Using fly genetics to dissect the cytoskeletal machinery of neurons during axonal growth and maintenance

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Summary
The extension of long slender axons is a key process of neuronal circuit formation, both during brain development and regeneration. For this, growth cones at the tips of axons are guided towards their correct target cells by signals. Growth cone behaviour downstream of these signals is implemented by their actin and microtubule cytoskeleton. In the first part of this Commentary, we discuss the fundamental roles of the cytoskeleton during axon growth. We present the various classes of actin- and microtubule-binding proteins that regulate the cytoskeleton, and highlight the important gaps in our understanding of how these proteins functionally integrate into the complex machinery that implements growth cone behaviour. Deciphering such machinery requires multidisciplinary approaches, including genetics and the use of simple model organisms. In the second part of this Commentary, we discuss how the application of combinatorial genetics in the versatile genetic model organism Drosophila melanogaster has started to contribute to the understanding of actin and microtubule regulation during axon growth. Using the example of dystonin-linked neuron degeneration, we explain how knowledge acquired by studying axonal growth in flies can also deliver new understanding in other aspects of neuron biology, such as axon maintenance in higher animals and humans.

Key words: Growth cone, Cytoskeleton, Axon, Drosophila, Actin, Microtubule, Brain disorders

Introduction
The cytoskeleton consists of actin, intermediate filaments (IFs) and microtubules (MTs), and has fundamental roles in virtually every function of cells, including cell division, motility, adhesion, signalling, endocytic trafficking and transport, as well as in the regulation of cell and organelle shapes and their distributions (Akhmanova and Stearns, 2013). Naturally, this also applies to the nervous system where the cytoskeleton has essential roles during the development, function, regeneration and degeneration of neurons. Of the more than 200 entries on the OMIM (Online Mendelian Inheritance in Man) database for genes encoding cytoskeleton-associated proteins, 44% have indexed links to human disorders, and 54% of these are linked to nervous system disorders, including neurodevelopmental disorders (e.g. lissencephalies, mental retardations), functional disorders (e.g. deafness) and a wide range of degenerative diseases (see supplementary material Table S1). Therefore, research of the cytoskeleton provides unique opportunities to unravel fundamental mechanisms of the nervous system, both in health and disease.

It is currently little understood how cytoskeleton-associated proteins contribute to cellular mechanisms of neurons. For example, the systemic importance of certain MT-binding proteins, such as motor proteins and the structural protein tau, has long been recognised from their involvement in a broad spectrum of neurodegenerative diseases, and we know their principal molecular functions. However, we have yet to explain conclusively how they function within normal or diseased neurons (Hirokawa et al., 2010; Morris et al., 2011). Here, we argue that this is best performed starting with processes for which there is already an extensive body of knowledge about the cytoskeleton, in particular the growth of axons.

Axons are slender neuronal extensions, which can be several metres long, and are often arranged into nerves or nerve tracts that provide essential ‘information highways’ in the nervous system. The proper function of nervous systems requires axons to grow and wire up correctly during development or regeneration, and these connections need to be maintained for decades in the ageing body. Here, we review the current concepts for the cellular roles that actin and MTs have in axons and discuss important gaps in our understanding of how the cytoskeleton is regulated to this end. We argue that genetics provides important means to advance this understanding, and explain how studies using the genetic model organism Drosophila melanogaster can make important contributions. We review recent advances with regard to actin and MT regulation during axonal growth in the fly and provide an example of how mechanistic understanding in one context can be used to identify mechanisms underpinning other processes, such as axon maintenance and neurodegeneration.

The organisation of the cytoskeleton in axons
Mature axons are the longest cellular entities that are produced by animals. They propagate electrical messages, which travel from the neuronal cell bodies towards the synaptic connections with their distant target cells, often several metres away. To maintain...
this unusual architecture, shafts of axons contain prominent bundles of parallel MTs, which provide structural support as well as the tracks for the vital long-distance cargo transport (Twelvetrees et al., 2012). Axonal MTs are discontinuous and are usually not attached to the centrosome (the main microtubule organizing centre of most cells); their plus ends mostly, but not entirely, point distally (Fig. 1A) (Ahmad et al., 1999; Baas and Lin, 2011; Basto et al., 2006; Stiess et al., 2010). The cortex of axon shafts is lined by short stabilised actin filaments (Fig. 1A), which are organised into repetitive rings by the scaffolding protein spectrin and linked to the axonal cell membrane through ankyrins (Xu et al., 2013). Axons also contain a number of different IF proteins that can regulate the specific diameters of different axon classes (Perrot and Eyer, 2009).

