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Formin' cellular structures

Physiological roles of Diaphanous (Dia) in actin dynamics

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Members of the Diaphanous (Dia) protein family are key regulators of fundamental actin driven cellular processes, which are conserved from yeast to humans. Researchers have uncovered diverse physiological roles in cell morphology, cell motility, cell polarity, and cell division, which are involved in shaping cells into tissues and organs. The identification of numerous binding partners led to substantial progress in our understanding of the differential functions of Dia proteins. Genetic approaches and new microscopy techniques allow important new insights into their localization, activity, and molecular principles of regulation.

Introduction

Cells evolved different actin nucleators that catalyze the nucleation reaction, the rate-limiting step in actin polymerization. Diaphanous (Dia) was originally identified by its essential function in cytokinesis[1] and constitutes a major branch of the formin family, which together with Arp2/3 and WH2 proteins represents the majority of actin nucleators in cells.[2] The formin protein family is defined by the presence of the formin homology domain 2 (FH2)[3] and is further classified according to the presence and arrangement of additional domains.[4] Dia proteins contain a RBD/FH3 region in their N-terminal part, which consists of a Rho-binding domain (RBD), 4 Arm repeats (also called Dia-inhibitory domain, DID), a dimerization domain (DD), and a putative coil-coiled region (CC, **Figure 1**). Phylogenetic sequence analysis suggests that the emergence of Dia predates the evolution of animals and thereby multicellularity.

Revisiting the evolutionary history of Dia

Analyses of the evolution of Dia have mostly been embedded in larger studies considering the complete formin family and thus focused on their common denominator, the FH2 domain. The position of the Dia subfamily varies in previous publications. In the initial phylogenetic classification by Higgs and Peterson, 7 metazoan groups were defined, which were separate from the formins in fungi, plants, and protists. Based on available non-FH2 sequences, Dia, DAAM, and FMNL subfamilies were proposed to be grouped together.[3,5] The position of non-yeast fungi was not resolved, and the yeast formins were the outgroup of all taxonomic groups including plants. Rivero et al. resolved the formin subfamilies but did not find sufficient bootstrap support for defining the relationship among them.[6] Out of the fungi genomes, only the yeast formins were included, which built a clade on their own. As a first evidence for a relationship among the subfamilies, Chalkia et al. grouped the FMNL and the DAAM subfamily together, when considering animals and choanoflagelates.[7] By focusing only on metazoan species, the clade consisting of FMNL and DAAM was further corroborated, with Dia and the inverted formins as sister clade. A first hint of a possible older evolutionary origin of Dia was shown by Grunt et al., who identified a protein from *Monosiga brevicollis*, which grouped together with metazoan Dia.[8] As in other studies, non-yeast and yeast fungal sequences formed their own clade. Interestingly, a phylogenetic tree based on the domains beside the FH2 supported a clade with DAAM and FMNL proteins.[6]

To revisit the relationship of the formin subfamilies and to define the position of the Dia subfamily (**Fig. 2A**), we combined the sequences of the N-terminal RBD/FH3 region common to the Dia subfamily (RBD, 4xArm/DID, DD, CC, **Figure 1**) and the FH2 domain to increase the phylogenetic resolution. Our analysis was therefore restricted to the Dia, DAAM, FMNL, and inverted formin subfamilies, as they are the only members containing a RBD/FH3 region. We extracted genes with this domain architecture from selected fungal and holozoan genomes. In the following, we merged the domains as identified by SMART (the DID/DD region is covered by the Hidden Markov Models (HMMs) for Drf_GBD and Drf_FH3; a single HMM exists for the FH2 domain).[9] Then, the sequences were aligned using MUSCLE[10] and the phylogenetic tree was reconstructed using PHYML after identification of the best model with PROTTEST.[11] In contrast to previous studies, we found a well-supported clade containing the metazoan inverted formins and fungal sequences. Thus, we identified possible fungal orthologs of the inverted formins. With a supported origin predating the divergence of fungi and Holozoa, we used this clade as outgroup. As in previous studies, Dia, DAAM, and FMNL were reconstructed as well-supported, monophyletic groups. Additionally, we were able to obtain support for the relationship between these subfamilies. Here, Dia is the oldest of the

