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Biochemical and Functional Characterization of Semaphorin6A-PlexinA Signaling in Zebrafish Eye Development

Riley St. Clair

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BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF
SEMAPHORIN6A-PLEXINA SIGNALING IN ZEBRAFISH EYE DEVELOPMENT

A Dissertation Presented

by

Riley St. Clair

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Neuroscience

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ABSTRACT

During embryonic development, cells respond to extracellular signals to establish proper tissue organization. Semaphorins (Semas) are a large class of secreted and transmembrane proteins that signal through Plexin (Plxn) receptors to guide migrating cells to their correct position and thus play critical roles in the development of various tissues including the nervous and cardiovascular systems. We have previously shown that Sema6A-PlxnA2 signaling is essential for visual system development, as decreasing endogenous Sema6A or PlxnA2 in zebrafish results in decreased cohesion of the early eye field, impaired retinal lamination, and smaller eye size. However, the molecular mechanisms governing these phenotypes are unknown. This dissertation describes the elucidation of functionally-relevant mechanisms of Sema6A-PlxnA signaling during eye development using biochemical and proteomic approaches in cell culture systems and the zebrafish as an in vivo vertebrate model of eye development.

We first describe our investigations on the receptor-proximal mechanisms of Sema6A-PlxnA signaling. The Src-family tyrosine kinase Fyn was known to bind to and phosphorylate PlxnA receptors. However, the specific sites of phosphorylation and their function were unknown. Using mass spectrometry, we identified highly-conserved, Fyn-induced PlxnA tyrosine phosphorylation sites. Mutation of these tyrosines to phenylalanine nearly eliminated Fyn-dependent PlxnA phosphorylation. Furthermore, unlike mRNA encoding wild type human PlxnA2, mRNA encoding the tyrosine-to-phenylalanine mutant PlxnA2 could not rescue the smaller eye size phenotype caused by endogenous PlxnA2 knockdown in zebrafish. This suggests that Fyn-dependent PlxnA2 phosphorylation is critical for proper vertebrate eye development.

Next, we report the discovery and functional characterization of a naturally-released soluble ectodomain of Sema6A (sSema6A). We show that sSema6A production is increased by PKC activity. The identification of several PKC-dependent phosphorylation sites in the intracellular region of Sema6A suggests a mechanism for PKC-dependent release of sSema6A. Importantly, we show that sSema6A is functional as it promotes the cohesion of zebrafish early eye field explants. This is the first report of a soluble ectodomain of the Sema6 class and suggests that Sema6A can have regulated, long-range signaling capacity in addition to its canonical contact-mediated functions.

Finally, we present our findings characterizing the role in eye development of CRMP2, a downstream effector of Sema-Plxn signaling. CRMP2 is known to be critical for lamination of the cerebral cortex, leading us to hypothesize that CRMP2 could also be involved in the lamination of the retina. Using morpholino-based knockdown of endogenous zebrafish Crmp2, we show that Crmp2 has a critical function in visual system development. Crmp2 knockdown results in smaller eye size, impaired retinal lamination and a weakened optic tract.

Together, this dissertation describes important novel Sema6A-PlxnA signaling mechanisms and places them in the context of vertebrate eye development.
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CHAPTER 1: INTRODUCTION

1.1. Dissertation Outline

This dissertation investigates the functionally-relevant mechanisms of Semaphorin-Plexin (Sema-Plxn) signaling in zebrafish eye development. Chapter 1 gives a literature review, beginning with a historical perspective, to introduce Semas as neuronal guidance cues and describes what is currently known in the field of Sema functioning and signal transduction. This chapter includes a mini-review on the phenomena of Sema ectodomain release from the cell surface. Chapter 2 characterizes the receptor proximal mechanisms of Sema signaling by describing phosphorylation of the Sema6A receptors PlxnA1 and PlxnA2. Chapter 3 characterizes a novel soluble Sema6A variant that is functional in early eye development. Chapter 4 investigates Sema6A-PlxnA downstream signaling by studying the microtubule-associated protein CRMP2 and reveals a novel CRMP2 role in eye development. A summary of the major research findings is found in Chapter 5, in addition to a critical analysis of the results. This chapter also includes a detailed discussion on the remaining questions and proposes future experiments to address them.

1.2. Significance of Study

During embryonic development, cellular responses to extracellular cues are critical, but not yet fully understood, in establishing the trillions of synaptic connections that form a functional nervous system. Studies have identified important signaling
molecules involved in establishing neuronal positioning and axonal pathfinding, critical processes for proper cortical brain development [1]. However, less is known about the signaling events required for correct development of non-cortical areas of the brain, such as the retina, as well as the functional consequences of disrupting signaling mechanisms involved in retinal development.

We have previously shown that one such signaling pathway, Semaphorin6A-PlexinA2 (Sema6A-PlxnA2), is critical for vertebrate eye development [2]. Current working models propose that the repulsive guidance cue Sema6A binds to PlxnA receptors on the growth cone of migrating neurons, which subsequently activates the tyrosine kinase Fyn. This initiates signaling cascades that ultimately regulate cellular adhesion and cytoskeletal dynamics, crucial events that navigate the axon to its proper synaptic partner. However, the majority of studies have investigated this signaling cascade out of the context of Sema6A binding. Furthermore, little is known about the in vivo molecular mechanisms underlying this critical signaling pathway. This dissertation aimed to elucidate the functionally-relevant mechanisms of Sema6A-PlxnA signaling during eye development.

Using biochemical and proteomic approaches in cell culture systems and the zebrafish as an in vivo model, the reports herein describe and characterize novel molecular mechanisms of Sema6A-PlxnA signaling and their functional significance in vertebrate eye development.
1.3. Extracellular Cues Guide the Migrating Axon

Proper nervous system development depends on correct cellular migration and synaptogenesis. Immature neurons are guided to their final location and synaptic targets by extracellular cues in a remarkably accurate fashion. The growth cone, a dynamic structure at the distal end of the axon, has the critical function to detect and respond to molecules in the environment. The growth cone is made up of actin and microtubule filaments with long extensions called filapodia that can extend 50 – 75 μm to explore the extracellular environment [3]. The growth cone has the machinery necessary to translate the extracellular signal to a change in the cytoskeleton, which guides the axon to its ultimate synaptic target by moving forward, collapsing, or changing direction. This complex cellular machinery includes transmembrane receptors, adaptor proteins, kinases and cytoskeletal regulatory proteins [4]. How the axon responds to extracellular cues and integrates these signals is critical in determining the ultimate synaptic location.

Ramón y Cajal first hypothesized that axon guidance is mediated by extracellular factors [5]. He applied the chemotaxis theory to migrating neurons and suggested that positive and negative chemotaxis could take place in the retina and other brain regions, and that these chemicals were secreted by other cells [5]. It was not until over fifty years later that direct evidence arose that gave credence to Ramón y Cajal’s hypothesis. These studies identified long-range cues as Ramón y Cajal suggested, as well as contact-mediated signals critical to axonal guidance.

Evidence of local, contact-mediated cues was found with the identification of
pioneer axons in the developing limb of grasshopper embryos. Bate (1976) observed a pair of pioneer neurons that establish peripheral nerve pathways [6]. Interestingly, these neurons did not take the shortest course to their target. Instead, they made distinct turns along the way after making contact with other cells. This seminal paper had two main conclusions: 1) something is guiding the pioneer axons to their correct target via repulsion from incorrect areas and 2) this guidance cue is acting at the local level. These findings triggered the field to identify these local guidance molecules. The next several decades resulted in the identification of numerous membrane-bound and secreted molecules, expressed in different locations and at different times, that are critical for axonal pathfinding.

Netrins were the first guidance molecules to be identified. In the 1970’s, Brenner studied the genome of *C. elegans* and screened and mapped the “uncoordinated” (*unc*) mutants [7]. The *unc* mutants affected movement and Brenner hypothesized that the corresponding genes have a role in the nervous system and he proposed that many of the *unc* mutants affected the formation of proper nervous system connections. For example, the *unc-5* mutant lacks the dorsal nerve cord [7]. The *unc-5* gene was further described and found to be a receptor for *unc-6*, and both are necessary for axonal guidance of dorsal migrating neurons in *C. elegans* [8]. The mammalian homologues to *unc-6*, netrin-1 and netrin-2, were characterized *in vitro* and *in vivo* and were found to promote axonal outgrowth of commissural axons [9, 10]. Netrins have since been shown to be secreted molecules that act bifunctionally: for example, as an attractant for retinal ganglion cells and as a repellant for trochlear motor neurons [11].
The bifunctionality of netrins occurs due to differential receptor expression. When netrin binds to the DCC receptor, the growth cone displays attractive growth. This is due to adaptor proteins on the DCC receptor that regulate the Rho family GTPases Cdc42 and Rac1, which promote actin nucleation and branching by activating N-WASP and Arp2/3 [12]. Conversely, if the growth cone expresses the unc-5 receptor, netrin binding will cause repulsion of the growth cone. The differential expression of receptors gives a relatively few number of guidance cues the flexibility to elicit multiple growth cone behaviors, including attraction and repulsion.

Slits are another family of secreted guidance cues that are involved in neuronal migration and axonal pathfinding. They act primarily as repulsive signals through the Robo receptor. Slit-Robo signaling inhibits Cdc42 through the GTPase activating protein (GAP) srGAP resulting in growth cone repulsion [12]. The repulsive role of slits is best characterized during midline crossing of commissural axons. Interestingly, Robo receptor expression levels vary as the growth cone of commissural axons moves through the extracellular environment. Robo is expressed in low levels as commissural axons cross the midline. Subsequent up-regulation of the Robo receptor prevents the commissural axons from incorrectly re-crossing the midline [13]. This illustrates the importance of spatiotemporal regulation of guidance cue receptors to achieve accurate axonal pathfinding.

Slits are also involved in establishing the location of the optic chiasm and directing retinal ganglion cells across the midline [14]. The retinal ganglion cells in
mutant mice lacking the *slit1* and *slit2* genes exhibit abnormal axon guidance and an ectopic optic chiasm develops [14]. This suggests that the repulsive signaling of slits provides a barrier for the optic nerve, keeping the retinal ganglion cells from aberrantly migrating along the pathfinding route.

In addition to long-range guidance cues such as netrins and slits, there are membrane-bound molecules that act via cell-cell contact, including ephrins. Ephrins are GPI-linked (ephrinA class) or transmembrane (ephrinB class) molecules. Ephrin signaling is involved in many developmental processes, including tissue organization, axon formation and growth cone guidance [15]. Ephrins regulate these processes via repulsion to maintain tissue patterns. For example, the developing hindbrain forms segmentations, rhombomeres, which later become cranial nerve origins [16]. The cells in adjacent rhombomeres must remain segmented in order for correct hindbrain development and this segmentation is facilitated by ephrin and Eph receptor expression. Rhombomere 5 expresses Eph receptors, while the adjacent rhombomeres, 4 and 6, express ephrin [17]. Thus, ephrin signaling is critical in tissue organization during development.

Ephrin signaling occurs when ephrins bind to Eph receptors, which are receptor tyrosine kinases. Eph receptors dimerize, autophosphorylate, and activate upon ligand binding. Eph receptor activation recruits adaptor proteins to transduce ephrin signaling. Ephexin is a unique GEF that, in the absence of Ephrins, activates the Rho-family of GTPases RhoA, Rac1 and Cdc42 [18]. However, upon ligand binding, Ephexin activity
toward RhoA is increased, while GEF activity toward Rac1 and Cdc42 is inhibited [18]. RhoA activation and Rac1/Cdc42 inhibition ultimately leads to actin depolarization and growth cone collapse. Interestingly, as the EphrinB molecules are transmembrane proteins, bidirectional signaling can occur [19]. Reverse signaling is transduced via Ephrin phosphorylation-dependent and/or PDZ-dependent protein recruitment [20]. Upon Eph binding, Src-family kinases (SFKs) become activated and mediate EphrinB tyrosine phosphorylation [21]. This subsequently recruits the adaptor protein Grb4 via its SH2 domain and initiates Pak1-mediated cytoskeletal reorganization [17]. PDZ-domain containing proteins, such as syntenin, can also be recruited to EphrinB proteins via a PDZ-binding motif on the C-terminus of EphrinB to initiate signaling and mediate synapse development [22, 23]. Bidirectional signaling, therefore, is an energy-efficient mechanism that enables tissues to produce multiple cellular outcomes from one ligand-receptor interaction.

Semaphorins (Semas) are another large family of guidance cues that are critical for nervous system development. There are currently 28 known Sema molecules, expressed in invertebrates and vertebrates (Figure 1.1). Semas are membrane-bound, transmembrane or secreted molecules that typically signal through Plexin (Plxn) receptors to regulate axonal pathfinding.

The Sema3A-PlxnA signaling pathway is the best characterized. PlxnA contains a GAP domain and influences actin and integrin dynamics through small GTPases [24]. Additionally, the cytosolic tyrosine kinase, Fyn, binds and phosphorylates PlxnA
receptors [25], which may transduce Sema3-PlxnA signaling. Fyn can also phosphorylate and activate the serine/threonine kinase Cdk5 that ultimately regulates microtubule dynamics [12]. Cdk5 phosphorylates the family of microtubule associated proteins collapsin response mediator proteins (CRMPs) [26, 27]. This initial phosphorylation event primes GSK3β to processively phosphorylate CRMPs, leading to a decrease in microtubule affinity and destabilization of the microtubule, ultimately resulting in growth cone collapse [26].

Although Semas are best known for their roles in axonal guidance, they also function in cellular processes such as immune system activation, proliferation, differentiation and angiogenesis [28, 29]. The various cellular functions of Semas are due to binding to distinct receptors [30-32]. Semas thus have diverse roles in development and Sema signaling can be transduced through combinations of multiple ligands and receptors on various cell types.

Identifying the cellular players and mechanisms involved in these signaling pathways is crucial to advancing our understanding of neuronal migration and represents one long-term research goal of this dissertation work. As Sema pathways play a critical role in axonal guidance during brain and eye development, the remainder of the chapter focuses on Sema signaling, with an emphasis on the major signaling proteins involved and the currently known molecular mechanisms underlying Sema signal transduction.
1.4. Semaphorin Signaling

1.4.1. The Semaphorin Family of Guidance Cues

The first Sema (Sema-1a) was characterized in the early 1990’s as an axon guidance cue in the developing limb bud of the grasshopper [33]. Since then, over twenty Semas have been discovered. These molecules are categorized into eight classes, based on C-terminal sequence homology (Figure 1.1) [34]. Sema-1 and Sema-2 are found exclusively in invertebrates, while classes 3-6 are expressed in vertebrates. There is an additional class found only in viruses (SemaV). Interestingly, while there are only four known invertebrate Semas, there are 22 Semas expressed in vertebrates [35], suggesting that Semas have critical and diverse roles in vertebrate development.

As Semas critically regulate the cytoskeleton, they have crucial roles for many cellular processes that depend on cytoskeletal dynamics including migration, proliferation, and differentiation. Thus, many Semas have overlapping roles in the nervous system, endothelial system, and immune system [29]. Nonetheless, different Sema classes have predominant roles in specific tissues, as summarized in Table 1.1. While typically repulsive, Semas act as inhibitory or permissive cues in neuronal and endothelial cell migration [29, 30]. The “immune Semas,” including Sema classes 4, 5 and 7, are involved in regulating immune cell responses [36].

The defining feature of all Semas is the presence of the semaphorin (sema) domain. The sema domain is an approximately 500 amino acid extracellular domain on
Figure 1.1. The Invertebrate and Vertebrate Semaphorins are Categorized based on Sequence Homology. A phylogenetic tree illustrating the similarities among the 28 Semas, classified into 8 classes based on sequence similarities. The letters in parentheses indicate the animal of the sequence used in the alignment. D, Drosophila; M, mouse; V, viral; Z, zebrafish (Sema4E has not yet been identified in mammals). Re-printed from Biomed Central (BMC) Open Access Publishing from Yazdani & Terman 2006. Authors used ClustalW software to generate the phylogenetic tree.
Table 1.1. Semas are a large class of invertebrate and vertebrate proteins that play roles in numerous systems. This table lists the 8 classes of Semas and each of the class members. Semas classes are expressed throughout embryonic development and the major systems are described in which the functional role of each Sema class has been highly characterized.

<table>
<thead>
<tr>
<th>Class</th>
<th>Invertebrate</th>
<th>Vertebrate</th>
<th>Viral</th>
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<tr>
<td></td>
<td>1</td>
<td>2</td>
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<td>3F</td>
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<td>3G</td>
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Major Systems

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the N-terminus [33]. It is a variant of the seven-blade β-propeller, composed of twisted β-sheets in a circular arrangement, and plays a role in protein-protein interactions [37]. The sema domain, although necessary for Sema activity, is not sufficient. Koppel & Raper (1998) found that Sema3A must be a dimer in order to have collapsing activity in embryonic chicken dorsal root ganglion explants [38]. It is now known that all Sema family members, secreted and membrane-bound, can form homodimers. The sema domain mediates specificity and receptor binding and is necessary for binding to Plxn receptors [37].

1.4.2. Plexins are the Major Semaphorin Receptors

Plexins are transmembrane proteins that are highly expressed in the developing nervous system [39]. There are nine mammalian Plxns, classified into four families, PlxnA-D [40] (Figure 1.2). PlxnA family members are the most widely studied and are expressed in the developing spinal cord, cortex, and retina [41]. PlxnA molecules are also expressed in the hippocampus and regulate guidance of mossy fibers to their synaptic targets, pyramidal cells [42]. PlxnA2 is the most common PlxnA in the developing cortex and is involved in the lamination and axonal guidance of cortical neurons [43]. While PlxnA molecules appear to be most highly expressed in nervous tissue, PlxnB-D family members are found primarily in bone and immune cells, and knockout mouse studies indicate these molecules are critical for proper immune response and bone development [44].
Figure 1.2. The Structural Domains of Vertebrate Semaphorins and their Receptors. Semaphorins are a large family of secreted, transmembrane, and GPI-linked molecules. The vertebrate secreted Sema class, Sema3, requires neuropilins as coreceptors. All Semas are characterized by an extracellular Sema and PSI domain and are classified into 8 classes based on the presence of other domains, including Ig-like domains and thrombospondin repeats. There are nine Plxn receptors characterized by an extracellular sema domain, PSI domain, and a glycine-proline-rich region and contain a split GAP domain in the intracellular region. Arrows indicate the Sema proteins that bind to the specified Plxn receptor. Sema: Semaphorin domain; PSI: plexin-semaphorin-integrin domain; G-P: glycine-proline rich domain; Ig: Immunoglobulin-like domain; CUB: complement binding domain; FV/FVIII: FV/FVIII coagulation factor-like domain; MAM: Meprin-A5-Mu domain. Re-printed with permission from Springer Nature Publishing from Negishi & Oinuma 2015 under license number 4510920424543. Adapted and modified by Sarah E. Emerson and Riley M. St. Clair.
Plxn family members have a highly conserved cytoplasmic domain, yet these evolutionary conserved molecules have little sequence similarity with other proteins [40]. This may suggest that novel cellular players or novel signaling mechanisms are involved in these pathways. Therefore, many cellular signaling biologists and developmental neuroscientists have been interested in elucidating the Sema-Plxn signaling pathways.

The Sema-Plxn structural interaction has been well characterized through crystal structure analysis and in vitro binding assays. Two independent research groups concurrently resolved the first structure of the mouse extracellular Sema6A-PlxnA2 complex as well as the unbound molecules [45, 46]. When unbound, Sema6A is in a “face-to-face” homodimer, which remains in this conformation upon binding to PlxnA2 (Figure 1.3A, C) [45, 46]. In contrast, unbound PlxnA2 is in an inhibitory “head-on” conformation (Figure 1.3B) [45]. Upon Sema6A binding, the PlxnA2 monomers are dissociated from each other and independently bind to the Sema6A homodimer via the sema domains of both Sema6A and PlxnA2 [45, 46]. Interestingly, the same binding interface is used for the PlxnA2-PlxnA2 interaction and the Sema6A-bound complex, suggesting that PlxnA2 undergoes a “partner exchange” to bind Sema6A [45]. Mutating residues in the interface disrupt Sema6A-PlxnA2 binding and thus no longer induce DRG growth cone collapse [45, 46].

While Plxns are the major signal transducing receptor for transmembrane Semas, class 3 secreted Semas require Neuropilin (Np) co-receptors [46, 47]. Nps are a small family of transmembrane molecules made up of Np1 and Np2 [47]. While Nps are
Figure 1.3. Crystal Structures of Sema-Plxn Signaling Complexes. Crystal structures of unbound and complexed Sema6A and PlxnA2 ectodomains, each with a cartoon schematic inset. A. Sema6A ectodomains form a “face-to-face” homodimer through their sema domains. B. PlxnA2 sema domains form a “head-on” homodimer. C. The Sema6A-PlxnA2 complex involves the two independent PlxnA2 molecules binding to the Sema6A homodimer. D. The Sema3A-PlxnA2-Np1 complex indicates that the Sema-Plxn binding interface is conserved in secreted Sema classes, while the Np1 co-receptor acts as a stabilizing bridge between Sema3A and PlxnA2 sema domains. Adapted from and reprinted with permission from Springer Nature Publishing from Nogi et al. 2010 (Figures 1.3A-C) and Janssen et al. 2012 (Figure 1.3D) under license numbers 4560951339161 and 4560960094307, respectively.
primarily expressed during neuronal development, they are also expressed during the development of non-neuronal tissues such as the cardiovascular system [47, 48]. It is now known that Nps can bind to various Plxn receptors to enable binding of various Sema3 class members [47]. Interestingly, Np1 was first identified as a necessary but not sufficient component of Sema3A-mediated repulsion [47-49]. Indeed, the transmembrane and short cytoplasmic tail of Np1 are not necessary for Sema3A binding or activity [50]. However, truncation mutants of PlxnA proteins lacking the cytoplasmic domain inhibits Sema3A-mediated growth cone collapse [47]. Together, these studies reveal that Plxns and Nps form a receptor complex for secreted Sema3 proteins, with Plxns mediating signal transduction.

Crystal structure analysis gives insight into the role of Nps in the Sema3 receptor complex. Mouse Sema3A-PlxnA2-Np1 was resolved to illustrate that Np1 forms a stabilizing bridge between the sema domains of Sema3A and PlxnA2 (Figure 1.3D) [51]. Without Np1, Sema3A has a weak affinity for PlxnA2 whereas Np1 can directly bind to both Sema3A and PlxnA2 in in vitro binding assays [51]. This suggests that Np1 and PlxnA2 can form complexes at the cell surface independent of Sema3A ligand binding. Indeed, the expression of Np co-receptors could confer Sema specificity to the same Plxn receptor. For example, PlxnA1 or PlxnA2 expression allows cells to be responsive to Sema6A and not Sema3A, while PlxnA1 or PlxnA2 co-expressed with Np1 enables Sema3A binding and responsiveness [47, 51, 52]. This difference occurs due to the reduced affinity of Sema3A for PlxnA2, while Sema6A has a strong affinity for PlxnA2 without the Np1 co-receptor [51]. The weak Sema3A-PlxnA2 interaction may
stem from subtle conformational differences between Sema3A and Sema6A: two extrusion loops of the Sema3A sema domain are larger than in the homologous extrusions of Sema6A, which enables Np1 binding [51]. Furthermore, amino acid differences in the Np1-binding interface result in a charge differences that could further support Sema3A-Np1 binding while inhibiting Sema6A-Np1 binding [51]. Importantly, the Sema3A-PlxnA2 binding interface is conserved in Np-independent Sema-Plxn interactions, such as Sema6A-PlxnA2 [51]. This suggests a conserved and canonical Sema-Plxn interaction across secreted and transmembrane Semas.

In addition to the Sema-Plxn interaction, the cellular players involved in PlxnA signaling have been proposed based on in vitro and explant studies as well as mouse genetic experiments. Signaling pathways induced by guidance molecules ultimately regulate the cytoskeleton. Sema signaling can regulate integrin and cytoskeletal dynamics, causing depolymerization of actin filaments, destabilization of microtubules and a decrease in focal adhesions, ultimately resulting in growth cone collapse.

**1.4.3. Sema-Plxn Signaling Regulates Adhesion and Cytoskeletal Dynamics**

By regulating integrin and cytoskeletal dynamics, Semas govern cellular morphology and migration. Interestingly, Sema7A can bind directly to integrin receptors through a unique RGD integrin-binding motif to play roles in axonal guidance, immune cell activation and endothelial cell migration [32, 53, 54]. Classically however, Semas indirectly influence integrin signaling by regulating the activity of small GTPases as all
Plxns contain a highly conserved intracellular GAP domain [55, 56]. This GAP domain is homologous to Ras GAPs in both sequence and structure and necessary for Plxn-mediated growth cone collapse [24, 57]. The intracellular region is comprised of two split GAP domains surrounding a Rho GTPase-binding domain (RBD), which is in an inactive conformation until ligand binding [45, 46]. Sema binding induces Plxn dimerization, however Rho GTPases such as Rac1 or Rnd1, must bind to the RBD for Plxn GAP activation to occur [55]. Interestingly, while the Ras-related proteins R-Ras and M-Ras have been shown to bind to the Plxn GAP domain in vitro, Plxn GAP activity is specific for the Ras homologue Rap [55]. Indeed, Rap inactivation is necessary for Sema3A-mediated growth cone collapse [55].

By regulating Rap activity, Plxn signaling can influence integrin-mediated adhesion and actin dynamics. When active, GTP-bound Rap1 binds the adaptor protein RIAM which acts as a scaffold for the recruitment of talin to the cytoplasmic domain of integrins [58] (Figure 1.4). This interaction increases integrin affinity to the extracellular matrix promoting adhesion [59]. Thus, Plxn-mediated Rap inhibition leads to de-adhesion by decreasing integrin-containing focal adhesions. Independent of Plxn, Rap1 inhibition can be opposed by C3G, a Src-Crk-dependent Rap1 GEF, which activates Rap1 leading to an increase in integrin-mediated adhesion [60]. Together, this illustrates the importance of a tightly regulated balance of GAPs and GEFs to regulate the activity of small GTPases to govern cellular adhesion and migration.

Plxn Rap GAP activity also regulates actin dynamics through multiple players
Figure 1.4. Working model of Sema-mediated regulation of cytoskeletal and integrin dynamics. Known and proposed effector proteins downstream of Plxn GAP activity or Plxn interactors. Proteins in green boxes have been shown to be involved in Sema-induced growth cone collapse in cell culture or explant assays. Proteins in black boxes have not yet been linked to Sema signaling, however are hypothesized to be downstream based on known protein interactions. The primary literature that elucidated the given proteins’ activation, interaction, or Sema-mediated function is included.
Rap has been shown to inhibit RhoA activity, thus Plxn GAP activity activates Rho [61]. Indeed, RhoA activation occurs downstream of Sema3A and is necessary for Sema3A-mediated growth cone collapse [61, 62]. RhoA-mediated collapse may be through ROCK/LIMK regulation of cofilin as cofilin inactivation is necessary for Sema3A-induced growth cone collapse of dorsal root ganglion cells [63]. When active, cofilin severs actin filaments and increasing the number of barbed ends, leading to increased actin polymerization and cellular motility [64, 65]. LIMK-mediated phosphorylation of cofilin results in decreased cofilin activity, reduced actin turnover and polymerization, and an inhibition of migration [64]. Rap inactivation also inhibits Cdc42-mediated WASP/Arp2/3 actin nucleation [66]. Thus, PlxnA GAP activity can mediate cytoskeletal dynamics and adhesion through small GTPases and kinases.

While extensive research has focused on actin regulation, the mechanistic details of Sema-mediated microtubule regulation are still poorly understood. At present, the CRMP family of microtubule associated proteins are the major cellular players implicated in Sema-induced microtubule destabilization. The tyrosine kinase Fyn associates with PlxnA receptors [25] and is thought to become active through autophosphorylation upon Sema binding. Fyn subsequently activates Cdk5 leading to Cdk5- and GSK3β-mediated CRMP phosphorylation [25, 26, 67] (Figure 1.4). However, it is currently unknown if Plxn GAP activity plays a role in this pathway. The function and mechanisms of CRMPs are discussed in detail in Section 1.5.

While much molecular work has been carried out in the context of the canonical
Sema, Sema3A, it is currently unknown if the downstream mechanisms are similar in Sema6A signaling. It is likely, however, that signaling is conserved as both Sema6A and Sema3A utilize overlapping PlxnA receptors (Figure 1.2). We have previously shown that Sema6A and PlxnA2 are expressed in the developing zebrafish visual system and are critical for proper visual system development [2, 68]. Yet, the molecular mechanisms governing Sema6A-PlxnA2-mediated functions in eye development are currently unknown and represent the major objective of this dissertation work.

