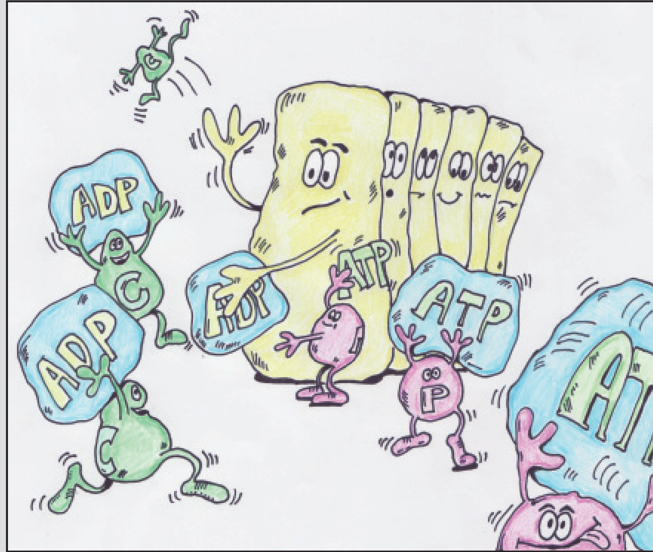

The Role of Cyclase-Associated Protein (CAP) in Actin Dynamics During Cell Motility and Morphogenesis

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ABBREVIATIONS

ABP	actin binding protein (general)
Abp1	actin binding protein 1
ADP	adenosinediphosphate
ADF	actin-depolymerizing factor
Aip1	actin-interacting protein 1
ATP	adenosinetriphosphate
Arp	actin related protein
cAMP	cyclic adenosine monophosphate
CAP	cyclase-associated protein
Cc	critical concentration
cc	coiled-coil domain
CH	calponin homology
CP	capping protein
E	embryonic day
Ena/VASP	enabled/vasodilator-stimulated phosphoprotein
ERM	ezrin/radixin/moesin
F-actin	filamentous actin
FH	formin homology
G-actin	globular actin (monomer)
GFP	green fluorescent protein
GST	glutathione S-transferase
GTP	guanosine triphosphate
IF	intermediate filament
NLS	nuclear localization signal
MAP	microtubule-associated protein
MT	microtubule
MIM	missing-in-metastasis
P1	first polyproline-rich sequence
P2	second polyproline-rich sequence
$PI(4,5)P_2$	phosphatidylinositol-(4,5)-bisphosphate
Pi	pyrophosphate, inorganic phosphate
Srv2	suppressor of RAS2-val19
WH2	WASP homology domain 2
WASP	Wiskott-Aldrich Syndrome Protein

ABSTRACT

The highly dynamic remodeling of the actin cytoskeleton is responsible for most motile and morphogenetic processes in all eukaryotic cells. In order to generate appropriate spatial and temporal movements, the actin dynamics must be under tight control of an array of actin binding proteins (ABPs). Many proteins have been shown to play a specific role in actin filament growth or disassembly of older filaments. Very little is known about the proteins affecting recycling i.e. the step where newly depolymerized actin monomers are funneled into new rounds of filament assembly. A central protein family involved in the regulation of actin turnover is cyclase-associated proteins (CAP, also called Srv2 in budding yeast). This 50-60 kDa protein was first identified from yeast as a suppressor of an activated RAS-allele and a factor associated with adenylyl cyclase. The CAP proteins harbor N-terminal coiled-coil (cc) domain, originally identified as a site for adenylyl cyclase binding. In the N-terminal half is also a 14-3-3 like domain, which is followed by central proline-rich domains and the WH2 domain. In the C-terminal end locates the highly conserved ADP-G-actin binding domain.

In this study, we identified two previously suggested but poorly characterized interaction partners for Srv2/CAP: profilin and ADF/cofilin. Profilins are small proteins (12-16 kDa) that bind ATP-actin monomers and promote the nucleotide exchange of actin. The profilin-ATP-actin complex can be directly targeted to the growth of the filament barbed ends capped by Ena/VASP or formins. ADF/cofilins are also small (13-19 kDa) and highly conserved actin binding proteins. They depolymerize ADP-actin monomers from filament pointed ends and remain bound to ADP-actin strongly inhibiting nucleotide exchange. We discovered that the ADP-actin-cofilin complex is able to interact directly with the 14-3-3 like domain at the N-terminal region of Srv2/CAP. The C-terminal high affinity ADP-actin binding site of Srv2/CAP competes for an actin monomer with cofilin. Cofilin can thus be released from Srv2/CAP for the subsequent round of depolymerization. We also revealed that profilin interacts with the first proline-rich region of Srv2/CAP and that the binding occurs simultaneously with ADP-actin binding to the C-terminal domain of Srv2/CAP. Both profilin and Srv2/CAP can promote nucleotide exchange of an actin monomer. Because profilin has much higher affinity to ATP-actin than Srv2/CAP, the ATP-actin-profilin complex is released for filament polymerization. While a disruption of cofilin binding in yeast Srv2/CAP produces a severe phenotype comparable to Srv2/CAP deletion, an impairment of profilin binding from Srv2/CAP results in much milder phenotype. This suggests that the interaction with cofilin is essential for the function of Srv2/CAP, whereas profilin can also promote its function without direct interaction with Srv2/CAP. We also show that two CAP isoforms with specific expression patterns are present in mice. CAP1 is the major isoform in most tissues, while CAP2 is predominantly expressed in muscles. Deletion of CAP1 from non-muscle cells results in severe actin phenotype accompanied with mislocalization of cofilin to cytoplasmic aggregates. Together, these studies suggest that Srv2/CAP recycles actin monomers from cofilin to profilin, and it therefore plays a central role in actin dynamics in both yeast and mammalian cells.

ORIGINAL PUBLICATIONS

This thesis is based on three original articles referred to in the text by their roman-numbers.

I. **Bertling, E.**, Quintero-Monzon, O., Mattila, P.K., Goode, B.L., Lappalainen, P. (2007) Mechanism and biological role of profilin-Srv2/CAP interaction. J. Cell. Sci. 120:1225-1234.

II. Quintero-Monzon, O., **Bertling, E.**, Johansson, E.M., Talarico, L., Chaudhry. F., Sihvo, M., Lappalainen P., Goode B.L. Reconstitution of the 600 kDa Srv2-actin complex reveals its architecture and mechanism for driving rapid actin turnover. (Submitted)

III. **Bertling, E.**, Hotulainen, P., Mattila, P.K., Matilainen, T., Salminen M., Lappalainen, P. (2004). Cyclase-associated protein 1 (CAP1) promotes cofilin-induced actin dynamics in mammalian nonmuscle cells. Mol. Biol. Cell. 15:2324-2334.

INTRODUCTION

The cytoskeleton

The cytoskeleton is a fundamental structure for all cells. Without the cytoskeleton, cells would just be loose sacks floating around in their environment, unable to maintain their shape, to move, feed or divide. The cytoskeleton is a dense and seemingly random cytoplasmic network of different kinds of filaments. They fill the space inside the cell and maintain the cell shape. Cytoskeletal components can generate mechanical forces: pushing, by arranging themselves into a certain direction, and pulling, by contracting along one another accompanied by loads of other proteins. These movements generate dramatic changes in cell shape needed for cell migration, endocytosis and cell division. The cytoskeleton also contributes to different kinds of translocation inside the cell by forming the tracks for motor proteins (Wade and Hyman, 1997, Godsel *et al*., 2008, Pollard and Borisy, 2003). In eukaryotic cells, the cytoskeleton is composed of three different filament types, microtubules, intermediate filaments and microfilaments.

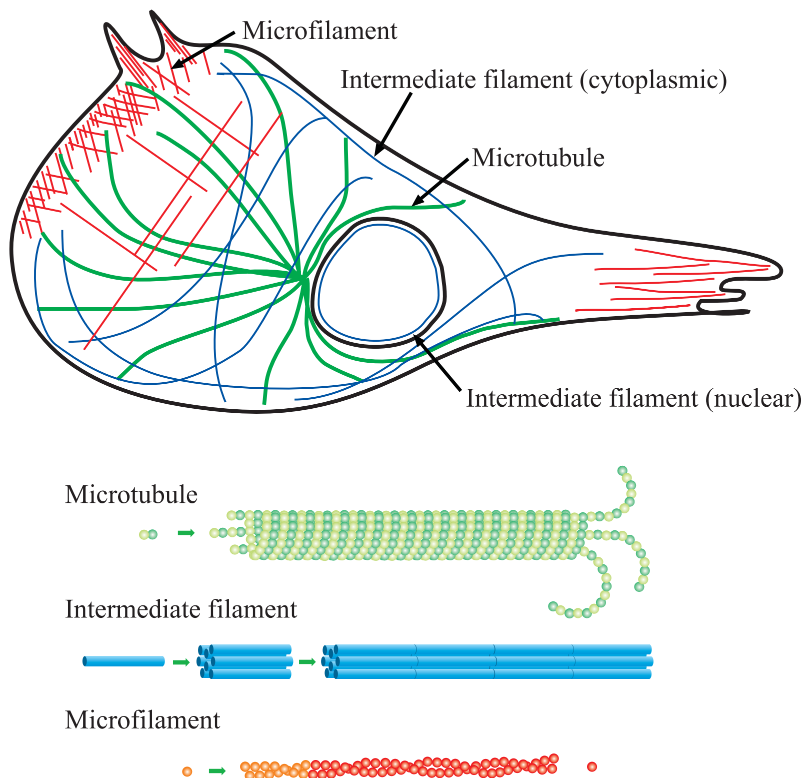

Figure 1. The cytoskeleton. Schematic picture illustrates the three components of the eukaryotic cytoskeleton. Microtubules (green) grow radial from centrosomes, plus- ends towards the plasmamembrane, growing and shrinking alternately. Intermediate filaments (blue) localize around the cell and in the nucleus next to the inner nuclear membrane. Microfilaments (actin filaments) (red) form fibrous structures to the growing front and to the shrinking tail of the cell.

Table 1. Cytoskeletal components. Cytoskeleton consists of three different filament types, microtubules, intermediate filaments and microfilaments. The subunit proteins and general functions are listed here.

Filament type	Protein	Function	Reference
Microtubules ∅ 24 nm	α/β-tubulin ~ 55 kD	Signaling, intracellular transport, chromosome segregation during cell division.	Wade and Hyman, 1997
Intermediate filaments ∅ 10 nm	I Keratins (Acidic) ~ 40-57 kD	Rigidity and signaling in epithelial cells.	Coulombe and Omary, 2002
	II Keratins (Basic) ~ 53-67 kD		
	III Vimentin, ~ 57 kD Desmin, ~ 53 kD Glial fibrillary acidic protein (GFAP) ~ 50 kD	Rigidity and signaling in astrocytes, mesenchymal, muscle and glial cells.	Herrmann and Aebi, 2004
	IV Neuro-filaments ~ 62-110kD	Neuronal Maintenance.	Barry *et al.,* 2007
	V Lamins ~ 67-70 kD	Nuclear envelope, mitosis	Haas and Jost, 2003
Microfilaments ∅ 7-9 nm	Actin ~ 45kD	Cell motility, morphogenesis, endocytosis, cell division	Pollard and Borisy, 2003

1. Microtubules

Microtubules represent the widest cytoskeletal filament type, being 24 nm in diameter. Highly homologous globular α- and β-tubulin monomers form heterodimers. Both monomers bind guanosine triphosphate (GTP), but only the one bound to β-tubulin is hydrolyzed during polymerization. The α- and β-tubulin heterodimers self-assemble to polar oligomer, resembling a cylindrical tube (Fig.1)(Nogales et al, 2006). β-subunits point towards the fast-growing plus-end, whereas the α-tubulins point towards the minus end, which is typically connected to the centromere (Nogales et al., 1999).

The organization and dynamics of microtubules are regulated by an array of microtubule-associated proteins (MAP)s, which can e.g. either stabilize or destabilize microtubules (Halpain and Dehmelt, 2006, Morrison, 2007). Recent studies have shown that also post-translational modifications play an important role in the regulation of microtubule function. α- and β-tubulin monomers can be acetylated, polyglycylated, polyglutamylated, tyrosinated, phosphorylated and palmitylated, which further increases microtubule diversity for variety of functions (Westermann and Weber, 2003).

Microtubules form a highly dynamic network inside the cell. The motor proteins dynein and kinesin can move along the microtubules and thus transport cargo across the cell (Caviston and Holzbaur, 2006). Microtubules also play a role in cell motility. The Rho

family GTPases, known to be key regulators in cell migration, contribute directly to the dynamics of microtubules by capturing and stabilizing them to the cell cortex (Watanabe et al., 2005). The most well characterized function of microtubules is the formation of the mitotic spindle, which drives chromosome segregation during cell division. The plus-ends of spindle microtubules attach to the kinetochore, which is a complex structure located at the centromere and is composed of >80 proteins. The dynamics of the spindle microtubules separates the daughter chromosomes after successful alignment of the all chromosomes and passing the spindle checkpoint (Cheeseman and Desai, 2008).

The growing family of MAPs also includes many oncogenes, tumor suppressors and regulators of apoptosis. This, together with the central role microtubules in cell division and survival, makes microtubules and their associated factors promising targets for a variety of cancer therapeutic drugs (Bhat and Setaluri, 2007).

2. Intermediate filaments

The second cytoskeletal filament type is the intermediate filament (IF). The principal role of these approximately 10 nm wide filaments is to maintain structural integrity. They also maintain the cell shape, position cell organelles, and organize cells to tissues by forming a variety of intercellular junctions. During cell division, intermediate filaments have also a signaling role in cell cycle control. In neurons, intermediate filaments are responsible for directionality of radial axon outgrowth (Herrmann *et al.,* 2003, Coulombe and Wong, 2004).

Intermediate filaments consist of ~65 different proteins (Hesse *et al.,* 2001). These proteins are expressed in distinct tissues and cell-types, depending on the structural and functional needs. Based on sequence homology, intermediate filaments can be divided into five classes listed in **Table 1.** In the general filament assembly model, the monomers first form a dimer, then a tetramer. Tetramers interact laterally to form unit-length filaments (ULFs), which can polymerize into long fibers (Fig. 1) However, different intermediate filaments display variations to this assembly model (Strelkov *et al.,* 2003).

Most IFs localize around the cytoplasm and form a rigid network supporting the cell under a mechanical stress, with an exception of the group V, lamins, which form a network inside the nucleus, next to the inner nuclear membrane. Lamins maintain the shape of the nucleus and play an important role during cell division (Haas and Jost, 2003). Intermediate filaments also have a structural function in positioning the cell organelles (Toivola *et al.,* 2005)

Unlike globular G-actin and tubulin, intermediate filament monomers are composed of a rod shaped central domain accompanied with globular N- and C- terminal domains (Fuchs and Weber, 1994). The central domains, more precisely the dimerization sites, are usually relatively well conserved, whereas the distal domains are more variable and harbor also many phosphorylation sites. Intermediate filaments are regulated through these specific phosphorylations. For example, during cell division or under certain cell stress, multiple phosphorylations of distal head domains induce depolymerization of the filaments. This property is important for the function of the intermediate filaments, which are thus rigid and relatively stable assemblies with a structural role, but can be quickly decomposed after adequate set of signals allowing the cell to undergo dramatic

changes in shape (Herrmann and Aebi, 2004). Mutations in the genes of intermediate filaments contribute to more than 30 diseases including muscular dystrophies, premature aging and skin and end-stage liver diseases (Herrmann and Aebi, 2004).

3. Microfilaments

Third filament type is the thinnest and is therefore called microfilament. These 7-9 nm wide filaments consist entirely of actin, which is the most abundant protein in many eukaryotic cell types. Probably the best-known function of actin is its ability to form contractile muscular units, i.e. sarcomeres, with a motor protein myosin II (Eddinger and Meer, 2007). In all eukaryotic cells, from cell-walled yeast to non-muscle cells of higher eukaryotes, actin with its unique ability to form polar filaments is responsible for a variety of important cellular functions, as discussed in the following chapters (Pollard and Borisy, 2003).

3.1. Actin

Actin monomer is a highly conserved 42-44 kDa globular protein consisting of two major domains which are further divided into four subdomains 1–4 (Kabsch *et al.,* 1990). Between the subdomains 2 and 4, there is a hydrophobic pocket which binds a nucleotide, either ADP or ATP (Fig.2). The ability to hydrolyze ATP and release the

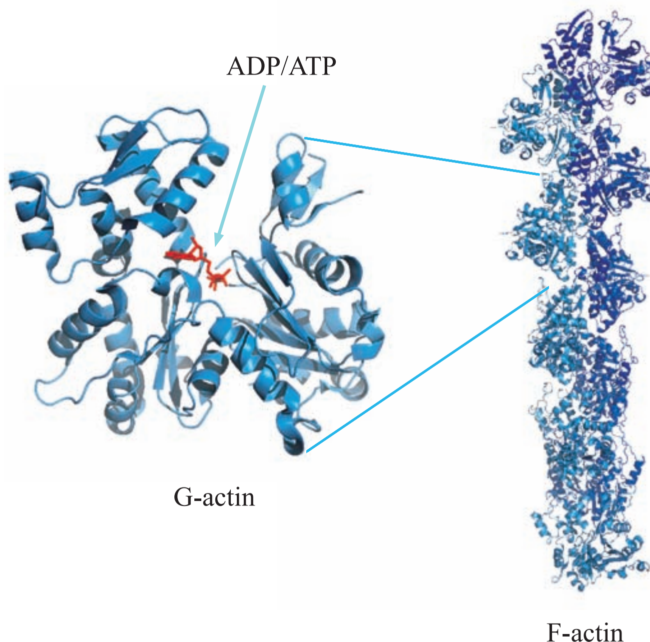

Figure 2. Three-dimensional structure of G- and F-actin. Crystal-structure of G-actin (monomer) reveals the cleft between two lobes where the nucleotide, either ADP or ATP and a divalent cation bind. Actin monomers can polymerize into polar F-actin (filament).

inorganic phosphate (Pi) makes actin protein a functional ATPase. Inside the domains, there is also a divalent cation, either Ca^{2+} or Mg^{2+} (Kabsch and Holmes 1995). On the opposite side of the protein, between the subdomains 1 and 3, locates a small cleft known to be a target for most actin-binding proteins (ABPs)(Dominguez *et al.,* 2004). The actin structure has been well studied, and the crystal structures of actin with an array of ABPs are available. The actin monomer is a flexible protein with the ability to change conformation from closed to more open depending on the prevailing circumstances, status of the nucleotide, interacting ABPs, the nature of the cation or the degree of oligomerization (Moraczewska *et al.,* 1999, Schüler, 2001).

