CHARACTERIZING THE MOLECULAR MECHANISMS OF AXON GUIDANCE: ACTIVATION AND REGULATION OF THE AXON GUIDANCE RECEPTOR PLEXIN A

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To Whom I Can Survive For the Past, the Present, and the Future

To My Family and Friends

To My Wife, Eun Sil Kim

and

To My Sons,

Albert Jinyoung Yang and Timothy Juyoung Yang

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CHARACTERIZING THE MOLECULAR MECHANISMS OF AXON GUIDANCE: ACTIVATION AND REGULATION OF THE AXON GUIDANCE RECEPTOR PLEXIN A

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Neuronal connectivity is precisely determined by axonal pathfinding during development. The navigating axons detect attractive and repulsive environmental cues by axon guidance receptors. However, the biochemical means through which multiple signaling pathways are integrated in navigating axons is poorly understood. Semaphorins are the largest family of axon guidance cues and utilize Plexin receptors to exert repulsive effects on axon extension. The intracellular region of Plexins contains a Ras GTPase activating protein (GAP) domain, which

is necessary for repulsive guidance effects. Previous studies suggest that activation of Plexin RasGAP requires interactions with both Semaphorin at the extracellular region and a Rho-family GTPase at the Rho family GTPase-binding domain (RBD). Interestingly, Semaphorin repulsion can be rapidly "turned-off" by other distinct cues and signaling cascades. However, the molecular mechanisms to activate or modulate Plexin RasGAP remain unclear. First, to further understand how the Plexin RasGAP is activated, I collaborated with the Zhang lab, and following determination of the crystal structure of the intracellular region of Plexin, I examined the roles of residues interfacing with the RasGAP domain using functional mutagenesis in the *Drosophila* model system. Our results demonstrate that Plexin exhibits an auto-inhibited conformation, and suggest that interaction among the previously uncharacterized juxtamembrane segment, the RBD, and the RasGAP domain is critical for Plexin RasGAP activation. Second, to better understand how Semaphorin/Plexin signaling is modulated, I characterized the results of a large-scale screen to look for proteins interacting with the cytoplasmic portion of Plexin and identified the phosphoserine binding protein 14-3-3ε as a specific Plexin-interacting protein. My results reveal that 14-3-3ε is specifically required for axon guidance during development. Moreover, Protein kinase A is found to phosphorylate Plexin in the RasGAP domain and mediates the 14-3-3ε interaction. Plexin-14-3-3ε interactions prevent Plexin from interacting with its Ras-family GTPase substrate,

which effectively switches Plexin-mediated axonal repulsion to Integrin-mediated adhesion. These findings uncover both a new molecular integration point between important axon guidance signaling pathways and a biochemical logic by which this guidance information is coalesced to steer the growing axon. Therefore, these new observations on activating and silencing specific signals that are repulsive to axon growth also illuminate new approaches to neutralize axonal growth inhibition and encourage axon regeneration.

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Yang, T. and Terman, J.R., (2011). 14-3-3ε couples protein kinase A to Semaphorin signaling and silences Plexin RasGAP-mediated axonal repulsion. *Neuron* In Press.

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LIST OF DEFINITIONS

AC	adenylate cyclase
ADP	adenosine diphosphate
AKAP	A kinase anchoring protein
Arf	ADP ribosylation factor
Arp2/3	actin-related protein 2/3
cAMP	cyclic adenosine monophosphate
Cdc42	Cell division control protein 42 homolog
CNS	central nervous system
DOCK	dedicator of cytokinesis
ECM	extracellular matrix
FAK	focal adhesion kinase
FasII	fasciclin II
FPLC	fast protein liquid chromatography
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GST	glutathione S-transferase
GTP	guanosine triphosphate
HA	hemagglutinin
ISN	intersegmental nerve
ISNb	intersegmental nerve b
Mical	molecule interacting with CasL
MLCK	myosin light chain kinase
Myc	myelocytomatosis viral oncogene homolog
Nck	non-catalytic region of tyrosine kinase adaptor protein
N-WASP	neuronal Wiskott-Aldrich Syndrome protein
Pak	p21 activated kinase
PDE	phosphodiesterase
PI3K	phosphatidylinositol-3 kinase
PI4P5K	phosphatidylinositol-4-phosphate 5-kinase
PIP2	phosphatidylinositol-4,5-biphosphate
PLC	phospholipase C
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PP2B	protein phosphatase 2B

PTEN	phosphatase and tensin homolog
Rab	Ras-related in brain
Rac	Ras-related C3 botulinum toxin substrate
Ran	RAs-related nuclear protein
Rap	Ras-related proteins
Ras	rat sarcoma
RBD	RhoGTPase binding domain
Rho	Ras homolog
RIAM	Rap1-GTP-interacting adaptor molecule
ROCK	Rho-associated protein kinase
R-Ras	related RAS
SNa	segmental nerve a
VNC	ventral nerve cord

CHAPTER ONE

General Introduction

Neuronal Circuit Formation and Axon Guidance

The nervous system is formed to coordinate an animal's reaction toward external stimuli and internal needs. To accomplish this goal, a hundred billion neurons in the human nervous system, for instance, are precisely wired in a specific way by connecting the nervous system including the brain and the spinal cord with different parts of body (Tessier-Lavigne and Goodman, 1996; Goodman and Shatz, 1993).

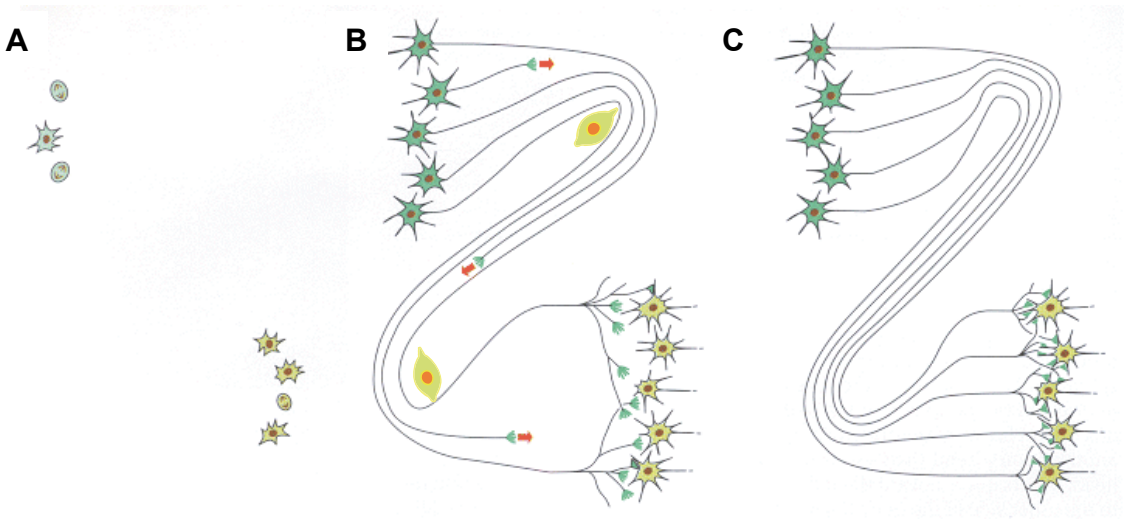

Figure 1.1 Three phases of neural development Neuronal connections generally develop through three developmental stages. Neurons are differentiated from neuroprogenitors (A) and send out axons which explore their environment to get to their targets (B, arrows). Axons make synapses and these synapses are further refined by neuronal activity throughout life (C). Modified from Alberts et al., 2002.

These complex networks of neurons generally develop through three different developmental stages (Raper and Mason, 2010; Alberts et al., 2002; Chedotal and Richards, 2010). First, neuroprogenitor cells proliferate and

differentiate into neurons (Figure 1.1A). Second, after a single axon is specified from multiple neurites, the axon extends to its targets (Polleux and Snider, 2010)(Figure 1.1B). Third, axons make synapses with targets and these synapses are strengthened or eliminated according to neuronal activity (Figure 1.1C). The goals of my dissertation research are to better understand how neurons are precisely connected and unravel underlying molecular mechanisms that build neuronal circuits.

Function of Growth Cones in Axon Guidance

At the stage of axonal navigation, pioneering axons extend first and "pave a road" for "follower" axons (Raper and Mason, 2010; Chedotal and Richards, 2010). To reach a target that is located a long distance away, these pioneering axons are attracted or repelled by guidance cues that are presented by intermediate "guide post cells" (Figure 1.1B) (Raper and Mason, 2010). These attractive and repulsive guidance cues influence growth cones over both short and long distance to direct axonal pathfinding (Tessier-Lavigne and Goodman, 1996; Huber et al., 2003; Dickson, 2002). In addition to these guidance cues, axons also require adhesive substrates such as the extracellular matrix, other axons, or target cells to attach and elongate on. Axons that extend during later stages follow the pioneering axons and adhere or fasciculate with them to increase the efficiency

and reliability of their pathfinding (Raper and Mason, 2010). These fasciculated axons also selectively defasciculate from one another at their target fields.

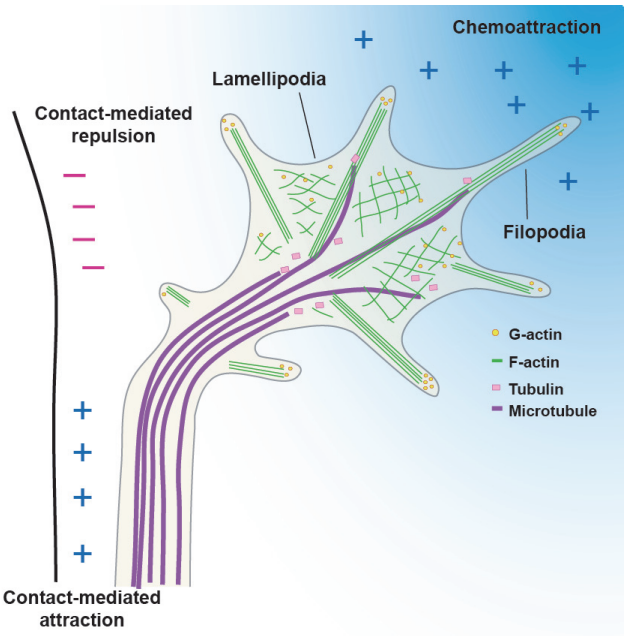

Figure 1.2 Function of the growth cone in axon guidance The growth cone at the tip of growing axons is a motile structure that navigates environments by integrating multiple guidance cues to reach target cells. This growth cone consists of filopodia and lamellipodia. These structures are supported by actin filaments and microtubules. Guidance receptors detect both attractive and repulsive guidance cues and integrate this antagonistic information into changes in cellular signaling. These signaling cascades regulate both the cytoskeletal and adhesive machinery in the growth cone and thereby "tell" axons to extend, turn, or stop. Modified from Dickson, 2002.

The leading edge of a growing axon, the growth cone, is a fan-shaped highly motile structure comprised of spike-like filopodia and web-like lamellipodia, that is susceptible to morphological changes in response to environmental cues (Figure 1.2) (Dent and Gertler, 2003; Dent et al., 2011). Filopodia are supported by bundled filamentous (F) actin and lamellipodia are

supported by mesh-like actin. In addition to these actin structures, microtubules strengthen the long axon shaft and also stabilize growth cones. Growth cones detect a range of attractive and repulsive guidance cues via guidance receptors that are located on the surface of filopodia. These opposing effects are transduced by intracellular signaling cascade that specify a change in cytoskeletal dynamics and axon-axon/axon-substrate adhesion (Bashaw and Klein, 2010; Suter and Forscher, 1998; Pollard and Borisy, 2003). Thus, by expressing different sets of guidance receptors, or by modulating cellular signaling states, each growth cone differentially regulates cytoskeletal dynamics and adhesion and so "chooses" to extend further, steer in another direction, or stop growing (Huber et al., 2003; Bashaw and Klein, 2010; Kolodkin and Tessier-Lavigne, 2011).

Molecular Mechanisms of Axon Guidance

More than half-a-century of intense study has now resulted in the identification of a number of axon guidance cues and their receptors (Dickson, 2002). More recently, the characterization of the downstream signaling molecules utilized by different guidance receptors has revealed common pathways that link guidance receptors to the cellular machinery that modulates growth cone movement and adhesion (Kolodkin and Tessier-Lavigne, 2011; Bashaw and Klein, 2010; Huber et al., 2003). These common pathways include small GTP binding proteins and secondary messengers such as cyclic nucleotides and calcium ions (Figure 1.3)

(Dickson, 2001; Hall and Lalli, 2010; Tojima et al., 2011; Gomez and Zheng, 2006; Song and Poo, 1999).

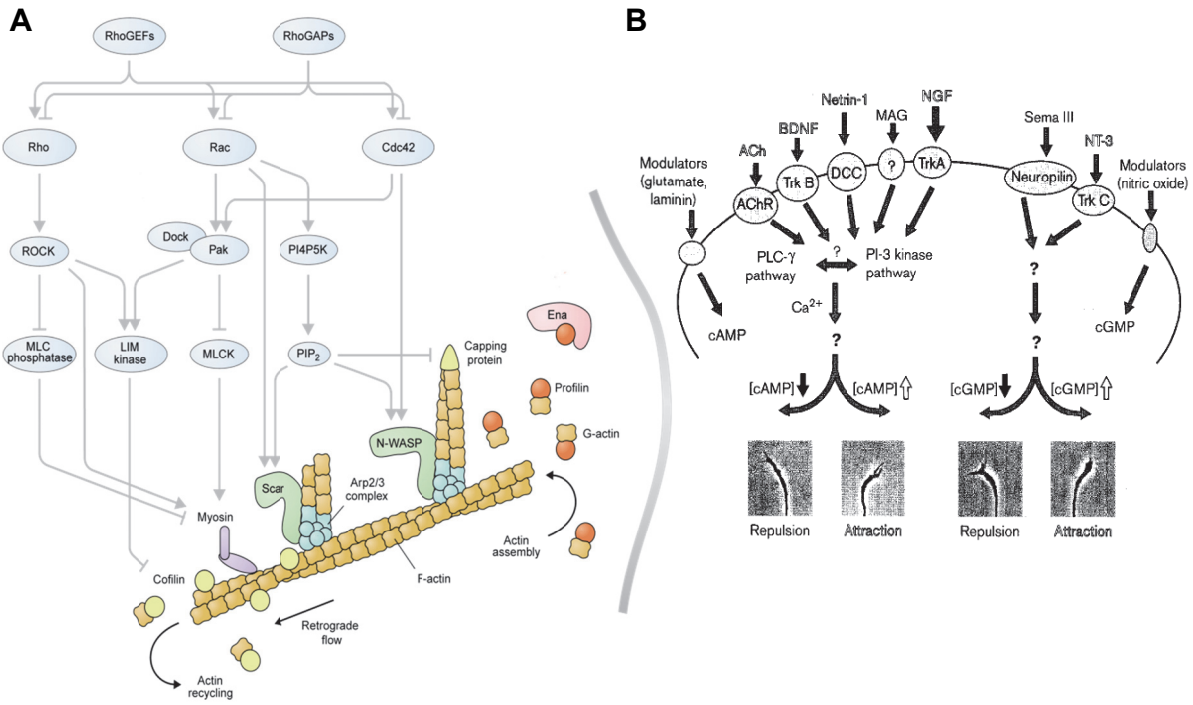

Figure 1.3 Signaling cascades that modulate the behaviors of growth cones responding to guidance cues (A) Rho family GTPase proteins regulate actin cytoskeleton dynamics, for example, actin filament elongation and branch formation. Adapted from Dickson, 2001. (B) Guidance cues are differently interpreted depending on the relative amount of cyclic nucleotides and calcium ions. Adapted from Song and Poo, 1999.

Function of Small GTPases in the regulation of cytoskeletal and adhesive dynamics Small GTP binding proteins (GTPases) have GTP hydrolysis activity, thereby, working as molecular switches that "turn-on" in the GTP bound form and "turn-off" in the GDP bound form (Hall and Lalli, 2010). GTP bound GTPases

preferentially associate with downstream effector molecules and activate their function either by recruiting effectors close to the membrane or by inducing conformational changes. The GTPase activity of GTPases is slow so that GTPase activating proteins (GAPs) accelerate the endogenous GTPase activity by controlling signal duration. In contrast, guanine nucleotide exchange factors (GEFs) stimulate the release of bound GDP, allowing for replacement with GTP, which is abundantly present in the cytoplasm. Therefore, GEFs "turn-on" and GAPs "turn-off" GTPase activity.

Among five Ras superfamily GTPases, such as Ras, Rho, Rab, Arf, and Ran, Rho family proteins have been best characterized as regulators of cytoskeletal structures (Hall, 1998; Dickson, 2001; Colicelli, 2004). For example, microinjection studies using fibroblast cells reveal that RhoA induces actin stress fibers and focal adhesion (Hall, 1998; Ridley and Hall, 1992). Likewise, Rac activation results in the formation of lamellipodia, as does Cdc42 for the formation of filopodia (Ridley and Hall, 1992). Although still far from completely understood, these Rho family GTPases influence cytoskeletal dynamics by activating downstream effector proteins (Dickson, 2001)(Figure 1.3A). For example, Rho is known to activate its effector Rho kinase (ROCK), which phosphorylates myosin light chain phosphatase and myosin light chain. These phosphorylation events subsequently increase the retrograde flow of actin filaments by enhancing actomyosin contractility (Dent et al., 2011). ROCK also

phosphorylates LIM kinase which phosphorylates cofilin, an actin dynamizing protein. Cofilin binds to both monomeric and filamentous actins with a preference to bind to ADP-bound actin. When bound to F-actin, cofilin severs actin filaments, increasing the rate of actin depolymerization. These cofilin activities lead to filament assembly by increasing available polymerization-competent barbed ends and monomeric actin. Therefore, inactivation of cofilin by phosphorylation by LIM kinase has been implicated in growth cone collapse (Hung and Terman, 2011). Likewise, Rac and Cdc42 share a common downstream effector, p21-activated kinase (PAK). PAK is known to activate LIM kinase, which inactivates myosin light chain kinase (MLCK), resulting in an inactivation of myosin and a decrease in actomyosin contractility. Rac also has been found to activate phosphatidylinositol-4-phosphate 5-kinase (PI4P5K). This PI4P5K generates phosphatidylinositol-4,5-biphosphate (PIP_2), which is used as a substrate for phospholipase C (PLC) or an activator of numerous downstream proteins including N-WASP (neuronal Wiskott-Aldrich Syndrome protein), an actin nucleation and branching factor working together with Arp2/3 (actin-related protein 2/3) complex. Cdc42 and PIP_2 cooperatively activate N-WASP by binding to an auto-inhibitory domain and activating Arp2/3.

Function of Small GTPases in the Integrin-mediated adhesion In addition to the regulation of cytoskeletal dynamics, Ras superfamily GTPases also regulate

cell-cell or cell-substrate adhesion by associating with Cadherins and Integrins (Giancotti and Ruoslahti, 1999; Kaibuchi et al., 1999; Fukata and Kaibuchi, 2001). Cadherins cluster together through Ca^{2+}- dependent homophilic dimerization, which results in cell-cell adhesion. The intracellular domain of Cadherins is linked to actin filaments through its association with α-catenin and β-catenin. These structures are further stabilized by Rac/Cdc42 activated IQGAP1 (Noritake et al., 2005). Likewise, Integrins are receptors for extracellular matrix (ECM) proteins including Laminins, Fibronectins, and Collagens (Giancotti and Ruoslahti, 1999; Humphries et al., 2006; Ridley et al., 2003). Integrins consist of α and β subunits. These subunits make heterodimers and bind to their ligands, which enables adherence to a substrate. These ECM components activate Integrins, which result in the reorganization of the actin cytoskeleton and initiate downstream signaling through diverse protein tyrosine kinases such as focal adhesion kinase (FAK). Integrins are also connected with the actin cytoskeleton by making protein complexes which involve talin, paxillin, and vinculin (Wiesner et al., 2005). Ras family GTPases, R-Ras and Rap1, have been known to promote Integrin-mediated cell adhesion (Zhang et al., 1996; Bos et al., 2001; Kinbara et al., 2003; Shattil et al., 2010). Although the molecular mechanism of how R-Ras activates Integrin-mediated adhesion is not completely understood (Kinbara et al., 2003), a possible mechanism suggests that R-Ras enhances Integrin activity by regulating endocytosis of Integrins through increasing membrane dynamics

(Conklin et al., 2010). Rap1 activates its effectors, RIAM and RAPL, which directly associate with Integrin complex and enhance Integrin activity (Kinbara et al., 2003; Mor et al., 2007). It should also be noted that Integrin signaling is bidirectional. For example, ECM binding triggers Integrin downstream signaling

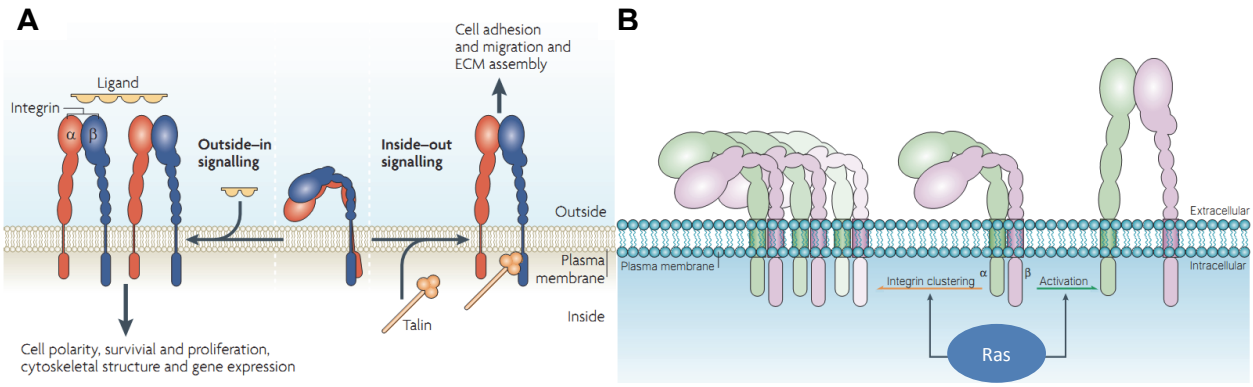

Figure 1.4 Bidirectional Integrin signaling and the activation by Ras family GTPase (A) Integrin signaling is bidirectional. ECM ligands cluster Integrins and induce classical "outside-in" receptor signaling. Intracellular activators, such as Talin, function as "inside-out" by inducing conformational changes of Integrins that result in increased affinity for ECM. Adapted from Shattil et al., 2010. (B) Ras family GTPases, can activate Integrin by inducing clustering of or changing conformations of Integrins. Adapted from Kinbara et al., 2003.

as "outside-in", and Integrin activation enhances Integrin's ability to bind to ECM as "inside-out" (Figure 1.4A) (Giancotti and Ruoslahti, 1999; Kinbara et al., 2003). The activation of Ras family GTPases is known to increase Integrin-mediated cell adhesion by inducing clustering of Integrins or conformational changes in the extracellular domains of Integrins, which consequently enhances avidity or affinity toward the ECM (Figure 1.4B) (Kinbara et al., 2003; Shattil et al., 2010).

Roles of adhesion in cellular migration It is noteworthy that cytoskeletal and adhesive complexes are closely connected in cell migration, such that their concerted activity is essential for the directional movement of growth cones during axon guidance (Hoang and Chiba, 1998; Kaibuchi et al., 1999; Suter and Forscher, 2000; Stevens and Jacobs, 2002; Nakamoto et al., 2004; Wiesner et al., 2005; Huang et al., 2007). A simplified view of cell migration which can also be applicable to growth cone guidance suggests that initial protrusions such as filopodia and lamellipodia develop by actin nucleation/polymerization (Pollard and Borisy, 2003; Ridley et al., 2003). Then, protrusions are stabilized by attaching to ECM or adjacent cells through Integrins, which are connected to the actin cytoskeleton (Wiesner et al., 2005). These adhesions provide "feet" for migration. The expansion of membrane at the leading edge results from the unbending of an elongated actin filament, not just by actin polymerization (Dickson, 2001; Dent et al., 2011). These steps cycle in the course of growth cone extension.

Function of second messengers in axon guidance As noted above, the activity of Rho and Ras family GTPases is subject to the regulation by multiple guidance receptors due to their broad influence on the regulation of the actin cytoskeleton and adhesion (Dickson, 2001; Hall and Lalli, 2010). For example, guidance receptors regulate the amount of active Rho GTPases by activation of GEFs or

GAPs, or recruit GTPases to the vicinity of active receptors via adaptor molecules such as Dock/Nck (Bos et al., 2007; Dickson, 2001; Kruger et al., 2005; Hall and Lalli, 2010). Moreover, growing evidence indicates that the response of growth cones to specific guidance cues can be converted or suppressed by the relative cellular levels of second messengers such as cyclic nucleotides and calcium ions (Song and Poo, 1999; Gomez and Zheng, 2006; Piper et al., 2007; Tojima et al., 2011). For example, the manner in which a growth cone responds to a particular guidance cues, is altered with increasing levels of cAMP (Figure 1.3B) (Song and Poo, 1999; Tojima et al., 2011). The increased level of these secondary messengers is thought to trigger the local or global activation of downstream effectors like protein kinases, phosphatases, or proteases (Tojima et al., 2011; Gomez and Zheng, 2006). Likewise, the coordinated activity of these proteins together with small GTPases and cell adhesion proteins regulates cytoskeletal dynamics, substrate adhesion, and asymmetric endocytosis/exocytosis of plasma membrane to result in growth cone turning (Bashaw and Klein, 2010; Huber et al., 2003).

Semaphorin/Plexin exerts Repulsive Guidance Responses

Semaphorin family The Semaphorin family of secreted and membrane-associated proteins is one of the largest families of guidance cues, which also function in other cellular processes such as migration, cytoskeleton

reorganization, and adhesion (Yazdani and Terman, 2006; Tran et al., 2007; Kruger et al., 2005). Semaphorins were first identified from two independent studies. One group while screening for molecules recognized by a monoclonal antibody identified Semaphorin as a new axonal glycoprotein important for normal growing of pioneering axons in the grasshopper (originally named Fasciclin IV) (Kolodkin et al., 1992). Another group found that chick brain membrane causes "collapse" of the growth cone structure of growing axons and using a biochemical purification scheme identified a Semaphorin (originally called Collapsin) as the molecule responsible (Fan et al., 1993; Raper and Kapfhammer, 1990; Luo et al., 1993). Further studies revealed that Semaphorins are phylogenetically conserved from nematodes to humans (and are also present in viruses), and comprise five different classes (Class 3 to Class 7) in humans, three classes (Classes 1, 2, and 5) in *Drosophila*, and Class V for viral encoded Semaphorins (Figure 1.5A) (Yazdani and Terman, 2006; Kruger et al., 2005).

Plexin family Plexin family transmembrane proteins serve as receptors for Semaphorins, exerting major downstream signaling in the growth cone (Negishi et al., 2005; Yazdani and Terman, 2006). Plexin was first identified as a calcium-dependent homophilic cell adhesion molecule involved in the formation of retinal

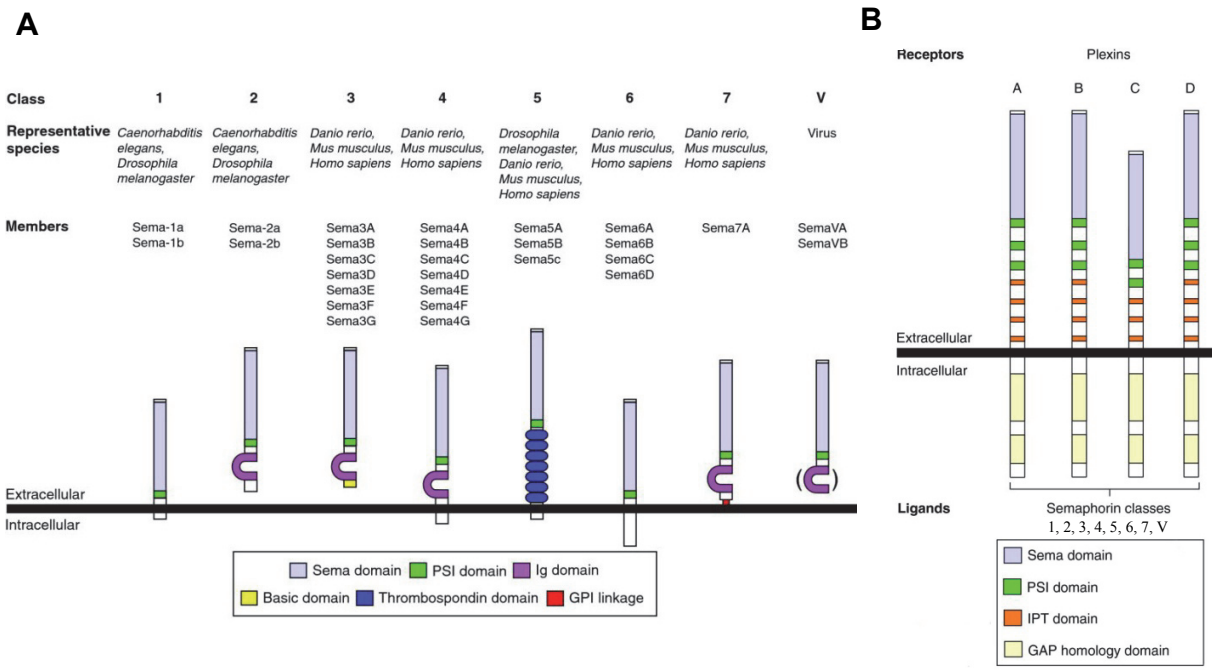

Figure 1.5 Classification and primary structure of members of the Semaphorin and Plexin protein families (A) Semaphorins are secreted or membrane-associated proteins with a conserved Sema domain at their amino-termini. Invertebrates encode Class 1, 2, and 5 Semaphorins, whereas vertebrates encode Class 3, 4, 5, 6, and 7 Semaphorins. Some DNA viruses also encode Class V Semaphorins, which are structurally similar to Class 7 Semaphorin. (B) Plexins are structurally similar to each other with a Sema domain at the amino-termini and a GAP homology domain at the carboxyl-termini. Plexins are known for their ability to bind to Semaphorin ligands. A Class Plexins interact with Class 1 and 6 Semaphorins, B Class Plexins with Class 2, 4, and 5 Semaphorins, C Class Plexins associate with Class 7 and V Semaphorins, and D Class Plexins with Class 3 Semaphorins (Kruger et al., 2005; Ayoob et al., 2006). A Class Plexins also associate with Class 3 Semaphorins in the presence of their ligand-biding neuropilin coreceptors. Adapted from Yazdani and Terman, 2006.

plexiform layers from a screen for molecules recognized by a monoclonal antibody in *Xenopus* (Ohta et al., 1992; Satoda et al., 1995; Ohta et al., 1995). The function of Plexin as a neuronal Semaphorin receptor was revealed by an in vivo study using *Drosophila* embryonic motor axons as a genetic model (Winberg et al., 1998b). Plexins are grouped into four classes and Semaphorin ligands for

each class of Plexins have been identified (Figure 1.5B) (Yazdani and Terman, 2006; Ayoob et al., 2006; Kruger et al., 2005). A growing literature now indicates that the primary function of Plexins is to mediate the repulsive effects of Semaphorins, which prevent growing axons from projecting into inappropriate areas (Tran et al., 2007; Kolodkin and Tessier-Lavigne, 2011; Kruger et al., 2005). Multiple combinations of Semaphorin and Plexin interactions have shown to be critical for the construction of major axon tracts including those originating in the olfactory bulb, the thalamus, the spinal cord, the retina, and the hippocampus (Chedotal and Richards, 2010; Koncina et al., 2007).

Molecular and biochemical mechanisms of Semaphorin/Plexin signaling The repulsive guidance effects of Semaphorins/Plexins on navigating axons have been well established using the *Drosophila* model neuromuscular system (Winberg et al., 1998a; Kolodkin et al., 1992; Winberg et al., 1998b). As previously mentioned, *Drosophila* Plexin A (PlexA) mediates repulsive guidance roles of the Semaphorin (Sema)-1a. Guidance defects found in *PlexA* loss of function (LOF) mutants resemble those seen in *Sema1a* LOF mutants (Yu et al., 1998; Winberg et al., 1998b). For example, in either *Sema1a* or *Plexin LOF* mutants, motor axons within the intersegmental nerve b (ISNb) frequently fail to defasciculate from the pioneering intersegmental nerve (ISN) due to decreased axon-axon repulsion (Yu et al., 1998; Winberg et al., 1998b). These defects are consistent with a role of

Sema-1a as a contact-mediated repulsive cue for ISNb axons. Molecular mechanisms mediating Sema-1a/PlexA guidance signaling have revealed a number of downstream proteins. Mical (molecule interacting with CasL) was identified as an interacting protein with the cytoplasmic region of PlexA from a

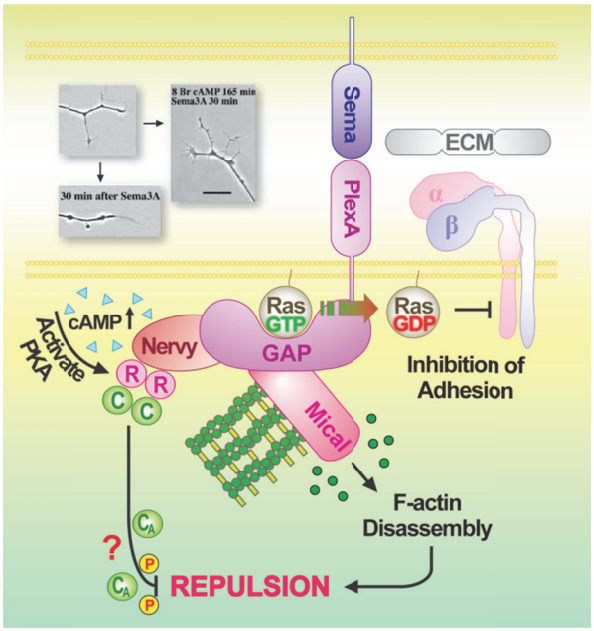

Figure 1.6 Working model of Semaphorin/Plexin-mediated repulsive guidance responses *Drosopihla* Sema-1a exerts respulsive guidance effects by activating PlexA. The cytoplasmic region of PlexA interacts with Mical, a novel F-actin depolymerization factor, and Nervy, an A kinase anchoring protein. These interactions mediate repulsive responses or modulate PlexA activity. Plexin family proteins have a GAP domain in the cytoplasmic region which inhibits Integrin-mediated cell adhesion by facilitating GTPase activity of its substrate Ras family GTPase. It is still unknown how PKA activity antagonizes Plexin-mediated repulsive guidance effects and how Plexin GAP activity is regulated. (Dontchev and Letourneau; Terman et al., 2002; Terman and Kolodkin, 2004; Oinuma et al., 2004; Oinuma et al., 2006; 2002 Hung et al., 2010)

yeast two-hybrid screen (Figure 1.6) (Terman et al., 2002). Mical activity is required for the propagation of PlexA-mediated axonal repulsion (Hung and

Terman, 2011, review) and a recent study indicates that Mical directly binds and disassembles F-actin (Hung et al., 2010). Hence, Mical directly links Semaphorin repulsive activity to actin reorganization. Moreover, PlexA also physically interacts with Nervy/MTG family adaptor/scaffolding proteins for protein kinase A (PKA), which are known as A kinase anchoring protein (AKAP) (Figure 1.6) (Fukuyama et al., 2001; Schillace et al., 2002; Terman and Kolodkin, 2004; Fiedler et al., 2010). *Nervy* LOF mutants, however, exhibit opposing effects to *PlexA* LOF, and increase defasciculation of ISNb axons, indicating that Nervy antagonizes PlexA-mediated axonal repulsion (Terman and Kolodkin, 2004). This PKA-involved antagonistic effect on PlexA-dependent repulsion is reminiscent of the finding that increasing cAMP can silence the repulsive effects of Sema3A on the cultured *Xenopus* and chick growth cones (Figure 1.6) (Nishiyama et al., 2003; Song et al., 1998; Song et al., 1997; Dontchev and Letourneau, 2002). This conserved role of cAMP signaling raises interesting questions about what are the candidate substrates of PKA and how PKA phosphorylation contributes to the inactivation of Semaphorin/Plexin repulsive signaling.

