

# Cytoskeletal dynamics in growth-cone steering

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## Summary

**Interactions between dynamic microtubules and actin filaments are essential to a wide range of cell biological processes including cell division, motility and morphogenesis. In neuronal growth cones, interactions between microtubules and actin filaments in filopodia are necessary for growth cones to make a turn. Growth-cone turning is a fundamental behaviour during axon guidance, as correct navigation of the growth cone through the embryo is required for it to locate an appropriate synaptic partner. Microtubule-actin filament interactions also occur in the transition zone and central domain of the growth cone, where actin arcs exert compressive forces to corral microtubules into the core of the growth cone and thereby facilitate microtubule bundling, a requirement for axon formation. We now have a fairly comprehensive understanding of the dynamic**

**behaviour of the cytoskeleton in growth cones, and the stage is set for discovering the molecular machinery that enables microtubule-actin filament coupling in growth cones, as well as the intracellular signalling pathways that regulate these interactions. Furthermore, recent experiments suggest that microtubule-actin filament interactions might also be important for the formation of dendritic spines from filopodia in mature neurons. Therefore, the mechanisms coupling microtubules to actin filaments in growth-cone turning and dendritic-spine maturation might be conserved.**

Key words: Actin filaments, Cytoskeleton, Filopodia, Growth cone, Microtubules, Pathfinding

## Introduction

The development of a properly connected, and hence functional, nervous system depends on the ability of neurons to guide their axons along appropriate routes in the embryo to locate a suitable synaptic partner, a process known as pathfinding. Axon guidance relies on the pathfinding abilities of the growth cone, which is a highly dynamic, sensory-motile structure at the tip of growing axons (Fig. 1). Growth cones pathfind by responding to molecular guidance cues that they encounter along their route. Guidance molecules attract or repel growth cones and are either locally tethered to the extracellular matrix or to a cell membrane, or encountered as a concentration gradient that diffuses from an intermediate or final target source (Chilton, 2006). Activation of receptors for guidance molecules in growth-cone plasma membranes drives intracellular signalling pathways that converge mainly at the growth-cone cytoskeleton (Kalil and Dent, 2005; Wen and Zheng, 2006; Zhou and Snider, 2006). The route that the growth cone must take to locate an appropriate synaptic partner is rarely a straight one and, at particular points along the route (choice points), growth cones must make steering manoeuvres to change direction – for example, when crossing the midline of the embryo. These steering manoeuvres necessitate reorganisation of the growth-cone cytoskeleton and thus it is not surprising that there is a convergence of signalling pathways at the growth-cone cytoskeleton. What these signalling pathways are and how they regulate the cytoskeleton has yet to be fully elucidated (Gordon-Weeks, 2004).

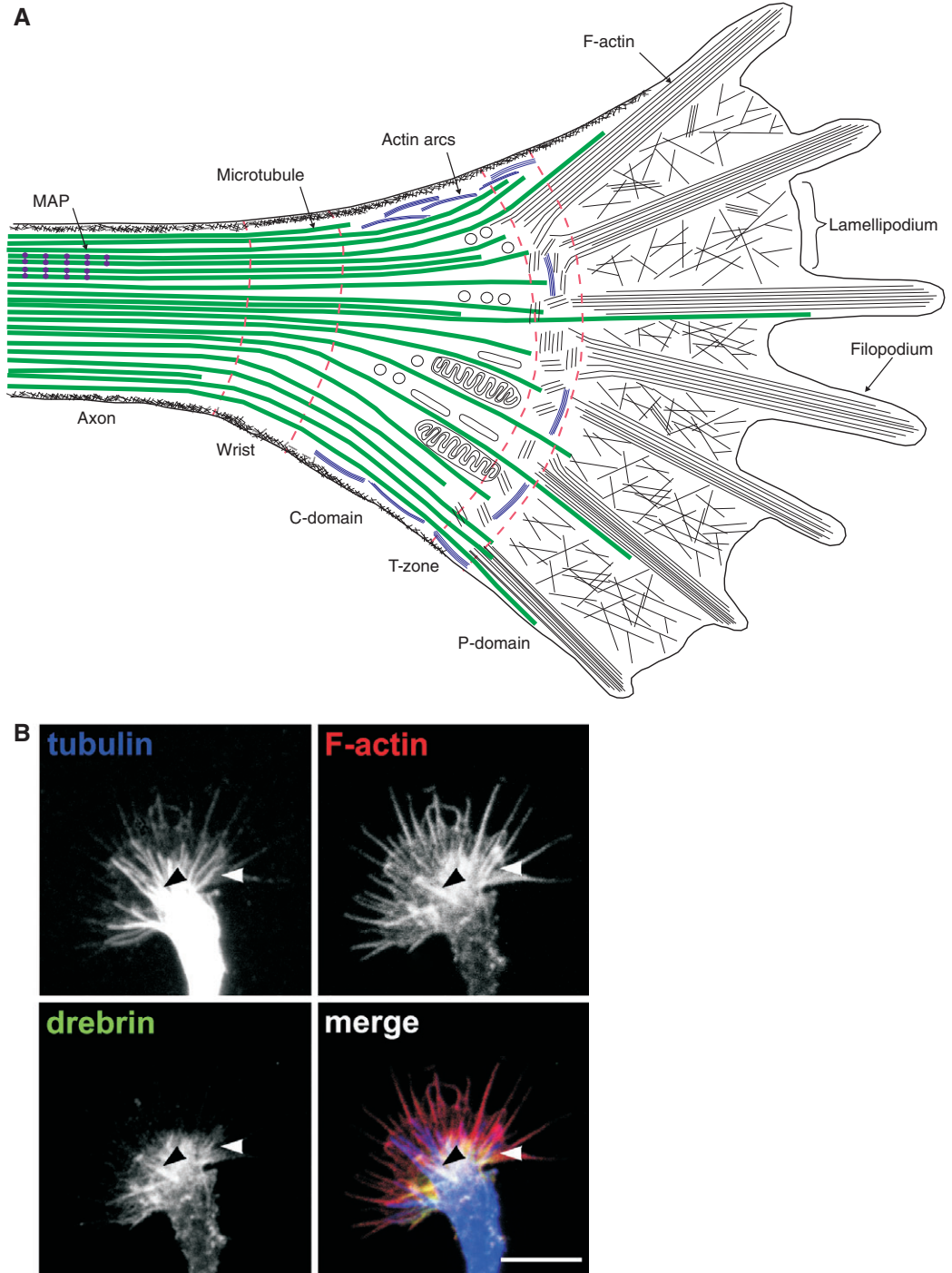
Growth cones have several distinct but contiguous morphological domains. At the end of the growing axon, the neurite expands into a relatively thick central (C)-domain that is filled with mitochondria, vesicles and reticulum (Fig. 1A). The C-domain connects to a more distal and relatively thin, motile region fringed with filopodia and lamellipodia, known as the peripheral (P)-domain. At the junction between the axon and the C-domain is the wrist of the growth cone,

