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INTEGRIN ACTIVITY REGULATION IN MECHANOSENSING

Integrin β1 activity and extracellular matrix crosstalk

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To my family

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Faculty of Medicine
Medical Biochemistry and Genetics
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MARTINA LERCHE: Integrin activity regulation in mechanosensing
Doctoral Dissertation, 215 pp.
Turku Doctoral Programme of Molecular Medicine
September 2021

ABSTRACT

Cell functions are highly dependent on the physical properties of the extracellular matrix (ECM). Integrins are transmembrane receptors providing the main molecular link that attaches cells to the ECM. The distinct biochemical and mechanical properties of integrins enable them to act as bi-directional signalling-hubs. By probing the ECM stiffness via integrin adhesions, cells respond to the rigidity of their environment by tuning proliferation, gene expression and even their epigenetic landscape. Furthermore, integrin adhesions allow cells to exert actomyosin-generated force on the ECM, enabling processes such as cell migration and ECM remodelling. Consequently, integrin activity needs to be thoroughly regulated on several levels. While the role of integrins as key cellular mechanosensors is well established, the link between mechanosensing and integrin activity regulation by integrin inactivators and newly synthesized integrins, is poorly understood. In this thesis, I have identified the integrin activity inhibitor SHARPIN as an important regulator of ECM remodelling in the developing mammary gland. By regulating the levels of the collagen-binding integrin $\alpha 11\beta 1$, SHARPIN affects the force generation capacity of mammary gland stromal fibroblasts and thereby their ability to remodel and assemble the ECM to support normal mammary gland development. Furthermore, I have investigated the trafficking and localization of newly synthesized integrin $\alpha 5\beta 1$. My results show that newly synthesized integrins are trafficked in a polarized manner to the tip of adhesions where they contribute to adhesion growth in a ligand-dependent manner. In addition, we find that a subset of newly synthesized integrin $\alpha 5$ can undergo unconventional secretion, to be rapidly trafficked to cell adhesions. Together, these results provide novel insights of how the crosstalk between the ECM and integrin activity regulates cell behaviour, and may be essential in understanding the pathological cell migration and ECM remodelling events in cancer.

KEYWORDS: Integrin, SHARPIN, ECM, mechanotransduction, adhesion

TIIVISTELMÄ

Soluväliaineen fyysiset ominaisuudet vaikuttavat eri solutoimintoihin, kuten solun liikkumiseen ja solunjakautumiseen. Integriinit läpäisevät solukalvon, tarjoavat soluille mekanismin sitoutua soluväliaineeseen ja toimivat kaksisuuntaisina signaalinvälittäjinä. Tunnustelemalla soluväliaineen jäykkyyttä integriinien kautta, solut reagoivat jäykkyyteen säätelemällä solunjakautumista, geenien ilmentymistä ja jopa epigeneettistä tilaa. Integriini-adheesiot välittävät myös solujen aktiini-tukirangan tuottamat voimat soluväliaineeseen, täten mahdollistaen toimintoja kuten solujen liikkumista ja soluväliaineen uudelleenmuokkaamista. Integriinien aktii-visuus ja siitä riippuvainen voimansiirto ovat tästä johtuen olennaisia solu-toiminnoissa, jotka liittyvät sekä yksilönkehitykseen että erilaisiin sairauksiin. Tästä syystä, integriinien aktiivisuus on tarkasti säädeltyä monella eri tasolla. Vaikka integriinien toiminta mekaanisina antureina tunnetaan hyvin, voimansiirron ja integriinien aktiivisuussäätelyn välinen yhteys tunnetaan huonosti. Tässä väitös-kirjatutkimuksessa olen tunnistanut integriiniaktiivisuusinhibiittorin SHARPIN:in tärkeänä säätelijänä soluväliaineen muokkaamisessa rintarauhasen kehityksessä. Säätelemällä kollageenia sitovan integriini α11β1 tasoja, SHARPIN vaikuttaa rintarauhasen tukikudossolujen voimaansiirtokapasiteettiin ja täten niiden kykyyn muokata soluväliainetta kehittyvässä rintarauhasessa. Lisäksi olen tutkinut vasta tuotettujen integriini α5β1-reseptorien kalvokuljetusta ja kohdentamista soluissa. Tulokseni osoittavat, että vasta tuotettujen integriinien kalvokuljetus on polari-soitunutta siten, että nämä integriinit lokalisoituvat adheesioiden päihin, edesauttaen adheesioiden kasvua ligandi-riippuvaisella tavalla. Osa vastatuotetuista integrii-neistä erittyy myös epätavanomaisesti solun pinnalle ohittaen Golgi-välitteisen erityksen kulkeutuen nopeasti adheesioihin. Väitöskirjani löydökset tarjoavat uutta tietoa integriinien ja soluväliaineen vuorovaikutuksesta ja näiden osuudesta solutoiminnan säätelyssä. Nämä löydökset saattavat auttaa paremmin ymmärtämään syövässä esiintyviä tautimekanismeja, kuten soluliikkumista ja soluväliaineen muokkaamista.

AVAINSANAT: Integriini, SHARPIN, ECM, voimansiirto, adheesio

Table of Contents

Abbreviations

ADMIDAS	adjacent to MIDAS
AFM	atomic force microscopy
Arp2/3	Actin related protein 2/3 complex
BMP-1	bone morphogenetic protein 1
BSA	bovine serum albumin
CCS	clathrin-coated structures
CDM	cell derived matrix
CO2	carbon dioxide
DAB	diaminobenzidine
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
EMCDD	electron-multiplying charged coupled device
EMT	epithelial to mesenchymal transition
ER	endoplasmic reticulum
F-actin	filamentous actin
FA	focal adhesion
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
FAT	focal adhesion targeting
FCS	fetal calf serum
FERM	4.1, ezrin, radixin and moesin
FN	fibronectin
FSP	fibroblast-specific protein
GAP	GTPase activating proteins
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein

GPCR	G protein-coupled receptor
GPI-AP	glycosylphosphatidylinositol-anchored proteins
GRASP	Golgi reassembling stacking proteins
GTP	guanosine triphosphate
HE	hematoxylin and eosin
HGF	hepatocyte growth factor
HRP	horseradish peroxidase
HRS	horse serum
IAC	integrin adhesion complex
ICAM	intercellular adhesion molecule
ICAP1	integrin cytoplasmic domain-associated protein 1
IF	immuno fluorescence
IGF2	insulin growth factor 2
IHC	immunohistochemistry
IL-2	interleukin-2
IRS-p53	insulin-receptor substrate p53
LAD	leucocyte adhesion deficiency syndromes
LAP	latency associated peptide
LINC	linker of nucleosceleton and cytoskeleton complex
LOX	lysyl oxidase
LSM	laser scanning microscope
LUBAC	linear ubiquitination assembly complex
MAPK	mitogen-activated protein kinase
MDGI	mammary-derived growth inhibitor
MEC	mammary epithelial cells
MEF	mouse embryonic fibroblast
MIDAS	metal ion dependent adhesion site
MLC	myosin light-chain
MMP	matrix metalloproteases
MRTF-A	myocardin-related transcription factor A
MSF	mammary gland stromal fibroblasts
NF-κβ	nuclear factor-κβ
PAA	polyacrylamide
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PFA	paraformaldehyde
PIP2	phosphatidylinositol (4,5)-bisphosphate
PLC	phospholipase C
PM	plasma membrane
pMLC	phospho myosin light chain

PSI   plexin-semaphorin-integrin
PTK2   protein tyrosine kinase 2
RIAM   RAS-related protein 1- Rap1-interacting molecule
RNA   ribonucleic acid
ROCK   rho-associated protein kinase
RUSH   retention using selective hooks
SBP   streptavidin binding peptide
SHANK   SH3 and multiple ankyrin repeat domains proteins
SHARPIN   Shank-associated RH domain protein
SHG   second harmonic generation
TEB   terminal end bud
TFM   traction force microscopy
TGF-β1   transforming growth factor β1
TIRF   total internal reflection fluorescence microscopy
UPS   unconventional protein secretion
VEGF   vascular endothelial growth factor
VEGFR2   vascular endothelial growth factor receptor 2
YAP   Yes-associated protein

List of Original Publications

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

I   Peuhu E., Kaukonen R.*, Lerche M.*, Saari M., Guzmán C., Rantakari P., De Franceschi N., Wärri A., Georgiadou M, Jacquemet G., Mattila E., Virtakoivu R., Liu Y., Attie Y, Silva K. A., Betz T., Sundberg J. P., Salmi M., Deugnier M-A., Eliceiri K. and Ivaska J. SHARPIN regulates collagen architecture and ductal outgrowth in the developing mouse mammary gland. *The EMBO Journal*, 2017; 36(2):165–182

II   Lerche M., Elosegui-Artola A., Kechagia J.Z., Guzmán C., Georgiadou M., Andreu I., Gullberg D., Roca-Cusachs P., Peuhu E[#]. and Ivaska J.[#]. Integrin binding dynamics modulate ligand-specific mechanosensing in mammary gland fibroblasts. *iScience*, 2020; 23(3):100907

III   Lerche M., Jacquemet G., Perez F. Boncompain G., Miserey-Lenkei S. and Ivaska J. Ligand-specific delivery of newly synthesized integrins regulates focal adhesions. *Manuscript*

* equal contribution
\# shared corresponding author

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1   Introduction

Animals are eukaryotes composed of up to trillions of cells. Together with the extracellular matrix (ECM), cells are the building blocks of tissue. Different kinds of tissues associate together to create the various organs that form organ systems, which in turn group together to form organisms such as human beings. As animals and their environment constantly undergo changes, also the cells and the ECM need to be dynamic to maintain a functioning organism. For timely and appropriate changes to take place, cells need to communicate with both other cells and their environment, and change their behaviour according to the received information. Cell communication is mediated by receptors, which are proteins that can be found both inside and on the surface of cells. These receptors receive signals in forms of ligands that acts as signalling molecules. When a ligand binds to a receptor it "hands over" information and the receptor can become activated and initiate a cellular response based on the received information. This cellular response can give rise to numerous different changes that in turn can modify both the cell and its surrounding and ultimately give rise to changes in the organism.

Integrins are transmembrane receptors that upon activation can recruit other intracellular proteins to form adhesion complexes. These adhesions serve as bi-directional signalling hubs and function as a physical contact site between the cell and the surrounding ECM. Signalling to and from the adhesions allows the cell to attach, remodel and move (migrate) within the ECM in a controlled manner. These processes all require physical forces to be connected and transmitted between the cell and its environment. Integrin receptors allows for these processes by connecting the intracellular actin cytoskeleton (the cell's skeleton and muscle) to the cell ECM. Integrin adhesions contain a variety of mechanosensitive molecules, which can undergo conformational changes in response to force. These force-induced conformational changes are converted into biochemical signalling events and give rise to changes in cell behaviour. This process, where cells sense the physical properties of their surrounding and alter their behaviour according to it, is called mechanotransduction.

The ECM undergoes changes throughout the animal life. Especially during developmental processes, the ECM is extensively deposited and remodelled to meet

the requirements of the next phase of the animal life. As the ECM affects integrin activity and integrin signalling reciprocally affects the ECM jointly regulating cell behaviour, deregulated integrin activity can lead to numerous pathologies such as fibrosis and cancer. By understanding how integrin activity regulates the ECM, and vice versa, we can learn more about the crosstalk between cells and their microenvironment in developmental and disease processes.

While the role of integrin activators in regulating ECM crosstalk have been studied to a great extent, the role of integrin activity inhibitors and newly synthesized integrins regulating these processes have been studied to a lesser degree. The aims of this study were to i) investigate the role of the integrin activity inhibitor SHARPIN in regulating mechanotransduction ii) investigate the role of ECM-crosstalk in regulating the activity of newly synthesized integrin. Here, I have studied the role of SHARPIN in modulating mechanotransduction in primary mammary gland stromal fibroblast. Furthermore, I have investigated how the altered mechanotransduction translates into disturbed ECM remodelling and mammary gland development in SHARPIN deficient mice. In addition, I have examined how the properties of the ECM can regulate integrin activity by influencing trafficking and maturation of newly synthesized integrins. The results presented in this thesis will expand our knowledge of integrin activity regulation in mechanotransduction and provide novel comprehension of the crosstalk between ECM and integrins.

2 Review of the Literature

2.1 Integrin receptors

Integrins are transmembrane receptors mediating cell contact to the extracellular matrix (ECM) and to cell counter receptors (Brakebusch, Fässler 2003). Since the classification of the integrin family of receptors in 1987 (R. Hynes 1987), 18 alpha subunits and 8 beta subunits have been identified in the metazoans. Integrins function as heterodimers and, to this day, the different subunits are known to form 24 different heterodimer pairs (in vertebrates) that each recognize, and bind to one or more extracellular ligand. As integrins are able to couple the extracellular ligands to the intracellular cytoskeleton and signalling proteins, integrins function as bidirectional signalling machines mediating transmembrane mechanical and biochemical signals. This enables cells to exert force and remodel their extracellular environment, during processes such as wound healing, migration and development, but also allows cells to respond to the environmental cues by activating signalling pathways to regulate key functions such as proliferation, survival and transcription (R. O. Hynes 2002).

2.1.1 A brief history of integrin receptors

During the 1970´s, scientists understood that there had to be something connecting the actin cytoskeleton to the extracellular matrix (ECM). Several studies provided evidence of intracellular fibres being physically connected to extracellular fibrils and the idea of a protein mediating this connection emerged. However, the identification and characterization of the integrin family was a long process. The main reason for this is that cells use many different receptors for cell-matrix adhesions, and attempts to block these adhesions therefore raised non-specific effects when non-integrin receptors also were blocked. Furthermore, many of the integrin ligands are large extracellular matrix molecules that in addition to integrins also bind several other molecules (Barczyk, Carracedo et al. 2010, R. O. Hynes 2004). The eventual identification of integrins was a result of multiple independent investigations taking place during this same time-period. Dissection of the fibronectin structure eventually led to the identification of the RGDS tetrapeptide sequence as the minimal cell-binding sequence (Pierschbacher, Ruoslahti 1984a). It was later discovered that the

RGD was the actual minimal cell-binding sequence as the substitution of the Serine to certain other amino acids also allowed cell attachment (Pierschbacher, Ruoslahti 1984b). As a result of the RGD sequence identification, the Ruoslahti group was furthermore able to discover several RGD binding proteins (Pytela, Pierschbacher et al. 1985a, Pytela, Pierschbacher et al. 1985b). In addition, optimization of RGD affinity chromatography protocols also led to the discovery that the manganese ion (Mn^{2+}) increases integrin activity (Gailit, Ruoslahti 1988). During the same time, two antibodies that blocked cell adhesion were discovered (Neff, Lowrey et al. 1982, Greve, Gottlieb 1982) and immunofluorescence images of these showed antigen alignment with both actin and fibronectin, which started a quest of "the fibronectin receptor" (Chen, Hasegawa et al. 1985, Damsky, Knudsen et al. 1985, R. O. Hynes 2004). These findings led to the isolation, characterization and sequence of the chick integrin β1 subunit encoding cDNA (Tamkun, DeSimone et al. 1986). Around the same time, several other groups were investigating and identifying different cell surface receptors involved in cell adhesions (Leptin, Aebersold et al. 1987, Springer, Miller et al. 1986, Hemler, Jacobson et al. 1985). The platelet membrane glycoproteins GPIIb and GPIIIa were for example found to be reduced or absent in platelets from Glanzmann thrombasthenia (bleeding disorder) patients. Furthermore, these glycoproteins were also found to form Ca^{2+}-dependent heterodimers in a 1:1 ratio and to be important for platelet aggregation and fibrinogen binding (Coller, Peerschke et al. 1983, Calvete 1995). It later became clear that these cell surface adhesion receptors were all related to the fibronectin receptor, and in 1987 the cell adhesion receptor family of integrins was described for the first time (R. Hynes 1987). The protein name originates from the structure revealing it being an integral membrane protein and the presumption (at the time) of the receptors integrating function of the actin cytoskeleton and the ECM, which later turned out to be accurate. During the following years, it was discovered that these proteins also serve as bidirectional signalling receptors (R. O. Hynes 1992). Since the classification of the integrin family of receptors in 1987, 18 alpha subunits and 8 beta subunits have been identified in the metazoans (R. O. Hynes 2002) and during the last decade an average of over 2000 integrin papers are published per year.

2.1.2   Integrin structure

Integrins function as heterodimers composed of one α- and β-subunit that associate via non-covalent bonds. Both subunits consist of several domains with flexible linkers between them. The different domains construct a receptor with a large extracellular "head" domain, containing a ligand-binding pocket, a transmembrane domain and two short cytoplasmic tails. The cytoplasmic tails of the α- and β-subunits bind to various intracellular adaptor proteins, some of which mediate a link

to the actin cytoskeleton or downstream signal transduction. The ectodomain of β-subunits consists of a β I-domain, inserted in a hybrid domain that in turn is inserted in a plexin-semaphorin-integrin (PSI) domain. These interconnected domains are followed by four epidermal growth factor (EGF) domains. The extracellular domain of α-subunits contains a β-propeller, a thigh and two calf-domains. In addition to these, half of the integrin α-subunits also have an α I-domain inserted in the β-propeller and for these integrins the ligand binding site resides solely in the α I-domain (Campbell, Humphries 2011, K. Zhang, Chen 2012). For the other integrins the ligand-binding site is formed jointly by the α- and the β-subunit ectodomains.

Integrins can exist in different conformational states that vary in ligand affinities. Generally, integrins are classified into three different conformational states: a bent "inactive state", a "primed state" and an extended, ligand-bound "active state" (Li, Su et al. 2017). In the inactive state, the extracellular integrin headpiece is bent down towards the membrane and interacts with a large interface of the integrin leg pieces. The α- and β-subunits' lower legs and transmembrane regions are connected, and the cytoplasmic tails associated with each other, which further stabilizes the bent structure. In this bent conformation, the ligand-binding domain is both in a low affinity state and in an unfavourable position for ligand interaction (Moore, Aaron et al. 2018, Park, Yuki et al. 2015). Many integrins are located at the plasma membrane in an "off-state", unable to bind ligands, until they undergo conformational changes that affects their ligand binding affinity. Especially divalent cation-induced conformational changes in the integrin I-domains are important for increasing the affinity towards the ligand (Campbell, Humphries 2011). Both the α and the β I-domains contain a metal ion-dependent adhesion site (MIDAS). In addition, the β I-domain also contains an adjacent to MIDAS (ADMIDAS) and a synergistic metal ion-binding site (SyMBS) (K. Zhang, Chen 2012). These sites are important binding sites for divalent cations and regulate interactions with the integrin ligands by affecting the receptor conformation (K. L. Brown, Banerjee et al. 2018). Calcium binds to MIDAS already in the endoplasmic reticulum (ER) and keeps the receptor in an inactive bent conformation until it is located to the cell membrane (Tiwari, Askari et al. 2011). At the cell surface, the extracellular cations Mg^{2+} and Mn^{2+} can replace the Ca^{2+}, which allows ligand binding to the receptor (K. Zhang, Chen 2012). In the primed state, also referred to as the extended closed state, the integrin headpiece is extended, with a favourable orientation for ligand binding, but the lower legs and transmembrane regions are still associated, which only allows low-affinity binding of the headpiece to the ligand (Sun, Costell et al. 2019). For integrins to become fully activated, the lower legs, the transmembrane regions and the cytoplasmic tails need to dissociate, allowing the head piece to undergo conformational changes that allows ligand binding with a >1000-fold higher affinity compared to the closed conformations (Li, Springer 2017).

2.1.3   Integrin activity regulation

Integrin activity can be regulated both through inside-out and outside-in signalling. External stimuli, such as ligand binding and mechanical stress, can induce clustering of activated integrins, which results in internal phosphorylation events of molecules such as Focal adhesion kinase and Src Family of Kinases, which induce downstream signalling (Mitra, Hanson et al. 2005, Mitra, Schlaepfer 2006, Welf, Naik et al. 2012). This process, where the environment of the cell induces integrin signalling and gives rise to a cell response, is called outside-in signalling. In contrast, various intracellular signalling cascades, such as signalling via growth factor receptors or G-protein coupled receptors, can promote binding of the integrin adaptor proteins talin and kindlin to the intracellular tail of integrins. Binding of these adaptor proteins to the intracellular integrin beta tail, results in a conformational change in the integrin head and a subsequent increased affinity for the ligand, allowing integrin binding to available ligands. This process is called inside-out signalling. Integrin-mediated downstream signalling events can be divided into three different temporal stages: the immediate response, the short-term effect, and the long-term effect. The immediate response comprises integrin binding to an immobilized ligand, resulting in integrin activation and phosphorylation events that affects the integrin-ECM link itself. These responses will in turn give rise to short-term effects that induces reorganization of the actin cytoskeleton. Finally, this reorganization of the cytoskeleton can generate long-term effects by activating signalling pathways or genetic programs that alter cell behaviour (Legate, Wickström et al. 2009).

Talin is a 270 kDa cytoplasmic and mechanosensitive protein with a C-terminal rod domain containing several vinculin and actin binding sites that can be revealed upon mechanical stretch. The talin N-terminal head consisting of four (F0-F3) subdomains, of which F1-F3 makes up the protein 4.1, ezrin, radixin and moesin (FERM) domain. The FERM domain contains binding sites both for filamentous actin (F-actin) and for the cytoplasmic tail of β integrins (Critchley, Gingras 2008). In its resting state, talin is in a closed and auto-inhibited conformation with the FERM domain interacting with the rod domain. Upon activation, by e.g. chemokines or phosphatidylinositol 4,5-bisphosphate (PIP2) at the plasma membrane, talin becomes extended and the FERM domain is now free to bind to the plasma membrane localised active small GTPase Rap1, which is a key regulator of talin recruitment to integrins (J. Yang, Zhu et al. 2014, L. Zhu, Yang et al. 2017, Camp, Haage et al. 2018). Talin FERM domain binds also to the membrane-proximal NPXY motif in the integrin β cytoplasmic tail. This binding leads to the dissociation of the integrin α- and β cytoplasmic tails and integrin activation (Jia-huai Wang 2012). If talin is simultaneously bound to the actin cytoskeleton, intracellular forces can be coupled to the ECM and sustained cell adhesion and cell spreading can take place (X. Zhang, Jiang et al. 2008).

Another important intracellular integrin activator is kindlin. Although kindlin also contains a FERM domain, it binds to a membrane-distal NPXY motif of the integrin β cytoplasmic tail and does thus not compete, but instead co-operates with talin to induce integrin activation. Kindlin cannot cause the separation of the two integrin tails, but by binding to integrin it promotes clustering of activated integrins and thereby promotes cell adhesion (Park et al. 2015). Kindlin also serves as a protein-protein interaction hub by recruiting adaptor proteins such as actin related protein (Arp2/3) and paxillin to the membrane that further initiate focal adhesion formation (Sun et al. 2019).

In addition to integrin activators, also integrin activity inhibitors are able to bind to the cytoplasmic tails of integrins. Docking protein 1 (Dok1), filamin and integrin cytoplasmic domain-associated protein 1 (ICAP1) have been identified as integrin β binding activity inhibitors (Pouwels, Nevo et al. 2012). Dok1 is a tyrosine rich cytoplasmic protein that is substrate for several tyrosine kinases (Yamanashi, Baltimore 1997). Dok1 binds to integrin β1, β2, β3, and β7 and inhibits their activation by competing with talin (Bachmann, Kukkurainen et al. 2019, Oxley, Anthis et al. 2008, Wegener, Partridge et al. 2007). In contrast, mammary-derived growth inhibitor (MDGI) and Shank-associated RH domain protein (SHARPIN) bind to the integrin α-subunits and stabilize the low-affinity conformation of the receptor (Pouwels et al. 2012). SHARPIN is a widely expressed 45 kDa-sized protein first identified in the rat brain where it interacts with SHANK-proteins via its C-terminal RH-domain (Lim, Sala et al. 2001). Since its discovery, SHARPIN has been found to be involved in several pathways that regulate the immune response, for instance by regulating T-cell receptor signalling and by serving as a component of the linear ubiquitination assembly complex (LUBAC) and thereby regulating nuclear factor-κβ (NF-κβ) signalling (Gerlach, Cordier et al. 2011, Redecke, Chaturvedi et al. 2016). In addition, SHARPIN inhibits integrin β1-activation by binding directly to the conserved region of the integrin α-subunit cytoplasmic tail and indirectly preventing integrin activators such as kindlin and talin from accessing the β1-subunit. Thus, its function as an integrin inactivator relies on its ability to keep integrins in their bent, inactive conformation (Rantala, Pouwels et al. 2011).

Also posttranslational modifications of integrins affect their activity. T cells that circulate in the blood need to be able to switch between inactive and active integrin states quickly. This fast switch in the activation state of the circulating cells, allows for adhesion to both other cells and to the endothelium when they encounter either antigen presenting cells or sites of inflammation. Fast activation is enabled by a high surface expression of inactive β2 integrins and a phosphorylation dependent activity switch in these unique cells. T cell receptor stimulation induces phosphorylation of integrin β2, which abrogates filamin binding but promotes binding of the 14-3-3 adaptor proteins. As filamin is an integrin activity inhibitor, while 14-3-3 proteins

are required for integrin β2 ligand binding to ICAM-1, this allows for a fast activation switch to mediate cell adhesion (Nurmi, Autero et al. 2007).

In addition to the conformational state of integrins, also the amount of integrins available for ligand binding at the plasma membrane affects cell behaviour, and thus needs to be modulated in a spatio-temporal manner. One way for cells to do so is to regulate the expression of integrins. Endothelial cells in the blood vessels, owing to their unique location, are constantly exposed to shear stress emanating from the blood flow. Upon shear stress, cells upregulate both their mRNA and protein expression of integrin α5β1, which results in stronger adhesions that are able to withstand the increased mechanical forces required for cell adhesion (Urbich, Walter et al. 2000). Integrins activity is also regulated by growth factors. Transforming growth factor β1 (TGF-β1) is known to increase the expression and cell surface levels of integrin β1 and several integrin α subunits as well as the expression of fibronectin and collagen I (Heino, Ignotz et al. 1989). Furthermore, by tuning the expression levels of specific αβ1 integrins, TGF-β1 can modulate the balance of active integrin heterodimers at the cell surface and thus cell behaviour. In osteogenic cells, TGF-β1 increases the levels of integrin α2β1, which increases the cell capability of collagen contraction (Riikonen, Koivisto et al. 1995). The importance of regulating cell surface availability of specific integrin heterodimers is highlighted by the collagen-binding integrins α2β1 and α1β1. While both integrins bind to collagen, they have opposite effects on collagen expression. Integrin α2β1 can induce collagen expression, whereas integrin α1β1 mediates downregulation of collagen expression (Ivaska, Reunanen et al. 1999, Langholz, Röckel et al. 1995).

Integrins and adhesions are important regulators of the exocytic machinery by capturing and stabilizing microtubules to adhesion sites via the cortical microtubule complex. By doing so, integrins regulate the traffic and release of newly synthesized proteins, such as ECM proteins, from the trans-Golgi network to areas in close proximity to adhesions at the plasma membrane (Nolte, Nolte-'t Hoen, Esther N. M. et al. 2021, Fourriere, Kasri et al. 2019). The targeted ECM protein exocytosis is especially important in blood platelets that need to adhere to sites of vascular injury and rapidly create strong adhesions to withstand the mechanical force from the blood flow. Initial adhesion to the endothelia, mediates localized secretion of adhesive matrix proteins such as fibronectin and fibrinogen in close proximity to these adhesion sites. This localized secretion promotes further integrin binding, platelet spreading and clot formation (Sakurai, Fitch-Tewfik et al. 2015, Nolte et al. 2021). In addition, cytotoxic T cells are known to release cytotoxic granules upon antigen stimulation to mediate killing of target cells. As mentioned before, integrin β2 activation in T cells is essential for adhesion to antigen presenting cells, but also for targeting the release of cytotoxic vesicles to these integrin β2 mediated adhesion sites. Engagement of integrin αLβ2 rearranges the cytoskeleton of the T cell to

promote polarization of the Golgi and the cytotoxic granules towards the adhesion sites. This polarization and subsequent targeting and release of granules to the vicinity of T-cell-target cell adhesion ensures effective killing of target cells while minimizing the off-target effects of the cytotoxic granules on the host tissue (Anikeeva, Somersalo et al. 2005).

