Profilin and the Microfilament System in Cultured Cells

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Abstract

The actin microfilament system plays a central function in eukaryotic cells. This force-generating machinery, where actin is the major component, undergoes rapid reorganizations due to the ability of actin to polymerize and depolymerize in response to different signals. Furthermore the filaments that are formed are often organized in various supramolecular arrangements like bundles or sheet-like structures adding to the dynamic character of the microfilament organization in most cells. This thesis deals with the organization of actin and the relation of microfilament dynamics, particularly with respect to the role of the actin binding protein profilin.

Profilin is a regulator of actin polymerization; it binds to actin monomers and forms the profilin:actin (P:A) complex and delivers actin monomers to the growing barbed end of the filament via elongation factors like Ena/VASP and the formins. Consequently profilin is found to accumulate at the plasma membrane of active cell edges. Interestingly, both actin and profilin are present in the nucleus, however it is unclear to what extent the profilin:actin complex is formed and functions in the nucleus.

Previous studies in our laboratory have shown that the distribution of profilin mRNA to the advancing cell edge is dependent on microtubules. The protein has been shown to co-localize with microtubules. This study provides further evidence for a connection between profilin/P:A and microtubules.

In addition to its force-generating activity in the cytoplasm actin is also influencing nuclear process. For example the transcription factor SRF which controls a large number of genes, including many encoding microfilament associated proteins like actin itself and profilin, ‘senses’ cytoplasmic changes in actin dynamics through its co-activator MRTF-A. MRTF-A is an actin monomer binding protein under constant nucleo-cytoplasmic shuttling. It competes with profilin for actin binding and upon serum stimulation it dissociates from actin and accumulates in the nucleus. Taking advantage of the competition between profilin and MRTF-A for actin, this thesis studies the connection between SRF/MRTF-A and actin dynamics with respect to profilin and indirectly P:A. Down-regulating the expression of isoforms profilin and I and II, respectively, both influences SRF/MAL-controlled transcription.

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CELL MOTILITY

Cell motility includes phenomena such as cell migration and intracellular trafficking of vesicles and macromolecular components. It is often activated in response to extracellular stimuli and is highly regulated. Three protein filament systems are generally coupled to these activities: the microfilament, microtubule and intermediate filament systems. Particularly the first two represent the major force-generating systems, and together, all three have a massive dynamic impact on the fine-structure of the cytoplasm in eukaryotic cells. This thesis deals primarily with the actin microfilament system, aspects of its regulation and relation to the microtubule system. Specific questions addressed are how the profilin:actin is transported to the growing ends of actin filaments at the cell periphery and how profilin is involved in the regulation of the transcription factor serum response factor.

Schematically, cell migration can be described as a series of actin-dependent motility events: polarization and outgrowth of protrusions in the form of sheet-like lamellipodia and spike-structured filopodia, adhesion to the extracellular matrix, followed by myosin-mediated translocation of the cell body and retraction of the rear (Ridley, 2011) and see (Fig. 1).

The directional movement of a cell corresponds to the cell´s leading edge and different cell types show different behavior in motility and directionality. For instance keratocytes have one large leading lamella and move rapidly (15 µm/s) across a surface, while fibroblasts can have several leading edges, high ruffling activity and many filopodia and move much slower (10 nm/s), e.g. see (Small et al., 2002).

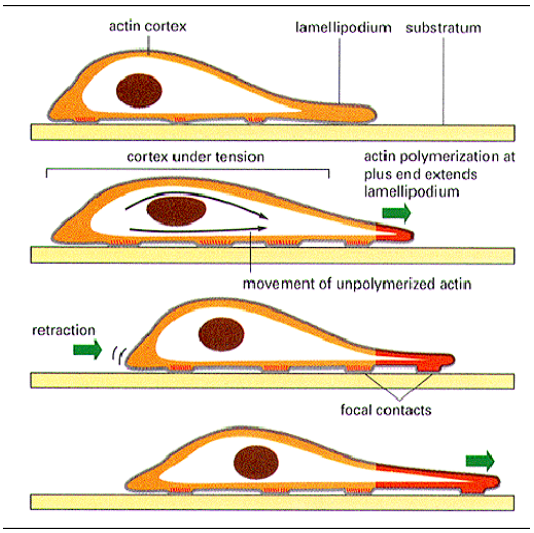

Figure 1. The steps involved in cell motility
The motility starts by extension of the cell edge due to controlled polymerization of actin filaments. The cell periphery attaches to the substratum via focal contacts and finally actomyosin dependent contractile activities mediate retraction of the trailing edge and cell body movement. Cartoon from Alberts et al., Molecular Biology of the Cell, Fifth edition

Force generation and intracellular trafficking

The microfilament system is formed by actin, myosin and a large number of regulatory proteins. It is essential to the eukaryotic cell. Dynamic actin filaments (microfilaments) and higher order structures of filamentous actin are found in practically all parts of the cell (Fig. 2a). The formation of such filaments by ordered (time and space) polymerization of actin is one important force-generating mechanism which contributes to a vast number of cellular behavior as well as intracellular organization, macromolecular transport etc. The polymerization is a salt-driven self-assembly process coupled with hydrolysis of the actin bound ATP and due to the latter in combination with the structural asymmetry of the actin molecule directionality of the growing filament is established.

The other force-generating mechanism exhibited by the microfilament system is generated by acto-myosin based contractions where actin and myosin filaments, organized in various contractile arrangements, interact under the expense of ATP to generate tension between different fix points. For instance during cell migration when the trailing edge of the moving cell is detached from the substratum and contracts this activity is in operation. However, the similar acto-myosin based mechanism is also powering many intracellular transportation phenomena although in those cases the myosin is not building filaments but rather operates as a single cargo-associated molecule together with the actin filament. The myosins are composed of a head, neck and tail domain. They bind to actin filaments through their head domain and undergo a series of conformational switches which requires ATP-hydrolysis and is the main force-generating step according to the classical cross-bridge theory of muscle contraction (Huxley and Simmons, 1981). This force generation is used for sliding of actin filaments of opposing polarity along conventional bipolar myosin polymers or for the movement of the unconventional non-filamentous myosins.

In addition to cell migration and adhesion, actin polymerization and actomyosin interactions are known to be involved in processes such as organelle transport, endo- and exocytosis, cytokinesis, transport of proteins and mRNPs. In several of these processes the microfilament system operates in parallel with the other force-producing machinery in the cell, i.e. the microtubule

system. This is built by heterodimers of α,β-tubulin that polymerize into tubular structures (microtubules), which just like actin filaments are asymmetric, displaying a fast and a slow polymerizing end. Similar to actin, the microtubules are under extensive reorganization in the cell. This is controlled by many microtubule interacting proteins as well as the tubulin-bound nucleotide (GTP/GDP), and it enables microtubule rearrangements to meet requirements for transportation of macromolecular complexes and organelles between the cell center and its periphery. Microtubule-dependent transport is based on two families of proteins, the dyneins and kinesins, which utilize ATP for motor-activity and the asymmetry of the microtubules for directionality. Thus dyneins are engaged in transport towards the cell center where the slow polymerizing end of the microtubules are anchored at the centrosome, while kinesins mediate transport towards the fast polymerizing ends at the cell periphery.

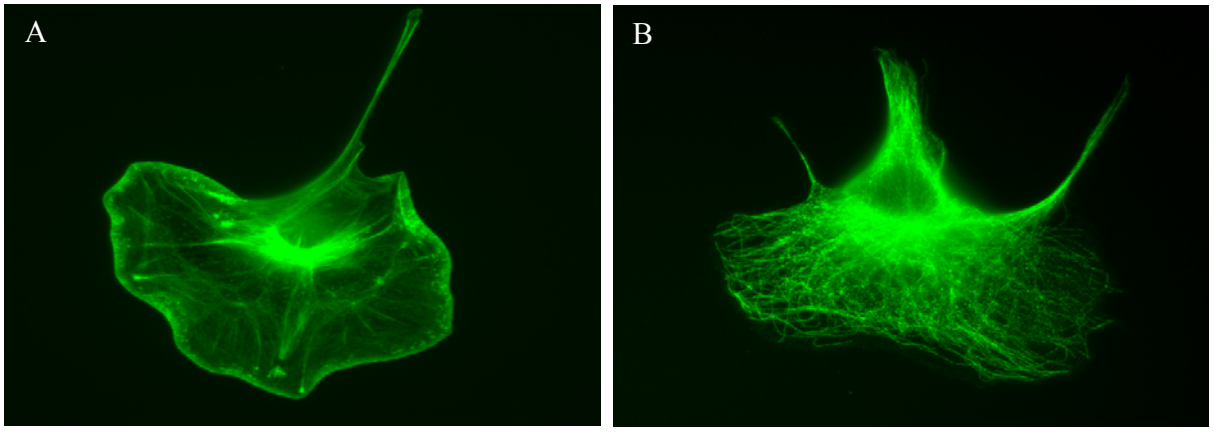

Figure 2.Fluorescence microscopy of actin microfilaments and microtubules in B16 mouse melanoma cells.
The distribution of filamentous actin (A) seen by FITC-phalloidin and of microtubules (B) using antibodies against alpha tubulin.

Actin - the main component of the microfilament system

Actin is a highly conserved 42 kDa weight ATPase. Each actin molecule binds ADP or ATP in complex with a cation, Mg^{2+} or Ca^{2+}, and exhibits a slow ATPase activity under low salt conditions. The cation stabilizes nucleotide binding to the protein and thereby the conformation of the actin molecule itself (Korn 1982, Schüler 2001). At physiological salt the actin rapidly polymerizes and its ATPase activity is dramatically stimulated in conjunction to the incorporation of new molecules into the filament, which occurs with different kinetics at the two ends of the filament reflecting the structural asymmetry of the actin molecule. Consequently the

filament expresses two facets of asymmetry: a structural and a biochemical defined by the nucleotide content of its subunits. Under stable, steady-state polymerizing conditions characterized where the concentrations of actin in unpolymerized and filamentous form remain constant, actin subunits in individual filaments are passed through the polymer from the fast polymerizing (+)-end to the slow polymerizing (-)-end in a process called treadmilling (Wegner 1982). The classical arrowhead pattern generated by myosin subfragment 1 (S1)-decoration (Huxley 1963) of actin filaments has been used to determine filament orientation in the cell by electron microscopy. It has revealed the filaments underneath the plasma membrane are all oriented with their fast polymerizing ends facing the membrane. In the cell, transitions between monomeric and filamentous actin are highly regulated, and involve many actin-binding proteins (Pollard et al., 2000).

Six actin isoforms have been identified in mammals; of these are four muscle specific (α-skeletal, α-cardiac, α-smooth, γ-smooth) and two (ß and γ-actin) are unique to non-muscle tissue (Vandekerckhove et al. 1978). The amino acid sequence differs in only a few positions between the isoforms, and in this respect the non-muscle actin´s are highly similar since only four of their 374 residues varies, i.e. while the very N-terminal sequence of β-actin is $D_2D_3D_4$---V_{10}, it is $E_2E_3E_4$---I_{10} in γ-actin. Interestingly, over the last couple of years it also been established that proteins functionally similar to actin are present in prokaryotes where they appear to be involved in force generating processes as well (Thanbicler et al. 2008).

Microfilament regulatory proteins

A large number of actin binding proteins are involved in controlling the arrangement and activities of the microfilament system. These involve both monomer and filament interacting molecules, whose interactions with actin are regulated by phosphorylation or by components such as lipids, ions and nucleotides, and often results from extracellular stimuli. The literature covering this area is vast, and here only some of these which are most relevant for my studies will be described.

Profilin

Profilin named after its ability to bind actin and keep it in a profilamentous form (Carlsson et al 1976), is a 14 kDa globular protein. Five mammalian isoforms of profilin are known. Profilin I is ubiquitously expressed, profilin IIa is most abundant in the brain but is also found in other tissues albeit in lower amounts (Honore et al. 1993; Witke et al. 2001), profilin IIb is expressed in kidney and is unique among the profilins in the sense that it interacts poorly with actin but has been found to associate with tubulin (Di Nardo et al. 2000). Profilin 3 is restricted to kidney and testis and profilin 4 to testis (Hu et al. 2001; Obermann et al. 2005). The sequence homology between the different profilin isoforms is relatively low, while their three-dimensional structure is highly conserved. Profilin was first isolated in complex with actin, the profilin:actin (P:A) -complex (Carlsson et al., 1977) and (Fig. 3).