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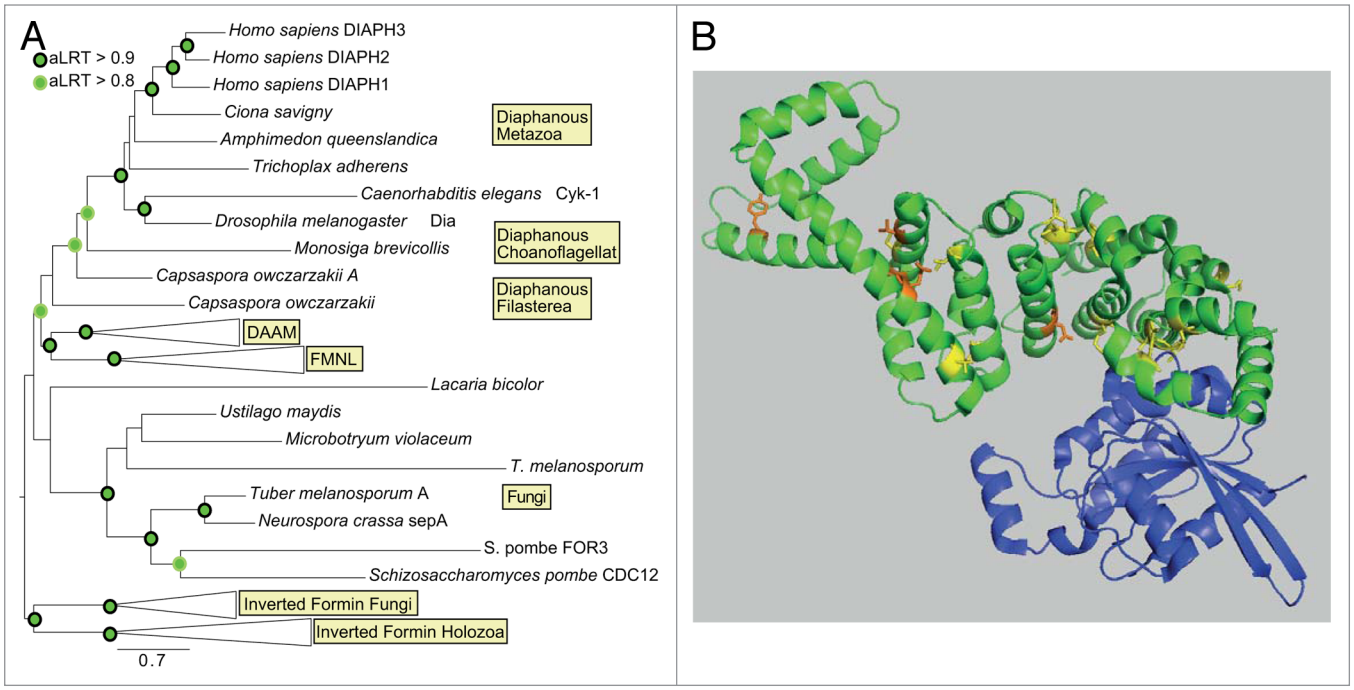

Figure 2. The evolutionary origin of *diaphanous*. (**A**) Phylogenetic tree based on the RBD/FH3 region and the FH2 domain. Green circles denote an approximate Likelihood Ratio (aLRT) support of > 0.8, a black circle indicates aLRT > 0.9. For accession numbers of Dia sequences see Supplemental Materials. (**B**) Differentially conserved sites between vertebrate Dia paralogs. Positions with a *z*score > 5 were mapped onto the structure of mDia1 (pdb: 3EG5; mDia1 in green, interacting Rho GTPase in blue). Sites conserved between Dia2 and Dia3 in yellow, differing sites in orange.

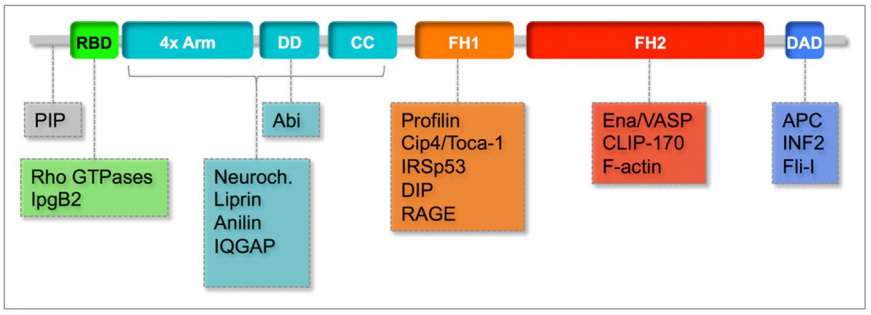

Figure 1. Domain structure and interactors of Dia. Dia consists of the formin homology domains 1 and 2 (FH1, FH2), Rho-binding domain (RBD), 4 Armadillo repeats (4x Arm, also called Dia inhibitory domain, DID), dimerization domain (DD), a predicted coiled-coil region (CC) and a dia autoregulatory domain (DAD). 4x Arm, DD, and CC are also referred to as FH3. Structure is shown for mDia1 (uniprot O08808,[41]). Dia interacting proteins are associated with the domains and regions they bind to.

3 subfamilies, and DAAM and FMNL evolved by a subsequent gene duplication. Within the Dia clade, the incorrect but well-supported placement of the arthropod and nematode sequences might be attributed to long-branch attraction. Unexpectedly, also sequences from the chonaoflagellat *Monosiga brevicollis* and the filasterean *Capsaspora owczarzakii* were placed with high support within the Dia clade. Thus, the emergence of Dia predates the evolution of animals and thereby multicellularity. Contrasting previous analyses, we retrieved a single fungal clade, covering yeast and non-yeast proteins including *Schizosaccaromyces pombe* FOR3 and CDC12 with sufficient support. Although the position of this clade as outgroup to the holozoan Dia, DAAM, and FMNL is not reliably supported, it might indicate that these fungal genes comprise the orthologs of the holozoan families. In this scenario, the last common ancestor of ophistikonts contained a single gene, which evolved by holozoan specific duplications to Dia, FMNL, and DAAM.