Plexin family proteins also exert repulsive guidance effects by endogenous GAP activity (Negishi et al., 2005; Kruger et al., 2005). The Plexin cytoplasmic region has a RasGAP homology domain which is divided by RhoGTPase binding domain (RBD) (Bell et al., 2011; Tong et al., 2009; He et al., 2009; Terman and

Kolodkin, 2004; Oinuma et al., 2004b; Zanata et al., 2002; Vikis et al., 2002; Hu et al., 2001; Driessens et al., 2001). Crystal structure of the Plexin cytoplasmic region reveals that the Plexin GAP structure is comparable to that of canonical RasGAPs in an autoinhibited conformation, which restricts association with its Ras substrate (Tong et al., 2009; He et al., 2009). The activation of the Plexin GAP is thought to require binding to both Semaphorin ligand and another Rho family GTPase to RBD in the cytoplasmic region (Oinuma et al., 2004a; Oinuma et al., 2004b). Although the complete understanding on how Semaphorin and GTPase contribute to the activation of Plexin RasGAP awaits further studies, it is thought that Semaphorin binding induces clustering of Plexins, and GTPase binding is necessary for the conformational change of Plexin and/or increasing the surface level of Plexin (Tong et al., 2009; He et al., 2009; Oinuma et al., 2004b; Oinuma et al., 2004a; Vikis et al., 2002; Vikis et al., 2000). RasGAP activity of Plexin has been shown to decrease the level of active GTP-bound R-Ras and its homologues (Saito et al., 2009; Toyofuku et al., 2005; Oinuma et al., 2004a). R-Ras, for example, is known to activate Integrin-mediated cell adhesion (Figure 1.6) (Zhang et al., 1996; Oinuma et al., 2006). Moreover, Plexin RasGAP activity also regulates other downstream proteins including phosphatidylinositol-3 kinase (PI3K) and phosphatase and tensin homolog (PTEN) (Oinuma et al., 2010; Ito et al., 2006), which results in growth cone collapse. Since a growth cone cannot extend toward its target in the presence of persisting repulsive guidance signaling

in vivo, the Plexin RasGAP activity needs to be tightly regulated. Therefore, molecular mechanisms underlying how Plexin GAP activity are turned-off will improve our understating of Semaphorin/Plexin-mediated axon guidance.

Diverse roles of Semaphorin/Plexin in cell migration While the molecular function of Semaphorin/Plexin signaling has been best characterized in axon guidance, cumulative evidence indicates that Sema/Plexin signaling also plays critical roles in diverse tissues and cellular events including dendrite growth/patterning, synaptogenesis, angiogenesis/vasculogenesis, immune system function, and in controlling cancerous growth (Saito et al., 2009; Wong et al., 2007; Tran et al., 2007; Toyofuku and Kikutani, 2007; Potiron et al., 2007; Neufeld et al., 2007; Koncina et al., 2007; Yazdani and Terman, 2006; Kruger et al., 2005). The molecular mechanisms underlying these diverse effects of Semaphorin/Plexin signaling converge onto a common function in the regulation of cell migration by regulating both cytoskeletal organization and Integrin-dependent adhesion (Kruger et al., 2005). For example, Class 3 Semaphorins regulate endothelial cell adhesion and migration, thereby promoting angiogenesis and vasculature formation as well as heart morphogenesis (Toyofuku and Kikutani, 2007; Neufeld et al., 2007). Likewise, Semaphorin/Plexin signaling modulates immune responses by inhibiting the migration of monocytes (Potiron et al., 2007). By taking advantage of this property, viruses encoding Semaphorin

A39R prevent immune surveillance by suppressing the migration of host immune-responsive cells (Potiron et al., 2007; Kruger et al., 2005). Moreover, because a loss of Semaphorin/Plexin signaling induces cell migration and angiogenesis, which are characteristics of cancer and metastasis, the deletions and mutations of genes encoding Semaphorin/Plexin have been reported in numerous types of cancer (Neufeld et al., 2007; Wong et al., 2007).

Drosophila Embryonic Neuromuscular System as an In vivo Model to Study Axon Guidance and Semaphorin/Plexin Signaling

Drosophila has long been implemented as a powerful in vivo model for studying the diverse aspects of neurobiology due to its wealth of genetic resources, relatively simple nervous system, and low redundant molecular pathways (Bate and Broadie, 1995; Araujo and Tear, 2003). The innervation of motor axons to their target muscles and CNS axons can be visualized by well-established monoclonal antibodies. For example, the Fasciclin II (FasII) antibody 1D4 (Van Vactor et al., 1993) reveals major motor axon tracts, which stereotypically innervate muscles in every hemisegment, and three parallel longitudinal axonal connectives on each side of the CNS midline (Figure 1.7). Among the five motor axon tracts, intersegmental nerve (ISN), ISNb, ISNd, segmental nerve a (SNa), and SNc, the ISNb and SNa axon tracts are easily identified and have been used as a model axon system for numerous studies (Van Vactor et al., 1993; Yu et al.,

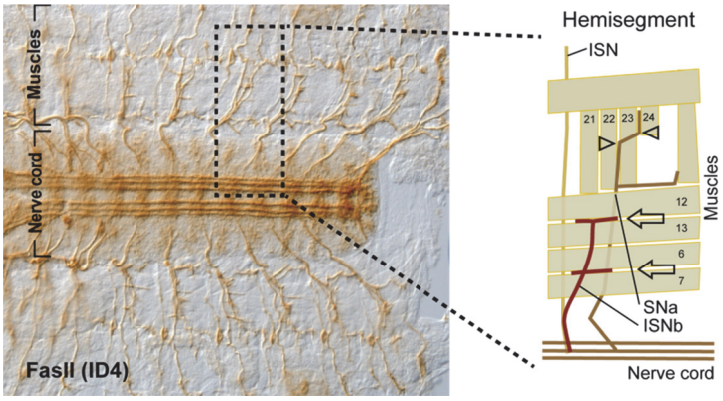

Figure 1.7 *Drosophila* embryonic neuromuscular system as an in vivo axon guidance model Major motor axon pathways and axons in the ventral nerve cord (VNC) are detected by axonal marker Fasciclin II (FasII) antibody 1D4. A filleted embryo at the stage of late 16 to 17 immunolabeled by ID4 exhibits three parallel longitudinal connectives on each side of nerve cord midline and stereotypic innervations of motor axons in the muscle field (Left). Axons within the ISNb tract innervate muscle clefts 6/7 and 12/13 (Right, arrows) and axons within the SNa project to dorsal muscles making two characteristic turns (Right, arrow heads). Anterior is on the left and ventral is at the middle.

1998; Winberg et al., 1998a; Winberg et al., 1998b; Terman et al., 2002; Terman and Kolodkin, 2004). For example, the axons within the ISNb axon tract originate from neurons located in the ventral nerve cord, and after defasciculating from the main ISN tract, they project to innervate ventral-lateral muscles 6/7 and 12/13 (Landgraf et al., 1997). Axons within the SNa tract take a different route (characterized by two easily observable turns) to innervate more dorsally located muscles. Abnormal pathfinding and target innervation of axons within these two tracts are frequently seen when Semaphorin/Plexin signaling is altered. Likewise, the parallel longitudinal tracts in the CNS are also disrupted in their organization when Semaphorin/Plexin signaling is altered. Thus, both motor and CNS axon

pathways serve as a sensitive in vivo model for further characterizing molecular mechanisms of the Semaphorin/Plexin-mediated repulsive guidance response.

CHAPTER TWO

Crystal Structure of The Plexin A3 Intracellular Region Reveals an Autoinhibited Conformation Through Active Site Sequestration

Previously published. He, H., **Yang, T.**, Terman, J.R., and Zhang, X. (2009). Crystal structure of the plexin A3 intracellular region reveals an autoinhibited conformation through active site sequestration. *Proc Natl Acad Sci U S A* 106, 15610-15615. Crystallization and structural determination of plexin A3 and COS-7 cell collapse assays were performed by He, H. and Zhang, X.

Abstract

Plexin cell surface receptors bind to semaphorin ligands and transduce signals for regulating neuronal axon guidance. The intracellular region of plexins is essential for signaling and contains a R-Ras/M-Ras GTPase activating protein (GAP) domain that is divided into two segments by a Rho GTPase-binding domain (RBD). The regulation mechanisms for plexin remain elusive, although it is known that activation requires both binding of semaphorin to the extracellular region and a Rho-family GTPase (Rac1 or Rnd1) to the RBD. Here we report the crystal structure of the plexin A3 intracellular region. The structure shows that the N- and C-terminal portions of the GAP homologous regions together form a GAP domain with an overall fold similar to other Ras GAPs. However, the plexin GAP domain adopts a closed conformation and cannot accommodate R-Ras/M-Ras in its substrate-binding site, providing a structural basis for the autoinhibited state of plexins. A comparison with the plexin B1 RBD/Rnd1 complex structure suggests that Rnd1 binding alone does not induce a conformational change in plexin,

explaining the requirement of both semaphorin and a Rho GTPase for activation. The structure also identifies an N-terminal segment that is important for regulation. Both the N-terminal segment and the RBD make extensive interactions with the GAP domain, suggesting the presence of an allosteric network connecting these three domains that integrates semaphorin and Rho GTPase signals to activate the GAP. The importance of these interactions in plexin signaling is shown by both cell-based and in vivo axon guidance assays.

Introduction

Plexins are a large family of type I transmembrane receptors for semaphorin axon guidance molecules. Repulsive signals from semaphorin-bound plexins are critical for proper axonal pathfinding and target innervation during neural development, and also play important roles in regulating cell migration, vascular patterning, and immune responses [reviewed in (Tran et al., 2007; Zhou et al., 2008; Neufeld et al., 2007)]. Malfunction of plexin signaling has been implicated in diseases such as neurological disorders and cancer, and plexins have emerged as new drug targets for these diseases (Yaron and Zheng, 2007).

All four classes of plexins (A, B, C, and D) share a similar domain structure, with an extracellular region that binds the semaphorin ligand, a single transmembrane helix and an approximately 600 residue-long intracellular region that transduces signals from the ligand-bound extracellular region to downstream pathways. Studies of the plexin intracellular region have led to the identification of a number of signaling pathways [reviewed in (Zhou et al., 2008; Tran et al., 2007; Yazdani and Terman, 2006)]. Of particular interest is the recognition that the plexin intracellular region exhibits sequence similarity to Ras GTPase-activating proteins (Ras GAPs), representing the first example of a transmembrane receptor containing a GAP domain (Hu et al., 2001; Rohm et al., 2000). R-Ras, a homologue of Ras involved in regulating cell adhesion and neurite outgrowth, is a substrate of the plexin GAP (Toyofuku et al., 2005;

Oinuma et al., 2004a). Conversion of R-Ras into the GDP-bound inactive form by the plexin GAP leads to decreased activity of integrin and loss of cell adhesion, contributing to repulsive axon guidance. A recent study shows that the plexin GAP also acts on another Ras homolog M-Ras, leading to remodeling of dendrite morphology (Saito et al., 2009). The GAP activity is important for plexin signaling, and plexin function is lost upon mutation of either of the two conserved catalytic arginine residues (Toyofuku et al., 2005; Oinuma et al., 2004a; Rohm et al., 2000; Oinuma et al., 2004b).

The GAP domain of plexins is unique in that it is divided into two segments, referred to as C1 and C2 respectively, by a domain of approximately 200 residues that does not show homology to Ras GAPs. The C1 and C2 regions each contain one of the two catalytic arginine residues (Scheffzek et al., 1996), and the mechanism through which these two arginines are positioned precisely in close proximity for catalysis is not known(Scheffzek et al., 1997). The domain between C1 and C2 in class A and B plexins binds Rho-family GTPases Rac1, Rnd1, or RhoD in their GTP-bound active form, and is called the Rho GTPase binding domain (RBD) (Rohm et al., 2000; Zanata et al., 2002; Driessens et al., 2001; Vikis et al., 2000). A recent study shows that plexin D1 RBD binds another Rho GTPase Rnd2, whereas the Rho GTPase specific for the class C plexin has yet to be identified (Uesugi et al., 2009). The structures of the core region of the plexin B1 RBD and its complex with Rnd1 have been solved [PDB ID: 2REX,

(Tong et al., 2007; Tong et al., 2008)]. The RBD core adopts an ubiquitin-like fold and binds Rnd1 through one edge of its β-sheet. Mutational analyses suggest that Rac1 and RhoD bind to the RBD in the same mode (Tong et al., 2007).

Plexins behave as logic "AND" gates during signal transduction, as their activation requires concomitant binding of semaphorin to the extracellular region and one of the Rho GTPases to the RBD (Saito et al., 2009; Oinuma et al., 2004b; Oinuma et al., 2004a; Zanata et al., 2002; Turner et al., 2004). The GAP activity of the plexin cytoplasmic domains can be induced by simultaneous binding of Rnd1 and an cluster-inducing antibody, whereas no activity was detected in the absence of either (Toyofuku et al., 2005; Oinuma et al., 2004b). While semaphorins may contribute to plexin activation by inducing a change in its oligomerization state (Toyofuku et al., 2005; Oinuma et al., 2004b; Klostermann et al., 1998; Takahashi et al., 1999), the mechanism by which the Rho GTPases facilitate the activation process remains unclear. A conformational change associated with GAP activation has been suggested by studies that show interactions between C1 and C2, and dissociation of the two upon RhoGTPase binding to the RBD (Oinuma et al., 2004b; Turner et al., 2004).

Central questions concerning plexin signaling mechanisms include how the separated C1 and C2 regions interact with each other to form a functional GAP domain, and how GAP activity is regulated by semaphorins and Rho GTPases. To address these questions we determined the crystal structure of the

intracellular domain of plexin A3, one of the most widely expressed plexins in the nervous system (Cheng et al., 2001). The structure shows that C1 and C2 of plexin fold in an intertwined manner to form a GAP domain that has an overall fold similar to other Ras GAPs (Scheffzek et al., 1996). Structural comparisons suggest that the plexin GAP is in an autoinhibited state, and Rho GTPase binding alone does not appear to be able to induce its activation. The structure also identifies a previously unnoted N-terminal segment. The GAP domain is coupled to both the N-terminal segment and the RBD through conserved inter-domain interactions, which were shown to be critical for signaling by our functional assays, providing insights into the plexin activation mechanism involving binding of both semaphorin and a Rho GTPase.

Results

Structure of the Intracellular Domain of Plexin A3

We have solved a 2.0-Å crystal structure of the intact intracellular region of plexin A3 (residues 1,247–1,872). Despite the unusual divided domain structure and low sequence similarity to other Ras GAPs (≈21% identity and ≈29% similarity to p120 Ras GAP) (Figure 2.1*A* and Figure S2.1), the two GAP homologous regions C1 and C2 of plexin A3 form a GAP domain with an overall fold similar to canonical Ras GAPs such as p120 Ras GAP (Figure 2.1*B* and *C*)(Oinuma et al., 2004b; Pena et al., 2008; Scheffzek et al., 1998a). The two catalytically critical arginine residues in plexin A3, Arg-1407 and Arg-1724, superimpose precisely with those in p120 Ras GAP (Figure 2.1*C*). The structure identifies unambiguously the boundaries of the C1 (residues 1,325–1,450) and C2 (residues 1,651–1,872) regions of plexin A3 (Figure S2.1). The plexin A3 GAP does show important structural differences when compared to other Ras GAPs, which will be discussed in detail below.

The RBD segment (residues 1,451–1,650) between C1 and C2 extends out of the GAP domain and adopts an independent ubiquitin-like fold, similar to that of the isolated plexin B1 RBD reported previously (Figure 2.1*A* and *B*) (Tong et al., 2007). The ubiquitin-like RBD core (residues 1,478–1,578) is connected to C1 and C2 by two linkers, the N-terminal linker (residues 1,451–1,477) and C-

terminal linker (residues 1,579–1,650), respectively (Figure 2.1*A*). The RBD interacts with the GAP domain through one edge but the area that binds Rho GTPases is entirely accessible (Figure 2.1*B* and see details below).

The structure reveals a previously unrecognized N-terminal segment (residues 1,246–1,324), which is not part of the GAP homology region and does not show noticeable sequence similarity to proteins outside of the plexin family (Figure 2.1*A* and *B* and Figure S2.1). This N-terminal segment adopts an extended helix-loop-helix-loop structure and makes extensive interactions with the GAP domain (Figure 2.1*B* and see details below).

Autoinhibition Mechanism of the Plexin GAP Domain.

The plexin A3 GAP domain adopts an overall elongated and curved shape similar to other Ras GAPs, with the active site containing Arg-1407 and Arg-1724 located at the concaved surface. However, the Ras substrate-binding cleft in the plexin A3 GAP is narrower and deeper than that of p120 Ras GAP (Figure 2.1*C*). Other Ras GAP structures such as synGAP and neurofibromin all adopt the same open, active conformation as that of p120 Ras GAP (Figure S2.2*B*). A recently deposited structure of the plexin B1 intracellular region (PDB ID: 3HM6) shows a closed conformation essentially identical to plexin A3 (Figure S2.2*A*), suggesting that this conformation is a favored resting state of the plexin family members.

The “closed” substrate-binding site of plexin A3 is mainly due to helices 4 and 5, which are taller than their counterparts in p120 Ras GAP, and large inward

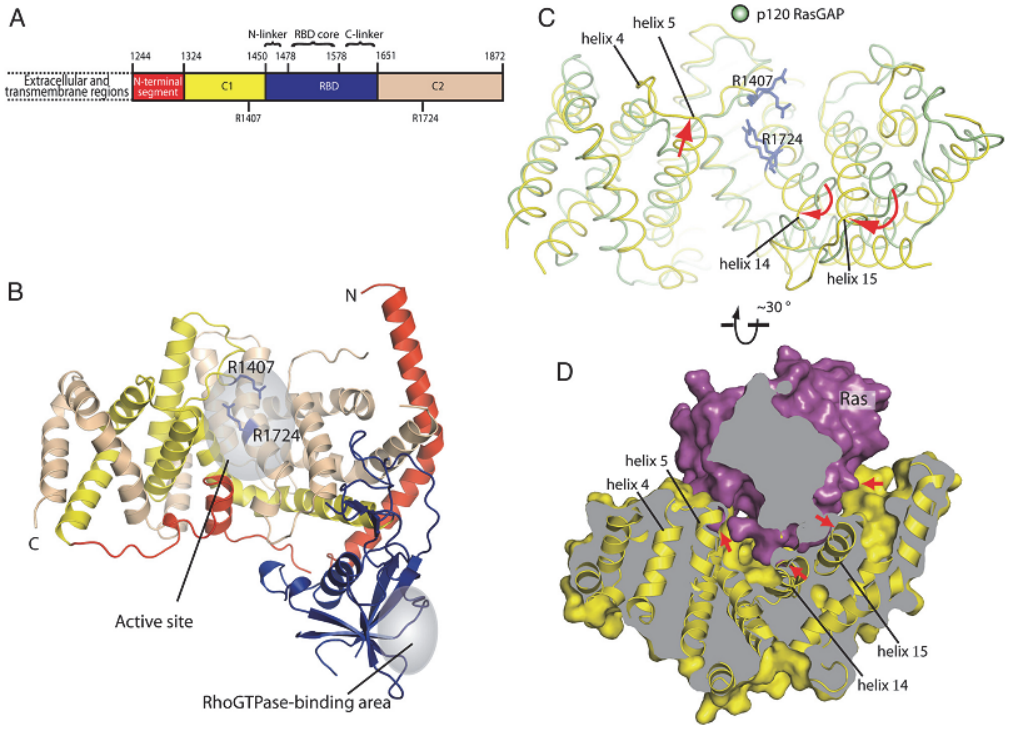

Figure 2.1. Overall structure of the plexin A3 intracellular region. (*A*) Schematic diagram of the plexin A3 intracellular region. The two catalytically critical arginines are shown. (*B*) Structure of the plexin A3 intracellular region. The color scheme is the same as that in *A*. (*C*) Superimposition of the GAP domains of plexin A3 and p120 Ras GAP (PDB ID: 1WQ1) suggesting an autoinhibited conformation of the plexin A3 GAP domain. Parts with significant differences between the two structures are highlighted with red arrows. (*D*) Cross-section view of the docking of Ras onto the plexin A3 GAP, based on the structure of the p120Ras GAP/Ras complex. Steric clashes between the GAP domain and Ras are highlighted with red arrows.

/downward shifts of helices 14 and 15 on the other side of the active site (Figure 2.1*C*). Docking a Ras molecule onto the plexin A3 GAP based on the structure of p120 Ras GAP/Ras complex results in many steric clashes (Figure 2.1*D*)

(Scheffzek et al., 1997). Given the high degree of structural similarity among Ras homologues, these clashes are likely to be present when R-Ras or M-Ras is docked. Therefore, this conformation of the plexin A3 GAP domain cannot bind its R-Ras/M-Ras substrate in a productive mode. This autoinhibited conformation explains the lack of GAP activity of plexins in the absence of upstream stimuli (Toyofuku et al., 2005; Oinuma et al., 2004b). A significant conformational change is required for the plexin GAP to convert to the active conformation as seen in p120 Ras GAP.

Oligomerization State of Plexin-Intracellular Domain.

Dimerization or higher order clustering plays an important role in regulating plexin signaling (Toyofuku et al., 2005; Oinuma et al., 2004b; Klostermann et al., 1998). The plexin intracellular regions weakly dimerize in solution (Figure S2.3). To gain insights into the nature of this dimer and its potential role in plexin regulation, we analyzed dimeric interactions in crystal structures of plexins. The plexin A3 crystals contain several putative dimers. Mutational analyses in combination with sequence considerations however suggest that none of these represents the plexin dimer seen in solution (Figure S2.3 and Table S2.1).

A dimer of the plexin B1 RBD core has been reported previously (Tong et al., 2007). The interface is mediated by the extra β-hairpin outside of the ubiquitin-like fold, which form a short four-stranded β-sheet between the two

monomers (Figure 2.2). The β-hairpin in the structure of the full length plexin A3 intracellular region does not make such a dimeric interaction. Instead, it forms an intra-molecular four-stranded β-sheet with a segment in the C-terminal linker (Figure 2.2), which is not present in the plexin B1 RBD core protein. We conclude that this dimer of the plexin B1 RBD core mimics the missing intra-molecular interaction, and the full-length plexin intracellular region is unlikely to form such a dimer. Consistently, the same intra-molecular interaction, instead of the dimer, is present in the structure of the full-length plexin B1 intracellular region (Figure S2.2*A*). The true nature of the preformed dimer of the plexin intracellular domain and its role in plexin signaling remain to be determined.

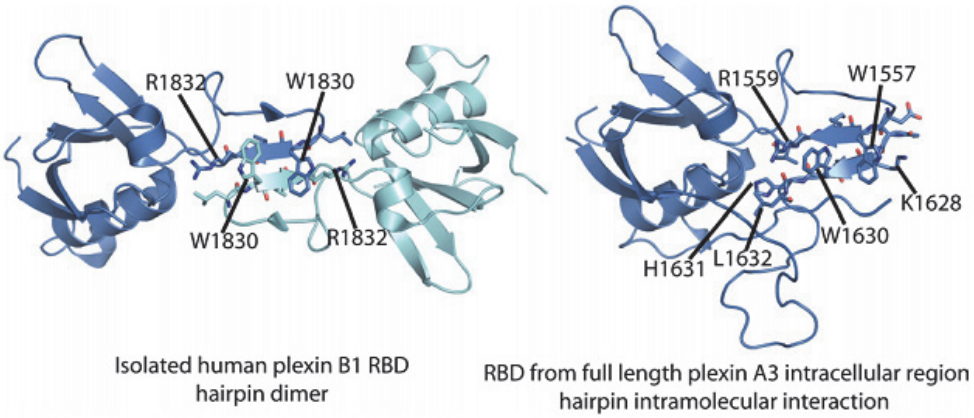

Figure 2.2. The dimer of the plexin B1 RBD core reported previously (2R2O) mimics an intra-molecular interaction between the RBD core and the C-terminal linker in plexin A3.

Binding of RhoGTPase to the RBD Alone Does Not Induce GAP Activation.

Our structure shows that the RBD is coupled to the GAP domain through extensive interactions made by both the N-terminal linker and the RBD core (Figure 2.3*A*). The N-terminal linker is tightly packed between the GAP domain and the RBD core. Leu-1464 in the N-terminal linker places its side chain into a hydrophobic pocket formed by helices 9, 11, 15, and 16 in the GAP domain. In addition, Ile-1453, Tyr-1462, and Leu-1469 also contribute to the RBD/GAP interface. The C-terminal linker is largely disordered, except for the segment between residues 1,626–1,636, which forms the short β-sheets with the extra loop from the RBD core mentioned above (Figures 2.2 and 2.3*A*). A conserved ^{1631}HLV^{1633} motif in this segment points their sidechains toward the domain interior, connecting interior packing in the N-terminal linker and the RBD core (Figure 2.3*A*). The RBD core also makes numerous contacts with the GAP domain, mainly through buried polar residues (Figure 2.3*A*). Most residues in the RBD/GAP interface are conserved, suggesting important functional roles for these interactions (Figure S2.1).

The RhoGTPase binding site in the RBD is located on the opposite side of the domain that interacts with the GAP domain. A superimposition of plexin A3 and the plexin B1 RBD/Rnd1 complex placed Rnd1 far away from the GAP domain (Figure 2.4*A*). Given the extensive interactions between the RBD and the GAP domain, it is unlikely that the relative orientation between the two can

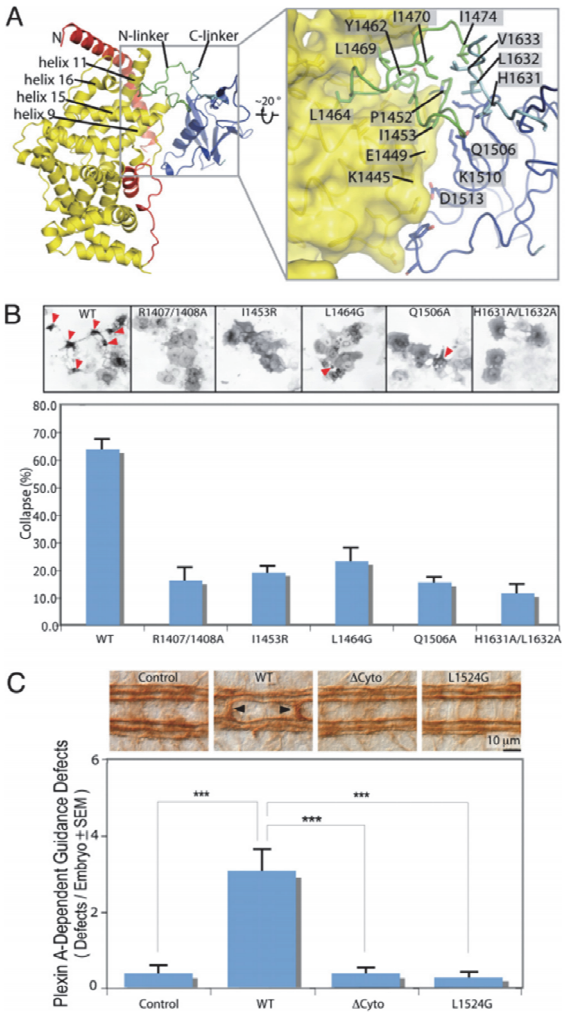

Figure 2.3. Interaction between the RBD and GAP domain. (*A*) Interface between the RBD and the GAP domain. The N-terminal linker (N-linker) and C-terminal linker (C-linker) are colored in green and cyan, respectively. (*B*) COS-7 cell collapse assays showing that mutations in the RBD/GAP interface abolish plexin-induced cell collapse. The percentages of collapse are average of three experiments. Error bars: standard deviations. Representative images of cells are shown and collapsed cells are marked with arrowheads. (*C*) In vivo axon guidance assay of the L1524G mutant in *Drosophila* plexin A. Increased expression of wild type (WT) plexin A induces axons to abnormally cross the midline of the central nervous system (arrowheads). Both truncation of the cytoplasmic domain (ΔCyto) and the L1524G mutation eliminate this effect. Genotypes: (Control) *ELAV-GAL4*,*UAS*:^{HA} *Plexin A*/+; (WT) *ELAV-GAL4*,*UAS*:^{HA} *Plexin A*/*UAS*:^{HA} *Plexin A*; (ΔCyto) *ELAV-GAL4*, *UAS*:^{HA} *Plexin A*/*UAS*: ^{HA} *Plexin A*$^{\Delta Cyto}$; (L1524G) *ELAV-GAL4*, *UAS*:^{HA} *Plexin A*/*UAS*:^{HA} *Plexin A* ^{L1524G}. ***$P < 0.0005$ by two-tailed Student's *t* test.

change sufficiently to allow Rnd1 to regulate the GAP domain directly. Instead, The RBD/GAP interactions may establish an allosteric network to facilitate the indirect communication between the RBD-bound Rho GTPase and the GAP domain. This model predicts that disruption of the RBD/GAP interface would prevent plexin activation.

We made four interface mutants of plexin A3 (I1453R and L1464G in the N-terminal linker, Q1506A in the RBD core and H1631A/L1632A in the C-terminal linker), and tested their activity by using a COS-7 cell-based collapse assay (Table S2.1) (Takahashi et al., 1999). Cells expressing the wild-type plexin A3 exhibit robust collapse (>60%) after stimulation by the Sema3F ligand (Figure 2.3*B*). The four mutants induce ≈20% cell collapse, close to a negative control mutant which contains a double R1407/1408A mutation in the catalytic site and is known to be incapable of signaling (Toyofuku et al., 2005; Oinuma et al., 2004b; Oinuma et al., 2004a; Rohm et al., 2000). We have also tested the L1464G-equivalent mutation in *Drosophila* plexin A (L1524G) in an in vivo axon guidance assay (Ayoob et al., 2006). Overexpressed neuronal wild-type plexin A generates axon guidance defects in *Drosophila*, whereas a plexin A mutant without the cytoplamic domain does not cause such effects (Figure 2.3*C*). Likewise, the L1524G mutation eliminates plexin A-induced guidance defects (Figure 2.3*C*), consistent with the results from the collapse assay. These results

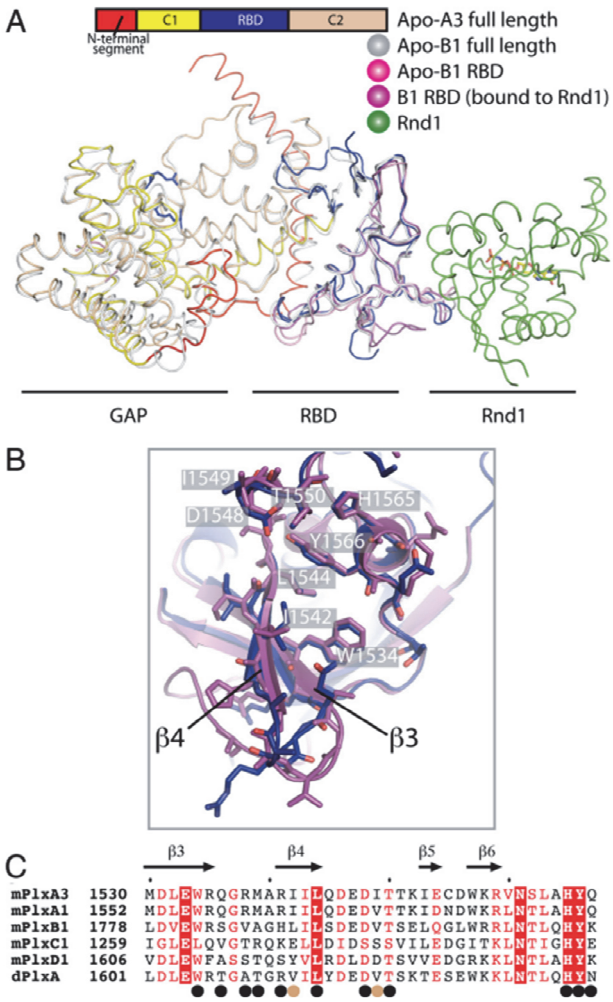

Figure 2.4. Rnd1 alone does not induce conformational changes in plexin. (*A*) Superimposition of the plexin A3 intracellular domain, the plexin B1 intracellular domain (PDB ID: 3HM6), the plexin B1 RBD core (PDB ID: 2R2O), and the plexin B1 RBD core/Rnd1 complex (PDB ID: 2REX). (*B*) Detailed view of the Rho GTPase binding site in the structures of plexin A3 and the plexin B1 RBD/Rnd1 complex. (*C*) Sequence alignment of the Rho GTPase-binding region in plexins. Residues involved in Rho GTPase binding are highlighted with circles. The two brown circles indicate the two residues that are conserved in class A and B plexins, but different in class C and D plexins. The secondary structure elements are based on the plexin A3 structure. m, Murine; d, Drosophila

together demonstrate that the interaction between the RBD and the GAP domain is critical for plexin signaling.

