

● PERSPECTIVE

## Localized regulation of the axon shaft during the emergence of collateral branches

The ability of the axon to form *de novo* collateral branches along its length is fundamental to the establishment of complex patterns of connectivity during development and is also a major response of many axonal populations following injury. The emergence of branches is under both positive and negative control by extracellular signals. How the site of branch formation is determined is a fundamental question regarding the formation of branches. In theory, the whole axon shaft has the potential to give rise to a branch, but yet branches form only at specific sites. This feature of the formation of branches is emphasized by studies in which nerve growth factor (NGF), covalently attached to 10 micron beads, was applied locally along embryonic sensory axons (Gallo and Letourneau, 1998). Although contact of a branch at the sites of contact, even after 3 hours of continuous contact only 46% of sites gave rise to a branch. Similarly, when NGF is bath applied to cultured sensory neurons, and thus the whole surface of the axon is exposed to NGF, axons usually only generate a maximum of 4–6 branches along the distal 100 microns of the axon, a response that is maximal by 30 minutes of treatment. Recent work, from our laboratory and others, has begun to shed light on why axons form branches at specific sites.

The formation of an axon branch is strictly dependent on both actin filaments and microtubules (Figures 1A and 2; see Kalil and Dent, 2014 for an excellent review). The first step in the formation of a branch, *in vivo* and *in vitro*, is the emergence of an axonal filopodium. Filopodia are finger-like projections from the surface of cells and are supported by a bundle of polymerizing actin filaments. The regulation of filopodia formation requires multiple regulatory mechanisms (Gallo, 2013), including Rho-family GTPases which are major regulators of the actin cytoskeleton (Spillane and Gallo, 2014). However, the majority of filopodia are transient and retracted back into the axon shaft without giving rise to a branch. For a filopodium to mature into a branch it must be invaded by microtubules, which provide structural stability and also allow for the transport of organelles and proteins into the nascent branch. However, as microtubule tips are dynamic, their entry into a filopodium is insufficient to promote the maturation of the filopodium into a branch and it is generally considered that a subsequent step involving the stabilization of the microtubule within the axonal filopodium is required for branch formation. Thus, in order to understand how a branch forms, and why it forms at a specific site, it is necessary to understand how the neuron locally regulates the dynamics and organization of actin filaments and microtubules.

Relative to the growth cone at the tip of an extending axon, the axon shaft exhibits low levels of actin filaments. In earlier work, we determined that the majority of axonal filopodia are generated from precursor structures termed axonal actin filament patches (Figure 1A and B). These actin patches are very dynamic and characterized by a meshwork of actin filaments which then generates the bundle of filaments that drives the elongation of a filopodium (Spillane et al., 2011). The formation of axonal actin patches is under regulation by NGF, which promotes the rate of formation of patches (patches/unit length

of axon/unit time) but does not affect the probability that a patch gives rise to a filopodium (Ketschek and Gallo, 2010). Thus, NGF increases the number of filopodia that form along the axon shaft by increasing the rate of formation of actin patch precursors.

Following NGF treatment, sites of patch formation correlate with the presence of the high affinity TrkA receptor for NGF (Ketschek and Gallo, 2010). However, TrkA receptors are found in clusters all along the axon and not all clusters give rise to patches, indicating that mere receptor localization is not sufficient to determine where patches form. However, patches are driven by the activity of phosphoinositide 3-kinase (PI3K) which exhibits localized signaling at sites of actin patch formation, as revealed by live imaging of a biosensor for PI3K activity (Ketschek and Gallo, 2010). These observations indicate that sites of actin patch formation in response to NGF require localized PI3K signaling though TrkA receptors, but not all sites populated by the receptors give rise to branches.

The above considerations indicate that the first step in determining where a branch will arise along the axon is determined by the localization of the formation of actin patches. In order for a cytosolic actin monomer to be polymerized into a filament it must be loaded with ATP. Following incorporation into a polymerizing filament, the activity of the ATPase domain of actin is greatly enhanced and hydrolyzes ATP to ADP. In studies addressing the role of axonal mitochondria, major sources of ATP generation, in the regulation of actin filament dynamics we observed that treatment with NGF induced a colocalization of sites of actin patch formation and the presence of stalled mitochondria, the respiration of which is required for patch formation (Ketschek and Gallo, 2010). Consistently, we and others have recently reported that axon branches and filopodia emerge preferentially from sites along the axon containing stalled mitochondria (Couchet et al., 2013; Spillane et al., 2013; Tao et al., 2014). The subcellular distribution of axonal mitochondria is thus a major determinant of sites of axon branching. However, as discussed above for the localization of TrkA receptors, not all sites of the axon containing stalled mitochondria give rise to an axon branch indicating that additional events must occur for a branch to form.

