



# Cytoskeletal Social Networking in the Growth Cone: How +TIPs Mediate Microtubule-Actin Cross-Linking to Drive Axon Outgrowth and Guidance

Garrett M. Cammarata, Elizabeth A. Bearce, and Laura Anne Lowery\*

*Department of Biology, Boston College, Chestnut Hill, Massachusetts*

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**The growth cone is a unique structure capable of guiding axons to their proper destinations. Within the growth cone, extracellular guidance cues are interpreted and then transduced into physical changes in the actin filament (F-actin) and microtubule cytoskeletons, providing direction and movement. While both cytoskeletal networks individually possess important growth cone-specific functions, recent data over the past several years point towards a more cooperative role between the two systems. Facilitating this interaction between F-actin and microtubules, microtubule plus-end tracking proteins (+TIPs) have been shown to link the two cytoskeletons together. Evidence suggests that many +TIPs can couple microtubules to F-actin dynamics, supporting both microtubule advance and retraction in the growth cone periphery. In addition, growing in vitro and in vivo data support a secondary role for +TIPs in which they may participate as F-actin nucleators, thus directly influencing F-actin dynamics and organization. This review focuses on how +TIPs may link F-actin and microtubules together in the growth cone, and how these interactions may influence axon guidance.** © 2016 Wiley Periodicals, Inc.

**Key Words:** +TIPs; axon guidance; growth cone; microtubule dynamics; cytoskeleton

## Introduction

### The Growth Cone

The growth cone is a dynamic structure found on the ends of growing axons, which allows axons to navigate through their environment by intercepting a myriad of

extracellular cues and translating them into mechanical behaviors [Lowery and Van Vactor, 2009]. The structure of the growth cone is thus suitably complex, containing several compartments each with unique cytoskeletal arrangements (Fig. 1). The structure of the growth cone is chiefly divided into three domains (central, transitional and peripheral), which contain distinct cytoskeletal arrangements of actin filaments (F-actin) and microtubules [Forscher and Smith, 1988]. Both of these polymers form complex arrays to create traction, transduce force, and facilitate trafficking, in order to permit proper elongation and navigation of the axon. Along the axon shaft, microtubules are tightly bundled [Yamada et al., 1971; Bunge et al., 1973; Bray et al., 1981; Tanaka and Kirschner, 1995]. Upon arrival at the growth cone central domain, their dynamic plus-ends splay more freely, growing and shrinking as they come into contact with F-actin arcs [Schaefer et al., 2002]. Many microtubule plus-ends are sequestered here and undergo looping and catastrophe. Some microtubules, commonly referred to as “pioneer microtubules”, break through to the peripheral domain, where they traverse through the criss-crossing F-actin lamella [Mitchison and Kirschner, 1988; Sabry et al., 1991; Tanaka and Kirschner, 1995; Schaefer et al., 2002]. Once they have reached the periphery, these pioneer microtubules intersect and then crosslink with F-actin bundles, which are thought to guide the microtubules out to the ends of filopodia [Zhou and Cohan, 2004] (Fig. 2A). As they journey out to the far reaches of the growth cone together, these interacting microtubule and F-actin networks are exposed to signaling events downstream of extracellular guidance cues.

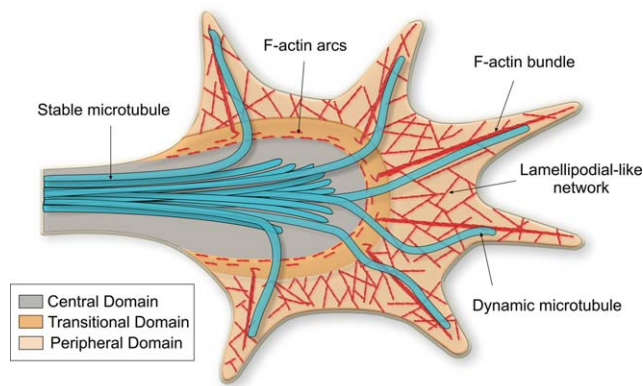
These guidance signaling pathways directly and indirectly modify filopodial actin and microtubule networks, catalyzing mechanical movements of the growth cone. Initially, precise mechanistic interactions between signaling molecules and the growth cone cytoskeleton were not clear, and early studies focused mainly on how these cascades may specifically impact the remodeling of F-actin [Bentley et al., 1994; Lin et al., 1994]. However, it had long been

\*Address correspondence to: Laura Anne Lowery, Department of Biology, Boston College, Chestnut Hill, Massachusetts.

E-mail: laura.lowery@bc.edu

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established that microtubules are also important for success-



**Fig. 1. Overview of the different compartments of the neuro-nal growth cone.** The growth cone is made up of several distinct compartments, each containing unique microtubule (blue) and F-actin (red) structural elements. The central domain (gray) is comprised of stable microtubules that have entered through the axon shaft and are consolidated. These stable microtubules are hindered by F-actin arcs; structures located in the transitional domain (tan) that act as barriers for microtubule growth. Microtubules that do emerge from the transitional domain into the peripheral domain (peach) are called “pioneer microtubules”. It is in this domain that pioneer microtubules are guided along F-actin bundles and traverse through a lamellipodial-like F-actin network. F-actin is orientated so that their barbed plus-ends are directed towards the leading edge of the growth cone while their pointed minus-ends are directed towards the central domain. These pioneer microtubules follow the F-actin tracks as they reach the ends of filopodia. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

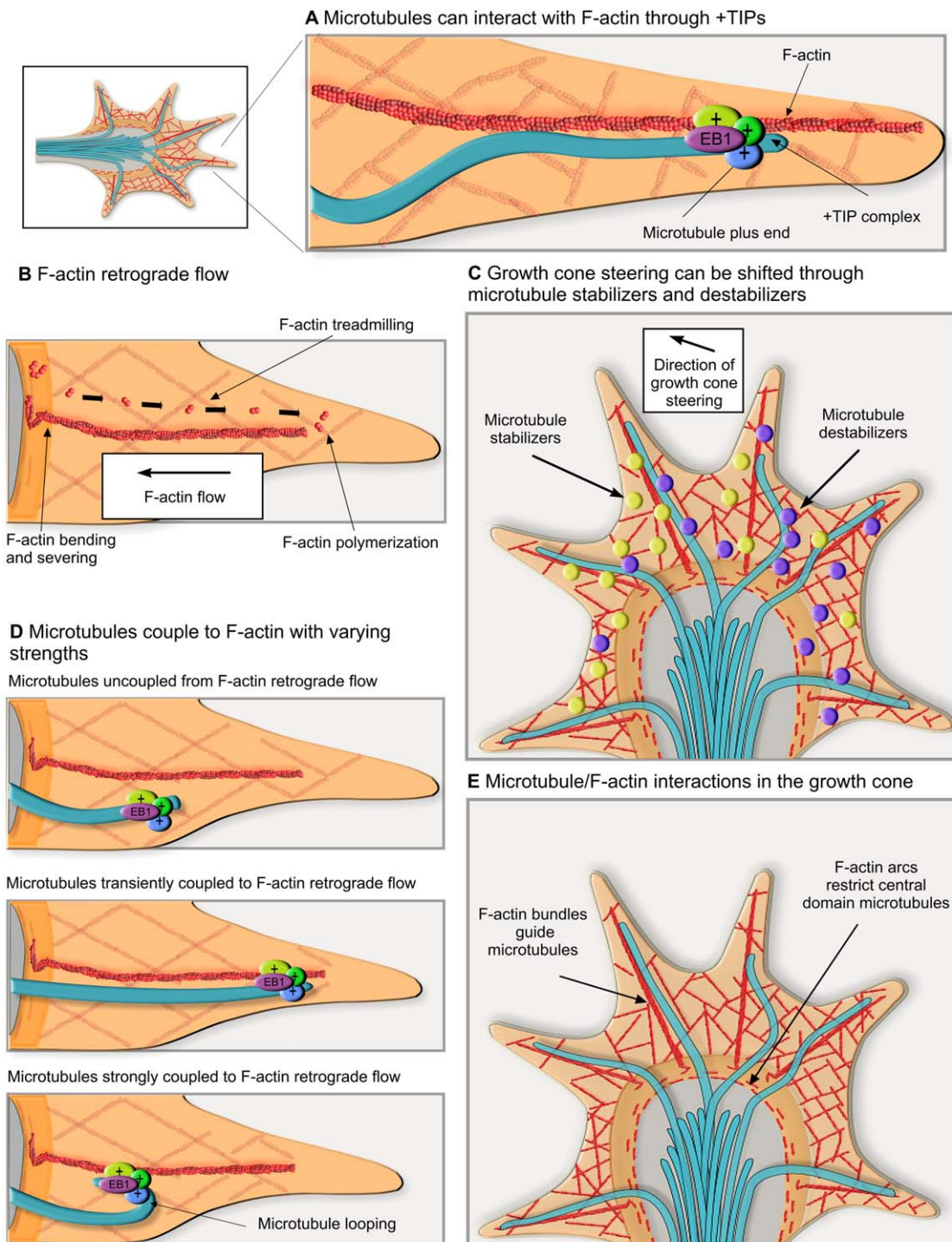
ful axon guidance [Marsh et al., 1984; Tanaka and Kirschner, 1991; Dreschel et al., 1992; Tanaka et al., 1995; Buck and Zheng, 2002]. Approximately a decade ago, an important set of studies revealed that guidance signaling pathways could evoke changes in growth cone guidance by direct modification of a specific set of proteins found on the distal tips of dynamic microtubules, the plus-end tracking proteins (+TIPs) (Fig. 2A) [Lee et al., 2004; Zhou et al., 2004]. Since then, increasing evidence has accumulated that +TIPs may be important for axon guidance and turning [Bearce et al., 2015]. However, their participation in these behaviors may stem from more than a single ability to interact with and regulate microtubules; many +TIPs interact with actin as well [Watanabe et al., 2004; Tsvetkov et al., 2007; Geraldo et al., 2008; Watanabe et al., 2009]. This affinity for both cytoskeletal networks makes +TIPs intriguing candidates as ‘bilingual’ networking facilitators, positioned to interact at the intersection of signaling pathway transduction and cytoskeletal remodeling. Understanding this precise junction is a dominant focus in the cell biological study of the growth cone; thus, more information is needed about how microtubules and actin work downstream of these signals and about how +TIPs may be regulating these behaviors.