To understand how Rnd1 or other RBD-binding Rho GTPases contribute to plexin activation through this coupling between the RBD and the GAP domain, we compared plexin A3 with structures of the full-length plexin B1 intracellular region, the plexin B1 RBD core, and its complex with Rnd1 (Figure 2.4) (Tong et al., 2007).

Rnd1 binds to one side of the RBD corresponding to strands β3, β4, the following short β-hairpin, and the surrounding loops in the plexin A3 intracellular region (Figure 2.4*A* and *C*). Residues in the RhoGTPase binding site are conserved among class A and B plexins, suggesting that they all bind Rnd1, Rac1, and RhoD in the same manner. Class C and D plexins however contain several distinct residues in the binding site (Figure 2.4*C*), explaining their different Rho GTPase specificities (Uesugi et al., 2009). The entire RBD core, including the Rho GTPase binding site, is essentially identical in the Rnd1-bound and the three apo-structures (Figure 2.4*A* and *B*), suggesting that binding of Rnd1 (and likely other Rho GTPases) alone cannot induce a significant conformational change in plexin. The RBD-bound Rho GTPase however may be able to facilitate the conformational change required for activation in the context of the plexin dimer/oligomer induced by semaphorin, allowing plexins to function as a logic "AND" gate in signal transduction (Zanata et al., 2002).

The Roles of the N-Terminal Segment in Regulating Plexin Activity.

The structure shows that the N-terminal segment also makes extensive interactions with the GAP domain, which may enable it to regulate the GAP conformation in response to semaphorin binding in the extracellular region. The first helix in the N-terminal segment interacts with the curved outer surface of one side of the GAP domain by undertaking a kink between residues Ala-1269 and Leu-1270 (Figure 2.5*B*).

Portions flanking this kink are anchored to helices 9, 11, and 16 in the GAP domain by both non-polar and electrostatic interactions (Figure 2.5*A* and *B*). Helices 9, 11, and 16 also participate in the hydrophobic pocket that binds Leu-1464 in the RBD (Figure 2.3*A*). These interactions therefore indirectly connect the N-terminal segment and the RBD. The loop following the first helix contains a ^{1295}IPFL^{1298} motif, which makes hydrophobic interaction with residues including Phe-1438 and Phe-1737 in the GAP domain (Figure 2.5*B*). Immediately following this loop is the second, shorter helix, which is located at the junction between the two sides that shape the concaved GAP active site. This amphiphathic helix interacts with the GAP domain through its hydrophobic side containing aromatic residues Tyr-1300, Tyr-1303, and Phe-1309.

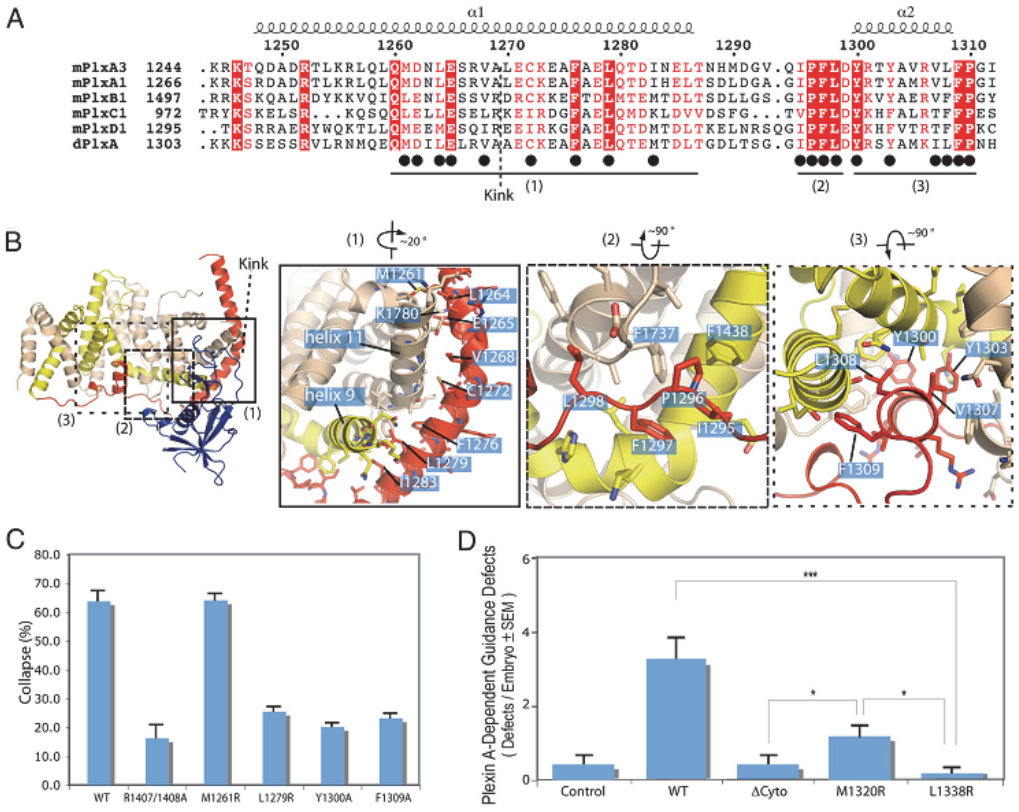

Figure 2.5. Interaction between the N-terminal segment and the GAP domain. (*A*) Sequence alignment of the N-terminal segment of plexins. Residues making contacts with the GAP domain are highlighted with black circles. m, Murine; d, Drosophila. (*B*) Interactions between the GAP domain and the N-terminal segment. The three right panels are exploded views of the correspondingly numbered boxes in the left panel. Changes of view angles are indicated by the rotation axis on the top of each panel. (*C*) Mutational analyses of the interface between the N-terminal segment and the GAP domain in the COS-7 collapse assay. (*D*) Mutational analyses of the corresponding interface residues in *Drosophila* plexin A using the *vivo* axon guidance assay. Genotypes: (M1320R) *ELAV-GAL4*, *UAS*:^{HA} *Plexin A*/*UAS*:^{HA} *Plexin A* ^{M1320R}; (L1338R) *ELAV-GAL4*, *UAS*:^{HA} *Plexin A*/*UAS*:^{HA} *Plexin A* ^{L1338R}; and as in Figure 3*C*. **P* < 0.05; ****P* < 0.001 by two-tailed Student's *t* test.

Most residues in the N-terminal segment contacting the GAP domain are conserved (Figure 2.5*A*), suggesting important functional roles for these inter-domain interactions. To test this, we performed mutational analyses using the same functional assays mentioned above. Four mutants, M1261R, L1279R, Y1300A, and F1309A, were examined in the cell collapse assay. While the M1261R mutation appears to have no effect, the other three mutations reduce the levels of collapse close to that of the R1407/1408A mutant (Figure 2.5*C*). We also analyzed the M1320R and L1338R mutations in *Drosophila* plexin A, corresponding to M1261R and L1279R in plexin A3, respectively, using the in vivo axon guidance assay. Consistent with the results from the collapse assay, the M1320R mutant remained partially active, and the L1338R mutation eliminated the repulsive effects of plexin A in axons (Figure 2.5*D*). These results together suggest that the N-terminal segment is not just a passive linker between the extracellular and the intercellular regions, but plays an important role in regulating plexin activity through its interactions with the GAP domain.

Discussion

The unusual divided architecture of the plexin GAP homology regions and their low sequence similarity to other Ras GAPs has raised the question of whether plexin is indeed a Ras GAP (Hu et al., 2001; Rohm et al., 2000; Pasterkamp, 2005). Our structure of plexin A3 shows that the C1 and C2 regions do form a canonical Ras GAP domain, providing a structural basis for the recently observed R-Ras/M-Ras GAP activity of plexin (Saito et al., 2009; Oinuma et al., 2004a). The GAP domain in this structure however exhibits an inactive conformation, representing an autoinhibited state of the plexin intracellular region. In contrast, structures of several other Ras GAP domains all show the active conformation (Scheffzek et al., 1997; Ayoob et al., 2006; Pasterkamp, 2005), and regulation of their activity often involves direct blocking of the active site by regulatory domains in the proteins (Bos et al., 2007). The observations of both active and inactive conformations suggest a distinct regulation mechanism for Ras GAPs. The GAP domain may exist in equilibrium between the two conformations, and other domains/proteins allosterically regulate its activity by trapping one conformation and shifting the balance between the two without interacting with the catalytic site directly.

A critical remaining question is how plexin is activated by semaphorin and a Rho GTPase binding. A well-studied signaling mechanism of receptors is ligand-induced dimerization of receptor tyrosine kinases, in which the two kinase

monomers activate each other by transphosphorylation of one or several regulatory tyrosines (Schlessinger, 2000). Dimerization can also promote inter-molecular interactions that allosterically induce the active conformation, as exemplified by the EGF receptor kinase (Zhang et al., 2006). Small GTPases trigger downstream signaling through recruiting their effectors to the proper location and/or inducing their active conformation (Vetter and Wittinghofer, 2001). In the case of plexins, however, neither semaphorin nor the Rho GTPases alone are sufficient for activation (Saito et al., 2009; Turner et al., 2004; Oinuma et al., 2004b; Oinuma et al., 2004a; Zanata et al., 2002). Our structural analyses support this by showing that the Rho GTPases do not appear to induce any conformational change in the GAP domain or interact with the GAP active site directly. Our data are consistent with an allosteric activation mechanism involving both of them, which is critically dependent on the tight coupling between the N-terminal segment, the GAP domain and the RBD in the plexin intracellular region. Elucidation of this allosteric mechanism requires further studies, especially structural analyses of plexin in the active conformation bound to both a Rho GTPase and the R-Ras/M-Ras substrate.

Materials and Methods

Protein Expression, Purification, and Characterization.

The coding sequence for the intracellular domain of mouse plexin A1 (residues 1,269–1,894) and A3 (residues 1,247–1,872) were cloned into a modified pET28 vector (Novagen), which expresses the target protein with a N-terminal His_6-Sumo protein tandem tag. Mutants of plexins were generated by Quickchange reactions (Stratagene). The proteins were expressed in the bacterial strain *ArcticExpress* (Stratagene) by following the manufacture's instruction. The protein was purified using a HisTrap 1-mL column (GE Healthcare). The His_6-Sumo tag was removed by treatment of a Sumo-specific protease Ulp1. The proteins were further purified by ion exchange chromatography, concentrated to approximately 10 mg/mL and stored under −70 °C. Seleno-methoinine replaced plexin A3 was expressed in the same bacterial strain using the protocol as described by Van Duyne et al. (Van Duyne et al., 1993). Native PAGE were performed using 10–20% Tris-HCl gradient gel (Bio-Rad).

Crystallization, Data Collection, and Structure Determination.

Initial crystallization trials of native plexin A3 at 5 mg/mL were performed through sitting drop vapor diffusion in the 96-well format. The initial condition (100 mM Bis-Tris propane, pH 7.5, 200 mM $NaNO_3$, and 20% PEG3350) was optimized by hanging drop vapor diffusion. Best crystals were obtained by mixing

plexin A3 (2–5 mg/mL) and the crystallization buffer (100 mM Bis-Tris propane, pH 7.75, 100 mM $NaNO_3$, and 15% PEG3350) at 1:1 volume ratio. Seleno-methionine replaced crystals were obtained in similar conditions. Both the native and seleno-methionine single-wavelength anomalous dispersion (SAD) datasets were collected at beamline 19BM in the Advanced Photon Source. The diffraction data were processed using the program HKL2000 (Otwinowski and Minor, 1997). The SAD dataset was used for solving the structure and ab initio model building using the Autosol and Autobuild Wizards in the Phenix package, respectively (Adams et al., 2002). The structure was then refined against the native dataset by iterative manual model building in Coot and refinement using the Phenix refinement module (Adams et al., 2002; Emsley and Cowtan, 2004). The statistics of data collection and structure refinement are summarized in Table S2.2.

COS-7 Cell Collapse Assay.

The cell collapse assay is based on the ability of plexins expressed on the surface of COS-7 cells to trigger cell collapse when activated by semaphorin (Takahashi et al., 1999). While plexin A3 failed to induce collapse in a previous study (Takahashi and Strittmatter, 2001), we observed robust collapse by increasing the concentrations of semaphorin and the duration of the stimulation. Full-length plexin A3 and and its co-receptor neuropilin 2 with a C-terminal myc-tag were cloned into the pIRES2-EGFP (Clontech) and pcDNA3.1 (Invitrogen) vectors,

respectively. Point mutations of plexin A3 were generated by QuikChange reactions (Stratagene). The plasmid encoding the plexin A3 ligand semaphorin 3F fused to alkaline phosphatase (AP-Sema3F) (a kind gift from Dr. Roman Giger) was transfected into HEK-293 cells for producing the ligand. COS-7 cells were seeded into six-well plates at a density of approximately 1.2×10^5 cells/well 1 day before transfection. Transfection were performed by using Fugene6 (Roche) with a ratio of Fugene: plexin: neuropilin at 4.5 μL:0.9 μg:0.6 μg. Two days after transfection, cells were treated with AP-Sema3F at 3–5 nM concentration at 37 °C for approximately 90 min. Cells expressing both plexin A3 and neuropilin 2 bound AP-Sema3F and were stained by the BCIP/NBT substrates. The staining results showed that both the wild-type plexin A3 and the mutants were expressed on the cell surface. The ratio of collapsed cells and the total number of stained cells was taken as the percentage of cell collapse. Each experiment was repeated three times and more than 200 cells were counted each time.

In Vivo Axon Guidance Assay.

Mutants of *Drosophila* plexin A were cloned into the *Drosophila* transformation vector ^{HA}plexin A pUAST (Terman et al., 2002). Multiple independent transgenic fly lines containing these HA-tagged plexin A proteins were generated (BestGene, Inc.) and examined for expression and in vivo function using standard approaches. Analysis of alterations in plexin A-dependent axon guidance was

performed as described previously, based on the observation that overexpressed plexin A in neurons using the *ELAV-GAL4* driver enhances axon-axon repulsion, leading to abnormal axon separation and crossing of the midline of the central nervous system (CNS) (Ayoob et al., 2006). Low level overexpression of plexin A (1 copy) in a wild-type background generate relatively minor CNS axon guidance defects. Under this sensitized genetic background, expression of an additional copy of plexin A robustly increases the number of defects, providing a sensitive in vivo assay for plexin A signaling. Stage 16 or later embryos expressing an additional copy of the plexin A or mutants were collected at 30 °C and immunostained to visualize CNS axons with the Fasciclin II antibody (1D4; Developmental Studies Hybridoma Bank). “Thick” axonal bundles exiting the longitudinals/crossing the CNS midline were counted in 10 embryos/genotype (≥ 90 segments).

CHAPTER THREE

14-3-3ε Couples Protein Kinase A to Semaphorin Signaling and Silences Plexin RasGAP-mediated Axonal Repulsion

Abstract

The biochemical means through which multiple signaling pathways are integrated in navigating axons is poorly understood. Semaphorins are among the largest families of axon guidance cues and utilize Plexin receptors to exert repulsive effects on axon extension. However, Semaphorin repulsion can be "turned-off" by other distinct cues and signaling cascades, raising questions of the logic underlying these events. We now uncover a simple biochemical switch that controls Semaphorin/Plexin repulsive axon guidance. Plexins are Ras family GTPase activating proteins (GAPs) and we find that the Plexin GAP domain is phosphorylated by the cAMP-dependent protein kinase (PKA). This Plexin phosphorylation generates a specific binding site for 14-3-3ε, a phospho-binding protein that we find to be necessary for axon guidance. These PKA-mediated Plexin-14-3-3ε interactions prevent Plexin from interacting with its Ras family GTPase substrate and antagonize Semaphorin repulsion. Our results indicate that these interactions switch repulsion to adhesion and identify a point of convergence for multiple guidance molecules.

Introduction

Neural connections form during development when neurons extend stalk-like axonal appendages that actively explore their environment, "looking" for specific signals that will guide them to their targets. Work over the past twenty years has identified a number of these extracellular signals, revealing that specific attractive and repulsive guidance cues control the cytoskeletal and adhesive machinery necessary for axon elongation (Tessier-Lavigne and Goodman, 1996; Dent et al., 2011). More recently, transmembrane receptors and intracellular signaling molecules have been found for many of these guidance cues, providing a further understanding of the molecular biology of axon guidance (Dickson, 2002; Huber et al., 2003; Bashaw and Klein, 2010). Yet, these fundamental discoveries have also raised important new questions regarding the biochemical mechanisms that enable growing axons to choose among this diverse array of guidance information, much of which is presented in concert, to precisely navigate to their targets.

Semaphorins are among the largest families of axon guidance cues and are best known for their ability to sculpt the nervous system by serving as axonal repellents (Tran et al., 2007). Semaphorins exert their repulsive effects by disassembling the actin and microtubule cytoskeletal elements necessary for axonal extension as well as by disrupting the adhesive interactions between an axon and its substrate (Hung and Terman, 2011). Semaphorins utilize Plexin

receptors to exert their cell biological effects and recently a number of signaling molecules have been identified that mediate Semaphorin/Plexin effects on the cytoskeleton (Zhou et al., 2008; Bashaw and Klein, 2010), including novel actin disassembly factors, the MICALs (Hung and Terman, 2011). Interestingly, Plexins also directly associate with small GTP-binding proteins and contain a GTPase activating protein (GAP) domain within their cytoplasmic portions (Rohm et al., 2000; Vikis et al., 2000; Driessens et al., 2001; Hu et al., 2001; Oinuma et al., 2004a; He et al., 2009; Tong et al., 2009). These observations have provided a direct link between Semaphorins/Plexins and small GTP-binding proteins, which are key regulators of cytoskeletal dynamics and cell adhesion (Dickson, 2001; Hall and Lalli, 2010). Indeed, in vitro work has revealed that Plexins exert repulsive/de-adhesive effects on growing axons by employing their RasGAP activity to inhibit Ras/Integrin-dependent axon-substrate adhesion (Serini et al., 2003; Oinuma et al., 2004a; Toyofuku et al., 2005; Oinuma et al., 2006; Uesugi et al., 2009; Tong et al., 2009).

Growing evidence also indicates that the repulsive effects of axon guidance cues can be silenced and even turned into attraction by raising the levels of specific signaling molecules like cyclic nucleotides. cAMP, for example, has emerged as a potent antirepellent that enables axonal growth and regeneration on repulsive/inhibitory substrates including Semaphorins (Song et al., 1998; Cai et al., 1999; Hopker et al., 1999; Dontchev and Letourneau, 2002; Neumann et al.,

2002; Qiu et al., 2002; Chalasani et al., 2003; Pearse et al., 2004; Han et al., 2007; Xu et al., 2010). The molecular and biochemical mechanisms of this cAMP antirepellent action are still poorly understood, but it is interesting that the cAMP-dependent protein kinase (PKA), which is activated by cAMP, has been found to associate in a complex with the Semaphorin receptor Plexin (Terman and Kolodkin, 2004; Fiedler et al., 2010) and antagonize Semaphorin-mediated repulsive axon guidance (Dontchev and Letourneau, 2002; Chalasani et al., 2003; Terman and Kolodkin, 2004; Parra and Zou, 2010). The targets of PKA and its biochemical role in regulating Semaphorin/Plexin repulsive axon guidance are unknown.

We now find that PKA phosphorylates a specific serine residue within the Plexin RasGAP domain and generates a binding site for a member of the 14-3-3 family of phospho-serine binding proteins, 14-3-3ε. Moreover, these PKA-mediated 14-3-3ε–Plexin interactions occlude the association between Plexin and its RasGAP substrate, Ras2, concomitantly making axons less responsive to Semaphorin-mediated repulsion and more responsive to Integrin-mediated adhesion. Our findings, therefore, uncover both a new molecular integration point between important axon guidance signaling pathways and a biochemical logic by which this guidance information is coalesced to steer the growing axon.

Results

14-3-3ε is a Specific PlexA-Interacting Protein

To search for components of the Semaphorin/Plexin repulsive axon guidance signaling cascade, we performed a yeast two-hybrid interaction screen using a portion of the Drosophila Plexin A (PlexA) cytoplasmic tail (Figure 3.1Aa; $PlexA^{Cyto2}$; (Terman et al., 2002)). Two critical Semaphorin/Plexin axon guidance signaling molecules, Mical and Nervy had previously been found in this screen (Terman et al., 2002; Terman and Kolodkin, 2004; Hung et al., 2010), so we wondered if either of the other two identified proteins might also be important for Semaphorin/Plexin axon guidance. Clone 135 was also a strong PlexA-interactor and molecular analysis revealed that it encoded the C-terminal region of the Drosophila 14-3-3ε protein (residues 98-261; Figure 3.1Ab). Interestingly, members of 14-3-3 protein family are important regulators of signal transduction through their ability to bind to phosphorylated serine/threonine residues within target proteins (Figure 3.1Ab; (Tzivion et al., 2001; Yaffe and Elia, 2001)). Our further analysis revealed that multiple different forms of PlexA and 14-3-3ε interact, but 14-3-3ε did not interact with the other Drosophila Plexin, PlexB (Figure 3.1Ac), suggesting a specificity among PlexA – 14-3-3ε interactions. Similarly, we also saw selective interactions between purified recombinant GST-14-3-3ε protein and full-length neuronally-expressed ^{HA}PlexA (Figure 3.1Ad). Moreover, Drosophila contains two highly conserved 14-3-3 family members, *14-*

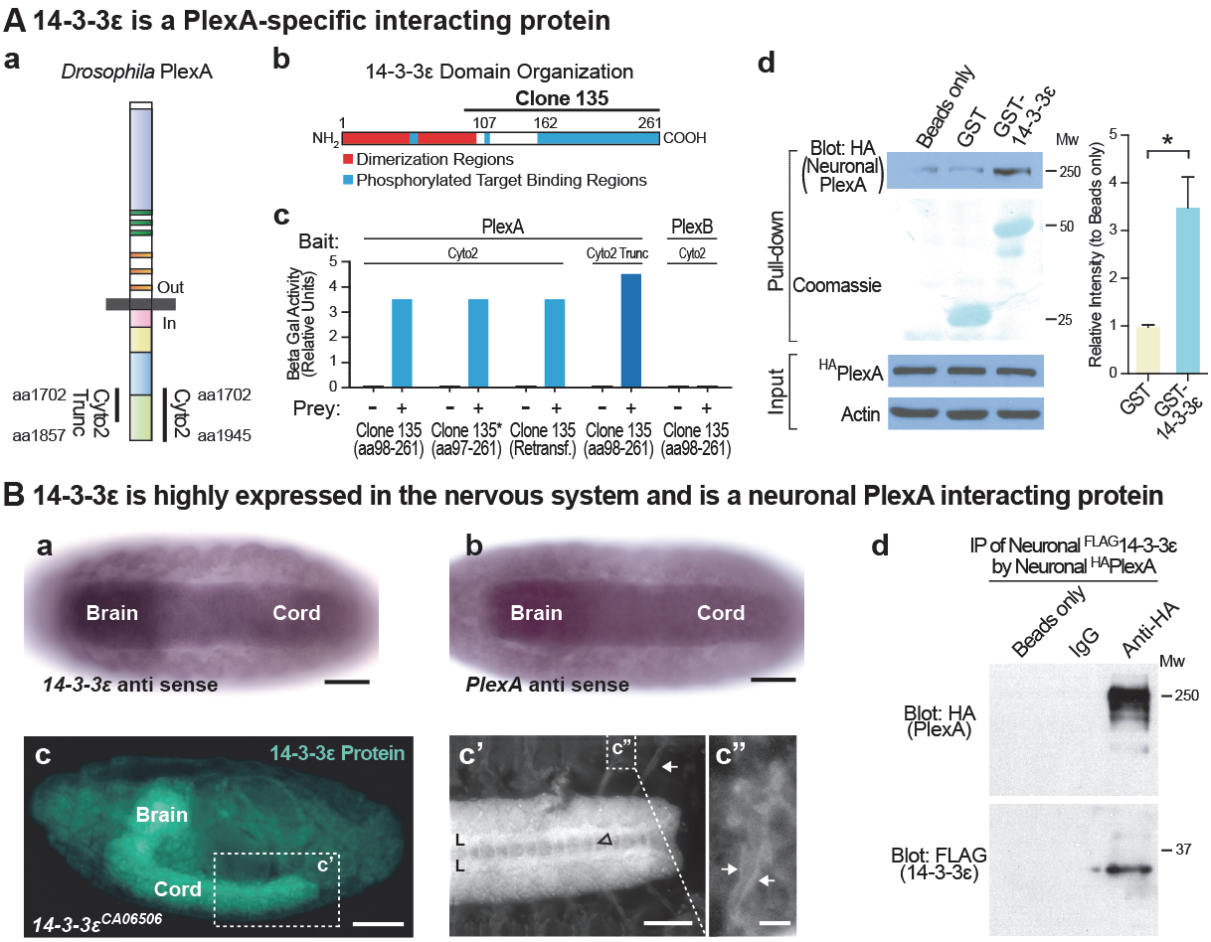

Figure 3.1. 14-3-3ε is a Neuronal PlexA Interacting Protein that is Highly Expressed in Navigating Axons (**A**) 14-3-3ε is a PlexA-specific interacting protein. (a) PlexA protein organization. The highly conserved "Cyto2 (C2)" region (amino acid (aa) residues 1702-1945) was the bait in a yeast two-hybrid screen. A bait containing a shortened version of the PlexA cytoplasmic region (Cyto2 Trunc; aa residues 1702-1857) was also employed. (b) 14-3-3ε protein organization. Clone 135 contains the C-terminus of 14-3-3ε which includes the phosphorylated target binding regions. (c) Clone 135 encodes a novel and specific PlexA-interacting protein. Clone 135 (prey) emerged from a yeast two-hybrid screen as a stong interactor with the Bait (the Cyto2 portion of PlexA) as measured by Beta-galactosidase activity (Beta Gal). Sequencing revealed that Clone 135 encoded aa 98-261 of 14-3-3ε. Similar interactions with PlexA^{Cyto2} were also observed with the related clone 135* and when Clone 135 was retransformed (retransf.) into yeast. No interactions were observed between Plexin B^{Cyto2} and Clone 135 but Clone 135 also strongly interacted with a truncated form of PlexA (PlexA$^{Cyto2\ Trunc}$). (d) Purified full length 14-3-3ε associates with PlexA. Purified recombinant GST-14-3-3ε protein, but not beads or GST protein only, added to Drosophila embryonic lysates robustly associates and "pulls down"

neuronally expressed ^{HA}PlexA. The purified GST-tagged proteins used in the GST pull-down experiments were stained (Pull-down, Coomassie) as were the lysates (Input) to confirm they were added in equal amounts. Molecular weight (MW) in kDa. The interaction compared to that of beads only was quantified. n=4; Error bar: SEM; *p<0.05 by Paired *t*-test. (**B**) 14-3-3ε is highly expressed in the nervous system and associates with neuronal PlexA. (a - b) In situ hybridization of *14-3-3ε* (a) and *PlexA* (b) in the Drosophila embryo using anti-sense RNA probes shows both transcripts are highly enriched in the central nervous system (brain and cord). (c, c', c'') 14-3-3ε protein is highly expressed in the Drosophila brain and nerve cord and localizes to CNS and motor axons. The *14-3-3ε*^{CA06506} (Figure S2A) is an enhancer trap fusion protein fly line expressing 14-3-3ε in its endogenous location fused to a green fluorescent protein (GFP) tag and revealed that GFP-14-3-3ε is highly expressed in the central nervous system (c), and localizes to longitudinal (L), commissural (e.g., arrowhead), and motor axons (e.g., arrows). The boxed region in c is seen at higher power in c' and likewise for c' in c''. Scale bars equal 50μm (Ba, Bb, Bc) and 25μm (Bc', Bc'') (d) PlexA and 14-3-3ε associate in vivo in neurons. Embryonic lysates from Drosophila embryos expressing ^{HA}PlexA and ^{FLAG}14-3-3ε in neurons were subjected to immunoprecipitation with antibodies against HA. Antibodies against ^{HA}PlexA (top) immunoprecipitated ^{FLAG}14-3-3ε (bottom) while controls (beads only or IgG antibody) did not. MW in kDa..

3-3ε and *14-3-3ζ/leonardo* (Figure S3.1A), but we found that 14-3-3ζ does not interact with PlexA in our yeast interaction assay (Figure S3.1B), suggesting that PlexA specifically and selectively interacts with a member of the 14-3-3 family of proteins.

14-3-3ε is Neuronal PlexA – interacting Protein that Localizes to Navigating Axons

To begin to determine whether 14-3-3ε might play a role in Semaphorin/Plexin axon guidance, we examined the expression of 14-3-3ε in Drosophila embryos. In situ hybridization analyses revealed that *14-3-3ε* mRNA is highly enriched in the central nervous system (CNS) at stages of axonal pathfinding in a similar

pattern to that for *PlexA* mRNA (Figure 3.1Ba-b). Likewise, antibodies to the 14-3-3ε protein (Tien et al., 1999; Su et al., 2001) as well as a Green Fluorescent Protein (GFP)–14-3-3ε protein fusion trap Drosophila transgenic line (Buszczak et al., 2007), revealed that 14-3-3ε is highly expressed in the embryonic brain and nerve cord (Figure 3.1Bc) and localizes strongly to longitudinal, commissural, and motor axons during stages of axonal pathfinding (Figure 3.1Bc' and 3.1Bc''). To directly test whether 14-3-3ε associates with PlexA in these neurons, we generated a fly line expressing both FLAG-tagged 14-3-3ε and HA-tagged PlexA in embryonic neurons using the GAL4-UAS system (Brand and Perrimon, 1993) and the neuron-specific driver, *ELAV-GAL4*. Performing co-immunoprecipitation experiments using HA antibodies consistently detected ^{FLAG}14-3-3ε in the immune complex immunoprecipitated by ^{HA}PlexA but not by control IgG antibodies (Figure 3.1Bd). These results, in combination with other related experiments (Figures 3.1Ad, 3.4D-E, 3.7B, S3.6E), indicate that PlexA and 14-3-3ε form a complex in vivo in neurons.

14-3-3ε is Required for Axon Pathfinding

To begin to explore the function of the PlexA – 14-3-3ε physical interaction, we turned to the Drosophila embryonic nervous system. Loss-of-function alleles of *PlexA* and its ligand *Sema1a* result in Drosophila embryonic motor axon pathfinding defects characterized by increased axonal fasciculation, stalling, and

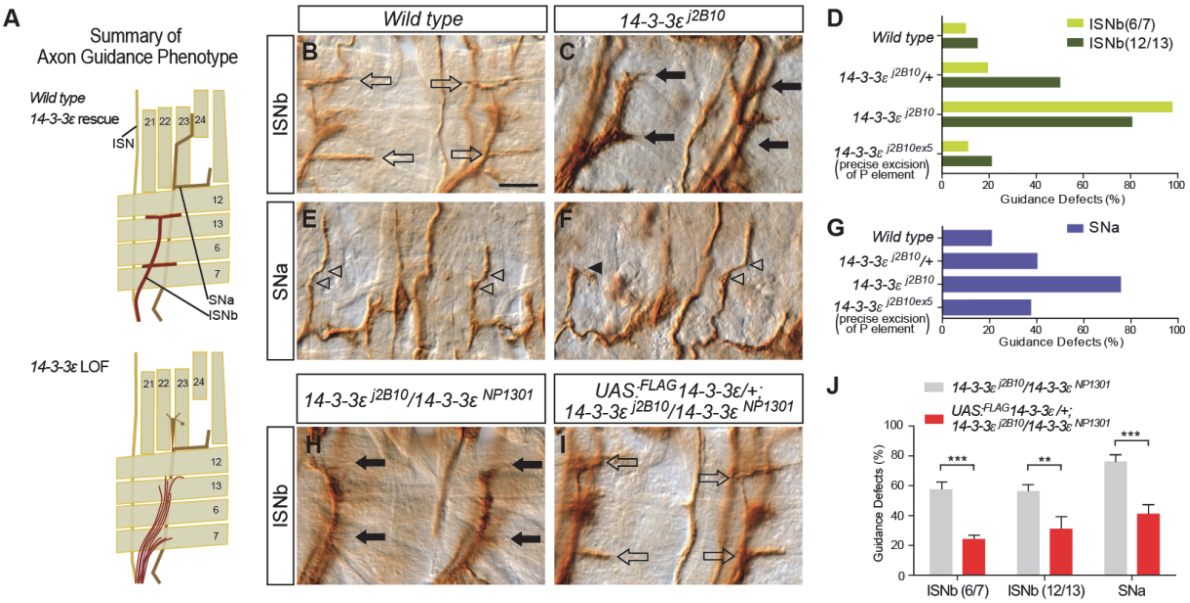

Figure 3.2. ***14-3-3ε* is Required for Normal Axon Guidance** (**A**) Summary diagrams of the typical ISNb and SNa motor axon pathways observed in different genetic backgrounds (top: *Wild type* and *14-3-3ε* rescue; bottom: *14-3-3ε* loss-of-function (LOF) mutants). (**B-C, E-F**) Representative hemisegments of filleted stage 16/17 embryos immunostained with the motor axon marker 1D4. The normal (*wild type*) axon guidance and innervation patterns of ISNb and SNa axons are shown in (**B**, open arrows) and (**E**, open arrowheads), respectively. In contrast, axons within the ISNb (**C**, closed arrows) and SNa (**F**, closed arrowhead) pathways of a *14-3-3ε* mutant (*14-3-3ε^{j2B10}*) exhibit abnormal pathfinding and muscle innervation defects. For example, ISNb axons were often less tightly fasciculated along their pathway (giving rise to thicker "bundles") and defasciculated at abnormal locations (**C**). Similar abnormal bundling and projection defects were seen with SNa axons (**F**). See also Figure S2. (**D, G**) The percentage of ISNb (**D**) and SNa (**G**) axon guidance errors in *wild type*, *14-3-3ε* heterozygous mutants *(14-3-3ε^{j2B10}/+), 14-3-3ε* homozygous mutants *(14-3-3ε^{j2B10}/14-3-3ε^{j2B10}),* and a line of flies in which the P element causing the mutation has been precisely excised (*14-3-3ε^{j2B10ex5}/14-3-3ε^{j2B10ex5}*). ISNb(6/7) = ISNb innervations at the muscle 6/7. ISNb(12/13) = ISNb innervations at the muscle 12/13. (n>100/genotype). (**H-J**) Expression of ^{FLAG}*14-3-3ε* significantly rescues the ISNb and SNa axon guidance defects that are present in *14-3-3ε* LOF mutants. *14-3-3ε^{NP1301}* expresses GAL4 under the control of the *14-3-3ε* genomic locus. n>90; Error bars: SEM; **p<0.01, ***p<0.001 by two-way ANOVA and Bonferroni post test. Scale bar equals 20μm for **B- C**, **E-F**, and **H-I**.

abnormal muscle innervation (Yu et al., 1998; Winberg et al., 1998b). Therefore, we wondered if *14-3-3ε* loss-of-function mutants exhibited motor axon guidance defects. A number of *14-3-3ε* loss-of-function alleles have been well-characterized (Figure S3.2A; (Chang and Rubin, 1997; Benton and St Johnston, 2003; Acevedo et al., 2007)) and have revealed that *14-3-3ε* mutants do not exhibit overt morphological defects within the nervous system or musculature (Acevedo et al., 2007). Indeed, it appears that both maternally supplied 14-3-3ε and compensation by 14-3-3ζ are sufficient for many developmental processes exhibit overt morphological defects within the nervous system or musculature (Acevedo et al., 2007). Indeed, it appears that both maternally supplied 14-3-3ε and compensation by 14-3-3ζ are sufficient for many developmental processes including cell fate specification and patterning (Chang and Rubin, 1997; Su et al., 2001; Acevedo et al., 2007; Krahn et al., 2009). However, neuronal expression of 14-3-3ε is necessary for normal embryonic hatching and adult viability for unknown reasons (Acevedo et al., 2007) and we now find that multiple combinations of five different *14-3-3ε* loss-of-function alleles generate motor and CNS axon guidance defects (Figures 3.2, S3.2).

