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Formins as effector proteins of Rho GTPases

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Abbreviations: FH2, formin homology 2; FH3, formin homology 3; GAP, GTPase activating protein; GBD, GTPase-binding domain; GEF, guanine nucleotide-exchange factor; Daam, Disheveled-associated activator of morphogenesis; Dia, Diaphanous-related formin; FHOD, FH1/FH2 domain-containing protein; FMNL, Formin-like protein

Formin proteins were recognized as effectors of Rho GTPases some 15 years ago. They contribute to different cellular actin cytoskeleton structures by their ability to polymerize straight actin filaments at the barbed end. While not all formins necessarily interact with Rho GTPases, a subgroup of mammalian formins, termed Diaphanous-related formins or DRFs, were shown to be activated by small GTPases of the Rho superfamily. DRFs are autoinhibited in the resting state by an N- to C-terminal interaction that renders the central actin polymerization domain inactive. Upon the interaction with a GTP-bound Rho, Rac, or Cdc42 GTPase, the C-terminal autoregulation domain is displaced from its N-terminal recognition site and the formin becomes active to polymerize actin filaments. In this review we discuss the current knowledge on the structure, activation, and function of formin-GTPase interactions for the mammalian formin families Dia, Daam, FMNL, and FHOD. We describe both direct and indirect interactions of formins with GTPases, which lead to formin activation and cytoskeletal rearrangements. The multifaceted function of formins as effector proteins of Rho GTPases thus reflects the diversity of the actin cytoskeleton in cells.

Introduction

Many cellular functions such as migration, adhesion, and changes in cell shape are regulated by remodeling of the actin cytoskeleton. The dynamic actin structures play key roles during tissue regeneration, immune responses, embryonic development, and wound healing in eukaryotic organisms. Among a wide array of cytoskeletal structures, three main categories of actin filament assemblies can be distinguished that play fundamental roles in cell migration of multicellular organisms. First, there is the lamellipodium as a veil-like membrane protrusion at the leading edge of a cell, which contains a meshwork of branched actin filaments. Secondly, filopodia and microvilli appear as finger-like outgrowths of the plasma membrane that are stabilized by an actin filament bundle of varying thickness. Lastly, actin stress fibers occur in the cytoplasm of the cell that can form at least three different assembly categories such as dorsal stress fibers, actin arcs, and ventral stress fibers. These actin structures are dynamically regulated by small GTPases of the Rho family, which has been phenotypically shown more than two decades ago.[1]

The assembly of actin monomers into filamentous structures does not occur spontaneously but requires factors which help to overcome the kinetic barrier of nucleation.[2] These actin nucleation factors can be classified into three groups: the Arp2/3 complex and its nucleation promoting factors, WH2 domain-containing nucleators, and formin proteins.[3-7] Members of these three groups employ different mechanisms to accomplish the nucleation and elongation of actin filaments. The Arp2/3 complex binds to the sides of pre-existing actin filaments and generates branched actin networks. Spir, as an example for WH2 domain-containing proteins, nucleates the assembly of straight actin filaments by its four WH2 domains.[8] The WH2 elements are lined up at defined distances to accomplish binding to one G-actin molecule each and doubled through Spir dimerization. Formins finally nucleate actin molecules from the barbed end and remain associated with the barbed end during filament elongation. In a landmark study, the formin mDia as the mammalian homolog of *Drosophila* Diaphanous was found as a downstream effector of Rho that selectively interacts with the triphosphate bound form of RhoA GTPase.[9] In this review we discuss the current knowledge on the structure, function, and activation mechanism of formins as downstream effectors of Rho GTPases.

Formin Effector Proteins of Rho-GTPases

In mammals, there are 15 formins that group into eight different sub-families based on their sequences and domain architectures.[5,10] A part of these formins were found to be autoinhibited, which gave rise to the classification as Diaphanous-related formins, or DRFs,[11,12] named after the product of the *Drosophila* gene *diaphanous*.[13] The intramolecular interaction

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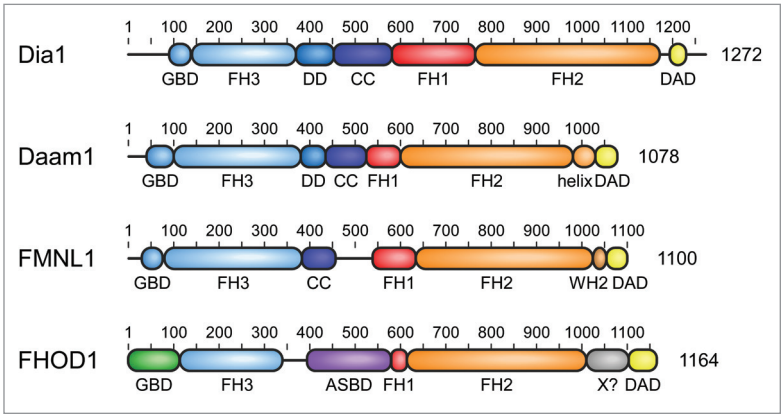

Figure 1. Domain architecture of mammalian Diaphanous-related formins. The multidomain proteins comprising more than 1000 amino acids contain a central proline rich FH1 domain followed by the actin polymerizing FH2 domain. DRFs contain in addition a C-terminal DAD autoregulation domain that interacts in the autoinhibited state with its N-terminal FH3 recognition domain. Additional dimerization elements DD (dimerization domain) and CC (coiled coil) contribute to the overall structure assembly of the formins. DRF activation occurs through interaction of the N-terminal GTPase-binding domain (GBD) with a Rho GTPase. Additional elements as the WH2 motif in FMNLs or the actin side-binding domain (ASBD) in FHOD1 contribute to the specificity of each DRF family.

between the C-terminal Diaphanous autoregulatory domain (DAD) and its N-terminal recognition domain, termed FH3 or DID, leads to the autoinhibition of DRF proteins.[11,12,14-16] For some of the DRFs it is now well established that the autoinhibition is relieved upon the interaction with an active Rho GTPase, such as Rho, Rac, or Cdc42.

Formins are multi-domain proteins of typically more than 140 kDa in weight that are defined by the presence of a formin homology 2 (FH2) domain. The flanking regions of the FH2 domain vary considerably between individual formins, reflecting the different cellular functions and regulatory mechanisms of the actin polymerization factors. A molecular scheme of the domain architecture of human DRFs is shown in **Figure 1**. The FH2 domain binds directly to G- and F-actin and has been shown for many formins to nucleate actin molecules and elongate actin filaments.[17] The approximately 400 amino acid long domain forms a doughnut-shaped head-to-tail dimer that remains associated with the fast-growing actin filament barbed end.[18] The formin thereby prevents binding of capping proteins during the elongation procedure.[19,20] In most formins a proline-rich FH1 domain that interacts with profilin for the recruitment of G-actin molecules precedes the FH2 domain, thus accelerating the actin polymerization rate of the formin.[21,22] N-terminal to the FH1 domain is the FH3 domain, which is the least conserved module in the overall domain architecture and involved in the regulation of formin activity. In the resting state of the formin, the FH3 domain recognizes the C-terminal DAD generating an intramolecular, autoinhibited complex. In some formins, the FH3 domain is N-terminally merged with a GTPase-binding domain (GBD), whereas an additional dimerization element can be found C-terminal to the FH3 domain. As DAD and FH3 domains are on average about 800 residues apart, it is not clear if the DAD binds intramolecular or intermolecular in the dimer assembly. A cartoon displaying the conformational changes from the autoinhibited to the active state of the DRF is shown in **Figure 2**.

The Diaphanous-related formins encompass the four mammalian families mDia, Daam, FMNL, and FHOD, that largely share a similar domain organization. Their interactions with Rho GTPases described today are listed in **Table 1** and will be discussed in the following.

