K. Hanks, and A.J. García. 2009. Focal adhesion kinase modulates cell adhesion strengthening via integrin activation. *Mol. Biol. Cell*. 20:2508–2519. doi:10.1091/mbc.E08-01-0076.

Mierke, C.T., B. Frey, M. Fellner, M. Herrmann, and B. Fabry. 2011. Integrin a5β1 facilitates cancer cell invasion through enhanced contractile forces. *J. Cell Sci.* 124:369–383. doi:10.1242/jcs.071985.

Miller, C.G., G. Budoff, J.L. Prenner, and J.E. Schwarzbauer. 2017. Minireview: Fibronectin in retinal disease. *Exp. Biol. Med.* 242:1–7. doi:10.1177/1535370216675245.

Millon-Frémillon, A., D. Bouvard, A. Grichine, S. Manet-Dupé, M.R. Block, and C. Albiges-Rizo. 2008. Cell adaptive response to extracellular matrix density is controlled by ICAP-1-dependent β1-integrin affinity. *J. Cell Biol.* 180:427–441. doi:10.1083/jcb.200707142.

Millon-Frémillon, A., M. Brunner, N. Abed, E. Collomb, A.S. Ribba, M.R. Block, C. Albiges□-Rizo, and D. Bouvard. 2013. Calcium and calmodulin-dependent serine/threonine protein kinase type II (CaMKII)-mediated intramolecular opening of integrin cytoplasmic domain-associated protein-1 (ICAP-1a)

negatively regulates β1 integrins. *J. Biol. Chem.* 288:20248–20260. doi:10.1074/jbc.M113.455956.

Milloud, R., O. Destaing, R. de Mets, I. Bourrin-Reynard, C. Oddou, A. Delon, I. Wang, C. Albigès-Rizo, and M. Balland. 2017. avβ3 integrins negatively regulate cellular forces by phosphorylation of its distal NPXY site. *Biol. Cell*. 109:127–137. doi:10.1111/boc.201600041.

Mitchison, T.J., G.T. Charras, and L. Mahadevan. 2008. Implications of a poroelastic cytoplasm for the dynamics of animal cell shape. *Semin. Cell Dev. Biol.* 19:215–223. doi:10.1016/j.semcdb.2008.01.008.

Miyamoto, M., S. Iwashita, S. Yamaguchi, and Y. Ono. 2009. Role of nm23 in the regulation of cell shape and migration via Rho family GTPase signals. *Mol. Cell. Biochem.* 329:175–179. doi:10.1007/s11010-009-0106-5.

Mogilner, A., and K. Keren. 2009. The Shape of Motile Cells. *Curr. Biol.* 19. doi:10.1016/j.cub.2009.06.053.

Monge, C., J. Almodóvar, T. Boudou, and C. Picart. 2015. Spatio-temporal control of LbL films for biomedical applications: From 2D to 3D. *Adv. Healthc. Mater.* 4:811–830. doi:10.1002/adhm.201400715.

Montanez, E., S. Ussar, M. Schifferer, M. Bösl, R. Zent, M. Moser, and R. Fässler. 2008. Kindlin-2 controls bidirectional signaling of integrins. *Genes Dev.* 22:1325–30. doi:10.1101/gad.469408.

Moore, S.W., P. Roca-Cusachs, and M.P. Sheetz. 2010. Stretchy proteins on stretchy substrates: The important elements of integrin-mediated rigidity sensing. *Dev. Cell*. 19:194–206. doi:10.1016/j.devcel.2010.07.018.

Morse, E.M., N.N. Brahme, and D.A. Calderwood. 2014. Integrin cytoplasmic tail interactions. *Biochemistry*. 53:810–820. doi:10.1021/bi401596q.

Mouw, J.K., G. Ou, and V.M. Weaver. 2014. Extracellular matrix assembly: a multiscale deconstruction. *Nat. Rev. Mol. Cell Biol.* 15:771–785. doi:10.1038/nrm3902.

Mulay, S.R., J. Desai, S. V. Kumar, J.N. Eberhard, D. Thomasova, S. Romoli, M. Grigorescu, O.P. Kulkarni, B. Popper, V. Vielhauer, G. Zuchtriegel, C. Reichel, J.H. Bräsen, P. Romagnani, R. Bilyy, L.E. Munoz, M. Herrmann, H. Liapis, S. Krautwald, A. Linkermann, and H.-J. Anders. 2016. Blebs lead the way: how to migrate without lamellipodia. *Nat. Commun.* 7:10274. doi:nrm2453 [pii]\r10.1038/nrm2453.

Muller, P.A.J., P.T. Caswell, B. Doyle, M.P. Iwanicki, E.H. Tan, S. Karim, N. Lukashchuk, D.A. Gillespie, R.L. Ludwig, P. Gosselin, A. Cromer, J.S. Brugge, O.J. Sansom, J.C. Norman, and K.H. Vousden. 2009. Mutant p53 Drives Invasion by Promoting Integrin Recycling. *Cell*. 139:1327–1341.

doi:10.1016/j.cell.2009.11.026.

Mullins, R.D., J. a Heuser, and T.D. Pollard. 1998. The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc. Natl. Acad. Sci. U. S. A.* 95:6181–6186. doi:10.1073/pnas.95.11.6181.

Murrell, M., P.W. Oakes, M. Lenz, and M.L. Gardel. 2015. Forcing cells into shape: the mechanics of actomyosin contractility. *Nat. Rev. Mol. Cell Biol.* 16:486–498. doi:10.1038/nrm4012.