During Drosophila embryonic development, motor axons grow within several well-characterized axonal pathways to reach their muscle targets (Keshishian et al., 1996; Araujo and Tear, 2003). For example, axons within the Intersegmental Nerve b (ISNb) normally defasciculate from the pioneering

Intersegmental Nerve (ISN) to innervate their muscle targets including muscles 6/7 and 12/13 (Figures 3.2A-B; (Van Vactor et al., 1993)). In contrast, we found that ISNb axons within Drosophila embryos containing the well-characterized *14-3-3ε*^{j2B10} loss-of-function (LOF) mutation (Figure S3.2A; (Chang and Rubin, 1997; Acevedo et al., 2007)), exhibit highly penetrant axon guidance defects including abnormal defasciculation, inappropriate pathway selection, and decreased muscle innervation (Figures 3.2A, C-D; S3.2D). These abnormal ISNb pathfinding defects were significantly rescued upon precise excision of the *j2B10* loss-of-function P element allele (*14-3-3ε*^{j2B10ex5}; Figures 3.2A, D; (Chang and Rubin, 1997; Acevedo et al., 2007)) and after restoring 14-3-3ε expression in *14-3-3ε* mutants using our ^{FLAG}*14-3-3ε* transgene (Figures 3.2A, H-J). We also observed similar ISNb axon guidance defects in other combinations of *14-3-3ε* loss-of-function mutants (Figure S3.2A-B, D) and axonal pathfinding errors within other motor axon pathways including the Segmental Nerve A (SNa) and the TN nerve (Figures 3.2A, E-G, J; S3.2B, D; data not shown) as well as in the CNS (Figure S3.2C). These results reveal for the first time that a member of the 14-3-3 family of phospho-serine binding proteins, 14-3-3ε, is required for axon guidance in vivo.

14-3-3ε Antagonizes Semaphorin/Plexin-mediated Repulsive Axon Guidance

We next compared 14-3-3ε–dependent axon guidance defects to those resulting from manipulating Sema-1a/PlexA signaling. Interestingly, we found that while some of the axon guidance defects we observed in *14-3-3ε* mutants (Figures 3.2; S3.2C-D) were similar to *Sema1a*, *PlexA*, and *Mical* mutants (Figure S3.2E), the majority resembled the effects of increasing PlexA axon guidance signaling (Figure S3.2E). Furthermore, we found that overexpressing ^{FLAG}14-3-3ε in neurons generated axon guidance defects (Figure S3.2B) that were more similar in nature to decreasing Sema-1a/PlexA/Mical repulsive axon guidance signaling (Figure S3.2E). These observations suggest that 14-3-3ε, like other components previously identified as functioning in Sema-1a/PlexA signaling such as Nervy and PKA (Figure S3.2E; (Terman and Kolodkin, 2004)), may antagonize Sema-1a/PlexA repulsive axon guidance. To further address this possibility, we turned to enhancer-supressor genetic assays. Sema-1a/PlexA repulsion affects the pathfinding ability of both motor and CNS axons and several robust, well-characterized in vivo axon guidance assays have been employed to characterize components of the Semaphrorin/Plexin axon guidance signaling cascade (Yu et al., 1998; Winberg et al., 1998b; Winberg et al., 2001; Terman et al., 2002; Terman and Kolodkin, 2004; Ayoob et al., 2004; Ayoob et al., 2006; He et al., 2009). For instance, increasing the levels of neuronal *PlexA* generates abnormally defasciculated axons that result in discontinuous CNS longitudinal connectives

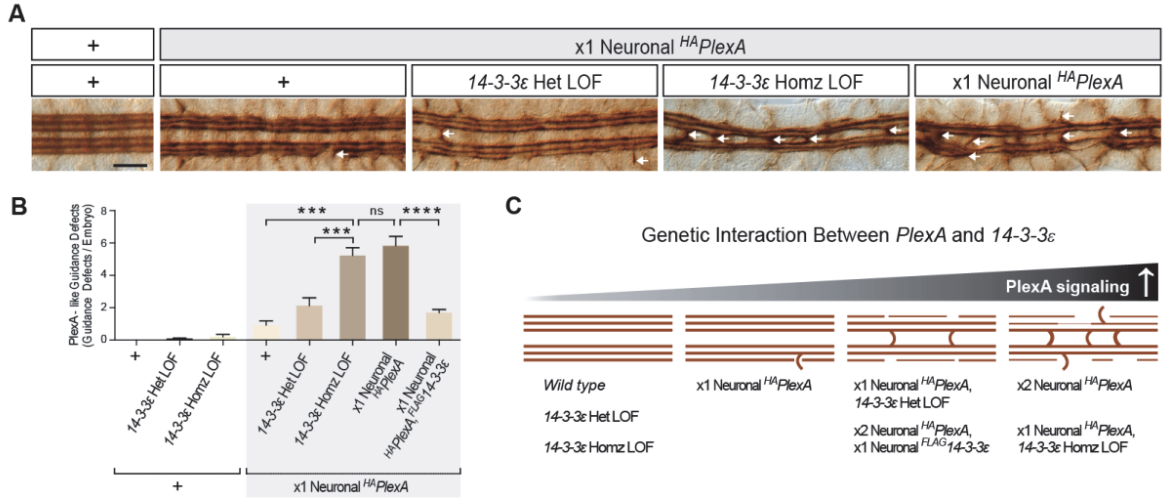

Figure 3.3. 14-3-3ε Antagonizes Semaphorin/PlexA Repulsive Axon Guidance (**A**-**B**) PlexA–dependent axon guidance defects are genetically modified by *14-3-3ε*. Increasing the neuronal levels of *PlexA* (x1 Neuronal ^{HA}*PlexA,* + [2nd image from left in **A**]) generates CNS axon guidance defects. In particular, note that specific longitudinal connectives are disrupted (e.g., arrow and in **B**). Decreasing the levels of *14-3-3ε* significantly enhances these *PlexA*-dependent guidance defects (x1 Neuronal ^{HA}*PlexA*, *14-3-3ε* Heterozygous (Het) LOF or x1 Neuronal ^{HA}*PlexA*, *14-3-3ε* Homozygous (Homz) LOF). Note, disrupted CNS longitudinals and axons projecting abnormally across the midline and into the periphery (e.g., arrows and in **B**) are similar to when the levels of *PlexA* are further increased in neurons (x1 Neuronal ^{HA}*PlexA,* x1 Neuronal ^{HA}*PlexA* [i.e., x2 Neuronal ^{HA}*PlexA*]). In contrast, increasing the levels of *14-3-3ε* suppresses these *PlexA*-dependent guidance defects (**B**, x2 Neuronal ^{HA}*PlexA*, x1 Neuronal ^{FLAG}*14-3-3ε*). Genotypes: x1 Neuronal ^{HA}*PlexA* is *ELAV-GAL4, UAS:*^{HA}*PlexA*/+; *14-3-3ε* LOF is *14-3-3ε*^{j2B10}; 1x Neuronal ^{FLAG}*14-3-3ε* is *UAS:*^{FLAG}*14-3-3ε*. Scale bar equals 50µm. n=90; Error bars: SEM; ***p<0.001 by one-way ANOVA, Tukey's multiple comparison test. (**C**) Summary. Genetic interaction analyses reveal that *PlexA*-dependent repulsive axon guidance is antagonized by *14-3-3ε*. Increasing PlexA axon guidance signaling induces defasciculation defects that result in defective longitudinal connectives in which axons project abnormally across the midline or into the periphery. Decreasing the levels of 14-3-3ε exacerbates these Semaphorin/Plexin-dependent guidance defects while increasing the levels of 14-3-3ε in neurons suppresses Semaphorin/Plexin-dependent guidance defects. See also Figure S3.

and axons crossing the midline or projecting abnormally into the periphery (Figure 3.3A; (Winberg et al., 1998b; Ayoob et al., 2004; He et al., 2009)). These axon guidance defects are dependent on Sema-1a/PlexA/Mical-repulsive axon guidance signaling (Figure S3.3B; (He et al., 2009; Ayoob et al., 2004)). Decreasing the levels of *14-3-3ε* significantly increased the axon guidance defects that resulted from increasing the neuronal levels of ^{HA}*PlexA*, further indicating that *14-3-3ε* antagonizes *PlexA* repulsive axon guidance (Figure 3.3A-C). Indeed we found that the guidance defects generated by low level increases in the expression of ^{HA}*PlexA* in neurons (1 copy of the transgene) in a *14-3-3ε* homozygous (Homz) LOF mutant were similar to those defects seen with high levels of neuronal ^{HA}*PlexA* (2 copies of the transgene) (Figure 3.3A-C). Likewise, we found that increasing neuronal 14-3-3ε in combination with neuronal PlexA significantly decreased the number of *PlexA*-dependent axon guidance defects (Figure 3.3B-C). These results along with other in vivo *Sema1a/PlexA*-dependent CNS and motor axon repulsive guidance assays (Figure S3.3A-C) indicate that *14-3-3ε* antagonizes *Sema1a/PlexA*-mediated repulsive axon guidance.

A Single Phosphorylated Serine Residue on PlexA Mediates its Interaction with 14-3-3ε

To investigate the mechanism by which 14-3-3ε antagonizes PlexA-mediated axon guidance, we first sought to determine the sites of interaction between PlexA and 14-3-3ε. Our yeast interaction screen identified that the C-terminal portion of the PlexA intracellular region specifically interacted with 14-3-3ε (Figure 3.1Aa, c), so we employed the yeast system to determine the region within PlexA responsible for this interaction. Yeast were cotransformed with baits comprising different portions of the PlexA cytoplasmic region and a prey containing the C-terminus of 14-3-3ε (Figure 3.4A). All PlexA constructs were expressed at a similar level in yeast and our analysis revealed that a region of PlexA containing residues 1774 through 1821 was necessary and sufficient for the interaction with 14-3-3ε (Figure 3.4A). Interestingly, we noticed a motif within residues 1774 through 1821 of PlexA that matched the consensus sequence for 14-3-3 binding proteins. In particular, amino acids 1791-1796 of Drosophila PlexA comprise a mode I 14-3-3 consensus binding motif, R/KSXpSXP, where p represents the phosphorylated serine (S) residue predicted to be critical for an interaction with 14-3-3 proteins (Figure 3.4B; (Yaffe et al., 1997; Rittinger et al., 1999)). To begin to determine if phosphorylation of a serine residue within the PlexA cytoplasmic region mediates the interaction between PlexA and 14-3-3ε we substituted selected serine (Ser) and threonine (Thr) residues within this consensus 14-3-3ε binding motif with alanine (Ala) (Figures 3.4C, S3.4A). Our mutatagenesis experiments revealed that the single Ser^{1794} residue was necessary

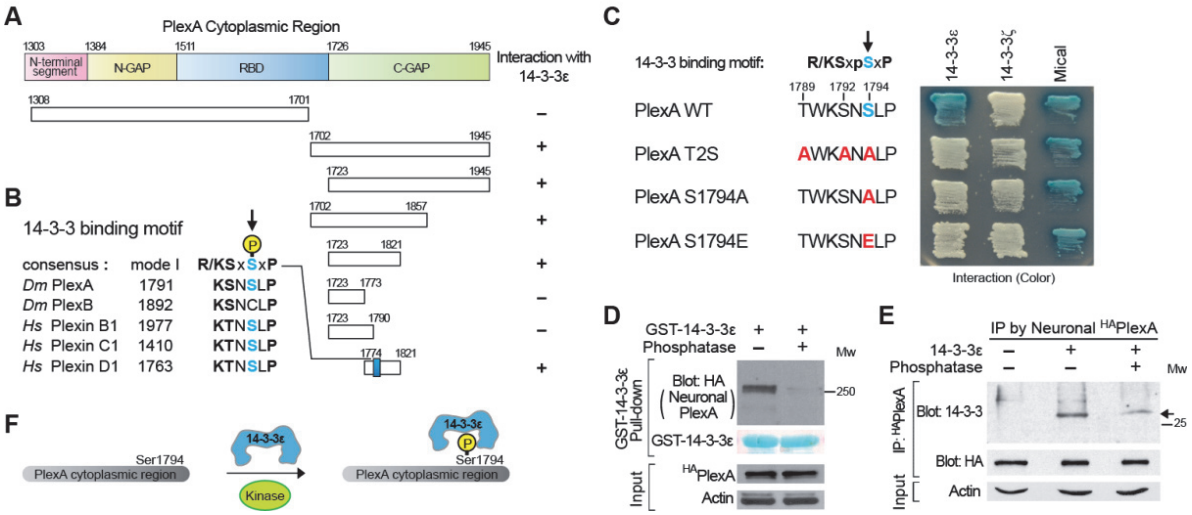

Figure 3.4. PlexA – 14-3-3ε Interactions Occur Through a Single Phosphoserine Residue Present in the PlexA Cytoplasmic Tail (**A-B**) Interaction analysis reveals that the region of PlexA that interacts with 14-3-3ε contains a 14-3-3 consensus binding motif. (**A**) Schematic representation of protein constructs containing different portions of the PlexA cytoplasmic region and their ability to interact with 14-3-3ε (Clone 135), as assessed using a yeast two-hybrid assay as in Figure 1A. "+" indicates that the two constructs interact, "–" indicates that they do not. The blue box indicates the position of the consensus 14-3-3 binding motif. The amino acid residues corresponding to the Plexin cytoplasmic tail are indicated above each construct. N-GAP: N-terminal GAP domain; RBD: Rho GTPase binding domain; C-GAP: C-terminal GAP domain (He et al., 2009). (**B**) The minimal region of PlexA required for an interaction with 14-3-3ε contains a consensus 14-3-3 binding motif where amino acid residues are in upper case (one-letter code), x is for any residue, and the consensus phosphorylation site is P in a circle. This consensus 14-3-3 binding motif is also conserved in mammalian Plexins that signal for Semaphorins within Classes 3, 4, 5, 6, and 7, but not in Drosophila PlexB (providing a basis for why we did not see an interaction between Drosophila PlexB and 14-3-3ε (Clone 135) (see Figure 1Ac)). *Dm Drosophila melanogaster*; *Hs Homo sapiens*. (**C**) Site-directed mutagenesis reveals that the Ser^{1794} residue in PlexA is critical for the interaction between PlexA and 14-3-3ε. A yeast two-hybrid assay as in (**A**) was used to look for interactions between PlexA ($PlexA^{Cyto2\ Trunc}$; residues 1702-1857) and 14-3-3ε (Clone 135) following substitutions of different Ser (S) or Thr (T) residues to Ala (A) or Glu (E) in the consensus 14-3-3 binding motif of PlexA. Ser^{1794} to Ala^{1794} or Glu^{1794} in PlexA (PlexA S1794A or S1794E) disrupted the interaction with 14-3-3ε but not with another Plexin interacting protein, Mical. Substituted residues are colored red. (**D-E**) Phosphatase treatment indicates that PlexA – 14-3-3ε interactions are phosphorylation dependent. (**D**) Purified GST-14-3-3ε protein pulled down ^{HA}PlexA from Drosophila embryonic lysates expressing neuronal ^{HA}PlexA, whereas incubating the lysates with a phosphatase (CIP) abolished the interaction. (**E**) Embryonic lysates containing neuronal ^{HA}PlexA were immunoprecipitated

with an HA antibody in the absence (–) or presence (+) of equal amounts of recombinant 14-3-3ε protein as well as a phosphatase (CIP). Phosphatase treatment decreased the coimmunoprecipitation between ^{HA}PlexA and 14-3-3ε (arrow). MW in kDa. (**F**) As diagrammed, our results indicate that the interaction between PlexA and 14-3-3ε is mediated by phosphorylation of PlexA Ser^{1794}.

for the observed PlexA interaction with 14-3-3ε, but not with one of Plexin's other interacting proteins, Mical (Figures 3.4C, S3.4A), indicating that the PlexA Ser^{1794} residue is a specific site of interaction with 14-3-3ε.

14-3-3 proteins typically bind to single phosphorylated serine or threonine residues on target proteins (Yaffe and Elia, 2001), so our results suggested that the Ser^{1794} residue on PlexA is phosphorylated and thereby provides the binding site for 14-3-3ε. To test this possibility, we first generated phospho-mimetic forms of Ser (Ser^{1794} to Glu^{1794} or Asp^{1794}), but found that as with other 14-3-3 interacting proteins (e.g., (Maudoux et al., 2000; Brummer et al., 2008)) adding one negative charge is not sufficient for the interaction between PlexA and 14-3-3ε (Figures 3.4C, S3.4A, data not shown). Therefore, to determine if phosphorylation mediates the interaction between PlexA and 14-3-3ε, we turned to in vitro binding assays with PlexA and purified 14-3-3ε. Our results revealed that a purified GST-14-3-3ε fusion protein "pulls down" neuronal ^{HA}PlexA from Drosophila embryonic lysates but not when the embryonic lysates were treated with a phosphatase (Figure 3.4D). Likewise, this phosphatase treatment disrupted the ability of neuronally expressed ^{HA}PlexA to immunoprecipitate 14-3-3ε (Figure

3.4E). Taken together, these results indicate that PlexA and 14-3-3ε associate via a single phosphoryated serine residue present in the cytoplasmic portion of the PlexA receptor (Figure 3.4F).

PKA Phosphorylates PlexA^{S1794} Enabling the PlexA – 14-3-3ε Interaction

Our results indicate that phosphorylation of PlexA^{S1794} mediates the interaction between PlexA and 14-3-3ε so we wondered which Ser/Thr kinase(s) phosphorylated PlexA. Interestingly, we first observed the interaction between PlexA and 14-3-3ε in yeast indicating that a serine/threonine kinase present in yeast is sufficient to phosphorylate PlexA. We also noticed that the 14-3-3ε binding site within PlexA contains a consensus phosphorylation site (R/KxxS; Figure S3.4B; (Pearson and Kemp, 1991; Amanchy et al., 2007)) for several kinases well-conserved from yeast to humans including the cAMP-dependent protein kinase (PKA), the Ca^{2+}-dependent protein kinase (PKC), and the cGMP-dependent protein kinase (PKG). Therefore, we employed in vitro kinase assays and incubated purified recombinant GST-tagged PlexA protein with purified proteins for these candidate serine/threonine kinases as well as other conserved serine/threonine kinases (Figure S3.4B). Our results revealed that the C-terminal portion of the PlexA cytoplasmic region (PlexA^{Cyto2}) is highly and specifically phosphorylated by PKA (Figure S3.4B). Likewise, we observed a specific PKA-mediated increase in incorporated ^{32}P over time within GST-PlexA^{Cyto2}, but not

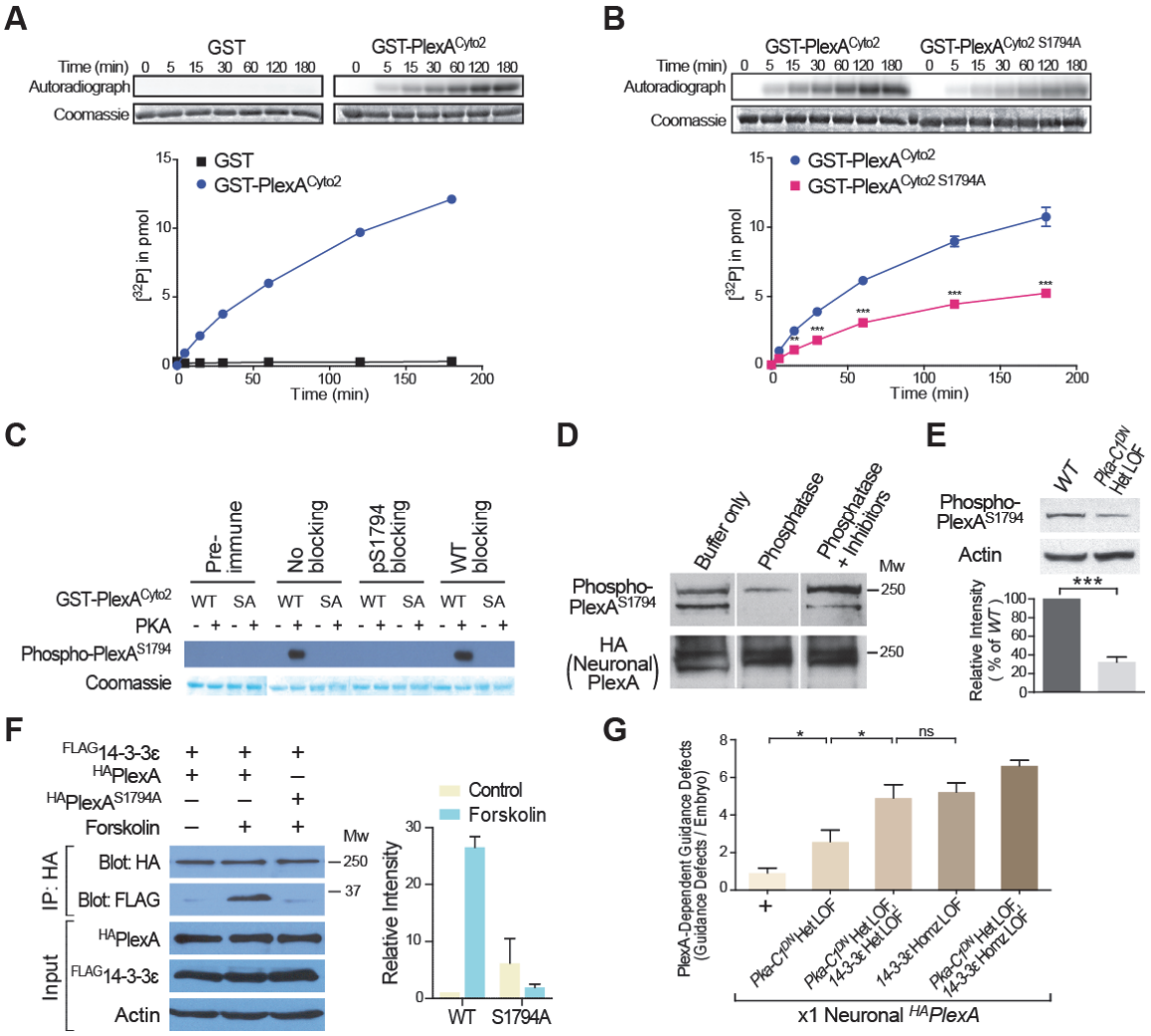

Figure 3.5. PKA Phosphorylates PlexA^{S1794} Mediating the PlexA – 14-3-3ε Interaction (**A-B**) PKA specifically phosphorylates the Ser^{1794} residue within the cytoplasmic portion of PlexA. In vitro kinase assays using purified PKA and recombinant proteins revealed that GST-PlexA^{Cyto2}, but not GST-only, was phosphorylated by PKA over time (**A**) but the amount of incorporated phosphate was significantly decreased following a substitution of Ser to Ala at the 1794 residue of PlexA (**B**; GST-PlexA$^{Cyto2\ S1794A}$). n=3; Error bar: SEM; **p<0.01, ***p<0.001 by two-way ANOVA, Bonferroni post test. Total amount of PlexA protein included in the assay is shown by Coomassie staining. (**C-E**) Generation of a phospho-PlexA^{S1794} specific antibody indicates that ^{HA}PlexA is phosphorylated by PKA in vivo in neurons. (**C**) The phospho-PlexA^{S1794} antibody recognizes purified GST-PlexA^{Cyto2} (WT) protein but only following pre-incubation with PKA. In contrast, the phospho-PlexA^{S1794} antibody does not recognize purified GST-PlexA$^{Cyto2\ S1794A}$ (SA) protein with or without incubation with PKA. Furthermore, preincubation of the phospho-

PlexA^{S1794} antisera with the pS1794 peptide, but not the wild-type (WT) peptide, abolishes immunorecognition of the PKA-phosphorylated GST-PlexA^{Cyto2} protein. The total amount of PlexA protein included in each experiment is shown by Coomassie staining. (**D**) ^{HA}PlexA is phosphorylated in vivo in neurons as examined with the phospho-PlexA^{S1794} antibody. The phospho-PlexA^{S1794} antibody recognized ^{HA}PlexA following immunoprecipitation of neuronally expressed ^{HA}PlexA (Buffer Only). Treatment of the membrane-bound immunoprecipitated ^{HA}PlexA with a phosphatase (lambda protein phosphatase [λPP]) decreased the phosphorylation level of ^{HA}PlexA, but not when phosphatase inhibitors (Na_3VO_4, NaF) were added. Total amount of immunoprecipitated ^{HA}PlexA was examined using an HA antibody (note that in this preparation and percentage gel, neuronal ^{HA}PlexA is observed as two different sized bands, as noted previously (Terman et al., 2002; Yu et al., 2010). MW in kDa. (**E**) PlexA phosphorylation in vivo is dependent on PKA activity. Drosophila embryos from wild type (WT) or dominant negative (DN) *Pka-C1* mutant (*Pka-C1^{DN}* Het LOF; (Pan and Rubin, 1995)) were lysed and examined using phospho-PlexA ^{S1794} antibody. Phosphorylation level of PlexA was significantly reduced in *Pka-C1^{DN}* mutant embryos compared to that of wild type embryos. n=8; Error bar: SEM; ***p<0.0001 by Paired *t*-test. (**F**) Increasing cAMP/PKA signaling induces a PlexA Ser^{1794} – dependent interaction between PlexA and 14-3-3ε. In a Drosophila Kc cell line, ^{FLAG}14-3-3ε robustly co-immunoprecipitates with ^{HA}PlexA, but not ^{HA}PlexA^{S1794A}, and the interaction is dependent on the addition of the cAMP/PKA activator, forskolin. n=4; MW in kDa. (**G**) PKA and 14-3-3ε work together to antagonize Plexin-mediated repulsive axon guidance in vivo. Employing the genetic interaction assay as in Figure 3 reveals that a mutation in *PKA-C1* (*Pka-C1^{DN}*) dominantly enhanced *PlexA*-dependent axon repulsion, which is further enhanced by *14-3-3ε* Het LOF (and generates guidance defects similar to *14-3-3ε* Homz LOF). Genotypes: x1 Neuronal *^{HA}PlexA* represents for *ELAV-GAL4,UAS:^{HA}PlexA/+*; *14-3-3ε* LOF for *14-3-3ε^{j2B10}*. n≥90; Error bars: SEM; *p<0.05; ***p<0.001 by one-way ANOVA, Tukey's multiple comparison test.

within GST, indicating that the Cyto2 region of PlexA is selectively phosphorylated by PKA (Figures 3.5A, S3.4C). We then purified recombinant PlexA protein containing a Ser^{1794} to Ala^{1794} substitution (GST-PlexA$^{Cyto2\ S1794A}$) and asked whether the PlexA^{S1794} residue, which is critical for 14-3-3ε binding, is phosphorylated by PKA. Notably, we observed a significant decrease in the amount of ^{32}P incorporated into GST-PlexA$^{Cyto2\ S1794A}$ at each time point compared to "wild-type" PlexA^{Cyto2} protein (Figure 3.5B), indicating that PKA phosphorylates PlexA Ser^{1794}. Interestingly, we also noticed that roughly half the

amount of ^{32}P was incorporated at each time point in GST-PlexA$^{Cyto2\ S1794A}$ compared to wild type PlexA^{Cyto2} protein (Figure 3.5B), suggesting that there may be two PKA phosphorylation sites in the PlexA^{Cyto2} region. Moreover, we found that Cdk5, a serine/threonine kinase previously found to associate in a complex with A Class mammalian Plexins (Sasaki et al., 2002), phosphorylated the PlexA^{Cyto2} region (Figures S3.4B), but similar levels of Cdk5-mediated phosphorylation were found on the purified PlexA$^{Cyto2\ S1794A}$ protein (Figure S3.4D). Together, these results reveal that PKA specifically and selectively phosphorylates the Ser^{1794} residue of PlexA.

We next sought to examine whether PlexA^{S1794} is phosphorylated in vivo, so we generated a rabbit polyclonal antibody against the phosphorylated form of PlexA^{S1794} (phospho-PlexA^{S1794}). Characterizing this phospho-PlexA^{S1794} specific antibody revealed its specific immunorecognition of peptides synthesized with a phosphorylated Ser^{1794} residue (pS1794), but not unphosphorylated (WT) or Ser^{1794} to Ala^{1794} (S1794A)-containing peptides (Figure S3.4E). Likewise, this phospho-PlexA^{S1794} antibody specifically recognized purified "wild-type" GST-PlexA^{Cyto2} (WT) protein, but not GST-PlexA$^{Cyto2\ S1794A}$ (SA) protein, following their in vitro phosphorylation by PKA (Figure 3.5C). Moreover, the ability of the phospho-PlexA^{S1794} antibody to recognize the phosphorylated GST-PlexA^{Cyto2} protein was blocked by pre-incubation with the pS1794 peptide but not with the WT peptide (Figure 3.5C). Therefore, in light of the specificity of this antibody

to the phosphorylated form of PlexA^{S1794} we examined whether PlexA^{S1794} is phosphorylated in vivo in neurons. Immunoprecipitating ^{HA}PlexA from embryonic neurons and immunoblotting with the phospho-PlexA^{S1794} – specific antibody revealed a protein band similar in size to ^{HA}PlexA (Figure 3.5D). Furthermore, phosphatase treatment of imunoprecipitated neuronal ^{HA}PlexA decreased this phospho-PlexA^{S1794} – specific band, but this effect was blocked in the presence of phosphatase inhibitors (Figure 3.5D). Moreover, the phosphorylation of PlexA^{S1794} in neurons was significantly reduced by a dominant-negative *PKA* mutant (Figure 3.5E), indicating that PKA also phosphorylates PlexA in vivo. Together, these results indicate that PlexA^{S1794} is phosphorylated in neurons and that this phosphorylation by PKA mediates the interaction between PlexA and 14-3-3ε. To further test this possibility, we asked whether forskolin, which activates PKA signaling by increasing cellular cAMP levels (Seamon et al., 1981), induced an association between ^{HA}PlexA and ^{FLAG}14-3-3ε. Using Drosophila tissue culture cells, we found little to no basal association between ^{FLAG}14-3-3ε and ^{HA}PlexA, but increasing cAMP levels significantly enhanced the association between ^{FLAG}14-3-3ε and ^{HA}PlexA (Figure 3.5F). In contrast, we observed little to no association between ^{FLAG}14-3-3ε and ^{HA}PlexA^{S1794A} in the presence of forskolin (Figure 3.5F), further indicating that phosphorylation of PlexA^{S1794} by PKA mediates the interaction between PlexA and 14-3-3ε.

PKA-mediated 14-3-3ε Interactions with PlexA^{S1794} Antagonize Semaphorin/Plexin Axon Repulsion

A protein complex containing PKA has been found previously to physically associate with the PlexA receptor (Terman and Kolodkin, 2004; Fiedler et al., 2010). This work in combination with our new biochemical results suggest a model in which inactive PKA is tethered to the PlexA receptor and upon cAMP-mediated activation, PKA specifically phosphorylates PlexA at Ser^{1794} and provides a binding site for 14-3-3ε. So what is the role of this PKA–14-3-3ε signaling pathway in Semaphorin/Plexin repulsive axon guidance? Like loss of *14-3-3ε*, decreasing PKA increased Sema-1a/PlexA repulsive axon guidance (Figures 5G, S3A, C; (Terman and Kolodkin, 2004)). These effects are further enhanced by simultaneously decreasing PKA and 14-3-3ε (Figures 3.5G, S3.3A), indicating that PKA and 14-3-3ε work together to antagonize PlexA repulsive axon guidance. These results also predict that disrupting the interaction between 14-3-3ε and PlexA might generate a hyperactive PlexA receptor so we generated two transgenic fly lines (^{HA}PlexA^{S1794A} and ^{HA}PlexA^{S1794E}; Figure 3.6A) containing single mutations that disrupt the assocation between PlexA and 14-3-3ε. Notably, although both the SA and SE Plexin receptors were expressed at or below the levels of "wild-type" ^{HA}PlexA (WT) in the embryonic neurons and on the surface of axons (Figures 3.6B, S3.5A-B), both mutations generated axon guidance defects consistent with increased PlexA repulsive axon guidance

signaling (Figure 3.6C-D). Therefore, disrupting the interaction between PlexA and 14-3-3ε generates defects consistent with hyperactive Semaphorin/Plexin-mediated repulsive axon guidance.

