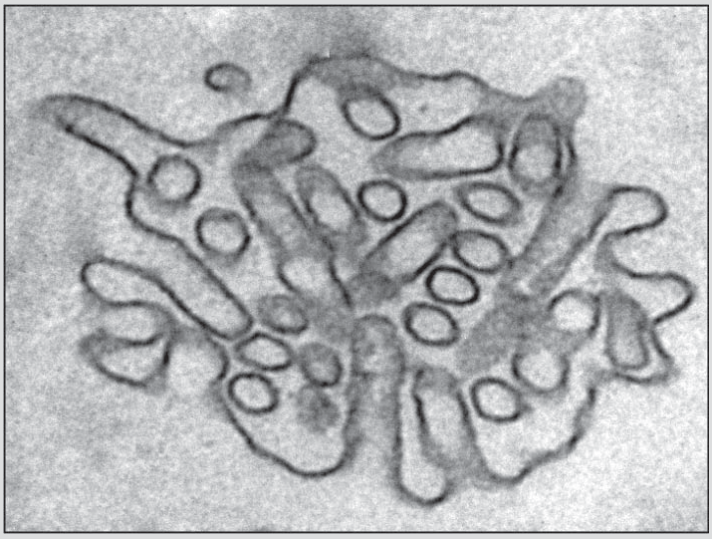

Missing-In-Metastasis (MIM) Regulates Cell Morphology by Promoting Plasma Membrane and Actin Cytoskeleton Dynamics

PIETA MATTILA

Institute of Biotechnology and
Department of Biological and Environmental Sciences
Division of Genetics
Faculty of Biosciences and
Viikki Graduate School in Biosciences
University of Helsinki

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Pieta Mattila

Institute of Biotechnology and
Department of Biological and Environmental Sciences
Division of Genetics
Faculty of Biosciences and
Viikki Graduate School in Biosciences
University of Helsinki
Finland

Academic dissertation

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Supervised by:
Professor Pekka Lappalainen
Institute of Biotechnology
University of Helsinki, Finland

Reviewed by:

Professor Elina Ikonen
Institute of Biomedicine
University of Helsinki, Finland

and

Professor John E. Eriksson
Department of Biology
Åbo Akademi University
Turku, Finland

Opponent:

Professor Giorgio Scita
IFOM Foundation
Institute FIRC of Molecular Oncology
University of Milan, Italy

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An EM micrograph displaying a lipid vesicle tubulated by MIM IMD.

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To my family

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ABBREVIATIONS

ADF-H	actin depolymerizing factor homology
ADP	adenosine diphosphate
ATP	adenosine triphosphate
Arp	actin related protein
BAR	Bin-Amphiphysin-Rsv
βT	β-thymosin fold
Cc	critical concentration
CP	heterodimeric capping protein
CRIB	Cdc42/Rac interactive binding
EM	electron microscopy
F-actin	filamentous actin
F-BAR	FCH BAR
G-actin	monomeric (globular) actin
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	GDP/GTP exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
I-BAR	inverse BAR domain (=IMD)
IF	intermediate filament
IMD	IRSp53/MIM homology (IM) domain
IRSp53	insulin receptor tyrosine kinase substrate p53
MIM	missing-in-metastasis
mRNA	messenger RNA
MT	microtubule
N-WASP	neural WASP
P_i	pyrophosphate, inorganic phosphate
PI	phosphoinositide
PIP	phosphatidyl inositol phosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5 -bisphosphate
$PI(3,4,5)P_3$	phosphatidylinositol 3,4,5 -trisphosphate
Ptc	patched
Rif	Rho in filopodia
RPTP	receptor protein tyrosine phosphatase
Shh	Sonic hedgehog
TEM	transmission electron microscopy
WASP	Wiscott-Aldrich syndrome protein
WAVE	WASP family Verprolin homologous
WH2	WASP homology domain 2
WIP	WASP interacting protein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on three original articles and one review article, which are referred to in the text by their roman numerals.

I Paunola, E., **P.K. Mattila**, and P. Lappalainen. (2002) WH2 domain: a small, versatile adapter for actin monomers. *FEBS Letters:* 513:92-7. (Review)

II **Mattila, P.K.,** M. Salminen, T. Yamashiro, and P. Lappalainen. (2003) Mouse MIM, a tissue-specific regulator of cytoskeletal dynamics, interacts with ATP-actin monomers through its C-terminal WH2 domain. *Journal of Biological Chemistry:* 278:8452-9.

III **Mattila, P.K.**, A. Pykäläinen, J. Saarikangas, V.O. Paavilainen, H. Vihinen, E. Jokitalo, and P. Lappalainen. (2007) Missing-in-metastasis and IRSp53 deform $PI(4,5)P_2$-rich membranes by an inverse BAR domain-like mechanism. *Journal of Cell Biology:* 176:953-64.

IV **Mattila, P.K.**, J. Saarikangas, M. Varjosalo, M. Bovellan, J. Hakanen, H. Savilahti, H. Sariola, J. Taipale, K. Sainio, M. Salminen, P. Lappalainen. Missing-In-Metastasis is required for kidney integrity but is dispensable for Sonic hedgehog signaling. *Submitted*

ABSTRACT

The cells of multicellular organisms have differentiated to carry out specific functions that are often accompanied by distinct cell morphology. The actin cytoskeleton is one of the key regulators of cell shape subsequently controlling multiple cellular events including cell migration, cell division, endo- and exocytosis. A large set of actin regulating proteins has evolved to achieve and tightly coordinate this wide range of functions. Some actin regulator proteins have so-called 'house keeping' roles and are essential for all eukaryotic cells, but some have evolved to meet the requirements of more specialized cell types found in higher organisms enabling complex functions of differentiated organs, such as liver, kidney and brain. Often processes mediated by the actin cytoskeleton, like formation of cellular protrusions during cell migration, are intimately linked to plasma membrane remodeling. Thus, a close cooperation between these two cellular compartments is necessary, yet not much is known about the underlying molecular mechanisms.

This study focused on a protein called missing-in-metastasis (MIM), which was originally characterized as a metastasis suppressor of bladder cancer. We identified MIM from sequence databases upon searches for new actin regulators containing the WH2 domain, which is a ubiquitous actin binding protein motif. Clear homologues of MIM were only identified in vertebrates. We demonstrated that MIM indeed regulates the dynamics of actin cytoskeleton via its C-terminal WH2 domain, and is expressed in a cell type-specific manner. Interestingly, further examination showed that the N-terminal IRSp53/MIM homology (IM) domain of MIM displays a novel membrane tubulation activity, which induces formation of filopodia in cells. Following studies demonstrated that this membrane deformation activity is crucial for cell protrusions driven by the full length MIM.

In mammals, there are five members of MIM/IRSp53 protein family, characterized by the conserved IM-domain. Functions and expression patterns of these family members have remained poorly characterized. To understand the physiological functions of MIM, we generated MIM knockout mice. MIM-deficient mice display no apparent developmental defects, but instead suffer from progressive renal disease and increased susceptibility to tumorigenesis. This indicates that MIM is not essential for embryonic development of mouse, but plays a role in the maintenance of specific physiological functions associated with distinct cell morphologies.

Taken together, these studies implicate MIM both in the regulation of the actin cytoskeleton and the plasma membrane. Our results thus suggest that members of MIM/IRSp53 protein family coordinate the actin cytoskeleton:plasma membrane interface to control cell and tissue morphogenesis in multicellular organisms.

REVIEW OF THE LITERATURE

1. Introduction to the cytoskeleton

All eukaryotic cells have a cytoskeleton, which allows the cells, for example, to maintain or remodel their morphology, resist pressure, move, divide, and efficiently take in or release substances. These complex processes rely on proteins that have the ability to form filamentous assemblies. Cytoskeletal filaments can be divided into three distinct classes: 1) actin filaments, also known as microfilaments, which are composed of a protein called actin, 2) microtubules (MT), which are composed of tubulin, and 3) intermediate filaments (IF), which can be formed by different proteins belonging to the diverse IF protein family (Bray, 2001).

Microtubules are tubular structures that have a peculiar character to grow from one end and then undergo a catastrophe, i.e. to suddenly shrink, from the same end. In cells, these long and stiff tubules are directed and stabilized to appropriate sites, where they are required to determine cell shape or function as tracks for vesicle movement. MTs also have a vital role in cell division, during which they form the mitotic spindle responsible for chromosome segregation. Kinesins and dyneins are MT motor proteins, which generate forces for movements along MTs either towards minus or plus ends (Bray, 2001).

Intermediate filaments are a diverse class of cytoskeletal structures that can be formed by different proteins, usually characteristic for the specific cell type. IFs are strong but elastic fibers mostly responsible for cells' resistance for mechanical forces. Special types of IFs are found in the nucleus, constituting the nuclear lamina, which has the important function to regulate the shape and protect the nucleus (Herrmann et al., 2007). Interestingly, recent studies have revealed new, non-mechanical functions for IFs, showing that these proteins are able to sequester or function as scaffolds for various signaling molecules in cells (Pallari and Eriksson, 2007).

2. Actin

Actin filaments, despite consisting of only one protein, form highly dynamic and divergent cytoskeletal assemblies. Actin filaments are thin and flexible, but with the help of numerous actin binding proteins they construct an outstanding variety of different structures ranging from contractile bundles to protruding networks. The actin cytoskeleton is essential for a variety of cell biological processes including cell movement, cytokinesis, endo- and exocytosis (Bray, 2001).

Actin is one of the most abundant proteins in our cells. Also, it is an incredibly conserved protein; yeast actin shares almost 90% identity with its human counterparts, and it appears that actins from all vertebrates can polymerize together and complement each other's functions to a large extent in cells. Lower eukaryotes have only one actin gene, but mammals have multiple isoforms that vary in their expression profiles between different tissues (dos Remedios, 2001).

Actin is a 43kDa globular protein and, accordingly, is called G-actin in its monomeric form. An actin molecule consists of four subdomains that roughly divide the protein into two halves with a cleft in the middle. A nucleotide, either ATP or ADP, and a divalent cation, usually Mg^{2+}, bind to this cleft (Kabsch et al., 1990; Otterbein et al., 2001). The

crucial feature of actin is its capability to polymerize into filaments, which are also called F-actin. Filaments are traditionally considered as the functional form of actin, since most of the activities of actin in cells are based on the structural properties of F-actin and involve polymerization (dos Remedios, 2001).

Growing evidence suggests that also prokaryotes have homologs of actin. Bacterial MreB is the first prokaryotic protein shown to display functional and structural similarities to actin, although the protein does not show any conservation to its eukaryotic counterparts at the level of amino acid sequence. MreB is important for the maintenance of cell shape of rod-like bacteria and for chromosome segregation (Doi et al., 1988; van den Ent et al., 2001). Also, a protein called ParM, which is involved in the localization of plasmids in bacteria, has been shown to have an atomic structure that resembles actin and to polymerize into filaments in an ATP-dependent manner. However, these filaments show MT-like dynamic instability (Garner et al., 2004; van den Ent et al., 2002).

2.1 Actin dynamics

Actin is capable of spontaneously polymerizing in an endwise manner into double helical filaments that are constantly turning over. The two ends of the filament present different molecular surfaces and thus have distinct biochemical characteristics. This results in divergence in their behavior and creates a fast growing barbed end (also known as a plus end) and a slowly growing pointed end (also known as a minus end), the nomenclature of which arrives from the arrowhead appearance of the filaments decorated by myosin (Craig et al., 1985).

In a test tube, as well as in cells, actin cycles between polymerized and unpolymerized forms. This cycle receives its energy from ATP hydrolysis. At steady state, actin monomers in the ATP-bound state incorporate into the barbed end of the filament, which has higher affinity for ATP-G-actin. Once the monomer is in the

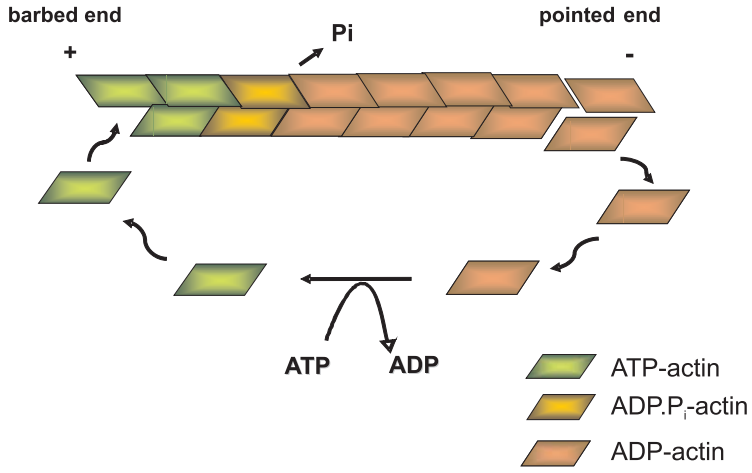

Figure 1. The treadmilling cycle of actin. Actin monomers assemble to the barbed end of actin filaments in their ATP-bound state. In the filament, nucleotide rapidly hydrolyzes into ADP.P_i, followed by liberation of inorganic phosphate. ADP-bound actin monomers prefer dissociation from the pointed end of the actin filament. To prime the monomers for new round of polymerization ADP is exchanged to ATP. This cycle, called actin treadmilling, occurs spontaneously in a test tube but is tightly regulated by myriad of actin binding proteins in cells.

filament, the nucleotide quickly hydrolyzes into ADP.P_i. Next, the inorganic phosphate (P_i) is released to leave rest of the filament in ADP-bound state. P_i release is thought to induce a structural change in the polymer, which makes it more unstable. Thus, filament pointed ends favor depolymerization. The ADP nucleotide of the depolymerized actin monomer is then replaced by ATP to prime the monomer for new round of polymerization (Figure 1) (Pollard, 1986; Wegner, 1982).

The above-described features of actin filaments result from distinct critical concentrations (Cc) for polymerization at each filament end: 0.1 μM for the barbed end and 0.7 μM for the pointed end. Between these concentrations a phenomenon called treadmilling takes place and the system reaches a steady state where depolymerization at the minus end balances polymerization at the plus end, stabilizing the actin monomer concentration close to 0.1 μM (Pollard, 1986). When actin is polymerized *in vitro* from G-actin there is a lag phase before fast polymerization begins. This is due to relatively unstable intermediates, actin dimers and trimers, serving as seeds for the filaments (Kasai et al., 1962). Net polymerization takes place until Cc is reached, and equilibrium between filamentous and monomeric actin exists.