In our recent work (Ketschek et al., 2015), we sought to further understand the role of axonal microtubules in the formation of axon branches. The axonal microtubule array, normally characterized by a bundle of aligned microtubules, undergoes splaying at sites where branches are present (Figure 2; Dent et al., 1999). We observed that treatment with NGF increased the number of sites of microtubule splaying along sensory axons. Importantly, the effects of NGF on microtubule splaying occurred maximally within 10 min of treatment and preceded the increase in the formation of actin patches and eventually branches. Thus, the splaying of axonal microtubules is one of the earliest features of the response of sensory axons as they begin to form branches following treatment with NGF. Consistently, analysis of the distribution of actin patches and filopodia along the axon revealed a correlation with sites of microtubule splaying. The functional significance of microtubule splaying remains a matter of conjecture, as there are currently no methods that would specifically block the splaying. However, the splaying may promote the accumulation of cellular components required for axon branching (*e.g.*, mitochondria and other organelles) or promote the targeting of microtubules tips into axonal filopodia. Consistent with the latter notion, we observed a strong correlation between sites of microtubule splaying and the targeting of microtubules into axonal filopodia. This indicates that sites of splaying are primed for the entry of microtubules

into axonal filopodia.

What regulates the ability of the axon to locally splay its microtubules? Through electron microscopic analysis we observed apparent contacts between axonal microtubules and actin filaments along axons. Thus, we considered the hypothesis that actin filaments and the associated force generating motor-protein myosin II may promote the splaying of microtubules by pulling them apart from each other. This hypothesis was readily falsified by experiments in which actin filaments were depolymerized or myosin II activity inhibited. In both cases, NGF-induced splaying was promoted and not inhibited. These data indicate that actin filaments and myosin II act to inhibit the ability of axonal microtubules to undergo splaying.

We next considered whether the microtubule associated protein1B (MAP1B) may be involved in the splaying of axonal microtubules. MAP1B was considered because prior studies determined that it is a negative regulator of axon branching. Analysis of the levels of microtubule associated MAP1B did not reveal any changes at sites of splaying relative to adjacent sites of the axon that did not exhibit splaying. However, we did obtain insights into the possible role of MAP1B during branching. Analysis of the distribution of microtubule associated MAP1B revealed that following treatment with NGF its association with microtubules was low during the early stages of branching, in filopodia and while the branch is maturing, but then achieved levels not distinguishable from the main axon shaft once the branch had fully matured. In contrast, in the absence of NGF, MAP1B association with microtubules was high during all stages of branching. Thus, a yet to be fully elucidated mechanism downstream of NGF signaling decreases the association of MAP1B with microtubules during the early stages of branching.

Insights into the possible mechanism regulating the association of MAP1B with microtubules during the early stages of branching came from investigation of the phosphorylated form of MAP1B at a glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) site (threonine 1265). Prior studies by other laboratories reported effects of NGF on GSK3 $\beta$  activity that are at face value in conflict. NGF treatment increases phosphorylation of MAP1B at the GSK3 $\beta$  site (e.g., Goold and Gordon-Weeks, 2003), indicating activation of GSK3 $\beta$ . In contrast, analysis of the levels of phosphorylated and inactivated GSK3 $\beta$  in sensory neurons following treatment with NGF indicated that NGF, at least partially, decreases GSK3 $\beta$  activity (e.g., Zhao et al., 2009). Prior studies used western blot analysis, which provides no subcellular resolution. In our study, we used an antibody to MAP1B phosphorylated at the GSK3 $\beta$  site and analyzed its distribution and levels in axons through quantitative immunocytochemistry. Consistent with studies observing NGF-induced increases in MAP1B phosphorylation, we found that NGF treatment increased the net levels of phosphorylated MAP1B within the axon shaft by extending the coverage of the axon shaft exhibiting phosphorylated MAP1B. However, we also observed that NGF treatment decreased the levels of phosphorylated MAP1B within axonal filopodia, consistent with the reported partial inactivation of GSK3 $\beta$ . These observations indicate that the effects of NGF on the phosphorylation of MAP1B at the GSK3 $\beta$  site are likely regulated at the sub-axonal level.

