Actin Dynamics in Muscle Cells

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ACADEMIC DISSERTATION

To be presented for public criticism,
with the permission of the Faculty of Biosciences of the University of Helsinki,
in the Auditorium (235) of the Viikki Infokeskus (Viikinkaari 11, Helsinki)
on the 26th of November 2009, at 10 A.M.

Helsinki 2009

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Cover figure: Localization of actin filaments (red) and α-actinin (green) in cardiomyocytes

ISSN 1795-7079
ISBN 978-952-10-5886-8 (paperback)
ISBN 978-952-10-5887-5 (PDF)
Press: Helsinki University Press
Helsinki 2009

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ABBREVIATIONS

ABP; actin binding protein
ACTA1; gene for skeletal muscle α-actin
ACTC1; gene for cardiac muscle α-actin
ACTN2; gene for α-actinin 2
ADF; actin-depolymerizing factor
ADP; adenosine diphosphate
ATP; adenosine triphosphate
CAPN3; gene for calpain-3
CFL2; gene for cofilin-2
Ca^{+2}; calcium ion
D; aspartic acid
DES; gene for desmin
DMD; gene for dystrophin
E; glutamic acid
F-actin; filamentous actin
FH; formin homology
FHL1; gene for four and a half LIM domain 1
FLIP; fluorescence lost in photobleaching
FLNC; gene for filamin-C, gamma (actin-binding protein 280)
FRAP; fluorescence recovery after photobleaching
G-actin; globular actin
GFP; green fluorescence protein
I; isoleucine
IF; intermediate filament
Ig; immunoglobulin
kb; kilobase pairs
KO; knock down organism
kDa; kilodalton
LDB3 (LIM domain binding 3); gene for ZASP/Cypher
Lmod; gene for leiomodin
Mg^{+2}; magnesium ion
mRNA; messenger RNA
MT; microtubule
MYH7; gene for slow beta myosin
MyHC; myosin heavy-chain
MYBPC3; gene for myosin binding protein-C
MYL3; gene for slow myosin essential light chain 3
MYL2; gene for slow myosin regulatory light chain 2
MYOT; gene for myotilin
NBD; 7-chloro-nitrobenz-2-oxa-1,3- diazole
NEB; gene for nebulin
NLS; nuclear localization signal
PCR; polymerase chain reaction
Pi; inorganic phosphate
PIP_2; phosphatidylinositol (4,5)-bisphospahte
Rho-GEF; Rho-guanine nucleotide exchange factor
RT-PCR; reverse transcription PCR

S; serine
SRF; serum response factor
SH3; Src (tyrosine kinase) homology 3 domain
T; threonine
TCAP; gene for telethonin
TIRF; total internal reflection microscopy
TnC; troponin C
TnI; troponin I
TNNI2; gene for fast, skeletal troponin I
TNNT2; gene for fast, skeletal troponin T
TPM1; gene for tropomyosin 1
TPM2; gene for tropomyosin 2
TPM3; gene fortropomyosin 3
TMOD; gene for tropomodulin
TTN; gene for titin
VCL; gene for vinculin
WH2; WASP-homology domain 2

GLOSSARY

a-band the area of the sarcomere spanned by thick filaments
cardiomyocyte heart muscle cell with a single nucleus
costamere set of proteins close to cell membrane providing membrane linkage between myofibril and sarcolemma
contractility ability of cell to shorten or shrink in respond to stimuli. Contractility is generated by the myosin motor proteins which use ATP hydrolysis as an energy source.
FRAP an imaging technique in which subset of fluorescent molecules are irreversibly quenched by an intense light. The time course of fluorescence recovery reflects the mobility of proteins.
I-band area on both sides of the Z-disc spanned by thin filaments that do not overlap with thick filaments
mechanotransduction sensing extracellular physical forces at the cell surface and its further convection into chemical signals within the cell.
myoblast muscle precursor cell with a single nucleus
myofibril highly organized bundle of contractile proteins or filaments
myofibrillogenesis transition form premyofibril to myofibril
myotube multinucleated skeletal muscle cell
premyofibril myofibril precursor, with some similarity to non-muscle cell stress fibers
sarcomere basic unit in striated muscle cells consisting mostly of think (myosin based) and thin (actin based) filaments sliding one over another generating mechanical force
sarcolemma muscle cell membrane
stress fibres array of parallel filaments consisting of filamentous actin and myosin II
Z-disc boundary of the sarcomere, where antiparallel thin filaments are anchored to α-actinin in association with many other proteins

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on four original articles which are referred in the text by their roman numerals.

I. **Skwarek-Maruszewska A.**, Hotulainen P., Mattila P.K., Lappalainen P. (2009), Contractility-dependent actin dynamics in cardiomyocyte sarcomers. ***Journal of Cell Science***, 122, 2119-26.

II. Chereau D., Boczkowska M., **Skwarek- Maruszewska A.**, Fujiwara I., Hayes D.B., Rebowski G., Lappalainen P., Pollard T.D. and Dominguez R. (2008), Leiomodin is an actin filament nucleator in muscle cells. ***Science***, 320, 239-243

III. **Skwarek-Maruszewska A.**, Boczkowska M., Zajac A.L., Kremneva E., Svitkina T., Dominguez R., Lappalainen P. Dynamic association of the tropomyosin-regulated actin filament nucleator, leiomodin, with actin filament pointed ends in mature cardiomyocyte myofibrils. *Manuscript*

IV. Nevalainen E.M., **Skwarek-Maruszewska A.**, Braun A., Moser M. and P. Lappalainen (2008), Two biochemically distinct and tissue-specific twinfilin isoforms are generated from mouse twinfilin-2 gene by alternative promoter usage. ***Biochemical Journal***, 417, 593-600

ABSTRACT

In every cell, actin is a key component involved in migration, cytokinesis, endocytosis and generation of contraction. In non-muscle cells, actin filaments are very dynamic and regulated by an array of proteins that interact with actin filaments and/or monomeric actin. Interestingly, in non-muscle cells the barbed ends of the filaments are the predominant assembly place, whereas in muscle cells actin dynamics was reported to predominate at the pointed ends of thin filaments. The actin-based thin filament pointed (slow growing) ends extend towards the middle of the sarcomere's M-line where they interact with the thick filaments to generate contraction. The actin filaments in muscle cells are organized into a nearly crystalline array and are believed to be significantly less dynamic than the ones in other cell types. However, the exact mechanisms of the sarcomere assembly and turnover are largely unknown. Interestingly, although sarcomeric actin structures are believed to be relatively non-dynamic, many proteins promoting actin dynamics are expressed also in muscle cells (e.g ADF/cofilin, cyclase-associated protein and twinfilin). Thus, it is possible that the muscle-specific isoforms of these proteins promote actin dynamics differently from their non-muscle counterparts, or that actin filaments in muscle cells are more dynamic than previously thought. To study protein dynamics in live muscle cells, I used primary cell cultures of rat cardiomyocytes. My studies revealed that a subset of actin filaments in cardiomyocyte sarcomeres displays rapid turnover. Importantly, I discovered that the turnover of actin filaments depends on contractility of the cardiomyocytes and that the contractility-induced actin dynamics plays an important role in sarcomere maturation. Together with previous studies those findings suggest that sarcomeres undergo two types of actin dynamics: (1) contractility-dependent turnover of whole filaments and (2) regulatory pointed end monomer exchange to maintain correct thin filament length. Studies involving an actin polymerization inhibitor suggest that the dynamic actin filament pool identified here is composed of filaments that do not contribute to contractility. Additionally, I provided evidence that ADF/cofilins, together with myosin-induced contractility, are required to disassemble non-productive filaments in developing cardiomyocytes. In addition, during these studies we learned that isoforms of actin monomer binding protein twinfilin, Twf-1 and Twf-2a localise to myofibrils in cardiomyocytes and may thus contribute to actin dynamics in myofibrils. Finally, in collaboration with Roberto Dominguez's laboratory we characterized a new actin nucleator in muscle cells - leiomodin (Lmod). Lmod localises towards actin filament pointed ends and its depletion by siRNA leads to severe sarcomere abnormalities in cardiomyocytes. The actin filament nucleation activity of Lmod is enhanced by interactions with tropomyosin. We also revealed that Lmod expression correlates with the maturation of myofibrils, and that it associates with sarcomeres only at relatively late stages of myofibrillogenesis. Thus, Lmod is unlikely to play an important role in myofibril formation, but rather might be involved in the second step of the filament arrangement and/or maintenance through its ability to promote tropomyosin-induced actin filament nucleation occurring at the filament pointed ends. The results of these studies provide valuable new information about the molecular mechanisms underlying muscle sarcomere assembly and turnover. These data offer important clues to understanding certain physiological and pathological behaviours of muscle cells. Better understanding of the processes occurring in muscles might help to find strategies for determining, diagnosis, prognosis and therapy in heart and skeletal muscles diseases.

REVIEW OF THE LITERATURE

1. The Cytoskeleton

Cells are enclosed by a lipid membrane, which serves as a semi-barrier between the outside environment and the internal cytoplasmic components. These components allow the cells to maintain their functions, integrity, communication and compartmentalization. One of the essential cell components is the cytoskeleton, which gives the cell its shape and acts as a stabilizing frame. Three key filament networks of the cytoskeleton - microtubules (MTs), microfilaments (MFs), and intermediate filaments (IFs) - allow cell also to coordinate the interplay between the internal and the external environments (Bray 2001).

The microtubular network is involved in intracellular movements of such structures as chromosomes, mitotic spindles, and other organelles inside the cells. The microfilaments are essential in the contraction, motility, cytokinesis, and endocytosis. Intermediate filaments are the tool of integration of the signals (mechanical stress) within two distinct compartments: nuclear-inner nucleus envelope (via lamins) and the cytoplasm-surrounding environment via cell junctions complexes (such as desmosomes, adherent junctions, gap junctions and tight junctions) situated at the plasma membrane.

Subunit proteins of MTs and MFs (tubulin and actin, respectively) are globular proteins that are able to polymerize into filaments and create highly coordinated and modulated structures. IFs are non-homogenous class of molecules (in humans encoded by at least 65 genes), which can be subdivided into three groups based on the method of assembly: keratins, vimentin-like proteins and lamins.

In contrast to intermediate filaments, microtubules and microfilaments have motor proteins (kinesins and dyneins for MTs, and myosins for MFs) which are responsible for generation of force for movement along filaments (Fuchs and Weber 1994; Herrmann et al., 2007; Howard and Hyman 2009).

2. Actin

Monomeric form of actin (43 kDa) is called G-actin (globular actin). Structural study of actin revealed that the protein can be divided by a horizontal and a vertical line to four domains: the small subdomains 1 and 2 and the large ones; 3 and 4. In comparison to the other three subdomains, the subdomain 2 has significantly smaller mass (Kabsch et al., 1990; Otterbein et al., 2001; Graceffa and Dominguez 2003). In the nucleotide-binding cleft between the domains resides a divalent metal ion (Mg^{+2} or Ca^{+2}) and an adenine nucleotide (ATP or ADP) (Figure 1). The four subdomains are stabilized and connected to the ion and the nucleotide by salt bridges and hydrogen bonds.

Actin monomers (G-actin) have the ability to polymerize into filamentous or fibrous (F) actin.