## Actin Networks in the Growth Cone

Actin dynamics have classically been credited with generating the major driving force behind growth cone steering and motility. Early on, the “clutch” hypothesis was put forth [Mitchison and Kirschner, 1988], later described as the substrate-cytoskeletal coupling model [Lin et al., 1994; Suter et al., 2000; Jay et al., 2000], which links actin dynamics to growth cone protrusion. F-actin bundles found in the peripheral domain are oriented such that their barbed plus-ends point towards the cell membrane, while their pointed minus ends are directed towards the central domain of the growth cone (Fig. 2B). F-actin lamellar networks are comprised of crisscrossed actin filaments which are located in the peripheral domain (Fig. 1). Under the effects of F-actin retrograde flow [Forscher and Smith, 1988; Lin and Forscher, 1995; Medeiros et al., 2006], the actin filaments are in a constant state of polymerization (at the plus-ends) and severing/de-polymerization (at the minus ends). This affects protrusion and retraction of the actin filaments and corresponding protrusion and retraction of the growth cone [Lin and Forscher, 1995; Dent et al., 2011] (Fig. 2B). While polymerization occurs in the peripheral domain of the growth cone, F-actin buckling and severing occurs in the transition zone, likely due to mechanical strain applied by either myosin II contraction or by the pushing of F-actin assembly. F-actin severing is followed by depolymerization of F-actin fragments [Medeiros et al., 2006], thus allowing free actin subunits to be recycled back to the plus-end (Fig. 2B). The interaction of actin with an adhesive substrate causes the filaments to be stabilized, reducing the rate of retrograde flow and allowing polymerization at the plus-end to dominate, resulting in net growth toward the edge of the cell [Suter et al., 2000]. However, this stability is counterbalanced by a need for dynamicity within the growth cone; developing and specified axons demonstrate an intrinsically less-stable actin network compared to developing dendrites, and this instability is critical for rapid axonal outgrowth. This unstable actin cytoskeleton facilitates extension of microtubules into the periphery of the growth cone [Bradke and Dotti, 1999].

## Microtubule Dynamics in the Growth Cone

Although it is widely accepted that the actin cytoskeleton provides a majority of the motile function in the growth cone, microtubules certainly do play an extensive role in axon guidance and outgrowth. This was initially shown by studies that demonstrated that microtubules were required for growth cone forward progression [Marsh et al., 1984; Tanaka et al., 1995]. Microtubules are dynamic structures by nature, constantly undergoing periods of polymerization followed by periods of shrinking [Kirschner et al., 1984], which seem to assist exploratory microtubules in “feeling” around the peripheral domain and making functional connections [Holy and Liebler, 1994]. Tubulin subunits ( $\alpha/\beta$



**Fig. 2. Microtubule and F-actin functioning in the periphery of the growth cone.** **A:** +TIP complexes allow pioneer microtubules to interact with F-actin bundles in the periphery. +TIP complex is not drawn to scale. **B:** F-actin polymerization and retrograde flow provide a constant treadmilling of actin towards the central domain. In the transitional domain, F-actin bundles are contracted and bent by myosin motor proteins while ADF/cofilin severs actin filaments into a pool of actin monomers. This pool provides G-actin that is then recycled back to the plus-end for F-actin polymerization. **C:** Microtubule stabilizers and destabilizers are able to control growth cone steering. Microtubules that bind stabilizers on a specific side of the growth cone will cause growth cone turning towards that axis (left). Conversely, destabilizing compounds, or regulated removal of stabilizing molecules, can cause repulsion or retraction from that area (right). **D:** Microtubule protrusion and advancement into the periphery can be altered through coupling with F-actin retrograde flow. When uncoupled from retrograde F-actin flow, microtubules may progress further into the periphery through polymerization and translocation/sliding. When microtubules are transiently coupled to F-actin retrograde flow, through +TIP complexes, they follow F-actin bundles towards the ends of the filopodia. However, if microtubules are coupled strongly enough, or undergo enduring coupling, then microtubules can be translocated retrograde flow, causing microtubule looping. **E:** Microtubules interact with several distinct F-actin structures throughout the growth cone. F-actin arcs surround the central domain and restrict microtubules from exploring the periphery. F-actin bundles provide tracks for growing microtubules to follow into growth cone filopodia. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]



tubulin heterodimers) are either added or removed at the microtubule plus-end by certain classes of +TIPs uniquely suited to this task (Fig. 2A) [Akhmanova and Steinmetz, 2008; Akhmanova et al., 2010]. (However, note that interaction of different types of +TIPs with microtubule plus-ends is quite complex and involves various processes and regulatory mechanisms. For review, see [Al-Bassam and Chang, 2011; Lowery and Van Vactor, 2009; Akhmanova and Steinmetz, 2008; Akhmanova and Hoogenraad, 2005; and Galjart, 2005]). Importantly, it was demonstrated that guidance cues could affect microtubule turning behaviors of the growth cone and predict overall direction of outgrowth [Sabry et al., 1991; Tanaka et al., 1995; Williamson et al., 1996; Challacombe et al., 1997; Zhou et al., 2002]. Following careful local application of a microtubule stabilizing drug, Taxol, or a depolymerizing drug, Nocodazole, a growth cone could be encouraged to turn towards or away from a specific axis [Buck and Zheng, 2002] (Fig. 2C). This led to the hypothesis that individual peripheral domain microtubules act as guidance sensors of the growth cone, helping to govern growth cone advancement and directionality [Gordon-Weeks, 2004].

