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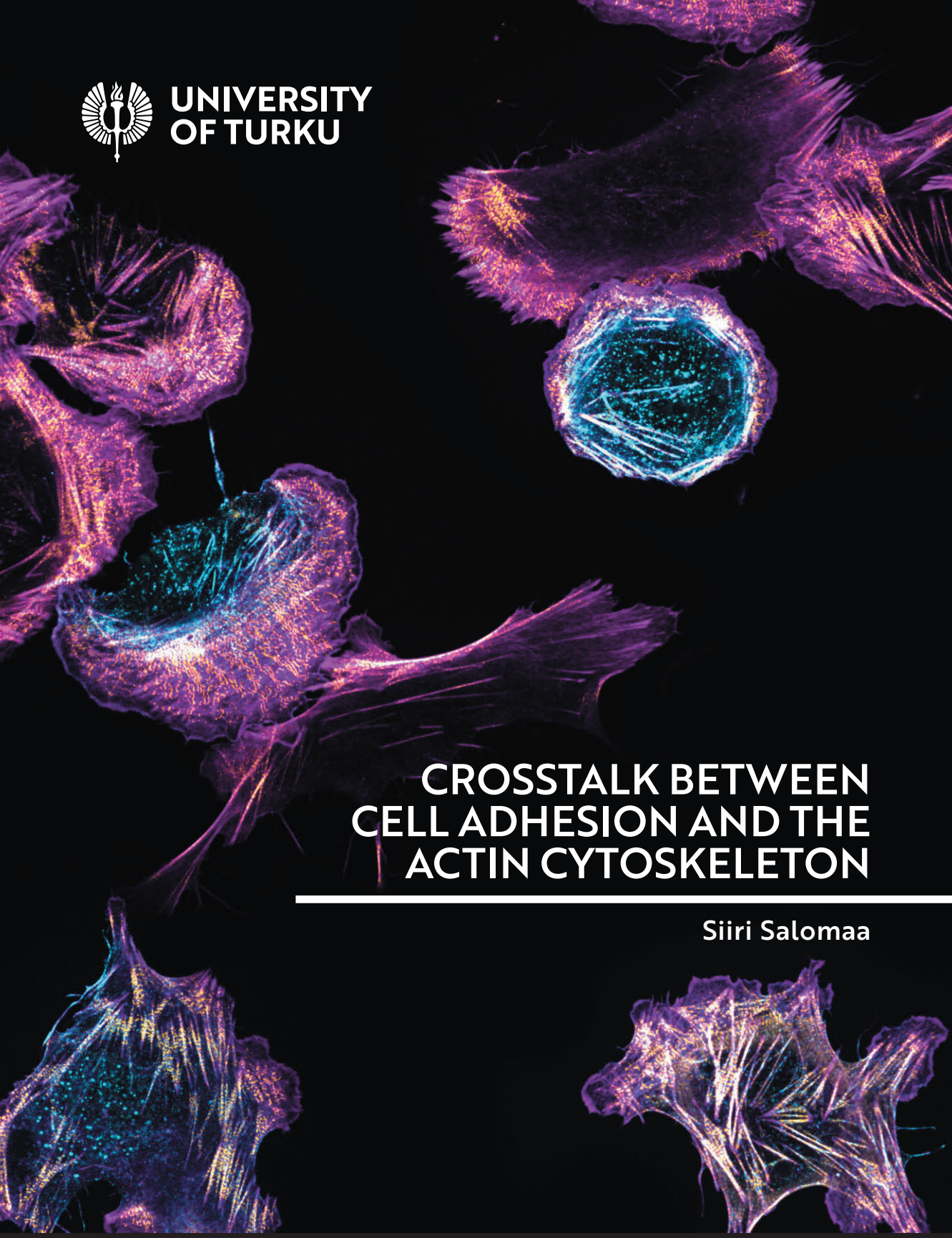

CROSSTALK BETWEEN CELL ADHESION AND THE ACTIN CYTOSKELETON

Siiri Salomaa

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ABSTRACT

In order to form tissues and to move, cells need to attach to the surrounding environment. Integrins are the major cell adhesion receptors that cells use to attach to the extracellular matrix on the outside of the cell, and to recruit a large adhesion complex on the intracellular side. As transmembrane proteins, integrins have an important role in mediating bidirectional signalling across the plasma membrane. Moreover, the integrin-based adhesions are linked to the actin cytoskeleton and thereby act as a link between the extracellular matrix and the actin cytoskeleton. The actin cytoskeleton is responsible for the cellular force generation, and integrin-based adhesions and the actin cytoskeleton create thereby a machinery, that cells can use for example to move. Moreover, integrins and the actin cytoskeleton can mediate reactions to extracellular cues and even alter gene expression. Both integrin activity and the actin cytoskeleton are carefully regulated, and mutations in genes encoding for integrin and actin regulators associate with plethora of diseases. However, less is known if integrin-based adhesions and the actin cytoskeleton are regulated by the same factors.

In this thesis, I have investigated the role of two known integrin inhibitors, SHANK3 and SHARPIN, in regulation of the actin cytoskeleton, and whether this occurs synergistically with regulation of integrins and cell adhesion. I have characterised novel interaction partners for both SHANK3 and SHARPIN, and defined their functions in regulating the cellular actin cytoskeleton, cell adhesion and cell migration. Furthermore, I have investigated how SHARPIN regulates integrin activity at tissue level and find that integrin inhibition can ameliorate the effects of SHARPIN loss *in vivo*. Importantly, the findings presented in my thesis provide novel insights that can be used to understand pathogenesis of cancer, neuropsychiathric disorders and psoriasis-like dermatitis.

KEYWORDS: actin, cancer, cell adhesion, cell migration, cytoskeleton, dermatitis, integrins, SHANK, SHARPIN

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TIIVISTELMÄ

Solut kiinnittyvät ympäristöönsä muodostaakseen kudoksia ja liikkuakseen. Integriinit ovat keskeisessä asemassa olevia solun tarttumisreseptoreja, joita solu käyttää kiinnityäkseen solun ulkopuolella soluväliaineeseen ja sisäpuolella muiden proteiinien sitomiseen ja siten monia proteiineja käsittävän adheesiokompleksin muodostamiseen. Integriinit läpäisevät solukalvon ja kykenevät siten välittämään signaaleja solun ulkopuolelta solun sisälle, ja solun sisäpuolelta ulkopuolelle. Lisäksi integriinit sitoutuvat välillisesti solun aktiinitukirankaan muodostaen siten yhteyden soluja ympäröivän soluväliaineen ja aktiinitukirangan välille. Solun aktiinitukiranka on vastuussa mekaanisten voimien tuottamisesta solussa, ja yhdessä integriinit ja aktiinitukiranka muodostavat koneiston, jonka avulla solu pystyy liikkumaan. Ne voivat myös yhdessä lukea solun ympäristön ominaisuuksia ja välittää siten säätelyä, jolla solu sopeutuu ympäristöönsä ja sen muutoksiin. Sekä integriinit että aktiinitukiranka vaativat tarkkaa säätelyä, ja mutaatiot niitä säätelevien proteiinien geeneissä ovat yhteydessä moniin erilaisiin sairauksiin.

Tutkin tässä väitöskirjassa miten kaksi integriinien säätelijöiksi tunnistettua proteiinia, SHANK3 ja sharpiini, vaikuttavat myös aktiinitukirankaan ja tapahtuuko tämä säätely yhdessä vai erikseen integriinien säätelyn kanssa. Kuvaan uusia, suoria vuorovaikutuksia sekä SHANK3:n että sharpiinin ja muiden solun proteiinien välillä. Lisäksi tutkin miten SHANK3 ja sharpiini vaikuttavat näiden uusien toimintojensa kautta solun aktiinitukirangan säätelyyn, solujen kiinnittymiseen ympäristöönsä ja solujen liikkumiseen. Lisäksi kartoitin miten sharpiini säätelee integriinien aktiivisuutta kudostasolla ja osoitin kuinka integriinien toiminnan estäminen voi lieventää sharpiinin geneettisen puutteen aiheuttaman tulehdusta. Väitöskirjani löydökset tarjoavat uutta tietoa, joka voi auttaa ymmärtämään syövän, neuropsykiatristen sairauksien ja psoriasikseksen tautimekanismeja paremmin.

AVAINSANAT: aktiini, integriinit, SHANK, sharpiini, solujen liikkuminen, solun tukiranka, syöpä, tarttumisreseptorit

Table of Contents

Abbreviations

Abi-1	Abeldon interacting protein 1
ABP	actin-binding protein
Abp1	actin-binding protein 1
Arp2/3	actin-related protein 2/3
ADF	actin depolymerizing factor
ADP	adenosine diphosphate
ARR	ankyrin repeat region
ASD	autism spectrum disorders
ATP	adenosine triphosphate
BSA	bovine serum albumin
CNS	central nervous system
cpdm	chronic hyperproliferative dermatitis
DMEM	Dulbecco's Modified Eagle's Medium
ECM	extracellular matrix
ERM	ezrin-radixin-moesin
FACS	fluorescence-activated cell sorting, flow cytometry
F-actin	filamentous actin
FAK	focal adhesion kinase
FBS	fetal bovine serum
FL	full-length
FRAP	fluorescence recovery after photobleaching
G-actin	globular actin
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GDP	guanosine diphosphate
GFP	green fluorescent protein
GST	glutathione S-transferase
GTP	guanosine triphosphate
HBSS	Hank's Balanced Salt Solution
HOIL-1	heme-oxidized IRP2 ubiquitin ligase 1
HOIP	HOIL-1 interacting protein

INF2	inverted formin 2
IRSp53	insulin receptor substrate of 53 kDa
kDa	kilodalton
KO	knockout
kPa	kilopascal
LUBAC	linear ubiquitin chain assembly complex
MEF	mouse embryonic fibroblast
MMP	matrix metalloproteinase
Myo10	Myosin-10
NF-κB	nuclear factor-κB
NPF	nucleation promoting factor
NZF	Npl4 zinc finger
PBS	phosphate buffered saline
PDZ	PSD-95/Discs large/ZO-1
PFA	paraformaldehyde
PH	pleckstrin homology
PLA	proximity ligation assay
pMLC	phospho myosin light chain
PP	polyproline
PSD	postsynaptic density
PTEN	phosphatase and tensin homolog
RFP	red fluorescent protein
RIAM	Rap1-GTP-interacting adaptor molecule
ROCK	Rho-associated protein kinase
SAM	sterile alpha motif
SCAR	suppressor of cAR
SH3	Src homology 3
SHANK	SH3 and multiple ankyrin repeat domains proteins
SHARPIN	SHANK Associated RH Domain Interactor
SPN	SHANK/ProSAP N-terminal
TCR	T-cell receptor
TIRF	total internal reflection fluorescence microscopy
TNFα	tumour necrosis factor α
TNFR	tumour necrosis factor receptor
UBL	ubiquitin-like
VASP	vasodilator-stimulated phosphoprotein
VCA	verprolin homology, central acidic
WASP	Wiscott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein
WT	wild type

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 SALOMAA, SI, Kremneva, E, Miihkinen, M, Lilja, J, Jacquemet, G, Vuorio, J, Hassani-Nia, F, Hollos, P, Isomursu, A, Vattulainen, I, Coffey, E, Kreienkamp, HJ, Lappalainen, P, Ivaska, J. SHANK3 binds actin and regulates filopodia formation and focal adhesions. *Manuscript*.

II Khan MH, SALOMAA SI, Jacquemet G, Butt U, Miihkinen M, Deguchi T, Kremneva E, Lappalainen P, Humphries MJ, Pouwels J. The Sharpin interactome reveals a role for Sharpin in lamellipodium formation via the Arp2/3 complex. *J Cell Sci.* 2017 Sep 15;130(18):3094-3107.

III Peuhu E, SALOMAA SI, De Franceschi N, Potter CS, Sundberg JP, and Pouwels J. Integrin beta1 inhibition alleviates the chronic hyperproliferative dermatitis phenotype of SHARPIN-deficient mice. *PLoS One*. 2017 Oct 17;12(10):e0186628.

1 Introduction

Cells are the building blocks of tissues, such as muscle, liver or brain tissue. In addition to cells, tissues consist of extracellular matrix (ECM), also known as connective tissue, which provides a framework for the tissue. The ECM is secreted by the cells themselves, and it consists mainly of different proteins and proteoglycans. In addition to working as a scaffold, the ECM has an important role in regulating cell signalling through engaging specific receptors or binding secreted soluble signalling mediators such as growth factors. The ECM composition also defines the physical parameters of the tissue cells and cells can exert mechanical forces to remodel the ECM. Importantly, the ECM acts as a template to which cells can adhere to and in which cells can migrate in (Hynes and Naba, 2012; Frantz et al., 2010).

Integrin adhesion receptors are transmembrane heterodimers that bind the ECM with their large extracellular domains, and recruit other proteins to their intracellular tails, which serve as binding platforms for integrin regulatory proteins and adhesion components. Importantly, integrin activation triggers formation of cell adhesions, which are large protein complexes that participate in force generation and signalling. As transmembrane proteins, integrins have a pivotal role in mediating signalling bidirectionally across the plasma membrane. Therefore, integrins participate in plethora of cellular signalling cascades (Michael and Parsons, 2020; Bouvard et al., 2013; Hynes, 2002). In this thesis, the focus will be on the role of integrin-based adhesions as interfaces between the ECM and the actin cytoskeleton, and how integrin activity is regulated at the tissue level.

Integrin-based adhesions consist of tens to hundreds of different proteins (Horton et al., 2015). Importantly, these adhesion-associated proteins connect integrins to the actin cytoskeleton. While the integrins make the initial contact with the environment, the actin cytoskeleton provides the cells a backbone and muscles. Importantly, adhesion-linked actin stress fibers can contract and mediate tension. Therefore, the actin cytoskeleton has a pivotal role in maintaining cell shape and mediating cell movement (Burridge and Wittchen, 2013; Livne and Geiger, 2016). Together, integrin-based adhesions and the connected actin cytoskeleton can read and react to the environmental cues, such as rigidity of the ECM, its protein composition and

topography, but also exert mechanical forces back to the environment (Geiger et al., 2001; Katz et al., 2000; Elosegui-Artola et al., 2016; Doyle et al., 2012).

Integrins can be both activated and inhibited by proteins that bind to their intracellular tails, and several proteins have been identified to either promote or suppress activity of integrins (Askari et al., 2009; Shattil et al., 2010). Function of these regulators is crucial, as dysregulated integrin activity associates with multiple diseases ranging from inflammatory diseases to bleeding disorders and cancer (Bouvard et al., 2013; Hynes, 2002). In addition, the actin cytoskeleton is regulated by multiple actin-binding proteins that organize cytoskeletal structures, crosslink actin filaments, regulate actin assembly and disassembly and contractility of the actin cytoskeleton. Consistently, dysregulated actin cytoskeleton associates with multiple diseases, including immune deficiencies and neuropsychiatric disorders (Joensuu et al., 2018; Yamaguchi and Condeelis, 2007; Machesky and Insall, 1998). Importantly, the diseases associated with dysregulated integrin activity, cell adhesion and/or actin cytoskeleton, are often caused by mutations in proteins regulating them. This highlights the importance of understanding the roles of integrin, adhesion and actin regulators.

Integrin-mediated cell adhesion and the actin cytoskeleton have been studied extensively, but there are still many unanswered questions. Especially crosstalk between integrin activity regulation and the actin cytoskeleton awaits further studies. In this thesis, I have investigated the role of two known integrin inhibitors, SHANK3 and SHARPIN, in regulation of the actin cytoskeleton. Furthermore, I have studied how SHARPIN-mediated integrin inhibition contributes to the chronic proliferative dermatitis phenotype that *Sharpin* null mice exhibit at the tissue level. The observations presented in this thesis will expand our knowledge on how distinct proteins can regulate both cell adhesion and the actin cytoskeleton. These findings will advance our current knowledge of regulation of integrins, cell adhesion and the actin cytoskeleton. Furthermore, they may provide novel information that can be used to develop new therapeutic targets in autism-spectrum disorders, cancer and psoriasis-like conditions.

2 Review of the Literature

2.1 Cell adhesion and the extracellular matrix

The tissue consists of cells and the extracellular matrix (ECM). The ECM is a non-cellular meshwork of fibrous proteins, such as fibronectin and collagen, and proteoglycans that surrounds the cells. It provides a physical scaffold for the tissues and it is the microenvironment of cells. Furthermore, cells themselves have an important role in formation and remodelling of the ECM, as they secrete proteins and other factors, such as ECM-modifying enzymes and growth factors, to the ECM (Frantz et al., 2010; Hynes and Naba, 2012). The ECM provides structure and form to a tissue, and its composition and stiffness varies greatly between different tissues (Frantz et al., 2010; Handorf et al., 2015). For example, while the brain tissue has an averege stiffness of 1-4 kilopascal (kPa), for the bone this is 15000-20000 kPa (Handorf et al., 2015).

Interactions between cells and the ECM have many distinct purposes. Cells adhere to the ECM to anchor themselves, but also to create movement and tensile forces. Furthermore, cell-matrix attachment has an important role in cell differentiation and mediating signalling to regulate the cytoskeleton and to trigger survival signalling for example in synergy with growth factor-dependent pathways. Both ECM composition and stiffness are known to regulate organization of the actin cytoskeleton and its contractility, cell cycle progression, differentiation of stem cells into specific cell types and tissue homeostasis (Frantz et al., 2010; Handorf et al., 2015).

2.1.1 Integrins are cell surface receptors that mediate bidirectional signalling across the plasma membrane

Integrins are major cell adhesion receptors, which have a pivotal role in how cells interact with their environment and surrounding cells. Importantly, cells use integrins to bind and make contacts to the surrounding ECM. Integrins form and function as heterodimers of an α and a β subunit, and the known 24 distinct heterodimers are combined from altogether 18 α and 8 β subunits. The different integrin heterodimers are further classified into RGD-, collagen-, laminin- and

leukocyte-specific receptors based on their binding substrates (Humphries et al., 2006; Hynes, 2002).

Regulation of integrin activity has a central role in how cells interact with the ECM, and dysregulated balance in integrin activity associates with various pathological conditions, including cancer, bleeding disorders and immunological diseases (Bouvard et al., 2013; Hynes, 2002). Importantly, integrins are transmembrane proteins, they mediate signalling bidirectionally, and activity of integrins can be regulated both from inside-out and outside-in (Hynes, 2002). Moreover, integrins can be both activated and inactivated through interactions with cytoplasmic integrin regulatory proteins (Bouvard et al., 2013; Calderwood, 2004; Gao et al., 2019; Pouwels et al., 2012; Shattil et al., 2010).

Integrins undergo conformational changes that regulate their affinity towards their ligands. Three major conformational stages have been well-characterised: a bent, inactive conformation, a primed, extended conformation and an open, fully activated conformation (Figure 1.) (Askari et al., 2009; Luo et al., 2007). Bent, inactive integrins are held in an inactive conformation through a salt bridge interaction between the cytoplasmic α- and β-tails (Campbell and Humphries, 2011). They can be switched into an extended primed-active conformation following binding of integrin activators to the cytoplasmic β-tail and separating the integrin cytoplasmic tails (Campbell and Humphries, 2011; Shattil et al., 2010). This is called inside-out activation, whereas outside-in activation is a process where primed, unoccupied integrin binds ECM ligand to become fully activated and ligand occupied (Askari et al., 2009; Luo et al., 2007; Shattil et al., 2010). Integrins can be inactivated and held in an inactivated conformation by recruitment of integrin inactivating proteins to the cytoplasmic α- and β-tails (Figure 1.) (Bouvard et al., 2013; Pouwels et al., 2012).

Talins are the most well-established integrin activators that bind to the integrin β-tail and promote recruitment of other adhesion-associated proteins to the site. For example, talins promote recruitment of kindlins to the integrin β-tail, and they co-operatively regulate integrin affinity (Moser et al., 2009; Sun et al., 2019). Furthermore, talin also recruits focal adhesion protein vinculin, and connects integrins to the actin cytoskeleton both through vinculin and by directly binding actin (Humphries et al., 2007; Sun et al., 2019). Importantly, talin acts as a mechanosensitive nexus in adhesion signalling (Goult et al., 2018). Small GTPase Rap1 and Rap1-GTP-interacting adaptor molecule (RIAM) recruit talin to the plasma membrane and relieve talin autoinhibition promoting thereby integrin and actin binding (Goult et al., 2018). The linkage between talin and contractile actin structures increases to stretching of talin promoting vinculin recruitment and increasing therefore force transmission between integrins and actomyosin structures (Yao et al., 2014). This force-induced unfolding of talin also induces RIAM

replacement with vinculin, and while integrin-talin-RIAM complex associates typically with initial cell protrusion tips, more mature adhesions that have vinculin are devoid of RIAM (Lagarrigue et al., 2015; Goult et al., 2018). In addition, the linkage between talin and the actin cytoskeleton also further increases integrin affinity in a mechanosensitive manner highlighting the importance of mechanical signalling in integrin activation (Sun et al., 2019).

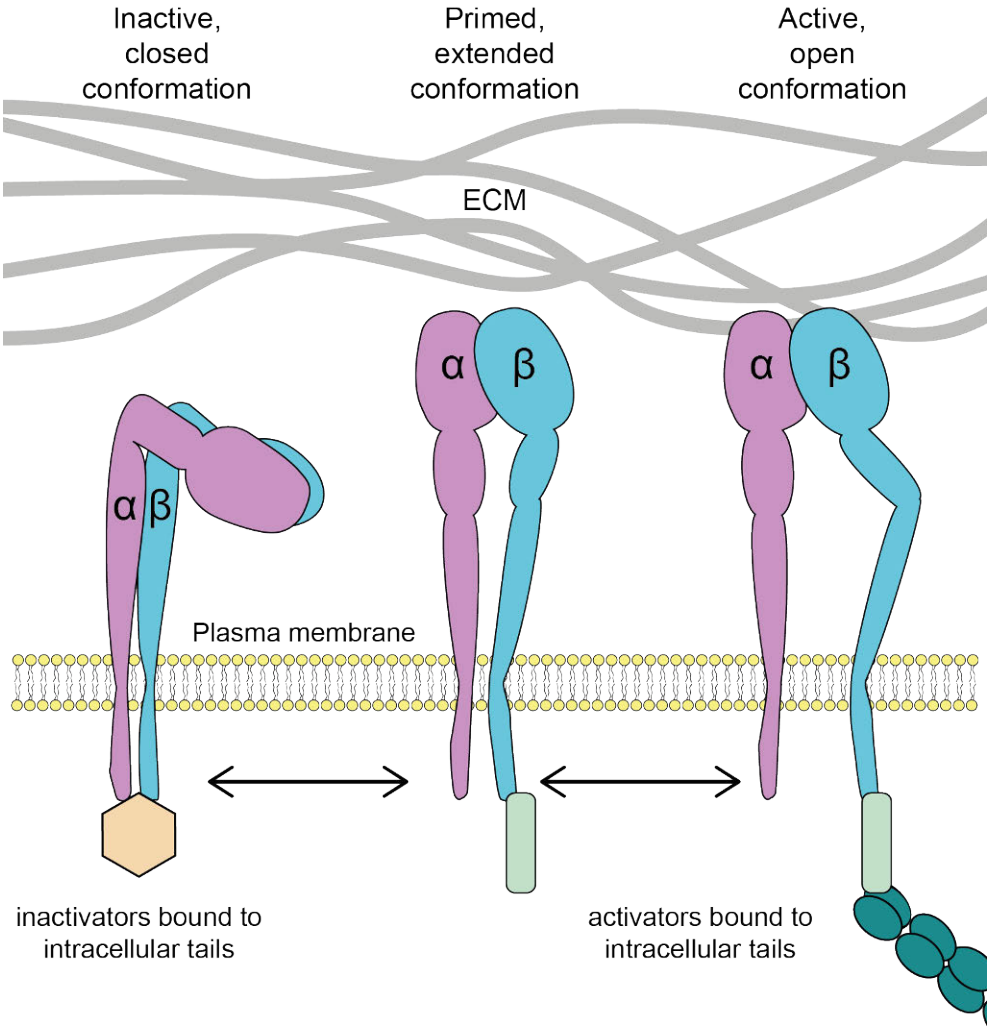

Figure 1. Integrins change conformation gradually from closed, inactive to open and fully active conformation. The intermediate primed conformation can be activated by binding to ECM ligands (outside-in) or by recruitment of intracellular activator proteins to the cytoplasmic integrin tails (inside-out). Furthermore, integrins can also be inhibited by binding of integrin inhibitors to the cytoplasmic tails. Image modified from (Isomursu et al., 2019; Luo et al., 2007; Moreno-Layseca et al., 2019)

Integrin inactivators are a diverse group of proteins that are known to bind both integrin α- and β-tails. For example, SHANK Associated RH Domain Interactor (SHARPIN) is known to bind to the integrin α-tail, whereas filamin is recruited to the β-tail (Rantala et al., 2011; Bouvard et al., 2013). In addition, SH3 and multiple ankyrin repeat domains protein 1 and 3 (SHANK1 and SHANK3) were recently shown to inhibit integrins indirectly by sequestering active Rap1 and limiting thereby talin-Rap1-RIAM-mediated integrin activation (Lilja et al., 2017). Taken together, thus far characterised integrin inactivators prevent the activation by limiting recruitment of integrin activators to the integrin β-tail (Bouvard et al., 2013; Pouwels et al., 2012). Interaction between integrin inhibitors and integrins may also be mechanosensitive, as for example interaction between filamin A and integrin β-tail has been shown to be regulated by actomyosin contractility and mechanical force (Ehrlicher et al., 2011; Pentikäinen and Ylänne, 2009). However, the role of mechanosignalling in regulation of integrin inhibitors have not been characterised in detail.

