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A requirement for filopodia extension toward Slit during Robo-mediated axon repulsion

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Axons navigate long distances through complex 3D environments to interconnect the nervous system during development. Although the precise spatiotemporal effects of most axon guidance cues remain poorly characterized, a prevailing model posits that attractive guidance cues stimulate actin polymerization in neuronal growth cones whereas repulsive cues induce actin disassembly. Contrary to this model, we find that the repulsive guidance cue Slit stimulates the formation and elongation of actin-based filopodia from mouse dorsal root ganglion growth cones. Surprisingly, filopodia form and elongate toward sources of Slit, a response that we find is required for subsequent axonal repulsion away from Slit. Mechanistically, Slit evokes changes in filopodium dynamics by increasing direct binding of its receptor, Robo, to members of the actin-regulatory Ena/VASP family. Perturbing filopodium dynamics pharmacologically or genetically disrupts Slit-mediated repulsion and produces severe axon guidance defects in vivo. Thus, Slit locally stimulates directional filopodial extension, a process that is required for subsequent axonal repulsion downstream of the Robo receptor.

Introduction

Growth cones are motile structures at the distal ends of axons that translate extracellular signals into directional responses, enabling axons to navigate to their proper targets (Lowery and Van Vactor, 2009). Morphologically, growth cones are characterized by two types of F-actin–supported structures: lamellipodia and filopodia. Lamellipodia are sheet-like protrusions supported by a branched actin network formed by the Arp2/3 complex, whereas filopodia are rod-like protrusions supported by unbranched parallel bundles of actin filaments formed by Ena/VASP and formin family proteins (Dent et al., 2011; Gomez and Letourneau, 2014). Ena/VASP proteins increase actin polymerization rates and promote the elongation of long, unbranched actin filaments by protecting the barbed end from capping (Bear et al., 2002; Hansen and Mullins, 2010; Winkelman et al., 2014). Although filopodia are assumed to play key roles in sensing and transducing guidance signals important for proper growth cone navigation (Bentley and Toroian-Raymond, 1986; Chien et al., 1993; Mattila and Lappalainen, 2008; Dent et al., 2011), their exact role in this process, as well as how guidance cues regulate filopodia formation and dynamics, are poorly understood.

Dorsal root ganglia (DRG) are sensory neurons that extend axon branches distally to the periphery and centrally into the spinal cord dorsal horn. DRG central projections enter the spinal cord at the dorsal root entry zone (DREZ), where they bifurcate and extend branches along the rostral–caudal axis (see Fig. 7 A). The abrupt change in axon orientation requires Slit1 and Slit2, ligands concentrated in the spinal cord midline that repel DRG axons expressing two of their cognate receptors, Robo1 and Robo2 (Kidd et al., 1998, 1999; Fricke et al., 2001; Hao et al., 2001; Long et al., 2004; Ma and Tessier-Lavigne, 2007). In vitro, stimulation of DRG neurons with Slit elicits a rapid reduction in growth cone area termed “collapse” caused, in large part, by actin depolymerization (Gallo and Letourneau, 2004). Repulsive growth cone turning is widely assumed to arise from localized actin depolymerization on the side of the growth cone exposed to the repulsive guidance cue (Lowery and Van Vactor, 2009; Dent et al., 2011; Vitriol and Zheng, 2012). Somewhat paradoxically, data from invertebrate models have demonstrated that Robo-dependent repulsion is mediated in part through Ena/VASP proteins (Bashaw et al., 2000; Yu et al., 2002), which promote actin polymerization (Bear and Gertler, 2009); however, how Slit-Robo signaling affects Ena/VASP-dependent actin assembly and membrane protrusion to enable axon repulsion from Slit remains unclear.

We investigated growth cone dynamics during Slit-Robo–mediated axonal repulsion. We find that axonal repulsion requires the asymmetric formation and extension of filopodia...
toward sources of the repulsive guidance cue Slit. Filopodium dynamics are regulated by formation of Robo:Ena–VASP complexes that are formed in response to Slit. DRG sensory afferents lacking all three Ena–VASP paralogs exhibit aberrant invasion of the spinal cord dorsal midline, a phenotype reminiscent of the defects observed in mouse embryos lacking SLIT1/2 or ROBO1/2 (Ma and Tessier-Lavigne, 2007). Although enhanced actin polymerization toward attractive guidance cues is well documented (Shekarabi and Kennedy, 2002; Lebrand et al., 2004; Tang and Kalil, 2005), these data demonstrate that, surprisingly, filopodia assemble and elongate toward the repulsive guidance cue Slit through the interaction of the Robo receptor with Ena–VASP proteins and that this dynamic regulation of filopodia is required for subsequent axonal repulsion.

