

Rac proteins and the control of axon development

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Rac GTPases and their effectors control cellular morphogenesis in a wide range of developmental contexts by regulating the structure and dynamics of the actin cytoskeleton. Although much is known about the biochemistry of Rac and Rac regulators, less is known about how Rac control cellular morphogenesis, including axon development, *in vivo*. Recent loss-of-function genetic studies using model organisms have shown that Rac and their effectors are required for multiple aspects of axon development, including axon outgrowth, axon guidance and axon branching. Interestingly, these studies have also revealed that Rac activity is required to prune spurious axons and branches. Analyses of Rac and their upstream and downstream effectors suggest that Rac signaling is complex. Different neurons utilize distinct combinations of upstream Rac regulators during axon development, possibly reflecting responses to different axon path-finding signals, and Rac use distinct downstream effectors to mediate different aspects of axon development, possibly reflecting differential regulation of the lamellipodial and filopodial growth-cone actin-cytoskeleton domains underlying axon developmental events.

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Abbreviations

Arp2/3	actin-related protein 2/3
CDM	CED-5, DOCK180, Myoblasticity
CNS	central nervous system
CRIB	Cdc42, Rac interactive binding
DH-GEF	disabled-homology GTP-exchange factor
GAP	GTPase-activating protein
GEF	GTP-exchange factor
LAR	leukocyte common antigen-related
LIMK	LIN-11, Islet-1, MEC-3 domain kinase
Mtl	MIG-2-like
Pak	p21-activated kinase
PDE	posterior deirid neuron
RPTP	receptor tyrosine phosphatase
SCAR	suppressor of cAMP receptor
SH	Scr homology
VNC	ventral nerve cord

Introduction

In nervous-system development, axonal projections from neurons extend long distances and make precise contacts with specific targets. Axons are guided to their targets by growth cones, dynamic motile structures at the distal tips of developing axons [1]. When extracellular guidance cues are detected by transmembrane receptors present on the growth-cone plasma membrane, a signal transduction cascade is initiated that leads to alterations in lamellipodial and filopodial actin-cytoskeleton morphology and to dynamics that mediate growth-cone outgrowth and steering in lamellipodia and filopodia, respectively [2,3].

Rac small GTPases of the Rho subfamily, which also includes Rho and Cdc42, control the structure and dynamics of the actin cytoskeleton [4]. Like other Ras-superfamily GTPases, Rac cycle between a GTP-bound active state and a GDP-bound inactive state. GTP-bound Rac hydrolyze GTP to GDP, rendering themselves inactive [5]. Rac activity is regulated by GTP exchange factors (GEFs), molecules that catalyze the exchange of GDP for GTP and activate Rac, and GTPase-activating proteins (GAPs), molecules that stimulate Rac GTPase activity and inactivate Rac [6].

Previous work has shown that gain-of-function constitutively active and dominant-negative Rac molecules perturb axon pathfinding and neuronal morphogenesis *in vivo* [7]. This review will focus on recent studies of loss of Rac activity that demonstrate that Rac and their upstream regulators and downstream effectors are normally required for multiple aspects of axon development *in vivo*.

Rac mediate axon guidance, outgrowth and branching as well as suppression of ectopic axon formation

The genomes of multicellular animals contain multiple Rac-like genes that function redundantly in many morphogenetic events including axon development [8^{••}–10^{••},11[•],12^{••}]. Rac-like molecules can be grouped into two families: the canonical Rac GTPases and the MIG-2-like (Mtl) GTPases. Mtls are similar to both Rac and Cdc42 and might define a new Rho GTPase family [13]. However, observations that Mtls have functional overlap with canonical Rac and that canonical Rac and Mtls share common regulators (see below) suggest that Mtls can be considered to be members of the Rac family [8^{••}–10^{••},11[•],12^{••}]. An overview of Rac nomenclature in *Caenorhabditis elegans* and *Drosophila* is given in Table 1.

Loss-of-function genetic studies in *C. elegans* and *Drosophila* demonstrate that Rac are required for multiple

Table 1

Rac nomenclature in *C. elegans* and *Drosophila*.

