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The role of PLXNA1 in visual system development of *Danio rerio*

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The role of PLXNA1 in visual system development
of *Danio rerio*

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Abstract

Plexin (PLXN) receptors, and their ligands, semaphorins (SEMA), traditionally mediate axon growth in the visual system by facilitating actin cytoskeletal rearrangements and initiating growth cone collapse through intracellular signaling mechanisms. A proposed novel mechanism for the development of the early eye involves the interaction of SEMA6A and PLXNA2 to direct migration and proliferation of retinal precursor cells (Ebert et al., 2014). However, this model is not fully explained by previous data; we propose that a related PLXNA family member, PLXNA1a, also plays a role in these processes. This research uses *in situ* hybridization and antisense morpholino gene knockdown to identify the expression and preliminary functional role of the uncharacterized PLXNA1a receptor in early zebrafish eye development. We hypothesize that *plexinA1a* is expressed in the same neuronal tissues as *plexinA2* and has a compensatory or parallel function.

Chapter 1: Introduction

During formation of the eye, neural plate cells evaginate bilaterally from the neural tissue that will become the brain to form the optic cup and optic stalk. Retinal progenitor cells continue to migrate into the optic cup from the optic stalk during development and will eventually become the neuronal cells that create the laminar structure of the retina (Figure 1) (Gestri et al., 2012; Ali & Sowden, 2011). These progenitor cells are guided to the proper destination within the eye by a multitude of guidance guides. Recently, it was discovered that signaling between SEMA6A and PLXNA2 is crucial for this migration process (Ebert et al., 2014). Proper guidance at this stage, as well as later in development, is critical because it is the basis of the retinotopic organization of the visual system in vertebrates, which creates an accurate perception of the environment from visual stimuli. Adequate proliferation during these early stages of eye development ensures there are enough retinal progenitors to generate the entire eye structure.

Plexins are single transmembrane-spanning receptors; their primary ligands are semaphorins, which are secreted or transmembrane glycoproteins that regulate cell motility (Janssen et al., 2010). 9 different plexins have been identified in vertebrates and categorized into 4 classes (A- D) and 8 classes of semaphorins (semas) have been described (1 being viral) (Waimey & Cheng, 2006; Krueger, Aurandt, & Guan, 2005). These molecules interact as homodimers within a sema binding domain on both proteins (Janssen et al., 2010) (Figure 2). PLXNA1 typically is associated with SEMA3A, 3F, and 6D and PLXNA2 has been associated with SEMA3A however there is a large variability in binding across tissue types and developmental stages (Figure 2) (Murakami et al., 2001).

