

PhD thesis

COORDINATION OF ACTIN AND MICROTUBULE  
DYNAMICS BY THE FORMIN PROTEIN  
DISHEVELLED-ASSOCIATED ACTIVATOR OF  
MORPHOGENESIS FORMIN

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## MOTIVATION TO MY THESIS WORK

Essential components of the eukaryotic cells are the polymer networks built from protein subunits; including microfilaments (or actin filaments, AF), intermediate filaments (IF) and microtubules (MT). These cytoskeletal protein arrays have fundamental roles in virtually all cellular functions; including but not limited to cell division, motility, adhesion, signaling, endocytic trafficking and transport. Proper cellular functionality; and thus health relies on the spatiotemporal regulation of the morphology and dynamics of these polymer networks, which is orchestrated by a large repertoire of associated proteins. Therefore, understanding the functional and structural principles of the regulation of these cytoskeletal polymers is basic for medicine and life sciences.

Formins are cytoskeletal polymer-associated regulatory proteins, which are defined by the presence of the evolutionary conserved formin homology (FH) regions; FH1 and FH2. The FH2 domain interacts with actin, while the FH1 domain can interact with the small actin-binding protein profilin. The tandem FH1-FH2 module can catalyze actin nucleation and processively elongate filaments from profilin actin. The Diaphanous-related formin (DRF) subfamily, in addition to the FH2 domains, contains N-, and C-terminally located elements, which can provide spatiotemporal control of the interactions of FH1-FH2 with actin in a RhoGTPase-dependent manner.

Disheveled-associated activator of morphogenesis (DAAM) formin belongs to the DRF subfamily and is involved in diverse morphogenetic processes mediated by the actin cytoskeleton. For example, DAAM plays a role in organizing apical actin cables that define the tracheal cuticle pattern in *Drosophila melanogaster* (Matusek, Djiane et al. 2006). It is also required for axonal growth and guidance by promoting filopodia formation in the growth cone (Matusek, Gombos et al. 2008), and DAAM is essential for sarcomerogenesis (Bao, Zhang et al. 2012; Molnar, Migh et al. 2014; Vogler, Liu et al. 2014). In our previous work we described the physico-chemical properties of the interaction of *Drosophila* DAAM FH1-FH2 and actin, and showed that DAAM FH1-FH2 is a profilin-gated actin assembly factor (Barko, Bugyi et al. 2010; Molnar, Migh et al. 2014).

### **Emerging novel activities of the C-terminal regions of formins**

Recent studies showed that – besides functioning as an autoregulatory element – the C-terminal regions of some formins from yeast to mammals (such as mouse Dia1, FMNL3, INF2, *Drosophila* Capuccino, human Daam, yeast Bni1 and Bnr1) can influence actin assembly mediated by the active FH2 domain (Chhabra and Higgs 2006; Gould, Maiti et al. 2011; Heimsath and Higgs 2012; Vizcarra, Bor et al. 2014). Biochemical studies indicate that isolated C-terminal regions can directly interact with actin, even in the absence of the FH2 domain. However, this interaction can have different functional consequences. The C-terminus of INF2 contains a WH2/DAD-like

motif, which in its isolated form sequesters monomeric actin and severs actin filaments (Chhabra and Higgs 2006). While the WH2-DAD-CT region of FMNL3 in its dimeric form nucleates actin and slows elongation (Heimsath and Higgs 2012). Similarly, the isolated dimeric DAD from Dia1 seems to be sufficient to promote actin nucleation (Gould, Maiti et al. 2011). In contrast, the tail region of Capuccino even if in its dimeric form, does not influence nucleation or elongation in the absence of FH2 (Vizcarra, Bor et al. 2014). These observations support that the activities of the C-terminal regions related to monomer binding and filament end interactions vary amongst formins, and raise the question how and which of the activities of the isolated C-terminal regions are transmitted to the functionality of each formin in the context of the FH1-FH2 domains.

### **Emerging novel activities of formins in the regulation of microtubule cytoskeleton**

According to the classical views, formins are key regulators of the actin cytoskeleton. Recent evidences, however, emphasize that the members of this protein family including mouse Dia1/2, INF1/2 and *Drosophila* Capuccino can interact with microtubules as well, and stabilize microtubule-based structures during cell migration, division and virus infection (Palazzo, Cook et al. 2001; Zhou, Leder et al. 2006; Bartolini, Moseley et al. 2008; Young, Thurston et al. 2008; Bartolini and Gundersen 2010; Gaillard, Ramabhadran et al. 2011; Roth-Johnson, Vizcarra et al. 2014). *In vitro* studies on mouse Dia1/2, INF2 and *Drosophila* Capuccino suggest that the microtubule-binding region comprises the FH2 domain, the same region that is essential for proper actin interaction. In contrast, isolated FH2 of INF1 does not bind microtubules, instead a novel C-terminal microtubule-binding domain is required for the interaction (Young, Thurston et al. 2008). Direct visualization of EGFP-mouse Dia2 FH1-FH2 further suggests that formins bind along the microtubule lattice (Bartolini, Moseley et al. 2008). The consequences of formin-microtubule interaction on microtubule dynamics have started to be described; mouse Dia1/2 can stabilize microtubules against cold-, and dilution-induced depolymerization, presumably by reducing subunit dissociation from the polymer (Bartolini, Moseley et al. 2008). The mouse Dia2, INF2 and *Drosophila* Capuccino can bundle microtubules (Bartolini, Moseley et al. 2008; Gaillard, Ramabhadran et al. 2011; Roth-Johnson, Vizcarra et al. 2014). Interestingly, INF2 can co-align microtubules with actin filaments (Gaillard, Ramabhadran et al. 2011). The structural details and the functional consequences of the formin-microtubule interaction have just started to be investigated. The observations raise the question which regions of formins are required for microtubule interaction and actin-microtubule co-alignment. Moreover, the intriguing possibility is raised that formins can interact simultaneously with the actin and microtubule cytoskeleton and can coordinate their dynamics. In conclusion, the C-terminal regions of formins can have a role in FH1-FH2-assisted actin assembly, on the other hand formins emerge as important regulators of the microtubule cytoskeleton, as well as novel coordinators of the actin-microtubule cytoskeleton.

