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REVIEW

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Intracellular Trafficking Mechanisms that Regulate Repulsive Axon Guidance

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Abstract—*Summary:* Friedrich Bonhoeffer made seminal contributions to the study of axon guidance in the developing nervous system. His discoveries of key cellular and molecular mechanisms that dictate wiring specificity laid the foundation for countless investigators who have followed in his footsteps. Perhaps his most significant contribution was the cloning and characterization of members of the conserved ephrin family of repulsive axon guidance cues. In this review, we highlight the major contributions that Bonhoeffer and his colleagues made to the field of axon guidance, and discuss ongoing investigations into the diverse array of mechanisms that ensure that axon repulsion is precisely regulated to allow for accurate pathfinding. Specifically, we focus our discussion on the post-translational regulation of two major families of repulsive axon guidance factors: ephrin ligands and their Eph receptors, and slit ligands and their Roundabout (Robo) receptors. We will give special emphasis to the ways in which regulated endocytic trafficking events allow navigating axons to adjust their responses to repellant signals and how these trafficking events are intimately related to receptor signaling. By highlighting parallels and differences between the regulation of these two important repulsive axon guidance pathways, we hope to identify key outstanding questions for future investigation.

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Keywords: Axon guidance, Repulsion, Slit-Robo, Ephrin-EphR, Endocytosis, Receptor trafficking.

INTRODUCTION

Nervous system wiring depends on the ability of axons to navigate long distances to find their appropriate synaptic targets. Axons are tipped with highly motile structures called growth cones, which guide their extension toward their final destinations through cytoskeletal processes. Growth cones are decorated with receptors for a diverse array of secreted and cell surface cues. Ligand-receptor interactions alter the membrane and cytoskeletal behavior of the growth cone, either drawing axons toward the source of the signal (attraction), or causing them to retreat or turn away from the source of the signal (repulsion). By integrating responses to a variety of these cues at the growth cone, axons travel along highly stereotyped paths toward their synaptic targets with remarkable precision.

From 1980 to 2010, the field of axon guidance exploded, encompassing three distinct but overlapping eras of research (Fig. 1). In the "pre-gene era", fundamental principles of axon guidance were initially described, and elegant *in vitro* axon guidance assays, which are still used today, were developed. During the "gene discovery era", the major families of axon guidance receptors and ligands were identified, cloned, and characterized. In the "regulation era", the first steps were taken to learn how these molecules are regulated to ensure that axons respond to the right cues at the right times. Efforts were also made to investigate how signals from these cues are transmitted intracellularly to coordinate remodeling of the growth cone plasma membrane and cytoskeleton.

Max Planck Institute scientist Friedrich Bonhoeffer was a titan in the field who made seminal discoveries during each of these periods. He and his trainees made major contributions to our understanding of the critical role for axon repulsion in nervous system wiring.

In the "pre gene era" of the 1980s and early 1990, the work from Bonhoeffer's laboratory illuminated many of the basic principles of how axons migrate toward their targets. One such principle is that axons prefer to migrate along certain cell types more than others. His

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Abbreviations: AP-2, Adaptor Protein 2; CC, Conserved Cytodomain, Conserved Cytoplasmic; EGFRs, Epidermal Growth factor receptors; FRET, fluorescence resonant energy transfer; MVB, multi-vesicular body; Ndfip1 and Ndfip2, Nedd4-family interacting proteins 1 and 2; Nrp2, neuropilin2; PDGFRs, Platelet-derived growth factor receptors; PlxnA1, PlexinA1; PRRG4, Proline Rich And Gla Domain 4; PTPs, Protein tyrosine Phosphatases; Robos, Roundabouts; Sema3B, Semaphorin3B.

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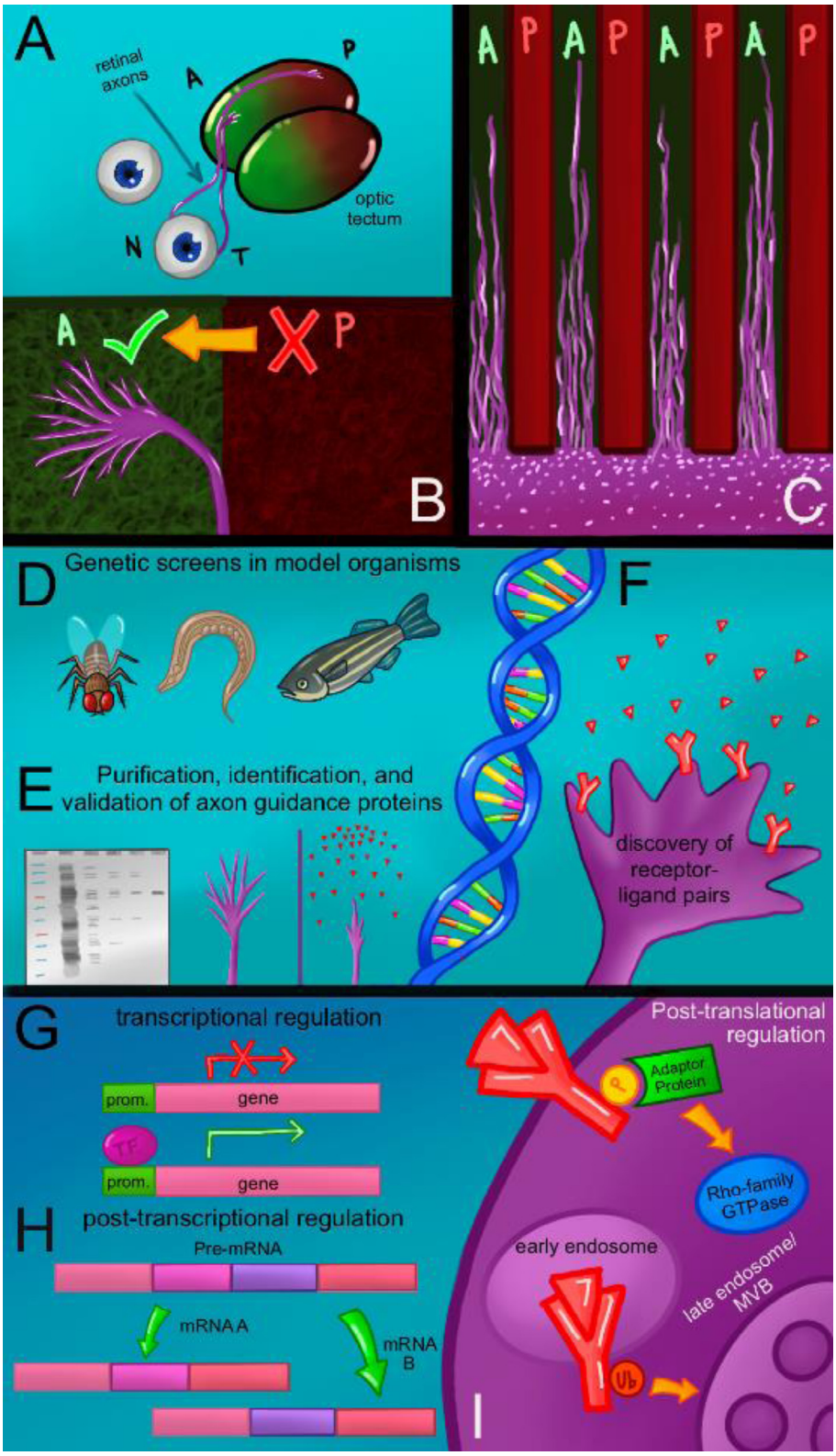
team found that, when offered two substrates, embryonic chick retinal axons were more likely to migrate over membranes of cells derived from their target tissue, the tectum, than over fellow retinal cell membranes (Bonhoeffer & Huf, 1980). Soon after, the Bonhoeffer group demonstrated that different types of axons within a structure are drawn to different target tissues. Using a similar substrate preference assay, they discovered that temporal retinal axons are more likely to migrate along cells derived from the anterior tectum, while nasal retinal axons migrated equally well across anterior and posterior tectal cells (Fig. 1A and B). These early assays developed by the Bonhoeffer lab were eventually refined into the famous stripe assay, in which axons travel along a carpet consisting of alternating stripes of two different types of cell membranes (Walter et al., 1987b;) (Fig. 1C). As well as testing axons' preference of substrate, this assay is used for a variety of applications including investigating whether certain substrates are attractive or repulsive (Walter et al., 1987a), and identifying critical molecules involved in axon guidance (Stahl et al., 1990; Drescher et al., 1995; Wang et al., 2004). With the stripe assay, Bonhoeffer and colleagues were able to significantly refine the understanding of how axons responded differentially to progressively more anterior regions of the tectum (Walter et al., 1987b) (Fig. 1C). This finding suggested that the guidance signal to which temporal axons respond is distributed in a gradient along the anterior-posterior axis of the tectum. Since then, many axon guidance cues have been found to be distributed in gradients (Tessier-Lavigne, 1992; Wu et al., 1999; Charron et al., 2003; Sloan et al., 2015). Bonhoeffer's stripe assay was also used in conjunction with axon collapse assays to demonstrate that temporal retinal axons respond to a repulsive signal from the posterior part of the tectum, as opposed to an attractive cue from the anterior (Walter et al., 1987a; Cox et al., 1990).

