Axon guidance: the cytoplasmic tail
Bharatkumar N Patel* and David L Van Vactor†

Recent advances in the study of axon guidance have begun to clarify the intricate signalling mechanisms utilised by receptors that mediate path-finding. Many of these axon guidance receptors, including Plexin B, EphA, ephrin B and Robo, regulate the Rho family of GTPases, to effect changes in motility. Recent studies demonstrate a critical role for the cytoplasmic tails of guidance receptors in signalling and also reveal the potential for a great deal of crosstalk between the various receptor-signalling pathways.

Introduction
Neurons in the developing central nervous system must make contacts with targets that may be thousands of cell-diameters away. The axon has to navigate through a terrain consisting of various cell types, neuronal processes and extracellular matrix molecules. Within this complex terrain are guidance cues that direct the growth cone, the motile tip of the axon, to its target. These cues consist of molecules that attract the extending axon to the appropriate target as well as molecules that repel it from inappropriate pathways. A large number of the molecular cues and their receptors that guide axons to their targets have been identified in different systems and in various organisms [1]. Among the most important families of receptors that regulate axon guidance are the receptor tyrosine kinases [2,3], the receptor tyrosine phosphatases [4], the immunoglobulin family of cell adhesion molecules [5], the cadherins [6], the plexins and neuropilins [7,8], the netrin receptors [9] and the roundabout (Robo) family of receptors (10); Figure 1.

With a large number of the receptors and their ligands identified, the focus has turned to examining the intracellular signalling mechanisms that transduce the signals at the cell surface into changes in growth cone motility. As the signalling pathways utilised by the individual guidance cues come into focus, the question of how the growth cone integrates the multiplicity of signals to produce the coordinated changes in cytoskeletal dynamics can be addressed. One of the emerging themes from the recent work is the major importance of the cytoplasmic domains of guidance receptors in signalling and the diverse mechanisms utilised by the cytoplasmic domains to regulate axon guidance. Because of space limitations, this review will focus primarily on major developments within the past year for a limited number of receptors whose signalling reveals a common theme of regulating GTPases to effect changes in motility. The reader is referred to the excellent reviews cited for further information on other receptor families.

Receptor regulation of GTPases
The importance of the Rho family of GTPases (including Cdc42, Rac and RhoA) in regulating cell motility and axon guidance is well established [11]. Investigations in many different systems indicate that attraction is mediated primarily by the Cdc42 and Rac GTPases, whereas repulsion is mediated by the Rho GTPases ([11]; Figure 2). In neuronal cell lines, activation of RhoA stimulates actinomysin contractility and stress fibre formation and leads to growth cone collapse. In contrast, activation of Cdc42 and Rac results in the extension of filopodia and lamellipodia, respectively [12]. The Rho GTPases are regulated by guanine nucleotide exchange factors (GEFs), which stimulate the GTPases by promoting GDP release, and by GTPase-activating proteins (GAPs), which inhibit the GTPases by promoting GTP hydrolysis [11]. By modulating the balance of activation of these GTPases, guidance cues can determine whether a growth cone is attracted or repelled [13]. Indeed, recent work demonstrates that a large number of guidance receptors regulate the Rho GTPases to effect changes in motility.

Plexins
Among the axon guidance receptors that regulate the Rho GTPases are the plexins, a family of receptors for the semaphorins — a large group of membrane-anchored and secreted axon-guidance molecules [7,8]. The plexins are required to mediate the growth cone collapsing activity of the semaphorins. The highly conserved and unique sex-plexin domain, found in the large cytoplasmic tail of the receptors, is likely to trigger the signalling cascade leading to growth cone collapse [14]. Indeed, the Plexin B cytoplasmic domain has been found previously to bind Rac directly [15,16].

