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Cell adhesion and invasion mechanisms that guide developing axons

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Abstract

Axon extension, guidance and tissue invasion share many similarities to normal cell migration and cancer cell metastasis. Proper cell and growth cone migration requires tightly regulated adhesion complex assembly and detachment from the extracellular matrix (ECM). In addition, many cell types actively remodel the ECM using matrix metalloproteases (MMPs) to control tissue invasion and cell dispersal. Targeting and activating MMPs is a tightly regulated process, that when dysregulated, can lead to cancer cell metastasis. Interestingly, new evidence suggests that growth cones express similar cellular and molecular machinery as migrating cells to clutch retrograde actin flow on ECM proteins and target matrix degradation, which may be used to facilitate axon pathfinding through the basal lamina and across tissues.

Introduction

Guidance of axons and dendrites to their correct synaptic target sites is a critical early step for the establishment of a functional adult nervous system and defects in neurite guidance have been attributed to many cognitive deficits [1, 2]. For proper guidance, growth cones at the tips of growing axons and dendrites must interact with molecules that promote or inhibit process outgrowth [3, 4]. A number of families of secreted and cell surface guidance molecules that are required for normal neuronal connectivity have been identified. Extensive research has focused on the molecular mechanisms that regulate the protrusion and retraction of growth cone filopodia and lamellipodia, which are important processes that influence the direction of planar axon outgrowth. Soluble, cell surface and substratum-associated extracellular ligands are known to activate intracellular biochemical signal transduction cascades that regulate motility. Most biochemical signals affect cytoskeletal dynamics and membrane trafficking directly, or indirectly through new protein translation, to control growth cone motility [5, 6]. However, in non-neuronal cell migration, it is widely accepted that the assembly of integrin-based adhesions to the ECM is critical for persistent

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cell migration [7]. Nascent focal adhesions (FAs) form at the leading edge of new protrusions and contain hundreds of proteins, many which are post-translationally modified and exchange as FAs mature. The complexity of FAs reflects the diversity of FA functions, ranging from rapid regulation of cell migration and tissue morphogenesis to long duration transcriptional control of cell differentiation. Growth cones also assemble integrin-based adhesions referred to as point contact (PC) adhesions, which share many components and functions to FAs, but likely serve functions that are unique to axon guidance and neuronal differentiation. A second important parallel recently identified between non-neuronal cell migration and growth cone migration is work showing that neuronal growth cones target MMP activities using invadopodia-like protrusions [8]. As guidance along pathways or entry into tissues likely requires local proteolytic cleavage of ECM molecules and other ligands or their receptors by MMPs [9–13], invadosome-dependent matrix degradation by growth cones is conceptually appealing. While MMP activities are widely studied in non-neuronal and cancer cell migration, very little is known about the role of ligand proteolysis during axon guidance. In this review, we compare recent advances to a deep literature on FAs and invadosomes in non-neuronal cell migration with what is known about growth cone adhesion and matrix remodeling during axon guidance.

Integrin adhesion complex components, dynamics and function

Integrins are transmembrane receptors involved in directional cell migration [14] that physically link cells to the extracellular matrix (ECM). Integrins recruit adaptor and signaling proteins to macromolecular complexes called FAs that connect to the actin cytoskeleton [15–17]. Proteomic techniques have identified hundreds of adaptor and signaling proteins that differentially and dynamically target to integrin adhesions [18], while nanoscopic imaging has begun to reveal their three-dimension organization within cells [19]. The structural composition of FAs is temporally regulated [20] and spatially ordered. First, along the axis of protrusion, phosphorylated paxillin is concentrated at distal tips toward the cell periphery, while actin binding proteins are more highly concentrated proximally where they attach to actin stress fibers [21]. More recent advances in super resolution microscopy have allowed the three dimensional architecture along the 200 nm axial depth of FAs be determined [19, 22]. This architecture can be divided into three nanodomains starting with the integrin signaling layer (ISL) within 10–20 nm of the plasma membrane, followed by the force transduction layer (FTL) and finally the actin regulatory layer (ARL), which begins ~60 nm from the membrane and extends up into the actin stress fibers [22]. These domains provide fine spatial and temporal regulation of protein function within adhesions that depends on mechanical force-induced changes, post-translational modifications and protein-protein interactions [23]. For example, vinculin is one key protein that has multiple FA functions from regulation of maturation to ECM mechanosensing [24, 25], and has over 14 binding partners, which differentially target to all three nanodomains. Extensive research has shown that FAs serve numerous and diverse functions within cells [17]. One of the best understood roles of FAs is the regulation of cell migration on ECM proteins by allowing dynamic linkages with anchored integrin receptors [26]. FA proteins such as talin and vinculin [25] link integrin receptors with the semi-rigid actin cytoskeleton, which restrains F-actin retrograde flow (RF) to power leading edge membrane protrusion [27, 28].