3.2. Actin dynamics

The most important feature of actin monomer (also called G-actin) is its ability to form dimers and to further oligomerize into long filaments called F-actin. In these structures, all actin monomers are facing into the same direction, thus forming a polar oligomer. Binding to other actin monomers also causes a conformational change within the monomers leading to altered biochemical properties. As a result, already the third monomer added to this growing filament has a preference to interact with certain end of the forming filament, called barbed or “fast growing” plus-end. The critical concentration (Cc) for polymerization at the barbed end is 0,1 μM under physiological ionic conditions. The other end is called pointed or “slow growing” minus-end and has a higher critical concentration for polymerization (Cc = 0,7 μM). In actin monomer concentrations between these two Cc´s, the barbed end favors filament formation (polymerization), whereas the actin monomers are dissociating from the pointed end. These differences cause actin pool never to find an equilibrium between G- and F-actin, but instead being in the constant circle of simultaneous growing and shrinking at the barbed and pointed ends, respectively. This phenomenon is called actin treadmilling (Fig 3.) (Wegner, 1982).

"Treadmilling"

Figure 3. ”Treadmilling”. Actin monomers undergo constant circulation. Under steady-state conditions, ATP-actin monomers are added to the filament barbed end (+). In the filament, ATP is hydrolyzed to ADP, which leads to more unstable structure of the filament. From pointed end (-), ADP-actin monomers are dissociated and funneled back to filament barbed end after nucleotide exchange.

The ability of actin monomers to bind to either ADP or ATP has important consequences in treadmilling. Filamentous actin has higher affinity to ATP-actin monomers than ADP-actin, so the monomers added to barbed ends are typically in the ATP-form (Pollard, 1986). In filaments, ATPase activity of actin is increased dramatically and it hydrolyses the inorganic phosphate from ATP to generate ADP-actin, causing also a conformational change within the protein (Pollard and Weeds 1984). Because the equilibrium drives monomer dissociation from pointed ends and ADP-actin dissociates from filament ends even more effectively, treadmilling rates are further increased (Fig 3.). The ability of actin to form filaments and to rapidly arrange them under strict spatial and temporal regulation of ABPs generates most key functions of actin in yeast and mammalian cells (Pollard *et al.* 2000).

3.3. The actin cytoskeleton in budding yeast

The actin cytoskeleton from budding yeast was first demonstrated already in 1984 (Kilmartin and Adams 1984, Adams and Pringle 1984). In *Saccharomyces cerevisiae,* actin is encoded by a single gene *ACT1*, which shows 80% sequence similarity with mammalian actin genes (Shortle *et al.,* 1982). The structural and biochemical properties of yeast actin are also notably similar to those of mammalian actin proteins. However, the need for F-actin structures in a yeast cell which is supported by a cell wall is highly different from the situation in mammalian non-muscle cells which maintain their shape solely with internal cytoskeleton.

In *S.cerevisiae* cells, F-actin staining visualizes three kinds of filamentous actin structures. The first of these are cortical spots, called “patches” which are more or less round structures found at the cell periphery, at the plasma membrane or somewhere near. During budding, these patches are concentrated to the growing daughter cell (Fig. 4A). The second clear structures are long actin filament bundles, called “cables” coursing longitudinally through the cell, and the third one is the contractile ring forming during cell division.

Early studies of yeast actin already suggested that the cortical patches were components of endocytic pathway (Kubler and Riezman 1993). This function was clarified by studies with endocytic vesicles (marked with FM4-64) colocalizing with actin marker Abp1-GFP (Huckaba *et al.,* 2003, Kaksonen *et al.,* 2003). Nowadays, actin patch dynamics can be visualized by using GFP-tagged actin binding proteins, and real-time movements can be recorded in living cells (Pelham and Chang 2001, Kaksonen *et al.,* 2003). It is known that patches form on plasma membrane, move inward and disappear. Patch movement can be divided into three stages according to the speed of the patch and the actin-binding proteins present. At the first stage, patches form and stay more or less non-motile near the membrane. In this stage, for example, the proteins Las17, Sla1, Sla2 and Pan1 are present (Kaksonen *et al.,* 2005). A recent study also localized an endocytic vesicle protein clathrin in these structures (Newpher *et al.,* 2005). At the second stage, polymerized actin appears, and the patch starts slow movement at the cortex (0,05 to 0,1 µm/s). At this stage, patches also contain Arp2/3, Abp1 and type I myosins, Myo3 and Myo5. These proteins contribute to actin filament assembly, accomplishing vesicle scission and generating pushing forces. At the third stage, the

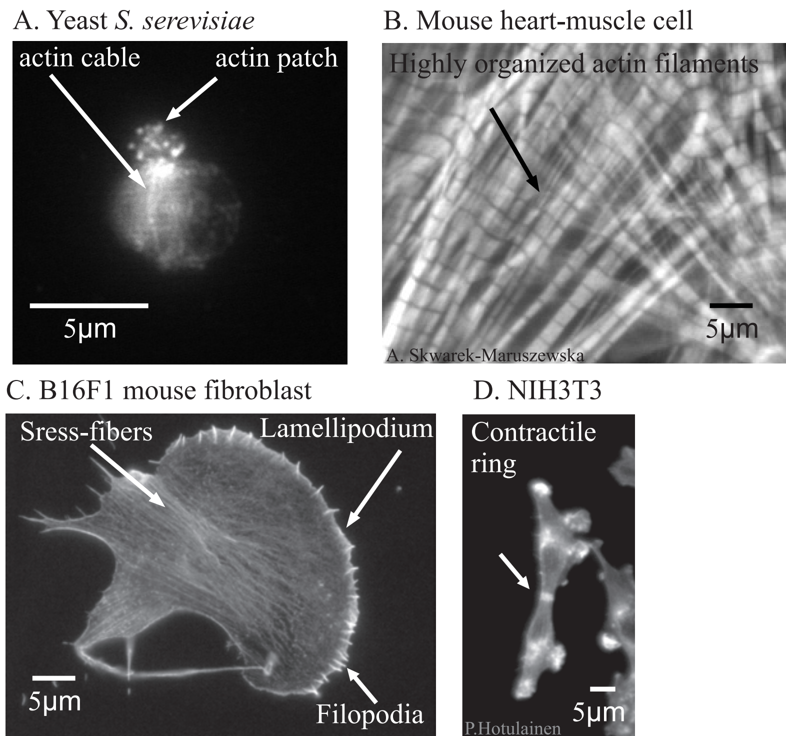

Figure 4. Actin structures in yeast and mammalian cells. (A) The yeast actin cytoskeleton consists of two structures visible by light microscopy: actin patches and actin cables. Patches are concentrated to the region of cell growth, whereas the cables usually course the cell longitudinally (B). Mouse heart muscle cell reveals the regular pattern of the actomyosin structures, called sarcomeres. (C) Mouse B16F1 melanoma cell have visible stress fibers, wide smooth lamellipodium, and several filopodia pointing out from lamellipodium. (D) Contractile ring in dividing NIH3T3 fibroblast.

patch starts fast, actin cable-based movement inward from the cortex (0,3 μm/s). Many early components are shed (Las17, Sla1, Sla2, Pan1, Myo3 and Myo5), while some actin binding proteins still remain associated with these structures (Kaksonen *et al.,* 2005).

At the last, rapid movement stage, endocytic patches move along actin cables. Actin cables are long directed actin bundles coursing the cell (Fig. 4A). Yeasts use these structures for polarized growth and as tracks for carrying cargo (Bretscher, 2003). These functions are closely related, the unbudded cells use the landmarks remaining from the previous budding events to orientate barbed ends of the actin cables towards to the new bud site and form polarity to direct next budding cycle (Moseley and Goode, 2006). Actin cables are polymerized by yeast formin-family proteins, Bni1 and Bnr1 (Evangelista *et al.,* 2002). Along these roughly parallel actin bundles, cells deliver organelles and cargo vesicles to daughter cells. Vesicles contain material for growth of the bud and are mainly delivered by type V myosins, Myo2 and Myo4 (Schott *et al.,* 2002).

The third, clearly separable actin structure in yeast cell is called actomyosin ring. This ring appears around the bud neck only during the late anaphase stage of the cell division. (Bi *et al.,* 1998, Lippincott and Li, 1998). The ring is formed in Arp2/3 dependent manner, and the contraction is generated by the interaction of actin filaments with type II myosin, Myo1 (Winter *et al.,* 1999, Watts *et al.,* 1987).

3.4. The actin cytoskeleton in mammalian cells

Mammals have at least six genes that code actin (Vandekerckhove *et al.,* 1978). They are divided into three groups based on their electrophoretic mobility, to α-, β- and γ-actins. These actin isoforms are selectively expressed in different tissues and cell types, four in skeletal muscles and β- and γ-actins mainly in non-muscle cells. These actin isoforms are >95 % identical to each other at amino acid level, and their biochemical properties are comparable. In mammalian cells, there are two types of actin-based movements. In the first type, the force is generated by motor protein myosin moving along the actin filaments. Such myosin driven forces play an important role in contraction of muscle sarcomeres (Fig. 4B) and stress fibers of non-muscle cells (Fig. 4C) as well as in contractile ring during cell division and in vesicle transport in endo- and exocytosis (Fig. 4D). In the second type, the movement results from growing filament barbed ends pushing the cellular membrane, for example during the formation of plasma membrane protrusions in cell migration (Fig. 4C) (Pollard and Borisy *et al.,* 2003).

Muscle cells have developed to carry out whole-cell contraction and generate forces to move the entire organism. In muscle cells, actin is arranged with motor protein type II myosin (together called actomyosin) into highly ordered structures i.e. sarcomeres (Fig. 4B). In order to form a functional muscle, sarcomeres must follow a continuous pattern throughout the cell and be firmly attached to the sarcomeres of the adjacent cell (Eddinger and Meer, 2007). Actin dynamics of mature muscle cells is highly different from that seen in non-muscle cells because most of the actin is reserved for these stable patterns. Nevertheless, also muscle cells contain a pool of “free” and dynamic actin which is perhaps responsible for other actin-based functions.

Staining of cultured non-muscle cells with an actin-binding drug phalloidin shows, most visibly, thick fibers coursing the cell (Fig. 4C). These structures are called stress fibers, and they are contractile actomyosin bundles responsible for maintenance of cell stiffness and contraction of the trailing edge during cell migration. Stress fibers are covered periodically by two proteins, α-actinin (bundling) and type II myosin (contraction). Stress fibers can be divided into three subtypes: ventral stress fibers must be attached to focal adhesions from both ends to generate forces to retract cell; dorsal stress fibers attached to the focal adhesions at one end in the growing lamellipodia, and transverse arcs, which are not directly attached to focal adhesions (Pellegrin and Mellers, 2007). A resent study revealed that dorsal stress fibers and transverse arcs are precursors of ventral stress fibers (Hotulainen and Lappalainen, 2006).

In motile animal cells, actin forms a structure called lamellipodium (Fig. 4C). Lamellipodium is a wide, dense network of branched actin filaments (Pollard and Borisy, 2003). It is formed to the edge of the cell, and it pushes the cell membrane forward during cell migration. In order to generate constant pushing force, lamellipodium must

simultaneously decompose from the back and funnel the released actin monomers to the growing front line. A core set of proteins involved in the formation of lamellipodium includes actin, Arp2/3 complex, profilin, capping protein (CP) and ADF/cofilins (Mullins *et al.,* 1998, Svitkina and Borisy 1999). Other structures related to lamellipodia include filopodia (Fig. 4C). These fingerlike protrusions consist of parallel unipolar bundles of actin filaments pointing out from the cell edge (Gupton and Gertler, 2007). In filopodia, there are no Arp2/3-based branched filaments. Instead, the nucleation and processive capping of actin filaments in filopodia is achieved by Ena/VASP- and formin –family proteins (Bear *et al.,* 2002, Schirenbeck *et al.,* 2005).

3.5. Actin in human diseases

Actin and actin-binding proteins play a central role in various cellular processes, and consequently mutations of these proteins are often associated to specific diseases. Mutations in skeletal α-actin gene (*ACT1)* in humans cause three different kinds of myopathies: actin myopathy (AM), intra-nuclear rod myopathy (IRM) and nemaline myopahy (NEM). The histopathological phenotype shows an accumulation of abnormal F-actin rods in muscle cells, and the clinical phenotype includes severe congenital muscle weakness and high mortality. More than 80 different point mutations in *ACT1* gene have been characterized to cause these diseases and usually these mutations disrupt specific actin interactions within the sarcomere structure (Sparrow *et al.,* 2003). Only few pathological phenotypes have been demonstrated for mutations in other actin genes. Point mutations in cardiac α-actin gene (*ACTC*) can cause hypertrophic cardiomyopathy (Mogensen *et al.,* 2004) whereas a specific mutation in non-muscle β-actin has been reported to alter depolymerization dynamics of actin and lead to developmental malformations, deafness and dystonia (Procaccio et al., 2006).

Actin interacts with a huge amount of proteins and also mutations in these can cause a plethora of actin-related sicknesses. Mutations in several actin-binding proteins, including α-actinin, nebulin, titin, ADF/cofilin, and tropomyosin have been linked to congenital myopathies (Laing, 2007). Disruption of ADF/cofilin's normal function in actin dynamics was also suggested play a role in several pathophysiological defects including Alzheimer's disease and ischemic kidney disease (Bamburg and Wiggan, 2002).

3.6. Actin binding proteins

Actin structures are regulated by numerous actin-binding proteins (ABPs). Some of them have a structural role, connecting filaments with one another (bundling, cross-linking etc.), to intracellular structures (organelles, vesicles, microtubules etc.), to plasma membrane, and through different kind of adhesions to environment (other cells, extracellular matrix, etc.). In addition, many ABPs function to mediate specific signals to modify actin structures. Although a plethora of ABPs is wide, actin interactions of these proteins are mediated through relatively few actin-binding domains. Six most ubiquitous actin binding motifs are the calponin homology (CH) domain, the WASP homology 2 (WH2) domain, the gelsolin homology domain, the actin-depolymerizing factor (ADF)

homology domain, the formin homology 2 (FH2) domain and the myosin motor domain (Gimona *et al.,* 2002, Paunola *et al.,* 2002, Lappalainen *et al.,* 1998, McGough *et al.,* 2003, Higgs *et al.,* 2005, Sellers *et al.,* 2000).

3.6.1. ABPs in actin dynamics

In the cells, the actin monomer concentration is high, even 100 μM, driving efficiently towards filament polymerization. Certain proteins, for example β-thymosins sequester efficiently actin monomers in 1:1 molar ratio, and thus maintain a pool of unpolymerized actin monomers. Monomers can be released to polymerization after suitable signals (Pollard and Borisy, 2003). The initial step in actin filament growth is called nucleation. This step takes place spontaneously, but can be greatly accelerated by nucleators such as formins and Arp2/3. Depending on the protein, the newly formed actin filaments can occur in parallel or branched conformation (Mullins *et al.,* 1998, Vavylonis *et al.* 2006, Krause *et al.,* 2003). Actin filaments continue to grow from their barbed ends as long as ATP-actin monomers are available. Small actin-binding protein, profilin, promotes nucleotide exchange of ADP-actin monomers and maintains adequate ATP-actin monomer pool (Witke *et al.* 2004).

Due to certain signals actin filaments either grow or are capped by various capping proteins (Wear *et al.,* 2003, McGough *et al.,* 2003). Unwanted actin filaments are severed and depolymerized from pointed ends by ADF/cofilins to maintain the pool of actin monomers (Bamburg *et al.,* 1999). However, structurally important actin filaments can be stabilized by for example tropomyosins (Blanchoin *et al.,* 2001), attached to membrane by ERM-proteins (Niggli and Rossy, 2007), or to other actin filaments by actin cross-linking proteins such as α-actinin and filamin (Edlund et al., 2001, Popowicz *et al.,* 2006). With motor protein myosin, actin filaments can also be used for intracellular trafficking and can promote contraction of the cell (Eddinger and Meer, 2007, Sellers, 2000). **Table 2**. shows a general overview of the actin binding proteins and their functions. Out of these proteins only profilin, ADF/cofilin and Cyclase-associated protein (CAP) are discussed here in more detail, because of their relevance in this study.

3.6.2. Profilin

One of the essential actin monomer binding proteins was originally identified as a DNAase inhibitor that co-crystalizes with actin (Lindberg 1966, Lazarides and Lindberg 1974). Later, it was named profilin according to its capability to keep actin in a monomeric or “profilamentous” form (Carlsson *et al.,* 1977). Profilin is a small (12-16 kDa) ubiquitous ATP-actin binding protein, and it can be found in a plethora of organisms, for example in yeast (Oechsner *et al.,* 1987), flies (Cooley *et al.,* 1992) and plants (Valenta *et al.,* 1991). Lower organisms, such as yeast, have only one gene encoding profilin (Magdolen *et al.,* 1988). An ameba *Dictyostelim discoideum* is an exception, since it has three profilin-coding genes, two of which have been characterized in detail (Haugwitz *et al.,* 1991). The number of profilin-coding genes seems to be larger in higher species, for example mammals have five: profilin I, IIa, IIb, III and IV. In general, the different isoforms have evolved to fulfill the needs of various cell

Table 2. **Actin binding proteins.** Some examples of the central actin binding proteins are presented here. Proteins are classified by their functions. Numbers after the protein name indicate the actin binding domain of the protein; 1. The calponin homology (CH) domain. 2. The WASP homology domain (WH2) domain. 3. The gelsolin homology domain. 4. The actin-depolymerizing factor (ADF) homology domain. 5. The formin homology 2 (FH2) domain 6. The myosin motor domain.