3. Actin binding proteins

The great diversity in the functions of the actin cytoskeleton is achieved via regulation by numerous actin binding proteins in response to various cellular signals. These proteins strictly govern nucleation, elongation, cross-linking, branching, and depolymerization of actin filaments in an orchestrated manner. Moreover, the concentration of monomeric actin in cells is high, even above 100 μM, which greatly exceeds the Cc for polymerization. This is enabled by actin sequestering proteins that maintain the large monomer pool releasing actin for polymerization only when required. The large amount of G-actin combined with nucleation and elongation promoting proteins ensure the fast rate of polymerization upon triggering signal (reviewed in Pollard et al., 2000). The most thoroughly characterized and conserved actin binding proteins are introduced in this chapter by categories defined by the main activities, that they are considered to exert on actin dynamics. Interestingly, it appears that while the functions of actin regulator proteins vary substantially, only a limited number of actin binding protein folds have been generated during evolution. One of the most widely used actin binding folds, the WASP homology 2 (WH2) domain, is introduced in detail.

3.1 WH2 domain

The WH2 domain is found in multiple regulators of the actin cytoskeleton that have exceptionally diverse functions and domain compositions. This short motif can be classified to the same family with the β-thymosin (βT) fold, which is found in the β-thymosin protein family of vertebrates. These small actin monomer sequestering proteins consist entirely of one βT domain. β-thymosins are found at very high intracellular concentrations (up to 500 μM) in most cells and are thus considered as the main G-actin sequestering agents in cells (reviewed in Hannappel, 2007). The WH2 domain is typically shorter than the βT fold, and due to the shorter extension of the binding surface on actin monomer does not sequester actin monomers (Figure 2) (Hertzog et al., 2004).

The important feature of WH2 domain is that it is capable of functioning in numerous different combinations, which allows vast variability in the regulation of actin dynamics carried out by WH2 domain containing proteins. Whereas β-thymosins sequester actin monomers, the WH2 domain is adjusted for actin filament nucleation in WASP/WAVE, Verprolin/WIP, and Spire protein families (chapter 3.2). Many complex multidomain regulators of the actin cytoskeleton, such as Srv2/CAP (chapter 3.4) and IRSp53/MIM proteins (chapter 6.2.3) also contain WH2 domains (reviewed in Dominguez, 2007). In addition, tandem WH2/βT repeat proteins are found in many lower metazoan proteins, such as cibulot, tetrathymosin and actobinding from *Drosophila melanogaster, Caenorhabditis elegans* and *Acanthamoeba castellanii,* respectively. The exact functions of these proteins are not known, but they appear to be crucial for neuronal development and reproduction (Van Troys, 2007).

3.2 Actin nucleating proteins

De novo nucleation of actin filaments is essential for generating new actin structures, but new barbed ends can be obtained also by uncapping, severing, or by forming branches on the existing filaments. In this chapter, the key nucleators of cellular F-actin, Arp2/3 complex with its activators (WASP/WAVEs) and formins, are described in more detail. However, recent studies have also identified new actin nucleating proteins. One of them is **Spire**, which nucleates actin filaments by clustering four actin monomers through its four tandem actin monomer binding WH2 domains. This results in the formation of a seed with four aligned actin monomers. Spire nucleates straight actin filaments and stays bound to the pointed end of the filament (Quinlan et al., 2005).

Arp2/3 is a complex of seven subunits, two of which are members of the actin related protein (Arp) family. Arp2/3 complex nucleates filaments by binding

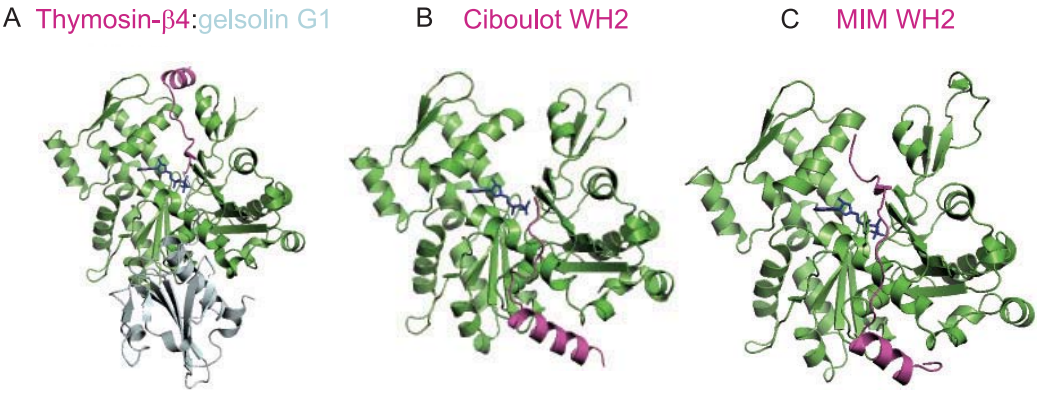

Figure 2. Structures of thymosin-β4 and the WH2 domains of Ciboulot and MIM in complex with actin. The Tβ/WH2 domains are shown in magenta, actin monomers in green and bound nucleotides in blue (ball-and-stick representation). Structures of **(A)** Thymosin-β4 fused to Gelsolin domain 1 (light cyan) (PDB ID:1T44), **(B)** WH2 domain D1 of Ciboulot (PDB ID:1SQK), and **(C)** WH2 domain of MIM (PDB ID:2D1K) in complex with actin. Please note the presence of an additional α-helix in thymosin-β4, which binds between actin subdomains 2 and 4, and is believed to be responsible for the monomer sequestering activity (Hertzog et al., 2004). The picture was created with program PyMOL (http://www.pymol.org).

to the sides of pre-existing filaments and creating a daughter strand in typical 70° angle to the mother filament (Mullins et al., 1998). So far, Arp2/3 complex is the only known factor promoting branching of actin filaments. Thus, Arp2/3 complex is believed to be mainly responsible for the nucleation of so-called dendritic actin meshwork typically found at the lamellipodium of a migrating cell (Figure 3 and chapter 4). According to current models, the two Arps of the Arp2/3 complex mimic an actin dimer and function as a seed for polymerization. However, on its own, Arp2/3 is a very weak nucleator and needs to be activated by other proteins (reviewed in Pollard et al., 2000).

Wiscott-Aldrich syndrome protein (**WASP**) and WASP family Verprolin homologous (**WAVE**) proteins are considered the main activators of the Arp2/3 complex. These proteins share a C-terminal catalytic so-called VCA module, composed of a WH2 domain, and central and acidic regions, which elicits interactions with actin monomers and the Arp2/3 complex leading to the actin branch nucleation. Upon activation of Arp2/3, the actin monomer bound to the WH2 domain of WASP is believed to be added as the first monomer to the nucleated filament (Mullins, 2000). This model is further supported by the comparison of Arp2/3 activation potential of WASP and WAVE proteins, showing that the tandem WH2 domains found in neural WASP (N-WASP) create the most efficient nucleator, assumingly by providing a longer filament seed (reviewed in Dominguez, 2007; Frittoli, 2007).

WASP is specifically expressed in hematopoietic cells and was initially identified as the causative gene for Wiscott-Aldrich syndrome (WAS), an immunological disorder. The close homologue, N-WASP, is highly abundant in neural tissue but its expression is, despite the name, ubiquitous. In cells, majority of N-WASP was recently shown to be bound to WASP interacting protein (**WIP**) / **verprolin** family members, which appear to stabilize the inactive conformation. However, additional molecules, such as Toca-1, are needed for activation downstream of a Rho family GTPase, Cdc42 (reviewed in Stradal and Scita, 2006). Mutations in the WIP binding site of WASP are found in many WAS patients, demonstrating the *in vivo* importance of this interaction (Burns et al., 2004). WIP/verprolin proteins contain two WH2 domains that are implicated in both F- and G-actin binding. The molecular mechanisms of actin binding are not clear, and also the *in vivo* functions of WIP/verprolin proteins are controversial, since they have been shown to inhibit WASP-Arp2/3 mediated actin nucleation, but to be essential for Cdc42-dependent filopodia formation (reviewed in Aspenstrom, 2005).

Interactions with Cdc42 and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) have been demonstrated to unfold the autoinhibited conformation of N-WASP leading to activation of the Arp2/3 complex (Prehoda et al., 2000). WAVE proteins, on the other hand, function in a multiprotein complex including Abi1, Sra1/PIR121, Nap/Kette, and HSPC300. Rac1 binds to WAVE complex, but how this leads to activation of Arp2/3 is still controversial (Eden et al., 2002; Innocenti et al., 2004). WASP and WAVE have been linked to formation of not only lamellipodia and filopodia of migrating cells, but also to membrane trafficking, podosome and phagocytic cup formation, cell adhesion, and pathogen

infections (reviewed in Stradal and Scita, 2006).

Formins are a large family of homodimeric actin nucleators that have a unique feature to induce formation of unbranched actin filaments by remaining associated to the elongating filament barbed end (Pruyne et al., 2002; Sagot et al., 2002). Formins nucleate filament growth by their highly conserved formin homology 2 (FH2) domain that homodimerizes into a flexible 'donut' –like structure, which processively moves with the growing filament end and simultaneously permits robust addition of new subunits (Xu et al., 2004). Adjacent to FH2 domain is the formin homology 1 (FH1) domain, which contains a proline rich sequence, capable of interacting with profilin (chapter 3.4) and facilitating the polymerization of profilin:actin (reviewed in Goode and Eck, 2007). Formins are able to protect the barbed end from capping proteins while they catalyze the filament elongation (Zigmond et al., 2003).

Mammals contain at least 15 different formins, which seem to function in generation of distinct actin structures and vary in their regulatory regions and binding partners. Probably the best characterized formins are diaphanous-related formins, such as mouse mDia1 and mDia2, which have been shown to be autoinhibited by the interaction between their diaphanous auto-regulatory domain (DAD) and diaphanous inhibitory domain (DID). Rho GTPase binds near the DID in N-terminal region of these proteins and releases the inhibition. Different formins appear to be activated by distinct subsets of the Rho family GTPases. Formins are used in various key cellular functions including cytokinesis, cell polarity, adhesion, endocytosis, and filopodia formation. Interestingly, formins are also implicated in the regulation of MT stability and mitotic spindle (reviewed in Goode and Eck, 2007).

3.3 Actin filament capping proteins

For efficient actin-induced movement actin filaments have to remain relatively short and rigid, which also allows rapid depolymerization. The length of actin filaments and the localization of fast growing barbed ends in cells are mastered by capping proteins. These proteins protect filament ends from addition or dissociation of actin monomers. If both ends of the filament are capped it is protected from depolymerization, but when only barbed end is capped, filament is destined for disassembly (reviewed in Pollard and Borisy, 2003). There are several actin filament capping proteins in cells, from which the best characterized are gelsolin and heterodimeric capping protein.

Heterodimeric **capping protein** (**CP**) is a highly conserved protein found in virtually all eukaryotic cells. This protein consists of two subunits, α and β, and it binds to the barbed ends of actin filaments with high affinity (0.1-1 nM). CP has been shown to bind several cellular components, such as $PI(4,5)P_2$ and CARMIL, both of which inactivate its filament capping activity (reviewed in Wear and Cooper, 2004). CP also binds twinfilin with high affinity, but the biological function of this interaction is unknown (Falck et al., 2004). CP is considered as a central component of the dendritic actin network, where Arp2/3 nucleated branched filaments are rapidly capped by CP to maintain the short filaments (Loisel et al., 1999).

Gelsolin is a ubiquitous filament barbed end capping protein that is composed of six homologous domains, G1-G6. In addition to capping, gelsolin is able to sever actin filaments in a Ca^{2+}-

dependent fashion. Gelsolin exists not only in the cytoplasm but is also secreted to the plasma of mammals, where it serves as an inhibitor of actin polymerization in blood. In fibroblasts, over-expression of gelsolin increases cell migration (reviewed in Silacci et al., 2004). During apoptosis, gelsolin is cleaved to G1-3 form, which has full activity independently of Ca^{2+}, causing disassembly of the actin cytoskeleton (Kothakota et al., 1997).

Only limited amount of data are available from more recently identified cappers including twinfilin (chapter 3.4), Eps8, actin interacting protein 1 (Aip1), and tropomodulins. The barbed end capping activity of **Eps8** remains auto-inhibited unless activated by Abi1 (Disanza et al., 2004). Furthermore, Eps8 appears to weakly cross-link actin filaments and has been suggested to activate F-actin bundling activity of IRSp53 (chapter 6.2.3) (Disanza et al., 2006). **Aip1**, on the other hand, binds F-actin very weakly by itself, but the presence of ADF/cofilins (chapter 3.4) increases its affinity for F-actin. This is proposed to lead both to filament severing and barbed end capping by Aip1 (reviewed in Ono, 2003). **Tropomodulins** specifically cap filament pointed ends and prefer tropomyosin-decorated actin filaments (chapter 3.5), which they cap with high affinity (≤0.05nM). Sarcomeric actin filaments require tropomodulins to define their length and provide stability (reviewed in Fischer and Fowler, 2003).

3.4 Actin monomer binding proteins

A set of actin monomer binding proteins regulate the cellular G-actin pool by sequestering and directing the monomers for polymerization according to the cellular requirements. Six actin monomer binding proteins are conserved throughout the eukaryotic evolution: profilin, ADF/cofilin, twinfilin, Srv2/CAP, WASP/WAVE, and Verprolin/WIP. WASP/WAVE and Verprolin/WIP proteins are also implicated in actin nucleation and were thus introduced above in chapter 3.2.

Profilin is a small actin monomer binding protein, which is highly abundant in cells and an essential component of many actin-dependent processes. Profilin binds ATP-G-actin with higher affinity than ADP-G-actin (K_D = 0.1 vs. 0.5 μM) and some isoforms catalyze the ADP/ATP nucleotide exchange of actin by 1000-fold (Goldschmidt-Clermont et al., 1992; Mockrin and Korn, 1980). Profilin also promotes the addition of actin monomers to the filament barbed ends, but in the absence of free barbed ends, profilin acts as a monomer sequestering protein. In addition to actin, profilin is known to interact with poly-(L)proline stretches found from several proteins including N-WASP, Ena/VASP, Arp2/3, and formins, all of which promote actin polymerization. Thus, profilin:actin is recognized by its interaction partners, which incorporate actin to the elongating filaments (reviewed in Witke, 2004). Furthermore, profilin was shown to interact with Srv2/CAP (Bertling et al., 2007).

Rapid depolymerization of undesired filamentous actin is essential for maintaining the cytoplasmic actin monomer pool that is used for construction of new actin assemblies and adapting the cytoskeleton to the constantly changing needs of the cell. The only protein family known to elicit this task is **ADF/cofilins**, which are responsible for high actin turnover rates *in vivo* and *in vitro*. These small proteins, comprised entirely of a single actin depolymerizing factor homology (ADF-H) domain, are

abundant in all eukaryotes and bind both filamentous and monomeric actin. ADF/cofilins bind to the sides of actin filaments, preferring ADP-actin, stimulate the dissociation of inorganic phosphate from F-actin, and concomitantly induce a twist in the filament structure leading to the promotion of depolymerization (reviewed in Bamburg, 1999; Paavilainen et al., 2004). Enhancement of P_i-release also results in shorter lifetime of Arp2/3-composed filament branches, since Arp2/3 complex has only weak affinity for ADP-actin filaments (Blanchoin et al., 2000). Furthermore, ADF/cofilins possess a weak filament severing activity, which also leads to increased amounts of filament ends and ultimately to enhanced depolymerization (Kiuchi et al., 2007). ADF/cofilins stay bound to the dissociating ADP-actin monomers, and subsequently release them to other actin monomer binding proteins. They also inhibit spontaneous nucleotide exchange on actin monomers, keeping the monomers in a polymerization incompetent state (reviewed in Bamburg, 1999; Paavilainen et al., 2004).