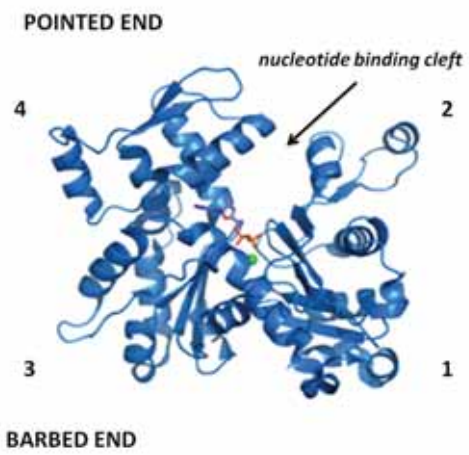


Figure 1

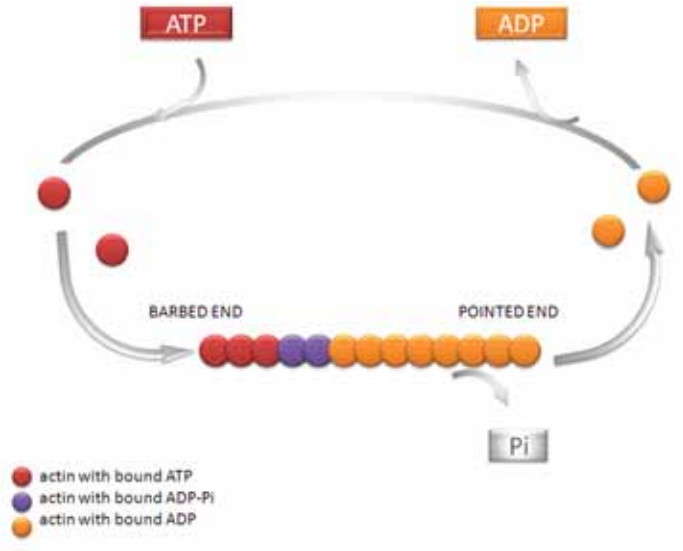
Structure of an actin monomer. The sub-domains 1 and 3 are oriented at the "barbed end" whereas the sub-domains 2 and 4 form the "pointed end" of the protein. In the nucleotide-binding cleft between them resides a divalent metal ion (Mg2+ or Ca2+; highlighted in green) and an adenine nucleotide, ATP or ADP (highlighted in red).

The filament has a polar structure: all subunits are oriented in the same direction. One end is known as the barbed end (plus) and the other one as the pointed end (minus). The subdomains 1 and 3 define the barbed end whereas the subdomains 2 and 4 form the pointed end. Polarity is important as the two ends differ in their affinities and kinetics of association of monomers and interact with different proteins regulating the assembly and architecture of the actin filaments. After polymerization, the conformational change in actin subdomain 2 accompanies the rapid hydrolysis of ATP to ADP and Pi. An additional consequence of the filament polarity is that the barbed end of the filament has higher affinity for ATP-actin than the pointed end. Monomers can be added or depolymerized from either end but both processes are ten-fold faster at the barbed end. The actin monomer net addition at the barbed end and net loss at the pointed end result in a process called treadmilling (Figure 2) (Wang, 1985).

An electron micrograph of actin filaments reveals that the structure consists of two strings of actin around each other in a double helix. The repeating unit consists of 27 subunits. *In vivo* structural plasticity of actin filaments has been recently postulated. This study shows that newly polymerized filaments shrink rapidly but switch to a stable state as they age (Kuech et al., 2008). Binding of proteins such as ADF/cofilin to actin causes a large structural change of actin subdomain 2, where so-called D-loop moves away from the actin hot spot cleft. This rearrangement affects interaction between the two actin strands by weakening their contacts and contributes to the weakening of the actin filament, inducing filament depolymerisation promoted by ADF/cofilin (Paavilainen et al., 2008).

Actin is a well-conserved protein and there is approximately 90% homology between yeast and mammalian actins. Mammals have six tissue-and functionally specific actin isoforms (Herman et al., 1993). Cytoplasmic actins β and γ are ubiquitous and the four muscle actins (skeletal (α-SKA), α-cardiac (α-CAA), α-smooth muscle (α-SMA) and γ-smooth muscle (γ-SMA)) can be found in different muscle types. The most variable region of this highly conserved molecule is the N-terminal region containing 10-20 amino acids (Table 1) (Khaitlina, 2001). Additionally, after synthesis the cytoplasmic isoforms (β and γ) are processed by acetylation and removal of the first methionine residue occurs. In addition, arginylation of 20% of β-actin in motile cells has been reported (Kashina, 2006; Karakozova et al., 2006).

Figure 2



Treadmilling of actin filament. Actin filament growth occurs in a polarised fashion. Addition of the ATP-actin monomers (red) occurs preferably at the fast-growing "barbed" (plus) end, whereas the removal of ADP-bound subunits (orange) occurs from slow-growing "pointed" (minus) end. The ADP moiety is then exchanged for ATP to set actin monomers for another round of polymerization. The net flow of newly acquired G-actin through the filament results in a dynamic filament turnover while the filament length is maintained.

Table 1

Major mammalian actin isoforms. The most variable region in the actin molecule is the N-terminal end. In human skeletal muscles, α-actin is the predominant isoform while α-cardiac actin is the most abundant isoform in the heart tissue. Likewise, α-smooth muscle actin is the major isoform in vascular tissues such as the aorta, while γ-smooth muscle actin predominates in the gastrointestinal and genital tracks. β and γ cytoplasmic actins are ubiquitously expressed.

Type of actin (source)	Abbreviation	N-terminal sequence
skeletal muscle actin	α-SKA	D-E-D-E-T...
cardiac actin	α-CAA	D-D-E-E-T...
smooth muscle actin (aorta)	α-SMA	E-E-E-D-S...
non-muscle tissue	β	D-D-D-I...
smooth muscle (stomach)	γ-SMA	E-E-E-T...
non-muscle tissue	γ	E-E-E-I...

Diversity of the isoforms, the mechano-chemical properties and the accessibility allows actin to be a key component in many cellular processes. Actin-based motile and adherent structures (lamellipodia, filopodia, "comet tails", invadopodia, podosomes) allow active cell migration that is necessary e.g. during wound healing, immunological responses, chemotaxis, pathogen infections and metastasis. In addition, actin-generated movements occur within the cell (reviewed in Pantaloni et al., 2001; Chhabra and Higgs, 2007; Koestler et al., 2008; Fehrenbacher et al. 2003; Gimona et al., 2008). The endocytic pathways in mammals, such as clathrin-mediated, caveolae/lipid raft-mediated, clathrin- and caveolae-independent endocytosis, fluid-phase endocytosis, and phagocytosis are actin-dependent processes (reviewed in Kaksonen et al., 2006).

Contractile actin-myosin structures are important for force generation in muscles as well as in non-muscle cells. For example, in non-muscle cells mechanical force is necessary for regulating cell and tissue morphogenesis during development end embryogenesis (Wozniak and Chen, 2009). In addition, nuclear actin contributes to the structural integrity and architecture of the nucleus and participates in the control of gene expression (reviewed in Vartiainen, 2008).

3. Actin Binding Proteins (Abps)

Spatio-temporal coordination of actin dynamics within the cells is key to its proper functionality. The set of actin binding proteins (ABPs), which bring together actin-based structures, can be divided into two main categories: proteins regulating F-actin assembly and disassembly and proteins regulating assembly of higher order F-actin structures (reviewed in Pollard and Borisy, 2003; Winder and Ayscough, 2005). To maintain controlled actin dynamics in cells the following steps are necessary: the nucleation of new filaments (e.g. by Arp2/3 or formins), nucleotide exchange on actin monomers (e.g. profilin), actin monomer sequestration (e.g. thymosins), monomer delivery and polymerization (e.g. Srv2/CAP and WASP), filament capping at the barbed and pointed ends (e.g. capZ and tropomodulin, respectively), depolymerisation and severing of filaments (e.g. ADF/cofilins) (reviewed in Paavilainen et al., 2004; Pollard, 2007). The cross-linking and bundling proteins (fascin, α-actinin, spectrin), rulers and stabilizers (nebulin, tropomyosin), and membrane anchorage proteins (annexin II, talin, vinculin) are responsible for controlling the three-dimensional organisation of the actin structures in cells (reviewed in dos Remedios et al., 2003).

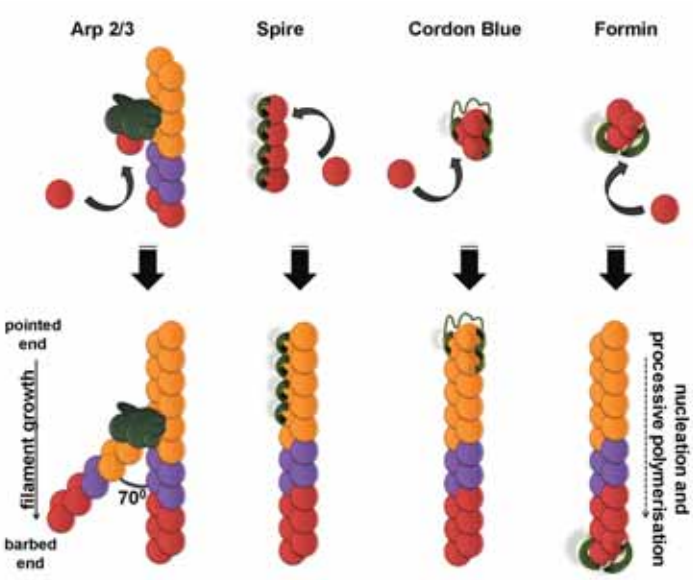


Figure 3

Mechanisms of actin filament nucleation. ATP-actin monomer (red) is brought to the existing actin nuclei stabilised by the nucleators (green). In contrast to other nucleators, formins are found at the barbed (plus) ends of actin filaments. Formin is said to be "processive barbed end cappers" because they remain bound to the barbed ends of actin filaments. The Arp2/3 complex is thought to mimic an actin dimer. Spire recruits and assembles a single stranded linear actin tetramer. Cordon bleu helps to assemble a trimeric actin nucleus to initate actin filament nucleation. ADP-Pi-actin and ADP-actin are violet and orange, respectively.

3.1 Actin Filament Nucleators

Actin polymerization can be accelerated by increasing the rate of filament growth or by generation of new filaments. *De novo* formation of actin filaments is an energetically unfavourable, slow process (reviewed in Qualmann and Kessels, 2008). Therefore, the initial "nucleation phase" (assembly of dimeric or trimeric actin complexes called nuclei) is powered by nucleation factors. So far, four major cellular nucleators of actin assembly have been identified, each overcoming these kinetic barriers via a different mechanism (Figure 3).

The first actin assembly factor identified was the **Arp2/3 complex**, composed of seven polypeptides (ARPC 1–5, Arp2 and Arp3). Arp2 and Arp3 are actin-like subunits - therefore, the Arp2-Arp3 dimer serves as a template for the monomer addition by mimicking the barbed end of a growing actin filament. Bound to the side of the pre-existing filament, the Arp2/3 complex nucleates a new filament and remains associated to its pointed end. Internal reflection fluorescence microscopy (TIRFM) study has showed that the complex preferentially attached near the barbed end of a pre-existing mother filament (reviewed in Amann and Pollard, 2001) creates branches at the characteristic angle of 70 degrees to the host filament. Repeated branching close to the leading edge of a motile cell creates a 'dendritic network' of short, stiff, branched filaments. The growing filaments push the membrane outwards resulting in plasma membrane protrusions. Therefore, the Arp2/3 complex is responsible not only for initiation of nucleation but also for interconnecting of the actin filaments. The Arp2/3 complex requires activation by one of several nucleation-promoting factors (NPFs) - including WASp, N-WASP, Scar/WAVE1, Scar/WAVE2 and Scar/WAVE3 in mammals (reviewed in Higgs and Pollard, 2001; Goley and Welch, 2006).

