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Staying in Shape with Formins

Review

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Formins constitute a diverse protein family present in all eukaryotes examined. They are defined by the presence of a formin homology 2 (FH2) domain, which possesses intrinsic and conserved functions regulating cytoskeletal dynamics. Over the past few years, formins have become recognized as potent nucleators of linear actin filaments that control a large variety of cellular and morphogenetic functions. Here, we review the molecular principles of formin-induced cytoskeletal rearrangements and their consequences for a growing number of biological processes.

Introduction

Eukaryotic cells require de novo nucleation of actin filaments from a large pool of monomeric actin bound to profilin in order to elicit temporal and spatial remodeling of the actin cytoskeleton, which underlies complex cellular functions such as the establishment of cell shape, cytokinesis, or cell motility. The rate-limiting step for actin filament polymerization is nucleation, and, so far, three major classes of actin nucleators are known to nucleate actin filaments in vivo: the Arp2/3 complex, Spire, and the formin-homology proteins (Mullins et al., 1997; Pruyne et al., 2002; Quinlan et al., 2005; Sagot et al., 2002b; Welch et al., 1997). However, despite their function as potent nucleators of actin polymerization, Arp2/3, Spire, and formins employ different mechanisms to accomplish their tasks. Arp2/3 binds to the sides of preexisting actin filaments and nucleates branched actin networks in the lamellipodia of motile cells. Spire nucleates the assembly of unbranched actin filaments and, like the Arp2/3 complex, remains bound to the pointed ends of the nucleated filaments (Quinlan et al., 2005). Pointed-end nucleators, such as Arp2/3 and Spire, will experience only limited growth before being capped at their barbed ends by capping proteins (Cooper and Pollard, 1985). Formins also nucleate linear filaments, but, unlike Arp2/3 and Spire, they do so from the barbed end. In fact, because formins remain associated with barbed ends during filament elongation, and because they antagonize capping proteins, they are ideal for efficient generation of long actin filaments in the presence of capping proteins (Harris and Higgs, 2004; Kovar, 2006; Moseley et al., 2004; Zigmond et al., 2003).

The term "formin" originates from a transgene insertion in the limb deformity locus that resulted in limb formation defects in mice (Mass et al., 1990; Woychik et al., 1990). However, targeted deletion of the Formin-1 gene did not reproduce the limb deformity phenotype (Chao et al., 1998; Wynshaw-Boris et al., 1997), and, in fact, a recent study demonstrated that, instead, the adjacent *Gremlin* gene is required for limb bud patterning (Zuniga et al., 2004). More recently, formins have emerged as a diverse family of ubiquitous, highly conserved multidomain proteins involved in a growing range of actin-based processes including the formation of actin cables in yeast and stress fibers in mammalian cells, the assembly of contractile rings during cytokinesis, and the formation of filopodia (Table 1). Here, we summarize the molecular properties of formins, describe new developments in the field, and discuss the cellular and organismal functions of these multifaceted cytoskeletal regulators.

Biochemical and Structural Properties of Formins

Formins are defined by a unique and highly conserved C-terminal formin homology 2 (FH2) domain of about 400 amino acid residues that is preceded by an N-terminally proline-rich FH1 domain (Figure 1) (for previous reviews, see Evangelista et al., 2003; Higgs, 2005; Kovar, 2006; Wallar and Alberts, 2003; Watanabe and Higashida, 2004; Zigmond, 2004). The FH2 core domain and the intervening linker region between the FH1 and FH2 domains are necessary and sufficient to nucleate actin polymerization from G-actin in vitro (Kovar et al., 2003; Pruyne et al., 2002; Sagot et al., 2002b). How formins modulate actin assembly at the molecular level is still not entirely understood, but, in most cases, their properties are changed considerably by the small G-actin binding protein profilin. The FH1 domain, composed of stretches of poly-proline residues, binds with low micromolar affinity to profilin (Evangelista et al., 1997; Watanabe et al., 1997) and is able to recruit and deliver new ATP-G-actin subunits from profilin-actin complexes to the FH2 domain for incorporation into growing filaments at their barbed ends (Chang et al., 1997; Sagot et al., 2002b). Binding of profilin to isolated FH1-FH2 fragments increases the elongation rates of formin bound filaments, but the effect that profilin has on formin-mediated actin polymerization differs greatly between various formin isoforms (Kovar et al., 2006; Kovar and Pollard, 2004; Romero et al., 2004). The conserved FH2 domain nucleates new actin filaments, most likely through stabilizing an actin dimer (Pring et al., 2003), and remains tightly bound with low nanomolar affinity to the barbed ends of the filaments (Moseley et al., 2004; Pruyne et al., 2002). In addition, the FH2 domains of the formins Bni1p, mDia1, dDia2, and FLR (the product of the formin-related gene in leukocytes) efficiently block the inhibitory activities of capping protein and gelsolin, which also interact with low nanomolar affinities with actin filament barbed ends (Harris and Higgs, 2004; Schirenbeck et al., 2005; Zigmond et al., 2003).

In contrast to capping proteins, which increase the critical concentration of actin by blocking the barbed ends, most formins, except fission yeast Cdc12

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Table 1. Locations and Proposed Functions of Selected Formins