Axons are usually maintained for the lifetime of an organism, i.e. for decades in humans. It is therefore not surprising that axonal decay is a characteristic feature in the ageing brain (Marner et al., 2011; Basto et al., 2006; Stiess et al., 2010). The cortex of the growth cone from where splayed MTs emanate into the actin-rich peripheral zone (Dent et al., 2011). The tip of the axonal MT bundle forms the central zone of the growth cone from where splayed MTs emanate into the actin-rich peripheral zone (Dent et al., 2011). In typical vertebrate neurons, several neurites are formed simultaneously on the cell body, but only one of these is selected as the axon. This selection process (referred to as neuron polarisation) involves signalling cascades leading to the targeted polymerisation events in mature axons (Kollins et al., 2009). Such dynamics might allow for structural plasticity, such as shortening or extension of the axon shaft (Küppers-Munther et al., 2004; Lamoureux et al., 2010; Rajagopalan et al., 2010), pruning and regeneration of axons or formation of new side branches (Letourneau, 2009), which are required for rewiring processes during learning and memory formation or the regeneration of damaged axons (Bradke et al., 2012; Saxena and Caroni, 2007).

**Cellular roles of the cytoskeleton during axonal development**

During development, axons are initiated as small neuritic protrusions on the surface of neuronal cell bodies. For this, MTs in the neuronal cell bodies become stabilised and push beyond the cell cortex (Brandt, 1998). This is assisted by dynamic rearrangements of the cortical actin cytoskeleton to remove barrier function (Flynn et al., 2012; Neukirchen and Bradke, 2011; Saengsawang et al., 2012) and the formation of filopodia, which provide membrane protrusions into which MTs can extend (Dent et al., 2007; Gonçalves-Pimentel et al., 2011). In typical vertebrate neurons, several neurites are formed simultaneously on the cell body, but only one of these is selected as the axon. This selection process (referred to as neuron polarisation) involves signalling cascades leading to the targeted

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**Fig. 1. Principal organisation and roles of actin and MTs in growing axons.** (A) The figure shows the principal distribution and organisation of MTs and F-actin networks in the soma, axon and growth cone of a neuron that is exposed to an attractive guidance cue. The tip of the axonal MT bundle forms the central zone of the growth cone from where splayed MTs emanate into the actin-rich peripheral zone (Dent et al., 2011). (B) Directed axon extension (growth cone turning). Splayed MTs become preferentially stabilised in a certain direction through guidance cues (or experimentally through unilateral exposure to MT-(de)-stabilising drugs or the application of an electrical field). Additional MTs follow in that direction, leading to engorgement of membrane structures in that area and, eventually, the establishment of a new axon segment (Buck and Zheng, 2002; Lowery and Van Vactor, 2009; McCaig et al., 2002; Sabry et al., 1991; Tanaka and Kirschner, 1995; Tanaka and Kirschner, 1991). (C) Growth cone turning requires splayed MTs: low doses of MT-stabilising drugs suppress the occurrence of splayed, side-stepping MTs (splayed MT). Growth cones that are treated this way fail to show normal turning behaviour at repulsive borders and stall instead (Challacombe et al., 1997; Williamson et al., 1996); they also no longer respond to unidirectional application of MT-stabilising drugs (Buck and Zheng, 2002). (D-E) Growth cone turning requires F-actin. When F-actin is destabilised through drugs (F-act-), MTs tend to be bundled and growth cones fail to turn at repulsive borders (Challacombe et al., 1996); they also no longer respond to unidirectional application of MT-stabilising drugs (Buck and Zheng, 2002). (F) Growth cone turning towards the cathode in electrical fields (galvanotropism) does not require F-actin (McCaig, 1989); this turning could be mediated by MTBPs, which are attracted to the cathode through positive net-charge of their MT-binding domains, thus dragging the MTs along with them.
localisation of cell polarity factors (Barnes and Polleux, 2009) and also depends on the specific stabilisation of MTs in the selected neurite (Brandt, 1998; Chen et al., 2013).

Once established, axons grow along stereotypic paths to their target cells, a process that is implemented by prominent hand-shaped growth cones at the axon tips. Growth cones are guided towards their specific target areas and cells through precise spatio-temporal patterns of chemical signals that are arranged along their paths (Tessier-Lavigne and Goodman, 1996). Many guidance cues and their receptors have been identified and shown to mediate repulsion or attraction (Araújo and Tear, 2003; Huber et al., 2003), thus controlling growth cone behaviours, such as growth velocity, pausing, turning, retraction or collapse. These morphogenetic movements downstream of guidance signalling are implemented by the prominent actin and MT cytoskeleton of growth cones (Fan et al., 1993), which is arranged in a particular spatial geometry.

The geometry of growth cones can be subdivided into an actin-rich peripheral zone and a MT-rich central zone (Fig. 1A), and the area in which MTs and F-actin prominently overlap is often referred to as the transition zone. The central zone of growth cones comprises the tips of the axonal MT bundles, and from here single MTs splay out into the peripheral zone. The peripheral zone consists of actin-rich membrane protrusions, which include finger-like filopodia (containing parallel F-actin bundles) and veil-like lamellipodia (containing F-actin lattices; Fig. 1A). Filopodia and lamellipodia sense guidance cues by presenting their receptors on their surfaces. They undergo constant shape changes, driven by continuous assembly of actin filaments at the leading edges, the retrograde flow of these filaments, and their depolymerisation and recycling further back in the transition zone (Box 1). This actin dynamics allow filopodia to reach out into the growth cone environment and act as signalling and adhesion sensors (Chien et al., 1993; Davenport et al., 1993; Dwivedy et al., 2007; Mattila and Lappalainen, 2008). They allow lamellipodia to act as dynamic sites of substrate adhesion and myosin-II-driven contraction and force generation, which are important in influencing MT behaviours during guided axon extension (Box 2).