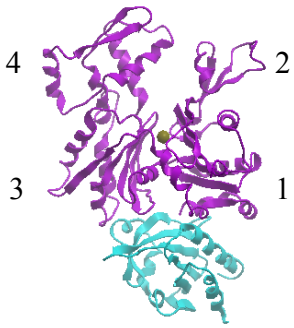

Figure 3. The profilin:β-actin complex. The ribbon structure of the actin monomer (purple) showing the locations of subdomain 1-4. The nucleotide and divalent cation are at the centre of the molecule. Profilin (turquoise) contacts the base of subdomain 1 and 3 on the actin molecule. (PDB file 2BTF, Schutt CE et al. 1993)

Extensive studies in many laboratories including ours have shown that profilin can influence actin in several ways see recent reviews (Witke 2004, Karlsson and Lindberg 2007, Jockusch et al., 2007). The original observation that profilin interferes with actin polymerization is now known to be the consequence of the ability of profilin to efficiently inhibit actin nucleation, however if actin filaments are present, the profilin:actin complex can add to their fast polymerizing end with similar kinetics as free monomeric actin (Carlier 2011), see (Fig 4). Furthermore, the binding of profilin to actin opens the actin nucleotide binding cleft and dramatically enhances exchange of ADP to ATP on the actin monomer.

Thus, actin in the P:A-complex is a polymerization competent ATP-actin, and profilin therefore is important not only for bringing this actin to the polymerizing filament end but also for the maintenance of the biochemical asymmetry of filamentous actin in the cell. The profilin:actin therefore represents a dynamic organization of unpolymerized actin and that it is the ultimate source of actin for filament growth in vivo has been demonstrated in experiments where a

covalently cross-linked variant of the complex (PxA) was microinjected into cells that had been stimulated by spreading or by exposure to a growth factor, or after infection with the intracellular pathogen *Listeria moncytogenes*; in all cases, microfilament-driven polymerization was rapidly interfered with (Grenklo et al., 2003). Consequently profilin is an essential protein in yeast, flies and mice (Balasubramanian 1994; Verheyen and Cooley 1994; Witke et al., 2001). Down-regulating profilin in cultured cells using siRNA affects the motile behavior of the cells in different ways (Bae et al., 2008 and Paper II), reflecting that under these experimental conditions actin is recruited for polymerization from other sources than profilin:actin.

In addition to actin, profilin also binds a number of other proteins. Of those the majority is involved in the actin assembly process and will be discussed below. Their interaction with profilin occurs via a unique poly-proline binding surface formed by residues in the N- and C-terminal helices on the profilin molecule (Björkegren et al 1993). The canonical sequence recognized by this binding surface is the glycine-pentaproline (GP5)-sequenced first recognized in Ena/VASP (Reinhard 1995a and see below). Later it was shown that also other proline-rich sequences containing stretches of prolines following one or more small aliphatic residues would bind profilin (Kang et al., 1997). Finally, the phosphoinositol lipids phosphatidylinositol-3,4-bisphosphate ($PI3,4P_2$), phosphatidylinositol-4,5-bisphosphate ($PI4,5P_2$) and phosphatidylinositol-3,4,5-trisphosphate ($PI3,4,5P_3$) represent another important group of interaction partners for profilin (Lassing and Lindberg 1985, Lu et al 1996). The $PI4,5P_2$ interaction has been mapped to two regions on the protein: one overlaps at least partially with the poly-proline binding surface and the other with the actin binding surface (Lambrechts et al 2002, Skare and Karlsson 2002). In vitro, the binding of $PI4,5P_2$ to profilin causes dissociation of the P:A, thereby releasing the actin monomer for polymerization (Lassing and Lindberg 1985). However, the in vivo relevance of this observation is still unclear, but studies of the actin binding protein cofilin (see below) suggest that this protein is sequestered at the inner leaflet plasma membrane via PI4,5P2 and is released to influence actin dynamics subsequent to receptor-stimulated PI4.5P2-hydrolysis (Van Rheenen et al., 2007). It is possible that a similar scenario occurs also for profilin.

Profilin also appears to be regulated by phosphorylation. This has been reported in several in vitro studies, which have led to the identification of protein kinase C and pp60Src as the responsible kinases which in both cases appears to target residues in the C-terminal helix of the molecule (Hansson et al 1988; Singh et al 1996, De Corte et al 1997, Björkegren-Sjögren 1997). Apparently, binding of PIP2 enhanced the availability of profilin for being targeted by the kinases, and it has also been reported that the modification interferes with poly-proline binding, (Björkegren et al, 1993). This is in agreement with the importance of the C-terminal helix of profilin for the PIP2-binding and poly-proline-interaction. For several years, it remained unclear whether phosphorylation of profilin occurred in vivo in mammalian cells. However, a recent study by (Shao et al., 2008) has shown that the kinase ROCK1 phosphorylates profilin in vivo in HEK293 and primary neurons.

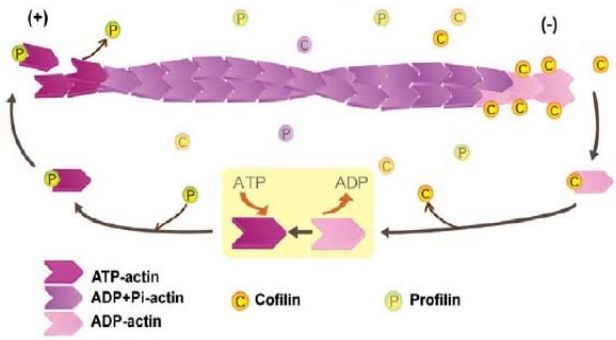

Figure 4. The role of profilin in actin polymerization. In cells actin filament treadmilling is regulated by actin binding proteins. Profilin accelerates nucleotide exchange from ADP to ATP on the actin monomer to promote the incorporation of ATP-bound monomers to filament (+)-end, whereas ADF/cofilin accelerates the dissociation of ADP bound actin from filament (-)-ends. (Lee and Dominguez, 2010).

Profilin:actin regulating proteins

Ena/VASP

The vasodilator-stimulated phosphoprotein (VASP) is the first characterized member of the Ena/VASP family of proteins; it is a profilin ligand, recognizing the poly-proline binding site on profilin (Reinhard et al 1995a), and operate as a filament elongator. In addition to profilin-binding, Ena/VASP interacts with a number of microfilament associated components through its N-terminal Wasp homology (WH)-1 domain (Reinhart et al., 1995b; Rebowski et al., 2010), formerly this binding domain was known as the Ena/VASP homology (EVH1) domain. Furthermore, Ena/VASP binds both monomeric and filamentous actin via a WH2-domain (formerly EVH2) closer to its C-terminus and finally also interacts with VASP itself through its tetramerization domain (Bachmann et al, 1999). Formation of a tetramer is required for

Ena/VASP to be able to work as a processive actin filament elongator. Recently, protein structure and biochemical studies of VASP points to a mechanism during actin polymerization where VASP transfers P:A via a series of binding sites towards the filament (+)-end (Ferron et al., 2007).

Ena/VASP localizes to the leading edge of migrating cells, and work underneath the plasma membrane by binding to proteins bound to the membrane such as lamellipodin via its WH1 domain. VASP has also been localized to the tip of the filopodia, possibly this location is the result of a myosin X-dependent transport (Tokuo and Ikebe, 2004). Its notable that myosin X has been shown to be involved in transport of integrins to filopodial tips (Sousa and Cheney, 2005) and similarly in Drosophila a unconventional myosin is operating to localize a cadherin to cell protrusions (Liu, 2008), supporting the view that also Ena/VASP undergoes intrafilopodial transport. Ena/VASP is also a prominent component of stress fibers and focal adhesions (Reinhard et al., 1992), where it appears to participate in the elongation of bundled actin filaments (Gertler et al) and in protein-protein interactions on the cytoplasmic side of cell attachments, respectively.

Ena/VASP contains four profilin-binding GP5-motifs (Reinhard et al., 1995); interestingly VASP has a higher affinity for profilin:actin compared to profilin alone (Chereau and Dominguez, 2006; Ferron et al., 2007), making it likely that it is profilin:actin that is recruited by VASP for filament elongation in vivo. The concentrations of VASP at the cell periphery have been seen to correlate with the velocity by which the cell edge advanced over the substratum (Rottner et al., 1999a). In a breast cancer cell line after profilin down regulation also the VASP concentration was decreased however the speed of cell migration increased with increasing localization of VASP at the cell periphery (Bae et al., 2009). This indicates that increased levels of VASP in the absence of profilin in some way can recruit actin for filament growth from other sources than profilin:actin.

WASP/WAVE-family of proteins

The Wiskott-Aldrich syndrome protein (WASP) and its ubiquitous variant N-WASP is another important actin nucleation and elongation promoting protein which also binds profilin. It is found

at the cell periphery and is particularly enriched in the filopodia. It has been reported that VASP binds to WASP and enhance actin polymerization (Castellano et al., 2001, Yarar et al., 2002). At the N-terminus the WASP homology 1 (WH1)-domain and the pleckstrin homology (PH)-domain, respectively mediate binding to a range of different proteins and to $PI(4,5)P_2$ in the plasma membrane. These interactions are important for the localization of the protein at the lipid bilayer as well for the control of its activity (Co et al., 2007). The later is also under strict regulation by the RhoGTPase Cdc42 which binds to the so called GBD/CRIB-domain and causes a conformational change (Kim et al., 2000). The binding of $PI(4,5)P_2$ and Cdc42 both contribute to the activity of N-WASP and is required for its nucleation promoting activity. It also contains a VCA-domain, which binds monomeric actin and the Arp2/3-complex. It has been reported that the VCA-domain could become phosphorylated at two serine residues and as a result increased affinity for Arp2/3 (Cory et al., 2003). It has been shown under in vivo conditions that the VASP/profilin-binding region in N-WASP is required for its participation in actin polymerization (Yarar et al., 2002), suggesting that also this protein recruits profilin:actin for actin polymerization.

Other members of the WASP family are WAVE/Scar, WASH, WHAMM and JMY (Rottner et al., 2010). Of these WAVE is the most well characterized (Miki et al., 1998). WAVE/Scar proteins lack the GBD/CRIB-domain. It has been shown that IRSp53 (a substrate for the insulin receptor tyrosine kinase), forms a trimeric complex with WAVE and activates Rac and that IRSp53 is essential for Rac induced ruffling (Miki et al., 2000).

The Arp2/3 complex

The Arp2/3 complex consists of seven subunits. It is often referred to as a nucleator of actin polymerization, however, this activity requires that it binds to different nucleation promoting factors (NPFs), like N-WASP. The interaction with N-WASP is at the C-terminal and results in the exposure of the two actin related molecules Arp2 and Arp3 such that they form a nucleation surface for incoming actin monomers by mimicking a (+)-end-free actin dimer (Firat-Karalar and Welch, 2011). In the case of N-WASP the Arp2/3 activity is under Rho GTPase regulation since the WASP-molecule is kept in an auto inhibited state until binding of activated Cdc42 occurs. As mentioned above, the regulation of this protein machinery involves several other

components such as: WASP interacting protein (WIP), Toca-1, and PI(4,5)P_2 (Takenawa and Suetsugu, 2007).

The Arp2/3 is essential for lamellipodia formation (Steffen et al., 2006). Other proteins involved in the process of Arp2/3 dependent filament formation are capping protein (CP) and ADF/cofilin which enhances disassembly of actin from the (-)-ends of the filament and also may cause severing of the filament, have been proposed to increase the rate of polymerization, whereby the elongation is concentrated to a lower number of available (+)-ends, making the cells more dynamic (Le Clainche and Carlier, 2008). The turnover rates of Arp2/3 and WAVE in conjunction with cofilin and CP compared to actin have been determined in FRAP experiments (Lai et al., 2008). Recovery of fluorescence was seen first at the tip of the lamellipodium, which then moved inwards demonstrating the constant turnover of actin monomers and also the existence of treadmilling in vivo. The Arp2/3 complex and the WAVE complex was shown to recover fluorescence similarly to actin, but while WAVE remained at the tip of the lamellipodium, the fluorescence of Arp2/3 moved inwards possibly as a consequence of the Arp2/3 complex being associated with the filament (-)-ends. In the same study cofilin doesn´t show the same pattern of actin and thereby proving that cofilin is not involved in polymerization, instead it is known that cofilin has an effect on actin turnover due to its ability to increase disassembly of actin filaments. CP had a similar turnover rate to actin and is most likely the major polymerization terminator (Lai et al., 2008). CP has a role to keep actin filaments short and in constant number in the lamellipodia and depletion of this protein causes a decrease in number of lamellipodia and increase of filopodia. The filopodia formed after CP knock down are dependent on Ena/VASP (Mejillano et al., 2004).