PlexA–14-3-3ε Interactions Occlude Plexin RasGAP-Mediated Repulsive Axon Guidance

We next sought to understand how the PKA-mediated interaction with 14-3-3ε might antagonize PlexA-mediated repulsion. One possiblitiy is that PlexA levels on growing axons might be down-regulated by a phosphorylation-mediated interaction with 14-3-3ε. However, the ^{HA}PlexA levels on the surface of axons were not increased in LOF *14-3-3ε* mutants (Figure S3.6A). Interestingly, we did notice that the Ser^{1794} residue that is critical for the interaction between 14-3-3ε and PlexA is located adjacent to one of the enzymaticaly critical arginine residues through which Plexins "turn-off" Ras^{GTP} signaling (Figure 3.7A; Arg^{1798}). In particular, the intracellular region of Plexins contains a GTPase activating protein (GAP) enzymatic domain that is structurally and functionally similar to GAPs for Ras superfamily GTPases (Figure 3.7A; (Oinuma et al., 2004a; He et al., 2009; Tong et al., 2009)). As a RasGAP, Plexin facilitates endogenous GTP hydrolysis by Ras family GTPases and thus functions to antagonize or "turn-off" Ras^{GTP} signaling (Oinuma et al., 2004a; Oinuma et al., 2006; Saito et al., 2009; He et al., 2009; Tong et al., 2009). In plexins, like other RasGAPs (Scheffzek et al., 1998b;

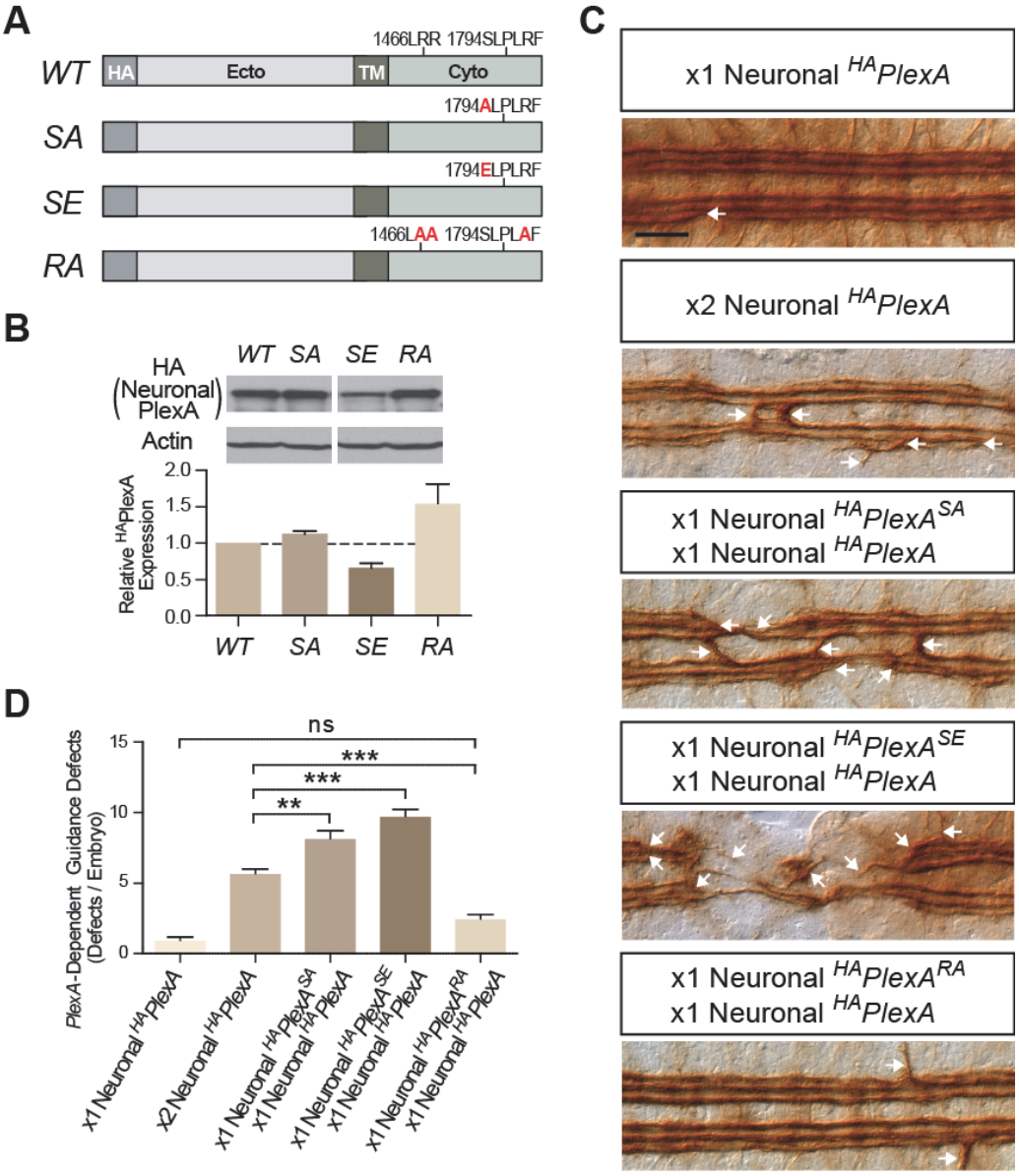

Figure 3.6. Disrupting the PlexA – 14-3-3ε Interaction Generates Hyperactive Plexin Repulsive Axon Guidance Signaling (**A**) Domain structures and names of the different *PlexA* transgenes. HA (2xHA tag), Ecto (extracellular region), TM (transmembrane region), Cyto (cytoplasmic region). Mutated residues are colored red. SA and SE are single substitutions of Ala^{1794} to Ser^{1794} or Glu^{1794}, respectively. RA represents an enzymatically GAP deficient PlexA by substituting Ala for enzymatically critical Arg residues ((Oinuma et al., 2004a); Figure S6D). (**B**) Disrupting the 14-3-3ε interaction site on PlexA did not significantly increase the abundance of

the different ^{HA}PlexA mutant proteins in Drosophila embryonic neurons. There was even a decrease in the abundance of the hyperactive (see Figures 6C-D) SE protein. The expression was quantified from Western Blots and compared to the value of wild type ^{HA}Plexin A (n=3). See also Fig. S5A-B. (**C-D**) Disrupting the PlexA – 14-3-3ε interaction significantly enhances *PlexA*-dependent repulsive axon guidance, while disrupting the GAP activity of PlexA suppresses *PlexA*-dependent guidance defects. Employing the sensitive genetic background dependent on the repulsive axon guidance effects of *PlexA* (as in Figure 3), reveals that disrupting the interaction between PlexA and 14-3-3ε (x1 Neuronal ^{HA}*PlexA*^{SA} or x1 Neuronal ^{HA}*PlexA*^{SE} with x1 Neuronal ^{HA}*PlexA*) significantly enhanced the repulsive effects of *PlexA* (compare to x2 Neuronal ^{HA}*PlexA*). In contrast, removing the PlexA cytoplasmic region significantly suppressed the repulsive effects of *PlexA* (^{HA}*PlexA*$^{\Delta Cyto}$; (He et al., 2009)) as did disrupting the GAP activity of PlexA (x1 Neuronal ^{HA}*PlexA*^{RA} with x1 Neuronal ^{HA}*PlexA*). Quantifying the number of defects, along with the expression data in **B** and Figures S5A-B, indicate that mutating Ser^{1794} of PlexA generates a hyperactive form of PlexA, whereas inactivating the GAP activity of PlexA generates a form of PlexA with reduced activity. Genotype: x1 ^{HA}*PlexA* represents for *ELAV-GAL4,UAS:*^{HA}*PlexA/+*; x1 Neuronal ^{HA}*PlexA*^{SA} for UAS: ^{HA}*PlexA*^{SA}/+; x1 Neuronal ^{HA}*PlexA*^{SE} for UAS: ^{HA}*PlexA*^{SE}/+; x1 Neuronal ^{HA}*PlexA*^{RA} for UAS: ^{HA}*PlexA*^{RA}/+. Scale bar equals 50μm. n>90; Error bars: SEM; **p<0.01, ***p<0.001 by one-way ANOVA, Tukey's multiple comparison test.

Ahmadian et al., 2003; Bos et al., 2007), arginine fingers cooperatively confer both GTPase binding and GAP activity, suggesting that the association of 14-3-3ε with the Ser^{1794} of PlexA would likely physically perturb the association between PlexA and its substrate GTPase (Figure 3.7A; (He et al., 2009; Tong et al., 2009)). To begin to test this mechanism of action, we wondered if the GAP activity of PlexA is important for PlexA-mediated repulsive axon guidance. The Plexin GAP activity has been shown to be critical for Plexin repulsive axon guidance in vitro (Rohm et al., 2000; Oinuma et al., 2004a), but its role in vivo has not been examined. Therefore, we followed well-characterized strategies (e.g., (Rohm et al., 2000; Oinuma et al., 2004a; He et al., 2009)) and made point mutations disrupting the catalytically important arginine fingers, thereby generating a GAP-

deficient PlexA transgenic Drosophila line ^{HA}PlexA^{RA} (Figure 3.6A). Our results revealed that unlike wild-type PlexA, neuronal expression of the PlexA GAP-deficient protein failed to rescue *PlexA*$^{-/-}$ mutant axon guidance defects (Figure S3.5C), and suppressed the ability of PlexA to mediate repulsive axon guidance (Figures 3.6B-D, S3.5B). These results indicate that the GAP activity of Plexin is important in vivo for repulsive axon guidance.

Ras family GTPases including R-Ras and M-Ras serve as substrates for Plexin family members (Oinuma et al., 2004a; Toyofuku et al., 2005; Saito et al., 2009), so we examined whether Drosophila Ras family members could also associate with Drosophila PlexA. Drosophila has a single Ras family GTPase, Ras2 (Ras64B), which is highly related to R-Ras and M-Ras (Figure S3.6B) and is thought to be their functional orthologue (Brand and Perrimon, 1993; Walker et al., 2006). Therefore, we generated recombinant protein for Ras2 (and also the Drosophila H-Ras/K-Ras orthologue Ras1 [Ras85D]) and found that ^{HA}PlexA preferentially associated with the GTP bound form of Ras2 (Figures 3.7B, S3.6C) and facilitated GTP hydrolysis of Ras2 that was dependent on the arginine fingers of PlexA (Figure S3.6D). Likewise, constituitively active Ras2, but not Ras1, suppressed the ability of PlexA to mediate repulsive axon guidance (Figure S3.3B), further indicating that Ras2 plays a specific role in PlexA repulsive signaling. Therefore, since both 14-3-3ε and Ras2 associate within the same region of the Plexin receptor, the RasGAP domain, we wondered if 14-3-3ε

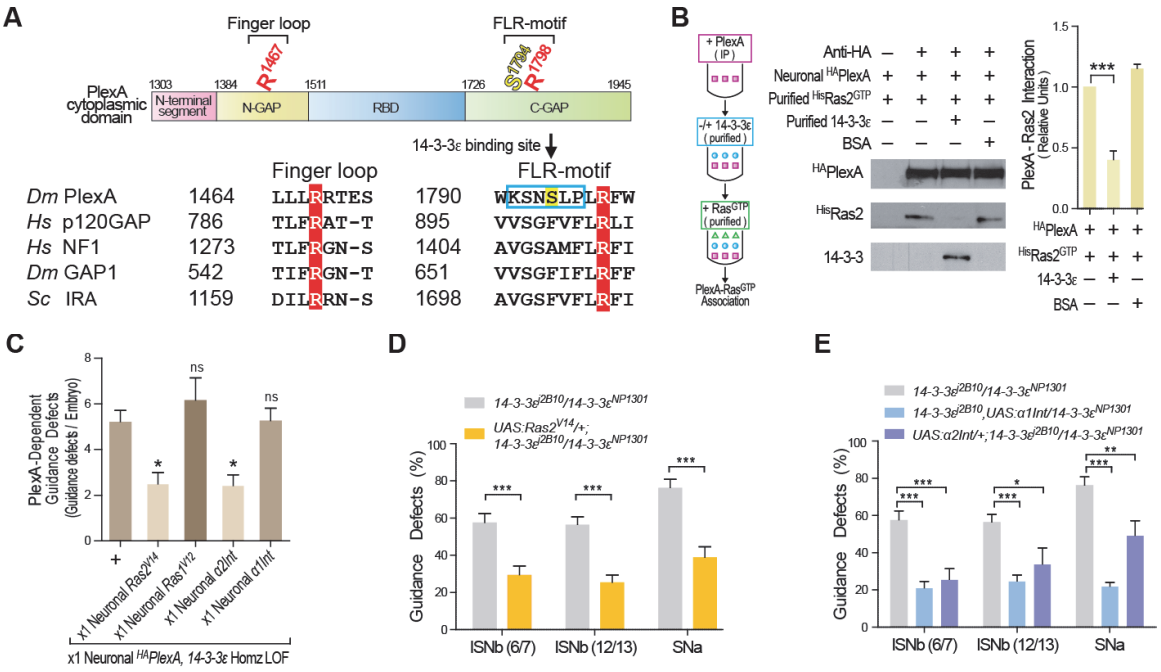

Figure 3.7. PlexA – 14-3-3ε Interactions Silence Plexin Ras-GAP-mediated Repulsive Axon Guidance (**A**) The 14-3-3ε binding site resides within the Plexin GAP domain. A schematic of the PlexA cytoplasmic region and sequence alignment of different GAP domains including PlexA reveal that the 14-3-3ε binding motif (blue box) resides in the FLR motif of PlexA, adjacent to one of the conserved Arg (R; colored red) residues critical for GAP activity. The critical 14-3-3ε binding Ser^{1794} residue of PlexA is highlighted in yellow (arrow). *Hs Homo sapiens*; *Dm Drosophila melanogaster*; *Sc Saccharomyces cerevisiae*. (**B**) Purified 14-3-3ε protein disrupts the association between PlexA and Ras2. Neuronally expressed ^{HA}PlexA was immunoprecipitated from embryonic lysates (square; left) and incubated with equal amounts of purified ^{His}Ras2, which was preloaded with GTPγS ($^{His}Ras2^{GTP}$, triangle; left), in the absence (–) or presence (+) of purified 14-3-3ε (circle; left) or BSA and associated proteins were detected by immunoblotting. $^{His}Ras2^{GTP}$ preferentially interacts with ^{HA}PlexA and this interaction is significantly decreased by 14-3-3ε, but not by BSA (middle and quantified in right). n=3; Error bar: SEM; ***p<0.001; paired *t*-test. (**C**) *Ras2*/*Integrin* receptor signaling counteracts *14-3-3ε* loss-of-function effects on *PlexA*-dependent repulsive axon guidance. Employing the *PlexA*-dependent in vivo repulsive axon guidance assay described in Figure 3 reveals that the increase in *PlexA*-dependent repulsive axon guidance seen when *14-3-3ε* is removed (*14-3-3ε* Homz LOF) is significantly suppressed by constitutively active $Ras2^{V14}$, but not by constitutively active $Ras1^{V12}$. Likewise, increasing the neuronal levels of an Integrin receptor (*α2Int*) suppressed PlexA-dependent repulsive guidance defects seen in *14-3-3ε* Homz LOF. Genotype: x1 Neuronal ^{HA}*PlexA* represents *ELAV-GAL4,UAS:*^{HA}*PlexA/+*; x1 Neuronal $Ras1^{V12}$ = $UAS:Ras1^{V12}$/+; x1 Neuronal $Ras2^{V14}$ = $UAS:Ras2^{V14}$/+; x1 Neuronal *α1Int* = *UAS: α1Int/+*; x1 Neuronal *α2Int* = *UAS: α2Int/+*; *14-3-3ε*

Homz LOF = *14-3-3ε*^{j2B10}. n≥90; Error bar: SEM; *p<0.05, ns: not significant; one-way ANOVA, Bonferroni post test. (**D**-**E**) Increasing the Ras2–Integrin adhesive signaling pathway rescues the axon guidance defects seen in *14-3-3ε* loss-of-function mutants. The quantification of motor axon guidance defects are as in Figure 2 and show that increasing the levels of *Ras2*^{V14} (**D**) or *α1* (*αPS1*) or *α2* (*αPS2*) *Integrins* (**E**) significantly rescues the ISNb and SNa motor axon guidance defects present in *14-3-3ε* LOF mutants. n≥90; Error bar: SEM; *p<0.05, **p<0.01, ***p<0.001 by two-way ANOVA, Bonferroni post test.

disrupted the PlexA – Ras2 association. To directly test this, we assessed the interaction between PlexA and Ras2 in the absence or presence of purified 14-3-3ε using immunoprecipitated neuronal PlexA. We found that neuronal ^{HA}PlexA interacts with purified ^{His}Ras2^{GTP}, but this interaction was significantly decreased by adding purified 14-3-3ε, but not purified BSA protein (Figure 3.7B). Furthermore, purified 14-3-3ε selectively decreased the ability of neuronal PlexA to facilitate GTP hydrolysis of Ras2 (Figure S3.6E). Moreover, using cell culture assays we found that phosphorylation alone did not alter the interaction between PlexA and Ras2 (Figure S3.6F), but that mutating Plexin's 14-3-3ε-binding site prevented 14-3-3ε from altering PlexA-Ras association (Figure S3.6G-H). Indeed, we found that raising the levels of active *Ras2* in neurons suppressed the hyperactive *PlexA* repulsive signaling caused by loss of *14-3-3ε* (Figure 3.7C), indicating that PlexA–14-3-3ε interactions function to "silence" Plexin RasGAP-mediated repulsive axon guidance.

A 14-3-3ε-dependent Switch Regulates Semaphorin/Plexin-Mediated Repulsion and Ras/Integrin-Mediated Adhesion

R-Ras signaling has been found to induce an increase in cellular adhesion and migration by activating integrins to bind to their ECM ligands (Zhang et al., 1996; Keely et al., 1999; Ivins et al., 2000; Oinuma et al., 2006). Plexins exert their repulsive/de-adhesive effects on growing axons by employing their GAP activity to inhibit specific Ras family GTPases and thereby "turn-off" Integrin-mediated substrate adhesion (Oinuma et al., 2004a; Oinuma et al., 2006). Thus, our results suggest that this Ras/Integrin-dependent adhesion can be turned-back-on through PKA-mediated phosphorylation of the Plexin RasGAP domain and subsequent binding of 14-3-3ε. Consistent with such a mechanism of action, integrins play important roles in establishing normal axonal trajectories and the loss of *integrins* generates axon guidance defects in Drosophila that resemble those seen following manipulations of Sema-1a/PlexA signaling (Hoang and Chiba, 1998; Huang et al., 2007; Stevens and Jacobs, 2002). Furthermore, employing several genetic assays we found that increasing the neuronal levels of integrins in vivo counteracted increased Sema-1a/PlexA repulsive signaling (Figure S3.3B), while decreasing integrin levels enhanced Sema-1a/PlexA repulsive axon guidance (Figure S3B-C). Moreover, we found that increasing specific integrins (*α2 Integrin* (*αPS2*); (Stevens and Jacobs, 2002)) in neurons suppressed the hyperactive *PlexA* repulsive signaling caused by the loss of *14-3-3ε* (Figure 3.7C). Therefore, we reasoned that if 14-3-3ε does function to increase Ras/Integrin-mediated adhesion then the axon guidance defects we observe in *14-3-3ε* mutants might be

significantly rescued by increasing Ras/Integrin signaling. Indeed, we found that the ISNb and SNa motor axon pathfinding defects present in *14-3-3ε* mutants were rescued by expressing constituitively active Ras2 (Figure 3.7D) or specific integrins (Figure 3.7E). Together, these results reveal that raising the levels of Ras/Integrin signaling counteracts the effects of decreasing 14-3-3ε, indicating a cAMP/PKA/14-3-3ε signaling pathway that directly controls the balance between Plexin RasGAP-mediated repulsion and Ras/Integrin-mediated adhesion.

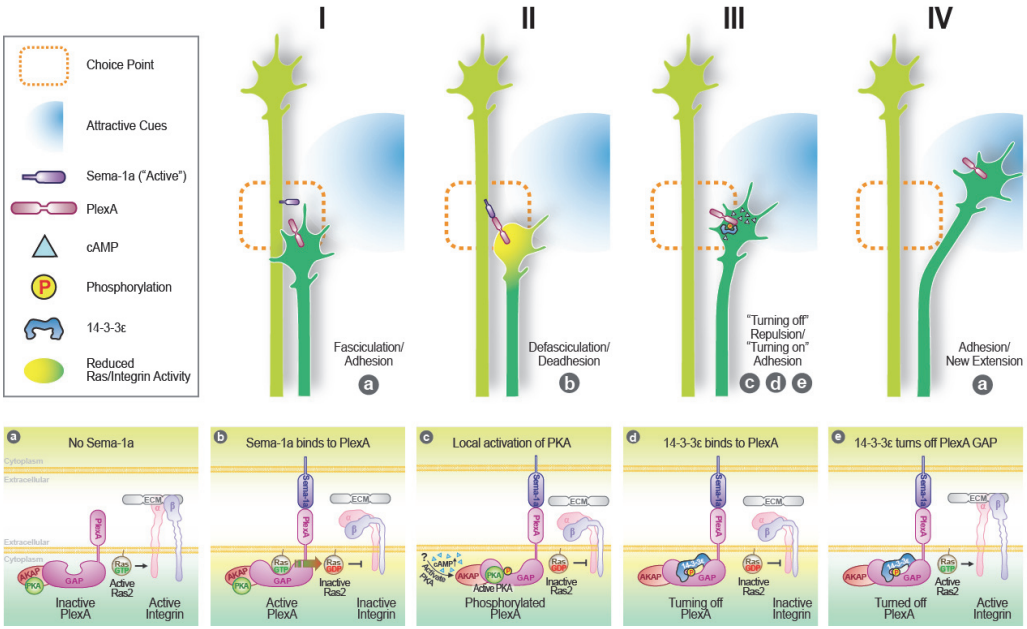

Figure 3.8. Working Model for Silencing of Plexin RasGAP-mediated Axon Repulsion by PKA/14-3-3ε This model based on our observations and previous results indicates that axon-axon fasciculation (e.g., (Yu et al., 2000)) and Integrin-mediated adhesion (e.g., (Hoang and Chiba, 1998; Huang et al., 2007)) allow axons to pathfind normally (I and a). Activated Sema/Plexin repellant signaling at choice points (e.g., (Yu et al., 1998; Winberg et al., 1998b), present study) induces defasciculation/de-adhesion (II and b). Then, to prevent stalling in the vicinity of the choice point because of an inability to extend on Integrin ligand substrates, cAMP/PKA/14-3-3ε signaling is required to "turn-off" Plexin RasGAP-mediated de-adhesion and restore Integrin-mediated adhesive signaling (III, c, d, and e). Axons now extend along Integrin-dependent adhesive substrates (IV, a).

Discussion

Axons rely on the activation of guidance receptors for correct navigation but receptor inactivation is also thought to be a means through which growth cones integrate both attractive and repulsive guidance signals. Our results now indicate that such a mechanism plays a critical role in Semaphorin/Plexin-mediated repulsive axon guidance. We find that PlexA uses its RasGAP activity to specify axon guidance but this activity is antagonized by a cAMP-dependent protein kinase (PKA)–mediated signaling pathway. PKA directly phosphorylates the GAP domain of PlexA and this phosphorylation provides a binding site for 14-3-3ε. 14-3-3ε is critical for axon guidance and disrupts the ability of Plexin to interact with its Ras GTPase substrate. These interactions effectively switch Plexin-mediated axonal repulsion to Integrin-mediated adhesion and provide a "simple" biochemical mechanism to integrate antagonistic axon guidance signals (Figure 3.8).

Our genetic experiments identify a critical role for 14-3-3ε proteins in directing axon guidance events during development. The 14-3-3 proteins are a phylogentically well-conserved family of cytosolic signaling proteins including seven mammalian members that play key roles in a number of cellular processes (Tzivion et al., 2001; Yaffe and Elia, 2001). Interestingly, 14-3-3 family proteins were first identified because of their high level of expression in the brain (Moore, 1967; Aitken, 2006), but despite considerable interest in their functions

(Skoulakis and Davis, 1998; Berg et al., 2003), their roles in the nervous system are still incompletely understood. For instance, 14-3-3 proteins are highly expressed in growing axons and have been found to modulate neurite extension and growth cone turning in vitro in a number of contexts (Boston et al., 1982; Tang et al., 1998; Graeser et al., 2002; Gehler et al., 2004; Rong et al., 2007; Taya et al., 2007; Kajiwara et al., 2009; Nozumi et al., 2009; Yoshimura et al., 2010; Ramser et al., 2010a; Kent et al., 2010; Ramser et al., 2010b). However, their necessity for directing axonal growth and guidance events in vivo are unknown as is the functional role of each family member in these neurodevelopmental processes. We now find that one of the two Drosophila 14-3-3 family members, 14-3-3ε, is required in vivo for axon guidance and plays specific roles in the pathfinding of motor and CNS axons. Moreover, previous mutant analysis has revealed that the other 14-3-3 family member in Drosophila, 14-3-3ζ (Leonardo), does not exhibit significant motor axon guidance or innervation defects (Broadie et al., 1997) but plays a critical role in synaptic transmission and learning and memory (Skoulakis and Davis, 1996; Broadie et al., 1997). These results indicate that individual 14-3-3 family members play specific roles in the development of the nervous system and in light of the requirement of 14-3-3ε in mammalian brain development and neuronal migration (Toyo-oka et al., 2003), and potential roles for 14-3-3ε in human neurological disease (e.g., (Ikeda et al., 2008; Bruno et al., 2010; Mignon-Ravix et al., 2010; Bi et al., 2009; Nagamani et al., 2009; Schiff et

al., 2010; Tenney et al., 2010)), future work will determine if 14-3-3ε's role in axon guidance is phylogenetically conserved.

Our genetic and biochemical experiments also identify a specific role for 14-3-3ε in regulating Semaphorin/Plexin-mediated repulsive axon guidance. Semaphorin/Plexin-mediated repulsive axon guidance is antagonized by increasing cAMP levels (Song et al., 1998; Dontchev and Letourneau, 2002; Chalasani et al., 2003; Parra and Zou, 2010), but the mechanisms underlying these cAMP-mediated effects are poorly understood. Interestingly, Plexins associate with the cAMP-dependent protein kinase (PKA) via MTG/Nervy family PKA (A kinase) anchoring proteins (AKAPs) (Fukuyama et al., 2001; Schillace et al., 2002; Terman and Kolodkin, 2004; Fiedler et al., 2010; Corpora et al., 2010). AKAPs position PKA at defined locations to allow for the spatially and temporally specific phosphorylation of target proteins in response to local increases in cAMP (Wong and Scott, 2004) and we now find that PKA phosphorylates the cytoplasmic portion of PlexA. Interestingly, our genetic and biochemical results suggest that this phosphorylation provides a binding site for a specific 14-3-3 family member, 14-3-3ε. 14-3-3 proteins are well-known as phosphoserine/threonine-binding proteins and have been found to utilize this ability to regulate the activity of specific enzymes (Yaffe and Elia, 2001; Tzivion et al., 2001). Indeed, we find that mutating the 14-3-3ε binding site on PlexA generates a hyperactive PlexA receptor, providing a better understanding of the

molecular and biochemical events through which cAMP signaling regulates Semaphorin/Plexin repulsive axon guidance. Future work will focus on identifying the "upstream" extracellular signal that increases cAMP levels, although it is interesting that the axonal attractant Netrin is known to increase cAMP levels (Corset et al., 2000) and antagonize Semaphorin-mediated axonal repulsion (Winberg et al., 1998a).

Our results also indicate that the GAP activity of Plexin is critical in vivo for repulsive axon guidance and that cAMP/PKA/14-3-3ε signaling regulates this Plexin RasGAP-mediated repulsion. Plexin receptors have within their cytoplasmic portions a GTPase activating protein (GAP) domain for Ras superfamily proteins (Rohm et al., 2000; Oinuma et al., 2004a; He et al., 2009; Tong et al., 2009). In vitro work has revealed that the RasGAP activity of Plexin is important for its signaling role (Oinuma et al., 2004a; Oinuma et al., 2006; Ito et al., 2006; Saito et al., 2009; Oinuma et al., 2010) and we now find that RasGAP activity is required in vivo in neurons for Plexin-mediated repulsive axon guidance. Moreover, our results indicate that 14-3-3ε binds to a single phosphoserine residue within the PlexA RasGAP domain and occludes this PlexA RasGAP–mediated axon guidance. Interestingly, positive regulation of GTPase signaling may be a conserved function for 14-3-3ε since it also increases the efficiency of Ras signaling during Drosophila eye development (Chang and Rubin, 1997) and 14-3-3 "turn-offs" the activity of other known GAPs

(e.g.,(Benzing et al., 2000; Feng et al., 2004)) and enhances Ras signaling (e.g., (Fantl et al., 1994; Irie et al., 1994; Gelperin et al., 1995; Roberts et al., 1997)) in other species. Therefore, our results along with those of others (e.g., (Oinuma et al., 2004a; He et al., 2009; Tong et al., 2009; Oinuma et al., 2006)) present a working model (Figure 3.8) in which Semaphorin/Plexin interactions activate Plexin RasGAP activity, which inactivates Ras and disables Integrin-mediated adhesion. However, our results also indicate that these Semaphorin/Plexin-mediated effects are subject to regulation, such that increasing cAMP levels activates Plexin-bound PKA to phosphorylate Plexin and provide a binding site for 14-3-3ε. These Plexin–14-3-3ε interactions occlude PlexA RasGAP-mediated inhibition of Ras and restore Integrin-dependent adhesion.

In conclusion, we have identified a simple mechanism that allows multiple axon guidance signals to be incorporated during axon guidance. Neuronal growth cones encounter both attractive and repulsive guidance cues but the molecular pathways and biochemical mechanisms that integrate these antagonistic cues and enable a discrete steering event are incompletely understood. One way in which to "integrate" these disparate signals is to allow different axon guidance receptors to directly modulate each other's function (e.g., (Stein and Tessier-Lavigne, 2001)). Another means is to tightly regulate the cell surface expression of specific receptors and thereby actively prevent axons from "seeing" certain guidance cues (e.g., (Kidd et al., 1998; Brittis et al., 2002; Keleman et al., 2002;

Nawabi et al., 2010; Chen et al., 2008; Yang et al., 2009; Kuwako et al., 2010)). Still further results are not simply explained by modulatory mechanisms like receptor trafficking, endocytosis, and local protein synthesis but indicate that interpreting a particular guidance cue is susceptible to rapid intracellular modulation by other, distinct, signaling pathways (e.g., (Song et al., 1998; Dontchev and Letourneau, 2002; Terman and Kolodkin, 2004; Parra and Zou, 2010; Xu et al., 2010)). Our results now indicate a new means to allow for such intracellular signaling cross-talk events and present a new logic by which axon guidance signaling pathways "over-ride" one another. Given this new molecular link between such key regulators of axon pathfinding as cyclic nucleotides, phosphorylation, and GTPases, our observations on silencing Semaphorin/Plexin-mediated repulsive axon guidance also suggest new approaches to neutralize axonal growth inhibition and encourage axon regeneration.

Materials and Methods

Yeast Two-Hybrid Screening and Assay

Yeast two-hybrid set-up, protein expression analyses, and screening were performed following standard procedures (Golemis, 2001; Terman et al., 2002; Terman and Kolodkin, 2004) and the initial screen was performed using a library containing Drosophila cDNAs (a kind gift of A. Kolodkin). Different portions of the PlexA cytoplasmic region, and the PlexB^{Cyto2} region (residues 1785-2051) were inserted into the bait vector (pEG202). The C-terminal region of 14-3-3ζ corresponding to Clone 135 was amplified from cDNA (a kind gift of D. St Johnston) and inserted into the prey vector (pJG4-5). All point muations in PlexA were generated using either the QuickChangeII Site-Directed Mutagenesis Kit (Stratagene) or standard PCR-based mutagenesis. All resulting constructs were sequenced on both strands. Bait and prey interactions were determined after plating on both Glucose and Galactose/Raffinose-containing medias containing 0.05mg/ml of X-gal and analyzing color development and growth rate from Day 3 to Day 5.

Sequence Analysis

Sequence alignment, consensus motif search and identification of homologs were performed by Web-based applications (BLAST, ScanProsite, ClustalW, Human

Protein Reference Database PhosphoMotif Finder, and HomoloGene) and manual analysis.

In Situ Hybridization

RNA in situ hybridization using digoxigenin-labeled RNA probes was performed as described using sense and antisense probes (Terman et al., 2002).

Drosophila Genetics, Phenotypic Characterization, and Immunolabeling

Drosophila husbandry and genetics were performed as described (Terman et al., 2002). For characterization of axon guidance phenotypes, embryos were collected and immunostained with the Fasciclin II monoclonal antibody 1D4 (1:4; Development Studies Hybridoma Bank; (Van Vactor et al., 1993)) as described (Terman et al., 2002; Ayoob et al., 2006; He et al., 2009). To our knowledge, no previously published reports have examined *14-3-3ε* mutants with 1D4. All motor axon pathfinding was examined by collecting embryos at 25ºC, while *PlexA*-dependent guidance defects were examined after raising embryos at 30ºC as previously described (Ayoob et al., 2006; He et al., 2009). The guidance defects were then determined using standard methods by either counting the total number of hemisegments with guidance defects for a particular genotype and presenting it as a percentage of the total number of hemisegments examined (Terman et al., 2002) or by counting the number of defects per embryo of a given

genotype to determine the average number of guidance defects per embryo (Ayoob et al., 2004). To generate transgenic lines for *14-3-3ε*, nucleotides encoding the open reading frame at the 5' end of 14-3-3ε were amplified from a cDNA clone (LD27892), digested with the EcoRI restriction endonuclease, and ligated using an endogenous EcoRI site with the 14-3-3ε cDNA from Clone 135 including 3'UTR in the p3XFLAG-CMV vector (Sigma). The ^{3XFLAG}14-3-3ε sequence was then moved to the Drosophila transformation vector pUAST and standard transformation approaches were employed to make transgenic flies. For mutant ^{HA}PlexA transgenic lines, point mutations were generated on pUAST-^{HA}PlexA (Terman et al., 2002) using the QuickChangeII Site-Directed Mutagenesis Kit (Stratagene) or standard PCR-based mutagenesis. Mutations were then confirmed by direct sequencing and restriction enzyme digestions and standard transformation approaches were used by BestGene, Inc. to make transgenic flies. All other stocks were described previously (Chang and Rubin, 1997; Yu et al., 1998; Terman et al., 2002; Hung et al., 2010; Terman and Kolodkin, 2004) or were obtained from the Bloomington Stock Center (including *Pka-C1*^{DN} (Pan and Rubin, 1995), *UAS:Ras1*^{V12} (Lee et al., 1996), *UAS:Ras2*^{V14} (Brand and Perrimon, 1993), and *βInt*^{mys1} (Bunch et al., 1992)) except *14-3-3ε*^{NP1301} (Drosophila Genetic Resource Center, Japan), *14-3-3ε*^{CA06506} (a kind gift of A. Spradling; (Buszczak et al., 2007)), and *UAS:α1Integrin*, *UAS:α2Integrin*, and *UAS:βIntegrin* (kind gifts of D. Brower; (Roote and Zusman, 1996)).