Results

Slit stimulates elongation of DRG growth cone filopodia

We imaged growth cones on axons extending from organotypic DRG explants by time-lapse microscopy to characterize their responses to Semaphorin3A (Sema3A) and Slit, two established repulsive guidance cues for DRG axons. Application of either Sema3A or the amino-terminal fragment of Slit2 (Slit), which contains the domain responsible for binding to Robo1 and Robo2 (Chédotal, 2007), caused a decrease in the area of which contains the domain responsible for binding to Robo1 or Slit2 (Slit), published repulsive guidance cues for DRG axons. Application of a Slit gradient induced a net repulsion of axons on extending from organotypic DRG explants. When a micropipette was positioned 100 µm from growth cones at a 45° angle, a mock gradient had no effect on filopodium orientation; filopodia were largely distributed evenly around the growth cone periphery, excluding the region occupied by the axon (Fig. 2 A). However, exposure to a Slit gradient produced a clear increase in the proportion of filopodia on the growth cone quadrant proximal to the higher Slit concentration (Fig. 2 A). This change in the orientation of filopodia toward the Slit gradient was caused by an increase in the number of filopodia facing the gradient, whereas the number of filopodia distal to the gradient remained essentially unchanged (Fig. 2 B). The increase in the number of filopodia was accompanied by a lengthening of filopodia proximal to the gradient: proximal filopodia were 37% longer after Slit exposure (10.5 ± 5.4 vs. 7.7 ± 3.6 µm) and were 17% longer than those on the distal growth cone quadrant, which were exposed to lower Slit concentrations (Fig. 2 C and Fig. S1). These data indicate that, in DRG explants, axonal growth cones encountering a spatially asymmetric Slit gradient respond by forming and elongating filopodia toward, rather than away from, higher concentrations of Slit.

Regulated filopodia dynamics are required for Slit chemorepulsion

The finding that filopodia extend toward a source of Slit was surprising, because it has conventionally been thought that repulsive guidance cues such as Slit mainly induce bulk actin depolymerization (Dent et al., 2011; Vitriol and Zheng, 2012). Therefore, we tracked growth cone trajectories to confirm that axons were in fact repelled by the Slit gradients, as would be expected. Growth cones in a mock gradient grew outward in a random distribution within ±45° from the median (Fig. 2 D). Application of a Slit gradient induced a net repulsion of axons (~9 ± 2° turning angle) compared with controls (0 ± 2°), with few “attractive” movements toward the micropipette (Fig. 2 E).

We next asked if Slit-mediated filopodium formation and elongation is required for axon repulsion. We measured the response of neurons in organotypic DRG explants isolated from embryos lacking all three Ena–VASP paralogs (Mena, VASP, and EVL; triple null is referred to hereafter as mve). When cultured in vitro, mve DRG growth cones are almost completely devoid of filopodia (Fig. 2 D, iv), consistent with previously published phenotypes of cortical neurons (Dent et al., 2007; Kwiatkowski et al., 2007) and fibroblasts (Bear et al., 2000; Mejillano et al., 2004) that lack Ena–VASP. When exposed to a Slit gradient, mve axons exhibited directionally persistent forward movement without deflecting away from Slit (Fig. 2 D). However, this defective response to Slit could be caused by loss of Ena–VASP function that is not directly related to filopodia. To determine if inhibiting filopodium dynamics in wild-type neurons would affect the turning response, we treated neurons...
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with cytochalasin D (CD), which caps the barbed ends of actin filaments in a stochastic manner at low concentrations (25 nM) known to inhibit filopodia without disrupting lamellipodia (Dent et al., 2007; Hansen and Mullins, 2015). Strikingly, wild-type neurons treated with CD at the onset of the Slit gradient failed to deflect away from Slit, essentially phenocopying the response of mve axons (Fig. 2 D). Thus, blocking the dynamic and spatially oriented formation and elongation of filopodia is sufficient to inhibit repulsive movement of DRG growth cones away from Slit.

Regulated filopodia dynamics are required for Slit-induced axon retraction

We noticed that exposing growth cones to high concentration Slit gradients, either by positioning the micropipette close to the axon or by loading a high concentration of Slit in the micropipette, could cause growth cone collapse followed by axon retraction (Fig. S3). Intriguingly, we never observed retraction in mve axons; however, as it was technically difficult to elicit this response reproducibly in controls, we sought a more reliable method of examining axon retraction. Bath application of Slit to control DRG neurons caused growth cone collapse (Fig. 3, A and B; and Video 4) and axon retraction in a dose-dependent manner: few axons retract at low (1 nM) Slit concentrations, whereas concentrations of Slit 4.5 nM or higher caused uniform axon retraction (Fig. 3 C). Although increasing concentrations of Slit progressively diminished the rate of mve axon extension, retraction of mve axons was never observed, even at the highest Slit concentration tested (30 nM; Fig. 3 C and Video 5; unpublished data). Indeed, some mve axons continued to extend even after stimulation with 6 nM Slit, a concentration that universally evoked retraction in controls (Fig. 3 C). Similarly, acute inhibition of filopodium dynamics with CD blocked axon retraction normally elicited by 6 nM Slit (Fig. 3 D). Although