Organism (Genes) Gene Products	Rho GTPase	Subcellular Location	Proposed Functions	References
A. thaliana (20)				
AtFH5	—	cell plate in endosperm	cytokinesis	(Ingouff et al., 2005)
S. cerevisiae (2)				
Bni1p[a]	Rho3p, Rho1p	bud tip and neck	cytokinesis, cell polarity, actin cables	(Imamura et al., 1997; Sagot et al., 2002a)
Bnr1p[a]	Rho3p, Rho4p	bud neck	cytokinesis, cell polarity, actin cables	(Evangelista et al., 2002; Imamura et al., 1997)
S. pombe (3)				
For3p[a]	Rho3p, Ccd42p	cell tip, polarisome	cell polarity, actin cables	(Feierbach and Chang, 2001)
Fus1p	—		cell polarity, mating	(Peterson et al., 1998)
Cdc12p	—	cleavage furrow	cytokinesis	(Chang et al., 1997; Pelham and Chang, 2002)
C. albicans (2)				
Bni1[a]	Cdc42	hyphal tip	cell polarity	(Li et al., 2005; Martin et al., 2005)
Bnr1[a]	n.d.		cytokinesis	(Martin et al., 2005)
A. nidulans (1)				
SepA[a]	—	hyphal tip, septation site	cytokinesis, cell polarity	(Harris et al., 1997; Sharpless and Harris, 2002)
D. discoideum (10)				
ForC	—	macropinosomes	endocytosis?	(Kitayama and Uyeda, 2003)
dDia2[a]	Rac1	filopodial tips	filopodium formation, cell adhesion	(Schirenbeck et al., 2005)
D. melanogaster (6)				
Diaphanous[a]	Rho1	furrow canal	cellularization;	(Grosshans et al., 2005)
			cytokinesis	(Castrillon and Wasserman, 1994)
			SRF, cell motility, and invasion	(Somogyi and Rorth, 2004)
Cappuccino	Rho1	oocyte cortex	cytoplasmic streaming	(Rosales-Nieves et al., 2006)
			cell polarity	(Emmons et al., 1995; Manseau and Schupbach, 1989)
DAAM	RhoA		tracheal development	(Matusek et al., 2006)
Formin-3	—		tracheal development	(Tanaka et al., 2004)
C. elegans (7)				
Cyk-1	—	cleavage furrow	cortical microfilaments, cytokinesis	(Severson et al., 2002; Swan et al., 1998)
X. laevis (14)				
DAAM1	RhoA		planar cell polarity	(Habas et al., 2001)
M. musculus (15)				
mDia1[a]	RhoA-C	membrane ruffles	stress fiber formation	(Watanabe et al., 1997, 1999)
			axon elongation	(Arakawa et al., 2003)
		stable microtubules	microtubule stabilization	(Palazzo et al., 2001)
			SRF	(Copeland and Treisman, 2002)
			adherens junction stability	(Sahai and Marshall, 2002)
		cell front	cell motility	
				(Goulimari et al., 2005; Vicente-Manzanares et al., 2003)
			cell cycle progression	(Mammoto et al., 2004)
			phagocytosis	(Colucci-Guyon et al., 2005)
			mitochondria distribution	(Minin et al., 2006)
mDia2[a]	Rif, Cdc42, and RhoA	filopodial tips	filopodium formation	(Pellegrin and Mellor, 2005; Peng et al., 2003)
			SRF	(Tominaga et al., 2000)
mDia3[a]	Cdc42, RhoA, and Rac1	cleavage furrow, metaphase, microtubules	microtubule attachment to kinetochores	(Yasuda et al., 2004)
Formin-1	—	cell-cell contacts	adherens junction formation	(Kobielak et al., 2004)
Formin-2	—		cytokinesis during oogenesis	(Leader et al., 2002)
FRL	Rac1		cell motility	(Yayoshi-Yamamoto et al., 2000)
Delphilin	—	Purkinje cell synapse	neurotransmission?	(Miyagi et al., 2002)
H. sapiens (14)				
hDia1[a]	n.d.		autosomal dominant deafness DFNA1, cochlear hair cell motility?	(Lynch et al., 1997)
hDia2[a]	n.d.		premature ovarian failure (cytokinesis)	(Bione et al., 1998)
hDia2C[a]	RhoD		endosome motility, endocytosis	(Gasman et al., 2003)
FHOD1 (FHOS)[a]	Rac1	stress fibers, lamellipodia	SRF, stress fiber formation, cell motility	(Gasteier et al., 2003; Koka et al., 2003; Westendorf, 2001)

Table 1. Continued

Organism (Genes) Gene Products	Rho GTPase	Subcellular Location	Proposed Functions	References
FHOS2[a]	n.d.	nestin filaments	stress fiber formation	(Kanaya et al., 2005)
FRL	n.d.		cell survival? leukemogenesis?	(Favaro et al., 2003)

Abbreviation: n.d., not determined.
[a] Diaphanous-related formins.

(Kovar et al., 2003), display no such effects and are therefore referred to as "leaky cappers" (Zigmond et al., 2003). These findings, together with immunoelectron microscopy and real-time imaging of FH2 bound filament growth, reveal that formins remain stably associated with the filament as actin monomers are inserted between the FH2 domain and the barbed end (Higashida et al., 2004; Kovar et al., 2006; Kovar and Pollard, 2004; Pruyne et al., 2002; Romero et al., 2004). Formins act as processive motors (Kovar and Pollard, 2004; Romero et al., 2004) that generate piconewton forces during the insertional assembly of actin filament barbed ends (Kovar and Pollard, 2004). Romero and colleagues (2004) suggested that processive mDia1-mediated actin polymerization requires profilin and is accompanied by ATP hydrolysis. However, a recent report with a variety of formin isoforms, including mDia1, demonstrates that this is rather unlikely (Kovar et al., 2006). Additionally, the FH2 domains from mammalian FRL1 and mDia2 were reported to bundle filaments (Harris et al., 2006).

A number of recently solved crystal structures of discrete formin domains provide new insights into the molecular details of formin-mediated actin assembly. The three-dimensional structure of the Bni1p FH2 domain revealed a flexible, tethered dimer architecture, in which two elongated actin binding heads are connected at either side to form a doughnut-shaped circular structure (Xu et al., 2004). This finding led to the intriguing hypothesis that, due to this architecture, formins may processively "stair step" at the ends of elongating actin filaments. Such a mechanism would imply considerable rotational movement of the formin around the helical actin filament. However, single molecule imaging of substrate-attached or Arp2/3 complex-nucleated filaments did not show evidence of supercoiling or rotation of the actin filaments as they are elongated by a formin (Kovar and Pollard, 2004), suggesting that the FH2 dimer slips on the barbed end during filament elongation (Shemesh et al., 2005). The crystal structure of the FH2 domain from mouse mDia1 turned out to be almost identical to that of yeast Bni1p (Shimada et al., 2004), implying that the core molecular mechanism of formin-mediated actin assembly has been evolutionarily preserved. A structure of the Bni1p FH2 domain complexed with tetramethylrhodamine (TMR)-actin revealed that each of the structural units in the FH2 dimer bind two actin subunits in an orientation similar to that in actin filaments, indicating that the FH2 domain indeed functions as a filament nucleus (Otomo et al., 2005b). Emerging models of formin-mediated actin assembly suggest that the FH2 dimer may oscillate between an "open" and "closed" conformational state, allowing or preventing incorporation of new subunits into the filament (Otomo et al., 2005b; Vavylonis et al., 2006).

A conserved subfamily of formins known as Diaphanous-related formins (DRFs) vastly increases the signaling complexity of formin proteins due to their ability to act as effectors of Rho family GTPases (Wasserman, 1998; Watanabe et al., 1997). In these proteins, the FH1 and FH2 domains are flanked by an array of regulatory domains at the N terminus and by a single C-terminal Diaphanous-autoregulatory domain (DAD) (Alberts, 2001). Whereas the DAD is composed of only a small stretch of amino acid residues, the much larger N-terminal regulatory region encompasses a GTPase binding domain (GBD) followed by an adjacent Diaphanous-inhibitory domain (DID) as well as a dimerization domain (DD) (Li and Higgs, 2005; Rose et al., 2005). A structurally less-defined region following the GBD containing both the DID and DD, previously referred to as FH3, has been implicated in subcellular localization of mDia proteins (Kato et al., 2001; Kitayama and Uyeda, 2003; Peterson et al., 1998). In the basal state, DRFs exist as autoinhibited proteins via intramolecular interactions between DID and DAD (Figure 1). This autoinhibition is relieved after binding of an active Rho GTPase to the GBD domain (Alberts, 2001; Li and Higgs, 2003; Watanabe et al., 1999). As revealed by the mDia1 crystal structure, the DID/DD region forms a stable dimer and associates together with GBD into a joined, all-helical folding unit containing armadillo repeats (Rose et al., 2005). The crystal structures of RhoC or RhoA in complex with the regulatory N terminus of mDia1 containing the GBD/DID region shows that Rho uses its switch I and II regions for interaction with both the GBD and DID domains (Otomo et al., 2005a; Rose et al., 2005).