2.1.2 Formation of adhesions and different adhesion types

The formation of adhesions is initiated by integrin activation and ECM-binding. Active integrins recruit cytoplasmic proteins to form nascent adhesions. Furthermore, activation of integrins induces integrin clustering within the plasma membrane recruiting additional integrins to the developing adhesion (Lawson and Schlaepfer, 2012; Sun et al., 2014). Integrin-based adhesions can be divided into different subtypes based on their location, shape and size (Figure 2.) (Conway and Jacquemet, 2019; Gardel et al., 2010; Zaidel-Bar et al., 2004; Zamir et al., 1999). Nascent adhesions are early adhesions, which are smaller in size and relatively short-lived. They are typically formed at the leading edge of a migrating cell or in proximity of protrusion sites (Zaidel-Bar et al., 2003). Nascent adhesions may mature into focal adhesions that are larger in size and more stable. Furthermore, they are more widely distributed and they are connected to contractile actin stress fibers (Oakes et al., 2012; Pasapera et al., 2010). Focal adhesions may further develop into elongated fibrillar adhesions depending on the ECM cues. Fibrillar adhesions are typically found in the cell center (Katz et al., 2000; Zaidel-Bar et al., 2004). Recently, filopodia adhesions have also been identified as their own adhesion type, as they have their distinct protein composition and localisation either at filopodia tip or along filopodia shaft. Moreover, filopodia adhesions can serve as precursors for nascent adhesion formation (Jacquemet et al., 2019). Early adhesion types, like for example filopodial and nascent adhesions, are often smaller in size and they have rapid turnover, whereas mature focal and fibrillar adhesions are larger in size, more stable and they persist for a longer time (Figure 2.) (Conway and Jacquemet, 2019; Gardel

et al., 2010; Choi et al., 2008; Webb et al., 2004; Doyle et al., 2012; Geiger and Yamada, 2011; Zamir et al., 1999).

Importantly, different adhesion types have also distinctive protein composition and they associate with different signature proteins. Microscopy-based studies have shown that filopodia adhesions are enriched in Myosin-10 (Myo10), talin, kindlin and p130Cas (Jacquemet et al., 2019). Nascent adhesions are in turn characterised by talin and paxillin, and they contain lower levels of vinculin, FAK (Zaidel-Bar et al., 2003) and α-actinin (Choi et al., 2008). Furthermore, newly formed nascent adhesions lack tensin and zyxin, which are typically detected in more mature adhesion types. In focal adhesions, levels of vinculin, FAK, vasodilator-stimulated phosphoprotein (VASP), zyxin and α-actinin are increased compared to nascent adhesions. While lower levels of zyxin are observed already in focal adhesions (Zaidel-Bar et al., 2003), it is highly prominent in fibrillar adhesions together with tensin (Figure 2.) (Katz et al., 2000; Zaidel-Bar et al., 2004; Zamir et al., 1999).

Both assembly and disassembly of focal adhesions needs to be carefully regulated, and dysregulation can lead to defective migration, e.g. loss of directionality, or problems with attachment and de-attachment (Pouwels et al., 2013), or disturb several other signalling pathways, including cell proliferation and survival (Bouvard et al., 2013; Wozniak et al., 2004). Adhesion assembly can be promoted for example by integrin clustering (Wiseman et al., 2004), actin polymerization (Zaidel-Bar et al., 2004, 2003) and increase in tension and actomyosin contractility (Gardel et al., 2010; Oakes et al., 2012; Zaidel-Bar et al., 2004). Dissassembly of focal adhesions can be triggered for example by protease activity (Franco et al., 2004), microtubules (Ezratty et al., 2005; Rafiq et al., 2019), cell division (Lock et al., 2018), integrin endocytosis (Moreno-Layseca et al., 2019) and focal adhesion kinase (FAK) signalling (Broussard et al., 2008; Tomar and Schlaepfer, 2009). Adhesion maturation is highly dependent on the actin cytoskeleton and the synergistic changes in the actin cytoskeleton and adhesion maturation and turnover are discussed in chapter 2.3.1.

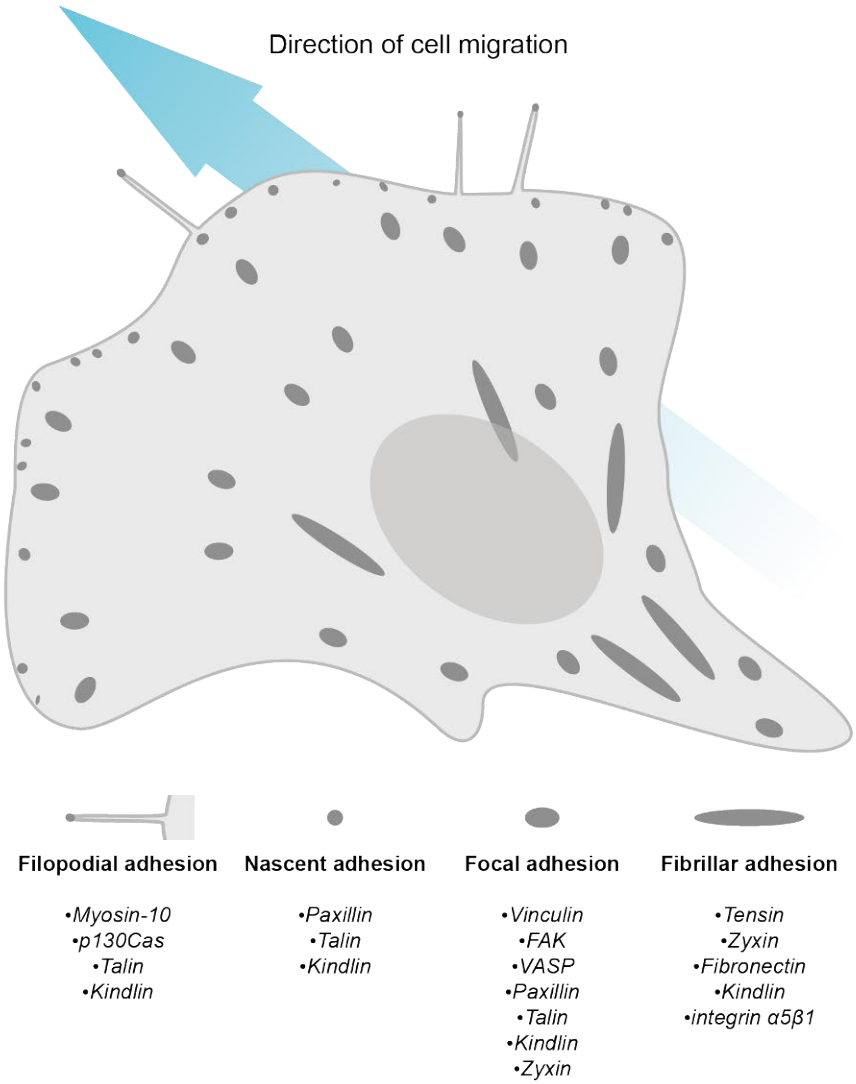

Figure 2. Four different adhesion types, their typical localisation and characteristic proteins that associate with each adhesion type. Image modified from (Conway and Jacquemet, 2019; Gardel et al., 2010)

2.1.3 Composition of integrin-based cell adhesions

Integrin-based adhesions are complexes that consist of tens to hundreds of associated proteins. The molecular composition of integrin-based adhesions have been investigated in several proteomic analyses studies (Horton et al., 2015; Zaidel-Bar et al., 2007). Different mass spectrometry screens have recognized altogether at least 2412 proteins that associate with integrin-based adhesions indicating that the molecular composition of adhesions may vary a lot. However, a "consensus adhesome" of 60 proteins has been defined by comparing the different studies, and it is considered to represent the core components of integrin-based adhesions (Horton et al., 2015).

Even though adhesions are composed of a large number of "adhesome" components, a general basic architecture has been proposed to be centered around a handful of key focal adhesion components (Kanchanawong et al., 2010). Futhermore, focal adhesions have been reported to consist of at least three conserved, regulatory layers that have partially overlapping components (Figure 3.). Integrin signalling layer is in close proximity of the plasma membrane and it contains for example paxillin and FAK. Integrin signalling layer is followed by an intermediate force-transduction layer that contains talin and vinculin that link integrins to the actin cytoskeleton. The innermost layer is an actin-regulatory layer containing both actin itself, and actin-regulatory proteins, such as α-actinin and zyxin (Kanchanawong et al., 2010; Stubb et al., 2019).

On top of the well-established adhesion constituents, the exact focal adhesion composition is regulated at least by the ECM ligands (Humphries et al., 2009), the integrin heterodimers involved (Bidone et al., 2019; Schiller et al., 2013), integrin activation status (Byron et al., 2015), adhesion size (Gardel et al., 2010; Zaidel-Bar et al., 2003), mechanical properties and actomyosin contractility (Choi et al., 2008; Oakes et al., 2012; Vicente-Manzanares et al., 2009), the maturation time (Horton et al., 2015; Gardel et al., 2010) and cell type (Conway and Jacquemet, 2019; Zaidel-Bar et al., 2004, 2003).

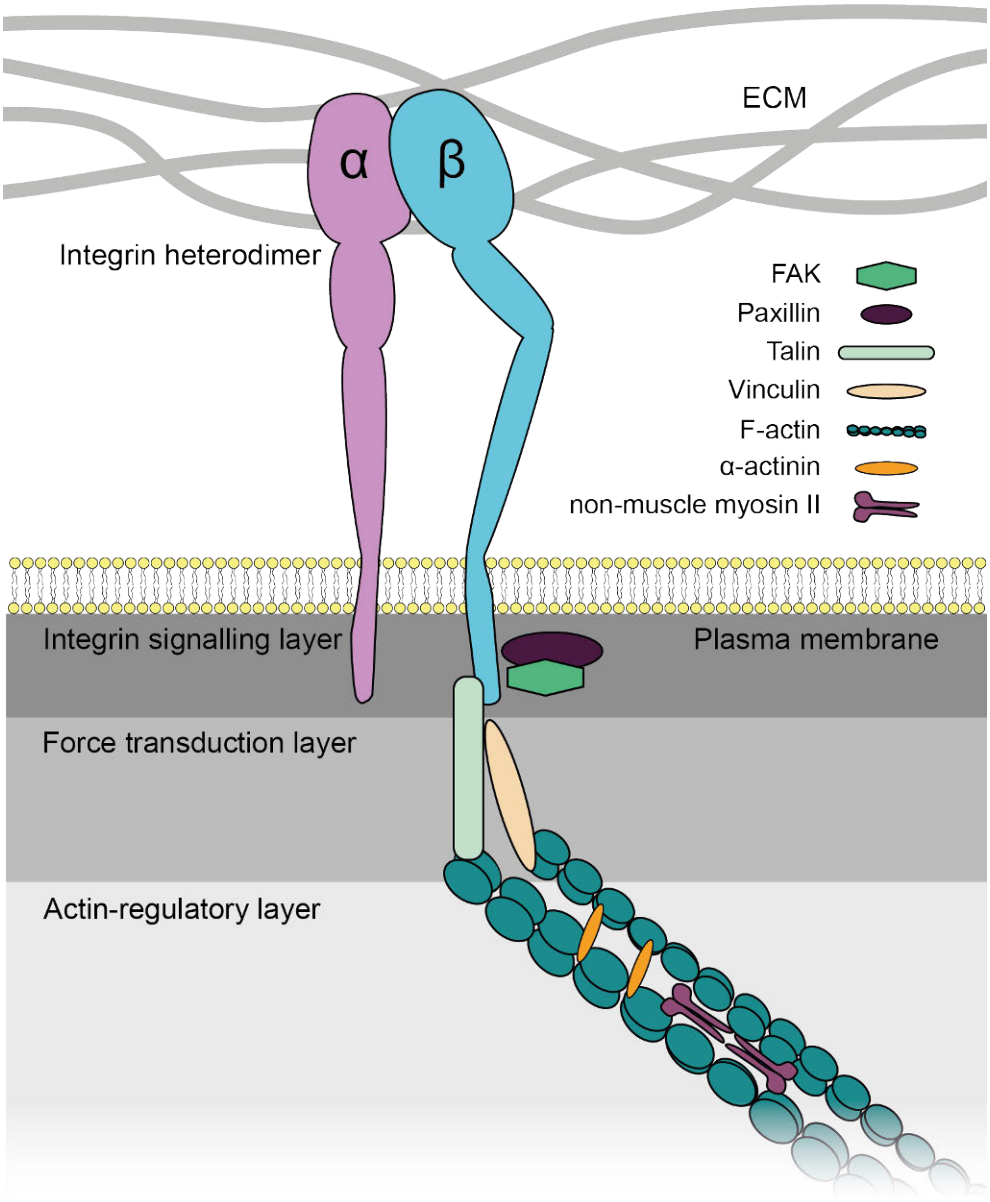

Figure 3. Integrin-based adhesions have three characteristic regulatory layers: integrin signaling layer, force transduction layer and actin-regulatory layer. Each of these layers have characteristic protein components that may partially overlap between layers. Image adapted from (Kanchanawong et al., 2010; Stubb et al., 2019)

2.2 The actin cytoskeleton

The cytoskeleton maintains cell shape, mediates intracellular transport and is essential for cell migration. The cytoskeleton consists of three main components; microtubules, intermediate filaments and actin. Microtubules are composed of α- and β-tubulins, which give rise to long and thick, cylindrical structures that have an important role in cell division and organelle transportation. Intermediate filaments are highly cell type specific structures that are composed of various different proteins, which are divided into four categories; keratins, vimentin and vimentin-related filaments, nuclear lamins and neurofilaments. However, their shared feature in different cell types and tissue are to provide mechanical support to the cell and protect it against external stresses. In this thesis, the focus will be on the actin cytoskeleton that is essential for cell morphology and movement of cells (Alberts et al., 2009; Hohmann and Dehghani, 2019). Actin is involved in plethora of cellular processes as it can both maintain the existing cytoskeletal structures and form protrusions towards any certain direction depending on the situation. Furthermore, actin-mediated changes in cells modulate polarity of the cell and cellular structures, transmit mechanical forces, alter cell shape, regulate endocytosis and mitochondrial dynamics, and mediate intracellular transport and transcription. Thereby, disturbed or abnormal regulation of the actin cytoskeleton associates with many developmental and physiological disorders, including cancer, neurodevelopmental disorders and myofibrillar myopathies (Alberts et al., 2009; Blanchoin et al., 2014; Hohmann and Dehghani, 2019).

2.2.1 Actin-binding proteins modulate the actin cytoskeleton

Actin is a 42 kilodalton (kDa) protein, which is one of the key components in the cytoskeleton of a cell. It can exist in two different forms: monomeric globular actin (G-actin) and linear polymeric, filamentous actin (F-actin), which forms double-helical structures (Holmes et al., 1990; Oda et al., 2009). Actin filaments are regulated in three central steps: nucleation, elongation and depolymerisation, and the process is powered by adenosine triphosphate (ATP) hydrolysis. The monomeric G-actin undergoes constant turnover between adenosine diphosphate (ADP)- and ATP-bound states. ATP-bound G-actin can form more stable oligomers via non-covalent interactions. These oligomers can form an actin nucleus, which is a stable multimer of G-actin that can serve as a precursor for actin polymersation (Pollard and Borisy, 2003; Xue and Robinson, 2013). Importantly, actin filaments are polar structures, and they have a fast-growing barbed end, and a slow-growing pointed end (Figure 4.). The ATP-bound G-actin monomers are added to the barbed end, but the ATP is hydrolysed into ADP towards the pointed end of an actin filament reducing the strength of the interaction between actin monomers and making it more prone to

depolymerisation (Pollard and Borisy, 2003; Xue and Robinson, 2013). Moreover, the dissociated ADP-bound actin monomers can be recharged through a nucleotide exchange back to ATP-bound actin monomers and added back to the barbed end. This constant addition of ATP-bound G-actin to the barbed end and dissociation of ADP-bound monomers from the pointed end forms a cycle called actin treadmilling (Figure 4.). Furthermore, this cycle maintains the cellular G-actin pool, that is essential for rapid re-organization of the actin cytoskeleton (Carlier and Shekhar, 2017; Suarez and Kovar, 2016).

Importantly, the actin cytoskeleton undergoes continuous reorganization, and it responds to both biochemical and mechanical signals (Blanchoin et al., 2014; Hohmann and Dehghani, 2019). The actin assembly and disassembly, formation and maintenance of the various actin structures and contacts between the actin cytoskeleton and other cellular structures require interaction between actin and multiple other proteins. Thereby, several different actin-binding proteins (ABPs) are needed to modulate the actin cytoskeleton. These ABPs have distinct functions, and they are divided into four sub-groups in this thesis: actin filament length regulators, actin-bundling proteins and actin crosslinkers, proteins that link actin to membranes and other cytoskeletal strucures, and contractility-related proteins. This thesis will introduce some of the major ABPs from each sub-group, but also plenty of other factors participate in regulation of actin dynamics.

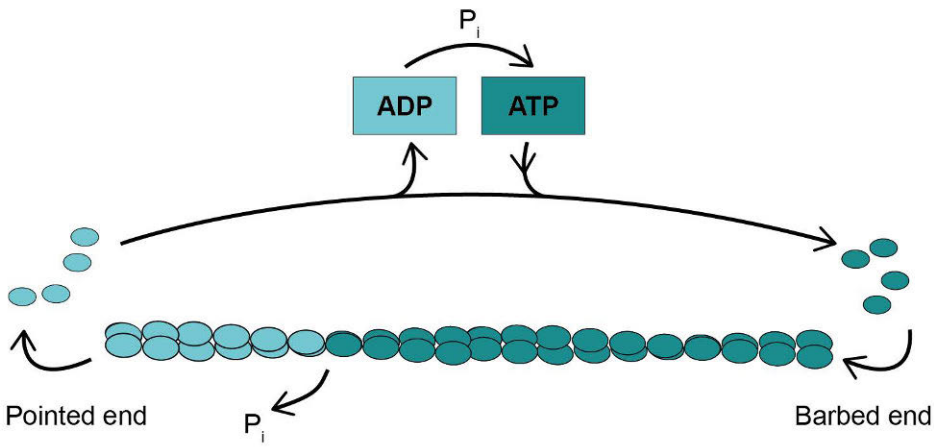

Figure 4. Actin treadmilling is a continuous process in which ATP-bound G-actin monomers are added to the barbed end of an actin filament, and ADP-bound G-actin monomers are dissociated from the pointed end. P_i = inorganic phosphate. Picture modified from Alberts et al., 2004.

2.2.1.1 The Arp2/3 complex nucleates branched actin filaments

Actin polymerization occurs naturally as such, but the process is very slow and actin nucleators and actin elongators are needed to enhance the assembly rate. The actin-related protein 2/3 (Arp2/3) complex is the major actin nucleator. It is found in all cell types where it helps to overcome the kinetic barriers of actin polymerization (Insall and Machesky, 2009; Rotty et al., 2013; Pollard, 2007). The Arp2/3 complex consists of seven subunits – Arp2, Arp3 and ArpC1-5 and all of its subunits are required to form a stable complex. Arp2 and Arp3 are the catalytic subunits, while ArpC1-5 act as stabilizers and scaffold the protein complex (Rotty et al., 2013). Moreover, Arp3 (Jay et al., 2000), ArpC1 (Abella et al., 2016) and ArpC5 (Millard et al., 2003) have been reported to have several isoforms mammalians, and different ArpC3 isoforms have been recognized in *Drosophila melanogaster* (Hudson and Cooley, 2002). The significance of the different isoforms has not been extensively studied, but it has been shown that some of the different isoforms have distinct cell type and tissue specific expression profiles (Millard et al., 2003; Jay et al., 2000). Furthermore, the isoform composition may also affect actin polymerization efficiency and how prone the produced actin filaments are to disassemble (Abella et al., 2016).

The Arp2/3 complex binds to the side of an existing actin filament (a mother filament), which triggers a conformational change in the Arp2/3 complex bringing the catalytic subunits Arp2 and Arp3 in close proximity. Importantly, the rearranged catalytic subunits resemble the barbed end of an actin filament promoting thereby recruitment of ATP-loaded G-actin monomers to the growing filament (Krause and Gautreau, 2014; Rotty et al., 2013) initiatiating formation of a new actin filament (a daughter filament) at a distinctive ~70º angle (Pollard, 2007).

The Arp2/3 complex has low intrinsic activity as such, and other proteins are needed to promote its activity. These activators are commonly known as nucleation promoting factors (NPFs), and they are divided into two classes: type I NPFs, which bind actin monomers, and type II NPFs, which bind actin filaments, in addition to binding to the Arp2/3 complex (Helgeson and Nolen, 2013; Machesky and Insall, 1998; Rotty et al., 2013). Type I NPFs have a characteristic verprolin homology, central acidic (VCA) domain, which binds the actin monomers and is required to promote the Arp2/3 complex activation efficiently (Kelly et al., 2006). The well-established members of type I NPFs include the Wiscott-Aldrich syndrome protein (WASP), suppressor of cAR (SCAR) and the WASP-family verprolin-homologous protein (WAVE) complex (Insall and Machesky, 2009; Machesky and Insall, 1998; Paavilainen et al., 2004; Rotty et al., 2013). Taken together, type I NPFs promote Arp2/3 activity by bringing together the Arp2/3 complex, a mother filament and actin monomers (Paavilainen et al., 2004; Pollard, 2007; Winder and Ayscough, 2005).

Type II NPFs have only low nucleation promoting activity compared to the type I NPFs as they lack the full VCA domain, but they are thought to have other functions that promote actin branching (Paavilainen et al., 2004; Rottner et al., 2017; Rotty et al., 2013). The most well-characterised type II NPF is cortactin (Helgeson and Nolen, 2013; Weed et al., 2000), which promotes formation of an actin branch by dislocating VCA-containing type I NPF from the Arp2/3 complex, stabilizing the growing branch and preventing debranching (Helgeson and Nolen, 2013; Weaver et al., 2001). Another type II NPF, actin-binding protein (Abp1), has been shown to promote Arp2/3-mediated actin nucleation through stabilizing branches by protecting filaments from branching-inhibiting glial maturation factor (GMF) (Gao et al., 2019). However, type II NPFs remain less well-characterised compared to type I NPFs.

Branched actin produced by the Arp2/3 complex is encountered in adherens junctions, endosomes, phagosomes and at the leading edge of migrating cells (Rotty et al., 2013). In this thesis the focus will be on the leading edge. Importantly, the Arp2/3-mediated polymerization of branched actin at the leading edge pushes the plasma membrane forward creating a wide, protrusive front that leads the cell migration. Thereby, the Arp2/3 complex has a pivotal role in promoting directional cell migration (Krause and Gautreau, 2014; Pollard and Borisy, 2003; Suraneni et al., 2012).

2.2.1.2 Formins elongate newly synthesized actin filaments

In addition to the Arp2/3 complex, other proteins can also nucleate actin filaments. While the Arp2/3 complex catalyses formation of branched actin, the formin protein family has an important role in nucleation and elongation of linear actin filaments (Breitsprecher and Goode, 2013; Higgs, 2005; Kovar, 2006). However, the exact mechanism how formins promote actin nucleation has not been unravelled in full detail, (Breitsprecher and Goode, 2013; Paul et al., 2008; Vavylonis et al., 2006), but they have been suggested to bind and stabilize actin monomers together forming thereby an initial actin nucleus (Pollard, 2007). Most famously, formins elongate actin filaments by adding actin monomers to the barbed end and by preventing binding of elongation terminating capping proteins (Paul et al., 2008; Pruyne et al., 2002; Vavylonis et al., 2006).

Formins are a large and heterogeneous family that has been shown to be encoded by at least 15 genes in mammals alone, and they have tissue- and cell type-specific expression patterns (Krainer et al., 2013). While formins are a diverse protein family, their function is mechanistically similar and they share highly conserved formin homology 1 (FH1) and formin homology 2 domains (FH2), which are sided by more various regulatory domains (Kovar, 2006). Importantly, formins form homodimers through FH2 domain adapting thereby a characteristic donut-shaped conformation (Xu et al., 2004), in which both subunits can bind two actin monomers (Pruyne et al., 2002).

