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ACTIN- AND ADHESION-MEDIATED REGULATION OF CELL STATES

From cancer to stem cells

Maria Taskinen

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University of Turku

Faculty of Medicine
Medical Biochemistry and Genetics
Turku Doctoral Programme of Molecular Medicine (TuDMM)
Turku Bioscience Centre

Supervised by

Professor, Johanna Ivaska
Turku Bioscience Centre
University of Turku and Åbo Akademi
Turku, Finland

Docent, PhD, Elisa Närvä
Institute of Biomedicine
University of Turku
Turku, Finland

Reviewed by

Professor, Cecilia Sahlgren
Faculty of Science and Engineering
Åbo Akademi
Turku, Finland

Docent, PhD, Minna Poukkula
Institute of Biotechnology
University of Helsinki
Helsinki, Finland

Opponent

Professor, James Bear
Department of Cell Biology and Physiology
University of North Carolina at
Chapel Hill School of Medicine
Chapel Hill, NC, USA

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To my family and friends

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ABSTRACT

Actin cytoskeleton and cell adhesion regulate cell shape, contractility and motility, all of which play important roles in development of normal tissues and diseases. Integrins are adhesion receptors that are connected to the actin cytoskeleton. However, it remains unclear how integrin-mediated adhesions and actin dynamics regulate different cell states in early development, stem cells and cancer progression. The aims of my thesis are to investigate on a mechanistic level how MASTL, a known cell cycle regulating kinase, regulates breast cancer cell adhesion and movement, and how it influences cancer stemness and pluripotency. Further, we wanted to investigate adhesion and actin-mediated transitions between pluripotent stem cell states.

This thesis reveals earlier unknown kinase-independent functions of MASTL as a transcriptional regulator of cancer cell spreading, actin organization and contractility, thus bringing new mechanistic insights into MASTL-mediated cancer progression. In more detail, MASTL facilitates activity of transcription factors SRF and MRTF-A by associating with and supporting the nuclear localization of MRTF-A. This leads to gene expression patterns that facilitate formation of adhesions, actin stress fibers and contractility. Further, we show that MASTL is highly expressed in cancer stem cells and pluripotent stem cells, and regulates expression of transcription factors OCT1 and OCT4, which are essential in stemness and pluripotency maintenance, respectively. In addition, MASTL supports TGF-β signaling, which is known to facilitate stemness maintenance. Finally, we reveal new functions of integrin β1 and actomyosin contraction in regulation of naïve and primed states in human induced pluripotent stem cells.

Taken together, this thesis reveals new adhesion- and actin-mediated mechanisms, that are important in cancer, stemness and pluripotency, and may help to guide development of new cancer treatment strategies and stem cell -based modeling of diseases and development.

KEYWORDS: Actin cytoskeleton, contractility, motility, adhesion, MASTL, cancer, stemness, hiPSC

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TIIVISTELMÄ

Aktiinitukiranka ja solujen adheesio säätelevät solujen muotoa, supistumiskykyä ja liikkumista, joista kaikki ovat tärkeitä tavallisten kudosten sekä sairauksien kehittymisessä. Integriinit ovat solujen tarttumisreseptoreita, jotka ovat yhteydessä aktiinitukirankaan. On kuitenkin vielä epäselvää, miten integriinivälitteiset adheesiot sekä aktiinidynamiikka vaikuttavat solujen tiloihin alkioiden, kantasolujen sekä syövän kehittymisessä. Tämän väitöskirjatyön tavoitteena on tutkia mekaanisella tasolla, miten MASTL, tunnettu solusykliä säätelevä kinaasi, vaikuttaa rintasyöpäsolujen adheesioon ja liikkumiseen, sekä syövän kantaisuuteen ja solujen monikykyisyyteen. Lisäksi halusimme tutkia adheesioiden sekä aktiinin säätelemää monikykyisten kantasolujen tilojen välistä siirtymistä.

Tämä väitöskirja paljastaa uusia aiemmin tuntemattomia kinaasi-riippumattomia MASTL:n tehtäviä, joilla se vaikuttaa syöpäsolujen leviämiseen, aktiinin järjestäytymiseen ja supistumiskykyyn transkriptionaalisella tasolla, ja tuo täten esiin uusia näkökulmia MASTL:n toimintaan syövän kehittymisen säätelijänä. Tarkemmin kuvailtuna, MASTL edesauttaa transkriptiotekijä SRF:n ja MRTF-A:n aktiivisuutta vuorovaikuttamalla MRTF-A:n kanssa ja edesauttamalla sen tuma-lokalisaatiota. Tämä johtaa geeni-ilmentymiskaavoihin, jotka edesauttavat adheesioiden, aktiinistressisäikeiden ja kontraktiliteetin muodostumisessa. Lisäksi osoitamme, että MASTL:ää ilmentyy runsaasti syöpäkantasoluissa sekä monikykyisissä kantasoluissa, joissa se säätelee kantaisuuden ylläpidossa tärkeiden transkriptiotekijöiden OCT1 and OCT4 ilmentymistä. Lisäksi MASTL tukee TGF-β-signalointia, joka on myös tärkeä kantaisuuden säätelyssä. Paljastamme myös uusia integriini β1:n ja aktomyosiinikontraktiliteetin vaikutuksia monikykyisten kantasolujen tilojen säätelyssä.

Yhteenvetona, tämä väitöskirja tuo esiin uusia adheesioiden ja aktiinin säätelemiä mekanismeja, jotka ovat tärkeitä syövässä, kantaisuudessa ja monikykyisyydessä, ja voivat auttaa uusien syöpähoitostrategioiden kehittämisessä ja tautien sekä aikaisen kehityksen mallintamisessa.

AVAINSANAT: Aktiinitukiranka, supistumiskyky, liikkumiskyky, adheesio, MASTL, syöpä, kantaisuus, hiPSC

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Abbreviations

ACTA2	Smooth Muscle α-actin
ADP	Adenosine diphosphate
AKT	Protein kinase B (also known as PKB)
ALDH1	Aldehyde dehydrogenase 1
AMOTL2	Angiomotin-Like 2
Arp2/3	Actin-related protein 2/3
ARPP19	cAMP-regulated phosphoprotein 19
ATP	Adenosine triphosphate
BCSC	Breast cancer stem cell
BSA	Bovine serum albumin
CAF	Cancer-associated fibroblast
CArG	$CC[AT]_6GG$
CCLE	Cancer cell line encyclopedia
CCN2	Cellular communication network factor 2
CSC	Cancer stem cell
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DOK1	Docking protein 1
DREAM	Dimerization partner, retinoblastoma-like, E2F and MuvB
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ELC	Essential light chain
EMT	Epithelial-mesenchymal transition
Ena/VASP	Enabled/vasodilator-stimulated phosphoprotein
ENSA	Endosulfine alpha
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
FAK	Focal adhesion kinase
FBS	Fetal bovine serum

FH1	Formin homolgy 1
FH2	Formin homology 2
FILIP1	Filamin A-Binding Protein 1
FLIP	Fluorescence loss in photobleaching
FACS	Fluorescence-activated cell sorting
GEF	Guanine nucleotide exchange factor
GEF-H1	Rho guanine nucleotide exchange factor 2
GFP	Green fluorescent protein
GKI-1	Greatwall kinase inhibitor-1
HBSS	Hanks' balanced salt solution
hESC	Human embryonic stem cell
hiPSC	Human induced pluripotent stem cell
ICAP1	Integrin cytoplasmic domain-associated protein 1
ICM	Inner cell mass
iMEF	Inactivated mouse embryonic fibroblasts
iPSC	Induced pluripotent stem cell
KLF17	Krüppel-like factor 17
KRAS	V-Ki-Ras2 kirsten rat sarcoma 2 viral oncogene homolog
LIMA1	LIM domain and actin-binding protein 1
LINC	Linker of nucleoskeleton and cytoskeleton
MASTL	Microtubule-associated serine/threonine kinase-like
MDGI	Mammary-derived growth inhibitor
MEF	Mouse embryonic fibroblast
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MRTF	Myocardin-related transcription factor
MRTF-A	Myocardin-related transcription factor A
MRTF-B	Myocardin-related transcription factor B
NM-2	Non-muscle myosin II
NM-2A	Non-muscle myosin II A
NM-2B	Non-muscle myosin II B
NM-2C	Non-muscle myosin II C
NPF	Nucleation promoting factor
OCT1	POU domain transcription factor 1
OCT4	POU domain transcription factor 4
PBS	Phosphate-buffered saline
PDGFB	Platelet derived growth factor subunit B
PFA	Paraformaldehyde
PHACTR-1	Phosphatase actin regulator-1
P_i	Inorganic phosphate

PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
pMLC	Phosphorylated myosin light chain
PP2A	Protein phosphatase 2A
PSC	Pluripotent stem cell
RGD	Arginine-glycine-aspartate
RLC	Regulatory light chain
RNAi	RNA interference
ROCK	Rho-associated coiled-coil containing protein kinase
ROCK1	Rho-associated coiled-coil containing protein kinase 1
ROCK2	Rho-associated coiled-coil containing protein kinase 2
ROS	Reactive oxygen species
SFRP2	Secreted frizzled related protein 2
SHANK1	SH3 and multiple ankyrin repeat domains 1
SHANK3	SH3 and multiple ankyrin repeat domains 3
SHARPIN	SHANK-associated RH domain-interacting protein
SILAC	Stable isotope labelling with amino acids in cell culture
SMAD3	SMAD family member 3
SOX2	Sex determining region Y-box 2
SRF	Serum response factor
STAT3	Signal transducer and activator of transcription 3
TCF	Ternary complex factors
TGFBR2	Transforming growth factor β receptor II
TGF-β	Transforming growth factor β
TIRF	Total internal reflection fluorescence
Tpm	Tropomyosin
VASP	Vasodilator stimulated phosphoprotein
WASP	Wiskott-Aldrich syndrome protein
ZIC2	Zic family member 2

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 Taskinen M.E.*, Närvä E.*, Conway J.R.W., Hinojosa L.S., Lilla S., Mai A., De Franceschi N., Elo L.L., Grosse R., Zanivan S., Norman J.C., Ivaska J. MASTL promotes cell contractility and motility through kinase-independent signaling. *Journal of Cell Biology,* 2020; 219(6): e201906204.

II Närvä E., Taskinen M.E., Lilla S., Isomursu A., Pietilä M., Weltner J., Isola J., Sihto H., Joensuu H., Zanivan S., Norman J. & Ivaska J. MASTL is enriched in cancerous and pluripotent stem cells and influences OCT1/OCT4 levels. *iScience*, 2022; 25(6): 104459.

III Taskinen M.E., Stubb A., Rasila P., Vahlman S., Sokka J., Trokovic R. & Ivaska J. Inhibition of integrin β1 activity and contractility support naïve-like state in human induced pluripotent stem cells. *Manuscript.*

* equal contribution

1 Introduction

Actin is a cytoskeletal protein that forms complex and dynamic networks that interact with extracellular space and other cells via different cell adhesion receptors. Integrins are a group of cell adhesion receptors that can bind various extracellular ligands using their extracellular head domains, and at the same time they use their cytosolic tail domains to bind to various scaffolding and signaling proteins, as well as actin filaments. This integrin-mediated interaction between extracellular and intracellular space enables the cells to sense and react to their environmental cues, such as biochemical composition of the extracellular matrix, tissue stiffness and other cells. The information the cells obtain from their environment can trigger intracellular signaling pathways and affect expression of genes that affect cell morphology, function, and survival. Such changes can cause a cell state transition – a process where a cell obtains certain gene and protein expression patterns, morphology and functional properties. Cell state transitions are important in embryonic development, tissue homeostasis and repair, differentiation of stem cells into specialized cell types, and in development of pathologies.

Dysregulation of cell adhesion and cytoskeleton can lead to drastic changes in cell function and signaling, which can trigger developmental defects and contribute to disease progression, for example in cancer. When cells are transformed into aggressive cancer cells, their integrin- and actin-related regulation becomes altered resulting in increased cell motility. This enables the cancer cells to invade and migrate individually or collectively, leading to cancer spreading and metastasis. In addition to this, cancer cells can obtain stem cell-like charasteristics, including ability to self-renew and differentiate into other cell types. This phenomena is known as cancer stemness, which is considered to be a possible driving force behind drug-resistance and recurrence in cancer.

In addition to cancer cell migration, cell movement is also important in embryonic development, wound healing and tissue development. Thus, actin and adhesions must be regulated in the correct way, at the right time and place to enable normal development and tissue homeostasis. Further, cell adhesion plays an important role in embryonic implantation, and is thus crucial for normal development. During implantation, the pluripotent embryonic stem cells go through

drastic changes regarding their gene expression patterns, signaling pathways and metabolism, leading to a state known as primed pluripotency, where the cells are ready to differentiate first into different germ layers and later into all different adult tissue types. The pluripotency states are mainly studied in mouse embryonic stem cells, but are less understood during human development.

This thesis aims to give new insigts into the regulation of cell adhesion and actin cytoskeleton during different steps of early human development and cancer progression.

2 Review of the Literature

2.1 The actin cytoskeleton

Actin is a highly conserved and abundant protein in all eukaryotes. Actin is a monomeric protein that can polymerize to form filaments, and plays an important role in eukaryotic cell development, structure and function. Together with other actin-binding proteins, actin forms a heterogeneous, complex, and dynamic network called the actin cytoskeleton. In humans, there are six actin isoforms: three distinct α-actins expressed in muscle cells, one β-actin expressed in non-muscle cells, one γ-actin expressed in smooth muscle and one γ-actin expressed in non-muscle cells. (Pollard, 2016) These different isoforms have their own specific roles in human, and mutations in their genes are associated with various developmental defects and diseases, from hearing loss and intellectual disabilities to muscle weakness and heart disease. (Parker et al., 2020). In non-muscle cells, actin generates force either by polymerization of actin filaments or by myosin-mediated movement when myosin motor proteins move along actin filaments. These forces enable the cells to contract, change shape, sense and interact with the environment, move, and divide. In order to achieve all these functions, actin and its dynamics are regulated by several accessory or regulatory proteins. (Svitkina, 2020) This chapter focuses on the assembly, regulation, and functions of β-actin structures in mammalian non-muscle cells.

2.1.1 Polymerization of actin filaments

The actin monomer, or globular actin (G-actin), is a 375 amino acid long polypeptide that folds into a protein with four subdomains. G-actin is able to bind ADP and ATP, and it can polymerize and form filamentous actin (F-actin). (Dominguez and Holmes, 2011; Pollard, 2016) The first and most rate-limiting step in the F-actin formation is the nucleation, meaning the formation of stable actin dimers and trimers. G-actin is often bound to sequestering proteins, such as profilin and thymosin-β4 that prevent actin polymerization. Further, actin dimers are very unstable, and thus actin-nucleating proteins are needed to aid the nucleation process. (Firat-Karalar and Welch, 2011; Pollard, 2016) After nucleation, G-actin molecules can bind the small

actin nucleus from both ends. The F-actin is growing more rapidly at one end of the filament, called the barbed (+) end, where actin is in an ATP-bound state. The ATP molecules are hydrolysed as the filaments grow, and ADP-bound actin monomers at the pointed (-) end are more rapidly dissociated from the filament compared to the actin depolymerisation at the barbed end. Before dissociation of actin monomer and inorganic phosphate (P_i) from the ATP, actin molecules are in an intermediate state (ATP-P_i-actin). Actin polymerization can reach a steady state called treadmilling, where actin is constantly polymerized at the barbed end and simultaneously dissociated at the pointed end (**Figure 1**). (Dominguez and Holmes, 2011; Wegner and Isenberg, 1983) The F-actin growth, stability, and depolymerisation are regulated by many actin associating proteins that are introduced in more detail in the chapter 2.1.2.

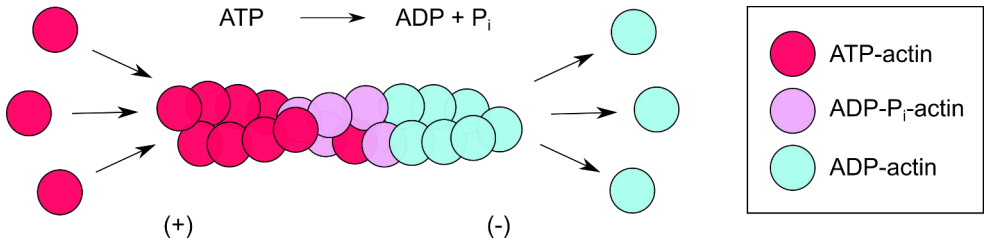

Figure 1. Illustration of actin treadmilling. ATP-bound actin (red) is polymerized at the barbed (+) end of an actin filament, where ATP is hydrolized into inorganic phosphate (P_i) and ADP, and ADP-bound actin (turquoise) at the pointed (-) end is dissociated from the filament.

2.1.2 Actin-associated proteins mediate actin filament assembly and disassembly

Actin filament assembly and disassembly are regulated by various actin-binding proteins that have different functions, including monomer binding, nucleation, branching, polymerization, severing, capping, cross-linking, and branching (Pollard, 2016). Since actin cytoskeletal regulation is essential in normal development and function of the cells, dysregulation of actin-associated proteins is often linked to developmental defects, such as cardiovascular and neurodevelopmental disorders (Labat-de-Hoz and Alonso, 2021) and diseases, including ischemic stroke (Labat-de-Hoz and Alonso, 2021), neurological diseases (Murk et al., 2021), Alzheimer's disease, and rheumatoid arthritis (Feldt et al., 2019), and cancers (Feldt et al., 2019; Izdebska et al., 2020; Labat-de-Hoz and Alonso, 2021; Pimm et al., 2020).

Profilin family proteins are small actin monomer binding proteins that inhibit spontaneous nucleation of actin (Pollard, 2016), but can facilitate actin polymerization by binding to proline-rich motifs in other actin regulators such as formin (Courtemanche, 2018; Funk et al., 2019) and Enabled/vasodilator-stimulated

phosphoprotein (Ena/VASP) (Ferron et al., 2007). Profilin competes actin monomer binding with thymosin-β4, a small actin sequestering protein that can inhibit actin nucleation and polymerization (Skruber et al., 2018).

Actin nucleating proteins help in the first steps of actin polymerization and filament formation. Actin-related protein 2/3 (Arp2/3) complex promotes actin nucleation and branching. It binds to an already existing actin filament and initiates new actin filament formation as a branch at about 70° angle relative to the pre-existing filament. Arp2/3 complex consists of seven subunits, including two actin-related proteins, Arp2 and Arp3, that together mimic an actin dimer, and five scaffolding subunits, Arpc1, Arpc2, Arpc3, Arpc4 and Arpc5, that mediate the interaction of the complex with actin filaments. (Gautreau et al., 2022) Arp2/3 complex is activated and recruited to the pre-existing actin filaments by nucleation promoting factors (NPF), such as Wiskott-Aldrich syndrome protein (WASP)-family NPFs (Alekhina et al., 2017). Another major actin nucleating protein group is formins. They are homodimeric proteins that can stabilize actin dimers and trimers by surrounding them with their formin homology 2 (FH2) domains (Courtemanche, 2018).

