

1 TITLE

2 The formin DAAM is required for coordination of the actin and microtubule cytoskeleton in
3 axonal growth cones

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5 RUNNING TITLE: Axonal actin/MT coordination

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24 SUMMARY STATEMENT

25 We provide novel insights into the mechanisms of actin-microtubule crosstalk in axonal
26 growth cones by describing the role of a formin protein in *Drosophila* neurons.

27 ABSTRACT

28 Directed axonal growth depends on proper coordination of the actin and microtubule
29 cytoskeleton in the growth cone. However, despite the relatively large number of proteins
30 implicated in actin-microtubule cross-talk, the mechanisms whereby actin polymerization is
31 coupled to microtubule stabilization and advancement in the peripheral growth cone remained
32 largely unclear. Here we identified the formin DAAM as a novel factor playing a role in
33 concerted regulation of actin and microtubule remodeling in *Drosophila* primary neurons. *In*
34 *vitro* DAAM binds to F-actin as well as microtubules and it has the ability to crosslink the
35 two filament systems. Accordingly, DAAM associates with the neuronal cytoskeleton, and a
36 significant fraction of DAAM accumulates at places where the actin filaments overlap with
37 that of microtubules. Loss of *DAAM* affects growth cone and microtubule morphology and
38 several aspects of microtubule dynamics, whereas biochemical and cellular assays revealed a
39 microtubule stabilization activity and binding to the microtubule tip protein EB1. Together
40 these data suggest that besides operating as an actin assembly factor, DAAM is involved in
41 linking filopodial actin remodeling to microtubule stabilization during axonal growth.

42

43 INTRODUCTION

44 Directional movement of axons, governed by their distally located growth cones, is elicited by
45 coordinated changes of peripheral F-actin (filamentous-actin) and central microtubule
46 networks (Dent and Gertler, 2003; Lowery and Van Vactor, 2009). F-actin in growth cone
47 periphery is localized to two main structures, filopodia and lamellipodia. Splayed
48 microtubules emanating from the central axonal microtubule bundles dynamically invade and
49 scan the F-actin rich periphery, often reaching deep into filopodia (Dent and Gertler, 2003;
50 Lowery and Van Vactor, 2009). Filopodial F-actin bundles can act as guides for these
51 dynamic microtubules, and it is thought that this actin-microtubule crosstalk followed by
52 microtubule and filopodia stabilization is a key step of growth cone advance. Most proteins
53 that have so far been linked to the molecular mechanisms of the interaction of actin and
54 microtubules belong to the microtubule plus-end tracking proteins (+TIPs), such as CLASP,
55 CLIP-170, APC, EB1/3, LIS1 and Spectraplakins/ACF7 (recently reviewed in (Bearce et al.,
56 2015; Cammarata et al., 2016; Coles and Bradke, 2015). Although the role of these proteins in
57 growth cone guidance is supported by genetic, biochemical and advanced microscopic assays,
58 these large proteins operate in complex interaction networks posing a great challenge for
59 dissecting how they precisely function.

60 Beyond the classical +TIPs, the number of proteins that may couple microtubules to F-
61 actin dynamics at plus-ends is still expanding (Coles and Bradke, 2015; Jiang et al., 2012).
62 For example, recent work identified members of the formin family as regulators of actin and
63 microtubule crosstalk in non-neuronal cells (Bartolini et al., 2008; Chesarone et al., 2010;
64 Gaillard et al., 2011; Rosales-Nieves et al., 2006; Young and Copeland, 2010). Formins are
65 well characterized actin assembly factors that promote the formation of unbranched actin
66 cables by facilitating their nucleation and elongation (Chesarone et al., 2010; Schonichen and
67 Geyer, 2010). These multidomain proteins contain two highly conserved signature domains,
68 the FH1 and FH2 formin homology domains. The dimeric FH2 nucleates actin filaments and
69 supports elongation by remaining processively attached to their barbed ends (Higashida et al.,
70 2004; Kovar and Pollard, 2004; Watanabe and Higashida, 2004), whereas the proline-rich
71 FH1 domain accelerates elongation by recruiting profilin-bound actin monomers (Li and
72 Higgs, 2003; Sagot et al., 2002). Some formins contain several other conserved regions
73 including the N-terminally located GBD (GTPase binding domain), DID (diaphanous
74 inhibitory domain), DD (dimerization domain), CC (coiled-coil region) and the C-terminal
75 DAD (diaphanous auto-regulatory domain) (Alberts, 2001; Chesarone et al., 2010; Li and

76 Higgs, 2003; Otomo et al., 2005; Rose et al., 2005). These domains are involved in the spatial
77 and temporal regulation of the actin assembly activity provided by the FH2 domain
78 (Chesarone et al., 2010). In addition, it has also been shown that a number of formins
79 influence microtubule (MT) stability and organization (Bartolini and Gundersen, 2010).
80 Consistently, many formins are able to bind to MTs *in vitro* through their actin binding FH2
81 domain and in some cases with their C-terminal region (Bartolini et al., 2008; Chesarone et
82 al., 2010; Gaillard et al., 2011; Roth-Johnson et al., 2014; Young et al., 2008; Zhou et al.,
83 2006). The presence of both an actin- and a microtubule-binding domain might allow these
84 proteins to crosslink the cytoskeletal components directly, such as Cappuccino does *in vitro*
85 and presumably in *Drosophila* oocytes (Rosales-Nieves et al., 2006). Conversely, instead of
86 or in parallel to direct binding, the Diaphanous-related formins (DRFs) mDia1, mDia2 and
87 mDia3 appear to stabilize MTs in fibroblast cells by +TIP binding through EB1, CLIP-170 or
88 APC (Cheng et al., 2011; Lewkowicz et al., 2008; Wen et al., 2004). Interestingly, mDia2 is
89 able to stabilize MTs and bind to +TIPs independently of its actin nucleation activity
90 (Bartolini et al., 2008). Finally, recent work revealed that CLIP-170 binds tightly to mDia1 to
91 increase the rate of actin polymerization from MT plus-ends (Henty-Ridilla et al., 2016).
92 Despite these advances, the *in vivo* contribution of formins to MT and F-actin interactions in
93 axonal growth cones remained elusive.

94 In this paper, we report the characterization of the role of the formin family protein
95 DAAM (Dishevelled associated activator of morphogenesis) in concerted regulation of actin
96 and MT remodeling in axons of *Drosophila* neurons. We show that *Drosophila* DAAM
97 partially colocalizes with both major cytoskeletal systems in neurons, and it is able to
98 crosslink actin filaments with MTs *in vitro*. DAAM stabilizes MTs against cold- or
99 **nocodazole**-induced depolymerization, and the absence of *DAAM* impairs neuronal MT
100 organization and dynamics. Although DAAM has the ability to bind MTs directly, it also
101 binds to an MT plus-end tracking protein, EB1, and often localizes to MT plus tips. This
102 suggests that, similar to migrating cells, a formin/+TIP dependent mechanism is crucial to
103 govern actin/MT coordination in growing axons.

