How to assemble contractile actomyosin bundles in cells?
How to assemble contractile actomyosin bundles in cells?

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Cover figure: Light-microscopy image of human osteosarcoma cell U2OS plated on crossbow micropattern with fluorescent fibronectin (Alexa 647), stained with Alexa 488-phalloidin to visualize the actin cytoskeleton and Alexa 568-vinculin to visualize focal adhesions.

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To the most precious people in my life, my wonderful boys, Dimitar and Viktor!

“DON’T PANIC!”
Douglas Adams
The Hitchhiker's Guide to the Galaxy
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ABBREVIATIONS

ADF  Actin depolymerizing factor
ADF-H Actin depolymerizing factor homology
Aip  Actin-interacting Protein
AMP  5’ AMP-activated protein
AMPK 5’ AMP-activated protein kinase
ARP  Actin-related protein
BAR  Bin-Amphipathin-Rsv
CaMK  Ca^{2+}/calmodulin-dependent protein kinase
Cdc42 Cell division control protein 42 homolog
CH  Calponin homology
D  Dimension
DAAM  Dishevelled associated activators of morphogenesis
DAD  Diaphanous autoregulatory domain
Dia  Diaphanous
DID  Diaphanous inhibitory domain
DRF  Diaphanous-related formins
Ena/VASP Enabled/vasodilator-stimulated phosphoprotein
Eps8 Epidermal growth factor receptor kinase substrate 8
ERM  Ezrin/radixin/moesin
EVH  EnA/VASP homology
EVL  Ena/VASP-like
FAB  F-actin binding
F-actin Filamentous actin
FH  Formin homology
FHOD  Formin homology domain
FMNL  Formin like protein
FRAP  Fluorescence recovery after photobleaching
GAB  G-actin binding
G-actin Monomeric (globular) actin
GAP  GTPase activating protein
GBD  GTPase-binding domain
GDI  GDP dissociation inhibitors
GEF  GDP/GTP exchange factor
GFP  Green fluorescent protein
GMF  Glia maturation factor
GST  Glutathione S-transferase
GTPase Guanosine triphosphatase
HMW  High molecular weight
IF  Intermediate filament
Ig  Immunoglobulin
INF  "Inverted" formin
JMY  Junction-mediating regulatory
LIM  Lin-1, Isl-1, Mec3 zinc finger domain
LIMK1 LIM domain kinase 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>LMW</td>
<td>Low molecular weight</td>
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<tr>
<td>MAL</td>
<td>Myocardin-related transcription factor</td>
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<tr>
<td>mDia</td>
<td>Mouse Dia</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light-chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>Myosin light-chain phosphatase</td>
</tr>
<tr>
<td>MRL</td>
<td>Mig10/RIAM/Lpd</td>
</tr>
<tr>
<td>NM</td>
<td>Non-muscle</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neural WASP</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAK</td>
<td>P21/Cdc42/Rac1-activated kinase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDZ</td>
<td>Psl-95,DlgA and ZO1 proteins</td>
</tr>
<tr>
<td>Pi</td>
<td>Pyrophosphate, inorganic phosphate</td>
</tr>
<tr>
<td>PK</td>
<td>Protein kinase</td>
</tr>
<tr>
<td>Rac</td>
<td>Ras-related C3 botulinum toxin substrate</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family member A</td>
</tr>
<tr>
<td>Rif</td>
<td>Rho in filopodia</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated coiled-coil forming kinase</td>
</tr>
<tr>
<td>RSK</td>
<td>Ribosomal s6 kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription PCR</td>
</tr>
<tr>
<td>SH3</td>
<td>SRC Homology 3 Domain</td>
</tr>
<tr>
<td>SLR</td>
<td>Spectrin-like repeats</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>STED</td>
<td>Stimulated emission depletion microscopy</td>
</tr>
<tr>
<td>TAZ</td>
<td>Transcriptional co-activator with PDZ-binding motif</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection fluorescence microscopy</td>
</tr>
<tr>
<td>Tks5</td>
<td>Tyrosine kinase substrate with five SH3 domains</td>
</tr>
<tr>
<td>TMBP</td>
<td>Tandem monomer binding protein</td>
</tr>
<tr>
<td>Tpm</td>
<td>Tropomyosin</td>
</tr>
<tr>
<td>U2OS</td>
<td>Human osteosarcoma</td>
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<tr>
<td>WASP</td>
<td>Wiscott-Aldrich syndrome protein</td>
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<tr>
<td>WAVE</td>
<td>WASP family Verprolin homologous</td>
</tr>
<tr>
<td>WH2</td>
<td>WASP homology domain 2</td>
</tr>
<tr>
<td>WHAMM</td>
<td>WASP homolog associated with actin, membranes, and microtubules</td>
</tr>
<tr>
<td>WIP</td>
<td>WASP interacting protein</td>
</tr>
<tr>
<td>YAP</td>
<td>Yes-associated protein</td>
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following five original articles, which are referred to in the text by their roman numerals (I-V):


Contribution:

I) G.G. designed the study together with P.L., executed the majority of the experiments, performed data analyses, and drafted the manuscript.

II) G.G. performed western blot experiments, rescue experiments, intensity measurements of protein incorporation, immunostaining experiments, and data analyses. G.G contributed to preparation of the figures, and to drafting of the manuscript.

III) G.G. cloned some of the constructs used in the study, performed *in vitro* TIRF experiments, data analyses, and helped draft the manuscript.

IV) G.G. performed western blot experiments and data analyses, and helped draft the manuscript.

V) G.G. designed the study together with P.L., executed the majority of the experiments, performed data analyses, and drafted the manuscript.

Manuscripts, which will not be included in this thesis:


ABSTRACT

The ability of cells to migrate, adhere, and contract is an essential feature, governing basically every process in the human body. These include fundamental functions such as development, muscle contraction, immune responses, wound healing, and a plethora of diseases including cancer metastasis, vascular diseases, and cardiomyopathies. Contractile actomyosin bundles are the central cellular structures, which enable the cell to perform all these processes, and thus it is crucial to understand how such structures are assembled in cells. Assembly of actomyosin bundles both in non-muscle and in muscle cells is regulated by a multitude of proteins, acting synchronously in a fascinating complexity. Many aspects of this process have remained mysterious, because of the sheer number of the involved actin-binding proteins, the intricate signaling pathways, and the complexity of the underlying interactions.

This thesis aimed at understanding of some of the fundamental processes behind the assembly of contractile structures in non-muscle cells (human osteosarcoma cells U2OS), and in heart muscle cells (primary rat cardiomyocytes). Previous studies revealed that contractile actomyosin bundles in U2OS cells, called ventral stress fibers, are generated from a network of precursors, named dorsal stress fibers and transverse arcs. This work revealed that the tropomyosin family proteins (Tpm) play central role in stress fiber assembly. Our studies on U2OS cells indicate that four functionally distinct tropomyosins orchestrate stress fiber assembly. Tpms 1.6/1.7, Tpm 2.1, and Tpms 3.1/3.2 stabilize specific stress fiber regions. In contrast, Tpm 4.2 regulates the recruitment of myosin II to diaphanous (Dia) 2 formin-nucleated actin filament population in stress fiber precursors. Our in vitro experiments revealed that these tropomyosin isoforms possess an intrinsic ability to segregate to different actin filaments and bind F-actin with distinct dynamics. Our results indicated that these tropomyosins determine some of the biochemical properties of actin filaments, such as their ability to associate with non-muscle myosin II and actin depolymerizing factor (ADF)/cofilin. Additionally, our studies demonstrated that the interaction between palladin and vasodilator-stimulated phosphoprotein (VASP) promotes dorsal stress fiber assembly. In contrast, the tension generated by transverse arcs inhibits this process. Tension also controls the length of ventral stress fibers and inhibits ADF/cofilin-mediated stress fiber disassembly. Furthermore, our studies in primary rat cardiomyocytes revealed that ADF/cofilins are also crucial for the length regulation and proper function of muscle sarcomeres.

Collectively these studies reveal important biochemical and mechanobiological principles that regulate the assembly of contractile actin bundles in non-muscle and in muscle cells.
REVIEW OF THE LITERATURE

1. Introduction to the cytoskeleton

Many fundamental cellular functions, including division, migration, endocytosis, and morphogenesis are dependent upon an intact cytoskeleton. The cytoskeleton can be defined as a network of protein filaments, found in the cytoplasm of many living cells. The cytoskeleton can be divided into three distinct networks, which are defined based on their protein compositions and diameters: 1) microtubules with an average diameter of 25 nm, composed of tubulins; 2) intermediate filaments (IF) with an average diameter of 10 nm, which are generated by multiple proteins, that belong to the IF protein family; and 3) actin filaments with an average diameter of 7 nm, also called microfilaments, which are composed by the protein actin (Alberts et al., 2014).

Microtubules are long tubular filaments that are formed by the polymerization of a dimer of two globular proteins, α- and β-tubulin. These highly dynamic structures are characterized by a process, called dynamic instability, which refers to the ability of one end of the microtubule to grow, reach a “catastrophe” event, and then shrink rapidly. Microtubules are essential for many processes including cilia and flagella-mediated migration, cytokinesis, vesicle and organelle transport, and development (Borisy et al., 2016).

Intermediate filaments are assembled from a family of proteins with similar structural and biochemical properties. These include e.g. vimentin, desmin, lamin, and keratin family proteins. In contrast to the actin filaments and microtubules, the intermediate filaments are heterogenous, more stable, apolar, and their subunits cannot bind nucleotides such as adenosine triphosphate (ATP) or guanosine triphosphate (GTP) (Goldman et al., 2012). Intermediate filaments are characterized by high tensile strength and elasticity, and they can resist mechanical forces, including twisting, bending, and compression. The intermediate filaments are mainly regarded as structural components, because of their mechanical properties and because they provide mechanical strength to the cell. Thus, intermediate filaments are important for the stabilizing of cell-cell and cell-substratum contacts, determination of cell and nuclear shape, and increase the mechanical stability of the cell (Alberts et al., 2014).

2. The actin cytoskeleton

Actin is among the most abundant intracellular proteins in eukaryotic cells and it can assemble into diverse networks that are involved in multiple processes (Fig. 1). Actin is highly conserved between different species with amino acids sequence identity of almost 90% between yeast and human (Alberts et al., 2014).

2.1. Actin isoforms and homologs

There is a great variety in the number of genes that encode actin proteins. Unicellular eukaryotes such as yeasts and amoebas have a single actin gene, whereas some plants have as many as 60 actin genes. Birds and mammals have six actin genes, and each encodes a different actin isoform with highly similar amino acid sequences (at least 93% between the isoforms). Four actin isoforms are expressed primarily in smooth (αsmooth-actin, and γsmooth-actin), skeletal (αskeletal-actin), and cardiac (αcardiac-actin) muscles, and two isoforms are ubiquitously expressed: βcyto-actin and γcyto-actin (Wickstead and Gull, 2011).

Many prokaryotes express homologs of cytoskeletal components: tubulin, intermediate filaments, and actin. The best studied bacterial actin homologs include MreB and ParM, which are involved in cell shape and polarity determination, and plasmid separation (Wickstead and Gull, 2011).

2.2. Actin polymerization

Actin filaments (also known as F-actin) are composed of two filament polymers that are wrapped around each other in a right-handed helix and are assembled from globular 43 kDa actin monomers (G-actin). Each actin monomer contains 4 subdomains, each of which is organized in two approximately equal-sized lobes, that are
separated by a deep cleft. The lobes and the cleft form the ATPase fold allowing the binding of a divalent cation, usually Mg\textsuperscript{2+} complexed with either ATP or adenosine diphosphate (ADP) nucleotide. The binding of ATP or ADP induces conformational change in G-actin, which in turn affects the ability of the monomers to polymerize into actin filaments. F-actin polymerization is triggered by cation addition (Mg\textsuperscript{2+}, K\textsuperscript{+}, or Na\textsuperscript{+}) and is accompanied by ATP hydrolysis to ADP and P\textsubscript{i}, which affects the polymerization kinetics (Alberts et al., 2014; Carlier and Shekhar, 2017).

It is important to note that in F-actin all the monomeric subunits have the same orientation, making the actin filament a polar structure with two different ends. The ends of the actin filament are referred to as the pointed (or minus) end and the barbed (or plus) end according to the arrow-like appearance of the myosin heads decorating the actin filament as visualized by electron microscopy. This intrinsic polarity of the actin filament plays an important role in actin polymerization and dynamics (Alberts et al., 2014; Carlier and Shekhar, 2017).

2.3. Actin dynamics

The addition of new actin monomers can occur at both filament ends, but because both ends present different structural and molecular surfaces and consequently have different biochemical properties, the filament grows faster at its barbed end, and slower at its pointed end.

Upon addition of new monomers to the actin filament, ATP hydrolysis due to the increased ATPase activity of F-actin occurs. This leads to the accumulation of ADP-bound subunits at the filament segment towards the pointed end. The instability of ADP-F-actin leads to the depolymerization of the filaments at their pointed ends. Then the ATP nucleotide on the depolymerized actin monomers can be exchanged to ATP, and the ATP-bound actin monomers can be added to the barbed end in a new round of polymerization events (Carlier and Shekhar, 2017). Barbed end elongation balances pointed end depolymerization, and this results in constant filament length and forward movement. This process is known as “treadmilling” and reveals how actin filaments can generate forces in the cell, such as the force required to push the membrane forward during cell migration (Carlier and Shekhar, 2017; Bugyi and Carlier, 2010).

2.4. Function of the actin cytoskeleton

Actin networks have a fundamental role in a myriad of cellular processes including endocytosis, vesicle and organelle movement, mitochondrial fission, establishment and maintenance of cell junctions, as well as cell polarity, division, and morphogenesis. Therefore, cells contain a large array of morphologically and functionally distinct actin filament structures (Fig. 1). Their assembly is dependant on the complex interactions of signalling pathways and multiple actin-binding proteins (Alberts et al., 2014; Pollard, 2016; Korobova et al., 2013). In addition to its function in cytoplasmic events, actin has structural roles and regulates processes, that take place in the nucleus. These include transcription by ribonucleic acid (RNA)-polymerases I, II, deoxyribonucleic acid (DNA) repair, chromatin remodeling, and gene expression, which are implicated in stem cell differentiation and development for example (Virtanen and Vartiainen, 2017; Misu et al., 2017; Yim and Scheetz, 2012).

Cell migration and adhesion are fundamental for multiple cellular processes, and are thus regulated by the formation of various actin-based structures with unique protein composition (Fig. 2). Such structures can be roughly divided into protrusive actin structures, such as lamellipodia, filopodia, blebs, and invadosomes, and contractile actin structures, such as stress fibers.

2.4.1. Protrusive actin structures

Protrusive actin structures generate force via actin polymerisation against the plasma membrane and thus drive forwards motion of adherens cells. The most prominent protrusive structures in cells on two-dimensional (2D) surfaces are the lamellipodia and the filopodia. These structures reside in the small region adjacent to the plasma membrane, which is described as the leading edge of the cell (Fig. 1). Invadosomes and blebs are not concentrated in the leading edge, but also contribute to cell migration through protrusive forces.

