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Neurodevelopmental Roles of Semaphorin6A/ PlexinA2 Signaling in Zebrafish

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NEURODEVELOPMENTAL ROLES OF SEMAPHORIN6A/PLEXINA2 SIGNALING
IN ZEBRAFISH

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ABSTRACT

A multitude of complex cellular changes are required throughout development in order for a single cell to transform into a fully functioning organism. Cellular events including proliferation, migration, and differentiation have to be carefully controlled in order for development to proceed correctly. In order to study such dynamic processes, *in vivo* models are often utilized. Using the zebrafish *(Danio rerio)* as a model system, we have investigated the role of an axon guidance signaling pair, Semaphorin6A (Sema6A) and PlexinA2 (PlxnA2), in neurodevelopment.

A previous investigation into the developmental expression patterns of *sema6A* and *plxnA2* in zebrafish, revealed overlapping expression in the developing eye. At this early stage, the cells in the optic vesicles are undifferentiated retinal precursor cells (RPCs) and therefore do not require Sema/Plxn signaling for their canonical axon guidance role. To understand what the function of this early expression was, we knocked down both *sema6a* and *plxna2* and observed 1) a loss of cohesion of RPCs within optic vesicles, and 2) a decrease in RPC proliferation (Ebert et al., 2014). Because these phenotypes were seen at an early stage and given that many developmental processes are dependent on genetic regulation, we hypothesized that Sema6A/PlxnA2 signaling could be regulating transcription of downstream target genes. To investigate this, we performed a microarray experiment and uncovered 58 differentially regulated genes (Emerson et al., 2017a). Prior to our study, it was not known that Sema/Plxn signaling led to changes in gene transcription.

In an effort to understand the contribution of identified candidate genes to early *sema6A/plxnA2* knockdown phenotypes, candidate genes with predicted functions in proliferation and migration were investigated. First, we show that *rasl11b* is important for regulation of RPC proliferation in the developing optic vesicles. Second, we show that *shootin-1* is important in optic vesicle migration, retinal pigmented epithelium formation and optic tract patterning. Furthermore, PlxnA2 regulation of *shootin-1* levels is important in sensory and motor axon patterning and branching in the peripheral nervous system.

Belonging to a large family of proteins with the ability to cross talk, Semas and Plxns rely on spatially and temporally differential expression patterns to perform their tissue-specific roles. Here, we used *in situ* hybridization to comprehensively uncover the neuronal expression patterns of the PlxnA family in the early developing zebrafish (Emerson et al., 2017b). In addition, we present for the first time that zebrafish have two genes for PlxnA1, A1a and A1b, which show divergent expression patterns.

Semas and Plxns are critical for many aspects of development and together, this body of work provides further insight into the downstream signaling mechanisms and roles of these essential developmental signaling proteins.

CITATIONS

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Emerson, S.E., Light, S.E., Ebert, A.M.. (2018). Neuronal expression patterns of the PlexinA family during zebrafish development. Gene expression patterns : GEP 27, 56-66.

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1.1. Zebrafish as a Model System for the Study of Development

1.1.1. Advantages of the Zebrafish

From the moment of fertilization, highly ordered and dynamic cellular events govern the processes of development. A multitude of complex cellular changes are required for the transformation of a single cell into a fully functioning organism. Cellular events including proliferation, differentiation, migration and patterning have to be carefully controlled in order for development to proceed correctly. Due to the complex and dynamic environments that cells encounter during development, *in vivo* studies are advantageous. There are many model systems that are used to study cellular developmental events and mechanisms. One such model system is the zebrafish (*Danio rerio*).

Zebrafish are an excellent model system for the study of vertebrate development for many reasons, including: large clutch sizes, external fertilization and high levels of genetic homology to humans. The complete zebrafish genome was sequenced in 2013, revealing a genome of 26,000 genes on 25 chromosomes (Howe et al., 2013). It was found that 71.4% of human genes have at least one zebrafish orthologue (Howe et al., 2013), making them an appropriate system to study general aspects of vertebrate development. Zebrafish are small freshwater fish native to the Himalayan region (Hamilton and Swaine, 1822). In the wild, they habituate low-flow streams and stagnant ponds and migrate to shallow rice paddies to mate. This mating behavior is easily recreated in the lab using shallow mating tanks and is triggered by daylight, so they are kept on a 14/10-hour light/dark cycle (Avdesh et al., 2012). A mating set of 5 fish can produce on average 100 embryos per week. Males are easily distinguished

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from females by morphology. Males tend to be darker pink in coloration with a slender body shape. Females are paler in color, have a rounder body shape and can be identified by the presence of an ovipositor (Avdesh et al., 2012). In the wild, zebrafish live in temperatures between 24.6-38.6°C (Engeszer et al., 2007) and embryos will develop faster at warmer temperatures. As a lab standard, embryos are raised in incubators set at 28.5°C or 25°C depending on the desired speed of development (Avdesh et al., 2012). They have a relatively short generation time, being sexually mature at three months post fertilization (Parichy et al., 2009). Zebrafish make ideal organisms for the study of development as all organ systems are present within just 72 hours post fertilization (hpf) (Parichy et al., 2009). Embryos are transparent, and any pigment that does develop can be blocked by the addition of phenylthiourea at 24 hpf (Karlsson et al., 2001), making morphological changes easy to observe using simple light microscopy.

1.1.2. Zebrafish as a Tool for the Study of Genetics

Zebrafish have been documented as a model system in labs since the 1930s (Creaser, 1934), but their potential as a system to study genetics was not realized until the 1980s (Streisinger et al., 1981). In 1993, a large forward mutagenesis screen using ethylnitrosourea (ENU) to mutagenize zebrafish sperm was undertaken in an event known in the field as "The big screen." This work lead to the identification of over 4000 genetic mutants (Driever et al., 1996) (Haffter et al., 1996) and pushed the potential of study in this model system forward substantially. Since then, many additional mutants have been generated using targeted reverse genetic techniques, most recently, CRISPR/Cas9 gene knockout has been used to efficiently create mutant stable lines (Liu et al., 2017). Zebrafish are able to readily regenerate fins, which makes DNA

genotyping of living fish possible. Gene knockdown is possible using Morpholino constructs (Nasevicius and Ekker, 2000), and mRNA can be administered via microinjection at the one-cell stage of development to observe the effects of overexpression or for rescue experiments.

There are a multitude of transgenic lines available for the study of development in zebrafish, with different cells and systems fluorescently labeled. Such lines have been genetically modified to express fluorescent proteins downstream of cell-specific enhancers/promoters. Some examples include: *kdrl* expressed in the vasculature; *rx3*, expressed in retinal precursor cells and the hypothalamus; *mnx1* expressed in motor neurons; and *ngn1*, expressed in sensory neurons (see Table 1.1). The generation of transgenic zebrafish lines have enabled developmental studies of specific cell types in specific systems, that otherwise would not be possible. There are several methods of transgenesis, with varying efficiencies. The first transgenic zebrafish was generated in 1988. DNA coding for Green fluorescent protein (GFP) was injected into a one cell stage embryo, was incorporated into the genome and expressed in the adult. The ability to express GFP was passed onto the germline, making it a heritable trait (Stuart et al., 1988). This first attempt was successful, but expression was weak, and the technique was inefficient and unreliable. Today more robust methods are utilized, a popular method being the *Tol2* transposon system (Kawakami, 2005). The *Tol2* transposase element was isolated from medaka fish, *Oryzias latipes,* (Allende et al., 1996) as a vertebrate transposable element that was active in zebrafish. To generate a transgenic zebrafish using the Tol2 system, the promoter region of a gene of interest is identified and isolated and cloned into a *Tol2* containing vector along with the sequence coding for GFP (or alternative fluorescent protein) downstream. This construct is co-injected

into the single cell of a fertilized embryo, along with the transposase enzyme mRNA. The coding sequences are incorporated into the genome, with the goal of heritable incorporation into germline cells. Incorporation can happen multiple times, 5-6 times on average (Kawakami, 2005). Once the construct is in the genome, the cell specificity of the promoter will allow GFP to be expressed only in the cells where the promoter is normally used. Embryos are grown up and screened for the presence of GFP in the desired cell types, and in-crossed to generate a stable transgenic line. The *Tol2* system increased transgenesis efficiency from 5% germline transmission when DNA was simply injected, to 50% germline transmission (Kawakami, 2005). There are alternative methods including: the synthetic transposon element, *sleeping beauty*, and elements from other organisms such as *mariner,* which was isolated from *Drosophila*.

1.2. Zebrafish Developmental Stages

Zebrafish development is rapid, and due to embryonic transparency, morphological events can be visualized using a basic light microscope. The developmental events that govern the early stages of the zebrafish have been well documented (Kimmel et al., 1995), (Figure 1.1). Early zebrafish development is described in 8 major stages. The zygote, cleavage, blastula, gastrula, segmentation, pharyngula, hatching and the early larva (Kimmel et al., 1995). Development begins after fertilization, when the sperm enters the egg via a specialized channel called the micropyle (Yanagimachi et al., 2017). The zygote stage lasts until the first cell cleavage at ~40 mins after fertilization. During this stage the protective outer chorion swells from the yolk, and the first cell is formed at the animal pole. Zebrafish development is unique in that all cell divisions happen on the surface of the yolk at the blastodisc. Cleavage follows, with cells multiplying every 15 minutes. Cell divisions are

Table 1.1. Table of Commonly used Transgenic Zebrafish

System	Developmental timeline	Transgenic line	Transgenic expression	Transgenic timeline
Eye	4 somites- eye field separation 12 somites- eye fields are separate and elongated 18 somites- invagination of optic cup begins (Ebert et al., 2014) 30 hpf- retinal ganglion cells differentiate (Ebert et al., 2014) 30-72hpf- retinal layers form (Almeida et al., 2014)	*rx3*:GFP *isl2b*:GFP SoFa1 *Atoh7*:gapRFP *Ptf1a*:cyt:GFP *Crx*:gapGFP	Retinal precursor cells and hypothalamus Retinal ganglion cells and cranial ganglia Retinal ganglion cells, horizontal cells, amacrine cells, photoreceptors Horizontal and amacrine cells Bipolar and photoreceptor	4 somites- 20 hpf 30 hpf- adult 30 hpf- adult
Circulatory system	22 hpf- heart tube begins to beat 30 hpf atria and ventricles form 36 hpf heart loops (Vogel and Weinstein, 2000)	*Kdrl*:mCherry	Blood vessels	5 somites- adult
Peripheral nervous system	24hpf- 48hpf sensory and motor neurons begin sending axons throughout the trunk Thanks to Christine Beattie, Ohio State and Uwe Strähle, KIT Germany.	*mnx1*:RFP x *ngn1*:GFP (SMo)	Motor and sensory neurons	48 hpf- adult
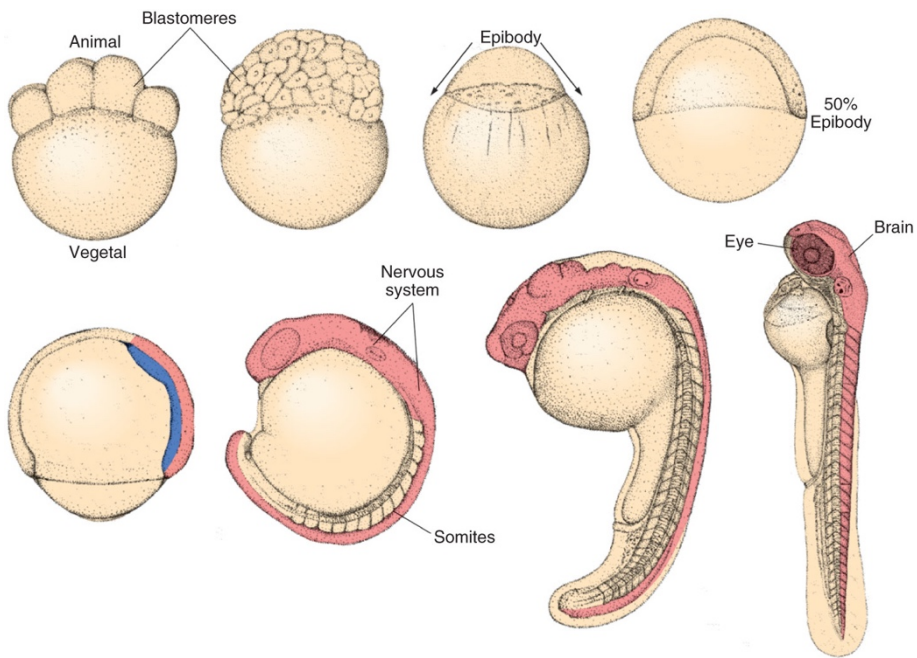
Figure 1.1. Development of the zebrafish embryo from the 4-cell stage to 72 hours post fertilization. Blue shows involution during gastrulation, neural tissue in red (Sanes, 2011). (Re-used with permission, license number 4557100694801.

synchronous at the first but by the tenth cell cycle, cells divide asynchronously as they start transcribing their own DNA and become motile in an event called the 'mid blastula transition' (Kane and Kimmel, 1993). The blastula period begins at 2 ¼ hours. Blastomeres begin the process of epiboly, and the cell layers begin to thin out and wrap around the yolk to completely cover it by the end of the gastrula period (Kimmel et al., 1995). Gastrulation is arguably to be the most important stage of development, with massive cell movements that begin the process of forming the three primary germ layers of the embryo, the ectoderm, endoderm and mesoderm. Involution begins at 50% epiboly, and a thickening of cells generates the morphologically distinctive shield stage (Kimmel et al., 1995). Involution creates two layers in the zebrafish embryo, the outer epiblast, which will become the ectoderm and the inner hypoblast, which will become both the mesoderm and endoderm (Kimmel et al., 1995). At 10 hpf, segmentation begins with the formation of the somites. These presumptive muscle blocks serve as easy morphological markers for staging purposes. A new somite pair forms posteriorly every 30 mins, and so can be counted to determine the age of the animal. During this time, the body axis thickens and the tail bud forms. The first body movements can be seen. Organ development begins with the kidneys and the notochord. The neural plate undergoes neurulation forming the nervous system. Most vertebrates undergo primary neurulation in which the neural folds at either side of the neural plate rise and fold together, forming a hollow neural tube. (This process is different in zebrafish, in which instead of forming a neural tube, a neural keel is formed due to midline involution of the ectoderm, which folds onto itself to form a solid neural rod). 'Secondary neurulation' then proceeds in which the rod is hollowed out during 'cavitation' (Papan

and Campos-Ortega, 1994). The pharyngeal period begins at 24 hpf, and it is defined by a complete set of somites and a bilateral body plan. Fins begin to form as well as the circulatory system. Pigment cells differentiate and are visible as black patches on the embryo. The head straightens out, and coordinated swimming is possible. The hatching period occurs between 48 and 72 hpf, and the embryo breaks through the protective chorion. During this time, fins jaws and gills rapidly develop. The early larval period marks the end of early development, in which fish have all organ systems present, are able to swim freely and mature into adults (Kimmel et al., 1995).

1.3. Development of the Vertebrate Central Nervous System.

1.3.1. Neural Induction

The development of the nervous system begins with specification of the ectoderm into neuroectoderm in a process called neural induction. Neural induction is driven in the dorsal ectoderm by the spatial and temporal expression of morphogens produced by the underlying mesoderm. The process of cell layering during gastrulation is essential for these interactions (Sanes, 2011). Hans Spemann and Hilde Mangold identified the 'Spemann organizer' region of the embryo at the dorsal blastopore lip. They discovered that when transplanted, this embryonic region was sufficient to induce and organize the formation of a second body axis with a duplicated nervous system in tadpoles (Hamburger, 1969). Scientists tried to uncover the factors expressed by the organizer, but it wasn't until 1993 that the first neural inducer, Noggin, was identified (Lamb et al., 1993). Chordin and Follistatin were later discovered to also act as neural inducers (Hemmati-Brivanlou et al., 1994; Sasai et al., 1994). The ectoderm is defaulted to become neuronal but repressed by the ubiquitous expression of Bone

Morphogenetic Protein (BMP). BMP signals though its TGFβ (nodal) receptor leading to epidermal cell fates. BMP is inhibited at the site of neural induction by the expression of antagonistic factors from the underling mesoderm (Weinstein and Hemmati-Brivanlou, 1999). Noggin, Chordin and Follistatin secreted from the dorsal mesoderm block BMP signaling by binding to it and sequestering it away from its TGFβ receptor (Piccolo et al., 1996; Sanes, 2011). This inhibits downstream activation of Smad, which when active inhibits transcription of Zic1. Zic1 activates downstream proneural genes, so when inhibited by BMP signaling, leads to epidermal cell fates (Figure 1.2).

Additional factors are also required for neuronal fate determination, including Fibroblast Growth Factors (FGFs), which can inhibit BMP signaling via Smad phosphorylation rendering it inactive. (Londin et al., 2005). In chick it has been shown that FGF binds to its FGF receptor to activate ERK signaling, activating the transcription of Zic3. Together genes such as Zic1 from the inhibition of BMP signaling and Zic3 from FGF signaling initiate transcription of neural progenitor genes such as Sox2 to promote neuronal cell fates (Marchal et al., 2009). In zebrafish, Sox1a/1b, 2, 3, and 19a/b have all been shown to be important for neuroectodermal fates (Dee et al., 2008; Schmidt et al., 2013). They are key in maintaining the proliferative progenitor pool by inhibiting differentiation factors such as *hexs1* and *her3*, and are downregulated prior to differentiation (Okuda et al., 2010). Once the neuroectoderm is specified, neurulation proceeds, as described above. The formation of the solid neural keel nerve rod, and the secondary hollowing of the structure to form the nerve cord (see section 1.2) (Kimmel et al., 1995). Neurogenesis occurs in clusters of

cells called proneural clusters, which are specified by the expression of proneural genes

such as *neurogenin1 (ngn1)* and *achaete-scute1 (asc)*. Cells within the proneural
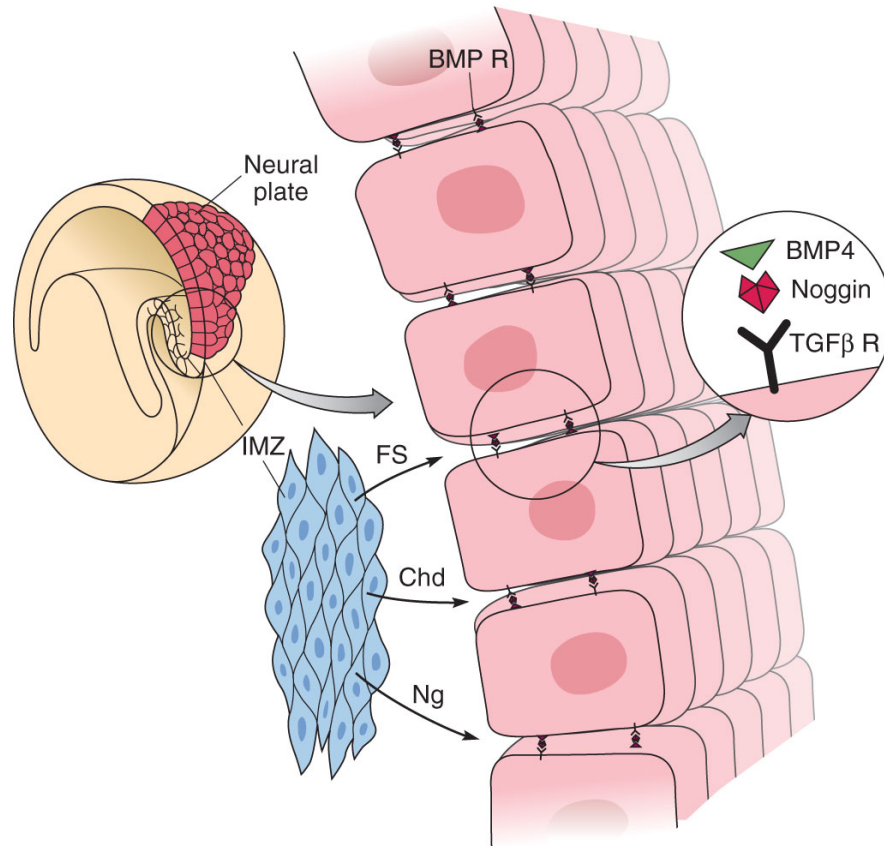
Figure 1.2. Model of neural induction. The involuting mesoderm releases follistatin, (FS), Chordin (Chd) and Noggin (Ng) to antagonize BMP signaling in the ectoderm, therefore inducing neuronal fates (Sanes, 2011). (Re-used with permission, license number 4557100694801).

clusters expressing the highest concentrations of proneural genes will become the neuroblasts. They are maintained by Delta/Notch signaling in a process called lateral inhibition. Cells expressing more Delta become neurons, which inhibits neuronal fates in neighboring cells expressing Notch. Notch and Delta are transmembrane proteins. When activated by its Delta ligand, Notch releases its intracellular portion into the cell. This intracellular fragment forms a complex with Suppressor of Hairless (SuH) to activate transcription of Enhancer of Split E(spl) proteins which act as repressors of *asc* proneural gene expression, decreasing levels of Delta, leading to an epidermal fate. In neighboring cells with higher levels of Delta, SuH is inhibited, and so Asc can drive gene expression of *delta* in a positive feedback loop generating a neuroblast (Castro et al., 2005; Hartley et al., 1987; Sanes, 2011).

1.3.2. Anterior-Posterior Patterning of the Nervous System

Once specified, the neural tube gives rise to the spinal cord and the brain. Rostrally, the neural tube enlarges to form the three primary brain vesicles, the prosencephalon, mesencephalon and rhombencephalon. These further segregate into 5 vesicles. The prosencephalon divides into the telencephalon and diencephalon, from which the eyes and hypothalamus originate. The rhombencephalon divides into the metencephalon (cerebellum) and myelencephalon (medulla) (Sanes, 2011). The anterior-posterior axis (head to tail, A-P) is governed initially by the organizer and transformed into more posterior fates by three main signal gradients, Wnt, retinoic acid (RA) and Fgf respectively. Hox gene expression is key to initial A-P regionalization of the embryo and is influenced by retinoic acid (RA) morphogen gradients. Higher levels of RA towards the posterior leads to transcription of more posterior Hox gene

expression and lower levels lead to more anterior Hox genes being expressed (Lewis, 1978). The most well documented study of Hox gene patterning in the nervous system is in the hindbrain. Different Hox gene combinations are expressed along the anterior-posterior axis, which lead to the very ordered boundaries of rhombomeres, which give rise to specific motor neuron types (Carpenter et al., 1993). In the forebrain, similar to Hox genes in the hindbrain, Pax gene expression patterns the forebrain. There are 9 Pax genes, expressed in an anterior-posterior order, and are essential for regionalization of the forebrain (Chalepakis et al., 1993). *Pax6* for example is a master regulatory gene that when activated in the anterior forebrain is necessary and sufficient to drive the formation of the eyes (Halder et al., 1995). The transcription factor Emx2 is expressed in an opposing gradient to Pax6 and patterns the posterior cortex (Muzio and Mallamaci, 2003).

1.3.3. Dorso-ventral Patterning of the Nervous System

Dorso-ventral patterning in the central nervous system has been studied most famously in the spinal cord. Sonic Hedgehog (Shh) is a key ventralizing factor. It is expressed by the notochord and floor plate and is important for the regionalization of motor neurons. BMPs and Wnts are expressed in the overlying ectoderm and signal to the dorsal spinal cord to generate sensory neurons. Shh was discovered to be expressed by the notochord and is presented to the neural tube at a gradient of concentrations, which activates differential gene expression along the D-V axis, to promote different neuronal progenitor fates (Jessell et al., 1989) (Figure 1.3). This is governed by different combinations of transcription factors that are activated along the gradient. For example, ventral fated neurons express Nkx2.2, intermediate progenitors express Olig2,
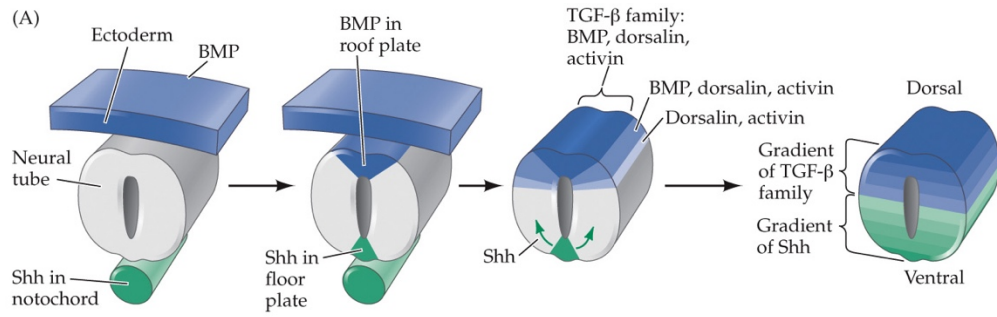
Figure 1.3. Patterning of the spinal cord. Dorso-ventral patterning is driven by a ventralizing gradient of Shh from the notochord and floor plate, and a dorsalizing presence of TGFb family members. High concentrations of Shh lead to the activation of differential combinations of transcription factor expression, leading to the formation of specific motor neurons along the D-V axis (Breedlove, 2017). (Re-used with permission, license number 9781605355795).

and dorsal progenitors express Pax7 due to the different concentrations of Shh that the cells are exposed to (Dessaud et al., 2008).

1.3.4. Development of the Sensory and Motor Systems

The spinal cord is home to the sensory and motor systems. Motor neuron cell bodies reside within the spinal cord, whereas sensory neuron cell bodies occupy dorsal root ganglia (DRG), and both are important for connecting the neural tube to the periphery. Sensory neurons are derived from neural crest cells. These cells delaminate from the ectoderm at the lateral borders the of neural plate as it enfolds to form the neural tube, in a process called an 'epithelial to mesenchymal transition' (Kuriyama and Mayor, 2008). These highly migratory cells give rise to many different cell types, such as: the cells of the sensory, enteric and autonomic ganglia; Schwann cells; smooth muscle cells; pigment cells; and the cells of the jaw bones and the skull (Sanes, 2011). In the zebrafish, mechanosensory Rohon-Beard neurons are the first neurons to differentiate and allow zebrafish to be able to sense their surroundings. These are a transient population of cells that undergo a long period of apoptosis until 5dpf and are replaced by sensory neurons (Reyes et al., 2004). Sensory neurons are born at 16hpf, and send axons from the dorsal root ganglia to the periphery (Kimmel et al., 1995). Sensory progenitors are specified by Wnt signaling, driving the expression of *ngn* (Lo et al., 2002). Motor neurons are born soon thereafter at around 18hpf. At 24 hpf, sensory-motor circuits become functional (Kimmel et al., 1995). They are organized into motor columns along the A-P axis, their organization being determined by FGF in the posterior, and RA in the anterior spinal cord leading to the differential expression of Hox genes as mentioned above. The motor columns innervate different regions of the

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adult organism. In zebrafish there are both primary and secondary motor neurons, primary motor neurons being of tree types, each projecting to a different direction. RoP, MiP and CaP, (rostral, middle and caudal), which project to ventral, dorsal and middle muscle respectively (Westerfield et al., 1986).

1.4. Zebrafish as a Model System for Eye Development.

1.4.1. Eye Field Specification

Zebrafish make an excellent model system for the study of neuronal eye development. Their retinas are proportionally large and make the study of neuronal connections and patterning accessible and contain all of the same neuronal cell types as humans. The eye field is first established in the developing nervous system at the anterior neural plate, prior to neural tube closure. Several evolutionarily conserved eye field transcription factors (EFTFs) drive eye specific cellular fates, and include *Pax6, Six3, Lhx2, Rx* and *Otx2* (Figure 1.4). These transcription factors are expressed at late gastrula stages, and mis-expression results in improper eye formation (Zuber et al., 2003). *Pax6* as a master regulatory gene for eye development and overexpression can result in ectopic eyes (Halder et al., 1995). The EFTFs have overlapping expression domains in the presumptive eye field and sequentially turn on to further specify more specific regions using feedback inhibition loops. Following the closure of the neural tube, the eye fields evaginate from the lateral diencephalon, forming two optic vesicles. *Shh* is expressed in the anterior midline of developing embryos and drives *pax2* expression, which in turn inhibits *pax6* expression. *Shh* expression is essential for turning off eye cell fates along the midline to allow for correct separation of the optic
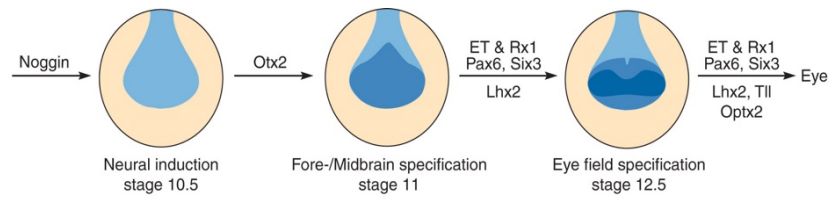
Figure 1.4. Eye Field Induction in the Anterior Neural Plate. Light blue shows area of *otx2* expression, dark blue shows the eye field. The sequential activation of different transcription factors delineates more specific areas of the eye fields (Sanes, 2011). (Re-used with permission, license number 4557100694801).

vesicles (Macdonald et al., 1995). Overexpression of *PAX6* along the midline, or a loss in *SHH* can result in holoprosencephaly in humans (Roessler et al., 1996). Once the optic vesicles have correctly separated and migrated bilaterally, they make contact with surface ectoderm of the head. This drives proliferation of the surface ectoderm, leading to development of the lens placode. The lens placode continues to proliferate and invaginates to form a separate vesicle of lens tissue. This invagination is synchronous to the invagination of the optic vesicles, by which point has developed polarity. The inner surface of the optic vesicle will develop into the neural retina, and the outer layer will become the retinal-pigmented epithelium (RPE). The posterior of the retina will become the optic stalk, replaced by the optic nerves once developed, connecting the eyes to the brain. The remaining surface ectoderm of the head adjacent to the lens will become the transparent cornea (Adler and Canto-Soler, 2007) (Figure 1.5).
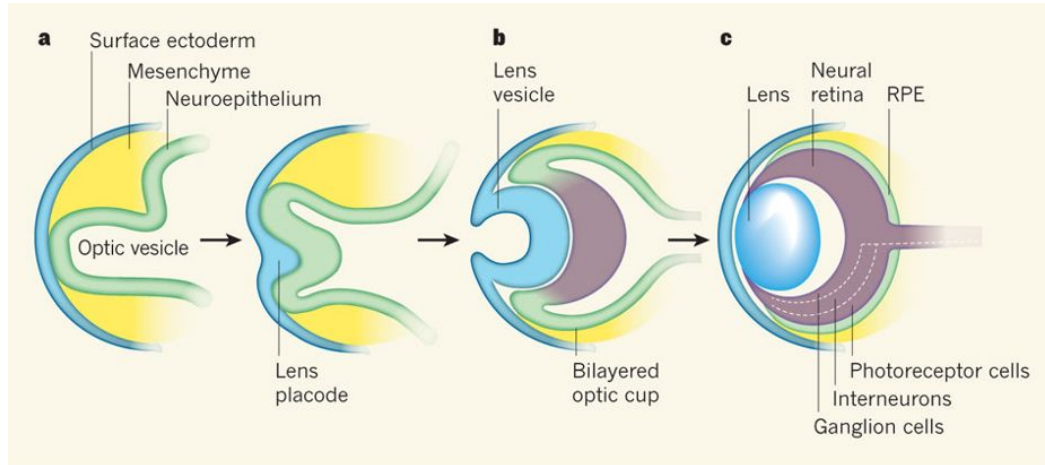
Figure 1.5. Eye Development from the Neural Plate. A) Eye-fated cells bud from the diencephalon to form the optic vesicles, which migrate bilaterally to reside at the side of the head. B) Once the vesicle reaches the surface ectoderm, it involutes forming a bi-layered cup. The lens is formed from the surface ectoderm. C) The inner layer becomes the neural retina, and the outer-most layer becomes the retinal pigmented epithelium (RPE). (Ali and Sowden, 2011). (Re-used with permission, license number 4540260928592).

1.4.2. The Structure of the Retina

The structure of the neural retina was first documented by Santiago Ramón y Cajal in 1893. He decided to try a Golgi silver stain on some tissue samples and discovered the beautifully organized laminar structure of the retina (Ramón y Cajal, 1893). The laminated structure of the retina is essential to its function and is delineated by different cell types and projections. At the inner basal side of the retina is the retinal ganglion cell layer. Retinal ganglion cells (RGCs) are the first cells to differentiate from retinal precursor cells (RPCs). They send out long axon projections from the retina contralaterally to the optic tecta in the brain. There are also misplaced amacrine cells in this layer. The next layer is the inner plexiform layer (IPL), consisting of axon projections to the retinal ganglion cell layer. The next cell layer moving apically is the inner nuclear layer (INL), containing bipolar cells, amacrine and horizontal cells. The outer plexiform layer (OPL) contains connecting axon projections linking to the most apical outer cell layer containing photoreceptor cells, rods and cones (Amini et al., 2018; Ramón y Cajal, 1893) (Figure 1.6). Müller glia span neural retina, with their cell bodies in the INL. Müller glia cells work to maintain a favorable environment for the neurons of the retina, by the uptake and removal of neurotransmitters such as GABA and acetylcholine (Sarthy and Lam, 1978). They also act as support scaffolds by connecting to the basal lamina. Without Müller glia, the retina loses its structure (MacDonald et al., 2015). The neural retina is encapsulated by the retinal pigmented epithelium (RPE) which has many roles, including photoreceptor maintenance by providing key metabolic nutrients, recycling of rhodopsin components, phagocytosing dying photoreceptor outer segments, which have a high turn-over rate, and absorbing
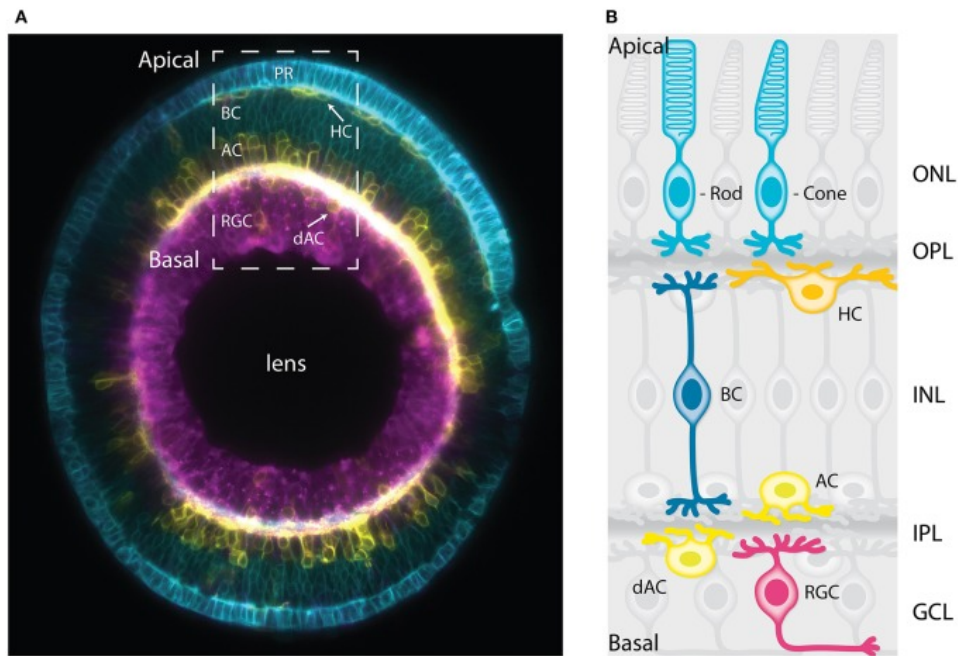
Figure 1.6. Cellular Organization of the Vertebrate Retina. A) triple transgenic SoFa-1 zebrafish retina showing the retinas laminar structure (*atoh7*:RFP, *ptf1a*:GFP, *crx*:CFP). B) The cells of the retina. The retina is consists of 3 cell body layer, and 2 plexiform layers. The ganglion cell layer (GCL) contains retinal ganglion cell (RGCs) and displaced amacrine cells (dAC). The inner nuclear layer (INL) houses bipolar (BC), horizontal (HC) and amacrine cells, and the outer nuclear layer (ONL) contains the rod and cone photoreceptor cells. The inner and outer plexiform layers (IPL/OPL) are the areas of synaptic connections between cells. (Amini et al., 2018). (Open access permission).

scattered light (Strauss, 2005). The RPE is an epithelial monolayer, which in zebrafish develops in two distinct phases between 8 somites/13 hpf and 26 somites/ 24 hpf. The first phase is one of rapid proliferation and expansion of the dorso-medial (future nasal) progenitor pool in an anterior direction from 8-16 somites. The second phase involves stretching of the cuboidal cells as the RPE expands to encapsulate the medial optic cup in a dorso-ventral pattern from 16-26 somites (Cechmanek and McFarlane, 2017). Impairments to the RPE are detrimental to photoreceptors and can result in retinal degeneration (Tschernutter et al., 2006).

1.4.3. Retinal Cell Fates

One of the big advantages of using zebrafish as a model system for development is that they develop very rapidly. All organ systems are present by 72 hpf, and the eyes are no exception. By 72 hpf, the retina is fully laminated with its three cell body layers, and two axonal plexiform layers. As mentioned, the first cells to differentiate are the RGCs. They differentiate from the multipotent retina progenitor pool, along with amacrine cells at the apical surface during interkinetic nuclear migration (Amini et al., 2018). Interkinetic nuclear migration is a process in which the nucleus migrates up and down the apical-basal plane, phasing with the cell cycle (Sauer, 1935). In the retina, progenitor cells project and attach to both apical and basal sides of the presumptive retina. Their nuclei translocate and migrate apically towards the pigmented epithelium to divide at M phase and return to the basal side near the lens during S phase. After cell division, one cell retains the basal process, and the other must generate a new one (Baye and Link, 2008). As RGCs and amacrine cells are born apically, but reside basally, they must migrate to their correct positions. This is achieved either by somal translocation as described, with basal processes attached, or

by multipolar migration, when apical and basal projections are lost, and the cell migrates 'freely' (Amini et al., 2018) (Figure 1.7).

Early born cells have flexible cell fates and have the potential to differentiate into any of the neuronal or glial cell types of the retina. They are influenced by intrinsic and extrinsic factors. After the initial RGCs and amacrine cells have differentiated from neuroepithelial progenitors, later cell fates become more restricted (Livesey and Cepko, 2001). The next cell types to be born are the horizontal cells and cones, followed by the rods and bipolar cells. The final cells to be born are the Müller glia (Wang and Cepko, 2016). Intrinsic and extrinsic factors are both important for cell fate determination within the retina. The environment that the cells develop in influences their fate. Experiments using dissociated cells taken from the retina across different time points were key in identifying this. Early born cells will differentiate into all cell types in the correct order if grown with other early cells. When early cells are mixed with later born cells, their fates will jump ahead to match the age of those they are grown with. Late cell fates are restricted, and so if mixed with early cells will not be able to 'retroactively' differentiate into early cell types, they will only become late cell types. This is thought to be driven by cell secreted factors, for example RGCs can express Shh and Gdf11 which control cell number and competency respectively (Sanes, 2011). Notch signaling is also important for controlling the number of cells allowed to differentiate at a time by controlling levels of pronounal genes within cells. Notch signaling promotes later cell fates such as rods (Perron and Harris, 2000), whereas inhibition leads to the expression of proneural transcription factors driving early fates, such as the transcription factor Ath5 that leads to RGC fates (Dorsky et al., 1997). Intrinsic cell fates are driven by the temporal expression of transcription factors. For
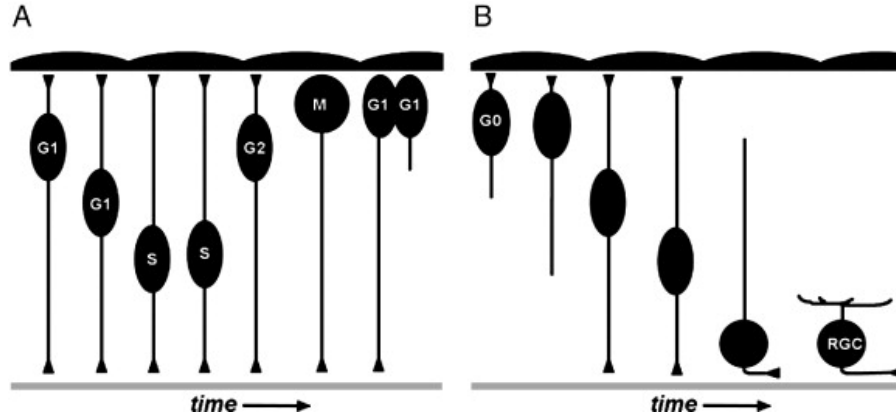
Figure 1.7. Interkinetic Nuclear Migration During Retinal Development. (A) Apical to basal movements during the cell cycle. M-phase is restricted to the apical surface near the RPE (Top of figure). (B) Model of nuclear translocation. Following cell cycle exit, progenitors maintain their apical and basal processes. These processes retract as the soma reaches the appropriate laminar position. (Apical RPE is up and basal is down) (Baye and Link, 2008). (Re-used with permission, license number 4540260630293).

example, *Vsx2* is initially expressed in all progenitors and then down-regulated in all cells except cells becoming bipolar and Müller cells (Clark et al., 2008). Vsx2 is a repressor and represses other key fate determining transcription factors, such as Ath5 and Foxn4 which restrict fates. Cells not expressing Vsx2 will have the potential to express Ath5 or Foxn2 and become other cell types (Vitorino et al., 2009). This is just one example of how cell fates are determined by transcription factors, each lineage will express different combinations, which will repress or activate others in a chain of events. This explains why later born cells cannot revert to earlier cell fates.

1.4.4. The Visual Pathway

The laminated structure of the retina is formed by the precise patterning and segregation of neuronal cell types. These layers are critical to set up the circuitry that allow for visual stimuli to be received by light striking the photosensitive retina, triggering chemical events that initiate nerve impulses that are processed in the visual centers of the brain. In zebrafish, RGC tracts converge and exit the eye to form the optic tracts. These cross over at the midline at the optic chiasm, and project to the optic tecta in the brain. In humans, RGCs project to additional centers in the brain for processing; the lateral geniculate body in the diencephalon, the superior colliculus (analogous to the optic tecta), the superior suprachiasmatic nucleus and the nucleus of the optic tract (Kumanogoh, 2015). Photoreceptors are unique in that at rest they are depolarized, due to Na+ channels being kept open by cGMP. Photoreceptors become hyperpolarized when light strikes them. Light induces an isomerization reaction to occur to retinal bound to opsin, where 11-*cis*-retinal is converted to all-*trans*-retinal. In turn this activates G-proteins which activate a phosphodiesterase, which inactivates cGMP, allowing Na+ channels to close. This hyperpolarizes the cells, leading to a

decrease in neurotransmitter release, which is interpreted in the brain as bright light. The cells of the RPE play a role in recycling all-*trans*-retinal back into 11-*cis*-retinal to be used again (Kumanogoh, 2015; Wald, 1945). Activated photoreceptors synapse with excitatory interneurons; the bipolar cells and inhibitory interneurons; the horizontal cells. Subsets of bipolar and amacrine cells converge onto one RGC. Subsets are of 2 types and are either excited by increases (ON) or decreases (OFF) of light. They terminate in different sublayers of the IPL, OFF apically and ON basally (Kumanogoh, 2015).

1.5. Axon Guidance Molecules and the Growth Cone in Development

1.5.1. The Growth Cone

Cellular migration during development is a highly regulated process, for example in neuronal development, differentiated neurons must send out axon projections which navigate a complex environment to reach their final target. Axons sense the environment via growth cones at the leading edge. Growth cones can respond to chemo-repulsive and chemo-attractive diffusible signals, and membrane-bound contact-repulsive and attractive signals in the environment to find their way to their synaptic targets. Growth cones were first described by Ramón y Cajal as 'battering rams' (Ramón y Cajal, 1893). Later, growth cones were more closely described as having amoeboid-like movements (Speidel, 1942). Growth cones are highly dynamic structures consisting of stiff microtubules in the center towards the axon linked via myosin to more flexible F-actin in the distal lamellipodia and finger-like filopodia projections (Heidemann, 1996) (Figure 1.8). Filopodial extensions project into the environment and sense positional cues via receptor-mediated signaling initiated at the

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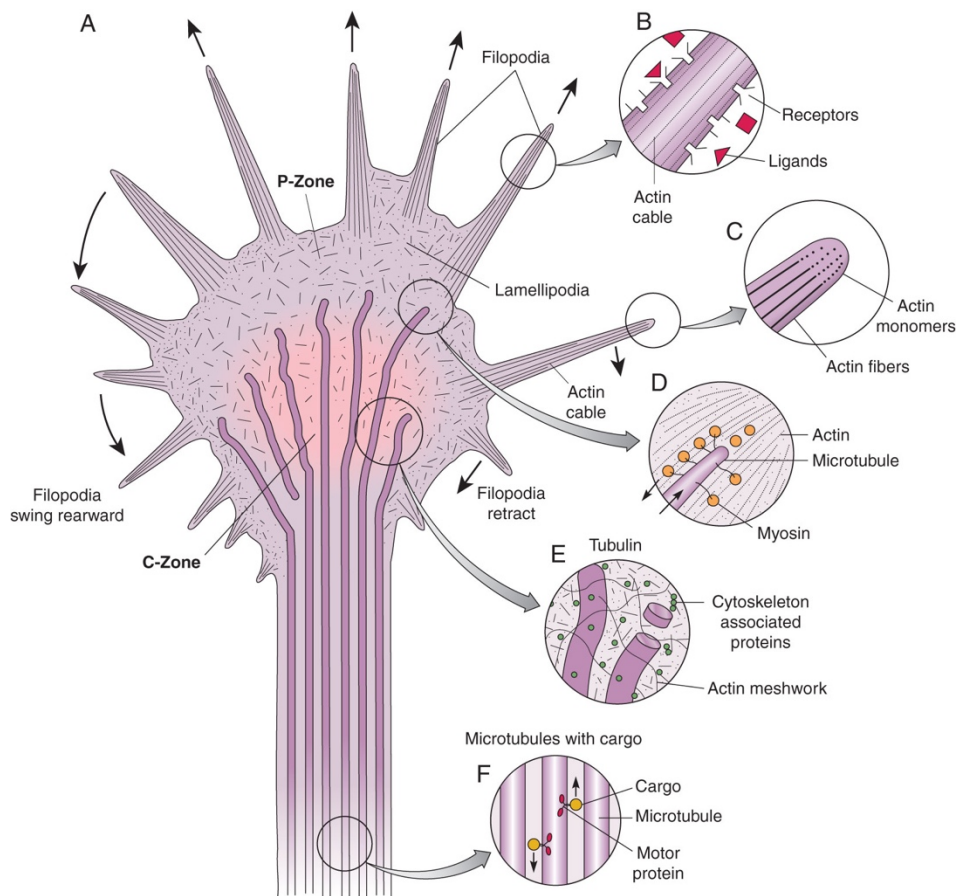
Figure 1.8. The Structure of the Growth Cone. The cytoskeletal arrangement of the growth cone allows for it to be dynamic to changing environments, which it senses through receptor interactions at the membrane surface. Actin polymerizes at the leading edge and pushes against the membrane to form filopodia. Filopodia eventually retract and move rear-ward. Between them are lamellipodia made of an actin meshwork. Actin is connected to the microtubules of the main axon via myosin interactions, allowing for traction and movement. Microtubules also act as 'highways' for the transport of cargo to and from the cell body. (Sanes, 2011). (Re-used with permission, license number 4557100694801).

surface membrane. Migratory axon projections are able to change directions rapidly due to the highly dynamic nature of the cytoskeletal elements make up the growth cone. At the leading edge, F-actin is constantly growing from polymerization of G-actin, and depolymerizing at the minus end. Actin and microtubule polymerization are promoted when the growth cone meets positive axon guidance cues, such as Netrins, growth factors and trophic factors. Conversely, growth cone collapse occurs when negative guidance cues are met, such as Semaphorins and Slits (Sanes, 2011). Guidance cues are able to influence cytoskeletal dynamics in the growth cone in many ways, one being to alter the activity of microtubule and actin associated binding proteins. The first repulsive axon guidance cue, termed 'collapsin' (now Sema3A) acts to destabilize microtubules, leading to filipodia retraction, and temporary paralysis of migration. Collapsin response mediator proteins (CRMPS) are microtubule associated proteins (MAPs) which stabilize microtubules within growth cones. Repulsive Sema3A has been shown to activate intracellular signaling cascades upon activation of their PlxnA receptors to destabilize CRMP2 attachments to microtubules via phosphorylation, leading to microtubule catastrophe and growth cone collapse (Goshima et al., 1995).

1.5.2. Pioneer Axons and Cell Adhesion Molecules

Pioneer axons are the first to migrate in development and form a scaffold for later neurons to follow. Pioneer neurons have help finding their way by attaching to guidepost cells. These cells are spaced close enough together along the route of migration, that the pioneer axon can easily send out filipodia to meet the next guidepost cell and follow the chain like stepping stones (Caudy and Bentley, 1986). Once the pioneer neurons have cut a path to their targets, new neurons can fasciculate with them and use them as a 'highway' to crawl along. Cell to cell attachments are typically via

homophilic cell adhesion molecules expressed on each axon surface, such as igCAMs and Cadherins (Walsh et al., 1997). Pioneer axons must crawl along the elements of the cytoskeleton and attach via integrins on their cell surface. Integrins form heterophilic dimers, and the expression of different integrin subunits determines which substrate they prefer (McKerracher et al., 1996). For example, neurons expressing integrin subunits α6 and β1 prefer to bind to Laminin, whereas neurons expressing α5

and β1 prefer Fibronectin. The expression levels of different integrin subunits can change throughout the migration of the axon, allowing for the migration through different environments (Cohen and Johnson, 1991).

1.6. Semaphorins and Plexins

1.6.1. The Semaphorin and Plexin Families and Structure

Semas and Plexins are a large family signaling proteins. Classically, Semas were discovered to mediate axonal migration during development as chemo-repulsive cues (Luo et al., 1993a). Using chick brain DRG neurons, Luo discovered the first secreted repulsive guidance cue and named it Collapsin (later to be named Sema3A), due to its role in growth cone collapse. When growth cones interact with a repulsive cue, filopodia retract and cause a temporary paralysis in migration until the cytoskeleton can recover and change direction (Buel et al., 2010; Schmidt and Strittmatter, 2007). Semas are secreted and transmembrane signaling molecules that bind and transmit signals primarily through Plxn receptors. The Sema family contains 21 genes and 8 additional genes found in invertebrates. There are 8 classes of Semas, 1-7, one viral Sema, V, and 4 classes of Plxns, A-D. Each have subfamilies and have different binding preferences to each other (Neufeld and Kessler, 2008) (Figure 1.9).
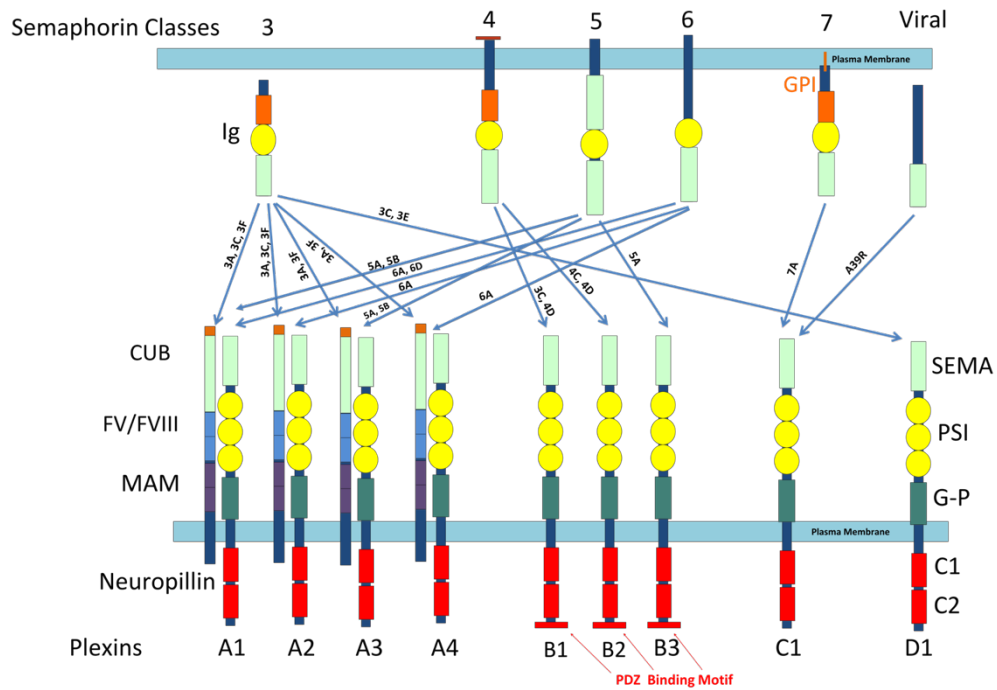
Figure 1.9. Semaphorin and Plexin Families. Semas are either transmembrane, secreted or GPI-anchored. Secreted Semas require an additional neuropillins co-receptor to signal. Semas and Plxns share a common Sema domain (light green) which they use for binding. There are high levels of cross talk between Semas and Plxns as indicated by the arrows which indicate binding partners. Ig-immunoglobulin-like, CUB- complement binding, FV/FVIII- coagulation factor-like, MAM- Meprin, A5, Mu, PSI- Plexin, Sema and integrin, G-P- Glycine-proline-rich, C1/C2- Catalytic 1/ 2. Adapted from Kumanogoh (2015). (Edited and re-used with permission, license number 4540261503517).

The unifying element of Plxns and Semas are their extracellular N-terminal Sema domains. The Sema domain is a large 500 amino acid, highly conserved extracellular domain and has an atypical 7-blade β propeller fold structure that allows for binding (Gherardi et al., 2004). The complex structure of the Sema domain enables binding to a variety of proteins; Sema and Plxn binding, dimerization of Plxns and Semas themselves and binding to other factors such as neuropillins, VEGFRs (vascular endothelial growth factor receptors) (Segarra et al., 2012) and MET receptors (Giordano et al., 2002). Semas can be secreted (Sema 2, 3, 6 & V), transmembrane (Sema 4-6), or GPI anchored (Sema 7). Secreted Semas require additional Neuropillin receptor binding to stabilize the Sema/Plxn complex (Janssen et al., 2012). All Semas contain the described Sema domain and an Ig (immunoglobulin like) domain. Plxns are more complex proteins in terms of domains, and contain an extracellular Sema domain, PSI domain (Plexin, Semaphorin, integrin), G-P rich domain and a c-terminal split cytoplasmic GAP domain (GTPase activating protein), that can regulate downstream Ras-family small GTPases (Negishi et al., 2005; Pasterkamp, 2005). Small GTPases act as molecular switches, 'on' when GTP bound, and 'off' when GDP bound, and influence downstream signaling cascades (Bos et al., 2007). Plxns are the first example of a receptor that contains a GAP domain within its structure (He et al., 2009). The GAP domain consists of two segments, C1 and C2, split by a 200 amino acid domain, known as the Rho GTPase binding domain (RBD). C1 and C2 each contain one of two arginine residues critical for GAP catalytic function (Scheffzek et al., 1996). C1 and C2 fold together to form and active GAP domain (He et al., 2009).The RBD of PlxnAs and Bs bind to Rac1, Rnd1 and RhoD in their GTP bound active forms. Rho GTPase binding to

the RBD is necessary, but not sufficient for Plxn activation. Plxns exist in an auto-inhibitory state, in which intracellular GAP domains are inactive (Takahashi and Strittmatter, 2001). Activation of Plxns is a two-step process. First, Rho GTPases bind to the RBD, rendering the C1-C2 GAP domain catalytic site accessible to small GTPases, by driving a conformational change, relieving inhibition by an 'activation segment'. (See Figure 1.10) (Wang et al., 2013). Next, receptor clustering by Sema ligands triggers GTP hydrolysis of small GTPases (Oinuma et al., 2004b). Investigations into the small GTPase that Plexin GAP domains regulate have been controversial. It was first thought to be R-Ras, and was logical as the GAP domain has high structural homology to Ras GAP domains (He et al., 2009). R-Ras is a homolog of the more well-known small GTPase, Ras, and is involved in cell adhesion via integrin activation, promoting migration (Toyofuku et al., 2005; Zhang et al., 1996). But later more detailed *in vivo* studies in mice refuted these findings, and instead found Rap to be regulated by Plxns (Pascoe et al., 2015; Wang et al., 2012). Raps are a subfamily of Ras homologs and have shown to bind the Plxn GAP domain and undergo GTP hydrolysis induced by Sema activation of Plxn. Raps are also key activators of integrin activation, so GAP conversion to its GDP-bound inactive form blocks integrin signaling, decreasing cell adhesion to the ECM, preventing cell migration. It is now accepted that Plxns have Rap GAP activity (Worzfeld et al., 2014), and it is speculated that R-Ras is indirectly regulated by this signaling pathway (Pascoe et al., 2015). Additional Plxn receptor-proximal signaling events are starting to be uncovered. For example, PlxnA C-terminal domains are phosphorylated by the non-receptor tyrosine kinase, Fyn, which is required for Plxn function in eye development as shown in zebrafish (St Clair et al., 2018).
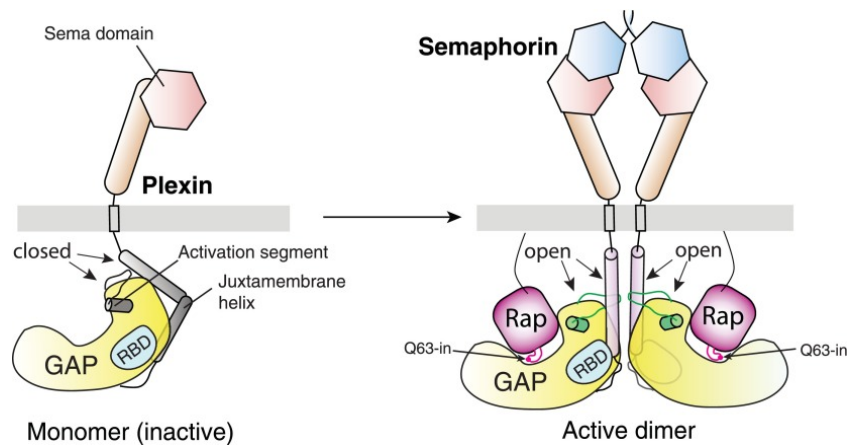
Figure 1.10. Model for the Activation of the Plexin RapGAP by Semaphorin-Induced Dimerization. Model based on the crystal structure of zebrafish PlxnC1 in complex with Rap. A) The Plexin intracellular domain exists in a closed auto-inhibitory state in the absence of Semaphorin. B) Semaphorin induced dimerization is necessary for the activation of the Plexin intracellular domain. Dimerization aided by Rho-GTPase binding to the RBD (Rho binding domain) drives an intracellular conformational change to Plxn, forming an active GAP domain, allowing for Rap binding. This stimulates the conversion of Rap-GTP to Rap-GDP, rendering it inactive. In terms of repulsive axon guidance, the inactivation of RAP leads to the inactivation of integrins, weakening cell-matrix adhesions, preventing further migration (Wang et al., 2013). (Open access permission).