The polymerization of microtubules, and/or their transport, into nascent branches is required for branch maturation. However, as noted above since microtubule tips are dynamic and undergo cycles of polymerization and depolymerization, the mere entry of a microtubule into an axonal filopodium is not sufficient for the formation of a branch. An additional step involving the stabilization of the microtubule in the filopodium is considered to be required for the maturation of the filopodium into a branch. As phosphorylation of MAP1B by GSK3 $\beta$  is

promotes microtubule tip polymerization and dynamics, these data indicate that the decreased phosphorylation of MAP1B in axonal filopodia may reflect a mechanism that contributes to the stabilization of microtubules during the early stages of branch formation.

Our prior work has determined that the actin-based component of NGF-induced axon branching is dependent on NGF-induced intra-axonal protein synthesis (Spillane et al., 2012, 2013). However, to date we have not yet found any effect of NGF on axonal microtubules which is dependent on intra-axonal proteins synthesis (Spillane et al., 2012). Indeed, the NGF-induced debundling of axonal microtubules is also protein synthesis independent (Ketschek et al., 2015). However, Feltrin et al (2012) report that MAP1B can be phosphorylated by a mechanism involving the intra-axonal synthesis of MKK7, and whether intra-axonal protein synthesis may regulate MAP1B phosphorylation in the context of NGF-induced axon branching remains to be determined.

Investigations into the mechanism of axon branching are beginning to shed light on the fundamental question of why branches are formed at specific sites along the axon. The mechanism of branching is quite complex and requires a convergence in space and time of multiple events involving localized signaling and localized regulation of cytoskeletal dynamics. It is tempting to hypothesize that the site of formation of an axon branch reflects a site along the axon where the multiple events required for branching (i.e., localized signaling, mitochondrial positioning, microtubule splaying, entry and stabilization into the filopodium, etc) have managed to be orchestrated in time and space. If this is correct, then the determination of the site of formation of an axon branch may have a stochastic component. One way to consider the issue at the conceptual level is that in order to be the site where a branch ultimately emerges from the axon is akin to playing multiple slot machines and winning on all of them (Figure 1C). While some segments of the axon may “win” X out of Y gambles, only the site that wins Y out of Y gambles is able to give rise to a branch. Live imaging of a multitude of variables along the axon will be required to test this hypothesis. While it is currently not technically feasible to simultaneously track all the necessary variables in space and time along the axon, with continued development of reporter systems and imaging technology it may ultimately be able to perform this multiplex analysis and directly test this hypothesis.

*This work has been supported by an NIH award (NS078030) to GG.*

Gianluca Gallo\*

Shriners Hospitals Pediatric Research Center, Temple University School of Medicine, Department of Anatomy and Cell Biology, Philadelphia, PA, USA

\*Correspondence to: Gianluca Gallo, Ph.D., tue86088@temple.edu.

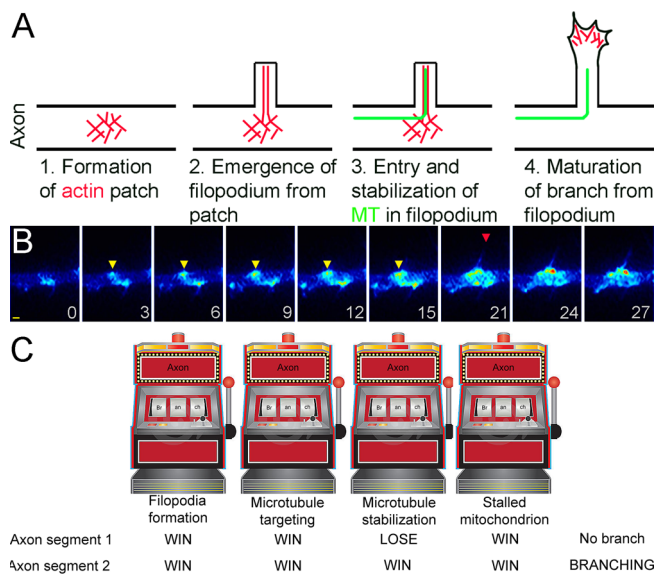
Accepted: 2015-06-17

doi:10.4103/1673-5374.162694

http://www.nrronline.org/  
Gallo G (2015) Localized regulation of the axon shaft during the emergence of collateral branches. *Neural Regen Res* 10(8):1206-1208.