### The Coupling of Microtubules to Actin Dynamics

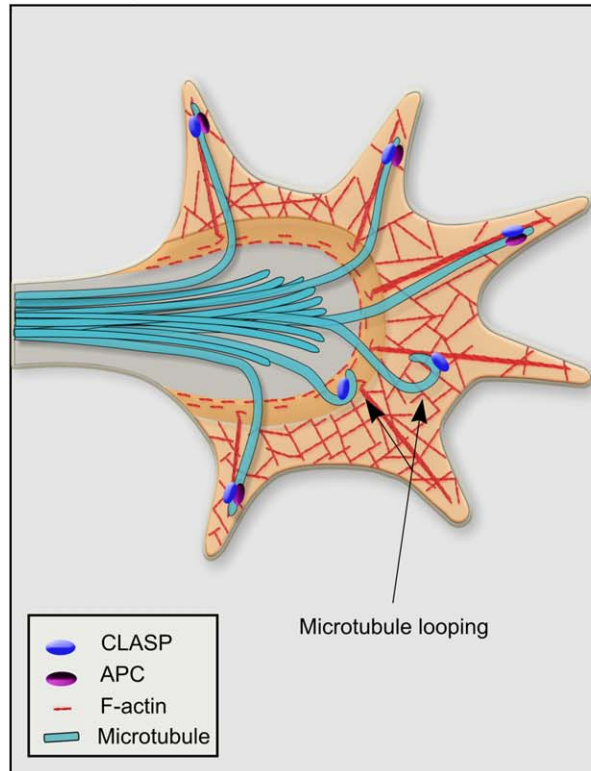
Several studies have shown a clear connection between the role of microtubules in growth cone steering and interaction with the actin cytoskeleton [O'Connor et al., 1993; Rodriguez et al., 2003; Zhou et al., 2004; Geraldo and Gordon-Weeks, 2009]. Microtubules usually follow the same path as F-actin bundles in the peripheral zone of the growth cone, leading to the idea that F-actin provides tracks for the microtubules to follow into the periphery [Letourneau, 1983] (Fig. 2E). When actin networks were manipulated with ML-7, a myosin kinase inhibitor that produces selective loss of actin bundles and resultant growth cone collapse without significantly disrupting actin veils [Zhou et al., 2002; Zhou and Cohan, 2001], microtubules seemed to be completely removed from the peripheral domain. In addition, growth cone turning in response to guidance cues was impaired when the F-actin structure was altered under treatment of ML-7 and cytochalasin D, an actin depolymerizer [Zhou et al., 2002]. By acting as predetermined tracks for microtubule growth, actin networks may regulate or even limit microtubule advancement into the periphery [Burnette et al., 2007; Geraldo and Gordon-Weeks, 2009] (Fig. 2E). Microtubules are transiently coupled to these F-actin tracks, leaving them susceptible to F-actin retrograde flow [Schaefer et al., 2002, 2008; Lee and Suter, 2008]. Coupling of exploratory microtubules to F-actin retrograde flow caused a reduction in the number of microtubules to advance into the periphery [Lin and Forscher, 1995; Zhou et al., 2002; Zhou and Cohan, 2004], and, furthermore,

caused looping of microtubules back towards the central domain [Lee and Suter, 2008]. In a separate study, uncoupling of F-actin-microtubule interactions caused microtubules to progress further into the periphery [Burnette et al., 2007]. The absence of F-actin bundles was not found to influence central domain microtubules; however, when F-actin arcs located in the transitional domain were disrupted, central domain microtubules were abnormally broad and irregular in appearance [Burnette et al., 2007]. This suggests that the F-actin arcs create a selective barrier in the transitional zone to limit microtubule escape into the periphery (Fig. 2E). These data indicate that F-actin-microtubule interactions can profoundly affect growth cone morphology and extension, which, in turn, suggest that regulation of these interactions may take place downstream of guidance cue interception. We postulate that +TIPs act as signaling pathway intermediates that can affect these cytoskeletal coupling events.

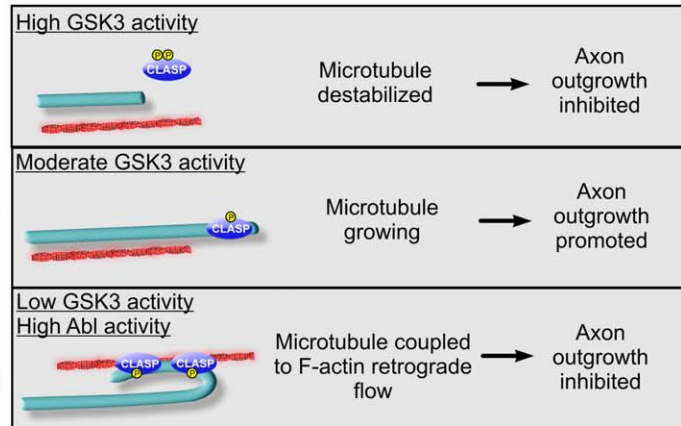
### +TIPs Act as Signaling Transduction Elements to Alter Cytoskeletal Dynamics

Within the context of closely-coupled actin and microtubule networks at the 'sensory' ends of growth cone filopodia, +TIPs are in the perfect location to act as cytoskeletal "social networking" agents. +TIPs can interact with a multitude of molecules and structures including microtubules, actin filaments, and regulatory kinases, which can induce microtubule-actin crosslinking [Alvez-Silva et al., 2012; Akhmanova and Steinmetz, 2008; Geraldo et al., 2008; Tsvetkov et al., 2007; Watanabe et al., 2004; Zhou et al., 2004]. In addition, +TIPs are very dynamic molecules and share a large interaction network with each other [Akhmanova and Steinmetz, 2008]. Furthermore, +TIPs have been demonstrated to mediate some of the F-actin-microtubule interactions that were shown to affect growth cone guidance in seminal works [Engel et al., 2014; Marx et al., 2013; Alvez-Silva et al., 2012; Hur et al., 2011; Geraldo et al., 2008; Tsvetkov et al., 2007]. +TIPs can orchestrate transient coupling events to F-actin during microtubule polymerization, thought to guide microtubules progressing into the periphery [Geraldo et al., 2008] (Fig. 2D). Excessive or enduring coupling to F-actin, through +TIP complexes, can cause microtubules to undergo retrograde flow, and thus effectively oppose microtubule outgrowth [Hur et al., 2011; Marx et al., 2013; Engle et al., 2014] (Fig. 2D). Asymmetric or spatially-regulated accumulations of these +TIPs could therefore be imagined to couple microtubules to F-actin in a way that leads to axial steering of the growth cone. In this review, we discuss some of the current evidence showing that +TIPs may work to influence growth cone guidance, in part, through the regulation of microtubule-F-actin interactions.

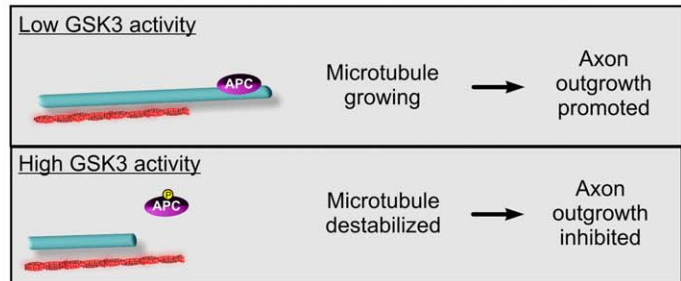
**A** Localization of CLASP and APC to the microtubule plus-end