## OBJECTIVES AND QUESTIONS

In my PhD work I aimed to investigate the activities of the C-terminal regions of *Drosophila* DAAM (dDAAM) in actin assembly, and the role of its FH2 and C-terminal regions in microtubule and actin-microtubule interactions. For this purpose, I planned to study the interactions of recombinantly produced proteins with actin and microtubules by using *in vitro* protein biochemical and biophysical approaches.

I addressed the following questions:

1. Is dDAAM autoregulated by its N-terminal and C-terminal domains, similarly to other DRFs, as predicted from its comparative sequence analysis?
2. Does the C-terminus of dDAAM influence the FH1-FH2-mediated actin assembly? If it does so, which regions are important for this activity?
3. Does the isolated C-terminal region of dDAAM interact with actin?
4. Does the dDAAM bind microtubules? If it does so, which regions are important for this interaction and what are the functional consequences?
5. Does dDAAM bind simultaneously to actin and microtubules? If it does so, which regions are important for this interaction?

## EXPERIMENTAL PROCEDURES

### Protein purifications

*Drosophila melanogaster* DAAM subfragments (DID: 115-356 aa cDAAM: 568-1153 aa, FH1-FH2: 568-1054 aa, cDAAM $\Delta$ CT: 568-1116, DAD-CT: 1083-1153 aa, DAD: 1083-1119 aa) and their mutated version (FH1-FH2<sup>I732A</sup>, cDAAM<sup>I732A</sup>, cDAAM<sup>R-A</sup>, DAD-CT<sup>R-A</sup>) were cloned into pGEX-2T vector (Amersham Biosciences) by our collaborator (József Mihály, Hungarian Academy of Sciences, Biological Research Centre, Szeged, Hungary) and were purified as Gluthation S-Transferase (GST) fusion proteins from *E. coli* BL21(DE3)pLysS strain (Novagen). Actin was extracted and purified from acetone-dried rabbit skeletal muscle powder (Feuer, Molnar et al. 1948) and modified with fluorescent probes according to standard protocols (Bugyi, Papp et al. 2006; Barko, Bugyi et al. 2010; Bugyi, Didry et al. 2010; Toth, Majoros et al. 2016). Unlabeled and Hylite Fluor<sup>TM</sup> 488 lyophilized tubulin was purchased from Cytoskeleton and were dissolved in BRB buffer (80 mM PIPES pH6.9, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 2 mM GTP, 1 mM DTT) according to the manufacturer's instructions. Recombinant mouse profilin 1 was purified and labeled by Alexa Fluor® C<sub>5</sub> 568 maleimide (Alexa568C, Invitrogen) as described previously (Perelroizen, Marchand et al. 1994). Recombinant mouse heterodimeric  $\alpha$ 1 $\beta$ 2 Capping Protein (CP) was purified as described previously (Bugyi, Didry et al. 2010).

### Steady-state fluorescence spectroscopy measurements

The steady-state anisotropy of monomeric actin (AlexaFluor® 488 NHS labeled G-actin, 0.2  $\mu$ M) was measured to study its interaction with dDAAM both in the absence and presence of profilin (0.8  $\mu$ M). The anisotropy measurements were performed using a Horiba Jobin Yvon spectrofluorometer. The dissociation equilibrium constant of the dDAAM:G-actin interaction was derived from the dDAAM concentration dependence of the anisotropy of Alexa488NHS-G-actin using the quadratic binding equation. Alternatively, to study the interaction of dDAAM DAD-CT and profilin:G-actin the anisotropy of Alexa Fluor® 568C<sub>5</sub> maleimide labeled profilin (Alexa568C-profilin) was measured.

The effects of dDAAM on the kinetics of actin assembly (2  $\mu$ M) in the absence and presence of profilin (6  $\mu$ M) were studied in pyrene (N-(1-pyrene)iodoacetamide) labeled actin based bulk polymerization experiments (Cooper, Walker et al. 1983; Pollard and Cooper 1984). The polymerization was initiated by the addition of 1 mM MgCl<sub>2</sub> and 50 mM KCl. The measurements were performed using a Safas Xenius FLX spectrofluorimeter. For quantitative analysis the bulk polymerization rate at each condition was derived from the slope of the pyrenyl trace at half-maximum polymerization. The antagonistic regulation of barbed end dynamics by dDAAM and CP was investigated as described (Bombardier, Eskin et al. 2015).