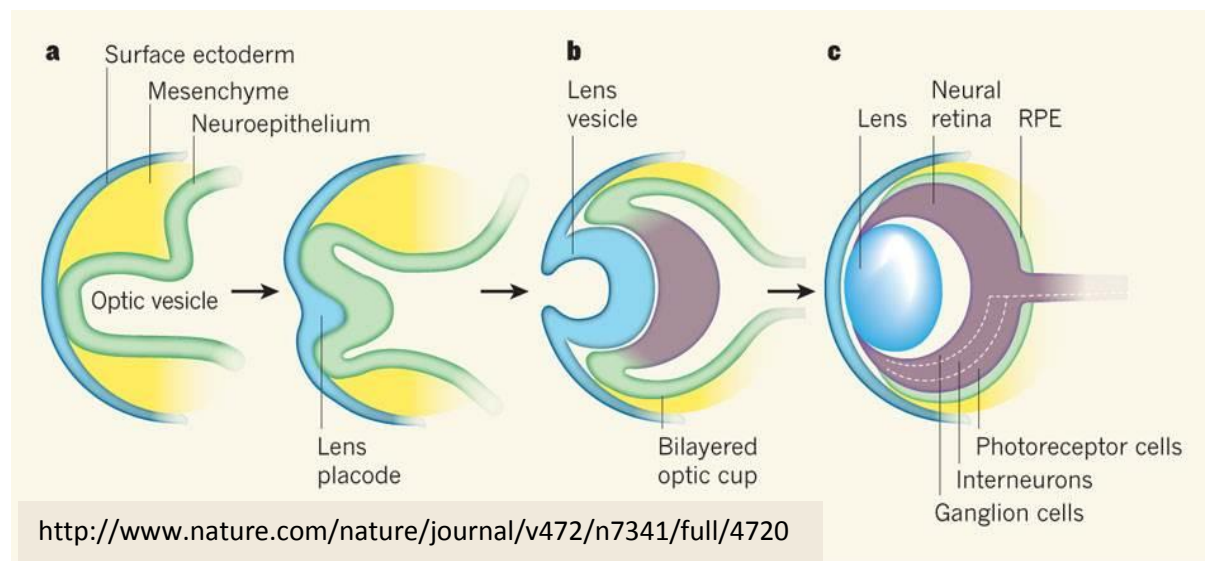