The second group of actin nucleators are the **formins**. The formins are involved in filopodia formation, cytokinesis, endocytosis as well as in cell polarity and adhesion (reviewed

in Wallar and Alberts, 2003). They not only stimulate the formation of new, unbranched filaments but also affect the rate of elongation at barbed ends. Formins are homodimers that always contain two conserved domains - formin homology 1 and 2 (FH1 and FH2). The FH1 domain contains polyproline repetitions that interact with SH3 domains and profilin (Evangelista et al., 1997; Higgs, 2005). The FH2 domain creates a dimeric ring which nucleates actin filaments by binding and stabilising two actin monomers (Pruyne et al., 2002). The FH2 domains remain associated with the elongating barbed end, providing an anchor and protection against capping. Profilin enhances the rate of elongation by concentrating actin on multiple sites located on the flexible FH1 domains and transferring actin rapidly to the end of the filament (Kovar, 2006).

Spire is the third actin nucleator identified. Similarly to the Arp2/3 complex, it remains bound to the pointed end of a newly formed actin filament, while not binding to the side of an existing filament and not generating a branched actin network. Instead, it promotes formation of linear filaments. The protein contains four closely spaced, linearly arranged actin-binding WH2 motifs and an actin binding linker (Kerkhoff, 2006). It is suggested that these domains work by stabilizing the association of four actin monomers along a single strand of two helical actin filaments (Baum and Kunda, 2005). Spire activity is enhanced by Cappucino formins - for example during establishment of polarity of *Drosophila melanogaster* oocytes (Quinlan et al., 2005; Dahlgaard et al., 2007). Their interaction is conserved also in mammals (Quinlan et al., 2007). Recently, the JMY protein was found to promote a Spire-like actin filament nucleation and to additionally promote actin branching by activation of Arp2/3 complex. Surprisingly, the JMY's role has been earlier associated with activation of p53-dependent transcription but not with regulation of the cytoskeleton (Zuchero et al., 2009).

An additional tissue-specific nucleator has also been found. **Cordon-blue (Cobl)** exists in neurons, especially in their neurites and growth cones and drives morphogenesis of the central nervous system. The protein contains three copies of a well-characterized actin-binding domain, WH2 motif. It binds to actin filaments, associates with their growing ends and promotes actin polymerization in a dose-dependent manner (reviewed in Renault et al., 2008). In addition, Cobl stabilizes a trimeric actin nucleus composed of a linear actin dimer bound to the first two closely spaced WH2 motifs of Cobl and a third actin monomer bound in cross-filament orientation to the third Cobl WH2 motif (Ahuja et al., 2007).

3.2 Actin Monomer Binding Proteins

Cells responding to the internal and external events require the rapid engagement of actin filament polymerization and/or depolymerisation. Because polymerization and depolymerisation can occur only at filament ends, the regulation of free filament ends controls the dynamics and organization of filaments. Also, accessibility and maintenance of the non-polymerized monomers is required and strictly regulated. A large fraction of non-polymerized actin is bound to various ABPs that regulate the amount and availability of monomers (Paavilainen et al., 2004). The key actin monomer-binding proteins that are highly abundant and evolutionary conserved are: profilin, ADF/cofilin, twinfilin, and SrV2/CAP.

Profilin, a small protein (12-16 kDa), has a higher affinity for ATP-G-actin than for ADP-G-actin. It forms a 1:1 complex with ATP-actin monomers (Jockusch et al. 2007). This abundant protein delivers ATP-actin monomers exclusively at the filament barbed ends; thus, it enhances the directionality of treadmilling (Schluter et al., 1997). In the absence of free barbed ends, profilin sequesters G-actin and inhibits filament nucleation. Additionally, the protein also interacts with phosphoinositides (Skare and Karlsson, 2002) and with poly-proline regions found in proteins promoting actin polymerization including formins and N-WASP (Suetsugu et al., 2002;

Evangalista et al., 2003; Miki and Takenawa, 2003). Gene silencing of profilin-1 in mammals led to significant reduction in the formation of actin filaments, focal adhesions and inhibition of cell migration demonstrating the physiological importance of this protein (Ding et al., 2006).

ADF/cofilin is a group of conserved, small (19 kDa) proteins, which bind to both momomeric (G) and filamentous (F) actin. In contrast to profilin, ADF/cofilin binds preferably to ADP-actin subunits that accumulate towards the pointed end of the filament, hence accelerating their dissociation. ADF/cofilins, bind cooperatively along the side of an actin filament at the 1:1 ratio, change the orientation of the F-actin subunits within the filaments - resulting in change of the filament twist (McGough et al., 1997). This conformational change promotes both fragmentation of the filament and increases the rate of subunit loss from the pointed end of the actin filament (Maciver et al., 1991; Carlier et al., 1997). Cofilin-decorated filaments are more flexible than native filaments - mostly due the dissociation of filament-stabilizing ions and reorganization of the actin subdomain 2. Thus, the mechanical asymmetry apparently promotes severing (McCullogh et al., 2008). Both properties can contribute to either increase in the turnover of the actin filaments or to promoting the growth from new filament ends, depending on the local availability of actin monomers. When bound to actin monomers, ADF/cofilin inhibits nucleotide exchange, consequently regulating the recycling of the monomer pool into ATP-actin (Bailly and Jones, 2003).

There are three ADF/cofilin isoforms in mammals: ADF, non-muscle cofilin-1 and muscle specific cofilin-2. Each of them displays distinct expression patterns and biochemical properties (Vartiainen et al., 2002). The ADF/cofilins are inactivated upon phosphorylation at Ser 3 through LIM or TES kinases, or upon binding to phosphoinositols (mainly PIP_2). Their activity is restored upon dephosphorylation - by Slingshot (or other phosphatases) or by PIP_2 hydrolysis (Arber et al., 1998; Ojala et al., 2001; Niwa et al., 2002).

Several studies have confirmed their important role in cytoskeletal remodelling. Cofilin in yeasts is essential for cell viability (Moon et al., 1993). Cofilin-1 depletion by siRNA from fibroblasts causes defects in cytokinesis and cell morphogenesis (Hotulainen et al., 2005). In addition, the cofilin-1 null mice are embryonic lethal due to the defects in neuronal tube morphogenesis and migration of the neural crest cells. Furthermore, the cells from cofilin-1 null mice have problems with the cell polarity and F-bundle formation (Gurniak et al., 2005). The role of cofilin in muscles has been confirmed by intensive studies of *C. elegans*. The nematode's *unc-60* is a homolog to the cofilin gene (McKim et al., 1994). It is expressed as two spliced variants: *unc-60A* and *unc-60B*. *Unc-60B* (referred to as a muscle-specific splice variant) is highly expressed in body wall cells and is necessary for proper assembly of myofibrils in muscles. The worms with altered *unc-60B* gene have disorganised thin filaments. Additionally, the filaments were not able to associate properly with myosin to generate the contraction force - resulting in paralysis of the animal (Ono et al., 1999).

ADF/cofilin is composed of one ADF-homology domain whereas **twinfilin** has two ADF homology domains separated by a short linker region. Twinfilin, similarly to cofilin, binds ADP-monomers with high affinity and prevents their assembly to filament ends (Goode et al., 1998, Vartiainen et al., 2000). In addition to the monomer sequestering function, it also works as barbed end capping protein (Helfer et al., 2006; Paavilainen et al., 2007).

However, the protein's biological function is not clear. Deletion of twinfilin in yeast leads to problems in cell polarization and regulation of the size of cortical actin patches (Goode at al., 1998). In the mammals, twinfilin over-expression causes loss of stress fibres (Vartiainen et al., 2000) whereas in the absence of twinfilin the *Drosophila* bristles show an uncontrollable growth (Wahlstrom et al., 2001).

Another abundant regulator of actin dynamics is the Srv2/CAP protein. Budding

yeast **Srv2** and its eukaryotic homolog **cyclase associated protein (CAP)** bind preferably ADP-actin monomers (Mattila et al., 2004). This multimeric protein (Balcer et al., 2003) accelerates cofilin-mediated severing/depolymerization of filaments. It helps in recycling newly depolymerized actin monomers from ADF/cofilin to profilin-bound ATP-actin monomers. Thus, Srv2/CAP works as an intermediate in actin monomer processing, catalyzes the nucleotide exchange on actin monomers and releases the cofilin for new rounds of filaments depolymerisation (Balcer et al., 2003; Paavilainen et al., 2007). Loss of CAP from mammalian cells results in diminished filament depolymerisation and defects in ADF/cofilin localization (Bertling et al., 2004).

4. CONTRACTILE PROTEINS IN MUSCLES

4.1 Structure and Function of Sarcomere

Human heart cells - cardiomyocytes - are constantly contracting cells; about three billion times during an average lifespan - to pump in average 7000 litres of blood per day (Severs, 2000). Contraction is generated by the single muscle functional unit - sarcomere, which is able to converse chemical free energy into mechanical work. Several aligned sarcomeres build up a myofibril. In this way the system powers force generation and myofibril shortening during muscle contraction (Holmes and Kabsch, 1991, Au, 2004).

Hundreds of protein filaments bundled together in a highly ordered manner create the striated appearance of the sarcomere. There are two major classes of the filaments: thick (about 15 nm in diameter) and thin (about 7nm) filaments composed of myosin and actin, respectively. In order to ensure the proper function of the whole array, hundreds of other proteins accompany the actin-myosin based filaments. Bands with different refraction index and density can be distinguished in polarized light and in electron micrograph sections of a single sarcomere (reviewed in Gregorio and Antin, 2000). The anisotropic myosin filaments are called A-bands (*Anisotropic* in polarised light) whereas the lighter and less polarized actin

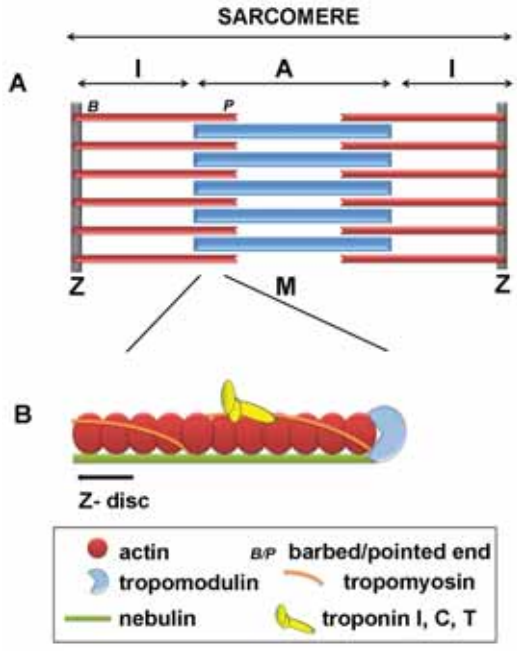


Figure 4

Major structures and components of the sarcomere. (**A**) A simplified schematic representation of the sarcomeric structure. Colour coding: blue; myosin filaments, grey; Z-discs, and red; actin filaments with barbed (B) and pointed (P) ends indicated. (**B**) The enlarged fragment of the thin filament showing the position of the major protein components.

filaments are called I-bands (*Isotropic* in polarised light). The myosin part which does not overlap with actin is called H-zone (for German *Helle* - light). Additionally, there are Z-discs (for German *Zwitter* - between) where actin from neighbouring sarcomeres overlaps, and M-regions (for German *Mittlescheibe* - central disc) where thick filaments are cross-linked in the centre of the sarcomere (Figure 4) (Squire, 1997).