In addition to demonstrating that axons respond to repulsive cues, Bonhoeffer's team unearthed other critical mechanistic aspects of axon guidance. In one study, they found that Xenopus retinal axons detached from their somas can still grow toward the appropriate tectal targets, suggesting that the growth cone contains most, if not all, of the machinery it needs to navigate to its destination (Harris et al., 1987). Before the major families of axon guidance cues were discovered, Godement and Bonhoeffer (1989) also demonstrated that axon guidance cues are conserved across species using clever mixed-species stripe assays. Finally, alongside Walter et al. (1990), Bonhoeffer developed conceptual frameworks describing how growth cones could read gradients of axon guidance signals, and how growth cone collapse could be linked to axon turning.

While Bonhoeffer and his group made invaluable 'pregenetic' discoveries about the nature of axon guidance, they swiftly adapted to the genetic era. The 1990s were a golden age for the discovery of the main receptor-ligand pairs involved in axon guidance (Fig. 1D–F). During this time, forward genetic screens were being conducted in invertebrates, most notably *C. elegans* (Hedgecock et al., 1990; McIntire et al., 1992) and *Drosophila* (Seeger et al., 1993) (Fig. 1D). Concurrently, other research groups used biochemical techniques (Fig. 1E) to isolate and identify proteins involved in axon guidance from vertebrate (Luo et al., 1993; Serafini et al., 1994) and invertebrate (Kolodkin et al., 1992) systems, and validated their activity *in vitro* (Luo et al., 1993; Kennedy et al., 1994; Serafini et al., 1994). In tandem, these two experimental approaches unearthed critical gene families such as *netrins* (Hedgecock et al., 1990; Ishii et al., 1992; Kennedy et al., 1994; Serafini et al., 1994), *semaphorins* (Kolodkin et al., 1992; Luo et al., 1993), and members of the *slit-roundabout (robo)* pathway (Kidd et al., 1998a; Kidd et al., 1999). Bonhoeffer and his group were the first to implicate ephrins, the ligands for Eph Receptor Tyrosine Kinase (RTK) receptors, in axon guidance. In a search to find the repulsive factors present in posterior tectal membranes, they found that treatment with Phospholipase C could abolish temporal axons' avoidance of posterior tectal tissue, suggesting that the repulsive factors in question were GPI-anchored (Drescher et al., 1995). Using an elaborate fractionation protocol and 2-D electrophoresis, they searched for GPI-anchored proteins whose expression was enriched in the posterior of the chick brain during the developmental time when retinal axons are guided to their target tissues. Through this method, they identified, purified, and cloned a 25 kDa molecule they called Repulsive Axon Guidance Molecule (RAGS, now known as ephrin-A5) (Drescher et al., 1995), which was the first ephrin discovered to play a role in axonal pathfinding.

In addition to biochemical approaches, Bonhoeffer and his group took advantage of the power of zebrafish transparency and genetic tractability, to design the first vertebrate genetic screen for genes involved in retinotectal pathfinding (Baier et al., 1996; Karlstrom et al., 1996; Trowe et al., 1996). In collaboration with the Nüsslein-Volhard laboratory in Tubingen, which had initiated a large-scale mutagenesis screen for genes involved in embryonic patterning, Bonhoeffer's group identified zebrafish homologs of RAGS(EphA5), Elf-1 (EphA2), Repulsive Guidance Molecule (RGM) (Brennan et al., 1997) and *astray*, the zebrafish homolog of Robo2 (Fricke et al., 2001). Through these studies and their work characterizing ephrins, the Bonhoeffer laboratory was a central force in the golden age of classical axon guidance gene discovery. Although new axon guidance genes continue to be discovered to this day, this golden age laid important groundwork for the field and ushered in a new era of research.

This third era of axon guidance research that we highlight began in the late 1990s, and is focused on investigating the regulation of axon guidance molecules and their downstream signaling mechanisms (Fig. 1G–I). During this time, Bonhoeffer's lab and collaborators identified a transcription factor that regulates ephrin-A3 and ephrin-A5 expression (Logan et al., 1996), and outlined the ways in which these ephrin ligands, through a combination of common and distinct functions, work together to properly wire the retinotectal map (Monschau et al., 1997). From Bonhoeffer's retirement in 2000 to the present day, many more regulatory



mechanisms for axon guidance gene families have been discovered (recently reviewed in Zang et al. (2021)). This collective effort has highlighted how a relatively small number of receptor-ligand pairs can produce such specific axon guidance events across many different developmental contexts. Numerous transcriptional, post-transcriptional and post-translational mechanisms controlling repulsive axon guidance pathways have been discovered, highlighting the importance of the Bonhoeffer group's research on the roles of axon repulsion in neural circuit wiring. In this review, we focus on the rapidly growing body of knowledge about how post-translational control of axon guidance receptor trafficking allows for the precise spatial and temporal control of axon repulsion. We will draw from recent literature on the Eph and Robo receptors to highlight common regulatory strategies and to identify outstanding questions for future investigation. The work highlighted in this review, and the research to come, is truly built on the back of a giant.

EPH AND ROBO RECEPTORS: OVERVIEW

Ephs are a large family of RTKs conserved across vertebrates and invertebrates, which function in diverse developmental events involving differential cell adhesion, including axon guidance, cell migration, and boundary formation between different tissue types. For an in-depth review on ephrin/Eph signaling, see Kania and Klein (2016). Eph receptors bind membrane-associated ephrin molecules and are classed into two subfamilies according to the type of ephrin they bind: the GPI-anchored ephrin-A's, or the ephrin-B's which contain a transmembrane domain and short cytoplasmic region. Ephrins and Ephs signal bidirectionally, meaning that either can act as ligand or receptor. The classic mode of ephrin-Eph signaling, known as "forward" signaling, occurs when ephrins act *in trans* as ligands for Eph receptors on a neighboring cell, and cause cell repulsion. "Reverse"

signaling in which Ephs act as *in-trans* ligands for ephrin receptors can also result in either repulsion or adhesion. Both modes of signaling depend on the clustering of Eph-ephrin pairs into large multimers. In forward signaling, this clustering facilitates Eph autophosphorylation which is essential for recruitment of downstream effector proteins. Meanwhile, reverse signaling involves phosphorylation of ephrin-B by Src family kinases. Often, both directions of signaling ultimately induce cytoskeletal remodeling, endocytosis, and repulsion.