Formins

Formins represent a family of proteins important for the control of actin polymerization and which operate differently compared to the WASP and WAVE family of proteins. Importantly the formins functions independently of the Arp2/3 complex. Typically these proteins contain two highly conserved so called formin homology 1 (FH1) and FH2 domains. The FH1 domain is poly-proline rich and is known to bind the profilin:actin complex, and the FH2 domain although it does not bind monomeric actin still nucleates actin in vitro. Precisely how the latter occurs is

unclear. Possibly the FH2 domain binds and stabilizes spontaneously formed actin nuclei, i.e. actin dimers and trimers and thereby stimulates polymerization by facilitating the elongation. Whether this is of relevance for the situation in vivo, however, is uncertain since as far as we understand today, monomeric actin in vivo is associated with profilin and thymosin and thereby inhibited to undergo spontaneous self-assembly. Thus, formins may not operate as nucleation factors in vivo. Many formins include a DAD-domain (Dia autoregulatory domain) at its C-terminus, which mediates autoinhibition by interaction with the N-terminus and is under control of the small GTPase RhoA (Romero et al., 2007).

Most studies of the formins have been done with mouse diaphanous 1 and 2 (mDia1 and mDia2). These assemble into homodimers and via the FH2 domain they form a ring-shaped structure around the (+)-end tip of the actin filament which moves processively with the growing (+)-end. In vitro studies of actin filament elongation from beads functionalized with mDia1 FH1-FH2 fragments demonstrated a dramatic increase in elongation rate in the presence of profilin compared to actin alone (Romero et al 2004). This led to the insight that the preferred actin source for formin-dependent filament formation is profilin:actin. To allow for the rapid processivity it is required that profilin is dissociated after the profilin:actin complex has been docked onto the growing filament end. If this occurs in conjunction to the hydrolysis of the actin bound ATP or not remains to be shown (Jégou et al., 2011; Pollard and Borisy, 2003).

Organization and the control of actin dynamics

Just underneath the plasma membrane the microfilament system forms a highly dynamic arrangement where actin polymerization and filament bundling results in formation of protruding structures like lamellipodia and filopodia some of which connect to the more internal stress fibers (Fig. 5). These are formed by actin under the control of several protein components some of which are mentioned above, and they are described below.

Lamellipodia consists of well ordered, densely packed actin filaments that often run in parallel towards the plasma membrane. This way, thin sheet-like structures are generated directly underneath the inner leaflet of the membrane forming a system of filaments with associated proteins that builds a dynamic support for the lipid bilayer (Höglund et al. 1980; Small et al.

1982, Urban 2010). As mentioned above, the filaments are all organized with their fast polymerizing end at the membrane. The incorporation of actin monomers at the tip of the lamellipodia and the rapid turnover of microfilament organization have been followed by using approaches based on fluorescent labeling of actin molecules in combination with techniques such as fluorescent recovery after photo-bleaching (FRAP) and fluorescent loss in photo-bleaching (FLIP). These experiments show that a major part of the actin monomers in the region interior to the lamellipodia called the lamella, is somehow transferred at high rate to the cell periphery to be used for filament elongation (Lai et al. 2008). At present it is unclear how this is accomplished in order to provide enough of monomeric actin at the polymerizing sites to support the extensive filament growth that brings the lamellipodial edge to advance.

Filopodia are spike-like protrusions extending from the cell surface at lamellipodia and sometimes also from other regions of the cell (Fig. 5). Like lamellipoda they are often very dynamic, especially at advancing edges where they form and retract in constantly new constellations before attaching either to the substratum or to neighboring cells. This way they appear to search the cell surroundings and direct its migration. Their core structure is a densely packed bundle of filaments organized with their fast polymerizing end towards the tip and whose number can vary quite extensively depending on cell type and activation; an average filament number based on published observations of the filopodial diameter is approximately 300 nm (Koestler et al., 2008) . This number, together with the velocity whereby the tip of the edge extend over the substratum (3.5 μm/min) and the increase in filament length per added actin monomer (2.7 nm) indicate that about 2200 actin molecules are needed per second for each filopodia.

The most extensive filament elongation at the cell periphery of migrating cells needs constant incorporation of profilin:actin complex. The profilin:actin complex is needed in a high concentration and ready to be fed onto the filament (+)-end. There is yet no explanation of how the transport of the monomeric actin and profilin or the profilin:actin complex is achieved to the polymerization sites, neither where the profilin:actin complex are formed. As mentioned above, there are observations indicating that myosin X can transport integrins and VASP along actin filaments within the protruding filopodia (Berg and Cheney 2002; Tokuo and Ikebe, 2004); it is

possible that monomeric actin is transported with a similar mechanism to the filopodia tip. Notably, studies in our lab show a staining pattern were cross-linked profilin:actin is partially co-distributed with microtubules, which indicate that profilin:actin might be transported along microtubules to actin polymerization sites (Grenklo et al. 2004), these observations will be discussed further in this thesis.

Another actin organized structure in cells is the stress fibers, which exists of bundles of 10-30 antiparallel actin filaments (Cramer et al. 1997), bipolar myosin II and actin-crosslinking proteins such as α-actinin, fascin, espin and filamin. In fibroblasts there are three types of stress fibers: ventral stress fibers, dorsal stress fibers and transverse arcs (Small et al. 1998). The ventral stress fibers are the most commonly observed, and that also can be seen in (Fig. 5). They are anchored at both sides by focal adhesions, protein complexes containing integrin receptors which contact the extracellular matrix.

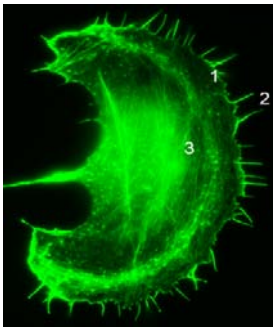

Figure 5. B16 cell labeled with FITC phalloidin to visualize actin filaments giving rise to: 1. lamellipodia generated at the cell periphery, underneath the plasma membrane, 2. during cell migration, cells extend fingerlike structures called filopodia to sense the environment and 3. Stress fibers which are built up by thick bundles of anti-parallel actin filaments and bipolar myosin II.

The microfilament system and signal transduction

The mechanisms that control the organization of actin are fundamental to understand the dynamics of cell motility. The actin binding proteins involved in the regulation of actin (described above), are all downstream targets of trans-membrane receptor signaling. Among the components recognized as central for actin reorganization are the Rho GTPases and the phosphoinositol lipids. The Rho GTPases belong to the Ras superfamily of small GTPases and switch between an active, GTP-bound state and an inactive, GDP-bound conformation (Ridley 2011). Rho GTPases can be activated by multiple different guanine exchange factors (GEFs) at

the leading edge, and switch back to their inactivated state by so called GTPase activating proteins (GAPs). Of the Rho GTPases, RhoA, Rac and Cdc42 are most well characterized involved in microfilament system regulation. They activate distinct pathways, generating specific actin organizations. RhoA causes stressfiber formation, activation of Rac leads to ruffling and lamellipodia formation and Cdc42 triggers generation filopodia. The WASP, WAVE and formin families of proteins are examples of actin nucleation and elongation factors regulated by the family of RhoGTPases. Another example is cofilin which is indirectly controlled by all three GTPases. In case of RhoA, a downstream effector is ROCK, a kinase whose substrate is the LIM-kinase (LIMK) which in turn phosphorylates cofilin, thereby blocking cofilin's ability to bind actin (Bernard 2007), and also Cdc42 and Rac can influence cofilin indirectly. In their case it is through their common target PAK, which acts similarly to ROCK by phosphorylating LIMK. As mentioned earlier, cofilin inactivated by phosphorylation is reported to be recycled back to the plasma membrane where it binds $PI(4,5)P_2$ (van Rheenen et al., 2009).

The Rho GTPases are also coupled to phospholipid signaling. Stimulation of cells with growth factors and other agonists leads to activation of the PI3-kinase leading to increased amounts of $PI(3,4,5)P_3$ and $PI(3,4)P_2$. Particularly $PI(3,4,5)P_3$ is important for activation of RacGEFs which in turn will stimulate Rac-dependent PI4-5-kinase in a positive feed-back loop, leading to increased formation of $PI(4,5)P_2$. The $PI(3,4,5)P_3$-producing activity of the PI3-kinase is balanced by PTEN, a phosphatase converting $PI(3,4,5)P_3$ to $PI(4,5)P_2$ (Ridley et al., 2003).
Cdc42 can also direct cell polarity by orienting the MTOC and Golgi apparatus in front of the nucleus (Lindberg et al., 2008).

Interaction between the microtubule and microfilament systems

Early cell biology studies demonstrated a close connection between the microtubule and microfilament systems, for instance drug treatments that disrupted the microtubule system changed the morphology and migratory behavior of fibroblasts from an asymmetric shape with leading lamellae to a more rounded form with large lamellipodiae expressing extensive ruffling more or less all around the perimeter of the cells (Dominia et al., 1977). Lately an increasing amount of evidence for crosstalk between the two force-generating systems in the cell has accumulated. For instance, high resolution confocal microscopy has pointed to a closely related

pattern of dynamics of at least a subset of microtubules and microfilament organizations at the periphery of migratory cells (Rodriguez et al., 2003; Schober et al., 2007; Krylyshkina et al., 2003). Another example is the activity of the mitotic spindle, where the microtubule dependent separation of the chromosomes is spatiotemporally coordinated with the development and contraction of the actomyosin containing contractile ring and involves microtubule-associated actin regulatory components (Gundersen et al., 2004). In this context it has been reported that the formins mDia1 and mDia2 decorate mid-bodies of dividing cells (Tominaga et al., 2000). Furthermore, the actin filament binding FH2 domain of mDia1 and 2 binds directly to three microtubule (+)-end binding proteins: the end-binding protein 1 (EB1), adenomatous polyposis coli (APC) and cytoplasmic linker protein (CLIP)-170 (Wen et al., 2004), and mDia1 appears to induce co-alignment of microfilament and microtubule arrays in HeLa cells (Ishizaki et al., 2001). Notably, members of the WASP family, the WASH and WHAMM proteins mentioned above, in addition to their interactions with actin and the Arp2/3 complex, also associate with microtubules and cause actin bundling when over-expressed (Campellone et al., 2008)

ACTIN IN TRANSCRIPTIONAL REGULATION - TOWARDS THE NUCLEUS

Gene expression through the transcription factor serum response factor (SRF) is regulated by at least two signaling pathways, controlling the SRF co-activators ternary complex factor (TCF) and myocardin-related transcription factor A (MRTF-A/MAL) see (Fig. 6). The former results from activation of G-protein coupled receptors (GPCRs) and receptor tyrosine kinases, which transmit signals via Ras to the mitogen-activated kinase (MAPK) cascade, resulting in TCF-dependent gene expression (Treisman, 1995). The other pathway leading to MRTF-A and SRF activation requires changes in actin dynamics (Sotiropoulos et al., 1999) for example after activation of the RhoGTPases RhoA or Rac (Posern et al., 2002).

The MRTF-A co-transcription factor binds directly to unpolymerized actin via its so called RPEL motifs at the N-terminus. The interaction surface on the actin is formed by subdomains 1 and 3 at the end of the actin monomer corresponding to the surface exposed at the (+)-end of the filament and therefore may compete with proteins like profilin and cofilin (Mouilleron et al 2008). Upon stimulation with Rho activators such as lysophosphadidic acid (LPA), actin

polymerization for instance as a consequence of formin activation (see above) causes release of MRTF-A from monomeric actin, allowing for its entrance to the nucleus where activation of SRF-controlled transcription then will take place (Vartiainen et al., 2007).

It is noteworthy that both profilin and actin are present in the nucleus (Bettinger et al 2004, Pederson et al 2005, Visa and Percipalle 2010). Although the precise role of these proteins in this compartment is far from fully understood it has been reported that actin functions in chromatin remodeling and transcription (Bettinger et al 2004, Pederson et al 2005). It has not been possible to fully establish if actin filaments are present in the nucleus, but since many of the components involved in regulating actin polymerization in the cytoplasm such as in addition to profilin, cofilin, VASP, WASP and formins to name few (Castano et al., 2010) together with the fact that also myosins are there, it seems reasonable that actin is present in the nucleus in filamentous form. In the case of profilin, it has been associated with splicing (Skare et al 2003) and transcription (Lederer et al 2005). The nuclear export protein, exportin 6, specifically transports profilin and actin from the nucleus to the cytoplasm (Stuven et al 2003) and this has led to the conclusion that the profilin:actin is a component of the nucleus (Stuven et al., 2003, Jockusch et al., 2007). This may not be the case however since the two proteins may have bound to the exportin molecule in succession and hence the complex formed at the stage of export out of the nucleus.