Semas can reverse signal (Yu et al., 2010), and can signal differently when expressed in *cis* or in *trans* with receptors. For example in mice DRGs, both sympathetic and sensory neurons express the Sema6A receptor, Plexin-A4, but only sympathetic neurons respond to it because Sema6a is also expressed on sensory neurons, and *cis*-inhibition of PlxnA4 occurs (Haklai-Topper et al., 2010a).

1.6.2. The Roles of Semaphorins and Plexins

Semas and Plxns are large families of proteins, each displaying different spatio-temporal expression patterns throughout development, allowing them to have varied roles (Ebert et al., 2012; Emerson et al., 2018). As mentioned, they were initially discovered to be axon guidance cues for developing neurons (Luo et al., 1993b), however it is being appreciated that they have much wider roles in development and are important across many systems. They have been shown to be important for vasculogenesis (Serini et al., 2003a), heart development (Toyofuku et al., 2004), immunity (Shi et al., 2000b), bone development (Behar et al., 1996a; Kang and Kumanogoh, 2013) and early eye development (Ebert et al., 2014; Emerson et al., 2017). Sema/Plxn signaling is important in some adult tissues, including neuronal regeneration and recovery after injury (Shim et al., 2012) and have been implicated in disease sates such as metastatic cancer (Gu and Giraudo, 2013) and neurological disorders (Van Battum et al., 2015), making them novel therapeutic targets.

1.6.3. Semaphorins and Plexins in the Central Nervous System

In neuronal development, documented roles of Semas and Plxns include long and short-range axon guidance, neural circuit formation and refinement. Semas are primarily repulsive guidance cues, and cause growth cone collapse and re-direction in

Plxn expressing growth cones. In the central nervous system, Semas and Plxns have documented roles in the developing eye and brain. In the developing brain, PlxnA2 and Sema6A guide neuronal positioning in the cerebellum (Renaud and Chetodal 2014, Renaud et al., 2008), lamination of the hippocampus (Tawarayama et al., 2010), and guidance of the corticospinal tract (Runker at al., 2008). Sema/Plxn signaling governs many aspects of eye development. The retina is highly laminated and requires the precise positioning of neuronal cell types into specified layers. Sema5A and 5B are expressed in the inner nuclear layer (INL) and form a repulsive limiting edge to PlxnA1 and A3 expressing axons from the inner plexiform layer (IPL), to keep them from over migrating. (Matsuoka et al., 2011a). Sema6A along with PlxnA2 and A4 control the stratification within the IPL of the retina. Specifically, Sema6A signaling controls the arborization of horizontal cell and on starburst amacrine cells (SAC) neurites, which govern direction-sensitive responses to motion by synapsing with direction-sensitive ganglion cells (Sun et al., 2013a). The inner plexiform layer in mice contains 5 sublayers, Off SAC projections express PlxnA2 and stratify in layer 2, and on SAC projections co-express Sema6A and PlxnA2, and target layer 4. Sema6A is expressed in the lower half of the IPL, and so repulses off SACs, and restricts them to the top layers, whereas inhibitory *cis* expression of Sema6A and PlxnA2 allows on SACs to occupy the bottom layers, as PlxnA2 is insensitive to Sema6A in *trans* when co-expressed in *cis* (Haklai-Topper et al., 2010b). Sema6D is important for guiding retinal ganglion cell projections in the optic nerve across the midline. Sema6D is expressed by glial cells in the optic chiasm. The Sema6D receptor, PlxnA1 is expressed by crossing RGCs (Kuwajima et al., 2012). Secreted Sema3D and 3E are expressed in tissues surrounding the optic chiasm and help to keep the RGCs projecting correctly (Sakai and Halloran, 2006). In early eye development it

has been shown that Sema6A and PlxnA2 work together to set up repulsive domains in optic vesicles for the organization and cohesion of retinal precursor cells (RPCs) during early migration (Ebert et al., 2014). Sema6A and PlxnA2 are important for proliferation of RPCs within optic vesicles by regulating transcription of key downstream target genes, including *rasl11b* (Emerson et al., 2017).

1.6.4. Semaphorins and Plexins in the Peripheral Nervous System

Sema/Plxn signaling also governs neuronal positioning in the peripheral nervous system. The first vertebrate Sema to be discovered, Sema3A was identified to stimulate growth cone collapse in sensory neurons (Luo et al., 1993b). Sema3A is expressed throughout the ventral spinal cord and restricts cutaneous sensory neurons to project only to the dorsal side. Early in development, Sema3A is transiently expressed dorsally, preventing premature innervation by sensory neurons expressing PlxnA3/A4 and Npn1 (Wright et al., 1995). Sema3A is also expressed in the periphery, and is important during embryonic development in axon pruning, fasciculation, branching and targeting of sensory and motor neurons (White and Behar, 2000). As previously described, motor axons project to skeletal muscle targets, and are grouped into motor columns along the A-P axis. Sema3A/Npn1 and Sema3F/Npn2 signaling are important in controlling the timing of motor innervation to the periphery. Npn1 is expressed in the lateral motor column, and Npn2 in the medial column. Spatially controlled Sema3 expression in the periphery ensures the motor nerves meet the correct synaptic targets (Huber et al., 2005). Sema6D is expressed in the dorsal horn of the spinal cord and guide PlxnA1 expressing proprioceptive sensory neurons towards the midline to project ventrally away from cutaneous sensory neurons, which do not express PlxnA1, at the dorsal side (Kumanogoh, 2015; Yoshida et al., 2006). Motor neuron sin zebrafish are patterned

within segments. There are three motor neurons per segment, RoP (rostral primary), MiP (middle primary) and CaP (caudal primary), which exit the spinal cord at a common exit point and innervate targets on myotomes. Sema3A is expressed in the myotome at different locations throughout development to guide motor axons to their targets. CaP neurons express Npn1 and is guided by Sema3A repulsion. Sema3A is initially expressed in the posterior half of the segment as a barrier wall to guide the axon projection along, as it projects ventrally. Npn1 mutants show mis-patterning and multiple axon exit points (Sato-Maeda et al., 2008). PlxnA4 has been shown to be necessary for sensory neuron branching, where PlxnA4 knockdown results in a reduction of branching, and excessive branching driven by Slit overexpression can be rescued by PlxnA4 knockdown (Miyashita et al., 2004).

1.7 Specific Aims

1.7.1. Background and Significance

Identification of Target Genes Downstream of Semaphorin6A/PlexinA2 in Zebrafish

In an investigation into the early expression patterns of *sema6A* and *plxnA2*, it was discovered that they were expressed in overlapping regions in the early developing eye fields. At this early stage the cells that make up the optic vesicles are retinal progenitor cells (RPCs), so have not yet differentiated, and so do not require Sema/Plxn signaling for their canonical axon guidance role. To investigate the early roles of this signaling pathway, Morpholino (MO) knockdown was utilized. Two striking phenotypes were uncovered in common to both knockdowns, at 18 somites, a loss of cohesion of cells within the optic vesicles, resulting in ectopic cells, and a decrease in RPC proliferation (Ebert et al., 2014). As these phenotypes were induced at such an early point

in development and given that many developmental processes are dependent on genetic regulation, it was hypothesized that Sema6A and PlxnA2 signaling could be regulating the transcription of downstream target genes. To investigate this, a microarray experiment was performed, uncovering 58 differentially regulated genes in common to both knockdown conditions. Prior to our study, it was not known that Sema/Plxn signaling led to changes in gene transcription. In an effort to understand the contribution of differential gene transcription to Sema6A/PlxnA2 knockdown phenotypes, target genes with predicted gene ontology functions in migration and proliferation were investigated. Of focus, one target gene, *Rasl11b* was investigated, in its role in the regulation of RPC proliferation.

The Role of Shootin-1 in Zebrafish Neurodevelopment

Shtn-1 was upregulated 1.21 LogFC in *sema6a* knockdowns, and 1.12 LogFC in *plxnA2* knockdowns (Emerson et al., 2017), with predicted gene ontology functions in cell migration. For this reason, the roles of Shtn-1 in zebrafish neuronal development were investigated. Shtn-1 is a neuronal polarization signal and clutch molecule. It links the intracellular actin cytoskeleton of the growth cone to the surrounding extracellular matrix, which enables traction forces for the cell to resist against in order to propagate forward. The increase in *shtn-1* expression seen in knockdowns was recapitulated to understand the role of PlxnA2 regulation of *shtn-1* expression in neurodevelopment.

Neuronal Expression Patterns of the PlexinA Family in Zebrafish Development

Cellular developmental environments are dynamic and in order to begin to investigate the roles of any developmental gene or protein, it is important to first understand where and when they are expressed. Being a large family of proteins with the ability to cross talk, Semas and Plxns rely on differential expression patterns to perform

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their individual roles. Occasionally Plxns can compensate for each other and we have shown that PlxnA1 can compensate for PlxnA2 (unpublished data). This aim uses *in situ* hybridization to comprehensively uncover the neuronal expression patterns of the PlxnA family in the early developing zebrafish. In addition to presenting for the first time that zebrafish have two genes for PlxnA1, A1a and A1b, which have divergent expression patterns.

1.7.2. Aims

Aim 1. Identification of *Rasl11b* as a downstream gene target of Semaphorin6A/ PlexinA2 signaling and its role in proliferation of retinal precursor cells in the developing zebrafish eye.

Aim 2. Investigating the role of *Shootin-1* in the developing zebrafish nervous system.

Aim 3. Elucidation of *plexinA* family member neuronal expression patterns during early zebrafish development.

1.8. References

Adler, R., Canto-Soler, M.V., 2007. Molecular mechanisms of optic vesicle development: complexities, ambiguities and controversies. Dev Biol 305, 1-13.

Allende, M.L., Amsterdam, A., Becker, T., Kawakami, K., Gaiano, N., Hopkins, N., 1996. Insertional mutagenesis in zebrafish identifies two novel genes, pescadillo and dead eye, essential for embryonic development. Genes & development 10, 3141-3155.

Almeida, A.D., Boije, H., Chow, R.W., He, J., Tham, J., Suzuki, S.C., Harris, W.A., 2014. Spectrum of Fates: a new approach to the study of the developing zebrafish retina. Development 141, 1971.

Amini, R., Rocha-Martins, M., Norden, C., 2018. Neuronal Migration and Lamination in the Vertebrate Retina. Frontiers in neuroscience 11, 742-742.

Avdesh, A., Chen, M., Martin-Iverson, M.T., Mondal, A., Ong, D., Rainey-Smith, S., Taddei, K., Lardelli, M., Groth, D.M., Verdile, G., Martins, R.N., 2012. Regular care and maintenance of a zebrafish (Danio rerio) laboratory: an introduction. Journal of visualized experiments : JoVE, e4196-e4196.

Baye, L.M., Link, B.A., 2008. Nuclear migration during retinal development. Brain research 1192, 29-36.

Behar, O., Golden, J.A., Mashimo, H., Schoen, F.J., Fishman, M.C., 1996. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. Nature 383, 525-528.

Bos, J.L., Rehmann, H., Wittinghofer, A., 2007. GEFs and GAPs: Critical Elements in the Control of Small G Proteins. Cell 129, 865-877.

Buel, G.R., Rush, J., Ballif, B.A., 2010. Fyn promotes phosphorylation of collapsin response mediator protein 1 at tyrosine 504, a novel, isoform-specific regulatory site. Journal of Cellular Biochemistry 111, 20-28.

Carpenter, E.M., Goddard, J.M., Chisaka, O., Manley, N.R., Capecchi, M.R., 1993. Loss of Hox-A1 (Hox-1.6) function results in the reorganization of the murine hindbrain. Development (Cambridge, England) 118, 1063-1075.

Castro, B., Barolo, S., Bailey, A.M., Posakony, J.W., 2005. Lateral inhibition in proneural clusters: cis-regulatory logic and default repression by Suppressor of Hairless. Development (Cambridge, England) 132, 3333-3344.

Caudy, M., Bentley, D., 1986. Pioneer growth cone steering along a series of neuronal and non-neuronal cues of different affinities. The Journal of neuroscience : the official journal of the Society for Neuroscience 6, 1781-1795.

Cechmanek, P.B., McFarlane, S., 2017. Retinal pigment epithelium expansion around the neural retina occurs in two separate phases with distinct mechanisms. 246, 598-609.

Chalepakis, G., Stoykova, A., Wijnholds, J., Tremblay, P., Gruss, P., 1993. Pax: gene regulators in the developing nervous system. Journal of neurobiology 24, 1367-1384.

Clark, A.M., Yun, S., Veien, E.S., Wu, Y.Y., Chow, R.L., Dorsky, R.I., Levine, E.M., 2008. Negative regulation of Vsx1 by its paralog Chx10/Vsx2 is conserved in the vertebrate retina. Brain Res 1192, 99-113.

Cohen, J., Johnson, A.R., 1991. Differential effects of laminin and merosin on neurite outgrowth by developing retinal ganglion cells. Journal of cell science. Supplement 15, 1-7.

Creaser, C.W., 1934. The Technic of Handling the Zebra Fish (Brachydanio rerio) for the Production of Eggs Which Are Favorable for Embryological Research and Are Available at Any Specified Time Throughout the Year. Copeia 1934, 159-161.

Dee, C.T., Hirst, C.S., Shih, Y.H., Tripathi, V.B., Patient, R.K., Scotting, P.J., 2008. Sox3 regulates both neural fate and differentiation in the zebrafish ectoderm. Developmental biology 320, 289-301.

Dessaud, E., McMahon, A.P., Briscoe, J., 2008. Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. Development (Cambridge, England) 135, 2489-2503.

Dorsky, R.I., Chang, W.S., Rapaport, D.H., Harris, W.A., 1997. Regulation of neuronal diversity in the Xenopus retina by Delta signalling. Nature 385, 67-70.

Driever, W., Solnica-Krezel, L., Schier, A.F., Neuhauss, S.C., Malicki, J., Stemple, D.L., Stainier, D.Y., Zwartkruis, F., Abdelilah, S., Rangini, Z., Belak, J., Boggs, C., 1996. A genetic screen for mutations affecting embryogenesis in zebrafish. Development (Cambridge, England) 123, 37-46.

Ebert, A.M., Childs, S.J., Hehr, C.L., Cechmanek, P.B., McFarlane, S., 2014. Sema6a and Plxna2 mediate spatially regulated repulsion within the developing eye to promote eye vesicle cohesion. Development 141, 2473-2482.

Ebert, A.M., Lamont, R.E., Childs, S.J., McFarlane, S., 2012. Neuronal expression of class 6 semaphorins in zebrafish. Gene expression patterns : GEP 12, 117-122.

Emerson, S.E., Light, S.E., Ebert, A.M., 2018. Neuronal expression patterns of the PlexinA family during zebrafish development. Gene expression patterns : GEP 27, 56-66.

Emerson, S.E., St Clair, R.M., Waldron, A.L., Bruno, S.R., Duong, A., Driscoll, H.E., Ballif, B.A., McFarlane, S., Ebert, A.M., 2017. Identification of target genes downstream of semaphorin6A/PlexinA2 signaling in zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 246, 539-549.

Engeszer, R.E., Patterson, L.B., Rao, A.A., Parichy, D.M., 2007. Zebrafish in the wild: a review of natural history and new notes from the field. Zebrafish 4, 21-40.

Gherardi, E., Love, C.A., Esnouf, R.M., Jones, E.Y., 2004. The sema domain. Current Opinion in Structural Biology 14, 669-678.

Giordano, S., Corso, S., Conrotto, P., Artigiani, S., Gilestro, G., Barberis, D., Tamagnone, L., Comoglio, P.M., 2002. The semaphorin 4D receptor controls invasive growth by coupling with Met. Nat Cell Biol 4, 720-724.

Goshima, Y., Nakamura, F., Strittmatter, P., Strittmatter, S.M., 1995. Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. Nature 376, 509-514.

Gu, C., Giraudo, E., 2013. The role of semaphorins and their receptors in vascular development and cancer. Experimental cell research 319, 1306-1316.

Haffter, P., Granato, M., Brand, M., Mullins, M.C., Hammerschmidt, M., Kane, D.A., Odenthal, J., van Eeden, F.J., Jiang, Y.J., Heisenberg, C.P., Kelsh, R.N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C., Nusslein-Volhard, C., 1996. The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development (Cambridge, England) 123, 1-36.

Haklai-Topper, L., Mlechkovich, G., Savariego, D., Gokhman, I., Yaron, A., 2010a. Cis interaction between Semaphorin6A and Plexin-A4 modulates the repulsive response to Sema6A. The EMBO Journal 29, 2635-2645.

Haklai-Topper, L., Mlechkovich, G., Savariego, D., Gokhman, I., Yaron, A., 2010b. Cis interaction between Semaphorin6A and Plexin-A4 modulates the repulsive response to Sema6A. The EMBO journal 29, 2635-2645.

Halder, G., Callaerts, P., Gehring, W.J., 1995. Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila. Science (New York, N.Y.) 267, 1788-1792.

Hamburger, V., 1969. Hans Spemann and the organizer concept. Experientia 25, 1121-1125.

Hamilton, F., Swaine, J., 1822. An account of the fishes found in the river Ganges and its branches. Hurst, Robinson, and Co., Edinburgh.

Hartley, D.A., Xu, T.A., Artavanis-Tsakonas, S., 1987. The embryonic expression of the Notch locus of Drosophila melanogaster and the implications of point mutations in the extracellular EGF-like domain of the predicted protein. The EMBO journal 6, 3407-3417.

He, H., Yang, T., Terman, J.R., Zhang, X., 2009. Crystal structure of the plexin A3 intracellular region reveals an autoinhibited conformation through active site sequestration. Proceedings of the National Academy of Sciences of the United States of America 106, 15610-15615.

Heidemann, S.R., 1996. Cytoplasmic mechanisms of axonal and dendritic growth in neurons. International review of cytology 165, 235-296.

Hemmati-Brivanlou, A., Kelly, O.G., Melton, D.A., 1994. Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. Cell 77, 283-295.

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J.C., Koch, R., Rauch, G.-J., White, S., Chow, W., Kilian, B., Quintais, L.T., Guerra-Assunção, J.A., Zhou, Y., Gu, Y., Yen, J., Vogel, J.-H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S.F., Laird, G.K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliott, D., Threadgold, G., Harden, G., Ware, D., Begum, S., Mortimore, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Lloyd, C., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D.,

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Auger, K., Wright, D., Collins, J., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J.D., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley, C.M., Ersan-Ürün, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberländer, M., Rudolph-Geiger, S., Teucke, M., Lanz, C., Raddatz, G., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Schuster, S.C., Carter, N.P., Harrow, J., Ning, Z., Herrero, J., Searle, S.M.J., Enright, A., Geisler, R., Plasterk, R.H.A., Lee, C., Westerfield, M., de Jong, P.J., Zon, L.I., Postlethwait, J.H., Nüsslein-Volhard, C., Hubbard, T.J.P., Crollius, H.R., Rogers, J., Stemple, D.L., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498.

Huber, A.B., Kania, A., Tran, T.S., Gu, C., De Marco Garcia, N., Lieberam, I., Johnson, D., Jessell, T.M., Ginty, D.D., Kolodkin, A.L., 2005. Distinct roles for secreted semaphorin signaling in spinal motor axon guidance. Neuron 48, 949-964.

Janssen, B.J., Malinauskas, T., Weir, G.A., Cader, M.Z., Siebold, C., Jones, E.Y., 2012. Neuropilins lock secreted semaphorins onto plexins in a ternary signaling complex. Nature structural & molecular biology 19, 1293-1299.

Jessell, T.M., Bovolenta, P., Placzek, M., Tessier-Lavigne, M., Dodd, J., 1989. Polarity and patterning in the neural tube: the origin and function of the floor plate. Ciba Foundation symposium 144, 255-276; discussion 276-280, 290-255.

Kane, D.A., Kimmel, C.B., 1993. The zebrafish midblastula transition. Development (Cambridge, England) 119, 447-456.

Kang, S., Kumanogoh, A., 2013. Semaphorins in bone development, homeostasis, and disease. Seminars in cell & developmental biology 24, 163-171.

Karlsson, J., von Hofsten, J., Olsson, P.E., 2001. Generating transparent zebrafish: a refined method to improve detection of gene expression during embryonic development. Marine biotechnology (New York, N.Y.) 3, 522-527.

Kawakami, K., 2005. Transposon tools and methods in zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 234, 244-254.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 203, 253-310.

Kumanogoh, A., 2015. Semaphorins. Springer Japan.

Kuriyama, S., Mayor, R., 2008. Molecular analysis of neural crest migration. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 363, 1349-1362.

Kuwajima, T., Yoshida, Y., Takegahara, N., Petros, T.J., Kumanogoh, A., Jessell, T.M., Sakurai, T., Mason, C., 2012. Optic chiasm presentation of Semaphorin6D in the context of Plexin-A1 and Nr-CAM promotes retinal axon midline crossing. Neuron 74, 676-690.

Lamb, T.M., Knecht, A.K., Smith, W.C., Stachel, S.E., Economides, A.N., Stahl, N., Yancopolous, G.D., Harland, R.M., 1993. Neural induction by the secreted polypeptide noggin. Science (New York, N.Y.) 262, 713-718.

Lewis, E.B., 1978. A gene complex controlling segmentation in Drosophila. Nature 276, 565-570.

Liu, J., Zhou, Y., Qi, X., Chen, J., Chen, W., Qiu, G., Wu, Z., Wu, N., 2017. CRISPR/Cas9 in zebrafish: an efficient combination for human genetic diseases modeling. Human genetics 136, 1-12.

Livesey, F.J., Cepko, C.L., 2001. Vertebrate neural cell-fate determination: lessons from the retina. Nature reviews. Neuroscience 2, 109-118.

Lo, L., Dormand, E., Greenwood, A., Anderson, D.J., 2002. Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. Development (Cambridge, England) 129, 1553-1567.

Londin, E.R., Niemiec, J., Sirotkin, H.I., 2005. Chordin, FGF signaling, and mesodermal factors cooperate in zebrafish neural induction. Developmental biology 279, 1-19.

Luo, Y., Raible, D., Raper, J.A., 1993a. Collapsin: A protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75, 217-227.

Luo, Y., Raible, D., Raper, J.A., 1993b. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75, 217-227.

Macdonald, R., Barth, K.A., Xu, Q., Holder, N., Mikkola, I., Wilson, S.W., 1995. Midline signalling is required for Pax gene regulation and patterning of the eyes. Development 121, 3267-3278.

MacDonald, R.B., Randlett, O., Oswald, J., Yoshimatsu, T., Franze, K., Harris, W.A., 2015. Muller glia provide essential tensile strength to the developing retina. The Journal of cell biology 210, 1075-1083.

Marchal, L., Luxardi, G., Thome, V., Kodjabachian, L., 2009. BMP inhibition initiates neural induction via FGF signaling and Zic genes. Proceedings of the National Academy of Sciences of the United States of America 106, 17437-17442.

Matsuoka, R.L., Chivatakarn, O., Badea, T.C., Samuels, I.S., Cahill, H., Katayama, K.-I., Kumar, S.R., Suto, F., Chédotal, A., Peachey, N.S., Nathans, J., Yoshida, Y., Giger, R.J., Kolodkin, A.L., 2011. Class 5 transmembrane semaphorins control selective Mammalian retinal lamination and function. Neuron 71, 460-473.

McKerracher, L., Chamoux, M., Arregui, C.O., 1996. Role of laminin and integrin interactions in growth cone guidance. Molecular neurobiology 12, 95-116.

Millet, S., Bloch-Gallego, E., Simeone, A., Alvarado-Mallart, R.M., 1996. The caudal limit of Otx2 gene expression as a marker of the midbrain/hindbrain boundary: a study using in situ hybridisation and chick/quail homotopic grafts. Development (Cambridge, England) 122, 3785-3797.

Miyashita, T., Yeo, S.Y., Hirate, Y., Segawa, H., Wada, H., Little, M.H., Yamada, T., Takahashi, N., Okamoto, H., 2004. PlexinA4 is necessary as a downstream target of Islet2 to mediate Slit signaling for promotion of sensory axon branching. Development (Cambridge, England) 131, 3705-3715.

Muzio, L., Mallamaci, A., 2003. Emx1, emx2 and pax6 in specification, regionalization and arealization of the cerebral cortex. Cerebral cortex (New York, N.Y. : 1991) 13, 641-647.

Nasevicius, A., Ekker, S.C., 2000. Effective targeted gene 'knockdown' in zebrafish. Nature genetics 26, 216-220.

Negishi, M., Oinuma, I., Katoh, H., 2005. Plexins: axon guidance and signal transduction. Cellular and molecular life sciences : CMLS 62, 1363-1371.

Neufeld, G., Kessler, O., 2008. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. Nature reviews. Cancer 8, 632-645.

Oinuma, I., Katoh, H., Negishi, M., 2004. Molecular dissection of the semaphorin 4D receptor plexin-B1-stimulated R-Ras GTPase-activating protein activity and neurite remodeling in hippocampal neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 24, 11473-11480.

Okuda, Y., Ogura, E., Kondoh, H., Kamachi, Y., 2010. B1 SOX coordinate cell specification with patterning and morphogenesis in the early zebrafish embryo. PLoS genetics 6, e1000936.

Papan, C., Campos-Ortega, J.A., 1994. On the formation of the neural keel and neural tube in the zebrafishDanio (Brachydanio) rerio. Roux's archives of developmental biology : the official organ of the EDBO 203, 178-186.

Parichy, D.M., Elizondo, M.R., Mills, M.G., Gordon, T.N., Engeszer, R.E., 2009. Normal table of postembryonic zebrafish development: Staging by externally visible anatomy of the living fish. 238, 2975-3015.

Pascoe, H.G., Wang, Y., Zhang, X., 2015. Structural mechanisms of plexin signaling. Progress in biophysics and molecular biology 118, 161-168.

Pasterkamp, R.J., 2005. R-Ras fills another GAP in semaphorin signalling. Trends in Cell Biology 15, 61-64.

Perron, M., Harris, W.A., 2000. Determination of vertebrate retinal progenitor cell fate by the Notch pathway and basic helix-loop-helix transcription factors. Cellular and molecular life sciences : CMLS 57, 215-223.

Piccolo, S., Sasai, Y., Lu, B., De Robertis, E.M., 1996. Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell 86, 589-598.

Ramón y Cajal, S., 1893. La rétine des vertébrés. . Cellule 9, 119–257.

Reyes, R., Haendel, M., Grant, D., Melancon, E., Eisen, J.S., 2004. Slow degeneration of zebrafish Rohon-Beard neurons during programmed cell death. 229, 30-41.

Roessler, E., Belloni, E., Gaudenz, K., Jay, P., Berta, P., Scherer, S.W., Tsui, L.C., Muenke, M., 1996. Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. Nat Genet 14, 357-360.

Sakai, J.A., Halloran, M.C., 2006. Semaphorin 3d guides laterality of retinal ganglion cell projections in zebrafish. Development (Cambridge, England) 133, 1035-1044.

Sanes, D.H., 2011. Development of the nervous system. Academic Press.

Sarthy, P.V., Lam, D.M., 1978. Biochemical studies of isolated glial (Müller) cells from the turtle retina. The Journal of cell biology 78, 675-684.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L.K., De Robertis, E.M., 1994. Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. Cell 79, 779-790.

Sato-Maeda, M., Obinata, M., Shoji, W., 2008. Position fine-tuning of caudal primary motoneurons in the zebrafish spinal cord. Development (Cambridge, England) 135, 323-332.

Sauer, F.C., 1935. Mitosis in the neural tube. 62, 377-405.

Scheffzek, K., Lautwein, A., Kabsch, W., Ahmadian, M.R., Wittinghofer, A., 1996. Crystal structure of the GTPase-activating domain of human p120GAP and implications for the interaction with Ras. Nature 384, 591-596.

Schmidt, E.F., Strittmatter, S.M., 2007. The CRMP Family of Proteins and Their Role in Sema3A Signaling, in: Pasterkamp, R.J. (Ed.), Semaphorins: Receptor and Intracellular Signaling Mechanisms. Springer New York, New York, NY, pp. 1-11.

Schmidt, R., Strähle, U., Scholpp, S., 2013. Neurogenesis in zebrafish - from embryo to adult. Neural development 8, 3-3.

Segarra, M., Ohnuki, H., Maric, D., Salvucci, O., Hou, X., Kumar, A., Li, X., Tosato, G., 2012. Semaphorin 6A regulates angiogenesis by modulating VEGF signaling. Blood 120, 4104-4115.

Serini, G., Valdembri, D., Zanivan, S., Morterra, G., Burkhardt, C., Caccavari, F., Zammataro, L., Primo, L., Tamagnone, L., Logan, M., Tessier-Lavigne, M., Taniguchi, M., Puschel, A.W., Bussolino, F., 2003. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature 424, 391-397.

Shi, W., Kumanogoh, A., Watanabe, C., Uchida, J., Wang, X., Yasui, T., Yukawa, K., Ikawa, M., Okabe, M., Parnes, J.R., Yoshida, K., Kikutani, H., 2000. The class IV semaphorin CD100 plays nonredundant roles in the immune system: defective B and T cell activation in CD100-deficient mice. Immunity 13, 633-642.

Shim, S.O., Cafferty, W.B., Schmidt, E.C., Kim, B.G., Fujisawa, H., Strittmatter, S.M., 2012. PlexinA2 limits recovery from corticospinal axotomy by mediating oligodendrocyte-derived Sema6A growth inhibition. Molecular and cellular neurosciences 50, 193-200.

Speidel, C.C., 1942. Adjustments of Nerve Endings: Harvey Lecture, January 16, 1941. Bulletin of the New York Academy of Medicine 18, 625-653.

St Clair, R.M., Emerson, S.E., D'Elia, K.P., Weir, M.E., Schmoker, A.M., Ebert, A.M., Ballif, B.A., 2018. Fyn-dependent phosphorylation of PlexinA1 and PlexinA2 at conserved tyrosines is essential for zebrafish eye development. The FEBS journal 285, 72-86.

Strauss, O., 2005. The Retinal Pigment Epithelium in Visual Function. 85, 845-881.

Streisinger, G., Walker, C., Dower, N., Knauber, D., Singer, F., 1981. Production of clones of homozygous diploid zebra fish (Brachydanio rerio). Nature 291, 293.

Stuart, G.W., McMurray, J.V., Westerfield, M., 1988. Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. Development (Cambridge, England) 103, 403-412.

Sun, L.O., Jiang, Z., Rivlin-Etzion, M., Hand, R., Brady, C.M., Matsuoka, R.L., Yau, K.-W., Feller, M.B., Kolodkin, A.L., 2013. On and off retinal circuit assembly by divergent molecular mechanisms. Science (New York, N.Y.) 342, 1241974-1241974.

Takahashi, T., Strittmatter, S.M., 2001. Plexina1 autoinhibition by the plexin sema domain. Neuron 29, 429-439.

Toyofuku, T., Yoshida, J., Sugimoto, T., Zhang, H., Kumanogoh, A., Hori, M., Kikutani, H., 2005. FARP2 triggers signals for Sema3A-mediated axonal repulsion. Nature neuroscience 8, 1712-1719.

Toyofuku, T., Zhang, H., Kumanogoh, A., Takegahara, N., Suto, F., Kamei, J., Aoki, K., Yabuki, M., Hori, M., Fujisawa, H., Kikutani, H., 2004. Dual roles of Sema6D in cardiac morphogenesis through region-specific association of its receptor, Plexin-A1, with off-track and vascular endothelial growth factor receptor type 2. Genes Dev 18, 435-447.

Tschernutter, M., Jenkins, S.A., Waseem, N.H., Saihan, Z., Holder, G.E., Bird, A.C., Bhattacharya, S.S., Ali, R.R., Webster, A.R., 2006. Clinical characterisation of a family with retinal dystrophy caused by mutation in the Mertk gene. The British journal of ophthalmology 90, 718-723.

Van Battum, E.Y., Brignani, S., Pasterkamp, R.J., 2015. Axon guidance proteins in neurological disorders. The Lancet. Neurology 14, 532-546.

Vergara, M.N., Canto-Soler, M.V., 2012. Rediscovering the chick embryo as a model to study retinal development. Neural Development 7, 1-19.

Vitorino, M., Jusuf, P.R., Maurus, D., Kimura, Y., Higashijima, S., Harris, W.A., 2009. Vsx2 in the zebrafish retina: restricted lineages through derepression. Neural development 4, 14.

Vogel, A.M., Weinstein, B.M., 2000. Studying Vascular Development in the Zebrafish. Trends in Cardiovascular Medicine 10, 352-360.

Wald, G., 1945. The chemical evolution of vision. Harvey lectures 41, 117-160.

Walsh, F.S., Meiri, K., Doherty, P., 1997. Cell signalling and CAM-mediated neurite outgrowth. Society of General Physiologists series 52, 221-226.

Wang, S., Cepko, C.L., 2016. Photoreceptor Fate Determination in the Vertebrate Retina. Investigative ophthalmology & visual science 57, ORSFe1-6.

Wang, Y., He, H., Srivastava, N., Vikarunnessa, S., Chen, Y.B., Jiang, J., Cowan, C.W., Zhang, X., 2012. Plexins are GTPase-activating proteins for Rap and are activated by induced dimerization. Science signaling 5, ra6.

Wang, Y., Pascoe, H.G., Brautigam, C.A., He, H., Zhang, X., 2013. Structural basis for activation and non-canonical catalysis of the Rap GTPase activating protein domain of plexin. eLife 2, e01279.

Weinstein, D.C., Hemmati-Brivanlou, A., 1999. Neural induction. Annual review of cell and developmental biology 15, 411-433.

Westerfield, M., McMurray, J.V., Eisen, J.S., 1986. Identified motoneurons and their innervation of axial muscles in the zebrafish. The Journal of neuroscience : the official journal of the Society for Neuroscience 6, 2267-2277.

White, F.A., Behar, O., 2000. The development and subsequent elimination of aberrant peripheral axon projections in Semaphorin3A null mutant mice. Developmental biology 225, 79-86.

Worzfeld, T., Swiercz, J.M., Senturk, A., Genz, B., Korostylev, A., Deng, S., Xia, J., Hoshino, M., Epstein, J.A., Chan, A.M., Vollmar, B., Acker-Palmer, A., Kuner, R., Offermanns, S., 2014. Genetic dissection of plexin signaling in vivo. Proceedings of the National Academy of Sciences of the United States of America 111, 2194-2199.

Wright, D.E., White, F.A., Gerfen, R.W., Silos-Santiago, I., Snider, W.D., 1995. The guidance molecule semaphorin III is expressed in regions of spinal cord and periphery avoided by growing sensory axons. The Journal of comparative neurology 361, 321-333.

Yoshida, Y., Han, B., Mendelsohn, M., Jessell, T.M., 2006. PlexinA1 signaling directs the segregation of proprioceptive sensory axons in the developing spinal cord. Neuron 52, 775-788.

Yu, L., Zhou, Y., Cheng, S., Rao, Y., 2010. Plexin a-semaphorin-1a reverse signaling regulates photoreceptor axon guidance in Drosophila. The Journal of neuroscience : the official journal of the Society for Neuroscience 30, 12151-12156.

Zhang, Z., Vuori, K., Wang, H., Reed, J.C., Ruoslahti, E., 1996. Integrin activation by R-ras. Cell 85, 61-69.

Zuber, M.E., Gestri, G., Viczian, A.S., Barsacchi, G., Harris, W.A., 2003. Specification of the vertebrate eye by a network of eye field transcription factors. Development 130, 5155-5167.

CHAPTER 2. IDENTIFICATION OF TARGET GENES DOWNSTREAM OF SEMAPHORIN6A/PLEXINA2 SIGNALING IN ZEBRAFISH

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Abbreviations Used:

dcdc2b-Doublecortin domain-containing 2b

ECM-Extracellular matrix

GAP-GTPase activating protein

GEF-Guanine nucleotide exchange factor

HPF-Hours post fertilization

mmp2-Matrix metalloproteinase 2

MO-Morpholino

Plxn-Plexin

rasl11b-RAS-like, family 11, member B

rbl2-Retinoblastoma-like protein 2

RPCs-Retinal precursor cells

Sema-Semaphorin

shtn-1-Shootin-1

Key Findings:

- Transcriptional changes downstream of Semaphorin6A (Sema6A) and PlexinA2 (PlxnA2) signaling regulate proliferation and unified migration of retinal precursor cells (RPCs) during early eye development.

- Microarray analysis has identified 58 transcriptional targets downstream of Sema6A/PlxnA2 signaling, 57 of which are significantly up-regulated in knockdown embryos.

- Functional characterization of *rasl11b,* a gene that is up-regulated in both *sema6A* and *plxnA2* knockdowns, has revealed its role in maintaining proliferation of RPCs during early zebrafish eye development.

2.1. Abstract

Background. Semaphorin (Sema)/Plexin (Plxn) signaling is important for many aspects of neuronal development, however the transcriptional regulation imposed by this signaling pathway is unknown. Previously, we identified an essential role for Sema6A/PlxnA2 signaling in regulating proliferation and cohesion of retinal precursor cells (RPCs) during early eye development. This study used RNA isolated from control,

Sema6A-deficient, and PlxnA2-deficient zebrafish embryos in a microarray analysis to identify genes that were differentially expressed when this signaling pathway was disrupted.

Results. We uncovered a set of 58 transcripts, and all but one were up-regulated in both *sema6A* and *plxnA2* knockdowns. We validated gene expression changes in subset of candidates that are suggested to be involved in proliferation, migration or neuronal positioning. We further functionally evaluated one gene, *rasl11b,* as contributing to disrupted proliferation in *sema6A* and *plxna2* knockdowns. Our results suggest *rasl11b* negatively regulates proliferation of RPCs in the developing zebrafish eye.

Conclusions. Microarray analysis has generated a resource of target genes downstream of Sema6A/PlxnA2 signaling, which can be further investigated to elucidate the downstream effects of this well-studied neuronal and vascular guidance signaling pathway**.**

2.2. Introduction

Neuronal migration and positioning are important aspects of nervous system development. During the early stages of neuronal development, guidance cues are essential to lead neurons and their processes to correct target tissues. One well-studied group of guidance cues are the Semaphorins (Semas). Semas are a family of transmembrane and secreted proteins that typically signal through Plexin (Plxn) receptors. There are 8 subclasses of Semas. Semas 1 and 2 are found in invertebrates, 3-7 are found in vertebrates and Sema V is found in the genome of non-neurotropic DNA

viruses (Neufeld and Kessler, 2008). The Plexin family is composed of 2 invertebrate Plxns, A and B, and 4 classes of vertebrate Plxns, A1-4, B1-3, C1 and D1 (Tamagnone et al., 1999). Semas and Plxns have tissue-specific expression patterns, and many Semas can signal through multiple Plxn family members. Semas were initially discovered with respect to their role as repulsive guidance cues for migrating axons (Luo et al., 1993a), although it is now appreciated that they have much broader roles in development. They have been implicated in vasculogenesis (Serini et al., 2003b), tumorigenesis (Neufeld and Kessler, 2008), immunity (Shi et al., 2000a), and bone development (Behar et al., 1996b). Here we focus specifically on the interaction and downstream effects of Sema6A and PlxnA2 in early eye development.

During eye development, retinal precursor cells (RPCs) are directed from the optic stalk into the correct domain of the developing retina (Rembold et al., 2006). We previously demonstrated that Sema6A and its receptor, PlxnA2, are necessary for the proliferation and cohesion of RPCs within the early embryonic eye vesicles (Ebert et al., 2014). More recently, Sema6A and PlxnA2 were shown to be required for interkinetic nuclear migration of RPCs within optic vesicles (Belle et al., 2016). In addition, Sema6/PlxnA signaling is required for neuronal positioning in the cerebellum (Renaud and Chedotal, 2014; Renaud et al., 2008), lamination of the hippocampus (Tawarayama et al., 2010), and guidance of the corticospinal tract (Rünker et al., 2008), among other roles. Several studies have begun to uncover the intracellular signaling mechanisms downstream of activated PlxnA receptors (He et al., 2009; Oinuma et al., 2004a; Toyofuku et al., 2005), however, prior to this study it was unknown what transcriptional regulation occurred downstream of Sema6A/PlxnA2 signaling.

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Microarray analysis using RNA extracted from 18 somite zebrafish embryos deficient in either Sema6A or PlxnA2 has enabled the identification of several downstream transcriptional targets of Sema6A/PlxnA2 signaling during early stages of neuronal development. Further characterization of one of these genes, RAS-like, family 11, member B (*rasl11b*), revealed its role in regulating RPC proliferation.

2.3. Results and Discussion

2.3.1. Microarray Analysis Identifies Transcriptional Changes Downstream of Sema6A/PlxnA2 Signaling

Confirming our previous results (Ebert et al., 2014), morpholino antisense oligonucleotides (MOs) targeted to either *sema6A* or *plxnA2* resulted in decreased proliferation and cohesion of RPCs within migrating optic vesicles compared to control embryos. To observe these phenotypes, we utilized the *rx3*:GFP zebrafish transgenic line which expresses GFP in RPCs and the hypothalamus (Rembold et al., 2006). Using confocal microscopy, we identified GFP-positive RPCs outside of the eye field and optic vesicles that were closer together in knockdown embryos (Fig. 2.1A-F).
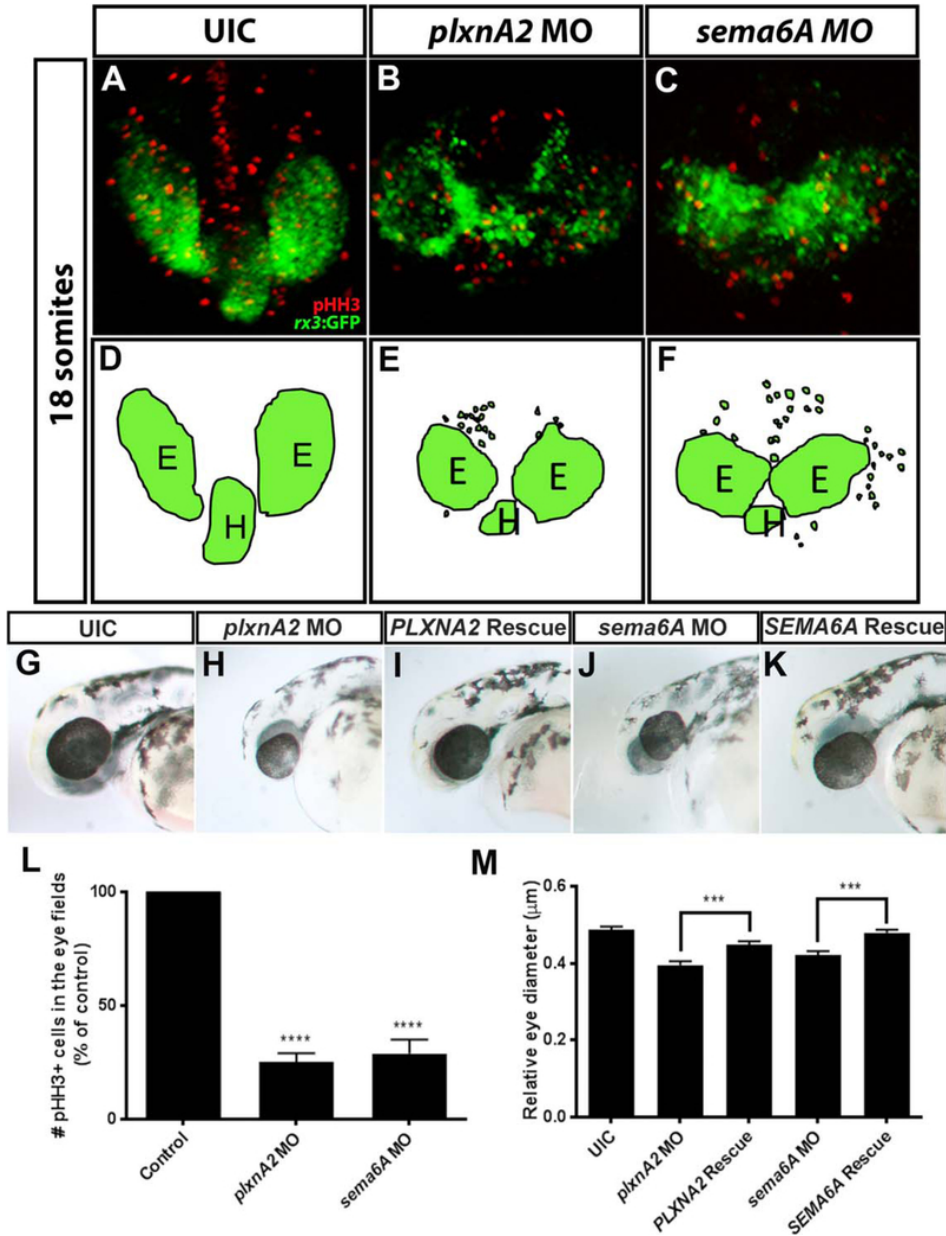
Figure 2.1. *Sema6A* and *plxnA2* **Knockdown Leads to Loss of Cohesion and Decreased Proliferation in Eye Vesicles. A–C:** Confocal dorsal views of 18 somite stage rx3:GFP embryos co-labeled with pHH3. **L:** Both sema6A and plxnA2 knockdowns display ectopic retinal precursor cells outside of the eye field as well as significantly reduced pHH3 positive cells in the developing eyes compared with controls, as quantified (control 100%, N = 3, n = 30; plxnA2 MO 25.11% ± 0.72%, N = 3, n = 30, ****$P < 0.0001$; sema6A MO 28.86% ± 1.12%, N = 3, n = 30; ****$P < 0.0001$, one-way ANOVA, multiple comparisons post-hoc test). **D–F:** Schematics of rx3:GFP dorsal view. **G,H,J,M:** Decreased proliferation is later observed as smaller eyes at 48 hpf (control 0.49 μm ± 0.008 μm, N = 3, n = 30; plxnA2 MO 0.40 μm ± 0.01 μm, N = 3, n = 29, ****$P < 0.0001$; sema6A MO 0.42 μm ± 0.009 μm, N = 3, n = 31; ****$P < 0.0001$, one-way ANOVA, multiple comparisons test). G,I,K,M: Full length human PLXNA2 and SEMA6A mRNA partially rescues smaller eye phenotypes (control 0.49 μm ± 0.008 μm, N = 3, n = 30; PLXNA2 rescue 0.45 μm ± 0.009 μm, N = 3, n = 32, ***$P < 0.001$; SEMA6A rescue 0.48 μm ± 0.008 μm, N = 3, n = 28; ***$P < 0.001$, one-way ANOVA, multiple comparisons test. Error bars indicate SEM). E, eye; H, hypothalamus.

Utilizing a mitotic marker, phospho-Histone H3 (pHH3), we observed less proliferation within the GFP-positive optic vesicles (Fig. 2.1 A-C, L, (control 100%, N=3, n=30; *plxnA2* MO 25.11% +/-0.72 N=3, n=30, P<0.0001; *sema6A* MO 28.86% +/-1.12, N=3, n=30; P<0.0001, one-way ANOVA, Multiple comparisons test)). As these embryos develop further, the decreased proliferation results in smaller eyes at 48 hours post fertilization (hpf) (Fig. 2.1G, H, J, M (Eye diameter relative to head length. Control 0.49 μm +/-0.008 μm, N=3, n=30; *plxnA2* MO 0.40 μm +/- 0.01 μm, N=3, n=29, P<0.0001; *sema6A* MO 0.42 μm +/-0.009 μm, N=3, n=31; P<0.0001, one-way ANOVA, Multiple comparisons test)). Off-target effects and specificity of both *sema6A* and *plxnA2* morpholinos have been addressed with secondary non-overlapping morpholino constructs, partial rescue of phenotypes via full-length mRNA injections, and penetrance verified by the absence of wildtype gene products by RT-PCR (Ebert et al., 2014). To re-address morpholino specificity in this study, co-injections of morpholino with full-length human *SEMA6A* or *PLXNA2* mRNA partially rescued eye size (Fig. 2.1 I, K, M (Eye diameter relative to head length. Control 0.49 μm +/-0.008 μm, N=3, n=30; *PLXNA2* rescue 0.45 μm +/-0.009μm, N=3, n=32, P<0.001; *SEMA6A* rescue 0.48 μm +/-0.008 μm, N=3, n=28; P<0.001, one-way ANOVA, Multiple comparisons test)). Overexpression of *SEMA6A* or *PLXNA2* human mRNA alone exhibited no significant developmental phenotypes (data not shown). Considering that eye development takes several hours, we wanted to investigate what transcriptional changes were occurring downstream of Sema6A/PlxnA2 signaling that could be controlling this process. We compared gene expression levels of 18 somite *plxnA2* knockdowns compared to uninjected control embryos (Fig. 2.2A) and 18 somite *sema6A* knockdowns compared to uninjected control

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embryos (Fig. 2.2B). In a microarray of 4885 zebrafish genes, we identified 58 significantly (greater than 2-fold)
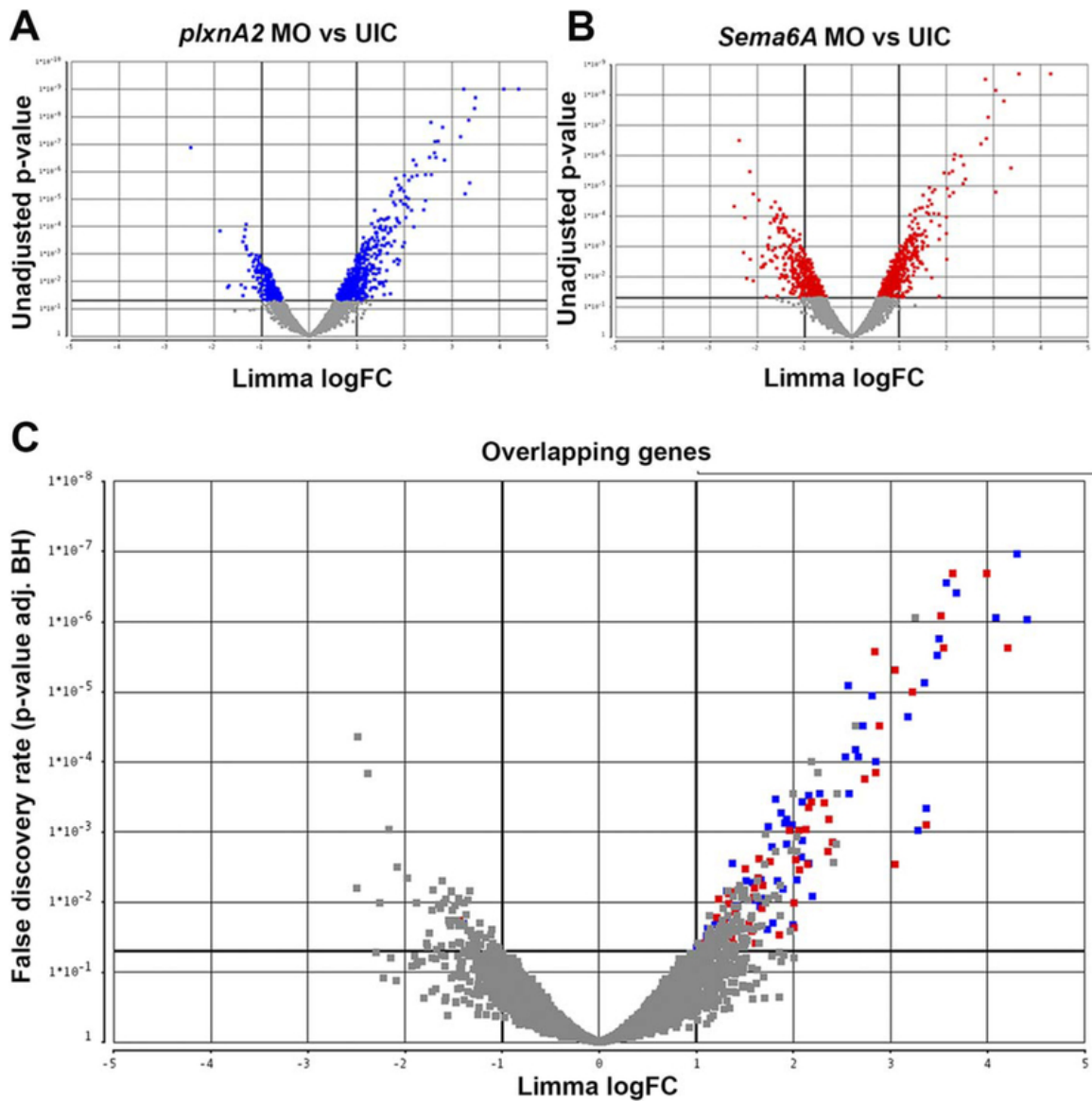
Figure 2.2. Volcano Plots Denoting Differential Gene Expression. Each point represents a gene in the microarray. **A:** Gene expression changes between plxnA2 MO and uninjected controls (UIC). **B:** sema6A MO compared with UIC. Gray are nonsignificant. **C:** Overlapping genes in both plxnA2 and sema6A plots. Blue squares indicate significantly different expression levels in plxnA2 MO-UIC, red squares in sema6A MOUIC. Gray are nonsignificant in at least one condition, with under a two-fold change difference in expression or $P > 0.05$.

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differentially regulated genes that were in common to both knockdowns (Table 2.1, Fig. 2.2C, GEO accession #GSE86246). Strikingly, of the 58 genes, 57 were up-regulated and only one down-regulated suggesting that Sema6A/PlxnA2 signaling is largely repressive to downstream transcriptional targets (Figs. 2.2C, 2.3). Using gene ontology information, we prioritized genes predicted to be involved in cell migration and proliferation for validation and further functional studies due to the phenotypes observed in knockdown embryos.
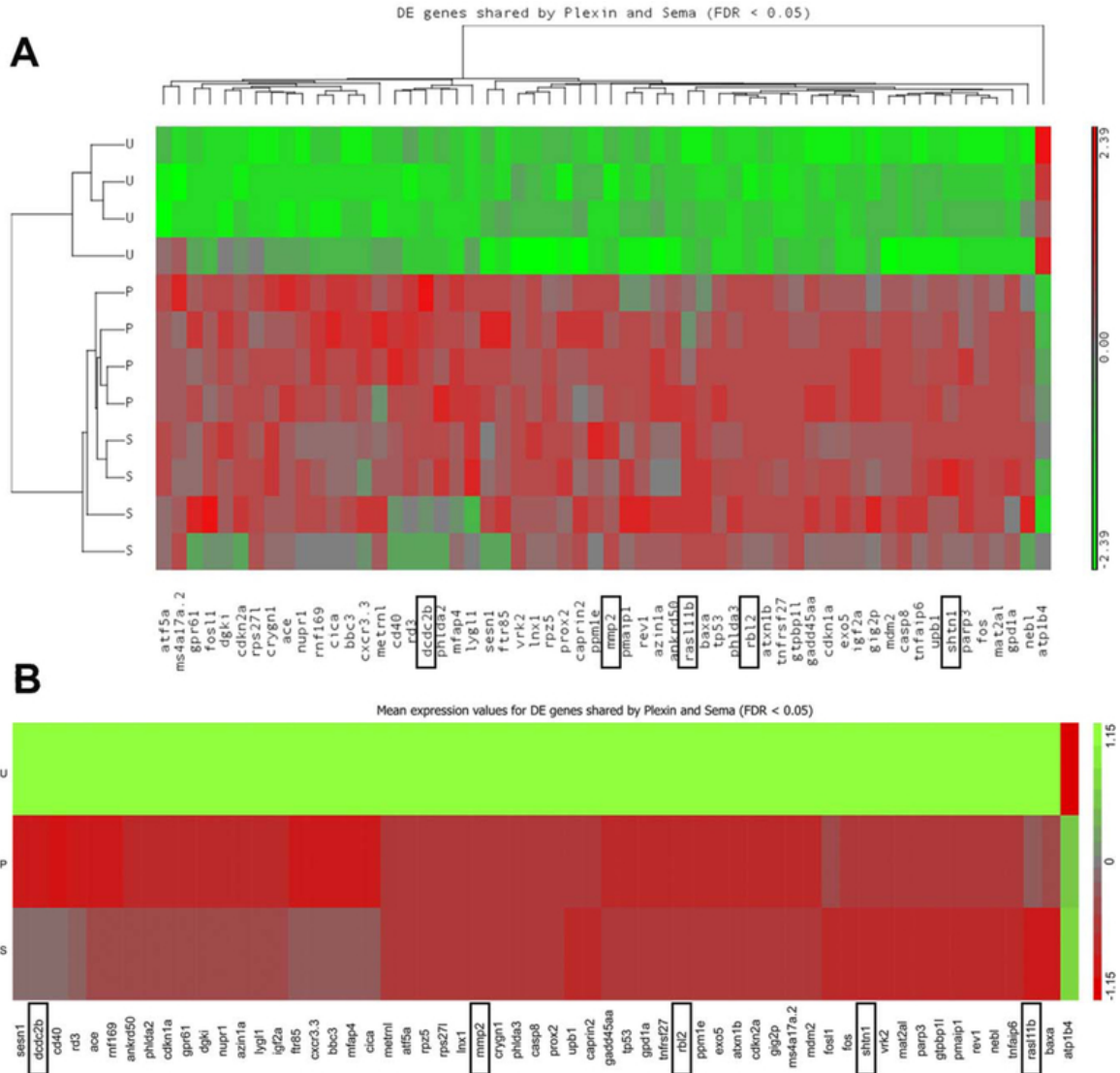
Figure 2.3. Microarray Heat Map of 58 Differentially Regulated Genes in Common to Both *sema6A* **MO-UIC and** *plxnA2* **MO-UIC.** Differential expression is represented on a green to red scale with expression values standardized to a mean of zero and standard deviation of one. **A:** Results from each of the four replicates are shown. **B:** Average expression change of four replicates. Boxes indicate validated genes. U, uninjected control; S, sema6A MO; P, plxnA2 MO.