## References

- Courchet J, Lewis TL Jr, Lee S, Courchet V, Liou DY, Aizawa S, Polleux F (2013) Terminal axon branching is regulated by the LKB1-NUAK1 kinase pathway via presynaptic mitochondrial capture. *Cell* 153:1510-1525.
- Dent EW, Callaway JL, Szebenyi G, Baas PW, Kalil K (1999) Reorganization and movement of microtubules in axonal growth cones and developing interstitial branches. *J Neurosci* 19:8894-8908.



**Figure 1 Overview of the mechanism of sensory axon collateral branching.**

(A) Sequence of cytoskeletal events underlying axon branching. The first step (1) is the formation of an actin patch, which serves as a precursor to the formation of an axonal filopodium (2; actin filaments shown in red). Next, the plus tip of an axonal microtubule (MT, green) must invade the filopodium and become stabilized (3). Finally, the filopodium undergoes maturation into a branch (4). During maturation the filopodium changes its morphology and actin distribution. The actin filament bundle that characterizes a filopodium is reorganized and the actin filaments become polarized to the tip of the nascent branch, which develops a small growth cone, and the shaft of the filopodium now contains microtubules. For actual examples of actin filament and microtubule distributions at the various stages of branch formation see **Figure 2**. (B) Time lapse sequence of actin patch formation and filopodia emergence as revealed by eYFP- $\beta$ -actin imaging along embryonic sensory axons. Time in seconds is shown in panels. Bar = 1  $\mu$ m. Between 3–15 seconds the actin patch (yellow arrowhead) forms and elaborates, *i.e.*, grows in size and intensity. Between 15–21 seconds a filopodium emerges from the patch (red arrowhead). (C) Proposed conceptual stochastic model for the determination of the site of axon branching. Multiple basic cellular events have to occur in a correct spatio-temporal sequence in order for a branch to form. In the interest of simplicity, the schematic represents a subset of the fundamental required events in branch formation; the formation of axonal filopodia, the entry and stabilization of microtubules into axonal filopodia, and the presence of a stalled mitochondrion. Each one of these basic events is in turn dependent on a complex multi-step biochemical mechanism. In the context of the Ketschek et al (2015) work, the regulation of the phosphorylation of MAP1B by GSK3 $\beta$  in axonal filopodia may contribute to the stabilization of microtubules in filopodia. In the panel, axon segment number 1 “wins” all but the stabilization of the microtubules and fails to generate a branch. In contrast, axon segment number 2 “wins” all and is able to generate a branch. Image of slot machine used as royalty free stock image from [www.dreamstime.com](http://www.dreamstime.com). eYFP: Enhanced yellow fluorescent protein; MAP1B: microtubule associated protein 1B; GSK3 $\beta$ : glycogen synthase kinase 3 $\beta$ .

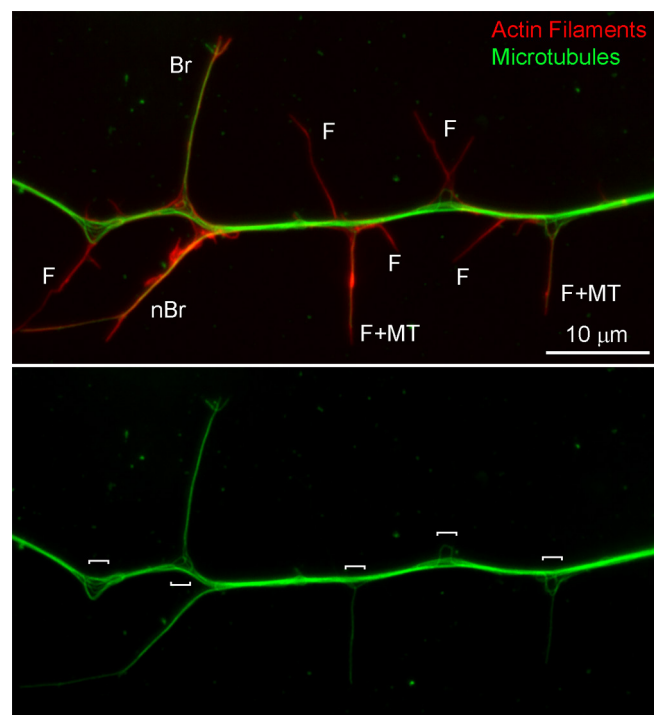
Feltrin D, Fusco L, Witte H, Moretti F, Martin K, Letzelter M, Fluri E, Scheiffele P, Pertz O (2012) Growth cone MKK7 mRNA targeting regulates MAP1b-dependent microtubule bundling to control neurite elongation. *PLoS Biol* 10:e1001439.