Naba, A., K.R. Clauser, S. Hoersch, H. Liu, S.A. Carr, and R.O. Hynes. 2012. The Matrisome: In Silico Definition and In Vivo Characterization by Proteomics of Normal and Tumor Extracellular Matrices. *Mol. Cell. Proteomics*. 11:M111.014647-M111.014647. doi:10.1074/mcp.M111.014647.

Nallamothu, G., J.A. Woolworth, V. Dammai, and T. Hsu. 2008. Awd, the homolog of metastasis suppressor gene Nm23, regulates Drosophila epithelial cell invasion. *Mol. Cell. Biol.* 28:1964–73. doi:10.1128/MCB.01743-07.

Naumanen, P., P. Lappalainen, and P. Hotulainen. 2008. Mechanisms of actin stress fibre assembly. *In* Journal of Microscopy. 446–454.

Nelson, C.M., R.P. Jean, J.L. Tan, W.F. Liu, N.J. Sniadecki, A.A. Spector, and C.S. Chen. 2005. Emergent patterns of growth controlled by multicellular form and mechanics. *Proc. Natl. Acad. Sci. U. S. A.* 102:11594–9. doi:10.1073/pnas.0502575102.

Ng, D.H.J., J.D. Humphries, A. Byron, A. Millon-Frémillon, and M.J. Humphries. 2014. Microtubule-dependent modulation of adhesion complex composition. *PLoS One*. 9:1–24. doi:10.1371/journal.pone.0115213.

Nishimura, T., and K. Kaibuchi. 2007. Numb Controls Integrin Endocytosis for Directional Cell Migration with aPKC and PAR-3. *Dev. Cell*. 13:15–28. doi:10.1016/j.devcel.2007.05.003.

Oakes, P.W., D.C. Patel, N.A. Morin, D.P. Zitterbart, B. Fabry, J.S. Reichner, and J.X. Tang. 2009. Neutrophil morphology and migration are affected by substrate elasticity. *Blood*. 114:1387–1395. doi:10.1182/blood-2008-11-191445.

Ochsner, M., M. Textor, V. Vogel, and M.L. Smith. 2010. Dimensionality controls cytoskeleton assembly and metabolism of fibroblast cells in response to rigidity and shape. *PLoS One*. 5. doi:10.1371/journal.pone.0009445.

Oskarsson, T., S. Acharyya, X.H.-F. Zhang, S. Vanharanta, S.F. Tavazoie, P.G. Morris, R.J. Downey, K. Manova-Todorova, E. Brogi, and J. Massagué. 2011. Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nat. Med.* 17:867–874. doi:10.1038/nm.2379.

VI. References

Oxley, C.L., N.J. Anthis, E.D. Lowe, I. Vakonakis, I.D. Campbell, and K.L. Wegener. 2008. An integrin phosphorylation switch: The effect of β3 integrin tail phosphorylation on Dok1 and talin binding. *J. Biol. Chem.* 283:5420–5426. doi:10.1074/jbc.M709435200.

Paluch, E., C. Sykes, J. Prost, and M. Bornens. 2006. Dynamic modes of the cortical actomyosin gel during cell locomotion and division. *Trends Cell Biol.* 16:5–10. doi:10.1016/j.tcb.2005.11.003.

Pankov, R., E. Cukierman, B.Z. Katz, K. Matsumoto, D.C. Lin, S. Lin, C. Hahn, and K.M. Yamada. 2000. Integrin dynamics and matrix assembly: Tensin-dependent translocation of α5β1 integrins promotes early fibronectin fibrillogenesis. *J. Cell Biol.* 148:1075–1090. doi:10.1083/jcb.148.5.1075.

Pankov, R., and K.M. Yamada. 2002. Fibronectin at a glance. *J. Cell Sci.* 115:3861–3863. doi:10.1242/jcs.00059.

Parri, M., and P. Chiarugi. 2010. Rac and Rho GTPases in cancer cell motility control. *Cell Commun. Signal.* 8:23. doi:10.1186/1478-811X-8-23.

Parsons, J.T., A.R. Horwitz, and M. a Schwartz. 2010. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nat. Rev. Mol. Cell Biol.* 11:633–643. doi:10.1038/nrm2957.

Paszek, M.J., C.C. DuFort, O. Rossier, R. Bainer, J.K. Mouw, K. Godula, J.E. Hudak, J.N. Lakins, A.C. Wijekoon, L. Cassereau, M.G. Rubashkin, M.J. Magbanua, K.S. Thorn, M.W. Davidson, H.S. Rugo, J.W. Park, D.A. Hammer, G. Giannone, C.R. Bertozzi, and V.M. Weaver. 2014. The cancer glycocalyx mechanically primes integrin-mediated growth and survival. *Nature*. 511:319–325. doi:10.1038/nature13535.

Paszek, M.J., N. Zahir, K.R. Johnson, J.N. Lakins, G.I. Rozenberg, A. Gefen, C.A. Reinhart-King, S.S. Margulies, M. Dembo, D. Boettiger, D.A. Hammer, and V.M. Weaver. 2005. Tensional homeostasis and the malignant phenotype. *Cancer Cell*. 8:241–254. doi:10.1016/j.ccr.2005.08.010.

Paul, N.R., J.L. Allen, A. Chapman, M. Morlan-Mairal, E. Zindy, G. Jacquemet, L. Fernandez del Ama, N. Ferizovic, D.M. Green, J.D. Howe, E. Ehler, A. Hurlstone, and P.T. Caswell. 2015. α5β1 integrin recycling promotes Arp2/3-independent cancer cell invasion via the formin FHOD3. *J. Cell Biol.* 210:1013–1031. doi:10.1083/jcb.201502040.