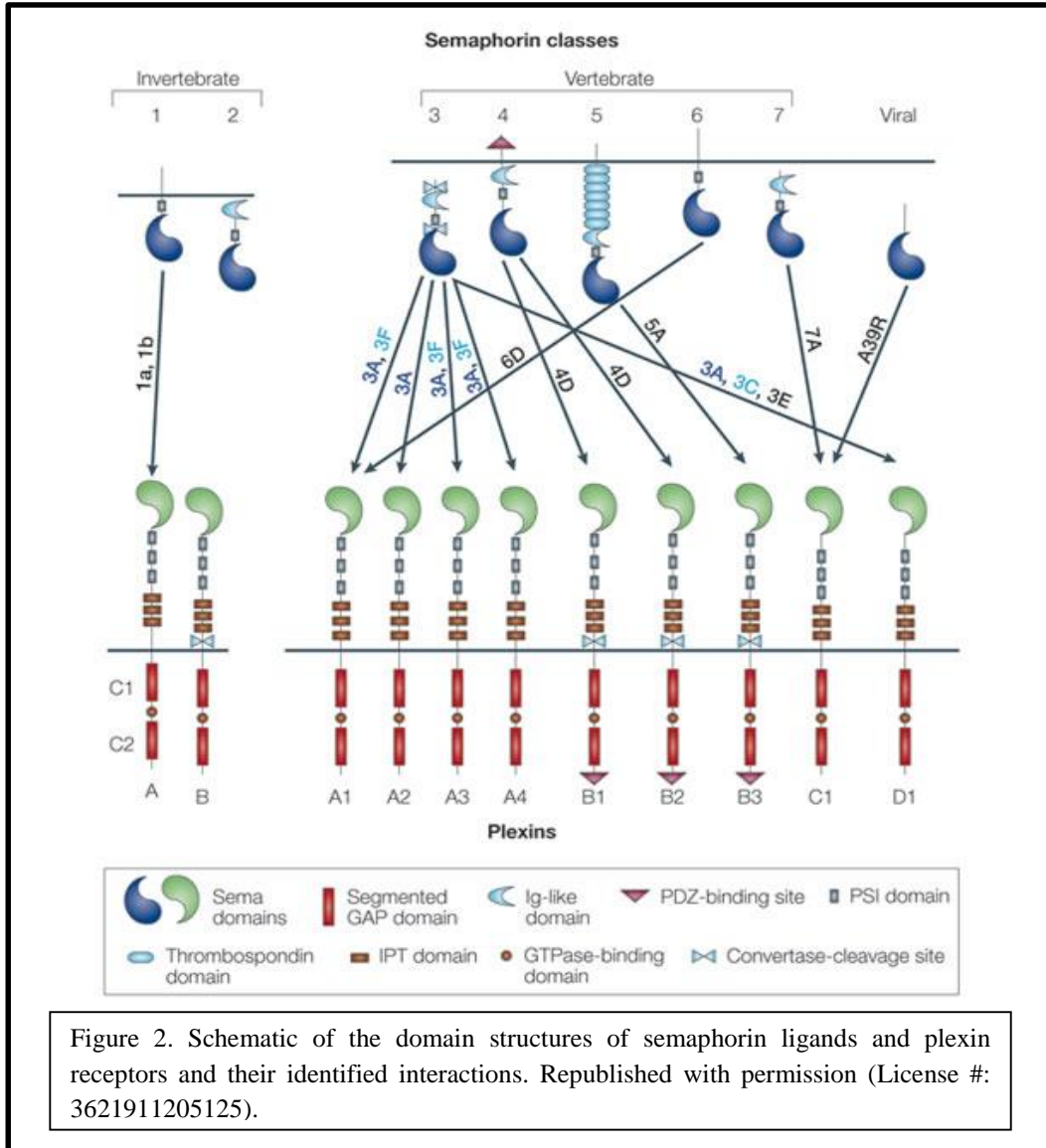