### **Total internal reflection fluorescence microscopy (TIRFM) experiments**

The separate effects of dDAAM constructs on the nucleation and elongation of actin filaments (0.5  $\mu\text{M}$   $\text{Mg}^{2+}$ -G-actin containing 10 % Alexa488NHS-G-actin) in the absence and presence of profilin (2  $\mu\text{M}$ ) were investigated by TIRFM. Actin filaments were attached to N-ethylmaleimide (NEM) labeled skeletal muscle myosin S1 functionalized glass surface to ensure that actin assembly can be visualized in the TIRF excitation field.

The actin filament-microtubule co-alignment ability of dDAAM constructs were studied by TIRFM microscopy. Mixtures of preassembled phalloidin-stabilized actin filaments (0.4  $\mu\text{M}$  containing 10 % Alexa568NHS-G-actin) and taxol-stabilized microtubules (0.4  $\mu\text{M}$  containing 10 % Hylite Fluor™ 488-tubulin) were visualized in the absence and presence of dDAAM. Images were captured with an Olympus IX81 microscope (491 nm and 568 nm laser-based TIRFM module, APON TIRF 60x NA1.45 oil immersion objective, Hamamatsu Orca-ER-1394 CCD camera). Image analysis was performed by Fiji.

### **Sedimentation experiments**

High-speed centrifugation (100.000 g, 20 min, 20°C) experiments were performed to investigate the binding of dDAAM constructs to the sides of actin filaments (2  $\mu\text{M}$ ). Supernatants and pellets were separated and analyzed by SDS-PAGE. To derive the dissociation equilibrium constant of the dDAAM:F-actin interaction data were analyzed as described (Shimada, Nyitrai et al. 2004).

Low-speed centrifugation (14000g, 5 min, 20°C) experiments were performed to study the actin filament/stabilized microtubule (1  $\mu\text{M}$ ) bundling/cross-linking activity of dDAAM constructs. The supernatants and pellets were carefully separated and the supernatants were processed for SDS-PAGE analysis. The relative amount of F-actin/microtubule in the supernatant was derived as the ratio of the amount of F-actin/microtubule in the presence of different dDAAM constructs to the amount of F-actin/microtubule in the absence of any other.

Low-speed centrifugation (4000 g, 10 min, 20°C) assays on a 30 % sucrose cushion were developed to study the F-actin:microtubule (2  $\mu\text{M}$ ) co-alignment activity of dDAAM (Elie, Prezel et al. 2015). Under the experimental conditions individual polymers, as well as F-actin bundles do not sediment, only larger filament complexes (MT bundles, F-actin:MT co-polymers) appear in the pellet. The pellets and supernatants were carefully separated and analyzed by SDS-PAGE.

### **Statistical analysis**

Data are derived from at least two independent experiments. Values are given as mean  $\pm$  standard deviation. Microscopy data were analyzed statistically by using two-sample T-test or Z-test considering the number of data and the variance (Excel, Microsoft). By convention:  $p \geq 0.05$  statistically not significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

## RESULTS AND DISCUSSION

### **dDAAM is *bona fide* DRF, the actin activities of its FH2 are auto-regulated through its N-terminal DID and C-terminal DAD domains**

Pyrenyl polymerization assays revealed that DID influences neither spontaneous actin polymerization nor actin assembly catalyzed by FH1-FH2. In contrast, cDAAM-mediated actin polymerization is inhibited by DID in a concentration-dependent manner. Since the only difference between dDAAM FH1-FH2 and cDAAM can be found at the C-terminal region, we concluded that DID negatively affects the actin polymerization activity of dDAAM by interacting with the C-terminus. The dissociation equilibrium constant was found to be ~ 30 nM, indicating a high-affinity interaction between DID and the DAD region of cDAAM. These findings suggest that the actin activities of *Drosophila* DAAM FH1-FH2 are autoregulated by its N-terminal DID and C-terminal DAD domains *in vitro*, thus dDAAM is a *bona fide* DRF formin and this mode of autoregulation is conserved between flies and humans (Liu, Sato et al. 2008).

### **cDAAM is more efficient in catalyzing actin assembly than FH1-FH2, which relies on its more potent nucleation promoting activity**

The polymerization promoting efficiencies of the FH1-FH2 region in the absence and presence of DAD-CT (FH1-FH2, cDAAM) were comparatively analyzed in pyrenyl polymerization experiments. cDAAM accelerates polymerization by ~ 36fold more efficiently than FH1-FH2. Steady-state fluorescence anisotropy measurements revealed that both dDAAM FH1-FH2 and cDAAM bind G-actin with dissociation equilibrium constant of a few tens of nM. These findings suggest that the different polymerization efficiencies of the DAD-CT lacking and possessing constructs do not arise from their different binding affinities. The analysis of TIRFM data revealed that, although the number of actin filaments formed in the presence of either FH1-FH2 or cDAAM is significantly higher than that is characteristic for spontaneous actin assembly, cDAAM is more efficient in filament production than the FH1-FH2. The elongation rate was affected quantitatively similarly by the two constructs. In conclusion, our observations clearly demonstrate that the more efficient actin assembly promoting activity of cDAAM is due to its more potent nucleating efficiency as compared to FH1-FH2. This suggests that DAD-CT of dDAAM – besides autoregulation – can tune the actin assembly activity of the active dDAAM FH1-FH2 region.