Comparison of the three-dimensional structures of the mDia1 N-terminal regulatory region in complex with Rho or DAD provides additional clues about the molecular details of Rho-mediated relief of autoinhibition (Lammers et al., 2005; Nezami et al., 2006). Although binding of Rho and DAD at the N-terminal fragment of mDia1 is mutually exclusive, the binding sites are only partially overlapping. Release of the autoinhibitory DAD interaction is mainly accomplished by Rho-induced restructuring of the adjacent GBD, which, in turn, interferes with binding of DAD to the neighboring DID domain (Nezami et al., 2006; Otomo et al., 2005a). Furthermore, the autoinhibition of DRFs via interactions of the N and C termini is supported by constitutively active DRF variants carrying either mutations in DAD or lacking the N-terminal regulatory region (Palazzo et al., 2001; Schönichen et al., 2006; Tominaga et al., 2000; Wallar et al., 2006; Watanabe et al., 1999).

Finally, it should be noted that addition of GTP bound RhoA does not fully activate mDia1 in vitro (Li and Higgs, 2005), suggesting that either the autoinhibited state may be formed between different mDia1 polypeptide chains,

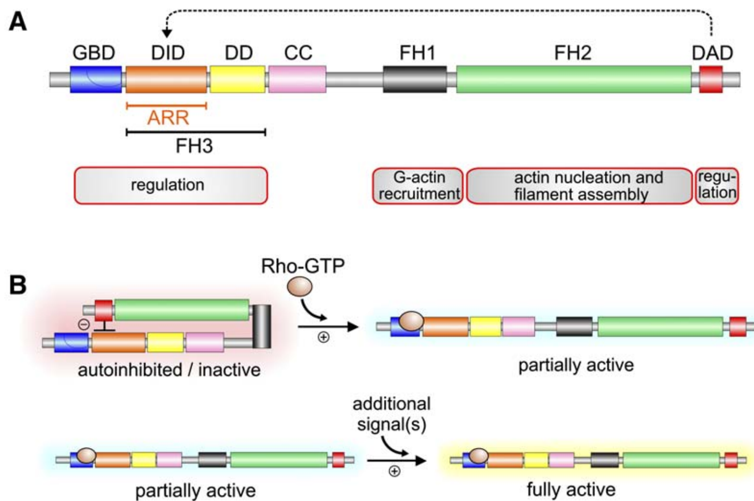

Figure 1. Domain Organization and Molecular Regulation of Diaphanous-Related Formins

(A) Schematic representation of the domain organization of a representative DRF such as mDia1. Abbreviations: GBD, GTPase binding domain; DID, Diaphanous-inhibitory domain; DD, dimerization domain; CC, coiled coil; FH1, formin homology 1 domain; FH2, formin homology 2 domain; FH3 formin homology 3 domain; ARR, armadillo-repeat region. The loosely defined FH3 region is based on sequence similarities to other DRFs and does not match true domain boundaries. Based on its structure, DID is also referred to as ARR.

(B) Autoinhibition of DRFs, caused by the interaction of DAD with DID, is partly relieved by association of an active, GTP bound Rho GTPase to GBD, allowing DID to adopt a structured conformation that, in turn, appears to induce release of DAD, leading to a partial activation of the DRF. An unknown additional signal(s) is required to fully activate the DRF.

or that additional signals discrete from activated Rho are required for full DRF activation. Either scenario provides opportunities for additional discoveries of the mechanisms for regulating formin activity. DRFs may be further regulated via their C-terminal DAD by yet unidentified factors. The regulation of formins lacking these regulatory domains is unknown and is likely to follow different mechanisms.

Cellular Functions and Organismal Roles of Formins

Formins Are Required for Cytokinesis

Cytokinesis is the final step of mitotic and meiotic cell division and results in the formation of two daughter cells. This highly regulated process is under the intricate control of Rho family GTPases acting through actin, nonmuscle myosin II, and a large number of accessory proteins (Glotzer, 2005; Hall, 1998). In most cell types, at the end of the cell cycle a contractile actomyosin ring is assembled at the site of cell separation and is believed to drive constriction of the plasma membrane by constriction of the actin filament ring under the influence of myosin II activities. Loss-of-function studies have shown that formins are involved in cytokinesis in a number of organisms including plants, budding and fission yeast, *Drosophila*, *C. elegans*, and vertebrates (Castrillon and Wasserman, 1994; Chang et al., 1997; Echard et al., 2004; Imamura et al., 1997; Ingouff et al., 2005; Peng et al., 2003; Swan et al., 1998; Tolliday et al., 2002; Tominaga et al., 2000). The use of actin-interfering drugs, such as cytochalasin D or latrunculin A, or the incorporation of Rhodamine-labeled G-actin in dividing cells, demonstrated that the contractile ring is a region of active actin filament barbed end assembly and rapid filament turnover (Noguchi and Mabuchi, 2001; Pelham and Chang, 2002; Tolliday et al., 2002; Wu et al., 2003). Ablation of Arp2/3 complex components in budding yeast, *Drosophila*, or *C. elegans* did not impair the formation of the contractile ring or prevent cytokinesis, suggesting that these specialized actin filaments are assembled by formins (Evangelista et al., 2002; Hudson and Cooley, 2002; Severson et al., 2002; Tolliday et al., 2002). Electron microscopy has shown that F-actin in the cleavage furrow is organized into bundles of predominantly linear filaments (Maupin and Pollard, 1986; Sanger and Sanger, 1980). Consistent with these findings, Rho1 and the formins Bni1p and Bnr1p, together with profilin, are required for assembly and maintenance of the actin ring in budding yeast (Tolliday et al., 2002).

In fission yeast, the function of the Arp2/3 complex in ring formation is still unclear. Like the cytokinesis formin Cdc12p, Arp2/3 is localized to the equatorial zone and is implicated for contractile ring formation. Thus, in fission yeast, both Cdc12p and Arp2/3 may contribute to ring assembly (Arai et al., 1998; Pelham and Chang, 2002), although it needs to be tested whether the latter is essentially required for cytokinesis. Recently, technical advances have enabled measurements of the global and local concentrations of cytoskeletal and signaling proteins as well as their order of appearance in the equatorial region during fission yeast cytokinesis (Wu et al., 2003; Wu and Pollard, 2005). These studies revealed that of the 600 total Cdc12p molecules in a single yeast cell about half concentrate in the contractile ring. Due to their similar affinities, capping proteins appear to compete with Cdc12p for actin filament barbed ends in vivo and in vitro (Kovar et al., 2005). Since capping proteins arrive at the cell division site after Cdc12p, they might gradually replace the formin from filament barbed ends, allowing subsequent filament disassembly during ring constriction (Kovar et al., 2005).