**B** Phosphorylation of CLASP by GSK3 and Abl kinases affects axon outgrowth



**C** Phosphorylation of APC by GSK3 affects axon outgrowth



**Fig. 3. Regulation of CLASP and APC- induced cytoskeletal coupling results in altered axon outgrowth events.** **A:** The +TIPs, CLASP and APC, are found on the plus-ends of microtubules located in the periphery of the growth cone. Asymmetric localization of both CLASP (dark blue) and APC (purple) can alter microtubule protrusion and interaction with F-actin (left panel). Together, APC and CLASP bind to the plus-end, resulting in extension of the microtubule to the ends of the filopodia. APC and CLASP plus-end activity are both associated with microtubule protrusion. Dissociation of APC from the plus-ends can impair MT protrusion. CLASP lattice-binding activity can additionally compromise microtubule progression, by coupling microtubules to retrograde flow, causing microtubule looping. **B** GSK3 and Abl kinase signaling can affect localization of CLASP. Under high concentrations of GSK3 kinase, CLASP is phosphorylated at two distinct sites. This allows detachment from the plus-end and destabilization of the microtubule, resulting in a reduction of axon outgrowth (upper panel). Under moderate GSK3 kinase signaling, CLASP is phosphorylated on one site and localizes to the plus-end, increasing microtubule protrusion and axon growth (middle panel). A low GSK3 kinase activity and a high Abl kinase activity causes CLASP to localize along the microtubule lattice, thereby allowing microtubules to be coupled to F-actin retrograde flow and inhibiting overall axon outgrowth (lower panel). **C** APC is regulated by GSK3 phosphorylation in a similar manner to CLASP. Subjected to low GSK3 kinase activity, APC remains unphosphorylated and associates with the plus-end of the microtubule. Microtubules are stabilized by APC allowing microtubule growth and overall axon outgrowth to be promoted (upper panel). High GSK3 kinase activity allows APC to be phosphorylated, dissociating from the plus-end, and destabilizing the microtubule. This results in a decrease in microtubule protrusion and an inhibition in axon outgrowth (lower panel).

## Body

### Linking Microtubules to F-Actin and Retrograde Flow

#### *The First Axon Guidance-Relevant +TIPs: CLASP/APC*

The first +TIPs that were identified in directing axon guidance mechanisms were CLASP and APC [Lee et al., 2004; Zhou et al., 2004]. Lee et al. showed that the CLASP1 homolog in *Drosophila* is downstream of Abl tyrosine kinase pathways [Lee et al., 2004], a signaling effector known to function downstream of repellent guidance cues. This initial study highlighted the localization of CLASP on

“pioneer microtubules” in the growth cone peripheral domain, which were previously exhibited to follow actin filaments [Mitchison and Kirschner, 1988; Sabry, 1991; Tanaka and Kirschner, 1995; Schaefer et al., 2002] (Fig. 3A). Furthermore, when CLASP was overexpressed in *Xenopus laevis* growth cones, microtubules remained sequestered in the central domain and exhibited a peculiar looping phenotype [Lee et al., 2004] (Fig. 3A). This finding suggested that Abl signaling, in addition to its known role in actin remodeling [Lanier and Gertler, 2000; Wills et al., 1999, Koleske et al., 1998], could affect microtubule dynamics in the growth cone periphery via its interaction with CLASP. However, whether there was any direct communication

between the actin and microtubule cytoskeletons by way of CLASP regulation was not completely clear.

Later studies alluded to a more direct link between CLASP, F-actin, and microtubule behaviors. Marx et al. first demonstrated that depletion of CLASP in *Xenopus* leads to slower velocities of microtubules growth as they exit the axonal shaft into the growth cone [Marx et al., 2013]. This phenotype was intriguing when coupled to evidence from earlier studies that had shown CLASP's ability to bind actin and microtubules through a shared domain, and CLASP's ability to undergo retrograde flow [Tsvetkov et al., 2007]. This led them to question whether it was CLASP that might be affecting microtubule coupling to F-actin retrograde flow, in turn affecting microtubule forward velocities. While speckle microscopy did not illustrate any marked change in actin retrograde flow following CLASP depletion, both microtubule advances in filopodial and actin network morphology in the lamellae were disrupted. This resulted in weakened F-actin structure as well as impaired axon outgrowth [Marx et al., 2013]. These experiments supported a functional role for CLASP-mediated microtubule-F-actin crosslinking in the growth cone.

Additional studies showed that CLASP-mediated cytoskeletal coupling could be regulated downstream of both Abl and GSK3 signaling mechanisms (Fig. 3B). Engel et al. indicated that Abl possesses the ability to phosphorylate CLASP within its microtubule and F-actin binding domain [Engel et al., 2014]. When a constitutively active form of Abl was overexpressed, CLASP localization to the plus-end of the microtubule was decreased, and its interaction with actin rich structures in the central domain increased. Thus, it was postulated that Abl activity could trigger a 'switch' between the microtubule and actin binding properties of CLASP; when CLASP is heavily phosphorylated, microtubule plus-end affinity could be reduced, and CLASP may then be targeted to the microtubule lattice where it could facilitate microtubule-F-actin crosslinking. Similarly, GSK3, a serine/threonine kinase and Wnt downstream effector, was also demonstrated to regulate CLASP localization and actin-microtubule binding in both a positive and negative manner. It was found that highly active GSK3 caused phosphorylation of multiple sites along CLASP, prompting CLASP to dissociate from the plus-ends and reduce overall axon outgrowth [Hur et al., 2011]. The same study indicated that a moderate GSK3 kinase activity allowed CLASP plus-end binding to promote axon elongation, while low GSK3 kinase activity promoted CLASP accumulation to the lattice, thereby promoting association with F-actin retrograde flow and causing characteristic microtubule looping. CLASP lattice localization could be effectively mitigated by treatment with blebbistatin, a myosin II inhibitor that affects F-actin retrograde flow, thus permitting microtubules to extend into the periphery once again [Hur et al., 2011]. Together, these studies illustrate a critical role for dynamic, +TIP-mediated cytoskeletal cross-

linking during axon outgrowth. Further evidence reveals that this role is not unique to CLASP, but is also shared by other +TIPs.

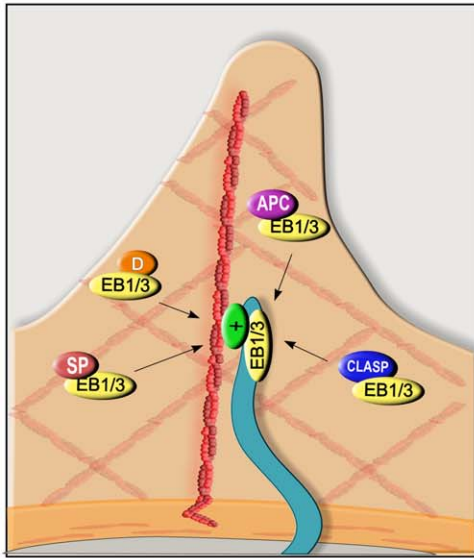
APC is another +TIP that emerged early on as an important mediator of microtubule-F-actin coupling during growth cone steering. APC promotes axon outgrowth by acting at the plus-end to stabilize microtubules [Zhou et al., 2004]. Also, APC undergoes phosphorylation by GSK3 in a CLASP-like manner, and its different phosphorylation states can change its interactions with the cytoskeleton. In one study, when GSK3 was inactive, APC bound to the plus-ends of microtubules, where it stabilized and promoted their outgrowth [Zhou et al., 2004] (Fig. 3C). This study demonstrated that APC-bound microtubules are located primarily in the actin-rich growth cone periphery, where GSK3 activity is repressed (Fig. 3A). Later observations indicated that this colocalization with actin structures may be a product of APC-mediated interaction, as APC was shown to interact with actin filaments in vitro and colocalize to actin structures in fibroblasts [Moseley et al., 2007]. Interestingly, APC's ability to interact with the microtubule plus-end or potentially facilitate microtubule-F-actin coupling may not only affect axon outgrowth, but also guidance and growth cone turning. APC's localization to a particular side of the growth cone caused axonal direction to change towards that side of the axis [Koester et al., 2007]. It could be speculated that spatially-restricted phosphorylation downstream of a localized signaling cascade may preferentially cause APC plus-end binding and microtubule stabilization, whereas separate signaling events on the opposite side of the growth cone may prompt microtubule-F-actin coupling and slowed microtubule velocities. Furthering this notion, loss of APC through GSK3 signaling presented large increases in microtubule looping and loss of growth cone progression [Purro et al., 2008]. While this data supports a role for APC in microtubule-F-actin coupling, the more intricate details of its regulation within the growth cone remain to be elucidated, as studies that have examined F-actin interactions with APC focused primarily on non-neuronal cells [Moseley et al., 2007; Marshall et al., 2011]. Looking beyond CLASP and APC, a number of +TIPs have also been affiliated with cytoskeletal coupling functions apart from their classical roles as microtubule regulators.