Figure 1. Slit induces filopodium elongation. (A) DIC images of DRG growth cones 15 s before (i and iii) and 10 min after addition of 1.5 collapsing units (CU) of either Slit (ii) or Sema3A (iv). One CU is the ligand concentration which induces ~50% growth cone collapse [Slit2, 400 ng/ml; Sema3A, 500 ng/ml]. Note the elongation of filopodia after stimulation with Slit [ii, white arrowheads mark filopodium tips], but not with Sema3A [iv, red arrowheads mark retraction fibers]. See Videos 1 and 2. Growth cone collapse (solid lines) and filopodium length were measured over a concentration range of Slit2 (v, blue) or Sema3A (vi, green); for ease of comparison, concentrations are given as CU. Greater than 40 growth cones were scored at each concentration per condition. *** P < 0.0001; n.s., P > 0.05; one-way ANOVA. (B) Live-cell DIC images of a growth cone during stimulation with 1.5 CU Slit. Filopodium tips are marked at 10s before (orange arrowheads) and at 170s after (blue arrowheads) Slit stimulation. Montages show time-lapse images of single filopodia, with the far-right panel showing an immunofluorescent image of the same filopodium after fixation at 180s post-Slit (phalloidin [F-actin], red; Mena, green). See Video 3. Bars, 10 µm. (C) Spontaneous and Slit-induced filopodium elongation (Elong.) kinetics were measured from DIC images captured every 5 s for 10 min before and after Slit addition. (i) Depicts length, rate, lifetime, and extension period measurements shown in panels ii–v (ii–v: preslit, n = 638 filopodia; postslit, n = 560 filopodia; 15 biological replicates). *** P < 0.0001; n.s., P > 0.05; two-tailed t test.
inhibiting filopodia with CD or ablating filopodia by genetic removal of Ena–VASP had profound effects on axon retraction, both of these conditions showed no significant differences in Slit-induced reduction in growth cone area compared with controls (Fig. 3, A and B). These data indicate that Robo signals are transduced in the absence of filopodia but that normal axonal responses to Slit require regulated filopodium dynamics.

**Slit promotes association of Ena–VASP with Robo**

We explored the possibility that Slit-elicited filopodium elongation involved Ena–VASP proteins, which (1) promote actin filament elongation (Bear and Gertler, 2009), (2) are concentrated at the tips of filopodia (Dent et al., 2011), (3) can bind to Robo in vitro (Bashaw et al., 2000; Yu et al., 2002), and (4) remain concentrated at the tips of filopodia undergoing Slit-induced elongation (Fig. 1 B). The *Drosophila melanogaster* orthologs of Robo1 and Mena were shown to exist in biochemical complexes in whole *Drosophila* embryo lysates (Bashaw et al., 2000); however, the cell biological consequences of this interaction, as well as its sensitivity to ligand stimulation, remain unclear. To determine if vertebrate Mena associates with Robo in a Slit-regulated manner, we immunoprecipitated endogenous Robo-1 from neuronal-like CAD cells (Byun et al., 2012). Under basal conditions, Mena was weakly detected in complexes with Robo-1; however, Slit treatment induced robust coimmunoprecipitation of Robo–Mena complexes (Fig. 4 A). The increased abundance of Robo–Mena complexes after Slit

![Diagram](image-url)
application occurred on a similar timescale as filopodium elongation (Figs. 1 and 2), consistent with the notion that dynamic regulation of Robo–Mena complex formation might regulate the increase in filopodium number and length in response to Slit.