1.4.4. Sema6A-PlxnA Signaling

Sema6A is highly expressed in developing brain, spinal cord, ovary and kidneys [69]. Mouse in situ hybridization assays and Northern blots show Sema6A mRNA expression in the developing cortex (layers IV and VI) and deep brain structures including thalamus, hypothalamus, and amygdala as well as the retina and olfactory system, cerebellum, and motor nuclei of the brain stem [70]. It is also expressed in the developing mouse ventral spinal cord, dorsal root ganglion cells, gastrointestinal tract, kidneys, skeletal muscle and lung [71]. In contrast, the close family member Sema6B is primarily expressed in developing muscles and spinal cord and, unlike many Semas, ubiquitously expressed in adult brain, heart, and lung [72].

Sema6AΔ mice studies have shown that the repulsive activity of Sema6A is critical for the in vivo guidance of migrating granule cells and corticospinal tract axons as well as cortical lamination [73-75]. In the hippocampus, PlxnA4-expressing mossy fibers synapse onto CA3 pyramidal neurons which express Sema6A throughout their soma and
projections [42]. Interestingly, cis expression of PlxnA2 with Sema6A in a specific pyramidal cell region antagonizes Sema6A-PlxnA4 signaling, resulting in a permissive environment for mossy fiber outgrowth and synapse formation [42]. This phenomenon, known as cis-inhibition, allows for accurate and precise targeting of mossy fiber-pyramidal cell synapse formation.

To determine the cellular effects and downstream mechanisms of Sema6A signaling, biochemical studies utilize a Sema6A-FC recombinant chimeric protein, of which the ectodomain is fused to the FC portion of IgG. This enables Sema6A to be secreted from the cell in a dimer [76], overcoming the technical obstacles of stimulating cells with a transmembrane protein. Similar to the hippocampus, cis-inhibition modulates Sema6A responsiveness of sympathetic and DRG neurons, both of which express PlxnA4 [77]. Neuron outgrowth of sympathetic neurons is impeded by Sema6A-FC in culture, whereas Sema6A is co-expressed with PlxnA4 on DRGs yielding DRG’s Sema6A-resistant [77].

Zebrafish and mouse expression data (from Zfin and Mouse Genomics Informatics, respectively) indicate that Sema6A is expressed in zebrafish and mouse retinas. Similarly, we have previously shown that Sema6A is expressed throughout early and late zebrafish eye development [78]. As retinal ganglion cells do not respond to Sema3A [79], this could indicate that Sema6A-PlxnA signaling is the major Sema pathway involved in eye development. Indeed, Sema6A is critical for retina lamination [80]. Sema6A is highly expressed in the ON sublayer of the inner plexiform layer
influencing neurite projection of ON retinal cells, which are excited by an increase in light [80]. PlxnA4-expressing OFF bipolar cells, excited by a decrease in light, are repelled from the ON sublayer to stratify the retina [80]. In contrast, OFF retinal ganglion cells do not express PlxnA4 and their dendrites can pass through the Sema6A-expressing ON layer to synapse with the appropriate bipolar cells in the OFF layer [80]. Similarly, Sema6A mediates synaptic targeting of amacrine cells through PlxnA2 [81]. PlxnA2-expressing OFF amacrine cells are repelled from entering the Sema6A-containing ON layer while ON amacrine cells do not express PlxnA2 and can thus migrate into the ON layer.

Interestingly, we have previously found a novel function of Sema6A-PlxnA2 signaling in zebrafish eye development. When Sema6A or PlxnA2 expression is decreased using antisense morpholino oligonucleotides (MO), the cohesion of the early eye field is impaired (Figure 1.5). Intriguingly, this phenotype occurs before retina precursor cells have differentiated and have not yet begun neurite outgrowth. Additionally, Sema6A or PlxnA2 MO results in a decrease in proliferative retinal precursor cells and a subsequent decrease in eye size later in development (Figure 1.5). These results suggest novel functions of Sema6A and PlxnA2, although the molecular mechanisms underlying these phenotypes are unknown.

1.4.5. Sema6 members act as receptors for reverse signaling
Figure 1.5. Sema6A and PlxnA2 are critical for zebrafish eye development. Endogenous Sema6A or PlxnA2 were decreased by injecting antisense morpholino oligonucleotides (MO) at the 1-cell stage. A-F. At the 18 somite stage, during early eye field migration, \textit{rx3}:GFP zebrafish expressing GFP in retinal precursor cells show ectopic cells when Sema6A or PlxnA2 is decreased. Proliferative cells were stained using pHH3 immunofluorescence, as shown in red and quantified in L. G-K. Brightfield images at 48 hours post fertilization show Sema6A or PlxnA2 MO results in smaller eye size, quantified in M. These phenotypes are specific to Sema6A or PlxnA2 as co-injecting WT mRNA constructs rescues the phenotypes. Re-printed with permission from Developmental Dynamics Journal (John Wiley and Sons Publishing) from Emerson et al. (2017) under license number 4510941106189.
Since the initial discovery of transmembrane Semas, scientists have been hypothesizing that they act as ligands, receptors, or both. However, most transmembrane Semas lack significant cytoplasmic domains [72], with the exception of the Sema6 class. Indeed, the first evidence of Sema6 class members exhibiting reverse signaling came from the discovery of Sema6B in mouse in 1997 [72]. Sequencing mouse Sema6B revealed proline-rich regions that are the preferred motifs of SH3 domains and \textit{in vitro} pull-down assays using GST-SH3 domains of various proteins show strong c-Src binding as well as nck, cAbl, and rasGAP binding [72]. Further analysis in cell culture systems shows that Sema6B co-IPs with c-Src and that this binding occurs through the Sema6B cytoplasmic domain at a c-Src SH3 binding consensus sequence, RXXPXXP [72].

Sequence analysis of the Sema6A cytoplasmic domain indicates a zyxin-like binding domain [70]. Zyxin is a proline-rich protein that regulates actin dynamics by binding to Ena/VASP proteins [82]. Thus, Klostermann et al. [70] first hypothesized that retrograde or reverse signaling can occur through the Sema6A cytoplasmic domain to regulate the actin cytoskeleton. Co-immunoprecipitation and pull-down assays in cell culture systems showed that the Ena/VASP family member EVL (Ena/VASP-like protein) can directly bind to the Sema6A C-terminus at an Ena/VASP preferred consensus binding motif DVPPKP (amino acid positions 1010-1015) [70]. Mena (mammalian enabled) and the tyrosine kinase Abl can also interact with Sema6A, with Abl being recruited to a specific region when stimulated with PlxnA2, similar to the PlxnA1 ligand-dependent Abl binding of Sema6D [83, 84].
Functionally, Sema6A reverse signaling maintains aggregation of cerebellular granule cell explants and inhibits neurite outgrowth and branching [83]. Mechanistically, PlxnA2 binding to Sema6A is thought to induce clustering of Sema6A, as forcing Sema6A intracellular domains to multimerize causes a decrease in neurite length similar to PlxnA2 stimulation [83]. Furthermore, Abl is necessary for Sema6A reverse signaling-mediated neurite outgrowth inhibition [83]. Sema6D has also been shown to be capable of reverse signaling in PlxnA1-mediated myocardial cell migration during heart development [84]. Additionally, the invertebrate Sema6 orthologue Sema-1a exhibits reverse signaling through the invertebrate PlxnA orthologue Plex-A, to mediate photoreceptor and olfactory neuron axon migration [84, 85].

As reverse signaling is conserved throughout invertebrates and vertebrates, cells may have developed ways to regulate the signaling direction. Interestingly, ectodomain release of transmembrane Semas has been illustrated in numerous cell types and may represent one mechanism of inhibiting reverse signaling. The following section contains a mini-review of what is currently known of the functions and mechanisms of Sema ectodomain release.

1.5. Soluble Isoforms of Transmembrane Semas

Semas are secreted and membrane-bound molecules classically acting as axonal guidance cues in nervous system development, although it is now widely appreciated that they have essential functions in the development of many systems including
cardiovascular, endothelial, and immune systems. While secreted forms enable diffusible or systemic roles, membrane-bound Semas mediate contact-dependent signaling. Interestingly, functional soluble ectodomains have been discovered in many of the vertebrate membrane-bound classes. While many such soluble Semas (sSemas) were first discovered to have critical roles regulating immune responses, emerging evidence shows that sSemas can govern axon guidance and endothelial cell migration (Table 1.2). This review highlights the discovery and function of sSema molecules as well as what is currently known about the mechanisms generating Sema ectodomain release. The potential roles and actions of Sema ectodomain shedding are discussed.

1.5.1. Sema4D

Sema4D is critical for immune and vasculature system function, playing roles in T-cell dependent antibody production, T and B cell proliferation, B cell aggregation, platelet activation, and endothelial cell migration [86-90]. Mouse genetic studies reveal Sema4D to be critical for T cell differentiation and the humoral immune response in addition to playing a role in arterial occlusion after vascular injury [88, 90, 91]. Sema4D signals through its low affinity receptor CD72 to mediate functions in lymphocytes and via its high affinity receptor PlxnB1 on endothelial cells [92, 93]. While Sema4D is predominantly expressed in hematopoietic tissue, Northern blot analysis indicates high expression in human skeletal muscle and lower levels in the brain, kidney, and small intestine [87]. Indeed, Sema4D has been shown to induce growth cone collapse of hippocampal neurons through PTEN activation by the R-Ras GAP activity of PlxnB1
<table>
<thead>
<tr>
<th>Transmembrane or Membrane-Bound Sema</th>
<th>Soluble Ectodomain?</th>
<th>Function of soluble ectodomain</th>
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<tbody>
<tr>
<td>Sema1 Sema-1a (invertebrate)</td>
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<tr>
<td>Sema-1b (invertebrate)</td>
<td>-</td>
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<tr>
<td>Sema4 Sema4A</td>
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<tr>
<td>Sema4C</td>
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<tr>
<td>Sema4D</td>
<td>Yes</td>
<td>Inhibits immune cell migration; Blocks pro-inflammatory cytokine production; Promotes endothelial cell migration and tumor blood vessel growth</td>
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<tr>
<td>Sema4E</td>
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<tr>
<td>Sema4F</td>
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<tr>
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<td>-</td>
<td></td>
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<tr>
<td>Sema5 Sema5A</td>
<td>Yes</td>
<td>rsSema5A: Promotes T cell and NK cell proliferation and proinflammatory cytokine production; Promotes endothelial cell proliferation</td>
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<tr>
<td>Sema5B</td>
<td>Yes</td>
<td>Induces collapse of DRG cell growth cones; Regulates synapse elimination in hippocampal neurons</td>
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<tr>
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<tr>
<td>Sema6D</td>
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<td>Possible functional roles in heart development</td>
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<tr>
<td>Sema7 Sema7A</td>
<td>Yes</td>
<td>rsSema7A: Inhibits megakaryocyte and platelet differentiation</td>
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</table>

Table 1.2. Summary of the transmembrane and membrane-bound Semas known to produce soluble ectodomains. This table lists all of the known invertebrate and vertebrate transmembrane or membrane-bound Semas and whether or not a soluble ectodomain has been shown to be released from the plasma membrane. The function of the soluble ectodomain fragment is listed, if known. rsSema indicates that a recombinant ectodomain was used in functional studies, rather than the naturally-released sSema product.
Sema4D was the first transmembrane Sema identified to have a functional soluble ectodomain [97-100]. The 120 kDa soluble Sema4D isoform (sSema4D) was discovered to be released from activated human lymphocytes and primary mouse lymphocyte cultures [97-99]. sSema4D is also present in vivo, as sSema4D was detected in the sera of mice immunized with a T cell-dependent antigen [99]. sSema4D stimulation inhibits migration of human monocytes and immature dendritic cells and blocks pro-inflammatory cytokine production in these cell types [100, 101]. sSema4D is shed from activated platelets and plays a role in thrombus formation [90]. Interestingly, while Sema4D ectodomain shedding can occur in neurons, it is not necessary for Sema4D-mediated synaptogenesis in the hippocampus [95].

Mechanistically, metalloproteases are implicated in sSema4D release, as the light metal chelators EDTA and EGTA inhibited greater than 50% of sSema4D shedding in a human T cell line and ADAM17 is necessary for Sema4D shedding in platelets [90, 98]. While general pharmaceutical inhibition of matrix metalloproteases (MMPs) did not affect shedding in lymphocytes, MT1-MMP is necessary to produce sSema4D in carcinoma cell lines and tumors [98, 102]. Inhibiting lysosome degradation does not impair sSema4D release, giving further evidence of ectodomain release occurring on the cell membrane [98]. This could suggest that Sema4D is cleaved at the plasma membrane and not in internal vesicles. Serine/threonine kinase inhibition with staurosporine
increases sSema4D production while the tyrosine phosphatase inhibitor vanadate has no effect, suggesting that Sema4D release may be regulated by phosphorylation [98]. This supports “sheddase” mechanisms as shedding enzymes often have structural requirements [103], and post-translational modifications can alter protein conformation, inferring differential sheddase susceptibility. Similarly, protein-protein interactions regulate Sema4D shedding tendency as calmodulin binds to a polybasic region of the Sema4D intracellular domain and this binding inhibits ectodomain shedding [104].

In addition to regulating physiologic responses, Sema4D ectodomain shedding is implicated in cancer and numerous diseases. Sema4D is highly expressed in carcinoma cell lines and tumors and is thought to promote tumor angiogenesis by MT1-MMP-mediated Sema4D shedding and endothelial cell migration through PlxnB1 [102, 105]. Furthermore, elevated serum sSema4D levels are correlated with autoantibody production, atrial fibrillation, heart failure, and postmenopausal osteoporosis in women [99, 106-108].

Soluble ectodomain production may be a general phenomenon of the Sema4 class, as large scale proteomic studies have detected Sema4B and Sema4C as substrates of BACE proteases [109]. Interestingly, the protease responsible may not be conserved among the Sema4 members suggesting that the while the shedding mechanisms vary across contexts, soluble Sema ectodomains may have a more predominant role than previously thought.
1.5.2. Sema5A

Vertebrate class 5 Semas, Sema5A and Sema5B, play roles in the nervous system and cardiovascular system. Sema5 members are structurally characterized by seven thrombospondin (TSP) type-1 repeats in the extracellular domain, which are thought to enhance ligand binding and Sema5A inhibitory potency [110]. Sema5A has a relatively broad expression pattern, being expressed in the adult brain, heart, kidney, muscle, and liver as well as during embryonic development [111]. Sema5A has bifunctional roles in the nervous system, attracting or repulsing axons via differential proteoglycan interactions through the TSP repeats, as well as functions in the vasculature and immune systems [30, 31, 112]. Sema5A acts as an inhibitory cue for retinal ganglion cell axons while it is a permissive cue for dorsal root ganglion cell axons and endothelial cells [31, 110, 113-115]. Sema5A positively regulates endothelial cell proliferation through PlxnB3 binding and Met tyrosine kinase activity and Akt phosphorylation [31]. There is also evidence that Sema5A plays a role in the immune response, as increased Sema5A expression in a human breast cancer cell line enhances proinflammatory genes including TNF-α and IL-8 [116].

An endogenous soluble Sema5A fragment was first discovered in the media of the pancreatic tumor cell line T3M4, which expresses high levels of endogenous Sema5A [109]. HeLa cells stably-expressing full-length Sema5A also release the soluble fragment [112]. Little is known of the cleavage site or mechanisms regulating sSema5A production, although ADAM17 has been shown to contribute to sSema5A production [112].
While the function of naturally released soluble Sema5A is not known, functional assays using recombinant Sema5A ectodomain (Sema5A-ECD) in culture and in vivo systems suggest that sSema5A plays roles in the vasculature and immune systems [109, 112]. Sema5A-ECD stimulation in cell culture increases proliferation of endothelial cells as well as increases expression of the pro-angiogenic factors IL-8 and VEGF [109]. sSema5A may also play roles in the immune system as stimulating cultured T cell or natural killer (NK) cells with Sema5A-ECD results in an increase in proliferation and the production of cytokines as well as promotes T cell differentiation [112]. Implanting Sema5A-ECD-expressing tumor cells in the pancreas of mice shows an increase in angiogenesis, suggesting a role of sSema5A in disease [31]. Interestingly, tumor cell metastasis may be mediated by autocrine signaling as Sema5A-ECD-expressing Panc1 tumor cells show increased invasiveness [117]. Furthermore, serum sSema5A levels are elevated in patients with Rheumatoid Arthritis (RA), suggesting that sSema5A could be a biomarker for RA and may contribute to RA pathogenesis [112].

Investigating the signaling mechanisms of Sema5A-ECD shows that recombinant sSema5A is mediated through PlxnA1 or PlxnB3 receptors in various systems. Knockdown of PlxnA1 or PlxnB3 in NK cells resulted in a significant decrease in Sema5A-ECD induced cytokine production [112]. PlxnB3 also mediates Sema5A-ECD-induced endothelial cell proliferation through Met tyrosine kinase and Akt activation [31, 114]. Future work is needed to investigate if the signaling mechanisms and function of the naturally released sSema5A is similar to that of the recombinant Sema5A ectodomain as well as further address the mechanisms of Sema5A shedding.
1.5.3. Sema5B

Similar to Sema5A, the homologous vertebrate family member Sema5B is critical for nervous system and cardiovascular development [118-121]. However, unlike the broad tissue expression pattern of Sema5A, Sema5B is highly expressed in the developing brain and spinal cord with lower expression in the embryonic heart and kidney [69, 111]. Within the brain, Sema5B is expressed in the cortex, hippocampus and olfactory bulbs, where it acts as an inhibitory guidance cue for cortical axons and mediates synapse elimination in hippocampal neurons [118, 119]. Similarly, Sema5B acts as a repellent cue for sensory neurons, collapsing growth cones and inhibiting DRG axon growth in culture [120, 122]. Sema5B is also expressed in endothelial cells, with expression levels being regulated by the transcription factor FoxP1, where it inhibits endothelial cell proliferation and migration [121].

Sema5B was first hypothesized to be proteolytically processed in 2009 with the observation of smaller Sema5B protein fragments being expressed in the neonatal brain as well as primary hippocampal cultures [119]. Later, the ectodomain of Sema5B was found to be released from Sema5B-expressing HEK cells [122]. Characterization of sSema5B identified it as a biologically active molecule, inducing collapse of cultured DRG cell axons and eliminating synaptic connections in hippocampal neuron cultures [119, 122].

The molecular mechanisms regulating sSema5B production remain to be elucidated, however, the putative Sema5B cleavage site was identified as a three amino
acid sequence in the ectodomain of Sema5B proximal to the transmembrane domain [122]. ADAM17 can induce cleavage of the Sema5B ectodomain, although other metalloproteases may also regulate sSema5B production as ADAM17 knockdown did not significantly decrease sSema5B production [122]. While cleavage produces a more potent repulsive protein for DRG axons, it is not necessary for Sema5B functioning as the non-cleavable deletion mutant is still able to induce axon repulsion [122].

### 1.5.4. Sema7A

Sema7A is a glycosylphosphatidylinositol (GPI) -anchored glycoprotein that has critical roles in the immune system [32, 123-125]. Sema7A is expressed in activated T cells, NK cells and B cells and is expressed on erythrocytes where it bears the JMH blood group antigen [126-128]. Sema7A activates chemotaxis in monocytes and induces monocyte pro-inflammatory cytokine production through the α1β1 integrin receptor [32, 124]. Sema7A is also expressed in the postnatal and adult brain and acts as a permissive cue for olfactory bulb axon outgrowth by signaling through integrins, focal adhesion kinase (FAK) and MAPK [53, 123]. Similarly, Sema7A stimulates angiogenesis in mouse corneas and promotes corneal nerve regeneration [54, 129].

Sema7A was first hypothesized to be shed from immune cells in 2002, as it is highly expressed in monocytes while also activating monocytes, suggesting autocrine signaling [124]. Later, Sema7A ectodomain shedding in monocytes was confirmed and ADAM17 was identified as the protease responsible [130]. A naturally released sSema7A was detected to be shed in activated platelets using mass spectrometry while biochemical
methods verified Sema7A shedding with ADAM17 as the predominant metalloprotease in Sema7A cleavage [130, 131].

Direct functional analysis of sSema7A is still necessary in normal physiological responses, however most studies use a recombinant soluble Sema7A to investigate the roles and mechanisms [53, 125]. However, sSema7A is implicated in chemotherapy-induced blood disorders. Thrombocytopenia is a blood disease characterized by low platelet count and chemotherapy patients have increased sSema7A plasma levels, being released by peripheral blood mononuclear cells (PBMCs), including T cells, B cells, NK cells and monocytes [125]. RA patients also have an increase in sSema7A plasma levels, leading to an increase in pro-inflammatory cytokine production in PBMCs, which may contribute to RA-mediated inflammation [130]. Treating a RA mouse model with Sema7A blocking antibodies, decreases pro-inflammatory cytokines and joint swelling, lending Sema7A as a possible RA drug target [130].

### 1.5.5. Proteases Involved in Sema Ectodomain Shedding

For most sSemas, a protease has been identified that is capable of or contributes to ectodomain release (Table 1.3). The zinc-dependent metalloprotease ADAM17 contributes to the cleavage of Sema4D, Sema5A, Sema5B, and Sema7A while the matrix metalloprotease MT1-MMP is necessary to cleave Sema4D in carcinoma cell lines and tumors [90, 102, 112, 122, 130, 131]. The aspartyl proteases βACE1 and βACE2 can cleave Sema4C and Sema4B, respectively [109, 132], although the biological activity of these soluble ectodomain remains unknown.
Table 1.3. Summary of the proteases known to shed Sema ectodomains. This table lists the known sSemas, what cell type(s) they are released from and the known proteases that are capable of producing sSemas.

<table>
<thead>
<tr>
<th>Semaphorin</th>
<th>Cell type shed from</th>
<th>Protease</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sema4B</td>
<td>Pancreatic islet β-cells</td>
<td>βACE2</td>
<td>Stutzer et al. 2013</td>
</tr>
<tr>
<td>Sema4C</td>
<td>HEK and HeLa cell lines</td>
<td>βACE1</td>
<td>Hemming et al. 2009</td>
</tr>
<tr>
<td>Sema4D</td>
<td>Activated human platelets</td>
<td>ADAM17</td>
<td>Zhu et al. 2007</td>
</tr>
<tr>
<td></td>
<td>Carcinoma cell lines and tumors</td>
<td>MT1-MMP</td>
<td>Basile et al. 2007</td>
</tr>
<tr>
<td>Sema5A</td>
<td>Pancreatic tumor cell line</td>
<td>Unknown</td>
<td>Sadanandam et al. 2012</td>
</tr>
<tr>
<td>Sema5A-expressing HeLa cells</td>
<td>ADAM17</td>
<td>Gras et al. 2014</td>
<td></td>
</tr>
<tr>
<td>Sema5B</td>
<td>Sema5B-expressing HEK cells</td>
<td>ADAM17</td>
<td>Browne et al. 2012</td>
</tr>
<tr>
<td>Sema7A</td>
<td>Activated human platelets</td>
<td>ADAM17</td>
<td>Fong et al. 2011</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>ADAM17</td>
<td>Xie &amp; Wang 2017</td>
</tr>
</tbody>
</table>
While there are many reasons for receptor cleavage, the function of Sema shedding remains elusive. Sema4D ectodomain release increases when dimerization occurs as well as receptor post-translational modification [97, 98], which may point to Sema shedding as one cellular mechanism of inhibiting receptor signaling by investing minimal cellular energy. Further fine-tuning of Sema activity could be achieved by differential expression of proteases. Additionally, shedding could allow paracrine or systemic signaling thus diversifying the roles of membrane-bound Semas. Investigating the circumstances of which Semas are cleaved will shed light onto the functional significance of sSemas and represents an important aspect of future research.

1.6. CRMPs

1.6.1. The CRMP family

CRMPs are critical downstream mediators of Sema-Plxn signaling. There are five vertebrate isoforms, CRMP1-5, of which CRMP1-4 share 75% identity [133]. CRMPs are uniquely expressed in the nervous system [133-135]. However, the different family members are expressed in discreet tissues and at specific times during development [134, 135]. CRMP2 is the most widely expressed CRMP, being present throughout the vertebrate cortex, hippocampus, cerebellum, retina and spinal cord as well as in the adult brain [135-137]. CRMP1 and CRMP4 are also expressed in the developing cortex and hippocampus and the ventral spinal cord [135]. CRMP3 is not expressed in the cortex, yet is expressed in the granule cell layer of the cerebellum throughout development and adulthood [135]. CRMP1-4 are also expressed in the peripheral nervous system, in the developing and adult dorsal root ganglia cells and CRMP1, CRMP2, and CRMP4 are
expressed in sympathetic ganglia [135]. In the adult, CRMP1, CRMP2 and CRMP5 are expressed in the post-mitotic neurons of the olfactory system [136].

At the subcellular level, CRMPs are expressed throughout neuronal cell bodies, dendrites and axons [136, 138]. CRMPs are most highly expressing during axonal outgrowth with CRMP2 expressed in growth cone lamellipodia [134]. Interestingly, CRMP2 is expressed during neuronal differentiation and is upregulated in response to neural induction cues such as noggin [134, 139]. Likewise, BMP4, a neural induction inhibitor and epidermalization signal suppresses CRMP2 expression, suggesting that CRMP2 plays roles in neuron differentiation and axonal outgrowth [139].

1.6.2. CRMPs are critical for axonal outgrowth and guidance

As CRMPs are highly expressed during neuronal differentiation and axon outgrowth, early studies hypothesized that CRMPs play critical roles in neuronal migration and axonal guidance. Indeed, CRMP2 was first identified by two separate research groups in 1995: one studying the genes involved in Sema3A-mediated growth cone collapse [137] and the other studying genes upregulated during neuron differentiation [134]. Furthermore, mutations in the invertebrate C. elegans CRMP homologue unc-33 cause neuronal guidance abnormalities, including premature or ectopic axon termination and branching in motor and sensory neurons [7, 140-142]. The vertebrate isoform CRMP2 is highly expressed in distal axons during axon outgrowth in culture and overexpression of CRMP2 leads to an increase in the number of axons [143]. CRMP2-mediated axon formation and elongation has also been demonstrated in the
immortal neuronal cell line NIE-115 and in DRG explants [144]. Interestingly, this function may be isoform specific, as CRMP1 and CRMP5 overexpression in NIE-115 cells does not increase neurite elongation [144].

CRMP1-deficient mice have defects in cortical migration and disorganization of dendrites [145]. Similarly, *in vivo* CRMP2 knockdown during embryonic development using *in utero* electroporation results in impaired radial migration of cortical neurons [146]. The CRMP2-silenced neurons are able to leave the ventricular zone, yet become stalled in the intermediate zone and do not migrate into the cortical plate [146]. Furthermore, the dendritic field is in an immature state, with a multipolar morphology instead of adapting the typical bipolar morphology of migrating pyramidal neurons [146, 147]. *Sema3A−/−* mice also exhibit abnormal pyramidal dendrite morphology [25], suggesting CRMP2 may be functioning downstream of Sema3A to mediate cortical development. Indeed, CRMP2 was first identified as a mediator of Sema3A signaling [137]. Sema3A signaling can be recapitulated in a COS7 collapse assay, as PlxnA1- and NP1-expressing COS7 cells collapse after 60 minutes of Sema3A stimulation [148]. When CRMP isoforms are co-transfected, Sema3A-mediated collapse occurs after only 10 minutes, suggesting that CRMPs accelerate Sema3A functions [148].

1.6.3. CRMPs are regulated by phosphorylation

CRMP2 is downstream of Sema3A signaling, as function-blocking antibodies to CRMP2 cause an absence in Sema3A-induced growth cone collapse [137]. Several amino acids have been identified that regulate CRMP affinity for microtubules. Activated Cdk5
phosphorylates CRMP2 \textit{in vitro} and \textit{in vivo} at Ser522 [67]. This initial phosphorylation event recruits another serine/threonyne kinase, GSK3\(\beta\), which phosphorylates CRMP2 at three residues, Thr509, Thr514, and Ser518 [26, 67]. These phosphorylation events cause a decrease in CRMP2 microtubule affinity, which may play a role in Sema-induced growth cone collapse (Figure 1.6) [26]. Indeed, CRMP2 phosphorylation is critical for its function in DRG explants as non-phosphorylatable mutants render DGRs insensitive to Sema3A [67].