### ***EB1/3 Can Connect Actin Binding Proteins to the Plus-End***

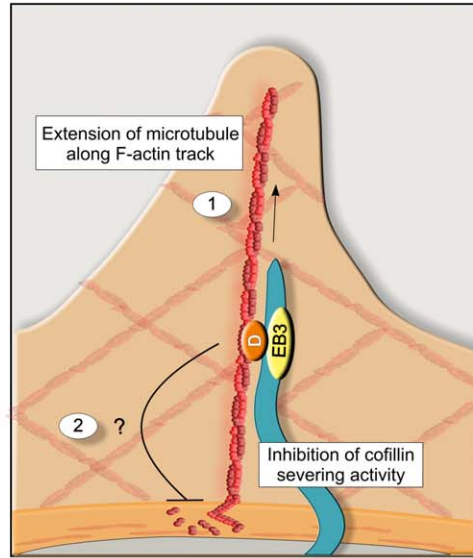
End-binding (EB) proteins are some of the best characterized +TIPs that regulate microtubule dynamics. The binding of EB proteins in an autonomous manner at the microtubule plus-end is sufficient to affect microtubule growth speeds and catastrophe rates [Komarova et al., 2009; Vitre et al., 2008]. However, perhaps their most powerful regulatory role centers on their ability to act as



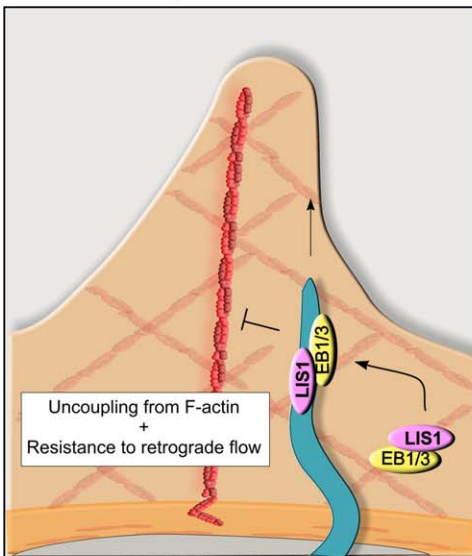
**A** EB1/3 localizes +TIPs and actin binding proteins to microtubule plus end



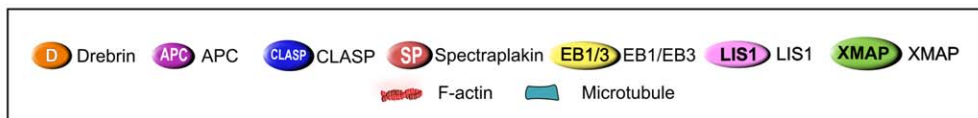
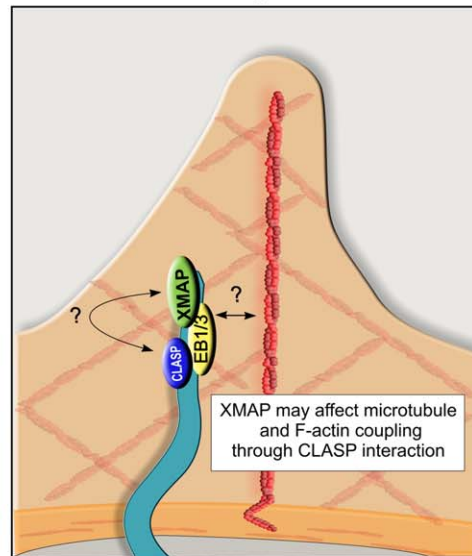
**B** EB3 and drebrin guide and extend microtubules along F-actin



**C** LIS1 uncouples microtubules from F-actin retrograde flow



**D** XMAP may alter the coupling state between microtubule and F-actin cytoskeletons



**Fig. 4. A wide variety of +TIP proteins have differential effects on the coupling state of microtubules and F-actin.** **A:** End Binding protein (EB1 and EB3) can localize numerous +TIPs as well as actin-binding proteins to the plus-end of the microtubule. Once guided and bound to the plus-end, +TIPs may alter the microtubule-F-actin coupling state, influencing microtubule protrusion and axonal outgrowth. **B:** EB3 interacts with actin binding protein, Drebrin, to couple microtubules to F-actin bundles. Microtubules are guided into the periphery, using drebrin bound actin filaments as tracks for further pathfinding. **C:** LIS1 uncouples microtubules from F-actin through a dynein dependent mechanism. When bound to microtubule plus-ends, LIS1 prevents microtubule binding to F-actin, and provides resistance to retrograde flow. **D:** XMAP215 may be involved in a CLASP-related mechanism, altering the binding of microtubules to F-actin in the growth cone periphery. Located beyond CLASP and EB1, XMAP215 may uniquely control coupling of microtubule to F-actin without the help of other +TIPs, although at the moment, this claim needs further support.

scaffolding proteins for other +TIPs, as well as some known actin binding proteins (Fig. 4A). This ability stems from EB1's well-known SXIP motif, which acts as a micro-

tubule tip localization signal for a variety of proteins [Hon-nappa et al., 2009]. Indeed, EB1 is necessary to recruit two of the aforementioned +TIPs, APC and CLASP, to the

plus-end, in addition to recruiting other known actin modulators, such as formins and spectraplakins [Akhmanova and Steinmetz, 2008; Honnappa et al., 2009]. In addition to +TIPs, EB1/3 have been recently shown in the growth cone to interact with the classic microtubule associated protein, MAP1B [Tortosa et al., 2013], which, in conjunction with Tau, have been shown to interact with actin filaments and alter actin dynamics [Cross et al., 1993; Pedrotti and Islam, 1996; Sharma et al., 2007]. Interaction with MAP1B caused sequestering of EB1 and EB3 into the cytosol of the growth cone. When overexpressed, MAP1B caused reduced EB binding to plus-ends, which in turn affected microtubule dynamics [Tortosa et al., 2013]. Therefore, it is important to note that EB proteins may play a central role in connecting classical MAPs and +TIPs to the regulation of actin dynamics in the growth cone.

There is also strong evidence that EB3 is involved in coupling microtubules to F-actin in the growth cone through an affiliation with the actin binding protein Drebrin [Geraldo et al., 2008; Worth et al., 2013; Tanabe et al., 2014]. EB3-bound microtubules were demonstrated to follow the tracks of F-actin bundles into peripheral filopodia, closely associating with Drebrin-bound actin (Fig. 4B). When a form of EB3 was expressed that could bind microtubules but lacked a Drebrin-interacting domain, growth cones showed arrested forward extension [Geraldo et al., 2008]. One recent study disputed this, indicating that a loss of Drebrin-binding caused only minimal effects [Dun et al., 2012]; but others have illustrated that a loss of this interaction could affect axon extension and actin-microtubule coupling in the growth cone [Geraldo et al., 2008; Worth et al., 2013; Tanabe et al., 2014; Sonogo et al., 2015]. These studies indicated that Drebrin is regulated downstream of Cdk5 and potentially PTEN phosphorylation [Kreis et al., 2013; Worth et al., 2013; Tanabe et al., 2014; Sonogo et al., 2015]. Cdk5 was shown to phosphorylate Drebrin on Ser142, causing an open conformation that allowed both binding to actin filaments and EB3-microtubule interactions. Phosphorylated Drebrin was associated to a larger extent with actin filaments in the filopodia of growth cones [Worth et al., 2013; Tanabe et al., 2014]. Not surprisingly, phosphomimetic mutants of Drebrin increased microtubule association with filopodial actin, while phospho-dead mutants reduced this association [Worth et al., 2013]. These data point towards an EB3-Drebrin interaction that may allow exploratory microtubules to be guided into the filopodia through their dynamic interface with F-actin bundles.

