The trip of the tip: understanding the growth cone machinery

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Abstract | The central component in the road trip of axon guidance is the growth cone, a dynamic structure that is located at the tip of the growing axon. During its journey, the growth cone comprises both 'vehicle' and 'navigator'. Whereas the 'vehicle' maintains growth cone movement and contains the cytoskeletal structural elements of its framework, a motor to move forward and a mechanism to provide traction on the 'road', the 'navigator' aspect guides this system with spatial bias to translate environmental signals into directional movement. The understanding of the functions and regulation of the vehicle and navigator provides new insights into the cell biology of growth cone guidance.

Chemotropic cue
An external chemical cue, often found in a gradient, that leads to a directional growth in response.

Protrusion
The stage of growth cone progression in which there is extension of filopodia and lamellipodia-like veils.

Engorgement
The stage of growth cone progression in which microtubules further invade into the growth cone, fixing the new axonal growth direction.

Consolidation
The stage of growth cone progression in which actin filaments at the neck of the growth cone depolymerize and the membrane shrinks to form a cylindrical axon shaft around the bundle of microtubules.

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During the development of the nervous system, each neuron extends an axon through a complex and changing environment to reach its final destination. At the tip of each axon is the growth cone (BOX 1). The highly dynamic behaviour of the growth cone and its responsiveness to multiple sources of spatial information allows it to find its target with an impressive level of accuracy. The growth cone 'vehicle' cannot move forward without a 'road' to travel along. For growth cones, this road comprises adhesive molecules that are either presented on a neighbouring cell surface (for example, transmembrane cell adhesion molecules (CAMs)[1]) or assembled into a dense extracellular matrix (ECM; for example, laminin and fibronectin[2]) (FIG. 1). These molecules provide defined surfaces to which growth cone receptors can adhere, but they also activate intracellular signalling pathways that are used by the growth cone guidance machinery. Additionally, anti-adhesive, surface-bound molecules (such as slits and ephrins[3,4]) can prohibit the advance of the growth cone and thus provide 'guard rails' that determine roadway boundaries. Finally, diffusible chemotropic cues represent the 'road signs' that present further steering instructions to the travelling growth cone (FIG. 1). These include a range of molecules, including factors that were initially identified explicitly in axon guidance assays[3,4], as well as morphogens[5], secreted transcription factors[6,7], neurotrophic factors[8,9] and neurotransmitters[10]. Whereas it was originally thought that some cues always function as attractive 'go' signals (for example, netrins) and others as repulsive 'stop' signals (for example, ephrins), it is now clear that the response to attraction or repulsion is not due to the intrinsic property of the cue, but rather to the specific receptors that are engaged in the growth cone and the internal signalling milieu of the growth cone. In particular, the 'navigator' function of the growth cone comprises the intracellular signalling elements that determine how environmental directions lead to a given guidance response[4].

Despite notable advances over decades of research, our current understanding of how the growth cone achieves its impressive road trip is far from complete. Here, we examine the basic cell biological features of growth cone guidance, focusing on the cytoskeletal mechanisms that the growth cone uses as its vehicle to move forward, as well as elements of the navigation system that convert spatial bias into steering by translating environmental guidance cues into localized cytoskeletal remodelling. Although changes in membrane dynamics, including the regulation of endocytosis and exocytosis, also have crucial roles in growth cone migration and are likely to be targets of guidance cue signalling[11,12], this topic is beyond the scope of this Review. We conclude by highlighting some of the key unsolved questions in growth cone dynamics, and propose explanations for how recent technological advances would allow future investigations to further knowledge in these areas.

The growth cone vehicle

The growth cone engages its cytoskeleton to drive forward and turn, continuously progressing through three stages of advance that are influenced by environmental factors: protrusion, engorgement and consolidation[13,14] (BOX 2). For the growth cone to navigate according to spatial landmarks, the motility machinery that drives forward movement must have the capacity to be biased

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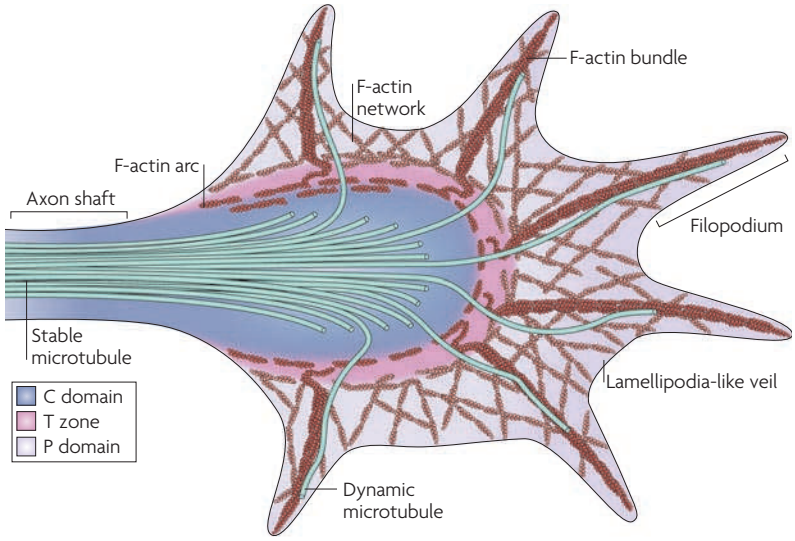
Box 1 | **The structure of the growth cone**



The structure of the growth cone is fundamental to its function. The leading edge consists of dynamic, finger-like filopodia that explore the road ahead, separated by sheets of membrane between the filopodia called lamellipodia-like veils (see the figure). The cytoskeletal elements in the growth cone underlie its shape, and the growth cone can be separated into three domains based on cytoskeletal distribution[14]. The peripheral (P) domain contains long, bundled actin filaments (F-actin bundles), which form the filopodia, as well as mesh-like branched F-actin networks, which give structure to lamellipodia-like veils. Additionally, individual dynamic 'pioneer' microtubules (MTs) explore this region, usually along F-actin bundles. The central (C) domain encloses stable, bundled MTs that enter the growth cone from the axon shaft, in addition to numerous organelles, vesicles and central actin bundles. Finally, the transition (T) zone sits at the interface between the P and C domains, where actomyosin contractile structures (termed actin arcs) lie perpendicular to F-actin bundles and form a hemicircumferential ring[33]. The dynamics of these cytoskeletal components determine growth cone shape and movement on its journey during development.

Actin treadmilling
The process by which the continual addition of actin subunits at the barbed end of an actin polymer and disassembly of the polymer at the pointed end ensures that the polymer stays of constant length, but individual subunits move along.

Filopodium
A thin, transient actin protrusion that extends from the cell surface and is formed by the elongation of bundled actin filaments in its core.

Lamellipodia-like veil
A thin, sheet-like extension of cytoplasm between filopodia that is formed by branched actin networks.

F-actin bundle
Long actin filaments that are crosslinked together in parallel, forming the core of filopodia.

spatially and to achieve accurate steering. In fact, the steering and drivetrain are intimately connected at a physical level. Therefore, if we are to fully grasp how guidance occurs, it is essential to understand the underlying cytoskeletal mechanisms that propel the vehicle forward and have the potential to be affected asymmetrically.