The expression levels of each ^{HA}*PlexA* mutant transgene was examined by lysing embryos in 3X Laemmli sample buffer by sonication followed by SDS-PAGE and Western analysis using rat HA monoclonal antibody (1:3000; 3F10, Roche). Actin levels were also examined using an actin monoclonal antibody (1:1000; MAB1501R, Chemicon) to provide controls and verify equal amounts of lysates were being examined containing each mutant ^{HA}PlexA protein. Signal was developed by chemiluminescent reagent (SuperSignal, Thermo Scientific) in dynamic range where signal is relatively linear. Films were scanned and band intensity was quantified using MetaMorph software (Molecular Dynamics) or ImageJ software (NIH). Surface expression of ^{HA}PlexA in embryonic nervous system was examined by immunohistochemistry using HA antibody (1:500; sc-805, Santa Cruz) or actin (1:1000; MAB1501R, Chemicon) and a secondary antibody conjugated with Alexa Fluor488 (Invitrogen). All processes including embryo collection, fixation, dissection and incubation with antibodies were performed in a detergent free condition as previously described (Williamson et al., 2010).

Co-immunoprecipitation

Neuronal ^{HA}PlexA and ^{FLAG}14-3-3ε embryonic lysates were prepared in RIPA buffer (50mM TrisHCl pH8.0, 150mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with complete protease inhibitor cocktail (Roche) and

1mM DTT as described (Terman et al., 2002; Terman and Kolodkin, 2004). Co-immunoprecipitation was performed by adding either mouse HA monoclonal antibody (5µg; 12CA5, Roche), mouse FLAG monoclonal antibody (5µg; M2, Sigma), or equal amounts of IgG to embryonic lysates, followed by incubation with GammaBind G Sepharose beads (GE Healthcare). Proteins were detected by Western analysis using HA antibody (1:3000; 3F10, Roche) or FLAG antibody (1:3000; M2, Sigma).

A Drosophila Kc cell line was maintained in Schneider's Drosophila Medium (Invitrogen) supplemented with 10% FBS and Penicillin/Streptomycin applying standard aseptic techniques (Cherbas, 1998; Cherbas, 2000). Plasmid DNAs including pUAST-^{HA}PlexA or pUAST-^{HA}PlexA^{S1794}, pUAST-^{FLAG}14-3-3ε, and pActGAL4 (a kind gift of J. Brugarolas) were co-transfected by Effectene transfection reagent (Qiagen) following the manufacturer's recommendation. After 48 hours of transfection, cells were treated with 10µM Forskolin (Sigma) and lysed in NP-40 lysis buffer (50mM TrisHCl pH7.4, 150mM NaCl, 0.5% NP-40) supplemented with complete protease inhibitor cocktail (Roche) and 5mM DTT. Co-immunoprecipitation and detection were performed using 2 µg of HA antibody as described above.

Protein Purification

For the purification of GST-14-3-3ε, GST-PlexA^{Cyto2}, GST-PlexA$^{Cyto2\ S1794A}$, and GST-RBD, the coding sequence for each protein was inserted into pGEX-4T expression vector (GE Healthcare). For GST-RBD, the sequence corresponding to the Ras binding domain (residues 128-212) of pole hole (D-Raf) was amplified from its cDNA clone (GH03557). For purification of ^{His}Ras1 and ^{His}Ras2, full length Ras1 or Ras2 sequences were amplified from cDNA clones (LD17536 for Ras1 and RE36103 for Ras2) and inserted into ppSumo expression vector (a kind gift of X. Zhang). For the purification of rat Inhibitor-1 to use as a control substrate for in vitro kinase assay, pET-15b-Inhibitor-1 was used (a kind gift of J. Bibb). Standard approaches were used to purify the proteins (Self and Hall, 1995; Hung et al., 2010). In short, proteins were expressed in ArcticExpress (DE3) competent cells (Stratagene) or Rosetta2(DE3)pLysS competent cells (Novagen), and purified using either GSTrapFF or HisTrapFF (GE Healthcare) affinity chromatography followed by ion-exchange chromatography using an ÄKTApurifier FPLC machine (GE Healthcare). To remove the GST tag, purified GST fusion proteins were incubated with Thrombin (Roche) following the manufacturer's instructions and after subjecting the samples to GSTrap chromatography, the unbound fractions (without the GST tag) were combined and concentrated.

GST Pull-down and In Vitro Binding Assays

Interaction between GST-14-3-3ε and ^{HA}PlexA was examined using a GST pull-down assay as described (Diekmann and Hall, 1995; Benard et al., 1999; Oinuma et al., 2004a). 50μg of recombinant GST-14-3-3ε fusion protein was incubated with neuronal ^{HA}PlexA embryonic lysates and "pulled down" by adding immobilized Glutathione beads (Thermo Scientific). Bound proteins were detected by Western analysis using an HA antibody (3F10) and the amount of input GST fusion protein was detected by staining the membrane with Coomassie Brilliant Blue. To look at the effect of phosphorylation on the interaction, embryonic lysates were pre-incubated with 400U of alkaline phosphatase (CIP, NEB) and then subjected to the GST pull-down assay.

To examine the interaction between ^{HA}PlexA and ^{His}Ras1 or ^{His}Ras2, ^{HA}PlexA was immunoprecipitated from neuronal ^{HA}PlexA embryonic lysates, and then incubated with GDP or GTPγS-preloaded ^{His}Ras1 or ^{His}Ras2 in binding buffer (25mM TrisHCl pH7.5, 40mM NaCl, 30mM $MgCl_2$, 0.5% NP-40, 1mM DTT) (Diekmann and Hall, 1995; Benard et al., 1999; Oinuma et al., 2004a; Toyofuku et al., 2005) in the presence or absence of equal molar amounts of purified 14-3-3ε or BSA (Pierce). After extensive washing, bound ^{His}Ras1 or ^{His}Ras2 was detected by Western analysis using a mouse His monoclonal antibody (1:3000; Anti-His_6, Roche). GDP and GTPγS-preloading on ^{His}Ras1 or ^{His}Ras2 was assessed by GST pull-down assay using GST-RBD proteins as described

(Diekmann and Hall, 1995; Benard et al., 1999) and bound ^{His}Ras1 or ^{His}Ras2 was detected by SDS-PAGE and Western analysis as decribed above. Developed films were scanned and band intensity was quantified as described above.

To examine the interaction between ^{HA}PlexA, ^{HA}PlexA^{SA}, or ^{HA}PlexA^{SE} and Ras2, each ^{HA}PlexA was immunoprecipitated from Kc lysates that in some cases had been subjected to Forskolin (Sigma) treatment as described above. Likewise, in some cases, ^{FLAG}14-3-3ε was co-transfected along with a plasmid containing one of the ^{HA}PlexA constructs. Then, the immunoprecipitated ^{HA}PlexA was incubated with Kc cell lysates expressing ^{Myc}Ras2 at 4 ºC. To prepare ^{Myc}Ras2 lysates, the sequence for full-length Ras2 was subcloned into the pAc5.1/V5-His vector (Invitrogen) with a Myc epitope (EQKLISEEDL) on its N-terminus. The ^{Myc}Ras2 construct was transfected into Kc cells and cells were lysed in lysis buffer (15mM TrisHCl pH7.5, 250mM Sucrose, 30mM $MgCl_2$, 150mM NaCl) (Khanna et al., 2010) supplemented with complete protease inhibitor cocktail (Roche) by sonication. Bound ^{Myc}Ras2 was detected using a Myc antibody (1:1000; 9E10, DSHB).

Plexin GAP Assays

Neuronal ^{HA}PlexA was immunoprecipitated from embryonic lysates as described above and precincubated with purified 14-3-3ε or BSA in binding buffer (25mM TrisHCl pH7.5, 40mM NaCl, 30mM $MgCl_2$, 0.5% NP-40, 1mM DTT). Kc cells

expressing ^{Myc}Ras2 were lysed in lysis buffer (15mM TrisHCl pH7.5, 250mM Sucrose, 30mM $MgCl_2$, 150mM NaCl) (Khanna et al., 2010) supplemented with complete protease inhibitor cocktail (Roche) by sonication. The ^{Myc}Ras2 lysates were incubated with immunoprecipitated ^{HA}PlexA at 30ºC, and separated from the ^{HA}PlexA-conjugated beads by centrifugation. These Plexin-treated lysates were then subjected to GST-RBD (50µg) pull-down to measure the amount of GTP bound ^{Myc}Ras2. The bound ^{Myc}Ras2 was examined by SDS-PAGE and Western analysis.

To examine RasGAP activity of ^{HA}PlexA^{RA}, constucts containing either wild type ^{HA}PlexA (WT) or ^{HA}PlexA^{RA} (RA) with ^{Myc}Ras2 were transfected into Kc cells and PlexA was activated by incubation with Sema-1a-Fc conditioned media. Kc cells were lysed in lysis buffer (15mM TrisHCl pH7.5, 250mM Sucrose, 30mM $MgCl_2$, 150mM NaCl) (Khanna et al., 2010) supplemented with complete protease inhibitor cocktail (Roche) by sonication and amount of GTP bound ^{Myc}Ras2 was measured by GST-RBD (50µg) pull-down assays (Oinuma et al., 2004a). Sema-1a sequence (residues 72-651) was inserted in pFUSE-hIgG1-Fc vector (InvivoGen) and IL2 signal sequence-Sema-1a-Fc cassette was subcloned into pAc5.1/V5-His vector (Invitrogen). Kc cells were transfected with Sema-1a-Fc construct and media were collected and used as Sema-1a-Fc conditioned media. Expression of Sema-1a-Fc was confimed by western analysis with human Fc_γ antibody (1:20000; Jackson ImmunoResearch).

In Vitro Kinase Assays

Phosphorylation of GST-PlexA^{Cyto2} and GST-PlexA$^{Cyto2\ S1794A}$ was determined by standard in vitro kinase assays using [γ-^{32}P] ATP (PerkinElmer) as a phosphate donor following the manufacturer's recommendation for each kinase and as described (Girault et al., 1989; Bibb et al., 1999). Aliquots were made at each time point and reactions were stopped by adding 3X Laemmli sample buffer and boiling. Proteins were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. Dried gels were exposed using a PhosphorImager screen and incorporated ^{32}P was quantified using a Storm PhosphorImager scanner (Molecular Dynamics). Each protein kinase was obtained as indicated: PKA, catalytic subunit (Promega); Akt2, CaMKIδ, CDK5/p25NCK, PAK1, PKCε, and ROCK1 (Cell Signaling Technology); PKG1 (Invitrogen). Similar results with PKA were also obtained using PKA, catalytic subunit from Cell Signaling Technology. The activity of each kinase was confirmed using known protein substrates, including Inhibitor-1 and Myelin Basic Protein (a kind gift of M. Cobb), and also peptide substrates (data not shown).

Generation and Validation of Phosphorylation Specific Antibody

A polyclonal antibody which specifically recognizes phosphorylated PlexA^{S1794} was generated by conjugating synthetic phosphopeptides (pS1794: NH_2-

CHTWKSN**p**S[1794]LPLRF-COOH; modified residues underlined, p as a phosphate group; UTSW peptide synthesis core facility) to KLH and injecting into 3 rabbits, which were initially screened from 6 rabbits for their low background (Covance) (Czernik, 1997; Harlow, 1999; Flavell et al., 2006). Antisera from different animals were screened for their ability to specifically recognize phosphopeptides (pS1794), controls (WT: NH_2-TWKSNSLPLR-COOH; S1794A: NH_2-TWKSNA[1794]LPLR-COOH; substituted residue underlined), and in vitro phosphorylated proteins by both spotting peptides on PVDF membrane using a Bio-Dot Microfiltration Apparatus (Bio-Rad) and using SDS-PAGE and Western blotting. The last bleeding sample from one animal was specific enough to recognize its target epitope without further purification. 400U of Lambda protein phosphatase (λPP; New England Biolabs) was treated on the membrane to dephosphorylate immunoprecipitated [HA]PlexA following the manufacturer's recommendation in the absence or presence of phosphatase inhibitors (10mM Na_3VO_4, 50mM NaF). To examine the phosphorylation of PlexA in vivo, embryos were collected from *wild type* (w^{1118}) or *Pka-C1* mutant ($Pka\text{-}C1^{DN}$ Het LOF), lysed using sonication, and examined as described above.

Microscopy, Imaging, and Software

Filleted Drosophila embryos were examined essentially as described (Huang et al., 2007; Hung et al., 2010) using an AxioImagerZ1 microscope (Zeiss) with DIC

optics and imaging was performed using an AxioCam HRc camera (Zeiss). Brightness, contrast and color balance of images were adjusted using Adobe Photoshop. GraphPad Prism 5 software was used for the statistical analyses.

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CHAPTER FOUR

Discussion

Summary of the Present Work

The Plexin family of large transmembrane proteins serves as a signal transducing component in the receptor complex for Semaphorins and it mediates repulsive axon guidance (Tran et al., 2007; Negishi et al., 2005; Kruger et al., 2005). The findings that Plexin has RasGAP homology domain in its cytoplasmic region (Hu et al., 2001; Rohm et al., 2000) and that Plexin indeed has GAP activity toward Ras family GTPases (Saito et al., 2009; Oinuma et al., 2004a; Toyofuku et al., 2005) raise questions about the importance of the RasGAP domain for Plexin function in axon guidance in vivo and how Semaphorins activate the Plexin RasGAP and what regulates GAP activity. My results now augment our understandings on these intriguing questions. First, genetic analyses reveal that a functional Plexin GAP domain is required in vivo for axon guidance (Chapter 3). Second, structural and functional analyses reveal that the GAP domain of Plexin has an autoinhibited conformation (He et al., 2009). The binding of both the Semaphorin ligand and a small GTPase to both the extracellular and intracellular portions of Plexin, respectively, is suggested to release the interaction between the GAP domain and the N-terminal segment and RBD domain of Plexin, which in turn enables the GAP domain to associate with its substrate. Third, genetic and biochemical results indicate how the potent antirepellant molecule cAMP inhibits Plexin GAP activity (Chapter 3). PKA directly phosphorylates the GAP domain of PlexA and this phosphorylation provides a binding site for 14-3-3ε. 14-3-3ε is

critical for axon guidance and disrupts the ability of Plexin to interact with its Ras GTPase substrate. These interactions effectively switch Plexin-mediated axonal repulsion to Integrin-mediated adhesion and provide a "simple" biochemical mechanism to integrate antagonistic axon guidance signals.

Activation of Plexin RasGAP

The autoinhibited conformation revealed from the crystal structure of the Plexin A3 intracellular region suggests that the Plexin RasGAP requires allosteric activation by binding to a Semaphorin and also a Rho-family GTPase (Oinuma et al., 2004a; He et al., 2009). Semaphorin dimerization is required for its biological function and activation of Plexin signaling (Klostermann et al., 1998; Nogi et al., 2010; Liu et al., 2010; Janssen et al., 2010). Recent crystal structures of a Semaphorin/Plexin complex indicate that a Semaphorin dimer and a Plexin dimer make a 2:2 heterotetramer (Nogi et al., 2010; Liu et al., 2010; Janssen et al., 2010). Plexins form 'head-on' homodimers, whereas Semaphorins form 'face-to-face' homodimers (Figure 4.1). When Plexins bind to Semaphorins, Plexin homodimers dissociate and switch their configuration to associate with Semaphorin in a 'head-to-head' position (Nogi et al., 2010). Semaphorin binding to Plexin has been proposed to trigger clustering of Plexins and stabilize the Plexin complex (Nogi et al., 2010; Liu et al., 2010; Janssen et al., 2010; Oinuma et al., 2004b; He et al., 2009). Nonetheless, how Semaphorin binding to the

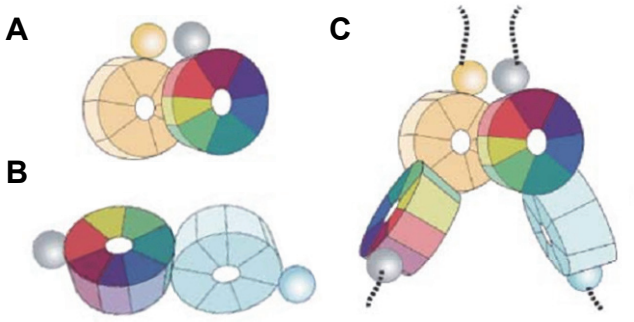

Figure 4.1 Configurational model of Semaphorin/Plexin interactions (A) Sema6A face-to-face homodimer. (B) Plexin A2 head-on dimer. (C) Sema6A-Plexin A2 2:2 heterotetramer. Adapted from Nogi et al., 2010

extracellular region of the Plexin receptor activates the intracellular GAP domain still requires further studies.

The Plexin RasGAP domain is unusual in that its RasGAP domain is divided by an RBD and direct binding of a GTPase to the RBD appears to be required for the activation of the Plexin RasGAP (Oinuma et al., 2004a). Different classes of Plexins have been found to interact with members of Rho GTPase family (Puschel, 2007). For example, Rnd1/2 are required for Plexin RasGAP activation (Saito et al., 2009; Oinuma et al., 2004a; Uesugi et al., 2009). Rac1 is known to promote surface expression of Plexin by directly binding to Plexin (Vikis et al., 2000; Vikis et al., 2002; Hu et al., 2001). RhoD competes with Rnd1 for binding to Plexin (Tong et al., 2007; Zanata et al., 2002). In addition, Plexin B binds to Rac and RhoA and this binding has been postulated to

sequester Rac from its downstream effector PAK and enhance the effects of RhoA, respectively (Hu et al., 2001).

Activity assays, crystal structures of the intracellular region of Plexins, and functional mutagenesis analyses suggest that the activation of the Plexin RasGAP is dependent on binding to both a Semaphorin and a small Rho family GTPase (Oinuma et al., 2004b; Oinuma et al., 2004a; Tong et al., 2009; He et al., 2009; Bell et al., 2011). This work suggests that Plexin RasGAP functions as a coincidence detector whose activity depends on both extracellular stimulus of a Semaphorin and intracellular status, which is regulated by other guidance cues and the cellular capacity to respond to those cues. It is also likely that the ability of Plexin to associate with other members of its signaling cascade (Hung and Terman, 2011) is also regulated by the extracellular stimulus of a Semaphorin, and that Plexin is normally kept in an "inactive" conformation.

Inhibition of Plexin RasGAP

Controlled inactivation of receptor signaling is critical for normal physiological function. Otherwise, the prolonged activation of a receptor will result in malfunction that can cause cancers and degenerative diseases (Hantschel and Superti-Furga, 2004; Holland et al., 1998; Wang and Qin, 2010). Likewise, growing axons require coordinated activation and inactivation of multiple guidance receptors to precisely connect the nervous system. Guidance receptors

are inactivated by various mechanisms including receptor endocytosis (Bashaw and Klein, 2010; Cowan et al., 2005; Williamson et al., 2010), ubiquitin-mediated degradation (Kim et al., 2005), decreases in surface level (Kidd et al., 1998; Nawabi et al., 2010; Keleman et al., 2002), and changes in receptor compositions (Stein and Tessier-Lavigne, 2001; Bashaw and Klein, 2010; Hong et al., 1999). In addition, the response to guidance cues is regulated by the relative amount of cyclic nucleotides inclucing cAMP and cGMP (Song and Poo, 1999; Dontchev and Letourneau, 2002; Nishiyama et al., 2003; Terman and Kolodkin, 2004; Tojima et al., 2011).

My genetic and biochemical analyses indicate one way in which cAMP/PKA signaling inactivates the Semaphorin/Plexin-mediated repulsive response. PKA phosphorylates the Plexin RasGAP domain and generates a binding site for 14-3-3ε. This 14-3-3ε binding to Plexin RasGAP inhibits Plexin RasGAP activity and my results indicate that this association converts Semaphorin-mediated repulsion to Integrin-mediated adhesion. Furthermore, my experiments indicate a mechanism for how this conversion from repulsion to adhesion occurs. In particular, Plexin RasGAP activity has been found to reduce R-Ras/Integrin-mediated adhesion (Oinuma et al., 2006; Oinuma et al., 2004a; Zhang et al., 1996; Ito et al., 2006), and my results reveal that the binding of 14-3-3ε to Plexin RasGAP domain blocks the association of substrate Ras GTPase to RasGAP domain, and results in an increased level of active R-Ras. This

relatively rapid second messenger/phosphorylation-mediated inhibition of Semaphorin/Plexin signaling also suggests that Semaphorin/Plexin-dependent repulsive responses may only be activated for a short period of time (i.e., the activity is tightly controlled in space and time). Furthermore, it will be interesting to determine if there are phosphatases that could dephosphorylate Plexin, and thus the GAP activity of Plexin could be flexibly prolonged at certain locations and development stages in vivo.

The mechanism of 14-3-3ε in the inhibition of the Plexin RasGAP, although very specific in nature, is in line with previous roles of 14-3-3 proteins in regulating the functions of enzymes in a phospho-specific manner (Skoulakis and Davis, 1998; Aitken, 2006; Berg et al., 2003; Yaffe, 2002; Tzivion et al., 2001). For example, the function of 14-3-3 as a regulator in multiple cellular processes can be summarized by its mechanism of actions. 14-3-3 can serve as a scaffolding protein by binding to two target proteins through homo/hetero dimerization. By binding to two sites in the same target protein, 14-3-3 can cause conformational change, which can result in the modulation of enzymatic activity. In particular, 14-3-3 binding can protect the phosphorylation level from dephosphorylation by phosphatases. Likewise, 14-3-3 binding can sequester the binding sites from association with other proteins or further post-translational modifications such as ubiquitination. 14-3-3 binding is also known to regulate surface expression of target proteins. These mechanisms are not necessarily

exclusive of each other, but depend on the function of target proteins. For example, it is interesting that 14-3-3ε does not change the surface expression level of Plexin on axons. Indeed, the competition between 14-3-3ε and Ras for binding to Plexin A and the proximity between 14-3-3ε binding Serine residue and a conserved Arginine residue for GAP activity indicates that 14-3-3ε binding sequesters the active site of the Plexin RasGAP domain. In this regard it is interesting that the RasGAP NF1, and a regulator of G protein signaling, RGS7, are also susceptible to a similar mechanism to regulate their GAP activity. In particular, the Ser424 residue in RGS7 is located near the contacting residues for the interaction with its substrate $G\alpha_i$ subunit and has been found to bind to 14-3-3τ, which silences the GAP activity (Benzing et al., 2000).

Interaction between Plexin A and 14-3-3ε

The association between Plexin A and 14-3-3ε is specific in that only one of four possible interactions between Plexin (PlexA, PlexB) and 14-3-3 (14-3-3ε, 14-3-3ζ) is observed. Although 14-3-3ε does not interact with PlexB, it would not be surprising to find that 14-3-3ε has a role in the regulation of PlexB function in vivo since PlexA and PlexB interact with each other (Ayoob et al., 2006). Likewise, it has not been determined whether *14-3-3ζ* can complement the in vivo guidance defects resulting from a loss of *14-3-3ε*. In light of possible

heterodimerization between 14-3-3ε and 14-3-3ζ, increased level of 14-3-3ζ may be able to compensate for a haploinsufficiency of 14-3-3ε.

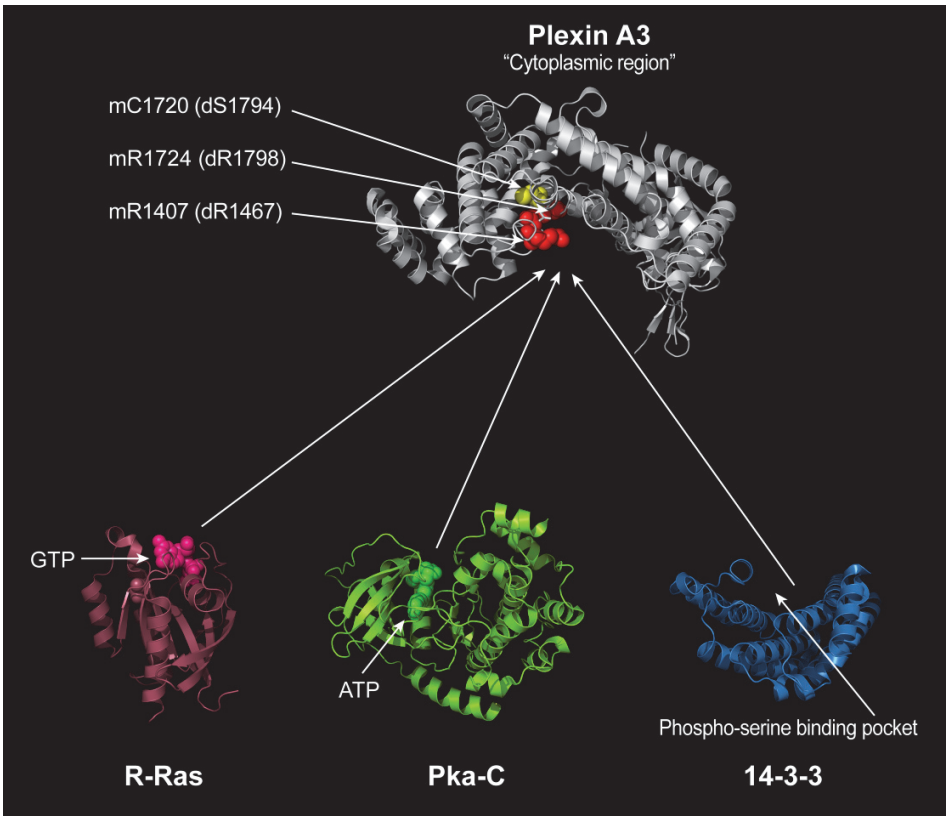

Figure 4.2 Structural Comparison of the Proteins Interacting with the Plexin RasGAP domain My results indicate that R-Ras, 14-3-3ε, and a catalytic subunit of PKA (Pka-C) share a binding site in the cytoplasmic region of Plexin A. The conserved arginine residues which are critical for a Plexin RasGAP activity are labeled in red. The residue corresponding to a 14-3-3ε binding site is labeled in yellow, which is veiled by the arginine residues. The amino acid residues for these arginine and serine in both mouse Plexin A3 (m) and Drosophila PlexA (d) are indicated by arrows. The structural model of the Plexin A3 "cytoplasmic region [some portions of the cytoplasmic region were not resolved structurally]" in the "inactive conformation" is oriented with the concave surface of RasGAP domain facing downward. Active sites for proteins that interact with the Plexin RasGAP domain are indicated such as GTP for R-Ras and ATP for Pka-C. The phospho-serine-binding pocket of 14-3-3 is also indicated (14-3-3 is thought to often function as a dimer and be able to bind two "different" phosphorylated residues. For clarity, I am only showing a monomer of 14-3-3). Protein data bank identification numbers: 3IG3 for the mouse Plexin A3, 2FN4 for the human R-Ras, 2BR9 for the human 14-3-3ε, and 2F7X for the cow catalytic subunit of PKA.

The Plexin cytoplasmic domain has been found to interact with several proteins, including Mical, Nervy, a Ras substrate, and 14-3-3ε (Terman et al., 2004, Terman and Kolodkin, 2006; Oinuma et al., 2006)(Chapter 3). In addition, a catalytic subunit of PKA interacts with Plexin cytoplasmic domain to phosphorylate PlexA (Chapter 3). Among these interacting proteins, a Ras substrate, 14-3-3ε, and a catalytic subunit of PKA share the same interacting region in the PlexA RasGAP domain. Therefore, it is interesting to speculate how the Ras substrate, 14-3-3ε, and a catalytic subunit of PKA interact with the Plexin A RasGAP domain based on the structural models of each protein (Figure 4.2). The current structural model of A Class Plexins indicates that the cytoplasmic region of Plexin has a "closed" conformation which cannot interact with its Ras substrate – and thus a conformational change needs to occur to allow Ras to bind to the Plexin RasGAP domain. Likewise, this "closed conformation" model indicates that the S1794 residue in PlexA is not exposed at the surface, suggesting that the Plexin RasGAP domain also needs to undergo a conformational change in order to be phosphorylated. It is thought that once Plexin is activated by binding to a Semaphorin and a Rho family GTPase, the conformation of the Plexin RasGAP domain changes and this allows access of the Ras GTPase to the Plexin RasGAP. At present, it is not known what causes the conformational change that allows the catalytic subunit of PKA to access the S1794 residue, but this could also be triggered by Semaphorin binding, Rho GTPase binding, and/or some

additional post-translational modification in the cytoplasmic region of PlexA (discussed below). Interestingly, structural examination of Ras, 14-3-3ε, and the catalytic subunit of PKA reveals that they are similar in size but have differences in their shapes (Figure 4.2), suggesting that each of them must access the Plexin RasGAP domain in a unique manner. For example, 14-3-3ε is "longer" and "narrower" than the "ball-shaped" Ras. Therefore, while the "ball-shaped" Ras may fit into the "open" RasGAP domain directly, the "saddle-shaped" 14-3-3ε may access the "open" RasGAP domain from the "side" (Figure 4.2). Future directions should be aimed at further exploring the conformational changes that occur when Plexin is activated and how these different proteins access the Plexin A RasGAP domain.

Possible Other Effects of 14-3-3ε on Plexin Function

Moreover, it is still possible that 14-3-3ε binding to Plexin may also result in the regulation of different aspects of Plexin A function. Although it is still not clear if 14-3-3ε binds to Plexin A as a monomer or dimer, 14-3-3ε may act as a dimer based on the observation that there is residual interaction between 14-3-3ε and mutant Plexin A S1794A (Figure S3.4). Furthermore, the carboxyl terminal region of Plexin A which interacts with 14-3-3ε has two consensus PKA phosphorylation sites including S1794 (Figure 3.5), suggesting that another unidentified phosphorylation residue may also provide a binding site for 14-3-3ε.

In addition, these two putative binding sites may not be equivalent in their affinity for 14-3-3ε. If 14-3-3ε binds to Plexin A as a dimer, one can predict other functions of 14-3-3ε depending on the site of second 14-3-3ε binding site. If the second site is present on the same Plexin A molecule containing the first 14-3-3ε binding site, the intramolecular binding of 14-3-3ε to both sites would be predicted to induce a conformational change to Plexin A. If the second site is on another Plexin A RasGAP domain, the intermolecular 14-3-3ε binding would efficiently turn off GAP activity of both Plexin A proteins. To test these possibilities, the identification of other phosphorylation sites (discussed below) and functional mutagenesis analyses are required. These conformational changes and/or the sequestration of active site may result in the inhibition of RasGAP activity and/or a decrease in the interactions with other Plexin A interacting proteins, such as Mical, Nervy, Plexin B, Otk, and Gyc76C (discussed below) (Ayoob et al., 2006; Ayoob et al., 2004; Winberg et al., 2001; Terman et al., 2002; Terman and Kolodkin, 2004).

Dual Function of Plexin A as a Repulsive Guidance Receptor

To avoid repulsive guidance cues, the growth cone shifts its orientation by reorganizing the actin cytoskeleton and decreasing the adhesion to the substrate on the side of the growth cone contacting repulsive cues. Plexin A appears to fulfill both of these hallmarks of a repulsive response through two different

signaling routes/mechanisms. First, Plexin mediates actin depolymerization by interacting with Mical, a novel actin depolymerization factor with oxidoreductase activity (Terman et al., 2002; Hung et al., 2010; Hung and Terman, 2011). Second, Plexin decreases adhesion by RasGAP activity which inhibits R-Ras/Integrin-mediated ECM adhesion (Oinuma et al., 2006; Oinuma et al., 2004a), whereas binding of 14-3-3ε to Plexin RasGAP domain inhibits RasGAP activity (Chapter 3). It is of interest whether one of these two functions of Plexin is predominantly required to mediate the repulsive response. The defects resulting from neuronal overexpression of *14-3-3ε* resemble those seen with a loss of *Mical*, suggesting that these two discrete molecular functions of Plexin may be dependent on each other. Alternatively, the loss of one of these two Plexin functions may result in same guidance defects because both actin depolymerization and loss of adhesion are necessary for the correct repulsive response. Nonetheless, if only one of the two functions of Plexin A can be manipulated, the effects of each function would be clearly distinguished. The motor and CNS axon guidance defects that result from loss of Plexin A are significantly rescued by wild type Plexin A transgene, but not by a 'GAP-deficient' Plexin A transgene (Figure S3.5), indicating that normal RasGAP activity is required for the repulsive effects of Plexin A. It is noteworthy that the embryos rescued by 'GAP-deficient' Plexin A transgene exhibit other guidance defects which resemble those resulting from gain of function *Mical* (data not

presented). This 'GAP-deficient' Plexin A can still mediate actin reorganization by interacting with Mical (data not presented), which suggests that loss of RasGAP activity in Plexin A may result in hyperactive Mical function in axons. Therefore, it is tempting to speculate that Plexin RasGAP activity and interaction with Mical are exclusive of each other, or these two functions are activated sequentially or require two different populations of Plexin complexes. If this is the case, these two functions will be differentially required and regulated depending on the different cellular contexts.

Phosphorylation of Plexin A

Silencing Plexin RasGAP activity by a phosphorylation-mediated interaction with 14-3-3ε suggests that distinct molecular pathways antagonize the repulsive response of Semaphorin/Plexin signaling by increasing the level of cAMP. Then, what other putative antagonizing pathways increase cAMP level? One candidate attractive cue is Netrin, which is expressed by muscles in developing *Drosophila* embryos (Winberg et al., 1998a; Mitchell et al., 1996). Netrin-dependent axon guidance requires cAMP signaling and the direct interaction between Netrin and adenosine A2b receptor increases the cAMP level (Corset et al., 2000; Ming et al., 1997). cAMP is produced by adenylate cyclase (AC). Among nine members of the AC family, AC1 and AC8 are known to be activated by calcium/calmodulin as well as heterotrimeric Gαs proteins (Dessauer, 2009). Intracellular calcium

signaling is an important mediator of growth cone guidance (Tojima et al., 2011; Gomez and Zheng, 2006) and Semaphorin activity is also regulated by calcium signaling (Nishiyama et al., 2011; Togashi et al., 2008; Nishiyama et al., 2003). Therefore, calcium-stimulated AC is another candidate for the inhibition of Plexin RasGAP (Xu et al., 2010). In addition, one provocative possibility is that Semaphorins may not only activate Plexin RasGAP but also inactivate Plexin as a 'feedback' inhibition through calcium signaling (Nishiyama et al., 2011), by which Semaphorin can further control the duration of repulsive signaling. Further in vivo studies will reveal which antagonizing factors decrease Semaphorin/Plexin repulsive signaling.