104

105

106 RESULTS

107 **DAAM localizes to axonal microtubule bundles and physically interacts with**
108 **microtubules**

109 Previously we have shown that DAAM plays an essential role in differentiation of the
110 embryonic nervous system (Matussek et al., 2008) and the adult brain (Gombos et al., 2015).
111 *In vitro* and *in vivo* studies revealed that DAAM behaves as a *bona fide* formin and it is
112 required for filopodia formation (Barko et al., 2010; Matussek et al., 2008), similarly to its
113 vertebrate orthologue, Daam1 (Jaiswal et al., 2013a). Furthermore, the observed cellular
114 phenotypes, i.e. the reduced filopodia number and length coincide with DAAM accumulation
115 along the shaft and on the tip of filopodia (Goncalves-Pimentel et al., 2011; Jaiswal et al.,
116 2013a; Matussek et al., 2008). Although formins are primarily considered as actin assembly
117 machineries, numerous studies demonstrated that some formins are able to interact with MTs
118 either directly or indirectly (Bartolini and Gundersen, 2010; Bartolini et al., 2008; Dahlgaard
119 et al., 2007; Gaillard et al., 2011; Gasteier et al., 2005; Rosales-Nieves et al., 2006; Roth-
120 Johnson et al., 2014; Shaye and Greenwald, 2015). Since the highly conserved FH2 was
121 identified as the main MT interacting domain in DRFs, it is reasonable to assume that DAAM
122 might also be able to interact with MTs. Therefore, to further elaborate on the functions of
123 DAAM, we reassessed its sub-cellular localization by performing immunostaining of
124 endogenous DAAM in cultured primary neurons. We found that, although DAAM primarily
125 localizes to F-actin rich structures, i.e. the filopodia, lamellipodia and cortical actin (Fig. S1
126 A-D, I, I'; Movie 1), a significant fraction of DAAM accumulated **in punctae** along the
127 axonal MTs (Fig. 1A-D, Fig. S1 E-H). To estimate the sub-cellular distribution of DAAM, we
128 quantified the protein-protein proximity index (PPI) (Wu et al., 2010; Zinchuk et al., 2011)
129 between DAAM and actin, and DAAM and tubulin, respectively. The analysis revealed that
130 ~47% (PPI: 0.47 ± 0.05) of DAAM is associated with the actin cytoskeleton. Surprisingly
131 however, another ~37% (PPI: 0.37 ± 0.05) exhibited an overlap with MTs (Fig. 1E). Most
132 notably, we detected frequent DAAM accumulation on axonal MT ends (36 ± 14 %) (Fig. 1F).
133 These observations were further verified with a second, independently created DAAM
134 antibody (Fig. S3 G), leading to very similar results (Fig. S1 E-H). Thus, besides actin,
135 DAAM is often localized to MTs in axonal growth cones pointing towards MT related
136 functions.

137 To begin to address whether DAAM interacts with MTs, GST-tagged DAAM
138 fragments were purified and subsequently used in an MT co-sedimentation and a GST pull-
139 down assay to test for direct interaction (Figs. 1G, H and S2 A-E). Both assays indicated that,
140 unlike the purified GST protein used as negative control, a DAAM fragment comprising of
141 the FH1 and FH2 domains was able to bind to taxol-stabilized MTs, while mutation of the
142 conserved amino acid (I732A) playing a critical role in actin interaction, did not affect the
143 interaction between GST::FH1FH2 and MTs. In addition to the FH2 domain, the C-terminal
144 region of some formins was also shown to contribute to MT-binding (Gaillard et al., 2011;
145 Roth-Johnson et al., 2014; Young et al., 2008), thus we also investigated the effect of the C-
146 terminal region of DAAM. It appeared that the DAD domain alone did not bind to MTs,
147 however the C-terminal tail (CT) region has an MT-binding capability. It has been shown that
148 MT binding by the tail region of different formins is mediated by ionic interactions between
149 the basic amino acids of the tail domain and the acidic MT surface (Gaillard et al., 2011;
150 Roth-Johnson et al., 2014). The CT domain of DAAM is also basic with a pI of 11.6., and in
151 line with former data, a mutant CT fragment (GST::DAD-CT^{R-A}) where the basic amino acids
152 were changed to alanines was not able to bind to taxol-stabilized MTs.

153 In addition to direct MT binding, several formins were shown to associate with MTs
154 through +TIP binding. Consistent with its MT plus end localization (Fig. 1F), DAAM was co-
155 immunoprecipitated with EB1 (an abundant +TIP in this system) from lysates of S2 cells
156 transfected with 3xFlag-tagged constitutively active DAAM, lacking the DAD domain (Fig.
157 1I). Collectively, these studies indicate that *Drosophila* DAAM, similarly to other formins,
158 has the ability to physically interact with MTs via its FH2 as well as CT domains. Besides
159 direct interaction, DAAM copurifies with EB1 suggesting an alternative mode of MT binding,
160 presumably restricted to MT plus ends.

161

162 **DAAM regulates the length and organization of axonal microtubule bundles**

163 The sub-cellular localization and the *in vitro* assays suggest that DAAM might play a role in
164 the regulation of MT organization during axon growth. Therefore, we next asked how loss of
165 DAAM affects MT morphology in primary neurons. Since the maternal DAAM product is
166 present in the embryos (Matusek et al., 2006), we generated primary neuronal cultures from
167 DAAM^{mat/zyg} mutant embryos (Matusek et al., 2008) in which maternal and zygotic DAAM
168 functions are both impaired. Previously we demonstrated that these neurons are able to
169 develop axons similar to the wild type control neurons, although the number and length of

170 growth cone filopodia are significantly decreased (Matusek et al., 2008). In the present study
171 we measured the length of the axonal MT bundles after tubulin staining, using the
172 semiautomatic NeuronJ plugin (Meijering et al., 2004), and revealed that axonal MT bundles,
173 and consequently, axons are significantly ($p=0.016$) shorter in the $DAAM^{mat/zyg}$ mutant neurons
174 ($9.67\pm 0.75\mu\text{m}$, $n=152$) as compared to wild type controls ($16.36\pm 2.2\mu\text{m}$, $n=235$) (Fig. 2A-B'
175 and F). Moreover, we also studied MT morphology (Fig. 2E and G), and noted that in
176 $DAAM^{mat/zyg}$ mutant neurons the frequency of looped and spread MTs is decreased, while the
177 frequency of bundled MTs is increased significantly ($p=0.0286$) (Fig. 2G).

178 To further elaborate on the role of DAAM, we overexpressed an activated form of
179 DAAM (CDAAM) lacking its N-terminal regions including the DID domain crucial for
180 autoinhibition (Liu et al., 2008), by using the pan-neuronal *elav-Gal4* driver. Similar to
181 $DAAM^{mat/zyg}$ mutant neurons, the axonal MT bundles were shorter ($p=0.0011$) in CDAAM
182 expressing cells ($14.88\pm 0.21\mu\text{m}$, $n=178$) as compared to controls ($21.51\pm 1.15\mu\text{m}$, $n=182$)
183 (Fig. 2C-D' and F). In contrast to $DAAM^{mat/zyg}$ neurons, the frequency of looped and spread
184 MTs increased, while the frequency of bundled MTs decreased in CDAAM expressing cells
185 (Fig. 2G). Thus, the loss- and gain-of-function studies both indicate a role in the regulation of
186 axonal microtubule length and organization.