Turning on the engine: F-actin retrograde flow. Growth cone motility and protrusion of the leading edge membrane depend on the dynamic properties of actin (BOX 3). Although actin might not be the only engine that powers axon elongation *per se* (axons that lack actin polymerization can still move forward, albeit with abnormal growth cone morphology and substratum selectivity)[15], actin is a central part of the mechanism that controls growth cone exploration. A combination of filamentous (F)-actin treadmilling and F-actin retrograde flow (the continuous movement of F-actin from the leading edge towards the centre of the growth cone) provide the 'motor' that keeps the growth cone engine idling (FIG. 2a) and available to drive movement in response to directional cues[16]. Following increased technological advances in live cell imaging, the past few years have seen substantial improvements in our molecular understanding of F-actin retrograde flow and how it relates to growth cone motility and protrusion.

It has been convincingly demonstrated that F-actin retrograde flow is driven both by contractility of the motor protein myosin II, which seems to be tethered through protein–protein interactions in the transition (T) zone (the region between the peripheral (P) and central (C) domains of the growth cone), and the 'push' from F-actin polymerization in the P domain (the region of the growth cone that includes filopodia and lamellipodia-like veils)[17]. Myosin II-driven compression across the T zone circumference causes buckling of the F-actin bundles (FIG. 2a), which might be enhanced by pushing from leading edge actin polymerization[17]. This leads to bundle severing near the proximal ends[17] and probably involves actin filament-severing proteins of the actin-depolymerizing factor (ADF)/cofilin family[18]. A recent paper suggests that myosin II might also actively depolymerize actin filaments[19]. After severing, the actin fragments are recycled into individual actin subunits and are available for transport to the periphery for further actin polymerization at the leading edge[20] (FIG. 2a).

Engaging the clutch and forming traction to push ahead. How does the growth cone use the actin engine to move forward? Mitchison and Kirschner first proposed the 'clutch' hypothesis[21], also called the substrate–cytoskeletal coupling model[22], which links growth cone protrusion to actin dynamics[16,23]. They suggested that growth cone receptor binding to an adhesive substrate leads to the formation of a complex that acts like a molecular clutch, mechanically coupling the receptors and F-actin flow, thus anchoring F-actin to prevent retrograde flow and driving actin-based forward protrusion of the growth cone on the adhesive substrate (FIG. 2a). Indeed, growth cone–substrate adhesions have long been shown to be important for growth cone migration[24] and, in fact, the generation of traction also requires myosin II[25].

Filopodia, in particular, are considered to be guidance sensors at the front line of the growth cone and might have a major role in establishing growth cone–substrate adhesive contacts during environmental exploration[26]. Studies show that filopodia function as points of attachment to the substrate and produce tension that is used for growth cone progression[27,28]. Whereas earlier studies that blocked filopodia formation using general F-actin inhibitors showed abnormal growth cone steering[29,30], a recent study that specifically targeted filopodial F-actin suggests that filopodia are dispensible for accurate growth cone guidance but are indeed required for normal growth cone motility[31], supporting their role in forming adhesive contacts.

Accumulating evidence in recent years supports the clutch model, in particular *in vitro* live growth cone imaging experiments that use APCAM, a neural CAM (NCAM) orthologue in *Aplysia californica*[32], a model system with large growth cones that allow the high resolution imaging of their cytoskeletal dynamics[33]. Following APCAM-mediated growth cone–substrate

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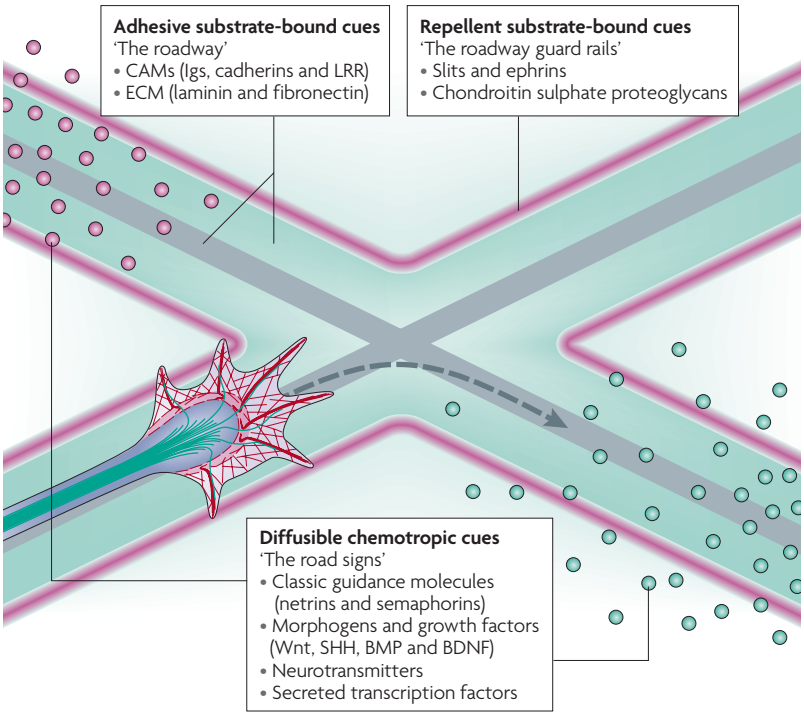

Figure 1 | **Directions for the trip.** The growth cone encounters many different types of cues in its environmental terrain. It travels on a 'road' that is made up of adhesive molecules that are either presented directly on a neighbouring cell surface (for example, transmembrane cell adhesion molecules (CAMs)[1]) or assembled into a dense and complex extracellular matrix (ECM; for example, laminin and fibronectin[2]). Additionally, anti-adhesive surface-bound molecules (such as slits, ephrins and chondroitin sulphate proteoglycans) can prohibit growth cone advance and thus provide the 'guard rails' that determine the road boundaries. Finally, diffusible chemotropic cues are the 'road signs' that present further steering instructions to the growth cone and include various diffusible chemotropic molecules (such as netrins and semaphorins[3,4]), as well as morphogens (such as Wnt, sonic hedgehog (SHH) and bone morphogenetic protein (BMP))[5] and growth or neurotrophic factors (such as brain-derived neurotrophic factor (BDNF))[5], secreted transcription factors[6–9] and neurotransmitters[10]. Whereas it was originally thought that some cues function as attractive 'go' signals (for example, netrins) and others as repulsive 'stop' signals (for example, ephrins), it is now clear that the response of attraction or repulsion is not due to the intrinsic property of the particular cue, but rather to the specific growth cone receptors that are engaged and the internal signalling of the growth cone. Green and red circles are interpreted as attractive and repulsive cues, respectively.

F-actin arc
An actomyosin contractile structure that is perpendicular to bundles of actin, forming a hemicircumferential ring in the transition zone.

Dynamic instability
The state used to describe microtubule polymer dynamics, in which microtubule polymers cycle through periods of growth, shrinkage and occasional pausing.

adhesion, dramatic local reorganization of actin occurs. Increased levels of localized actin assemble at the site of adhesion, followed by regional slowing of retrograde flow and growth cone protrusion[16,34,35]. Subsequently, F-actin bundles disappear between the adhesion site and the C domain (the region of the growth cone in which the microtubules (MTs) from the axon shaft enter the growth cone), and F-actin arcs reorientate from the C domain towards the adhesion site, creating a corridor between the two regions. Actomyosin-driven tension builds up between the actin assembly at the adhesion site and the actin arcs with their associated MTs, and the growth cone undergoes engorgement as the C domain moves forward[35] (BOX 2).