Endocytosis of integrins is another way of regulating integrin receptor availability at the plasma membrane, and both active and inactive integrins undergo continuous endocytosis. While some integrins are targeted for degradation, a large fraction is trafficked back to the plasma membrane to permit assembly of new adhesions. In a soft environment, mammary epithelial cells decrease their integrin β1 expression in a stiffness-correlating manner by promoting endocytosis and lysosomal degradation of integrins. This process is dependent on the lipid raft protein caveolin-1 and its phosphorylation state (Yeh, Ling et al. 2017). Non-phosphorylated caveolin-1 localizes to caveolae in the retracting part of cells where they mediate endocytosis, while phosphorylated caveolin-1 is relocalized to protruding areas of cells to mediate polarized cell migration by stabilizing lipid rafts at focal adhesion (Parat, Anand-Apte et al. 2003, Salanueva, Cerezo et al. 2007). On stiff substrates, integrin β1 induces Src-mediated phosphorylation of caveolin-1, which further stabilizes lipid rafts at focal adhesions. As substrate stiffness and actomyosin contractility decreases, also the level of caveolin-1 phosphorylation reduces, which allows integrin endocytosis and adhesion disassembly (Salanueva et al. 2007, Yeh et al. 2017). Integrin endocytosis and recycling is especially important in migrating cells that need to both assemble and disassemble adhesions during this process (Paul, Jacquemet et al. 2015). In addition to endocytosis, caveolae can function to guide cell migration in a stiffness-dependent manner. When cells migrate up a stiffness gradient, in a 3D environment, the low membrane-tension at the cell rear promotes caveolae formation at the retracting rear of the cell. Caveolae can in turn recruit a RhoA GEF to activate RhoA that organizes F-actin contractility needed for rear-retraction (Hetmanski, de Belly et al. 2019). Microtubule-mediated protein delivery have also a crucial role in the process of adhesion turnover as it provides delivery of matrix metalloproteases to adhesion sites and thus allows for disruption of integrin-ECM connections (Stehbens, Paszek et al. 2014). In addition, microtubules provide adhesions with proteins such as Numb, that are important for internalization and endocytosis of the (released) integrins. Numb localizes to clathrin-coated structures and binds directly to several cargo proteins, including integrins, and links them to the endocytic machinery. In migrating cells, Numb localizes at the cell front close to focal adhesions and engages with inactive integrins (not talin bound), residing in regions closely behind the lamellipodia to drive endocytosis and recycling of these unengaged integrins (Nishimura, Kaibuchi 2007). Inactive integrins have been shown to undergo rapid recycling via the short-loop recycling pathway in an F-actin-

and Rab4-dependent manner and to be targeted to protrusions at the plasma membrane (Arjonen, Alanko et al. 2012). Together this suggests that internalized inactive integrins located behind the lamellipodia are rapidly recycled to protrusions in the leading edge to facilitate cell migration. The recycling kinetics of integrins can also be influenced by growth factor receptors and kinases. Platelet-derived growth factor (PDGF) stimulates rapid recycling of integrin αvβ3 to newly forming adhesions in a process that depends on association of the kinase PDK1 and the cytoplasmic tail of integrin β3 (Roberts, Barry et al. 2001, Woods, White et al. 2004). Another protein involved in clathrin-mediated endocytosis of integrins is Dab2. Dab2 localizes to clathrin-coated pits and mediates bulk endocytosis and recycling of inactive integrins to maintain an intracellular pool of integrins that can be recycled to the leading edge of the cell to assist in adhesion assembly and cell migration (Teckchandani, Toida et al. 2009). While ligand-bound active integrins have a high net endocytosis rate compared to inactive integrins, recycling of these integrins is slower and some of these ligand-bound integrins are targeted for lysosomal degradation (Arjonen et al. 2012, Lobert, Brech et al. 2010). In contrast, active but non-ligand bound integrins can be endocytosed together with talin and the adhesion kinases FAK and Src. This co-endocytosis, allows integrins to remain in the active conformation and associated to talin during endocytosis and recycling. Once recycled, these active talin-bound integrins contribute to adhesion assembly at the leading edge of cells and allows for polarized adhesion formation and migration (Nader, Ezratty et al. 2016). Alternatively, active and ligand-fragment bound integrins can recruit FAK to the endosomes to induces anoikis-suppression inside-in signalling in fibroblasts and cancer cells (Alanko, Mai et al. 2015). Taken together, integrin activity can regulate endocytosis and recycling of integrins to promote assembly and disassembly of adhesions. Furthermore, integrin-mediated adhesions are involved in organizing targeted exocytosis needed for various integrin-dependent processes such as ECM remodelling, cell migration and immune responses. However, whether integrin activity also contributes to localized delivery of newly synthesized integrins is still not known.

To conclude, integrin activity is regulated at several levels, which allows for spatio-temporal cell behaviour. Furthermore, the regulation of integrin activity depends on both extracellular and intracellular signals and extensive crosstalk between other signalling pathways and integrin activity.

2.1.4 Integrin heterodimers and ligands

The ligand binding site of integrins is formed in an interface between the β-propeller of the integrin α-subunit and the βI-domain of the integrin β-subunit. Integrin α-subunits that contain an αI-domain (α1, α2, α10, α11, αL, αM, αX and αD) mediate

ligand binding either exclusively via this domain or in addition to the β-propeller, together with the β-subunit (Luo, Carman et al. 2007). The 24 integrin heterodimers, are generally divided into four families based on their ligand specificity: the laminin-binding, collagen-binding, RGD-recognizing integrins and leukocyte integrins (illustrated in Figure 1) (Takada, Ye et al. 2007). Here, a few examples of integrin-ligand interactions from each subfamily are discussed as well as the consequent cellular response.

2.1.4.1   Leukocyte specific integrins

Leukocytes are part of the immune system and circulate in the blood from where they are recruited to sites of injury or inflammation, to participate in immune functions. This process includes leukocyte adhesion to the endothelium, activation of leukocytes that results in slow rolling and adhesion, leukocyte crawling on the endothelium and finally transmigration of the leukocytes through the endothelium. Several molecular players are involved in this process such as selectins, glycoproteins and integrins (Mitroulis, Alexaki et al. 2015). While there are several different type of leukocytes, they all express at least one integrin β2 heterodimer. In addition to these integrins, leukocytes also express other members of the integrin receptor family in a time- and signal-dependent manner. The leukocyte specific receptors are thus αLβ2, αMβ2, αXβ2 and αDβ2. Leukocyte receptor ligands belong to the intercellular adhesion molecule (ICAM) family. While a general receptor binding sequence has not been identified in ICAMs, they all present acidic residues that are positioned in flexible loops, which enables them to coordinate Mg^{2+} and Mn^{2+} ions on the integrin receptor thus allowing receptor-ligand interaction. (E. S. Harris, McIntyre et al. 2000).

Integrins at the cell surface of circulating leukocyte are kept in an inactive quiescent state that allows for circulation of leukocytes. Upon inflammation, leukocytes arrest and integrins are activated which allows for leukocyte adhesion and transendothelial migration. This process is highly controlled and called the leukocyte adhesion cascade. The process is initiated by inflammation, which induces an enhanced expression of selectins at the luminal or apical membrane of endothelial cells. Selectins bind to and interact with leukocytes in the blood flow, which will progressively slow down and start rolling on the endothelium. The selectin interaction activates various intracellular signalling of leukocytes that co-operate with chemokine signals detected from the inflamed endothelia to trigger integrin activation in an inside-out manner. As an example, G protein-coupled receptors (GPCRs) on leukocytes bind to chemokines, which stimulate these receptors causing rapid activation of phospholipase C (PLC). This results in elevated intracellular Ca^{2+} levels and production of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate.

Elevated Ca^{2+} and DAG activate the small GTPase RAS-related protein 1- Rap1-interacting molecule (Rap1-RIAM) complex. Activated Rap1-RIAM can then interact with talin and mediate talin-induced inside-out activation of integrins (Zarbock, Kempf et al. 2012, Katagiri, Kinashi 2012, Patsoukis, Bardhan et al. 2017). Activation of integrins leads to binding to ICAM, which mediates slow rolling and outside-in activation of integrins leading to cell spreading and further adhesion maturation via Src signalling (discussed later). Once arrested, leukocytes crawl on the endothelia in an integrin β2-dependent process called locomotion, which allows leukocytes to identify a proper site for transmigration of the endothelial monolayer. Transmigration is the final step of the leukocyte arrest cycle and depends on integrin αLβ2 and αMβ2 interacting with their ligands ICAM-1 and ICAM-2 (Mitroulis et al. 2015). The importance of integrin β2 in the immune system is highlighted in patients with leucocyte adhesion deficiency syndromes (LAD). Leukocytes from these patients express no, or highly reduced surface levels of the integrin β2 receptor and display severe adhesion and motility defects, which causes recurrent severe bacterial and fungal infections in the patients (E. S. Harris et al. 2000, Etzioni, Frydman et al. 1992).

2.1.4.2   Laminin-binding integrins

Integrin-mediated cell adhesion to laminin gives rise to cellular responses that differ from responses mediated by other ECM-binding integrins. While adhesion to collagen and fibronectin can generate formation of large focal adhesions and fibrillar adhesions connected to stress fibres (discussed in section 2.2), laminin-mediated adhesions are generally smaller and connected to fewer actin stress fibres. As the ligand-binding affinities for laminin isoforms range from 1–20 nM and thus do not vary much from the dissociation constant reported for the integrin-fibronectin affinity, the functional differences between laminin-binding and fibronectin-binding integrins is most likely due to variations in the activated downstream signalling pathways (Stipp 2010, Nishiuchi, Takagi et al. 2006, Takagi, Strokovich et al. 2003). In line with this, laminin-mediated adhesion results in activation of Rac, which promotes cell migration. In contrast, fibronectin-mediated adhesions preferentially activates RhoA that enhances stress fibre assembly and focal adhesion formation (discussed later), suggesting a distinct ligand-receptor dependent activation of Rho family GTPases to dictate cell responses (Gu, Sumida et al. 2001). Laminins are trimeric glycoproteins composed of an α, β and γ chain and the expression of the individual laminin chains is tissue and cell type-specific. In mammals, 5 α, 3 β and 3 γ chain have been identified and together they give rise to 16 different isoforms (Yamada, Sekiguchi 2015). Laminins are ECM proteins and are the major components of the basement membrane. Integrin α3β1, α6β1, α6β4, and α7β1

associate with the C-terminal domain of laminins (Yamada, Sekiguchi 2015). The laminin ligand specificity and affinity varies considerably among these integrins, and the activation of these different receptors also generates different signalling activities (Ramovs, te Molder et al. 2017). In addition to these classical laminin-binding integrins, also the collagen-binding integrins α1β1 and α2β1 recognize and bind to specific laminins via the N-terminal domains of laminin (Stipp 2010, Yamada, Sekiguchi 2015). Like laminins, integrin heterodimers also exhibit an expression pattern that is cell type and tissue-specific. While knockout of integrin β1 is lethal as it is expressed throughout the body and has several different binding α-subunit partners (Fässler, Meyer 1995) mutations of different α-subunits are not necessarily as severe due to their limited expression and redundancy between some integrin α-subunits. Integrin α7β1 is mainly expressed in skeletal and cardiac muscle, and thus mutations in integrin α7 give rise to muscle defects (Hayashi, Chou et al. 1998). In contrast, integrin α3β1, α6β1 and α6β4 mediate adhesion of epithelial cells to the laminins in the basement membrane and are mostly expressed in the skin, stomach, lung, kidney, intestine and bladder (Ramovs et al. 2017). In the mammary gland epithelia, integrin α3β1, α6β1 and α6β4 are expressed and crucial for regulating cell polarity and alveogenesis during pregnancy (Romagnoli, Bresson et al. 2020). Furthermore, laminin is required for induction of lactogenic differentiation (Streuli, Schmidhauser et al. 1995) and integrin β1 has been shown to be crucial for controlling cell proliferation and development of the mammary gland (Faraldo, Deugnier et al. 1998).

Integrin α6β4 is an exceptional integrin, as its β4 subunit consists of a long cytoplasmic tail, in contrast to the other integrin subunits´ short cytoplasmic tails. This long β4 tail contains a signalling domain that is able to bind to keratins, which is required for hemidesmosome assembly (Spinardi, Ren et al. 1993). Integrin α6β4 is mainly expressed in epithelial cells were it locates to the basal side of the cells and bind laminin in the ECM and mediates stable adhesions to the basal membrane via hemidesmosome formation. The distinct structure of integrin α6β4 further allows for nucleation of the cytokeratin and basal lamina connection, via its interactions to plectin, BP230 and collagen XVII (Stewart, O'Connor 2015). The importance of these stable hemidesmosomal connections can be seen in epidermolysis bullosa patients, where mutations in the gene encoding for integrin β4 causes alteration in integrin α6β4 protein expression and hemidesmosomal dysfunction resulting in severe skin blistering (Pulkkinen, Kim et al. 1998). Likewise, mouse models with depletion of either subunit of this integrin give rise to severe skin defects and perinatal death as a result of the hemidesmosomal dysfunctions (Ramovs et al. 2017, Georges-Labouesse, Messaddeq et al. 1996). During wound closure, hemidesmosomes dissociate to allow cell migration and α6β4 is translocated to lamellipodia and filopodia where it instead binds to the actin cytoskeleton and

mediates cell migration. This process is induced by growth factor signalling and direct phosphorylation of the β4 tail (Stewart, O'Connor 2015). Upon activation, these multiple phosphorylation sites can act as docking sites for adaptor proteins to activate downstream pathways such as PI3K and MAPK that regulate cell fate decisions such as proliferation and survival (Ramovs et al. 2017). While this process is controlled in normal cells, deregulated integrin α6β4 activity is observed in several cancers where it promotes tumour cell proliferation, invasion and metastasis (Stewart, O'Connor 2015). In addition, integrin α6β4 can associate with receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and ErbB2. Furthermore, association of integrin α6β4 to ErbB2 amplifies its signalling ability to enable mammary tumour progression (Guo, Pylayeva et al. 2006). A similar amplifying effect by integrin α6β4 can be seen for c-Met, a tyrosine kinase receptor for hepatocyte growth factor (HGF), where activation of c-MET by HGF stimulation leads to phosphorylation of the integrin β4 tail. This phosphorylation leads to recruitment of adaptor proteins, which can enhance the HGF-induced signalling by c-MET (Trusolino, Bertotti et al. 2001, Organ, Tsao 2011)

2.1.4.3   RGD-recognizing integrins

The RGD-binding integrins α5β1, α8β1, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8 and αIIbβ3 all share the ability to recognize ligands containing the arginine-glycine-aspartic acid (RGD) sequence. The RGD ligands bind to an interface between the β-propeller of the integrin α-subunit and the I-domain of the integrin β-subunit (Kononova, Litvinov et al. 2017). Because of its small size, RGD sites can easily be reproduced with peptides. These peptides can promote cell adhesion when coated onto a surface, or prevent adhesion if introduced to cells in a suspension as they then block the ligand-binding site of integrins. RGD sequences can be found in thousands of proteins but since the RGD sequence is not always available at the protein surface for integrin binding, only a fraction of these proteins mediates integrin binding. Some well-known RGD-containing integrin ligands are fibronectin, fibrinogen, vitronectin, von Willebrand factor, thrombospondin, laminin, and under some conditions, collagens (Ruoslahti 1996). The RGD-binding integrins are the most undiscriminating ones of the integrin receptor family with several integrins having multiple binding partners that they also share with other integrins from this subfamily. However, the ligand affinity can vary significantly between different integrins due to the differences in the fitting of the ligand to the integrin α-β binding pocket (Humphries, Byron et al. 2006). Furthermore, the cellular response mediated by integrins also varies depending on the specific ligand-integrin interaction (Tobias G. Kapp, Florian Rechenmacher et al. 2017). All RGD-binding integrins except for αvβ5 and αvβ8 bind to fibronectin, and in addition, integrin α4β1 is capable to bind

fibronectin in an RGD-independent manner via the V-region of fibronectin (Takada et al. 2007, Sechler, Cumiskey et al. 2000).

Fibronectin (FN) is broadly expressed by multiple cell types and is abundantly found in the blood plasma. In addition, FN is an important part of the ECM where it not only provides elastic structure to the ECM and an attachment site for cells, but also regulates collagen architecture and acts as the key regulator of intra- and extracellular communication via integrin receptors to regulate multiple cell activities. FN is secreted as a dimer made up of monomers that contains three different types of repeating units called FN repeats. One FN monomer contains 12 type I repeats, two type II repeats and 15-17 type III repeats that together make up for about 90 % of the protein sequence (Pankov, Yamada 2002). The FN repeats comprise functional domains that mediate FN self-assembly, cell surface receptor binding and interactions with other ECM components (Y. Mao, Schwarzbauer 2005). The RGD motifs can be found in the Type III repeats. In addition to binding integrins, fibronectin also binds to other ECM molecules such as collagen, heparin and fibrin. Even though only one gene encodes for fibronectin in humans, as many as 20 different variants of FN can be found due to alternative splicing of the pre-mRNA. The solubility of FN varies, and the proteins can therefore be sub-divided into plasma FN (pFN) which is soluble, and cellular FN (cFN) which is less soluble. While pFN has a relative simple splicing pattern and is predominantly produced by hepatocytes in the liver, cFN is produced by many cell types such as fibroblasts, endothelial cells and myocytes, and therefore more heterogenous due to cell-type specific splicing patterns. Furthermore, the cFN can self-associate into aggregates and fibrils that are insoluble and part of the ECM. These fibrils can further be assembled by cells into fibrillar networks in an integrin-dependent process. This process is called FN fibrillogenesis or FN matrix assembly. As cell-type specific splicing patterns generates FN molecules that differ in receptor and ligand-binding affinities and solubility, cells are able to alter their ECM composition in a tissue and developmental-specific manner (Pankov, Yamada 2002, Y. Mao, Schwarzbauer 2005). The matrix assembly process is a step-wise and complex process that involves binding domains from all three FN repeats and interactions with both other FN molecules and integrins. The process is initiated when newly secreted FN dimers bind to integrin α5β1 located in focal adhesions. These integrins exert force on the FN dimer, by sliding towards the cell body along actin bundles, to unfold the dimer (Pankov, Cukierman et al. 2000). While this process is primarily induced by integrin α5β1, also α4β1 and αvβ3 integrins are involved under activated circumstances (Sechler et al. 2000, Danen, Sonneveld et al. 2002). When force is exerted on the integrin α5β1 receptor, it can undergo a mechanical switch that has a higher receptor-ligand bond strength (i.e. catch-bond, discussed later). This switch is dependent on the engagement of the FN synergy site, which resides in close proximity to the RGD

site. Upon integrin α5β1 binding to the synergy site, FAK activation increases (Friedland, Lee et al. 2009), which allows for increased tension on the FN molecule. This stretch induces unfolding of FN and exposes FN binding sites to allow FN self-assembly and fibril formation. Fibrils are further stabilized inside fibrillar networks via noncovalent interactions giving rise to an insoluble FN matrix. As both α4β1 and αvβ3 lack the ability of binding to the synergy site but are capable of initiating fibrillogenesis after manipulated FAK activation, it is thought that fibrillogenesis is induced by the high tension mediated by integrin α5β1 (Gudzenko, Franz 2015, Singh, Carraher et al. 2010). In addition, integrin adhesion activity is needed also for matrix maintenance as perturbation of Src kinase activity (discussed later) after matrix assembly causes a reduction of FN incorporation into stable matrix (Wierzbicka-Patynowski, Mao et al. 2007). Degradation and removal of ECM proteins is crucial for ECM homeostasis and involved in several processes such as tissue repair and development. ECM degradation is thought to occur via ECM protein degradation by enzymes and via cell internalization and degradation. While many different proteases contribute to ECM degradation, the matrix metalloproteinases are the major ECM degradation enzymes. These enzymes can further be divided into a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) and matrix metalloproteinase (MMPS) families (P. Lu, Takai et al. 2011). Integrin β1 clustering at the invasive front of cells induces polarized exocytosis of MMPs, which induces matrix degradation in front of the cell, enabling cell migration. In addition, integrin α5β1 has been shown to mediate endocytosis of matrix bound FN. Integrin-fibronectin interactions are thus important regulators of ECM turnover and migration (Shi, Sottile 2008, Bravo-Cordero, Marrero-Diaz et al. 2007).

Integrin αIIbβ3 is the most abundantly expressed integrin in platelets and up to 80 000 receptors can be found on the cell surface. In resting platelets, these integrins are in an inactive bent conformation. As a result of tissue injury, sub endothelial ECM becomes exposed and allows for integrin dependent adhesion and activation of blood platelets (Grüner, Prostredna et al. 2003). Platelets are also activated via other non-integrin platelet receptors (e.g. protease-activated receptors). This causes inside-out activation of integrin αIIbβ3 via proteins like kindlin and talin, which induces a conformational switch of the receptor that allows effective binding to soluble fibrinogen and other ligands. Integrin αIIbβ3 fibrinogen-binding by adjacent platelets causes aggregation of platelets and outside-in signalling of integrins mediated by phosphorylation events (discussed later). The outside-in signalling then enables essential platelet functions such as fibrinogen assembly, cell spreading and aggregation (Durrant, van den Bosch, Marion T. et al. 2017, Huang, Li et al. 2019). Mutations in either ITGA2B or ITGB3 leading to defects in integrin αIIbβ3 expression can be seen in Glanzmann thrombasthenia, a severe bleeding disorder

where a reduced number of (or dysfunctional) αIIbβ3 on platelets causes aggregation failure (Nurden, Fiore et al. 2011). Because of its central role in platelet aggregation, integrin αIIbβ3 blocking drugs can be used in treatment. Abciximab, eptifibatide, and tirofiban, three integrin αIIbβ3 antagonists are for example used as anti-thrombotics drugs. Because of their high efficacy, bleeding is a common and severe side effect of these drugs. Studies with more specific antagonists of integrin αIIbβ3 display promising results for the possibility of developing second-generation antagonists with milder side effects (Kuo, Chung et al. 2019).

The assembly of fibrin and fibronectin by platelet integrins in the blood clot, together with chemokines released by the platelets, can further recruit fibroblasts and endothelial cells to aid in matrix assembly at the injured area (Darby, Laverdet et al. 2014). Fibroblasts do not only remodel the ECM, but are also able to secrete and activate growth factors (Mueller, Fusenig 2004). The ECM contains several bound and latent growth factors acting as pro-fibrogenic cytokines. One important growth factor is the transforming growth factor beta (TGF-β). The latency of TGF-β depends on a latency-associated peptide (LAP) which prevents non-activated receptor binding. TGF-β can be activated by the RGD-binding integrins αvβ1, αvβ3, αvβ5 αvβ6 and αvβ8, binding to LAP via their RGD sequence and exerting force on the LAP-TGF-β complex resulting in liberation of TGF-β from LAP and thus allow TGF-β receptor activation and signalling (Khan, Marshall 2016, Robertson, Rifkin 2016). TGF-β signalling causes phosphorylation of Smad2 and Smad3, which can oligomerize with Smad4 and translocate to the nucleus where Smad2/3/4 acts as a transcriptional complex and initiates myofibroblast differentiation. These myofibroblast express high levels of collagen and collagen-binding integrins and are highly contractile. TGF-β receptors can also form a complex together with integrin α3β1 and E-cadherin in epithelial cells, which causes a β-catenin-Smad2 complex to mediate epithelial to mesenchymal transition (Margadant, Sonnenberg 2010, N. F. Brown, Marshall 2019).

Integrin αvβ3 is one of the most promiscuous integrins with over 12 potential ligand partners. In addition to fibronectin, it also binds e.g. vitronectin, laminin, osteopontin and fibrinogen (Humphries et al. 2006). Under many circumstances integrin αvβ3 preferentially binds to vitronectin, this is especially pronounced under low force conditions. This preference for vitronectin is abolished when mechanical load is exerted on the receptor. The mechanical load causes a conformational switch and a gradual decrease in ligand selectivity, allowing binding to other ligands such as fibronectin and fibrinogen. This property of the receptor enables it to mediate mechanosensitive responses in a wide variety of surroundings (Bachmann, Schäfer et al. 2020). Its exceptional number of ligand interactions is also reflected in the numerous ways it can affect cancer progression (Nieberler, Reuning et al. 2017). Viruses can enter their host cell in various manners and many viruses display RGD-

motifs on their viral envelope via which they can bind to cells and activate integrin-signalling pathways. Human adenovirus, Epstein-Barr virus and Human immunodeficiency virus have all for example been shown to use integrin αvβ3 (among other integrins) to promote cell entry or cell attachment to support viral infection (Hussein, Walker et al. 2015). Integrin $\alpha_v\beta_3$ is also highly expressed in glioblastoma where it promotes tumour cell migration, invasion and angiogenesis (Malric, Monferran et al. 2017). In addition to its several ligand interactors, integrin αvβ3 also interacts with MMPs, insulin and several different growth factors (Liu, Wang et al. 2008). Angiogenesis is a regulated process that occurs through an angiogenic switch that depends on the balance between pro-angiogenic factors such as growth factors and anti-angiogenic molecules such as thrombospondin. Endothelial cell responses depend on growth factor and integrin signalling cross-talk to regulate adhesion, migration and proliferation required for endothelial tube network formation in angiogenesis. Vascular endothelial growth factor and cell adhesion to vitronectin and fibrinogen induces phosphorylation of integrin αvβ3 and subsequent Src activation. Src activation is in turn essential for the activation of the vascular endothelial growth factor receptor 2 (VEGFR2) and its association with integrin αvβ3. This activation and association further activates both receptors on endothelial cells to drive adhesion and migration in angiogenesis. The crosstalk of VEGF, integrin αvβ3 and VEGFR2 is thus an important part of activating pro-angiogenic factors to both induce and drive angiogenesis (Somanath, Malinin et al. 2009, Liu et al. 2008, Mahabeleshwar, Feng et al. 2007).

To conclude, the large family of RGD containing ligands and their integrin receptors contribute to several different cellular processes important at various stages of life. The tissue specific expression of both ligands and integrins, together with variations in ligand-binding capacity and affinity, generates a vide repertoire for regulating specific cellular responses in a site- and time-dependent manner.
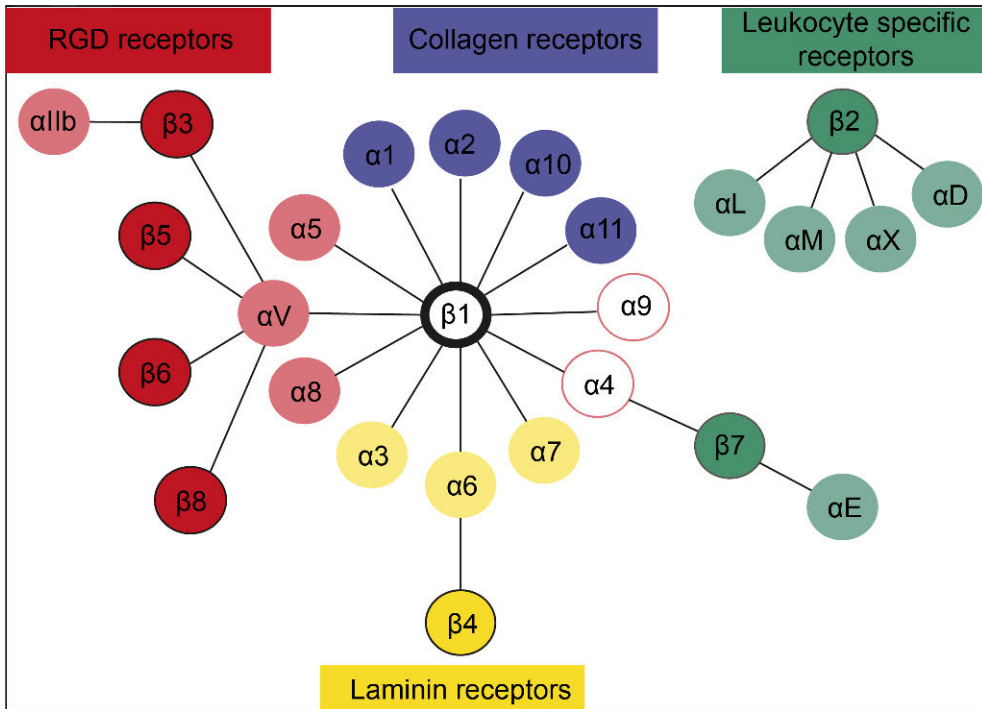
Figure 1. The integrin receptor family and the pairing of integrin α and β subunits into ligand-binding heterodimers. Modified from (R. O. Hynes 2002).

2.1.4.4   Collagen-binding integrins

While many cell-interactions with the collagen matrix occur indirectly via matrix glycoproteins, cells can also directly bind to, and interact with collagen via integrins. All collagen-binding integrins (α1β1, α2β1, α10β1, α11β1) are composed of a β1-subunits pairing with an α-subunit that contains an I-domain (Barczyk et al. 2010). The first identified collagen-binding integrins were integrin α1β1 and α2β1 originally found in activated T-cells (hence their alternative names very late antigen complex 1 and -2 (Hemler et al. 1985)) and their expression has since then been detected in several other cell types including fibroblast, epithelial and vascular cells. The later found integrin α10β1 mostly interacts with collagen type II and is therefore mostly restricted to cartilage tissue where it participates in cartilage development (Lundgren-Åkerlund, Aszòdi 2014). Integrin α11β1 expression is primarily restricted to fibroblasts (tissue specific) and mesenchymal stem cells (Musiime, Chang et al. 2021). Like the RGD-recognizing integrins, collagen-binding integrins also recognize a specific amino acid sequence that they bind to via their I-domain. This is the GFOGER sequence (or variants of it) in native triple helical collagens where "O" stands for hydroxyproline (Knight, Morton et al. 1998, Knight, Morton

et al. 2000). In addition, collagen also contains other recognition sequences, which can be recognized by integrins α5β1, αvβ3 and αvβ1. Due to collagen assembly into complex structure *in vivo* these sequences are quite often hidden or unavailable for receptor binding but can however become exposed and available as a result of proteolysis (Heino 2007).