Interestingly, CRMPs can also be phosphorylated at S555, however this phosphorylation event is not mediated by Sema3A [149, 150]. Instead, this occurs downstream of the collapsing cues, LPA and EphrinA5 and mediated through Rho-ROCK [149]. Suggesting that CRMPs are important mediators of cellular collapse and multiple pathways converge to activate CRMPs. Yet, not much is known about these phosphorylation events \textit{in vivo}. Cdk5-dependent phosphorylation at S522 is necessary for proper dendritic patterning, as mouse knock-in of S522A mutant results in an increase in the number of basal dendrites of cortical pyramidal cells as well as impaired dendrite morphology and extension [147]. Interestingly, this mutant phenotype is exaggerated with a \(CRMP1^{-/}\) background, giving evidence that CRMP isoforms work together.

1.6.4. CRMPs regulate microtubule dynamics

Functionally, CRMPs are heterotetramer microtubule associated proteins that regulate the cytoskeleton [148, 151]. CRMP1-4 interact with tubulin dimers and this interaction is mediated by the C-terminus of CRMPs [27]. Furthermore, CRMP2
Figure 1.6. Proposed Model of Sema6A-PlxnA2 Microtubule Regulation. A. In the absence of Sema6A, the PlxnA2 homodimer is in an auto-inhibitory “head-on” conformation. Although Fyn can still bind to PlxnA2, it has lower kinase activity and no downstream signaling occurs. Unphosphorylated CRMP2 binds and stabilizes microtubules. B. Upon Sema6A binding to PlxnA2, Fyn is activated and phosphorylates CDK5, which in turn phosphorylates CRMP2. This initial phosphorylation event recruits GSK3β, which further phosphorylates CRMP2, lowering CRMP2 microtubule affinity. This leads to microtubule destabilization and contributes to growth cone collapse.
promotes an increase of tubulin assembly into microtubules *in vitro* [27]. Cellular assays also indicate that CRMP2 co-localizes with microtubules in fibroblasts and promotes axon formation in NIE-115 cells and cultured hippocampal neurons [27]. CRMP2 also associates with the mitotic spindle in dividing HeLa cells as well as microtubules in N2A cells and cultured chick retinal ganglion cells [26, 152, 153].

CRMP2 phosphorylation status regulates its affinity for microtubules and thus is a critical mechanism of CRMP2-mediated microtubule dynamics. A phospho-mimic CRMP2 mutant, T514D has a lower affinity for tubulin and T514D, S518D, or S522D mutants are no longer associated with the mitotic spindle in HeLa cells [26, 67]. In contrast alanine mutants localize to the spindle similarly to WT CRMP2 [26]. Functionally, aspartate mutants are not able to induce the CRMP2-mediated axon elongation and branching in culture hippocampal neurons nor can they induce Sema3A-mediated DRG growth cone collapse [26, 67].

In conclusion, CRMPs are critical in mediating Sema signaling throughout nervous system development. Of the five isoforms, CRMP2 has the widest expression pattern and has been the most characterized, although the high homology between CRMP1-4 suggests conserved mechanisms. CRMP2 phosphorylation regulates its affinity for microtubules and tubulin dimers, thus influencing cytoskeletal dynamics and playing a critical role in Sema-mediated growth cone collapse and cortical cell migration. Future work is needed to elucidate the roles and mechanisms of CRMP2 outside of cortical development.
1.7. Zebrafish as a Model Organism

This dissertation work used zebrafish as a model organism to study functionally-critical molecular mechanisms of vertebrate eye development. Zebrafish are a strong model to study development due to external fertilization and transparency of the embryos, the short generation time of 2 – 3 months, and the high fecundity [154]. Embryonic development is rapid, completing at 72 hours [155]. Furthermore, there is high genetic similarity between zebrafish and humans. Approximately 70 percent of human genes have a zebrafish orthologue [156]. Many of these genes are involved in conserved processes such as proliferation, differentiation, and migration. Our genes of interest, including Sema6A, PlxnA1/A2, Fyn, and CRMP2 are expressed in the zebrafish and are genetically similar to their human orthologues (Table 1.4).

Visual system development is a highly conserved process across vertebrates [5]. Retinal cellular differentiation and lamination occurs in a highly organized and stereotypical manner [157]. There are six neuronal cell types in the vertebrate retina, including retinal ganglion cells, amacrine cells, bipolar cells, horizontal cells, and photoreceptors (rods and cones), as well as one type of glial cell, known as Müller cells. Lamination of the retina occurs similar to cortical lamination, in which differentiation and migration occurs in an inside-to-outside fashion. The zebrafish inner retina differentiates first, with retinal ganglion cells at 26 hours post fertilization (hpf), while the photoreceptors and Müller glia differentiate last around 72 hpf [157]. Retinal ganglion cell axons form the optic nerve to innervate the optic tectum. In zebrafish, the pathfinding of retinal ganglion cell axons begins at 34-36 hpf, when retinal ganglion cell
Table 1.4. Percent Identity of Human Sema6A-PlxnA Signaling Protein to Model Organisms. This table displays a comparison of Sema6A, PlxnA1, PlxnA2, Fyn, and CRMP2 sequences across multiple species, including mammals, vertebrates, and invertebrates. If a model organism does not have the given protein, the closest homolog is written. Sequence information was acquired from NCBI and Uniprot and aligned using MUSCLE Sequence Alignment (EMBL-EBI). Human protein reference numbers are as follows: Sema6A: NP_065847.1; PlxnA1: NP_115618; PlxnA2: EAW93457.1; Fyn: NP_002028.1; CRMP2: NP_001377.1.

<table>
<thead>
<tr>
<th>Human Protein</th>
<th>Zebrafish (D. rerio)</th>
<th>Mouse (M. musculus)</th>
<th>Rat (R. norvegicus)</th>
<th>Chicken (G. gallus)</th>
<th>Fruit Fly (D. melanogaster)</th>
<th>Round Worm (C. elegans)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sema6A</td>
<td>69%</td>
<td>95%</td>
<td>94%</td>
<td>90%</td>
<td>Sema1a: 35%</td>
<td>Sema1a: 31%</td>
</tr>
<tr>
<td>PlxnA1</td>
<td>PlxnA1a: 82% PlxnA1b: 80%</td>
<td>96%</td>
<td>96%</td>
<td>88%</td>
<td>PlexA: 44%</td>
<td>Plx-1: 37%</td>
</tr>
<tr>
<td>PlxnA2</td>
<td>82%</td>
<td>97%</td>
<td>97%</td>
<td>89%</td>
<td>PlexA: 42%</td>
<td>Plx-1: 35%</td>
</tr>
<tr>
<td>Fyn</td>
<td>FynA: 92% Fynb: 91%</td>
<td>99%</td>
<td>99%</td>
<td>93%</td>
<td>c-Src: 51%</td>
<td>Src: 53%</td>
</tr>
<tr>
<td>CRMP2</td>
<td>90%</td>
<td>99%</td>
<td>99%</td>
<td>98%</td>
<td>CRMP: 46%</td>
<td>unc-33: 37%</td>
</tr>
</tbody>
</table>
axons leave the retina. The axons cross the optic chiasm at 36-40 hpf, reach the tectum at 46-48 hpf and innervate the tectum cells at 70-72 hpf, completing the visual system circuitry [158].

The rapid development of the zebrafish visual system makes zebrafish an optimal model for studying the vertebrate visual system. Retinal ganglion cell axons can be used as a model for axonal pathfinding. The transgenic zebrafish line, isl2b:GFP, expresses green fluorescent protein (GFP) in retinal ganglion cell bodies and processes that allows for visualization of the optic nerve and tectum innervation. Zebrafish are highly visual animals and the eye and tectum are relatively large [157]. This allows for robust morphological screens. Hematoxylin and Eosin staining (H & E), for example, visualizes all retinal cell types and retinal lamination. Additionally, in situ hybridization techniques allow for a qualitative analysis of transcript expression patterns.

In addition to modeling visual system development and functioning, the zebrafish visual system is an excellent in vivo tool to study vertebrate Sema6A-PlxnA2 signaling mechanisms. There is high homology between the human and zebrafish proteins that are involved in Sema6A-PlxnA2 signaling (Table 1.4). Decreasing Sema6A or PlxnA2 give characteristic and reproducible visual system phenotypes, including impaired early eye field cohesion and smaller eye size [2, 68] (Figure 1.4). Therefore, we can use the zebrafish visual system as a tool to investigate the mechanisms of Sema6A-PlxnA2 signaling. Rescue experiments can answer questions about the functionally relevant phosphorylation sites. By injecting morpholinos to decrease endogenous proteins, such as
PlxnA2 and CRMP2, and simultaneously co-injecting various non-phosphorylatable mRNA mutant constructs, we can determine which phosphorylation events are necessary for vertebrate eye development.

In summary, the zebrafish visual system is a model system to 1) better understand and functionally characterize Sema6A-PlxnA2 signaling molecules in the vertebrate visual system and 2) elucidate in vivo mechanisms, such as phosphorylation events, of Sema6A-PlxnA2 signaling.

1.8. Questions Remaining

Semas function in numerous cell types during embryonic development of the nervous system, cardiovasculature, and immune systems. While many of the cellular players have been identified, a better understanding of the functionally significant in vivo molecular mechanisms of Sema signaling is required. Much work has characterized Sema-mediated roles in the context of cortical and hippocampal development. Yet, less is known about Sema signaling functions in other tissues such as the visual system. This dissertation aimed to fill this gap by investigating Sema6A-PlxnA signaling mechanisms using the zebrafish visual system.

The complexity of Sema-Plxn signaling results in numerous downstream pathways being activated to govern cytoskeletal dynamics and adhesion as well as non-canonical roles such as proliferation and transcriptional regulation. Investigating the receptor proximal mechanisms can begin to elucidate the activation steps required to
initiate various signaling cascades. The tyrosine kinase Fyn constitutively binds to PlxnA1 and PlxnA2 and can induce PlxnA phosphorylation [25]. Yet, the functional consequences of PlxnA phosphorylation are not understood. Herein, we report that Fyn induces PlxnA1 and PlxnA2 phosphorylation at two tyrosine residues and that this phosphorylation is critical for zebrafish eye size. Future work will elucidate the cellular and molecular functions of Fyn-mediated PlxnA phosphorylation in Sema6A signaling.

Traditionally, Semas have been classified into either secreted or membrane-bound classes. Yet, many of the transmembrane and membrane-anchored Sema classes have been shown to undergo ectodomain cleavage to produce functional soluble ectodomains. However, a functional soluble ectodomain of the Sema6 class has not been characterized in the nervous system. We have previously identified Sema6A peptides in human and rat embryonic cerebrospinal fluid [159]. In this dissertation, we report that Sema6A does indeed produce a soluble form which is naturally released in addition to being regulated by the serine/threonine kinase PKC. Importantly, we show that sSema6A is functional, as it plays a role in maintaining cohesion of zebrafish early eye explants. Future work will further characterize the regulation and roles of Sema6A ectodomain cleavage.

While CRMP functions have been well established in cell culture systems, less is known about the in vivo roles. The relatively few in vivo studies have investigated CRMP2 function in cortical lamination [146, 147]. Less is known about the roles of CRMP2 in non-cortical laminated brain regions, such as the retina. However, CRMP2 is known to be expressed and associated with microtubules in chick retinal ganglion cells.
Herein, we report that CRMP2 has a novel \textit{in vivo} role in visual system development, mediating lamination and axonal migration in a similar manner to its function in cortical lamination. Future work will determine if Cdk5- and GSK3β-mediated CRMP2 phosphorylation is critical for CRMP2 \textit{in vivo} functions during visual system development.

Together, this dissertation describes critical and novel Sema6A-PlxnA signaling mechanisms that are functionally relevant in vertebrate eye development.
1.9. References


CHAPTER 2: FYN-DEPENDENT PHOSPHORYLATION OF PLEXINA1 AND PLEXINA2 AT CONSERVED TYROSINES IS ESSENTIAL FOR ZEBRAFISH EYE DEVELOPMENT

2.1. Abstract

Plexins (Plxns) are semaphorin (Sema) receptors that play important signaling roles, particularly in the developing nervous system and vasculature. Sema-Plxn signaling regulates cellular processes such as cytoskeletal dynamics, proliferation, and differentiation. However, the receptor-proximal signaling mechanisms driving Sema-Plxn signal transduction are only partially understood. Plxn tyrosine phosphorylation is thought to play an important role in these signaling events as receptor and non-receptor tyrosine kinases have been shown to interact with Plxn receptors. The Src-family kinase Fyn can induce the tyrosine phosphorylation of PlxnA1 and PlxnA2. However, the Fyn-dependent phosphorylation sites on these receptors have not been identified. Here, using mass spectrometry-based approaches, we have identified highly-conserved, Fyn-induced PlxnA tyrosine phosphorylation sites. Mutation of these sites to phenylalanine results in significantly decreased Fyn-dependent PlxnA tyrosine phosphorylation. Furthermore, in contrast to wildtype human PLXNA2 mRNA, mRNA harboring these point mutations cannot rescue eye developmental defects when co-injected with a plxnA2 morpholino in zebrafish embryos. Together these data suggest that Fyn-dependent phosphorylation at two critical tyrosines is a key feature of vertebrate PlxnA1 and PlxnA2 signal transduction.
2.2. Introduction

Plexins (Plxns) are semaphorin (Sema) receptors that are highly expressed during development where sema-plxn signaling plays important roles in nervous system, cardiac, vasculature and bone development [1, 2]. At the cellular level, Sema-Plxn signaling regulates cytoskeletal dynamics, integrin-mediated adhesion, proliferation, differentiation, transcription, and programmed cell death [3-7]. With a wide array of functions, the signaling outcome of Semas depends on a variety of factors, including receptor subtype and the effector proteins associated with the cytosolic regions of both Plxns and transmembrane Semas [2, 8]. The intracellular signaling milieu in which Plxns and Semas act is highly dynamic and determined by differential protein expression driven by changing cell types and developmental timing [9-11].

In mammals, there are nine Plxns classified into four subfamilies: PlxnA1-A4, B1-3, C1, and D1 [12]. PlxnA family members, receptors for Sema3 and Sema6 classes, are the most highly expressed Plxns throughout nervous system development, with PlxnA2 being the most highly expressed PlxnA in the cortical plate [12-16]. Furthermore, PlxnA2 and the close family member PlxnA1 are also expressed in non-cortical regions of the nervous system, including the retina [14, 17]. We have previously shown the importance for Sema6-PlxnA signaling in early vertebrate eye development, as decreasing PlxnA2 or Sema6A in the zebrafish results in reduced retinal precursor cell proliferation, loss of eye field cohesion, and impaired retinal lamination [7, 17]. Together, these results show that Sema6-PlxnA signaling initiates a variety of functional outcomes depending on cellular and organismal context.
Although the nature of Sema-Plxn binding has been well characterized [18, 19], the receptor proximal events that initiate specific functional outcomes of Sema-Plxn signaling are only partially understood. Plxn phosphorylation by tyrosine kinases is thought to be one way by which Plxns mediate intracellular signaling in response to Sema binding [20]. Receptor-type and cytoplasmic tyrosine kinases are known to associate with Plxn family members [16, 21-23]. In fact, there is evidence through large-scale proteomic screens that the intracellular domain of all nine Plxn family members can be tyrosine phosphorylated at least to some degree [24]. However, currently only PlxnB, based on mutagenesis studies, has been shown to be phosphorylated at specific sites by a specific kinase (ErbB2) [25] and to our knowledge no kinases have been identified to phosphorylate specific PlxnA tyrosine residues. Nonetheless, PlxnA receptors have been shown to be tyrosine phosphorylated when overexpressed in standard human cell lines [12] and the Src family tyrosine kinase (SFK) Fyn was later discovered to constitutively associate with and phosphorylate the intracellular region of PlxnA1 and PlxnA2 [21]. Furthermore, the cytosolic tyrosine kinase Fes has been shown to bind and phosphorylate PlxnA1 [22], and the receptor tyrosine kinase (RTK) VEGFR2 has been shown to associate with PlxnA1 [16, 23].

In spite of these known associations between tyrosine kinases and PlxnA receptors, no tyrosine phosphorylation sites have been identified to be regulated by specific tyrosine kinases. As Fyn has been implicated in PlxnA phosphorylation and is critical to Sema-induced dendritic maturation in cultured cortical neurons [21, 26], we focused our study on Fyn-dependent PLXNA phosphorylation. To begin to elucidate the
signaling role of tyrosine phosphorylation sites on Plxns, we set out to ascertain Fyn-dependent phosphorylation sites of PLXNA2 and PLXNA1. Using mass spectrometry and biochemical approaches, we have identified two novel Fyn-induced PLXNA phosphorylation sites that are conserved across vertebrates and invertebrates. Site-specific mutants of PLXNA2 and PLXNA1 verified that these tyrosine residues are the major sites of Fyn-dependent phosphorylation. Using zebrafish as a model organism, we investigated the in vivo functional significance of these sites and found that Fyn-dependent PlxnA2 phosphorylation is critical for zebrafish eye size. We end with a discussion on the possible signaling roles of these phosphorylation events at the molecular and cellular levels.

2.3. Results

2.3.1. Fyn Induces PLXNA2 and PLXNA1 Tyrosine Phosphorylation

To recapitulate the Fyn-dependent PlxnA2 and PlxnA1 phosphorylation found by Sasaki et al. 2002, we first transfected HEK293 cells with expression plasmids encoding Flag-tagged PLXNA2 and either Fyn wildtype (WT) or a kinase dead (KD) point mutant of Fyn. PLXNA2 showed prominent tyrosine phosphorylation when Fyn WT was co-expressed and this phosphorylation was absent when Fyn KD was co-expressed (Figure 2.1A). Similarly, Flag-tagged PLXNA1 tyrosine phosphorylation was present when Fyn WT was co-expressed and absent when Fyn KD was co-expressed (Figure 2.1B). These results are consistent with the findings of others (Sasaki et al., 2002)
Figure 2.1. Fyn Induces PLXNA2 and PLXNA1 Phosphorylation. A-B. HEK293 cells were transfected with expression plasmids encoding PLXNA2-Flag (A), PLXNA1-Flag (B), Fyn wildtype (WT) or Fyn kinase dead (KD) as indicated. Cell extracts were subjected to immunoprecipitation with α-Flag resin. Immune complexes and whole cell extracts were subjected to SDS-PAGE and immunoblotting with the indicated antibodies.
and demonstrate tyrosine phosphorylation events on PlxnA2 and PlxnA1 that are induced by Fyn kinase activity. To understand the molecular mechanisms of how PlxnA tyrosine phosphorylation might regulate PlxnA receptors, we next sought out to identify the tyrosine residues on PLXNA2 and PLXNA1 that are phosphorylated in a Fyn-dependent manner.

2.3.2. Bioinformatics Reveals Conserved Tyrosine Phosphorylation Sites on PLXNA2 and PLXNA1

As an initial approach to identify potential Fyn-dependent tyrosine phosphorylation sites we asked if PlxnA family members were known to be tyrosine phosphorylated at specific sites as determined by large-scale phosphoproteomic analyses. PhosphoSitePlus [24] is an online database that curates post-translational modification information, including phosphorylation, from both large-scale and site-specific studies. PhosphoSitePlus listed eight intracellular tyrosine residues found to be phosphorylated on PLXNA2 in large-scale proteomic screens. Seven out of these eight residues were found phosphorylated two or fewer times. However, Y1605 has been found phosphorylated in 20 independent proteomic screens (Figure 2.2A). This site is conserved across vertebrates and invertebrates as well as within PLXNA1 (Figure 2.2B). Indeed, PhosphoSitePlus shows nine intracellular tyrosine residues to be phosphorylated on PLXNA1, with eight of these sites only being found to be phosphorylated once (Figure 2.2A). However, Y1608 in PLXNA1 is homologous to PLXNA2 Y1605 and has been found to be phosphorylated 58 independent times. These data suggest there may be an evolutionarily
conserved role for this phosphorylation event in PlxnA signaling. Importantly, the kinase that phosphorylates this site has not been identified and we hypothesized that Fyn could be the tyrosine kinase responsible for phosphorylating PlxnA2 and PlxnA1 on Y1605 and Y1608, respectively.

### 2.3.3. Targeted Mass Spectrometry Finds Y1605 as a Fyn-Dependent Phosphorylation Site on PLXNA2

To determine if Fyn phosphorylates PLXNA2 at Y1605 we made use of synthetic, stable isotope-containing peptides harboring phosphorylated and unphosphorylated Y1605 as references to determine the expected elution time of the native PLXNA2 peptides as well as their MS2 spectral patterns. HEK293 cells were transfected with expression plasmids encoding PLXNA2 WT-Flag alone or with either Fyn WT or Fyn KD. PLXNA2 was then immunopurified from extracts of each group. A portion of each immunoprecipitation was used for immunoblotting to confirm PLXNA2 expression and to confirm Fyn-induced PLXNA2 tyrosine phosphorylation (Figure 2.3A). The majority of each immunoprecipitation was subjected to SDS-PAGE and coomassie staining (Figure 2.3B). From these gels, the control and PLXNA2 bands running at approximately 200 kDa were excised and subjected to in-gel tryptic digestion. Extracted peptides were supplemented with unphosphorylated and phosphorylated Y1605-containing peptides (QTSSYNIPASASISR) containing $^{13}$C$_5$- and $^{15}$N-labeled proline. A targeted approach was used to trigger MS/MS scans on the $m/z$ values of the doubly-charged precursor ions for the native tryptic peptides of PLXNA2 containing unphosphorylated and phosphorylated Y1605. Using the synthetic stable isotope-
Figure 2.3. Targeted Mass Spectrometry Reveals Y1605 as a Fyn-Induced Phosphorylation Site on PLXNA2. HEK293 cells were transfected with expression plasmids encoding PLXNA2-Flag, Fyn wildtype (WT) or Fyn kinase dead (KD) as indicated. Whole cell extracts were subjected to immunoprecipitation with α-Flag resin. A. Whole cell extracts and a small fraction of the immune complexes were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. B. The majority of the immune complexes for each sample was subjected to SDS-PAGE and protein bands were visualized by coomassie staining. The regions of the gel where PLXNA2 bands run were excised and digested with trypsin and the tryptic peptides were prepared for mass spectrometry. Unphosphorylated and phosphorylated Y1605-containing synthetic reference peptides with a $^{13}$C$_5$- and $^{15}$N-labeled proline (P*) were added to the native extracted peptides. A targeted approach was used to subject the appropriate m/z values of the doubly-charged tryptic peptides containing either the unphosphorylated or phosphorylated Y1605 to Collision-Induced Dissociation in a linear ion trap-orbitrap mass spectrometer. C-D. The unphosphorylated native PLXNA2 peptide containing Y1605 was found across treatment groups, but the phosphorylated native peptide was only found in the PLXNA2 + Fyn WT group. Shown are the MS/MS spectra of the phosphorylated Y1605 synthetic peptide (C) and the Y1605 phosphorylated native peptide (D).
containing peptides as a reference allowed us to readily detect the phosphorylated and unphosphorylated Y1605-containing PLXNA2 tryptic peptides (the MS2 spectral pattern of the phosphorylated synthetic peptide is shown in Figure 2.3C). The unphosphorylated peptide was found across treatment groups. However, the phosphorylated Y1605 PLXNA2 peptide was only detected in cells expressing both PLXNA2 and Fyn WT (Figure 2.3D). This supports the hypothesis that active Fyn can induce phosphorylation of PLXNA2 at Y1605 in cells.

2.3.4. A SILAC Mass Spectrometry Approach Identifies Y1677 as a Novel Fyn-Dependent Phosphorylation Site on PLXNA2

To ascertain if additional Fyn-dependent PLXNA2 phosphorylation sites could be identified, we used Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC), a proteomics approach that utilizes cells grown in media containing heavy-labeled amino acids [27]. The advantage of a quantitative labeling method such as SILAC is that it allows different treatment types to be combined and handled equally in the preparation of peptides for LC-MS/MS. HEK293 cells expressing PLXNA2 WT-Flag and Fyn WT were grown in DMEM containing no heavy labeled amino acids (Light condition, Figure 2.4A). Cells expressing PLXNA2 WT-Flag and Fyn KD were grown in DMEM containing $^{13}$C- and $^{15}$N- labeled arginine and lysine (Heavy condition), resulting in newly synthesized proteins incorporating heavy labels. PLXNA2 was immunoprecipitated using $\alpha$-Flag resin and both PLXNA2 groups were combined and loaded onto a SDS-PAGE gel and stained with coomassie dye (Figure 2.4B). The
Figure 2.4. Y1677 is a Novel Fyn-Dependent PLXNA2 Phosphorylation Site. A. Diagram showing the SILAC design to identify PLXNA2 phosphorylation sites dependent on Fyn kinase activity. B. Coomassie-stained gel of proteins immunoprecipitated from SILAC cell extracts. The region of the gel corresponding to approximately 200 kDa was cut out from experimental and control lanes, subjected to in-gel tryptic digestion and run on a linear ion trap-orbitrap mass spectrometer. C-D. MS1 mass spectra highlighting the isotopic envelopes of the tryptic peptides containing unphosphorylated PLXNA2 Y1677 (C) and phosphorylated PLXNA2 Y1677 (D). The blue dashed line (bottom) indicates the expected value of the phosphorylated PLXNA2 peptide, which was not present ($m/z = 601.282$). The black dashed line in both spectra represents the mean heavy:light fold change (mean = 2.87 +/− 0.48 fold change; n = 71, errors bars indicate s.d.) of all other fully tryptic PLXNA2 peptides identified. E. Multiple sequence alignment showing the position of Y1677 across several model organisms for both PlxnA2 as well as PlxnA1.
PLXNA2 band as well as the untransfected control band were cut and prepared for mass spectrometry analysis.

Following a SEQUEST search and an automated calculation of the heavy:light ratio [28] of PLXNA2 peptides, we determined the distribution of all PLXNA2 fully tryptic peptides identified which covered amino acid sequences for which we did not find a phosphorylation event. As the relative abundance of these peptides was not likely altered by the treatment, they would define the relative amount of PLXNA2 immunoprecipitated from the two conditions. The average heavy:light ratio for the 71 peptide pairs we identified was 2.9 with a standard deviation of 0.5. This indicated that in this experiment the amount of PLXNA2 immunoprecipitated from the heavy state with Fyn KD expressed was nearly three times the amount immunoprecipitated from the light state with Fyn WT expressed. Only one phosphotyrosine-containing peptide was identified in our analysis and, importantly, it was found only in the light state. Surprisingly, we did not identify phosphotyrosyl-1605 in the SILAC analysis. The identified phosphopeptide contained tyrosine phosphorylation at Y1677. The stoichiometry of phosphorylation at Y1677 can be inferred by the decrease in the unphosphorylated form of this peptide relative to the average SILAC ratio. The SILAC ratio of the unphosphorylated form is approximately 15% less when Fyn WT is co-expressed compared to the mean SILAC ratio of PLXNA2 peptides suggesting 15% phosphorylation at that site (Figure 2.4C). As the precursor ion for the phosphopeptide was not observable in the heavy state, a signal-to-noise (S/N) calculation was used to approximate its minimum fold increase. While the total amount of the phosphopeptide
might in fact be zero, the S/N calculation describes the lowest possible observable signal and serves as a baseline for the minimum fold increase. The lowest possible signal is a peptide ion in the full MS1 mass spectrum with an observable isotopic envelope as compared to surrounding noise peaks which do not have observable isotopic envelopes and therefore are considered noise from the solvent alone. In this case the signal of the observed light phosphopeptide was 9.4 times the noise. Given that less light PLXNA2 was immunoprecipitated compared to heavy PLXNA2, the total fold increase of this phosphopeptide induced by Fyn WT was even greater.

Interestingly, a multiple species alignment indicates that Y1677 is conserved between PlxnA2 and PlxnA1 across species, including vertebrates and invertebrates (Figure 2.4E). Indeed, this tyrosine residue is in a highly conserved region of the cytoplasmic domain across all Plxn family members [20], which may point to an evolutionarily important function.