Whereas many studies that investigated the clutch hypothesis focused on the cell adhesion molecule APCAM in *A. californica*, recent studies have begun to provide a molecular basis for similar clutch linkages between actin and other CAMs and ECM receptors in growth cones. In non-neuronal systems, the focal adhesion proteins talin and vinculin provide a prototypic molecular clutch that is mediated by the integrin ECM receptors[36]. More recently discovered examples of growth cone-specific clutch machinery include catenins, which mechanically couple N-cadherin receptors and F-actin flow in rat neurons[37], and the novel protein shootin 1, which mediates linkage between L1CAM receptors and F-actin flow[38]. A complete molecular understanding of the clutch mechanism will allow the transition from *in vitro* findings to embryonic systems and will provide a framework for understanding the overall logic that governs forward progression of the growth cone *in vivo*.

MTs help steer the vehicle. Although actin structures are rapidly remodelled in response to guidance cues, actin is not the only component of the vehicle, as growth cones cannot move forward without MT function[39]. Whereas the mechanisms that control MTs have generally received less attention than those of actin, recent studies have confirmed early seminal experiments[40] by showing that MTs have major roles in the process of growth cone steering in two complementary ways: individual P domain MTs act as guidance sensors, whereas bulk C domain MTs steer growth cone advance[41].

First, before growth cone protrusion (BOX 2), a population of individual MTs actively explore the P domain[42] (FIG. 2b) using their property of dynamic instability (BOX 3). Because the introduction of a localized adhesive cue leads to an increase in the number of exploratory MTs that interact with the adhesion site[35], it has been proposed that these MTs might act as guidance sensors (FIG. 2b). This might occur either by carrying signals involved in steering to and/or from the cortical membrane, or by acting as a scaffold for the localized recruitment of key signalling components needed for navigation[43]. For example, dynamic MTs are required for the localized accumulation of active Src family kinase signalling at sites of adhesion, which is necessary for the turning response of a growth cone to an adhesive substrate[43], and MTs probably bring other signalling molecules as well (for example, Rho family GTPase regulators).

The second major role of MTs in steering occurs during engorgement (BOX 2), after initial actin remodelling in response to cues. At this point, stable, bundled C domain MTs move into the area of new growth, as consolidation of a new region of axon shaft occurs behind them, thereby fixing the axonal direction[16,44]. Further supporting the instructive role of MTs in growth cone steering, the inhibition of MT dynamics prevents growth cone turning in response to guidance cues, whereas localized MT stabilization induces turning[44].

MT interactions with actin. The role of MTs during growth cone steering clearly requires the participation of, and interaction with, actin[45,46]. Recent live-imaging studies show that the function of actin dynamics might be to provide spatio-temporal guidance to MTs to steer

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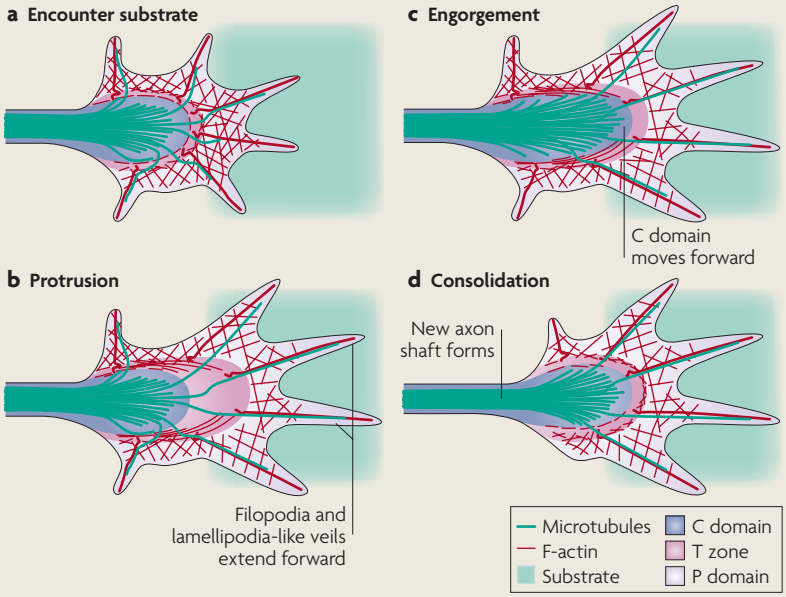
Box 2 | **Stages of axon outgrowth**



A traditional description of the axon outgrowth process separates it into three stages: protrusion, engorgement and consolidation[13,14]. These occur upon encountering attractive, adhesive substrates. This sequence during growth cone progression provides a framework for understanding detailed molecular mechanisms, and we assume that some of the same mechanistic events are used in response to diffusible chemotropic cues.

The distal end of the growth cone contacts an adhesive substrate (see the figure, part **a**). The binding of growth cone receptors activates intracellular signalling cascades and begins the formation of a molecular 'clutch' that links the substrate to the actin cytoskeleton. During protrusion, this clutch strengthens, resulting in regional attenuation of filamentous (F)-actin retrograde flow. This anchors the actin with respect to the substrate so that, as F-actin polymerization continues in front of the clutch site, the lamellipodia-like veils and filopodia of the peripheral (P) domain move forward to extend the leading edge (see the figure, part **b**) (see REF. 124 for a discussion of the molecular ratchet model for membrane protrusion). Engorgement occurs after actin clears from the corridor between the adhesion and the central (C) domain, perhaps as F-actin behind the clutch is severed and removed (see the figure, part **c**). F-actin arcs reorientate from the C domain towards the site of new growth[16,34,35], followed by the invasion of C domain microtubules (MTs) into this region, which are guided by transition (T) zone actin arcs and C domain actin bundles. Finally, consolidation of the recently advanced C domain occurs as the proximal part of the growth cone compacts at the growth cone neck to form a new segment of axon shaft (see the figure, part **d**). The myosin II-containing actin arcs compress the MTs into the newly localized C domain (followed by MT-associated protein stabilization). Retraction of the filopodia away from the area of new growth occurs as F-actin protrusive activity is suppressed in these regions (also promoted by myosin II activity[52]), further promoting axon shaft consolidation. These three continuous and overlapping stages occur during the formation of nascent axons, and also when new growth cones form from an axon shaft during axon branching[14,125].

the growth cone in the right direction. In particular, actin has a pivotal role in determining MT localization in the growth cone, acting as both a barrier to premature MT invasion and as a guide to MTs during their advance[45,47]. Furthermore, local perturbation of actin structures leads to the redistribution of MTs and a change in the direction of growth[48]. Here, we discuss two important interactions between MTs and actin: between P domain MTs and F-actin bundles, and between C domain MTs and actin arcs (FIG. 2c).

As dynamic MTs preferentially explore the growth cone periphery, they usually follow the trajectories of F-actin bundles[42], which are thought to guide MT advance into the P domain[41,45]. A recent study, however, showed that these F-actin bundles are not required for MT advance; indeed, they inhibit MT penetration into the P domain when the MTs are coupled to F-actin bundle-specific retrograde flow[47] (by MT–actin crosslinking proteins). As MT coupling to F-actin retrograde flow directly affects the ability of MTs to explore the P domain, it seems likely that regulation of MT–actin coupling and uncoupling (the release of MTs from F-actin retrograde flow) would have an effect on MT dynamics. This prediction was shown in a more recent study that examined P domain MTs[35]. Not only does increased MT–actin uncoupling allow dynamic MTs to explore growth cone sides more frequently than central regions under uniform conditions (which might account for increased sensitivity to guidance cues that are not on the current axon outgrowth path), but it also allows an increased number of MTs to explore sites of APCAM-mediated adhesion[35]. It is possible that repellent guidance cues induce an opposite response in the actin–MT coupling state, thus leading to an opposite effect on the cytoskeletal machinery.