2.4.1.1. Lamellipodia

The lamellipodium is a thin sheet-like structure, that is several micrometers wide and 200 nm thick. The lamellipodium is important for haplotaxis, regulation of cell adhesion (Wu et al., 2012; Kiosses et al., 2001; Ehrlich et al., 2002),
**Figure 1. Actin cytoskeleton based structures in metazoan cells.** Actin is involved in multiple processes as shown in a hypothetical migrating cell, attached to a neighboring cell on the left. On the right is a side view of a migrating cell illustrating that cells contain dorsal structures such as ruffles, and ventral structures such as invadosomes, which enable cell migration and invasion. This figure illustrates the main actin-based structures in metazoan cells, but it is plausible that actin is involved also in additional, currently unidentified processes. The picture is modified from Chhabra and Higgs, 2007.
epithelial wound healing (Farooqui and Fenteany, 2005), and endothelial barrier function (Breslin et al., 2015). The lamellipodium contains relatively short branched actin filaments oriented with their growing barbed ends towards the cell edge (Yang and Svitkina, 2011; Vinzenz et al., 2012). This so called “dendritic network” can generate force via polymerization against the plasma membrane, and is the main driving force for generating leading edge protrusion and pushing adherens cells forwards (Svitkina and Borisy, 1999; Krause and Gautreau, 2014). Behind the leading edge, aged filaments are debranched, disassembled and depolymerized. This results in recycling of the actin monomers, which are constantly added to the actin filament barbed ends at the plasma membrane. The loosening of the dendritic network behind the leading edge also creates space for the contractile stress fiber network (discussed in chapter 2.4.2.1.). This process results in the phenomenon of “actin retrograde flow”, which describes that the velocity of the cell protrusion is slower than the speed of actin polymerization, this results in sliding of actin filaments backwards relative to the substrate (Krause and Gautreau, 2014; Shemesh et al., 2012; Le Clainche and Carlier, 2008).

2.4.1.2. Filopodia
Filopodia are thin finger-like cellular protrusions, composed from parallel actin bundles. Filopodia are involved in several processes including environmental sensing, guidance to chemoattractants, cell migration and adhesion, phagocytosis, wound healing, neuronal growth cone orientation, embryonic development, and cancer cell invasion (Jacquemet et al., 2017; Jacquemet et al., 2015; Arjonen et al., 2011; Mattila and Lappalainen, 2008; Gupton and Gertler, 2007). Although filopodia architecture, dynamics, and size vary greatly depending on the cell type, filopodia generally consist of straight actin filaments, oriented in parallel bundles (Gupton and Gertler, 2007; Young et al., 2015). In migratory cells, filopodia can be found at the leading edge, where they push against the membrane and regulate cell velocity, directionality, and path-finding (Mattila and Lappalainen, 2008).

2.4.1.3. Invadosomes
Podosomes (in normal cells) and invadopodia (in cancer cells) also collectively known as invadosomes, are dynamic actin-rich cellular protrusions. Invadosomes are present in many cell types and regulate pericellular proteolytic activity, extracellular matrix (ECM) remodelling and degradation. Therefore, invadosomes contribute to cell migration and adhesion-dependent processes including hematopoietic cell migration, development, angiogenesis, and metastasis (Paterson and Courtneidge, 2017; Schachtnet et al., 2013).

2.4.1.4. Blebs
Blebs emerge as spherical expansions of the membrane of migrating cells, both in cell culture conditions and in vivo (Charras, 2008; Charras and Paluch, 2008). Blebbing can be utilized together or instead of other protrusion types such as lamellipodia depending on the cell environment, intracellular signalling, actin polymerisation, contractility, and substrate adhesiveness (Diz-Muñoz et al., 2016; Tozuoğlu et al., 2013; Bergert et al., 2012). Blebbing is fundamental especially for three-dimensional (3D) cell migration, and has been implicated in several processes, including cell spreading, tumor dissemination, invasion pathfinding, force transmission, apoptosis and cytokinesis, gastrulation and development, and pathogen invasion (Sanz-Moreno and Marshall, 2010; Charras and Paluch, 2008; Paluch and Raz, 2013).

2.4.2. Contractile actin structures
Unlike protrusive actin structures, the force production in contractile structures, such as stress fibers (in non-muscle cells, Fig. 3) or myofibrils (in muscle cells, Fig. 4) is not dependant on actin treadmilling. In contractile structures force is generated by the ATP-driven movement of the bipolar bundles of myosin II motor domains on bipolar arrays of actin filaments, which results in the contraction of the actomyosin bundle (Alberts et al., 2014).

2.4.2.1. Actin stress fibers
Stress fibers are thick actin bundles, prominent in various cells types and involved in fundamental processes such as cell migration and adhesion, wound healing and morphogenesis (Tójkander et al., 2012).

2.4.2.1.1. Focal adhesions as linkers of stress fibers and ECM
Attachment to neighbouring cells via cell-cell adhesions, such as adherens junctions, or to the substrate via cell-substrate adhesions, such as
Figure 2. *Filamentous actin structures involved in cell migration*. Schematic illustration of the major F-actin structures involved in cell migration of a hypothetical cell, moving upwards. These structures include lamellipodia, filopodia, blebs, invadosomes, and stress fibers. Each structure has distinct molecular composition and the boxes in this figure list the most prominent players involved. Focal adhesions are shown as yellow structures at the ends of the stress fibers. ADF, actin depolymerizing factor; Arp2/3, actin-related protein complex 2/3; BAR, Bin-Amphiphysin-Rvs; CP, capping protein; ERM, ezrin/radixin/moesin; GMF, glia maturation factor; Mena, mouse enabled; NPFs, nucleation promoting factors; Tks5, tyrosine kinase substrate with five SH3 domains; VASP, vasodilator-stimulated phosphoprotein. The picture is modified from Le Clainche and Carlier, 2008.
focal adhesions, are crucial for the function and force generation of contractile structures.

Focal adhesions are protein plaques that are central for ECM-cytoskeleton linkage and attachment of the stress fibers to the substrate (Fig. 2, Fig. 3) (Gardel et al., 2010). This attachment is critical for stress fiber function as it enables the force transmission between the actin cytoskeleton and the cell environment. It is suggested that focal adhesions act as "molecular clutch" during cell movement (Swaminathan and Waterman, 2016). Focal adhesions are complex networks of cytoplasmic proteins and transmembrane integrins (Bouvard et al., 2013; Ivaska, 2012; Pellinen et al., 2006). Focal adhesions vary in size, dynamics, and protein composition depending on their maturation stage and tension generated by myosin II-generated and external forces (Gardel et al., 2010; Riveline et al., 2001).

3D super-resolution fluorescence microscopy revealed a stratified organization of focal adhesions with distinct strata composed of integrins, integrin signalling layer (integrin cytoplasmic tails, focal adhesion kinase and paxillin), intermediate force-transduction layer (talin and vinculin), a top actin-regulatory layer (zyxin, VASP and α-actinin), and finally actin stress fibers (Kanchanawong et al., 2010). It is suggested that talin serves as template/organizer of the focal adhesion, and together with vinculin regulates the force transduction between the cell and the environment (Kanchanawong et al., 2010).

Because of their function as a linker of the actin cytoskeleton and the ECM, focal adhesions are heavily implicated in mechanotransduction (Swaminathan and Waterman, 2016). Maturation of focal adhesions is regulated by utilization of dorsal stress fibers as a structural template, stress fiber-generated tension, matrix rigidity, and tyrosine-kinase signalling (Geiger et al., 2009; Oakes et al., 2012; Elseguel-Artola et al., 2016; Prager-Khoutorsky et al., 2011). There is no consensus regarding the traction forces generated by focal adhesions, as it appears that such forces are dependent not only on focal adhesion size, but also on the different cell systems (Burridge and Guilluy, 2016).

### 2.4.2.1.2. Stress fiber morphology

Stress fibers have been studied using two different experimental setups (Burridge and Guilluy, 2016). Ridley and Hall observed that some cell types disassemble their stress fibers and focal adhesions upon serum deprivation. This phenomenon permitted the analysis of focal adhesion and stress fiber assembly upon re-addition of the serum and subsequent activation of Ras homolog gene family member A (RhoA), especially in stationary and confluent cells (Ridley and Hall, 1992).

Some cell types were found to be resistant to serum starvation, thus the majority of more recent studies have been done in cells spreading and migrating on coverslips that are coated with ECM components, such as fibronectin (Tojkander et al., 2012). Currently, the human osteosarcoma cell line (U2OS) is the best and most-widely established model for such studies based on the dynamic properties and suitability for live cell analysis of their stress fiber network (Hotulainen and Lappalainen, 2006). Stress fibers in U2OS can be divided into three distinct types based on their morphology, myosin content, and attachment to focal adhesions (Fig. 3) (Small et al., 1998; Tojkander et al., 2012). Dorsal stress fibers are attached to focal adhesions at their distal ends and cannot contract intrinsically, because they do not contain myosin II. The transverse arcs are curved actomyosin bundles roughly parallel to the leading edge. They are connected to focal adhesions only through interactions with dorsal stress fibers. The region, where the dorsal stress fibers and arcs are present is frequently called "lamella". Important property of arcs is their ability to flow from the leading edge towards the cell center in migrating cells (Heath, 1983; Small et al., 1998; Hotulainen and Lappalainen, 2006), that is suggested to be driven by continuous arc contractions (Zhang et al., 2003). Dorsal stress fibers and transverse arcs serve as precursors that give rise to ventral stress fibers during an intricate maturation process. The ventral stress fibers are the truly contractile actin fibers, because they contain highly organized α-actinin/myosin II units and they are directly connected to focal adhesions at each end (Tojkander et al., 2012).

Furthermore, some cells display a fourth category of stress fibers, called the perinuclear actin cap. These fibers are connected to especially mechanosensitive focal adhesions at both ends and resemble ventral stress fibers, which wrap over and connect to the nucleus (Khatou et al., 2009; Kim et al., 2012).
2.4.2.1.3. Molecular composition and dynamics of stress fibers

All stress fiber types have complex molecular compositions with over 20 actin-binding proteins that regulate their assembly, dynamics, and function (Table 1) (Tojkander et al., 2012). Several studies have reported via fluorescence recovery after photobleaching (FRAP) experiments, that the interactions of most actin-binding proteins with stress fibers are highly dynamic, which in turn suggests that stress fibers are dynamic structures (Schmidt and Nichols, 2004; Hotulainen and Lappalainen, 2006; Endlich et al., 2009).

2.4.2.1.4. Stress fiber assembly and disassembly

Studies from U2OS cells have revealed a crucial role for actin polymerisation in de novo assembly of stress fibers in migrating cells. Live cell microscopy revealed two distinct mechanisms involved in stress fiber assembly (Hotulainen and Lappalainen, 2006): i) formin (mDia1/DRF1)-driven actin polymerization at focal adhesions behind the leading edge to generate dorsal stress fibers, and ii) endwise annealing of myosin bundles and actin-related protein complex 2/3 (Arp2/3)-nucleated actin bundles at the lamella to generate transverse arcs.

During cell migration, the leading edge oscillates between extension and retraction cycles and transverse arcs precursors form through the condensation of actin and myosin bundles in the lamellipodium during the retraction phase (Hotulainen and Lappalainen, 2006; Burnette et al., 2011). Importantly, dorsal stress fibers and transverse arcs anneal together and serve as precursors for the generation of the mature ventral stress fibers.

The polarity of the actin filaments within the fibers has great significance for the contractile properties of these structures and thus many studies have been devoted to this aspect (Pellegrin and Mellor, 2007). Unlike the constant, alternating polarity of contractile units within the muscle sarcomeres, there are substantial polarity variations in different stress fibers with alternating (sarcomeric), graded, uniform, or random polarity present (Cramer et al., 1997; Svitkina et al., 1997; Pellegrin and Mellor, 2007). A majority of the stress fibers in migrating cells appear to have an overall graded polarity that is characterized by uniform polarity at the filament ends with the barbed ends pointing outwards and mixed polarity in the filament center (Cramer et al., 1997). In dorsal stress fibers the filaments are unipolar with the barbed ends closest to the adhesion and mixed polarity at their proximal ends (Cramer et al., 1997). Dorsal stress fibers grow by vectorial actin polymerization at focal adhesions: addition of new ATP-actin monomers to the barbed ends, that results in rapid elongation of the barbed ends facing the focal adhesion. Resembling the pattern of muscle sarcomeres, contractile stress fibers (e.g. arcs and ventral stress fibers) display periodic pattern of α-actinin and myosin localization (Langanger et al., 1986).

Myosin incorporates into dorsal stress fibers by displacement of α-actinin, and this myosin bundle moves towards the cell center due to actin polymerisation at the focal adhesion and elongation of the dorsal stress fiber. In contrast, the transverse arcs are generated by the endwise assembly of short bundles containing either α-actinin or myosin (Hotulainen and Lappalainen, 2006).

Stress fiber disassembly is induced by inhibition of RhoA (Lamb et al., 1988; Lampugnani et al., 1990) and loss of tension, which have a direct effect on cofilin-mediated severing and disassembly of F-actin (Hayakawa et al., 2011). It is suggested that dorsal stress fibers and arcs are also required for the assembly the perinuclear actin fibers, as these require the interconnection of dorsal stress fibers with arcs at spots rich in α-actinin-1 (Maninová and Vomastek, 2016).
<table>
<thead>
<tr>
<th>Stress fiber component</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-actinin</td>
<td>F-actin crosslinking, signal transduction</td>
<td>(Lazarides and Burridge, 1975; Sjobom et al., 2008)</td>
</tr>
<tr>
<td>Caldesmon</td>
<td>Regulation of contractility, cell motility and SF stability</td>
<td>(Mayanagi and Sobue, 2011)</td>
</tr>
<tr>
<td>Calponin</td>
<td>Regulation of contractility, motility, stability, signal transduction</td>
<td>(Rozenblum and Glimona, 2008)</td>
</tr>
<tr>
<td>Coactosin</td>
<td>F-actin binding</td>
<td>(Hou et al., 2009)</td>
</tr>
<tr>
<td>Cysteine-rich protein 1 (CRP1)</td>
<td>F-actin bundling</td>
<td>(Tran et al., 2005)</td>
</tr>
<tr>
<td>Fascin</td>
<td>F-actin bundling</td>
<td>(Hashimoto et al., 2011)</td>
</tr>
<tr>
<td>PH1/FH2 domain-containing protein 1 (FHOD1)</td>
<td>Stress fiber stabilization</td>
<td>(Jurmeister et al., 2011)</td>
</tr>
<tr>
<td>Filamin</td>
<td>Filament crosslinking, mechanosensing</td>
<td>(Brotschi et al., 1978; Ehrlicher et. al., 2011)</td>
</tr>
<tr>
<td>Myosin phosphatase Rho-interacting protein (MPRIP)</td>
<td>Control of MLC phosphorylation through MLCP</td>
<td>(Mulder et al., 2003; Koga and Ikebe, 2005)</td>
</tr>
<tr>
<td>Myosin II</td>
<td>Stress fiber contraction</td>
<td>(Vicente-Manzanares et al., 2009)</td>
</tr>
<tr>
<td>Palladin</td>
<td>Actin crosslinking, cytoskeletal scaffold</td>
<td>(Otey et al., 2005)</td>
</tr>
<tr>
<td>PDZ-LIM proteins</td>
<td>Signal transduction</td>
<td>(Vallenius et al., 2000; Krcmery et al., 2010)</td>
</tr>
<tr>
<td>Septin 2 (SEPT2)</td>
<td>Regulation of myosin II activity, stress fiber organization</td>
<td>(Joo et al., 2007; Dolat et al., 2014)</td>
</tr>
<tr>
<td>Transgelin</td>
<td>Stabilization of stress fibers, regulation of contraction</td>
<td>(Assinder et al., 2009)</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>Stabilization of stress fibers, regulation of contraction</td>
<td>(Gunning, 2008)</td>
</tr>
<tr>
<td>Vasodilator-stimulated phosphoprotein (VASP)</td>
<td>Stress fiber assembly</td>
<td>(Boukhelfa et al., 2004)</td>
</tr>
<tr>
<td>V-Zyxin</td>
<td>Stress fiber stability and repair</td>
<td>(Smith et al., 2013)</td>
</tr>
<tr>
<td>Kinases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine/threonine protein kinase 35 (CLK1 or STK35)</td>
<td>Signal transduction</td>
<td>(Vallenius and Makela, 2002)</td>
</tr>
<tr>
<td>NUA1 family SNF-like kinase 1 and 2 (NUA1 and NUA2)</td>
<td>Regulation of MLC phosphorylation</td>
<td>(Vallenius et al., 2011)</td>
</tr>
<tr>
<td>Rho-associated protein kinase (ROCK)</td>
<td>MLC phosphorylation</td>
<td>(Kawabata et al., 2004)</td>
</tr>
</tbody>
</table>
2.4.2.1.5. Stress fiber regulation

Stress fiber assembly, morphology, and properties are regulated both by chemical signalling and by mechanotension. The major regulators of the biochemical signalling are the small GTPases RhoA, Ras-related C3 botulinum toxin substrate 1 (Rac1), and cell division control protein 42 homolog (Cdc42) (Heasman and Ridley, 2008). RhoA directly promotes stress fiber assembly through mDia1 and Rho-associated coiled-coil forming kinase (ROCK), which activates LIM domain kinase 1 (LIMK1) and inhibits ADF/cofilin-mediated disassembly (Leung et al., 1996; Watanabe et al., 1999; Watanabe et al., 1997; Maekawa et al., 1999). Furthermore, the transcription of genes that encode cytoskeletal proteins is regulated by RhoA signaling through myocardin-related transcription factor (MAL, also known as MKL1 and MRTF-A) and serum response factor (SRF) pathway, which regulates the composition of the actin cytoskeleton (Hill et al., 1995; Miralles et al., 2003). Importantly, Rho/ROCK pathway and the Ca^{2+}/calmodulin-dependent pathway control the phosphorylation of the myosin light chain (MLC), which is crucial for myosin II ATPase activity and stress fiber contractility (Vicente-Manzanares et al., 2009; Katoh et al., 2001; Amano et al., 1996; Chrzanowska-Wodnicka and Burridge, 1996; Kimura et al., 1996; Watanabe et al., 1999). The RhoA-related GTPase RhoH, which is known as ARHF and Rho in filopodia (Rif) might contribute to stress fiber assembly via the regulation of the formins mDia1 and mDia2 (Fan et al., 2010). Rac1 regulates dorsal stress fiber assembly via α-actinin 1 and lamellipodia formation via Arp2/3 (Kovac et al., 2013; Koestler et al., 2013). Cdc42 regulates the formin mDia2 and filopodia (Pollard, 2007) and it has been suggested that filopodial protrusions can be recycled for the generation of stress fibers (Nemethova et al., 2008; Anderson et al., 2008).