and at the junction between the C-domain and the P-domain is the transition (T)-zone (Fig. 1A). As the growth cone advances, leaving behind a growing axon, the P-domain is converted into the C-domain through a process that involves stabilisation of filopodia against retraction and the engorgement of lamellipodia with organelles (Goldberg and Burmeister, 1986; Aletta and Green, 1988). The C-domain, in turn, gives rise to the axon by constriction at the growth-cone wrist and bundling of microtubules.

Growth-cone motility is associated with the extension and retraction of filopodia and the ebb (retraction) and flow (extension) of lamellipodia between the filopodia. Filopodia have a central role in axon guidance because they are the first part of the growth cone to come into contact with guidance molecules; filopodia are equipped with the molecular machinery to detect and respond to these signals (Gordon-Weeks, 2004; Gupton and Gertler, 2007). Filopodia respond to guidance cues either by stabilising when they encounter an attractive guidance molecule, or by retracting on contact with a repellent guidance molecule. If this occurs differentially on one side of the growth cone, then the growth cone will turn towards an attractive guidance molecule or away from a repellent guidance molecule. Such growth-cone turning is a key behavioural event in pathfinding and occurs specifically at choice points. Thus, the growth cone translates the information conveyed by guidance molecules into changes in motile behaviour that result in steering manoeuvres and, consequently, a change of route during pathfinding.

Dynamic interactions between microtubules and actin filaments (F-actin) are important for enabling a wide range of cellular events (Rodriguez et al., 2003). In this Commentary, we discuss the dynamics and regulation of microtubule-actin filament interactions in the context of growth-cone advance and steering during axon guidance. We focus on the role of filopodia in axon guidance and review evidence that coupling of dynamic microtubules to actin filaments in filopodia is an essential event that underlies neuritogenesis, growth-cone advance and growth-cone turning,

**Fig. 1.** (A) Organisation of the growth-cone cytoskeleton. The growth cone is an expanded, motile structure at the tip of the axon and is divided into several morphological regions (as indicated by dashed red lines). In the axon shaft, microtubules (green lines) are organised into parallel bundles by MAPs (purple dumbbells), whereas in the C-domain they become defasciculated and extend individually through the T-zone and into the P-domain, where they become aligned alongside the F-actin bundle (black lines) in filopodia. In the P-domain, the F-actin in filopodia is organised into parallel bundles that extend rearwards across the P-domain to terminate in the T-zone, where they are severed into short filaments by a mechanism that is incompletely understood. F-actin in the lamellipodia of the P-domain is organised into a branched dendritic network. In the T-zone and at the periphery of the C-domain there are so-called actin arcs (blue lines) composed of anti-parallel bundles of F-actin and myosin II. Actin arcs produce compressive forces in the C-domain that corral the unbundled microtubules and thereby facilitate microtubule bundling in the growth-cone wrist. (B) Immunofluorescence confocal images of microtubule (tubulin) and actin filament (F-actin) distribution in a growth cone from an embryonic cortical neuron in culture. The growth cone was simultaneously fixed and detergent-extracted to remove soluble proteins and preserve the cytoskeleton. F-actin is labelled with phalloidin and microtubules are identified with an antibody specific for tubulin. Microtubules extend individually into the P-domain, where they align alongside the bundles of F-actin in filopodia (white arrowheads). Drebrin predominantly localises to the T-zone but also associates with the proximal region of the F-actin in those filopodia where microtubules are also commonly inserted (black arrowheads). In the merged image, microtubules are blue, F-actin is red and drebrin is green.



which are fundamental behaviours during axon guidance. The intracellular signalling pathways that regulate this cytoskeletal interaction are also discussed and, finally, we briefly touch on the relevance to other cellular processes of microtubule-actin filament interactions found in growth-cone steering. Other aspects of axon guidance, such as axon growth and branching, have been reviewed recently (Kornack and Giger, 2005).

### The growth-cone cytoskeleton

There are two cytoskeletal filaments that are invariably present in growth cones: microtubules and actin filaments (Fig. 1B). The

dynamic behaviour of these filaments, which has been extensively studied in growth cones (reviewed by Dent and Gertler, 2003; Rodriguez et al., 2003; Gordon-Weeks, 2004; Zhou and Cohan, 2004; Kalil and Dent, 2005; Conde and Cáceres, 2009; Lowery and Van Vactor, 2009), produces distributions of these two cytoskeletal components within the growth cone that are, to some extent, mutually exclusive (Fig. 1B). However, the regions where filament overlap occurs, and therefore where microtubule-F-actin interactions take place, are the most significant for axon growth and growth-cone turning. In the axon shaft, microtubules are bundled by microtubule-associated proteins (MAPs), whereas in the