Class	Name	Function	Reference
Structural proteins	α-actinin[1] filamin[1]	- Cross-linking, F-actin bundling	Edlund *et al. 2001* Popowicz *et al.,* 2006
Protein connecting actin and plasma membrane	IMD-domain proteins (MIM)[2] ERM proteins	- Binds both lipids and actin and deforms plasma membrane - Binds both membranes and actin	Mattila *et al.* 2007 Niggli and Rossy, 2007
Motor proteins	myosin II[6] myosin I, V[6]	- In actomyosin structures - Vesicle trafficking	Eddinger and Meer, 2007 Sellers, 2000.
Regulators - Monomer binding	profilin ADF/cofilins[4] twinfilin[4] thymosin β4[2] Srv2/CAP	- Nucleotide exchange - Filament depolymerization - Sequestering of ADP-actin - Sequestering of ATP-actin -Binds ADP-actin	Witke *et al.* 2004 Bamburg *et al.,* 1999 Vartiainen *et al.* 2000 Paunola *et al.* 2002 Mattila *et al.,* 2004
- Nucleating	Arp2/3 Formins [5 (+2)] Ena/VASP	- Branched filaments (activated by WASP[2] and WAVE[2]) - Nucleate parallel filaments, control barbed end growth - Uncapping, elongation promotion	Mullins *et al.,* 1998 Vavylonis *et al.* 2006 Krause *et al.*2003
- Capping	capping protein gelsolin[3]	- Barbed end capping - Barbed end capping and filament severing	Wear *et al.,* 2003 McGough *et al.*2003
- Filament binding	Abp1/drebrin[4] Aip1 tropomyosin	- Binds filaments, activates Arp2/3 - Binds filaments, activates ADF/cofilins - stabilize filaments	Goode *et al.* 2001 Mohri and Ono 2003 Blanchoin *et al.* 2001

types of multicellular organisms. Although the functional properties and structure of profilins seem to be highly conserved, sequence similarities between different profilins are surprisingly low (Witke *et al.,* 2004). The crystal-structures of profilin revealed a seven-stranded anti-parallel β-pleated sheet with two α-helices derived from N- and C-terminal sequences on the top and two other α-helices on the bottom side of the plate (for structure see Fig.6)(Schutt *et al.,* 1993, Cedergren-Zeppezauer *et al.,* 1994).

3.6.2.1. Biochemical properties of profilin

Biochemically, profilin interacts with actin monomers, preferring ATP-actin (Kd $\approx$0,1 μM) over ADP-actin (Kd $\approx$0,5 μM), polyprolines and phosphatidylinositol-(4,5)-bisphosphate (PIP_2) (Lu and Pollard, 2000, Lassing and Lindberg, 1985). Upon binding to actin monomer, many profilins accelerate the rate of nucleotide exchange by more than 1000-fold and stay bound to ATP-actin monomers (Mockrin *et al.,* 1980, Goldscmidt-Clermont 1992). Formins, a family of processive filament cappers, can use these profilin-actin complexes for barbed-end growth. Formins contain proline-rich region, which binds profilin with relatively high affinity (Suetsugu *et al.,* 1998, Vavylonis *et al.,* 2006). The ability of profilins to bind polyproline sequences has been known for long (Lindberg *et al.,* 1988). Nowadays, more than 50 new ligands for profilin have been characterized, among many formins (Witke *et al.,* 2004). The binding surface for polyproline locates in the C-and N-terminal α-helices of profilin, whereas the actin-binding site lies on the opposite side of the molecule. This suggests that profilin can bind these ligands simultaneously (Mahoney *et al.,* 1997). Profilin is also known to interact with phosphatidylinositol-(4,5)-bisphosphate (PIP_2) trough two separate binding sites. The other binding site overlaps with the actin binding site, leading to dissociation of actin in the presence of PIP_2 (Lassing and Lindberg, 1985). Other PIP_2 binding site is interlaced with polyproline binding surface and PIP_2 binding is thus also suggested to perturb interaction between profilin and proline-rich sequences (Lambrechts *et al.,* 2002, Skare and Karlsson, 2002). These functions make PIP_2 a central inhibitory element in the regulation of profilin function.

3.6.2.2. Physiological role of profilin

In all organisms studied, deletion of profilin yields a lethal or severely impaired phenotype with growth defects and visible actin disorganization. In yeast *Saccharomyces cerevisiae,* severe phenotypes in profilin null (*pfy1*Δ) and point mutant strains, having a defect in actin binding (e.g. *pfy1-4*), show the importance of profilin, and especially its actin binding property, for growth, morphology and actin organization (Hareer *et al.,* 1990, Wolven *et al.*, 2000). It also seems that an important function of yeast profilin *in vivo* is its capability to accelerate actin's nucleotide-exchange rate and promote actin turnover. This is further supported by the observation that the cellular defects of *pfy1-4* can be rescued with actin mutant having increased intrinsic rate of nucleotide exchange (*act1-15*) (Wolven *et al.,* 2000). In mammalian cells, profilins localize to the sites of rapid actin remodeling, for example to the cell periphery, but also to the nucleus and the perinuclear area (Buss *et al.,* 1992). Profilin-1 knockout mice arrest at early stages

of development suggesting an importance for profilin function also in mammalian cells (Witke *et al.,* 2001).

3.6.3. ADF/cofilin

ADF/cofilins are small (13-19 kDa) actin binding proteins. These proteins were initially identified as actin-depolymerizing factor (ADF) and cofilamentous (cofilin) agent from chick and porsine brain, respectively (Bamburg *et al.,* 1980, Maekawa *et al.,* 1984). Subsequentially, ADF/cofilin genes have been identified from all eukaryotic organisms studied (e.g. Iida *et al.,* 1993, Gunsalus *et al., 1995,* McKim *et al., 1995*). In the budding yeast, there is only one cofilin gene (*COF1*), whereas in mammals, there are three isoforms, cofilin-1, cofilin-2 and ADF expressed (Moon et al., 1993, Vartiainen *et al.,* 2002). The structure of cofilin reveals a four-stranded mixed β-sheet sandwiched between two pairs of α-helices. Loop regions combining secondary structure elements perform most of the variability between cofilin isoforms (for structure see Fig.6)(Hatanaka *et al.,* 1996, Fedorov *et al.,* 1997).

3.6.3.1. Biochemical properties of ADF/cofilins

ADF/cofilins bind both monomeric and filamentous actin, preferring ADP-actin over ATP-actin. The main function of cofilin is to depolymerize actin filaments from their pointed ends. This is a rate-limiting step in actin treadmilling. As a result actin turnover can occur 25-fold faster in the presence of ADF/cofilin *in vitro* (Carlier *et al.,* 1997). On the other hand, ADF/cofilin, when bound to monomeric actin, inhibits spontaneous nucleotide exchange and may actually, under certain concentrations, decelerate treadmilling (Hawkins *et al.,* 1993). Amino acids responsible for actin interactions form two separate binding sites around the helix number three. One of them is responsible for actin monomer binding, whereas the other one binds an adjacent actin subunit during interaction with actin filament (Lappalainen *et al.,* 1997). Binding of ADF/cofilin to F-actin changes the twist of actin filament by ~5° between two actin monomers and the resulting thermodynamic change is believed to lead to enhanced filament severing and dissociation of subunits from pointed end (McGough. *et al.,* 1997). ADF/cofilin also binds to phosphatidylinositol-(4,5)-bisphosphate (PI(4,5)P_2) through residues lying next to the actin binding surfaces, thus producing an effective negative regulation for cofilin´s actin binding (Yonezava *et al.,* 1991). As another regulatory element, ADF/cofilin harbors conserved phosphorylation site in the far N-terminus (Ser3 in animal ADF/cofilins). Phosphorylation dramatically (20-30 fold) decreases ADF/cofilin´s affinity to actin (Ressad *et al.,* 1998). ADF/cofilin have a nuclear localization sequence (NLS), but the possible role of this characteristic remains elusive (Matsuzaki *et al.,* 1988).

14-3-3 proteins form a large group of highly conserved approximately 30 kDa proteins. These dimeric proteins are widely expressed in different organisms and tissues. 14-3-3 proteins have reported to be involved in cell signaling, cell cycle progression, intracellular trafficking, cytoskeletal regulation and transcription. The interactions between these proteins with their ligands are usually highly specific and influenced by phosphorylations of the ligand and the dimerization of the 14-3-3 protein (Aitken *et al.,* 2006). Phosphorylated ADF/cofilins have been proposed to interact with a 14-3-3

protein family member 14-3-3ζ, but the physiological role of this interaction has not been studied (Birkenfeld *et al.,* 2003).

3.6.3.2. Physiological roles of ADF/cofilins

A deletion of cofilin gene from yeast *Saccharomyces cerevisiae* is lethal (Moon *et al.,* 1993). A systematic screen of point mutations in yeast *COF1* gene revealed the *in vivo* importance of functions determined biochemically. As expected, actin binding and depolymerization activities are important for yeast growth and normal actin distribution. Nevertheless, dramatic *in vivo* phenotypes of two yeast cofilin mutants (Cof1-5 and Cof1-9) cannot be explained by defects in actin binding. Thus, in addition to actin binding, also other protein interactions appear to be essential for the function of cofilin in yeast (Lappalainen *et al.,* 1997).

In cultured mouse fibroblasts, cofilin-1 localizes to the sites of rapid actin turnover for example to lamellipodia (Yonezawa *et al.,* 1987). Knocking down the cofilin-1 and/or ADF (cofilin-2 is not expressed in these cells) causes increased content of F-actin structures and disrupts actin-based functions such as cell migration and division (Hotulainen *et al.,* 2005). Regardless of the biochemical, structural and cell biological similarity of mouse ADF/cofilin isoforms, the expression patterns and knockout phenotypes reveal some diversity among these proteins in mammals. Cofilin-1 mRNA is uniformly expressed already during development at E9.5 and subsequently in all adult tissues with an exception of skeletal muscles. Cofilin-2 mRNA, in addition to being expressed in heart, liver and testis, is the only isoform expressed in adult skeletal muscles. On the other hand, mRNA of ADF is mainly found in brain and epithelial tissues during late embryonic stages and in adult mice (Vartiainen *et al.* 2002). Transgenic mice lacking cofilin-1 die at E10.5 as a result of an inability to close the neural tube (Gurniak *et al.,* 2005), whereas ADF knockout mice are viable, but develop irregular thickening of the corneal epithelium and become blind before reaching the age of one month (Ikeda *et al.,* 2003).

3.6.4. Cyclase-associated protein (CAP)

One of the essential proteins regulating actin cytoskeleton in both yeast and mammalian cells are the cyclase-associated proteins (CAPs), also called Srv2 in budding yeast (In “introduction” referred to only as CAP). These proteins were originally found from budding yeast as a suppressor of Ras allele (Fedor-Chaiken *et al.,* 1990) and in another study as a part of the adenylyl-cyclase complex (Field *et al.,* 1990). Afterwards, homologues of CAP have been identified from all organisms studied (e.g. *S.pombe*: Kawamukai *et al.,* 1992, *Dictyostelium discoideum*: Gotwald *et al.,* 1996, Mouse: Votjek and Cooper 1993, Rat: Zelicof *et al.,*1999, Pig: Gieselmann and Mann 1992, Human: Mattviw *et al.,* 1992, *Arabidopsis thaliana*: Barrero *et al.,* 2002). A second CAP isoform has also been identified from rat and humans and named CAP2 (Swiston *et al.,* 1995, Yu *et al.,* 1994, Hubberstey *et al.,* 1996). In general, CAP1 orthologues in animals share high degree (90%) of sequence identity to each other, while CAP2 proteins show only ~60% amino acid sequence similarity to CAP1 from the same species (Hubberstey and Motillo, 2002).

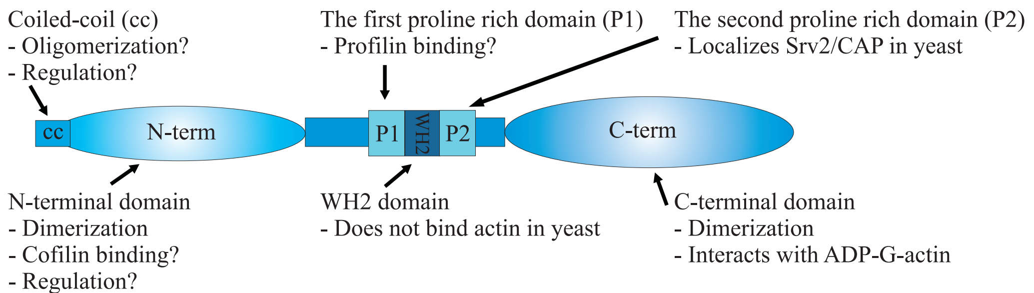

Figure 5. The domain structure of Srv2/CAP. The N-terminal end of Srv2/CAP contains the coiled coil structure, followed by the N-terminal 14-3-3 like domain. The middle region of Srv2/CAP harbors two proline-rich domains and WH2 domain between them. In the C-terminal end of Srv2/CAP locates a highly conserved ADP-G-actin binding domain. Previously suggested, but not confirmed, functions of the domains are marked in to the figure with question marks.

3.6.4.1. Biochemical properties of CAP

CAPs are 54-56 kD proteins with a highly conserved domain structure (Fig. 5). In the N-terminal end, there is a conserved coiled-coil (cc) structure known to mediate the adenylyl cyclase binding in yeast (Nishida *et al.,* 1998) the importance of which was long considered controversial (Wang *et al.,* 1995). This interaction was subsequently confirmed and CAP was suggested to form another Ras-binding site to adenylyl cyclase and mediate Ras-dependent activation (Shima *et al.,* 1999). CAP has been proposed to also function as an important link in Ras-signaling induced apoptosis in *Saccharomyces cerevisiae* (Gourlay and Ayscough 2006). Interaction with adenylyl cyclase is not conserved in mammals, most likely due to the differences between adenylyl cyclases of yeast and mammals (Hubberstey and Motillo, 2002). Although the CAP proteins have also been linked to cAMP signaling and adenylyl cyclase also in *Dictyostelium discoideum* (Noegel *et al.,* 2000, 2004), a possible interaction of Ras with mammalian CAP remains elusive.

After the coiled-coil domain follows the N-terminal domain, the function of which remains a mystery. This domain harbors the most diverse sequences in CAP proteins between the different species. The NMR structure of this domain (amino acids 51-226 in *D.discoideum* CAP) reveals six antiparallel helices (for structure see Fig.6)(Ksiazek *et al.,* 2003, Mavoungou *et al.,* 2004). Biochemical studies showed strong antiparallel dimerization of positively charged clusters on the surface of helices number 4 (Yosof *et al.* 2005, 2006). The structure of the N-terminal domain resembles 14-3-3 domain, which is known to interact with phosphoserine containing proteins like cofilin (Aitken, 2006, Birkenfield *et al.,* 2003). Biochemical studies propose that human CAP may indeed interact with cofilin through its N-terminal domain and accelerate cofilin-dependent actin turnover (Moriyama and Yahara, 2002). However, the mechanism and possible biological relevance of this interaction remains to be shown.

In the middle region of the CAP protein, there are two proline-rich regions. The first one, being highly conserved, harbors still unknown function, while the second one is responsible for correct subcellular localization of the CAP1 in yeast trough interaction

with SH3 domain of Abp1 (actin-binding protein 1). Abp1 localizes to cortical actin cytoskeleton through direct interaction with actin filaments (Freeman, 1996, Lila and Drubin, 1997, Yu *et al.,* 1999). However, Abp1 appears not to bind CAP in mammalian cells, probably due to low sequence conservation of the second proline-rich region, the factor responsible for correct sub-cellular localization of CAP in mammals remains to be identified. Studies with synthetic peptides and a high throughput yeast 2-hybrid screen suggest that CAP may also interact with profilin through its polyproline regions (Lambrech *et al.,* 1997, Drees *et al.,* 2001). Between the proline-rich regions lies the WH2 domain. In other proteins, WH2 domains are known to bind actin monomers (Paunola *et al.,* 2002), but at least in yeast Srv2/CAP, this domain does not contribute to the actin binding (Mattila *et al.,* 2004)

The highly conserved C-terminal domain of CAP binds actin monomers and strongly inhibits actin polymerization (Freeman *et al.,* 1995). The monomer sequestering activity of *Dictyostelium* CAP has also been proposed to be inhibited by PIP_2, but only in the presence of N-terminal domain (Gottwald *et al.,* 1996). The C-terminal domain of CAP prefers ADP-actin monomers (Kd 0,02-0,05 μM) over ATP-actin (Kd 1,9 μM) (Mattila *et al.,* 2004) and it has also been observed to facilitate filament elongation at the barbed end by stimulating ADP/ATP-exchange on actin monomers (Moriyama and Yahara, 2002). Crystal-structure of the domain (amino acids 370-526 of *S.cerevisiae* Srv2/CAP) revealed six coils of right-handed β-helix flanked by antiparallel β-strands. This domain forms an unusual dimer by domain swapping of the last stands of each monomer (for structure see Fig.6)(Dodatko *et al.,* 2004). A mutation analysis revealed an actin binding surface locating at the C- terminal end of the β-strand structure (Mattila *et al.,* 2004).