Therefore, the rate of mesenchymal cell migration is inversely proportional to the rate of RF in cells on ECM, such that cells migrate rapidly when RF is slow due to strong clutching to FAs [29].

Growth cone point contact (PC) adhesions regulate axon outgrowth and guidance downstream of soluble cues

Similar to FAs of non-neuronal cells, growth cones form integrin-dependent adhesions with ECM proteins termed point contact (PC) adhesions [30–33] (Fig 1). PC adhesions typically assemble within extending filopodia after integrin receptors bind ECM ligands leading to receptor clustering and recruitment of adhesion proteins such as talin, paxillin, and vinculin [34]. PCs stabilize filopodial protrusions and remain fixed in position during growth cone advance, but turnover quickly in rapidly extending axons [34, 35]. Similar to mesenchymal migration, the formation and dynamic turnover of PC adhesions at the growth cone periphery correlates with the rate of axon outgrowth and is regulated by both the cell substratum and soluble growth factors [4, 36]. Many axon guidance cues are known to operate by regulating integrin-mediated adhesion [37–44]. Moreover, conditions that stimulate the rapid assembly and turnover of growth cone PCs promote fast axon extension and localized assembly and turnover in gradients of growth factors can lead to axon turning [35]. In contrast, cell substrata and guidance cues that prevent PC assembly or turnover, or promote PC disassembly, reduce axon outgrowth [32, 45, 46]. While there has been some tantalizing evidence that integrin-mediated adhesions may control some axon guidance decisions *in vivo* [47–50], the roles of adhesion dynamics in axon guidance *in vivo* is not understood.

Growth cones exhibit F-actin RF similar to cells migrating on ECM [51–53] and clutching of RF is thought to regulate the rate and direction of axon extension [54–59]. Evidence from several laboratories has shown that the rate of RF is directly related to substratum traction forces [60–62] and inversely related to the rate of growth cone advance [54, 63]. However, it is unclear whether the rate of RF depends on PC adhesions and if local clutching to adhesions is responsible for growth cone turning. To address these questions, we recently measured RF in growth cones on a variety of substrata with F-actin speckle microscopy [64]. RF is slowest in neurons cultured on laminin (LN) where rapidly migrating growth cones make many dynamic PC adhesions [34]. Moreover, acute stimulation with LN accelerates axon outgrowth over a time course that correlates with PC formation and reduced RF. We also show that RF is attenuated locally at paxillin-containing PCs and at sites of membrane protrusion. Further, using micro-patterns of PDL and LN, we demonstrate that individual growth cones have differential RF rates while interacting with two distinct substrata. Lastly, we find that treatment of growth cones with soluble axon guidance cues, which increase or decrease PC adhesions, have corresponding effects on RF rates. Together our results suggest that local differences in clutching of RF may control axon guidance on ECM proteins downstream of axon guidance cues. It is also important to note that similar to FAs, it is likely that PC adhesions serve many additional function roles beyond clutching RF, such as regulation of actin polymerization.