Stress fibers are both tension-generating and mechanosensitive structures, thus the mechanobiological aspect of their regulation is as complex as the biochemical signalling (Burridge and Guillevin, 2016). Importantly, stress fibers and focal adhesions are typically present only in cells that have been plated on rigid substrata or subjected to high levels of shear stress, which leads to RhoA activation (Franke et al., 1984; Wozniak et al., 2003; Paszek et al., 2005; Burridge and Wittchen, 2013). Conversely, detachment of the cells induces stress fiber disassembly (Mochitate et al., 1991; Discher et al., 2005). Tension has a central role in stress fiber morphology and alignment as stress fiber orientation is regulated by fluid shear and cyclic stretch through Src/Fyn/Yes kinase signaling and p130Cas (Niedieke et al., 2012; Ohashi et al., 2017). Also stress fiber repair and restoration of force transmission through MAPK-mediated zyxin activation is tension-dependent (Yoshigi et al., 2005; Smith et al., 2010; Hoffman et al., 2012). During wound closure, mechanical tension induces stress fiber assembly in epithelial cells, which then differentiate into myoepithelial cells (Pellegrin and Mellor, 2007). Furthermore, it has been suggested that tension promotes mDia/formin-mediated actin polymerization at the focal adhesions (Riveline et al., 2001; Schiller et al., 2013). Tension-mediated activation of RhoA leads to the activation of ROCK and myosin II, and the activated myosin II results in increased contractility and stress-fiber generated tension, thus reinforcing a positive-feedback loop (Amano et al., 1996).

It is noteworthy that ventral stress fibers are typically under higher mechanical tension than dorsal stress fibers and arcs (Soine et al., 2015). Therefore, it has been suggested that tension might be more important for the regulation of ventral stress fibers (Burridge and Guillevin, 2016).

2.4.2.1.6. Stress fiber functions

Stress fibers have been observed in many cultured animal cells, including fibroblasts, smooth muscle, endothelial, epithelial, and some cancer cell lines, but there is a considerable variation in stress fiber morphology and properties depending on the cell line (Tojkander et al., 2012).

Some cells contain strong ventral stress fibers, whereas others might display only very thin stress fibers, or the stress fiber network might be missing completely. In principle, there is an inverse correlation between rate of migration and ventral stress fibers, as less motile cells have more ventral stress fibers (Couchman and Rees, 1979). Apart from their function in cell adhesion, stress fibers are important for tail retraction of migrating cells (Chen, 1981; Crowley and Horwitz, 1995), and cell shape determination (Nakata et al., 2016). Moreover, stress fibers can act as rulers to define the long cell axis, thus controlling persistence of migration (Rid et al., 2005).

In the tissue environment, stress fibers have been implicated in several processes linked to cell
migration, such as dorsal closure in Drosophila embryos (Jacinto et al., 2002), embryonic wound healing (Martin and Lewis, 1992), mesenchymal cell migration (Vallenius, 2013), endothelial cell branching in 3D environment (Fischer et al., 2009), and metastasis (Anekal et al., 2015; Goreczny et al., 2017). Interestingly, stress fibers have been also involved in processes that are not directly connected to cell motility. These include cell proliferation via the Hippo pathway (Wada et al., 2011) and stiffening and proliferation of pre-invasive breast cancer cells (Tavares et al., 2017).

The ability of stress fibers to contract and resist tension does not only affect cell migration, but also enables the contraction of myoepithelial cells around epithelial ducts (Haguenau, 1959; Olins and Brenner, 1982), the resistance to fluid shear stress, cyclic stretch or hypertension in endothelial cells lining the blood vessels and in vascular smooth muscles (Franke et al., 1984; Wong et al., 1983; Sato and Ohashi, 2005). Stress fibers in adjacent endothelial cells (human umbilical vein endothelial cells) are physically connected through discontinuous adherens junctions, and thus maintain the integrity of endothelial barriers by providing support against tensile stress and regulate barrier function, permeability, and vascular stretch (Prasain and Stevens, 2009; Millán et al., 2010; Birukov et al., 2003). In wound granulation tissues stress fibers are involved in the contraction of wound and scar tissue (Gabbiani et al., 1972; Tomasek et al., 2002), and dermal wound closure (Sandbo and Dulin, 2011).

Dorsal stress fibers have been implicated in cell migration and cell spreading via Rac-1 (Kovac et al., 2013), ECM remodelling through fibrillar adhesions (Skau et al., 2015), flattening of the lamella and cell motility (Burnette et al., 2014), and renal epithelial motility together with arcs via septins (Dolat et al., 2014). Transverse arcs are present in neuronal growth cones (Geraldo and Gordon-Weeks, 2009; Schaefer et al., 2008) and immunological synapses of T lymphocytes, where they have been implicated in the movement and dynamics of receptor clusters, which results in reduced T cell receptor signalling (Yi et al., 2012; Murugesan et al., 2016). Furthermore, arcs are implicated in leading edge advancement and directed cell motion (Burnette et al., 2011). Importantly, stress fiber like precursors, resembling transverse arcs, serve as templates for the formation of myofibrils during myofibrillogenesis in striated muscle (Sanger et al., 2009).

Nuclear structure and gene transcription are affected by mechanical forces transmitted from the ECM to the nucleus (Maniotis et al., 1997, Discher et al., 2005; Wang et al., 2009) and the perinuclear actin fibers and their especially mechanosensitive focal adhesions have been implicated in this process (Khatau et al., 2009). The key functions of the perinuclear actin cap include regulation of nuclear shape and positioning, as well as protection against DNA damage (Gomes et al., 2005; Luxton et al., 2010; Nagayama et al., 2011). The actin cap regulates also persistent cell migration (Kim et al., 2014), and promotes cell survival and metastasis during confined migration (Skau et al., 2016).

2.4.2.2. Striated muscles

Similarly to the stress fibers, muscle sarcomeres are also composed of actomyosin bundles and regulated by many different actin-binding proteins. Muscles are essential for the force generation, tissue contraction, and movement at the whole organism scale, as they can convert chemical energy into mechanical work. In contrast to smooth muscle, striated muscle tissues (heart and skeletal muscle) contain repeating functional units (sarcomeres), which assemble in higher structures: myofibrils and myotubes (Alberts et al., 2014). Improper assembly and alignment of sarcomeres and the subsequent higher structures results in several muscle diseases such as cardiomyopathies, myopathies, and muscular dystrophies (Laing and Nowak, 2005).

2.4.2.2.1. Organization of sarcomeres in myofibrils

Myofibrils, composed of repetitive sarcomeric units, are the contractile structures in striated muscle cells, which generate force in a highly directional way (Gautel and Djonovic-Carugo, 2016). Sarcomeres are highly organized structures, where the force is generated by the ATP-driven sliding of myosin II-containing bundles (thick myofilaments) along actin containing bundles (thin myofilaments) (Fig. 4) (Cooke, 2004). Myosin binding to the actin filaments is regulated by Ca²⁺ dependent interaction between skeletal troponin (Tpm 1.1) in concert with the troponin complex (Flicker et al., 1982; McKillop and Geeves, 1993). The highly regular, repetitive order of actin and myosin arrays in the sarcomere and sarcomere units in
Figure 4. Organization of the muscle sarcomere. Muscle sarcomeres are the building blocks of myofibrils in striated muscles. This figure illustrates the complex organization of the sarcomere and the principle of muscle contraction. For simplicity, majority of the sarcomeric proteins are omitted and only the thick filaments (myosin) and the thin filaments (F-actin) are shown. The sarcomere is flanked by two regions (Z-lines) acting as anchoring points for the actin filaments. Surrounding the Z-line is the I-band (thin filaments not overlapping with the thick filaments). Next is the A-band containing the entire length of the thick filaments, the A-band includes the M-line, which is the center of the sarcomere. Upon influx of Ca$^{2+}$ and hydrolysis of ATP, the myosin bundle can move towards the barbed (+) ends of the F-actin filaments, which are anchored at the Z-disk. This generates a sliding contraction, that is responsible for force generation in striated muscles. The picture is modified from Alberts et al., 2014.
the myofibril, and the coordinated function of millions of sarcomeres permits the amplification of contraction and force along the muscle fibre over long distances (Gautel and Djinovic-Carugo, 2016).

2.4.2.2.2. Molecular composition of sarcomeres
The building blocks of the sarcomere need to contain filaments of uniform length to enable the high order of the sarcomere. Therefore, multiple actin-binding proteins are involved in the organization of this precise structure. Besides myosin, the main actin-binding proteins in sarcomeres are: i) barbed-end capping protein: CapZ; ii) pointed-end capping proteins: tropomodulins; iii) filament stabilizing proteins: tropomyosins; iv) regulators of myosin binding: troponin, tropomyosin; v) nucleators: leiomodins and forms; v) cross-linkers: α-actinin isoforms 2 and 3; vi) scaffolding proteins: titin and nebulin; vii) accessory/regulatory proteins: myotilin, myopalladin (Gautel and Djinovic-Carugo, 2016; Fowler and Dominguez, 2017).

2.4.2.2.3. Assembly and dynamics of sarcomeres
The mechanisms governing sarcomere assembly during muscle development are much less understood than those involved in muscle contraction (Lemke and Schnorrer, 2017). It is suggested that during myofibrillogenesis leiomodins and forms nucleate actin filaments, which are subsequently capped by tropomodulins. Next, muscle specific scaffolding proteins orientate many of the remaining sarcomeric proteins (Lemke and Schnorrer, 2017).

In contrast to non-muscle cells, filament elongation in muscle occurs mainly at the pointed end, which dictates very unique F-actin polymerization/depolymerization specifics. The continuous exchange of actin subunits indicates that the filament pointed ends might contain a mixture of ATP/ADP-Pi- and ADP-actin subunits (Littlefield et al., 2001). It has been suggested that tropomodulin capping regulates actin subunit exchange and thus controls actin assembly and filament length (Fowler and Dominguez, 2017). Furthermore, although muscle actin filaments do not appear to undergo treadmilling, some treadmilling regulators such as ADF/cofilins, twinfilin, and CAP are expressed in muscle (Lemke and Schnorrer, 2017).

Although, it has long been believed that actin filaments in sarcomeres are very stable with slow actin monomer exchange occurring only at their ends, it was recently demonstrated that some actin filaments in cardiomyocyte sarcomeres display rapid turnover rates, which depends on cardiomyocyte contractility (Ono, 2010). Furthermore, it was demonstrated that these filaments do not contribute to contractility, and interplay of ADF/cofilins and myosin contractility was required for the disassembly of non-productive filaments (Skwarek-Maruszewska et al., 2009).

Three models have been suggested for myofibrillogenesis: i) premyo fibrils model: premyo fibrils are assembled from short bipolar non-muscle myosin filaments and short α-actinin cross-linked actin filaments, which is followed by gradual exchange of non-muscle for muscle myosin in the mature myofibrils at an early developmental stage (Rhee et al., 1994; White et al., 2014); ii) latent protein complexes model: independent separate assembly of thick myosin containing filament precursors and thin containing α-actinin cross-linked actin filaments (I-Z-I bodies) is followed by co-assembly of these structures in a second step (Rui et al., 2010); iii) tension driven self-organisation model: tension generated by bipolar myosin filaments pulling on bipolar cross-linked actin filaments and involving mechanosensitive proteins (titin, talin, and integrins) locally orients the actin and myosin filaments, and coordinates the assembly across the entire structure, as tension is present at muscle-tendon junctions and along developing and mature myofibrils (Lemke and Schnorrer, 2017). However, additional work is required to elucidate how myofibrils are assembled in muscle cells, and how their organization and dynamics are regulated.

3. Actin-binding proteins
To enable the vast diversity of actin-based structures, cells express a multitude of actin-binding proteins, which regulate the actin cytoskeleton in response to cellular signalling. Actin-binding proteins can accelerate the treadmilling and nucleation rates of actin filaments. These activities are important in vivo, because treadmilling and filament nucleation of actin in the absence of other proteins are too slow to account for the fast movements observed in
cells (Carlier and Shekhar, 2017; Bugyi and Carlier, 2010). Specific proteins and protein families orchestrate actin filament nucleation, elongation, cross-linking, branching, depolymerization, and recycling of ADP to ATP in monomers to regulate the diverse functions of actin in cells (Lappalainen, 2016; Pollard, 2016). The following section introduces a subset of the most thoroughly characterized and conserved actin-binding proteins, divided into subgroups based on their effects on actin assembly.

### 3.1. Actin monomer binding proteins

The ionic conditions in the cytoplasm, the critical concentration of G-actin, and the estimated total concentration of cytosolic actin favor F-actin polymerisation, thus one would expect that basically all actin in the cell should be polymerized. However, measurements of G- and F-actin levels show that nearly 40% of actin is present as a monomer in the cell (Lodish et al., 2016). This leads to the conclusion that additional factors keep the concentration of free ATP G-actin below the critical concentration required for polymerization of pure actin to prevent spontaneous nucleation.

The actin-binding proteins, β-thymosin and profilin, play a critical role in regulating G-actin levels in cells, due to their ability to bind actin monomers and control their addition into filament ends. Beta-thymosin 4 forms a 1:1 complex with ATP-actin monomers, inhibit nucleotide exchange and prevent the assembly of actin monomers into filaments (Goldschmidt-Clermont et al., 1992, de la Cruz et al., 2000; Carlier et al., 1993).