In current models of growth cone guidance (Fig. 1B) (Dent et al., 2011; Lowery and Van Vactor, 2009), external signals trigger the directional stabilisation of single MTs in the peripheral zone. These stabilised MTs are then extended by the extension of the bulk of MTs from the central domain (a process referred to as engorgement), thus giving rise to a new axon segment. This elongation step is completed when lamellipodia and filopodia relocate distally to the newly formed axon tip (Fig. 1B). In this model, MTs are the essential implementers of axon extension, whereas F-actin is required for the directionality of this extension (through influencing MT behaviours; Box 2). Accordingly, blocking MT dynamics inhibits axon growth (Letourneau and Ressler, 1984; Sánchez-Soriano et al., 2010; Tanaka et al., 1995), whereas the pharmacological or genetic inhibition of actin polymerisation, either in cultured neurons or in vivo, does not usually suppress axonal growth per se, but abrogates growth cone turning and pathfinding (Fig. 1D,E) (Buck and Zheng, 2002; Challacombe et al., 1996; Chien et al., 1993; Kaufmann et al., 1998; Marsh and Letourneau, 1984; Sánchez-Soriano et al., 2010). This said, a directional movement of growth cones does not always require F-actin (Fig. 1F), as some signalling mechanisms can target MTs directly. For example, GSK3-mediated phosphorylation of the MTBPs APC1, MAP1B and/or CLASP is known to directly regulate MT stability in growth cones (Hur et al., 2011; Lucas et al., 1998; Purro et al., 2008).
In vitro F-actin networks generate membrane protrusions on all sides of growth cones that can be invaded by MTs in any direction. This facilitates lateral extension and stabilisation of MTs as an important prerequisite for growth cone turning (see also Fig. 1B).

F-actin networks can influence MT behaviours mechanically (Lee and Suter, 2008; Schaefer et al., 2002) (see also Fig. 1A). Thus, MT extension is antagonised by F-actin backflow or through the formation of transverse bundles (arcs) that form ‘road-blocks’. MT extension can be promoted and guided through the formation of radial F-actin bundles as tracks for MT elongation. MTs can be pushed laterally or medially through actomyosin contractions. Finally, MT extension can be channelled into certain directions through coordinated loss of F-actin from local spots within lamellipodia.

Actin networks form scaffolds that serve as a platform for ABPs. Some of these ABPs influence MT behaviours through mediating actin-MT linkage. This linkage can be direct through ABPs that also contain MT-binding domains [e.g. MAP1B, spectraplakins, coronin 7 (POD1 in Drosophila), cytoplasmic linker-associated proteins (CLASPs) or adenomatous polyposis coli (APC) (Bouquet et al., 2007; Moseley et al., 2007; Rothenberg et al., 2003; Sánchez-Soriano et al., 2009; Tsvetkov et al., 2007)]. Other ABPs are involved in indirect crosstalk with MTs by interacting with MTBPs. Reported examples are the ABP drebrin that is linked to the MTBP EB3, the ABP PPP1R9A (better known as spinophilin) that binds to DCX, IQGAP that interacts with CLIP-170, or functional interactions between the actin-binding motor protein myosin II and the MT-associated dynein motor protein complex (Bielas et al., 2007; Geraldo et al., 2008; Myers et al., 2006; Swiech et al., 2011).

Some ABPs can tether actin networks to transmembrane receptors when they are engaged with their extracellular ligands. This has been demonstrated for different classes of transmembrane receptors (Giannone et al., 2009; Moore et al., 2009; Moore et al., 2010). Such linkage of F-actin to the extracellular environment generates mechanical forces across the membrane, which can trigger intracellular signalling events (referred to as the ‘clutch’ mechanism) that then can influence actin and MT dynamics (Suter and Fischer, 2000; Suter and Miller, 2011).

The importance and challenge of understanding cytoskeletal machinery

Clearly, the cytoskeleton has essential roles during axonal growth, but we still do not understand how it is regulated to perform these functions. Different classes of actin-binding proteins (ABPs) and MTBPs are known to regulate cytoskeletal dynamics (Box 2); many of these are expressed in neurons (Table 1), and we often have a reasonable understanding of their molecular mechanisms, at least in vitro. However, we do not understand how these individual regulators functionally integrate into the common cytoskeletal machinery that ultimately implements neuronal behaviours. This deficit also leaves us with little means to explain the cellular aberrations caused by mutations of genes encoding ABPs or MTBPs that have been linked to brain disorders, including disorders of neurodevelopment and axonal growth (supplementary material Table S1) (Nugent et al., 2012; Tischfield et al., 2011).