Recent data in non-neuronal cells points to another possible role for coupling of EB3 and Drebrin to F-actin. Namely, Drebrin has recently been found to compete with the actin severing protein, cofilin. Although not demonstrated *in vivo* or in neuronal growth cones presently, Drebrin was able to compete with cofilin for binding to actin filaments in several studies [Sharma et al., 2011; Mikati

et al., 2013; Grintsevich et al., 2014]. Mikati et al. indicated that using the neuron specific isoform, Drebrin A, could alter actin polymerization dynamics *in vitro*. One likely role of cofilin in the growth cone is to contribute to F-actin recycling following the severing of F-actin bundles, a process which is promoted through myosin II contractility in the transition zone and through mechanical forces created during F-actin assembly [Medeiros et al., 2006]. Therefore, coupling of EB3 and Drebrin to F-actin in the growth cone may potentially inhibit cofilin severing activity (Fig. 4B). This might indirectly affect how coupled microtubules may progress into the periphery and into filopodia, though this speculative function still needs rigorous growth cone-specific substantiation, as growth cone cytoskeletal dynamics may undergo unique regulation compared to other types of directed cell migration.

### ***LIS1 Interacts with Various Molecules to Uncouple Microtubule/F-Actin Cytoskeletons***

LIS1 is a +TIP which also has links to axon formation and augmentation of microtubule-F-actin uncoupling. LIS1 was recognized early for its impacts on neural development; indeed, its name stems from findings that demonstrated that hemizygous mutations in *LIS1* contributed to the severe brain malformation lissencephaly [Reiner et al., 1993]. Studies have shown that in rat and chick neurons, LIS1 can alter the microtubule-F-actin coupling state through a dynein-mediated interaction [Grabham et al., 2007]. When LIS1 and dynein were inhibited, linkage to F-actin retrograde flow was increased and microtubules were prevented from reaching the periphery. However, treatment with the myosin II inhibitor, blebbistatin, led to the rescue of microtubule extension [Grabham et al., 2007]. These data suggest that LIS1 can uncouple microtubules from the actin cytoskeleton and provide resistance to actin retrograde flow (Fig. 4C). LIS1 was also found to interact in mouse neurons with IQGAP1, a molecule widely known for its actin and microtubule cross-linking abilities in certain cell types [Kholmanskikh et al., 2003; Kholmanskikh et al., 2006]. Precisely how this LIS1-IQGAP interaction functions in coupling is not yet clear, as IQGAP's role in axon pathfinding has not been closely examined. However, as IQGAP expression has been linked with modulation of important signaling molecules, such as RhoA, Rac1, and Cdc42 [Fukata et al., 1999; Kuroda et al., 1996], an interaction between LIS1 and IQGAP could present a plausible link between microtubule-F-actin coupling and more complex downstream signaling events. With its verified role in cytoskeletal uncoupling, LIS1 offers some promise as a “balancing” mediator of other microtubule-F-actin coupling +TIPs, and its regulation and interplay with additional microtubule regulators warrants further examination.



## **Spectraplakins Uniquely Bind Actin Filaments to the Microtubule Lattice**

Spectraplakins are a class of proteins long shown to interact with both microtubules and actin cytoskeletons [Leung et al., 1999; Fuchs and Karakesisoglou, 2001; Kodama et al., 2003; Applewhite et al., 2010; Stroud et al., 2011; Suozzi et al., 2012]. However, such interactions have only recently been documented to occur at microtubule plus-ends [Slep et al., 2005; Applewhite et al., 2010; Alvez-Silva et al., 2012] and shown to do so within the growth cone [Alvez-Silva et al., 2012]. Several studies have demonstrated that Spectraplakins are required for microtubule protrusion and axon outgrowth [Alvez-Silva et al., 2012; Margaron et al., 2013; Ka et al., 2014], as well as filopodial formation [Sanchez-Soriano et al., 2009]. Structure-function studies of one such Spectraplakins, actin crosslinking factor 7 (ACF7), showed that it can impact microtubule organization in the growth cone, an effect that requires both microtubule and actin interaction domains [Sanchez-Soriano et al., 2009]. Spectraplakins are guided to the plus-ends of microtubules by EB1 [Alvez-Silva et al., 2012], and furthermore, actin-microtubule interactions are controlled by Spectraplakins binding through this EB1 interaction [Alvez-Silva et al., 2012; Applewhite et al., 2013] (Fig. 4A). EB1 interactions at the plus-end conformationally change Spectraplakins shape, thus opening domains for microtubule attachment, and once in its conformationally-active form, Spectraplakins preferentially bind to the microtubule lattice, where it may interact with actin filaments [Alvez-Silva et al., 2012; Applewhite et al., 2013]. While there is much known about how Spectraplakins regulate F-actin-microtubule interactions, several aspects of this specific mechanism have not been explicitly examined in growth cones.

While interactions of Spectraplakins with other classes of +TIPs have not been rigorously examined in neurons, non-neural data indicate that such interactions may take place to modulate F-actin-microtubule coupling. There is evidence that ACF7 may act in a manner upstream of the +TIP, CLASP [Drabek et al., 2006]. Furthermore, a deficiency in ACF7 resulted in disorganization of peripheral microtubule bundles [van Vactor et al., 1993; Wu et al., 2008], an effect that was comparable to a CLASP deficiency [Stehbens and Wittmann, 2012]. Similarly, Spectraplakins were also shown to interact with APC near cortical focal adhesions [Zaoui et al., 2010]. Taken together with the F-actin-microtubule coupling role of these proteins individually, these data may support a more elaborate or collaborative mechanism for Spectraplakins in regulating cytoskeletal coordination.

## **The Microtubule Polymerase, XMAP215, May Have Secondary Actin Mediatory Functions**

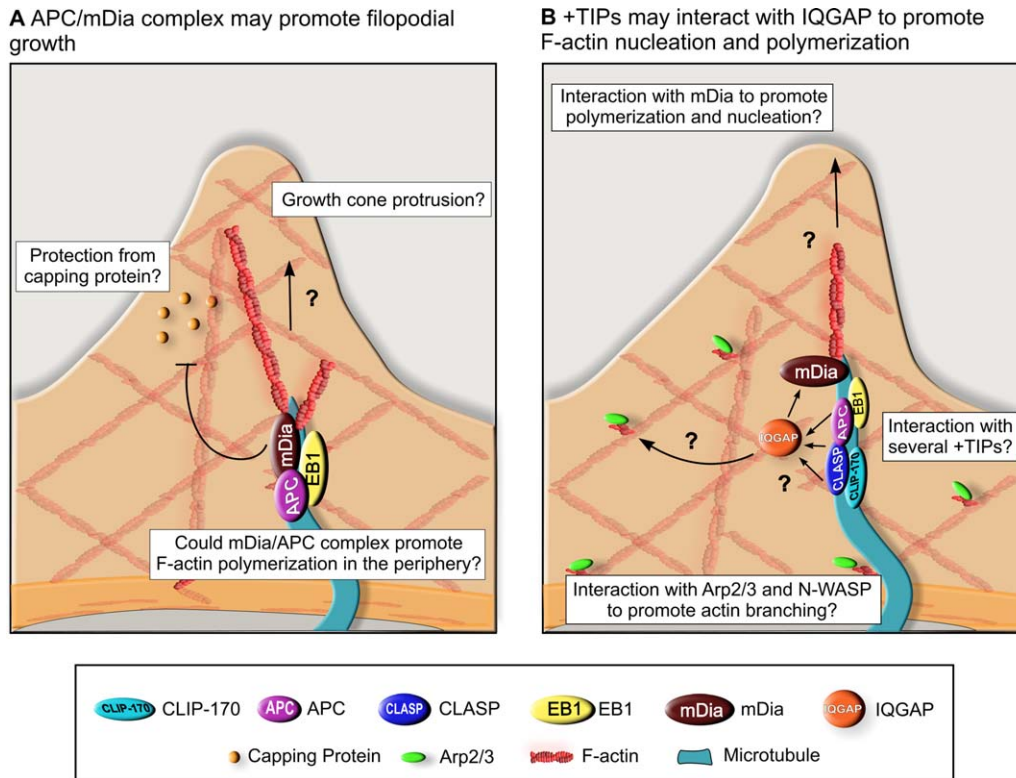
XMAP215 was classically characterized as a microtubule growth enhancement protein [Gard and Kirschner, 1987]