### 2.3.5. Y1605 and Y1677 are the Major Fyn-dependent Sites of PLXNA2 Tyrosine Phosphorylation

To determine if Y1605 and Y1677 were the major sites of Fyn-dependent PLXNA2 tyrosine phosphorylation we made single and double tyrosine-to-phenylalanine substitutions in PLXNA2 at these sites (Y1605F, Y1677F, and Y1605F/Y1677F (Y2F)). Co-transfection of expression plasmids encoding PLXNA2 Y2F-Flag and Fyn WT showed a significant decrease in Fyn-dependent PLXNA2 tyrosine phosphorylation (Figure 2.5A, quantified in Figure 2.5C). However, some Fyn-dependent tyrosine
phosphorylation remained on the Y2F mutant suggesting the presence of at least one additional Fyn-dependent tyrosine phosphorylation site on PLXNA2. To determine the predominant Fyn-induced PLXNA2 phosphorylation site, the single point mutants were co-expressed in HEK293 cells with Fyn WT. We found that Fyn-induced tyrosine phosphorylation was reduced for both Y1605F and Y1677F mutants (Figure 2.5B, quantified in Figure 2.5C). Interestingly, each PLXNA2 mutant appeared to decrease in tyrosine phosphorylation signal equally, suggesting Fyn shows roughly equal preference for phosphorylating Y1605 and Y1677.

2.3.6. Fyn-Dependent Phosphorylation Sites are Conserved Between PLXNA1 and PLXNA2

To ascertain if Fyn phosphorylates PLXNA1 at sites homologous to those identified on PLXNA2, we co-transfected HEK293 cells with expression plasmids encoding Fyn WT and either PLXNA1 WT-Flag or PLXNA1 Y2F-Flag. The Y2F allele of PLXNA1 has tyrosine-to-phenylalanine substitutions at Y1608 and Y1679, the sites homologous to Y1605 and Y1677 in PLXNA2. As hypothesized, PLXNA1 tyrosine phosphorylation decreased significantly when both Y1608 and Y1679 were unable to be phosphorylated (Figure 2.6A, quantified in Figure 2.6B). In fact, it appears that the combination of Y1608 and Y1679 constitute almost all of the Fyn-dependent tyrosine phosphorylation on PLXNA1 (Figure 2.6A).

2.3.7. Fyn-dependent PlxnA2 Phosphorylation Sites are Critical for Zebrafish Eye Development
Figure 2.5. Y1605 and Y1677 are the Major Sites of Fyn-Induced PLXNA2 Tyrosine Phosphorylation. A-B. HEK293 cells were co-transfected with Fyn WT and Flag-tagged expression constructs for either PLXNA2 WT or the PLXNA2 tyrosine-to-phenylalanine mutants Y2F (Y1605F/Y1677F), Y1605F, or Y1677F. PLXNA2 was immunopurified by α-Flag resin and subjected to SDS-PAGE and immunoblotting with the indicated antibodies. C. The relative decrease in overall tyrosine phosphorylation normalized to PLXNA2 WT + Fyn WT was quantified for each sample type. Histograms show the average of three independent experiments. *Students T-Test, p < 0.05.
Figure 2.6. Y1608 and Y1679 are the Major Fyn-Induced Sites of PLXNA1 Tyrosine Phosphorylation. A. HEK293 cells were co-transfected with Fyn WT or Fyn KD and Flag-tagged expression constructs for either PLXNA1 WT or the PLXNA1 tyrosine-to-phenylalanine Y2F mutant (Y1608F/Y1679F). PLXNA1 was immunopurified by α-Flag resin and subjected to SDS-PAGE and immunoblotting with the indicated antibodies. B. The relative decrease in overall tyrosine phosphorylation normalized to PLXNA1 WT + Fyn WT was quantified for each sample type. Histograms show the average of three independent experiments. *Students T-Test, p < 0.05.
To investigate the functional importance of \textit{in vivo} PlxnA2 phosphorylation, we turned to the zebrafish model as we have previously characterized the function of PlxnA2 in zebrafish eye development [7, 17]. \textit{PlxnA2} is expressed in the retina and is critical to zebrafish eye development, as decreasing endogenous PlxnA2 using a morpholino anti-sense oligonucleotide (MO) targeted to \textit{plxnA2} results in decreased proliferation of retinal precursor cells (RPCs), loss of RPC cohesion within migrating optic vesicles, impaired retinal lamination, and decreased eye size [7, 17]. Importantly, these phenotypes are rescued by co-injecting the \textit{plxnA2} MO with human \textit{PLXNA2-WT} mRNA at the one-cell stage of development, supporting the evolutionary conservation of PlxnA2 among vertebrates [7, 17]. The \textit{plxnA2} MO construct used has been previously validated to address knock-down specificity and any potential off-target effect [7, 17]. If PlxnA phosphorylation is critical to Sema6A signaling and vertebrate eye development, we hypothesized that the \textit{plxnA2} morphant phenotypes would not be rescued upon co-injection of \textit{plxnA2} MO with human \textit{PLXNA2 Y2F} mRNA. Indeed, while co-injecting \textit{plxnA2} MO with \textit{PLXNA2 WT} mRNA rescued the small eye phenotype at 48 hours post fertilization (hpf), co-injection of \textit{PLXNA2 Y2F} mRNA did not (Figure 2.7A-D). To control for any variations in overall embryo size we normalized eye diameter to head length (quantified in Figure 2.7G). Furthermore, overexpression of human \textit{PLXNA2 WT} or \textit{Y2F} mRNA resulted in no obvious phenotype, nor was eye size significantly different than uninjected controls (UIC) (Figure 2.7E-F; quantified in 2.7G). As it was formally possible that \textit{PLXNA2 Y2F} did not rescue the \textit{plxnA2} MO phenotype due to improper subcellular localization, we conducted immunofluorescence on HEK293, COS7, and NIE-115 cells expressing \textit{PLXNA2 WT} and \textit{Y2F} (Figure 2.7H-O). HEK293 and COS7
Figure 2.7. Fyn-dependent PlxnA2 tyrosine phosphorylation is essential for zebrafish eye development. A-F. Brightfield images of zebrafish embryos at 48 hours post fertilization (hpf). PlxnA2 morphants showed a reduction in eye size (A, B, G) compared to uninjected control (eye diameter relative to head length (Control 0.513 μm +/-0.003 μm, n=40 N=4; PlxnA2 MO 0.439 μm +/-0.009 μm, n=40 N=4, ****P<0.0001)). Co-injection of 2 ng plxna2 MO with 200 pg full-length human PLXNA2 WT mRNA rescued the small eye phenotype (C, D, G), however, full-length human PLXNA2 Y2F mRNA was unable to rescue eye size when co-injected with 2 ng plxna2 MO (Control 0.513 μm +/-0.003 μm, n=40 N=4; WT rescue 0.488 μm +/-0.008 μm, n=34 N=4, n.s; Y2F rescue 0.437 μm +/-0.007 μm, n= 40 N=4, ****P<0.0001). No observed difference in eye size was seen with overexpression of 200 pg WT or Y2F full-length human mRNA (E, F, G) (Control 0.513 μm +/-0.003 μm, n=40 N=4; 200 pg WT 0.493 μm +/-0.005 μm, n=30 N=3, n.s; 200 pg Y2F 0.502 μm +/-0.005 μm, n=30 N=3, n.s). One-way ANOVA, multiple comparisons test. Error bars indicate s.e.m. UIC: uninjected control, MO: morpholino, hpf: hours post fertilization. (H-K) PLXNA2 WT and Y2F show similar subcellular localization in both HEK293 and COS7 cells. Green is PLXNA2 and blue is DAPI.
cells were relevant given they have been used to investigate PlxnA2 signaling in biochemical and cell-based assays [12, 15, 18, 19]. COS7 cells are heterogeneous and we focused here on cells with extended processes as Sema-PlxnA signaling is known to localize to neuronal processes and to regulate axon and dendrite guidance [12, 29-31]. NIE-115 is a heterogeneous cell line that is responsive to semaphorins, as Sema3A stimulation results in neurite retraction [32]. We show immunofluorescence of PLXNA2 WT and Y2F expressed in NIE-115 cells displaying long processes (Figure 2.7L,M) as well as numerous short processes (Figure 2.7N,O). In all cases PLXNA2 WT and Y2F appeared to have similar subcellular expression patterns (Figure 2.7H-O), suggesting that the Y2F mutation did not affect protein localization. Together, these results suggest that the Fyn-dependent PlxnA phosphorylation sites identified in this study have important, conserved in vivo functions.

2.4. Discussion

Here we show that Fyn induces the phosphorylation of two conserved sites on PLXNA1 and PLXNA2. Mechanistically, PlxnA tyrosine phosphorylation by SFKs could, via structural alterations, modulate the GAP activity of the receptors as the conserved sites of tyrosine phosphorylation are in or near the split GAP domain of the PlxnA receptors [33]. Additionally, PlxnA tyrosine phosphorylation could be involved in recruiting effectors of signal transduction. Tyrosine phosphorylation can regulate protein-protein interactions by providing docking sites for SH2 or PTB domain-containing proteins. Precedence for these signaling mechanisms have been established in Sema-Plxn signaling with Sema4D-induced tyrosine phosphorylation of PlxnB1 leading to the
recruitment of the p85 subunit of PI3K and subsequent Akt activation. Stimulation of this signaling axis in endothelial cells was shown to promote endothelial migration [34]. While PI3K is not predicted to bind to PLXNA1 and PLXNA2, a Scansite [35] analysis of the intracellular domains predicts a possible function for one of the two sites we have identified here. Scansite suggests that PLXNA2 Y1605 phosphorylation could provide a binding site for the SH2 domain of the adaptor molecule Crk, or its relative CrkL, which preferentially bind to phosphorylated YXXP motifs [36]. The Rap1-GEF C3G binds to the SH3 domain of Crk and CrkL, and is involved in many cellular processes including cell-cell interactions, integrin-mediated adhesion, and differentiation [37]. Thus, Crk and CrkL binding to phosphorylated PlxnA2 could mediate some of the individual roles of Sema signaling. In spite of the fact that SFKs themselves harbor the name-sake SH2 domain, they do not appear to use their SH2 domains to bind to PlxnA receptors as Fyn has been reported to be constitutively bound to PlxnA2 independent of Fyn’s kinase activity, and by extension, the tyrosine phosphorylation state of PlxnA2 [21]. Future work will work to identify the proteins that bind to PLXNA proteins at the Fyn-induced phosphorylation sites.

Although the mechanisms downstream of these phosphorylation sites are unknown, the identification of Fyn-dependent PlxnA phosphorylation sites has been anticipated for some time [20]. Here, we have identified the two predominant sites and have found them to be functionally relevant to PlxnA signaling, setting the stage for future steps in delineating the role of tyrosine phosphorylation in PlxnA signaling pathways.
2.5. Materials and Methods

2.5.1. Bioinformatics and Multiple Sequence Alignments

Previously reported human PLXNA2 and PLXNA1 tyrosine phosphorylation from large-scale proteomic screens was collected from PhosphoSitePlus [24] (Cell Signaling Technology, Danvers, MA, USA). The conservation of PlxnA2 Y1605 and Y1677 was determined by aligning PlxnA2 and PlxnA1 for vertebrates and invertebrates: human (PLXNA2: NP_079455.3; PLXNA1: NP_115618.3), mouse (PlxnA2: NP_032908.2; PlxnA1: NP_032907.1), rat (PlxnA2: NP_001099458.2; PlxnA1: XP_002729444.2), chicken (PlxnA2: XP_015154528.1; PlxnA1: XP_414370.4), zebrafish (PlxnA2: BAD35133.1; PlxnA1a: XP_003201265.4; PlxnA1b: NP_001103480.1), fruit fly (PlxnA: NP_524637.2), and roundworm (PlxnA: BAB85224.1). Alignments were done using MUSCLE [38] in Geneious version 10.1 (Biomatters, Auckland, New Zealand).

2.5.2. Plasmids, Cell Culture, and Transfections

The human PLXNA2 construct (Origene Technologies, Rockville, MD, USA) harbored human PLXNA2 (NP_079455) containing a C-terminal Myc and Flag tag in pCMV6-Entry. The cloning sites were SgfI and MluI. BioBasic (Markham, Ontario, Canada) produced the single and double point mutant PLXNA2 constructs: Y1605F, Y1677F, and Y1605F/Y1677F. BioBasic also synthesized human PLXNA1-WT and PLXNA1-Y1608F/Y1679F (Y2F) containing a C-terminal Myc and Flag tag and cloned them into the vector pUC57. BioBasic cloned PLXNA1-WT into pCMV6-Entry vector
using SgfI and MluI. To develop a pCMV6-PLXNA1-Y2F construct, we used MluI and BssHII (New England Biolabs, Ipswich, MA, USA) to subclone a portion of PLXNA1 containing Y1608F/Y1679F from pUC57-PLXNA1-Y2F into pCMV6-Entry-PLXNA1-WT. Human Fyn-WT (plasmid #16032) and kinase dead mutant L299M Fyn (plasmid #16033) constructs were purchased from Addgene (Cambridge, MA, USA), which were originally cloned into pRK5 and deposited by Dr. Filippo Giancotti [39]. HEK293 cells were maintained in DMEM with L-glutamine, 4.5 g/L glucose and sodium pyruvate (MediaTech/Corning Life Sciences, Tewksbury, MA, USA) supplemented with 5% Fetal Bovine Serum (Hyclone, Logan, UT, USA), 5% Cosmic Calf Serum (Hyclone), 50 units/mL penicillin and 50 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C and 5.0% CO₂. Calcium phosphate-based transfections were conducted on cells grown to 75% of confluence. Following a 6-hour incubation, the cells were washed with warm PBS and incubated for an additional 18-20 hours in full medium prior to lysis.

2.5.3. Cell Lysis, Immunoprecipitation, and Western Blotting

After transfection, cells were placed on ice, washed with cold PBS, and lysed in lysis buffer (25 mM Tris pH 7.4, 137 mM NaCl, 10% glycerol, 1% Igepal) containing protease inhibitors (5 µg/mL Pepstatin, 10 µg/mL Leupeptin, 1 mM PMSF) and phosphatase inhibitors (1 mM NaVO₃, 25 mM NaF, 10 mM Na₂H₃P₂O₇). Cell extracts were prepared for SDS-PAGE, immunoblotting and immunoprecipitation as previously described [40]. A Bradford Assay [41] was performed to determine protein concentration and extracts were normalized with lysis buffer. Depending on the experiment, between 20 and 30 µg of total protein extract was used for immunoblotting. Depending on the
experiment, between 750 and 1,000 μg total protein was used for immunoprecipitations and was incubated overnight at 4 °C with 10 μl of a 50% slurry of α-Flag M2 affinity resin (Sigma-Aldrich Corp., St. Louis, MO, USA) pre-washed with lysis buffer. Immune complexes were washed three times with lysis buffer, drained, and then denatured in protein sample buffer at 95 °C for 5 minutes prior to SDS-PAGE separation. The following primary antibodies and dilutions were used: α-Fyn (rabbit mAb, 1:2000, Cell Signaling Technology), α-Flag M2 (mouse mAb, 1:2000, Sigma-Aldrich Corp.), α-phosphotyrosine 4G10 (mouse mAb; 1:1000, EMD Millipore, Billerica, MA, USA), and α-Src pY416 (rabbit mAb, 1:5000, Cell Signaling Technology). The following secondary antibodies were used: α-rabbit-HRP (goat IgG, 1:15,000, EMD Millipore), α-mouse-HRP (goat IgG, 1:5,000, EMD Millipore), or for immunoprecipitation samples α-mouse-HRP Light Chain Specific (goat IgG, 1:10,000, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunoblotting was performed as described previously [40].

2.5.4. Enhanced Chemiluminescence, Densitometry Analysis, and Statistics

Proteins from Western Blotting were detected using enhanced chemiluminescence (ThermoFisher Scientific, Waltham, MA, USA) and film was developed using a Medical Film Processor SRX-101A (Konica Minolta Medical & Graphic, Tokyo, Japan). The raw film scans were converted to grayscale and inverted in Adobe Photoshop for densitometry analysis using the mean histogram tool. The background (“untransfected control” group) signal was subtracted from all bands and the tyrosine phosphorylated PLXNA2 or A1 (as measured by α-phosphotyrosine 4G10) levels were made relative to PLXNA2 or A1 + Fyn WT in each experiment by setting this
value as 100%. Total PLXNA2 or A1 protein levels were made relative in a similar fashion using α-Flag M2 lanes. Relative α-phosphotyrosine 4G10 levels were then divided by the relative α-Flag M2 levels to calculate the PLXNA2 or A1 tyrosine phosphorylation per sample type. To account for relative Fyn activity, α-phosphotyrosine (4G10) or α-Src pY416 levels for Fyn (as a proxy for kinase activity) were made relative with the levels in the co-transfected Fyn with PLXNA1 or PLXNA2 WT set as 100% in each experiment. The relative PLXNA2 or A1 tyrosine phosphorylation levels were then divided by the relative levels of phosphotyrosyl-Fyn to calculate the final relative tyrosine phosphorylation levels of PLXNA2 or A1. These values were then Log2 transformed and a Student’s T-Test pair-wise comparison was performed on the data from three independent experiments using JMP Pro 12 Statistical Software (SAS Institute, Cary, NC, USA).

2.5.5. Sample Preparation for Mass Spectrometry and Mass Spectrometry Data Analysis

HEK293 cells either untransfected or expressing PLXNA2-WT-Flag and either Fyn WT or Fyn KD were lysed and extracts were subjected to immunoprecipitation with α-Flag M2 affinity resin as described above. Samples were loaded onto 10% SDS-PAGE gels and proteins were visualized using coomassie stain (0.1% coomassie brilliant blue R-250, 20% glacial acetic acid, 40% methanol). The gel region containing the visible PLXNA2-Flag band and corresponding to approximately 200 kDa was cut out, diced, and prepared for tryptic digestion as previously described [42] with the reduction and alkylation steps being omitted. Proteins were subjected to in-gel digestion with 6 ng/μL
sequencing-grade modified trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate at 37 °C for 6-9 hours. The tryptic peptides were resuspended in Solvent A (2.5% acetonitrile, 0.15% formic acid) and separated via HPLC prior to MS/MS analysis on a linear ion trap-orbitrap (LTQ-Orbitrap) mass spectrometer controlled with Thermo Xcalibur 2.1 software. Peptides were loaded onto 15 cm x 100 µm columns packed with 5 µm C18 packing material (Bruker Daltonics, Billerica, MA, USA, pore size = 200 Å) over 15 minutes in Solvent A and eluted using a 0–50% gradient of Solvent B (99.85% acetonitrile, 0.15% formic acid) over 38 minutes and electrosprayed (2.1 kV) into the mass spectrometer. This gradient was followed by 3 minutes of a 50-100% gradient of Solvent B. This was followed by 4 minutes at 100% Solvent B before a 10-minute equilibration in 100% Solvent A. The precursor scan (scan range = 360–1700 m/z, resolution = 3.0x10⁴, scan speed = 0.3 Hz) was followed by four collision-induced dissociation (CID) tandem mass spectra. CID spectra were acquired for the top two ions in the precursor scan (isolation window = ±2.0 m/z, collision energy = 35 eV), followed by two targeted scans. The targeted scans targeted the average mass (+/- 1.6 m/z) of the doubly-charged tryptic peptide ions harboring: (i) the unphosphorylated PLXNA2 Y1605 peptide (791.859 m/z, QTSSYNIPASASISR); and (ii) the phosphorylated (pY, underlined) PLXNA2 Y1605 peptide (831.842 m/z, QTSSpYNIPASASISR). Dynamic exclusion of precursor ions for MS/MS was enabled with a repeat count of 2, repeat duration of 30 seconds, and exclusion duration of 120 seconds. Theoretical m/z values were determined using the Fragment Ion Calculator from the Proteomics Toolkit (Institute for Systems Biology, Seattle, WA, USA). Synthetic tryptic peptides (Cell Signaling Technology) harboring unphosphorylated and phosphorylated Y1605 were
added prior to the LC-MS/MS runs. The synthetic peptides were distinguishable from the native given they contained a $^{13}$C$_5$, $^{15}$N-proline residue increasing the mass by 6.0138 Da. Both manual examination using Thermo Xcalibur’s Qual Browser as well as SEQUEST searches were used to interrogate tandem mass spectra. SEQUEST searches queried the forward and reversed concatenated human 2011 Uniprot proteome. The search parameters required tryptic peptides, permitted a precursor $m/z$ tolerance of +/- 20 ppm and allowed amino acid modifications of serine, threonine, and tyrosine (+79.9663 Da for phosphorylation), cysteine (+71.0371 Da for acrylamidation), and methionine (+15.9949 Da for oxidation). SEQUEST peptide identifications were required to be within a false discovery cutoff of 1%.

For the Stable-Isotope Labeling of Amino Acids in Cell Culture (SILAC) experiment, HEK293 cells were grown in $^{13}$C$_6$, $^{15}$N$_4$-labeled arginine and $^{13}$C$_6$, $^{15}$N$_2$-labeled lysine (“heavy condition”) as previously described [43]. These cells were transfected as described above with expression plasmids encoding PLXNA2 WT-Flag and Fyn KD. HEK293 cells grown in DMEM containing no heavy-labeled amino acids (“light condition”) were transfected with expression plasmids encoding PLXNA2 WT-Flag and Fyn WT. Following lysis, PLXNA2-Flag was immunoprecipitated from each cell state using α-Flag resin. Immune complexes from the two states were combined and subjected to SDS-PAGE. The coomassie-stained PLXNA2 band and the corresponding control derived from untransfected cells were cut and prepared for mass spectrometry analysis as detailed above. Peptides were subjected to LC-MS/MS in a linear ion trap-orbitrap run as previously described [44]. SEQUEST search parameters allowed a
precursor ion mass tolerance of 30 ppm, required peptides to be tryptic and allowed for the same amino acid modifications as described above with the addition of arginine (+10.008 for the heavy label) and lysine (+8.014 for the heavy label). Following an automated calculation of the heavy:light ratio [28], the average heavy:light fold change was calculated from unphosphorylated peptides that were fully tryptic and contained no internal arginines or lysines.

2.5.6. Immunofluorescence in Cell Culture

HEK293 cells were transfected in 6 cm dishes at 75% of confluence with 0.25 µg of either PLXNA2-WT-Flag or PLXNA2-Y2F-Flag using calcium phosphate precipitation as previously described [45]. COS7 cells were seeded onto glass coverslips in 6-well plates and transfected at 20% of confluence using polyethylenimine [46] and incubated at 37 °C and 5.0% CO₂ for 48 hours. NIE-115 cells were seeded onto poly-L-lysine treated coverslips and transfected at 90% of confluence with Lipofectamine 2000 (ThermoFisher Scientific). Both COS7 and NIE-115 cells were transfected with 2 µg of either PLXNA2-WT-Flag or PLXNA2-Y2F-Flag and incubated at 37 °C and 5.0% CO₂ for 48 hours. HEK293, NIE-115, and COS7 cells were then processed for immunofluorescence as previously described [45]. The primary antibody was incubated at room temperature for 1 hour in a humidified chamber: α-Flag M2 (mouse mAb, 1:5000, Sigma-Aldrich) in 1.5% BSA in PBS. The secondary antibody was incubated for 45 minutes at 4 °C in a humidified chamber: α-mouse Alexa Fluor 488 (1:10,000, Molecular Probes/Invitrogen). Coverslips were mounted onto microscope slides using VectaShield Hardset mounting media with DAPI (Vector Laboratories, Burlingame, CA,
Cells were imaged at 40X magnification using a Nikon Eclipse Ti inverted confocal microscope (Nikon Instruments, Melville, NY, USA). Raw image files were converted to TIFs using ImageJ version 1.48a (National Institutes of Health, USA) and color processing was done in Adobe Photoshop CS6 (San Jose, CA, USA).

2.6.7. Zebrafish husbandry, Morpholino and mRNA Rescue Experiments

Zebrafish embryos were developmentally staged as previously described [47]. The zebrafish used in this study (isl2b:GFP transgenic line) were generously provided by Dr. Chien (University of Utah, USA). All procedures were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC). For morpholino injections, 2 ng of a splice-blocking morpholino (MO) (Gene Tools, Philomath, OR, USA) targeting plxnA2 and/or 200 pg of full-length human PLXNA2 or PLXNA2 Y2F mRNA was injected at the one-cell stage as previously described [7]. The plxnA2 MO sequence was as follows: AAAAGCGATGTCTTTCTCACCTTCC, which targeted the exon 2-intron 2 boundary. The plxnA2 MO knock-down efficiency has been previously validated using RT-PCR, and off-target effects and MO specificity have been addressed by rescue of morphant phenotypes with full length human PLXNA2 mRNA and co-injection of plxnA2 and p53 MOs [7, 17]. A second non-overlapping MO construct reproduced the same phenotypes [17]. Capped and tailed full-length mRNA was generated from the pCMV6-Entry-PLXNA2-WT and -Y2F plasmids by linearizing the plasmids using PmeI (New England Biolabs), T7 RNA polymerase (mMESSAGE mMACHINE kit, Ambion, Austin, TX, USA), and the ThermoFisher PolyA Tailing Kit. These constructs were injected or co-injected with MO into zebrafish embryos at the one-
cell stage [47]. Zebrafish embryos were incubated at 28.5 °C for 2 days until the 48 hours post fertilization (hpf) stage [47]. Measurements and brightfield images taken at 6.3X magnification were obtained using SPOT imaging software version 5.2 (Sterling Heights, MI, USA). To account for any variations in overall embryo size, eye size was calculated as eye diameter normalized to head size, which was defined as the distance from the anterior tip of the head to the posterior otic placode [7]. Figures were compiled using Adobe Photoshop CS6, where image brightness was optimized. Graphs and statistical analyses were obtained using Prism 6 (GraphPad Software, La Jolla, CA, USA).
2.6. References


39. Mariotti A, Kedeshian PA, Dans M, Curatola AM, Gagnoux-Palacios L & Giancotti FG (2001) EGF-R signaling through Fyn kinase disrupts the function of


CHAPTER 3: PKC INDUCES RELEASE OF A FUNCTIONAL ECTODomain
OF THE GUIDANCE CUE SEMAPHORIN6A

3.1. Abstract

Semaphorins (Semas) are a family of secreted and transmembrane proteins that play critical roles in development. Of the four vertebrate transmembrane or membrane-bound Sema classes, all but the Sema6 class has been shown to be capable of producing functional soluble ectodomains. Herein, we show that the soluble ectodomain of Sema6A, sSema6A, exhibits natural and PKC-induced release from cells. We also show sSema6A release correlates with PKC induced Sema6A phosphorylation. Finally, this soluble variant is functional as it promotes the cohesion of zebrafish early eye field explants. This report of the first soluble ectodomain of the Sema6 class that is functional in the nervous system suggests that in addition to its canonical contact-mediated functions, Sema6A may have regulated, long-range, forward-signaling capacity.

3.2. Introduction

Semaphorins (Semas) are a family of secreted and transmembrane proteins that play crucial roles in the developing nervous system. Although Semas are best characterized as repulsive axonal guidance cues [1, 2], it is now appreciated that Semas also play diverse developmental and homeostatic roles in numerous tissue types including endothelial cells, bone cells, and cells of the cardiovascular and immune systems [3-5]. Semas also participate in pathogenic states such as metastatic cancers and neurodegenerative diseases [4, 6, 7]. Semas bind primarily to plexin (Plxn) receptors [8,
and regulate many cellular processes including cytoskeletal dynamics, integrin-dependent adhesion, proliferation, differentiation, and transcription [3, 10-13]. There are 20 vertebrate Semas identifiable by their conserved extracellular, Plxn-binding sema domain encompassing ~500 amino acids near the N-terminus [14, 15] followed by a cysteine-rich plexin–semaphorin–integrin (PSI) domain of ~50 amino acids. Vertebrate Semas can be both transmembrane and secreted and have been grouped into five classes (Sema3-7). Membrane-bound Semas (Semas4-7) act locally and regulate cells via cell-cell contact while secreted Semas (Sema3) can act over longer distances and typically require neuropilins as Plxn co-receptors [16].