Regulation and function of invadosomes as modulators of cell invasion

Podosomes and invadopodia are filopodia-like protrusions that emerge from the apical and basal surfaces of migratory cells that were first described in metastatic cancer cells and later discovered in non-transformed cells [65]. The terms podosome and invadopodia typically refer to protrusions in normal and cancer cells, respectively. While recent evidence suggests that important distinctions exist between invadopodia and podosomes, such as dependence on scaffolding proteins Nck versus Grb2 [66, 67], they are often referred to collectively as invadosomes. Similar to filopodia, invadosomes are composed of an F-actin rich core together with several actin bundling proteins and regulators of actin polymerization (Fig 1). For example, invadopodia contain the actin bundling proteins filamin, fascin and α-actinin; the actin regulatory proteins Cdc42, cortactin, N-WASP and Arp2/3; as well as the kinases Abl/Arg, Src, protein kinase C (PKC) and the lipid phosphatidylinositol-3,4-bisphosphate ($PI(3,4)P_2$); and certain adhesion and growth factors receptors [65, 68–71]. However, invadosomes are distinct from filopodia in several ways. For example, invadosomes are long-lived protrusions that typically form on the basal surface of cells away from the leading edge. In 3D environments, invadosomes penetrate into the underlying ECM to promote cell migration through tissues or 3D matrices. To facilitate ECM degradation, invadosomes target both cell surface and secreted MMPs and ADAMs to their tips [72, 73]. Finally, two key scaffolding proteins that distinguish invadosomes are the adaptor proteins, tyrosine kinase substrate with four SH3 domains (Tks4) and its homolog, Tks5 (also known as SH3PXD2A and Fish).

Conditions that promote assembly, maturation, MMP secretion and disassembly of invadosomes are important unresolved questions. Invadosomes often form near sites of FA disassembly and common components and several signals have been implicated in this transition [74]. Invadosome assembly is induced by growth factors that activate Src, Abl and Arg tyrosine kinases, as well as PI3K and PKC, which may function in cooperation with integrin signaling [70, 71, 75]. Phosphorylation of key invadosome-associated proteins, such as Tks5 and cortactin, initiates invadosome formation, resulting in tissue invasion. Sequential production of phosphatidylinositol lipids may target key factors for invadosome formation and stabilization. $PI(4,5)P_2$ activates actin regulators like N-WASP and cofilin [76], but $PI(4,5)P_2$ is subsequently converted into $PI(3,4,5)P_3$ by PI3K, which stimulates invadosomes through Akt [74, 77]. More recently, the Condeelis laboratory showed that cortactin, N-WASP, cofilin and F-actin arrive together to form an invadosome precursor, followed by Tks5, which stabilizes nascent invadosomes [78]. Targeting of Tks5 occurs via interaction of its PX domain with $PI(3,4,)P_2$, which accumulates at the invadosome core through local SHIP2 5′ phosphatase hydrolysis of $PI(3,4,5)P_3$. Regulation of MMP delivery and secretion is another key step for invadopodia function and this is controlled in part by trafficking of Rab-associated vesicles along microtubules [79], while secretion is mediated by exocytic components like cortactin, Exocyst complex proteins and VAMP7 ([80–82]. Ultimately, invadosomes must disassemble, which was recently shown to involve Rac1 activation of Pak1, which phosphorylates cortactin at Ser113 leading to release of cortactin from F-actin [83].

Many growth factors and chemokines have been shown promote invadosome formation and function on a variety of cell types both *in vitro* and *in vivo*. For example, transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), hapatocyte growth factor (HGF) and stromal derived factor 1α (SDF1α) regulate invadopodia or podosome formation by a variety of transformed cancer cells, as well as normal vascular, smooth muscle and immune cells [76]. However, the conditions of the cellular substrata, such as the composition, density and rigidity of the underlying basal lamina, likely influence how cells respond to growth factors [84, 85], which is consistent with a mechanosensory function of invadosomes [86]. Interestingly, many intracellular signals that modulate invadosome formation and function downstream growth factors are used to control embryonic development, including axon guidance.