We explored this interaction further by pull-down assays of GFP-tagged fragments of rat Robo1 using immobilized recombinant mouse Ena–VASP homology 1 (EVH1) domain, which mediates protein–protein interactions with several Ena–VASP binding partners (Niebuhr et al., 1997; Peterson and Volkman, 2009). We observe a robust interaction between EVH1 and a segment of Robo containing the Conserved Cytoplasmic 2 (CC2) motif (Fig. 4 C, asterisk), which includes a canonical EVH1 binding site (F/L followed by four prolines, abbreviated as LP4; Niebuhr et al., 1997) previously identified in Drosophila Robo1 (Bashaw et al., 2000). This interaction was dependent on the LP4 sequence, as a leucine-to-alanine substitution disrupted EVH1 binding (CC2m; Fig. 4 D). Interestingly, we found that the near-full-length Robo intracellular domain showed no binding to EVH1, despite containing CC2 (Fig. 4 C). Indeed, EVH1 binding was inversely correlated with the length of the construct containing CC2 (Fig. 4 E). We reasoned that EVH1 binding sites might be obscured in the context of the full-length protein in the absence of ligand and that ligand binding could relieve this putative autoinhibition. If this were the case, it would be possible that other EVH1 binding sites existed within the Robo intracellular domain that would not be detected using our pull-down approach; we therefore probed arrays of overlapping peptides spanning the entire intracellular portion of Robo1 with purified recombinant protein containing the Mena EVH1 domain. This analysis confirmed binding to CC2 and also identified an additional potential binding site in CC3 (Fig. S2 A). Sequence analysis revealed that although the LP4 motif in CC2 is highly conserved evolutionarily, the CC3 LP4 motif exhibits considerably less conservation among Robo family members and homologs (Fig. S2 B) and showed no interaction with EVH1 in our pull-down assays (Fig. 4 C).
Together, these data support a model in which Slit induces a conformational change in Robo that is required for robust binding of CC2 to the EVH1 domains of Ena–VASP proteins.

Ena–VASP binding to Robo CC2 is required for ligand-induced filopodium elongation

To determine whether Slit-induced filopodium elongation requires direct interaction of Mena with Robo, we tested if disrupting EVH1 binding sites in the intracellular domain of Robo affected growth cone responses. To facilitate analysis of Robo mutants, we used a previously developed chimeric receptor in which the extracellular portion of the hepatocyte growth factor (HGF) receptor, Met, is fused to the transmembrane and intracellular sequence of Robo1; upon stimulation with HGF, Met-Robo transduces repulsive signals normally elicited by Slit without activating endogenous Robo receptors (Stein and Tessier-Lavigne, 2001). For these experiments, constructs were cotransfected with a GFP reporter into primary cultures of dissociated DRG neurons. Filopodia in Met-Robo–expressing neurons appeared similar to GFP-transfected controls in number, length, and dynamics and displayed normal responses when stimulated by Slit (robust filopodium elongation and decreases in growth cone area; Fig. 5, B and C). Upon stimulation with 50 ng/ml HGF, Met-Robo–expressing growth cones exhibited filopodium elongation similar to that elicited by Slit (Fig. 5 B). GFP-transfected DRG neurons show no apparent response to HGF, indicating that HGF stimulates filopodium dynamics solely through the expressed Met-Robo chimera (Fig. 5, A and B). Interestingly, mutation of the EVH1 binding site in CC2 (CC2m) of the Met-Robo chimera completely disrupted filopodium elongation elicited by HGF; whereas an analogous mutation in CC3 (CC3m) did not interfere with HGF-elicited filopodium elongation (Fig. 5 B). HGF stimulation of any of the Met-Robo chimera–transfected (MR, CC2m, or CC3m) neurons resulted in decreases in growth cone area (∼50%) similar to what is typically seen after stimulation with Slit (Fig. 5 C). These data indicate that all the chimeric receptors tested were capable of mediating some ligand responses and that lamellipodium dynamics can be regulated independently of filopodia. Thus, a functional Ena–VASP binding site in CC2 is dispensable for the regulation of lamellipodia but is required for the induced elongation of growth cone filopodia after activation of the Robo receptor.

Robo requires a functional Ena–VASP binding site in CC2 to mediate axonal repulsion

The finding that the Ena–VASP binding site in the Robo CC2 motif was required for filopodium elongation, but dispensable for growth cone collapse (Fig. 5), allowed us to determine if these activities are required for axonal repulsion. We transfected Met-Robo chimeras or a GFP control into wild-type DRG neurons and examined the response of filopodia (length and number) and axon turning to a gradient of HGF. As expected, we saw no response to HGF in the GFP-transfected controls (Fig. 6). Similar to wild-type neurons responding to a Slit gradient (Fig. 2), we observed an increase in the number of filopodia oriented toward the HGF gradient in Met-Robo–transfected growth cones (Fig. 6 A; 0–45°). Additionally, a ∼30% increase in filopodium length was observed after the onset of the HGF gradient only in the Met-Robo–transfected growth cones (before, 5.7 ± 2.7 µm; after, 7.4 ± 4.5 µm; Fig. 6 B). Again, similar to filopodia in a Slit gradient (Fig. 2), the filopodia proximal to the HGF gradient showed a stronger response...
in Met-Robo–expressing growth cones than those located distal to the gradient (Fig. 6 C). However, growth cones transfected with the CC2m construct actually exhibited a slight decrease in the number of filopodia both proximal and distal to the HGF gradient (Fig. 6 A). Additionally, mutation of the Ena–VASP binding site in CC2 abrogated the ligand-induced increase in filopodium length evoked in the Met-Robo–expressing growth cones (Fig. 6, B and C). Importantly, axonal repulsion away from the HGF gradient in the Met-Robo–expressing neurons (−18.5 ± 15.7°) was not observed in either GFP controls (0.2 ± 13.8°) or in neurons expressing CC2m (4.8 ± 16.6°) (Fig. 6 D). Together, these data indicate that the Ena–VASP binding site in the Robo CC2 motif is required to mediate both the spatially oriented formation and elongation of filopodia toward a repulsive ligand (Fig. 6, A–C) and the eventual movement of the axon away from the source of the repulsive cue (Fig. 6 D).
Abnormal DRG axon extension toward the dorsal midline by mve axons in vivo

