How Actin Filaments and Microtubules Steer Growth Cones to Their Targets

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ABSTRACT: **Guidance molecules steer growth cones to their targets by attracting or repelling them. Turning in a new direction requires remodeling of the growth cone and bending of the axon. This depends upon reorganization of actin filaments and microtubules, which are the primary cytoskeletal components of growth cones. This article discusses how these cytoskeletal components induce turning. The importance of each component as well as how interactions between them result in axon guidance is discussed. Current evidence shows that microtubules are influenced by both the organization and dynamics of actin filaments in the peripheral domain of growth cones. Cytoskeletal models for repulsive and attractive turning are presented. Molecular candidates that may link actin filaments with microtubules are suggested and potential signal transduction pathways that allow these cytoskeletal components to affect each other are discussed.** © 2003 Wiley Periodicals, Inc. J Neurobiol 58: 84–91, 2004

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Growth cones are key cytoskeletal structures at the tips of growing axons that determine the direction of axon growth in response to various guidance cues during development, and they also mediate axon regeneration after nerve injury. It is broadly agreed that the cytoskeletal reorganization of the growth cone is the conversion point of most, if not all, guidance-cue-induced signaling cascades during axon guidance (Baas and Luo, 2001). Studying the mechanism of how guidance cues steer the growth cone will help us to better understand how the extremely complex patterns of neuronal wiring are generated during development. Moreover, it will also allow the development of potential therapies for nerve injury because cues that act to repel growth cones during development may act as factors that inhibit CNS regeneration in adults.

INDISPENSABLE ROLE OF ACTIN FILAMENTS AND MICROTUBULES DURING AXON GUIDANCE

Actin filaments are major cytoskeletal elements that determine the structure and motility of growth cones. At the growth cone peripheral domain (P-domain), an actin meshwork is concentrated at the leading edge and actin bundles project radially throughout the growth cone into filopodia. Microtubules are the other major structural element of the growth cone that support growth-cone-mediated axon extension. They project from the axon shaft to the central region (C-domain) of the growth cone in a bundled form. At

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the end of the microtubule bundles, individual microtubules splay apart and some of them invade the actin-rich P-domain and display dynamic instability (Zhou et al., 2002; Kabir et al., 2001).

Both actin filaments and dynamic microtubules in the P-domain have been known to play a vital role in growth cone motility during axon guidance. Early studies have shown that when neurons are cultured on poly-lysine, cytochalasin treatment that depolymerizes actin filaments causes axons to grow in a wandering pattern (Marsh and Letourneau, 1984). Similarly, *in vivo* application of cytochalasin abolishes directed axon growth towards targets (Chien et al., 1993), indicating an important role of actin filaments in axon guidance. Vinblastin and taxol, drugs that regulate microtubule assembly, when used at low doses diminish microtubule dynamics without significantly affecting microtubule assembly or actin dynamics. Treating the growth cone with low doses of vinblastin or taxol leads to loss of directed axon growth in response to a repulsive guidance cue without interfering with axon growth (Williamson et al., 1996; Challacombe et al., 1997). Although it is generally accepted that both cytoskeletal components act in a coordinated manner, these studies manipulated actin filaments or microtubules individually and therefore they did not provide any direct evidence for interactions between these components.

ACTIN-MICROTUBULE INTERACTION DURING GROWTH CONE TURNING

The importance of actin-microtubule interactions in the growth cone during axon guidance was suggested by recent studies. First, coordinated actin polymerization at the leading edge and microtubule protrusion in the direction of growth cone turning have been observed when one growth cone contacts and extends over another growth cone (Lin and Forscher, 1993). On the contrary, low concentration of cytochalasin that diminishes actin structures prevents growth cone turning at the border of an inhibitory substrate (Challacombe et al., 1996). In such growth cones, microtubules are closer to the leading edge and are evenly distributed when growth cones meet the border, suggesting a regulatory effect of actin filaments on microtubule behavior during growth cone turning. However, how these two cytoskeletal components interact with each other is unknown.