The need to understand cytoskeletal regulation at the cellular level in neurons and non-neuronal cells alike is a current problem (Fletcher and Mullins, 2010; Insall and Machesky, 2009). This is clearly reflected by the number of review articles that attempt to propose models explaining the functional integration of cytoskeletal regulators (e.g. Conde and Cáceres, 2009; Dent et al., 2011; Gallo, 2013; Lowery and Van Vactor, 2009; Pak et al., 2008). For example, there are mechanistic models aiming to explain how different classes of ABPs functionally combine during filopodia formation (Mattila and Lappalainen, 2008) and models based on end-binding (EB) proteins that propose how an increasing number of MT-plus-end-associating proteins can regulate MT dynamics (Akmanova and Steinmetz, 2008; Akmanova and Steinmetz, 2010) (see also Fig. 2). Currently, such models depend on knowledge gained from a broad range of different neuronal systems, and they therefore remain hypothetical and are difficult to test experimentally.

In this Commentary, we promote strategic considerations that will help to overcome some of the current shortcomings in explaining cytoskeletal machinery. We believe that stronger emphasis must be given to studying the various parts of cytoskeletal machinery consistently in the same neuron systems, so that their functional links and interfaces can be directly assessed. These neuronal systems should not only be amenable to biochemical, biophysical and cell biological approaches, but also to the use of combinatorial genetics, therefore allowing a systematic assessment of different gene manipulations or combinations of mutations in the same cells. Such a strategy will help us to integrate the knowledge we have about single cytoskeletal regulators so that we can develop higher-order concepts about the governance of the cytoskeletal machinery during axon growth or in the context of brain disorders.

Drosophila as a model to study cytoskeletal machinery during axonal growth

One organism in which combinatorial genetics has been and continues to be most successfully applied to the study of complex biological problems is the fruit fly Drosophila melanogaster (Keller, 1996; Roote and Prokop, 2013). Work in Drosophila is low cost, impressively fast, and particularly amenable to genetic manipulations and unbiased genetic screens (Sánchez-Soriano et al., 2007). Accordingly, Drosophila research has been an incubator for new ideas and concepts in neurobiology, and work in the fly revealed many fundamental molecular and cellular mechanisms that have turned out to be conserved in higher animals (Bellen et al., 2010). Work in Drosophila has made important contributions to the understanding of axon growth, including the discovery and analysis of proteins involved in pathfinding, and the discovery of mechanisms underpinning pioneer guidance, nerve branching, target cell recognition and axonal transport (Araújo and Tear, 2003; Astigarraga et al., 2010; Brochtrup and Hummel, 2011; Landgraf and Thor, 2006; Sánchez-Soriano and Prokop, 2005; Sánchez-Soriano et al., 2007; Zala et al., 2013).

To study the cytoskeletal machinery during axon growth, fly neurons offer a number of useful features. First, most of the Drosophila ABPs and MTBPs display a high level of sequence conservation with their mammalian counterparts, and they are usually expressed in the nervous system (Table 1) (Sánchez-Soriano et al., 2007). Second, cytoskeletal regulators tend to

Box 2. How F-actin networks influence MT dynamics in growth cones

- F-actin networks generate membrane protrusions on all sides of growth cones that can be invaded by MTs in any direction.
- F-actin networks can influence MT behaviours mechanically (Lee and Suter, 2008; Schaefer et al., 2002) (see also Fig. 1A). Thus, MT extension is antagonised by F-actin backflow or through the formation of transverse bundles (arcs) that form ‘road-blocks’. MT extension can be promoted and guided through the formation of radial F-actin bundles as tracks for MT elongation. MTs can be pushed laterally or medially through actomyosin contractions. Finally, MT extension can be channelled into certain directions through coordinated loss of F-actin from local spots within lamellipodia.
- Actin networks form scaffolds that serve as a platform for ABPs. Some of these ABPs influence MT behaviours through mediating actin-MT linkage. This linkage can be direct through ABPs that also contain MT-binding domains [e.g. MAP1B, spectraplakins, coronin 7 (POD1 in Drosophila), cytoplasmic linker-associated proteins (CLASPs) or adenomatous polyposis coli (APC) (Bouquet et al., 2007; Moseley et al., 2007; Rothenberg et al., 2003; Sánchez-Soriano et al., 2009; Tsvetkov et al., 2007)]. Other ABPs are involved in indirect crosstalk with MTs by interacting with MTBPs. Reported examples are the ABP drebrin that is linked to the MTBP EB3, the ABP PPP1R9A (better known as spinophilin) that binds to DCX, IQGAP that interacts with CLIP-170, or functional interactions between the actin-binding motor protein myosin II and the MT-associated dynein motor protein complex (Bielas et al., 2007; Geraldo et al., 2008; Myers et al., 2006; Swiech et al., 2011).
- Some ABPs can tether actin networks to transmembrane receptors when they are engaged with their extracellular ligands. This has been demonstrated for different classes of transmembrane receptors (Giannone et al., 2009; Moore et al., 2009; Moore et al., 2010). Such linkage of F-actin to the extracellular environment generates mechanical forces across the membrane, which can trigger intracellular signalling events (referred to as the ‘clutch’ mechanism) that then can influence actin and MT dynamics (Suter and Fischer, 2000; Suter and Miller, 2011).
Table 1. Examples of evolutionary conserved MTBPs and ABPs in human and fly