While membrane-bound Semas canonically govern contact-mediated cellular events, it is appreciated that regulated processing of Semas at the plasma membrane results in soluble Sema (sSema) ectodomains that can function similarly to their traditional membrane-bound counterparts [17-21]. These sSemas, notably sSema4D and sSema7A, were first identified to be functional in the mammalian immune system [17, 18, 22]. However, sSema5B has been shown to be produced and functional in the nervous system, acting as a neuronal and axonal guidance cue [19]. Currently, at least one member of each vertebrate membrane-bound or transmembrane Sema class has been shown to produce a functional soluble variant except for the Sema6 class. However, in proteomics analyses we and others found evidence for additional sSemas, including possible variants of Sema6A and Sema6D, that were produced in vivo and released into mammalian embryonic cerebrospinal fluid (CSF) or plasma [23-25].
We have been studying the molecular mechanisms and developmental function of Sema6A-PlxnA signaling. Previously, we reported that Sema6A is highly expressed in retinal precursor cells in the early eye field and is critical in maintaining early eye field cohesion [11, 26, 27]. Given that our proteomics analyses identified Sema6A in CSF [23], we were prompted to ask if Sema6A might generate a naturally-released ectodomain. This would have important implications as Sema6A could then signal at a distance and could act as a cue that would be effectively separated from, or even antagonistic to, its known reverse signaling roles [28-30].

Herein, we show that the Sema6A ectodomain can be naturally released and that Protein Kinase C (PKC) activity enhances its release. Further, consistent with the regulatory roles of kinases driving the release of other sSemas [22], we find that sSema6A release correlates with PKC-induced Sema6A phosphorylation at intracellular serines. Finally, we show that sSema6A is functional as it promotes the cohesion of early zebrafish eye fields in cultured explants, a phenotype known to be induced by Sema6A-FC [26]. To our knowledge, these data constitute the first characterization of a natural and regulated release of an ectodomain for the Sema6 class that is functional in the nervous system. These results suggest that Sema6A may be acting at a distance in addition to its canonical contact-mediated effects. As Sema6 class members can also reverse signal, ectodomain release may also be a novel mechanism to regulate unidirectional signaling.
3.3. Results

3.3.1. Semaphorin ectodomains are present in embryonic cerebrospinal fluid and sSema6A is produced by cultured cells

We have previously shown, by proteomic analysis, that there are numerous signaling molecules present in human and rat embryonic cerebrospinal fluid (CSF) during critical stages of development [23]. Of these, Sema proteins from several Sema classes were identified, including Sema4 and Sema6 [23]. We performed a SEQUEST analysis of the raw data from this study using the original parameters to identify the location of the peptides on the Sema proteins (Figure 3.1A-B). Proteins that were identified by two or more unique peptides were retained and of these, Sema6A, Sema6D, Sema4B, and Sema5A were detected in rat and/or human embryonic CSF. Mapping the peptides to the protein location showed that only peptides corresponding to the ectodomain of the Sema proteins were identified. While protein secretion through exosomes is a possibility, many vertebrate transmembrane and membrane-bound Sema proteins have been identified to be proteolytically processed to release a functional ectodomain [17-19, 21, 22, 31]. Of the Sema proteins detected, Sema4B and Sema5A can be cleaved by BACE2 and ADAM17, respectively, to produce soluble ectodomains [20, 31, 32]. We have been investigating the roles of Sema6A in eye development and have shown it to be critical for early eye field cohesion and retinal precursor cell proliferation [11, 26]. Although the Sema6 class has not yet been shown to have soluble, released ectodomains, we asked the question if a soluble form of Sema6A can be naturally-released from Sema6A-expressing cells. To begin to address this, we first transfected HEK293 cells with an expression construct
encoding full-length human Sema6A. Following a recovery in full media, cells were rinsed and incubated with OPTI-MEM reduced media for 18-24 hours and the conditioned media was collected. Cell lysate and conditioned media samples were denatured and subjected to SDS-PAGE and immunoblotted with an extracellular-targeted α-Sema6A (Figure 3.1C). Compared to the full-length protein found in whole cell extracts, a smaller protein was detected by the antibody in the conditioned media of Sema6A-expressing cells. We also found that the soluble Sema6A can be produced in the neuronal cell line N2A (Figure 3.1D).

3.3.2. Mass spectrometry analysis identifies a soluble Sema6A ectodomain

To determine the identity of the Sema6A fragment contained in the conditioned media of Sema6A-expressing cells, we transfected HEK293 cells with an expression plasmid that encoded for human Myc-Sema6A, with the Myc tag located at the N-terminus, following the signal sequence [28]. After overnight recovery in full media, cells were incubated in OPTI-MEM reduced serum and the conditioned media was collected after 18-24 hours. Sema6A protein expression was verified in whole cell extracts via immunoblotting (Figure 3.2A, left). Sema6A was immunoprecipitated using α-Myc from cell lysates and the conditioned media. A minor portion of the immune complexes was subjected to SDS-PAGE and immunoblotted with α-Myc to confirm immunoprecipitation efficiency of full-length and soluble Sema6A (Figure 3.2A, right). Interestingly, the banding pattern of the soluble Sema6A variant, sSema6A, suggests that it could contain post-translational modifications such as glycosylation. The major portion of each immune complex was run on SDS-PAGE and coomassie stained (Figure 3.2B). Although not
Figure 3.1. Semaphorin ectodomain peptides identified in Zappaterra et al. in human and rat embryonic cerebrospinal fluid. A. Schematic illustrating the semaphorin (Sema) proteins that we previously identified in human and rat embryonic cerebrospinal fluid (eCSF). Asterisks indicate positions of peptides discovered in rat CSF while triangles indicate positions of peptides discovered in human CSF. Of note, all peptides identified were in the ectodomains. B. Table summarizing the number of peptides identified in each sample. If a semaphorin protein is not listed, it was not identified. C-D. HEK293 cells (C) and N2A cells (D) cells overexpressing an expression construct encoding human Sema6A were incubated in OPTI-MEM reduced serum media overnight. The conditioned media was collected and cells were lysed. Samples were run on SDS-PAGE and immunoblotted with α-Sema6A. A smaller protein of approximately 95 kDa was detected in the conditioned media (arrowhead) of cells expressing full-length (FL) Sema6A (arrow).
detectable by coomassie staining, the region of the gel corresponding to the molecular weight of the soluble Sema6A product, 85-110 kDa, was excised, digested with trypsin, and tryptic peptides were analyzed using LC-MS/MS. Mapping the peptide coverage of the soluble Sema6A fragment, we found only peptides of the ectodomain (Figure 3.2C, Supplemental Table 3.1) consistent with the sSema6A variant encompassing the ectodomain alone. As Semas are known glycoproteins and glycosylation can hinder peptide detection, we conducted glycosylation prediction analyses using three separate prediction software programs for both O- and N-linked glycosylation (Supplemental Table 3.2). The ectodomain residues that were predicted to be glycosylated in at least two prediction programs were mapped to the Sema6A protein (Figure 3.2C, labeled as “O” and “N”). In an attempt to determine the C-terminus of sSema6A, the last tryptic peptide detected was LTFEQDIER at amino acid positions 551-559. A SEQUEST analysis with no enzyme specified identified the peptide GNTDGLGDCHN at amino acid positions 560-570 (Supplemental Figure 3.1). With the discovery of the possible C terminus of sSema6A, we hypothesize that the ectodomain is released by proteolytic cleavage after asparagine 570, approximately 80 amino acids distal to the transmembrane domain.

As we purified the full length and ectodomain Myc-Sema6A proteins via immunoprecipitation, we also conducted mass spectrometry analysis to determine binding partners of full-length cell-bound Sema6A and the sSema6A ectodomain released into the media. We identified 373 proteins interacting with full-length Sema6A (Supplemental Figure 3.3). Of these, tubulin and cadherin binding proteins were enriched as analyzed by GO Molecular Functions and Cellular Components using Metascape.
Figure 3.2. Mass spectrometry analysis reveals the Sema6A ectodomain as the soluble Sema6A product. HEK cells were transfected with an expression plasmid encoding Myc-SEMA6A. Immunoprecipitation with α-Myc was used to purify the full-length cell-bound Sema6A, and the soluble Sema6A contained in the media. A. Whole cell extracts and a small portion of the immune complexes were subjected to SDS-PAGE and western blotting to verify SEMA6A expression and a successful immunoprecipitation. Arrows indicate the full length protein and arrowheads indicate the smaller soluble Sema6A form. The multiple bands of Sema6A may indicate post-translational modifications such as glycosylation. B. The major portion of the immune complexes was subjected to coomassie staining. Each sample lane was excised according to molecular weight, digested with trypsin, and prepared for tandem mass spectrometry. C. The tryptic peptides from bands corresponding to a molecular weight of the soluble Sema6A variant, approximately 85-110 kDa, were analyzed and mapped onto human Sema6A. Gray bars indicate the location and spectral count of the tryptic peptides identified. The starred bar indicates the non-tryptic peptide detected which is predicted to be the cleavage site that produces sSema6A. As peptide coverage is negatively affected by glycosylation, we also mapped potential glycosylation sites. The letters “O” and “N” indicate predicted O- or N-linked glycosylation sites, respectively, that were identified from at least two independent glycosylation prediction programs.
enrichment analyses (Supplemental Figure 3.2). For the sSema6A ectodomain binding partners, 24 proteins were identified (Supplemental Table 3.4). Of these, proteins involved in binding kinases and inhibiting enzymes were enriched (Supplemental Figure 3.3).

3.3.3. Naturally-released sSema6A can be regulated by PKC

To verify that sSema6A production does not occur simply due to over-expression or cell death, we determined if sSema6A can be produced in a regulated manner. To examine this, we first conducted a time course analysis of sSema6A release into the media. After 24 hours, transfected HEK cells were incubated with OPTI-MEM reduced media for various amounts of time, from 0 minutes to 18 hours (Figure 3.3A-B). We were able to detect sSema6A in as little as 2 hours and, although variable, consistently detected sSema6A at 6 hours, and observed a plateau at 18 hours. As proteases and kinases are known to regulate the release of ectodomains [33-35], we asked whether the activation of PKC through phorbol 12-myristate 13-acetate (PMA) stimulation could enhance sSema6A production. Indeed, a 30 minute PMA stimulation (100 nM) at the end of the 6 hour conditioned media incubation of Sema6A-expressing cells caused a significant increase of sSema6A (Figure 3.3C-D). To verify that PMA-induced sSema6A release is occurring through PKC activation, we pre-treated cells with the specific PKC inhibitor Bisindolylmaleimide 1 (BIM1, 5 µM, 30 minutes) before stimulating cells with PMA. PKC inhibition using BIM1 resulted in a significant decrease of sSema6A and brings sSema6A levels back to baseline (Figure 3.3E-F). Lastly, to determine if PKC-induced sSema6A production is occurring through the Mek/Erk pathway, a well-known
Figure 3.3. Naturally released sSema6A can be regulated by PKC.
Figure 3.3. Naturally released sSema6A can be regulated by PKC. HEK293 cells were transfected with an expression construct encoding human Myc-Sema6A. Following overnight growth, HEK293 cells were incubated in OPTI-MEM reduced-serum media for the indicated amount of time or 6 hours if not indicated. The conditioned media was then collected and the cells were lysed. Proteins were concentrated from the conditioned media using TCA precipitation. Protein samples were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. A. The time course of detectable sSema6A production, which was normalized to 18 hours, quantified in B. As sSema6A was consistently detected at 6 hours, this time point was selected for subsequent pharmacological experiments. C. Sema6A-expressing cells were treated with the PKC activator PMA (100 nM, 30 minutes) or DMSO vehicle alone during the last 30 minutes of the 6-hour conditioned media incubation. The Log2 fold change in sSema6A production with PMA treatment was normalized to the Sema6A untreated group and quantified in D ($p < 0.0001$). E. PMA-induced sSema6A production is due to PKC activation. Sema6A-expressing cells were pre-treated with the selective PKC inhibitor BIM1 (5 µM) for 30 minutes prior to PMA stimulation. Log2 fold change sSema6A levels were normalized and quantified in F (+PMA/-BIM1 compared to +PMA/+BIM1 $p < 0.018$; -PMA/+BIM1 compared to +PMA/-BIM1 $p < 0.039$). G. PMA/PKC-induced sSema6A production is independent of the Mek/Erk pathway. Sema6A-expressing cells were pre-treated with the Mek inhibitor U0126 (10 µM) for 30 minutes prior to PMA stimulation. Log2 fold change sSema6A levels were normalized and quantified in H ($p < 0.012$).
downstream target of PKC, we pre-treated cells with the specific Mek inhibitor U0126 (10 µM, 30 minutes) before stimulating cells with PMA (Figure 3.3G-H). Although the Mek inhibitor blocked PMA-induced Erk activation, we observed no significant decrease in sSema6A release in Mek-inhibited cells, suggesting that PKC-induced sSema6A production occurs through a Mek/Erk-independent mechanism.

### 3.3.4. PKC-induced phosphorylation of Sema6A

To begin to address how PKC can be inducing Sema6A ectodomain release, we hypothesized that a conformational change, possibly due to phosphorylation, could be occurring which allows Sema6A to be more susceptible to ectodomain processing. We asked if Sema6A phosphorylation can be induced by PKC activation. Indeed, we detected basal Sema6A phosphorylation using a phospho-motif-specific antibody, α-RXXpS/T, which recognizes the phosphorylated minimal target motif of PKC as well as several AGC family kinases. This phospho-signal can be inhibited when PKC is blocked with BIM1 (Figure 3.4A). This phosphorylation increases when PKC is activated with PMA and this increase can be blocked by pre-treatment with BIM1 (Figure 3.4A, quantified in 3.4B). While we cannot rule out indirect PKC-dependent phosphorylation, the major kinase targeting RXXS/T sites downstream of PKC is RSK, and RSK is dependent on Mek-Erk signaling [36, 37]. Bioinformatics show that the Sema6A intracellular domain contains multiple AGC kinase/PKC phosphorylation target motifs (Figure 3.4C). To identify phosphorylated RXXpS motifs, we subjected Myc-Sema6A immunoprecipitation samples to mass spectrometry analysis. We identified three phosphorylation sites: S952, S988, and S1005 within PKC-preferred RXXS motifs (Supplemental Figures 3.4-6).
While future validation is required, bioinformatics and mass spectrometry data suggest that these sites could be phosphorylated by PKC which may be one mechanism of regulating sSema6A production.

3.3.5. Naturally released sSema6A promotes cellular cohesion of early eye fields

To determine if spontaneously released sSema6A was functional, we collected conditioned media from Sema6A-expressing and untransfected HEK cells. Sema6A-PlxnA2 signaling is known to be required for proper eye vesicle integrity, as decreasing levels of either Sema6A or PlxnA2 in zebrafish embryos results in ectopic cells and loss of eye field cohesion [11, 26]. We have previously developed a cohesion-based assay to test the function of secreted forms of Semas in zebrafish eye explants (Figure 3.5A) [26]. Sema6A-FC is known to promote cellular cohesion of early eye fields as stimulating whole eye explants at 18 hours post fertilization (hpf) with Sema6A-FC results in less ectopic cells [26]. We used this assay to determine if sSema6A can regulate cells of the early eye field. We used eye explants from rx3:GFP zebrafish, which drives GFP expression in retinal precursor cells, enabling observation of the integrity of the early eye field. Consistent with published findings, stimulating 18 hpf eye explants with sSema6A-containing conditioned media for 6 hours resulted in increased eye field cohesion, compared to conditioned media from untransfected control cells, as sSema6A resulted in a significant decrease in the number of GFP-positive ectopic cells (Figure 3.5B-E, quantified in 3.5F). The presence of sSema6A in the Sema6A conditioned media was verified via Western Blotting (Figure 3.5G).
Figure 3.4. PKC-induced phosphorylation of Sema6A. A-B. HEK293 cells expressing Myc-Sema6A were stimulated with PMA or DMSO vehicle control, with or without pre-treatment of BIM1. Sema6A was purified via immunoprecipitation with α-Myc and immune complexes were subjected to SDS-PAGE and immunoblotting with a PKC/AGC kinase phospho-motif specific antibody, α-RXXpS/T. A. Untreated cells show baseline RXXpS/T Sema6A phosphorylation while PMA stimulation increases this phosphorylation. BIM1 abolishes the baseline and PMA-induced phosphorylation, as quantified in B. Phosphorylation levels and total protein levels were normalized to Sema6A +PMA/+BIM1 and the Log2 fold change was calculated using the fraction phosphorylated/total Sema6A (-PMA/-BIM1 compared to +PMA/+BIM1 \( p < 0.0087 \); +PMA/-BIM1 compared to +PMA/+BIM1 \( p < 0.0042 \)). Dashed line indicates cropping together due to a tear in the gel. See Figure 3E for whole cell extract Myc-Sema6A expression levels and RXXpS/T protein levels. C. Bioinformatics indicate four RXXS sites predicted (ScanSite) and/or observed to be phosphorylated in large-scale proteomic studies (PhosphoSitePlus). Mass spectrometry analysis of Sema6A identified three of these sites to be phosphorylated (Supplementary Figures 3.4-3.6).
Figure 3.5. Naturally released sSema6A promotes cellular cohesion of early eye fields. A. Eyes were dissected from 18 hpf embryos of \textit{rx3}:GFP transgenic zebrafish, which have GFP-positive retinal precursor cells. Individual eye explants were cultured in individual wells of a 96-well plate containing conditioned media from either untransfected or Sema6A-expressing cells for 6 hours. B-E. After incubations in conditioned media, brightfield (B, C) and fluorescent (D, E) images of the eye explants were taken and eye field cohesion was assessed by counting the number of GFP-positive ectopic cells, quantified in F, \( n=26 \) embryos incubated in control media and \( n=23 \) embryos incubated in sSema6A media, \( p = 0.009 \). Scale bar represents 75 \( \mu \)m. G. Cell extracts and conditioned media of untransfected or Sema6A-expressing cells were subjected to SDS-PAGE and immunoblotted with \( \alpha \)-Sema6A to verify protein expression and the presence of sSema6A in the conditioned media.
3.4. Discussion

We report here that the transmembrane Sema6A can be naturally released from the cell as a soluble ectodomain fragment and that this sSema6A functions in maintaining the integrity of the early eye field in explant studies. We show that sSema6A can be produced in common cell lines, including a neuronal cell line, and that this release is a regulated process that can be induced by PKC activation. This increase in sSema6A levels is correlated with an increase in full-length Sema6A phosphorylation at intracellular PKC phosphorylation motifs, suggesting this could be one mechanism regulating Sema6A’s susceptibility to ectodomain processing. Similarly, there is evidence that Sema4D ectodomain shedding may be regulated by phosphorylation [22] and future work will investigate if Sema6A phosphorylation is necessary for ectodomain release.

To our knowledge, Sema6A is the first Sema6 class capable of being converted naturally by cells into a soluble ectodomain that is functional in the nervous system. With the addition of this report, it is now clear that ectodomain release is common to each of the vertebrate and membrane-bound Sema classes (Figure 3.6). The transmembrane Sema4D and the GPI-linked Sema7A have soluble, released forms that are involved in monocyte and B cell migration and stimulating monocyte cytokine production, respectively [17, 18, 21, 22, 38]. Additionally, sSema5B is released by ADAM-17 and functions as a repulsive guidance cue in dorsal root ganglion explants [19]. Furthermore, the Sema6D ectodomain is released from cells expressing full-length Sema6D, with possible functional roles in cardiac development [39]. While the protease responsible for cleaving Sema6A remains unknown, mass spectrometry analysis suggests cleavage
occurs between amino acids 570 and 571. The proteases involved in producing all of the known sSemas have not yet been identified. However, membrane-type 1 MMP has been shown to cleave Sema4D while ADAM-17 is sufficient to cleave Sema5B and Sema7A [19, 38, 40]. Interestingly, substrate O-glycosylation can influence ADAM-mediated ectodomain processing [41] and our bioinformatics analyses show that Sema6A may be heavily glycosylated near the predicted cleavage sequence.

The signaling mechanisms of sSema6A have yet to be determined. Semas act as homodimers that bind to Plxn receptors and the secreted Sema3 class requires neuropilin co-receptors. Thus, the nature of how sSema6A binds to its receptor and whether or not it is different from transmembrane Sema6A remains to be deciphered. The roles of recombinant Sema6A ectodomain proteins have previously been studied and suggest that it acts as a repulsive cue, blocking endothelial cell migration and regulating apoptosis in lung cancer cells [42, 43]. We hypothesize that the soluble form is a way to propagate the effects of Sema6A over greater distances. Sema6A cleavage could also allow for autocrine signaling or to down-regulate surface expression. Additionally, sSema6A may be regulating the direction of signaling. As a transmembrane Sema, Sema6A exhibits reverse signaling resulting in collapse of Sema6A-expressing cells, possibly through interactions with Mena and Ena/VASP-like protein (EVL) [28, 44]. The discovery of a naturally released Sema6A that is functional in nervous system development suggests that Sema6A can have both contact-mediated and long-range effects and sSema6A may be one process by which cells control the activation of forward-only signaling, or control reverse signaling in a dominant-negative fashion. This report opens the field to
Figure 3.6. Schematic depicting the structural domains of Semas and indicating Semas whose ectodomains can be naturally released from cells. There are 2 invertebrate classes and 5 vertebrate classes of Semas (*class 5 Semas are found in vertebrates and invertebrates). ‡Although Semas can have broad expression patterns and diverse functions, this figure focuses on those systems in which the functional role of the indicated Sema class has been highly characterized. The major domains of each Sema class are shown. The specific Sema family members known to produce a soluble ectodomain are labeled. ^Sema4B and Sema4C have been shown to be substrates of BACE proteases, although a naturally-released ectodomain has not yet been characterized [32, 45]. †Some viral Semas do not have an Ig-like domain. The schematic was inspired in part by a previous review [46].
investigate the nature of sSema6A binding and the mechanisms of sSema6A processing and signaling.

3.5. Materials and Methods

3.5.1. Plasmids and Molecular Cloning

The untagged human Sema6A cDNA (Genbank reference BC032619.1 and IMAGE reference 5578066) in pCMV-Sport6 was acquired from Open Biosystems/Thermo Scientific (Huntsville, AL, USA). The post-signal sequence, N-terminally tagged human Myc-Sema6A in pCAGGS vector was published previously [28] and generously provided by Kevin J. Mitchell at Trinity College (Dublin, Ireland) and Alain Chédotal, Céline Heitz and Yvrick Yager from the Institut de la Vision, INSERM (Paris, France).

3.5.2. Cell Culture and Transfections

HEK293 cells were maintained in DMEM with sodium pyruvate, L-glutamine, and 4.5 g/L glucose (MediaTech/Corning Life Sciences, Tewksbury, MA, USA). This media was supplemented with 5% Fetal Bovine Serum (Hyclone, Logan, UT, USA), 5% Cosmic Calf Serum (Hyclone), 50 units/mL penicillin and 50 µg/mL streptomycin (Pen Strep, Invitrogen, Carlsbad, CA, USA). Cells were cultured to 75% of confluence at 37 °C and 5% CO₂ and were then transfected with 5-7.5 µg Sema6A expression plasmids using calcium phosphate precipitation.

3.5.3. Conditioned Media, Pharmacological Treatments, and Western Blotting
Following transfection, cells were incubated at 37 °C and 5% CO₂ for 6 hours and then washed with warm PBS and incubated overnight in full medium. The cells were then washed again with warm PBS and incubated for the indicated amount of time in OPTI-MEM (ThermoFisher Scientific, Waltham, MA, USA) supplemented with Pen Strep. For pharmacological studies, cells were incubated in OPTI-MEM for 6 hours, with or without stimulation with 100 nM PMA (Biomol, Enzo Life Sciences, Plymouth Meeting, PA, USA) for the final 30 minutes. Pre-treatment of 5 μM BIM1 (EMD/Millipore Sigma, Burlington, MA, USA) or 10 μM U0126 (Biomol) occurred for 30 minutes immediately prior to PMA stimulation. The conditioned media was collected and clarified by centrifugation at 4,000 rpm for 5 minutes.

Cells lysis was as previously described [47] in lysis buffer (25 mM Tris pH 7.4, 137 mM NaCl, 10% glycerol, 1% Igepal) containing protease inhibitors (5 μg/mL Pepstatin, 10 μg/mL Leupeptin, 1 mM PMSF) and phosphatase inhibitors (1 mM NaVO₃, 25 mM NaF, 10 mM Na₂H₂P₂O₇). For immunoblotting, 20-30 μg total protein extract was denatured in protein sample buffer (125 mM Tris pH 6.8, 7.5% glycerol, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and bromophenol blue at 0.02%) at 95 °C for 5 minutes and subjected to SDS-PAGE as previously described [47]. Proteins were precipitated from the conditioned media using 15% trichloroacetic acid (TCA) as previously described [48] before resuspension in sample buffer. The primary antibodies, α-Sema6A (rabbit pAb, 1:1000, Novus Biologicals, Littleton, CO, USA) or α-Myc (rabbit mAb, 1:1000, Cell Signaling Technology, Beverly, MA, USA), were diluted in 10 mL of 1.5% BSA in TBST containing 0.0005% sodium azide and incubated with the
membranes overnight, rocking at 4 °C. The secondary antibody was α-rabbit (goat IgG, 1:15,000, EMD Millipore, Billerica, MA, USA). Protein bands were detected using enhanced chemiluminescence (ThermoFisher Scientific) and film, which was developed using a Medical Film Processor SRX-101A (Konica Minolta Medical & Graphic, Tokyo, Japan). The raw film scans were converted to grayscale and brightness was optimized in Adobe Photoshop CS2 (San Jose, CA, USA). Densitometry analysis was conducted on the raw film scans using the mean histogram tool in Photoshop. The sSema6A levels were normalized to the untreated sSema6A α-Myc band and the Log2 fold change was calculated. For Sema6A phosphorylation experiments, densitometry analysis was conducted as previously described [49] by first subtracting the background film signal then normalizing the phosphorylated Sema6A (α-RXXpS/T) and total Sema6A (α-Myc) to the +PMA/+BIM1 group. The Log2 fold change was calculated using the fraction of normalized phosphorylated Sema6A over the normalized total Sema6A levels. A student’s T-test comparison was performed on the data from 2-3 independent experiments using GraphPad Prism 7 (La Jolla, CA, USA).

3.5.4. Mass Spectrometry

To identify the tryptic peptides of the Sema proteins detected in human and rat embryonic cerebrospinal fluid previously published [23], the original raw mass spectrometry results were subjected to a SEQUEST search using the forward and reverse concatenated International Protein Index (IPI) human and rat databases. The same filter parameters were used as in the previous study except proteins were retained if two or more unique peptides were identified, as opposed to three or more.
The conditioned media of HEK293 cells either untransfected or expressing Myc-Sema6A was collected and cells were lysed. Sema6A protein from extracts and conditioned media was purified by immunoprecipitation with 7.5 µL α-Myc resin (BioLegend, San Diego, CA, USA) supplemented with 1 µg α-Myc (Cell Signaling) and 2.5 µL each of Protein A and G beads, rocking at 4 °C overnight. Immune complexes were washed three times with lysis buffer, drained completely, and denatured in sample buffer. Boiled samples were loaded onto a 10% SDS-PAGE gel. After running, gels were stained with coomassie (0.1% coomassie brilliant blue R-250, 20% glacial acetic acid, 40% methanol). The entire gel lanes were excised and prepared for mass spectrometry as previously described [50]. Briefly, gel regions were diced and proteins were reduced and alkylated prior to being subjected to in-gel digestion with 6 ng/ul trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate at 37 °C for 9-12 hours. The tryptic peptides were resuspended in 2.5% acetonitrile, 0.15% formic acid and separated via HPLC prior to MS/MS analysis on a Q Exactive Plus mass spectrometer controlled with Thermo Xcalibur 4.0 software (ThermoFisher Scientific). Peptides were eluted and analyzed as previously described [51]. A SEQUEST search of the mass spectrometry data was performed using the forward and reverse concatenated 2011 Uniprot Human Protein database requiring tryptic peptides and permitting phosphorylation of serine, threonine and tyrosine (+79.9663 Da), oxidation of methionine (+15.9949 Da), and acrylamidation and carbamidomethylation of cysteine (+71.0371 and +57.0215, respectively). XCorr filters for peptide identification were set at 1.8, 2.0, 2.2, 2.4, and 2.6 for +1, +2, +3, +4, and +5 charged peptides, respectively. Proteins were filtered by excluding those proteins
that were not identified by three or more unique peptides. Proteins were considered to be binding partners after subtracting non-specific proteins found in untransfected control groups.

The peptide coverage of the soluble Sema6A product detected in the conditioned media was determined by analyzing the peptides corresponding to the molecular weight 85-110 kDa. A SEQUEST search was performed as described above and Sema6A was identified with a false discovery rate (FDR) of 0.00%. To identify additional Sema6A peptides, the XCorr filters were relaxed to 0.8, 1.1, 1.4, 1.7, and 2.0 for +1, +2, +3, +4, and +5 charged peptides, respectively. This did not increase the FDR and those additional peptides that emerged were manually validated. To identify the putative C-terminus/cleavage site of sSema6A, a similar SEQUEST search using the forward and reverse human Sema6A Uniprot sequence was performed but without requiring tryptic peptides (no enzyme search).