Most biochemical studies so far have been carried out with purified domains of CAP. Many laboratories have reported dramatic problems, aggregation and autoproteolysis, while trying to purify the full length CAP (see e.g. Ksiazek *et al.,* 2003). First insights into the function of full-length protein were reported during this thesis study. CAP was proposed to function as a multimeric complex and enhance actin turnover by recycling actin monomers and cofilin (Moriyama and Yahara, 2002, Balcer *et al.,* 2003).

3.6.4.2. Biological role of CAP

Already in the first studies concerning this protein, CAP was noticed to be important for actin organization in yeast (Fedor-Chaiken *et al.,* 1990). *Saccharomyces cerevisiae* Srv2/CAP localizes to patch-like structures that partially overlap actin patches (Freeman *et al.,* 1996). Mutants lacking Srv2/CAP displayed four phenotypes that appear to be unrelated to Ras-signaling, the inability to grow on rich medium (YPD), temperature sensitivity on minimal medium, sensitivity to nitrogen starvation, and swollen cell morphology (Field *et al.,* 1990). In light microscopy analysis, Srv2/CAP mutants also showed depolarized actin patches and diminished actin cables. The phenotype of Srv2/CAP deletion in yeast can be partially rescued by expression of profilin (Votjek *et al.,* 1991), which together with the biochemical data suggests that these two proteins may have a functional link. Loss of CAP from *Dictyostelium discoideum* results in altered cell morphology, development and motile behavior (Noegel *et al.,* 1999). Later, CAP was also found to play an important role in endocytosis of *Dictyostelium discoideum*

through direct interaction with the N-terminal half of CAP and V-ATPase (Sultana *et al.,* 2005).

CAP proteins are likely to display similar activities in plants. High functional homology is suggested by studies in which yeast CAP deletion is rescued by *Arabidopsis thaliana* CAP1 and over-expression of CAP in tobacco plants causes severe growth defects (Barrero *et al.,* 2002, 2003). Deletion of CAP from *Arabidopsis t.* causes decreased germination efficiency of pollen and actin filament disruption in the growth zone of the root hairs (Deeks *et al.,* 2007). Plant CAPs were also reported to bind actin similarly to yeast and mammalian proteins (Barrero *et al.,* 2002). Interestingly, later studies suggest that *Arabidopsis t.* CAP can promote nucleotide exchange of actin, but unlike yeast CAP, it has equal affinity to both ATP- and ADP-actin monomers (Chaudry *et al.,* 2007).

In *Drosophila,* loss of CAP results in uncontrolled actin polymerization in developing oocytes, causing problems in cell polarity and proper asymmetric distribution of mRNA determinants (Baum *et al.,* 2000). CAP has an important role also later in development, because it prevents excess actin polymerization in developing eyedisc (Benlali *et al.,* 2000). These studies suggest the role of CAP in sequestering actin monomers and preventing polymerization rather than promoting it.

In mammalian cells, CAP1 was initially observed to localize to membrane ruffles and actin stress fibers. In contrast to findings in *Drosophila* cells, microinjection of purified CAP induced formation of stress fiber-like filaments in serum starved Swiss 3T3 cells (Freeman and Field, 2000). The role of CAP in mammalian cells has not been comprehensively demonstrated and this analysis is further complicated by the presence of two isoforms. However, these isoforms may be tissue specific, because rat CAP1 and CAP2 were found to be expressed in partially different tissues by RT-PRC (Swiston *et al.,* 1995).

AIMS OF THE STUDY

CAP protein was initially identified from budding yeast in the year 1990 (Fedor- Chaiken *et al.,* 1990, Field *et al.,* 1990). However, the role of CAP in cytoskeletal dynamics has been largely unknown. In addition to G-actin, CAPs have been suggested to interact with profilin and ADF/cofilin, but the physiological roles and mechanisms of these interactions were not known.

The aims of this study were:

- To dissect the mechanisms by which CAP interacts with profilin and ADF/cofilin, as well as to map the binding sites of these proteins in CAP.

- To study the importance of profilin and ADF/cofilin interactions of CAP in yeast.

- To reveal the expression patterns and biological roles of the two mouse CAP isoforms.

- To generate a model for how CAP contributes to actin dynamics.

MATERIALS AND METHODS

The methods used in this study are listed below. The detailed descriptions can be found from original publications at in the end of this thesis. The roman numbers of the publications are indicated after the method.

Method	**Publication**
Actin binding assay (NBD-assay)	I, II
Cloning and plasmid construction	I, II, III
Culture and transfection of mammalian cells	III
FRAP (Fluorescent recovery after photobleaching)	III
Generation and affinity purification of antibodies	III
Immunofluorescence of mammalian cells	III
Labeling actin with NBD	I, II
Latrunculin A-assay	III
Light-microscopy techniques	I, III
Northern blot-analysis	III
Purification of rabbit muscle actin	I, II
Recombinant protein expression and purification	I, II, III
SDS-PAGE	I, II, III
Supernatant depletion pull-down assay	I, II
Tryptophan fluorescence assay	I
Radioactive *in situ*-hybridization	III
RNAi-methods	III
Western blotting	I, III
Yeast immunofluorescence	I
Yeast co-immunoprecipitation	I

Table 3. Yeast strains used in this study. Here are listed all phenotypes of yeast strains and features of the mutant proteins used in the original publications included in this thesis.

Name	Mutation	Phenotype	Feature	Reference
Srv2/CAP				
SRV2	None	Wild-type	Wild-type	Freeman *et al.,* 1996
Srv2Δ	Deletion	Severe actin and growth phenotype	Srv2/CAP null	Gerst *et al.,* 1991
Srv2-201	P278A, P279A, P280A	Enlarged cell size	Profilin binding	I
Srv2-90	F189A, W199A	Actin and growth phenotype	Cofilin-G-Actin binding	II
Srv2-91	R202A, L204A, K205A, E206A	Actin and growth phenotype	Cofilin-G-Actin binding	II
Srv2-92	R208A, E209A, D211A	Wild-type	Not tested	II
Srv2-93	E216A, K219A, K220A	Wild-type	Not tested	II
Srv2-94	K233A, K234A	Wild-type	Wild-type	II
Srv2-ΔCC	Deletion of residues 1-50	Actin and growth phenotype	Hexamerization	II
Srv2-Δ1-72	Deletion of residues 1-72	Actin and growth phenotype	Not tested	II
Profilin				
Pfy1-4	K66A	Conditional (25-34°C)	Actin binding	Wolven *et al.*, 2000
Pfy1-14	Y119A, Y125A	Conditional (25-34°C)	Polyproline binding	Wolven *et al.*, 2000
Cofilin				
Cof1-5	D10A, E11A	ts$^-$ (14-25°C), actin phenotype	Srv2/CAP binding*	Lappalainen *et al.,* 1999, II*
Cof1-9	D34A, K36A, E38A	Lethal	Srv2/CAP binding*	Lappalainen *et al.,* 1999, II*
Cof1-19	R109A, R110A	Wild-type	Not tested	Lappalainen *et al.,* 1999
Aip1				
Aip1Δ	Deletion	Thickened actin cables	Aip1 null	Rodal *et al.,* 1999

RESULTS AND DISCUSSION

4. Characterization of Srv2/CAP-profilin interaction (I)

Profilin is known to interact with polyproline stretches, and Srv2/CAP harbors two of them in the central region of the protein. Direct interaction between these two proteins was previously suggested in high throughput yeast-2-hybrid assays and by studies using synthetic peptides (Lambrech *et al.,* 1997, Drees *et al.,* 2001), but the mechanism and *in vivo* role of this possible interaction had not been investigated.

4.1. Srv2/CAP interacts directly with profilin.

In order to study the possible interaction between Srv2/CAP and profilin, we purified recombinant profilin and C-terminal fragment of the Srv2/CAP containing proline-rich central domains and C-terminal actin-binding domain. We carried out a series of pull-down assays and revealed that these proteins, indeed, do interact with each other *in vitro*. In order to determine the exact dissociation constant, we purified the central domain of Srv2/CAP that does not contain any tryptophans and performed quantitative tryptophan fluorescence assay with recombinant wild-type profilin. This assay revealed a Kd of 1,3 μM for the interaction between profilin and Srv/CAP. When we used a profilin mutant lacking polyproline binding site (Pfy1-14)(Wolven *et al.,* 2000) in the same assays, we did not detect any binding, which suggests that profilin interacts with a polyproline region of Srv2/CAP (Fig. 6).

In order to reveal the profilin binding site in Srv2/CAP, we carried out mutagenesis analysis on proline residues of both polyproline regions. By a series of supernatant depletion pull-down assays and tryptophan fluorescense assays of Srv2/CAP, we concluded that Srv2/CAP binds profilin through its first polyproline region. In order to ensure that mutations do not interfere with Srv2/CAP's actin binding, we carried out actin binding assays and measured the interaction parameters by fluorescence change of NBD-labeled ADP-G-actin. By replacing three prolines with alanines in this region, the affinity of Srv2/CAP to actin was retained similar to the wild type protein. We also tested whether profilin interferes with Srv2/CAP's actin binding or actin excludes profilin from Srv2/CAP. Both assays suggested that these two proteins are able to interact with Srv2/CAP simultaneously without competition.

These biochemical assays demonstrate that two highly abundant actin binding proteins, Srv2/CAP and profilin, interact directly. Previous biochemical studies show that a sequence consisting of ten prolines is optimal for profilin binding (Perelroizen *et al.,* 1994). Yeast Srv2/CAP contains nine prolines in the first (P1) and five in the second (P2) proline-rich region. In previous fluorescence spectroscopy studies, profilin was shown to interact with a dissociation constant of 5 μM with a 15-residue long synthetic peptide that corresponds to the first polyproline region (P1) of mammalian CAP (Lambrech *et al.,* 1997). By tryptophan fluorescence assay, we measured approximately four-fold higher affinity for profilin for the 120 residue long central fragment of yeast Srv2/CAP. This difference may result from the differences between mammalian and yeast proteins or more likely from the fact that also the adjacent residues around the proline-rich region of Srv2/CAP are important for binding.

In yeast Srv2/CAP, the profilin binding is located in the first proline-rich region (P1), whereas mutations in P2 had no effect (Fig. 6). The mammalian P1 region is highly conserved with yeast protein but contains higher number of prolines. (e.g. P1 of mouse CAP1 contains 14 prolines). Yeast and mammalian profilins are highly similar and the binding surface in Srv2/CAP is conserved to mammalian CAP1. This suggests interaction also between mammalian CAP and profilin. Similar series of assays could perhaps show if the interaction is conserved to mammals and lighten the comparable functions of yeast and mammalian Srv2/CAP proteins.

The second polyproline stretch (P2) mediates binding to the SH3 domain of Abp1 and regulates the localization of Srv2/CAP in yeast. In mammals, P2 region is poorly conserved and does not contain a consensus sequence for SH3-domain binding (Freeman *et al.,* 1996). As a result, the role of the P2 region in mammalian CAP and the mechanism regulating the subcellular localization of this protein remains elusive.

Our data show that Srv2/CAP can simultaneously bind both profilin and ADP-G-actin, thus suggesting a ternary complex formation. This is supported by previous studies showing the ability of profilin to bind polyproline and actin simultaneously (Mahoney *et al.,* 1997). Together these data propose that ADP-actin first binds to the high affinity site in the C-terminal region of Srv2/CAP, while profilin binds to P1 region of the Srv2/CAP and establishes a close contact with the ADP-actin monomer. At the following stage, nucleotide exchange of actin is stimulated by profilin, Srv2/CAP or by both. Because Srv2/CAP has low and profilin high affinity to ATP-G-actin, profilin receives ATP-actin monomer from Srv2/CAP and subsequently dissociates from Srv2/CAP (Witke *et al.,* 2001, Mattila *et al.,* 2004). After this, the profilin-ATP-G-actin complex can be used for dynamic growth of actin filaments promoted by for example formins (Vavylonis *et al.,* 2006). We propose that the Srv2/CAP, which binds both profilin and ADP-actin, could function as a “loading station” for profilin-ATP-actin that will be subsequently used for spatially and temporally controlled actin polymerization. In yeast cells, Srv2/CAP maintains a sufficient ATP-actin monomer pool ready for rapid actin polymerization. Thus, the loss of Srv2/CAP would result in a diminished amount of ATP-G-actin and subsequent defects in actin-based cellular functions.

4.2. *In vivo* role of the Srv2/CAP-profilin interaction

We next examined whether profilin binds Srv2/CAP also *in vivo* and whether this interaction has any physiological role in yeast cells. We generated a yeast strain *srv2-201* harboring mutations that change 3 prolines to alanines at the P1 site of Srv2/CAP. These mutations efficiently deplete profilin binding in biochemical assays without interfering with interaction with actin. Co-immunoprecipitation analysis demonstrated that direct interaction between Srv2/CAP and wild-type profilin does occur in yeast cells. Profilin was not detected in immunoprecipitations of *srv2-201* cell extracts, suggesting that P1 region of Srv2/CAP is essential for profilin interaction also *in vivo*.

In order to investigate the significance of Srv2/CAP’s profilin binding *in vivo,* we carried out cell biological analysis of the *srv2-201* yeast cells. Similarly to wild type cells, in *srv2-201* cells, mutant Srv2/CAP localizes to patch-like structures, partially

overlapping with actin patches (Freeman *et al.,* 1996). Thus, the P1 region is not important for the correct subcellular localization of Srv2/CAP. A deletion of Srv2/CAP from *Saccharomyces cerevisiae* (*srv2*Δ) shows reduced growth rates in all temperatures tested, depolarized actin patches and cables, and enlarged cell size (Gerst *et al.,* 1991, Votjek *et al.,* 1991). Importantly, *srv2-201* yeast strain did not display obvious defects in growth or actin distribution. However, *srv2-201* yeast strain showed statistically significant enlargement in cell size suggesting that Srv2/CAP-profilin interaction is more supportive than crucial in regulation of cell growth and morphology. Because of the high general importance of Srv2/CAP and profilin in actin dynamics, the direct interaction would be assumed to have more severe phenotype. The test modes for yeast strain phenotypes in laboratory conditions are quite limited. It is thus possible that specific conditions (e.g. certain types of stress), not included in this study, exist and where Srv2/CAP-profilin interaction may become more crucial and *srv2-201* strain, without a normal loading of ATP-actin-profilin monomers, could fail to tolerate it. We also tested *srv2-201* mutant yeasts for possible genetic interactions with *pfy1-4*, *cof1-19* and *aip1*Δ, each of which is synthetic lethal with *srv2*Δ. *srv2-201* did not have any additional defects when crossed with *cof1-19* and *aip1*Δ strains. However, *srv2-201* partially suppressed the phenotype caused by *pfy1-4,* suggesting specific genetic interaction between these two genes. *pfy1-4* mutant cells have greatly diminished actin cable staining, depolarized distribution of actin patches and inability to concentrate patches to the growing bud. In *pfy1-4 srv2-201* yeasts patch polarization and enrichment to the bud was partially rescued, whereas restoration of cables was not detected.

Together, these data suggest that Srv2/CAP interacts with profilin also *in vivo.* At least in yeast, profilin plays an important role by promoting nucleotide exchange of ADP-actin monomers (Wolwen *et al.,* 2000, Lu and Pollard, 2001). As a result, ATP-actin or ATP-actin-profilin complexes can be used for new rounds of actin polymerization. Reducing profilin's affinity to ATP-actin in *pfy1-4* cells results in decrease in generation of functional ATP-G-actin from ADP-G-actin (Wolven *et al.,* 2000). We propose that in yeast cells profilin interacts with P1 region of Srv2/CAP and receives the actin monomer from the high affinity actin binding site of Srv2/CAP. Pfy1-4 has reduced affinity for actin, which may result in Pfy1-4 getting stuck to the Srv2/CAP. In *srv2-201* strain, the profilin sequestering activity of Srv2/CAP is depleted and Pfy1-4 remains free to perform its role in actin dynamics more effectively, resulting in a partial rescue of the phenotype. An alternative explanation for the genetic interaction with *pfy1-4* and *srv2-201* is that interaction with mutant profilin Pfy1-4 may hinder the nucleotide exchange function of Srv2/CAP. With profilin-free Srv2-201 protein, the nucleotide exchange of actin may be more efficient and that would rescue the patch phenotype, whereas restoration of formin-nucleated cables would fail due to the reduced amount of profilin-actin complexes. To test this hypothesis, one could set up a more sensitive nucleotide exchange assay. For this assay the nucleotide exchange function of Srv2/CAP should be specifically depleted by mutagenesis from both wild-type and Srv2-201. Then one could test whether the mutant profilin Pfy1-4 changes nucleotide more effectively when bound or not bound to Srv2/CAP (which does not interfere the measurement by promoting the nucleotide exchange).

5. Characterization of Srv2/CAP-cofilin interaction (II)

The rate-limiting step of actin turnover is the dissociation of ADP-actin monomers from filament pointed ends (Pollard 1986). ADF/cofilins (referred to as cofilin henceforth) play an essential role in accelerating monomer dissociation from filaments and releasing actin monomers for new rounds of polymerization (Carlier, 1997). After dissociation from filaments, cofilin remains bound to ADP-actin, and inhibits its nucleotide exchange. Srv2/CAPs have been proposed to function as a high molecular weight complex that would play an important role in displacing cofilin from ADP-actin to sustain efficient turnover (Moriyama and Yahara, 2002, Balcer *et al.,* 2003).

5.1. Generating the Srv2/CAP-actin complex

We purified a full-length recombinant Srv2/CAP from *E.coli* cells and reconstituted a Srv2/CAP-actin complex. This approximately 600 kD complex consists of six Srv2/CAP and six ADP-actin molecules. The full-length Srv2/CAP and the N-terminal half of the protein can form a hexamer also in the absence of actin. C-terminal half and the full-length protein lacking only the N-terminal coiled-coil structure form dimers.