187

188 **DAAM regulates axonal microtubule dynamics**

189 As deviations in the organization of axonal MTs are often linked to impaired MT dynamics,
190 we next sought to examine MT dynamics *in vivo*, in the absence of DAAM. Microtubule
191 dynamics were quantified by using ImageJ's TrackMate plugin (Tinevez et al., 2016),
192 following live acquisition of high-resolution images of neurons (6-9 hours *in vitro*, HIV)
193 expressing EB1::GFP, a marker of MT plus ends (Morrison et al., 2002; Stepanova et al.,
194 2003) (Figs. 3A, B and S3 H). Time lapse recordings were deconvolved and pre-filtered with
195 a custom made difference of Gaussian filter to enhance the signal of interest and to exclude
196 false positive hits (Fig. S3 A-B''; Movie 2). The analyses of EB1::GFP track velocity in wild
197 type neurons revealed that MTs tend to slow down upon entering the growth cones, showing a
198 slight, but consistent growth velocity difference between the growth cone ($6.54\mu\text{m}\times\text{min}^{-1}$)
199 and the axonal shaft MTs ($8.12\mu\text{m}\times\text{min}^{-1}$) (Fig. 3C). For this reason, in the following studies
200 MT dynamics in growth cones and axonal shafts were analyzed separately. Because EB1 is
201 known to promote MT growth by preventing MT catastrophes (Komarova et al., 2009), as a
202 further control we compared MT growth velocity in cultured neurons expressing either

203 EB1::GFP or Jupiter::GFP (another widely used marker of microtubule dynamics) (Karpova
204 et al., 2006). We measured the dynamic instability parameters of individual MTs in the
205 growth cone and found no significant differences between EB1::GFP or Jupiter::GFP
206 (6.47 ± 1.5 , $n=8$) expressing neurons (Fig. S3 C-F), and hence, during our subsequent studies
207 we used EB1::GFP to monitor MT dynamics.

208 First we compared microtubule growth velocities in control and *DAAM*^{mat/zyg} mutant
209 neurons. We found that the median growth velocity measured in *DAAM*^{mat/zyg} mutant neurons
210 was ~91% higher in the growth cones and ~80% higher in the axon shaft compared to the
211 controls ($p < 0.0001$ for both) (Fig. 3D; Movie 3). Parallel to that, lifetime measurements of
212 growth cone MTs by utilizing the Kaplan-Meier estimator revealed a reduction in MT lifetime
213 of the mutant neurons ($p < 0.0001$) (Fig. 3E). The presence of DAAM seems to reduce MT
214 growth rate and to enhance lifetime by reducing catastrophe frequency. Thus, these
215 experiments showed that *DAAM* affects MT dynamics in neurites, which is likely to be the
216 cause of the altered MT organization phenotypes observed previously. In line with former
217 observations of Bartolini et al., 2008 for mDia2, it appears that increased MT dynamics is
218 paralleled with reduced lifetime that would be consistent with a role in MT stabilization.

219

220 **DAAM is involved in retrograde translocation of microtubules in growth cone filopodia**

221 To better understand the mechanisms of how DAAM affects MT dynamics, we focused our
222 attention to the individual pioneer microtubules, which extend into the growth cone periphery.
223 Pioneer microtubules are of particular importance, since their entry into the filopodia and their
224 subsequent stabilization is essential prior to growth cone turning (Buck and Zheng, 2002) and
225 axon branching (Dent and Kalil, 2001). Expression of EB1::GFP is a suitable tool to follow
226 the plus end of these MTs (Fig. 4A) as in most cases it is obvious when they enter filopodia,
227 where they subsequently disappear at some point along their length, presumably because the
228 MT has either stopped growing or depolymerized. To follow pioneer MT growth, we recorded
229 kymographs along the axis of filopodia, then we calculated the directionality distributions of
230 these graphs to differentiate between anterograde displacement, pause and retrograde
231 translocation (Figs. 4B-C', E-F' and S3 H-L). We observed that the EB1::GFP signal
232 frequently paused and/or moved rearwards in the growth cone filopodia of wild type cells
233 (Fig. 4B, B'). Given that EB1 decorates only the polymerizing end of MTs, it suggests that
234 these MTs underwent retrograde translocation which is a commonly observed phenomenon in
235 the growth cone periphery (Schaefer et al., 2008). It is thought that MTs tend to couple to the
236 retrograde actin flow through transient interactions and they are transported back as they

237 assemble (Marx et al., 2013) (consistently, in our primary neurons the retrograde MT
238 translocation rate appears similar to that of retrograde actin flow, $\sim 5.3\mu\text{m}\cdot\text{min}^{-1}$). Therefore,
239 the retrograde actin flow poses a dynamic barrier to pioneer microtubules and removes them
240 from the growth cone periphery (Schaefer et al., 2002; Schaefer et al., 2008). Remarkably,
241 however, in *DAAM^{mat/zyg}* mutant neurons we rarely observed pause or retrograde translocation
242 of MTs in the filopodia (Fig. 4C, C'). The frequency of these events was significantly lower
243 as compared to control cells ($p=0.02$) (Fig. 4D-F'). Therefore, we conclude that, at least in the
244 pioneer MTs, the lack of DAAM reduces the retrograde translocation rate of MTs which is
245 consistent with the increased MT growth velocity.

246

247 **Direct versus indirect effects on microtubule dynamics upon loss of DAAM**

248 Since formins were originally described as actin cytoskeleton regulators, the question arises as
249 to whether the microtubule cytoskeleton related phenotypes are direct effects of the absence
250 of DAAM or the consequence of the impaired actin cytoskeleton. To elucidate the role of the
251 actin cytoskeleton on MT dynamics, we disturbed the actin cytoskeleton by latrunculin A, a
252 drug which sequesters G-actin and promotes depolymerization (Coue et al., 1987; Yarmola et
253 al., 2000). Primary neurons expressing Actin5C::GFP treated with 200nM latrunculin A
254 showed signs of actin cytoskeleton breakdown in the growth cone as early as 30-60 seconds
255 after treatment. This depolymerization plateaus after ~ 5 minutes when the stereotypic
256 searching movements of filopodia completely disappear (Fig. S4 A-D; Movie 4). After
257 establishing the F-actin breakdown protocol, we applied the same conditions to primary
258 neurons expressing EB1::GFP, and measured MT growth dynamics in the 5-20 minute zone
259 after treatment. We found that median growth velocity is increased both in the axon shaft and
260 the growth cone region (Fig. 3D; Movie 5). Although this increase was similar to the one
261 observed in *DAAM^{mat/zyg}* neurons, we noted the formation of long, slender microtubule
262 containing protrusions, which was not observed in *DAAM^{mat/zyg}* neurons (Fig. S4 E-I; Movie
263 5). These results indicate that the F-actin network forms a barrier to MT growth in axons
264 which was already reported in growth cones (Burnette et al., 2007; Forscher and Smith, 1988;
265 Zhou and Cohan, 2004) but seems to apply for the shaft as well. Although the latter may
266 imply the existence of an unexplored actin/MT crosstalk in axonal shafts, subsequently we
267 focused our studies on the peripheral growth cone.