As β-thymosin, profilin is a G-actin-binding protein, that is critical for the maintenance of a pool of actin monomers in solution (Kaiser et al., 1999; Pernier et al., 2016). An important functional difference between the two actin binding proteins is that profilin promotes nucleotide exchange from ADP to ATP at the G-actin and can thus enhance actin filament assembly (Goldschmidt-Clermont et al., 1992; Pantaloni and Carlier, 1993). Moreover, although profilin prevents spontaneous nucleation of actin filaments, it does not inhibit actin polymerization at filament barbed ends (Tobacman, 1983; Pollard and Cooper, 1984). Thus, profilin enhances actin filament assembly by the addition of actin monomers to the filament barbed ends and interacts with the poly-(L) proline stretches of several proteins, including formins, Arp2/3, enabled/vasodilator-stimulated phosphoprotein (Ena/VASP), and neural Wiscott-Aldrich syndrome protein (N-WASP) to accelerate actin polymerization. It was shown recently that profilin competes with Arp2/3-activating nucleation promoting factors and funnels monomers to formin or VASP-driven actin polymerization, profilin thus favors the assembly of formin-nucleated versus Arp2/3-nucleated actin filament networks (Rotty et al., 2015; Suarez et al., 2015).

**Twinfilin** is a multidomain, multifunctional protein, that can bind actin monomers and filaments. Twinfilin consists of two ADF-homology (ADF-H) domains that are connected by a short linker. It binds ADP monomers with a higher activity than ATP G-actin and prevents their assembly into filaments. In addition to actin monomer sequestering, twinfilin also interacts with capping protein, caps actin filament barbed ends (Poukkula et al., 2011), and accelerates actin filament depolymerization (Johnston et al., 2015).

Similarly to twinfilin, **Srv2/cyclase-associated protein** (Srv2/CAP) is a multifunctional protein, which binds actin monomers (Ono, 2013). Srv2/CAP accelerates actin dynamics by promoting actin filament disassembly and catalyzing ADP to ATP nucleotide exchange on actin monomers. Srv2/CAP may also function as a scaffolding protein by interacting with multiple partners, including cofillin-actin complex, profilin, and twinfilin (Ono, 2013; Makkonen et al., 2013).

**ADF/cofilins** belong to a family of actin monomer and filament binding proteins that have a central role in F-actin disassembly and thus they promote actin filament treadmilling (dos Remedios and Thomas, 2001; Kanellos and Frame, 2016). Proteins from the ADF/cofilin family are very abundant, highly conserved and are essential for viability in eukaryotic cells. Vertebrates express three ADF/cofilin isoforms: ADF/cofilin-1, and cofilin-2 with overlapping expression patterns, but they have distinct functions as demonstrated by studies in knockout mice (Agrawal et al., 2012; Gurniak et al., 2014). All ADF/cofilins consist of a single ADF-H domain and can bind both actin monomers and filaments (Lappalainen, 2007). The majority of ADF/cofilins shows a preference for binding ADP-G-actin over ATP-G-actin with the exception of muscle-specific cofilin-2, which binds ATP-G-actin and ADP-G-actin with similar activities (Vartiainen et al., 2002). As β-thymosin 4, ADF/cofilins inhibit the exchange of the actin-bound nucleotide. It has therefore been suggested that ADF/cofilins can...
sequester actin monomers and prevent them from binding to F-actin (Nishida, 1985; Carlier et al., 1997).

However, the major function of ADF/cofilins appears to be the disassembly of aged ADP-actin filaments. ADF/cofilins bind to actin filaments in a cooperative manner, twist the filaments, and induce a conformational change (McGough et al., 1997). This leads to the fragmentation of the filament at the boundaries between bare and ADF/cofilin decorated filament segments (Hawkins et al., 1993; Elam et al., 2013). Furthermore, ADF/cofilins stimulate the dissociation of Pi from ADP-Pi-actin, which results in the accumulation of ADP-actin filament segments (Blanchoin and Pollard, 1999). Additional functions of ADF/cofilins include filament stabilization at saturating conditions (Gressin et al., 2015) and the creation of more polymerization-capable barbed ends, resulting from the severing events, induced by ADF/cofilins (Ghosh et al., 2004; Bamberg et al., 1999; Lappalainen, 2007). ADF/cofilins also accelerate actin filament depolymerization at their pointed ends (Wioland et al., 2017; Shekhar and Carlier, 2017). ADF/cofilins interact with Arp2/3 complex-nucleated networks, which leads to their reorganization and dissociation of the Arp2/3 complex (Chan et al., 2009), and cooperates with actin-interacting protein (Aip1) to disassemble fully all actin filament networks (Gressin et al., 2015). All these functions establish ADF/cofilins as major regulators of actin turnover rates both in vitro and in vivo (Kanellos and Frame, 2016).

3.2. Actin nucleating proteins and elongation factors

Actin filament nucleation, e.g. the formation of stable complex of monomers called a nucleus, is the kinetically unfavorable, rate-limiting step in filament polymerization. Therefore, several proteins that catalyze de novo nucleation in cells have been implicated as playing a critical role in actin filament polymerization (Chhabra and Higgs, 2007). Mammalian cells express three main groups of actin nucleation factors: i) actin-related proteins 2 and 3 complex (Arp2/3) and its activators; ii) tandem monomer binding proteins (TMBPs); and iii) formins (Skau and Waterman, 2015).

The Arp2/3 complex, which consists of seven proteins, is the only nucleator, which can induce the assembly of branched actin filaments (Yang and Svitkina, 2011; Svitkina and Borisy, 1999). Arp2 and Arp3 proteins constitute the core of the Arp2/3 complex and are structurally similar to actin monomers and function as nucleation sites for the new filament. Importantly Arp2/3 complex requires binding to an existing “mother” filament to nucleate a new filament. Furthermore, the Arp2/3 complex requires activation by nucleation promoting factors, which include proteins from several different families such as Wiskott–Aldrich syndrome protein (WASP)/neural-WASP (N-WASP), WASP-family verprolin-homologous protein (WAVE), WASP and Scar homologue (WASH), WASP homolog associated with actin, membranes, and microtubules (WHAMM), and junction-mediating regulatory protein (JMY) family proteins (Marchand et al., 2001; Higgs and Pollard, 2000; Blanchoin et al., 2000; Machesky et al., 1999; Svitkina and Borisy, 1999; Skau and Waterman, 2015). Inhibition of Arp2/3 complex can involve several proteins such as arpin, coronin, and glia maturation factor (GMF), which can act on their own or synergistically with each other (Sokolova et al., 2016; Dang et al., 2013; Cai et al., 2007; Cai et al., 2008; Ydenberg et al., 2013; Poukkula et al., 2014). After nucleation, the Arp2/3 complex remains associated with the pointed end and does not affect the assembly rates at the barbed end. Thus, the Arp2/3 complex is crucial for the assembly of branched, dendritic actin filament networks in cells (Skau and Waterman, 2015).

In contrast, nucleators that belong to the remaining two groups, generate straight, parallel actin filaments. The TMBPs group encompasses proteins that have WH2 domains and can bind at least three actin monomers; thus mimicking stable actin trimers. Members of the group include Spire, Cordon-bleu, leiomodin, JMY, and VopL/F (Skau and Waterman, 2015). JMY can nucleate filaments at its own and activate Arp2/3 complex. It is suggested therefore that JMY promotes the assembly of branched actin networks by nucleation of mother filaments, followed by activation of the Arp2/3 complex and subsequent branching (Zuchero et al., 2009). Similarly to the Arp2/3 complex, the majority of the TMBPs are suggested to cluster actin monomers, generate linear actin filaments and remain associated with the pointed filament ends, although conflicting studies have been published about the Spire protein (Ito et al., 2011; Quinlan et al., 2005).

The third group of actin nucleation factors is
composed of the formin protein family (Chhabra and Higgs, 2007). Formins nucleate actin filaments and remain associated with the growing barbed ends, and thus promote the assembly of unbranched actin filaments. There are 15 different formins expressed in humans (Schönichen and Geyer, 2010). Formins can be divided into 7 groups based on their structure (Skau and Waterman, 2015): i) Diaphanos-related formins (DFR): Dia1, Dia2, and Dia3; ii) Dishevelled associated activators of morphogenesis: DAAM1 and DAAM2; iii) the “inverted” formins: INF1 and INF2; iv) the formin homology domain-containing proteins: FHOD1 and FHOD2; v) formin-like proteins: FMNL1, FMNL2 and FMNL3; vi) the founding members of the formin family: FMN1 and FMN2; and vii) delphilin. All members of the formin family are characterized by the presence of two specific domains: formin homology 1 and 2 (FH1 and FH2). FH2 is the most well conserved domain among the different formin groups. It binds G- and F-actin and induces actin filament nucleation from free actin monomers (Pruyne et al., 2002; Sagot et al., 2002; Zigmond et al., 2003), enhances processive barbed end elongation and inhibits barbed end capping (Harris et al., 2004; Kovar et al., 2005; Moseley et al., 2004). The FH2 domain homodimerizes into a donut-shape structure, which moves with the growing actin filament and thus permits the processive addition of new G-actin subunits (Xu et al., 2004). The FH1 domain is located next to the FH2 domain. It contains poly-proline stretches that can bind profilin-actin monomers, and subsequently deliver these to the barbed end, which accelerates the filament elongation rate (Romero et al., 2004).

Apart from the FH1 and FH2, formins from different groups contain characteristic domains. The GTPase-binding domain (GBD) can interact with Rho-family GTPases (discussed in chapter 5.1.), and is important for formin activation. The GBD is present in four formin groups: DRF Dia, Daam, FHOD, and FMNL (Schönichen and Geyer, 2010). A regulatory domain, called Diaphanos Inhibitory Domain (DID), can be found in DRF Dia, Daam, FHOD, and FMNL, and the presence of this motif is often accompanied by the expression of C-terminal Diaphanos Autoregulatory Domain (DAD). Binding between the DID and the DAD domains induces autoinhibition and can be released by activation by Rho GTPases, thus presenting an important regulatory step in formin function (Li and Higgs, 2005; Li and Higgs, 2003). The only formin that contains DID domain but no DAD is INF2 (Chhabra and Higgs, 2006). It contains an actin-binding WH2 domain instead, and can accelerate both actin polymerization and depolymerization (Gurel et al., 2015; Gurel et al., 2014; Chhabra and Higgs, 2006). Given the large size of the formin family and their domain diversity, it is not surprising that formins have different regulatory mechanisms and cellular functions. Formins regulate actin filament nucleation and elongation, severing and depolymerization, and are thus critical for many processes, such as cell migration and adhesion, cell polarity, endocytosis, and cytokinesis (Skau and Waterman, 2015; Chhabra and Higgs, 2007).

The in vitro nucleation activity of the vasodilator-stimulated phosphoprotein (VASP) is probably less relevant in vivo compared to its elongation activity, thus VASP is usually not considered as a traditional nucleation factor. The activity of VASP is critical for the assembly of straight actin filaments, and therefore the properties of these proteins are discussed here. The ENA/VASP Homology (EHV) protein family includes three proteins that are found in mammals: enabled/mouse enabled (Ena/Mena), Ena/VASP-like (EVL), and VASP. The proteins of the EVH family have several domains (listed from N- to C-terminus) as following: i) EVH1 domain binds to a motif consisting of the consensus sequence (D/E)FP PPPX (D/E) (D/E), and mediates protein interactions; ii) proline-rich region mediates the binding to profilin and SRC Homology 3 Domain- (SH3) and WW-domain-containing proteins; and iii) EVH2 domain includes G-actin binding (GAB) and F-actin-binding (FAB) sites, followed by a C-terminal coiled-coil motif that is responsible for tetramerization (Krause et al., 2003; Sechi and Wehland, 2004; Bear and Gertler, 2009). It has been proposed that VASP promotes filament elongation via the binding to profilin-G-actin, which allows the actin monomer to be transferred to the G-actin binding site in VASP and thus added to the filament barbed end (Ferron et al., 2007; Chereau and Dominguez, 2006).

In addition to a role in actin filament elongation, it has been suggested, that VASP can act as actin filament crosslinking and bundling factor, due to its ability to form tetramers. Furthermore, it has been suggested that VASP can promote filament elongation via competition with capping protein, although no "uncapping" activity has been directly demonstrated. Rather, it is suggested, that Ena/VASP can associate with or
near the barbed end of growing filaments and thus inhibits capping protein binding (Bear et al., 2002; Furman et al., 2007; Reinhard et al., 1992; Barzik et al., 2005).

Two recent studies demonstrated that profilin inhibits Arp2/3 complex-mediated nucleation and filament branching, actions that direct G-actin either to formin-mediated (Suarez et al., 2015) or VASP-mediated actin filament assembly (Rotty et al., 2015) instead. These studies and also the observation that Ena/VASP inactivation results in shorter actin branches, denser dendritic network, and increased cell motility speed (Bear et al., 2002), lead to the assumption that after Arp2/3 complex-initiated nucleation and branching, Ena/VASP is recruited to the daughter filament barbed end and it mediates its elongation using profilin-G-actin (Rotty and Bear, 2014).

The activities of EVH proteins are regulated via several mechanisms including alternative splicing (Philippar et al., 2008), preference for specific profilin-actin isoforms (Dugina et al., 2009; Mouneimne et al., 2012), targeting to specific cellular locations, and post-translational modifications (Barzik et al., 2005). The binding partners of VASP, which are involved in protein targeting and thus mediate VASP activity include: i) the focal adhesion-associated protein zyxin that recruits VASP to focal adhesions and ventral stress fibers, that are under tensile stress (Hoffman et al., 2006; Smith et al., 2010; Hoffman et al., 2012), ii) the MRL (Mig10/RIAM/Lpd) family protein Lamellipodin that delivers Ena/VASP proteins to the growing barbed ends and enhances their processivity via tethering them to actin filaments and then clustering them at the plasma membrane (Hansen and Mullins, 2015); and iii) Abi1, a WAVE complex component that binds the EVH1 domain of Ena/VASP proteins via its SH3 domain and targets these to the cell edge (Chen et al., 2014).

The most common post-translational modification of Ena/VASP proteins is phosphorylation, which is mediated by several kinases (Döppler and Storz, 2013). VASP is phosphorylated at residue Y39 (EVH1 domain) by the Abi kinase which affects dorsal ruffling and cell migration. The phosphorylation of S157 in the proline-rich stretch by protein kinases A, G, and D1 (PKA, PTK, and PKD1) serves as a targeting signal to the membrane or leading edge localization. The majority of the phosphorylation sites are located in the EVH2 domain: S239 is phosphorylated by PKA, PTK, and S3 AMP-activated protein kinase (AMPK), T278 is phosphorylated by ribosomal s6 kinase 1 (RSK1), PKA, PTK, and AMPK, and S332 is phosphorylated by PKD1 and AMPK. Phosphorylations at the EVH2 domain regulate anti-capping, F-actin binding and bundling activities at VASP, and consequently modulate filopodia, stress fiber and focal adhesion organization and cell motility (Döppler and Storz, 2013).

3.3. Actin filament capping proteins

An important way to regulate cytoskeletal dynamics is to cap the filament ends, as this prevents the addition or dissociation of filament subunits. Capping of both filament ends prevents filament polymerization and depolymerization, and protects the filaments from disassembly, which in turn leads to greater filament stability and maintains filament length. On the other hand, the capping of the barbed filament end only, desinates the filament for disassembly.

Many barbed end capping proteins, including the heterodimeric capping protein and its muscle homolog CapZ, gelsolin superfamily proteins, and Aip1, have been characterized so far. In contrast, only few pointed end capping proteins have been identified: the tropomodulin family proteins (Shekhar et al., 2016; Fowler and Dominguez, 2017). Tropomodulins bind tropomyosins (discussed in chapter 3.5.2) and prefer tropomyosin-decorated actin filaments, that enhance their capping activity. The tropomodulins play a critical role especially in maintaining the length, turnover, and architecture of sarcomeric actin filaments (Fowler and Dominguez, 2017). Based on their conservation from yeast to higher eukaryotes and their intracellular concentrations, it is assumed that heterodimeric capping protein family proteins are the major barbed-end cappers in non-muscle cells. Capping proteins bind actin filaments barbed ends with very high affinity and prevent association and dissociation of monomers from capped ends (Shekhar et al., 2016). Capping protein is a versatile protein, which can interact also with other actin-binding proteins, including capping protein, Arp2/3 and myosin-1 linker (CARMIL), and twinfilin, that can modulate its activities (Shekhar et al., 2016). Capping protein is important component of the Arp2/3-nucleated branched networks, as it quickly caps and maintains the length of Arp2/3-nucleated filaments (Shekhar et al., 2016). Furthermore, it
has been suggested, that capping protein promotes Arp2/3 mediated nucleation by local enrichment of actin monomers (Akin and Mullins, 2008).