Almost universally accepted theory for muscle contraction is the sliding-filament hypothesis proposed by two independent teams (Huxley et al., 1954a; Huxley et al., 1954b). Contraction is caused by sliding of the thin and thick filaments past each other resulting in shortening of the sarcomeres. Simultaneously, shortening of many sarcomeres leads to development of macromolecular force resulting in muscle contraction (reviewed by Cooke, 2004). The force is generated by the myosin heads, which undergo an actin-activated ATPase cycle during which they form transient cross-bridges between thin filaments in the regions of overlap (reviewed by Geeves and Holmes, 1999).

4.2 Sarcomeric Proteins

4.2.1 Thick filament proteins

The thick filaments in muscle cells are composed mostly of the **myosin II**, which is also called conventional myosin. This large protein (~ 500 kDa) is build from 6 polypeptides chains: two heavy chains (~ 200 kDa each) and two pairs of light chains (~ 20 kDa each). The C-terminal heavy chain forms the tail of the protein, whereas the N-terminal sequence forms the globular head. Each head has an actin-binding side and is able to hydrolyze ATP. The light chains are associated with the heads and they have a regulatory role. The heads lie on the surface, where they can interact with actin during contraction (reviewed by Geeves and Holmes, 1999).

In addition to principal thick filament component - myosin - the sarcomeres also contain several associated proteins that are involved in assembly, maintenance of structural integrity, and regulation of contractile activity.

One group are **myosin binding protein-C** (**MyBP-C**) and its smaller counterpart **myosin binding protein-H** (**MyBP-H**) (reviewed in Winegrad, 1999 and Flashman et al., 2004). MyBP-C is expressed both in heart and skeletal muscles whereas MyBP-H exists only in skeletal muscle. Both proteins are localised in the A-band of the sarcomere. The cardiac isoform is composed of eight immunoglobulin-like domains and three fibronectin 3-like domains. MyBP-H possesses homology (50% of identity) to the four C-terminal domains of MyBP-C. MyBP-C contributes to the thick filament structure via interactions at its C-terminus with myosin and titin. *In vitro*, *in situ*, and *in vivo* experiments indicate that MyBP-C may play a role in the thick filament stability but is not absolutely essential for sarcomere formation and assembly (reviewed in Flashman et al., 2004).

Myomesin plays an important role in the maintaining of the thick filament lattice. It has been shown that antiparallel myomesin dimers link the thick filaments within the M-band, a role analogous to that of α-actinin in the Z-disc (Lange et al., 2005). Myomesin consists mainly of immunoglobulin-like domains and fibronectin 3-like domains. Those domains might function as reversible "shock absorbers" by sequential unfolding in the case of extremely high or long-sustained stretching forces. These complex visco-elastic properties of myomesin might be crucial for stability of the sarcomere (Schoenauer et al. 2005, reviewed in Agarkova and Perriard, 2005).

4.2.2 Thin filament proteins

Actin is the major component of the thin filaments in the muscle (Holmes and Kabsch, 1991). Similarly to filaments in non-muscle cells also muscle actin filaments display polarity and contain the fast-growing (barbed) end and the slow-growing (pointed) end. The thin filament pointed ends extend

toward the middle of the sarcomere's M-line where they interact with the thick filaments to generate contraction. Actin dynamics at both ends of the filament is strictly regulated by a set of actin-binding proteins with distinct functions (reviewed by Clark et al., 2002).

Tropomodulin (**Tmod**) is a tissue specifically expressed protein. The muscle-specific isoform Tmod 1 (43 kDa) is a multifunctional protein: its pointed-end actin filament-capping activity prevents thin filament elongation, whereas its interaction with tropomyosin prevents thin filament depolymerization (Weber et al., 1994; Almenar-Queralt et al., 1999). Decreasing tropomodulin levels in cardiomyoctytes lead to marked elongation of actin filaments, whereas overexpression lead to abnormally short actin filaments (Gregorio et al., 1995). Regulation of pointed ends has important consequences for higher-order functions in multicellular organisms. For example, in mice, deletion of the gene encoding Tmod1 results in embryonic lethality caused by abnormal cardiac development and hemolytic anemia (Chu et al., 2003). Deletion of the *Drosophila* Tmod isoform, *Sanpodo,* results in abnormal neuronal development caused by defects in asymmetric cell division (Dye et al., 1998; Skeath and Doe, 1998).

Tropomyosin (**TM**) is also a multi-isoform protein. There are more than 40 Tm isoforms found in different tissues, generated by alternative mRNA splicing of four genes (reviewed by Gunning et al., 2008). In the striated muscles, these rod-shaped molecules create a double helix along the actin filament (reviewed by Gimona, 2008). Each tropomyosin molecule is in contact with seven actin units. The N-terminal part protruding at the pointed end of the actin filament is required for interactions with Tmod (Sung and Lin 1994, Vera et al. 2000, Greenfield and Fowler 2002). Both proteins form a tight cap preventing addition or loss of G-actin (Almenar-Queralt et al., 1999). Additionally, TM also increases the actin filament stiffness by copolymerizing along their lengths to prevent filament fragmentation and bending (Broschat et al., 1989; Adami et al., 2002; Kostyukova et al., 2004).

Tropomyosin also enhances actin filament assembly and physically protects thin filaments from the depolymerizing effects of ADF/cofilin and gelsolin (Ono and Ono, 2002; Nyakern-Meazza et al., 2002). *In vivo* studies have revealed a critical role of tropomyosin in proper muscle function. For example, homozygous tropomyosin-null mice are embryonic lethal, whereas heterozygous knockout mice show no obvious phenotype (Blanchard et al., 1997; Rethinasamy et al., 1998). In *Drosophila*, mutations of one muscle tropomyosin isoform (*Tm1*) result in disruption of peripheral (but not central) myofibrillar organization, as well as in alterations of force-generating properties (Kreuz et al., 1996). Studies on *Caenorhabditis elegans* demonstrate that suppression of tropomyosin expression leads to disorganized sarcomeric actin filaments and muscle paralysis (Ono and Ono, 2002). Another well-established role of tropomyosin is to cooperate with the troponin complex in regulating the Ca^{2+}-dependent actomyosin interaction (reviewed by Geeves and Lehler, 2002).

The Troponin (Tn) complex - unlike tropomyosins - is present only in striated muscles. It comprises three subunits (TnI, TnC, TnT) defined on the basis of their functions (inhibitory, calcium binding and tropomyosin binding). Troponin I (TnI) has the ability to bind to actin and is responsible for holding the complex together. This globular protein is responsible for inhibition of the actin-myosin interaction. Troponin C (TnC) is a calcium-binding protein which undergoes large conformational changes when binding a calcium ion (reviewed by Solaro and Rarick, 1998; Gordon et al., 2001; Paul et al., 2009). Troponin T (TnT) has a regulatory role during the muscle contraction (Hinkle at al., 2003; Hinkle and Tobacman, 2003).

Tmod regulates the thin filament length at the pointed (slow) end, whereas the barbed (fast) end is capped by **capping protein (CapZ)**. The capping protein prevents depolymerisation and elongation of actin filaments at the Z-disc. Inhibition of CapZ alters thin filament assembly during myofibrillogenesis (Schafer et al., 1995).

Recently, Pappas et al. 2008 presented a model of Z-disc architecture in which nebulin interacts with CapZ of a thin filament from an adjacent sarcomere. This effectively cross-links the thin filaments within the Z-disc, directly joining the two sarcomeres together and preventing growth of the barbed ends into the adjacent sarcomere.

4.2.3 Z- disc proteins

The densely packed structure of Z-discs defines the lateral boundary of sarcomeres within the myocyte cytoskeleton. Its main role is to provide an architectural framework for alignment and anchoring of the thin filament system. In addition, some components of the Z-disc are also involved in signalling processes that may regulate muscle development and degradation. They also link the contractile functions of the muscle sarcomeres to the membrane system and allow for integration via costamers with the extracellular matrix (reviewed in Pyle and Solaro, 2004).

The most widely used Z-disc marker in sarcomers is **α-actinin**. Out of 4 isoforms - the muscle-specific ones are: α-actinin 2 and α-actinin 3. The cardiac α-actinin 2 is an anti-parallel homodimer that cross-links actin/ nebulin filaments in the myofibrils (Chan et al., 1998). α–actinin also binds the amino terminal end of a giant protein - titin - that extends from the middle of the A-band to the Z-band (Sorimachi et al., 1997). In addition, α-actinin binds myotilin (Salmikangas et al., 1999), FATZ (Faulkner et al., 2000), and cypher (Zhou et al., 1999). During myofibrillogenesis a scaffolding protein that organizes α-actinin and actin into symmetrical I-Z-I structures is the muscle-specific **N-RAP**. N-RAP associates with newly forming actin filaments before incorporation of α-actinin (Manisastry et al., 2009).

Myotilin (myofibrillar titin-like protein) is an α-actinin interacting protein in skeletal and cardiac muscle cells. It also binds and cross-links F-actin in thin filaments. It is composed of Ig - like domains located at the C-terminal region of the protein whereas the N-terminal part of myotilin is unique, consisting of a serine-rich region with no homology to the known proteins (reviewed in Otey et al., 2005). Transgenic mice with mutated myotilin develop muscle dystrophy. Surprisingly, myotilin gene disruption in mice does not cause significant alteration in the muscles, the heart or other organs function of newborns or adults (Moza et al., 2007).

Another protein from the novel subfamily, which contains immunoglobulin-like domains, is **myopalladin**. This muscle-restricted protein has multiple roles in Z-disc and thin filaments assembly (reviewed in Otey et al., 2005). Together with titin it might have signalling roles in targeting and orientating nebulin to the Z-line during sarcomere assembly (Ma and Wang, 2002). Overexpression of myopalladin's NH(2)-terminal, CARP-binding region in live cardiac myocytes results in severe disruption of all sarcomeric components studied, suggesting that the myopalladin-CARP complex in the central I-band may have an important regulatory role in maintaining the sarcomeric integrity. It might also be involved, together with CARP (Bang et al., 2001) and Ankrd2 (Miller et al., 2003), in sensing stress signals and linking these to the muscle genes regulation.

4.2.4 Giant muscle proteins

Nebulin in humans and mice is encoded by a single gene (localized at the chromosome 2) that exhibits extensive alternative splicing, which produces isoforms ranging from 500 to 900 kDa (Pelin et al., 1997). It is a filamentous protein of modular organization. Most of the modules are arranged in the highly ordered super-unit repetitions, each containing seven modules. A single nebulin molecule associates along the thin filament with the C-terminus anchored to the Z-disc (Wright et al., 1993) and the N-terminus located at the pointed end of the thin filament (Millevoi et al., 1998; McElhinny et al., 2001). The central region of the protein is composed of up to 185 repeats, each containing 35 amino acids (Labeit and Kolmerer, 1995b). A single nebulin module interacts across sarcomeres with actin monomers, troponin-tropomyosin complex

(Pfuhl et al., 1996), desmin (Bang et al., 2002) and tropomodulin (McElhinny et al., 2001).

