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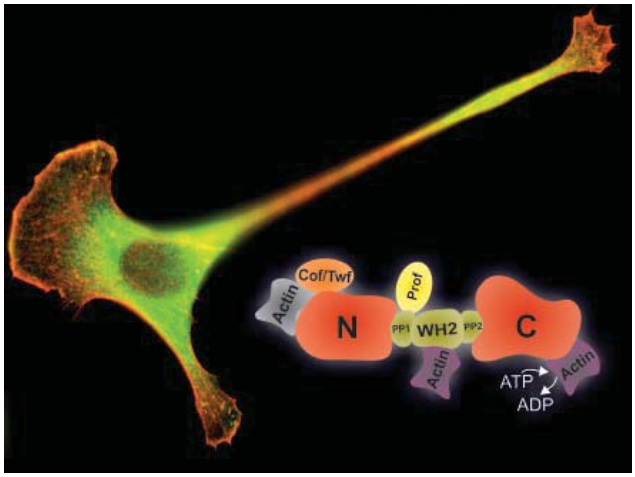
MAARIT MAKKONEN

Interplay between Cyclase-Associated Protein, Cofilin, Profilin and Twinfilin in Actin Dynamics



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Interplay Between Cyclase-Associated Protein, Cofilin, Profilin and Twinfilin in Actin Dynamics

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ACADEMIC DISSERTATION

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Cover figure: A snapshot of a mouse NIH3T3 fibroblast cell expressing GFP-CAP1 (green) and stained with Alexa Fluor 568 phalloidin to visualize filamentous actin (red) and a drawing illustrating the interactions of CAP.

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”Askel ja pysähdys kerrallaan
yksi nurin yksi oikein
etsi tietä joka hehkuu,
polkua joka puhuu”

- A. W. Yrjänä

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on three original articles (I-III) which are referred to in the text by their roman numerals.

I. Quintero-Monzon, O., Jonasson, E. M., Bertling, E., Talarico, L., Chaudhry, F., **Sihvo, M.,** Lappalainen, P., and Goode, B. L. (2009) Reconstitution and dissection of the 600-kDa Srv2/CAP complex: roles for oligomerization and cofilin-actin binding in driving actin turnover. *The Journal of biological chemistry* 284, 10923-10934

II. **Makkonen, M.,** Bertling, E., Chebotareva, N. A., Baum, J., and Lappalainen, P. (2013) Mammalian and malaria parasite cyclase-associated proteins catalyze nucleotide exchange on G-actin through a conserved mechanism. *The Journal of biological chemistry* 288, 984-994

III. Kremneva, E., **Makkonen, M.**, Skwarek-Maruszewska, A., Gateva, G., Michelot, A., Dominguez, R., and Lappalainen, P. (2013) Cofilin-2 controls actin filament length in muscle sarcomeres. *submitted manuscript*

In addition, some unpublished data "Mammalian CAP1 interacts with twinfilin-ADP-G-actin complex" (**Makkonen** et al., unpublished) will be presented.

Contributions:

I. Maarit Makkonen, together with Enni Bertling, carried out experiments for figure 2 (C-D) and figure 5 (D-G) and purified proteins used in these experiments. Maarit Makkonen together with Enni Bertling also cloned some of the plasmids used in assays mentioned above.

II. Maarit Makkonen planned, conducted and analysed the results of >80% of the experiments and prepared all figures. Maarit Makkonen wrote the manuscript together with Pekka Lappalainen.

III. Maarit Makkonen planned, performed the experiments and analysed the results for figures 1 (A-B), 2 (C), 6 (A-D) and S6 (A-B) and prepared the corresponding figures.

ABSTRACT

Several fundamental biological processes rely on actin. The ability of actin to form dynamic networks is crucial for processes including cell migration, endocytosis and cell division in unicellular and multicellular organisms. Furthermore, in sarcomeres of muscle cells, actin and myosin form interdigitating networks responsible for muscle contraction.

Actin is highly conserved and abundant protein in all eukaryotic cells. It exists as monomeric (G-actin) and filamentous (F-actin) forms, which are in balance and strictly regulated by plethora of actin binding proteins. These proteins regulate the assembly, disassembly, branching and capping of actin filaments to produce actin networks that are able to undergo changes rapidly according to cell's needs. Among the most central actin binding proteins are cyclase-associated protein (CAP), actin depolymerizing factor (ADF)/cofilin, profilin and twinfilin, which are under investigation in this study. CAP was first identified as a protein associated with adenylyl cyclase. Later it was realized that this function is not conserved from yeast to mammals and that CAP has more important role related to actin dynamics. Loss of CAP in yeast cells results in abnormalities in actin distribution, and in CAP-deficient mammalian cells decreased cell motility is observed. In nonmuscle cells CAP localizes to dynamic actin structures like lamellipodia together with actin and cofilin. Furthermore, lack of the muscle-specific isoform of mammalian CAP results in disruption of sarcomeric structure and dilated cardiomyopathy in knockout mice.

CAP is known to bind G-actin and accelerate actin turnover together with ADF/cofilin, which is the severing and depolymerizing agent in actin filament regulation. CAP is suggested to release cofilin from actin monomers, thus recycling it for new rounds of depolymerization. Furthermore, CAP has been shown to accelerate nucleotide exchange in actin monomers and this is crucial for building dynamic actin structures. Profilin sequesters actin monomers and promotes their assembly to the filament. It also catalyzes the nucleotide exchange on actin monomers to promote their assembly to filament ends. CAP is known to interact with profilin, but the exact mechanism how these proteins work together is not understood. Twinfilin sequesters actin monomers and caps actin filament ends preventing their growth. It also binds another filament capping protein (CP) but the overall mechanism how twinfilin regulates actin turnover has remained elusive.

In this study, we reveal that interactions with actin monomers, ADF/cofilin and profilin are conserved in CAPs from yeast to mammals. Unexpectedly, we observed that mammalian CAP has higher affinity for ATP-actin than yeast CAP, and that mammalian CAP has two independent profilin binding sites whereas yeast CAP has only one. We also demonstrate a novel function for the 'mini-CAP' from apicomplexan parasite as a nucleotide exchange promoting factor. The malaria parasite CAP comprises only the C-terminal ADP-actin binding site suggesting that this domain is crucial and harbors the most conserved function of CAPs. These findings together enlighten our knowledge of how CAPs regulate actin dynamics together with ADF/cofilin and profilin.

We also revealed that CAP, twinfilin and ADP-actin form a ternary complex. We demonstrate this novel interaction by two independent assays, native PAGE and supernatant depletion pull-down assay. We demonstrate that both ADF-homology (ADF-H) domains of twinfilin are capable of forming a complex with CAP and actin. The mechanism and biological role of this interaction remain to be solved in future.

In addition to studies with nonmuscle proteins, we expanded our research to muscle-specific ADF/cofilin. Many actin-binding proteins have muscle-specific isoforms in addition to nonmuscle ones, but very little is known about these isoforms. We studied the muscle-specific

cofilin-2 and noted that the levels of cofilin-2 increased during sarcomere maturation while cofilin-1 amounts remained constant. We compared the actin binding abilities of muscle-specific cofilin-2 and nonmuscle cofilin-1 and show that cofilin-2 binds ATP-actin with higher affinity than cofilin-1. Importantly, we identified a specific cluster of residues on the surface of cofilin-2 that is responsible for its high-affinity interactions with ATP-actin. Thus, our studies suggest that this region functions as a 'nucleotide sensor' in ADF/cofilins. Therefore, a specific ADF/cofilin isoform with high affinity for ATP-actin evolved to regulate actin dynamics in thin filaments of sarcomeres. The roles of other muscle-specific proteins are under particular interest and subject of future research.

In summary, the findings of this study reveal the mechanisms by which CAP regulates actin dynamics together with ADF/cofilin, profilin and twinfilin. Furthermore, this study elucidates yet rather unknown actin regulation by muscle-specific cofilin-2.

ABBREVIATIONS

Abp1	Actin binding protein 1
ADF	Actin depolymerizing factor
ADF-H	Actin depolymerizing factor homology
ADP	Adenosine diphosphate
Aip1	Actin interacting protein 1
Arp	Actin related protein
ATP	Adenosine triphosphate
BAR	BIN/Amphiphysin/Rvs
CAP	Cyclase-associated protein
cDNA	Complementary DNA
Cobl	Cordon-bleu
Cof	Cofilin
CP	Capping protein
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FH	Formin homology
G-actin	Globular actin
GMF	Glia maturation factor
GRABP	Gelsolin-related actin binding protein
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
hnRNPs	Heterogenous nuclear ribonucleoproteins
IMC	Inner membrane complex
K_d	Dissociation constant
kD	Kilodalton
Lmod	Leiomodin
MAL	Megakaryocytic acute leukemia
MKL1	Megakaryoblastic leukemia 1
mRNA	Messenger RNA
MRTF-A	Myocardin related transcription factor A
NBD	7-Chloro-4-nitrobenzeno-2-oxa-1,3-diazole
NPF	Nucleation-promoting factor
PAGE	Polyacrylamide gel electrophoresis
PfCAP	*Plasmodium falciparum* CAP
P_i	Inorganic phosphate
$PI(4,5)P_2$	Phosphatidylinositol 4,5-bisphosphate
PP1	Polyproline region 1
PP2	Polyproline region 2
Prof	Profilin
Ras	Rat sarcoma
RhoA	Ras homolog gene family member A
RNA	Ribonucleic acid
RNAi	RNA interference

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH3	Src homology 3 domain
Src	Sarcoma
SRF	Serum response factor
TIRF	Total internal reflection fluorescence
WASP	Wiskott-Aldrich syndrome protein
WH2	WASP homology domain 2
WIP	WASP-interacting protein
XAC	*Xenopus laevis* ADF/cofilin

1. INTRODUCTION

1.1. Actin

Actin is highly conserved protein found in all eukaryotic cells. It is among the most abundant proteins in most cells. Striated muscle cells have the highest amounts of actin, which can constitute 20% of total muscle protein. Actin is restricted to only eukaryotes, but it has been suggested that an ancestral actin exists in prokaryotes because bacterial proteins such as MreB show striking similarity to actin when comparing their filamentous structures (reviewed in dos Remedios et al., 2003). In eukaryotic cells, the ability of actin to form polarized filaments and to interact with a number of actin-binding proteins allows it to perform different functions dependent on the cell type, as will be discussed in chapters 1.2. and 1.3.

1.1.1. Structure of actin

Actin exists in monomeric (G-actin) and filamentous (F-actin) forms in cells. Each actin monomer comprises of two domains, which can be further subdivided into two subdomains yielding four subdomains altogether (Kabsch et al., 1990). Each domain consists of similar motif with alternating β-sheets and α-helixes, suggesting that a gene duplication might have taken place (Kabsch et al., 1990).

Actin monomer binds either ADP or ATP complexed with divalent cation Mg^{2+} or Ca^{2+} through a nucleotide binding cleft located in the center of the monomer, between the four subdomains (reviewed in Kabsch & Holmes, 1995). Binding of nucleotide and divalent cation affects the conformation of the actin molecule by domain rotations. This is also case with numerous ligands that actin has, including all the actin-binding proteins which control the conformation of actin and regulate the actin filament turnover (reviewed in Schuler, 2001). The affinity of actin for ATP is higher than for ADP. Ca^{2+}-actin binds ATP with 200-fold affinity compared to ADP whereas Mg^{2+}-actin binds ATP with 4-fold better affinity than ADP (Kinosian et al., 1993). Considering the fact that cells have much higher concentration of Mg^{2+} compared to Ca^{2+}, monomeric actin mainly exists as Mg^{2+}-ATP-actin inside the cells.

1.1.2. Actin filament dynamics

The ability to polymerize from monomers into filaments is the key process involved in all actin structures and processes. Polymerization from scratch is unfavorable since the newly formed actin dimers are more likely to dissociate than associate with another monomer to form a trimer. However, once the trimer has been formed, actin rapidly polymerizes and filament elongates. The conditions inside the cells including high salt (KCl > 50 mM) and Mg^{2+} concentrations favour polymerization. The ends of one filament differ from each other as actin monomers are orientated so that their subdomains 1 and 3 are facing towards the other end and subdomains 2 and 4 towards the other. These ends are named as barbed and pointed ends, respectively, according to their arrowhead-like pattern when decorated with myosins (reviewed in dos Remedios et al., 2003).

Except for associating, actin monomers also dissociate continuously from both ends establishing a steady state where both filaments and monomers are in equilibrium. ATP-actin monomers associate more likely to barbed ends of the filaments than pointed ends, resulting in filament elongation mainly from the barbed end. In steady state, the actin concentration left in solution as monomers is called the critical concentration, and it is 0.1 μM at the barbed end and 0.6 μM at the pointed end (reviewed in Pollard et al., 2000).

In actin filament, ATP-actin monomers rapidly undergo ATP hydrolysis yielding ADP-P_i-actin, which later become ADP-actin when γ-phosphate dissociates. ADP-actin subunits then dissociate at the pointed end and monomeric ADP-actins undergo a nucleotide

exchange process where they are "re-charged" with ATP. ATP-actin monomers are then ready for new rounds of polymerization where they are added to the barbed ends of filaments (reviewed in Pollard & Borisy, 2003). This ATP-hydrolysis driven cycle is called treadmilling and illustrated in Figure 1. Treadmilling provides actin filaments characteristic to perform their multiple dynamic functions in cellular processes. Treadmilling *per se* is comparatively slow process and different steps of the cycle are regulated by an array of actin binding proteins, which will be discussed in more detail in chapter 1.4.

1.2. Actin based processes in animal cells

Many biological processes are actin-dependent. In muscle cells, actin cooperates with myosin in sarcomeres to constrict the cell, and in nonmuscle cells actin constitutes the skeletal framework determining cell shape and movement. Besides actin, the cytoskeleton also includes microtubules and intermediate filaments. The cytoskeletal actin filaments provide mechanical strength and motility for animal cells, whereas microtubules take care of vesicle transport and intermediate filaments function to resist mechanical forces. Also vesicle internalization and cell division are actin-dependent. All these processes are somewhat complicated including numerous proteins involved, but actin is one of the key players in all of these actions and they are introduced below. Furthermore, additional functions of actin in nucleus are discussed in chapter 1.2.5.

1.2.1. Muscle cell contraction

Striated myofibrils found from muscle cells in heart and skeletal muscle are examples of extremely well organized actin networks. In these cells, actin and myosin, accompanied by plethora of other proteins, are organized into specialized units called sarcomeres, which produce force for muscle contraction. Muscle contraction is a result of molecular interactions between these muscle-specific

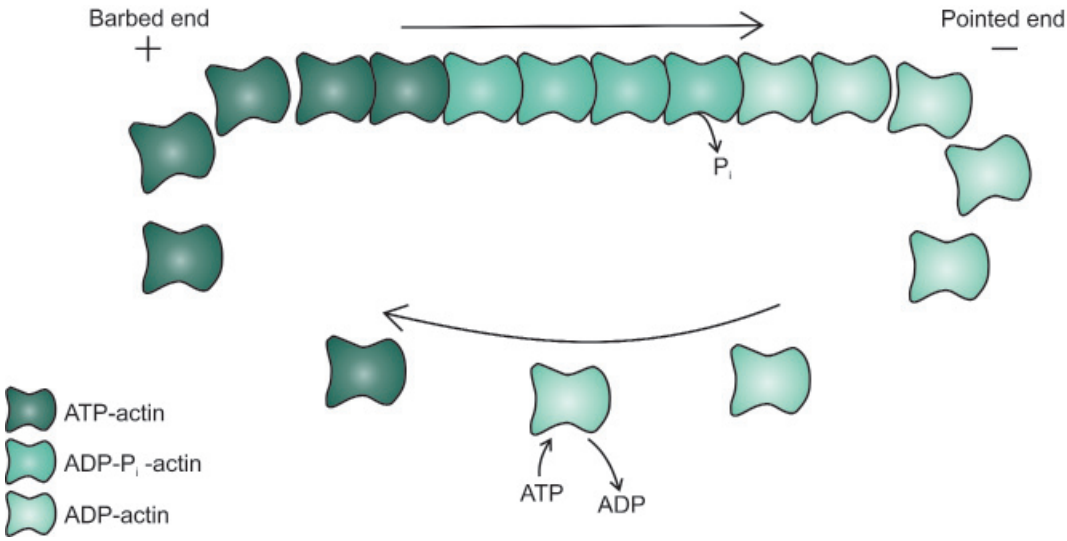

Figure 1. Treadmilling of actin. ATP-actin monomers associate to the barbed (fast-growing) end of the actin filament and undergo rapid ATP hydrolysis. Subsequently the phosphates of the ATP-P_i-actins dissociate, followed by ADP-actin monomer disassembly at the pointed (slow-growing) end of the filament. The nucleotides in ADP-actin momomers are exchanged to ATPs, resulting newly "charged" ATP-actin monomer pool to be added to the barbed end.

proteins that are precisely aligned and tightly regulated, but not static, since they constantly undergo dynamic turnover (reviewed in Sanger & Sanger, 2008).