Table 2.1. List of 58 genes that are differentially regulated downstream of Sema6a/Plxna2 signaling at 18 somites, common to both knockdowns compared to uninjected controls. GEO accession # GSE86246.

zfin symbol	ensembl gene ID	logFC Sema-UIC	logFC Plexin-UIC
ace	ENSDARG00000079166	2.02	2.67
ankrd50	ENSDARG00000007077	1.59	1.99
Atf5a	ENSDARG00000068096	2	2
Atp1b4	ENSDARG00000053262	−1.44	−1.4
Atxn1b	ENSDARG00000060862	3.99	4.3
Azin1a	ENSDARG00000052789	1.36	1.6
baxa	ENSDARG00000020623	1.35	1
bbc3	ENSDARG00000069282	2.37	3.35
caprin2	ENSDARG00000020749	1.36	1.29
casp8	ENSDARG00000058325	3.55	3.5
cd40	ENSDARG00000054968	1.57	2.84
Cdkn1a	ENSDARG00000076554	2.13	2.53
cdkn2a	ENSDARG00000037262	3.05	3.28
cica	ENSDARG00000071150	1.28	1.88
Crygn1	ENSDARG00000087437	2.31	2.27
cxcr3.3	ENSDARG00000070669	1.09	1.51
dcdc2b	ENSDARG00000053744	1.25	2.16

zfin symbol	ensembl gene ID	logFC Sema-UIC	logFC Plexin-UIC
Dgki	ENSDARG00000063578	1.55	1.55
exo5	ENSDARG00000042727	3.22	3.48
Fos	ENSDARG00000031683	3.05	2.81
fosl1	ENSDARG00000015355	2.4	2.03
ftr85	ENSDARG00000034707	1.5	2.09
gadd45aa	ENSDARG00000043581	2.85	3.18
gig2p	ENSDARG00000088260	1.23	1.32
gpd1a	ENSDARG00000043701	1.64	1.74
gpr61	ENSDARG00000003900	1.85	2.2
Gtpbp1l	ENSDARG00000042900	2.84	2.56
igf2a	ENSDARG00000018643	1.51	1.82
Inx1	ENSDARG00000043323	1.4	1.41
Lygl1	ENSDARG00000056874	1.38	1.66
mat2al	ENSDARG00000063665	2.89	2.64
mdm2	ENSDARG00000033443	1.76	1.91
metrnl	ENSDARG00000007289	1.67	1.71
mfap4	ENSDARG00000090557	2.73	4.09
mmp2	ENSDARG00000017676	1.33	1.31
ms4al7a.2	ENSDARG00000093546	1.61	1.73
nebl	ENSDARG00000021200	2	1.79
nupr1	ENSDARG00000094300	1.64	1.93

zfin symbol	ensembl gene ID	logFC Sema-UIC	logFC Plexin-UIC
parp3	ENSDARG00000003961	1.96	1.77
phlda2	ENSDARG00000042874	1.28	1.63
Pmaip1	ENSDARG00000089307	2.15	1.89
phlda3	ENSDARG00000037804	3.64	3.57
ppm1e	ENSDARG00000026499	1.06	1.1
prox2	ENSDARG00000041952	1.69	1.67
rasl11b	ENSDARG00000015611	2.18	1.58
rb12	ENSDARG00000045636	3.52	3.68
rd3	ENSDARG00000031600	1.21	1.93
rev1	ENSDARG00000018296	1.6	1.41
mf169	ENSDARG00000042825	2.05	2.71
rps27l	ENSDARG00000090186	2.06	2.08
rpz5	ENSDARG00000075718	3.37	3.37
sesn1	ENSDARG00000020693	1.49	2.57
shtn1	ENSDARG00000039697	1.21	1.12
tnfaip6	ENSDARG00000093440	1.33	1.19
tnfrsf27	ENSDARG00000079403	4.21	4.4
tp53	ENSDARG00000035559	1.23	1.37
upb1	ENSDARG00000011521	2.16	2.09

2.3.2. *In situ* Hybridization and RT-PCR Validation of Microarray Results

Zebrafish embryos were injected with either *sema6A* or *plxnA2* morpholinos at the one cell stage, and fixed at the 18 somite stage, dehydrated and processed for *in situ* hybridization. Digoxigenin (DIG)-labeled antisense riboprobes were generated for RAS-like, family 11, member B *(rasl11b),* shootin-1 *(shtn-1),* doublecortin domain-containing 2b (*dcdc2b),* matrix metalloproteinase 2 *(mmp2),* and retinoblastoma-like protein 2 *(rbl2)* and *in situ* hybridization was performed on control and knockdown embryos. Probe intensity for all five candidate genes was consistent with microarray results and offered additional spatial information (Fig. 2.4A-E). Transverse sections of *rasl11b* and *shtn-1* labeled embryos show neuronal expression, including in the developing eye, at this early stage (Fig. 2.4A, B right columns). We observed overlap between previously reported *plxnA2* and *sema6A* expression (Ebert et al., 2014) and the candidate genes, supporting a relationship between Sema6A/PlxnA2 signaling and these genes. We generated cDNA from uninjected control, *sema6A* knockdown, and *plxnA2* knockdown embryos at 18 somites and performed RT-PCR (Fig. 2.4A'-E'). The results are consistent with the microarray and *in situ* expression data, showing increases in transcriptional levels of the candidate genes in both knockdowns compared to uninjected control. (Fig. 2.4A'-E' (*rasl11b* control 0.1137 +/-0.04, N=3*, sema6A* MO 0.34 +/-0.02, N=3, P<0.01, *plxnA2* MO 0.32 +/-0.01, N=3, P<0.01; *shtn-1* control 0.08 +/-0.01, N=3, *sema6A* MO 0.28 +/-0.01, N=3, P<0.01, *plxnA2* MO 0.24 +/-0.03, N=3, P<0.01; *dcdc2b* control 0.06 +/-0.004, N=3, *sema6A* MO 0.13 +/-0.02, N=3, P<0.05, *plxnA2* MO 0.24 +/-0.02, N=3, P<0.001; *mmp2* control 0.004 +/-0.001, N=5, *sema6A* MO 0.21 +/-0.07, N=5, P<0.05, *plxnA2* MO 0.27 +/-0.06, N=5, P<0.01; *rbl2* control 0.01 +/-0.002, N=4, *sema6A* MO 0.17 +/-0.02,

N=4, P<0.001, *plxnA2* MO 0.15 +/-0.03, N=4, P<0.01; one-way ANOVA, Multiple comparisons test)).
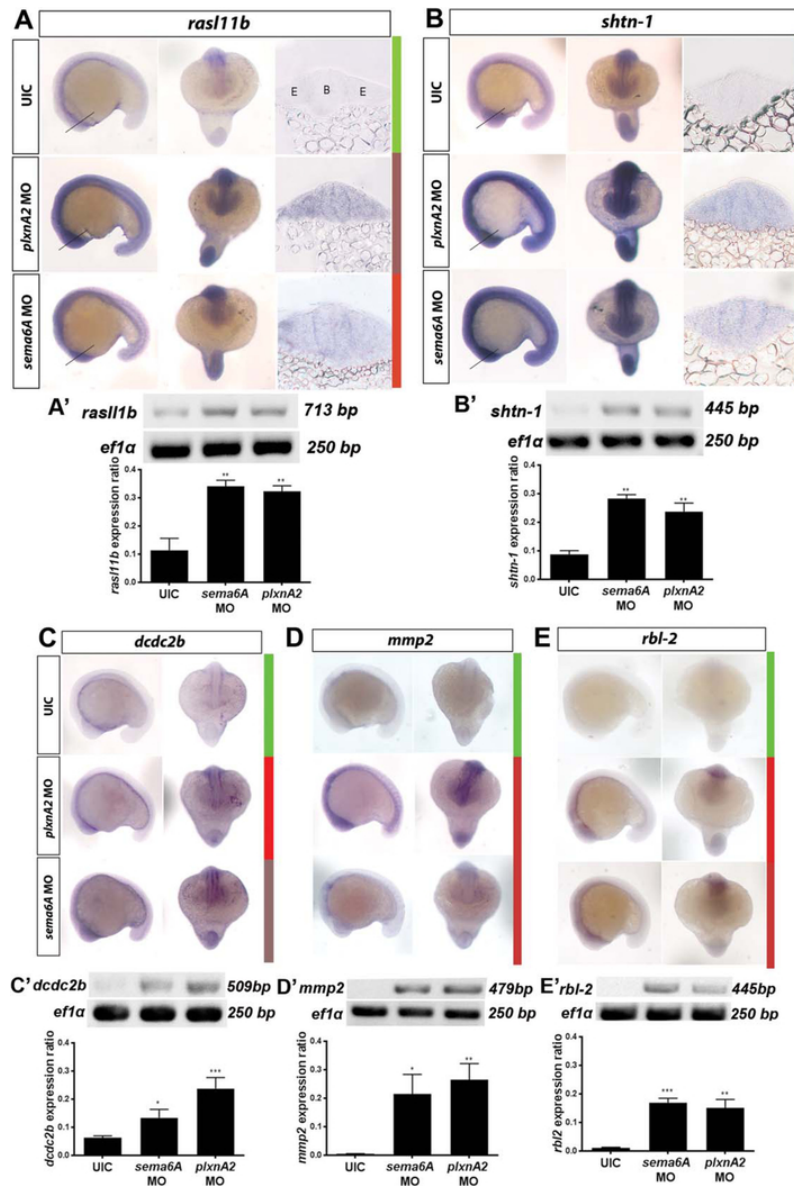
Figure 2.4. Microarray Validation. *In situ* hybridization and RT-PCR validation of differential gene expression in plxnA2 and sema6A knockdowns at 18 somites. A–E: Top, middle, and bottom panels show uninjected control, plxnA2 MO injected, and sema6A MO injected embryos, respectively. Left and right panels show lateral and dorsal views, respectively, for each probe under each condition. Colored bars show corresponding heat map expression changes for each gene. UIC, uninjected control. A, B: Additional right panels show transverse 20 μm sections through 18 somite stage brain and eye vesicles processed for *in situ* hybridization with rasl11b and shtn-1 probes. Lines in whole-mounts indicate corresponding section locations. A′–E′: RT-PCR gels and quantification of gene expression changes for each gene as a ratio to ef1α control levels (rasl11b control 0.1137 ± 0.04, N = 3, rasl11b sema6A MO 0.34 ± 0.02, N = 3, **P < 0.01; rasl11b plxnA2 MO 0.32 ± 0.01, N = 3, **P < 0.01; shtn-1 control 0.08 ± 0.01, N = 3, shtn-1 sema6A MO 0.28 ± 0.01, N = 3, **P < 0.01; shtn-1 plxnA2 MO 0.24 ± 0.03, N = 3, **P < 0.01; dcdc2b control 0.06 ± 0.004, N = 3, dcdc2b sema6A MO 0.13 ± 0.02 N = 3, *P < 0.05; dcdc2b plxnA2 MO 0.24 ± 0.02, N = 3, ***P < 0.001; mmp2 control 0.004 ± 0.001, N = 5, mmp2 sema6A MO 0.21 ± 0.07, N = 5, *P < 0.05; mmp2 plxnA2 MO 0.27 ± 0.06, N= 5, **P < 0.01; rbl2 control 0.01 ± 0.002, N = 4, rbl2 sema6A 0.17 ± 0.02, N = 4, ***P < 0.001; rbl2 plxnA2 MO 0.15 ± 0.03, N = 4, **P < 0.01; one-way ANOVA, multiple comparisons test. Error bars indicate SEM). B, brain. E, eye.

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2.3.3. Overexpression Of *rasl11b* Leads to Decreased Proliferation In Optic Vesicles

Microarray results indicated that *rasl11b* has a 2.18 logFC in *sema6A* knockdowns and a 1.58 logFC in *plxnA2* knockdowns (Table 2.1). We chose to further investigate this gene because of its predicted role in cell proliferation and expression in the developing eye field. To determine if increased *rasl11b* expression contributes to decreased proliferation of RPCs, we injected full-length zebrafish *rasl11b* mRNA into single-cell embryos. We visualized proliferation (pHH3) in the early eye field (*rx3*:GFP) of 18 somite embryos injected with 200 pg or 400 pg *rasl11b* mRNA. Overexpression of *rasl11b* resulted in decreased RPC proliferation in a dose-dependent manner (Fig. 2.5A-D (control 100%, N=3, n=30; 200pg *rasl11b* 72.87% +/-4.25%, N=3, n=38, $P<0.0001$; 400 pg *rasl11b* 62.19% +/-4.01%, N=3, n=31, $P<0.0001$, one-way ANOVA, Multiple comparisons test)). At 48 hpf, overexpression of *rasl11b* resulted in smaller eyes (Fig. 2.5 E-G) (Eye diameter relative to head length, control 0.48 μm +/-0.005 μm, N=3, n=30; 400 pg *rasl11b* 0.39 μm +/-0.01 μm, N=3, n=36, $P<0.0001$, one-way ANOVA, Multiple comparisons test)). We show that *rasl11b* is negatively regulated downstream of Sema6A/PlxnA2 signaling and when overexpressed, decreases RPC proliferation and eye size.
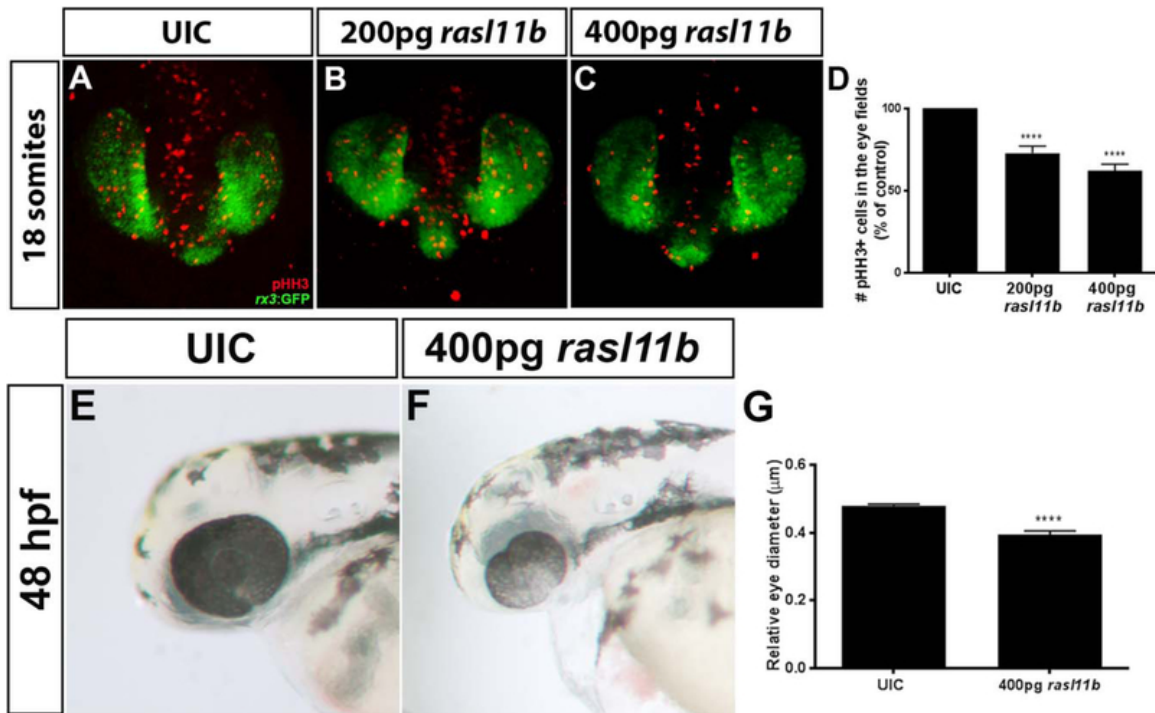
Figure 2.5. Overexpression of *rasl11b* Decreases Proliferation of RPCs. A–C: Confocal dorsal views of 18 somite rx3:GFP embryos co-labeled with pHH3. **D:** Increasing doses of rasl11b resulted in decreased proliferation within the GFP positive eye field (control 100%, N = 3, n = 30; 200 pg rasl11b 72.87% ± 4.25% N = 3, n = 38, ****P < 0.0001; 400 pg rasl11b 62.19% ± 4.01%, N = 3, n = 31, ****P < 0.0001; one-way ANOVA, multiple comparisons test; error bars indicate SEM). **E–G:** Overexpression of rasl11b results in smaller eyes at 48 hpf (eye diameter relative to head length; control 0.48 μm ± 0.005 mm N = 3, n = 30; 400 pg rasl11b 0.39 μm ± 0.01 μm, N = 3, n = 36; ****P < 0.0001; one-way ANOVA, multiple comparisons test; error bars indicate SEM).

2.3.4. Proposed Signaling Mechanism of Rasl11b Impinging on Ras Signaling

Rasl11b is a small atypical GTPase that has been implicated in prostate cancer (Louro et al., 2004a). In zebrafish, Rasl11b inhibits mesendodermal and prechordal plate formation during embryogenesis, and interacts with the one-eyed pinhead signaling pathway (Pezeron et al., 2008). Rasl11b is highly conserved in vertebrates and is atypical to most Ras-like family members in two ways. Firstly, it is cytosolic and lacks carboxy terminal lipid modification sites which allow for membrane anchoring (Pezeron et al., 2008). Secondly, it has a lower GTPase activity than Ras, and is more often in its active GTP-bound state (Colicelli, 2004). Ras proteins are well known to be involved in the MAPK pathway, therefore we hypothesize that Rasl11b acts as a negative regulator of MAPK by outcompeting Ras for its effectors such as Raf, leading to decreases in RPC proliferation seen in knockdown embryos.

It is known that Plexins have an intracellular split GAP (GTPase activating protein) domain that can regulate Ras-family small GTPases (Negishi et al., 2005; Pasterkamp, 2005). Small GTPases act as molecular switches: "on" when GTP-bound, and "off" when GDP-bound (Bos et al., 2007). GAPs increase GTP hydrolysis and thereby increase the "off", GDP-bound form of the protein. Plxn intracellular GAP domains are inactive when Plxns are in inactive, open conformations. Upon Sema binding, PlxnAs undergo a conformational change, which forms an active GAP domain, in addition to activating downstream effector proteins (He et al., 2009). When Sema6A/PlxnA2 signaling is disrupted, *rasl11b* expression increased (Table 2.1 & Fig. 2.4A). We propose that when non-membrane bound Rasl11b is present, it inhibits membrane-tethered Ras signaling through its lower GTPase activity and its ability to

recruit either Raf or the small Ras activating guanine nucleotide exchange factor (GEF), son of sevenless (SOS), away from the membrane. This could sequester Raf or SOS away from Ras, therefore Ras is predominantly found in its GDP-bound state, reducing signal propagation, and resulting in decreased pro-proliferative effects of the MAPK signaling cascade.

2.3.5. Proposed Contributions of *shtn-1, dcdc2b, rbl2* and *mmp2* to Knockdown Phenotypes

Additional validated genes may also contribute to knockdown phenotypes. Microarray results demonstrated that *shtn-1* has a 1.21 logFC in *sema6A* knockdowns and a 1.12 logFC in *plxnA2* knockdowns. Shtn-1 is involved in the formation of cellular asymmetric polarization signals, which direct the cell during migration, as seen in the orientation of neurite outgrowth of cultured hippocampal neurons (Toriyama et al., 2006a). To polarize, cells need to have carefully regulated amounts of Shtn-1. Overexpression of Shtn-1 in rat hippocampal neurons leads to its impaired asymmetric distribution and induces the formation of surplus axons (Toriyama et al., 2006a). Alternatively, Shtn-1 may control the actin cytoskeleton in migrating cells, in that Shtn-1 couples f-actin retrograde flow to L1-CAM aiding with "clutch engagement" at the leading edge of the extending axons of rat hippocampal neurons in culture (Shimada et al., 2008b). Thus, we hypothesize that an increase in Shtn-1 could impair the coordinated migration of RPCs, as individual cells fail to either receive the correct polarization signals and/or regulate their actin cytoskeleton.

Dcdc2b has a 1.25 logFC in *sema6a* and a 2.16 logFC in *plxnaA2* knockdowns (Table 2.1). It is one of a family of 11 DCX domain-containing proteins. DCX domain-containing proteins are atypical microtubule-associated proteins. They bind to tubulin via

their DCX domains to regulate microtubule stabilization and also couple microtubule-actin binding through various accessory proteins (Dijkmans et al., 2010). In zebrafish, mutations in *dcdc2b* have uncovered a role in the function of microtubules within cilia of hair cells, correlating with deficits that lead to deafness in humans (Grati et al., 2015). It is known that cytoskeletal microtubule dynamics at the trailing edge of migratory cells are critical for cellular movement, in addition to generating cellular polarity, both of which guide the direction of migration (Ganguly et al., 2012). We hypothesize that the increase in expression of *dcdc2b* in *sema6A* and *plxnA2* knockdowns is partially responsible for the abnormal RPC migration seen within optic vesicles. Increases in *dcdc2b* could stabilize microtubules, inhibiting microtubule depolymerization at the trailing edge of migrating RPCs. Stabilization would potentially not only slow normal cellular migration, but disrupt the normal direction of migration, and explain why RPCs within knockdown optic vesicles do not migrate as a tightly unified group.

Rbl2, sometimes referred to as p130, is a member of the pocket protein family, along with p107 and retinoblastoma (Rb) (Cobrinik, 2005). Mutations to the *Rb* family of genes can lead to a rare form of retinal cancer, retinoblastoma, in which cell cycle regulation is lost (Friend et al., 1986; Murphree and Benedict, 1984). Rbl2 has a 3.52 logFC in *sema6a* and a 3.68 logFC in *plxnaA2* knockdowns (Table 2.1). These proteins are known to bind to members of the E2F family of transcription factors to regulate the cell cycle. Rbl2 binds to E2F4 to form a repressor complex in mitotic cells to block cell cycle progression (Dingar et al., 2012). The ability of Rbl2 to repress transcription is in part because it can recruit methyltransferases to histones, causing chromatin condensation, and therefore decreasing the accessibility of transcriptional machinery to the promoters of genes that promote the cell cycle (Lai et al., 2001). We therefore

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hypothesize that increases in *rbl2* levels in knockdown embryos contributes to impaired proliferation of RPCs by blocking the cell cycle.

Matrix metalloproteinases (MMP) are well known for their role in central nervous system development and amphibian regeneration through their ability to degrade extracellular matrix (ECM) proteins (Brinckerhoff and Matrisian, 2002). *mmp2* has a 1.33 logFC in *sema6a* and a 1.31 logFC in *plxnaA2* knockdowns (Table 2.1). Recently, *mmp2* was shown to be important for degrading non-permissive ECM to allow for zebrafish retinal ganglion cell axon outgrowth during recovery from injury (Lemmens et al., 2015). Increases in MMP2 could lead to increased degradation of the ECM surrounding RPCs, and removal of key proteins required for cell migration. Alternatively, increases in MMP2 at the leading edge of migrating RPCs could allow for the ease of pseudopodia through surrounding tissue and cause RPCs to over-migrate.

2.4. Conclusions

We confirm through morpholino knockdown, that Sema6A/PlxnA2 signaling regulates proliferation and cohesive migration of RPCs in developing optic vesicles in zebrafish. Here we provide a resource of genes that are transcriptionally regulated downstream of Sema6A/PlxnA2 signaling. Initial characterization of one gene, *rasl11b*, uncovered its contributing role to the proliferation of RPCs, providing insight into the mechanisms that control this key developmental process. The broadness of Sema/Plxn signaling is only just becoming widely appreciated, and further research into downstream targets and signal propagation is necessary to fully understand the diverse roles of this signaling pair.

2.5. Experimental Procedures

2.5.1. Zebrafish Husbandry

*rx3:*GFP zebrafish embryos were generously provided by Jochen Wittbrodt, University of Heidelberg, Germany and developmentally staged as previously described (Kimmel et al., 1995). All procedures were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC).

2.5.2. Injections and Rescue Constructs

Antisense morpholino oligonucleotides (MOs) (Gene Tools, Philomath, OR. USA), or mRNA were injected at the one-cell stage (Kimmel et al., 1995). Morpholino concentrations and sequences were as follows: *plxnA2* e2i2, 2 ng (AAAAGCGATGTCTTTCTCACCTTCC); *sema6A*, 4 ng (TGCTGATATCCTGCACTCACCTCAC). Both *sema6A* and *plxnA2* morpholino constructs have been previously validated using RT-PCR to show successful knockdown (Ebert et al., 2014). Off target effects and specificity of morpholinos was addressed by rescue by full length human mRNA and co-injection of *sema6A* or *plxnA2* and *p53* morpholino. Second non-overlapping morpholino constructs reproduced the same phenotypes, see (Ebert et al., 2014). Capped and tailed full-length mRNA, (mMESSAGE mMACHINE kit, Ambion, Austin, TX, cat# AM1344; Poly(A) tailing kit, Invitrogen Cat# AM1350) was generated from plasmids containing human *plxnA2* (Origene, Cat# RC221024) or *sema6A* (Invitrogen, Cat# MDR1734-202803771) and injected into embryos at the one cell stage for a final concentration of 200pg or 400pg/embryo. Full-length, zebrafish *rasl11b* mRNA was generated from pCS2+-DR-Rasl11b generously provided by Dr. Philippe Mourrain (Paris, France).

2.5.3. Microarray Assay

Uninjected control, *sema6A* knockdown, and *plxnA2* knockdown embryos were staged at 18 somites and total RNA was isolated from 100 embryos using TRIzol (Invitrogen) followed by ethanol precipitation. Each treatment had four replicates generated from separate injection experiments. Data collection and analyses performed by the IBEST Genomics Resources CORE at the University of Idaho was supported by grants from the National Center for Research Resources (P30GM103324) and the National Institute of General Medical Sciences (8 P20 GM103397-10) from the National Institutes of Health. RNA from 18 somite stage control, *sema6A* and *plxnA2* knockdown embryos was analyzed using the NimbleGen Zebrafish 12x135k Array based on Zv9 [090818_Zv7_EXPR], following the NimbleGen arrays user's guide version 5.1.

2.5.4. Microarray Data Analysis

NimbleScan software (NimbleGen. Madison, WI) was used to align a chip-specific grid to control features and extract raw intensity data for each probe and each array. Chip images were then visually checked for each array and verified not to contain any significant spatial artifacts. Raw intensity data was then read into the R statistical computing environment and checked for quality. Chip intensity distributions, boxplots and hierarchical clusters were compared and checked for any unusual global patterns. Each array was then background corrected and normalized using the quantile normalization procedure and finally each probeset was summarized using the median polish procedure as described with the robust multichip average (RMA) procedure (Irizarry et al., 2003a) (Bolstad et al., 2003) (Irizarry et al., 2003b). The median polish procedure is a robust method for summarizing all probes contained within each probeset to a single expression value for each gene, taking into account individual probe effects. Probesets with low levels of expression variation across all samples (IQR < 0.5) were

removed from further analysis, reducing the overall number of statistical tests to be performed. Differential expression was then assessed using a linear model with an empirical Bayesian adjustment to the variances (Smyth, 2005) and comparisons of interest were extracted using contrasts. The Benjamini and Hochberg (BH) method was used to control for the expected false discovery rate given multiple tests (Benjamini and Hochberg, 1995). Probesets were considered statistically differentially expressed with a BH adjusted p-value of < 0.05. Both raw intensity data (.pair files) and RMA-normalized expression values are available in the National Center for Biotechnology Information Gene Expression Omnibus database (NCBI GEO accession no. GSE86246).

2.5.5. Whole-mount *In Situ* Hybridization

Zebrafish embryos were staged at 18 somites, fixed in 4% paraformaldehyde overnight at 4ºC, and stored at -20ºC in 100% methanol until use. *In situ* hybridization was performed as described previously (Thisse and Thisse, 2008). Digoxigenin (DIG)-labeled antisense RNA probes were generated using primers listed in Table 2.2 (IDT Coralville, IA). PCR products were cloned into pCR 2.1 TOPO vector (Invitrogen, Carlsbad, CA) and sequencing at the UVM Cancer Center Advanced Genome Technologies Core, Burlington, VT. Embryos were oriented in 4% methyl cellulose on depression slides and imaged using a Nikon SMZ800 dissecting light microscope at 50x magnification.

2.5.6. RT-PCR

cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) from isolated control, *plxnA2* knockdown or *sema6A* knockdown total RNA (described above). Gene specific primers (Table 2.2) were used to amplify approximately 500 base pair amplicons in 10 μl PCR reactions from 1μg cDNA using PCR Mastermix (Thermo).

ef1α primers were used as parallel control reactions. PCRs were run on a 1% agarose gel (Invitrogen). Gels were imaged using a Syngene INGENIUS3 system and GENEsys V1.5.5.0 imaging software. Densitometry was performed using Adobe Photoshop CS6. Band intensities were normalized to background, and a ratio of gene band intensity to *ef1α* control levels was obtained for each sample. At least three independent replicates were averaged. Graphs and statistical analyses were obtained using Prism 6.

2.5.7. Sectioning, Imaging, Cell Counts and Eye Measurements

Embryos were dehydrated in 100% ETOH for 30 minutes prior to embedding using a JB4 Embedding Kit (Polysciences, Inc., Warrington, PA) and sectioned on a Leica RM2265 microtome at 20μm. Brightfield images were obtained using an Olympus iX71 inverted light microscope and figures were assembled using Adobe Photoshop CS6, in which image brightness and contrast were optimized. Eye size measurements were obtained using SPOT imaging software version 5.2. To control for any changes in overall embryo size, eye size was calculated as a ratio of eye diameter divided by the distance from the anterior tip of the head to the posterior otic placode for each embryo. Proliferating cell counts were recorded when pHH3 positive cells were seen within GFP positive optic vesicles only. For confocal imaging, embryos were mounted in 0.5% low-melt agarose on glass bottom dishes (CLS-1811 ChemGlass, NJ) and imaged at 20X using 2 μm steps on a Nikon Eclipse Ti inverted microscope. Z Stacks were subjected to a Kalman stack filter in Image J and were presented as maximum intensity projections. RGB images were generated in Adobe Photoshop CS6.

Table 2.2. Primer sequences and accession numbers used for antisense riboprobe generation and RT-PCR.

Name	Forward primer	Reverse primer	Accession #
rasl11b	ATGCGTCTGATCCAGAACATG	TAATACGACTCACTATAGGGGTCACACTGAAGTGACGGTGC	NM_200140.1
dcdc2b	TGCTTCGTACTGGAGCTGTG	TGCTTCGTACTGGAGCTGTG	NM_001037689.2
shtn-1	TTTAGGTGACACTATAGAAGAGAGGCCTTACGGAAGCTGAAT	TAATACGACTCACTATAGGGGAGATGCCCAAGAGCTTCTTTTGT	NM_001198761.1
rbl2	TTTAGGTGACACTATAGAAGGGAGATCAAACCCTTCGCTCTG	TAATACGACTCACTATAGGGGAGAAAGAAGCCGTTGATCAGCAT	XM_002666954.5
mmp2	TTTAGGTGACACTATAGAAGGGCCCTTCAAATTCTTGGGTGA	TAATACGACTCACTATAGGGGAGACAATTTTCTCCGGAAGCTCA	NM_198067.1

2.5.8. Whole-mount Immunohistochemistry

18 somite stage embryos were fixed in 4% paraformaldehyde overnight at 4°C, rehydrated in PBST (PBS + 1% Triton), incubated in immuno block (PBST + 10% NGS) for 1 hour at room temperature, primary antibody in immuno block overnight at 4°C, secondary antibody for 2 hours at room temperature and stored in PBST until imaged. Antibodies used: phospho-Histone H3, pHH3 (1:1,000, Cell Signaling, Cat# 3377, Danvers, MA), anti-rabbit Alexa Flour 555 (1:1000, Cell Signaling, Cat# 4413S).

2.6. Acknowledgements

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Behar O, Golden JA, Mashimo H, Schoen FJ, Fishman MC. 1996. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. Nature 383:525-528.

Belle M, Parray A, Belle M, Chedotal A, Nguyen-Ba-Charvet KT. 2016. PlexinA2 and Sema6A are required for retinal progenitor cell migration. Dev Growth Differ 58:492-502.

Benjamini Y, Hochberg Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological) 57:289-300.

Bolstad BM, Irizarry RA, Astrand M, Speed TP. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19:185-193.

Bos JL, Rehmann H, Wittinghofer A. 2007. GEFs and GAPs: Critical Elements in the Control of Small G Proteins. Cell 129:865-877.

Brinckerhoff CE, Matrisian LM. 2002. Matrix metalloproteinases: a tail of a frog that became a prince. Nat Rev Mol Cell Biol 3:207-214.

Cobrinik D. 2005. Pocket proteins and cell cycle control. Oncogene 24:2796-2809.

Colicelli J. 2004. Human RAS Superfamily Proteins and Related GTPases. Science's STKE : signal transduction knowledge environment 2004:RE13-RE13.

Dijkmans TF, van Hooijdonk LW, Fitzsimons CP, Vreugdenhil E. 2010. The doublecortin gene family and disorders of neuronal structure. Cent Nerv Syst Agents Med Chem 10:32-46.

Dingar D, Konecny F, Zou J, Sun X, von Harsdorf R. 2012. Anti-apoptotic function of the E2F transcription factor 4 (E2F4)/p130, a member of retinoblastoma gene family in cardiac myocytes. J Mol Cell Cardiol 53:820-828.

Ebert AM, Childs SJ, Hehr CL, Cechmanek PB, McFarlane S. 2014. Sema6a and Plxna2 mediate spatially regulated repulsion within the developing eye to promote eye vesicle cohesion. Development 141:2473-2482.

Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM, Dryja TP. 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323:643-646.

Ganguly A, Yang H, Sharma R, Patel KD, Cabral F. 2012. The Role of Microtubules and Their Dynamics in Cell Migration. Journal of Biological Chemistry 287:43359-43369.

Grati Mh, Chakchouk I, Ma Q, Bensaid M, Desmidt A, Turki N, Yan D, Baanannou A, Mittal R, Driss N, Blanton S, Farooq A, Lu Z, Liu XZ, Masmoudi S. 2015. A missense mutation in DCDC2 causes human recessive deafness DFNB66, likely by interfering with sensory hair cell and supporting cell cilia length regulation. Human Molecular Genetics 24:2482-2491.

He H, Yang T, Terman JR, Zhang X. 2009. Crystal structure of the plexin A3 intracellular region reveals an autoinhibited conformation through active site sequestration. Proceedings of the National Academy of Sciences of the United States of America 106:15610-15615.

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. 2003a. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 31:e15.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. 2003b. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4:249-264.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of embryonic development of the zebrafish. Dev Dyn 203:253-310.

Lai A, Kennedy BK, Barbie DA, Bertos NR, Yang XJ, Theberge M-C, Tsai S-C, Seto E, Zhang Y, Kuzmichev A, Lane WS, Reinberg D, Harlow E, Branton PE. 2001. RBP1 Recruits the mSIN3-Histone Deacetylase Complex to the Pocket of Retinoblastoma Tumor Suppressor Family Proteins Found in Limited Discrete Regions of the Nucleus at Growth Arrest. Molecular and Cellular Biology 21:2918-2932.

Lemmens K, Bollaerts I, Bhumika S, De Groef L, Van Houcke J, Darras VM, Van Hove I, Moons L. 2015. Matrix metalloproteinases as promising regulators of axonal regrowth in the injured adult zebrafish retinotectal system. J Comp Neurol.

Louro R, Nakaya HI, Paquola AC, Martins EA, da Silva AM, Verjovski-Almeida S, Reis EM. 2004. RASL11A, member of a novel small monomeric GTPase gene family, is down-regulated in prostate tumors. Biochem Biophys Res Commun 316:618-627.

Luo Y, Raible D, Raper JA. 1993. Collapsin: A protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75:217-227.

Murphree AL, Benedict WF. 1984. Retinoblastoma: clues to human oncogenesis. Science 223:1028.

Negishi M, Oinuma I, Katoh H. 2005. Plexins: axon guidance and signal transduction. Cell Mol Life Sci 62:1363-1371.

Neufeld G, Kessler O. 2008. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. Nat Rev Cancer 8:632-645.

Oinuma I, Ishikawa Y, Katoh H, Negishi M. 2004. The Semaphorin 4D Receptor Plexin-B1 Is a GTPase Activating Protein for R-Ras. Science 305:862-865.

Pasterkamp RJ. 2005. R-Ras fills another GAP in semaphorin signalling. Trends in Cell Biology 15:61-64.

Pezeron G, Lambert G, Dickmeis T, Strahle U, Rosa FM, Mourrain P. 2008. Rasl11b knock down in zebrafish suppresses one-eyed-pinhead mutant phenotype. PLoS One 3:e1434.

Rembold M, Loosli F, Adams RJ, Wittbrodt J. 2006. Individual cell migration serves as the driving force for optic vesicle evagination. Science 313:1130-1134.

Renaud J, Chedotal A. 2014. Time-lapse analysis of tangential migration in Sema6A and PlexinA2 knockouts. Mol Cell Neurosci 63:49-59.

Renaud J, Kerjan G, Sumita I, Zagar Y, Georget V, Kim D, Fouquet C, Suda K, Sanbo M, Suto F, Ackerman SL, Mitchell KJ, Fujisawa H, Chedotal A. 2008. Plexin-A2 and its ligand, Sema6A, control nucleus-centrosome coupling in migrating granule cells. Nat Neurosci 11:440-449.

Rünker AE, Little GE, Suto F, Fujisawa H, Mitchell KJ. 2008. Semaphorin-6A controls guidance of corticospinal tract axons at multiple choice points. Neural Development 3:34.

Serini G, Valdembri D, Zanivan S, Morterra G, Burkhardt C, Caccavari F, Zammataro L, Primo L, Tamagnone L, Logan M, Tessier-Lavigne M, Taniguchi M, Puschel AW, Bussolino F. 2003. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature 424:391-397.

Shi W, Kumanogoh A, Watanabe C, Uchida J, Wang X, Yasui T, Yukawa K, Ikawa M, Okabe M, Parnes JR, Yoshida K, Kikutani H. 2000. The Class IV Semaphorin CD100 Plays Nonredundant Roles in the Immune System: Defective B and T Cell Activation in CD100-Deficient Mice. Immunity 13:633-642.

Shimada T, Toriyama M, Uemura K, Kamiguchi H, Sugiura T, Watanabe N, Inagaki N. 2008. Shootin1 interacts with actin retrograde flow and L1-CAM to promote axon outgrowth. The Journal of Cell Biology 181:817-829.

Smyth GK. 2005. limma: Linear Models for Microarray Data. In: Gentleman R, Carey VJ, Huber W, Irizarry RA, Dudoit S, editors. Bioinformatics and Computational

Biology Solutions Using R and Bioconductor. New York, NY: Springer New York. pp 397-420.

Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, Tessier-Lavigne M, Comoglio PM. 1999. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99:71-80.

Tawarayama H, Yoshida Y, Suto F, Mitchell KJ, Fujisawa H. 2010. Roles of Semaphorin-6B and Plexin-A2 in Lamina-Restricted Projection of Hippocampal Mossy Fibers. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7049-7060.

Thisse C, Thisse B. 2008. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat. Protocols 3:59-69.

Toriyama M, Shimada T, Kim KB, Mitsuba M, Nomura E, Katsuta K, Sakumura Y, Roepstorff P, Inagaki N. 2006. Shootin1: a protein involved in the organization of an asymmetric signal for neuronal polarization. The Journal of Cell Biology 175:147-157.

Toyofuku T, Yoshida J, Sugimoto T, Zhang H, Kumanogoh A, Hori M, Kikutani H. 2005. FARP2 triggers signals for Sema3A-mediated axonal repulsion. Nat Neurosci 8:1712-1719.

CHAPTER 3. SHOOTIN-1 IS REQUIRED FOR NERVOUS SYSTEM DEVELOPMENT IN ZEBRAFISH.

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Abbreviations:

GFP – green fluorescent protein

Hpf – hours post fertilization

RFP – red fluorescent protein

RPC – retinal precursor cells

RPE – retinal pigmented epithelium

Shtn-1 – Shootin-1

Sema – Semaphorin

Plxn – Plexin

3.1. Abstract

Coordinated development relies on the migration of cells through complex and changing environments as they navigate to their correct destinations. The interactions that link the intracellular cytoskeleton of the migrating cell to the surrounding extracellular matrix (ECM) are critical to provide traction forces for movement. Shootin-1 (shtn-1) is a key proponent of cell migration, being a 'clutch molecule', it provides critical links between the ECM and F-Actin retrograde flow in neuronal growth cones by binding to both the cell adhesion molecule, L1-CAM and Cortactin (Kubo et al., 2015; Shimada et

al., 2008a; Toriyama et al., 2006b). Shtn-1 is primarily neuronally expressed and was discovered to be an asymmetrical signal that drives cell polarization during development. In differentiating rat hippocampal neurons, it was found that the competitive accumulation of an available pool of Shtn-1 in neurites breaks cell symmetry, leading to the formation of a single axon, driving cell polarization (Toriyama et al., 2006b). Furthermore, studies have uncovered the importance of careful Shtn-1 regulation, as both overexpression and knockdown in cultured rat hippocampal neurons leads to abnormalities in neuronal development. Overexpression of *shtn-1* disrupts polarization and leads to longer neurites and surplus axons, whereas RNAi knockdown prevents cellular polarization and leads to shorter neurites (Shimada et al., 2008a; Toriyama et al., 2006b).

It is known that Netrin signaling positively regulates Shtn-1 via phosphorylation events. Netrins activate Pak-1 via Cdc42 and Rac-1, which phosphorylates Shtn-1 at Ser101 and Ser249. Phosphorylation of Shtn-1 enhances binding to F-Actin via Cortactin and the generation of traction forces, promoting axon outgrowth (Kubo et al., 2015; Toriyama et al., 2013). We previously showed that Sema6A and PlxnA2 signaling negatively regulates *shtn-1* gene expression (Emerson et al., 2017) and this signaling pathway may work antagonistically to Netrin signaling to control the precise levels of Shtn-1 in a mechanism of 'checks and balances.'

Here we investigate the roles of PlxnA2-regulated *shtn-1* expression in zebrafish neurodevelopment and show that *in vivo*, *shtn-1* is important for early eye patterning, pathfinding of the optic tracts to the tecta and axon branching of sensory and motor neurons of the peripheral nervous system.

Keywords. Retina, eye development, peripheral nervous system, microarray.

3.2. Introduction

During development, cells must navigate through complex and changing environments as they migrate to their correct destinations. As cells crawl along the components of the extracellular matrix (ECM) or the scaffolds of neighboring cells, they are led by an array of guidance cues that mediate cell migration in the appropriate direction. Neurons must often travel long distances during development and send out lengthy axon projections to connect to their synaptic counterparts. Axon migration is led by a highly sensitive and dynamic growth cone that responds to guidance cues in the environment that stimulate cellular signaling events to mediate internal cytoskeleton dynamics. Changes to the cytoskeleton and components that control neuronal cell migration can alter directional movement, which ultimately leads to the patterning of the nervous system.

An important group of guidance cues in neuronal development are the Semaphorins (Semas), which typically bind to and signal through Plexin (Plxn) receptors. Originally identified to be involved in repulsive axon guidance (Luo et al., 1993b), Sema and Plxn signaling is now known to play roles in many aspects of development, including cell proliferation and cohesion during eye development (Ebert et al., 2014; Emerson et al., 2017), vasculogenesis (Serini et al., 2003a), bone development (Behar et al., 1996a), kidney development (Perala et al., 2011), tumorigenesis (Neufeld and Kessler, 2008) and heart development (Epstein et al., 2015), among others. Semas and Plxns are both large families of proteins. There are 8 classes of Semas; Semas1 and 2 are found in invertebrates, Semas 3-7 are found in vertebrates, and SemaV is a viral form. There are 4 classes of vertebrate Plxns, (A1-4, B1-3, C1 and D1) in addition to invertebrate PlxnA and B (Tamagnone et al., 1999). Semas are transmembrane, membrane-bound or secreted

proteins, and typically signal through transmembrane Plxn receptors (Comeau et al., 1998; Perälä et al., 2012), secreted Semas require an additional Neuropillin co-receptor to signal (Chen et al., 1997). Semas can bind to multiple different Plxn receptor family members, and binding partners are determined by differential spatial and temporal expression patterns during development (Ebert et al., 2012; Emerson et al., 2018).

We have previously shown that *sema6A* and *plxnA2* are expressed in overlapping regions in the early zebrafish eye and play roles in early eye development. Knock-down of either *sema6A* or *plxnA2* results in decreased proliferation and a loss of overall cohesion of retinal precursor cells (RPCs) within optic vesicles (Ebert et al., 2014; Emerson et al., 2017). In an effort to better understand the downstream signaling mechanisms of *sema6A/plxnA2* signaling that could contribute to our observed knockdown phenotypes, we performed a microarray screen that aimed to identify Sema6A- and PlxnA2-dependent transcriptional targets. Among 58 target genes that were differentially regulated in both knockdown conditions vs. control, *Shootin-1* (*shtn-1*) was identified and hypothesized to contribute, in part, to some of the early eye phenotypes that we saw including disorganization of RPCs and loss of cohesive optic vesicles (Emerson et al., 2017).

Shtn-1 was originally described to be brain specific, and to accumulate in the growth cones of developing neurons (Toriyama et al., 2006b). More recently, a novel isoform was discovered in mice, (*Shtn-1b),* and has been shown to be additionally expressed in peripheral tissues (Higashiguchi et al., 2016; Minegishi et al., 2018). Shtn-1 was discovered through proteomic screens using differentiating rat hippocampal neurons in an effort to identify neuronal polarizing signals (Toriyama et al., 2006b). It was found that competitive accumulation of Shtn-1 in neurites can break neuronal symmetry and

initiate axon formation (Toriyama et al., 2006b). Shtn-1 is a 'clutch molecule' and links cytoskeletal actin filament retrograde flow of the migrating growth cone to the ECM through binding to the cell adhesion molecule L1-CAM, essential to build traction forces for cell movement (Shimada et al., 2008a). Experiments using cultured rat hippocampal neurons have shown that it is critical for the levels of Shtn-1 to be tightly regulated during development. Overexpression of *shtn-1* can disrupt polarization leading to surplus axons. Conversely, when inhibited, cell polarization is inhibited and L1-dependent axon outgrowth is impaired, leading to fewer, shorter projections (Shimada et al., 2008a; Toriyama et al., 2006b). *Shtn-1b* KO mice show problematic development of the olfactory bulb due to impaired rostral migration of olfactory interneurons (Minegishi et al., 2018), and display dysgenesis and mis-projection of commissural axons in the developing forebrain (Baba et al., 2018), showing that Shtn-1 is critical for neuronal cell migration *in vivo*.

Netrins are attractive axon guidance cues and have been shown to positively regulate Shtn-1 mediated traction forces. Netrins activate Pak-1 via Cdc42 and Rac-1, which phosphorylates Shtn-1 at Ser101 and Ser249 (Toriyama et al., 2013). Phosphorylation of Shtn-1 in XTC fibroblasts enhances binding to F-actin via cortactin and the generation of traction forces, promoting migration (Kubo et al., 2015). Recently, it has been shown that spatially regulated Shtn-1 phosphorylation in cultured rat hippocampal neuron growth cones leads to axon turning in response to Netrin-1 gradients due to the promotion of the L1-CAM interaction (Baba et al., 2018).

The literature supports a critical role for Shtn-1 in neuronal cell polarization and migration, and that Shtn-1 needs to be carefully regulated for appropriate neuronal differentiation to occur. Although the signaling pathway downstream of Netrins to

positively regulate Shtn-1 is well documented, the negative regulation of Shtn-1 is poorly understood. Here we show that Sema6A/PlxnA2 signaling represses *shtn-1* expression at the transcriptional level and could work antagonistically to Netrin signaling to control the precise levels of Shtn-1 in a mechanism of 'checks and balances.' We investigate the importance of this regulation in zebrafish neurodevelopment, expanding on the roles of Shtn-1 *in vivo* in a novel system. Our results indicate that PlxnA2 repression of *shtn-1* expression is important for optic vesicle migration, patterning of the optic tract, in addition to the patterning and branching of sensory neurons and motor axons in the peripheral nervous system of the developing zebrafish.

3.3. Materials and Methods

3.3.1. Zebrafish Husbandry

Rx3:GFP embryos were generously provided by Jochen Wittbrodt, University of Heidelberg, Germany. *isl2b*:GFP/*kdrl*:mcherry zebrafish embryos were provided by the late Chi-Bin Chien, University of Utah and Jau-Nian Chen, UCLA. *mnx2*:RFP/*ngn-1*:GFP zebrafish embryos were provided by Christine Beattie, Ohio State University and Uwe Strähle, KIT Germany. Zebrafish were bred and embryos were developmentally staged as previously described (Kimmel et al., 1995). Pigmentation was blocked by addition of 0.003% phenylthiourea (Fisher Scientific, cat # 207250050) at the 18 somite stage. All procedures were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC protocol # 15-031).

3.3.2. Injections

Antisense Morpholino oligonucleotides (MO) (Gene Tools, Philomath, OR), or full-length mRNAs were injected at the one-cell stage (Kimmel et al., 1995). *plxnA2* e2i2

MO (AAAAGCGATGTCTTTCTCACCTTCC) was injected for a final concentration of 2 ng. *plxnA2* MO constructs have been previously validated using RT-PCR to show successful knockdown (Ebert et al., 2014).Off-target effects and specificity of MOs have been addressed by rescue by full-length human mRNA and co-injection of *plxnA2* and *p53* MOs. In addition, second non-overlapping MO constructs reproduced the same phenotypes, previously published data, see (Ebert et al., 2014) also (Emerson et al., 2017). Capped and tailed full-length mRNA, (mMESSAGE mMACHINE kit, Ambion, Austin, TX, cat# AM1344; Poly(A) tailing kit, Invitrogen Cat# AM1350) was generated from plasmids containing human *plxnA2* (Origene, Cat# RC221024) or full-length *Danio rerio shtn-1* cloned by PCR using zebrafish cDNA following RNA extraction. PCR Forward T7 primer: TAATACGACTCACTATAGGGGAGAATGG CGTCAAAAGGAAAGAAGAATAAAG, reverse primer: GTCACAGTGATTTCATAA CAGACTCTG. Constructs were sequence-verified at The Vermont Integrative Genomics Resource DNA Facility and was supported by University of Vermont Cancer Center, Lake Champlain Cancer Research Organization, and the UVM Larner College of Medicine prior to RNA generation. mRNAs were injected into embryos at the one cell stage for a final concentration of 400pg/embryo.

3.3.3. Whole-mount *in situ* Hybridization

Zebrafish embryos were staged as described (Kimmel et al., 1995), fixed in 4% paraformaldehyde overnight at 4°C, and stored at -20°C in 100% methanol until use. *In situ* hybridization was performed as described previously (Thisse and Thisse, 2008). DIG-labeled antisense RNA probes were generated using the following primers: *shtn-1* forward SP6 primer: TTTAGGTGACACTATAGAAGAGAGGCCTTACGGAGGCT GAAT, reverse T7: TAATACGACTCACTATAGGGGAGATGCCCAAGAGCTTCTT

TTGT; *plxnA2* forward primer ATGTGATACAGGAGCCGAGG, reverse primer: AGAGTCAGAAGGCTGTCGGA (IDT, Coralville, IA). PCR products were cloned into pCR 2.1 TOPO vector (Invitrogen, Carlsbad, CA) and sequenced at The Vermont Integrative Genomics Resource DNA Facility and was supported by University of Vermont Cancer Center, Lake Champlain Cancer Research Organization, and the UVM Larner College of Medicine. Embryos were oriented in 4% methyl cellulose on depression slides and imaged using a Nikon SMZ800 dissecting light microscope at 50x magnification.

3.3.4. Sectioning, Embedding and H&E Staining

Embryos were dehydrated in 100% ETOH for 30 min before embedding using a JB4 Embedding Kit (Polysciences, Inc., Warrington, PA) and sectioned on a Leica RM2265 microtome at 20 μm. Sections were stained using Hematoxylin and Eosin B (Fisher Cat#S25347 and #548-2423 respectively). Brightfield images were obtained using an Olympus iX71 inverted light microscope and figures were assembled using Adobe Photoshop CS6, in which image brightness and contrast were optimized.

3.3.5. Imaging and Quantification

Images of *rx3*:GFP optic vesicles, *isl2b*:GFP optic tracts and *ngn-1*:GFP/*mnx2*:RFP sensory and motor neurons were obtained using an Olympus iX71 inverted light microscope in conjunction with SPOT imaging software version 5.2 for quantification of optic vesicle distances, the presence and projection of optic tracts, sensory axon projection angle and motor neuron branch numbers respectively. For confocal imaging of *rx3*:GFP, embryos were mounted in 0.5 % low-melt agarose on glass bottom dishes (CLS-1811 ChemGlass, NJ) and imaged at 20X using 2 μm steps on a Nikon Eclipse Ti inverted microscope. Z Stacks were subjected to a Kalman stack filter

in Image J and were presented as maximum intensity projections. RGB images were generated in Adobe Photoshop CS6.

3.4. Results

3.4.1 *Shtn-1* is Negatively Regulated by PlxnA2 Signaling and is Important for Optic Vesicle Migration

Our previous work in zebrafish revealed a novel role for Sema6A/PlxnA2 signaling in early eye development. Both *sema6A* and *plxnA2* knockdown embryos display decreased cohesion (Ebert et al., 2014) and proliferation (Emerson et al., 2017) of retinal precursor cells (RPCs) within optic vesicles. In an effort to better understand the downstream events of Sema/Plxn signaling, we performed a microarray, which revealed Sema6A/PlxnA2-dependent transcriptional regulation of target genes. *Shtn-1* was significantly upregulated in *sema6a* and *plxnA2* knockdown embryos, compared to uninjected controls and is expressed in overlapping regions of the optic vesicles with *plxnA2* (Emerson et al., 2017) (Fig. 3.1A, B). To investigate the functional consequence of repression of *shtn-1* on *sema6a* and *plxnA2* knockdown phenotypes, we deliberately overexpressed full-length *Danio rerio shtn-1* mRNA at the one cell stage. We hypothesize that this will mimic the increase in expression seen in *plxnA2* and *sema6A* knockdown embryos at 18 somites (Fig. 3.1A-C). Co-injection of full-length human *PLXNA2* mRNA rescued the overexpression phenotypes of *shtn-1*, reinforcing that *shtn-1* expression is negatively regulated downstream of PlxnA2 signaling (Fig. 3.1D). Importantly, overexpression of full-length human *PLXNA2* alone does not significantly alter *shtn-1* levels from control (Fig. 3.1E).