PKA phosphorylates the S1794 residue in the Plexin cytoplasmic region, but the other PKA phosphorylation site in the carboxyl- half of Plexin A has not been determined yet, nor has the one Cdk5 phosphorylation site. The S1794 residue resides next to the second Arginine residue (R1798), which is critical for enzymatic activity of RasGAP. When these two residues are compared in the crystal structure of the mouse Plexin A3 cytoplasmic region, C1720 of Plexin A3, which corresponds to *Drosophila* S7194, is adjacent to both enzymatically critical Arginine residues, R1407 and R1724. However, this C1720 is beneath the surface of the current (inactive and unable to associate with Ras family GTPases) structural model of Plexin A3, which suggests that S1794 needs to be exposed on the surface in order to make S7194 accessible for the PKA phosphorylation.

Therefore, it is likely that Plexin A needs to undergo a conformational change prior to the phosphorylation of S1794. Interestingly, one of the well-conserved PKA phosphorylation consensus sequence in the cytoplasmic region of Plexin A, $RGS^{1759}ALP$ (this sequence is also conserved in the vertebrate Class A Plexins), is located in outer surface of RasGAP domain, on the opposite side of the RasGAP active site. If this S1759 is another PKA phosphorylation site, it would be interesting that this residue is positioned in a flexible linker to connect two arms of a RasGAP domain. In addition, this RGSALP sequence corresponds to a mode I 14-3-3 binding consensus motif (RxpSxxP), which suggests that this site may be another unknown 14-3-3 binding site. Phosphorylation of this S1759 residue may be able to distort the GAP domain so that S1794 will be exposed. Likewise, phosphorylation of Plexin A by Cdk5 needs more validation in vivo for its roles in axon guidance. Further studies on the phosphorylation of Plexin A would provide an additional understanding of its role in the regulation of Plexin GAP activity and/or the interaction with other proteins.

Plexins interact with members of the Nervy/MTG family of A kinase anchoring proteins (AKAPs) (Fiedler et al., 2010; Terman and Kolodkin, 2004). AKAPs serve as scaffolding proteins which convey information and regulate signaling pathways by interacting with many signaling components such as kinases, phosphatases, GTPases, phosphodiesterases (PDE), actin, 14-3-3, and membrane-associated receptors and channels (Wong and Scott, 2004). If

phosphorylation of Plexin A is dynamically regulated during the course of axon guidance, it is tempting to speculate that Nervy may also interact with particular phosphatases such as PP1, PP2A or PP2B (calcineurin). This is not a radical idea in that phosphorylation is a reversible modification and reactivation of the Plexin RasGAP by dephosphorylation would be a more energy and time-efficient method than synthesizing another Plexin complex. If a phosphatase is identified as a signaling component of Semaphorin/Plexin repulsive guidance response, it would also be interesting to determine when the phosphatase activity is required. My current working model suggests that the Plexin RasGAP is activated by Semaphorin binding and inactivated by PKA/14-3-3ε-mediated inhibition, such as [off (no ligand binding) – on (ligand binding) – off (ligand binding with phosphorylation/14-3-3 binding)]. Sema-1a is expressed in the major motor axon pathways (discussed below) (Yu et al., 1998), suggesting that some Plexin receptors may always interact with the Semaphorin ligand during axon guidance. To prevent this possible tonic activation of Plexin RasGAP, phosphorylation-mediated inhibition may be required and reactivation of Plexin may occur by dephosphorylation, such as [off (ligand binding with phosphorylation/14-3-3 binding) – on (ligand binding with dephosphorylation) – off (ligand binding with phosphorylation/14-3-3 binding)]. However, arguing against this possibility is that very little is known of how much/how rapidly 14-3-3 proteins dissociate from their targets. One possibility is suggested by the experiments of (Tzivion et al.,

2000; Margolis et al., 2006) in which 14-3-3 proteins appear to preferentially "choose" other (more abundant) target proteins to associate with, and these proteins have been termed "14-3-3 binding sinks". In such as model, if 14-3-3 were to release from Plexin and preferentially bind these "14-3-3 binding sinks", a possible phosphatase could dephosphorylate the target site. Another possibility suggested by the experiments of Margolis and colleagues is that additional post-translational alterations to the target protein (Plexin) may induce 14-3-3 to dissociate from Plexin and thereby allow phosphatase access (Margolis et al., 2006). To distinguish among these different possibilities, the spatio-temporal regulation of Semaphorin/Plexin and PKA signaling needs to be examined during the course of axon guidance.

Semaphorin-Plexin Interaction During the Growth Cone Guidance

There are many mechanisms by which repulsive guidance cues sculpt the nervous system. Repulsive guidance cues at times appear to surround axon bundles by generating a channel through which axons can go through. Likewise, repulsive cues are secreted from intermediate 'guide-post cells' to cordon off areas where growth cones cannot extend (Lowery and Van Vactor, 2009; Tessier-Lavigne and Goodman, 1996). Interestingly, Sema-1a is expressed in the major motor axon pathways and CNS axons during the development of *Drosophila* embryonic nervous system (Yu et al., 1998). Embryonic motor axons express both repulsive

cues (Sema-1a) and receptors (Plexin A) but they are selectively defasciculated. How does Sema-1a enable selective defasciculation of motor axons? Previous results suggest that Sema-1a/Plexin A establishes a basal level of axon-axon repulsion. This basal repulsion is overcome by axon-axon attraction provided by cell adhesion molecules, such as Fasciclins, which results in fasciculation. At choice points, this balance between attraction and repulsion shifts to repulsion due to a decrease in axon-axon attraction, which leads to defasciculation (Winberg et al., 1998b; Yu et al., 2000). In addition, Plexin decreases Ras/Integrin-mediated cell adhesion through its RasGAP activity. Therefore, activation of the Plexin RasGAP at a choice point also reduces axon-substrate adhesion and may make growth cones more susceptible to other attractive cues enabling defasciculation. However, a prolonged decrease in axon-substrate adhesion may cause axons to stall after defasciculation (Huang et al., 2007). My results support a model in which in order to restore Integrin/Ras-mediated axon-substrate adhesion, Plexin RasGAP is inactivated by PKA phosphorylation and subsequent 14-3-3 binding to the Plexin RasGAP domain (Chapter 3).

Plexin was first identified as a homophilic cell adhesion molecule (Ohta et al., 1995; Ohta et al., 1992), suggesting that Plexins interact with each other and mediate adhesion in the *trans* configuration. However, Plexins cannot initiate a repulsive response in the *cis* homodimer configuration (Nogi et al., 2010). In addition, transmembrane Semaphorins can initiate 'reverse' signaling through its

short cytoplasmic tail (Kruger et al., 2005; Komiyama et al., 2007; Yu et al., 2010). Therefore, all the possible interactions between Semaphorins and Plexins, such as Semaphorin/Plexin (*trans*), Semaphorin/Plexin (*cis*), Plexin/Plexin (*trans*), Plexin/Plexin (*cis*), Semaphorin/Semaphorin (*trans*), and Semaphorin/Semaphorin (*cis*), add further complexity to a full understanding of Semaphorin/Plexin signaling in axon guidance. One possible mechanism to resolve this seemingly complex mechanism is suggested by evidence that the *cis* interaction between ligand and receptor silences receptor activity (Haklai-Topper et al., 2010; Carvalho et al., 2006). In the developing mouse olfactory system, olfactory receptor neurons expressing the same olfactory receptor converge onto the same glomeruli in the olfactory bulb. To construct this topographic map, axons from olfactory receptor neurons are presorted by complementary expression of Semaphorin 3F and its receptor Neuropilin 2 gradients (Imai et al., 2009; Takeuchi et al., 2010). This example suggests that differentially expressed ligand and receptor separate axons from each other depending on the relative activity of receptor signaling. The gradient expression of Semaphorin is also important to establish dorso-ventral axis for the innervation of *Drosophila* embryonic sensory axons (Zlatic et al., 2009). Therefore, it will be of interest to test whether Semaphorin/Plexin interactions in the *cis* configuration and the relative expression levels of Semaphorin/Plexin contribute to the guidance of *Drosophila* motor axons.

APPENDIX A

Supplemental Information for Chapter Two

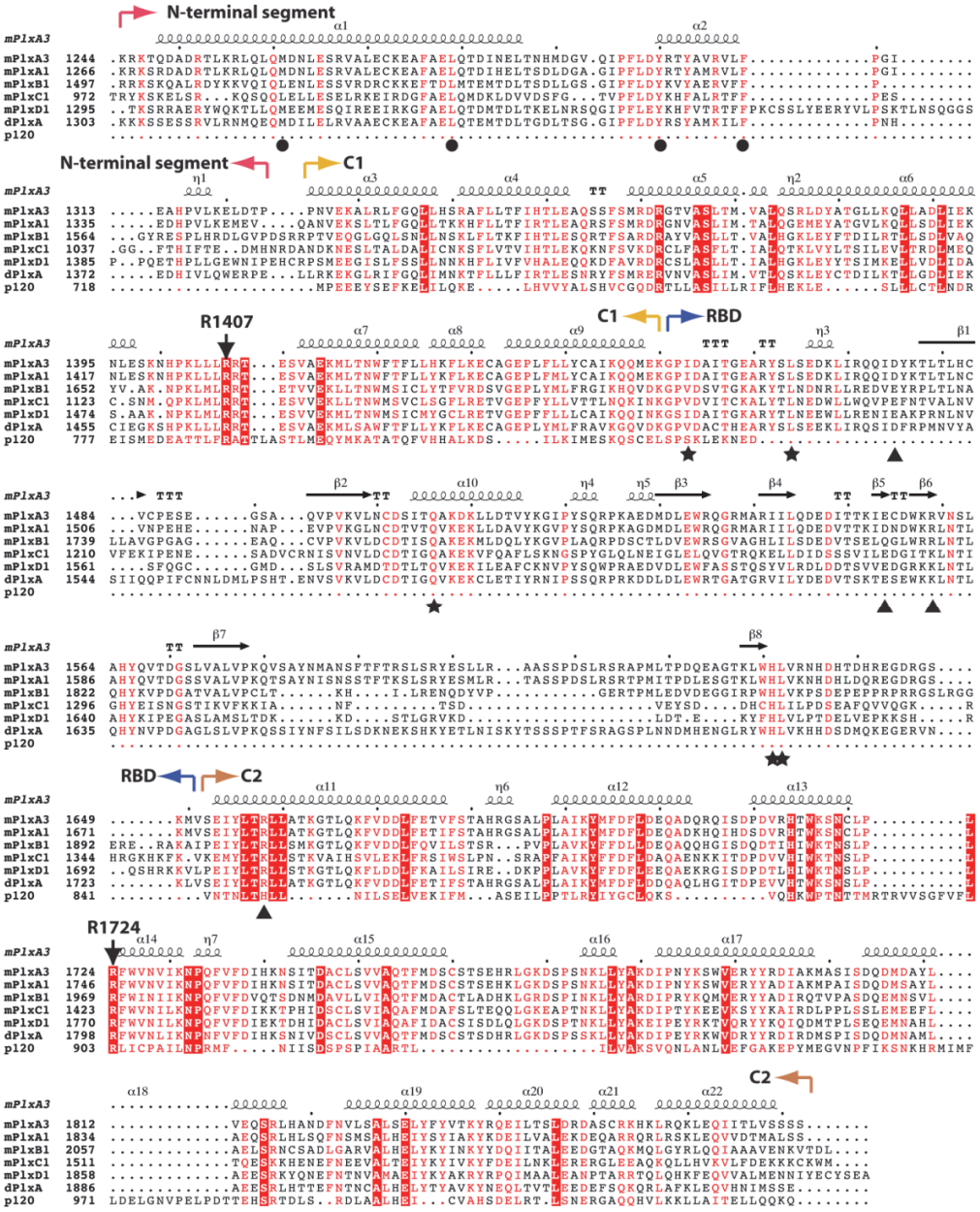

Figure S2.1. Sequence alignment of the intracellular region of plexins and the p120 Ras GAP domain. The p120 Ras GAP domain is aligned to plexin A3 based on a superimposition of their structures. The secondary structural elements of plexin A3 are shown on the top. Mutations tested in the paper are highlighted with circles (the interface between the N-terminal segment and the GAP domain), stars (the interface between the RBD and the GAP domain) and triangles (the crystallographic dimer interface seen in the plexin A3 crystal).

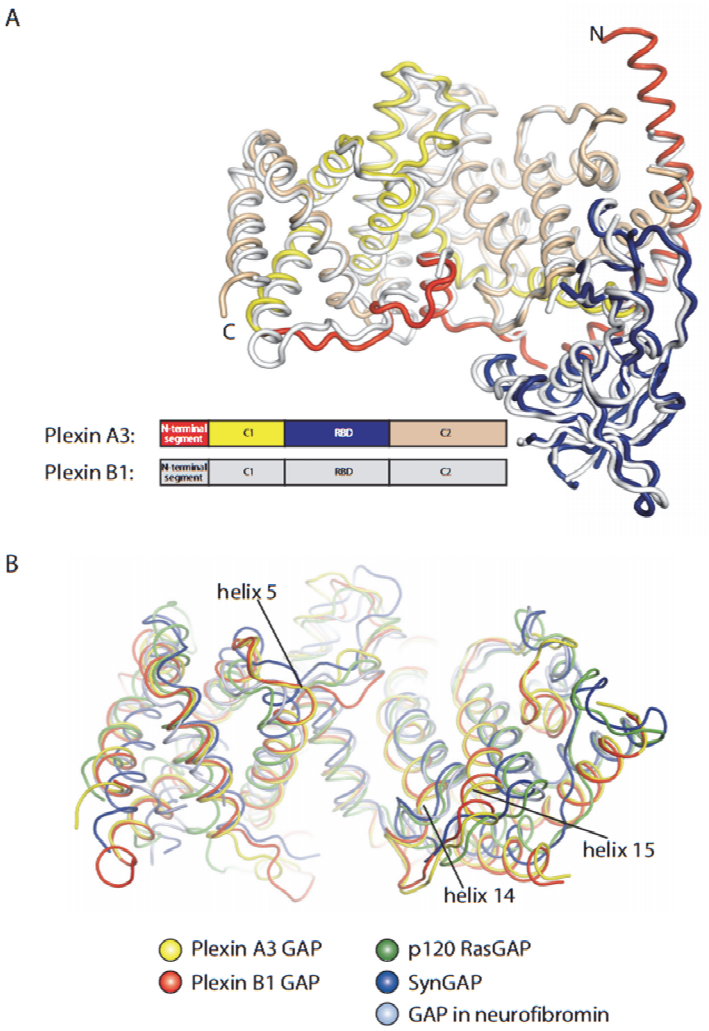

Figure S2.2. Structural comparison of the intracellular regions of plexin A3 and plexin B1 and other Ras GAPs. (*A*) Superimposition of the structures of the intracellular domains of plexin A3 (this work) and plexin B1 (PDB ID: 3HM6, structure in the PDB), showing that the two are essentially identical, with minor differences present in loop regions. (*B*) Superimposition of the GAP domains of plexin A3, plexin B1, p120 Ras GAP (PDB ID: 1WQ1), SynGAP (PDB ID: 3BXJ), and neurofibromin (PDB ID: 1NF1). The GAP domains of p120 Ras GAP, SynGAP, and neurofibromin all adopt the same active conformation. Plexin A3 and B1 are very similar to each other, but deviate substantially in helices 4, 5, 14, and 15 when compared with the other three, resulting in a more ‘‘closed’’ active site.

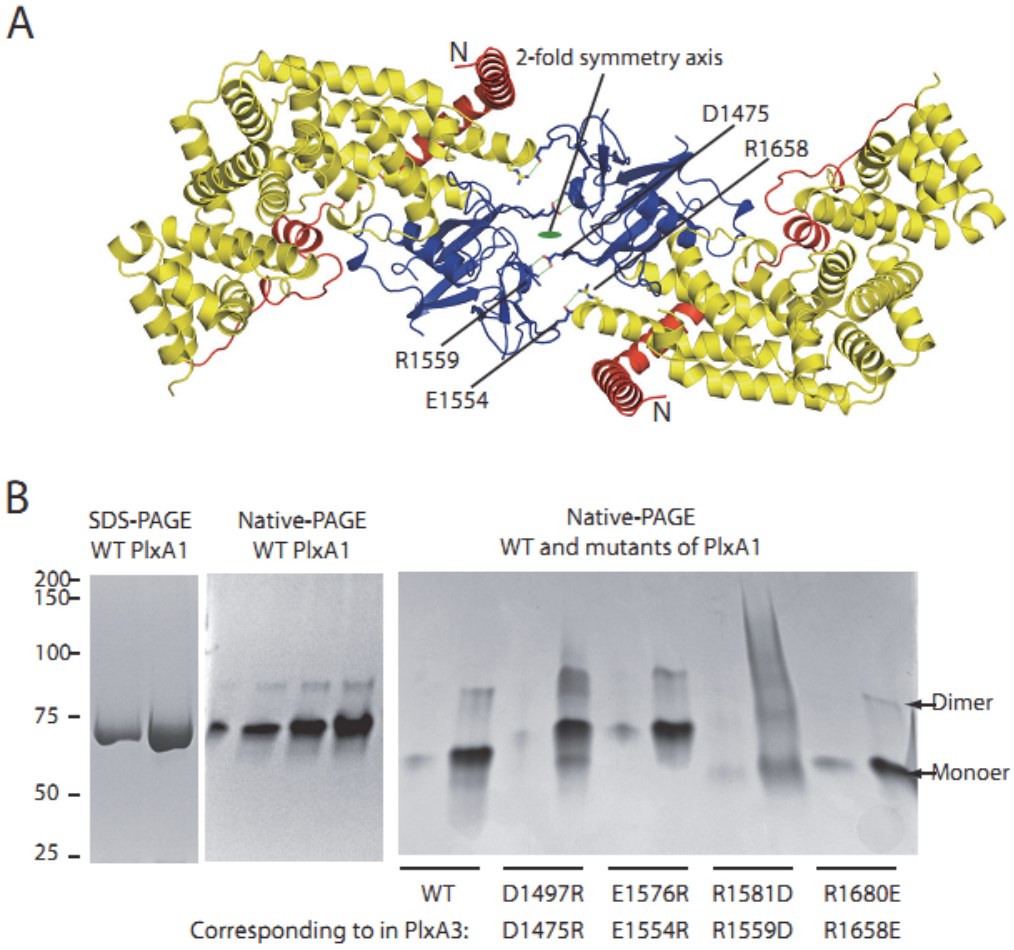

Figure S2.3. Dimerization of plexin intracellular regions. (*A*) A dimer observed in the plexin A3 crystal structure. While several symmetric crystallographic dimers are formed in plexin A3 crystals, only the one shown here has a conserved dimer interface. The two conserved ion-pairs in the interface are highlighted. We performed mutational analyses of this dimer, considering that the other dimers in the crystal with unconserved interfaces are not likely to be functionally important. (*B*) Native gel analyses of Plexin A1 dimerization. The wild-type plexin A1 dimerizes weakly, mutations of the residues corresponding to the ion-pairs in *A* do not affect dimerization, suggesting that this crystallographic dimer does not represent the plexin dimer seen in solution. The differences in mobility between the mutants and the wild type are due to charge differences of the proteins.

Mouse Plexin A3	Mouse Plexin A1	Drosophila Plexin A	Location	Rational
M1261R		M1320R	Interface between the N-terminal segment and the GAP domain	Disruption of the interface by a bulky charged residue
L1279R		L1338R		Disruption of the interface by a bulky charged residue
Y1300A				Removal of the interactions between the aromatic residues in the interface
F1309A				Removal of the interactions between the aromatic residues in the interface
I1453R			Interface between the RBD and the GAP domain	Disruption of the interface by a bulky charged residue
L1464G		L1524G		Disruption of the interface by removing the entire side chain
Q1506A				Disruption of the interface by replacing the large side chain with a smaller one
H1631A/L1632A				Disruption of the interface by replacing the large side chain with a smaller one
	D1497R		Equivalent to D1475R in Plexin A3, crystallographic dimer interface	Disruption of the crystallographic dimer interface by charge reversal mutations (Figure S3)
	E1576R		Equivalent to E1554R in Plexin A3, crystallographic dimer interface	
	R1581D		Equivalent to R1559D in Plexin A3, crystallographic dimer interface	
	R1680E		Equivalent to R1658E in Plexin A3, crystallographic dimer interface	
R14071408A			Active site	Negative control
		ΔCyto	Entire cytoplasmic region	Negative control

Table S2.1. List of mutations tested in this study

Dataset	Seleno-Met SAD	Native
Data collection		
Space group	*C*2	*C*2
Unit cell parameters		
a, Å	147.7	147.8
b, Å	46.9	47.2
c, Å	101.2	101.2
β, °	118.7	118.7
Wavelength, Å	0.97926	0.97926
Resolution, Å	50–2.75 (2.85–2.75)[a]	50–2.00 (2.07–2.00)
No. of reflections	150,460	195,739
No. of unique reflections	16,221	41,961
Completeness, %	99.8 (99.9)	99.6 (97.4)
I/σ	20.3 (4.5)	26.3 (3.0)
R_{sym}, %[b]	11.1(45.8)	5.0 (35.5)
Refinement		
R_{work}, %		18.8
R_{free}, %		22.4
Molecules per asymmetric unit		1
No. of protein atoms		4,479
No. of non-protein atoms		371
rmsd bond length, Å		0.004
rmsd bond angle, °		0.719
Ramachandran plot		
Favored, %		93.1
Allowed, %		6.9
Disallowed, %		0

Numbers in parenthesis refer to the highest resolution shell. $R_{sym} = \Sigma|I - \langle I\rangle|/\Sigma I$, where I is the observed intensity of a reflection, and $\langle I\rangle$ is the average intensity of all the symmetry related reflections.

Table S2.2. Data collection and refinement statistics

APPENDIX B

Supplemental Information for Chapter Three

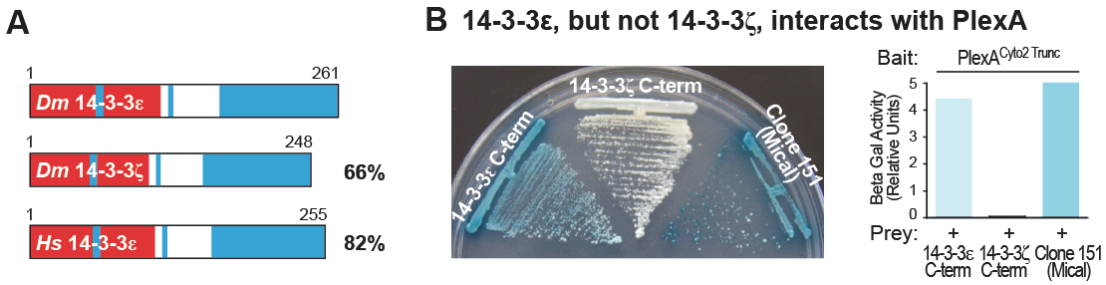

Figure S3.1. 14-3-3ε Specifically Interacts with PlexA, Related to Figure 3.1. (**A**) Sequence comparisons show the amino acid identity between different Drosophila (*Dm*) and Human (*Hs*) 14-3-3 family members and *Dm*14-3-3ε. (**B**) 14-3-3ε, but not 14-3-3ζ, associates with PlexA. The results of a yeast two-hybrid interaction assay show that PlexA associates (Blue color = Beta-galactosidase activity that indicates an interaction (Golemis, 2001)) with the C-terminus of Drosophila 14-3-3ε but not the C-terminus of the other Drosophila 14-3-3 family member, 14-3-3ζ. Mical served as a positive control for a Plexin-interacting protein (Terman et al., 2002).

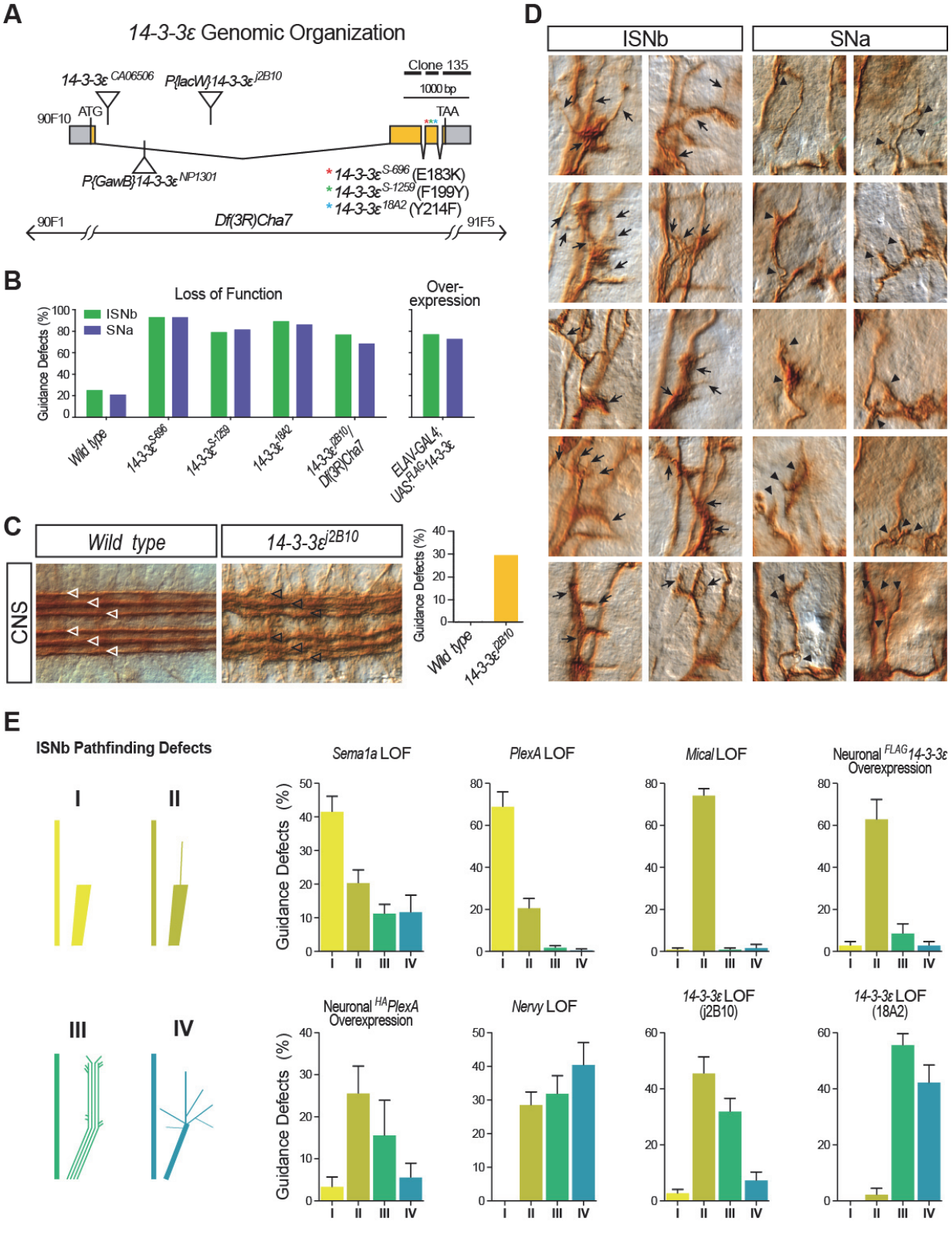
A
14-3-3ε Genomic Organization
14-3-3ε CA06506
P{lacW}14-3-3ε j2B10
Clone 135
1000 bp
90F10
ATG
TAA
P{GawB}14-3-3ε NP1301
*14-3-3ε S-696 (E183K)
*14-3-3ε S-1259 (F199Y)
*14-3-3ε 18A2 (Y214F)
90F1
Df(3R)Cha7
91F5
B
Loss of Function
Over-expression
Guidance Defects (%)
ISNb
SNa
Wild type
14-3-3ε S-696
14-3-3ε S-1259
14-3-3ε 18A2
14-3-3ε j2B10/ Df(3R)Cha7
ELAV-GAL4; UAS FLAG 14-3-3ε
C
Wild type
14-3-3ε j2B10
CNS
Guidance Defects (%)
Wild type
14-3-3ε j2B10
D
ISNb
SNa
E
ISNb Pathfinding Defects
I
II
III
IV
Sema1a LOF
PlexA LOF
Mical LOF
Neuronal FLAG 14-3-3ε Overexpression
Neuronal HA PlexA Overexpression
Nervy LOF
14-3-3ε LOF (j2B10)
14-3-3ε LOF (18A2)
Guidance Defects (%)

Figure S3.2. Further Characterization of the Axon Guidance Defects in *14-3-3ε* Loss-of-Function and Gain-of-Function Mutants and Comparison to Sema-1a/PlexA Axon Guidance Signaling Components, Related to Figure 3.2. (**A**) Genomic organization of Drosophila *14-3-3ε* and the different alleles used for this study. Each exon is boxed, the coding regions are indicated in orange, and the corresponding genomic sequence of Clone 135 is demarcated. The insertion positions of the three P element alleles are also indicated as are the three published point (missense) mutation alleles (asterisks). (**B**) Quantification of the percentage of ISNb and SNa pathway defects revealed that all three point mutant alleles (*14-3-3ε*$^{S-696}$, *14-3-3ε*$^{S-1259}$, and *14-3-3ε*^{18A2}) exhibit axon guidance defects. *Df(3R)Cha7* is a deficiency of the genomic region containing the *14-3-3ε* locus. Overexpressing a ^{FLAG}*14-3-3ε* transgene using pan-neuronal driver *ELAV-GAL4* also generated ISNb and SNa guidance defects (*ELAV-GAL4; UAS:* ^{FLAG}*14-3-3ε*). n≥90. (**C**) In wild-type embryos, three major evenly-spaced longitudinal axon tracts are detected with the 1D4 antibody on each side of the embryonic midline (white arrowheads). In *14-3-3ε* mutants, however, we noticed that at times these CNS longitudinal axon tracts exhibited abnormal organization. In this example, the normally tightly fasciculated longitudinal axons (compare white and black arrows) are thicker/more defasciculated than normal and are discontinuous with axons extending as a part of smaller bundles within the CNS. Our analyses indicated that the majority of CNS defects present in *14-3-3ε* mutants were consistent with increased axonal defasciculation and were more reminiscent of PlexA GOF than Sema1a/PlexA LOF (see also Figures 3, S3A). Defects reminiscent of increased defasciculation were also present in the motor axon pathways of *14-3-3ε* mutants (Figures 2, S2D-E). n≥100/genotype. (**D**) Analyses of multiple combinations of five different *14-3-3ε* loss-of-function alleles reveals numerous defects that are indicative of increased axonal defasciculation (See also Figure 2C and F). For example, ISNb axons were seen to separate inappropriately along their pathway and remain as an abnormally thick bundle of defasciculating axons or split prematurely into different projections that extended abnormally across the muscle field (arrows). Similar abnormal defasciculation/splitting of axon bundles into multiple projections were also seen for SNa axons (arrowheads). These types of defects are consistent with an increased sensitivity to axon-axon repulsive cues (e.g., (Winberg et al., 1998b; Terman et al., 2002; Hung et al., 2010; Terman and Kolodkin, 2004)). See also Figure S2E. (**E**) Altering *14-3-3ε* levels generates axon guidance defects that are related but qualitatively and quantitatively distinct from similar alterations to Sema-1a/PlexA levels. Extensive in vivo studies using Drosophila motor axons have revealed that axons require a number of different influences to make it to their targets including axon-axon adhesion/fasciculation, axon-axon repulsion, axon-substrate adhesion, target attraction, and non-target repulsion (Araujo and Tear, 2003). For example, it is known that motor axons fasciculate with other motor axons through the action of adhesion molecules like NCAM (Fasciclin II) and use these axons to guide them into the vicinity of their targets (Van Vactor, 1998). Then, at particular choice points repulsive guidance cues like Sema-1a and its receptor PlexA allow particular motor axons to defasciculate from other motor axons (Yu et al., 1998; Winberg et al., 1998b). Integrins responding to their ligands in the extracellular matrix are also necessary for motor axons to respond at this choice point and to grow to their muscle targets (Hoang and Chiba, 1998; Huang et al., 2007), as are attractants such as netrin which are made by muscle targets (Winberg et al., 1998a). Disrupting any of these influences leads to related axon guidance defects – and this fits with observations from a number

of different systems in vivo and in vitro that a loss of axon-axon repulsion looks similar to the inability of an axon to adhere/grow on a particular substrate. The requirement for this integration of adhesive and repulsive pathways are also thought to be the reasons why the defects that result from loss-of-function and overexpression of some molecules/signaling pathways such as Plexins (Winberg et al., 1998b; Hu et al., 2001; Ayoob et al., 2006) and Integrins/Cas (Hoang and Chiba, 1998; Huang et al., 2007) can look similar in appearance. Nonetheless, indepth analysis of these guidance defects often reveals quantifiable variations among these related phenotypes (e.g., (Winberg et al., 1998b; Terman et al., 2002; Terman and Kolodkin, 2004)) and we find that *14-3-3ε* loss-of-function mutants exhibit a distinct pattern of guidance defects compared to that of loss of Sema-1a/PlexA repulsive signaling components. For example, both *Sema1a* (*Sema1a*^{P1}/*Sema1a*^{P1}) and *PlexA* (*PlexA*$^{Df(4)C3}$/*PlexA*$^{Df(4)C3}$) loss-of-function (LOF) mutants exhibit a higher percentage of what we have now called Class I and II defects (see also (Winberg et al., 1998b; Yu et al., 1998)), whereas overexpression of PlexA in neurons (neuronal ^{HA}PlexA overexpression: *ELAV-GAL4,UAS:*^{HA}*PlexA/+*) exhibit a higher percentage of Class II, III, and IV defects. In a similar manner, loss of function mutations in *Nervy* (*Nervy*^{PDFKG1}/*Nervy*^{PDFKG1}), which genetically antagonize the repulsive response of Sema-1a/PlexA exhibit a majority of Class II, III, IV defects (i.e., they resemble neuronal overexpression of PlexA; see also (Terman and Kolodkin, 2004)). Like *Nervy* mutants and neuronal PlexA overexpression, *14-3-3ε* LOF mutants (e.g., scored for two different *14-3-3ε* LOF mutants [*14-3-3ε*^{j2B10}/*14-3-3ε*^{j2B10} and *14-3-3ε*^{18A2}/*14-3-3*^{18A2}*]*) exhibit a majority of Class II, III, and IV defects. In contrast, neuronal overexpression of *14-3-3ε* (*ELAV-GAL4/ELAV-GAL4;UAS:*^{FLAG}*14-3-3ε/UAS:*^{FLAG}*14-3-3ε*) generates a majority of defects that resemble loss of *Sema1a, PlexA,* and *Mical*. The data are presented as the percentage of hemisegments that exhibited each class of guidance defects in each different genotype; n≥100 hemisegments (10 embryos)/genotype; Error bar: SEM. Assignment of the defects into the different classes was determined as follows on 1D4 immunostained embryos: (Hung et al., 2010; Terman et al., 2002; Terman and Kolodkin, 2004; Yu et al., 1998; Winberg et al., 1998b). Class I represents ISNb axons that separate from the ISN, but stall together in a tightly fasciculated manner around muscle 6/7. Class II represents ISNb axons that separate from the ISN, exhibit some stalling around muscle 6/7, but also extend a few axons towards muscle 12/13. Class III represents ISNb axons that do not stall around muscle 6/7 but extend towards muscles 12/13 in a thick bundle of abnormally defasciculating axons. Class IV represents ISNb axons that exhibited excessive "splitting" and defasciculation in different directions. Class I defects would be consistent with too much fasciculation with what appears to be a continuum towards Class III and IV defects which are consistent with too much defasciculation (see also (Winberg et al., 1998b; Yu et al., 1998; Terman et al., 2002; Terman and Kolodkin, 2004)). Likewise, as previously described (e.g., (Hung et al., 2010; Terman et al., 2002; Terman and Kolodkin, 2004)), this type of characterization revealed that all mutants examined exhibited a spectrum of different classes, but certain classes of defects were more prevalent than others. These observations complement our additional genetic assays (Figures 3, 5G, 6, 7C-E, and S3) that indicate that 14-3-3ε (like PKA, Nervy, Integrins, and Ras2) antagonizes Sema repulsive signaling and does not promote (like Plexin and Mical) Sema repulsive signaling.