By light microscopy, several types of stripes are visible in muscle cells resulting from different filament systems linked together (reviewed in Clark et al., 2002). These include dark A bands containing thick myosin II filaments and light I bands containing thin filaments composed of actin but not myosin. In the middle of the I bands are Z discs, which connect adjacent sarcomeric units together thus serving as boundaries between them. α-actinin is the best characterized and major component of the Z disc, but to date, also several other proteins have been identified, which all together form a complex network that not only have a structural role in sarcomeres but are also involved in signal transduction (reviewed in Faulkner et al., 2001 and Clark et al., 2002). The structure of muscle sarcomere is illustrated in Figure 2.

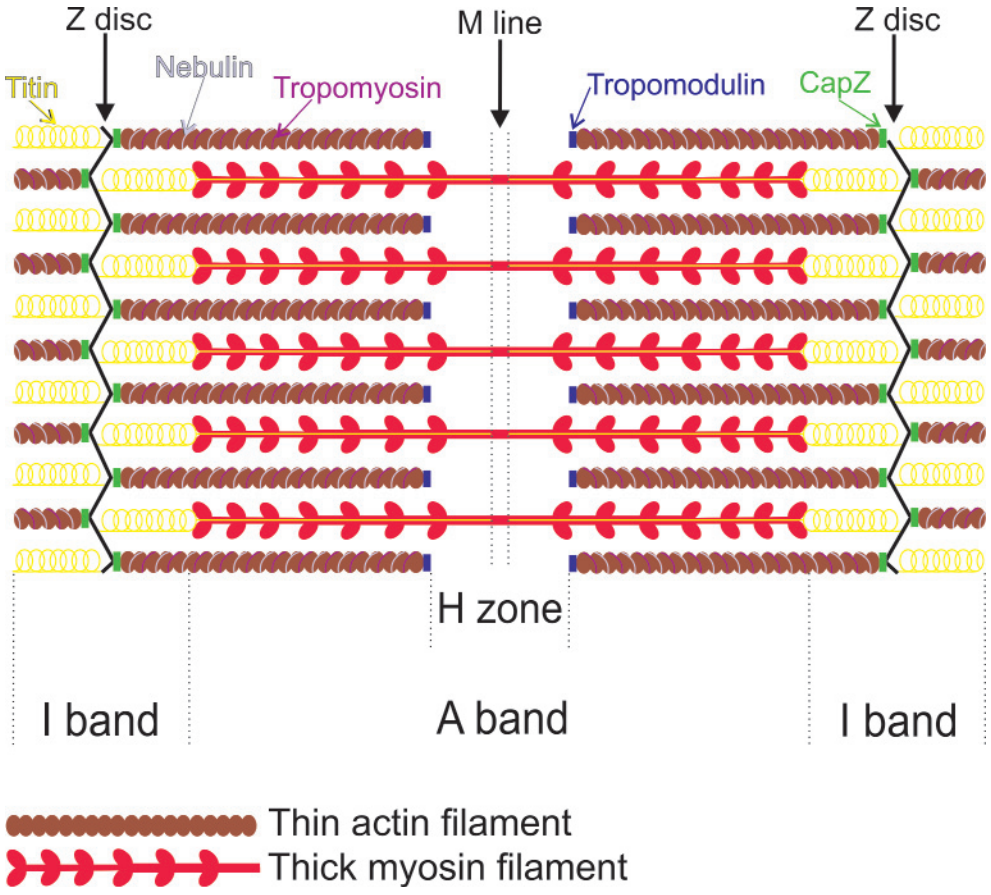

Figure 2. The organization of muscle sarcomeres. Sarcomere is a functional unit stretching from Z disc to another Z disc. The interdigitating thin actin filaments and thick myosin filaments constitute the main components of the sarcomere. Actin filament barbed ends are facing the Z discs and pointed ends are towards the M line. Other proteins including tropomyosin, nebulin, tropomodulin, CapZ and titin regulate the dynamics of the thin and thick filaments to produce muscle contraction. In contracting sarcomere, myosin heads walk along the actin filaments moving them towards the M line and decreasing the H zone width.

The thick myosin filaments interdigitate with thin actin filaments in A band, except in the middle region, which is called H zone. Furthermore, special proteins in the H zone form M line in the middle of the sarcomere. In addition to actin and myosin, giant proteins titin and nebulin form additional filament systems, which are parallel to thin filaments and attached to the Z discs. Titin is anchored to Z discs via its N-terminus, spanning the I- and A bands, whereas its C-terminal end overlaps with the M line (Wang et al., 1979). The enormous size and elastic elements enables titin to act as a molecular spring in maintaining the sarcomeric structure. Nebulin, in turn, extends from Z discs to the pointed ends of the thin filaments and has been suggested to determine the lengths of the thin filaments (Kruger et al., 1991).

Thin actin filaments extend from Z discs to the H zone. They are accompanied by troponins and tropomyosins, which together regulate the force-generating interactions between myosin and actin in a Ca^{2+}-dependent manner (reviewed in Vale & Milligan, 2000 and Craig & Lehman, 2001). Tropomyosins also stabilize thin filaments preventing their depolymerization from the pointed ends (Broschat 1990). The heads of the myosin II molecules at the A band are bipolarly orientated and interact with actin filaments to drive muscle contraction. The tail regions of myosins form M line, which is free from thin filaments. However, several proteins located in the M line have been identified, e.g. myomesin and M-protein (Obermann et al., 1996).

Thin filaments are capped at barbed ends by CapZ (Casella et al., 1987; Hug et al., 1992), which is also called as capping protein (CP) in nonmuscle cells. At pointed ends, filaments are capped by tropomodulin, which binds to tropomyosin and actin molecules blocking elongation and depolymerization at pointed ends of thin filaments (Weber et al., 1994). The lengths of the thin filaments are extremely precise and constant within a myofibril, and several mechanisms have been suggested to regulate their length including capping proteins and 'nebulin ruler' (reviewed in Littlefield & Fowler, 2008 and Gokhin & Fowler, 2013). Also other molecules, e.g. actin polymerizing, depolymerizing, severing and/or sequestering proteins might be involved in determining the thin filament length. In consequence of many proteins involved, the mechanism of this regulation remains still unsolved.

1.2.2. Cell migration

It is crucial for many cell types to perform directional motility. For example, in developing embryo, closure of wound, immune responses, neuronal path-finding and metastatic cancer cells the ability to migrate is essential. In nonmuscle cells, actin is able to produce force without any motor proteins by polymerizing against the cell membrane to push it forward (reviewed Rottner and Stradal, 2011).

The mechanism by which cells migrate is universal and includes four steps (reviewed in Ridley et al., 2003). First, cell polarizes and produces a protrusion at the leading edge. Protrusions can be large lamellipodia or thinner filopodia oriented towards the direction of migration. Second, the protrusions are attached to the substratum, either extracellular matrix or other cells, via adhesion molecules. Third, the rear of the cell is retracted and fourth, it is detached from substratum. This cycle is repeated continuously in crawling-type of motility, although some details may differ according to cell type.

At the leading edge, actin filaments are oriented differently depending on the protrusion type. In filopodia, the filaments are assembled as parallel bundles, whereas in lamellipodia they form branched networks (reviewed in Ridley, 2011). The actin dynamics at the leading edge of moving cell is explained by a dendritic-nucleation model (reviewed in Pollard et al., 2000). In the model, new actin filaments are created by the Arp2/3 complex,

which is activated by Wiskott-Aldrich syndrome protein (WASP) family proteins. The elongating filaments push the plasma membrane forward establishing a protrusion. The elongation of individual filaments is soon restricted by capping protein, and new Arp2/3 complexes are needed for initiating new branches to the actin "bush". Hydrolysis of ATP and dissociation of phosphate in actin filaments triggers ADF/cofilins to sever and depolymerize older filaments yielding ADP-actin monomers, which are converted to ATP-actin monomers by profilin. Maintaining a large pool of monomeric actin is essential for rapid filament growth and some actin binding proteins are involved in maintenance of the large monomer pool. This is important, because the concentration of actin monomers needed is above the critical concentration for actin filament assembly. This task is taken care by e.g. profilin and thymosin-β4, which both bind actin monomers and compete with each other for binding. However, from these two proteins only profilin is able to elongate actin filament barbed ends when bound to ATP-actin (reviewed in Pollard et al., 2000). In continuously moving cells, the assembly and disassembly of actin filaments are in balance, controlled by mechanisms described above.

The attachment of protrusion to the substratum is mediated by specific receptors found in cells. The major family of these receptors are integrins, which link the extracellular matrix to actin filaments via adaptor proteins. Adhesions serve as traction points or "feet" by which cell is able to grab the substratum. Actin together with myosin generates contractile force that is transmitted to adhesion sites, allowing cell to move forward (reviewed in Ridley et al., 2003 and Rottner and Stradal, 2011). Finally, to be able to proceed, cell needs to disassemble the adhesions at rear to promote rear retraction (reviewed in Webb et al., 2002).

1.2.3. Endocytosis

Cellular uptake is fundamental process for the normal function of many cell types. Cells use endocytosis e.g. to dispose of pathogens or damaged cells, signaling with other cells and take up nutrients or potential antigens. Endocytosis includes many types of cellular uptake such as phagocytosis, macropinocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis, from which the clathrin-mediated endocytosis will be discussed here in more detail, because it is the best characterized form of endocytosis (reviewed in Doherty & McMahon, 2009).

Clathrin-mediated endocytosis is a complex process involving many proteins with different properties, including endocytic coat proteins and regulators of actin assembly. Endocytosis includes several processes that occur sequentially, specific proteins functioning in particular phases. First, endocytic proteins, including clathrin, are recruited on the plasma membrane forming an endocytic coat, which is surrounded by proteins that activate Arp2/3 to nucleate new actin filaments. Next, actin polymerization starts and filaments are crosslinked and attached to the coat, and this is accompanied by increase in curvature of membrane and elongation of the invagination. Barbed ends of actin filaments are pointing towards the membrane, and promote invagination, elongation and fission. It is not understood how actin filaments generate force to invaginate the vesicle and what is the role of myosins in this process, as they are found to localize to endocytic sites. After elongation, membrane scission occurs. In mammalian cells, dynamin together with actin, several actin-binding proteins and BIN/Amphiphysin/Rvs (BAR) domain proteins seem to be the key players in this event. In yeast, dynamin is less critical while another protein, Vps1, is suggested to play more important role triggering the scission (reviewed in Kaksonen et al., 2006 and Mooren et al., 2012).

Although it is evident that actin is crucial in clathrin-mediated endocytosis in yeast, the need of actin in endocytosis in mammalian cells is less clear. There is evidence that actin may play a role in several stages of endocytosis but, some data suggest that this role is not obligatory (Yarar et al., 2005; Fujimoto et al., 2000). Yeast cells have higher turgor pressure due to their cell walls, which has been suggested to result in the different requirements for actin in yeast and mammals (Aghamohammadzadeh & Ayscough, 2009). Supporting the need of actin in endocytosis in mammalian cells, actin has been shown to elongate tubular necks of clathrin pits together with BAR domain proteins (Ferguson et al., 2009). Actin filaments also form actin cortex associated with the plasma membrane, generating cortical tension. This might inhibit endocytosis but, however, the results of the role of cortical tension and actin in endocytosis are somewhat confusing and need still further investigations (reviewed in Mooren et al., 2012).

1.2.4. Cell division

Cytokinesis is the last step in the cell cycle, where two cells are physically separated from each other. In eukaryotes, cytokinesis occurs by an universal mechanism involving actomyosin contractile ring, but there are differences between different species like yeast and animal cells. The budding yeast cytokinesis is discussed briefly in chapter 1.3.1. while the cytokinesis in metazoan cells is discussed here.

The first phase of cytokinesis is to determine the place for the cleavage furrow. This occurs slightly differently depending on the cell type, but it takes place in the beginning of anaphase by astral microtubules, the central spindle or both (reviewed in Glotzer, 2004). The formation of cleavage furrow also requires activation of GTPase RhoA, although it seems not to be critical in determining the division plane (reviewed in Balasubramanian et al., 2004).

The next step is the formation of actomyosin ring. The activation of RhoA results in polymerization of actin by formins and activation of myosin by phosphorylation by either inhibiting of myosin phosphatase or phosphorylating myosin light chain (Kawano et al., 1999; Piekny & Mains, 2002; Watanabe et al., 2008). Also other actin binding proteins like α-actinin and filamin are found to be involved in formation of contractile ring (Fujiwara et al., 1978; Nunnally et al., 1980). The actin filaments are orientated towards several directions in contractile ring, that is, they have mixed polarities. The constriction of the ring is suggested to occur via mechanism related to muscle sarcomeres including several contractile modules in series around the ring, but more information is needed to build a reliable model for constriction. This is because contractile rings differ from muscle sarcomeres being more complicated with non-organized actin and myosin (reviewed in Pollard, 2010). In addition, the ring apparatus is disassembled during constriction, most likely by ADF/cofilins (Gunsalus et al., 1995).

After constriction, cytokinesis is finalized by membrane remodeling event, where the plasma membrane of the dividing cell is subdivided into two. This requires delivery of new membranes by targeted secretion to compensate the increased cell surface area of the daughter cells (reviewed in Balasubramanian et al., 2004).

1.2.5. Additional functions of actin in animal cells

Actin is traditionally considered as a cytoplasmic protein, but it is important to note that actin is also found from the nucleus, where it has a potentially crucial role in regulation of gene expression. Several functions have been described for actin in transcription, including binding to chromatin remodeling complexes together with actin related proteins (Arps), associating with all three RNA polymerases and binding to specific heterogenous nuclear ribonucleoproteins

(hnRNPs) which regulate transcription and pre-mRNA processing (reviewed in Farrants, 2008 and Skarp & Vartiainen, 2010). Actin has also a force-generating role in nucleus and it has been suggested to drive the movement of individual chromosomal loci or even the whole chromosomes. This force-generated movement is based on either actin polymerization or cooperation with nuclear myosin I (reviewed in Skarp & Vartiainen, 2010).