<i>C. elegans</i> Racs	
CED-10	Similar to canonical Racs
RAC-2/3*	Similar to canonical Racs
MIG-2	Member of the MIG-2-like Rac-related family
<i>Drosophila</i> Racs	
Rac1	Similar to canonical Racs
Rac2	Similar to canonical Racs
Mtl	MIG-2-like, member of the MIG-2-like Rac-related family

*The *rac-2/3* locus is a direct duplication of a *rac* gene resulting in two coding regions that can give rise to the nearly identical proteins RAC-2 and RAC-3 [8**].

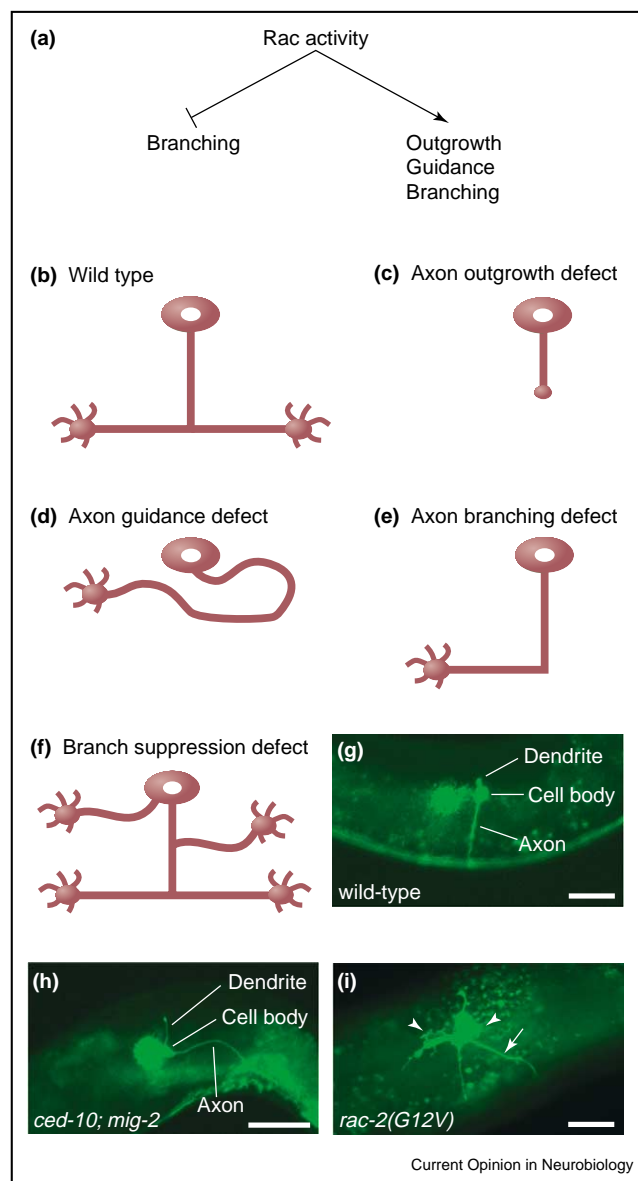
aspects of axon development including axon outgrowth, axon guidance and axon branching, as well as for the suppression of spurious axon branching (Figure 1). In *C. elegans*, two canonical Racs, CED-10 and RAC-2/3, and one Mtl, MIG-2, have overlapping roles in axon development: reduction of function of any one gene results in few axon defects whereas each pairwise double mutant combination displays synthetic axon defects [8**,14*]. *Rac* double mutants display defects in axon pathfinding, including premature axon termination and axon misguidance [14*]. In *Drosophila*, two canonical Racs, Rac1 and Rac2, and one Mtl also have partially overlapping roles in axon guidance and outgrowth [9**,10**] and are also required for normal axon branch formation [9**]. Branch formation defects are also seen in *C. elegans* *Rac* double mutants (EA Lundquist, unpublished data). These studies of loss of Rac function indicate that Racs are required for axon guidance, outgrowth and branch formation. *C. elegans* *Rac* double mutants also display ectopic axons and axon branches [8**,14*], suggesting that Racs are normally involved in the suppression of superfluous axon and branches (Figure 1).