After nucleation, actin filament polymerization is regulated by formins and Ena/VASP. Formins bind to the barbed end of an actin filament with their FH2 domain, and bind and recruit profilin-bound actin monomers to the growing filament with their tentacle-like formin homology 1 (FH1) domains (Courtemanche, 2018). Ena/VASP are tetrameric proteins that promote actin polymerization by binding and recruiting free G-actin or profilin-bound actin molecules to the F-actin's barbed end. In addition, Ena/VASP can prevent capping protein (CP) from binding to F-actin barbed ends, and thus keeps the filament available for new actin monomers. (Faix and Rottner, 2022) In addition to heterodimeric CP, gelsolins cap F-actin from barbed ends, and tropomodulins, in turn, cap F-actin pointed ends (Pollard, 2016).

Cofilins and gelsolins are two major actin severing protein families that bind and disassemble actin filaments. Cofilins prefer binding to ADP-bound actin monomers, and are thus considered to disassemble aged actin filaments (Poukkula et al., 2011). In addition, cofilin induces F-actin aging by promoting inorganic phosphate (P_i) dissociation from an intermediate state between ATP and ADP (ATP-P_i) (Blanchoin et al., 2000). Gelsolins are multifunctional proteins that are able to bind G- and F-actin. When activated by calcium, gelsolins can severe and cap actin filaments, or sequester actin monomers. (Feldt et al., 2019)

In order to form actin bundles or networks, actin filaments are connected by actin crosslinking proteins, such as α-actinin that connects actin filaments into bundles (Sjöblom et al., 2008), and filamin that organizes actin filaments into orthogonal networks and functions as a scaffold by binding to various proteins, including integrins and other receptors (Nakamura et al., 2011). Further, tropomyosins can

bind along actin filaments and stabilize them. (Manstein et al., 2020) Tropomyosins are discussed in more detail in chapter 2.1.3.2.

2.1.3 Actin structures in mammalian cells

Mammalian cells have many different actin-based structures that mediate cell shape and many crucial processes, such as cell division, endocytosis and movement. The actin cortex (**Figure 2**) is a thin actin network connected to the intracellular face of the plasma membrane and is present in nearly all animal cells. The actin cortex consists of branched actin filaments, myosin motor proteins, and several other actin binding proteins. Together these components mediate cell morphogenesis in various cellular processes, including polarization, division and migration. (Chugh and Paluch, 2018; Svitkina, 2020) During cell division, actin plays an important role in cell rounding, microtubule orientation and spindle assembly, and later forms a contractile actin ring (**Figure 2**) that separates the two daughter cells from each other (Gibieža and Petrikaitė, 2021; Heng and Koh, 2010; Kunda and Baum, 2009). Actin filaments can also be found from cell protrusions, such as microvilli, stereocilia and filopodia that are used for sensing the extracellular environment. Microvilli (**Figure 2**) are thin cell membrane protrusions found in various cell types, including polarized epithelial cells and immune cells. Parallel actin bundles, consisting of 10-30 actin filaments, support the microvilli structure. Microvilli increases the cell surface area, and thus for example facilitates absorption of nutrients in intestinal cells. (Orbach and Su, 2020; Pelaseyed and Bretscher, 2018) Stereovilli (**Figure 2**), also known as stereocilia, are microvilli-like, actin filament supported membrane protrusions found in the hearing cells in the inner ear. They are thicker and longer compared to microvilli, and bend as a response to sound. (Pelaseyed and Bretscher, 2018) Filopodia (**Figure 2**) are thin and dynamic actin-rich protrusions that can extend when actin filaments polymerize towards the cell membrane. These actin filaments are bundled together by actin bundling proteins, such as fascin. Myosin proteins are able to slide along the actin bundles and transport proteins to the filopodium tip. Filopodia play important roles in cell adhesion, extracellular matrix (ECM) sensing, and cell migration during developmental processes and cancer. (Gallop, 2020; Jacquemet et al., 2015) Another important actin structure affectin cell migration is the lamellipodium (**Figure 2**), a thin, wide protruding structure in migrating cell's leading edge. Lamellipodia are formed by Arp2/3 mediated actin polymerization that leads to a branched actin network formation. (Schaks et al., 2019) Actin can also form contractile bundles called actin stress fibers, which are described in the next section.

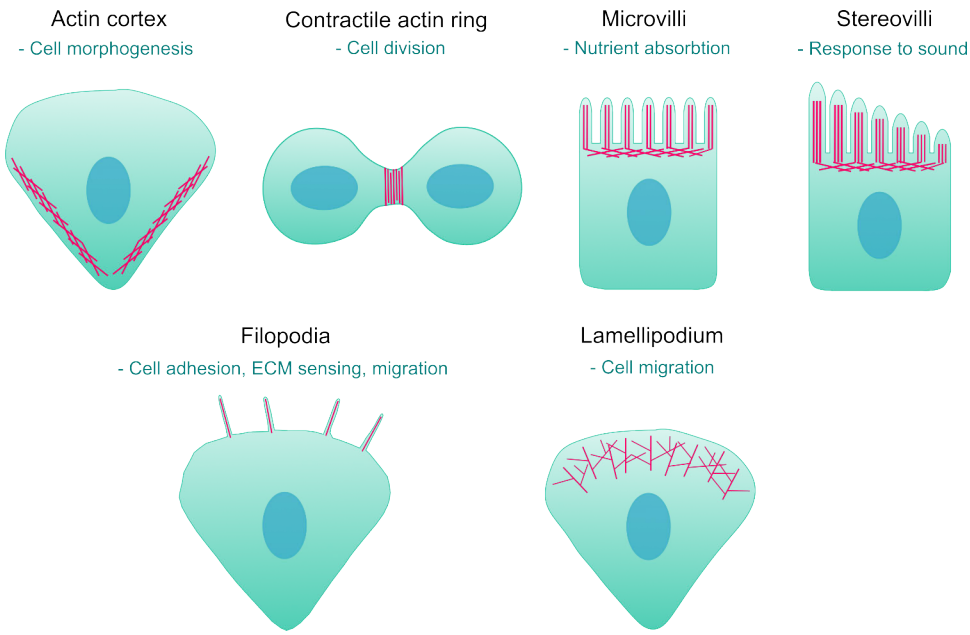

Figure 2. Illustration of different actin structures and their main functions in non-muscle cells. The actin structures are drawn using red colour, and the nucleus is shown as blue.

2.1.3.1 Actin stress fibers

Actin stress fibers are contractile structures found in many cultured non-muscle mammalian cells, and they play an important role in cell shape and movement in 2D. They usually consist of 10-30 actin filaments that are linked together by a bundling protein α-actinin, and these bundles are moved by a motor protein non-muscle myosin 2 (NM-2). (Pellegrin and Mellor, 2007; Tojkander et al., 2012) Stress fibers are often connected to the extracellular matrix via integrin-containing protein complexes called focal adhesion. Integrins and focal adhesions are described in more detail in chapter 2.2. Actin stress fibers can be divided into five types based on their composition, morphology, localization, and connection with focal adhesions (**Figure 3**), and together they form a diverse and dynamic actin network. The most studied stress fiber types are ventral stress fibers, dorsal stress fibers, transverse arcs, and perinuclear actin cap (Tojkander et al., 2012). In addition, cortical stress fibers were recently identified (Lehtimäki et al., 2021).

Dorsal stress fibers (**Figure 3**) are linear actin bundles that are connected to focal adhesions at one end, usually close to the cell edge. Unlike other stress fibers, dorsal stress fibers lack NM-2B, and thus are not able contract. (Tojkander et al., 2012) Dorsal stress fibers are formed by actin polymerization at focal adhesions, a process mediated by Dia1 formin and vasodilator stimulated phosphoprotein (VASP) (Gateva et al., 2014; Hotulainen and Lappalainen, 2006; Watanabe et al., 1999).

Trasverse arcs (**Figure 3**) are long, curved and contractile bundles consisting of α-actinin crosslinked actin bundle regions that are linked together by NM-2 bundles. These structures are located at the lamella, but are not directly connected to focal adhesions like other stress fibers. (Tojkander et al., 2012) Transverse arcs are formed at the cell leading edge by re-organization of Arp2/3-nucleated branched actin network (Hotulainen and Lappalainen, 2006) or recycled filopodial actin bundles (Anderson et al., 2008; Nemethova et al., 2008). In migrating cells, transverse arcs can translocate from the cell leading edge towards the cell centre by a process called retrograde flow (Anderson et al., 2008; Hotulainen and Lappalainen, 2006; Ponti et al., 2004).

Ventral stress fibers (**Figure 3**) are thick and contractile actomyosin bundles, which interact with focal adhesions at their both ends. They are important in cell adhesion, contraction and movement, and generate the force to retract migrating cell's trailing edge. (Burridge and Guilluy, 2016; Tojkander et al., 2012) Ventral stress fibers are formed by fusion of already existing dorsal stress fibers and transverse arcs (Hotulainen and Lappalainen, 2006). The contraction of the arcs pulls the connected dorsal fibers closer together, whereas the focal adhesions in the dorsal fibers force the arcs closer to the ventral side of the cells, resulting in a flattened lamella (Burnette et al., 2014).

Perinuclear caps (**Figure 3**) are similar to ventral stress fibers, since also their both ends are connected to focal adhesions. However, unlike in ventral stress fibers, perinuclear cap's one end is located at the cell leading edge, the middle part above the nucleus, and the other end at the cell rear in a migrating cell. (Khatau et al., 2009) Perinuclear caps are connected to the nucleus via the linker of nucleoskeleton and cytoskeleton (LINC) complex (Khatau et al., 2009), and regulate the nuclear positioning in migrating cells (Maninova et al., 2017; Maninová and Vomastek, 2016).

Cortical stress fibers (**Figure 3**) are recently identified structures where thin actomyosin filaments are connected to nascent focal adhesions at their both ends. They are similar to ventral stress fibers by molecular composition but are shorter and generate weaker traction forces. Cortical stress fibers are usually located under or next to the nucleus and are in close proximity to the ventral surface of the cell. They are formed by NM-2-mediated reorganization of ventral actin cortex independently of actin polymerization on focal adhesions or fusion of other pre-existing stress fibers. (Lehtimäki et al., 2021)

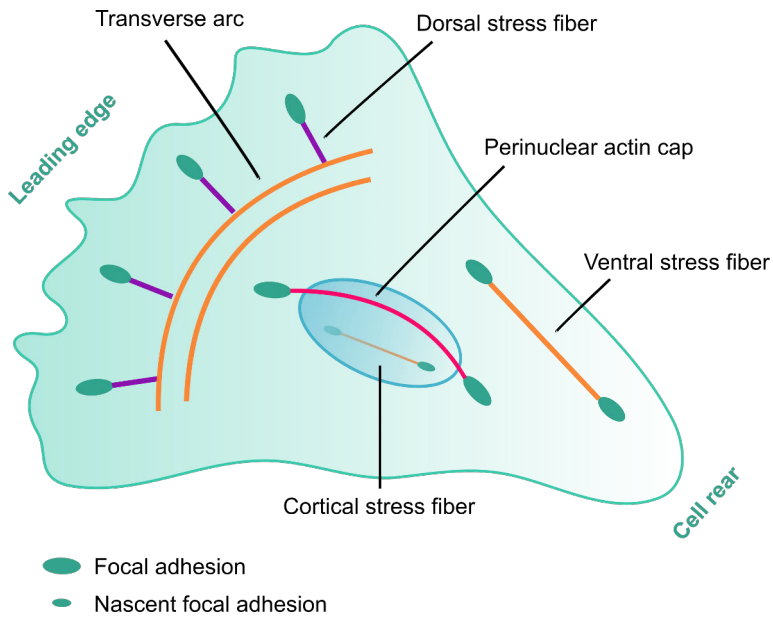

Figure 3. Illustration of five actin stress fiber types in a migrating cell: dorsal, ventral and cortical stress fibers, perinuclear actin cap and transvers arcs.

2.1.3.2 NM-2 and tropomyosins regulate contractile actin structures

NM-2 and tropomyosins are actin binding proteins that play important roles in actin dynamics and contractility (Heissler and Manstein, 2013; Manstein et al., 2020). NM-2 is an essential force-generating protein that mediates actomyosin contraction in non-muscle cells. Each hexameric NM-2 molecule consist of two essential light chains (ELC), two regulatory light chains (RLC), and a coiled-coil tail formed by two heavy chains. NM-2 molecules form bipolar filaments that typically consist of approximately 28 NM-2 molecules. In order to form filaments and generate force to move along F-actin, NM-2 has to be activated by phosphorylation of the RLC. (Heissler and Manstein, 2013) Several kinases are known to phosphorylate RLC, including Rho-associated protein kinase (ROCK) and myosin light chain kinase (MLCK) that activate NM-2, and protein kinase C (PKC) that, in turn, inactivates NM-2 (Vicente-Manzanares et al., 2009).

RhoA is a Rho GTPase family member that plays a key role in myosin regulatory light chain (MLC) phosphorylation and actomyosin contraction. Rho GTPases are guanine nucleotide-binding proteins that can cycle between an inactive, GDP-bound state and an active, GTP bound state (Jaffe and Hall, 2005). The activity of Rho GTPases is regulated by guanine nucleotide exchange factors (GEFs) that activate Rho GTPases and GTPase-activating proteins (GAPs) that, in turn, inactivate Rho GTPases (Bos et al., 2007). Rho guanine nucleotide exchange factor 2 (GEF-H1) is

specialized in activating RhoA. Once activated, RhoA is able to activate its downstream targets, including Rho-associated coiled-coil containing protein kinases 1 and 2 (ROCK1 and ROCK2). After this, ROCK1/2 phosphorylates MLC, leading to NM-2 activation and generation of contractile forces in actomyosin structures. (Joo and Olson, 2021) This GEF-H1-RhoA-ROCK-pMLC-signaling cascade (**Figure 4**) is important especially in generating contractile force to retract the trailing edge of a migrating cell (Joo and Olson, 2021).

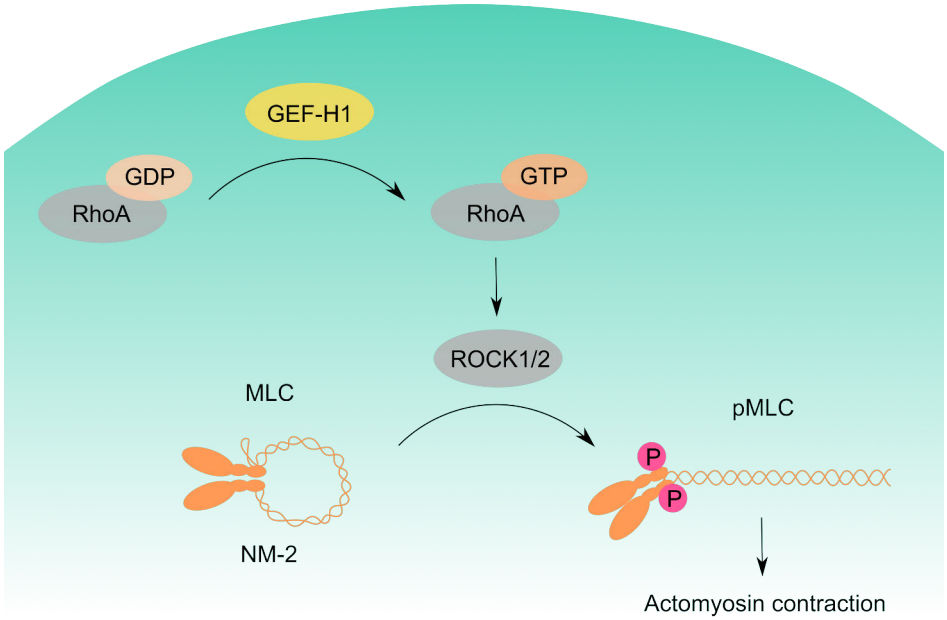

Figure 4. Illustration of non-musle myosin light chain (MLC) phosphorylation via GEF-H1-RhoA-ROCK-signaling pathway.

There are three different mammalian NM-2 isoforms – non-muscle myosin II A (NM-2A) encoded by *MYH9*, non-muscle myosin II B (NM-2B) encoded by *MYH10*, and non-muscle myosin II C (NM-2C) encoded by *MYH14* gene (Heissler and Manstein, 2013). These isoforms are reported to have both, isoform-specific and overlapping, functions and subcellular localization patterns. NM-2A and NM-2B isoforms are broadly expressed in various cell types from fetal to adult tissues, whereas NM-2C expression is low in smooth muscle-containing tissues and either absent or lowly expressed in fetal tissues (Golomb et al., 2004; Shutova and Svitkina, 2018). In migrating cells, NM-2A has been shown to facilitate formation of transverse arcs and dorsal stress fibers at the cell leading edge, whereas NM-2B has been shown to promote ventral stress fiber formation at the cell rear. Thus, the ratio

of NM-2A and NM-2B plays an important role in cell polarity and migratory behaviour. (Shutova et al., 2017; Shutova and Svitkina, 2018). In addition, NM-2A and NM-2B isoforms are able to copolymerize (Beach et al., 2014; Shutova et al., 2014) and jointly regulate cell motility (Shutova et al., 2017).

Tropomyosins (Tpm) are a group of dimeric, rod-like F-actin binding proteins that are thought to stabilize actin filaments, and regulate binding of myosins and other proteins to actin stress fibers. Tpms were first identified as muscle contraction regulators, but were later shown to have important functions also in non-muscle cells. (Manstein et al., 2020) There are four different mammalian Tpm genes, *TPM1*, *TPM2*, *TPM3* and *TPM4*, and altogether 28 different isoforms that have distinct roles in various biological processes from morphogenesis to metabolism. Tpm functions and binding properties to different actin structures are often isoform-specific. (Gunning et al., 2015; Manstein et al., 2020) For instance, Tpm4.2 and Tpm1.7 can be found at ventral stress fibers and transverse arcs, but not at dorsal stress fibers in human osteosarcoma cells (Tojkander et al., 2011). Further, Tpm1.8/9 isoforms have been shown to locate at the leading edge of migrating cells where they facilitate the re-organization of branched actin networks into stable actin filaments, a process aided by actin binding proteins cofilin and coronin 1B (Brayford et al., 2016). Different Tpm isoforms can also have distinct properties regarding their actin binding dynamics, and ability to recruit and activate NM-2. Tpm1.6 and Tpm1.7 can stably bind actin filaments and protect them from actin severing protein cofilin, whereas more dynamic Tpm3.1, Tpm3.2 and Tpm4.2 are specialized in mediating NM-2 activation, but are not able to effectively protect actin filaments from cofilin (Gateva et al., 2017).