**Table 1. Dynamic properties of microtubules in growth cones**

Fluorescent molecule	Average orthograde growth rate ( $\mu\text{m}/\text{min}$ )	Orthograde translocation rate ( $\mu\text{m}/\text{min}$ )	Retrograde translocation rate ( $\mu\text{m}/\text{min}$ )	Neuron type	Species	Reference
Rho-tubulin	7.14 $\pm$ 0.33			Bag cell	<i>Aplysia</i>	(Kabir et al., 2001)
Rho-tubulin	6.9 $\pm$ 0.3			Bag cell	<i>Aplysia</i>	(Schaefer et al., 2002)
Rho-tubulin	8.4 $\pm$ 0.4 (central) 7.8 $\pm$ 0.4 (peripheral)	4.5 $\pm$ 0.4	4.7 $\pm$ 0.2	Bag cell	<i>Aplysia</i>	(Lee and Suter, 2008)
Tubulin-GFP	9.80 $\pm$ 0.47			Spinal cord	<i>Xenopus</i>	(Ma et al., 2004)
Rho-tubulin	10.5 $\pm$ 1.9			Spinal cord	<i>Xenopus</i>	(Tanaka and Kirschner, 1991)
EB1-GFP	10.22 $\pm$ 0.5			Spinal cord	<i>Xenopus</i>	(Ma et al., 2004)
EB3-GFP	12			Hippocampal	Mouse	(Stepanova et al., 2003)
EB3-GFP	8.7 $\pm$ 0.5			Hippocampal	Rat	(Grabham et al., 2007)
EB3-GFP	5.7 $\pm$ 0.3			SCG	Rat	(Myers et al., 2006)
Rho-tubulin	7.7 $\pm$ 0.9			Cerebral cortical	Hamster	(Dent et al., 1999)
CLASP-GFP	9.7 $\pm$ 2.1			Spinal cord	<i>Xenopus</i>	(Lee et al., 2004)

Rho, rhodamine; SCG, superior cervical ganglion.

growth-cone C-domain, they are spread apart and single microtubules extend through the T-zone and as far as the filopodia in the P-domain (Fig. 1A).

Microtubules are intrinsically polarised filaments with structurally and functionally distinct ends (Howard and Hyman, 2003). At the so-called plus end, tubulin assembly takes place preferentially, and at the minus end, tubulin disassembles or the end is capped, most commonly by a microtubule-organising centre such as the centrosome. Growth cones do not have centrosomes and very little is known about the dynamics of microtubule minus ends in growth cones. Most microtubules in growth cones are oriented with their plus end pointing distally, i.e. towards the front of the growth cone. The exceptions are the looped or highly curved microtubules that are occasionally found in the C-domain (see below). The main mechanism for distal microtubule extension in growth cones is plus-end assembly; there is some evidence for distal (anterograde) polymer translocation, possibly driven by the microtubule motor cytoplasmic dynein (Dent et al., 1999; Zhou et al., 2002; Schaefer et al., 2002; Ma et al., 2004; Myers et al., 2006). Retrograde translocation of microtubules, by contrast, is commonly observed (see below) (Schaefer et al., 2008).

Microtubules in growth cones exhibit dynamic instability, alternating between relatively slow growth from their plus end and rapid plus-end disassembly (catastrophe), which might be followed by recovery of plus-end assembly (rescue). Microtubules grow from their plus ends at average rates of between 6 and 10  $\mu\text{m}/\text{minute}$  (Table 1), a growth rate that enables them to traverse the growth cone from back to front in just a few minutes – even in *Aplysia*, which has the largest known growth cones – and is an order of magnitude faster than the forward translocation speed of the growth

cone itself (Table 2) [see also table 1.1 in *Neuronal Growth Cones* (Gordon-Weeks, 2005)]. Microtubules growing toward the front of the growth cone pass through the T-zone and into the P-domain. This behaviour brings them into contact with the F-actin cytoskeleton in the P-domain, particularly the F-actin bundles in filopodia (Fig. 1). In fact, microtubules cross the P-domain by associating preferentially with the sides of filopodia F-actin bundles. However, their extension into the P-domain does not depend on filopodia (Burnette et al., 2007); rather, filopodia ensure that there is a radial array of microtubules across the P-domain. Thus, the cytoskeleton of the C-domain is largely composed of dynamic microtubules that extend into the P-domain. However, there is a subset of microtubules in the C-domain that are relatively stable. These microtubules are generally restricted to the C-domain and are often more highly curved than the dynamic microtubules. Their numbers increase when growth cones stall or pause (Tsui et al., 1984; Lankford and Klein, 1990; Sabry et al., 1991; Tanaka and Kirschner, 1991; Dent et al., 1999), or when glycogen synthase kinase 3 (GSK3) is inhibited (Lucas et al., 1998; Goold et al., 1999; Kim et al., 2006; Purro et al., 2008). The function of these more stable microtubules, and hence their relation to dynamic microtubules, is unclear. They might represent, in part, the population of microtubules that become incorporated into the growing axon (see below).

In contrast to the C-domain, the P-domain is supported by F-actin, the organisation of which differs between lamellipodia and filopodia (Fig. 1A). In lamellipodia, F-actin is predominantly organised into a branched dendritic network, whereas in filopodia the F-actin is present as parallel bundles. These two different types of F-actin organisation require the action of F-actin-bundling proteins in

**Table 2. Dynamic properties of F-actin (filopodia) in growth cones**

Fluorescent molecule or mode of visualisation	Average orthograde speed ( $\mu\text{m}/\text{min}$ )	Average retrograde speed ( $\mu\text{m}/\text{min}$ )	Neuron type	Species	Reference
Alexa 594-Phall	4-6	6.2 (in P-domain) 0.8 (in T-zone)	Bag cell	<i>Aplysia</i>	(Medeiros et al., 2006)
Phall-actin		4.7 $\pm$ 0.2	Bag cell	<i>Aplysia</i>	(Lee and Suter, 2008)
Actin-GFP		4.68 $\pm$ 0.24 (stiff substrate) 6.3 $\pm$ 0.42 (soft substrate)	Forebrain	Chick	(Chan and Odde, 2008)
Actin-GFP	11.6 $\pm$ 0.9	9.2 $\pm$ 1.2	Cerebral cortical	Rat	(Geraldo et al., 2008)
Polystyrene beads		0.063 $\pm$ 0.002	SCG	Rat	(Myers et al., 2006)
Actin-GFP/caged Q-Rho-actin	1.0	0.63	Neuroblastoma cells (NG108)	Mouse	(Mallavarapu and Mitchison, 1999)

Phall, phalloidin; Rho, rhodamine; SCG, superior cervical ganglion.

filopodia and of F-actin-branching proteins in lamellipodia. Several F-actin-bundling proteins, including  $\alpha$ -actinin (Sobue and Kanda, 1989) and fascin (Edwards and Bryan, 1995), have been found in filopodia, and the F-actin-nucleating and F-actin-branching protein complex, Arp2/3 (actin-related protein 2/3), is present in lamellipodia (Goldberg et al., 2000; Mongui et al., 2007; Korobova and Svitkina, 2008). However, exactly how filopodia form in cells is not entirely clear (Gupton and Gertler, 2007; Mattila and Lappalainen, 2008). There is some conflicting evidence as to what extent F-actin in the dendritic network of lamellipodia can contribute to the parallel bundles of F-actin in filopodia, and this might vary between cell types (Mongui et al., 2007; Korobova and Svitkina, 2008).