The primary function of nebulin is supposedly to serve as a thin filament ruler that determines the pointed-end thin filaments length in striated muscles (McElhinny et al., 2005). A study performed on the nebulin KO mice indicates that nebulin in skeletal muscles is indeed required for specifying and maintaining the thin filament lengths with the precision achieved *in vivo* (Witt et al., 2006). In addition, the same authors showed that in skeletal muscles nebulin's functions extend beyond the pointed-end thin filament length control (as previously suspected) and include control of the thin filament barbed-end capping, Z-disc structure, and contractility. This study excluded a role of the nebulin in myofibrillogenesis. Nebulin knockout mice are viable up to the postnatal day 20 and assembly of the nebulin-free myofibrils occurs. However, over time the mice develop progressive weakness and skeletal muscle myopathy. Recent studies suggested that nebulin is just a component of the combinatorial process that defines the precise filament length (Littlefield and Fowler 2008, Castillo et al., 2009).

It has been suggested that other mechanisms determine actin filament lengths in cardiac tissues, whereas the initial studies failed to detect nebulin mRNA or protein in the heart muscle. However, a recent study revealed that the N-terminal, C-terminal and central regions of nebulin are expressed in the mouse heart but at levels ranging from 0.4-3.3% of the amount of the skeletal muscle (Kaziemierski et al., 2003). Due to low levels nebulin can not be a stable structural component of most actin filaments in cardiac muscles (McElhinny et al., 2003). A good candidate for such protein in heart is a small (107 kDa) homologue of the nebulin - nebulette, although this protein is unable to extend fully along the length of the cardiac thin filaments (Moncman and Wang, 1995; Moncman and Wang, 2002).

Another giant (700 - 3700 kDa), multifunctional structural protein in heart and skeletal muscle cells is **titin**. A single titin molecule stretches from the Z-disc (N-terminus) through the I-band, to the M-band (C-terminus) (Fürst et al., 1989; reviewed by Labeit and Kolmerer, 1995a). The N-terminal part works as an elastic "spring" consisting of two regions of tandem Ig domains. The C-terminal part, acting as a protein-ruler, contains a mixture of immunoglobulin and fibronectin repeats, and possesses kinase activity (Gregorio et al., 1999).

The protein provides mechanical stability and has a key role in the structural integrity of the sarcomere during contraction myofibrillogenesis (Agarkova et al., 2003; Agarkova and Perriard, 2005). During maturation of the sarcomeres, titin's N-terminus is localized in Z-bodies (Sanger et al., 2000; reviewed by Tskhovrebova and Trinick, 2003). The M-line region has been implicated in sarcomere assembly through the titin kinase domain and its substrate - telethonin (Weinert et al., 2006).

Loss of titin's M line ends results in impaired stability of the muscle fibres and disassembly of the existing filaments. Thus, Z-disc and M-band have a predominantly mechanical function, withstanding the strain on the contractile filaments during passive stretch and active contraction (Agarkova and Perriard, 2005). Apart the mechanical functions, titin performs signalling function by interacting with telethonin (also known as t-cap) at the Z-disc, MURF1 and MURF2, obscurin, and DRAL/FHL-2 at the M-band (reviewed in Lange et al., 2006).

Obscurin (approximately 800 kDa) is the third member of a family of giant proteins expressed in vertebrate striated muscle. It is a multidomain protein composed of tandem adhesion modules like fibronectin-III domains and signaling domains like Rho-guanine nucleotide exchange factor (Rho-GEF). The N-terminus of obscurin interacts with the M-band proteins titin and myomesin, whereas the C-terminus mediates interactions with membrane proteins - ankyrins. Obscurin is not present within sarcomeres but surrounds them close to the Z-disc and M-line (reviewed in Kontrogianni-Konstantopoulos et al., 2009).

Obscurin knockout mice display centralized nuclei in skeletal muscles as a sign of mild myopathy, but have normal sarcomeric structure and preserved muscle function. Lack of obscurin in cross-striated muscles leads to changes in sarcoplasmic reticulum architecture (Lange et al., 2009). This result confirms the role of obscurin as a mediator of the interactions between the sarcoplasmic reticulum and the myofibrils (Kontrogianni-Konstantopoulos et al., 2006).

4.3 Actin Dynamics in Muscle

Actin-based thin filaments in sarcomere (similarly to actin in non-muscle cells) also possess an intrinsic polarity. The fast-growing (barbed) end is anchored to the Z-disc and the filament extends towards the middle of the sarcomere's M-line, where it is capped by tropomodulin at the pointed end. Regulation of the dynamics at those ends is achieved in part by capping proteins that block the association and dissociation of monomers from the filament (Clark et al., 2002).

In contrast to the rapid polymerization at the filament barbed ends in the lamellipodia of non-muscle cells (reviewed by Cooper and Schafer, 2000), the filament elongation in muscle cells was proposed to occur mainly at the pointed end. Study performed by Littlefield et al. 2001 on the cardiac myocytes showed that pointed ends are the major sites of actin incorporation in muscle thin filaments and that treadmilling does not account for the maintenance of uniform actin filament lengths in myofibrils. Instead, Tmod dynamics at pointed ends controls the extent of actin assembly and directly determines thin filament lengths in striated muscles (reviewed by Fisher and Fowler, 2003).

The pointed ends of actin filaments in muscles transiently capped by tropomodulin allow for continuous actin subunit exchange, thus helping to control the length of the muscle thin filaments. In addition, a study performed on *Drosophila* confirmed that thin filaments elongate from their pointed ends during the *de novo* myofibril assembly in muscle development (Mardahl-Dumesnil and Fowler, 2001). The regulation of the pointed end capping by SPOD (homolog of the mammalian tropomodulin) is critical for specification of final thin filament lengths in mature myofibrils. On the other hand, the thin filament lengths in *Drosophila* indirect flight muscle (IFM) are not predetermined to a particular length; multiple thin filament lengths are possible suggesting several mechanisms regulating and controlling the length of the filaments (Mardahl-Dumesnil and Fowler, 2001).

Untill now, several mechanisms have been proposed to explain the strikingly uniform lengths in striated muscle sarcomeres. In vertebrates, most of the attention has been focused on nebulin working as a ruler or cap locator (Fowler et al., 2006). However, recently nebulin's role as a ruler has been criticised. It is debated that nebulin in skeletal muscle (and nebullete in cardiac muscle) might not control the pointed-end actin dynamics directly, but instead it could stabilize the large core region of the thin filaments (Littlefield and Fowler, 2008).

It is also important to note, that although actin filaments in muscle cells do not appear to undergo treadmilling (Littefield et al., 2001), many actin binding proteins that in non-muscle cells promoted filament treadmilling are also expressed in muscle cells. Those include e.g. ADF/cofilins, CAP and twinfilin (Vartiainen et al., 2002; Bertling et al., 2004; Nevalainen et al., 2008). Thus, it is possible that in addition to pointed ends subunit exchange also other type(s) of actin dynamics exist in muscle.

5. Aberrations Of Contractile Proteins And Myofibril Abnormalities

Several muscle disorders (heart and skeletal) occur due to improper functioning of sarcomeres or sarcomere-associated proteins. Muscle diseases can be associated with mutations not only in structural proteins but also in signalling molecules, enzymes etc. Such phenotypes can occur due the expression of

Table 2

Selected components of the skeletal muscle myofibre involved in muscle diseases. The abbreviations and short descriptions of the diseases are found in the text.

Protein	Disease
CONTRACTILE AND CONTRACTILE-ASSOCIATED PROTEINS	
α-actin, skeletal *(ACTA1)*	AM, NM,
α-actin, cardiac *(ACTC1)*	DCM, HCM
slow beta myosin *(MYH7)*	CCD, CM
slow myosin essential light chain 3(*MYL3*) and slow myosin regulatory light chain 2 *(MYL2)*	HCM with myopathy
tropomyosin 1 *(TPM1)*	HCM
tropomyosin 2 (TPM2) and tropomyosin 3 *(TPM3)*	NM
fast troponin I *(TNNI-2)* and fast troponin-T *(TNNT-2)*	HCM
myosin binding protein-C *(MYBPC3)*	HCM, CM
four and a half LIM domain 1 *(FHL1)*	EDMD
GIANT PROTEINS	
titin *(TTN)*	LGMD, TMD, HCM
nebulin *(NEB)*	NM
Z-DISC PROTEINS	
α-actinin 2 *(ACTN2)*	DCM
telethonin *(TCAP)*	LGMD
myotilin *(MYOT)*	LGMD, MFM
ZASP/Cypher *(LDB3)*	MFM, DCM
SARCOLEMMA AND CYTOSKELETON ASOCIATED PROTEINS	
dystrophin *(DMD)*	DMD, BDM, DCM
cofilin 2 *(CFL2)*	NM
vinculin *(VCL)*	DCM
calpain 3 *(CAPN3)*	LGMD
desmin *(DES)*	DCM
filamin-C, gamma (FLNC)	MFM

a mutant protein at the sarcolemma, in the extracellular matrix, in the sarcomere or in the nuclear membrane (reviewed in Hernandez et al., 2001; Mudd and Kass, 2008). This leads to development of abnormalities of muscle contraction and relaxation.

For clarity I will focus only on the alterations in the sarcomere proteins. Table 2 lists components of the muscle myofibrils involved in muscle dysfunctions. The abbreviations and short descriptions of the diseases are listed below. More information can be found at http://www.musclegenetable.org/.

Studies of the muscle functions are expected to advance our understanding of the processes occurring in muscles at the physiological and pathological stages. The expanded knowledge of the molecular mechanisms and their bases will allow more focused diagnoses (including prenatal or pre-symptomatic ones), more accurate prognoses, and targeted exploration of possible treatments based on the actual disease mechanisms (reviewed in Laing and Nowak, 2005).

- Becker Muscular Dystrophy and ***Duchenne Muscular Dystrophy*** **(BDM)/(DMD)** - most common types of muscular dystrophies. Rapidly progressing skeletal and cardiac muscle weaknesses. Phenotypically variable but around puberty patients are wheelchair-bound and rarely reach 30 years of age (reviewed in Davies and Nowak, 2006).

- Central Core disease **(CCD)** - a type of cardiomyopathy with characteristic "core" regions in the muscles composed of the structured or amorphous masses of proteins (reviewed in Laing and Nowak, 2005).

- Congenital myopathies **(CM)** - clinical and genetic heterogeneous disorders characterized by skeletal muscles weakness. The major forms have been indentified: actin myopathy (**AM**) with the excess of thin filaments deposits, intranuclear rod myopathy (**IRM**) with characteristic rods inside the nucleus, and nemalin myopathy (**NM**) with cytoplasmic nemaline bodies (rods). Nemaline myopathy is the most common type of the myopathies (reviewed in Goebel et al., 1997; Clarkson et al., 2004; Laing and Nowak, 2005).

- ***Dilated cardiomyopathy*** **(DCM) -** disease in which the ventricles (mainly the left ventricle) are dilated, thin walled and contract poorly (reviewed in Towbin et al., 1999).

- ***Emery-Dreifuss muscular dystrophy (EDMD)*** - a rare disorder characterized by early joint contractures, muscular dystrophy, and cardiac involvement with conduction defects and arrhythmias. It can be either autosomaly dominant or X-linked (reviewed in Davies and Nowak, 2006).

- ***Hypertrophic cardiomyopathy*** **(HCM) -** disease in which the ventricular walls and interventricular septum are thick (hypertrophic) (reviewed in Towbin et al., 1999; Keren et al., 2008). Often associated with ventricular hypertrophy (Kimura et al., 1997).

- ***Limb Girdle Muscular Dystrophy*** **(LGMD) -** heterogeneous disorder occurring at different ages (childhood to adulthood), severity (severe to mild) and inheritance (dominant and recessive). Mostly affects shoulder girdle and pelvic muscle. Necrosis of muscle fibres occurs (reviewed in Danièle et al., 2007; Selcen and Carpen, 2008).