A recent report from Goodman and colleagues [17•] now suggests that Plexin B mediates motor axon guidance by...
inhibiting active Rac and enhancing RhoA signalling in *Drosophila*. The researchers found that Plexin B directly interacts with Rac *in vitro* and, at high molar excess, inhibits Rac’s ability to associate with and activate its downstream effector, the serine/threonine kinase PAK1, by activating LIM kinase and inhibiting myosin light chain kinase, which limits depolymerisation and the retrograde flow of actin filaments [18,19]). The biochemical experiments also demonstrated that Plexin B binds to and activates RhoA. Although the biochemical interactions demonstrated *in vitro* have not been confirmed *in vivo*, dosage-sensitive genetic interactions between Plexin B and the GTPases suggest that Plexin B mediates repulsion by inactivating Rac and activating RhoA (Figure 3). Because a Plexin B loss-of-function mutant is not yet available, future experiments may address some remaining issues, including clarifying the role of Plexin B *in vivo* through an analysis of loss-of-function mutants and examining whether Plexin A, which also plays a role in motor axon guidance in *Drosophila* [7,8,20], signals in a similar manner.
Ephrin/Eph signalling

Another major group of axon guidance molecules that utilise members of the Rho family of GTPases to regulate cell and growth cone motility is the Eph receptor family. Like other members of the receptor tyrosine kinase family, Eph receptors are transmembrane proteins with an extracellular ligand-binding domain and an intracellular cytoplasmic region having a tyrosine kinase domain [21,22]. The Eph receptors and their ligands, the ephrins, mediate cell-contact-dependent signalling and play important roles in the establishment of tissue patterns primarily by mediating repulsion [21,22].

Ephrin A ligands (ephrin A1–A5) are glycosylphosphatidylinositol-anchored molecules that bind EphA receptors (EphA1–A8) and play a critical role in axonal patterning in the nervous system, which includes the formation of the retinotopic map in vertebrates [23–25]. In contrast to the A ephrins, the B ephrins (ephrin B1–B3) are transmembrane proteins with a cytoplasmic tail and bind to EphB (EphB1–B6) and EphA4 receptors [21,22]. The B ephrins and their receptors play important roles in axonal patterning in the midline, including the formation of the anterior commissure [26], sorting of retinal axons at the optic chiasm [27] and the crossing of corticospinal tract neurons in the spinal cord [28].

Whereas considerable progress has been made in understanding how ephrin/Eph expression profiles regulate patterning in the nervous system [21,22], the intracellular signalling mechanisms that transduce the signals into changes in growth cone motility are still poorly understood; however, a number of recent reports have begun to shed light on the molecular pathways involved in ephrin/Eph signalling.
**Ephexin**

Recently, Greenberg and co-workers [29**] identified a GEF that interacts directly with the EphA4 receptor cytoplasmic domain. This protein called ephexin (for Eph-interacting exchange factor) acts as a direct link between Eph receptors and Rho GTPases. Ephexin is homologous to the Dbl family of GEFs and contains a unique amino-terminal region, a DH/PH domain (Dbl/plextrin homology region), which is required for binding to EphA4, and a Src homology 3 (SH3) domain. In the absence of stimulation, ephexin is constitutively bound to the EphA4 receptor cytoplasmic domain and is in a position to activate Cdc42 and Rac1 [29**]. In this state, growth cone extension is promoted. When EphA4 receptors are stimulated with a ligand (ephrin A1), ephexin activates RhoA while reducing the activation of Cdc42 and Rac1. The net effect is to induce growth cone collapse. Further studies should help determine whether ephexin is required for signalling by the other EphA receptors, or possibly even the EphB receptors.