Perturbing filopodia or the interaction of Robo with Ena–VASP proteins produced severe defects in Robo-mediated axon guidance in our in vitro assays, prompting us to examine DRG axon guidance in mve embryos (Fig. 7). To confirm that mve growth cones lack filopodia in vivo, spinal cord explants cultured ex vivo were infected with herpes simplex virus encoding soluble tdTomato. Using this approach, we could clearly trace axonal projections from the DRG and readily observed filopodia on control growth cones inside the DREZ but failed to detect filopodia on mve growth cones (Fig. 7 B).

We performed whole-mount immunofluorescence on fixed embryos to examine axon tracts; horizontal optical sections across the spinal column at the level of the DREZ (Fig. 7 A, gray plane) clearly showed that DRG central projections in embryonic day 10.5 (E10.5) control embryos had made the rostral–caudal turn and formed a prominent lateral axon tract called the oval bundle of His (OBH; Fig. 7, C and D). However, mve embryos exhibited a severely disrupted axonal organization, with DRG central projections aberrantly extending toward the dorsal midline and failing to form a recognizable OBH at E10.5 (Fig. 7, C and D). Although lateral axon tracts formed in mve embryos by E12.5 (Fig. 7 C), the OBH appeared broad and loosely fasciculated (Fig. 7, C and D, compare areas outlined with red dashed lines). Additionally, neurofilament-positive axons could be observed invading the dorsal midline in mve embryos, but not in littermate controls (Fig. 7, C and E; and Fig. S4). Indeed, axons in mve embryos could be observed extending nearly to the posterior median septum and were tipped by growth cones appearing to lack discernible filopodia (Fig. 7, C, vii). The failure of mve axons to properly orient in the DREZ at E10.5, and the aberrant invasion of axons into the dorsal midline at E12.5 (Fig. 7 and Fig. S4), are similar to defects observed for DRG axons in the DREZ of embryos lacking either Slit or Robo (Ma and Tessier-Lavigne, 2007). Consistent with our earlier study implicating Ena–VASP function downstream of Netrin (Lebrand et al., 2004), we also observed evident defects in commissural axon crossing in the ventral midline of mve embryos compared with littermate controls (TAG-1 positive axons; Fig. S4 C). Together, these findings suggest that Ena–VASP proteins are required for proper responses to Slit–Robo signaling in vivo, mirroring the results of our in vitro assays.

Discussion

We conclude that the repulsive guidance cue Slit induces both the formation of DRG growth cone filopodia, as well as a rapid, robust elongation of filopodia, by promoting formation...
of complexes containing the Robo receptor and Ena–VASP proteins. Filopodia preferentially form and elongate toward Slit gradients, and genetically or pharmacologically disrupting filopodium formation or dynamics blocks repulsion away from Slit sources. In vivo, we find that Ena–VASP–deficient growth cones, which lack discernible filopodia, are unable to accomplish the rostral–caudal turn normally made by DRG axons as they enter the DREZ around E10.5, similar to defects observed in embryos lacking either Slit (Slit 1/2−/−) or Robo (Robo 1/2−/−; Ma and Tessier-Lavigne, 2007).

Despite the established dosage-sensitive genetic interactions between mutations in the Robo and Ena–VASP homologs in Drosophila and Caenorhabditis elegans, complete loss of Ena–VASP proteins produces only mild midline axon guidance phenotypes in these invertebrate model systems (Bashaw et al., 2000; Yu et al., 2002) compared with defects observed in embryos lacking either Slit (Slit 1/2−/−) or Robo (Robo 1/2−/−; Ma and Tessier-Lavigne, 2007).