Under normal conditions, most microtubules in the growth cone are confined to the C-domain with a few of them splaying apart and penetrating into the P-domain. A previous study (Forscher and Smith, 1988) showed that depletion of actin filaments in the P-domain with cytochalasin increased microtubule protrusion into the otherwise microtubule-poor region. This early observation suggested that actin filaments in the P-domain acted as a physical barrier to contain microtubules in the C-domain. On the other hand, increased actin polymerization was associated with directed microtubule protrusion when beads were coupled to actin filaments through membrane adhesion proteins on the surface of growth cones (Suter and Forscher, 1998). Localized stabilization of microtubules with taxol induced attractive growth cone turning only when actin dynamics were intact (Buck and Zheng, 2002). Experiments using a gradient of cytochalasin caused repulsive rather than attractive turning (Yuan et al., 2003), which suggests that microtubule stabilization required for attractive turning depends on the presence of actin filaments. EM studies have shown that many dynamic microtubules invading the P-domain are coupled with actin bundles (Gordon-Weeks, 1991). These observations support the idea that actin filaments interact with microtubules, stabilizing them in some conditions but acting as a barrier in other conditions.

Taken together, these studies point out the importance of coordinated actin polymerization and microtubule protrusion in growth cone turning. Two fundamental questions concerning these studies are whether actin filaments play a positive or negative role in regulating microtubule assembly and how actin polymerization at the leading edge is coordinated with microtubule protrusion near the C-domain. These questions must be addressed before a cytoskeletal model of growth cone turning can be developed.

ACTIN DYNAMICS AND ORGANIZATION BOTH INFLUENCE MICROTUBULES

Studies from several labs have shown, surprisingly, that actin filaments can act as both a barrier and a guide for microtubules. Although this seems contradictory, the effect depends upon two important features of actin networks, the dynamics of retrograde flow and the organization of actin into bundles and meshwork. The early cytochalasin experiments suggested that actin filaments blocked microtubule extension. However, recent studies show more specifically that microtubules are repelled by the retrograde flow of actin filaments rather than by actin filaments per se (Zhou et al., 2002). When actin retrograde flow was slowed or stopped by inhibition of myosin activity in

growth cones, extensive microtubule protrusion into the P-domain occurred (Zhou et al., 2002). These microtubules extended in the presence of the dense actin meshwork. Thus, although cytochalasin removes all actin filaments and causes microtubule protrusion into the P-domain, it is necessary only to block retrograde flow of actin filaments to cause microtubule protrusion. The presence of retrograde flow provides a major force that prevents microtubules from invading the P-domain. Similar observations of microtubules repelled by actin retrograde flow were made in nonneuronal cells. In newt lung epithelial cells, microtubules with different orientations were carried backwards by actin filaments at the same rate, suggesting a physical interaction between them rather than trapping of microtubules by the actin network (Waterman-Storer et al., 1997; Salmon et al., 2002).

Together with retrograde flow, actin filaments that are oriented parallel to the leading edge and that are located in the region of the transition zone between P- and C- domains may also inhibit microtubule extension. Parallel-oriented actin filaments are present in normal growth cones (Schaefer et al., 2002). Moreover, an arc-shaped structure composed of actin filaments forms in the transition zone after actin bundle loss. This presumably is due to filaments from dissociated actin bundles that are carried back by retrograde flow (Zhou and Cohan 2001). These parallel-oriented filaments may act as a physical barrier for microtubules both in normal growth cones as well as those exposed to guidance molecules. They may limit the opportunity for dynamic microtubules to extend from the C-domain into the P-domain.

Retrograde flow and parallel-oriented actin filaments may establish a condition that prevents extension of most microtubules from the C-domain into the growth cone periphery. How do microtubules penetrate into the periphery? Similar to other cells, microtubules in growth cones display dynamic instability characterized by cycles of growth and shortening. The net growth of microtubules is determined by the lifetime of their growth phase. Several studies have shown directly that in growth cones the rate of microtubule polymerization is significantly faster than the rate of actin retrograde flow (Kabir et al., 2001; Zhou et al., 2002). This provides the means by which microtubule growth can overcome retrograde flow and thus invade the growth cone P-domain.