- Myofibrillar myopathy **(MFM) -** myofibrillar dissolution and degradation, skeletal muscle weakness associated with cardiac conduction blocks, arrhythmias, and restrictive heart failure. Characteristic disorganization of the fibre architecture, accumulation of degraded filamentous material in larger aggregates in cardiac and skeletal muscle cells (reviewed in Clarkson et al., 2004; von Nandelstadh et al., 2009).

- *Tibial Muscular Dystrophy* **(TMD)** - mild adulthood disease affecting distal muscles of the body, such as anterior compartments of the legs (reviewed in Laing and Nowak 2005).

AIMS OF THE STUDY

The actin cytoskeleton in non-muscle cells is highly dynamic and the actin filaments are constantly assembled. In contrast, actin filaments in muscles are organized into nearly crystalline arrays (sarcomeres) and are believed to be significantly less dynamic than the ones in other cell types. Although sarcomeric actin structures are believed to be relatively non-dynamic, many proteins promoting actin dynamics are expressed also in muscle cells, e.g. ADF/cofilin and twinfilin. Additionally, the mechanism promoting actin nucleation in muscles has been not known. Therefore, in our studies we aimed to:

1. Reveal the mechanisms underlying the *per se* actin dynamics in muscle cells.

2. Understand the roles and differences between ADF/cofilins and twinfilins in muscles as compared to non-muscle cells.

3. Identify and characterize protein(s) responsible for promotion of actin filament formation in muscle cells.

MATERIALS AND METHODS

The methods used in this study are listed in the table below. The detailed description can be found from articles at the end of this thesis book. The roman numbers of the publications are indicated after the method. Methods personally applied during study are highlighted.

Method	Publication
actin polymerisation assay	II, IV
affinity purification of antibodies	II
confocal image analysis	II, III
contractility assay	I, III
cardiomyocyte primary cell culture	I, II, III, IV
FRAP microscopy	I, III
FLIP microscopy	III
FRAP/FLIP data analysis	I, III
immunofluorescence microscopy	I, II, III, IV
in vitro light microscopy	I
inhibition of cell contraction	I, III
Latrunculin- B assay	I, III
NBD-actin assay	IV
Recombinant protein expression and purification	II, III, IV
siRNA study	I, II, III
sedimentation velocity assay	II
Recombinant DNA techniques (cloning)	I, II, III, IV
RT-PCR	IV
transfections of primary cells	I, II, III, IV
TIRF microscopy	II
Western blotting	I, II, III

RESULTS AND DISCUSSION

The study, which results are presented and discussed below, utilize heart muscle cells (cardiomyocytes) as a model allowing to follow up the processes involved in and underlying the maintenance and dynamics of the sarcomeric structures. The cells used in the studies - cardiomyoctyes from neonatal rat heart (NRC) - maintain the integral myofibrillar apparatus, as verified by their beating. Application of live-imaging microscopy techniques (quantitative and qualitative), accompanied by biochemical studies, allowed us to elucidate how actin filaments assemble, how the processes of sarcomere maintenance occurs, and to determine which regulatory mechanisms underlay these processes.

6. Actin dynamics in heart muscle cells

6.1 Contractility-dependent actin dynamics in cardiomyocyte sarcomeres (I)

The mechanisms controlling the turnover of sarcomere components are poorly understood. We applied the fluorescence recovery after photobleaching (FRAP) technique to follow and visualize protein motility in live, beating cells. This technique allows us to measure not the simple steady-state protein distribution but rather provides information about the kinetic properties of the molecules (Phair and Misteli 2001). In the cells expressing GFP-tagged actin we bleached a small area of a cell. The movement of unbleached molecules from the neighbourhood to the bleached area was measured. Based on that data we determined that the mobile fraction of actin in myofibrils is approximately 30% and the half-time of recovery ($t_{1/2}$) of this mobile fraction is approximately 60 seconds (I, Fig. 3C and Fig. S4). The small mobile fraction (30%) in muscle cells, as compared to the large mobile fraction (close to 90%) in typical non-muscle cells, might be caused by very slow dynamics of majority of actin filaments in myofibrils. Another possible reason might be the tight binding and interaction of a fraction of the actin pool with other proteins and/or membranes leading to no or restricted mobility.

Interestingly, in the cardiomyocyte cultures we noticed a small (approx. 5-10%) population of cells that were not contracting. This group showed no GFP-actin recovery in myofibrils after photobleaching (I, Fig. 1A). To correlate this effect with contraction, we applied inhibitors of contractility: BDM and a more specific myosin II inhibitor, blebbistatin. In both cases, the lack of contraction lead to the lack of recovery (I, Fig. 1C and Fig. 2A). Thus, we concluded that myofibril contraction is a necessary process to the maintain actin turnover in muscle cells. However, the mechanism of how the contractility induces the actin dynamics in muscle cells remains unclear. One possibility is that myosin II itself may induce filament depolymerization as recently demonstrated *in vitro* (Haviv et al., 2008).

6.2 Dynamic actin pool is composed of filaments that do not contribute to the contractility (I)

Latrunculin-B is a wildly used compound, which alters actin polymerization (Coue et al., 1987; Ayscough et al., 1997). This reagent has an ability to sequester free actin monomers thus causing disassembly of the dynamic actin filament pool while the stable filaments remain intact. Results of previous experiments suggested that myofibrils are stable in the presence of latrunculin-A, but the authors did not concentrate on the possible effects of this compound on sarcomere morphology and contractility (Wang et al., 2005). Our experiments involving latrinculin-B revealed three interesting features of F-actin dynamics in sarcomeres.

First, after a short (10 – 30 min) latrunculin-B treatment, the sarcomeric actin structures became more regular, as characterized by the wider and more visible M-band region

(I, Fig. 5A). The cells also displayed less F-actin staining outside the actual sarcomeric structures. These data suggest that sarcomeric actin filaments are relatively stable, whereas the non-sarcomeric actin structures undergo a relatively rapid turnover.

Secondly, the actin kinetics analysis *in vivo* (FRAP analysis of GFP-β-actin expressing cells) demonstrated that majority of dynamic actin pool in myofibrils was depleted after latrunculin-B treatment. However, surprisingly, the lack of this pool did not affect the number of spontaneously beating cells (I, Fig. 5B). Thus, the dynamic, fast recovering pool does not contribute to generation of the contraction.

Thirdly and finally, although the lack of actin monomer pool did not affect the ability of the cells to contract, in the long run we observed less synchronous beating as compared to the non-treated cells. This suggests that cell-substrate adhesion complexes (costamers) or specialized cell-cell junctions (intercalated discs) in cardiomyocytes may depend on the dynamics of the non-sarcomeric actin structures (I, Fig. 5C and Supplementary video 1 and 2).

Collectively, these results suggest that the contractility-dependent actin turnover induces depolymerization of non-productive actin filaments in cardiomyocyte myofibrils, whereas the correctly aligned sarcomeric actin filaments, contributing to contractility, are significantly more stable.

6.3 Proper assembly of actin filaments in cardiomyocytes depends on contractility (I and III)

Myofibrillogenesis, the sequence of events involved in the assembly of contractile proteins into myofibrils, has been intensively studied to date. The most common and accepted model of myofibril assembly contains the following three steps: premyofibrilis, nascent myofibrils and mature myofibrils (reviewed in Sanger et al., 2000; Gunning et al., 2008). The earliest precursors appear near cell periphery as immature fibrils containing punctuated α-actinin bodies, α-actin and muscle tropomyosin. Premyofibrils resemble actin stress fibres in non-muscle cells because they exhibit the same irregular α-actinin and non-muscle myosin II periodicity (Hotulainen and Lappalainen, 2006; Sanger, 2005). Over the time, the myofibrils begin to align with one another and other proteins are recruited (titin, muscle myosin II) and eliminated (e.g. non-muscle myosin II) (Sanger et al., 2000).

Important questions related to the muscle differentiation are how the striated muscles produce myofibrils with such regular arrays of sarcomeres and which mechanisms and pathways are involved in the control of this event. One of the models proposes integrin-dependent cell-matrix adhesion as the starting point for myofibrillogenesis (reviewed in Sparrow and Schock, 2009). Another requirement for proper myofibril assembly is contractility. Inhibition of contraction in skeletal muscles by channel blockers causes myofibril disassembly (De Deyne, 2000) whereas inhibition of the contractility in cadiomyocytes causes disassembly of costameres (Sharp et al., 1997). Our studies also reveal this tendency.

The cells were monitored at various intervals in order to follow the changes in the proteins distribution. After isolation and plating, the myofibrils displayed uniform F-actin staining without visible lack of F-actin in the M-band region. This fibroblast-like array of actin structures remained untouched until the cells spontaneously started to contract (I, Fig. 6A). Between 24 and 44 hours after plating, the cells began to beat and this was accompanied by the appearance of regular sarcomeres with a lack of visible F-actin staining in the M-band (I, Fig. 6B). Furthermore, tropomodulin displayed a relatively diffused localization in newly plated cardiomyocytes, while this filament-pointed end capping protein concentrated at the pointed ends/M-band region in well-organized sarcomeres of contractile cells (I Fig S5).

Also, the actin nucleator - Lmod - appears relatively late during the myofibril assembly as compared to its counterpart - Tmod - and the M-line protein - myomesin (III, Fig.1 and S1). About 24 hours after plating

the cardiomyocytes begin to contract. This is accompanied by the maturation of premyofibrils into myofibrils displaying typical periodic F-actin organization. To confirm that contractility is indeed important for the proper assembly of myofibrils in neonatal cardiomyocytes, we inhibited contractility with blebbistatin or BDM 24 hours after plating (I, Fig. 6B). Importantly, in the presence of these myosin inhibitors myofibrils failed to mature and did not gain the regular sarcomeric F-actin pattern. Also, the Z-disc organization was affected - α-actinin became diffuse and punctate. Nevertheless, the cells were viable because they began to contract after BDM washout. These data show that the maturation of myofibrils is accompanied by a more regular appearance of sarcomeric actin filament arrays and that the reorganization of sarcomeric actin arrays during maturation is dependent on contractility.

We also tested whether localization of Lmod to myofibrils would be affected by cardiomyocyte contractility. Interestingly, prevention of contractility did not immediately affect the striated Lmod localization in myofibrils. However, after longer blebbistatin treatment, loss of Lmod localization to myofibrils was observed in the majority of the cells (III, Fig. 6B and C). These data suggest that although Lmod localization to myofibrils is not directly dependent on myosin II induced contractility, the lack of contractility induces displacement of Lmod from sarcomeres after longer periods of time.

7. Proteins involved in actin rearrangements in muscle cells

Regulation of actin-filament organization and dynamics is important to power such cell functions as motility and contractility. In contrast to the non-muscle cells, where actin filaments turn over rapidly to produce motile actin filaments, in muscle cells they remain relatively stable and display regular organization. However, even such structures must undergo rearrangement and/or maintenance (see also I). In our studies, we focused on the characterization of the novel actin nucleator leiomodin in the muscle cells, and the roles of ADF domain proteins: ADF/cofilin (one domain) and twinfilins (two domains) in muscles. While Lmod is the first identified nucleator in muscle cells, the two other groups of proteins have been studied extensively in non-muscle cells. We have extended previous work to study the muscle cell aspects of those two proteins.