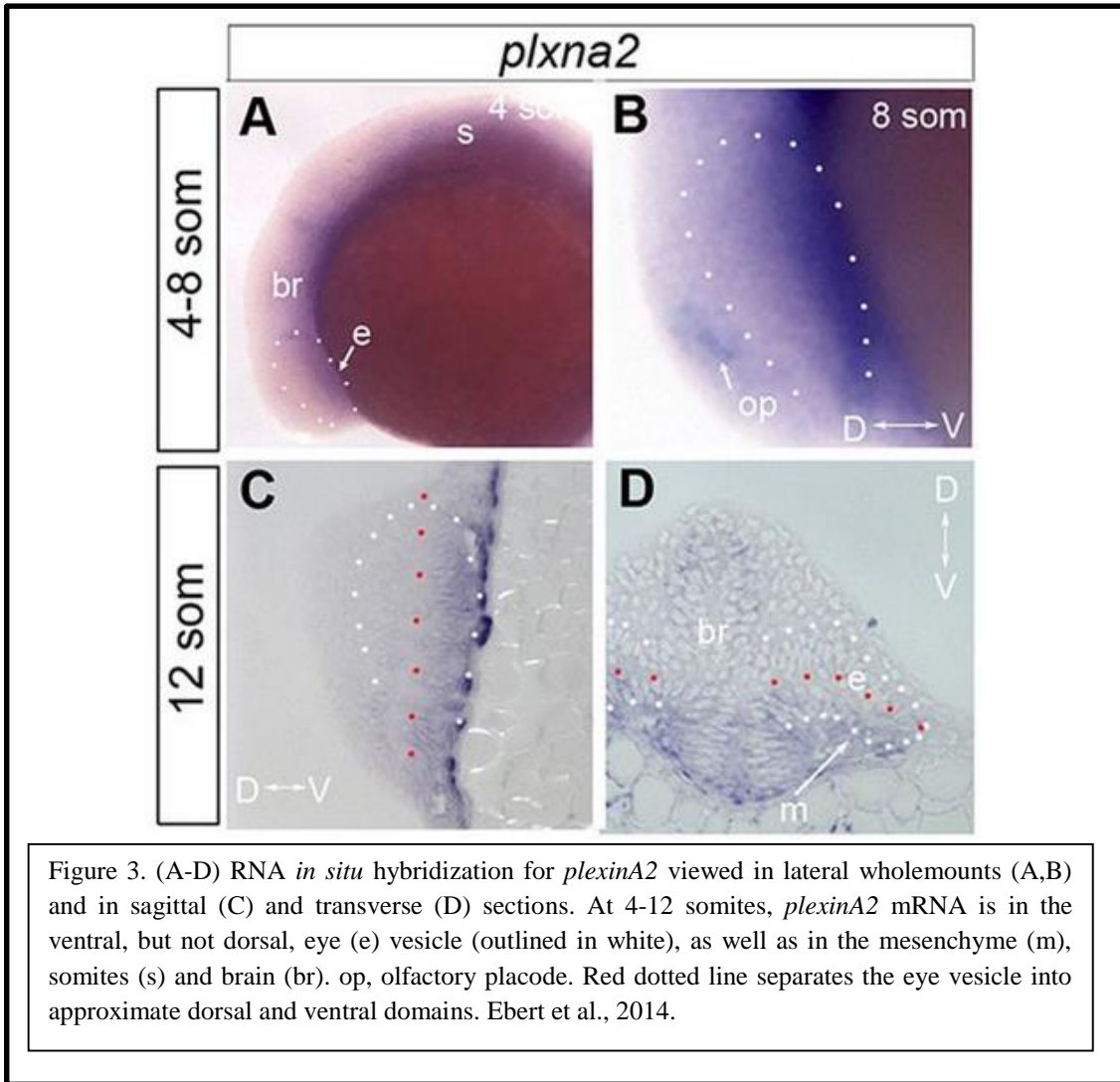
Figure 1: Early eye development in vertebrates. (A) Optic vesicles evaginate from the diencephalon towards the surface ectoderm. (B) Optic vesicles invaginate forming the optic cup and pull in epithelium to form the primitive lens. (C) The developed eye consists of a laminated neural retina containing all neurons and glia, retinal pigmented epithelium (RPE), and lens. Republished with permission (License #: 3621911069173).