The faster rate of microtubule growth alone is not sufficient to allow dynamic microtubules to advance towards the growth cone leading edge. Recent experiments using fluorescent speckle microscopy to study cytoskeletal dynamics (Schaefer et al., 2002; Cohan and Waterman-Storer, 2002) indicate that retrograde flow of actin filaments is coupled to rearward translocation of microtubules. When radial actin bundles were lost from the P-domain, microtubules also lost their radial orientation and they became bent and buckled and were swept rearward. Coupling of microtubules to actin retrograde flow prevented microtubules from extending into areas of the P-domain devoid of actin bundles, apparently because microtubules could not extend over the actin meshwork. However, microtubules that contacted actin bundles in the P-domain appeared to align to and track along the bundles. During their growth phase, these microtubules can advance toward the leading edge. Thus, treatments that disrupted actin bundles without affecting retrograde flow impeded the penetration of dynamic microtubules toward the leading edge. Actin bundles may act to stabilize a subset of dynamic microtubules in the P-domain, allowing microtubules in their growth phase to extend in a radial direction toward the leading edge.

A balance between retrograde flow and microtubule net growth would determine the position of microtubule plus ends. Any signaling event that decreased retrograde flow or increased microtubule growth lifetime could promote microtubule extension. Indeed, it was shown (Kabir et al., 2001) that activation of protein kinase C (PKC) increased microtubule growth lifetimes, which led to microtubule extension in *Aplysia* growth cones. A likely mechanism to increase microtubule growth lifetime is through stabilizing the assembled microtubules without compromising microtubule polymerization (without capping effect). Microtubules aligned with actin bundles in the growth cone flow retrogradely at the same rate as the actin bundles. In addition, microtubules that contact actin meshwork or actin arc filaments in the growth cone also undergo retrograde flow at the same rate (Schaefer et al., 2002). These observations suggest a physical connection between actin filaments and microtubules. Given the fact that most growing microtubules in the P-domain are along polarized actin bundles, a microtubule-binding complex that mediates the interaction between actin filaments and microtubules may act to stabilize polymerized microtubules. The identification of this proposed complex will be of great interest in future investigations. An alternative explanation for coaligned microtubules and actin bundles is that they are both targeted to the same place in the growth cone periphery, such as substrate contact points, similar to that observed in fibroblasts (Kaverina et al., 1999). However, we

think this is unlikely because targeting microtubules to focal contacts in fibroblasts results in dissociation of both focal contacts and actin bundles (stress fibers) and leads to lamella retraction.

ACTIN POLYMERIZATION, LEADING EDGE ACTIN, AND MICROTUBULES

Actin polymerization at the growth cone leading edge also is an important event that must be coordinated with microtubule assembly near the C-domain. Actin bundles are the only cytoskeletal structures that span the whole width of the growth cone. Their interaction with dynamic microtubules makes them the ideal candidates to couple actin polymerization at the leading edge with microtubule extension. Indeed, treatment that specifically disrupts actin bundles decreases actin filament concentration at the leading edge and causes growth cone collapse (Zhou and Cohan, 2001). This suggests that organizing actin filaments into bundles may promote actin polymerization at the leading edge. Extensive studies about the mechanism of actin polymerization have used non-neuronal cells as model systems (Pollard and Borisy, 2003). In many such motile cells, Arp2/3 plays an important role in actin assembly at the leading edge (Pollard and Borisy, 2003). Most of these cells have few actin bundles and Arp2/3-mediated actin assembly appears largely independent of actin bundling. In contrast, growth cones have prominent actin bundles that are essential for filopodia. Moreover, growth cones collapse when actin bundles are disrupted (Zhou and Cohan, 2001). It is possible that actin assembly is regulated by additional molecules at the growth cone leading edge, which also may involve actin bundling (Steketee and Tosney, 2002). A family of proteins named formins has been identified to play a direct role in regulating nucleation and polarization of unbranched, filamentous actin structures, such as actin bundles, independent of Arp2/3 (Pruyne et al., 2002). As another actin nucleating factor, formins may play a role in growth cones together with Arp2/3 to regulate actin assembly at the leading edge.