Furthermore, FH2 domain binds actin, while FH1 domain may bind actin monomer binding protein profilin that brings ATP-bound actin monomers to the barbed end of an actin filament (Paul et al., 2008; Pruyne et al., 2002; Vavylonis et al., 2006). Some formins, such as Diaphanous-related formins, are regulated by autoinhibition, and they have a characteristic diaphanous inhibitory domain (DID) and diaphanous auto-regulatory domain (DAD) (Breitsprecher and Goode, 2013; Higgs, 2005). In addition, these autoinhibited formins have a GTPase-binding domain (GBD), which is a binding site for different Rho GTPases (Otomo et al., 2005; Rose et al., 2005). Autoinhibitory mechanism of formins is elementary, as for example point mutations that disrupt function of the DID and DAD domains of inverted formin 2 (INF2) lead to uncontrolled actin filament elongation and associate with Charcot-Marie-Tooth disease and focal segmental glomerulosclerosis (A et al., 2019).

In addition to having tissue- and cell type specific expression patterns (Krainer et al., 2013), distinct formins are also known to associate with different cellular actin structures. For example, Diaphanous-related formin dDia2 (Schirenbeck et al., 2005) and formin-like protein 2 (FMNL2) (Block et al., 2012) have been shown to promote filopodia formation (Schirenbeck et al., 2005), mammalian formin mDia1 catalyses formation of dorsal actin stress fibers (Hotulainen and Lappalainen, 2006) and INF2 has been shown to associate with endoplasmatic reticulum (Chhabra et al., 2009) and promote thereby mitochondrial dynamics (Chakrabarti et al., 2018).

2.2.1.3 Cofilin and other actin-severing proteins promote actin disassembly

In general, actin polymerisation at the barbed end occurs faster than dissociation of G-actin monomers at the pointed end. Therefore, different ABPs inhibit actin polymerisation and filament elongation, and induce actin depolymerisation (Dos Remedios et al., 2003). Disassembly of actin filaments can be induced by actin-severing proteins, such as cofilin-1. The actin depolymerizing factor (ADF)/cofilin family proteins are the best-characterised proteins that mediate actin turnover and disassembly. In mammalians, the ADF/cofilin family has been reported to consist of three proteins: ADF (also known as destrin), cofilin-1 and cofilin 2 (Maciver and Hussey, 2002), which have tissue and cell type specific expression profiles. Cofilin-1 is found in most cells, while ADF is mainly expressed neuronal and epithelial cells (Vartiainen et al., 2002). Cofilin-2 is expressed in muscle tissues, and it is also less efficient in depolymerising actin filaments compared to ADF and cofilin-1 (Vartiainen et al., 2002). Furthermore, ADF and cofilin-1 can compensate each other in some extent (Hotulainen et al., 2005) and they have higher affinity for ADP-bound actin monomers, but cofilin-2 can also interact with ATP-bound actin monomers with higher affinity (Kremneva et al., 2014). ADF/cofilin family proteins can both

severe actin filaments and promote dissociation of ADP-bound G-actin monomers from the pointed (Kanellos and Frame, 2016; Paavilainen et al., 2004). As ADF/cofilin dissociates actin monomers from the pointed end of an actin filament, the released monomers can be re-charged with ATP and recycled to the barbed end where the filament grows faster maintaining the dynamic actin turnover. Furthermore, this treadmilling may promote membrane protrusion and cell migration, as the fast-growing end pushes the plasma membrane forward (Kanellos and Frame, 2016). Indeed, ADF and cofilin-1 knockdown cells have been shown to migrate poorly (Hotulainen et al., 2005).

Actin filament length can also be modulated by different capping proteins and actin monomer binding proteins. Capping proteins that bind to the growing barbed end block addition of new G-actin monomers, and inhibition of capping proteins leads to aberrant lamellipodia formation and induced filopodia formation (Mejillano et al., 2004). Some barbed end-capping proteins, such as gelsolin, can also severe actin filaments and induce actin depolymerisation (Winder and Ayscough 2005). Therefore, capping proteins participate in regulation of the cellular G-actin monomer pool. In addition, cytoplasmic actin monomer binding proteins, such as twinfilin, can suppress actin polymerization by sequestering free actin monomers and/or by keeping them in GDP-loaded state (Xue and Robinson, 2013; Palmgren et al., 2002).

2.2.1.4 Actin-bundling proteins and crosslinkers organize higher-order actin structures

Proteins that crosslink and bundle simple actin filaments are important in creating higher-order F-actin structures. Actin-bundling proteins bring actin filaments together and align them in a parallel manner, while crosslinkers organise actin filaments together in more various ways, including for example centripetal or branched alignment. In order to bundle or crosslink actin filaments, a protein needs to have either two or more, distinct actin-binding sites or it needs to dimerize or oligomerize (Winder and Ayscough, 2005). For example, actin bundling protein fascin has two actin-binding sites (Jansen et al., 2011), whereas another actin bundling protein α-actinin dimerises in order to bundle filaments (Meyer and Aebi, 1990). Also actin crosslinkers come in various shapes and sizes, as they organize filaments in various structures. For example, a large actin crosslinker, spectrin, has several actin-binding sites, whereas transgelin, a small actin crosslinker, is known to dimerise to crosslink actin (Winder and Ayscough, 2005). Different actin bundling proteins and actin crosslinkers have an important role in formation of distinct cellular actin structures. For example, actin bundling proteins have an important role in assembly of actin stress fibers and filopodia, whereas actin crosslinkers organize dense, branched actin network at the leading edge (Matsudaira, 1994; Tseng et al., 2005).

2.2.1.5 Proteins that link actin to other cytoskeletal structures, the plasma membrane and membrane proteins

The actin cytoskeleton acts as a mechanical framework and scaffold for many other proteins. Importantly, many proteins bind to actin without having actin modulation as their primary function. These proteins include for example proteins that create anchorage to membrane proteins and directly to the plasma membrane, and proteins, which link actin to other cytoskeletal elements. While the actin cytoskeleton forms an important part of the cytoskeleton, it is linked to other cytoskeletal elements, namely microtubules and intermediate filaments, through other proteins. For example, plectin links actin filaments to both microtubules and intermediate filaments (Winder and Ayscough, 2005). In addition, the actin cytoskeleton is connected to the plasma membrane via linker proteins, such as Myosin 1 and ezrin-radixin-moesin (ERM) family proteins (McClatchey, 2014). Furthermore, actin is also indirectly linked to transmembrane proteins integrins through adhesion proteins talin and vinculin (Geiger et al., 2001; Humphries et al., 2007).

2.2.1.6 Contractility-related proteins

In order for a cell to move or divide, actin filaments need to be able to transmit forces and contract. While actin forms the base of the "skeleton and muscles" needed for a cell to move and change shape, it needs ABPs in order to contract and create force. Importantly, myosin family of motor proteins are crucial in generation of force and movement. There are roughly 20 classes of different myosins and they differ in their structure and specified functions. Two-headed myosin II has a pivotal role in function of the actin stress fibers, where it participates in creation of contractile forces and tension. In addition, myosins use actin as a route for moving different cargo ranging from vesicles to other proteins. Myosin II decorates actin filaments in a bipolar manner and it contracts the filaments powered by ATP hydrolysis (Tojkander et al., 2011, 2015; Vicente-Manzanares et al., 2009). In addition to myosins, several different proteins and protein families have been observed to modulate contractility of F-actin. For example actin-bundling proteins, such as α-actinin, have an important role in stress fibers, that are major contractile actin structures of mesenchymal cells (Tojkander et al., 2012).

2.2.2 Actin structures of a cell

The actin cytoskeleton acts as a functional scaffold for many cellular processes, and it has a crucial role in cell morphogenesis and intracellular architecture. As actin participates in plethora of cellular events, also cellular actin structures are diverse. Here, the focus will be on actin structures that are needed for integrin-mediated cell

adhesion and lamellipodial (also known as mesenchymal) cell migration in 2D environments. Importantly, polarization of a cell is a prerequisite for cell migration. The front of a migrating cell is referred to as the leading edge, which is the protrusive front of the cell, and the back of a cell is called the trailing edge (Figure 5.) (Conway and Jacquemet, 2019; Petrie and Yamada, 2012). Whilst this chapter introduces actin structures characterised in 2D environments, it is important to acknowledge that these structures are also adapted in 3D environments (Caswell and Zech, 2018).

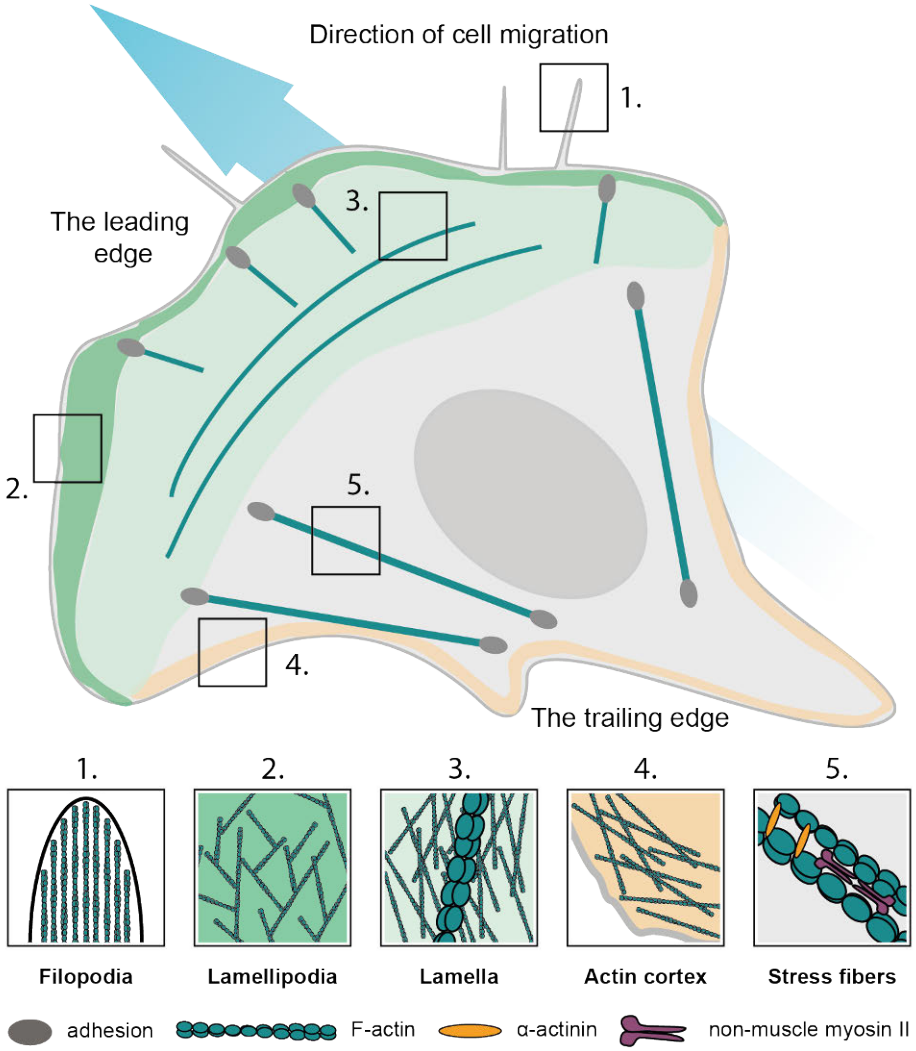

Figure 5. A simplistic illustration describing different actin structures typically found in a mesenchymal cell in 2D.

2.2.2.1 Filopodia are thin protrusions that probe the environment

Filopodia, also known as microspikes, are actin-rich, finger-like protrusions at the cell front that sense the surrounding environment (Figure 5.). In normal conditions, filopodia formation has a role in neuronal growth cone guidance, fusion of epithelial sheets and embryogenesis, but they also have a central role in some pathological conditions, such as cancer cell migration (Jacquemet et al., 2015; Mattila and Lappalainen, 2008; Gallop, 2020). In addition to interacting with the ECM, filopodia have also been shown regulate directional cell migration through sensing chemokines (Boer et al., 2015; Meyen et al. 2015). Therefore, findings from both developmental (Boer et al., 2015; Meyen et al. 2015) and cancer studies highlight the role of filopodia in guiding cell migration also in 3D environments (Caswell and Zech, 2018; Jacquemet et al., 2015).The length of a filopodium varies a lot based on a cell type and the cellular context. Filopodia consist of parallel actin bundles, that are packed tightly together by actin-bundling proteins, such as fascin (Faix et al., 2009; Mattila and Lappalainen, 2008; Vignjevic et al., 2006). Several different mechanisms have been proposed for filopodia formation. In the *de novo* nucleation model, filopodia are initiated by formins (Faix et al., 2009; Kage et al., 2017; Yang and Svitkina, 2011; Mellor, 2010). In the convergent elongation model, actin filaments of the filopodia originate from the lamellipodial actin network, and are thereby originally initiated by Arp2/3-mediated actin nucleation and branching (Korobova and Svitkina, 2008; Svitkina et al., 2003). However, neither of these mechanisms seems to be essential, as downregulation or knockout of the Arp2/3 complex is known to induce formation of filopodia while the lamellipodia formation is reduced showing that Arp2/3 mediated actin nucleation is not necessary for formation of filopodia. Furthermore, filopodia are also observed after silencing or knockout of formins (Rottner et al., 2017; Steffen et al., 2013; Wu et al., 2012). Taken together, these findings indicate that there are multiple, mutually exclusive ways how filopodia can be formed, and the filopodia formation mechanisms may be cell type-specific (Young et al., 2015).

2.2.2.2 Lamellipodia consists of branched actin network

A lamellipodium is a wide, veil-like zone of branched actin at the leading edge of a cell (Figure 5.). Depending on the cell type, lamellipodium length has been reported to be approximately 1-5 µm starting from the leading edge (Ponti et al., 2004; Small et al., 2002). Furthermore, the composition and stiffness of the surrounding environment and the ECM also affect lamellipodia formation. Strong lamellipodia are formed especially in cells migrating on rigid 2D surfaces (Krause and Gautreau, 2014; Small et al., 2002; Wu et al., 2012). In 3D matrices, lamellipodia are typically smaller, more elongated and less uniform in shape (Caswell and Zech, 2018; Petrie

et al., 2012). In addition to lamellipodia, cells utilize also other types of leading edge structures that will be discussed further in chapter 2.3.2.

Importantly, formation of the lamellipodial branched actin network is dependent on the Arp2/3 complex and its activators, NPFs (Krause and Gautreau, 2014; Rotty et al., 2013; Suraneni et al., 2012; Wu et al., 2012). However, also other ABPs regulate the lamellipodial actin network. For example, actin crosslinkers, such as filamin, assemble the branched actin network (Ydenberg et al., 2011), capping proteins regulate the length of the formed actin filaments (Akin and Mullins, 2008) and actin disassembly factors, such as cofilin-1, maintain actin turnover and thereby modulate protrusion formation (Hotulainen et al., 2005; Kanellos and Frame, 2016). In fact, the rate of lamellipodial actin assembly is dependent on available G-actin pool (Rottner et al., 2017).

Growing lamellipodia extends towards the plasma membrane generating pushing forces and promoting membrane protrusion. However, the Arp2/3-dependent actin branching has a poor efficiency in generating mechanical force and the rate of actin polymerization at the lamellipodia edge is not proportional to protrusion efficiency or force generation. Importantly, formin-induced actin filaments have been observed to induce force generation at the lamellipodia highlighting the role of other ABPs in regulation of lamellipodial dynamics (Kage et al., 2017).

A lamellipodium is followed by a lamellum, which is a zone of densely packed actin network and curved, contractile actin stress fibers that are aligned parallel to the leading edge (Figure 5.). Lamella are created by condensation of lamellipodial branched actin through actin retrograde flow (discussed in chapter 2.3.1). Importantly, lamella has a far greater force generation capability compared to lamellipodia as it contains contractile actin structures (Ponti et al., 2004; Giannone et al., 2004; Mendoza et al., 2015; Burnette et al., 2011).

2.2.2.3 Actin-associated motor proteins generate force and increase contractility of actin stress fibers

Actomyosin is the basis of contractile structures, such as myofibrils of muscles, cytokinetic ring during cell division and stress fibers of non-muscle cells (Michelot and Drubin, 2011). Importantly, actin stress fibers (Figure 5.) have a central role in cell adhesion, cell migration, force generation and cell morphogenesis. Stress fibers are thick bundles of multiple actin filaments that have been reported to consist of up to 30 actin filaments (Cramer et al., 1997). Contractility-associated ABPs, such as as non-muscle myosin II, have a crucial role in formation and function of stress fibers as they generate force along the stress fibers (Vicente-Manzanares et al., 2009; Tojkander et al., 2011). In fact, stress fibers appear to be dependent on tension and contraction as inhibition of myosin II leads to disassembly of stress fibers (Smith et

al., 2010; Humphries et al., 2007; Burridge and Guilluy, 2016). However, while actin and myosin II are the central components of stress fibers, they also employ a variety of other ABPs. For example, α-actinin is known to bundle and crosslink the parallel actin filaments together (Pellegrin and Mellor, 2007; Meyer and Aebi, 1990) and formins have been reported to nucleate and elongate stress fibers (Watanabe et al., 1999; Tojkander et al., 2011; Hotulainen and Lappalainen, 2006). In addition to ABPs, actin stress fibers also associate with several focal adhesion-associated proteins, such as vinculin and talin. Importantly, focal adhesion-related proteins may modulate stress fiber assembly and their turnover (Choi et al., 2008; Chrzanowska-Wodnicka and Burridge, 1996; Pasapera et al., 2010).

Prominent stress fibers are not typical for all cell types. In cultured mammalian cells, strong stress fibers are found especially in fibroblasts, endothelial cells and some cancer cell lines. The type of stress fibers may also vary a lot: in highly motile cells, stress fibers are typically more dynamic and thinner, whereas in non-motile cells they can be very stable and thick (Pellegrin and Mellor, 2007; Tojkander et al., 2012). Furthermore, extracellular cues including both biochemical and mechanical signals modulate stress fiber number, structure and organization. Importantly, stress fibers are most abundant in tissues and localizations where they encounter high mechanical stresses. Culture on rigid 2D surfaces, such as typical cell culture plastic, may induce stress fiber number and thickness, and in turn, cells grown on softer substrates have more delicate stress fibers (Discher et al., 2005). Taken together, these observations highlight the role of mechanosensing in regulation of stress fibers. Consistently, experiencing mechanical stress, such as fluid shear stress, may induce stress fiber formation in endothelial cells (Malek and Izumo, 1996; Noria et al., 2004). Moreover, stress fibers are also observed in 3D environments (Petrie et al., 2012; Gateva et al., 2014).

2.2.2.3.1 Stress fibers can be divided into four different subtypes

Stress fibers exist in multiple forms, and the distinct types of stress fibers associate differently with focal adhesions and localize differently within a cell. Stress fibers are divided into three or four subcategories, depending on the source (Figure 6.). Dorsal stress fibers originate from the leading edge and they are oriented radially to the edge. Importantly, dorsal stress fibers are linked to a focal adhesion from one end, and they do not typically contain contractility-inducing myosin II (Hotulainen and Lappalainen, 2006; Tojkander et al., 2011, 2012). Dorsal stress fibers are followed by curved transverse arcs that are a part of lamella and originate from lamellipodia. Transverse arcs move towards the center and posterior part of a migrating cell due to actin retrograde flow. Transverse arcs contain myosin II and they are thereby contractile. However, transverse arcs are not connected to focal

adhesions to begin with, but they may fuse together with dorsal stress fibers and create additional contacts to focal adhesions maturing eventually into ventral stress fibers (Ponti et al., 2004; Burnette et al., 2011; Hotulainen and Lappalainen, 2006). Ventral stress fibers are anchored to focal adhesions from both ends and they occur typically in the central and posterior parts of a cell (Pellegrin and Mellor, 2007; Burridge and Guilluy, 2016; Tojkander et al., 2012). Perinuclear actin cap has been proposed to be a fourth subtype of stress fibers. It resembles structurally ventral stress fibers as it also consists of actomyosin bundles that are linked to focal adhesions from both ends. However, perinuclear actin cap reaches above the nucleus and it is thought to provide mechanical protection to the nucleus and regulate its shape and position. The perinuclear actin cap may also regulate nuclear shape in interphase cells, but it has also been proposed to participate in mechanotransduction between cell's environment and the nucleus (Khatau et al., 2009).

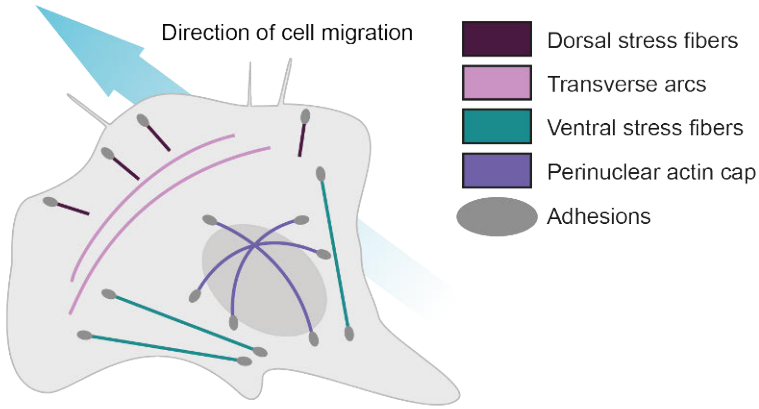

Figure 6. Actin stress fibers can be divided into four distinct categories: dorsal stress fibers, transverse arcs, ventral stress fibers and perinuclear actin cap. Each of these stress fiber types has its characteristic localization and association with adhesions.

2.2.2.4 The actin cortex regulates cell shape and membrane tension

Actin filaments are most concentrated just beneath the plasma membrane where they form a thin layer called the actin cortex, which is the main regulator of cell shape (Figure 5.) (Chalut and Paluch, 2016; Chugh and Paluch, 2018). Cortical actin filaments form a densely packed meshwork, which is organized by actin crosslinkers (e.g. α-actinin), myosin motor proteins (e.g. myosin II) and proteins that anchor the actin filaments to the plasma membrane (e.g. ERM family proteins) (Salbreux et al., 2012; McClatchey, 2014; Chugh and Paluch, 2018). The cortical actin network is generated at least by the Arp2/3 complex and formin mDia1 (Chugh and Paluch, 2018). The actin cortex undergoes a constant turnover, which allows the cell to adapt

to external cues and stresses. Thereby, the actin cortex can accommodate rapid, mechanical changes in the cell shape in response to different stimuli. Furthermore, the actin cortex regulates membrane tension, which has an important role in various cellular processes, including cell migration (Charras and Paluch, 2008; Sanz-Moreno and Marshall, 2010), cell division and tissue morphogenesis (Chalut and Paluch, 2016; Salbreux et al., 2012). Local plasma membrane-actin cortex detachment or disassembly of the cortical actin may give rise to bleb formation, which can be used as a leading edge structure instead of lamellipodia in different migration modes (Fackler and Grosse, 2008; Diz-Muñoz et al., 2016; Charras and Paluch, 2008), which will be discussed further in Chapter 2.3.2.

2.2.2.5 Podosomes and invadopodia create contacts to the ECM

Podosomes and invadopodia are actin-rich, adhesive structures that cells use to interact with the surrounding ECM. While podosomes are structures that occur in normal, healthy cells, such as macrophages, invadopodia are typical for cancer cells and they are induced through activation of Src family kinases (Kelley et al., 2010; Tehrani et al., 2007) and protein kinase C (PKC) (Bowden et al., 1999; Murphy and Courtneidge, 2011). Podosomes have an actin core, which consists of branched actin filaments surrounded by an integrin-based ring structure (Hoshino et al., 2013; Branch et al., 2012). Formation of the branched filaments is dependent on Arp2/3 activity, which is typically activated by WASP, an actin-regulator specifically found in podosomes, but not focal adhesions or invadopodia (Hoshino et al., 2013; Murphy and Courtneidge, 2011). In addition, the ring of integrins and other adhesion-related proteins, such as vinculin and talin, which frames the actin core, is specific to podosomes. A cap in the cytosolic part of podosomes contains formins and organizes longer actin filaments that extend towards the ring and link to integrins thereby allowing podosomes to act as mechanosensors (Hoshino et al., 2013; Murphy and Courtneidge, 2011; Branch et al., 2012).

The actin core of invadopodia is also dependent on the Arp2/3 complex and its activator N-WASP (Oser et al., 2009). The actin network is anchored to the membrane and adhesion proteins similarly to podosomes, the growing invadopodium maybe dissociated from actin polymerization before it matures altering turnover rate (Murphy and Courtneidge, 2011). Both Arp2/3 activity and formins are required for the growth of invadopodia and formation of protrusions. Integrins have a pivotal role in invadopodia formation, as they often create the first contact between the cell and the ECM (Linder et al., 2011; Branch et al., 2012; Murphy and Courtneidge, 2011), however invadopodia lack a clear spatially defined integrin ring structure and may be more puncta-like (Clark and Weaver, 2008). Importantly, invadopodia are protrusions that cells use especially in context of cancer to establish connections with the ECM

and to remodel it. For example, invadopodia utilize matrix metalloproteases (MMPs) to degrade the surrounding ECM and to promote cancer cell invasion (Linder, 2007; Linder et al., 2011; Basbaum and Werb, 1996). Taken together, the protrusiveness and lifetime are the main difference between podosomes and invadopodia - podosomes are short lived structures (minutes) characteristic to migrating cells and invadopodia are long lived matrix degrading structures that can persist for hours (Li et al., 2010; Basbaum and Werb, 1996).