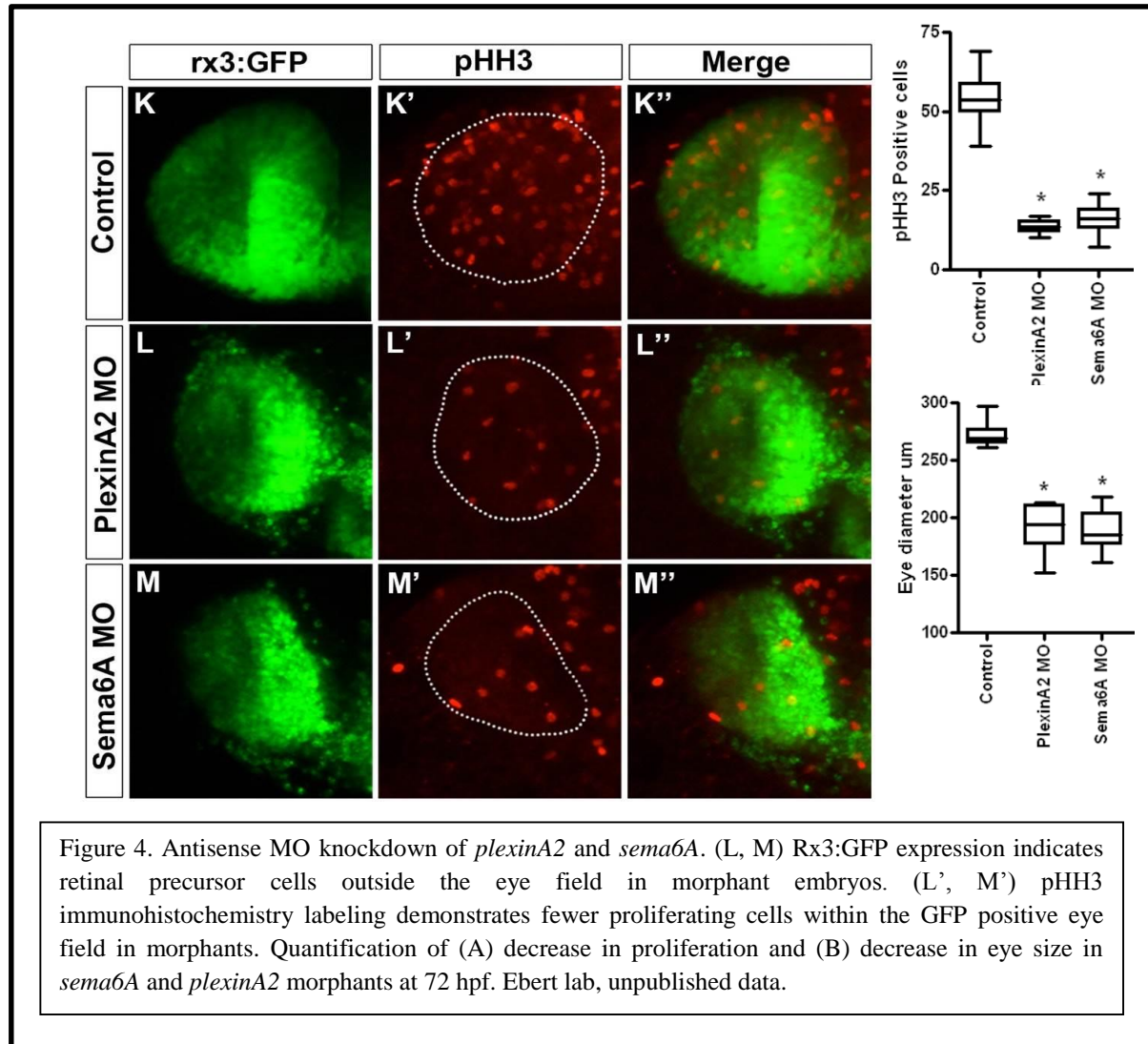


Both semas and plexins are expressed in the developing nervous system in high abundance but their expression is quite low in adult tissues, with the exception of the hippocampus. There is evidence however that they may be involved in regeneration after trauma (Hota & Buck, 2012). Semaphorins traditionally act as repulsive cues in the environment that direct axon growth in the developing nervous system by activating intracellular signaling mechanisms via its interaction with a plexin receptor. The result of this interaction is alteration of the stability of the actin cytoskeleton and initiation of growth cone collapse (Negishi et al., 2005). Activation of Rho-family small GTPases is responsible for this remodeling (Hota & Buck, 2012). Additionally, cell adhesion molecules (CAMs) are often part of this cascade and mediate adhesion and proliferation (Waimey & Cheng, 2006).

Preliminary research from the Ebert lab has shown that *plexinA2* is present in the early eye vesicle and is interacting with SEMA6A in a novel tissue autonomous manner. Specifically, mRNA for the *plexinA2* receptor is expressed in the ventral eye and the surrounding head mesenchyme during early stages of development (Figure 3) (Ebert et al., 2014). The presence of *plexinA2* in this region is acting with *sema6A* as a ventral repellent mechanism to guide retinal progenitors.

The result of this interaction is direction of migrating progenitors and promotion of eye vesicle cohesion. The downstream signaling mechanisms of this interaction may or may not be the same as the classical plexin/semaphorin pathway described above. *PlexinA2* antisense morpholino knockdown resulted in decreases in proliferation of retinal precursor cells causing smaller eyes and a loss of cohesion of the retinal precursor cells within the eye field (Figure 4). This phenotype is copied by knockdown of SEMA6a, the receptor for PLXNA2. This data led to





the current model which is that retinal progenitor migration into the ventral eye is directed by SEMA6a repulsion with PLXNA2 and cells that enter the incorrect domain are removed from the eye field (Ebert et al., 2014). In addition, this signaling mechanism is involved in maintenance of proper levels of proliferation in the migrating eye field. However, this model is not fully explained simply by the interaction between PLXNA2 and SEMA6a alone and other plexin receptors may also be involved. For example, a dorsal directionality cue in the eye vesicle has yet to be identified.