Racs independently control multiple aspects of axon development

In *C. elegans*, each *Rac* double mutant displays the entire spectrum of axon defects described above [14*]. By contrast, Rac activity in *Drosophila* appears to be dose-sensitive, as sequential removal of Rac function reveals progressively different defects in axon development: branch formation is the first to be disrupted, followed by guidance and finally outgrowth [9**]. These *Rac* mutant defects reflect distinct Rac-controlled processes rather than sequentially more severe deficits in the same process. The different *rac* mutant defects appear independently

(Figure 1 Legend) Racs are required for multiple aspects of axon development. (a) Rac activity promotes axon outgrowth, guidance and branch formation and inhibits the formation of ectopic axons and axon branches. (b–f) Axon defects caused by *Rac* loss of function are represented in schematics of the *C. elegans* posterior deirid neuron

Figure 1



(PDE). (b) In the wild type, the PDE axon grows ventrally to the ventral nerve cord (VNC), where the axon branches and extends anteriorly and posteriorly in the VNC. (c) Foreshortened axons indicate axon outgrowth defects. (d) Axons that retain the ability to extend but that are misguided (i.e. fail to reach the VNC and wander laterally) indicate axon-guidance defects. (e) Axons that fail to branch at the VNC indicate axon-branching defects. (f) The presence of extra axons and axon branches in *Rac* mutants indicates that Racs are required to suppress the formation of spurious axons and branches. (g–i) The micrographs show *C. elegans* PDE neurons visualized with green fluorescent protein. (g) A wild-type PDE neuron. A single unbranched axon extends ventrally to the VNC, where it branches and extends anteriorly and posteriorly (out of focus). (h) A PDE neuron from a *ced-10(n1993); mig-2(mu28)* *rac* loss-of-function double mutant. The axon is misguided and extends posteriorly instead of ventrally to the VNC. (i) A PDE from an animal with neuron-specific expression of constitutively active RAC-2 harboring the G12V mutation. The neuron displays an ectopic axon (arrow) as well as ectopic lamellipodial and filopodial structures (arrowheads). Scale bars in (g–i) represent 10 micrometers.

of one another in both *Drosophila* and *C. elegans* [9**,14*]. For example, in *C. elegans*, misguided and terminated axons are apparent both with and without ectopic axon branches, and in *Drosophila*, branch formation defects occur in the absence of outgrowth or guidance defects. More convincing is the observation that Rac's utilize distinct downstream effectors to mediate different axon development events. In *Drosophila*, CRIB-domain-containing Rac downstream effectors (where CRIB stands for 'Cdc42, Rac interactive binding') such as p21-activated kinase (Pak) are involved in axon guidance and branch formation and but not axon outgrowth [9**]. Thus, Rac activity is apparently involved in multiple, separate processes during axon development, including axon outgrowth, axon guidance, and axon branch formation as well as in the suppression of ectopic axon branches.

The conclusions drawn from the *Rac* loss-of-function studies are supported by analyses of dominant, constitutively active Rac mutants. The glycine-12-to-valine (G12V) mutation, which is canonical for constitutive activation of all Ras-superfamily GTPases, results in a molecule that cannot hydrolyze GTP and is therefore constitutively active [5]. In *C. elegans*, neuron-specific expression of mutant Rac(G12V) molecules results in extensive axon branching and the formation of ectopic lamellipodia and filopodia [14*], possibly reflecting the role of Rac activity in the formation of structures that are normally involved in axon guidance, outgrowth and branching. Furthermore, Rac(G12V) activity causes weak axon outgrowth and guidance defects [14*], possibly reflecting the normal role of Rac in the suppression of growth-cone structures. Thus, apparent loss-of-function defects induced by constitutively active Rac's might reflect the role of Rac's in both the formation and the suppression of actin-based morphogenetic structures.