Actin is also linked to several proteins that regulate transcription. These proteins include e.g. myocardin related transcription factor A (MRTF-A, also known as megakaryocytic acute leukemia, MAL, or megakaryoblastic leukemia 1, MKL1) and gelsolin family proteins (reviewed in Skarp & Vartiainen, 2010). MAL is a co-activator of the serum response factor (SRF), which controls many immediate-early genes encoding e.g. signaling and cytoskeletal proteins. Actin regulates MAL by controlling its nuclear import, export and activation in nucleus, which eventually affect the expression of cytoskeletal proteins including actin itself (Vartiainen et al., 2007). Gelsolin and several gelsolin-related actin binding proteins (GRABPs) regulate actin dynamics in the cytoplasm. However, they also act as co-activators for several nuclear receptors e.g. androgen, estrogen and thyroid hormone receptors which mediate the expression of their target genes (reviewed in Archer et al., 2005).

Actin distribution between nucleus and cytoplasm is tightly regulated and highly dynamic. Controlling nuclear actin levels in cells is important for efficient transcription. Actin can rapidly move in and out of the nucleus and the transport in both directions is active. Exportin 6 mediates the export of actin from nucleus whereas importin 9 is required for importing actin to the nucleus. Interestingly, importin 9 also interacts with cofilin, and this interaction appears to be crucial for actin import (Dopie et al., 2012).

The organization of actin in the nucleus is not completely understood yet. Studies where actin polymerization was inhibited suggested that polymerization is needed for transcription but not necessarily for all nuclear functions of actin. Based on several studies, actin is suggested to exist in three different populations in the nucleus (Huet et al., 2012). The two populations with fastest and second fastest turnover rates have been implicated to correspond to free actin monomers and polymers, respectively. The third and largest (~ 60% of total nuclear actin) population with slowest turnover rate is suggested to be involved in chromatin remodeling, transcription regulation and mRNA processing. However, more investigations are needed to reveal the significance and mechanism of nuclear actin in different cellular processes in distinct cell types.

1.3. The actin cytoskeleton in unicellular organisms

1.3.1. The actin cytoskeleton in budding yeast

In 1984, it was established in two publications that yeast cells have two distinct actin structures, cortical dots called patches and cytoplasmic fibers called cables (Adams & Pringle, 1984; Kilmartin & Adams, 1984). Adams and coworkers also found actin to be concentrated in the neck region connecting the mother cell and the bud, which was later confirmed as third actin structure, contractile actomyosin ring (Bi et al., 1998; Lippincott & Li, 1998).

Actin patches are now known to mediate endocytosis (discussed in chapter 1.2.3.). These actin structures mature during different endocytic stages and they display different types of movement depending on the stage of endocytosis (Kaksonen et al., 2003). After first actin-independent phase of endocytosis, recruitment of endocytic patch components to the cell cortex, follows the slow actin-dependent movement. In this stage, Arp2/3-dependent actin polymerization occurs

driving vesicle formation and internalization (Kaksonen et al., 2003). After slow motility phase, patch undergoes transition to rapid movement stage, in which the newly formed vesicle is released and moves inwards from the cell cortex (Kaksonen et al., 2003). The vesicle is transported to endosomal sorting compartments along actin cables, which mediate the retrograde flow in yeast cells (Huckaba et al., 2004).

In yeast cells, the polarized growth and organelle segregation are mediated by the actin cytoskeleton, unlike in larger eukaryotic cells, in which microtubules perform this function. Pruyne et al. (1998) showed, by using mutant strain defective for tropomyosin, that the actin cables mediate the delivery of secretory vesicles for polarization of cell growth. It is important to note that although both actin cables and cortical patches are polarized in yeast cells, it is the cables which are required for targeted secretion, not the patches (Pruyne et al., 1998). Type V myosins function as motors to deliver different cargos along actin cables (reviewed in Bretscher, 2003). Transport of post-Golgi secretory vesicles, which facilitate polarized cell growth, is dependent on Myo2. Also transport of cell organelles including vacuole, Golgi, nucleus and peroxisomes to daughter cell occurs in Myo2-dependent manner. Another type V myosin expressed in budding yeast, Myo4, uses actin cable tracks similarly to Myo2 but instead of vesicles and organelles it transports daughter-specific mRNA into the bud (reviewed in Bretscher, 2003). Unlike actin patches, cables are assembled in Arp2/3-independent manner by actin nucleating proteins formins and profilin (reviewed in Moseley & Goode, 2006).

In yeast cytokinesis, two different F-actin structures at the bud neck are visible during anaphase. First, actin cables are orientated towards the bud neck in mother and daughter cell, targeting the secretion along the axis of polarity (reviewed in Pruyne et al., 2004). Second, a contractile actomyosin ring forms when a ring of F-actin associates with Myo1p ring, which appears already before bud emergence (Bi et al., 1998; Lippincott & Li, 1998). The actomyosin ring constricts to facilitate the neck closure and then disappears. F-actin ring, as well as patches and cables, undergo rapid turnover demonstrated by studies using an actin monomer sequestering drug, latrunculin A. Thus, a set of actin-binding proteins including e.g. ADF/cofilins, actin interacting protein 1 (Aip1), CAP, profilin and twinfilin are involved promoting the actin turnover in yeast (reviewed in Moseley & Goode, 2006). The *S. cerevisiae* actin-binding proteins that are known to be involved in actin dynamics are listed in Table 1.

1.3.2. The actin cytoskeleton in apicomplexan parasites

The phylum Apicomplexa includes several protozoan parasites that require a host cell in order to survive and replicate. Invading to host cells results in severe diseases in host organism depending on the parasite, e.g. toxoplasmosis (*Toxoplasma*), enteritis (*Cryptosporidium*) or malaria (*Plasmodium*) (reviewed in Frénal & Soldati-Favre, 2009). *Plasmodium falciparum,* which is responsible of causing malaria, is transmitted to humans via mosquitoes. Parasite moves through bloodstream to liver where it multiplies. Later, the parasite enters to red blood cells where they proliferate and further spread the infection (reviewed in Cowman & Crabb, 2006).

Apicomplexan parasites display a unique type of movement called gliding motility, which is based on actomyosin system, to access the host cells. Apicomplexans have multi-layered membrane-structure including plasma membrane and inner membrane complex (IMC) that consists of the inner and outer membranes. The compartment between the plasma membrane and IMC contains most of the proteins generating the gliding motility, including actin and myosin, which are linked to both membrane systems. The movement is suggested to occur by IMC-bound myosins walking along actin filaments, which are

Table 1. Comparison of actin binding proteins found from yeast *S. cerevisiae* and malaria parasite *P. falciparum*.

Functional class	Homologue	Protein in *S. cerevisiae*	Protein in *P. falciparum*
Monomer treadmilling	Aip1	Aip1	
	CAP	Srv2	PfC-CAP
	Cofilin	Cof1	PfADF1, PfADF2
	Gelsolin/Villin		*
	GMF	GMF	
	Profilin	Pfy1	PfPfn
	Twinfilin	Twf1	
	(unknown)	Bud6/Aip3	
Nucleation	Arp2/3 complex	Arp2/3 complex	**
	Eps15	Pan1	
	Formin	Bni1, Bnr1	PfFormin1, PfFormin2
	mAbp1	Abp1	
	WASp	Las17/Bee1	
F-actin capping	Capping protein	Cap1/Cap2	PfCPα, PfCPβ
Crosslinking and bundling	Calponin	Scp1	
	Coronin	Crn1	Pf coronin
	Fimbrin	Sac6	
	IQGAP	Iqg1/Cyk1	
	Tropomyosin	Tpm1, Tpm2	
	(unknown)	Abp140	
Myosin	Myosin	Myo1 – Myo5	Pfmyo-A – Pfmyo-F
Other	HIP1R	Sla2	
	WIP	Vrp1	

* *P. falciparum* has two gelsolin-like domains which are most likely functionally unrelated. ** ARPC1 subunit only. Data for the table has been collected from reviews by Baum et al. (2006), Moseley & Goode (2006) and Schüler & Matuschewski (2006). *P. falciparum* myosins were described in Chaparro-Olaya et al. (2005) and GMF was characterized by Gandhi et al. (2010) and Nakano et al. (2010).

linked to cell surface through adhesion molecules (reviewed in Baum et al., 2006).

The actin filaments in apicomplexan parasites are somewhat different compared to eukaryotes. Studies on *T. gondii* revealed that *in vitro*, their actin filaments are 10 times shorter than rabbit actin filaments and unstable, probably due to instability of filaments itself and actin-binding proteins (Schmitz et al., 2005; Sahoo et al., 2006). The cytosolic actin in parasites most probably exists primarily as monomeric form (Dobrowolski et al., 1997). However, *T. gondii* actin has 3-4-fold lower critical concentration than conventional actins and thus polymerizes readily into filaments (Sahoo et al., 2006). These characteristics

are most likely applied to all apicomplexan parasites, making their actins well suited for gliding motility where rapid turnover of filaments is needed.

The protein repertoire regulating actin in apicomplexan parasites is remarkably limited compared to that of other eukaryotes (reviewed in Baum et al., 2006). The actin binding proteins found from apicomplexan *P. falciparum* compared to ones found from *S.cerevisiae* are listed in Table 1. However, it is still possible that parasites perform their cell processes by some compensatory proteins that are still unknown. Three main classes of proteins regulating actin dynamics are found from apicomplexan and include ADF/cofilin, profilin, and CAP (reviewed in Olshina et al., 2012). The lack of Arp2/3 complex and its regulators in apicomplexan parasites suggests that the most probable filament nucleator in these organisms is formin (Baum et al., 2008). Coronin is the only known crosslinking protein found from apicomplexan, whereas numerous actin bundling proteins found from yeasts and animals are absent. Filament barbed end capping is most likely taken care by heterodimeric CapZ since it is the only known capping protein found from apicomplexan (reviewed in Baum et al., 2006).

P. falciparum has two ADF/cofilin isoforms, PfADF1 and PfADF2, from which only PfADF1 is expressed throughout the life cycle (Schüler et al., 2005). Unlike other eukaryotic ADF/cofilins, PfADF1 was detected to bind actin monomers and not filaments, and stimulate nucleotide exchange on actin monomer instead of inhibition, which is characteristic to other ADF/cofilins (Schüler et al., 2005). However, recent studies provided evidence that ADF1 in *P. falciparum* and *T. gondii* are capable of severing actin filaments (Mehta & Sibley, 2010; Wong et al., 2011).

Apicomplexan seem to have only a single profilin, which binds and sequesters actin monomers, as shown for *P. falciparum* and *T. gondii* profilins (Kursula et al., 2008; Plattner et al., 2008). *T. gondii*, *P. falciparum* and *C. parvum* profilins also promote actin assembly at barbed ends (Plattner et al., 2008). There is very little information whether apicomplexan profilins catalyze nucleotide exchange on actin monomers similarly to other profilins. There is one study demonstrating that *T. gondii* profilin, unexpectedly, reduces nucleotide exchange instead of promoting it (Kucera et al., 2010).

Apicomplexans have one CAP homologue, which completely lacks the N-terminal and WASP homology domain 2 (WH2) domains that are present in other eukaryotes (Hliscs et al., 2010). *C. parvum* CAP has been shown to sequester actin monomers inhibiting actin polymerization (Hliscs et al., 2010), but other possible activities of *C. parvum* or other apicomplexan CAPs are unknown.

1.4. Actin binding proteins

Actin filament treadmilling allows constant reorganization of the filamentous networks that produce force and allow cell movement. However, actin filament treadmilling and filament reorganization are very slow processes without any cooperative proteins. A plethora of actin binding proteins have been identified so far, and they are able to speed up the treadmilling process by ~200-fold (reviewed in Dos Remedios et al., 2003). These proteins can affect the filament reorganization by various mechanisms, like generating free barbed ends for polymerization (by uncapping, severing or nucleating), capping existing filament ends, maintaining actin monomer pool (actin sequestering proteins) or crosslinking actin filaments.

1.4.1. Actin filament nucleating proteins

New actin filaments are needed in cells in order to build actin networks in cytoplasm. The formation of nuclei required for initiating actin filament elongation is kinetically unfavorable process, which is also inhibited by various actin monomer sequestering proteins.

Specific nucleating proteins are thus needed for speeding up the nucleation process.

Formins are a large family of actin-binding proteins that are able to nucleate new actin filaments. They also alter the filament elongation rate and "walk" along the growing filament remaining at the barbed end. The ability to alter the elongation or depolymerization varies between different formins. All formins compete with barbed-end capping proteins by inhibiting their function and thus allowing filament elongation at the barbed ends (reviewed in Higgs, 2005).

Formins contain a formin homology 1 (FH1) domain, which is variable in length and rich in proline, and a highly conserved formin homology 2 (FH2) domain. Together these domains regulate actin filament elongation (Castrillon & Wasserman, 1994; Higgs & Peterson, 2005). FH2 domain is a dimer forming a donut-shape structure that wraps around actin polymer in the barbed end and moves along the filament during elongation (Xu et al., 2004). FH1 is suggested to be unstructured and it serves as a binding site for profilin. Binding of profilin to actin inhibits nucleation and monomer addition to the pointed ends but not to the barbed ends. However, profilin binding to the FH1 domain of formin enhances addition of profilin-actin to the barbed end resulting filament elongation (reviewed in Higgs, 2005).

In contrast to formins, Arp2/3 is able to produce branched actin filaments. The branched actin networks are needed at the leading edge of the moving cell to push the cell membrane forward. Arp2/3 consists of seven subunits, from which two are actin-related proteins 2 and 3 (Arp2, Arp3) that are stabilized by five other subunits. Arp2 and Arp3 mimic actin monomers and serve as a nuclei for the new daughter filament which branches off from mother filament at a 70° angle (reviewed in Pollard, 2007). Arp2/3 alone is inefficient in nucleating new filaments, but binding to existing actin filament enhances its nucleation activity. Activation of Arp2/3 is initiated by nucleation-promoting factors (NPFs), which is a group of proteins belonging to several protein families (reviewed in Campellone & Welch, 2010). Also phosphorylation in Arp2 is needed for activation by NPFs (LeClaire et al., 2008).

The more recently identified actin nucleation proteins include Spire, Cordon-bleu (Cobl) and Leiomodin (Lmod), which all contain WH2 domains that bind G-actin. Spire has four WH2 domains and linker, which cooperatively bind to total of four actin monomers (Quinlan et al., 2005). Cobl has three WH2 domains and polyproline regions and recently it has been shown to have only weak filament nucleating and strong filament severing activity (Ahuja et al., 2007; Husson et al., 2011). Lmod is a muscle-specific nucleating protein that has a domain organization resembling that of tropomodulins, except the C-terminus which has a WH2 domain and polyproline region. Lmod has been shown to be a strong actin nucleator in muscle cells and important for sarcomere organization in cardiomyocytes (Chereau et al., 2008).

1.4.2 Actin filament capping proteins

Actin filament capping is essential for cell movement. Capping of filament barbed ends results in shorter filaments, which are more efficient in pushing cell membrane. Furthermore, capping of filaments can target filament assembly towards desired direction (reviewed in Pollard & Borisy, 2003). Several barbed-end capping proteins are known, from which gelsolin and heterodimeric capping protein are the best characterized and discussed here. Only one family of pointed-end capping proteins, tropomodulins, is known to exist. Tropomodulins have low affinity for F-actin alone, but the affinity increases when F-actin is decorated by tropomyosin (Weber et al., 1994).

Eight proteins belonging to gelsolin superfamily have been characterized in mammals (reviewed in Nag et al., 2013). These all contain variable number (three to

six) of gelsolin domains, which are structures including β-sheet sandwiched between two α-helixes (Burtnick et al., 1997). Binding of calcium ions changes the conformation of gelsolin and activates it (Choe et al., 2002). There are three major actin-binding sites in gelsolin, which are exposed in activation by calcium ions (reviewed in Nag et al., 2013). In addition to capping, gelsolin is also able to sever actin filaments. Thus, gelsolin is efficient protein to dissolve actin gels by severing filaments and remaining in the filament end as a cap therefore preventing filament growth and reannealing (reviewed in Dos Remedios et al., 2003).