PROPOSED CYTOSKELETAL MODEL FOR TURNING

As the growth cone moves forward in a straight path, actin bundles are uniformly distributed throughout the lamellipodium and an actin meshwork is concentrated near the leading edge (Fig. 1). Actin assembly occurs at the leading edge of the lamellipodium and at filopodial tips and the actin bundles and meshwork display retrograde flow. During their growth phase, dynamic microtubules in the C-domain polymerize at a rate faster than actin retrograde flow and extend into the P-domain. Microtubules that contact actin bundles advance in a linear path along the bundles toward the leading edge whereas other microtubules are swept back towards the C-domain (Fig. 1). As they pause or shorten, microtubules are removed from the P-domain. The symmetric array of radial actin bundles, ongoing retrograde flow, and the stochastic dynamics of microtubules allow a subset of dynamic microtubules to distribute symmetrically into the P-domain on radial paths adjacent to actin bundles. In this setting, a repulsive signal can initiate the complex cytoskeletal changes necessary to redirect the path of the axon simply by inducing the loss of actin bundles in one region of the growth cone (Zhou and Cohan, 2002). Focal loss of actin bundles is followed by focal loss of dynamic microtubules. Meanwhile, loss of actin bundles also is associated eventually with focal loss of meshwork at the leading edge and focal collapse of the lamellipodium. Protrusion of the lamellipodium occurs in a new direction where actin bundles remain. Subsequently, microtubules redistribute over the area of remaining actin bundles, which changes the trajectory of the axon. In this model, actin bundles play a crucial role in coordinating lamellipodial protrusion and direction of axon extension.

In contrast to repulsive signals, focal exposure to an attractive signal must initiate a series of cytoskeletal changes toward the stimulus. Experiments analyzing spreading of one growth cone on another and bead-induced protrusion in *Aplysia* growth cones suggest that attractive signals induce a focal increase in actin assembly. This is followed by focal extension of microtubules. It is possible that attractive cues might also cause a regional increase in actin bundling or increase the duration of the microtubule growth phase. These events could increase the number of microtubules in the direction of the guidance signal. Attractive turning also requires loss of the lamellipodium on the side opposite the attractive signal. The mechanism responsible for that is unknown.

In the above models, actin filaments are key cytoskeletal structures that initiate subsequent changes in growth cone shape and axon trajectory. Actin bundles orchestrate both actin polymerization at the leading edge and microtubule extension from the C- domain. Focal loss of the actin bundles is sufficient to initiate repulsive growth cone turning (Zhou et al., 2002). Focal increases in actin assembly can increase microtubule extension that may cause attractive turning. To understand these changes completely, it will

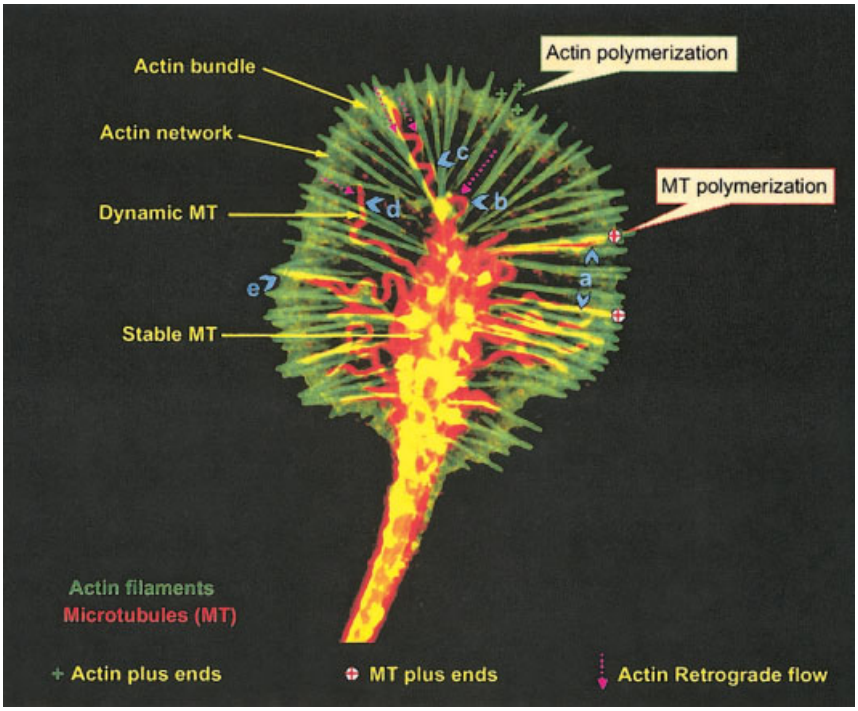

Figure 1 A *Helisoma* growth cone that has been stained to show actin filaments (green) and microtubules (red). A subset of individual, dynamic microtubules extends from the central domain into the peripheral domain composed of actin filaments. (a) A microtubule tracks along an actin bundle, extending toward the leading edge as its growth rate exceeds the rate of retrograde flow. (b) A microtubule forms a loop when its plus end is carried rearward to the central domain by retrograde flow. (c) A microtubule attached to an actin bundle buckles under the force of actin retrograde flow. (d) A microtubule detached from an actin bundle orients parallel to the leading edge due to the influence of the retrograde flow of the actin network. (e) Two microtubules appear to attach to the same actin bundle by their tips. Reproduced from *The Journal of Cell Biology*, 2002, Vol. 157(5), 2002-cover, by copyright permission of The Rockefeller University Press.