Regulation of Diaphanous Related Formins

The resting, autoinhibited complex

An autoinhibitory intramolecular interaction between the C- and N-terminal regions has been described for all mammalian DRFs,[12,23-26] but only the autoregulatory interaction of mDia1 is known at structural detail to date.[15,16] The C-terminal DAD of mDia1 is composed of an amphipathic helix with the central consensus motif MDxLLxxL followed by an unstructured, basic region of variable sequence and length (**Fig. 3**).[12,27] While the DAD helix is essential for the binding to a hydrophobic surface patch at the concave side of the FH3 armadillo repeat structure,[15,16] the basic region seems to be important for the affinity of the interaction.[15,27] The interaction of the basic region with the FH3 domain has not been determined by structural means yet, but large, negatively charged patches were identified in mDia1 and FHOD1 adjacent to the MDxLLxxL recognition site.[15,28] The basic region of the DAD likely interacts with an acidic groove located between the FH3 domain and the elongated α-helix at the C-terminus of the FH3 domain in mDia1, which connects the FH3 domain with the DD domain.[29,30] In addition, the DAD has been shown to exhibit dual functions in autoinhibition and actin assembly as it enhances actin nucleation by recruiting actin monomers.[31] This function is achieved without altering the filament elongation rate of the FH2 domain and independently of the FH1 domain.[31]

The activating complex

The interaction of Rho GTPases with formin effectors has been first described for mDia1 by the Narumiya laboratory.[9,11] The specific binding of active RhoA•GTP to mDia1 was confirmed in the following years exhibiting dissociation constants in the nanomolar affinity range.[32,33] The structural characterization of the activating complex of mDia1 with RhoC revealed that formin binding is mediated essentially through the switch regions of the GTPase, similar to that of other Rho effectors.[32] While the switch I region (also named the "effector loop") exclusively interacts with the GBD, the switch II region forms contacts with the GBD and the FH3 domain (**Fig. 4**). The GTPase interacts with the mDia1 GBD through a complementary hydrophobic surface, whereas mainly electrostatic driven interactions are formed with the first armadillo motif on the concave side of the FH3 domain.[32,34] Since all formin-interacting residues in the switch regions of Rho GTPases are conserved, the specificity of the GTPase–formin

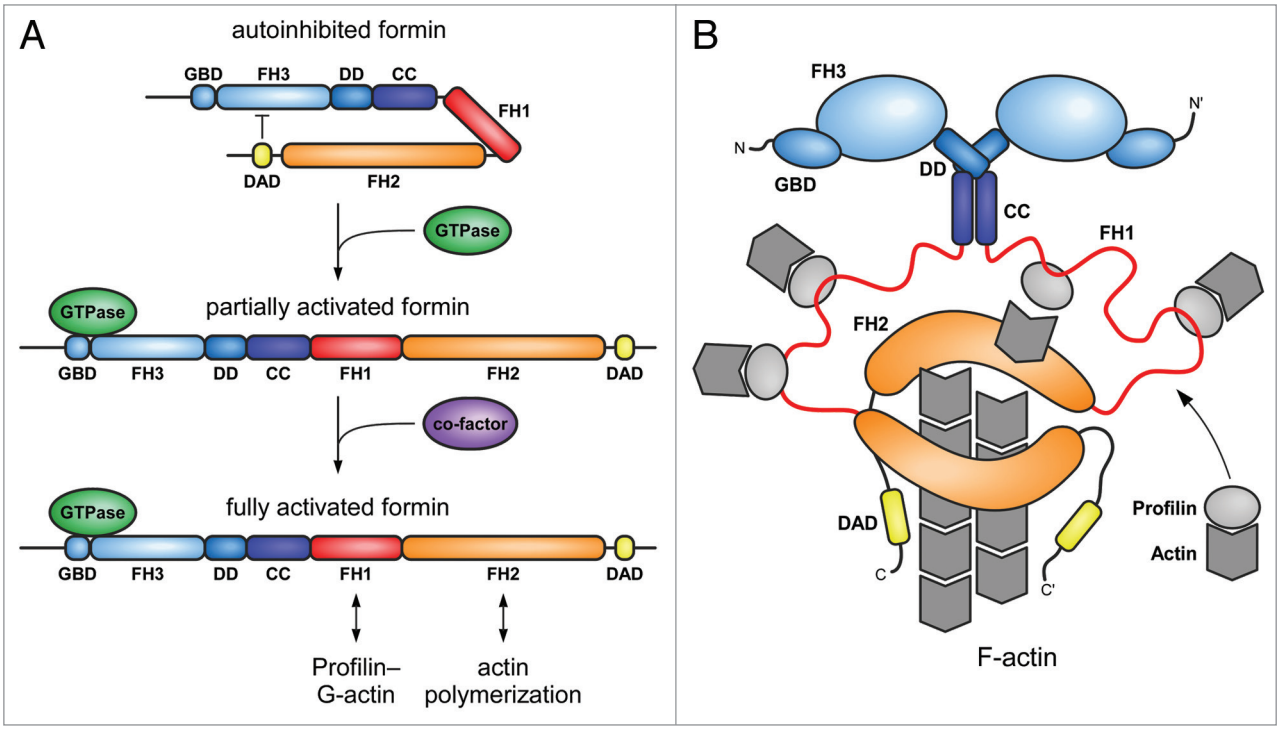

Figure 2. Cartoon of the regulation of a Diaphanous related formin. (**A**) In the autoinhibited state, the C-terminal DAD interacts with the N-terminal FH3 domain. Binding to a GTP-bound Rho GTPase leads to relief of the autoinhibited state by a partial displacement of the DAD and formin activation. Possible co-factors as e.g., kinases for formin phosphorylation, additional interaction factors of the DAD (as described for Daam), or interactions with membrane compartments for proper orientation might be required for full activation of the formin. GBD, GTPase-binding domain, FH1/2/3, formin-homology domains, DD, dimerization domain, CC, coiled coil domain, DAD, Diaphanous-autoregulation domain. (**B**) Cartoon of the activated formin dimer. The proline-rich FH1 domain recruits profilin–actin complexes in close proximity to the FH2 domain. G-actin molecules are polymerized to F-actin by the dimeric FH2 domain.

interaction remains elusive. Two aromatic residues C-terminal to the α3 helix of the GTPase were shown to be involved in binding and contribute to the specificity of the interaction.[34] Mutation of three interacting asparagine residues located in the first Armadillo repeat of mDia1 from ^{164}NNN to the corresponding residues TSH found in mDia2 and mDia3 increased the binding affinity to Cdc42.[34]

Activation through displacement

The mechanism how Rho GTPases displace the autoregulatory DAD domain from the FH3 domain is not fully understood today. Our molecular insights are currently based on the available complex structures of the N-terminus of mDia1 with either the DAD or active RhoC, respectively. Although the binding interfaces displayed on the surfaces of the FH3 domain for both, the DAD and the GTPase, only slightly overlap (**Fig. 5**), a simultaneous binding is excluded.[15,16,32,35] Therefore, a two-step binding mechanism to abolish the autoinhibition has been suggested.[15] The Rho GTPase might first bind in an initially weak complex to the GBD of the mDia1 formin, followed by the formation of a stronger interaction with the GBD-FH3 interface, which subsequently could result in the dislocation of the DAD from the FH3 domain.[15]

However, there is increasing evidence that binding of a Rho GTPase is not sufficient for full activation of a DRF. Whereas constitutively active RhoA is able to completely displace a small DAD peptide from an N-terminal construct of mDia1,[15,32] such active GTPase only partially relieved the autoinhibited complex between the dimeric N-terminus (GBD-FH3-DD or GBD-FH3-DD-CC) and the C-terminus (FH2-DAD).[14,35] In vitro polymerization assays using near to full-length mDia1 protein exhibited only partial activation even in the presence of a three orders of magnitude higher excess of constitutively active RhoA.[36] These observations led to the conclusion that additional formin family-specific regulation mechanisms might be required for full activation.[36,37] For example, two studies demonstrated that phosphorylation events could interfere with the FH3-DAD interaction contributing to DRF regulation,[38,39] and likewise association to membrane compartments is suggested to strengthen the active state. These additional activators are displayed as co-factors bridging the partially activated to the fully activated state of the DRFs shown in **Figure 2A**.