Heterodimeric capping protein (CP) is found in almost all eukaryotic cells. It consists of α and β subunits, which have similar secondary and tertiary structures despite the lack of amino acid sequence similarity (Yamashita et al., 2003). CP caps filament barbed ends with high affinity (K_d ~ 0.1–1nM) by two independent actin binding sites located in the C-temini of the α and β subunits (Wear et al., 2003). CP is known to interact with another actin-binding protein twinfilin, but the interaction between these two proteins does not affect their actin binding properties (Falck et al., 2004).

1.4.3. Actin filament crosslinking proteins

Connecting actin filaments together is important for cell to obtain rigid three-dimensional actin structures. A variety of proteins have been identified to crosslink actin filaments in cells, including for example α-actinin, fascin, fimbrin, filamin, some myosins and spectrin. From these proteins, α-actinin is one of the best characterized and therefore discussed here as an example.

α-actinin has a molecular structure of an anti-parallel dimer bearing high elasticity and strength. Each monomer consists of an N-terminal actin binding domain followed by four spectrin repeats and two EF-hand regions (reviewed in Broderick & Winder, 2002). In muscle cells, α-actinin localizes to the Z discs of sarcomers linking together actin filaments of adjacent sarcomers. In nonmuscle cells, α-actinin is found from stress fibers, lamellipodia and different adhesion sites in cell-cell and cell-matrix contacts. In addition to actin, α-actinin interacts with a variety of different proteins in stress fibers and adhesion sites including e.g. transmembrane proteins and receptors. Also several regulatory molecules like phophotidylinositol lipids and tyrosine kinases bind to α-actinin, regulating its activity (reviewed in Otey & Carpen, 2004).

1.4.4. Actin monomer pool regulating proteins

1.4.4.1. Actin depolymerizing factor (ADF)/ cofilins

The first protein belonging to the ADF/cofilin family was identified from chick embryo brain as a 19 kD protein that was observed to depolymerize actin filaments (Bamburg et al., 1980). After this, a large group of homologous proteins with various names (ADF, cofilin, destrin, actophorin, coactosin, twinstar, unc-60, XAC) have been identified from numerous eukaryotic organisms studied (reviewed in Bamburg, 1999). Studies on different organisms show that ADF/cofilins are fundamental for all eukaryotes since deficiency of ADF/cofilin causes lethality and defects in cytokinesis and muscle assembly in *Drosophila* and *C. elegans* (reviewed in Poukkula et al., 2011). Today the mammalian ADF/cofilin protein family is considered to consist of three kind of proteins, ADF, cofilin-1 and cofilin-2, from which cofilin-1 is expressed in most cell types whereas cofilin-2 is expressed mainly in striated muscles and ADF in epithelial cells (Ono et al., 1994; Vartiainen et al., 2002).

ADF/cofilins consist of a single ADF-H domain, which is known to bind both G-actin and F-actin (Lappalainen et al., 1998). By binding to actin monomers ADF/cofilins inhibit the nucleotide exchange (Nishida et al., 1985; Hayden et al., 1993; Kardos et al., 2009). Most ADF/cofilins prefer ADP-G-actin binding over ATP-G-actin (Kd = 0.02-0.15

μM and 0.6-8.0 μM, respectively), although mammalian cofilin-2 and chick ADF bind both ADP- and ATP-G-actin with similar affinities (reviewed in Poukkula et al., 2011).

ADF/cofilins bind cooperatively to filamentous actin, preferentially ADP-F-actin, and enhance the filament treadmilling rate by disassembling actin filaments (Figure 3). The mechanism of filament disassembly is controversial, since there are studies showing cofilin-induced depolymerization of actin from the pointed ends and filament fragmentation by severing of ADP-actin filaments (Carlier et al., 1997; Andrianantoandro & Pollard, 2006; Gandhi et al., 2009). Binding of ADF/cofilin changes the actin filament structure by inducing a twist in the filament, which enhances filament's fragmentation (McGough et al., 1997; Galkin et al., 2001). ADF/cofilins also contribute to "filament aging" by accelerating the P_i release from ADP-P_i-actin and this activity has been shown to spread allosterically to distal sites that are not occupied with ADF/cofilins (Blanchoin & Pollard, 1999; Suarez et al., 2011). In addition, ADF/cofilins are able to contribute actin filament organization by promoting debranching of Arp2/3-induced daughter filaments and also potentially nucleating of new filaments by stabilizing actin dimers (Blanchoin et al., 2000; Andrianantoandro & Pollard, 2006).

The activity of ADF/cofilins is regulated by pH, phosphorylation and binding of phosphoinositides and other proteins including Aip1, coronin and cyclase-associated protein (Rodal et al., 1999; Cai et al., 2007; Gandhi et al., 2009; Moriyama & Yahara, 2002).

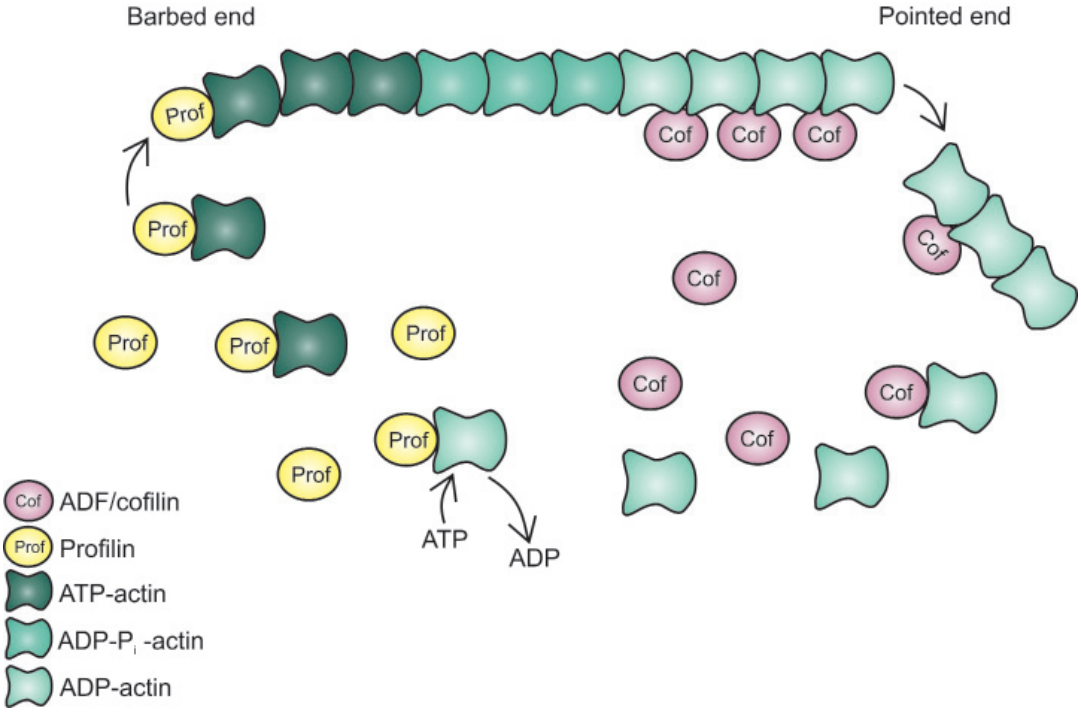

Figure 3. The functions of ADF/cofilin and profilin in actin dynamics. ADF/cofilins bind to actin filaments and sever and depolymerize them into ADP-actin monomers. ADF/cofilins are dissociated from ADP-actin monomers and recycled for new rounds of severing/depolymerization. Profilins catalyze nucleotide exchange from ADP to ATP on actin monomers and sequester ATP-actin monomers. In the presence of barbed ends, profilin assembles actin monomers to the fast-growing ends of the filaments.

ADF/cofilin is pH-sensitive since elevation of pH above 7.1 enhances its depolymerizing activity (Yonezawa et al., 1985; Hayden et al., 1993). Inactivation of ADF/cofilins can occur by two different mechanisms, either binding of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) or by phosphorylation of Ser-3 (Yonezawa et al., 1990; Gorbatyuk et al., 2006; Morgan et al., 1993; Agnew et al., 1995; Moriyama et al., 1996).

In budding yeast, mutations in *cofilin* causes enlarged cells with abnormal cortical actin patches and defects in actin polymerization (Lappalainen & Drubin, 1997). In mammalian cells, cofilin displays dynamic localization at the lamellipodium (Lai et al., 2008). Inactivation of ADF or cofilin-1results in abnormal stress fibers, defects in cytokinesis and impaired cell movement as well as diminished actin turnover, demonstrating that ADF/cofilins are crucial for the normal function of the cell (Hotulainen et al., 2005).

1.4.4.2. Twinfilins

First twinfilin isoform was identified as a novel tyrosine kinase named A6, which seemed to be conserved in different species (Beeler et al., 1994). However, subsequent studies could not confirm the protein kinase activity but instead, a homologous protein from yeast was shown to sequester actin monomers (Goode et al., 1998). To date, there are three different twinfilin isoforms found from mammals. *Twinfilin-1* and *twinfilin-2a* are expressed in most tissues, whereas *twinfilin-2b* expression is restricted to skeletal muscle and heart (Vartiainen et al., 2003; Nevalainen et al., 2009).

Twinfilins consist of two ADF-H domains separated by a central linker region and followed by a C-terminal tail region (reviewed in Palmgren et al., 2002). All twinfilin isoforms bind actin monomers with higher affinity for ADP-G-actin than for ATP-G-actin (Palmgren et al., 2001; Ojala et al., 2002; Nevalainen et al., 2009). Both ADF-H domains of twinfilin bind actin independently, but the C-terminal ADF-H domain has ~ 10-fold higher affinity for ADP-G-actin compared to N-terminal ADF-H domain (Ojala et al., 2002). Twinfilins bind heterodimeric capping protein through their C-terminal tail region which, at least in yeast, is essential for correct localization of twinfilin, but does not affect the actin monomer binding of twinfilin (Palmgren et al., 2001; Falck et al., 2004). Interestingly, twinfilins have been reported to sever actin filaments in low pH and capping protein seems to inhibit this action (Moseley et al., 2006). Mammalian twinfilin, but not yeast or *Drosophila* twinfilin, also caps actin filament barbed ends and both ADF-H domains are needed for this activity (Helfer et al., 2006; Paavilainen et al., 2007). The biochemical functions of twinfilin described above are illustrated in Figure 4. Twinfilins are regulated by PI(4,5)$P_{2,}$ which seems to inhibit actin binding but not CP binding of twinfilin (Palmgren et al., 2001; Vartiainen et al., 2003; Falck et al., 2004).

In budding yeast, twinfilin localizes mainly to the cytoplasm but is also enriched in cortical actin patches (Goode et al., 1998; Palmgren et al., 2001). Yeast cells deficient for twinfilin are viable, but the combination of twinfilin deficiency with *cofilin* or *profilin* mutants results in synthetic lethality (Goode et al., 1998; Wolven et al., 2000). Similarly, in mammalian cells, twinfilin shows strong cytoplasmic localization but in addition, twinfilin is found from actin-rich cell processes and filopodia (Vartiainen et al., 2000; Vartiainen et al., 2003). Twinfilin localization is regulated by small GTPases Cdc42 and Rac1, which target twinfilin-1 to cell-cell contacts and membrane ruffles, respectively (Vartiainen et al., 2000; Vartiainen et al., 2003). All twinfilin isoforms are present in cardiomyocytes where twinfilin-1 and twinfilin-2b are found to enrich in myofibrils (Nevalainen et al., 2009).

Twinfilin has also been studied in *Drosophila*, where *twinfilin* and *cofilin* have been shown to have genetic interaction. Mutations in *Drosophila twinfilin* resulted in defects in bristle morphology, axon growth,

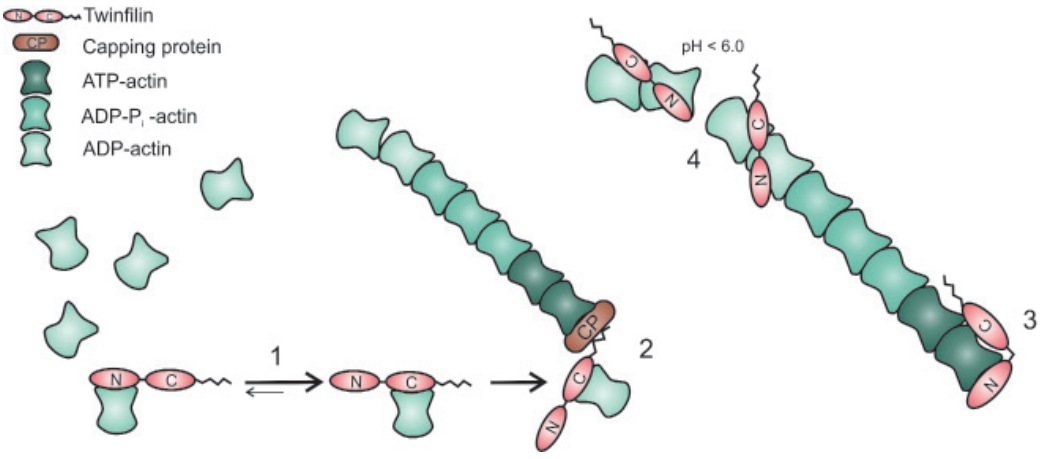

Figure 4. The functions of twinfilin in actin dynamics. The N-terminal ADF-H domain of twinfilin binds ADP-actin monomers with ~10-fold lower affinity than C-terminal ADF-H domain. ADP-actin monomer is suggested to first bind to the N-terminal ADF-H domain of twinfilin, resulting in conformational change and then transferring to C- terminal ADF-H domain (1). Interaction with capping protein is crucial for the localization of twinfilin at least in yeasts. Twinfilin binds capping protein through C-terminal tail region, and this interaction does not inhibit the ADP-actin monomer binding of twinfilin (2). Twinfilin also caps barbed ends of the actin filaments through its both ADF-H domains, the N-terminal ADF-H domain binding to terminal actin subunit and C-terminal ADF-H to the side of the filament (3). In acidic conditions, twinfilin has been shown to sever actin filaments but the mechanism for this activity has not been clarified (4).

neurotransmission and border cell migration (Wahlström et al., 2001; Wang et al., 2010).

1.4.4.3. Profilins

Profilin was first characterized from calf spleen as a protein that co-crystallizes with actin and inhibits actin monomers to polymerize (Carlsson et al., 1977). Later profilins have been identified in all organisms studied and several isoforms are found to exist. *Profilin I* is expressed in most tissues except skeletal muscle, whereas *profilin II* is expressed mostly in brain but also in skeletal muscle, uterus and kidney (Honore et al., 1993; Witke et al., 1998). At least mouse profilin II has two isoforms due to alternative splicing; profilin IIA which binds actin similarly to profilin I, and profilin IIB which does not bind actin (Di Nardo et al., 2000). The less characterized *profilin III* and *profilin IV* are testis-specific (Hu et al., 2001; Obermann et al., 2005). Profilins have multiple binding partners and functions related to actin dynamics, membrane trafficking, nuclear transport and neuronal plasticity (reviewed in Birbach, 2008).

Profilin has binding sites for actin and polyproline sequences, which are distinct from each other allowing simultaneous binding of these two ligands (Schutt et al., 1993; Mahoney et al., 1997). Profilins also bind phosphoinositides and PI(4,5)P_2 is known to regulate both actin and polyproline binding of profilin (Lassing & Lindberg, 1985; Lambrechts et al., 1997).