Pelham, R.J., and Y.L. Wang. 1998. Cell locomotion and focal adhesions are regulated by the mechanical properties of the substrate. *Biol. Bull.* 194:348–350. doi:10.1073/pnas.95.20.13661.

Peyton, S.R., and A.J. Putnam. 2005. Extracellular matrix rigidity governs smooth muscle cell motility in a biphasic fashion. *J. Cell. Physiol.* 204:198–209.

doi:10.1002/jcp.20274.

Pietras, A., A.M. Katz, E.J. Ekström, B. Wee, J.J. Halliday, K.L. Pitter, J.L. Werbeck, N.M. Amankulor, J.T. Huse, and E.C. Holland. 2014. Osteopontin-CD44 signaling in the glioma perivascular niche enhances cancer stem cell phenotypes and promotes aggressive tumor growth. *Cell Stem Cell*. 14:357–369. doi:10.1016/j.stem.2014.01.005.

Plotnikov, S. V., A.M. Pasapera, B. Sabass, and C.M. Waterman. 2012. Force fluctuations within focal adhesions mediate ECM-rigidity sensing to guide directed cell migration. *Cell*. 151:1513–1527. doi:10.1016/j.cell.2012.11.034.

Pollard, T.D. 2016. Actin and actin-binding proteins. *Cold Spring Harb. Perspect. Biol.* 8. doi:10.1101/cshperspect.a018226.

Pollard, T.D., and G.G. Borisy. 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*. 112:453–465. doi:10.1016/S0092-8674(03)00120-X.

Psaila, B., and D. Lyden. 2009. The metastatic niche: adapting the foreign soil. *Nat. Rev. Cancer*. 9:285–293. doi:10.1038/nrc2621.

Puklin-Faucher, E., and M.P. Sheetz. 2009. The mechanical integrin cycle. *J. Cell Sci.* 122:179–186. doi:10.1242/jcs.049544.

Régent, M., E. Planus, A.P. Bouin, D. Bouvard, M. Brunner, E. Faurobert, A. Millon-Frémillon, M.R. Block, and C. Albiges-Rizo. 2011. Specificities of β1 integrin signaling in the control of cell adhesion and adhesive strength. *Eur. J. Cell Biol.* 90:261–269. doi:10.1016/j.ejcb.2010.09.006.

Ren, X.D., W.B. Kiosses, and M.A. Schwartz. 1999. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* 18:578–585. doi:10.1093/emboj/18.3.578.

Renkawitz, J., K. Schumann, M. Weber, T. Lämmermann, H. Pflicke, M. Piel, J. Polleux, J.P. Spatz, and M. Sixt. 2009. Adaptive force transmission in amoeboid cell migration. *Nat. Cell Biol.* 11:1438–1443. doi:10.1038/ncb1992.

Renz, M., C. Otten, E. Faurobert, F. Rudolph, Y. Zhu, G. Boulday, J. Duchene, M. Mickoleit, A.-C. Dietrich, C. Ramspacher, E. Steed, S. Manet-Dupé, A. Benz, D. Hassel, J. Vermot, J. Huisken, E. Tournier-Lasserve, U. Felbor, U. Sure, C. Albiges-Rizo, and S. Abdelilah-Seyfried. 2015. Regulation of β1 integrin-Klf2-mediated angiogenesis by CCM proteins. *Dev. Cell*. 32:181–90. doi:10.1016/j.devcel.2014.12.016.

Reticker-Flynn, N.E., D.F.B. Malta, M.M. Winslow, J.M. Lamar, M.J. Xu, G.H. Underhill, R.O. Hynes, T.E. Jacks, and S.N. Bhatia. 2012. A combinatorial extracellular matrix platform identifies cell-extracellular matrix interactions that correlate with metastasis. *Nat. Commun.* 3:1122. doi:10.1038/ncomms2128.

VI. References

Ridley, A.J., M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons, and A.R. Horwitz. 2003. Cell migration: integrating signals from front to back. *Science*. 302:1704–9. doi:10.1126/science.1092053.

Riento, K., and A.J. Ridley. 2003. Rocks: multifunctional kinases in cell behaviour. *Nat. Rev. Mol. Cell Biol.* 4:446–456. doi:10.1038/nrm1128.

del Rio, A., R. Perez-Jimenez, R. Liu, P. Roca-Cusachs, J.M. Fernandez, and M.P. Sheetz. 2009. Stretching single talin rod molecules activates vinculin binding. *Science*. 323:638–641. doi:10.1126/science.1162912.

Riveline, D., E. Zamir, N.Q. Balaban, U.S. Schwarz, T. Ishizaki, S. Narumiya, Z. Kam, B. Geiger, and A.D. Bershadsky. 2001. Focal contacts as mechanosensors: Externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. *J. Cell Biol.* 153:1175–1185. doi:10.1083/jcb.153.6.1175.

Robertson, J., G. Jacquemet, A. Byron, M.C. Jones, S. Warwood, J.N. Selley, D. Knight, J.D. Humphries, and M.J. Humphries. 2015. Defining the phospho-adhesome through the phosphoproteomic analysis of integrin signalling. *Nat. Commun.* 6:6265. doi:10.1038/ncomms7265.