Formin inhibiting co-regulators often bind directly to the FH2 domain and thereby block the actin polymerization activity as it has been shown for the interaction of DIP-1 with mDia1.[40] In contrast, activating co-regulators may prevent the autoinhibitory interaction between the C-terminal DAD and the N-terminal FH3 domain. For example, the competitive binding of Anillin to the N-terminus of mDia2 effects its DAD release resulting in formin activation.[41] On the other hand, the activating Fli-1 protein has been reported to bind

Table 1. Interactions between formins and Rho GTPases

Formin	binding domain	Rho GTPase	References
mammalia			
mDia1, mDia2	GBD-FH3	RhoA	9, 11, 131
mDia1, mDia2	GBD-FH3	RhoB	11, 70
mDia1	GBD-FH3	RhoC	11
mDia2, mDia3	GBD-FH3	Cdc42	71, 131, 132
mDia1, mDia2	GBD-FH3	Rac1,2	34, 69
hDia2	n.d.	RhoD	133
mDia2	n.d.	Rif	66
Daam1	N-terminus (aa 41–477)	RhoA, -B, -C	33, 75
Daam1	N-terminus (aa 1–698)	Rac1	79
Daam1	n.d.	Cdc42	56
FMNL1	n.d.	Rac1	86, 88, 100
FMNL1	N-terminus (aa 1–450)	Cdc42	37
FMNL2	N-terminus (aa 27–276)	RhoC	102
FMNL2	GBD-FH3 (aa 1–379)	Cdc42	95
FMNL3	n.d.	RhoC	110
FHOD1	helical domain-FH1 (aa 422–717)	Rac1	113, 134
INF2	FH3 (aa 1–340)	Cdc42	135
Drosophila melanogaster			
Capu	N-terminus (aa 125–250)	Rho1	136, 137
dmDia	N-terminus (aa 1–464)	Rho1	138
dmDAAM	n.d.	RhoA	76
Dictyostelium discoideum			
ForH (dDia2)	n.d.	Rac1A	139
Schizosaccharomyces pombe (fission yeast)			
Cdc12p	N-terminus (aa 1–524)	Cdc15p	140
For3p	N-terminus (aa 149–488)	Rho3p	141
For3p	N-terminus (aa 149–488)	Cdc42p	141, 142
Saccharomyces cerevisiae (Baker's yeast)			
Bni1p	GBD-FH3 (aa 90–343)	Rho1p	143, 144
Bni1p	n.d.	Rho3p	145, 146
Bni1p	n.d.	Rho4p	146, 147
Bni1p	n.d.	Cdc42p	148, 149
Bnr1p	n.d.	Rho4p	146, 147

n.d., not determined

to the C-terminal DAD and to interfere with the FH3–DAD autoinhibition of mDia1.[42] As another regulation mechanism, the autoinhibition of mDia2 is reversed by ROCK1-mediated posttranslational phosphorylation near the DAD domain, which leads to formin activation.[39]

Overall domain assembly

First studies addressed the assembly of full-length mDia1 in the autoinhibited state, yet the overall structure of the 140 kDa protein is not fully understood.[29,30,36] Structural assemblies of the N- and C-terminal regions lacking the GBD and the FH1 domain reveal a tetrameric conformation[29,30] that might result from crystal packing as full-length mDia1 is a dimer in solution.[29,30,36] First insights into the structure of the dimeric, almost full-length mDia1 formin in the autoinhibited state were reported by Maiti et al. using electron microscopy.[36] In this reconstruction the dimeric, fork-shaped N-terminus folds over the doughnut-shaped FH2 domain and inhibits F-actin elongation by steric hindrance of actin filament binding. Likewise, the mDia1 FH2 domain in the autoinhibited state of the crystal structure seems to be accessible for G-actin, but not F-actin due to steric hindrance.[29] In the activated state, the elongated mDia1 molecule might not easily drop back into the autoinhibited conformation, due to large conformational transitions between the active and inactive conformations of the formin. It is indeed conceivable that the DAD interacts in the activated state with the F-actin filament preventing renewed autoinhibition, as interaction of the mDia1 DAD with actin has been described.[31]

Subcellular localization

Besides GTPase-mediated activation, the subcellular localization of the formin is also part of its regulation. This has been first described for the *S. pombe* formin Fus1,[43] followed by mammalian formins FMNL1 and mDia1.[37,44] As early as 2001, it has been assumed that the FH3 domain of mDia1 regulates its subcellular recruitment.[45] Membrane recruitment of formins occurs either through the direct interaction with a prenylated, membrane-associated Rho GTPase or includes other, GTPase independent localization mechanisms. mDia1 and mDia2, but not mDia3, contain an additional membrane binding motif composed of polybasic clusters N-terminal to the GBD that is thought to sustain the interaction with phospholipids through electrostatic interactions.[46,47] A Rho GTPase independent localization mechanism was confirmed for the N-terminus of Daam1 and the yeast formins Cdc12p and Bnr1p.[48-50] Meanwhile, it has been shown that a region inside the FH3 domain of mDia1 mediates binding to the scaffolding protein IQGAP1.[51] Furthermore, scaffolding proteins that contain membrane-anchored BAR domains represent crucial interfaces between signal transduction and actin cytoskeleton dynamics.[52,53] Consequently, some formins were described to be recruited by FH1–SH3 interactions with BAR proteins to specific membranes, such as mDia by IRSp53[54,55] or Daam by Cip4/Toca-1.[56]

Overall, the combination of formin localization either by intrinsic targeting motifs or external recruitment factors and the interaction with activating factors of the Rho family GTPases determines the regulation of DRFs in cells. An overview about the expression profiles, specific function in actin remodeling, cellular functions, and binding interactions and localization of the DRFs is provided in **Table 2**. In the following we will describe the four mammalian DRF families with regard to their function and