**Aip1** barbed-end capping enhances coflin-mediated actin filament disassembly and turnover (Gressin et al., 2015). **Gelsolin**-mediated severing and filament capping leads to the generation of short capped filaments that depolymerize at their pointed ends (Harris and Weeds, 1984; Burtnick et al., 1997). Thus, actin filament capping proteins are critical of the dynamics and turnover of actin filaments *in vivo* (Shekhar et al., 2016).

### 3.4. F-actin cross-linking proteins

An important mechanism of actin filament organization into more complex structures that permit the cell to generate force, is the bundling/cross-linking of actin filaments. Actin filament cross-linking proteins are characterized either by the presence of two actin binding domains (ABD) or by a single ABD combined with dimerization ability. Thus, actin cross-linking proteins, which bind to the sides of the actin filaments, can cross-link single actin filaments into complex bundles. Many actin cross-linking proteins have been identified so far, including fibrin, villin, filamin, fascin, plectin, and α-actinin (Alberts et al., 2014; Foley and Young, 2014).

**Alpha-actinins** are evolutionarily conserved actin crosslinkers, which generate actin filament bundles in non-muscle cells, and in Z-discs of muscle sarcomeres (Murphy and Young, 2015; Sjöblom et al., 2008). Mammals have four α-actinins genes and can generate several α-actinin splice variants that have tissue-dependent expression patterns. All α-actinins have an N-terminal ABD that is composed of two calponin homology (CH) motifs, followed by a rod domain that encompasses four spectrin-like repeats (SLRs) that are responsible for protein dimerization. The calmodulin-like (CaM) domain with EF-hand motifs is located at the C-terminus and can bind intracellular calcium and regulate the activity of the ABD domain (Burridge and Fermannisco, 1981; Tanget al., 2001). Alpha-actinins assemble into antiparallel homo- or heterodimers, which in mammals are formed by the non-muscle isoforms, α-actinin 1 and 4, or by the muscle isoforms 2 and 3. Alpha-actinins are multitasking proteins, that are involved in wide variety of cellular processes, and that interact with plethora of other actin-binding proteins (Murphy and Young, 2015).

**Palladin** is an F-actin-binding and bundling protein (Parast and Otey, 2000; Mykkänen et al., 2001; Dixon et al., 2008). It can enhance actin polymerisation (Gurung et al., 2016), and has been suggested to act as a molecular scaffold (Otey et al., 2005). There are multiple palladin isoforms (major ones: 90-92 kDa, 140 kDa, and 200 kDa), generated by alternative splicing from a single palladin gene, and expressed in tissue and developmental stage dependent manner (Fig. 5) (Otey et al., 2005). The other two members of this protein family include myotilin and myopalladin, which are expressed in skeletal and cardiac muscle (Otey et al., 2005).

Originally, palladin was identified as a stress-fiber associated protein, important for stress fiber organization (Parast and Otey, 2000; Mykkänen et al., 2001). Furthermore, palladin is implicated in the organization of other actin-based structures such as focal adhesions (Parast and Otey, 2000), podocytes (Endlich et al., 2009), invadopodia (Goicoechea et al., 2014), dorsal ruffles and podosomes (Goicoechea et al., 2006), and adherens junctions (Tay et al., 2010). Thus, palladin isoforms are involved in many physiological processes including embryonic development, development and motility of smooth muscle cells, fibroblasts, and primary neurons (Parast and Otey, 2000; Boukhelifa et al., 2001; Jin et al., 2009; Liu et al., 2017). Palladin is implicated in the regulation of cell motility and metastasis, but whether it promotes (von Nandelier et al., 2014; Goicoechea et al., 2009) or inhibits (Chin and Toker, 2010) migration is a matter of debate.

All palladin isoforms contain N-terminal polyproline regions, flanked by several serine residues, and 3-5 C-terminal tandem immunoglobulin C2 (IgC2) domains. These different domains enable the interactions of palladin with many actin-binding proteins including α-actinin (Rönty et al., 2004), ezrin (Mykkänen et al., 2001), VASP (Boukhelifa et al., 2004), a PDZ-LIM family member CLP36 (Maeda et al., 2009), epidermal growth factor receptor kinase substrate 8 (Eps8) (Goicoechea et al., 2006), the Arp2/3 complex (Qian et al., 2013), and profilin (Boukhelifa et al., 2006) (Fig. 5). The IgC2 domains have been implicated in F-actin binding and bundling (Dixon et al., 2008; Gurung et al., 2016; Vattepu et al., 2015), which is enhanced by dimerization (Vattepu et al., 2015) and phosphorylation (Chin and Toker, 2010), and
Figure 5. Major palladin isoforms and interaction partners. Schematic illustration of the most abundant and well studied palladin isoforms (top panel) and the interaction sites of the major binding partners of palladin (bottom panel). As indicated in the bottom panel palladin interacts with a plethora of proteins: α-actinin (Rönty et al., 2004), Akt1 (Chin and Toker, 2010), CLP36 (Maeda et al., 2009), Ena/VASP (Boukheleifa et al., 2004), Eps8 (Goicoechea et al., 2006), ezrin (Mykkänen et al., 2001), F-actin (Dixon et al., 2008), integrin (Liu et al., 2007), LASP/Nebulin (Rachlin and Otey, 2006), profilin (Boukheleifa et al., 2006), Src (Rönty et al., 2007). PP, proline-rich domain; IgC2 immunoglobulin C2 domain.
inhibited by phosphoinositides (Yadav et al., 2016).

Many of the binding partners of palladin are associated with actin structures such as focal adhesions and stress fibers, but the implications of these interactions for stress fiber organization and assembly have remained largely unknown.

3.5. F-actin side binding proteins

The actin filament is characterized by a large binding surface along its sides. Thus, proteins that bind to the filament sides, such as tropomyosins and myosins, are important regulators of F-actin stability and dynamics.

3.5.1. Myosin family proteins

Members of the myosin superfamily that contains 18 subclasses of myosins, are characterized by directional movement along the sides of the actin filament, typically towards the barbed filament end, and are thus crucial for the generation of contractile force in the cell (Masters et al., 2017). Generally, members of this family comprise three characteristic domains: i) the motor domain with ATPase activity, which is important for F-actin interactions; ii) the regulatory neck domain; iii) the tail domain, which docks the myosin to specific cargoes and promotes self-association of myosin into dimers of larger bipolar bundles in the case of myosin II (de la Cruz and Ostap, 2004). Myosin family members are characterised by varying grades of processivity. Importantly, the step size and frequency of the myosins, i.e. myosin processivity, is determined by the ATPase cycle step rates, which allows myosins to make just one or many subsequent steps before dissociation from the actin filament. These properties relate to the specific biological function of a myosin with fast contractions such as those that result from very brief association (few milliseconds) and subsequent pulling on F-actin by myosin in muscle and a much longer association with F-actin, which results in "hand over hand" walking over long distances in the case of the transport myosin V, for example (Geeses, 2016). Conventional myosins of class II are indispensable for the organization and contractility of high order actin structures such as lamellipodia, stress fibers, and muscle sarcomeres.

3.5.2. Tropomyosin family proteins

The major proteins that bind along the entire side of the actin filament, are the tropomyosins (Tpm). It is suggested that the majority of F-actin in the cell is decorated by tropomyosins (Gunning, 2008; Lehman et al., 2000). Tropomyosins are involved in plethora of actin-based processes both in muscle, and in non-muscle cells, including sarcomere contractility, cell motility, adhesion, apoptosis, and cytokinesis (Gunning, 2008).

The tropomyosin family is characterized by great protein variety and consists of over 40 different isoforms in mammals, generated via alternative splicing from four different genes (Gunning et al., 2008). The tropomyosins can be divided into HMW (high molecular weight) or LMW (low molecular weight) isoforms based on differences in their length and sequence. All tropomyosins are characterized by the presence of pseudo-repeats of ~40 amino acids, which corresponds to one actin subunit of the actin filament (Domínguez, 2011; Gunning, 2008). The HMW or long tropomyosin isoforms have seven such pseudo-repeats and thus one tropomyosin molecule can interact with seven subunits of the actin filament. The LMW or short tropomyosin isoforms have four to six pseudo-repeats and can thus interact with shorter fragment of the actin filament (Hitchcock-DeGregori and An, 1996; Gunning, 2008). The largest sequence variability among the Tpm isoforms can be found within pseudo-repeat 2 and at the C-terminus. Importantly, genetic studies on mice suggest that Tpm isoforms have nonredundant functions, indicating that different Tpm isoforms might have different effects on actin filaments, which in turn regulate different physiological processes (Gunning et al., 2005).

Tropomyosins are double-stranded α-helical rod-shaped coiled-coil proteins and form hetero- or homo-dimers, which interact with actin through ionic interactions and align in a head-to-tail fashion along the two long pitch helices of the actin filament (von der Ecken et al., 2015; Jancó et al., 2013). Similarly to the actin filaments, the tropomyosin filaments are polar with their N-terminal end directed towards the pointed end and the C-terminal end directed towards the barbed actin filament end (Tobacman, 2008). Each tropomyosin helix consists of two tropomyosin molecules, entwined in a parallel fashion along the actin filament, although it has been reported that fragments of tropomyosin isoforms can form antiparallel coiled-coil dimers (Rao et al., 2012).
The C-terminal coiled coil opens and thus allows insertion of the N-terminal coiled coil, which results in cooperative binding and head-to-tail alignment of tropomyosin molecules to form a polymeric complex along the actin filament (Frye et al., 2010; Greenfield et al., 2006). The formation of head-to-tail polymers is critical for the proper binding of Tpms to actin filaments (Tobacman, 2008), because Tpm dimers bind F-actin only with a low affinity (dissociation constant $K_d \sim 10^{-3} \text{ M}$) (Wegner, 1979).

Tropomyosins can undergo several protein modifications, which affect their conformation and activity (Gunning, 2008). The acetylation of the N-terminal methionine in both chains of the Tpm dimer is among the most important tropomyosin modifications in the majority of Tpms, because it enhances coiled coil formation, head-to-tail polymerization, and binding to F-actin (Hitchcock-DeGregori and Heald, 1987; Urbancikova and Hitchcock-DeGregori, 1994; Skoumlha et al., 2007). Furthermore, in the fission yeast *Schizosaccharomyces pombe* acetylation has important role in Tpm segregation and has profound effect on the ability of Tpm to regulate myosin motor activity (Skoumlha et al., 2007; Coulton et al., 2010).

Because Tpms decorate the entire length of the actin filament, it is suggested that different Tpms can regulate the interaction of multiple actin-binding proteins with F-actin in an isoform dependent manner (Gunning et al., 2015). The interaction between Tpms and myosin is extensively studied (Manstein and Mulvihill, 2016), and beside the role of Tpm isoforms in myosin II regulation (Hundt et al., 2016), Tpms have been implicated also in the regulation of Myo1b, Myo1c and Myosin V (Gunning et al., 2015). At least certain Tpms isoforms can protect actin filaments against ADF/cofilin-mediated disassembly (Ono and Ono, 2002), gelsolin-mediated severing (Ishikawa et al., 1989), and prevent depolymerization at pointed ends (Broschat et al., 1989). Tpms further stabilize the pointed ends of actin filaments via their interaction with tropomodulins (Gunning et al., 2015). In addition to the filament severing proteins, Tpms might also regulate also the F-actin binding of crosslinking factors such as fascin (Creed et al., 2011) and fimbrin (Skau and Kovar, 2010). Some Tpm isoforms inhibit actin nucleation by the Arp2/3 complex (Blanchin et al., 2001), regulate formin-nucleated actin filaments (Wawro et al., 2007; Ujfalusi et al., 2009; Ujfalusi et al., 2012), and together with profilin promote formin dependent nucleation in *Saccharomyces cerevisiae* (Alioto et al., 2016). Apart from their effects on actin nucleators, Tpms can regulate actin polymerisation by mechanically stabilizing actin filaments (Hitchcock-DeGregori et al., 1988) and decreasing actin depolymerisation at the pointed end (Broschat et al., 1989).

Interestingly, the binding of Tpms to F-actin is regulated by several nucleation factors. At least in *S. pombe* and *S. cerevisiae* formins can govern the sorting of different Tpms to different actin structures (Johnson et al., 2014; Alioto et al., 2016). The binding of the *Drosophila* Tpm (non-muscle *Drosophila* Tm1A) to the pointed ends of F-actin is inhibited by Arp2/3 complex and enhanced by cofolin, which generates new pointed ends and primes the Arp2/3 networks for Tpm binding (Hsiao et al., 2015).

All these findings demonstrate the complex interactions of Tpms with F-actin and actin-binding proteins, and suggest that Tpms can regulate the functional properties of actin filaments.

### 4. Sorting of actin filament populations

The function of tropomyosins has been extensively discussed in the context of generating different actin filament populations, e.g. priming actin filaments for interaction with specific actin-binding proteins and thus, regulating the molecular composition of actin networks and their distinct functions in the cell (Michelot and Drubin, 2011; Gunning et al., 2015). The question of protein sorting into different networks is central to the field of actin research, because of the wide variety of actin-binding proteins and actin networks in a common cytoplasm (Michelot and Drubin, 2011). Besides sorting by tropomyosin decoration (Gunning et al., 2015), it has been suggested that recruitment of specific nucleation factors to specific cell locations (Yeh et al., 2007; Levskaya et al., 2009) and the structure/conformation of the actin filament (Galkin et al., 2012) might also play a role in generation of specific actin filament populations in the cell.

It has been shown that nucleation factors such as Arp2/3 complex and formins are indeed capable of generating actin networks with distinct architecture both in *in vitro* and *in vivo* assays:
branched filaments in the case of activation of Arp2/3 (Cameron et al., 1999; Michelot et al., 2010), and parallel filaments in the case of formins (Romero et al., 2004; Michelot et al., 2007). As mentioned earlier, elegant studies done by the Bear and Kovar lab demonstrated that profilin can act as a gatekeeper of G-actin distribution by directing actin monomers to the VASP or formin-derived parallel filaments instead of the Arp 2/3 derived branched networks (Rotty et al., 2015; Suarez et al., 2015).

There is also evidence that F-actin exists in several structural states (Galkin et al., 2010). As mentioned earlier, the actin filament can adopt three different states based on the nucleotide bound. It has been suggested that naked actin filaments in vitro exist in multiple discrete structural states and the helical twist of the actin filament can vary along its length (Galkin et al., 2012; Galkin et al., 2010; Greene et al., 2009). Additionally, several actin-binding proteins bind preferably to specific actin filament conformations in vitro (Michelot et al., 2011; Hild et al., 2010; Michelot and Drubin, 2011). All these suggest that the specific actin filament conformation itself might define how proteins interact with it, which could culminate in actin filament sorting.

Decoration by a specific tropomyosin isoform has been suggested as important mechanism for filament sorting based on their non-overlapping functions in multiple cellular processes, isoform-specific effects on many actin-binding proteins, and intracellular sorting in a number of cell types (Gunning et al., 2008; Gunning et al., 2015). However, the significance of different tropomyosin isoforms for stress fiber assembly and any isoform-specific differences in their association with single actin filaments in vitro have remained a mystery.

5. Regulation of the actin cytoskeleton

Regulation of the actin cytoskeleton is a complex process that involves both biochemical, and mechanobiological regulatory mechanisms to orchestrate the activities of plethora of actin-binding proteins.