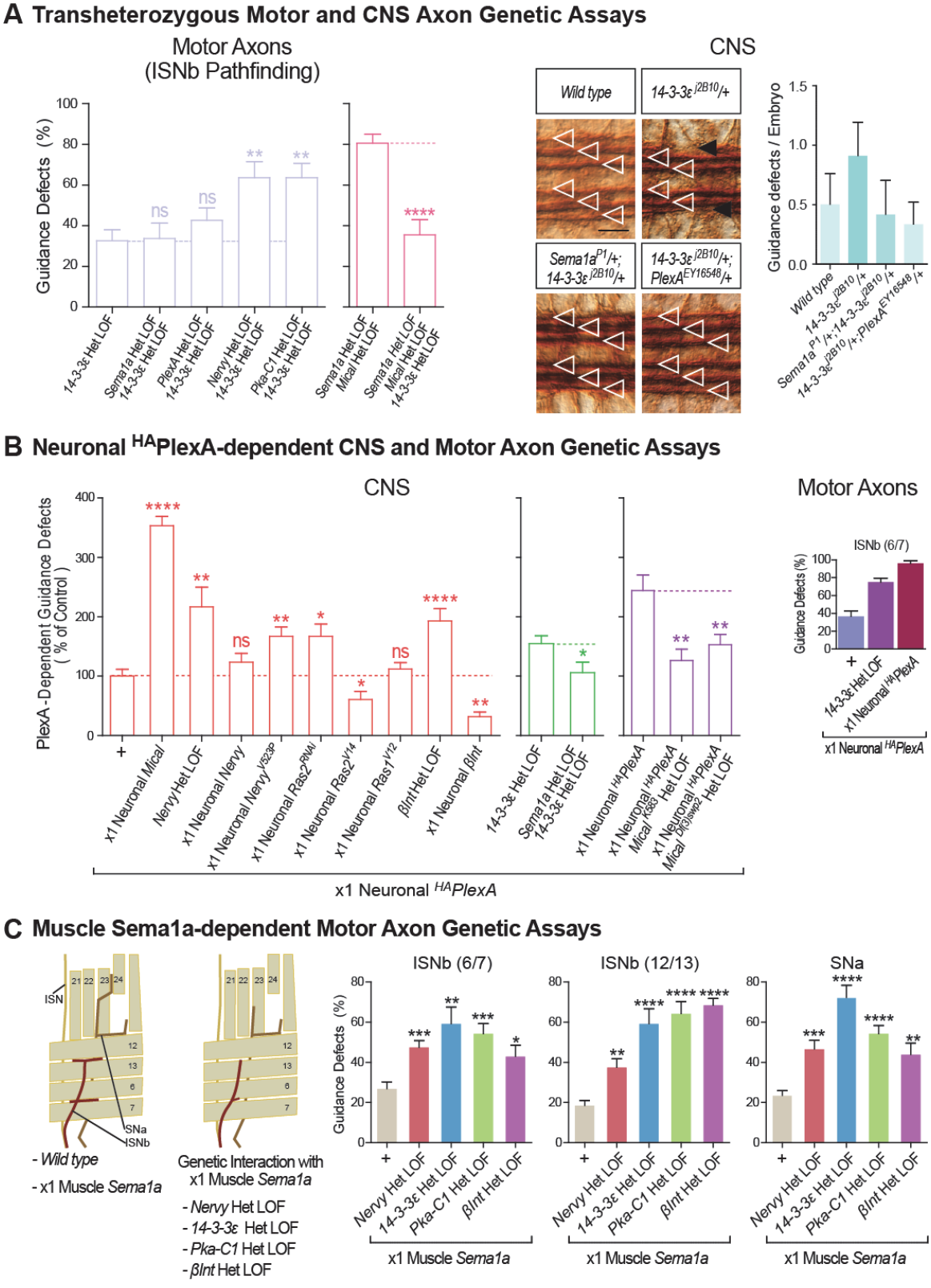
A Transheterozygous Motor and CNS Axon Genetic Assays
Motor Axons
(ISNb Pathfinding)
Guidance Defects (%)
CNS
Wild type
14-3-3ε j2B10/+
Sema1a P1/+; 14-3-3ε j2B10/+
14-3-3ε j2B10/+; PlexA EY16548/+
Guidance defects / Embryo
B Neuronal HAPlexA-dependent CNS and Motor Axon Genetic Assays
CNS
PlexA-Dependent Guidance Defects (% of Control)
x1 Neuronal HAPlexA
Motor Axons
ISNb (6/7)
Guidance Defects (%)
C Muscle Sema1a-dependent Motor Axon Genetic Assays
ISN
SNa
ISNb
- Wild type
- x1 Muscle Sema1a
Genetic Interaction with
x1 Muscle Sema1a
- Nervy Het LOF
- 14-3-3ε Het LOF
- Pka-C1 Het LOF
- βInt Het LOF
ISNb (6/7)
ISNb (12/13)
SNa
Guidance Defects (%)
x1 Muscle Sema1a

Figure S3.3. Multiple Different Genetic Interaction Assays Indicate that 14-3-3ε Antagonizes Sema-1a/PlexA-mediated Repulsive Axon Guidance, Related to Figure 3.3. (**A**) Dominant genetic interactions indicate that 14-3-3ε does not facilitate Sema-1a/PlexA-mediated repulsive axon guidance, but functions to antagonize Sema-1a signaling. The pathfinding of motor and CNS axons are sensitive to changes in the dosage of genes in axon guidance pathways. For example, dominant (transheterozygous) interactions have been observed between *Sema1a* and its downstream mediators of repulsive axon guidance including *PlexA*, *OTK*, *Gyc76c*, and *Mical* (Yu et al., 1998; Winberg et al., 2001; Terman et al., 2002; Ayoob et al., 2004). Therefore, motor and CNS axon pathways were analyzed to look for genetic interactions between *14-3-3ε* and *Sema1a/PlexA* signaling components. The pathfinding defects seen in *14-3-3ε* heterozygotes (*14-3-3ε^{j2B10}*/+; motor axons, left, Blue) were not enhanced by a loss of one copy of *Sema1a* (*Sema1a^{P1}*/+; motor axons, left, Blue) or *PlexA* (*PlexA$^{Df(4)C3}$*; motor axons, left, Blue). In contrast, *Nervy* (*Nervy^{PDFKG38}*/+; motor axons, left, Blue) or *Pka-C1* (*Pka-C1^{DN}*/+; motor axons, left, Blue) heterozygotes significantly enhanced the ISNb pathfinding defects that were present in *14-3-3ε* heterozygotes. Likewise, consistent with an antagonistic role for 14-3-3ε in Sema-1a/PlexA-mediated repulsive axon guidance, *14-3-3ε* heterozygotes suppressed the ISNb pathfinding defects that were present in embryos heterozygous for both *Sema1a* and *Mical* (*Sema1a^{P1}*/+;*Mical$^{Df(3)swp2}$*/+; motor axons, right, Pink). n≥100 hemisegments (10 embryos)/genotype; Error bar: SEM ; **p<0.01 by one-way ANOVA and Bonferroni post test for Blue, ****p<0.0001 by Student's *t*-test for Pink. In addition to looking for transheterozygous interactions using motor axons, we also examined transheterozygous genetic interactions using CNS axons. *Wild-type* embryos exhibit three straight and parallel FasII-positive longitudinal connectives on both sides of the midline (CNS, open arrowheads). In contrast, we noticed that the longitudinal connectives of heterozygous *14-3-3ε^{j2B10}* are not continuous in some hemisegments (CNS, closed arrowheads), providing a sensitive assay for looking for dominant genetic interactions. However, this phenotype was not enhanced (and was slightly suppressed) in embryos transheterozygous for both *14-3-3ε^{j2B10}* and *Sema1a^{P1}* or transheterogyzous for both *14-3-3ε^{j2B10}* and *PlexA^{EY16548}* (n≥100 hemisegments (10 embryos)/genotype; Error bars: SEM; one-way ANOVA). These results complement those in Figure 3 and others in Figure S3 that indicate that *14-3-3ε* antagonizes *Sema1a/PlexA* repulsive axon guidance. **(B)** *PlexA*-dependent repulsive axon guidance is modulated differently by Semaphorin and Ras/Integrin signaling components. (CNS) Previously employed (Ayoob et al., 2004; He et al., 2009) CNS axon guidance defects that result from increasing the levels of Neuronal *^{HA}PlexA* as in Figure 3 were used to further characterize genetic interactions. (Red) Defects that resulted from low level increases in PlexA (x1 Neuronal *^{HA}PlexA*; see also Figure 3) were also genetically modified by changes in Sema/Plexin signaling components: they were enhanced by increased Mical (x1 Neuronal *Mical*), enhanced by decreased Nervy (*Nervy* Het LOF), and enhanced by increased expression of a dominant negative form of Nervy (x1 Neuronal *Nervy^{V523P}*; (Terman and Kolodkin, 2004). Likewise, these Plexin A repulsive guidance defects were enhanced by decreased Ras2 (x1 Neuronal *Ras2^{RNAi}*) and decreased βIntegrin (*βInt* Het LOF), while they were suppressed by increased Ras2 (x1 Neuronal *Ras2^{V14}*) and increased βIntegrin (x1 Neuronal *βInt*). (Green) Using related genetic backgrounds, it was found that the enhanced *PlexA*-dependent guidance defects caused by *14-3-3ε* Het LOF (see also Figure 3) were suppressed by decreased Sema-1a (*Sema1a* Het LOF, *14-3-3ε* Het LOF).

(Purple) Using related genetic backgrounds, it was also found that the defects that resulted from high level of PlexA in neurons (x2 Neuronal ^{HA}*PlexA*; see also Figure 3) were suppressed by decreasing *Mical* (*Mical*^{K583}*Het LOF or Mical*$^{Df(3)swp2}$ *Het LOF;* (Hung et al., 2010; Terman et al., 2002; Beuchle et al., 2007)). The data are presented as a percentage of the control (x1 Neuronal ^{HA}*PlexA, +*) and statistical comparisons were made to the value represented by the dotted line in each colored graph; n≥90 hemisegments (10 embryos)/genotype; Error bar: SEM; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Student's *t*-test. Taken together with our other genetic interaction data using this assay (Figures 3B, 5G, 6D and 7C), these *PlexA*-dependent guidance defects indicate that PlexA repulsive axon guidance requires Sema-1a and Mical but is antagonized by 14-3-3ε, PKA, Nervy, Ras2, and Integrins. (Motor Axons) *PlexA*-mediated guidance defects in motor axon pathways are enhanced by decreasing the levels of 14-3-3ε. Increasing the levels of Neuronal ^{HA}*PlexA* (+: *ELAV-GAL4,UAS:*^{HA}*PlexA/+*) also generated motor axon guidance defects in ISNb target innervation at muscle 6/7. These defects are enhanced by *14-3-3ε* heterozygotes (*14-3-3ε* Het LOF: *ELAV-GAL4,UAS:*^{HA}*PlexA/+;14-3-3ε*^{j2B10}*/+*). Compare also to the motor axon guidance defects that occur when high levels of ^{HA}*PlexA* are expressed in neurons (i.e., adding another copy of neuronal ^{HA}PlexA; x1 Neuronal ^{HA}*PlexA*: *ELAV-GAL4,UAS:*^{HA}*PlexA/ELAV-GAL4,UAS:*^{HA}*PlexA*). n≥100 hemisegments (10 embryos)/genotype. (**C**) Guidance defects due to Sema-1a repulsion are enhanced by decreasing 14-3-3ε/PKA/Integrin signaling. Sema-1a is normally expressed in neurons (Yu et al., 1998). When Sema-1a is ectopically expressed in muscles, ISNb axons are repelled by their muscle targets in a PlexA-dependent manner (Winberg et al., 1998b; Yu et al., 1998). Consistent with previous reports (Terman and Kolodkin, 2004), heterozygous LOF *Nervy* mutants increased ISNb innervation defects in embryos expressing muscle Sema-1a. Likewise, heterozygous LOF *14-3-3ε*, heterozygous LOF *Pka-C1*, and heterozygous LOF *βInt* mutants enhanced these guidance defects caused by muscle Sema-1a repulsion. n≥100 hemisegments (10 embryos)/genotype; Error bar: SEM; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Student's *t*-test. Genotypes: Muscle Sema-1a for *UAS:Sema 1a/+;24B-GAL4/+*; *Nervy* Het LOF for *nervy*^{PDFKG1}*/+*; *14-3-3ε* Het LOF for *14-3-3ε*^{j2B10}*/+*; *βInt* Het LOF for *mys*^{1}*/+*.

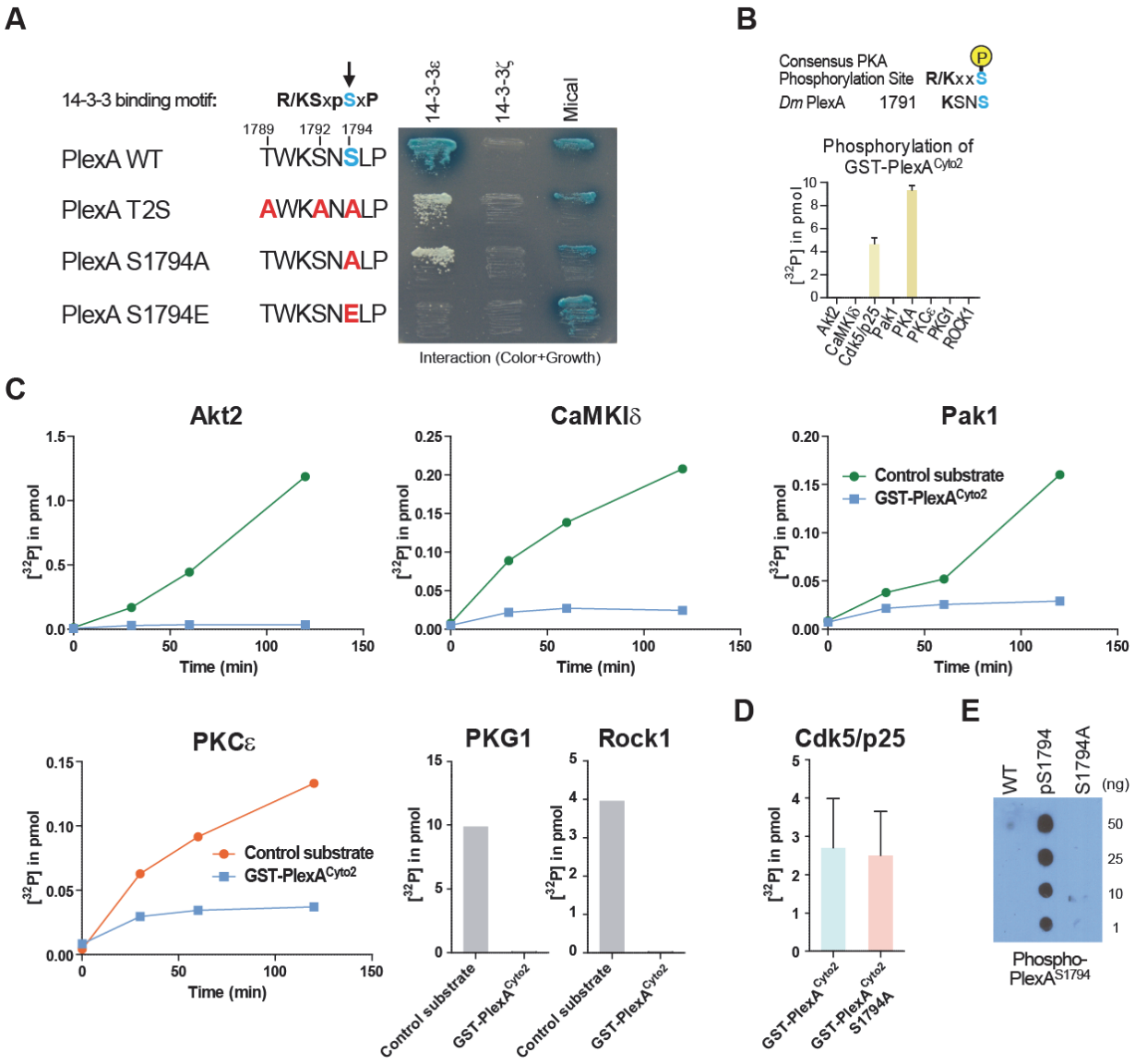

Figure S3.4. In Vitro Kinase Assays Reveal that Cdk5 Does Not Phosphorylate the PlexA Ser[1794] Residue, Related to Figure 3.4 and 3.5. (**A**) Site-directed mutagenesis reveals that the Ser[1794] residue in PlexA is critical for the interaction between PlexA and 14-3-3ε. A yeast two-hybrid assay as in Figure 4C was used to look for interactions between PlexA (PlexA$^{Cyto2\ Trunc}$; residues 1702-1857) and 14-3-3ε (Clone 135) following substitutions of different Ser (S) or Thr (T) residues to Ala (A) or Glu (E) in the consensus 14-3-3 binding motif of PlexA. In this additional assay to that in Figure 4C, Ser[1794] to Ala[1794] or Glu[1794] in PlexA (PlexA S1794A or S1794E) strongly diminished the interaction with 14-3-3ε. Substituted residues are colored red.

(**B**) The 14-3-3 binding site of PlexA contains a consensus Protein Kinase A (PKA) phosphorylation site (top) and the cytoplasmic portion of PlexA is highly and specifically phosphorylated by PKA (bottom). The employed in vitro kinase assay also revealed that both PKA and Cdk5, but not any of the other serine-threonine kinases tested, phosphorylated the cytoplasmic portion of PlexA, as determined by incorporation of ^{32}P into purified protein corresponding to the $PlexA^{Cyto2}$ region. n≥ 3/kinase. (**C**) Examples of the quantification ^{32}P into substrates by different kinases as in Figures 5A-B reveal that Akt2, CaMKIδ, Pak1, PKCε, PKG1, and Rock1 can phosphorylate a known control substrate but show little to no phosphorylation of GST-$PlexA^{Cyto2}$. Protein phosphatase inhibitor-1 (Inhibitor-1; blue) or Myelin basic protein (MBP; red) were used as control substrates. Autophosphorylation of PKG1 and Rock1 provided a control for their activity. (**D**) Cdk5 phosphorylates GST-$PlexA^{Cyto2}$, but not on Ser^{1794} (GST-$PlexA^{Cyto2\ S1794A}$). The phosphorylation levels of $PlexA^{Cyto2}$ and $PlexA^{Cyto2\ S1794A}$ were similar and together with other results (Figure S4B) indicate that Cdk5 phosphorylates PlexA in vitro but not on Ser^{1794}. (**E**) Dot blot anaylsis demonstrates that antisera generated against a peptide containing a phosphate group at PlexA Ser^{1794} (phospho-$PlexA^{S1794}$) specifically recognizes the phosphorylated Plexin peptide (pS1794), but not a wild-type Plexin peptide (WT) or a peptide containing a Ser to Ala substitution at the 1794 residue of PlexA (S1794A). The amount of peptide blotted on the membrane is shown in ng.

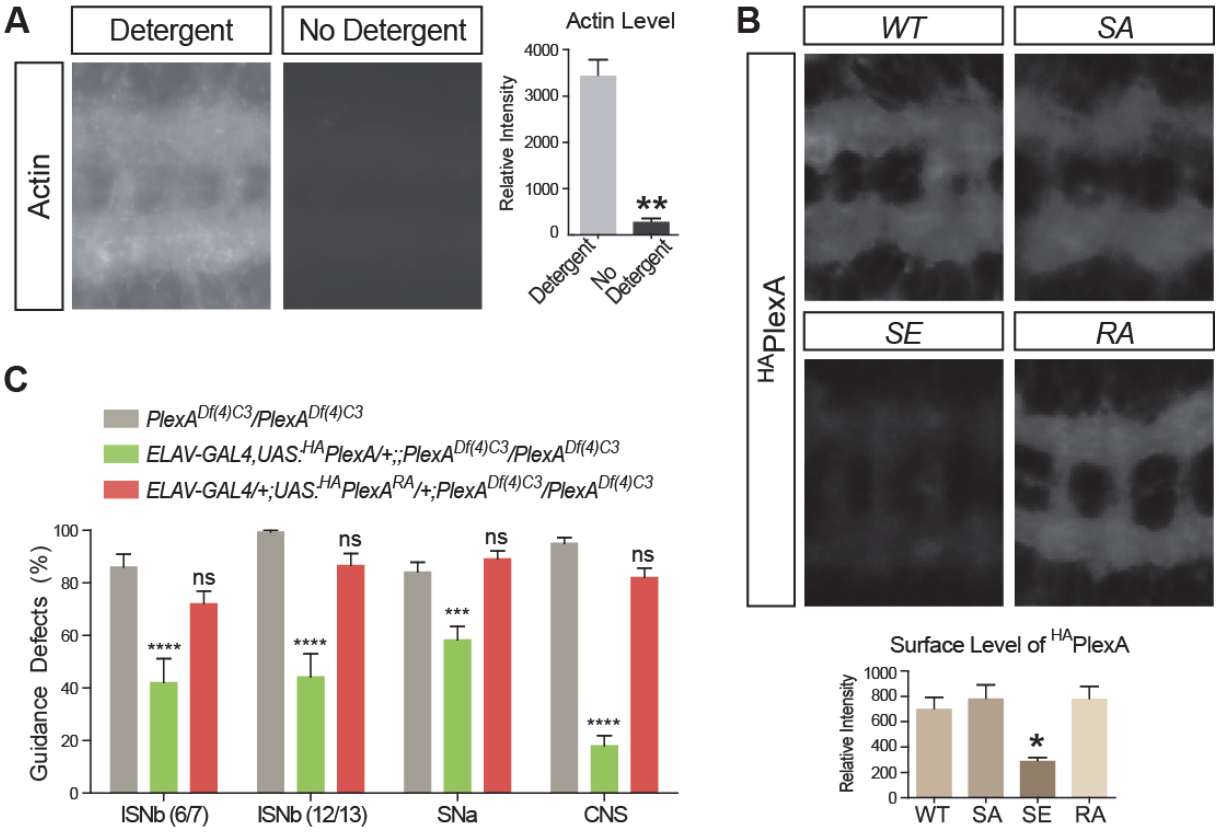

Figure S3.5. Additional Characterization of ^{HA}PlexA Mutant Transgenes, Related to Figure 3.6. (**A-B**) Standard approaches were used to examine the surface expression of different forms of PlexA (Williamson et al., 2010). (**A**) As a control, wild type embryos were immunostained for actin (a cytoplasmic protein). Actin was only detected in the presence of detergent, indicating that antibodies are not able to gain appreciable intracellular access in the absence of a detergent. n=4; Error bar: SEM; **p<0.01 by Student's *t*-test. (**B**) All mutant ^{HA}PlexA transgenes were expressed on the surface of axons at or below the wild type ^{HA}PlexA. Surface expression level of ^{HA}PlexA transgenes were examined using detergent-free conditions and an antibody that recognizes the HA epitope of ^{HA}PlexA that is present extracellularly. One copy of each transgene was expressed using the neuronal driver *ELAV-GAL4*. n≥5; Error bar: SEM; **p<0.01 by 1 way ANOVA. (**C**) A RasGAP deficient form of PlexA is unable to rescue the motor and CNS axon guidance defects that result from loss of *PlexA*. Loss of function *PlexA* generates defects in the guidance of ISNb (abnormal target innervations at muscle 6/7 and 12/13), SNa, and FasII-positive outer longitudinal CNS axons (Winberg et al., 1998b). Expressing ^{HA}PlexA in neurons significantly rescues these defects, while disrupting the enzymatically critical Arg residues (^{HA}PlexA^{RA}) for the RasGAP makes ^{HA}PlexA^{RA} unable to rescue the defects. n≥100 hemisegments (10 embryos)/genotype; Error bar: SEM; ***p<0.001, ****p<0.0001 by one-way ANOVA and Bonferroni post test.

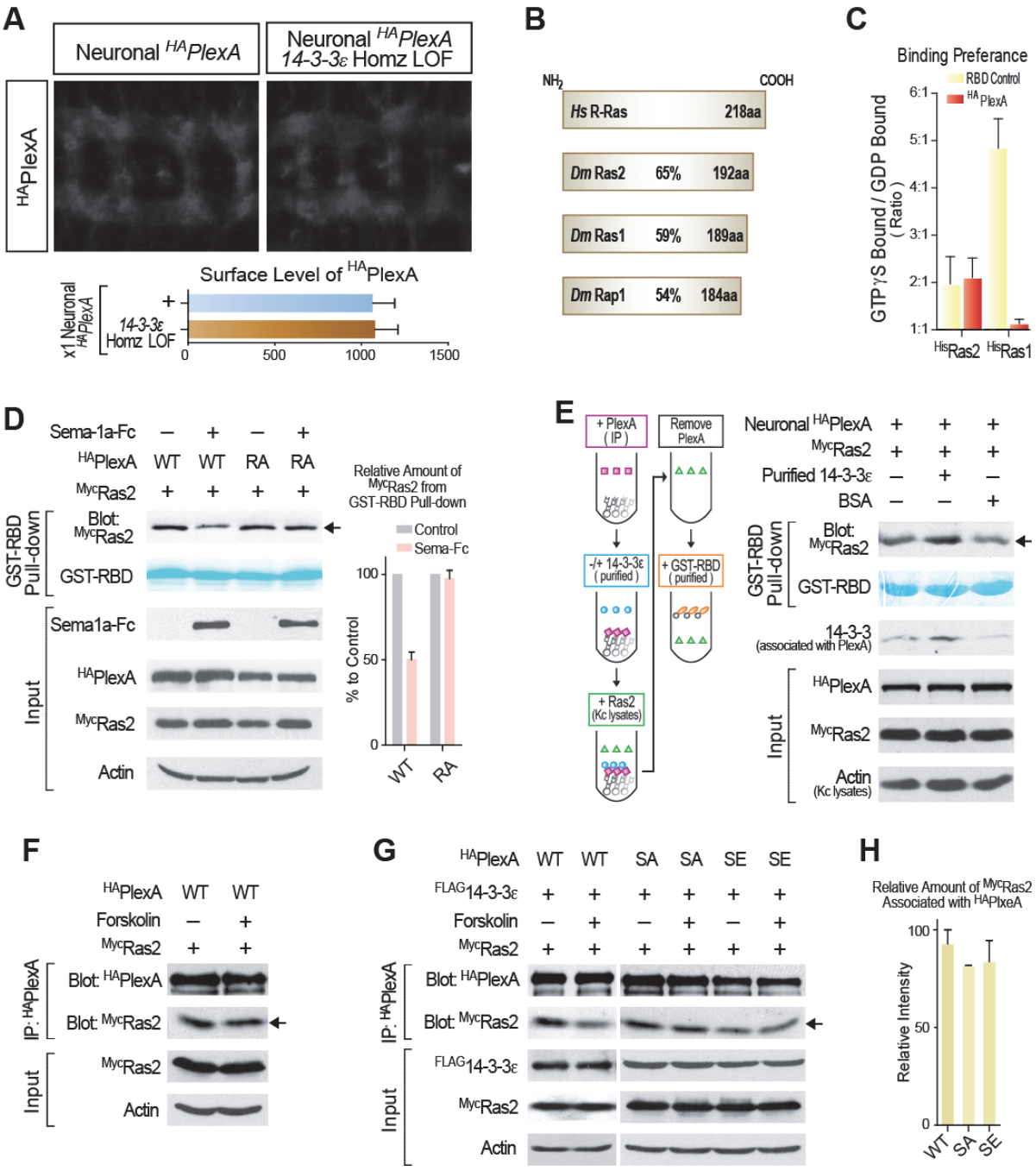

Figure S3.6. 14-3-3ε Antagonizes PlexA by Blocking PlexA RasGAP Function, Related to Figure 3.7. (**A**) Loss-of-function of *14-3-3ε* does not increase the surface expression of PlexA on axons. ^{HA}PlexA was expressed using the neuronal driver *ELAV-GAL4* in *wild type* or homozygous

LOF *14-3-3ε* embryos. Expression of ^{HA}PlexA on the surface of axons was examined as in Figures S5A-B and the expression level was not altered by LOF *14-3-3ε*. (**B**) Sequence comparison between Ras superfamily proteins indicates that Ras2 is most similar to Human R-Ras among different Ras family members in Drosophila. (**C**) ^{HA}PlexA preferentially interacts with ^{His}Ras2$^{GTP\gamma S}$. Interactions between PlexA and GTPγS or GDP-bound forms of Ras2 or Ras1 proteins were examined using neuronally expressed ^{HA}PlexA and purified ^{His}Ras proteins and results are presented as the ratio of the GTPγS-bound Ras versus the GDP-bound Ras that bound to ^{HA}Plexin. The Ras binding domain (RBD) of Raf was used as a control because of its preferential binding to GTP-bound Ras2 and Ras1 (Oinuma et al., 2004a). (**D-H**) Additional characterization of PlexA and 14-3-3ε. (**D**) Consistent with previous results using mammalian Plexins and other GAPs ((Bos et al., 2007; Oinuma et al., 2004a)), mutations to conserved Arg residues in the PlexA GAP domain alter RasGAP activity. PlexA RasGAP activity was examined by GST-RBD pull-down (the Ras binding domain (RBD) of Raf; see Figure S6C) to measure the amount of GTP-bound ^{Myc}Ras2 in Kc cells after transfection with either wild type (WT) or GAP mutant (RA) ^{HA}PlexA and ^{Myc}Ras2, and then subsequent activation of PlexA with Sema-1a-Fc conditioned media. The amount of GTP-bound ^{Myc}Ras2 was not decreased by ^{HA}PlexA^{RA}. (**E**) Association of 14-3-3ε with PlexA inhibits PlexA RasGAP activity. PlexA RasGAP activity was examined by GST-RBD pull-down to measure the amount of GTP-bound ^{Myc}Ras2 in Kc cell lysates after incubation of immunoprecipitated neuronal ^{HA}PlexA from embryonic lysates with Kc cell lysates expressing ^{Myc}Ras2. Pre-incubation of ^{HA}PlexA with purified 14-3-3ε increased the amount of ^{Myc}Ras2 bound to GST-RBD. The association ("pull-down") of 14-3-3ε with ^{HA}PlexA was also examined following removal of ^{HA}PlexA from the lysates. Note that some endogenous 14-3-3ε is present in these cells (and it was "pulled-down" by ^{HA}PlexA). (**F**) Forskolin treatment does not disrupt the interaction between ^{Myc}Ras2 and ^{HA}PlexA. ^{HA}PlexA was immunoprecipitated from Kc cells after transfection and Forskolin treatment, then the immunoprecipitated ^{HA}PlexA was incubated with Kc cell lystaes expressing ^{Myc}Ras2. (**G**) 14-3-3ε does not disrupt the interaction between ^{Myc}Ras2 and mutant ^{HA}PlexA proteins which do not interact with 14-3-3ε. Different ^{HA}PlexA proteins (see Figure 6A) were transfected along with ^{FLAG}14-3-3ε into Kc cells. Cells were then subjected to treatment with or without Forskolin and immunoprecipitation using an HA antibody. Immunoprecipitated Plexins were then incubated with Kc cell lysates expressing ^{Myc}Ras2. In contrast to wild type ^{HA}PlexA (WT), the interaction between ^{HA}PlexA^{SA} or ^{HA}PlexA^{SE} and ^{Myc}Ras2 was not altered by co-transfection with 14-3-3ε and Forskolin treatment. (**H**) ^{HA}PlexA (WT), ^{HA}PlexA^{SA}, and ^{HA}PlexA^{SE} show no significant differences in their ability to associate with ^{Myc}Ras2. Relative amount of ^{Myc}Ras2 bound to each mutant ^{HA}PlexA (as in Figure S6G) was normalized to the amount of immunoprecipitated ^{HA}Plexins and compared to that bound to wild type ^{HA}PlexA. Our results from these biochemical experiments are also supported by our genetic assays that reveal that altering Plexin Ras-GAP activity (the RA mutant) gives rise to very different effects on Plexin repulsion than do the Plexin A-14-3-3ε binding mutants S1794A and S1794E (Figure 6) – further indicating that the S1794A and S1794E mutants do not decrease Ras binding or Ras-GAP activity.

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