Type I collagen is the most abundantly expressed protein in vertebrates and incorporated in the ECM of various tissues such as tendons, skin and bones, where it provides the tissue with form and mechanical strength (Sweeney, Orgel et al. 2008). In vertebrates, over 30 different types of collagens have been identified, all of which consists of three α chains making up a triple helix that can be either a homotrimer or a heterotrimer. The different types of collagens assemble into various supramolecular structures and collagen can be further subdivided into the following subfamilies based on their structures: collagen networks, anchoring fibrils, collagen fibrils and beaded filaments (Ricard-Blum 2011). In the basal lamina, underlying the epithelial cells, type IV collagen assembles into a unique two-dimensional network. This network acts as a scaffold and allows for interactions with other basement membrane proteins such as laminins and proteoglycans to assemble the mature basement membrane (K. L. Brown, Cummings et al. 2017). Collagen fibrils are the most common structures, stabilized by covalent bindings, and composed of different types of collagens depending on which tissue they reside in. The process in which collagens together with non-collagenous proteins or proteoglycans assemble into these macromolecular alloys, is called fibrillogenesis (Mienaltowski, Birk 2014, Kadler 2017). One of the primary functions of collagen matrix is to shield the cells from mechanical forces, which vary in both magnitude and form (shear, compression, tension) depending on the tissue. As such, the collagen fibril arrangement also needs to vary to be able to meet the mechanical requirements of the tissue. In cartilage, where swelling causes pressure, narrow fibrils of primarily type II collagen are assembled into an open network, while parallels of closely packed ruffled fibres are observed in tendons to allow transmission of muscle generated forces (Holmes, Lu et al. 2018). While the assembly and organization of the collagen matrix *in vivo* is still incompletely understood, a growing appreciation of fibroblasts taking part in this process has emerged. Collagens can self-assemble and form fibrils *in vitro*, and it was earlier believed that collagens also self-assemble *in vivo*. It is now accepted that the assembly process *in vivo* requires interactions and incorporation of other matrix molecules such as fibronectin and heparin. Fibronectin matrix has been shown to be important for the early steps of collagen assembly by acting as an assembly scaffold for the secreted pro-collagen and the collagen C-propeptide proteinase bone morphogenetic protein 1 (BMP-1) needed for collagen cleavage (Saunders, Schwarzbauer 2019). Furthermore, this scaffold was earlier thought to regulate collagen deposition and to act as a nucleator of the cleaved pro-

collagens to induce collagen fibrillogenesis and mediate cell forces via integrins pulling on the fibronectin scaffold. Fibroblast are now known to exert mechanical forces also directly on collagens via integrin-collagen interactions, which aid in collagen assembly and organization (Musiime et al. 2021, Zeltz, Gullberg 2016). The earlier idea of fibroblasts participating in collagen matrix assembly and organization only by producing matrix components and by exerting force via fibronectin is now challenged, as increasing studies support the idea of a direct integrin-collagen interaction taking part in this process (Kadler, Hill et al. 2008). Integrin α2β1 and integrin α11β1 expression in fibroblasts has been shown to enhance fibrillogenesis. Moreover, expression of these integrins in fibronectin knock-out fibroblasts (4D cell line that also lacks collagen-binding integrins) allows for assembly of short collagen fibres in close proximity to the cell surface also in the absence of fibronectin, suggesting that integrins can act as collagen fibre nucleators in the absence of fibronectin. However, the formation of a collagen fibril network requires a fibronectin scaffold (Velling, Risteli et al. 2002).

Wound healing is a process highly dependent on fibroblasts. Upon tissue injury, fibroblasts generate thicker and longer collagen fibres, which they organize and translocate to generate a compact ECM closing the wound. The organization and translocation of the fibres is mediated by fibroblast locomotion exerting tractional forces generated by actomyosin (discussed later) on loosely packed thin fibres into growing collagen fibres to pack them closely together, thus generating the dense matrix characteristic to scar tissue (Ehrlich, Hunt 2012). In mice, integrin α2 and -α11 expression is increased in both tissue and fibroblasts after tissue injury (Zweers, Davidson et al. 2007). Integrin α11 knockout mice display impaired wound contraction, attributed to both a reduced migration ability of fibroblast and to a reduced myofibroblast differentiation causing a fibroblast contractility and collagen-remodelling defect in the α11 knockout mouse (Schulz, Zeltz et al. 2015). Furthermore, knockdown of integrin α11β1 alters the myofibroblast phenotype of liver specific myofibroblast and reduces adhesion, migration and collagen contraction in these cells. On the other hand, integrin α11β1 induced expression correlates with increasing fibrogenesis in both mice and human fibrotic tissue. Elevated expression of integrin α11β1 can be induced by TGF-β activation, which is one of the key growth factors mediating disease progression by activated fibroblasts (Bansal, Nakagawa et al. 2017). Moreover, integrin α11β1 over-expression is also common in non-small-cell lung carcinoma (NSCLC), where an increased stromal expression of integrin α11β1 promotes tumorigenicity. The pro tumorigenic effect of integrin α11β1 is partially attributed to its regulatory role of insulin growth factor 2 (IGF2) expression in fibroblast (C. Zhu, Popova et al. 2007).

While integrin α1β1 can bind to several different collagen types, it has a preference for the non-fibrillar collagen type IV. For integrin α2β1 the opposite is

true; the receptor has a clear preference for fibrillar collagen I, for which it also has a significant higher binding affinity compared to type IV collagen (Zeltz, Gullberg 2016). This is also reflected in the different integrin expression patterns of CD4 and CD8 T cells and their responses to influenza infection in the lung. While both subset of T cells express both integrin α1β1 and α2β1, their localization in the infected lung differs. CD4 T cells, with higher expression of integrin α2β1, tends to localize to interstitial spaces rich in collagen I and only rarely to the airways or blood vessels with a collagen IV rich basement membrane. In contrast, CD8 T cells with high expression of integrin α1β1 mostly localizes to collagen IV rich areas. In addition to regulating cell specific localization of T cells within the inflamed tissue, these integrins also have different roles in the immune response. CD8 T cells are important for the secondary immune response and need to remain attached to the ECM at the inflamed tissue to generate a memory specific protection. Accordingly, integrin α1β1 inhibition increases CD8 T cell localization from the lung tissue to the spleen and thus compromises the secondary immune response by abrogating the CD8 T cell-ECM attachment and retention in the tissue (Richter, Ray et al. 2007, Ray, Franki et al. 2004). While the role of integrin α2β1 in T cells is less understood, integrin α2β1 has been shown to regulate leukocyte recruitment to inflammation sites by mediating both extravasation of leukocytes from the vasculature and migration of leukocytes in extravascular tissue, thus allowing them to reach sites of inflammation (Werr, Johansson et al. 2000). The fact that integrin α2β1 mediates both of these processes in leukocytes, further highlights the ability of integrins to bind different types of collagen presented either in the blood vessels and ECM respectively.

Taken together, collagen remodelling by integrins is important for tissue regeneration, and regulated by both integrin and collagen production as well as complex integrin growth factor cross-talk. Deregulated integrin expression, implicated in both abrogation of wound healing as well as with fibrosis, indicates a need for a balanced integrin activity in tissue homeostasis.

2.2   Mechanotransduction

Cells are continuously exposed to different kinds of mechanical stimuli from their environment. This stimuli can be caused, for example, by a matrix filament pushing onto the cell when it is migrating through the matrix, causing compressive force, a neighbouring cell pulling at a cell-cell junction giving rise to tensile forces, or an endothelial cell experiencing shear force from the blood flow (Charras, Yap 2018). Regardless of the nature or origin of the force, cells need to interpret the forces and respond to them, to allow proper cell function in the continuously dynamic microenvironment. Cells do so by a process known as mechanotransduction, where mechanical stimuli is interpreted and translated into biochemical signals that give

rise to changes in cell behaviour (Martino, Perestrelo et al. 2018). Mechanotransduction is crucial for several different processes such as development and tissue homeostasis, and therefore tightly controlled, involving coordinated regulation of both extracellular and intracellular proteins and signalling. Perturbation of mechanotransduction can cause cellular and tissue dysfunction such progeria, deafness and cancer (Jaalouk, Lammerding 2009).

2.2.1   Integrin adhesion complexes and focal adhesions

Cells perceive and interpret mechanical stimuli via different mechanosensitive molecules at the cell membrane including G protein coupled receptors, ion channels and integrins among others. One of the main mechanotransduction hubs of cells are focal adhesions where mechanical cues from the viscoelastic extracellular matrix are transmitted to the intracellular cytoskeleton and reciprocally intracellular tension is exerted onto different components of the ECM (Sun, Guo et al. 2016). Cytoskeleton contractility is generated by the motor protein myosin II pulling on F-actin fibres that slide in response to the pulling. Crosslinking proteins such as α-actinin and filamin, organize these proteins into larger stress fibres (Martino et al. 2018). The force a focal adhesion can transmit is thought to correlate with its size and the amount of adaptor proteins recruited to the adhesion (Goffin, Pittet et al. 2006). Integrin-ECM binding initiates integrin clustering and recruitment of adaptor proteins causing formation of dynamic macromolecular integrin adhesion complexes (IACs). These complexes are generally divided into four different subtypes depending on their maturation state, size, subcellular localization and shape (Conway, Jacquemet 2019). Over 200 different proteins have been reported to regulate, or to be located to the adhesion nexus, of which 60 are thought to make up the "core adhesome", identified from multiple mass spectrometry datasets (Horton, Humphries et al. 2016). Here, some of the key adhesion proteins are described, along with their function in the different adhesion structures.

The first adhesion subtypes to arise from integrin-ECM link formations are filopodial and nascent adhesions. Rap1 and Rap1-GTP-interacting adaptor molecule (RIAM) binding to talin promotes talin localization to the plasma membrane and a conformational change of talin that unmasks the integrin binding site, enabling integrin binding and activation (J. Yang et al. 2014, Han, Lim et al. 2006, L. Zhu et al. 2017, Camp et al. 2018). Filopodial adhesions are incredibly dynamic and probe the cell environment for mechanical cues at the filopodia tip. Myosin-X triggered actin assembly together with insulin-receptor substrate p53 (IRSp53) deformation or tabulation of the plasma membrane can initiate filopodia assembly. Actin crosslinking proteins, such as α-actinin bundles actin filaments together, and protrusion of these bundled filaments is initiated by formins and/or actin regulators.

Myosin-X can then transport integrins to the filopodia shaft and tip. At these sites, unclustered but active integrins and talin connects the parallel actin bundles of filopodia to the ECM and create adhesion sites to probe the ECM (Jacquemet, Hamidi et al. 2015). Moreover, p130Cas which can become phosphorylated upon mechanical stretch (Sawada, Tamada et al. 2006) co-localizes with Myosin-X in filopodial tips, suggesting that it can act as a mechanosensors at these tips (Jacquemet, Stubb et al. 2019). Once the lamellipodia advances, adhesions at the filopodia shaft can mature into focal adhesions (Fischer, Lam et al. 2019).

Nascent adhesions typically form 1-2 µm away from the plasma membrane in the lamellipodial region where actin undergoes rapid retrograde flow. The formation of nascent adhesions requires actin polymerization but not non-muscle myosin II activity. These adhesions are approximately 100 nm wide and immobile to their nature. They generally consists of around 50 integrins together with integrin-actin linker proteins such as talin and kindlin along with signalling proteins like focal adhesion kinase (FAK) and p130CAS. Their small size, along with their short lifetime has resulted in relatively few studies of nascent adhesions. It is thought that nascent adhesions form as a result of integrin conformational change, where either ligand binding or intracellular adaptor protein binding causes a shift from the inactive, low ligand binding affinity conformation to the active, high affinity conformation. The conformational switch is followed by integrin clustering into nascent adhesions (Henning Stumpf, Ambriović-Ristov et al. 2020). Integrins can indirectly recruit paxillin, a large scaffolding protein, which is also one of the earliest proteins recruited to adhesions (Laukaitis, Webb et al. 2001). This is most likely because of its ability to bind to several cytoskeletal proteins and tyrosine kinases (Schaller 2001). In addition, Paxillin is able to recruit GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), and thereby regulate the activation of signalling molecules at adhesions (López-Colomé, Lee-Rivera et al. 2017). This protein recruitment is needed for adhesion maturation and paxillin is furthermore recognized as one of the most connected proteins within the core adhesome (Horton et al. 2016). When the rear of the lamellipodium, where actin disassembly takes place, approaches the nascent adhesions, they either mature into focal complexes and -adhesions or disassemble. The fate of the nascent adhesions is thought to depend on the protein composition, mechanical properties of the substrate and coupling of the nascent adhesion to the actomyosin network (Vicente-Manzanares, Horwitz 2011).

If substantial forces are built-up on the actin-integrin-ECM axis in the nascent adhesions, adhesion maturation into focal complexes takes place. Focal complexes are around 500 nm wide and are located more rearward than nascent adhesions, at the interface of the lamellipodia and lamella. They are rich in phospho-paxillin, talin, FAK, and α-actinin and more long-lived than nascent adhesions. Whereas some

undergo rapid disassembly, others mature into focal adhesions that reside outside of the lamellipodia (Gardel, Schneider et al. 2010, Zaidel-Bar, Cohen et al. 2004). While focal complexes are associated with a loose actin network, focal adhesions can associate with the ends of actin stressfibers in the lamella (Le Clainche, Carlier 2008). Focal adhesions are often located near the cell periphery at the lamellum and are flat and elongated with an area that can be several microns in size. They are able to anchor bundles of actin microfilaments through the flat plaque of adhesion proteins to mediate strong adhesion to the substrate (Geiger, Bershadsky et al. 2001). The transformation of nascent adhesions into focal complexes and focal adhesions requires a switch in the relative activities of the Rho GTPases RhoA and Rac1. While Rac1 activity is needed for early adhesion, RhoA activity is required to activate downstream effectors such as ROCK and mDia1 in focal adhesion. In nascent adhesions, RhoA is inactive but the increased tension in the growing adhesion mediates RhoA activation (Lawson, Burridge 2014). ROCK can activate myosin II driven cell contractility by myosin light chain phosphorylation, this pathway can however be bypassed if external tension is applied. mDia1 in turn, induces actin polymerization and can also target microtubule for protein delivery to the growing adhesion (Geiger, Bershadsky 2001). The increased actomyosin contraction triggers unfolding of talin and can expose one or several of the 11 cryptic vinculin binding sites that resides in the talin rod domains. Vinculin binding to talin induces a strengthening of the connection to actin and drives adhesion maturation (Goult, Yan et al. 2018, Yao, Goult et al. 2016). Furthermore, actomyosin triggered force also causes RIAM dissociation from several talin domains and induces an exchange of RIAM for vinculin. As the magnitude of force on talin dictates both the level of talin unfolding and the number of RIAMs to disassociate, also the biochemical output will depend on the magnitude of force as it controls the possible binding partners to talin (Vigouroux, Henriot et al. 2020).

Focal adhesions are furthermore important phosphorylation platforms and in addition to protein unfolding, force can induce protein phosphorylation to activate adhesion proteins, as in the case for FAK. FAK is a non-receptor tyrosine kinase and scaffold protein, containing an N-terminal FERM domain, a C-terminal focal adhesion targeting (FAT) domain and a central kinase domain. The FAT domain mediated interaction with both paxillin and talin allows recruitment of the auto-inhibited FAK from the cytoplasm to adhesions (Acebrón, Righetto et al. 2020). At the adhesion, FAK can additionally bind via its FERM domain to the clustered phosphatidylinositol 4,5-bisphosphate (PIP2) at the plasma membrane. Forces generated by the actin cytoskeleton causes the C-terminus of FAK to be pulled away from the membrane, which results in force induced exposure of both FAKs auto-phosphorylation site and the Src phosphorylation site. Recruitment of the Src-kinase to the auto-phosphorylated FAK phosphorylates the remaining tyrosine residues

leading to full activation of FAK (Bauer, Baumann et al. 2019). Active FAK can by binding to Src family kinases and other signalling molecules, trigger multiple downstream pathways to regulate processes such as cell survival, proliferation and migration. Overexpression and activation of FAK has been observed in several human cancers and FAK is thus an attractive target for cancer therapies (Zhao, Guan 2009, Murphy, Rodriguez et al. 2020). Activated FAK can also phosphorylate paxillin, which induces vinculin recruitment to the FA. This results in phosphorylated paxillin acting as mechano-adaptor, although recruitment of paxillin itself to adhesions is not contractility dependent (Pasapera, Schneider et al. 2010).

Vinculin is thought to stabilize adhesions by both locking the unfolded conformation of talin, thereby preventing it from re-folding, and by binding to actin and talin, thus supporting the force load from actin (Yao, Goult et al. 2014). Vinculin is one of the key focal adhesion proteins and its position at the actin-talin interface is ideal for coordinating force-induced signal. Furthermore, the recruitment of vinculin to FAs correlates with force, and these forces also directly act on vinculin (Carisey, Tsang et al. 2013). The structure of vinculin comprises a head domain that binds to talin, a tail domain binding to both F-actin and paxillin and a flexible linker that separates the two domains (Bakolitsa, Cohen et al. 2004, Ziegler, Gingras et al. 2008). Because of its ability to both bind actin and stabilize talin, it is also an important part of the "molecular clutch" (discussed later) and thus important for proper force generation (Hu, Ji et al. 2007). This is also observed by its localization to the leading edge of cells where high forces are exerted to allow cell migration (Ji, Lim et al. 2008). The importance of vinculin in force generation was demonstrated in vinculin depleted cells displaying a clear reduction in both cell spreading and migration (Mierke, Kollmannsberger et al. 2008).

The important role of focal adhesions as mediators of cell adhesion, cytoskeleton regulation, force generation and signalling machineries (Wozniak, Modzelewska et al. 2004) is reflected by the large number of focal adhesion proteins, of which only a few have been discussed here, taking part in mediating these processes. The complex interplay and dynamics between the different components has been extensively studied but for a long time the architecture of the flat <200 nm focal adhesion plaque remained unknown due to the limitations in vertical and horizontal resolution. About 10 years ago, the development of the super resolution imaging technique iPALM revolutionized the field by allowing three dimensional imaging and mapping of the specific adhesion molecules with nanoscale resolution (Shtengel, Galbraith et al. 2009, Kanchanawong, Shtengel et al. 2010). This resulted in identification of distinctly organized but overlapping functional layers inside of the focal adhesions (Figure 2). The uppermost layer of the focal adhesion, the actin regulatory layer, consist of actin, zyxin, vasodilator-stimulated phosphoprotein (vasp) and α-actinin, all proteins involved in reinforcement of stress fibres (Hoffman,

Jensen et al. 2012), suggesting that this FA-layer regulates FA-strengthening and the cytoskeleton. The lowest layer, spanning the plasma membrane, is the integrin signalling layer, which in addition to integrins is enriched in paxillin and FAK, which both can affect gene expression upon integrin binding (M. C. Brown, Turner 2004, Mitra et al. 2005). The intermediate layer, spanning both into the integrin signalling layer and the actin regulatory layer, thus mediating a link between the actin cytoskeleton and the ECM-bound integrins, is enriched in vinculin and talin. The position of these proteins, together with their mechanosensitive nature suggest that this layer functions as a force-transduction layer. The vertical organization is well in line with the different binding partners of the adhesion proteins and furthermore reveals a protein strata that bridges the gap between the integrin cytoplasmic tails and the actin cytoskeleton (Kanchanawong et al. 2010). Also the horizontal distribution of adhesion proteins is organized into nanostructures. Structured illumination microscopy studies have revealed that proteins in the integrin signalling layer, such as paxillin, tend to localize to the membrane proximal region of focal adhesions. In contrast, proteins from the actin regulatory layer, such as zyxin, distributes in the membrane distal layer of the adhesions. Interestingly, vinculin does not only co-localize with the actin regulatory proteins in the distal part of the adhesions, but also form a distal tip protrusion of the adhesion where it can engage with the actin retrograde flow (Legerstee, Abraham et al. 2021). This could be attributed to mechanical force stretching talin, and revealing further vinculin binding sites, thus allowing active vinculin to be positioned higher up in the adhesion. In line with this, inactive vinculin has been shown to co-localize with paxillin while active vinculin positions higher up in z-resolution (Case, Baird et al. 2015). Furthermore, co-localization studies of focal adhesion proteins have shown that loss of actomyosin contractility decreases co-localization of different proteins within adhesions, suggesting that mechanical force is important for regulating the nanoscale architecture of adhesions (Xu, Braun et al. 2018). In addition, a proximity-dependent adhesome, generated in situ based on multiplexed proximity biotinylation of adhesion proteins, identified spatial associations and topological organization of the adhesome that is consistent with the 3D architecture of the FA (Chastney, Lawless et al. 2020).
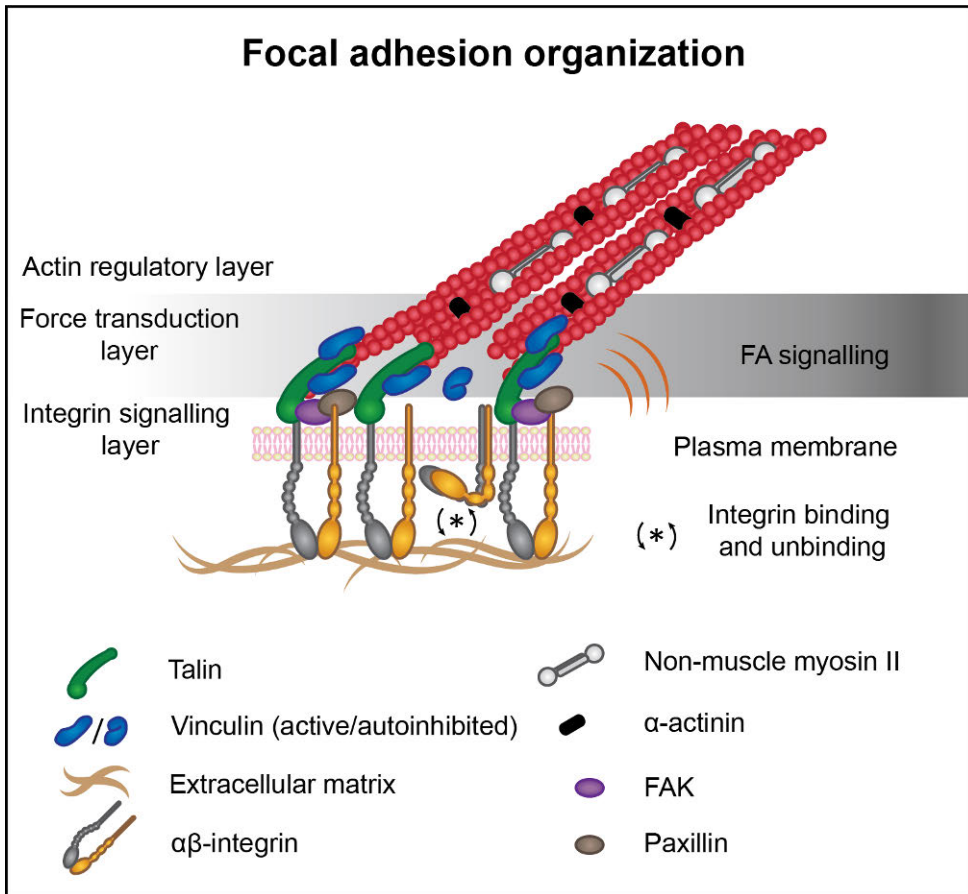
Figure 2. Simplistic illustration of the focal adhesion organization and its regulatory layers. Modified from (Kanchanawong et al. 2010, Isomursu, Lerche et al. 2019).

In contrast to focal adhesions, fibrillar adhesions are located more centrally in cells. These ECM contacts are more elongated and are associated with ECM fibrils and remodelling of the ECM. As these adhesions mediate assembly and reorganize fibronectin in the ECM, to assemble fibronectin fibrils, they are typically enriched with the fibronectin receptor integrin α5β1 and tensin (Geiger et al. 2001). Tensin is a cytoplasmic protein that can bind both actin and integrin in a similar fashion as talin (McCleverty, Lin et al. 2007). Increase in matrix stiffness is also known to promote growth of fibrillar adhesions in a tensin dependent manner (Barber-Pérez, Georgiadou et al. 2020). Tensin has furthermore been shown to be important for force generation as depletion of tension leads to reduced traction force generation in cells plated on fibronectin (Georgiadou, Lilja et al. 2017). Fibronectin-bound integrin α5β1 continuously translocates parallel to actin stress fibres from the cell peripheral focal adhesions towards the cell centre to form fibrillar adhesions

(Conway, Jacquemet 2019). Moreover, intact focal adhesions, initially composed of various basement membrane binding integrin heterodimers, have been shown to slide inwards in a winch-like mechanism in association with shortening stress-fibres. As these adhesions mature, the original integrins mediating the cell-ECM attachment are replaced by integrin α5β1 to mediate extensive fibronectin assembly (J. Lu, Doyle et al. 2020). In addition to mediate relocation of integrins and the bound fibronectin, the tensile force that is mediated from the actin cytoskeleton to the extracellular fibronectin molecules via integrins, also causes stretch-induced unfolding of the fibronectin molecule to reveal cryptic binding sites needed for fibrillogenesis (Geiger et al. 2001). As phosphorylation of the integrin β tail inhibits its binding to talin, but does not affect its binding to tensin, it is possible that phosphorylation events in the focal adhesion could induce a switch in integrin-binding preference. This switch, from talin to tensin could promote migration of the integrin-tensin complex out of the focal adhesions, to initiate fibrillar adhesion formation (McCleverty et al. 2007, Clark, Howe et al. 2010).

In addition to the phosphorylation and activation events of the individual adhesion proteins triggering downstream signalling, several other players are important in mechanotransduction. The tension and dynamics of the actin cytoskeleton, the actin mediated mechanical link between the adhesions and the nucleus and nuclear mechanoresponses are all taking part in translating biophysical cues into biological responses (Isomursu et al. 2019). Actomyosin contractility can mediate opening of nuclear pore complexes to allow nuclear translocation of proteins that are transcriptionally active. On stiff substrates, ECM-nuclear mechano-coupling causes nuclear pores to stretch which increases nuclear translocation of Yes-associated protein (YAP), a mechanosensitive transcriptional regulator to the nucleus (Elosegui-Artola, Andreu et al. 2017). YAP can then bind to transcription factors to initiate transcription of genes involved in development, proliferation, migration and cancer metastasis (Heng, Zhang et al. 2021). Matrix stiffening has been shown to induce epithelial to mesenchymal transition (EMT) as a result of actin polymerization and reorganization, and subsequent nuclear localization of Myocardin-related transcription factor A (MRTF-A) (Dai, Qin et al. 2008). Actomyosin contractility can also affect gene transcription by regulating the accessibility of genes via the linker of nucleoskeleton and cytoskeleton complexes (LINC). In keratinocytes, integrin adhesion mediated tension on the nuclear lamina and the associating chromatin has been shown to repress epidermal differentiation complex gene expression. When tension on the LINC complex was depleted, the gene expression was activated unwarrantedly, suggesting a role for adhesion mediated regulation of time relevant gene expression (Carley, Stewart et al. 2021). Also ion channels play a role in mechanotransduction. Shear stress from the blood flow can for example activate Piezo1, a plasma membrane ion pump, to induce a

calcium influx that via downstream signalling affects the spatial organization of endothelial cells (Li, Hou et al. 2014). Traction forces mediated by myosin II can also generate local membrane tension, which in turn induces a spatially restricted calcium influx via Piezo1 allowing for spatio-temporal activation of downstream signalling (Ellefsen, Holt et al. 2019).

2.2.2 The molecular clutch model

Cells are constantly exerting and transmitting forces to their environment and the mechanical rigidity of the surrounding tissues influences many cellular processes such as gene expression, cell proliferation and migration. These processes are not only important in normal tissue homeostasis but also implicated in development and cancer (Chanet, Martin 2014, Lauffenburger, Horwitz 1996). Thus, understanding the mechanism of how forces are generated and transmitted by cells, and how they affect cell functions have been of high interest for a long time.

Mathematical modelling has together with biophysical and -chemical experiments been used to describe the mechanisms of how cells sense and respond to their environment through integrin adhesions. These models are based on the molecular clutch hypothesis, first proposed by Mitchison and Kirschner in 1984, to explain cell movement and extension in neuronal growth cones. They proposed that the extension and movement of a cell is a result of adhesion receptors linking the underlying substrate to the myosin driven rearward flow of F-actin via linker proteins, generating a tension by slowing down the actin flow. The linkage of the F-actin to the underlying substrate in turn, would result in cell protrusion and forward movement as a result of the actin polymerization taking place at the barbed end of actin filaments. Furthermore, they speculated that a variable slippage between linker proteins, adhesion receptors and the actin cytoskeleton must take place, since cells exhibit continues myosin driven rearward flow of actin but only fluctuating forward movements. Thus, the coupling of the adhesion receptor and the F-actin was proposed to function as a molecular-clutch, allowing forward movement of the cell only when the substrate bound receptors are engaged to the myosin driven rearward flowing actin cytoskeleton, stalling the flow and causing forward movement with the growing actin bundle (Mitchison, Kirschner 1988).