2.1.4 Transcriptional regulation of actin cytoskeleton

Actin cytoskeleton is constantly remodelled to enable cells to change their shape and move. In order to do this, the cells have to adjust their gene expression patterns and produce new actin cytoskeletal components and regulator proteins to enable the desirable cytoskeletal changes. (Pollard and Cooper, 2009) Serum response factor (SRF) and myocardin-related transcription factors (MRTF) are key transcription factors that regulate many genes involved in actin cytoskeleton formation and regulation. SRF regulates gene transcription by binding to a CC[AT]$_6$GG (CArG) consensus sequence, that can be found in the promoters of a wide range of genes. (Gau and Roy, 2018; Olson and Nordheim, 2010) SRF transcriptional activity is directed by many transcriptional coregulators, such as ternary complex factors (TCF), MRTFs, homeobox-domain transcription factors and zinc-finger transcription factors. (Gau and Roy, 2018) MRTF and SRF are jointly specialized in promoting expression of cell adhesion-, actin dynamics- and actomyosin

contraction-related genes, such as SRF itself, integrin β1, vinculin, different actin isoforms, cofilin, gelsolin, myosins and tropomyosins. (Olson and Nordheim, 2010) MRTF/SRF activity is mainly mediated by Rho-GTPase signaling, that can be induced by activation of different transmembrane receptors, including integrins, transforming growth factor β (TGF-β) receptors and Frizzled proteins (Corda et al., 2017; Esnault et al., 2014; Olson and Nordheim, 2010; Winter et al., 2001).

While MRTF and SRF regulate actin cytoskeleton and actomyosin contractility, actin polymerization can, reciprocally, regulate MRTF/SRF activity. G-actin/F-actin ratio plays an important role in this process. (Gau and Roy, 2018) G-actin can bind MRTF, which leads to MRTF inhibition and enhanced nuclear export (Vartiainen et al., 2007). When cells are cultured in serum-deprived conditions, MRTF is mainly cytoplasmic and bound to G-actin. Serum stimulation, in turn, induces actin polymerization and release of G-actin from MRTF. After this, MRTF can be transported to the nucleus where it can be transcriptionally active. (Vartiainen et al., 2007)

In addition to co-regulation of actin dynamics with SRF, MRTF can also regulate cell motility by interacting with other transcriptional regulators, including yes-associated protein (YAP) (Kim et al., 2017) and signal transducer and activator of transcription 3 (STAT3) (Liao et al., 2014; Xing et al., 2015). Further, MRTF has been shown to promote Slug expression and epithelial-mesenchymal transition (EMT) by interacting with SMAD family member 3 (SMAD3) – a process that initiates invasive and migratory behaviour in cancer cells (Morita et al., 2007).

2.2 Integrin-mediated adhesions

Integrins are transmembrane cell surface receptors that enable cells to sense chemical and physical properties in their environment, and react to them by inducing various biochemical intracellular signaling pathways. (Hynes, 2002; Kechagia et al., 2019) Integrin-mediated regulation is essential to a wide range of biological processes from embryogenesis to normal tissue development and homeostasis. Thus, integrin dysregulation can trigger numerous developmental defects, such as embryonic lethality and defects in cardiovascular development, and diseases, such as cancer, multiple sclerosis and fibrosis (Danen and Sonnenberg, 2003; Slack et al., 2022). Integrins mediate adhesion to the extracellular matrix (ECM) ligands and other cells and are connected to the intracellular actin cytoskeleton via focal adhesion protein complexes. These integrin-mediated connections are important in regulation of cell shape, contractility and motility. (Conway and Jacquemet, 2019; Hynes, 2002) In addition to the integrin-ECM-mediated signaling initiated at the plasma membrane, endocytosed integrins are able to activate intracytoplasmic signaling pathways (Moreno-Layseca et al., 2019). This chapter gives an overview of integrin structure

and function, and describes how integrins interact with the ECM and the actin cytoskeleton.

2.2.1 Integrin structure and ligand specificity

Integrins are a large group of heterodimers consisting of distinct α and β subunits. There are 8 different β subunits and 18 different α subunits, and these can give rise to altogether 24 different αβ heterodimers. (Hynes, 2002) The ~1000 amino acid long α subunits and ~750 amino acid long β subunits consist of several domains that are connected via flexible linkers. Each integrin subunit is composed of a short cytoplasmic tail, a single helix crossing the plasma membrane, and ectodomains that reside in the extracellular space. (Campbell and Humphries, 2011) Integrin heterodimers contain an extracellular ligand binding part, 'the head', and two flexible 'legs', that both go through conformational changes when integrins are either inactivated or activated. (Campbell and Humphries, 2011)

Integrin heterodimers can be divided into four partly overlapping groups based on their extracellular ligand binding specificity – collagen binding integrins, laminin binding integrins, leukocyte-specific integrins, and integrins that recognize the arginine-glycine-aspartate (RGD) sequence in extracellular ligands, such as fibronectin and vitronectin (Chastney et al., 2021; Hynes, 2002). Integrin β1 is the most common β-subunit in integrin heterodimers. It can form heterodimers with altogether 12 different α-subunits (α1, α2, α3, α4, α5, α6, α7 α8, α9, α10, α11 and αV), where the α-subunit determines the heterodimer specificity to different ligands, including collagen, laminin and fibronectin (Hynes, 2002). Integrin β1 is essential in normal embryonic development, since in the absence of integrin β1 embryos are not able to gastrulate and develop upon implantation (Fässler et al., 1995; Stephens et al., 1995). The second most-frequently presented integrin subdomain within the 24 heterodimers, is αV that can form heterodimers with 5 different β-subunits (β1, β3, β5, β6 and β8). These can bind to RGD motif containing ligands, such as fibronectin and vitronectin. (Chastney et al., 2021; Hynes, 2002)

In addition to the interaction between integrin head and ECM ligands, the integrin cytoplasmic tail can bind intracellular proteins, such as talin and kindlin. Even though integrin itself lacks enzymatic activity, they recruit intracellular kinases, small GTPases and different scaffold proteins to activate downstream signaling in response to extracellular ligand binding. Furthermore, the binding of intracellular proteins to the integrins regulates their activity from within the cell and enables integrin-mediated signal transduction. (Campbell and Humphries, 2011) This bidirectional signaling will be explained in more detail in the following chapter.

2.2.2 Integrin activation and bidirectional signaling

Integrin conformational changes play a central role in integrin-mediated signaling. Integrin activation can be promoted by intracellular proteins that induce extended and open integrin conformation and facilitate ECM ligand binding – a process known as inside-out signaling. On the other hand, integrin attachment to the extracellular ligands can induce binding of various intracellular molecules to the integrin tail, and activation of biochemical signaling pathways – a process called outside-in signaling. (Hynes, 2002)

When integrins are inactive, their ligand binding is hindered due to a bent conformation, where the head domains are pointed towards the integrin legs. (Campbell and Humphries, 2011) This bent conformation is supported by integrin inactivators – intracellular proteins that directly bind to the integrin tails and block the binding of integrin activating proteins. Filamin, integrin cytoplasmic domain-associated protein 1 (ICAP1) and docking protein 1 (DOK1) are integrin inactivators that bind to the integrin β subunit tails, whereas SHANK-associated RH domain-interacting protein (SHARPIN) and mammary-derived growth inhibitor (MDGI) inhibit integrins by binding to their α-tails (Bouvard et al., 2013; Calderwood et al., 2003; Chang et al., 1997; Kiema et al., 2006; Nevo et al., 2010; Rantala et al., 2011). In addition to integrin regulation by direct binding, intracellular proteins can affect integrin activation also indirectly. For example, SH3 and multiple ankyrin repeat domains 1 and 3 (SHANK1 and SHANK3) have been shown to inhibit integrin activation by interacting with Ras and Rap GTPases (Lilja et al., 2017).

Integrin activation involves conformational changes from bent to extended, and further, open conformation. In the latter, α- and β-legs are separated from each other while the head domains remain bound together. (Campbell and Humphries, 2011) Talin is a key integrin activator that binds to the integrin β-tail and induces the extended and open conformation of integrin heterodimers (Tadokoro et al., 2003). Talin-induced integrin activity can be further enhanced by tensin1 and tensin 3 that help to maintain integrin activity during integrin adhesion maturation. (Georgiadou et al., 2017; Georgiadou and Ivaska, 2017). Kindlin is another integrin activator that directly binds integrin β-tail and facilitates integrin's ligand binding affinity. (Rognoni et al., 2016)

While integrins are active and bound to the extracellular ligands, they can be connected to the actin cytoskeleton via talin and tensins. (Georgiadou and Ivaska, 2017) Further, integrins can be clustered into adhesion complexes where a wide range of proteins can be recruited to the cytoplasmic side of the adhesions, where they induce different 'outside-in' signaling cascades. (Kechagia et al., 2019) Among other signaling inducers, focal adhesion kinase (FAK) and Src promote phosphorylation and activation of adhesome components, and are thus important regulators of integrin-mediated signaling. (Chastney et al., 2021)

2.2.3 Focal adhesions – links between ECM and actin cytoskeleton

Focal adhesions are integrin-mediated complexes consisting of multiple scaffolding and signaling proteins, that together function as a mechanoresponsive link between the ECM and the intracellular actin cytoskeleton (**Figure 5**). Focal adhesions are important regulators of actin dynamics, cytoskeletal organization and signaling. On the other hand, actin can regulate focal adhesion maturation, as well as expression of focal adhesion components. Together focal adhesions and actin cytoskeleton generate forces that regulate cell shape, contractility and movement. (Geiger et al., 2009)

To generate focal adhesions, integrins first form smaller and less stable nascent adhesions. Talin is present in the nascent adhesions where it binds and activates integrins, and links them to actin filaments. (Tadokoro et al., 2003) This is followed by binding of other proteins to the adhesion site. Vinculin binds both talin and actin, which induces integrin clustering, and strengthens the connection between the integrins and actin (Humphries et al., 2007). Further, NM-2 mediated actomyosin contraction at the adhesion sites facilitates the adhesion formation and maturation (Vicente-Manzanares et al., 2009).

Focal adhesion nanostructure has been studied in human osteosarcoma cells and mouse embryonic fibroblasts using three dimensional super-resolution microscopy (Kanchanawong et al., 2010). Based on this approach, the focal adhesion components between the integrins and actin stress fibers form protein-specific layers. FAK and paxillin interact with integrin tails close to the plasma membrane. In the next layer, more distal from the plasma membrane, is the so-called force-transduction layer where talin and vinculin are located. Finally, furthest away from the cell-ECM interface, is the actin-regulatory layer consisting of actin polymerase VASP, actin crosslinker α-actinin, and zyxin that has been shown to recruit VASP and α-actinin to the stress fibers (Kanchanawong et al., 2010; Smith et al., 2010).

Focal adhesion nanostructure has been studied also in mouse embryonic stem cells (ESC) (Xia et al., 2019) and human induced pluripotent stem cells (hiPSCs) (Stubb et al., 2019) using a similar super-resolution microscopy approach. Even though ESCs and hiPSCs have similar focal adhesion components compared to differentiated cells, the focal adhesion architecture is distinct in stem cells. For example, vinculin has a stem cell-specific, head-above-tail orientation and is located further away from the plasma membrane in hiPSCs compared to somatic cells (Stubb et al., 2019; Xia et al., 2019). These findings suggest that focal adhesion structures may vary in different cell types and developmental stages. Integrins and focal adhesions in hiPSCs are described in more detail in the chapter 2.4.3.

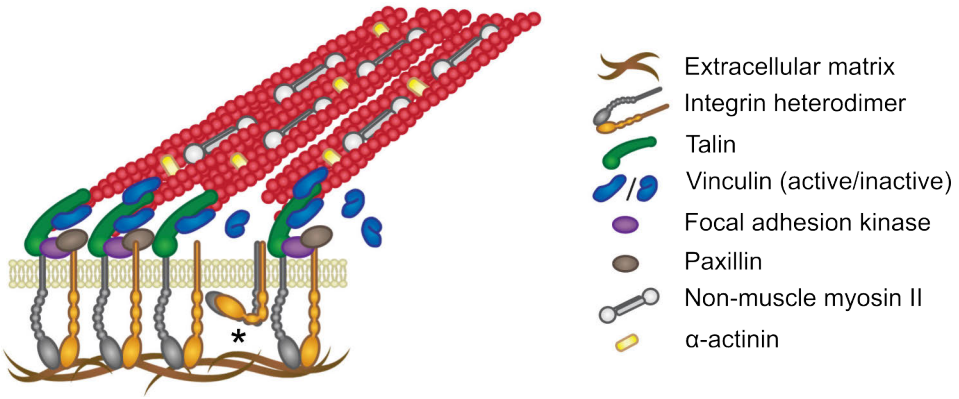

Figure 5. Simplified illustration of active integrin heterodimers interacting with the extracellular matrix, and intracellular focal adhesion components and actin stress fibers (shown as red). The integrin hetreodimer marked with a star (*), illustrates an incative integrin heterodimer in a bent conformation. Modified from (Isomursu et al., 2019).

2.3 Cancer

Cancer is a large group of diseases, where normal cells' regulation gets disrupted giving rise to uncontrolled cell proliferation. Cancer cells can disseminate from tumors, from where they can invade to the surrounding tissues, migrate, and form metastases into other parts of the body. Various intracellular signaling pathways related to cell cycle, cell adhesion and motility are dysregulated in cancer. (Hanahan, 2022) Thus, integrins and actin cytoskeleton, that are key regulators of cell adhesion and motility, are central players during various steps in cancer progression. (Hamidi and Ivaska, 2018; Olson and Sahai, 2009; Yamaguchi and Condeelis, 2007) In addition, stem cell like properties in cancer cells have been shown to promote cancer progression and recurrence (Aponte and Caicedo, 2017). This chapter focuses on cell adhesion and actin in cancer cell migration and stemness and introduces a new potential cancer drug target MASTL.

2.3.1 Adhesions and actin mediate cancer cell motility

Cell migration plays an important role in various biological processes, including tissue development, wound healing and immune response. Further, cancer cell migration can be an indicator of cancer aggressiveness. Cells can adopt different migration modes depending on the cell type, environmental cues and intracellular signaling. According to the current understanding, cells can adopt four distinct migration modes – mesenchymal, collective, amoeboid, and lobopodial (**Figure 6**). (Yamada and Sixt, 2019)

In mesenchymal cell migration (**Figure 6**), cells first form membrane protrusions at the leading edge, followed by formation and maturation of adhesions. After this, cells contract by using their actin stress fibers, enabling the cell rear to move closer to the cell leading edge. This protrusion-adhesion-contraction cycle is constantly repeated as the cells move, and is highly dependent on front-to-rear cell polarity. (Case and Waterman, 2015; Krause and Gautreau, 2014; Ridley et al., 2003) Actin polymerization mediates the formation of cell protrusions that are important in 2D and 3D cell migration. Arp2/3 mediates the lamellipodia formation both in 2D and 3D (Caswell and Zech, 2018). Further, actin polymerization is needed in the formation of filopodia, which are typically short-lived and taken over by lamellipodia in 2D. In contrast, in 3D environments, filopodia and other actin-rich protrusions, such as linear invadopodia and actin spikes, are extensively used in cancer cell migration (Jacquemet et al., 2015). Invadopodia are known to facilitate cancer cell invasion to the surrounding tissue by mediating degradation of the ECM (Murphy and Courtneidge, 2011).

Collective cell migration (**Figure 6**) is typical to cancer cells and invading tumors, where a group of cells that are connected together via cell-cell adhesions, move together and are led by the leading cells that form adhesions and forces to the ECM. (Friedl et al., 2012) Cancer-associated fibroblasts (CAFs) are thought to function as guides for collective tumor invasion. CAFs can remodel the ECM in order to create paths for the migrating cells that are connected to CAFs via heterophilic, N- and E-cadherin containing cell-cell adhesions. (Gaggioli et al., 2007; Labernadie et al., 2017)

Amoeboid cell migration (**Figure 6**) is mainly mediated by forces that are generated by actomyosin contraction. The cells are usually round and show very low amounts of adhesions to the ECM. (Yamada and Sixt, 2019) Further, membrane blebbing is typical in amoeboid migrating cells. The blebs are formed when connections between the actin cortex and the plasma membrane are disrupted, a process facilitated by actin cortex contraction. (Charras and Paluch, 2008)

Lobopodial cell migration (**Figure 6**) has characteristics from both mesenchymal and amoeboid cell migration, and is common in 3D migration. The cells that are using this migration mode, form adhesions and pulling forces to the ECM, but also generate bleb-like membrane protrusions at the leading edge. (Yamada and Sixt, 2019) Importantly, cancer cells are able to adjust to their environment by switching their mode of migration between lobopodial, mesenchymal and amoeboid (Callan-Jones and Voituriez, 2016; Liu et al., 2015).

In addition to the actin reorganization, integrin trafficking and adhesion turnover are important in cancer cell migration. While the cells move, they constantly form new adhesions at the leading edge, and disassemble adhesions at the cell rear. This kind of adhesion turnover is facilitated by integrin trafficking, where integrins are

endocytosed and recycled to another place at the cell membrane. (Hamidi and Ivaska, 2018; Paul et al., 2015) Different integrin heterodimers can mediate distinct migration modes, even when exposed to similar extracellular stimuli. For instance, when cells are plated on fibronectin, integrin αVβ3 recycling has been associated with directionally persistent cell migration, whereas integrin α5β1 recycling has been shown to promote random cell movement and invasive behaviour (Jacquemet et al., 2013; White et al., 2007).

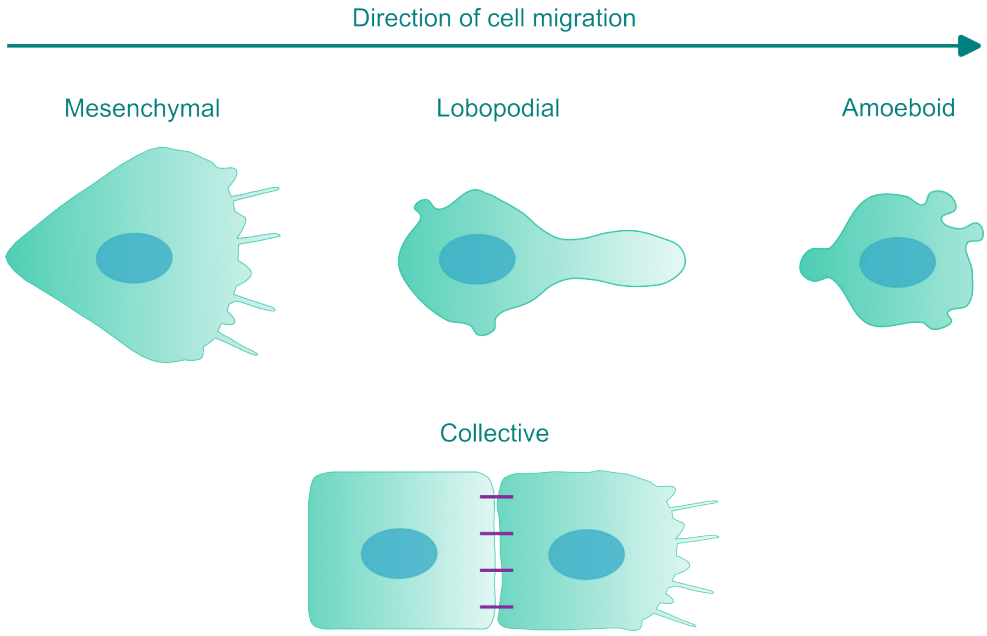

Figure 6. Simplified illustration of cells in different migration modes – mesenchymal, lobopodial, amoeboid and collective. Purple lines demonstrate the cell-cell-contacts.