Roca-Cusachs, P., N.C. Gauthier, A. Del Rio, and M.P. Sheetz. 2009. Clustering of alpha(5)beta(1) integrins determines adhesion strength whereas alpha(v)beta(3) and talin enable mechanotransduction. *Proc. Natl. Acad. Sci. U. S. A.* 106:16245–16250. doi:10.1073/pnas.0902818106.

Roca-Cusachs, P., A. del Rio, E. Puklin-Faucher, N.C. Gauthier, N. Biais, and M.P. Sheetz. 2013. Integrin-dependent force transmission to the extracellular matrix by -actinin triggers adhesion maturation. *Proc. Natl. Acad. Sci.* 110:E1361–E1370. doi:10.1073/pnas.1220723110.

Rossier, O., and G. Giannone. 2016. The journey of integrins and partners in a complex interactions landscape studied by super-resolution microscopy and single protein tracking. *Exp. Cell Res.* 343:28–34. doi:10.1016/j.yexcr.2015.11.004.

Rossier, O., V. Octeau, J.-B. Sibarita, C. Leduc, B. Tessier, D. Nair, V. Gatterdam, O. Destaing, C. Albigès-Rizo, R. Tampé, L. Cognet, D. Choquet, B. Lounis, and G. Giannone. 2012. Integrins β1 and β3 exhibit distinct dynamic nanoscale organizations inside focal adhesions. *Nat. Cell Biol.* 14:1057–1067. doi:10.1038/ncb2588.

Rottner, K., A. Hall, and J. V. Small. 1999. Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr. Biol.* 9:640–648. doi:10.1016/S0960-9822(99)80286-3.

Ruprecht, V., P. Monzo, A. Ravasio, Z. Yue, E. Makhija, P.O. Strale, N. Gauthier, G.

V. Shivashankar, V. Studer, C. Albiges-Rizo, and V. Viasnoff. 2017. How cells respond to environmental cues – insights from bio-functionalized substrates. *J. Cell Sci.* 130:51–61. doi:10.1242/jcs.196162.

Sabass, B., M.L. Gardel, C.M. Waterman, and U.S. Schwarz. 2008. High resolution traction force microscopy based on experimental and computational advances. *Biophys. J.* 94:207–220. doi:10.1529/biophysj.107.113670.

Saez, A., A. Buguin, P. Silberzan, and B. Ladoux. 2005. Is the Mechanical Activity of Epithelial Cells Controlled by Deformations or Forces? *Biophys. J.* 89:L52–L54. doi:10.1529/biophysj.105.071217.

Sahai, E., R. Garcia-Medina, J. Pouyssegur, and E. Vial. 2007. Smurf1 regulates tumor cell plasticity and motility through degradation of RhoA leading to localized inhibition of contractility. *J Cell Biol*. 176:35–42. doi:jcb.200605135 [pii]10.1083/jcb.200605135.

Salaita, K., P.M. Nair, R.S. Petit, R.M. Neve, D. Das, J.W. Gray, and J.T. Groves. 2010. Restriction of receptor movement alters cellular response: physical force sensing by EphA2. *Science*. 327:1380–1385. doi:10.1126/science.1181729.

Sawada, Y., and M.P. Sheetz. 2002. Force transduction by Triton cytoskeletons. *J. Cell Biol.* 156:609–615. doi:10.1083/jcb.200110068.

Sawada, Y., M. Tamada, B.J. Dubin-Thaler, O. Cherniavskaya, R. Sakai, S. Tanaka, and M.P. Sheetz. 2006. Force Sensing by Mechanical Extension of the Src Family Kinase Substrate p130Cas. *Cell*. 127:1015–1026. doi:10.1016/j.cell.2006.09.044.

Scales, T.M.E., and M. Parsons. 2011. Spatial and temporal regulation of integrin signalling during cell migration. *Curr. Opin. Cell Biol.* 23:562–568. doi:10.1016/j.ceb.2011.05.008.

Schaefer, A., M. Nethe, and P.L. Hordijk. 2012. Ubiquitin links to cytoskeletal dynamics, cell adhesion and migration. *Biochem. J.* 442:13–25. doi:10.1042/BJ20111815.

Schiller, H.B., C.C. Friedel, C. Boulegue, and R. Fässler. 2011a. Quantitative proteomics of the integrin adhesome show a myosin II-dependent recruitment of LIM domain proteins. *EMBO Rep.* 12:259–266. doi:10.1038/embor.2011.5.

Schiller, H.B., C.C. Friedel, C. Boulegue, and R. Fässler. 2011b. Quantitative proteomics of the integrin adhesome show a myosin II-dependent recruitment of LIM domain proteins. *EMBO Rep.* 12:259–266. doi:10.1038/embor.2011.5.

Schiller, H.B., M.-R. Hermann, J. Polleux, T. Vignaud, S. Zanivan, C.C. Friedel, Z. Sun, A. Raducanu, K.-E. Gottschalk, M. Théry, M. Mann, and R. Fässler. 2013. β1- and av-class integrins cooperate to regulate myosin II during rigidity sensing of fibronectin-based microenvironments. *Nat. Cell Biol.* 15:625–36.

doi:10.1038/ncb2747.

Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y.J.-Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, K. Liceiri, P. Tomancak, and C. A. 2012. Fiji: An open source platform for biological image analysis. *Nat. Methods*. 9:676–682. doi:10.1038/nmeth.2019.Fiji.

Schwartz, M.A. 2010. Integrins and extracellular matrix in mechanotransduction. *Cold Spring Harb. Perspect. Biol.* 2. doi:http://dx.doi.org/10.2147/CHC.S21829.