Interestingly, the absence of F-actin bundles does not lead to the inappropriate advance of C domain MTs[47], suggesting that although F-actin bundles regulate exploratory P domain MTs during protrusion, stable C domain MT movement into the growth cone during engorgement might be regulated by another mechanism, namely the actin network and actin arcs. Indeed, another study showed this to be the case. Disruption of actin arcs results in the failure of MT consolidation during axon outgrowth, leading to an abnormally broad C domain[49]. During engorgement, as the C domain advances towards an adhesion site, actin arcs on the sides of the C domain become more prominent (BOX 2) and mechanical connectivity between extracellular adhesion, actin arcs and the C domain is apparent[49]. Thus, actin arcs normally form a barrier around the C domain that regulates MT advance by capturing MTs on the sides of the growth cone and transporting them into the C domain. In a separate study, it was shown that myosin II, which mediates the contraction of antiparallel actin filaments found in actin arcs, has an important role in actively transporting MTs from the sides into the C domain, compressing them into bundles and perhaps securing them[50] until they are stably crosslinked by MT-associated proteins (MAPs) in the growth cone neck[51]. Myosin II in the growth cone neck has also been shown to suppress F-actin protrusion to allow axon shaft consolidation[52], and this function might contribute to its function during growth cone turning[53].

Therefore, distinct classes of actin structures seem to regulate different populations of MTs. Whereas F-actin bundles can inhibit the protrusive activities of P domain MTs when they are coupled together, F-actin arcs regulate the engorgement and consolidation activities of C domain MTs (FIG. 2c). Therefore, an obvious question is: how are MT–actin interactions regulated in a spatiotemporal manner in response to guidance cues? This topic is addressed below.

Actin network
Actin filaments that are crosslinked in a branched pattern, forming the structure of lamellipodia-like veils.

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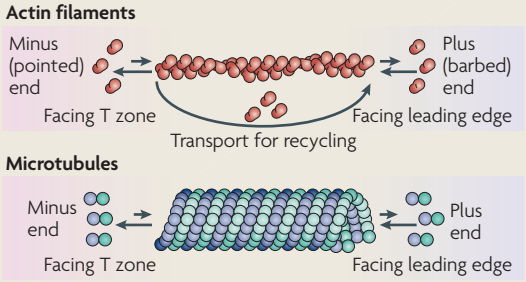
Box 3 | **Cytoskeletal dynamics**



Actin filaments are polarized polymers that are composed of actin monomers. Their formation, stability and destruction are carefully regulated at every stage in the growth cone. Actin monomers can be added to either end (see the figure), but changes in the equilibria of polymerization dynamics depend on whether ATP or ADP is associated with actin. In the growth cone, ATP–actin is usually added to the plus (or barbed) end that points towards the cell membrane, ATP hydrolyses to form ADP–actin, and ADP–actin disassembles at the minus (or pointed) end that faces the transition (T) zone. Monomer-binding proteins then transport actin back to the leading edge to support further growth. Other actin-binding proteins include nucleation factors that create new actin plus ends for growth, capping proteins that block growth or disassembly, antagonists of capping proteins, actin filament-severing proteins and filament-stabilizing proteins, such as those that assemble F-actin into higher-order structures (for example, bundles and networks) and those that anchor F-actin to specific regions of the membrane (reviewed in REF. 126).

Microtubules (MTs) are polarized structures that are composed of α- and β-tubulin dimers and are assembled into linear arrays. A linear array of alternating α- and β-tubulin subunits form a protofilament, 11–15 of which form the wall of the MT. GTP–tubulin dimers are added to the plus end, and GDP–tubulin dimers dissociate from the minus end following GTP hydrolysis (see the figure). In growth cones, MT plus ends, which face outwards towards the periphery, exhibit dynamic instability — they cycle through periods of growth, shrinkage and occasional pausing[127]. Numerous proteins bind to MTs: some proteins stabilize MTs (for example, MT-associated protein 1B (MAP1B)[111]), some act as MT motors (for example, dynein and kinesin[128]) and others are part of a family called plus-end tracking proteins (+TIPs), which have been implicated in dynamic control of plus end MTs and linking MTs with actin- or membrane-associated structures (for example, end-binding proteins (EBs), adenomatous polyposis coli (APC) and CLASP (cytoplasmic linker protein-associated protein[97,98])).

The growth cone as a navigator

Thus far, we have described how changes in the cytoskeletal machinery drive the forward progression of the growth cone vehicle. However, growth cone pathfinding obviously does not consist solely of moving forward; it is a dynamic process in which the growth cone progresses, pauses, turns and retracts as it navigates through the embryonic landscape and encounters various directions for its trip. Spatial bias in a given direction can occur through either positive cues that increase protrusion (towards the side of new growth) or negative cues that decrease protrusion (occurring on the side away from new growth). For spatial discontinuities in the environment to drive growth cone steering and, in particular, to accurately interpret numerous cues simultaneously, the growth cone requires a navigation system that can translate multiple environmental directions and integrate separate signalling pathways to locally modulate the dynamics of the cytoskeletal machinery. The overall logic that governs this process is still emerging. There is a vast literature that describes specific aspects of the growth cone navigation system, but many studies focus on individual pathways that are engaged by particular cues or receptors. Although there are numerous signal transduction molecules that convey guidance information, including kinases[54,55], phosphatases[56] and calcium ions[57], our most comprehensive understanding is of the Rho family of GTPases, a class of molecules that control cytoskeletal dynamics downstream of nearly all guidance signalling receptors[58,59] (FIG. 3).

Although increasing numbers of studies have analysed the functions of Rho GTPases and their cytoskeletal effectors in the growth cone, much of our understanding of these molecules still comes from non-neuronal systems (for example, fibroblasts, neutrophils and *Dictyostelium discoideum*[60,61]). Because substantial differences exist in molecular content between cell types, we must be careful not to assume that the systems act identically. Nonetheless, numerous studies do suggest certain parallels in cytoskeletal signalling between neuronal and non-neuronal cells[60,61]. Additionally, common cell biological mechanisms underlie growth cone guidance and regulation of other aspects of axon biology, such as axon initiation and modelling of secondary axonal branches[62]. Thus, studies on cytoskeletal signalling in other systems might provide insights into the mechanisms of growth cone guidance (and vice versa).