In order to observe the phenotypic effects of *shtn-1* overexpression on early eye development, we utilized the *rx3*:GFP transgenic line, which expresses GFP in RPCs and hypothalamus. During early eye development, RPCs evaginate from the diencephalon to form the optic vesicles and migrate bilaterally to reside at either side of the head. When *shtn-1* levels are increased in either *plxnA2* knockdown or *shtn-1* overexpression embryos, the optic vesicles do not migrate as far apart as control embryos by 18 somites (Fig. 3.1A'-C'). This migration defect can be rescued by co-injection of full-length *PLXNA2* (Fig. 3.1D'). *PLXNA2* alone showed no significant phenotype (Fig. 3.1E'). In some cases, *shtn-1* overexpression resulted in no separation between optic vesicles, and embryos were cycloptic (Supplemental Fig. 3.6). These data suggest that PlxnA2 repression of *shtn-1* expression is required for optic vesicle separation. The relationship between *shtn-1* and PlxnA2 is reinforced when evaluating their expression patterns. At 18 somites, expression patterns are predominantly neuronal, with co-expression in the optic vesicles (Fig. 3.1 F-G''''). *PlxnA2* is expressed throughout the optic vesicle with stronger ventral expression (Fig. 3.1F-F'''') and *shtn-1* is expressed ubiquitously throughout the optic vesicles (Fig. 3.1G-G''''). This overlap in expression domains, and the change in expression levels of *shtn-1* with manipulated *plxnA2* levels supports the hypothesis that PlxnA2 and Shtn-1 are involved in the same pathway to contribute to early eye development.
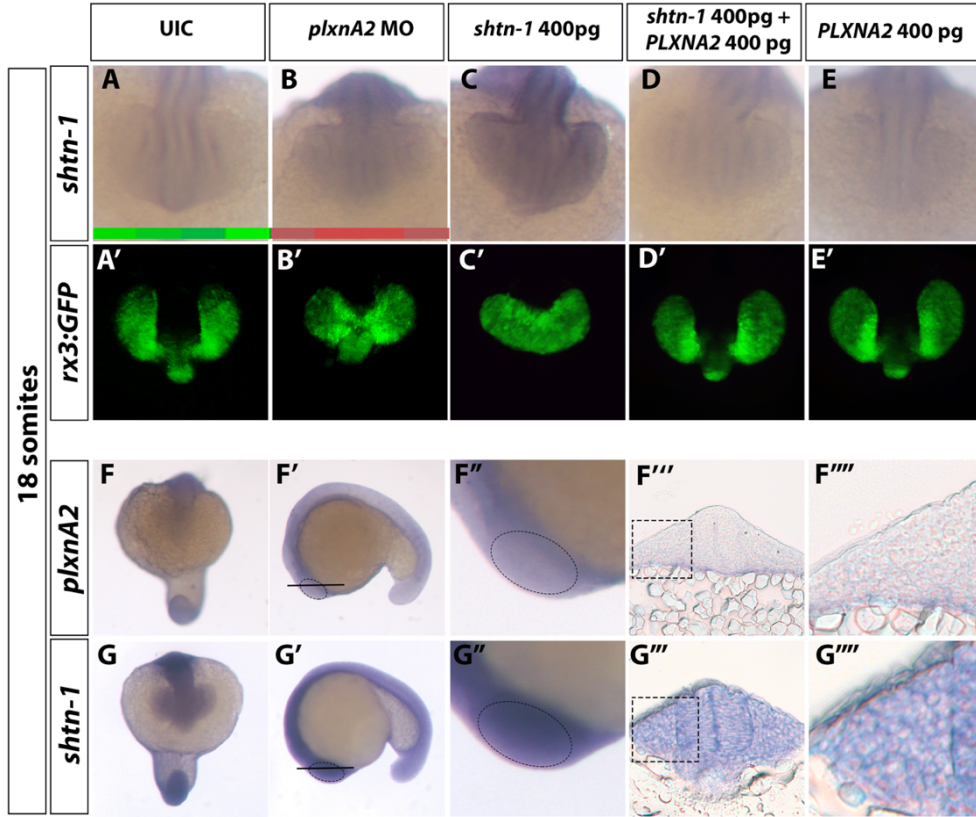
Figure 3.1. PlxnA2 Negatively Regulates *shtn-1* Expression to Control Optic Vesicle Migration. A-E *In situ* hybridization showing manipulation of *shtn-1* expression levels in the eye fields at 18 somites. A'-E' Dorsal confocal images of *rx3*:GFP embryos at 18 somites. A&A', Uninjected control, B&B' *PlxnA2* MO injected, C&C', 400 pg *Shtn-1* overexpression, D&D', Co-injection of 400pg *shtn-1* and 400 pg human *PLXNA2*, E&E', 400 pg human *PLXNA2* alone. F (dorsal) & F'(lateral) wholemount *in situ* expression of *plxna2* at 18 somites, F'' zoom of eye. F'''&F'''' transverse section at line shown in F'. G-G' whole mount *in situ* expression of *shtn-1* at 18 somites, G'' zoom of eye. G'''&G'''' transverse section at line shown in G'.

3.4.2. PlxnA2 Regulation of *shtn-1* Expression is Important for the Development of the Retinal Pigmented Epithelium.

To investigate the role of PlxnA2 repression of *shn-1* expression in later retinal development, we looked at the lamination patterns in the retina in transverse sections of 72 hpf embryos stained with H&E (Fig. 3.2A-D'). When observing the expression patterns of both *plxnA2* and *shtn-1* throughout development, we observed both co-expressed in similar tissues, including the retina at 72 hpf (Fig. 3.2E & F). During the formation of the retina, RPCs undergo interkinetic nuclear migration to divide apically and then must migrate to their target layer. Therefore, we hypothesized that *shtn-1* overexpression might lead to migration defects and result in improper retinal lamination. To our surprise, we found that overexpression of *shtn-1* resulted in no lamination defects, even in cycloptic embryos (Supplemental Fig. 3.6). However, formation of the retinal pigmented epithelium (RPE) was significantly impaired at the midline (Fig. 3.2B, B') and can be rescued by co-injection of human *PLXNA2* (Fig. 3.2C, C'). Injection of *PLXNA2* alone showed no phenotype (Fig. 3.2D, D') (Percent of normal RPE coverage, UIC 100% ± 0%, N = 2, n = 16; *shtn-1 400pg* 87.39% ± 5.82%, N = 2, n = 16, $P < 0.5$; *shtn-1 400pg* + *PLXNA2* 400 pg 100% ± 0%, N = 2, n = 16; n.s.; *PLXNA2* 400 pg 100% ± 0%, N = 2, n = 16; n.s). Together, this shows that *shtn-1* is not involved in retinal lamination but does play an important role in the development of the RPE, specifically at the midline.
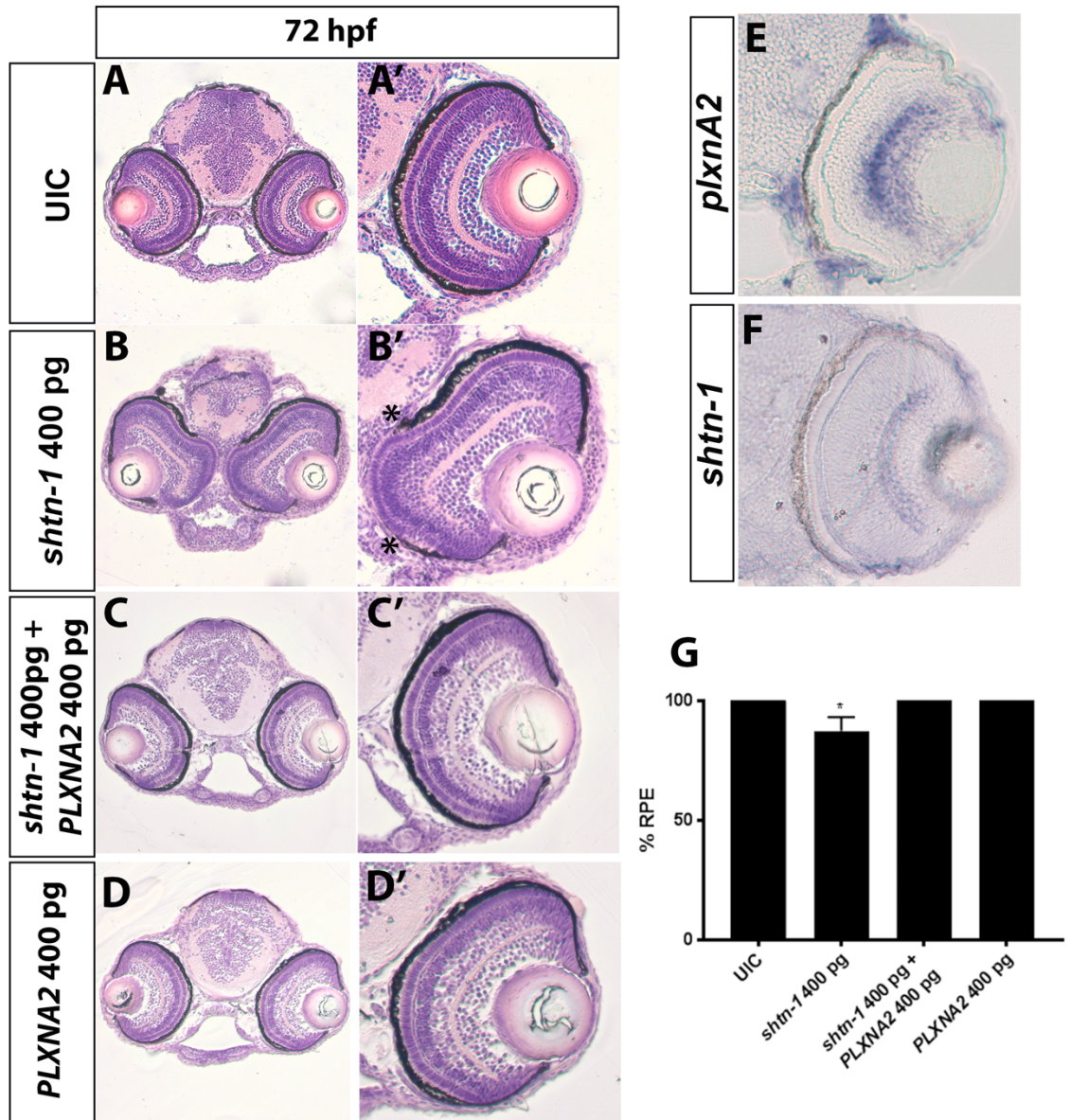
Figure 3.2. The Regulation Of *shtn-1* Expression is Important for RPE Development, but not Retinal Lamination. A-D H&E stained transverse sections of the eyes and forebrain at 72 hpf. A'-D' zoom of right eye. A&A', Uninjected control, B&B' 400 pg *shtn-1* overexpression, C&C' Co-injection of 400pg *shtn-1* and 400 pg human *PLXNA2*, D&D' 400 pg human *PLXNA2* alone. E&F *in situ* hybridization of *plxnA2* and *shtn-1* at 72 hpf in the eye. G. Quantification of percent intact retinal pigmented epithelium (RPE) as seen in A-D'. UIC 100% ± 0, N = 2, n = 18; *shtn-1 400pg 87.39% ±* 5.824% , N = 2 n = 18, $P < 0.05$; *shtn-1 400pg + PLXNA2* 400 pg 100% ± 0, N = 2, n = 18; n.s.; *PLXNA2* 400 pg 100% ± 0, N = 2, n = 18; n.s. * In B' indicate site of missing RPE.

3.4.3. PlxnA2 Regulation of *shtn-1* Levels is Important for Optic Tract Formation

During eye development, the RGCs of each retina project their axons out of the eye as the optic nerve, cross at the optic chiasm in the ventral brain, and project contralaterally to the optic tecta. As Shtn-1 is important for axon formation and migration, we investigated if PlxnA2-regulated *shtn-1* expression was important for optic tract formation. Utilizing the *isl2b*:GFP transgenic, which labels RGCs and their axonal processes, we observed that *shtn-1* overexpression leads to tract projections to the optic tecta that are weaker (Fig. 3.3A, B). This phenotype can be rescued by co-injection of full-length *PLXNA2* (Fig. 3.3C). *PLXNA2* alone has no effect on tract strength (Fig. 3.3D), as quantified in Fig. 3.3E. (Tract strength score: strong = 1, weak = 0.5, absent = 0. UIC 1 ± 0, N = 3, n = 15; *shtn-1 400pg* 0.83 ± 0.06, N = 3, n = 15, $P < 0.01$; *shtn-1 400pg* + *PLXNA2* 400 pg 0.967 ± 0.03, N = 3, n = 15; n.s.; *PLXNA2* 400 pg 1 ± 0, N = 3, n = 15; n.s).

When observing the tracts from a ventral view as they cross-over at the optic chiasm, *shtn-1* overexpression leads to inappropriate fasciculation and targeting to the tecta (Fig. 3.3A' & 3.3B'), which can also be rescued by co-injection of full-length *PLXNA2* (Fig 3.2C'). *PLXNA2* alone has no effect (Fig. 3.3D'), quantified in (Fig. 3.3F). (UIC: 100% normal projections N = 3, n = 15; *shtn-1* 400 pg: 46.6% normal projections N = 3, n = 15; *shtn-1 400pg* + *PLXNA2* 400 pg; 90% normal projections N = 3, n = 15.; *PLXNA2* 400 pg: 86.6% normal projections N = 3, n = 15). *Shtn-1* overexpression often results in tracts that project nasally, away from the tecta. Together, this data shows that excess *shtn-1* leads to mis-patterning of the optic tracts and highlights the importance of the regulation of *shtn-1* in eye development.
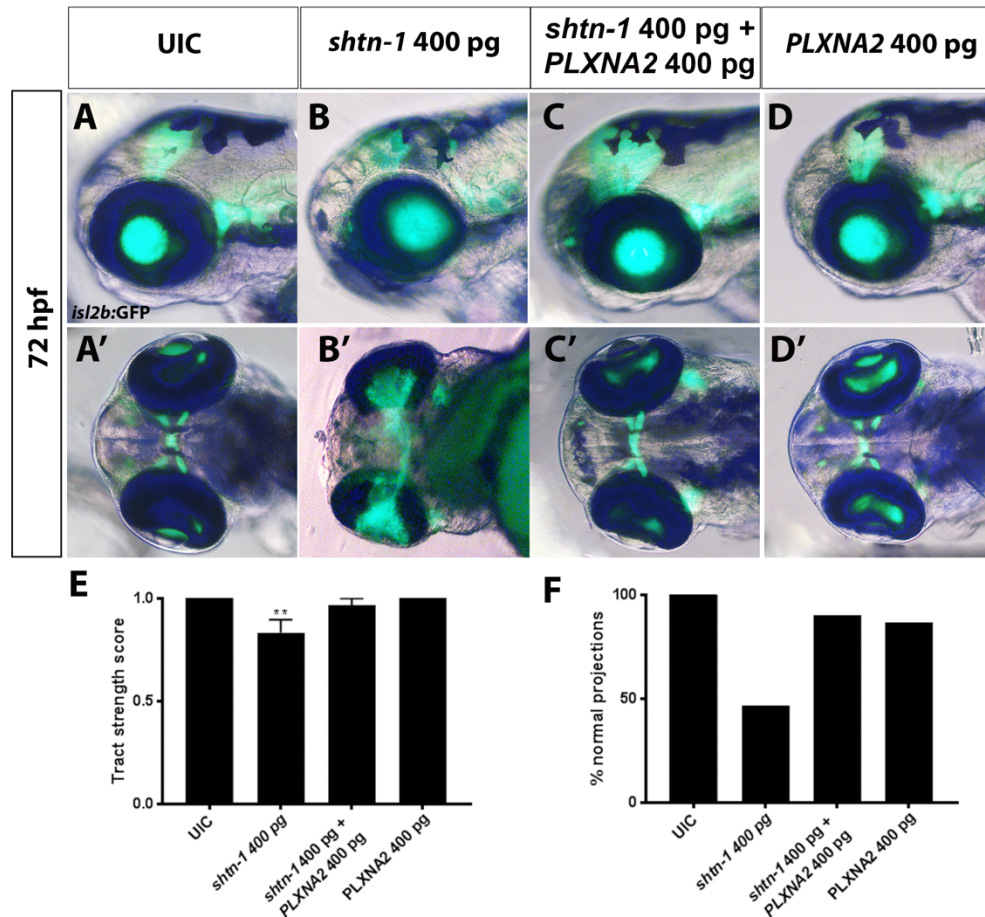
Figure 3.3. Plxna2 Regulation of *shtn-1* Levels is Important for Optic Tract Crossing. A-D Lateral view of *isl2b*:GFP embryos at 72 hpf. A'-D' Dorsal views. A&A', Uninjected control, B&B', 400 pg *shtn-1*, C&C', Co-injection of 400pg *shtn-1* and 400 pg human *PLXNA2*, D&D', 400 pg human *PLXNA2* alone. E quantification of tract projection strength, as seen in A-D. Tracts were scored 1 for strong, 0.5 for weak and 0 for absent. UIC 1 ± 0, N = 3, n = 15; *shtn-1 400pg* 0.83 ± 0.06, N = 3, n = 15, $P < 0.01$; *shtn-1 400pg + PLXNA2* 400 pg 0.967 ± 0.03, N = 3, n = 15; n.s.; *PLXNA2* 400 pg 1 ± 0, N = 3, n = 15; n.s. F. quantification of percentage of tracts that have normal projections. Tracts crossing the optic chiasm were scored 1 for normal, 0 for abnormal, as seen in A'-D'. UIC 100% normal projections N = 3, n = 15; *shtn-1* 400 pg 46.6% normal projections N = 3, n = 15; *shtn-1 400pg + PLXNA2* 400 pg 90% normal projections N = 3, n = 15.; *PLXNA2* 400 pg 86.6% normal projections N = 3, n = 15.

3.4.4. PlxnA2 Regulation of *shtn-1* Expression is Important for Sensory and Motor Neuron Patterning

While observing embryos in their dish, we saw behavioral phenotypes in embryos with increased levels of *shtn-1*. Control embryos at 72 hpf display a stereotypical sinusoidal swimming pattern, propelling them in a forward direction. However, embryos with an excess of *shtn-1* spin rapidly around in circles, and do not move forward. When we observed this swimming behavior in slow motion, we could see that the fish moved with a jerky motion, their tails contracting to one side and releasing repeatedly. As some *shtn-1* overexpression embryos show malformed body axes, we controlled for musculature and skeletal defects leading to this behavior by observing embryos with less severe body axis curvatures and scored phenotypic differences in a straight part of the body axis. *Shtn-1* is expressed in similar areas to *plxnA2* in the spinal cord (Fig. 3.4A-B'), and as *shtn-1* is expressed primarily in neurons, we utilized the *ngn-1*:GFP/*mnx2*:RFP double transgenic which labels sensory and motor neurons with GFP and RFP respectively to observe the effect of *shtn-1* overexpression on peripheral axon patterning.

Typically, dorsal root ganglia sensory neurons project axons from their cell bodies ventrally and evenly spaced in pairs along each side the tail (Fig. 3.4C-D'''). In embryos with excess *shtn-1*, axons displayed severe disorganization, with inappropriate anteroposterior angles of projection, towards neighboring axons. Furthermore, often there were misplaced cell bodies out of the region of the spinal cord (Fig. 3.4E-E'''). *Shtn-1* overexpression can be rescued by co-injecting *PLXNA2* (Fig. 3.4F-F'''), and injections of *PLXNA2* alone shows no significant phenotype (Fig. 3.4G-G''').
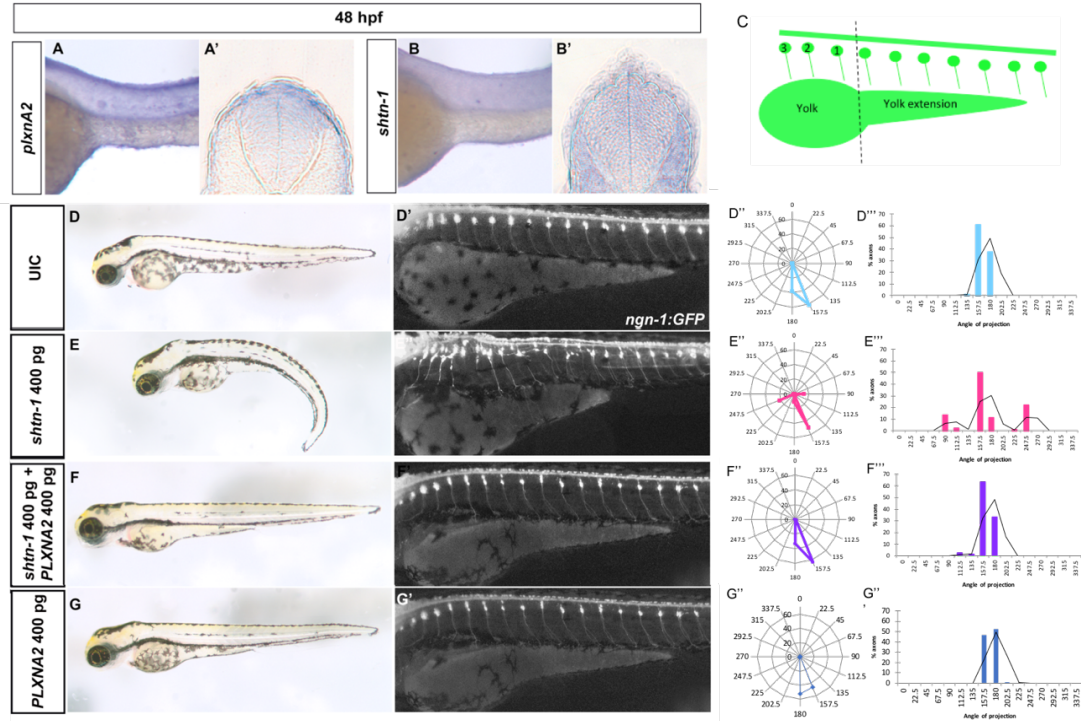
Figure 3.4. PlxnA2 Regulation of *shtn-1* Levels is Important for Sensory Neuron Patterning. A-B' *in situ* hybridization of *plxna2* (A-A') and *shtn-1* (B-B') in the trunk at 48 hpf. A&B lateral wholemount image showing yolk extension. A'&B' in section. C. Trunk schematic of a control embryo at 72 hpf (anterior to the left). The first three DRG axons shown anterior to the beginning of the yolk extension were evaluated for each fish. D-G Brightfield lateral views at 72 hpf. D'-G' epifluorescnt lateral trunk zoom images of *ngn1*:GFP embryos at 72hpf. D''-G'' Radar plots showing initial angle of axon projection from the DRG. D'''-G''' histogram plots also displaying angles of projection. D-D''', Uninjected control, E&E''' *shtn-1* overexpression, F&F''', Co-injection of 400pg *shtn-1* and 400 pg human *PLXNA2*, G-G''', 400 pg human *PLXNA2* alone.

Overexpression of *shtn-1* lead to defects in motor axon patterning in the trunk with a doubling of the number of primary axon branches compared to control embryos (Fig. 3.5A-3.5E). Branch number can be rescued by co-injection of full-length *PLXNA2* (Fig. 3.5C, C'). Only a minor phenotype was observed in embryos injected with *PLXNA2* alone (Fig. 3.5D-D'). Branches were counted as branch points from the main axon above the dotted lines as shown (Fig 3.5A-D). Quantified in (Fig. 3.5E). (UIC 4.78 ± 0.17, N = 5, n = 69; *shtn-1 400pg* 7.99 ± 0.23, N = 5, n = 69, $P < 0.0001$; *shtn-1 400pg + PLXNA2* 400 pg 5.51 ± 0.16, N = 5, n = 63; $P < 0.05$; *PLXNA2* 400 pg 5.65 ± 0.14, N = 5, n = 69; $P < 0.01$). Taken together, these results show that excess *shtn-1* leads to mis-patterning of the sensory and motor systems, which could explain observed impairments in swimming ability seen in *shtn-1* overexpression embryos. This again reinforces the importance of the correct balance of *shtn-1* in the developing embryo, controlled at least in part by PlxnA2 signaling.
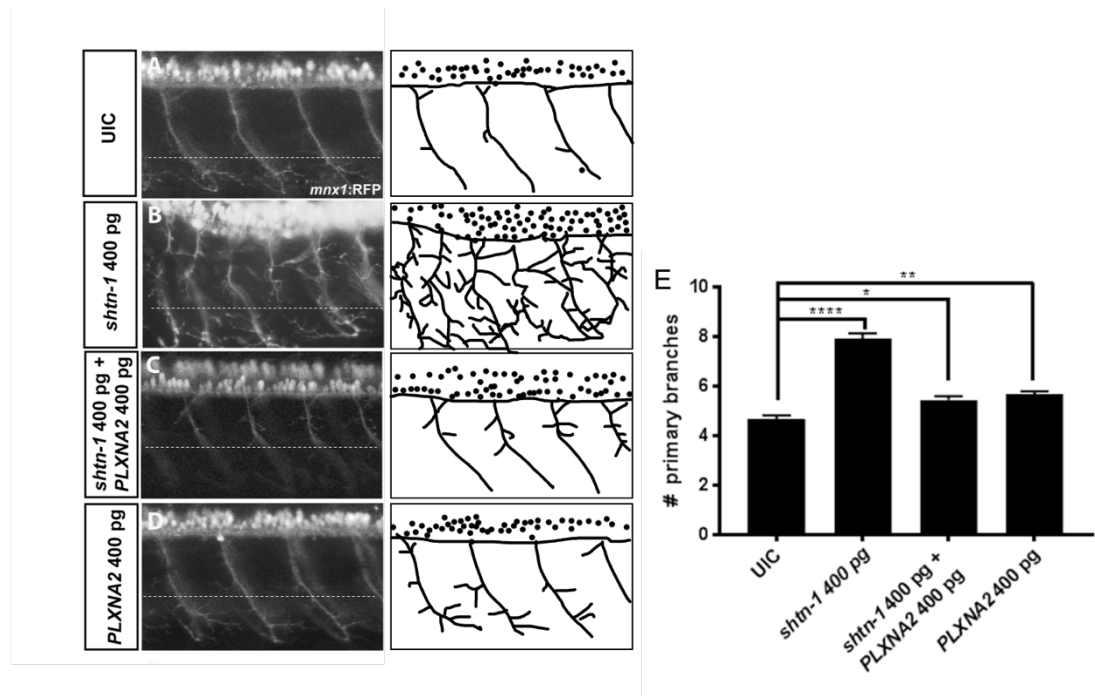
Figure 3.5. Overexpression of *shtn-1* Leads to Surplus Motor Axon Branching and can be Rescued by *PLXNA2* Overexpression. A-D Lateral epi-fluorescent images of *mnx1*:GFP motor axons in the trunk (anterior to the left). A'-D' Schematics of branching patterns seen in A-D. A&A', Uninjected control, B&B', 400 pg *shtn-1* overexpression, C&C', Co-injection of 400pg *shtn-1* and 400 pg human *PLXNA2*, D&D', 400 pg human *PLXNA2* alone. F Quantification of numbers of primary branches. UIC 4.78 ± 0.17, N = 5, n = 69; *shtn-1 400pg* 7.99 ± 0.23, N = 5, n = 69, $P < 0.0001$; *shtn-1 400pg + PLXNA2* 400 pg 5.51 ± 0.16, N = 5, n = 63; $P < 0.05$; *PLXNA2* 400 pg 5.65 ± 0.14, N = 5, n = 69; $P < 0.01$.

3.5. Discussion

Previous studies have revealed that overexpression of *shtn-1* leads to polarization defects and surplus axons in rat hippocampal neurons (Toriyama et al., 2006b) in addition to the promotion of neurite outgrowth in XTC fibroblasts (Shimada et al., 2008a). Our data show that overexpression of *shtn-1* in the developing zebrafish supports previous cellular findings and leads to developmental abnormalities at the organismal level. We show that with dysregulated *shtn-1* expression, eye development and the patterning of the peripheral nervous system goes awry. Specifically, we see improper migration of RPCs during optic vesicle separation, and defects in optic tract formation and guidance. In addition, we observed disorganized axon projections of sensory neurons and surplus branching and disorganization of motor neuron axons, which we hypothesize contributes to the observed impaired swimming behavior.

Shtn-1 is upregulated during neuronal polarization and has been shown to be a key factor during axon determination and formation in rat hippocampal neurons. (Toriyama et al., 2006b). During axon determination, the available pool of *shtn-1* is spread out between neurites. It has been postulated that stochastic increases of *shtn-1* levels between neurites leads to asymmetric accumulation and positive feedback loops in competitive neurites. Eventually, Shtn-1 reaches threshold concentrations within a single neurite, which drives increased migration and elongation, leading to axon formation (Toriyama et al., 2006b). We believe that excess Shtn-1 acts in a similar way to polarize RPCs, in that excess Shtn-1 disrupts the ability of a single filopodia to accumulate more than others, preventing RPC polarization, so that migration in the appropriate direction is impaired. This directional confusion of RPCs may result in a lack of coordinated

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movement and separation of the eye field to form two individual optic vesicles. Excess *shtn-1* could also potentially inhibit migration by forming surplus actin:ECM connections, leading cells to become slowed or stalled, although further experiments would have to be performed to uncover the mechanism behind optic vesicle abnormalities.

We hypothesize that mis-polarization of cells due to excess *shtn-1* also explains the disorganized axon trajectories seen in the optic tracts of overexpression embryos, especially in tracts that project nasally. It is also possible that the lack of RPE at the optic disc could be causing the loss of axon fasciculation as the RGC axons exit the eye. Netrins are expressed specifically in neuroepithelial cells surrounding the optic disc and in glial cells surrounding the optic stalk, and aid fasciculation of axons in the optic tract. In *Netrin* mutant mice, the optic disc is smaller with optic nerve hypoplasia (Deiner et al., 1997). Netrins positively regulate Shtn-1, and Shtn-1 overexpression leads to loss of fasciculation and disorganization of the optic tracts, the opposite of what is seen with *Netrin-1* knockout. This supports our model for the necessity for careful regulation of Shtn-1, positively by Netrins and negatively by PlxnA2.

Observed defects in the retinal pigmented epithelium could be due to many factors. One hypothesis is that in *shtn-1* overexpression embryos, the optic vesicles don't achieve adequate separation, therefore the RPE fated cells do not receive the appropriate signals from the tissue that would normally be at the midline. It is known that Hh signaling is required for RPE determination, and that sonic hedgehog (shh) and tiggy-winkle hedgehog (twhh) are both expressed at the midline and by the RPE (Stenkamp et al., 2000). Shh inhibition by cyclopamine results in a loss of RPE differentiation (Perron et al., 2003) and the effects look similar to those seen with the overexpression of *shtn-1*.

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Hh signaling is a well-known mediator of midline development. A loss of Shh in the midline, which usually represses Pax6, results in cycloptic embryos, which we also see in embryos with excess *shtn-1*. Further experiments are required to address any involvement of Shh signaling in RPE and midline abnormalities with respect to *shtn-1*.

Overexpression of *shtn-1* leads to abnormal swimming behaviors. We discovered that this un-coordinated behavior might be due to excess *shtn-1* in driving mis-patterning of sensory and motor neurons in the peripheral nervous system. Sensory neurons showed mis-targeting and disorganization of axons, in addition to ectopic cell bodies. We believe this to be an effect of disrupted polarization, leading to axons forming inappropriately and projecting from the cell body at the wrong angle. Surplus branching of axons was also seen in motor neurons. It is known that several guidance cues are important for the regulation of axon branching. Overexpression of Netrins increases cortical axon branching by 50% (Dent et al., 2004), supporting the model of Shtn-1 regulation of axon branching.

It is clear that *shtn-1* expression must be maintained at a precise level in order for stochastic increases in neurites to be realized and appropriately reinforced to drive polarization, axon formation and cell migration. It has been shown that Netrins act as positive regulators of Shtn-1 via Cdc42, Rac-1 and Pak-1 activation. Pak-1 phosphorylates Shtn-1 at ser101 and ser249 to enhance binding to actin retrograde flow, in turn promoting axon out-growth (Toriyama et al., 2013). Here we show that PlxnA2 signaling acts as a negative regulator of *shtn-1* expression. It should be noted that the levels of regulation are different in this proposed model. Netrin signaling leads to activation of Shtn-1 at the protein level by Pak-1 mediated post-translational phosphorylation. We show that PlxnA2 signaling represses *shtn-1* at the transcriptional

level. We believe that this also allows for a degree of temporal regulation. Netrin signaling leads to an immediate activation of Shtn-1, whereas PlxnA2 signaling could lead to a slower repression of *shtn-1* by limiting the pool of available Shtn-1 protein being transcribed. This mechanism of 'checks and balances' between Netrin/DCC positive regulation and Sema6/PlxnA2 negative regulation could be essential to maintain the narrow concentration range required for Shtn-1 function.

3.6. Supplemental Figures
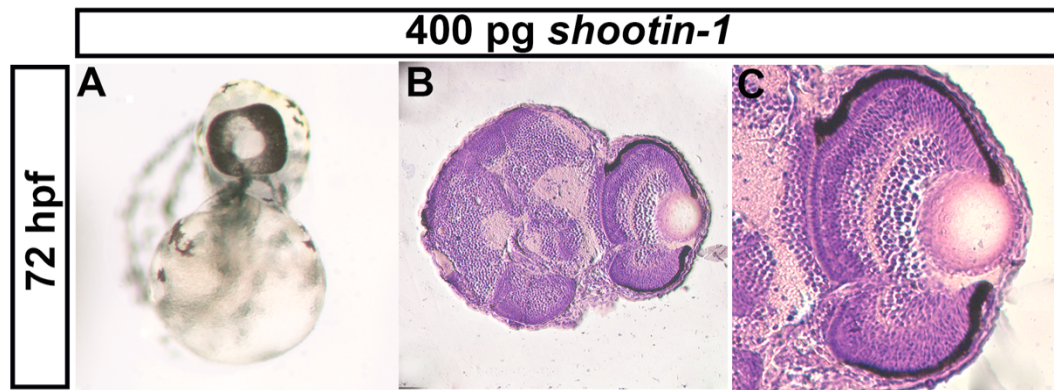
Figure 3.6. Supplemental Fig. 1. Overexpression of *shtn-1* can Induce Cyclopia. A) Brightfield image at 48 hpf. B&C (zoom) H&E stained sections of the retina of A.
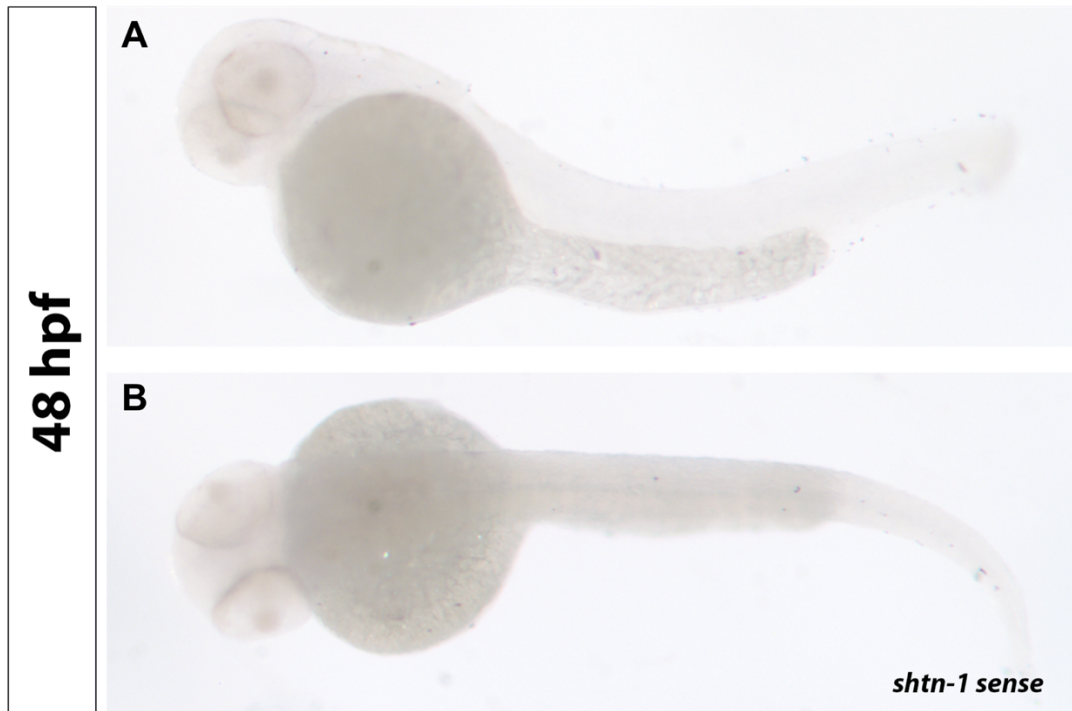
48 hpf

shtn-1 sense

Figure 3.7. Supplemental Fig 2. *Shtn-1* Sense Probe. Whole-mount *in situ* hybridization at 48 hpf. A. Lateral view. B. Dorsal view.

3.7. References

Baba, K., Yoshida, W., Toriyama, M., Shimada, T., Manning, C.F., Saito, M., Kohno, K., Trimmer, J.S., Watanabe, R., Inagaki, N., 2018. Gradient-reading and mechano-effector machinery for netrin-1-induced axon guidance. eLife 7.

Behar, O., Golden, J.A., Mashimo, H., Schoen, F.J., Fishman, M.C., 1996. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. Nature 383, 525-528.

Chen, H., Chedotal, A., He, Z., Goodman, C.S., Tessier-Lavigne, M., 1997. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron 19, 547-559.

Comeau, M.R., Johnson, R., DuBose, R.F., Petersen, M., Gearing, P., VandenBos, T., Park, L., Farrah, T., Buller, R.M., Cohen, J.I., Strockbine, L.D., Rauch, C., Spriggs, M.K., 1998. A poxvirus-encoded semaphorin induces cytokine production from monocytes and binds to a novel cellular semaphorin receptor, VESPR. Immunity 8, 473-482.

Deiner, M.S., Kennedy, T.E., Fazeli, A., Serafini, T., Tessier-Lavigne, M., Sretavan, D.W., 1997. Netrin-1 and DCC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia. Neuron 19, 575-589.

Dent, E.W., Barnes, A.M., Tang, F., Kalil, K., 2004. Netrin-1 and semaphorin 3A promote or inhibit cortical axon branching, respectively, by reorganization of the cytoskeleton. The Journal of neuroscience : the official journal of the Society for Neuroscience 24, 3002-3012.

Ebert, A.M., Childs, S.J., Hehr, C.L., Cechmanek, P.B., McFarlane, S., 2014. Sema6a and Plxna2 mediate spatially regulated repulsion within the developing eye to promote eye vesicle cohesion. Development (Cambridge, England) 141, 2473-2482.

Ebert, A.M., Lamont, R.E., Childs, S.J., McFarlane, S., 2012. Neuronal expression of class 6 semaphorins in zebrafish. Gene expression patterns : GEP 12, 117-122.

Emerson, S.E., Light, S.E., Ebert, A.M., 2018. Neuronal expression patterns of the PlexinA family during zebrafish development. Gene expression patterns : GEP 27, 56-66.

Emerson, S.E., St Clair, R.M., Waldron, A.L., Bruno, S.R., Duong, A., Driscoll, H.E., Ballif, B.A., McFarlane, S., Ebert, A.M., 2017. Identification of target genes downstream of semaphorin6A/PlexinA2 signaling in zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 246, 539-549.

Epstein, J.A., Aghajanian, H., Singh, M.K., 2015. Semaphorin signaling in cardiovascular development. Cell metabolism 21, 163-173.

Higashiguchi, Y., Katsuta, K., Minegishi, T., Yonemura, S., Urasaki, A., Inagaki, N., 2016. Identification of a shootin1 isoform expressed in peripheral tissues. Cell and tissue research 366, 75-87.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 203, 253-310.

Kubo, Y., Baba, K., Toriyama, M., Minegishi, T., Sugiura, T., Kozawa, S., Ikeda, K., Inagaki, N., 2015. Shootin1-cortactin interaction mediates signal-force transduction for axon outgrowth. The Journal of cell biology 210, 663-676.

Luo, Y., Raible, D., Raper, J.A., 1993. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75, 217-227.

Minegishi, T., Uesugi, Y., Kaneko, N., Yoshida, W., Sawamoto, K., Inagaki, N., 2018. Shootin1b Mediates a Mechanical Clutch to Produce Force for Neuronal Migration. Cell reports 25, 624-639.e626.

Neufeld, G., Kessler, O., 2008. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. Nature reviews. Cancer 8, 632-645.

Perala, N., Jakobson, M., Ola, R., Fazzari, P., Penachioni, J.Y., Nymark, M., Tanninen, T., Immonen, T., Tamagnone, L., Sariola, H., 2011. Sema4C-Plexin B2 signalling modulates ureteric branching in developing kidney. Differentiation 81, 81-91.

Perälä, N., Sariola, H., Immonen, T., 2012. More than nervous: The emerging roles of plexins. Differentiation 83, 77-91.

Perron, M., Boy, S., Amato, M.A., Viczian, A., Koebernick, K., Pieler, T., Harris, W.A., 2003. A novel function for Hedgehog signalling in retinal pigment epithelium differentiation. Development (Cambridge, England) 130, 1565-1577.

Serini, G., Valdembri, D., Zanivan, S., Morterra, G., Burkhardt, C., Caccavari, F., Zammataro, L., Primo, L., Tamagnone, L., Logan, M., Tessier-Lavigne, M., Taniguchi, M., Puschel, A.W., Bussolino, F., 2003. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature 424, 391-397.

Shimada, T., Toriyama, M., Uemura, K., Kamiguchi, H., Sugiura, T., Watanabe, N., Inagaki, N., 2008. Shootin1 interacts with actin retrograde flow and L1-CAM to promote axon outgrowth. The Journal of cell biology 181, 817-829.

Stenkamp, D.L., Frey, R.A., Prabhudesai, S.N., Raymond, P.A., 2000. Function for Hedgehog Genes in Zebrafish Retinal Development. Developmental biology 220, 238-252.

Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G.I., Song, H., Chedotal, A., Winberg, M.L., Goodman, C.S., Poo, M., Tessier-Lavigne, M., Comoglio, P.M., 1999. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99, 71-80.

Toriyama, M., Kozawa, S., Sakumura, Y., Inagaki, N., 2013. Conversion of a signal into forces for axon outgrowth through Pak1-mediated shootin1 phosphorylation. Current biology : CB 23, 529-534.

Toriyama, M., Shimada, T., Kim, K.B., Mitsuba, M., Nomura, E., Katsuta, K., Sakumura, Y., Roepstorff, P., Inagaki, N., 2006. Shootin1: a protein involved in the organization of an asymmetric signal for neuronal polarization. 175, 147-157.

CHAPTER 4. NEURONAL EXPRESSION PATTERNS OF THE PLEXINA FAMILY DURING ZEBRAFISH DEVELOPMENT

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Abbreviations used:

CG- cranial ganglia

CMZ- cilliary marginal zone

DT- dorsal thalamus

FB- fin bud

Fbr- forebrain

GAP- GTPase activating protein

HB- hindbrain

HG- hatching gland

Hi- intermediate hypothalamus

Hpf- hours post fertilization

INL- inner nuclear layer

IPL- inner plexiform layer

L- lens

LF- lateral forebrain

lfb- lateral forebrain bundle

lTeO- lateral optic tectum

MO- medulla oblongata

NR- neural retina

OpV- optic vesicle

OV- otic vesicle

P- pons

Plxn- Plexin

Po- pre-optic region

PTd- dorsal part of posterior tuberculum

PTv- ventral part of posterior tuberculum

R- retina

RBD- Rho binding domain

RGC- retinal ganglion cell layer

RPC- retinal precursor cell

SC- spinal chord

Sema- Semaphorin

T- thalamus

TeO- optic tectum

V- ventricle

VEGFR2- vascular endothelial growth factor receptor 2

V3- 3rd ventricle

Highlights

- *plexinA* members family show unique expression patterns during zebrafish development

- *plexinAs* are expressed in distinct neuronal tissues in the developing brain and eye

- *plexinA1* has two genes, *A1a* and *A1b*, which show distinct expression patterns

4.1. Abstract

Plexins (Plxns) and Semaphorins (Semas) are key signaling molecules that regulate many aspects of development. Plxns are a family of transmembrane protein receptors that are activated upon extracellular binding by Semas. Activated Plxns trigger intracellular signaling cascades, which regulate a range of developmental processes, including axon guidance, neuronal positioning and vasculogenesis. Semas are a large family of both transmembrane and secreted signaling molecules, and show subtype specific binding to different Plxn family members. Each Plxn can play different roles in development, and so tightly regulated temporal and spatial expression of receptor subtypes is critical to ensure appropriate signaling. Here we elucidate the expression

profiles of the *plxnA* family, *plxnA1a, A1b, A2, A3 and A4* at 18, 24, 36, 48, 60 and 72 hours post fertilization in the developing zebrafish. We show that PlxnA family members are expressed in neuronal tissues during zebrafish development, but exhibit key differences in expression within these tissues. We also highlight that *plxnA1* has two genes in zebrafish, *A1a* and *A1b*, which show divergences in expression patterns during early development.

4.2. Introduction

Plexins (Plxns) and Semaphorins (Semas) are essential developmental signaling proteins. They were initially identified as axon guidance cues, mediating actin dynamics at developing axonal growth cones (Luo et al., 1993a). It is now appreciated that Plxns and Semas play additional roles in development across multiple systems, including vasculogenesis (Serini et al., 2003b), early eye development (Ebert et al., 2014; Emerson et al., 2017), immunity (Shi et al., 2000a) and bone development (Behar et al., 1996b), among others. Plxns are a large family of transmembrane protein receptors, which signal upon binding by specific members of the Sema family. There are 2 invertebrate Plxns (PlxnA and B) and 9 vertebrate Plxns (PlxnA1-A4, B1-3, C1 and D1) (Tamagnone et al., 1999). Due to a whole genome duplication event in zebrafish, many genes have two copies (Glasauer and Neuhauss, 2014). An in-depth search for all plxnA family members revealed that zebrafish have two *plxnA1* genes (*plxnA1a* and *plxnA1b*). There are 8 subclasses of Semas, Sema1 and 2 are invertebrate forms, 3-7 are vertebrate forms and V is virally expressed (Neufeld and Kessler, 2008). There are transmembrane (Sema classes 4, 5 & 6), GPI anchored (Class 7), and secreted Semas (Class 3 and V) (Kolodkin et al., 1993). Secreted Semas require additional neuropilin co-receptors in a complex with Plxns

in order to signal (He and Tessier-Lavigne, 1997; Janssen et al., 2012; Kolodkin et al., 1997).

All Plxns share a common 500 amino acid extracellular SEMA domain that mediates Sema and Plexin binding. The SEMA domain has an atypical 7-blade propeller structure, and binds to other SEMA domains in a head to head conformation (Love et al., 2003). The SEMA domain is highly conserved, yet varies subtly between the different family members (Koppel et al., 1997), leading to restricted combinations of possible Plxn/Sema complexes. Extracellular Plxn domains include 3 repeating PSI (plexin, semaphorin, integrin) domains and multiple IPT (Ig-like, plexins, transcription factor) domains (Bork et al., 1999). Inside the cell, Plxns have a characteristic split GTPase activating protein (GAP) domain (C1 and C2) (Rohm et al., 2000), with a Rho binding domain (RBD) in-between (Oinuma et al., 2004a). Once activated, folded GAP domains initiate multiple intracellular signaling cascades to ultimately regulate many cellular events, including but not limited to: cell migration via mediating cytoskeletal dynamics (Curreli et al., 2016; Deo et al., 2004; Rosslenbroich et al., 2005; Schmidt and Strittmatter, 2007; Serini et al., 2003b), integrin-mediated adhesion (Basile et al., 2007; Choi et al., 2014; Walzer et al., 2005), transcriptional regulation (Emerson et al., 2017), cell proliferation (Ebert et al., 2014; Emerson et al., 2017) and apoptosis (Bagnard et al., 2001; Gagliardini and Fankhauser, 1999; Wehner et al., 2016).

The same Sema classes can activate multiple different Plxn family members. In this paper, we focus on the PlxnA family, which can be activated by both class 3 and class 6 Semas (Renaud et al., 2008; Suto et al., 2005; Suto et al., 2007). Despite the high level of cross talk within the PlxnA family, each member has distinct roles in vertebrate development. PlxnA1 plays roles in axon guidance for retinal axons crossing the optic

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chiasm midline with Sema6D and Nr-CAM (Kuwajima et al., 2012). Additionally, PlxnA1 with Sema6D controls endothelial cell migration during heart development in combination with VEGFR2 (Toyofuku et al., 2004). PlxnA2/Sema6A signaling has been shown to be important for axon migration in the cerebellum (Renaud and Chedotal, 2014; Renaud et al., 2008), guidance of the corticospinal tract (Rünker et al., 2008), patterning of the retina (Sun et al., 2013b) and the maintenance of cohesion and proliferation of retinal precursor cells (Ebert et al., 2014; Emerson et al., 2017), among others. PlxnA3 (also referred to as *sidetracked* in zebrafish) is important for the guidance of pioneer intraspinal motor neurons as they exit the spinal cord (Palaisa and Granato, 2007), facial branchiomotor nerve axon guidance in conjunction with PlxnA4 (Schwarz et al., 2008), and pruning of hippocampal axon branches during brain development (Bagri et al., 2003). PlxnA4 is important for axon guidance during development. It guides mossy fibers of the hippocampus, (Suto et al., 2007) and both sympathetic and sensory neurons in dorsal root ganglia (Haklai-Topper et al., 2010a). Furthermore, in retinal development, PlxnA4 is important for guiding axons to their correct sub-laminar targets to correctly pattern the inner plexiform layer (Matsuoka et al., 2011b).

It is clear that PlxnA/Sema signaling is essential in development, and although some of the functions of the PlxnA family members are known, limited data exists for their expression. Here we elucidate the specific expression patterns of each PlxnA across the early developmental stages of the zebrafish. We show that there are distinct temporal and spatial differences in expression patterns of each family member, including the two novel *plxnA1* paralogs in zebrafish, *A1a* and *A1b*. This data will enable a platform from which the developmental roles of specific plxnA genes can be determined.

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4.3. Results

4.3.1. PlxnA Family Phylogenetic Tree

A neighbor–joining consensus phylogenetic tree of full-length amino acid sequences was generated in Geneious R10 (Kearse et al., 2012). Analysis shows that each PlxnA family member clusters in the same clade as expected across species (Fig. 4.1). Zebrafish (*D. rerio*) have two *plxnA1* genes, *plxnA1a* and *A1b,* due to a whole genome duplication (Glasauer and Neuhauss, 2014), resulting in two protein products. These group with orthologous PlxnA1s of other species. Protein alignments for the SEMA and Ras-GAP domains of the plxnA family show that although family members are overall well conserved, the majority of differences occur in the SEMA binding domains (Supplemental Fig.4.8).
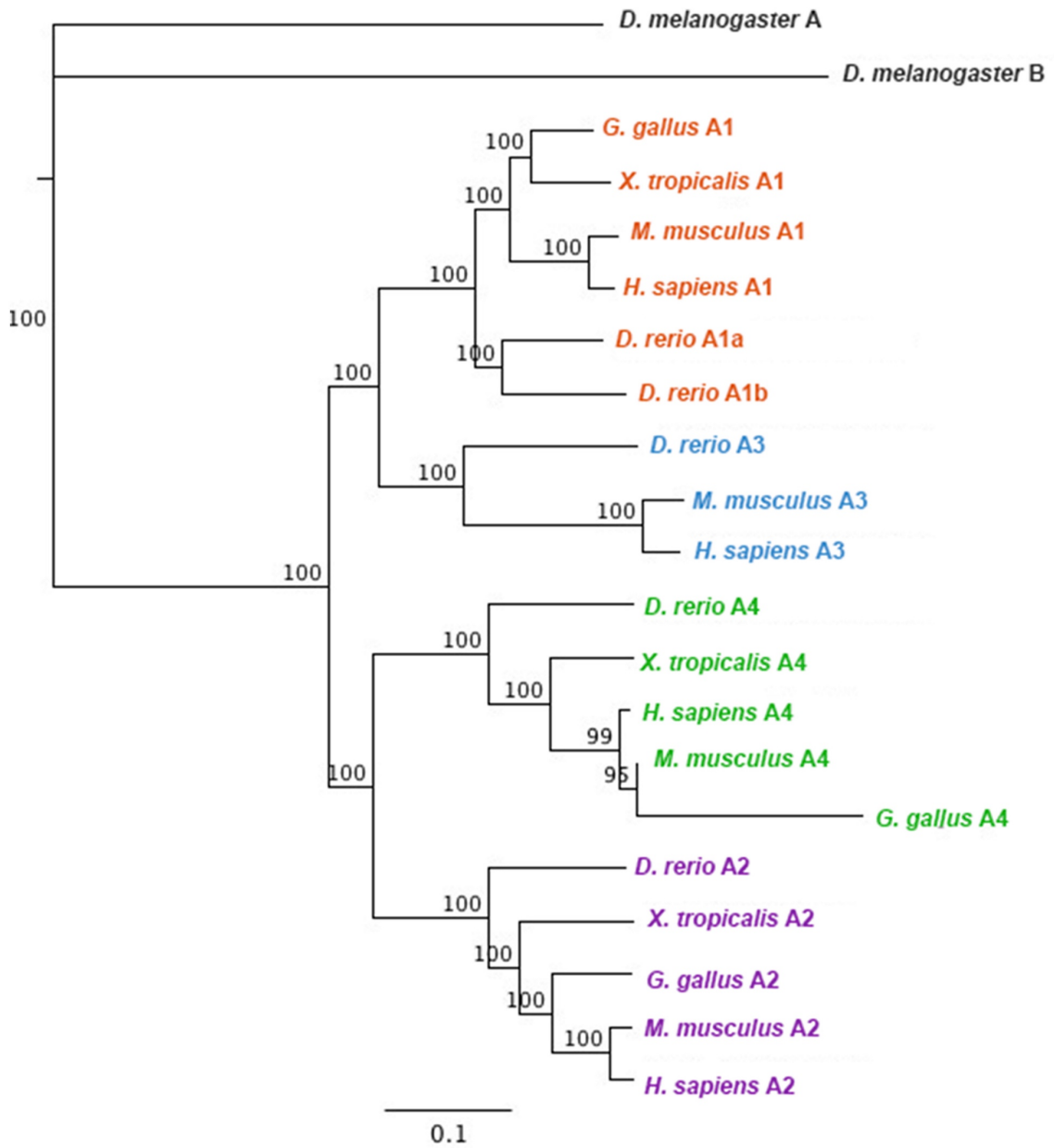
Figure 4.1. Phylogeny of PlxnA Family Members. Phylogenetic tree displaying the relationships of PlxnA family member full-length amino acid sequences. Numbers indicate maximum likelihood bootstrap values.

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4.3.2. *plxnA1a* Expression

plxnA1a is expressed in the undifferentiated retinal precursor cells of the optic vesicles (OpV), and the forebrain (FBr) at 18 hpf (Figs. 4.2A-A''). At 24 hpf, staining is seen adjacent to the lens in the neural retina (NR) and in the forebrain ventricle (V) (Figs. 4.2 B-B''). *plxnA1a* is expressed in the optic tectum (TeO) at 36 and 48 hpf (Figs. 4.2C''', 4.2D'', 4.2D'''). In the developing eye, *plxnA1a* is expressed in the retinal ganglion cell layer (RCG) and the inner plexiform layer (IPL) of the retina at 60 and 72 hpf (Figs. 4.2E'', 4.2 F''). Staining is seen in the lateral forebrain at 60hpf and around the optic commissure (oc) at 72 hpf (Fig. 4.2F''). *plxnA1a* is expressed in the otic vesicle (OV) of the developing ears during all stages of development shown (Figs. 4.2 C''''-F'''').

Figure 4.2. *plxnA1a* **Expression.** Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A-F) Whole-mount lateral, (A'-F') whole-mount dorsal. Brightfield sections (A''-F'') forebrain, (A'''-F''') midbrain and (C''''-F'''') hindbrain. Embryos were imaged at different developmental time points. (A-A''') 18 hpf, (B-B''') 24 hpf, (C-C'''') 36 hpf, (D-D'''') 48 hpf, (E-E'''') 60 hpf, and (F-F'''') 72 hpf. Lines in (A-F) indicate locations of the sections shown at that time-point. Inset shows sense probe control. Hpf-hours post fertilization, OpV- optic vesicles, FBr-forebrain, V-ventricle, NR-neural retina, L-lens, TeO- optic tectum, OV- otic vesicle, CMZ-cilliary marginal zone, lTeO- lateral optic tectum, MO- medulla oblongata, IPL-inner plexiform layer, RGC- retinal ganglion cell layer, FB- fin bud.

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4.3.3. *plxnA1b* Expression

plxnA1b is expressed in the retinal precursor cells of the optic vesicles (OpV) and the forebrain (FBr) at 18 hpf (Figs. 4.3A-A''). Staining persists in the neural retina (NR) close to the developing lens at 24 hours post fertilization (hpf) (Figs. 4.3B-B''). *plxnA1b* is expressed in the inner plexiform layer (IPL) at 60 hpf (Fig. 4.3E'') and retinal ganglion cell layer (RGC) at 72 hpf (Fig. 4.3F''). As the forebrain develops, *plxnA1b* is expressed first dorsally at 36 hpf (Fig. 4.3C'') and can be distinctly seen in the lateral forebrain bundle (lfb) by 48 hpf (Fig. 4.3D''). At 60 hpf, staining can be seen more ventrally around the pre-optic region (Po) (Fig. 4.3E''). Additionally, staining is seen in the lateral optic tectum (lTeO) at 60 and 72 hpf (Figs. 4.3E'', F''-F'''). During development *plxnA1b* is distinctly expressed in the developing ear and in the otic vesicles (OV) (Figs. 4.3B'', 4.3C''''-F''''). At 36 and 48 hpf *plxnA1b* is expressed in the hypothalamus (H) (Figs. 4.3C''', D''') and in the medulla (MO) at 60 hpf (Fig. 4.3E'''). The cranial ganglia (CG) also express *plxnA1b* at 60 and 72 hpf (Figs. 4.3E, E', F, F').