The reconstituted Srv2/CAP-actin complex co-migrated in gel filtration assay and had same hydrodynamic properties than the native yeast Srv2/CAP-actin complex. This complex also promoted nucleotide exchange of cofilin-bound actin in a similar manner to the native Srv2/CAP complex (Balcer *et al.,* 2003). We also revealed that full-length Srv2/CAP displays nearly identical affinities to ADP- and ATP-G-actin and effects on nucleotide exchange rates as previously shown for the C-terminal half of yeast Srv2/CAP (Balcer *et al.,* 2003, Mattila *et al.,* 2004). In Pi-release assay, in which the overall effects of Srv2/CAP to the cofilin-mediated actin turnover are detected, full-length Srv2/CAP protein and its separate domains combined together induced strong increase in actin turnover. However, separate Srv2/CAP domains alone or mutant protein lacking the coiled-coil domain displayed severe defects in promoting actin turnover.

In vivo, Srv2/CAP is suggested to form a high molecular weight complex (Balcer *et al.,* 2003). Already early co-immunoprecipitation studies showed that oligomerization or at least dimerization of Srv2/CAP occurs also *in vivo* (Hubberstey *et al.,* 1996). Studies on full length Srv2/CAP have been difficult due to problems arising from auto-proteolysis (Ksiazek *et al.,* 2003) and aggregation of recombinant CAP (Yosof *et al.,* 2006). Absence of the physiological components of the complex may also result in the unstable nature of the purified recombinant proteins. For these reasons, most studies with CAP have been carried out with purified fragments of this protein. Both C- and N-terminal (without the coiled-coil region) domains crystallize as homodimers (Dodatko *et al.,* 2004, Ksiazek *et al.,* 2003, Mavoungou *et al.,* 2004). In our assays, C-terminal domain was a dimer, whereas full-length proteins form a hexamer. These data suggest that in an intact hexameric complex, N- and C-terminal domains interact with only one other domain, thus forming a homodimer, whereas hexamerization occurs through the coiled coil region (Fig. 6). Interestingly, in the structure of N-terminal CAP (Fig 6.) the C-and N- terminal ends of the amino acid chain point to the same direction, which is controversial to the model we suggest. However, both coiled-coil and the central domain missing from the structure are so long that they could easily reach to the orientation

proposed in our model. In future, it would be very informative to solve the high resolution structure of the full-length CAP and the intact hexamer.

Our analysis also suggests that hexamerization of Srv2/CAP alters the biochemical properties of this protein. The C-terminal half of Srv2/CAP has similar nucleotide-exchange and ADP-actin binding properties as the full-length protein, regardless of the different oligomerization state. However, in Pi release assay, only a full-length protein or N- and C-terminal domain combined together promoted a concentration-dependent enhancement of cofilin-induced actin turnover. The separate domains alone or the full-length protein lacking coiled-coil domain required for hexamerization displayed severe defects in actin dynamics. Pi-release assay does not only measure a separate protein function such as actin-binding or nucleotide-exchange assays, but reflects the function of this protein in more comprehensive way. Together these data suggest that both functional C- and N-terminal domains of Srv2/CAP as well as its hexamerization are needed for optimal enhancement of cofilin-induced actin turnover *in vitro*.

5.2. Yeast Srv2/CAP interacts with cofilin *in vitro*

Earlier studies suggested that mammalian CAP interacts with cofilin-actin complex (Moriyma and Yahara 2002), but the mechanism and cellular role of this interaction were not known. By supernatant depletion pull-down assay, we demonstrated that yeast cofilin binds to full-length yeast Srv2/CAP, but only in the presence of ADP-actin monomers. In order to rule out the possibility of this interaction occurring through actin, we also repeated the experiment with the N-terminal half of the Srv2/CAP, which does not bind actin. Also this domain efficiently interacted with cofilin-actin. The N-terminal region of Srv2/CAP harbors a 14-3-3-like domain that in other proteins is capable to bind cofilin (Birkenfield *et al., 2003*). In order to map the cofilin binding site of Srv2/CAP, we introduced several mutations to the 14-3-3-like domain of yeast Srv2/CAP. Two of the mutants, Srv2-90 and Srv2-91, showed clear defect in an interaction with cofilin-actin complex. This data clearly points the interaction site to the conserved surface residues depleted from mutants Srv2-90 and Srv2-91 (Fig. 6). Furthermore, in a Pi-release assay the mutants Srv2-90 and Srv2-91 did not enhance cofilin-mediated actin turnover.

Next, we wanted to map the Srv2/CAP binding site of cofilin. Previous systematic mutagenesis of yeast cofilin identified two mutants, Cof1-5 and Cof1-9, which display severe phenotypes in yeast, but do not show defects in actin interactions *in vitro* (Lappalainen *et al.,* 1997). Therefore, we tested whether these two mutants display defects in Srv2/CAP binding. In pull-down assays Cof1-5 and Cof1-9 failed to interact with Srv2/CAP even in the presence of ADP-actin. We also learned that addition of the N-terminal domain of Srv2/CAP increased affinity of cofilin to ADP-actin, indicating formation of a ternary complex. Importantly, Cof1-5 and Cof1-9 bound to ADP-G-actin with normal affinity even in the presence of excess of N-Srv2. These data provide further support for the model that the Srv2/CAP binding site lies in the surface residues mutated in Cof1-5 and Cof1-9 (Fig. 6).

In early studies, Srv2/CAP was proposed to sequester actin monomers and suppress actin polymerization (Freeman *et al.,* 1995, Gieselmann and Mann 1992, Hubberstey *et al.,* 1996, Zelicof *et al.,* 1996). However, more recent studies suggested that Srv2/CAP instead plays an important role in accelerating cofilin mediated actin turnover

(Balcer *et al.,* 2003, III). Identification of a direct interaction between yeast Srv2/CAP and cofilin-actin provides further support to this. Importantly, without ADP-actin, Srv2/CAP is able to bind cofilin only marginally. This confirms that only actin-bound cofilin is delivered to Srv2/CAP and enhances recycling of cofilin for new depolymerization events, while the ADP-G-actin is transferred to high affinity C-terminal domain of Srv2/CAP (Mattila *et al.,* 2004). The mutants that were defective in cofilin-binding also failed to accelerate cofilin-induced actin turnover in the context of the full-length Srv2/CAP, suggesting that direct interaction between Srv2/CAP and cofilin is required to efficiently deliver ADP-actin monomers from cofilin to the high affinity actin binding site at the C-terminal region of Srv2/CAP. This function could provide an effective way of regulation. If cells could, for example, inhibit the interaction between Srv2/CAP and cofilin by phosphorylation, this could lead to a reduced depolymerization rates of actin. But this needs further studies with signal cascades regulating the function of Srv2/CAP.

The important role of Srv2/CAP-cofilin interaction is further supported by strong growth phenotypes of *cof1-5* and *cof1-9* mutants (Lappalainen *et al.,* 1997), which in our assays failed to interact with Srv/CAP *in vitro.* In a three-dimensional structure, the residues mutated in Cof1-5 and Cof1-9 are located next to the G-actin binding site of cofilin, but do not overlap with it. In the three-dimensional model of the cofilin-actin complex (Wriggers *et al.,* 1998), the residues important for Srv2/CAP interaction are optimally located to mediate binding to other proteins. These residues also do not overlap with the F-actin binding site of cofilin, but the possible interaction of Srv2/CAP with the cofilin-F-actin complex needs to be studied. To test this, one could set up a high speed co-sedimentation assay and see if the Srv2/CAP also binds cofilin/ADP-F-actin. This should also be tested with mammalian CAP. The localization signal of mammalian CAP is currently unknown. Binding to F-actin bound cofilin would perhaps localize CAP to sites of rapid actin turnover similarly to yeast Abp1 mediated localization. This needs further investigation.

5.3. Interaction of Srv2/CAP with cofilin *in vivo*

In a clear correlation to *in vitro* studies, mutant strains *srv2-90* and *srv2-91* showed similar phenotype to *srv2*Δ, whereas *srv2-94,* which did not affect cofilin binding *in vitro,* was indistinguishable from wild type cells. As an independent assay *SRV2, srv2-90, srv2-91* and *srv2-94* were crossed to *pfy1-4* and *cof1-19*, both synthetically lethal with *srv2*Δ (Wolven *et al.* 2000, Balcer *et al.,* 2003). *srv2-90* and *srv2-91* showed strong synthetic phenotypes with both alleles, interaction with *pfy1-4* being more severe. *srv2-94* showed unaltered phenotype in these genetic backgrounds, again suggesting *srv2-94* strain to be comparable to wild-type strain.

Deletion of *SRV2* gene from *Saccharomyces cerevisiae* results in a severe phenotype with enlarged cell size, loss of visualized actin cables and unpolarized patch distribution (Fedor-Chaiken *et al.,* 1990, Field *et al.,* 1990). This phenotype reveals the importance of Srv2/CAP in yeast actin dynamics. By mutating the previously mentioned cofilin-actin binding site from Srv2/CAP (*srv2-90* and *srv2-91*) the phenotype is almost as severe. Depletion of the Srv2/CAP binding site from cofilin (*cof1-5* and *cof1-9)* also results in obvious actin phenotype (Lappalainen and Drubin, 1997 and Lappalainen *et al.,* 1997).

Together, these data suggest that the recycling of cofilin is crucial for the function of the Srv2/CAP in yeast cells. In the future, it remains to be studied whether this interaction is also important in CAP proteins of other organisms. In cultured mammalian cells this could be studied by depleting the endogenous CAP by RNAi and replacing it by transfecting specifically mutated CAP constructs to the cells.

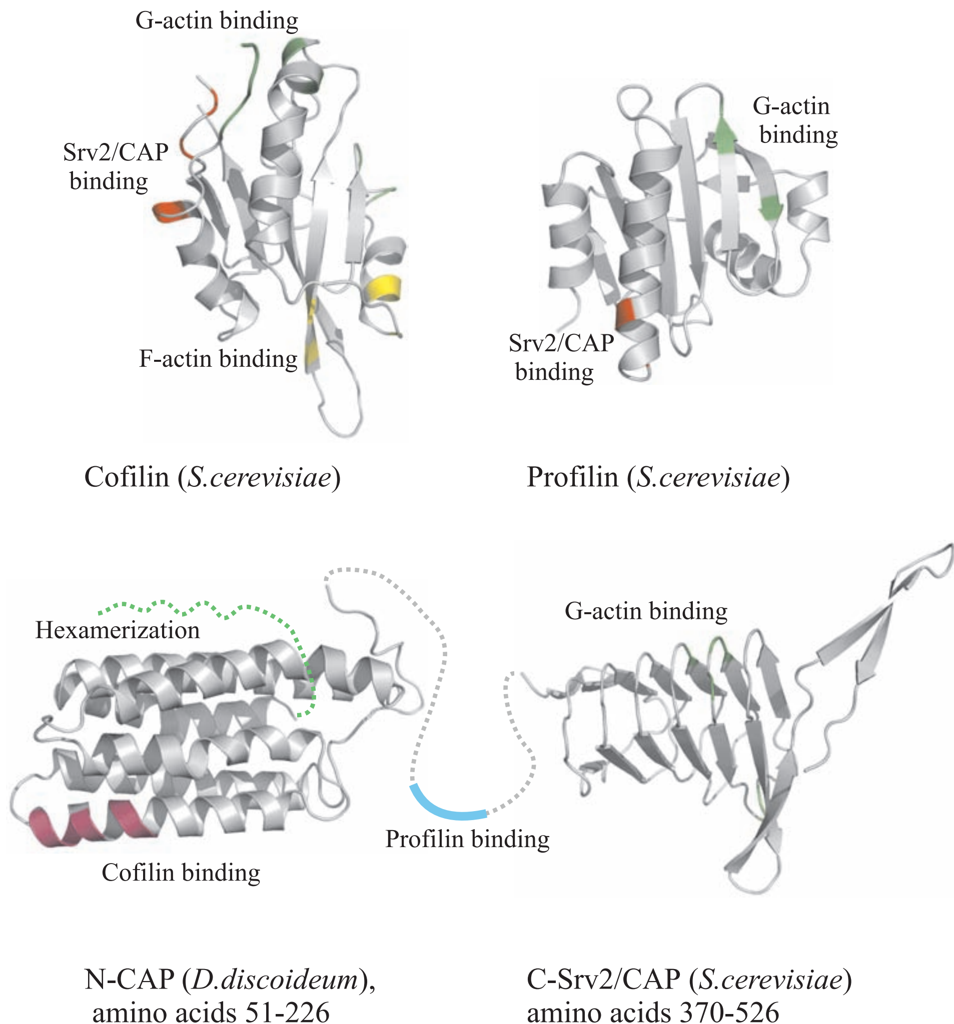

Figure 6. Structures of profilin, cofilin and CAP. Interaction sites are indicated with colours, green: G-actin binding, yellow: F-actin binding, red: Srv2/CAP binding, purple: cofilin binding and blue: profilin binding. Dashed lines show currently unsolved structural domains of Srv2/CAP. The coordinates for yeast cofilin, profilin, C-terminal Srv2/CAP and *Dictyostelium discoideum* N-terminal CAP were obtained from Wriggers *et al*., 1998, Eads *et al.,* 1998, Mavoungou *et al.,* 2004 and Dodatko *et al.,* 2004.

6. Role of the CAP in mammalian cells (III)

CAP proteins have been characterized in all eukaryotic organisms studied. Yeasts and other single cell organisms have only one isoform of CAP, whereas all mammals studied so far have two isoforms (Yu *et al.,* 1994, Swiston *et al.* 1995). Possible differences in expression patterns of CAP isoforms in mammalian tissues have been studied only by RT-PCR method (Swiston *et al.* 1995). Also subcellular localization of CAP in mammalian cells and its contribution to actin dynamics have remained elusive.

6.1. Identification of two mouse CAP isoforms

It has been reported that at least humans and rats have two different isoforms of CAP (Yu *et al.,* 1994, Swiston *et al.,* 1995). We wanted to identify the other CAP isoform from mice and to further investigate possible differences in their expression patterns. By performing sequence database searches we found mouse CAP2, which is approximately 60% identical to the previously identified mouse CAP1 (Votjek and Cooper, 1993). We carried out northern blot and *in situ*-hybridization assays to elucidate the expression of these two isoforms in different tissues. CAP1 mRNA was expressed widely during all stages of embryogenesis and in all adult tissues except in skeletal muscles. The *in situ*-hybridizations also provided evidence that CAP1 mRNA is the major isoform in most tissues. CAP2 mRNA signal was weak in samples trough embryogenesis, but was strongly expressed in adult heart, brain and skeletal muscles, being also the only isoform in adult striated skeletal muscles. This was further supported by *in situ*-hybridization analysis showing that CAP2 expression is restricted to developing myotubes and to adult skeletal muscles. In adult mice, CAP2 was also expressed in certain areas of the brain distinct from those in which CAP1 is expressed. We also generated specific, non-cross reacting antibodies against CAP1 and CAP2, and learned that CAP2 was not expressed in any of the non-muscle cell lines tested. On the other hand, CAP1 was highly abundant, being expressed at levels equal to those of cofilin-1 in NIH3T3- and B16F1-cells. In these cells, CAP1 localized diffusely to cytoplasm, but also clearly concentrated to actin-rich membrane-ruffles and showed a uniform staining in lamellipodia of migrating fibroblasts. In both NIH3T3 and B16F1 cells, CAP1 also colocalized with cofilin-1 antibody staining.

Together these studies revealed that at least one CAP isoform is expressed in all tissues during embryogenesis and in adult mice, suggesting that CAP is a central regulator of actin dynamics in all cell-types and stages of development. Interestingly, CAP2 is expressed specifically in striated muscles. In muscle cells, majority of actin is organized with motor protein myosin into highly stabile structures called sarcomeres, and the fraction of actin maintaining other cellular functions is much smaller than in non-muscle cells (Eddinger and Meer, 2007). We propose, that in order to maintain the actin dynamics in this highly specialized cell-type, muscle-specific CAP isoform has evolved. Same phenomenon has also been observed with other actin binding proteins such as ADF/cofilin, twinfilin and capping protein (Ono *et al.,* 1994, Vartiainen *et al.,* 2003, Schafer *et al.,* 1994,). Whereas the muscle-specific ADF/cofilin isoform, cofilin-2, is known to promote actin filament turnover less efficiently than non-muscle isoforms

(Vartiainen *et al.* 2002), the biochemical differences of CAP isoforms have not been examined. All functional domains identified from Srv2/CAP (I, II, Mattila *et al.,* 2004) appear to be conserved in CAP1 and CAP2, suggesting at least similar functions, whereas the dynamics or regulatory steps can be different between these isoforms. A recent study demonstrated that CAP2 has a functional actin binding site like CAP1, and proposed that CAP2 is localized in the nuclei of undifferentiated myoblasts and to M-line in mature myotubes (Peche *et al.,* 2007). CAP2 was also reported to be upregulated in hepatocellular carsinoma in humans, but the cell biology behind this finding remains elusive (Shibata *et al.,* 2006).

In non-muscle cells, CAP1 localizes to cell periphery at the sites of rapid actin turnover. Similar localization to cortical cytoskeleton was previously reported for *Dictyostelium* CAP (Noegel *et al.,* 1999). According to previous reports, mammalian CAP1 localizes to the sites of rapid actin turnover (Moriyama and Yahara, 2002) and to stress fibers (Freeman and Field, 2000). However, we only detected a very faint localization of CAP1 to stress fibers in a fraction of NIH3T3 cells. In yeast, Srv2/CAP localizes partially to actin patches, which are sites of rapid actin dynamics similar to lamellipodia in mammalian cells (Freeman *et al.,* 1996). In mammalian non-muscle cell, the uniform lamellipodial staining observed here for CAP1 is common for many proteins involved in rapid remodeling of actin structures, such as Arp2/3 and twinfilin (Svitkina and Borisy, 1999, Vartiainen *et al.,* 2000). Unlike cofilin-1 (Yonezawa *et al.,* 1987), CAP1 did not display localization to nucleus. However, clear colocalization between CAP1 and cofilin-1 in lamellipodia in all cell-types tested suggest a functional link between these proteins also in mammalian cells.