The Roundabouts (Robos) are a family of receptors for the slit ligand. They are single-pass transmembrane proteins whose extracellular domains consist of five type-C2 IG domains and three type III fibronectin repeats. Their intracellular domains contain three to four conserved cytoplasmic (CC) motifs. As these receptors have no autocatalytic activity, the majority of their signaling depends on the recruitment of cytoplasmic adaptor proteins, especially those modulating the actin cytoskeleton. (For an in-depth review of Robo signaling and its various roles in development, see Blockus and Chedotal (2016)). Robo receptors are involved in a broad range of developmental processes including axon guidance, cell migration, organogenesis, and stem cell regulation. They are, however, best known for their role in repulsive axon guidance at the embryonic midline. In both vertebrates and invertebrates, Robo1 prevents midline crossing in ipsilateral neurons, and coordinates the repulsive events necessary for midline crossing in commissural neurons (Dickson & Gilestro, 2006). In *Drosophila*, Robo2 works together cell-autonomously with Robo1 to mediate repulsion in response to slit, and also has a non-autonomous role in the negative regulation of Robo1 signaling in pre-crossing commissural axons (Dickson & Gilestro, 2006; Evans et al., 2015). In *Drosophila* both Robo2 and Robo3 control lateral positioning of commissural axons after they cross the midline (Rajagopalan et al., 2000; Simpson et al., 2000). In vertebrates, Robo1 and Robo2 also regulate lateral positioning and fasciculation in post-crossing commissural axons (Blockus & Chedotal, 2016). It is important to emphasize that all vertebrate Robo receptors are related to *Drosophila* Robo1, and Robo2 and Robo3 in insects have distinct structures and functions. The intracellular trafficking system regulates Robo1 function in a myriad of ways. In this review, we will concentrate mostly on the trafficking of Robo1, called Robo for the rest of this review. While less is known about the regulation of the other Robo family receptors, it is possible that the same trafficking machinery can also regulate them due to their shared Conserved Cytodomain (CC) motifs and other structural similarities in their cytodomains with Robo1.

REGULATION OF RECEPTOR TRAFFICKING

Across many model systems and neuronal contexts, a number of receptor trafficking events control axon responses to extracellular cues. First, axon guidance receptors must be delivered to the axon surface. Based on studies of other transmembrane neuronal proteins, axonal targeting of these receptors is likely controlled by one of the three major sorting pathways for axonal membrane proteins: (1) direct sorting from the Trans-Golgi Network (TGN), (2) transcytosis from the somatodendritic compartment to the axon or (3) uniform plasma membrane targeting followed by selective removal/retention (For detailed reviews of these pathways, see Winckler and Mellman (2010) and Winckler and Yap (2011)). While specific targeting sequences and axon-targeting pathways have not been explored in great detail for Ephs and Robos, it is clear that in the context of spinal commissural axon guidance, delivery of repulsive receptors to the growth cone membrane is tightly regulated. For example, receptors for the midline repellants Semaphorin3B (Sema3B) and slit are trafficked to the growth cone surface at distinct times during commissural axon guidance (Pignata et al., 2019). Tracking growth cone delivery using phluorin-tagged receptors that only fluoresce when they are on the cell surface reveals that while neuropilin2 (Nrp2) is expressed on the growth cone surface during the entire process of midline crossing, PlexinA1 (PlxnA1) and Robo are only trafficked to the cell surface after commissural axons have entered the floor plate, and occupy different spatial domains of the growth cone (Pignata et al., 2019). Since Sema3B response requires both PlxnA1 and the co-receptor Nrp2, a delay of PlxnA1 surface insertion until axons have crossed the midline may explain how commissural axons are prevented from prematurely responding to Sema3B. A previous study provides further evidence for this mechanism, observing low PlxnA1 expression in pre-crossing axons and demonstrating that Calpain proteases downregulate PlxnA1 expression via proteolytic cleavage (Nawabi et al., 2010). In contrast to these findings, another research group observed high PlxnA1 expression in pre-crossing commissural axons (Hernandez-Enriquez et al., 2015). It remains unclear, however, whether the PlxnA1 observed is indeed located at the cell surface. In addition, it is possible that the antibody utilized in this

Fig. 1. Schematic of the three ‘‘Eras” of axon guidance research. (A–C) The ‘‘Pre-Gene” era, during which fundamental principles of axon guidance were discovered and *in vitro* assays were developed. **(A)** Retinal axons traveling toward their synaptic targets in the optic tectum. Nasal axons target the posterior tectum, while temporal axons target the anterior. **(B)** A temporal retinal axon is repelled from posteriorly-derived tectal tissue and therefore, driven toward anteriorly-derived tectal tissue. **(C)** Bonhoeffer’s stripe assay, with temporal axons migrating along strips of anterior tectal tissue and avoiding posterior tectal tissue. **(D–F)** The ‘‘Gene Discovery” era, when several classical axon guidance receptors and their ligands were discovered. D). Model organisms used in axon guidance genetic screens in the 1990s. *Drosophila*, *C. elegans*, and Zebrafish. **(E)** Fractionation and purification of a hypothetical axon guidance protein and its in-vitro validation as a repulsive guidance molecule using an axon collapse assay. **(F)** A generalized axon guidance receptor and its ligand. **(G–I)** The ‘‘Regulation Era,” which began in the late 1990s and continues into the present. **(G)** An example of transcriptional regulation via a transcription factor binding to the promoter of a gene. **(H)** An example of post-transcriptional regulation via mRNA splicing. **(I)** Post-translational modifications affecting a generic axon guidance receptor’s signaling and trafficking. These include phosphorylation allowing for the recruitment of a downstream adaptor proteins, and ubiquitination shunting the receptor toward the late endosome.

study detected cleaved PlxnA1 fragments as well as intact protein.

While there remains much to be discovered about the mechanisms of repulsive axon guidance receptor delivery to the growth cone surface, we will predominantly focus for the remainder of our discussion on endo-lysosomal trafficking of these receptors after they have been delivered to the axonal membrane. We will, however, highlight one interesting exception where Robo receptors appear to be transiently negatively regulated, by direct shunting from the TGN to an endo-lysosomal degradative pathway (Keleman et al., 2002). The different steps of the endo-lysosomal trafficking pathway, including endocytosis, recycling, and lysosomal degradation, are each critical points for regulating axons' responses to repulsive cues. Endocytosis of ligand-activated guidance receptors plays important roles in their function, by promoting axon repulsion through membrane detachment, as well as through activating downstream signaling events. Repulsive receptors can also undergo endosomal recycling to the plasma membrane and this regulated recycling can lead to sensitization or desensitization of axons to their respective ligands (Fiederling et al., 2017; Kinoshita-Kawada et al., 2019). In addition, lysosomal degradation prevents premature surface expression (Keleman et al., 2002) of repulsive receptors, and terminates their signaling (Sabet et al., 2015). Growing evidence also indicates that repulsive receptors can continue signaling from endosomes (Boissier et al., 2013; Chance & Bashaw, 2015) and lysosomes (Valenzuela & Perez, 2020), suggesting that the control of receptors' movement through the endosomal system could serve to regulate the duration of signaling.

ROBO ENDO-LYSOSOMAL SORTING: NEGATIVE REGULATION OF MIDLINE REPULSION

In bilateral organisms, activity on the left and right halves of the organism must be coordinated. This coordination occurs via a population of neurons whose axons cross the midline and project to the contralateral side of the body (Evans & Bashaw, 2010; Nawabi & Castellani, 2011; Gorla & Bashaw, 2020). Midline glia simultaneously secrete attractive and repulsive ligands, so an axon's behavior at the midline is highly dependent on the set of receptors expressed on the surface of its growth cone at a particular developmental timepoint. Initially, commissural axons are sensitive to attractive ligands, allowing for entry into the midline; however, upon reaching the midline axons become sensitive to repulsive cues including slit. This sensitivity propels them out of the midline and prevents re-crossing (Fig. 2). To enter the midline, it is imperative for commissural axons to prevent premature sensitivity to slit. In both vertebrates and invertebrates, this is achieved by keeping Robo levels low on the growth cone surface until axons have crossed the midline (Fig. 2B, E). In *Drosophila*, Commissureless (Comm) plays a central role in downregulating Robo in pre-crossing commissural neurons (Tear et al., 1996; Kidd et al., 1998b; Keleman et al., 2002). Comm expression