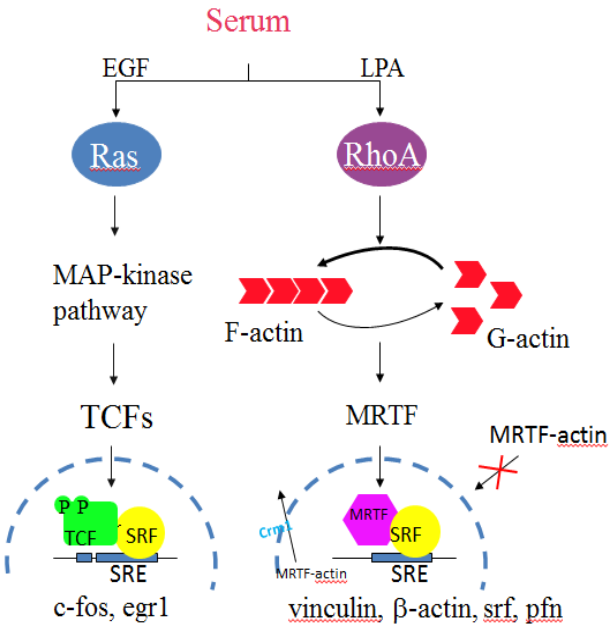

Figure 6. Signaling pathways and activation of SRF by its co-activators TCF and MRTF. The MAPK cascade results in activation of TCF and the Rho signaling pathway results in actin polymerization and consequently accumulation of nuclear MRTF. When MRTF is bound to actin it cannot enter the nucleus and start transcription, while MRTF-actin in the nucleus is needed for its export out from the nucleus. The activation of SRF by its two co-transcription factors TCF and MRTF is mutually exclusive.

PRESENT INVESTIGATION

The overall aim of my research is to understand the role of profilin during cell migration and proliferation. As part of this object I have addressed the following aspects of the function of profilin in this thesis.

1. The connection between profilin/profilin:actin and microtubules in B16 mouse melanoma cells (Study I).

2. The connection between SRF/MRTF-A and actin dynamics with respect to the role of profilin I and II (Study II).

Summary of present investigations

Study I

Since profilin:actin is the principal precursor form of actin for filament formation, and recognized by several of the actin polymer-forming machineries in the cell it is important to learn where and when the complex forms and how it reaches the cell periphery. Previous studies in our laboratory pointed to a role of the microtubule system in this context (Grenklo et al., 2004, Johnsson and Karlsson 2010). In Study I the *Proximity Ligation Assay* (Gustafsdottir et al., 2005) was used to further analyze these observations, and this led to the conclusion that profilin and actin co-distribute with the microtubule system. This was also supported by western blot analysis of microtubule-enriched cell extracts. The component(s) linking profilin and/or profilin:actin to the microtubules remains to be identified. Possibly this is connected to ongoing translation of profilin from microtubule-associated profilin mRNA (Johnsson and Karlsson 2010 EJCB 89, 394-401).

Study II

Actin is indirectly involved in gene expression by controlling the activity of the transcription factor SRF, which controls a large number of genes, including many encoding microfilament associated proteins like actin itself and profilin. Changes in the monomeric actin pool is sensed through the SRF co-activator MRTF-A. MRTF-A is an actin monomer binding protein under constant nucleocytoplasmic shuttling. It competes with profilin for actin binding and upon serum stimulation it dissociates from actin and accumulates in the nucleus.

In Study II it is shown that the SRF/MRTF-A pathway depends on the presence of profilin I and II. Down-regulation of profilin I expression using siRNA interferes with the nuclear accumulation of MRTF-A fused to GFP, and siRNA-depletion of profilin I and II represses SRF/MRTF-A-controlled transcription as seen using a plasmid reporter system. Moreover, profilin-depleted cells showed a more directional migration albeit their movement occurred with reduced speed.

Future experiments

Study I

Our aim is now to continue with biochemical experiment to further analyse the connection between profilin and microtubules. By the microtubule partitioning assay we have been able to follow profilin after stabilization and destabilization of microtubules in Western Blot. We are now in the process of performing co-immunoprecipitations with tubulin and its motor protein kinesin to see if there is a direct linkage between these two proteins and profilin. In another approach we intend to test whether incubation of whole cell extracts on glascover slips to which microtubules have been pre-added would result in the capture of endogenous profilin or profilin after over-expression. These experiments will be performed in combination with addition of the non hydrolysable ATP-analog AMP-PNP which interferes with the dissociation of kinesin from the microtubules. Successful entrapment of profilin would then open up for the incorporation of previously characterized mutant profilin constructs in order to identify the nature of the connection to the microtubules.

Study II

Experiments in progress are a qPCR analysis to provide more information about the expression of profilin I and II and their influences on each other in B16-F1 cells after profilin siRNA. We also intend to analyze the relative distribution of MAL in the cytoplasm and the nucleus after down regulation of the two profilin isoforms. We are going to establish the SRF-luciferase assay for analysis of the actin binding capacity of different fusion variants of profilin, as well as the use of this assay as a method to measure the status of actin polymerization after different treatments of cultured cells.

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Profilin is Associated with Microtubules in Cultured Cells

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Summary

Characterization of new members of the actin nucleation factor families and recent progress in membrane trafficking suggest that actin polymerization can take place from microtubule-associated protein platforms. In this study we have combined profilin and tubulin antibodies for proximity ligation in cultured B16 mouse melanoma cells and reveal that a fraction of cellular profilin is associated with microtubules as defined by this technique. This result is further supported by biochemical analysis using a microtubule partitioning assay. Together these observations suggest that microtubule-coupled polymerization of actin depends on recruitment of profilin:actin.

Introduction

Actin polymerization – the directional growth of actin filaments as a consequence of ordered addition of new actin molecules to the favored (+)-end of the filament – is a fundamental and tightly regulated process and a prerequisite for numerous cell biology phenomena. Not least during cell migration rapid filament growth plays an essential role in advancing the leading edge with lamellipodia and filopodia forward over the substratum [1]. The structural and biochemical asymmetry of the filament form the basis for the directionally of the force generation and is maintained by hydrolysis of ATP on the incoming actin subunit at the filament (+)-end, e.g. [2-3].

The rapid elongation of the assembled filaments building the core structure of the lamellipodia and filopodia requires a rapid continuous supply of subunits to the very tip of the extending protrusions. There is strong evidence for actin molecules to be re-circulated from the inwards pointing (-)-end of the filaments to sites of polymerization at the front [4]. Actin dissociating from the (-)-ends is sequestered by cofilin and, via a not fully understood process involving the cyclase associated protein and profilin [5-7], the actin-bound ADP is replaced by ATP resulting in formation of polymerization competent ATP-actin bound to profilin, i.e. profilin:actin, which in turn is recruited to the growing (+)-ends by various nucleation and elongation factors [3, 8]. Additionally, newly translated actin from messenger RNA at the periphery is thought to contribute to the local maintenance of the actin rich zone immediately underneath the inner leaflet of the plasma membrane [9-10].

Despite a wealth of information about the regulation and dynamics of actin organization in lamellipodia and filopodia it still remains to understand how the necessary amounts of non-filamentous ATP-bound actin is brought to the polymerization sites at the tip of the protrusions. Based on the drastic interference with actin polymer formation seen after microinjection of a covalently coupled, non-dynamic profilin:actin complex [11] into spreading and growth stimulated cells as well as after infection with the intracellular pathogen Listeria monocytogenes [12-13], we have previously suggested that profilin:actin is a major source for polymerization-competent actin in the cell [3]. Like actin mRNA, also profilin mRNA is located to regions exhibiting intensive turn-over of the submembraneous actin

arrangement, but in contrast to the actin encoding messenger, the profilin mRNA depends on microtubules for its distribution [14]. A possible scenario is that actin picked up by profilin at the inner edge of the lamellipodium from the cofilin/CAP-complex or after translation and folding and then is actively transported to protrusion tips. Precedence for this exists, for instance integrins are known to be trafficked by myosin X to the most distal end of filopodia [15]. Similarly the actin elongator protein VASP has been reported to undergo intrafilopodial trafficking in a myosin X-dependent process and there are also observations that Drosophila cadherin is transported to filopodial tips by an unconventional myosin [16-17].

To further understand how profilin:actin is supplied to the leading edge we have decided to reinvestigate and enhance the analysis of previous results [18], suggesting that a fraction of profilin and possibly also profilin:actin consistently co-localizes with microtubules that extend through the cytoplasm towards the cell periphery. Whether this profilin results from translation of microtubule associated profilin mRNA while under distribution to the cell periphery is unclear but possible [19]. Notably in this context, new members of the WASP family of proteins such as WASH [20] have led to the insight that for instance endosomal membrane transports are under combined control by the microtubule and microfilament systems and may actually be driven by actin nucleation factors, which uses the microtubule system as a platform further emphasizing the close connection between the two force-generating systems in the eukaryotic cell, e.g. [21]. Here we have used the so called proximity ligation technique [22] to further analyze profilin's association with microtubules, and note a significant association of profilin with the microtubule system.

Materials and Methods

Cell culturing

Mouse melanoma B16-F1 cells were cultured in DMEM (Thermo Scientific) supplemented with 10% fetal calf serum (FCS) at 37°C in the presence of 5% CO_2. The cells were cultured over-night on glass coverslips in 24-well plates (17 000 cells/well) pre-coated with 25µg/ml laminin (Sigma, cat no: L2020) for 2 hours at 37°C. In some experiments the cells were exposed to nocodazole (Fluka, cat no 74151) as indicated prior to fixation.

Proximity Ligation Assay (PLA) and Fluorescence Microscopy

Cells cultured on coverslips as above were fixed for 20 minutes at 37°C with 4% formaldehyde (Sigma) in PBS, pH 6.8, containing 5mM EGTA to stabilize the microtubules and then demembranated with 0.1% Triton-X in PBS for 10 minutes at RT. The coverslips were pre-washed in PBS-EGTA, and incubated with the PLA-blocking solution (Olink Biosciences) for 30 minutes 37°C. Samples were then incubated with primary antibodies A_I, A_{II}, P_I and P_{II} (generated in hens against cross-linked profilin:actin [11] and affinity purified as described in [18, 23]; all diluted 1:20), anti-α-tubulin generated in mice (Abcam, cat no: ab7291; 1:200), anti-α-tubulin rabbit (Abcam, cat no: ab18251; 1:200) and anti-β-arrestin 1 (Santa Cruz, cat no: sc9182; 1:20) for 1 hour at RT. Secondary antibodies conjugated with oligonucleotides (PLA probe MINUS and PLA probe PLUS) were added to the reaction and incubated for 35 minutes at 37°C, followed by addition and incubation with the hybridization solution containing oligonucleotides for 15 minutes at 37°C. The ligase (Ligation solution) was added and incubated for 15 minutes at 37°C, followed by nucleotides and polymerase and allowing for rolling-circle amplification for 35 minutes at 37°C. Finally, the product was detected by incubation with fluorescent oligonucleotides for 50 minutes at 37°C. This solution also contained the FITC-labeled secondary antibody (Jackson ImmunoResearch laboratories, cat no: 705-095-003; cat no: 711-095-152) added for visualization of microtubules simultaneously with the PLA-signal. The coverslips were mounted in ProLong Gold (Molecular Probes) and microscopy performed using a Leica DMLB microscope equipped with 63x objective lens and a DC350F CCD camera (Leica Microsystems). The PLA signals were visible as fluorescent dots. Documentation was performed using the same exposure time within each series of samples keeping gain unchanged.