At the base of the vertebrates, further duplications gave rise to Dia1, 2, and 3. Usually, a gene retained after a gene duplication undergoes either neofunctionalization (i.e., one of the genes evolves a new function) or subfunctionalization (i.e., each gene retains a subset of the original function).[12] In the case of Dia, one of the described functional differences is their affinity to small GTPases of the Rho family. mDia1 has a small number of interactors, whereas mDia2 and 3 are more promiscuous. Structural analyses revealed that this functional difference can be attributed to a small motif consisting of 'NNN' in mDia1, which is substituted to 'TSH' in mDia2 and 3.[13] These positions are conserved throughout the vertebrate Dia1 and Dia2/3, respectively. This pattern is a showcase of class II functional divergence, where a position is conserved in both subfamilies, but harbors subfamily specific amino acids.[14] As the existence of such sites might enable to home in on specificity determining sites, we aligned the mDia paralogues of different vertebrates und performed an SDPfox analysis[15] (**Table S1**). Indeed, the

Table 1. Dia binding partners and their proposed functions

binding partner	binding domain	physiological function	biochemical function	references
Rho1/RhoA	RBD	activation of nucleation and elongation	release of autoinhibition	13,16,19,41,63,110
IpgB2	RBD			158
PIP2, phospholipids	N-basic	apical/membrane localizaton		47-49,158,161
Profilin	FH1		required for elongation activity	17-19
Cip4/Toca/FBP17	FH1	antagonizes Dia mediated membrane stabilization and cytokinesis	inhibition of nucleation and elongation	22
IRSp53	FH1	filapodia induction		24,81,162
DIP/Wish	FH1	blebbing in amoeboid migratory cells	mDia2 dependent filament assembly and bundling	23,25,163-165
RAGE	FH1	cell migration		28,166
Ena/Vasp	FH2	lamellipodia, SRF activity		34,36,37
Clip-170	FH2	phagocytosis		35
Neurochondrin	FH3	unknown		45
Abi	DD	junctions in MDCK cells		44
Liprin	DID-DD	antagonises membrane localizaton		42
Anillin	DID	cytokinesis, localization of mDia2		43
IQGAP	DID	phagocytosis, caveolae membrane insertion		46,167
APC	DAD		synergistic actin polymerization	51-53,64
INF2	DAD	lamellipodia in podocyte	actin polymerization and SRF activation	56,168
Fli-I	DAD		promotes release of Dia autoinhition	54
Hck		neutrophil chemotaxis		148
HDAC6		MT deacetylation		169
PKD2	N-terminal	spindle localization of PKD2		73
Exportin 6		nuclear export		170
Importin-a	N-terminal	nuclear import		171
Crm1	C-terminal	nuclear export		171

already known motif was significantly identified (i.e., *z*-score < 5). Additionally, further positions, mostly in the N-terminus of Dia, were predicted. To estimate the relevance of these sites, we mapped them onto the structure of mDia1 (pdb:3EG5) (**Fig. 2B**).[13] Indeed, further sites in addition to the NNN–TSH motif were predicted in proximity to the bound GTPase. This includes positions 210 and 262, which belong to the DAD interaction interface.[16] Further sites were identified when comparing mDia2 and mDia3. This includes position 422, which harbors a tyrosine in mDia1 and 2 and a phenylalanine in mDia3. This position is part of the dimer forming area[16] and might therefore be involved in the homodimerization of Dia proteins. The additional sites might be a good starting point for further analyses of functional differences between the Dia subfamilies. Interestingly, when comparing Dia2 and Dia3, 1 of the 2 kept the amino acid present in mDia1, whereas a substitution happened in the other (**Table S1**). This might indicate that both have retained parts of the functionality of Dia1.

Dia a multimodular actin regulator—a matter of the binding partner

Members of the Dia protein family are multimodular proteins that interact with numerous actin regulators, adapters, and signaling components (**Table 1**, **Figure 1**). Beside the formin homology domains FH1 and FH2, the presence of additional domains, i.e., RBD/FH3 and the C-terminal Dia-autoregulatory domain (DAD), is the characterizing feature of the Dia subfamily. The FH1 domain is proline rich and binds to a range of proteins. Most importantly, the FH1 domain binds Profilin,[17-19] which is required for the elongation activity of Dia, albeit not for nucleation.[20,21] Among the interactors of the FH1 domain are proteins with SH3 domains, such as the F-BAR protein Cip4/Toca-1, the I-BAR protein IRSp53, and the Dia interacting protein DIP/Wish.[22-24] The F-BAR protein Cip4 contains an SH3 domain at its C-terminal end, which is necessary for colocalization of Cip4 and Dia in *Drosophila* S2 cells.[22] This interaction is also involved in controlling Dia-catalyzed actin polymerization. Cip4/Toca-1 protein inhibits the Profilin-independent nucleation as well as the Profilin-dependent elongation. Single molecule analysis by TIRF microscopy revealed that about 90% of elongating filaments were suppressed by Cip4. However, about one-tenth of the filaments showed increased elongation speed, suggesting that these filaments escaped inhibition.[22] The inhibition of nucleation and elongation by Cip4 seems to be mediated by the interaction of SH3 and FH1 domains, since the isolated SH3 domain was also inhibitory.[22] The SH3 domain containing DIP inhibits actin polymerization in vitro,[25] and interestingly also mDia2 dependent actin bundling.[25-27] The inhibitory mechanism and the role of the SH3-FH2 interaction has remained unclear. The function of a third SH3 containing protein, IRSp53, on actin polymerization in vitro has not been reported yet. Given that the SH3 domain of Cip4 on its own showed inhibitory activity,[22] it is likely that IRSp53 inhibits filament nucleation and elongation in a similar manner.

The FH1 domain has also been reported to bind to the intracellular part of the RAGE receptor protein (receptor for advanced glycation end products) and by this may mediate RAGE-dependent Rac1 and Cdc42 activation and cell migration.[28] The molecular mechanism of Dia mediated RAGE signal transduction and its potential role on actin polymerization is unknown, however.