Rho GTPases behind the wheel. Rho GTPases, which include RhoA, RAC1 and CDC42, are signalling nodes that couple upstream directional cues and downstream cytoskeletal rearrangements to either enhance actin polymerization for protrusion or promote disassembly and actomyosin contraction for retraction[58,59] (FIG. 3). If regulation of Rho GTPase activity is to convey guidance information, its upstream regulators must be activated in a spatially specific manner, internally reflecting the extracellular environment. Upstream regulators include the proteins that activate Rho GTPases (guanine nucleotide-exchange factors (GEFs)) and those that inactivate them (GTPase-activating proteins (GAPs))[58,63] (FIG. 3; a further, newly implicated method of Rho GTPase regulation is the local translation downstream of guidance signalling[64], which is briefly discussed in BOX 4). Many Rho GTPase regulators have been studied in non-neuronal systems, but our understanding of their specific functions in the growth cone is not as advanced[63]. Growth cone guidance receptors can contain their own GTPase regulatory

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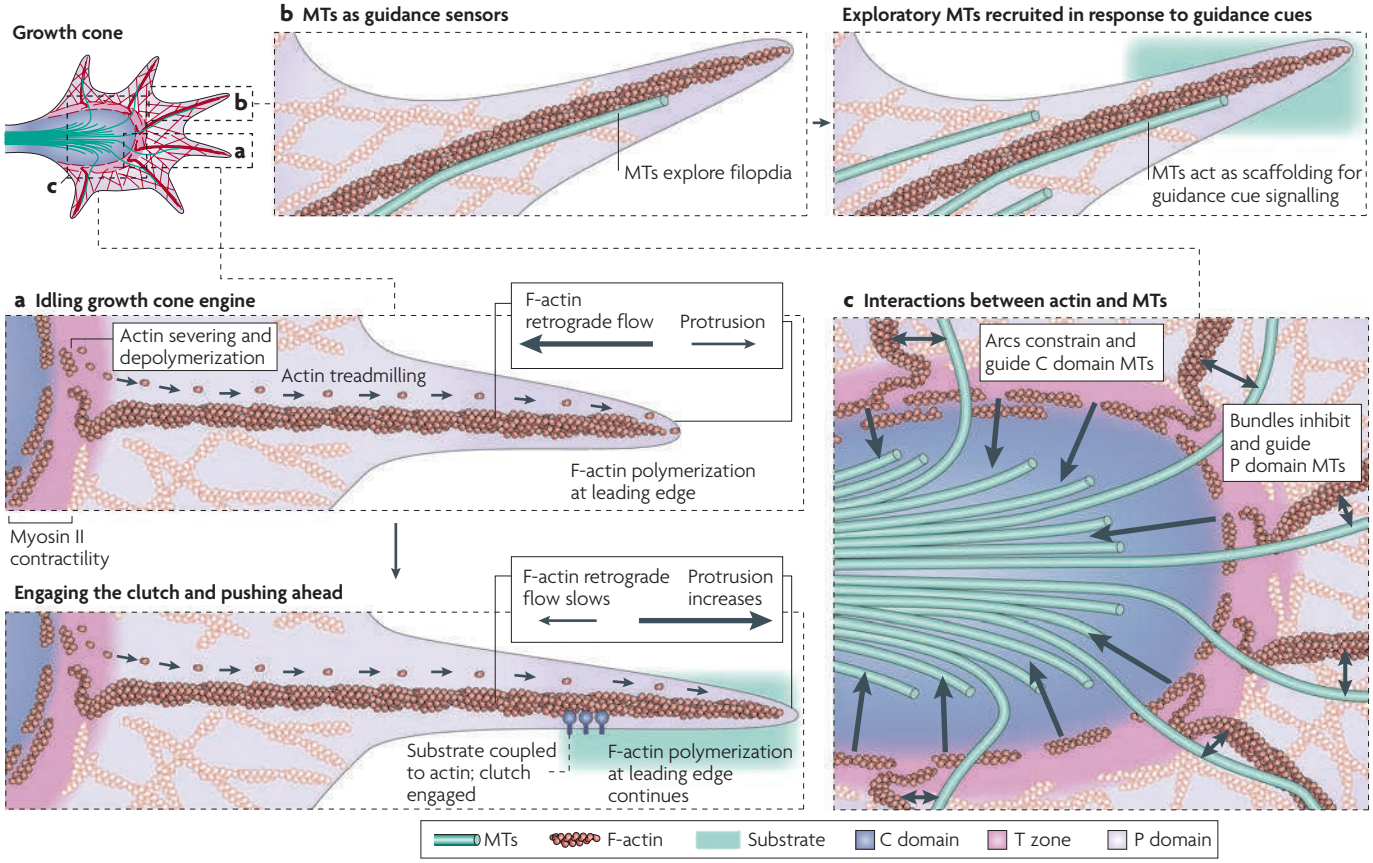

Figure 2 | **The growth cone 'vehicle'.** Boxed regions of the growth cone are shown in subsequent panels. **a** | Together, filamentous (F)-actin treadmilling (in which F-actin is polymerized at the leading edge and severed at the transition (T) zone, with the subunits recycled back to the leading edge) and F-actin retrograde flow (the continuous movement of F-actin from the leading edge towards the centre of the growth cone) keep the growth cone engine idling. When retrograde flow and polymerization forces are balanced, no protrusion occurs. When filopodia encounters an adhesive substrate, growth cone receptors bind to the substrate and are coupled to F-actin through 'clutch' proteins. This engages the clutch, anchoring F-actin with respect to the substrate and attenuating F-actin retrograde flow. Further F-actin polymerization pushes the membrane forward, which results in growth cone protrusion. **b** | Peripheral (P) domain microtubules (MTs) explore filopodia along F-actin bundles and might act as guidance sensors. As a filopodium encounters a guidance cue, exploratory MTs might act as scaffolding for further signalling, and additional MTs are recruited to the region. **c** | Actin has a role in determining MT localization in the growth cone. Actin arcs constrain and guide central (C) domain MTs (single arrows), and F-actin bundles inhibit and guide P domain MTs (double arrows).

domains, such as the Plexin family of receptors for the semaphorins[65], but many other receptors transduce their activation state through separate regulators that are recruited by cytoplasmic domains. For example, the guidance cue ephrin B3 binding to the EphA4 receptor activates the regulatory RacGAP α-chimerin (also known as N-chimaerin) to inhibit growth cone extension[66]; EphA receptors can trigger the activation of the RhoGEF ephexin to activate RhoA but inhibit CDC42 and RAC1 to induce growth cone collapse[67]; and the guidance cues slit and netrin can both signal through the RacGEF and RhoGEF TRIO to regulate growth cone dynamics[68,69].

A potentially confusing feature of Rho GTPase signalling is that the possible signalling network combinations of GEFs, GAPs and GTPases are numerous and complex. Multiple GTPases (that have antagonistic functions) can be activated in response to the same guidance cue. For example, ephrin A4 can lead to RhoA activation, Rac protein inactivation or Rac activation, depending on which receptors and GEFs or GAPs are engaged[58], and over 70 GEFs and 80 GAPs have been described in mammals[70]. Many of them regulate several different Rho GTPases, and a particular GTPase might be regulated by numerous GEFs and GAPs that all reside in the same cell. How can this complex network of interactions be functionally explained in the growth cone? A recent profiling study of the proteome in neuroblastoma cells suggests that Rho GTPase spatial localization and activation might be the answer, which is a popular idea that is supported by previous studies and speculation[71]. This particular profiling study found that specific GEFs and GAPs are differentially localized between the cell body and the axonal process[72]; of the 14 GEFs that are expressed in neuroblastoma cells, 11 are enriched in axonal processes compared with the cell body, as

Neuroblastoma
A tumour derived from primitive ganglion cells that can partially differentiate into cells that have the appearance of immature neurons.