Microtubule partitioning assay and western blot

Cells grown overnight were trypsinized (Thermo Scientific), suspended in DMEM (Gibco) supplemented with 10% FCS and adjusted to a final concentration of 2x 10^6 per ml, followed by treatment with either Taxol (Paclitaxel; Sigma cat no T7402) for 4 minutes at 37°C or Nocodazole (Fluka; cat no 74151) for 15 minutes at 37°C and then centrifuged at 1200g in an Eppendorf centrifuge for 30 seconds. The pelleted cells were resuspended in 200µl 80 mM PEM (Pipes 100mM, 1mMEGTA, 1mM $MgCl_2$) pH 6.9, supplemented with 0.5% Tx-100, 4 mM EGTA, 5µl/ml Leupeptin (Sigma cat no: L0649). The resulting extracts were centrifuged at 1800xg for 30 seconds, followed by acetone precipitation over night at - 20°C while the pelleted material was left at - 20°C over night. The acetone precipitated supernatant material was centrifuged resuspended in 200 µl of SDS-PAGE sample buffer and boiled immediately, and the pellet material was boiled in same volume of sample buffer. The samples were loaded immediately after boiling onto a 12% SDS-polyacrylamide gel. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Hybond-C Extra; Amersham Bioscience) at 5mA/cm^2 for 2 hours. The membrane was incubated in 10% skim milk in Tris-buffered saline with Tween-20 (TBST) overnight, followed by primary antibodies: anti-α-tubulin (Abcam, cat no: ab7291; 1:2000), anti-β-actin (Sigma, cat no: A5441 "AC-15"; 1:4000), anti-GAPDH (Abcam, cat no: ab8245; 1:40 000), anti-profilin I (own laboratory, raised in rabbits against human profilin I; 1:2000). After incubation with HRP-conjugated secondary antibodies against mouse (Dako, cat no: P0447) and rabbit IgG (Pierce, cat no: 1858415) with dilution of 1:2000, the membrane was developed using West Dura HRP substrate (Pierce). Images were captured using the Quantity One software (Bio-Rad).

Results

Antibody labeling of profilin in cells of the highly motile mouse melanoma cell line B16, result in a fenestrated pattern of fine dots that accumulate towards the perinuclear region, Fig 1. Also at the very tip of the broad lamellipod often displayed by these cells it was sometimes possible to observe a thin 'band' of intensified fluorescence that is interpreted to mark the inner lining of the plasma membrane. A similar pattern of distribution has been reported for fibroblasts as well as other cultured cells [18, 23-24], and co-distributions of fractions of profilin staining with both actin stress fibers and microtubules have been observed, e.g. [18, 24].

Although variable, the profilin containing dots are closely packed, causing a rather intense general fluorescence of the cell and posing problems to visualize changes in the distribution of the microtubule-associated fraction of profilin, compare with the microtubule array in Fig 1. Therefore we decided to use the proximity ligation assay (PLA) to investigate whether the profilin antibodies would signal a co-distribution with microtubules as defined by this technique, which is reported to result in a positive signal for any pair of antibodies aimed for that are not separated by more than 40 nm. Since profilin and profilin:actin display a general distribution indicating their presence in all areas of the cell, antibodies to a protein with a similar general distribution but not reported to bind microtubules were necessary to be introduced as a control of the specificity of the labeling. The choice fell on β-arrestin, which distributes in a dot-like pattern all over the cell much like the profilin, Fig 2.

Upon combination of profilin antibodies and antibodies versus tubulin, PLA typically resulted in a large number of discrete red signals signaling a separation of only 40 nm or less for the two antibodies. After optimization of the technique, introducing two different secondary antibodies for the tubulin antibody it was possible to visualize the microtubule array simultaneously with the PLA-signal as described in the materials and methods section. This demonstrated that virtually all PLA-signals were localized to microtubules in agreement with the detection of a microtubule associated antigen, Fig 3. There was weak tendency of the PLA-signals to accumulate towards the outer parts of the lamellae although microtubule associated signals were observed in most parts of the cells. The result with the profilin-tubulin pair of antibodies was in striking contrast to control experiments where the β-arrestin and

tubulin antibodies were combined, Fig 3B, suggesting that the PLA-signal in the first case reflected the close co-localization of profilin to microtubules. Microtubule depolymerization by nocodazole treatment of the cells before fixation and antibody incubation led to reduced PLA-signal, while the β-arrestin-tubulin antibody pair resulted in the similar signal level as with the intact microtubule system, Fig 4.

If the profilin detected by PLA to be associated with microtubules is a translation product of microtubule-associated profilin mRNA it is clear that the co-distribution reflects an indirect association of profilin with the microtubule system, and although tubulin has been captured from a brain tissue extract on a profilin column [25] there are no evidence for a direct interaction between profilin and tubulin. Based on this and the supposition that profilin mRNA is trafficking to sites of actin polymerization on microtubules under the influence of kinesin [14], we decided to employ PLA to study co-localization of profilin and kinesin, Fig 5. As before the distribution of the microtubule system was simultaneously displayed and thus provided an additional internal control by enabling a direct correlation with the distribution of the tubulin polymers. As for the profilin-tubulin antibody pair described above we observed a dramatic difference when comparing the PLA-signal resulting from the profilin-kinesin labeling with that from the β-arrestin-kinesin control, and again practically all of the positive signals were associated with microtubules.

The above results were obtained with the P_{II} antibodies which have been generated against covalently coupled profilin:actin [11] and then affinity purified against profilin [13, 18]. However, qualitatively the same result was obtained with the P_I antibodies, which were generated by immunizing another animal and then purified as P_{II}. Thus, two different profilin antibody preparations let to the same observation, strongly suggesting that a fraction of profilin in fact is associated with microtubules. Moreover, in the course of the isolation of the P_I and P_{II} antibodies the highly specific actin antibodies denoted A_I and A_{II} were obtained by affinity purification from the corresponding total antisera [18, 23] and when combined with the tubulin antibodies for PLA again reproduced the result obtained with P_I and P_{II}, pointing to the possibility that at least some of the profilin detected as microtubule-associated in fact represents profilin:actin, [23].

To analyze the profilin/profilin:actin association with microtubules further we used a more biochemical approach, where B16 cells held in suspension were incubated with the

microtubule stabilizing and destabilizing drugs Taxol and Nocodazole, respectively, prior to lysis. The resulting extracts were then centrifuged to partition microtubules with their associated components from the rest of the material present in the extracts. Western blotting of the samples revealed a rather extensive co-sedimentation of profilin with the microtubules in samples resulting after Taxol-treatment, Fig 6. In contrast, the corresponding samples of non-drug treated cells or cells exposed to nocodazole contained dramatically less profilin in the pelleted fraction, essentially confirming the immunohistochemical result of a profilin-microtubule interaction. Densitometry of the western blot result demonstrated a 10 times increased amount of the transferred profilin into the pelleted material after Taxol-treatment compared to untreated cells, Fig 6B. Interestingly, the same pattern of co-partitioning with microtubules was invariably seen for GAPDH. The nature behind this result is unclear but since GADPH has been reported to associate with microtubules [26], possibly in a multi-protein complex that includes Rab2 and atypical protein kinase C [27], our observation points to profilin as having one or more microtubule-linking molecules in common with GAPDH.

Discussion

Cell motility is a highly combinatorial phenomenon involving numerous signaling and force-generating processes with the dynamically organized microtubule and microfilament systems as the key players controlling directionality and generating force. These two filament systems co-exist in most cells and it has become increasingly clear that their activities are highly coordinated during migration and cell growth in a productive way for tissue maintenance and development, e.g. [28-30]. In the context of this study where profilin and possibly profilin:actin is recognized to be microtubule-associated, it is interesting that the WASH and WHAMM members of the WASP family of Arp2/3-dependent actin nucleation factors directly binds microtubules [20]. These proteins are coupled to different intracellular membrane trafficking and endosomal receptor recycling processes, e.g. [21]. Furthermore, WAVE2 an isoform member of the canonical WAVE/SCAR family that operates together with Arp2/3 at lamellipodial tips to maintain actin dependent protrusion at the cell edge has been reported to be transported along microtubules in a kinesin dependent process [31-32]. Finally cortactin, also an Arp2/3-dependent actin nucleator, has been coupled to endosomal protein sorting and recycling [33].

With this rather extensive literature of actin nucleating factors being associated with microtubules and/or membrane trafficking it appears reasonable that a protein such as profilin, which is a central regulatory component of actin polymerization is associated with these processes. Since profilin:actin is a major source of actin for filament growth it might be that most of the microtubule-associated profilin detected with the proximity ligation technique in fact is profilin:actin. The fact that also the A_I and A_{II} antibodies resulted in positive PFA-signals when combined with tubulin antibodies argues in favor of this supposition, and since A_{II} specifically recognize actin in filamentous form [18] it appears that we are detecting actin filament growth from a microtubule-associated platform that recruits profilin:actin to feed the polymerization. It is noteworthy that the P_I and P_{II} antibodies in a recent paper [23] were proposed to recognize profilin:actin in addition to profilin and, correspondingly, the A_I antibodies appear to target the profilin:actin complex as well as actin in other non-filamentous constellations. Unfortunately, because all four of these antibody preparations are of avian origin (IgY) it is not possible to combine P_I or P_{II} with A_I for a PLA to investigate their possible co-distribution along microtubules as were done with kinesin. It remains to be seen

whether it is possible to combine the profilin-IgY with AC15, a mouse derived peptide antibody against non-filamentous β-actin (Grenklo 2004) that would enable specific localization of profilin:actin.

Transport of mRNA to specific sites in the cytoplasm, where the corresponding proteins are translated in juxtaposition to their proper location of action is a common phenomenon particularly for actin and actin regulatory proteins operating at the very cell edge, [9, 34]. Profilin is one of those whose distribution of messenger RNA depends on an intact microtubule system [14]. A tentative model based on this and the observations made here would be that the profilin mRNA is translated at sites along the microtubules, and the resulting protein then combines with actin to form profilin:actin to be used for filament formation by the microtubule-associated nucleation promoting factors. Protein translation in conjunction to microtubule-dependent mRNP transportation has been reported for the intermediary protein peripherin [19], providing precedence for a general model by which protein translation from a specific mRNA can occur at different cytoplasmic sites by trafficking of the specific mRNP.

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Figures and Legends

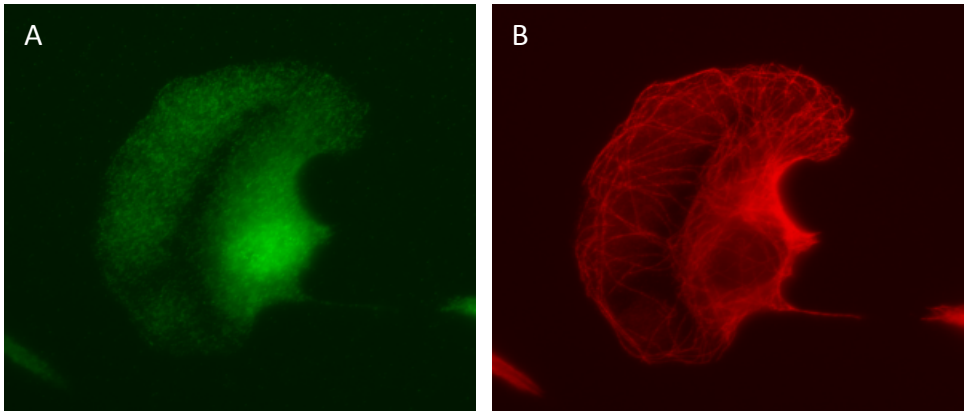


Figure 1: The distribution of microtubules and profilin in B16 cells as seen by antibody-labeling.

Mouse melanoma B16 cells cultured on glass cover slips were stained with antibodies against profilin (A) and tubulin (B). Co-distribution of the two patterns can be discerned in certain area however mostly it is difficult to evaluate this due to the general distribution of the fine dots representing profilin (and possibly profilin:actin). Clearly though it is only a fraction of the profilin that is associated with microtubules, see also [18].

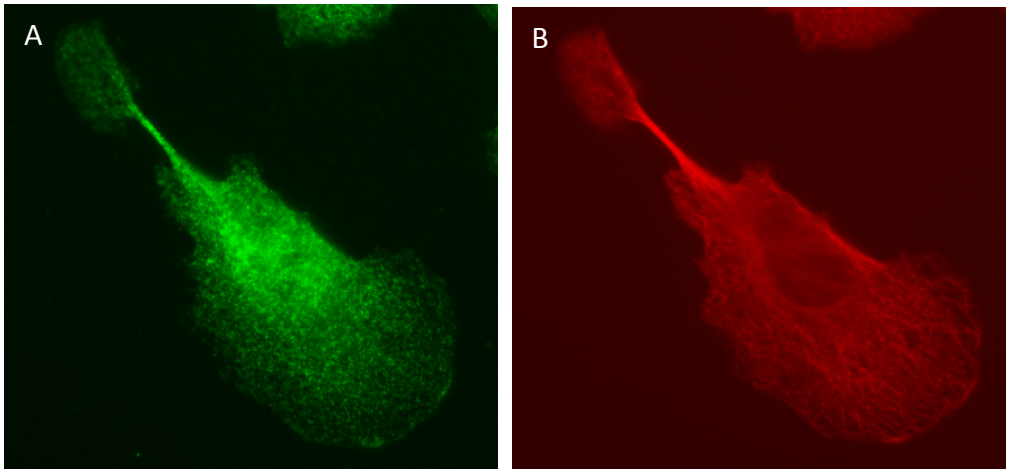


Figure 2: The distribution of β-arrestin in B16 cells.