### **The proper nucleation promoting activity of dDAAM relies on the presence of both functional FH2 and C-terminus**

Steady-state anisotropy measurements revealed that DAD-CT can bind to G-actin even in the absence of FH2, however, the binding affinity was relatively weak ( $K_d$  ~ few  $\mu$ M), strongly salt-dependent and was not influenced significantly by profilin. DAD and DAD-CT<sup>R-A</sup> showed extremely weak interactions in anisotropy measurements. Pyrenyl polymerization and TIRFM measurements revealed that neither C-terminal construct

can influence significantly actin assembly – neither filament number nor filament growth rate – in the absence of FH1-FH2. In conclusion, although isolated DAD-CT is able to bind to monomeric actin it has no significant influence on the dynamics of actin assembly in the absence of the FH2 domain, even in its dimeric form that is maintained by the GST-tag. This indicates that dDAAM DAD-CT interacts with G-actin independently from the FH2 domain but does not influence actin dynamics in the absence of the FH2 domain, thus it possesses an FH2-dependent activity in actin dynamics.

To investigate the contribution of the FH2 domain and each C-terminal element – DAD and CT – to the functionality of dDAAM, mutated and truncated versions of dDAAM were studied (FH1-FH2<sup>I732A</sup>, cDAAM<sup>I732A</sup>, cDAAM $\Delta$ CT and cDAAM<sup>R-A</sup>). In pyrenyl polymerization and TIRFM measurements we found that cDAAM $\Delta$ CT and cDAAM<sup>R-A</sup> are more potent than FH1-FH2 in accelerating actin assembly but less potent than the native cDAAM, which originates from the more potent nucleation ability of these constructs as compared to FH1-FH2. We also found that the I732A mutation impairs not only the monomer binding and filament end interactions of dDAAM, but also abolishes the nucleation activity of dDAAM. In conclusion, functional coordination between FH2 and CT is essential to reconstruct the proper functionality – in terms of actin nucleation and filament end interaction – of dDAAM.

### **dDAAM antagonizes with capping proteins and DAD-CT contributes to this activity**

Formins are known to compete with classical Capping proteins (CP) to maintain barbed end dynamics (Romero, Le Clainche et al. 2004; Bombardier, Eskin et al. 2015). We tested in competition-based pyrenyl polymerization experiments whether the C-terminal DAD-CT regions can contribute to this activity. We found that cDAAM compete with CP more efficiently as compared to FH1-FH2 (IC<sub>50</sub> values: FH1-FH2: 47.7 ± 16.97 nM, cDAAM: 345.9 ± 27.60 nM). Truncations/mutations in the CT region partially reduces the efficiency of cDAAM to uncap CPs bound barbed ends, yet these constructs are still more effective than FH1-FH2. Neither cDAAM<sup>I732A</sup>, FH1-FH2<sup>I732A</sup> nor can compete with CPs, also DAD-CT in the absence of FH1-FH2 fails to uncapped CP-bound filament ends. These results suggest that the wild-type FH2 is necessary for the antagonistic action between dDAAM and CPs, however, this activity is tuned by the DAD-CT region. In conclusion, our data reveal a novel role of the C-terminal region as the part of the anti-capping module of formins.

### **dDAAM binds to the side of actin filaments and possesses F-actin bundling activity**

In high-speed sedimentation experiments we found that DAD-CT binds to the sides of actin filaments ( $K_d = 38.9 \pm 3.2 \mu\text{M}$ ), albeit with lower efficiency as compared to the FH1-FH2 domain ( $K_d = 2.1 \pm 0.5 \mu\text{M}$  (Barko, Bugyi et al. 2010)). Truncation/mutation in the C-terminus (DAD, DAD-CT<sup>R-A</sup>) significantly reduces the filament binding ability ( $K_d > 100 \mu\text{M}$ ). Low-speed sedimentation revealed that both FH1-FH2 and cDAAM are



able to bundle actin filaments with approximately the same efficiency, while the bundling ability of the native, truncated/mutated C-terminus was extremely weak, consistently with the low affinity F-actin interaction. These observations show that, besides interacting with actin monomers, isolated DAD-CT can decorate the side of actin filaments, and as a functional consequence it arranges actin filaments into higher-order bundled structures. Our data also suggest that the main side-binding/bundling element of DAAM is the FH2 domain, while the DAD-CT region has minor contribution to this activity.