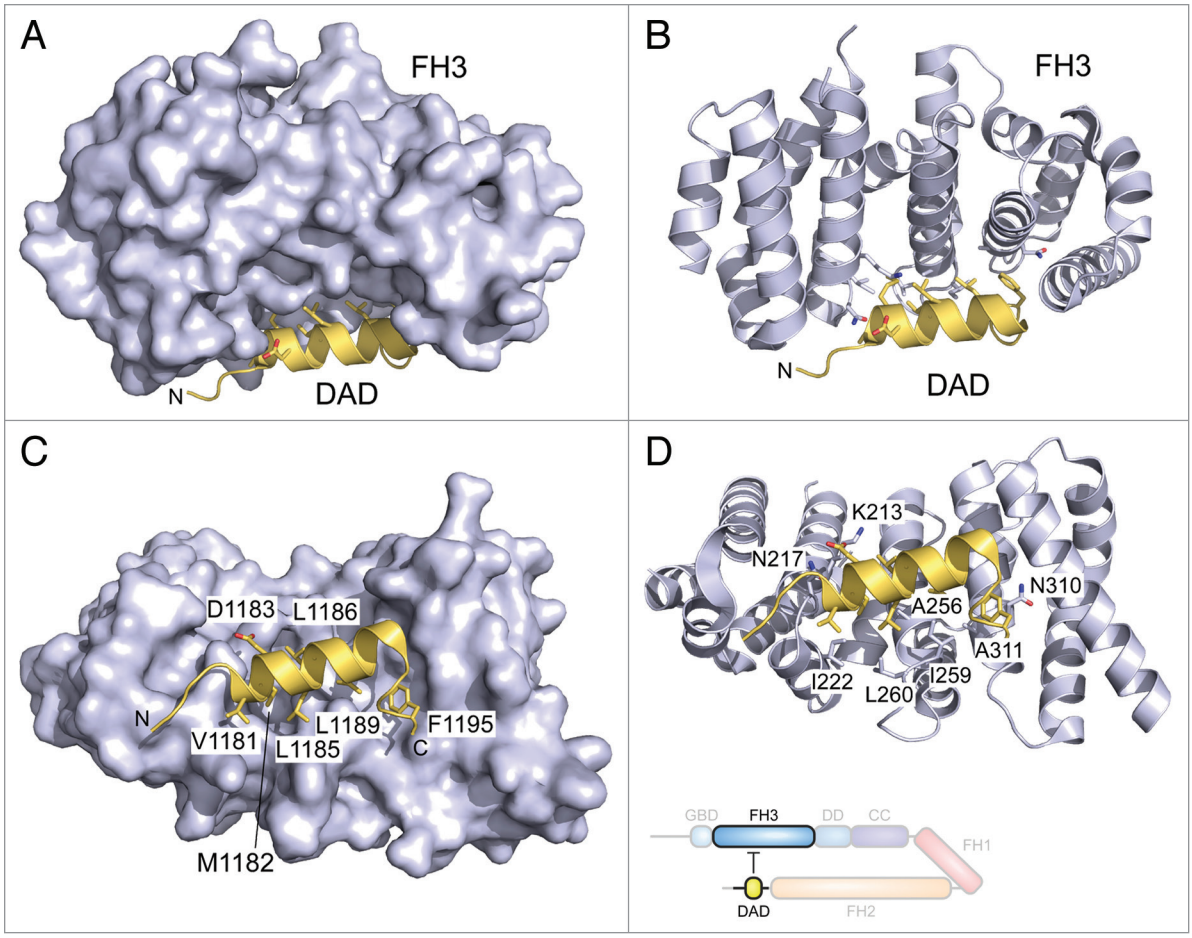

Figure 3. Structure of the autoinhibited FH3–DAD complex of mDia1. The helical DAD binds into the concave site of the FH3 domain armadillo repeat structure. (**A**) The DAD consensus motif MDxLL extends to VMDxLLxxLx$_5$F in the binding interface to the FH3 domain. (**B**) All five armadillo repeats participate in the interaction of the N-terminal FH3 domain with the C-terminal autoregulatory domain. Mutation of the central A256 residue to aspartate on the last turn of the third armadillo repeats leads to relief of the autoinhibition and activation of the formin.[15] Displayed is the structure 2F31 (ref. 16). A cartoon of the interaction scheme is shown below the atomistic model.

mechanism of activation by Rho GTPases as known to date. A model figure summarizing the function and localization of DRFs in cells is shown in **Figure 6**.

mDia

The mammalian Dia formin family with the three isoforms mDia1, mDia2, and mDia3 is a major effector of Rho GTPases.[9,11] mDia proteins induce actin filaments upon activation and cooperatively work with ROCK (Rho-associated coiled-coil kinase) to regulate the formation of actin stress fibers in cultured cells. mDia1 is the mouse ortholog of human Dia1 or DRF1 that shares 90.3% sequence identity to its human counterpart. mDia1 binds to the barbed ends of actin filaments and promotes strong polymerization activity, as seen by the processive movement of mDia molecules at the filament barbed ends in living cells.[57] In a recent study Breitsprecher and colleagues used single-molecule fluorescence microscopy techniques to image actin filament polymerization in vitro by differentially labeling the adenomatous polyposis coli (APC) and the FH1-FH2-DAD domain assembly of mDia1.[58] Upon filament polymerization, the complexes separated as visualized in the fluorescence images, with mDia1 moving processively on growing barbed ends while APC remained at the site of nucleation.

The best studied isoform of the Dia family is mDia1, which is involved in a variety of cellular processes including mechanotransduction,[59-61] cell polarization and migration of certain cell lines,[51,62] axonal outgrowth in primary cell cultures of cerebellar granule neurons,[63] and exocrine vesicle secretion in the apical membrane.[64] mDia2 instead is involved in filopodia formation,[65-67] and cytokinesis in cultured cells.[68] mDia2 was also ascribed a function in the formation of the contractile ring during asymmetric cell division of erythroblasts and endosome trafficking in fibroblasts.[69,70] mDia3 finally was shown to be required for chromosome alignment in HeLa cells,[71] potentially by phosphorylation and regulation through the kinase Aurora B.[72] A comprehensive overview of mDia function and the phenotypes resulting from mDia1 and mDia3 knockout in mice was recently provided by the Narumiya laboratory.[73] Diseases