Racs act downstream of multiple guidance receptors

Axon guidance signals are detected by transmembrane receptors on the growth-cone plasma membrane. Many guidance receptors and their ligands have been identified [3], two of which have recently been shown to act upstream of Rac signaling in axon development. The cytoplasmic tail of the semaphorin receptor Plexin B binds directly to Rac-GTP and inhibits Rac activity by sequestering Rac-GTP away from its effector Pak, contributing to Plexin-B-mediated axon repulsion [15,16]. Furthermore, in *C. elegans*, mutation of the *Rac* gene *ced-10* suppresses the effects of an activated form of the netrin receptor UNC-40 on axon development, indicating that CED-10 Rac acts downstream of UNC-40 [17*]. Interestingly, CED-10 Rac but not MIG-2 acts downstream of UNC-40, and CED-10 Rac acts in parallel to a second redundant pathway downstream of UNC-40 involving the cytoskeletal effector molecule UNC-34 Enabled [17*]. Thus, Rac's can have redundant functions with one

another as well as with other cytoskeletal signaling pathways that regulate axon development.

The UNC-73 Trio GTP-exchange factor is a key regulator of Rac activity in axon development

Although Rac's can interact directly with the cytoplasmic tail of the Plexin B guidance receptor, other receptors undoubtedly utilize intermediate adaptor molecules to control Rac activity. Candidates for such adaptor molecules include the GEFs of the disabled-homology family (DH-GEFs). Recent studies implicate the UNC-73/Trio DH-GEF as a key regulator of Rac activity during axon development. In *Drosophila* and *C. elegans*, mutations in *unc-73/trio* lead to axon defects that resemble *Rac* loss-of-function [8**,14*,18–22]. *Racs* and *unc-73* interact genetically in *C. elegans*: weak *unc-73* mutations are enhanced by mutations in each of the three *racs*, indicating that UNC-73 acts with all three Rac's in axon development [8**,14*]. Furthermore, *unc-73* mutations are suppressed by Rac overactivation, indicating that Rac's act downstream of *unc-73* [12**,14*]. Moreover, overactivation of the Rac-GEF domain of *Drosophila Trio* is suppressed by *Rac* loss-of-function [10**]. Additionally, biochemical evidence from vertebrates, *C. elegans* and *Drosophila* indicates that UNC-73 Trio acts as a GEF for both canonical Rac's and Mtls [12**,21,22]. Together, these results clearly define UNC-73 Trio as a key regulator of Rac activity in axon development. UNC-73/Trio probably controls the Rac's in many aspects of axon development, as *C. elegans unc-73* mutants display the full range of axon development defects (outgrowth, guidance, branch formation and branch suppression defects), and each of these defects is enhanced by *rac* loss of function [14*].

Human Trio interacts with the cytoplasmic tail of a LAR receptor tyrosine phosphatase (RPTP) [23]. Mutations in *Drosophila Dlar*, which encodes a LAR RPTP, cause axon path-finding defects and enhance *Trio* mutations [18,24], suggesting that Trio acts downstream of Dlar RPTP in axon path-finding. Whether UNC-73 Trio acts downstream of other guidance receptors remains unclear. Unlike *ced-10* mutations, *unc-73* mutations do not suppress the effects of an activated UNC-40 guidance receptor [17*], suggesting that UNC-73 does regulate CED-10 Rac downstream of the netrin receptor UNC-40 in *C. elegans* ventral axon path-finding. However, this experiment is complicated by the fact that the *unc-73* mutations used were not null, leaving open the possibility that UNC-73 might participate with CED-10 downstream of UNC-40.

A combinatorial model of Rac regulation during axon development

A complex model of Rac function in axon development is emerging. First, although UNC-73/Trio probably controls multiple Rac's in axon development, other upstream regulators control specific Rac's. For example, CED-5, a