2.3 At the interface of cell adhesion and the actin cytoskeleton

There is an enormous amount of crosstalk between cell adhesion and the actin cytoskeleton. Importantly, they function in an orchestrated manner in many biological processes, such as cell migration and tissue maintenance (Discher et al., 2005; Geiger et al., 2001). In this chapter, the interdependent function of cell adhesion and actin dynamics will be discussed using cell migration as an example.

2.3.1 The actin retrograde flow and adhesion maturation

Actin retrograde flow comprehends a continuous cycle driven by actin polymerisation and actomyosin contractility (Vallotton et al., 2004). In this cycle actin is polymerized at the leading edge, from which it flows towards the cell body forming lamella and stress fibers, which in turn connect to focal adhesions increasing contractility and eventually translocate closer to the trailing edge of the cell until adhesions at the trailing edge are disassembled (Swaminathan et al., 2017). Therefore, actin retrograde flow has a crucial role in adhesion maturation. As described in chapter 2.1.2, adhesions can be divided into at least four different subtypes. The initial adhesions are smaller filopodia and/or nascent adhesions, which recruit other adhesion proteins, such as talin and vinculin, while they undergo maturation (Conway and Jacquemet, 2019). Furthermore, connection to talin and vinculin links adhesions to the actin cytoskeleton and importantly, to contractile actin stress fibers. These actomyosin bundles transmit increased tension and forces to integrin-based adhesions promoting their maturation into focal and fibrillar adhesions changing their alignment and orientation. Taken together, actin retrograde flow is dependent on actin polymerization and contractility, and it regulates adhesion formation and maturation (Ponti et al., 2004; Schwarz and Gardel, 2012; Swaminathan et al., 2017; Alexandrova et al., 2008; Burnette et al., 2011).

Engagement with the actin cytoskeleton has a critical role in regulation of the morphological and compositional maturation of adhesions. Formation of early filopodia adhesions is initiated by integrin-ECM engagement at the filopodia tip.

Paxillin is recruited to tips of stable filopodia, from where it translocates to filopodia shafts and starts forming adhesion-like clusters. These clusters may give rise to nascent and focal adhesions when lamellipodia advances (Jacquemet et al., 2019). So far, filopodia adhesions remain less well characterised compared to other adhesion types. Formation of nascent adhesions is driven by actin polymerization at the leading edge, but it does not depend on myosin II-mediated contraction (Choi et al., 2008; Vicente-Manzanares et al., 2009). Moreover, lamellipodial protrusion dynamics have been shown to regulate protein composition and stabilization of nascent adhesions as inhibition or arrest of lamellipodial protrusion triggers zyxin recruitment to nascent adhesions inducing adhesion growth and focal adhesion maturation (Zaidel-Bar et al., 2003).

Mature focal adhesions are linked to contractile actomyosin stress fibers and increased actomyosin contractility promotes adhesion growth and maturation (Chrzanowska-Wodnicka and Burridge, 1996; Humphries et al., 2007; Vicente-Manzanares et al., 2009; Choi et al., 2008). Importantly, myosin II-activity has been shown to promote the compositional maturation of adhesions by promoting vinculin recruitment to adhesions by inducing FAK-mediated paxillin phosphorylation (Pasapera et al., 2010). Furthermore, vinculin recruitment induces focal adhesion growth by promoting clustering of activated integrins and slowing down integrin turnover (Humphries et al., 2007).

Focal adhesion maturation is a mechanosensitive process, but while increased tension and contractility clearly promote focal adhesion maturation, myosin II activity is not absolutely essential for focal adhesion maturation. Focal adhesions have been shown to mature even in cells expressing motor-domain lacking myosin II (Legate et al., 2009) and while treatment with myosin II inhibitor or inhibitor of myosin phosphorylating Rho-associated protein kinase (ROCK) reduces number of mature focal adhesions, some mature adhesions are still observed (Pasapera et al., 2010; Even-Ram et al., 2007; Humphries et al., 2007). These findings indicate that while actomyosin contractility induces focal adhesion maturation, it is not sufficient as such. It has been proposed that association with actin itself might be the elementary factor triggering maturation of adhesions (Oakes et al., 2012). Importantly, myosin II-mediated contractility and tension are essential for stress fibers, as myosin II inhibition leads to disassembly of stress fibers and thereby decreased association between focal adhesions and actin (Humphries et al., 2007; Smith et al., 2010). Interestingly, while fibrillar adhesions associate with actin, they persist even when myosin function is inhibited suggesting they are more dependent on the ECM topography than tension (Katz et al., 2000; Zamir et al., 1999; Biggs and Dalby, 2010). Taken together, these observations show that formation and maturation of adhesions and dynamics of the actin cytoskeleton are highly synergistic and different actin structures associate with distinct adhesion types.

2.3.2 Cell migration is essential for tissue development and maintenance

Cell migration is an essential part of maintenance of healthy tissues, as it is crucial in many physiological processes, including embryonic development and organogenesis, immune system function and wound healing. However, dysregulated cell migration associates with different diseases, including inflammation and cancer (Shaw and Martin, 2016; De Pascalis and Etienne-Manneville, 2017).

Cell migration can be triggered and modulated by various cues, such as growth factors and cytokines. In addition, the ECM is an imperative factor in cell migration, and the ECM composition, rigidity and topology are known to regulate cell migration speed and migration direction (Park et al., 2018; Doyle et al., 2012, 2015; Wolf et al., 2013). Interaction with the surrounding environment and coordinated attachment and de-attachment are a prerequisite for cell migration. Cells attach to the surrounding ECM and form adhesions that obtain varying strengths and last for varying times. The connected actin cytoskeleton generates force that is a prerequisite for the movement. Importantly, cells can also remodel the ECM to ease the migration. For example, cells exert the integrin-based adhesions and the actin cytoskeleton pushes and pulls the ECM (De Pascalis and Etienne-Manneville, 2017). Furthermore, cells can also express and secrete proteolytic enzymes, such as MMPs, that degrade the ECM to promote cell migration, and in case of cancer, invasion (Levental et al., 2009; Paszek et al., 2005; Linder, 2007; Friedl and Wolf, 2009). In addition to the secreted MMPs, a subgroup called membrane-type matrix metalloproteinases (MT-MMPs), such as MT1-MMP, remain tethered to the plasma membrane either through a transmembrane domain or by a glycosylphosphatidylinositol (GPI)-anchor (Itoh, 2015). MT-MMPs have been shown promote cancer cell migration, invasion and metastasis through digesting the ECM and by activating precursors of other secreted proteases (Itoh, 2015; Sato et al., 1994). For example cancer associated fibroblasts (CAFs) are known to remodel the surrounding ECM and thereby to promote cancer cell migration and proliferation (Labernadie et al., 2017; Glentis et al., 2017; Attieh et al., 2017).

While cells can migrate both as single cells and collectively, in this thesis the focus will be on single cell migration.

2.3.2.1 Cell adhesion and the actin cytoskeleton function synergistically in cell migration

Cell migration includes polarization of cells so that they obtain a leading edge and a trailing edge. The leading edge and the trailing edge are characterised by distinct adhesive and cytoskeletal structures, and coordinated function of cell adhesion and re-organization of the actin cytoskeleton is a prerequisite for cell migration. (Charras

and Paluch, 2008; Ridley, 2011; Petrie and Yamada, 2012). The main example used in this thesis is lamellipodial cell migration, which occurs in four stages: adhesions hold a cell in place, actin polymerisation pushes the membrane forward extending protrusions, new adhesions are formed under the protrusions and the cell will gradually roll forward when the actomyosin structures contract and the trailing edge is released (Figure 7.) (Mattila and Lappalainen, 2008; Pollard and Borisy, 2003; Giannone et al., 2004). In addition, the process may be accompanied by proteolytic activity, which remodels the ECM and promotes migraton (Wolf et al., 2013; Friedl and Wolf, 2009). All in all, cell migration is a carefully regulated interplay between adhesion and actin dynamics.

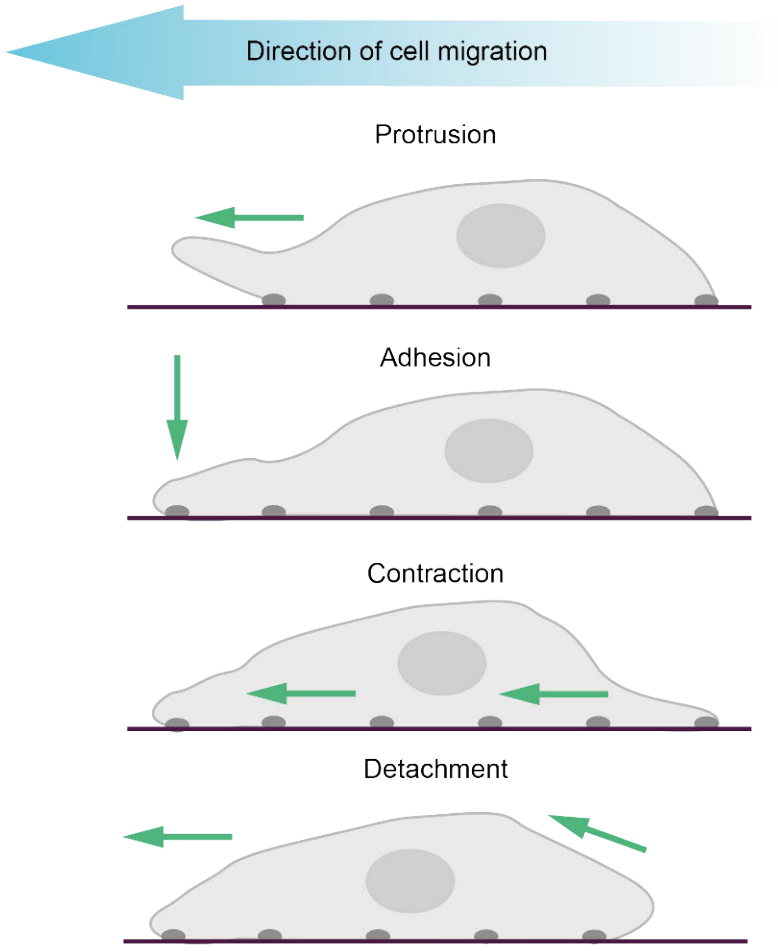

Figure 7. Cell migration occurs in four stages: protrusion, adhesion, contraction and detachment. Image modified from (Mattila and Lappalainen, 2008).

Cell adhesion receptors, such as integrins, have an important role in signal transduction between a cell and its surroundings. In order to migrate, a cell needs to grab the ECM at the leading edge through integrin receptors and assemble adhesions, but at the same time, it needs to let go of the grip at the trailing edge (Pouwels et al., 2013; Broussard et al., 2008; Huttenlocher et al., 1996; Doyle et al., 2015). This highlights the importance of adhesion assembly and disassembly regulation. Furthermore, integrin-based adhesions modulate directional cell migration by mediating signalling involved in chemotaxis (migration towards a soluble ligand), durotaxis (migration towards stiffer environment), and haptotaxis (migration towards higher ECM concentration) (Charras and Sahai, 2014; Conway and Jacquemet, 2019; Krause and Gautreau, 2014).

In addition to adhesions, also different cytoskeletal structures have an important role in directing cell migration. For example, Arp2/3-mediated lamellipodia formation is essential for haptotaxis (King et al., 2016). However, it is non-essential for chemotactic cell migration (Wu et al., 2012). Furthermore, actin polymerisation at the leading edge is needed to extend the cell and form protrusions and cell-matrix adhesions (Zaidel-Bar et al., 2003; Krause and Gautreau, 2014; Caswell and Zech, 2018; Wu et al., 2012). In addition, the actin cytoskeleton has an imperative role in force generation, which is essential for cell movement. In protrusive actin network, such as filopodia and lamellipodia, force is generated through actin polymerization and retraction, and the forces are generally modest compared to forces generated by actomyosin structures, such as stress fibers (Jacquemet et al., 2015; Le Clainche and Carlier, 2008). Lamellipodia are followed by lamella, in which nascent adhesions are coupled to transverse arcs promoting adhesion maturation and creating contractile actomyosin machinery needed for the trailing edge retraction (Burnette et al., 2011). Thereby, myosin II-containing actin stress fibers have an important role in pulling the cell forward and promoting detachment of the the trailing edge (Burnette et al., 2014; Vicente-Manzanares et al., 2009; Gardel et al., 2010; Burridge and Guilluy, 2016). Taken together, both dynamic regulation of cell adhesion and different actin structures are essential for cell migration and they act in an interdependent manner.

2.3.2.2 Different migration modes

In tissues, cells migrate within a 3D environment consisting of surrounding cells and the ECM. This requires a lot of plasticity – ability to adjust the cell shape and mode of migration. All migration modes discussed in this thesis share similar features, including polarization, protrusion, and contraction, but the cell shape and velocity during migration can vary a lot. For single cells migrating in 3D, there are many characterized modes of migration, including amoeboid (blebby), lamellipodial

(mesenchymal; described above for 2D) and lobopodial/pseudopodial migration (Figure 8.) (Krause and Gautreau, 2014; Charras and Paluch, 2008; Small et al., 2002; Petrie et al., 2012; Sanz-Moreno and Marshall, 2010). While lamellipodial cell migration is the main example used in this thesis, also different migration modes will be introduced briefly.

Lamellipodial migration is characterized by a leading edge that has a dense layer of branched actin filaments, a lamellipodium, followed by actin stress fibers that pull the trailing edge forward (Figure 8.). These actin structures associate with different kind of integrin-based adhesions, as described earlier in chapter 2.1.2. Lamellipodially migrating cells in 3D matrices are dependent on integrins and proteolytic activity (Wolf et al., 2003; Oudin et al., 2016). They can also employ other actin-rich, adhesive structures, such as filopodia and invadopodia. Importantly, while filopodia occur more typically in the proximity of the leading edge, invadopodia are commonly detected under the nucleus in 2D models, or ahead of the nucleus in 3D matrices (Wolf et al., 2007; Infante et al., 2018; Ferrari et al., 2019). Furthermore, while filopodia can push and pull the ECM, they lack proteolytic activity, which is pivotal for invadopodia (Linder et al., 2011; Jacquemet et al., 2015; Caswell and Zech, 2018; Shibue et al., 2013; Eddy et al., 2017).

While cells employing lamellipodial migration are typically elongated, amoeboid migration is more typical to cells that are rounded. This is linked to the fact that amoeboid cell motility is integrin-independent and the protrusive structures involved in amoeboid cell migration are typically blebs of different sizes (Figure 8.). Importantly, while cells employing amoeboid migration have less obvious polarization compared to very elongated cells, polarization and a blebby leading edge is also needed to promote amoeboid migration (Charras and Paluch, 2008; Petrie and Yamada, 2012; Sanz-Moreno and Marshall, 2010). Blebbing is induced by increase in intracellular pressure and weakening of the actin cortex (Charras and Paluch, 2008; Sanz-Moreno and Marshall, 2010; Charras et al., 2005). Interestingly, lamellipodially migrating cells can be induced to switch to amoeboid migration by inhibiting integrins or proteolytic activity (Wolf et al., 2003). Consistently, amoeboid cell migration, does not depend on proteolytic activity (Wolf et al., 2003; Sahai and Marshall, 2003; Sabeh et al., 2009).

Cells can also employ lobopodia to migrate. Lobopodia are blunt, cylindrical protrusions that are suggested to be a “hybrid version” between lamellipodial and amoeboid cell migration (Figure 8.). Lobopodia are actin-rich structures, but their formation is thought to be more dependent on hydrostatic pressure rather than actin polymerization, in contrast to lamellipodia, filopodia or invadopodia. Furthermore, lobopodially migrating cells are dependent on integrin-ECM adhesion (Petrie et al., 2012, 2014; van Helvert et al., 2018).

Taken together, the leading edge morphology is imperative for the migration mode used. While different cell types may prefer using certain migration modes, one and the same cell can also utilize many different migration modes depending on the cellular context. The surrounding environment regulates the leading edge structures, and while lamellipodial migration may be the most common mode seen on rigid 2D surfaces, cells utilize various leading edge structures to migrate in 3D environments (Charras and Paluch, 2008; Petrie and Yamada, 2012, 2016; Friedl and Wolf, 2010). Migration in tight spaces requires protease activity, while more loosely organized ECM may favour amoeboid migration (Sabeh et al., 2009; Charras and Paluch, 2008; Wolf et al., 2003; Friedl and Wolf, 2009). Taken together, the migration mode and leading edge structures depend on the properties of the surrounding ECM, including the ECM composition, stiffness, elasticity and topography (Charras and Sahai, 2014; Doyle et al., 2012, 2015; van Helvert et al., 2018).

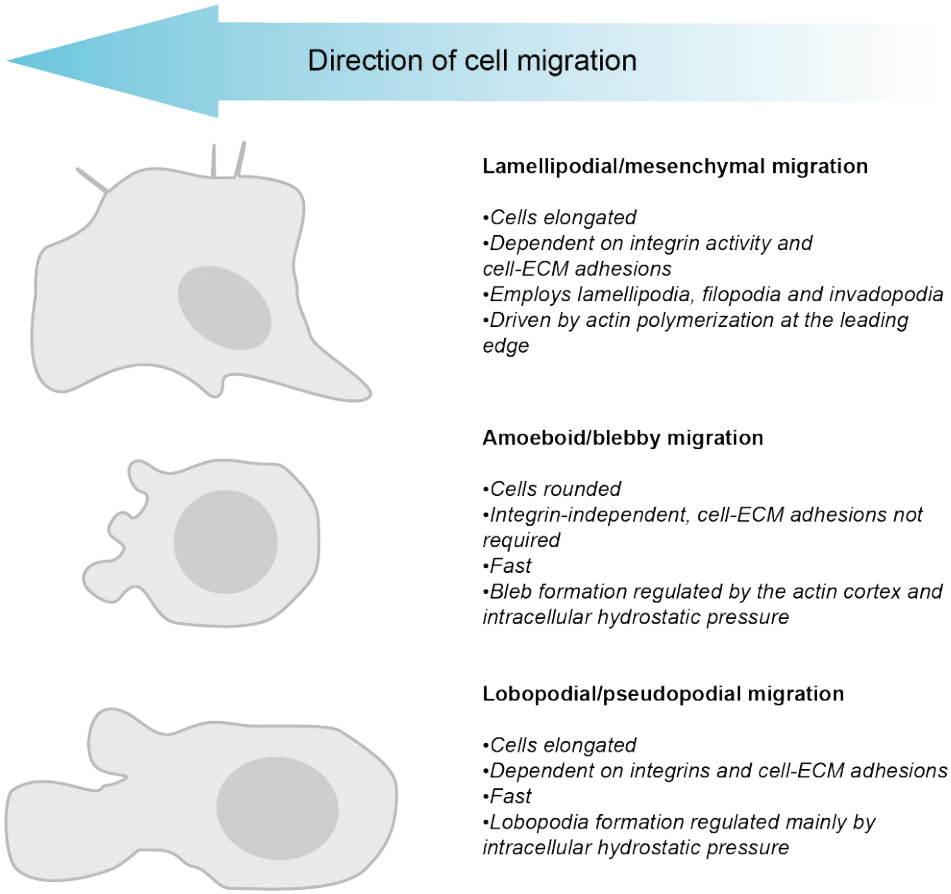

Figure 8. Cell have different leading edge structures depending on the migration mode.

2.4 The SHANK family of multidomain scaffold proteins

The SH3 and multiple ankyrin repeat domains protein (SHANK) family consists of SHANK1, SHANK2 and SHANK3, and they were first identified from the postsynaptic density (PSD) of excitatory neurons where they act as scaffold proteins and mediate signalling (Lim et al., 1999; Naisbitt et al., 1999). SHANKs are large in size, and the full-length proteins consist of approximately 2000 amino acid residues. However, SHANKs have multiple splice variants, and thereby the detected sizes of proteins have been reported to range at least between 120-240 kDa (Lim et al., 1999; Naisbitt et al., 1999). SHANKs were first characterized to include five functional domains: ankyrin repeat region (ARR), Src homology 3 domain (SH3), PSD-95/Discs large/ZO-1 domain (PDZ), polyproline region (PP) and sterile alpha motif (SAM) (Figure 9.). Later on, the very N-terminus of the protein, which was earlier considered to be a part of ARR, was recognized as an independent Shank/ProSAP N-terminal (SPN) domain (Mameza et al., 2013). SHANK1 and SHANK3 share a higher similarity, while SHANK2 most commonly appears to lack the N-terminal ARR part. While SHANK proteins were originally identified in the central nervous system (CNS) (Naisbitt et al., 1999), they are also observed in other tissues (Boeckers et al., 2002; Lim et al., 1999). Importantly, SHANK family proteins have different expression profiles. It was originally reported that in adult rat, SHANK1 is expressed exclusively in the brain, whereas SHANK2 is expressed in kidneys and liver in addition to the expression in brain, and SHANK3 is mainly expressed in heart, brain and spleen (Lim et al., 1999). Since that, SHANKs have also been observed to be expressed in most of the tissues in human body, while the expression levels remain the highest in the brain (Lilja et al., 2017).

The major function of SHANK proteins is to interact with other proteins and thereby affect organization and signalling (Baron et al., 2006). As multidomain scaffold proteins, SHANKs can for example connect different types of glutamate receptor complexes to the actin cytoskeleton of dendritic spines. Furthermore, SHANK proteins can oligomerize through the C-terminal SAM-domain providing additional opportunities in scaffolding and organizing cellular structures and protein complexes (Baron et al., 2006; Lim et al., 1999; Naisbitt et al., 1999). However, while SHANKs have been studied extensively in the CNS, the knowledge of the role of SHANKs in other tissues remains scarce. Recently SHANK1 and SHANK3 were shown to have a function outside the CNS as they inhibit integrin function in cancer cells through sequestering active Rap1 and R-Ras (Lilja et al., 2017). This highlights the need of further studies of SHANK function outside the nervous system. In this thesis, the focus will be on SHANK3, which is most commonly spliced so that it includes the complete N-terminal part, and which is also expressed widely outside

the CNS (Lilja et al., 2017; Lim et al., 1999; Sheng and Kim, 2000; Naisbitt et al., 1999).

2.4.1 The N-terminal part of SHANK3

Functions and significance of the SHANK3 N-terminal segment remained elusive for long, while C-terminal PDZ, PP and SAM domains were the main focus of research. The N-terminal part of SHANK3 consists of the SPN and ARR domains. Especially the knowledge of the SPN domain function has remained scarce. The ARR domain has several known interaction partners, including SHARPIN (Lim et al., 2001), α-fodrin (Böckers et al., 2001) (Figure 9.) and hyperpolarization-activated cyclic nucleotide–gated (HCN) family ion channels (Yi et al., 2016). The SPN domain was first identified to interact with the ARR domain through intramolecular interactions and to restrict thereby interaction between ARR and other proteins (Mameza et al., 2013). However, recently SPN has also been shown to have functions that appear to be independent of ARR domain (Lilja et al., 2017).

The SPN domain is quite small, and is comprised of approximately 90 amino acids. Recently, the structure of SPN and ARR domains was resolved using X-ray crystallography, and the SPN domain was identified as a novel interaction site for active small GTPases Ras and Rap1. In addition, it was shown that SHANK1 and SHANK3 inhibit integrin function by sequestering R-Ras and Rap1 (Lilja et al., 2017).

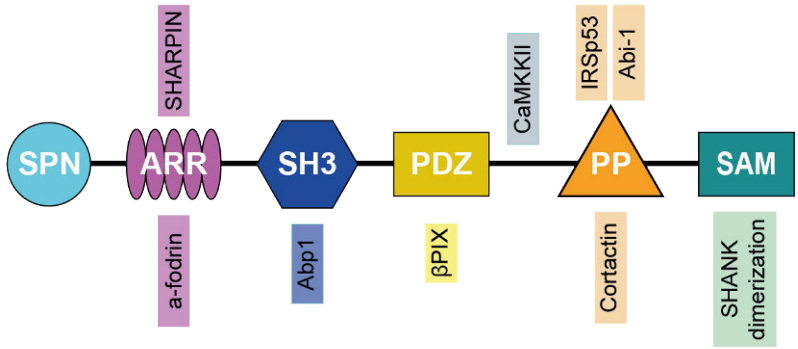

Figure 9. A schematic illustration of SHANK3 domains and actin-associated proteins that bind directly to SHANK3.