and, more recently, as a processive microtubule polymerase [Brouhard et al., 2008]. However, data over the last few years have given rise to a possible actin mediatory function of XMAP215. First, *mmps* (*minispindles*), a *Drosophila* orthologue to XMAP215, was shown to genetically interact with and antagonize the activity of the actin binding +TIP, CLASP, during axon outgrowth events [Lowery et al., 2010]. Subsequent studies led to the discovery that chTOG, the XMAP215 mammalian orthologue, interacted with the +TIPs, SLAIN1/2, CLASP and CLIPs, to increase microtubule growth rates and reduce catastrophe rates [van der Vaart et al., 2011; van der Vaart et al., 2012] and that the +TIP, TACC3, promoted the microtubule polymerization function of XMAP215 [Nwagbara et al., 2014]. However, one study indicated that partial knockdown of XMAP215 in *Xenopus* led to increased EB1-GFP velocities specifically in growth cones. These findings were at odds with previous experiments since XMAP215 knockdown was expected to lead to decreased microtubule growth rates [Lowery et al., 2013]. However, it was demonstrated through quantitative fluorescent speckle microscopy (QFSM) that microtubule polymer translocation/sliding was altered with XMAP215 knockdown, resulting in a reversal of microtubule translocation from retrograde to anterograde movement. Given that microtubule retrograde sliding is suggested to result from frequent transient coupling with F-actin retrograde flow [Myers et al., 2006; Lu et al., 2013], it is possible that XMAP215 directly or indirectly mediates the coupling and uncoupling of microtubules to actin flow (Fig. 4D). However, whether this function could happen independently or through interactions with CLASP, a known XMAP215 and actin interactor, is still unclear. Additionally, it is also possible that microtubule translocation could be affected through microtubule-microtubule sliding or other mechanisms. Thus, a role for XMAP215 in mediating interactions with F-actin currently remains speculative.

## **Expanding Roles for +TIPs: Evidence for Participating as F-Actin Nucleators**

Emerging evidence suggests that +TIPs may additionally have a novel function at microtubule plus-ends: facilitating formation of “seeding sites” for new F-actin nucleation. Although not yet demonstrated in growth cones, mounting data links +TIPs to actin nucleation both directly and indirectly (through interactions with additional microtubule-F-actin coupling proteins). These non-neural studies indicate that +TIPs possess more diverse roles than previously thought in controlling actin interactions and dynamics.

Evidence suggests that actin nucleation from microtubule plus-ends could be facilitated by interactions between APC and mDia (Fig. 5A), a formin tied to a large set of functions related to actin remodeling, such as nucleation from actin barbed ends, elongation, and bundling [Gupton et al.,



**Fig. 5. +TIPs may possess secondary functions as actin nucleators in the growth cone.** **A:** APC has been shown to interact with mDia, a formin, in order to promote actin filament assembly in fibroblast cells. However, a similar mechanism may occur in the periphery of neuronal growth cones. Upon binding to the plus-end, APC may stimulate F-actin polymerization and nucleation through mDia. APC/mDia complex has also been shown to protect F-actin from capping protein. If a similar mechanism does occur in growth cones, then APC/mDia could potentially promote F-actin assembly from microtubule plus-ends and, therefore, filopodial growth. **B:** Several +TIPs have been shown to interact with IQGAP, an actin binding and nucleation-promoting protein, in fibroblasts, epithelial cells, and macrophages. If similarly regulated in neuronal growth cones, +TIPs may function with IQGAP to bridge signaling cascades (i.e., CLASP and APC phosphorylation, and resultant microtubule behaviors) with F-actin nucleation. IQGAP may promote branching of actin filaments through Arp2/3 and N-WASP. Similarly, IQGAP could interact with mDia in growth cones, promoting nucleation and F-actin polymerization. IQGAP function has largely been shown in non-neuronal cells, and these interactions are not yet confirmed in growth cones. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

2007; Brandt et al., 2007; Okada et al., 2010]. Additionally, mDia has been demonstrated to contain a microtubule binding domain, and it can also influence microtubule stability and organization [Palazzo et al., 2001; Ishizaki et al., 2001; Bartolini et al., 2008]. Although not necessarily considered to be a +TIP, mDia is known to interact with EB1 and with the +TIPs CLIP-170 and APC [Wen et al., 2004; Lewkowicz et al., 2008; Jaiswal et al., 2013]. In fibroblasts, EB1 and APC can complex with mDia to stabilize microtubules at the cell cortex, and this complex can be regulated downstream of the Rho GTPase, RhoA [Wen et al., 2004]. Recent studies have highlighted the importance of this complex, showing evidence of a conserved mechanism that stimulates actin filament assembly [Okada et al., 2010; Jaiswal et al., 2013]. It will be intriguing to discover whether this mechanism is conserved in filopodia of neuronal growth cones, as this may provide new understanding of how +TIPs that interact with filopodial F-actin bundles could also facilitate additional F-actin extensions and, thus, growth cone protrusion.

CLIP-170, CLASP, and APC may also be involved in actin nucleation, by way of interactions with the actin binding protein IQGAP [Fukata et al., 2002; Watanabe et al., 2004; Galjart et al., 2005; Watanabe et al., 2009]. IQGAP is known to modulate numerous functions in actin remodeling; it interacts with Arp2/3 in epithelial and fibroblast cells to regulate actin branching [Bensenor et al., 2007; Le Clainche et al., 2007], and with mDia1 to mediate nucleation in macrophages and keratinocytes [Brandt et al., 2007; Wickstrom et al., 2010]. Furthermore, a number of studies have shown that IQGAP participates in actin crosslinking [Erickson et al., 1997; Fukata et al., 1997; Ho et al., 1999; Mateer et al., 2002] and also interacts with CLIP-170 to promote initial neurite extension and dendrite arborization [Fukata et al., 2002; Wang et al., 2007; Swiech et al., 2011]. It should also be noted that, as explained earlier, knockdown of CLASP in growth cones showed disruption in F-actin networks [Marx et al., 2013], a result which could have occurred due to altered F-actin nucleation dynamics. These +TIPs, which recruit IQGAP to pioneer

**Table I. An Overview of the Possible Functions +TIPs Use to Link Microtubules and F-Actin in the Neuronal Growth Cone**