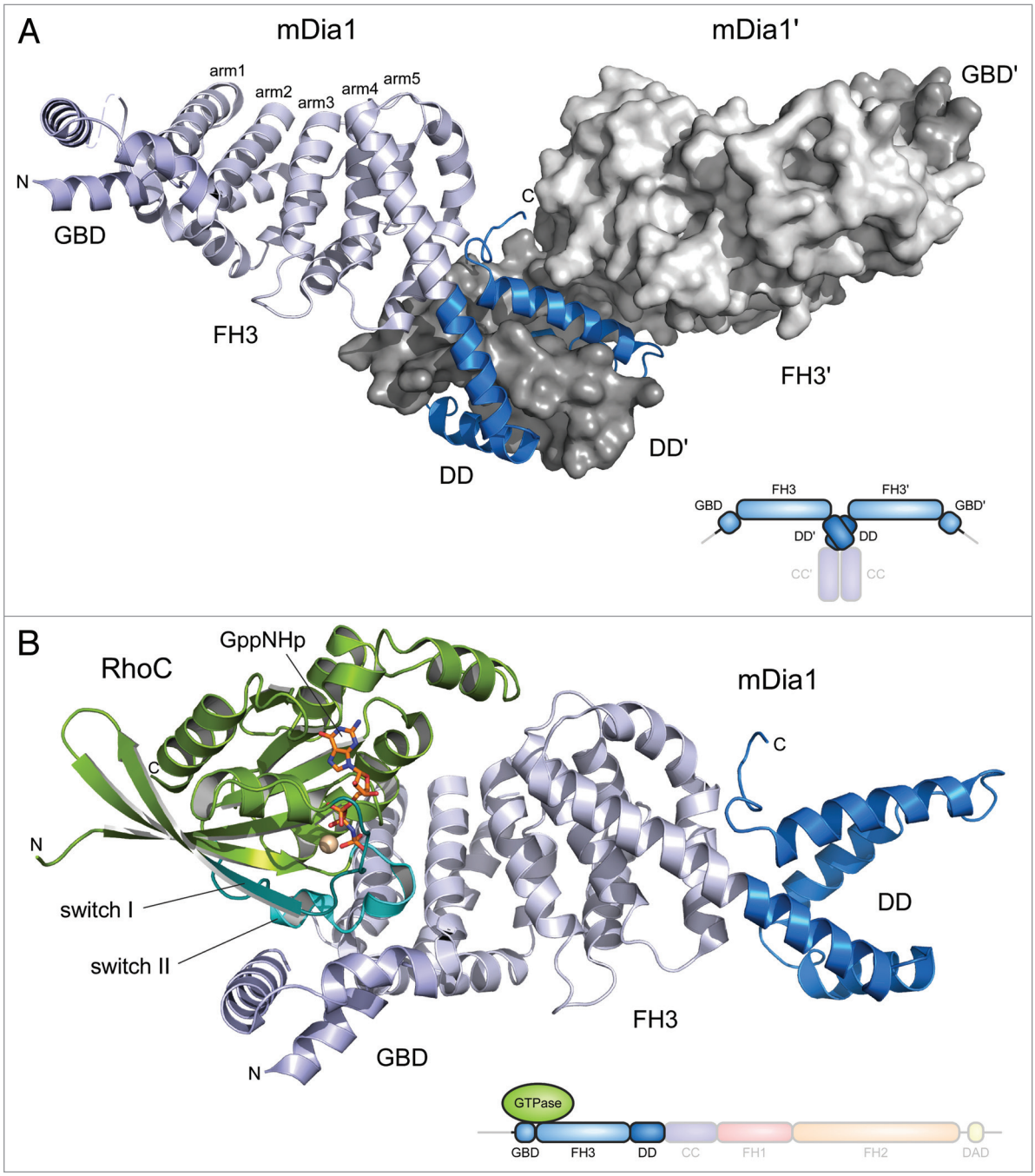

Figure 4. Structure of the RhoC–mDia1 complex. (**A**) Assembly of the N-terminal dimer structure in mDia1. The GBD-FH3-DD subdomains are displayed as cartoon representation in blue shadings. The five armadillo repeats of the central FH3 domain are labeled. The last armadillo repeat leads into a bundle of four interweaved helices forming the dimerization domain. The second molecule of the dimer is shown as surface representation. (**B**) Complex structure between RhoC•GppNHp and mDia1. RhoC mostly interacts with hydrophobic residues in the GBD of the formin. The two switch regions of the GTPase whose conformation is changed upon the nucleotide change are highlighted. Displayed is the structure 1Z2C (ref. 32). A cartoon of the interaction schemes is shown below each atomistic model.

associated with the roles of formins in cell division, migration, immunity, and microvesicle formation imply various types of cancer, deafness, and mental retardation.[74] The misregulation of formins is suggested to loosen adhesion of cancer cells, migration and ultimately invasion.[74]

The majority of biochemical and structural data to this day results from mDia1-Rho GTPase interactions. The FH3 domain (also called DID for "Diaphanous inhibitory domain"), encompassing amino acids 133–377 in mDia1, is located in the N-terminal regulatory part of DRF proteins. Up to now,

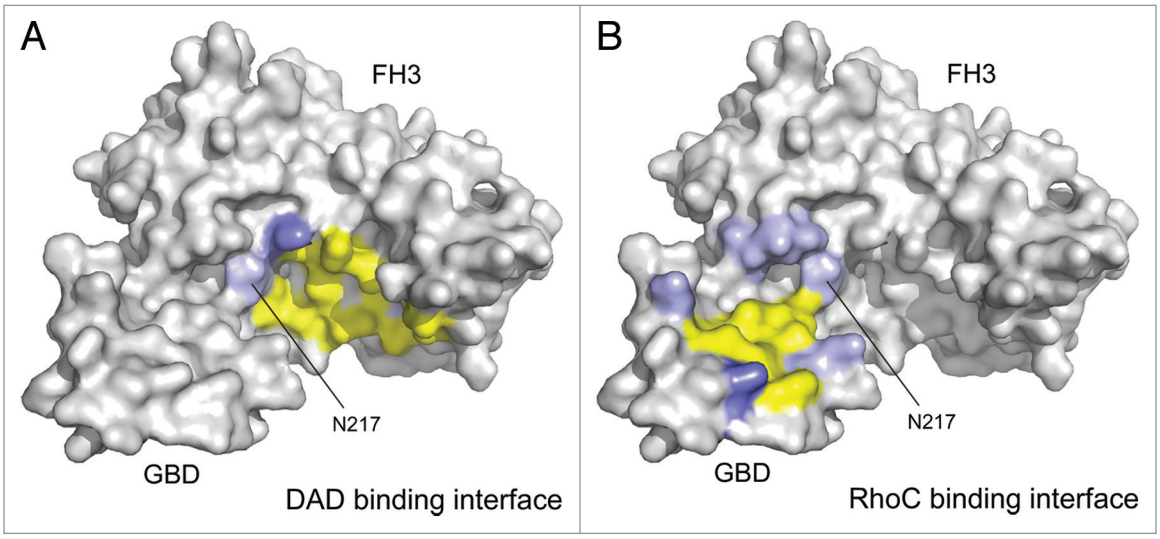

Figure 5. Display of the autoinhibitory and activating binding interfaces on mDia1 GBD-FH3 domains. (**A**) DAD binding interface on mDia1. Displayed are interacting residues derived from the mDia1 FH3–DAD complex structure 2F31 (ref. 16) and highlighted on the GBD-FH3 structure 1Z2C (ref. 32). Hydrogen bonds are formed between N217, N310, and Q352 (colored light blue) of the FH3 domain and the DAD. A salt bridge to D1183 of the DAD is mediated by K213 (colored blue) and hydrophobic interactions to the DAD motif are contributed by I222, K252, L253, A256, I259, L260, Q307, A311, T314, V351, and V355 (colored yellow) of the FH3 domain. (**B**) Display of the RhoC binding interface on mDia1 GBD-FH3 based on the evaluation provided in the 1Z2C structure.[32] Polar interactions to the GTPase are formed by K100 and Q118 of the GBD and N164, N165, N166, and N217 of the FH3 domain. A salt bridge is mediated by K107 and hydrophobic interactions are formed by M90, M94, N95, L96, P103, L104, and M115. Only N217 on the second armadillo repeat of the FH3 domain is in the intersection of the binding interface between the inhibiting and activating complex.

FH3 domain structures of mDia1[16,32,35] and FHOD1[28] have been determined. The FH3 domain is exclusively helical and composed of five armadillo (ARM) repeats (**Fig. 4**).[28,35] ARM repeats consist of three α helices arranged in a rectangular triangle, with each repeat rotated against another by 15–20° forming an elongated, banana-shaped structure with a convex and concave site of the superhelical domain fold. High sequence variations make a prediction of the helical assembly of other DRFs based on the structures of mDia1 or FHOD1 difficult and hence the prediction of interaction sites speculative. In mDia1, the extended helix of the last ARM motif leads into the following dimerization domain (DD, aa 377–452) that consists of three α-helices with two helices of each dimer chain forming a four helix bundle (**Fig. 4A**).[32,35] A helical region that displays sequence features of a coiled coil structure is located C-terminal to the dimerization domain (CC, aa 452–570). It has been demonstrated that the DD of mDia1 is sufficient for dimerization and that the N-terminus of mDia1 represents a constitutive dimer which might only dissociate through unfolding.[35] Not all DRFs necessarily contain this second dimerization element in addition to the C-terminal head-to-tail arrangement of the FH2 domain, as the N-terminus of FHOD1 was shown to contain a flexible linker region instead (**Fig. 1**).[28] N-terminal to the FH3 domain of mDia1 is the GTPase-binding domain (GBD, aa 73–131). This short segment is constructed of three triangularly arranged helices that are connected by a short linker to the FH3 domain (**Fig. 4**).[32,34] In the absence of the activating Rho GTPase, the GBD is presumably loosely folded but moves freely in solution,[35] representing therefore a subdomain rather than an independent structural unit.