3.5.5. Bioinformatics methods

To predict O- and N-linked glycosylation sites on the ectodomain of human Sema6A, the following glycosylation prediction software programs were used: NetOGlyc 4.0 [52], GlycoEP [53], YinOYang 1.2 [54] for O-glycosylation sites and ExPASy ScanProsite [55], GlycoEP [53], and NetNGlyc 1.0 [56] for N-glycosylation sites. If a site was predicted to be glycosylated in two or more prediction programs, it was considered likely to be glycosylated.
Metascape [57] (http://metascape.org) was used to determine the Gene Ontology (GO) classifications and enrichment scores of the Sema6A-FL and sSema6A binding partners. Of the 373 binding partners identified for Sema6A-FL, those proteins identified by the top quartile of peptides (9+ peptides, n = 101 proteins) were used in the analyses. All of the 24 binding partners of sSema6A were used in the analyses. Proteins were classified based on GO Molecular Functions and GO Cellular Components and an enrichment analysis was conducted using the human genome as background with a minimum enrichment of 1.5 and a p value less than 0.01.

PKC phosphorylation motifs, RXXpS, on Sema6A were identified by using ScanSite 4.0 [58] (MIT, Cambridge, MA, USA). PhosphoSite Plus [59] (Cell Signaling Technology) was used to identify phospho-serine residues in RXXS motifs that were previously found by large-scale proteomics studies.

3.5.6. Zebrafish Husbandry and Eye Explant Experiments

Zebrafish embryos were developmentally-staged as previously described [60]. The rx3:GFP transgenic zebrafish line, which expresses GFP in retinal precursor cells, was generously provided by Dr. Joachim Wittbrodt at the University of Heidelberg, Germany. All procedures were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC). Zebrafish embryos were collected at the one-cell stage and incubated at 25 °C for approximately 24 hours until the 18 somite stage was reached [60]. At 18 somites, whole eyes were dissected from GFP-positive embryos as previously described [26] using a Nikon SMZ800 brightfield dissecting microscope.
with an add-on GFP fluorescent lamp and filter set (NightSea, Hatfield, PA, USA). Dissected eyes were transferred to individual wells of a 96-well plate containing 100 µL of either untransfected control conditioned media or Sema6A conditioned media and incubated at 28 °C and 5.0% CO₂ for 6 hours. After conditioned media treatment, eye explants were imaged at 40X magnification using SPOT imaging software version 5.2 (Sterling Heights, MI, USA) on an Olympus inverted epifluorescent microscope. Figures were generated using Adobe Photoshop CS6, where image brightness was normalized and optimized. Quantification of eye explants was performed in Image J version 1.48a (National Institutes of Health, USA) by counting the number of ectopic GFP-positive precursor cells that had dissociated from the eye field following 6 hours of conditioned media incubation. Images were blinded prior to quantification and the average number of ectopic cells in each treatment was analyzed using GraphPad Prism 6 (La Jolla, CA, USA).
3.6. References


3.7. Supplemental Tables and Figures

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**Supplemental Table 3.1. Tryptic peptides detected for Sema6A in the conditioned media of Sema6A-expressing cells.** This table shows the location, sequence, charge, and SEQUEST cross-correlation value (XCorr), a peptide confidence score, of the 17 unique and 41 total tryptic peptides found in the 85-110 kDa range of the conditioned media sample. **The non-tryptic peptide at amino acid positions 560-570 is shown that was identified using SEQUEST analysis with no enzyme specified. Residue modifications are shown as follows: # acrylamidation of cysteine, * oxidization of methionine, ^ carbamidomethylation of cysteine.**
### Supplemental Table 3.2. Predicted O- and N-linked glycosylation sites on extracellular Sema6A

A. Three O-linked glycosylation prediction software programs (NetOGlyc, GlycoEP, and YinOYang) predicted the serines and threonines to be glycosylated. B. Three N-linked glycosylation prediction software programs (ExPASy ProSite, GlycoEP, and NetNGlyc) predicted the asparagines to be glycosylated. Those sites that were predicted to be glycosylated in at least two of the prediction software programs were considered likely to be glycosylated and indicated in the Final Sites column. *N434 has been found to be glycosylated in a large-scale murine proteomics study (PhosphoSitePlus).

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#### B. N-linked Glycosylation Predictions

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Supplemental Table 3.3. Sema6A binding partners. HEK293 cells were transfected with expression constructs encoding full-length Myc-Sema6A. After 48 hours, cells were lysed and Sema6A was immunoprecipitated using α-Myc. Immune complexes were run on a SDS-PAGE gel and subjected to commassie staining. The entire lane was cut and proteins were digested with trypsin and prepared for mass spectrometry. After subtracting non-specific binding partners from mock-transfected cells, 373 proteins were detected to interact with Sema6A. Proteins are sorted by spectral count, the number of peptides that were detected for each protein.
**Supplemental Table 3.4. The Binding Partners of the Soluble Sema6A Ectodomain.**

The conditioned media of HEK293 cells expressing full-length Myc-Sema6A was collected and soluble Sema6A was immunoprecipitated using α-Myc. Immune complexes were run on a SDS-PAGE gel and subjected to commassie staining. The entire lane was cut and proteins were digested with trypsin and prepared for mass spectrometry. After subtracting non-specific binding partners from collected media of mock-transfected cells, 25 proteins were detected to interact with sSema6A. Proteins are sorted by spectral count, the number of peptides that were detected for each protein.

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Supplemental Figure 3.1. Amino acid sequence GNTDGLGDCHN is a potential C-terminus of the soluble Sema6A ectodomain. Conditioned media from Myc-Sema6A-transfected HEK293 cells was collected and the soluble Sema6A ectodomain was purified by immunoprecipitation with α-Myc. Immune complexes were subjected to SDS-PAGE and coomassie staining. The region of the gel corresponding to the molecular weight of sSema6A, 85-110 kDa, was excised, digested with trypsin and prepared for mass spectrometry. A SEQUEST search with no enzyme specified enabled the detection of a peptide indicating the potential C-terminus. The spectra (A) and predicted and observed b/y ion masses (B) of one representative peptide identified (3 total peptides identified). ^ carbamidomethylation
Supplemental Figure 3.2. Gene Ontology Annotation and Enrichment Analysis of Sema6A-FL binding partners. HEK293 cells expressing Myc-Sema6A-FL were lysed and FL Sema6A was purified by immunoprecipitation with α-Myc. Immune complexes were subjected to SDS-PAGE and coomassie staining. The entire lane was excised, digested with trypsin and prepared for mass spectrometry. A SEQUEST search identified peptides and proteins. Non-specific binding partners were removed by subtracting those proteins present in the untransfected control immunoprecipitation group. Of the 373 binding proteins with three or more peptides identified, the top 25% were subjected to GO annotation analysis and enrichment analysis relative to the entire human genome via Metascape Software. A-B are analyses using GO Molecular Functions. B-C are analyses using GO Cellular Components.
Supplemental Figure 3.3. Gene Ontology Annotation and Enrichment Analysis of sSema6A-binding partners. sSema6A was purified via α-Myc immunoprecipitation from the conditioned media of HEK293 cells expressing Sema6A-FL. Immune complexes were subjected to SDS-PAGE and coomassie staining. The entire lane was excised, digested with trypsin and prepared for mass spectrometry. A SEQUEST search identified peptides and proteins. Non-specific binding partners were removed by subtracting those proteins present in the untransfected control immunoprecipitation group. The 24 binding proteins identified by three or more peptides were subjected to GO annotation analysis and enrichment analysis relative to the entire human genome via Metascape Software. A-B are analyses using GO Molecular Functions. B-C are analyses using GO Cellular Components.
Supplemental Figure 3.4. CID MS/MS spectra of peptides with phosphorylated and unphosphorylated RXXpS site S952 found on the intracellular domain of Sema6A.
Supplemental Figure 3.5. CID MS/MS spectra of peptides with phosphorylated and unphosphorylated RXXpS site S988 found on the intracellular domain of Sema6A.
Supplemental Figure 3.6. CID MS/MS spectra of peptides with phosphorylated and unphosphorylated RXXpS site S1005 found on the intracellular domain of Sema6A.
Supplemental Figures 3.4-3.6. CID MS/MS spectra of peptides with phosphorylated and unphosphorylated RXXpS sites found on the intracellular domain of Sema6A. HEK293 cells expressing Myc-Sema6A were lysed and Sema6A was purified via immunoprecipitation with α-Myc. Immune complexes were subjected to SDS-PAGE and coomassie staining. The band corresponding to full-length Sema6A was excised, diced, digested with trypsin, and analyzed via LC-MS/MS. A SEQUEST analysis and manual verification of the spectra found three sites to be phosphorylated within the PKC/AGC kinase-preferred motif RXXS: S952 (Supplemental Figure 4), S988 (Supplemental Figure 5), and S1005 (Supplemental Figure 6). The spectra of the phosphorylated peptides (A) are presented with their corresponding unphosphorylated peptides (B). A bracket (on Supplemental Figure 6) indicates the isotopic envelope and is labeled with the monoisotopic mass.
CHAPTER 4: THE PLXNA DOWNSTREAM EFFECTOR PHOSPHOPROTEIN
CRMP2 IS ESSENTIAL FOR ZEBRAFISH EYE DEVELOPMENT

4.1. Abstract

Plexins (Plxns) are a family of semaphorin (Sema) receptors that play essential roles in the developing nervous system and cardiovasculature as well as the activation of the immune response. We have previously shown that the transmembrane Sema6A and its PlxnA2 receptor are critical in vertebrate eye development, as morpholino-based knockdowns of Sema6A and PlxnA2 in zebrafish result in decreased early eye field cohesion, smaller eye size, and impaired retinal lamination. Yet, the molecular mechanisms downstream of Sema6A-PlxnA signaling are only partially understood. Collapsin response mediator proteins (CRMPs) are a family of microtubule-associated proteins that are regulated by phosphorylation initiated by Sema-Plxn signaling. Here, we show a novel role for Crmp2 in the development of the zebrafish visual system. Using in situ hybridization, we show that crmp2 mRNA is expressed in the retinal ganglion cell layer of the retina. Knockdown of Crmp2 results in smaller eye size, impaired optic tract formation, and disrupted retinal lamination. The effects of CRMPs may be mediated by phosphorylation on the C-terminal tail, which in turn is hypothesized to regulate microtubule dynamics. To test the role of CRMP2 phosphorylation, we made phospho-mimetic and non-phosphorylatable mutants. Using the zebrafish, we show preliminary data characterizing the in vivo role Crmp2 phosphorylation plays in vertebrate visual system development. Future work will test the functional significance of Crmp2 phosphorylation in visual system development using crmp2<sup>−/−</sup> CRISPR/Cas9 zebrafish.

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4.2. Introduction

Semaphorins (Semas) are extracellular cues that play critical roles in the development of numerous tissues, including the nervous system and cardiovascular [1]. Semas are a large family of secreted and membrane-bound ligands that typically signal through Plexin (Plxn) receptors to mediate cellular and axonal migration [1-3]. Sema-Plxn signaling is mediated through small GTPases and kinases that ultimately regulate integrin-dependent adhesion and cytoskeletal dynamics to cause growth cone collapse [4]. The microtubule associated protein CRMP2 (collapsin response mediator protein 2) is a necessary downstream effector protein of the canonical secreted Sema, Sema3A [5, 6]. CRMP2 is a phosphoprotein that binds to tubulin and microtubules to promote polymerization and stabilization in vitro and in cell culture systems [7, 8]. When phosphorylated, CRMP2 has a lower affinity for microtubules and is thought to destabilize microtubules leading to growth cone collapse [8, 9]. The serine/threonine kinases Cdk5 and GSK3β are activated downstream of Sema3A signaling and subsequently phosphorylate CRMP2 [8-10]. Cdk5 phosphorylates CRMP2 at S522, which primes CRMP2 to be processively phosphorylated by GSK3β at T509, T514 and S518. These phosphorylation steps are critical for Sema3A-mediated collapse, as alanine mutations rendering CRMP2 unable to be phosphorylated results in dorsal root ganglion cells resistant to Sema3A-induced growth cone collapse [9].

CRMP2 is expressed in the vertebrate brain, spinal cord and retina throughout nervous system development [5, 11, 12]. In the brain, CRMP2 is expressed in the cortex, hippocampus and cerebellum and in vivo studies have investigated CRMP2 function in
cortical lamination and pyramidal cell migration [11-14]. Little is known of the in vivo functions of CRMP2 in non-cortical laminated regions such as the retina. CRMP2 is expressed in the retinal ganglion cell (RGC) layer [5, 15] and has been shown to colocalize with microtubules in dissociated chick RGCs [16]. CRMP2 is a known downstream mediator of Sema3A [5, 6], which utilizes the PlxnA receptors PlxnA1-4 in complex with neuropilin co-receptors [17]. Similarly, the transmembrane Sema6A can signal through PlxnA2 and PlxnA4 [17, 18]. We have previously shown that Sema6A and PlxnA2 to be critical for vertebrate eye development, as anti-sense oligonucleotide-based knockdown of Sema6A or PlxnA2 in the zebrafish results in decreased early eye field cohesion, impaired retina lamination and smaller eye size [19, 20]. Therefore, we hypothesized that CRMP2 may play an important mediator of Sema6A-PlxnA2 signaling during zebrafish eye development. We show that crmp2 is expressed in the zebrafish retina throughout embryonic development, in similar cell layers as PlxnA2. Using an anti-sense oligonucleotide targeting crmp2, we herein report a novel role for Crmp2 in the developing zebrafish retina. We show that knockdown of Crmp2 expression results in smaller eye size, disrupted retinal lamination, and impaired optic tract formation. As phosphorylation is critical for CRMP2 functions in vitro, cell culture systems and in mouse cortical development [8, 9, 14], we hypothesize that CRMP2 phosphorylation downstream of Sema6A signaling is critical in these described novel roles. Using site-directed mutagenesis, we mutated the Cdk5- and GSK3β- dependent residues to alanine, rendering them unable to be phosphorylated at these four sites (CRMP2 4A: T509A, T514A, S518A, S522A). Future work will investigate the function of Crmp2
phosphorylation in vertebrate visual system development using \( crmp2^{+/+} \) CRISPR zebrafish.

### 4.3. Results

#### 4.3.1. Sema6A signaling molecules are expressed in the developing zebrafish retina

CRMP2 is expressed in the chick and zebrafish retinal ganglion cell layer and its expression pattern partially overlaps with PlxnA4 in the zebrafish brain and spinal cord [5, 15]. To recapitulate these findings and to compare the expression pattern to Sema6A and PlxnA2 in the retina, we performed \textit{in situ} hybridization to \( crmp2 \) (Figure 4.1). At 48 hours post fertilization (hpf), Crmp2 is expressed in the RGC layer which is maintained at 72 hpf, where it is also expressed in the inner nuclear layer (INL) which includes bipolar cell bodies (Figure 4.1C, F). We have previously reported the retina expression patterns of Sema6A and PlxnA2 [21, 22]. At 48 and 72 hpf, Sema6A is expressed in the RGC layer (Figure 4.1A, D). PlxnA2 is expressed primarily in the INL in addition to lower expression in the RGC layer (Figure 4.1B, E). Comparing the expression patterns indicates the PlxnA2 and Crmp2 have overlapping expression and Sema6A is expressed in adjacent cells. This suggests that Crmp2 may be downstream of PlxnA2 and involved in Sema6A-mediated functions in eye development.

To further determine the expression pattern of \( crmp2 \) throughout zebrafish embryonic development, \textit{in situ} hybridization was conducted at the 18 somite (18 hpf), 24 hpf, 36 hpf, 48 hpf, 60 hpf and 72 hpf stages (Figure 4.2). \( Crmp2 \) mRNA expression was observed in the brain and spinal cord throughout nervous system development.
Figure 4.1. Sema6A signaling molecules are expressed during zebrafish visual system development. *In situ* hybridization using anti-sense probes targeting Sema6A (A, D), PlxnA2 (B, E), and Crmp2 (C, F) mRNA at 48 hours post fertilization (hpf) (A-C) and 72 hpf (D-F) representing mid-late embryonic development. Zebrafish were fixed and embedded for sectioning. The transverse sections illustrated are at or near the center of the eye, where the optic tract leaves the retina. While mRNA-specific staining occurs, all three transcripts are expressed in the developing retina, specifically in the retinal ganglion cell layer (RGC) and inner nuclear layer (INL). TeO: optic tectum; DT: dorsal thalamus. The Sema6A and PlxnA2 figures (A, D, E) are re-printed with permission from *Gene Expression Patterns* Journal (Elsevier Publishing) from Ebert et al. (2012) under license number 4532031503659 and from Emerson et al. (2017) under license number 4532040354318, respectively.
Figure 4.2. CRMP2 is highly expressed in the cortex and retina throughout development. *In situ* hybridization using an anti-sense oligonucleotide crmp2 targeted probe shows crmp2 mRNA expression in zebrafish throughout embryonic development, at the 18 somite stage (A), 24 hpf (B), 36 hpf (C), 48 hpf (D), 60 hpf (E), and 72 hpf (F) stages. Lateral views (A-F) and dorsal views (A’-F’) of whole-mount *in situ* are shown. Crmp2 is highly expressed in the nervous system, including the forebrain (FBr), midbrain (MB), hindbrain (HB), ear (Ea) and Rohan Beard neurons (RBN). Transverse sections (C”-F”') of the retina show specific staining in the retinal ganglion cell layer (RGC) and inner nuclear layer (INL).
Specific staining was seen in the developing forebrain (FBr), ear (Ea) and Rohan Beard neurons (RBN), which are early sensory neurons of the spinal cord, at the 18 somite and 24 hpf stages (Figure 4.2A-B’). Crmp2 expression becomes more discreet at 36 hpf (Figure 4.2C-C”), with visible staining in the neural retina by 48 hpf and remaining throughout retina development until 72 hpf (Figure 4.2D-F”). Transverse sections show that crmp2 retina expression is confined to the RGC and INL layers (Figure 4.2D”-F”).

4.3.2. Crmp2 is necessary for zebrafish eye size

As Sema6A and PlxnA2 are necessary for zebrafish eye size [20], we first asked the question if Crmp2 has a similar role. As an initial assessment of Crmp2 function in the zebrafish visual system, we visualized gross morphological changes upon knockdown of endogenous Crmp2 expression. We injected increasing doses of a splice-blocking anti-sense oligonucleotide (MO) targeting crmp2 at the one-cell stage of TL or AB zebrafish (Figure 4.3A, left). To verify crmp2 knockdown efficiency, RNA was isolated from uninjected (UIC) or MO embryos at 24 hpf and a cDNA library was made using RT-PCR. Primers recognizing mature crmp2 mRNA were used for semi-quantitative analysis of knockdown efficiency (Figure 4.3A, right). Zebrafish were developmentally staged at 72 hpf and eye size was measured, normalized to head size. Normalized eye size decreased in a dose-dependent manner (Figure 4.3B-C). To determine if this phenotype occurs due to a general developmental delay, UIC and crmp2 morphant embryos were developmentally staged at 5 days post fertilization (dpf) and normalized eye size was measured. At 5 dpf, crmp2 morphant embryos still have a significantly smaller eye size.
Figure 4.3. Crmp2 is necessary for zebrafish eye size. A. Zebrafish were injected at the one-cell stage with varying amounts of crmp2 morpholino (MO). B. Endogenous knockdown occurs via blocking intron 1 splicing, thus removing exon 2 and encoding for a truncated protein. C. The efficiency of endogenous crmp2 knockdown was determined by detecting mature crmp2 mRNA levels using semi-quantitative RT-PCR. D. Eye size was measured at 72 hpf and quantified by normalizing eye size to head size in two independent experiments, quantified in E. Brightfield images are of representative embryos from each group. The black circle overlay shows the relative eye size of an uninjected control (UIC) embryo. UIC = 63 n, 2 ng MO = 40 n, 4 ng MO = 54 n, 6 ng MO = 56 n. **p = 0.0003. ****p < 0.0001.
(Figure 4.4A-B) suggesting that the observed decreased eye size is not due to a developmental delay.

4.3.3. Crmp2 is necessary for retina lamination

As Crmp2 is necessary for proper cortical lamination [13, 14], and we observed smaller eye size in crmp2 morphants, we hypothesized that Crmp2 may play a role in non-cortical lamination, such as in the retina. Increasing doses of crmp2 MO were injected at the one-cell stage. Embryos were developmentally staged at 72 hpf, fixed, embedded and sectioned transversely through the retina. The cells of the retina were visualized using hemotoxylin and eosin (H & E) staining, which showed retinal lamination defects in a dose-dependent manner as well as a decrease in retina area at higher doses of MO (Figure 4.5A-C). The retinal abnormalities in crmp2 morphants appeared to be due to a lack of the inner and outer plexiform layers as evident in the H & E staining. Quantifying the number of retina layers using the plexiform layers as boundaries showed that higher doses of crmp2 MO results in less retina layers due to weakened or absent inner and outer plexiform layers (Figure 4.5B). Morpholinos have been shown to have off-target effects by activating p53-mediated apoptosis [23]. To ensure that the crmp2 MO does not non-specifically activate p53, the highest dose of crmp2 MO was co-injected with a p53-targeted MO. Although more variable, co-injecting the p53 MO does not fully rescue the crmp2 MO-induced defect in retina layers, suggesting that the non-specific p53 activation plays little role in eye size (Figure 4.5B). In contrast, co-injecting p53 MO rescues the crmp2 MO-induced decrease in retina area (Figure 4.5C).
**Figure 4.4. Impaired retinal development by decreasing crmp2 expression is not due to a developmental delay.** Zebrafish were injected with 8 ng crmp2 morpholino (MO) at the one-cell stage. A. Brightfield images of representative embryos at 5 days post fertilization (dpf). B. Eye size was quantified and normalized to head size in two independent experiments. UIC = 34 n, MO = 30 n. p <0.0001.
To qualitatively investigate the specific retina cell layers, we repeated the experiment using Spectrum of Fates (Sofa1) transgenic zebrafish which have RFP+ retinal ganglion cells (RGCs), GFP+ amacrine and horizontal cells and CYP+ bipolar cells and photoreceptors [24]. At higher doses of MO, photoreceptors and horizontal cells were absent while the RGC layer appeared thinner (Figure 4.5D-E).

4.3.4. Crmp2 is critical for optic tract development

With a thinner RGC layer of crmp2 morphant Sofa1 embryos, we asked whether or not the optic tract was impaired when Crmp2 is decreased. We repeated the experiment using isl2b:GFP zebrafish, which expresses GFP in RGC cell bodies and axons. At 72 hpf, the RGC layer and optic tract was impaired with increasing doses of crmp2 MO, as shown by confocal Z-stacks of a lateral view of the eye (Figure 4.6A). Qualitatively, crmp2 morphants appeared to have thinner GFP+ RGC layers, compared to UIC (Figure 4.6A). A small sample size rendered it difficult to quantify the thickness of the RGC layer. Crmp2 knockdown also resulted in a weakened optic tract, as visualized using isl2b:GFP zebrafish (Figure 4.6A-B). To account for fluorescent variability, the fluorescent optic tract intensity was normalized to the cranial ganglia intensity.

4.3.5. Exogenous Crmp2 WT mRNA does not rescue the crmp2 morphant eye size phenotype

We next sought to investigate the in vivo mechanism of Crmp2 in zebrafish eye development. CRMPs are phosphoproteins regulated by the serine/threonine kinases
Figure 4.5. Crmp2 is necessary for proper retinal lamination. Zebrafish were injected with an anti-sense oligonucleotide (MO) at the one-cell stage to knockdown endogenous Crmp2 expression. A. 72 hours post fertilization, 6 ng MO-injected zebrafish were fixed, embedded in resin, and sectioned transversely through the retina. Hemotoxylin and eosin (H & E) staining show retinal lamination defects in crmp2 morphant zebrafish. B. The number of retina layers was quantified by using the eosin-positive inner and outer plexiform layers (IPL, OPL) to demarcate the nuclear layers. UIC vs 4 ng, \( p < 0.0002 \); UIC vs. 8 ng, \( p < 0.0001 \); UIC vs 8 ng+p53 MO, \( p < 0.0001 \); 8 ng vs 8 ng+p53 MO, \( p < 0.008 \). UIC = 12 n, 2 ng = 12 n, 4 ng = 13 n, 8 ng = 11, 8 ng+p53 MO = 13 n. C. The retina area was measured from the transverse sections and normalized to the mean UIC area. UIC vs 8 ng, \( p < 0.04 \). UIC = 10 n, 2 ng = 12 n, 4 ng = 12 n, 8 ng = 11, 8 ng+p53 MO = 11 n. D-E. Further analysis of individual retinal cell types was observed using the Spectrum of Fates (Sofa1) zebrafish transgenic line. D. A schematic representing an uninjected control Sofa1 zebrafish retina at 72 hpf. E. Increasing doses of crmp2 MO result in impaired retinal ganglion cells (Ath5 marker), amacrine and horizontal cells (Ptf1a marker), and bipolar cells and photoreceptors (Crx marker).
Figure 4.6. Crmp2 is critical for the proper development of the optic tract. Transgenic *isl2b*:GFP zebrafish, which express GFP in retinal ganglion cell (RGC) bodies and axons, were injected with varying amounts of *crmp2* morpholino (MO) at the one-cell stage to knockdown endogenous Crmp2 expression. A. At 72 hours post fertilization (hpf), zebrafish were anesthetized and the RGC layer and optic tract was visualized using confocal z-stack imaging. At 4-8 ng MO, the RGC layer appears qualitatively thinner and the optic tract (OT) is impaired as measured by the optic tract fluorescent intensity normalized to the cranial ganglia (CG) intensity, quantified in B. UIC = 2 n; 2 ng = 4 n; 6 ng = 2 n; 8 ng = 3 n; 8 ng+p53 MO = 4 n. UIC vs 4 ng, *p* < 0.009. UIC vs 8 ng, *p* < 0.02.
Cdk5 and GSK3β, which are known to be activated downstream of Sema3A [8, 9]. Most CRMP phosphorylation studies have been conducted in cell culture or explant studies using Sema3A, while the S522 phosphorylation event has been studied in mouse cortical development [14]. To investigate whether or not Crmp2 phosphorylation is critical for Crmp2-mediated functions in zebrafish retina development, we mutated the four residues that are phosphorylated by Cdk5 and GSK3β to alanines using site-directed mutagenesis in the rat CRMP2 sequence: T509A, T514A, S518A and S522A (CRMP2 4A). 100 pg of rat CRMP2 WT or 4A constructs were co-injected into zebrafish embryos with 4 ng crmp2 MO at the one-cell stage. Normalized eye size was measured at 72 hpf and neither CRMP2 WT nor 4A rat mRNA constructs were able to rescue crmp2 MO-induced smaller eye size (Figure 4.7A). To ensure that the mRNA was not contributing to this phenotype, eye size was measured for 72 hpf embryos injected with WT or 4A alone and we observed no difference to UIC (Figure 4.7A).

As a control, WT mRNA should be able to rescue endogenous RNA knockdowns [25]. Although rat CRMP2 is 90% identical to zebrafish, the small difference may have caused the rat CRMP2 mRNA to be unable to rescue. Therefore, zebrafish crmp2 WT DNA was amplified from embryonic cDNA using RT-PCR and cloned into TOPO II vector. mRNA was produced from this plasmid and co-injected with crmp2 MO at the one-cell stage. The zebrafish crmp2 WT mRNA was unable to rescue the crmp2 morphant phenotype (Figure 4.7B-C). As we are unable to rescue the eye size phenotype with zebrafish crmp2 mRNA, we cannot at this time make any conclusions about the necessity of Crmp2 phosphorylation in eye development. Future work will address the
Figure 4.7. Overexpressing Crmp2 WT mRNA does not rescue the crmp2 morphant phenotype. One-cell-stage zebrafish embryos were co-injected with 4-6 ng crmp2 MO and 100 pg MO-resistant Crmp2 mRNA. Eye size phenotype was observed and quantified at 72 hours post fertilization (hpf). A. Co-injecting rat, Rattus norvegicus, Crmp2 wildtype (WT) mRNA constructs (90% identity to zebrafish) results in no rescue of crmp2 morphant eye size. Similarly, co-injecting a phospho-null Crmp2 mutant (4A) results in no rescue, although no mechanistic inferences can be made due to WT not rescuing. Eye size was normalized to head size in two independent experiments. UIC = 60 n, MO = 33 n, MO + WT = 12, MO + 4A = 15, WT alone = 30, 4A alone = 28. p < 0.0001. B. Co-injecting zebrafish, Danio rerio, crmp2 WT mRNA construct results in no rescue of crmp2 morphant eye size. Quantification of eye size was conducted in one independent experiment, UIC = 23 n, MO = 26 n, Rescue = 18, p < 0.0001. C. Brightfield images of representative embryos at 72 hpf.
obstacles of MO-based knockdowns to further investigate the mechanisms governing Crmp2 function.