6.2. The role of CAP1 in mammalian non-muscle cells

We examined the role of CAP1 in B16F1 and NIH3T3 cells by depleting it using RNAi-method (Elbashir *et al.* 2001). The cells were transfected with fluorescein-labelled RNAi-oligonucleotides to distinguish RNAi transfected cells from non-transfected ones. Immunofluorescence analysis showed that the CAP1 RNAi transfected cells did not contain detectable levels of CAP1. The knock-down cells exhibited slightly enlarged and depolarized morphology and contained abnormal accumulation of F-actin structures. In order to further investigate the effects of CAP1 knock-down, we conducted an analysis on cell migration, endocytosis and actin turnover. CAP1 knock-down cells were generally unable to migrate directionally. Lamellipodial ruffles were motile, but the migration speed was decreased to half as compared to wild-type. Also receptor-mediated endocytosis of transferrin was decreased to 40% in knock-down cells. Uptaken transferrin was also diffusely distributed around the cell, in comparison to perinuclear localization typical to wild-type cells.

In order to study the actin turnover rates in CAP1 knock-down cells, we used FRAP (Yoon *et al.,* 2002). After bleaching the stress-fibers in GFP-actin expressing cells, the recovery was monitored by live cell imaging. Recovery rates were clearly decreased in CAP1 depleted cells suggesting diminished actin turnover rates. To further elucidate the reason for reduced actin turnover, we tested the actin depolymerization rates in wild-type and CAP knockdown cells by Latrurunculin-A based assay (Lappalainen and Drubin,

1997). Latrunculin-A sequesters monomers dissociated from filaments and prevents their addition to growing ends (Coue *et al.,* 1987). After the Latrunculin-A treatment, actin filaments were rapidly lost from wild-type cells, whereas in the CAP1 knock-down cells, F-actin structures disappeared more slowly. These data suggest that CAP1 promotes actin filament depolymerization in mammalian cells. Interestingly, after CAP1 knock-down the major ADF/cofilin isoform in these cells, cofilin-1, was lost from its normal localization and aggregated to cytoplasm together with G-actin.

Similar actin phenotypes with abnormal F-actin structures as revealed for CAP1 knock-down cells have also been observed in *Drosophila* and *Dictyostelium* CAP mutants (Noegel *et al.,* 1999, Baum *et al.,* 2000, Benlali *et al.,* 2000). CAP1 knock-down cells had also a defect in receptor-mediated endocytosis. Vesicle trafficking is known to be dependent on actin organization in mammalian cells (Fujimoto *et al.,* 2000), and Srv2/CAP is linked to vesicle trafficking in yeast through synthetic lethality with the endocytic protein Sla2 (Lila and Drubin 1997). In theory, thick disorganized actin fibers filling the cytoplasm of the CAP1 knock-down cells may have a physical influence in endocytosis without a direct link between CAP1 and any compartment of endocytic pathway. Nevertheless, recent findings have linked *Dictyostelium discoideum* CAP directly to the dynamics of endo-lysosomal system (Sultana *et al.,* 2005).

Interestingly, the actin phenotype of CAP1 knock-down cells clearly resembles to the ones reported for ADF/cofilin knock-down in the same cell lines (Hotulainen *et al.,* 2005). Similar phenotype was also detected from cells in which ADF/cofilins were phosphorylated by over-expression of LIM-kinase. Phosphorylation effectively inactivates ADF/cofilin leading to an accumulation of actin filaments in the cells (Arber *et al.,* 1998, Yonezava *et al.,* 1991). In CAP1 knock-down cells, cofilin-1 aggregates with actin to abnormal cytoplasmic structures and thus loses its normal activity. Our recent findings with yeast cofilin and Srv2/CAP proteins indicate that Srv2/CAP releases cofilin from newly depolymerized ADP-actin (II). The fact that protein domains with relevance to this function are highly conserved in all Srv2/CAP proteins suggest that this function is probably also conserved in both mammalian CAPs (Moriyama and Yahara, 2002). CAP1 and CAP2 are highly similar proteins. CAP2 may have a similar role in recycling muscle specific ADF/cofilin isoform, cofilin-2, in muscle cells. Mutations in cofilin-2 gene in humans are known to cause a nemaline myopathy. Reduced depolymerization rate of actin filaments by cofilin-2 mutant causes accumulation of rod-like actin-rich structures called “nemaline bodies” to the muscles of the patients (Pankaj et al., 2007). Loss of CAP2 could also reduce the depolymerization rates of cofilin-2 and cause similar, but probably milder, defects, regarding to the phenotypes detected in cofilin-1 and CAP1 deficient cells.

Thus, we propose that in cells lacking functional CAP protein, cofilin accumulates with ADP-G-actin, which results in similar phenotype as seen in cells lacking ADF/cofilin. When functional CAP1 is present, cofilin-1 is effectively recycled to new rounds of depolymerization and ADP-actin monomers are funneled to profilin and subsequently to growing actin filament ends. These functions increase the overall actin treadmilling rates and affect cells ability to remodel its actin cytoskeleton and maintain directional migration.

7. Model: Role of Srv2/CAP in actin turnover

Based on present results, we suggest a model for the function of Srv2/CAP in cells. A schematic picture of how Srv2/CAP contributes to remodeling of the actin cytoskeleton by accelerating turnover rate or in more detail, funnels actin monomers from pointed end depolymerizing factor cofilin to profilin and further to barbed end growth, is shown in Figure 7. For clarity only one molecule of Srv2/CAP1 is drawn, although our studies show that hexamerization of Srv2/CAP further optimizes its function.

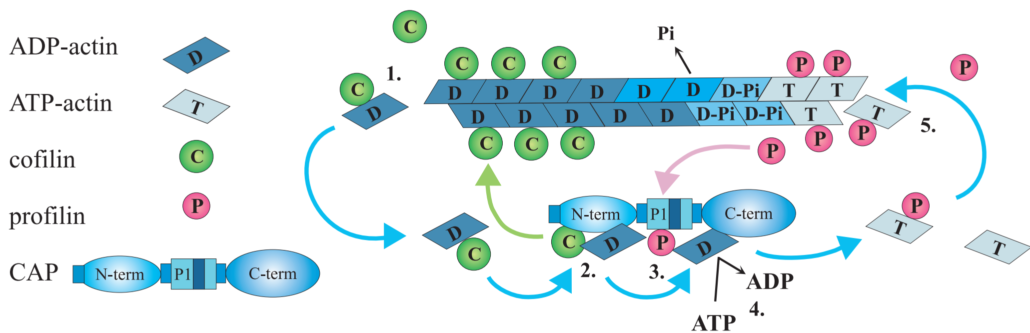

Figure 7. Model: The mechanism of CAP-function in actin turnover.

1. Cofilin binds to the ADP-actin in the filament. Cofilin twists the filament and promotes breakage and subunit dissociation. Cofilin remains bound to the ADP-actin monomer and strongly inhibits nucleotide exchange.
2. Cofilin-ADP-G-actin complex binds to the N-terminal 14-3-3 like domain of Srv2/CAP and forms a three-protein complex. In budding yeast, Srv2/CAP localizes to F-actin structures via interaction with Abp1, a protein binding filamentous actin. In mammalian cells, this interaction is not conserved, and currently unknown interaction localizes CAP1 to the sites of rapid actin turnover. For clarity reasons, Abp1 is excluded from the picture.
3. ADP-G-actin is transferred from cofilin to the high-affinity binding site of the C-terminal Srv2/CAP. Another alternative is that the nucleotide of actin is exchanged when the monomer is still bound to cofilin. In any case, the correct three-dimensional structure and close proximity of N- and C-terminal domains of either same or adjacent Srv2/CAP subunits in the hexamer is needed for the function. Due to the low affinity of N-terminal domain of Srv2/CAP to actin-free cofilin, it probably dissociates rapidly from Srv2/CAP. This releases cofilin for new rounds of actin filament depolymerization. In yeast cells deletion of the interaction site needed for cofilin-actin-Srv2/CAP complex either from cofilin (*cof1-5, cof1-9*) or Srv2/CAP (*srv2-90, srv2-91*) results in severe actin disorganization similar to deletion of Srv2/CAP. In mammalian non-muscle cells, from which CAP1, the only isoform expressed, is depleted, cofilin is not efficiently released from ADP-G-actin and it forms abnormal aggregates with actin at the cytoplasm. This results in reduced actin filament turnover rates and accumulation on F-actin structures inside the cell.
4. Profilin binds to the first polyproline-rich region of Srv2/CAP while the ADP-actin monomer is bound to the C-terminal actin-binding site. Both profilin and Srv2/CAP can promote nucleotide exchange of G-actin, but this possibly synergistic function needs further investigation. Nevertheless, actin is converted efficiently into ATP-G-actin form. Because Srv2/CAP has much lower affinity to ATP- than to ADP-actin monomers and conversely profilin binds ATP-G-actin with high affinity, the monomer is transferred to profilin. If the interaction between these two proteins is excluded (*srv2-201)*, the actin monomer can still be transferred to profilin or the nucleotide of ADP-actin are exchanged by Srv2/CAP, providing adequate amount of ATP-actin monomers in cells. Thus, under normal growth conditions, a loss of this function causes only a mild enlargement of cell size.
5. Profilin-ATP-actin complex is released from Srv2/CAP and it is now free to be added into growing barbed ends of the filaments.

FUTURE PROSPECTS

This study demonstrates that CAP proteins play an important role in accelerating actin filament turnover in both yeast and mammalian cells. We propose that CAP binds newly depolymerized cofilin-ADP-G-actin complexes and assists the release of cofilin to further perform its function. CAP also promotes nucleotide exchange of actin and thus charges profilin-ATP-G-actin complexes for subsequent rounds of filament polymerization. However, several steps of this model need further investigation to reveal the exact mechanism by which CAP promotes actin dynamics.

An interesting result from these studies is the fact that hexamerization is required for optimal function of CAP proteins. In future, it will be important to obtain structural data of the complex, in order to understand why hexamerization is important for the function of CAP. CAP is also likely to harbor several binding sites for currently unknown ligands. These could be, for example, members of Ras-signaling pathway, as suggested in studies with yeast. In order to examine this, one could carry out pull-down assays to identify CAP's interaction partners from the cell lysate and identify the co-purifying partners by mass-spectrometry. Identification of these binding partners that could, for example, regulate the localization of mammalian CAPs would bring important new information about the protein. It is also important to note that there are some differences in the actin dynamics between yeast and mammalian systems. Although the core functions of the CAP seem to be well conserved, the possible differences between mammalian and yeast CAPs remain to be studied. Also the specific functions of the CAP2, developed to fulfill the needs of muscle cells, will be important to examine. These assays require purification of similar mammalian recombinant proteins, both CAP1 and CAP2, and setup a similar series of experiments as carried out in this study for yeast Srv2/CAP.

Very little is known of the pathways regulating the activity of Srv2/CAP proteins. As in the cases of many other actin binding proteins, phosphorylaton and interactions with PIP_2 may regulate CAPs. Thus, the possible phosphorylation status and phosphorylation sites of CAPs should be identified. Furthermore, the possible PIP_2 binding of CAPs should be confirmed by fluorometric methods and vesicle pull-down assays. Also studies of currently unknown post-translational modifications of Srv2/CAP proteins should be tested. Adding these regulatory mechanisms to the model would lead us closer in understanding the exact role of CAP in actin dynamics.

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REFERENCES

Adams, A. E. and Pringle, J. R. 1984. Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. *J. Cell Biol.* 98:934-945.

Aitken, A. 2006. 14-3-3 proteins: A historic overview. Review. *Seminars in Cancer Biology.* 16:162-172.

Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O. and Caroni, P. 1998. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature.* 393:805 – 809.

Balcer, H. I., Goodman, A. L., Rodal, A. A., Smith, E., Kugler, J., Heuser, J. E. and Goode, B. L. 2003. Coordinated regulation of actin filament turnover by a high-molecular-weight Srv2/CAP complex, cofilin, profilin, and Aip1. *Curr. Biol.* 13:2159-2169.

Bamburg, J.R., Harris, H.E. and Weeds, A.G. 1980. Partial purification and characterization of an actin depolymerizing factor factor from brain. *FEBS Letters.* 121(1):178-182.

Bamburg, J.R. 1999. Proteins of the ADF/cofilin family: Essential Regulators of Actin Dynamics. Review. *Annu. Rev. Cell Dev. Biol.* 15:185-230.

Bamburg, J.R. and Wiggan, O.P. 2002. ADF/Cofilin and actin dynamics in disease. Review. *Trends in Cell Biol.* 12:598-605

Barrero, R.A., Umeda, M., Yamamura, S. and Uchimiya, H. 2002. *Arabidopsis* CAP regulates the actin cytoskeleton necessary for plant cell elongation and division. *The Plant Cell*. 14:149-163.

Barrero, R.A., Umeda, M., Yamamura, S. and Uchimiya, H. 2003. Over-expression of *Arabidopsis* CAP causes decreased cell expansion leading to organ size reduction in transgenic tobacco plants. *Annals of Botany.* 91:599-603.

Barry, D.M., Millecamps, S., Julien, J.P. and Garcia, M.L. 2007. New movements in neurofilament transport, turnover and disease. Review. *Exp. Cell Res*. 313(10):2110-20.

Baum, B., Li, W. and Perrimon, N. 2000. A cyclase-associated protein regulates actin and cell polarity during *Drosophila* oogenesis and in yeast. *Curr. Biol*. 10:964-973.

Bear, J.E., Svitkina, T.M., Krause, M., Schafer, D.A., Loureiro, J.J., Strasser, G.A., Maly, I.V., Chaga, O.Y., Cooper, J.A., Borisy, G.G. and Gertler, F.B. 2002. Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. *Cell.* 109:509-521.

Benlali, A., Draskovic, I., Hazelett, D. and Treisman, K. 2000. Act up controls actin polymerization to alter cell shape and restrict Hedgehog signaling in the *Drosophila* eye disc. *Cell*. 101:271-281.

Bhat, K.M. and Setaluri, V. 2007. Microtubule-associated proteins as targets in cancer chemotherapy. Review. *Clin. Cancer Res*. 15;13(10):2849-54.

Bi, E., Maddox, P., Lew, D.J., Salmon, E.D., McMillan, J.N., Yeh, E. and Pringle, J.R. 1998. Involvement of an actomyosin contractile ring in *Saccharomyces serevisiae* cytokinesis. *J. Cell Biol.* 142:1301-1304.

Birkenfield, J., Betz, H. and Roth, D. 2003. Identification of cofilin and LIM-domain-containing protein kinase 1 as a novel interaction partners of 14-3-3ζ. *Biochem. J.* 369:45-54.

Blanchoin, L., Pollard, T.D. and Hitchcock-DeGregori, S.E. 2001. Inhibition of the Arp2/3 complex-nucleated actin polymerization and branch formation by tropomyosins. *Curr. Biol.* 11:1300-1304.

Bretscher, A. 1999. Regulation of cortical structure by ezrin-radixin-moesin protein family. *Curr. Opin. Cell. Biol.* 11:109-116.

Bretscher, A. 2003. Polarized growth and organelle segregation in yeast: the tracks, motors, and receptors. *J. Cell Biol.* 160:811-6.

Buss, F., Temm-Grove, C., Henning, S. and Jockusch, B.M. 1992. Distribution of profilin in fibroblasts correlates with the presence of highly dynamic actin filaments. *Cell Motil. Cytoskeleton.* 37(2):51-61.

Caviston, J.P. and Holzbaur, E.L. 2006. Microtubule motors at the intersection of trafficking and transport. Review. *Trends Cell Biol*. 16(10):530-7.

Carlier, M.F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G.X., Hong, Y., Chua, N.H. and Pantaloni, D. 1997. Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. *J. Cell Biol.* 136:1307-1322.

Carlsson, L., Nyström, L. E., Sundkvist, I., Markey, F. and Lindberg, U. 1977. Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. *J. Mol. Biol.* 115:465-83.

Cedergren-Zeppezauer, E.S., Goonesekere, N.C., Rozycki, M.D., Myslik, J.C., Dauter, Z., Lindberg, U. and Schutt, C.E. 1994. Crystallization and structure determination of bovine profilin at 2.0 Å resolution. *J. Mol. Biol.* 240(5):459-475.

Chaudhry, F., Guérin, C., von Witsch, M., Blanchoin, L. and Staiger, C. 2007. Identification of *Arabidopsis* cyclase-associated protein 1 as the first nucleotide exchange factor for plant actin. *Mol. Biol. Cell.* 18:3002-3014.

Cheeseman, I.M. and Desai, A. 2008. Molecular architecture of the kinetochore-microtubule interface. Review. *Mol. Cell Biol.* 9:33-46.

Cooley, L., Verheyen, E. and Ayers, K. 1992. Chikadee encodes a profilin required for intercellular cytoplasm transport during *Drosophila* oogenesis. *Cell.* 69(1):173-184.

Coue, M., Brenner, S.L., Spector, I., and Korn, E.D. 1987. Inhibition of actin polymerization by latrunculin A. *FEBS Lett.* 213:316-318.

Coulombe, P.A. and Omary, M.B. 2002. 'Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. *Curr. Opin. Cell Biol*. 14(1):110-22.

Coulombe, P.A. and Wong, P. 2004. Cytoplasmic intermediate filaments revealed as dynamic and multipurpose scaffolds. *Nat. Cell Biol.* 6:699-706

Deeks, M.J., Rodrigues, C., Dimmock, S., Ketelaar, T., Maciver, S.K., Malhó, R. and Hussey, P.J. 2007. *Arabodopsis* CAP1 -a key regulator of actin organization and development. *J. Cell. Sci.* 120:2609-2618.