2.4.2 SHANK3 is an important actin regulator

SHANK3 has been identified as an important actin regulator, as it modulates the synaptic actin cytoskeleton. So far there has been no reports of a direct interaction between SHANK3 and actin, but SHANK3 regulates the actin cytoskeleton through other ABPs and actin-related proteins, such as cortactin (Naisbitt et al., 1999), β-PIX (Park et al., 2003), actin-binding protein 1 (Abp1) (Haeckel et al., 2008), insulin receptor substrate of 53 kDa (IRSp53) (Bockmann et al., 2002), Ca^{2+}/calmodulin (CaM)-dependent protein kinase IIα (CaMKKIIα) (Stephenson et al., 2017) and Abeldon interacting protein 1 (Abi-1) (Qualmann, 2004) (Figure 9.). Furthermore, other actin regulators, such as Cdc42 (Park et al., 2003), Rac1 and cofilin (Duffney et al., 2015), are downstream targets of SHANK3. Abnormal interactions between SHANK3 and its binding partners has been shown to lead to alterations in the synaptic actin cytoskeleton and dendritic spine morphology (Durand et al., 2012; Sarowar and Grabrucker, 2016). Not surprisingly, both knockdown and knockout of *Shank3* reduce dendritic spine density and maturation (Grabrucker et al., 2011; J. et al., 2011; Wang et al., 2016, 2011; Bozdagi et al., 2010), whereas SHANK3 overexpression promotes dendritic spine development (Zhou et al., 2016; Arons et al., 2012; Speed et al., 2015; Kouser et al., 2013; Verpelli et al., 2011; Jaramillo et al., 2016, 2017; Mei et al., 2016). Taken together, these studies demonstrate that SHANK3 has an important role in normal development of dendritic spines through regulation of the actin cytoskeleton.

2.4.3 SHANK mutations associate with autism spectrum disorders and other neurological disorders

Genes encoding all three SHANK family members have been identified as altered in autism spectrum disorders (ASD) (Berkel et al., 2010; Durand et al., 2007; Gauthier et al., 2009; Moessner et al., 2007). Furthermore, mutations and deletions of genes encoding SHANKs also result in autistic phenotype in mouse models (Duffney et al., 2015; Monteiro and Feng, 2017; Mei et al., 2016).

As SHANKs are major scaffold proteins in the synapses, mutations altering SHANK function may associate with ASD through several different routes. Dysregulation and lack of SHANK3 has been linked especially to changes in dendritic spine morphogenesis and activity of synapses. Importantly, this has been reported to be caused, at least in part, by changed actin dynamics (Duffney et al., 2015; Durand et al., 2012; Mei et al., 2016; Monteiro and Feng, 2017; Sarowar and Grabrucker, 2016). Consistently, SHANK-mediated changes in actin have been shown to alter many pivotal processes and signalling cascades in the dendritic spines (Sarowar and Grabrucker, 2016). Furthermore, stem cell derived neurons from autistic donors having *Shank3* microdeletions have also abnormal actin cytoskeleton

(Kathuria et al., 2018). In line with these observations, the autistic phenotype caused by *Shank3* depletion in mice can be rescued by inhibiting cofilin or activating Rac or its downstream effector p21-activated kinase (PAK) highlighting importance of SHANK3 as an actin regulator (Duffney et al., 2015).

In addition to ASD, SHANK3 mutations associate also with other neurological and neurodevelopmental disorders. SHANK3 22q13 chromosome deletion leads to Phelan-McDermid syndrome, which is characterized by severe intellectual deficits (Bonaglia et al., 2006; Phelan and McDermid, 2012) and SHANK3 mutations that disrupt synapto-nuclear shuttling of SHANK3 are known to associate with schizophrenia (Grabrucker et al., 2014). Taken together, these observations highlight the importance of SHANK3 in normal development and function of synapses.

2.4.3.1 Mutations in the N-terminal part of SHANK3 that are found in ASD-patients

ASD-linked missense mutations have been recognized in all of the functional domains of SHANK3 and several mutations have been located in the N-terminal part of the protein (Boccuto et al., 2012; Durand et al., 2007; Gauthier et al., 2009). These N-terminal mutations include R12C, L68P, R300C and Q321R, but also other less-studied mutations that have been found in patients with ASD, such as P141A, A198G and S341L (Table 1.) (Boccuto et al., 2012; Hassani Nia and Kreienkamp, 2018). The genetic relevance of SHANK3 mutations in ASD has been difficult to uncover, as some mutations, such as R300C, can be inherited from healthy parents. There is also indications that certain mutations may have different neurological effects depending on the environment and other factors. For example autism-linked mutation L68P was inherited from an epilectic father in the initial study that recognized the mutation (Gauthier et al., 2009). Altogether, the so far characterized mutations do not have a dominant effect on the disease. While most of the SHANK3 mutations are very rare, R300C has a higher prevalence also in healthy individuals suggesting that it may rather be a gene polymorphism instead of being a pathogenic mutation (Hassani Nia and Kreienkamp, 2018). The ASD-associated mutations in the N-terminal SHANK3 and their molecular outcomes are summarized in Table 1.

Table 1. Known ASD patient mutations within the N-terminal SHANK3.

MUTATION	LOCATION	MOLECULAR OR CELLULAR OUTCOMES	REFERENCE
R12C	SPN	Reduced Ras- and Rap1-binding followed by reduced integrin inhibition, alterations in dendritic spines and their density, changes in actin	(Durand et al., 2012, 2007; Lilja et al., 2017)
L68P	SPN	Interaction with Ras and Rap1 lost, unable to inhibit integrin function. Increased α-fodrin and SHARPIN binding. Intramolecular interaction with SPN domain lost.	(Gauthier et al., 2009; Lilja et al., 2017; Mameza et al., 2013)
P141A	ARR	Not determined so far	(Boccuto et al., 2012; Hassani Nia and Kreienkamp, 2018)
A198G	ARR	Not determined so far	(Durand et al., 2007)
R300C	ARR	Alterations in dendritic spines and their density, changes in actin	(Durand et al., 2012, 2007)
Q321R	ARR	Alterations in dendritic spines, actin accumulation in spines, altered neuronal growth cone motility	(Durand et al., 2012)
S341L	ARR	Not determined so far	(Moessner et al., 2007)

2.5 SHARPIN is a multifunctional adaptor protein

SHARPIN (SHANK-Associated RH Domain Interactor) is a multifunctional adaptor protein that is approximately 45 kDa in size (Lim et al., 2001), and it consists of three functional domains: a pleckstrin homology (PH) domain (Stieglitz et al., 2012), a ubiquitin-like (UBL) domain and a Npl4 zinc finger (NZF) domain (Figure 10.) (Ikeda et al., 2011; Stieglitz et al., 2012). Interestingly, SHARPIN was originally identified as a SHANK interactor from the PSD of neurons (Lim et al., 2001). Indeed, the SHANK3 ARR domain binds to the SHARPIN UBL domain, but the function of this interaction remains obscure, and it may be that SHARPIN only interacts with certain SHANK splice variants (Lim et al., 2001; Mameza et al., 2013). Moreover, SHARPIN is ubiquitously expressed in most of the human and rodent tissues (Seymour et al., 2007) and in several human cancer cell lines (Rantala et al., 2011). Since its original discovery, SHARPIN has been shown to bind many other proteins and to regulate their activity, including caspase-1 inactivation in sepsis (Nastase et al., 2016), phosphatase and tensin homolog (PTEN) inhibition (He et al., 2010) and T cell antigen receptor (TCR) complex inactivation (Park et al., 2016). However, the most studied SHARPIN functions are its role in integrin activity regulation (Pouwels et al., 2013; Rantala et al., 2011; Gao et al., 2019; Kasirer-Friede

et al., 2019) and nuclear factor kappa B (NF-κB)-signalling pathways (Figure 10.) (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011).

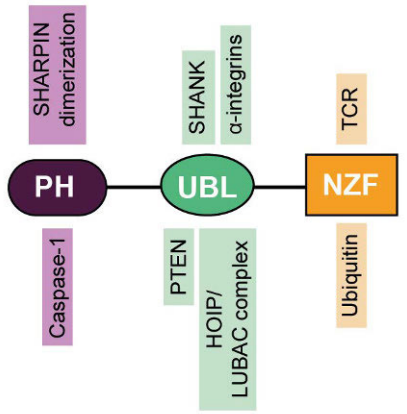

Figure 10. A schematic illustration of SHANK3 domains and actin-associated proteins that bind directly to SHANK3.

2.5.1 SHARPIN inhibits integrins and promotes NF-κB signalling as a part of LUBAC complex in a mutually exclusive manner

SHARPIN binds to the cytoplasmic tails of α-integrins and it has been shown to reduce thereby recruitment of integrin activators, talin and kindlin, to the integrin β1-tail (Rantala et al., 2011). Importantly, SHARPIN binds to a highly conserved sequence within the α-tail, and thereby it is expected to inhibit all β1-subunit containing integrin heterodimers (Figure 10.) (Rantala et al., 2011). Furthermore, SHARPIN has been shown to inhibit β2-integrins in lymphocytes (Pouwels et al., 2013) and αIIbβ3 integrins in platelets (Kasirer-Friede et al., 2019). Interestingly, a recent study also showed that SHARPIN may also bind to β1-integrin tails and form a complex with the β1-integrin and kindlin preventing talin recruitment and integrin activation (Gao et al., 2019). This brings up the question whether SHARPIN would be able to bind both integrin cytoplasmic tails at the same time through different domains, but this remains to be unravelled, as the binding site for integrin β1 has not been mapped in detail. Importantly, both SHARPIN and SHANK have been shown to inhibit integrins independently of each other (Lilja et al., 2017).

The linear ubiquitin chain assembly complex (LUBAC) mediates the canonical NF-κB signalling pathway downstream of tumour necrosis factor α (TNFα), which is an important receptor upstream of inflammation, immune responses and cell death (Gerlach et al., 2011; Sasaki and Iwai, 2015). The LUBAC complex is an E3 ligase

that mediates NF-κB signalling by adding linear polyubiquitin chains to proteins, such as NEMO, which is an adaptor protein for IkB kinase (IKK) complex, an activator of NF-κB transcription factor. Importantly, aberrant NF-κB signalling associates with cancer and inflammatory disorders (Gerlach et al., 2011; Niu et al., 2011; Sasaki and Iwai, 2015). The LUBAC complex consists of three proteins: SHARPIN, heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-L1) and HOIL-1 interacting protein (HOIP). HOIP acts as the catalytic subunit of the LUBAC complex, and while SHARPIN does not have its own enzymatic activity in the LUBAC complex, it has an important role as an adaptor protein. SHARPIN is needed to promote LUBAC function and NF-κB pathway signalling, and lack of SHARPIN has been observed to cause dysregulation of NF-κB signalling and cell death (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). Interestingly, while SHARPIN was originally reported to bind only HOIP, not HOIL-1L, a recent study shows that based on a X-Ray crystal structure of the complex, SHARPIN and HOIL-1L could interact with newly identified LUBAC-tethering motifs (LTMs) (Figure 10.) (Fujita et al., 2018). Taken together, SHARPIN and HOIL-L1 act as LUBAC complex adaptors and mediate stabilization of the complex, and all three complex members are needed for normal LUBAC function (Fujita et al., 2018; Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011).

Importantly, the binding sites of α-integrins and HOIP have been mapped to overlap partially in the SHARPIN UBL domain. However, the interactions have been shown to be mutually exclusive. SHARPIN appears to be able to bind only either α-integrin tail or HOIP at the time, but disruption of interaction between SHARPIN and α-integrins does not disrupt the HOIP binding site, and *vice versa* (De Franceschi et al., 2015).

2.5.2 Spontaneus SHARPIN null mice develop psoriasis-like chronic proliferative dermatitis

A spontaneous *Sharpin* null mutant mouse line has been identified (Seymour et al., 2007). Importantly, these mice develop a chronic hyperproliferative dermatitis, and they are thereby referred to as *cpdm* mice. Furthermore, *cpdm* mice suffer from cutaneous and systemic eosinophilic inflammation, skin blistering and epidermal hyperproliferative thickening and defective, lymphoid organ development (Potter et al., 2014; Seymour et al., 2007). Importantly, lack of SHARPIN seems to impair LUBAC-mediated NF-κB activation, and cells isolated from *cpdm* mice are very sensitive for TNF-induced cell death (Gerlach et al., 2011; Ikeda et al., 2011). Importantly, simultaneous knockout of the *Tnf* gene can rescue the inflammatory phenotype of *cpdm* mice (Gerlach et al., 2011). *Hoil-1L* KO mice do not exhibit similar skin phenotype as *cpdm* mice and *Hoil-1L* KO mice and cells isolated from

them exhibit milder versions of similar phenotypes as *cpdm* mice, such as sensitivity to TNF-induced cell death (Ikeda et al., 2011; Tokunaga et al., 2009, 2011). Furthermore, simultaneous genetic ablation of both *Sharpin* and *Hoil-1L* has a strong, combinatory effect (Ikeda et al., 2011). Lack of HOIP, the catalytic subunit of the LUBAC complex, is embryonically lethal in mice (Fujita et al., 2018).

Especially the epidermis, the outermost skin layer, is heavily affected in *cpdm* mice. Importantly, *cpdm* mice exhibit hyperkeratosis and high number of apopototic keratinocytes (Seymour et al., 2007). Furthermore, it has been shown that at least the TNF-dependent cell death induces skin inflammation in *cpdm* mice (Kumari et al., 2014; Rickard et al., 2014). However, since SHARPIN is a multifunctional adaptor protein, also other SHARPIN interactors may contribute to the *cpdm* phenotype. For example, integrins have an important role in keratinocytes, that are the main cell type in the epidermis and other types of stratified, squamous epithelia (Watt, 2002).

In normal, healthy epidermis, integrin expression is limited to the basal layer that connects the basal lamina to the epidermis (Hotchin et al., 1993, 1995; Watt, 2002), while in psoriasis-like conditions and hyperproliferative dermatitis, integrins are also expressed in suprabasal layers (Watt, 2002). Importantly, a transgenic mouse model overexpressing β1-integrins suffers from inflammatory, psoriasis-like skin phenotype, epidermal hyperplasia and abnormal keratinocyte differentiation (Carroll et al., 1995). Taken together, integrin β1-overexpressing mouse model exhibits very similar phenotype to SHARPIN null *cpdm* mice, which also have increased integrin activity in the epidermis (Rantala et al., 2011). However, the actual status and reasons behind integrin activation in *cpdm* mice remains to be unravelled, as inflammation may also trigger integrin activation. Importantly, dysregulated integrin activity has been shown to associate with skin blistering, inflammatory diseases and immune system malfunction (Bouvard et al., 2013; Watt, 2002) and integrin function blocking therapies have been shown to alleviate psoriasis-like phenotypes both in human (Ley et al., 2016) and mice (Conrad et al., 2007). Taken together, the *cpdm* mice phenotype shares many similarities with mouse models that have increased integrin activity, and the role of integrin activation in the *cpdm* phenotype awaits further investigation.

2.5.3 The role of SHARPIN in cancer

Interestingly, SHARPIN has been observed to be highly expressed in multiple different cancers, including human esophageal (Zhang et al., 2019), breast (De Melo and Tang, 2015; Zhuang et al., 2017), prostate (Zhang et al., 2014; Li et al., 2015), skin (Tamiya et al., 2018) and liver cancers (Table 2.) (Tanaka et al., 2016). Importantly, SHARPIN may promote cancer cell proliferation, survival and invasion through NF-κβ-signalling and its downstream mechanisms (Zhang et al., 2014; Li et al., 2015), and disruption of interaction between SHARPIN and other LUBAC

complex members have been shown to function as a potential target in LUBAC-dependent cancers (Fujita et al., 2018; Yang et al., 2014). However, the mechanisms through which SHARPIN promotes cancer progression and tumorigenesis are various, and SHARPIN has also been shown to promote oncogenesis through other mechanisms than LUBAC- and NF-κB signalling (Table 2.). For example, SHARPIN has been shown to promote melanoma growth by enhancing activity of an epigenetic regulator protein, arginine methyltransferase 5 (PRMT5) (Tamiya et al., 2018). In addition, SHARPIN has been observed to promote estrogen-dependent cancer progression by stabilizing estrogen receptor α (Zhuang et al., 2017), and it is known to inhibit phosphatase and tensin homolog (PTEN) (He et al., 2010) and to promote degradation of p53, which are both tumour supressors (Yang et al., 2017). SHARPIN has also been shown to participate in ECM remodelling through upregulation of Versican, a chondroitin sulfate proteoglycan, and NF-κβ-mediated MMP expression (Tanaka et al., 2016).

Interestingly, while SHARPIN is upregulated in many cancers and seems to promote oncogenesis, it has also been shown to inhibit cancer progression in esophageal cancer through inhibition of Hippo pathway signalling by poly-ubiquitinylating yes-associated protein (YAP) and targeting it for proteosomal degradation (Zhang et al., 2019). Taken together, SHARPIN associates with multiple different human cancers, but there is no consensus what the mechanism would be how SHARPIN drives cancer progression. As SHARPIN is a multifunctional protein, it may promote oncogenesis through several different mechanisms and in a cancer type specific manner highlighting the need of further studies regarding the role of SHARPIN in cancer.

Table 2. How SHARPIN associates with different cancers.

CANCER TYPE	PROMOTES / INHIBITS CANCER PROGRESSION	DOWNSTREAM TARGET / MOLECULAR MECHANISM	REFERENCE
Breast	Promotes	Not defined / MDM2-mediated induction of p53 degradation / estrogen receptor α stabilization	(De Melo and Tang, 2015; Yang et al., 2017; Zhuang et al., 2017)
Cervical	Promotes	Loss of PTEN	(He et al., 2010)
Esophageal	Inhibits	YAP inhibition	(Zhang et al., 2019)
Liver	Promotes	Upregulation of versican, NF-κβ-mediated MMP upregulation	(Tanaka et al., 2016)
Prostate	Promotes	NF-κβ-mediated effects	(Li et al., 2015; Zhang et al., 2014)
Skin/melanoma	Promotes	Induction of PRMT5 activity	(Tamiya et al., 2018)

3 Aims

Integrins are the major cell surface adhesion receptors that mediate a plethora of cellular processes including cell proliferation, cell differentiation and signal transmission between a cell and its surrounding ECM. They are transmembrane proteins that mediate signalling bidirectionally, from the cell interior to regulate integrin-ECM interaction and in response to integrin-ECM adhesion to activate cellular signalling pathways, and connect the ECM to the actin cytoskeleton. Regulation of integrin activity has a pivotal role in several signalling pathways and not surprisingly, dysregulation of integrin function is implicated in a variety of disorders including cancer and inflammatory diseases (Bouvard et al., 2013; Hynes, 2002). Importantly, our group has identified SHANKs (Lilja et al., 2017) and SHARPIN (Rantala et al., 2011) as novel integrin inhibitors. While both SHANK3 and SHARPIN were originally identified from the CNS (Lim et al., 2001; Naisbitt et al., 1999), they have been shown since to be expressed also in many other tissues (Lilja et al., 2017; Rantala et al., 2011). As the integrin-based cell adhesion and the actin cytoskeleton are highly interdependent, I wanted to characterise how SHANK3 and SHARPIN affect cytoskeletal structures, such as filopodia and lamellipodia. In addition, since integrins are critical regulators of the homeostasis of the epidermis, I wanted to investigate the role of integrin activity in the hyperproliferative dermatitis characteristic to SHARPIN-deficient *cpdm* mice (Seymour et al., 2007). Although increased integrin activity is observed in the epidermal layer of *cpdm* skin (Rantala et al., 2011), it has remained enigmatic whether it is secondary to the systemic inflammation in mice.

The specific aims of my thesis are:

I. Characterization of the role of SHANK3 SPN domain in filopodia formation
II. Identification of novel SHARPIN interactors and characterization of its role in lamellipodia formation
III. Investigation of how increased integrin activity caused by lack of SHARPIN modulates the hyperproliferative dermatitis phenotype in *Sharpin*$^{cpdm/cpdm}$ mice

4 Materials and Methods

This chapter describes the methods and experimental procedures in which I have been personally involved with. Precise details, such as catalogue numbers, and microscope objective and camera information, can be found in original articles. Roman numerals (I, II and III) indicate in which original articles each method was used.

4.1 Cell lines (I, II)

Multiple different cancer and non-cancer cell lines were used in the original articles, including CHO (chinese hamster ovary), HEK-293 (hamster embryonic kidney), HeLa (human cervical cancer), MEF (mouse embryonic fibroblast), NCI-H460 (human non-small lung adenocarcinoma) and U2OS (human osteosarcoma) cells (Table 3.). All cell culture reagents were obtained from Sigma or GIBCO. All cell lines were obtained either from American Type Culture Collection (ATCC) or Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ). Cells were regularly tested negative for mycoplasma infection. All cell lines were grown in a humified incubator at 37 ºC supplemented with 5 % CO_2.

Table 3. Cell lines used in the original articles. DMEM = Dulbecco's Modified Eagle's Medium, FBS = fetal bovine serum, MEM = minimum essential medium, pen-strep = penicillin-streptomyocin.

CELL LINE	CULTURE MEDIUM	ORIGINAL ARTICLE
CHO	α-MEM base, 5 % FBS, 2 mM L-glutamine, 1 % pen-strep	I
HEK-293	DMEM base, 10 % FBS, 2 mM L-glutamine, 1 % pen-strep	I, II
HeLa	DMEM base, 10 % FBS, 2 mM L-glutamine, 1 % sodium pyruvate, 1 % non-essential amino acids, 2 % HEPES, 1 % pen-strep	II
MEF (CPDM & WT)	DMEM base, 10 % FBS, 1 % sodium pyruvate 1 % non-essential amino acids, 1 % pen-strep, 0.001% β-mercapto-ethanol	II
NCI-H460	RPMI1640 base, 10 % FBS, 2 mM L-glutamine, 1 % non-essential amino acids, 1% sodium pyruvate and 1% glucose, 1 % pen-strep	II
U2OS	DMEM base, 10 % FBS, 2 mM L-glutamine, 1 % pen-strep	I, II

4.1.1 Generation of SHARPIN-knockout cell lines using CRISPR (II)

Sharpin knockout NCI-H460 cell lines were created using CRISPR genome engineering technique. GuideRNAs were designed with MIT CRISPR Designer (http://crispr.mit.edu/), and two guideRNAs (5'-TGGCTGTGCACGCCGCGGTG-3', 5'-TCAGTTTCCTACACCATCCG-3') were obtained from Sigma. GuideRNAs were cloned into pSpCas9(BB)-2A-GFP (PX458) described earlier in (Ran et al., 2013). Both guideRNA plasmids were transfected into cells. GFP expressing cells were sorted into single cell cultures with FACSaria IIu Cell Sorter (BD Biosciences). Single cell cultures were grown and screened using PCR. For DNA samples, cells were trypsinized, spun down, resuspended in distilled water and boiled for 10 min. Forward primer used was 5'-GTGTCCATTTGTGGGCAAAG and reverse primer 5'-GGCACTGACCATTCTGTCCT. PCR was done with Kod XtremeTM Hot Start DNA Polymerase (EMD Millipore, #71975). Length of the PCR product was approximately 900 bp, but in the case of cleavage with both guideRNA plasmids the product was approximately 500 bp shorter due to cleavage of sequence between them. We selected two knockout cell lines, that were cleaved with two guideRNA plasmids based on PCR, and did not produce SHARPIN protein measured with western blot. Control cell line was one of the flow cytometry-sorted single cell cultures that still produced long PCR product and expressed normal amount of SHARPIN based on western blot detection.

4.2 Transient transfections, plasmids and siRNAs (I, II)

Lipofectamine 3000 and P3000 Enhancer reagent (Thermo Fisher Scientific) were used to transiently transfect plasmids into cells, and Hiperfect (Qiagen) and RNAiMAX (Thermo Fisher Scientific) were used to introduce RNAi oligonucleotides into cells for silencing gene expression. The plasmids used in this thesis are listed in Table 4. Point mutations were introduced to original wild type (WT) plasmids using QuickChange II mutagenesis kit (Agilent) or ordered from Gene Universal Inc. siRNAs targeting human SHARPIN and a scramble control were obtained from Qiagen, and siRNAs against Arp3 and HOIP were obtained from Dharmacon.

Table 4. Plasmids used in the original articles. FL = full-length protein, GFP = green fluorescent protein, GST = glutathione S-transferase, RFP = red fluorescent protein, WT = wild type.