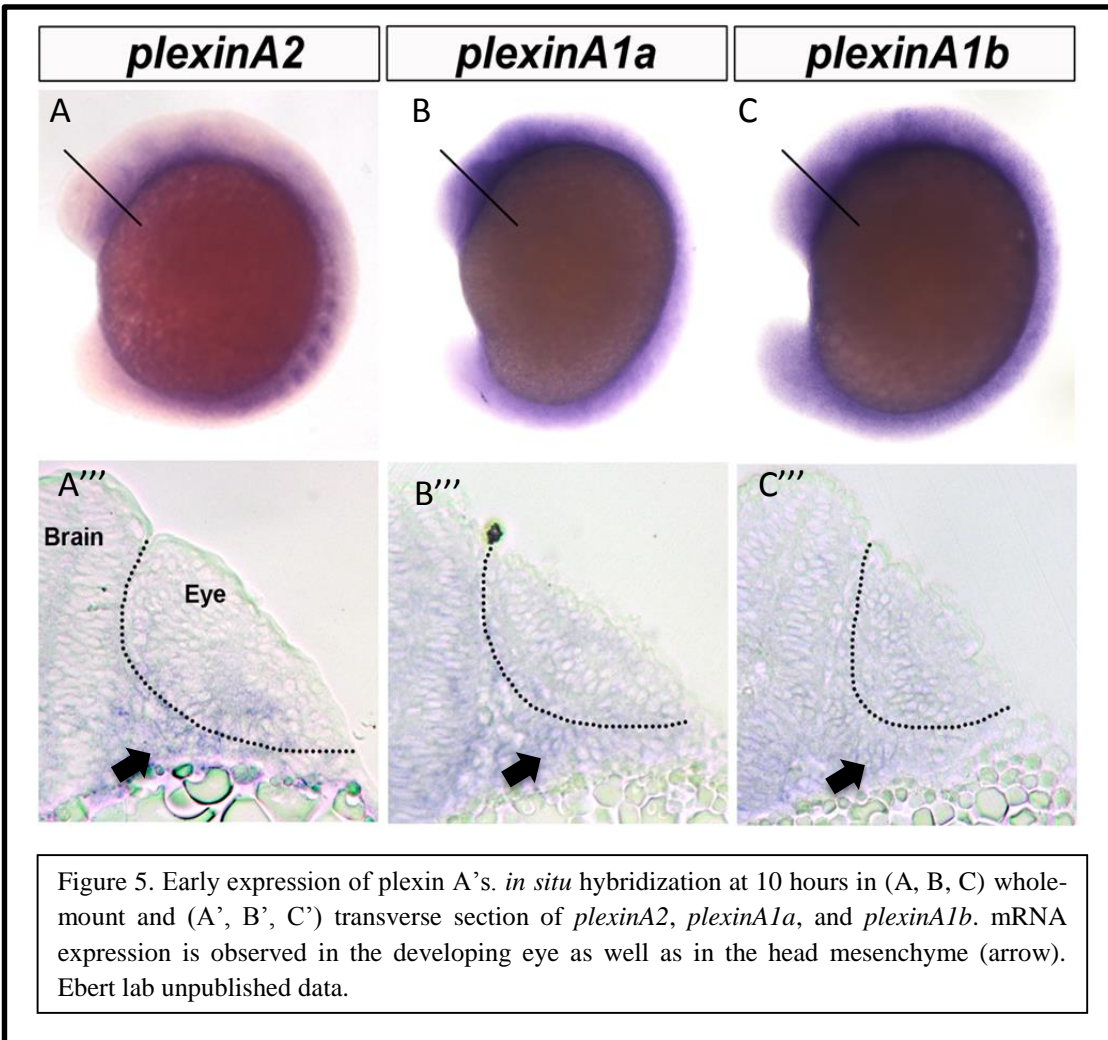
Semas and plexins are abundantly expressed in the developing vertebrate visual system and are highly involved in maintenance of correct neuronal development. Another member of the plexin A family, *plexinA1*, is expressed in the medial periphery of the neural retina of mice at the site of ganglion cell accumulation (Murakami et al., 2001). Additionally, SEMA3A, which can bind PLXNA1, is found along the pathway of migrating retinal ganglion cells (RGCs) in mice and at their target during early development (30-60 hours post fertilization (hpf)) and in the inner nuclear layer of the retina during late development (72 hpf) (Callander et al., 2007). The current state of the literature has very little to say about the PLXNA1 receptor in zebrafish, its expression patterns, or its functional role, and how it relates to PLXNA2. Additionally, although two subtypes of the PLXNA1 receptor, A1a and A1b have been identified, no expression and functional differences between the two have emerged from any study.