### **Sequence characteristics and possible binding modes of DAD-CT on actin**

We found that profilin does not affect significantly the binding of dDAAM DAD-CT to actin and its actin activities, either, which suggests that profilin and DAD-CT can bind simultaneously to monomeric actin and form a ternary complex. This result predicts that the binding site of the two proteins on actin largely differ. In contrast, the WH2 domains of Sarcomere Length Short (SALS) protein can displace DAD-CT from G-actin, as indicated by the results of anisotropy experiments. These results suggest that the binding of SALS-WH2 interferes with that of DAD-CT, indicating that the main binding sites of these proteins overlap. The binding site of profilin on actin monomers is found to be on the hydrophobic cleft between subdomain 1 and 3 at the barbed face (Schutt, Myslik et al. 1993). The N-terminal part of the WH2 folds into an amphipathic  $\alpha$ -helix that interacts with the hydrophobic cleft of actin, the interaction is mediated by a conserved hydrophobic amino acid triplet LLxxI of WH2. The binding is further strengthened by the downstream LKKT/V motif. While their C-terminus extends along the negative surface patch of the actin molecule towards the pointed face, through interactions mainly controlled by electrostatic forces. Apparently, our bioinformatics analysis revealed that the DAD-CT of DAAM contains the conserved LLxxI motif, however the consensus LKKT/V motif that fundamentally strengthens actin interactions of WH2 domains is absent. Altogether our data support that the weak actin binding of dDAAM DAD may be due to the interaction of the WH2-like amino acid triplet LLxxI with the hydrophobic cleft, and the complex is fundamentally strengthened by the CT region that may mediate the connections between the negative stretch of G-actin towards the pointed end in a similar manner as the C-terminal extension of WH2 domains.

### **dDAAM interacts with microtubules and organizes these polymers into bundled/cross-linked**

In low-speed sedimentation experiments we found that cDAAM is able to induce the formation of bundled/cross-linked microtubule arrays. Interestingly, FH1-FH2 lacking the C-terminal regions does not possess this activity. Isolated DAD-CT also fails to bundle/cross-link microtubules in the absence of FH1-FH2. These observations indicate that isolated FH1-FH2 and DAD-CT regions alone are not sufficient to bundle/cross-link these polymers. The efficient bundling/cross-linking requires the

simultaneous binding of both of these regions of dDAAM to microtubules, and our data suggest that this activity of dDAAM relies on cooperative interactions.

### **dDAAM simultaneously interacts with actin filaments and microtubules and co-aligns these polymers**

dDAAM can interact with both F-actin and microtubules, which raises the intriguing possibility that dDAAM has the ability to physically interact with the two polymers simultaneously. To test this possibility, we developed a low-speed sedimentation protocol that offers conditions, which allow to separate the F-actin:MTs complexes from other polymer species (e.g. individual polymers or bundled polymers). To achieve such conditions, the centrifugation is performed through a 30 % sucrose gradient at very low speed (4000 g). Under these conditions individual or bundled (e.g. by FH1-FH2, cDAAM) actin filaments remain in the supernatant, while larger protein complexes (e.g. bundled/cross-linked microtubules by cDAAM) can be selectively sedimented and appear in the pellet. Intrinsic to this strategy, actin filaments can sediment and appear in the pellet only if they are physically linked to microtubules. Our results revealed that a significant fraction of F-actin appeared in the pellet with microtubules in the presence of cDAAM, implying a simultaneous interaction between cDAAM and the two polymers. In contrast, actin filaments do not sediment in the presence of microtubules and FH1-FH2, DAD-CT, DAD or DAD-CT<sup>R-A</sup>. Results from low-speed sedimentation assays were fully supported by TIRFM-based co-localization experiments. These observations suggest that both FH2 and CT regions are needed for the physical co-alignment of actin filaments and microtubules, however neither is sufficient alone, indicating a cooperative nature of this activity.