Profilin binds ATP-G-actin with higher affinity (K_d = 0.1μM) than ADP-G-actin (K_d = 0.5 μM) and many profilins are able to accelerate the exchange of nucleotide bound to actin monomer thus "recharging" actin monomers for new rounds of polymerization

(Vinson et al., 1998; Mockrin & Korn, 1980). In the absence of barbed ends, profilin sequesters actin monomers but when barbed ends are available, profilin-actin complex associates with growing filament ends promoting actin filament elongation, as demonstrated in Figure 3 (Tilney et al., 1983; Pantaloni & Carlier, 1993). In addition, these multi-functional proteins inhibit the nucleation of new actin filaments and change the critical concentration of actin, an issue that has been widely discussed and debated in literature over the years (reviewed in Yarmola & Bubb, 2009). Moreover, profilin interacts through its polyproline-binding domain with a wide variety of ligands that cooperate with profilins in different cellular processes (reviewed in Witke, 2004).

The importance of profilin function in actin-based motility has been shown both *in vitro* by reconstituting the motility machinery and also *in vivo* in different organisms (Loisel et al., 1999). In single cell organisms, the decreased levels of profilin lead to reduced growth and cell motility, abnormal phenotype and defects in actin distribution and cytokinesis (Haarer et al., 1990; Haugwitz et al., 1994). Studies in cultured fibroblasts and *Drosophila* cells show that profilin localizes to actin-rich and highly dynamic lamellipodia and is needed in lamellipodia formation (Buss et al., 1992; Rogers et al., 2003). In multicellular organisms fly and mouse, disruption of *profilin* gene is lethal (Verheyen & Cooley, 1994; Witke et al., 2001).

1.4.5. Cyclase-associated proteins (CAPs)

CAP (also called Srv2 in budding yeast) was first identified from *S. cerevisiae* as a 70 kD protein associated with adenylyl cyclase (Field et al., 1988). This protein was named as cyclase-associated protein and although it seemed to be needed for adenylyl cyclase activation by Ras, also other functions were suspected (Field et al., 1990; Fedor-Chaiken et al., 1990). Later, the importance of CAP in interaction between Ras and adenylyl cyclase has been questioned and since this interaction is not conserved in mammals CAPs are instead thought to have a more important function as actin cytoskeleton regulating proteins (Wang et al., 1992; reviewed in Hubberstey and Mottillo, 2002). To date, CAPs are found from all eukaryotes studied including yeast, plants and mammals and they regulate actin dynamics through a complicated mechanism, which still needs to be defined.

1.4.5.1. Structure of CAP

CAPs in eukaryotes are composed of N-terminal and C-terminal domains, which are separated by a central region containing two polyproline-rich repeats and a WH2 domain (Figure 5). The N-terminal domain is the least conserved region in CAPs over the species, while C-terminal domain is the most conserved. Identities between *S.cerevisiae* and human N-terminal, middle and C-terminal domains are 25%, 43% and 38%, respectively (Matviw et al., 1992).

The structures of N-terminal domain obtained from *Dictyostelium discoideum* CAP revealed that amino acids 51-226 of N-CAP form a bundle of six antiparallel α-helixes while the structure of the first 50 amino acids could not be solved, perhaps because of the random-coil conformation (Ksiazek et al., 2003, Mavoungou et al., 2004). However, this most extreme N-terminal sequence has been suggested to have a coiled-coil structure based its amino acid sequence (Nishida et al., 1998). Studies on yeast and human CAPs showed that the C-terminal domain of the protein consists of antiparallel β-stands, which form a six-coil β-helix that dimerizes by domain swapping between the last two strands (Dodatko et al., 2004). Also the N-terminal domains are found to dimerize and full-length CAP oligomerizes forming high molecular weight complexes (Hubberstey et al., 1996; Ksiazek et al., 2003; Dodatko et al., 2004; Yusof et al., 2005; Yusof et al., 2006; Zelicof et al., 1996).

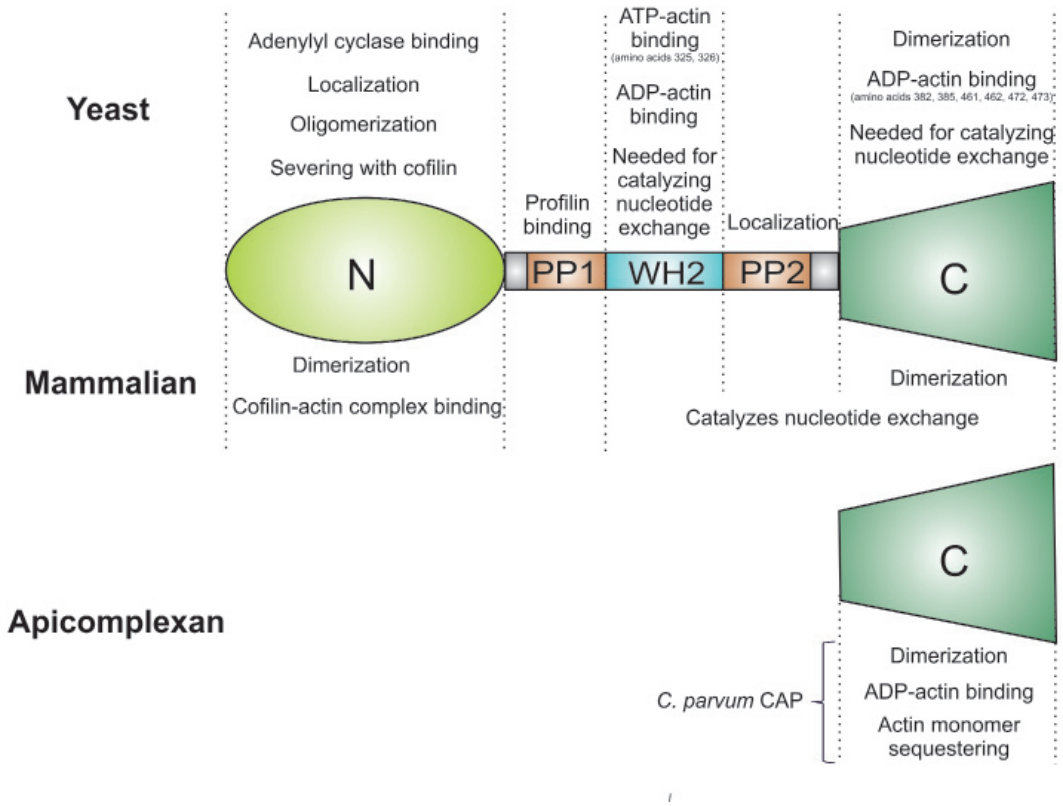

Figure 5. The domain structure and known functions of yeast, mammalian and apicomplexan CAPs. Most CAPs have a similar domain structure in which the N-terminal and C-terminal halves constitute independent α-helical and β-sheet structures, respectively. Between these domains lie two polyproline regions (PP1 and PP2) and a WH2 domain. However, apicomplexan CAPs consist only of the β-sheet domain. The known functions of each domain are listed in the figure.

Interestingly, CAPs found in apicomplexan parasites are small 'mini-CAPs' that resemble the C-terminal domain of other CAPs (Hliscs et al., 2010).

In the middle of the two domains lies the WH2 domain, which is generally known to bind monomeric actin and is highly conserved in CAPs from yeast to mammals (reviewed in Paunola et al., 2002). WH2 domain is flanked by two polyproline regions (PP1 and PP2), from which the first is more conserved than the latter.

1.4.5.2. Biochemical properties of CAP

Both *S. cerevisiae* and *S. pombe* CAPs are known to have a unique property to interact with adenylyl cyclase via their N-terminal domains (Gerst et al., 1991; Kawamukai et al., 1992). However, the significance of this interaction has been questioned (Wang et al., 1992). In *S. cerevisiae* CAP, the first 36 amino acids in N-terminal domain forming a coiled-coil structure are responsible of adenylyl cyclase binding (Nishida et al., 1998). Human or *S. pombe* CAPs cannot suppress the phenotype associated with Ras-adenylyl cyclase pathway that results from loss of N-terminal *S. cerevisiae* CAP, suggesting

limited functional conservation of this domain (Matviw et al., 1992). Since the binding to adenylyl cyclase has not been conserved in mammals, the role of coiled-coil domain in mammalian CAPs remains unsolved.

The N-terminal domain was shown to interact with cofilin-actin complex whereas it neither binds actin nor cofilin alone (Moriyama and Yahara, 2002). The N-terminal domain of CAP has been shown to accelerate the turnover of F-actin mediated by cofilin and thus CAP has been proposed to dissociate cofilin from cofilin-actin complexes for further severing and depolymerization (Moriyama and Yahara, 2002; Balcer et al., 2003). In recent studies, *S. cerevisiae* and human CAPs were also suggested to assist cofilin in severing actin filaments (Chaudhry et al., 2013; Normoyle & Brieher, 2012). However, the mechanism how these properties link together remains unknown.

Downstream of CAP's N-terminal domain are located the two polyproline regions and the WH2 domain. The first polyproline region is highly conserved between species. In yeast CAP, PP1 but not PP2, binds profilin, and mutation of only two prolines at PP1 region disrupt this binding (Bertling et al., 2007). In addition, binding site of profilin does not overlap with the ADP-actin binding site, suggesting that profilin, ADP-actin and CAP form a complex (Bertling et al., 2007). Before the present study, it has not been shown whether the PP1 in mammalian CAP binds profilin similarly than yeast CAP.

In *S. cerevisiae*, the second polyproline region has been shown to interact with the Src homology 3 domain (SH3) domain of actin binding protein 1 (Abp1), and this interaction localizes CAP to the cortical actin cytoskeleton (Freeman et al., 1996; Lila & Drubin, 1997). Abp1 also links CAP to actin filaments at least in *S. cerevisiae* (Balcer et al., 2003). There are no reports of this interaction in mammals, probably because the PP2 region seems not to be highly conserved from yeast to higher eukaryotes.

In the middle of the polyproline regions lies the WH2 domain, which is generally known to bind actin monomers in different proteins (reviewed in Paunola et al., 2002). However, there are conflicting results about actin binding by the yeast CAP WH2 domain. In a study carried out by Mattila et al. (2004), only weak actin binding was detected for the WH2 domain, and mutations in WH2 domain in C-terminal CAP caused a small decrease in ATP-actin binding. On the contrary, Chaudhry et al. (2010) showed that WH2 domain binds both ATP- and ADP-actin with same affinity and is important for the function of CAP *in vitro* and in cells. Before the present study, there was no information about actin binding properties of mammalian CAP1 WH2 domain.

The C-terminal half of CAP sequesters actin monomers and accelerates nucleotide exchange from ADP to ATP on actin monomers (Freeman et al., 1995; Moriyama & Yahara, 2002). The C-terminal domain of *S. cerevisiae* CAP binds ADP-actin with high affinity (K_d = 0.02 μM) and the ADP-actin binding residues were mapped to the C-terminal β-sheet structure, although some additional residues towards the N-terminal domain enhance the binding affinity (Mattila et al., 2004). It remains still unknown if these residues responsible for ADP-actin binding are conserved in mammalian CAP and whether mammalian CAP binds also ATP-actin, since yeast C-terminal CAP has been shown to bind ATP-actin only with modest affinity (K_d 1.9 μM, Mattila et al., 2004). The domain structure and function of each domain of CAP are summarized in Figure 5. The figure shows a comparison of known functions between yeast, mammalian and apicomplexan CAPs.

1.4.5.3. Cellular role of CAP

Since CAP was initially found from *S. cerevisiae*, the very first studies on CAP in living cells were performed with yeast cells. Deletion of *CAP* from yeast was observed to yield two independent phenotypes: one

is loss of responsiveness to activated Ras and the other includes abnormalities in cell morphology and growth (Field et al., 1990; Gerst et al., 1991). The N-terminal domain of CAP seems to be needed for Ras responsiveness while C-terminal domain was observed mainly to be responsible for normal cell morphology and growth (Gerst et al., 1991; Kawamukai et al., 1992). The loss of *CAP* in yeast results also random budding and abnormal actin distribution (Vojtek et al., 1991).

In mouse fibroblasts, CAP localizes to lamellipodia and dorsal ruffles together with actin and cofilin (Vojtek & Cooper, 1993; Moriyama & Yahara, 2002; Bertling et al., 2004). Knockdown of *CAP1* from mouse cells resulted in larger and less polarized cells containing thick stress fibers and decreased cell motility (Bertling et al., 2004). Also endocytosis and actin turnover were diminished and cofilin was observed to aggregate together with actin (Bertling et al., 2004). On the contrary, studies conducted with HeLa cells showed that knocking down of *CAP1* results in an increased cell movement (Zhang et al., 2013). However, authors also found CAP1 to interact with focal adhesion kinase (FAK), which may explain the conflicting results, since HeLa cells are not strongly adherent and thus an increase in adhesion molecules can enhance cell motility (Zhang et al., 2013).

1.4.5.4. Physiological role of CAP

In *Dictyostelium*, CAP localizes to the edges of moving cells. When *CAP* expression is restricted, cells show abnormal size and defects in cytokinesis, growth, endocytosis, cell movement, polarization and F-actin organization (Gottwald et al., 1996; Noegel et al., 1999; Noegel et al., 2004).

CAPs are also found in plants. In cotton (*Gossypium hirsutum*), *CAP* is expressed mostly in young fibers and it might be involved in cell elongation in the fibers (Kawai et al., 1998). In *Arabidopsis*, *CAP* is expressed in roots, leaves, flowers and stems and its overexpression results growth abnormalities such as smaller leaves and petioles (Chaudhry et al., 2007; Barrero et al., 2002). *Arabidopsis* CAP has been detected to bind both ADP- and ATP-actin and accelerate nucleotide exchange on actin monomers (Chaudhry et al., 2007).

There are two CAP isoforms in *C. elegans*, named CAS-1 and CAS-2. CAS-1 localizes to the M lines in *C. elegans* body wall muscle and its depletion causes aggregation of F-actin and cofilin (Nomura et al., 2012). The biochemical properties of CAS-1 and CAS-2 have been described to differ from each other at some extent. Both C-terminal halves are able to bind G-actin, but only C-CAS-2 is able to promote nucleotide exchange independently since C-CAS-1 requires some additional parts in the N-terminal domain (Nomura et al., 2012; Nomura & Ono, 2013). The N-terminal halves of both CAS-1 and CAS-2 bind G-actin, and in addition CAS-1 binds cofilin-actin complex while this property has not been reported for CAS-2 (Nomura et al., 2012; Nomura & Ono, 2013).

CAP proteins are also known to play developmental role in *Drosophila*. *CAP* is needed in *Drosophila* oocyte polarization and eye disc development by preventing excess actin polymerization (Benlali et al., 2000; Baum et al., 2000).

In mammals, two CAP isoforms (named CAP1 and CAP2) are found and they share 64% identity at the amino acid level (Yu et al., 1994). In rat and mouse, *CAP1* is expressed in most tissues except skeletal muscle while *CAP2* expression is mainly restricted to heart, skeletal muscle and brain (Swiston et al., 1995; Bertling et al., 2004). In developing cardiomyocytes and undifferentiated cardiac muscle cells, CAP2 was observed to localize to the nucleus whereas in mature muscle cells CAP2 localizes to M lines and I bands in myofibrils (Peche et al., 2007). Deletion of *CAP2* in mouse results in heart problems with disarrayed sarcomers and dilated cardiomyopathy (Peche et al., 2013).

2. AIMS OF THE STUDY

The actin binding properties of CAP, ADF/cofilin and profilin have been studied for a few decades already, but the overall understanding how these proteins cooperate together in regulating actin dynamics is still elusive. Moreover, the cellular functions of these proteins have been studied mainly in nonmuscle cells and whole organisms, but their muscle-specific isoforms are still poorly characterized. This study aims to reveal how CAP, ADF/cofilin, profilin and twinfilin together regulate actin dynamics *in vitro* and also broaden our understanding on the functions of the muscle-specific isoforms of actin-binding proteins.