2.3.2 Cancer stemness

Cancer stem cells (CSC) are a small population of cells within a tumor that have stem cell-like characteristics, such as ability to self-renew and differentiate. CSCs are considered to be a driving force in cancer progression, tumor heterogeneity, metastasis, treatment resistance and cancer recurrence. (Atashzar et al., 2020; Batlle and Clevers, 2017; Najafi et al., 2019; Peitzsch et al., 2017) CSCs are thought to originate from adult stem cells, somatic cells or differentiated cells, and the transformation into CSCs is suggested to be caused by mutations, cell fusion, environmental stimuli or metabolic reprogramming (Atashzar et al., 2020).

CSC can be identified and isolated based on their biomarkers that are often cancer type-specific. Cell surface markers, such as aldehyde dehydrogenase 1

(ALDH1), and glycoproteins CD44, CD24 and CD133, can be used in CSC recognition in many cancers. (Atashzar et al., 2020) For instance, high ALDH1 and CD44 levels have been linked to CSC in breast cancer, where ALDH1 has been shown to promote cell motility and metastasis, and CD44 is mediating cell proliferation and tumor growth (Li et al., 2017). In addition to cell surface markers, high expression of certain transcription factors is linked to cancer stemness. Pluripotency factors POU domain transcription factor 4 (OCT4), sex determining region Y-box 2 (SOX2) and NANOG are transcription factors that are highly expressed in pluripotent stem cells, but have been linked also to cancer stemness (Liu et al., 2013). Further, POU domain transcription factor 1 (OCT1) has been established as a stemness marker in CSC and somatic stem cells (Maddox et al., 2012).

Integrins, including integrin α6, β1, β3 and β4, are often enriched in CSCs and have been associated with essential CSC functions in several cancers. (Seguin et al., 2015). Integrin α6 is associated with cancer stemness and drug resistance in many cancers, including glioblastoma, breast and prostate cancer, whereas integrin β4 is used as a stemness marker in pancreatic, prostate and breast cancer (Xiong et al., 2021; Zhu et al., 2019). Further, fascin-mediated integrin β1 expression has been shown to promote breast CSC maintenance (Barnawi et al., 2019). Even though integrin-mediated signaling is typically promoted by integrin-ECM interactions, ligand-independent integrin signaling has been shown to support anchorage-independent growth and other cancer stemness functions. For instance, αVβ3 integrins are able to cluster at the cell membrane without binding to ECM ligands, and promote stemness via V-Ki-Ras2 kirsten rat sarcoma 2 viral oncogene homolog (KRAS) (Seguin et al., 2014) or anchorage-independent growth via Src signaling (Desgrosellier et al., 2009).

Further, signaling pathways that drive and support cancer stemness have been established, including Notch, Wnt/β-catenin, Hedgehog and TGF-β signaling (Bellomo et al., 2016; Fodde and Brabletz, 2007; Takebe et al., 2011). Notch signaling mediates communication between cells and is activated when transmembrane Notch receptors bind to Delta or Jagged receptors on the surface of a neighboring cell. Notch signaling leads to transcription of stemness related genes, such as Myc, and regulates metabolism, self-renewal and maintenance of CSCs. (Landor et al., 2011; Takebe et al., 2011) Wnt/β-catenin, Hedgehog and TGF-β signaling pathways are activated when secreted proteins bind transmembrane proteins Frizzled, Ptch or transforming growth factor β receptor (TGFBR), respectively. This leads to activation of transcription factors and expression of genes that support CSC survival and maintenance. (Bellomo et al., 2016; Takebe et al., 2011) Inhibition of the abovementioned pathways is a potential strategy for CSC targeting, however, the crosstalk of these and other signaling pathways has created

challenges to this cancer treatment approach. (Huang et al., 2021; Takebe et al., 2015) In addition to this complexity, TGF-β signaling has a dual role in cancer. It has tumor suppressive functions during early stages of cancer, but promotes cancer progression, EMT, invasiveness, and metastasis during later stages of cancer. (Lebrun, 2012)

2.3.3 MASTL – a potential target in cancer therapy

Microtubule-associated serine/threonine kinase-like (MASTL) is a cell cycle regulator that is also associated with cancer progression (Marzec and Burgess, 2018). MASTL is a human orthologue of Greatwall kinase in *Drosophila*, and is highly conserved from arthropods to vertebrates (Burgess et al., 2010; Voets and Wolthuis, 2010). Further, MASTL is essential for early mouse embryonic development (Álvarez-Fernández et al., 2013), yet the role in early human development has not been studied. MASTL is mainly located in the nucleus during interphase, but once the cell has entered mitosis, MASTL is translocated to the cytoplasm before nuclear envelope breakdown (Álvarez-Fernández et al., 2013). The most well understood function of MASTL is its ability to facilitate mitotic entry by phosphorylating its substrates endosulfine alpha (ENSA) and cAMP-regulated phosphoprotein 19 (ARPP19). Once phosphorylated, ENSA and ARPP19 can bind and inhibit protein phosphatase 2A (PP2A), which leads to activation of other mitotic kinases, and thus enables the mitotic entry and maintenance (Vigneron et al., 2016).

High MASTL expression is associated with cancer progression and poor patient prognosis in many cancers, including breast, oral and gastric cancer (Marzec and Burgess, 2018), yet the molecular mechanisms behind this is not well understood. MASTL-mediated Protein kinase B (AKT, also known as PKB) signalling has been proposed as a mechanism behind MASTL-induced cancer progression (Marzec and Burgess, 2018). Overexpression of MASTL has been shown to increase AKT signalling according to two independent studies (Rogers et al., 2018; Vera et al., 2015), yet the molecular mechanism behind this has not been studied in detail. Further, a recent study shows that MASTL promotes pancreatic cancer progression by affecting epidermal growth factor (EGF) signaling (Fatima et al., 2021). Recent studies have also linked MASTL to CSC associated characteristics, such as anchorage-independent growth (Rogers et al., 2018; Vera et al., 2015; Yoon et al., 2018) and radioresistance in breast cancer (Yoon et al., 2018). However, the role of MASTL in cancer stemness requires further investigation.

Since MASTL is so largely associated with cancer progression and poor outcome, inhibitors against MASTL have been under development. Greatwall kinase

inhibitor-1 (GKI-1) (Ocasio et al., 2016), MASTL kinase inhibitor-1 (MKI-1) (Kim et al., 2020) and MASTL kinase inhibitor-2 (MKI-2) (Kang et al., 2021) are the first developed inhibitors against MASTL kinase activity. In addition, compounds from natural and synthetic origins have been identified as potential MASTL inhibitors based on an *in silico* screening study (Ammarah et al., 2018).

So far, only two substrates, ENSA and ARPP19, for MASTL have been identified. However, recent studies have identified new potential MASTL substrates using phosphoproteomic assays (Hermida et al., 2020) and stable isotope labelling with amino acids in cell culture (SILAC)-based kinase screen (Marzec et al., 2022). These, together with the earlier identified substrates, ENSA and ARPP19, might open new windows in understanding the mechanisms behind MASTL-mediated cancer progression.

In addition to abovementioned findings, MASTL has been shown to influence cell migration. When MASTL is overexpressed in breast epithelial cells that have low endogenous MASTL levels, their ability to collectively migrate gets disrupted (Rogers et al., 2018). On the other hand, overexpression of MASTL in breast cancer cells increases mesenchymal cell migration (Vera et al., 2015). The mechanism of how MASTL regulates cell motility has not been studied. Interestingly, however, there are some indications of MASTL being linked to actin cytoskeleton and integrins, which are known to be important in cell migration. Thrombocytopenia-associated patient mutations in MASTL have been shown to cause abnormal actin cytoskeleton in platelets (Hurtado et al., 2018). Further, a RNA interference (RNAi) screening-based study, that was done to predict previously unknown integrin regulators, suggests that MASTL is a potential regulator of integrin β1 activity (Pellinen et al., 2012). The possible link between MASTL, actin cytoskeleton and cell adhesion in the regulation of cell motility and cancer progression remains to be studied.

2.4 Pluripotent stem cells

Pluripotent stem cells (PSC), that have the ability to self-renew and differentiate into almost any adult cell type, can be derived from two different origins. First, embryonic stem cells (ESC) are PSCs that are derived from the inner cell mass (ICM) of a blastocyst. Second, PSCs can be made artificially by reprogramming adult cells into an embryonic stem cell-like state. (Liu et al., 2020) This chapter focuses on human induced pluripotent stem cells (hiPSC) that are reprogrammed from human somatic cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006), and are promising tools in regenerative medicine and studying of early human development (Yamanaka, 2020).

2.4.1 Human induced pluripotent stem cells

HiPSCs are human embryonic stem cell-like cells that are reprogrammed from somatic cells by overexpressing four essential pluripotency factors, OCT3/4, SOX2, KLF4 and c-Myc, also known as the Yamanaka factors (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). HiPSCs can be differentiated back to somatic cells, and thus they can be potentially used in various clinical applications. For example, hiPSC technology can be used for generation of healthy tissue that originates from patients own somatic cells. There are currently several clinical trials involving hiPSC technology, including trials related to cancer immunology, eye- and cardiovascular diseases. (Ilic and Ogilvie, 2022; Yamanaka, 2020) Further, hiPSCs have been recently used in generation of blastoids to model human blastocysts function and development (Kagawa et al., 2022).

2.4.2 Naïve and primed states of hiPSCs

During blastocyst implantation, the ICM cells go through drastic changes regarding gene transcription patterns, metabolism and signaling pathways. These changes during implantation are predominantly studied in mice, where the pre-implantation ICM cells are thought to be in a naïve state, whereas the post-implantation ICM is considered to be in a primed state. (Nichols and Smith, 2009) The cells in naïve and primed states are thought to have distinct differentiation plasticity – when the naïve PSCs can differentiate into embryonic and extraembryonic lineages, the primed cells are prone to differentiate into ectoderm, endoderm and mesoderm germ layers that give rise to different adult tissue types. The transition between these states is a complex and stepwise process, where different intermediate states between naïve and primed pluripotency have been demonstrated. (Endoh and Niwa, 2022; Wang et al., 2021)

Due to ethical considerations, the pre- and post-implantation stages are challenging to study in human. Thus, the usage of hiPSCs in modelling of early embryonic development in human has raised a lot of interest recently. HiPSCs that are reprogrammed using the Yamanaka factors, are thought to be in a primed-like state that resembles the post-implantation PSCs. During the past years several protocols for reversion of primed hiPSCs into a naïve-like state have been developed. (Collier et al., 2022; Hassani et al., 2019; Taei et al., 2020) Primed and naïve hiPSCs have distinct gene expression patterns and signalling pathways that maintain the pluripotency states. (Lynch et al., 2020; Martinez-Val et al., 2021; Nichols and Smith, 2009; Sim et al., 2017; Takashima et al., 2014; Weinberger et al., 2016) One key signaling pathway that is active in primed hiPSCs but inhibited during reversion into naïve-like state, is extracellular signal-regulated kinase (ERK) signaling (Takashima et al., 2014). Further, naïve and primed hiPSCs can

be identified by several cell state-specific markers, such as naïve markers krüppel-like factor 17 (KLF17) and T-Box Transcription Factor 3 (TBX3), and primed markers, including Zic family member 2 (ZIC2) and secreted frizzled related protein 2 (SFRP2).

2.4.3 Adhesions and actin structures in hiPSCs

Primed hiPSCs typically grow as round and rather tightly packed two dimensional colonies in vitro. The survival and maintenance of hiPSCs and human embryonic stem cells (hESCs) in vitro is highly dependent on anchorage to the ECM via integrins (Braam et al., 2008; Meng et al., 2010; Rodin et al., 2014; Rowland et al., 2010). Thus, hiPSCs are routinely cultured on integrin-binding matrixes, such as matrigel, vitronectin and laminin. HiPSCs and hESCs are not able to survive as single cells due to ROCK-induced over-activation of actomyosin contraction (Chen et al., 2010; Ohgushi et al., 2010; Watanabe et al., 2007). Thus, ROCK inhibition is routinely used when hiPSCs are freezed, thawed or passaged as single cells.

Recent studies have shown that the actin and protein composition in the colony edge differs from the colony centre (Y. Kim et al., 2022; Närvä et al., 2017; Stubb et al., 2019). Primed hiPSCs have been reported to have unique actin and focal adhesion structures. The colony edge is decorated with thick and contractile actin stress fibers that have large focal adhesions, at both ends of the stress fibers – structures also known as contractile actin fence and cornerstone focal adhesions, respectively (**Figure 7**) (Närvä et al., 2017). The hiPSC specific focal adhesions have unique molecular architecture, including head-above-tail orientation of vinculin, and accumulation of integrin β5 and talin at the edge of the focal adhesions (Stubb et al., 2019).

In addition to actin and focal adhesions, also other actin-related proteins, such as Angiomotin-Like 2 (AMOTL2) and LIM domain and actin-binding protein 1 (LIMA1), are accumulated at the hiPSC colony edge (**Figure 7**) where the cells are more prone to differentiate compared to the cells in the middle part of hiPSC colonies (Y. Kim et al., 2022). A recent study demonstrates that LIMA1 is more highly expressed in naïve hiPSCs compared to primed hiPSCs, and suppresses membrane blebbing (Duethorn et al., 2022). Further, unlike many somatic cells, hiPSC are lacking perinuclear actin cap. However, when hiPSCs start to differentiate, perinuclear actin cap is formed upon laminA/C expression and LINC complex formation. (Khatau et al., 2012)

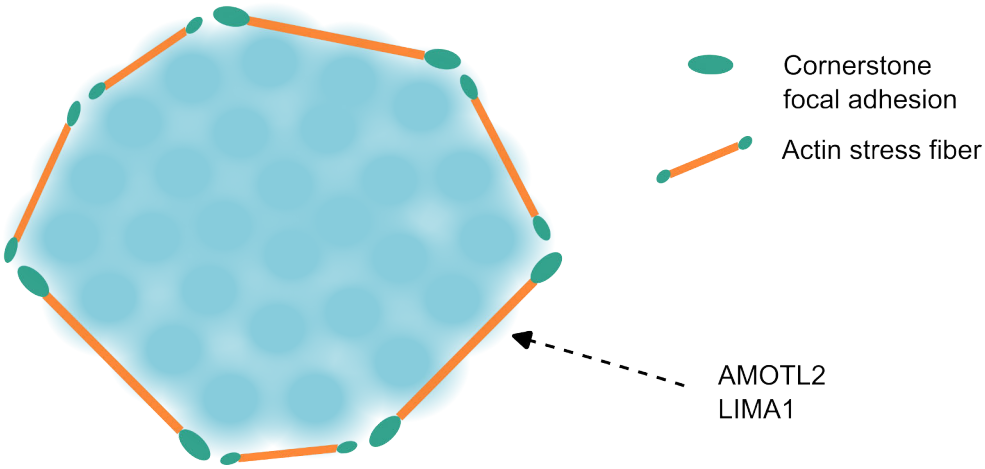

Figure 7. Simplified illustration of cornerstone focal adhesions and actin stress fibers surrounding hiPSCs colonies.

3 Aims

Integrin-mediated adhesions and actin cytoskeleton are important in cancer progression and differentiation, however their distinct roles in cancer and pluripotent stem cells remain incompletely understood. MASTL is a cell cycle regulator that has been linked to cancer progression. Further, earlier studies suggest that MASTL promotes cancer stemness-like properties, such as anchorage-independent growth and drug resistance, and may be involved in the regulation of integrins. However, the molecular mechanisms behind MASTL-mediated regulation of cancer progression and stemness remain largely unknown.

The specific aims of my thesis are:

I. To investigate the role of MASTL in breast cancer cell adhesion, actomyosin contraction and motility.

II. To determine MASTL mediated regulation of stemness in breast cancer and pluripotent stem cells.

III. To study integrin β1 and actomyosin contractility in the regulation of the primed and naïve states of human induced pluripotent stem cells

4 Materials and Methods

The methods used in original publications I-III are described here, keeping the focus on the experimental procedures where I have taken part in planning, conducting and practical implementation. The more detailed descriptions of all methods can be found from the original publications.

4.1 Cell culture methods and transfections

4.1.1 Cell lines and culture methods (I-III)

Breast cancer cell lines

Human triple-negative breast adenocarcinoma MDA-MB-231 cells (ATCC, original publication I and II) were cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% MEM non-essential amino acid solution and 2 mM l-glutamine. Human breast adenocarcinoma MCF7 cells (ATCC, original publication I) were cultured in DMEM supplemented with 10% FBS and 2 mM l-glutamine. Human breast adenocarcinoma MDA-MB-436 cells (ATCC, original publication II) were cultured in DMEM supplemented with 10% FBS, 10 µg/ml insulin, 2 mM l-glutamine and 1% penicillin-streptomycin. All aforementioned cell lines were cultured in + 37°C, 5% CO_2. For passaging, MDA-MB-231 and MCF7 cells were detached using Trypsin-EDTA solution, and MDA-MB-436 cells were detached by scraping.

Human epithelial cell lines

Human breast epithelial MCF10A cells (original publication I and II) were cultured in DMEM/F-12 supplemented with 10% horse serum, 100 ng/ml cholera toxin, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin and 1% penicillin-streptomycin in + 37°C, 5% CO_2. For passaging, cells were detached using 0.25% Trypsin-EDTA in Hanks' Balanced Salt Solution (HBSS).

Primed hiPSC lines

HiPSC lines HEL11.4 (original publication II), HEL62.4 (original publication II) and HEL24.3 (original publication III) were a kind gift from Timo Otonkoski (University of Helsinki). The cells were cultured on Matrigel (Corning) coated culture dished in Essential 8 (StemCell Technologies) medium. AICS-0016 cells (Allen institute; original publication III) were cultured on Matrigel coated plates in mTeSR1 medium (StemCell Technologies). All hiPSCs were cultured in in + 37°C, 5% CO_2, and medium was changed daily from all hiPSCs. For passaging, cell colonies were detached using 50 mM EDTA in PBS. Parental human fibroblasts used for generating HEL11.4 cell line, were cultured in DMEM/F-12 (11320033, Gibco) medium supplemented with 10% FBS in + 37°C, 5% CO_2, and detached using 0.25% Trypsin-EDTA in (HBSS) for passaging of the cells.