Dodatko, T., Fedorov, A.A., Grynberg, M., Patskovsky, Y., Rozwarski, D.A., Jaroszewski, L., Aronoff-Spencer, E., Kondraskina, E., Irving, T., Godzik, A. and Almo, S.C. 2004. Crystal structure of the actin binding domain of the cyclase-associated protein. *Biochemistry*. 43:10628-10641.

Dominguez, R. 2004. Actin-binding proteins- A unifying hypotesis. *Trends biochem. Sci.* 29(11):572-578.

Drees, B., Sundin, B., Brazeau, E., Caviston, J., Guang-Chaou, C., Wei, G., Korminski, K., Lau, M., Moskow, J., Tong, A., Sckenkman, L., McKenzie, A., Brennwald, P., Longtine, M., Bi, E.,

Chan, C., Novick, P., Boone, C., Pringle, J., Davis, T., Fields, S. and Drubin, D.G. 2001. A protein interaction map for cell polarity development. *J. Cell Biol*. 3:549-571.

Eads, J.C., Mahoney, N.M., Vorobiev, S., Bresnick, A.R., Wen, K.K., Rubenstein, P.A., Haarer, B.K. and Almo, S.C. 1998. Structure determination and characterization of *Saccharomyces cerevisiae* profilin. *Biochemistr*y. 11;37(32):11171-81.

Eddinger, T.J. and Meer, D.P. 2007. Myosin II isoforms in smooth muscle: heterogeneity and function. Review. *Am. J. Physiol. Cell Physiol*. 293(2):C493-508.

Edlund, M., Lotano, M.A. and Otey, C.A. 2001. Dynamics of alpha-actinin in focal adhesions and stress fibers visualized with alpha-actinin-green fluorescent protein. *Cell Motil. Cytoskeleton.* 48:190-200.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* 411:494-498.

Evangelista, M., Pruyne, D., Amberg, D.C., Boone, C. and Bretscher, A. 2002. Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. *Nat. Cell Biol.* 4:260-269.

Fedor-Chaiken, M., Deschenes, R.J. and Broach, J.R. 1990. *SRV2*, a gene required for RAS activation of adenylate cyclase in yeast. *Cell.* 61:329-340.

Fedorov, A.A., Lappalainen, P., Fedorov, E.V., Drubin, D.G. and Almo, S.C. 1997. Structure determination of yeast cofilin. *Nat. Struct. Biol*. 4(5):366-9.

Field, J., Vojtek, A., Ballester, R., Bolger, G., Colicelli, J., Ferguson, K., Gerst, J., Kataoka, T., Michaeli, T., Powers, M., Riggs, L., Rodgers, L., Wieland, I., Wheland, B. and Wigler, M. 1990. Cloning and characterization of *CAP*, the *S.cerevisiae* gene encoding the 70 kd adenylyl-cyclase associated protein. *Cell.* 61:319-327.

Fuchs, E. and Weber, K. 1994. Intermediate filaments: structure, dynamics, function, and disease. Review. *Annu. Rev. Biochem*. 63:345-82.

Freeman, N. and Field, J. 2000. Mammalian homolog of the yeast cyclase-associated protein, Srv2/CAP, regulates actin filament assembly. *Cell Motil. Cytoskeleton.* 45:106-120.

Freeman, N.L., Chen, Z., Horenstein, J., Weber, A. and Field, J. 1995. An actin monomer binding activity localizes to the carboxyl-terminal half of the *Saccharomyces cerevisiae* cyclase-associated protein. *J. Biol. Chem.* 270:5680-5685.

Freeman, N.L., Lila, T., Mintzer, K., Chen, Z., Pahk, P., Ren, R., Drubin, D. and Field, J. 1996. A conserved proline-rich region of the *Saccharomyces cerevisiae* cyclase-associated protein binds SH3 domains and modulates cytoskeletal localization. *Mol. Cell. Biol.* 16(2):548-556.

Fujimoto, L.M., Roth, R., Heuser, J.E. and Schmid, S.L. 2000. Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells. *Traffic.* 1:161-171.

Gieselmann, R. and Mann, K. 1992. ASP-56, a new actin sequestering protein from the pig platelets with homology to CAP, an adenylate cyclase-associated protein from yeast. *FEBS Lett.* 298:149-153.

Gimona, M., Djinovic-Carugo, K., Kranewitter, W.J. and Winder, S.J. 2002. Functional plasticity of CH domains. *FEBS Lett.* 513(1):98-106.

Godsel, L.M., Hobbs, R.P. and Green, K.J. 2008. Intermediate filament assembly: dynamics to disease. *Trends Cell Biol.* 18(1):28-37.

Goldschmidt-Clermont, P.J., Furman, M.I., Wachsstock, D., Safer, D., Nachmias, V.T. and Pollard, T.D. 1992. The control of actin nucleotide exchange by thymosin beta 4 and profilin. A potential regulatory mechanism for actin polymerization in cells. *Mol. Biol. Cell.* 3:1015-1024.

Goode, B.L., Rodal, A.A., Barnes, G. and Drubin, D.G. 2001. Activation of the Arp2/3 coplex by the actin filament binding protein Abp1p. *J. Cell Biol.* 153:627-634.

Goode, B.L., and Eck, M.J. 2007. Mechanisms and function of formins in the control of actin assembly. *Annu. Rev. Biochem*. 76:593-627.

Gottwald, U., Brokamp, R., Karakesisoglou, I., Schleicher, M. and Noegel, A.A. 1996. Identification of a cyclase-associated protein (CAP) homologue in *Dictyostelium discoideum* and characterization of its interaction with actin. *Mol. Biol. Cell.* 7: 261-72.

Gourlay, C.W. and Ayscough, K. 2006. Actin-induced hyperactivation of the Ras-signaling pathway leads to apoptosis in *Saccharomyces cerevisiae. Mol. Cell. Biol.* 26(17):6487-6501.

Gunsalus, K.C., Bonaccorsi, S., Williams, E., Verni, F., Gatti, M. and Goldberg, M.L. 1995. Mutations in twinstar, a Drosophila gene encoding a cofilin/ADF homologue, result in defects in centrosome migration and cytokinesis. *J. Cell Biol.* 131(5):1243-59.

Gupton, S.L. and Gertler, F.B. 2007. Filopodia: the fingers that do the walking. *Sci. STKE.* 2007: re5.

Gurniak, C.B., Perlas, E. and Witke, W. 2005. The actin depolymerizing factor cofilin-1 is essential for neural tube morphogenesis and neural crest cell migration. *Dev. Biol.* 278(1):231-241.

Haarer, B.K., Lillie, S.H., Adams, A.E., Magdolen, V., Bandlow, W. and Brown, S.S. 1990. Purification of profilin from *Saccharomyces cerevisiae* and analysis of profilin-deficient cells. *J. Cell Biol.* 110:105-14.

Haas, M. and Jost, E. 2003. Functional analysis of phosphorylation sites in human lamin A controlling lamin disassembly, nuclear transport and assembly. *Eur. J. Cell Biol.* 62:237-247.

Halpain, S. and Dehmelt, L. 2006. The MAP1 family of microtubule-associated proteins. Review. *Genome Biol*. 7(6):224.

Hatanaka, H., Ogura, K., Moriyama, K., Ichikawa, S., Yahara, I. and Inagaki, F. Tertiary structure of destrin and structural similarity between two actin-regulating protein families. *Cell*. (7):1047-55.

Haugwitz, M., Noegel, A.A., Rieger, D., Lottspeich, F. and Schleicher, M. 1991. *Dictyostelium discoideum* contains two profilin isoforms that differ in structure and function. *J. Cell Sci.* 100:481-489.

Hawkins, M., Pope, B., Maciver, S.K. and Weeds, A.G. 1993. Human actin depolymerizing factor mediates a pH-sensitive destruction of actin filaments. *Biochemistry.* 32(38):9985-93.

Herrmann, H., Hesse, M., Reichenzeller, M., Aebi, U. and Magin, T. 2003. Functional complexity of intermediate filament cytoskeletons: From structure to assembly to gene ablation. *Int. Rev. Cytol.* 223:83-175.

Herrmann, H. and Aebi, U. 2004. Intermediate filaments: molecular structure, assembly mechanism, and integration into functionally distinct intracellular scaffolds. Review. *Annu. Rev. Biochem*. 73:749-89.

Hesse, M., Magin, T.M. and Weber, K. 2001. Genes for intermediate filament proteins and the draft sequence of the human genome: novel keratin genes and a surprisingly high number of pseudogenes related to keratin genes 8 and 18. *J. Cell Sci.* 114(Pt 14):2569-75.

Higgs, H.N. 2005. Formin proteins: A domain based approach. *Trends biochem. Sci.* 30(6):342-353.

Hotulainen, P., Paunola, E., Vartiainen, M.K. and Lappalainen, P. 2005. Actin-depolymerizing factor and cofilin-1 play overlapping roles in promoting rapid F-actin depolymerization in mammalian non-muscle cells. *Mol. Biol. Cell.* 16:649-664.

Hotulainen, P. and Lappalainen, P. 2006. Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. *J. Cell Biol.* 173:383-394.

Hubberstey, A., Yu, G., Loewith, R., Lakusta, C. and Young, D. 1996. Mammalian CAP interacts with CAP, CAP2, and actin. *J. Cell. Biochem*. 60:459-466.

Hubberstey, A.V. and Mottillo, E.P. 2002. Cyclase-associated proteins: CAPacity for linking signal transduction and actin polymerization. *FASEB J.* 6:487-499.

Huckaba, T.M., Gay, A.C., Pantalena, L.F., Yang, H.C. and Pon, L.A. 2003. Live cell imaging of the assembly, disassembly, and actin cable-dependent movement of endosomes and and actin patches in the budding yeast, *Saccharomyces serevisiae. J. Cell. Biol.* 167:519-530.

Iida, K., Moriyma, K., Matsumoto, S., Kawasaki, H., Nishida, E. and Yahara, I. 1993. Isolation of yeast essential gene, *COF1*, that encodes a homologue of mammalian cofilin, a low-Mr actin-binding and depolymerizing protein. *Gene.* 124:115-120.

Ikeda, S., Cunningham, L.A., Boggess, D., Hawes, N., Hobson, C.D., Sundberg, J.P., Naggert, J.K., Smith, R.S. and Nishina, P.M. 2003. Aberrant actin cytoskeleton leads to accelerated proliferation of corneal epithelial cells in mice deficient for destrin (actin deolymerizing factor). *Hum. Mol. Genet.* 12(9):1029-1037.

Kabsch, W. and Holmes, K.C. 1995. The actin fold. *FASEB J.* 9:167-174.

Kabsch, W., Mannherz, D., Suck, D., Pai, E.F. and Holmes, K.C. 1990. Atomic structure of the actin:DNaseI complex. *Nature.* 347:37-44.

Kaksonen, M., Sun, Y. and Drubin, D.G. 2003. A pathway for association of receptors, adaptors, and actin during endocytic internalization. *Cell.* 115:475-87.

Kaksonen, M., Toret, C.P. and Drubin, D.G. 2005. A modular design for the clathrin- and actin-mediated endocytosis machinery. *Cell.* 123:305-320.

Kawamukai, M., Gerst, J., Field, J., Riggs, M., Rodgers, L., Wigler, M. and Young, D. 1992. Genetic and biochemical analysis of the adenylyl cyclase-associated protein, cap, in *Schizosaccharomyces pombe*. *Mol. Biol. Cell.* 3:167-80.

Kilmartin, J. and Adams, A. 1984. Structural rearrangements of tubulin and actin during the cell cycle of *Saccharomyces cerevisiae. J. Cell. Biol.* 98:922-933.

Krause, M., Dent, E.W., Bear, J.E., Loureiro, J.J. and Gertler, F.B. 2003. Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. Review. *Annu. Rev. Cell Dev. Biol.* 19:541-64.

Ksiazek, D., Bandstetter, H., Israel, L., Bourenkov, G.P., Katchalova, G., Janssen, K.P., Bartunik, H.D., Noegel, A.A., Schleicher, M. and Holak, T. 2003. Structure of the N-terminal domain of the adenylyl cyclase-associated protein (CAP) from *Dictyostelium discoideum*. *Structure*. 11:1171-1178.

Kubler, E. and Riezman, H. 1993. Actin and fimbrin are required for the internalization step of endocytosis in yeast. *The EMBO J.* 12:2855-2862.

Laing, N.G. 2007. Congenital myopathies. Review. *Curr. Opin. Neurol.* 20(5):583-9.

Lambrech, A., Verschelde, J-L., Jonckheere, V., Goethals, M., Vandekerckhove, J. and Ampe, C. 1997. The mammalian profilin isoforms display complementary affinities for PIP2 and proline rich sequences. *The EMBO J.* 3:484-494.

Lambrechts, A., Jonckheere, V., Dewitte, D., Vandekerckhove, J. and Ampe, C. 2002. Mutational analysis of human profilin I reveals a second PI(4,5)-P2 binding site neighbouring the poly(L-proline) binding site. *BMC Biochem*. 28;3:12.

Lappalainen, P. and Drubin, D.G. 1997. Cofilin promotes rapid actin filament turnover *in vivo*. *Nature*. 388:78-82.

Lappalainen, P., Fedorov, E.V., Fedorov, A.A., Almo, S.C. and Drubin, D.G. 1997. Essential functions and actin-binding surfaces of yeast cofilin revealed by systematic mutagenesis. *EMBO J*. 16:5520-5530.

Lappalainen, P., Kessels, M.M., Cope, M.J. and Drubin, D.G. 1998. The ADF homology (ADF-H) domain: A highly exploited actin-binding module. *Mol. Biol. Cell.* 9:1951-1959.

Lassing, I. and Lindberg, U. 1985. Specific interaction between phosphatidyl 4,5-bisphosphate and profilactin. *Nature*. 314:472-474.

Lazarides, E. and Lindberg, U. 1974. Actin is the naturally occurring inhibitor of deoxyribonuclease I. *Proc. Natl. Acad. Sci. USA*. 71(112):4742-4746.

Lila, T. and Drubin, D. 1997. Evidence for physical and functional interactions among two *Saccharomyces cerevisiae* SH3 domain proteins, an adenylyl cyclase-associated protein and the actin cytoskeleton. *Mol. Biol. Cell*. 8:367-385.

Lindberg, M.U. 1966. Crystallization from calf spleen of two inhibitors of deoxyribonuclease. *J. Biol. Chem.* 241(5):1246-1249.

Lindberg, U., Schutt, C.E., Hellsten, E., Tjäder, A.C. and Hult, T. 1988. The use of poly(L-proline)-sepharose in the isolation of profilin and profilactin complexes. *Biochim. Biophys. Acta.* 967(3):391-400.

Lippincott, J. and Li, R. 1998. Sequential assembly of myosin II, an IQGAP-like protein, and filamentous actin to ring structure involved in budding yeast cytokinesis. *J. Cell. Biol.* 140: 355-366.

Lu, J. and Pollard, T.D. 2001. Profilin binding to poly-L-proline and actin monomers along with ability to catalyze actin nucleotide exchange is required for viability of fission yeast. *Mol. Biol. Cell*. 12(4):1161-75.

Maekawa, S., Nishida, E., Ohta, Y. and Sakai, H. 1984. Isolation of low molecular weight actin-binding protein from porsine brain. *J. Biochem.* 95(2):377-385.

Magdolen, V., Oechsner, U., Müller, G. and Bandlow, W. 1988. The intron containing gene from yeast profilin (*PFY*) encodes a vital function. *Mol. Cell. Biol.* 8:5108-5115.

Mahoney, N.M., Janmey, P.A. and Almo, S.C. 1997. Structure of the profilin-poly-L-proline complex involved in morphogenesis and cytoskeletal regulation. *Nat. Struct. Biol.* 4(11):953-960.

Matsuzaki, F., Matsumoto, S., Yahara, I., Yonezawa, N., Nishida, E. and Sakai, H. 1988. Cloning and characterization of porcine brain cofilin cDNA. Cofilin contains the nuclear transport signal sequence. *J. Biol. Chem.* 263(23):11564-11568.

Mattila, P.K., Quintero-Monzon, O., Kugler, J., Moseley, J.B., Almo, S.C., Lappalainen, P. and Goode, B.L. 2004. A high affinity interaction with ADP-actin monomers underlies the mechanism and *in vivo* function of Srv2/cyclase-associated protein (CAP). *Mol. Biol. Cell*. 15:5158-5171.

Mattila, P.K., Pykäläinen, A., Saarikangas, J., Paavilainen, V.O., Vihinen, H., Jokitalo, E. and Lappalainen, P. 2007. Missing-in-metastasis and IRSp53 deform PI(4,5)P2-rich membranes by an inverse BAR domain-like mechanism. *J. Cell Biol.*176(7):953-64.

Matviw, H., Yu, G. and Young, D. 1992. Identification of a human cDNA encoding a protein that is structurally and functionally related to the yeast adenylyl cyclase-associated CAP proteins. *Moll. Cell. Biol.* 12(11):5033-5040.

Mavoungou, C., Israel, L., Rehm, T., Ksiazek, D., Krajewski, M., Popowicz, G., Noegel, A., Scleicher, M. and Holak, T.A. 2004. NMR structural characterization of the N-terminal domain of the adenylyl cyclase-associated protein (CAP) from *Dictyostelium discoideum. J. Biomol. NMR.* 29:73-84.

McGough, A., Pope, B., Chiu, W. and Weeds, A. 1997. Cofilin changes the twist of F-actin: implications for actin filament dynamics and cellular function. *J. Cell Biol.* 138:771-781.

McGough, A.M., Staiger, C.J., Min, J.G. and Simonetti, K.D. 2003. The gelsolin family of actin regulatory proteins: Modular structures, versatile functions. *FEBS Lett.* 552(2-3):75-81.