The specific aims of the study are:

1. To reveal whether the ADF/cofilin, profilin and actin binding sites are conserved in CAPs from yeast to mammals.
2. To elucidate the mechanism of CAP-promoted nucleotide exchange on actin.
3. To reveal the mechanism that is responsible for the high-affinity ATP-actin binding of the muscle-specific ADF/cofilin isoform, cofilin-2.
4. To test whether CAP also interacts with twinfilin, which like ADF/cofilins is composed of ADF-H domains.

3. MATERIALS AND METHODS

The methods Maarit Makkonen personally used in this study are listed below. The publications in which the methods have been used and described in detail are indicated below by roman numbers.

Method	Publication
Site-directed mutagenesis and plasmid construction	I, II, III
Recombinant protein expression and purification	I, II, III, unpublished data
Rabbit skeletal muscle actin purification	I, II, III, unpublished data
7-Chloro-4-nitrobenzeno-2-oxa-1,3-diazole(NBD)-G-actin binding assay	I, II, III
Nucleotide exchange assay	I, II
Supernatant depletion pull-down assay	I, II, unpublished data
Tryptophan fluorescence spectroscopy	II
Pyrene-labeled actin polymerization assay	II
Native PAGE	II, unpublished data
Isolation of neonatal rat cardiomyocytes	III
Western blotting	III

4. RESULTS AND DISCUSSION

4.1. CAPs interact with ADF/cofilins, profilin and twinfilin to regulate actin dynamics

Efficient treadmilling of actin filaments requires rapid recycling and "recharging" of actin monomers. This can be executed by ADF/cofilin, profilin, CAP and twinfilin in concert. ADF/cofilins are known to disassemble actin filaments through depolymerization and/or severing (Carlier et al., 1997; Gandhi et al., 2009). Profilin catalyzes the nucleotide exchange on actin monomers and also adds actin monomers to the growing barbed ends of filaments (Mockrin & Korn, 1980; Tilney et al., 1983). Among other activities, twinfilin binds actin monomers preferring ADP-G-actin over ATP-G-actin (Palmgren et al., 2001). We suggest that the missing link connecting all these proteins is CAP, which brings together cofilin, profilin, twinfilin and actin, thus ensuring sufficient treadmilling rate required for actin-dependent cellular processes.

4.1.1. CAPs bind ADF/cofilin, profilin and actin through conserved mechanisms (I, II)

4.1.1.1. Identification of ADF/cofilin binding site in yeast CAP (I)

An earlier study showed that the N-terminal region of CAP interacts with cofilin, but the exact location at the actin-binding site was unknown (Moriyama & Yahara, 2002). Another study on yeast CAP showed that it binds ADP-actin with high affinity and ATP-actin with low affinity, and pinpointed the ADP-actin binding activity to the C-terminal region of CAP (Mattila et al., 2004).

We studied the function of N-terminal region of yeast CAP by reconstituting CAP-actin complex (publication I, Figure 1). We showed that the N-terminal coiled coil domain mediates oligomerization of yeast CAP (publication I, Figures 2A-B and 6). However, oligomerization is not critical but optimizes the function of CAP *in vitro* (publication I, Figure 2E-F). *In vivo* studies on budding yeast showed that deletion of the coiled coil domain resulted in partial defects in cell growth and morphology (publication I, Figure 6).

Consistent with an earlier study with yeast C-CAP (Mattila et al., 2004), we found that full length yeast CAP binds ADP-actin with high affinity (K_d = 56 nM) and ATP-actin with low affinity (K_d = 2 μM) (publication I, Figure 2C). The actin binding activity is in the C-terminal region of CAP, whereas the N-terminal region does not have any effect on nucleotide exchange in actin monomer (publication I, Figure 2D). We mutated several solvent-exposed residues at the N-terminal region of yeast CAP and observed impaired cell growth and abnormal cell morphology with two alleles Srv2-90 and Srv2-91 (publication I, Figures 3 and 4). We purified Srv2-90 and Srv2-91 mutant proteins from *E. coli* and showed that these mutants had also diminished effect on promoting actin turnover *in vitro* (publication I, Figure 5A-C). Srv2-90 and Srv2-91 mutant proteins also displayed impaired binding ability for cofilin-actin complex in supernatant depletion pull-down assays (publication I, Figure 5D-F). This shows that the N-terminal domain of yeast CAP binds cofilin-actin complex, consistent with the earlier study with human CAP (Moriyama & Yahara, 2002). Furthermore, we identified specific residues in yeast CAP, which are critical for this interaction (publication I, Figure 5F).

Reciprocally, we also mapped the CAP-binding site on cofilin by testing the ability of two cofilin mutants to bind yeast CAP in presence of ADP-actin. In an earlier study these mutations, Cof1-5 and Cof1-9, caused growth defects in budding yeast without affecting actin binding (Lappalainen et al., 1997). Thus, the authors of this study suggested that these residues are important for some other unknown activity of cofilin. We revealed by supernatant depletion pull-down assay that these mutations abolish the CAP-

binding activity of cofilin indicating that these residues, which are non-overlapping with the actin binding surface of cofilin (Lappalainen et al., 1997), are responsible for interaction of cofilin with yeast CAP (publication I, Figure 5G-H). This confirms that cofilin can bind both actin and CAP simultaneously, thus forming a ternary complex to accelerate actin turnover.

Several studies have provided evidence to support the idea that the main function of CAP is to accelerate cofilin-mediated actin turnover by recycling cofilin from ADP-actin monomers and catalyzing the nucleotide exchange in actin monomers (Moriyama & Yahara, 2002; Balcer et al., 2003; Chaudhry et al., 2007). Our findings showing that N-terminal region of yeast CAP has an important role in actin turnover by forming a complex together with cofilin and ADP-actin fits in this model very well. However, two recent studies suggested that CAP also directly severs actin filaments together with cofilin (Normoyle & Brieher, 2012; Chaudhry et al., 2013) indicating that the function of this protein may be more complex.

4.1.1.2. Identification of cofilin-actin and actin binding site in mammalian CAP1 (II)

Since both human CAP1 and yeast CAP bind cofilin-actin complex via N-terminal α-helical domain (Moriyama & Yahara, 2002; publication I, Figure 5) and the residues critical for the binding have been mapped in yeast CAP (publication I, Figure 5), we asked whether the corresponding surface-exposed residues would be responsible for cofilin-actin binding also in mouse CAP1. We thus mutated four conserved amino acids on the surface of α-helical domain of mouse CAP1 (publication II, Figure 1), which were shown to be critical for cofilin-actin binding in yeast CAP in publication I. Mutations in these residues also disrupted the cofilin-mediated filament severing activity of CAP in yeast (Chaudhry et al., 2013). Our supernatant depletion pull-down assay shows that, like human and yeast CAPs, mouse N-CAP1 binds cofilin-actin complexes but not cofilin alone without actin (publication II, Figure 7). Furthermore, the mutated N-CAP1 showed no binding activity for cofilin-actin complexes confirming that the cofilin-actin binding site of CAP is conserved from budding yeast to mammals (publication II, Figure 7).

4.1.1.3. Identification of two independent profilin binding sites in mammalian CAP1 (II)

Yeast CAP is known to interact with profilin through its first polyproline region and mutation of only two prolines abolishes this binding activity (Bertling et al., 2007). We tested whether this binding site is conserved in mouse CAP1 by using a fragment of CAP1 containing the two polyproline regions. This CAP1 fragment bound profilin with similar affinity (K_d ~ 1 μM; publication II, Figure 8A) as previously reported for yeast C-CAP (Bertling et al., 2007). However, the three proline-to-alanine substitutions (mutPP1) did not affect significantly profilin binding (publication II, Figure 8B). The first polyproline region of mouse CAP1 contains more prolines than yeast CAP and thus we tested if mutation of another three prolines (mutPP2) downstream of mutPP1 would affect the profilin binding of CAP1. Interestingly, neither mutation mutPP1 nor mutPP2 affected profilin binding alone, but when combined together, they almost completely abolished profilin binding of CAP1 (publication II, Figure 8B-D). These data show that, unlike yeast CAP, mouse CAP1 has two adjacent profilin binding sites that can bind profilin independently from each other. Whether one CAP molecule can bind one or two profilins at a time, needs still further investigation.

4.1.2. Mammalian CAP binds ATP-actin with high affinity (II)

Yeast CAP has been shown to bind ADP-actin with high affinity (K_d = 18 nM) whereas its affinity for ATP-actin is only modest (K_d =

1.4-1.9 μM) (Mattila et al., 2004; Chaudhry et al., 2010). We studied the actin binding properties of mouse CAP1 and found that CAP1 binds ADP-actin with high affinity (K_d = 50 nM), similarly to yeast CAP (publication II, Figure 2A-B). Interestingly, we also revealed that its affinity for ATP-actin is considerably higher (K_d = 380 nM) compared to yeast CAP (publication II, Figure 3A). Several specific residues responsible for ADP-actin binding have been mapped in yeast C-CAP and mutations in the WH2 domain of yeast CAP were shown impair the weak ATP-actin binding (Mattila et al., 2004; Chaudhry et al., 2010). We tested if the corresponding mutations in mouse CAP1 would affect the actin binding properties, and found that mutation of six residues in C-CAP1 β-sheet structure abolished ADP-actin binding while ATP-actin binding was not affected (publication II, Figures 2D and 3C). Furthermore, we found that mutation of only one amino acid in WH2 domain (K271A) disrupted the ATP-actin binding of CAP1 while ADP-actin binding ability was similar to the one of wild type CAP1 (publication II, Figures 3B and 2C). These data shows that unlike yeast CAP, mouse CAP1 binds both ADP-and ATP-actin with high affinity. Furthermore, these data reveal that the ADP-actin binding site is conserved in C-terminal region of yeast and mammalian CAPs and that the ATP-actin binding site is located at the WH2 domain at mouse CAP1.

Next we examined more closely the WH2 domain itself and tested whether this domain alone, without C-terminal β-sheet structure, would bind ATP-actin. The construct containing merely WH2 domain and both polyproline regions was found to bind ATP-actin with relatively high affinity (K_d = 730 nM) suggesting that WH2 domain alone is sufficient for ATP-actin binding in mouse CAP1 (publication II, Figure 3D). The WH2 domain of CAP1 also inhibited nucleotide exchange from ε-ATP to ATP in actin monomer (publication II, Figure 3F), which is in line with previous observations with yeast CAP (Chaudhry et al., 2010). Since previous studies showed that the WH2 domain of yeast CAP inhibits actin polymerization (Chaudhry et al., 2010), we tested the effect of mouse CAP1 WH2 domain on actin polymerization. In contrast to WH2 domain of yeast CAP, the WH2 domain of mouse CAP did not affect actin polymerization thus allowing actin monomers to be added to barbed ends meanwhile CAP1 was bound to the monomer (publication II, Figure 3E). This contradictory result may arise from different experimental setups, but more likely and intriguing option is that yeast and mammalian CAPs have slightly different ways to assist the actin filament turnover.

4.1.3. Catalyzing nucleotide exchange is the most conserved function of CAP

4.1.3.1. The C-terminal domain of CAP1 is sufficient for catalyzing nucleotide exchange (II)

The C-terminal half of CAP is known to catalyze nucleotide exchange from ADP to ATP on actin monomers (Moriyama & Yahara, 2002; Chaudhry et al., 2010). In order to study the roles of ADP- and ATP-actin binding sites on nucleotide exchange activity of CAP1, we performed the nucleotide exchange assay from ADP to ATP with our wild-type and mutated C-CAP1 constructs. As expected, wild type C-CAP1 accelerated the nucleotide exchange on actin monomers (publication II, Figure 4A). Interestingly, mutation in WH2 domain ATP-actin binding site did not affect this nucleotide exchange activity. On the contrary, mutations in β-sheet domain ADP-actin binding site diminished the effect of C-CAP1 to catalyze nucleotide exchange (publication II, Figure 4B). This is somewhat contradictory to earlier results with yeast CAP, which was proposed to require also the WH2 domain for accelerating nucleotide exchange on cofilin-bound actin monomer (Chaudhry et al., 2010). However, the constructs used in studies by Chaudhry et al. (2010) were slightly different

than in ours since they used full length yeast CAP. Furthermore, the mutations that diminished the nucleotide exchange activity were more extensive, including replacement of four amino acids to alanines, whereas in our study only one lysine-to-alanine substitution was needed to abolish the ATP-actin binding.

To further confirm the dispensable role of WH2 domain on nucleotide exchange, we performed experiments with the C-terminal region of yeast CAP lacking the amino acids critical for ATP-actin binding. Importantly, also this deletion construct was sufficient in accelerating nucleotide exchange on actin monomers, albeit in slightly higher concentration (publication II, Figure 4C). This could be explained by an earlier report of yeast CAP, where the β-sheet structure alone was found to be sufficient for moderate affinity ADP-actin binding, whereas additional sequences upstream of this domain were needed for high-affinity binding (Mattila et al., 2004). Collectively, these results show that C-terminal β-sheet structure of CAP is needed and sufficient for nucleotide exchange from ADP to ATP on actin monomers in yeast and mammals, while the ATP-actin binding activity of the WH2 domain is not required for this activity.

*4.1.3.2. **Plasmodium falciparum** CAP catalyzes nucleotide exchange on actin monomers (II)*

Compared to yeast, plants and animals, CAP-proteins found from apicomplexan parasites are much smaller comprising only the C-terminal β-sheet domain. Apicomplexan CAPs are not well characterized yet, but the structure of *Cryptosporidium parvum* CAP has been solved and the protein was shown to sequester G-actin (Vedadi et al., 2007; Hliscs et al., 2010). However, the role of apicomplexan CAPs in actin dynamics is largerly unknown.

We produced and purified *Plasmodium falciparum* CAP (PfCAP) and examined its activity in actin dynamics *in vitro*. For our surprise, PfCAP did not migrate properly on SDS-PAGE, instead most of the protein remained on the top of the gel (publication II, Figure 5A, left panel). This could provide an explanation for problems reported in purification of PfCAP in earlier study (Hliscs et al., 2010). When loaded on nondenaturating PAGE, PfCAP migrated as a single band (publication II, Figure 5A, right panel) and the identity of PfCAP was confirmed by mass spectrometry. Our analytical ultracentrifugation experiment showed that PfCAP dimerizes in solution (publication II, Figure 5B), consistent with earlier reports on the C-terminal domains of yeast, mammalian and *Cryptosporidium parvum* CAPs (Zelicof et al., 1996; Hubberstey et al., 1996; Hliscs et al., 2010).

We were unable to detect the direct binding of PfCAP to G-actin purified from rabbit, probably because rabbit and *P. falciparum* actins share only ~ 76 %, identity. However, PfCAP was found to catalyze the nucleotide exchange from ADP to ATP on actin monomers and from this assay we were also able to determine that PfCAP binds rabbit actin with ~ 370 nM affinity (publication II, Figure 6). Although the affinity of PfCAP for rabbit actin is somewhat lower compared to yeast and mammalian CAPs, these results show that PfCAP, lacking the N-terminal domains of yeast and animal CAPs, can efficiently catalyze nucleotide exchange on actin monomers.