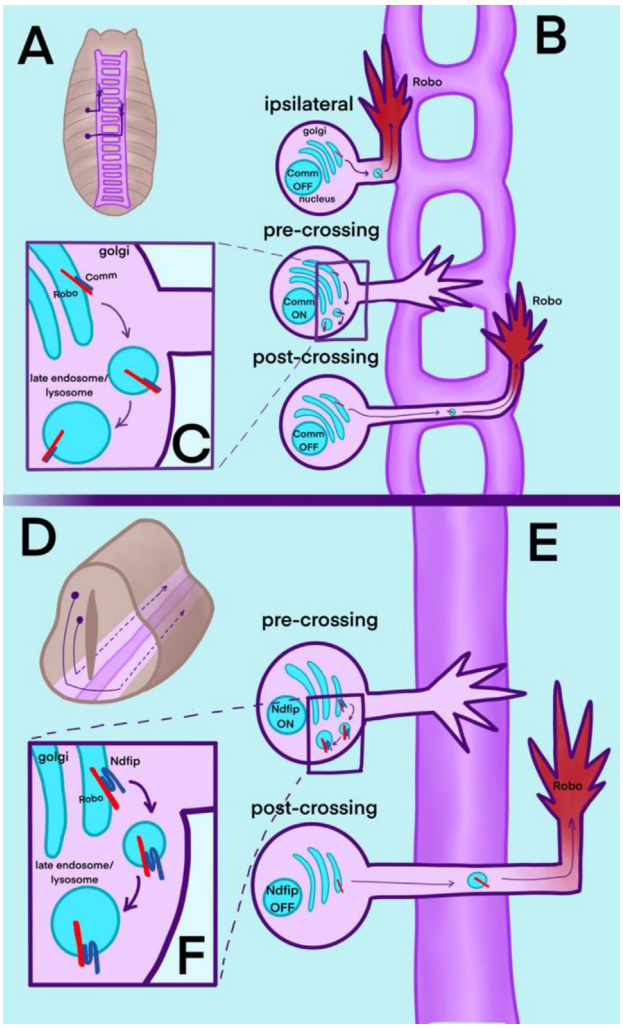


Fig. 2. Robo insertion at the growth cone membrane is prevented via an endo-lysosomal degradative pathway. (A) Drosophila embryo, showing the navigational pathways of one commissural axon and one ipsilateral axon in the nerve cord. **(B)** An ipsilateral axon, pre-crossing commissural axon, and post-crossing commissural axon at the midline. Robo is present on the growth cone surface of both the ipsilateral and post-crossing commissural axons, rendering these axons sensitive to the repulsive Slit cue. This Slit sensitivity prevents midline crossing for the ipsilateral axon, and ensures that the post-crossing axon only crosses the midline once. The pre-crossing commissural axon, however, expresses Comm but does not have Robo on its growth cone surface. This axon is therefore insensitive to Slit, allowing it to travel toward and cross the midline. **(C)** Comm interacts with newly-synthesized Robo in the Golgi, and shunts it directly toward the late endosome. This prevents Robo from reaching the growth cone surface. **(D)** Embryonic mouse spinal cord, showing the navigational pathways of one commissural axon and one ipsilateral axon. **(E)** A pre-crossing commissural axon and post-crossing commissural axon at the midline. Ndfip is expressed in pre-crossing commissural neurons, preventing Robo from being inserted into the growth cone membrane. Ndfip is not expressed in post-crossing commissural neurons, but Robo is present on the growth cone surface. **(C)** Ndfip prevents Robo surface insertion by directing it to an endo-lysosomal degradative pathway.

is under tight spatial and temporal control, such that it is turned on in a particular subsets of commissural neurons specifically when it is time for that population to extend their axons across the midline (Keleman et al., 2002) (Fig. 2B). Comm expression is induced, in part, by the

attractive axon guidance receptor Frazzled (the Drosophila homolog of Deleted in Colorectal Cancer (Dcc)), whose non-canonical signaling pathway involves cleavage of its intracellular domain which then enters the nucleus to act as a transcription factor for the Comm gene (Neuhaus-Follini & Bashaw, 2015).

Various *in vitro* and *in vivo* studies demonstrate that Comm acts as an endocytic sorting receptor, diverting newly synthesized Robo away from the plasma membrane and toward late endosomes/lysosomes, presumably to be degraded (Fig. 2C). In COS-7 cells transfected with Robo alone, Robo is observed at the plasma membrane (Keleman et al., 2002; Myat et al., 2002). When co-expressed with Comm, however, Robo and Comm co-localize in intracellular vesicles positive for late endosomal markers (Keleman et al., 2002). Antibody feeding assays demonstrate that Robo found with Comm in endo-lysosomal compartments is not derived from surface internalization (Keleman et al., 2002), suggesting instead that it is directly sorted from the Golgi. This finding is corroborated by live-imaging in *Drosophila*, which demonstrates that Comm prevents Robo transport down axons, thus inhibiting its ability to reach the growth cone surface (Keleman et al., 2005). Both *in vivo* (Kidd et al., 1998b) and *in vitro* (Gilestro, 2008), Comm not only alters Robo localization but also lowers its overall protein levels. Therefore, Comm downregulates Robo not through endocytosis, but by shunting it from the synthetic pathway to an endo/lysosomal degradative pathway (Fig. 2C).

Comm's ability to downregulate Robo stability and surface localization is likely dependent on cells' ubiquitination machinery. Two PY motifs (PPCY at amino acids 220–223, and LPSY at amino acids 229–232) are present in the Comm cytoplasmic tail (Myat et al., 2002). In other proteins these motifs are known to bind Nedd4-family HECT ubiquitin ligases. Mutation of these motifs disrupts numerous aspects of Comm function including its trafficking to endosomes (Keleman et al., 2002), diversion of Robo away from the cell surface (Keleman et al., 2002; Keleman et al., 2005), ability to promote midline crossing (Keleman et al., 2002; Keleman et al., 2005), and downregulation of Robo protein levels (Dickson & Gilestro, 2006). Therefore, it is highly likely that Comm regulates Robo through interaction with one or more of the three Drosophila Nedd4-family ligases: Nedd4, Sud(x), and Smurf. The precise mechanism of this downregulation remains unknown.

An early structure–function analysis of Comm suggested that it must be ubiquitinated by Nedd4 to negatively regulate Robo function (Myat et al., 2002). This idea, however, was challenged by the finding that an un-ubiquitinatable Comm (CommKR), in which all cytoplasmic lysines were converted to arginines, effectively shunts Robo to endosomes *in vitro* and promotes midline crossing *in vivo*[53] when overexpressed in the nerve cord. It is possible that while Comm's ubiquitination status is unimportant for its ability to downregulate Robo, interaction with Nedd4 family ligases may still be important for this process. Perhaps, instead of recruiting Nedd4-family ligases for its own ubiquitination, Comm serves as a scaffold to bring them into proximity with Robo. Indeed, Robo ubiquitination has been implicated as a negative regulator of its repulsive function. Upon slit stimulation, Robo recruits de-ubiquitinating enzyme USP33 in cultured mouse commissural spinal neurons, and USP33 knockdown abolishes growth cone collapse in response to slit (Yuasa-Kawada et al., 2009). As Robo must be de-ubiquitinated to respond to slit, Comm may downregulate Robo by facilitating its ubiquitination, triggering its subsequent trafficking to late endosomes and degradation.

As Comm is not conserved outside of insects but Robo must still be tightly regulated in other animal families, efforts have been made to identify a functional analog of Comm in vertebrates. Attempts to identify the vertebrate Comm analog were driven by searching for vertebrate proteins sharing some sequence similarity to important domains of the Comm protein. One such protein is the Proline Rich and Gla-domain containing Protein 4 (PRRG4) (Justice et al., 2017), which shares sequence similarity to the LPSY motif-containing region of Comm. In COS-7 cells, PRRG-4 is able to divert mammalian Robo away from the plasma membrane. Unlike Comm, which traffics Robo to late endosomes and lysosomes, PRRG4 appears to trap Robo in the endoplasmic reticulum and Golgi (Justice et al., 2017). In cultured breast cancer cells, overexpression of PRRG4 is able to decrease Robo protein levels in a manner dependent on PY motifs. Unexpectedly, this Robo turnover appears to be driven primarily by the proteasome as opposed to the lysosome (Zhang et al., 2020). A role for PRRG4 in midline crossing in the mammalian spinal cord has not yet been tested; however, based on the results described above, it appears that PRRG4′s mechanism of action is likely to be distinct from Comm.