268 We reasoned that the changes in MT dynamics upon loss of DAAM might result from
269 a slower actin retrograde flow or a change in the coupling of microtubules to the retrograde
270 flow, or a combination of these two effects. To begin to test these alternatives, we compared

271 the retrograde actin flow in filopodia of wild type and *DAAM* mutant neurons. First, we
272 followed growth-cone motility by measuring filopodial dynamics in neurons expressing
273 Actin5C::GFP (Movie 6). Live recordings (7-9 HIV) revealed that the Actin5C::GFP signal
274 was slightly fainter in *DAAM^{mat/zyg}* neurons as compared to wild type neurons, and in
275 accordance with previous findings (Matusek et al., 2008), filopodia number and length were
276 reduced (Fig. 5B, C). To quantify the dynamic properties of growth cone filopodia, we
277 recorded kymographs along the axis of the filopodia and calculated their extension and
278 retraction velocity based on their tip displacement as a function of time. The mean extension
279 velocity elevated by 28% (not significant) whereas the mean retraction rate decreased by 33%
280 ($p=0.0096$) in the *DAAM^{mat/zyg}* mutant neurons as compared to wild type (Fig. 5E, F).
281 Moreover, by analyzing the fluorescent speckle pattern (Waterman-Storer and Salmon, 1998)
282 (Fig. 5D, D') of the Actin5C::GFP signal, we determined that the mean velocities of the
283 retrograde actin flow remained constant as the filopodia extended or retracted (Bornschlögl et
284 al., 2013). The kymographs also revealed that the rate of the retrograde flow highly correlates
285 with the retraction rate of the filopodia, both in wild type and *DAAM^{mat/zyg}* neurons. Consistent
286 with the reduced filopodia retraction rate, retrograde flow is decreased by 37.5% (not
287 significant) in the *DAAM^{mat/zyg}* neurons (Fig. 5G). Since the extension and retraction rates of
288 the growth cone filopodia depend on the retrograde flow and the growth rate of the filopodial
289 F-actin bundle, i.e. the polymerization rate of newly incorporated actin at the filopodial tip,
290 we could calculate the plus end dynamics of the actin filaments during filopodia extension
291 and retraction (Fig. 5A, H). We found that during filopodia extension the plus end growth of
292 actin filaments exceeds the retrograde flow, and this rate is 20% faster (not significant) in
293 *DAAM^{mat/zyg}* neurons as compared to wild type (Fig. 5I). In most filopodia the plus end of the
294 actin filament appears to be stalled during retraction both in wild type and *DAAM^{mat/zyg}*
295 neurons (Fig. 5J), therefore the retraction completely depends on the retrograde flow.

296 In summary, we revealed that although the actin depolymerizing drug, latrunculin A,
297 had similar effects on MT growth dynamics as to the lack of *DAAM*, it also induced the
298 formation of long, filopodia-like protrusions, never observed in *DAAM* mutant neurons, and
299 henceforth these effects are clearly not identical. Moreover, we found that the absence of
300 *DAAM* has a very weak effect on the retrograde actin flow, while the retrograde MT
301 translocation frequency exhibits a considerable reduction in the mutant neurons. Therefore,
302 these data suggest that the effect of *DAAM* on MT dynamics is unlikely to be an indirect (or
303 largely indirect) effect due to altered actin dynamics (i.e. reduced retrograde actin flow),
304 instead they may indicate a failure in proper coordination of actin and MT dynamics.

305

306 **DAAM stabilizes microtubules *in vitro* and *in vivo***

307 Consistent with findings for some other members of the formin protein family (Bartolini et
308 al., 2008; Chesarone et al., 2010; Gaillard et al., 2011), DAAM appears to promote MT
309 stabilization in primary neurons as suggested by our MT dynamics studies. To test it more
310 directly, we examined whether DAAM protects against MT destabilization in two different
311 conditions. Firstly, an MT co-sedimentation assay was used to measure the effect of cold
312 treatment on preassembled MTs in the presence or absence of purified GST::FH1FH2 protein.
313 In accordance with our expectation, the presence of FH1FH2 was able to protect MTs against
314 cold-induced depolymerization indicated by the elevated tubulin level in the pellet (Fig. 6D,
315 E). As a parallel approach, we next asked how loss of DAAM affects MT stability. To this
316 end we induced MT depolymerization by nocodazole treatment of *DAAM^{mat/zyg}* primary
317 neuronal cultures and compared them to control treated neurons. Control cells treated with
318 nocodazole exhibited only a few areas along the axon which were devoid of microtubules
319 (Fig. 6A-A' and C). In contrast, nocodazole treated *DAAM^{mat/zyg}* primary neurons had a two
320 fold increase in the number of brakes along axonal microtubules (Fig. 6B-B' and C). In
321 conclusion, DAAM behaves as a potent MT stabilizing factor in two diverse assays, and its N-
322 terminally truncated, FH1FH2 form already seems sufficient for stabilization, at least in *in*
323 *vitro* conditions.

324

325 **DAAM mediates the co-alignment of microtubules and actin filaments**

326 To test the possibility of DAAM mediated coupling between MTs and F-actin, we first
327 performed *in vitro* low speed sedimentation experiments adapted from Elie et al. 2015 (Fig.
328 7A-C). Phalloidin-stabilized F-actin and/or taxol-stabilized MTs were centrifuged on a
329 sucrose cushion in the absence or presence of DAAM constructs. This strategy ensures that
330 individual polymers (either F-actin or MTs) and F-actin bundles assembled by DAAM remain
331 in the supernatant, only larger polymer complexes can sediment and appear in the pellet (Elie
332 et al., 2015). Accordingly, in the presence of GST::CDAAM MTs were detected in the pellet,
333 whereas in the presence of both F-actin and MTs a fraction of F-actin cosedimented with
334 microtubules in the GST::CDAAM containing samples (Fig. 7C). Intrinsic to the experimental
335 conditions, F-actin can only appear in the pellet if it is physically linked to MTs, therefore
336 these observations imply a simultaneous interaction between CDAAM and that of the two
337 polymer systems. In contrast to CDAAM, actin or MT polymers did not sediment in the

338 presence of GST::FH1-FH2 or the C-terminal DAD-CT, DAD or DAD-CT^{R-A} constructs (Fig.
339 7C and S4 J), suggesting that the FH2 and the CT regions are both required for F-actin and
340 MT crosslinking yet neither is sufficient alone.

341 To further test the F-actin/MT coordinating ability of DAAM, TIRFM experiments
342 were carried out with fluorescently labeled (Alexa568NHS) F-actin and MTs (labeled with
343 HiLyte488) (Fig. 7D-F'''). For quantification the F-actin area co-localized with MTs, as well
344 as the MT area co-localized with F-actin were derived (Elie et al., 2015) (Fig. 7G, H). In
345 control samples lacking DAAM, ~ 13 % of F-actin and MTs appeared co-aligned (n = 19)
346 (Fig. 7D-D'''), and the presence of GST::FH1-FH2 did not change this ratio (n = 18) (Fig.
347 7F-F'''). On the contrary, GST::CDAAM increased the fraction of co-aligned polymers to ~
348 40 % (n = 22) (Fig. 7E-E'''), corroborating that DAAM has the ability to promote actin and
349 MT co-alignment. In agreement with this, our actin/microtubule/DAAM colocalization
350 studies revealed that ~22% (PPI: 0.224±0.04) of DAAM is associated with both the actin and
351 MT cytoskeleton (Fig. 8A-D), which means that the majority of MT associated DAAM
352 (~37%) is also colocalized to the actin cytoskeleton. Together the capacity of DAAM to
353 simultaneously bind the two cytoskeletal systems *in vitro* and its axonal localization along the
354 overlapping regions strongly suggest that DAAM is involved in the coordination of the actin
355 and MT cytoskeleton.