The FH2 domain, which forms a dimeric torus, is sufficient for actin nucleation. It can bind G-actin and the barbed end of filaments.[29-31] As actin dimer and trimer formation is the kinetic barrier of actin polymerization, nucleation may be triggered by binding of actin mono-, di-, or trimers to the FH2 torus through weak actin binding sites.[29] In addition to nucleation, Dia also elongates existing actin filaments, which depends on the G-actin binding protein Profilin.[20,32] The FH2 torus remains bound to the barbed end of F-actin and may rotate during elongation.[33] For elongation, one of the subunits of the dimeric torus loses its link to the barbed end, accepts a new actin monomer from a Profilin-actin complex, and adds it to the barbed end of the filament. In the next step, the other subunit of the FH2 torus dissociates from the barbed end and incorporates a new monomer. This stepwise mechanism is referred to as processive capping. A second, indirect mechanism contributes to elongation in vivo. The FH2 torus prevents binding of capping proteins to the barbed end, which counteracts elongation.

Ena/Vasp and Clip-170 have been described to physically interact with the FH2 domain of Dia.[34-37] Although it has not been addressed, if and how binding of Ena/Vasp or Clip-170 interferes with nucleation and elongation and barbed end binding, it is attractive to speculate that binding to the FH2 domain may block sites important for the catalytic mechanism and suppress one or more of these activities. Future in vitro experiments, especially single molecule TIRF assays, will reveal such potential activities.

The RBD/FH3 domain is not a single entity in structural terms but includes a dimerization domain (DD), a coiled-coil domain (CC), and a domain with 4x Arm repeats.[38-40] On the N-terminal side is the RBD that interacts with activated RhoGTPases (**Fig. 1**). As the autoregulatory domain at the C-terminal part (DAD) interacts with the 4x Arm domain and part of the RBD and thus prevents binding of activated RhoGTPases, this region is often refered to as Dia-inhibitory domain (DID).[13,16,38,41] A number of structurally diverse proteins (Neurochondrin, Abi, Liprin, Anilin, IQGAP) bind to the FH3 domain.[42-46] For none of these binding proteins a direct effect on actin polymerization has been reported yet. However, some of the FH3 interactors, Liprin, Anilin, and IQGAP, for example, seem to be involved in subcellular localization of Dia.[42,43,46]

In addition to these FH3 interactors, basic regions at the N-terminus or C-terminus of Dia are involved in controlling membrane association and binding of phosphoinositols (PIP). The N-terminal part contains a stretch of basic residues that is important for membrane association of Dia.[47-49] In polarized *Drosophila* tracheal cells and Madin-Darby canine kidney (MDCK) cells, restriction of Dia to the apical domain is controlled by a combination of these mechanisms. On the one side, the RBD domain binds to apically activated and membrane attached Rho; on the other side, the basic N-terminal region of Dia binds to PI(4,5)P2, which is enriched in the apical membrane.[47] The combination of these mechanisms not only mediates apical restriction but also controls activation of actin polymerization. Besides the release of DID-DAD autoinhibition by Rho-GTP binding, the interaction with PI(4,5)P2 may also contribute to activation. Such a dual mechanism is consistent with the previous report that addition of liposomes affects actin polymerization in a PIP2 concentration dependent manner.[49]

The DAD domain at the C-terminus tightly binds to the N-terminal DID domain to form a dormant Dia dimer.[50] Only after opening of the intramolecular loop by binding of Rho GTPases to RBD, the FH2 torus becomes accessible to actin monomers and filaments, which allows nucleation and elongation. Similar to the disruption of the DID-DAD interaction by RhoGTPases, other proteins binding to the DAD domain may also release autoinhibition. APC (adenomatous polyposis coli) synergistically activates actin polymerization by binding to the DAD domain.[51-53] Interestingly, APC and Dia together nucleate filaments but become separated upon elongation of the filament as demonstrated by single molecule TIRF microscopy. While Dia sits at the growing barbed end, APC remains associated with the pointed end.[52] Flightless-1 (Fli-1), a member of the gelsolin family, is another example of an activating DAD interactor, which acts synergistically to Rho. As Rho-GTP only partially relieves autoinhibition, addition of Fli-1 to such an in vitro assay further activates Dia.[54] Similar to proteins binding to the RBD or DAD, a synergistic activation may be achieved by phosphorylation of the serine or threonine residues within the DAD domain. Phosphorylation of mDia2 at 2 conserved residues (T1061 and S1070) by Rho dependent kinase (Rok) increases activation.[55] DID and DAD domains of different members of the formin family may interact. Recent studies further showed that the DID domain of INF2 (inverted formin 2) binds to the DAD domain of mDia, allowing cross-regulation among formins. Such an interaction may be functionally relevant, as binding of INF2's DID domain reduces the rate of actin polymerization by mDia1 in vitro.[56]

Physiological functions of Dia in single cells and in multicellular context

Essential role of Dia in cell cycle and cytokinesis

Dia was initially identified in screens for genes required for spermatogenesis in male flies.[1,57,58] Counterparts of Dia in yeast, Bni1, Cdc12, and *C. elegans*, Cyk-1, were found because of their essential function in cytokinesis,[18,59-63] demonstrating the conservation of Dia's function in cytokinesis.[22,43,64-69] Dia is involved in setting up, positioning, and constriction of the contractile ring (**Fig. 3**). Loss of Dia function leads to failure of cytokinesis and results in multinuclear cells. In addition to cytokinesis, Dia is involved in diverse aspects of mitosis. Dia is required for proper segregation of centrosomes shortly before mitosis in *Drosophila* blastoderm embryos.[70] Incomplete centrosome separation probably reflects an indirect function of Dia, as no obvious centrosomal localization of Dia has been reported yet. Dia has been implicated in the attachment of metaphase chromosomes to kinetochores, their alignment, as well as the orientation of the mitotic spindle and localization of spindle proteins.[71-75] Dia may also be involved in linking cell growth with cell cycle progression at the G1/S transition.[76,77] In the