PLASMID	ORIGINAL ARTICLE
eGFP-C1	I, II
GFP-Arp2	II
GFP-Cofilin-1	I
GFP-Rap1 Q63E	I
GFP SHANK3 SPN WT & point mutants R12C and Q37A/R38A	I
GFP SHANK3 FL WT & point mutants R12C, Q37A/R38A and N52R	I
GFP SHARPIN WT & point mutant V240A/L242A	II
GFP-Talin	I
GST SHANK3 SPN WT and Q37A/R38A	I
GST SHANK3 SPN-ARR WT and N52R	I
Kindlin-2-GFP	I
mCherry-SHARPIN	II
mEmerald-Lifeact	II
mRFP-N1	I, II
mRuby-Lifeact	I
Myo10-mCherry	I
pspCas9(bb)-2a-GFP (px458), (Ran et al. 2013)	II
SHANK3-mRFP FL and fragments of different lengths	I
SHANK3 SPN-ARR-mRFP, WT and N52R	I
SUMO SHANK3 SPN WT	I
SUMO SHANK3 SPN-ARR WT	I

4.3 Mice (III)

The spontaneous SHARPIN null *cpdm* mouse strain (Seymour et al., 2007), C57BL/KaLawRij-*Sharpincpdm*/RijSunJ mouse strain, hereafter referred to as *Sharpin*$^{cpdm/cpdm}$, $^{cpdm/+}$ or $^{+/+}$), was obtained from The Jackson Laboratory (#007599, Bar Harbor, ME, USA). *Tnfr1-Sharpin* double knockout *Tnfrsf1a*^{tm1Imx} *Sharpin*$^{cpdm/cdpm}$ mice, *Tnfrsf1at*^{m1Imx} Stock No: 003242; hereafter referred to as Tnfr1$^{-/-}$), was obtained from Prof. H. Walczak (University College London, UK). DNA samples were extracted from skin pieces gotten from ear marking using KAPA Mouse Genotyping Kit (KK7302). TaqMan SNP Genotyping Assay Mix (5793982, Applied Biosystems) and TaqMan Universal PCR Master Mix were used to detect the *Sharpin*^{cpdm} mutation. The *Tnfr1* phenotype was determined based on PCR amplification. Both *Sharpin*$^{cpdm/?}$ and *Sharpin*$^{cpdm/?}$-*Tnfr1*$^{+/?}$ colonies were maintained through breeding of heterozygous mice. Single and/or double knockout mice and their WT or heterozygous littermate controls were selected to experiments based on genotyping results.

4.3.1 Isolation of primary keratinocytes from mouse epidermis (III)

6-8-week old *Tnfr1*$^{-/-}$-*Sharpin*$^{cpdm/cpdm}$ and WT or heterozygous litter mate controls were sacrificed, dorsal skin was shaved with a scalpel and a skin piece was harvested in cold PBS (phosphate buffered saline) supplemented with penicillin and streptomycin. The piece of skin was incubated overnight in 0.25 % porcine trypsin (Sigma) in Ca^{2+}-free Hank's Balanced Salt Solution (HBSS) at +4 ºC on a shaker (~90 rpm). Next day, dermis was removed from the epidermis using forceps. The remaining epidermis was minced mechanically into finer pieces and incubated in 0.2 % Collagenase XI (Sigma) in HBSS at 37 ºC for 30 min. During the incubation, the mixture was resuspended and stirred every 5-10 min to promote dissociation of keratinocytes from tissue. The suspension was filtered through a cell strainer (70 µm; BD Biosciences) to separate keratinocytes from remaining tissue material. The separated keratinocytes were places on ice and incubated with 20 U/ml DNase I (Roche) in HBSS for 5 min, after which DNAase was removed by centrifugation and cells were further treated according to downstream assays in question. The cells were grown in a humified incubator at 32 ºC supplemented with CO_2.

4.3.2 Proliferation assay of keratinocytes and treatment with β1-integrin blocking antibody (III)

After isolation, equal amounts of mouse primary keratinocytes were plated on 96-well plate coated with 20 µg/ml Collagen Type I. Cells were grown in FAD medium (3.5:1.1 DMEM and Ham's F12 Nutrient Mixture) containing 10 % chelated FBS, 100 µg/ml penicillin-streptomycin, 5 µg/ml insulin, 100 pM choleratoxin, 10 ng/ml epidermal growth factor (EGF), 100 U/ml sodium pyruvate, and 0.5 µg/ml hydrocortisone. On the first day after the plating, the media was replaced with fresh media containing either with 10 µg/ml anti-Itgb1 (anti-mouse CD29 Armenian hamster IgG, Biolegend) or isotype IgG control antibody (Biolegend). The treatment media was replaced with fresh media every other day. The effect of treatment on keratinocyte proliferation was quantified using Cell Proliferation Reagent WST-1 tetrazolium salt (Roche) according to manufacturer's instructions. Detection was performed by analysing absorbance at 450 nm with BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader. Samples were subjected to WST-1 assay as duplicates and the relative proliferation was calculated by normalizing proliferation to amount of cell at different time points to day zero.

4.4 Antibodies and fluorescent dyes

Antibody-based detection was used in flow cytometry, immunofluorescence microscopy and western blot. Staining protocols are described together with the aforementioned methods. Primary antibodies and their associated details are listed in table 5. The secondary antibodies AlexaFluor 488-, 555-, 568, and -647 IgG against mouse and rabbit were used in flow cytometry, immunofluorescence and immunohistochemistry microscopy, and obtained from Invitrogen, Life Technologies. Secondary antibodies IRDye680 and IRDye800 against mouse and rabbit used in western blot were obtained from (LI-COR). Details of directly fluorochrome-conjugated actin dyes can be found from Table 6. DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) was used to visualize nuclei in microscopy.

Table 5. Primary antibodies used in the original articles. FACS = flow cytometry, IF = immunofluorescence, WB = western blot.

ANTIGEN	MANUFACTURER	PRODUCT CODE	APPLICATION	DILUTION	ORIGINAL ARTICLE
Active integrin β1 (clone 9EGeg7)	PD Pharmingen	553715	FACS / IF	1:50 / 1:100	III
Arp2	Abcam	ab47654	IF / WB	1:100 / 1:1000	II
β-actin	Sigma	A1978	WB	1:1000	I
CD29-647 (total integrin β1)	BioLegend	102210	FACS	1:50	III
CD31-Pacific Blue (PECAM-1)	BioLegend	102422	FACS	1:50	III
CD45-Pacific Blue (PTPRC)	BioLegend	103125	FACS	1:200	III
CD49f-488 (integrin α6)	BioLegend	313608	FACS	1:20	III
Cortactin	Merck Millipore	05-180	IF / WB	1:100 / 1:1000	II
GAPDH	HyTest	5G4MaB6C5	WB	1:10000	II, III
GFP	Abcam	ab1218 + ab69507	IF / WB	1:100 / 1:1000	I, II
GST	CST	91G1	WB	1:1000-5000	I, II
Integrin α6	Serotec	NKI-GoH3	IF	1:200	III
Keratin 14	Biosite	PRB-155P	IF / WB	1:600 / 1:1000	III
Non-muscle myosin IIA	BioLegend	90980	IF	1:1000	I
PB1 (integrin α5)	Developmental studies hybridoma bank	-	FACS	1:7,5	I
Phospho myosin light chain	CST	3674	IF	1:100	I
SHARPIN	Abcam	ab69507	IF	1:100	II
Sharpin	Proteintech	14626-1-AP	WB	1:1000	II, III
Vimentin	CST	D21H3	WB	1:1000	III

Table 6. Directly conjugated actin dyes used in the original articles.

DYE	MANUFACTURER	PRODUCT CODE	DILUTION	ORIGINAL ARTICLE
Atto-phalloidin-647	Sigma	65906	1:500	I, II
Atto-phalloidin-740	Sigma	07373	1:75	I
Phalloidin Alexa Fluor 488	Invitrogen	A12379	1:200	I
Phalloidin Alexa Fluor 647	Invitrogen	A22287	1:200	I
Sir-actin-647	Spirochrome	SC001	1:5000	I

4.5 Other reagents, drugs and inhibitors (I, II, III)

Fibronectin from bovine plasma and collagen type I from rat tail were obtained from Merck-Millipore and used to coat cell culture plates and glass-bottom dishes. The Arp2/3 complex inhibitor CK666 was obtained from Sigma.

4.6 Flow cytometry (fluorescence-activated cell sorting, FACS) (I, III)

To analyse integrin activity in primary mouse keratinocytes, cells were isolated as described above, kept on ice and fixed with 2 % paraformaldehyde (PFA) in PBS for 10 min at RT. PFA was removed by centrifugation, followed by suspending the cells in cold Tyrodes buffer (10 mM Hepes-NaOH pH 7.5, 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$, 11.9 mM NaHCO3, 5 mM glucose and 0.1 % bovine serum albumin (BSA)). The cells were stained with directly fluorochrome-conjugated primary antibodies (described in Table 5) diluted in Tyrodes. After the antibody incubations, cells were washed with Tyrodes and finally resuspended in PBS before the analysis.

To analyse the role of SHANK3 SPN mutants in integrin activity, GFP-tagged SPN constructs were expressed in CHO cells. The cells were detached gently using Hyclone® HyqTase (Thermo Fisher Scientific). The cells were pre-incubated with 5 mM EDTA (integrin-inhibiting negative control) or PBS, after which they were incubated with Alexa Fluor 647-labelled fibronectin 7-10 fragment. After the incubation, cells were washed with cold Tyrodes buffer and fixed with 2 % PFA in PBS. The cells were stained for total α5β1-integrin for 40 min followed by incubation with Alexa Fluor-conjugated secondary antibody. Samples were washed with cold Tyrodes and resuspended in PBS before analysis.

All FACS samples were analysed using BD Biosciences LSRFortessa and FlowJo and Flowing Software analysis softwares.

The integrin activity index was calculated with the following equation:

Integrin activity index = $(F-F_0) / (F_{integrin})$

- F = geometric mean fluorescence intensity of fibronectin fragment binding
- F_0 = is the mean fluorescent intensity of fibronectin fragment binding in negative control sample
- $F_{integrin}$ = the normalized average mean fluorescent intensity of total αβ1 integrin (PB1)

4.7 Protein expression and purification (I)

Recombinant proteins were expressed in competent *E. coli* BL21 strain and grown in Luria-Bertani (LB) broth supplemented with selection antibiotics. The production of transformed proteins was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG), and the proteins were purified from the prepared bacterial lysate with either glutathione sepharose (GE Healthcare) or Protino Ni-Ted resin (Macherey-Nagel) depending on if the protein in question has a glutathione S-transferase (GST) or a polyhistidine tag. Proteins were eluted from beads with reduced L-glutathione or imidazole, respectively. Dialysis of proteins and buffer exchange was performed overnight at +4C using Thermo Scientific Slide-A-Lyzer™ Dialysis Cassettes. The exact details of buffer recipes can be found from the original articles I.

4.8 SDS-PAGE gel electrophoresis, Coomassie Blue staining and western blotting (I, II, III)

SDS-PAGE gel electrophoresis was used to separate purified proteins and protein extracts based on size under denaturing conditions. Coomassie Blue staining was used to analyze different steps of protein production and purification. After run, the gel was washed briefly with tap water and incubated in Expedeon Instant Blue Coomassie Protein Stain overnight at RT. After incubation, the gel wash washed with plenty of tap water. For western blot detection, SDS-PAGE gel was transferred to Trans-Blot® Turbo™ nitrocellulose membranes using with Bio-Rad Trans-Blot® Turbo™ Transfer System. Membranes were blocked for 1 h at RT either with 5 % milk in TBST or with Thermo Scientific Pierce™ StartingBlock™ Blocking Buffers diluted in PBS according to manufacturer's protocol. Primary antibodies and secondary antibodies were diluted in the blocking buffer. Primary antibodies were incubated overnight at +4C, and secondary antibodies were incubated for 1 h at RT.

Blocking and incubations were done under constant rotation or shaking. Antibodies used and their dilutions are listed in Table 5. Both Coomassie gels and western blot membranes were scanned with either Odyssey infrared scanner (LI-COR) or Bio-Rad Chemidoc.

4.9 Microscopy sample preparation

Different microscopy techniques were used to image cells and tissue samples in all original articles included in this thesis. For the sample preparation, cells were plated on either on glass coverslips, glass-bottom dishes (MatTek and Cellvis) or 8-well µ-slides (Ibidi) for times indicated in the original articles. For given experiments the cell culture plates and glass surfaces were coated with fibronectin or collagen. Samples were imaged either as fixed with 4 % PFA or live. After fixing, samples were stained either blocked with 10 % horse serum in PBS or quenched with 1 M glycine in PBS. Antibodies and actin dyes were either diluted directly to PBS or in 10 % horse serum in PBS. Both primary (Table 5) and secondary antibodies or actin dyes (Table 6.) were incubated for 30 min at RT or overnight at + 4C. Glass coverslips were mounted on glass slides using Mowiol mounting media, and glass-bottom dishes were imaged as filled with PBS. For live-imaging, cells were kept in their normal culture medium supplemented with 10 mM HEPES.

Mouse skin samples were prepared from O.C.T TissueTek (Sakura)-embedded frozen tissue sections plated on glass slides and ringed with PapPen. The sections were fixed with 4 % PFA and blocked and permeabilised with 2 % BSA and 0.1 % Triton-X in PBS. Primary (Table 5.) and secondary antibodies were diluted in 2 % BSA in PBS at RT for 1 h. After the secondary antibody, samples were washed 3 times with PBS, and one time with distilled water. The washed samples were mounted with Vectashield mounting medium.

4.9.1 Micropatterns

For some experiments, cells were confined to certain shapes, such as crossbow shape) using micropatterns. Micropatterns were created on glass coverslips as described earlier (Azioune et al., 2009). The created patterns were coated with 50 µg/ml fibronectin. Furthermore, 555-labelled BSA and Alexa Fluor 488- or 647-labelled fibrinogen were used to visualize the patterns. Cells were plated on patterns for 3-4 hours, after which they were fixed, stained and imaged as described above.

4.10 Microscopes and image analysis

Different confocal microscopes (3i Marianas Spinning disk confocal microscope, Carl Zeiss LSM780 laser scanning confocal microscope and Carl Zeiss LSM880 laser scanning confocal microscope LSM880 with AiryScan module) were used to image selected focal plains and to get high-resolution image data of cellular structures throughout the cell or tissue samples. Furthermore, they were used for fluorescence recovery after photobleaching (FRAP) experiments. Total internal reflection fluorescence microscopy (TIRF) microscope (Carl Zeiss Laser-TIRF 3 Imaging System) was used for imaging bottom plane structures, such as adhesions and actin, from both fixed and live samples. Widefield microscopes (Zeiss AxioVert 200 M and Nikon Eclipse Ti2-E) were used to image overall cell morphology and localization of different proteins in cells, as well as live cell migration experiments. Detailed information about the cameras and objectives the microscopes were supplemented with can be found from the original articles I, II and III.

Most of the image analysis was performed with ImageJ and its different plug-ins, including, Chemotaxis, coloc 2 and MTrackJ. Live-cell videos were prepared with ImageJ. FRAP data was analysed using SlideBook 6 software and Carl Zeiss Zen Black FRAP analysis modules. FilamentSensor software (Eltzner et al., 2015) was used to quantify thick actin bundles.

4.10.1 Wound healing assay

Equal amounts of cells were seeded to Essen BioScience IncuCyte™ ImageLock™ 96-well plate. The day after splitting when cells had formed a confluent monolayer, a scratch wound was made using Essen BioScience WoundMaker™. After making the wound, wells were washed to remove detached cells and fresh media was added. Imaging was started directly after making the wound using Essen BioScience IncuCyte™ and images were taken by IncuCyte Zoom™ System (Essen BioScience) every two hours until the wound was closed. The wound closure was analyzed with the IncuCyte Zoom software.

4.11 Statistical analysis

GraphPad Prism 7 software was used for all statistical analyses. Comparison between two groups was done Student's t-test with Welch's correction or Mann-Whitney T-test depending if the data was normally distributed. One sample t-tests were further subjected to Bonferroni sequential correction. Kruskal-Wallis non-parametric test with Dunn's multiple comparison test was used when comparing several sample groups.

5 Results and discussion

5.1 SHANK3 inhibits filopodia formation by sequestering active Rap1 through its N-terminal SPN domain and modulating integrin activation (I)

At the time when this study was initiated, very little was known about the function of SHANK3 outside the CNS. Importantly, our group had recently identified a novel function for SHANK1 and SHANK3 as inhibitors of integrins using X-ray protein crystallography. The structure revealed that the N-terminal SPN-domain of SHANK3 adopted a Ras-association domain (RA) like fold, suggesting that it could function as a novel interaction site for Rap1- and Ras-family small GTPases. Furthermore, our group showed that the SPN domain has a high affinity for active, guanosine triphosphate (GTP)-bound forms of Rap1 and Ras-family proteins, but it does not bind inactive, guanosine diphosphate (GDP)-bound variants (Lilja et al., 2017). In conclusion, SHANKs inhibit integrin function by sequestering the integrin activating GTP-bound Rap1 and R-Ras through its SPN-domain limiting their bioavailability at the plasma membrane. Interestingly, autism-linked SHANK3 patient mutations, R12C and L68P (Durand et al., 2007; Gauthier et al., 2010), are within the SHANK3 SPN domain, and we found that these mutations have also reduced ability to inhibit integrin activation (Lilja et al., 2017).

Migrating cells use filopodia to probe the environment, and maturation of filopodia tip adhesions to focal adhesions directs cell migration (Jacquemet et al., 2016, 2019). Importantly, filopodia formation requires Rap1/talin-mediated integrin activation axis (Jacquemet et al., 2016; Lagarrigue et al., 2015), but the role of integrin inactivators in filopodia regulation remains poorly defined. As we recently recognized SHANK1 and SHANK3 as integrin inhibitors and showed that they inhibit cancer cell migration, we wanted to characterise their role in filopodia formation. We focused on SHANK3, as it is widely expressed also outside the CNS (Lilja et al., 2017). Filopodia are dynamic structures, and their formation can be induced by multiple proteins. Here, we used expression of myosin 10 (Myo10) to promote filopodia formation in U2OS human osteosarcoma cells. U2OS cells have a low Myo10 expression as such, and they are thereby a good model for filopodia

induction (Young et al., 2018). In line with our hypothesis, cells co-expressing fluorescently tagged Myo10-mCherry and GFP SHANK3 had significantly lower number of Myo10-positive filopodia compared to cells co-expressing Myo10-mCherry and GFP control (I, Fig. 1B-C). Since we showed in our previous publication that the N-terminal SHANK3 SPN domain was responsible for integrin inhibition, we hypothesized that expression of the SPN domain alone would be sufficient to inhibit filopodia formation. Consistently, co-expression of GFP SPN and Myo10-mCherry reduced number of Myo10-positive filopodia significantly (I, Fig. 1D-E), but we were surprised to see that GFP SPN also overlapped with filamentous structures throughout the cell (I, Fig. 1D).

5.1.1 The SHANK3 SPN domain colocalises with actin and binds it directly

SHANK3 has been shown to interact with and to modulate function of many actin regulators (Figure 9., I, Fig. 2A), but there has been no reports of a direct interaction between SHANK3 and actin so far. Furthermore, to date the only identified binding partners of the very N-terminal SHANK3 SPN domain are limited to Ras family proteins and Rap1 (Lilja et al., 2017), and the knowledge of the SPN domain function remains scarce. We found, using immunofluorescence confocal microscopy, that GFP SPN colocalised with F-actin staining (I, Fig. 1D, F-G). Furthermore, in a GFP co-immunoprecipitation assay, GFP SPN pulled down β-actin from cell lysate indicating that the SPN domain associates with actin (I, Fig. 3F). However, colocalisation and co-immunoprecipitation assays can also detect indirect association as protein complexes. Therefore, to determine if the SPN domain interacts directly with actin, we employed actin co-sedimentation assay with recombinant SPN protein and purified F-actin. The actin co-sedimentation assay is based on centrifugation with speeds at which F-actin will form a pellet. Proteins that bind F-actin should thereby also be present in the pellet fraction after the centrifugation. We observed that recombinant SPN protein co-sedimented with F-actin and was present in the pellet, but when it was centrifuged without actin (negative control) it remained in the supernatant fraction confirming that SPN only localised to the pellet fraction when bound to F-actin (I, Fig. 2C-D, S2C). Taken together, these data indicate that the SPN domain binds actin directly.

5.1.2 Creation and validation of an actin-binding deficient SPN mutant

The X-ray crystal structure from our previous publication revealed that the SHANK3 SPN domain has a high similarity to F0 motif of talin four-point-one, ezrin, radixin,

moesin (FERM) domain (I, Fig. S3A) (Goult et al., 2010; Lilja et al., 2017). Talin F0 is similar to kindlin F0 domain (I, Fig. S3B) (Goult et al., 2010), and interestingly, kindlin-2 F0 domain has been reported to bind actin directly (Bledzka et al., 2016). Bledzka et al. mapped potential kindlin-2 F0 actin-binding sites, to surface residues L47 and K48 based on sequence similarity to other known ABPs, and showed that mutation of these residues into alanines substantially reduced actin binding (Bledzka et al., 2016). This inspired us to compare the SPN domain with kindlin-2 F0 domain to identify potential actin-binding residues. Sequence alignment and structure superimposition of the kindlin-2 F0 and the SHANK3 SPN domains indicated that Q37 and R38 would be the corresponding amino acids in the SPN domain (I, Fig. 3A-B, S3C). We introduced the Q37A/R38A point mutations into GFP SPN and GST SPN expression vectors, and studied how these point mutations affected interaction between the SPN domain and actin. In a GFP co-immunoprecipitation assay, GFP SPN Q37A/R38A pulled down less β-actin compared to GFP SPN WT (I, Fig. 3F). In line with this, GST SPN Q37A/R38A co-sedimented approximately 40 % less with F-actin compared to WT (I, Fig. 3G-H). Taken together, these data indicate that Q37A/R38A point mutation interferes with the SPN-actin-binding site such that the actin-binding is significantly reduced. However, it is likely that the full actin-binding site of the SHANK3 SPN domain involves also other amino acid residues, as the actin binding is not completely abolished. Nevertheless, the Q37A/R38A point mutant provides a valuable tool for studying effects of reduced interaction between the SHANK3 SPN domain and actin.

Consistent with the reduced actin binding, GFP SPN Q37A/R38A did not colocalise with actin when expressed in cells (I, Fig. 3C-E). As the SHANK3 SPN domain also sequesters active, integrin-activating Rap1, we wanted to investigate if previously characterized Rap1-binding deficient mutant R12C (Lilja et al., 2017) would have altered colocalisation with actin. However, we observed that GFP SPN R12C expressing cells had very similar phenotype with GFP SPN WT, and they both colocalised strongly with F-actin (I, Fig. 3C-E). These findings indicate that interaction between SPN and actin does not depend on interaction with active Rap1 and integrin inhibitory function.

5.1.3 The SPN domain binds actin directly, but it does not bundle actin filaments together

As we observed a strong colocalisation between GFP SPN WT and thick actin filaments (I, Fig. 3C-E), we speculated whether the SPN domain could bundle actin filaments together and promote thereby stress fiber formation. We performed a low-speed actin co-sedimentation assay in which actin only goes to pellet in bundled form. Our results show that while recombinant SPN protein and actin are present in

pellet after high-speed centrifugation (I, Fig. 2C), they both remain in the supernatant after a low-speed centrifugation (I, Fig. 2D). These results indicate that while SPN domain binds F-actin, it does not bundle actin filaments together. Furthermore, we did not observe that recombinant SPN protein would stabilize or de-stabilize actin filaments in spontaneous or cofilin-induced depolymerisation assay *in vitro* (I, Fig. S3H-I).

5.1.4 The role of SPN-actin interaction in integrin activity and focal adhesions

To confirm that the SPN Q37A/R38A mutant is only deficient in actin binding, and not in integrin inhibition, we employed a FACS-based integrin activity assay that measures the level of integrin activation relative to total cell surface β1-integrins. In our previous publication, we showed, using the same experimental setting, that expression of GFP SPN WT, but not R12C, inhibited integrin activity in CHO cells (Lilja et al., 2017). Interestingly, here we observed that while expression of GFP SPN WT reduced integrin activity, GFP SPN Q37A/R38A expressing cells had even lower integrin activity (I, Fig. 4F). Altogether, our data shows that Q37A/R38A point mutation only disrupts the actin binding, but retains the integrin inhibitory functions of the SHANK3 SPN domain. To study further how actin binding affects interaction between the SPN domain and active Rap1, we performed a modified actin co-sedimentation assay in presence of active, GTP-analogue-loaded Rap1. Interestingly, we observed that presence of active Rap1 inhibited interaction between the SPN domain and actin (I, Fig. 4G-H, S4G). Addition of active Rap1 reduced proportion of actin-bound recombinant SPN protein compared to control sample without Rap1 (I, Fig. 4G-H). Taken together, these data indicate that active Rap1 may compete with actin from binding the SPN domain and that disruption of actin binding may even enhance integrin inhibition due to increased availability towards sequestering active Rap1.

In previous work from our group, SHANK3 silencing was shown to increase number of nascent adhesions and to reduce average adhesion size (Lilja et al., 2017). Here, we observed that in addition to colocalising with actin stress fibers (I, Fig. 3C-D), GFP SPN WT-expressing cells had very large, vinculin-positive adhesions (I, Fig. 4A-B). Taken together, these data suggest that SHANK3 and its SPN domain may regulate focal adhesion size and maturation by sequestering active Rap1 and by altering thereby recruitment of talin to cytoplasmic tails of integrins. However, focal adhesions are also physically connected to actin stress fibers and adhesion size has been shown to be regulated by actomyosin contractility (Burridge and Guilluy, 2016; Chrzanowska-Wodnicka and Burridge, 1996). Thereby, we wanted to investigate how the distinct roles of the SHANK3 SPN domain in actin- and Rap1-binding affect

focal adhesions. To address this, we analysed the number and size of focal adhesions from cells expressing GFP control, GFP SPN WT, actin-binding deficient GFP SPN Q37A/R38A and Rap1-binding deficient GFP SPN R12C. Interestingly, only GFP SPN WT induced large focal adhesions, and both Q37A/R38A and R12C remained similar to our negative GFP control (I, Fig. 4A-C). The average cell area and adhesion count remained unchanged in all conditions (I, Fig. 4A, D-E). Taken together, these data indicate that GFP SPN WT interacts with both actin and active Rap1 to modulate focal adhesion size and maturation.