**PDZ–RGS3**

Although much work has been performed on the signalling mechanisms downstream of the Eph receptors, the ephrin B ‘ligands’ have also been shown to be capable of signal transduction in what has been termed ‘reverse signalling’ [30–32]. Recently, Flanagan and co-workers [33**] have shown that reverse signalling is in part mediated by a novel protein that interacts with the cytoplasmic domain of ephrin B. This protein, PDZ–RGS3, contains a PDZ (PSD-95/Dlg/ZO-1) domain required for binding to ephrin B, and an RGS (regulator of G-protein signalling) domain, which interacts with heterotrimeric G proteins [34]. Proteins containing RGS domains have been shown previously to act as GAPs for the heterotrimeric G proteins that are associated with a large number of transmembrane receptors, including the chemokine receptor CXCR4 [35].
In an elegant series of experiments, the researchers demonstrate that the binding of EphB to ephrin B activates the RGS domain of PDZ–RGS3, which acts as a GAP towards heterotrimeric G proteins associated with G-protein-coupled receptors. This inhibits signalling downstream of the chemokine receptor CXCR4, thereby leading to a loss of chemotaxis towards its ligand, SDF-1. (b) Ephrin B activation can also lead to the recruitment of Grb4, an SH2/SH3 adaptor protein. Grb4 can interact with a number of different proteins that can remodel the actin cytoskeleton, including Abi-1, CAP and PAK1. The signalling pathways activated by Grb4 are likely to lead to actin depolymerisation and growth cone collapse. PDZ–RGS3 and Grb4 appear to bind distinct regions of the ephrin B cytoplasmic tail; however, it is not known if the effectors can bind simultaneously or if the binding of one effector might affect the binding of the other.

**Grb4**
Cowan and Henkemeyer [37••] recently found that the adapter protein Grb4 also transduces ephrin B reverse signals. Grb4 is an adapter protein closely related to, but distinct from, Nck/DOCK, which has been shown to play an important role in linking tyrosine kinases to intracellular signalling pathways, including those involving the Rho GTPases [38–40]. Grb4 contains an SH2 domain, which is required for binding to the cytoplasmic domain of ephrin B1, as well as three SH3 domains. Stimulation by soluble EphB2 leads to tyrosine phosphorylation of the ephrin B1 cytoplasmic domain, which in turn recruits Grb4 through its SH2 domain. Ephrin B1 activation results in a loss of polymerised actin in transfected cells and an increase in focal adhesion kinase (FAK) activity. The loss of actin polymerisation was inhibited by co-transfecting cells with a mutant Grb4 containing only the SH2 domain, which suggests that the SH3 domains are required for ephrin B1-mediated collapse. A yeast two-hybrid screen and GST-pulldown assays identified a number of proteins that interact with the SH3 domains of Grb4. All of these proteins have been implicated in cytoskeletal reorganisation [18,19,41,42], which suggests that Grb4 is likely to be a **bona fide** regulator of the cytoskeleton and axon guidance. The large number of proteins that Grb4 interacts with also suggests the possibility of a great deal of crosstalk between various signalling pathways.

**Robo**
Another receptor that has been shown recently to regulate the Rho family of GTPases is Robo, which plays a major role in axon guidance at the midline of the nervous system. In the midline of vertebrates, insects and nematodes,
Commissural axons are attracted by netrin proteins secreted by midline cells that activate a receptor of the DCC (deleted in colorectal cancer) family on growth cones [43]. After crossing the midline, the axons cease to be attracted to the midline and are instead repelled from it; thus, ensuring that they do not recross. The repulsion is mediated, in part, by an increased sensitivity to repellents on the portion of the axon that has crossed the midline so that the axon is now responsive to repellents such as Slit and semaphorin [44–48]. Axons that cross the midline also lose their responsiveness to the netrin attractant, although they still continue to express the DCC receptor [48]. In *Drosophila* and vertebrates, the Robo family of Slit receptors mediates repulsion and plays an important role in the decision of axons to cross the midline [48–53]. In *robo* mutant *Drosophila* embryos, too many axons cross the midline where the ligand for the Robo receptors, Slit, is produced by glial cells [46–49,53].