Feeder cells

Inactivated mouse embryonic fibroblasts (iMEF; A24903, Life Technologies) were cultured on 0.1 % gelatin (07903, Stemcell Technologies) coated dishes in DMEM/F12 (11320033, Gibco) medium supplemented with 10 % FBS (Sigma-Aldrich) at +37˚C, 5 % CO_2. The cells were washed twice with PBS and medium was replaced with naïve hiPSC culture medium before used as feeder cells for naïve hiPSCs. Alternatively, in-house iMEF feeder layers were produced by stopping the proliferation of confluent *cpdm* MEFs (Rantala et al., 2011) by treating them with Mitomycin C (M4287, Sigma) for 3h at 37˚C followed by washing with PBS.

Generation of naïve hiPSC lines

Control and anti-β1 naïve hiPSCs (original publication III) were reverted from primed HEL24.3 hiPSCs using NaïveCult Induction Kit (StemCell Technologies). The hiPSCs were cultured on inactivated mouse embryonic fibroblasts (iMEF; A24903, Life Technologies) in + 37°C, 5% O_2, 5% O_2 during the reversion. For generation and maintenance of the anti-β1 naïve hiPSC line, the culture medium was supplemented with function blocking antibody for integrin β1 (MAb13; anti-β1) throughout the reversion process, except when passaging and re-plating the cells.

Naïve hiPSC maintenance

The reverted naïve hiPSCs were cultured on iMEFs, Matrigel, vitronectin or laminin-521, in NaïveCult Expansion medium (StemCell Technologies) or home-made culture medium (full protocol in original publication III), in + 37°C, 5% O_2, 5% O_2. When passaging, freezing and thawing the cells, 10 μM ROCK inhibitor Y-27632

(StemCell Technologies) was added in the medium to ensure the survival of single cells.

4.1.2 Mammosphere formation (II)

For enriching cancer stem cells, breast cancer cells were cultured as mammospheres in MammoCult medium (StemCell Technologies). In order to do this, breast cancer cells were detached with trypsin and separated into single cells using a cell strainer. Cells were washed with MammoCult medium and plated in low-adhesion 96-well plates. The medium was changed on the following day, and the spheres were let to grow another 3-5 days. The living cells were stained with cell-permeable Calcein AM dye, imaged with Nikon Eclipse Ti-E widefield microscope, and the sphere number and size were analysed using ImageJ.

4.1.3 Plasmid transfections (I-II)

All transient plasmid transfections in the human breast and epithelial cells were performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. The DNA constructs used in this study are listed in the Table 1.

Table 1. DNA constructs used in transient transfections. WT = wild type.

DNA construct	Source	Original publication
pcDNA EGFP-C2	In house	I, II
pcDNA EGFP MASTL WT (wild type, siRNA resistant)	In house	I, II
pcDNA EGFP MASTL G44S (kinase dead, siRNA resistant)	In house	I, II
pcDNA EGFP MASTL E167D (patient mutation, siRNA resistant)	In house	I
pIND20-MRTF-A-GFP	Robert Grosse (University of Marburg, Germany)	I
mEmerald-paxillin-22	Michael Davidson (Addgene, #54219)	I
pcDNA3.1 EGFP ARHGEF2	Michael Sixt (IST, Austria)	I
pCGN-Oct-1	Winship Herr (Addgene, #53308)	II

4.1.4 siRNA transfections and shRNA transductions (I-II)

In order to silence target genes, siRNAs were transfected into cells using RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. Lentiviral shRNA transductions were performed according to the manufacturer's instruction (AMSBIO). All siRNAs and shRNAs used in this study are listed in the Table 2.

Table 2. siRNAs and shRNAs used in RNAi.

RNAi reagent	Source	Identifier	Original publication
siRNA			
Allstars negative control siRNA	Qiagen	1027281	I, II
Hs_MASTL_6 FlexiTube siRNA	Qiagen	SI02653014	I, II
Hs_MASTL_7 FlexiTube siRNA	Qiagen	SI02653182	I, II
Hs_MASTL_11 FlexiTube siRNA	Qiagen	SI04441066	I
Hs_MKL1_7 (MRTFA) FlexiTube siRNA	Qiagen	SI04172028	I
MRTFA siRNA	Sigma-Aldrich	PDSIRNA5D	I
Hs_SRF_5 FlexiTube siRNA	Qiagen	SI02757622	I
Hs_POU2F1_5 FlexiTube siRNA	Qiagen	SI03071908	II
Hs_TGFBR2_5 FlexiTube siRNA	Qiagen	SI00301910	II
Hs_TGFBR2_6 FlexiTube siRNA	Qiagen	SI02223179	II
shRNA			
pLenti-H1-shRNA negative control (RFP-PURO)	AMSBIO		II
pLenti-H1-shRNA against MASTL #3 (RFP-PURO) Rsv	AMSBIO		II

4.2 Protein and gene expression measurements

4.2.1 Western blot (I-III)

Cells were washed with phosphate-buffered saline (PBS) on ice, and lysed with TX lysis buffer (TXLB; 50 mM Tris-HCl, pH 7.5, 0.5% Triton-X, 150 mM NaCl, 0.5% glycerol, 1% SDS, Complete protease inhibitor [Sigma-Aldrich], and phos-stop tablet [Sigma-Aldrich]). Cells were collected into eppendorf-tubes by scraping,

boiled for 5 min and homogenized by sonication. Protein concentrations were measured using DC Protein assay (Bio-Rad) and normalized by adding a required amount of TXLB. SDS sample buffer was added in each sample, and samples were boiled for 5 min prior to loading on precast Tris-Glycine-eXtendet SDS-PAGE gels with a 4–20% gradient (Bio-Rad). After separation, the proteins were transferred on nitrocellulose membranes with a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked by using 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST), followed by incubation with primary antibodies at +4°C, overnight. Membranes were washed three times with TBST, incubated with secondary antibodies (indicated in Table 3) at room temperature for 1h. Membranes were washed again three times with TBST, and scanned using Odyssey infrared system (LI-COR Biosciences). The protein band intensities were analysed using ImageJ.

4.2.2 RNA sequencing (III)

RNA was extracted from cells that were treated with function blocking antibodies, using Nucleospin RNA kit (#740955.25 Macherey-Nagel). Sample quality was verified with Agilent Bioanalyzer 2100 and sample concentrations were determined by using Qubit®/Quant-IT® Fluorometric Quantitation kit (Life Technologies). Stranded mRNA Ligation kit (Illumina) was used in the library preparation to amplify RNA, and sequencing was carried out using NovaSeq 6000 S4 instrument, v1.5 (Illumina). The RNA sequencing analysis is described in the original publication III.

4.2.3 qPCR (I-III)

Cells were washed with ice-cold PBS, and total RNA was isolated using a Nucleospin RNA kit (#740955.25 Macherey-Nagel) according to manufacturers' protocol. RNA was synthesized into complementary DNA by using high capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), and the expression levels of target genes were measured with QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific). Total mRNA expression levels were normalized to GAPDH or ATCB mRNA levels.

4.3 Cell adhesion, spreading and migration assays

4.3.1 Real-time cell adhesion assay (I)

Cell adhesion was studied using xCELLigence real-time cell analyzer (RTCA) (Roche), which detects the cell index indicating the impedance of electron flow

between gold microelectrodes on the bottom of E-plate wells. The magnitude of the cell index is affected by the number and area of adherent cells on the bottom of the wells. 96-well E-plate wells were first coated with 5 µg/ml collagen, fibronectin or bovine serum albumin (BSA) overnight at + 4°C, followed by blocking with 0.1% BSA in PBS for 3h at + 37°C. The BSA coated wells were used as a negative control. The wells were washed with PBS, cell growth medium was added on each well and background readings were measured using the xCELLigence analyzer. Cells were detached using trypsin, and 2,000 cells were diluted in serum-free medium and added on each E-plate well. The attachment of the cells was followed in real time using the xCELLigence analyser by measuring impedance.

4.3.2 Cell spreading assay (I)

Plasmid-transfected or siRNA treated cells were plated in full growth medium on glass bottom plates (MatTek) or µ-slide chambered coverslips (Ibidi), that had been coated with 5 µg/ml collagen overnight at + 4°C. The cells were let to adhere for 2h, fixed with 4% paraformaldehyde (PFA) and washed with PBS. The cells were permeabilized using 0.1% Triton-X in PBS, followed by immunofluorescence staining. MDA-MB-231 and MCF7 cells were stained with Phalloidin Atto-674N (Sigma-Aldrich) to visualize F-actin and 4′,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei, in 1% BSA in PSB for 45min at room temperature. MCF10A cells were first stained with primary antibodies (indicated in the original publication I) in 1% BSA in PBS, overnight at +4°C, followed by incubation with Alexa Fluor secondary antibodies, Phalloidin Atto-647 and DAPI in 1% BSA in PBS for 45min at room temperature. The cells were washed with PBS and imaged with a 3i Spinning disk confocal microscope. The cell spreading area was analysed based on F-actin staining, using ImageJ.

4.3.3 Focal adhesion analysis (I)

SiRNA treated MCF10A cells were plated as single cells on collagen coated glass-bottom plates and the cells were let to adhere for 2h in full growth medium. The cells were fixed with 4% PFA, washed with PBS and permeabilized with 0.1% Triton-X. The cells were stained with paxillin or vinculin primary antibodies in 1% BSA in PBS overnight at +4°C, washed with PBS and incubated with Alexa Fluor secondary antibodies, Phalloidin Atto-647 and DAPI for 45min at room temperature. The cells were imaged using a 3i Spinning disk confocal microscope, and the focal adhesion number and area was analysed based on paxillin or vinculin staining using ImageJ.

4.3.4 Cell migration assay (I)

Cells that were treated with siRNAs and/or transfected with plasmid DNA, were plated on collagen-coated 24-well plate wells in full growth medium. Collagen coating was performed by incubating the wells with 5 µg/ml collagen in PBS overnight at + 4°C and washed with PBS prior to plating the cells. Free movement of the cells was followed by imaging the cells with a Nikon Eclipse Ti-E widefield microscope every 10 min for 10 h. The cell migration was quantified using ImageJ software, and Manual tracking and Chemotaxis tool plugins.

4.3.5 Integrin function blocking assays (III)

In order to study how integrin activity affects hiPSC properties and function, the cell culture medium was supplemented with 5 µg/ml integrin function blocking antibodies. Rat anti-integrin β1 (mAb13, produced in house) was used to inhibit integrin β1, mouse anti-integrin αVβ5 (MAB1961Z, Sigma-Aldrich) was used to inhibit integrin αVβ5, and rat Rat IgG2α (ab18450, Abcam) was used as an isotype control. Cells were incubated with the antibodies for 12-48h, and the integrin inhibition affect was studied by live cell imaging, immunofluorescence, RNA sequencing or western blot.

4.4 Flow cytometry-based cell cycle analysis (I)

Cells treated with siRNAs were detached using trypsin, suspended in full medium and centrifuged at 200 x g for 3 min. Cells were washed with PBS, fixed with 70% ice-cold EtOH, centrifuged at 800 x g for 4 min, and washed with 2 % FBS in PBS. Cells were re-suspended in PBS, and DNA staining was performed by incubating the cells with 50 µg/ml propidium iodide (Sigma) and 100 µg/ml RNAase for 20 min. Cells were washed with PBS and the fluorescence-activated cell sorting (FACS) was performed using FACS Calibur (BD Biosciences). The cell cycle profiles were analysed using FlowJo (BD Biosciences) software.

4.5 Methods to study actin structures

4.5.1 Micropatterning and stress fiber analysis (I)

Crossbow shaped micropatterns were produced by first coating glass coverslips with poly-L-lysine-grafted polyethylene glycol to prevent protein binding, and after that by exposing the coated coverslips to UV-light through a micropatterned photomask in order to break the poly-L-lysine-grafted polyethylene glycol bonds and thus allow

proteins to bind on the UV-exposed areas (Azioune et al., 2009). The micropatterned coverslips were coated with 5 µg/ml collagen overnight at +4°C and washed with PBS. MDA-MB-231 cells were plated on micropatterns for 2h and the cells were fixed with 4% PFA. The cells were permeabilized using 0.2 % Triton in PBS and stained with phosphorylated myosin light chain (pMLC) antibody overnight at +4°C. The cells were washed three times with PBS, and stained with Alexa Fluor 488 secondary antibody, Atto Phalloidin 647 to visualize the F-actin, and with 4′,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei, and imaged with a total internal reflection fluorescence (TIRF) microscope.

4.5.2 F- and G-actin fractionation (I)

MCF10A cells were treated with siRNAs for 48h as descriped earlier, or with F-actin polymerizing and stabilizing reagent jasplakinolide (0.1 µM), or DMSO for 30 min. Cells were washed twice with ice-cold PBS, lysed with actin lysis buffer (1 mM EDTA, 20 mM HEPES, pH 7.9, 50 mM NaCl, 0.5% Triton X-100 and EDTA-free protease inhibitors in PBS) on ice for 15 min (Grosse et al., 2003; Posern et al., 2002), and cells were collected by scraping. Fractionation was performed by ultrasentrifugation at 100,000 x g for 1 h at + 4°C, resulting in F-actin containing pellet and G-actin containing supernatant. The pellet was suspended with actin lysis buffer to reach the same volume with the G-actin fraction. SDS-PAGE loading buffer was added in both fractions, followed by protein separation by SDS-PAGE, transferring of the proteins to a nitrocellular membrane and immunoblotting with β-actin antibody to determine actin protein levels in each fraction (original publication I).

4.6 Protein translocation assays

MRTF-A translocation assay with live cell imaging (I)

MCF10A cells with stable expression of inducible pIND20-MRTF-A-GFP were treated with siRNAs for 48 h, followed by treatment with 333 ng/ml doxycycline for 24 h to induce MRTF-A-GFP expression. Prior to live-cell imaging, the cells were cultured without serum overnight. The cells were imaged using a LSM 880 (Zeiss) confocal microscope, and serum stimulation was performed under the microscope by adding 20% horse serum on the cells. The MRTF-A translocation upon serum stimulation was followed by imaging the cells every 10 s. The nuclear MRTF-A intensity was analysed by using ImageJ.

MRTF-A translocation assay with fixed cells (I)

MCF10A cells were plated on glass-bottom plates and transfected with EGFP control or EGFP MASTL WT, G44S or E167D plasmids without serum for 24 h. Serum stimulation was performed by adding 20% horse serum in the dishes, and the cells were fixed with 4% PFA 0, 2 or 5 min after adding the serum. The cells were permeabilized using 0.1% Triton in PBS, followed by incubation with MRTF-A primary antibody overnight at + 4°C. The cells were washed with PBS, incubated with Alexa Fluor 555 secondary antibody, Atto Phalloidin 647 for F-actin staining and DAPI for nuclear staining, and imaged using a 3i CSU-W1 spinning disk confocal microscope. The nuclear/cytoplasmic ratio of MRTF-A intensity was analysed using ImageJ.

4.7 Microscopy (I-III)

Cell imaging in the original publications I-III was performed using the following microscopes: 3i Marianas CSU-W1 Spinning disk confocal microscope (Intelligent Imaging Innovations, 3i Inc) with Orca Flash 4 sCMOS camera (2,048 × 2,048; Hamamatsu Photonics) and 20x (NA 0.8 air, WD 0.55 mm, Plan Apo, DIC; Zeiss) objective or 63x (NA 1.4, WD 0.19 mm, Plan-Apochromat, Zeiss) oil objective (I-III); LSM880 confocal laser scanning microscope (Zeiss) with 63x (NA 1.4, WD 0.14 mm, C Plan-Apochromat, Zeiss) objective (I); and Nikon Eclipse Ti-E widefield microscope with Hamamatsu Orca C13440 Flash 4.0 ERG (b/w) sCMOS camera, and 20x (Plan Apo lambda, NA 0.75, WD 1,000 μm) objective or 20x (Nikon CFI S Plan Fluor ELWD, NA 0.45, WD 8,200 μm) objective (I). Further details can be found from the original publications (I-III). Quantitative image analysis was performed by using ImageJ software.

4.8 Antibodies (I-III)

All primary and secondary antibodies used in western blot, immunofluorescence, flow cytometry and immunohistochemistry experiments in the original publications I-III are listed in the Table 3.

Table 3. Primary antibodies used in fluorescence-activated cell sorting (FACS), immunofluorescence (IF), immunohistochemistry (IHC) and western blot (WB).