356

357

358 DISCUSSION

359 Proper coordination of the actin and microtubule cytoskeleton is thought to be key to growth
360 cone advance, yet the molecular mechanisms of actin-MT crosstalk during axonal growth
361 remained largely elusive. Here we report our findings suggesting that the formin family
362 protein DAAM is an important novel factor of actin-microtubule coordination in neuronal
363 growth cones. Formins are extensively characterized for their ability to interact with G-actin
364 and promote their assembly into F-actin. These studies established that the FH2 domain binds
365 actin strongly, whereas some formins contain an additional, albeit much weaker actin binding
366 motif in their DAD-CT region (Gould et al., 2011; Vizcarra et al., 2014). In accordance with
367 recent findings for other formins, we found that, besides actin binding (Barko et al., 2010), the
368 FH2 domain of DAAM is also able to interact with MTs. Moreover, we showed that the
369 positively charged CT region but not the DAD domain is also capable of MT binding.
370 Considering the dimeric nature of the formin proteins, the full length DAAM protein contains
371 multiple actin and MT binding sites. However, how these interacting elements act in a
372 concerted fashion is still elusive. The CDAAM fragment that contains all the mapped
373 cytoskeleton interacting domains of DAAM, exhibits an F-actin bundling activity *in vitro*, and
374 it is also able to crosslink actin filaments with MTs. In the concentration range where purified
375 CDAAM is able to co-align F-actin and MTs, neither FH1-FH2 nor DAD-CT possesses these
376 activities. This suggests a synergic action of these regions, for which the polymer binding
377 ability of the FH2 domain seems to be substantial. Based on these *in vitro* data the question
378 arises as to which of these activities are relevant *in vivo*, particularly, in growth cones. With
379 regard to actin bundling, although not distributed uniformly, DAAM is detected along the
380 filopodial actin bundles that would be in harmony with a role in filament bundling. Whereas
381 majority of the DAAM protein associates either with actin filaments or with MTs, it is
382 remarkable that a large population displays a DAAM/actin/MT triple colocalization in growth
383 cones, possibly indicating a role in crosslinking or co-aligning. In further support of this, the
384 effect of *DAAM* on MT dynamics and organization can also be explained by assuming that in
385 the absence of *DAAM* the two cytoskeletal systems are decoupled from each other.

386 Organization of the actin and MT cytoskeleton is intimately linked to each other in
387 most cellular processes examined. Therefore, perturbation of any of the two systems is likely
388 to indirectly affect the other one as well. It follows that actin regulators, such as formins, may
389 affect cellular MT organization by indirect mechanisms besides or instead of direct effects.
390 This has so far been a relatively poorly treated, yet a critical question when studying the role

391 of formins in MT regulation. We attempted to address this issue by comparing the effect of an
392 actin depolymerizing drug to the lack of DAAM in primary neurons. We found an increased
393 MT growth speed upon Lat-A treatment in the growth cone which was very similar to what is
394 observed in the absence of *DAAM*. However, we also noticed that Lat-A treatment induced
395 the formation of long, filopodia-like protrusions that has never been detected in *DAAM*
396 mutant cells. Thus it appears that partial depolymerization of the neuronal actin cytoskeleton
397 has a comparable, yet not identical, effect on MT dynamics as to the loss of formin function,
398 suggesting that an indirect effect cannot be excluded. Nevertheless, a number of observations
399 argue against that the effect of DAAM on growth cone MT organization and dynamics would
400 be exclusively or largely indirect. First, DAAM is often colocalized with MTs and it clearly
401 has the ability to bind to MTs. Second, DAAM is able **to associate with** MTs even when its
402 actin binding is compromised. Third, the loss of *DAAM* strongly reduces the retrograde MT
403 translocation frequency but only weakly affects the retrograde actin flow. These results,
404 together with that of the actin/MT crosslinking activity detected *in vitro* and the
405 DAAM/actin/MT triple colocalization observed in growth cones, strongly suggest that the
406 major function of DAAM is related to the coordination of actin and MT dynamics.

407 One intriguing characteristic of the interaction between DAAM and microtubules is
408 the ability to bind MTs directly as well as to associate with MTs through +TIPs. The major
409 MT related effects of DAAM, such as MT stabilization and actin-MT crosslinking, can
410 potentially be explained by direct MT binding that would consequently impact on MT
411 dynamics. In addition, DAAM, bound to MTs, may interact with other microtubule associated
412 proteins that would offer further regulatory possibilities. On the other hand, +TIP binding is
413 equally exciting, particularly in light of recently discussed models of growth cone advance
414 (Bearce et al., 2015; Cammarata et al., 2016), and data revealing how MT plus-ends trigger
415 formin-dependent rapid actin assembly *in vitro* and in motile cells (Henty-Ridilla et al., 2016;
416 Jaiswal et al., 2013b; Okada et al., 2010). While in the classical models of growth cone
417 guidance actin dynamics has been credited as the major driving force of motility and as the
418 primary target of guidance signaling, subsequent studies suggested that the peripheral pioneer
419 MTs also act as guidance sensors, leading to the hypothesis that the closely coupled regulation
420 of actin and MT dynamics is at the heart of guidance signaling (Bearce et al., 2015;
421 Cammarata et al., 2016; Coles and Bradke, 2015). Meanwhile, +TIPs, many of which are able
422 to interact both with F-actin and MTs, were shown to act as signal transducers during growth
423 cone guidance, and they emerged as key regulators of actin/MT coordination downstream of

424 axon guidance signaling. Parallel to this, it has been established that some +TIPs collaborate
425 with the formin mDia1, that enables MT plus-ends to govern localized actin assembly *in vitro*
426 and in fibroblast cells (Henty-Ridilla et al., 2016; Jaiswal et al., 2013b; Okada et al., 2010),
427 and that the interaction of CLIP170 with an unknown formin is important for dendritic arbor
428 formation in primary neurons (Henty-Ridilla et al., 2016). Given that DAAM is present at MT
429 plus-ends and it associates with EB1 in S2 cells, we propose that DAAM is a strong candidate
430 to promote actin assembly in concert with +TIPs. Thus, our data reinforces the importance of
431 a formin/+TIP module as a general mechanism of linking actin and MT dynamics, and most
432 importantly, we provide the first *in vivo* evidence that such a mechanism is at work in axonal
433 growth cones.

434 In addition to the potential to promote actin polymerization at MT plus-ends, we found
435 that DAAM retains an MT stabilizing activity both *in vitro* and in primary neurons, and the
436 FH2 domain seems sufficient for MT stabilization. This is similar to the case of several other
437 formins and therefore it might be a shared feature of this protein family. Although the
438 physiological importance of formin mediated MT stabilization in the context of neuronal
439 growth remains largely unknown, DAAM clearly appears to possess multiple capabilities to
440 influence the cytoskeleton due to its actin nucleation and elongation, MT stabilization, MT
441 plus end binding and actin-MT crosslinking activities. Strikingly, perhaps with the exception
442 of actin assembly, many other +TIPs exhibit related biochemical properties. Based on this we
443 propose that the unique or key function of DAAM in orchestrating the growth cone
444 cytoskeleton is facilitating the formation of new actin filaments at the plus-ends of pioneer
445 MTs (Fig. 8 E-G). We think that this step is controlled by the navigation cues which also
446 coordinate a multitude of cytoskeletal responses, including other transient actin-MT
447 interactions, through other actin- and/or MT-binding proteins, such as (+) TIPs, to guide
448 axons in a complex cellular environment. Whether DAAM is an essential factor in all neurons
449 or formins act redundantly in this context, awaits future elucidations.