Twinfilin is a conserved protein that is composed of two ADF-H domains connected by a short linker region. Twinfilin binds ADP-actin monomers with high affinity and prevents their assembly to filament ends. In addition to this actin monomer sequestering activity twinfilin has been shown to bind capping protein (reviewed in Palmgren et al., 2002). Interestingly, recent studies revealed that twinfilin also functions as a filament barbed end capping protein (Helfer et al., 2006; Paavilainen et al., 2007). Twinfilin is involved in developmental processes in *Drosophila* and has been linked to endocytosis in mammalian and yeast cells, but how different biochemical activities of twinfilin contribute to these processes is not yet known (Helfer et al., 2006; Wahlstrom et al., 2001).

Srv2/CAP directly interacts with actin monomers and depletion of Srv2/CAP leads to the defects in the organization of the actin cytoskeleton. The proposed core function of Srv2/CAP is to serve as a molecular hub recruiting multiple other actin binding proteins, such as ADF/cofilin, profilin, and Abp1, to recycle actin monomers and ADF/cofilin for new rounds of filament assembly and disassembly, respectively (reviewed in Goode, 2007). Srv2/CAP prefers binding to ADP-actin monomers over ATP-actin and, at least in the yeast protein, the main actin binding region is located at the C-terminal β-strand domain (Mattila et al., 2004).

3.5 F-actin side binding proteins

A variety of F-actin side binding proteins have evolved to modulate the properties of actin filaments, thereby generating distinct sets of F-actin networks for different purposes.

Tropomyosins decorate actin filaments and physically protect them from depolymerization by ADF/cofilin, severing and capping by gelsolin, and branch formation by Arp2/3 (Blanchoin et al., 2001). Tropomyosins interact with gelsolin, dissociating it from F-actin and are also capable of annealing short actin filaments (Ishikawa et al., 1989). Interestingly, a recent study showed that tropomyosins regulate the barbed end dynamics by activating formins to stimulate rapid elongation of unbranched actin filaments (Wawro et al., 2007). The classical function of tropomyosins is in the sarcomeres of muscle cells, where they decorate actin filaments with troponin and play a key role in Ca^{2+}-regulated muscle contraction by controlling myosins' sliding

along F-actin (reviewed in Gong et al., 2005). In yeast, tropomyosins are essential for actin cable formation and thus, for cell polarization (Pruyne et al., 1998).

Myosins are a large superfamily of actin-based molecular motors that walk along actin filaments in a directed fashion. Typically, myosins are barbed end directed motors, but one isoform, myosin VI, has been shown to move towards the filament pointed end (Wells et al., 1999). Generally, myosins consist of three domains: the motor domain, which interacts with actin and possesses the ATPase activity, the regulative neck domain, and the tail region that anchors myosins to specific cargo molecules. The cargoes of myosins vary from another actin filament to plasma membrane, messenger RNA (mRNA), or membrane vesicles. Myosins are divided to 15 classes, of which conventional myosins of class II form filaments both in muscle and in non-muscle cells. In non-muscle cells, myosin II promotes, for example, stress fiber contractility and lamellipodial retrograde actin flow. On the other hand, multiple unconventional myosins, including classes Ia, VI, VIIa, X, and XVa, have been linked to the formation of pseudopodia of motile cells and to generation of parallel actin bundle structures of cells (reviewed in Faix and Rottner, 2006; Sellers, 2000).

3.5.1 Actin cross-linking proteins

Actin bundling/cross-linking proteins bind to the sides of actin filaments and contain either two independent actin binding sites or are oligomers of two or more actin binding proteins. A plethora of actin bundling proteins including α-actinin, fascin, filamin, vinculin, fimbrin, espin, and spectrin have been identified so far. Many of these proteins are specialized for particular actin structures, such as cortical F-actin network or parallel actin bundles of filopodia (chapters 4 and 5).

α-actinin is perhaps the best characterized actin bundling protein. It is an evolutionarily conserved protein belonging to the spectrin superfamily. In non-muscle cells, α-actinin is present in stress fibers, lamellipodia, and cell-cell and cell-matrix adhesions (reviewed in Otey and Carpen, 2004). α-actinin is a dimer, in which each monomer is composed of an N-terminal actin binding region followed by four central spectrin-like repeats creating the rod domain responsible for dimerization, and a C-terminal calmodulin-like domain with EF-hand motifs providing sensitivity for Ca^{2+} (Witke et al., 1993). In muscle myofibrils, α-actinin localizes to the z-disks, where it cross-links opposing actin filaments from adjacent sarcomeres. Four isoforms of α-actinin exist in mammals, of which the muscle-specific isoforms are insensitive for Ca^{2+} due to nonfunctional EF-hands (reviewed in Virel and Backman, 2004).

4. Actin in cells

Actin manifests its actions via a range of three-dimensional filamentous assemblies in cells. There is almost an infinite number of different actin based structures constantly adjusted to fulfill varying cellular requirements. The actin cytoskeleton reaches the whole cytoplasm, where it functions, for instance, in supporting many molecular complexes and cell organelles, and localizing mRNA molecules. However, the most clearly defined actin structures are often found at the cell periphery. A traditional example of a process depending on a well-organized but highly dynamic actin cytoskeleton is cell migration, illustrated in Figure 3.

Protrusive actin structures (lamellipodia and filopodia) lead the migration while contractile stress fibers, linked to focal adhesions, mediate the attachment to the substratum and support the cell. Finally, the rear of the cell is pulled forward (Bray, 2001). In addition to migration, highly ordered actin processes operate, for example, in cytokinesis and endo- and exocytosis. Here, an overview is provided on major actin based structures. However, filopodia and related structures are discussed in more detail in chapter 5.

4.1 Lamellipodia

Cells move mostly with the help of protruding actin structures collectively called pseudopodia. The most prominent structure in the leading edge of the cell is lamellipodium. Finger-like F-actin bundle structures, filopodia, are often

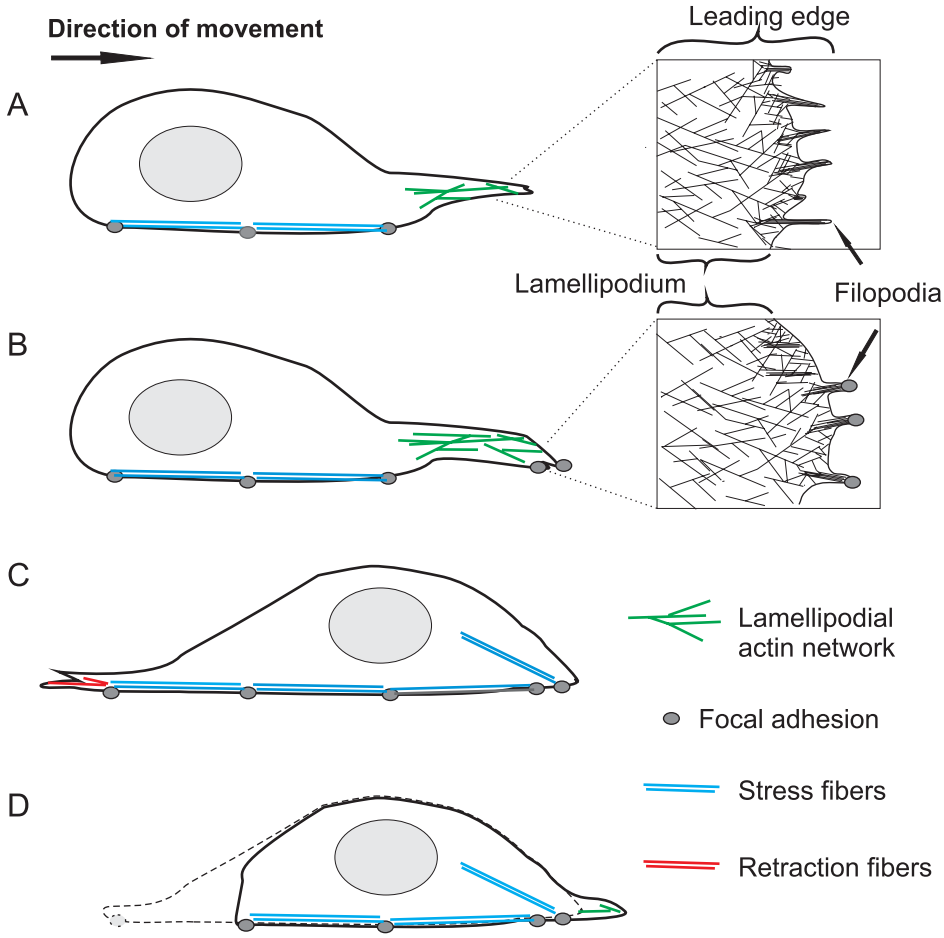

Figure 3. A schematic representation of cell migration on 2-D substratum. A. Cell motility is initiated by actin-dependent protrusion of the leading edge. Leading edge is composed of lamellipodia and filopodia (insets), which contain actin filaments with elongating barbed ends oriented towards plasma membrane. **B.** After extension, leading edge forms adhesions with the substratum. **C.** Nucleus and cell body are translocated forward through acto-myosin based contraction forces. **D.** Finally, the adhesions at the rear of the cell disassemble and trailing edge retracts.

found embedded in and protruding out from the lamellipodia. They are clearly distinguishable, although they spread their roots into lamellipodia (Figure 3, insets). All these structures at the cell front are composed of actin filaments that point their growing barbed ends towards the plasma membrane (Svitkina et al., 2003).

Lamellipodium is a thin (200 nm) leaflet with a width of several micrometers (Small and Resch, 2005). It is composed of branched actin meshwork with typical 70° angles between the filaments resulting from Arp2/3 mediated branching (Svitkina and Borisy, 1999). Branching frequency is highest in the close proximity to the plasma membrane resulting in very short filaments pushing towards the membrane. Behind the leading edge, debranching and depolymerization of filaments makes the network less dense and ultimately the structure is disassembled and replaced by other supporting structures, such as stress fibers (chapter 4.2). Lamellipodial structures that have no firm attachment to the substratum are often called membrane ruffles. In moving fibroblasts, the speed of actin polymerization is typically faster than the velocity of the cell protrusion, which leads to sliding of actin filaments backwards with respect to the substratum. This phenomenon is called actin retrograde flow. New actin monomers are constantly added to the barbed ends at the plasma membrane and depolymerization occurs from the pointed ends after branch dissociation or severing of the filaments (reviewed in Small and Resch, 2005; Welch et al., 1997).

The type of branched actin meshwork found in lamellipodia is also known as the dendritic actin array. The forces of dendritic actin polymerization have been reproduced *in vitro* using purified proteins to form so-called actin 'comet tail' structures (chapter 4.3). These studies provided a list of essential components for dendritic nucleation that includes Arp2/3 and its activators, capping protein, ADF/cofilin, and profilin. This study demonstrated that the power of actin polymerization is the main driving force of leading edge protrusion, and that no motor proteins are required for the movement (Loisel et al., 1999).

Lamellipodia are not only found at the leading edge of the cell, but also, for instance, in the growth cones of migrating neurites and during formation of the phagocytic cup of macrophages. It is clear that a plethora of components, in addition to the minimal crew of proteins promoting dendritic nucleation, is required to fine-tune and regulate lamellipodial extensions and enable the adoption of this structure for multiple cellular functions.

4.2 Contractile actin structures

Contractile actin structures are composed of antiparallel arrays of actin filaments associated with myosins. The pivotal role of actin in sarcomeres, the contractile machinery of striated muscle cells, has been evident for a long time. In sarcomeres actin filaments are strictly organized into tight bundles with myosin filaments lying next to the actin filaments. Through series of attachments and dissociations myosins walk along F-actin, which results in muscle contraction. This way, molecular events are transformed into large-scale movements of the body. The sarcomeric F-actin is considered to be highly stable, and even the most stable actin structures in non-muscle cells are believed to display much higher turnover rates as compared to sarcomeres (Bray, 2001).

Also no-muscle cells contain various contractile acto-myosin structures, such as stress fibers, adhesion belts

in epithelium, and contractile rings of dividing cells that separate the two daughter cells in cytokinesis. Stress fibers resemble sarcomeres in a sense that they are composed of antiparallel arrays of F-actin interspersed by bipolar myosin II filaments. Stress fibers connect the cytosplasmic structures to the substratum by interacting with adhesion complexes (focal adhesions) (Figure 3) and are important for changes in cell morphology. Contractility produced by special stress fibers, called retraction fibers, provides traction forces to pull the rear of the cell forward during cell migration (reviewed in Mitchison and Cramer, 1996; Small and Resch, 2005). A recent study revealed that stress fibers are generated both through formin-mediated actin polymerization at focal adhesions and from Arp2/3 nucleated lamellipodial actin network (Hotulainen and Lappalainen, 2006).

4.3 Other actin-based structures

Typical features of eukaryotic cells include a so-called actin cortex lying beneath the plasma membrane. The role of this three-dimensional actin meshwork is to give support and strength to the cell. The specific breakdown or remodeling of actin cortex is involved, for example, in cell motility, endocytosis, phagocytosis, and secretion of vesicles (Bray, 2001).

Actin polymerization is also the driving force for many vesicular movements both in mammalian and yeast cells. Formation of actin 'comet tails' has been shown to move endocytic vesicles and phagosomes in the cytoplasm. In addition, actin is considered to be essential for the pinching of vesicles from the plasma membrane during endocytic internalization (reviewed in Kaksonen et al., 2006).

Certain cytosolic parasites, such as *Listeria monocytogenes, Shigella flexineri* and Vaccinia virus, utilize similar actin comet tails as the rocketing endosomes for their movements inside the cells. *Listeria* is a widely studied and an outstanding example of this type of motility. This pathogen hijacks the actin polymerization machinery of the cell to form an actin comet tail that propels the parasite in the cytoplasm and eventually slings it to the neighboring cell (reviewed in Gouin et al., 2005). *Listeria* has served as a model for studying components necessary or affecting actin-induced movements in cells or in cell extracts. Furthermore, mimicking lipid vesicles or particles (typically, containing nucleation promoting factors, such as N-WASP) have been used to set up an *in vitro* system to reconstitute the motility with purified protein components (reviewed in Kaksonen et al., 2006).