Other candidates for the vertebrate Comm analog are the Nedd4-family interacting proteins 1 and 2 (Ndfip1 and Ndfip2) (Gorla et al., 2019), which act as adaptor proteins to recruit Nedd4-family ubiquitin ligases to their substrates (Fig. 2D–F). Both Ndfips 1 and 2 have a domain that shares sequence similarity with a region of Comm conserved in *Drosophila* and mosquito, including PPXY and LPXY motifs. Interestingly, while *Drosophila* have their own Ndfip gene, it is only known to participate in larval developmental events where it regulates Notch, such as wing disc patterning (Dalton et al., 2011) and neuronal stem cell maintenance in the brain (Li et al., 2018). At this point in development, the nerve cord has already finished wiring and commissural axons have crossed the midline. In vertebrates, both Ndfip proteins are expressed in commissural neurons in the mouse spinal cord as they are projecting to and across the midline. Like Comm, they physically interact with Robo and can divert it away from the plasma membrane in COS-7 cells, instead routing it to late endosomes. Loss of both Ndfips has been shown to inhibit midline crossing in the mouse spinal cord. Spinal cords of *Ndfip1*$^{-/-}$, *Ndfip2*$^{-/-}$ mice have reduced commissure thickness, and open book preparations of these spinal cords reveal midline crossing defects such as inappropriate ipsilateral projection and floor plate stalling. In addition, mutation of Ndfips 1 and 2 elevates Robo levels

in pre-crossing commissural neurons (Gorla et al., 2019). Taken together, mammalian Ndfips appear to regulate Robo levels in pre-crossing commissural neurons using a mechanism very similar to that of Comm (Fig. 2D–F). Whether this mechanism of preventing the delivery of receptors to the growth cone to control the timing of axon responses to their respective ligands is unique to Robo receptors, or if instead it represents a more general strategy to control axon responsiveness remains to be determined. In this context, it is interesting to note that like Robo, the delivery of PlxnA1 to commissural axon growth cones is prevented until after axons have entered the floor plate, raising the possibility that similar regulation of PlxnA1 trafficking may occur.

RECEPTOR ENDOCYTOSIS IS REQUIRED FOR AXON REPULSION: EPH-EPHRIN

For receptors on the cell surface, endocytosis is the means by which they first enter into the endocytic compartment. Endocytosis and cytoskeletal reorganization are intimately intertwined, and both processes are necessary to generate ephrin/Eph-induced repulsive responses (Fig. 3). Ephrins and ephs bind to one another with very high affinity, creating an adhesive interaction between neighboring cells and neurites that must be disrupted for repulsion to occur. One way this interaction can be broken is through proteolytic cleavage of the ephrin ectodomain (Hattori et al., 2000; Janes et al., 2005). Alternatively, the interaction can be disrupted through removal of the intact ligand-receptor complex from the contacting cell surfaces. This can occur via a process called trans-endocytosis, in which complexes of receptor, membrane-bound ligand, and a small piece of the neighboring cell's membrane are internalized (Fig. 3A, E). In cells cultured with variants of Eph and ephrins that cannot be internalized, the normally repulsive Eph-ephrin interaction becomes adhesive. Trans-endocytosis occurs bidirectionally (Mann et al., 2003; Zimmer et al., 2003; Parker et al., 2004) and is necessary for cell–cell separation in cell lines, as well as growth cone repulsion in cultured neurons (Mann et al., 2003; Marston et al., 2003). Trans-endocytosis is heavily dependent on a cell's actin cytoskeletal machinery. Internalization of Eph-ephrin complexes, as well as subsequent repulsive events, can be ablated with the actin polymerization inhibitor cytochalasin D. In addition, dominant-negative versions of actin regulatory proteins including the Rho family GTPases and Scar, a component of the Wave regulatory complex that promotes ARP2-3-dependent branched actin polymerization, can attenuate ephrin-Eph internalization and repulsion (Marston et al., 2003).

Many downstream effectors of ephrin-Eph signaling modulate activity of Rho-family GTPases, which drive the shape changes at the plasma membrane underlying ephrin-Eph endocytosis and subsequent repulsion (Fig. 3B, C). Rho-family GTPase-dependent actin polymerization is regulated by these effectors through a variety of mechanisms. For example, Src homology 2 (SH2) domain containing inositol polyphosphate

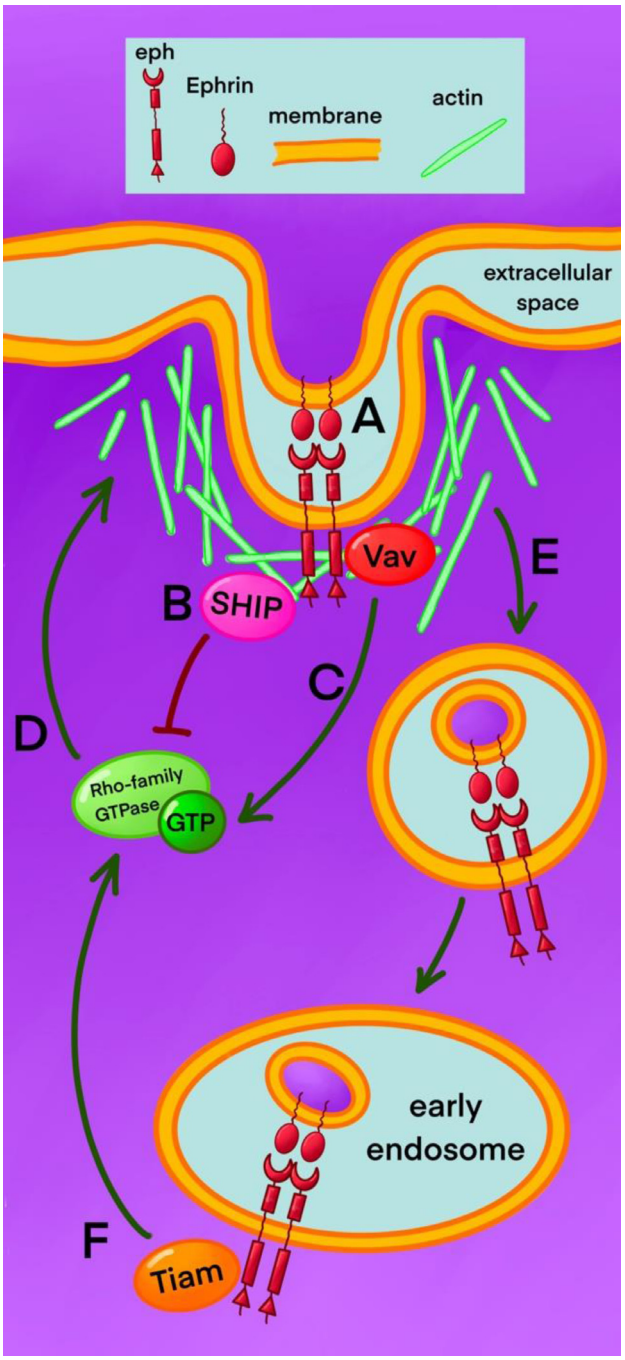


Fig. 3. Eph receptors interact with the actin cytoskeleton to drive endocytosis. (A) EphRs bind ephrins on the surface of a neighboring cell. **(B, C)** Interaction with ephrins causes Ephs to cluster and adopt an active conformation at the plasma membrane, which allows them to recruit downstream effector proteins which affect the ability of Rho-family GTPases to remodel the actin cytoskeleton **(B)** SHIP phosphatases negatively regulate Rho-family GEFs by decreasing intracellular levels of PIP_3. **(C)** Vav Rho-GEFs positively Rho-family GTPases by catalyzing exchange of GDP for GTP. **(D)** Rho-family GTPases modulate the actin cytoskeleton, causing membrane involution. **(E)** The Eph-ephrin complex is undergoes trans-endocytosis, in which the intact receptor-ligand pair and a piece of the neighboring cell's membrane is internalized. **(F)** When the Eph-ephrin complex is in the early endosome, it interacts with the Tiam family of Rho-family GEFs. Tiams activate Rho-family GEFs which encourages further cytoskeletal remodeling and endocytosis.