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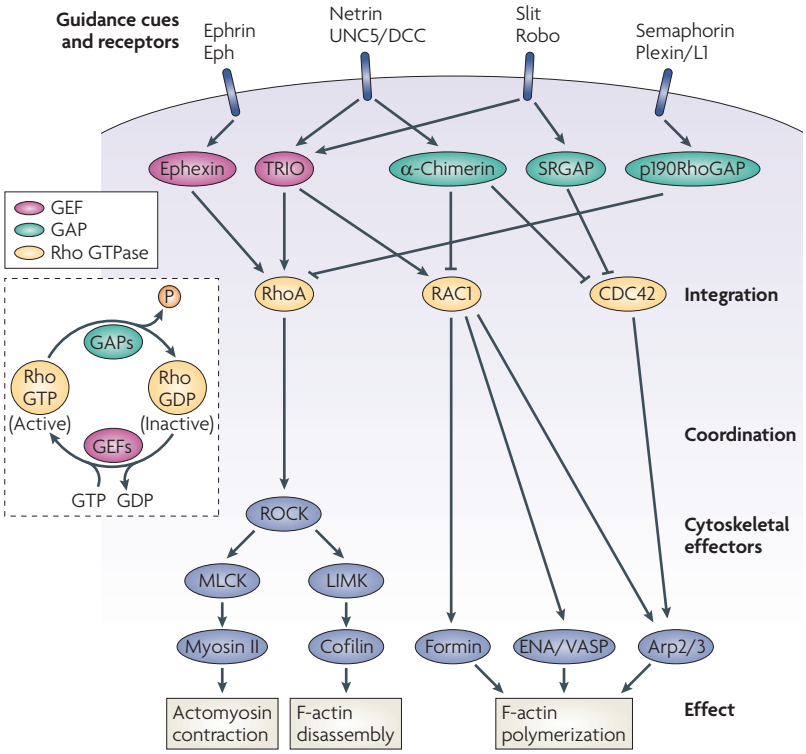

Figure 3 | **The growth cone as a 'navigator'.** Rho family GTPases act as key navigation signalling nodes to integrate upstream directional cues and coordinate downstream cytoskeletal rearrangements. The activation of receptors by guidance cues leads to the activation of Rho GTPase regulators. These include guanine nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs), which activate and inactivate Rho GTPases, respectively. Rho GTPases integrate the responses of upstream pathways and coordinate downstream effects by modifying the function of cytoskeletal effectors. Activation or inactivation of cytoskeletal effectors leads to responses such as actomyosin contraction, filamentous (F)-actin disassembly or F-actin polymerization. The resulting growth cone turning response depends on the localization of the guidance signalling inside the growth cone. Only some of the known examples of guidance cues and receptors, GEFs, GAPs and cytoskeletal effectors that are downstream of Rho GTPases are shown in the figure. Arrows do not necessarily denote direct interaction. The boxed inset shows the Rho GTPase activation–inactivation cycle, in which GAPs lead to the hydrolysis of GTP to GDP, whereas GEFs catalyse the exchange of GDP for GTP. Arp2/3, actin-related protein2/3; ENA/VASP, enabled/vasolidator-stimulated phosphoprotein; LIMK, LIM domain kinase; MLCK, myosin light chain kinase; ROCK, Rho kinase; SRGAP, slit–robo GAP; UNC5, uncoordinated protein 5.

are 6 of the 7 neuroblastoma GAPs. The authors propose that spatial compartmentalization of Rho GTPase regulators might allow the same GTPase to be regulated by distinct GEFs or GAPs in different locations throughout the growth cone. Moreover, time-lapse microscopy after individual knockdown of these GEFs or GAPs showed distinguishable axonal phenotypes. Whereas depletion of several regulators (including the GAP of RAC1 and CDC42 — ARHGAP30 — and dedicator of cytokinesis 4 (DOCK4), the GEF of RAC1) led to an increase in axon extension on an ECM substrate but normal cytoskeletal structure, silencing of others led to changes in axon extension along with distinct and obvious perturbations in the actin cytoskeleton[72]. For example, knockdown of the GAP of CDC42, slit–robo GAP 2 (SRGAP2), resulted in increased filopodia and cell spreading, knockdown of breakpoint cluster region (BCR), the GAP of RAC1, led to increased filopodia without cell spreading, and loss of TRIO, the GEF of RhoA and RAC1, led to long but unstable filopodia. Thus, even though RAC1 could be targeted by seven different GEFs and three different GAPs, and CDC42 could be targeted by four GEFs and five GAPs, all of which are located in the same cell, each upstream regulator is probably required for distinct cellular functions. These data suggest that the same GTPase might control various aspects of growth cone cytoskeletal dynamics, such as F-actin assembly, disassembly and retrograde flow, by receiving different upstream inputs of GEFs and GAPs in time and space.

Following activation, how do distinct Rho GTPases mediate downstream growth cone responses to affect growth cone steering? Intriguingly, activation of the same GTPase can lead to opposite responses of the growth cone: for example, whereas RhoA activation leads to growth cone retraction (by promoting myosin II contractile activity)[73], it can also be required for axon outgrowth[74] (by inhibiting the actin filament-severing protein family ADF/cofilin)[75]. Again, as with the upstream regulators, an explanation for this discrepancy is that Rho GTPases have different functions in the growth cone depending on their localization and, specifically, depending on which downstream effector molecules are activated. Numerous Rho GTPase effectors have been identified[76,77], but only a few (such as Rho kinase (ROCK)) have been well studied in the growth cone (see REF. 59 for a review). Following activation by Rho GTPases, these effectors either directly or indirectly regulate numerous downstream targets to modify the cytoskeleton to direct the growth cone vehicle in a spatially biased manner.

Control of actin dynamics at the leading edge. Rho GTPase cytoskeletal effectors are known to regulate all of the aspects of the actin cycle that affect growth cone steering, including F-actin assembly at the periphery, F-actin retrograde flow towards the C domain, and disassembly and recycling of actin at the T zone.

Actin polymerization at the leading edge must occur for the engine to run. This process is controlled by multiple regulators, including the actin nucleators, actin-related protein (Arp)2/3 complex and the formins, and the F-actin polymerization factors enabled/vasolidator-stimulated phosphoprotein (ENA/VASP). The Arp2/3 complex is a major effector of RAC1 and CDC42 and is thought to control the nucleation of F-actin polymerization and F-actin branching by binding to existing F-actin[78]. Several studies have shown that Arp2/3 is required for guidance[79,80], but whether this complex functions in a similar way in neuronal and non-neuronal cells has been questioned[79]. However, it was recently shown that Arp2/3 is present in the branched F-actin networks of growth cones and does affect their protrusion dynamics[81,82]. Inhibition of Arp2/3 in neurons blocks protrusion of both lamellipodia-like veils and filopodia and also increases RhoA activity[81], but future studies will be needed to determine its full role in growth cone motility. Downstream of Rho GTPase signalling, formins nucleate and then remain continuously associated with

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Box 4 | **Regulation of localized protein translation and degradation in the growth cone**