Since then, the molecular clutch model and our understanding of the molecular machinery driving force generation has extensively evolved even though the fundamental basics of the hypothesis has remained the same. To explain how substrate stiffness affects the cellular molecular clutch, the clutches and the substrate have been treated as Hookean springs in a stochastic model of the molecular clutch. In such a model, myosin motors pull on an actin bundle with a certain force at a certain velocity. Molecular clutches reversibly engage the F-actin bundle with

binding and unbinding rates to resist the retrograde flow. If tension is applied to a clutch, the unbinding rate will increase exponentially with the force exerted on the bond according to Bell's law (Bell 1978). When engaged to the F-actin, the force generated by myosin pulling backwards will load tension with a spring constant onto the engaged clutches as they are stretched by the bound F-actin moving backwards. This will cause engaged clutches to stretch and transmit a force induced strain on the compliant substrate they are bound to. As the substrate is compliant and can undergo deformations as a result of forces, it will also behave as a spring. Myosin generated backward flow of the actin will thus exert tension and deformation on the substrate until the clutches fail with a force dependent unbinding rate. Thus, the mechanical resistance to force loading is determined by the mechanical stiffness of both the clutches and the substrate they are bound to. This gives rise to two phenotypes of traction force dynamics on compliant substrates: *a frictional-slippage*, with high retrograde flow velocity and low force generation on stiff substrates, and a *load-and-fail* on soft substrates, with low actin velocities and high traction forces. On stiff substrates, the clutches will engage to the F-actin but unbind shortly after, as the lack of compliance of the stiff substrate causes rapid tension building on the clutch thus shortening its binding lifetime (Chan, Odde 2008). This will cause the F-actin bundle to continuously slip backwards towards the cell centre and away from the point of contact at a relatively constant velocity. This slippage behaviour of actin can be observed on glass (stiff) substrates (Hu et al. 2007). On soft substrates, the high compliance of the substrate will prolong the clutch binding lifetime since the tension on individual clutches will build up slowly. The long engagement time allows neighbouring clutches to share the mechanical load as the tension increases with increasing substrate strain. This in turn, causes increased resistance to the myosin motor force, which will slow down the retrograde flow. The force load will eventually become so high that a stochastic loss of one clutch will cause a cascade of failing clutches and force unloading from the substrate allowing it to return to its original position. The traction dynamics of soft substrates will therefore be oscillatory, characterized by relatively long periods of increasing tensions and the subsequent traction forces, followed by rapid decreases in the built-up tension and relaxation of the substrate. If the substrate is too soft, the model enters a second regime with faster actin flow and lower forces, this time because tension is built up too slow on the clutches because of the high deformability of the substrate, causing clutches to fail spontaneously before motors can load enough tension on the substrate. Also the load-and-fail phenotype on soft substrates has been observed experimentally by Chan and Odde who proposed the stochastic model of the molecular clutch. Furthermore, they observed a transition from the load-and-fail dynamics on soft substrates into frictional slippage-dynamics on stiffer substrates when they investigated cell behaviour at different stiffness's proposing that changes

in clutch dynamics may function as a foundation for cells to sense the stiffness of their environment (Chan, Odde 2008). Thus, according to the stochastic molecular clutch model, where clutches and substrate are assumed to behave like Hookean springs, force transmission is maximized for a specific value of rigidity.

2.2.2.1   Force prediction by the molecular clutch

As force transmission through the molecular clutch is more effective when the different components are more engaged to each other, the maximum force transmission will occur within the load-and-fail regime where more clutches will simultaneously have time to engage and share the load. Furthermore, the maximum (peak) transmission will occur at a stiffness when the time for all clutches to bind equals the substrates load-and-fail cycle time. At this stiffness, all possible clutches are engaged and loaded to their fullest and therefor the motors are also resisted to their fullest extent and the retrograde flow at its lowest speed (B. Bangasser, Rosenfeld et al. 2013). Above this rigidity value, forces on clutches load too fast, causing disengagement of clutches before additional clutches can bind, and below this rigidity force loading is too slow causing spontaneous clutch disengagement before high forces are ever reached (Figure 3). This model thus suggests a bi-phasic relationship between substrate stiffness and force generation and an inverse relationship between force generation and actin retrograde flow (Chan, Odde 2008).

As all components of the molecular clutch participate in force transmission, changes in any of the parameters that define the motor-clutch model will also cause a shift in the optimal force transmission stiffness. This explains why different cell types have different optimal stiffness's (B. L. Bangasser, Shamsan et al. 2017). An increase in the number of clutches will for instance cause a shift of the optimal stiffness to higher rigidities while a decrease in clutch number will shift it to lower rigidities (B. Bangasser et al. 2013). Furthermore, simulations of the molecular clutch with both single parameter and dual parameter changes, have shown that changing dual parameters simultaneously can cause magnitudes of shift of the optimal stiffness, while changes in only one parameter has limited ability to shift the stiffness optimum. In addition, changes in the clutch parameters (e.g. bond rupture force and unloaded off-rate) generally causes larger shifts in the optimal stiffness than changes in the motor parameters (e.g. number of myosin motors). Furthermore, increases in motor parameters tends to decrease the optimal stiffness while increases in clutch parameters shifts it to higher rigidities as a result of creating a stronger clutch, with the exception of the unloaded clutch-off rate which increase makes the clutch weaker (B. Bangasser et al. 2013).
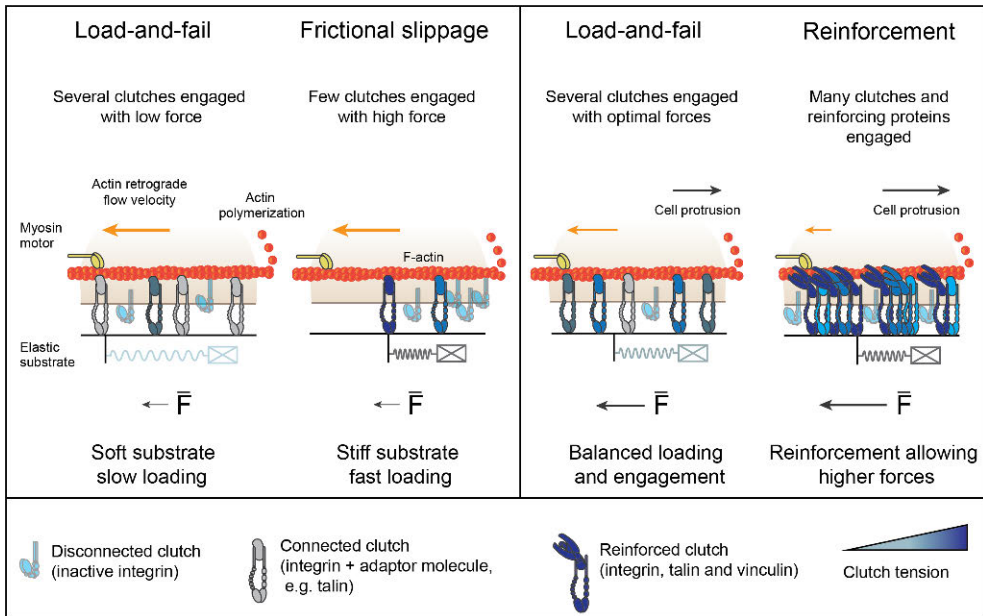
Figure 3. Illustration of the molecular clutch and its different traction force dynamics on compliant substrates. Modified from (Chan, Odde 2008, Isomursu, Park et al. 2020).

In addition to the number of clutches present, also the quality of the clutches affects the clutch engagement time and the subsequent force transmission. This is because different integrin heterodimers exhibit different ligand-bond kinetics even when binding to the same ligand. Integrin αvβ3 has been shown to have a three-fold increase of unbinding rates and a five-fold increase in binding rates to fibronectin compared to integrin α5β1, causing a shift of the force optimum to higher rigidities when cells bind to fibronectin substrates via integrin αvβ3 (Elosegui-Artola, Bazellières et al. 2014).

For cells to be able to transduce force, in other words to translate the force into biochemical signals that will affect cell functions as a response to stiffness, the force in the clutch system will need to be transmitted through at least two mechanosensors with different properties (Elosegui-Artola, Oria et al. 2016). In the classical ECM-integrin-talin-actin-myosin clutch this could be provided by talin and integrin. While talin unfolds according to the Bell model (Bell 1978, Yao et al. 2014), integrin can act as catch-slip bonds. In a catch-slip bond, the lifetime of the binding first decreases with force (slip-bond) until it reaches a threshold after which it increases with force (catch-bond) to a maximum life-time to then again decrease with force (catch-slip bond). In integrins, this catch-behaviour is due to force-assisted activation of the integrin headpiece (F. Kong, García et al. 2009). These distinct force properties lead to an intersection of the two force-lifetime curves and causes talin to unfold only

after a certain rigidity: when integrin unbinding is slower than talin unfolding (Elosegui-Artola, Trepat et al. 2018).

2.2.2.2 Reinforcement of the molecular clutch

One important aspect of mechanotransduction is reinforcement. Reinforcement is a result of some clutches engaging long enough for mechanotransduction and adhesion growth to take place. In addition to the adaptor proteins directly connecting integrins to F-actin filaments, such as talin (Ringer, Weißl et al. 2017), α-actinin (Roca-Cusachs, del Rio et al. 2013), tensin (Clark et al. 2010) and kindlin (Theodosiou, Widmaier et al. 2016), also indirect linkages between actin and integrins affect force transmission and transduction through the molecular clutch. These indirect linkages includes vinculin, FAK and paxillin among others (Sun, Tseng et al. 2016, Carisey et al. 2013). Talin unfolding leads to cryptic binding sites to vinculin to become exposed, and allows vinculin to bind to the adhesion (Yao et al. 2016). This binding of vinculin not only strengthens the talin-actin bond but also induces clustering of activated integrins and recruitment of other FA proteins (Humphries, Wang et al. 2007). The resulting adhesion growth will further alter the cell behaviour and force response as an increased number of clutches (e.g. integrins) will now be sharing the force load and prevent increasing force load on individual clutches that in other cases would cause clutch failure on higher rigidities (Elosegui-Artola et al. 2018). This force reinforcement thus allows adhesions to grow in response to stiffness, which also explains why most cell types exhibit a monotonic increase in traction forces as a response to increasing stiffness's. In a scenario where talin is depleted from the molecular clutch system, adhesion reinforcement becomes inhibited, and cells that otherwise show a monotonic stiffness-force relationship switch to a bi-phasic force relationship consistent with the first molecular-clutch predictions of force transmission (Elosegui-Artola et al. 2016).

To conclude, the dynamic nature of the molecular clutch enables cells to sense and respond to changes in the rigidity via the ECM-integrin-talin-adaptor protein-actin cytoskeleton molecular-clutch. While changes in any of the parameters of the molecular clutch components will have an effect on the force transmission, the clutch is more sensitive to changes in clutch parameters than to changes in the motor parameters. Furthermore, the different layers of force transmission and the numerous components playing a part in force transduction offers the cell a wide variety of ways to sense and alter its behaviour as a result to changes in the environment. In addition, the molecular clutch model provides a useful tool for understanding how the actin cytoskeleton generated forces are transmitted to the ECM and mediates cell migration. Furthermore, the model aids us in understanding how alterations to specific components of the clutch would affect force

transmission and transduction, since simulations of force transmission allows manipulations of the different parameters and the prediction of the resulting force changes.

3   Aims

Integrins are transmembrane receptors that mediate a physical connection of the cell interior to the cell exterior. By binding to extracellular ligands and recruiting a plethora of intracellular adaptor proteins, integrins form adhesion complexes that connect the intracellular actin cytoskeleton to the extra cellular matrix (ECM). These adhesion complexes are rich in mechanosensitive proteins, and thus allow the cell to probe the stiffness of the ECM in addition to exert actomyosin generated forces on the ECM. The rigidity of the ECM can also affect cell behaviour as intracellular mechanosensitive proteins can undergo force induced conformational changes to induce biochemical signalling events. Integrin mediated adhesions are thus vital for both regulating mechanosensitive processes such as migration and gene expression, but also important in regulating tissue remodelling events, as for instance ECM remodelling during development or wound healing. In solid tumors the stromal cells in the tumor microenvironment induce extensive remodelling of the ECM, a process that shares similarities with physiological remodelling processes such as wound healing and during embryonic development. However, in cancer, the remodelling is often uncontrolled owing to deregulated integrin activity as well as other mechanisms. Thus, by understanding the interplay between integrin activity regulation and the ECM remodelling processes, we can learn more about the crosstalk between cancer cells and their microenvironment.

While many studies have investigated the role of stiffness in inducing integrin activation and the role of integrin activators in these processes, not much is known about the interplay between integrin inactivators, integrins and the ECM. Our group has previously identified SHARPIN as an endogenous inhibitor of integrin activity. However, the role of SHARPIN in regulating integrin mediated mechanosensitive processes has remained unknown. In this thesis, I have therefore addressed the role of SHARPIN in mammary gland development and further investigate how SHARPIN affects mechanotransduction by fibroblasts.

During cell migration, adhesion turnover is facilitated by integrin unbinding, plasma membrane diffusion as well as integrin endocytosis and recycling. Integrin endocytosis is highly affected by the activity state of integrins and the integrin-ECM mediated cytoskeletal- and membrane tension. This allows for spatially controlled

recycling of integrins to the protruding area of cells where new adhesions are formed and thus supporting directed cell migration. What the role of plasma membrane delivery of newly synthesized integrins in polarised cell protrusion and migration is currently not understood. Furthermore, the role of extracellular ques in influencing the trafficking and maturation of newly synthesized integrins is yet to be defined, along with their potential role in mechanosensing. By utilizing novel methodology, I have investigated the role of newly synthesized integrins in the above-mentioned processes.

The specific aims of this thesis are:

I. Characterization of the role of SHARPIN in the developing mammary gland

II. Investigate the effect of SHARPIN mediated integrin activity regulation in mechanotransduction

III. Identify the role of newly synthesized integrins in mechanosensing and investigate the role of ECM in regulating the maturation and localization of newly synthesized integrins

4 Materials and Methods

4.1 Animals (I, II)

The inbred mouse strain C57BL/KaLawRij-*Sharpin*[cpdm]/RijSunJ, with a spontaneous mutation leading to the complete loss of SHARPIN (HogenEsch, Gijbels et al. 1993, Seymour, Hasham et al. 2007) was acquired from The Jackson Laboratory (Stock number: 007599, Jackson Laboratory, Bar Harbour, USA). C57BL/KaLawRij-+/*Sharpin*[cpdm], were crossed to generate C57BL/KaLawRij-*Sharpin*[cpdm]/*Sharpin*[cpdm], C57BL/KaLawRij+/+, and C57BL/KaLawRij-+/*Sharpin*[cpdm] mice. Heterozygotes were mated for maintaining the colony and mice were genotyped for the *Sharpin*[cpdm] mutation. KAPA Mouse Genotyping Kit (Merck, KK7301) was used to extract DNA and the *Sharpin*[cpdm] mutation detected using 40x genotyping assay mix (TaqMan SNP Genotyping Assays, 5793982, Applied Biosystems) and TaqMan Universal PCR Master Mix (4304437, ThermoScientific). Mice were examined for mating plug appearance to time mating, and sacrificed at age P15. C57BL/KaLawRij-*Sharpin*[cpdm]/*Sharpin*[cpdm], female mice (homozygous for the *cpdm* allele, from here on *Sharpin*[cpdm]) and their wild-type (C57BL/KaLawRij+/+, from here on wt) or heterozygotic (C57BL/KaLawRij-+/*Sharpincpdm*, with a wt phenotype, from here on also included as wt) littermates were used in the experiments, at indicated ages. As the *Sharpin*[cpdm] and wt mice had not established an oestrus cycle by the time samples were collected (not older than 49 days) they were not synchronised for oestrus.

A conditional SHARPIN knockout strain was created at The Jackson Laboratory. Briefly, transgenic mice with a S100 calcium binding protein A4 (S100a4, also known as fibroblast-specific protein 1, FSP-1) promoter for *cre* (BALB/c-Tg*(S100a4-cre)*1Egn/YunkJ, stock no. 012641, The Jackson Laboratory) where mated with conditional *Sharpin* null mice (B6(Cg)-*Tyr*[c–2J] *Sharpin*[tm1Sun]/Sun, stock number 012641, The Jackson Laboratory). First generation mice, heterozygous for both *Sharpin* and *cre*-recombinase, were crossed to produce a second generation that were genotyped for *Sharpin* and *cre*-recombinase. Detailed description of the generated mouse strain and its phenotype have been described in (Sundberg, Pratt et al. 2020).

All mice were housed under standard conditions with 12 h light/dark cycles and food and water available *ad libitum.* All animal experiments were authorized and ethically assessed in accordance with The Finnish Act on Animal Experimentation by the National Animal Experiment Board (Animal licence number 7522/04.10.03/2012) and The Jackson Laboratory Animal Care and Use Committee (approval number 07005).

4.2 Mammary gland cell isolation and culture (I, II)

Utilization of primary cells in research is both more expensive and experimentally more demanding than using stable cell lines. Primary cells often tend to proliferate slower and to a limited extent *in vitro*. They are also more heterogeneous which results in more variation when comparing repetitive experimental results. On the other hand, primary cells are closer replicating the *in vivo* setting and the heterogenous cell population allows for a more accurate insight of the biological events taking place in an organism. The limited proliferation capacity also results in a lower amount of genetic alterations that can cause confounding results when using stable cell lines (Hughes, Marshall et al. 2007). In addition to spontaneous gene mutations taking place over time, also environmental factors can affect the gene profile over time. Most available stable cell lines have a history of being cultured on stiff plastic or glass surfaces, which are magnitudes stiffer than the *in vivo* environment. As cells have the ability to adapt to substrate stiffness by altering gene expression of proteins such as integrins (Yeung, Georges et al. 2005), the gene profile of stable cell lines cultured on plastic can differ to quite an extent from the gene profile of primary cells and are hence not an optimal model for studying mechanotransduction events. In this thesis, primary cells from the mouse mammary gland have been used to study the role of SHARPIN in regulating integrin activity and how this affects the mechanotransduction of the developing mammary gland (Original publication I, II). In addition to being more expensive and sensitive to work with, primary cells tend to have a lower transfection efficiency than stable cell lines, which can sometimes result in methodological limitations. As all experiments performed in Original publication III required transient transfection of an integrin construct, primary cells were not utilized in that project.

The 2nd, 3rd, 4th and 5th mammary glands from 2–4 wt and *Sharpin^{cpdm}* virgin mice 6–7 week-old were collected for cell isolation. Mammary glands were removed aseptically, without lymph nodes, and minced extensively by using surgical scalpel blades. To further digest the tissue, incubation in 25–30 ml of digestion media [DMEM (Dulbecco's modified Eagle's medium)/F12, 5% foetal calf serum (FCS), 5 μg/ml insulin, 50 μg/ml gentamicin] containing 2 mg/ml collagenase type XI (SCR103, Sigma) was carried out in a 37 °C shaker (120 rpm) for 2-3 h. The digested

tissue was centrifuged 10 min at 400 g and floating fat cells discarded. The cell pellets were resuspended in isolation media (DMEM/F12, 50 µg/ml gentamicin, 1 % penicillin/streptomycin) with 20 U/ml DNase I (Roche) and incubated for 3 min at room temperature with occasional shaking. Cells were pelleted and resuspended in isolation media by pipetting up and down 10 times to disaggregate cells. Disaggregated cells were pulse centrifuged to 1500 rpm followed by resuspension and disaggregation 4 times. The supernatant was collected after each round of pulse centrifugation for mammary gland stromal fibroblast isolation. After the final pulse centrifugation, the pellet containing mammary epithelial cells (MEC) was collected. To obtain single cells, MECs were trypsinised and pushed through a 70 µm cell strainer (352350, BD Biosciences). The collected supernatants containing MSFs was pelleted and cells were resuspended in fibroblast growth medium (DMEM/F12, 5% FCS, 1% L-glutamine, 1% penicillin/streptomycin). To remove dead cells, medium was replaced the following day. Passages 1–4 of the primary MSFs were used in experiments if not indicated otherwise. A minimum of two independent isolates of MSFs per genotype were used in each experiment.

4.3   Cell culture (I, II, III)

Multiple cell lines were used for the studies, all cell lines and their growth medium are listed in Table 1. All cell lines were grown in a humified incubator at 37 ºC supplemented with 5 % CO_2. All non-primary cell lines were routinely tested for mycoplasma contamination.

4.3.1   Transient transfections of cells, siRNAs and plasmids

To deplete gene expression in MSFs Lipofectamine RNAiMax (13778150, Thermo Fisher) was used to introduce RNAi oligonucleotides into cells. Briefly a mixture in 4:3 ratio (µl RNAiMax: µl siRNA) was prepared in 200 µl Opti-MEM and incubated for 20 min before adding to adhered cells grown on a 12-well plate in 400 µl fresh media and cells incubated for 48 h. Allstars negative control siRNA (1027281, Qiagen) was used as a control siRNA in all experiments. The SHARPIN targeting siRNA (5′-GCUAGUAAUUAAAGACACAd(TT)-3′) was custom ordered from Qiagen. ON-TARGETplus siRNA SMARTpools were ordered from Dharmacon: Mouse Itga1 (109700);
(5'-CUUUAAUGACGUCGUGAUU-3', 5′-GCCUAUGACUGGAACGGAA-3', 5′-CCACAAUUGACAUCGACAA-3', 5'-AGGGCAAGGUGUACGUGUA-3') and Mouse Itga11 (319480); (5'-AUGGAUGAGAGGCGGUAUA-3′, 5'-UCAGAAGACAGGAGACGUA-3', 5'-GCAUCGAGUGUGUGAACGA-3', and 5'-CCAGCGACCCUGACGACAA-3').

To deplete gene expression in U2OS cells, Lipofectamine 3000 or Lipofectamine RNAiMax were used to introduce oligonucleotides into cells. Reverse transfection was performed for transfections done with Lipofectamine 3000. GRASP65 and GRASP55 were downregulated with Flexitube siRNAs (GS64689 and GS26003 respectively, Qiagen). In addition, custom ordered siRNA oligonucleotides targeting GRASP65 and GRASP55 where ordered from Qiagen:

GRASP65: (5′-AAGGCACUACUGAAAGCCAAU-3') and

GRASP55: (5′- AACUGUCGAGAAGUGAUUAUU-3')

Western blotting of total cell lysates was carried out to determine the knockdown efficiency at protein level. Transient transfection of plasmids into cells was carried out by using Lipofectamine 3000 (L3000075, ThermoFisher), Lipofectamine 2000 (11668019, ThermoFisher), jetPRIME® (114-75, Polyplus transfection) or DNAIn (GST-2131, MTI Global Stem) according to manufacturer's instructions. Cells were incubated for 24 h before carrying out experiments. Plasmids used in this thesis are listed in Table 2.

4.4 Preparation and imaging of cell samples (I, II, III)

Several different microscopes were used for the work included in this thesis, the 3i Marianas Spinning disk confocal microscope was most often chosen for its long working distance, ability to capture several focal planes and channels fairly rapidly. This was an advantage especially while performing live cell imaging of co-transfected cells and when imaging samples on polyacrylamide hydrogels which increases the thickness of the samples. For studying basal adhesion structures on flat surfaces, the Total internal reflection fluorescence microscopy (TIRF) microscopes were chosen because of their excellent signal-to-noise ratio. Most of the image analysis was performed in ImageJ as it is an open source image processing platform with thousands of useful plugins. The specific details of the imaging set-up, microscopes and objectives used, along with image analysis of each experiments can be found in the original articles I, II and III.

4.4.1 Ligand-coating, antibodies and fluorescent dyes (I, II, III)

Coverslips used for immunofluorescence experiments were acid washed and coated with 10 µg/ml Poly-D-Lysine (A003E, Sigma) for 30 minutes followed by PBS wash. In addition to coverslips, 35 mm imaging dishes with a polymer coverslip (80136, Ibidi) were used. Ligand coating (20 µg/ml) was performed for 1h at RT or overnight at +4 °C with either collagen type I (08-115, Merck-Millipore) or

fibronectin (341631, Merck-Millipore). Immunolabelling with antibodies and fluorescent dyes was employed in several methods throughout the project. The specific labelling protocols used for each method are described with the below-mentioned methods. Primary antibodies are listed in Table 3. HRP-linked secondary antibodies against rat, rabbit and mouse IgG were obtained from GE Healthcare and used in immunolabelling and Western blotting. Also IRDye680 and IRDye800 (LI-COR) secondary antibodies against mouse and rabbit were used in Western blotting. AlexaFluor 488-, 555-, 568, and -647 IgG secondary antibodies against rat, mouse and rabbit were all obtained from Invitrogen, Life Technologies and used in flow cytometry, immunofluorescence and immunohistochemistry. Nuclei were stained with DAPI (D1306, Life Technologies) and Phalloidin–Atto 647N (65906, Sigma) was used for F-actin staining.

4.4.2   Live cell imaging

Cells were seeded and allowed to spread for a minimum of 2 h on ligand-coated dishes or coverslips prior to imaging of live cell samples, unless otherwise stated. Live cell imaging was carried out at 37 °C in presence of 5% CO_2 (or in HEPES buffered media when CO_2 was not applicable).

4.4.3   Imaging of fixed samples

Cells were seeded and allowed to spread for a minimum of 2 h on ligand coated dishes or coverslips prior to fixation and permeabilisation with 4 % PFA and 0.1 % Triton X-100. To inhibit unspecific binding of the antibodies, samples were blocked with 10 % HRS and 1 M Glycine was used to quench background signal caused by free aldehydes. All primary and secondary antibodies were diluted in 10 % HRS and incubated for a minimum of 1 h at RT. Samples were washed in between and after antibody incubations. F-actin and nuclei were co-labelled with phalloidin and DAPI in indicated cases. All antibodies used are listed in Table 3, with the exception of the mouse anti-integrin α11 antibody, which was developed and provided by Donald Gullberg (Zeltz, Alam et al. 2019). When this antibody was used, samples were fixed with methanol, instead of PFA, for 10 min at -20 °C, the antibody was diluted in a 1:200 ratio.

4.5   Flow cytometry (I, II, III)

All cell samples were immediately placed on ice and cells were detached using HyQtase™ (SV30030.01, GE Healthcare). To label cell surface proteins, pelleted cells were resuspended and incubated with primary antibodies diluted in Tyrode's

buffer (10 mM HEPES-NaOH at pH 7.5, 137 mM NaCl, 2.68 mM KCl, 1.7 mM MgCl2, 11.9 mM NaHCO3, 5 mM glucose, 0.1% BSA). In cases were fluorochrome-conjugated primary antibodies were not used, samples were washed and incubated with secondary antibodies also diluted in Tyrode's buffer. Depending on the experiment, cells were either fixed prior to, or after antibody labelling, all samples were fixed with PFA and washed prior to being analysed using the BD LSRFortessa flow cytometer. FSC-A/SSC-A dot blot was used to gate live cells. Flowing software version 2 (Cell Imaging Core of the Turku Bioscience Centre) was used for data analysis. All antibodies used are listed in Table 3.

4.6   Pulldowns and immunoblotting (I, II, III)

Pulldowns were performed by lysing cells expressing GFP-constructs with immunoprecipitation-lysis buffer (40 mM Hepes-NaOH, 75 mM NaCl, 2 mM EDTA, 1% NP40, protease and phosphatase inhibitors) for 30 min at +4 °C. Lysates were pelleted and supernatant was incubated with GFP-Trap beads (gtak-20, ChromoTek) for 55 min at +4 °C. The GFP-Trap binding pulldown complexes were washed with wash-buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % NP-40) and denatured in reducing Laemmli buffer. Pulldown with biotinylated peptides was carried out by incubating N-terminally biotinylated peptides (custom ordered from Genescript) with streptavidin conjugated Dynabeads (65001, ThermoFisher) for 30 min at RT. The peptide-bead complexes were then incubated for 2 h at +4°C with supernatant from EGFP-GRASP65 overexpressing cells (prepared as described above for GFP-pulldown samples), followed by washes with wash-buffer (50 mM Tris-HCl pH 7.5m, 150 mM NaCl and 1 % NP-40) and denaturation.

   Total protein extracts were likewise prepared in denaturing buffer, and both pulldown samples and total protein samples were run on SDS–PAGE for separation. Separated samples were transferred to the nitrocellulose membrane with Trans-Blot Turbo Transfer Pack (170-4159, Biorad) followed by 1 h block with LI-COR blocking solution (LI_COR, bioscience) or in 5% milk in Tris Buffered Saline and 0.1% Tween 20 (TBST). Blocked membranes were incubated for a minimum of 1 h with primary and secondary antibodies. Membranes were washed 3 times with TBST in between the incubations and before scanning, all antibodies were diluted in blocking solutions and are listed in Table 3. Membranes were scanned with BioRad ChemiDoc MP Gel Analyzer, an infrared imaging system (Odyssey; LI-COR Biosciences) or Azure Sapphire RGBNIR Biomolecular Imager. Acquired images and protein band intensities were further analysed in ImageJ.

4.7   Methods for studying mechanotransduction

While a large repertoire of cell biological tools exist for studying gene and protein activities, methods for investigating mechanical forces applied by cells or onto cells are much more limited. This is partially because cell generated forces are very small to their nature and therefore challenging to measure directly. These forces can however be measured indirectly by examining cell generated deformation of substrates or even molecules (Polacheck, Chen 2016, Y. Zhang, Ge et al. 2014). The methods available vary greatly in preciseness of output, ease of use, and required recourses. In addition, there is often an inverse correlation between force resolution and ease of use. One of the most simple methods for studying substrate deformation is by collagen contraction assays. In this assay, cells are embedded inside a 3D collagen matrix with a known surface area. The contractility of the cells can then be measured over time by examining the change in surface area of the matrix over time. This method does not require high-end microscopes or complex data analysis but on the other hand, the method only provides macroscopic measurements of deformation (usually over days) and does not allow for determination of forces neither the contraction generated by individual cells. In contrast, several different tension sensors, such as DNA hairpin tension probes, have been developed to measure and map forces with as low as pico Newton sensitivity. While these tension sensors provide magnitudes higher resolution of forces, they require high expertise and sample preparation time.