Schwarz, U.S., N.Q. Balaban, D. Riveline, A. Bershadsky, B. Geiger, and S.A. Safran. 2002. Calculation of Forces at Focal Adhesions from Elastic Substrate Data : The Effect of Localized Force and the Need for Regularization. *Biophys. J.* 83:1380–1394. doi:10.1016/S0006-3495(02)73909-X.

Scita, G., and P.P. Di Fiore. 2010. The endocytic matrix. *Nature*. 463:464–473. doi:10.1038/nature08910.

Serebriiskii, I., J. Estojak, G. Sonoda, J.R. Testa, and E. a Golemis. 1997. Association of Krev-1/rap1a with Krit1, a novel ankyrin repeat-containing protein encoded by a gene mapping to 7q21-22. *Oncogene*. 15:1043–1049. doi:10.1038/sj.onc.1201268.

Sharma, V.P., D. Entenberg, and J. Condeelis. 2013. High-Resolution Live-Cell Imaging and Time-Lapse Microscopy of Invadopodium Dynamics and Tracking Analysis. *In* Methods in molecular biology (Clifton, N.J.). 343–357.

Shattil, S.J., C. Kim, and M.H. Ginsberg. 2010. The final steps of integrin activation: the end game. *Nat. Rev. Mol. Cell Biol.* 11:288–300. doi:10.1038/nrm2871.

Sheetz, M.P. 2001. Cell control by membrane–cytoskeleton adhesion. *Nat. Rev. Mol. Cell Biol.* 2:392–396. doi:10.1038/35073095.

Sheetz, M.P., J.E. Sable, and H.G. Döbereiner. 2006. Continuous membrane-cytoskeleton adhesion requires continuous accommodation to lipid and cytoskeleton dynamics. *Annu Rev Biophys Biomol Struct*. 35:417–434. doi:10.1146/annurev.biophys.35.040405.102017.

Shi, F., and J. Sottile. 2008a. Caveolin-1-dependent 1 integrin endocytosis is a critical regulator of fibronectin turnover. *J. Cell Sci.* 121:2360–2371. doi:10.1242/jcs.014977.

Shi, F., and J. Sottile. 2008b. Caveolin-1-dependent beta1 integrin endocytosis is a critical regulator of fibronectin turnover. *J. Cell Sci.* 121:2360–71. doi:10.1242/jcs.014977.

Shibata, A.C.E., T.K. Fujiwara, L. Chen, K.G.N. Suzuki, Y. Ishikawa, Y.L. Nemoto, Y. Miwa, Z. Kalay, R. Chadda, K. Naruse, and A. Kusumi. 2012. Archipelago

architecture of the focal adhesion: Membrane molecules freely enter and exit from the focal adhesion zone. *Cytoskeleton*. 69:380–392. doi:10.1002/cm.21032.

Shroff, H., C.G. Galbraith, J.A. Galbraith, H. White, J. Gillette, S. Olenych, M.W. Davidson, and E. Betzig. 2007. Dual-color superresolution imaging of genetically expressed probes within individual adhesion complexes. *Proc. Natl. Acad. Sci.* 104:20308–20313. doi:10.1073/pnas.0710517105.

Smith, L., S. Cho, and D.E. Discher. 2017. Mechanosensing of matrix by stem cells: From matrix heterogeneity, contractility, and the nucleus in pore-migration to cardiogenesis and muscle stem cells in vivo. *Semin. Cell Dev. Biol.* doi:10.1016/j.semcdb.2017.05.025.

Stehbens, S., and T. Wittmann. 2012. Targeting and transport: how microtubules control focal adhesion dynamics. *J. Cell Biol.* 198:481–9. doi:10.1083/jcb.201206050.

Stehbens, S.J., M. Paszek, H. Pemble, A. Ettinger, S. Gierke, and T. Wittmann. 2014. CLASPs link focal-adhesion-associated microtubule capture to localized exocytosis and adhesion site turnover. *Nat. Cell Biol.* 16:561–573. doi:10.1038/ncb2975.

Stroeken, P.J.M., B. Alvarez, J. Van Rheenen, Y.M. Wijnands, D. Geerts, K. Jalink, and E. Roos. 2006. Integrin cytoplasmic domain-associated protein-1 (ICAP-1) interacts with the ROCK-I kinase at the plasma membrane. *J. Cell. Physiol.* 208:620–628. doi:10.1002/jcp.20699.

Stroka, K.M., and H. Aranda-Espinoza. 2009. Neutrophils display biphasic relationship between migration and substrate stiffness. *Cell Motil. Cytoskeleton*. 66:328–341. doi:10.1002/cm.20363.

Su, Y.-T., C. Gao, Y. Liu, S. Guo, A. Wang, B. Wang, H. Erdjument-Bromage, M. Miyagi, P. Tempst, and H.-Y. Kao. 2013. Monoubiquitination of filamin B regulates vascular endothelial growth factor-mediated trafficking of histone deacetylase 7. *Mol. Cell. Biol.* 33:1546–60. doi:10.1128/MCB.01146-12.

Tabdanov, E., N. Borghi, F. Brochard-Wyart, S. Dufour, and J.P. Thiery. 2009. Role of E-cadherin in membrane-cortex interaction probed by nanotube extrusion. *Biophys. J.* 96:2457–2465. doi:10.1016/j.bpj.2008.11.059.

Takagi, J., B.M. Petre, T. Walz, and T.A. Springer. 2002. Global conformational earrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell*. 110:599–611. doi:10.1016/S0092-8674(02)00935-2.