5-phosphatase 2 (SHIP2), a member of the SHIP family of phosphatases which dephosphorylates PIP_3, can down regulate Rac1 activity by decreasing intracellular levels of Phosphatidylinositol 3,4,5 triphosphate (PIP_3). SHIP2 binds the SAM motif of EphA2 and negatively regulates ephrin-A1-EphA2 endocytosis by lowering intracellular PIP_3 levels, thereby suppressing Rac1-driven cytoskeletal remodeling (Fig. 3B) (Zhuang et al., 2007). In contrast to reduced PIP_3 levels, Rho-family GTPases can be positively regulated by Rho-family GEFs, which in turn promotes ephrin-Eph endocytosis (Cowan et al., 2005). For example, members of the Vav family of Rho GEFs interact with the intracellular domains of ephrin-activated Ephs via their SH2 domains, resulting in a phosphorylation-dependent conformational change that leads to Vav activation (Fig. 3C). Mice lacking Vav2 and Vav3 display aberrant axon pathfinding in processes involving ephrin-Eph based repulsion, such as ipsilateral retinal axon projection to the dorsal lateral geniculate nucleus. Furthermore, neurons cultured from *Vav2*$^{-/-}$, *Vav3*$^{-/-}$ double mutant mice are unable to collapse their growth cones in response to ephrin signaling (Cowan et al., 2005). In addition to the Vavs, the Tiams are another class of Rho-family GEFs that regulate ephrin-Eph endocytosis (Yoo et al., 2010; Boissier et al., 2013; Gaitanos et al., 2016) and repulsion. These GEFs induce cytoskeletal remodeling specifically through Rac. Tiam1 interacts with phosphorylated tyrosines on the juxtamembrane region of activated EphA receptors, and this interaction stimulates Tiam1 Rac-GEF activity (Boissier et al., 2013) (Fig. 3F). Downregulation of endogenous Tiam1 activity in renal cell lines reduces Rac activity and efficient endocytosis of both the ephrin-A5-EphA8 (Yoo et al., 2010) and ephrin-A1-EphA2 complexes (Boissier et al., 2013). Tiam2 has been similarly implicated as an important regulator of ephrin-B-EphB trans-endocytosis. Constitutively active Tiam2 increases bidirectional internalization of the ephrin-B2-EphB1 complex in neighboring SKN cells, while a dominant-negative Tiam2 produces the opposite effect (Gaitanos et al., 2016). Taken together, various downstream effectors of ephrin-Eph signaling modulate Rho-GTPase activity through a variety of mechanisms, driving cytoskeletal rearrangement which is necessary for subsequent ephrin-Eph internalization.

Rab-GTPase-mediated intracellular trafficking and the formation of clathrin-coated vesicles also regulate internalization of the ephrin-Eph complex. Rin1, a Rab5 GEF, was demonstrated to bind ephrin-B3 stimulated EphA4 via its Sh2 domain. Following this interaction, Rin1 GEF activity is responsible for driving endocytosis of the ephrin-B3-EphA4 complex in both HeLa and SKN cells. In accordance with these cell line experiments, depletion of Rin1 in mouse primary neurons prevents effective EphA4 internalization (Deininger et al., 2008). This study indicates that Rab5 plays a role in Eph receptor endocytosis. In 293T cells, internalized ephrin-A8-EphA5 complexes colocalize with transferrin (Yoo et al., 2010), a glycoprotein that is taken up via clathrin-mediated endocytosis. In addition, blocking clathrin cage assembly through potassium depletion inhibits EphB-ephrin-B reverse endocytosis in CHO cells. Dynamin, a GTP-ase involved in pinching off clathrin-coated pits, is also an important player in endocytosis of ephrin-Eph complexes. Blocking dynamin function prevents ephrin-B-EphB endocytosis in both forward (Marston et al., 2003; Zimmer et al., 2003) and reverse (Parker et al., 2004) directions in cell lines. As well as promoting endocytosis, clathrin and dynamin play an important role in repulsive cell behavior. Accordingly, a dominant-negative form of dynamin and the clathrin coat assembly inhibitor PAO are each capable of preventing ephrin-B2-stimulated growth cone collapse in primary neurons (Srivastava et al., 2013). Taken together, pathways involved in building and pinching off clathrin-coated vesicles help to internalize ephrin-Eph complexes.

Researchers have begun to flesh out the mechanisms by which ephrin-Eph complexes are internalized in clathrin-coated pits, both in the forward and reverse directions. In a recent study (Evergren et al., 2018), EphB2 forward trans-endocytosis was found to depend on the endocytic scaffolding protein Eps15R. Eps15R interacts with EphB2 through the adaptor protein Numb, and binds directly to other proteins facilitating clathrin coat assembly. One such protein is the clathrin Adaptor Protein 2 (AP-2), which is a major AP for recruiting cargoes into clathrin-coated pits. In addition, through a series of strategic Esp15R truncations, a non-canonical motif (DPFxxLDPF) that binds clathrin heavy chains was identified and shown to be required for the ability of EphB2 and ephrin-B1-expressing cells to separate from one another (Evergren et al., 2018). In contrast to forward endocytosis which requires additional proteins to link Eph to APs, EphB-ephrin-B reverse endocytosis likely occurs via direct interaction between APs and ephrin. Although direct interaction has not yet been observed biochemically, the ephrin-B1 cytoplasmic tail contains a putative AP-binding motif (YXXΦ) (Parker et al., 2004). Together, these studies highlight that, alongside modulators of the actin cytoskeleton, Rab-dependent trafficking and clathrin coat machinery are other important regulators of ephrin-Eph endocytosis.

RECEPTOR ENDOCYTOSIS IS REQUIRED FOR AXON REPULSION: ROBO

As with ephrins, the ability of Robo to produce a repulsive response is highly dependent on its endocytosis. A variety of endocytosis genes have been shown to genetically interact with the slit-Robo pathway using the *slit,robo*$^{/+}$ sensitized background in Drosophila (Chance & Bashaw, 2015). Decreasing the *slit* and *robo* gene doses by half causes a partial loss of repulsion and, as a result, a small subset of neurons from the ipsilateral FasII + neuron population ectopically cross the midline. In this background, mutations of genes involved in clathrin-mediated endocytosis (α-adaptin and endophilinA), and endosomal trafficking (rab5 and rab7) increase ectopic crossing (Chance & Bashaw, 2015). In addition to these genetic interactions, Robo and various elements of endocytic trafficking machinery interact functionally. Rab5, Rab7, and dynamin are observed to positively regulate Robo

endocytosis *in vitro*. In accordance with *in vitro* findings, mutation of these genes causes ectopic Robo expression in commissures as well as inappropriate midline crossing in the embryo. AP-2 is another important regulator of Robo endocytosis. Mutating two AP-2 binding motifs in the Robo C-terminal region inhibits Robo internalization and signaling *in vitro*. In addition, overexpressing Robo with mutant AP-2 binding motifs fails to produce excessive midline repulsion *in vivo*. Instead, this AP-binding mutant Robo acts like a dominant negative and competes for endogenous slit, desensitizing axons to the repulsive signal (Chance & Bashaw, 2015).

SIGNALING FROM THE EARLY ENDOSOME: ROBO AND EPH

After internalization, receptor-ligand complexes make their first stop in the early endosome, which is characterized by the presence of Rab5 or Eea1 (for a review on endosomal trafficking pathways, see Cullen and Steinberg (2018)). The early endosome is an important signaling hub for RTKs including Epidermal Growth factor receptors (EGFRs) (Pennock & Wang, 2003) and Platelet-derived growth factor receptors (PDGFRs) (Wang et al., 2004), provided they remain active and attached to their ligands. While relatively few studies have directly shown Eph receptors signaling from the early endosome, they have been observed to stay ligand-bound (Boissier et al., 2013) and phosphorylated (Marston et al., 2003; Boissier et al., 2013) in these compartments. In addition, ephrin-A2-stimulated EphA1 associates with and activates its downstream effector Tiam1 in the early endosome, but not at the plasma membrane. This suggests that an initial ephrin-Eph internalization event creates a positive feedback loop in which internalized Eph receptors associate with and activate Tiam1, and Tiam1 induces further endocytosis through modulation of the cytoskeleton (Fig. 3F). It is likely that Tiam 1 concurrently drives repulsion by inducing structural changes to growth cone projections, though this has not been investigated as thoroughly as its role in driving endocytosis.