4.4 Nuclear actin

In addition to cytosolic structures, actin and various actin binding proteins are also found in the nucleus. The role of nuclear actin has been questioned for a long time, mostly because the F-actin binding drug, phalloidin, that is widely used to visualize actin in cells, does not recognize nuclear actin. This might suggest that nuclear actin is not performing its main functions in filamentous form or that the filaments are structurally different from their cytosolic counterparts. Lately, nuclear actin was shown to be required for efficient transcription by RNA-polymerases I, II, and III (Fomproix and Percipalle, 2004; Hofmann et al., 2004; Hu et al., 2004; Philimonenko et al., 2004). Particularly, actin has been suggested to be important for the transcription initiation complex assembly and transcription elongation,

where actin polymerization by N-WASP may be involved (Hofmann et al., 2004; Wu et al., 2006). Also, actin is implicated in chromatin-remodeling complexes and nuclear lamina (reviewed in Bettinger et al., 2004). Moreover, a recent study demonstrates a role for nuclear actin in the regulation of a transcription cofactor MAL. Direct interaction with actin monomers, taking place both in the cytoplasm and nucleus, controls the localization and activity of this transcription cofactor in a manner dependent on actin monomer levels (Vartiainen et al., 2007).

4.5 Signaling to the actin cyto-skeleton – the Rho family GTPases

Small GTP binding proteins are molecular switches that cycle between a generally inactive GDP-bound and active GTP-bound forms. These proteins bind their downstream effectors typically in the GTP-bound form and return to the inactive state due to their intrinsic GTPase activity, which is often enhanced by GTPase activating proteins (GAPs). The change of GDP for GTP is assisted by GDP/GTP exchange factors (GEFs). Some GTPases have a third class of regulators called GDP dissociation inhibitors (GDI), which inhibit the exchange of nucleotide and keep the proteins in an inactive conformation (reviewed in Ridley, 2006).

The Rho subfamily of GTPases consists of 22 members, which play a leading role in the regulation of the actin cytoskeleton. Indeed, they have been shown to interact with, and regulate multiple actin binding proteins. In addition, these GTPases regulate microtubules, thereby coordinating the functions of the two cytoskeletal systems. Rho GTPases are typically activated on cellular membranes by their GEFs and thus direct their downstream targets on the membranes as well. Typically Rho GTPases contain lipid binding polybasic motifs or lipid modifications, such as prenylation or palmitoylation, to strengthen the localization to the membranes. An important aspect of, for example, cell protrusion activation by Rho GTPases is to localize the correct actin polymerization machinery to specific regions of the plasma membrane (reviewed in Ridley, 2006).

The most thoroughly studied mammalian Rho GTPases are Rac1, Cdc42 and RhoA. Many studies have demonstrated that Cdc42 promotes filopodia, but this GTPase also induces lamellipodia formation in some cell types. Rac1 is most often linked to the activation of the Arp2/3 complex and formation of the lamellipodial actin network. Inhibition of either Cdc42 or Rac1 was shown to reduce the processivity of leading edge extension (reviewed in Ridley, 2006). Cdc42 functions via interactions with multiple proteins, but the most clearly demonstrated activities include the induction of Arp2/3-dependent nucleation by activating WASP and N-WASP (Stradal and Scita, 2006). Similarly to Cdc42, also Rac1 has been clearly assigned to the activation of the Arp2/3 complex, via relieving the inhibition of WAVE proteins (Eden et al., 2002).

RhoA induces the formation of stress fibers and focal adhesions. RhoA inactivates ADF/cofilins by activating ROCK and LIM kinases (Maekawa et al., 1999). Also, downstream of RhoA, ROCK kinase phosphorylates myosin II and inactivates myosin light chain phosphatases, resulting in increased stress fiber formation and myosin II-based contraction (reviewed in Bresnick, 1999).

Direct regulation of actin binding proteins by RhoA is found among formins.

RhoA interacts with at least mDia1 and mDia2 activating them. It appears that multiple signaling pathways from various Rho GTPases, including also Cdc42 and Rif, lead to the activation of multiple formins in cells. Rho GTPases signal to the actin cytoskeleton also via associating with PI(4)P 5-kinases that catalyze the formation of $PI(4,5)P_2$ (chapter 6.1) (reviewed in Ridley, 2006).

5. Filopodia

Filopodia have been implicated in a number of cellular processes, such as migration, wound healing, adhesion to the extracellular matrix, guidance towards chemoattractants, neuronal growth cone pathfinding, and embryonic development. Filopodia are thin finger-like protrusions composed of parallel F-actin bundles that can vary greatly in their length, dynamics, and location. Typically, filopodia are found in cells that sense external gradients of chemoattractants or at the leading edge of the protruding cell where they may arise from the lamellipodial actin network (Figures 3 and 4A). However, in many cell types filopodia form without underlying dendritic actin array, so divergence in molecular mechanisms forming these protrusions is expected, and indeed, many contradictory findings have been reported (reviewed in Faix and Rottner, 2006; Gupton and Gertler, 2007). In addition, many other cellular extensions morphologically resemble filopodia as they consist similarly of parallel F-actin bundles, but these are considerably less dynamic and have specialized functions and molecular compositions. These structures include microvilli of enterocytes and lymphocytes, and stereocilia of cochlear cells. Comparative studies are warranted to determine the molecular and functional similarities between these structures.

5.1 Functions of filopodia and related structures

5.1.1 Filopodia in migration and chemotaxis

Generally, filopodia have been assigned as ‘antennas’ for cells to probe their microenvironment and serve as pioneers during protrusion, but the roles of

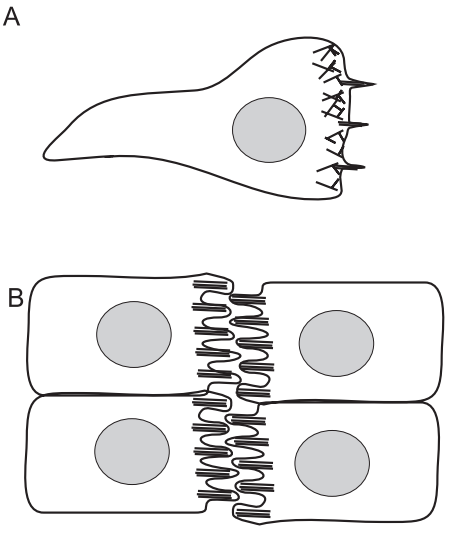

Figure 4. Examples of filopodia and related parallel actin bundle containing cellular protrusions. A. Typical filopodia are found at the leading edge of a migrating fibroblast. **B.** Filopodia of epithelial sheets play a role during wound healing and as precursors for adherens junction formation (adhesion zipper). **C.** Microvilli are found on the apical surface of many epithelial cell types and form, for example, intestinal brush border. **D.** Stereocilia of inner ear cells function in detecting sound waves.

filopodia are diverse and still, in many cases, remain vague. Filopodia have been implicated in several fundamental physiological processes, of which cell migration is among the best studied systems. Interestingly, ablation of filopodia at the leading edge of a migrating cell or a neuronal growth cone does not abolish migration, but in many cases affects the velocity, chemoattractant sensing, and path-finding properties (reviewed in Gupton and Gertler, 2007).

The main function of filopodia is considered to be sensing cell's surroundings and acting as sites for signal transduction. Filopodia have been shown to contain receptors for diverse signals and adhesion structures to the extracellular matrix. Many adhesion molecules, such as integrins and cadherins, are localized to the filopodia tips or along the shafts, and it has been suggested that filopodia function in sensing permissive substrates to allow adhesion or locomotion (Galbraith et al., 2007; Steketee and Tosney, 2002).

In neuronal growth cones, that are actin and MT rich structures at the ends of neurites, filopodia have been shown to play an important role in orienting the growth cones towards guidance cues, and thus leading to correct neurite outgrowth and maturation of one axon and multiple dendrites (reviewed in Gupton and Gertler, 2007). However, some studies suggest that filopodia are not essential for all types of neurite guidance. Retinal ganglion cells depleted of filopodia were able to migrate along the optic tract, although slowly, but failed to establish terminal arborizations (Dwivedy et al., 2007).

Interestingly, filopodia seem to play an important role in cell-cell adhesion, as implicated in would healing, dorsal closure in *Drosophila* embryo, and in the formation of adherens junctions of epithelial cells. In common to all these processes is that filopodia, which protrude from opposing cells, help the sheets of cells to align and adhere together (Figure 4B) (Wood et al., 2002). This 'adhesion zippering' of filopodia leads to the formation of mature adherens junctions between keratinocytes in a Ca^{2+}-dependent manner, suggesting a specific mode of regulation, important e.g. in wound healing (Vasioukhin et al., 2000).

5.1.2 Dendritic filopodia

In addition to neurite outgrowth, it was recently realized that the first developmental phase of dendritic spine formation involves filopodia. Dendritic spines are postsynaptic regions of most excitatory neuronal synapses that play an important role in higher brain functions, such as learning and memory. Expression of constitutively active Rac1 induces formation of dendritic spines and this provided the first evidence of the importance of the actin cytoskeleton in spine formation (Luo et al., 1996). Spines continuously change morphology by modulating their underlying actin machinery that plays a pivotal role in the spine plasticity and integrity. Filopodial precursors of spines have been suggested to dynamically grow, reach for the presynaptic partner and either stabilize and mature to a spine or, without a proper signal, shrink back to the dendrite backbone (reviewed in Sekino et al., 2007). However, only limited data are available on the role of the actin cytoskeleton in dendritic filopodia and spine formation, and further studies are thus required to elucidate whether these filopodial structures are generated through a similar mechanism as filopodia in motile cells.

5.1.3 Filopodia in phagocytosis and immune recognition

In macrophages, several filopodia typically explore the environment. After finding a pathogen, filopodia bind to it and retract towards the cell body. Filopodia and underlying lamellipodia then transform into a phagocytic cup. Interestingly, a recent study revealed the traction forces developed by macrophage filopodia upon capture of pathogen-mimicking particle. Surprisingly strong forces mediated over distances as large as 10 μm were found, demonstrating that filopodia can play a crucial role in pathogen capture (Vonna et al., 2007).

Upon antigen recognition, T lymphocytes rapidly form a filopodia-rich lamellipodium that spreads over the antigen-presenting cell. The exact role of filopodia here is yet to be confirmed, but it is likely that they enhance the efficiency of the protrusion of actin sheet and subsequent cell conjugation. This dramatic reorganization of the lymphocyte cytoskeleton leads to the formation of the immunological synapse and lymphocyte activation (reviewed in Dustin and Cooper, 2000). The formation of immunological synapse employs components of the dendritic actin network, such as Arp2/3 and WASP, but also the Ena/VASP family proteins are critical to enable efficient spreading, suggesting a key role for filopodia in this event (Krause et al., 2000). Interestingly, in the absence of the Arp2/3 complex, T cells are still capable of conjugating with antigen presenting cells by extending filopodia over the target cell (Gomez et al., 2007).

5.1.4 Microvilli

Typically, filopodia undergo constant growing and shrinking, in contrast to very similar structures, called microvilli, that are maintained relatively constant in length. Microvilli are found at the apical surface of many epithelial cells but also in lymphocytes and some sensory cells (Figure 4C). Microvilli form the brush border of intestine and kidney tubules, where their main function is to increase the surface of the epithelium and participate in nutrient absorption. Intestinal microvilli are 1-2 μm long and consist of approximately 20 parallel, highly ordered and tightly bundled actin filaments. Compared to filopodia microvilli are stable and uniform, yet actin filaments of microvilli constantly turn over (Loomis et al., 2003) and can be rapidly disassembled according to specific stimuli (reviewed in Bartles, 2000; Revenu et al., 2004).

Villin is the major actin cross-linking protein in the brush border. However, redundancy with other actin cross-linkers, such as fimbrin and small epsin, which are also found in microvilli, is likely, since villin knockout mice show only subtle changes in microvillar structure. Interestingly, upon lesions of intestine epithelium villin-deficient mice show reduced epithelial-mesenchymal transition, which is suggested to result form the lack of Ca^{2+}-induced severing activity of villin, facilitating microvilli break-down and acquirement of fibroblast-like morphology (reviewed in Bartles, 2000). Over-expression of villin in fibroblasts results in the formation of filopodia or microvilli on the dorsal cell surface (Friederich et al., 1989).

Proteins of ezrin-radixin-moesin (ERM) family function at the membrane: actin interface at the cell cortex and have, among other functions, an essential role in the development and stability of microvilli. ERM proteins are essential for the morphogenesis of the apical

domain in epithelial cells of various tissues, but details how ERMs regulate membrane proteins and coordinate actin polymerization still remain enigmatic. ERM proteins are found in the cytoplasm in an inactive conformation, where both membrane and actin binding sites are masked. Binding to $PI(4,5)P_2$ and subsequent phosphorylation relieve the intramolecular inhibition (reviewed in Fievet et al., 2007).

The actin bundling protein espin is proposed to play an important role in microvilli of sensory cells, such as taste receptor and chemoreceptor cells. This Ca^{2+}-insensitive F-actin cross-linker seems to be specialized for the extensions dedicated to sense bending or chemical ligands. Espins also have multiple accessory functions, including actin monomer binding via WH2 domain, and interactions with $PI(4,5)P_2$, and profilin (reviewed in Sekerkova et al., 2006).

Circulating T and B lymphocytes contain multiple microvilli on their surface. It is thought that these protrusions are important for the segregation of surface receptors and that they mediate the labile adhesions essential for cell crawling in the capillary walls and contact formation under flow (von Andrian et al., 1995). Upon lymphocyte migration into tissues or upon antigen recognition, microvilli are rapidly downregulated or concentrated to the rear of the cell (reviewed in Dustin and Cooper, 2000). Upon treatment with the actin monomer sequestering drug, latrunculin A, lymphocyte microvilli disassemble within 2 minutes, demonstrating the high dynamics of these structures. Lymphocyte microvilli do not nucleate via WASP-Arp2/3 pathway, in contrast to the filopodia of immunological synapse, demonstrating the differences in their regulation (Majstoravich et al., 2004).

5.1.5 Stereocilia

Stereocilia, the hearing organs of hair cells of inner ear, are considered as highly specialized microvilli. Hair cells are mechanosensory cells that detect sound waves via their highly organized, staircase-like collection of stereocilia on top of the cells (Figure 4D). Upon bending of stereocilia, ion channels of the surrounding plasma membrane open and downstream signaling, which leads to neurotransmitter release, is triggered. Stereocilia are derived from microvillar precursors, but in mature state are considerably longer and contain more actin filaments, which are densely cross-linked by fimbrin and espin to form a paracrystalline actin core. Despite of their apparent stability and highly defined length, actin treadmilling in stereocilia core (approximately 0.002-0.04 monomers/s) is required for maintenance of the structure and is proportional to the stereocilia length so that synchronous turnover in stereocilia of a hair cell is obtained (Rzadzinska et al., 2004).