Takagi, J., K. Strokovich, T.A. Springer, and T. Walz. 2003. Structure of integrin a5β1 in complex with fibronectin. *EMBO J.* 22:4607–4615. doi:10.1093/emboj/cdg445.

VI. References

Takahashi, I., K. Onodera, Y. Sasano, I. Mizoguchi, J.-W. Bae, H. Mitani, M. Kagayama, and H. Mitani. 2003. Effect of stretching on gene expression of beta1 integrin and focal adhesion kinase and on chondrogenesis through cell-extracellular matrix interactions. *Eur. J. Cell Biol.* 82:182–192. doi:10.1078/0171-9335-00307.

Takala, H., E. Nurminen, S.M. Nurmi, M. Aatonen, T. Strandin, M. Takatalo, T. Kiema, C.G. Gahmberg, J. Ylänne, and S.C. Fagerholm. 2008. 22 integrin phosphorylation on Thr758 acts as a molecular switch to regulate 14-3-3 and filamin binding. *Blood*. 112:1853–1862. doi:10.1182/blood-2007-12-127795.

Tamkun, J.W., D.W. DeSimone, D. Fonda, R.S. Patel, C. Buck, A.F. Horwitz, and R.O. Hynes. 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell*. 46:271–282. doi:10.1016/0092-8674(86)90744-0.

Tang, C.-H., R.-S. Yang, T.-H. Huang, S.-H. Liu, and W.-M. Fu. 2004. Enhancement of fibronectin fibrillogenesis and bone formation by basic fibroblast growth factor via protein kinase C-dependent pathway in rat osteoblasts. *Mol. Pharmacol.* 66:440–9. doi:10.1124/mol.66.3.

Teckchandani, A., N. Toida, J. Goodchild, C. Henderson, J. Watts, B. Wollscheid, and J. a. Cooper. 2009. Quantitative proteomics identifies a Dab2/integrin module regulating cell migration. *J. Cell Biol.* 186:99–111. doi:10.1083/jcb.200812160.

Thery, M., and M. Bornens. 2006. Cell shape and cell division. *Curr Opin Cell Biol*. 18:648–657. doi:10.1016/j.ceb.2006.10.001.

Tilghman, R.W., C.R. Cowan, J.D. Mih, Y. Koryakina, D. Gioeli, J.K. Slack-Davis, B.R. Blackman, D.J. Tschumperlin, and J.T. Parsons. 2010. Matrix rigidity regulates cancer cell growth and cellular phenotype. *PLoS One*. 5. doi:10.1371/journal.pone.0012905.

Tomar, A., and D.D. Schlaepfer. 2009. Focal adhesion kinase: switching between GAPs and GEFs in the regulation of cell motility. *Curr. Opin. Cell Biol.* 21:676–683. doi:10.1016/j.ceb.2009.05.006.

Totsukawa, G., Y. Wu, Y. Sasaki, D.J. Hartshorne, Y. Yamakita, S. Yamashiro, and F. Matsumura. 2004. Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts. *J. Cell Biol.* 164:427–439. doi:10.1083/jcb.200306172.

Totsukawa, G., Y. Yamakita, S. Yamashiro, D.J. Hartshorne, Y. Sasaki, and F. Matsumura. 2000. Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J. Cell Biol.* 150:797–806. doi:10.1083/jcb.150.4.797.

Tse, J.R., and A.J. Engler. 2010. Preparation of hydrogel substrates with tunable mechanical properties. *Curr. Protoc. Cell Biol.* doi:10.1002/0471143030.cb1016s47.

Ulrich, T.A., E.M. De Juan Pardo, and S. Kumar. 2009. The mechanical rigidity of the extracellular matrix regulates the structure, motility, and proliferation of glioma cells. *Cancer Res.* 69:4167–4174. doi:10.1158/0008-5472.CAN-08-4859.

Upla, P., V. Marjomäki, P. Kankaanpaa, J. Ivaska, T. Hyypiä, V.D.G. F.G., and J. Heino. 2004. Clustering Induces a Lateral Redistribution of 2 1 Integrin from Membrane Rafts to Caveolae and Subsequent Protein Kinase C-dependent Internalization. *Mol. Biol. Cell*. 15:625–636. doi:10.1091/mbc.E03-08-0588.

Valdembri, D., and G. Serini. 2012. Regulation of adhesion site dynamics by integrin traffic. *Curr. Opin. Cell Biol.* 24:582–591. doi:10.1016/j.ceb.2012.08.004.

Vicente-Manzanares, M., J. Zareno, L. Whitmore, C.K. Choi, and A.F. Horwitz. 2007. Regulation of protrusion, adhesion dynamics, and polarity by myosins IIA and IIB in migrating cells. *J. Cell Biol.* 176:573–580. doi:10.1083/jcb.200612043.

De Vries, W.N., A. V Evsikov, B.E. Haac, K.S. Fancher, A.E. Holbrook, R. Kemler, D. Solter, and B.B. Knowles. 2004. Maternal beta-catenin and E-cadherin in mouse development. *Development*. 131:4435–4445. doi:10.1242/dev.01316.

Wang, H.-R., Y. Zhang, B. Ozdamar, A.A. Ogunjimi, E. Alexandrova, G.H. Thomsen, and J.L. Wrana. 2003. Regulation of Cell Polarity and Protrusion Formation by Targeting RhoA for Degradation. *Science (80-.).* 302:1775–1779. doi:10.1126/science.1090772.