In addition to their function in assembly of the contractile ring and polarized endocytosis during cell division (Gachet and Hyams, 2005), formins may also function in mitotic spindle positioning. Kato and colleagues (2001) reported localization of mDia1 at the mitotic spindle, indicating an important role in cytokinesis. However, in mouse embryonic stem cells, disruption of the *DRF1* gene, which encodes mDia1, did not lead to defects in cytokinesis (Peng et al., 2003). This may be due to a compensatory increase in expression of mDia2, suggesting that mDia1 and mDia2 could exert overlapping functions during cytokinesis (Peng et al., 2003). More recently, Cdc42 and its downstream effector mDia3 were shown to regulate microtubule (MT) attachment

to kinetochores during mitosis (Yasuda et al., 2004). Although Cdc42-mDia3 signaling is not involved in the initial attachment of MTs to kinetochores, this signaling pathway appears to be critical in subsequent, stable, biorientated MT attachment for proper chromosome alignment and segregation.

Filopodium Formation

Filopodia are rod-like cell surface protrusions composed of bundles of parallel actin filaments that grow by actin monomer addition at their tips and shrink upon slower polymerization and continuous actin retrograde flow, followed by filament disassembly within the cell body (Mallavarapu and Mitchison, 1999; Small et al., 1978). These highly dynamic structures appear to be used by many cell types as sensory tools to explore environmental cues to guide cell migration or axon extension (Dent and Gertler, 2003; Wood and Martin, 2002). Filopodia have also been implicated in a wide range of other important cellular processes including cell-substrate adhesion and phagocytosis (Schirenbeck et al., 2005; Tuxworth et al., 2001), as well as the zippering and fusion of epithelial sheets during morphogenesis of *Drosophila* and *C. elegans* (Perez-Moreno et al., 2003; Wood and Martin, 2002). Furthermore, at the onset of an immune response, long filopodia-like structures participate in the formation of the immunological synapse between T cells and antigen-presenting cells (Hogg et al., 2003; Salazar-Fontana et al., 2003). Cocrosslinking of CD9 ligand and Fcγ receptors by monoclonal antibodies activates macrophages and leads to filopodium extension, indicating that these structures may also have important functions during infection and inflammation (Kaji et al., 2001). Despite their biological significance, our knowledge of the molecular mechanisms underlying filopodia formation is still incomplete (Faix and Rottner, 2006).

Since filopodia frequently emanate from lamellipodial structures, it has been proposed that lamellipodia serve as precursor structures that can develop into filopodia after receiving appropriate stimuli (Biyasheva et al., 2004; Small et al., 1999; Svitkina and Borisy, 1999). An attractive model termed "the convergent elongation model of filopodia formation" suggests that they arise from the dendritic network of Arp2/3-nucleated actin filaments by selective elongation, coalescence, and bundling by proteins of the filopodium tip complex (Svitkina et al., 2003). The latter contains Ena/VASP proteins, which were proposed to promote the growth of long filaments by inhibiting the capping process (Bear et al., 2002; Mejillano et al., 2004). However, other studies indicate that VASP neither interacts with filament barbed ends nor displays any detectable anticapping activity (Samarin et al., 2003; Schirenbeck et al., 2006). Instead, Schirenbeck and colleagues (2006) showed that the bundling activity of VASP is required for filopodium formation in *Dictyostelium*. Furthermore, very recent loss-of-function studies of Arp2/3 components or one of its activators (WAVE-complex) by RNA interference in mouse B16-F1 melanoma cells revealed that filopodia formation was not affected, despite the absence of lamellipodia. These results were substantiated by gene disruptions in *Dictyostelium* amoebae for the WAVE-complex components Nap1 and Scar, which also showed no effect on filopodium formation (Steffen et al., 2006). Together, these findings suggest that the Arp2/3 complex is not required for the assembly of filopodial actin filaments. Instead, recent evidence points toward a pivotal role of formins in the assembly of filopodial actin filaments. Ablation of mDia1 in murine embryonic stem cells resulted in higher expression of mDia2 and led to increased formation of lamellipodia, microspikes, and filopodia, resembling characteristic changes in the actin cytoskeleton after activation of Rho family GTPases such as Cdc42 (Peng et al., 2003). Microinjection of anti-mDia2 antibody or the expression of a dominant-negative mDia2 blocked Cdc42-induced actin reorganization, suggesting a potential role of mDia2 as an effector of Cdc42 (Peng et al., 2003). Although Cdc42 can efficiently induce filopodium formation (Hall, 1998), recently it was shown that it is not essential for this process (Czuchra et al., 2005). Notably, a second mDia2 pathway leading to the formation of filopodia has been established for the Rho GTPase Rif (Rho in filopodia), suggesting that mDia2-mediated filopodia can be induced by at least two distinct pathways (Pellegrin and Mellor, 2005). A crucial role for the DRF dDia2 for filopodium formation has been demonstrated in *Dictyostelium* cells by gene disruption and accumulation of GFP-tagged as well as of endogenous dDia2 at the distal tips of filopodial actin filaments (Schirenbeck et al., 2005). Together, these findings suggest that the basic principle and mechanism of formin-mediated filopodium formation are conserved in many eukaryotes (Figure 2).

Overexpression of EGFP-tagged mDia1 lacking its N-terminal regulatory region in *Xenopus* fibroblasts led to a massive formation of actin fibers and, most notably, to filopodia-like structures that were labeled at their tips with the EGFP fusion protein (Higashida et al., 2004). This mDia1 localization has yet to be confirmed with either GFP-tagged full-length constructs or by antibody labeling of endogenous protein. Hence, it cannot be formally ruled out that the N-terminally truncated mDia1 is mislocalized due to its high affinity for actin filament barbed ends. However, the potential involvement of additional formins in filopodium formation in mammalian cells is quite likely, since filopodium-forming NIH3T3 fibroblasts reportedly do not express mDia2 (Tominaga et al., 2000).