be necessary to identify the signaling pathways that control actin assembly and bundling and the molecules that allow microtubules to interact with actin filaments.

POTENTIAL CANDIDATES THAT LINK ACTIN FILAMENTS WITH MICROTUBULES AND COORDINATE THEIR REORGANIZATION

As discussed above, interaction between actin filaments and microtubules is at the heart of coordinating growth cone motility in response to guidance cues. Any molecules that have the capacity to link these two cytoskeletal systems, either directly or indirectly, are potential key players in regulating growth cone motility. Currently, there are many new molecules that may serve to mediate the interaction between actin filaments and microtubules. Among them, plakin family members are proteins that directly bind both microtubules and actin filaments, thereby regulating coordinated reorganization of two cytoskeletal systems (see detailed review of Leung et al., 2002). *Drosophila* plakin Kakapo/short-stop has been shown to be required for sensory and motor neuron axon growth during development (Lee et al., 2000), indicating an important role for microtubule-actin coupling in axon growth as well.

Other proteins that may link microtubules and F-actin in an indirect manner include the tumor-suppressor protein adenomatous polyposis coli (APC), which was recently shown to bind both actin filaments and microtubules (Dikovskaya et al., 2001). APC associates with plasma membrane in an actin-dependent manner in cultured polarized mammalian epithelial cells (Rosin-Arbesfeld et al., 2001). More strikingly, APC was also found to cluster at the plus ends of the polymerizing microtubules through another microtubule plus-end binding protein, EB-1 (Mimori-Kiyosue

et al., 2000). This localization of APC/EB-1 complex at the microtubule distal tips can promote microtubule polymerization and protect microtubules from shortening (Munemitsu et al., 1994; Nakamura et al., 2001). The physiological relevance of the observed cytoskeleton association of APC is uncertain. Its binding to the microtubule plus ends may act to regulate cell motility by controlling microtubule assembly and stabilization. What is the function of the actin-associated APC in cell motility regulation? Studies from yeast and *Drosophila* mitotic cells found that APC (or its yeast counterpart Kar9) binds microtubules via EB-1 (or an additional unknown linker in *Drosophila*) and membrane cortex (actin dependent) at the same time (Bienz, 2002). This allows membrane-bound APC to capture microtubules and thereby modulate their dynamics. A plausible hypothesis is that in motile cells APC may also serve to connect cortical actin (or other actin structures) with growing microtubules and thus regulate cell motility. Indeed, APC molecules were observed to be mainly clustered at the microtubules that contact the plasma membrane in dynamic protrusions of motile cells (Nathke et al., 1996).

Another interesting protein is formin-homology protein mDia, which is also an effector of Rho GTPase. Overexpression of a constitutively activated mDia without the Rho binding domain causes alignment of microtubules parallel to actin bundles (Ishizaki et al., 2001). Further study showed that formin homology domain 2 (FH2) of mDia mediated this microtubule alignment with actin bundles by positioning microtubule tips to the sites of mDia accumulation. The molecular mechanism underlying this is still unclear. However, the mDia homologue in yeast, Bni1p, has been shown to play an important role in regulating microtubule orientation through controlling the position of Kar9p, a microtubule-capturing protein similar to APC (Miller and Rose, 1998). Formin also can directly mediate actin bundle formation using its FH1 domain by recruiting profilin (Lew, 2002; Sagot et al., 2002). An intriguing possibility is that formin proteins may not only mediate actin polymerization at the growth cone leading edge by initiating actin bundles but they may also capture dynamic microtubules via APC.

Many of the above-mentioned proteins have not been localized or studied in nerve growth cones. This information will be important in determining their ability to coordinate actin filament and microtubule interactions in growth cones and their potential role in axon guidance.