Note that the β-arrestin antibody staining (A) reveals a general dot-like pattern reminiscent of that seen after labeling with profilin antibodies (Fig 1). Panel B displays the microtubules.

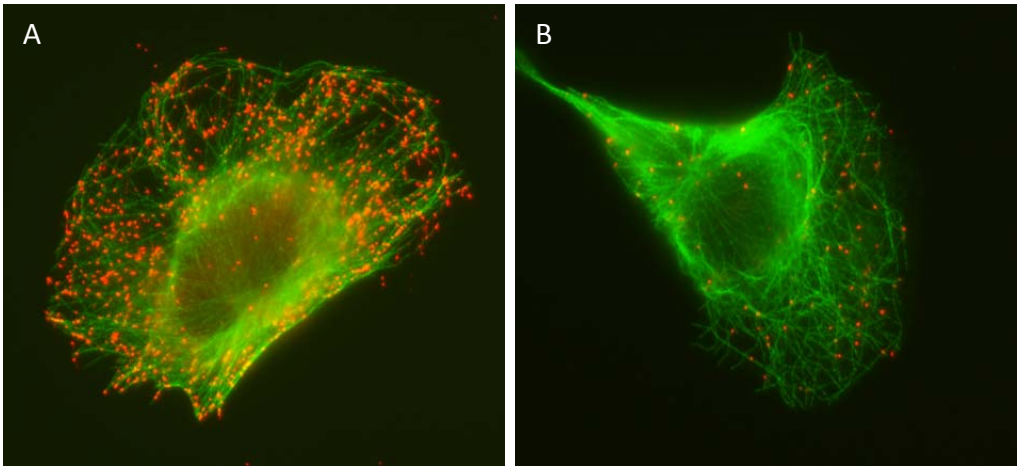


Fig 3: Localization of profilin in close vicinity (< 40 nm) of microtubules by the proximity ligation technique

Panel A shows the distribution of profilin to microtubule-close sites and in panel B the similar technique was employed with antibodies against β-arrestin for control. The drastic difference in density of the PLA-signal from the two experiments is obvious and suggests that the profilin-tubulin pair of antibodies detects a localization of profilin to the microtubules. Panel A, displays the result after combining tubulin and profilin antibodies; and panel B shows the result of a similar experiment using antibodies to tubulin and β-arrestin.

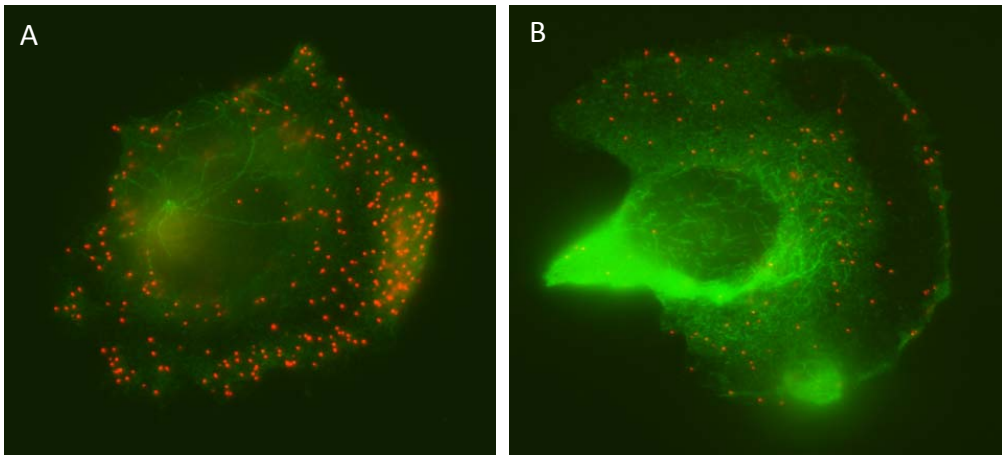


Fig 4: Localization of profilin (A) and β-arrestin (B) with PLA as in Figure 3 but after nocodazole-induced depolymerization of the microtubules. B16 cells cultured as in previous experiments were exposed to 2 μg/ml nocodazole for 15 minutes before fixation and preparation for microscopy as described in materials and methods.

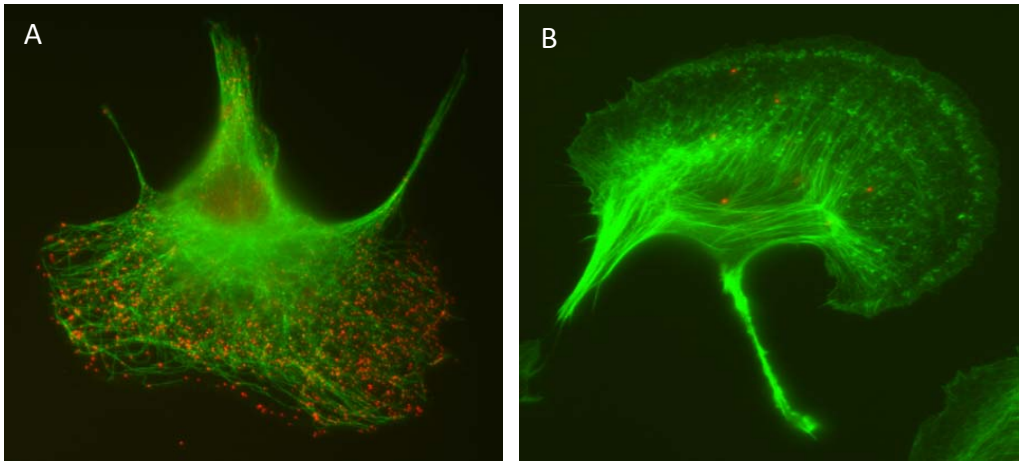
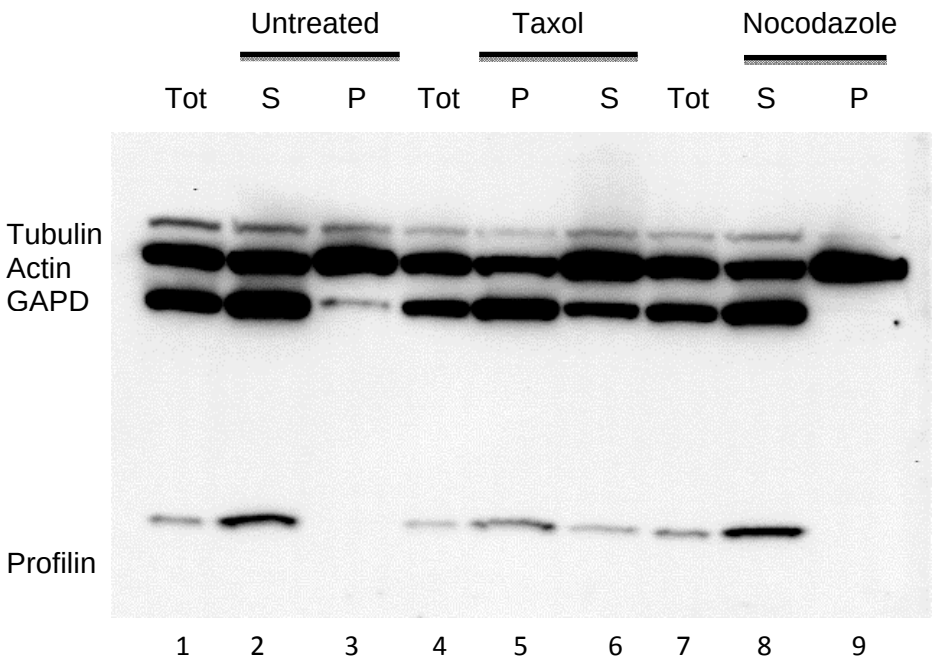


Fig 5: Co-localization of kinesin with profilin (A) and β-arrestin (B) using PLA in combination with fluorescence labeling of the microtubules. Note that the PLA-positive signals resulting from the profilin-kinesin antibody pair in A display extensive microtubule localization.

A



B

	Untreated			Taxol			Nocodazole		
	Tot	S	P	Tot	S	P	Tot	S	P
Tubulin	739	1009	570	653	978	514	694	1197	246
GAPDH	1233	2671	52	862	2812	663	1017	3064	7
Profilin	475	2280	24	420	2138	275	820	4349	5

Fig 6: Western blot analysis of profilin in samples after microtubule partitioning in extracts of drug-treated cells.

Panel A: Extracts of B16 cells after microtubule stabilization and depolymerization, respectively, by exposure to Taxol or Nocodazole, were centrifuged and the resulting supernatants (S) and pellets (P) were then analyzed for presence of profilin, actin, tubulin and GAPDH by western blot. Non-drug treated cells were included as controls (untreated). In all experiments involving partitioning of tubulin by centrifugation the same number of cells ($2x10^6$) was analyzed, while analysis of the total extracts (Tot) represents 2.5x less material ($80x10^3$ cells). Note that in Taxol-treated cells the tublin is shifted into the pellet fraction in comparison with untreated and nocodazole treated cells. Profilin and GAPDH both show a

similar distribution and are enriched in the pellet after Taxol treatment and in the supernatant after nocodazole. This result is typical of six independent experiments.
Panel B: Densitometry values of the bands displayed in Panel A, using the most optimal exposure for each protein and normalizing all values to the value for actin in the middle Tot lane (lane 4). Note the similarity in distribution between profilin and GAPDH in supernatant and pellet.

Profilin I and II are both influencing SRF-dependent signaling in B16 melanoma cells and loss of Profilin I interferes with cell migration

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Running title: Depletion of profilin I and II in mouse melanoma

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Abstract

Profilin is a central component of the microfilament system. Its involvement in controlling actin polymerization associated with transmembrane signaling through its interaction with phosphatidylinositol lipids and a plethora of protein components carrying proline-rich sequence motifs has inspired to a large number of studies. Despite these efforts, however, its function is still not well understood. Therefore we have initiated a study of cultured cells after down-regulation of profilin expression using siRNA. Here we report that depletion of the profilin I and II isoforms in B16 mouse melanoma cells interferes with MAL/SRF-dependent transcription and that loss of profilin reduces their speed of migration as well as their ability to fine-tune their control of the migratory apparatus.

Introduction

The actin microfilament system is controlled by a diverse set of components that transduces information relayed from the cell surface into force-generating remodeling of actin organization with subsequent changes of the cell motility as immediate consequence (1-2). This activity is also intimately linked to signaling pathways that leads to the nucleus and gene expression control.

Profilins represent a family of proteins whose activity is coupled to the control of actin polymerization. In mammals five isoforms are known, profilin I, IIa and b, and profilin III, and IV, of which profilin I and profilin IIa are the best characterized (3). While profilin I is ubiquitously expressed, the tissue distribution of the IIa isoform is primarily restricted to brain and kidney although low amounts are also reported for cells of other tissue origin (4). Apart from actin, these isoforms bind polyL-proline (PLP)-sequences present in a diverse array of proteins, and they also interact with phosphatidylinositol lipids, primarily phosphatidylinositol (4,5)-bisphosphate (PIP2). Most studies characterizing these binding properties have been performed with profilin I, the first isoform to be isolated and therefore considered the archetype member of the family (5-6).

The profilin-actin interaction occurs at the surface of the actin monomer corresponding to the exposed surface at the fast polymerizing (+)-end of the actin filament (7-8), leaving the PLP-binding site on profilin accessible for interactions with other components (9). This ability of profilin to link unpolymerized actin to specific proteins expressing the critical proline-rich sequence motif is a property of fundamental cell biological importance and reflects profilins multifaceted influence on actin dynamics. For instance, spontaneous filament nucleation of profilin-sequestered actin is efficiently inhibited but docking of the complex via the free (-)-end of the actin monomer to available fast polymerizing filament ends or exposed nucleation surfaces of similar structure such as the activated Arp2/3-complex is simultaneously allowed for (2, 6, 10). Furthermore, the actin in the complex is held by profilin in an “open state” (11) characterized by a reduced nucleotide affinity. Thus profilin drastically increases nucleotide exchange on the actin monomer, rapidly causing replacement of ADP for ATP and simultaneously it interferes with the actin-ATPase (12-14). Since ATP-bound actin is the preferred state for filament elongation, the profilin-actin complex therefore carries a polymerization-competent form of actin to sites for filament growth where profilin-PLP interactions with different nucleation and elongation promoting factors (NEPF) such as N-WASP, Ena/VASP and the formins (15-16) ensure proper recruitment of the actin monomer.