A report from Wong et al. [54••] now demonstrates that mammalian Robo1 transduces part of the signal for repulsion, by directly interacting with srGAPs. The binding of Slit to Robo1 recruits srGAP to the receptor’s cytoplasmic tail. This is associated with the activation of RhoA and the inhibition of Cdc42. This shift in the balance of GTPase activation may lead to growth cone collapse and repulsion. Robo1 can also silence signalling by the netrin receptor DCC in a direct form of crosstalk between the two receptors. The binding of Slit to Robo1 induces an interaction between the cytoplasmic domains of Robo1 and DCC (*). This involves the CC1 domain in Robo1 and the P3 domain in DCC. This direct interaction prevents DCC from transmitting the signal for attraction upon netrin binding. At present, it is not known whether Robo1 can transduce the srGAP-mediated signal for repulsion and silence attraction by DCC simultaneously. In addition, it is not known how phosphorylation of the Robo CC1 domain by the tyrosine kinase Abl (dashed arrow) may affect signalling by the srGAP pathway or the ability of Robo to silence attraction by DCC.
GTPases participate in axon guidance downstream of Robo \textit{in vivo}.

Whereas the Rho GTPases play a role in mediating Robo’s output, a recent report from Stein and Tessier-Lavigne [55••] demonstrates that Robo can silence attraction to the midline as well, in a direct form of crosstalk with the netrin receptor DCC. These investigators found that the Robo cytoplasmic domain can inhibit signalling by DCC [55••]. In transfected \textit{Xenopus} neurons, the binding of Slit to Robo induces a direct interaction between the cytoplasmic domains of Robo and DCC. This interaction prevents DCC from transmitting the signal for attraction upon netrin binding. The interaction of the cytoplasmic tails of the two receptors is mediated by short conserved domains in each receptor — CC1 in Robo and P3 in DCC (Figure 6b).

Stein and Tessier-Lavigne [55••] were able, with a pair of chimeric receptors, to mimic the physical association and the silencing effect. These receptors were activated by two completely different ligands and associated through different interaction domains, providing further evidence that the silencing occurs at the level of the cytoplasmic domains [55••]. These results suggest that the activation of Robo by Slit not only serves to repel axons from the midline, but also switches off attraction to it; thus, ensuring that they do not recross (Figure 6). It is not yet known if Robo-mediated silencing of netrin attraction occurs \textit{in vivo}; however, the Robo family of receptors in mammals and \textit{Drosophila} are highly conserved, with all three members in \textit{Drosophila} (Robo1–3) containing the conserved CC1 domain [49–52,55••]. In addition, the \textit{Drosophila} netrin receptor Frazzled is highly homologous to DCC and also contains the cytoplasmic P3 domain [56]; thus, this provides the opportunity to perform genetic studies in \textit{Drosophila} and to examine if Robo-mediated silencing of attraction to the midline occurs \textit{in vivo}.

**The cytoplasmic domain in signalling and crosstalk**

These and other studies (e.g. [57,58*]) highlight the importance of the cytoplasmic domain in transmitting guidance cues. They also emphasise that the cytoplasmic domains are the sites where signals are not only transmitted but are regulated as well. As an increasing number of cytoplasmic signalling partners are identified for each class of axon guidance receptor, the next wave of questions will address the interactions that occur between the various signalling pathways. This will help clarify how receptor activation leads to an integrated output that translates the multiple axon guidance cues at the cell surface into coordinated changes in growth cone motility.

Future work should shed light on the regulation of receptor signalling and the cross talk that occurs between the various signalling pathways. For example, it is important to examine in which contexts Grb4 or PDZ–RGS3 signalling predominates during ephrin B reverse signalling. Although Grb4 and PDZ–RGS3 appear to bind distinct regions of the ephrin B cytoplasmic domain [33••,37••], it is not known if the binding of the two effectors is mutually exclusive or if both can bind the receptor simultaneously (Figure 5). Furthermore, these interactions are likely to be regulated by EphB binding, because phosphorylation of the ephrin B cytoplasmic domain, which occurs upon binding to EphB, recruits Grb4 through its SH2 domain. In addition, it will be worth investigating if PDZ–RGS3 can regulate other heterotrimeric G proteins that may play a role in axon guidance [59,60].