Figure 7. DRG axon guidance defects in mve mutant embryos. (A) Diagram of DRG projections in an embryonic spinal cord. Gray planes indicate the orientation and location of optical sections shown in B and C. (B) Confocal images of spinal cord explants infected with HSV-tdTomato to visualize DRG projections. Filopodia are observed on growth cones in control, but not mve, embryos [compare growth cones marked by red arrowheads in ii and iii]. (C) Confocal micrographs of whole-mount embryos stained with neurofilament antibody to label axons. Only the right half of the spinal cord is shown for clarity. Boxed areas in i and ii are enlarged in iii and iv. OBH is outlined in red dashed lines in C and D. Boxed region in vi is enlarged in vii, revealing a growth cone lacking filopodia near the ventral midline. (D) Schema of axonal projection patterns observed in control and mve embryos at E10.5 and E12.5. (E) Quantification of central projection defects in wild-type (m+/+v+/+e+/+), littermate ([m+/+v+/+e+/+]/[m−/−v−/−e−/−]), and Ena/VASP null ([m−/−v−/−e−/−]) embryos. Thoracic spinal levels in six embryos [corresponding to 156 DRG] were scored for each condition; a spinal level was scored as defective if no recognizable OBH was present [C, iv] or if NF-positive axons extend past the OBH toward the dorsal midline [C, vii]. Bars: [B, i–iii; and C, i–vi] 50 µm; [C, vii] 10 µm. GAP43, growth associated protein 43; NF, neurofilament; DIV, days in vitro; WT, wild type. Circular objects in C are autofluorescent cells.

Actin dynamics in repulsive axon guidance

A common model for axon guidance proposed by many laboratories (including our own) posited that growth cone turning is initiated by opposing actions of attractive and repulsive guidance cues on the actin cytoskeleton: attractive cues promote actin polymerization, whereas repulsive cues cause proximal actin filament disassembly (Dent et al., 2011; Vitriol and Zheng, 2012). This model is intuitively appealing and is based on strong experimental evidence showing that attractive cues promote actin polymerization (Shekarabi and Kennedy, 2002; Lebrand et al., 2004; Tang and Kalil, 2005; Marsiek et al., 2010), whereas bath application of repulsive cues induce actin disassembly and growth cone collapse (Raper and Kaphammer, 1990; Cox et al., 1990; Luo et al., 1993; Fan et al., 1993; Brose et al., 1999; Nguyen Ba-Charvet et al., 1999; Niclou et
Filopodia are required for Slit-Robo-mediated axon repulsion

The regulation of lamellipodia and filopodia downstream of Robo appear to be separable activities: neurons expressing a Met-Robo chimera lacking a functional Ena–VASP binding site fail to elongate filopodia in response to ligand yet still exhibit a ligand-dependent decrease in growth cone area (Fig. 5, B and C). Additionally, Slit treatment of growth cones lacking filopodia elicits a robust decrease in growth cone area similar to controls (Fig. 3, A and B). This decrease in growth cone area is one of the primary attributes of “growth cone collapse,” a descriptive phrase that is frequently used to describe, and assay for, the repulsive activity of guidance cues (Bray et al., 1980; Kaphammer et al., 1986; Cox et al., 1990; Piper et al., 2006; Hata et al., 2009; Yue et al., 2013). However, we find that although growth cones lacking filopodia exhibit signs of “collapse” after Slit stimulation, they exhibit marked defects in Slit-elicited axon retraction (Fig. 4, C and D) and axon repulsion (Fig. 2, D–F). Furthermore, disruption of the Robo-Ena–VASP interaction does not affect collapse (Fig. 5 C), but it does block Robo-mediated effects on filopodium length (Fig. 5, A and B; and Fig. 6, A–C) and axonal repulsion (Fig. 6 D). Thus, collapse of the growth cone lamellipodial veil, a process that normally precedes, or occurs concomitantly with axon repulsion, is insufficient for successful Robo-mediated axon guidance in the absence of Slit-elicited changes in filopodium dynamics.

Why would growth cones elaborate filopodia in response to a repulsive guidance cue such as Slit? One potential function for this behavior is that the area a growth cone samples increases with the square of its radius (Gallo and Letourneau, 2004); thus, increasing the number and/or length of filopodia is an efficient way for migrating axons to sample their environment. For example, the filopodium elongation observed toward Slit gradients (~37%; Fig. 2 C) increased the area sampled by growth cones by nearly 70%. In the context of an extracellular gradient, this increase in sampling area effectively magnifies differences between low and high concentrations, potentially enabling better resolution of shallow gradients. This model has been invoked to explain why growth cones become larger and more “complex” (ratio of perimeter/area) at choice points in vivo (Raper et al., 1983; Tosney and Landmesser, 1985; Waxman et al., 1995). In addition to their potential sensory function, filopodia have also been shown to play key roles in forming adhesions to substrates, directing microtubule exploration of the growth cone periphery, serving as organizers of directional endo- and exocytosis, and as sites of localized signaling (Robles et al., 2003; Dent et al., 2011; Ros et al., 2015). It was recently found that in the case of axonal attraction toward Netrin, extension of filopodia toward a Netrin gradient is regulated by changes in VASP monoubiquitination (Menon et al., 2015). Thus, regulation of filopodia by different guidance receptors can use distinct mechanisms that produce disparate outcomes: filopodial extension induced by Slit is required for repulsion, whereas filopodial extension induced by Netrin causes attraction. A key goal of future studies will be to examine how filopodia integrate these diverse cellular processes in defined spatiotemporal patterns in response to extracellular cues to enable proper axon guidance.