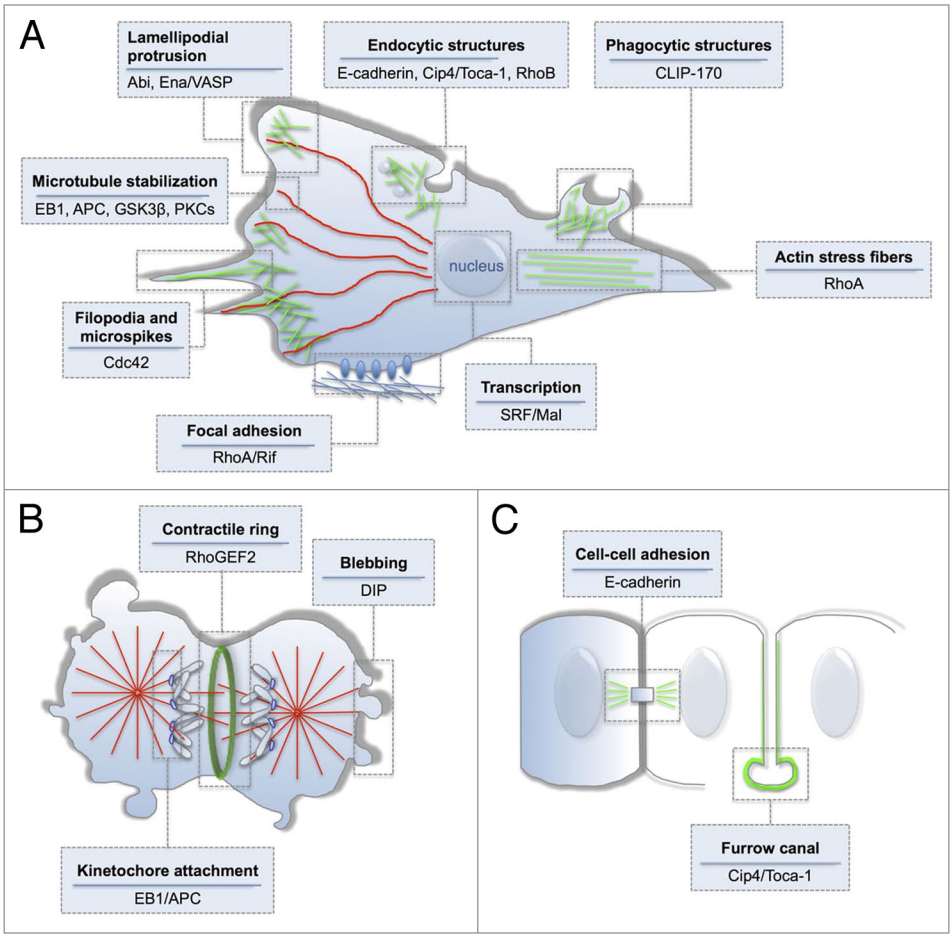

Figure 3. Dia dependent cellular structures and functions. Members of Diaphanous (Dia) protein family play important roles in (**A**) migrating, (**B**) in dividing, and (**C**) in the epithelial cells. Dia proteins mainly act on the assembly of actin filaments (marked in green) but also contribute to the regulation of microtubules dynamics (marked in red). Diverse cellular structures and functions are differentially regulated by a combinatorial use of numerous Dia binding partners (depicted in the boxes).

early *Drosophila* embryo, Dia is required for centrosome induced formation of pole cells, the germ cell precursors at the posterior pole of the embryo.[69]

The role of Dia in regulating membrane protrusions and cell migration

Actin polymerization provides the mechanical force to drive membrane protrusions such as lamellipodia and filopodia. Formins, like Dia, nucleate linear actin filaments in filopodial protrusions (**Fig. 3**), whereas the Arp2/3 complex is thought to be the key nucleator generating a branched actin network during lamellipodia formation. Recent studies indicate that both protrusive structures require an intricate crosstalk between central actin nucleators. In the absence of the Arp2/3 complex or its major activator, the WAVE regulatory complex (WRC), mDia2-dependent filopodia are induced.[78] By contrast, loss of mDia2 not only inhibits filopodia formation but also severely affects lamellipodia structure.[79] All active mDia proteins (mDia1–3) are able to induce filopodia, although the underlying pathways seem to be different.[80-82] An essential role of Dia in regulating lamellipodial-filopodial balance has been observed during *Drosophila* dorsal closure.[83] Like mDia proteins, active *Drosophila* Dia localizes to filopodial tips and increases filopodial lifetime. In addition to such a direct function, Dia can also promote lamellipodia protrusions by recruiting Ena/VASP to the leading edge.[83] Ena/VASP directly binds a central WRC subunit, Abi, the interactor of Abelson kinase (Abl),[84] suggesting that filopodial assembly might originate from the lamellipodial network through the convergent-elongation mechanism as proposed previously.[85] Supporting this notion, loss of function studies in different systems revealed conserved roles of Dia proteins in cell spreading and in cell migration including fibroblasts, epithelial, neuronal, and blood cells.[79,83,86-88]