Future work should also clarify how Robo signalling is modulated and the crosstalk that occurs with other signalling pathways. In addition to regulating the Rho GTPases, Robo has been shown to directly interact with the non-receptor tyrosine kinase Abelson (Abl) as well as its substrate Enabled (Ena), which is involved in mediating Robo signalling, and with Abl antagonising Robo’s output [58*]. The ability of Abl to inhibit the repulsive output of Robo is likely to be due to its ability to phosphorylate the cytoplasmic tail of the receptor. Indeed a mutant Robo lacking an Abl-phosphorylation site in the CC1 domain leads to a hyperactive receptor [58*]. Future experiments should address if the phosphorylation of the Robo cytoplasmic domain by Abl affects the interaction of srGAP with Robo, and its ability to modulate GTPase activity and mediate repulsion. Additionally, Abl-mediated phosphorylation might also affect the ability of Robo to silence attraction by the netrin receptor, as silencing requires the CC1 domain in Robo; thus, it will be worthwhile examining how phosphorylation affects Robo’s repulsive output and its ability to silence attraction at the midline (Figure 6).

Intriguingly, the overall plan in Robo signalling is very similar to that of the receptor tyrosine phosphatase DLAR, which plays an important role in photoreceptor and motor axon guidance in \textit{Drosophila} [61–64]. Akin to the Robo pathway, Ena is required for DLAR’s output, whereas Abl is an antagonist of the receptor [62]. Furthermore, DLAR also genetically interacts with a GTPase, Rac1 and the GEF Trio [63–66]. Although it is not known if Robo interacts with either Rac1 or Trio, the similarity between the Robo and DLAR signalling pathways suggests that a convergence between Abl and GTPase pathways may be a theme common to numerous guidance receptors. It will be very interesting to see whether this translates into crosstalk between the Robo and phosphatase signalling pathways.

**Conclusions**

Considerable work is required to decipher the elaborate signalling mechanisms used by the various receptors to finely regulate axon guidance. Nonetheless, a combination of biochemical, cell culture and \textit{in vivo} experiments, such as that described here, should contribute to a better understanding of the signalling mechanisms that regulate axon guidance. In addition to the GTPases, many other effectors are likely to be found downstream of the
guidance receptors, such as a multitude of non-receptor tyrosine and serine/threonine kinases and proteins that effect the growth cone cytoskeleton [67].

A great deal of crosstalk between the different receptor signalling pathways is also likely, which has been emphasised by the recent work. Future work should reveal further examples of crosstalk, at the level of receptor-cytoplasmic domains as well as at the level of downstream mediators. Ultimately, a better understanding of the intricate receptor signalling mechanisms will help reveal how a multiplicity of cues at the cell surface are translated into the stereotyped changes in growth cone responsiveness and motility that are necessary during the development of the nervous system.

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
- of special interest
- • of outstanding interest


This study demonstrates that the semaphorin receptor Plexin B inhibits Rac through a direct interaction of the receptor's cytoplasmic domain with the GTPase. Plexin B was also found to activate RhoA. The authors suggest that the combined inhibition of Rac and activation of RhoA leads to growth cone collapse.


The authors identify a novel guanine nucleotide exchange factor, ephexin, which interacts directly with the EphA receptor cytoplasmic domain. When EphA receptors are stimulated with ephrin A1, ephexin activates RhoA while reducing the activation of Cdc42 and Rac1. This shift in the balance of GTPase activation is thought to lead to growth cone collapse.


The authors identify a novel protein, PDZ-GRS, which associates with the ephrin B cytoplasmic domain and is activated upon EphB binding. This leads to the inhibition of signalling through the G-protein-coupled receptor CXCR4, and the loss of chemotaxis towards its ligand SDF-1.
Identification of Grb4/Nckbeta, a Src homology 2 and 3 domain-containing adapter protein having similar binding and biological properties to Nck.


The authors show that the tyrosine kinase Abelson (Abi) and its substrate Enabled (Ena) act downstream of Robo. Ena is found to be part of Robo's output, whereas Abi antagonises Robo signalling. In vitro, Abi phosphorylates the Robo C1 domain; and a Robo receptor lacking the tyrosine phosphorylation site is hyperactive, which suggests that Abi-mediated phosphorylation of Robo negatively regulates Robo signalling.


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