Gallo G, Letourneau PC (1998) Localized sources of neurotrophins initiate axon collateral sprouting. *J Neurosci* 18:5403-5414.

Gallo G (2013) Mechanisms underlying the initiation and dynamics of neuronal filopodia: from neurite formation to synaptogenesis. *Int Rev Cell Mol Biol* 301:95-156.

Goold RG, Gordon-Weeks PR (2003) NGF activates the phosphorylation of MAP1B by GSK3 $\beta$  through the TrkA receptor and not the p75(NTR) receptor. *J Neurochem* 87:935-946.

Kalil K, Dent EW (2014) Branch management: mechanisms of axon branching in the developing vertebrate CNS. *Nat Rev Neurosci* 15:7-18.



**Figure 2 Examples of the distribution of actin filaments and microtubules along a sensory axon.**

The sample was simultaneously fixed and extracted to only reveal the polymeric cytoskeleton and stained with antibodies to  $\alpha$ -tubulin and rhodamine-phalloidin which specifically labels actin filaments, F: filopodium (step 2 in **Figure 1**). F+MT: filopodium containing one or more microtubules (step 3 in **Figure 1**). Br: mature branch with polarized actin filaments (step 4 in **Figure 1**). nBr: nascent branch containing microtubules, but the actin filaments have not yet fully polarized to the tip of the branch. [ : sites along the axon exhibiting varying degrees of microtubule splaying.

Ketschek A, Gallo G (2010) Nerve growth factor induces axonal filopodia through localized microdomains of phosphoinositide 3-kinase activity that drive the formation of cytoskeletal precursors to filopodia. *J Neurosci* 30:12185-12197.

Ketschek A, Jones S, Spillane M, Korobova F, Svitkina T, Gallo G (2015) Nerve growth factor promotes reorganization of the axonal microtubule array at sites of axon collateral branching. *Dev Neurobiol* doi: 10.1002/dneu.22294.

Spillane M, Ketschek A, Jones SL, Korobova F, Marsick B, Lanier L, Svitkina T, Gallo G (2011) The actin nucleating Arp2/3 complex contributes to the formation of axonal filopodia and branches through the regulation of actin patch precursors to filopodia. *Dev Neurobiol* 71:747-758.

Spillane M, Ketschek A, Donnelly CJ, Pacheco A, Twiss JL, Gallo G (2012) Nerve growth factor-induced formation of axonal filopodia and collateral branches involves the intra-axonal synthesis of regulators of the actin-nucleating Arp2/3 complex. *J Neurosci* 32:17671-17689.

Spillane M, Ketschek A, Merianda TT, Twiss JL, Gallo G (2013) Mitochondria coordinate sites of axon branching through localized intra-axonal protein synthesis. *Cell Rep* 5:1564-1575.

Spillane M, Gallo G (2014) Involvement of Rho-family GTPases in axon branching. *Small GTPases* 5:e27974.

Tao K, Matsuki N, Koyama R (2014) AMP-activated protein kinase mediates activity-dependent axon branching by recruiting mitochondria to axon. *Dev Neurobiol* 74:557-573.

Zhao Z, Wang Z, Gu Y, Feil R, Hofmann F, Ma L (2009) Regulate axon branching by the cyclic GMP pathway via inhibition of glycogen synthase kinase 3 in dorsal root ganglion sensory neurons. *J Neurosci* 29:1350-1360.