Many profilins are also known to catalyze nucleotide exchange, but they bind ADP-actin with lower affinity compared to CAPs (K_d = 0.5 μM for profilin, K_d = 0.02-0.05 μM for CAPs, Vinson et al., 1998; Mattila et al., 2004; publication II). Furthermore, it is not likely that only profilins would be responsible for exchanging the nucleotide since e.g. yeast profilins exhibit low nucleotide exchange activity and plant profilins have no detectable activity (Perelroizen et al., 1996; Eads et al., 1998). Thus, these data together with our results support our hypothesis that nucleotide exchange activity, which resides

on the C-terminal β-sheet domain of CAP, is the most conserved function of CAPs over the species. Furthermore, CAPs rather that profilins probably have the main responsibility for recharging ADP-actin monomers with ATP in living cells.

4.1.4. Mammalian CAP1 interacts with twinfilin-ADP-G-actin complex (Makkonen et al., unpublished)

Twinfilins bind ADP-G-actin through specific residues located in their two ADF-H domains and these sites correspond to ADP-G-actin sites in ADF/cofilins (Palmgren et al., 2001; Paavilainen et al., 2008). Since twinfilin seems to have somewhat higher affinity for ADP-G-actin than ADF/cofilins and cells have relatively high cellular concentration of twinfilin, it has been suggested that twinfilin competes with ADF/cofilin for ADP-G-actin (Ojala et al., 2002). Based on these observations combined with results showing that CAPs bind cofilin-actin complexes (Moriyama & Yahara, 2002, publication I, publication II), we hypothesized that N-terminal domain of CAP could possibly bind also twinfilin-actin complexes. To strengthen our hypothesis, the searches in Molecular INTeraction database (MINT) suggested that these proteins interact, based on *in vivo* screen in *S. cerevisiae* (Tarassov et al., 2008). To test our assumption, we produced and purified full-length mouse twinfilin and tested whether it binds mouse N-CAP1 in the presence of ADP-G-actin by native PAGE. We loaded these proteins alone and mixed together on nondenaturating gel. Importantly, the mixture containing all three proteins migrated as a single band, whereas in the absence of actin, both twinfilin and CAP migrated as separate bands. This demonstrates that in the presence of actin, N-CAP1 indeed binds twinfilin and actin forming a ternary complex (Figure 6A, indicated by two adjacent asterisks). We confirmed the ternary complex formation in physiological salt conditions by a supernatant depletion pull-down assay. A clear decrease of twinfilin in the supernatant was visible in the samples containing N-CAP1 and actin, compared to the samples lacking actin (Figure 6B, marked by an asterisk). This further confirms that N-CAP1 binds twinfilin-ADP-actin complex. However, the significance of this binding and possible relation on cofilin-ADP-G-actin-N-CAP1 complex formation remains unresolved.

Both ADF-H domains of twinfilin are able to bind actin (Goode et al., 1998; Palmgren et al.,. 2001), so next we asked which ADF-H domain is responsible for the ternary complex formation. We produced and purified both N-terminal and C-terminal ADF-H domains of twinfilin and tested the complex formation by native gel assay. Our results revealed that both ADF-H domains are able to form a complex with N-CAP1 and ADP-actin independently from each other (Figure 7A). In the case of N-terminal ADF-H domain, the complex between actin and twinfilin was not clearly visible (Figure 7A, left panel, lane 4), probably due to lower affinity of the N-terminal ADF-H domain of twinfilin for ADP-actin compared to the C-terminal domain (Ojala et al., 2002).

To compare the affinities of full-length twinfilin and its N- and C-terminal ADF-H domains for CAP1, we performed a supernatant depletion pull-down assay for N- and C-terminal twinfilin domains. We noted that the decrease of either N- or C-twinfilin in supernatant was less clear, although some decrease was visible (Figure 7B and 7C, decreased twinfilin band is marked by an asterisk). We quantified the twinfilin band intensities on gels and according to these results, the full-length twinfilin is efficiently depleted leaving only ~ 20 % of twinfilin in supernatant, whereas ~ 60-70 % of both of the N- and C-terminal ADF-H domains remained in the supernatant (Figure 7D). This shows that although the both halves of twinfilin are able to form ternary complex with N-CAP1 and ADP-actin, they are less efficient in complex formation compared to full-length twinfilin. Further investigations are needed to

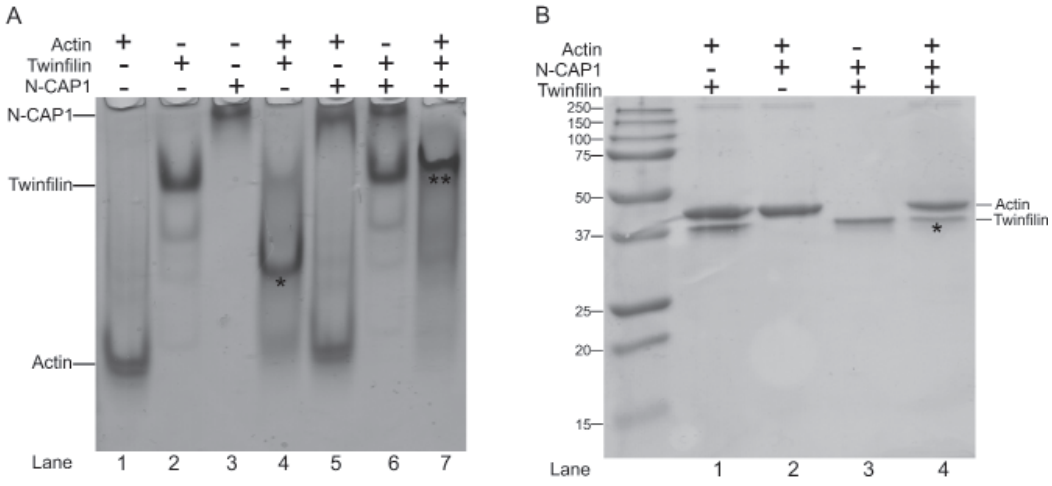

Figure 6. Interaction of N-CAP1 with twinfilin-actin complex. (A) Native gel demonstrating the formation of ternary complex between N-CAP1, ADP-actin and full-length twinfilin. Proteins were mixed together in G-buffer (5 mM Tris pH 8, 0.1 mM $CaCl_2$, 0.2 mM DTT, 0.2 mM ADP) and loaded on 6% nondenaturating PAGE. The bands were visualized by Coomassie staining. Lane 1, actin. Lane 2, twinfilin. Lane 3, N-CAP1. Lane 4, actin and twinfilin. Lane 5, actin and N-CAP1. Lane 6, twinfilin and N-CAP1. Lane 7, actin, twinfilin and N-CAP1. The concentrations of proteins were 5 μM. The complex formed between actin and twinfilin is marked with an asterisk on lane 4 and the ternary complex formed by actin, twinfilin and N-CAP1 is marked by two adjacent asterisks on lane 7. (B) Supernatant depletion pull-down assay showing the interaction of N-CAP1 with twinfilin-actin complex. Purified ADP-actin, full length twinfilin and N-CAP1-GST beads were mixed in F-buffer (10 mM Tris pH 7.5, 0.2 mM $CaCl_2$, 0.5 mM DTT, 0.2 mM ADP, 1 mM $MgCl_2$, 100 mM KCl), incubated for 20 minutes and centrifuged 16000g. Supernatants were collected and loaded on SDS-PAGE. Proteins were visualized by Coomassie stain. Lane 1, actin and twinfilin. Lane 2, actin and N-CAP1-GST beads. Lane 3, N-CAP1-GST beads and twinfilin. Lane 4, actin, N-CAP1-GST beads and twinfilin. The decrease of twinfilin in supernatant by N-CAP1-GST beads in the presence of actin is shown by an asterisk.

elucidate the mechanism and biological role of this complex formation.

4.1.5. Model how CAPs regulate actin dynamics in nonmuscle cells

Taken into consideration the data presented above, a new model for the function of CAP in actin dynamics is presented in Figure 8. CAPs are suggested to function as platforms for several binding partners including cofilin, profilin and twinfilin, which all work together to promote actin turnover. In addition to their function as a rather stationary platform, CAPs also have a more active role in catalyzing nucleotide exchange, activity which is the best conserved function in CAPs between apicomplexan parasites, yeasts and animals.

4.2. Actin filament length in muscle sarcomeres is controlled by cofilin-2

Many actin-binding proteins that regulate actin dynamics in nonmuscle cells are also expressed in muscle cells. Importantly, muscle-specific isoforms of many proteins including CAP2, profilin II, twinfilin-2b and cofilin-2 are found in mammals (Bertling et al., 2004; Witke et al., 1998; Nevalainen et al., 2009; Vartiainen et al., 2002). Contrary to actin-dependent processes in nonmuscle cells,

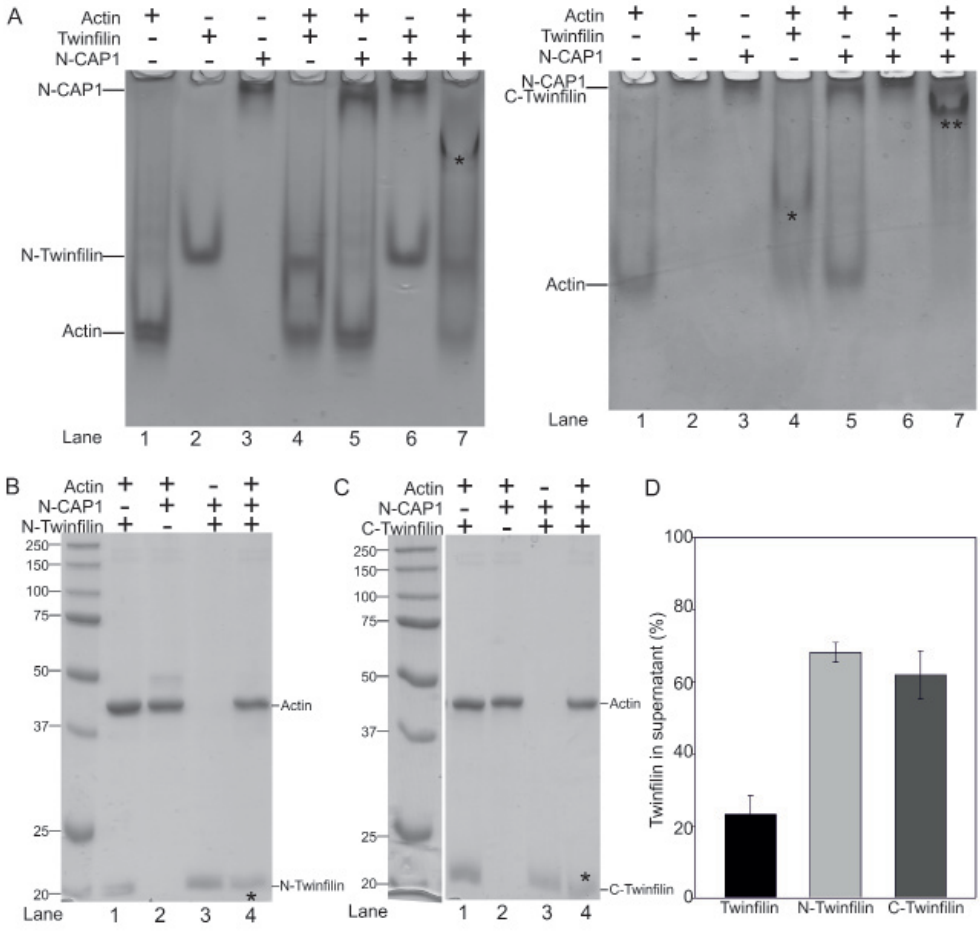

Figure 7. The abilities of N- and C-terminal ADF-H domains of twinfilin to form complex with N-CAP1 and actin. (A) Native gel assays demonstrating that both N-terminal and C-terminal ADF-H domains are able to form a complex with N-CAP1 and actin. Proteins were mixed together in G-buffer (5 mM Tris pH 8, 0.1 mM $CaCl_2$, 0.2 mM DTT, 0.2 mM ADP) and loaded on 6% nondenaturating PAGE. The bands were visualized by Coomassie staining. The concentrations of proteins were 5 μM. Left gel shows the result of the assay carried out with N-terminal ADF-H domain of twinfilin, N-CAP1 and ADP-actin and the gel on the right hand side represents data from C-terminal ADF-H domain of twinfilin, N-CAP1 and ADP-actin. In left gel, the complex formed by actin, N-twinfilin and N-CAP1 is indicated by an asterisk on lane 7. In gel on the right side, the faint band in lane 4 demonstrates the complex formed by actin and C-twinfilin and is marked by an asterisk, whereas the complex formed by actin, C-twinfilin and N-CAP1 is marked with two adjacent asterisks on lane 7. (B,C) Supernatant depletion pull-down assay showing the interaction of N-CAP1 with ADP-actin and N-twinfilin (B) or C-twinfilin (C). Purified ADP-actin, twinfilin and N-CAP1-GST beads were mixed in F-buffer (10 mM Tris pH 7.5, 0.2 mM $CaCl_2$, 0.5 mM DTT, 0.2 mM ADP, 1 mM $MgCl_2$, 100 mM KCl), incubated for 20 minutes and centrifuged 16000g. Supernatants were collected and loaded on SDS-PAGE. Proteins were visualized by Coomassie stain. Lane 1, actin and twinfilin. Lane 2, actin and N-CAP1-GST beads. Lane 3, N-CAP1-GST beads and twinfilin. Lane 4, actin, N-CAP1-GST beads and twinfilin. The decrease of twinfilin in supernatant by N-CAP1-GST beads in the presence of actin is shown by an asterisk. (D) Quantification of the amounts of full length, N- and C-twinfilin in the supernatant in pull-down assays shown in Figures 6B (full length), 7B (N-twinfilin) and 7C (C-twinfilin). Error bars represent SEM of three independent experiments.

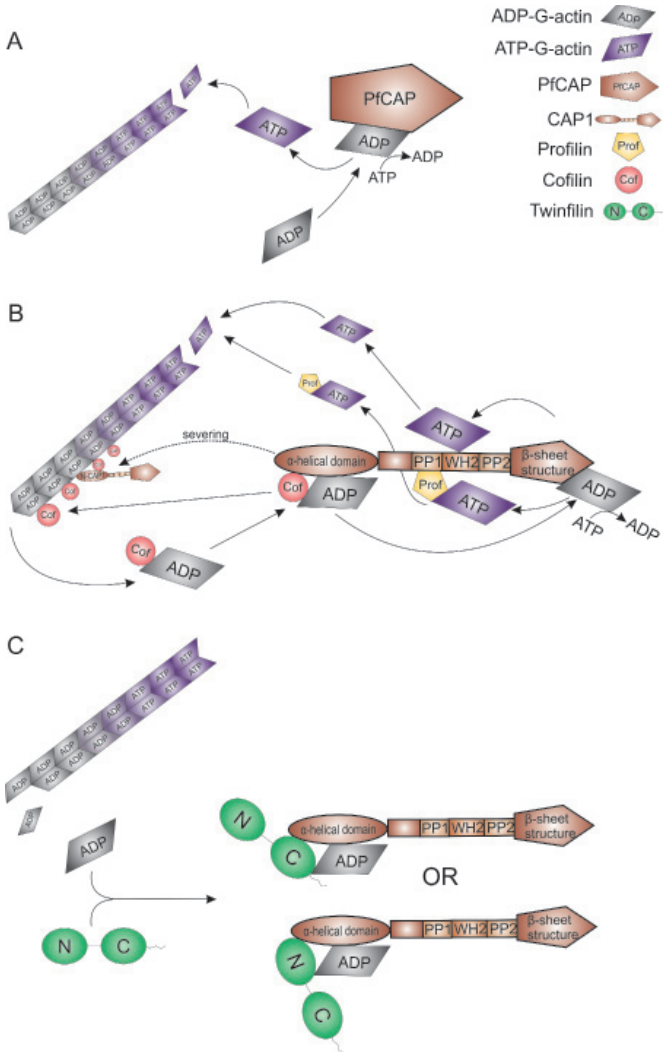

Figure 8. A model for CAP function in actin dynamics. (A) Apicomplexan 'mini-CAPs', consisting only of the β-sheet domain, catalyze nucleotide exchange from ADP to ATP on actin monomer generating ATP-actin monomers for polymerization. (B) Mammalian CAPs are multifunctional proteins promoting actin turnover in concert with cofilin and profilin. CAP binds cofilin-ADP-actin complexes through the N-terminal domain or, as suggested for yeast CAP, the N-terminal domain might accelerate cofilin-mediated actin filament severing. ADP-actin is transferred from N-terminus to C-terminus, releasing cofilin for new rounds of depolymerization/severing. C-terminal domain of CAP catalyzes the exchange of nucleotide in actin monomer and newly "recharged" actin is transferred to the central WH2 domain, which binds ATP-actin with high affinity. Alternatively, ATP-actin can be handed to profilin, which binds to first polyproline region of CAP. Thus ATP-actin monomer can be transported to the filament barbed end by either profilin or CAP. (C) Mammalian CAP interacts also with twinfilin-ADP-actin complexes through N-terminal domain. Twinfilin binds ADP-actin monomers and transfers them to CAP forming a ternary complex similarly as cofilin. Both ADF-H domains of twinfilin are capable to form the complex independently. Followed by complex formation, C-terminus of CAP most likely binds the ADP-actin monomer thus releasing twinfilin from N-CAP, similarly as cofilin, and the subsequent events are most likely to occur similarly as demonstrated in panel B. The figure is modified version from publication II.

actin dynamics in muscle sarcomeres seems not to be regulated by treadmilling. Instead, sarcomeric actin filaments undergo exchange of actin subunits at both barbed and pointed ends (Littlefield et al., 2001). Multiple actin-binding proteins have been suggested to be involved in thin filament length regulation in muscle sarcomeres, but the overall mechanism has not been clarified yet. Thus, we studied the function of mouse cofilin-2 in actin dynamics in context of thin filament length regulation in rat cardiomyocytes.