| Cytoskeleton regulator class | MTBPs | ABPs |
|-----------------------------|-------|------|
| Nucleators                  | γ-tubulin ring complex (γ/Tub23C and other components); doublecortin (DCX-EMAP) | Arp2/3 complex (Arpc1, Arp3, etc.); formins (DAAM, Fhos, CG32183, dia) |
| Plus-end-binding proteins   | MAP4F/EB1 (EB1); MT-plus-end-tracking proteins (TIPs); doublecortin (DCX-EMAP); CAP5/CHTOG/XMAP215 | WASP/SCAR/WAVE* (SCAR); WASL/N-WASP* (WASP); APC* (Apc); β4-thymosin/TMSB4X (β4); profilin* (Chic); twinfilin* (Tufl) |
| Mono- or oligomer-binding proteins | Stathmin/SCG10* (Stai); DYSL2/CRMP2* (CRMP2); CAP5/CHTOG/XMAP215* (msps); tub-specific chaperone E* (tsec); CLASP* (chb) | Fasiclin* (Sv); α-actinin* (Actn); plastin/timbrin* (Fim); filamin* (Cher); tropomyosin* (Tm1); moesin*, ezrin*, radixin* (Moes); β/β'-spectrin* (β/β'-Spec); drebrin* (CG10083); coronin* (coro, podl); MLLT4/Afdadin-6* (cono); CTTN* (Cortactin); PPP1R9A* (spindlin) |
| Shaft stabilisers and cross-linkers | MAP2T* (tau); MAP2P* (tau); MAP1B* (futsch); MACF1*; dystonin* (shot) | Tropomodulin* (tmod); Arp2/3 complex (Arpc1, Arp66B, etc.) |
| Minus-end stabilisers | γ-tubulin ring complex (γ/Tub23C and other components); CAMSAP1* (Patronin) | Myosin XVA* (Myo1A4); myosin VA* (idiam); myosin II (zip, sqh) |
| Plus-end motors | Type 1 kinesins (Khc, Klc, Pair); type 2 kinesins (Klp64D, Klp68D, Kf3C, Kap3); type 3 kinesins (unc-104, Khc-73) | Myosin V* (jar) |
| Minus-end motors | cytoplasmic dynein/dynactin (Dhc64C, cip, sw, Gl, etc.); type 13 kinesins/KIF2C/MCAK* (Klp10A, Klp59C, Klp59D) | Cofilin* (tsr); gelsolin* (Gel) |
| Severs, de-polymerisers | katanin* (Kar60); spastin* (spas); type 13 kinesins/KIF2C/MCAK* (Klp10A, Klp59C, Klp59D) | ATP hydrolysis; potentially acetylation, arginylation, phosphorylation, etc. |
| Post-translational modifications (enzymes not listed) | GTP hydrolysis; acetylation; (poly-) glycylation; (poly-) glutamylation; de-tyrosination; de-glutamylation (Δ2 tubulin) |

Some proteins fulfil more than one function and are repeated in different positions; terms highlighted with an asterisk are listed in OMIM, other terms are not declared as orthologues in FlyBase in spite of partial sequence homologies.