Cell Adhesion and Motility

Dynamic rearrangement of the actin cytoskeleton is essential for cellular shape as well as for regulation of cell-substrate and cell-cell adhesion. Mammalian formins have been shown to be critically involved in these processes. Derivatives of mDia1 that lack the N-terminal regulatory domain cause the formation of long, parallel stress fibers, which become disorganized upon ROCK inhibition, suggesting that the activities of these two downstream Rho effectors are balanced to control actin fibers of different thickness and density (Watanabe et al., 1999). In fibroblasts and other cell types, bundles of actin fibers extend toward the cell periphery into areas of focal adhesions, which represent plasma membrane anchorage points of cells attached to a solid surface substratum. Focal adhesion assembly requires functional RhoA (Ridley and Hall, 1992). Interestingly, the activity of the RhoA effector mDia1 appears to be sufficient for tension force-induced formation of dynamic adhesions, as cells in which RhoA is inhibited by the C3 exoenzyme still generate focal contacts when active mDia1 is

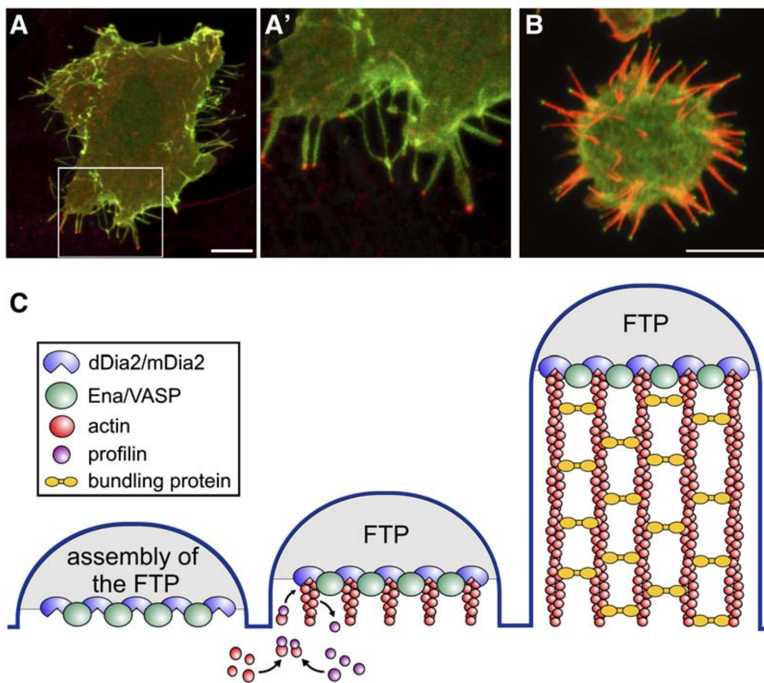

Figure 2. The Localization of Formins at Filopodial Tips Is Conserved in Organisms as Evolutionary Diverse as Mouse and *Dictyostelium*

(A) Localization of GFP-tagged full-length mDia2 (red) in an NIH 3T3 fibroblast expressing constitutively active Rif-QL mutant (green) illustrates the concentration of mDia2 at filopodial tips.

(A′) Higher magnification of the inset shown in (A).

(B) A *Dictyostelium* cell expressing GFP-tagged full-length dDia2 stained with anti-GFP antibodies to visualize dDia2 (green) and with rhodamine-conjugated phalloidin to visualize F-actin (red). The merged image shows prominent dDia2 accumulation at the distal tips of filopodial actin filaments. The three-dimensional reconstruction was computed from confocal sections. The scale bars represent 10 μm in (A) and 5 μm in (B). (A) and (A′) were reprinted from an article by Pellegrin and Mellor (2005), with permission from Elsevier.

(C) A possible mode of filopodium formation. During the initiation phase (left), a filopodium tip complex (FTP) containing formins such as dDia2/mDia2 and members of the Ena/VASP family is assembled at the plasma membrane. Formins may drive de novo actin nucleation as shown here or, alternatively, elongation of preexisting filaments. However, lamellipodial filaments are not essential for filopodium formation. Filaments elongated by dDia2/mDia2 are subsequently bundled and stabilized by VASP, as demonstrated recently in *Dictyostelium* (middle). Assuming that filopodial filaments are anchored in the cortical cytoskeleton, it is likely the coordination of both protein activities, nucleation, and rapid stabilization of the actin filaments by physical crosslinking that provides the appropriate cytoskeletal architecture and force required to push the membrane outward. Actin-bundling proteins such as fascin that are not restricted to the filopodial tip subsequently replace VASP and stabilize the elongating actin filaments along the entire shaft of the growing filopodium (right).

expressed (Riveline et al., 2001), indicating that focal adhesion formation depends on local actin polymerization by formins. However, zyxin-CFP-enriched focal adhesions are still formed in human osteosarcoma cells depleted of mDia1 (Hotulainen and Lappalainen, 2006). Related DRFs could be substituting for mDia1 function, but these studies may indicate that mDia1 is required for actin polymerization from preassembled dorsal focal adhesions. As yet, none of these studies have demonstrated the presence of a formin in focal adhesions.

In mammalian epithelia, cells are organized in a polar fashion by forming adherens junction complexes containing E-cadherins plus α- and β-catenins. Two recent studies have established a role for formins in the formation and maintenance of intercellular adhesions. Microinjection of active mDia1 promoted the appearance of E-cadherin and α-catenin clusters at cell-cell contacts and at the cell periphery (Sahai and Marshall, 2002); furthermore, by employing yeast two-hybrid screens, Formin-1 was identified as a direct binding partner for α-catenin, which recruits it to adherens junctions (Kobielak et al., 2004). A Formin-1 mutant lacking the actin-polymerizing FH2 domain but still capable of binding α-catenin displaced E-cadherin from intercellular junctions and perturbed cell-cell adhesion in keratinocytes (Kobielak et al., 2004). Thus, directed actin polymerization through formins is likely to play a fundamental role in dynamically reshaping the plasma membrane.

Actin-based cell motility requires de novo synthesis of actin filaments. Several previous studies have shown that specific DRFs are involved differently in cell motility processes, probably due to their distinct modes of GTPase binding and regulation. mDia1 is the most thoroughly studied formin with regard to cell motility and migration. An earlier study reporting the identification of mDia1 showed that it colocalizes with profilin and RhoA in membrane ruffles of highly motile fibrosarcoma cells (Watanabe et al., 1997). It could be demonstrated that mDia1 in fact colocalizes with the active form of RhoA at the front of migrating fibroblasts, further supporting a role for mDia1 function during directed cell locomotion (Goulimari et al., 2005). This activity of mDia1 might be tightly balanced, as cells microinjected with constitutively active forms of mDia1 failed to polarize the Golgi apparatus and were mostly left behind the wound edge in scratch-wound assays (Magdalena et al., 2003). However, these results may be difficult to interpret, as one cannot rule out that excessive stress fiber formation caused by transient expression of deregulated active mDia1 derivatives contributes to this observation (Copeland and Treisman, 2002; Watanabe et al., 1999). Other work with constitutively active forms of mDia1 demonstrated the formation of polarized, stable microtubules before the onset of migration in scratch-wounded fibroblasts (Gundersen et al., 2004; Palazzo et al., 2001; Wen et al., 2004), indicative of a critical role of mDia1 in cell motility. Moreover, RNA interference used to deplete mDia1 efficiently prevented fibroblast migration in both scratch-wound assays (Goulimari et al., 2005) and studies involving chemokine-induced axon elongation in cerebellar granule neurons (Arakawa et al., 2003), clearly demonstrating the requirement of mDia1 for directional cell motility. Nevertheless, which of the known mDia1 functions, such as actin

polymerization, microtubule stabilization, and/or MAL/SRF activation (see below), are responsible for the essential role of mDia1 in cell motility is not yet clear.