SIGNAL TRANSDUCTION BETWEEN ACTIN FILAMENTS AND MICROTUBULES

In addition to direct physical interaction, as discussed above, indirect interactions also occur between actin filaments and microtubules via signal transduction pathways. Actin filaments and microtubules are not only structural components of the growth cone that are passive targets of extracellular signals, but they are also signaling adaptors that are vital for coordinated cytoskeletal rearrangement. Several studies have shown in both growth cones and non-neuronal motile cells that changes in microtubule dynamics can affect actin organization (Gallo, 1998). In fibroblasts, microtubule disruption induces the formation of stress fibers and focal adhesions, possibly by activating Rho GTPase (Enomoto, 1996; Liu et al., 1998). In contrast, microtubule growth induced by removal of a microtubule-destabilizing drug caused rac activation and led to actin-mediated lamellipodial protrusions (Waterman-Storer et al., 1999). The role of rac was confirmed by over-expression of the dominant negative Rac, which antagonized the microtubule growth-induced membrane protrusion. However, in another study, it was shown that targeting of microtubules to focal adhesions promoted their relaxation and dissociation that led to edge retraction (Kaverina et al., 1999). Consistent with this, more frequent excursions of microtubules were observed at the rear of motile cells, where retraction occurred, whereas at the extending leading edge fewer microtubules targeted focal contacts. It was suggested in this study that inactivation of Rho may be responsible for these events. Together, microtubule growth may mediate both membrane protrusion and retraction depending on their spatial localization and what signals they are delivering to the cell edges. This provides a potential way for microtubules to regulate directed cell migration through regulating polarized edge dynamics. More importantly, localized microtubule assembly in the growth cone also promotes lamellipodial extension (Buck and Zheng, 2002), presumably by Rac activation as well. It is worth noting that in both studies dynamic instability was required for microtubule-mediated membrane protrusion and retraction because stabilization of microtubule dynamic instability without microtubule disassembly suppressed cell motility (Liao et al., 1995). This also reinforces the importance of microtubules in the growth cone P-domain that display dynamic instability. This not only provides an efficient way for microtubules to sample the P-domain for signals from actin filaments, but also

allows precise temporal-spatial regulation of actin dynamics by microtubules.

The specific sequestering of Rho GEF-H1 by microtubules may explain both microtubule-disruption-induced Rho activation and microtubule-growth-induced Rho inactivation (Krendel et al., 2002). How microtubule growth activates Rac remains to be identified. The fact that only growing microtubules can promote membrane dynamics argues against the common view that microtubules only passively serve as tracks for the directed delivery of materials or signals necessary for lamellipodial protrusion. The most likely possibility is the association of Rac-activating molecules with a growing microtubule plus-end binding complex, such as CLIP-CLASP or EB1-APC complex. Thus, the delivery of such Rac activating signals to the leading edge would depend on new microtubule growth. Indeed, a RacGEF protein named Asef has been identified as a binding partner for the microtubule-binding molecule APC, which only binds to growing plus ends (Kawasaki et al., 2000, 2003). In summary, microtubules regulate actin dynamics mainly through an indirect signaling cascade. Whether actin filaments affect microtubules through similar signaling events remains an open question.

Actin-based structures, especially filopodia, are at the leading edge of the growth cone, where extracellular guidance cues are first encountered. Therefore it is plausible that signals from guidance cues first induce actin reorganization, which then leads to microtubule changes that complete the turning process. Recent studies have demonstrated that attractive and repulsive effects of guidance cues depend upon Rho, Rac, and Cdc42 activity (Patel and Van Vactor, 2002; Yuan et al., 2003). However, microtubules may also act as primary targets of guidance cues and thus affect growth cone turning (Buck and Zheng, 2002). Cues such as semaphorin may affect microtubule dynamics directly through collapsin response mediator protein-2 (CRMP-2; Goshima et al., 1995), which has been shown to play a vital role in microtubule assembly (Fukata et al., 2002). Reciprocal interactions between actin filaments and microtubules may form a feedback signaling loop that reinforces the initiating signal stimulated by guidance cues, whether actin or microtubules are the primary targets. Thus, molecules that regulate interactions between actin filaments and microtubules may be the key controlling elements that allow guidance cues to steer growth cones to their targets.

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