Use of the fly to unravel new ABP functions during axonal growth

Within a few years, work in Drosophila growth cones has identified at least four new ABPs as actin regulators in neurons, including Shot, DAAM, Psdin, and Canoe. Shot is the only Drosophila member of the spectraplakin family of actin- and MT-binding proteins with two close homologues in mammals, MACF1 (also known as AC7) and dystonin (DST, also known as BPAG1) (Suozzi et al., 2012). Both Shot and AC7 promote filopodia formation (Sánchez-Soriano et al., 2009). In Shot, this function is relevant for axon guidance at the CNS midline, and it requires binding of the two EF-hand domains to the putative translational regulator Extra bases (Exba, also known as elf5C) (Fig. 2A) (Lee et al., 2007; Sánchez-Soriano et al., 2009), thus potentially contributing to local translation events in growth cones (Van Horck and Holt, 2008). DAAM (DAAM1 in mammals) is one of four Drosophila formins (Diaphanoous, DAAM, Fhos and CG32183) reported to be present in the nervous system (Paswan et al., 2008; Prokop et al., 2011; Sánchez-Soriano et al., 2007). DAAM has now been shown to be the essential formin in growth cones of embryonic neurons, where it promotes filopodia formation and axonal pathfinding in neurons after only 6 hours in culture (Prokop et al., 2012; Sánchez-Soriano et al., 2010). This said, alternative mechanisms to other ABPs may contribute to the regulation of formin-mediated growth cone dynamics (Mattie et al., 2010; Pawson et al., 2008). This repertoire can be further expanded when making manipulations of cytoskeletal regulators can be combined with a use of the fly to unravel new ABP functions during axonal growth.
the central nervous system (CNS) (Gonçalves-Pimentel et al., 2011; Matusek et al., 2008). Psidin (known as NAA25 in mammals) is the auxiliary subunit of the NatB acetylation complex required for protein acetylation (Stephan et al., 2012). In addition, it has been shown that Psidin can act outside this complex as an ABP that regulates actin dynamics both in neurons and in non-neuronal cells of Drosophila (Kim et al., 2011; Stephan et al., 2012). These studies show that Psidin antagonises F-actin stabilisation mediated by Tropomyosin 1 in lamellipodia, which is required for the pathfinding of olfactory neurons in the fly brain. Canoe and its mammalian homologue MLLT4 (also known as afadin, AFD6) are PDZ-domain-containing ABPs that localise to adherens junctions and have been shown to interact with profilin (Boettner et al., 2000; Lorger and Moelling, 2006; Sawyer et al., 2009). In Canoe-deficient embryos, axonal pathfinding at the CNS midline is impaired, and this aberration correlates with two growth cone phenotypes: an increase in filopodia numbers and a failure to localise Robo proteins (receptors that are important regulators of midline pathfinding) to filopodia (Slováková et al., 2012). In this scenario, Canoe

Fig. 2. Roles of Shot in MT regulation at the interface with other classes of ABPs and MTBPs. (A) The functional domains of Shot (below the structure) and their reported molecular interactions (above the structure). CH, calponin homology domains; plakin, plakin-like domain; SR-rod, spectrin repeat rod; EF, EF-hand motifs; GRD, Gas2-related domain; MtLS, microtubule tip localisation sequence; C-tail, C-terminal tail. (B) A close-up of a growth cone showing a single MT and three different aspects of the cytoskeletal machinery (boxed and numbered), all of which are being studied in fly neurons. (1) Regulation of MT plus-end dynamics; it includes end-binding proteins (here EB1) which directly bind MT plus ends and recruit MT-plus-end-tracking proteins (+TIPs) (blue arrows; see Akhmanova and Steinmetz, 2008; Etienne-Manneville, 2010). Shot has to compete with other +TIPs for binding to a limited pool of EB1; when bound to EB1, it links MT plus ends to F-actin (via its N-terminal CH domains) and guides polymerising MT plus ends in the direction of axon growth. The proper localisation of Shot at MT plus ends also requires direct association with MTs through its GRD and C-terminal tail. CLASPs and XMAP bind tubulin and promote MT polymerisation; CLASPs are +TIPs whereas XMAP215 (XMAP) binds MT plus-ends either directly or through +TIPs: Small spindles 2 (Ssp2) in Drosophila and Slains in mammals (not shown; Al-Bassam and Chang, 2011; Currie et al., 2011; van der Vaart et al., 2012; Li et al., 2011; Lowery et al., 2010). Stathmins (STMN) sequester tubulin (Manna et al., 2009), and doublecortin (DCX) binds MT plus ends independently (Bechstedt and Brouhard, 2012). (2) Actin dynamics. The Shot–actin linkage is influenced by actin regulators (red stippled arrows) that coordinate the dynamics and structure of F-actin networks (cortex, lamellipodia, filopodia; Pak et al., 2008; Xu et al., 2013). In the context of filopodia formation, Shot contributes to the regulation of F-actin through binding of Exba/eIF5C at its EF-hand motifs (Lee et al., 2007; Sánchez-Soriano et al., 2009; Alves-Silva et al., 2012). Other MT shaft binders are Tau which protects MTs against the MT-severing protein Katanin in axons (not shown; Qiang et al., 2006), and mammalian MAP1B can cross-link MTs as well as mediate actin-MT linkage during axon growth (Bouquet et al., 2007; Riederer, 2007). Tau, MAP1B and Shot (in an EB1-independent mode) influence MT polymerisation kinetics through yet unknown mechanisms (stippled grey arrow; Alves-Silva et al., 2012; Feinstein and Wilson, 2005; Tymanskyj et al., 2012).
might regulate the number of filopodia directly through its function as an ABP, or indirectly through acting as a scaffold for Robo receptors, since loss of Robo has been previously shown to lead to an increase in the number of filopodia (Murray and Whittington, 1999).

Use of the fly to study the functional integration of ABPs during filopodia regulation

*Drosophila* growth cones have been used to carry out loss-of-function analyses of other ABP-encoding genes, including *Arpc1* (ARP2), *Arpc3* (ARP3), *capping protein A* (CAPZA), *capping protein B* (CAPZB), chickadee (profilin), enabled (ENAH, also known as VASP), *singed* (fascin) and *tropomyosin 1* (TPM1) (human homologues are given in the brackets) (Gonçalves-Pimentel et al., 2011; Kraft et al., 2006; Sánchez-Soriano et al., 2010). All these data and tools can now be combined to understand the functional networks of ABPs, as is best illustrated for studies of filopodia formation. Filopodia are prominent actin-based structures, and data from numerous studies in a wide range of cellular models have led to the formulation of two composite models of filopodia formation (Mattila and Lappalainen, 2008).