As mentioned previously, *plexinA2* has a very specific ventral early eye field expression pattern. Interestingly, both *plexinA1a* and *plexinA1b* mRNA are also expressed in similar or surrounding regions at similar levels, suggesting that there may be an interaction or relationship

between these three receptors, in terms of functional activity (Figure 5B, C). Coexpression of these receptors in same domains as SEMA6A indicates that they may share this ligand partner. This project aims to investigate the role of both the PLXNA1a and PLXNA1b receptors in the early eye. Furthermore, we will speculate as to which semaphorin(s) PLXNA1a and PLXNA1b may be interacting with during the early stages of eye development. Interestingly, our preliminary data imply that the two zebrafish *plexinA1* homologs share early expression patterns in some tissues, however, later in development, they have divergent expression patterns. The second phase of this project is an investigation into the role of PLXNA1a specifically as it relates to PLXNA2 in formation and integrity of the early eye. Preliminary *in situ* expression data show that this homolog has a strong expression in the early neuronal tissues and surrounding head mesenchyme.

In light of the preliminary data collected, we hypothesize that *plexinA1a* and *A1b* will show similar early expression patterns to *plexinA2* and will be expressed in the developing eye and surrounding mesenchyme. Additionally, we hypothesize that the knockdown of *plexinA1* will result in a similar phenotype to that seen for knockdown of *plexinA2*, that being a loss of proliferation and cohesion in the early eye. Furthermore, we hypothesize that *plexinA1* can partially compensate for the loss of *plexinA2* in morphants.

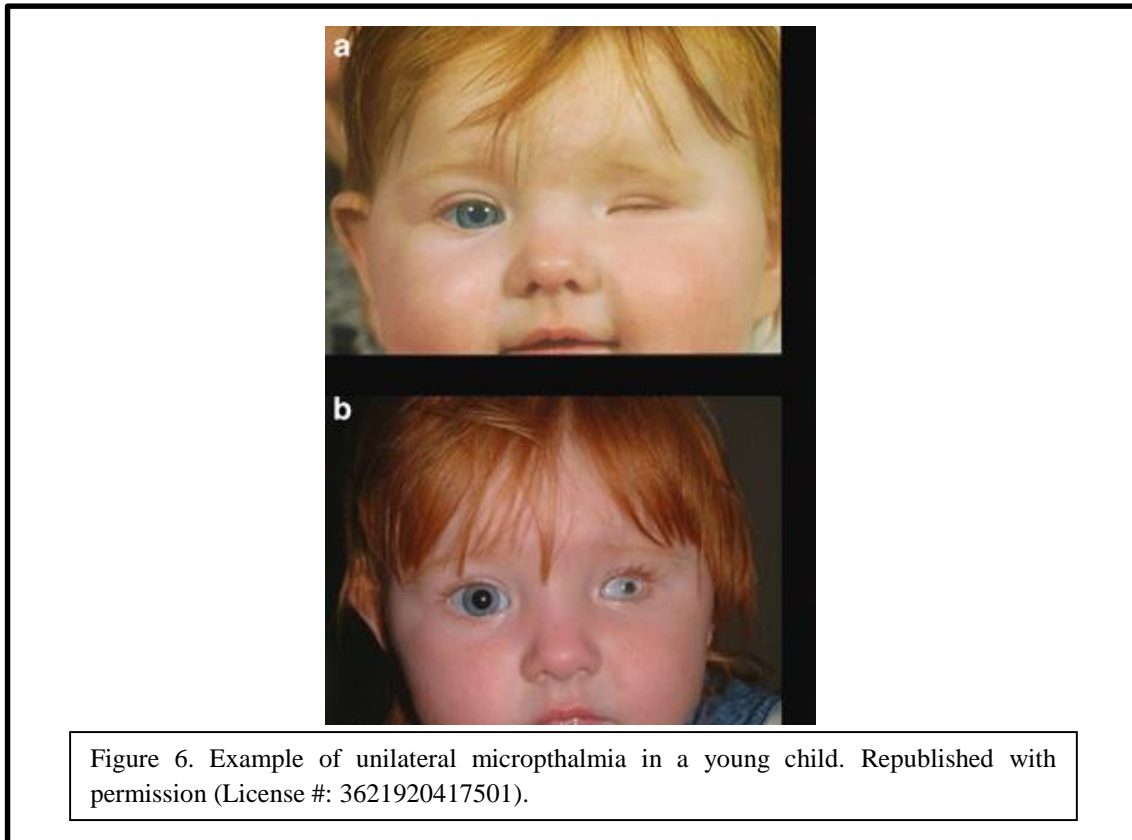
Danio rerio (zebrafish) are an ideal model organism in developmental research because the embryos are externally fertilized, fairly large, can be genetically manipulated, and are easily visualized due to their transparency. Additionally, they have an accelerated period of early development with all major organs patterned within three days. Juveniles reach sexual maturity at three months of age. Development of the visual system in zebrafish is important immediately



after fertilization because eyesight will be a major way for the zebrafish to escape predation. A fully functioning visual system is intact within three days post fertilization (Gestri et.al, 2012).