Studies of stereocilia formation in *Xenopus* revealed an important role for Xevl, a homologue of Ena/VASP proteins that play a key role in filopodium formation. However, further work is required to reveal the mechanisms of stereocilia formation, because Xevl does not localize in stereocilia but rather to the anchoring structure, cuticular plate (Wanner and Miller, 2007). Stereocilia, similarly to sensory microvilli, contain espins as key actin bundling proteins consistent with the specialized role of these cross-linkers in cellular extensions implicated in signaling (reviewed in Lin et al., 2005a; Sekerkova et al., 2006).

5.1.6 Neurosensory bristles of *Drosophila*

Neurosensory bristles found on the thorax of *Drosophila melanogaster* are comparable to hair cell stereocilia in a sense that also these projections serve as mechanosensory organs. Each *Drosophila* mechanosensory cell contains only one long (typically 250-300 μm) bristle, which is curved and consists of 12-18 plasma membrane associated F-actin bundles. Bristle formation involves a unique feature of end-to-end joining of preformed F-actin bundles, which is likely to be important for the construction of curvature and length. Two F-actin bundling proteins, forked and fascin, are shown to be important in bristle formation. Forked acts during the initiation of bristle formation, while fascin provides final stiffness to the structure. There is evidence that cross-linking proteins also regulate the turn-over of F-actin in bristles. Turn-over is highest at the tip, where only forked is present and decreases proportionally closer to the base, where fascin predominates (reviewed in Tilney and DeRosier, 2005).

5.2 Filopodium structure

Studies on filopodia have revealed plenty of variation in dynamics, length, and positioning of filopodia in different cells, indicating distinct or differently regulated machineries generating discrete sets of filopodia. Fibroblast lamellipodia and nerve growth cone filopodia rarely exceed 10 μm in length, but in sea urchin embryos they extend up to 40 μm (Welch and Mullins, 2002). Very short filopodia of cultured cells are often called microspikes and are almost completely embedded in the cell cortex or the leading edge. Filopodia can be found either at the cell periphery, as typically in the case of migration, or on the apical cell surface, resembling microvilli, where their function often remains tentative.

Electron microscopy (EM) experiments from various cell types also revealed distinct architectures of filopodia. Platinum replica transmission EM (TEM) of the leading edge suggested that filopodia arise from lamellipodial F-actin network, probably through bundling and uncapping of these filaments. A continuous actin bundle was seen to span from the root to the tip of filopodia (Svitkina et al., 2003). This study provided key evidence for the so-called 'convergent elongation' model, in which filopodial actin filaments are nucleated by the Arp2/3 complex in the dendritic actin array. In contrast, cryo-electron tomography of *Dictyostelium* filopodia revealed a discontinuous F-actin bundle in the filopodium core, and short individual filaments converging into the 'terminal cone'. The differences compared to mammalian leading edge filopodia are proposed to optimize the faster dynamics of *Dictyostelium* processes (Medalia et al., 2007).

5.3 Signaling to filopodia

Small GTPases of the Rho superfamily are linked to the regulation of cell morphology and, especially, the actin cytoskeleton. From the well-established members, particularly Cdc42 has been implicated in the formation of filopodia. Cdc42 interacts with WASP and N-WASP and this, together with $PI(4,5)P_2$ binding, relieves the autoinhibited conformation of WASP leading to the activation of the Arp2/3 complex. It was demonstrated that expression of Cdc42/Rac interactive binding (CRIB) domain of WASP blocks induction of filopodia by Cdc42, suggesting that Cdc42 exerts its function mainly via WASP:Arp2/3 pathway

(Pellegrin and Mellor, 2005). However, it was also demonstrated that cells devoid of N-WASP and WASP are able to produce filopodia upon Cdc42 stimulation, showing that multiple pathways must exist in the process. One of the possible alternative pathways could involve a Cdc42-interacting protein IRSp53, which binds WAVE2 and Ena/VASP protein Mena, and induces filopodia and lamellipodia formation (chapter 6) (reviewed in Gupton and Gertler, 2007).

The three-dimensional structure and relatively slow dynamics of filopodia raise questions of importance of actin nucleator proteins after the initial burst of nucleation. In lamellipodia, constant nucleation by Arp2/3 is essential but, in contrast, filopodial F-actin could, in principle, exist for long periods of time by just treadmilling. In addition, Cdc42 is not essential for triggering filopodia in all cells or all situations, since filopodia formation was demonstrated also in cells depleted from Cdc42 (reviewed in Gupton and Gertler, 2007).

Another small GTPase, called Rho in filopodia (Rif), also stimulates filopodia formation upon over-expression. Rif exerts its function via activating mDia2 formin (Pellegrin and Mellor, 2005). Additionally, Rho GTPases TC10 and RhoT have been demonstrated to induce filopodia formation (Abe et al., 2003). It is clear that multiple Rho GTPases are able to induce cellular protrusions when over-expressed but yet the roles under physiological conditions remain unclear. Plasmalemmal phosphoinositides are also found to activate filopodia formation by localizing actin polymerization machinery (through $PI(4,5)P_2$) and by spatially activating Rho GTPases (through $PI(3,4,5)P_3$) (chapter 6.1).

RhoA and Rac1 antagonistically regulate the phosphorylation state and activity of ERM proteins in microvilli. Activation of PI(4)P 5-kinase via RhoA and subsequent localization of ERM proteins to the $PI(4,5)P_2$-rich membrane domains leads to the phosphorylation of ERMs and induces microvilli formation, while Rac1 activation leads to the dephosphorylation of ERM proteins and rapid loss of the apical protrusions (Louvet-Vallee, 2000; Nijhara et al., 2004).

5.4 Molecular composition of filopodia

The architecture of filopodia and lamellipodia are dramatically different, although filopodia have been suggested to arise from and spread their roots into lamellipodia (Svitkina et al., 2003). Also, F-actin in filopodia was shown to be rather stable, turning over in 20 min (Mallavarapu and Mitchison, 1999), as compared to filaments in lamellipodia, which turn over in 1 min (Theriot and Mitchison, 1992). Clearly, quite distinct sets of molecular machineries are needed to establish these protrusions.

A convergent elongation model has been proposed to explain the filopodia formation from underlying lamellipodial actin meshwork. In this model, the Arp2/3 nucleated filaments are protected from capping to continue elongation and are cross-linked together to form a bundle that protrudes against plasma membrane. Here, Ena/VASP proteins that localize to the tips of filopodia, are thought be in a key role. Association of Ena/VASPs with the barbed ends of the filaments could mark these filaments for filopodial elongation by their multiple activities, including inhibition of barbed end capping, enhancement of filament elongation, and F-actin bundling (reviewed in Gupton and Gertler, 2007; Welch and Mullins, 2002). Ena/VASPs also contain the profilin binding poly-

(L)proline sequence, and profilin has been demonstrated to enhance Ena/VASP-mediated barbed end protection from capping proteins (Barzik et al., 2005).

Ena/VASPs are also critical for the formation of 'adhesion zippers', filopodia-based contacts preceding adherens junction formation in epithelial cells. Interestingly, in this study Ena/VASP function was dispensable for filopodium protrusion, but required for membrane sealing. Ena/VASPs localized to the filopodia tips via interactions with vinculin, zyxin and α-catenin. During adhesion zipper formation, F-actin on the shafts of filopodia was mainly cross-linked by α-actinin and not by a more commonly utilized filopodium-specific actin bundling protein, fascin (Vasioukhin et al., 2000).

Actin filament bundling proteins are essential to establish and maintain the tight F-actin bundles of filopodia. Fascin is found in many filopodial structures, and it is specialized for making stiff and parallel filament bundles. Also α-actinin and ABP-280 are present in filopodia, but they seem to be more flexible cross-linkers and are utilized also in antiparallel and more loose F-actin structures. Fascin is not an efficient bundler of preformed filaments, but is a master in bundling filaments as they polymerize or are already loosely linked together (reviewed in Welch and Mullins, 2002).

Although filopodia are often found at the leading edge, it is evident that lamellipodium is not always the platform for filopodia. Many studies have demonstrated filopodia formation by a mechanism different from convergent elongation, often involving the formin Dia2. Since formins are known to induce straight actin filaments by processive barbed end nucleation, it is not surprising that they have been linked to filopodia formation. Dia2 over-expression induces filopodia in many cell types and, importantly, knockout studies in *Dictyostelium* have demonstrated a vital role of dDia2 in filopodia formation in this organism (reviewed in Gupton and Gertler, 2007). Interestingly, dDia2 cooperates with the single VASP orthologue in filopodia formation, which suggests interdependency of these two filopodia initiation models, and warrants further studies on the mechanisms (Schirenbeck et al., 2006).

Myosins have also been implicated in the formation of filopodia, although their role during this process is not well understood. Over-expression of unconventional myosin X induces filopodia formation and the protein localizes to the filopodia tips. Myosin X appears to move up and down filopodia shafts probably by walking towards the filament barbed ends and sliding down with the retrograde actin flow (Berg and Cheney, 2002; Berg et al., 2000).

In addition to F-actin, myosin X interacts with VASP, $PI(3,4,5)P_3$, integrins, and MTs, and thus multiple roles for myosin X in filopodia formation have been suggested. The general proposition is that myosin X acts as a transporter for other filopodial components, such as Ena/VASP proteins and integrins, to the dense tip of the filopodium. Indeed, the motor domain is required for filopodia formation by myosin X, but integrin binding does not have a crucial function, as demonstrated by myosin X-induced filopodia formation on the apical surface of cells, i.e. without substratum attachments. Also, deletion of the integrin binding domain does not affect filopodia formation (Bohil et al., 2006). Interestingly, the related myosins 7a, 7b, and 15a localize to stereocilia and microvilli and are demonstrated to be

Table I. Key proteins involved in the formation of lamellipodia and filopodia (reviewed in Faix and Rottner, 2006; Gupton and Gertler, 2007; Revenu et al., 2004).

Protein	Localization	Proposed activities and functions
α-actinin	Filopodial F-actin bundle, especially rear, and stress fibers	Bundling of actin filaments.
Arp2/3	Lamellipodia	Nucleation of branched actin filaments upon the activation by several regulator proteins.
Capping Protein	Lamellipodia	Barbed end capping.
Cdc42	Plasma membrane	Signaling, especially to filopodia formation.
Cofilin	Lamellipodia, behind the very front	Depolymerization of actin filaments, filament severing.
Ena/VASP	Tips of filopodia, leading edge	Uncapping, bundling, and elongation promotion of filaments. Part of the so-called tip complex of filopodia, thought to destine actin filaments to filopodia by binding to barbed ends.
Fascin	Along the filopodia shafts, lamellipodia	Considered as the main F-actin bundling protein of filopodia.
Formins (dDia2, mDia2)	Lamellipodia and filopodia tips	Nucleate unbranched actin filaments upon the activation by Rho GTPases and control barbed end growth.
Gelsolin	Lamellipodia	Severing, barbed end capping.
Myosin X	Filopodia tips	Transport to filopodia, anchorage of filopodial integrins.
Profilin	Diffuse, enriched at cell periphery	Promotes nucleotide exchange and addition of actin monomers to barbed ends. Profilin:actin is considered to be the major form of polymerization competent actin.
Rac1	Plasma membrane	Signaling, especially to lamellipodia formation.
Rif	Tips of filopodia	Signaling to filopodia.
Srv2/CAP	Lamellipodia	Recycles ADP-actin monomers from cofilin to profilin for new rounds of polymerization.
Twinfilin	Lamellipodia	Sequesters actin monomers, caps ADP-F-actin barbed ends.
WASP	Lamellipodia	Activates Arp2/3 complex.
WAVE	Lamellipodia and filopodium tip (WAVE2/3)	Activates Arp2/3 complex.

critical for the integrity of these structures (reviewed in Sousa and Cheney, 2005).

Additional mechanisms and players in the formation of filopodia are also emerging. Interestingly, a recent study identified an integral membrane protein, lipid phosphatase-related protein 1 (LPR1), in the induction of filopodia in a manner independent of Arp2/3 complex and Ena/VASPs. This protein seems to be catalytically inactive compared to its family members, lipid phosphatases, and thus, has evolved a distinct function that leads to filopodia formation by mechanisms not resolved to date. A tempting idea is that this membrane protein could function as

a link between the plasma membrane and the core of filopodia (Sigal et al., 2007).

The forces developed by the polymerization of packed actin filaments are traditionally thought to result in an extension of the plasma membrane, while the Brownian motion of the filament ends allows space for addition of new subunits and consequently, pushes the plasma membrane forward. Recently, N-WASP was demonstrated to physically attach the growing filament barbed ends to the plasma membrane. This activator of Arp2/3 nucleation machinery contains a polybasic patch that interacts with lipids. According to this study, the WH2 domain of the membrane bound N-WASP feeds the barbed ends with actin monomers and simultaneously links the filament ends to the membrane (Co et al., 2007). Future studies will reveal if similar linkage between filament barbed ends and the plasma membrane is also necessary for filopodia formation.

6. Interplay between the actin cytoskeleton and the plasma membrane

Many actin-dependent processes, including formation of cellular protrusions, cell division, and vesicle trafficking, are coupled to the remodeling of the plasma membrane or other cellular membrane compartments. During endocytosis, forces developed by the actin cytoskeleton are coupled to the invaginating membrane pit that develops into an endocytic vesicle. In adhesion complexes that mediate the attachments of the cell to the substratum or neighboring cells, plasma membrane receptors are linked to the actin cytoskeleton, such as stress fibers. In addition to the physical linkage between the membrane and the cytoskeleton, recent studies have clearly demonstrated that plasmalemmal phosphoinositides mediate signals to the actin cytoskeleton.

6.1 PI(4,5)P_2 and PI(3,4,5)P_3

For controlled cell migration and extension of pseudopods, actin polymerization machinery has to be localized to the plasma membrane and elongating filaments have to be protected from capping and depolymerization. Activities and localization of myriad regulators of actin cytoskeleton are controlled by phospholipid moieties at the plasma membrane. Most potent signaling lipids to the actin cytoskeleton include phosphoinositides PI(4,5)P_2 and PI(3,4,5)P_3 (reviewed in Hilpela et al., 2004).

Approximately 5 % of total phosphoinositides (PI) in cells are phosphorylated at 4- and 5-positions, and PI(4,5)P_2 comprises about 1 % of total phospholipids in cells (McLaughlin et al., 2002; Rameh and Cantley, 1999). The estimated local concentration of PI(4,5)P_2 is approximately 5 mM (Lemmon and Ferguson, 2000). Traditionally, PI(4,5)P_2 has been mainly considered to function as a precursor for the second messenger molecules inositol(1,4,5)trisphosphate (IP_3) and diacylglycerol (DAG). However, research carried out during the past decade has revealed that PI(4,5)P_2 interacts with a plethora of actin binding proteins and regulates their activity. Thus, PI(4,5)P_2 is currently regarded as a general regulator of actin dynamics at the plasma membrane. Intriguingly, it was shown that PI(4,5)P_2 or PI(3,4,5)P_3-rich lipid vesicles induce actin polymerization in *Xenopus laevis* cell extracts (Ma et al., 1998). Also, over-expression of PI(4)P 5-kinase induces formation of F-actin structures in many

cell types and sequestration of $PI(4,5)P_2$ at the plasma membrane by over-expressing the $PI(4,5)P_2$ –binding PLCδ PH domain inhibits actin polymerization (reviewed in Hilpela et al., 2004; McLaughlin et al., 2002).