5.1.5 Interaction between the SPN domain and actin is regulated by the SPN-ARR fold

Our data thus far indicates that the SPN domain interacts directly with actin. However, imaging of longer SHANK3 fragments (with additional SHANK-domains) revealed that the presence of the adjacent ARR-domain seemed to interfere with the SPN–actin-colocalisation (I, Fig. 2B). The two first N-terminal SHANK3 domains, SPN and ARR, interact with each other through intramolecular interactions (Mameza et al., 2013; Lilja et al., 2017). In addition, this interaction has been shown to inhibit SHARPIN and α-fodrin from binding to the SHANK3 ARR domain (Mameza et al., 2013). Importantly, this suggests that some of the SPN actin-binding surface may be sandwiched between the two domains in longer SHANK3 fragments. Therefore, we performed actin co-sedimentation assay with both recombinant SPN and SPN-ARR proteins, and observed that while recombinant SPN bound to F-actin and was present in the pellet fraction (I, Fig. 2C-D, S2C), SPN-ARR remained in the supernatant (I, Fig. 5A, S5C-D) indicating that the SPN-ARR fold interferes with the putative actin-binding site.

We used the available X-ray crystallography data (Cai et al., 2019; Lilja et al., 2017) to predict potential ways to trigger opening of the SPN-ARR fold. Importantly, we identified N52 as potentially critical amino acid residue in the interface between the two domains (I, Fig. 5B-C), and mutated it into positively charged arginine to reveal additional interaction surfaces at the SPN-ARR interface. Moreover, our collaborators performed molecular simulations to detect if N52R point mutation would induce conformational changes in the SPN-ARR fold. Interestingly, they showed that charge repulsion introduced by the N52R mutation would induce opening of the fold, which in the WT protein remained closed in their tested time scales (I, Fig. 5D-F). We introduced the N52R point mutation into recombinant SPN-ARR protein, and strikingly, approximately 90 % of SPN-ARR N52R bound to F-actin in co-sedimentation assay, while WT did not bind actin (I, Fig. 5I-J). Furthermore, when expressed in cells, SPN-ARR N52R-mRFP colocalised very strongly with F-actin in microscopy experiments, while most of the SPN-ARR WT

localised diffusely in the cytoplasm (I, Fig. 5G-H). These data validate our hypothesis that the SPN-ARR fold interferes with actin binding, and the fold opening dynamically regulates interaction between the SPN domain and actin.

5.1.6 The role of SHANK3-actin binding in filopodia formation

While GFP SPN WT colocalises with F-actin in cells, the full-length GFP SHANK3 is present throughout the cytoplasm with a slight intensification at the plasma membrane (I, Fig. 2B). We introduced the actin-binding disrupting Q37A/R38A and actin-binding promoting N52R point mutations into full-length GFP SHANK3 to study if altered actin binding would also affect SHANK3 localisation in cells. Both GFP SHANK3 WT and the Q37A/R38A mutant localised similarly throughout the cell (I, Fig. 6A-B), whereas N52R mutant had a strikingly different localisation (I, Fig. 6A). GFP SHANK3 N52R was heavily concentrated in the cell center and almost absent from the cell periphery and cell edges. Furthermore, GFP SHANK3 N52R overlapped with F-actin staining in the center of the cell (I, Fig. 6A). This suggests that the N52R point mutation forces the SPN-ARR fold to stay open promoting interaction between full-length SHANK3 and F-actin.

Filopodia are regulated by actin dynamics, bundling and polymerisation as well as integrin activity. As we observed that interaction with both actin and active Rap1 was required for regulation of focal adhesions (I, Fig. 4A-E), we wanted to investigate whether that would also have a role in regulation of filopodia formation. Intriguingly, we observed that Rap1-binding deficient R12C and, based on our findings with the SPN and ARR domains, actin-binding deficient Q37A/R38A and actin-binding dominant active N52R were all unable to inhibit formation of Myo10-positive filopodia (I, Fig. 6E-F). Importantly, only GFP SHANK3 WT inhibited filopodia formation when co-expressed with Myo10-mCherry (I, Fig. 6E-F). Taken together, these data indicate that SHANK3 needs to be able to interact dynamically with both actin and active Rap1 to inhibit filopodia formation.

5.1.7 Discussion

In this study, we describe a novel direct interaction between SHANK3 and actin. In addition, we mapped that interaction between SHANK3 and actin occurs through the less-studied N-terminal SHANK3 SPN domain and showed that interaction between the SPN domain and actin is dynamically regulated by SPN-ARR fold opening. Importantly, we show that while the binding sites of actin and the other known SPN interactor active Rap1 are distinct, the SHANK3 SPN domain may still need to interact with both of them in some cellular contexts to regulate for example adhesion

size and filopodia formation. This suggests that the actin binding may be a scaffolding function critically enabling SHANK3 to coordinate distinct activities in cells.

In our groups previous work, we showed that SHANK3 inhibited cancer cell migration and invasion through sequestering active Rap1 and limiting thereby integrin activation (Lilja et al. 2017). Importantly, filopodia promote cancer cell migration and invasion (Jacquemet, Hamidi, and Ivaska 2015), and number of filopodia has been shown to be regulated by integrin activity (Jacquemet et al., 2016). Moreover, expression of integrin activating talin and constitutively active Rap1 promote filopodia formation (Jacquemet et al., 2016; Lagarrigue et al., 2015). Consistently, cells plated on anti-β1 integrin antibodies, which lock integrins either into active or inactive conformation, exhibited high number of Myo10-positive filopodia when integrins were activated, and a low number when integrins were kept inactive (Jacquemet et al., 2016). Taken together, observations from our group and others highlight the role of integrin-induced filopodia formation in cancer and cell migration.

Here I addressed if expression of integrin-inhibiting SHANK3 regulated filopodia formation. As expected, full-length SHANK3 and the active Rap1-sequestering SPN domain alone reduced the number of Myo10-positive filopodia. However, I also discovered that the SPN domain binds actin directly and addressed if it contributed to inhibiton of filopodia formation. My data indicate that while both actin- and Rap1-binding sites are located in the SPN domain, they are distinct and disrupting actin binding does not prevent sequestration of Rap1, and *vice versa*. My flow cytometry assays indicated that the actin-binding deficient GFP SPN Q37A/R38A inhibited integrins even more efficiently than SPN WT, which lead me to hypothesise that full-length GFP SHANK3 Q37A/R38A would be a very efficient inhibitor of filopodia formation as well. However, this was not the case, as GFP SHANK3 Q37A/R38A did not reduce the number of filopodia when compared to control. My data suggests that SHANK3 needs to interact with both actin- and Rap1 to inhibit filopodia formation. Currently, it is not clear why this is the case. We do not detect WT SHANK3 protein localising to filopodia tips (Guillaume Jacquemet, personal communication), suggesting that it might not act directly in filopodia to limit active Rap1 availability, but would rather function in the cell body affecting the overall pool of active Rap1. In fact, when we compared how GFP SHANK3 WT, actin-binding deficient Q37A/R38A, actin-binding dominant active N52R and Rap1-binding deficient R12C regulated Myo10-positive filopodia, only WT inhibited filopodia formation. Importantly, these findings suggest that dynamic interaction between SHANK3 and actin may be necessary in facilitating other SHANK3 functions. However, it is important to recognize that the SPN domain alone cannot be used to replace in the full-length SHANK3 in functional studies, and we have

only confirmed changes in SPN-actin binding in shorter SHANK3 fragments. Studying actin binding of the full-length SHANK3 is complicated by the fact that it is unlikely to be similarly folded when expressed in *Escherichia coli*, and when expressed in mammalian cells, other actin-related SHANK3 interactors complicate the result interpretation. While the SPN domain alone is a valuable tool in characterizing molecular interactions, its functions may differ in full-length SHANK3 where other functional domains and their interactors also affect the protein function.

Although we focus on the role of integrin activity in regulation of filopodia formation, the possibility that SHANK3 regulates filopodia formation through its other actin-related binding partners remains unexplored. In neurons, SHANK3 has been shown to bind to and associate with many proteins that are known as filopodia regulators outside the CNS. For example, the SHANK3 PDZ domain binds β-PIX (Park et al., 2003), which is a guanosine exchange factor (GEF) for filopodia-promoting GTPases Cdc42 and Rac1. Furthermore, the SHANK3 PP region binds IRSp53 (Bockmann et al., 2002), which also promotes filopodia formation through the Arp2/3 complex and as a Cdc42 effector (Lim et al., 2008). However, as all of the above mentioned SHANK3 interactors are known to promote filopodia formation in non-neuronal cells rather than inhibit it, and my data shows that the Rap1-sequestering SPN domain alone is sufficient to inhibit filopodia formation, I hypothesise that the inhibitory effect I observed in my experiments, is due to reduced integrin activity.

Firstly, it is important to consider the differences between cell types and developmental stages. In neurons, SHANK3 has been identified as an important scaffold protein for multiple actin regulators and it is considered to recruit them to the PSD and growth cones promoting cytoskeletal development (Bockmann et al., 2002; Durand et al., 2012; Lim et al., 2008; Naisbitt et al., 1999; Park et al., 2003; Sarowar and Grabrucker, 2016; Wang et al., 2019). Importantly, the cellular context is thereby very different from cancer cells that I have used in my studies. For example, cortactin, which binds to the SHANK3 PP region (Naisbitt et al., 1999), has been shown to stabilize filopodia in neuronal growth cones (Yamada et al., 2013), but in cancer cells it promotes invadopodia formation (Linder et al., 2011). Importantly, there is a lack of studies comparing the role of interaction between SHANK3 and its actin-related binding partners in the CNS and in non-neuronal cells.

Secondly, our data suggests that active Rap1 and actin may compete with each other from binding the SPN domain. Our *in vitro* data suggests that presence of active Rap1 reduces interaction between purified F-actin and recombinant SPN protein. So far, this has only been addressed in the context of the SPN domain alone (instead of longer SHANK3 fragments) and with purified proteins, which is a very simplistic set-up compared to all factors that are present in the cytoplasm of

a cell, such as the other actin regulators SHANK3 is known to interact with. However, these data lead me to hypothesise that the SPN domain may regulate whether SHANK3 is destined to promote actin polymerization and actin remodelling, or if it focuses on regulating bioavailability of active GTPases and integrin activity. I hypothesise that when the SPN domain is bound to F-actin, SHANK3 may regulate organisation of the actin cytoskeleton by bringing several actin regulators together or in close proximity of each other. The assumption that SPN-actin binding has a role in scaffolding is also supported by the fact that we did not observe SPN-actin interaction to modify the actin itself. We did not observe that SPN domain alone would have bundled actin, induced actin polymerization or affected actin stability. In turn, when the SPN domain is bound to active Rap1, interaction with F-actin and other actin regulators may be suppressed. Intriguiginly, excess of active Rap1 has been shown to lead to long and thin dendritic spines in neurons (McAvoy et al., 2009; Pak et al., 2001), where as SHANK3 has been shown to do the opposite: to promote spine maturation and spine head formation through regulation of the actin cytoskeleton (Durand et al., 2012; Sarowar and Grabrucker, 2016). Therefore, I hypothesise that SHANK3 may act as a counterbalance for increased Rap1 activity by sequestering it, bringing it to close contact with Rap1-inactivating GTPase activating proteins (GAPs), such as spine-associated RapGAP SPAR, that binds to the SHANK3 PDZ domain (Spilker et al., 2008). Once active Rap1 is no longer abundant, SHANK3 may continue to recruit and scaffold actin regulators promoting spine head enlargement and maturation. Taken together, the SPN domain may act as a major switch regulating which signalling events and cellular processes SHANK3 participates.

In this study, we also demonstrated with a proof-of-concept point mutant N52R, that SPN-ARR fold opening regulates the SPN-actin interaction. However, the exact physiological signal that triggers the fold opening remains to be discovered. Post-translational modifications, such as phosphorylation, occurring in the close proximity of the interface could potentially induce the fold opening. Importantly, phosphorylation of SHANK3 S685, which is located in the SHANK3 PP domain, has been shown to regulate binding of Abi1 and recruitment of the WAVE complex to the PSD promoting thereby actin polymerisation. Furthermore, S685I has been identified as an ASD-linked patient mutation highlighting importance of both post-translational modifications in SHANK3 function and the importance of SHANK3 mediated actin re-organisation (Wang et al., 2019). However, the only so far reported phosphorylation site in the N-terminal SHANK3 identified in several phosphoproteome screens is Y122 (Phosphosite.org database & (Wang et al. 2019)). As Y122 is not localised close to the SPN-ARR interface, we did not see it as an obvious candidate for triggering the SPN-ARR fold opening. However, the possible role of phosphorylation and other post-translational

modifications as regulators of the SPN-ARR fold remains to be studied. Furthermore, the role of N-terminal SHANK3 patient mutations (Table 1.) in the SPN-ARR fold opening and actin binding remains to be investigated. Here, I found that R12C does not disrupt interaction with actin, and we know based on previous observations that another autism-linked patient mutation, L68P, disrupts the SPN-ARR fold severely making it non-functional (Lilja et al. 2017; Mameza et al. 2013). Altogether, our data indicate that dynamic interaction between SPN and actin is essential, but the physiological signalling events that trigger the fold opening are yet to be identified.

Taken together, this study describes a novel, direct interaction between the SHANK3 SPN domain and F-actin. Intriguingly, we demonstrate that the actin-binding site is cryptic and its availability is regulated by the SPN-ARR fold opening. Furthermore, our data indicates that the SPN domain may serve as an interaction hub, that determines whether SHANK3 participates in Rap1 and integrin signalling or in remodelling of the actin cytoskeleton. Importantly, our data reveals new information of SHANK3 function that may create a platform for further investigations of the role of SHANK3 in cancer cell migration and dendritic spine morphogenesis. Importantly, understanding how interaction between the SHANK3 SPN domain and actin and/or active Rap1 and discovering ways how to control it may lead to identification of novel therapeutic targets in both cancer and neurological disorders.

5.2 Identification of novel SHARPIN interactors (II)

SHARPIN has been shown to bind multiple proteins, such as SHANKs (Lim et al., 2001), the LUBAC complex (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011), caspase-1 (Nastase et al., 2016) and PTEN (He et al., 2010) (Figure 10.), and it is thought to mediate and modulate their function as an adaptor protein. Importantly, our group also identified SHARPIN as an integrin inhibitor (Rantala et al., 2011). However, we are far from complete understanding of SHARPIN-mediated signalling. With this project, we set out to further characterize SHARPIN function and identify new SHARPIN interactors. Therefore, our collaborators performed a mass spectrometry screen to identify SHARPIN-linked cellular processes and potential novel interactors of SHARPIN. In order to identify especially proteins that would interact with SHARPIN in an integrin-dependent manner, our collaborators performed the screen with both GFP SHARPIN-expressing cells in suspension (without engaging integrins to the ECM) and with adherent cells plated on fibronectin. However, there was no major differences between the data sets from cells in suspension or adherent cells (II, Fig. S1A-B, Table S1). Importantly, the mass spectrometry screen identified known SHARPIN interactors, such as the

LUBAC complex members HOIP (*RNF31*) and HOIL-L1 (*RBCK1*), validating the quality of the screen (II, Fig. S1C). The identified hits were further classified based on confidence of the hit, and furthermore, the hits were divided into clusters based on gene ontology and biological processes they associate with (II, Fig. 1A, Table S2) (Huang et al., 2009; Merico et al., 2010). The gene ontology analyses identified SHARPIN to associate with many cytoskeletal regulators (II, Fig. 1B-C, Table S3). Importantly, two members, ArpC2 and ArpC5, of the seven subunit Arp2/3 complex were amongst the identified hits (II, Fig. 1C). Encouraged by presence of several Arp2/3 subunits in the list, we decided to confirm interaction between SHARPIN and the Arp2/3 complex to validate our mass spectrometry-based SHARPIN interactome.

5.2.1 Establishment of interaction between SHARPIN and the Arp2/3 complex

After confirming the interaction between SHARPIN and the Arp2/3 complex with different techniques, such as proximity ligation assay (II, Fig. 2A, S2A-B) and co-immunoprecipitation assays with both cell extracts (II, Fig. 2B-C) and recombinant or purified proteins (II, Fig. 2F, S2F), we characterised which domain of SHARPIN would be the interaction site. SHARPIN has three functional domains, the N-terminal PH domain, the central UBL domain and the C-terminal NZF domain (Figure 10.). Importantly, our group's earlier work identified the UBL domain as an interaction site for integrin α-tail and the LUBAC complex. Furthermore, the binding sites were shown to be partially overlapping, but mutually exclusive (De Franceschi et al., 2015). We used fluorescence resonance energy transfer (FRET) combined with fluorescence lifetime imaging microscopy (FLIM) and GFP co-immunprecipitation assay to map interaction between SHARPIN and the Arp2/3 complex to occur through the UBL domain (II, Fig. 3A-B). In our previous work, we modelled potential binding surfaces in the UBL domain that were evolutionary conserved in similarly folded proteins, and created six single or double point mutants by replacing identified amino acids with alanines (De Franceschi et al., 2015). We used FRET-FLIM to study energy transfer between GFP SHARPIN WT and mutant constructs and Arp3-mRFP, and detected that three of our GFP SHARPIN mutants, V240A/L242A, V267A and L276A, exhibited reduced interaction with Arp3-mRFP (II, Fig. 3C). As we found in our groups earlier work that V267A and L276A had also altered interaction with α-integrins and the LUBAC complex, we selected V240A/L242A as our tool to study specifically the role of SHARPIN-Arp2/3 interaction.

5.2.2 SHARPIN localisation to lamellipodia is not specific, but secondary to increased cytoplasm in membrane ruffles

Once we had confirmed the interaction between SHARPIN and the Arp2/3 complex, we employed different microscopy techniques to study how interaction between SHARPIN and the Arp2/3 complex affects cell morphology and cytoskeletal structures (II, Fig. 4A-C). Furthermore, we wanted to study if SHARPIN and the Arp2/3 complex colocalise in these structures (II, Fig. S4A-C). The Arp2/3 has a pivotal role in catalysing formation of branched actin network at the leading edge of the cell and it has been shown to localise in lamellipodia (Suraneni et al., 2012; Welch et al., 1997). Interestingly, SHARPIN has also been reported to localise at the cell edge (Rantala et al., 2011). To study this further, we performed live-cell imaging with GFP SHARPIN-expressing NCI-H460 non-small lung adenocarcinoma cells. We observed that GFP SHARPIN-expressing cells had typically multiple lamellipodia-like ruffles, and GFP-SHARPIN localised especially in these structures (II, Movie S1). We also imaged SHARPIN- and Arp3-silenced (disrupts the entire Arp2/3 complex) cells expressing mEmerald-Lifeact (to visualize actin) with total internal reflection fluorescence microscopy (TIRF) to determine their contribution to actin dynamics (II, Movie S2-4). Importantly, while control silenced cells were polarized and they had typically multiple, lamellipodia-like structures, both SHARPIN- and Arp3-silenced cells had a round shape and they lacked lamellipodia. Furthermore, both SHARPIN- and Arp3-silenced cells exhibited more filopodia, which has been observed earlier in response to Arp2/3 downregulation (II, Movie S2-4) (Beli et al., 2008; Wu et al., 2012).

We observed that both endogenous SHARPIN and Arp2 localised at lamellipodia-like structures and at the leading edge when stained from fixed HeLa cervical cancer cells and NCI-H460 cells (II, Fig. S4A, C). However, our further analysis indicated that SHARPIN localisation at the leading edge may not be specific to the Arp2/3 complex. We employed line scans to plot SHARPIN, Arp2 and cytoplasmic protein p65 (an irrelevant protein for lamellipodia formation used as a negative control) intensities across the lamellipodia. These revealed that SHARPIN and our negative control p65 had similar intensity plots suggesting that the intensity peak at the leading edge might be unspecific (II, Fig. S4C-D). Furthermore, we did not observe SHARPIN staining at the leading edge of U2OS cells, whereas Arp2 peaked strongly at their lamellipodia (II, Fig. S4E). Taken together, these findings raised a question whether SHARPIN localisation at the leading edge would be due to cell shape and thickness of the cytoplasm. For example, NCI-H460 cells make quite thick ruffles at the leading edge, whereas U2OS cells are very flat and they have only a little cytoplasmic thickening at lamellipodia. To study further if SHARPIN was recruited to lamellipodia primarily together with the Arp2/3 complex

or if it localises there due to cytoplasmic thickening, we employed fluorescence recovery after photobleaching (FRAP) live-cell imaging. Importantly, the Arp2/3 complex members have been shown to recover at lamellipodia after fluorescent photobleaching starting from the cell edge, in a similar manner with retrograde actin treadmilling (Lai et al., 2008). We bleached similar rectangular areas across lamellipodia of NCI-H460 cells that had thick ruffles. As we anticipated, we could not observe any particular direction in the recovery of GFP SHARPIN, but it recovered very rapidly and diffusely (II, Fig. S4F). Taken together, these data indicate that SHARPIN localisation at the leading edge may be dependent on other factors, such as amount of cytoplasmic thickening at the leading edge, rather than association with the Arp2/3 complex.

5.2.3 SHARPIN supports Arp2/3-mediated lamellipodia formation

As we observed, with live-cell imaging, that SHARPIN-silenced cells were similar to Arp3-silenced cells, and they both lacked lamellipodia-like structures (II, Movie S3-4), we wanted to study further if SHARPIN promoted Arp2/3-mediated lamellipodia formation. To address this, we quantified F-actin- and cortactin-positive lamellipodia from control-, SHARPIN- and Arp3-silenced NCI-H460 cells. Similarly to what we observed with live-cell imaging, both SHARPIN- and Arp3-silenced cells were mostly round and they had significantly reduced number of lamellipodia-like ruffles (II, Fig. 4A, S5A, D). Furthermore, we quantified lamellipodia from HOIP-silenced cells to distinguish whether SHARPIN promotes lamellipodia formation specifically due to interaction with the Arp2/3 complex, or do also SHARPINs other roles, such as the LUBAC complex, modulate lamellipodia formation. Importantly, HOIP-silencing did not inhibit lamellipodia formation suggesting that lack of lamellipodia, caused by downregulation of SHARPIN, would be due to interaction with the Arp2/3 complex (II, Fig. 4A). Furthermore, we established two SHARPIN KO cell lines from single cell clones using CRISPR. Consistently, these SHARPIN KO cell lines exhibited significantly reduced number of lamellipodia (II, Fig. 4B, S5C). Importantly, we were able to rescue lamellipodia formation in SHARPIN-silenced and SHARPIN KO NCI-H460 cell lines by re-expressing GFP SHARPIN WT (II, Fig. 5A-B). Furthermore, we showed that our previously identified LUBAC-binding mutants (De Franceschi et al., 2015) rescued the lamellipodia formation similarly to GFP SHARPIN WT, but the Arp2/3-binding deficient GFP SHARPIN V240A/L242A did not induce lamellipodia formation in SHARPIN-depleted cells (II, Fig. 5A-B). Altogether, our findings indicate that SHARPIN promotes Arp2/3-mediated lamellipodia formation indepent of the LUBAC complex.

5.2.4 The role of SHARPIN in lamellipodia-driven cell migration

The Arp2/3 complex has an important role in lamellipodial migration, and Arp2/3-depleted cells lack a strong leading edge and migrate inefficiently (Suraneni et al., 2012; Wu et al., 2012). Thereby, we performed a wound healing assay with silenced HeLa cells. However, while Arp3-silenced samples had significantly wider wounds 36 h post wounding, SHARPIN silenced samples exhibited similar wound healing speed with control silenced samples (II, Fig. S6C). This suggests that lack of SHARPIN may promote wound healing through altering function of its other binding partners, such as through increased integrin activity. To address how interaction between SHARPIN and the Arp2/3 complex specifically modulates cell migration, we performed a random migration assay where we rescued SHARPIN null mouse embryonic fibroblasts isolated from *cpdm* mice (*cpdm* MEFs) with re-expression of GFP control, GFP SHARPIN WT and the Arp2/3-binding deficient GFP SHARPIN V240A/L242A. We tracked random migration of single cells overnight, and showed that expression of both GFP SHARPIN WT and V240A/L242A promoted relative migration speed compared to cells expressing GFP control. However, rescue with GFP SHARPIN WT was slightly more efficient than rescue with V240A/L242A (II, Fig. 6A-B). Taken together, these observations indicate that SHARPIN modulates cell migration, but it may be only partially mediated by Arp2/3-driven lamellipodia formation.