Localized translation in response to guidance cues has emerged as an important mechanism that mediates cytoskeletal dynamics, including RhoA signalling, during growth cone steering events[129–131]. In particular, attractive cues, such as netrins and brain-derived neurotrophic factor (BDNF), induce the asymmetric localization of mRNA and translation of cytoskeletal components, such as β-actin, on the side of the growth cone in which new filamentous (F)-actin polymerization (and thus growth cone extension) occurs[132,133]. Furthermore, repulsive cues, such as slit, induce the asymmetric translation of proteins that break down the cytoskeleton, such as actin-depolymerizing factor (ADF)/cofilin (a family of actin filament-severing proteins that disassemble F-actin filaments)[134] as well as β-thymosin (an actin monomer-sequestering protein that inhibits actin polymerization)[135]. The microtubule (MT)–F-actin crosslinking protein short stop, which is required for growth cone steering, also binds directly to a newly discovered translation inhibitor, krasavietz (also known as extra bases)[136]. Furthermore, the plus-end tracking protein (+TIP) family member adenomatous polyposis coli (APC), which also binds to MT plus ends and has a role in steering, has recently been shown to be required for RNA localization to cell protrusions in non-neuronal migrating cells[137] and, thus, it might function in a similar way in growth cones. In addition to cytoskeletal elements, local translation of the signalling molecule RhoA GTPase is required for the collapsing response of *Xenopus laevis* growth cones to the repulsive cue semaphorin 3A[64], suggesting that localized translation is a common theme for many molecules involved in growth cone steering.

Finally, localized protein degradation might also have a role in the regulation of growth cone dynamics. This has been shown to be true for RhoA at the leading edge in migrating fibroblasts[138], and seems also to be true in neuronal cells, during their outgrowth[139,140].

elongating F-actin barbed ends[83]. Formin is required for growth cone filopodia formation, and the *Drosophila melanogaster* formin DAAM was recently shown to act together with Rac GTPases and ENA during axonal growth regulation[84]. The novel actin nucleator, cordon-bleu, is also highly enriched in rat brain and might have a role during growth cone guidance[85].

ENA/VASP proteins are a family of proteins that enhance F-actin elongation by several methods, including binding to F-actin barbed ends at the leading edge to antagonize capping proteins (which inhibit F-actin elongation), a process that is also observed for the formins, and also by recruiting actin subunit complexes to the P domain for further polymerization[86]. Although ENA/VASP proteins are mainly thought to be regulated by protein kinases downstream of guidance cue signalling (such as the cyclic nucleotide-activated kinases, including protein kinase A (PKA)[86]), there are genetic interactions between ENA and TRIO in *D. melanogaster*[87], and the ENA regulator Ableson tyrosine kinase functions through Rac and Rho GTPases in both neuronal and non-neuronal cells[88,89], suggesting that there is crosstalk between Rho GTPase signalling and ENA/VASP proteins.

Additionally, cytoskeletal effectors that are downstream of RhoA GTPase have crucial roles in regulating F-actin retrograde flow and disassembly of actin in the T zone. ROCK, one of the most widely studied downstream effectors of RhoA, has multiple phosphorylation targets that are implicated in actin dynamics in the growth cone, including myosin light chain kinase (MLCK), LIM domain kinase (LIMK) and ezrin–radixin–moesin (ERM) proteins. Phosphorylation of MLCK induces myosin II activity and promotes its association to F-actin, leading to actomyosin contraction and driving F-actin retrograde flow[59,90]. Active LIMK inactivates the actin filament-severing protein ADF/cofilin by phosphorylation, thereby stabilizing actin filaments and promoting the forward progression of the vehicle[75]. Finally, ERM proteins are another group of actin-binding proteins that have an important but unclear role in growth cone actin dynamics. Not only are they directly downstream of ROCK[91], they can interact with specific growth cone receptors (such as L1CAM)[92], suggesting that they might have a role in crosstalk between different signalling pathways. Activated ERM proteins are asymmetrically localized in response to guidance cues in the growth cone and promote growth cone motility in response to the guidance cue semaphorin 3A[93], but how they function to remodel actin dynamics has not been clarified.

These examples are just a few of the many effectors of actin dynamics that are downstream of Rho GTPase signalling. Additionally, there are other important actin effectors that are not clearly linked to the Rho GTPases. The complete picture of how guidance cues affect all aspects of actin dynamics on a global growth cone scale is still emerging.

Steering and MT–F-actin interactions

As with actin, there are many aspects of MT dynamics that can be controlled, including nucleation, polymerization, stabilization and translocation along F-actin, all of which have roles in steering the growth cone vehicle and which are coordinated by cytoskeletal effectors downstream of guidance signalling. However, although numerous studies have elucidated the functions of a multitude of actin-binding proteins, fewer studies have analysed the detailed functions of MAPs. Whereas Rho GTPase signalling has a central role in regulating actin dynamics, this is not as evident for MTs, although there are several examples of MT-specific RhoGEFs in non-neuronal cells, such as RHOGEF2 in *D. melanogaster*[94] and XLFC in *Xenopus laevis*[95], and RAC1 can regulate MT dynamics in non-neuronal cells[96]. However, this connection has not yet been clearly shown in growth cones. Based on recent studies, it is clear that the ability of MTs to explore the growth cone periphery and enter into filopodia (an important early step that is necessary for proper steering in response to environmental cues) is highly dependent on how MTs interact with the F-actin bundles and network, and thus Rho signalling at least indirectly affects MT dynamics.

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A central focus of studies on MT dynamics in the growth cone revolves around the control of MT–actin interactions; specifically, the coupling and uncoupling of MTs and F-actin retrograde flow. For example, positive guidance cues, such as the adhesive molecule APCAM, increase the frequency of MT–actin uncoupling, leading to increased MT exploration at sites of cue binding[35]. By contrast, repellent cues might increase coupling between MTs and actin, and thus reduce MT exploration and increase MT 'looping', because the growing plus ends of MTs are transported back towards the C domain by their linkage to F-actin retrograde flow, thereby forming MT loops in the growth cone. Thus, one function of the growth cone navigation system is to locally control MT–actin interactions in response to asymmetric guidance cues. The detailed signalling pathways by which this occurs are still unclear, but it is obvious that components of the navigation system can control MT dynamics by directly or indirectly modulating the activity of MAPs, and, in particular, those that function as MT–actin crosslinking proteins.

Revealing the roles of MAPs in growth cone dynamics. One group of MAPs that has recently been shown to have a crucial role in growth cone dynamics is the family of plus-end tracking proteins (+TIPs; including end-binding proteins (EBs) and adenomatous polyposis coli (APC)), which bind specifically to the plus ends of MTs[97,98]. These proteins were originally implicated in affecting the stabilization of MT plus ends, but they also mediate MT crosslinking to actin, either in a positive or negative way. Interestingly, the +TIP EB1 (also known as MAPRE1) is required for RHOGEF2 association with MT plus ends in non-neuronal cells[94], which suggests the intriguing possibility that navigator signalling molecules, such as Rho GTPase regulators, might actually harness MT dynamics to move themselves into areas of guidance cue signalling[99]. This could be one explanation for how exploratory MTs are required early for steering.