Name	Possible functions	References
A: Microtubule and F-actin coupling		
CLASP	Binds directly to actin filaments; Influences filopodial and F-actin networks structure; Can couple microtubules to F-actin regulated through Abl and GSK3 kinase signaling	[Tsvetkov, 2007; Marx, 2013; Hur, 2011; Engel, 2014]
CLIP-170	Shown to be involved in interactions with CLASPs and IQGAP	[Fukata, 2002; Galjart, 2005; Akhmanova and Steinmetz, 2008]
APC	Microtubule stabilizer; Steering of growth cone based on axis of binding; Promotes decoupling of F-actin from microtubules through GSK3 kinase signaling	[Zhou, 2004; Koester, 2007; Purro, 2008]
End Binding (EB1/3)	Guides +TIPs to the microtubule plus-end; EB3 interacts with Drebrin to couple F-actin structures to microtubules; EB3/Drebrin guide microtubules along F-actin bundles; EB3/Drebrin may inhibit cofilin activity	[Akhmanova and Steinmetz, 2008; Honnappa, 2009; Geraldo, 2008; Worth, 2013; Tanabe, 2014; Sharma, 2011; Mikati, 2013; Grintsevich, 2014]
LIS1	Can uncouple microtubules from F-actin through dynein mediated mechanism and provide resistance to F-actin retrograde flow; Shown to interact with IQGAP in neurons	[Myers, 2006; Grabham, 2007; Kholmanskih, 2003; Kholmanskih, 2006]
Spectraplakins	Interacts with microtubules and F-actin through EB1 dependent mechanism; Required for proper microtubule protrusion/organization and axon outgrowth; Required for proper filopodial formation; May interact upstream of CLASP	[Alvez-Silva, 2012; Applewhite, 2013; Margaron, 2013; Ka, 2014; Sanchez-Soriano, 2009; Drabek, 2006]
XMAP215	May alter coupling state of microtubules and F-actin (potentially through CLASP); Interacts with +TIPs, SLAIN1/2, CLASP and CLIPs	[Lowery, 2013; Lowery, 2010; Van der Vaart, 2011; Van der Vaart, 2012]
B: Speculative F-actin nucleation functions		
APC/mDia	Could facilitate actin nucleation and bundling of F-actin through interactions at microtubule plus-end; mDia can interact with EB1, APC and CLIP-170; mDia can influence microtubule stability and organization	[Jaiswal, 2013; Okada 2010; Lewkowicz, 2008; Wen, 2004; Bartolini, 2008; Palazzo, 2001; Ishizaki, 2001]
IQGAP	Interacts with Arp2/3 to regulate actin branching; Interacts with mDia to mediate nucleation; Interacts with +TIPs CLIP-170, CLASP and APC; Shown to crosslink actin filaments	[Le Clainche, 2007; Bensenor, 2007; Brandt, 2007; Wickstrom, 2010; Fukata, 2002; Watanabe, 2004; Galjart, 2005; Watanabe, 2009; Erickson]
A Several +TIPs that have been demonstrated to couple microtubules to F-actin structures directly, or indirectly through actin binding proteins and other +TIPs. B +TIPs may possess a secondary function as promoters of actin nucleation dynamics, shown through interactions with mDia and IQGAP in non-neuronal cells.		

microtubule plus-ends, could facilitate similar mechanisms to nucleate actin structures in the growth cone.

### An Early Model for How +TIP-Directed Microtubule and F-Actin Interactions Control Guidance

Although evidence for how +TIPs control axon guidance through microtubule/F-actin interaction remains in its early stages, a basic model may be put forward. While microtubules actively extend into the periphery of the growth cone, they are in regular contact with F-actin. Depending on the specific configuration of +TIPs localizing to the plus-end [Zhou et al., 2004; Tsvetkov et al., 2007; Akhmanova and Steinmetz, 2008; Geraldo et al., 2008; Alvez-Silva et al.,

2012], as well as the accumulation of localized signaling molecules [Tanaka et al., 1995; Sabry et al., 2001; Zhou et al., 2002], microtubules may couple with F-actin retrograde flow through both F-actin bundles in the periphery and F-actin arcs in the transitional domain [Schaefer et al., 2002; Zhou et al., 2002; Burnette et al., 2007; Lee and Suter, 2008; Geraldo and Gordon-Weeks, 2009]. While prevention of microtubule extension through the coupling to retrograde flow would cause an inhibition of forward growth cone progression [Lin and Forscher, 1995; Zhou et al., 2002; Zhou and Cohan, 2004], it may be that an asymmetrical coupling of microtubules to F-actin throughout the growth cone causes some microtubules to progress forward while others retract, which can result in altered



steering of the growth cone [Buck and Zheng, 2002]. Some +TIPs that do couple microtubules to actin, like EB1 and the actin binding protein drebrin, may not cause microtubule looping back to the central domain, but instead allow transient coupling of microtubules along F-actin bundles [Geraldo et al., 2008]. This may effectively guide microtubules out alongside F-actin, thereby allowing polymerization to dominate and extend microtubules further into the periphery. There is some evidence that the appropriate balance of these persistent or transient coupling behaviors (or alternatively, complete dissociation from the plus-end) is influenced by +TIP modifications downstream of signaling kinases [Lee et al., 2004; Zhou et al., 2004; Hur et al., 2011; Engel et al., 2014], thus linking microtubule and actin behaviors to the guidance molecules encountered by the remodeling growth cone. However, as future studies investigate additional details of microtubule and F-actin interactions, more advanced models will undoubtedly be put forward.

## Conclusions and Perspectives

The growth cone requires collaborative and dynamic coordination of the F-actin and microtubule cytoskeletons in order to properly navigate the extracellular cues presented to it within the developing embryo. Coupling and uncoupling mechanisms between the two cytoskeletons can alter localization of microtubules, thus promoting their retraction or protrusion. Similarly, closely-linked microtubules and F-actin can be exposed to the same local signaling cascades, allowing them to undergo coordinated remodeling. +TIPs are increasingly demonstrated to act in this capacity, as they can serve as both modulators of plus-end dynamics and microtubule-F-actin cross linkers, which can be dynamically regulated by phosphorylation events downstream of guidance cue signaling pathways. Extracellular signaling cues can therefore be translated through actin/microtubule binding into growth cone outgrowth and turning events. A secondary mechanism +TIPs may employ in growth cones is their ability to stimulate actin nucleation, a function that may be especially relevant in the far reaches of the growth cone filopodia. While significant strides have been made in unraveling how certain +TIPs may act on both F-actin and microtubule cytoskeletons, and the ramifications that these interactions lend to growth cone behaviors, several chief broad questions remain unanswered.

Although we know many details about certain +TIPs and how they can affect microtubule/actin dynamics, much of this research was not conducted in neurons. There is evidence that +TIPs can serve growth cone-specific functions, rather than behaving consistently across different cell types [Stepanova et al., 2003; Lowery et al., 2013]. Thus, it is both valuable and necessary to continue studying the functions of +TIPs both in and outside of the neural context. Future studies of +TIPs such as the formins and Spectra-

plakins, which have been examined chiefly in fibroblasts and epidermal cells, may reveal that they function in unique roles during axon guidance.

It is also important to note that our known library of +TIPs is still expanding (Table I). The more recently identified small kinetochore-associated protein, SKAP [Dunsch et al., 2011], was also shown to bind to EB1 and IQGAP in order to mediate microtubule steering and protrusion in HEK cells [Cao et al., 2015]. However, it remains to be seen whether this association with IQGAP may also indicate a role in microtubule-F-actin crosslinking events. In addition to SKAP, mitotic interactor and substrate of Plk-1 protein, MISP, has been recently demonstrated to interact with EB1 as well as to colocalize with the actin cytoskeleton and focal adhesion proteins [Maier et al., 2013]. Although its ability to act as a +TIP on microtubule plus-ends has not been confirmed, it would be interesting to see the potential role it could play in mediating microtubule and F-actin interactions. As identification of TIPs increases, we must examine their interactions with F-actin and focal adhesion proteins, with signaling cascades, and with one another, in order to fully understand their roles in guidance behaviors.

Over the last several years there have been significant advances in the types of techniques used to study the cell biological interactions within growth cones. Techniques like live brain imaging [Leung and Holt, 2012] allow researchers to follow growth cones in living tissue. Also, recent biochemical assays employing the protein TIPact, a minimalist molecule containing microtubule and actin binding domains, could potentially be used to uncover more information on how these two cytoskeletons interact [Lopez et al., 2014]. Furthermore, the use of super resolution microscopy techniques has also increased, allowing for image resolution that extends beyond the diffraction limit of light and confocal microscopes. Specifically, recent experiments on photoactivatable complimentary fluorescent (PACF) proteins have shown precise super resolution images of EB1 tracking along the microtubule plus-ends [Xia et al., 2014]. These techniques may vastly benefit our understanding of the inner workings of actin-microtubule interactions.

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