7.1 Identification of novel actin nucleator in muscle cells - leiomodin (II and III)

In contrast to the formins and Arp2/3 complex, well characterized in non-muscle cells, little was known concerning mechanisms controlling actin assembly in muscle cells. Sequence analysis by Roberto Dominguez's laboratory suggested that leiomodin-2 (the cardiac and skeletal muscle isoform), containing three actin-binding sites, could possibly recruit three actin monomers to form a polymerization nucleus.

Previously, leiomodins were regarded as larger homologues of the filament pointed end–capping protein - Tmod, due to the sequence similarity (Conley et al., 2001). Lmod shares with Tmod two actin-binding sites, a flexible N-terminal region and the leucine-rich repeating domain. Lmod also contains a C-terminal extension of 150 residues. Roberto Dominguez's laboratory revealed that Lmod is indeed a strong actin filament nucleating protein. The smallest fragment with strong nucleation activity includes the leucine-rich repeat and the C-terminal extension. The N-terminal region displays three-fold enhancement of the nucleation activity and recruits tropomyosin (II, Fig. 1A).

Biochemical analysis using recombinant proteins performed in Dominguez laboratory confirmed the significant ability of the protein to promote the filament assembly. Application of TIRF (total internal reflection fluorescence) microscopy allowed direct observation of the Lmod-induced actin nucleation. The number of filaments increased exponentially with the Lmod concentration, whereas Lmod had no

effect on the elongation rate of the filament ends. This suggests that Lmod stimulates polymerization by nucleating filaments rather than increasing their elongation rates (II, Fig. 3C). In addition, monitoring the fluorescence increase of pyrene-labled actin upon polymerization confirmed that even nanomolar concentrations of Lmod dramatically stimulate the nucleation/ polymerization, as compared to the spontaneous actin assembly (II, Fig. 3A). Further analysis focused on the potential role of tropomyosin (TM) in modulation of Lmod nucleation activity and revealed that addition of 1μM TM increases the nucleation activity of Lmod (III, Fig. 4C).

7.2 Localization and dynamics of leiomodin in muscle cells (II and III)

FRAP analysis - using GFP-tagged Lmod and Tmod - allowed us to reveal the molecular mobility of those proteins in beating cardiomyocytes. Both proteins display dynamic localization to myofibrils (half-life of ~8 s) with high level of exchangeability (approx. 90%) of the proteins (III, Fig. 3A-C).

To study Lmod localization in cardiomyocytes we applied antibody staining and expression of the enhanced green fluorescent protein EGFP–Lmod fusion protein. In both cases, we noticed that the Lmod localizes close to the M-line, near the pointed ends of the actin filaments (II, Fig. 1B and C). Further analysis using different model systems (stretched chicken cardiomyocytes or zebra fish skeletal muscle myocytes) showed double bands of endogenous Lmod.

Double staining with myomesin antibody confirmed that the Lmod bands flanked the M-lines (III, Fig. 2D), which is consistent with the Lmod localizing to the filament pointed ends. Surprisingly, however, Lmod does not show strong co-localization with Tmod (III, Fig. 2E). While Tmod forms narrow bands that are only occasionally resolved as doublets, the bands of Lmod are broader and better separated. Moreover, Lmod typically localizes farther away from the M-lines than Tmod. In some sarcomeres, Lmod doublets could not be resolved, but Lmod still formed much broader bands than Tmod. The zone of Lmod-positive staining overlapped significantly with myosin II thick filaments. Furthermore our studies using actin monomer sequester - latrunculin-B - revealed that Lmod localization to myofibrils requires the availability of polymerization-competent actin monomers, whereas the localization of Tmod does not (III, Fig. 7A-C).

7.3 Role of Lmod in sarcomere assembly and/or maintenance (II and III)

To study the role of Lmod in sarcomere assembly we applied RNA interference (RNAi). To knockdown the Lmod expression in cardiomyocytes we transfected the cells with Lmod-small interfering RNA oligonucleotides and followed the changes in the protein level and distribution over the time. After 36 hours of Lmod silencing we observed a reduced level of the protein (II, Fig. 2 and Fig. S4) and lack of the organized, striated pattern observed in the control cells transfected with the scrambled oligonucleotide (II Fig. 2 and S5). In Lmod-siRNA cells, the Z-disc protein α-actinin concentrated in small spots, in contrast to the typical striated pattern observed in the control cells (II, Fig. S5). The adhesion and spreading of the Lmod knockdown cells were also diminished compared to wild-type cells.

Thus, Lmod plays a role in sarcomere assembly and organization. Even though Lmod's over-expression and depletion lead to defects in sarcomere organization it was not possible to conclude whether it contributes to the formation of pre-myofibrils and/or regulates the actin dynamics in mature myofibrils.

Our resent analysis provided evidence against the role of Lmod as an initial actin nucleator during myofibrillogenesis (III). Due the fact that Lmod appears during the late phase of the myofibrillogenesis, it is not expected to be an actin nucleator at early stages of myofibrillogenesis (III, Fig. 1A-C, Fig. S1).

Therefore, we assume that Lmod does not contribute to the initial actin filament nucleation during premyofibril formation, but rather contributes to organization and/or

maintenance of mature myofibrils through a mechanism that requires its interaction with tropomyosin. In line with this hypothesis, our recent studies revealed that a subpopulation of actin filaments in cardiomyocyte myofibrils undergoes relatively rapid turnover, which may contribute to the maintenance of correct thin filament organization in mature myofibrils (I). Unfortunately, our attempts to examine the role of Lmod in this contractility-dependent actin dynamics were unsuccessful due to technical limitations.

Thus, the question remains open: which protein or proteins are the key players in the actin filament nucleation at the initial stages of premyofibril assembly? The SALS (sarcomere length short) protein, containing WH2-actin binding domain, has been identified during thin filament assembly in primary muscle cells of *Drosophila*. This protein is required for assuring the proper sarcomere size (Bai et al., 2007). The authors postulated a two-steps model of the filament assembly process. The first step does not require SALS for the initial thin filaments assembly. However, the SALS protein is necessary in the second step for lengthening of the filaments from their pointed ends. A similar, two-step process has also been suggested in the myofibrillogenesis of vertebrate striated muscles (Gregorio and Antin, 2000).

7.4 ADF/cofilins are essential for the correct organization of sarcomeric actin arrays (I)

Despite the suggested slow turnover of actin filaments in myofibrils, many proteins that promote rapid actin dynamics in non-muscle cells are also present in muscles (Ono et al., 1994; Vartiainen et al., 2002; Bertling et al., 2004; Yamashiro et al., 2008). In our studies we focused on ADF/cofilins whose role in underlying actin dynamics in non-muscle cells is relatively well understood. However, the role of these proteins in striated muscles remains unclear (Vartiainen et al., 2002).

Two ADF/cofilin isoforms are expressed in striated muscles - cofilin-1 in heart muscle and cofilin-2 in heart and skeletal muscles. The mutation in cofilin-2 gene, which is the dominant isoform in mature muscles, is associated with nemaline myopathy (Agrawal et al., 2007). Furthermore the mutations in UNC-60B, the *C. elegans* muscle specific homologue of ADF/cofilins, lead to defects in actin organization in the body wall muscles in worms (Ono et al., 1999).

To evaluate whether ADF/cofilins promote actin dynamics in sarcomeres or if they only contribute to dynamics of non-sarcomeric actin structures in muscle cells, we depleted cofilin-1 and cofilin-2 simultaneously from cardiomyocytes by RNAi. The simultaneous transfection resulted in decrease in the cellular cofilin-1/2 levels, as detected by Western blotting (I, Fig. S6A) and immunofluorescence analysis (I, Fig. 7A-D).

As compared to the non-transfected neighbouring cells, the cofilin-1/2 knockdown cells typically had severely disorganized sarcomeric actin filaments (I, Fig. 7B and E) and displayed lack of beating and fluorescence recovery. These conclusions were derived by analysis of the cells expressing GFP-actin (I, Fig. S6B and C). Analysis of the cells transfected only with oligonucleotides specific to either one of the isoforms suggested that depletion of cofilin-2 leads to more severely disorganized sarcomeric actin cytoskeleton than depletion of cofilin-1. However, this has to be confirmed in the future with the aid of isoform-specific antibodies for cofilin-1 and cofilin-2.

These data support the idea that the actin filaments in myofibrilis undergo remodelling and that actin-binding proteins like ADF/cofilins take part in this process. One of hypothetical role of the ADF/cofilins is depolymerisation of the filaments non-productive in contraction. An excess of filaments intended for depolymerisation might also be generated during the myofibrillogenesis. Pre-myofibrilis retain the stress-fibre like structure in the early stages of sarcomeres formation. However, these actin structures must undergo remodelling in order to gain the mature myofibril actin organisation (Dlugosz et al., 1984). In addition to this role(s) in myofibrilis, ADF/cofilins

may also contribute to remodelling of non-sarcomeric cytoskeleton (e.g. costameres) in muscle cells (Ervasti, 2003).

7.5 Localization of twinfilin isoforms in muscle cells (IV)

Twinfilin (Twf), an evolutionary conserved regulator of actin dynamics exists in one isoform in yeast, worms and flies, whereas mammals have two twifilin genes (*Twf1* and *Twf2*) with approximately 75% amino acid identity to each other. Both isoforms bind actin monomers and capping proteins; however, their tissue distribution varies. Mouse twinfilin-1 and -2 are abundant proteins and localize mostly diffusely in the cytoplasm, but are sometimes enriched in filopodia and lamellipodia (Vartiainen et al., 2003).

Because mammals have two splice variants of twinfilin-2 (referred as Twf2a and Twf2b), we decided to analyse their localization in cardiomyocytes. Twf-2b is expressed exclusively in the muscle and heart tissues, whereas Twnf 2a is ubiquitously expressed. Using myc-tagged twinfilin-1, -2a and -2b expressed in rat cardiomyocytes, we observed mostly punctuated cytoplasmic localization for each isoform. Occasionally, we observed enrichment of twinfilin-1 and twinifilin-2b in myofibrils (IV, Fig. 6A and B).

These data, supported by the biochemical analysis, revealed differences between the two splice variants of twinfilin-2. However, the exact role of twinfilins in regulating actin dynamics in muscle cells remains to be determined. Because twinfilins contribute to actin dynamics by sequestering actin monomers and capping filament barbed ends (Ojala et al., 2002; Hefler et al., 2006), it is possible that twinfilins are able to maintain the proper cytoplasmic actin monomer pool in muscle cells. In support of this model, previous studies demonstrated that inactivation of *Twf1* gene in *Drosophila* results in abnormal actin filament assembly in the bristle actin filament bundles (Wahlstrom et al., 2001).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The major myofibril proteins have typically half-lives between 3 and 10 days, meaning that every sarcomere is re-created every 2-3 weeks (Martin, 1981). On the other hand, it has been thought that this highly organized paracrystal protein lattice is stable and does not undergo any dynamics. However, it remains puzzling how this dense and well-organized structure remains dynamic with contractile proteins being synthesized and incorporated into sarcomeres to replace older and potentially damaged proteins ("sarcomere maintenance") (Michele et al. 1999)(Figure 5).

Barbed-end assembly and the pointed-end disassembly (Cooper and Schafer, 2000) drive the highly dynamic actin turnover in non-muscle cells. Thus, the length of the actin filaments is influenced by the termination of growth at the barbed-end and prevention of the shortening at the pointed end. In contrast, in muscle cells the "active" site controlling the filament length is the pointed-end (Littlefield et al., 2001). This occurs primarily due to pointed end capping action of Tmod (Littlefield and Fowler, 1998), which is accompanied by a set of other proteins such as tropomyosins, ADF/cofilins and nebulin. However, little has been known about protein(s) responsible for promoting actin filament nucleation/elongation in muscle cells.