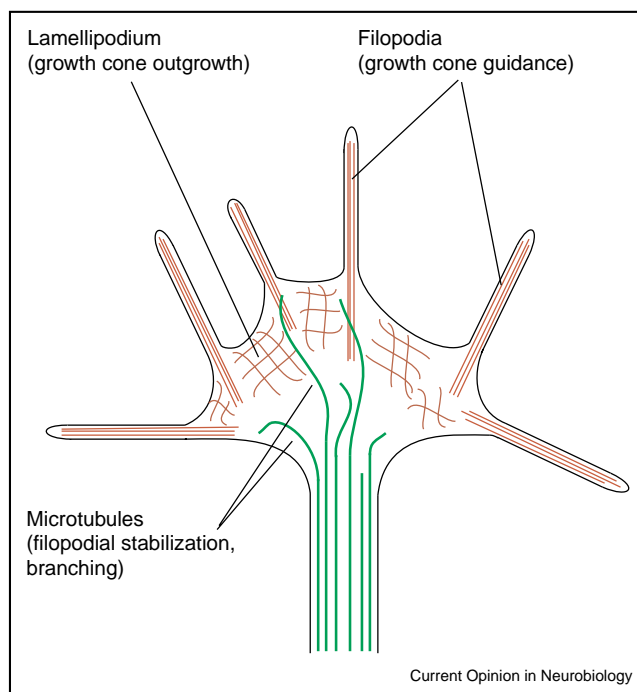
CDM family member that is thought to modulate Rac GTP exchange, acts in the MIG-2 pathway but not in the CED-10 or RAC-2/3 pathways in *C. elegans* axon development [8**,12**,25]. Second, Rac might use different regulators in response to different axon-guidance cues and receptors. The CDM protein CED-5 and the CrkII-like SH2-SH3 domain containing the receptor-adaptor protein CED-2 act with MIG-2 in motor-neuron axon development but not in amphid sensory-neuron axon development in *C. elegans* [12**]. Thus, Rac activity in different axon path-finding events is controlled by distinct combinations of guidance receptors and Rac regulators. An important future task will be to decipher the combinations of molecules that regulate Rac during axon development as well as to determine how these signaling pathways relate to the different guidance-receptor systems that mediate axon development.

Racs might control distinct growth-cone actin-cytoskeleton domains during axon development

The morphogenetic effects of Rac molecules are mediated in part by their regulation of the structure and dynamics of the actin cytoskeleton [4]. The growth-cone actin cytoskeleton (Figure 2) consists of multiple domains that mediate different aspects of growth-cone outgrowth (see Figure 2; [2]). The lamellipodial actin cytoskeleton consists of a network of branched and cross-linked actin filaments that is thought to mediate growth-cone motility and outgrowth, and the filopodial actin cytoskeleton consists of bundled actin filaments that protrude into the filopodial extensions and is thought to mediate growth cone steering in response to guidance signals [2,26]. Additionally, axon-shaft microtubules extend into the growth cone and interact with filopodial actin bundles in a process that is thought to stabilize filopodia and consolidate outgrowth decisions [27,28].

Rac mutations affect both axon outgrowth and axon guidance, suggesting that Rac controls both the lamellipodial and filopodial actin cytoskeleton of the growth cone. Rac is also required for suppression of ectopic axons and branches. Studies in *C. elegans* indicate that a migrating growth cone at a guidance choice-point extends multiple filopodia, all but one of which is retracted before outgrowth proceeds along the remaining filopodium [29]. When filopodial retraction is blocked by mutation of the *unc-119* gene, ectopic axon branching results [30]. Ectopic axons observed in *Rac* mutants could be the result of a failure in filopodial retraction, which might indicate that Rac normally plays a role in the destabilization of filopodia. Rac might control the formation or collapse of axons via the Rac-GTP/GDP cycle: Rac-GTP might mediate the formation of axons whereas Rac-GDP might control their collapse. However, constitutively active Rac causes defects in axon guidance and outgrowth, suggesting that Rac-GTP activity controls both axon formation and collapse.