Regulation and function of invadosomes as moderators of axon guidance

There has been some experimental evidence for invadosome function in neuronal development *in vivo.* For example, maturation of post-synaptic membrane acetylcholine receptor clusters of developing myotubes involves podosome-mediated ECM remodeling [87] and evidence also indicates that MMPs regulate dendritic spine development [88]. In addition, neural crest cells require invadosomes for proper migration and targeting in developing zebrafish [89]. More recently, we demonstrated that neuronal growth cones in 2D and 3D environments form invadosomal protrusions that contain many essential proteins for invadosome function, such as cortactin and Tks5 [8]. Growth cones from all neuronal types and species examined, including a variety of mouse and human neurons form invadosomes *in vitro* and invadosomes were also observed in *Xenopus* growth cones *in vivo*. Growth cone invadosomes contain dynamic F-actin, several actin regulatory proteins and matrix metalloproteases (MMPs), which locally degrade the matrix. When viewed with 3D super resolution microscopy, F-actin foci often extend together with microtubules within orthogonal protrusions emanating from the growth cone central domain. Finally, inhibiting Tks5 function both reduced matrix degradation *in vitro* and disrupted motoneuron (MN) axons exiting the spinal cord into the periphery. Together, our results suggest that growth cones use invadosomes to target protease activity during axon guidance through tissues.

Many important open questions remain concerning how and where invadosome-dependent activities regulate axon guidance behaviors *in vivo*. There is good evidence that a number of extracellular ligands and receptors are proteolytically cleaved by enzymes expressed on the surface or secreted from growth cones. Studies performed both *in vitro* and *in vivo* suggest that proteolytic cleavage is directed toward specific ligands in the environment, as well as receptors on growth cones to activate or terminate motility [90–96]. For example, the extracellular domain of DCC is cleaved by an unknown metalloprotease, which may terminate or modulate growth cone sensitivities to Netrin and other secreted axon guidance cues [97, 98]. In contrast, kuzbanian is a disintegrin and metalloproteases metalloprotease (ADAM), which cleaves Robo receptors to activate cell signaling to promote axon repulsion [99]. Moreover, recent evidence suggests that MMPs and ADAMs can reveal cryptic sites in molecules or generate mature forms of proteins that are otherwise inert or even switch receptor affinity [100–103]. Invadosomes have been shown to direct the activity of several

types of MMPs, including MMP2, MMP9, MT1-MMP and several ADAMs [8, 65, 73, 104, 105]. Intriguingly, each of these proteases have been linked to axon outgrowth and guidance functions [13, 93, 94, 99, 106–109].

Our findings suggest that several classes of spinal neurons may using invadosomes to target MMP activities during axon guidance (Fig 2A). For example, MN axons use invadosomes to exit the spinal cord (Fig 2B) [8] and we suspect the nascent peripheral processes of Rohan Beard (RB) sensory neurons also use invadosomes to target MMP degradation of ECM proteins for exiting (Fig 2A). In addition, *in vivo* imaging shows that RB growth cones in the skin form peripherally directed protrusions that resemble invadosomes (Fig 2d), but the function of these protrusions is not known [8]. Interestingly, we also observe F-actin foci within commissural interneuron (CI) growth cones at the midline (Fig 2C) [8, 110], which may be invadosomes that are necessary to detect or terminate sensitivities to midline cues. 3D imaging of CI growth cones may resolve if F-actin foci are protrusions that extend dorsally into the floor plate or ventrally toward the notochord. It is not clear what environmental factors promote or stabilize invadosomes to support tissue invasion or signaling detection. However, a number of growth factor, chemokines and ECM proteins found in the environment of developing neurons activate SFKs, protein kinase C (PKC) and PI3K [4, 111, 112], which may phosphorylate several key invadosome-associated proteins and lipids to promote invadosome formation (Fig 1). In the future, it will be important to determine the specific extracellular factors that control invadosome assembly, stabilization and function *in vivo* and identify both the location of invadosome activity and targets of MMP proteolysis.