Most of our knowledge of how axon guidance cues function comes from genetic or biochemical screens that identified signaling pathway components and downstream effectors; however, relatively few subsequent studies have examined the contribution of these pathways to regulating growth cone cytoskeleton dynamics at high spatial and temporal resolution. The unexpected finding that Slit promotes the formation and extension of DRG filopodia highlights the need for more detailed study of the regulation of cytoskeleton network dynamics by...
axon guidance cues, particularly those that mediate repulsion. Bringing new approaches and technologies to bear on investigating the dynamics of these signaling pathways promises to yield informative and unexpected insight into the mechanisms regulating nervous system development.

Materials and methods

Western blotting

All reagents were obtained from Sigma-Aldrich unless noted otherwise. Protein samples were resolved with SDS-PAGE using 10% gels (Bio-Rad Laboratories). For immunoblotting, proteins were transferred to nitrocellulose membranes (80 V, 3 h) at 4°C. Stock solutions of antibodies were diluted 1:1,000 in PBS-T (137 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, and 0.1% Tween-20, pH 7.2) before use and detected with Alexa Fluor 680– or 800–conjugated secondary antibodies (1:20,000) imaged using an Odyssey scanning system (LI-COR Biosciences). Primary antibodies used included: anti-GFP (Takara Bio, Inc.), anti-GST (Sigma-Aldrich), anti-Mena219743, anti-Mena219743, anti-β3-tubulin (Promega), anti-Robo1 (Abcam), and anti-neurofilament (EMD Millipore).

Plasmids and reagents

Met-Robo cDNA was kindly provided by Elke Stein (Yale University, New Haven, CT) (Stein and Tessier-Lavigne, 2001). RoboCC domains were amplified from Met-Robo cDNA using Phusion polymerase (New England Biolabs, Inc.). Amplified products were inserted into pC113 (Cheeseman and Desai, 2005; gift from I. Cheeseman, Massachusetts Institute of Technology, Cambridge, MA) between the EcoRI-Sall sites. Mena EVH1 (amino acids 1–115) was amplified by PCR from mouse cDNA and inserted into pGEX2TK (GE Healthcare). Recombinant GST-EVH1 protein was produced in BL-21 Escherichia coli and purified using standard methods. Slt2N and Sema3A were purchased from PeproTech.

EVH1 binding assays

For pull-down experiments, GFP-tagged CC domains from rat Robo1 were expressed in HEK293 cells. Cells were washed with PBS, lysed in 1mL ice-cold lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Nonidet-P 40, and 1 mM Pefabloc) and centrifuged at 18,000 g for 10 min. The supernatant was transferred to a new tube and incubated with 2 µg GST-Mena-EVH1 at 4°C for one hour. A total of 5 µl packed glutathione-agarose resin (Thermo Fisher Scientific) was added and incubated for an additional hour. Resin was pelleted (1,000 g, 5 min), 100,000 cells were resuspended in 20 µl of Amaxa SCN basic neuron buffer to which 1 µg of plasmid DNA had been added. Nucleofection was performed with an Amaxa Nucleofector II device, using program SCN 5.

Immunofluorescence and imaging

Whole-mount neurofilament staining. Embryos were collected at E10.5 or E12.5 and processed according to Hua et al. (2013). In brief, embryos were bleached (60% MeOH, 20% DMSO, and 20% H2O2) at 4°C for 24 h, washed five times in MeOH, and postfixed (80% MeOH and 20% DMSO) at 4°C overnight. Embryos were rehydrated using a MeOH series in PBS dilutions. After rehydration, embryos were incubated in blocking solution (PBS, 0.5% Triton X-100, 20% DMSO, and 5% normal donkey serum) at 4°C overnight. The following day, anti-NF and anti-GAP43 antibodies were added at 1:200 and incubated for 5 d at RT with end-over-end rotation. Embryos were washed at least six times for 1 h in PBS (PBS plus 0.5% Triton X-100) and then incubated with Alexa Fluor–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:500 in blocking buffer and 20% DMSO at 4°C for 24 h, washed five times in MeOH, and postfixed (80% MeOH and 20% DMSO) at 4°C overnight. The following day, anti-NF and anti-GAP43 antibodies were added at 1:200 and incubated for 5 d at RT with end-over-end rotation. Embryos were washed at least six times for 1 h in PBS (PBS plus 0.5% Triton X-100) and then incubated with Alexa Fluor–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:500 in blocking buffer for 1 d. After staining, embryos were washed five times in PBS, dehydrated in EtOH, and either stored at −20°C or imaged after clearing in BABB (33% benzyl alcohol and 67% benzyl benzoate). Optical sections were acquired using a laser scanning confocal (Fluoview 1200; 30x/1.05 NA UPlanApo, Si immersion objective; Olympus).