## CONCLUSIONS AND WORKING MODELS

### **Mechanistic view of the enhancement of FH2-mediated actin assembly by the C-terminal of formins**

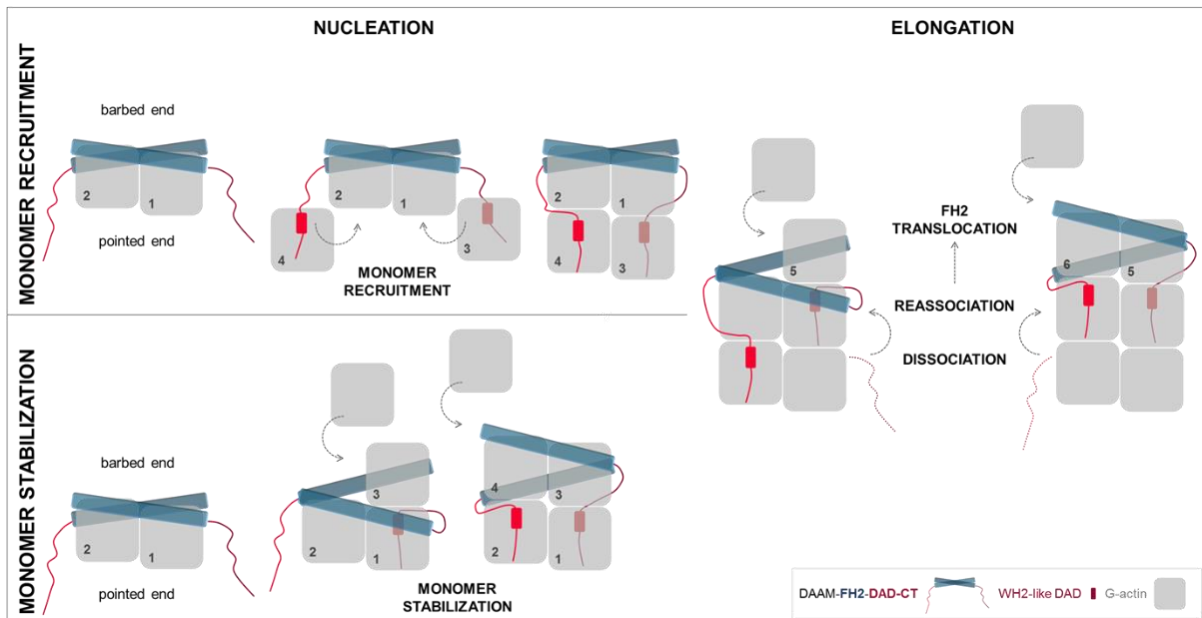
The main findings of this work are that dDAAM DAD-CT makes the FH1-FH2 region a more potent nucleator, it also contributes to the strengthening of FH1-FH2 interactions with filament ends. How can we relate and interpret the structural and functional behavior of DAD-CT? Analysis of the role of the N-terminally located FH1 domain of FMNL3 in the antagonism of FH2 with capping proteins revealed that FH1 increases the ability of FH2 to maintain filament elongation in the presence of CPs. Since FH1 does not interact directly with actin, this observation led to the proposal that FH1 increases the stability of the FH2 dimer, which makes it a more efficient elongator (Gould, Maiti et al. 2011; Thompson, Heimsath et al. 2013). One possible explanation of our findings is that DAD-CT adopts a similar mode of action, it can make the FH2 dimer a more efficient nucleator by stabilizing its structure. In this way, DAD-CT would contribute indirectly – independent from its own actin binding ability – to the core activities of FH2.

Considering that isolated dDAAM DAD-CT can bind monomeric actin independently from the FH2 domain, an alternative proposal can be envisaged. We suggest that DAD-CT contribute to the enhanced nucleation activity of FH2 by its direct interactions with actin. We performed alignments of known X-ray structures of actin, FH2 domains and formins to investigate the possible actin binding modes of DAD-CT in the FH1-FH2-DAD-CT dimer (*Figure 32*). Structural data predicts that each of the DAD-CT regions in the FH1-FH2-DAD-CT dimer can establish contacts with an actin monomer, in addition to the monomers bound by the FH2 dimer. Considering these, DAD-CT can directly interact with actin in their complexes with FH1-FH2-DAD-CT, which lead to the stabilization of nucleation intermediers, thus enhanced nucleation. We propose the ‘monomer stabilization model’ to explain the contribution of the C-terminus of formins to FH2-mediated nucleation. According to this model, the actin monomers that are captured by the FH1-FH2 dimer are subsequently bound and stabilized by DAD-CT in their complexes with dDAAM. In this scenario, the concerted binding of actin by FH1-FH2 and DAD-CT domains in the dDAAM dimer would result in the stabilization of four actin subunits by dDAAM, which would completely overcome the structural and kinetic barrier of actin assembly imposed by the nucleation phase.

A recent model proposes that the FH1-FH2-C-terminus of formins forms a tripartite machinery, in which the C-terminus serves as a monomer recruitment motif that captures monomers and subsequently assembles to the FH1-FH2-bound dimer (Gould, Maiti et al. 2011). The ‘monomer recruitment’ model implies that the DAD-CT bound actin monomers incorporate at pointed ends, which would be inhibited by profilin, yet we do not detect inhibition of the activities of DAD-CT by profilin. On the other hand, one has to consider that the actin affinity of dDAAM and other formins is relatively weak in the absence of the FH2 domain. Efficient monomer recruitment would require reasonable actin binding strength. Considering this, the ‘monomer recruitment’

model implicates that the affinity of the C-terminus of formins in the FH1-FH2-C-terminus is substantially strengthened. This might occur by FH2-mediated structural changes in the C-terminal regions, or the FH2 domain by bringing actin subunits into the close proximity of DAD-CT could increase the apparent affinity of the C-terminus. In this scenario, the low-affinity C-terminal regions of formins may be involved in the stabilization of actin monomers captured by the FH2 dimer, while high-affinity C-terminal domains can mediate monomer recruitment.

Besides nucleation DAD-CT also supports the interaction of FH2 with the filament ends, as well as its anti-capping efficiency. This is manifested possibly through interactions of the DAD-CT with the sub-terminal actin subunits, consistently with the proposed structural model. In the presence of the C-terminal regions, the stair stepping of formins requires the dissociation and re-association of both FH2 and DAD-CT, which can influence the processive mode of filament end tracking, as suggested by our data and other work.