Similarly to microtubules, actin filaments are also polarised with one end at which monomeric, globular (G)-actin is preferentially assembled (the so-called barbed end), because of its tenfold lower critical concentration (100 nM vs 1  $\mu$ M) compared to the other end (the so-called pointed end). As is the case with microtubules, the actin filaments in filopodia are oriented with their barbed ends pointing distally, and G-actin is added near, or at the tip, of the filopodium (Forscher and Smith, 1988; Lin et al., 1996). Plus-end addition of G-actin produces a force on the F-actin bundle that can move the bundle rearwards (known as retrograde flow; Table 2), or a force on the plasma membrane that extends the filopodium. Which of these two outcomes occurs depends partly on the strength of the adhesion of the filopodium to the substratum that the growth cone is growing on (Bard et al., 2008). This mechanism has been likened to a 'clutch', where engagement of the clutch represents the molecular linkage between the cytoskeleton, membrane proteins and proteins in the extracellular matrix that resists the plus-end assembly-driven retrograde flow of F-actin (Suter and Forscher, 2000; Chan and Odde, 2008; Bard et al., 2008). However, the molecular details of the clutch mechanism are not clear.

The rate of filopodia extension and retrograde flow can vary independently of each other and might therefore be independently regulated (Mallavarupu and Mitchison, 1999). The actin filaments in the lamellipodia are also predominantly oriented with their barbed ends distal, and move retrogradely. The retrograde flow of F-actin is augmented by the action of non-muscle myosin II, as demonstrated by the finding that specific inhibition of myosin II ATPase by the small molecule inhibitor blebbistatin attenuates F-actin retrograde flow (Medeiros et al., 2006; Burnette et al., 2008; Geraldo et al., 2008) and enhances filopodia extension (Rösner et al., 2007). All known isoforms of myosin II (IIA, IIB and IIC) are present in growth cones (Turney and Bridgman, 2005) and, as blebbistatin inhibits the activity of all three, it is not clear from these experiments which isoforms are important in retrograde flow. Experiments with knockout mice might help to address this problem; however, retrograde flow rate in growth cones from myosin-IIB-knockout mice is higher than in wild-type mice, possibly because of compensation by myosin IIA (Brown and Bridgman, 2003). This functional redundancy between isoforms complicates the interpretation of these results (Bridgman et al., 2001; Brown et al., 2009). Growth cones from myosin-IIB-knockout mice turn less efficiently at sharp substrate borders in culture than growth cones that contain all isoforms of myosin II, whereas treatment of wild-type growth cones with blebbistatin severely inhibits turning, suggesting that more than one isoform is involved (Turney and Bridgman, 2005). Myosin II is also involved in the axon retraction that follows the growth-cone collapse that can be induced in vitro by axon severing (Gallo, 2004) or by growth-cone-collapsing factors

such as semaphorin 3A (Gallo et al., 2002; Wylie and Chantler, 2003; Gallo, 2006). There is some evidence from knockdown experiments with antisense oligodeoxynucleotides that myosin IIA is involved in neurite retraction and that myosin IIB is involved in neurite extension (reviewed by Chantler and Wylie, 2003).

A third type of F-actin assembly has been described in growth cones of *Aplysia* bag cells (Schaefer et al., 2002; Zhang et al., 2003; Burnette et al., 2007). Here, the F-actin is bundled into arcs that are present in the T-zone, where they are oriented perpendicular to the radial filopodia, and in the wrist region of the growth cone, where their orientation is more longitudinal (Fig. 1A) (Burnette et al., 2008). Axon retraction might be mediated by contraction of the longitudinally oriented actin arcs in the C-domain (Fig. 1A). Actin arcs probably form from pre-existing actin filaments of the dendritic network in lamellipodia, near or possibly within the T-zone; in addition, a contribution from the proximal ends of filopodia F-actin bundles might also occur (Burnette et al., 2007).

As the growth cone advances, leaving behind the growing axon, microtubules in the growth-cone wrist are corralled together by compressive forces that constrict the wrist into the narrower axon shaft. Corraling of the microtubules facilitates their crosslinking into bundles, which is the predominant organisation of microtubules in the axon shaft (Fig. 1A). There is some evidence that the F-actin arcs and myosin II activity are involved in generating the compressive forces, at least in invertebrate (*Aplysia*) growth cones (Schaefer et al., 2002; Burnette et al., 2008; Schaefer et al., 2008). Myosin II is localised in the growth cone mainly in the T-zone (Lewis and Bridgman, 1992; Rochlin et al., 1995; Bridgman, 2002; Loudon et al., 2006), and blebbistatin inhibits microtubule bundling (Burnette et al., 2008). Myosin II is a two-headed, barbed-end-directed myosin that can form bipolar filaments and therefore contract anti-parallel F-actin bundles. In growth cones, myosin II is present as bipolar filaments (Bridgman, 2002) and, because actin arc movement is rapidly and completely blocked by blebbistatin (Burnette et al., 2008), this suggests that actin arcs are composed of anti-parallel F-actin bundles, although this has yet to be demonstrated directly. Contraction of the anti-parallel bundles of actin arcs could provide the compressive forces for corraling microtubules (Burnette et al., 2008), although these would most probably need to be circumferentially oriented to compress the microtubules that are oriented longitudinally. Although the actin arcs in the T-zone are circumferential, in the wrist they are longitudinal (Fig. 1A) (Burnette et al., 2008). Corraling of microtubules presumably brings them within range of microtubule-bundling MAPs (see below).

Microtubules and actin filaments have reciprocal but overlapping distributions in growth cones that reflect their dynamic behaviour. As growth cones advance, they construct the axon that forms behind them; this process is dependent on coordination between the two sets of filaments within growth cones.