Our study identified a novel actin nucleator in muscle cells - leiomodin. The activity of this mobile, pointed-end localizing protein is enhanced by interactions with tropomyosin. However, its role in primary filament formation is unlikely due the fact that this protein is present only in the late phases of

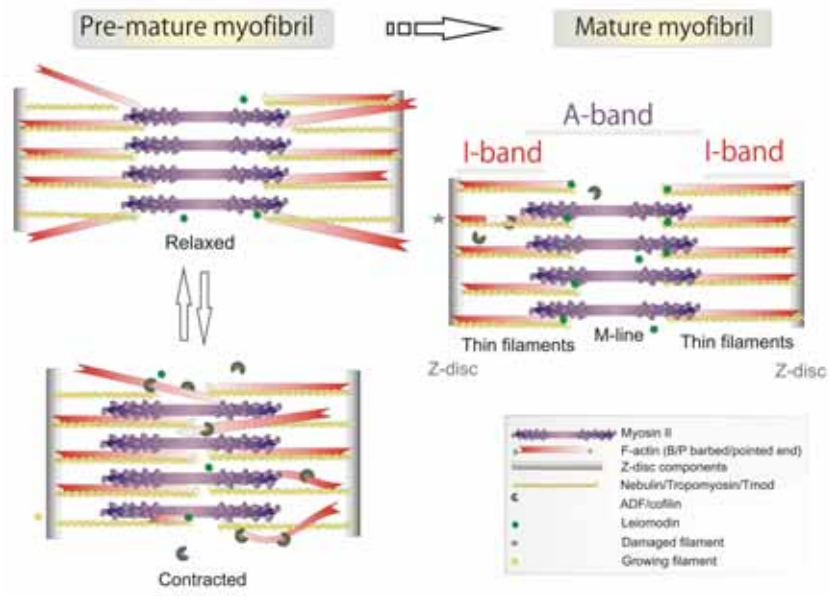


Figure 5

A working model for contractility-induced actin dynamics in cardiomyocyte sarcomeres. In addition to correctly aligned actin filaments, sarcomeres contain mis-aligned actin filaments that do not contribute to contractility. These non-productive actin filaments must be depolymerised, e.g. by ADF/cofilins. The depolymerization of mis-aligned filaments is dependent on contractility. Mis-aligned filaments are subsequently replaced by correctly aligned filaments and in 'mature' cardiomyocytes the population of mis-aligned/damaged filaments is consequently smaller as compared to 'pre-mature' cardiomyocytes. The formation of the new filaments might be catalysed by the muscle specific actin filament nucleator, leiomodin.

myofibrillogensis, when the actin filaments are already present but not yet defined in length. Thus, Lmod appears to be involved in later stages of myofibrils organization/ maintenance.

However, the primary nucleator responsible for the initial actin filament polymerisation still has to be identified. Formins, which are actin nucleators associated with the barbed ends, might be good candidates. Moreover, obscurin has been suggested to act as a molecular scaffold during myofibrillogenesis by providing binding sites for sarcomeric proteins and coordinating the sarcomeric alignment of nearby structures (e.g. the sarcoplasmic reticulum). In addition, obscurin is likely to play an important role in signaling cascades that control homeostasis and muscle gene expression.

Importantly, our studies showed that sarcomeres undergo two types of actin dynamics: (1) contractility-dependent turnover of whole filaments and (2) regulatory pointed end monomer exchange maintaining correct thin filaments length. Our studies bridge the two-steps model of the filament assembly observed in *Drosophila* with the suggestion that a similar process occurs also in vertebrate muscle cells.

The first step is formation of the short thin filaments (this might be formin-driven) and subsequently the filaments are organized to more regular structures (this step could be Lmod- and contractility-dependent). However, the exact sequence of events during the *de novo* formation of actin filaments is still not known. The open questions are: (1) what is the exact mechanisms regulating the stoichiometry of the thin filament (e.g. 1:7 tropomyosin:actin monomer), (2) when and how the newly synthesised proteins are incorporated into the continuously contracting sarcomeres, and (3) is the incorporation process ordered or stochastic (Michele et al. 1999)?

Most of results related to the *de novo* formation of muscle sarcomeres, the organization of the structures into sarcomeres and the key regulators of these events come from intensive studies of embryonic, neonatal and differentiating muscle cell lines. However, it is not well understood if the same processes occur in fully mature muscle sarcomeres or if they are driven by (at least partially) different mechanisms.

Studies of the sarcomere maintenance in adult tissues are still limited due to the technical reasons (e.g. problems in isolation and proper culturing of the cells, limitations in transfection, lack of established alternative methods such as artificial sarcomere-like system mimicking the cell behaviour).

Another important element to define is the similarities and differences between sarcomeres from the cardiac muscle cells and the skeletal muscle cells. It is well known that these two cell types express different sets of proteins (e.g. muscle nebulin versus nebulette in cardiac cells), in different intervals, at different levels, and different isoforms variants. In addition, the mechanisms regulating contraction in muscles and heart are different (electrical and chemical, respectively). This suggests that it is not always possible to employ the same approaches to both systems. Further studies are also necessary to investigate the differences between the protein isoforms expressed in muscle and non-muscle cells. Even thought the proteins belong to the same protein families they are expressed in different tissues and/ or cell compartments where they could play overlapping or tissue-specific roles. Thus, further biochemical, immunochistochemical and functional studies should be carried out to reveal the differences between tropomodulin, ADF/cofilin and twinfilin isoforms.

In the context of studies concerning actomyosin-based cell contractility, it is also worth to mention mechanotransduction and its growing regulatory role in physiological and pathological processes. The forces that are generated by contractile activity regulate embrionic development, morphogenesis, development programming and strengthening of skeletal muscles tendons and bones (reviewed in Wozniak and Chen, 2009) - raging from humans to insects. External, mechanical forces are transduced into changes in intracellular biochemistry and gene expression.

Thus, forces applied at the surface not only modulate the cytoskeletal network, but also promote structural rearrangements in the nucleus. Forces that act on the nucleus might promote changes in shape, folding or kinetics of proteins and/or might modify the higher-order chromatin organization and thereby alter nuclear protein self-assembly, gene transcription and RNA-processing (Wang et al., 2009). For example, it has been demonstrated that stretching of cells, such as cardiomyocytes, causes induction of immediate-early genes followed by strong growth response (Sadoshima and Izumo, 1993). Mechanical tension regulates MAL- and SRF-mediated gene expression in *Drosophila*. Expression of these genes is required for cytoskeletal integrity and for the robust actin cytoskeleton necessary for cell migration and cellular differentiation (Somogyi and Rorth, 2004). It might be interesting (but also challenging) to study the possible nucleoskeletal functions of sarcomeres proteins.

Interestingly, a set of proteins investigated by us has links to the nucleus. For example, leiomodin, similarly to tropomodulin, possesses NES (nuclear export signal) and lack of this part leads to protein accumulation in the nucleus (Kong and Kedes 2004 and III). Interestingly, reduction of the accessibility of the actin monomer pool in cardiomyocytes by the LatB not only displaces leiomodin from myofibrils but also induces its accumulation to the nucleus and in the perinuclear zone (III, Fig. 7C). Lmod possesses probably also NLS (nuclear localization signal) in the C terminal part and the mutation in this region indicates its role in the cyto-nuclear shuttling (III). In addition, cofilin-2 might co-exist in the cytoplasm and in the nucleus as revealed recently by our immunofluorescent studies (Skwarek-Maruszewska et al, unpublished data). A new actin nucleator - JMY - was recently identified. Interestingly, this protein not only activates the Arp2/3 and direct assembly of actin filaments using a Spire-like mechanism, but also shuttles between the cytoplasm and the nucleus. The activity of JMY is regulated, at least in part, by sequestration in the nucleus (Zuchero et al., 2009).

Thus, further biochemical, biophysical and cell biological studies are required to understand the role of various actin-binding proteins in muscle cells and to reveal the cytoplasmic and possible nuclear functions of those proteins. Such studies would provide important clues to understanding certain physiological and pathological behaviours of muscle cells. Better understanding of the processes occurring in muscles might help to find strategies for determining, diagnosing, prognosis and therapy of heart and skeletal muscule diseases.

ACKNOWLEDGEMENTS

This study was carried out during 2004-2009 at the Institute of Biotechnology, University of Helsinki, with financial support from the Viikki Graduate School in Biosciences.

I wish to express my deepest gratitude to my supervisor Professor Pekka Lappalainen for taking this risk and offering me the opportunity to join his lab, for his continuous interest and openness toward my projects and his help in organising my life in Finland. Docent Pirta Hotulainen is warmly thanked for her lab supervision, scientific discussions, all helpful advices and friendliness.

I wish to thank Professors Mart Saarma (former) and Tomi Mäkelä (present), the directors of the Institute of Biotechnology for the provision of the excellent working research and core facilities. The Viikki Graduate School in Biosciences, especially Docent Eeva Sievi and Doctor Sandra Falck, are acknowledged for excellent courses and support during my thesis's studies. I wish to thank the head of the division of Genetics, Professor Tapio Palva, for his positive attitude and help during obtaining my degree.

Professor Olli Carpén and Docent Johan Peränen are warmly thanked for the careful examination of this Thesis and for their timely and valuable comments that helped me to improve it. The input from Johan and Docent Jussi Jäntti as members of my follow-up committee is also warmly appreciated.

My collaborators and co-authors, especially Professor Roberto Dominguez as well people working in his group, are warmly acknowledged for their invaluable contributions to the publications included in the Thesis. Special thanks go to Pieta for bringing to the lab "cardiomyocytes' know-how", to Pirta for teaching me the microscope techniques, to Elena for taking over my projects and to Elisa for incorporating me into her "twinfilin-story".

Thanks go to the great IT support team, the media kitchen, the equipment care facilities and to the other technical services in the Institute. Big hugs for Riitta, Petri, RePe, Iikka, Hannu, Anna-Liisa for making my life easier and solving my everyday's problems.

During my stay in Pekka's laboratory I came in contact with several fascinating people with whom I shared enjoyable, fruitful and inspiring time. Many, many thanks and sincerest gratitude (in chronological order) to Pirta, Enni, Pieta, Elisa, Ville, Perttu, Juha, Miia, Ansku, Maarit, Hongxia, Markku, Sari, Elena, Martina, Geri for creating a great atmosphere, for understanding my weaknesses and for the good teamwork over the years. Your presence, enthusiasm and never-ceasing energy made my work so much easier and the daily efforts in the lab enjoyable. You were my Family - I will not forget you!

Big hugs to the "5th floor people" for the great spirit on the floor, the enjoyable parties, dining and cooking together and the corritor/bench/centrifuge/printers talks on the way. Big hugs go especially to Marion for her openness and friendship.

Thanks, Emidio for the unforgettable trips and for bringing Italian temperament and pasta to my Finnish life.

The person to keep my body and soul in good shape and accompany me in endless Stockmann visits was Jan - thanks for your long - lasting friendship beyond the borders.

Last but certainly not least - I would like to let my family know how much they mean to me and that without their support and encouragement I would have not come that far. Thanks to my parents Zofia and Kazimierz, my brother Michal, father-in-law Wladyslaw and the fantastic aunt - Wanda.

I am especially thankful to my beloved husband Krzysztof for his continuous support, encouragement and staying behind me in all circumstances. Without your help and openness I could not follow my passion and develop myself. I am greatly indebted to you, for things I can never thank enough for.

Helsinki, 2009

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