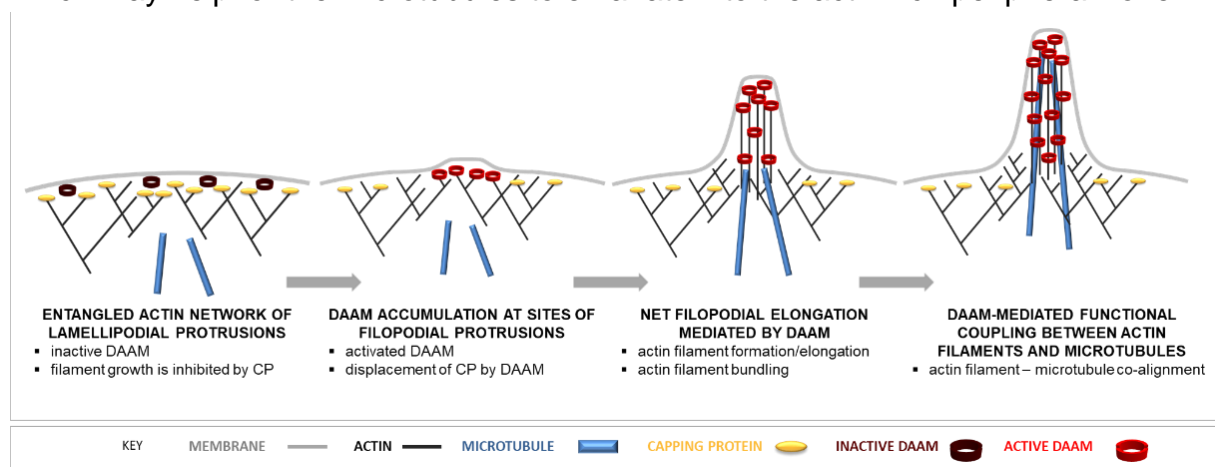


**Alternative model of the concerted monomer recruitment and filament end interaction by the FH2 and C-terminal regions of formins.**

Schematic representation of the 'monomer recruitment' (Gould, Maiti et al. 2011) and 'monomer stabilization' models, as alternative scenarios to explain actin nucleation and elongation mediated by FH1-FH2-DAD-C-terminus.

## Mechanisms of the coordinated regulation of the actin and microtubule cytoskeleton by dDAAM

Besides its actin interactions, in this work we identified novel interactions and activities of dDAAM: it can bundle/cross-link microtubules, and simultaneously interacts with both actin filaments and microtubules and co-align the two cytoskeletal polymers. Interestingly, the main actin interacting FH2 domain is not sufficient for microtubule bundling/cross-linking and actin-microtubule co-alignment, these activities essentially require the presence of the C-terminus. Thus, our data reveal a novel interaction of the DAD-CT region of dDAAM. Our collaborator revealed that these interactions and activities have biological relevance in *Drosophila* primary neurons (Szikora, Foldi et al. 2017). In growth cones dDAAM co-localizes with both the actin and the microtubule cytoskeleton, and a significant fraction of the protein (~ 20 %) co-aligns with both polymer networks. As a functional relevance of these interactions, dDAAM is crucial for proper growth cone filopodia formation and dynamics. Albeit more work has to be done to truly understand the molecular choreography underlying the actin-microtubule cytoskeleton-related neuronal functions of dDAAM, based on our *in vitro* data we propose a working hypothesis of the role of dDAAM in growth cone filopodia formation. At the actin-rich peripheral zones, the entangled actin array of lamellipodial protrusions is maintained by coordinated action of the Arp2/3 complex machinery and Capping proteins, which determine the force production and mechanical properties of the network (Blanchoin, Boujemaa-Paterski et al. 2014). Capping proteins by binding to the filament ends inhibit their elongation. At sites of filopodia formation dDAAM is activated by binding to RhoGTPases, which in turn relieves the autoinhibitory interaction between DID and DAD, and exposes the FH1-FH2 module to actin. dDAAM by competing with Capping proteins can maintain directed and sustained profilin:actin incorporation at filament ends, which results in net filopodial lengthening. On the other hand, dDAAM can bundle filopodial actin filaments that may contribute to the mechanical integrity of the array. Due to its ability to simultaneously interact with both actin filaments and microtubules, dDAAM can co-align the two to polymer networks, which may help for the microtubules to emanate into the actin-rich peripheral zone.



**Working model of the dDAAM-mediated filopodia formation in neurons.**

## SUMMARY

In my PhD work I was interested in the role of *Drosophila* Dishevelled-associated activator of morphogenesis (dDAAM) formin in cytoskeleton dynamics regulation. DAAM is essential in diverse biological processes and was found to be a key component of the regulatory machinery of actin and microtubules remodeling in neurons. Previously our group described the actin activities of the FH1-FH2 of dDAAM. Recent studies suggested that the C-terminal regions of formins can also influence actin dynamics. Also, *in vivo* data demonstrated that dDAAM can interact with the neuronal microtubule cytoskeleton.