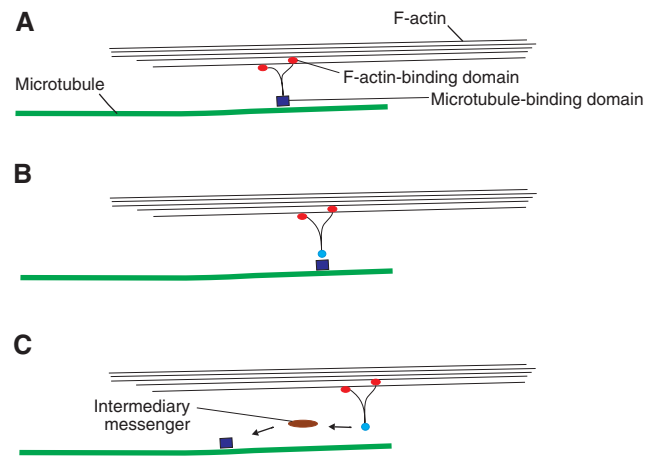
### Microtubule–F-actin coupling underlies growth-cone turning

It appears that the dynamic behaviour of microtubules and F-actin in growth cones is coordinated in some way. This reciprocity of behaviour was first shown in the seminal experiments of Forscher and Smith in which they noticed, in *Aplysia* growth cones, that microtubules extend further into the P-domain than normal when F-actin retrograde flow is attenuated by application of cytochalasins (small molecules that block F-actin plus-end assembly) (Forscher and Smith, 1988). A similar effect is also seen when F-actin

retrograde flow is attenuated either by global inhibition of myosin II with blebbistatin or by local application of microbeads coated with cell-adhesion molecules to *Aplysia* growth cones (Schaefer et al., 2008; Medeiros et al., 2006). Conversely, under circumstances in which retrograde F-actin flow is increased, as in the myosin-IIIB-knockout mouse, microtubules penetrate filopodia less successfully (Brown and Bridgman, 2003). Direct observation of microtubule dynamic behaviour following microinjection of rhodamine-tubulin has revealed that some microtubules move retrogradely at the same rate as the retrograde flow of F-actin (compare Tables 1 and 2) (Schaefer et al., 2002; Schaefer et al., 2008). Localised depolymerisation of filopodial F-actin on one side of the growth cone inhibits microtubule extension on that side and is associated with growth-cone turning away from the inhibited side (Zhou et al., 2002). Complete removal of filopodia almost doubles the number of microtubules in the P-domain and allows them to extend to the front of the lamellipodium, although they are abnormally curled (Burnette et al., 2007). Despite the continued extension of microtubules into the P-domain, lamellipodia lacking filopodia are unable to form a neurite (Dent et al., 2007). These results show that filopodial F-actin retrograde flow continually transports microtubules rearwards and thereby clears dynamic microtubules from the P-domain. Microtubules also leave the P-domain by plus-end disassembly and, consequently, when microtubule catastrophe is reduced, microtubules extend more frequently and farther into the P-domain (Kabir et al., 2001). This reciprocal correlation between microtubule and F-actin dynamics suggests that these two cytoskeletal elements are physically linked to each other. Whether this is a direct physical interaction between the two types of filament, or one mediated by specific proteins, has not been determined.

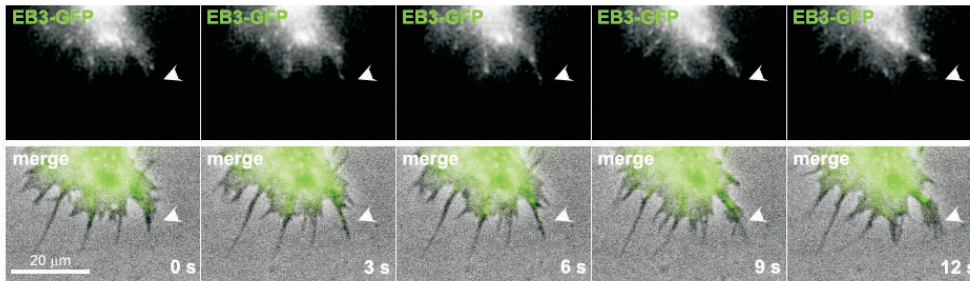
The idea that microtubules are physically coupled to F-actin suggests, significantly, that this interaction might underlie growth-cone turning. In this model, dynamic microtubules become coupled to F-actin in those filopodia that have stabilised against retraction following an encounter with an attractive guidance molecule. Microtubule 'capture' by stabilised filopodia is therefore seen as an essential step in growth-cone turning and the vectorial growth that follows turning. The first evidence to support this model came from pioneering observations made by Sabry and co-workers of microtubules in living growth cones of grasshopper limb bud explants (Sabry et al., 1991), and by Tanaka and Kirschner of microtubules in growth cones from *Xenopus* spinal cord neurons turning at sharp borders between permissive and non-permissive substrates in culture (Tanaka and Kirschner, 1991; Tanaka and Kirschner, 1995) (see also Challacombe et al., 1996). These studies revealed that, as growth cones make a turn and filopodia become stabilised on the side of the turn, their dynamic microtubules become oriented and stabilised preferentially in the direction of the turn, consistent with the idea of coupling between microtubules and F-actin. That such coupling underlies turning was subsequently shown by several independent observations in which either the dynamic instability of microtubules was inhibited globally, thereby preventing them from entering the P-domain (Williamson et al., 1996; Challacombe et al., 1997), or microtubules were artificially either destabilised or stabilised on one side of the growth cone (Buck and Zheng, 2002).