4.7.1   Preparation of polyacryl-amide hydrogels (I, II)

Most cell biological studies are performed in 2D settings on glass or plastic substrates but a growing appreciation for using various substrates with stiffness's resembling the *in vivo* stiffness has emerged during the past decades. Substrate stiffness affects various cell properties and processes such as morphology, gene expression, migration and differentiation (Y. Yang, Wang et al. 2017). In these studies we have utilized polyacrylamide hydrogels in some of the experiments to allow for *in vitro* studies in an environment that partially resembles the *in vivo* environment of cells. While 2D polyacryl-amide gels allows for a partially *in vivo* mimicking environment it still lacks the 3D effect of the ECM along with the rheology and interplay of the different ECM components such as collagen fibrils. However, the utilization of 2D gels is technically easier and more versatile than the utilization of 3D models. Thus, culturing cells on 2D polyacrylamide hydrogels allows for a middle ground between unphysiologically stiff plastic or glass substrates and more complex 3D cultures that are methodologically more limited and challenging for studying integrin-ligand interactions in response to stiffness.

Polyacryl-amide hydrogels were prepared in-house by treating 35 mm glass bottom dishes with 14 mm bottom wells (Cellvis, catalog number D35-14-1-N) with 1 ml Bind Silane solution (7.14% Plus One Bind Silane (GE17-1330-01, Sigma), 7.14% acetic acid in 96% ethanol) for 30–60 minutes and washed twice with 96 % ethanol. The glass bottom dishes were left to dry completely while the hydrogel mixture was prepared. To obtain hydrogels ranging in stiffness from 0.7–13 kPa, a mixture containing 4-18 % acrylamide solution (A4058, Sigma) and 0.03-0.4 % bis acrylamide solution (M1533, Sigma) was prepared and diluted in PBS up to a final volume of 500 µl. To initiate polymerization of the mixture, 5 µl 10 % ammonium persulfate (1610700, BioRad) and 1 µl N,N,N′,N′-Tetramethylethylenediamine (T9281, Sigma) was added and the mixture was quickly vortexed. A drop of 11.8 µl hydrogel mixture was added on top of the dry glass bottom dish, a 13 mm glass coverslip was placed carefully on top of the drop, and the gel left to polymerize for a minimum of 1 h at RT. After polymerization, 1 ml PBS was added to the dish and the coverslip carefully removed from the polymerized gel. The precise Young's modulus of the polyacryl-amide hydrogels were confirmed with atomic force microscopy (AFM). The gel surface was activated to allow ligand binding. The gels were incubated for 30 min with Sulfo-SANPAH activation solution (0.2 mg/ml Sulfo-SANPAH (803332, Sigma), 2 mg/ml N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (03450, Sigma) in 50 mM Hepes) on slow agitation and shielded from light in RT, followed by 10 min UV-light activation. Activated gels were washed three times with PBS and coated with ECM ligand. Activated 2 kPa hydrogels were coated with fibronectin (341631, Merck-Millipore) or collagen I (08-115, Merck-Millipore) at a range of concentrations (0.1–50 µg/ml) to determine a saturating concentration of ECM coating. Coated gels were blocked with 5 % bovine serum albumin (A3294, Sigma) and then incubated with anti-fibronectin (F3648, Sigma) or anti-Collagen I (NB600-408, Novus Biologicals) primary antibodies. Washed samples were incubated with the secondary antibody Alexa fluor 568 anti-rabbit (A10042, Thermo Fisher) and the intensity imaged by confocal microscopy. Subtraction of background fluorescence (measured from non-ligand coated, stained samples) was done and the relative fluorescence intensity was calculated for each ligand. Based on the results, a saturating concentration of 20 µg/ml for each ligand was used for coating of in-house prepared polyacrylamide hydrogels.

4.7.2   Atomic force microscopy (I, II)

The stiffness of different materials such as skin tissue or cell culture substrates can be measured with various methodologies. The elastic modulus of large samples, as for example skin, can be tested by both compressing or stretching a tissue sample

while recording the extension and load (Griffin, Premakumar et al. 2016). In contrast, particle-tracking micro rheology can be used to measure the elasticity of cells. This method allows for high spatial resolution but at the same time the injected and tracked particles needed for the measurements, might affect the mechanical properties of the cell (Thomas, Burnham et al. 2013). In these studies, we chose to use atomic force microscopy (AFM) as it can be used to measure the stiffness of not only small tissue samples, but also the stiffness of different regions of a specimen or even a cell (Engler, Rehfeldt et al. 2007). All AFM measurements were carried out using a JPK NanoWizard II AFM with a CellHesion module (JPK Instruments), mounted on a Carl Zeiss confocal microscope Zeiss LSM510 (Carl Zeiss AG). Force indentation was performed on four hydrogel samples obtained from two independent preparations. Three repetitions of 100 indentation curves distributed in a 10×10 grid (100×100 μm^2) were performed for each hydrogel. Triangular cantilevers of silicon nitride and with a spring constant of 0.1 Nm^{-1} were custom fitted with 4.5 μm (in diameter) broad borosilicate glass spheres (Novascan Tech) and calibrated before each experiment using the thermal noise method (Hutter, Bechhoefer 1993). Gels were indented at 37 °C in PBS. For indentations of tissue sections, the fourth mammary glands of female mice were snap-frozen, embedded in O.C.T.-compound in TissueTEK® standard plastic moulds and cut with Cryostat Leica CM 1950 into 30 μm sections directly on 22-mm coverslips and refrozen. Indentation was performed in 3–6 sections of 20 μm thickness. Totally, 21 mammary gland slices from 6 animals were analysed, coming from three independent mice per condition. 192 indentation curves per slice, distributed in three regions, were performed with an 8×8 point grid (100×100 μm2) in each region. Measurements were carried out at 37 °C in DMEM supplemented with 10% FCS. Cantilevers with a 0.06 Nm^{-1} were fitted with glass spheres with a 5.0 μm diameter. Measurements were carried out at 37 °C in DMEM supplemented with 10% FCS. The deflection sensitivity was determined using glass substrates as an infinitely stiff reference material. The Hertz model (Hertz 1896) of impact was used to determine the elastic properties of the sample when a calibrated force of 5 nN was applied onto the sample. The JPK data processing software (JPK DP Version 4.2) with an input Poisson's ratio of 0.5 was used to calculate the Young's elastic modulus.

While AFM is widely used to determine the stiffness of both substrate, tissue and cell samples, it is important to remember that sample properties also largely affect the result. When measuring the elastic modulus of gels, it is important to consider the gel thickness as the stiffness of the underlying substrate might contribute to the result if very thin gels of a soft nature are used. Furthermore, the homogeneity of the sample also affects the measurements (Frey, Engler et al. 2007). While PAA gel substrates can be fairly homogenous in stiffness, this is rarely the case for tissue sections where both cell and ECM properties vary greatly within the sample. For

heterogeneous samples, it is better to use spherical cantilever tips instead of triangular shaped tips as the point of contact with the sample will be greater and a more accurate bulk stiffness of the sample can be detected. On the other hand, triangular tips, with a small sample-contact area, have the benefit of improving the accuracy and resolution of the measurement (Engler et al. 2007). The heterogeneity of the sample should therefore be taken into consideration when planning the experiment, i.e. the shape of the cantilever tip (sharp or spherical), and the number and locations of sample indentations.

4.7.3   Traction force microscopy (I, II)

Cellular traction forces can be measured by various methods such as microfabricated thin films or micropillars. While these two methods are equally useful for measuring cell generated forces, and to some extent even easier to employ than traction force microscopy, their fabrication requires specific expertise. In these projects we have employed 2D traction force microscopy (TFM) to study the force generation capacity of MSFs. TFM is based on tracking cell generated deformations of elastic substrates to measure cellular force. In 2D and 2.5D TFM, cells are seeded on top of a gel that is embedded with fluorescent beads. Cell contractions will deform the substrate and the fluorescent beads will move in position as a result of this deformation. By acquiring images of the beads within the gel both in a relaxed state (without a cell contracting the gel) and in a stressed state (with cells contracting the gel), cell generated forces can be calculated based on the displacement of beads and the stiffness of the gel. As the bead displacement is critical for calculating the forces, TFM is also very prone to errors. This is because small shifts in bead locations caused by other factors then cell contractions (e.g. sample drifting during imaging), can result in large errors in force calculations. It is therefore important to carefully examine the acquired relaxed and stressed images before force calculations, and to apply image processing (e.g. image alignment) when needed. In 2.5D TFM, the traction field is calculated not only based on the x, y displacement of the beads but also the z-plane displacement is taken into consideration. In this system, the cells are embedded on top of the 2D gels and the adhesions are thus not resembling the *in vivo* adhesions any more than in 2D TFM (Polacheck, Chen 2016, Legant, Choi et al. 2013). In 3D TFM, cells are embedded within the gel, allowing cells to establish adhesions more closely replicating *in vivo* adhesions than on 2D. While this method is more accurate than the 2D TFM, the computational power and expertise required to resolve the force maps is much more demanding (Legant, Miller et al. 2010). While all the above mentioned TFM methods provides valuable information about the spatio-temporal force generation of cells, the set-ups of the systems all still lack the precise composition and rheology of *in vivo* ECM. Therefore, TFM can be

considered when studying the effect of different components in the mechanotransductive pathway and how these affect force generations of cells. The absolute force values from different TFM set-ups and experiments should however not be given too much value as many factors affects the force calculations and might give rise to false force calculations.

In-house polyacryl-amide hydrogels were prepared as above, with the addition of FluoSphere™ bead solution (0.2 μm, 505–515 nm; F8811, Invitrogen) to 8% of the volume before initializing the polymerization. Cells were seeded on hydrogels 4 h before performing experiments. Images of single cells and the fluorescent beads in the underlying gel were acquired with either a Nikon Ti Epifluorescence microscope with a 40x objective (N.A. 0.6), or a Spinning-disk confocal 3i (Intelligent Imaging Innovations, 3i Inc) microscope with a Yokogawa CSU-W1 scanner and back illuminated 10 MHz EMCDD camera (Photometrics Evolve) with a 40x objective (N.A. 1.1). Cells were detached by adding 10x trypsin or 10% Triton-X, and a second set of images of the beads, now in the relaxed gels as the cells were detached, was acquired. As in-house gels do not have a perfectly even surface, Z stacks of 15–30 images with a distance of 1 μm were performed and the best focus planes were chosen to create an average intensity projection in ImageJ to ensure good quality of fluorescent bead analysis. Bead positions in the deformed versus relaxed positions were compared, and a map of gel displacement was measure by using a custom particle-imaging-velocimetry software (Bazellières, Conte et al. 2015). Assuming that the gel displacement were caused by cells exerting forces on the gel-cell contact area, forces were measured using Fourier transform algorithm, previously described in (Butler, Tolić-Nørrelykke et al. 2002, Oria, Wiegand et al. 2017) and the average force per unit area was measured of each cell.

4.7.4 Actin retrograde flow measurements (II)

mEmerald-Lifeact-7 (GFP-LifeAct) transfected cells were seeded on 2 kPa collagen coated polyacryl-amide hydrogels for 45–105 minutes prior to imaging. Images of the actin cytoskeleton were acquired every second for 125 seconds. Kymographs were generated by drawing lines along actin fibres close to the cell periphery at the leading edge of the cell. The actin flow per cell was calculated based on the slope of the actin signal in the generated kymograph.

4.7.5 Bead recruitment and magnetic tweezer experiments (II)

AFM, optical tweezers and magnetic tweezers are commonly used methods to investigate binding forces between two molecules. In this study we have investigated

the binding forces between integrins and their ligands by the use of magnetic tweezers. While force measurements with magnetic tweezers don´t have as good spatial or temporal resolution as optical tweezers, they are more fitted for live cell experiments as magnetic tweezers do not cause photo damage. In addition, as live cell experiments require cells to be in growth media, both AFM and optical tweezer experiments can suffer from unspecific interaction with e.g. cell debris possibly present in the growth media, while magnetic tweezer experiments are less sensitive to these (Neuman, Nagy 2008).

To quantify integrin β_1 recruitment to ligands, 3 µm carboxylated silica beads (Kisker Biotech) were coated with either biotinylated pentameric FN7-10 (a four-domain segment of fibronectin containing the RGD motif (Coussen, Choquet et al. 2002)) or with biotinylated GFOGER (a synthetic triple-helical collagen peptide with high affinity for collagen-binding integrins (W. Zhang, Käpylä et al. 2003) diluted 1:200 in biotinylated BSA. Cells were seeded and allowed to spread where after coated beads were deposited onto cells and allowed to attach for 30 minutes. Non-attached beads were washed off and samples were fixed and stained for integrin β1. Immunofluorescence imaging was performed and the average intensity of both beads and surrounding area was quantified. The integrin recruitment was defined as the difference between those two values (González-Tarragó, Elosegui-Artola et al. 2017). To measure the detachment time of integrin-ligand bonds, 3 µm carboxylated magnetic beads (Invitrogen) were coated and introduced to cells as described above. After bead attachment, beads at the lamellipodia of cells were pulled with a 1 nN pulsatory force (1 Hz) and the bead detachment time was measured.

4.8   Prediction of force transmission by mathematical modelling (II)

Modelling of the forces were performed using the molecular clutch model described previously in detail (Elosegui-Artola et al. 2014). The model considers the number of myosin motors pulling on an actin fibre. The actin fibre can bind to a set of ECM ligands through integrins and adaptor proteins that together form molecular clutches. The ECM ligands are connected to the substrate through a spring constant that represents substrate stiffness. Molecular clutches bind to the ECM ligand with an effective binding rate, and unbind with an unbinding rate. The unbinding rate values used here are based on previously experimentally determined values for the FN-$\alpha5\beta1$ single molecule bond, where the unbinding rate depends on force with a catch bond behaviour (F. Kong et al. 2009). As clutches only transmit forces to the substrate when they are bound, the overall force transmission will critically depend on the binding dynamics. Modelling was carried out by keeping some of the parameters constant, while changing others, based on both our experimental set-ups and the

obtained results (in original publication II). As there, to our knowledge, are no reported measurements of the systematic force-life-time at the single molecule level for integrin α11β1 (or other collagen-binding integrins), we used the catch bond dependence reported for integrin α5β1 in (F. Kong et al. 2009). All parameters used for modelling are listed in Original Publication II, Table S1.

4.9   Micropatterning (I, III)

Cells were seeded and grown on micropatterns to allow investigation of different processes in an environment where cell spreading is confined to controlled shapes and areas. Micropatterns were produced as described earlier by (Azioune, Storch et al. 2009). In the case of dual-coated micropatterns, the PLL-*g*-PEG was replaced with biotinylated PLL-*g*-PEG (PLL(20)-*g*[3.5]-PEG(2)/PEG(3.4)-biotin(50%), SuSoS). After the coating of the micropatterned area, the coverslips were blocked with 3% BSA for 1h at RT. Coverslips were then washed and a second round of coating, now of the non-patterned area, was performed using streptavidin conjugated ligand fragments. Streptavidin conjugation was performed using the FastLink Streptavidin Labeling Kit (KA1556, Abnova) according to manufacturer´s instruction.

For studying collagen degradation in MSFs, cells were plated for 4 h on crossbow shaped micropatterns coated with type I collagen from rat tail (20 µg/ml; 08-115, Millipore) or DQ™ collagen type I fluorescein conjugate (100 µg/ml; D12060, Life Technologies) and AlexaFluor 647-conjugated human plasma fibrinogen (5 µg/ml; Molecular Probes). Samples were fixed in 4 % PFA for 10 min, followed by staining with AlexaFluor 546 phalloidin (Life Technologies) and DAPI. Stained samples were washed and finally mounted in Mowiol containing DABCO (10981, Sigma) before imaging with a Zeiss LSM780 laser scanning confocal microscope. The fluorescent micropattern was used to align and assemble images of all cells in one condition into one stack to generate average intensity maps of the DQ™ collagen (Théry, Racine et al. 2006, Vonaesch, Cardini et al. 2013). A heat map was then applied to the Z-projection of the image to allow examination and interpretation of the results.

4.10   Collagen contraction (I)

While collagen contraction assays do not allow for the measurement of forces per se, it is one of the most straightforward methods for investigating cell-generated contraction of substrates (Polacheck, Chen 2016). Because of its ease of use, fast sample analysis and low requirement on laboratory equipment, it is a popular method for studying cell-generated contraction of ECM. In this study we used the collagen

contraction assay as a first method to study whether SHARPIN affects the ability of MSFs to contract collagen. MSFs were detached, counted and 1×10^5 cells were resuspended in 30 µl medium and mixed with 170 µl PureCol® EZ Gel Bovine Collagen Solution, type I in DMEM/F-12 medium (5074, Advanced Biomatrix). The MSF-Collagen I mixture was seeded on a 48-well plate (V= 170 µl/well) or a 24-well plate (V= 500 µl/well) and allowed to polymerize for 1–2 h at 37 °C. After polymerization, MSF growth medium was added and the collagen plug was gently detached from the cell culture well edges. The collagen plug area was imaged with Bio-Rad ChemiDoc MP gel analysis instrument after 24 h (48-well) or 72 h (24-well) and the plug area quantified in ImageJ. Average plug areas per experiment were normalised to wt or ctrl siRNA transfected cells.

4.11   Production of cell-derived matrix (I)

Cell-derived matrices (CDM) were prepared as previously described (Cukierman, Pankov et al. 2001, Kaukonen, Jacquemet et al. 2017). Briefly, coverslips were coated at RT for 1 h with 0.2% gelatin (G1393, Sigma) in PBS and gelatin was cross-linked with 1% glutaraldehyde for 30 min at RT followed by 20 min incubation with 1 M glycine at RT. Low-passage wt or *Sharpin^cpdm* MSFs were seeded at a density of 5×10^4 per gelatin-coated coverslip and grown to confluency. MSFs were then treated with 50 µg/ml ascorbic acid containing MSF growth medium to promote collagen synthesis. The ascorbic acid treatment was continued over 10 days and the medium changed every day. To produce acellular CDMs, extraction buffer (0.5% Triton X and 20 mM NH_4OH in PBS) was used to extract the cells followed by 1 h treatment with 10 µM DNAse (11284932001, Roche) at 37 °C. CDMs where then fixed in 4% PFA for 10 min and blocked in 30% Horse Serum (HRS) at RT for 10 min. CDMs were immunolabelled with the Collagen I antibody (NB600-408, Novus) for 1 h at RT, followed by 3 washes and incubation of goat Anti-Rabbit IgG Alexa Fluor 568 or 488 secondary antibody (A-11011 and A-11008, ThermoFisher). Labelled CDMs were mounted with Mowiol and imaged with Zeiss Axiovert 200M with Yokogawa CSU22 spinning disc confocal microscope unit with Hamamatsu Orca ER CCD camera (Hamamatsu Photonics K.K.), 3i CSU-W1 spinning disc confocal microscope (Intelligent Imaging Innovations) with Hamamatsu CMOS Orca Flash 4 or with Carl Zeiss LSM780 laser scanning confocal microscope using 40×/1.2 W objective. Presence of collagen bundles was evaluated from maximum intensity projection images, all results were normalized to wt sample.

4.12 Soluble collagen assay (I)

MSFs were grown to confluency on a 24-well plate and culture medium was replaced to serum-free medium. The conditioned medium from parallel samples was collected after 2, 5 and 7 days of MSF culture. Samples were centrifuged to remove cell debris and supernatants stored at -20 °C until analysis. After the final sample collection, the relative cell number per well was quantified with Cell Proliferation Reagent WST-1 (Roche) and analysed for absorbance at 450 nm with a Multiscan Ascent plate reader (Thermo Scientific). Sirius red (365548, Sigma) assay was performed to quantify soluble collagen (Marotta, Martino 1985). Briefly, 1 ml of 50 µM Sirius red in 0.1 M acetic acid was added to each sample (100 µl) (medium collected from triplicate samples) or collagen standard sample (0–12 µg/ml collagen type I) and samples were incubated at RT for 30 min and mixed every 5 min by inverting the sample tubes. Samples were centrifuged (16,000 g 10 min), to pellet the precipitated collagen and pellets were washed once with acetic acid. The absorbance of the drained, resuspended pellet (in 0.1 M KOH) was analysed at 570 nm with Multiscan Ascent plate reader. To determine the soluble collagen, the standard curve normalized to cell amount was used for each sample. All data were further normalized to the soluble collagen concentration of the wt samples.

4.13 RNA sequencing and bioinformatics (I, II)

MSFs were isolated and grown to confluency without passaging and lysed in RNA extraction lysis buffer. The Nucleospin® RNA kit (740955.10, Macherey-Nagel) was used to DNase treat and extract RNA, purity of sample was analysed with the 2100 Bioanalyzer Instrument (Agilent). To analyse RNA expression, TruSeq RNA Library Preparation Kit v2 (Illumina) was used and RNA sequencing libraries were sequenced with HiSeq2500 (Illumina) TruSeq v3 sequencing chemistry. Twelve samples were run on one lane and single-read was performed with 1x50 basepair read length, followed by 6 base pair index run. The technical quality of the sequencing was good and cluster amount matched expectations. Base calling was performed using standard bcl2fastq software (Illumina) and more than 80% of all bases were above Q30. A reference genome originating from the UCSC database was downloaded from Illumina's iGenomes website (https://support.illumina.com/sequencing/sequencing_software/igenome.html). Tophat (v. 2.0.1) was used to align the raw sequencing reads to mm10 reference genome. HTSeq (v 0.5.4p3) was used for genewise read counting based on RefSeq gene annotations. R (v.3.1) and Bioconductor (v.2.14) was used for downstream data analysis. For raw count value normalization the TMM method of the edgeR package was used. Normalized expression values were further summarized into reads per kilobase of exon per million reads mapped (RPKM), however, these

values were not used in statistical testing. Statistical testing was performed applying voom transformation and the pairing of the samples were taken into account by applying limma package. The differentially expressed genes were filtered in Ingenuity Pathway Analysis software with (P-value < 0.05 and fold-change > 1.5). The differently expressed genes with RNA expression above 0.2 in the most significantly altered canonical pathways were visualized by generation of a heat map with GENE-E matrix visualization and analysis platform (http://www.broadinstitute.org/cancer/software/GENE-E/index.html). The RNA sequencing data set was added to Gene Expression Omnibus (GEO) database repository (GSE83795).

4.14 Evaluation of lineage-specific expression of SHARPIN in the mammary gland (I)

Inguinal mammary glands were collected from adult (P15) virgin wt BALB/c female mice. MSFs were isolated and incubated with fluorescent labelled primary antibodies for 20 min at RT. Labelling was performed based on the lineage-specific expression profiles described previously (Di-Cicco, Petit et al. 2015). Labelled cells were sorted on a FACSVantage flow cytometer (BD Biosciences, USA), and data were analysed using FlowJo software. Purified cell populations were studied for lineage-specific marker genes (basal, *Keratin 5*; luminal, *Keratin 18*; stromal, *Pdgfra*) and *Sharpin* mRNA expression levels determined by qPCR. Moloney Murine Leukemia Virus Reverse Transcriptase, RNase H Minus (M3681, Promega) was used for RNA reverse transcription into cDNA. Quantitative PCR was performed by real time monitoring of the increase in fluorescence of the SYBR® Green dye (S1816, Sigma) in a LightCycler® 480 Real-Time PCR System (Roche Applied Science). The primers used for qPCR analysis: *Sharpin, Krt5*, *Krt18*, *Pdgfr* and *Gapdh* were all purchased from SABiosciences/Qiagen.

4.15 Whole-mount staining and quantification (I)

The fourth mammary glands were whole-mounted by placing them on object glass and left to briefly adhere followed by submerging and fixation overnight at +4 °C in Carnoy´s medium (60% EtOH, 30% chloroform, 10% glacial acetic acid). Tissue was rehydrated in decreasing ethanol series the following day. To allow detection of the epithelial structures in the adipose tissue of the mammary gland, whole mounts were stained over night at room temperature (RT) with Carmine Alum (0.2% carmine, 0.5% aluminium potassium sulphate dodecahydrate). Stained samples were dehydrated followed by 2–3 days of bleaching in xylenes and finally mounted in DPX Mountant for histology (06522, Sigma). The stained wholemounts were

imaged with a Zeiss SteREO Lumar V12 stereomicroscope (NeoLumar 0.8× objective, Zeiss AxioCam ICc3 colour camera). Several images of the same whole mount were combined into a mosaic picture automatically in Photoshop.

To quantify the mammary gland ductal outgrowth, the area covered by the ductal tree in mosaic images of the whole-mounts was measured in ImageJ. The ductal tree was traced manually (of virgin mice) and analyzed with the plug-ins "skeletonise" and "analyse skeleton" in ImageJ to measure branch number in whole-mounts. Terminal end-bud number was measured individually from each whole-mount image in ImageJ.

4.16   Mouse tissue samples (I)

Mouse mammary gland tissue sections that had been Carnoy´s medium-fixed or formalin fixed and paraffin-embedded, were deparafinised, rehydrated and conventionally hematoxylin and eosin stained to allow visualization of the nuclei and the ECM respectively. To allow antibodies access to their epitope (that can be masked during formalin fixation) in immunohistochemistry (IHC) samples, epitope unmasking was performed in citrate buffers using the 2100 Antigen Retriever (Aptum, UK). The unmasked tissue samples were blocked for 45 min with 0.5% Fetal Calf Serum (FCS) in PBS and incubated with primary antibodies (diluted in blocking buffer) over night. Stained samples were washed and incubated for 2 h at RT with fluorochromeconjugated secondary antibodies. To generate cryosections, snap frozen mammary gland tissues embedded in Tissue-Tek® O.C.T. compound (4583, Sakura) were cut in 6 μm sections for HE and IF staining. HE sections were imaged with the inverted wide-field microscope Zeiss Axiovert 200M. Cryosections for IF were further fixed with 4% Paraformaldehyde in PBS, followed by 20 min RT combined block and permeabilization in 2% bovine serum albumin (BSA), 0.1% Triton™ X-100 in PBS. Samples were incubated with primary antibodies in 2% BSA/PBS for 1 h at RT, washed and incubated with fluorochrome conjugated secondary antibodies for 1 h at RT. Finally, samples were washed and incubated with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, D1306, Thermo Fisher) and mounted in Mowiol containing DABCO (10981, sigma) to prevent fading. Samples were imaged with Zeiss Axiovert 200M with spinning disc confocal microscope unit with Hamamatsu Orca ER CCD camera (Hamamatsu Photonics K.K.).

4.17   Second harmonic imaging of mouse tissue samples (I)

To investigate collagen fibres in mouse mammary gland tissue samples, second harmonic generation (SHG) microscopy was employed. Carmine alum-stained

mammary gland whole-mount samples from 6- to 7-week-old wt and *Sharpin^cpdm* mice were imaged with a Leica SP5 MP multiphoton microscope system on DM6000 CFS upright microscope, with a 20× 1.0 W objective, and two photon excitation set to 890 nm wavelength (Leica Microsystems, Mannheim, Germany). To observe a common tissue structure non-filtered emission signal was used, and a 440/20 emission filter was used to separate SHG signal. A z-axis step size of 5.0 μm was used to image individual terminal end buds (TEB) over a whole-tissue volume. Four representative and continues slices, for all TEB stacks were made into maximum projection images, per mouse. 5-13 TEBs from 9 wt mice and 5-12 TEBs from 6 *Sharpin^cpdm* mice were imaged. Some images contained artificial fibres, originating from membrane structures or blood vessels, to exclude these from the analysis, only fibres in manually defined regions of interest were considered. To quantify the collagen fibre phenotype (as clear collagen bundles vs mesh-like, curly collagen fibres), two people performed blind scoring of the maximum projection images that were provided in a randomized order. The percentage of images with the two collagen fibre phenotypes were then calculated and analysed for each mouse.

4.18   Human tissue samples (I)

An Institutional Review Board of the Helsinki University Central Hospital approved the study and formalin-fixed paraffin-embedded human mammary gland tissues were collected from the archives of the Department of Pathology, Helsinki University Central Hospital, Helsinki, Finland. Samples were deparaffinised and rehydrated, endogenous peroxidase activity blocked with 3% hydrogen peroxide followed by epitope unmasking with 10 mM sodium-citrate buffer (pH 6.0). The sample was incubated over night at 4 °C with primary SHARPIN antibody diluted in PowerVision blocking solution (PV6122, Leica). Samples were washed and incubated with the secondary anti-rat antibody Histofine® Simple Stain (414141F, Nichirei Biosciences) for 30 min at RT. Following washes, antibody complexes were counterstained with ImmPACT™ 3, 3 -diaminobenzidine (DAB) Peroxidase Substrate (LS-J1075, LSBio). As the Histofine® Simple Stain contains peroxidases; the DAB-HRP will produce a dark brown precipitate. Nuclei were co-stained with haematoxylin. Following dehydration, samples were mounted and studied with an Olympus BX43 microscope and Lumenera Lt425 camera using a 20× objective (200× magnification).

4.19   Cleared fat pad transplantation (I)

Transplantation was carried out on 3-week-old wt host animals under isoflurane anaesthesia and analgesia (Temgesic, Rimadyl). Mammary gland pieces

(approximately 1 mm^3) isolated from 7-week-old wt or *Sharpin^{cpdm}* female donor mice were transplanted to the fourth pad of the host animal (wt and *Sharpin^{cpdm}* transplants to each side) after clearing the fat pad up to, and including, the lymph node. To confirm complete removal of the host epithelium, the removed pad was fixed and stained. Transplants were analysed after 7–11 weeks in virgin mice (n = 9) or after 13 weeks on day 15 of the first pregnancy (P15; n = 8). Carmine alum whole-mount stainings of the transplanted fourth mammary glands were prepared to allow analyses of the growth. Terminal differentiation of the second and third mammary gland (P15) was monitored as a control sample. Growth take-on-rate was calculated from P15 transplant and virgin samples (n = 17). Evaluation of the fat pad filling rate was performed from the virgin mouse transplants that had initially begun to grow (n = 7–10).