Figure 4.3. *plxnA1b* **Expression.** Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A-F) Whole-mount lateral, (A'-F') whole-mount dorsal. Brightfield sections (A''-F'') forebrain, (A'''-F''') midbrain and (C''''-F'''') hindbrain. Embryos were imaged at different developmental time points. (A-A''') 18 hpf, (B-B''') 24 hpf, (C-C'''') 36 hpf, (D-D'''') 48 hpf, (E-E'''') 60 hpf, and (F-F'''') 72 hpf. Lines in (A-F) indicate locations of the sections shown at that time-point. Inset shows sense probe control. Hpf-hours post fertilization, OpV- optic vesicle, Fbr, forebrain, SC-spinal cord, NR-neural retina, V- ventricle, HB- hindbrain, OV- otic vesicle, L-lens, Hi-intermediate hypothalamus, lfb-lateral forebrain bundle, Po-pre-optic region, T-thalamus, lTeO- lateral optic tectum, MO- medulla oblongata, RGC- retinal ganglion cell layer, IPL-inner plexiform layer, CG-cranial ganglia.

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4.3.4. *plxnA1a* and *plxnA1b* Comparisons

At 18 somites and 24 hpf, *plxnA1a* and *plxnA1b* are expressed in similar areas. They both show staining throughout the developing optic vesicles (OpV) and developing forebrain (FBr) at 18 somites (Figs. 4.2A'', 4.2A''', 4.3A'' and 4.3A'''), in addition to the ventricle (V) and neural retina (NR) at 24 hpf (Figs. 4.2B'', 4.2B''', 4.3B'' and 4.3B'''). However, *plxnA1a* and *plxnA1b* differ in expression patterns by 36 hpf. *plxnA1a* is expressed in the optic tectum (TeO), whereas *plxnA1b* is seen in the intermediate hypothalamus (Hi) (Figs. 4.2C''', 4.3C'''). At 48 hpf, *plxnA1a* and *plxnA1b* are both expressed in the developing lateral forebrain (lfb) (Figs. 4.2D'' and 4.3D''), although *plxnA1a* staining is more diffuse and can be seen more ventrally than *plxnA1b*, expanding into the ventral thalamus (T). At 60 hpf, *plxnA1a* and *plxnA1b* are both expressed in similar areas throughout the developing forebrain and midbrain (Figs. 4.2E'', 4.3E''), however they differ greatly in the developing ventral hindbrain where *plxnA1a* is ubiquitously expressed, and *plxnA1b* is absent (Figs. 4.2E'''', 4.3E''''). Both genes are expressed in the developing otic vesicles (OV) throughout the stages of development shown (Figs. 4.2C''''- 4.2F'''' and 4.3C''''- 4.3F''''). *PlxnA1a* is strongly expressed in the fin buds, especially at 60hpf (Fig. 4.2E'), whereas *plxnA1b* only shows very faint staining, if any, at all developmental stages shown (Figs. 4.3C' - F').

4.3.5. *plxnA2* Expression

plxnA2 is expressed in the ventral optic vesicles (OpV) and the neighboring mesenchyme at 18 hpf (Figs. 4.4A-A''), but is not strongly expressed at 24 hpf (Figs. 4.4B-B'''). Later in development, *plxnA2* is again expressed in the developing eye. It is expressed markedly in the cilliary marginal zone (CMZ) at 36 hpf (Fig. 4.4C'') and in the

retinal ganglion cell layer (RGC) and inner nuclear layer (INL) from 48 hpf to 72 hpf (Figs. 4.4D''-F''). Staining can also be seen in the optic chiasm (oc) and optic tectum (TeO/lTeO) at 72 hpf (Figs. 4.4F''-F'''). At 36 hpf, staining can be seen in distinct nuclei of the thalamus (Fig. 4.4C''), moving ventrally as development proceeds (Figs. 4.4D''-F''). By 36 hpf, *plxnA2* is expressed in the intermediate hypothalamus (Hi) and the medulla (MO) (Figs. 4.4C''', D''', F''').
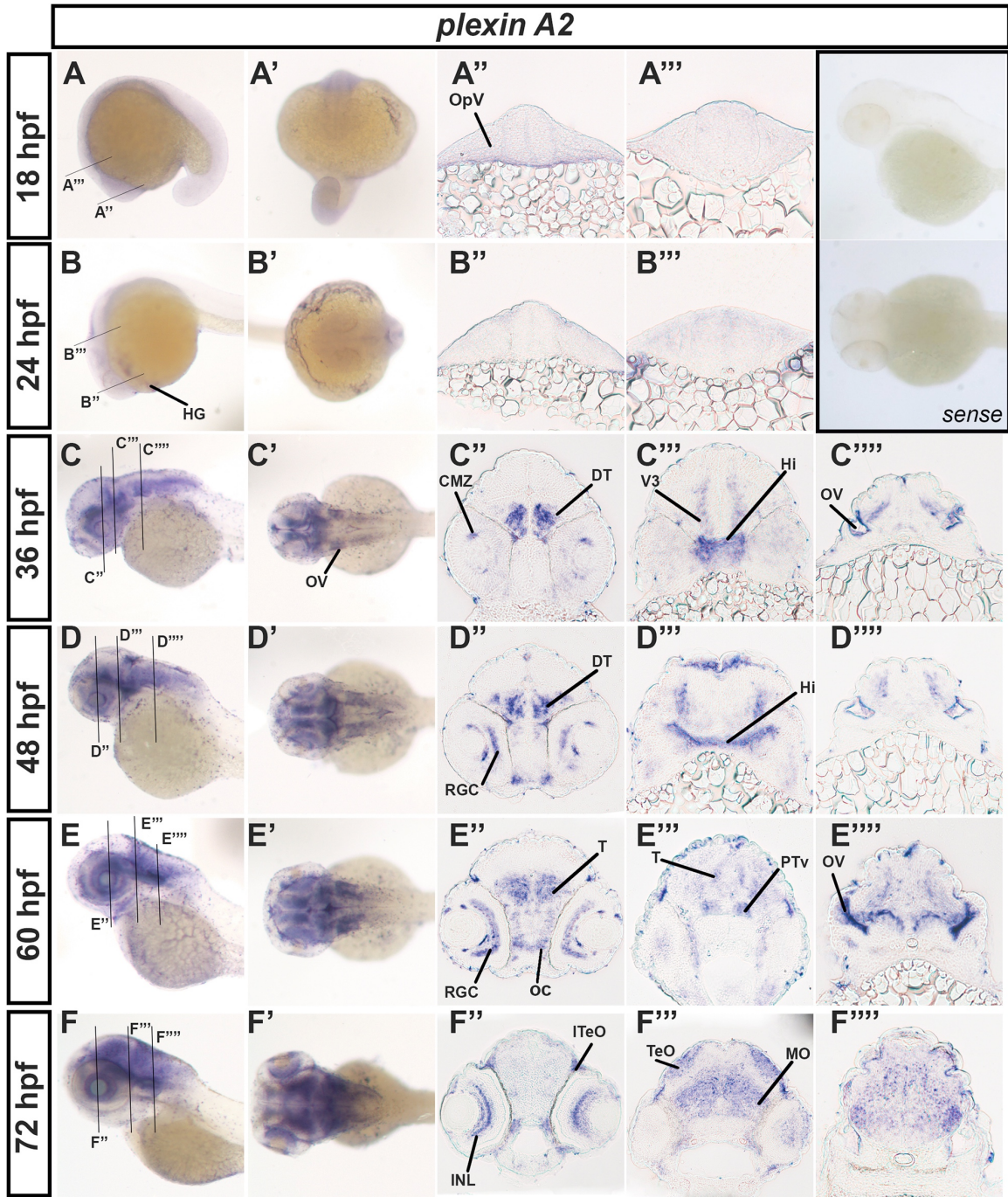
Figure 4.4. *plxnA2* **Expression.** Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A-F) Whole-mount lateral, (A'-F') whole-mount dorsal. Brightfield sections (A''-F'') forebrain, (A'''-F''') midbrain and (C''''-F'''') hindbrain. Embryos were imaged at different developmental time points. (A-A''') 18 hpf, (B-B''') 24 hpf, (C-C'''') 36 hpf, (D-D'''') 48 hpf, (E-E'''') 60 hpf, and (F-F'''') 72 hpf. Lines in (A-F) indicate locations of the sections shown at that time-point. Inset shows sense probe control. Hpf-hours post fertilization, OpV- optic vesicle, HG-hatching gland, CMZ-cilliary marginal zone, DT-dorsal thalamus, V3- 3rd ventricle, Hi-intermediate hypothalamus, OV-otic vesicle, oc-optic chiasm, RGC- retinal ganglion cell layer, INL-inner nuclear layer, T-thalamus, PTv-ventral part of posterior tuberculum, lTeO- lateral optic tectum, MO- medulla oblongata.

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4.3.6. *plxnA3* Expression

plxnA3 is not expressed in the developing zebrafish nervous system until 36 hpf (Figs. 4.5A-B'''). At 36 hpf, *plxnA3* is expressed in the early neural retina (NR) (Fig. 4.5C'') and persists to 72 hpf in the retina adjacent to the lens (R) (Figs. 4.5D''-F''). Expression is also seen in the developing optic system in the optic tectum (TeO) at 48 and 60 hpf (Figs. 4.5D''', E'''), but is absent by 72 hpf (Fig. 4.5F'''). Furthermore, *plxnA3* is expressed throughout the developing lateral forebrain (LF), dorsal thalamus (DT), and the ventral tuberculum (PTv) from 36 hpf onwards (Figs. 4.5C''-F''). Staining can be seen in the medulla at 60 and 72 hpf (Figs. 4.5E'''', F''', F''''), and the developing ear from 36 to 72 hpf (Figs. 4.5C'''''-F''''). Prolonged staining shows expression in motor neurons along the tail, and in the cranial ganglia (Supplemental Fig. 4.9).
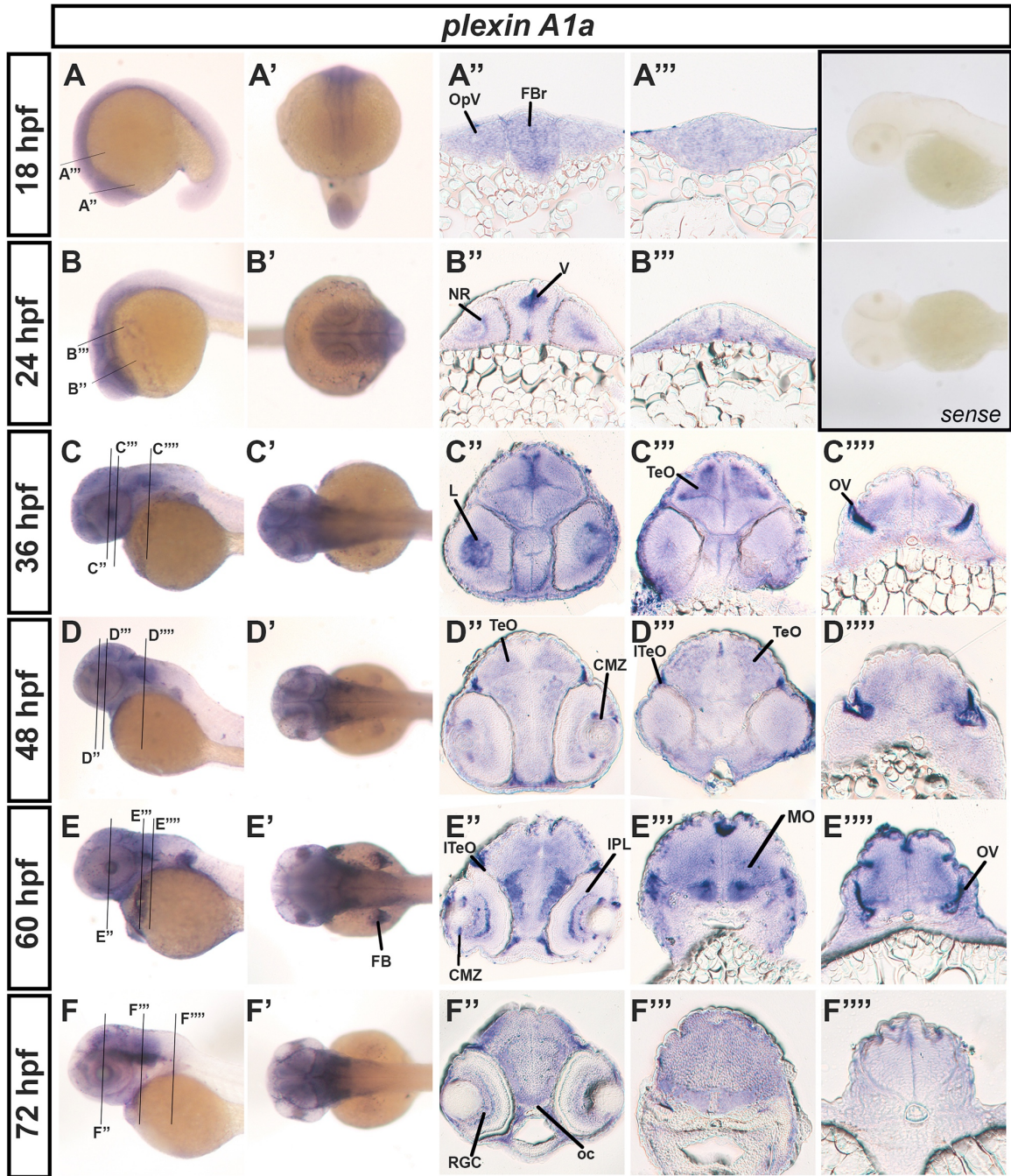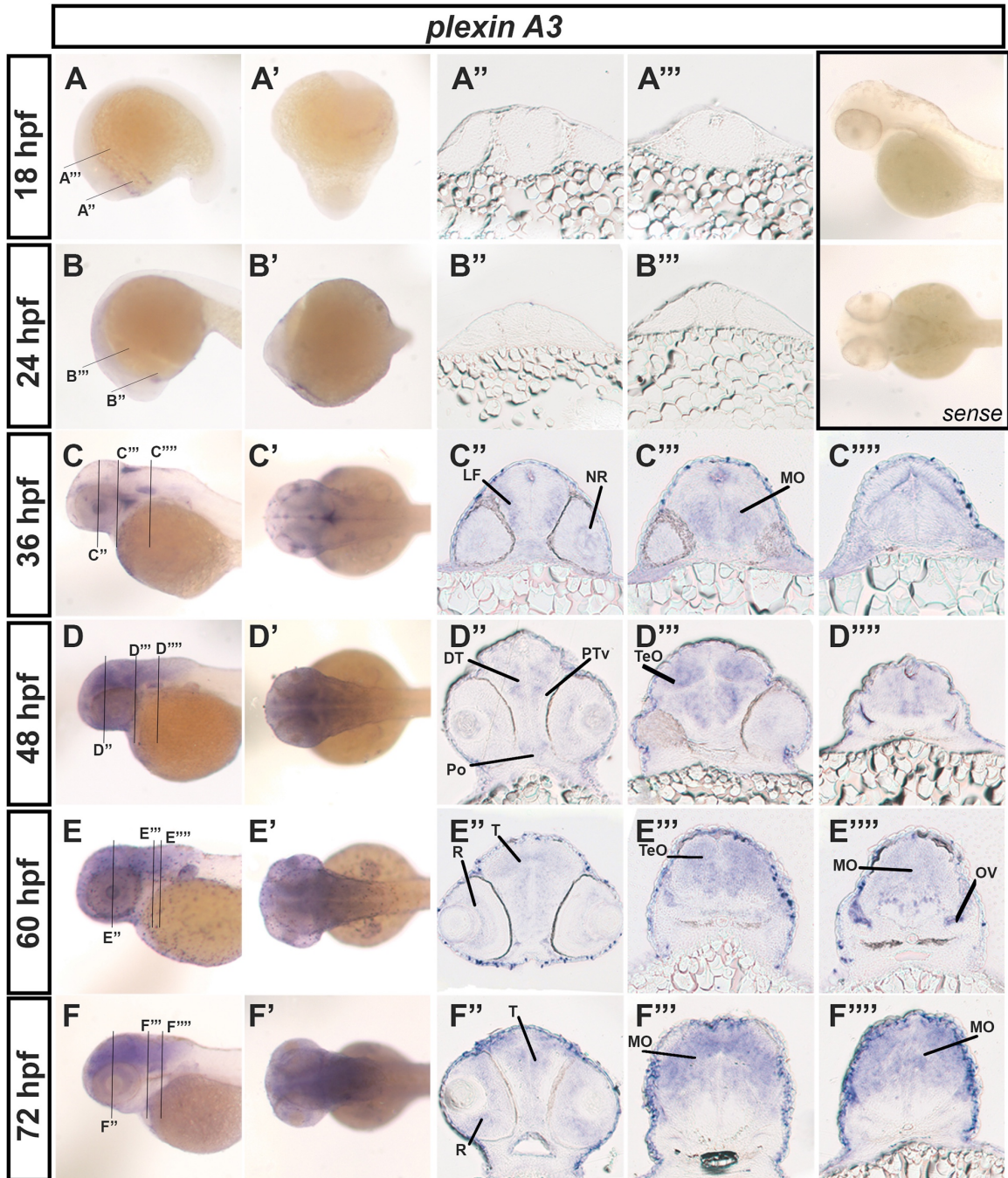
Figure 4.5. *plxnA3* **Expression**. Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A-F) Whole-mount lateral, (A'-F') whole-mount dorsal. Brightfield sections (A''-F'') forebrain, (A'''-F''') midbrain and (C''''-F'''') hindbrain. Embryos were imaged at different developmental time points. (A-A''') 18 hpf, (B-B''') 24 hpf, (C-C'''') 36 hpf, (D-D'''') 48 hpf, (E-E'''') 60 hpf, and (F-F'''') 72 hpf. Lines in (A-F) indicate locations of the sections shown at that time-point. Inset shows sense probe control. Hpf-hours post fertilization, LF- lateral forebrain, NR-neural retina, MO- medulla oblongata, DT-dorsal thalamus, PTv-ventral part of posterior tuberculum, Po-pre-optic region, TeO- optic tectum, T-thalamus, OV- otic vesicle, R-retina.

4.3.7. *plxnA4* Expression

At 18s *plxnA4* is expressed throughout the developing eye field in the optic vesicles (OpV) (Fig. 4.6A''). *plxnA4* has very distinct staining in the lateral forebrain (lfb) from 36 hpf (Fig. 4.6C''), spreading dorso-ventrally throughout the thalamus and the tuberculum (T/PTd/PTd) towards the pre-optic region (Po) at 48 hpf (Fig. 4.6D''), and is most ventrally in the optic chiasm (oc) at 60 and 72 hpf (Figs. 4.6E''-F''). *plxnA4* is expressed in the developing visual system from 24 hpf in the retinal precursor cells of the neural retina (NR) (Fig. 4.6B''), the lens (L) at 36 hpf (Fig. 4.6C'') and the inner plexiform layer (IPL) at 60 hpf (Fig. 4.6E''). *plxnA4* is strongly expressed in the hindbrain dorsal to the otic vesicles (OV) at 48 hpf (Fig. 4.6D''''), in the medulla (MO) (Fig. 4.6E''') and in the otic ganglia (OG) at 60 hpf (Fig. 4.6 E''''). *plxnA4* is localized to the otic vesicles (OV) during the stages of early development shown (Figs. 4.6A''', B''', C''''-F'''').
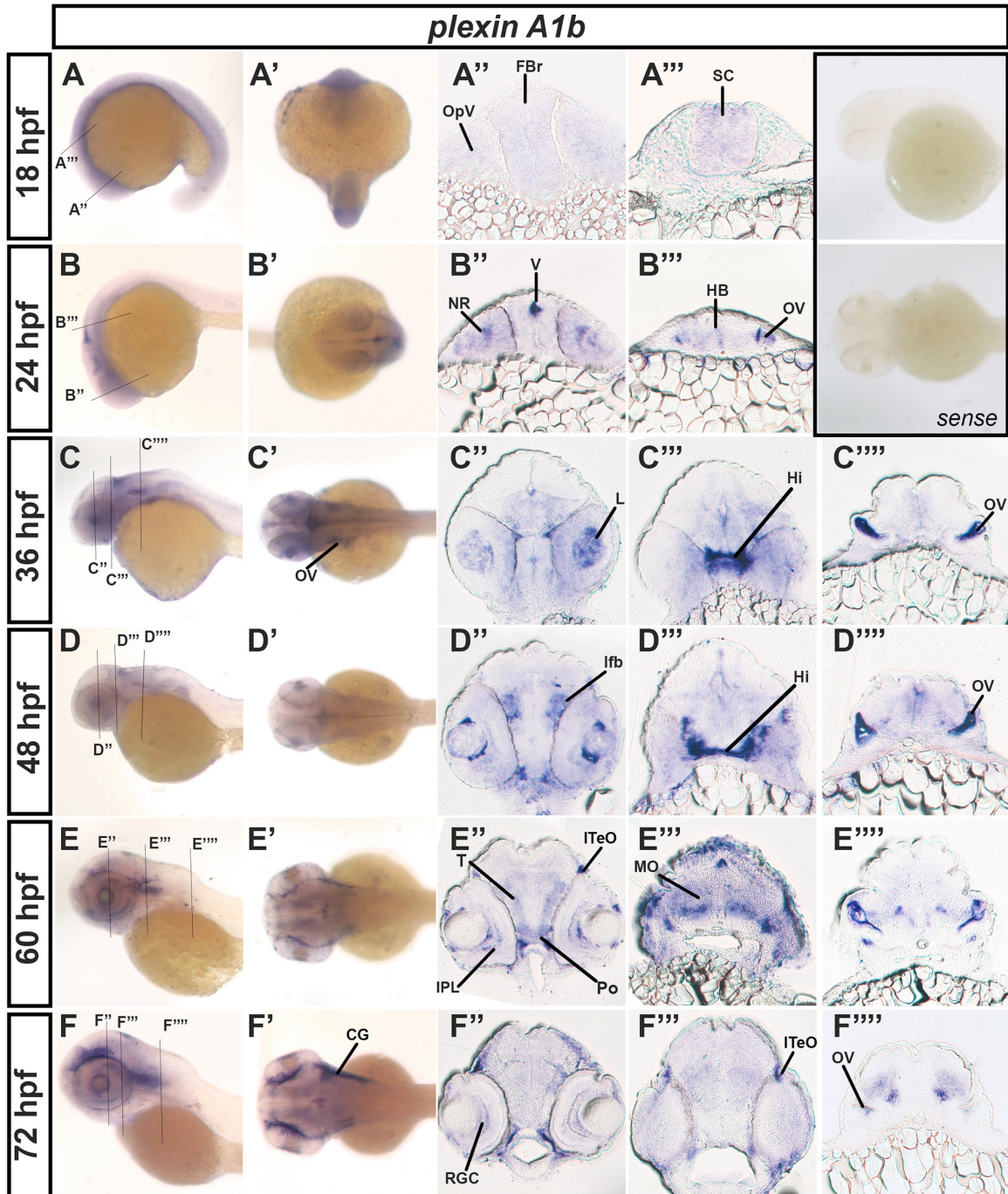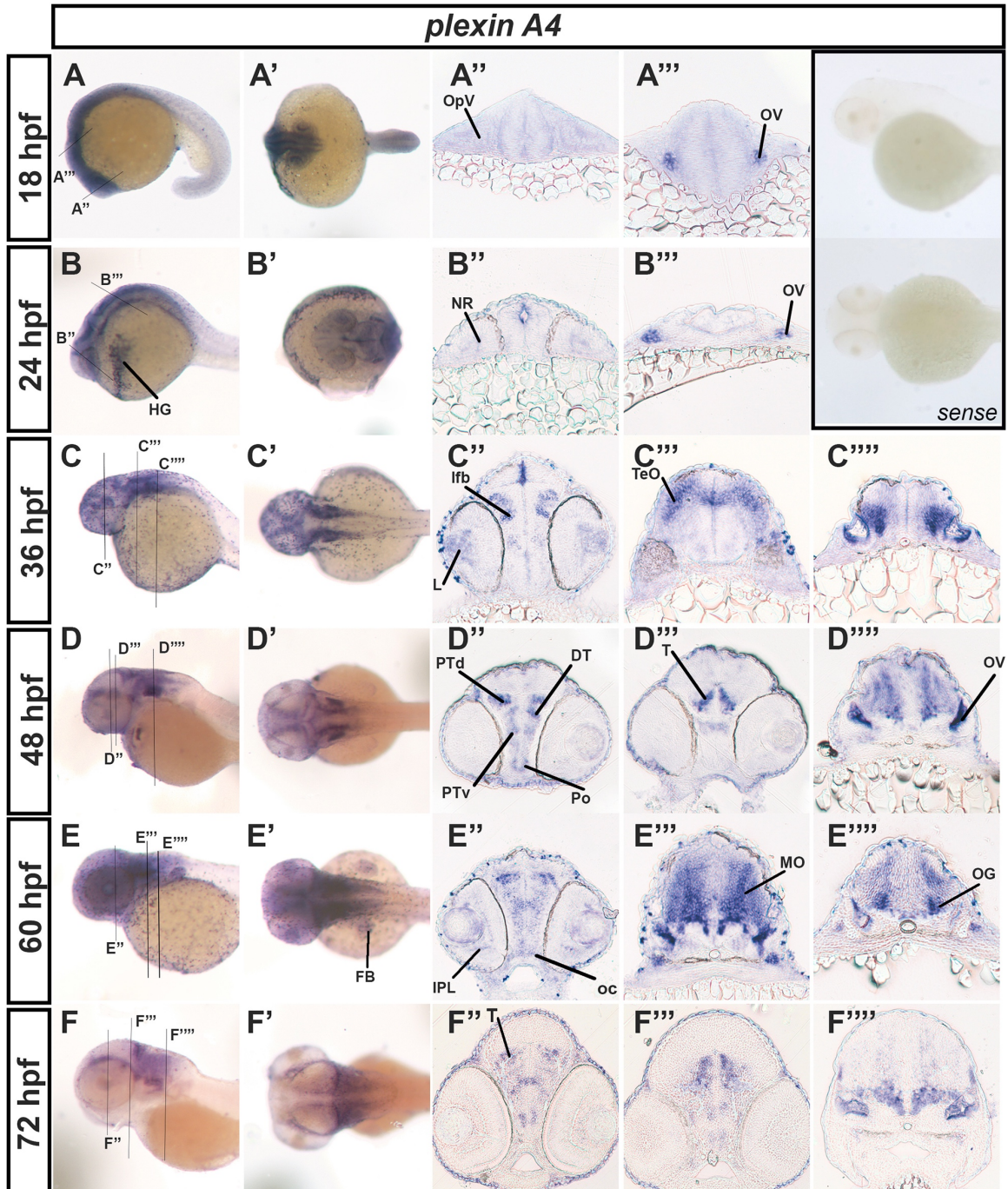
Figure 4.6. *plxnA4* **Expression.** Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A-F) Whole-mount lateral, (A'-F') whole-mount dorsal. Brightfield sections (A''-F'') forebrain, (A'''-F''') midbrain and (C''''-F'''') hindbrain. Embryos were imaged at different developmental time points. (A-A''') 18 hpf, (B-B''') 24 hpf, (C-C'''') 36 hpf, (D-D'''') 48 hpf, (E-E'''') 60 hpf, and (F-F'''') 72 hpf. Lines in (A-F) indicate locations of the sections shown at that time-point. Inset shows sense probe control. Hpf-hours post fertilization, OV- otic vesicle, NR-neural retina, L-lens, lfb-lateral forebrain bundle, TeO- optic tectum, PTd-dorsal part of posterior tuberculum, IPL-inner plexiform layer, T-thalamus, DT-dorsal thalamus, PTv-ventral part of posterior tuberculum, Po-pre-optic region, and MO- medulla oblongata, oc-optic chiasm, OG-otic ganglion, FB- fin bud, HG-hatching gland.

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	plxnA1a	*plxnA1b*	*plxnA2*	*plxnA3*	*plxnA4*
Optic vesicle	■	■	■		■
Early neural retina	■	■		■	■
Retinal ganglion cell layer	■	■	■		
Cilliary marginal zone	■		■		
Inner plexiform layer	■	■			■
Inner nuclear layer			■		
Otic vesicles	■	■	■	■	■
Hypothalamus		■	■		
Optic tectum	■		■	■	■
Lateral optic tectum	■	■	■		
Thalamus	■	■	■	■	■
Optic chiasm			■		■
Medulla oblongata	■	■	■	■	■

Figure 4.7. Expression Patterns of *plxnA1a, A1b, A2, A3* and *A4* in Specific Neuronal Tissues in Zebrafish. Purple boxes indicate positive expression of the corresponding gene at any time-point between 18 hpf and 72 hpf in the developing zebrafish.

4.4. Discussion

Our results show that the PlxnA family have some overlap in expression, but also have distinct differences, as summarized in (Fig. 4.7). It is not surprising that our results show areas of co-expression, as it is well documented that different Plxns can regulate each other. Sema6A can activate both PlxnA2 and A4, which often share spatial and temporal expression patterns (Haklai-Topper et al., 2010a; Renaud et al., 2008; Suto et al., 2005; Suto et al., 2007). PlxnA2 and A4 have been shown to work together to guide migrating axons to their correct targets, via *cis* inhibition with Sema6A (Haklai-Topper et al., 2010a; Suto et al., 2007).

Comparing the expression patterns of the PlxnAs to their ligands further supports our findings. As mentioned earlier, Sema3 and Sema6 are ligands for the PlxnA family, and here we are able to confirm that their expression domains overlap in certain areas during development. For example in the retina, *sema6A* and *plxnA2* are expressed in overlapping and neighboring neuronal layers at 72 hpf, *sema6A* in the RGC layer, and *plxnA2* in the RGC layer and the INL (Figs. 4.4E''-F''), as previously observed (Ebert et al., 2014). *sema3Aa, 3Fa, 3Fb, 3Gb, 6A* and *6D* are expressed in the optic tectum at 50 hpf onwards (Callander et al., 2007; Ebert et al., 2012) which correlates with the expression of *plxnA1a, A2, A3* and *A4* later in development (Figs. 4.2 & 4.4-4.6). Additionally, *Sema3A, 3C 3Fa, 3Fb, 3Ga, 3Gb, 6Ba* and *6D* are expressed in the dorso-lateral thalamus at 40-50 hpf (Callander et al., 2007; Ebert et al., 2012), matching the spatial and temporal expression of the *plxnAs* (Figs. 4.2-4.6). It is known that *sema3Aa* and *3E* are expressed in the optic chiasm, and that *3Fa* and *3GB* are expressed dorsally to the optic chiasm, forming a repulsive border to migrating axons, guiding RGC axons as they cross the midline (Callander et al., 2007). We show that *plxnAs* are also expressed in

this area, *plxnA2 and A4* in the optic chiasm (Fig. 4.4 and 4.6), and *plxnA1a and A1b* in neighboring tissues (Figs. 4.2 and 4.3). Of the limited expression data in zebrafish, our findings corroborate published results. As mentioned, *plxnA2* is expressed in the ventral optic vesicles at 15 hpf, and the RGC layer and INL in zebrafish at 72 hpf (Ebert et al., 2014), matching our results (Figs. 4.4A'', D''-F''). We also show that *plxnA4* is expressed in the same neuronal areas in whole-mount at 48 hpf (Fig. 4.6D), as in (Christie et al., 2006). *plxnA3* expression in 24-36 hpf zebrafish has been previously investigated, and expression is seen in developing motor neurons and in the cranial ganglia (Tanaka et al., 2007). We show similar staining patterns at the same ages (Supplemental Fig. 4.9). In order to observe distinct staining in specific brain regions, staining was stopped before the heads became too dark, this resulted in the staining in the tail to be faint, suggesting that *plxnA3* expression levels in the developing trunk are significantly lower compared to the dark staining observed in the brain, eye and ear. Staining was therefore prolonged to show that our probes do indeed show similar patterns to previously published results in the tail. (Supplemental Fig. 4.9). The expression of the *plxnA* family has been previously investigated in mice (Murakami et al., 2001; Perälä et al., 2005) and although precise timing is hard to compare, we see consistencies in neuronal expression patterns between these two species. For example, *PlxnA1-3* are expressed in the developing mouse hypothalamus (Murakami et al., 2001), and we show similar staining in that area in zebrafish for *plxnA1b* and *A2* (Figs. 4.3C''', 4.3D''', 4. 4C''' and 4.4D'''). In the developing mouse retina, *A1* and *A3* are expressed in the RGC layer, and *A2* is expressed in the RGC and INL layers (Murakami et al., 2001), matching the expression patterns seen in zebrafish (Figs. 4.2F'', 4.3F'', 4.4D''-4.4F', 4.3E'' and

4.3F''). As a key model system for development, our work in zebrafish provides a much-needed data set for future studies in the field.

Our work is the first to document a distinction between the two *plxnA1* homologs *plxnA1a* and *A1b* in zebrafish. Due to whole genome duplication, many zebrafish genes have two transcripts. After a whole genome duplication event, copies of genes can either undergo neo-functionalization, sub-functionalization or a loss of function. Here we show that *plxnA1a* and *A1b* have some overlapping and some unique domains of expression during development, suggesting that the two genes may have evolved independent roles in development.

In this body of work, we comprehensively elucidate the spatial and temporal expression patterns of the *plxnA* family during zebrafish development. Each family member shows both overlapping and divergent expression domains in the developing nervous system, which correlates with the temporal and spatial patterns of their ligands, and expression patterns in mice. We have also uncovered novel expression patterns for two transcripts of *plxnA1*, *A1a* and *A1b*. Future work will address the functional relevance of the two *plxnA* transcripts, to determine if they display any level of redundancy or if they have evolved independent functions.

4.5. Experimental Procedures

4.5.1. Zebrafish Husbandry

TL embryos were raised at 28.5°C and developmentally staged as previously described (Fishman et al., 1997; Kimmel et al., 1995). Embryos were raised in egg water and pigmentation was blocked by addition of 0.003% phenylthiourea (PTU) at 24 hpf.

All procedures were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC), protocol number 15-031.

4.5.2. Phylogeny and Protein Alignments

Full-length *plxnA1a* and *A1b* nucleotide sequences were initially found by searching the zebrafish genome in the NCBI database. NCBI BLAST was used to establish that they had disparities in sequence homology. Geneious R10 software (http://www.geneious.com) (Kearse et al., 2012) was used to generate a neighbor-joining consensus tree based on alignments of the full-length amino acid sequences referenced in Table 4.1. 'Majority greedy clustering' was used, rooted to *D. melanogaster*. Numbers indicate bootstraps of percent consensus support. SEMA and Ras-GAP protein domain alignments were also generated using Geneious R10 software (http://www.geneious.com) (Kearse et al., 2012), after importing domain sequences identified using the NCBI Conserved Domain Finder tool(https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Table 4.1. Accession numbers for PlexinA family member amino acid sequences across different species, used for phylogenetic analysis. PlexinA family member denoted after species name.

Species	Accession number
D. melanogaster A	NP_524637
D. melanogaster B	NP_524616.2
G. gallus A1	XP_015148852.1
X. tropicalis A1	NP_001090760.1
M. musculus A1	AA138024.1
H. sapiens A1	NP_115618.3
D. rerio A1a	XP_003201265.4
D. rerio A1b	NP_001103480.1
D. rerio A3	NP_001091959.1
M. musculus A3	NP_032909.2
H. sapiens A3	NP_059984.3
D. rerio A4	XP_005164747.1
X. tropicalis A4	XP_002931894.2
H. sapiens A4	NP_065962
M. musculus A4	NP_786926.2
G. gallus A4	XP_015147312.1
D. rerio A2	XP_689780.5
X. tropicalis A2	AA161575.1
G. gallus A2	XP_015154528.1
M. musculus A2	AAH68155.1
H. sapiens A2	EAW93457.1

4.5.3. Whole-mount *in situ* Hybridization

Zebrafish embryos were developmentally staged at 18, 24, 36, 48, 60 or 72 hpf, fixed in 4% paraformaldehyde overnight at 4ºC, and stored at -20ºC in 100% methanol until use. *In situ* hybridization was performed as described previously (Thisse and Thisse, 2008). Digoxygenin (DIG)-labeled antisense and sense (control) RNA probes were generated using primers listed in Table 4.2 (IDT Coralville, IA). A second probe was generated for *plxnA3* to confirm previously published expression data in the tail (Tanaka et al., 2007) (Supplemental Fig. 4.9). PCR products were sequenced at the UVM Cancer Center Advanced Genome Technologies Core, Burlington, VT prior to probe generation.

Table 4.2. Primers and Accession Numbers for PlexinA Family Gene-specific Antisense Probe Generation.

Name	Forward primer	Reverse primer	Accession #
plxna1a	GCAGCTGGATGAACCCCTGC	TGTAATACGACTCACTATAGGGGCTGATTGTGAGCAAGATCC	XM_003201217.4
plxna1b	CTCAGCCGGAAAACACATGG	TGTAATACGACTCACTATAGGGGAACTTCACCTCCGGGTTTC	NM_001110010.1
plxna2	ATGTGATACAA1qGGAGCCGAGG	AGAGTCAGAAGGCTGTCGGA	XM_684688.7
plxna3	ACCCGACCTTTGAACCTCTT	TGTCATTCGTCAGCTCTTGG	NM_001098489.1
plxna3 *(2⁰ target)*	ATTTAGGTGACACTATAGCACCGAGAGTCCAGGAGAAG	TAATACGACTCACTATAGGGTTGCAAGAACTGCT	NM_001098489.1
plxna4	ATTTAGGTGACACTATAGGGAGACAAACCCTGTGCATT	TAATACGACTCACTATAGGGTTCTCCACCTGCTCCTGTCT	XM_005164690.3

4.5.4. Embedding, Sectioning, Imaging and Annotation

Post *in situ* hybridization, embryos were oriented in 4% methyl cellulose and imaged using a Nikon SMZ800 dissecting light microscope at 5X magnification. Embryos were dehydrated in 100% ETOH overnight prior to embedding using a JB4 Embedding Kit (Polysciences, Inc., Warrington, PA). Embedded embryos were sectioned on a Leica RM2265 microtome at 20μm and mounted on slides for imaging. Sections were imaged on an Olympus iX71 inverted light microscope at 20X magnification. Figures were cropped, adjusted for brightness and contrast and assembled using Adobe Photoshop CS6. Anatomical annotations were informed with the use of the zebrafish developmental atlases (Mueller, 2005) and (Bryson-Richardson et al., 2012).

Author conflict of interest: none.

A

B

C

	A1a SEMA	A1b SEMA	A2 SEMA	A3 SEMA	A4 SEMA		A1a Ras-GAP	A1b Ras-GAP	A2 Ras-GAP	A3 Ras-GAP	A4 Ras-GAP
A1a SEMA		85.2%	54.0%	66.6%	54.4%	A1a Ras-GAP		94.4%	84.1%	85.9%	81.6%
A1b SEMA	85.2%		53.5%	66.7%	54.2%	A1b Ras-GAP	94.4%		82.1%	85.0%	81.0%
A2 SEMA	54.0%	53.5%		49.8%	60.6%	A2 Ras-GAP	84.1%	82.1%		81.0%	84.8%
A3 SEMA	66.6%	66.7%	49.8%		51.2%	A3 Ras-GAP	85.9%	85.0%	81.0%		79.2%
A4 SEMA	54.4%	54.2%	60.6%	51.2%		A4 Ras-GAP	81.6%	81.0%	84.8%	79.2%	

D
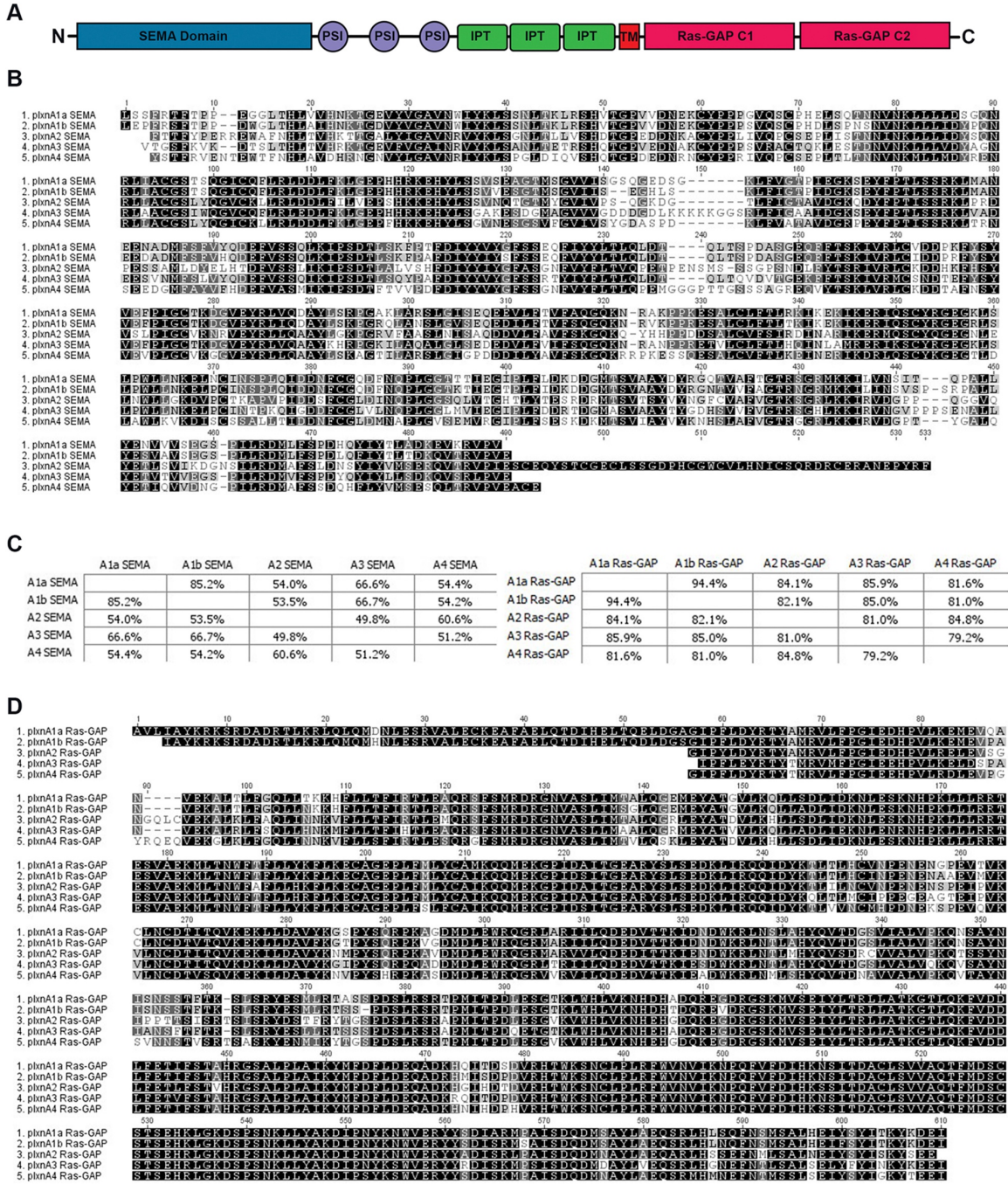
Figure 4.8. Supplemental Fig. 1. Conservation of Zebrafish PlxnA1 Family Member Domains. A. Schematic of typical plxnA protein domains. B. Alignment of the SEMA binding domain of all zebrafish PlxnA1 family members. C. Percent homology between zebrafish plxnA family member SEMA and Ras-GAP domains. D. Alignment of the Ras-GAP binding domain of all zebrafish plxnA1 family members. PSI- plexin-semaphorin and integrin domain, IPT- Ig-like, plexins, transcription factors domain, TM-transmembrane domain, Ras-GAP- Ras GTPase activating-protein domain.
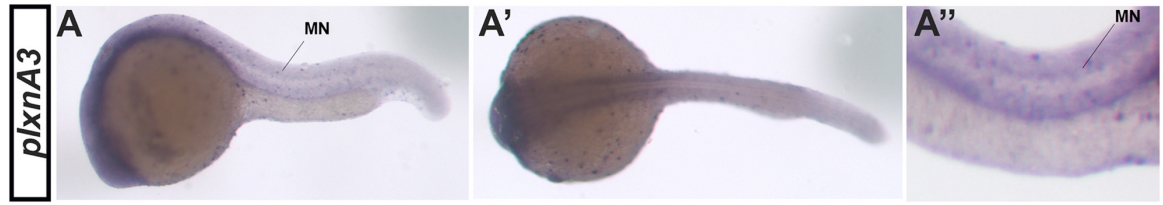
Figure 4.9. Supplemental Fig. 2. *plxna3* **Trunk Expression.** Whole-mount *in situ* hybridization showing plxnA3 expression. A. Lateral, A''. Dorsal and A'''. Dorsal-zoom views at 24 hpf.

4.7. References

Bagnard, D., Vaillant, C., Khuth, S.T., Dufay, N., Lohrum, M., Puschel, A.W., Belin, M.F., Bolz, J., Thomasset, N., 2001. Semaphorin 3A-vascular endothelial growth factor-165 balance mediates migration and apoptosis of neural progenitor cells by the recruitment of shared receptor. The Journal of neuroscience : the official journal of the Society for Neuroscience 21, 3332-3341.

Bagri, A., Cheng, H.J., Yaron, A., Pleasure, S.J., Tessier-Lavigne, M., 2003. Stereotyped pruning of long hippocampal axon branches triggered by retraction inducers of the semaphorin family. Cell 113, 285-299.

Basile, J.R., Gavard, J., Gutkind, J.S., 2007. Plexin-B1 utilizes RhoA and Rho kinase to promote the integrin-dependent activation of Akt and ERK and endothelial cell motility. J Biol Chem 282, 34888-34895.

Behar, O., Golden, J.A., Mashimo, H., Schoen, F.J., Fishman, M.C., 1996. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. Nature 383, 525-528.

Bork, P., Doerks, T., Springer, T.A., Snel, B., 1999. Domains in plexins: links to integrins and transcription factors. Trends Biochem Sci 24, 261-263.

Bryson-Richardson, R., Berger, S., Currie, P., 2012. Chapter 5 - 48 Hours, Atlas of Zebrafish Development. Academic Press, San Diego, pp. 33-54.

Callander, D.C., Lamont, R.E., Childs, S.J., McFarlane, S., 2007. Expression of multiple class three semaphorins in the retina and along the path of zebrafish retinal axons. Developmental dynamics : an official publication of the American Association of Anatomists 236, 2918-2924.

Choi, Y.I., Duke-Cohan, J.S., Chen, W., Liu, B., Rossy, J., Tabarin, T., Ju, L., Gui, J., Gaus, K., Zhu, C., Reinherz, E.L., 2014. Dynamic control of beta1 integrin adhesion by the plexinD1-sema3E axis. Proceedings of the National Academy of Sciences of the United States of America 111, 379-384.

Christie, T.L., Starovic-Subota, O., Childs, S., 2006. Zebrafish collapsin response mediator protein (CRMP)-2 is expressed in developing neurons. Gene Expression Patterns 6, 193-200.

Curreli, S., Wong, B.S., Latinovic, O., Konstantopoulos, K., Stamatos, N.M., 2016. Class 3 semaphorins induce F-actin reorganization in human dendritic cells: Role in cell migration. Journal of leukocyte biology 100, 1323-1334.

Deo, R.C., Schmidt, E.F., Elhabazi, A., Togashi, H., Burley, S.K., Strittmatter, S.M., 2004. Structural bases for CRMP function in plexin-dependent semaphorin3A signaling. The EMBO Journal 23, 9-22.

Ebert, A.M., Childs, S.J., Hehr, C.L., Cechmanek, P.B., McFarlane, S., 2014. Sema6a and Plxna2 mediate spatially regulated repulsion within the developing eye to promote eye vesicle cohesion. Development 141, 2473-2482.

Ebert, A.M., Lamont, R.E., Childs, S.J., McFarlane, S., 2012. Neuronal expression of class 6 semaphorins in zebrafish. Gene expression patterns : GEP 12, 117-122.

Emerson, S.E., St Clair, R.M., Waldron, A.L., Bruno, S.R., Duong, A., Driscoll, H.E., Ballif, B.A., McFarlane, S., Ebert, A.M., 2017. Identification of target genes downstream of semaphorin6A/PlexinA2 signaling in zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 246, 539-549.

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Fishman, M.C., Stainier, D.Y., Breitbart, R.E., Westerfield, M., 1997. Zebrafish: genetic and embryological methods in a transparent vertebrate embryo. Methods in cell biology 52, 67-82.

Gagliardini, V., Fankhauser, C., 1999. Semaphorin III can induce death in sensory neurons. Molecular and cellular neurosciences 14, 301-316.

Glasauer, S.M., Neuhauss, S.C., 2014. Whole-genome duplication in teleost fishes and its evolutionary consequences. Molecular genetics and genomics : MGG 289, 1045-1060.

Haklai-Topper, L., Mlechkovich, G., Savariego, D., Gokhman, I., Yaron, A., 2010. Cis interaction between Semaphorin6A and Plexin-A4 modulates the repulsive response to Sema6A. The EMBO Journal 29, 2635-2645.

He, Z., Tessier-Lavigne, M., 1997. Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell 90, 739-751.

Janssen, B.J., Malinauskas, T., Weir, G.A., Cader, M.Z., Siebold, C., Jones, E.Y., 2012. Neuropilins lock secreted semaphorins onto plexins in a ternary signaling complex. Nature structural & molecular biology 19, 1293-1299.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics (Oxford, England) 28, 1647-1649.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 203, 253-310.

Kolodkin, A.L., Levengood, D.V., Rowe, E.G., Tai, Y.T., Giger, R.J., Ginty, D.D., 1997. Neuropilin is a semaphorin III receptor. Cell 90, 753-762.

Kolodkin, A.L., Matthes, D.J., Goodman, C.S., 1993. The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell 75, 1389-1399.

Koppel, A.M., Feiner, L., Kobayashi, H., Raper, J.A., 1997. A 70 Amino Acid Region within the Semaphorin Domain Activates Specific Cellular Response of Semaphorin Family Members. Neuron 19, 531-537.

Kuwajima, T., Yoshida, Y., Takegahara, N., Petros, T.J., Kumanogoh, A., Jessell, T.M., Sakurai, T., Mason, C., 2012. Optic chiasm presentation of Semaphorin6D in the context of Plexin-A1 and Nr-CAM promotes retinal axon midline crossing. Neuron 74, 676-690.

Love, C.A., Harlos, K., Mavaddat, N., Davis, S.J., Stuart, D.I., Jones, E.Y., Esnouf, R.M., 2003. The ligand-binding face of the semaphorins revealed by the high-resolution crystal structure of SEMA4D. Nature structural biology 10, 843-848.

Luo, Y., Raible, D., Raper, J.A., 1993. Collapsin: A protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75, 217-227.

Matsuoka, R.L., Nguyen-Ba-Charvet, K.T., Parray, A., Badea, T.C., Chedotal, A., Kolodkin, A.L., 2011. Transmembrane semaphorin signalling controls laminar stratification in the mammalian retina. Nature 470, 259-263.

Mueller, T., 2005. Atlas of Early Zebrafish Brain Development: A Tool for Molecular Neurogenetics, 1st ed. ed. Elsevier, Amsterdam ; Boston, Mass.

Murakami, Y., Suto, F., Shimizu, M., Shinoda, T., Kameyama, T., Fujisawa, H., 2001. Differential expression of plexin-A subfamily members in the mouse nervous system.

Developmental dynamics : an official publication of the American Association of Anatomists 220, 246-258.

Neufeld, G., Kessler, O., 2008. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. Nature reviews. Cancer 8, 632-645.

Oinuma, I., Ishikawa, Y., Katoh, H., Negishi, M., 2004. The Semaphorin 4D Receptor Plexin-B1 Is a GTPase Activating Protein for R-Ras. Science (New York, N.Y.) 305, 862-865.

Palaisa, K.A., Granato, M., 2007. Analysis of zebrafish sidetracked mutants reveals a novel role for Plexin A3 in intraspinal motor axon guidance. Development 134, 3251-3257.

Perälä, N.M., Immonen, T., Sariola, H., 2005. The expression of plexins during mouse embryogenesis. Gene Expression Patterns 5, 355-362.

Renaud, J., Chedotal, A., 2014. Time-lapse analysis of tangential migration in Sema6A and PlexinA2 knockouts. Molecular and cellular neurosciences 63, 49-59.

Renaud, J., Kerjan, G., Sumita, I., Zagar, Y., Georget, V., Kim, D., Fouquet, C., Suda, K., Sanbo, M., Suto, F., Ackerman, S.L., Mitchell, K.J., Fujisawa, H., Chedotal, A., 2008. Plexin-A2 and its ligand, Sema6A, control nucleus-centrosome coupling in migrating granule cells. Nature neuroscience 11, 440-449.

Rohm, B., Rahim, B., Kleiber, B., Hovatta, I., Puschel, A.W., 2000. The semaphorin 3A receptor may directly regulate the activity of small GTPases. FEBS Lett 486, 68-72.

Rosslenbroich, V., Dai, L., Baader, S.L., Noegel, A.A., Gieselmann, V., Kappler, J., 2005. Collapsin response mediator protein-4 regulates F-actin bundling. Experimental Cell Research 310, 434-444.

Rünker, A.E., Little, G.E., Suto, F., Fujisawa, H., Mitchell, K.J., 2008. Semaphorin-6A controls guidance of corticospinal tract axons at multiple choice points. Neural Development 3, 34.

Schmidt, E.F., Strittmatter, S.M., 2007. The CRMP Family of Proteins and Their Role in Sema3A Signaling, in: Pasterkamp, R.J. (Ed.), Semaphorins: Receptor and Intracellular Signaling Mechanisms. Springer New York, New York, NY, pp. 1-11.

Schwarz, Q., Waimey, K.E., Golding, M., Takamatsu, H., Kumanogoh, A., Fujisawa, H., Cheng, H.J., Ruhrberg, C., 2008. Plexin A3 and plexin A4 convey semaphorin signals during facial nerve development. Dev Biol 324, 1-9.

Serini, G., Valdembri, D., Zanivan, S., Morterra, G., Burkhardt, C., Caccavari, F., Zammataro, L., Primo, L., Tamagnone, L., Logan, M., Tessier-Lavigne, M., Taniguchi, M., Puschel, A.W., Bussolino, F., 2003. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature 424, 391-397.

Shi, W., Kumanogoh, A., Watanabe, C., Uchida, J., Wang, X., Yasui, T., Yukawa, K., Ikawa, M., Okabe, M., Parnes, J.R., Yoshida, K., Kikutani, H., 2000. The Class IV Semaphorin CD100 Plays Nonredundant Roles in the Immune System: Defective B and T Cell Activation in CD100-Deficient Mice. Immunity 13, 633-642.

Sun, L.O., Jiang, Z., Rivlin-Etzion, M., Hand, R., Brady, C.M., Matsuoka, R.L., Yau, K.W., Feller, M.B., Kolodkin, A.L., 2013. On and off retinal circuit assembly by divergent molecular mechanisms. Science (New York, N.Y.) 342, 1241974.

Suto, F., Ito, K., Uemura, M., Shimizu, M., Shinkawa, Y., Sanbo, M., Shinoda, T., Tsuboi, M., Takashima, S., Yagi, T., Fujisawa, H., 2005. Plexin-A4 Mediates Axon-Repulsive Activities of Both Secreted and Transmembrane Semaphorins and Plays Roles in Nerve Fiber Guidance. The Journal of Neuroscience 25, 3628.

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Suto, F., Tsuboi, M., Kamiya, H., Mizuno, H., Kiyama, Y., Komai, S., Shimizu, M., Sanbo, M., Yagi, T., Hiromi, Y., Chedotal, A., Mitchell, K.J., Manabe, T., Fujisawa, H., 2007. Interactions between plexin-A2, plexin-A4, and semaphorin 6A control lamina-restricted projection of hippocampal mossy fibers. Neuron 53, 535-547.

Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G.I., Song, H., Chedotal, A., Winberg, M.L., Goodman, C.S., Poo, M., Tessier-Lavigne, M., Comoglio, P.M., 1999. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99, 71-80.

Tanaka, H., Maeda, R., Shoji, W., Wada, H., Masai, I., Shiraki, T., Kobayashi, M., Nakayama, R., Okamoto, H., 2007. Novel mutations affecting axon guidance in zebrafish and a role for plexin signalling in the guidance of trigeminal and facial nerve axons. Development 134, 3259-3269.

Thisse, C., Thisse, B., 2008. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat. Protocols 3, 59-69.

Toyofuku, T., Zhang, H., Kumanogoh, A., Takegahara, N., Suto, F., Kamei, J., Aoki, K., Yabuki, M., Hori, M., Fujisawa, H., Kikutani, H., 2004. Dual roles of Sema6D in cardiac morphogenesis through region-specific association of its receptor, Plexin-A1, with off-track and vascular endothelial growth factor receptor type 2. Genes Dev 18, 435-447.

Walzer, T., Galibert, L., Comeau, M.R., De Smedt, T., 2005. Plexin C1 engagement on mouse dendritic cells by viral semaphorin A39R induces actin cytoskeleton rearrangement and inhibits integrin-mediated adhesion and chemokine-induced migration. Journal of immunology (Baltimore, Md. : 1950) 174, 51-59.

Wehner, A.B., Abdesselem, H., Dickendesher, T.L., Imai, F., Yoshida, Y., Giger, R.J., Pierchala, B.A., 2016. Semaphorin 3A is a retrograde cell death signal in developing sympathetic neurons. Development 143, 1560-1570.