When microtubules are globally prevented from interacting with F-actin in filopodia by application of microtubule-depolymerising or microtubule-stabilising compounds, growth cones are unable to turn at sharp substrate borders in culture (Williamson et al., 1996; Challacombe et al., 1997; Buck and Zheng, 2002). Similarly,



**Fig. 2.** Models of the molecular mechanisms of microtubule–F-actin coupling in growth cone filopodia. The diagrams show microtubules (green lines) located to one side of the proximal end of a filopodial bundle of F-actin (black lines). (A) The simplest mechanism for crosslinking microtubules to F-actin involves a protein that has both F-actin-binding (red blobs) and microtubule-binding (purple box) domains. Examples of such proteins found in growth cones include members of the spectraplakin family such as shortstop. It is assumed that the F-actin-binding domains recognise adjacent F-actin in the bundle, and that this confers specificity of binding to bundled F-actin. (B) A development of the simplest mechanism for crosslinking microtubules to F-actin is to have two proteins that bind to each other – one that binds to F-actin and the other to microtubules. The F-actin-binding protein drebrin and the +TIP protein EB3 are present in growth cones and have these properties. (C) A final possibility for coupling microtubules to F-actin is to have information conveyed through intermediary messengers (brown oval). In this model there is no direct physical interaction between microtubules and F-actin. The direction of information flow is depicted from F-actin to microtubules but could also occur in the opposite direction.

stabilising microtubules in *Aplysia* growth cones prevents appropriate steering responses on contact with a microbead that is coated with cell-adhesion molecules (Suter et al., 2004). Knocking down the expression of dynein heavy chain using small interfering RNAs suppresses microtubule insertion into filopodia and this is also associated with inhibition of growth-cone turning (Myers et al., 2006) (see also Grabham et al., 2007). By contrast, disassembly of microtubules on one side of the growth cone by an externally applied gradient of the microtubule-depolymerising compound nocodazole causes the growth cone to turn away from that side (Buck and Zheng, 2002). Conversely, when microtubules are stabilised on one side of the growth cone, either by locally activating caged taxol, or by exposing growth cones to a gradient of taxol, the growth cone turns towards that side (Buck and Zheng, 2002). Inhibition of the microtubule motor kinesin-5 has recently been found to prevent growth-cone turning at sharp substrate borders in vitro by preventing microtubules entering, and therefore becoming stabilised on, the growth-cone turning side (Nadar et al., 2008). The consequences of microtubule capture by filopodia are that the microtubule becomes stabilised against depolymerisation and provides a track for the delivery of organelles for lamellipodial engagement, which leads to axon growth and enables growth-cone turning. Some evidence suggests that the first stabilised microtubule can recruit additional microtubules by crosslinking them into bundles (e.g. Sabry et al., 1991), thereby consolidating the stabilisation. Therefore, there might be two regions in the growth cone where microtubule bundling occurs: continually at the wrist



**Fig. 3.** EB3 surfs on the growing end of dynamic microtubules in growth cones. Consecutive video frames [GFP fluorescence (upper panels), and merged fluorescence and phase contrast (lower panels)] from an embryonic cortical neuron growth cone expressing EB3-GFP. EB3-GFP comets can be seen entering the proximal region of emerging filopodia at the periphery of the growth cone (arrowheads).

in the process of forming the axon and, during growth-cone turning, in the P-domain.

### Molecular mechanisms of microtubule–F-actin coupling

What is the molecular mechanism of coupling between dynamic microtubules and F-actin in growth-cone filopodia? A priori, the simplest mechanism would be to crosslink microtubules and F-actin with a single protein that simultaneously binds both microtubules and F-actin; for example, members of the spectraplaklin family, such as kakapo/short-stop, which have microtubule- and F-actin-binding domains, required for axon extension (Fig. 2A) (Lee et al., 2000; Sonnenberg and Liem, 2007), the adenomatous polyposis coli protein (APC) (Zumbrunn et al., 2001; Dikovskaya et al., 2001), or *Drosophila* Pod-1 (Rothenberg et al., 2003). A variation of this mechanism would be the involvement of separate proteins that have these two functions and, in addition, have binding domains for each other (Fig. 2B). Alternatively, rather than physically bridging the two cytoskeletal elements, the interaction could be transient but instructive, and lead to the separate stabilisation of each filament (Fig. 2C). For microtubules coupling to parallel bundles of F-actin, as in growth cone filopodia, the actin-binding protein would most likely be a side-binder, perhaps with two or more actin-binding domains that recognise parallel, but not anti-parallel, F-actin bundles, e.g. fascin (Cohan et al., 2001). In addition, it is possible to speculate that there are regulatory controls superimposed on these basic binding properties that confer an ability to respond to the actions of guidance molecules: for example, an ability to recognise F-actin bundles in filopodia stabilised by an attractive guidance molecule, or to respond directly to intracellular signals that arise from the activation of guidance-molecule receptors (see below). This last consideration raises the issue of whether there is a basal level of microtubule–F-actin coupling that supports unguided growth-cone advance – that is, that which occurs without the action of guidance molecules.

Growth cones of cultured neurons tend to grow in straight lines, which suggests that microtubule capture occurs predominantly by filopodia at the front, and not at the sides, of the growth cone. However, the growth cones of some neurons, such as those of dorsal root ganglion neurons, have a tendency to divide into equal halves in culture and give rise to two daughter axons. This could conceivably come about by the simultaneous capture of microtubules on two sides of the growth cone.

The microtubule-binding proteins that are most likely to mediate microtubule–F-actin interactions in growth-cone filopodia are the microtubule tip-tracking (+TIP) proteins that bind to and form protein complexes at the plus ends of growing microtubules (Kalil and Dent, 2004; Akhmanova and Steinmetz, 2008). At the core of these plus-end complexes are two members of the end-binding (EB)

family of archetypical +TIP proteins, EB1 and EB3. EB1 is ubiquitously expressed, whereas EB3 is highly enriched in the nervous system, and both are expressed in growth cones (Stepanova et al., 2003). EB1 and EB3 associate only with growing microtubule plus ends, and promote continuous microtubule growth by preventing microtubule catastrophes (Komarova et al., 2009). Both proteins have C-terminal binding domains for other +TIP proteins, including APC, p150<sup>glued</sup> (a component of the dynein-dynactin complex) and cytoplasmic linker protein (CLIP)-170. Recently, it has been shown that CLIP-170 is unable to bind directly to microtubule plus ends but is recruited to them by EB1 (Bieling et al., 2008; Dixit et al., 2009). When expressed in neurons as GFP-fusion proteins, EB1 and EB3 ‘surf’ on the plus ends of growing microtubules in growth cones, appearing as ‘comets’ with tails that point toward the minus end of the microtubule (Fig. 3) (Morrison et al., 2002; Stepanova et al., 2003; Ma et al., 2004; Myers et al., 2006; Geraldo et al., 2008). EB3-GFP comets can be seen entering filopodia where they usually disappear at some point along their length, presumably because the microtubule has either stopped growing or has depolymerised (Fig. 3).