Daam

The protein Dishevelled-associated activator of morphogenesis 1 (Daam1) was identified as interaction factor of Dishevelled (Dvl), which mediates the non-canonical Wnt/PCP (planar cell polarity) signaling pathway.[75] Early functional studies of Daam1 in lower species suggested an essential role in *Xenopus* gastrulation and *Drosophila* trachea formation.[75,76] Daam1 localizes to the plasma membrane and cytoplasmic vesicles, and this pattern is tightly regulated by Wnt and Dvl.[75,77-79] Recent studies in mammalian systems underline the role of the two Daam proteins, Daam1 and Daam2, in cell development.

Daam1 is highly expressed in developing murine organs, including the heart. Consistent with this expression pattern, Daam1-deficient mice show cardiac defects, including ventricular noncompaction, double outlet right ventricles, and ventricular septal defects. These animals die during embryonic development or at early postnatal days.[80] The role of Daam1 in the nervous system has been analyzed in zebrafish.[81] Here, Daam1 is enriched in the dorsal part of the asymmetric habenular neuropil. Loss of Daam1 in zebrafish embryos resulted in disturbed asymmetry and reduced neuropil formation. This can be explained by the finding that Daam1 regulates outgrowth of neuronal axons and dendrites.[81] Another Daam1-dependent process is the closure of the neural tube during embryogenesis. This process involves a regulating cadherin, Dvl, Daam1, and the PDZ-RhoGEF to upregulate Rho kinase.[82] Cellular forces of the ROCK stimulated actomyosin-dependent contraction promote the polarized bending of the neural plate.

Table 2. Expression profiles, functions, and interactions of DRFs

	mDia1/DRF1	mDia2/DRF3	mDia3/DRF2	Daam1, Daam2
Expression	several cell types and tissues	several cell types and tissues	several cell types and tissues	Daam1: expressed in early developmental stages[112] Daam2: high expression in neuronal cells,[112] high expression in later development stages of central nervous system[112]
Actin regulation	F-actin nucleation, elongation[14,22]	F-actin nucleation, elongation[17,22]	F-actin nucleation, elongation, bundling[163,164]	F-actin polymerization[33,167,168]
Actin structures	Stress fibers[11,150]	Filopodia[65-67,160,169]	Stress fibers[71]	Filopodia[79]
Function	Mechanotransduction[59-61] Cell polarization[62,151] Cell migration[51,151] Phagocytosis[51] Cell motility of T cells[152] Axogenesis of neurons[63] Endosome trafficking[153] Exocrine vesicle secretion[64] Microtubule stabilization[154-156] Cell signaling, transcriptional regulation[157]	Cytokinesis[41, 68,161] Nucleation of erythroblasts[69] Cell movements during gastrulation[162] Endosome trafficking[70]	Cell mitosis[165] Mitotic chromosome alignment[71,72] Endosome trafficking[133] Apical-basal polarity of neuroepithelial cells[166]	Non-canonical Wnt/PCP pathway[75] Cell development: Vertebrate gastrulation[75,169] Tracheal development[76] Axonal morphogenesis[79] Asymmetric morphogenesis[81] Neural-tube closure during embryogenesis[82] Spinal cord development[83] Heart morphogenesis[80]
Interactions, localization	Rho GTPase RhoA[9,158] Polybasic N-terminal clusters[46] IQGAP1[51] IRSp53[54,55] CLIP-170[159] Nuclear localization[172]	Rho GTPase Rif[47] Polybasic N-terminal clusters[47] Abi1[67] Anillin[41] Nuclear localization[172]	n.d.	Cip4[56] Toca-1[56]
	FMNL1	**FMNL2, FMNL3**	**FHOD1**	**FHOD3**
Expression	Macrophage-enriched[86] Hematopoietic cells and tissues (thymus, spleen, peripheral blood leukocytes)[98,99] Overexpressed in lymphoma cells[98,99]	Cells of nervous system, epithelium, lymphoid tissue[94] Overexpressed in colorectal carcinoma[101]	high expression in several cell types[112] mesenchymal cells[119]	low average expression levels, specific expression in skeletal and cardiac muscle[112] highly expressed in heart[123,170,171]
Actin regulation	F-actin polymerization, severing, bundling[106]	F-actin polymerization, bundling[95,109]	F-actin bundling, capping[115]	F-actin acceleration[123]
Actin structures	Lamellipodium, filopodia[86]	Lamellipodium, filopodia[94,95,107]	Stress fibers[113] enriched in transversal actin arcs, mature stress fibers[116]	Stress fibers[124,170]
Function	Cell proliferation[100] Cell adhesion, growth, and migration[86,100] Centrosome polarity[88] Cytotoxic T cell activation[88] Recognition of the antigen presenting cell[88] Phagocytosis[37,89] Regulation of podosomes[90] Golgi complex stabilization[91] Non-apoptotic membrane blebbing[92]	Cell motility and cell migration[94,95,97,101-103,110] Cell proliferation[101,105] Endothelial cell elongation during angiogenic morphogenesis[96]	Cell division[118] Cell migration[119]	Regulation of sarcomere organization[122] Heart developement[125] Myofibril maintenance[123]
Interactions, localization	Rho GTPase Cdc42[37] srGAP2[111]	Rho GTPase Cdc42[95] N-terminal myristoylation[95]	Recruitment by Rho GTPase Rac1[113] Phosphorylation by ROCK[38,126] Association with Nesprin-2-giant[173]	Phosphorylation by CK2[123]