Like Eph receptors, Robo's internalization is ligand-dependent and necessary for its ability to signal through downstream effectors. One such effector is Son of Sevenless (Sos) which is a dual-specificity GEF regulating both Ras and Rho family GTP-ases. In the Drosophila nerve cord, Robo-mediated axon repulsion requires interaction with Sos, which activates Rac1 to induce cytoskeletal rearrangements necessary for repulsive responses (Yang & Bashaw, 2006). In S2R+ cells treated with slit-conditioned media, Sos is recruited to Robo in early endosomes. This slit-dependent recruitment, however, is absent in cells expressing a mutant form of Robo incapable of endocytosis (Chance & Bashaw, 2015). While Sos-driven cytoskeletal remodeling drives morphological changes at the growth cone, it may also be able to drive a feedback loop of endocytosis and further signaling like Tiam1 does for Ephs, though this possibility has not yet been investigated. Taken together, internalization into the early endosome allows both Eph and Robo receptors to recruit the downstream effectors that are necessary to produce changes in axon behavior. Thus, growth cone repulsion depends on the intricate coordination between the actin rearrangements that drive receptor endocytosis and the additional cytoskeletal rearrangements driven by receptor signaling. A major challenge for the field is resolving the spatial and temporal sequence of events that together produce the membrane and cytoskeletal reorganization required for robust axonal responses.

ENDOSOMAL RECYCLING: EPHRIN AND EPH

Upon entry into the endocytic pathway, internalized receptors can take at least two different routes: recycling back to the plasma membrane, or ubiquitination and shunting to later endo-lysosomal compartments for degradation. Both ephrins and Ephs have been reported to undergo recycling. Following ligand stimulation and endocytosis, a subset of EphA2 receptors return to the plasma membrane via two different recycling pathways mediated by Rab11 and Rab4 (Boissier et al., 2013). The Rab4-dependent or "fast pathway" recycles receptors directly from the early endosome, while the Rab11-dependent or "slow" pathway returns receptors to the plasma membrane from perinuclear recycling endosomes. Just as with Ephs, Rab11 is involved with ephrin recycling. A recent study reported that the Rab11 adaptor protein Rab11fip5 can form a complex with ephrin-B1 and GTP-bound Rab11, and its rab-binding domain is critical for recycling ephrin-B1 to the cell surface in Xenopus neuroectoderm explants (Yoon et al., 2021). Ephrin-B1 recycling appears to be important for proper development of the embryonic frog brain, as knockdown of rab11fip reduced telencephalon size in a manner similar to ephrin-B1 knockdown (Yoon et al., 2021).

Receptor recycling is a critical means by which ephrin-Eph signaling can be regulated. One way recycling can control ephrin-Eph signaling is by suppressing extraneous signal from autonomously activated Eph receptors. Autonomously activated receptors are a small population of the total receptor pool that signal in a ligand-independent manner due to events such as random receptor collision or spontaneous conformational change in their catalytic domains (Sabet et al., 2015). In COS-7 cells, ephrin-independent Eph activation was observed using a fluorescence resonant energy transfer (FRET) sensor that produces signal when the Eph kinase domain adopts its active conformation and becomes auto-phosphorylated. Protein tyrosine Phosphatases (PTPs) dephosphorylate activated RTKs, making them an important counterbalance against this ligand-independent autophosphorylation. PTPB1 is an ER-localized PTP which exhibits low phosphatase activity near the plasma membrane and high activity in the perinuclear region, raising the possibility that such dephosphorylation happens in the Rab11-dependent recycling pathway. Indeed, autonomously activated Eph receptors are shunted to the rab11 positive pericentriolar recycling endosome, placing them in an area with high PTPB1

activity (Sabet et al., 2015). This change in localization facilitates Eph deactivation via dephosphorylation, ultimately leading to the return of these receptors to the plasma membrane.

The recycling endosome also plays an important role in the ability of axons to adapt to changing levels of ephrin-Eph signaling on their path toward a synaptic target. Chick retinal ganglion cells can become habituated to and re-sensitized to ephrin-A-EphA signaling, in both forward and reverse directions (Fiederling et al., 2017). Desensitization to prolonged signaling occurs through removal of ephrins and Ephs from the growth cone surface via clathrin-dependent endocytosis, while re-sensitization depends on recycling of these molecules to the cell membrane. The recycling endosome coordinates this process of adaptation by providing a temporary storage area for internalized ephrins and Ephs, allowing them to be reinserted into the growth cone membrane at a later time (Fiederling et al., 2017). In summary, the recycling endosome is a critical organelle for regulating ephrin-Eph signaling, allowing cells to suppress inappropriate receptor activation as well as adapt to a changing extracellular environment.

ENDOSOMAL RECYCLING: ROBO

As with ephrin-Eph signaling, endosomal recycling allows growth cones to alter their sensitivity to slit-Robo signaling. Robo is initially deposited into the growth cone membrane in response to a floor-plate derived signal, possibly slit, as floor plate-conditioned media triggers Robo insertion at the cell surface in cultured neurons (Pignata et al., 2019). Following this initial insertion of Robo onto the growth cone surface, axons receive their first exposure to slit cues secreted by the midline. Upon reaching the midline, the initial slit exposure triggers a positive feedback loop of repulsive Robo signaling that helps propel them out of the floor plate and keeps them restricted to the contralateral side of the body. This positive feedback loop is dependent on both Robo receptor endocytosis and recycling of Robo back to the growth cone surface. Exposure to slit increases axonal Robo levels in mouse commissural neurons and post-crossing commissural neurons exhibit stronger slit-induced axon collapse than their slit-naïve pre-crossing counterparts (Kinoshita-Kawada et al., 2019). To understand the origin of this increase in Robo levels and repulsive response, antibody feeding assays were used to visualize the dynamics of total, surface, internalized, and freshly membrane-inserted pools of Robo receptor in dorsal spinal cord neurons. Interestingly, unliganded Robo is constitutively targeted for degradation via the proteasome, while slit-stimulated Robo is endocytosed and recycled back to the growth cone (Kinoshita-Kawada et al., 2019). Although slit had a stabilizing effect on Robo, however, an increase in Robo surface levels was not observed when compared to slit-naïve cells. In addition, the antibody feeding paradigm used in this study was unable to directly demonstrate the mobilization of internalized Robo to the surface. With that said, Robo recycling and stabilization following initial slit exposure can still affect axons' response to future slit stimulus and serve as one possible means among others which eventually lead to increased Robo surface levels in post-crossing axons.

Several components of endosomal trafficking machinery are involved in the process of Robo stabilization and recycling at the growth cone following initial slit exposure. Knockdown of Rab5 or Rab11 reduces Robo surface upregulation as well as axon responsiveness to slit in cultured neurons (Kinoshita-Kawada et al., 2019). Similarly, pharmacological treatment blocking clathrin-mediated endocytosis and dynamin eliminate slit response. In addition, Arf6, a GTP-ase involved in both endocytosis and recycling, as well as its activators, Cytohesins, were identified as important players in elevating Robo levels and slit sensitization in post-crossing commissural neurons. Cytohesins bind the CC2 and CC3 motifs in the Robo cytodomain and knockdown of Cytohesins 1 or 3 eliminates slit responsiveness as well as slit-induced Arf6 activation *in vitro*. In the spinal cord, Arf6 mutation, as well as Cytohesin 1 or 3 knockdown lead to axon stalling in the floor plate or at its contralateral edge, reminiscent of the Robo mutant phenotype (Kinoshita-Kawada et al., 2019). Taken together, this data points to a model of slit sensitization in which slit-stimulated Robo activates Arf6 via Cytohesins 1 and 3. Through Arf 6, as well as Rab5 and 11, ligand activated Robo is protected from degradation and is targeted for clathrin-mediated endocytosis and later recycling back to the growth cone surface. When recycled to the surface, Robo increases growth cone sensitivity to slit, allowing for expulsion of the axon out of the floor plate.