Conclusions

While many thousands of studies over the last 4 decades have examined the molecular basis of axon guidance, our understanding of how the immense complexity of the nervous system forms so reliably is far from complete. It is clear that many diverse intracellular signals are activated within growth cones by adhesion and guidance cue receptors to control cytoskeletal and membrane dynamics. Less is understood about how secreted guidance cues and growth factors regulate adhesive interactions of growth cones with the basal lamina and other cells. Most cells use actin-based protrusion to power cell migration and it is clear that growth cone migration and guidance share many common mechanisms. For example, migrating non-neuronal cells assemble dynamic integrin adhesions to the ECM with complex molecular architecture and diverse functions. Growth cones assemble similar integrin adhesions that are regulated by axon guidance cues, but much remains unknown regarding how point contact adhesions are regulated and the diversity of their functions. More recently, we found that growth cones also form apical and basal protrusions that resemble invadopodia of invasive cells in many ways. We showed that growth cones form actin rich protrusions from their central domain that target MMP-mediated ECM degradation and are necessary for proper MN exiting the spinal cord. While the importance of MMP activities in developing neurons has been known for many years, the molecular mechanisms involved in MMP targeting by growth cones have not been appreciated.

The identification of invadosome-like protrusions on growth cones provides a new molecular target of control by axon guidance cues. Regulation of axon guidance through 3D space and across tissue barriers is poorly understood and many open questions remain. For example, what conditions support growth cone invadosome formation, maturation and turnover? We suspect that specific growth factors or chemokines secreted from distal sources likely promote the disassembly of point contact adhesions and redirect actin regulatory signals toward invadosomal protrusions. It is also unclear the specific MMPs and ADAM proteins targeted to growth cone invadosomes, their targeting mechanisms and the ligands that are cleaved or degraded by MMPs.

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Highlights

- Compare and contrast fibroblast focal adhesions with growth cone point contact adhesions.
- Compare and contrast invadosomes of invasive cancer cells with analogous protrusions recently found on growth cones.
- Discuss important future directions.