n.d., not determined

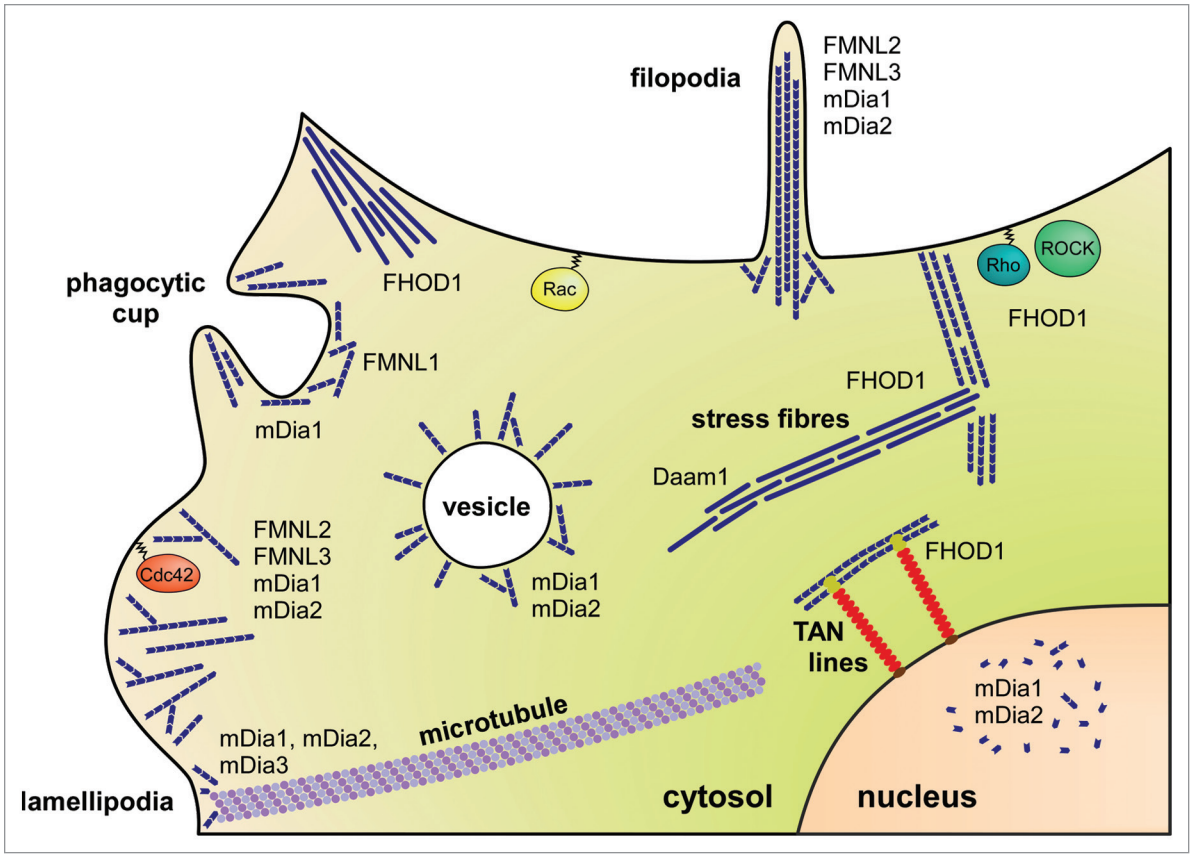

Figure 6. Model of Diaphanous-related formin function and localization in cells. Shown are formins involved in filopodia and lamellipodia generation, the stabilization of actin stress fibers, interactions with microtubuli cytoskeletal structures or transmembrane actin-associated (TAN) lines, and vesicle formation and trafficking. The activation by Rho family GTPases as known today is indicated. Of note, FHOD3 is highly expressed in cardiac and skeletal muscle tissue and not displayed in this model scheme.

Like Daam1, Daam2 seems to be involved in developmental processes regulated by Wnt-signaling. Two studies describe that Daam2 is important for asymmetric cell behavior. At first, loss-of-function studies revealed that Daam2 is required for dorsal progenitor identities and canonical Wnt signaling by its interaction with Dvl3, which modulates Wnt signal transduction during spinal cord development.[83] In addition, initiation of a leftward tilt in gut morphogenesis is likewise a critical aspect of asymmetric cell behavior that was found to be modulated by Daam2. Effectors of the transcription factor Pitx2 responsible for the transfer of left-right information from early gastrulation to morphogenesis were found to mediate Wnt signaling to activate Daam2.[84]

The activation mechanisms of the two Daam proteins remain not well understood on a molecular level. The Daam proteins are autoinhibited by a C-terminal DAD, similarly as found in Dia formins, but activation is achieved through the interaction of the DAD with the PDZ domain of Dishevelled, releasing the autoinhibited state.[26] As active Daam1 was reported to lead to RhoA activation, a positive feedback loop that amplifies the levels of active GTPase has been proposed.[26,75] For Daam1 it has been speculated that either a RhoGEF is recruited to active Daam1 to increase the pool of GTP-loaded RhoA or that a RhoGAP might be silenced such that less RhoA-GTP is hydrolyzed. Yet, another study described that Daam1 does neither regulate cytoskeletal organization through RhoA nor Rac1 or Cdc42.[80] Active Daam1 however is found in nonadhesive regions of cells bridging fibronectin-coated adhesive strips where it associated with actin networks containing myosin II and the cross-linker filamin A.[85]

FMNL

The "Formin-like" (FMNL) protein family represents the third family of mammalian DRFs and includes the members FMNL1 (alternative name FRL1), FMNL2 (also named FRL3), and FMNL3 (also named FRL2) with a total of eight splicing isoforms. The multidomain FMNL formins have been initially described as formin-related genes in leukocytes (FRL)[86] and share about 23% sequence identity with Dia1.[5] The functional roles of FMNL formins seem to be diverse and are only partly defined to date. The different members of the FMNL family appear to regulate similar processes during development based on overlapping expression patterns, but also seem to have independent functions based on distinct tissue expression.[87] The macrophage-enriched FMNL1 is involved in the regulation of cell adhesion, growth and migration through the reorganization

of the lamellipodial and filopodial actin cytoskeleton.[86] In T lymphocytes, this formin has been identified as essential regulator of centrosome polarity and exhibits crucial functions in the activation of cytotoxic T cells.[88] FMNL1 proteins display unique patterns circular around centrosomes and localize at the tip of filopodial structures that have been developed during the recognition of the antigen presenting cell.[88] It has been furthermore described that FMNL1 is recruited to the phagocytotic cup and involved in the Fcγ receptor-mediated phagocytosis.[37] FMNL1 also accumulates at the pseudopods of macrophages and regulates macrophage coiling phagocytosis since its depletion reduces the uptake of invading borrelia.[89] Recently published studies have shown that FMNL1 may be involved in the regulation of podosomes and the structural stabilization of the Golgi complex.[90,91] Activated FMNL1 might cause polarized non-apoptotic membrane blebbing independent of ROCK or Src activity.[92] Additionally, first hints from proteomic screens point to a potential involvement of FMNL1 in calcium-dependent membrane plasticity.[93]

The second member of the FMNL formin family, FMNL2, generates protruding actin structures at the leading edge of a migrating cell, the lamellipodium and filopodia.[94,95] FMNL3 in contrast regulates endothelial cell elongation during angiogenic morphogenesis by microtubule alignment[96] and seems to be involved in cell motility.[97] Both, FMNL2 and FMNL3 are predominantly associated with the plasma membrane and this localization depends on their N-terminal myristoylation (**Table 2**).[95]

The misregulation of FMNL formins has been implicated with severe diseases. FMNL1 is enriched in hematopoietic cells and tissues such as thymus, spleen and peripheral blood leukocytes and is overexpressed in malignant lymphomas of patients with chronic lymphocytic leukemia as well as in T cells from patients with malignant non-Hodgkin lymphoma.[98,99] The depletion of FMNL1 reduces cell proliferation as well as migration of leukemia cells and tumor growth.[100] The *fmnl2* gene is expressed in many tissues with the highest expression level found in cells of the nervous system, the gastrointestinal as well as the breast epithelium and the lymphoid tissue.[94] Overexpression of FMNL2 in cells of colorectal carcinoma causes a more aggressive tumor behavior associated with increased proliferation, motility, invasion, and metastasis.[101] In breast melanoma cells, FMNL2 is likewise involved in their invasive cell migration.[102] Furthermore, FMNL2 promotes the epithelial mesenchymal transition, which is associated with the loss of cell adhesions and enhanced migration ability.[103] Besides a link to cancer progression, FMNL2 could also be involved in diseases of the nervous system. A sporadic 3.9 Mb deletion in gene locus 2q23.3 of an infant caused severe mental retardation, early onset of puberty, reduced stature and hand anomalies.[104] This locus encompasses five genes including *fmnl2*. As a possible reason for these symptoms a morphological change of the dendritic spines based on disturbances of the actin cytoskeleton has been proposed.[104] Like the other two FMNL proteins, also FMNL3 seems to participate in the proliferation of malignant tumor cells.[105]

Actin filament polymerization and bundling activity of FMNL formins was reported by several groups, while FMNLs also might sever actin filaments.[25,95,106-108] It has been shown for FMNL1 that the dimeric FH2 domain associates with the barbed end of actin filaments, processively elongates them in the presence of profilin, reduces the elongation rate in the absence of profilin, and prevents binding of capping proteins.[106] An FH1-FH2 protein construct of FMNL3 induces filopodia formation and accumulates at their tips, while the corresponding construct of FMNL1 does not.[107] The region C-terminal to the FH2 domain accelerates the actin assembly activity of FMNL3 and this activity is mediated by an actin monomer and F-actin barbed end binding WASP homology 2 (WH2)-like sequence.[109] FMNL2 represents an actin filament elongation factor promoting cell migration rather than a nucleation factor.[95]

FMNL1, -2 and -3 are autoinhibited by interactions between the N- and C-termini[25,37,95] and also hetero-dimeric complex formation between N- and C-terminal domains of FMNL2 and FMNL3 appeared to be possible.[25] The specificity of Rho GTPases for individual FMNL formins is still under debate as contradictory studies were reported in the past (**Table 1**). A nucleotide-independent binding mode of the N-terminus of FMNL1 to Rac1 has been described,[86] as well as the nucleotide-dependent interaction with active GTPases Cdc42,[37,95] Rac1,[100] and Rho.[88,102,110] RhoC, but not RhoA, was shown to specifically interact with FMNL3, which promotes polarized migration through FMNL3 by restricting lamellipodial broadening.[110] In addition, Cdc42-induced recruitment of FMNL1 and FMNL2 to the plasma membrane has been demonstrated.[37,95] FMNL1 might interact with the iBAR domain-containing protein srGAP2 and co-localizes with it at the phagocytotic cup of macrophages.[111] srGAP2 is a Rac1-specific RhoGAP and might represent an inhibition mechanisms of formin activity.