$PI(4,5)P_2$ concentrations in cells are relatively constant, but undergo small changes upon stimulation of cell migration. Furthermore, $PI(4,5)P_2$ is suggested to be enriched in plasmalemmal rafts and, for example, at the leading edge. Enzymes producing $PI(4,5)P_2$, PIP-kinases, are shown to be targeted to the sites of actin polymerization, thereby providing a spatial signal for polymerization. There are different isoforms of PIP-kinases targeted to different actin-rich structures, such as leading edge or focal adhesions. PIP-kinases are localized to the plasma membrane and activated by the Rho family GTPases. For example, RhoA induces the formation of microvilli and ARF6 promotes membrane ruffle formation via PIP-kinases (reviewed in Hilpela et al., 2004; Ling et al., 2006). A recent study carried out in yeast *Saccharomyces cerevisiae* revealed that $PI(4,5)P_2$ levels decrease and increase during the different stages of clathrin-dependent endocytosis and suggested that $PI(4,5)P_2$ turnover is required for productive clathrin- and actin-dependent vesicle internalization (Sun et al., 2007).

Generally, $PI(4,5)P_2$ inactivates many negative regulators of actin polymerization, such as filament capping, severing, and depolymerizing proteins. Correspondingly, $PI(4,5)P_2$ localizes and activates multiple positive regulators, which enhance actin nucleation or filament elongation. Binding to $PI(4,5)P_2$ triggers WASP and N-WASP, activators of the Arp2/3 complex (Prehoda et al., 2000), and dissociates CP and gelsolin from the barbed ends of filaments, leading to actin polymerization in close proximity to the plasma membrane (Liepina et al., 2003; Schafer et al., 1996). Furthermore, the activity of profilin is regulated by phosphoinositides. Binding to $PI(4,5)P_2$ down-regulates the interactions with poly(L)-proline sequences and both $PI(4,5)P_2$ and $PI(3,4,5)P_3$ were shown to release profilin:actin complex. However, since profilin:actin is competent for polymerization, the biological role of this inhibition remains unclear. Profilin is also able to regulate phosphoinositides by protecting them from hydrolysis by activating PIP 3-kinase (reviewed in Witke, 2004). Also, ADF/cofilins are inhibited by $PI(4,5)P_2$ and other phosphoinositides (reviewed in Bamburg, 1999; Paavilainen et al., 2004). This nicely fits with their function in depolymerizing aged actin filaments behind the leading edge of the cell.

$PI(4,5)P_2$ has also been demonstrated to activate the ERM family of proteins, which link actin filaments to membrane proteins. $PI(4,5)P_2$ facilitates the interactions of ERM proteins both with membrane proteins and with F-actin. In addition, $PI(4,5)P_2$ participates in the activation of focal adhesion proteins, talin and vinculin, and regulates multiple actin cross-linking proteins in variable ways. For example, the actin bundling activity of α-actinin is strongly activated by $PI(4,5)P_2$, whereas that of filamin and cortexillin is inhibited by $PI(4,5)P_2$ (reviewed in Hilpela et al., 2004).

In addition to modulating the activity of proteins, phosphoinositides also function as localization signals. Due to the rather high concentration and relatively even distribution, $PI(4,5)P_2$ is argued to control actin polymerization generally at the plasma membrane. In contrast, the more defined localization of

$PI(3,4,5)P_3$, the less abundant derivative of $PI(4,5)P_2$, would function as a sharp spatial marker for signaling pathways, leading to polymerization activation (Insall and Weiner, 2001). Only less than 0.25 % of the total phosphoinositides are phosphorylated at position 3 (Rameh and Cantley, 1999) and even after stimulation, the $PI(3,4,5)P_3$ concentration remains at approximately 20 times lower level than that of $PI(4,5)P_2$, reaching an estimated local concentration of 200 μM (Lemmon and Ferguson, 2000).

PIP 3-kinase activity, which phosphorylates $PI(4,5)P_2$ to generate $PI(3,4,5)P_3$, is necessary and sufficient for many cellular processes involving actin assembly. Increase in $PI(3,4,5)P_3$ is demonstrated to lead to enhanced actin polymerization, cell migration and invasiveness. $PI(3,4,5)P_3$ is generated strictly in space and time by growth factor stimuli that activate the PIP 3-kinases. For example Rho GTPases are effectors of $PI(3,4,5)P_3$ and transmit the signal further. To achieve an immediate and transient signal, PIP-5-phosphastases rapidly dephosphorylate $PI(3,4,5)P_3$. While the majority of the effects of $PI(3,4,5)P_3$ to the actin cytoskeleton are mediated via Rho GTPases, also myosin X was shown to directly interact with $PI(3,4,5)P_3$ (reviewed in Hilpela et al., 2004).

6.2 BAR domain superfamily

The interplay between membrane deformation and remodeling of the actin cytoskeleton is demonstrated by a critical role of actin dynamics during endocytosis and localization of actin and actin regulatory proteins to the sites of vesicle formation. However, the underlying mechanisms are far from understood.

Research carried out during the last decade has revealed an exceptional set of proteins that are able to drive, sense, and maintain membrane curvature via their Bin-Amphiphysin-Rvs (BAR) and FCH BAR (F-BAR) domains. BAR and F-BAR domain proteins function particularly in endocytosis and are closely linked to the actin cytoskeleton, because many of these proteins were shown to interact with the regulators of actin assembly. Surprisingly, a BAR-related, all-helical dimeric structure was also observed in the IRSp53/MIM homology (IM) domain (Figure 5), which was demonstrated to function as an F-actin bundling domain (reviewed in Dawson et al., 2006). Future structural analyses will reveal if the BAR-like fold is even more widely exploited in the eukaryotic kingdom.

6.2.1 BAR domain

BAR domains are found in a variety of proteins typically implicated in membrane traffic, regulation of the actin cytoskeleton, and signaling. The common feature of BAR domains is that they form dimers and are able to deform liposomes into narrow tubules. This translates into the formation of tubular invaginations from the plasma membrane upon over-expression of BAR domain proteins in cells. BAR domains were also demonstrated to bind small GTPases, including Rac1 (reviewed in Habermann, 2004; Itoh and De Camilli, 2006).

BAR domains are ‘banana-shaped’ dimers that interact with negatively charged lipids through the patches of positively charged residues at the concave face of the crescent (Figure 5A). The membrane deformation and curvature sensing are driven by the intrinsic curvature of the rigid domain. A subset of BAR domains, called N-BARs, contain an amphipathic N-terminal helix that inserts into the lipid bilayer and drives

stronger membrane deformation. Protein families that contain N-BAR, such as endophilins and amphiphysins, are thought to drive membrane deformation in cells, while simple BAR domains without the amphipathic helices, found e.g. in oligophrenins and sorting nexins, are probably curvature sensing modules that recruit other proteins to the sites of membrane invagination or to endocytic vesicles (Gallop et al., 2006; Masuda et al., 2006; Peter et al., 2004).

6.2.2 F-BAR domain

F-BAR domain is another class of protein modules capable of binding and tubulating liposomes similarly to BAR domains. Sequence comparisons and structural predictions suggested that this domain, also known as extended FCH (EFC) domain, shares homology with the BAR domain (Itoh et al., 2005).

Recently, three F-BAR domain structures, from proteins called FBP17, CIP4, and FCHo2, have been solved confirming a clear, although rather distant, relation to BAR-domains. Distinct from BAR, F-BAR features much longer helices and shallower degree of curvature (Figure 5B), which correlates well with the wider diameter of membrane tubules induced by F-BAR domains. Thus, it is likely that BAR and F-BAR domains function in separate steps of endocytosis shaping different parts of the nascent vesicle. Interestingly, one F-BAR monomer contains 5 α-helices as compared to canonical 3 helices of BAR domains (Henne et al., 2007; Shimada et al., 2007). FCHo2 F-BAR contains a kink perpendicular to the membrane binding surface that is suggested to lead to different tangential positioning of the domain at the membrane and subsequently to the formation of 60 to 150 nm diameter tubules (Henne et al., 2007). F-BAR domains of FBP17 and CIP4 were demonstrated to form filaments resulting in a spiral-like lattice on the membrane, which is likely to drive the constriction of the tubule from

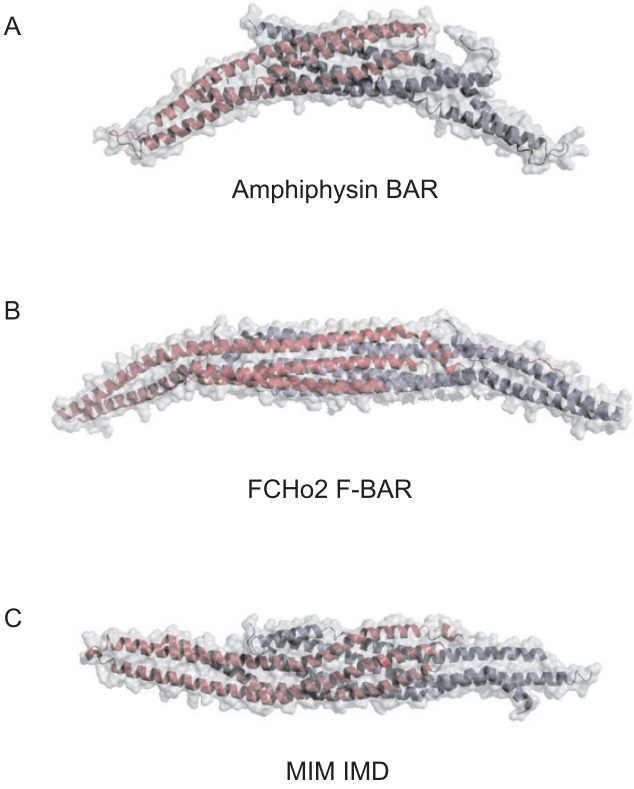

Figure 5. Comparison of three subfamilies of the BAR domain superfamily. One monomer of each dimer is in magenta and the other in blue. Surfaces are shown in grey. **A.** N-BAR domain of amphiphysin (PDB ID:1URU). **B.** F-BAR domain of FCHo2 (PDB ID:2V0O). **C.** IM-domain of MIM (PDB ID:2D1L). The picture was created with program PyMOL (http://www.pymol.org).

the parental membrane (Shimada et al., 2007).

F-BAR domains can be found in isolation as in FCHo2, or with other protein domains, such as in syndapin and members of the *Pombe* Cdc15 homology (PCH) family of proteins like FBP17 and CIP4. PCH proteins are key players in endocytosis and are closely linked to the actin cytoskeleton because they bind, for example, to N-WASP (Itoh et al., 2005).

6.2.3 IM-domain - IRSp53/MIM protein family

IM-domain (IMD), a conserved protein domain, found from cytoskeletal regulator proteins missing-in-metastasis (MIM) and insulin receptor substrate p53 (IRSp53), induces drastic formation of filopodia upon over-expression in cells (Yamagishi et al., 2004). Several studies have demonstrated that IM-domains cross-link F-actin (Bompard et al., 2005; Disanza et al., 2006; Gonzalez-Quevedo et al., 2005; Millard et al., 2005; Yamagishi et al., 2004), but this activity has also been a matter of some discrepancy (Lee et al., 2007). IM-domains from MIM and IRSp53 bind Rac1 and MIM was also demonstrated to activate Rac1 (Bompard et al., 2005; Miki et al., 2000). Intriguingly, when this domain was crystallized and the atomic structure solved, a remarkable similarity to the BAR domain was revealed (Figure 5C) (Lee et al., 2007; Millard et al., 2005). In contrast to BAR and F-BAR, the IM-domain has 'zeppelin-shape' rather than 'banana-shape' and no involvement in endocytosis or membrane trafficking has been revealed. This has raised the question of how a BAR-like fold is exploited in the formation of filopodia.

MIM was initially characterized as a potential metastasis suppressor in bladder cancer (Lee et al., 2002). The following studies have not confirmed the role specifically in metastasis, however, changes in MIM expression, down- or upregulation, have been linked to the formation of different cancers in multiple studies (Callahan et al., 2004; Loberg et al., 2005; Ma et al., 2007; Wang et al., 2007a). MIM was suggested to enhance Arp2/3-mediated actin polymerization through interaction with cortactin but to inhibit WASP-mediated actin polymerization (Lin et al., 2005b). Interestingly, MIM was also characterized as a Sonic hedgehog (Shh) responsive gene and furthermore, to potentiate Shh-induced transcription via direct interactions with transcription factors Gli1 and 2 (Callahan et al., 2004; Gonzalez-Quevedo et al., 2005). The biological functions of different activities of MIM in cells and animals have remained obscure.

IRSp53 has been more extensively studied than MIM, but also the role of IRSp53 in cells remains elusive. Many interaction partners have been identified for IRSp53, and thus it has been suggested to function as a scaffold for the actin polymerization machinery. IRSp53 interacts with Cdc42 and Rac1 through its N-terminal region, and with WAVE2 and Ena/VASP family protein Mena through its central SH3 domain. It is proposed that IRSp53 would recruit Mena downstream of Cdc42 to filopodia tips and, on the other hand, activate WAVE2 downstream of Rac1 resulting in Arp2/3-promoted lamellipodium formation (Krugmann et al., 2001; Miki and Takenawa, 2002).

Interestingly, a predominant proportion of IRSp53 was demonstrated to reside at the cell membranes (Suetsugu et al., 2006). Eps8, an actin filament capping protein, was shown to bind to IRSp53 and promote its F-actin bundling

activity, leading to increased cell motility and Rac1 activation (Disanza et al., 2006; Funato et al., 2004). IRSp53 has also been implicated in the dendritic spine formation with multiple interaction partners, but whether this protein is involved in the filopodial stage of spine formation or in the generation of mature spines is not known (Choi et al., 2005; Hori et al., 2005).

AIMS OF THE STUDY

Upon database searches for putative actin binding WH2 domains, we identified several previously uncharacterized proteins containing the domain. We selected one of these novel proteins for further characterization. During our analysis the corresponding mRNA was linked by others to the formation of bladder cancer metastases and thus named as missing-in-metastasis (MIM) (Lee et al., 2002). In addition to the WH2 domain, MIM contains a novel N-terminal IM-domain shared with IRSp53 (Yamagishi et al., 2004). Also, MIM was recently suggested to be a Sonic hedgehog (Shh) responsive gene and to potentiate Gli transcription factor mediated transcription of the Shh pathway (Callahan et al., 2004). However, the mechanisms by which MIM regulates cell morphogenesis as well as its role(s) in animal tissues have been unknown. Specific aims of this work were:

1. To characterize the expression pattern and possible actin binding activities of MIM.

2. To reveal the molecular mechanism underlying filopodia formation by IM-domains of MIM and IRSp53.

3. To study the physiological role of MIM by generating and analyzing knockout mice and to analyze the role of MIM in Sonic hedgehog signaling.