5.2.5 Discussion

Since its original discovery (Lim et al., 2001), the role of SHARPIN has been mostly studied in the context of NF-κβ-signalling and the LUBAC complex (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). However, SHARPIN has also been identified as a binding partner for various other proteins (He et al., 2010; Nastase et al., 2016; Park et al., 2016; Rantala et al., 2011), that do not appear to be connected with the LUBAC complex, or each other. Our study contributes to this list by identifying multiple potential SHARPIN interactors. Furthermore, our data indicates that SHARPIN interacts with the Arp2/3 complex and influences Arp2/3-driven processes in cells without any obvious links to other established SHARPIN functions.

We observed with different experiments and in different cell lines that SHARPIN promotes lamellipodia formation. However, the exact mechanism how SHARPIN promotes Arp2/3-mediated lamellipodia formation remains unclear. Our data indicates that downregulation of SHARPIN abolishes lamellipodia formation similarly to Arp3 silencing (Suraneni et al., 2012; Wu et al., 2012). In addition, the Arp2/3-binding deficient GFP SHARPIN V240/L242A could not rescue

lamellipodia formation, in contrast to GFP SHARPIN WT. Furthermore, silencing of SHARPINs other binding partners, such as HOIP, did not affect the lamellipodia formation. Taken together, these data indicate that SHARPIN promotes lamellipodia formation in an Arp2/3-dependent manner. However, while we showed that recombinant SHARPIN protein binds the purified Arp2/3 complex, we did not observe that recombinant SHARPIN would promote Arp2/3-mediated actin polymerization in a gold-standard pyrene polymerization assay (II, Fig. 2G). This suggests that SHARPIN may need additional factors to promote Arp2/3 activation, or that SHARPIN may promote lamellipodia formation by bringing the Arp2/3 complex together with another protein that promotes Arp2/3 function.

Interestingly, our SHARPIN interactome identified also other Arp2/3-related proteins, such as Arpin (Dang et al., 2013; Gorelik and Gautreau, 2015), growth factor receptor-bound protein 2 (Grb2) (Giubellino et al., 2008), and Rho GTPases Cdc42 and Rac1 (Ridley, 2011) as potential SHARPIN interactors. This suggests that SHARPIN could promote Arp2/3-mediated lamellipodia formation through bringing Arp2/3 activating factors in contact with the Arp2/3 complex, or by sequestering Arp2/3 inhibiting factors. Furthermore, my data shows that SHARPIN-silenced cells have a slow response to serum-stimulated lamellipodia formation (II, Fig. 4C), indicating that SHARPIN could indeed promote growth factor-mediated response of the Arp2/3 complex. However, the possibility that SHARPIN promotes Arp2/3 activation by recruiting other factors awaits to be investigated.

In addition, SHARPIN has an important role in ubiquitin signalling, as it is known to bind ubiquitin through its C-terminal NZF domain (Ikeda et al., 2011), and participate in the LUBAC E3 ubiquitin ligase complex through its UBL domain (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). The potential role of SHARPIN in other E3 ligase complexes has not been studied while there are indications that it could also promote ubiquitinylation of its binding partners independent of the LUBAC complex (De Melo et al., 2014). However, it is not clear whether SHARPIN-mediated ubiquitinylation has a role in the lamellipodia formation, and there are no reports that would describe the LUBAC complex members localising at lamellipodia. Studies describing the role of ubiquitinylation in regulation of the actin cytoskeleton remain scarce, but interestingly, Arp2/3 activators WAVE1 (Jessick et al., 2013) and WAVE2 (Huang et al., 2009) have been shown to be regulated by ubiquitinylation linking ubiquitin signalling to actin polymerization. Furthermore, E3 ubiquitin ligase complexes have been shown to regulate endosomal actin assembly (Hao and Potts, 2014) highlighting the role of ubiquitinylation and different E3 ubiquitin ligase complexes in cytoskeletal regulation. However, both the role of SHARPIN in other E3 ligase complexes and the role of E3 ligases in regulation of the actin cytoskeleton await for further characterization. Another possible theory how SHARPIN could promote Arp2/3

function is by increasing its stability, as we observed that also Arp3 protein levels were slightly reduced in SHARPIN-silenced cells.

We were not able to fully decipher how SHARPIN-Arp2/3 interaction regulates cell migration. Importantly, addressing the role of SHARPIN in cell migration is greatly complicated by the fact that it also regulates cell migration through regulation of integrin activity (Pouwels et al., 2013; Rantala et al., 2011). I observed that while Arp3-silencing expectedly reduced wound closure in scratch wound assay, SHARPIN-silenced cells healed the wound similarly to control cells. This is in line with reports showing that silencing of SHARPIN (Rantala et al., 2011) and another integrin inhibitors SHANK1 and SHANK3 promotes migration velocity (Lilja et al., 2017). Importantly, our data suggests that integrin- and Arp2/3-related SHARPIN functions would be distinct. Treatment with an Arp2/3-inhibitor CK666 (Nolen et al., 2009) abolished interaction between SHARPIN and Arp2, but did not interfere with SHARPIN-α2-integrin interaction in a PLA assay (II, Fig. S3A-B). Furhtermore, CK666 treatment did not reduce activity of cell surface β1-integrins in a FACS assay (II, Fig. S3E). One possible way to study the role of SHARPIN-Arp2/3 in migration would be to balance integrin activation levels in SHARPIN-silenced and control cells by plating them on poly-L-lysine coating that does not promote integrin-mediated signalling, or on antibodies that lock integrins either in active or inactive conformation (Jacquemet et al., 2016). Importantly, actin polymerisation and adhesion assembly are highly interdependent in migrating cells, and thereby it is challenging – and possibly even physiologically less important – to investigate them exclusively in case of SHARPIN.

Taken together, our study highlights that SHARPIN has a role as an adaptor protein in multiple different cellular contexts and SHARPIN function is not limited to integrin- and LUBAC-mediated signalling. Moreover, we identify a long list novel potential SHARPIN interactors, and characterise how SHARPIN promotes lamellipodia formation through the Arp2/3 complex independent of regulation of integrin activity and LUBAC function. Importantly, our SHARPIN interactome opens a door for multiple other investigations regarding the role of SHARPIN in different biological processes.

5.3 Investigation of reasons and outcomes of increased integrin activity in the epidermis of SHARPIN null *cpdm* mice (III)

Earlier work from our group identified SHARPIN as an integrin inhibitor. Importantly, SHARPIN binds to integrin α-tail preventing thereby recruitment of integrin activators to integrin β-tail (Rantala et al., 2011). Spontaneous SHARPIN null *cpdm* mice (*Sharpin*$^{cpdm/cpdm}$) have been identified earlier, and importantly, they

suffer from chronic proliferative dermatitis, hence the abbreviation *cpdm* (Seymour et al., 2007). In our previous work, we observed that active β1-integrin levels were elevated in skin sections from *Sharpin*$^{cpdm/cpdm}$ mice compared to WT samples (Rantala et al., 2011). Our findings are in line with report showing that integrin β1-overexpression leads to psoriasis-like skin phenotype in mice (Carroll et al., 1995). However, the increased overall inflammation and epidermal thickening due to keratinocyte proliferation has also been linked to SHARPIN's role in the NF-κβ-signalling mediating LUBAC complex and sensitization of cells to TNF-induced cell death and inflammation (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). Therefore, the role of increased integrin activity in the *cpdm* skin phenotype has remained ambiguous.

5.3.1 Integrin activity is elevated also in epidermis of *Tnfr1*$^{-/-}$ *Sharpin*$^{cpdm/cpdm}$ mice

To address, whether the increased integrin activity in *Sharpin*$^{cpdm/cpdm}$ epidermis is caused by SHARPIN depletion or if it is secondary to augmented TNF-induced cell death and inflammation, we used a TNF-receptor (TNFR) null *Tnfr1*$^{-/-}$ *Sharpin*$^{cpdm/cpdm}$ double knockout (KO) mouse strain. *Tnfr1*$^{-/-}$ *Sharpin*$^{cpdm/cpdm}$ mice do not suffer from proliferative dermatitis and they lack most of the systemic inflammation phenotypes (Kumari et al., 2014; Rickard et al., 2014). In line with earlier observations, we found that the skin of *Tnfr1*$^{+/+}$ *Sharpin*$^{cpdm/cpdm}$ mice was significantly thicker, indicating inflammatory hyperproliferation (Kumari et al., 2014; Rickard et al., 2014). Furthermore, the active β1-integrin staining (9EG7) was also suprabasally present in the epidermis (III, Fig. 1A) (Rantala et al., 2011) while in healthy tissues it should be confined to the basal keratinocyte layer (Hotchin et al., 1993, 1995; Watt, 2002). In contrast, skin sections from *Tnfr1*$^{-/-}$ *Sharpin*$^{cpdm/cpdm}$ mice were of normal thickness, healthy looking and the active β1-staining was limited to the basal layer (III, Fig. 1A). Moreover, we confirmed also with keratin 14- (a keratinocyte marker) and integrin α6-staining that integrins were only expressed in the basal layer of *Tnfr1*$^{-/-}$ *Sharpin*$^{cpdm/cpdm}$ epidermis (III, Fig. 1B).

We used a FACS-based assay to further analyse the integrin activation specifically in keratinocytes. We isolated cells from the skin and gated out all other residual cell types, such as endothelial cells (positive for CD31, Platelet And Endothelial Cell Adhesion Molecule 1 (PECAM1)) or leukocytes (positive for CD45, Protein Tyrosine Phosphatase, Receptor Type C (PTPRC)), and included only cells positive for α6-integrin (CD49f) labelling (III, Fig. 1C). The α6-integrin-positive cells were further analysed for active (9EG7) and total (HMβ1–1) β1-integrin staining. Importantly, our data indicates that integrin activity was significantly increased in *Tnfr1*$^{-/-}$ *Sharpin*$^{cpdm/cpdm}$ keratinocytes compared to

keratinocytes isolated from *Tnfr1*$^{-/-}$ *Sharpin*$^{+/?}$ mice. As expected, *Tnfr1*$^{+/?}$ *Sharpin*$^{cpdm/cpdm}$ keratinocytes exhibited even higher integrin activity compared to cells from double KO mice (III, Fig. 1D). In addition, the total integrin β1 levels were slightly, but not significantly, increased in *Tnfr1*$^{+/+}$ *Sharpin*$^{cpdm/cpdm}$ mice (III, Fig. 1E). Altogether, these data indicate that lack of SHARPIN increases integrin activity in epidermal keratinocytes even in absence of inflammation (such as in *Tnfr1*$^{-/-}$ *Sharpin*$^{cpdm/cpdm}$ mice), but integrin activity increases also secondary to inflammation (such as in *Tnfr1*$^{+/+}$ *Sharpin*$^{cpdm/cpdm}$ mice).

5.3.2 Integrin β1 function-blocking antibody treatment reduces hyperproliferation of *Tnfr1*$^{+/?}$ *Sharpin*$^{cpdm/cpdm}$ cells

As integrin function-blocking therapies have been reported to ameliorate psoriasis-like hyperproliferative dermatitis (Conrad et al., 2007; Ley et al., 2016), we tested if integrin β1 function-blocking antibody treatment would attenuate the *Sharpin*$^{cpdm/cpdm}$ skin phenotype. However, while the antibody treatment reduced epidermal thickening and hyperproliferation (III. Fig. 2A-B), it did not reduce infiltration on inflammatory cells in the *Sharpin*$^{cpdm/cpdm}$ skin (III. Fig. 3A-F). In addition, we developed a method to isolate primary keratinocytes from mouse skin for cell culture. The purity of the isolated population was controlled by western blot and staining for keratin-14 and vimentin (a fibroblast marker) (III, Fig. 2F, S1E). In line with our other observations, treatment with integrin β1 function-blocking antibody reduced relative keratinocyte proliferation also in isolated cells (III, Fig. 2G-H). However, the treatment reduced proliferation also in *Sharpin*$^{+/?}$ control cells (III, Fig. 2H). Taken together, these data indicate that integrin activity is increased in *Tnfr1*$^{-/-}$ *Sharpin*$^{cpdm/cpdm}$ skin even in absence of inflammation, and that inhibition of integrin activity partly rescues the epidermal hyperproliferation phenotype in *Sharpin*$^{cpdm/cpdm}$ skin and in isolated keratinocytes. Moreover, as treatment with β1 function-blocking antibody inhibits the hyperproliferation, but does not reduce infiltration of inflammatory cells in *Sharpin*$^{cpdm/cpdm}$ skin, the chronic inflammation phenotype is more likely due to LUBAC function than integrin activity.

5.3.3 Discussion

As a multifunctional adaptor protein, SHARPIN is known to bind directly and modulate the function of many other proteins. The most well-established interaction partners of SHARPIN are integrins (Gao et al., 2019; Rantala et al., 2011; Kasirer-Friede et al., 2019) and the LUBAC complex (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). SHARPIN null *cpdm* mice suffer from hyperproliferative

dermatitis and multiorgan inflammation, which have been linked to aberrant NF-κB-signalling and induced TNF-mediated apoptosis as a consequence of abrupted LUBAC function (Ikeda et al., 2011; Tokunaga et al., 2009, 2011). However, it has remained unclear how increased integrin activity contributes to the *Sharpin*^{cpdm} skin phenotype, or is it just a secondary outcome of the systemic inflammation.

Both lack of SHARPIN and another LUBAC-complex member HOIL-1L has been separately shown to sensitize cells to TNF-induced cell death. However, *Hoil-1l*-depleted cells exhibit more moderate phenotypes compared to *Sharpin*^{cpdm}-cells, and most importantly, *Hoil-1l* KO mice do not exhibit similar hyperproliferative dermatis as *Sharpin*^{cpdm} mice (Ikeda et al., 2011; Tokunaga et al., 2009, 2011). While especially interaction with the LUBAC complex has been proposed to underlie the skin phenotype of *Sharpin*^{cpdm} mice by sensitizing the cells to TNF-induced cell death (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011), our group showed that levels of active β1-integrin are elevated in the epidermis of *Sharpin*^{cpdm} mice. Furthermore, integrin expression was not confined to the basal layer of the epidermis in *Sharpin*^{cpdm} mice (Rantala et al., 2011), as it is expected to be under normal conditions. Encouraged by reports describing similar phenotype of integrin β1-overexpressing mouse strain (Carroll et al., 1995), we hypothesised that increased integrin activity would also contribute to the *Sharpin*^{cpdm} mice skin phenotype, in addition to the LUBAC complex. Here, we were able to show that while increased inflammation increases integrin activity in the skin, we also detect increased integrin β1-activity in almost completely inflammation free *Tnfr1*$^{-/-}$ *Sharpin*$^{cpdm/cpdm}$ mice. Furthermore, others have shown that integrin function blocking antibodies ameliorate psoriasis-like phenotypes in human (Ley et al., 2016) and mice (Conrad et al., 2007). In line with these observations, we see reduction in epidermal hyperproliferation upon treatment with integrin β1 function-blocking antibody. However, as the antibody treatment is not sufficient to reduce infiltration of inflammatory cells in the skin tissue, these findings suggest that while increased integrin activity contributes to the *cpdm* phenotype, it does not explain it solely. Moreover, other SHARPIN interactors, such as the LUBAC complex, contribute at least to the development of the systemic inflammation.

In addition to binding integrins and the LUBAC complex members, SHARPIN is also known to interact with multiple other proteins, which may also contribute to the *Sharpin*^{cpdm} skin phenotype. For example, SHARPIN has been shown to directly bind and inhibit inflammation and cell death-driving caspase-1 in a LUBAC-independent manner. Furthermore, *Sharpin*^{cpdm} mice have been shown to exhibit reduced survival upon sepsis, while caspase-1 inhibition ameliorated the phenotype (Nastase et al., 2016). Another study has shown that caspase-1 and -11 are highly upregulated in *Sharpin*^{cpdm} skin sections. Interestingly, induction of caspase-1 and -11 were observed in the *Sharpin*^{cpdm} skin already before the actual onset of the

dermatitis. Moreover, caspase-1 and -11 depletion delayed onset of the dermatitis, but onset of systemic inflammation remained unchanged indicating that caspase-1 and -11 may contribute to the *Sharpin*^{cpdm} skin phenotype independent of the LUBAC-complex-mediated systemic inflammation (Douglas et al., 2015). Furthermore, caspase-1 and -11 participate in inflammasome signalling, that is activated upon innate immune responses to tissue damage, invading micro-organisms and metabolic perturbations. In addition to downregulation of caspase-1 and -11, genetic ablation of another inflammasome sensor, leucine-rich repeat (LRR)-containing protein 3 (NLRP3), also alleviated the *Sharpin*^{cpdm} skin phenotype (Douglas et al., 2015). Altogether, these findings suggest that responses to tissue damage and inflammasome activation may be altered in *Sharpin*^{cpdm} mice, and they could also contribute to the *cpdm* phenotype. Intriguingly, as NLRP3 inflammasome has been shown to be activated downstream of β1 integrins upon micro-organism infection integrins (Jun et al., 2012; Thinwa et al., 2014), it is tempting to speculate that the increased integrin activity and inflammasome activation in *Sharpin*^{cpdm} mice are connected.

There is a lack of studies describing the role of SHARPIN in development and other diseases than dermatitis as *Sharpin*$^{cpdm/cpdm}$ mice develop severe dermatitis early on, they need to be sacrificed already when they are 8-week old. Importantly, *Tnfr1*$^{-/-}$ *Sharpin*$^{cpdm/cpdm}$ mice that do not exhibit signs of major inflammation, have a longer lifespan and they may provide a novel way to study the role of SHARPIN in other developmental stages and in other diseases, such as cancer. Interestingly, SHARPIN upregulation and gene amplification have been shown to associate with multiple human cancers (De Melo and Tang, 2015; Li et al., 2015; Tamiya et al., 2018; Tanaka et al., 2016; Zhang et al., 2014; Zhuang et al., 2017). Therefore, the dermatitis-free *Tnfr1*$^{-/-}$ *Sharpin*$^{cpdm/cpdm}$ mice may be a valuable tool in cancer research. Furthermore, *Tnfr1*$^{-/-}$ *Sharpin*$^{cpdm/cpdm}$ and *Sharpin*$^{cpdm/cpdm}$ mice may be used as a model system in psoriasis studies and in investigations of the role of integrin activity in skin disorders.

6 Summary

The purpose of the work presented in this thesis was to further characterise two proteins that our group has identified as inhibitors of integrin cell adhesion receptors: SHANK3 (Lilja et al., 2017) and SHARPIN (Rantala et al., 2011). Importantly, as there is an immense amount of crosstalk between cell adhesion and the actin cytoskeleton, we wanted to investigate if and how SHANK3 and SHARPIN also regulate the actin cytoskeleton and cytoskeletal structures, such as filopodia and lamellipodia. Furthermore, we set out to unravel a question that was left unanswered in our previous work: does increased integrin activity contribute to the hyperproliferative dermatitis phenotype of SHARPIN null *cpdm* mice, or is it driven by the role of SHARPIN in the LUBAC complex and NF-κβ-signalling. Here, we identify novel roles for SHANK3 and SHARPIN in regulation of the actin cytoskeleton in cells, and characterise the role of SHARPIN-mediated integrin inhibition *in vivo*.

6.1 SHANK3 is a novel actin-binding protein that regulates filopodia formation

We set out to investigate the role of SHANK3 in filopodia formation through inhibition of integrins. However, we found that instead of regulating filopodia exclusively through integrins, SHANK3 has previously unappreciated actin-binding activity. SHANK3 binds actin directly with its N-terminal SPN domain, which was previously shown to regulate integrin activation by sequestering active Rap1 (Lilja et al., 2017). Through site-directed mutagenesis, biochemistry and cell imaging, we mapped the Rap1- and actin-binding sites in the SPN domain and demonstrated that they are distinct. Furthermore, we showed that the two N-terminal SHANK3 domains, SPN and ARR are folded in a way that covers the actin-binding site in the interface between the two domains. Thereby, opening of the SPN-ARR fold dynamically regulates the interaction between SHANK3 and actin. Importantly, we showed that while the SPN domain can interact with active Rap1 and actin separately, the full-length SHANK3 may require ability to interact dynamically with both active Rap1 and actin to inhibit filopodia formation. Importantly, our data indicates that SHANK3 may have an important role in regulation of both cell

adhesion and the actin cytoskeleton as it balances between scaffolding its multiple binding partners, such as Rap1, actin and actin regulatory proteins. Importantly, our data indicate that the N-terminal SPN domain may act as a switch that determines in which cellular processes and signalling cascades SHANK3 participates. These novel roles of SHANK3 may have implications in both neuronal and non-neuronal cells, as cell adhesion and actin re-organization have pivotal role in both cancer cell migration and dendritic spine morphogenesis. Furthermore, understanding how interaction between SHANK3 and its different binding partners can be manipulated may lead to identification of novel therapeutic approaches to treat ASD and cancer.

6.2 SHARPIN promotes lamellipodia formation

In this part of the thesis, we presented the first mass spectrometry-based SHARPIN interactome and demonstrate that it associates with a versatile set of proteins implicated in many different biological processes. We observed that several putative SHARPIN interactors associate with cytoskeletal regulation, and confirmed a direct interaction between SHARPIN and the Arp2/3 complex. Our data indicate that SHARPIN promotes Arp2/3-dependent formation of branched actin networks at the leading edge of the cell. Furthermore, we mapped that the interaction with the Arp2/3 complex occurs through same SHARPIN domain (the UBL domain) to which also α-integrins and the LUBAC complex binds. However, we showed that the Arp2/3 binding site is distinct from them and created an Arp2/3-binding deficient SHARPIN mutant. Importantly, we showed that the interaction between SHARPIN and the Arp2/3 complex promotes lamellipodia formation independent of the LUBAC-complex and integrins. These data indicate that the UBL-domain is a central activity hub in SHARPIN, but it is unclear how the different UBL-interations and functions are regulated or coordinated in cells. As the binding sites for integrins, the LUBAC complex and the Arp2/3 complex are all in close proximity, it may be that SHARPIN is only able to interact with one of them at the time. Therefore, interaction between SHARPIN and its different binding partners could be induced by different cellular challenges, such as inflammation, TNF- or growth factor-mediated signalling. Furthermore, the possibility that SHARPIN interacts with different proteins in spatially distinct locations remains to be studied. Importantly, this study highlights that SHARPIN is likely to have multiple functions and interaction partners in addition to integrin- and LUBAC-mediated signalling which have been the focus of most SHARPIN studies so far. Understanding how SHARPIN interacts with its different binding partners in different cellular contexts and different cellular locations may improve our understanding of molecular mechanisms driving cancer progression and/or inflammation.

6.3 SHARPIN-deficiency increases integrin activity and keratinocyte proliferation also in absence of inflammation

Several studies have shown that SHARPIN interacts with integrins (Rantala et al., 2011; Pouwels et al., 2013; Gao et al., 2019; Kasirer-Friede et al., 2019) and is a member in the NF-κβ-signalling pathway mediating LUBAC complex (Gerlach et al., 2011; Tokunaga et al., 2011; Ikeda et al., 2011). While SHARPIN has been shown to inhibit integrins independent of the LUBAC complex in cells, the role of SHARPIN-mediated integrin inhibition *in vivo* has remained more obscure. Importantly, SHARPIN null *cpdm* mice suffer from an inflammatory skin phenotype that has been linked to increased TNF-mediated apoptosis due to altered LUBAC function. In addition, intergrin activity has been shown to be increased in the *Sharpin*$^{cpdm/cpdm}$ skin (Rantala et al., 2011) raising a debate what part of the increased integrin activity in these mice is a secondary effect to the overall increased inflammation and what is the contribution of the lack of SHARPIN. We addressed this by looking specifically into epidermis of *cpdm* mice. Importantly, we distinguished *cpdm* phenotype features that appear to be more dependent on integrin activity or the LUBAC complex, respectively. We showed that while integrin activation is partly caused by the general inflammation in *cpdm* mice, integrin activity is also elevated in the absence of inflammation, as shown by simultaneous depletion of TNFR1 receptor. Furthermore, while the LUBAC complex and altered NF-κβ-signalling appear to drive inflammation in the *cpdm* skin, the increased integrin activity contributes to hyperproliferation and epidermal thickening. Taken together, our findings suggest that both aberrant integrin- and LUBAC-mediated signalling contribute to the *cpdm* phenotype. This introduces an intriguing possibility that induction of integrin-mediated adhesion and -signalling pathways may contribute to or even account for some of the *cpdm* phenotype features that have so far been linked solely to TNF- and NF-κβ-signalling. Furthermore, there may also be crosstalk between increased TNF- and integrin-mediated signalling that contributes to the *cpdm* phenotype, but this remains to be investigated. Taken together, this study expands our current knowledge of molecular mechanisms underlying the SHARPIN *cpdm* skin phenotype and the systemic inflammation. Importantly, investigation of how the distinct binding partners of SHARPIN function at tissue level may improve the current understanding of how integrin activity and the LUBAC complex regulate inflammation and contribute to psoriasis-like conditions.

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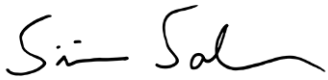

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