FHOD

The mammalian FHOD family comprises two proteins, FHOD1 and FHOD3 (the name FHOD2 has been misleadingly assigned to a protein of a different formin family and is thus discontinued). Both FHOD1 and FHOD3 show considerably different expression profiles in cells. In a recent study, 22 different human cell and tissue types were analyzed by quantitative real-time PCR, showing on average highest expression levels for FHOD1 among all 15 formins.[112] In contrast, FHOD3 was lowest on average but with a very specific expression profile in cardiac and skeletal muscle, outbalancing here its sister homolog FHOD1.

Expression of active FHOD1 leads to a phenotype of F-actin stress fibers.[113] The protein contains an N-terminal F-actin side binding element and localizes to cellular stress-fiber structures.[114] Yet FHOD1 is thought to poorly elongate actin filaments but rather acts as an actin bundling factor with capping activity toward the filament barbed end.[115] FHOD1 thus stabilizes actin filaments by protecting barbed ends from depolymerization with

its dimeric FH2 domain, whereas the region N-terminal to the FH1 domain mediates F-actin bundling by binding to the sides of adjacent F-actin filaments. The protein moves with the actin retrograde flow and enriches in actin arcs and more mature stress fibers,[115] rather than staying at the leading edge and expanding cell migration as Dia and FMNL. FHOD1 stimulates the spatio-temporal organization of transversal arcs that are formed by fusion of short antiparallel actin filaments, which is critical for stress fiber maturation.[116] The GBD-FH3 domains of FHOD1 are responsible for stress fiber association and co-localization with Myosin.[116]

FHOD1 was recently described to be recruited to integrin clusters, which results in actin assembly.[117] Integrin binding to matrix ligands provides critical signals for cell growth or differentiation. Targeting of FHOD1 to the integrin sites depends on the direct interaction with Src family kinases and is upstream of the activation by Rho kinase. Functional studies showed that retention of the mitotic kinase Aurora-B at the cortex depends on FHOD1, which becomes phosphorylated by the kinase.[118] Modulation of FHOD1 activity by Aurora-B thereby contributes to daughter cell spreading after mitosis. FHOD1 also appeared to be markedly upregulated upon epithelial-to-mesenchymal transition in cancer cells contributing to cell migration and invasion.[119] FHOD1 in conjunction with Rac1 was furthermore described as novel regulators of vaccinia actin tail formation.[120] Vaccinia virus thus integrates the activity of the N-WASP-ARP2/3 and Rac1-FHOD1 pathways to display robust actin-based motility. FHOD1 and Arp2/3 were also shown to cooperate in *Salmonella* invasion where both factors occupy distinct microdomains at the invasion site and control distinct aspects of membrane protrusion formation.[121]

FHOD3 was first described to regulate sarcomere organization in cardiomyocytes where it localizes to thin actin filaments in a striated pattern.[122] Its depletion by siRNA resulted in a marked reduction in filamentous actin and disruption of the sarcomeric structure. A splice variant of FHOD3 specific for striated muscles promotes the polymerization of actin filaments in cardiomyocytes and downregulation of this isoform severely affects myofibril integrity.[123] This specific FHOD3 variant is phosphorylated by casein kinase 2 (CK2), which is required for proper targeting of muscle FHOD3 to the myofibrils in embryonic cardiomyocytes being in the mature state restricted to the Z-disc proper in the adult heart.[124] Knockout of *fhod3* in mice resulted in disturbed myofibril maturation and embryonic lethality due to problems in heart development.[125] Together, these studies demonstrate the different functions of FHOD1 and FHOD3 in cells, which is reflected by their different expression profiles.

Although FHOD is considered a DRF according to its domain architecture (**Fig. 1**), its interaction with GTPases and mechanism of activation remains still elusive. FHOD1 is autoinhibited by a C-terminal DAD[24] and truncation of the C-terminus leads to an active phenotype.[24,113] Structural studies showed that FHOD1 contains an N-terminal GTPase-binding domain composed of an ubiquitin superfold, yet a direct interaction of the GBD or the GBD-FH3 unit to Rac1 could not be confirmed.[28] Instead FHOD1 was shown to become phosphorylated at three specific sites within the C-terminal DAD by the Rho effector kinase ROCK.[38,126] This interaction places FHOD1 as a downstream effector of Rho, which is in line with the phenotype attributed to this GTPase and the observed function of active FHOD1 in stress fiber formation (**Fig. 6**). The ubiquitin superfold found in FHOD1 is known as GTPase-binding domain from Ras family effectors such as Raf, PI3 kinases or RalGDS.[28] A similar N-terminal domain structure is found as F0 domain in Kindlin and Talin,[127,128] moving the FHOD domain assembly close to integrin co-factors. The *Dictyostelium discoideum* protein Formin C (ForC) as the closest homolog to mammalian FHOD1 contains a similar N-terminal ubiquitin domain structure, whose positively charged surface area mediates localization to specific membrane patches.[129] Likewise, ForC binds to actin filaments and crosslinks them into loose bundles of mixed polarity.[130] The association of FHOD1 with the growing actin filament as bundling and capping factor however makes a stable interaction with a GTPase unlikely but fits well to the activation mechanism via phosphorylation, e.g., by ROCK. The possible interaction partners for recruitment and activation of FHOD1 and FHOD3 are not yet clear.

Conclusions

The analysis of the interaction between Rho GTPases and formin effector proteins is only at the beginning, as the specificity (or promiscuity) of these GTPases for effectors of the actin cytoskeleton is not yet well understood. For some formin families such as Dia and FMNL, all three major Rho GTPase subfamilies Rho, Cdc42, and Rac have been reported to interact with these effectors (**Table 1**). However, as different biochemical techniques were employed for the analyses of these interactions, some of these results are difficult to compare. It is supposed that GTPase activation by guanine nucleotide exchange factors occurs at lipid membranes. The Rho, Cdc42, and Rac subfamilies all contain C-terminal prenylation motifs as either farnesylation or geranylgeranylation that target these signaling proteins to membrane compartments. The targeting of formins to specific cellular membranes is therefore a major determinant of function. This mechanistic condition correlates well with the observed phenotypes of some DRFs, as e.g., for the generation of filopodia and lamellipodia at the leading edge of a cell (**Fig. 6**). The spatial positioning of these cytoskeletal membrane protrusions appears secured by the association and activation of the complex at membranes. Other formins such as FHOD instead are activated through phosphorylation, which only indirectly requires Rho as the upstream factor of ROCK kinase. Likewise the combined interaction of a Rho GTPase and a DAD binding factor as in Daam might be required for full activation of the formin. This diversity requires the individual characterization of each formin and the consideration of multiple co-factors. Future functional and structural studies are therefore required to shed light on the versatile aspects of the modulation

of the actin cytoskeleton by formins as downstream effectors of Rho GTPases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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