METHODS

The most important preparative and experimental methods that I have used in this study are summarized in the table below. Detailed descriptions are found in the original publications as indicated.

Table II. Methods personally applied in this study.

Method	Publication
Co-sedimentation assays	II, III
Dynamic light scattering	III
Generation of knockout mice	IV
Histological analysis	IV
Immunofluorescence light microscopy	II, IV
In vitro light microscopy	III
Mammalian cell culture	II, IV
Mu transposition	IV
NBD-actin assay	II, III
Northern blotting	II
Preparation of synthetic lipid vesicles	III
Purification of rabbit muscle actin	II, III
Pyrenyl-actin polymerization assays	II, III
Recombinant protein expression and purification	II, III
Reverse transcription polymerase chain reaction	IV
SDS-PAGE	II, III
Site-directed mutagenesis and plasmid construction	II, III, IV
Southern blotting	IV
Transmission electron microscopy	III
Urea denaturation assay	II
Whole Mount X-gal staining	IV

RESULTS AND DISCUSSION

7. MIM regulates actin dynamics

7.1 Identification of a novel WH2 domain protein, MIM (I)

By early 21st century, the sequences of human genome, as well as of several model organisms, such as mouse, fruit fly, and bakers yeast, became available through the development of new powerful sequencing methods. This provides us with excellent opportunities to explore and compare protein sequences from diverse organisms. This way we are able to analyze the conservation and postulate on the evolution of a variety of protein modules. On the other hand, sequencing projects have uncovered plenty of new protein sequences without functional information.

We mined databases and carried out a comparison of sequences resembling short monomeric actin binding motifs WH2 and β-thymosin (I, Fig. 2). We postulated that these motifs would have similar folds and bind actin through a conserved mechanism despite the relatively low sequence similarity (I). Later, this hypothesis was confirmed by solving the atomic structures of both classical WH2 domain and β-thymosin in complex with monomeric actin (Figure 2) (Hertzog et al., 2004; Irobi et al., 2004). These domains are typically unstructured in solution, but upon interaction with actin adopt a very similar, mostly α-helical structure that extends from the barbed end to the side of the monomer, where the main interaction site resides (Dominguez, 2007).

In our database searches, we found several new WH2 domain containing putative actin binding proteins. We chose one of these proteins, which had a well-conserved WH2 domain in its very C-terminus, for further characterization. This protein appears to exist only in vertebrates, although proteins with limited sequence similarity to the N-terminal region of this protein are encoded in the *Drosophila melanogaster* and *Caenorhabditis elegans* genomes. During our analyses, a partial mRNA sequence corresponding to this protein was found in a screen for new factors associated with metastatic behavior of bladder cancer cells. This mRNA was down-regulated in several highly metastatic bladder cancer cell lines and tumor samples as compared to less aggressive counterparts and, accordingly, it was named missing-in-metastasis (MIM) (Lee et al., 2002).

7.2 MIM binds ATP-G-actin via its WH2 domain (II)

In order to study the possible actin binding activities of MIM, the C-terminal half of MIM containing the WH2 domain, MIM-CT (amino acids 400-759), was expressed and purified for biochemical analyses. Actin binding was analyzed with a fluorometric assay using NBD-labeled ATP or ADP-actin monomers that change their intrinsic fluorescence upon interactions with many proteins. Indeed, MIM-CT bound to NBD-actin and displayed five times stronger interaction with ATP-actin monomers (K_D = 60 nM) as compared to ADP-actin monomers (K_D = 300 nM). Importantly, when we deleted the WH2 domain from MIM-CT no binding was observed, demonstrating that MIM interacts with actin via its WH2 domain (II, Fig. 4).

Next, we studied how MIM affects dynamics of actin polymerization. To this end, we performed a series of pyrene actin polymerization assays to follow

actin polymerization kinetics. These experiments showed that MIM inhibits *de novo* nucleation of actin filaments but allows bound monomers to incorporate into barbed ends of filaments, although the kinetics of the polymerization reaction was reduced by MIM-CT. Again, the mutant protein with deleted WH2 domain did not affect actin polymerization (II, Fig. 6).

Our findings on MIM WH2 domain are in line with the literature of other WH2 domains. All WH2 domains analyzed to date prefer to interact with ATP-actin monomers (Carlier et al., 1993; Hertzog et al., 2002) or have been shown to function in promoting actin filament polymerization, which utilizes ATP-G-actin (Dominguez, 2007). Recent structural analyses have provided an explanation for why some βT/WH2 domains promote actin filament assembly, while others inhibit polymerization. These properties seem to be mainly controlled by the length of the amino acid sequence following the main actin interaction site, LKKT-like sequence (Figure 2). Recently, the structure of WH2 domain of MIM in complex with actin was solved. A shorter stretch of amino acids following the main actin-binding site as compared to β-thymosins, but longer than in the prototypical WH2 domains (from WASP and Spire), was found. Interestingly, additional contacts with actin after the main interaction site were also revealed (Figure 2C) (Lee et al., 2007).

In addition to the somewhat exceptional structure, the WH2 domains in MIM and in the related protein, IRSp53, are found in isolation in the C-termini of these proteins. In contrast to many other WH2 domain proteins, MIM and IRSp53 do not possess polymerization activating domains next to the WH2 domain. Thus, in MIM and IRSp53 WH2 domains were suggested to have a scaffolding function rather than actin polymerization activation function (Lee et al., 2007). These conclusions are compatible with our findings that MIM WH2 domain does not sequester actin monomers or nucleate formation of actin filaments. The affinity of MIM to ATP-G-actin is, however, relatively high, suggesting that the majority of MIM in cells is in complex with actin monomers. Thus, MIM clearly has the potential to control the elongation of actin filaments and undesired nucleation (II).

Our studies revealed that MIM interacts with actin and controls its dynamics *in vitro*. To study if MIM regulates the actin cytoskeleton *in vivo*, we over-expressed full-length MIM fused to green fluorescent protein (GFP) in cultured animal cells. Indeed, over-expression of MIM in NIH 3T3 fibroblasts resulted in the loss of certain F-actin structures, especially stress fibers, and simultaneous appearance of abnormal F-actin structures, such as microspikes (II, Fig. 8). Similar results were concurrently obtained by others (Woodings et al., 2003). Together, our biochemical characterization of MIM-CT and cell biological analyses demonstrated that MIM is a new regulator of actin cytoskeleton that utilizes its C-terminal WH2 domain to interact with polymerization competent actin monomers and control their assembly into filaments.

8. IM-domains induce negative membrane curvature

Interestingly, a new conserved protein domain was identified from the N-terminal region of MIM, IRSp53, and three uncharacterized proteins (Yamagishi et al., 2004). This domain, named IRSp53/MIM homology (IM) domain (IMD), was shown to bundle actin filaments *in vitro* and induce drastic formation of filopodia when expressed in cells. Surprisingly, the

crystal structure of this domain showed a clear homology to the BAR domains, which are known to promote plasma membrane curvature during endocytosis (Lee et al., 2007; Millard et al., 2005).

8.1 IM-domains do not bundle F-actin at physiological conditions (III)

While IM-domains were shown to function as potent inducers of filopodia in cells, our goal was to understand the molecular mechanism leading to this phenotype. Surprisingly, our assays revealed that the F-actin bundling activity of IM-domains, which was proposed to underlie the filopodia formation, was hardly detectable at physiological ionic conditions. Importantly, actin cross-linking assays carried out at sub-physiological ionic conditions and dynamic light scattering analysis revealed that MIM IMD forms aggregates in low-salt conditions, which leads to substantial actin bundling activity (III, Fig. 5). Furthermore, detailed confocal microscopy analysis of IMD-induced filopodia showed that MIM IMD did not localize in the F-actin bundle of filopodia, where actin cross-linking proteins are expected to reside. Instead, MIM IMD localized to the plasma membrane surrounding the actin bundle (III, Fig. 4). These results disputed the biological role of the previously reported F-actin bundling by IM-domains (Yamagishi et al., 2004).

Another activity demonstrated in the literature for IM-domains is interaction with the small GTPase Rac1 (Bompard et al., 2005; Miki et al., 2000). Next, we examined whether Rac1 would influence the filopodia formation by IM-domains. Interestingly, our studies characterized two splice variants for MIM IMD, which remarkably differed in their Rac1 binding capability. While the shorter splice variant clearly bound to Rac1, no binding was detected with the longer splice variant. Quantification of the number of the filopodia formed by these splice variants when expressed in cells revealed no differences, indicating that IM-domains can induce filopodia without interaction with Rac1 (III, Fig. 6). Together, our results strongly suggest that another mechanism, distinct from F-actin cross-linking and Rac1 binding activities, must promote filopodia formation by IM-domains.

8.2 IM-domains bind and tubulate PI(4,5)P_2-rich membranes (III)

As mentioned above, the atomic structure of IM-domains resembles BAR domains, which possess a well-characterized membrane binding and deforming activity (Lee et al., 2007; Millard et al., 2005). To test the functional relatedness between these two domains, we analyzed the lipid binding properties of MIM IMD. A native PAGE electrophoresis assay revealed an interaction between MIM IMD and PI(4,5)P_2. Also co-sedimentation analysis demonstrated that IM-domains from both MIM and IRSp53 bind PI(4,5)P_2-rich synthetic lipid vesicles. Significantly weaker affinity to PI(3,4,5)P_3-rich vesicles, which are more negatively charged, provided evidence for the specificity of the interaction between IMD and PI(4,5)P_2. Importantly, these experiments suggested that the IM-domain has evolved from a BAR domain-like ancestor and retained its lipid binding capability (III, Fig. 1).

To map the PI(4,5)P_2 binding site on MIM IMD, we performed an alanine-scanning mutagenesis. This revealed that a relatively large positively charged region at each end of the dimeric IMD is important for lipid binding. Moreover, the same region was also found to be important

for F-actin binding, demonstrating that these binding sites overlap on the surface of MIM. However, in this mutagenesis we also identified hydrophobic residues that contributed exclusively to $PI(4,5)P_2$ binding (III, Fig. 2).

In order to study the biological importance of the lipid-binding by IM-domains, we analyzed the filopodia formation activity of the MIM IMD mutant (L145,147,170A), which show moderate defects in $PI(4,5)P_2$ binding but normal F-actin binding activity *in vitro*. Importantly, a significant decrease in filopodia induction by this mutant suggests that interaction with membranes, and not F-actin bundling activity, is critical for filopodia formation by IM-domains (III, Fig. 3).

We then extended our analysis to possible activities that IM-domains could display in the context of lipid membranes. With transmission electron microscopy (TEM), we discovered that MIM IMD induced dramatic deformation of $PI(4,5)P_2$-rich lipid vesicles that in semi-thick sections (120 nm) showed distinguished tubular characteristics. By performing electron tomography analysis we found that these tubules invaginated towards the interior of lipid vesicles (III, Fig. 1). This is the opposite direction as compared to the BAR domains that induce the formation of long and separate membrane tubules outwards from the vesicle. It is important to note that this is the first example of induction of membrane deformation to this direction, also known as negative curvature.

To explain the molecular mechanism of the observed membrane tubulation, we analyzed the structure of the lipid binding interface of MIM IMD in more detail. We realized that although the $PI(4,5)P_2$ binding surface is conserved with membrane binding surface of BAR domains, it is convex as compared to the concave shape of BAR domains (Peter et al., 2004). Furthermore, extrapolation of the curvature of the lipid-binding site of MIM IMD results in a circle with diameter of 95 nm (III, Fig. 7). This is in good agreement with the diameter of membrane tubules induced by MIM IMD *in vitro* (78 nm, SD = 7 nm).

Together, these data propose that the previously described F-actin bundling activity of IM-domains is an *in vitro* artifact resulting from aggregation of the domain. Although the dimeric IM-domains bind F-actin, they display only relatively low affinity to actin (Yamagishi et al., 2004). It is important to note that actin filaments contain prominent negative charge on their surface, which has been suggested to lead to unspecific binding and bundling by proteins rich in positive charge (Tang and Janmey, 1996). The significance of the hydrophobic residues for the $PI(4,5)P_2$-interaction, specificity towards $PI(4,5)P_2$ over $PI(3,4,5)P_3$, and remarkable membrane tubulation activity strongly suggest that the main physiological ligand for IM-domains is not actin filaments, but cellular membranes enriched with $PI(4,5)P_2$.

8.3 MIM regulates cell morphology via IMD-mediated membrane deformation (IV)

To understand the biological significance of IMD-dependent membrane binding and G-actin binding mediated by the WH2 domain, we performed a cell biological analysis of full-length MIM. We used COS-7 cells, which have endogenous MIM (unpublished data), and should thus contain the physiological binding partners of MIM enabling correct regulation of the protein. COS-7 cells also display clear phenotype after MIM

over-expression, namely predominant formation of filopodia in majority of the cells and formation of membrane ruffles in minority of the cells. Similar phenotypes have also been reported by others, with slight variations between experimental set-ups (Bompard et al., 2005; Lin et al., 2005b; Wang et al., 2007b; Woodings et al., 2003). Localization of MIM was examined in COS-7 cells that expressed minimal detectable amounts of MIM-GFP. MIM was seen to strongly concentrate at the plasma membrane in the cell periphery and in actin-rich ruffles (IV, Fig. 3).

Interestingly, deletion of WH2 domain had no detectable effect on the sub-cellular localization or over-expression phenotype of MIM. This suggested that interaction with actin monomers does not play a major role in protein localization or regulation of cell morphology by MIM, but more likely serves a fine-tuning role under more challenging environments. However, we observed that MIM with inactivated IMD did not induce any protrusions when expressed in COS-7 cells. Also, we observed no filopodia upon co-expression of MIM with pleckstrin homology (PH) domain of PLC-δ_1, which is known to bind PI(4,5)P_2 with high specificity and affinity, and consequently sequester PI(4,5)P_2 at the plasma membrane (Lemmon and Ferguson, 2000; Stauffer et al., 1998; Varnai and Balla, 1998). Furthermore, the localization of MIM to the plasma membrane was lost upon IMD-inactivation and clearly reduced in cells co-expressing wild-type MIM and the PH domain. These results demonstrate that IMD-mediated interaction with PI(4,5)P_2 at the plasma membrane is essential for correct sub-cellular localization and regulation of cell morphology by MIM (IV, Fig. 3).

However, the drastic filopodia formation by IM-domains alone as compared to rather subtle and variable phenotypes of full-length MIM suggest that also other MIM domains than IMD regulate filopodia formation in cells. Additional interaction partners have been reported for MIM, such as cortactin and RPTPδ (Gonzalez-Quevedo et al., 2005; Lin et al., 2005b; Woodings et al., 2003). However, while the binding sites for these proteins are not currently known, it was not feasible to inactivate these interactions without a risk of destroying the conformation of MIM. Clearly, the robust activity of the IM-domain must be tightly regulated in cells and further studies are warranted to reveal these mechanisms.