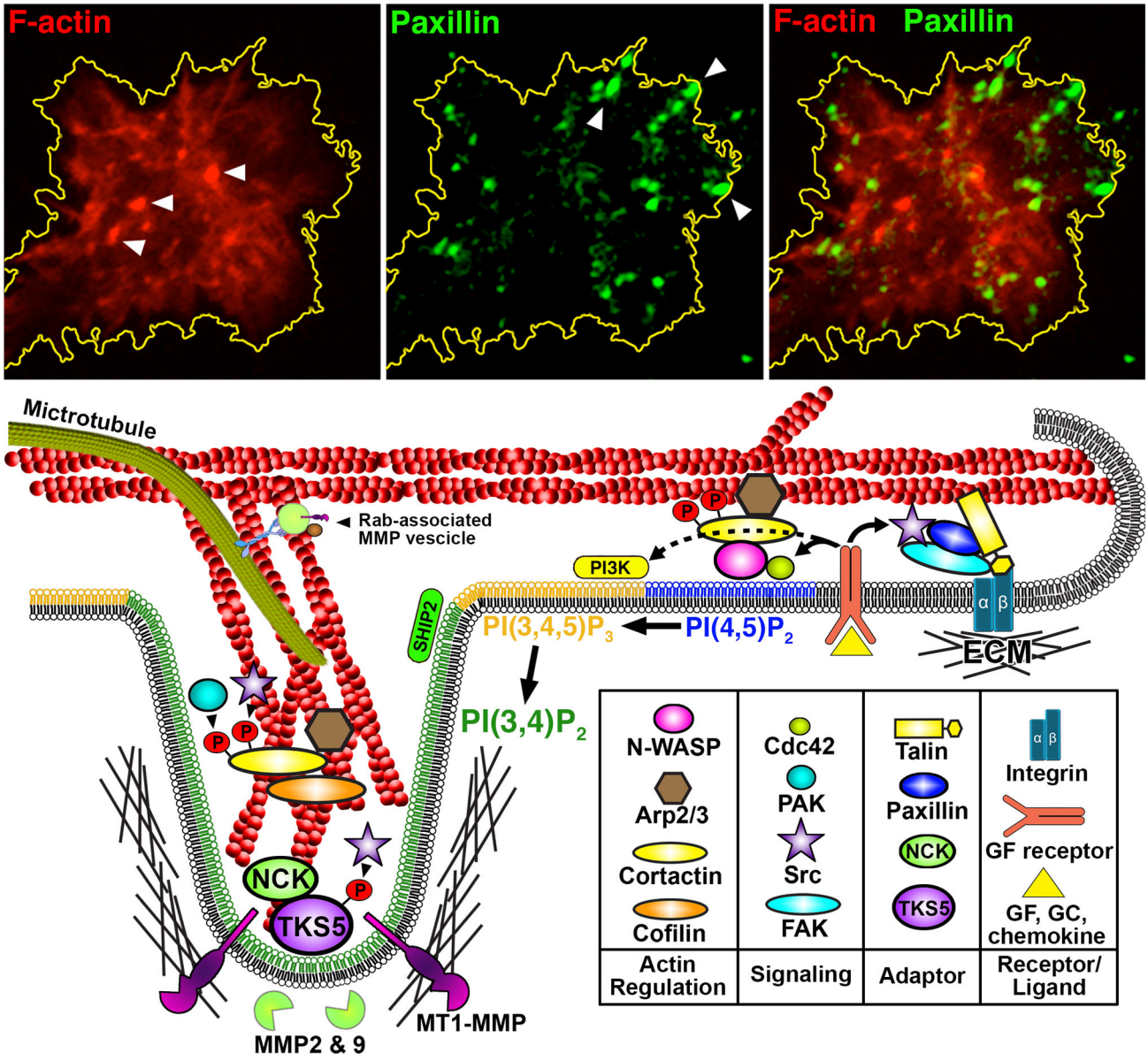

Figure 1. Regulation of axon guidance by ECM adhesion and degradation

A representative neuronal growth cone (above) labeled for F-actin (red) and paxillin (green), which indicate invadosomes in the central domain (F-actin, arrowhead) and peripheral point contact adhesions (paxillin, arrowhead), respectively. The schematic drawing below shows common signaling pathways that regulate peripheral point contact adhesions and basally directed invadosomal protrusions. Integrin adhesions and invadosomes are regulated by extracellular signals from ECM proteins, growth factors (GF), guidance cues (GC) and chemokines in the environment. Receptors on growth cones activate signaling pathways that modulate adhesion site dynamics and peripheral actin polymerization to support planar axon extension upon the ECM. However, activation of key lipid modulators and other signaling pathways by specific GFs likely redirects actin polymerization regulators and adaptor proteins toward basal invadosomal protrusions. Mature invadosomes are targeted by

microtubules, which support Kinesin motor and Rab-dependent trafficking of MMP containing vesicles. Targeted secretion of MMPs leads to local degradation of ECM, allowing developing axons to penetrate ECM barriers to cross into new tissues. Image by Miguel Santiago-Medina.