CHAPTER 5. DISCUSSION

5.1. Significance

Semas and Plxns were initially discovered for their role in axon and vascular guidance, however it is becoming appreciated that they are important for multiple aspects of development. We previously identified a novel role for Sema6A/PlxnA2 in early eye development regulating proliferation and cohesion of optic vesicles. With the appreciation that Sema/Plxn signaling is requied for many developmental processes, it is important to understand how these critical proteins work. Some of the receptor-proximal signaling events are beginning to be uncovered, however prior to our study, it was unknown if there was any transcriptional regulation downstream of Sema6A/PlxnA2 signaling. We show 58 target genes transcriptionally regulated by Sema6A/PlxnA2 signaling. Interestingly, 57/58 genes are significantly up-regulated in knockdown animals, therefore under normal conditions, this signaling pair is repressive to expression of target genes. We investigated the roles of two candidate genes, *rasl11b* and *shtn-1*, based on predicted roles in proliferation and migration. We show that Sema6A/PlxnA2 repression of *rasl11b* is important for the proliferation of RPCs, and negative regulation of *shtn-1* is important for RPC migration and separation of optic vesicles, as well as optic, motor and sensory nerve patterning. There is cross-talk between different Sema ligands and Plxn receptors, however they often play tissue-specific roles in development. This led us to question where other signaling partners are expressed throughout early development, and how this might contribute to their specificity. We comprehensively investigated the spatial and temporal expression patterns of PlxnA1a, 1b, 2, 3 and 4 in early zebrafish development, providing a key resource of information to those interested

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in the study of the PlxnA family. Furthermore, this was the first identification of the duplication of zebrafish PlxnA1, PlxnA1a and PlxnA1b, and their divergent expression patterns. Our research therefore presents a significant finding to the field and has the potential to be applicable to Sema/Plxn signaling in other systems.

5.2. Proposed Mechanism of Sema6A/PlxnA2 Transcriptional Repression of Target Genes

It is unusual to observe such a dramatic one-sided result from a microarray experiment, in that 57/58 genes were up-regulated in both Sema6A and PlxnA2knockdown embryos. This warrants further investigation into the mechanism of repression that is being activated under normal signaling conditions. Transcriptional regulation is highly dynamic, and complex. Transcription factor (TF) availability and dynamics, combinatorial effects, histone modifications and chromatin remodeling influencing enhancer, promoter and silencer accessibility are just some of the elements that are involved in the control of gene expression. One hypothesis is that Sema6A/PlxnA2 signaling controls developmental gene expression programs via activation of specific transcriptional effectors and/or regulation of chromatin accessibility. To test this, RNA seq could be performed on FACs-sorted *rx3*:GFP control, *plxnA2* and *sema6A* knockdown embryos at 18 somites. Techniques such as chromatin accessibility analysis by ATAC sequencing (assay for transposase accessible chromatin followed by high throughput sequencing) and chromatin structure analysis by histone modification, ChIP sequencing (chromatin immunoprecipitation followed by high throughput sequencing) are commonly used to address these types of questions. ATAC seq would analyze differences in open chromatin footprints between control and

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knockdown embryo eye-enriched cells. ATAC-seq motifs can be annotated for patterns that signify TF binding sites and enhancers, transcriptional start sites, and repressed regions (Hoffman et al., 2012)**.** ChIP-seq could be utilized as a technique to annotate areas of chromatin that are transcriptionally repressed or activated via differences in histone modifications. Antibodies against two histone modification markers, H3K27me3, a marker of areas of repression, (Young et al., 2011) and H3K27ac, a marker of active regions of transcription, would initially be used. Regulatory patterns between control and knockdown embryos could be compared. RNA seq, ATAC seq and ChIP seq could be compared in parallel to identify key *cis* regulatory modules, and transcription factor binding sites in order to build an image of the regulatory landscape downstream of Sema6A/PlxnA2 signaling in eye-enriched cells. These experiments would not only uncover transcriptional effectors and regulatory mechanisms of chromatin remodeling downstream of Sema6A/PlxnA2 signaling in the early eye, but also potentially uncover much broader mechanisms shared by other Semas and Plxns that control multiple aspects of development. In identifying common downstream transcription factors, we could also link yet unknown upstream effectors by pathways that are known to regulate these transcription factors, uncovering a more complete intracellular signal transduction pathway.

5.3. Potential of Other Sema/Plxn Signaling Partners to Regulate Transcription

Uncovering the ability of Sema6A/PlxnA2 signaling to drive transcriptional regulation poses the natural question of whether this is a universal Sema/Plxn signaling mechanism. Currently, it is unknown if other Sema/Plxn signaling partners also regulate gene transcription, however it is not unreasonable. The PlxnA family is structurally highly similar, especially within their intracellular domains, making it more likely that

they interact with similar downstream effectors. We could perform additional microarray experiments to analyze the potential of different Sema/Plxn signaling partners to regulate gene transcription and compare the differences in downstream target genes for specificity for each family member. Cross talk exists between Semas and Plxns, in that Semas can activate multiple Plxns, and Plxns can bind and be activated by multiple Semas. For example, PlxnA2 can bind to Sema3A, 3C, 3F and 6A, and Sema6A can bind to PlxnA1, A2 and A4. Differences in gene regulation between these different signaling partners could be important in the diversification of their temporal and spatially disctinct developmental roles.

5.4. Proposed Mechanism of Rasl11b Signaling

We show that *rasl11b* is a downstream target of Sema6A/PlxnA2 signaling and is important in the regulation of proliferation of RPCs within the developing optic vesicles. Being only one of 58 differentially regulated target genes, others with predicted roles in cell cycle regulation and proliferation, it is surprising that *rasl11b* overexpression can recapitulate the Sema6A/PlxnA2 knockdown phenotype of decreased proliferation. This suggests that *rasl11b* plays a large role in the regulation of proliferation of this cell type.

We found that *rasl11b* is upregulated 4.54 fold in *sema6A* knockdown animals and 2.95 fold in *plexinA2* knockdown animals, and when intentionally upregulated, *rasl11b* leads to decreased proliferation of RPCs within optic vesicles at 18 somites. Rasl11b is a small atypical GTPase that has been implicated in prostate cancer (Louro et al., 2004). Due to its relatedness to Ras, which promotes cell-proliferation, it was surprising that overexpression lead to the opposite phenotype in RPCs. Rasl11b is highly conserved in vertebrates and is atypical to most Ras-like family members in two ways. First, it is cytosolic and lacks carboxy terminal lipid modification sites which allow for

membrane anchoring (Pezeron et al., 2008). Second, it has a lower GTPase activity than Ras, and is more often in its active GTP-bound state (Colicelli, 2004). Ras proteins are well known to be involved in the mitogen-activated protein kinase (MAPK) pathway, therefore, we hypothesize that Rasl11b acts as a negative regulator of MAPK by outcompeting Ras for its effectors such as Raf, leading to decreases in RPC proliferation seen in knockdown embryos.

Activation of Ras leads to the activation of the MAPK cascade, canonically in the order of Ras, Raf, Mek and Erk. Erk activation leads to transcription factor activation (e.g. Creb and Myc), which impacts the cell cycle. We propose that Sema6A/PlxnA2 signaling regulates the MAPK pathway in two ways to mediate cell proliferation (outlined in Figure 5.1). 1) Activation of PlxnA2 drives an intracellular conformational change, leading to the formation of an active GAP domain. This inhibits the small GTPase Rap-1, releasing its inhibition on Raf-1, which can then activate the downstream effectors of the MAPK cascade in response to to normal cell proliferation signals. In PlxnA2 knockdown animals, Rap-1 is in its active GTP bound state, and can out-compete Ras for Raf, therefore inhibiting the MAPK cascade, inhibiting cell proliferative signaling. 2) We propose that Rasl11b indirectly inhibits Ras signaling via its ability to outcompete Ras for its upstream activating proteins, such as Raf or the small Ras activating GEF, son of sevenless (SOS). Typically, Ras is activated by SOS, which is constitutively active, and cytoplasmic. It is recruited to the membrane to meet membrane tethered Ras by Grb2. By having a lower GTPase activity, rasl11b is more often found in its GTP-bound active state, and therefore can bind to Raf or SOS, sequestering them away from the membrane and away from Ras. If Ras is not able to be activated, it would be predominantly found in its GDP-bound state, reducing signal propagation, and

resulting in decreased pro-proliferative effects of the MAPK signaling cascade. Therefore, in normal Sema6A/PlxnA2 signaling, *rasl11b* expression is inhibited, and not able to inhibit proliferation via the MAPK cascade, although in PlxnA2 knockdowns, *rasl11b* expression is increased, inhibiting the MAPK cascade, leading to decreased proliferation.
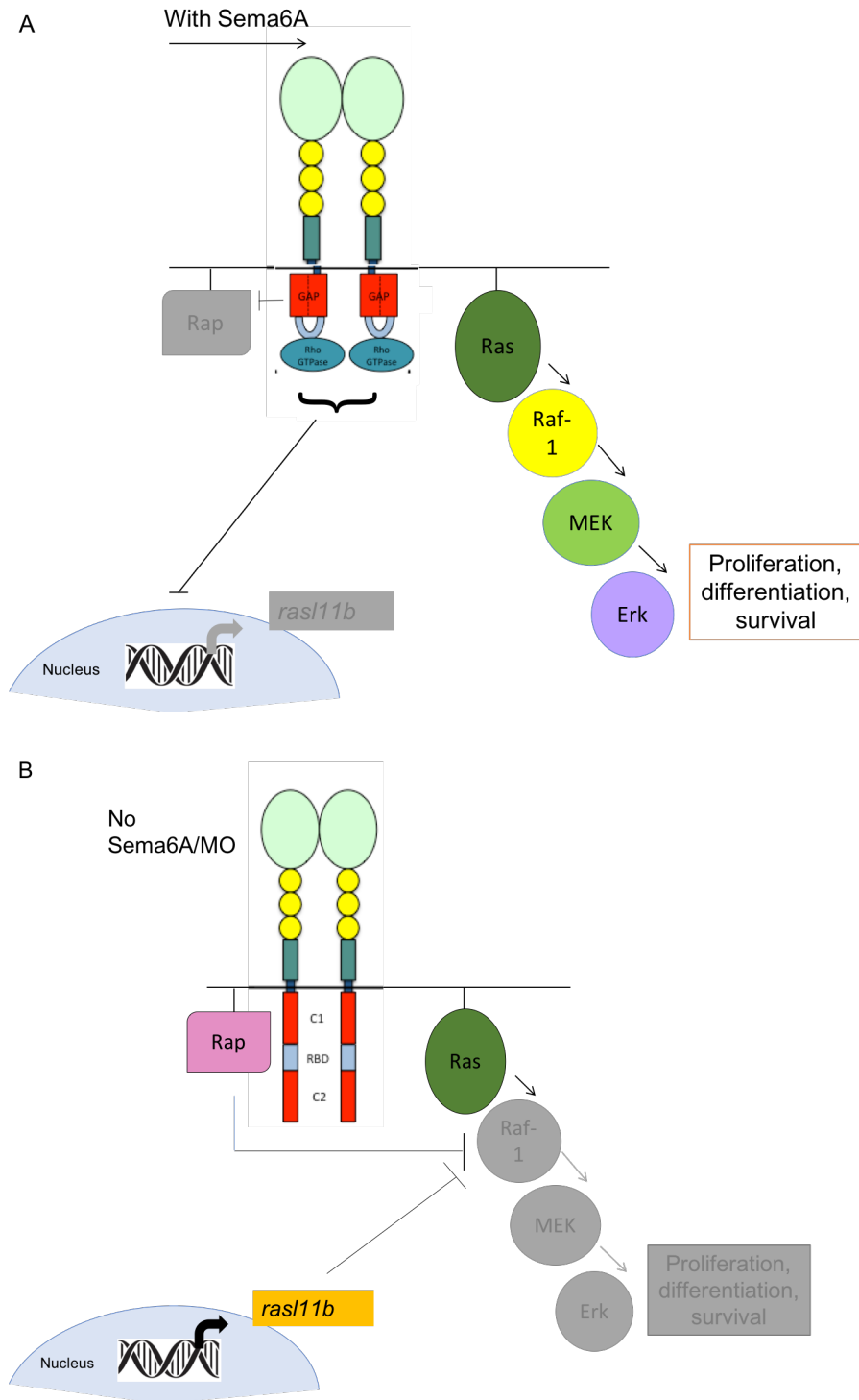
Figure 5.1. Proposed Mechanism of Rasl11b Signaling. When PlxnA2 is activated by Sema6A, *rasl11b* transcription is inhibited, in addition, the functional GAP domain that is formed inactivates the small GTPase Rap, allowing for MAPK proliferative signaling. B. When PlxnA2 is inactive, or impaired, an increase in *rasl11b* expression leads to the inhibition of the MAPK cascade, in addition, Rap is active, and can also repress the MAPK cascade, leading to decreased proliferation.

To investigate the effect of rasl11b on the MAPK pathway, we performed western blots for activated pERK, using HEK293 cells transfected with HA-ERK2 and Flag-tagged DR rasl11b. Increasing doses of *rasl11b* (5, 7.5, 10 μg) showed a *trend* of decreasing pERK levels, suggesting that Rasl11b indeed negatively regulates the MAPK pathway. This preliminary data needs to be repeated, potentially with higher concentrations to get a more robust result. To confirm the mechanism of repression of Rasl11b, activation of additional MAPK effectors could be analyzed, such as Raf-1 and Mek, with overexpression of *rasl11b*. If Rasl11b inhibits the activation of either one, they would show decreased phosphorylation via western blots, along with decreased pERK in a similar experiment to our preliminary pERK experiments. Furthermore, levels of pERK should be able to be rescued by co-transfecting constitutively active phosphomimmeticic forms of the protein *rasl11b* is found to be activating.

Closely related Rasl11a has been shown to be downregulated in prostate and primary breast tumors (Louro et al., 2004b), suggesting that Rasl11b inhibition of cell proliferation may be a conserved mechanism between the two genes. Our double-repression model of PlxnA2 repressing transcription of *rasl11b*, which itself represses MAPK signaling, is similar to a published role for Rasl11b in endodermal and prechordal plate development in zebrafish. It was shown that *oep* (one-eyed pinhead) negatively regulates Rasl11b, which itself negatively regulates endodermal and prechordal plate development. *oep* mutants have increased *rasl11b*, however knocking out *rasl11b* using MO in *oep* mutants rescued the *oep* phenotype. This mechanism of double repression is matched in our model. Therefore, if we knocked down *rasl11b* using a MO in *plxnA2*

mutants, we should see a rescue of RPC proliferation. There are constitutively active zebrafish constructs available, Rasl11b^{G12V} and Rasl11b^{Q61L}, based on Ras mutations found in tumors (Pezeron et al., 2008). The group documented that no effect was seen with overexpression of the constitutively active constructs alone, but they were not investigating RPC proliferation, and did not describe doses used, so perhaps a higher dose could have an effect. Due to our hypothesis, if Rasl11b was constitutively active, it would bind and sequester upstream Erk effectors, and decrease MAPK signaling, resulting in decreased proliferation.

5.5. Proposed Mechanism of *shtn-1* Signaling

Netrins have been shown to be positive regulators of Shtn-1, via Pak-1 phosphorylation, increasing binding affinity to Cortactin, stabilizing the link between the ECM and the cytoskeleton and promoting traction forces for migration. We add an additional negative regulatory component to this model, in that PlxnA2 signaling represses *shtn-1* gene expression (Fig. 5.2). It is important to appreciate the difference in the level of regulation at each point of our model, Netrins activating Shtn-1 at the protein level, and PlxnA2 repressing *shtn-1* at the transcript level. Therefore, PlxnA2 can control the available pool of *shtn-1* transcript, which can later be regulated by Netrin signaling once translated. It is unknown if Netrins and PlxnA2 communicate to regulate the overall active levels of Shtn-1, and it would be interesting to investigate if either could activate or inactivate each other in a feed-back loop.
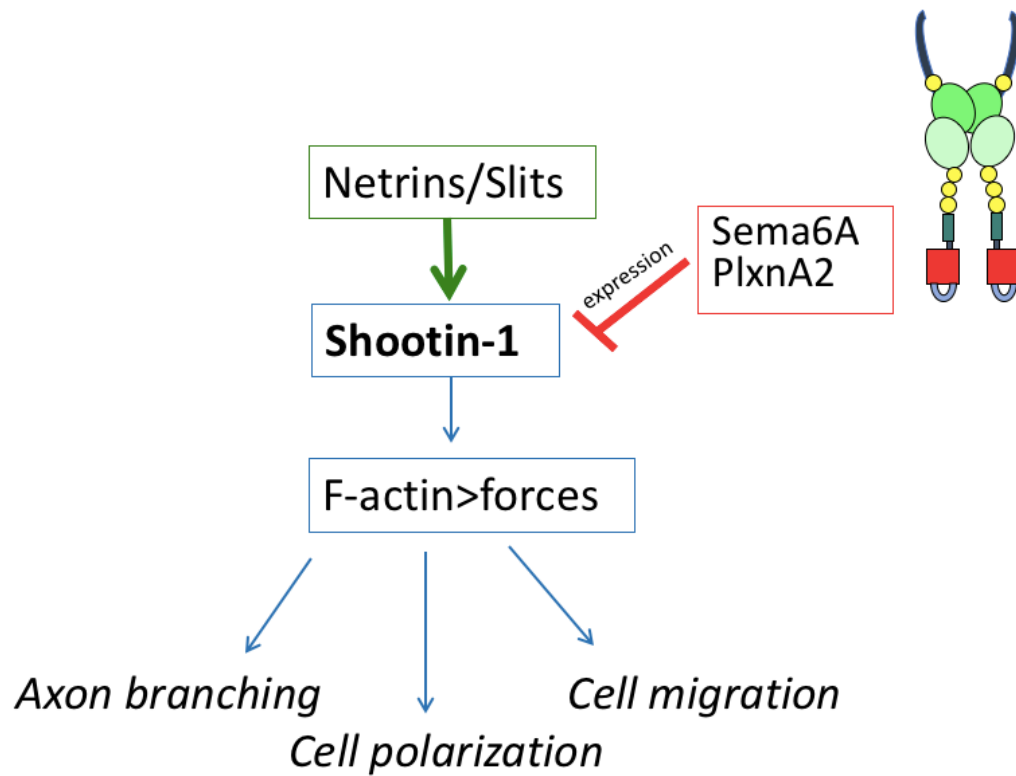
Figure 5.2. Proposed Mechanism of Shtn-1 Signaling. Netrins are known to positively regulate Shtn-1 via phosphorylation by Cdc42 and Pak-1, leading to increased binding to actin, increased traction forces, promoting cell migration, polarization and axon branching. PlxnA2/Sema6A signaling represses *shtn-1* expression, negatively regulating the effects of Shtn-1 signaling.

As Shtn-1 is a polarization signal for migrating cells, we hypothesize that with excess *shtn-1*, a lack of unified directional migration results in the inability of the optic vesicles to separate normally. As a neuron is differentiating, stochastic differences in Shtn-1 levels between neurites lead to competition for the highest levels, which drives the formation of an axon. In the same respect, we hypothesize that the same mechanism could be occurring in the filopodia of RPCs as they are migrating.

It would be interesting to perform time-lapse experiments of developing *rx3*:GFP early eyes, to observe migration events and compare *shtn-1* overexpression embryos with control, and *PLXNA2* and *shtn-1* co-injection 'rescues.' In time lapse movies of control embryos, waves of coordinated cell movements can be seen, whereas in *plxnA2* knockdowns, cells are disorganized and do not move together in wave like streams (Ebert et al., 2014). We would expect similar phenotypes to be seen in *shtn-1* overexpression embryos. Software such as optical flow using Fiji can generate a directional color gradient for cell movements, which would allow for the evaluation of mis-guided migration of RPCs with excess *shtn-1*. There are several markers of polarity that can be probed for within the optic vesicles, such as *foxgx1a* and *tbx5* which are expressed dorsally (future nasal) and *vax2*, which is expressed ventrally. Preliminary *in situ* experiments at 12 somites have shown that they are decreased in expression in *shtn-1* overexpression embryos compared to control embryos. This would need to be repeated, with rescues and *PLXNA2* alone controls, however is suggestive of a loss of correct polarity cues within the optic vesicle, which could be due to a disorganization of the vesicles or could be driving the disorganization. At a cellular level, single cell polarity

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markers could be probed for, such as *f-actin* at the leading edge, to evaluate polarity errors within individual cells with excess *shtn-1*.

Retinas have an amazing ability to re-aggregate once dissociated in culture. It has been shown that eyes dissected from 24 hpf embryos can be dissociated, and plated, and dissociated retinal cells will migrate towards each other to re-form into an organoid within ~3 hours (Eldred et al., 2017). It would be interesting to perform cell migration assays on eyes dissected form *shtn-1* overexpression embryos to see if migration during reaggregation was impaired and if *PLXNA2* co-injection could rescue normal migration of dissociated cells. Furthermore, eyes dissected from *shtn-1* mutant embryos could be used to see how migration is impaired, and if you could rescue this by additionally knocking out PlxnA2, to increase production of functional *shtn-1*.

It is intriguing that at 72 hpf, the ventral-medial RPE is missing in embryos with excess *shtn-1*. Several transcription factors are key to the development of the RPE. Otx2, Mitf and Pax6 are all required for RPE fates, as well as inhibition of Fgf8 and Sox2, which drive retinal fates. Hh and Wnt signaling are also important for driving RPE specification. It has been shown that a loss of RPE-specific transcription factors leads to transdifferentiation of the RPE into neural retina. The inverse is also true, for example, if Wnt signaling is not repressed by Six3 during early eye field specification, the entire optic vesicle becomes RPE (Fuhrmann et al., 2014). It could be that *shtn-1* overexpression represses or inactivates one or more RPE specific transcription factors, leading to transdifferentiation of the RPE into retinal cell fates, projecting into the midline. This could be investigated by *in situ* hybridization and immunohistochemistry, probing for the presence of each TF, using control and *shtn-1* overexpression embryos to look for changes in expression, and then using MO knockdown of candidate TFs to

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recapitulate the RPE phenotype. It is also possible that the RPE requires a signal from the midline to develop in that region, and due to the eyes being closer together in *shtn-1* overexpression embryos, the signal is not being produced, as there is missing tissue in the forebrain region. It is interesting that Hh signaling is required for RPE determination, (Shh and Tiggy-winkle hedgehog twhh) are both expressed by the RPE, most highly in the center (Stenkamp et al., 2000). Shh inhibition by cyclopamine results in a loss of RPE differentiation (Perron et al., 2003) and the effects look similar to those seen with the overexpression of *shtn-1*. Hh signaling is a well-known mediator of midline development. A loss of Shh in the midline, which usually represses Pax6, results in cycloptic embryos, which we also see in embryos with excess *shtn-1*. It would be worth investigating if you could rescue *shtn-1* overexpression phenotypes by also overexpressing Shh. If a rescue of RPE formation occurred, but the eyes still remained closer together it would suggest that Shtn-1 negatively impacts Shh signaling in the RPE specifically, whereas if we saw a rescue of the development of midline structures, and eye vesicle separation, it could suggest that Shtn-1 is acting on Shh at the midline, and the RPE is receiving signals from midline structures in order to develop correctly. It is possible that the reduction of fasciculation seen in *shtn-1* overexpression embryos is due to missing RPE at the exit point of the optic nerve. This might also be seen to rescue if indeed Shh signaling is driving the impairment at the midline. Another way to tease apart the role of the midline from the RPE itself would be to overexpress *shtn-1* specifically in the RPE. this could be achieved by driving the expression of *shtn-1* downstream of an RPE-specific promoter such as Best1. Using this technique, if the RPE develops normally, it could be concluded that the RPE impairments seen in *shtn-1* overexpression embryos are due to the surrounding tissues.

Interestingly, Shh, Netrins and Shtn-1 have been shown to be important for the development of RGC projections from the eye and have been shown in different pathways to regulate each other. Shh can positively regulate Netrins, Netrins can positively regulate Shtn-1, however, we do not yet know if Shh can regulate Shtn-1 or vice versa. Shh is expressed in the RGC layer, with its highest concentrations at the center of the retina, and is important for the guidance of RGC projections to the optic disc (Kolpak et al., 2005). Overexpression or inhibition of Shh leads to loss of centrally projecting RGCs at the optic disc (Kolpak et al., 2005). Netrins are expressed specifically in neuroepithelial cells surrounding the optic disc and in glial cells surrounding the optic stalk. The Netrin receptor, DCC is expressed by RGCs (Deiner et al., 1997). In *Netrin* mutant mice, the optic disc is smaller with optic nerve hypoplasia due to improper axon guidance in the retina (Deiner et al., 1997). Netrins positively regulate Shtn-1, and Shtn-1 overexpression leads to loss of fasciculation and disorganization of the optic tracts, the opposite of what is seen with *Netrin-1* knockout. This supports our model for the necessity for careful regulation of Shtn-1 levels by Netrins and PlxnA2 and suggests that the optic tract phenotype seen could be due to an axon migration issue. It also raises the question of if Shh signaling is also involved in Shtn-1 signaling.

H&E sections show that neuronal lamination is conserved in embryos with excess *shtn-1* levels, which was surprising, as Shtn-1 is involved in cell migration, and the cells of the retina need to migrate to their correct layers during development. It would be interesting to use SoFa-1 transgenic fish to investigate if the correct cell types are indeed in the correct layers of the eye. Additionally, it is unknown if embryos with excess *shtn-1* would be able to see. Optokinetic chambers could be used to test if the fish are able to track correctly. As we see impaired optic tract projections to the optic tecta in terms of

loss of fasciculation and improper directional targeting, it is hypothesized that the ability to track would be low in embryos with excess *shtn-1*.

It is known that several guidance cues are important for the regulation of axon branching. Netrins are secreted positive axon guidance cues and have been shown to increase cortical axon branching. Sema3A can either inhibit or promote axon branching dependent on the tissue (Dent et al., 2004). Here we show that PlxnA2 regulation of *shtn-1* is important for correct axon branching, as overexpression of *shtn-1* leads to surplus motor axon branches and can be rescued by co-injecting *PLXNA2*. There are two main mechanisms of axon branching, both reliant on changes to cytoskeletal dynamics. When repellant Sema3A causes growth cone collapse, it pauses before recovery. When a growth cone pauses after collapse, they enlarge due to the formation of microtubule loops. Pausing behaviors leave remnants behind, giving rise to interstitial branches. Alternatively, branching doesn't always require pausing events, as with Netrin-induced branching, localized accumulation of actin filaments in filapodial extensions drive branching, forming a smoother branch transition point (Dent et al., 2004). Overexpression of Netrins increases axon branching by 50%, which supports our model of Shtn-1 regulation of axon branching in that Netrins positively regulate Shtn-1 activation and we have shown excess *shtn-1* to promote axon branching. Therefore, in our model, Sema6A signaling via PlxnA2 represses *shtn-1* expression and decreases branching. This would be testable in cell culture using neurons with excess *shtn-1*. Excess *shtn-1* should lead to more branching, and experiments with bath applied Sema-FC would test to see if branching numbers would be lower due to PlxnA2 activation and *shtn-1* transcriptional repression. It would also be interesting to investigate the cytoskeletal dynamics of Shtn-1-driven axon branching, using immunolabelled

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microtubules and actin, with tubulin and phalloidin antibodies respectively. As Netrins have been shown to drive interstitial branches due to increased actin polymerization, we would hypothesize to see the same with *shtn-1* overexpression, as opposed to enlargements of branching points and an accumulation of microtubules forming loops.

We have shown what happens to neurodevelopment with increasing levels of *shtn-1*. It would be interesting to take this idea further, first by knocking down/out *shtn-1* using MO or CRISPR to see the effects *in vivo*. We would expect to see an inability of neurons to polarize and differentiate axons, and potentially similar lack of migration in the optic vesicles, due to an inability of RPCs to polarize and migrate in a uniform direction. CRISPR stable lines take at least 9 months to generate, so to investigate this more rapidly, CRISPR interference (CRISPi) could be used, which recruits a dead Cas9 enzyme to the target site on the gene of interest to prevent transcription.

5.6. Potential Study of Additional Downstream Target Genes

We have characterized two downstream transcriptional targets of Sema6A/PlxnA2 signaling, each playing important roles in neurodevelopment. The microarray identified 58 target genes, therefore providing a resource for many future projects. Some interesting potential projects include an investigation into the target gene MMP2, matrix metalloproteinase 2, and if it has roles in aiding cell migration, leading to the impaired cohesion phenotype that we observe in PlxnA2 and Sema6A knockdown optic vesicles at 18 somites. In addition to the target gene, Rbl2 (retinoblastoma like 2), which is a cell cycle inhibitor, therefore increased expression in knockdowns could contribute to the observes decrease in proliferation of RPCs. Dcdc2b (double-cortin domain containing 2b) is a microtubule associated protein and stabilizes microtubules when bound. Increased expression of *dcdc2b* could disrupt normal RPC migration by

over-stabilizing microtubules, preventing the necessary depolymerization required for cell movement, therefore contributing to the lack of unified migration of RPCs in optic vesicles, driving the loss of cohesion phenotype that we see in knockown animals. As we see such robust and easily testable phenotypes in knockdown embryos, it is conceivable to investigate the contributions of many of the downstream target genes to them. As PlxnA2 and Sema6A are present in many other tissues, the investigation could also be expanded to additional systems throughout development.

5.7. Generation of PlxnA2 CRISPR Mutant

We generated a PlxnA2 CRISPR knockout zebrafish line, in order to have a stable mutant line for future experiments. This line was generated using Cas9 nanos, a form of Cas9 that is targeted to the germline, resulting in high levels of efficiency in terms of heritable gene-specific mutations (Moreno-Mateos et al., 2015). The mutation has been sequence-verified to be a nonsense mutation in exon 2 (**TTT**-T**AG**) that results in a premature STOP codon (**U**AG), in place of a Phenylalanine (**TTT**) at amino acid site 401, leading to a truncated protein (Figure 5.3A). Preliminary data show that PlxnA2 mutants phenocopy PlxnA2 knockdown embryos at 18 somites with respect to decreased cohesion of RPCs within the optic vesicles, resulting in ectopic cells, and decreased separation of optic vesicles (Figure 5.3B).
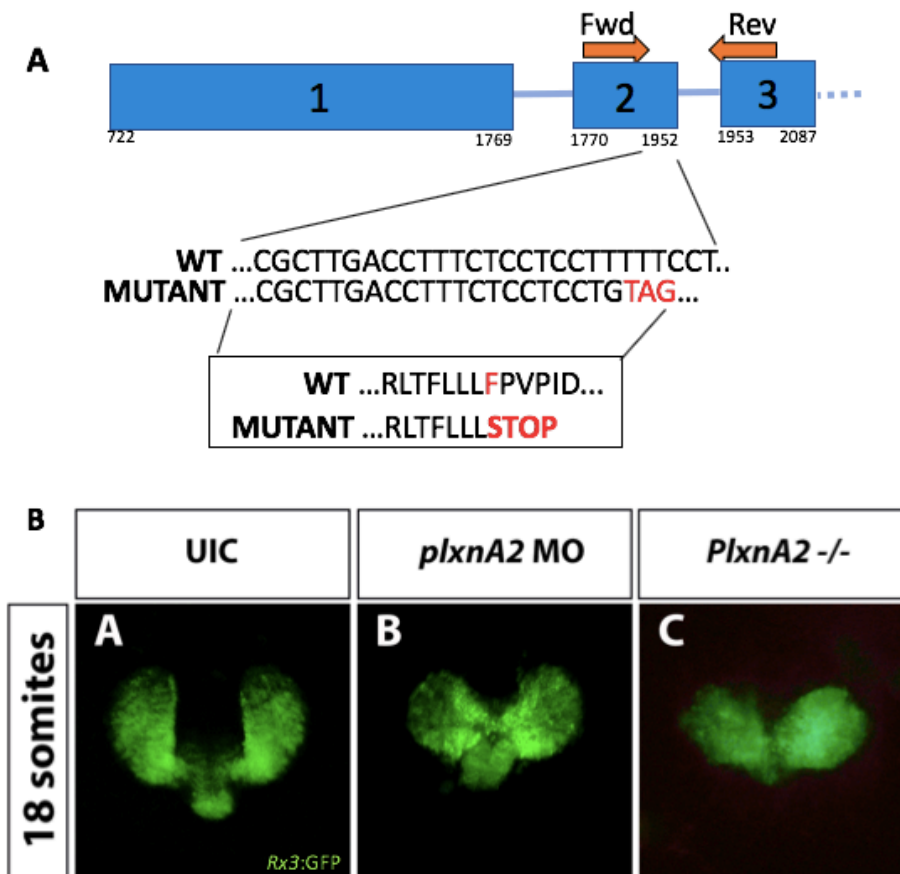
Figure 5.3. *Zebrafish PlxnA2* **CRISPR Mutant Phenocopies** *PlxnA2* **Knockdown** A) Schematic of mutation site within exon 2 of *plxnA2*. Orange arrows show location of sequence validation primers. The mutation is a non-sense mutation (**TTT-TAG**), Phenylalanine (F)-STOP at amino acid site 401 in Exon 2, which results in a truncated protein. Numbers indicate base pair regions of each exon B). Dorsal 18 somite *rx3*:GFP images showing the optic vesicles of A) uninjected control, B) *plxna2* knockdown and C) *plxna2* knockout embryos.

Embryos in wholemount have visible cell death in the eyes and brain, smaller eyes and heart edemas, also seen in knockdown embryos. Future experiments would investigate if there is decreased proliferation of RPCs as seen in knockdown embryos. Embryos only survive for ~72 hpf, before the mutation is lethal. It is presently unknown if the homozygous null condition is embryonic lethal and the phenotypes seen are due to a heterozygous mutation. When two known heterozygous adults are in-crossed, we see ~25% embryonic death, suggesting an embryonic lethal null, however sequencing of embryonic fin clips needs to be performed on potential mutants to resolve this question.

We performed an *in situ* hybridization experiment to see how the levels of *shtn-1* changed due to *plxnA2* knockout to see if we would get the same result as *plxnA2* knockdown. We were surprised to see that even though levels increased in mutants as expected at 18 somites, expression levels did not get as high as with *plxnA2* knockdown. We believe that this could be due to the ability of Plxns to compensate for each other over time, therefore not allowing *shtn-1* levels to get as high.

5.8. PlxnA Family Expression in Optic Vesicles

In zebrafish, the PlxnA family consists of PlxnA1a, A1b, A2, A3 and A4, and showing both overlapping and divergent expression patterns during early development. As Sema6A can activate multiple members of the PlxnA family, (A1, A2 and A4) we wanted to determine the expression patterns of each family member to see if additional PlxnAs might be involved in Sema6A-mediated early eye development. We show that all PlxnAs apart from PlxnA3 are expressed in the early optic vesicles at 18 somites, each with different expression domains. At 18 somites, *plxnA1a* is expressed throughout the optic vesicle, with the strongest expression at the anterior. *A1b* and *A2* are expressed throughout the vesicle, with highest expression levels at the ventral side. *A4* is expressed

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uniformly throughout the optic vesicle. Preliminary results have uncovered the potential of PlxnA1b and A2 to compensate for each other. This needs to be further investigated and validated but is supported by their shared expression patterns. It is known that when Sema and Plxn are expressed on the same cells, Semas can *cis* inhibit Plxn *trans* signaling. This is the reasoning behind the published working model of cell organization within optic vesicles (Ebert et al., 2014). As Sema6A is expressed throughout the early eye vesicle, there is potential for repellant Sema signaling to be negated in ventral cells that also express PlxnA1b and A2 in *cis*, allowing Sema-sensitive RPCs to reside ventrally. Cells entering the optic vesicle domain that are insensitive to Sema6A can reside dorsally. This model is further complicated with the knowledge that additional *plxnA* family members are expressed throughout the eye vesicle. There is overlap throughout the optic vesicle, but if we look at the areas of highest expression, *plxnA1a* is anterior, *A2* and *A1b* ventral and *A4* is throughout but highest out of all *plxnAs* dorsally. It could be that additional *semas* are expressed by different groups of RPCs that act as ligands for PlxnA1 and A4, as Sema3's are ligands for these PlxnA family members, and there are multiple Sema6 family members. Additional *in situ* experiments would have to be performed to validate expression of potential *sema* ligands at this early stage, to tease apart the organization domains of the optic vesicles.

As stated above, it should be noted that in PlxnA2 mutant embryos *shtn-1* levels do not increase as much as levels seen in PlxnA2 knockdown embryos. This could be due to compensation of different PlxnA family members in mutant embryos. We have previously shown that PlxnA1b can compensate for PlxnA2 knockdown in terms of regulation of proliferation of RPCs and eye size at 18 somites. This highlights an

advantage of the use of morpholino knockdown in the ability to analyze the effect of a manipulating a single gene, without giving the genome time to adapt over generations.

5.9. Summary

Sema/Plxn signaling is important for many aspects of development, from vasculogenesis, heart development, bone development, immunity, cell proliferation and axon guidance. Improper Sema/Plxn signaling has been implicated in several disorders such as neurodegenerative diseases including Alzheimer's disease, amyotrophic lateral sclerosis (ALS) and Multiple sclerosis, psychological disorders such as schizophrenia and multiple metastatic cancers. Being such critical signaling proteins, it is important to understand how they work. Studying Sema6A and PlxnA2 in the zebrafish could uncover signaling mechanisms that can be applied to other Semas and Plxns in multiple systems and organisms, which could lead to the discovery of potential therapeutics for developmental and degenerative diseases.

5.10. References

Deiner, M.S., Kennedy, T.E., Fazeli, A., Serafini, T., Tessier-Lavigne, M., Sretavan, D.W., 1997. Netrin-1 and DCC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia. Neuron 19, 575-589.
Dekker, J., Marti-Renom, M.A., Mirny, L.A., 2013. Exploring the three-dimensional organization of genomes: interpreting chromatin interaction data. Nature reviews. Genetics 14, 390-403.
Dent, E.W., Barnes, A.M., Tang, F., Kalil, K., 2004. Netrin-1 and semaphorin 3A promote or inhibit cortical axon branching, respectively, by reorganization of the cytoskeleton. The Journal of neuroscience : the official journal of the Society for Neuroscience 24, 3002-3012.
Ebert, A.M., Childs, S.J., Hehr, C.L., Cechmanek, P.B., McFarlane, S., 2014. Sema6a and Plxna2 mediate spatially regulated repulsion within the developing eye to promote eye vesicle cohesion. Development (Cambridge, England) 141, 2473-2482.
Eisen, J.S., Smith, J.C., 2008. Controlling morpholino experiments: don't stop making antisense. Development (Cambridge, England) 135, 1735-1743.

Eldred, M.K., Charlton-Perkins, M., Muresan, L., Harris, W.A., 2017. Self-organising aggregates of zebrafish retinal cells for investigating mechanisms of neural lamination. Development (Cambridge, England) 144, 1097.

Fuhrmann, S., Zou, C., Levine, E.M., 2014. Retinal pigment epithelium development, plasticity, and tissue homeostasis. Experimental eye research 123, 141-150.

Gehrke, A.R., Schneider, I., de la Calle-Mustienes, E., Tena, J.J., Gomez-Marin, C., Chandran, M., Nakamura, T., Braasch, I., Postlethwait, J.H., Gómez-Skarmeta, J.L., Shubin, N.H., 2015. Deep conservation of wrist and digit enhancers in fish. Proceedings of the National Academy of Sciences 112, 803-808.

Gnyszka, A., Jastrzebski, Z., Flis, S., 2013. DNA methyltransferase inhibitors and their emerging role in epigenetic therapy of cancer. Anticancer research 33, 2989-2996.

Hoffman, M.M., Ernst, J., Wilder, S.P., Kundaje, A., Harris, R.S., Libbrecht, M., Giardine, B., Ellenbogen, P.M., Bilmes, J.A., Birney, E., Hardison, R.C., Dunham, I., Kellis, M., Noble, W.S., 2012. Integrative annotation of chromatin elements from ENCODE data. Nucleic Acids Research.

Kok, F.O., Shin, M., Ni, C.W., Gupta, A., Grosse, A.S., van Impel, A., Kirchmaier, B.C., Peterson-Maduro, J., Kourkoulis, G., Male, I., DeSantis, D.F., Sheppard-Tindell, S., Ebarasi, L., Betsholtz, C., Schulte-Merker, S., Wolfe, S.A., Lawson, N.D., 2015. Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. Developmental cell 32, 97-108.

Kolpak, A., Zhang, J., Bao, Z.-Z., 2005. Sonic Hedgehog Has a Dual Effect on the Growth of Retinal Ganglion Axons Depending on Its Concentration. The Journal of Neuroscience 25, 3432.

Louro, R., Nakaya, H.I., Paquola, A.C.M., Martins, E.A.L., Silva, A.M.d., Verjovski-Almeida, S., Reis, E.M., 2004. RASL11A, member of a novel small monomeric GTPase gene family, is down-regulated in prostate tumors. Biochemical and Biophysical Research Communications 316, 618-627.

Perron, M., Boy, S., Amato, M.A., Viczian, A., Koebernick, K., Pieler, T., Harris, W.A., 2003. A novel function for Hedgehog signalling in retinal pigment epithelium differentiation. Development (Cambridge, England) 130, 1565-1577.

Pezeron, G., Lambert, G., Dickmeis, T., Strahle, U., Rosa, F.M., Mourrain, P., 2008. Rasl11b knock down in zebrafish suppresses one-eyed-pinhead mutant phenotype. PloS one 3, e1434.

Robu, M.E., Larson, J.D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S.A., Ekker, S.C., 2007. p53 activation by knockdown technologies. PLoS genetics 3, e78.

Stenkamp, D.L., Frey, R.A., Prabhudesai, S.N., Raymond, P.A., 2000. Function for Hedgehog Genes in Zebrafish Retinal Development. Developmental biology 220, 238-252.

Suryamohan, K., Halfon, M.S., 2015. Identifying transcriptional cis-regulatory modules in animal genomes. Wiley interdisciplinary reviews. Developmental biology 4, 59-84.

Tamara, V., Peggy, P., Greetje, E., Vera, R., 2004. Trichostatin A - like Hydroxamate Histone Deacetylase Inhibitors as Therapeutic Agents: Toxicological Point of View. Current Medicinal Chemistry 11, 1629-1643.

Young, M.D., Willson, T.A., Wakefield, M.J., Trounson, E., Hilton, D.J., Blewitt, M.E., Oshlack, A., Majewski, I.J., 2011. ChIP-seq analysis reveals distinct H3K27me3 profiles that correlate with transcriptional activity. Nucleic Acids Research.

6. COMPREHENSIVE BIBLIOGRAPHY

Ali, R.R., Sowden, J.C., 2011. DIY eye. Nature 472, 42.

Allende, M.L., Amsterdam, A., Becker, T., Kawakami, K., Gaiano, N., Hopkins, N., 1996. Insertional mutagenesis in zebrafish identifies two novel genes, pescadillo and dead eye, essential for embryonic development. Genes & development 10, 3141-3155.

Almeida, A.D., Boije, H., Chow, R.W., He, J., Tham, J., Suzuki, S.C., Harris, W.A., 2014. Spectrum of Fates: a new approach to the study of the developing zebrafish retina. Development (Cambridge, England) 141, 1971.

Amini, R., Rocha-Martins, M., Norden, C., 2018. Neuronal Migration and Lamination in the Vertebrate Retina. Frontiers in neuroscience 11, 742-742.

Arenzana, F.J., Carvan, M.J., 3rd, Aijon, J., Sanchez-Gonzalez, R., Arevalo, R., Porteros, A., 2006. Teratogenic effects of ethanol exposure on zebrafish visual system development. Neurotoxicology and teratology 28, 342-348.

Avdesh, A., Chen, M., Martin-Iverson, M.T., Mondal, A., Ong, D., Rainey-Smith, S., Taddei, K., Lardelli, M., Groth, D.M., Verdile, G., Martins, R.N., 2012. Regular care and maintenance of a zebrafish (Danio rerio) laboratory: an introduction. Journal of visualized experiments : JoVE, e4196-e4196.

Baba, K., Yoshida, W., Toriyama, M., Shimada, T., Manning, C.F., Saito, M., Kohno, K., Trimmer, J.S., Watanabe, R., Inagaki, N., 2018. Gradient-reading and mechano-effector machinery for netrin-1-induced axon guidance. eLife 7.

Bagnard, D., Vaillant, C., Khuth, S.T., Dufay, N., Lohrum, M., Puschel, A.W., Belin, M.F., Bolz, J., Thomasset, N., 2001. Semaphorin 3A-vascular endothelial growth factor-165 balance mediates migration and apoptosis of neural progenitor cells by the recruitment of shared receptor. The Journal of neuroscience : the official journal of the Society for Neuroscience 21, 3332-3341.

Bagri, A., Cheng, H.J., Yaron, A., Pleasure, S.J., Tessier-Lavigne, M., 2003. Stereotyped pruning of long hippocampal axon branches triggered by retraction inducers of the semaphorin family. Cell 113, 285-299.

Basile, J.R., Gavard, J., Gutkind, J.S., 2007. Plexin-B1 utilizes RhoA and Rho kinase to promote the integrin-dependent activation of Akt and ERK and endothelial cell motility. J Biol Chem 282, 34888-34895.

Baye, L.M., Link, B.A., 2008. Nuclear migration during retinal development. Brain research 1192, 29-36.

Beebe, S.J., Øyen, O., Sandberg, M., Frøysa, A., Hansson, V., Jahnsen, T., 1990. Molecular Cloning of a Tissue-Specific Protein Kinase (Cγ) from Human Testis—Representing a Third Isoform for the Catalytic Subunit of cAMP-Dependent Protein Kinase. Molecular Endocrinology 4, 465-475.

Behar, O., Golden, J.A., Mashimo, H., Schoen, F.J., Fishman, M.C., 1996a. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. Nature 383, 525-528.

Behar, O., Golden, J.A., Mashimo, H., Schoen, F.J., Fishman, M.C., 1996b. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. Nature 383, 525-528.

Belle, M., Parray, A., Belle, M., Chedotal, A., Nguyen-Ba-Charvet, K.T., 2016. PlexinA2 and Sema6A are required for retinal progenitor cell migration. Dev Growth Differ 58, 492-502.

Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological) 57, 289-300.

Blader, P., Strahle, U., 1998. Ethanol impairs migration of the prechordal plate in the zebrafish embryo. Developmental biology 201, 185-201.

Bolstad, B.M., Irizarry, R.A., Astrand, M., Speed, T.P., 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics (Oxford, England) 19, 185-193.

Bork, P., Doerks, T., Springer, T.A., Snel, B., 1999. Domains in plexins: links to integrins and transcription factors. Trends Biochem Sci 24, 261-263.

Bos, J.L., Rehmann, H., Wittinghofer, A., 2007. GEFs and GAPs: Critical Elements in the Control of Small G Proteins. Cell 129, 865-877.

Breedlove, S.M., 2017. Foundations of neural development. Oxford university press.

Brinckerhoff, C.E., Matrisian, L.M., 2002. Matrix metalloproteinases: a tail of a frog that became a prince. Nat Rev Mol Cell Biol 3, 207-214.

Bryson-Richardson, R., Berger, S., Currie, P., 2012. Chapter 5 - 48 Hours, Atlas of Zebrafish Development. Academic Press, San Diego, pp. 33-54.

Buel, G.R., Rush, J., Ballif, B.A., 2010. Fyn promotes phosphorylation of collapsin response mediator protein 1 at tyrosine 504, a novel, isoform-specific regulatory site. Journal of Cellular Biochemistry 111, 20-28.

Butcher, R.W., Sutherland, E.W., 1962. Adenosine 3',5'-Phosphate in Biological Materials: I. PURIFICATION AND PROPERTIES OF CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE AND USE OF THIS ENZYME TO CHARACTERIZE ADENOSINE 3',5'-PHOSPHATE IN HUMAN URINE. Journal of Biological Chemistry 237, 1244-1250.

Callander, D.C., Lamont, R.E., Childs, S.J., McFarlane, S., 2007. Expression of multiple class three semaphorins in the retina and along the path of zebrafish retinal axons. Developmental dynamics : an official publication of the American Association of Anatomists 236, 2918-2924.

Carpenter, E.M., Goddard, J.M., Chisaka, O., Manley, N.R., Capecchi, M.R., 1993. Loss of Hox-A1 (Hox-1.6) function results in the reorganization of the murine hindbrain. Development (Cambridge, England) 118, 1063-1075.

Castro, B., Barolo, S., Bailey, A.M., Posakony, J.W., 2005. Lateral inhibition in proneural clusters: cis-regulatory logic and default repression by Suppressor of Hairless. Development (Cambridge, England) 132, 3333-3344.

Caudy, M., Bentley, D., 1986. Pioneer growth cone steering along a series of neuronal and non-neuronal cues of different affinities. The Journal of neuroscience : the official journal of the Society for Neuroscience 6, 1781-1795.

Cechmanek, P.B., McFarlane, S., 2017. Retinal pigment epithelium expansion around the neural retina occurs in two separate phases with distinct mechanisms. 246, 598-609.

Chalepakis, G., Stoykova, A., Wijnholds, J., Tremblay, P., Gruss, P., 1993. Pax: gene regulators in the developing nervous system. Journal of neurobiology 24, 1367-1384.

Chen, H., Chedotal, A., He, Z., Goodman, C.S., Tessier-Lavigne, M., 1997. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron 19, 547-559.

Choi, Y.I., Duke-Cohan, J.S., Chen, W., Liu, B., Rossy, J., Tabarin, T., Ju, L., Gui, J., Gaus, K., Zhu, C., Reinherz, E.L., 2014. Dynamic control of beta1 integrin adhesion by the plexinD1-sema3E axis. Proceedings of the National Academy of Sciences of the United States of America 111, 379-384.

Christie, T.L., Starovic-Subota, O., Childs, S., 2006. Zebrafish collapsin response mediator protein (CRMP)-2 is expressed in developing neurons. Gene Expression Patterns 6, 193-200.

Clark, A.M., Yun, S., Veien, E.S., Wu, Y.Y., Chow, R.L., Dorsky, R.I., Levine, E.M., 2008. Negative regulation of Vsx1 by its paralog Chx10/Vsx2 is conserved in the vertebrate retina. Brain Res 1192, 99-113.

Clegg, C.H., Correll, L.A., Cadd, G.G., McKnight, G.S., 1987. Inhibition of intracellular cAMP-dependent protein kinase using mutant genes of the regulatory type I subunit. Journal of Biological Chemistry 262, 13111-13119.

Cobrinik, D., 2005. Pocket proteins and cell cycle control. Oncogene 24, 2796-2809.

Cohen, J., Johnson, A.R., 1991. Differential effects of laminin and merosin on neurite outgrowth by developing retinal ganglion cells. Journal of cell science. Supplement 15, 1-7.

Colicelli, J., 2004. Human RAS Superfamily Proteins and Related GTPases. Science's STKE : signal transduction knowledge environment 2004, RE13-RE13.

Colledge, M., Scott, J.D., 1999. AKAPs: from structure to function. Trends in Cell Biology 9, 216-221.

Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E., Goodman, H.M., 1986. A cyclic AMP- and phorbol ester-inducible DNA element. Nature 323, 353-356.

Comeau, M.R., Johnson, R., DuBose, R.F., Petersen, M., Gearing, P., VandenBos, T., Park, L., Farrah, T., Buller, R.M., Cohen, J.I., Strockbine, L.D., Rauch, C., Spriggs, M.K., 1998. A poxvirus-encoded semaphorin induces cytokine production from monocytes and binds to a novel cellular semaphorin receptor, VESPR. Immunity 8, 473-482.

Creaser, C.W., 1934. The Technic of Handling the Zebra Fish (Brachydanio rerio) for the Production of Eggs Which Are Favorable for Embryological Research and Are Available at Any Specified Time Throughout the Year. Copeia 1934, 159-161.

Curreli, S., Wong, B.S., Latinovic, O., Konstantopoulos, K., Stamatos, N.M., 2016. Class 3 semaphorins induce F-actin reorganization in human dendritic cells: Role in cell migration. Journal of leukocyte biology 100, 1323-1334.

Dee, C.T., Hirst, C.S., Shih, Y.H., Tripathi, V.B., Patient, R.K., Scotting, P.J., 2008. Sox3 regulates both neural fate and differentiation in the zebrafish ectoderm. Developmental biology 320, 289-301.

Deiner, M.S., Kennedy, T.E., Fazeli, A., Serafini, T., Tessier-Lavigne, M., Sretavan, D.W., 1997. Netrin-1 and DCC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia. Neuron 19, 575-589.

Della Fazia, M.A., Servillo, G., Sassone-Corsi, P., 1997. Cyclic AMP signalling and cellular proliferation: regulation of CREB and CREM. FEBS Letters 410, 22-24.

Dent, E.W., Barnes, A.M., Tang, F., Kalil, K., 2004. Netrin-1 and semaphorin 3A promote or inhibit cortical axon branching, respectively, by reorganization of the

cytoskeleton. The Journal of neuroscience : the official journal of the Society for Neuroscience 24, 3002-3012.

Deo, R.C., Schmidt, E.F., Elhabazi, A., Togashi, H., Burley, S.K., Strittmatter, S.M., 2004. Structural bases for CRMP function in plexin-dependent semaphorin3A signaling. The EMBO Journal 23, 9-22.

Dessaud, E., McMahon, A.P., Briscoe, J., 2008. Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. Development (Cambridge, England) 135, 2489-2503.

Diez-Roux, G., Banfi, S., Sultan, M., Geffers, L., Anand, S., Rozado, D., Magen, A., Canidio, E., Pagani, M., Peluso, I., Lin-Marq, N., Koch, M., Bilio, M., Cantiello, I., Verde, R., De Masi, C., Bianchi, S.A., Cicchini, J., Perroud, E., Mehmeti, S., Dagand, E., Schrinner, S., Nurnberger, A., Schmidt, K., Metz, K., Zwingmann, C., Brieske, N., Springer, C., Hernandez, A.M., Herzog, S., Grabbe, F., Sieverding, C., Fischer, B., Schrader, K., Brockmeyer, M., Dettmer, S., Helbig, C., Alunni, V., Battaini, M.A., Mura, C., Henrichsen, C.N., Garcia-Lopez, R., Echevarria, D., Puelles, E., Garcia-Calero, E., Kruse, S., Uhr, M., Kauck, C., Feng, G., Milyaev, N., Ong, C.K., Kumar, L., Lam, M., Semple, C.A., Gyenesei, A., Mundlos, S., Radelof, U., Lehrach, H., Sarmientos, P., Reymond, A., Davidson, D.R., Dolle, P., Antonarakis, S.E., Yaspo, M.L., Martinez, S., Baldock, R.A., Eichele, G., Ballabio, A., 2011. A high-resolution anatomical atlas of the transcriptome in the mouse embryo. PLoS Biol 9, e1000582.

Dijkmans, T.F., van Hooijdonk, L.W., Fitzsimons, C.P., Vreugdenhil, E., 2010. The doublecortin gene family and disorders of neuronal structure. Central nervous system agents in medicinal chemistry 10, 32-46.

Dingar, D., Konecny, F., Zou, J., Sun, X., von Harsdorf, R., 2012. Anti-apoptotic function of the E2F transcription factor 4 (E2F4)/p130, a member of retinoblastoma gene family in cardiac myocytes. Journal of molecular and cellular cardiology 53, 820-828.

Dooley, K., Zon, L.I., 2000. Zebrafish: a model system for the study of human disease. Current opinion in genetics & development 10, 252-256.

Dorsky, R.I., Chang, W.S., Rapaport, D.H., Harris, W.A., 1997. Regulation of neuronal diversity in the Xenopus retina by Delta signalling. Nature 385, 67-70.

Driever, W., Solnica-Krezel, L., Schier, A.F., Neuhauss, S.C., Malicki, J., Stemple, D.L., Stainier, D.Y., Zwartkruis, F., Abdelilah, S., Rangini, Z., Belak, J., Boggs, C., 1996. A genetic screen for mutations affecting embryogenesis in zebrafish. Development (Cambridge, England) 123, 37-46.

Ebert, A.M., Childs, S.J., Hehr, C.L., Cechmanek, P.B., McFarlane, S., 2014. Sema6a and Plxna2 mediate spatially regulated repulsion within the developing eye to promote eye vesicle cohesion. Development (Cambridge, England) 141, 2473-2482.

Ebert, A.M., Lamont, R.E., Childs, S.J., McFarlane, S., 2012. Neuronal expression of class 6 semaphorins in zebrafish. Gene expression patterns : GEP 12, 117-122.

Eldred, M.K., Charlton-Perkins, M., Muresan, L., Harris, W.A., 2017. Self-organising aggregates of zebrafish retinal cells for investigating mechanisms of neural lamination. Development (Cambridge, England) 144, 1097.

Emerson, S.E., Light, S.E., Ebert, A.M., 2018. Neuronal expression patterns of the PlexinA family during zebrafish development. Gene expression patterns : GEP 27, 56-66.

Emerson, S.E., St Clair, R.M., Waldron, A.L., Bruno, S.R., Duong, A., Driscoll, H.E., Ballif, B.A., McFarlane, S., Ebert, A.M., 2017. Identification of target genes downstream

of semaphorin6A/PlexinA2 signaling in zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 246, 539-549.

Engeszer, R.E., Patterson, L.B., Rao, A.A., Parichy, D.M., 2007. Zebrafish in the wild: a review of natural history and new notes from the field. Zebrafish 4, 21-40.

Epstein, J.A., Aghajanian, H., Singh, M.K., 2015. Semaphorin signaling in cardiovascular development. Cell metabolism 21, 163-173.

Facciol, A., Tsang, B., Gerlai, R., 2019. Alcohol exposure during embryonic development: An opportunity to conduct systematic developmental time course analyses in zebrafish. Neuroscience and biobehavioral reviews 98, 185-193.

Fishman, M.C., Stainier, D.Y., Breitbart, R.E., Westerfield, M., 1997. Zebrafish: genetic and embryological methods in a transparent vertebrate embryo. Methods in cell biology 52, 67-82.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M., Dryja, T.P., 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323, 643-646.