Through its interaction with PIP2, profilin is associated with initial processes during receptor signaling and this lipid binding may also be a mechanism for its recruitment to the plasma membrane. The interaction occurs at two separate sites and dissociates the profilin-actin complex in vitro (17) albeit a trimeric PIP2-profilin-actin complex can form if the profilin-actin interaction is stabilized by covalent cross-linking (18). The role of the PIP2-profilin interaction in vivo is still unclear but in the light of the rapid actin remodeling, occurring after receptor activation with subsequent consequences for cell motility and the fact that the activity of several actin-binding proteins is under control by PIP2 suggest that also this activity contributes to connect actin dynamics with signaling. Notably, profilin interferes with phospholipase Cγ-dependent hydrolysis of PIP2 unless the enzyme has been activated by a tyrosine phosphorylation (19-20), and it can also influence PIP2 turnover via its interaction with the regulatory subunit p85 of phosphatidylinositol 3-kinase (21) further emphasizing its connection to receptor signaling.

The microfilament system is well established as immediate target downstream activated surface receptors rapidly changing the motility of stimulated cells (2, 10). In addition to altered cell behavior, the increase in actin turnover resulting from such receptor signaling is affecting gene expression via the megakaryocytic acute leukaemia (MAL)/serum response factor (SRF)-pathway (22-23). The co-transcription factor MAL is present at the cell periphery and binds monomeric actin at a site overlapping with the interaction surface for several actin monomer binding proteins (24) and is therefore considered to effectively compete with proteins such as profilin and cofilin for monomeric actin in vivo. Non-bound to actin, MAL rapidly shuttles to the nucleus (25) where it combines with SRF to initiate expression of a large number of genes important for cell migration and differentiation (26). Hence, MAL/SRF-dependent transcription represents a nuclear process directly integrated with actin dynamics at the plasma membrane and incorporates processes involving nuclear actin. Profilin, like actin is also a component of the nucleus where it has been implicated to function in transcription and pre-mRNA processing (27-28). Central to the studies presented here was to investigate whether profilin, in addition to its connection to signaling mechanisms in the immediate nearness of the activated receptor at the plasma membrane influences gene expression mechanisms dependent on the actin-controlled MAL/SRF-pathway. For this we employed siRNA-technique to reduce the expression of profilin isoforms I and II in B16 mouse melanoma cells transfected with a SRF-dependent reporter system. In parallel, the motility of the cells was documented.

Materials and Methods

Cell culturing and siRNA treatments

Mouse melanoma B16-F1 cells were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum (FCS) at 37°C in the presence of 5% CO_2. Prior to transfection with the siRNA duplexes, matching the *Mus musculus* sequence of Profilin I 5´-AGAAGGTGTCCACGGTGGT-3´, and Profilin IIa 5´-GCAAAATACTTGAGAGACT3´ (Dharmacon), 40 000 cells were plated into culture dishes of 3.5 cm in diameter. Transfection was carried out with Lipofectamine 2000 (Invitrogen), using 50 nM profilin siRNA. For mock transfection, the control siRNA (Dharmacon, cat no: D-001910-03) was used. After 7 hr, the cultures were washed with medium, and then cultured for 24, 48 and 72 hours. In some experiments AlF_4^--stimulation was performed with 50µM $AlCl_3$ and 30µM NaF as described by (29). For the imaging experiments (below), transfection was performed with Lipofectamine as above except that the cells were cultured in a 24-well plate and that each siRNA was mixed with 5 nM siGlo (Dharmacon, cat no: D-001630-02) to enable identification of transfected cells. The two different profilin isoform specific siRNAs were transfected in sequence, with the siRNA for profilin I and II being transfected 3 days and 2 days, respectively, before imaging. Cells targeted for profilin I knockdown were treated with control siRNA two days before imaging, and the control siRNA was combined with siGlo prior to transfection.

Western Blot analyses

Cell cultures were washed with phosphate buffered saline (PBS), lysed by incubation with passive lysis buffer (PLB; Promega, cat no: E1941, see luciferase assay below) for 15 minutes at room temperature, and centrifuged at 13 000x rpm in an Eppendorf centrifuge for 15 minutes. The supernatant was collected and precipitated with acetone overnight in -20°C. The precipitate was collected by centrifugation at 4°C, dissolved in water and after addition of PAGE-sample buffer it was boiled. The samples were loaded onto a 12% SDS-polyacrylamide gel, adjusting sample volumes for protein content as estimated by absorbance measurement at 595 nm after addition of Bradford reagent (Bio-Rad). After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Hybond-C Extra; Amershan Bioscience) at 5mA/cm^2 for 2 hours. The membrane was incubated in 10% skim milk in Tris-buffered saline tween-20 (TBST) overnight, followed by primary antibodies: anti-alpha tubulin (Abcam, cat no: ab7291; 1:2000), anti-GAPDH (Abcam, cat no: ab8245; 1:40 000), anti-profilin I (own laboratory, raised in rabbit against human profilin I; 1:2000) and anti-profilin II (Santa Cruz, cat no: sc-100955; 1:250). After incubation with HRP-conjugated secondary antibodies against mouse (Dako, cat no: P0447) and rabbit IgG (Pierce, cat no: 1858415) with dilution of 1:2000, the membrane was developed with West Dura HRP substrate (Pierce), and images captured using the Quantity One software (Bio-Rad).

Luciferase reporter assay

Cells in a 24-well plate (14 000 cells/well) were transfected with a plasmid mixture containing the SRF reporter p3DA.luc (8 ng) encoding the firefly luciferase and the reference reporter ptk-RL (20 ng) encoding *Renilla* luciferase. For each experiment, one well containing the control plasmid SRF-VP16 (20 ng) was included. The plasmid system was a generous gift by M. Vartiainen, Helsinki (25). When appropriate, cells were transfected with profilin siRNA (see above), cultured for 72 h, lysed in PLB according to the manufacturer's instructions after which luciferase activities were determined. For each measurement, 20 µl of the samples were used together with the substrates for the firefly and *Renilla* luciferase, respectively (Promega, cat no: E1910). The luminescence was measured in a luminometer

(Turner BioSystem). Firefly luciferase activity was normalized to Renilla luciferase activity. Data were normalized to reporter activation by SRF-VP16 and lysates of cells transfected with control siRNA.

Imaging

For live cell imaging cells were plated on laminin coated 50 mm glass bottom culture dishes (MatTek) on the day before imaging, and were observed at 37^{o}C in the presence of 5% CO_2 in a Zeiss Axiovert 200M inverted fluorescence microscope (Carl Zeiss, Germany) equipped with a climate chamber, an EC-Plan-Neofluar 10x/0.3 Ph1 objective lens and a DG-4 light source (Sutter Instrument Novato, CA). Images were captured using an AxoCam MRm camera (Carl Zeiss) and SlideBook 5.0 software (3i). One hour time-lap sequences (one exposure/minute) were acquired. Images for siGlo were only acquired for the first time point. To measure migration distance, cells exhibiting siGlo signal, indicating that they were transfected, were tracked using Image J14.3h software with the plug in 'manual tracking'. The cells were tracked with the centre of the nucleus as reference point and the distance migrated during each minute was measured and added to retrieve the whole distance travelled. The coordinates retrieved for the first and last position allowed for determination of the net movement during the time period (termed displacement).

Results and Discussion

a. Depletion of profilin I and II

Profilin I is the major profilin isoform in B16 melanoma cells and part of the control apparatus of actin organization. To investigate more precisely its involvement in cell motility and influence on MAL/SRF-dependent transcription, which correlate with the cellular levels of monomeric actin we used small interference (si)-RNA to down-regulate profilin production.

After transfection of the siRNA duplex the cells were cultured for 24, 48 and 72 hr before analyzed for profilin expression by western blot of lysates prepared from the different cultures, Fig 1. A semi-quantitative assessment of the antibody-blotted profilin band using densitometry with tubulin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for normalization, demonstrated a significant depletion of profilin already after 24 hr. On average, less than 20% of the profilin concentration in samples of cells transfected with control siRNA remained after this treatment and a low expression level persisted over the entire period, Fig 1 A slight but consistent recovery of the amount of profilin was observed after 72 hr, probably as a consequence of a dropping effect of the transfected siRNA as the cell culture gradually increased in density. This and all western blot assessments in the following are average values based on results obtained in four independent experiments.

Since an increased expression of the profilin II isoform might be compensating for the loss of profilin I, the amount of isoform II was monitored in parallel with profilin I, using profilin II-specific antibodies. However, the result suggested that rather than being increased, the level of profilin II was also reduced, though the numbers were more variable compared to the corresponding values measured for profilin I, possibly this was due to the lower signal detected for isoform II, Fig 1. This was surprising, and since the profilin I siRNA was designed to be unable to bind profilin II mRNA, it was necessary to analyze the background to this result in more detail, and in particular to see if a modulation of profilin II expression also was influencing the levels of profilin I. Therefore parallel experiments with siRNA depletion of profilin II were performed, Fig 1, panels AC. The result showed that profilin I expression was negatively affected by a depletion of profilin II by approximately 25% of control values at early time points after transfection and that the protein level had recovered completely after 72 hr. In an analogous analysis, determining the isoform II expression after siRNA transfections, nearly 90% depletion of profilin II after 24 and 48 hr and a small but somewhat larger recovery than the corresponding observation for profilin I at the last time point was seen after transfection of the profilin II siRNA, Fig 1, panels BD. Depletion of profilin I similarly caused a reduced isoform II expression of approximately 50% after 24 hrs. For unknown reasons this value then recovered completely after 48 hr only to drop again to approximately 70% at the last time point measured after transfection. Thus, essentially this supported the view that expression of the two profilin isoforms mutually influenced each other. Notably, the result is also in agreement with the identification of profilin I as being under control of MAL/SRF and most likely this accounts also for the expression of profilin II (26; see further below).

To ensure equal loading of the samples analyzed in the above experiments, they were adjusted for protein content in addition to the normalization of the final densitometry values against the average value obtained by measuring the blotting signal from tubulin and GAPDH. However,

a full account for possible isoform interdependence remains to be seen until a qPCR determination of the corresponding isoform mRNAs has been established

b. MAL/SRF-signaling

According to the view that the MAL/SRF-pathway is controlled through the MAL-actin interaction, reducing the cellular concentration of profilin would increase the monomeric actin available for sequestration of MAL and this would then interfere with MALs activation of SRF. To analyze to what extent profilin depletion in fact affected this signaling pathway, and if both isoform I and II would have an effect, a luminescence based SRF-dependent reporter system (25) was used. Like many tumor cell lines B16 cells are characterized by autocrine stimulation (30), and attempts to include serum stimulation after a period of serum starvation in combination with the siRNA-treatment to augment signaling did not result in the typical drastic activation seen for instance with fibroblasts.

A significantly enhanced effect (by approximately 54%) in the SRF-reporter read out was however observed when the cells were treated with AlF_4, which trap nucleotide binding proteins in a conformation mimicking the tri-nucleotide bound state and therefore is thought to augment actin based motility through massive activation of RhoGTPases (29). In agreement with the hypothesis and fully concurrent with the described integration of the SRF-pathway with actin turnover, down-regulation of profilin caused a drastic reduction in MAL/SRF-dependent expression, Fig 2. With profilin I siRNA, the reporter activity was lowered with about 90% for cells growing under homeostasis as well as for those being treated with AlF_4, and also targeting profilin II led in both cases to a similar lowering (with ca 65% and 50%, respectively).

Combining the two siRNAs led to an increased effect observed with AlF_4-exposed cells, with 11% and 48% compared to separate down-regulation of isoform I and II, respectively. Cells grown under homeostasis showed no significant variation from treatment with siRNA I in this respect while the activity was reduced with 22% compared to cells transfected with siRNA II only. Copeland and Treisman have reported that RhoA-activated mDia stimulates SRF-signaling through its induction of actin polymerization (31). Together with the fact that profilin-actin is the source of actin for formin-induced filament formation this suggests that RhoGTPase-dependent actin remodeling in the AlF_4-activated cells was the reason to the augmented effect of reducing both profilin isoforms.