Antibody	Source	Identifier	Method	Original publication
Primary antibodies				
Goat anti-NANOG	R&D Systems	Cat# AF1997	IF, WB	II, III
Mouse anti-AKT	Cell Signaling	Cat# 2920	WB	II
Mouse anti-active β1 integrin (12g10)	In house	N/A	IF	III
Mouse anti-CD24, Alexa Fluor 647	BD Biosciences	Cat# 561644	FACS	II
Mouse anti-ERK	Cell Signaling	Cat# 4696	WB	III
Mouse anti-GAPDH	HyTest	Cat# 5G4MAB6C5	WB	I, II
Mouse anti-integrin β1	BD Biosciences	Cat# 610468	WB	I, II
Mouse anti-MRTF-A	Santa Cruz	Cat# sc-390324	IF	I
Mouse anti-NM-2B	Abcam	Cat# ab684	WB	I
Mouse anti-paxillin	BD Biosciences	Cat# 612405	IF, WB	I, III
Mouse anti-SSEA-5	Millipore	Cat# MABD88	IF	II
Mouse anti-SOX2	R&D Systems	Cat# MAB2018	IF, WB	II
Mouse anti-TGFBR2	Santa Cruz	Cat# sc-17799	WB	II
Mouse anti-vimentin	Santa Cruz	Cat# sc-6260	WB	II
Mouse anti-vinculin	Sigma-Aldrich	Cat# V9131	IF, WB	I
Mouse anti-α-tubulin	Hybridoma bank	12g10	IF, WB	I, II
Mouse anti-β-actin	Sigma-Aldrich	Cat# A1978	WB	I, III
Rabbit anti-AKT	Cell Signaling	Cat# 9272	WB	II
Rabbit anti-CD44, FITC	BD Biosciences	Cat# 553133	FACS	II
Rabbit anti-E-cadherin	Cell Signaling	Cat# 3195	IF	I
Rabbit anti-GEFH1	Cell Signaling	Cat# 4076	WB	I
Rabbit anti-GEFH1	Abcam	Cat# ab155785	IF	I
Rabbit anti-integrin β3	Abcam	Cat# ab179473	WB	II
Rabbit anti-integrin β3	Abcam	Cat# ab75872	IHC	II
Rabbit anti-KLF17	Sigma-Aldrich	Cat# HPA024629	IF	III
Rabbit anti-MASTL	Sigma-Aldrich	Cat# HPA027175	WB, IHC	I, II
Rabbit anti-MASTL	Abcam	Cat# ab86387	WB	I, II

Rabbit anti-MLC 2	Cell Signaling	Cat# 3672	WB	I
Rabbit anti-OCT1	Proteintech	Cat# 10387-1-AP	WB	II
Rabbit anti-OCT3/4	Santa Cruz	Cat# sc-9081	WB	II, III
Rabbit anti-phospho-AKT (S473)	Cell Signaling	Cat# 9271	WB	II
Rabbit anti-phospho-ERK (T202/Y204)	Cell Signaling	Cat# 4370	WB	III
Rabbit anti-phospho-GEFH1 (S886)	Cell Signaling	Cat# 14143	WB	I
Rabbit anti-phospho-paxillin (Y118)	Cell Signaling	Cat# 2541	WB	I
Rabbit anti-phospho-MLC 2 (T18/S19)	Cell Signaling	Cat# 3674	IF, WB	I, III
Rabbit anti-phospho-MLC (S20)	Abcam	Cat# ab2480	WB	I
Rabbit anti-phospho-SMAD3 (S423/425)	Cell Signaling	Cat# 9520	WB	II
Rabbit anti-SMAD3	Abcam	Cat# ab28379	WB	II
Rabbit anti-tropomyosin 4.2 (Delta Tm 9d)	Prof. P. Gunning (UNSW, Sydney, Australia)	(Schevzov, et al., 2011)	IF, WB	I
Rabbit anti-β-catenin	Abcam	Cat# ab32572	IF	I
Secondary antibodies				
Donkey anti-mouse IgG (H+L), highly cross-adsorbed, Alexa Fluor 568	Invitrogen	Cat# A10037	IF	I, II, III
Donkey anti-mouse IgG (H+L), highly cross-adsorbed, Alexa Fluor 488	Invitrogen	Cat# A-21202	IF	I, II, III
Donkey anti-mouse IgG (H+L), IRDye 680RD	LI-COR Biosciences	Cat# 926-68072	WB	I, II, III
Donkey anti-rabbit IgG (H+L), highly cross-adsorbed, Alexa Fluor 488	Invitrogen	Cat# A-21206	IF	I, II, III
Donkey anti-rabbit IgG (H+L), highly cross-adsorbed, Alexa Fluor 568	Invitrogen	Cat# A10042	IF	III
Donkey anti-rabbit IgG (H+L), IRDye 800CW	LI-COR Biosciences	Cat# 926-32213	WB	I, II, III

5 Results

5.1 MASTL kinase-independently regulates cell motility, contractility and stemness (I, II)

5.1.1 MASTL regulates cell spreading and migration in a cell cycle- and kinase-independent manner (I)

MASTL has been linked to cancer invasiveness and metastasis *in vivo* (Rogers et al., 2018; Vera et al., 2015), yet the molecular mechanisms have not been studied in detail. A previous RNA interference (RNAi) screen that was performed in order to predict new integrin activity regulators, demonstrated that MASTL is a potential regulator of integrin β1 activity (Pellinen et al., 2012). Since integrins are key regulators of cell motility, we wanted to further investigate how MASTL regulates integrin-mediated cell adhesion and cell migration in cancer cells. First, we investigated how MASTL depletion affects cancer cell morphology and motility. We silenced MASTL using siRNAs in MDA-MB-231 and MCF7 breast cancer cells that have high endogenous MASTL expression (I, Figure S2A). We discovered that MASTL depletion increased cell spreading on collagen coated dishes in both cell lines (I, Figure 1, S1, S2B-C). In contrast, EGFP MASTL overexpression decreased the cell spreading compared to the control EGFP transfected cells (I, Figure 1D-E). Interestingly, the cell spreading phenotype after MASTL silencing was rescued when the MASTL silenced MDA-MB-231 cells were transfected to re-express either wild-type (WT) or kinase-dead (G44S) mutant EGFP MASTL (I, Figure 1F-I and S1F). This demonstrates that the cell spreading phenotype is not an artefact caused by RNAi off-targets, and indicates that MASTL regulates breast cancer cell spreading in a kinase-independent manner. In addition, MASTL silencing increased the spreading of breast epithelial MCF10A cells when cultured as single cells (I, Figure 2A-B) or as a monolayer (I, Figure 3A-C). The MASTL silenced monolayer cells were more flat compared to the control cells (I, Figure 3B), and occasionally the cell-cell junctions were disrupted resulting in gaps in the cell monolayer (I, Figure 3D).

Next we studied if the cell spreading upon MASTL silencing affected the cell motility. We plated the control and MASTL silenced MDA-MB-231 breast cancer cells on collagen, and followed the free cell movement using live-cell imaging with

a wild-field microscope. While the control cells migrated rapidly and changed their shape constantly, the MASTL silenced cells remained stationary, round and flat (I, Figure 7A-C, Video 1 and 2). In line with this, MASTL silencing also decreased MDA-MB-231 cell invasion into collagen gels (I, Figure 10D-E).

Integrin-mediated focal adhesion formation and dynamics are important in regulation of cancer cell migration and invasion (Hamidi and Ivaska, 2018). Thus, we wanted to investigate whether MASTL could affect the focal adhesion assembly and dynamics. Surprisingly, MASTL depletion did not have any effect on the focal adhesion size based on paxillin immunofluorescence staining (I, Figure 2C). To further investigate the focal adhesion dynamics, we performed live-cell imaging on MDA-MB-231 cells stably expressing m-Emerald paxillin by using a confocal microscope. Interestingly, the focal adhesion assembly, disassembly or lifetime were not altered upon MASTL silencing (I, Figure 7D-G). In addition, we could not detect any changes in the integrin β1 activity (I, Figure 2E-H and S2D-G) or paxillin phosphorylation (I, Figure 7H-I), which is known to regulate focal adhesion assembly (Zaidel-Bar et al., 2007).

Since MASTL is a known cell cycle regulator (Castro and Lorca, 2018), we wanted to ensure that the increased cell spreading and impaired cell motility after MASTL silencing are not due to changes in the cell cycle regulation. We were able to show that the 48 h MASTL silencing time, which was used in all of our experiments, did not affect the cell cycle profile (I, Figure 2I and S2H-I). Taken together, these findings indicate that MASTL regulates cancer cell spreading and motility independently of focal adhesion dynamics, cell cycle or MASTL's kinase activity.

5.1.2 MASTL affects actin cytoskeletal structures (I)

To understand the mechanisms behind MASTL-mediated cell spreading and motility, we performed a genome-wide transcriptome analysis and a stable isotope labelling with amino acids in cell culture (SILAC) -based mass spectrometry proteomic analysis in MASTL silenced MDA-MB-231 cells. This transcriptomic and proteomic profiling revealed that MASTL regulates several genes related to cell adhesion, motility and actin cytoskeleton (I, Figure 4 and 5A). One of the downregulated hits after MASTL silencing was Rho guanine nucleotide exchange factor 2 (GEF-H1, *ARHGEF2*), that is an important regulator of actin stress fiber and actin meshwork formation (Birkenfeld et al., 2008; Joo and Olson, 2021). MASTL silencing also decreased actomyosin contraction regulators, such as a motor protein non-muscle myosin II B (NM-2B, *MYH10*) that moves actin filaments, and Tropomyosin 4.2 (Tpm4.2, *TPM4*), which binds to actin filaments, and recruits and activates non-muscle myosins at the actin stress fibers (Manstein et al., 2020). We

were able to validate the downregulation of GEF-H1, NM-2B and Tpm4.2 upon MASTL silencing also on mRNA level by using qPCR (I, Figure 5B), and on protein level by using western blot (I, Figure 5C-H and S4A) and immunofluorescence (I, Figure S4B) in MDA-MB-231 cells. Re-expression of siRNA resistant EGPF MASTL WT overturned the downregulation of GEF-H1, NM-2B and Tpm4.2 in the MASTL silenced cells (I, Figure 5I), confirming that the altered gene expression is not an RNAi off-target effect. Further, expression of EGFP GEF-H1 in MASTL depleted cells decreased the cell spreading (I, Figure 5J-K), indicating that GEF-H1 can regulate cell contractility independently of MASTL. In addition to cancer cells, we observed reduced GEF-H1 (I, Figure S4C) and Tpm4.2 (I, Figure S4D) levels also in MCF10A cells upon MASTL silencing, implying that MASTL-mediated actin regulation is not only restricted to cancer cells.

Next, we wanted to study how actin structures are affected upon MASTL silencing. We plated control and MASTL depleted MDA-MB-231 cells on collagen coated micropatterns that mimic the shape of a migrating cell, and performed an immunofluorescence staining of F-actin and phosphorylated myosin light chain (pMLC), which is an essential in actomyosin contraction (Murrell et al., 2015). We observed pMLC-positive actin stress fibers across the control cells, but the amount of these structures was significantly decreased after MASTL silencing (I, Figure 6A-B). In contrast, the MASTL depleted cells had wider lamellipodia-like structures compared to the control cells (I, Figure 6A and 6C). The pMLC protein levels were significantly decreased after MASTL silencing based on western blot analysis (I, Figure 6D-E), and the reduction of pMLC was reversed after re-expression of siRNA resistant EGFP MASTL WT in the MASTL silenced MDA-MB-231 cells (I, Figure 6F). These results indicate that MASTL facilitates actin stress fiber formation and contraction by affecting transcription of actin cytoskeleton regulators.

5.1.3 MASTL mediates cell contractility via transcription factors MRTF-A and SRF (I)

SRF and MRTF-A are mechanoresponsive transcription factors that jointly regulate transcription of genes related to cell motility and actin cytoskeleton (Gau and Roy, 2018; Onuh and Qiu, 2021). Interestingly, GEF-H1 has been shown to regulate SRF activity (Itoh et al., 2014), and Tpm4.2 is one of SRF target genes (Esnault et al., 2014). This prompted us to study the possible effect of MASTL in SRF transcriptional activity. Silencing of MASTL in MCF10A cells reduced the mRNA levels of vinculin and FOS, which are known SRF target genes (I, Figure 8A). Vinculin protein levels were also significantly decreased upon MASTL silencing based on western blot analysis (I, Figure 8B-C). Consistently, we detected less vinculin immunofluorescence staining at the focal adhesions (I, Figure S5B), and

also the total vinculin intensity was decreased after MASTL silencing in MCF10A cells (I, Figure S5C).

To study the SRF transcriptional activity, we silenced MASTL and SRF in MCF10A cells stably expressing luciferase reporter with MRTF-SRF specific promoter, and followed the luciferase activity upon serum stimulation. MASTL silencing prevented SRF transcriptional activity similarly to the SRF silencing (I, Figure 8D). In contrast, overexpression of wild-type or kinase-dead (G44S) mutant EGFP MASTL significantly increased the SRF transcriptional activity in MCF7 cells (I, Figure 8E). We also wanted to investigate thrombocytopenia-associated MASTL E167D patient mutation, since it has been linked to cytoskeletal dysregulation in platelets (Hurtado et al., 2018). Interestingly, overexpression of E167D mutant EGFP MASTL did not affect SRF activity in MCF7 cells (I, Figure S5D).

MRTF-A and SRF activity are highly dependent on the G-actin concentration within the cells. High G-actin concentration promotes cytoplasmic accumulation of MRTF-A, whereas the decrease of G-actin concentration upon actin polymerization facilitates MRTF-A nuclear localization and SRF transcriptional activity. (Gau and Roy, 2018) To study how MASTL affects G-actin concentration, we performed a G-actin/F-actin fractionation assay in MASTL-silenced MCF10A cells. While an actin polymerizing drug jasplakinolide, which was used as a positive control, increased the F-actin/G-actin ratio in the control cells, MASTL silencing did not affect the F-actin/G-actin ratio under normal culture conditions (I, Figure S5E-F). However, MASTL depletion blocked the nuclear translocation of MRTF-A upon serum stimulation in MCF10A cells, based on live cell imaging (I, Figure 9A). In contrast, overexpression of wild-type and kinase-dead EGFP MASTL accelerated the MRTF-A nuclear transport after serum stimulation, whereas the E167D patient mutant MASTL did not affect the MRTF-A transport rate (I, Figure 9B). These results imply that MASTL facilitates the MRTF-A nuclear localization and SRF activity without affecting the F-actin/G-actin ratio.

Since MASTL has been reported to mainly localize into the nucleus during interphase (Álvarez-Fernández et al., 2013), we wanted to investigate whether MASTL could associate with MRTF-A. We performed a GFP pull-down assay in MCF7 cells overexpressed with EGFP (control), wild-type or kinase-dead EGFP MASTL, and observed an association of MRTF-A with MASTL wild-type and kinase-dead mutant, but not with the EGFP control (I, Figure 9C). Further, to study the nuclear retention of MRTF-A, we performed a fluorescence loss in photobleaching (FLIP) analysis in GFP-MRTF-A expressing MCF7 cells after MASTL silencing and serum stimulation. Repeated bleaching of the cytoplasmic MRTF-A caused an enhanced loss of nuclear MRTF-A on the MASTL depleted cells compared to the control cells (I, Figure 9D-F). Taken together, these results indicate

that MASTL associates with MRTF-A in a kinase-independent manner, and that MASTL facilitates the nuclear retention of MRTF-A (**Figure 8**).

Finally, we compared the effects of MASTL and MRTF-A depletion in 2D and 3D environments. We found that MRTF-A silencing induced cell spreading similarly to MASTL silencing in MCF10A cells plated on 2D collagen coated dishes (I, Figure 10A-B). Our western blot analysis revealed that MRTF-A depletion decreased the protein levels of vinculin, a known SRF target gene (Miralles et al., 2003), but not paxillin or GEF-H1, which are not SRF targets (Itoh et al., 2014; Ly et al., 2013) (I, Figure 10C). These results were similar in MASTL depleted cells, except that GEF-H1 levels were decreased upon MASTL silencing (I, Figure 2A and 8B). In addition, MRTF-A depletion reduced breast cancer cell invasion and induced cell rounding in 3D collagen I matrix similarly to MASTL depletion (I, Figure 10C-G). Importantly, MASTL silencing also caused cytoplasmic accumulation of MRTF-A in breast cancer cells in 3D (I, Figure 10H-I). These results imply that MASTL is essential for MRTF-A-mediated cancer cell invasion in 3D environments and regulates GEF-H1 through, yet an unknown SRF-independent mechanism (**Figure 8**).

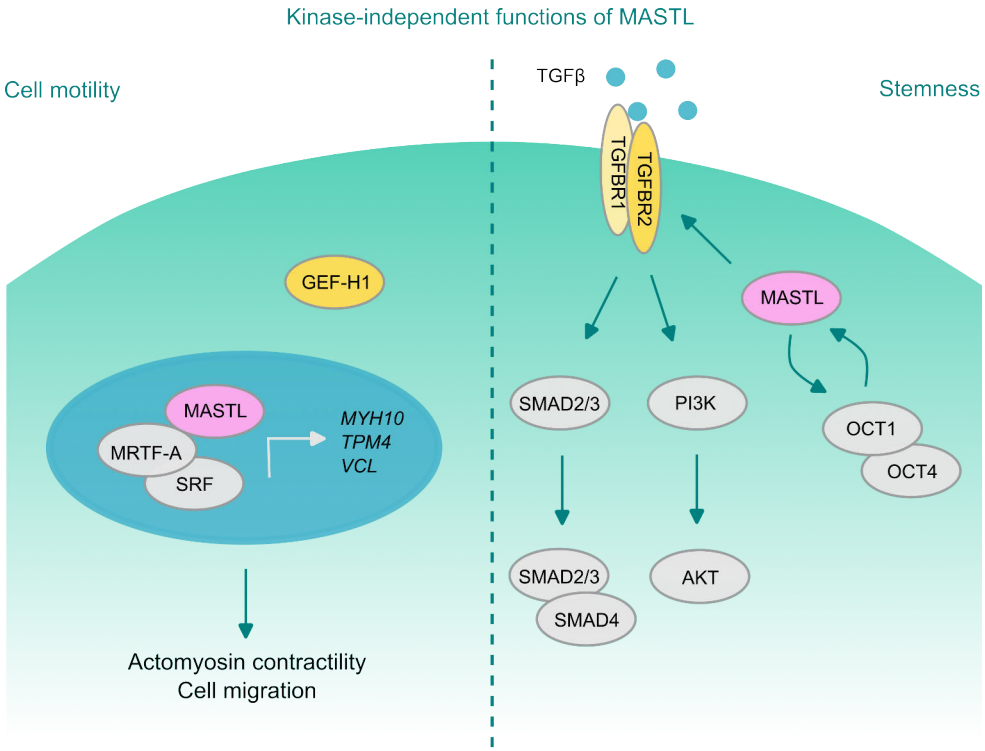

Figure 8. Schematic illustration of kinase-independent functions of MASTL in cell motility (original publication I) and stemness (original publication II). *Modified from original publication II*.

5.1.4 MASTL supports stemness in cancer cells and pluripotent stem cells (II)

MASTL upregulation has been linked to recurrence and poor patient outcome in many cancers. (Conway et al., 2020; Marzec and Burgess, 2018) Cancer stem cells are considered to be a potential driving force in cancer recurrence and treatment resistance (Najafi et al., 2019). Interestingly, MASTL has been shown to facilitate anchorage-independent growth (Rogers et al., 2018; Vera et al., 2015; Yoon et al., 2018), a characteristic associated with CSCs. In addition, MASTL has been reported to promote radiation resistant breast cancer stem cell (BCSC) formation (Yoon et al., 2018). Since the molecular mechanisms behind this, however, have remained elusive, we studied how MASTL could influence cancer stemness. We studied breast cancer patient samples collected from a large randomized FinHer trial (Joensuu et al., 2009) using immunohistochemistry, and discovered a correlation between expression of MASTL and integrin β3 (II, Figure 1D), which is associated with breast cancer stemness and drug resistance (Lo et al., 2012; Seguin et al., 2015; Vaillant et al., 2008). This prompted us to further study MASTL and integrin β3 expression in different breast cancer cell lines using the Cancer Cell Line Encyclopedia (CCLE) database. We found that MASTL expression was significantly higher in cell lines with high integrin β3 expression compared to cell lines with low integrin β3 levels (II, Figure S2A). In order to study MASTL and integrin β3 expression in cancer stem cells, we cultured breast cancer cells as mammospheres, a technique used for BCSC enrichment (Dontu et al., 2003; Ponti et al., 2005). Interestingly, MASTL and integrin β3 levels were significantly increased in sphere cultures compared to regular 2D cultured MDA-MB-231 cells (II, Figure 2A-B). In contrast, MASTL depletion decreased the size of mammospheres derived from MDA-MB-231 and MDA-MB-436 triple negative breast cancer cells (II, Figure 2I-J, S2E-H).