In addition to Arf6/Cytohesin-mediated trafficking, another recycling-based mechanism upregulates Robo surface expression in axons that have entered the floor plate. RabGDI and calsyntenin-1 cooperate in these axons to insert Robo into the growth cone membrane from recycling endosomes (Philipp et al., 2012; Alther et al., 2016). RabGDI is a component of vesicle fusion machinery and is involved in regulating Rab-GDP conversion in to Rab-GTP, while Calsyntenin1 links Rab11-positive recycling endosomes to kinesin, the motor for forward transport down an axon. Robo, RabGDI, rab11 and calsyntenin all have partially overlapping expression domains in the chick spinal cord (Alther et al., 2016). Knockdown of either RabGDI or calsyntenin-1 inhibits Robo insertion into the growth cone surface in cultured chick commissural neurons (Alther et al., 2016). Accordingly, low dose RNA against calsyntenin-1 and RabGDI, which reduces expression of both genes to hypomorphic levels, induces a floor plate stalling phenotype, suggesting an insensitivity to slit signaling. In summary, these results indicate that Rab GDI and calsyntenin-1 may work together to enable midline exit by modulating crossing axons' sensitivity to slit through delivery of Robo to the growth cone membrane. Taken together, endocytosis and later recycling to the membrane, help sensitize growth cones to slit signaling. Whether recycling endosomes are also involved in de-sensitizing and habituating to Slit, however, remains to be investigated.

LYSOSOMAL DEGRADATION

Lysosomal degradation is another pathway that ligand-activated Eph receptors can take upon reaching the early endosome. Cargoes destined for degradation remain in the early endosome, which matures into a late endosome and ultimately a multi-vesicular body (MVB). Over the course of this maturation, the interior of the endosome becomes increasingly acidic and the endosome membrane buds inward to form intraluminal vesicles. Finally, the MVB fuses with the lysosome and proteases degrade its cargo. The lysosomal degradative pathway is critical for maintaining normal cell physiology and homeostasis, by removing unwanted or damaged proteins and recycling important cellular building blocks like amino acids. In general, the degradative pathway is critical for downregulating and limiting the duration of ephrin-Eph signaling.

Ubiquitination is an important switch regarding a protein's fate in the early endosome, often targeting a protein for degradation. As with Robo, ubiquitination is an important signal for sorting activated Ephs to lysosomes. Cbl (named after Casitas B-lineage Lymphoma), a RING finger E3 ubiquitin ligase, is known to regulate a variety of RTKs including Ephs. In CHO cells, stimulation with ephrin causes Cbl recruitment to activated EphB1 receptors. Cbl binds to EphB1 via its tyrosine kinase binding (TKB) domain, and intact EphB1 kinase activity is required for this interaction. Once recruited to EphB2, Cbl is phosphorylated by Src kinase, and this activating phosphorylation allows it to ubiquitinate EphB2, targeting it for degradation (Fasen et al., 2008). Cbl also ubiquitinates and downregulates EphAs (Boissier et al., 2013; Sabet et al., 2015). In this context, Cbl is recruited to ephrin-stimulated EphA2 and binds to a phosphorylated tyrosine in the Cbl docking sequence (YXXXP) in the EphA2 cytoplasmic domain. This interaction leads to EphA2 ubiquitination and subsequent trafficking to late endosomes.

In addition to ubiquitin ligases, a cell's ESCRT (endosomal sorting complexes required for transport) machinery is critical for regulating whether Ephs are shunted down a degradative pathway. ESCRTs are complexes that recognize ubiquitinated cargoes and route them into the forming intraluminal vesicle of a late endosome in the process of maturing into an MVB. Several components of the ESCRT machinery were identified in a recent proteomics screen for EphB2 interactors, including the ESCRT regulatory protein HT-PTP (Lahaie et al. 2019). HT-PTP binds ligand-activated EphB2 and protects it from lysosomal degradation. In addition, loss of HT-PTP caused aberrant ephrin-B-EphB-mediated axon guidance in lateral motor column neurons, demonstrating that regulation of Ephs via ESCRT machinery is important for proper neuronal wiring. Taken together ubiquitin ligases and proteins that interact with ubiquitinated cargos serve as switches to determine whether Eph receptors enter recycling or degradative pathways.

While transport to late endosomes and beyond usually marks the end of a receptor's signaling lifetime, there are notable exceptions. In certain special cases, Eph and ephrin receptors can maintain the ability to signal after entering the late endocytic pathway. For example, Ephs can signal through exosomal vesicles (EVs), which derive from the intraluminal vesicles of MVBs and whose production relies on ESCRT machinery. Instead of fusing to the lysosome, certain MVBs fuse with the plasma membrane, releasing their intraluminal vesicles into the extracellular space as EVs. For example, EphB2 can be secreted in EVs, both in 293Tcells and cultured cortical neurons (Gong et al., 2016). Interestingly, Ephb2-containing exosomes are able to induce reverse signaling and growth cone collapse in ephrin-B2 expressing neurons, indicating that not all Eph-ephrin signaling requires cell contact and that signaling may occur at longer ranges than originally thought.

Not only is signaling range longer than previously believed, but signaling duration is as well. While lysosomes are usually associated with quenching intracellular signaling, a recent study (Valenzuela & Perez, 2020) demonstrated that ephs can retain signaling ability all the way to the lysosome in HeLa and MBD-MBA231 cells. Notably, a trans-endocytosed complex of ephrin-A2-EphA1 was shown to remain intact throughout the late endosomal pathway, allowing EphA2 to signal intracellularly. Interestingly, the majority of ephrin-A1-EphA2 complexes are routed to lamp1-positive lysosome-like compartments which are acidic but non-degradative. These compartments, dubbed "signaling lysosomes" serve as long-term storage containers for ligand bound, activated Eph receptors and persist in daughter cells following mitosis, suggesting the intriguing possibility that ephrin signaling could persist over a few cell generations. Overall, research on the fate of ephrins and Ephs in the lysosome reveals fascinating mechanisms of signaling for these molecules and opens up a rich area of study for other families of axon guidance receptors.

FUTURE DIRECTIONS

The last 25 years of research have highlighted the importance of receptor trafficking as a means of regulating ephrin-Eph and slit-Robo signaling. Nevertheless, there remain many open questions. While a large body of research documents the trafficking of Robo and EphRs following internalization, much less is known about the mechanisms governing the timing and spatial distribution of their expression on the growth cone surface. The late endosomal/lysosomal pathway is another fertile ground for inquiry. While recent studies have observed interesting mechanisms of ephrin-Eph signaling through a late endosomal pathway *in vitro*, it remains unknown whether these unconventional signaling methods are important for axon guidance or other *in vivo* developmental events. In addition, the possible ability of Robo and other repulsive receptors to signal from late endosomes/lysosomes has not yet been investigated. Whether these receptors can remain ligand-bound and in active structural conformations in the lysosome, and whether they can interact with downstream effectors from this compartment, are

questions which remain open for testing. Furthermore, while ubiquitination and lysosomal degradation play a large role in preventing early Robo expression at the growth cone membrane, the role of these processes in terminating Robo signaling has not been studied. Finally, it remains to be seen whether cytoskeletal remodeling regulates Robo endocytosis in a similar manner to Eph endocytosis. As many of Robo's effectors such as Sos (Yang & Bashaw, 2006) and the Wave Regulatory Complex (Chaudhari et al., 2021) modulate the actin cytoskeleton, it is possible that these effectors drive repulsion through a combination of endocytosis and their better known functions of inducing structural changes to growth cone processes. While we have been investigating the regulation of repulsive axon guidance factors for the last quarter century, the numerous questions remaining in the ''regulation era'' of research will keep us occupied for many years to come. Indeed, the work of Bonhoeffer, his team, and his contemporaries has set up a strong foundation on which we will continue to build.

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