5.1. Biochemical regulation of the actin cytoskeleton

The biochemical regulation of the actin cytoskeleton is governed by GTPases and kinases. The Rho family of GTPases features about 20 proteins, which regulate multiple processes, including cell migration, cell polarity, cytokinesis, and morphogenesis (Hodge and Ridley, 2016; Ridley, 2015). In their active form, the GTPases contain a GTP molecule and bind their downstream effectors. The GTPases undergo a conformational change upon hydrolysis of the GTP to GDP, which leads to their inactivation. The hydrolysis is due to their intrinsic GTPase activity, which can be enhanced by GTPase activating proteins (GAPs). Apart from the GAPs, the cycling of the GTPases is further controlled by GDP/GTP exchange factors (GEFs), which convert the GDP to GTP. GDP dissociation inhibitors (GDI) keep the GTPases in an inactive conformation by inhibiting nucleotide exchange (Alberts et al., 2014).

Upstream regulators of the GAPS and GEFs include phosphorylation and phosphoinositide signalling at the plasma membrane (Hodge and Ridley, 2016). The most prominent members of the Rho GTPases that are involved in actin cytoskeleton regulation, include Cdc42, Rac, and RhoA. Typically different kinases regulate specific higher order actin structures, thus: i) Cdc42 induces filopodia; ii) Rac1-activated pathways result in the formation of lamellipodia and membrane ruffles; and iii) RhoA induces stress fibers and focal adhesions assembly via its downstream effector ROCK and mDia1 (Hodge and Ridley, 2016).

ROCK is a serine-threonine kinase from the AGC (PKA/PKG/PKC) family and has been implicated in the activation of several formins, including mDia1 (Watanabe et al., 1999) and FHOD1 (Takeya et al., 2008), both of which promote stress fiber generation. ROCK phosphorylates profilin at serine 137 (Ser137), thus inhibiting its monomer binding activity (Shao et al., 2008; da Silva et al., 2003). ROCK phosphorylates several other downstream effectors, which include myosin light chain (MLC), MLC phosphatase (MLCP), and LIM kinase (Hodge and Ridley, 2016). Phosphorylation of MLC combined with the inhibition of the MLC phosphatase by phosphorylation increases the myosin II ATPase activity, which leads to increased contractility (Hodge and Ridley, 2016). The active,
phosphorylated LIM kinase phosphorylates and thus inactivates the actin-depolymerizing activity of ADF/cofilin, which results in reduced actin filament disassembly (Maekawa et al., 1999). Thus, activation of ROCK and its downstream effectors by Rho induce actin stress fiber assembly and focal adhesion formation.

The Myosin light-chain kinase (MLCK) is another serine-threonine kinase, which is activated upon Ca\(^{2+}\) binding to calmodulin and deactivated by myosin light-chain phosphatase (MLCP). MLCK phosphorylates the regulatory light chain of myosin II primarily at Ser19, which is an important mechanism in the regulation of smooth muscle contraction (Sellers et al., 1981). Other important kinases that regulate actin-binding proteins include the AMPK, activated by decreased intracellular ATP levels (Mihaylova and Shaw, 2011), the Src, a non-receptor tyrosine kinase (Kirkbride et al., 2011), and the cAMP- and cGMP dependent protein kinases PKA and PFK. All three kinases phosphorylate VASP at Ser239 and threonine 278 (Thr278) (Butt et al., 1994; Blume et al., 2007). Furthermore, AMPK loss increases β1-integrin activity and thus promotes cell adhesion, matrix formation, and mechanotransduction (Georgiadou et al., 2017; Georgiadou and Ivaska, 2017).

### 5.2. Mechanobiological regulation of the actin cytoskeleton

It is noteworthy that different cell environments present not only different chemical properties, but also specific mechanical dimensions. Cells utilize sensor molecules (mechanosensors) to translate extra- and intercellular mechanical forces into biochemical signaling to be able to respond to the mechanical properties of their environment. This process is called mechanotransduction and leads to changes in cell shape and morphology, and modulation of actin cytoskeleton organization, which in turn regulates many biological processes including cell polarity, migration, differentiation, and wound healing (Iskratsch et al., 2014).

Several actin-binding proteins are regulated by tension or undergo conformational changes directly as a result of mechanical forces in the range of 10–50 pN and thus can mediate mechanical stimuli to the actin cytoskeleton. Such proteins include focal adhesion-associated proteins p130 Crk-associated substrate (p130Cas) (Sawada et al., 2006), β-integrin (Puklin-Faucher et al., 2006), filamin (Ehrlicher et al., 2011), zyxin (Yoshigi et al., 2005), VASP (Leerberg et al., 2014), and talin (del Rio et al., 2009). Tensile forces (<3 pN) also accelerate the polymerisation rates of forms such as mDia1 (Jégou et al., 2013) and Bni1p (Courtemanche et al., 2013), and computational models suggest that their profilin-actin binding sites are revealed under tension (Bryant et al., 2016). Furthermore, mDia1 regulates mechanosensitive actin polymerization, which is modulated by F- and G-actin homeostasis and is independent of kinase signalling (Higashida et al., 2013).

Importantly tension and stretching can modulate the properties of the actin filament itself and can induce changes in its helical structure and twist (Huxley and Brown, 1967; Wakabayashi et al., 1994). This alters the interactions of several actin-binding proteins with F-actin. Stretching of the actin filament by tension (>2 pN) leads to a decrease of the binding rate of coflin to F-actin and subsequently inhibits (or delays) its severing activity (Hayakawa et al., 2011). Furthermore, myosin II motor domain has increased affinity to the stretched actin filaments (Uyeda et al., 2011). Additionally, some actin-binding proteins, including coflin, mDia1, and gelsolin, can twist and modulate the flexibility of the actin filament, generating long-range conformational changes in actin filaments (Galkin et al., 2001; McCullough et al., 2008; Pfändner et al., 2010; Papp et al., 2006; Prochnowicz et al., 1996).

The significance of the interplay of biochemical and mechanobiological signaling is demonstrated by the regulation of several GTPases and kinases (e.g. Rac1/Cdc42, RhoA, and AMPK) by mechanical stress (Ohashi et al., 2017; Blair et al., 2009). The interplay between mechanical signals and nuclear transcription has been demonstrated in the context of the transcriptional coactivators and Hippo effectors YAP/TAZ, which play an important role in mechanotransduction in various tissues (Dupont et al., 2016).

Despite the wealth of studies on these signalling pathways, the precise biochemical and mechanobiological principles, that regulate the assembly of actin stress fibers and other complex actomyosin bundles have remained elusive.
AIMS OF THE STUDY

Prior to the research conducted for this thesis, it was known that actin stress fibers constitute the main contractile machinery in many non-muscle cells, including endothelial, epithelial, and fibroblast cells (Tojkander et al., 2012). Importantly, the organization of α-actinin and myosin into a periodic pattern in stress fibers, suggests that studies on the assembly mechanisms of actin fibers in non-muscle cells can therefore further our understanding of the general mechanisms behind the generation of contractile actin bundles also in muscle cells. Despite this observation, little was known about the general mechanisms behind the generation of contractile actin bundles. Therefore, the goal of this study was to investigate how stress fibers assemble in the human osteosarcoma U2OS cell model and thus shed light on the fundamental question: “How to assemble a contractile actomyosin bundle in the cell?”. To tackle this problem, I focussed on the following specific aims:

1. To characterize the mechanisms of stress fiber assembly in the context of mechanosensing and the actin-binding protein palladin.
2. To reveal if stress-fiber associated tropomyosin isoforms can specify distinct actin filament populations in vitro and in cells.
3. To elucidate similarities and differences in the assembly and dynamics of actomyosin bundles in non-muscle and muscle cells.
**METHODS**

Table 2. Experimental methods personally applied in this study. More detailed descriptions can be found in the original publications, which are indicated with roman numbers.

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RESULTS AND DISCUSSION

Contractile actin fibers, including stress fibers and muscle sarcomeres, are important for fundamental cellular processes, including migration, endocytosis, morphogenesis and therefore their assembly and dynamics need to be tightly regulated (Tojkander et al., 2012). Stress fiber assembly is regulated by various actin-binding proteins (Table 1), but the exact mechanisms for this remain elusive. Therefore, studies on the functional properties of stress fiber components are expected to further our understanding of how contractile structures are built in cells.

Historically, the studies on the mechanisms behind stress fiber assembly and dynamics have been hampered by the observation, that these structures are either very thick and non-dynamic (in non-motile cells) or very few and thin (in motile cells), which in both cases makes them unsuitable for live cell imaging studies. To bypass these limitations, we used the human osteosarcoma cell line (U2OS), which displays stress fiber structures, which are thick enough to be imaged and also dynamic enough to allow the analysis of assembly processes (Hotulainen and Lappalainen, 2006). Thus, the intrinsic properties of their actin filament network makes U2OS cells an excellent model for live cell imaging and consecutively assembly and dynamics studies of actin stress fibers in vivo.

Previous studies have shown that two not truly contractile structures, dorsal stress fibers and transverse arcs, can serve are precursors for the generation of contractile ventral stress fibers (Hotulainen and Lappalainen, 2006). Ventral stress fibers represent the mature contractile stress fiber type and can exert a force on the substrate and the cell body. Interestingly, periodic distribution of stress fiber components including α-actinin and myosin in ventral stress fibers and transverse arcs resembles the organization of muscle sarcomeres. This resemblance implies, that studies of stress fiber assembly could reveal the general mechanisms that govern the generation and maturation of contractile actin structures (Tojkander et al., 2012).

6. Dorsal stress fiber assembly in U2OS cells is regulated by interplay between palladin and VASP (I)

6.1. Palladin regulates dorsal stress fiber assembly in U2OS cells

In the early 2000’s a novel stress fiber component, named palladin was identified in mouse (Parast and Otey, 2000), as well in human cells (Mykkänen et al., 2001). In both studies, palladin was described as colocalizing with known stress fiber and focal adhesion components α-actinin (Parast and Otey, 2000) and ezrin (Mykkänen et al., 2001). Furthermore, the main functional domains of the protein were identified (Fig. 5) (Otey et al., 2005). Although it was suggested that palladin is important for stress fiber and focal adhesion organization, the exact mechanisms of palladin function were unknown. The multiple binding partners of palladin (Otey et al., 2005) and its actin bundling activity (Dixon et al., 2008), raises the question about whether palladin acts as a scaffolding protein or as a bundling factor, and this prompted us to study the role of palladin in stress fiber dynamics and assembly.

Previous studies demonstrated that palladin affects the overall organization of the actin network. Since dorsal stress fibers and transverse arcs serve as precursors for the mature stress fiber network (ventral stress fibers), it was important to determine if palladin contributes specifically to the generation of a particular stress fiber type.

We used colocalization and RNAi depletion studies, combined with micropatterning technology to show that palladin contributes mainly to the assembly of dorsal stress fibers without major effects on the transverse arcs and ventral stress fibers (I, Fig. 1 and 2), a phenotype that was later confirmed by others (Azatov et al., 2016). Furthermore, depletion of palladin compromises the assembly of stress fiber-like bundles, and results in altered cell morphology and diminished contractility of cells cultivated in 3D collagen matrices (I, Fig. 3 and Supl. Figs. S3 and S4).
6.2. Palladin recruits VASP to dorsal stress fibers

Importantly, palladin can not only act as actin bundling factor, but also as a scaffolding protein and thereby bind to many regulators of the actin cytoskeleton (Otey et al., 2005). Therefore, it was important to reveal, if palladin mediates dorsal stress fiber assembly via its function as bundling factor or as a scaffold. Interestingly, we observed that overexpression of palladin, which is known to generate thick actin bars (Boukhelifa et al., 2003; Rönty et al., 2004), resembled the overexpression phenotype of actin nucleating/polymerizing factors, including mDia1 and VASP, rather than the overexpression phenotype of actin bundling factors such as α-actinin1 (I, Fig. 4 and Supl. Fig. S5). This observation and the known association between palladin and VASP (Boukhelifa et al., 2004), prompted us to investigate this interaction in more detail. We revealed that palladin regulates dorsal stress fiber assembly via VASP recruitment to this stress fiber type (Fig. 6). First, we showed that VASP did not incorporate into dorsal stress fibers in palladin knockdown background (I, Fig. 5). Second, VASP depletion by RNAi compromised dorsal stress fiber assembly, which was similar to the phenotype observed in the palladin knockdown cells (I, Fig. 5). Third, a palladin mutant construct with disrupted VASP interaction sites, did not result in the thick actin bars when over-expressed in cells and in contrast to the WT-palladin construct could not rescue the lack of dorsal stress fibers in palladin knockdown cells (I, Fig. 6 and 7). Fourth, palladin and VASP displayed similar rapid dynamics in dorsal stress fibers, which indicates that both proteins might associate with dorsal stress fibers in complex with each other (I, Fig. 8).

It is important to note that both palladin (Dixon et al., 2008; Vattepu et al., 2015) and VASP (Schirenbeck et al., 2006) have strong actin bundling activity. Palladin stimulates actin polymerization via the elimination of the lag phase of the slow nucleation step of polymerization (Gurung et al., 2016). VASP inhibits the binding of capping proteins and enhances processive filament elongation at the filament barbed end (Breitsprecher et al., 2011; Hansen and Mullins, 2010). We speculate that palladin and VASP regulate dorsal stress fiber assembly via their polymerisation/elongation activity because their overexpression phenotypes resemble the phenotypes of actin nucleators such as mDia1 and not of actin bundling factors such as α-actinin1. Furthermore, a recent study indicated that the actin-binding and bundling Ig-like domain of palladin enhances actin polymerization and reduces depolymerization in vitro (Gurung et al., 2016). The data from that study further strengthens our findings, which demonstrated that the primary palladin function in dorsal stress fibers is to enhance actin polymerization and assembly.

Besides interaction with VASP, palladin can also bind directly to profilin via a poly-proline rich motif (Boukhelifa et al., 2004). Palladin has two distinct poly-proline rich binding sites for VASP (Boukhelifa et al., 2004) and the second binding site lies within the region, which has been implicated in profilin binding (Boukhelifa et al., 2006). This raises the question if palladin can bind VASP and profilin simultaneously. However, it is highly likely that binding of VASP to palladin sterically inhibits palladin’s binding to profilin, and profilin bound to palladin likely also compromises the interaction between VASP and palladin. VASP interacts with palladin likely via its EVH1 domain (Boukhelifa et al., 2004) and binds to profilin via its central proline-rich motifs (Reinhard et al., 1995; Ferron et al., 2007), this raises the possibility that VASP might bind palladin and profilin simultaneously. Profilin has been implicated in channeling actin monomers from the Arp2/3 pathway to formin and VASP nucleated actin filaments (Rotty et al., 2015; Suarez et al., 2015). This aligns well with the role of formin mDia1 and VASP in actin polymerization at focal adhesions-associated dorsal stress fibers (Hotulainen and Lappalainen et al., 2006; II), the structural details of their interaction with profilin (Kursula et al., 2008), and the localization of profilin to stress fibers (Boukhelifa et al., 2006; Buss et al., 1992). Therefore, it is possible that palladin can deliver profilin-actin monomers to VASP. Another interesting binding partner of palladin, which binds to the same poly proline rich region as VASP, is Eps8, which has been implicated in barbed end capping (Disanza et al., 2004; Goicoechea et al., 2006). This indicates that Eps8 might have antagonistic function to VASP, since it caps the filament barbed ends and prevents filament polymerisation. This further contributes to the importance of palladin as dorsal stress fiber regulator, as it suggests that palladin could either enhance actin polymerization via binding to VASP and its own polymerisation activity, or inhibit
Figure 6. A working model for the function of palladin in stress fiber assembly. (A) In wild-type cells (left panel), palladin interacts with VASP and recruits the complex to dorsal stress fibers to promote actin filament assembly in these structures. In palladin knock-down cells (right panel), absence of palladin results in mislocalization of VASP and thus, diminished actin assembly along the length of dorsal stress fibers. (B) Palladin regulates the assembly of dorsal stress fibers in 2D environment (left panel) and the general organization of stress-fiber like structures in 3D environment (right panel). The absence of palladin in cells, that are plated in 3D collagen matrices, results in loss of the actin stress fiber network and leads to defects in cell elongation. Yellow indicates focal adhesions, red indicates α-actinin, blue indicates myosin II, magenta indicates F-actin structures in knock-down cells in 3D cultures. The picture is modified from I.
actin polymerisation via binding to Eps8 and possible regulation of filament capping. Anyway, all these interesting possibilities could be assessed with further biochemical and cell-biological experiments.