Fuhrmann, S., Zou, C., Levine, E.M., 2014. Retinal pigment epithelium development, plasticity, and tissue homeostasis. Experimental eye research 123, 141-150.

Gagliardini, V., Fankhauser, C., 1999. Semaphorin III can induce death in sensory neurons. Molecular and cellular neurosciences 14, 301-316.

Ganguly, A., Yang, H., Sharma, R., Patel, K.D., Cabral, F., 2012. The Role of Microtubules and Their Dynamics in Cell Migration. Journal of Biological Chemistry 287, 43359-43369.

Garcia, G.R., Noyes, P.D., Tanguay, R.L., 2016. Advancements in zebrafish applications for 21st century toxicology. Pharmacology & therapeutics 161, 11-21.

Gherardi, E., Love, C.A., Esnouf, R.M., Jones, E.Y., 2004. The sema domain. Current Opinion in Structural Biology 14, 669-678.

Giordano, S., Corso, S., Conrotto, P., Artigiani, S., Gilestro, G., Barberis, D., Tamagnone, L., Comoglio, P.M., 2002. The semaphorin 4D receptor controls invasive growth by coupling with Met. Nat Cell Biol 4, 720-724.

Glasauer, S.M., Neuhauss, S.C., 2014. Whole-genome duplication in teleost fishes and its evolutionary consequences. Molecular genetics and genomics : MGG 289, 1045-1060.

Goshima, Y., Nakamura, F., Strittmatter, P., Strittmatter, S.M., 1995. Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. Nature 376, 509-514.

Grati, M.h., Chakchouk, I., Ma, Q., Bensaid, M., Desmidt, A., Turki, N., Yan, D., Baanannou, A., Mittal, R., Driss, N., Blanton, S., Farooq, A., Lu, Z., Liu, X.Z., Masmoudi, S., 2015. A missense mutation in DCDC2 causes human recessive deafness DFNB66, likely by interfering with sensory hair cell and supporting cell cilia length regulation. Human Molecular Genetics 24, 2482-2491.

Gu, C., Giraudo, E., 2013. The role of semaphorins and their receptors in vascular development and cancer. Experimental cell research 319, 1306-1316.

Haffter, P., Granato, M., Brand, M., Mullins, M.C., Hammerschmidt, M., Kane, D.A., Odenthal, J., van Eeden, F.J., Jiang, Y.J., Heisenberg, C.P., Kelsh, R.N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C., Nusslein-Volhard, C., 1996. The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development (Cambridge, England) 123, 1-36.

Haklai-Topper, L., Mlechkovich, G., Savariego, D., Gokhman, I., Yaron, A., 2010a. Cis interaction between Semaphorin6A and Plexin-A4 modulates the repulsive response to Sema6A. The EMBO Journal 29, 2635-2645.

Haklai-Topper, L., Mlechkovich, G., Savariego, D., Gokhman, I., Yaron, A., 2010b. Cis interaction between Semaphorin6A and Plexin-A4 modulates the repulsive response to Sema6A. The EMBO journal 29, 2635-2645.

Halder, G., Callaerts, P., Gehring, W.J., 1995. Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila. Science (New York, N.Y.) 267, 1788-1792.

Hamburger, V., 1969. Hans Spemann and the organizer concept. Experientia 25, 1121-1125.

Hamilton, F., Swaine, J., 1822. An account of the fishes found in the river Ganges and its branches. Hurst, Robinson, and Co., Edinburgh :.

Hammerschmidt, M., Bitgood, M.J., McMahon, A.P., 1996. Protein kinase A is a common negative regulator of Hedgehog signaling in the vertebrate embryo. Genes Dev 10, 647-658.

Hartley, D.A., Xu, T.A., Artavanis-Tsakonas, S., 1987. The embryonic expression of the Notch locus of Drosophila melanogaster and the implications of point mutations in the extracellular EGF-like domain of the predicted protein. The EMBO journal 6, 3407-3417.

He, H., Yang, T., Terman, J.R., Zhang, X., 2009. Crystal structure of the plexin A3 intracellular region reveals an autoinhibited conformation through active site sequestration. Proceedings of the National Academy of Sciences of the United States of America 106, 15610-15615.

He, Z., Tessier-Lavigne, M., 1997. Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell 90, 739-751.

Heidemann, S.R., 1996. Cytoplasmic mechanisms of axonal and dendritic growth in neurons. International review of cytology 165, 235-296.

Hemmati-Brivanlou, A., Kelly, O.G., Melton, D.A., 1994. Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. Cell 77, 283-295.

Higashiguchi, Y., Katsuta, K., Minegishi, T., Yonemura, S., Urasaki, A., Inagaki, N., 2016. Identification of a shootin1 isoform expressed in peripheral tissues. Cell and tissue research 366, 75-87.

Hoffman, M.M., Ernst, J., Wilder, S.P., Kundaje, A., Harris, R.S., Libbrecht, M., Giardine, B., Ellenbogen, P.M., Bilmes, J.A., Birney, E., Hardison, R.C., Dunham, I., Kellis, M., Noble, W.S., 2012. Integrative annotation of chromatin elements from ENCODE data. Nucleic Acids Research.

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J.C., Koch, R., Rauch, G.-J., White, S., Chow, W., Kilian, B., Quintais, L.T., Guerra-Assunção, J.A., Zhou, Y., Gu, Y., Yen, J., Vogel, J.-H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S.F., Laird, G.K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliott, D., Threadgold, G., Harden, G., Ware, D., Begum, S., Mortimore, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Lloyd, C., Stevens, C.,

Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D., Collins, J., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J.D., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley, C.M., Ersan-Ürün, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberländer, M., Rudolph-Geiger, S., Teucke, M., Lanz, C., Raddatz, G., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Schuster, S.C., Carter, N.P., Harrow, J., Ning, Z., Herrero, J., Searle, S.M.J., Enright, A., Geisler, R., Plasterk, R.H.A., Lee, C., Westerfield, M., de Jong, P.J., Zon, L.I., Postlethwait, J.H., Nüsslein-Volhard, C., Hubbard, T.J.P., Crollius, H.R., Rogers, J., Stemple, D.L., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498.

Huang, Y., Roelink, H., McKnight, G.S., 2002. Protein kinase A deficiency causes axially localized neural tube defects in mice. J Biol Chem 277, 19889-19896.

Huber, A.B., Kania, A., Tran, T.S., Gu, C., De Marco Garcia, N., Lieberam, I., Johnson, D., Jessell, T.M., Ginty, D.D., Kolodkin, A.L., 2005. Distinct roles for secreted semaphorin signaling in spinal motor axon guidance. Neuron 48, 949-964.

Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B., Speed, T.P., 2003a. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 31, e15.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., Speed, T.P., 2003b. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics (Oxford, England) 4, 249-264.

Ito, T., Ando, H., Suzuki, T., Ogura, T., Hotta, K., Imamura, Y., Yamaguchi, Y., Handa, H., 2010. Identification of a primary target of thalidomide teratogenicity. Science (New York, N.Y.) 327, 1345-1350.

Janssen, B.J., Malinauskas, T., Weir, G.A., Cader, M.Z., Siebold, C., Jones, E.Y., 2012. Neuropilins lock secreted semaphorins onto plexins in a ternary signaling complex. Nature structural & molecular biology 19, 1293-1299.

Jessell, T.M., Bovolenta, P., Placzek, M., Tessier-Lavigne, M., Dodd, J., 1989. Polarity and patterning in the neural tube: the origin and function of the floor plate. Ciba Foundation symposium 144, 255-276; discussion 276-280, 290-255.

Ji, L., Chauhan, V., Flory, M.J., Chauhan, A., 2011. Brain region-specific decrease in the activity and expression of protein kinase A in the frontal cortex of regressive autism. PloS one 6, e23751.

Joore, J., van de Water, S., Betist, M., van den Eijnden-van Raaij, A., Zivkovic, D., 1998. Protein kinase A is involved in the induction of early mesodermal marker genes by activin. Mech Dev 79, 5-15.

Kane, D.A., Kimmel, C.B., 1993. The zebrafish midblastula transition. Development (Cambridge, England) 119, 447-456.

Kang, S., Kumanogoh, A., 2013. Semaphorins in bone development, homeostasis, and disease. Seminars in cell & developmental biology 24, 163-171.

Karlsson, J., von Hofsten, J., Olsson, P.E., 2001. Generating transparent zebrafish: a refined method to improve detection of gene expression during embryonic development. Marine biotechnology (New York, N.Y.) 3, 522-527.

Kawakami, K., 2005. Transposon tools and methods in zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 234, 244-254.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics (Oxford, England) 28, 1647-1649.

Kemp, B.E., Graves, D.J., Benjamini, E., Krebs, E.G., 1977. Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. J Biol Chem 252, 4888-4894.

Kim, C., Xuong, N.-H., Taylor, S.S., 2005. Crystal Structure of a Complex Between the Catalytic and Regulatory (RIα) Subunits of PKA. Science (New York, N.Y.) 307, 690.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 203, 253-310.

Kirschner, L.S., Yin, Z., Jones, G.N., Mahoney, E., 2009. Mouse models of altered protein kinase A signaling. Endocrine-related cancer 16, 773-793.

Kolodkin, A.L., Levengood, D.V., Rowe, E.G., Tai, Y.T., Giger, R.J., Ginty, D.D., 1997. Neuropilin is a semaphorin III receptor. Cell 90, 753-762.

Kolodkin, A.L., Matthes, D.J., Goodman, C.S., 1993. The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell 75, 1389-1399.

Kolpak, A., Zhang, J., Bao, Z.-Z., 2005. Sonic Hedgehog Has a Dual Effect on the Growth of Retinal Ganglion Axons Depending on Its Concentration. The Journal of Neuroscience 25, 3432.

Koppel, A.M., Feiner, L., Kobayashi, H., Raper, J.A., 1997. A 70 Amino Acid Region within the Semaphorin Domain Activates Specific Cellular Response of Semaphorin Family Members. Neuron 19, 531-537.

Kubo, Y., Baba, K., Toriyama, M., Minegishi, T., Sugiura, T., Kozawa, S., Ikeda, K., Inagaki, N., 2015. Shootin1-cortactin interaction mediates signal-force transduction for axon outgrowth. The Journal of cell biology 210, 663-676.

Kumanogoh, A., 2015. Semaphorins. Springer Japan.

Kuo, M.W., Lou, S.W., Chung, B.C., 2014. Hedgehog-PKA signaling and gnrh3 regulate the development of zebrafish gnrh3 neurons. PLoS One 9, e95545.

Kuriyama, S., Mayor, R., 2008. Molecular analysis of neural crest migration. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 363, 1349-1362.

Kuwajima, T., Yoshida, Y., Takegahara, N., Petros, T.J., Kumanogoh, A., Jessell, T.M., Sakurai, T., Mason, C., 2012. Optic chiasm presentation of Semaphorin6D in the context of Plexin-A1 and Nr-CAM promotes retinal axon midline crossing. Neuron 74, 676-690.

Lai, A., Kennedy, B.K., Barbie, D.A., Bertos, N.R., Yang, X.J., Theberge, M.-C., Tsai, S.-C., Seto, E., Zhang, Y., Kuzmichev, A., Lane, W.S., Reinberg, D., Harlow, E., Branton, P.E., 2001. RBP1 Recruits the mSIN3-Histone Deacetylase Complex to the

Pocket of Retinoblastoma Tumor Suppressor Family Proteins Found in Limited Discrete Regions of the Nucleus at Growth Arrest. Molecular and Cellular Biology 21, 2918-2932.

Lamb, T.M., Knecht, A.K., Smith, W.C., Stachel, S.E., Economides, A.N., Stahl, N., Yancopolous, G.D., Harland, R.M., 1993. Neural induction by the secreted polypeptide noggin. Science (New York, N.Y.) 262, 713-718.

Lemmens, K., Bollaerts, I., Bhumika, S., De Groef, L., Van Houcke, J., Darras, V.M., Van Hove, I., Moons, L., 2015. Matrix metalloproteinases as promising regulators of axonal regrowth in the injured adult zebrafish retinotectal system. J Comp Neurol.

Lewis, E.B., 1978. A gene complex controlling segmentation in Drosophila. Nature 276, 565-570.

Liu, J., Zhou, Y., Qi, X., Chen, J., Chen, W., Qiu, G., Wu, Z., Wu, N., 2017. CRISPR/Cas9 in zebrafish: an efficient combination for human genetic diseases modeling. Human genetics 136, 1-12.

Livesey, F.J., Cepko, C.L., 2001. Vertebrate neural cell-fate determination: lessons from the retina. Nature reviews. Neuroscience 2, 109-118.

Lo, L., Dormand, E., Greenwood, A., Anderson, D.J., 2002. Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. Development (Cambridge, England) 129, 1553-1567.

Londin, E.R., Niemiec, J., Sirotkin, H.I., 2005. Chordin, FGF signaling, and mesodermal factors cooperate in zebrafish neural induction. Developmental biology 279, 1-19.

Louro, R., Nakaya, H.I., Paquola, A.C., Martins, E.A., da Silva, A.M., Verjovski-Almeida, S., Reis, E.M., 2004a. RASL11A, member of a novel small monomeric GTPase gene family, is down-regulated in prostate tumors. Biochemical and biophysical research communications 316, 618-627.

Louro, R., Nakaya, H.I., Paquola, A.C.M., Martins, E.A.L., Silva, A.M.d., Verjovski-Almeida, S., Reis, E.M., 2004b. RASL11A, member of a novel small monomeric GTPase gene family, is down-regulated in prostate tumors. Biochemical and Biophysical Research Communications 316, 618-627.

Love, C.A., Harlos, K., Mavaddat, N., Davis, S.J., Stuart, D.I., Jones, E.Y., Esnouf, R.M., 2003. The ligand-binding face of the semaphorins revealed by the high-resolution crystal structure of SEMA4D. Nature structural biology 10, 843-848.

Luo, Y., Raible, D., Raper, J.A., 1993a. Collapsin: A protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75, 217-227.

Luo, Y., Raible, D., Raper, J.A., 1993b. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75, 217-227.

Macdonald, R., Barth, K.A., Xu, Q., Holder, N., Mikkola, I., Wilson, S.W., 1995. Midline signalling is required for Pax gene regulation and patterning of the eyes. Development 121, 3267-3278.

MacDonald, R.B., Randlett, O., Oswald, J., Yoshimatsu, T., Franze, K., Harris, W.A., 2015. Muller glia provide essential tensile strength to the developing retina. The Journal of cell biology 210, 1075-1083.

Marchal, L., Luxardi, G., Thome, V., Kodjabachian, L., 2009. BMP inhibition initiates neural induction via FGF signaling and Zic genes. Proceedings of the National Academy of Sciences of the United States of America 106, 17437-17442.

Masai, I., Yamaguchi, M., Tonou-Fujimori, N., Komori, A., Okamoto, H., 2005. The hedgehog-PKA pathway regulates two distinct steps of the differentiation of retinal

ganglion cells: the cell-cycle exit of retinoblasts and their neuronal maturation. Development 132, 1539-1553.

Matsuoka, R.L., Chivatakarn, O., Badea, T.C., Samuels, I.S., Cahill, H., Katayama, K.-I., Kumar, S.R., Suto, F., Chédotal, A., Peachey, N.S., Nathans, J., Yoshida, Y., Giger, R.J., Kolodkin, A.L., 2011a. Class 5 transmembrane semaphorins control selective Mammalian retinal lamination and function. Neuron 71, 460-473.

Matsuoka, R.L., Nguyen-Ba-Charvet, K.T., Parray, A., Badea, T.C., Chedotal, A., Kolodkin, A.L., 2011b. Transmembrane semaphorin signalling controls laminar stratification in the mammalian retina. Nature 470, 259-263.

McKerracher, L., Chamoux, M., Arregui, C.O., 1996. Role of laminin and integrin interactions in growth cone guidance. Molecular neurobiology 12, 95-116.

Minegishi, T., Uesugi, Y., Kaneko, N., Yoshida, W., Sawamoto, K., Inagaki, N., 2018. Shootin1b Mediates a Mechanical Clutch to Produce Force for Neuronal Migration. Cell reports 25, 624-639.e626.

Miyashita, T., Yeo, S.Y., Hirate, Y., Segawa, H., Wada, H., Little, M.H., Yamada, T., Takahashi, N., Okamoto, H., 2004. PlexinA4 is necessary as a downstream target of Islet2 to mediate Slit signaling for promotion of sensory axon branching. Development (Cambridge, England) 131, 3705-3715.

Moreno-Mateos, M.A., Vejnar, C.E., Beaudoin, J.-D., Fernandez, J.P., Mis, E.K., Khokha, M.K., Giraldez, A.J., 2015. CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. Nature methods 12, 982-988.

Mueller, T., 2005. Atlas of Early Zebrafish Brain Development: A Tool for Molecular Neurogenetics, 1st ed. ed. Elsevier, Amsterdam ; Boston, Mass.

Murakami, Y., Suto, F., Shimizu, M., Shinoda, T., Kameyama, T., Fujisawa, H., 2001. Differential expression of plexin-A subfamily members in the mouse nervous system. Developmental dynamics : an official publication of the American Association of Anatomists 220, 246-258.

Murphree, A.L., Benedict, W.F., 1984. Retinoblastoma: clues to human oncogenesis. Science (New York, N.Y.) 223, 1028.

Muzio, L., Mallamaci, A., 2003. Emx1, emx2 and pax6 in specification, regionalization and arealization of the cerebral cortex. Cerebral cortex (New York, N.Y. : 1991) 13, 641-647.

Nasevicius, A., Ekker, S.C., 2000. Effective targeted gene 'knockdown' in zebrafish. Nature genetics 26, 216-220.

Nedvetsky, P.I., Zhao, X., Mathivet, T., Aspalter, I.M., Stanchi, F., Metzger, R.J., Mostov, K.E., Gerhardt, H., 2016. cAMP-dependent protein kinase A (PKA) regulates angiogenesis by modulating tip cell behavior in a Notch-independent manner. Development 143, 3582.

Negishi, M., Oinuma, I., Katoh, H., 2005. Plexins: axon guidance and signal transduction. Cellular and molecular life sciences : CMLS 62, 1363-1371.

Neufeld, G., Kessler, O., 2008. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. Nature reviews. Cancer 8, 632-645.

Oinuma, I., Ishikawa, Y., Katoh, H., Negishi, M., 2004a. The Semaphorin 4D Receptor Plexin-B1 Is a GTPase Activating Protein for R-Ras. Science (New York, N.Y.) 305, 862-865.

Oinuma, I., Katoh, H., Negishi, M., 2004b. Molecular dissection of the semaphorin 4D receptor plexin-B1-stimulated R-Ras GTPase-activating protein activity and neurite

remodeling in hippocampal neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 24, 11473-11480.

Okuda, Y., Ogura, E., Kondoh, H., Kamachi, Y., 2010. B1 SOX coordinate cell specification with patterning and morphogenesis in the early zebrafish embryo. PLoS genetics 6, e1000936.

Palaisa, K.A., Granato, M., 2007. Analysis of zebrafish sidetracked mutants reveals a novel role for Plexin A3 in intraspinal motor axon guidance. Development 134, 3251-3257.

Papan, C., Campos-Ortega, J.A., 1994. On the formation of the neural keel and neural tube in the zebrafishDanio (Brachydanio) rerio. Roux's archives of developmental biology : the official organ of the EDBO 203, 178-186.

Parichy, D.M., Elizondo, M.R., Mills, M.G., Gordon, T.N., Engeszer, R.E., 2009. Normal table of postembryonic zebrafish development: Staging by externally visible anatomy of the living fish. 238, 2975-3015.

Pascoe, H.G., Wang, Y., Zhang, X., 2015. Structural mechanisms of plexin signaling. Progress in biophysics and molecular biology 118, 161-168.

Pasterkamp, R.J., 2005. R-Ras fills another GAP in semaphorin signalling. Trends in Cell Biology 15, 61-64.

Perala, N., Jakobson, M., Ola, R., Fazzari, P., Penachioni, J.Y., Nymark, M., Tanninen, T., Immonen, T., Tamagnone, L., Sariola, H., 2011. Sema4C-Plexin B2 signalling modulates ureteric branching in developing kidney. Differentiation 81, 81-91.

Perälä, N., Sariola, H., Immonen, T., 2012. More than nervous: The emerging roles of plexins. Differentiation 83, 77-91.

Perälä, N.M., Immonen, T., Sariola, H., 2005. The expression of plexins during mouse embryogenesis. Gene Expression Patterns 5, 355-362.

Perron, M., Boy, S., Amato, M.A., Viczian, A., Koebernick, K., Pieler, T., Harris, W.A., 2003. A novel function for Hedgehog signalling in retinal pigment epithelium differentiation. Development (Cambridge, England) 130, 1565-1577.

Perron, M., Harris, W.A., 2000. Determination of vertebrate retinal progenitor cell fate by the Notch pathway and basic helix-loop-helix transcription factors. Cellular and molecular life sciences : CMLS 57, 215-223.

Pezeron, G., Lambert, G., Dickmeis, T., Strahle, U., Rosa, F.M., Mourrain, P., 2008. Rasl11b knock down in zebrafish suppresses one-eyed-pinhead mutant phenotype. PloS one 3, e1434.

Piccolo, S., Sasai, Y., Lu, B., De Robertis, E.M., 1996. Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell 86, 589-598.

Rall, T.W., Sutherland, E.W., 1958. FORMATION OF A CYCLIC ADENINE RIBONUCLEOTIDE BY TISSUE PARTICLES. Journal of Biological Chemistry 232, 1065-1076.

Ramón y Cajal, S., 1893. La rétine des vertébrés. . Cellule 9, 119–257.

Rembold, M., Loosli, F., Adams, R.J., Wittbrodt, J., 2006. Individual cell migration serves as the driving force for optic vesicle evagination. Science (New York, N.Y.) 313, 1130-1134.

Renaud, J., Chedotal, A., 2014. Time-lapse analysis of tangential migration in Sema6A and PlexinA2 knockouts. Molecular and cellular neurosciences 63, 49-59.

Renaud, J., Kerjan, G., Sumita, I., Zagar, Y., Georget, V., Kim, D., Fouquet, C., Suda, K., Sanbo, M., Suto, F., Ackerman, S.L., Mitchell, K.J., Fujisawa, H., Chedotal, A., 2008. Plexin-A2 and its ligand, Sema6A, control nucleus-centrosome coupling in migrating granule cells. Nature neuroscience 11, 440-449.

Reyes, R., Haendel, M., Grant, D., Melancon, E., Eisen, J.S., 2004. Slow degeneration of zebrafish Rohon-Beard neurons during programmed cell death. 229, 30-41.

Roessler, E., Belloni, E., Gaudenz, K., Jay, P., Berta, P., Scherer, S.W., Tsui, L.C., Muenke, M., 1996. Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. Nat Genet 14, 357-360.

Rohm, B., Rahim, B., Kleiber, B., Hovatta, I., Puschel, A.W., 2000. The semaphorin 3A receptor may directly regulate the activity of small GTPases. FEBS Lett 486, 68-72.

Ross, E.M., Gilman, A.G., 1980. Biochemical properties of hormone-sensitive adenylate cyclase. Annual review of biochemistry 49, 533-564.

Rosslenbroich, V., Dai, L., Baader, S.L., Noegel, A.A., Gieselmann, V., Kappler, J., 2005. Collapsin response mediator protein-4 regulates F-actin bundling. Experimental Cell Research 310, 434-444.

Rünker, A.E., Little, G.E., Suto, F., Fujisawa, H., Mitchell, K.J., 2008. Semaphorin-6A controls guidance of corticospinal tract axons at multiple choice points. Neural Development 3, 34.

Sakai, J.A., Halloran, M.C., 2006. Semaphorin 3d guides laterality of retinal ganglion cell projections in zebrafish. Development (Cambridge, England) 133, 1035-1044.

Sanes, D.H., 2011. Development of the nervous system. Academic Press.

Sarthy, P.V., Lam, D.M., 1978. Biochemical studies of isolated glial (Müller) cells from the turtle retina. The Journal of cell biology 78, 675-684.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L.K., De Robertis, E.M., 1994. Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. Cell 79, 779-790.

Sato-Maeda, M., Obinata, M., Shoji, W., 2008. Position fine-tuning of caudal primary motoneurons in the zebrafish spinal cord. Development (Cambridge, England) 135, 323-332.

Sauer, F.C., 1935. Mitosis in the neural tube. 62, 377-405.

Scheffzek, K., Lautwein, A., Kabsch, W., Ahmadian, M.R., Wittinghofer, A., 1996. Crystal structure of the GTPase-activating domain of human p120GAP and implications for the interaction with Ras. Nature 384, 591-596.

Schmidt, E.F., Strittmatter, S.M., 2007. The CRMP Family of Proteins and Their Role in Sema3A Signaling, in: Pasterkamp, R.J. (Ed.), Semaphorins: Receptor and Intracellular Signaling Mechanisms. Springer New York, New York, NY, pp. 1-11.

Schmidt, R., Strähle, U., Scholpp, S., 2013. Neurogenesis in zebrafish - from embryo to adult. Neural development 8, 3-3.

Schwarz, Q., Waimey, K.E., Golding, M., Takamatsu, H., Kumanogoh, A., Fujisawa, H., Cheng, H.J., Ruhrberg, C., 2008. Plexin A3 and plexin A4 convey semaphorin signals during facial nerve development. Dev Biol 324, 1-9.

Segarra, M., Ohnuki, H., Maric, D., Salvucci, O., Hou, X., Kumar, A., Li, X., Tosato, G., 2012. Semaphorin 6A regulates angiogenesis by modulating VEGF signaling. Blood 120, 4104-4115.

Serini, G., Valdembri, D., Zanivan, S., Morterra, G., Burkhardt, C., Caccavari, F., Zammataro, L., Primo, L., Tamagnone, L., Logan, M., Tessier-Lavigne, M., Taniguchi,

M., Puschel, A.W., Bussolino, F., 2003a. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature 424, 391-397.

Serini, G., Valdembri, D., Zanivan, S., Morterra, G., Burkhardt, C., Caccavari, F., Zammataro, L., Primo, L., Tamagnone, L., Logan, M., Tessier-Lavigne, M., Taniguchi, M., Puschel, A.W., Bussolino, F., 2003b. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature 424, 391-397.

Sheng, T., Chi, S., Zhang, X., Xie, J., 2006. Regulation of Gli1 Localization by the cAMP/Protein Kinase A Signaling Axis through a Site Near the Nuclear Localization Signal. Journal of Biological Chemistry 281, 9-12.

Shi, W., Kumanogoh, A., Watanabe, C., Uchida, J., Wang, X., Yasui, T., Yukawa, K., Ikawa, M., Okabe, M., Parnes, J.R., Yoshida, K., Kikutani, H., 2000a. The Class IV Semaphorin CD100 Plays Nonredundant Roles in the Immune System: Defective B and T Cell Activation in CD100-Deficient Mice. Immunity 13, 633-642.

Shi, W., Kumanogoh, A., Watanabe, C., Uchida, J., Wang, X., Yasui, T., Yukawa, K., Ikawa, M., Okabe, M., Parnes, J.R., Yoshida, K., Kikutani, H., 2000b. The class IV semaphorin CD100 plays nonredundant roles in the immune system: defective B and T cell activation in CD100-deficient mice. Immunity 13, 633-642.

Shim, S.O., Cafferty, W.B., Schmidt, E.C., Kim, B.G., Fujisawa, H., Strittmatter, S.M., 2012. PlexinA2 limits recovery from corticospinal axotomy by mediating oligodendrocyte-derived Sema6A growth inhibition. Molecular and cellular neurosciences 50, 193-200.

Shimada, T., Toriyama, M., Uemura, K., Kamiguchi, H., Sugiura, T., Watanabe, N., Inagaki, N., 2008a. Shootin1 interacts with actin retrograde flow and L1-CAM to promote axon outgrowth. The Journal of cell biology 181, 817-829.

Shimada, T., Toriyama, M., Uemura, K., Kamiguchi, H., Sugiura, T., Watanabe, N., Inagaki, N., 2008b. Shootin1 interacts with actin retrograde flow and L1-CAM to promote axon outgrowth. The Journal of Cell Biology 181, 817-829.

Smyth, G.K., 2005. limma: Linear Models for Microarray Data, in: Gentleman, R., Carey, V.J., Huber, W., Irizarry, R.A., Dudoit, S. (Eds.), Bioinformatics and Computational Biology Solutions Using R and Bioconductor. Springer New York, New York, NY, pp. 397-420.

Somers, G.F., 1960. Pharmacological properties of thalidomide (alpha-phthalimido glutarimide), a new sedative hypnotic drug. British journal of pharmacology and chemotherapy 15, 111-116.

Speidel, C.C., 1942. Adjustments of Nerve Endings: Harvey Lecture, January 16, 1941. Bulletin of the New York Academy of Medicine 18, 625-653.

St Clair, R.M., Emerson, S.E., D'Elia, K.P., Weir, M.E., Schmoker, A.M., Ebert, A.M., Ballif, B.A., 2018. Fyn-dependent phosphorylation of PlexinA1 and PlexinA2 at conserved tyrosines is essential for zebrafish eye development. The FEBS journal 285, 72-86.

Stenkamp, D.L., Frey, R.A., Prabhudesai, S.N., Raymond, P.A., 2000. Function for Hedgehog Genes in Zebrafish Retinal Development. Developmental biology 220, 238-252.

Strauss, O., 2005. The Retinal Pigment Epithelium in Visual Function. 85, 845-881.

Streisinger, G., Walker, C., Dower, N., Knauber, D., Singer, F., 1981. Production of clones of homozygous diploid zebra fish (Brachydanio rerio). Nature 291, 293.

Stuart, G.W., McMurray, J.V., Westerfield, M., 1988. Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. Development (Cambridge, England) 103, 403-412.

Sun, L.O., Jiang, Z., Rivlin-Etzion, M., Hand, R., Brady, C.M., Matsuoka, R.L., Yau, K.-W., Feller, M.B., Kolodkin, A.L., 2013a. On and off retinal circuit assembly by divergent molecular mechanisms. Science (New York, N.Y.) 342, 1241974-1241974.

Sun, L.O., Jiang, Z., Rivlin-Etzion, M., Hand, R., Brady, C.M., Matsuoka, R.L., Yau, K.W., Feller, M.B., Kolodkin, A.L., 2013b. On and off retinal circuit assembly by divergent molecular mechanisms. Science (New York, N.Y.) 342, 1241974.

Suto, F., Ito, K., Uemura, M., Shimizu, M., Shinkawa, Y., Sanbo, M., Shinoda, T., Tsuboi, M., Takashima, S., Yagi, T., Fujisawa, H., 2005. Plexin-A4 Mediates Axon-Repulsive Activities of Both Secreted and Transmembrane Semaphorins and Plays Roles in Nerve Fiber Guidance. The Journal of Neuroscience 25, 3628.

Suto, F., Tsuboi, M., Kamiya, H., Mizuno, H., Kiyama, Y., Komai, S., Shimizu, M., Sanbo, M., Yagi, T., Hiromi, Y., Chedotal, A., Mitchell, K.J., Manabe, T., Fujisawa, H., 2007. Interactions between plexin-A2, plexin-A4, and semaphorin 6A control lamina-restricted projection of hippocampal mossy fibers. Neuron 53, 535-547.

Takahashi, T., Strittmatter, S.M., 2001. Plexina1 autoinhibition by the plexin sema domain. Neuron 29, 429-439.

Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G.I., Song, H., Chedotal, A., Winberg, M.L., Goodman, C.S., Poo, M., Tessier-Lavigne, M., Comoglio, P.M., 1999. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99, 71-80.

Tanaka, H., Maeda, R., Shoji, W., Wada, H., Masai, I., Shiraki, T., Kobayashi, M., Nakayama, R., Okamoto, H., 2007. Novel mutations affecting axon guidance in zebrafish and a role for plexin signalling in the guidance of trigeminal and facial nerve axons. Development 134, 3259-3269.

Tawarayama, H., Yoshida, Y., Suto, F., Mitchell, K.J., Fujisawa, H., 2010. Roles of Semaphorin-6B and Plexin-A2 in Lamina-Restricted Projection of Hippocampal Mossy Fibers. The Journal of neuroscience : the official journal of the Society for Neuroscience 30, 7049-7060.

Thisse, C., Thisse, B., 2008. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat. Protocols 3, 59-69.

Toriyama, M., Kozawa, S., Sakumura, Y., Inagaki, N., 2013. Conversion of a signal into forces for axon outgrowth through Pak1-mediated shootin1 phosphorylation. Current biology : CB 23, 529-534.

Toriyama, M., Shimada, T., Kim, K.B., Mitsuba, M., Nomura, E., Katsuta, K., Sakumura, Y., Roepstorff, P., Inagaki, N., 2006a. Shootin1: a protein involved in the organization of an asymmetric signal for neuronal polarization. The Journal of Cell Biology 175, 147-157.

Toriyama, M., Shimada, T., Kim, K.B., Mitsuba, M., Nomura, E., Katsuta, K., Sakumura, Y., Roepstorff, P., Inagaki, N., 2006b. Shootin1: a protein involved in the organization of an asymmetric signal for neuronal polarization. 175, 147-157.

Toyofuku, T., Yoshida, J., Sugimoto, T., Zhang, H., Kumanogoh, A., Hori, M., Kikutani, H., 2005. FARP2 triggers signals for Sema3A-mediated axonal repulsion. Nature neuroscience 8, 1712-1719.

Toyofuku, T., Zhang, H., Kumanogoh, A., Takegahara, N., Suto, F., Kamei, J., Aoki, K., Yabuki, M., Hori, M., Fujisawa, H., Kikutani, H., 2004. Dual roles of Sema6D in cardiac morphogenesis through region-specific association of its receptor, Plexin-A1, with off-track and vascular endothelial growth factor receptor type 2. Genes Dev 18, 435-447.

Tschernutter, M., Jenkins, S.A., Waseem, N.H., Saihan, Z., Holder, G.E., Bird, A.C., Bhattacharya, S.S., Ali, R.R., Webster, A.R., 2006. Clinical characterisation of a family with retinal dystrophy caused by mutation in the Mertk gene. The British journal of ophthalmology 90, 718-723.

Van Battum, E.Y., Brignani, S., Pasterkamp, R.J., 2015. Axon guidance proteins in neurological disorders. The Lancet. Neurology 14, 532-546.

Visel, A., Thaller, C., Eichele, G., 2004. GenePaint.org: an atlas of gene expression patterns in the mouse embryo. Nucleic Acids Res 32, D552-556.

Vitorino, M., Jusuf, P.R., Maurus, D., Kimura, Y., Higashijima, S., Harris, W.A., 2009. Vsx2 in the zebrafish retina: restricted lineages through derepression. Neural development 4, 14.

Vogel, A.M., Weinstein, B.M., 2000. Studying Vascular Development in the Zebrafish. Trends in Cardiovascular Medicine 10, 352-360.

Wald, G., 1945. The chemical evolution of vision. Harvey lectures 41, 117-160.

Walsh, D.A., Perkins, J.P., Krebs, E.G., 1968. An Adenosine 3',5'-Monophosphate-dependant Protein Kinase from Rabbit Skeletal Muscle. Journal of Biological Chemistry 243, 3763-3765.

Walsh, F.S., Meiri, K., Doherty, P., 1997. Cell signalling and CAM-mediated neurite outgrowth. Society of General Physiologists series 52, 221-226.

Walzer, T., Galibert, L., Comeau, M.R., De Smedt, T., 2005. Plexin C1 engagement on mouse dendritic cells by viral semaphorin A39R induces actin cytoskeleton rearrangement and inhibits integrin-mediated adhesion and chemokine-induced migration. Journal of immunology (Baltimore, Md. : 1950) 174, 51-59.

Wang, B., Fallon, J.F., Beachy, P.A., 2000. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell 100, 423-434.

Wang, S., Cepko, C.L., 2016. Photoreceptor Fate Determination in the Vertebrate Retina. Investigative ophthalmology & visual science 57, ORSFe1-6.

Wang, Y., He, H., Srivastava, N., Vikarunnessa, S., Chen, Y.B., Jiang, J., Cowan, C.W., Zhang, X., 2012. Plexins are GTPase-activating proteins for Rap and are activated by induced dimerization. Science signaling 5, ra6.

Wang, Y., Pascoe, H.G., Brautigam, C.A., He, H., Zhang, X., 2013. Structural basis for activation and non-canonical catalysis of the Rap GTPase activating protein domain of plexin. eLife 2, e01279.

Wehner, A.B., Abdesselem, H., Dickendesher, T.L., Imai, F., Yoshida, Y., Giger, R.J., Pierchala, B.A., 2016. Semaphorin 3A is a retrograde cell death signal in developing sympathetic neurons. Development 143, 1560-1570.

Weinstein, D.C., Hemmati-Brivanlou, A., 1999. Neural induction. Annual review of cell and developmental biology 15, 411-433.

Westerfield, M., 2000. The zebrafish book a guide for the laboratory use of zebrafish (Danio rerio).

Westerfield, M., McMurray, J.V., Eisen, J.S., 1986. Identified motoneurons and their innervation of axial muscles in the zebrafish. The Journal of neuroscience : the official journal of the Society for Neuroscience 6, 2267-2277.

White, F.A., Behar, O., 2000. The development and subsequent elimination of aberrant peripheral axon projections in Semaphorin3A null mutant mice. Developmental biology 225, 79-86.

Worzfeld, T., Swiercz, J.M., Senturk, A., Genz, B., Korostylev, A., Deng, S., Xia, J., Hoshino, M., Epstein, J.A., Chan, A.M., Vollmar, B., Acker-Palmer, A., Kuner, R., Offermanns, S., 2014. Genetic dissection of plexin signaling in vivo. Proceedings of the National Academy of Sciences of the United States of America 111, 2194-2199.

Wright, D.E., White, F.A., Gerfen, R.W., Silos-Santiago, I., Snider, W.D., 1995. The guidance molecule semaphorin III is expressed in regions of spinal cord and periphery avoided by growing sensory axons. The Journal of comparative neurology 361, 321-333.

Yanagimachi, R., Harumi, T., Matsubara, H., Yan, W., Yuan, S., Hirohashi, N., Iida, T., Yamaha, E., Arai, K., Matsubara, T., Andoh, T., Vines, C., Cherr, G.N., 2017. Chemical and physical guidance of fish spermatozoa into the egg through the micropyledagger,double dagger. Biology of reproduction 96, 780-799.

Yoshida, Y., Han, B., Mendelsohn, M., Jessell, T.M., 2006. PlexinA1 signaling directs the segregation of proprioceptive sensory axons in the developing spinal cord. Neuron 52, 775-788.

Young, M.D., Willson, T.A., Wakefield, M.J., Trounson, E., Hilton, D.J., Blewitt, M.E., Oshlack, A., Majewski, I.J., 2011. ChIP-seq analysis reveals distinct H3K27me3 profiles that correlate with transcriptional activity. Nucleic Acids Research.

Yu, L., Zhou, Y., Cheng, S., Rao, Y., 2010. Plexin a-semaphorin-1a reverse signaling regulates photoreceptor axon guidance in Drosophila. The Journal of neuroscience : the official journal of the Society for Neuroscience 30, 12151-12156.

Zetterqvist, O., Ragnarsson, U., Humble, E., Berglund, L., Engstrom, L., 1976. The minimum substrate of cyclic AMP-stimulated protein kinase, as studied by synthetic peptides representing the phosphorylatable site of pyruvate kinase (type L) of rat liver. Biochemical and biophysical research communications 70, 696-703.

Zhang, Z., Vuori, K., Wang, H., Reed, J.C., Ruoslahti, E., 1996. Integrin activation by R-ras. Cell 85, 61-69.

Zuber, M.E., Gestri, G., Viczian, A.S., Barsacchi, G., Harris, W.A., 2003. Specification of the vertebrate eye by a network of eye field transcription factors. Development 130, 5155-5167.

7. APPENDIX 1. DEVELOPMENTAL EXPRESSION PATTERNS OF PROTEIN KINASE A CATALYTIC SUBUNITS IN ZEBRAFISH.

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Abbreviations used:

AMP- adenosine monophosphate

AKAPs- A kinase anchoring proteins

cAMP- cyclic AMP

CDK- cyclin dependent kinase

CREB- cAMP response element binding protein

FB- fin buds

FBr- forebrain

HG- hatching gland

Hi- intermediate hypothalamus

INL- inner nuclear layer

lTeO- lateral optic tectum

MB- midbrain

MO- medulla oblongata

NR- neural retina

OPL- outer plexiform layer

OpV- optic vesicles

OV- otic vesicles

PKA- protein kinase A

PRKAC- protein kinase A catalytic subunit

RGC- retinal ganglion cell layer

R subunits- regulatory subunits

Shh- sonic hedgehog

T- thalamus

TeO- optic tectum

Highlights

- *prkac* subunits are expressed throughout the developing zebrafish nervous system.

- Zebrafish have 4 protein kinase A catalytic subunit genes; *prkcαa, αb, βa, and βb*.

- Each *prkac* subunit shows unique expression patterns in zebrafish development.

7.1. Abstract

Protein kinase A (PKA), also known as cAMP dependent protein kinase, is an essential component of many signaling pathways, many of which regulate key developmental processes. Inactive PKA is a tetrameric holoenzyme, comprised of two catalytic (PRKAC), and two regulatory subunits. Upon cAMP binding, the catalytic subunits are released and thereby activated. There are multiple isoforms of PKA catalytic subunits, but their individual roles are not well understood. In order to begin studying their roles in zebrafish development, it is first necessary to identify the spatial and temporal expression profiles for each *prkac* subunit. Here we evaluate the expression profiles for the four zebrafish *prkacs*: *prkacαa*, *αb, βa,* and *βb*, at key developmental time points: 24, 48 and 72 hours post fertilization. We show that zebrafish *prkacs* are expressed throughout the developing nervous system, each showing unique expression patterns. This body of work will inform future functional studies into the roles of PKA during development.

7.2. Introduction

Ordered developmental events are dependent on dynamic, yet tightly regulated cellular signaling environments. Common methods of cellular signal transduction involve the production of second messengers, such as cyclic AMP (cAMP), which propagate signals by further activation of downstream effectors (Rall and Sutherland, 1958). One of the most widely characterized targets of cAMP is cAMP-dependent protein kinase, or protein kinase A (PKA). Initially identified as the mediator of epinephrine stimulation

and a key regulator of metabolic responses, PKA is activated via cAMP in response to a variety of environmental cues and is involved in a vast number of biological systems, including many aspects of development (Kirschner et al., 2009).

PKA exists as tetrameric holoenyzme composed of two regulatory (R) subunits each bound to a catalytic (C) subunit, known as the R_2C_2 complex. In mammalian systems there are four R subunits: RIα, RIβ, RIIα, RIIβ and three C subunits: Cα, Cβ & Cγ (Ji et al., 2011). Cγ is specific to the testis (Beebe et al., 1990), so here we focus on Cα and Cβ. In zebrafish, due to a whole genome duplication event, there are two genes for each *prkac* subunit, *αa*, *αb*, *βa* and *βb*. Multiple factors work together to provide dynamic and sensitive PKA-mediated signaling responses to stimuli. Activation of PKA occurs upon the co-operative binding of two cAMP molecules per R subunit. cAMP binding drives a conformational change, which frees the C subunits from the R_2C_2 complex (Clegg et al., 1987; Kim et al., 2005). Active C subunits can then phosphorylate serine/threonine residues of cytoplasmic (Walsh et al., 1968) and nuclear (Comb et al., 1986) target proteins at exposed R-R-X-S sites (Kemp et al., 1977; Zetterqvist et al., 1976). The persistence, range and localization of cAMP levels are dependent on both production of cAMP by adenylate cyclases (Ross and Gilman, 1980) and degradation by phosphodiesterases, which catalyze the conversion of cAMP to adenosine monophosphate (AMP) (Butcher and Sutherland, 1962). Localization of PKA is achieved through A-kinase anchoring proteins (AKAPs), which anchor PKA regulatory subunits to cellular components (Colledge and Scott, 1999).

PKA plays key roles in the regulation of many essential developmental processes. One of the major targets of PKA are the cAMP response element binding protein (CREB) family of transcription factors, which regulate cellular differentiation and

proliferation in multiple tissues throughout development (Della Fazia et al., 1997). PKA is a negative regulator of Sonic Hedgehog (Shh) signaling, which is crucial for many aspects of development, including embryonic patterning (Hammerschmidt et al., 1996). The careful regulation of Shh signaling events involves posttranslational modifications of downstream Gli transcription factors. PKA binds and phosphorylates Gli1, Gli2 and Gli3 at multiple sites to alter their activity. Gli1 and 2 are typically transcriptional activators, while Gli3 is a repressor. Increased PKA activity has been shown to inhibit Gli1 driven gene transcription via phosphorylation and cytoplasm sequestration (Sheng et al., 2006). PKA is crucial to setting up anterior to posterior gradients of Gli3 in the developing vertebrate limbs to allow for correct patterning (Wang et al., 2000). Disrupted Gli3 gradients can cause human limb defects such as Greig Cephalopolysyndactyly syndrome (GCPS) and Pallister Hall Syndrome (PHS). In zebrafish, increased PKA activity has been shown to decrease differentiation of Hedgehog controlled gonadotrophin-releasing hormone 3 (*gnrh3)* neurons, which are important for reproductive system development (Kuo et al., 2014). In mice, it has been shown that PKA in the endothelium is essential for angiogenesis and vascular development (Nedvetsky et al., 2016). PKA was shown to inhibit retinal neurogenesis progression, upstream of cyclins and cdk (cyclin dependent kinase) inhibitors (Masai et al., 2005). Moreover, Prcacα-/- cβ-/+ or Prcacα -/+ cβ-/- mice result in phenotypes including severe underdevelopment, neural tube defects leading to spina bifida and exencephaly while Prcacα -/- cβ -/- mice lead to increased embryonic mortality (Huang et al., 2002). In addition, mice with deficiencies in PKA catalytic subunit isoforms showed diminished responses to cued fear conditioning and displayed severe brain defects including decreased tissue in the amygdala, hippocampus and cortex (Kirschner et al., 2009). In zebrafish, PKA has also been shown to be involved in activin

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signaling. Activin is a member of the TGF-β superfamily of transmembrane serine/ threonine kinase receptors, and requires PKA for activation of downstream gene targets such as *goosecoid* and *no tail*, which are important for zebrafish mesoderm development (Joore et al., 1998), neuronal maturation and cell-cycle exit of retinoblasts of the developing eye (Masai et al., 2005).

PRKACs are clearly important to many key developmental processes. Although in adult mice the alpha and beta catalytic subunits display tissue specific expression, suggesting that they have divergent roles, there is no data comparing the expression patterns of each subunit throughout early development. Here we use zebrafish to observe and compare the temporal and spatial expression patterns of *prkacs*, We provide expression data for each *prkac* subunit at 24, 48, 72 hours post fertilization (hpf) that will allow future functional studies to be modeled in zebrafish.

7.3. Results

7.3.1. PRKAC Phylogenetic Tree

Full-length amino acid sequences were used to generate a neighbor–joining consensus phylogenetic tree using Geneious R10 software (Kearse et al., 2012) (Fig. 7.1). It can be seen that across species, PRKACαs and PRKACβs cluster independently. Importantly, zebrafish (*D.rerio*) α and β gene duplicate protein products cluster with the respective α and β groups. Full-length protein alignments and percent similarity can be seen in supplemental data (Fig. 7.6).
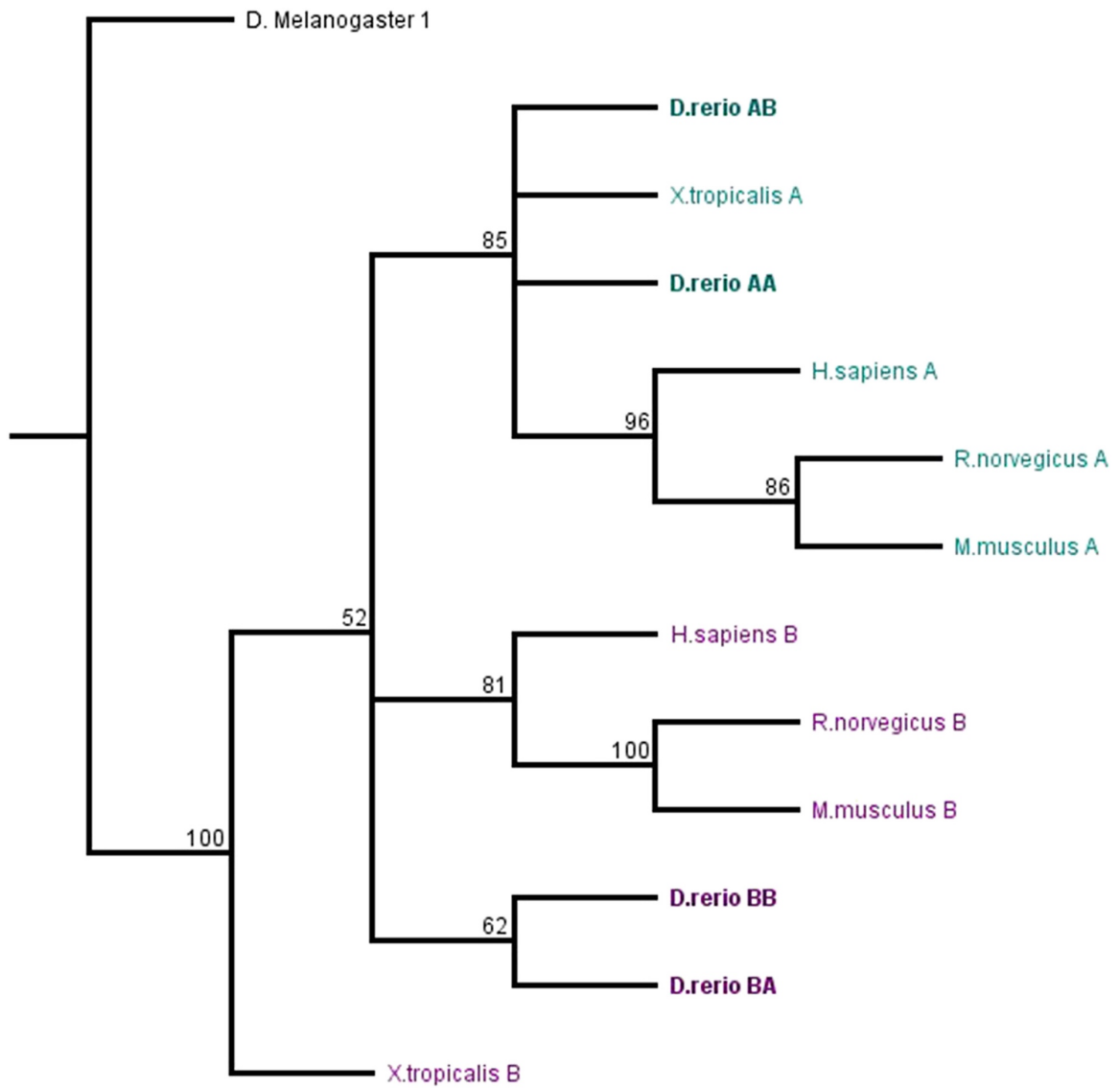
Figure 7.1. PRKAC Phylogeny. Phylogenetic tree based on full-length protein sequence alignments. PRKACα subunits and PRCAβ subunits cluster independently of one another across species. Zebrafish (D. rerio) have duplicated α and β subunits, which cluster consistently with the α and β subunits across other species.

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7.3.2. *prkacαa* Expression

prkacαa is expressed strongly throughout the early developing zebrafish nervous system. From 24 hpf, staining can be seen in the neural retina (NR) adjacent to the lens (Fig. 7.2A''), at 48 hpf *prkacαa* is expressed throughout the retina (Fig. 7.2B'') and at 72 hpf it can be seen in the retinal ganglion cell (RGC) and inner nuclear layers (INL) (Fig. 7.2C''). Expression is also seen in the optic tectum (TeO) at 48 hpf (Fig. 7.2B''). *prkacαa* is expressed throughout the developing thalamus (DT, VT) (Figs. 7.2B'', 7.2C''), midbrain (MB) (Fig. 6.2B'''), and medulla oblongata (MO) (Figs. 7.2B'''', 7.2C'''-7.2C''''). *prkacαa* is expressed in the developing ear, in the otic vesicles (OV) from 24 to 72 hpf (Figs. 7.2A''', 7.2B'''', 7.2C''''). At 24 hpf, staining can be seen in the hatching gland (HG) (Figs. 7.2A-7.2A'), and in the fin buds (FB) at 48 hpf (Fig. 7. 2B').

Figure 7.2. *prkacaa* **Neuronal Expression Patterns.** Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A–C) Whole-mount lateral, (A′-C′) whole-mount dorsal. Brightfield sections (A″-C″) forebrain, (A‴-C‴) midbrain and (B‴‘-C‴‘) hindbrain. Embryos were imaged at different developmental time points. (A-A‴) 24 hpf, (BB‴‘) 48 hpf, (CC‴‘) 72 hpf. Lines in (A–C) indicate locations of the sections shown at that time-point. Hpf-hours post fertilization, NR-neural retina, OV otic vesicle, E−ear, FB fin bud, DT-dorsal thalamus, TeO optic tectum, Popre-optic region, PTv-ventral part of the posterior tuberculum, INL-inner nuclear layer, MB- mid brain, VT-ventral thalamus, RGC- retinal ganglion cell layer, MO- medulla oblongata, n.s-non-specific. Inset shows sense probe control.

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7.3.3. *prkacab* Expression

prkacab is ubiquitously expressed throughout the early developing nervous system, at 24 hpf staining is visible in the forebrain (FBr) (Fig. 7.3A''), and by 48 hpf in the thalamus (T) and medulla oblongata (MO), persisting through to 72 hpf (Figs. 7.3B'', 7.3B'''', 7.3C''' and 7.3C''''). *prkacab* is also expressed in the developing visual system. At 24 hpf, it is expressed throughout the optic vesicles (OpV) (Fig. 7.3A'') and can be seen throughout the retina as it undergoes lamination. At 48hpf, dark staining is seen in the outer plexiform layer (OPL) (Fig. 7.3B'') and the retinal ganglion cell (RGC) and inner nuclear layers (INL) at 72 hpf (Figs. 7.3B'' and 7.3C''). *prkacab* is strongly expressed in the otic vesicles (OV) at 72 hpf (Fig. 7.3C''''). At 48 hpf additional staining is seen in the fin buds (FB) (Fig 7.3B').
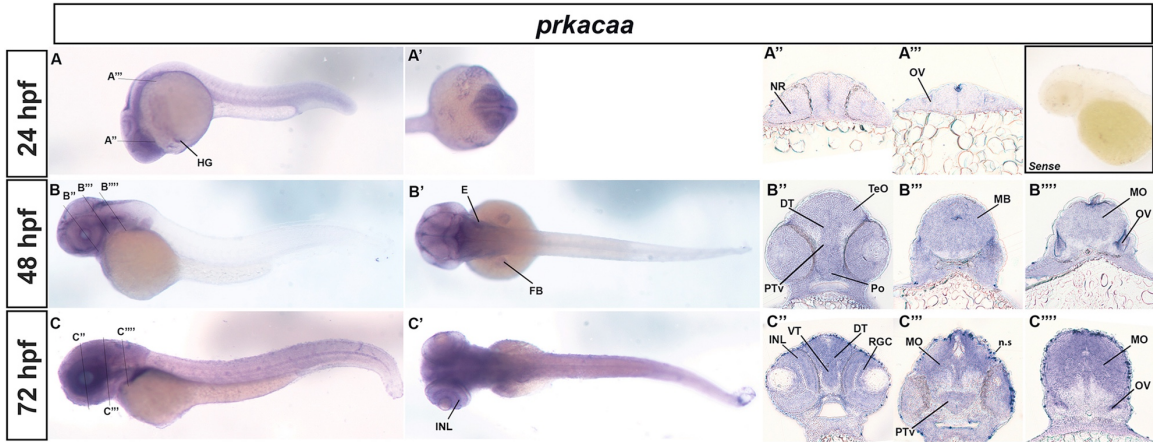
Figure 7.3. *prkacab* **Neuronal Expression Patterns.** Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A–C) Whole-mount lateral, (A′-C′) whole-mount dorsal. Brightfield sections (A″-C″) forebrain, (A‴-C‴) midbrain and (B⁗‘-C⁗‘) hindbrain. Embryos were imaged at different developmental time points. (A-A‴) 24 hpf, (BB⁗‘) 48 hpf, (CC⁗‘) 72 hpf. Lines in (A–C) indicate locations of the sections shown at that time-point. Hpf-hours post fertilization, FB fin bud, OpV- optic vesicle, FBrforebrain, T-thalamus, TeO optic tectum, Popre-optic region, MO- medulla oblongata, INL-inner nuclear layer, RGC- retinal ganglion layer, T-thalamus, OV otic vesicle, n.s-non-specific. Inset shows sense probe control.