In the ‘convergent elongation model’, Arp2/3 generates actin filaments in lamellipodia, and ENAH/VASP aggregates their plus ends to give rise to elongating filopodial bundles. In the ‘de novo nucleation model’, formin molecules aggregate to generate clusters of new actin filaments, which then directly elongate into parallel filopodial bundles.

*Drosophila* neurons were used to test whether these two models co-exist in neurons. Single-mutant analyses of the factors essential in these models (i.e. Enabled, the formin DAAM and different Arp2/3 complex components) all caused a partial but significant reduction in filopodia numbers, in agreement with the potential co-existence of both models (Gonçalves-Pimentel et al., 2011; Matussek et al., 2008). When loss of the Arp2/3 component Sop2 was combined with functional loss of DAAM in the same neurons (DAAM<sup>+/−</sup> Sop2<sup>−/−</sup> double-mutant neurons), filopodia were completely absent and F-actin levels were severely depleted (Gonçalves-Pimentel et al., 2011). To our knowledge, a genetic condition that depletes F-actin to this degree has not been reported for other eukaryotic systems. It indicates that Arp2/3 and DAAM are the prevailing nucleators in embryonic *Drosophila* neurons. The fact that the two nucleators enhance each other’s phenotypes indicates that they work in distinct complementary functional pathways during filopodia formation. This is consistent with the biochemical evidence that Arp2/3 and formins nucleate actin filaments through different molecular mechanisms (Chesarone and Goode, 2009; Mattila and Lappalainen, 2008).

However, it does not yet resolve whether these two nucleators contribute to two entirely separate processes of filopodia formation.

To make this distinction, genetic interaction studies using partial loss of gene functions are very helpful. For example, in heterozygous gene constellation (i.e. one mutant and one normal gene copy), the levels of the proteins they encode tend to be only moderately reduced, and, typically, this reduction is not sufficient to cause a phenotype. Accordingly, neurons that are heterozygous for DAAM, Sop2 or ena show normal filopodia numbers (Gonçalves-Pimentel et al., 2011). However, any combinations of these heterozygous conditions (transheterozygous mutant neurons: DAAM<sup>+/−</sup> Sop2<sup>−/+</sup> or DAAM<sup>+/−</sup> ena<sup>−/+</sup> or Sop2<sup>−/+</sup> ena<sup>−/+</sup>) become functionally rate-limiting and lead to a strong reduction in filopodia numbers (Gonçalves-Pimentel et al., 2011).

These findings are inconsistent with regard to whether two completely separate modes of filopodia formation exist, but do suggest that the pathways downstream of the two nucleators converge at some point. Therefore, these authors suggested a more general model of convergent elongation, in which not only Arp2/3-derived but also DAAM-derived actin filaments can be used by Ena to be transformed into filopodial actin bundles (Gonçalves-Pimentel et al., 2011). These examples illustrate how systematic genetic analyses used in *Drosophila* neurons can contribute to the formulation of new mechanistic models of cytoskeletal regulation.

Use of the fly to study mechanisms of actin–MT linkage during axonal growth and maintenance

Actin–MT crosstalk is an important aspect of cytoskeletal regulation in growing axons, but its mechanisms are little understood (Box 2). Promising contributions have been made by work on the above mentioned spectraplakins Shot and ACF7. Besides filopodia-associated phenotypes, we have shown that loss of Shot in fly neurons and loss of the mouse homologue ACF7 in mouse neurons result in another conserved phenotype – disorganised axonal MT bundles that is coupled with reduced axon growth (Sánchez-Soriano et al., 2009). Detailed work on Shot has shown that these functions depend on actin–MT linkage. Structure–function analyses have revealed that functions of Shot in MT organisation depend on three simultaneous molecular interactions (Fig. 2): (1) binding of its N-terminal calponin homology domains to F-actin, (2) association to MTs through two C-terminal domains (the Gas2-related domain and the positively charged C-terminal tail), and (3) binding of C-terminal tail to EB1 (end-binding protein 1) at MT plus ends (Alves-Silva et al., 2012; Bottenberg et al., 2009; Lee and Kolodziej, 2002; Sánchez-Soriano et al., 2009). These findings suggest a model in which Shot binds to the plus ends of polymerising MTs and guides them along actin structures in the direction of axon growth (Alves-Silva et al., 2012). This model is further supported by the observation that frequent off-track extensions of MTs are seen during live imaging of *shot* mutant neurons; this can explain the observed MT disorganisation and diminished axon growth (off-track MTs are less likely to contribute to axon extension). The guidance model is also supported by studies of ACF7-like MT-disorganisation phenotypes observed in EB1-deficient, as well as in *shor<sup>−/−</sup> eb1<sup>−/−</sup>* transheterozygous mutant neurons (Alves-Silva et al., 2012), and, furthermore, is consistent with proposed roles of ACF7 in guiding MT extension along actin stress fibres towards focal adhesions in non-neuronal cells (Kodama et al., 2004).