Wang, H.B., M. Dembo, S.K. Hanks, and Y. Wang. 2001. Focal adhesion kinase is involved in mechanosensing during fibroblast migration. *Proc. Natl. Acad. Sci. U. S. A.* 98:11295–300. doi:10.1073/pnas.201201198.

Wang, H.B., M. Dembo, Y.L. Wang, S. Ghassemi, G. Meacci, S. Liu, A. a Gondarenko, A. Mathur, P. Roca-cusachs, M.P. Sheetz, J. Hone, J.L. Leight, M. a Wozniak, S. Chen, M.L. Lynch, and S. Christopher. 2013. Substrate flexibility regulates growth and apoptosis of normal but not transformed cells. *Am. J. Physiol. Cell Physiol.* 279:C1345--50. doi:11029281.

Wang, N., J.D. Tytell, and D.E. Ingber. 2009. Mechanotransduction at a distance: mechanically coupling the extracellular matrix with the nucleus. *Nat. Rev. Mol. Cell Biol.* 10:75–82. doi:10.1038/nrm2594.

Webb, D.J., K. Donais, L.A. Whitmore, S.M. Thomas, C.E. Turner, J.T. Parsons, and A.F. Horwitz. 2004. FAK–Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat. Cell Biol.* 6:154–161. doi:10.1038/ncb1094.

Webb, D.J., J.T. Parsons, and A.F. Horwitz. 2002. Adhesion assembly, disassembly and turnover in migrating cells – over and over and over again. *Nat. Cell Biol.*

4:E97–E100. doi:10.1038/ncb0402-e97.

Wehrle-Haller, B. 2012. Assembly and disassembly of cell matrix adhesions. *Curr. Opin. Cell Biol.* 24:569–81. doi:10.1016/j.ceb.2012.06.010.

White, D.P., P.T. Caswell, and J.C. Norman. 2007. avβ3 and a5β1 integrin recycling pathways dictate downstream Rho kinase signaling to regulate persistent cell migration. *J. Cell Biol.* 177:515–525. doi:10.1083/jcb.200609004.

von Wichert, G., G. Jiang, A. Kostic, K. De Vos, J. Sap, and M.P. Sheetz. 2003. RPTP-alpha acts as a transducer of mechanical force on alphav/beta3-integrin-cytoskeleton linkages. *J. Cell Biol.* 161:143–53. doi:10.1083/jcb.200211061.

Wirtz, D., K. Konstantopoulos, and P.C. Searson. 2011. The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nat. Rev. Cancer*. 11:512–522. doi:10.1038/nrc3080.

Wiseman, P.W., C.M. Brown, D.J. Webb, B. Hebert, N.L. Johnson, J. a Squier, M.H. Ellisman, and a F. Horwitz. 2004. Spatial mapping of integrin interactions and dynamics during cell migration by image correlation microscopy. *J. Cell Sci.* 117:5521–5534. doi:10.1242/jcs.01416.

Worth, D.C., K. Hodivala-Dilke, S.D. Robinson, S.J. King, P.E. Morton, F.B. Gertler, M.J. Humphries, and M. Parsons. 2010. avβ3 integrin spatially regulates VASP and RIAM to control adhesion dynamics and migration. *J. Cell Biol.* 189:369–383. doi:10.1083/jcb.200912014.

Wu, C., A.J. Fields, B.A. Kapteijn, and J.A. McDonald. 1995. The role of alpha 4 beta 1 integrin in cell motility and fibronectin matrix assembly. *J. Cell Sci.* 108 (Pt 2:821–9.

Xie, C., J. Zhu, X. Chen, L. Mi, N. Nishida, and T.A. Springer. 2010. Structure of an integrin with an alphaI domain, complement receptor type 4. *EMBO J.* 29:666–79. doi:10.1038/emboj.2009.367.

Xiong, J.-P. 2001. Crystal Structure of the Extracellular Segment of Integrin alpha Vbeta 3. *Science (80-.).* 294:339–345. doi:10.1126/science.1064535.

Xiong, J.-P., T. Stehle, R. Zhang, A. Joachimiak, M. Frech, S.L. Goodman, and M.A. Arnaout. 2002. Crystal structure of the extracellular segment of integrin alpha Vbeta3 in complex with an Arg-Gly-Asp ligand. *Science*. 296:151–155. doi:10.1126/science.1069040.

Yamazaki, D., S. Kurisu, and T. Takenawa. 2009. Involvement of Rac and Rho signaling in cancer cell motility in 3D substrates. *Oncogene*. 28:1570–1583. doi:10.1038/onc.2009.2.

Yap, C.C., and B. Winckler. 2015. Adapting for endocytosis: roles for endocytic sorting adaptors in directing neural development. *Front. Cell. Neurosci.* 9:119.

doi:10.3389/fncel.2015.00119.

Ye, F., G. Hu, D. Taylor, B. Ratnikov, A.A. Bobkov, M.A. McLean, S.G. Sligar, K.A. Taylor, and M.H. Ginsberg. 2010. Recreation of the terminal events in physiological integrin activation. *J. Cell Biol.* 188:157–173. doi:10.1083/jcb.200908045.

Ye, F., A.K. Snider, and M.H. Ginsberg. 2014. Talin and kindlin: the one-two punch in integrin activation. *Front. Med.* 8:6–16. doi:10.1007/s11684-014-0317-3.

Yeung, T., P.C. Georges, L. a. Flanagan, B. Marg, M. Ortiz, M. Funaki, N. Zahir, W. Ming, V. Weaver, and P. a. Janmey. 2005. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil. Cytoskeleton*. 60:24–34. doi:10.1002/cm.20041.