To better understand the molecular mechanism underlying the biological functions of DAAM in the nervous system, I analyzed the interactions of different regions of dDAAM with actin and microtubules. My results are summarized below:

- dDAAM is *bona fide* DRF, it is autoregulated through its N-terminal DID and C-terminal DAD domains.
- The DAD-CT containing cDAAM fragment is more efficient in catalyzing actin assembly as compared to FH1-FH2, due to its more potent actin nucleation activity.
- The DAD-CT containing cDAAM fragment is more efficient in maintaining filament end dynamics in the presence of Capping proteins as compared to FH1-FH2, which points towards a novel role of DAD-CT, as part of the filament elongation machinery and the anti-capping formin module.
- The isolated dDAAM DAD-CT can bind actin monomers and filaments, independently from the FH2 domain, and the interaction is mainly mediated by electrostatic forces.
- The binding of dDAAM DAD-CT to monomeric actin is not influenced significantly by profilin, in contrast WH2 domain proteins can disrupt the interaction.
- Despite of the ability of dDAAM DAD-CT to bind actin, it fails influence actin dynamics in the absence of FH2, thus this region possesses FH2-dependent activities.
- dDAAM can assemble actin filaments into bundled structures, the FH1-FH2 is necessary and sufficient for this activity.
- dDAAM can organize microtubules into higher-order bundled structures, which requires the simultaneous binding of both FH2 and the DAD-CT regions to microtubules, presumably via cooperative interactions.
- dDAAM is able to bind actin filaments and microtubules simultaneously and co-align the two polymer systems, which relies on both the FH2 and DAD-CT regions.

Altogether, my data provide novel insight into the mechanistic view of the regulation of the actin and co-regulation of the actin-microtubule cytoskeleton by dDAAM, as well as broadens of our understanding of the regulation of these polymers by each region of formins.

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### Publications related to my PhD work

1. **Andrea Teréz Vig**, István Földi, Szilárd Szikora, Ede Migh, Rita Gombos, Mónika Ágnes Tóth, Tamás Huber, Réka Pintér, Gábor Csaba Talián, József Mihály, Beáta Bugyi (2017) The activities of the C-terminal regions of the formin protein dishevelled-associated activator of morphogenesis (DAAM) in actin dynamics. *Journal of Biological Chemistry*, J Biol Chem. 2017 Aug 18;292(33):13566-13583. doi: 10.1074/jbc.M117.799247. Epub 2017 Jun 22.  
IF: 4.258, Q1/D1
2. Szilárd Szikora, István Földi, Krisztina Tóth, Ede Migh E, **Andrea Teréz Vig**, Beáta Bugyi, József Maléth, Péter Hegyi, Péter Kaltenecker P., Natalia Sanchez-Soriano, József Mihály J (2017) The formin DAAM is required for coordination of the actin and microtubule cytoskeleton in axonal growth cones. *Journal of Cell Science*, J Cell Sci. 2017 Aug 1;130(15):2506-2519. doi: 10.1242/jcs.203455. Epub 2017 Jun 12.  
IF: 4.431, Q1/D1, Independent citation: 1

### Conference talks related to my PhD work

1. **Andrea Vig**, Péter Gaszler, Mónika Ágnes Tóth, Beáta Bugyi: The CT region of DAAM has a supporting role in FH2-mediated actin dynamics regulation, 2016.05.27-29. 5<sup>th</sup> Interdisciplinary Doctoral Conference, Pécs
2. **Andrea Vig**, Andrea Majoros, Tamás Huber, Ede Migh, József Mihály, Miklós Nyitrai and Beáta Bugyi: DAAM formin's C-terminal end in the actin-formin interaction, Membrán Transzport Konferencia 2015.05.19-22. Sümeg

### Conference posters related to my PhD work

1. Pintér Réka, Fórizs Judit Viktória, Huber Tamás, **Vig Andrea Teréz**, Bugyi Beáta (2017): *A Disheveled-Associated Activator of Morphogenesis (DAAM) formin szerepe az aktin dinamikájában.* (2017. 08. 22–25. A Magyar Biofizikai társaság XXVI. Kongresszusa, Szeged)
2. Fórizs Judit Viktória, Pintér Réka, **Vig Andrea Teréz**, Huber Tamás, Bugyi Beáta (2017): *A DAAM (Disheveled-Associated Activator of Morphogenesis) formin szerepe az aktin-mikrotubulus dinamika koordinációjában.* (2017. 08. 22–25. A Magyar Biofizikai társaság XXVI. Kongresszusa, Szeged)
3. Szilárd Szikora, István Földi, Krisztina Tóth, **Andrea Vig**, Ede Migh, Beáta Bugyi, József Mihály (2017): *The formin DAAM coordinates the actin and the microtubule cytoskeleton during axonal development.* (2017. 03. 31–04. 02. Hungarian Molecular Life Sciences, Eger)
4. István Földi, Szilárd Szikora, Krisztina Tóth, **Andrea Vig**, Beáta Bugyi, József Mihály (2017): *Characterization of the interaction between a Drosophila formin DAAM and microtubules.* (2017. 03. 31–04. 02. Hungarian Molecular Life Sciences, Eger)
5. Péter Gaszler, Judit Viktória Fórizs, Tamás Huber, **Andrea Teréz Vig**, Beáta Bugyi (2017): *Coordination of actin-microtubule dynamics by Disheveled-*

- Associated Activator of Morphogenesis (DAAM) formin.* (2017. 03. 31–04. 02. Hungarian Molecular Life Sciences, Eger)
6. Péter Gaszler, Judit Viktória Fórizs, **Andrea Teréz Vig**, Beáta Bugyi (2016): *Coordination of actin-microtubule dynamics by DAAM formin.* (2016. 08. 28–31. Annual Conference of the Hungarian Biochemical Society, Szeged)
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