450

451 MATERIALS AND METHODS

452

453 **Fly stocks and genetics**

454 For mutant analysis *DAAM^{Ex1}* or *DAAM^{Ex1};elav-Gal4,UAS-EB1::GFP* and
455 *DAAM^{Ex68},Ubi::GFP/ Y^{Dp(1;Y)Sz280}* lines were crossed as described previously (Matusek et al.,
456 2008). In addition we used the following stocks: *w¹¹¹⁸*, *w;elav-Gal4*, *w;UAS-DADm-*
457 *DAAM::GFP* (Matusek et al. 2008), *w; UAS-CDAAM*, *Jupiter::GFP* (Karpova et al., 2006),
458 *w;elav-Gal4,UAS-EB1::GFP* and *UAS-Actin5C::GFP* (Roper et al., 2005).

459

460 **Molecular biology and antibody production**

461 For S2 cell expression of Δ DAD-DAAM we used a pAWF- Δ DAD-DAAM destination clone
462 created by standard methods. For bacterial protein expression the DAD, DAD-CT and
463 FH1FH2^{1732A} constructs were generated by using standard cloning and *in vitro* mutagenesis
464 methods in the pGex2T vector. The DAD-CT^{R-A} mutant construct was created by gene
465 synthesis (Sangon Biotech), and then cloned into pGex2T (Fig. S2 F). Primers are listed in
466 supplementary Table 1.

467 The novel DAAM antibody (Rb#4938) was generated in rabbit after immunization
468 with purified FH1FH2 produced in bacteria. The sera were collected, and the IgG fraction was
469 purified on ProteinG agarose beads. Specificity of the antibody was confirmed by Western
470 blot analysis (Fig S3 G).

471

472 **Cell cultures and immunohistochemistry**

473 *Drosophila* primary neuronal cells were obtained from stage 11 embryos as published in
474 Sanchez-Soriano et al., 2010. S2 cells were grown in Schneider's media supplemented with
475 10% heat inactivated FBS.

476 For transfection of S2 cells, 2×10^6 cells were seeded in 6-well plates and then
477 transfected with pAct5C- Δ DAD-DAAM::3xFlag construct using Effectene transfection
478 reagent (Qiagen).

479 Cultured neurons were fixed at 6 HIV, stained as described in Matusek et al., 2008.
480 The following primary antibodies were used: mouse anti- α -tubulin (1:1000), rabbit anti-
481 dDAAM (1:1000, Matusek et al. 2006), chicken anti-GFP (1:1000, Abcam). Actin was
482 labelled with either Alexa-488- or Alexa-546-phalloidin (ThermoScientific).

483 For treatments with the microtubule destabilizing drug nocodazole, neurons were
484 plated onto coverslips coated with 0.5 mg/ml Concanavalin A (Sigma) and kept at 22 °C for

485 12 h. After 12 h, neurons were treated with either nocodazole (100 μ M; Sigma) or vehicle
486 (both diluted in Schneider's medium) for 4 h at 22 °C.

487

488 **Co-immunoprecipitation and Western blots**

489 Co-immunoprecipitation was carried out as described previously (Gombos et al., 2015).
490 Lysates of transfected S2 cells were incubated with rabbit anti-EB1 antisera, then samples
491 were incubated with protein-A magnetic beads (Bio-Rad). Eluted proteins were analyzed by
492 Western blot using a standard procedure. Rabbit anti-EB1 (1:2000, gift from H. Ohkura),
493 mouse anti-Flag (1:1000, M2, Sigma-Aldrich), mouse anti- α -tubulin (1:10,000, DM1A,
494 Sigma-Aldrich), anti-rabbit IgG-HRP (1:10000, Jackson) and anti-mouse IgG-HRP (1:5000,
495 Dako) were used for Western blots.

496

497 **Protein expression and purification**

498 *Drosophila* DAAM constructs were expressed and purified as GST-tagged proteins as
499 described (Barko et al., 2010). Actin was purified from rabbit skeletal muscle and labeled
500 with Alexa Fluor® 568 carboxylic acid succinimidyl ester (Alexa568NHS, Invitrogen)
501 (Kellogg et al., 1988; Spudich and Watt, 1971). Mg²⁺-ATP-actin filaments (F-actin) and
502 phalloidin-stabilized F-actin were prepared as described (Toth et al., 2016). Taxol-stabilized
503 MTs were prepared from unmodified and/or Hilyte FluorTM 488 labeled tubulin
504 (Cytoskeleton, Inc.).

505

506 **MT/DAAM-binding assays**

507 *MT co-sedimentation*

508 MTs were prepared from tubulin protein (Cytoskeleton, Inc.) following the vendor's
509 instructions. Purified GST (4 μ M) or GST-tagged dDAAM fragments (0.25 - 4 μ M) were
510 incubated with taxol-stabilized MTs (0.5 μ M) for 30 min at room temperature in MT binding
511 buffer (MBB; 10 mM HEPES pH 7.0, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 20 μ M taxol,
512 0.5 mM thesit). Samples were centrifuged at 100,000 g for 25 min. The pellets were washed
513 in MBB then resuspended in SDS-PAGE sample buffer. Proteins in the supernatants and
514 pellets were resolved by SDS-PAGE and stained by colloidal Coomassie blue.

515

516 *GST pull-down assay*

517 GST and GST-tagged DAAM fragments were expressed and purified from a small volume
518 culture (2 mL) as described above. The proteins were not eluted from glutathione beads,
519 instead the beads were incubated with pre-assembled MTs (0.5 μ M) for 30 min in MBB.
520 Beads were washed in MBB then the proteins were eluted and analyzed by SDS-PAGE and
521 Western blot.

522

523 **MT stability assay**

524 The cold-induced depolymerization assay was carried out as described (Bartolini et al., 2008).
525 Tubulin was polymerized in the presence of 4 μ M GST or GST::FH1FH2 proteins. After
526 polymerization matching aliquots of samples were centrifuged as described for MT co-
527 sedimentation. The remaining samples were incubated on ice, then centrifuged at 4 °C.
528 Proteins in the supernatants and pellets were resolved by Coomassie-stained SDS-PAGE and
529 the gels were quantified by using densitometry (ImageJ).

530

531 **MT/F-actin crosslinking assay**

532 Since both CDAAM and FH1-FH2 induces F-actin bundles, which sediment at very
533 low speed, we adapted the sedimentation protocol used by Elie et al 2015 to test the ability of
534 DAAM to interact simultaneously with F-actin and MTs. Phalloidin-stabilized F-actin (2 μ M)
535 and/or taxol-stabilized MTs (2 μ M) were incubated with DAAM constructs (9 μ M) in BRB-K
536 at room temperature for 40 min. Samples were loaded onto a 30 % sucrose cushion and
537 centrifuged (4000 g, 10 min, 25°C). Under these conditions individual polymers, as well as F-
538 actin bundles do not sediment, only larger filament complexes (MT bundles, MT-F-actin
539 copolymers) appear in the pellet (Elie et al., 2015). Pellets and supernatants were analyzed by
540 SDS-PAGE. In control experiments based on high-speed centrifugation (100.000 g, 20 min,
541 25°C) both F-actin and MTs appeared in the pellet, which confirms that both polymers exist
542 under the applied experimental conditions, and the lack of sedimentation of the polymers is
543 not due to depolymerization.