Importantly, this study also expanded our understanding behind the mechanisms that govern dorsal stress fiber assembly and demonstrated that actin filament assembly is required both along the entire length of the actin filament bundles, and, as previously demonstrated, at the focal adhesions (Hotulainen and Lappalainen, 2006). Vectorial actin filament polymerization at focal adhesions is driven by mDia1 and VASP, and we determined in this present study, that the interaction between VASP and palladin governs the actin assembly along the entire dorsal stress fiber length.

Furthermore, this study demonstrated that myosin-containing stress fiber-like bundles, attached at focal adhesion-like structures, are also present in U2OS cells cultivated in 3D collagen matrixes. The profound effects of palladin depletion on these structures indicated that proper precursor generation is crucial for the assembly of such stress-fiber-like structures in 3D environment. Interestingly, in cells cultivated on 2D environment ventral stress fibers still assembled despite the disturbed dorsal stress fiber generation. This indicates that when the canonical pathway for ventral stress fiber is disturbed (Hotulainen and Lappalainen, 2006), then alternative pathways like the fusion of filopodia-derived dorsal stress fibers (Nemethova et al, 2008) ensure the formation of ventral stress fibers. Nevertheless, an exciting open question is if ventral stress fibers derived from alternative pathways have different molecular composition and properties, for example stability and contractility. Thus, the more pronounced effect of palladin depletion in 3D environment suggests that the alternative pathways compensating for disturbances of the canonical pathways are also affected by palladin depletion. Another plausible explanation for the stronger effects in cells cultured in 3D compared to 2D is that in 3D palladin regulates structures, that are important for cell invasion and matrix degradation in 3D (Najm and El-Sibai, 2014; von Nandelstadh et al., 2014). This might result in differences of general matrix stiffness, which in turn might promote or inhibit the formation of stress-fiber like bundles in 3D.

7. Mechanobiological regulation of stress fiber assembly (II)

Biochemical signalling has long been established as a key mechanism in the actin cytoskeleton regulation (Hodge and Ridley, 2016). In contrast, the mechanical regulation of actin filament assembly has remained elusive. Mechanobiology has emerged as a critical mechanism for regulation of many cellular processes and for example stress fibers and focal adhesions have been described as being mechanosensitive structures (Mochitate et al., 1991; Discher et al., 2005; Pelham and Wang, 1999). However, the details of the mechanobiological regulation of stress fiber assembly were unknown.

7.1. Ventral stress fiber assembly is the mechanosensitive phase of stress fiber assembly and is regulated by AMPK-mediated VASP phosphorylation

We used multiple imaging methods, colocalization studies, drug-treatments, and traction force microscopy to examine how mechanical forces regulate the assembly of the contractile network in U2OS cells (Fig. 7). We identified ventral stress fiber generation as the mechanosensitive step in the stress fiber assembly process (II, Fig. 1). Transverse arc fusion during centripetal flow leads to an increase in their contractility, which ultimately results in turning of the distal dorsal stress fibers and attached focal adhesions, and subsequent alignment of the entire structure into straight contractile bundle, a ventral stress fiber (II, Fig. 1, Fig. 2, Supl. Fig. 1). We showed that the increased contractility, which correlated with myosin II-driven tension, inhibits vectorial actin polymerization at focal adhesions via VASP, and results in fundamental decrease of the actin polymerization rates at ventral stress fibers compared to dorsal stress fibers (II, Fig. 2, Fig. 3, Fig. 4, and Fig. 7). Furthermore, our data demonstrated that the myosin II-derived tension activates AMPK, which in turn phosphorylates and inactivates VASP and leads to the mechanosensitive inhibition of vectorial actin polymerization at the focal adhesion. This results in the assembly and alignment of ventral stress fibers (II, Fig. 4, Fig. 5, and Fig. 6).
Figure 7. A working model for the mechanosensitive assembly of ventral stress fibers in U2OS cells. (A) Vectorial actin polymerization from focal adhesions located at the leading edge of the cell drives dorsal stress fiber elongation. Multiple transverse arcs intersect the dorsal stress fibers to form a network. Vectorial actin polymerization and dorsal stress fiber elongation is regulated by mDia1 and VASP. (B) Arcs move towards the cell center through retrograde flow and fuse with each other to form thicker actomyosin bundles. (C) Actomyosin contractions in the arcs generates force, which is transmitted via their connection with the dorsal stress fibers to the focal adhesions. This leads to the enlargement of the focal adhesion and to the alignment of the dorsal stress fibers, that are located terminally to the arc. The tension generated by the myosin II-driven contractility of the arcs results in VASP phosphorylation and inactivation, and consequently inhibits vectorial actin polymerization in the “terminal” focal adhesions. (D) Cofilin-1 is tension-sensitive and promotes the disassembly of the relaxed dorsal stress fibers. These fibers are not connected to the arc ends and thus, do not contribute to ventral stress fiber assembly. (E) In contrast to the non-productive, relaxed dorsal stress fibers, the contractile stress fibers are protected from cofilin-1–induced severing. These steps lead to the formation of a contractile ventral stress fiber, that is connected to mature focal adhesions at each end and is aligned perpendicularly to the cell edge. The figure is modified from Il.
7.2. Tension-sensitive regulation of dorsal stress fiber assembly

We showed that disruption of the contractile transverse arc network resulted in abnormally elongated dorsal stress fibers. Furthermore, live cell microscopy revealed that weaker myosin-generated forces resulted in dorsal stress fibers disassembly (II, Fig. 8). This prompted us to study the mechanosensitive regulation of dorsal stress fibers. Actin depolymerizing factors ADF/cofilins are among the most prominent and well studied regulators of F-actin disassembly (Poukkula et al., 2011). Importantly, ADF/cofilins localize preferably to the non-contractile/relaxed actin fibers, disassemble the flexible actin filaments in vitro, and increase the bending flexibility of actin filaments (Hayakawa et al., 2011; McCullough et al., 2008; Elam et al., 2013). In line with these studies our data suggested that cofilin1 promotes disassembly of non-productive dorsal stress fibers, which are not subjected to the myosin II generated tension, that is mediated by the transverse arcs (II, Fig. 9). In contrast, contractile actin bundles (e.g. transverse arcs and ventral stress fibers) are under constant tension, and are thus protected from cofilin-1 mediated disassembly.

Collectively, this study revealed that mechanobiological regulation plays as important role in stress fiber assembly as biochemical signalling. In the future, it will be important to determine the effects of both external force and myosin II generated tension also on other actin-binding proteins that regulate stress fiber assembly. It is likely, that also other proteins are involved in regulation of vectorial actin polymerization, as depletion of VASP did not result in complete inhibition of the process (II, Fig. 7).

Good candidates for mediating biochemical and mechanical signalling to stress fibers are formins Dia1 and Dia2, as these are both, involved in stress fiber assembly (IV; Hutulainen and Lappalainen, 2008), and regulated by tension (Jégou et al., 2013; Courtemanche et al., 2013). Since tension increases the polymerisation rates of formins, it will be interesting to determine if polymerisation rates of Dia1 at focal adhesions and Dia2 at transverse arcs is affected by tension generated by myosin II driven stress fiber contractions. Furthermore, formins, e.g. mDia2, and VASP are known to generate actin structures such as filopodia, characterized by significantly distinct properties (Barzik et al., 2014). Thus, formins may promote the turnover of additional focal adhesion-associated actin filament populations independent of VASP, but the significance and regulation of such functions remains to be elucidated.

Importantly, VASP is still present at focal adhesions, even when its actin polymerization activity is inhibited by phosphorylation (II, Fig. 4). Therefore, it is possible that VASP contributes to focal adhesion integrity also via its actin filament bundling activity. These data combined with the findings from publication I of this thesis indicate that VASP may have three distinct roles in stress fiber assembly: i) actin filament bundling to maintain focal adhesions; ii) regulation of vectorial actin polymerisation at focal adhesions; and iii) regulation of actin polymerization along the entire length of dorsal stress fibers.

Our work also found that cells that lack dorsal stress fibers can still form ventral stress fiber-like structures. However, the organization of these actomyosin bundles in cells is abnormal compared to wild-type cells. This suggests that dorsal stress fibers may function as ‘rails’, which support the fusion and consequent maturation of the transverse arcs. We showed that myosin II-driven contractions result in the condensation of transverse arcs. In the future, it will be important to determine, if this intrinsic property of transverse arcs is supported by additional proteins, which might enhance the alignment of transverse arcs.

8. Cofilin-2 regulates actin filament length in muscle sarcomeres (III)

Recent studies suggest that similarly to stress fibers in U2OS cells, actin filaments in muscle sarcomeres undergo dynamic turnover. The dynamic turnover of sarcomeric actin filaments is regulated either via myosin contractility (Skwarek-Maruszewska et al., 2009), or by means of actin subunit exchange at filament barbed and pointed ends (Littlefield et al., 2001; Golkin and Fowler, 2013). Importantly, muscle cells contain specific isoforms of many actin-binding proteins. Although defects in sarcomere actin fiber length and alignment due to mutations and misregulated expression of the muscle specific protein isoforms can lead to multiple heart and skeletal muscle diseases, the precise role of many of these proteins in sarcomere dynamics has remained elusive. The
ADF/cofilins are essential for the regulation of actin dynamics in cells and the different cofilin isoforms cannot functionally compensate for each other in vivo (Bellenchi et al., 2007; Gurniak et al., 2014; Agrawal et al., 2007; Ockeloen et al., 2012). We, therefore, aimed to determine what is the function of the muscle-specific ADF/cofilin isoform, cofilin-2.

Immunofluorescence and RNAi knockdown experiments in cultured neonatal rat cardiomyocytes revealed that cofilin-2 localizes close to the pointed ends of the actin filaments and the M-line in sarcomeres, and is critical for regulation of the proper actin filament length (III, Fig. 1, Fig. 2, and Fig. 3). Cosedimentation and actin depolymerization assays indicated that cofilin-2 mediates these effects via its unique ability to disassemble efficiently also ATP-actin filaments (III, Fig. 4). Furthermore, our data revealed specific cluster of residues in cofilin-2 that are important for ATP-actin binding (III, Fig. 5, Fig. 6, and Fig. 7). These residues are crucial for cofilin-2 function in cells (III, Fig. 6), and likely act as a nucleotide state sensor in ADF/cofilins.

Importantly, the barbed ends of actin filaments of non-muscle cells contain ATP- or ADP-Pi-actin, whereas the pointed end is populated by ADP-actin. The monomer exchange kinetics in muscle sarcomeres, however are partially reversed, and filament assembly also occurs at the pointed end (Littlefield et al., 2001). This suggests that the pointed ends of actin filaments in sarcomeres may contain a mixture of ADP- and ATP/ADP-Pi actin. Thus, the ability of cofilin-2 to promote the disassembly of both ADP- and ATP/ADP-Pi-actin filaments corresponds well to the properties of muscle sarcomeres, where actin monomer exchange occurs also at the pointed ends. The proposed function of cofilin-2 in maintenance of filament length in mature sarcomeres aligns also well with its maturation-dependent expression in cardiomyocytes (III, Fig.1), its increased expression in mature skeletal muscle tissues (Ono et al., 1994; Vartiainen et al., 2002), and its depletion/mutation phenotypes in mice and human. In mice, cofilin-2 depletion leads to accumulation of abnormal filamentous actin structures and disrupts sarcomeric organization (Agrawal et al., 2012; Gurniak et al., 2014). In humans, cofilin-2 mutations result in nemaline myopathy and abnormal filament organization in muscle myofibrils (Agrawal et al., 2007; Wallgren-Pettersson et al., 2011).

The suggested tension-dependent model for sarcomere alignment (Lemke and Schorrer, 2017) and our recent findings, that cofilin disassembles stress fibers, which are not under tension (II) raise an interesting possibility that cofilin-2 might also be regulated by tension in muscle sarcomeres. It is plausible, that the actin filaments in properly aligned sarcomeres are subjected to higher tension, which would inactivate cofilin-2 and protect them against disassembly. In contrast, improperly aligned filaments and non-productive filaments would be disassembled by cofilin-2.

9. Six tropomyosin isoforms are essential and non-redundant for stress-fiber assembly in U2OS cells (IV)

Tropomyosin was discovered in the late 1940s, when it was isolated from muscle (Bailey 1946; Bailey, 1948), and thus it is among the best studied actin-binding proteins in muscles. Tropomyosin is known to stabilize actin filaments in muscle sarcomeres (Cooper, 2002), where it plays a critical role in regulating contraction (Greaser and Gergely, 1971). Importantly, there are over 40 different tropomyosin isoforms in mammals (Gunning et al., 2005), but their precise functions in non-muscle cells are still largely unknown. Because of the large isoform variety and their critical role in muscle cells, we set on to dissect the possible functional differences of tropomyosins in stress fibers using U2OS cells.

9.1. U2OS express a set of six different tropomyosin isoforms

Our data indicate that U2OS cells express a set of six different tropomyosin isoforms: three high molecular weight isoforms (HMW): Tpm 2.1 (Tm1), Tpm 1.6 (Tm2), and Tpm 1.7 (Tm3), and three low molecular weight isoforms (LMW): Tpm 3.1 (Tm5-NM1), Tpm 3.2 (Tm5-NM2), and Tpm 4.2 (Tpm4) (Fig. 8 and IV, Fig. 1). Importantly, we revealed that these tropomyosin isoforms display distinct localization patterns along dorsal stress fibers close to focal adhesions, which are the sites of active actin polymerisation (IV, Fig. 2). Their localizations can be divided into three subgroups: i) the distal part of the dorsal stress fiber, colocalizing with the focal adhesions: Tpm 2.1,
Results and discussion

Figure 8. Gene structures and scheme of the stress fiber localization of the stress-fiber associated tropomyosin isoforms. The panel on the left shows a schematic representation of the intron-exon organization of the four human tropomyosin genes and the six stress-fiber-associated tropomyosin isoforms expressed from these genes. The panel on the right illustrates the distribution of the tropomyosin isoforms within the stress fiber network of U2OS cells. The picture is modified from V.

Tpm 3.1, and Tpm 3.2; ii) along the entire stress fiber: Tpm 1.6; and iii) beneath the focal adhesion at the sites of myosin incorporation: Tpm 1.7, and Tpm 4.2 (Fig. 8, and IV, Fig. 2).

9.2. Tropomyosins have essential and nonredundant roles in stress fiber assembly

Depletion of Tpm 2.1, Tpms 1.6/1.7, and Tpms 3.1/3.2 independently disrupted the stress fiber network and Tpm 4.2 depletion lead to formation of curly stress fibers (IV, Fig. 3 and Fig. 4). This indicates that these tropomyosin isoforms have essential, and at least partially nonredundant functions in stress fiber assembly in U2OS cells. The focal adhesion localization of isoforms Tpm 2.1, Tpm 3.1, and 3.2 and the overexpression phenotype of Tpm 3.1., which leads to the stabilization and enlargement of focal adhesions (Bach et al., 2009), suggest that these isoforms stabilize actin filament populations at distal ends of dorsal stress fibers and focal adhesions (IV, Fig. 1, Fig. 2, and Fig. 3). In contrast, Tpm 1.6 localizes along the entire dorsal stress fiber, therefore Tpm 1.6 possibly stabilizes actin filaments along the stress fibers (IV, Fig. 1, Fig. 2, and Fig. 3). The Tpm 4.2 appears to be different from the other Tpms, as it regulates the incorporation of myosin II into Dia2 formin-nucleated actin filaments, which serve as transverse arcs precursors (IV, Fig. 3, Fig. 4, Fig. 5, and Fig. 6). This aligns well with its role in premyofibrils formation (Vlahovich et al., 2008).