Figure 2

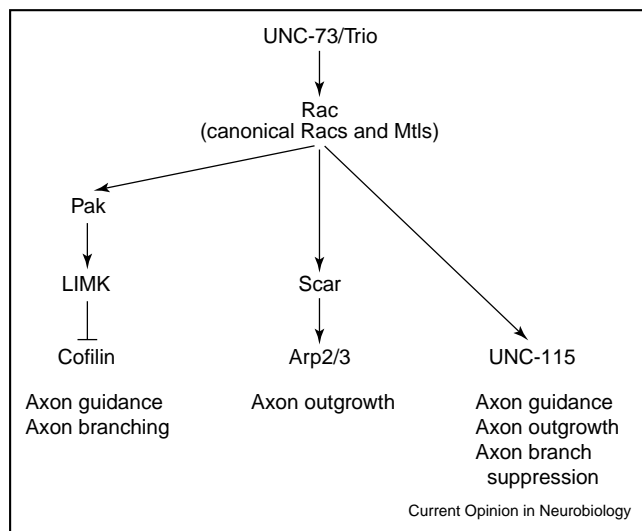


Racs might influence multiple cytoskeletal domains of the growth cone during growth cone outgrowth, guidance and branching. Actin filaments are red and microtubules are green. The growth cone lamellipodium, which contains a branched network of actin filaments, mediates growth cone outgrowth. Growth cone filopodia, which contain bundles of actin filaments, mediate growth cone steering. Axon shaft microtubules interact with and stabilize specific filopodia, which consolidates guidance decisions and mediates axon branching. Microtubules are also involved in the formation of secondary growth cones from existing axons. Rac molecules might control these different cytoskeletal domains during different aspects of axon development.

Rac control of the CRIB-domain effector Pak might mediate axon guidance

Genetic studies suggest that distinct downstream Rac effectors are used to regulate different axon development events. *Drosophila* Rac1 carrying the Y40C mutation, which blocks Rac interaction with CRIB-domain-containing effectors such as Pak, can rescue axon outgrowth defects in *Rac* mutants but not guidance or branch formation defects [9**], indicating that Rac interaction with this type of effector is not required for axon outgrowth but is required for axon guidance and branch formation (Figure 3). Pak, a serine/threonine kinase, is activated by Rac [31,32] and is involved in *Drosophila* axon development [33]. Pak activity stimulates LIM kinase (LIMK), which in turn phosphorylates and inhibits the actin-filament-severing and depolymerizing protein cofilin [34-36]. Cofilin activity has been shown to stimulate neurite extension [37]. Together, these results indicate that Rac might utilize Pak to influence the growth-cone actin cytoskeleton during axon guidance and branch

Figure 3



Rac activity might influence the actin cytoskeleton by multiple pathways. UNC-73/Trio, a Rac-specific DH-GEF, controls Rac activity, including both canonical Rac and Mtl, during axon development. *Drosophila* Rac1 might utilize the CRIB-domain-containing effector Pak to control axon guidance and branch formation. Pak activates LIMK, which phosphorylates and inhibits the actin-severing and depolymerizing protein cofilin. The Arp2/3 complex and one of its activators, SCAR, are involved in *Drosophila* axon development. The Arp2/3 complex nucleates actin filament branches from preexisting actin filaments and is involved in formation of the lamellipodial cytoskeleton, suggesting that Arp2/3 activity might be involved in axon outgrowth. To date, no interaction of Rac activity with SCAR or the Arp2/3 complex during axon path-finding has been found. However, Rac activates SCAR *in vitro*, suggesting that Rac activity might also regulate SCAR and the Arp2/3 complex during axon path-finding. The F-actin binding protein UNC-115 acts downstream of Rac signaling during axon guidance, outgrowth and branch suppression in *C. elegans*. Interestingly, UNC-115 might act downstream of the canonical Rac CED-10 and RAC-2/3 and not MIG-2, the Mtl Rac. Although the activity of UNC-115 on actin dynamics is not well understood, it is intriguing to speculate that UNC-115 modulates lamellipodial and filopodial dynamics in response to Rac signaling during axon guidance, outgrowth and branch suppression.

formation via LIMK and cofilin (Figure 3). Pak activity might be involved specifically in filopodial dynamics, as CRIB domain Rac effectors are important in axon guidance and branch formation but not in axon outgrowth. Pak is not the only CRIB-domain effector of Rac; another one, such as the insulin-receptor substrate p53 [38], might act downstream of Rac in axon guidance and branch formation.