4.20 Statistical analysis (I, II, III)

All statistical analyses were performed with the GraphPad Prism 7 software. The names and/or numbers of individual statistical tests, samples and data points are indicated in figure legends. Unless otherwise stated, all results are representative of three independent experiments and P values <0.05 are shown in graphs.

4.21 Other reagents and drugs (I, II, III)

Lysosomal degradation was disrupted by treating cells with 100 nM Bafilomycin A1 (196000, Merck-Millipore) for 6 h. The release of the RUSH cargos was induced by addition of 3mM of D-biotin (B4501, Sigma-Aldrich). Conventional protein secretion was blocked by incubating the cells with 10 μM Golgicide A (G0923, Sigma-Aldrich) 30 minutes prior to imaging.

Table 1. Cell lines and culture medium used in the original articles. DMEM = Dulbecco's Modified Eagle's Medium, FCS = fetal bovine serum, EGF = epidermal growth factor.

Cell line	Culture medium	Original Publication
Talin1$^{-/-}$ MEF	DMEM, 15 % FCS, 1% L-glutamine, 1% penicillin/streptomycin	I
MSF	DMEM/F12, 5% FCS, 1% L-glutamine, 1% penicillin/streptomycin	I, II
MEC	DMEM/F12, 10% FCS, 5 ug/ml insulin, 1 ug/ml hydrocortisone, 10 ng/ml mouse EGF, 50 ug/ml gentamycin, 1% L-glutamine, 1% penicillin/streptomycin	I
U2OS	DMEM, 10 % FCS, 1% L-glutamine, 1% penicillin/streptomycin	III
HEK293FT	DMEM, 10 % FCS, 1% L-glutamine, 1% penicillin/streptomycin	III
HeLa	DMEM, 10 % FCS, 1% L-glutamine, 1% penicillin/streptomycin	III
CHO	HAM'S F12,10 % **FCS** and 1 % L-glutamine	III

Table 2. Plasmids used in the original articles.

Plasmid	Original Publication
GFP-Paxillin	I
pBJ1 human integrin alpha 11 (ITGA11)-EGFP	II
mEmerald-Lifeact-7 (GFP-LifeAct)	II
Str-KDEL_SBP-EGFP-ITGA5 (RUSH-$\alpha 5$)	III
Str-KDEL_SBP-mCherry-CD59 (RUSH-CD59)	III
pmKate2-paxillin	III
ERoxBF	III
pEGFP-GRASP65	III

Table 3.   Primary antibodies used in the original articles.

Antigen	Species	Manufacturer	Catalog number	Original Publication
Integrin α6	rat	Serotec	MCA699	I
E-cadherin	rabbit	Cell Signaling	3195S	I
ACTA2	mouse	Sigma	A5228	I
Keratin 8	rat	Hybridoma Bank	AB_531826	I
SHARPIN	rabbit	Proteintech	14626-1-AP	I, II
Collagen I alpha 1	rabbit	Novus	NB600-408	I
Tubulin	mouse	Hybridoma Bank	AB_1157911	I
Vimentin	rabbit	Cell signaling	5741S	I
GAPDH	mouse	Hytest	5G4MaB6C5	I
S100a4	rabbit	Abcam	ab27957	I
APC-CD45 (30-F11)	rat	Biologend	103111	I
PE-CD54 (YN1/1.7.4)	rat	Biologend	116107	I
APC-CD31 (MEC13.3)	rat	Biologend	102509	I
Brilliant Violet 421™-CD24	rat	Biologend	101825	I
FITC-CD24	rat	BD Biosciences	553261	I
PEcy7-CD49f	rat	Biologend	313621	I
Fibronectin	rabbit	Sigma	F3648	II
Vinculin	mouse	Sigma	V9131	II
LAMP-1 (1D4B)	rat	Abcam	Ab25245	II
YAP	mouse	SantaCruz	Sc-101199	II
Integrin α11	rabbit	Provided by D. Gullberg, University of Bergen		II
Integrin β1 (MB1.2)	rat	Millipore	LV1766450	II
Integrin β1	mouse	BD Biosciences	610468	III
GRASP55	rabbit	Sigma	HPA035274	III
GRASP65	rabbit	Sigma	HPA056283	III
GFP	rabbit	Abcam	Ab290	III
Integrin α5β1 (HA5)	mouse	Millipore	MAB 1999	III
GFP	mouse	Abcam	Ab1218	III
Integrin α5 (SNAKA51)	mouse	Millipore	MABT 201	III

5   Results

5.1   Deregulated integrin activity diminishes mechanotransduction and impairs mammary gland development (I, II)

5.1.1   SHARPIN-deficient mice display reduced mammary ductal outgrowth during puberty

To understand how integrin activity regulation affects mammary gland development, we studied the pubertal mammary ductal outgrowth in a mouse strain harbouring a spontaneous deletion of the integrin inactivator SHARPIN (Sharpin[cpdm/cpdm], from here on Sharpin[cpdm]), and wild type (Sharpin^{+/+} or Sharpin[+/cpdm], from here on wt) female mice. The mammary gland contains highly proliferative structures at their ends called terminal end buds (TEBs) that drive mammary ductal invasion through the mammary fat pad and regulates the formation of the ductal tree (Paine, Lewis 2017). From carmine alum-stained mammary gland whole mounts, we observed a much smaller ductal tree area together with a reduced number of ductal branches and TEBs in pubertal (age 5–7 weeks) Sharpin[cpdm] mice compared to wt mice (I, Fig. 2A-C and E-F). In pre-pubertal mice (age 3–4 weeks) the area of the ductal trees were similar in wt and Sharpin[cpdm] mice, indicating a reduced outgrowth of the ductal tree during puberty (I, Fig 2A-B). A similar cellular organization of TEBs and a similar polarity of the mammary ductal cell layers, were observed in wt and Sharpin[cpdm] mice by examining histological sections from 7-week-old mice by hematoxylin-eosin (HE) staining and by immunolabelling of luminal (CDH1, KRT8) and basal (ITGA6, ACTA2) epithelial cells (I, Fig. 2D, G). Furthermore, we observed similar levels of BrdU-positive and cleaved caspase 3-positive cells in wt and Sharpin[cpdm] mammary gland TEBs, indicating that the observed differences in mammary gland growth were not due to differences in cell polarity, cell proliferation or cell death (I, Fig. EV 2D). In addition, we verified that Sharpin[cpdm] mice had a normal puberty onset (evaluated by vaginal opening; I, Fig. EV 2B) as well as normal hormone production by studying the oestrogen and progesterone expression levels in the mammary gland (I, Fig. EV 2C). Therefore, the delayed mammary gland development during puberty was not secondary to hormonal differences. These results indicate that the integrin

activity inhibitor SHARPIN affects the invasion and outgrowth of the developing mammary gland.

5.1.2 Stromal expression of SHARPIN regulates mammary gland development

Immunohistochemical (IHC) analysis of SHARPIN in paraffin-embedded human tissue sections showed that SHARPIN is expressed both in the epithelial and stromal cells of the mammary gland (I, Fig. 1A). Furthermore, co-staining experiments showed that the SHARPIN expressing stromal cells were mainly spindle-shaped, vimentin positive fibroblasts (I, Fig. EV1A). Western blot analyses of isolated mouse mammary gland stromal fibroblasts (MSFs) and mammary epithelial cells (MECs, identified by co-expression of CDH1) also confirmed the protein expression of SHARPIN in these cell populations (I, Fig. 1B, EV 2A). On the basis of surface level expression of CD24 and ICAM (Di-Cicco et al. 2015), cells were further FACS-based sorted into mature luminal epithelial (ICAM^{neg}, CD24^{high}), luminal progenitor (ICAM^{int}, CD24^{high}), basal epithelial (ICAM^{high}, CD24^{int}) and stromal cells (CD24^{neg}) (I, Fig. 1C). Quantitative PCR of the sorted cells was performed to determine mRNA expression of lineage specific marker genes (basal, *Keratin 5*; luminal, *Keratin 18*; stromal, *Pdgfra*) (I, Fig. EV 1B). When *Sharpin* mRNA expression levels were compared in these four cell types, we found that while all cell types expressed *Sharpin*, basal epithelial cells expressed *Sharpin* to a much lower extent than luminal epithelial (progenitors and mature) and stromal cells (I, Fig. 1D). These results made us wonder if the reduced mammary ductal outgrowth in pubertal SHARPIN deficient mice (I, Fig. 2A–C) could result from defects in the epithelial regeneration and differentiation. MECs from wt and Sharpin^{cpdm} mice were isolated and cell populations sorted, based on CD24 expression in combination with the basal markers CD29 (integrin beta 1) or CD49f (integrin alpha 6), and the levels of luminal and basal epithelial cells quantified. These results showed no difference in the proportions of basal and luminal populations between wt and Sharpin^{cpdm} MECs (I, Fig. EV 2E). As these results suggested that expression of SHARPIN in the stromal, and not the epithelial compartment, could affect mammary gland development, we next performed transplantation of small pieces of wt or Sharpin^{cpdm} mammary epithelium into cleared (epithelium-free) mammary fat pads of virgin wt recipients (I, Fig. 3A). Analyses of the ductal outgrowth 7–11 weeks after transplantation, revealed that Sharpin^{cpdm} epithelium was able to both regenerate and invade into the wt stroma to the same extent as wt epithelium (I, Fig. 3A–C). Furthermore, studies of mammary gland whole mounts from pregnant wt mice that had undergone epithelium transplantation (both from wt and Sharpin^{cpdm}, 10 weeks pre-mating) showed that the mammary epithelium derived from both genotypes was able to

regenerate and undergo tertiary branching and alveologenesis in the context of wt stroma (I, Fig. 3D, EV 2F). As Sharpin[cpdm] mice have a short lifespan (Potter, Wang et al. 2014) we were not able to perform wt epithelial transplantation into Sharpin[cpdm] mice. Instead, we generated a conditional *Sharpin* knockout in stromal cells (*S100a4-Cre;Sharpin^{fl/fl}*) by using the S100 Calcium Binding Protein 4 (*S100a4*) promoter for *Cre* expression (Cheng, Bhowmick et al. 2005) (I, Fig. EV4A-C). Whole mount staining of mammary glands from control (*S100a4-Cre;Sharpin^{fl/+}*) and conditional knock-out mice (*S100a4-Cre;Sharpin^{fl/fl}*) confirmed that stromal SHARPIN expression regulates the ductal outgrowth as both the number of TEBs and the outgrowth area was reduced in *S100a4-Cre;Sharpin^{fl/fl}* mice (I, Fig 3E-G). As stromal immune cells also are known to affect mammary gland development (Reed, Schwertfeger 2010), we next analysed the immune cell populations in the mammary gland stroma (I, Fig. EV 3A). Quantification of the different cell populations revealed more leukocytes (CD45^{+}), eosinophils (CD11b^{+} Siglec F^{high}), neutrophils (CD11b^{+}Ly6G^{high}), and macrophages (CD11b^{+}F4/80^{+}) in the pubertal mammary gland of Sharpin[cpdm] compared to wt animals (I, Fig. EV 3B). The increased amount of immune cells in the mammary gland is consistent with the previously reported multi-organ inflammation phenotype of Sharpin[cpdm] mice (HogenEsch et al. 1993). Furthermore, elevated levels of eosinophils and macrophages were observed around the TEBs in Sharpin[cpdm] animals while no differences were observed between *S100a4-Cre;Sharpin^{fl/fl}* and wt animals (I, Fig. EV 4D). However, when we studied the angiogenesis we observed no differences between wt and Sharpin[cpdm] animals, as measured by the area covered by PECAM1 (CD31) in IHC stained mammary gland sections (I, Fig. EV 3C).

Together, these results show that expression of SHARPIN in the stromal, but not epithelial, cells is required for mammary gland development.

5.1.3 Lack of SHARPIN causes impaired adhesion assembly and cell spreading on collagen

As fibroblasts play a major role in matrix deposition during mammary gland development we decided to study the MSFs in more detail *in vitro*. Cells from both wt and Sharpin[cpdm] were isolated and their viability confirmed with WST-1 assay (I, Fig. EV 5A–B). Since SHARPIN is an inactivator of integrin β1 (Rantala et al. 2011) (confirmed in I, Fig. EV 5C and II, Fig. 1A, S1A) we first studied how the reduced integrin inactivation would affect focal adhesion (FA) formation and cell spreading of MSFs. We prepared polyacrylamide (PAA) hydrogels of different stiffness and coated them with the ECM ligands fibronectin (FN), which becomes increasingly deposited (3-fold) during puberty (Woodward, Mienaltowski et al. 2001), or collagen I (Coll) that is abundant around larger mammary ducts (Keely, Wu et al.

1995). A saturating concentration of 20 µg/ml was used for each ligand (II, Fig. S1B). MSFs from wt and Sharpin[cpdm] mice were plated on hydrogels and immunolabelled for vinculin (a mature FA marker) and co-stained for actin (II, Fig. 1B, S1C). In line with the observed increase in relative integrin β1 activity (II, Fig1 A, S1A), Sharpin[cpdm] MSFs displayed larger cell area (2–13 kPa) and larger or a greater number of adhesions (2–4 kPa) on FN coated hydrogels when compared to wt cells (II, Fig 1B–C and S1D-F). Surprisingly, when MSFs were plated on 2 kPa collagen coated substrates we observed a reduced cell spreading and decreased FA length in Sharpin[cpdm] MSFs (II, Fig 2B–F and S1C). In addition, we observed that both wt and Sharpin[cpdm] MSFs displayed mature FAs on soft substrates and that maturation maxima was reached already at a stiffness of 2–4 kPa (II, Fig 1F, S1F). This is much lower to previously reported stiffness thresholds for FA maturation (Elosegui-Artola et al. 2016). Thus the observed mature FAs on soft substrates suggested that these primary cells would be adapted to the soft growth environment in the mammary gland (Plodinec, Loparic et al. 2012). When we studied the nuclear localization of the mechanosensitive transcription factor Yes-associated protein (YAP) on 2 kPa collagen coated substrates we observed that both wt and Sharpin[cpdm] MSFs exhibited nuclear localization of YAP (II, S1G). Furthermore, this localization was reduced in Sharpin[cpdm] MSFs, in line with the reduced cell spreading observed on 2 kPa collagen coated substrates (II, Fig. 1B–C and S1G–H).

As the MSFs showed differences in cell spreading and adhesion formation in a stiffness and ligand dependent manner, we next asked if SHARPIN could affect integrin-ligand binding dynamics. To assess this, we performed bead recruitment and magnetic tweezers experiments with beads coated with fibronectin or collagen (II, Fig 2A). These experiments showed both an increased integrin β1 recruitment, as well as a faster detachment time from collagen-coated beads in Sharpin[cpdm] MSFs when compared to wt MSFs (II, Fig. 2B–C). In contrast, no differences in binding dynamics of wt and Sharpin[cpdm] MSFs were observed with fibronectin-coated beads (II, Fig. 2B–C). To investigate if the faster receptor-ligand binding dynamics would influence adhesion dynamics, we studied GFP-Paxillin transfected wt and Sharpin[cpdm] MSFs plated on collagen-coated coverslips by live cell total internal reflection (TIRF) imaging. In line with the faster binding dynamics, Sharpin[cpdm] MSFs also displayed increased assembly and disassembly rates of adhesions, these paxillin adhesions were also larger compared to the adhesions in wt MSFs (I, Fig. 5J–K). The faster adhesion dynamics were also confirmed by enhanced phosphorylation of protein tyrosine kinase 2 (PTK2) in Sharpin[cpdm] MSFs (I, Fig. EV5D–E). Together these results demonstrate that SHARPIN deficiency causes both faster binding and unbinding rates of integrin to collagen, resulting in more rapid collagen adhesion dynamics in Sharpin[cpdm] MSFs.

5.1.4 SHARPIN-deficiency downregulates collagen-binding integrin α11

To understand why the lack of SHARPIN, as an inactivator of integrin β1 that can bind both collagen and fibronectin through forming specific heterodimers with different integrin α-subunits, only affected adhesion dynamics on collagen we performed RNA sequencing and analysed the expression levels of all matrix binding integrins (II, Fig. S3A). As *Itga1* and *Itga11* were the only collagen-binding α-subunits expressed, that form a heterodimer with β1, we decided to analyse their surface expression. Interestingly, we found markedly reduced levels of both integrin α1 and integrin α11 in Sharpin[cpdm] MSFs compared to wt MSFs (II, Fig. 4A). As these results could explain the contradicting results of reduced cell spreading of Sharpin[cpdm] MSFs plated on soft collagen matrices (II, Fig.1 B-C) we next studied the cell spreading of wt and Sharpin[cpdm] MSFs with siRNA-mediated downregulation of integrin α1 and -α11 (II, Fig. S3D). While silencing of either of them had no effect on the spreading area of Sharpin[cpdm] MSFs, integrin α11 downregulation reduced the spreading area of wt MSFs to the same level as control silenced Sharpin[cpdm] MSFs (II, Fig. 5A), indicating a role for integrin α11 in cell spreading. Reduced levels of integrin α11 expression in Sharpin[cpdm] MSFs were further confirmed with both western blot analyses and immunofluorescence staining (II, Fig. 4B–D). As *Itga11* mRNA levels where equal in wt and Sharpin[cpdm] MSFs (II, Fig. S3A) we investigated if the lack of SHARPIN could cause the downregulation of integrin α11 on a protein level and indeed, we observed reduced protein expression following SHARPIN downregulation in wt MSFs (II, Fig. 4E–F). Furthermore, we also excluded the possibility of an integrin α11 downregulating effect of the *in vitro* culture condition on stiff fibronectin-rich substratum (plastic in the presence of serum fibronectin) by examining the integrin α11 protein levels from cells plated on soft collagen-coated substrates directly after isolation (II, Fig. S3B–C). We next wondered if the SHARPIN-induced downregulation of integrin α11 could be a result of increased degradation of integrin α11, as integrin receptor activity status is known to regulate the endocytosis and degradation of the receptors (Arjonen et al. 2012, Rainero, Howe et al. 2015). Co-localization analyses of MSFs immunolabelled with integrin α11 and the lysosomal marker Lamp1 showed higher levels of integrin α11 co-localizing with Lamp1 in Sharpin[cpdm] MSF (II, Fig. 4G–H). Furthermore, Bafilomycin A1 treatment, which disrupts the lysosomal degradation, increased the co-localization of integrin α11 and Lamp1 in both wt and Sharpin[cpdm] MSF (II, Fig. 4G–H), suggesting that integrin α11 is downregulated in Sharpin[cpdm] MSF via lysosomal degradation. To further validate that the observed Sharpin[cpdm] MSF spreading phenotype is a function of the reduced integrin α11 levels, we studied cell spreading in integrin α11-EGFP overexpressing MSF on collagen-coated soft substrates (II, Fig. 5B). While overexpression of exogenous integrin α11 did not affect the

spreading area of wt MSFs, it increased the spreading area of Sharpin[cpdm] MSF (II, Fig. 5B–C). In addition, introduction of integrin α11-EGFP to Sharpin[cpdm] MSF was also proficient to increase the detachment time of collagen-coated beads (II, Fig. 5D).

Together these results suggest that Sharpin[cpdm] MSF downregulate integrin α11 by increasing degradation of integrin α11 as a consequence of the increased relative integrin β1 activity in the absence of SHARPIN. Furthermore, re-introduction of integrin α11β1 is sufficient to rescue the faster adhesion dynamics and cell spreading phenotype in Sharpin[cpdm] MSF, suggesting that SHARPIN is essential in regulating correct integrin activity and expression levels required for proper adhesion dynamics and cell spreading on collagen at a stiffness range relevant to the *in vivo* tissue.

5.1.5   SHARPIN regulates force transduction of MSFs

Fibroblast are capable of exerting high forces which can drive cell adhesion, migration and rearrangement of the ECM (Wasserman, Weitz et al. 2009, A. K. Harris, Wild et al. 1981, Scott, Mair et al. 2015). We therefore sought to investigate the role of SHARPIN in MSF force generation. Force generation is depending on the different components of the contractility machinery often referred to as the molecular clutch (Case, Waterman 2015, Chan, Odde 2008). Clutch dynamics can be modelled based on parameters of the different components to predict force generation. The different parameters of the clutch affecting force generation are; substrate compliance, the number and binding dynamics of integrin-ECM bonds, reinforcement of the integrin-actin link through vinculin recruitment, and actomyosin contractility (Elosegui-Artola et al. 2016). This led us to measure the remaining parameter of actomyosin contractility, to be able to predict the force transmission. MSFs were grown on different stiffnesses and the level of phosphorylated myosin light-chain 2 (pMLC2) was analysed as it is known to regulate actomyosin contractility. Interestingly, pMLC2 levels correlated with stiffness, while no differences between wt and Sharpin[cpdm] MSF were observed (II, Fig. 3A, S2A–B). As all clutch parameters, except for the integrin-collagen dynamics (II, Fig. 2C), were similar in wt and Sharpin[cpdm] MSF, we next performed computational modelling of the molecular clutch to predict force transmission in wt and Sharpin[cpdm] MSF. The model predicts that the increased binding and unbinding rates of integrins to collagen should cause a shift in the traction force peak towards higher rigidities (II, Fig. 3B). Moreover, as a result of MSFs being able to form mature adhesions already at low stiffness's, and re-inforcement of the integrin-actin link therefore not increasing with increasing stiffness (II, Fig. 1E–F) the model also predicts a biphasic force/rigidity relationship, even in situations where pMLC levels increases with stiffness (II, Fig. 3A–B).

We then tested the model prediction by performing traction force microscopy (TFM). In line with our observations of comparable binding and unbinding dynamics on fibronectin, the wt and Sharpin[cpdm] MSF displayed similar traction forces on fibronectin (II, Fig. 3D). However, we did not observe any traction peak, which could result from the peak residing at a stiffness higher than the range experimentally measured here (0.6–14.5 kPa). For collagen, the model predicted a shift of the traction peak towards higher stiffness in conditions with faster integrin binding dynamics. As predicted, when forces were measured on collagen we found that wt MSFs exhibited a bi-phasic traction peak at a stiffness range around 2 kPa that was absent in Sharpin[cpdm] MSF (II, Fig. 3C, S2C and I, Fig. 5F–G). Again, the absence of the peak could be a result of the peak being shifted towards higher stiffness's. The observed differences in the force maxima on different ECM ligands is consistent with the view that different integrin heterodimer-ligand bonds can withstand forces to different degrees. Furthermore, TFM measurements of MSFs ectopically expressing mCherry-SHARPIN on soft collagen coated substrates, verified the role of SHARPIN in regulating force transmission as re-introduction of SHARPIN to Sharpin[cpdm] MSF rescued traction force generation in these cells (I, Fig 5H–I).

The TFM experiments on collagen displayed for the first time in mammalian primary cells the biphasic force/rigidity relationship that is the fundamental prediction of the molecular clutch model. Previously, the biphasic response has not been observed as it is almost always masked by the reinforcement of the integrin-actin link that takes place on higher rigidities. The observed biphasic force response would be a result of the adhesions not undergoing further reinforcement and thus decoupling the adhesion maturation from force generation. To further confirm that the biphasic force response is adhesion maturation dependent we compared adhesion maturation and actin contractility to *talin 1* knockout mouse embryonic fibroblasts (talin1^{-/-} MEF), which display a wild-type phenotype due to a compensatory upregulation of talin 2. These cells have previously been shown to display a monotonic force-rigidity response as a result of adhesion maturation. These cells displayed immature adhesions on soft matrices and displayed a monotonic stiffness-actin contractility relationship that would be in line with adhesion maturation on higher stiffness's (II, Fig. S2A and F). Together, these results indicate that the observed biphasic rigidity response in MSFs is a result of mature adhesions formed already at very low stiffness's. As the molecular clutch model is based on integrins linking the ECM to the rearward-flowing actin cytoskeleton and thereby enabling cells to exert forces on the environment, it also predicts an inverse relationship between traction forces and actin retrograde flow. Comparison of the actin retrograde flow of wt and Sharpin[cpdm] MSF on 2 kPa collagen-coated gels showed that in addition to Sharpin[cpdm] MSF exerting less forces on soft collagen matrices, they also displayed the predicted increased actin flow (II, Fig. 3E–F). In addition, comparison

of flow in stably adhered (plated for 4 h on 2 kPa collagen) talin1$^{-/-}$ MEFs to wt and Sharpin^{cpdm} MSF revealed very slow rearward flow (beyond detection limit) in MSFs, further demonstrating their unique ability to form mature adhesions and stress fibres on low stiffness ranges (II, Fig. S2D). To further evaluate the observed mechano adaption of MSFs to soft collagen-coated substrates we compared the cell surface levels of integrins in MSFs to MEFs and found that MSFs express notably higher amounts of integrin α1, α2 and α11 and almost 2-fold more integrin β1 (II, Fig. S2E), suggesting that these cells are adapted to a soft environment by upregulation of integrin receptors.

To understand how SHARPIN-mediated force regulation in MSFs could affect mammary gland development, we next examined how MSFs collectively transmit forces by seeding cells inside a 3D floating collagen plug. In line with the reduced traction forces exerted by individual cells, Sharpin^{cpdm} MSFs also contracted collagen to a lesser extent than wt MSFs, determined by the larger area of the collagen plug 3-days post cell-culture (I, Fig. 5C). The reduced collagen contraction was also achieved in wt MSFs upon partial silencing of SHARPIN (I, Fig. 5D–E). Together, these results show that MSFs are mechanically adapted to the soft *in vivo* environment of the mammary gland, enabling them to form mature adhesions already at low stiffness's with a stiffness optimum around 2 kPa on collagen. Furthermore, lack of SHARPIN shifts this stiffness optimum to higher rigidities as a consequence of the faster binding dynamics to collagen. In addition to the observed reduction in force generation on a single cell level, SHARPIN also affects the capacity of collective force transmission, as Sharpin^{cpdm} MSFs display a reduction of collagen 3D plug contraction.

5.1.6 SHARPIN-deficiency reduces collagen fibre assembly in the mammary gland stroma

Fibroblast can affect ECM organization in multiple ways, including producing, degrading, and aligning the ECM components (Tschumperlin 2013). Furthermore, integrin α11 has been shown to be important for collagen fibril assembly and collagen remodelling (Schulz et al. 2015). This prompted us to compare the mRNA expression pattern of freshly isolated wt and Sharpin^{cpdm} MSFs (I, Fig 5A and Appendix Table S1) with Ingenuity Pathway Analysis. As we discovered both up- and down-regulation of several collagen and collagenase genes, we next studied the ability of these cells to degrade collagen by plating them on DQ™ Collagen-coated micropatterns (I, Fig. 5B). When we measured the amount of degraded collagen 4 h post cell plating, revealed by loss of fluorescence quenching of collagen upon protease cleavage, we observed reduced proteolytic activity in the Sharpin^{cpdm} MSFs (I, Fig. 5B). We next analysed the capability of wt and Sharpin^{cpdm} MSFs to convert

soluble collagen into fibrillar collagen by Sirius red-assay (Marotta, Martino 1985) that allows measurement of the soluble collagen and thereby indirectly measures the insoluble fibrillar collagen. We initially observed a reduced amount of soluble collagen production by Sharpin[cpdm] MSFs compared to wt MSFs, despite this the amount of soluble collagen was higher in culture media collected 7 days later from Sharpin[cpdm] MSFs compared to wt MSFs (I, Fig. 6A). This indirectly indicates that Sharpin[cpdm] MSFs have a reduced capacity to assemble the produced soluble collagen into fibrillar collagen effectively removing it from solution. To further evaluate the role of SHARPIN in ECM production we analysed cell derived matrices (CDM), which closely resembles the *in vivo* ECM (Cukierman et al. 2001, Kaukonen et al. 2017), produced by MSFs. Immunolabelling of Collagen I in the CDMs revealed reduced collagen bundling ability of Sharpin[cpdm] MSFs (I, Fig. 6B-C). Based on these findings, together with our results of SHARPIN deficient MSFs displaying impaired force generation and a reduced capacity of collagen contraction, we next investigated the impact of SHARPIN on stromal collagen organization. To this end, we utilized multiphoton laser scanning microscopy and second harmonic generation (SHG) imaging to allow for label free visualisation of the stromal collagen fibres surrounding the invading mammary epithelium in mammary gland whole mounts from wt and Sharpin[cpdm] mice (I, Fig. 4A). Close examination of collagen-fibres near the TEBs, revealed a reduced amount of collagen fibres that were in contact with the TEBs in the SHARPIN deficient mammary gland (I, Fig. 4B). In addition, analyses of the length and thickness of these collagen fibres revealed a trend of slightly shorter and thicker fibres in Sharpin[cpdm] mammary gland stroma (I, Fig. EV4E–G). As collagen organization has been shown to correlate with stiffness (Maller, Hansen et al. 2013) we next performed atomic force microscopy (AFM) indentations to measure the stiffness of the mammary gland tissue and found that the Sharpin[cpdm] mammary gland stroma was clearly softer than the wt stroma (I, Fig. 4C). However, when we compared the stiffness of pre-pubertal mice (age 4 weeks), we did not observe a difference in the stromal stiffness (I, Fig. EV4H) and can therefore not exclude the possibility of a reduced stiffness as a result of the reduced mammary ductal outgrowth. Together, these results indicate a crucial role for SHARPIN in regulating collagen production, degradation and assembly in the mammary gland stroma.