The role of Dia in regulating endocytosis and endosome dynamics

Actin polymerization reshapes the plasma membrane but has also been proposed to drive membrane invaginations and propel endocytic vesicles during a variety of morphogenetic events. In the *Drosophila* embryo, Dia and non-muscle Myosin II (Myo II) control the initiation of E-cadherin endocytosis by regulating the recruitment of clathrin and the Adaptor Protein 2 (AP2).[89] Thus, the function of Dia-dependent linear F-actin appears to be different from Arp2/3 induced branched actin filaments that promotes endocytosis. Unlike Dia, Arp2/3 mediated branched actin filament nucleation by WASP/WAVE proteins facilitates

Dynamin-dependent vesicular scission.[90,91] Recent live-imaging analyses of *dia* mutant embryos revealed an increased endocytic activity and thus suggests an inhibitory function of Dia on tubular membrane invaginations. This inhibitory role of Dia is based on an antagonistic interaction with the F-BAR protein Cip4, a known activator of the WASP/WAVE-Arp2/3 pathway.[22,90-92] Since Cip4 inhibits actin nucleation by Dia in vitro, a model has been proposed in which Cip4 controls 2 different pools of actin filaments: activation of branched filaments by its WASP/WAVE interaction and suppression of linear filaments by inhibition of Dia.[22] The strong co-localization of Dia and Cip4 at newly formed endocytic vesicles further suggests an additional role of Dia in vesicle trafficking.[22] In mammals, a conserved function of Dia proteins in regulating endosome movement has been found. mDia1 and mDia2 are recruited to endosomes by activated RhoB.[93-95] Endosomal F-actin induced by Dia associates with cortical actin stress fibers, which might control further transport of endosomes to microtubules.[95] A similar function of mDia2 and Abl has recently been postulated for the formation of stress-fiber-linked caveolae.[96]

In addition to trafficking, Dia regulates the morphology of the Golgi apparatus.[97] Activated Dia leads to dispersion of Golgi membrane stacks, which is based on repressed fusion of small Golgi stacks into larger compartments as well as increased formation of Rab6 positive transport vesicles. As Dia colocalized with the Rab6 vesicles, at least partially, their number may be directly controlled by Dia.[97] Mitochondria are also affected by Dia. Activation of Dia leads to increased anchoring to actin filaments and loss of mobility of mitochondria, whereas RNAi mediated depletion of Dia elevated mobility.[98]

The role of Dia proteins on microtubule dynamics

Over the last decade, growing evidence has emerged that Dia not only acts on actin polymerization but also contributes to the regulation of microtubule dynamics.[99] The first hints came from overexpression experiments with constitutively active mutant proteins lacking their autoregulatory domains. Expression of active mDia1 induces bipolar elongation of HeLa cells, in which microtubules are aligned in parallel with F-actin stress fibers.[100] At the same time, Gregg Gunderson's group observed that expression of active mDia2 is sufficient to generate and orient stable, detyrosinated microtubules.[101] Subsequent work of the same group identified mDia2 in a complex with EB1 and APC, 2 important microtubule plus-end tracking proteins, suggesting a role for mDia2 in microtubule capping.[102] The molecular complexity of mDia induced microtubule stabilization was further increased by the observation that mDia1 regulates the glycogen synthetase kinase-3β (GSK3β) through novel PKCs to promote microtubule stabilization.[103]

The molecular mechanism of microtubule stabilization by mDia proteins remained completely unclear for a long time. The first experimental evidence against an unspecific effect of mDia induced actin assembly on microtubule dynamics came from the finding that the microtubule stabilization activity can be separated from its actin nucleation activity.[104] Actin nucleation-defective FH2 fragments of mDia1, mDia2, and mDia3 retain their ability to bind to microtubules, EB1, and APC, and more importantly, they are still capable to induce microtubule stabilization upon overexpression in serum-starved NIH3T3 cells.[72,104] Thus, the FH2 domain seems to function in both actin nucleation and microtubule stabilization. Interestingly, re-expression of actin-nucleation-deficient mDia3 in mDia3 knockdown cells fully rescue the chromosome misalignment phenotype, suggesting that mDia3 directly acts on microtubules at the kinetochore.[72,75] Since the activity of mDia2 for actin is in the nanomolar range, whereas that for microtubules is in the micromolar range, a competition model has been proposed, in which a redistribution of the mDia proteins to microtubules can only occur, if the affinity for microtubules is significantly increased.[104] Supporting this idea, recent work from the Gundersen group identified an actin capping protein that promotes microtubule stabilization by antagonizing mDia1.[105] Comparative in vitro studies with different recombinant formins further revealed a reciprocal inhibition between actin and microtubule dynamics.[106] Microtubules strongly inhibit actin polymerization by mDia2, whereas actin monomers inhibit in turn microtubule binding/bundling by inverted formins such as INF2.[106] Based on the observation that stable microtubules preferentially form at the leading edge of migrating cells,[107] the current model proposes a sequential action of mDia proteins on actin filaments and microtubules. mDia proteins are released from actin filaments by competition through capping proteins to promote microtubule stabilization required for cell polarization. Despite increasing in vitro evidence, a physiological role of the microtubule stabilizing activity of Dia proteins has not yet been found in vivo. In *Drosophila*, Dia directly binds both fly APC's (APC1 and APC2), but unlike in vertebrates, the APC-Dia complexes seem to affect actin directly rather than through an EB1-dependent effect on microtubules.[51,64] However, known formin-dependent fundamental processes such as axonal outgrowth and growth cone motility, meiosis in mammalian oocytes or cytoplasmic streaming of *Drosophila* oocytes require a tightly regulated, spatiotemporal coordination between actin and microtubule dynamics. Thus, future studies will be required to further decipher the microtubule stabilizing activity of Dia proteins in the context of a living animal.