4.4. Discussion

Here we show that Crmp2 is expressed in the RGC layer during zebrafish embryonic development. A comparison with Sema6A and PlxnA2 retina expression indicates that Crmp2 is expressed in similar cell layers to PlxnA2 in the developing zebrafish retina and in adjacent cell layers to Sema6A expression. This supports previous findings as Crmp2 has previously been shown to be expressed in the RGC layer of chick and zebrafish [5, 15]. Interestingly, Sema6A has been shown to be expressed in the mouse IPL [26, 27] in contrast to our findings of RGC expression. We show that Sema6A is most highly expressed in the RGC layer while PlxnA2 and Crmp2 are expressed in the RGC and INL. The PlxnA2- and Crmp2-expressing INL cells could be repelled from ectopically innervating the RGC layer through Sema6A signaling. Furthermore, CRMP2 plays critical roles in initiating axon formation in hippocampal neurons and dendritic patterning in cortical neurons [13, 14, 28, 29]. Early Crmp2 RGC expression could initiate axon outgrowth while late Crmp2 expression could function to properly guide RGC processes. Indeed, we also report that Crmp2 functions in proper optic tract development. However, a weaker optic tract may be due to a corresponding thinning in the RGC layer. Increasing our sample size and performing RGC cell counts would allow us to distinguish if the weak optic tract is due to a decrease in RGC number or due to the impaired guidance of RGC axons. Furthermore, observing the process formation and
guidance of dissociated UIC or crmp2 morphant RGCs in culture would give better insight into the cellular effects of Crmp2 on RGC cellular migration.

We also show that knockdown of Crmp2 in the zebrafish results in smaller eye size and impaired retina lamination. The lamination defects are phenotypically similar to PlxnA2 morphants [19], suggesting that Crmp2 could be mediating PlxnA2 signaling. While Sema-CRMP2 studies have used the canonical secreted Sema3A, both Sema3A and Sema6A share PlxnA2 as a receptor. Therefore it is likely that the downstream mechanisms of Sema3A and Sema6A are conserved and CRMP2 could be an important mediator of Sema6A signaling. CRMP2 mediates the lamination and dendritic morphology of cortical neurons [13, 14]. As crmp2 morphants have impaired lamination, particularly in the inner and outer plexiform layers in which dendritic fields reside, our work gives evidence that Crmp2 has a similar function in the retina. Transgenic lines, such as the Zebrabow which enables individual cells and their processes to be distinguished [30], or immunofluorescent studies using synaptic markers would allow the observation of dendrite morphology in the retina.

Interestingly, the p53 MO control rescued the crmp2-MO induced decrease in retina area. While it is possible that the crmp2 MO has off-target effects, it is unlikely as co-injecting the p53 MO did not rescue the retina lamination phenotype. In combination with the eye size phenotype, this could point to Crmp2 having functions in proliferation or apoptosis. Future experiments will investigate if the small eye size phenotype is due to a decrease in proliferation or an increase in apoptosis.
Initial analysis of the molecular mechanisms governing \textit{in vivo} Crmp2 function showed that MO-based experiments cannot determine the role of Crmp2 phosphorylation in our system. This could be due to a number of reasons. First, this could be due to technical or experimental reasons. The expression levels of endogenous and exogenous mRNA may be crucial in governing Crmp2-mediated phenotypes. Optimizing the amount of injected MO and mRNA may enable WT RNA to rescue. Furthermore, Crmp2 may be tightly spatiotemporally regulated and perhaps driving Crmp2 expression in a tissue-specific manner, for example under the \textit{isl2b} promoter, may overcome this obstacle. Similarly, photo-MOs could be used to precisely control temporal and spatial gene expression \[31\]. Lastly, CRMPs form heterotetramers which may introduce a biological challenge to using MOs. The \textit{crmp2} MO splices out exon 2, yet this is predicted to produce a truncated protein as exon 3 is still in frame (Figure 4.3A) and RT-PCR analysis could verify this. If so, the observed phenotypes could be due to a dominant negative instead of a knockdown. It is possible that exogenous zebrafish \textit{crmp2} WT RNA is therefore unable to rescue the functional consequences of the dominant negative protein. These potential problems could be addressed by using the CRISPR/Cas9 system to produce \textit{crmp2} zebrafish to avoid the technical challenges introduced by MO use in our system. Future work will use \textit{crmp2} to study the mechanisms of Crmp2 phosphorylation in zebrafish visual system development.

4.5. Materials and Methods

4.5.1. Zebrafish husbandry
Zebrafish embryos were raised at 28.5 °C and developmentally staged as previously described [32]. Embryos were raised in E3 solution (5 mM NaCl, 0.17 mM KCl, 0.50 mM CaCl₂, 8.42 mM MgCl₂) with 0.0001% methylene blue. For fluorescent analyses, pigmentation was blocked by addition of 0.003% phenylthiourea (PTU) at 24 hours post fertilization (hpf). The following zebrafish lines were used in this study: isl2b:GFP, Sofa1, TL (Tüpfel long fin) and AB. The isl2b:GFP and Sofa1 lines were generously provided by Chi-Bin Chien (University of Utah, Salt Lake City, Utah, USA) and William Harris (University of Cambridge, Cambridge, UK) respectively. The TL and AB lines were purchased from the Zebrafish International Resource Center (ZIRC, Eugene, OR, USA). All procedures were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC), protocol number 15-031.

4.5.2. In situ hybridization

Zebrafish embryos were developmentally staged at 48 or 72 hpf, fixed in 4% paraformaldehyde overnight at 4 °C and stored at -20 °C in 100% methanol until use. Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense RNA probes as previously described [33]. The CRMP2 (accession #: NM_001020517) antisense probe was generated from the Crmp2 zebrafish plasmid pCMVSport6-crmp2 (IMAGE clone 7062180). The Sema6A and PlxnA2 antisense probes are published and previously described [21, 22]. For whole-mount in situ visualization, embryos were embedded in 4% methyl cellulose and imaged using a Nikon SMZ800 dissecting light microscope at 6x magnification. Embryos were embedded in resin using the JB4 Embedding Kit (Polysciences, Warrington, PA, USA) and sectioned on a Leica RM2265
microtome at 20 µm. Sections were imaged on an Olympus iX71 microscope at 20x magnification. Figures were compiled using Adobe Photoshop CS6, where image brightness was optimized.

4.5.3. CRMP2 morpholino

Endogenous CRMP2 was knocked down using antisense morpholino (MO) oligomers (Gene Tools, Philomath, OR, USA) targeting the intron 1-exon 2 boundary. Knockdown efficiency was verified by semi-quantitative RT-PCR using the following forward and reverse primers: TAGATGTCGGCACAAGAACGA and TCCTCCATTGCTTCTGAAT.

4.5.4. mRNA rescue experiments

Rat (*Rattus norvegicus*) CRMP2 RNA was amplified from the pcDNA3.1-CRMP2 construct generously provided by Alyson Fournier (McGill University, Montreal, QC, CA). The CRMP2 4A construct was generated using site-directed mutagenesis to create serine to alanine or threonine to alanine point mutations at the following residues: T509, T514, S518 and S522. DNA was linearized using Stu1 (New England Biolabs, Ipswich, MA, USA) followed by *in vitro* RNA production using T7 polymerase with the mMessage mMachne T7 kit (Invitrogen, Carlsbad, CA, USA).

Zebrafish (*Danio rerio*) CRMP2 WT RNA was made by amplifying CRMP2 from 48 hpf cDNA from the forward primer ATGTCTGGCTATCAGGGCAAG and the reverse primer TTAGCCCAGGCTGGTGATG using Q5 high-fidelity DNA polymerase
The zebrafish CRMP2 PCR product was cloned into pCR-Blunt II Topo using the Zero Blunt Topo PCR cloning kit (Invitrogen). DNA was linearized using BamH1 (New England Biolabs) followed by *in vitro* RNA production using the mMMessage mMMachine T7 kit.

RNA was stored in single-use aliquots at -80 °C until immediately before use. 100 pg RNA was injected or co-injected with Crmp2 MO at the one-cell stage. RNA was injected directly into the cell, while the MO was injected into the yolk.

### 4.5.5. Eye size, retinal lamination, and optic tract quantification

Zebrafish were developmentally staged at 72 hpf [32], when embryonic development is complete. To measure eye size, zebrafish were first imaged using a Nikon SMZ800 dissecting light microscope at 6x magnification. Eye diameter was measured using SPOT software version 5.2 (Sterling Heights, MI, USA). To account for embryo variability, eye diameter was normalized to head diameter as previously described: as measured by the length between the anterior tip of the head and the posterior otic placode [20, 34].

To measure retinal lamination, 72 hpf UIC or Crmp2 MO zebrafish were fixed in 4% paraformaldehyde overnight and dehydrated in 100% ethanol. Zebrafish were embedded in resin as described above and sectioned on a Leica RM2265 microtome at 8 μm. Sections were stained with hematoxylin, rinsed and stained with eosin. Sections were imaged on an Olympus IX71 microscope at 20x magnification. Lamination was
quantified by counting the number of layers present as defined by the eosin-predominant inner and outer plexiform layers. Retina area was also measured using SPOT software, excluding the lens and retinal pigmented epithelial regions. Figures were compiled using Adobe Photoshop CS6, where image brightness was optimized. To further observe specific retina cell layers, the Sofa1 transgenic line was used for a Crmp2 morpholino dose response experiment. The Sofa1 line uses fluorescent markers to visualize the photoreceptor, horizontal, bipolar, amacrine and retinal ganglion cell layers [24].

To visualize the optic tract, the isl2b:GFP transgenic line was used, which have GFP+ retinal ganglion cell bodies and processes. At 72 hpf, UIC or Crmp2 MO zebrafish were embedded in 4% methyl cellulose and lateral view Z-stack images were taken at 20x magnification using a Nikon Eclipse Ti inverted confocal microscope (Nikon Instruments, Melville, NY, USA). Raw image files were compressed and converted to TIFs using ImageJ version 1.48a (National Institutes of Health, Bethesda, MA, USA) and color was optimized in Adobe Photoshop. The optic tract was quantified on raw images in Photoshop by measuring the intensity of the optic tract region. To account for fluorescent variability among individual zebrafish, the optic tract intensity value was normalized to the cranial ganglion intensity for each zebrafish. A one-way ANOVA and multiple comparisons test was performed using Prism 6 (GraphPad Software, La Jolla, CA, USA).
4.6. References


CHAPTER 5: BROADER IMPACTS AND FUTURE DIRECTIONS

5.1. Sema6A-PlxnA signaling in vertebrate eye development

The reports described in this dissertation aimed to elucidate the functionally significant molecular mechanisms of Sema6A signaling in vertebrate eye development. While Sema6A is critical for the development of the nervous system and cardiovasculature, the mechanisms initiating signal transduction remain poorly understood. We show that the tyrosine kinase Fyn induces the phosphorylation of PlxnA1 and PlxnA2 receptors at two specific tyrosine residues and that this phosphorylation event is critical for zebrafish eye development. Furthermore, some transmembrane and membrane-bound Semas can produce functional soluble ectodomains and we show here, for the first time, that the Sema6A ectodomain is naturally released from the cell surface. This soluble Sema6A, sSema6A, can be regulated through PKC activation and is biologically active in maintaining eye field cohesion in zebrafish early eye explants. Lastly, we show that the microtubule associated protein Crmp2, which is known to be downstream of Sema-Plxn signaling, is critical in zebrafish eye development by regulating retinal lamination, optic tract formation and overall eye size.

5.1.1. Broader Impacts

In summary, we have identified and characterized novel mechanisms in Sema6A-PlxnA signaling that are critical in governing vertebrate visual system development. Semas and Plxns are highly conserved molecules that play roles in the development and homeostasis of numerous tissues including the nervous system,
cardiovasculature and immune system, in addition to bone and organ development [1, 2]. Thus, the functionally-significant mechanisms of Sema-Plxn signaling we have identified in the visual system may be relevant to other developmental contexts.

We have shown that Fyn-mediated PlxnA1 and PlxnA2 phosphorylation occurs at two critical tyrosines. Multiple Sema molecules can bind to PlxnA1 and PlxnA2 including the Sema3, Sema5, and Sema6 classes (see Figure 1.2). Interestingly, Sema6A is not known to bind PlxnA1, while Sema6B and Sema6D can signal through the PlxnA1 receptor [3]. Sema6B is expressed in the spinal cord in addition the adult brain, heart and lungs [4] while Sema6D-PlxnA1 signaling mediates heart development [5]. Sema5A and 5B play roles in the nervous system, vasculature and immune system, with PlxnA1 as one of their receptors [5, 6]. Many Sema3 class members can interact with PlxnA1 and PlxnA2, which have important roles in the nervous and vasculature systems as well as mediating immune responses [7]. Thus, it is possible that PlxnA1/A2 phosphorylation may be a conserved mechanism that is crucial for transducing signals from numerous Sema classes in multiple systems.

An alignment of the human Plxn proteins indicates that the PlxnA2 Y1605 and Y1677 sites are conserved in the PlxnA family and in all eight Plxn molecules, respectively (Figure 5.1). The crystal structure of the PlxnA intracellular domain [8] may give insights into the potential reasons for this. The functionally-critical Plxn GAP domain is conserved in all Plxns and consists of two split GAP domains surrounding a Rho GTPase-binding domain (RBD) [8, 9]. The Y1677 site resides in the C-terminal split
Figure 5.1. The Fyn-mediated PlxnA2 phosphorylated tyrosine residues are conserved in the Plxn family. An alignment of the human Plxn proteins indicates that the PlxnA2 Y1605 site is conserved in the PlxnA class while the PlxnA2 Y1677 site is highly conserved in all Plxn proteins. The numbers correspond to the PlxnA2 protein. The sequence information was acquired from NCBI and aligned using MUSCLE Sequence Alignment (EMBL-EBI). The protein reference numbers are as follows: PlxnA1: NP_115618.3; PlxnA2: NP_079455.3; PlxnA3: NP_059984.3; PlxnA4: NP_065962.1; PlxnB1: XP_016962119.1; PlxnB2: NP_036533.2; PlxnB3: NP_005384.2; PlxnC1: NP_005752.1; PlxnD1: NP_055918.2.
GAP domain (C2) while the Y1605 site is in the C-terminal linker region between the RBD and C2 [8]. Indeed, Y1677 is in a highly conserved region (Figure 5.1) and phosphorylation of this residue may be critical in regulating Plxn GAP activity. Perhaps the PlxnA class-specific Y1605 is involved in recruiting specific effector proteins. Intriguingly, Fyn mediates the phosphorylation of both Y1605 and Y1677 at equal preference (see Figure 2.5B). This could suggest that Fyn plays a general role for all Plxns while also mediating PlxnA-specific signaling functions.

We have also shown that the transmembrane Sema6A produces a naturally-released and PKC-induced soluble ectodomain that functions in early zebrafish eye development. This report suggests that ectodomain release is a phenomenon common to all vertebrate transmembrane and membrane-bound Sema classes. Future work will determine if this is common to the other three Sema6 class members. Indeed, a large-scale protease screen for βACE1 substrates identified Sema6A and Sema6D although βACE1-mediated cleavage of Sema6A and Sema6D has not been validated [10]. As the Sema6 class members are closely related, this could point to a conserved mechanism and may suggest that cleavage in this region is common to the Sema6 proteins. As Sema6 class members are expressed throughout the body in numerous tissues, this could have broad implications with Sema6 acting through cell-contact or paracrine and autocrine manners.

Through discovering a soluble functional form of Sema6A, we have also characterized a useful tool to study Sema6A-mediated signaling mechanisms. Most of the
molecular mechanisms have been elucidated by stimulating cells with the secreted Sema3A, while transmembrane and GPI-anchored Sema mechanisms have been addressed by using FC chimeric forms. This allows for the dimerization and secretion of Semas yet may have less biological relevance. With the addition of this report, studies can now be performed using concentrated and purified naturally-released sSema6A to investigate Sema6A-mediated mechanisms. For example, cell culture systems have shown that CRMP2 phosphorylation occurs upon Sema3A stimulation [11, 12]. Furthermore, genetic evidence suggests that Fyn is downstream of Sema3A as Sema3A−/− and Fyn−/− mice have similar phenotypes and Sema3A+/−; Fyn+/− mice show no additive phenotypic effects [13]. Using sSema6A can be one way to determine if these proteins are also regulated by Sema6A binding.

Finally, we have shown that the microtubule associated protein Crmp2 has a novel role in governing zebrafish eye development. We report that Crmp2 functions in retinal lamination, optic tract formation and eye size. This study indicates that CRMP2 has in vivo roles outside of the traditionally studied cortex and opens the field to investigate the molecular mechanisms governing CRMP2-mediated functions in the vertebrate visual system.

In addition to having critical roles in development, Sema signaling molecules are also implicated in pathogenesis and thus there is clinical significance in determining the mechanisms of Sema-Plxn signal transduction. Sema signaling is crucial for axonal guidance and may play a role in axonal guidance deficits such as congenital synkinesis.
and congenital cranial dysinnervation disorders [14]. Many Sema molecules are implicated in neurodegeneration disorders, cancers and autoimmune disorders [6, 15-17]. With their roles in governing endothelial migration, Sema signaling has been implicated in cancer metastasis by promoting the neovascularization of tumors [18]. Furthermore, downstream molecules of Sema signaling, including Fyn and CRMP2, are associated with diseases. Fyn is overexpressed or hyperactive in many cancer types, including glioblastomas, melanomas, and breast cancers [19]. Hyperphosphorylated CRMP2 is found in Alzheimer’s disease, and may contribute to early pathogenesis [20]. Furthermore, CRMP2 is overexpressed in neuropsychiatric disorders, such as schizophrenia [21, 22]. Therefore, determining the mechanisms of these proteins can give a better understanding of the pathogenesis of diseases and cancers, which could lead to the identification of possible therapeutic targets.

In conclusion, Sema-Plxn signaling is conserved and functions in numerous and diverse tissue development as well as in pathologic conditions. Therefore, the impacts of elucidating the mechanisms of Sema6A and PlxnA2 signaling in vertebrate eye development can be applied to multiple systems and cellular processes in healthy and disease states.

5.1.2. Working model of Sema6A-PlxnA signaling in zebrafish eye development

Together, the studies described in this dissertation have elucidated molecular mechanisms of Sema6A-PlxnA signaling that are critical for zebrafish eye development. Adding these insights to the field of existing literature allows for a more comprehensive
working model of the regulatory mechanisms governing Sema6A-PlxnA signaling (Figure 5.2). This model describes the receptor proximal events and major downstream cellular processes that occur in forward and reverse signaling. With the discovery of Sema6A ectodomain release, it also illustrates one mechanism of regulating signal direction.

When PlxnA receptors are unbound, the extracellular and intracellular domains are in autoinhibitory, inactive conformations [8, 23, 24]. However, upon binding of transmembrane Sema6A, PlxnA receptors change conformation such that the intracellular Rap1 GAP domain is active and forward signaling occurs [9, 23, 24]. This leads to the regulation of small GTPases, which governs integrin-mediated adhesion and cytoskeleton regulation [25]. We have shown that Fyn phosphorylates PlxnA1 and PlxnA2 in the GAP domain and the RBD linker region [26], however it is currently unknown if this phosphorylation event mediates GAP domain activity. Furthermore, it remains to be determined if Fyn-induced PlxnA phosphorylation occurs upon Sema6A ligand binding. Numerous cellular processes are known to be regulated by Sema6A-PlxnA signaling, including proliferation, differentiation, apoptosis and transcription [1, 27], however the downstream mechanisms are not well understood. The Sema6A-PlxnA binding interaction also initiates reverse signaling on the Sema6A-expressing cell. The tyrosine kinase Abl is recruited to the intracellular domain of Sema6A upon PlxnA binding and this interaction is necessary for PlxnA2-induced cellular collapse [28]. Ena/VASP proteins including Mena and EVL also interact with the intracellular domain of Sema6A [29] and could promote cellular collapse by regulating adhesion and migration.
Figure 5.2. Working Model of Sema6A-PlxnA Signaling in Zebrafish Eye Development. This schematic illustrates various signaling paradigms resulting from the Sema6A-PlxnA interaction. When Sema6A does not bind to PlxnA receptors, PlxnA is in an autoinhibitory conformation and forward signaling does not occur. When transmembrane Sema6A binds to PlxnA, bidirectional signal can occur, with forward signaling resulting in cellular processes such as integrin and cytoskeletal regulation, transcriptional regulation, as well as proliferation, differentiation and apoptosis. Less is known about the processes governed by reverse signaling, however, it is hypothesized that integrins and cytoskeletal dynamics are regulated. The ectodomain of Sema6A can be naturally released or via PKC activation, resulting in a functional soluble product, sSema6A. This would enable forward signaling with the absence of reverse signaling and represents one mechanism to regulate the direction of signaling. See Section 5.1.2. for more detailed information.
While transmembrane Sema6A binding to PlxnA can initiate bidirectional signaling, our discovery of a functional soluble Sema6A ectodomain proposes a mechanism to regulate the direction of signaling. We have shown that sSema6A can be produced naturally and through PKC activation, in a Mek/Erk-independent manner. This is hypothesized to activate a protease which subsequently sheds the ectodomain from the cell surface. Ligand-dependent ectodomain release could be another mechanism of inhibiting reverse signaling. We have also shown that the intracellular domain of Sema6A is phosphorylated at numerous PKC consensus motifs, suggesting direct-PKC phosphorylation and could present a mechanism of conferring protease resistance to Sema6A. In vivo experiments have begun to elucidate the functional importance of forward and reverse signaling during zebrafish eye development. We have shown that sSema6A is sufficient to maintain early eye field cohesion, although the specific PlxnA receptor transducing this signal remains unknown. Unpublished data from our lab also indicates that a Sema6A-Δcyt mutant, lacking the intracellular domain, rescues the Sema6A-morphant-induced decrease in retinal precursor cell proliferation (data from Caroline Dumas). Together, these results provide insight into the processes that are mediated by Sema6A-PlxnA forward signaling, including eye field cohesion and retinal precursor proliferation. Future investigations will determine if there are specific processes governed by reverse or bidirectional signaling, such as retinal lamination and optic tract formation.

5.1.3. Sema6A and PlxnA2 have closely related family members to govern numerous biological processes
While this dissertation has primarily focused on Sema6A-PlxnA2 signaling, the high conservation among the large Sema and Plxn families, as well as the high promiscuity between Sema and Plxn classes, suggests that the mechanisms we have elucidated may be conserved to regulate cellular processes in numerous systems. For instance, PlxnA2 is a receptor for Sema3, Sema5 and Sema6 class members, while the Sema6 molecules bind to PlxnA class members [3]. As these Sema classes regulate nervous system, cardiovascular and bone development, as well as immune system activation [1, 15], elucidating PlxnA2 receptor proximal mechanisms has broad implications. Interestingly, both Sema and Plxn families have expanded throughout evolution, as there are only five and two invertebrate Sema and Plxn molecules, respectively, and 21 and nine vertebrate Sema and Plxn molecules, respectively [30]. Determining if the invertebrate molecules have similar regulatory mechanisms, such as Plex-A phosphorylation and Sema-1 ectodomain release, would give insight on when these mechanisms arose throughout evolution.

Through duplication and divergence, the expansion of protein families can allow for increases in biological complexity and substrate specificity. As invertebrate Sema-Plxn signaling is essential for neuronal and axonal guidance in nervous system development, vertebrate Sema-Plxn signaling has evolved to use similar downstream mechanisms to regulate cellular guidance in numerous tissues [5]. Although promiscuous, slight modifications in the sema domain allow for substrate specificity [31]. This would enable a single migrating cell to express multiple classes of receptors to be responsive to numerous ligands in various environments. By sequestering or inhibiting receptors, the
cell can spatiotemporally regulate its responsiveness to environmental signals in a rapid and energy efficient manner.

The addition of vertebrate co-receptors confers further specificity of Sema ligands. For example, PlxnA1 and PlxnA2 are both receptors for Sema3A and Sema6A, however, PlxnA1/A2 can only bind Sema3A in the presence of the Neuropilin (Np) co-receptor Np1 [32, 33]. Furthermore, PlxnA2 transducing Sema3A and Sema3C signals, with the ligand specificity dependent on the Np expressed: Np1 allows for PlxnA2 to bind to Sema3A and Np2 allows for PlxnA2 to bind to Sema3C [32]. Together, this illustrates how the expression of specific Nps allows for the discrimination within the secreted Sema3 class while the presence or absence of Nps confers specificity between secreted and transmembrane Semas. As Nps are found only in vertebrates [5], this represents an additional mechanism of increasing biological complexity and substrate specificity of vertebrate Sema-Plxn signaling.

5.1.4. Linking Sema6A, PlxnA and CRMP2

We show that the cellular players of Sema6A-PlxnA signaling, including PlxnA2 and Crmp2 are critical for zebrafish eye development. Although we have investigated these mechanisms in separate studies, pairing our results with what is currently known about Sema-Plxn signaling allows us to make inferences to link the molecular mechanisms of Sema6A, PlxnA, and CRMP2. By comparing knockdown phenotypes and expression profiles, we can hypothesize how these mechanisms cooperate to transduce Sema6A signaling. Future experiments can directly determine the link between the
molecular mechanisms of these cellular players by using sSema6A in biochemical experiments or through genetic in vivo studies.

We show that sema6A, plxnA2 and crmp2 are expressed in the developing retina. Specifically, sema6A is expressed in the RGC layer while plxnA2 and crmp2 are expressed in the retinal ganglion cells (RGC) and inner nuclear layer (INL) (see Figure 4.1). In contrast, Sema6A has been found to be primarily expressed in the mouse INL layer, specifically in the ON sublayer [34, 35]. However, the expression and function of Sema6A and PlxnA2/A4 in the mouse retina can provide insight into the possible functions of Sema6A-PlxnA2 signaling in the zebrafish. Murine Sema6A is expressed in the ON sublayer of the INL, repelling PlxnA4-expressing OFF bipolar cells and PlxnA2-expressing OFF amacrine cells for proper retinal lamination [34, 35]. Sema6A-PlxnA2 signaling may be acting similarly, with Sema6A-expressing RGCs repelling the PlxnA2- and Crmp2-expressing INL cells from ectopically migrating into the RGC layer. Indeed, qualitative analysis of Sofa1 zebrafish indicates that crmp2 morphants have an impaired INL (see Figure 4.5E, Ptf1a panel).

We have previously shown that plxnA2 knockdown in zebrafish results in impaired lamination and smaller eye size [36]. Our findings indicate that crmp2 knockdown phenocopies plxnA2 morphants. Sema6A is also expressed later in development, yet the role of Sema6A in late zebrafish eye development, such as retinal lamination, is not yet known. Similarly, Sema6A and PlxnA2 are essential in maintaining early eye field cohesion [27, 36]. Future experiments will also investigate if Crmp2 is
necessary for the early eye functions by observing \textit{crmp2} morphants or null zebrafish during earlier time points. The expression of CRMP2 is confined to the nervous system and thus it may be that Crmp2 does not play a role in early eye development before 26-28 hpf, when neuronal differentiation begins. Indeed, \textit{crmp2} is not expressed at 36 hpf and \textit{in situ} retina sections can determine if \textit{crmp2} is expressed at earlier time points (see Figure 4.2). Establishing the role of \textit{crmp2} in early eye development as well as Sema6A functioning during retinal lamination and optic tract formation would begin to give insight into the \textit{in vivo} pathway of Sema6A signaling during zebrafish eye development.

If \textit{sema6A}, \textit{plxnA2} and \textit{crmp2} morphants show similar phenotypes, this could suggest that these molecules may be working in the same pathway. If, for example, \textit{crmp2} morphants do not have impaired early eye field cohesion, this would point to a Crmp2-independent pathway that is being regulating by Sema6A-PlxnA2 signaling.