Evidence suggests that at least two +TIP members, lissencephaly 1 (LIS1; also known as PAFAH1B1) and APC, promote MT–actin uncoupling in the growth cone. In particular, LIS1 cooperates with the MT motor protein dynein to allow the uncoupling of dynamic MTs from actin retrograde flow in chick and rat neurons plated on a laminin substrate[100,101]. Inhibition of dynein and LIS1 prevents MT advance into the periphery and reduces the ability of MTs to resist F-actin retrograde flow[101]. Consequently, the growth cone cannot accurately steer along a laminin border[100]. This is further confirmed by the blockage of retrograde flow using the myosin II inhibitor blebbistatin, which leads to the recovery of MT extension into the P domain in dynein-depleted growth cones and demonstrates that actin retrograde flow can prevent MT exploration[100]. Whereas the pathways by which guidance cues lead to LIS1 and dynein activity in the growth cone have not been elucidated, LIS1 can specifically interact with the RAC1 and CDC42 regulator IQGAP1 in migrating mouse neurons[102], and LIS1 deficiency is associated with dysregulation of CDC42, RAC1 and RhoA[103]. Additionally, connections exist between dynein and Rho GTPases in other systems (for example, the dynein regulator nudel binds to and inhibits the Rho GTPase regulator CDC42GAP in migrating mouse cells[104]), suggesting that Rho GTPases control LIS1 and dynein-dependent uncoupling downstream of guidance cues. Interestingly, a recent study showed that the motor protein kinesin 5, working antagonistically with dynein, has a key role in controlling MT extension into the P domain during growth cone turning and is specifically phosphorylated on the side opposite the invasion of MTs before turning[105], further demonstrating that motor proteins are likely targets of guidance cue signalling.

Similar to LIS1, APC is another +TIP that promotes MT–actin uncoupling in the growth cone and also directly interacts with IQGAP1 (REF. 106). APC binds to a subset of MTs in the growth cone and its binding location indicates the future growth direction of the axon. Local enhancement of APC association with MTs leads to growth cone steering towards that axis[107], possibly by preventing MT binding to F-actin. This speculation is supported by a recent study that shows that APC loss from MT plus ends leads to increased MT looping and the prevention of growth cone progression[108]. APC loss occurs in response to the morphogen WNT3A, which acts through the intracellular Wnt signalling pathway member dishevelled 1 and can induce axonal remodelling in mouse dorsal root ganglia neurons. In this case, the Wnt signalling pathway might be regulating APC–MT association by modifying the APC phosphorylation state. When APC is lost from the MT plus ends, this might allow other MT–actin crosslinkers to bind to the plus ends and couple MTs to F-actin retrograde flow, leading to MT looping. For example, the +TIP member CLASP (cytoplasmic linker protein-associated protein), promotes MT looping in the growth cone when overexpressed[109] and can link MT ends to actin in non-neuronal cells[110], although its actin-binding activity in the growth cone has not been examined. If this function holds true in growth cones, CLASP might be a +TIP member that drives MT–actin coupling and MT looping behaviour downstream of environmental 'stop' signals. Interestingly, glycogen synthase kinase 3β (GSK3β), which is downstream of a number of guidance cues, including Wnt, netrin and semaphorin signalling[111], can regulate whether CLASP binds to the MT plus ends or along the entire MT, and this change in localization might regulate its function[112].

MAP1B, which is considered to be a scaffold protein that stabilizes MTs but can also bind actin, also regulates growth cone steering and motility of mouse neurons by controlling MT–actin dynamics[113]. In particular, the role of MAP1B in growth cones might be to couple MTs and F-actin in response to guidance cues, as MAP1B function is required for the MT looping that occurs in response to treatment with lysophosphatidic acid (LPA; a phospholipid derivative that triggers axonal process retraction and growth cone collapse)[113]. As MAP1B function can be modulated by phosphorylation[111], this suggests a mechanism by which the ability of MAP1B to couple MT and actin depends on upstream guidance cue signalling. For example, MAP1B is a phosphorylation substrate of GSK3β[114], as is CLASP.

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Whereas coupling MTs to F-actin retrograde flow prevents MT extension to the periphery in these cases, interactions between MTs and F-actin can also promote MT extension. For example, a recent study showed that the F-actin-associated protein drebrin binds directly to the +TIP protein EB3 in embryonic rat growth cones, an interaction that occurs specifically in the proximal region of filopodia when EB3-bound MTs enter and align alongside drebrin-bound F-actin bundles[115]. Strikingly, loss of drebrin function prevents MT extension into filopodia, suggesting that the drebrin–EB3 interaction is important for allowing MT exploration of filopodia[115].

These recent studies that examine the growth cone functions of MT–actin regulatory proteins are beginning to uncover the complete and detailed mechanisms by which environmental cues are translated to changes in the cytoskeleton, and, especially, they seek to answer the intriguing question of how MTs interact functionally with the actin network during growth cone steering. However, there is still much to understand of the role of MTs and MT–actin interactions in the growth cone, and this is a promising area of growth cone guidance research for the future.

Conclusions and future perspectives

Like an experienced driver, the growth cone navigation system can integrate multiple environmental variables, including road conditions, stop lights and street signs, to direct the vehicle and reliably reach its destination. Navigation system signalling through Rho GTPases and downstream cytoskeletal effectors is superimposed on the cytoskeletal mechanisms of the vehicle, including F-actin retrograde flow and F-actin guidance of MTs, to introduce spatial bias for steering the growth cone in the right direction. Understanding how multiple cytoskeletal effectors work in concert to achieve this, in combination with the elucidation of additional signalling pathways that mediate growth cone navigation, will give an integrated picture for the logic of growth cone dynamics over time.

Whereas much work has investigated how individual cues are interpreted, the next target will be to understand how the growth cone interprets multiple overlapping gradients of cues. Previously, it has been difficult to examine complex effects on the growth cone cytoskeletal machinery, in part owing to technical limitations of experimental assays. Recent work that uses microfluidic devices has allowed the production of stable, precisely controlled gradients in various combinations of both diffusible and substrate-bound factors[116,117]. These types of combined cues can more faithfully recapitulate the complexity of the *in vivo* environment and, thus, future experiments that use this method, in combination with high-resolution cytoskeletal imaging, hold considerable potential for refining our understanding of the growth cone guidance mechanism.

In the past decade, there have been great advances in high-resolution imaging methods that can be used for analysing growth cone cytoskeletal dynamics, including fluorescent speckle microscopy[118] and total internal reflection fluorescence microscopy[119]. Combinations of these microscopy methods with new cross-correlation speckle-tracking algorithms for quantitatively measuring the details of cytoskeletal dynamics have provided further insights into the roles of actin and MTs during normal vehicular function of the growth cone[36,120], including the finding that exploratory MTs might have a role in early signalling from guidance cues, whereas actin dynamics guide and control more stable MTs to fix the direction of new growth. The same types of quantitative methodology will need to be applied to understand protein–protein interactions and catalytic activations of signalling molecules, through advanced fluorescent sensors and tags[121–123]. This is an exciting time for studying growth cone dynamics, with new techniques and microscopy tools that are now providing further opportunities to explore the outstanding questions of the growth cone vehicle and its navigation.

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DATABASES
UniProtKB: http://www.uniprot.org
α-chimerin | APC | APCAM | ARHGAP30 | CDC42 | CLASP | DAAM | DOCK4 | EB1 | EphA4 | ephrin B3 | ephexin | IQGAP1 | kinesin 5 | L1CAM | LIS1 | LIMK | MLCK | NCAM | netrin | RAC1 | RhoA | RHOGEF2 | ROCK | semaphorin 3A | shootin 1 | slit | SRGAP2 | talin | TRIO | vinculin

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