To further investigate MASTL in cancer stemness, we studied how MASTL affects ROS signaling. Low ROS levels are an indicator of cancer stem cells (Diehn et al., 2009). Silencing of MASTL promoted ROS activity in MDA-MB-231 cells (II, Figure 2G-H). Further, silencing of MASTL decreased cell surface levels of CD44 (II, Figure 2M-N), an established cancer stem cell marker (Najafi et al., 2019). Together these findings indicate that high MASTL levels support breast cancer stemness.

In order to broaden our understanding in MASTL-mediated regulation of stemness in general, we studied MASTL expression in pluripotent stem cells. According to the Amazonia gene expression database, MASTL expression was significantly higher in pluripotent stem cells compared to fibroblasts or other somatic cells (II, Figure 3A). Consistently, MASTL expression was downregulated when hiPSCs were differentiated into fibroblasts or embryonic bodies (II, Figure 3B, 3D),

and MASTL levels were significantly higher in hiPSCs compared to their parental fibroblasts (II, Figure 3I). Further, 48h MASTL depletion in hiPSCs led to significant downregulation of key pluripotency regulators OCT4 and NANOG (II, Figure 3J-L). HiPSCs were also more sensitive to MASTL inhibitor GKI-1 compared to the parental fibroblasts (II, Figure S2B-C). However, 48h incubation with low doses of GKI-1 did not alter pluripotency factor OCT4 or NANOG levels (II, Figure S3D), suggesting that MASTL might support stemness in a kinase-independent manner.

5.1.5 MASTL affects transcriptional stemness regulators OCT1 and OCT4 (II)

Transcription factor OCT1 is an established stemness regulator in somatic and cancer stem cells (Maddox et al., 2012). In line with our abovementioned findings of MASTL supporting stemness, MASTL depletion in breast cancer cells led to a significant downregulation of OCT1 (II, Figure 2C-D, 2K-L). Conversely, OCT1 overexpression increased MASTL levels in MDA-MB-231 cells (II, Figure 2E-F). Further, OCT4, a homologous transcription factor with OCT1, was downregulated after MASTL depletion in hiPSCs (II, Figure 3J-K). OCT4 is an essential factor in pluripotency maintenance and generation of hiPSCs (Obinata et al., 2022). In addition, OCT1 and OCT4 are known to share a similar DNA binding site sequence, an octamer motif 5'-ATGCAAAT-3'(Schöler et al., 1989; Vázquez-Arreguín and Tantin, 2016). Interestingly, we detected three OCT1/OCT4 specific octamer motifs in the MASTL promoter sequence (II, Figure S2D). These results indicate that MASTL regulates OCT1 levels in cancer stem cells and OCT4 levels in pluripotent stem cells, and suggest that OCT1 and OCT4 might regulate MASTL gene transcription (**Figure 8**).

5.1.6 MASTL supports TGF-β signaling in cancer and pluripotent stem cells (II)

Since cell surface proteins are important in cancer stem cell and pluripotent stem cell function and characterization (Kim and Ryu, 2017), we studied how MASTL affects cell surface proteins using a proteomic approach. We performed a SILAC-based surfaceome analysis in MASTL silenced MDA-MB-231 cells and discovered that MASTL silencing decreased the surface levels of several proteins related to tissue development (II, Figure 4). One of the downregulated hits, transforming growth factor β (TGF-β) receptor II (TGFBR2), caught our attention, since it has been linked to stemness. TGF-β binding to TGFBR2 can induce canonical and non-canonical signaling pathways that are important in breast cancer stem cell and pluripotent stem cell maintenance (James et al., 2005; Lo et al., 2012; Zhang, 2009). TGFBR2 levels

were also decreased at the mRNA and total protein level upon MASTL silencing (II, Figure S4A) according to our earlier transcriptome and total proteomic analysis done in MDA-MB-231 cells (original publication I). Further, we validated the downregulation of TGFBR2 in MASTL depleted MDA-MB-231 cells using qPCR and western blot (II, Figure 5A-C). We also studied expression of the other TGF-β family members, TGFBR1 and TGFBR3. TGFBR1 levels were not altered upon MASTL depletion in MDA-MB-231 cells (II, Figure S5A). TGFBR3 mRNA levels were downregulated after MASTL silencing with one siRNA (II, Figure S5B), however, the total protein levels were not altered (original publication I). Further, when MASTL was overexpressed in MCF10A cells, both, the wild-type and the kinase dead mutant (G44S) EGFP MASTL increased TGFBR2 protein levels (II, Figure 5F-G). In line with this, MASTL kinase function inhibition with GKI-1 did not affect TGFBR2 levels in MDA-MB-231 cells (II, Figure 5H-I). These results indicate that MASTL positively regulates TGFBR2 expression in a kinase-independent manner.

In addition to our findings on MASTL-mediated TGFBR2 expression, we studied how MASTL influences TGF-β signaling pathways. TGF-β can activate many signaling pathways via TGFBR2, including non-canonical phosphoinositide 3-kinase (PI3K) – protein kinase B (AKT, also known as PKB) pathway (Zhang, 2009), that is important in the maintenance of pluripotency and cancer stemness (Yu and Cui, 2016). MASTL has been linked to AKT activation (Rogers et al., 2018; Vera et al., 2015), but the mechanism behind this has remained unknown. MASTL depletion decreased AKT phosphorylation in MDA-MB-231 cells (II, Figure 6A-B). However, MASTL inhibition with GKI-1 did not alter AKT activation upon TGF-β stimulation (II, Figure 6C-D), implying that MASTL kinase function is not needed for MASTL mediated AKT activation.

In addition to non-canonical signaling, TGFBR2 can also activate canonical SMAD signaling pathways that are linked to stemness (Massagué, 2012). Especially SMAD2 and SMAD3 have been shown to be essential pluripotency regulators (Mullen and Wrana, 2017). MASTL depletion significantly decreased SMAD3 activation in MDA-MB-231 cells (II, Figure 6E-F). In addition, silencing of TGFBR2 in hiPSCs decreased OCT4 and NANOG levels to the same extent as MASTL silencing (II, Figure 6G). Taken together, these findings indicate that MASTL supports stemness kinase-independently by regulating TGFBR2 levels, and by facilitating downstream non-canonical PI3K/AKT and canonical SMAD2/3 signaling (**Figure 8**).

5.2 Integrin β1 and the actin cytoskeleton regulate human induced pluripotent stem cell's naïve and primed states (III)

5.2.1 Integrin β1 activity varies in primed and naïve hiPSCs on different ECMs (III)

Integrins play important roles in embryonic development and regulation of cell states in different biological contexts (Hynes, 2002). Integrin β1 has been shown to be essential in the post-implantation mouse embryo development and survival, but is not required for the pre-implantation ICM formation (Molè et al., 2021; Stephens et al., 1995). According to a recent cell surface proteomic study, primed hiPSCs, that resemble the post-implantation ICM cells, have more integrin β1 on the cell surface compared to naïve hiPSCs that resemble the pre-implantation ICM cells (Wojdyla et al., 2020). However, the regulation and activity of integrin β1 in the naïve and primed states has not been studied. In order to investigate this, we plated primed and naïve PSCs on Matrigel, vitronectin and laminin 521 – ECMs frequently used in iPSC culture, and performed immunofluorescence staining of active integrin β1 (III, Figure 1A-C, S1A). In the primed hiPSCs, β1 activity was highest in the cells plated on Matrigel and laminin 521, and lowest on vitronectin (III, Figure 1A-B). Active β1 integrin was also differently distributed on these matrices. On vitronectin, active integrin β1 was located at large focal adhesions at the colony edge, as reported earlier (Närvä et al., 2017; Stubb et al., 2019). On Matrigel and laminin 521, integrin β1 was located mainly at the edge but also to some extent at the middle part of the colonies (III, Figure 1A-B). Surprisingly, naïve hiPSC, that were derived from primed hiPSCs using an earlier published chemical reversion protocol (Guo et al., 2017), had higher active integrin β1 levels compared to primed hiPSCs on all matrices (III, Figure 1A-B). We observed high integrin β1 activity on the same matrices also in naïve hESCs that were obtained from primed hESCs using the same reversion protocol (III, Figure S1A). Despite the high integrin activity, naïve PSCs lacked focal adhesion structures on all matrices, and integrins were distributed evenly throughout the whole colony area (III, Figure 1A, S1A). In addition, naïve hiPSCs lost their typical tightly packed colony morphology when plated on laminin 521, whereas on vitronectin, where the integrin β1 activity was lowest, the colonies remained compact (III, Figure 1A-C). These results suggest that the ECM-integrin connections might have a great impact on the naïve hiPSCs colony morphology.

5.2.2 Focal adhesions and stress fibers are lost during reversion into naïve-like state in hiPSCs (III)

As already mentioned, primed hiPSCs have prominent focal adhesions and actin stress fibers surrounding the colonies (III, Figure 1A, 2D) (Närvä et al., 2017; Stubb et al., 2019), whereas naïve PSCs lack these structures (III, Figure 1A, S1A). To study how the focal adhesions are changed during reversion into naïve-like state, we collected small amounts of cells at different time points during the 48-day reversion, and plated them on vitronectin, Matrigel and laminin 521. We performed an immunofluorescence staining of F-actin, KLF17, a naïve PSC marker, and paxillin, a main focal adhesion component. On the 10th day of the reversion, some hiPSCs had already started to express KLF17 (III, Figure 2E, S2C-D). Interestingly, these KLF17-positive cells did not have clear focal adhesions or actin stress fibers, whereas the KLF17-negative cells on the same culture dishes had large focal adhesions and thick actin stress fibers that are typical for primed hiPSCs (III, Figure 2E, S2C-D). This suggests that the transition from primed to naïve state involves loss of focal adhesions and dramatic changes in actomyosin structures.

5.2.3 Integrin β1 inhibition induces naïve-like colony morphology and gene expression (III)

To further study the impact of integrin β1 activity in hiPSC colony morphology, we treated the primed hiPSCs with an integrin β1 function-blocking antibody (Mab13, anti-β1). A short-term (12h) incubation with anti-β1 did not alter pluripotency factor OCT4 levels in primed hiPSCs (III, Figure S1B-C), but had a significant impact on the colony morphology. While the control primed hiPSC colonies were round and flat, integrin inhibition decreased the colony area (III, Figure 1D-E), but increased the cell clustering (III, Figure 1F-G) and colony height (III, Figure 1F-G). Overall, the integrin β1 inhibition triggered a tightly packed and dome-like colony morphology, which resembled the morphology typical for naïve hiPSCs (**Figure 9**).

Earlier studies show that primed hiPSCs have a strong contractile actin fence and large 'cornerstone' focal adhesions surrounding the colony (Närvä et al., 2017), and that integrin β1 and αVβ5 are differently located within these focal adhesions (Stubb et al., 2019). Thus, we wanted to study how inhibition of integrin αVβ5 with a function blocking antibody affects the hiPSC colony morphology and actomyosin contraction. Integrin β1 and αVβ5 blocking antibodies did not alter OCT4 protein levels in primed hiPSCs after 24h incubation (III, Figure S3A-B). While integrin β1 inhibition induced cell clustering, inhibition of integrin αVβ5 did not have any clear effect on the colony morphology in primed hiPSCs (III, Figure 3A-B). We wanted to study if the integrin β1 inhibition induced cell clustering could be caused by increased contractility. Surprisingly, the integrin β1-inhibited primed hiPSC colonies

had less phosphorylated MLC compared to the control cells, whereas αVβ5 inhibition did not have a significant effect on pMLC levels (III, Figure 3A-C). We obtained similar results also when using another primed hiPSC line (III, Figure S3C-E). These findings indicate that integrin β1 inhibition induces cell clustering but decreases actomyosin contractility in primed hiPSC colonies.

To study how integrin β1 inhibition affects naïve hiPSC morphology and function, we generated an anti-β1 naïve hiPSC line by keeping the anti-β1 function blocking antibody in the culture medium throughout and after the reversion (III, Figure 2A). The hiPSCs reached a typical naïve-like colony morphology in the presence of anti-β1 (III, Figure 2B). Interestingly, integrin β1 inhibition increased the proliferation of hiPSCs during the reversion when compared to control hiPSC that were reverted without anti-β1 (III, Figure S2A-B). Further, the primed hiPSC markers *ZIC2* and *SFRP2* were lost in control and anti-β1 naïve hiPSCs as expected, whereas the relative expression of naïve markers *KLF17* and *TBX3* was higher in the anti-β1 naïve hiPSCs compared to the control naïve hiPSCs (III, Figure 2C). These results suggest that integrin β1 inhibition may support the reversion into naïve-like state, but further investigation is needed to confirm this.

To study how integrin inhibition affects naïve hiPSC morphology and actomyosin contractility, we treated the control and anti-β1 naïve hiPSCs with IgG control or anti-β1 function blocking antibody (III, Figure 3D). Interestingly, replacement of anti-β1 with IgG control in the anti-β1 naïve hiPSCs, that were reverted and cultured in the presence of anti-β1, triggered a flattening of the colonies after 48 h (III, Figure 3E). Integrin inhibition had no effect on the control naïve hiPSC colony morphology, whereas cell clustering was decreased in the IgG treated anti-β1 naïve hiPSCs (III, Figure 3F-H). However, integrin inhibition did not alter the pMLC levels in the control or the anti-β1 naïve hiPSCs (III, Figure 3F-I).

Finally, we performed an unbiased genome-wide transcriptome analysis to get a better understanding of the possible mechanisms leading to changes in colony morphology and actomyosin contractility in primed and naïve hiPSCs. The analysis revealed that short-term (12 h) integrin β1 inhibition in primed hiPSCs altered expression of 10 genes (III, Figure 4A). 9 of the differentially expressed genes were downregulated upon integrin β1 inhibition, including *PDGFB* and *CCN2* that are positive regulators of extracellular signal-regulated kinase (ERK) signaling according to gene ontology annotations. In contrast, *DUSP8*, that was upregulated after integrin β1 inhibition, has been shown to negatively regulate ERK (Ding et al., 2019). In line with these results, integrin β1 inhibition decreased phosphorylated ERK protein levels in primed hiPSCs (III, Figure 4B-C). Since inhibition of ERK signaling has been shown to support naïve state in mouse embryos and human PSCs (Nichols and Smith, 2009; Takashima et al., 2014), our findings regarding the

attenuated ERK signaling support the idea that integrin inhibition induces naïve-like features in hiPSCs.

We also compared the control and anti-β1 naïve hiPSCs in our transcriptome analysis, which revealed 36 differentially expressed genes (III, Figure 4D). Continuous integrin β1 inhibition caused downregulation of 5 and upregulation of 31 genes. We found that three actin cytoskeleton related genes, *PHACTR-1*, *ACTA2* and *FILIP1* (Allain et al., 2012; Jarray et al., 2011; Nagano et al., 2002; Schildmeyer et al., 2000; Wiezlak et al., 2012), were upregulated in the anti-β1 naïve hiPSCs. This may partly explain the different colony morphology properties between the control and anti-β1 naïve hiPSCs (III, Figure 3). In addition, many genes related to embryonic development and differentiation were differentially expressed. Integrin β1 inhibition decreased the expression of *HOXB1* and *GLI2* that are linked to the pattern specification process, and increased the expression of *SIX2* and *PRAME* that negatively regulate cell differentiation (III, Figure 4E). These findings imply that continuous integrin β1 inhibition might support the naïve-like state by affecting the transcriptional patterns in hiPSCs (**Figure 9**).

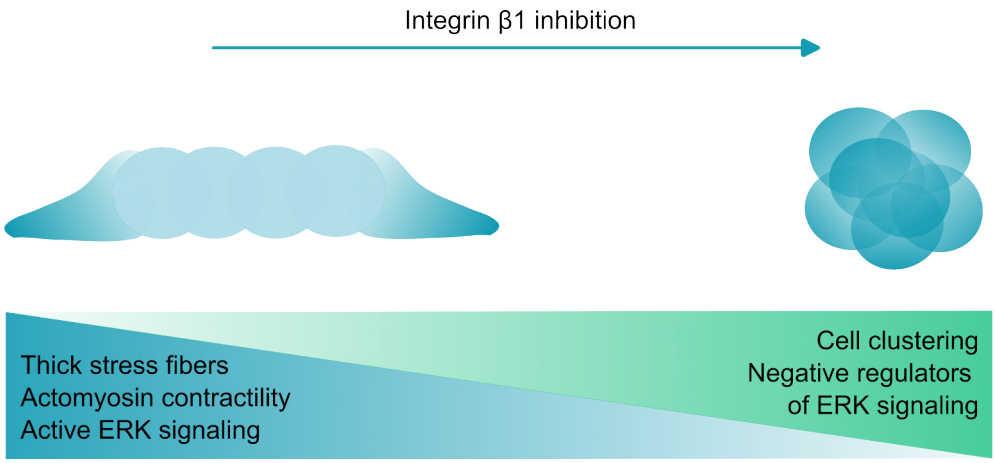

Figure 9. Simplified illustration of cornerstone focal adhesions and actin stress fibers surrounding hiPSCs colonies.

6 Discussion

6.1 MASTL in regulation of the actin cytoskeleton and cell motility (I)

Although MASTL kinase function in cell cycle regulation has been extensively studied (Castro and Lorca, 2018; Vigneron et al., 2016), the cell cycle-independent and kinase-independent functions of MASTL have remained unclear. Our studies (original publication I) revealed previously unknown kinase-independent functions of MASTL as a transcriptional regulator of genes involved in regulation of actin cytoskeleton, contractility and cell motility. MASTL depletion induced cell spreading while decreasing actin stress fiber formation, contractility, invasion and migration. Further, we show that MASTL regulates transcription of GEF-H1 (ARHGEF2) and MRTF-A/SRF target genes, such as Tpm4.2 (TPM4) and NM-2B (MYH10). MASTL associated with MRTF-A, and facilitated MRTF-A/SRF transcriptional activity independently of MASTL kinase activity.