In directionally migrating, non-neuronal cells in culture, there is evidence for the involvement of three +TIP protein complexes in microtubule capture and stabilisation by cortical actin in lamellipodia: APC and EB1 bind to the formin mDia (Wen et al., 2004); CLIP-associating proteins (CLASPs) couple microtubules to cell adhesions via the spectraplaklin ACF7 and LL5 $\beta$ , a phosphatidylinositol (3,4,5)-trisphosphate sensor (Drabek et al., 2006; Lansbergen et al., 2006; Wu et al., 2008); and CLIP-170 interacts with Rac1, IQGAP1 and components of the dynein-dynactin complex (Fukata et al., 2002; Lansbergen et al., 2004). To what extent these +TIP complexes are also involved in microtubule–F-actin interactions in growth cones is unclear.

There is conflicting evidence of a role for APC in axon growth and growth-cone turning. APC binds to microtubules, accumulating at their plus ends independently of EB1 and increasing their stability and bundling (Barth et al., 2008). Although APC is concentrated in the C-domain in growth cones, it is bound to the plus ends of a subset of microtubules in the P-domain and in membrane-associated patches in lamellipodia (Zhou et al., 2004; Gärtner et al., 2006; Kim et al., 2006; Koester et al., 2007; Purro et al., 2008). EB3 is absent from those microtubule plus ends to which APC is bound, suggesting that these microtubules are stable and not elongating (Koester et al., 2007); this is consistent with the function of APC in non-neuronal cells (Barth et al., 2008). Functional studies of APC in vitro suggest that it is important for growth-cone advance and turning (Koester et al., 2007; Purro et al., 2008). Inactivation of APC on one side of the growth cone using micro-CALI, a technique that uses laser light to locally inactivate antibody-targeted proteins by free radical generation, can induce turning (Koester et al.,

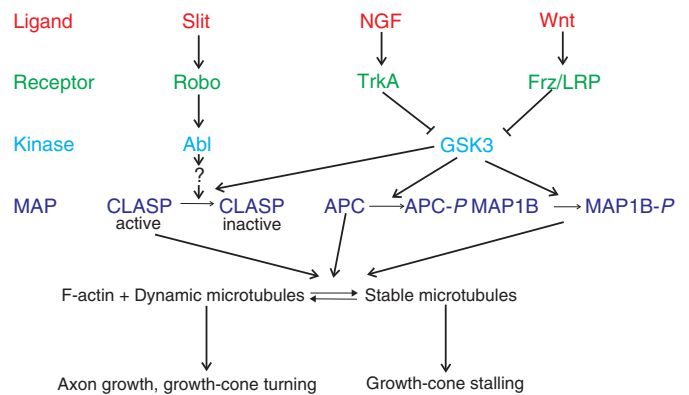
2007). The global loss of APC from microtubule plus ends that is induced by Wnt signalling causes growth cones to stall and enlarge and increases the number of curved microtubules (Purro et al., 2008). Thus, APC might link stabilised microtubules to cortical actin patches in growth cones, although in epithelial cells, APC that is localised to microtubules is in a distinct pool from that associated with membrane actin patches (Rosin-Arbesfeld et al., 2001). Such patches might include those associated with focal adhesive contacts in the C-domain (Gomez et al., 1996).

Despite these *in vitro* findings with invertebrate neurons, null mutants of the two APC genes in *Drosophila* show no axon extension or growth-cone pathfinding defects (Rusan et al., 2008). The reason for this discrepancy is not clear, and the issue will probably not be resolved until mice with a conditional knockout of the *apc* gene are developed. In *Drosophila*, the CLASP orthologue Orbit/MAST is present in growth cones, presumably at the plus end of microtubules, and is downstream of the non-receptor tyrosine kinase Abl and the growth-cone guidance molecule Slit (Lee et al., 2004). Flies carrying mutations of Orbit/MAST phenocopy those carrying mutations in Abl; this phenotype is associated with pathfinding errors in growth cones at the midline choice point, where Slit acts as a repellent through the Robo receptor. *Drosophila* growth cones are too small to determine the relationship between Orbit/MAST and microtubules, but in cultured *Xenopus* spinal cord motor neurons, CLASP-GFP localises to the growing tips of a subset of dynamic microtubules that extend into the P-domain and track alongside the F-actin in filopodia (Lee et al., 2004). Whether this is an example of a guidance-signalling pathway that directly regulates microtubule dynamics, or whether F-actin dynamics are upstream of microtubules, is unclear.

More recently, the F-actin side-binding protein drebrin has been shown to bind specifically to EB3 (Geraldo et al., 2008) and to localise to the T-zone (Geraldo et al., 2008; Mizui et al., 2009) and the proximal part of some growth-cone filopodia that invariably have a microtubule inserted (Fig. 1B) (Geraldo et al., 2008). The interaction between drebrin, which is bound to the proximal filaments of the F-actin bundle of filopodia, and EB3, which surfs on the tips of microtubules entering filopodia, was shown to be important for growth-cone formation and axon growth (Geraldo et al., 2008). However, whether this interaction is also necessary for growth-cone turning has yet to be determined.

### Signalling pathways that regulate microtubule–F-actin coupling

There are several key steps in the reorganisation of the growth-cone cytoskeleton during pathfinding for which signalling pathways have been uncovered. These steps include the plus-end assembly of microtubules and filopodia F-actin, retrograde F-actin flow and microtubule–F-actin coupling (Fig. 4). Several signalling pathways, all including kinases, have been identified that appear to regulate microtubule dynamics directly in growth cones (Lee et al., 2004; Zhou et al., 2004; Purro et al., 2008) (reviewed by Kalil and Dent, 2004). In many of these pathways, the serine/threonine kinase GSK3 is emerging as a master regulator of microtubule dynamics in growth cones: it phosphorylates several MAPs including APC (Kim et al., 2006), MAP1B (Lucas et al., 1998; Goold et al., 1999; Kim et al., 2006), collapse response mediator proteins (CRMPs) (Cole et al., 2004; Arimura et al., 2005; Yoshimura et al., 2005) and CLASPs (Wittmann and Waterman-Storer, 2005; Kumar et al., 2009). GSK3 is, in general, constitutively active, and its inhibition by several growth-cone guidance pathways (including those