Yu, C., N.B.M. Rafiq, F. Cao, Y. Zhou, A. Krishnasamy, K.H. Biswas, A. Ravasio, Z. Chen, Y.-H. Wang, K. Kawauchi, G.E. Jones, and M.P. Sheetz. 2015. Integrin-beta3 clusters recruit clathrin-mediated endocytic machinery in the absence of traction force. *Nat. Commun.* 6:8672. doi:10.1038/ncomms9672.

Yusko, E.C., and C.L. Asbury. 2014. Force is a signal that cells cannot ignore. *Mol. Biol. Cell*. 25:3717–25. doi:10.1091/mbc.E13-12-0707.

Zaari, N., P. Rajagopalan, S.K. Kim, A.J. Engler, and J.Y. Wong. 2004. Photopolymerization in microfluidic gradient generators: Microscale control of substrate compliance to manipulate cell response. *Adv. Mater.* 16:2133–2137. doi:10.1002/adma.200400883.

Zaidel-Bar, R., and B. Geiger. 2010. The switchable integrin adhesome. *J. Cell Sci.* 123:1385–1388. doi:10.1242/jcs.066183.

Zaidel-Bar, R., S. Itzkovitz, A. Ma'ayan, R. Iyengar, and B. Geiger. 2007. Functional atlas of the integrin adhesome. *Nat. Cell Biol.* 9:858–867. doi:10.1038/ncb0807-858.

Zamir, E., B.Z. Katz, S. Aota, K.M. Yamada, B. Geiger, and Z. Kam. 1999. Molecular diversity of cell-matrix adhesions. *J. Cell Sci.* 112 (pt11):1655–1669.

Zamir, E., M. Katz, Y. Posen, N. Erez, K.M. Yamada, B.Z. Katz, S. Lin, D.C. Lin, A. Bershadsky, Z. Kam, and B. Geiger. 2000a. Dynamics and segregation of cell-matrix adhesions in cultured fibroblasts. *Nat. Cell Biol.* 2:191–6. doi:10.1038/35008607.

Zamir, E., M. Katz, Y. Posen, N. Erez, K.M. Yamada, B.Z. Katz, S. Lin, D.C. Lin, a Bershadsky, Z. Kam, and B. Geiger. 2000b. Dynamics and segregation of cell-matrix adhesions in cultured fibroblasts. *Nat. Cell Biol.* 2:191–196. doi:10.1038/35008607.

Zawistowski, J.S., I.G. Serebriiskii, M.F. Lee, E. a Golemis, and D. a Marchuk. 2002.

KRIT1 association with the integrin-binding protein ICAP-1: a new direction in the elucidation of cerebral cavernous malformations (CCM1) pathogenesis. *Hum. Mol. Genet.* 11:389–396.

Zhang, H., F. Landmann, H. Zahreddine, D. Rodriguez, M. Koch, and M. Labouesse. 2011. A tension-induced mechanotransduction pathway promotes epithelial morphogenesis. *Nature*. 471:99–103. doi:10.1038/nature09765.

Zhang, J., R.E. Clatterbuck, D. Rigamonti, D.D. Chang, and H.C. Dietz. 2001. Interaction between krit1 and icap1alpha infers perturbation of integrin beta1-mediated angiogenesis in the pathogenesis of cerebral cavernous malformation. *Hum. Mol. Genet.* 10:2953–2960.

Zhang, X.A., and M.E. Hemler. 1999a. Interaction of the integrin β1 cytoplasmic domain with ICAP-1 protein. *J. Biol. Chem.* 274:11–19. doi:10.1074/jbc.274.1.11.

Zhang, X.A., and M.E. Hemler. 1999b. Interaction of the integrin beta1 cytoplasmic domain with ICAP-1 protein. *J. Biol. Chem.* 274:11–9.

Zhang, X., G. Jiang, Y. Cai, S.J. Monkley, D.R. Critchley, and M.P. Sheetz. 2008. Talin depletion reveals independence of initial cell spreading from integrin activation and traction. *Nat. Cell Biol.* 10:1062–1068. doi:10.1038/ncb1765.

Zhong, C., M. Chrzanowska-Wodnicka, J. Brown, A. Shaub, A.M. Belkin, and K. Burridge. 1998. Rho-mediated contractility exposes a cryptic site in fibronectin and induces fibronectin matrix assembly. *J. Cell Biol.* 141:539–551. doi:10.1083/jcb.141.2.539.

Zhu, J., B.-H. Luo, T. Xiao, C. Zhang, N. Nishida, and T.A. Springer. 2008a. Structure of a complete integrin ectodomain in a physiologic resting state and activation and deactivation by applied forces. *Mol. Cell*. 32:849–61. doi:10.1016/j.molcel.2008.11.018.

Zhu, J., B.H. Luo, T. Xiao, C. Zhang, N. Nishida, and T.A. Springer. 2008b. Structure of a Complete Integrin Ectodomain in a Physiologic Resting State and Activation and Deactivation by Applied Forces. *Mol. Cell*. 32:849–861. doi:10.1016/j.molcel.2008.11.018.

Zschenker, O., T. Streichert, S. Hehlgans, and N. Cordes. 2012. Genome-wide gene expression analysis in cancer cells reveals 3D growth to affect ECM and processes associated with cell adhesion but not DNA repair. *PLoS One*. 7. doi:10.1371/journal.pone.0034279.

VI. References