Live-cell imaging. E12.5 DRG explants were cultured for 12–18 h after plating; explant media was changed to phenol-red–free Leibovitz’s L15 media (Gibco) supplemented with 0.35% BSA before imaging. Differential interference contrast (DIC) videos were acquired on a Nikon Ti microscope equipped with a 37°C environmental chamber, motorized x/y stage, and a Perfect-Focus system to prevent sample drift. Images were acquired using a water immersion objective (40x/1.15 NA Apo WD; Nikon), and a CoolSnap HQ camera (Roper Scientific) controlled by Nikon Elements software. For analysis of growth cone motility and filopodium lifetime, images were acquired every 15 s; for detailed analysis of filopodium protrusion, images were collected every 5 s. Image stacks were corrected for lateral drift in the image plane using the Template Matching plugin for ImageJ (National Institutes of Health, v1.47m; Q. Tseng, Université Joseph Fourier, Grenoble, France). Only growth cones that were not in contact with other cells and had extended more than 0.3 mm away from the explant were chosen for imaging. Quantification of growth cone area (Fig. 3) was performed by manually outlining the growth cone using a Wacom tablet and measured using ImageJ. Growth cone extension or retraction (Fig. 3) was characterized by generating a kymograph along the direction of growth cone movement using the multiple kymograph plugin for ImageJ (J. Rietdof and A. Seitz, European Molecular Biology Laboratory, Heidelberg, Germany); the x/y coordinates of the leading edge over time were manually traced in ImageJ and exported to MATLAB (R2013b; MathWorks). Data were normalized so that the normal alleles of Mena, VASP, and EVL were used as an additional control. Wild-type and littermate (m+/−v−/−e−−)/m+/−v−/−e−−) DRG explants performed similarly in all assays tested.
Growth cone positions were tracked using the Manual Tracking ImageJ
delivering 100-kPa pulses of 16-ms duration at a 0.8-Hz repetition rate.

Slit gradients used the same media with imaging media (L15; Life Technologies); Slit gradients used the same media with the addition of 5 nM Slit-2 (Slit2N; PeproTech); HGF gradients used the same media with the addition of 200 ng/ml HGF (PeproTech). Gradients were generated using a PicospritzerIII (Parker Hannifin) set to deliver 100-kPa pulses of 16-ms duration at a 0.8-Hz repetition rate. Growth cone positions were tracked using the Manual Tracking ImageJ plugin (F.P. Cordelieres, Curie Institute, Paris, France) with the point where the axon inserts into the growth cone used as the point of reference; raw data were exported to MATLAB for analysis.

Online supplemental material
Fig. S1 shows characterization of growth cone turning in response to Slit gradients. Fig. S2 shows evolutionary conservation of Ena/VASP binding sites in Robo receptors. Fig. S3 shows characterization of growth cone collapse and retraction in response to a Slit gradient. Fig. S4 shows axon projections in spinal cord coronal and transverse sections. Video 1 shows a wild-type growth cone undergoing Slit-induced filopodium elongation. Video 2 shows a wild-type growth cone undergoing Sema3A-induced collapse without filopodium elongation. Video 3 shows correlative live-cell DIC and immunofluorescence of a wild-type growth cone undergoing Slit-induced filopodium elongation. Video 4 shows a wild-type neuron undergoing Slit-induced filopodium elongation and neurite retraction. Video 5 shows an Ena/VASP null neuron, which lacks filopodia and does not display Slit-induced filopodium formation or elongation or neurite retraction. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201509062/DC1.

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Author contributions: R.E. McConnell characterized Slit-induced filopodium dynamics and performed growth cone turning assays. R.E. McConnell and M. Vidaki performed biochemical analyses. R.E. McConnell and A.S. Meyer characterized Slit-induced axon retraction. J.E. van Veen performed spine explant and peptide array overlay experiments. R.E. McConnell performed whole-mount embryo imaging analyses. A.V. Kwiatkowski made the initial observation of midline defects in Ena–VASP null embryos. J.E. van Veen made the initial observation of Slit-induced filopodium elongation. R.E. McConnell and F.B. Gertler wrote the manuscript. All authors read and commented on the manuscript.

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