Additional DRFs implicated in cell migration, such as FRL, appear to be Rac regulated (Yayoshi-Yamamoto et al., 2000). Regardless of the upstream signal, DRFs have diverse roles in cell migration. While overexpression of FHOD1 (formin homology 2 domain containing protein) enhances cell movement (Koka et al., 2003), cell migration is impaired by overexpression of dDia2 in *Dictyostelium* amoebae (Schirenbeck et al., 2005). However, the latter is likely due to a greatly increased cell-substrate adhesion caused by the expression of this formin. The nature of these differences as well as the roles of other formins in cell motility events constitute an emerging field of study and are far from being fully understood.

Endocytosis

Endocytosis involves delivery of internalized molecules into early endosomes for recycling or compartmental transport. Endosomal motility is controlled by the small GTPase RhoD and requires dynamic interaction of endosomal organelles with the actin cytoskeleton (Murphy et al., 1996; Taunton, 2001). Recently, formins have been implicated in regulation of endocytosis, probably via their actin-polymerizing activity. In an attempt to search for novel effectors of the small GTPase RhoD, a splice variant of hDia2, named hDia2C, was identified (Gasman et al., 2003). This isoform of hDia2 contains a seven amino acid insertion bearing the GTPase binding domain, which targets hDia2C to early endosomes to regulate their motility along actin filaments in a Src-dependent manner (Gasman et al., 2003). The localization of DRFs on endosomes appears to be a more general phenomenon, as reported earlier for mDia1 and mDia2 (Tominaga et al., 2000). A recent study in HeLa cells proposed that active RhoB recruits mDia1 to endosomes and further showed that constitutively active mDia1 induces the formation of actin coats on endosomal membranes to inhibit their transfer to microtubules (Fernandez-Borja et al., 2005). In apparent contradiction to these findings, expression of a similarly active mDia1 mutant did not alter the motility of GFP-Rab5-labeled endosomes in living HeLa cells (Gasman et al., 2003). However, both reports agree that constitutively active mDia1 or hDia2C lacking the Rho binding domain still target efficiently to endosomes in a Rho-independent fashion by mechanisms that remain unclear (Fernandez-Borja et al., 2005; Gasman et al., 2003). Another open question is whether the different DRFs that colocalize with endosomes fulfill separate functions to regulate specific aspects of endocytic vesicle transport. In the case of complement receptor-mediated phagocytosis in macrophages, both mDia1 and mDia2 are thought to play critical roles, although the underlying molecular mechanism remains to be elucidated (Colucci-Guyon et al., 2005).

Cell Polarity

Both budding and fission yeast have been used extensively as model systems to study the mechanisms that control the development and establishment of cell polarity. During yeast cell division, actin cables and microtubules are redirected toward marked cortical growth zones in a tightly controlled spatiotemporal manner in order to segregate organelles and to assure cell cycle progression (Bretscher, 2003; Chang and Peter, 2003). Over the past few years, it has become increasingly clear that several formins exert specific and essential roles for polarized cell growth in yeast.

In budding yeast, the DRFs Bni1p and Bnr1p interact with profilin and rearrange the actin cytoskeleton into cable-like actin structures at the bud cortex (Evangelista et al., 1997, 2002; Imamura et al., 1997; Sagot et al., 2002a). This apparently requires the localized activity of formins, as a GFP-Bni1p fusion protein was dynamically recruited to the bud tip and neck at cortical sites of cell growth throughout the cell cycle (Ozaki-Kuroda et al., 2001). The polarized localization seems to be specifically controlled by distinct cortical landmark proteins, which are believed to regulate formin function through positive feedback loops. Comparison of Bni1p and Bnr1p showed that they differentially bind to cell polarity factors such as Bud6 (Figure 3A). These findings could explain why Bni1p and Bnr1p assemble different forms of actin cables (Pruyne et al., 2004), although this may also be due to the fact that Bnr1p additionally bundles actin filaments, whereas Bni1p does not (Moseley and Goode, 2005).

In the rod-shaped fission yeast, the DRF For3p localizes at the cell end or tip, where it controls actin cable formation in interphase cells (Feierbach and Chang, 2001; Nakano et al., 2002). How is the polarized regulation of formin-dependent actin polymerization initiated and timed during cell growth? Recently, Chang and colleagues were able to provide some cues to this puzzle. They showed that the microtubule-associating factor Tea1p is transported to microtubule plus ends toward the cell tip to interact with a polarity factor complex ("polarisome") including For3p (Feierbach et al., 2004). By screening for Tea1p binding proteins, a microtubule plus end factor (Tea4p) was identified that interacts directly with For3p to facilitate actin cable assembly and polarized cell growth (Martin et al., 2005). Thus, microtubule plus ends dynamically deliver proteins to cortical cell growth zones for the spatial regulation of actin polymerization through formins (Figure 3B). Further research will hopefully clarify whether similar mechanisms are at work in mammalian cell systems.

Serum Response Factor Activity/Transcription

The ability of formins to reorganize the actin cytoskeleton also has transcriptional consequences since such changes regulate the actin binding transcriptional coactivator MAL, also known as MRTFa and MKL1 (Miralles et al., 2003). MAL dimers form complexes with serum response factor (SRF), leading to the induction and upregulation of many cytoskeletal target genes, including both α- and β-actin (Figure 4) (Miralles et al., 2003; Sun et al., 2006). SRF was initially isolated as a nuclear protein binding to the serum response element (SRE) to mediate transcriptional activation of immediate early genes, such as c-*fos*, as well as cytoskeletal actin genes (Treisman, 1987). Interestingly, SRF appears to be essential for cell adhesion, spreading, and stress fiber formation, since embryonic stem cells deficient for SRF display strongly reduced expression of focal adhesion proteins such as vinculin, talin, or zyxin (Schratt et al., 2002). The recently identified SRF cofactor MAL is associated with a pool of monomeric actin (G-actin) that, when polymerized by mDia1, causes the coactivator to