7.3.4. *prkacβa* Expression

At 24 hpf, *prkacβa* is expressed diffusely in the early developing eye and ear, in the optic vesicles (OpV) (Fig. 7.4A''), and the otic vesicles (OV) (Fig. 7.4A'''). At 48 hpf, anterior staining can be seen in the developing retinal ganglion cell layer (RGC) of the retina, the lateral optic tectum (lTeO) and lateral forebrain (lfb) (Fig. 7.4B''), more posterior staining is seen in the intermediate hypothalamus (Hi) (Fig. 7.4B''') and the otic vesicles (OV) (Fig. 7.4B''''). Staining persists in the developing eye and ear at 72 hpf and can be seen in the retinal ganglion cell (RGC) and inner nuclear layers (INL) of the retina, the optic tectum (TeO) and the otic vesicles (OV) (Figs. 7.4C'', 7.4C'''). Additional staining is also seen in the developing thalamus (T) at 72 hpf (Fig. 7.4C''), whereas staining is absent from this area earlier in development (Fig. 7.4B''). *prkacβa* is also expressed diffusely throughout the midbrain and medulla oblongata (MO) in the hindbrain at 72 hpf (Fig 7.4C''''). At 24 hpf staining is seen in the hatching gland (HG) (Figs. 7.4A, 7.4A') and in the fin buds (FB) at 48 and 72 hpf (Figs. 7.4B', 7.4C').
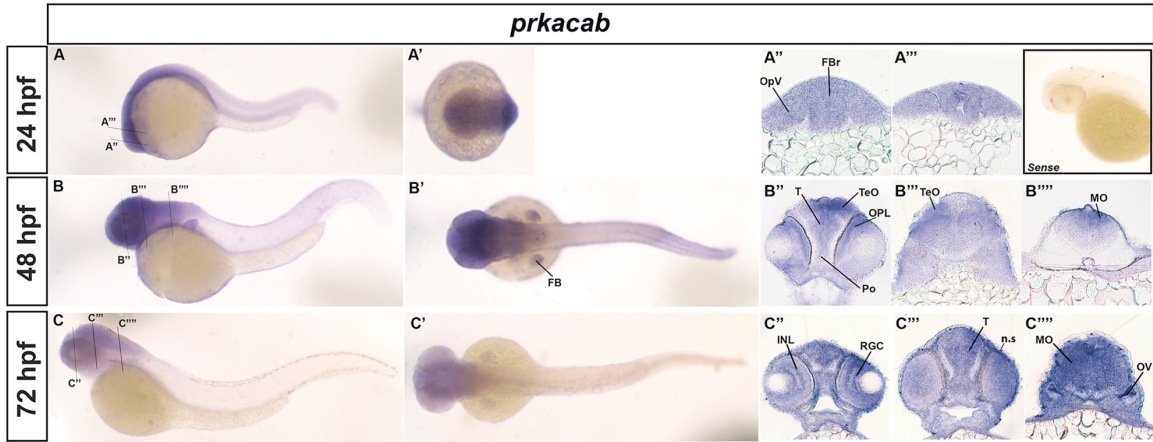
Figure 7.4. *prkacβa* Neuronal Expression Patterns. Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A–C) Whole-mount lateral, (A′-C′) whole-mount dorsal. Brightfield sections (A″-C″) forebrain, (A‴-C‴) midbrain and (B‴′-C‴′) hindbrain. Embryos were imaged at different developmental time points. (A-A‴) 24 hpf, (BB‴′) 48 hpf, (CC‴′) 72 hpf. Lines in (A–C) indicate locations of the sections shown at that time-point. Hpf-hours post fertilization, FB fin bud, OpV- optic vesicle, OV otic vesicle, lTeO- lateral optic tectum, RGC- retinal ganglion cell layer, lfb-lateral forebrain bundle, Hi-intermediate hypothalamus, INL-inner nuclear layer, T-thalamus, TeO optic tectum, MO-medulla oblongata, n.s-non-specific. Inset shows sense probe control.

7.3.5. *prkacβb* Expression

prkacβb is absent from the developing nervous system at 24 hpf (Fig. 7.5A-A'''), but by 48 hpf, it is expressed in the first differentiated retinal ganglion cells (RGCs) in the retina (Fig. 7B''). Additionally, at 48 hpf, *prkacβb* is expressed in the intermediate hypothalamus (Hi) (Fig. 6.5B''') and the developing otic vesicles (OV) (Fig. 7.5B''''). At 72 hpf *prkacβb* is expressed throughout the thalamus (T), and the retina, including the retinal ganglion cell (RGC) and inner nuclear layers (INL) but is absent from the optic tectum (Fig. 7.5C''). Additionally, at 72 hpf, staining is seen throughout the medulla oblongata (MO) (Fig. 7.5C'''-7.5C''''). Expression is also seen in the fin buds (FB) at 48 and 72 hpf (Figs. 7.5B', 7.5C').
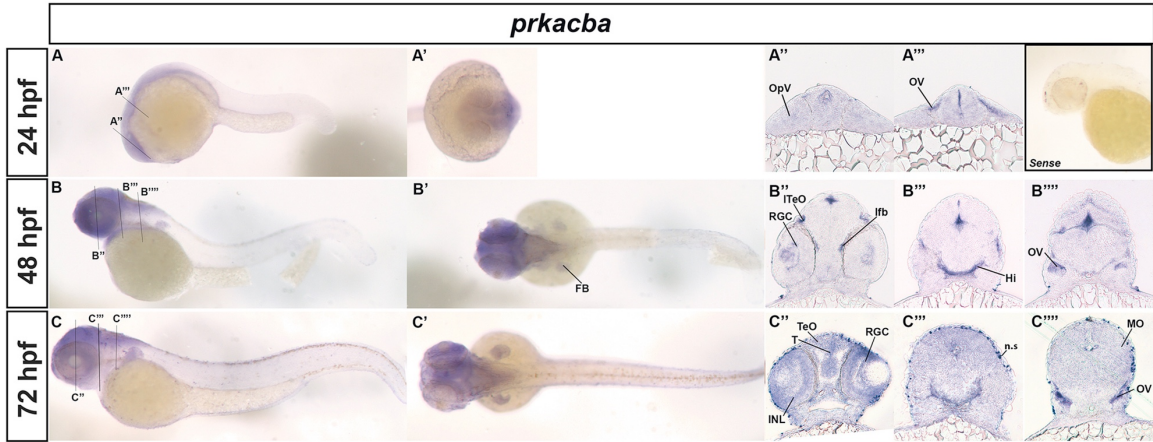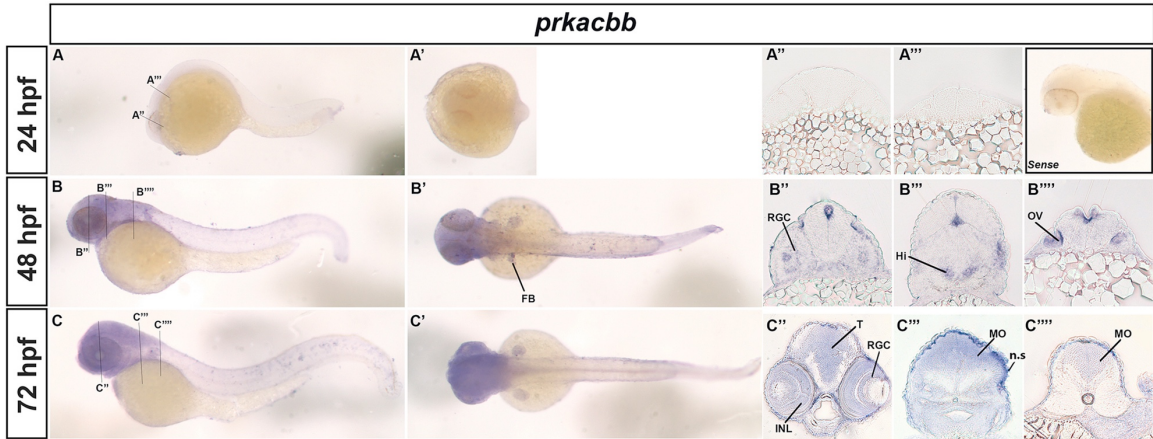
Figure 7.5. *prkacβb* **Neuronal Expression Patterns.** Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A–C) Whole-mount lateral, (A′-C′) whole-mount dorsal. Brightfield sections (A″-C″) forebrain, (A‴-C‴) midbrain and (B‴‘-C‴‘) hindbrain. Embryos were imaged at different developmental time points. (A-A‴) 24 hpf, (BB‴‘) 48 hpf, (CC‴‘) 72 hpf. Lines in (A–C) indicate locations of the sections shown at that time-point. Hpf-hours post fertilization, FB fin bud, RGC- retinal ganglion cell layer, Hi-intermediate hypothalamus, OV otic vesicle, T-thalamus, INL-inner nuclear layer, MO- medulla oblongata, n.s-non-specific. Inset shows sense probe control.

7.4. Discussion

PKA is well documented to be important for many aspects of development, and the limited data available has hinted at divergences in expression between different *prkac* subunits. Here we investigated these possible differences and show that expression patterns of each *prkac* subunit in zebrafish show dynamic and unique expression patterns in development. Of the limited data available for *prkac* subunit expression, it has been shown previously in zebrafish that *prkacab* is expressed in the developing central nervous system at 18 and 20 somites, which matches our findings (Thisse and Thisse 2004), (Fig. 7.3). In mice, expression data exists for *prkacα* and *cβ at E14.5.* Similar to our results, *cα* is expressed strongly throughout the nervous system (Diez-Roux et al., 2011) (Figs. 7.2&6.3), whereas *cβ* shows greater specificity in expression, and is restricted to punctate areas in the brain (Visel et al., 2004), (Figs. 7.4&7.5).

Our results are the first to document *prkac* subunit expression throughout a developmental time-course. We show in that *prkac* subunits are expressed predominantly in the developing nervous system, and that each display important temporal differences in expression patterns. Furthermore, we report that although there is spatial overlap in expression domains between subunits, there are also some unique differences, which suggest possible divergent roles in nervous system development. This body of work will inform future functional studies in the field involving the roles of *PRKAC* subunits in development.

7.5. Experimental Procedures

7.5.1. Zebrafish Husbandry

AB strain zebrafish embryos were raised at 28.5 °C in egg water as described (Fishman et al., 1997). Pigmentation was blocked by addition of 0.003% phenylthiourea (PTU) to embryos at 24 hpf. All procedures were approved by the University of Vermont Animal Care and use committee (IACUC).

7.5.2. Phylogenetic Tree and Protein Alignments

A neighbor-joining consensus tree was generated, based on alignments of the full-length amino acid sequences referenced in Table 6.1, using Geneious R10 (http://www.geneious.com) (Kearse et al., 2012). 'Majority greedy clustering' was used, and the tree was rooted to *D. melanogaster prkac 1*. Numbers indicate bootstraps of percent consensus support. Full-length amino acid alignments and distances were additionally generated using Geneious R10 software (http://www.geneious.com) (Kearse et al., 2012).

Table 7.1. Accession numbers PRKAC full-length amino acid sequences used for

phylogenetic tree and alignments.

Species	Accession number
D. melanogaster 1	NP_995672.1
D.rerio αA	XP_021326630.1
D.rerio αB	NP_001003470.1
D.rerio βA	NP_957317.1
D.rerio βB	XP_005171151
X.tropicalis α	AA170936.1
X.tropicalis β	XP_012815837.1
M. Musculus α	AAR54834.1
M. Musculus β	AARH54533.1
R. Norvegicus α	NP_001094392.1
R. Norvegicus β	NP_001071113.1
H. Sapiens α	XP_016882437.1
H. Sapiens β	XP_006710821.1

7.5.3. Whole-mount *in situ* Hybridization

Zebrafish embryos were staged at 24, 48 and 72 hpf, fixed in 4% paraformaldehyde overnight at 4°C, and stored at -20°C in 100% methanol until use. *In situ* hybridization was performed as described previously (Thisse and Thisse, 2008). Digoxigenin-labeled antisense and sense RNA probes were generated using primers listed in Table 6.2 (IDT Coralville, IA). PCR products were sequence verified at the UVM Cancer Center Advanced Genome Technologies Core, Burlington, VT, prior to probe generation. Probe sequences were analyzed for sequence similarity using BLAST multiple sequence alignment. When probe sequences were individually analyzed using BLAST, only the gene of interest was identified, showing that each probe is gene specific.

Table 7.2. Primer sequences used for PRKAC probe generation

	NCBI Reference Sequence	Forward Primer	Reverse Primer	Probe location (Bp)
PRKA CαA	NM_00108 2840	TTTAGGTGACACTA TAGAAGGGACCAT TTTGAGCGGTTGAA G	TAATACGACTCACTA TAGGGGAGAGGTGC CAGGTACTCAGGTGT	700-1201
PRKA CαB	NM_00100 3470.1	TTTAGGTGACACTA TAGAAGGGGCAGT ACGTTCAGGGAGG AG	TAATACGACTCACTA TAGGGGAGACCGTT CTTGTTTCCGTA	795-1287
PRKA CβA	NM_20102 3.1	TTTAGGTGACACTA TAGAAGGGACAAG CGGTCTCCTTTCCT T	TAATACGACTCACTA TAGGGAGACCGTTCT TGAGGTTTCCGTA	520-1011
PRKA CβB	NM_00103 4976.1	TTTAGGTGACACTA TAGAAGGGCGAAT CGGGAGGTTTAGT GA	TAATACGACTCACAT TAGGGGAGAGTTGT GGCAAACCATTTGTG	426-907

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7.5.4. Sectioning and Imaging

Post *in situ* hybridization embryos were oriented in 4% methyl cellulose on depression slides and imaged in whole-mount using a Nikon SMZ800 dissecting light microscope at 5x magnification. Embryos were dehydrated in 100% ETOH for 2 hours prior to embedding using a JB4 Embedding Kit (Polysciences, Inc., Warrington, PA). Embedded embryos were sectioned on a Leica RM2265 microtome at 0.20μm and resulting sections were heat fixed onto slides for visualization. Brightfield images were obtained using an Olympus iX71 inverted light microscope and figures were assembled using Adobe Photoshop CS6, in which image brightness and contrast were optimized. In order to visualize internal structures distinctly, embryos for sectioning were stained for a longer time period than embryos for whole-mount imaging. This resulted in some superficial non-specific staining at 72 hpf. Anatomical annotations were informed with the use of the zebrafish developmental atlases (Mueller, 2005) and (Bryson-Richardson et al., 2012).

7.6. References

Beebe, S.J., Øyen, O., Sandberg, M., Frøysa, A., Hansson, V., Jahnsen, T., 1990. Molecular Cloning of a Tissue-Specific Protein Kinase (Cγ) from Human Testis—

Representing a Third Isoform for the Catalytic Subunit of cAMP-Dependent Protein Kinase. Molecular Endocrinology 4, 465-475.

Bryson-Richardson, R., Berger, S., Currie, P., 2012. Chapter 5 - 48 Hours, Atlas of Zebrafish Development. Academic Press, San Diego, pp. 33-54.

Butcher, R.W., Sutherland, E.W., 1962. Adenosine 3',5'-Phosphate in Biological Materials: I. PURIFICATION AND PROPERTIES OF CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE AND USE OF THIS ENZYME TO CHARACTERIZE ADENOSINE 3',5'-PHOSPHATE IN HUMAN URINE. Journal of Biological Chemistry 237, 1244-1250.

Clegg, C.H., Correll, L.A., Cadd, G.G., McKnight, G.S., 1987. Inhibition of intracellular cAMP-dependent protein kinase using mutant genes of the regulatory type I subunit. Journal of Biological Chemistry 262, 13111-13119.

Colledge, M., Scott, J.D., 1999. AKAPs: from structure to function. Trends in Cell Biology 9, 216-221.

Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E., Goodman, H.M., 1986. A cyclic AMP- and phorbol ester-inducible DNA element. Nature 323, 353-356.

Della Fazia, M.A., Servillo, G., Sassone-Corsi, P., 1997. Cyclic AMP signalling and cellular proliferation: regulation of CREB and CREM. FEBS Letters 410, 22-24.

Diez-Roux, G., Banfi, S., Sultan, M., Geffers, L., Anand, S., Rozado, D., Magen, A., Canidio, E., Pagani, M., Peluso, I., Lin-Marq, N., Koch, M., Bilio, M., Cantiello, I., Verde, R., De Masi, C., Bianchi, S.A., Cicchini, J., Perroud, E., Mehmeti, S., Dagand, E., Schrinner, S., Nurnberger, A., Schmidt, K., Metz, K., Zwingmann, C., Brieske, N., Springer, C., Hernandez, A.M., Herzog, S., Grabbe, F., Sieverding, C., Fischer, B., Schrader, K., Brockmeyer, M., Dettmer, S., Helbig, C., Alunni, V., Battaini, M.A., Mura,

C., Henrichsen, C.N., Garcia-Lopez, R., Echevarria, D., Puelles, E., Garcia-Calero, E., Kruse, S., Uhr, M., Kauck, C., Feng, G., Milyaev, N., Ong, C.K., Kumar, L., Lam, M., Semple, C.A., Gyenesei, A., Mundlos, S., Radelof, U., Lehrach, H., Sarmientos, P., Reymond, A., Davidson, D.R., Dolle, P., Antonarakis, S.E., Yaspo, M.L., Martinez, S., Baldock, R.A., Eichele, G., Ballabio, A., 2011. A high-resolution anatomical atlas of the transcriptome in the mouse embryo. PLoS Biol 9, e1000582.

Fishman, M.C., Stainier, D.Y., Breitbart, R.E., Westerfield, M., 1997. Zebrafish: genetic and embryological methods in a transparent vertebrate embryo. Methods in cell biology 52, 67-82.

Hammerschmidt, M., Bitgood, M.J., McMahon, A.P., 1996. Protein kinase A is a common negative regulator of Hedgehog signaling in the vertebrate embryo. Genes Dev 10, 647-658.

Huang, Y., Roelink, H., McKnight, G.S., 2002. Protein kinase A deficiency causes axially localized neural tube defects in mice. J Biol Chem 277, 19889-19896.

Ji, L., Chauhan, V., Flory, M.J., Chauhan, A., 2011. Brain region-specific decrease in the activity and expression of protein kinase A in the frontal cortex of regressive autism. PloS one 6, e23751.

Joore, J., van de Water, S., Betist, M., van den Eijnden-van Raaij, A., Zivkovic, D., 1998. Protein kinase A is involved in the induction of early mesodermal marker genes by activin. Mech Dev 79, 5-15.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the

organization and analysis of sequence data. Bioinformatics (Oxford, England) 28, 1647-1649.

Kemp, B.E., Graves, D.J., Benjamini, E., Krebs, E.G., 1977. Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. J Biol Chem 252, 4888-4894.

Kim, C., Xuong, N.-H., Taylor, S.S., 2005. Crystal Structure of a Complex Between the Catalytic and Regulatory (RIα) Subunits of PKA. Science (New York, N.Y.) 307, 690.

Kirschner, L.S., Yin, Z., Jones, G.N., Mahoney, E., 2009. Mouse models of altered protein kinase A signaling. Endocrine-related cancer 16, 773-793.

Kuo, M.W., Lou, S.W., Chung, B.C., 2014. Hedgehog-PKA signaling and gnrh3 regulate the development of zebrafish gnrh3 neurons. PLoS One 9, e95545.

Masai, I., Yamaguchi, M., Tonou-Fujimori, N., Komori, A., Okamoto, H., 2005. The hedgehog-PKA pathway regulates two distinct steps of the differentiation of retinal ganglion cells: the cell-cycle exit of retinoblasts and their neuronal maturation. Development 132, 1539-1553.

Mueller, T., 2005. Atlas of Early Zebrafish Brain Development: A Tool for Molecular Neurogenetics, 1st ed. ed. Elsevier, Amsterdam ; Boston, Mass.

Nedvetsky, P.I., Zhao, X., Mathivet, T., Aspalter, I.M., Stanchi, F., Metzger, R.J., Mostov, K.E., Gerhardt, H., 2016. cAMP-dependent protein kinase A (PKA) regulates angiogenesis by modulating tip cell behavior in a Notch-independent manner. Development 143, 3582.

Rall, T.W., Sutherland, E.W., 1958. FORMATION OF A CYCLIC ADENINE RIBONUCLEOTIDE BY TISSUE PARTICLES. Journal of Biological Chemistry 232, 1065-1076.

Ross, E.M., Gilman, A.G., 1980. Biochemical properties of hormone-sensitive adenylate cyclase. Annual review of biochemistry 49, 533-564.

Sheng, T., Chi, S., Zhang, X., Xie, J., 2006. Regulation of Gli1 Localization by the cAMP/Protein Kinase A Signaling Axis through a Site Near the Nuclear Localization Signal. Journal of Biological Chemistry 281, 9-12.

Thisse, C., Thisse, B., 2008. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat. Protocols 3, 59-69.

Visel, A., Thaller, C., Eichele, G., 2004. GenePaint.org: an atlas of gene expression patterns in the mouse embryo. Nucleic Acids Res 32, D552-556.

Walsh, D.A., Perkins, J.P., Krebs, E.G., 1968. An Adenosine 3',5'-Monophosphate-dependant Protein Kinase from Rabbit Skeletal Muscle. Journal of Biological Chemistry 243, 3763-3765.

Wang, B., Fallon, J.F., Beachy, P.A., 2000. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell 100, 423-434.

Zetterqvist, O., Ragnarsson, U., Humble, E., Berglund, L., Engstrom, L., 1976. The minimum substrate of cyclic AMP-stimulated protein kinase, as studied by synthetic peptides representing the phosphorylatable site of pyruvate kinase (type L) of rat liver. Biochemical and biophysical research communications 70, 696-703.

7.6. Supplemental Material

A
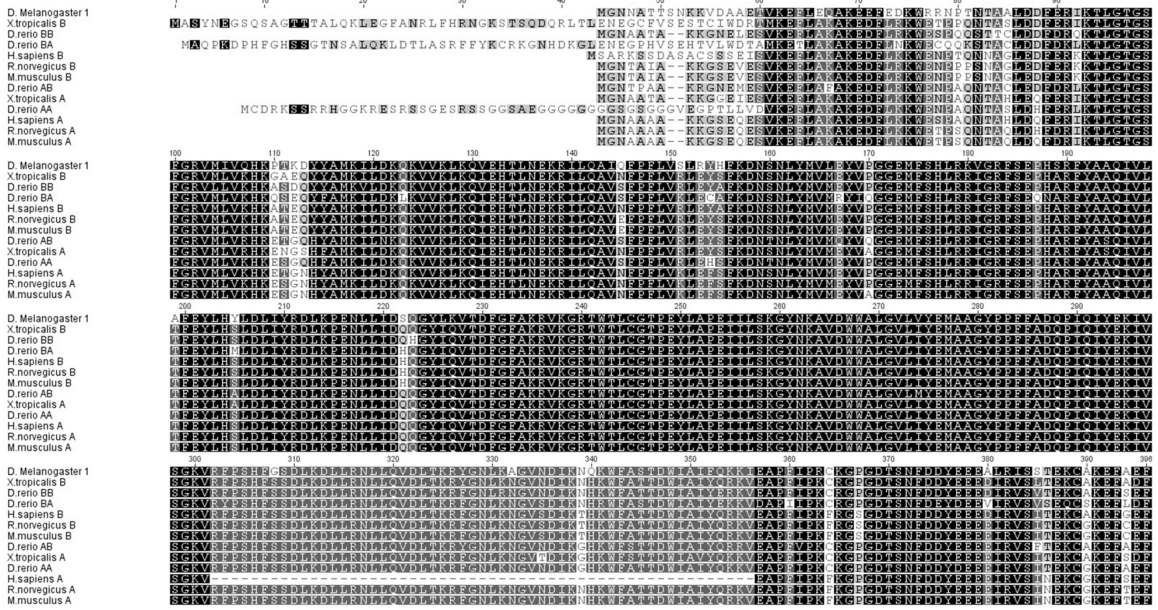
B

	X.tropicalis B	X.tropicalis A	R.norvegicus B	R.norvegicus A	M.musculus B	M.musculus A	H.sapiens B	H.sapiens A	D.rerio BB	D.rerio BA	D.rerio AB	D.rerio AA	D. Melanoga...
X.tropicalis B		87.5%	89.0%	89.0%	89.0%	88.7%	89.3%	73.7%	88.7%	77.2%	87.0%	81.0%	82.4%
X.tropicalis A	87.5%		90.0%	92.0%	90.0%	92.6%	87.5%	79.2%	88.9%	80.2%	90.9%	89.8%	81.3%
R.norvegicus B	89.0%	90.0%		91.7%	100%	91.5%	93.2%	77.2%	90.9%	81.9%	89.2%	88.1%	81.6%
R.norvegicus A	89.0%	92.0%	91.7%		91.7%	99.4%	88.7%	82.3%	90.9%	81.6%	89.2%	90.7%	81.9%
M.musculus B	89.0%	90.0%	100%	91.7%		91.5%	93.2%	77.2%	90.9%	81.9%	89.2%	88.1%	81.6%
M.musculus A	88.7%	92.6%	91.5%	99.4%	91.5%		88.4%	82.3%	90.6%	81.6%	89.2%	90.1%	81.6%
H.sapiens B	89.3%	87.5%	93.2%	88.7%	93.2%	88.4%		74.2%	88.1%	81.6%	86.1%	87.9%	79.9%
H.sapiens A	73.7%	79.2%	77.2%	82.3%	77.2%	82.3%	74.2%		75.8%	67.1%	74.9%	75.6%	69.1%
D.rerio BB	88.7%	88.9%	90.9%	90.9%	90.9%	90.6%	88.1%	75.8%		83.0%	88.6%	87.3%	81.6%
D.rerio BA	77.2%	80.2%	81.9%	81.6%	81.9%	81.6%	81.6%	67.1%	83.0%		79.9%	75.8%	75.1%
D.rerio AB	87.0%	90.9%	89.2%	89.2%	89.2%	89.2%	86.1%	74.9%	88.6%	79.9%		88.1%	79.9%
D.rerio AA	81.0%	89.8%	88.1%	90.7%	88.1%	90.1%	87.9%	75.6%	87.3%	75.8%	88.1%		80.2%
D. Melanogaster 1	82.4%	81.3%	81.6%	81.9%	81.6%	81.6%	79.9%	69.1%	81.6%	75.1%	79.9%	80.2%	

Figure 7.6. Supplementary figure 1. A. Full-length PRKACα and PRKACβ protein sequence alignments across different species, used for phylogenetic tree generation. B. Table of percent similarity between sequences.

8. APPENDIX 2. PROVIDING STUDENT-LED, HYPOTHESIS-DRIVEN ZEBRAFISH MODULES TO UNDERGRADUATE INSTITUTIONS IN VERMONT

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8.1. Abstract

The Zebrafish (*Danio rerio*) is an excellent model system to study development. With large clutch sizes, ease of manipulation, rapid external development, and a range of readily available transgenic lines, zebrafish are a useful tool for applied learning. With the University of Vermont (UVM) being the only undergraduate institution in Vermont with a zebrafish facility, we aimed to share our techniques and tools with neighboring universities and colleges that would otherwise not have access to this scientifically important model organism. Here we describe a student-lead, hypothesis-driven laboratory module that we have successfully employed. The described module acts as a framework for a multiple-week lab that can be adapted to suit different abilities and areas of interest. The lab is based on toxicology screens to observe how different chemicals interfere with embryonic zebrafish development over 72 hours post fertilization. Students learn first-hand how different biological systems should correctly form, and how highly regulated developmental processes are disrupted by exogenous compounds. We have introduced this module to four institutions testing various chemicals: (1) At Johnson State College in Vermont, students tested chemicals found in local mine run off. (2) At UVM, students

focused on chemicals that bypass the water treatment systems. (3) At Saint Michael's College students led an open-ended investigation to test commonly used health, beauty and cleaning products hypothesized to interfere with early developmental processes. (4) At Green Mountain College students tested the developmental effects of Tamoxifen treatment. This outreach program has been successful in its ability to engage students by integrating local ecology and environmental issues with developmental biology. Furthermore, the nature of the module encourages students to work independently and experience the scientific process first-hand.

Key words: Outreach; development; toxicology; Danio rerio; transgenic; fluorescent; microscopy.

8.2. Introduction

The zebrafish (*Danio rerio*) is a powerful vertebrate model organism that is widely used to study various aspects of biology, including toxicology and development (Dooley and Zon, 2000; Garcia et al., 2016). Particular advantages include: (1) external fertilization; (2) large clutch sizes; (3) rapid development of organ systems within 72 hours post fertilization (hpf); (4) small size and ease to raise under laboratory conditions; (5) suitability for genetic manipulation; (6) high genetic homology to humans (approximately 80% of zebrafish genes have human orthologs) (7) availability of numerous transgenic reporter lines; (8) regenerative capabilities of various organ systems; (9) transparency of developing embryos that can be facilitated using phenylthiourea (Karlsson et al., 2001). Within the classroom, developing organ systems can be observed using conventional bright-field microscopy. If transgenic reporter lines

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are used, organ systems can be observed in more detail using fluorescent microscopy. Mutant and transgenic lines are readily available from vendors such as ZIRC (Zebrafish International Resource Center, (www.zebrafish.org) or other zebrafish labs throughout the US.

One of the most convincing arguments that highlight the power and importance of zebrafish toxicology screening for human development has been demonstrated in the context of Thalidomide. Initially tested on rodents, this compound was previously approved for human consumption to alleviate symptoms of nausea and sleeplessness experienced by women during early pregnancy (Somers, 1960). The steep increase of children born with abnormal limbs after the approval of Thalidomide, however, quickly raised concern and led to the ban of the drug for this purpose. The effects of Thalidomide on zebrafish development have since been shown to be similar to those observed in humans and confirmed the teratogenic nature of this drug, especially with regard to abnormal limb development and angiogenesis (Ito et al., 2010). This example is just one of many in zebrafish that have since led to important discoveries with human relevance (Dooley and Zon, 2000). Introducing students to this powerful screening technique who would otherwise not have access to this vertebrate model system is therefore relevant and important. With UVM being the sole undergraduate institution in Vermont with a zebrafish facility, we aimed to share our resources with the wider undergraduate community. In the following article, we outline the learning objectives, experimental design, setup, procedures and provide examples for student-led experiments that have resulted in interesting findings.

8.3. Student Learning Objectives

In this module students learn to conduct, execute and document an independent, hypothesis-driven experiment with special emphasis is on the following topics: (1) understanding of the importance of model organisms, (2) how to perform primary literature research and develop a testable hypothesis, (3) experience handling zebrafish embryos and learn how to identify different developmental stages, (4) design and write an experimental protocol, (5) perform a dose response experiment using a graded series of dilutions, (6) appreciate the importance of experimental controls; (7) identify abnormal developmental phenotypes throughout all developmental stages; (8) utilize bright-field and fluorescence microscopy including image acquisition; (9) compose scientific figures and document findings in form of a lab report.

Our module is based on a systematic toxicology screen, similar to those utilized in high-throughput screening at specialized institutions (Garcia et al., 2016). Fertilized zebrafish eggs are treated with selected chemical compounds to investigate possible effects on development. We have taken our module to cell biology, genetics, neuroscience and developmental biology classes at undergraduate institutions in Vermont, and it can easily be tailored to adjust the level of desired student independence. For example, treating fertilized zebrafish embryos with 1%, 2% or 3% ethanol is a well-known inducer of cyclopia and other developmental abnormalities by 72 hpf (Blader and Strahle, 1998) (Arenzana et al., 2006; Facciol et al., 2019). This experiment can be utilized as a complete stand-alone lab in which all of the learning objectives are met and the outcome is predictable. We have employed this activity to familiarize students with the nature of toxicity screening prior to them designing their own experiments, thus facilitating the effectiveness of their independent, individually designed experiments. The

zebrafish model has also been used to benefit high school students who take Accelerated Program Biology classes and are often required to complete biology-based internships. During their residencies the students carried out basic experimental procedures using fixed transgenic animals, learned anatomical and developmental aspects of the fish, fluorescence microscopy and composed figures of their work to present in their classes. Here we describe how zebrafish development classes can be successfully taught in upper and lower level classrooms as well at the high school level.

8.4. Materials and Methods

8.4.1. Target Student Populations.

Since 2015 we have implemented this module at four different undergraduate institutions and adapted the experimental design for each school to meet course-related requirements. At Northern Vermont University in Johnson, students exposed zebrafish eggs to chemicals found in local mine run off. Students at UVM in Burlington tested the effects of chemicals that bypass the water treatment systems and are thus present in Lake Champlain. In our collaboration with Saint Michael's College in Colchester we have established a regular lab component in which students test chemical compounds that are commonly found in households such as artificial food supplements, cleaners, drugs and cosmetic products including soaps, shampoo and other hair products. At Green Mountain College in Poultney, students tested the developmental effects of treating embryos with a common chemotherapeutic drug, Tamoxifen. In general students are most engaged when they designed, researched, planned, executed, evaluated and presented their own experiments. The integration of transgenic fish lines allows students to study not only

general developmental abnormalities such as head or limb formation but monitor specific organ systems and employ fluorescence microscopy.

This module has been used in upper-level undergraduate Neuroscience and Developmental Biology classes but is adaptable to all levels of undergraduate education. Student numbers per class ranged typically between 15-20, with 2-4 students per group. Students who lacked previous knowledge of microscopy, received assistance with the visualization and image-acquisition process.

8.4.2. Duration and Setup of Lab Module

This lab is usually run over a 4-lab period but can be adapted to a 3-lab module depending on the duration of the individual lab sessions (Table 8.1). In the first lab, students are introduced to zebrafish as a model system. They learn about the relevance behind their experiments, their upcoming schedule and are asked to research and plan experiments they would like to conduct. During this first lab students also study wild type embryos of different developmental stages and learn to identify specific characteristics. Students perform a practice ethanol dose response experiment on wild type embryos and subsequently form groups to discuss their own experiments. In lab 2 students evaluate and document the results of their ethanol experiments using bright-field dissecting microscopes. Students finalize a hypothesis to test and develop a protocol. In the third lab, students set up and start their own experiments. In the fourth lab they image their embryos using bright-field and fluorescence microscopy. Throughout the 72-hour period during which the fish develop students record the development of the embryos at regular time intervals (e.g. every morning and evening). Students summarize their experiments including goal and hypothesis, materials and methods, results and discussion in form of a

lab report. Alternatively, students may be asked to present their experiments in form of a PowerPoint presentation.

8.5. Lab Procedures

8.5.1. Zebrafish Husbandry

All of our breeding stocks are approved by the University of Vermont animal care and use committee (IACUC). Any zebrafish breeding stocks and protocols using embryos kept over 72 hpf require IACUC approval. Through collaboration and outreach, we were able to supply embryos and tools to schools that did not have equipment. Zebrafish can be housed and maintained very easily using basic supplies, and obtained from companies such as ZIRC. Wild type zebrafish can also be purchased at local pet stores. Fish can be kept in a range of freshwater systems from small 25-gallon tanks to fully automated institutional systems. Adult fish are fed a diet of brine shrimp and fish flakes, (Brine shrimp direct, Ogden, UT) and juveniles are fed a larval formula (Zebrafeed <100, Sparos, Olhão, Portugal) from seven days post fertilization to four weeks. Embryos do not require feeding prior to 7 days, as they are provided with nutrition by the yolk, and can be kept in glass petri dishes at 28.5°C. Detailed instructions regarding zebrafish care have been published by (Westerfield, 2000). To enhance the student experience for their own experiments, different transgenic zebrafish lines were used in conjunction with fluorescent microscopy. These lines are genetically modified to drive the expression of fluorescent markers downstream of a desired promoter in order to highlight specific developing systems. To date, we have used two lines of transgenic animals for this module. The SoFa1 transgenic line expresses various fluorescent proteins in the neural retina (Table 2) and is suitable to observe development of the visual system.

Table 8.1. Schedule based on a 4-week lab.

Lab 1.
- **Introduction, including background, schedule and expectations**
- **Students form groups of 2-4**
- **Fixed embryo staging experiments**
- **Ethanol dose response experiment set up**
- **Students leave with an idea of a chemical to test**

Lab 2.
- **Observe ethanol experiment results at 48-72 hpf**
- **Finalize testable hypothesis**
- **Design protocol**
- **Students must arrive at next lab with an approved, written-up protocol**

Lab 3.
- **Perform experiments**

Lab 4.
- **Observe experimental results at 48-72 hpf**
- **Image phenotypes and make figures**
- **Students then generate a lab report**

Table 8.2. Potential biological systems of investigation, time-points of interest, and example transgenic lines.

System	Developmental timeline	Transgenic line	Transgenic expression	Transgenic timeline
Eye	4 somites- eye field separation 12 somites- eye fields are separate and elongated 18 somites- invagination of optic cup begins (Ebert et al., 2014) 30 hpf- retinal ganglion cells differentiate (Ebert et al., 2014) 30-72hpf- retinal layers form (Almeida et al., 2014)	*rx3*:GFP *isl2b*:GFP SoFa1 *Atoh7*:gapRFP *Ptf1a*:cyt:GFP *Crx*:gapGFP	Retinal precursor cells and hypothalamus Retinal ganglion cells and cranial ganglia Retinal ganglion cells, horizontal cells, amacrine cells, photoreceptors Horizontal and amacrine cells Bipolar and photoreceptor	4 somites- 20 hpf 30 hpf- adult 30 hpf- adult
Circulatory system	22 hpf- heart tube begins to beat 30 hpf atria and ventricles form 36 hpf heart loops (Vogel and Weinstein, 2000)	*Kdrl*:mCherry	Blood vessels	5 somites- adult
Peripheral nervous system	24hpf- sensory and motor neurons begin sending axons throughout the trunk Thanks to Christine Beattie, Ohio State and Uwe Strähle, KIT Germany.	*mnx1*:RFP x *ngn1*:GFP	Motor and sensory neurons	48 hpf- adult

This line was generously provided by Bill Harris, University of Cambridge, United Kingdom. We can visualize the peripheral nervous system in transgenic zebrafish lines expressing red fluorescent protein (RFP) in motor neurons and green fluorescent protein (GFP) in sensory neurons (Table 2). The animals were kindly provided by Christine Beattie, Ohio State and Uwe Strähle, KIT Germany.

8.5.2. Fixation and Staging

For initial staging experiments, wild type Tüpfel long fin (TL) embryos were raised in egg water at 28.5 °C and developmentally staged at 1000 cell, shield, 18, 24, 36, 48, 60 or 72 hpf as previously described (Fishman et al., 1997; Kimmel et al., 1995). The embryos were fixed in 4% paraformaldehyde overnight at 4 °C, and stored at −20 °C in 100% methanol until use. Students were given copies of the developmental stage chart published by (Kimmel et al., 1995) to use as a reference.

8.5.3. Dilutions and Treatments

Newly fertilized wild type and transgenic zebrafish embryos were provided by the Ebert Lab at UVM and distributed to student groups at different undergraduate institutions. They were separated into individual wells of a 24 well plate with 6-8 embryos per well. Each student group used a control well containing 500 μl egg water and 3 dose response treatment wells with 500 μl of test chemical diluted in egg water. If the drug had to be diluted in a carrier solution such as alcohol or DMSO, an additional carrier control group was used. Embryos were incubated at 28.5 °C until the appropriate developmental stage. For the ethanol experiment the control wild type embryos were kept in a well with 500 μl egg water. The experimental animals were kept for 24 hours in 500

μl of either 1%, 2% or 3% ethanol diluted in egg water from the "high stage" of development (approx. 3 hpf). After 24 hours the ethanol solution was replaced by regular egg water for the remainder of their embryonic development.

8.5.4. Microscopy and Image Acquisition

Embryos were dechorionated, using #5 Dumont forceps and imaged using bright field dissecting microscopes. Images were either captured via microscope-mounted cameras, or cell phones. Embryos were transferred to glass depression slides for fluorescent imaging. Figures were compiled using Adobe Photoshop.

8.5.5. Euthanasia

Zebrafish were either cooled down in ice water and frozen or treated with a lethal dose of 4% Tricaine for 20 minutes or until all movement ceased, followed by a secondary method of euthanasia by incubation in ice water at 0-4 °C for 20 minutes. Zebrafish should be disposed of according to institutional guidelines.

8.5.6. Pre-Lab Preparation

Fish were separated by sex in mating tanks the over-night before the experiments and 'released' in the morning to mate. After the spawning event embryos were collected in glass petri dishes containing egg water (Westerfield, 2000). A sufficient amount of egg water was prepared for all of the required experimental solutions and controls.

8.6. Materials

Zebrafish embryos (6-8/well per group)

24 well plates

Glass petri dishes

Transfer pipettes

Egg water

Plastic pipettes

Glass beakers

Drugs/ chemicals of choice

100% Ethanol

28.5 °C incubator

Dissecting microscope with image acquisition capabilities

Fluorescent microscope with image acquisition capabilities

Camera

Imaging software (for example: SPOT Version2.1 /Adobe Photoshop CS7) (optional).

8.7. Results

8.7.1. Staging Lab

Students identified fixed zebrafish embryos at different stages up to 72 hpf using the provided developmental key, took images of the embryos and created figures to describe and recognize the developing organs and structures (Fig. 7.1A). These images were also used for comparison with experimental animals to identify developmental abnormalities.

8.7.2. Ethanol-Treated Animals

Animals treated with 1% and 3% ethanol as well as control animals were removed from their chorion 72 hpf. Development was compared between the different groups. The developmental abnormalities in the animals were easily recognized, which makes this lab component very suitable as an initial experiment. Compared to control
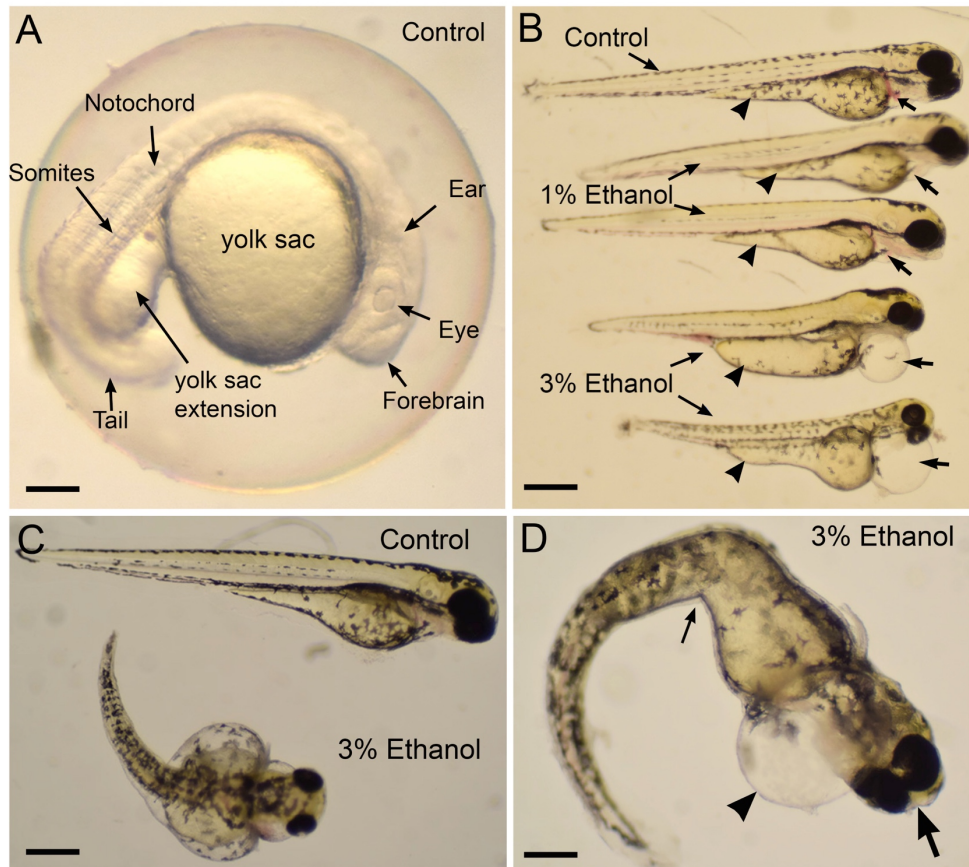
Figure 8.1. Early Development of Zebrafish Embryos (*Danio rerio*). (A) Wild type embryo at approximately 21 hpf shows the differentiation of organ systems such as eyes, ears, brain, somites and the notochord that induces the dorsally located neural tube. (B, C) Comparison of zebrafish 72 hpf that were exposed to either conventional egg water (controls) or egg water containing 1% or 3% ethanol. Compared to controls animals the ethanol-exposed embryos showed stunted growth, enlarged yolk sac extensions (*arrowheads*) and enlarged pericardial sacs (*arrows*). (D) Ethanol exposed animal with malformed spinal column (*small arrow*), enlarged pericardium (*arrowhead*) and narrowing of the eyes (*large arrow*). Scale bars: *A*: 150 μm, *B*: 400 μm, *C*: 320 μm, *D*: 250 μm.

animals, both 1% and 3% embryos showed stunted growth along the anterior posterior axis (Fig. 7.1B-D). Embryos of the 3% ethanol group showed significant abnormalities. Common phenotypic defects seen in compromised embryos included heart and brain edemas, a kinked or shortened body axis, small brains and heads, fin defects, pigmentation defects, small and malformed eyes, and lethality. Additionally, areas of cell death appear black under a dissecting microscope. The students captured images and created figures and described their findings (Fig. 8.1).

8.7.3. Student-Designed Teratogen/Toxicology Experiments

Students at St. Michael's College tested a variety of cleaning products that are commonly used in households and accumulate in wastewater or may be inhaled in small amounts, especially when spray bottles are used. Tested concentration gradients typically varied between 0.001% and 0.1% depending on the chemical. Examples of tested chemicals include: liquid hand soap, air fresheners, surface and window cleaners, artificial food supplements, and medications such as cough or cold syrup.

In some experiments the embryos did not develop, whereas in others no obvious developmental abnormalities could be observed. In experiments where students observed phenotypic abnormalities the most common phenotypes were abnormal development of the spinal column (scoliosis), smaller eyes or enlarged pericardium similar to the ethanol experiments.

In one example where students tested liquid hand soap in 100% of the test wells only the clear chorion of the eggs remained, whereas the developing embryo in the inside had dissolved. The students formulated the hypothesis that the hand soap may have contained proteolytic enzymes. In their report the students discussed that such enzymes
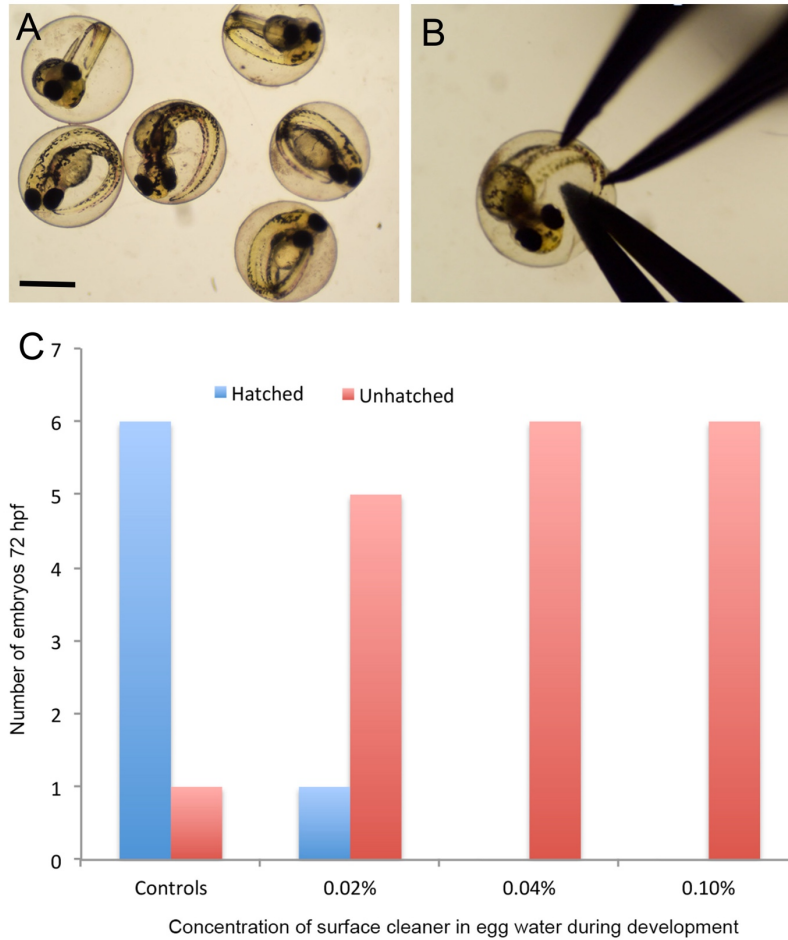
Figure 8.2. Zebrafish Embryos Exposed to 0.02%, 0.04% and 0.1% of Surface Cleaner for 24 hours During Early Development. (A) Unhatched embryos in experimental groups. (B) Removal of the chorion from experimental embryos was performed with fine forceps. (C) Whereas the majority of embryos in the control well hatched, only one embryo in the 0.02% experimental group hatched. All other embryos failed to hatch 120 hpf. Scale bar: 600 μm.

may also be found in laundry detergents and if disposed of into water streams, it may contribute to the decline of species in affected bodies of water. In this case we were unable to test the hypothesis further, however such observations may lead to student research projects at undergraduate institutions. In another example, embryos were exposed to a common brand of household surface cleaner. Initially the embryos did not show any obvious signs of phenotypic developmental abnormalities in any of the tested concentrations (0.02%, 0.04% and 0.1%). However, in the control group, six out of seven embryos (86%) hatched out of their chorion as expected around 72 hpf (Fig. 8.2). In contrast only one of the six embryos in the 0.02% test group and none of the embryos in the 0.04% (n=6) or 0.1% (n=6; Fig. 8.2) hatched. Students formulated several hypotheses for this observation. One hypothesis was that the cleaner caused abnormal muscle development that prevented the animals from moving and hatching out of the chorion. A second hypothesis was that the cleaner interfered with the consistency of the chorion and that enzymes released by the embryos to open the chorion during the hatching process were ineffective. Students tested the muscle hypothesis by opening the chorion manually using fine forceps (Fig. 8.2). All of the embryos showed normal muscle movement, allowing the students to reject the tested hypothesis. When transgenic animals were used, students also used fluorescent microscopy to visualize the transgenic cell lines and investigate whether abnormal development of the relevant organ systems could be observed (Fig. 8.3).

8.7.4. High School Internship Programs

To provide internship opportunities to high school students who are enrolled in AP Biology classes, faculty provided students with fixed sensory and motor transgenic
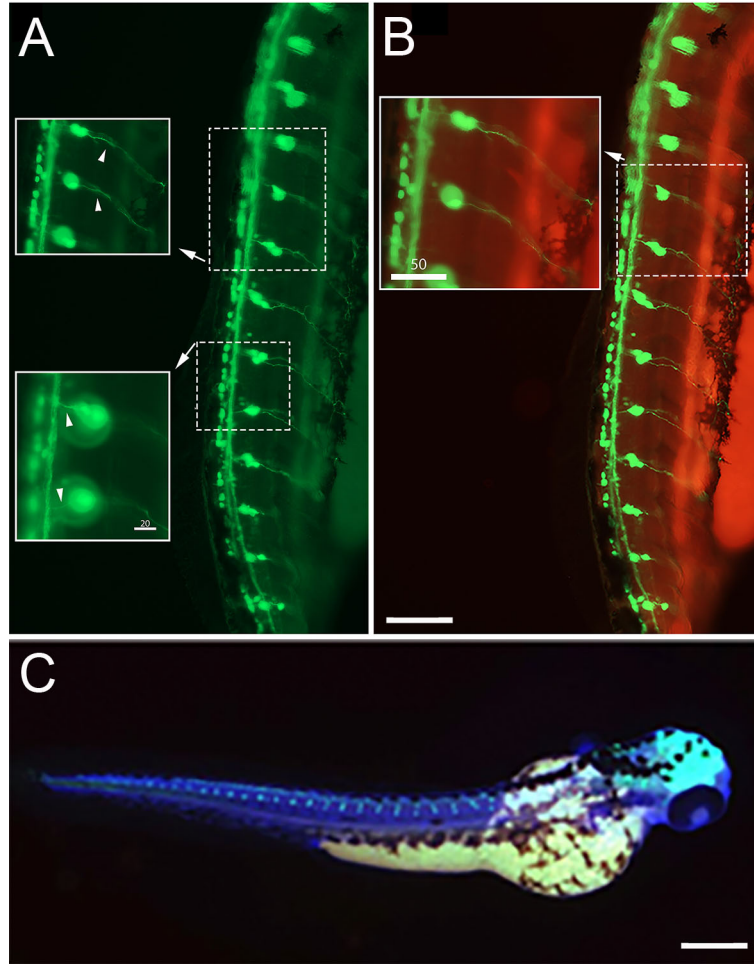
Figure 8.3. Figures of Fluorescence Microscopy of Sensory and Motor Transgenic Zebrafish Imaged and Created by Undergraduate and High School Students. (A, B) Green fluorescent protein expressing sensory neurons are clearly visible in the developing embryos; arrowheads: neurites. Images and figure by Brian Eldridge Saint Michael's College, Class of 2018. (C) Low magnification of embryo that was additionally stained with Hoechst Blue nuclear stain. Image by Dorcas Lohese, Colchester High School, Class of 2018. Scale bars: *(A)* 20 μm, *(B)* inset: 50 μm; 100 μm; *(C)* 600 μm.

zebrafish. Similar to the college program, students first learned about the model system and developmental stages. Students then learned how to perform basic lab procedures such as staining nuclei within cells with a fluorescent blue stain (Hoechst Blue Sigma H 6024). Students learned how to mount the fish for microscopy and investigate the transgenic cell lines (in this case motor neurons in red and sensory neurons in green, (Fig. 8.3) using a florescent Zeiss AxioImager microscope. Students learned how to acquire images and document their findings in form of professional scientific figures.

8.8. Discussion

Our outreach module has proven successful in exposing the benefits of using zebrafish as a model organism for development to undergraduate institutions in Vermont that would otherwise not have access to. Our module can be adapted for each institution depending on the class level, topic and focus. Students can learn about their local environments and investigate potential hazards that may impact ecosystems and the biodiversity around them or have medical implications to human health.

Our goal to promote independent thinking and strengthen the abilities of students to conduct independent research is achieved by this module. In selecting a chemical compound to study, students perform primary literary research and generate a hypothesis to test. They learn how to design appropriate protocols for their experiments, including the use of controls. In evaluating any developmental phenotypes generated by the addition of a chemical compound, students learned bright-field and fluorescent microscopy techniques, how to capture representative images, and generate publication

quality figures to include in a lab report. Our module allows students to design independent experiments, giving them exposure to the process of the scientific method. Cookie-cutter labs allow instructors to plan for well-predicted outcomes, which are sometimes necessary in teaching labs, but it is not always a realistic experience for students. Students should made aware that experiments won't always have the predicted outcome. This is a great opportunity to talk about scientific resilience, troubleshooting and adaptability. These important lessons are hard to teach in a regimented lab, and without them, students can develop unrealistic expectations of scientific research practices.

This module has the potential to include student exposure to thinking about ethical scientific practices. Using an animal model warrants discussion of best practices and the importance of following animal care approved protocols. Ethical data collection can also be discussed, for example, how to capture representative images and how image manipulation can inappropriately be misused.

We have received positive feedback from students and collaborators who experienced this lab module. Through our outreach program, we hope to continue to give more students the opportunity to work with new tools and equipment, and gain skills that they wouldn't otherwise have been exposed to during their undergraduate education.

8.9. References

Almeida, A.D., Boije, H., Chow, R.W., He, J., Tham, J., Suzuki, S.C., Harris, W.A., 2014. Spectrum of Fates: a new approach to the study of the developing zebrafish retina. Development (Cambridge, England) 141, 1971.

Arenzana, F.J., Carvan, M.J., 3rd, Aijon, J., Sanchez-Gonzalez, R., Arevalo, R., Porteros, A., 2006. Teratogenic effects of ethanol exposure on zebrafish visual system development. Neurotoxicology and teratology 28, 342-348.

Blader, P., Strahle, U., 1998. Ethanol impairs migration of the prechordal plate in the zebrafish embryo. Developmental biology 201, 185-201.

Dooley, K., Zon, L.I., 2000. Zebrafish: a model system for the study of human disease. Current opinion in genetics & development 10, 252-256.

Ebert, A.M., Childs, S.J., Hehr, C.L., Cechmanek, P.B., McFarlane, S., 2014. Sema6a and Plxna2 mediate spatially regulated repulsion within the developing eye to promote eye vesicle cohesion. Development (Cambridge, England) 141, 2473-2482.

Facciol, A., Tsang, B., Gerlai, R., 2019. Alcohol exposure during embryonic development: An opportunity to conduct systematic developmental time course analyses in zebrafish. Neuroscience and biobehavioral reviews 98, 185-193.

Fishman, M.C., Stainier, D.Y., Breitbart, R.E., Westerfield, M., 1997. Zebrafish: genetic and embryological methods in a transparent vertebrate embryo. Methods in cell biology 52, 67-82.

Garcia, G.R., Noyes, P.D., Tanguay, R.L., 2016. Advancements in zebrafish applications for 21st century toxicology. Pharmacology & therapeutics 161, 11-21.

Ito, T., Ando, H., Suzuki, T., Ogura, T., Hotta, K., Imamura, Y., Yamaguchi, Y., Handa, H., 2010. Identification of a primary target of thalidomide teratogenicity. Science (New York, N.Y.) 327, 1345-1350.

Karlsson, J., von Hofsten, J., Olsson, P.E., 2001. Generating transparent zebrafish: a refined method to improve detection of gene expression during embryonic development. Marine biotechnology (New York, N.Y.) 3, 522-527.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 203, 253-310.

Somers, G.F., 1960. Pharmacological properties of thalidomide (alpha-phthalimido glutarimide), a new sedative hypnotic drug. British journal of pharmacology and chemotherapy 15, 111-116.

Vogel, A.M., Weinstein, B.M., 2000. Studying Vascular Development in the Zebrafish. Trends in Cardiovascular Medicine 10, 352-360.

Westerfield, M., 2000. The zebrafish book a guide for the laboratory use of zebrafish (Danio rerio).

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