By using specific antibodies generated against cofilin isoforms (publication III, Figure 1A), we revealed that cofilin-2 localizes near M lines while cofilin-1 localization was mostly diffuse in cardiomyocyte myofibrils (publication III, Figure 1C-D). We also detected cofilin-2 levels to increase during sarcomere maturation in newly plated cardiomyocytes while the amount of cofilin-1 remained constant (publication III, Figure 1B). This is in line with earlier observations from mouse cells and embryos demostrating that *cofilin-2* mRNA is upregulated during myofibrillogenesis (Ono et al., 1994; Vartiainen et al., 2002). However, the role of cofilin-2 in myofibrillogenesis is somewhat controversial. An earlier study with *cofilin-2* knockout mouse showed that this protein is not needed in myofibrillogenesis but instead, is crucial for maintaining the developed muscles (Agrawal et al., 2012). Yet, another study showed that in cultured chicken skeletal muscle cells, depression of cofilin-2 caused defects in actin organization only if it was depleted in the early stage of myofibrillogenesis (Miyauchi-Nomura et al., 2012).

To assess the role of cofilin-2 in sarcomeres, we depleted cofilin-2 in cardiomyocytes by RNAi. Previous studies demonstrated that depletion of the muscle-specific cofilin in *C.elegans* and mouse results in defects in actin organization and accumulation of actin aggregates (Ono et al., 2003; Agrawal et al., 2012). Furthermore, depletion of both cofilin isoforms leads to disorganized actin filaments and lack of contractility in rat cardiomyocytes (Skwarek-Maruszewska et al., 2009). Our RNAi studies revealed that cofilin-2 is important for maintenance of regular actin filament lengths in muscle sarcomeres. This is because myofibrils of *cofilin-2* knockdown cardiomyocytes displayed a loss of F-actin free zones at the M line region (publication III, Figures 2 and S2). This observation is further supported by the abnormally diffuse localization of pointed end capping protein tropomodulin 1 in cofilin-2 knockdown cells (publication III, Figure 3B). However, the overall sarcomere organization was not disrupted, since M band protein myomesin showed normal striated localization in cofilin-2 knockdown cardiomyocytes which lack the F-actin free zones (publication III, Figure 3A). The depletion of cofilin-2 also resulted in increase of α-actinin-2 (publication III, Figure S1) which is in line with previous results showing the increase of other sarcomeric proteins in cofilin-2 deficient mouse muscles (Agrawal et al., 2012).

4.2.1. Identification of the ATP-G-actin binding site at cofilin-2 (III)

Previous studies showed that both cofilin-1 and cofilin-2 bind ADP-actin monomers with high affinity, whereas cofilin-2 shows higher affinity for ATP-actin monomers than cofilin-1 (Vartiainen et al., 2002). We compared the affinities of cofilin isoforms to filamentous actin, and discovered that both cofilins bind ADP-F-actin with similar high affinities (K_d ~ 0.2 μM) and they can co-assemble on the same filament, as shown by total internal reflection fluorescence (TIRF) microscopy (publication III, Figures 4A and S3). However, cofilin-2 displayed higher affinity for ADP-actin filaments than cofilin-1 when tropomyosin was present (publication III, Figure S5). Experiments with ATP-F-actin analog ADP·BeF_x-F-actin show that cofilin-2 has higher affinity for ADP·BeF_x-F-actin (K_d = 0.5 μM) than cofilin-1 (K_d > 2 μM) (publication

III, Figure 4B). Furthermore, cofilin-2 more efficiently disassembles ADP·BeF_x-F-actin than cofilin-1, suggesting that cofilin-2 is more efficient in interacting with ATP-actin filaments compared to cofilin-1 (publication III, Figures 4C-D and S4A).

Next, we wanted to identify the residues responsible for different actin binding properties of cofilin-1 and cofilin-2. We found that replacement of three conserved residues on the actin binding surface of cofilin-1 by corresponding cofilin-2 residues (E141D, E142D, V143I) increased the cofilin-1 disassembly activity for ADP·BeF_x-F-actin (publication III, Figures 5 and S4B). Next we tested whether this "cofilin-1 to cofilin-2" mutation would affect the ATP-actin monomer binding ability. Importantly, our experiments revealed that these mutations significantly enhanced the ATP-actin monomer binding affinity of cofilin-1 (from K_d ~ 230 nM to K_d ~ 20 nM; publication III, Figure 6A-B). This increase in ATP-G-actin binding was even further enhanced by replacing one additional cofilin-1 residue by a cofilin-2 specific residue (A137V). We also tested these replacements vice versa, replacing residues 141-143 on cofilin-2 by cofilin-1-specific residues, and observed diminished binding affinity for ATP-G-actin (from K_d ~ 30 nM to K_d ~ 110 nM; publication III, Figure 6C-D). Thus, a specific cluster of residues is important for the high affinity of cofilin-2 for ATP-actin.

These results, together with observations of cofilin-2 localization to near M line and phenotype resulting from depletion of cofilin-2, suggest cofilin-2 to be well suited for promoting disassembly of actin filaments from pointed ends in muscle sarcomeres. Due to both actin filament disassembly and assembly taking place at the M line region, actin filament ends at these sites are expected to contain both ATP- and ADP-subunits. Thus, cofilin-2, with its specific ATP-actin binding activity is well suited to drive actin dynamics at these sites. It still remains examined, how cofilin-2 localizes to the M line region in myofibrils. One candidate for localizing cofilin-2 is CAP, which has a nonmuscle isoform CAP1 and muscle specific isoform CAP2 (Bertling et al., 2004). Since CAP1 is known to interact with cofilin-1 in nonmuscle cells (Moriyama & Yahara, 2002) and CAP2 has been found to localize to M lines in cardiomyocyte myofibrils (Peche et al., 2007), it is possible that CAP2 interacts with cofilin-2 in muscle sarcomeres thus localizing it near M lines.

4.2.2. Cofilin-2 does not show actin isoform specificity in ATP-G-actin binding (III)

Since cofilin-2 has higher affinity for ATP-actin than cofilin-1, and the actin we used in our assays was α-actin from skeletal muscle, we wanted to assess whether this specific actin isoform caused different binding preferences of cofilins. We tested the binding of β/γ-ATP-G-actin versus α-ATP-G-actin and noted that cofilin-2 binds both actin isoforms with higher affinity than cofilin-1 (publication III, Figure S6). However, the affinities of both cofilins for muscle actin were somewhat higher than for nonmuscle actin. These results show that variations in specific cofilin residues have not evolved to increase the affinities of cofilins towards certain actin isoforms, but rather to distinguish between ATP- and ADP-nucleotide bound to actin monomers.

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This study provides new evidence for actin regulation in cells conducted by CAP, cofilin, profilin and twinfilin. In publications I and II we identified the conserved residues on the surface of yeast and mammalian CAPs that are involved in cofilin binding and also mapped the binding site of CAP on the surface of yeast cofilin. The complex formation between CAP, cofilin and ADP-actin has been shown earlier by Moriyama and Yahara (2002), but the exact binding sites were not known before this study. Localizing the binding interfaces allows further investigations and speculations about the mechanism and biological significance of the complex formation. In future, structural studies will be needed to reveal the mechanism by which these proteins interact with each other. In addition, rescue experiments in mammalian cells would shed light into the cellular roles of these protein interactions.

Since CAPs are multifunctional proteins with several binding partners, the significance of different activities in different organisms is difficult to elucidate. In publication II we show that C-terminal halves of yeast, mouse and malaria parasite *P. falciparum* CAPs are capable and sufficient for exchanging nucleotide on actin monomers. This characteristic is the first, and so far the only known function for PfCAP. From these results, we hypothesize that promoting nucleotide exchange on actin monomers is the most conserved function of CAPs. Profilin is traditionally considered to be responsible for exchanging nucleotide on actin monomers. However, this assumption needs to be reconsidered now and we suggest that mainly CAP is responsible for nucleotide exchange to promote actin turnover in cells. We also reveal that profilin binding by CAP is conserved from yeast to mammals, as shown in publication II. However, in contrast to the yeast protein, mammalian CAP1 has two independent profilin binding sites. Further studies will be needed to reveal how profilin and CAP function together and what is the significance of each protein in actin dynamics and whether they cooperate or compensate each other's actions.

For our surprise, mammalian CAP unlike yeast CAP, binds also ATP-actin with high affinity through WH2 domain as shown in publication II. This finding is unexpected and leads us to speculate whether yeast and mammalian CAP functions differ from each other. This study is the first one to show that mammalian CAP1 binds also ATP-actin with high affinity and this can be crucial for its function. Furthermore, we show in publication II that unlike yeast CAP, mammalian CAP1 WH2 domain allows actin polymerization when bound to actin monomer. These findings suggest that, before further investigations, yeast and mammalian CAPs should not be assumed to share exactly same functional mechanisms in actin regulation. Although the main function of CAPs in cells is most probably to accelerate actin turnover, CAPs from different species might carry out this action through slightly different ways.

Our unpublished data (Makkonen et al., unpublished) revealed a completely new interaction partner, twinfilin, which binds CAP together with ADP-actin in complex. Importantly, this is first study to show that these two proteins interact together directly. This interaction is notable since the functions of CAP and twinfilin are not completely understood yet. In our unpublished data we show that N-CAP1 forms ternary complex together with twinfilin and ADP-G-actin, and that both ADF-H domains of twinfilin are capable of the complex formation. However, full-length twinfilin containing both ADF-H domains is more effective in forming this complex. This interaction is a useful starting point to create new hypothesizes when aiming to understand the roles of CAP and twinfilin in regulating actin dynamics. Crystallization and structure determination would reveal the binding mechanism and importance of two

separate ADF-H domains of twinfilin in the complex formation.

Another important question is whether the binding site for N-CAP1 on surface of twinfilin corresponds to conserved N-CAP1 binding site on ADF/cofilin surface (publication I). To test this, we plan to design a twinfilin construct bearing mutations that correspond to cofilin mutants Cof1-5 and Cof1-9 that are defective in yeast CAP binding (publication I) and test whether such mutants are able to form a complex with ADP-G-actin and N-CAP1. Vice versa, it would be interesting to investigate whether the binding site for twinfilin-actin complex on the surface of N-CAP1 corresponds to the binding site mapped for cofilin-actin complex (publication I, publication II). This could be tested by our mouse mutation construct N-CAP1 mutα (publication II) e.g. by supernatant depletion pull-down assay.

It would be also useful to determine whether cofilin-actin and twinfilin-actin compete with each other for binding to N-CAP1. The role of twinfilin-actin binding to CAP1 should also be investigated more closely since the cellular roles of cofilin and twinfilin seem to be somewhat overlapping. This is supported by observations made with yeast, where deletion of twinfilin alone did not result strong phenotype but, when combined to cofilin mutant, resulted in synthetic lethality (Goode et al., 1998). It has been shown earlier that human N-CAP1 accelerates the nucleotide exchange from ADP to ATP on actin monomer in the presence of cofilin (Moriyama and Yahara, 2002). The possible overlapping roles of twinfilin and cofilin could be examined similarly by observing the effect of N-CAP1 to accelerate the nucleotide exchange in the presence of twinfilin. Also, it would be interesting to perform an F-actin turnover assay with N-CAP1, cofilin and twinfilin and test whether addition of twinfilin would make a difference in the turnover rate accelerated by CAP1 and cofilin.

We show that cofilin-2 localizes near M lines and disassembles actin filaments on muscle sarcomeres thus regulating the filament length (publication III). There is very little information about the muscle-specific isoforms of actin binding proteins and how they regulate actin dynamics in muscle cells. Cofilin is a fundamental actin cytoskeleton regulating protein in nonmuscle cells and we show that cofilin-2 has also a crucial function in muscle cells. The regulation of sarcomeric length is essential for proper function of sarcomere in contracting muscle and thus the mechanism and proteins involved in this regulation are necessary to elucidate. This study broadens our understanding about the thin filament length regulation, but still the overall mechanism remains largerly unknown and additional studies are needed to fully understand how the actin dynamics is regulated in muscle cells. For example, other muscle-specific actin binding protein isoforms should be examined more closely.

It is already known that mammalian CAP2, as well as *C.elegans* muscle-specific CAP, localize to M lines (Peche et al., 2007; Nomura et al., 2012). Recently published results from *CAP2* knockout mouse showed that in the absence of CAP2, mice have disarrayed sarcomeres in cardiomyocytes and they develop severe heart defects and dilated cardiomyopathy (Peche et al., 2013). The interaction of CAP2 and cofilin-2 in muscle cells would be important to study. First, the expression of CAP1 and CAP2 proteins in muscle cells should be tested and this could be studied by Western blot. It would be interesting to find out if these CAP isoforms are differentially expressed during maturation like we showed for the two cofilin isoforms. If both isoforms are present, it would be informative to determine if they localize similarly to cofilin isoforms. To find out if muscle-specific isoforms cofilin-2 and CAP2 interact with each other and whether they prefer the muscle-specific isoform over nonmuscle isoform, one could apply similar

pull-down experiments as used for cofilin-1 and CAP1 in publication II of this study. These experiments would perhaps give us more information about cofilin-2 localization and function near M lines.

The roles of twinfilin-2b and profilin II in muscle cells would be also interesting to reveal. Since yeast and mammalian CAPs are known to interact with profilin (Bertling et al., 2007; publication II) and we show in our unpublished data that mammalian CAP1 interacts with twinfilin (Makkonen et al., unpublished) it would be enlightening to examine the potential interactions of muscle-specific profilin II and twinfilin-2b with CAP2, as described above for cofilin-2 and CAP2 interaction. Further studies concerning these interactions could give us valuable information and further widen our knowledge about actin filament regulation in muscle cells.

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