544

545 **Microscopy and image analysis**

546 Confocal images were captured either on a Zeiss LSM880 or on an Olympus FV1000 LSM.
547 Images were restored using the Huygens Professional software (Scientific Volume Imaging).
548 To visualize the F-actin and MT structures induced by DAAM, phalloidin-stabilized F-actin
549 (0.4 μ M containing 10 % Alexa568NHS-actin) and taxol-stabilized MTs (0.4 μ M containing
550 10 % Hilyte FluorTM 488-tubulin) were incubated with DAAM constructs (1 μ M) in BRB-K

551 at room temperature for 30 min. Samples were diluted in BRB-K* (BRB-K supplemented
552 with 0.2 % (w/v) methylcellulose, 0.5 % (w/v) BSA, 50 mM 1,4-diazabicyclo-[2,2,2]octane
553 (DABCO) and 100 mM DTT), applied onto poly-L-lysine-treated (Sigma Aldrich) coverslips
554 and visualized by TIRF microscopy (Olympus IX 81). Microtubule-F-actin co-localization
555 was quantified as described (Elie et al., 2015) (3 – 4 independent experiments at each
556 conditions, 18-22 images). For nocodazole treatment in primary neurons, images were
557 captured with a Nikon Eclipse 90i microscope equipped with a high resolution CCD Camera
558 (Retiga 3000). Images were processed using ImageJ software.

559 MT dynamics were analyzed from EB1::GFP time lapse recordings, using TrackMate
560 (v3.3.0). To allow particle detection and faithful tracking the spatial and temporal resolution
561 of the live recordings were set to 100 nm/voxel and 0.9 sec/frame, respectively. Filopodial
562 actin dynamics measurements were performed on 7-9 HIV neurons expressing Actin5C::GFP.
563 Imaging of the neurons was performed in glass bottom petri dishes (MatTek corporation) in
564 growth media. Filopodia with recognizable extension and retraction events were selected for
565 further analysis.

566

567 **Statistics and figures**

568 Statistical analysis was carried out using Prism 5 (GraphPad Software Inc.). D'Agostino-
569 Pearson omnibus test was used to assess the normality of the data. Pairwise comparisons were
570 made using either the Student's t test or the Mann-Whitney U test according to the normality.
571 ANOVA was used for multiple comparisons; $p < 0.05$ was considered as statistically
572 significant. Figures and cartoons were edited in Illustrator CS4 (Adobe).

573

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578

579 COMPETING INTERESTS

580 No competing interests declared.

581

582 AUTHOR CONTRIBUTIONS

583 P.H., B.B. and J.M. conceived and supervised the study; S.S., I.F., K.T., E.M.,A.V., B.B.,
584 **P.K.** and J.M. performed the experiments; S.S., I.F., B.B., **N.S.S.** and J.M. analyzed data;
585 S.S., I.F., B.B. and J.M. wrote the manuscript.

586

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599

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778

779

780 FIGURE LEGENDS

781 **Figure 1. Axonal localization and physical interaction of DAAM with microtubules.**

782 (A-D) Growth cone of a primary neuron (6 HIV), labeled against F-actin, tubulin and DAAM.
783 Asterisk marks filopodial DAAM localization. Arrows point to DAAM puncta localized at the
784 end of microtubules. **Arrowheads show DAAM localization along the MT bundles.** (E)
785 Scatterplots show the protein-protein proximity index measured between DAAM and F-actin,
786 and DAAM and tubulin. Grey dots represent values measured on individual cells (DAAM-
787 Actin, 0.46 ± 0.14 , mean \pm s.d. n=40; DAAM-Tubulin, 0.38 ± 0.15 , mean \pm s.d. n=40). Black dots
788 represent the median of the independent experiments with their median. (F) Bar diagram
789 shows the frequency of microtubule end localization of DAAM ($36.18 \% \pm 13.57 \%$,
790 mean \pm s.d.; measured on **100 MT ends** from three independent experiments). (G) Domain
791 structure of full length DAAM and DAAM fragments used in this study. (H) **MT co-**
792 **sedimentation assay shows that MTs physically interact with the FH1FH2 fragment of**
793 **dDAAM.** (I) Co-IP shows that Δ DAD-DAAM::3xFlag co-precipitates specifically with EB1
794 from S2 cell lysates.

795 **Figure 2. DAAM affects axonal microtubule length and morphology.**

796 (A-D) Representative images of primary neurons (6 HIV) derived from wild type (*white*¹¹¹⁸)
797 (A, A'), *DAAM*^{mat/zyg} (see materials and methods) (B, B'), *Elav-Gal4* (*w;elav-Gal4/+*) (C, C')
798 or *CDAAM* (*w;elav-Gal4,UAS-CDAAM/+*) (D, D') expressing embryos. (E) Examples for
799 classification of growth cone microtubules as bundled, looped or spread. (F) Scatterplots
800 show the length of the axonal microtubules of primary neurons. Grey dots represent values
801 measured on individual cells (wild type: $15.42 \mu\text{m}$, n=235; *DAAM*^{mat/zyg}: $9.61 \mu\text{m}$, n=152;
802 *Elav-Gal4*: $20.3 \mu\text{m}$, n=182; *CDAAM*: $13.8 \mu\text{m}$, n=178; median with interquartile range).
803 Black dots represent the median of the independent experiments with their median. (G)
804 Boxplots show the frequency of axonal microtubule morphology classes. Unpaired *t*-tests
805 were used for statistical analysis.

806 **Figure 3. The effect of DAAM on axonal microtubule dynamics.**

807 (A) Representative image of a primary neuron expressing EB1::GFP (6 HIV) (time-lapse
808 projection). The axon and the growth cone are distinguished by dotted rectangles. (B) Time-
809 lapse projection of recognized EB1::GFP tracks. The tracks are color coded according to their
810 median velocity (from yellow to cyan, $0 \mu\text{m} \times \text{min}^{-1}$ – $30 \mu\text{m} \times \text{min}^{-1}$). (C) Scatterplots show

811 significant velocity difference between the axonal and growth cone EB1::GFP comets (growth
812 cone: $6.54 \mu\text{m}\times\text{min}^{-1}$, n=64; axon: $8.12 \mu\text{m}\times\text{min}^{-1}$, n=60; median with interquartile range).
813 **(D)** Scatterplots show the median velocity of EB1::GFP tracks measured on primary neurons
814 derived from EB1::GFP expressing wild type and *DAAM^{mat/zyg}* embryos. It is also compared to
815 EB1::GFP expressing wild type neurons treated with latrunculin A (growth cone - wild type:
816 $6.54 \mu\text{m}\times\text{min}^{-1}$, n=64; *DAAM^{mat/zyg}*: $12.53 \mu\text{m}\times\text{min}^{-1}$, n=57; latrunculin A treated: 11.16
817 $\mu\text{m}\times\text{min}^{-1}$, n=18) (axon - wild type: $8.12 \mu\text{m}\times\text{min}^{-1}$, n=60; *DAAM^{mat/zyg}*: $14.64 \mu\text{m}\times\text{min}^{-1}$,
818 n=52; latrunculin A treated: $13.85 \mu\text{m}\times\text{min}^{-1}$, n=15; median with interquartile range). Dots
819 represent the median velocity of the EB1::GFP tracks measured on individual cells. Mann
820 Whitney test was used to compare the respective pairs. **(E)** Survival analysis was used to
821 compare microtubule lifetime of wild type and *DAAM^{mat/zyg}* neurons expressing EB1::GFP.
822 Short tracks (below 3.6 second) were discarded from the analysis and the threshold is marked
823 with a dotted line. The Mantel-Cox test was used to compare the survival curves (white,
824 n=219; *DAAM^{mat/zyg}*, n=161).