In summary, the data included in these two manuscripts collectively indicate, that in the absence of SHARPIN, MSFs have higher relative amounts of integrin β1 causing an increased degradation of integrin α11β1. The decreased integrin α11β1 levels causes faster unbinding of the collagen ligand, leading to reduced traction force generation of MSFs. As traction forces are important for collagen fibre bundling and alignment this causes deregulated rearrangement of the developing mammary gland in SHARPIN deficient mouse.

5.2 Ligand-dependent targeting of newly synthesized integrins and their contribution to focal adhesion growth (III)

5.2.1 Ligand-dependent maturation and localization of newly synthesized integrins to the plasma membrane

To study the trafficking of newly synthesized integrins we utilized the retention using selective hooks (RUSH) system (Boncompain, Divoux et al. 2012). The two-state assay is based on the interaction of two fusion proteins: a fluorescently tagged protein of interest fused to a streptavidin binding peptide (SBP) and a hook protein fused to streptavidin. As a protein of interest, we used integrin α5 and for the hook protein, we used the ER retention signal sequence "KDEL" (RUSH-α5). In steady state, the integrin α5 will be bound to the hook protein because of the SBP-streptavidin interaction and therefore anchored in the ER. This interaction can be disrupted by the addition of biotin, which induces a synchronized release of the integrin α5 from the ER. We chose to study the integrin α5 as it has been extensively studied in the field and is the main fibronectin receptor expressed in many cell types. To avoid interfering with the integrin cytoplasmic tail interactions (Morse, Brahme et al. 2014) we inserted the enhanced green fluorescent protein (EGFP)-tag, the IL-2 signal peptide and the SBP in the extracellular head domain of integrin α5. As the N-terminus of integrin α5 localizes between the α5 and β1 subunit and away from the RGD-binding site (in the published crystal structures), we chose the N-terminus as the site for the insertion (Nagae, Re et al. 2012). To confirm that the ligand binding function of the RUSH-α5 was intact, we plated RUSH-α5 transfected U2OS cells on coverslips coated with fibronectin or collagen and allowed the cells to spread. The RUSH-α5 construct was released by the addition of biotin, and cells were fixed and immunolabelled with an antibody recognizing the active integrin α5 (SNAKA51). Immunofluorescence imaging revealed co-localization of RUSH-α5 with the SNAKA51 staining in structures resembling focal- and fibrillar adhesions on fibronectin (III, Supplementary figure 1D). In contrast, the RUSH-α5 was more diffusely localized along the plasma membrane in cells plated on collagen (III, Supplementary figure 1D). These ligand specific results indicate that the activity and ligand binding capacity of the RUSH-α5 construct was intact. To investigate the release in more detail, we studied the cell surface levels of RUSH-α5 with flow cytometry. Transfected cells were stained with anti-integrin α5β1 and anti-GFP antibody either prior or post release of the construct and cell surface levels were compared. While the release of the RUSH-α5 construct only modestly increased the integrin α5β1 cell surface levels, due to the endogenously expressed integrin α5 in these cells, the GFP surface levels were clearly increased following release of the

construct (III, Figure 1A). Integrin β1-subunits are expressed in an excess ratio by most cell types and retained in their immature form in the ER until they assemble with newly synthesized α-integrins (Heino et al. 1989, Strooper, Leuven et al. 1991, Lenter, Vestweber 1994). By performing GFP-pulldown experiments with RUSH-α5 or EGFP-transfected cells, we co-precipitated endogenous integrin β1 and studied whether the immature (faster migrating, lower band) or mature (slower migrating, upper band) integrin β1 interacted with the RUSH-α5. Before release, only the immature form of the integrin β1 interacted with RUSH-α5, while the RUSH-α5 predominantly interacted with the mature integrin β1 2 h after release from the ER (III, Supplementary figure 1A, left panel). Furthermore, the integrin β1 maturation state correlated with the RUSH-α5 release time (III, Supplementary figure 1A, right panel). These experiments indicate that the construct was retained by the hook protein in the ER before the biotin induced release, and indicates receptor maturation in the Golgi. Next, to study if the maturation rate of integrin β1 was ECM ligand dependent, we compared the ratios of mature and immature integrin β1 in cells plated on collagen or fibronectin coated substrates. The mature proportion of integrin β1 interacting with RUSH-α5 increased with time after release, and receptor maturation displayed a trend for faster maturation on fibronectin-coated substrates over collagen-coated substrates (III, Figure 1B and C). A faster maturation trend of the β1 receptor was also observed on stiff (50 kPa) compared to soft (0.5 kPa) fibronectin-coated substrates (III, Supplementary figure 1B and C). As these results suggested that the maturation rate of integrins is influenced by the ECM, we next studied if the secretion of newly synthesized integrins would be influenced by the ECM ligand. For this, we co-transfected cells with RUSH-α5 and a control cargo protein (glycosylphosphatidylinositol-anchored proteins (GPI-APs) cluster of differentiation 59 (CD59); RUSH-CD59). Co-transfected cells were plated on either collagen or fibronectin and allowed to spread for 2–4 h before time-lapse imaging of the ER release of the constructs was performed. On both collagen and fibronectin, a clear ER localization of the RUSH-α5 was observed before the induced release. Addition of biotin resulted in prominent, synchronized release of the construct, which quickly localized to puncta like structures resembling the Golgi complex where it remained for 15–20 minutes (III, Figure 1D). The kinetics of the RUSH-α5 were thus similar to what has been reported previously for other RUSH-constructs (Boncompain et al. 2012). On fibronectin, small adhesion like structures of RUSH-α5 started appearing after 15–20 minutes of release and these grew in size and number as more RUSH-α5 was delivered from the Golgi (III, Figure 1D). In contrast, the RUSH-α5 localization to the plasma membrane was more evenly distributed on collagen, and the RUSH-α5 seemed to diffuse along the plasma membrane rather than form any adhesion like structures (III, Figure 1D). As the plasma membrane localization of the co-transfected RUSH-CD59 construct was similar on both ligands

(III, Supplementary figure 1E), these results suggest that targeting of newly synthesized integrins to the plasma membrane is ligand-specific. Together, these results show that the RUSH-α5 construct is a functioning integrin α5 subunit that can form an active heterodimer with integrin β1. In addition, maturation kinetics of integrins tend to be faster in conditions where an active integrin is needed.

5.2.2 Newly synthesized integrin α5 are secreted in a polarized and ligand-dependent manner to the protruding edge of the cell

To further validate the ligand-specific delivery of integrins to the plasma membrane, we plated RUSH-α5 and RUSH-CD59 co-transfected cells on dual-coated line micropatterns where FN-coated thin lines alternates with GFOGER (a synthetic collagen peptide with high affinity for collagen-binding integrins (W. Zhang et al. 2003). Following release, the RUSH-α5 preferentially localized to FN-coated lines at the cell edges (III, Figure 1E). This was further highlighted when line profiles were plotted in a 90° angle to the fibronectin-coated lines and intensity profiles compared. The RUSH-α5 intensity profile followed the pattern of the FN intensity profile, while the profile of the RUSH-CD59 did not correlate with the FN intensity (III, Figure 1E lower panel). Since many of the release-induced adhesion structures we observed localized to protruding areas of the cell (III, Figure 1D), we next investigated whether the trafficking of RUSH-α5 would be polarized. RUSH-α5 transfected cells were plated and allowed to spread on 9 μm wide micropatterned lines coated with either collagen or fibronectin and an anti-GFP nanobody, followed by time-lapse imaging of the RUSH-α5 ER release. The N-terminally positioned EGFP-tag of RUSH-α5 allows the anti-GFP nanobody to trap the RUSH-α5 at the initial secretion position and thus prevents diffusion and further traffic of the construct (Fourriere et al. 2019). While the cells clearly preferred to target the RUSH-α5 release to one cell edge over the other when plated on fibronectin, the secretion on collagen was more evenly distributed to both cell edges (III, Figure 1F). The polarized secretion was quantified by measuring the RUSH-α5 intensity from both an area in the predominantly protruding edge and an area in the retracting edge of the cell (III, Figure 1F schematic). The intensity was equal in both edges of the cell for the first 20 minutes after release, followed by a prominent, polarized intensity increase in the predominantly protruding area of FN plated cells (III, Figure 1G). On collagen-coated lines, the secretion was equally distributed to both cell edges for the first 35 minutes after which a modest preference towards the predominantly protruding area could be observed (III, Figure 1F-H). While the observed increase of RUSH-α5 intensity on fibronectin could partially be a result of an increased number of adhesions forming in the protruding edge of cells, and thus contribute to

the intensity increase, cells on collagen only displayed a modest polarized intensity increase. Even if cells on collagen do not form new integrin α5 adhesions, these cells should equally form new adhesions to the protruding area of the cells to which RUSH-α5 would be distributed and trapped by the anti-GFP nanobody even if integrin-ligand bonds are not formed. These results suggests that trafficking of newly synthesized integrin α5 is polarized and ligand-dependent. Furthermore, the ligand-dependent polarized secretion of newly synthesized integrins to the protruding edge of the cell indicate that integrins are targeted to areas of the cell where new adhesions are formed.

5.2.3 Newly synthesized integrin α5 are targeted to protruding areas of the cell to mediate adhesion formation and cell protrusion in a ligand-dependent manner

To study the localization of RUSH-α5 to the adhesion like structures in detail, we performed time-lapse imaging of RUSH-α5 and pmKate2-paxillin co-transfected U2OS cells plated on fibronectin- or collagen-coated dishes. Paxillin adhesions were segmented, and the RUSH-α5 secretion to these adhesions studied. On collagen, the localization to these adhesions was diffuse, with a slight increase in intensity observed following 45 minutes of release (III, Figure 2A-B). This slight increase was most likely a consequence of the re-localization of the RUSH-α5 from the ER to the plasma membrane as also the intensity in the areas surrounding the adhesions seemed slightly increased following release (III, Figure 2A). On fibronectin, the release of the RUSH-α5 led to a slight intensity increase in the adhesions already 20 min after release and 45 min post release RUSH-α5 was mainly detected in these adhesions and a high increase in the RUSH-α5 recruitment was observed (III, Figure 2A-B). To assess whether the targeted localization of integrins would contribute to adhesion formation or growth, we measured the effect of RUSH-α5 release on the paxillin adhesion area in cells. The release of RUSH-α5 increased the adhesion area on fibronectin, while it had no effect on the collagen adhesions (III, Figure 2C). Furthermore, the increased adhesion area observed already 20 min after release suggests that at least a fraction of the integrins are trafficked directly to the adhesion vicinity where they contribute to adhesion growth. In line with this, we observed a slightly higher release induced increase of the cell area at this time point (III, Figure 2D) which could indicate that these adhesions mediate cell spreading. This was supported by the observation of fibronectin plated cells forming longer RUSH-α5 protrusions after release compared to cells plated on collagen (III, Figure 2E). As our line micropattern experiments suggested a ligand-dependent polarized secretion of RUSH-α5 in a two-directional setting, we were curious to explore whether the

secretion would be polarized in a setting allowing free cell spreading. To quantify the polarized secretion in cells, we generated spatiotemporal tracking maps of the cells based on the paxillin signal to be able to unbiasedly identify protruding and retracting regions of the cells (III, Figure 2F). Comparison of the RUSH-α5 secretion to these areas, confirmed the earlier observation of a ligand-dependent polarized secretion taking place on fibronectin (III, Figure 2G). While we did observe a slight increase of the RUSH-α5 recruitment also to the protruding areas of cells plated on collagen 45 min after release, this recruitment was significantly lower compared to cells plated on fibronectin. In addition, we also observed an increase in the recruitment to the retracting areas of cells plated on collagen, which the cells plated on fibronectin lacked. Together these results indicate a polarized and ligand-dependent secretion of newly synthesized integrins to the protruding areas of cells where they participate in adhesion formation and cell protrusion.

5.2.4 Newly synthesized integrin α5 localizes to the tip of newly formed adhesions

Prompted by the observation of RUSH-α5 contributing to adhesion growth, we next evaluated the recruitment of RUSH-α5 to individual adhesions. RUSH-α5 and pmKate2-Paxillin co-transfected cells where plated and allowed to spread on fibronectin coated coverslips, and RUSH-α5 recruitment to adhesions was imaged by total internal reflection fluorescence (TIRF) microscopy (III, Figure 3A). Individual paxillin- and integrin-positive adhesions, with a minimum lifetime of 15 minutes, were identified and divided into four areas (Area 1–4). These areas were further classified based on their position in the adhesion in relation to the cell, with Area 4 being the most membrane proximal area, (closest to the cell edge) and Area 1 the most membrane distal area (closest to the cell body) (III, Figure 3B). To identify the spatio-temporal localization of RUSH-α5 in adhesions, we compared the intensity of RUSH-α5 in each area relative to the intensity observed in the same area 2.5 minutes before the first RUSH-α5 signal was detected in the adhesion (III, Figure 3C). There was a clear increase in RUSH-α5 intensity in the most membrane proximal areas (Area 4 and 3) at the time of "adhesion birth", followed by an intensity drop in these areas simultaneously with an intensity increase in the membrane distal areas (Area 1 and 2) (III, Figure 3D). These results would be compatible with two scenarios: 1) either there is membrane proximal to membrane distal directionality of RUSH-α5 recruitment to the adhesions or alternatively 2) RUSH-α5 slides backwards from the adhesion tip towards the cell body as the adhesion matures. To distinguish between these possibilities, we performed the same experiment on coverslips coated with FN and an anti-GFP nanobody. Trapping of the RUSH-α5 construct abolished both the intensity drop in the membrane proximal

areas, and reduced the intensity increase in the most membrane distal areas confirming a sliding mediated re-localization of the RUSH-α5 in growing adhesions (III, Figure 3E–F). These results suggest that integrins are initially secreted to the plasma membrane at the tip of adhesions from where the adhesion grows backwards towards the cell body as it matures.

5.2.5 Unconventional secretion of integrins to adhesions and protruding areas of cells

In several of the live cell imaging experiments, we observed a small number of secreted vesicles being trafficked to the cell membrane at early time points after the RUSH-α5 release, these vesicles furthermore seemed to be secreted from areas outside of the Golgi (III, Figure 4A). In addition, the line micropattern experiments displayed small RUSH-α5 adhesion structures at the FN line already 10 minutes after the release while the RUSH-CD59 was located in the Golgi (III, Figure 1E). Together, these results urged us to investigate whether a fraction of RUSH-α5 could be secreted directly from the ER to the plasma membrane in a process known as unconventional protein secretion. Unconventional protein secretion, where proteins are directly secreted from the ER to the plasma membrane without entering the Golgi, is often induced by different kinds of cell stress (Rabouille 2016). To study the possible unconventional secretion of newly synthesized integrins, we inhibited the conventional Golgi secretion by treating cells with Golgicide A (Saenz, Sun et al. 2009) and studied the secretion of RUSH-α5 to the protruding areas of cells. While the treatment with Golgicide A clearly abolished the conventional secretion taking place 20 minutes after release and onwards (III, Figure 4B), it did not affect the "early" release of RUSH-α5 observed at 15 minutes after the release (III, Figure 4B and C). Early secretion of RUSH-α5 to adhesions was also observed in Golgicide A treated cells co-transfected with pmKate2-Paxillin (III, Figure 4D). Together these results implicate that a fraction of the newly synthesized integrins are targeted directly from the ER to focal adhesions via a secretion pathway circumventing Golgi traffic.

5.2.6 GRASP protein mediate unconventional secretion via the integrin PDZ-recognizing motif

Unconventional protein secretion (UPS), can be mediated by Golgi reassembling stacking proteins (GRASPs) that interact via their PDZ-domain with various cargo proteins (Gee, Noh et al. 2011, Gee, Kim et al. 2018). During drosophila development, dGRASP (the drosophila homologue to the mammalian expressed GRASP65 and GRASP55) has been proposed to mediate unconventional secretion

of integrins upon the mechanical stress that takes place during epithelial remodeling (Schotman, Karhinen et al. 2009, Schotman, Karhinen et al. 2008). This prompted us to investigate whether the observed UPS of RUSH-α5 could be GRASP-mediated. We first performed immunostaining of GRASPs in RUSH-α5 transfected cells that were fixed after various time points of RUSH-α5 release. Immunofluorescence imaging revealed a large fraction of GRASP proteins in the vicinity of Golgi but strikingly also GRASP puncta that seemed to co-localize with RUSH-α5 adjacent to adhesion structures at early time points after release (III, Supplementary figure 2A). Next, we performed GFP-pulldown experiments with GFP-control transfected or RUSH-α5 transfected cells, which confirmed an interaction between GRASP and RUSH-α5 (III, Figure 4E). To investigate whether GRASP proteins are implicated in the early secretion of RUSH-α5, we studied the recruitment of RUSH-α5 to FN dots on dual coated (FN and GFOGER) micropatterns in cells where GRASP had been downregulated with siRNA (III, Supplementary figure 2B and C). Silencing of GRASPs reduced the delivery of secreted RUSH-α5 to FN dots 10-15 minutes after release, however it also drastically reduced the conventional secretion observed 20 minutes after release (III, Supplementary figure 2C). The reduction in conventional secretion of RUSH-α5 following GRASP depletion was most likely a result of impeding Golgi stack formation (Xiang, Wang 2010). Downregulation of GRASPs have furthermore been linked to downregulation of integrin α5β1 (Ahat, Xiang et al. 2019) and could thus also affect the lifetime of our exogenous RUSH-α5 construct. As integrin α5 harbors PDZ-recognizing motifs in their cytoplasmic tail (Tuomi, Mai et al. 2009), we sought to investigate whether one of these could mediate the interaction with GRASP and if we thus could interrupt the interaction of the two proteins without disrupting the important Golgi linked processes of GRASPs. The integrin α5 cytoplasmic tail sequence PPATSDA, harbors one canonical PDZ-recognizing motif (SDA)(El Mourabit, Poinat et al. 2002) and one non-canonical that is generated by the two prolines (PP) inducing an internal β hairpin structure to function as a PDZ-recognition motif (Tuomi et al. 2009). Pulldown experiments with biotinylated α5 wt peptide (PPATSDA), a control β1 biotinylated peptide, and biotinylated peptides where either the canonical SDA sequence was deleted (delta SDA, PPAT), or the non-canonical motif disrupted (PPAA, AAATSDA), revealed that the canonical SDA sequence mediates the interaction between GRASP and integrin α5 (III, Figure 4F). This led us to study the impact of GRASPs on early secretion of integrin α5 with a RUSH-α5 construct from where the SDA sequence was deleted (RUSH-α5 dSDA). Recruitment of RUSH-α5 dSDA to adhesions was investigated by time-lapse imaging of RUSH-α5 dSDA and pmKate2-paxillin co-transfected cells on fibronectin. The disruption of the GRASP-integrin interaction abolished the early secretion of RUSH-α5 dSDA while it was observed for RUSH-α5 (III, Figure 4G T<25 min). However, by only affecting the GRASP-integrin

connection, the conventional secretion (that was abolished in GRASP downregulated cells) was able to induce a burst of RUSH-α5 dSDA traffic to adhesions, as observed in an increased "late" recruitment of RUSH-α5 dSDA to the adhesions (III, Figure 4G T≥25 min). The slightly lower recruitment of RUSH-α5 dSDA compared to RUSH-α5 by the end of the experiment, could be a result of the unconventional and conventional secretion summing up to a greater total recruitment while the RUSH-α5 dSDA is a result of the conventional secretion alone (III, Figure 4G, T 25–45 min). These results provide evidence for a GRASP mediated fast, unconventional secretion of integrin α5 to adhesions, mediated via the classical C-terminal PDZ-recognizing sequence "SDA" in integrin α5.

6 Discussion

6.1 Deregulated integrin activity impairs mammary gland development via MSF mediated force transmission (I, II)

Here we identified SHARPIN as an important regulator of stromal collagen remodelling in the mammary gland. Our studies demonstrate that the integrin inactivator SHARPIN orchestrates mammary gland development by regulating the collagen-binding integrin α11β1 levels in MSFs, thereby affecting their force generated remodelling of the mammary gland stroma. SHARPIN-deficiency results in downregulation of integrin α11β1, which causes faster integrin-collagen-binding dynamics in MSFs *in vitro*. Faster receptor-ligand dynamics causes alterations in the force generation capacity of MSFs and could thus lower their ability to remodel and assemble the ECM, causing reduced ductal outgrowth in the developing mammary gland. In addition, the primary MSFs studied here display high integrin expression levels, mature focal adhesions and nuclear localization of the mechanosensitive transcription factor Yap at low stiffness ranges, suggesting a mechanoadaption to their naturally soft *in vivo* environment. Furthermore, as a result of the mature adhesions and thereby a lack in adhesion reinforcement with increasing stiffness, these cells display the rarely observed biphasic traction-stiffness relationship, which is the fundamental molecular clutch model prediction.

The mammary gland is composed of epithelial cells, fibroblasts, adipocytes, immune-, lymphatic- and vascular cells. All of these cell types play an important role and work together to maintain a functional organ during the different stages of the mammary gland life cycle. The mammary gland is a highly regenerative organ that undergoes the first stage of development prenatally, but only fully develops during puberty in response to hormonal changes and other factors. In addition to these developmental stages, the mammary gland further develops during pregnancy, lactation and involution (Inman, Robertson et al. 2015). During pubertal development, a process called branching morphogenesis takes place and the mammary epithelium invades into the collagen rich adipose tissue. The increased levels of especially oestrogen and growth factors promotes cell division and formation of terminal end buds (TEBs). TEBs are unique structures directing the

ductal elongation and branching through the fat pad (Paine, Lewis 2017, Paavolainen, Peuhu 2021). Integrins mediates the interaction of the mammary epithelial cells and the ECM, and this interaction is important for regulating the developmental process. Both luminal and basal epithelial cells of the mammary gland express integrins. These integrins do not only mediate cell adhesion, but also detect local ques of the ECM such as stiffness and growth factors, that together enables context-dependent signalling check-points regulating cell fate decisions (Katz, Streuli 2007, Paavolainen, Peuhu 2021). As the most drastic changes during the developmental stages take place in the epithelial cells, the majority of mammary gland research has focused on these cells and less is known about the stromal-ECM changes during development. Integrins are important mechanosensitive receptors regulating the ECM and the physical properties of the ECM also affect integrin signalling (Keely 2011, Delcommenne, Streuli 1995, Civitarese, Talior-Volodarsky et al. 2016). The ECM undergoes changes during breast cancer progression but also during the developmental cycle of the mammary gland. Processes such as ECM remodelling, re-initiation of cell proliferation, angiogenesis and invasion are important during mammary gland development and likewise associated with tumor progression (Wiseman, Werb 2002). The developing mammary gland is thus a good model for studying how the ECM affects integrin activity and vice versa, to better understand the crosstalk between the tumor and its microenvironment. The role of integrin activity regulation in mammary gland development has not been investigated in detail. Moreover, while many studies have looked at integrin activators and ECM crosstalk, integrin inactivators have received much less focus. Here, we studied the role of SHARPIN, an endogenous inactivator of integrin β1 (Rantala et al. 2011), in modulating mechanosensing in primary mammary gland stromal fibroblast. Furthermore, we examined how deregulated integrin activity in MSFs translates into disturbed ECM remodelling and mammary gland development in SHARPIN deficient mice.

6.1.1 SHARPIN modulates adhesion dynamics by regulating integrin α11β1 levels

Integrin activation is crucial for assembling and strengthening of focal adhesions to induce cell spreading (Gallant, Michael et al. 2005, Michael, Dumbauld et al. 2009, Cavalcanti-Adam, Volberg et al. 2007). Here we found that while Sharpin[cpdm] MSFs, with a higher relative integrin β1 activity, spread more than their wt counterparts on fibronectin, they quite counterintuitively displayed reduced cell spreading on soft collagen matrices (II, Fig. 1A-C). This ligand and stiffness dependent spreading phenotype was traced back to downregulation of the collagen receptor integrin α11 in the absence of SHARPIN (II, Fig. 4A-F). Integrin function is tightly regulated on

several levels. In addition to changes in protein expression and activity status of the receptor, trafficking of integrins regulates the receptor availability at the plasma membrane and endocytosis of integrins have been shown to mediate focal adhesion disassembly (Ezratty, Bertaux et al. 2009, Kechagia, Ivaska et al. 2019). Our observations of similar *Itga11* mRNA levels in wt and Sharpin[cpdm] MSFs, together with increased co-localization of integrin α11 with Lamp1 in Sharpin[cpdm] MSFs (II, S3A and Fig 4G) suggests that SHARPIN affects integrin α11 levels indirectly by increasing the degradation of the receptor. This hypothesis is in line with previous findings showing higher endocytosis rate of active integrin receptors, and increased lysosomal degradation of ligand-bound integrins (Arjonen et al. 2012, Lobert et al. 2010). However, the exact mechanisms of the SHARPIN-mediated downregulation of integrin α11 warrants further investigations.

The reduced levels of integrin α11 is also likely the main reason for the increased integrin-collagen-binding dynamics observed in Sharpin[cpdm] MSFs (II, Fig. 2B-C), as ligand-unbinding rates were rescued by ectopic expression of Itga11-EGFP (II, Fig. 5D). While the exact reason for the increased integrin-collagen unbinding rates in Sharpin[cpdm] MSFs remains unknown, one possibility could be integrin α1 replacing α11 in the collagen adhesions resulting in weaker adhesions, as there are variations in integrin-ligand bond strengths (Seetharaman, Etienne-Manneville 2018, Isomursu et al. 2019). Knockdown experiments of integrin α1 and integrin α11 have shown that while integrin α11 deficiency causes drastic reduction of cell adhesion and migration, downregulation of integrin α1 has almost no effect on cell adhesion or migration (Popov, Radic et al. 2011). These results are in line with our finding of integrin α11, but not α1, being indispensable for proper cell spreading on collagen (II, Fig 5A–C). Furthermore, the faster binding dynamics observed in Sharpin[cpdm] MSFs, with lower integrin α11, are directly mirroring their increased turnover of collagen adhesions (I, Fig 5 J–K and EV5D–E).

6.1.2 Increased integrin-collagen-binding dynamics results in deregulated force generation of MSFs at *in vivo* corresponding rigidities

As actin contractility does not change as a result of SHARPIN deficiency, but instead displays a stiffness correlating increase in MSFs (II, Fig. 3A, S2A–B) the only altered features capable of affecting force transmission in Sharpin[cpdm] MSFs are the increased binding dynamics. By only changing the parameters of binding and unbinding dynamics in the molecular clutch model, the model predicts both the lower traction force generation and faster actin retrograde flow observed in Sharpin[cpdm] MSFs on soft collagen substrates (II, Fig. 3B–F, I, Fig. 5F–I). In addition, the model also predicted the fundamental biphasic relationship between force and stiffness as

a result of adhesion maturation not taking place at higher rigidities (II, Fig. 1E–F, 3B). In situations where the number of integrin-ligand clutches are constant an increase in myosin motors pulling on these clutches would result in failure of these clutches to withstand the increased force loading after a certain threshold and disengage, causing a reduction in traction forces after the traction peak has been reached (Chan, Odde 2008, Case, Waterman 2015). This biphasic relationship is almost always masked by the reinforcement of the adhesions (Elosegui-Artola et al. 2016) and was observed by us for the first time in mammalian primary cells. Both the model prediction and our experimental traction force measurements showed no differences in traction force generation on fibronectin (II, Fig. 3B, D) which is consistent with no differences in the binding dynamics to fibronectin (II, Fig. 2B–C). However, our model was not able to predict the final increase in traction forces displayed by wt MSFs on stiff collagen substrates. The increase could be due to other cellular events, which are not considered in the clutch model, taking place at focal adhesions and influencing force transmission. As an example, perturbation of formin, an actin nucleator, has been shown to inhibit stress fibre assembly at adhesions without completely disrupting force transmission at adhesion sites (Oakes, Beckham et al. 2012). In addition to the mature adhesions and biphasic stiffness-force relationship, the MSFs also displayed mature actin stress fibre and significantly slower actin flow at low stiffness compared to talin1$^{-/-}$ MEFs. This could be a result of the soft collagen rich *in vivo* environment of the mammary gland increasing the expression levels of certain integrins in the MSFs (II, Fig. S2D–E). As different integrin expression patterns determines which ECM ligands integrins bind to, the expression pattern also influences the forces a cell can withstand as a result of the different integrin-ligand bond strengths (Seetharaman, Etienne-Manneville 2018, Elosegui-Artola et al. 2014).