Genetic manipulations in eye explant experiments would yield direct evidence of PlxnA2 and Crmp2 being involved in Sema6A-mediated functions in zebrafish eye development. For example, PlxnA4 is another known Sema6A receptor and is also expressed during eye development, playing roles in retinal lamination \cite{34, 35}. As PlxnA2 has a similar cohesion phenotype to Sema6A, it is the likely Sema6A receptor for this process. Future experiments can determine which PlxnA receptor is predominantly mediating Sema6A-dependent early eye field cohesion. Treating early eye field explants with sSema6A results in an increase in cohesion as measured by less ectopic cells (see Figure 3.5). If PlxnA2 is mediating this process, I hypothesize that sSema6A would not be able to maintain cohesion in \textit{PlxnA2} morphant eye explants. A similar experiment
using eye explants from *crmp2* morphant or null zebrafish would establish the contribution of Crmp2 in sSema6A-mediated functions.

Interestingly, we have also shown that decreasing Sema6A in zebrafish results in less proliferative cells in the developing retina (see Figure 1.5). At the cellular level, Sema signaling is known to regulate migration, differentiation and proliferation [1]. While all of these processes depend on cytoskeletal dynamics, it is not fully understood how Sema signaling governs these processes. Investigating the role of Crmp2 in the proliferation of retina precursor cells could give an understanding of how Sema6A mediates proliferation. If Sema6A influences proliferation via regulating cytoskeletal dynamics, decreasing Crmp2 may show a similar proliferation phenotype to Sema6A. However, if Sema6A is acting through a novel pathway to initiate proliferation, which may be the case as Crmp2 may not be expressed during this time, then Crmp2 may not be essential for this process. We have previously shown that the small GTPase Ras-like 11b is involved in Sema6A-mediated retina precursor cell proliferation [27], which could suggest that Sema signaling is regulating MAPK pathways.

In conclusion, we have shown that Sema6A, PlxnA2, and Crmp2 play critical roles in the development of zebrafish visual system. Linking this pathway in eye development *in vivo* is an important field of study moving forward. Additionally, we have identified functionally-significant molecular mechanisms of Sema6A and PlxnA2 in eye development. Future experiments can address the cellular contexts and signaling roles of these mechanisms. Specifically, the cellular and molecular roles of PlxnA
phosphorylation events in the context of Sema6A binding, the mechanisms and functions of Sema6A ectodomain release, and the cellular and molecular role of Crmp2 phosphorylation in zebrafish eye development. The remaining sections of this chapter focus on how future experiments can address these questions.

5.2. Determining the roles and contexts of PlxnA phosphorylation

5.2.1. Does Fyn-mediated PlxnA phosphorylation occur upon Sema6A ligand binding?

While we have shown that PlxnA1 and PlxnA2 phosphorylation is mediated by Fyn, it is still not yet known whether or not this phosphorylation occurs in the context of Sema signaling. To address this, we can take advantage of sSema6A, the functional soluble ectodomain of Sema6A. Stimulating PlxnA2-expressing cells with sSema6A and measuring PlxnA2 tyrosine phosphorylation levels via immunoblotting will establish if PlxnA2 phosphorylation is occurring upon ligand binding. To further investigate this molecular process, we can determine if sSema6A simulation activates Fyn, if phosphorylation occurs at Y1605 and Y1677 and if Fyn is necessary for Sema6A-induced PlxnA2 phosphorylation. HEK293 cells have endogenous Fyn and pharmacological Fyn inhibition or the Src/Yes/Fyn-knockout cell line SYF mouse embryonic fibroblast (MEF) cells [37] would address if Src-family kinases mediate sSema6A-induced PlxnA2 phosphorylation. Similar experiments could be conducted using bath application of the secreted Sema3A, a known ligand of the PlxnA1-NP1 and PlxnA2-NP1 receptor
complexes, to determine if PlxnA1 and PlxnA2 are phosphorylated upon Sema3A binding.

5.2.2. What role does PlxnA phosphorylation play on PlxnA functioning?

PlxnA sequence analysis may give clues as to how phosphorylation may be affecting PlxnA functioning. The cytoplasmic domains of Plxns are predominantly made up of split GAP domains flanking a Rho GTPase-binding domain (RBD). Plxns are in an autoinhibitory conformation without ligand binding. Upon Sema binding as well as Rho-family GTPase binding to the RBD, the intracellular domain of PlxnA adopts an open conformation that activates the GAP domain [8]. Y1677 is in the C-terminal split GAP domain. With this in mind, PlxnA phosphorylation could regulate the conformation and thus activity of the GAP domain, or recruit effector proteins for signal transduction.

To test the hypothesis that PlxnA phosphorylation is necessary for Rap GAP activity, an in vitro Rap activity assay can be conducted. A photometrically-based assay was specifically designed to test Plxn-mediated Rap GAP activity [38]. When Rap hydrolysis occurs inorganic phosphate is released. Thus an in vitro assay that includes a modifiable substrate can measure Rap activity. The non-phosphorylated substrate MESG can be detected at 320 nm and when it reacts with phosphate, it is absorbed at 360 nm [38]. We could add GTP-bound Rap to WT or non-phosphorylatable (Y-F) mutant PlxnA2 constructs in addition to the MESG substrate and measure the absorbance values. Comparing WT and Y-F reactions would allow us to determine if phosphorylation is necessary for the PlxnA2 GAP activity.
Additionally, PlxnA tyrosine phosphorylation may recruit effector proteins to enable signal transduction. SH2 domain-containing proteins, including kinases and adaptors, can interact with other proteins through phosphorylated tyrosine residues. To test if proteins are differentially binding, immunoprecipitation (IP) and mass spectrometry analyses can identify interacting partners of PlxnA2 WT and Y2F mutants. We have previously attempted this experiment and found no differences, however the cell line or amount of protein could have contributed to this negative result. Using a neuronal cell line such as N2A and increasing the amount of protein analyzed may allow non-specific binding partners to be detected. Fyn can act as a positive control as it is known to bind to PlxnA2 [13]. Alternatively, PlxnA protein interactions may be too transient to detect using traditional co-IP methods. Recent technology has developed a cloning technique called proximity labeling to identify transient interactors [39, 40]. By fusing a H$_2$O$_2$-activatable biotin ligase to PlxnA, we could label proteins that come in proximity of PlxnA WT or Y2F and thus likely to interact [40]. Purifying biotin-labeled proteins using streptavidin beads and identifying them using mass spectrometry may be an alternative, more sensitive, method of identifying transient binding partners.

5.2.3. Is PlxnA phosphorylation necessary for downstream signaling?

As PlxnA phosphorylation is necessary for zebrafish eye development, we hypothesize that is it crucial for initiating downstream signaling. Biochemical experiments could address this question by using known Sema-induced molecular outputs such as CRMP2 phosphorylation. A constitutively active PlxnA1 (CA-PlxnA1) has been developed that does not include the inhibitory extracellular region and can be used for
biochemical assays without ligand stimulation [41]. This would be especially useful as we do not yet know if PlxnA phosphorylation is mediated by ligand binding. Overexpressing CA-PlxnA1 results in growth cone collapse and inhibits axon outgrowth [41], implicating CRMP2-mediated functions. Therefore, it is likely that expressing CA-PlxnA1 leads to the phosphorylation of CRMP2. Transfecting a CA-PlxnA1 Y2F mutant will enable us to determine the effect of PlxnA phosphorylation has on the activity level of CA-PlxnA1. Comparing CRMP2 phosphorylation via Western Blotting in CA-PlxnA1 Y2F-expressing cells would allow us to determine if PlxnA phosphorylation is necessary for downstream signaling. We could also use growth cone collapse and axon outgrowth as cellular outputs for PlxnA Y2F signaling.

**5.2.4. What cellular processes are regulated by PlxnA phosphorylation?**

Lastly, it is important to determine the cellular processes that may be regulated by PlxnA phosphorylation, including proliferation, migration and transcription. PlxnA2 phosphorylation at Y1605 and Y1677 is necessary for zebrafish eye development, as the Y2F mutant cannot rescue the PlxnA2 morpholino-induced decrease in eye size. The smaller eye size could suggest impaired proliferation or increased cell death of retina precursor cells. Repeating this experiment using proliferation markers such as pHH3 or apoptosis markers such as cleaved-Caspase3 during early eye development would determine if proliferation is impaired or cell death is aberrantly increased in plxnA2 MO + Y2F embryos. Similarly, investigating the early eye field cohesion and retinal lamination phenotypes in these embryos would give insight into what cellular processes PlxnA2 phosphorylation is mediating. Future experiments could also use cell culture
systems to determine if migration is impaired when PlxnA2 is unable to be phosphorylated at these sites. Lastly, we have previously shown that Sema6A-PlxnA2 signaling regulates transcription of numerous genes involved in proliferation and migration [27]. Microarray studies could investigate if PlxnA phosphorylation is critical in regulating transcription by comparing the transcriptome of WT versus PlxnA2 Y2F mutant embryos.

5.3. Investigating the nature of Sema6A ectodomain release

5.3.1. Is sSema6A a dimer or monomer?

A major question currently left unanswered is whether or not sSema6A is a dimer or monomer. To determine this, purified sSema6A from the conditioned media of Sema6A-expressing HEK cells can be denatured and subjected to SDS-PAGE with and without reducing agents. Observing an approximately doubled molecular weight in the non-reducing conditions would support a dimer.

Sema dimerization is classically thought to be necessary for their functions [42], which suggests that sSema6A may be a dimer. However, recombinant soluble Sema6A ectodomains (rsSema6A) have been shown to be monomers [43, 44], where activity may depend on rsSema6A concentration and/or cell type. The monomeric rsSema6A (10 µg/mL) has an inhibitory effect on VEGF-induced endothelial cell migration [43], while it showed no effect (approximately 5 ng/mL) on sympathetic growth cone collapse [44]. In contrast, the secreted dimerized chimeric protein Sema6A-FC can collapse
sympathetic growth cones at approximately 20 pg/mL. Thus, one hypothesis is that dimerization positively affects activity. Would the monomeric rsSema6A be able to function on sympathetic growth cones if tested at much higher concentrations, in the µg/mL range? For our eye field cohesion assay, it would be interesting to do a dose response curve comparing the activities of sSema6A and Sema6A-FC. Determining if naturally-released sSema6A is a dimer or monomer would be interesting and important as Sema dimerization is traditionally thought to be necessary for signaling, yet rSema6A has been shown to be functional as a monomer in specific contexts.

5.3.2. Does sSem6A mediate cellular collapse?

The dimerized, secreted chimera Sema6A-FC can induce sympathetic neuron growth cone collapse [45] and binds to PlxnA4- or PlxnA2 -expressing COS7 cells [28, 45], yet these studies do not measure cellular collapse. Sema3A can cause PlxnA1/NP1-expressing COS7 cells to collapse within 60 minutes [12]. Interestingly, we have not been able to show sSema6A-induced cellular collapse by bath applying sSema6A conditioned media. This may be due to a low concentration of sSema6A. Likewise, different concentrations may be needed to produce an effect in different experimental conditions, as we show that the same amount of sSema6A maintains cohesiveness of early eye explants. Centrifugal concentration methods or adding a His tag and Ni²⁺ purifying could be some ways to increase the concentration and thus provide better tools to stimulate cells. Interestingly, Sema3E-induced collapse of PlxnD1-expressing COS7 cells was mediated by decreasing integrin-containing focal adhesions and thus only
collapsed when plated on fibronectin-coated coverslips [46]. Similarly, fibronectin may be necessary for Sema6A-PlxnA1-mediated COS7 collapse.

An alternative hypothesis is that sSema6A may function through different receptors or require neuropilin co-receptors, which are cell-type specific. Perhaps sympathetic cells do not express the necessary machinery for rsSema6A to be functional. Support for this hypothesis could come from a negative collapse result at high rsSema6A concentrations, while being responsive to Sema6A-FC. However, testing sympathetic growth cone collapse using a COS co-culture method shows some functionality of the monomeric rsSema6A, in comparison of the negative collapse result seen in rsSema6A bath applications [44]. The authors suggest that this is a more sensitive system or that the culture conditions may be promoting dimerization [44]. With these studies in mind, it will be important to determine the receptors or co-receptors in which sSema6A signals. Recombinant sSema5A has been shown to signal through the transmembrane Sema5A receptors PlxnA1 and PlxnB3 [6, 47], while the secreted Sema3 class requires neuropilin (Np) co-receptors [48]. As we currently have a robust assay to test sSema6A function, we can use morpholinos or CRISPR to transiently reduce or stably knockout known Sema6A receptors, such as PlxnA1, PlxnA2, and PlxnA4 to determine which Plxn(s) are required for sSema6A-induced early eye field cohesion as well as determine if the neuropilins Np1 and Np2 are necessary. For example, if Np1 is required for sSema6A-mediated eye field cohesion, stimulating Np1 morphant eye fields with sSema6A would inhibit the sSema6A-induced increase in eye field cohesion.
5.3.3. What is the protease responsible for producing sSema6A?

While we show that sSema6A can be produced spontaneously as well as induced by PKC activity, the protease responsible is currently unknown. Based on other sSema studies, ADAM17 or βACE1 are likely candidates (see Table 1.3, pg. 32). ADAM17 has been shown to be sufficient in cleaving multiple Sema ectodomains, including Sema4D, Sema5A, Sema5B and Sema7A [6, 49-52]. Co-transfecting ADAM17 with Sema6A full-length (FL) and measuring the amount of sSema6A detected in the CM would determine if ADAM17 is sufficient to produce sSema6A. Alternatively, aspartyl-proteases may be involved in Sema6A shedding as large-scale proteomic screens of βACE substrates identified Sema6A and Sema6D [10]. However, only one peptide for each of these proteins was identified and βACE-mediated Sema6 cleavage was not validated. It is also likely that many proteases are sufficient to cleave Sema6A, as knocking down ADAM17 is not sufficient to inhibit Sema5B ectodomain release and multiple proteases have been shown to cleave Sema4D [49, 50, 53].

5.3.4. Is Sema6A phosphorylation necessary for cleavage?

We show that Sema6A is phosphorylated in a PKC-dependent manner. Furthermore, PKC activation by PMA increases sSema6A production (see Figure 3.3). Therefore, it is possible that phosphorylation is one mechanism that PKC activity leads to Sema6A ectodomain release. To test this, we can mutate the RXXS serine residues predicted by mass spectrometry to alanines to render them unable to be phosphorylated. Comparing the sSema6A levels from FL and S-A mutants would allow us to determine if intracellular Sema6A phosphorylation is a mechanism to regulate cleavage. Additionally,
unpublished findings from our lab show that Sema6A lacking the cytoplasmic domain exhibits a drastic increase in sSema6A production compared to full-length Sema6A. This supports the hypothesis that the intracellular domains may be in a conformation that protects Sema6A from protease targeting. Indeed, serine/threonine phosphorylation status of Sema4D regulates sSema4D release, as staurosporine treatment increases sSema4D shedding [54]. Furthermore, Sema4D binds calmodulin through a specific binding site and dissociation of this complex is sufficient to initiate cleavage [55]. Using the Calmodulin Target Database [56] to predict potential calmodulin binding motifs in Sema6A shows no intracellular preference for calmodulin binding. However, Abl is known to bind to the intracellular domain of Sema6A [28] and Abl binding may confer cleavage resistance in a similar manner as calmodulin and Sema4D. In summary, Sema6A phosphorylation could confer cleavage susceptibility based on either conformation or through binding other effector proteins. This is an interesting avenue to pursue as regulating cleavage susceptibility is a crucial mechanism to constrain sSema6A production to specific spatiotemporal contexts.

5.3.5. What is the effect of shedding Sema6A from the cell surface?

Protein ectodomain release is a highly regulated process that only occurs in about 2% of cell surface-associated proteins, including receptors, growth factors and cell adhesion molecules [57]. Shedding can occur to allow autocrine or paracrine signaling, increase protein turnover and inhibit signaling. As Sema6A can signal bidirectionally, ectodomain shedding could also regulate the direction of signaling by inhibiting reverse signaling and only allowing forward signaling to occur. Shedding of the Sema6A
ectodomain would render cells non-responsive to reverse signaling. This leads to consider how much of the phenotypes observed in Sema6A zebrafish morphants are due to reverse signaling. We have recently shown that forward signaling primarily regulates Sema6A-mediated early eye field cohesion, as a Sema6A-Δcyt can rescue the sema6A morphant-induced early eye field cohesion and retinal precursor cell proliferation phenotypes (data from Abagael Lasseigne and Caroline Dumas). Interestingly, Sema-1a is known to exhibit both forward and reverse signaling simultaneously to mediate photoreceptor axon migration in *Drosophila* [58]. It would be interesting to investigate Sema6A reverse signaling in late zebrafish eye development to determine the roles of bidirectional Sema6A signaling in different spatiotemporal contexts.

**5.3.6. What is the in vivo contribution of sSema6A?**

We have previously shown that sSema6A is produced during development using proteomic analysis of human and rat embryonic cerebrospinal fluid (CSF) [59]. It would be interesting to determine the predominant form of Sema6A *in vivo*. Does Sema6A predominantly act through the full-length transmembrane Sema6A or the naturally-released soluble Sema6A variant? In fact, the relative *in vivo* contribution is not known for any of the soluble Semas.

One way to address this is by mutating the cleavage site and expressing it in *sema6A*-deficient or null zebrafish. Sema6A is necessary for early eye field cohesion, retinal precursor cell proliferation and eye size [27, 36]. If Sema6A ectodomain release is critical, we would predict that the un-cleavable mutant would be unable to rescue the
phenotypes caused by decreasing or knocking out Sema6A. Determining the \textit{in vivo} functional contribution of sSema6A will have broad impacts on the field, as this would determine the necessity of ectodomain processing of Semas in development as well as challenge the canonical view that membrane-bound Semas act through cell-cell contact.

5.4. Investigating the Crmp2 mechanisms critical in eye development

5.4.1. What are the mechanisms underlying \textit{crmp2} MO-induced optic tract impairment?

We show that \textit{crmp2} morphant zebrafish embryos exhibit impaired optic tract formation. However, we also observe impairments in the retinal ganglion cell (RGC) layer. Thus, there could be multiple reasons for this phenotype. Is impaired optic tract formation due to aberrant RGC axon guidance or stalling, ectopic tectum innervation, or is the weakened optic tract simply due to a thinning of the RGC layer? To determine if the optic tract phenotype is due to a reduced number of RGCs, we can do RGC cell counts to conclude if the decrease in optic tract intensity is similar to a decrease in RGC number.

Alternatively, the weakened optic tract could be due to a decrease in axon initiation and outgrowth. Cell culture studies have implicated Crmp2 function in axon initiation and outgrowth [60-62]. Dissociated retina cell cultures from uninjected control (UIC) or \textit{crmp2} morphant \textit{isl2b}:GFP embryos could determine if the GFP+ RGC cells have fewer and/or shorter processes. It is also possible that the RGC axons do not
innervate the optic tectum appropriately. In *C. elegans*, *unc-33* encodes for the CRMP2 invertebrate homolog [11]. *unc-33* mutants exhibit axonal guidance defects in sensory and motor neurons, leading to aberrant axonal branching, ectopically migrating axons and stalling axons [63, 64]. In the vertebrate visual system, axonal guidance is crucial for topographic mapping of the tectum. For example, nasal and temporal RGC axons innervate the posterior and anterior tectum, respectively [65]. This spatial organization is mediated by Ephrin signaling [65], which is known to induce Crmp2 phosphorylation via ROCK in DRG growth cone collapse [66]. Thus, Crmp2 may be crucial in proper patterning of the tectum, which occurs between 60-72 hpf in zebrafish visual system development [67]. Transgenic zebrafish lines such as the Zebrabow or lipophilic tracers such as DiI could be used to visualize individual cells and their axons to determine if RGC axons are migrating to their proper location in the tectum.

### 5.4.2. What are the mechanisms underlying Crmp2-mediated retina lamination?

We have shown that injecting a morpholino targeting *crmp2* leads to impaired retina lamination. *In vivo* studies investigating Crmp2 function in the mouse cortex can give insights into the possible mechanisms of Crmp2-mediated retina lamination in the zebrafish. Silencing *Crmp2* expression impairs radial migration of cortical neurons as well as causes abnormal dendritic morphology [68, 69]. Qualitatively, *crmp2 Sofa1* morphants do not show severe migration phenotypes as the retina layers appear in appropriate locations (see Figure 4.5E). However, the amacrine cells (*Ptf1a* marker) and RGCs (*Ath5* marker) layers show ectopic cells as well as horizontal cells (*Ptf1a* marker) and photoreceptor cells (*Crx* marker) are absent with higher doses of morpholino. These
cells are among the last to differentiate in the developing retina and observing the retina layers of *Sofa1 crmp2* morphants after 72 hpf would determine if the absent cell layers are due to a developmental delay.

Alternatively, Crmp2 could be mediating dendritic patterning in the retina. Knockdown and knockout mice studies show that Crmp2 is crucial for the neurite morphology of migrating neuron and the organization of cortical cell dendrites [68, 69]. Crmp2 may have a similar function in the retina. This hypothesis is supported by the loss of the inner and outer plexiform layers (IPL and OPL), as visualized by H & E staining. The IPL and OPL are the primary synaptic layers of the retina and are thinner or absent in *crmp2* morphants (see Figure 4.5A). As *crmp2* mRNA is expressed in the RGC and INL cell layers during IPL and OPL formation, at around 60-72 hpf [67], immunofluorescence experiments could determine if Crmp2 is expressed in the RGC and INL cell dendrites. Furthermore, the *Zebrabow* line could be used to observe the dendritic morphology of individual retina neurons.

### 5.4.3. Is Crmp2 phosphorylation necessary for zebrafish eye development?

While we have shown that Crmp2 is critical for zebrafish eye development, investigating the molecular mechanisms of Crmp2 functioning is an important area of future study. CRMP interactions with microtubules are regulated by phosphorylation and we hypothesize that phosphorylation at critical residues are necessary for Crmp2-mediated functions in eye development. There are four sites that are phosphorylated downstream of Sema3A signaling, including the Cdk5-mediated priming of Ser522 and
the subsequent GSK3β-directed phosphorylation of S518, T514, and T509 [20, 62]. We hypothesize that these regulatory mechanisms are conserved in Sema6A-PlxnA2 signaling and necessary for zebrafish eye development. To this end, we have developed mutant constructs in which these sites are mutated to alanines (CRMP2 4A), rendering them unable to be phosphorylated, or mutated to aspartate (CRMP2 4D) to mimic phosphorylation. If Crmp2 phosphorylation is necessary for eye development, I predict that introducing CRMP2 4A mRNA in crmp2 deficient or null embryos will be unable to rescue Crmp2 mediated functions. Similarly, CRMP2 phosphorylation may be tightly regulated and the phosphomimic 4D mutant may be unable to rescue Crmp2-mediated functions as well.

Initial experiments testing the necessity of Crmp2 phosphorylation in zebrafish eye development used morpholinos to decrease endogenous Crmp2 (see Figure 4.7). A crucial control in this experiment was the rescue with Crmp2 WT mRNA as exogeneous, morpholino-resistant mRNA should rescue the phenotypes observed in crmp2 morphant zebrafish. Indeed, this is the gold-standard control for indicating that a given morpholino has no off-target effects [70]. As indicated in the Chapter 4 discussion, there are many potential reasons as to why zebrafish Crmp2 WT mRNA cannot rescue endogenous crmp2 knockdown-induced phenotypes. With this in mind, a crmp2 null zebrafish line may be one way to overcome the challenges with using morpholinos. We are in the process of generating crmp2 null zebrafish using the CRISPR/Cas9 system (Figure 5.3). Injecting CRMP2 WT RNA into CRISPR embryos will determine if this approach will be a successful tool to study the in vivo function and importance of Crmp2.
Figure 5.3. Generating *crmp2* CRISPR zebrafish. This schematic illustrates the steps in developing stable *crmp2* CRISPR zebrafish line. The germline-specific endonuclease nanos-Cas9 is co-injected with guide RNA (gRNA) targeted to exon 3 of the *crmp2* gene. The gRNA recruits Cas9, which results in double-stranded breaks. Non-homologous end joining occurs in an attempt to repair the double-stranded break. This imprecise process generally leads to insertions or deletions (INDELs), resulting in no expression of functional Crmp2. This process occurs in the gametes of the F₀ generation, where it is likely that each gamete encodes for a different mutation. Out-crossing F₀ zebrafish leads to a mosaic population, where each heterozygous zebrafish expresses a different mutant *crmp2*. Validation testing occurs here and individual F₁ zebrafish with specific and known mutations are out-crossed to produce a stable F₂ line. Experiments will in-cross the F₂ generation and collect the homozygous null embryos for experimental use.
phosphorylation. If successful, it will be interesting to investigate the phenotype of injecting CRMP2 4A RNA into crmp2 CRISPRs. As PlxnA2 signaling is important in lamination while Ephrin signaling is involved patterning the optic tectum [36, 65], 4A may be unable to rescue the lamination phenotype but still able to rescue the optic tract impairments if they are mediated by Ephrin-Crmp2 signaling, which leads to CRMP2 phosphorylation at S555 via ROCK, leading to a decreased affinity to tubulin [66, 71].

Interestingly, the other Crmp isoforms cannot offset the effects of transiently decreasing Crmp2 using morpholinos, suggesting that Crmp2-containing heterotetramers are critical for optic tract formation, retina lamination and overall eye size. In situ hybridization for Crmp3 and Crmp4 indicates that Crmp3 has a similar expression pattern to Crmp2, being expressed in the RGCs while Crmp4 is expressed in the RGCs and INL at 48 hpf and exclusively in the INL by 72 hpf (data by Morgan McNellis). As knockout animals have been known to have compensation effects, Crmp2 CRISPR zebrafish lines may show less severe phenotypes due to the possible upregulation of Crmp3 and Crmp4.

5.4.4. How is Crmp2 phosphorylation regulating cellular microtubule dynamics?

Cell culture systems and mouse genetic studies have demonstrated that CRMP2 phosphorylation is crucial for its function in neurite outgrowth and morphology [20, 62, 68, 69]. Additionally, immunofluorescence assays and immunoprecipitation experiments have shown that CRMP2 phosphorylation regulates its affinity to tubulin and microtubules [20, 62]. Furthermore, CRMP2 can promote microtubule polymerization in vitro [72]. While it is known that CRMP2 phosphorylation regulates its affinity to bind to
mitotic spindles [62], currently no high-resolution cellular studies have been conducted to determine how CRMP2 phosphorylation affects microtubule polymerization in the context of a migrating neuron. To determine how CRMP2 phosphorylation affects growth cone microtubule dynamics, we have started a collaboration with the lab of Laura Anne Lowery (Boston College). Her lab uses *Xenopus* neural tube or eye explants to investigate growth cone dynamics using high-resolution live cell imaging. By injecting GFP- or mCherry- tagged fusion constructs into *Xenopus* embryos and culturing tissue later in development, we can visualize both microtubules and a protein of interest during growth cone migration using spinning disk confocal microscopy. Preliminary results have shown that injecting *CRMP2 WT* or mutant RNA into Xenopus embryos yields no significant differences between WT, 4A and 4D mutants in polymerization rates in growing axons. However, the constructs that were used in this study were untagged; using GFP-tagged proteins will ensure that the CRMPs are being translated and trafficked to the growth cone machinery appropriately. Future work will use CRMP2-GFP constructs to better visualize where phosphorylated or unphosphorylated CRMP2 is localizing on microtubules and how it is affecting microtubule polymerization in growth cones.

5.5. Final Remarks

In conclusion, the reports described in this dissertation have elucidated functionally-significant molecular mechanisms of Sema6A-PlxnA2 signaling governing vertebrate visual system development. We have enhanced the understanding of the molecular processes that regulate Sema6A- and PlxnA2- mediated functions in early and late eye development. We have identified Fyn-mediated PlxnA phosphorylation sites that
are critical for PlxnA2 functioning as well as discovered a novel Sema6A variant, sSema6A, that is functional in early eye development. We have also characterized a novel role of Crmp2 in late eye development, mediating retina lamination and optic tract formation.

Future work will extend our investigations into the mechanisms governing Sema6A-PlxnA2 signaling in vertebrate eye development. We will study the context of PlxnA phosphorylation in Sema6A signaling and the cellular processes it may govern; further characterize sSema6A and the in vivo functional role of sSema6A production; and investigate the mechanisms of Crmp2 in mediating its novel visual system functions. These exciting future directions will establish the molecular, cellular and developmental consequences of Sema6A-PlxnA2 signaling mechanisms.
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