Previous studies have demonstrated that MASTL influences cell motility. MASTL overexpression has been shown to increase MDA-MB-231 breast cancer cell migration (Vera et al., 2015), and disrupt collective cell migration in MCF10A breast epithelial cells (Rogers et al., 2018). However, the molecular mechanisms behind MASTL mediated cell motility have not been studied in detail. Actomyosin contractility is a major player in regulation of cell shape and motility (Pollard and Cooper, 2009). We show that MASTL depletion induces cell spreading (I, Figure 1, 2, S2), and reduces cell migration in breast cancer cells (I, Figure 7). Further, we demonstrate a drastic decrease in contractile actin stress fibers upon MASTL silencing (I, Figure 6), which may largely explain the disrupted cell shape and migratory behavior. Importantly, our findings indicate that MASTL mediated regulation of cell spreading is independent of MASTL kinase activity (I, Figure 1G-I). In line with our results, a previous study shows that overexpression of MASTL kinase dead mutant (G44S) in MDA-MB-231 cells does not affect cell migration (Vera et al., 2015). This finding might be due to high endogenous MASTL levels in MDA-MB-231 cells, yet the direct effect of kinase activity or inactivity in cell migration needs to be investigated further. Interestingly, MASTL overexpression has also been shown to disturb cell-cell contacts in MCF10A breast epithelial cells that

have low endogenous MASTL expression (Rogers et al., 2018). Our study demonstrated that also MASTL depletion leads to partial loss of cell-cell contacts in MCF10A monolayers (I, Figure 3), suggesting that regulation of MASTL protein expression levels are important in normal breast epithelial tissue maintenance. These results imply that regulation of MASTL levels may have a great impact in the normal tissue homeostasis.

Transcriptional regulators MRTF-A and SRF are key mediators of cell motility, since they regulate expression on various genes related to cell adhesion, actin cytoskeleton and actomyosin contractility (Gau and Roy, 2018). Our study reveals that MASTL regulates MRTF-A/SRF activity kinase-independently, by associating with MRTF-A, and by facilitating its cytoplasmic/nuclear translocation and nuclear retention (I, Figure 8, 9). This finding provides previously unknown mechanistic insights for MASTL-mediated cell motility. Further, these findings are in line with previous studies showing that high MASTL expression correlates with cancer invasiveness and metastasis *in vivo* (Rogers et al., 2018; Vera et al., 2015). These and our supportive findings about MASTL silencing-induced reduction of cell invasion in 3D highlight the importance of MASTL-mediated cell motility in different environments and biological settings. In addition, MRTF-A and SRF have been shown to respond to actin dynamics caused by mechanical stimuli (Gau and Roy, 2018; Montel et al., 2019), and cancer cells are known to be exposed to various mechanical stresses during cancer progression (Northcott et al., 2018). Since our results indicate that MASTL regulates MRTF-A and SRF, it would be interesting to study whether MASTL could also have a mechanoresponsive role in cancer cells.

GEF-H1-RhoA-ROCK-signaling pathway regulates MLC phosphorylation, and is important especially in generating contractile actomyosin forces in the rear of migrating cells (Joo and Olson, 2021). We demonstrated that MASTL affects expression of GEF-H1 (I, Figure 5), which is not a SRF target gene. GEF-H1 is known to bind microtubules, and microtubule disassembly is known to promote GEF-H1 release and activation (Joo and Olson, 2021). We investigated if MASTL silencing influences microtubule formation, but could not observe any obvious changes that would have encouraged further exploration in this direction. Thus, the mechanisms behind MASTL mediated GEF-H1 expression remains unknown. Interestingly, however, GEF-H1 has been shown to positively regulate SRF activity (Itoh et al., 2014). These findings indicate that MASTL facilitates SRF activity in two distinct ways – by increasing SRF regulator GEF-H1 levels and by associating with MRTF-A. Further, GEF-H1 has been shown to be involved in multiple biological processes, including vesicle trafficking, immune response, cancer progression and neurodevelopment (Joo and Olson, 2021). Thus, our findings that link MASTL to GEF-H1 expression, suggest that in addition to cancer and cell cycle regulation, MASTL might have unknown roles in various biological contexts.

NM-2A and NM-2B have both distinct and overlapping roles in migrating cells, and the ratio and copolymerization of these isoforms have a great impact on the cell polarization and migratory behavior (Shutova et al., 2017; Shutova and Svitkina, 2018). Our study shows that MASTL depletion reduces the protein levels of NM-2B (I, Figure 5) and Tpm4.2 (I, Figure 5), which has been shown to enhance actomyosin contraction by recruiting NM-2A molecules to the actin stress fibers (Tojkander et al., 2012, 2011), and by activating the NM-2A ATPase activity (Gateva et al., 2017). Thus, we can conclude that MASTL affects non-muscle myosins directly by regulating NM-2B expression, and potentially indirectly by affecting Tpm4.2 levels. Further, MASTL might affect the NM-2A/NM-2B ratio, which may also partly explain the cell spreading, loss of cell polarity and reduced cell migration upon MASTL depletion in breast cancer.

In addition to cell motility, actin cytoskeleton and contractility play important roles also in cell division (Gibieža and Petrikaitė, 2021; Heng and Koh, 2010; Kunda and Baum, 2009). MASTL has been shown to regulate the cell cycle by inhibiting PP2A via MASTL's substrates ENSA and ARPP19 (Castro and Lorca, 2018; Vigneron et al., 2016). Our findings show that MASTL also plays an important role in actin cytoskeleton organization and contraction in interphase cells. However, it remains to be studied whether MASTL could also regulate the actin dynamics and contractile actin ring formation during cell division.

6.2 MASTL in regulation of stemness (II)

Previous studies have linked MASTL with CSC related features, such as anchorage independent growth (Rogers et al., 2018; Vera et al., 2015; Yoon et al., 2018), cancer recurrence and resistance to cancer treatment (Conway et al., 2020; Marzec and Burgess, 2018; Yoon et al., 2018). However, a direct link between MASTL and cancer stemness has not been studied. We demonstrated in the original publication II that MASTL supports stemness in BCSCs and pluripotent stem cells by regulating transcription factors OCT1 and OCT4, respectively, and by facilitating TGF-β signaling via TGFBR2.

TGF-β binding to its receptors on the cell surface induces activation of SMAD2/3 and PI3K/AKT pathways, which are essential in the maintenance of pluripotency (Singh et al., 2012). In fact, SMAD regulates transcription of NANOG, a key pluripotency factor (Xu et al., 2008). Further, TGF-β signalling has been shown to regulate cancer stemness (Bellomo et al., 2016). We showed that MASTL regulates TGFBR2 expression in MDA-MB-231 cells (II, Figure 4, 5), and facilitates downstream SMAD2/3 and AKT signalling (II, Figure 6). Importantly, our results indicated that the regulation of TGFBR2 levels, and activation of SMAD2/3 and AKT was independent of MASTL kinase activity (II, Figure 5, 6), thus revealing a

novel kinase-independent function of MASTL in cancer. However, the direct mechanism of how MASTL regulates TGFBR2 needs further investigation.

Our earlier described study (original publication I) revealed that MASTL regulates the actin cytoskeleton via transcriptional regulators MRTF-A and SRF. Abnormal regulation of the actin cytoskeleton and TGF-β signalling are both important in cancer progression, however, it is not fully understood how they are linked together (Melchionna et al., 2021). Interestingly, SRF and MRTF have been linked to TGF-β mediated signalling. SRF depletion has been shown to reduce TGFBR2 levels in neutrophils (Taylor et al., 2014). Further, TGF-β has been shown to induce nuclear localization of MRTF-A and MRTF-B, which together with SMAD3 regulate expression of Slug, an important regulator of EMT (Morita et al., 2007). Here we show that overexpression of MASTL wild-type or kinase-dead mutant increases TGFBR2 levels in epithelial cells, whereas inhibition of MASTL kinase activity has no effect in the TGFBR2 levels in breast cancer cells (II, Figure 5). Our findings in original publications I and II imply that MASTL facilitates MRTF-A/SRF signalling kinase-independently by associating with MRTF-A, and by affecting TGFBR2 levels.

AKT signaling has been shown to promote actin cytoskeletal remodelling, EMT and cell motility in embryonic development and cancer progression (Xue and Hemmings, 2013). Further, previous studies show that MASTL overexpression promotes AKT signalling in breast cancer cells (Rogers et al., 2018; Vera et al., 2015), but the molecular mechanism have remained unknown. As explained above, our study (original publication II) demonstrates that MASTL facilitates AKT signaling via regulating TGFBR2 levels in cancer and stem cells, and thus provides new mechanistic insight to MASTL mediated AKT signaling. However, a direct link between MASTL mediated cytoskeletal regulation (original publication I) and AKT activity in cancer and embryogenesis remains to be studied in detail.

We have demonstrated that MASTL can regulate the actin cytoskeleton-related transcription factors MRTF-A and SRF (original publication I), as well as stemness-related transcription factors OCT1 (II, Figure 2) and OCT4 (II, Figure 3). However, the transcriptional regulation of MASTL itself remains unclear. Our results indicate that MASTL is a potential OCT1 target gene, since OCT1 silencing decreased the MASTL protein levels, whereas OCT1 overexpression promoted the MASTL expression in breast cancer cells (II, Figure 2). Further, we identified a potential binding site for OCT1 and OCT4 in the MASTL promoter (II, Figure S2). Interestingly, MASTL has also been identified as a potential target gene of the dimerization partner, retinoblastoma-like, E2F and MuvB (DREAM) complex, which is a key regulator of cell cycle related gene expression (Fischer et al., 2016), and E2F transcription factor 8 (E2F8), which has been linked to cancer cell proliferation (Tian et al., 2017). However, further studies are needed to understand

the transcriptional regulation of MASTL in stemness and during different steps of cell cycle and cancer progression.

6.3 MASTL's kinase-independent functions in cancer (I,II)

Previous studies related to MASTL in the regulation of cancer progression have been focused on MASTL kinase activity in cell proliferation and tumor growth, and MASTL mediated AKT signaling (Marzec and Burgess, 2018). Thus, inhibition of MASTL kinase activity has interested many researchers as a potential cancer treatment strategy (Kang et al., 2021; Kim et al., 2020; Ocasio et al., 2016) However, we have revealed kinase-independent functions of MASTL that are important in regulation of cancer cell motility and stemness (original publications I and II), pointing out the possibility that MASTL promotes cancer invasion and metastasis in a kinase-dependent manner. It has been previously reported, that also other cell motility-related kinases, such as epidermal growth factor receptor (EGFR), have kinase-independent functions, including serving as a scaffold for protein complexes, sequestering other proteins, and directly interacting with transcription factors or DNA (Rauch et al., 2011). Interestingly, inhibitors against invasive and metastatic functions have raised interest as potential treatment against cancer (Gandalovičová et al., 2017). Our results showed that the association of MASTL with MRTF-A is important in cancer cell motility (original publication I). Thus, it would be interesting to study whether inhibition of this MASTL-MRTF-A association could prevent cancer invasion and metastasis. Further, inhibitors against TGF-β signalling have been extensively developed (Colak and Ten Dijke, 2017; Huang et al., 2021). Since our findings indicate that MASTL is as a kinase-independent mediator of TGF-β signalling in cancer cells and stemness (original publication II), raises a question whether combination of MASTL and TGF-β inhibition could be an effective cancer treatment strategy.

Tumors are typically heterogeneous, meaning that cancer cells in different parts of the tumor have distinct characteristics and gene expression patterns, that are affected by genomic instability and environmental cues (Dagogo-Jack and Shaw, 2018). Thus, it would be interesting to study how MASTL and its activity are distributed within tumors. It remains to be studied whether MASTL expression is altered in the invasive parts of tumors compared to the middle parts of the tumor. In addition, further investigation is needed to understand how the kinase-dependent and kinase-independent functions of MASTL are coordinated in order to maintain proliferation, invasion, metastasis and stemness in cancer.

6.4 Integrins and actin cytoskeleton in regulation of pluripotency states (III)

Although integrin β1 is known to be essential in post-implantation embryonic development (Fässler et al., 1995; Stephens et al., 1995), its role during earlier steps of development or in the transition between pluripotency states has not been studied in detail. In this study (original publication III) we show that integrin β1 is active in primed and naïve human PSCs when the cells are cultured on Matrigel, vitronectin and laminin, which are commonly used in stem cell culture. Further, acute integrin β1 inhibition induced naïve-like features in primed hiPSCs regarding the colony morphology and gene expression patterns, and reduced actomyosin contractility and ERK signalling. In naïve hiPSCs, continuous β1 inhibition supported naïve-like state by positively influencing reversion from primed into naïve-like state, and by affecting gene expression patterns.

Modelling early human development by chemically resetting hiPSCs into a naïve-like state has recently raised interest among researchers, and many resetting protocols have been developed (Hassani et al., 2019; Taei et al., 2020). However, the effect of different matrixes in naïve hiPSCs culture have not been studied in detail, and a clear consensus of culture medium and other conditions in the maintenance of naïve-like state in hiPSCs is still lacking. We demonstrated that integrin β1 activity varies in primed and naïve hiPSCs when plated on different matrixes that are commonly used in hiPSC culture (III, Figure 1, S1). Further, when naïve hiPSCs were plated on laminin, they lost their typical compact and dome-shaped colony morphology, whereas on Matrigel and vitronectin the colonies remained as typical looking naïve-like colonies. These findings bring up the importance of careful selection of ECM in hiPSCs culture *in vitro*. Further, a recent study shows that integrin β1 inhibition facilitates a proper mouse ICM organization when the cells are cultured in 3D Matrigel (E. J. Y. Kim et al., 2022). This is in line with our findings about integrin β1 induced naïve-like colony morphology of hiPSCs that are cultured on Matrigel (III, Figure1, 3). Together these findings imply that integrin β1 inhibition may facilitate the maintenance of early stage ICM cells when cultured *in vitro*.

Previous studies have demonstrated a special actin cytoskeletal organization in primed hiPSCs, where the colony edges are decorated by contractile and thick actin stress fibers that terminate into large focal adhesions (Närvä et al., 2017; Stubb et al., 2019). Further, a RNA sequencing based study where the cells on the edge and middle part of the hiPSC colonies were separated and studied, revealed and accumulation of actin regulators, such as LIMA1 and AMOTL2, on the colony edge where the cells were also more prone to differentiate (Y. Kim et al., 2022). We show that integrin β1 inhibition in primed hiPSCs disrupts actin organization and actomyosin contractility, and promotes cell clustering typical to naïve hiPSCs (III, Figure 1, 3, S3). Further, our RNA sequencing analysis revealed downregulation of

AMOTL2 upon integrin inhibition in primed hiPSCs (III, Figure 4). AMOTL2 has been shown to negatively regulate mechanoresponsive transcription factor YAP in adult cells (Wang et al., 2011; Zhao et al., 2011). YAP, again, is an important regulator of actin organization during human PSC differentiation into mesodermal lineage (Pagliari et al., 2021). The interplay between integrins, AMOTL2 and YAP in regulation of different pluripotency states and differentiation in hiPSCs remains to be studied.

Downregulation of ERK signaling has been shown to support a naïve-like state in human PSCs and mouse ESCs (Nichols et al., 2009; Takashima et al., 2014). Interestingly, our transcriptome analysis revealed that in primed hiPSCs, integrin β1 inhibition downregulated PDGFB and CCN2, which are positive regulators of ERK signalling, and in contrast, upregulated DUSP8 (III, Figure 4), which is negative regulator of ERK signalling (Ding et al., 2019). Further, we observed a downregulation of pERK levels upon integrin β1 inhibition in primed hiPSCs. These findings suggest that integrin inhibition pushes the primed hiPSCs towards a naïve-like state by affecting gene expression patterns and ERK activity.

Our transcription analysis also revealed that integrin β1 inhibition in naïve hiPSCs increases expression of smooth muscle α-actin (ACTA2), and actin regulators phosphatase actin regulator-1 (PHACTR-1) and filamin A-binding protein 1 (FILIP1) (III, Figure 4). PHACTR-1 is an actin binding protein that regulates actin stress fiber and lamellipodium formation (Allain et al., 2012; Jarray et al., 2011; Wiezlak et al., 2012), and FILIP1 regulates cell migration via filamin A (Nagano et al., 2002). It remains to be studied how these actin cytoskeletal regulators function in naïve hiPSCs and whether they have a role in the maintenance and differentiation of naïve pluripotent stem cells.

7 Conclusions

The purpose of this thesis was to gain mechanistic insight into regulation of integrin-mediated cell adhesions and actin cytoskeleton in cancer, stemness and pluripotency. We revealed previously unknown, kinase-independent functions of MASTL in transcriptional regulation of cancer cell adhesion, actomyosin contractility, motility and stemness. Further, we discovered that MASTL supports signaling pathways that are essential in maintenance of pluripotent stem cells. Finally, we made significant findings of how integrin β1 activity and actomyosin contractility mediate human induced pluripotent stem cell functions and transition between naïve and primed states.

Original publication I

We discovered in the original publication I novel kinase-independent and cell-cycle-independent functions of MASTL in regulation of actin cytoskeleton, contractility and motility in cancer cells. We show that MASTL facilitates MRTF-A/SRF mediated transcription of genes that are important in cell adhesion, actin organization and actomyosin contraction, including vinculin, Tpm4.2 and NM-2B. Further, we demonstrate that MASTL promotes expression of GEF-H1, which is known to regulate actomyosin contraction and facilitate SRF activity. In more detail, MASTL association with MRTF-A facilitates MRTF-A nuclear localization and transcriptional activity, and hinders MRTF-A nuclear export. These findings explain the spread out cell morphology, reduction in contractile actomyosin structures and decreased cell migration, which we observed upon MASTL silencing. Taken together, this study brings new mechanistic insight into MASTL mediated cell motility and cancer progression.

Original publication II

This study (original publication II) revealed further kinase-independent functions of MASTL as a regulator of stemness in cancer and pluripotent stem cells. We show that MASTL is highly enriched in BCSCs and human PSCs, where MASTL increases transcriptional stemness regulators OCT1 and OCT4 levels, respectively.

Interestingly, we also observed a potential binding site for OCT1/OCT4 in the MASTL promoter, indicating that MASTL is a potential OCT1/OCT4 target gene. Further, we show that MASTL supports stemness by positively regulating TGF-β signalling and downstream SMAD and AKT activation via TGFBR2. These findings provide mechanistic insight into previously observed MASTL mediated AKT signalling in cancer. Further, revealing MASTL functions in cancer stemness partly explain why high MASTL levels correlate with cancer metastasis, drug resistance and recurrence in many cancers. Further, this is the first study linking MASTL in maintenance of pluripotency.

Original publication III

Here (original publication III) we demonstrate novel functions of integrin β1 activity and actomyosin contractility in regulation of primed and naïve hiPSCs. We show that integrin β1 inhibition induces naïve-like features in primed hiPSCs, regarding colony morphology, loss of actomyosin contractility, gene expression patterns and decreased ERK activity. Further, integrin β1 inhibition supports naïve-like state by facilitating cell clustering and fine-tuning gene expression patterns in naïve hiPSCs. In addition, our transcriptomic analysis revealed changes in multiple actin cytoskeleton regulators in primed and naïve hiPSCs, which may help to understand actin regulation in different states of pluripotency in human. Taken together this study reveals previously unknown functions of integrins in regulation of PSC states, and suggests that integrin inhibition is a potential tool in fine-tuning PSC properties and functions *in vitro*.

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