Rac regulation of the actin-related protein 2/3 complex might mediate axon outgrowth

A distinct downstream cytoskeletal effector of Rac is the actin-related protein 2/3 (Arp2/3) complex, a seven-molecule conglomerate that nucleates actin filaments from the sides of preexisting actin filaments [39,40]. Arp2/3 activity is thought to contribute to the branched actin network of lamellipodia [41,42]. Several molecules that activate

Arp2/3-dependent actin nucleation have been identified, and canonical Rac activity stimulates at least two of these, Suppressor of cAMP receptor (SCAR) and cortactin, *in vitro* [42,43]. SCAR is a member of the WASP-homology-2/central/acidic-region family of Arp2/3 activators (where WASP stands for Wiscott–Aldrich syndrome protein). Mutations in *Drosophila* Scar and in *Arp3* and *Arpc1*, which encode components of the Arp2/3 complex, cause axon defects in the CNS, including axon displacement and failure to form axon commissures [44], indicating that SCAR and the Arp2/3 complex are involved in axon development. Although a direct functional link between Rac and the Arp2/3 complex has not been made *in vivo*, Scar, Arp3 and Arpc1 defects resemble those caused by Rac mutation in the CNS [10,44]. It might be that the Arp2/3 complex modulates lamellipodial actin dynamics and growth-cone outgrowth in response to Rac signaling (Figure 3).

Rac regulation of UNC-115/abLIM might mediate multiple aspects of axon development

Recent experiments in *C. elegans* have identified an additional cytoskeletal effector downstream of Rac: the F-actin-binding protein UNC-115, which is similar to vertebrate abLIM. *unc-115* mutations are associated with low-penetrance axon-guidance defects and ectopic axon branching [14,45]. Furthermore, a dominant-negative form of abLIM/Limatin perturbs retinal ganglion cell axon path-finding in the vertebrate visual system [46]. Genetic analyses in *C. elegans* indicate that UNC-115 acts in the canonical Rac pathways but not in the MIG-2 Mtl pathway to mediate axon outgrowth and guidance [14]. In fact, UNC-115 function is required for the ectopic lamellipodia and filopodia induced by constitutively active RAC-2 [14], indicating that UNC-115 is normally required for the Rac-dependent formation of lamellipodia and filopodia. UNC-115 is an actin-binding protein, but the precise effect of UNC-115 on actin dynamics is not understood. It is intriguing to speculate that UNC-115 might modulate lamellipodial and filopodial actin dynamics in response to Rac signaling during axon guidance, outgrowth and branch suppression (Figure 3).

Do Racs regulate microtubule dynamics in axon development?

Some aspects of axon development that are dependent on Rac also involve microtubules. As diagrammed in Figure 2, axon-shaft microtubules extend into the growth cone where they are thought to interact with and stabilize filopodial actin bundles. This interaction might consolidate guidance and outgrowth decisions and mediate growth-cone branching [27,28]. Furthermore, microtubules are involved in secondary growth-cone initiation, which leads to axon branching [47,48]. A direct link between Rac signaling and microtubules is suggested by the IQGAP protein, a Rac GAP with an IQ domain (IqxxxRGxxxR)

that also interacts with CLIP-170 and stimulates CLIP-170-mediated microtubule-end-capturing activity [49,50]. Furthermore, Pak phosphorylates and inhibits the microtubule catastrophe factor stathmin in response to Rac signaling [51]. The roles of IQGAP and stathmin in axon development remain to be determined, but it might be that IQGAP and stathmin influence growth-cone microtubules in response to Rac signaling during axon outgrowth, guidance and branch formation.

Conclusions

Rac GTPases have for some time been known to regulate the actin-cytoskeleton dynamics underlying cellular morphogenesis. The work summarized here represents the early stages in the dissection of the roles of Rac and their upstream regulators and downstream effectors in axon development. The emerging picture is complex. Multiple Rac molecules have functional overlap with one another and with other cytoskeletal signaling pathways during axon development. Furthermore, distinct combinations of upstream guidance receptors and cytoplasmic Rac regulators control the Rac in different axon path-finding events. Finally, Rac might utilize different downstream cytoskeletal effectors to influence the distinct filopodial and lamellipodial actin-cytoskeleton domains involved in axon development. An important future endeavor will be to identify and characterize the different combinations of guidance receptors and Rac regulators involved in specific guidance decisions and to understand the molecular mechanisms by which Rac mediate growth-cone actin-cytoskeleton change in response to guidance signals. Also, it will be important to understand how Rac interact with other cytoskeletal signaling pathways, particularly the other Rho GTPases Rho and Cdc42, during growth-cone path-finding in the developing nervous system.

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