Roles of Dia in shaping tissues during development

Dia is required for embryonic development in multiple species. In zebrafish, Dia controls cell movements and convergent extension during gastrulation possibly as an effector of the *wnt* signaling pathway.[108,109] In embryos depleted of Dia2, cells of the deep marginal layer, prechordal plate, and lateral epidermis lose protrusions and blebs. In *Drosophila* embryogenesis, formation of the first epithelial cell layer is controlled by Dia.[69,110] During cellularization of *dia* mutant embryos, the array of invaginating furrows is disrupted and incomplete, and adjacent nuclei are frequently incorporated in the same cell. As the speed of invagination of formed furrows is not affected, Dia seems to stabilize newly formed furrows. In addition, the typical epithelial compartmentalization is impaired in *dia* mutants, as lateral and basal domains are not separated.[22] In this process, Dia is controlled by RhoGEF2-Rho1 signaling and acts together with the non-receptor tyrosine kinase Abl and Ena (Enabled) in controlling actin filament formation in the apical microvillous structure of the plasma membrane.[110,111] Later in embryonic development, Dia is involved in tissue morphogenesis. Invagination of the mesoderm, which relies on a stereotypic

series of cell shape changes starting with apical constriction, is impaired in *dia* mutants, as the level of apical non-muscle myosin is reduced and the maturation of adherens junctions is disturbed.[83] Specifically, Dia assembles F-actin filaments that suppress E-Cadherin localization in the medial region and connect contractile medial actomyosin filaments with adherens junctions.[112] In dynamic junctions during cell intercalation, Dia promotes the turnover of E-Cadherin and thus contributes to establishing the anisotropy of actomyosin activity needed for directional intercalation. It was proposed that Dia controls formation of stable cortical actin patches, which lead to lateral clustering of E-Cadherin.[89,113] Segmentation of *Drosophila* embryos becomes visible by segmental grooves, which are formed by infoldings of the epidermis. In this process, Dia may be involved in stabilization of adherens junctions but less so by triggering apical actomyosin contraction.[114] Beside embryonic tissues, imaginal discs of *Drosophila* are an excellent system to analyze tissue remodelling and junction dynamics. It has been reported that Dia sustains apical tension during differentiation in the pupal eye disc.[115]

The function of Dia in formation, positioning, and maturation of cellular junctions has also been studied in cultured cells. Dia localizes to adherens junctions and is needed for maintenance and strengthening of adherens junctions downstream of Rho1 and counteracting Rho kinase.[116-118] The requirement of Dia for adherens junctions may change during tumorigenesis. While Dia is involved in the assembly of tangential junctions in non-transformed cells, radial junctions are not Dia-dependent in transformed cells.[116] Dia function may be coordinated by Arp2/3 through its direct interaction with Abi, a well-characterized WAVE/Arp2/3 activator.[44] Studies of mice lacking mDia1 and mDia3 confirmed these results from cultured cells, showing that neuroepithelial cells have an attenuated apical actin belt and lost adherens junctions.[119]

Recent studies further highlighted the importance of apical targeting of Dia activity as a conserved feature of all epithelial cells forming different tubular organs in 3D cyst of cultured MDCK cells and in *Drosophila* trachea, salivary glands, hindgut, and Malpighian tubules.[47,120] Remarkably, in the absence of apical actin polymerization, the apical-basal polarity of these tubular epithelial cells is not affected but secretion via the apical surface to the tube lumen is blocked.[120] Apical secretion requires Myosin V (Myo V) motor protein, which transports secretory vesicles along polarized actin filaments nucleated by Dia.[120]

In addition to polarization, Dia is involved in the formation and the maturation of cell-cell and cell-matrix junctions. *Drosophila* neuromuscular junctions grow in a Dia-dependent manner after initial contact formation. Dia acts on the presynaptic side and is regulated by the receptor tyrosine phosphatase Dlar and the guanine nucleotide exchange factor Trio.[121] Dia is also involved in the interaction and stimulation of dendritic cells by T cells as shown with bone-marrow derived dendritic cells from mDia1 deficient mice.[122] Furthermore, Dia functions in focal adhesions and cell-matrix interactions. This function of Dia may be developmentally regulated and depends on the specific composition of FAs. During oogenesis in *Drosophila*, the follicle epithelium switches from a columnar to squamous morphology. This morphology change is associated with a switch in integrin subtypes. Analysis of mutant clones showed that integrin downregulated Dia and Profilin levels.[123] Dia may be involved in maturation of focal adhesions as a mechano-sensor. The increased F-actin assembly and FA growth in focal adhesions induced by external force depends on mDia1.[124] In addition, Dia may control assembly of the stress fibers that are linked to FA involving signaling by Rho or Rif GTPases.[125-128]