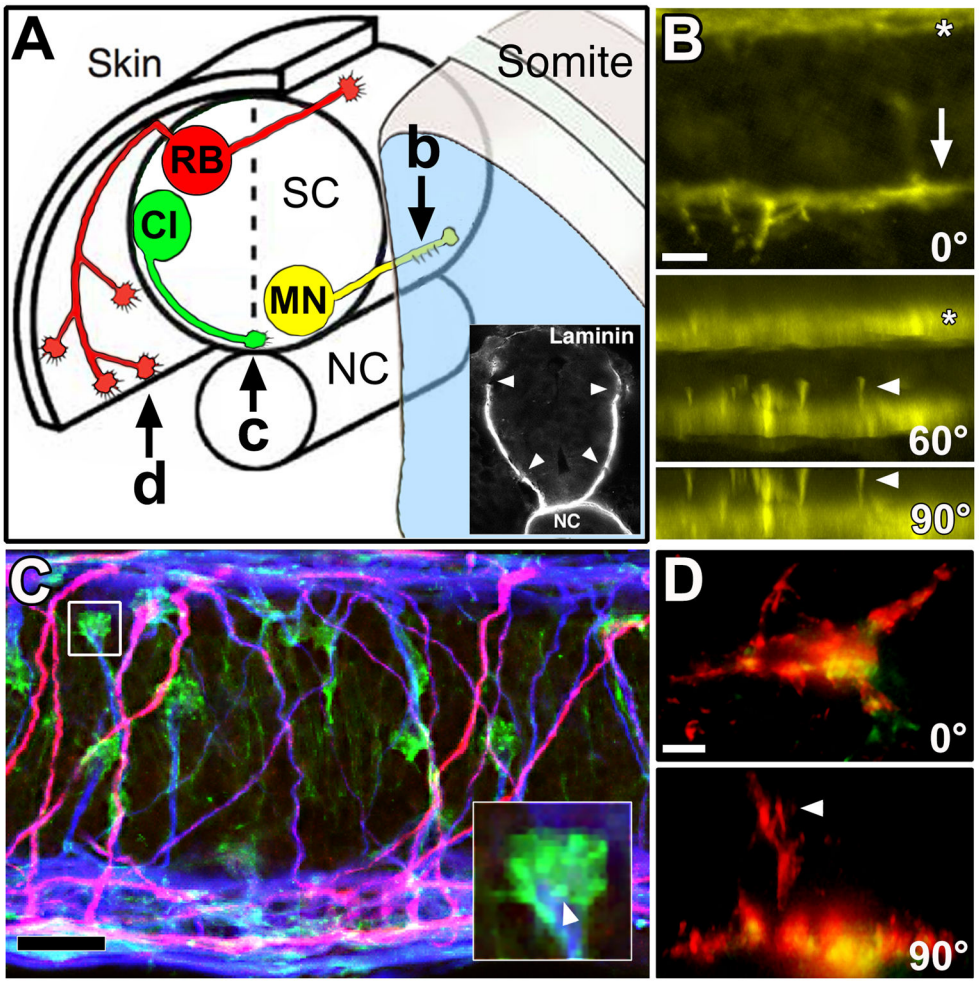

Figure 2. Potential sites of invadosome-mediated axon guidance of spinal neurons

A. Schematic drawing of the developing *Xenopus* spinal cord (SC) with Rohan Beard (RB) sensory neurons with peripheral process in the skin, a commissural interneuron (CI) with a growth cone at the midline and a motoneuron (MN) with a central axon prior to branching into the periphery. Inset shows LN ensheathing the spinal cord. Note, breaks in LN labeling at RB and MN exit points (arrowheads). NC=notochord. **B–D**. Immmunolabeled processes at each position indicated in (A). **B**. Confocal 3D rendering of the lateral surface of the spinal cord labeled for Synaptotagmin-2 (yellow) displayed at 3 different projection angles. Asterisks indicate the dorsal fascicle. Arrow indicates a MN terminal growth cone, while arrowheads in x-axis rotations indicate invadosomal protrusions along MN axon. Scale, 10 μm. **C.** Confocal Z-series projection of the ventral surface of the spinal midline labeled for F-actin (green), neurofilaments (red) and MTs (blue). Inset, 3X zoom. Note F-actin foci in CI growth cones at midline (arrowhead). Scale, 20 μm. **D.** Confocal 3D rendering of RB peripheral process growth cone in the skin labeled for N-CAM (red) and cortactin (green). Side view of the growth cone shows an apical protrusion (arrowhead) extending toward the peripheral skin. Scale, 3 μm.