McKim, K.S., Matheson, C., Marra, M.A., Wakarchuk, M.F. and Baillie, D.L. 1994. The *Caenorhabditis elegans* unc-60 gene encodes proteins homologous to a family of actin-binding proteins. *Mol. Gen. Genet*. 242(3):346-57.

Mockrin, S. C. and Korn, E. D. 1980. *Acanthamoeba* profilin interacts with G-actin to increase the rate of exchange of actin-bound adenosine 5'-triphosphate. *Biochemistry.* 19:5359-5362.

Mogensen, J., Perrot, A., Andersen, P.S., Havndrup, O., Klausen, I.C., Christiansen, M., Bross, P., Egelblad, H., Bungaard, H., Osterziel, K.J., Haltern, G., Lapp, H., Reinecke, O., Gregersen, N. and Børglum, A.D. 2004. Clinical and genetic characteristics of α cardiac actin gene mutations in hypertrophic cardiomyopathy. *J. Med. Genet.* 41:e10.

Mohri, K. and Ono, S. 2003. Actin filament disassembling activity of *Caenohabtidus elegans* actin-interacting protein 1 (UNC-78) is dependent on filament binding by a specific ADF/cofilin isoform. *J. Cell. Sci.* 116:4107-4118.

Moon, A.L., Janmey, P.A., Louie, K.A. and Drubin, D.G. 1993. Cofilin is an essential component of the yeast cortical cytoskeleton. *J. Cell Biol.* 120:421-435.

Moraczewska, J., Wawro, B., Seguro, K. and Strzelecka-Golaszewska, H. 1999. Divalent cation-, nucleotide-, and polymerization-dependent changes in the conformation of subdomain 2 of actin. *Biophys. J.* 77(1):373-85.

Moriyama, K. and Yahara, I. 2002. Human CAP1 is a key factor in the recycling of cofilin and actin for rapid actin turnover. *J. Cell Sci.* 115:1591-601.

Morrison, E.E. 2007. Action and interactions at microtubule ends. Review. *Cell. Mol. Life Sci.* 64(3):307-17.

Moseley, J. and Goode, B. 2006. Review. The actin cytoskeleton: from cellular functions to biochemical mechanism. *Microbiol. Mol. Biol. Reviews.* 17(3):605-645.

Mullins, R.D., Heuser, J.A. and Pollard, T.D. 1998. The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping and formation of branching networks of filaments. *Proc. Natl. Acad. Sci. USA*. 95:6181-6186.

Newpher, T.M., Smith, R.P., Lemmon, V. and Lemmon, S.K. 2005. *In vivo* dynamics of clathrin and its adaptor-dependent recruitment to the actin based endocytic machinery in yeast. *Dev. Cell.* 9:87-98.

Niggli, V. and Rossy, J. 2007. Ezrin/radixin/moesin: Versatile controllers of signaling molecules and of the cortical cytoskeleton. *Int. J. Biochem. Cell Biol*. Feb 22.

Nishida, Y., Shima, F., Sen, H., Tanaka, Y., Yanagihara, C., Yamawaki-Kataoka, Y., Kariya, K-I. and Kataoka, T. 1998. Coiled-coil interaction of N-terminal 36 residus of cyclase-associated protein with adenylyl cyclase is sufficient for it's function in *Saccharomyces cerevisiae* Ras pathway. *The J. Biol. Chem.* 273:28019-28024.

Noegel, A.A., Rivero, F., Albrecht, R., Janssen, K-P., Köhler, J., Parent, C. and Schleicher, M. 1999. Assessing the role of ASP56/CAP homologue of *Dictyostelium discoideum* and requirements for subcellular localization. *J. Cell. Sci.* 112:3195-3203.

Noegel, A.A., Blau-Wasser, R., Sultana, H., Müller, R., Israel, L., Schleicher, M., Patel, H. and Weijer, C.J. 2004. The cyclase-associated protein CAP as regulator of cell polarity and cAMP signaling in *Dictyostelium*. *Mol. Biol. Cell*. 15:934-945.

Nogales, E. 1999. A structural view of microtubule dynamics. Review. *Cell Mol. Life Sci.* 1;56(1-2):133-42.

Nogales, E. and Wang, H.W. 2006. Structural mechanisms underlying nucleotide-dependent self-assembly of tubulin and its relatives. Review. *Curr. Opin. Struct. Biol*. 16(2):221-9.

Oechsner, U., Magdolen, V. and Bandlow, W. 1987. The cDNA and deduced amino acid sequence of profilin from *Saccharomyces cerevisiae. Nucleid Acid Res.* 15:9078.

Ono S., Minami, N., Abe, H. and Obinata, T. 1994. Characterization of novel cofilin isoform that is predominantly expressed in mammalian skeletal muscle. *J. Biol. Chem.* 269(21): 15280-15286.

Paavilainen, V.O., Bertling, E., Falck, S. and Lappalainen, P. 2004. Regulation of cytoskeletal dynamics by actin monomer binding proteins. Review. *Trends Cell Biol.* 14:386-394.

Paunola, E., Mattila, P. and Lappalainen, P. 2002. WH domain: a small, versatile adapter for actin monomers. Review. *FEBS Letters*. 513:92-97.

Peche, V., Shekar, S., Leichter, M., Korte, H., Schröder, R., Schleicher, M., Holak, T.A., Clemen, C.S., Ramanath-Y, B., Pfitzer, G., Karakesisogglou, I. and Noegel, A.A. 2007. CAP2, cyclase-associated protein 2, is a dual compartment protein. *Cell. Mol. Life. Sci.* 1-14.

Pelham, R.J. and Chang, F. 2001. Role of actin polymerization and actin cables in actin-patch movement in *Schizosaccharomyces pombe. Nat. Cell. Biol.* 3:235-244.

Pellegrin, S. and Mellor, H. 2007. Actin stress fibres. Review. *J. Cell Sci*. 120(Pt 20):3491-9.

Perelroizen, I., Marchand, J.B., Blanchoin, L., Didry, D. and Carlier, M.F. 1994. Interaction of profilin with G-actin and poly(L-proline). *Biochemistry*. 33:8472-8478.

Pollard T.D. 1986. Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. *J. Cell Biol.* 103(6 Pt 2):2747-54.

Pollard, T.D. and Borisy, G.G. 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell.* 112:453-465.

Pollard, T.D. and Weeds A.G., 1984. The rate constant for ATP hydrolysis by polymerized actin. *FEBS Lett*. 170(1):94-8.

Pollard, T.D., Blanchoin, L. and Mullins, R.D. 2000. Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu. Rev. Bhiophys. Biomol. Struct.* 29:545-576.

Popowicz, G.M., Schleicher, M., Noegel, A.A. and Holak, T.A. 2006. Filamins: promiscuous organizers of the cytoskeleton. Review. *Trends Biochem. Sci.* 31(7):411-9.

Procaccio, V., Salazar, G., Ono, S., Styers, M., Gearing, M., Davila, A., Jimenez, R., Juncos, J., Gutekunst, C.-A., Meroni, G., Fontanella, B., Sontag, E., Sontag, J.M., Faundez, V. and Wainer, B.H. 2006. A Mutation of β-actin that alters depolymerization dynamics associated with autosomal dominant developmental malformations, deafness and dystonia. *Am. J. Hum. Genet.* 78:947-960.

Ressad, F., Didry, D., Xia, G.X., Hong, Y., Chua, N.H., Pantaloni, D. and Carlier, M.F. 1998. Kinetic analysis of the interaction of actin-depolymerizing factor (ADF)/cofilin with G- and F-actin. *J. Biol. Chem.* 273(33):20894-20902.

Rodal, A.A., Tetreault, J.W., Lappalainen, P., Drubin, D.G. and Amberg, D.C. 1999. Aip1p interacts with cofilin to disassemble actin filaments. *J.Cell. Biol.* 145:1251-1264.

Schafer, D.A., Korshunova, Y.O., Schroer, T.A. and Cooper, J.A. 1994. Differential localization and sequence analysis of capping protein beta-subunit isoforms of vertebrates. *J. Cell Biol.* 127:453-465.

Schirenbeck, A., Bretschneider, R., Arasada, M., Schleicher, M. and Faix, J. 2005. The diaphanous-related forming dDia2 is reguired for the formation and maintenance of filopodia. *Nat. Cell. Biol.* 7:619-625.

Schott, D.H., Collins, R.N. and Bretscher, A. 2002. Secretory vesicle transport velocity in living cell depends on the myosin-V lever arm length. *J. Cell. Biol.* 156:35-39.

Schüler, H. 2001. ATPase activity and conformational changes in the regulation of the actin. *Biochim. Biophys. Acta.* 1549(2):137-147.

Schutt, C.E., Myslik, J.C., Rozycki, M.D., Goonesekere, N.C. and Lindberg, U. 1993. The structure of crystalline profilin-beta-actin. *Nature.* 365(6449):810-816.

Sellers, J.R. 2000. Myosins: A diverse superfamily. *Biochim. Biophys. Acta.* 1496(1):3-22.

Shibata, R., Mori, T., Du, W., Chuma, M., Gotoh, M., Shimazu, M., Uada, M., Hirohashi, S. and Sakamoto, M. 2006. Overexpression of cyclase-associated protein 2 in multistage hepatocarsinogenesis. *Clin. Cancer Res.* 12(18)5363-5368.

Shima, F., Okada, T., Kido, M., Sen, H., Tanaka, Y., Tamada, M., Hu, C-D., Yamawaki-kataoka, Y., Kariya, K-I. and Kataoka, T. 2000. Association of yeast adenylyl cyclase with cyclase-associated protein CAP forms a second Ras-binding site which mediates its Ras-dependent activation. *Mol. Cell. Biol.* 20(1):26-33

Shortle, D., Haber, J. and Botstein, D. 1982. Lethal distruption of yeast actin gene by integrative DNA transformation. *Science*. 217:371-373.

Skare, P. and Karlsson, R. 2002. Evidence for two interaction regions for phosphatidylinositol(4,5)-bisphosphate on mammalian profilin I. *FEBS lett.* 522:119-24.

Sparrow, J.C., Nowak, K.J., Durling, H.J., Beggs, A.H., Wallgren-Pettersson, C., Romero, N., Nonaka, I. and Laing, N.G. 2003. Muscle disease caused by mutations in the skeletal muscle alpha-actin gene (*ACT1*). Review. *Neuromuscular Disorders.* 13:519-531.

Strelkov, S.V., Herrmann, H. and Aebi, U. 2003. Molecular architecture of intermediate filaments. Review. *Bioessays*. 25(3):243-51.

Suetsugu, S., Miki, H. and Takenawa, T. 1998. The essential role of profilin in the assembly of actin for microspike formation. *EMBO J.* 17(22):6516-6526.

Sultana, H., Rivero, F., Blau-Wasser, R., Schwager, S., Balbo, A., Bozzaro, S., Schleicher, M. and Noegel, A.A. Cyclase-associated protein is essential for the functioning of the endo-lysosomal system and provides a link to the actin cytoskeleton. *Traffic.* 6:930-946.

Svitkina, T.M and Borisy, G.G. 1999. Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. *J. Cell. Biol.* 145:1009-1026.

Swiston, J., Hubberstey, A., Yu, G. and Young, D. 1995. Differential expresson of CAP and CAP2 in adult rat tissues. *Gene.* 165:273-277.

Toivola, D.M., Tao, G.Z., Habtezion, A., Liao, J. and Omary, M.B. 2005. Cellular integrity plus: organelle-related and protein-targeting functions of intermediate filaments. Review. *Trends Cell Biol.* 15(11):608-17.

Valenta, R., Duchene, M., Ebner, C., Valent, P., Sillaber, C., Deviller, P., Ferreira, F., Tejkl, M., Edelmann, H. and Kraft, D. 1992. Profilins constitute a novel family of functional plant pan-allergens. *J. Exp. Med.* 175(2):377-385.

Vandekerckhove, J. and Weber, K. 1978. At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence of the amino-terminal tryptic peptide. *J. Mol. Biol*. 25:783-802.

Vartiainen, M.K., Mustonen, T., Mattila, P.K., Ojala, O.J., Thesleff, I., Partanen, J. and Lappalainen, P. 2002. The three mouse actin-depolymerizing factor/cofilins evolved to fulfill cell-type-specific requirements for actin dynamics. *Mol. Biol. Cell*. 13(1):183-94.

Vartiainen, M., Ojala, P.J., Auvinen, P., Peränen, J. and Lappalainen, P. 2000. Mouse A6/twinfilin is an actin monomer-binding protein that localizes to the regions of rapid actin dynamics. *Mol. Cell Biol.* 20:1772-1783.

Vartiainen, M.K., Sarkkinen, E.M., Matilainen, T., Salminen, M. and Lappalainen, P. 2003. Mammals have two twinfilin isoforms whose subcellular localizations and tissue distributions are differentially regulated. *J. Biol. Chem.* 278:34347-34355.

Vavylonis, D., Kovar, D., O'shaughnessy, B. and Pollard, T.D. 2006. Model of formin-associated actin filament elongation. *Mol. Cell* 21:455-466.

Vojtek, A.B. and Cooper, J.A. 1993. Identification of a cDNA encoding mouse CAP: a homolog of the yeast adenylyl cyclase associated protein. *J. Cell Sci.* 105:777-785.

Vojtek, A., Haarer, B., Field, J., Gerst, J., Pollard, T. D., Brown, S. and Wigler, M. 1991. Evidence for a functional link between profilin and CAP in the yeast *S. cerevisiae*. *Cell.* 66:497-505.

Wade, R.H. and Hyman, A.A. 1997. Microtubule structure and dynamics. Review. *Curr. Opin. Cell Biol*. 9(1):12-7.

Wang, J., Suzuki, N. and Kataoka, T. 1995. The 70-kilodalton adenylyl cyclas-associated protein is not essential for interaction of *Saccharomyces cerevisiae* adenylyl cyclase with RAS proteins. *Mol. Cell. Biol.* 12(11):4937-494.

Watanabe, T., Noritake, J. and Kaibuchi, K. 2005. Regulation of microtubules in cell migration. Review. *Trends Cell Biol.* 15(2):76-83.

Watts, F.Z., Shieks, G. and Orr, E. 1987. The yeast *MYO1* gene encoding a myosin-like protein required for cell division. *EMBO J*. 6:3499-3505.

Wear, M.A., Yamashita, A., Kim, K., Maeda, Y. and Cooper, J.A. 2003. How capping protein binds the barbed end of the actin filament. *Curr Biol*. 13(17):1531-7.

Westermann, S. and Weber, K. 2003. Post-translational modifications regulate microtubule function. Review. *Nat. Rev. Mol. Cell Biol.* 4(12):938-47.

Wegner, A. 1982. Treadmilling of actin at physiological salt concentrations. An analysis of the critical concentrations of actin filaments. *J. Mol. Biol.* 161(4):607-15.

Winter, D.C., Choe, E.Y. and Li, R. 1999. Genetic dissection of the budding yeast Arp2/3 complex: a comparison of the *in vivo* and structural roles of individual subunits. *Proc. Natl. Acad. Sci. USA*. 96:7288.7293.

Witke, W., Sutherland, J.D. and Sharpe, A. 2001. Profilin I is essential for cell survival and cell division in early mouse development. *Proc. Natl. Acad. Sci. USA*. 98(7):3832-3836.

Witke, W. 2004. The role of profilin complexes in cell motility and other cellular processes. Review. *Trends in Cell Biol*. 14:461-468.

Wolven, A.K., Belmont, L.D., Mahoney, N.M., Almo, S.C. and Drubin, D.G. 2000. *In vivo* importance of actin nucleotide exchange catalyzed by profilin. *J. Cell Biol.* 150:895-904.

Wriggers, W., Tang, J.X., Azuma, T., Marks, P.V. and Janmey, P. 1998. Cofilin and gelsolin segment-1: molecular dynamics simulation and biochemical analysis predict a similar actin binding mode. *J. Mol. Biol.* 282:921-932.

Yoon, Y., Pitts, K. and McNiven, M. 2002. Studying cytoskeletal dynamics in living cells using green fluorescent protein. Review. *Mol. Biotechnol.* 21(3):241-50.

Yonezawa, N., Homma, Y., Yahara, I., Sakai, H. and Nishida, E. 1991. A short sequence responsible for both phosphoinositide binding and actin binding activities of cofilin. *J. Biol. Chem.* 266:17218-17221.

Yonezawa, N., Nishida, E., Koyasu, S., Maekawa, S., Ohta, Y., Yahara, I. and Sakai, H. 1987. Distribution among tissues and intracellular localization of cofilin, a 21 kDa actin-binding protein. *Cell Struct. Funct.* 12:443-452.

Yusof, A., Hu, N.-J., Wlodawer, A. and Hofmann, A. 2005. Structural evidence for variable oligomerization of the N-terminal domain of cyclase-associated protein (CAP). *Proteins: Structure, Function, and Bioinformatics*. 58:255-262.

Yusof, A., Jaenicke, E., Pedersen, J.S., Noegel, A.A., Schleicher, M. and Hofmann, A. 2006. Mechanism of oligomerisation of cyclase-associated protein from *Dictyostelium discoideum* in solution. *J. Mol. Biol*. 362:1072-1081.

Yu, G., Swiston, J. and Young, D. 1994. Comparison of human CAP1 and CAP2, homologs of the yeast adenylyl cyclase-associated proteins. *J. Cell Sci.* 107:1671-1678.

Yu, J., Wang, C., Palmieri, S.J., Haarer, B.K. and Field, J. 1999. A cytoskeletal localizing domain in the cyclase-associated protein, CAP/Srv2p, regulates access to a distant SH3-binding site. *J. Biol. Chem*. 274:19985-19991.

Zelicof, A., Gatica, J. and Gerst, J.E. 1999. Molecular cloning and characterization of rat homolog of CAP, The adenylyl cyclase-associated protein from *Saccharomyces serevisiae. J. Biol. Chem.* 268(18):13448-13453.