Dia-linking cytoskeleton with mechanics and transcription

Cells are able to respond to mechanical stimuli, such as stretching during cell migration by changes in transcription. The serum response factor (SRF) and its cofactor Mal constitute a pathway that mediates such a response and that is dependent on the pool of G-actin. SRF/Mal can be triggered by cytoplasmic and nuclear Dia in cultured cells.[30,37,129-132] In response to forces, mDia1 promotes α-SMA (α smooth muscle actin) promoter activity as a result of the release of MRTF-A, a transcriptional co-activator of SMA from actin monomers. Conversely, force-induced α-SMA expression is blocked in mDia1 knockdown cells.[133] Thus, differentiation of myofibroblasts depends on mDia1.[133] The SRF/Mal pathway has been also investigated in its physiological context in collective migration of border cells in *Drosophila* oogenesis. Here, SRF/Mal provides a feedback mechanism for re-enforcing cytoskeletal strength. SRF/Mal signaling is essential for border cell migration, as *mal* mutant cells fail to migrate. Furthermore, accumulation of Mal in border cell nuclei is triggered by cell streching and activated Dia.[134]

Dia can sense and respond to mechanical stimuli.[135] This may rely on indirect signaling mechanisms or availability of globular actin.[136] In addition, the mechanism of progressive capping by the FH2 dimer appears to be inherently sensitive to forces in a piconewton range.[32,137,138] This prediction has recently been tested experimentally with microfluidic devices, in which calibrated piconewton forces can be applied to actin filaments. The measurements showed that the elongation rate of polymerization by Dia increased by up to a factor of 2 when filaments were pulled.[139] Such a force dependent activity may be a common feature of formins, as yeast Bni1 shows an elevated elongation rate after force application on filaments.[140] Controlled mechanical deformations of the cell cortex induced processive F-actin assembly by Dia in a manner independent of Rho or Ca^{2+} ions but dependent on LIM kinase. In this system, the initial event that senses the mechanical changes may be an increased amount of globular actin monomers, which may in turn increase polymerization rate by Dia.[136]

Conversely to sensing, Dia may also directly control the mechanical properties of cells. The stiffness of cells is controlled by the actin cortex beneath the plasma membrane. Expression of activated Dia leads to an increased stiffness of cells[141] and expression of Dia-interacting protein (DIP) induced mDia2 dependent blebbing of the plasma membrane,[25] indicating a role of Dia in assembly of the actin cortex.

Functions in mammalian physiology

Most studies on Dia functions were focused on molecular and cellular aspects. Based on findings from human genetics and analyses in different genetic model organisms, unexpected specific functions in complex physiological processes have been identified. Very early on in the study of Dia, a link of Dia to deafness in humans was established. DFNA1, characterized by a fully

penetrant sensorineural hearing loss and malfunction of hair cells in the inner ear, is linked to a mutation in Dia that leads to a small C-terminal truncation.[142,143] A corresponding auditory dysfunction was observed in *Drosophila dia* mutants,[144] indicating a conserved function. Conversely, hyperactivation of Dia/Dia3 in the auditory system also impairs hearing in *Drosophila* and mice and leads to auditory neuropathy (AUNA1) in humans.[145,146] Another insight from human genetics was provided by the mapping of premature ovarian failure (POF) to the Dia3 gene.[147] Although the consequences of the mutation in humans have not been reported yet, similarities to the function of Dia in the male and female germline of *Drosophila*[1,69] may be expected.

In cells of the hematopoetic system, a number of specific functions of Dia have been revealed. Dia mediates adhesive and migratory behavior of dendritic cells and their interaction with T cells in the lymphnodes.[122] Similarly, T cells and neutrophils deficient for mDia1 showed a poor adherence to the extracellular matrix and chemotactic migration behavior.[25,148-150] These observations point to a role of Dia activation in T cell response.[151]

Dia is also important in the erythrocyte lineage. A unique feature of erythropoesis is the loss of the nucleus during maturation of erythroblasts. In a type of asymmetric cell division, the pyknotic nucleus moves to one "daughter" cell separated from the main, anuclear cell body by a contractile actin ring. Closure of the actin ring leads to extrusion of the nucleus. Dia2 and Rac GTPases control the formation of the contractile actin ring, while Dia depletion blocked enucleation.[152] In platelets, Dia participates in thrombin-induced reorganization of the actin cytoskeleton.[153,154] In macrophages, Dia is involved in phagocytosis. Dia together with formin-like protein 1 (FMNL1) controls the formation of Borrelia-induced pseudopods that capture and enwrap the pathogene,[155] possibly by a mechanism that requires IQGAP and CLIP170 functions.[39,46,156] A requirement of Dia has been shown during pathogen infection, such as Shigella. The virulence protein IpgB2 binds the Rho binding domain of Dia and is involved in protrusion formation and spread of bacteria within the host cell.[157,158]

Finally, a surprising link of Dia to cortisol hormones was identified in adrenocortical cells.[159,160] In response to adrenocorticotropin (ACTH) steroids are synthesized with the reaction pathway partly located in the endoplasmic reticulum and mitochondria. As interference with Dia1 lead to decreased mitochondria mobility and decreased cortisol but increased adrenal androgene synthesis, Dia1 seems to be involved in interorganelle substrate transfer by dynamic trafficking of mitochondria.

Conclusions and Perspectives

In summary, Dia proteins control diverse fundamental biological processes that are mediated by combinatorial interactions with different binding partners. The nature of some interactions including well-known actin regulators such as Abi proteins or members of the Ena/VASP proteins is still unclear. Future genetic approaches including detailed structure-function analyses in the mutant background will significantly increase our understanding of these interactions in vivo. Additional quantitative multi-wavelength single-molecule imaging approaches and new super-resolution microscopy approaches will shed light on these interactions how and where these conserved regulatory networks act on cellular structures and cell dynamics. The use of advanced optogenetic tools for the manipulation of endogenous proteins in single cells will further complement our knowledge about these evolutionarily conserved modules in actin nucleation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/cib/article/27634

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