**Fig. 4.** Intracellular signalling pathways in growth cones converging on the cytoskeleton. The balance between dynamic and stable microtubules is regulated via several growth-cone guidance pathways, including signalling pathways activated by the binding of Slit to the Robo receptor, by the neurotrophin nerve growth factor (NGF) binding to the TrkA receptor, and by Wnt ligands binding to the Frizzled receptor (Frz) and its co-receptor low density lipoprotein receptor-related protein (LRP). Through the activity of kinases such as Abl and GSK3, these pathways converge at microtubule-associated proteins (MAPs) that bind directly to microtubules and alter their dynamic instability. Localised changes in the stability of microtubules modify their interactions with F-actin and regulate axon growth and growth-cone pathfinding.

stimulated by neurotrophins and Wnts) leads to changes in both microtubule and F-actin dynamics (Fig. 4) (Lucas et al., 1998; Eickholt et al., 2002; Owen and Gordon-Weeks, 2003; Zhou et al., 2004; Kim et al., 2006; Purro et al., 2008).

Phosphorylation of APC by GSK3 regulates its microtubule binding (Zumbrunn et al., 2001). When GSK3 is inhibited, following the activation of TrkA receptors by nerve growth factor, APC phosphorylation is reduced, allowing it to bind to microtubules; in turn, this enhances microtubule assembly and stimulates axon growth (Zhou et al., 2004). By striking contrast, inhibition of GSK3 by activation of the Wnt signalling pathway leads to a loss of APC from microtubule plus ends, an increase in curved microtubules and an increase in growth-cone stalling (Purro et al., 2008). A possible explanation for these apparently conflicting results is that different degrees of GSK3 inhibition might produce a differential response: strong, global inhibition arrests axon growth by stalling growth cones, whereas weak, localised inhibition increases axon growth (Kim et al., 2006). Notably, although these findings implicate signalling pathways in regulating the role of APC in axon growth, they do not address the role of APC in growth-cone turning. MAP1B, however, which is a downstream target of TrkA signalling via GSK3 phosphorylation (Goold and Gordon-Weeks, 2005), has been implicated in microtubule stabilisation and growth-cone turning (Mack et al., 2000) (reviewed by Lowery and Van Vactor, 2009).

During the conversion of the C-domain into the axon, microtubule bundling in the growth-cone wrist is facilitated by compressive forces (Burnette et al., 2008), which might be generated by contraction of F-actin arcs (Schaefer et al., 2002) that corral microtubules to within crosslinking range of microtubule-bundling MAPs. A candidate MAP that might crosslink microtubules in the wrist of the growth cone is the microtubule-bundling and microtubule-stabilising protein doublecortin (Dcx) (Gleeson et al., 1999; Friocourt et al., 2003; Deuel et al., 2006; Bielas et al., 2007). Mutations in Dcx in humans cause X-linked lissencephaly, in which

neuronal migration in the cortex is abnormal and neurite formation is compromised (Guerrini and Parrini, 2009). Dcx is phosphorylated by cyclin-dependent kinase 5, which decreases the capacity of Dcx to bind to microtubules, and is dephosphorylated by protein phosphatase 1 (PP1). Microtubule bundling by dephosphorylated Dcx in the growth-cone wrist requires the formation of a complex between Dcx, PP1 and the PP1 adaptor molecule spinophilin, as shown by analysis of cultured neurons from Dcx- and spinophilin-knockout mice (Bielas et al., 2007).

Several signalling pathways that regulate microtubule and F-actin dynamics in growth cones have been identified, but understanding how they are integrated with each other to orchestrate growth-cone pathfinding, and exactly which pathways regulate microtubule–F-actin coupling, is still unclear.

### Microtubule–F-actin coupling and dendritic-spine development

The major excitatory input onto cortical neurons is through synapses on dendritic spines, which develop from dendritic filopodia following axon contact during synaptogenesis. Until recently, it was widely believed that, although microtubules are present in the dendritic shaft, they do not penetrate into spines. However, using fluorescent-protein-tagged EB3 as a marker for growing microtubule tips, it has been shown that microtubules do insert into dendritic filopodia and spines; this was probably missed previously because the insertion is transient and rapid, and only a small proportion of spines are targeted at any one time (Gu et al., 2008; Hu et al., 2008; Jaworski et al., 2009). Microtubule insertion is functionally coupled to spine maturation because it is enhanced by factors that induce maturation, and because preventing microtubule insertion attenuates maturation (Hu et al., 2008; Jaworski et al., 2009). The entry of microtubules into dendritic spines is associated with changes in spine morphology that are EB3-dependent and, as it is well established that F-actin dynamics underlie these changes, this suggests that microtubule–F-actin interactions are involved in dendritic-spine plasticity. In support of this idea, Jaworski and colleagues found a biochemical link between EB3 and the F-actin-binding protein cortactin that regulates dendritic-spine F-actin polymerization (Jaworski et al., 2009). In addition, the F-actin side-binding protein drebrin, which has been shown to mediate interactions between dynamic microtubule plus ends and F-actin in filopodia by binding directly to EB3 in growth cones (Geraldo et al., 2008), has a well known role in regulating F-actin in dendritic spines (Sekino et al., 2007). Therefore, there might be common elements between microtubule–F-actin coupling in growth cones and dendritic spines.

### Conclusions and perspectives

It is now clear that dynamic microtubule–F-actin interactions in growth cones play major roles in the cytoskeleton reorganisation that underlies axon growth and growth-cone pathfinding. However, we do not currently have a complete description of the guidance signalling pathways that regulate and enable these interactions in growth cones, and many questions about the molecular mechanisms remain unanswered. For example, is the interaction between microtubules and F-actin mediated by physical coupling, or is it a ‘pass-the-parcel’ situation in which the interaction is transient but instructive? Another major area of enquiry is identification of the intracellular signalling pathways that regulate the key steps of cytoskeletal reorganisation that underlie axon growth and growth-

cone pathfinding. It is probable that many of these questions will be addressed by direct observation of cytoskeletal dynamics in living growth cones as they respond to guidance cues using high resolution techniques such as total internal reflection microscopy (TIRM) and by the generation of mouse models that are conditional knockouts for genes central to these processes.

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