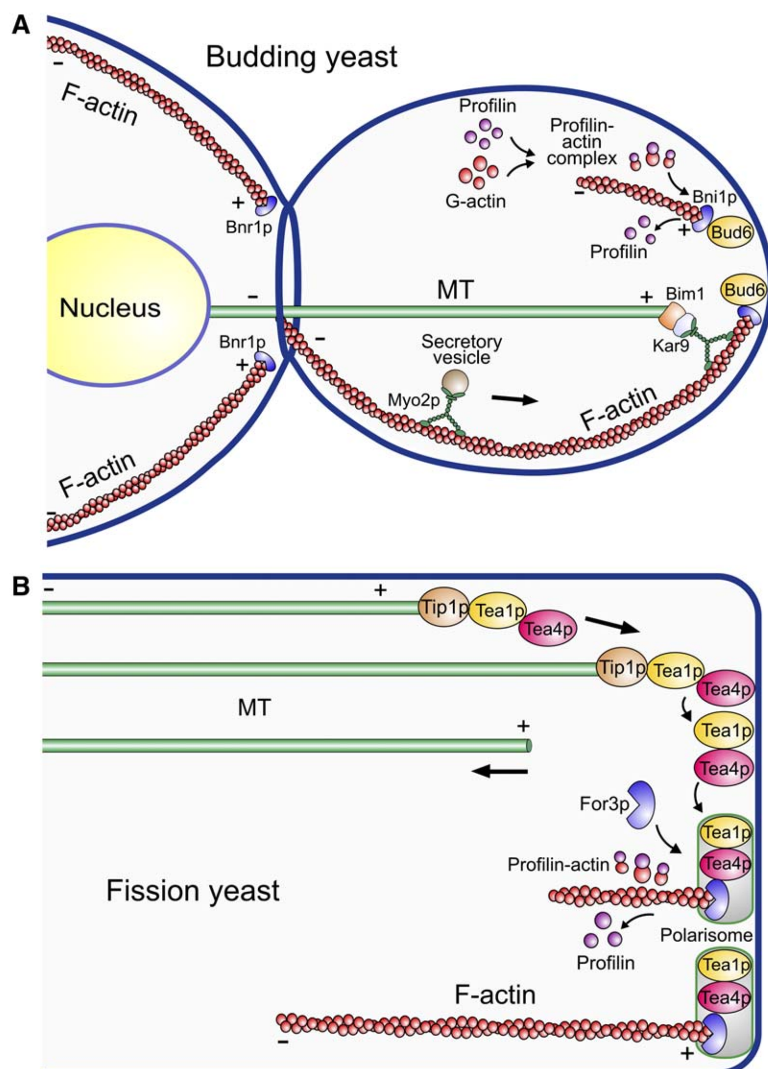

Figure 3. Regulation of Cell Polarity by Formins in Yeast

(A) In budding yeast, the two DRFs Bni1p and Bnr1p control localized actin polymerization at the bud tip and bud neck, respectively. The cell polarity factor Bud6p directly stimulates the activity of Bni1p. DRF-induced actin polymerization is required for Myo2p-mediated transport of secretory vesicles along actin bundles into the bud in order to deposit the compound for polarized cell growth. This type of Myosin V-based transport system is also employed to deliver the microtubule plus end proteins Bim1 (mammalian EB1) and Kar9 (mammalian APC) for polarized microtubule stabilization (capture) at the cell cortex during mitotic cell division.

(B) In fission yeast, a protein complex including the CLIP170 homolog Tip1p as well as the factors Tea1p and Tea4p is transported on growing microtubule plus ends until they become deposited at the cell tips after microtubule shrinkage. Subsequently, several polarity factors are recruited at the cell cortex into a large protein complex called the polarisome. There, Tea4p directly binds to and regulates the DRF For3p to promote actin filament assembly during "new end take off" (NETO) and polarized cell growth.

translocate from the cytoplasm into the nucleus, where it activates SRF-dependent transcription from the SRE (Figure 4) (Cen et al., 2004; Miralles et al., 2003; Posern et al., 2004). It is not clear whether the MAL/G-actin complex still allows interaction and regulation by profilin. Several proteins such as ROCK, VASP, N-WASP, and Lim kinase, which regulate actin dynamics, are able to stimulate MAL/SRF; however, thus far, mDia1 appears to be the most efficient, at least in simple transfection assays (Miralles et al., 2003; Sotiropoulos et al., 1999). In the case of mDia1, SRF stimulatory activity correlates directly with the ability to polymerize actin and depends on the FH2 domain as well as on the cooperative function of VASP (Copeland and Treisman, 2002; Grosse et al., 2003). Other DRFs known to activate SRF include mDia2, FHOD, and Diaphanous (Gasteier et al., 2003; Somogyi and Rorth, 2004; Tominaga et al., 2000; Westendorf, 2001), and this list is likely to grow in the future. Induction of MAL/SRF target genes very likely provides an essential feedback mechanism for regulating cytoskeletal dynamics during processes like migration, invasion, and adhesion (Sun et al., 2006). For example, during *Drosophila* border cell migration, the SRF coactivator MAL, which responds to Diaphanous, is critical for efficient cell invasion; border cells that cannot migrate lack nuclear localization of MAL (Somogyi and Rorth, 2004). SRF activity is also required for neuronal migration during forebrain development in mice (Alberti et al., 2005).

Morphogenesis

The integrity and orientation of cells within the epithelial plane are regulated by a process referred to as planar cell polarity (PCP), which involves frizzled (*fz*) and dishevelled signaling as well as the downstream function of RhoA. While searching for novel binding partners of dishevelled, Habas et al. (2001) identified a DRF that binds to and regulates RhoA to control *Xenopus* gastrulation and was consequently named Dishevelled-associated activator of morphogenesis (Daam1). *Xenopus* embryos injected with morpholinos to block endogenous Daam1 protein synthesis exhibited severe gastrulation abnormalities characteristic of defective PCP signaling (Habas et al., 2001), suggesting that Daam1 is crucial for this process. By contrast, a recent genetic study showed that the only *Drosophila* homolog, Daam, has either no essential or a redundant role in PCP establishment (Matusek et al., 2006). In *Drosophila* tracheal cells, Daam appears to act upstream of src family kinases to organize the actin cytoskeleton of the respiratory system