825 **Figure 4. The effect of DAAM on retrograde MT translocation**

826 **(A)** The cartoon illustrates a growth cone filopodia invaded by a microtubule where the
827 growing plus end is labeled with EB1::GFP. **(B-C')** Representative kymographs demonstrate
828 microtubule plus end displacement along growth cone filopodia in wild type **(B, B')** and
829 *DAAM^{mat/zyg}* **(C, C')** neurons expressing EB1::GFP. Red arrowheads mark the point where
830 microtubules stall or undergo retrograde translocation. Dotted lines indicate where pioneer
831 microtubules enter the growth cone filopodia. **(D)** Boxplot shows the frequency of
832 microtubule retrograde translocation in wild type and *DAAM^{mat/zyg}* neurons (*white*: 18.9 ± 6.4 ,
833 n=219; *DAAM^{mat/zyg}*: 6.3 ± 2.5 , n=161; mean \pm s.d, from four independent experiments). **(E-F')**
834 Summary of extracted kymographs from wild type **(E)** and *DAAM^{mat/zyg}* **(F)** neurons
835 expressing EB1::GFP. **(E', F')** Histograms show the summarized directionality distribution,
836 measured on the extracted kymographs.

837 **Figure 5. Effect of DAAM on axonal filopodia and actin dynamics.**

838 **(A)** A cartoon to illustrate filopodia dynamics (extension and retraction) and the underlying
839 actin dynamics (actin polymerization, retrograde flow). **(B, C)** Axons of Actin5C::GFP
840 expressing **(B)** wild type and **(C)** *DAAM^{mat/zyg}* neurons (6 HIV). The growth cones are marked
841 by a dotted red circle. **(D)** Filopodia with unevenly incorporated Actin5C::GFP; Scale bar:
842 200nm. **(D')** A kymograph revealing the actin retrograde flow meanwhile the filopodia

843 extends and retracts. **(E-G)** Scatterplots show filopodia extension (wild type: $5.28 \mu\text{m}\times\text{min}^{-1}$,
844 $n=134$; $DAAM^{mat/zyg}$: $6.75 \mu\text{m}\times\text{min}^{-1}$, $n=64$), retraction (wild type: $6.82 \mu\text{m}\times\text{min}^{-1}$, $n=91$;
845 $DAAM^{mat/zyg}$: $3.38 \mu\text{m}\times\text{min}^{-1}$, $n=117$) and actin retrograde flow (wild type: $5.64 \mu\text{m}\times\text{min}^{-1}$,
846 $n=114$; $DAAM^{mat/zyg}$: $2.93 \mu\text{m}\times\text{min}^{-1}$, $n=97$) rates measured in wild type and $DAAM^{mat/zyg}$
847 neurons expressing Actin5C::GFP. Grey dots represent the values of individual events. Black
848 dots represent the median of independent experiments. Mann Whitney test was applied for
849 statistical analysis. **(H)** Scheme of a hypothetical kymograph recorded along filopodia
850 depicted on panel A. **(I, J)** Scatterplots show actin plus end dynamics during filopodia
851 extension (wild type: $5.28 \mu\text{m}\times\text{min}^{-1}$, $n=134$; $DAAM^{mat/zyg}$: $6.75 \mu\text{m}\times\text{min}^{-1}$, $n=64$) and
852 retraction rates (wild type: $6.82 \mu\text{m}\times\text{min}^{-1}$, $n=91$; $DAAM^{mat/zyg}$: $3.38 \mu\text{m}\times\text{min}^{-1}$, $n=117$)
853 measured in wild type and $DAAM^{mat/zyg}$ neurons expressing Actin5C::GFP. Grey dots
854 represent the values of individual events calculated based on the actin retrograde flow and
855 filopodia extension and retraction rates.

856 **Figure 6. DAAM stabilizes MTs *in vivo* and *in vitro***

857 **(A-B')** Images show axonal MT bundles of DMSO (A, B) or nocodazole (A', B') treated
858 control and $DAAM^{mat/zyg}$ primary neurons (12 HIV). Arrows point to gaps along axonal
859 MT bundles. **(C)** Quantification of the number of breaks along the MT bundles of
860 control (DMSO treated: 1 ± 0.2 , mean \pm s.e.m., $n=213$; nocodazole treated: 6.7 ± 0.8 ,
861 mean \pm s.e.m., $n=206$) and $DAAM^{mat/zyg}$ primary (DMSO treated: 2.3 ± 0.4 , mean \pm s.e.m.,
862 $n=157$; nocodazole treated: 12 ± 1.3 , mean \pm s.e.m., $n=137$) neurons, measured in 3
863 independent experiments. **(D, E)** Densitometric analysis of SDS-PAGE gels **(D)** show
864 that the amount of tubulin in the pellet is higher in samples with GST::FH1FH2 **(E)**
865 after cold-induced depolymerization as compared to controls (mean \pm s.d., $n=3$, **
866 $P<0.01$).

867 **Figure 7. F-actin and microtubule co-alignment mediated by DAAM.**

868 **(A-C)** Representative Coomassie-stained SDS-PAGE gels from low-speed centrifugation
869 experiments showing the amount of MTs and F-actin in the supernatants (S) and in the pellets
870 (P) in the absence or presence of either GST::CDAAM or FH1-FH2, as indicated. **(D-F)**
871 Representative fluorescence micrographs of F-actin (cyan) and MTs (red) in the absence or
872 presence of GST::CDAAM and GST::FH1FH2, as indicated. Yellow regions on the merged
873 images highlight the overlapping F-actin and MT regions. Binary images show the
874 overlapping polymer area. **(G, H)** Ratio of the co-aligned F-actin and MT area in the absence

875 or presence of GST::CDAAM or GST::FH1FH2 (average of 3–4 independent experiments is
876 shown).

877 **Figure 8. DAAM colocalizes with overlapping F-actin and microtubules.**

878 **(A-D)** A representative growth cone showing DAAM puncta on overlapping F-actin and MTs
879 (white rectangle). **(B)** The yellow dotted line marks the outline of the growth cone. **(C)** Image
880 shows microtubules (red) and microtubules overlapping with actin filaments (grey). Inlet
881 shows DAAM puncta (magenta) localized on overlapping F-actin and microtubules. **(D)**
882 Scatterplots show the protein-protein proximity index measured between DAAM and the
883 overlapping F-actin and microtubules. Grey dots represent values measured on individual
884 cells (0.22 ± 0.1 , mean \pm s.d. $n=40$). Black dots represent the median of the independent
885 experiments with their median. **(E) Schematic model of the growth cone cytoskeleton. (F,**
886 **G) Localization and proposed functions of DAAM in growth cones based on former**
887 **studies and the current study: 1) actin barbed end binding, actin assembly, 2) F-actin**
888 **bundling, 3) MT side binding (stabilization), 4) EB1 binding, 5) F-actin and MT**
889 **coalignment. Our current work suggests that the key function of DAAM is to**
890 **facilitate/boost the formation of new actin filaments at the MT plus-ends through**
891 **interactions with EB1.**