9. MIM is dispensable for embryonic development and Shh-signaling

9.1 Cell type-specific expression of MIM (II)

Detectable MIM or IRSp53 homologues are not found for example in yeast, but all mammalian genomes appear to contain several proteins with sequence homology to these proteins. This indicates that different members of the IM-domain protein family may function in different tissues of multicellular organisms. Furthermore, the lack of clear MIM homologues in invertebrates suggests that MIM is not essential for all eukaryotic cells but may function in more complex and specialized cellular events of vertebrates. To analyze the tissue distribution of MIM, we performed Northern blot and *in situ* hybridization analyses on various mouse tissues. We found that during development MIM is highly expressed in muscles and post-mitotic neurons, and in adult mice the expression is prominent in kidney, liver, and Purkinje cells of the cerebellum (II, Fig. 2 and 3). Later, similar results were obtained by others, using RT-PCR (Loberg et al., 2005) and Western blotting (Wang et al., 2007a).

9.2 Generation of MIM knockout mice (IV)

In order to study the physiological role of MIM, we generated MIM knockout mice. We found that $MIM^{+/-}$ and $MIM^{-/-}$ mice were viable and normal in appearance, demonstrating that MIM is not playing a key role during embryonic development. Histological analysis of several tissues from young $MIM^{-/-}$ animals, including kidney, liver, brain, skin, and bladder did not reveal differences as compared to wild-type littermates (IV).

The IRSp53/MIM protein family consists of five members in mammals, expression patterns of which, in most cases, are not well known. A closest homologue of MIM, ABBA, has a distinct expression profile from MIM, being predominant, for example, in glial structures (Saarikangas et al., submitted manuscript). However, we ensured by *in situ* hybridizations that ectopic expression of ABBA does not rescue MIM-deficiency (IV). The possibility still remains that IRSp53-related proteins compensate for MIM-deficiency in mice, and future work characterizing these family members is required to reliably assess the possible key roles of IM-domain-containing proteins during development.

9.3 MIM does not regulate Shh-signaling (IV)

MIM has been suggested to activate the Sonic hedgehog (Shh)-signaling pathway via direct interaction with transcription factors Gli1 and 2 (Callahan et al., 2004). Shh is a potent morphogen that controls fly and vertebrate embryonic development, including tissue/organ patterning, and is often dysregulated in tumors. Our finding that MIM-deficient mice are viable with no developmental abnormalities is incompatible with the substantial role of MIM in this pathway during development (IV).

Shh elicits transcriptional responses via its receptor Patched (Ptc) that upon ligand binding relieves the repression of Smoothened, which allows the signal transduction leading to activation of Gli transcription factors (Varjosalo and Taipale, 2007). Consequently, in $Ptc^{-/-}$ mice Shh pathway is constitutively active, which leads to embryonic lethality at E9 due to the failure in neural tube closure (Goodrich et al., 1997). We crossed $MIM^{-/-}$ mice with a $Ptc^{+/-}$ strain, to study the phenotype of double knockout embryos. Deletion of a Shh pathway activator should improve the $Ptc^{-/-}$ phenotype by lowering the levels of transcription. However, $MIM^{-/-}Ptc^{-/-}$ E9.5 embryos displayed as severe and similar malformations than $Ptc^{-/-}$ embryos (IV, Fig. 2). These analyses demonstrate that MIM is not a Gli-activator during mouse embryonic development.

Next, we analyzed the transcriptional synergy between MIM and Gli1 and 2 in a luciferace reporter gene assay (Taipale et al., 2000). We did not detect any effect of MIM on Gli-mediated transcription (IV, Fig. 2). Furthermore, we could not detect Gli1 or 2 in complex with MIM-GFP in co-immuno precipitation experiments (IV).

Our analyses show that, in contrast to the earlier report (Callahan et al., 2004), MIM plays no role in Shh-induced transcription. It was also suggested that MIM is a Shh responsive gene (Callahan et al., 2004; Gonzalez-Quevedo et al., 2005). Our studies do not exclude this possibility, and interestingly, we found that less $Ptc^{+/-}MIM^{-/-}$ mice were born than expected by Mendelian distribution. This suggests additional synergic function of these proteins during development (IV).

Shh is capable of directing cellular behavior, such as cell migration and

differentiation, but how these signals are transmitted to the actin cytoskeleton, the key player in these processes, is poorly understood. MIM may be one of the proteins transmitting the signal eventually to the changes in the cytoskeleton and plasma membrane, but MIM clearly is not a critical factor during the embryonic development.

10. MIM-deficiency leads to renal failure and increased susceptibility to tumors (IV)

Because MIM knockout mice developed into adulthood without apparent abnormalities, we next extended our analysis to aged animals in order to reveal possible progressive phenotypes. Interestingly, the histological examination of 11-15 months old mice revealed drastic morphological changes in the kidneys of $MIM^{-/-}$ mice. More detailed examination showed degeneration of kidney structures, particularly renal fibrosis, dilated tubules, and collapsed glomeruli with glomerulosclerosis (IV, Fig. 4). The first signs of this progressive nephrotic disease ultimately leading to the end stage renal failure were seen at age of 6-7 months.

Podocytes are specialized glomerular cells responsible for the primary urine filtration in kidney glomeruli. Key structure in the regulation of urine filtration are actin-based podocyte protrusions, called foot processes, interposed with unique structures called slit diaphragms, which establish the barrier to urinary protein loss (Somlo and Mundel, 2000). Electron microscopic analysis of 8 months old $MIM^{-/-}$ mice revealed focal abnormalities in the glomerular basement membrane (unpublished data), fusions of podocyte foot processes and thus loss of slit diaphragms (IV, Fig. 4).

Interestingly, a recent study described a role for IRSp53 in kidney podocytes, where it was shown to interact with synaptopodin. Synaptopodin is an actin-associated protein essential for podocyte morphology. Interaction of synaptopodin with IRSp53 negatively regulates the formation of cell protrusions by disrupting the Cdc42:IRSp53:Mena complex (Yanagida-Asanuma et al., 2007). The emerging view is that the dynamic actin cytoskeleton of podocyte foot processes is of critical importance to the maintenance of glomerular filtration and, for example, mutations in α-actinin-4 have been linked to glomerulosclerosis. Also, fusion and effacement of podocyte foot processes are among the first structural changes associated with glomerular dysfunction that, if not reversed, lead to the development of glomerulosclerosis and end stage renal failure (reviewed in Somlo and Mundel, 2004).

In addition to the development of renal disease, we observed that $MIM^{-/-}$ and $MIM^{+/-}$ mice have increased susceptibility to neoplasia, especially in liver, where strong MIM expression is also found (II, Fig. 2; IV). Approximately 12 % of both $MIM^{-/-}$ and $MIM^{+/-}$ mice of age of 12-20 months developed liver tumors, whereas no tumors were found from wild-type littermates in this analysis. Our results are in good agreement with the findings that MIM expression levels are often decreased in human hepatocellular carcinomas (Ma et al., 2007). In addition, down-regulation of MIM has been reported in bladder carcinomas and in metastasive bladder cancer cell lines (Lee et al., 2002; Wang et al., 2007a). In the future, more comprehensive analysis with larger quantities of animals is needed to fully evaluate the role of MIM and related proteins in carcinogenesis and metastasis.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Multiple cellular events, such as cell migration and remodeling of the cell shape, depend on the dynamic changes of the actin cytoskeleton and concomitant transformations at the plasma membrane. In this study, we examined a protein called missing-in-metastasis (MIM), which was initially identified as a tumor suppressor of bladder carcinoma (Lee et al., 2002). However, the biochemical mechanisms by which MIM functions in cells and the physiological role of this protein in animals were unknown.

We found that MIM is a cell type-specific protein that binds ATP-actin monomers with high affinity. This interaction is mediated via its C-terminal conserved WH2 domain, which thus enables MIM to regulate actin polymerization. In addition to the actin monomer binding WH2 domain, MIM contains an N-terminal IM-domain, which is shared by five members of the so-called MIM/IRSp53 protein family (Yamagishi et al. 2004). Our analyses revealed that IM-domains bind the plasma membrane through specific interactions with $PI(4,5)P_2$ and that this interaction is critical for the formation of filopodia by IM-domains in cells. Furthermore, we found that IM-domains possess a unique BAR domain-like, but inverted membrane tubulation activity that we propose to facilitate the formation of plasma membrane protrusions in cells (Figure 6). Importantly, our analyses also demonstrate that the interaction with $PI(4,5)P_2$ at the plasma membrane is essential for the correct sub-cellular localization of full-length MIM and for MIM-induced remodeling of cell morphology.

Further work will be necessary to reveal in detail the mechanisms how IM-domains induce membrane curvature. A fundamental question is also whether IM-domains function as membrane curvature sensing, stabilizing, or inducing modules in cells. This is particularly important, because the general view, although never challenged in cells, has been that the formation of the outward curvature at the plasma membrane is a passive event promoted by the elongation of underlying actin filaments. Additional membrane for the extension is considered to both diffuse from the surroundings and be transported in the form of secretory vesicles to the site of protrusion.

Moreover, it is possible that MIM may connect the elongating actin filament barbed ends to the plasma membrane (Figure 6). Such activity was lately demonstrated for the WASP family proteins (Co et al., 2007). Further studies are also required to analyze whether IM-domain proteins function in the context of other cellular membranes, such as multivesicular bodies or viral spherules, which harbor topologically identical membrane curvature as compared to plasma membrane protrusions (Williams and Urbe, 2007). Indeed, our preliminary light microscopy studies have visualized MIM-GFP on moving vesicular structures (unpublished data).

In addition to biochemical characterization of MIM, we generated and analyzed MIM knockout mice to understand the biological role of this vertebrate-specific protein. MIM is the first member of MIM/IRSp53 protein family, from which knockout animals are described. We found that MIM is dispensable for embryonic development, but required for the maintenance of proper kidney architecture and function.

MIM$^{-/-}$ mice develop progressive nephrotic disease ultimately leading to renal failure. In addition, MIM-deficient mice show substantially increased susceptibility to liver tumors. Our ongoing studies further investigate MIM null mice for other possible phenotypes, such as behavioral and metabolic disorders.

ABBA, which we also identified in our original database searches for WH2 domain containing proteins, is a close homologue of MIM that is strongly expressed in radial glia. Depletion of ABBA from a glial cell line, C6R, results in the impairment of lamellipodial persistency and cell process outgrowth (Saarikangas et al., submitted manuscript). Furthermore, it has been shown that IRSp53 knockdown neurons are defective in dendritic spine formation (Choi et al., 2005; Hori et al., 2005). These studies demonstrate a role for MIM/IRSp53 proteins in the formation of cellular protrusions. Correspondingly, the renal failure caused by MIM-deficiency may derive from defects in glomerular podocyte foot processes. Also, impaired plasma membrane dynamics may result in defects in other highly polar kidney epithelial cells and explain alterations observed in hepatocytes.

Deletion of actin binding proteins from mice have often resulted either in severe embryonic lethality at early stages of development, as for profilin 1 deletion (Witke et al., 2001), or in relatively mild phenotypes in specific cellular systems, as

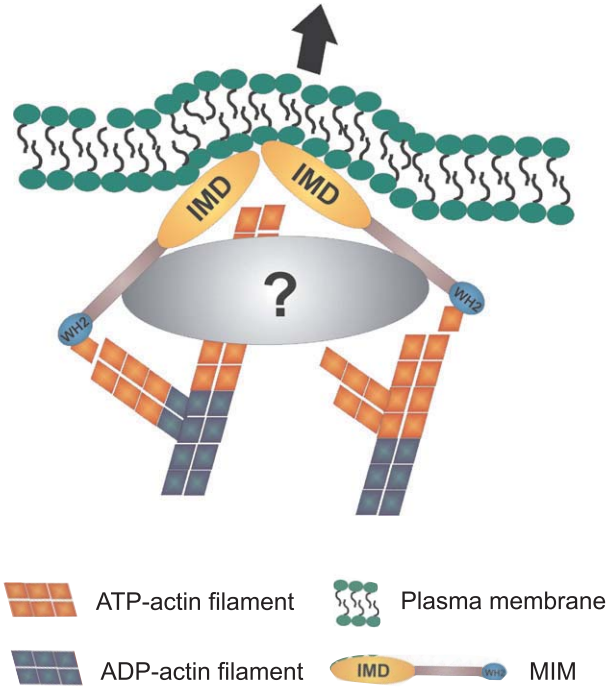

Figure 6. Schematic model for functions of MIM in formation of cell protrusions. IM-domain of MIM dimerizes and binds to the plasma membrane, where it senses and/or creates curvature. The C-terminal WH2 domain of MIM binds ATP-actin monomers and regulates their assembly to barbed ends of actin filaments. Currently uncharacterized interacting proteins activate MIM. MIM facilitates the formation of cell protrusion by creating membrane curvature and/or by recruiting other proteins, such as cortactin, involved in actin polymerization machinery to correct location by sensing the negative (outwards) curvature of plasma membrane.

demonstrated by defects in T cell function in formin mDia1 (Eisenmann et al., 2007) or WASP null mice (Snapper et al., 1998) and subtle behavioral changes in profilin 2 null mice (Pilo Boyl et al., 2007). The subtle phenotypes are often a result from high redundancy of multiple isoforms and related proteins in mammals, which ensures the adjustability and functionality of vital cellular processes. In the future, it will be important to generate and analyze mice deficient in multiple MIM/IRSp53 family proteins in order to reliably asses the biological importance of the unique membrane deformation activity of IM-domains. On the other hand, lower model organisms often contain much less redundancy. Our database searches have revealed only single putative IM-domain protein, a distant homologue of IRSp53, in *Dictyostelium* and *C. elegans*, and future studies investigating the roles of these proteins are also warranted to provide us with better understanding on cellular events requiring MIM/IRSp53 proteins.

Our data suggest a model, where MIM interacts with curved PI(4,5)P_2-rich regions at the plasma membrane, typically at the cell periphery. The curvature of the membrane may be one of the localization cues for MIM. Upon formation of cell protrusions, the underlying actin cytoskeleton is activated and membrane deformation is assisted by IM-domain. MIM and IRSp53 have been found to interact with various proteins, such as cortactin and WAVE proteins that are regulators of actin nucleation by Arp2/3 complex (Krugmann et al., 2001; Miki and Takenawa, 2002). Although the roles and mechanisms of these interactions are poorly established, it is likely that they have important contributions to the activity of MIM/IRSp53 proteins and also, as evidence has emerged, vice versa. Thus, MIM/IRSp53 proteins probably function as parts of larger protein complexes that upon a triggering signal recruit the machinery necessary for directed actin polymerization and induction of membrane protrusions (Figure 6).

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