6.1.3   Deregulated force transmission in Sharpin^{cpdm} MSFs causes reduced stromal collagen organization

The deregulated force transmission on 2D (TFM) in SHARPIN deficient cells was also translated into reduced large scale force generation on a multicellular and 3D level, as Sharpin^{cpdm} MSFs displayed reduced capacity to contract a 3D collagen gel (II, Fig. 5C–E). Indicating an important role of integrin activity regulation in processes where cells exert forces on their environment. Collagen is the most abundant protein in the human body and fibroblast secrete procollagen that assembles into fibrillar collagen bundles in the ECM. Our study found that MSFs lacking SHARPIN secrete less soluble collagen and also have a reduced capacity to convert newly synthesized collagen into fibrillar collagen (I, Fig. 6A). Collagens can polymerize and undergo self-assembly *in vitro*, and it was long thought that this self-

assembly, guided by precise interactions between collagen molecules, would take place also *in vivo*. However, it is now known that several other factors, such as other ECM components also influence collagen polymerization (J. R. Mao, Taylor et al. 2002, Graham, Holmes et al. 2000). The reduced ability of Sharpin[cpdm] MSFs to polymerize collagen fibres *in vitro* is in line with our findings of SHARPIN deficiency causing downregulation of integrin α11, as the collagen-binding integrins α1 and α11 have been shown to promote fibrillogenesis of type I and III collagen (Velling et al. 2002). Furthermore, Sharpin[cpdm] MSFs also displayed reduced assembly of collagen fibres *in vitro* (I, Fig. 6B–C), which could be a result of lower capacity to exert forces, as mechanical tension provided by fibroblasts organizes and assembles the collagen network. Reciprocally, the rigidity of the collagen network determines the mechanical load the cells experience and thereby affects their cytoskeletal phenotype (Grinnell 2000). The reduced capacity of Sharpin[cpdm] MSFs to exert forces at stiffness ranges resembling the *in vivo* stiffness of the mammary gland (Plodinec et al. 2012) is also consistent with the aberrant collagen fibre organization observed in the mammary gland of Sharpin[cpdm] mice (I, Fig. 4A–B). Movement of collagen fibres by fibroblasts in 3D requires high contractile forces (Wei, Adelstein et al. 2005). As Sharpin[cpdm] MSFs exert lower forces this would translate into a reduced capacity to transport and organize collagen fibres. In addition to displaying less organized collagen fibres, the fibres also tended to be shorter in Sharpin[cpdm] (II, Fig. EV4 E-F), which is in line with our observations of reduced capability of Sharpin[cpdm] MSFs to polymerize collagen *in vitro*. The shorter length of the collagen fibres could also affect the collagen network organization. Indeed, we did observe a reduced stiffness of the Sharpin[cpdm] mammary gland (I, Fig. 4C) and stromal organization of collagen fibres have been shown to directly correlate with stiffness in the cornea (Leonard, Cosert et al. 2019). In addition to affect the stromal stiffness, the reduced fibrillar collagen deposition observed in Sharpin[cpdm] could also affect the clutch dynamics of MSFs *in vivo*. As a reduced availability of ligands could result in a reduced amount of clutches interacting with the ECM, this could result in a weaker clutch and hence faster unbinding rates of the clutch (B. Bangasser et al. 2013, Chan, Odde 2008). This would in turn further enhance the increased integrin-collagen binding dynamics of Sharpin[cpdm] MSFs observed on equal ligand density *in vitro* that stems from the lower integrin α11β1 levels compared to wt MSFs (II, Fig. 2A–C). However, further studies of the impact of reduced ligand availability, for example by varying the ligand concentration or density of the substrate, when comparing the force generation capacities of wt and Sharpin[cpdm] MSFs would be required to confirm this hypothesis.

Another important regulator of collagen crosslinking and fibrillogenesis is lysyl oxidase (LOX) (Herchenhan, Uhlenbrock et al. 2015), which has been shown to be upregulated by TGF-β (Sethi, Mao et al. 2011). As integrin α11 downregulation has

been shown to reduce TGF-β expression (Talior-Volodarsky, Connelly et al. 2012), the reduced mammary gland stiffness could also be a result of an altered LOX mediated cross-linking of the matrix. However, whether the observed reduced stiffness we observed in the SHARPIN deficient mammary gland is a direct result of the collagen network or attributed to other alterations in the Sharpin[cpdm] mammary gland warrants further investigation. While our mRNA profiling recognized several differentially regulated ECM remodelling genes, such as matrix metalloproteases, in Sharpin[cpdm] MSFs, the reduced collagen fibres identified here are most likely not due to an increased proteolytic activity in Sharpin[cpdm] MSFs as these cells displayed a reduced collagenolytic activity (I, Fig. 5A–B). Together, these results show that SHARPIN regulates the mammary gland stromal assembly and organization. While the exact mechanism behind the altered stroma in Sharpin[cpdm] mammary gland still remains to be investigated, our data suggests that the downregulation of integrin α11 deregulates the force transmission of MSFs and thereby diminishes the assembly and organization of the collagen fibres in the mammary gland stroma.

6.1.4 SHARPIN regulates mammary ductal outgrowth

The development of the mammary gland is unique as the final stages takes place postnatally during puberty. Hormonal changes induces branching morphogenesis, a process in which epithelial cells in the highly proliferative terminal end buds invade into the fat pad (Macias, Hinck 2012, Hinck, Silberstein 2005). The mammary gland of Sharpin[cpdm] mice displayed reduced ductal outgrowth and a reduced number of both branches and TEBs, while the morphology of the TEBs, and the cellular organization of the mammary gland was comparable to wt mice (I, Fig. 2). In addition to its role as an integrin inactivator, SHARPIN is also a component of the LUBAC complex with the potential to activate NF-κB signalling (Tokunaga, Nakagawa et al. 2011). As increased NF-κB signalling has been shown to trigger epithelial proliferation and ductal branching (Brantley, Chen et al. 2001) the intuitive reason for reduced mammary ductal outgrowth would be decreased proliferation of the Sharpin[cpdm] epithelial cells, as these undergo extensive proliferation and differentiation during the mammary gland development. While SHARPIN was expressed in both stromal and epithelial cells (I, Fig. 1A-B), our results show no difference in apoptotic or proliferative cells in wt and Sharpin[cpdm] TEBs (I, Fig. EV 2D). Furthermore, the epithelial expression of SHARPIN is dispensable for the mammary gland morphogenesis as transplanted Sharpin[cpdm] mammary epithelia into the cleared fat pad of wt mice developed normally (I, Fig. 3A–D). While we were unable to transplant wt epithelia into the Sharpin[cpdm] mouse due to the short lifespan of this mouse strain, we were able to create a stromal deletion of *Sharpin* in wt mice by using the *S100a4* promoter for *Cre* expression (Bhowmick, Chytil et al. 2004).

S100a4 (also known as fibroblast specific protein 1, FSP1) is widely used as a fibroblast targeting promoter as FSP1 is considered a fibroblast marker (Strutz, Okada et al. 1995). S100a4 protein expression could be detected in both wt and Sharpin[cpdm] MSFs by Western blotting, and in wt mammary gland stroma by immunohistochemistry labelling (I, Fig. EV 4A and C). Furthermore, S100a4-Cre; Sharpin^{fl/fl} mice displayed a clear reduction in stromal *Sharpin* mRNA in the mammary gland and both mammary ductal outgrowth as well as number of TEBs were reduced in S100a4-Cre; Sharpin^{fl/fl} mice compared to S100a4-Cre; Sharpin^{fl/+} mice (I, Fig. EV 4B and Fig. 3E–G). However, there is no fibroblast specific promoter for Cre-recombinase expression and the S100a4 promoter has been shown to be active also in hematopoietic cells (P. Kong, Christia et al. 2013). Immune cells in the mammary gland are known to take part in regulating the proliferation and outgrowth of TEBs (Reed, Schwertfeger 2010). In line with this, we observed an increased immune cell infiltration in the *Sharpin[cpdm]* mammary gland (I, Fig. EV3A–B and EV4D), which could indicate a deregulation of the mammary gland by immune cells. However, while the S100a4-Cre driven gene deletion led to reduced mammary ductal outgrowth, the S100a4-Cre; Sharpin^{fl/fl} mice harboured similar levels of immune cell infiltration at the TEBs as wt mice (I, Fig. EV4D).

These results suggest that the stromal expression of SHARPIN regulates the mammary gland development. Furthermore, aligned collagen fibres in the stroma have been shown to orient and direct epithelial branching (Brownfield, Venugopalan et al. 2013) and the abundance of collagen fibres, but not collagen concentration, promotes epithelial invasion of mammary epithelial organoids (Nguyen-Ngoc, Ewald 2013). In addition, fibrillar collagen I induces proliferation and invasion of breast cancer cells (Reyes-Ramos, Álvarez-García et al. 2021). In contrast, in mammary glands where collagen I fibres are not linearized, the fibres have a suppressing effect on cell invasion (Maller et al. 2013). Together, this suggests that the reduced number and organization of collagen fibres in the Sharpin[cpdm] stroma causes a reduction of the epithelial cell invasion of the TEBs, and thereby affects the mammary gland development.

6.2 Newly synthesized integrins are secreted in a polarized and ligand-dependent manner and contribute to adhesion growth (III)

Here we show that trafficking of newly synthesized integrins adds another layer to the cell repertoire of integrin activity regulation. By targeting the secretion of newly synthesized receptors to the tip of focal adhesions where they mediate adhesion formation and maturation, cells can generate protrusion in a spatially controlled and ligand-dependent manner. For this study, we generated an N-terminally-tagged

integrin α5 RUSH construct, which allows for synchronized release of newly synthesized integrins without interfering with its ligand binding capacity or possible C-terminal interactions. This allowed us to identify a previously unknown mechanism of GRASP-mediated fast secretion of newly synthesized integrins to focal adhesions in mammalian cells.

Integrin function regulation takes place on several different levels because of their important roles as bi-directional signalling mediators between the cell and the ECM, and their subsequent ability to regulate cell functions such as proliferation, migration and gene expression. By regulating endo- and exocytosis of integrins, cells are able to tune the amount of available receptors at the cell surface (Kechagia et al. 2019).

Integrin endocytosis and the subsequent recycling of the receptor has been identified as a major regulator of polarized focal adhesion formation and cell migration (Paul et al. 2015, Roberts et al. 2001, Woods et al. 2004). The activity state of integrin α5β1 is known to affect its recycling destination with active integrins being targeted to the cell rear and inactive integrins to the cell front where they can participate in generating new adhesions required for cell migration (Dozynkiewicz, Jamieson et al. 2012). However, the role of newly synthesized integrins in focal adhesion assembly and cell migration has remained unknown along with the possible effect of ECM properties regulating the maturation and localization of these integrins. This is mainly due to the previously limited methods available for live-cell studies of newly synthesized receptor maturation and trafficking. Although pulse-chase metabolic labelling methods and the use of conformation-specific antibodies have identified some aspects of the integrin maturation pathway (Heino et al. 1989, Tiwari et al. 2011) these methods have their own limitations, and do not allow for real-time visualization of the integrin delivery to the plasma membrane.

6.2.1 Development of RUSH-α5 identified ligand-dependent maturation and trafficking of newly synthesized integrin α5

By utilizing the RUSH-assay (Boncompain et al. 2012) and the available crystal structure of the α5β1 ectodomain (Nagae et al. 2012), we created a functional N-terminally tagged RUSH-α5 construct that allows for synchronized release, maturation and visualization of newly synthesized integrin α5 (III, Figure 1A, D and Supplementary figure 1A, D). Previous studies have shown that cells maintain a tensional homeostasis by for example upregulating integrin α5β1 expression in response to increased shear force to allow increased adhesion formation in situations where stronger cell adhesions are required (Urbich et al. 2000). In contrast, cells can also down-regulate the amount of integrins in situations where lower forces and cell-

adhesions are required (Yeh et al. 2017). These results suggest that cells regulate the surface levels of integrin α5β1 according to their environment. In line with this, our results revealed a trend of faster integrin α5β1 receptor maturation in a stiffness and ligand-dependent manner (III, Figure 1B–C and Supplementary figure 1B–C). Furthermore, the ECM ligand composition also affected the localization of newly synthesized integrins, by mediating a polarized secretion targeted to the protruding areas of the cell, but more prominently under circumstances where an active integrin-ECM bond could be established (III, Figure 1D–H, 2F–G). These results suggest that a cross-talk between integrins at the cell-ECM interface and the newly synthesized integrins in the ER could regulate the localization of newly synthesized integrins in a polarized ligand-dependent manner. However, whether the lack of a polarized localization of RUSH-α5 on collagen is due to a loss of polarized secretion of these cells on collagen, or a result of inactive RUSH-α5 integrins being rapidly endocytosed and recycled (Arjonen et al. 2012, Nishimura, Kaibuchi 2007), remains to be investigated. Experiments with a collagen-binding integrin RUSH-construct displaying the opposite ligand-dependent phenotype or by performing experiments under conditions where endocytosis is blocked would provide some answers to these questions although rapid diffusion of inactive integrins along the plasma membrane could be challenging to prevent. However, diffusion of inactive integrins are most likely not the cause of the ligand-dependent phenotype as the difference was observed also under conditions where an anti-GFP nanobody was used to trap the secreted RUSH-α5 construct at its initial secretion position at the plasma membrane (III, Fig 1F–H).

6.2.2 Ligand-dependent activation of integrin α5 at the focal adhesions contribute to FA growth and cell protrusion

Integrins are known to indirectly capture and stabilize microtubules at adhesion sites and thus regulate the secretion of various newly synthesized proteins to so-called exocytic hot-spots at close vicinity of focal adhesions (Fourriere et al. 2019, Nolte et al. 2021). Here we found that also newly synthesized integrins are targeted to focal adhesions and mediate adhesion growth in a manner that depends on the extracellularly presented ligand (III, Figure 2A–C). The observed ligand-specific recruitment could be a result of two different scenarios. The first, and most likely one, involves all newly synthesized integrins (and other membrane proteins) being targeted to the adhesions, but RUSH-α5 is only able to become trapped on fibronectin as it can bind this ligand and thus experience mechanical force to become activated and stably incorporated in the adhesion (Chan, Odde 2008, F. Kong et al. 2009). The second scenario would be that a high level of active, endogenous integrin α5β1 in fibronectin adhesions, are communicating to the cell interior that more

integrins of this same, specific heterodimer are needed to mediate tension homeostasis at adhesions. This communication could take place in a similar manner as shear flow mediates a specific increase of integrin α5β1 expression in endothelial cells (Urbich et al. 2000). Further studies with intracellular force disrupting agents such as blebbistatin, or experiments with an α5 knock-out cell line in addition to exploiting integrin α5β1 blocking antibodies, could reveal some answers to these possible scenarios. The increased adhesion area, trend for a faster increase of the cell area and longer protrusions observed on fibronectin (III, Figure 2C–E) are furthermore in line with the molecular clutch model predictions of force transduction with an increased number of integrin receptors. The model predicts that an increased amount of clutches in the adhesion would results in a stronger clutch, as the mechanical load on the individual clutches would decrease when more clutches share the force load (B. Bangasser et al. 2013, Chan, Odde 2008). This shared force load would in turn result in a slower force build-up on the individual clutches, allowing talin and vinculin more time to unfold and reinforce the clutch before clutch failure (Elosegui-Artola et al. 2018). An increased reinforcement of the clutch will cause an increase in the traction forces the cell generates and slow down the actin retrograde flow, leading to an increased capacity for cell protrusion, migration and cell spreading (B. Bangasser et al. 2013, Nisenholz, Rajendran et al. 2014, Case, Waterman 2015). Thus, our results suggest an additional level of integrin activity regulation by targeted exocytosis of newly synthesized receptors controlling cell protrusion and adhesion in a ligand-dependent manner. Further studies on how ligand availability affects exocytosis of newly synthesized integrins and their contribution to cell protrusion and adhesion, with for example an integrin α5 knock-out cell line or varying ligand concentrations would shed further insight into this hypothesis. In addition, our results show that integrins first localize to the membrane proximal tip of the adhesion from where they flow backwards towards the cell body (III, Figure 3). This finding is consistent with previous observations of integrin β1 moving backwards in focal adhesions and the notion of integrins that are coupled to ECM and F-actin, are being dragged backwards by the myosin generated actin retrograde flow (Case, Waterman 2015, Rossier, Octeau et al. 2012, Chan, Odde 2008).

6.2.3   GRASP mediated interaction with the integrin α tail allows for rapid secretion of newly synthesized integrins to adhesions

While most plasma membrane and secretory proteins are synthesized in the endoplasmic reticulum (ER) and transported to the plasma membrane (PM) via the conventional secretion pathway, some proteins undergo unconventional protein

secretion (UPS) and are directly targeted from the ER to the plasma membrane (Rabouille 2016). In conventional secretion, proteins containing either a signal peptide or a transmembrane domain are transported from the ER to the Golgi where they are further processed (e.g. glycosylated), sorted into secretory vesicles and finally transported along the cytoskeleton to the PM with which the vesicles fuse. UPS can be mediated for two types of proteins: 1) leaderless cytosolic proteins that lack a signal peptide and 2) transmembrane proteins containing a signal peptide but that bypass at least a part of the Golgi. Different sorts of cellular stress such as inflammation, nutrient-, ER- and mechanical stress are the major triggers of UPS (Rabouille 2017). Proteins can undergo UPS via four different pathways. Cytoplasmic leaderless proteins can reach the extracellular space directly from the cytoplasm by translocating across the PM through plasma membrane pores (Type I) or via ABC transporter proteins (Type II). These two pathways are often triggered by inflammation and allows for extracellular release of cytokines. The Type III pathway of UPS is typically induced by cellular stress. This stress causes membrane-bound organelles to become secretory and allows leaderless proteins to be packed into autophagy-associated vesicles followed by PM secretion. Transmembrane proteins or proteins containing a signal peptide can undergo Type IV, Golgi-bypassing UPS. These proteins bypass at least part of the Golgi complex, are always transported via tubulovesicular system and reach the plasma membrane via e.g. Hsp70, Rab8 or GRASP-mediated transport (Kim, Gee et al. 2018). Here, we found that part of the newly synthesized integrins were trafficked to the plasma membrane very rapidly after biotin-induced release from the ER, indicating a Golgi circumventing transport of these integrins (III, Figure 4A). Furthermore, this rapid or "early" secretion of integrins to the plasma membrane and focal adhesions was insensitive to Golgicide A inhibition of the classical secretory pathway, suggesting that integrins can undergo unconventional protein secretion (III, Figure 4B–D). GRASP proteins were originally identified as post-mitotic Golgi reassembling proteins, that through their PDZ domain oligomerize to tether the Golgi membranes together (Barr, Puype et al. 1997). During mitosis, phosphorylation of GRASP induces monomerization of the GRASP oligomers, which allows for Golgi disassembly and division into the two daughter cells (Feng, Yu et al. 2013, Sengupta, Linstedt 2010). In addition, GRASPs have been identified as mediators of UPS in mammalian cells (Gee et al. 2011, Kim, Noh et al. 2016) and this process requires a phosphorylation dependent monomerization of GRASPs as it makes the PDZ domain of GRASP proteins available for interactions with their carrier proteins (Kim et al. 2016). In this study, we found that integrin α5 interacts with GRASP proteins and that this interaction is mediated via the PDZ-recognizing motif "SDA" in the integrin α5 cytoplasmic tail (III, Figure 4E–F, Supplementary figure 2A). Furthermore, siRNA downregulation of GRASPs or disruption of the PDZ mediated

interaction with GRASP abolished the early secretion of integrin α5 (III, Figure 4G, Supplementary Figure 2B–C). While ER stress can induce UPS, and overexpression of our construct could result in such a stress, we find it unlikely as we did not observe UPS of the co-transfected RUSH-CD59 construct. In addition, mechanical stress has earlier been linked to UPS of integrins in drosophila. During drosophila development, mechanical stretching causes epithelial flattening which results in reduced cell-cell contacts and increased cell-ECM contacts on the basolateral side of the epithelia. In this process, mechanical stretch induces an upregulation of GRASP that induces UPS of epithelial integrins, which localize to the basolateral plasma membrane to form focal adhesions and stabilize the basal adhesion (Schotman et al. 2008). Furthermore, elevated levels of cytosolic Ca^{2+} can induce GRASP phosphorylation by activating PKCα, which would allow for monomerization of GRASP to induce UPS (Ireland, Ramnarayanan et al. 2020). Since mechanical stretch can increase the cytosolic Ca^{2+} levels by opening up ion channels at the plasma membrane (Garcia, C S N B, Prota et al. 2006), it is possible that tension also via this mechanism induce GRASP mediated UPS. As another trigger of UPS is inflammation, which also mediates UPS of cytokines that in turn can activate integrins, it would be interesting to know whether a crosstalk between integrins and cytokines is involved in regulation of this process (Mezu-Ndubuisi, Maheshwari 2020, Daniels, Brough 2017). Further studies in low tension environments together with intracellular Ca^{2+} elevating reagents such as Thapsigargin and integrin activation blocking antibodies would provide some answers to these questions. Together these results suggest that integrins can be rapidly secreted to focal adhesions via an unconventional protein secretion pathway that circumvents the Golgi. This secretion depends on the classical PDZ-recognizing motif in the integrin α5 tail mediating an interaction with GRASP. While the underlying mechanism triggering this secretion remains unknown, it is tempting to speculate that increased forces at the plasma membrane, mediated by integrin-adhesions, triggers intracellular signalling events mediating a fast secretion of integrins to the plasma membrane to increase adhesion formation in a spatio-temporal manner.

7 Conclusions

The purpose of this thesis was to study different modes of integrin activity regulation in mechanosensing. While the role of integrin activators and ECM stiffness in mechanosensing has been studied extensively, there is a gap in the literature regarding our knowledge about the role of integrin inactivators in this process. Our group has previously identified SHARPIN as an important inhibitor of integrin β1 activity (Rantala et al. 2011) and we were thus prompted to investigate the role of SHARPIN in mechanosensing. Furthermore, our group and others have studied how the integrin activity state affects trafficking of the integrin β1 receptor (Arjonen et al. 2012) and the subsequent mechanosensitive processes such as cell spreading and migration (Ratcliffe, Sahgal et al. 2016). However, the role of newly synthesized integrins in mechanosensing has not been investigated in detail, largely owing to lack of suitable methodology. By collaborating with the developers of the RUSH-assay (Boncompain et al. 2012) we developed an integrin α5-construct that allows for real-time imaging of synchronized release of newly synthesized integrin α5 to study the contribution of newly synthesized integrins in mechanosensing.

7.1 SHARPIN regulates force transmission in mammary gland stromal fibroblast (I, II)

In this study we show that SHARPIN deficient mice (Sharpin[cpdm]) display impaired mammary gland development due to reduced stromal re-arrangement. We furthermore show that SHARPIN regulates force transmission of mammary stromal fibroblasts (MSFs), the key regulators of ECM remodeling. The reduced force generation of Sharpin[cpdm] MSFs could be traced back to increased activity of integrin β1 molecules at the surface of SHARPIN null or depleted cells, resulting in increased lysosomal degradation of the collagen-binding receptor integrin α11β1. Reduced integrin α11β1 levels resulted in faster integrin-collagen-binding kinetics in Sharpin[cpdm] MSFs. In silico modeling, based on the molecular clutch model, predicted faster binding kinetics resulting in lower force generation. Concordant with this prediction, we observed reduced force generation capacity in the Sharpin[cpdm] MSFs at low ECM rigidity. This was specifically due to loss or SHARPIN triggering reduced integrin α11β1 expression, since we were able to rescue the ligand-binding

kinetics and the force generation phenotype of Sharpin[cpdm] MSFs by ectopic expression integrin α11β1 or SHARPIN to the cells. Together, these results point to an important role for SHARPIN in mechanotransduction by regulating the activity level of integrin β1.

7.2 Newly synthesized integrins are secreted in a ligand-dependent manner and contribute to adhesion growth (III)

By using RUSH-α5, an N-terminally EGFP-tagged integrin α5-construct that allows for synchronized release of newly synthesized integrins from the endoplasmic reticulum (ER), we identified a polarized secretion of integrin α5 to the plasma membrane. This polarized secretion depended on the ECM ligand presented at the cell surface, and targeted secretion was observed to a greater extent when cells were plated on the integrin α5 ligand fibronectin than on collagen which does not bind integrin α5. We found that integrin α5 displays a trend for faster maturation under circumstances where an active receptor is needed, and is trafficked to the protruding areas of the cell in a ligand-dependent manner. Furthermore, newly synthesized integrins are localized to the tip of focal adhesions where they contribute to adhesion growth in a ligand-dependent manner. In addition, we propose a previously unappreciated mode of unconventional protein secretion (UPS) of mammalian integrin α5, a process where newly synthesized integrins are circumventing at least part of the Golgi. This UPS, allowed for rapid trafficking of newly synthesized integrins from the ER to adhesions. Moreover, UPS of integrin α5 is dependent on a PDZ-binding motif in the cytoplasmic tail of integrin α5, interacting with the Golgi reassembling stacking proteins (GRASPS). Together, these results points to an ECM-dependent secretion of newly synthesized integrins to polarized regions of the cell, where they can play an active part in mechanotransduction.

Acknowledgements

I would like to express my gratitude to the pre-examiners of this thesis, Aki Manninen and Staffan Strömblad. I sincerely thank you for your insightful comments, criticism and suggestions that all improved this work. I would also like to thank Patrick Caswell for accepting the invitation to be my opponent and I truly look forward to the scientific discussion with you.

I am extremely grateful for all the great collaborators and co-authors that I have had the pleasure to work with and learn from. Science at its best is a team effort and you are all All-stars players. Thank you Emilia Peuhu, Riina Kaukonen, Markku Saari, Camilo Guzmán, Pia Rantakari, Nicola De Franceschi, Anni Wärri, Maria Georgiadou, Guillaume Jacquemet, Elina Mattila, Reetta Virtakoivu, Yuming Liu, Youmna Attieh, Kathleen A Silva, Timo Betz, John Sundberg, Marko Salmi, Marie-Ange Deugnier, Kevin Eliceir, Alberto Elosegui-Artola, Jenny Kechagia, Ion Andreu, Donald Gullberg, Pere Roca-Cusachs, Franck Perez, Gaelle Boncompain and Stéphanie Miserey-Lenkei. In addition, I would like to express my gratitude to the Institute for Bioengineering of Catalonia and Institut Curie for hosting me during my research visits to these excellent institutions.

It takes a village, Ivaska Lab, thank you for raising me and for creating such a lovely work environment! I could not have wished for better lab mates: Aki, Aleksi, Anja, Antti, Camilo, David, Elisa, Emmi, Gautier, Guillaume, Hellyeh, Hussein, Ilkka, Iman, James, Jaroslav, Jasmin, Jenni, Johanna J., Johanna L., Jonna, Kerstin, Maria G., Maria R., Maria T., Mathilde, Megan, Mika, Mitro, Nicola, Nicolas, Niklas, Nuria, Paula, Paulina, Petra, Pranshu, Reetta, Riina, Siiri, Taru, Tiina and Valentijn. I am grateful for the inspiring scientific discussions and for all the laughs and shared moments both in the lab and outside of work. I feel extra grateful for the talented Postdocs of Ivaska lab. Thank you especially Emmi for bringing me onto the shared projects. For teaching me mouse work and for having patience with me during the different phases of my learning process. Guillaume, you are a rockstar in imaging and image analysis! Thank you for sharing your knowledge, for always being happy to help, being a great teacher, your endless ideas on how to improve projects and helping me turn cool images into numbers. Camilo, thank you for being a great co-worker already while in the Abankwa lab, explaining physics in a way that even I could understand, your valuable input in our project and being a friend. Thank you also Markku for your guidance and assistance with the microscopes and cheering me up when the spinning disk refused to co-operate with me. I would also like to express a special gratitude to Jenni and Petra, for always being so helpful and cheerful in the lab, as well as offering free therapy sessions in the coffee corner. Hellyeh, you are a true hero wearing a smile instead of a cape, thank you for your endless support with the day-to-day tasks! A special shout-out to the *Wannabe PhD* group of the Ivaska lab, I love you guys! Thank you for the numerous dinner nights, Christmas porridges and cottage trips. For proofreading and helping with the

translations of applications, moral support and sharing the ups and downs over a beer (or two), and always being there for each other. Aki, there are no words, you made the time in the Ivaska lab wonderful. Thank you for being the best travel company, the shared ideas and the problem solving, whether they took place over a beer or during a run they were always fun and inspiring. Thank you also Anni and Albert for the dinner nights and the zoom calls during Corona that made life feel joyful and almost normal again!

In addition to all the scientific skills I have gained in Biocity, I have also been lucky to meet some amazing people over the years. I would therefore like to thank my "second family" in Biocity: Marika, Daniel, Sofia, Iris, Johanna, Rasmus, Josef, Elina and Sebastian. Thank you for the support, company, game nights and making Biocity feel like a second home filled with friends. Daniel, thank you for sharing the same odd sense of humour as me, the much needed wining and dining events and the endless jokes and laughs. Marika, you are the big sister I never had. Thank you for helping me already during my master's thesis and coming over just to give me a hug when I locked myself in to write my thesis. Thank you for knowing what I need before I open my mouth and for always cheering me up. Sofia, thank you for the fun times we spent in the lab together as undergrads, being a great friend and solid as a rock. For the endless times you have helped with James when I have been travelling or working late nights!

Thank you Kaisa, for your support, encouragement and being there for me through the highs and the lows. For picking up the phone in the middle of the night when things did not go as planned in Barcelona, being James' best friend all those times I was stuck in the lab and believing in me when I didn't do it. Joska and Paukku, my dear old friends, you have been there for me since I was a kid in a red soccer jersey. Thank you for all the shared moments on the field, helping me find that inner "agre" and never being further than a phone call away.
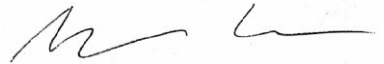
My greatest gratitude goes out to my family. Thank you for the endless support and love, always encouraging us to ask questions and to be curious. Thank you for taking care of James and providing him with a second home. Martin, you have walked by my side from day one, you have pushed me forward but still always had my back, thank you for making me fall in love with competition and being proud of me! Thank you Dad for your comforting words and warm hugs that have always been a safe-haven to me. For raising my curiosity towards the nature that surrounds us. For taking me along for the hunting and fishing trips as a kid and explaining the anatomy of fish, birds and deer and answering the endless amount of questions. **Mom, my super hero and role model**, **this book is for you!** You have showed me that it is possible to aim high and achieve anything. Thank you for setting an example, taking us to the lab when we were kids and letting me fall in love with biology on my own and not out of pressure. Thank you for listening and

understanding when I have been struggling in the lab. I hope I can make you as proud of me one day as I am proud of you.

Turku, September 2021
Martina Lerche

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