Clearly these experiments demonstrated that a reduced expression of either profilin isoform had a detrimental effect on SRF-dependent gene expression. Co-transfection with a plasmid expressing green fluorescent protein (GFP)-MAL revealed a reduced nuclear distribution of GFP-fluorescence in cells depleted for profilin (not shown). Thus, the reduced expression downstream of SRF in profilin depleted cells was likely to result from interference with SRF-activation by the co-transcription factor MAL. This in turn most likely was due to an increased sequestration of MAL by monomeric actin being freed from profilin.

Early studies aiming to compare the biochemical properties of the profilin I and II isoforms led to somewhat confusing results due to unawareness of the existence of two splicing variants of profilin II (a and b) in mammalian tissue (32-33). However, later studies have eliminated this confusion (4, 34) and, although a direct comparison of profilin isoforms from the same species with respect to their interaction with non-muscle actin still remains to be seen, it is generally considered that profilin I and IIa binds actin with similar affinity while profilin IIb hardly is an actin binding protein (3). The result observed here with respect to the

effect on the MAL/SRF-activity therefore suggests that the B16 cells express a considerable portion of its total profilin as the IIa isoform. Consequently the profilin-actin pool in these cells not only consists of two non-muscle actin isoforms, β and γ, but also two profilin variants, I and IIa. Clearly qPCR-data will provide more information on this issue. However, based on the present observations, data generated by single profilin I or IIa isoform depletion using siRNA, warrants concern unless the cellular content of the alternative isoform is carefully analyzed, particularly if the aim is to analyze isoform function in the context of cell behavior and signalling.

c. Migration analysis

Profilin-depleted B16 cells were also studied with respect to migration properties on laminin coated glass by videoing individual cells for periods of 60 minutes with exposures being made every minute, Fig 3. Image analysis revealed that during this period cells transfected with the control siRNA migrated on average 58.2 μm (n=166), i.e. their average speed was just under 1 μm/minute. After profilin I siRNA transfection this number was reduced with 20 % to 46.6 μm (0.78 μm/minute; n=130) while combining the siRNA duplexes to deplete both isoforms had a less severe effect (13%; 50.7 μm; 0.85 μm/minute; n=206). Thus it is obvious that in the B16 cells a depletion of at least 80% of the total profilin pool formed by profilin I and II did not seriously impair the capacity of the cells to translocate over the substratum. Nor did the overall morphology appear to be altered. Therefore we conclude that actin polymerization processes which are directly linked to cell migratory mechanisms like lamellipodial extension can occur by the channeling of actin molecules for filament elongation from other sources than profilin-actin. Thymosin-actin could be one variant of sequestered non-filamentous actin that is recruited (35).

Another interesting feature of the migratory properties of profilin-depleted cells resulting from the above data was realized when instead of considering the actual length the cells had migrated their average displacement was measured, i.e. the point-to-point movement from start to end of session. It was found that this value did not differ to any large extent, 19.6 μm for the control cells while the once depleted for profilin I had altered their position with 19.5 μm and for those lacking both isoforms the average displacement was 20.3 μm, Fig 3. Combining this with the fact that the profilin-depleted cells moved with a slower speed it appears that these cells were less prone to change direction of movement compared to the control cells. Despite the small variation from the control cells, this conclusion was indeed supported when the pattern of migration was scrutinized and the resulting path was plotted for each cell as displayed in Fig 3. An analogous observation has been reported for a breast cancer cell line, which shows slower but more stable lamellipodial protrusions upon down-regulation of profilin expression (36). A possible explanation is that loss of profilin results in difficulties to fine-tune the activities of the microfilament system in response to the array of incoming signals that emanates from cell-substratum contacts and other surface receptor activations. This input complexity together with context-dependent variations due to the use of different cell types may be the reason why profilin depletion has been reported to both decrease and increase cell motility (36-37). The autoregulatory enhancement of reduced profilin expression through the MAL/SRF-dependent feed-back mechanism as depicted here and which is likely to influence also several other microfilament proteins including actin itself adds a further complication to this issue. The lack of an added effect on migration when profilin II was depleted in cells already lacking profilin I suggests that the former isoform plays a less important role in motility. This is a puzzling finding, considering the significant influence on MAL/SRF-dependent expression upon depletion of this isoform, which requires

that it binds actin forming profilin-actin. The solution to this enigma will need to await future experimentations.

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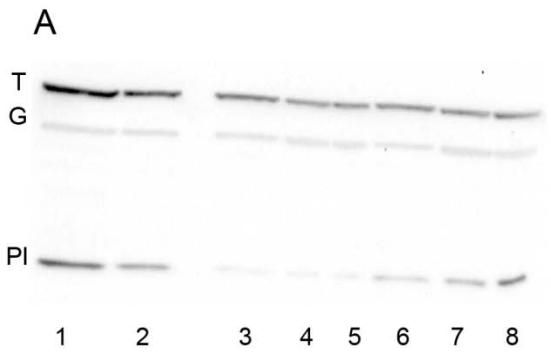
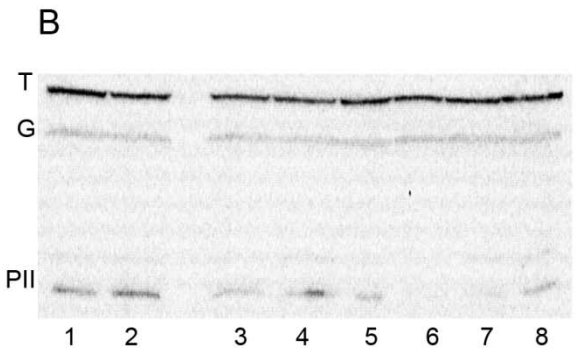
Figures and Legends





C

Pfn I	24 hr	48 hr	72 hr
Pfn I siRNA	16%	11%	19%
Pfn II siRNA	48%	90%	150%

D

Pfn II	24 hr	48 hr	72 hr
Pfn I siRNA	47%	99%	33%
Pfn II siRNA	10%	10%	28%

Figure 1. Expression of profilin after siRNA depletion. Western Blot analysis of cell extracts for A) profilin I and B) profilin II. Sample loading was adjusted to protein concentration as determined by the Bradford assay. The different lanes display: 1, lysates of untreated cells; 2, control siRNA transfected cells; 3, 4, and 5 profilin I siRNA transfected cells, 24, 48 and 72 hr of culture after transfection; and 6,7, and 8 the similar analysis after profilin II siRNA transfection. The membranes were simultaneously blotted for α-tubulin (T), GAPDH (G), profilin I (PI, panel A only) and profilin II (PII, panel B only). Panels C) and D) demonstrate the result of densitometry of the bands illustrated in panels A) and B), using exposures in the linear range of the instrument as defined by the software (Quantity One, Bio-rad), and after normalization against tubulin and GAPDH (see text) and expressed as percentage of the corresponding values in the samples prepared from cells transfected with control siRNA. Note that the values in panels C and D refer to single measurements of the samples displayed in A and B. Therefore some deviate from the average values stated in the text, i.e. panel C, Pfn II siRNA 24 and 48 hr; and panel D, Pfn I siRNA 72 hr.

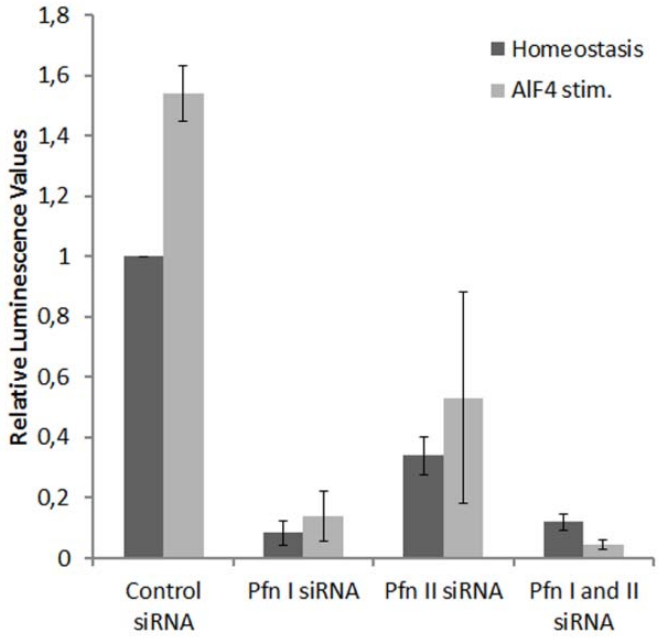


Figure 2. Profilin depletion represses SRF activities. Cells were transfected with the SRF reporter system (see text), and SRF dependent expression was measured for cultures growing under homeostasis (dark grey) as well as under AlF_4^--stimulation for 15 min (light grey). Data of 2 different experiments with SEM (double samples in each experiment) are shown relative to the corresponding activity measured in cells transfected with the control siRNA.

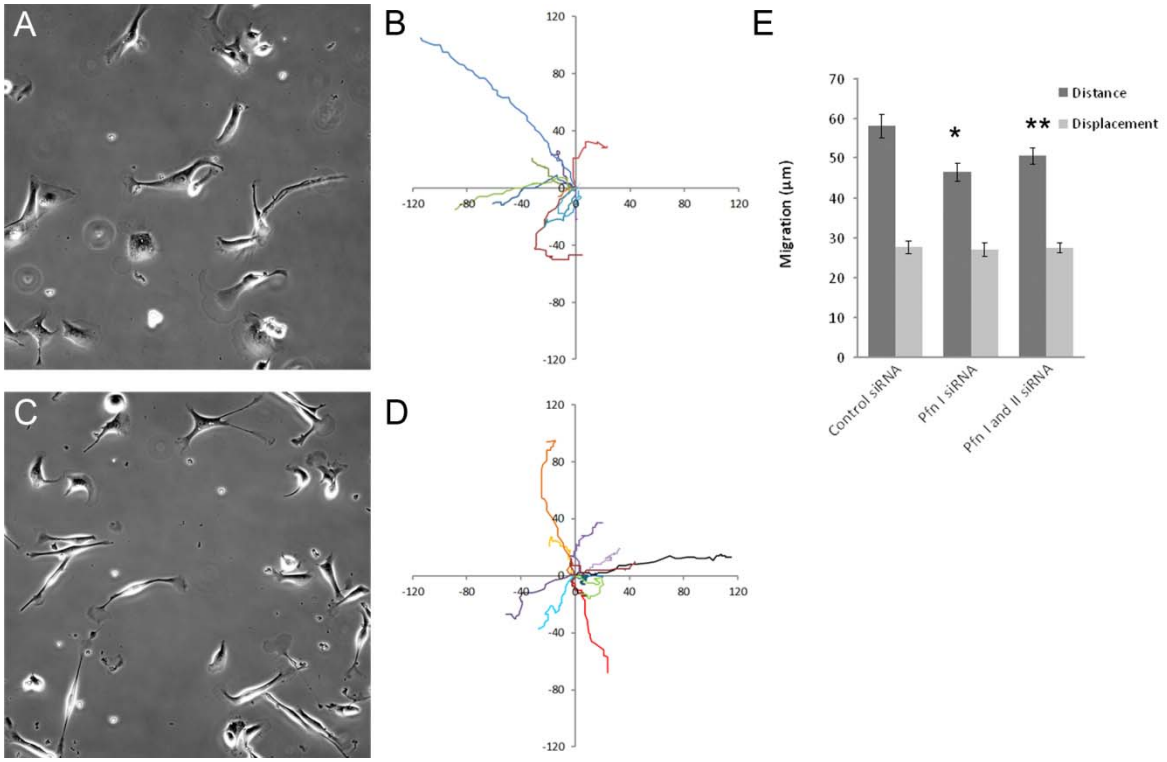


Figure 3. Silencing of profilin affects migration speed while net displacement is unchanged. Cells in (A) were transfected with control siRNA, and those in (C) with a mixture of profilin I and II siRNA for 72h. The images show the first exposure of a one hour time-laps sequence while (B and D) display the trajectories from the entire sequence of exposures captured from the cells in A and C respectively (one minute intervals). The y- and x-axis both show migration in µm. Each transfected cell that stayed within the field of view was tracked based on the presence of siGlo signal (not shown). (E) A bar chart representing the total distance migrated and the net displacement from start to end-point for cells transfected with control siRNA, profilin I siRNA as well as profilin I and II siRNAs simultaneously. (*) represent p= 0.0024 and (**) p=0.055 relative to cells transfected with control siRNA, data based on measurement of a total of 130-200 cells for each condition during three independent experiments, bars represent SEM.