Notably, the MT guidance model offers a promising molecular platform for further research. On the one hand, it can be used to understand the roles of other cytoskeletal regulators during MT guidance, for example of ABPs or the various proteins that bind and regulate MT plus ends (see details in Fig. 2). On the other hand, the MT guidance model suggests a potential mechanism contributing to the maintenance of axons in the mature nervous system, as will be explained in the following.

The second mammalian homologue of Shot, dystonin, is strongly expressed in peripheral neural ganglia (Leung et al., 2001), which, in dystonin mutant mice, undergo a severe neurodegeneration at postnatal stages that is referred to as HSAN (hereditary sensory autonomic neuropathy) (Duchen et al., 2001).
1963; Duchen et al., 1964). The underlying mechanisms remain inconclusive and were proposed to be associated with roles of dystonin in intermediate filament organisation and axonal transport, and in ER or Golgi dysfunction (Ferrier et al., 2013; Young and Kothary, 2007). We propose instead that HSAN pathology might be primarily caused by defects in MT guidance and stabilisation on the basis of the following argument. Axonal MT bundles are believed to be stabilised through structural MTBPs, such as tau or MAP1B (Fig. 2) (Chilton and Gordon-Weeks, 2007). At the same time, there appears to be a steady and dynamic MT turnover, as suggested by continued MT polymerisation events in mature axons (Kollins et al., 2009). Spectraplakins can regulate both functions through two functionally conserved domains at their C-terminus: the Gas2-related domain can stabilise MTs against depolymerisation, whereas the C-terminal tail mediates the aforementioned binding to EB1 required for MT guidance. These domains jointly mediate a robust association along the shaft and at the tip of MTs that is required for both MT guidance and stabilisation (Fig. 2) (Alves-Silva et al., 2012; Honnappa et al., 2009; Sun et al., 2001). Absence of either domain in Shh causes severe MT disorganisation and loss of MT stability in axons of developing fly neurons (Alves-Silva et al., 2012; Sánchez-Soriano et al., 2009) and, in addition, mature sensory neurons of dystonin mutant mice, which are prone to degeneration display severe MT disorganisation and loss of MT stability (Ferrier et al., 2013; Yang et al., 1999). Consistently, a recently reported frame-shift mutation in the human dystonin gene functionally deletes the Gas2-related domain and the C-terminal tail and causes a severe form of HSAN that mirrors the degenerative pathology of dystonin mutant mice (Edvardson et al., 2012). These data suggest that spectraplakins perform similar MT-regulating functions during the development of fly neurons and the maintenance of sensory mammalian neurons. Severe MT disorganisation and instability caused by absence of spectraplakins can, therefore, be expected to affect vital functions such as axonal transport and impact negatively on neuronal survival (Sheng and Cai, 2012).

This example shows how functional studies of cytoskeletal regulators during axon growth in the fly can provide new ideas and testable hypotheses for research on other aspects of neuronal cytoskeleton, even in other animals.

Conclusions and future perspectives

In this Commentary, we have discussed how systematic and combinatorial genetic studies of different ABPs and MTBPs in the neuronal system of the fly can improve our understanding of cytoskeletal regulation during a biological process such as axon growth. Considering the high degree of evolutionary conservatism of actin, tubulin and their regulators, the enormous similarities of cytoskeletal dynamics in fly and vertebrate neurons, and the similarities of mutant phenotypes reported so far, we feel that insights gained in the fly system will be informative for cytoskeletal research in mammalian or other vertebrate neurons.

Understanding the cytoskeletal regulation that underpins cellular behaviours will have important implications and applications. For example, we still have little understanding of how signalling mechanisms instruct the cytoskeleton to influence growth cone behaviours (Huber et al., 2003). Knowing how the various ABPs and MTBPs that are targeted by particular signalling events contribute to the cellular cytoskeletal machinery will greatly help to close this knowledge gap. Understanding these cellular roles of cytoskeletal regulators is also the first step on the long path to developing mathematical models that can describe the cytoskeletal dynamics that are at the basis of different cellular processes (Oelz et al., 2008), and it will facilitate the introduction of systems approaches for cell biology research (Liberali and Pelkmans, 2012). Furthermore, the principal strategies described here for axon growth can also be applied to the investigation of other roles and regulation of the cytoskeleton in other relevant areas, such as synapse formation, axonal pruning and Wallerian degeneration, axon regeneration and neurodegeneration. Notably, for all of these processes, suitable *Drosophila* models have already been established, thus hopefully paving the way for rapid progress (Bosson et al., 2012; Fang and Bonini, 2012; Gistelink et al., 2012; Goellner and Aberle, 2012; Jaiswal et al., 2012).

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