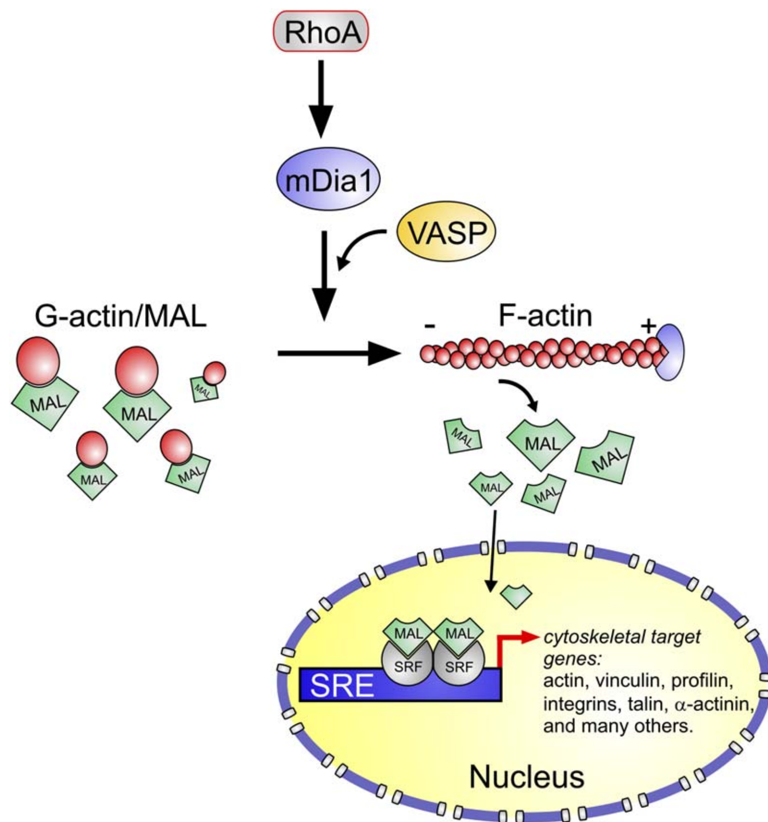

Figure 4. Transcriptional Activation of MAL/SRF by mDia1

A pool of monomeric G-actin is directly bound to MAL, thereby retaining the coactivator in the cytoplasm. Upon activation of mDia1, the MAL/G-actin pool becomes depleted through polymerization and stabilization of actin filaments (F-actin) requiring the cooperative activity of the actin-bundling protein VASP, which physically interacts with mDia1. The conformational changes of actin during filament nucleation and polymerization releases MAL, which, in turn, rapidly accumulates in the nucleus to drive serum response factor (SRF)-dependent gene transcription from the serum response element (SRE). These target genes include immediate early genes such as c-*fos*, but they also include actin, filamins, cofilins, and many other cytoskeletal regulators that are involved in various cellular processes such as motility, adhesion, and shape change. This likely provides an efficient feedback mechanism between signal-controlled actin dynamics and the transcriptional upregulation of actin itself as well as actin-regulating factors.

(Matusek et al., 2006), displaying some similarity to mDia2 and hDia2C, which have also been implicated to act upstream of src tyrosine kinases in mammalian cells (Gasman et al., 2003; Tominaga et al., 2000). Additionally, formin-3 (form3) was shown to be required for tracheal development in *Drosophila* by controlling actin reorganization in fusion cells at the branch tip to allow tracheal fusion (Tanaka et al., 2004).

Drosophila genetics was applied early on to study the in vivo functions of Diaphanous, the close homolog of mDia1. Diaphanous was demonstrated to be essential for cytokinesis during spermatogenesis and oogenesis, and it was subsequently demonstrated to be essential for fertility (Castrillon and Wasserman, 1994). This function of formins appears to be conserved across species, as mutations in the human *DIA* gene lead to premature ovarian failure, most likely as a result of defective cell division during ovarian follicle differentiation (Bione et al., 1998). In addition, targeted deletion of formin-2 results in oocyte metaphase I block and hypofertility in mice (Leader et al., 2002). In *Drosophila* embryos, Diaphanous further controls plasma membrane invagination during cellularization, a process that requires reorganization of the cortical actin cytoskeleton through Rho/Diaphanous-induced actin polymerization (Afshar et al., 2000; Grosshans et al., 2005).

In addition to DRFs, which are defined by their N-terminal regulation through Rho-like GTPases, the *Drosophila* homolog of mammalian formin-2, cappuccino (capu), was recently demonstrated to bind Rho at its N terminus despite the absence of any previously characterized predictive domain (Rosales-Nieves et al., 2006). Although capu appears not to be regulated via intramolecular autoinhibition, Rho binding to capu may nevertheless regulate its actin-polymerizing activity by an unknown mechanism (Rosales-Nieves et al., 2006). To add further complexity to this scenario, the actin-nucleating protein Spire was shown to bind to the FH2 domain of capu, thereby inhibiting its F-actin/microtubule crosslinking activity, but independent of its actin nucleation activity, to time the onset of microtubule-dependent cytoplasmic streaming during germline development in *Drosophila* (Rosales-Nieves et al., 2006).

Microtubule Stabilization

In addition to their actin-polymerizing activity, some DRFs promote the formation of polarized, detyrosinated microtubules, which represent a microtubule subpopulation with a long half-life (>1 hr) and therefore are named "stable microtubules" (Gundersen et al., 2004). In directionally migrating fibroblasts, mDia1 appears to be sufficient as well as necessary for the generation of orientated, stable microtubules, as demonstrated by microinjection of active mDia1 derivatives or by applying RNA interference against mDia1 (Goulimari et al., 2005; Palazzo et al., 2001). Formation of stable microtubules is mediated by the interaction of mDia1 with the microtubule tip proteins adenomatous polyposis coli (APC) and EB1, thereby capturing microtubules at the cell cortex (revealed by using TIRF microscopy; [Wen et al., 2004]). Furthermore, the spectraplakin family member ACF7 (actin crosslinking family 7), which binds along microtubules, was shown to be required for microtubule stabilization downstream of active mDia, as demonstrated in ACF7 knockout endodermal cells (Kodama et al., 2003). Intriguingly, as is the case for actin polymerization and SRF activation, the FH2 domain was found to be

essential for stable microtubule formation, suggesting that actin-dependent processes may nevertheless be involved (Wen et al., 2004). Thus, DRFs mediate important crosstalk between the actin and tubulin cytoskeletons. The precise mechanism by which mDia forms stable MTs is far from complete and requires further study. Also, it is not known whether this activity is restricted to certain formins or if it represents a general property of this protein family.

Concluding Remarks

Our present understanding of the molecular functions of formins has grown immensely over the past few years from detailed structural insights to complex and diverse cellular roles. It is somewhat puzzling, however, that even though over 15 known formins exist in mammals, some of them, such as mDia1, have been reported to be critically involved in numerous yet strikingly different cellular mechanisms ranging from cell cycle regulation to organelle distribution (Table 1). Thus, the known molecular roles for formins to date already cover almost the entire repertoire of cellular events. This of course raises the question of whether the 15 mammalian formins have largely overlapping functions or if some have very specific ones. We have just begun to discover the importance of formins in physiological and pathophysiological processes in complex organisms. Future studies, for example with mice deficient for specific formins, will further our understanding as to which of the reported cellular functions are in fact relevant under in vivo situations in various tissues and organs. The continued structural analysis of formins will be similarly important for understanding their precise molecular mechanisms. Currently, it is unclear what the exact nature of the complete full-length formin structure is or how these proteins associate and function as homodimers during actin polymerization. It will be exciting to unravel these structural challenges, which will most certainly further accelerate our scientific interest in these potent cytoskeletal regulators. One exciting future prospect is that formins may represent a family of attractive drug targets and may provide novel possibilities for the treatment of actin-dependent processes such as inflammation, metastasis, and invasive diseases.

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