These data and previously published results (Hotulainen and Lappalainen, 2006) suggest that the transverse arcs are assembled from at least two distinct actin filament pools: 1) Dia2-nucleated, Tpm 4.2 decorated actin filaments, containing myosin II, and 2) Arp2/3-nucleated, α-actinin-crosslinked filaments (IV, Fig. 7). Importantly, this indicates that, in contrast to filopodial or lamellipodial structures, the contractile stress fibers are assembled via a pathway that requires a set of several different tropomyosin isoforms and at least two distinct actin filament populations, that are nucleated by Arp2/3 and Dia2 formin.

10. Biochemical functions of tropomyosins: To elucidate the mechanisms of generation of actin filament populations (V)

Their non-overlapping cell biological functions, mode of binding to the actin filament, and isoform variety suggest that tropomyosin proteins could play a critical role in defining distinct actin filament populations and filament sorting in a common cytoplasm (Michelot and Drubin, 2011; Gunning et al., 2015). This prompted us to study the possible biochemical differences between these isoforms and their effects on major stress fiber components.
10.1. Tropomyosin isoforms bind actin filaments with distinct dynamics and segregate to specific filament segments

To test the hypothesis that tropomyosins can define specific F-actin populations, we purified the six different isoforms, which we found to be essential and non-redundant for stress fiber assembly: Tpm1.6, Tpm 1.7, Tpm 2.1, Tpm 3.1, Tpm 3.2, and Tpm 4.2, as non-tagged or sfGFP/mCherry-N-terminally-tagged fusion proteins (V, Fig.1). The in vitro FRAP experiments with sfGFP-tropomyosins on either single actin filaments or on actin filament bundles revealed that the HMW isoforms (Tpm 1.6, Tpm 1.7, and Tpm 2.1) displayed only very slow recovery on actin filaments, which suggested stable association with actin filaments (V, Fig.1 and Suppl. Fig. S2). In contrast, the LMW Tpms (Tpm 3.1, Tpm 3.2, and Tpm 4.2) displayed rather fast recovery, which indicated dynamic association with the actin network (V, Fig.1 and Suppl. Fig. S2). This finding correlates with a recent study, which reported that the HMW isoforms display stronger F-actin binding cooperativity in comparison to the LMW isoforms (Janco et al, 2016).

We also tested if the tropomyosin isoforms have the intrinsic property to segregate into different actin filaments or different parts of the same filament by visualizing the localizations of sfGFP- and mCherry-tropomyosin pairs on actin filaments in in vitro TIRF experiments (V, Fig.2, Suppl. Fig. S1, Suppl. Fig. S2, and Suppl. Fig. S3). We observed that different tropomyosins often segregated into different segments of the actin filament (V, Fig.2 and Suppl. Fig. S3). Moreover, we observed that generally tropomyosin isoforms generated from different genes do not co-polymerize with each other. The exception to this was Tpm 2.1, which appears to co-polymerize with Tpm 3.1 and Tpm 3.2, but not with Tpm 1.6 and Tpm 1.7. These data suggest that different tropomyosin isoforms have the intrinsic ability to segregate into different parts of the actin filament, and could thus generate actin filament populations, decorated by a specific tropomyosin isoform.

10.2. Decoration by specific tropomyosin isoforms confers distinct functional properties to actin filaments

Further biochemical experiments revealed that actin filament populations that are decorated by a specific tropomyosin isoform have distinct functional properties regarding their ability to protect F-actin against coflin-mediated disassembly, and to stimulate myosin II ATPase activity (Fig. 9 and V, Fig. 3, Fig. 4). Only Tpm 1.6 and Tpm 1.7 were able to protect actin filaments from coflin-mediated disassembly, and these isoforms appeared to be the most efficient in competing with the coflin for actin binding as visualized by in vitro TIRF assays (V, Fig. 3 and Supl. Fig. 4). Furthermore, coflin displaced tropomyosin and extended cooperatively along actin filaments towards the pointed end (V, Fig. 3 and Supl. Fig. 4). This correlates with recent study, that demonstrated that coflin clusters grow towards the filament pointed end, because coflin induces a unidirectional conformational change towards the pointed end of the actin filament (Ngo et al, 2015).

Tropomyosins are known to regulate myosin II activity in muscle sarcomeres (Gunning, 2008). Furthermore, MgATPase activity and myosin motility along actin filaments are regulated by some tropomyosins in non-muscle cells (IV; Gunning, 2008). Therefore, we investigated if there is a functional difference between the different stress fiber-associated tropomyosin isoforms regarding myosin II activation. Quantification of the actin-activated ATPase activity of non-muscle myosin IIa meromysosin (NMIIa-HMM) during steady-state demonstrated that only the LMW isoforms, Tpm 4.2, Tpm 3.1, and Tpm 3.2, efficiently increased the ATPase activity of NMIIa-HMM (V, Fig. 4).

Together, these data lead to the following conclusions regarding the function of non-muscle tropomyosin isoforms (Fig. 9). First, different tropomyosin isoforms have an intrinsic ability to segregate into different actin filament populations, and thus define the functional properties of the filaments. Second, the HMW isoforms, Tpm 1.6 and Tpm 1.7 protect the actin filament populations, which they decorate, from coflin disassembly, but do not activate the ATPase activity of NMIIa-HMM. Third, the LMW isoforms, Tpm 4.2, Tpm 3.1, and Tpm 3.2 increase the
Results and discussion

![Diagram showing Tpm 1.6/1.7, Tpm 2.1, Tpm 3.1/3.2, and Tpm 4.2]

**Figure 9. A working model for the role of tropomyosins in sorting of actin filament populations.** Schematic representation of the segregation of stress fiber-associated tropomyosin isoforms to different actin filaments and their role in the determination of the functional properties of distinct actin filament populations. Different isoforms bind F-actin with distinct dynamics and regulate the interaction of non-muscle myosin II and coflin-1 with actin filaments. The picture is modified from V.

ATPase activity of NMIla-HMM, but cannot protect the decorated filaments from coflin-mediated disassembly. Last, the dynamics of F-actin binding of the isoforms corresponds to their specific functions: Tpm 1.6, Tpm 1.7, and Tpm 2.1 associate very stably with actin filaments. In contrast, Tpm 4.2, Tpm 3.1, and Tpm 3.2 interact dynamically with actin filaments. It is important to note that the HMW isoform Tpm 2.1 could not effectively protect the actin filaments from coflin-mediated disassembly or regulate the ATPase activity of NMIla-HMM, which indicates that this isoform might regulate the activity of other actin-binding proteins within the stress fiber network. Previous data demonstrate that Tpm 2.1 localizes strongly with the focal adhesions in the U2OS cells (IV) and regulates mechanosensitive actomyosin-based sarcomere-like contractile units at the integrin adhesions (Wolfenson et al., 2016). Therefore, it is plausible that Tpm 2.1 regulates the activity of some focal-adhesion associated proteins, and possibly interacts with mechanosensitive proteins such as talins, p130Cas, and zyxin.

The precise mechanisms of tropomyosin isoform segregation remain to be elucidated in the future. Possible mechanisms include: i) conformational changes on the actin filament, induced by tropomyosin binding; ii) lack of head-to-tail association between different isoforms; and iii) preference of different isoforms for a particular actin isoform or actin conformation (ATP or ADP-bound).

The presence of two structurally identical tropomyosin-binding grooves along the actin filament indicates the need for coordination between the two tropomyosin-binding grooves in the case of decoration by only one Tpm isoform. This does not support the notion that differences in the N- and C-termini and head-to-tail interactions are solely responsible for the observed tropomyosin segregation. Furthermore, this at least partially explains, why isoforms with very different N-termini (e.g. Tpm2.1 and Tpm3.1/2) do not segregate on different actin filaments.

Structural data from tropomyosin-decorated actin filaments revealed that tropomyosin (Tpm1.1) interacts via its main-negatively charged surface with a positively charged groove on F-actin (von der Ecken et al., 2015). Interestingly, this tropomyosin displays the striking ability to change its position rapidly across the actin filament at low energy cost.
Moreover, the position of this tropomyosin does not obscure any major regions of the myosin-binding site, and myosin binding to the filament results in movement of the tropomyosin. It is possible, that similar mechanism occurs also in stress fibers, with some Tpm isoforms displaying slight shift on F-actin and blocking important myosin binding sites.

Supporting the last possibility, it was shown that non-muscle Drosophila Tm1A preferentially binds to ADP-bound regions of the filament near the pointed end, and then polymerizes from this “nuclei” to the rest of the filament (Hsiao et al., 2015). The same study showed that the Arp2/3 complex blocks pointed ends, which diminishes Tm1A recruitment to these sites. An interesting possibility is that different Tpm isoforms might compete for binding to specific filament segments, including the pointed ends.

Furthermore, it has been suggested that despite their very high sequence identity (only 4 amino acids difference N-terminally) β- and γ-actin isoforms segregate according to the stress fiber type (Dugina et al., 2009). Beta-actin is preferentially distributed in arcs and ventral stress fibers, in contrast, γ-actin is more common in dorsal stress fibers. The same study indicated that β-actin has a more pronounced role in regulation of cell contraction in comparison to γ-actin, which aligns well with the stress fiber distribution of the actin isoforms. This raises the interesting possibility that different tropomyosin isoforms could display stronger preference for a certain actin isoform. This would be especially interesting to test for Tpm4.2, which does not localize along dorsal stress fibers, does not bind to single actin filaments composed of a mixture of β- and γ-actin, but regulates myosin II-driven contractions.

Besides the precise mechanism of tropomyosin sorting on filaments, another interesting question raised by this study is how tropomyosin isoforms are targeted to specific structures in cells. Formins regulate the segregation of tropomyosin isoforms in yeast (Alioto et al., 2016; Johnson et al., 2014). Furthermore, tropomyosins stabilize formin-nucleated filaments and regulate formin-driven polymerization (Ujfalusi et al., 2009; Ujfalusi et al., 2012; Wawro et al., 2007), and together with profilin promote formin-driven nucleation (Alioto et al., 2016). Together these studies suggest that at least the stress-fiber associated formin isoforms (mDia1, mDia2, FHOD1, INF2, and DAAM1) might regulate the recruitment of different tropomyosin isoforms to the stress fiber network (II; Hotulainen and Lappalainen, 2006; Schönichen et al., 2013; Schulze et al., 2014; Skau et al., 2015; Ang et al., 2010).

Therefore, studies on the regulation of tropomyosin targeting to different subcellular locations, and their interactions with other stress fiber-associated proteins will provide important new insights into the mechanisms of stress fiber assembly and the generation of distinct actin populations in cells.
CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This thesis work focussed on the molecular mechanisms behind the assembly of contractile structures in non-muscle and muscle cells. Our studies demonstrated the importance of mechanosensing and the interplay of several actin-binding proteins in stress fiber assembly and revealed the significance of ADF/cofilins for length maintenance in muscle sarcomeres.

It has long been assumed that ventral stress fibers and muscle myofibrils might have very similar assembly patterns and therefore it has been suggested that studies on stress fiber assembly might shed light on general mechanisms of actomyosin bundle assembly. However, it is also important to consider the differences in stress fiber and myofibril organization, and force application to the substrate (Livne and Geiger, 2016). Unlike sarcomeres, stress fibers/fibers are generally poorly aligned and cannot contract as efficiently as myofibrils. Thus, stress fibers generate seemingly isometric and lower forces compared to myofibrils (Deguchi et al., 2006). It would be interesting to understand, if this is a consequence only of the less uniform organization of the stress fiber “sarcomeric” units, or if it is due to the differences in protein composition and dynamics between stress fibers and myofibrils. A crucial difference between stress fibers and myofibrils is the way of myosin activation. The lack of the troponin complex in stress fibers might have drastic effects on the kinetics of myosin activation, and on the synchronization of myosin head binding to the actin filament. Our data suggest that myosin activation in U2OS cells is dependent on a specific tropomyosin isoform, but the precise molecular mechanisms remain mysterious. In order to explain why some isoforms behave as they do, it would be necessary to obtain atomic coordinates of their precise binding position on the actin filament. It is plausible that either the kinetic differences in F-actin binding or an isoform-dependent shift in the binding position along F-actin may define why some tropomyosin isoforms activate myosin, whereas others do not.

Another interesting aspect is how the different tropomyosins segregate along actin filaments and what intrinsic signals guide this phenomenon. As previously mentioned, actin filaments have varying twists (Galkin et al., 2012). This, combined with the presence of different actin isoforms and their reported functional differences (Dugina et al., 2009), represents a possible mechanism for tropomyosin sorting. In the future it will be important to test, if β- and γ-actin have isoform-specific effects, and if the twist of the actin filament is affected by the associated tropomyosin isoforms.

Additionally, formins have been implicated in tropomyosin sorting in yeast (Alioto et al., 2016; Johnson et al., 2014), and thus it will be important to test if mammalian formins can contribute to the sorting of mammalian tropomyosin isoforms (Fig. 10). Importantly, formins affect the conformation of actin filaments (Bugyi et al., 2006), and thus it will be interesting to test if formins could regulate tropomyosin sorting by altering the filament twist or other conformational changes. Filament twisting may also play pivotal role in regulation of the cofilin-tropomyosin interactions.

Mechanosensing is a rapidly developing topic in the actin field. Recent data indicate that many actin-binding proteins can act as mechanosensors, thus expanding the complexity of the interplay of mechanobiological and biochemical signalling pathways. Besides actin filament populations and filament sorting, an important question regarding actin stress fiber and myofibril assembly is how the different precursors and building blocks are aligned together (Fig. 10). As previously mentioned (Lemke and Schnorrer, 2017), force has been suggested to play central role in sarcomere alignment, but majority of the molecular details remain mysterious. In stress fibers, the building blocks of tropomyosin-decorated myosin containing F-actin units and α-actinin decorated Arp2/3-nucleated F-actin units need to be aligned for proper stress fiber contractility. It is a mystery, which proteins are fundamental for connecting these building blocks and how the dorsal stress fibers are connected to the arcs (Fig. 10). Moreover, whether mechanosensitive proteins could act as linkers between these stress fiber units, and if other mechanosensitive pathways besides the AMPK-VASP regulate stress fiber assembly are important open questions for future studies.

In conclusion, this work revealed an important mechanism for filament sorting based on the interactions between specific tropomyosin isoforms and actin filaments. Additionally, these studies shed light on the significance of several central actin-binding proteins including palladin, VASP, and ADF/cofilins for actomyosin bundle
Concluding remarks and future perspectives

**Figure 10. Important future questions regarding stress fiber assembly.** Schematic representation of our current knowledge and highlights of future research questions regarding stress fiber assembly. In the past, we identified that two distinct F-actin actin populations contribute to the assembly of the stress fiber network in U2OS cells. (A) Arp2/3-nucleated filaments contribute to the generation of α-actinin-crosslinked actin filament populations (1 and 2). In a different pathway, formin-nucleated filaments generate tropomyosin (Tpm4.2)-decorated actin filament populations (3). Myosin II is subsequently recruited to the Tpm4.2-decorated (formin-nucleated) actin filaments (4). Panel A highlights an important open question regarding this pathway, namely: what drives tropomyosin segregation. (B) Transverse arcs are generated through the endwise annealing of myosin II-containing actin filament populations and α-actinin-crosslinked actin filament populations (5). The transverse arcs interact with the proximal ends of dorsal stress fibers (6). In the future it will be important to determine which proteins regulate the annealing of the distinct actin filament populations and mediate transverse arc assembly and maturation. (C) Transverse arc maturation and formation of the contractile stress fiber network. Interesting question for future studies is how the dorsal stress fibers and the arcs connect with each other and how this affects for example the force transmission from the arcs to the dorsal stress fibers. The picture is modified from IV.

assembly both in muscle and in non-muscle cells. Furthermore, an interplay between mechanobiological and biochemical signalling proved fundamental for regulation of stress fiber alignment and assembly. Future studies on the precise pathways behind these phenomena will be crucial for our understanding on the general mechanisms of actomyosin bundle assembly, and thus will shed light on the fundamental principles of multiple biological and pathological processes.
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