Zebrafish research is relevant to mammalian biology because there is high homology between the zebrafish and mammalian genomes. Furthermore, zebrafish and mammals share common mechanisms of development. However, experimental design must take into account that zebrafish underwent a chromosomal duplication during the course of their evolution (Woods et al., 2000). This means that it is necessary to confirm that the zebrafish genes we are working with are orthologous to mammalian genes, and that they play a similar role.

Studies of the mechanisms of zebrafish eye development have important implications for the generation of therapy techniques for many human diseases of the eye. For example, microphthalmia is a condition that arises before birth and results in an abnormally small size of one or both eyeballs, which is usually accompanied by a significant loss of vision (Figure 6) (Genetics Home Reference, 2014). An understanding of the process by which the early eye is formed through guidance and cell proliferation is crucial to development of therapies that can address this condition and other common problems in this process.



Chapter 2: Materials and Methods

ZEBRAFISH HUSBANDRY

All procedures were approved by the University of Vermont Animal Care Committee (Protocol #12-055). Zebrafish embryos were developmentally staged as previously described (Kimmel et al., 1995). Embryos were treated with 0.0003% phenylthiourea (PTU) (Sigma) at 24 hpf to block pigmentation, fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline with 0.1% tween (PBT) overnight, and stored in 100% methanol (MeOH) at -20°C or PBT at 4°C.

SEQUENCING AND ALIGNMENTS

mRNA and protein sequences were taken from the NCBI database (PLXNA2: XP_689780.5; PLXNA1a: NP_001103480.1; PLXNA1b:XP_003201265.3). Alignments were completed using the ClustalW website (Kyoto University Bioinformatics Center) and the BLAST program (National Center for Biotechnology Information). Protein domains and structures were identified using Phosphosite (Cell Signaling Technologies).

RNA ANTISENSE PROBES

Primers were designed to create a DNA product of approximately 500-600 base pairs with a SP6 RNA polymerase promoter sequence on the forward primer and a T7 RNA polymerase promoter sequence on the reverse primer (Table 1). The DNA products were amplified at annealing temperatures of 63°C for *plexinA1a* and 55°C for *plexinA1b*. PCR products were verified on a 1% agarose gel and 5 uL of sodium acetate (CH₃COONa) was added along with two volumes of 100% ethanol (EtOH) to precipitate overnight at -20°C. Digoxigenin labeled antisense RNA probes were generated from 1ug of the DNA product with T7 RNA polymerase using the DIG

RNA labeling kit from Roche as per manufacturer's protocol. Probes were stored in hybridization buffer at -20°C (Thisse & Thisse, 2008). Control *in situ* were done with no addition of RNA probe.

	Forward Primer	Reverse Primer
<i>plexinA1a</i>	5' CTCAGCCGGAAAACACATGG 3'	5' GAACTTCACCTCCGGGTTTC 3'
<i>plexinA1b</i>	5' GGGACGCTGCAGATCTACTC 3'	5' AGCTGTCATGATGAGCGATG 3'
<i>plexinA2</i>	5' CTTTGAACCACTCAGCACCA 3'	5' CGTATTCCAGTCGACCCTGT 3'

Table 1. Primer sequences for *plexinA1a*, *plexinA1b*, and *plexinA2* mRNA probes

RNA IN SITU HYBRIDIZATION AND SECTIONING

In situ protocol adapted from Thisse & Thisse (2008). Embryos were rehydrated in 100% PBT, permeabilized with proteinase K, and incubated with 1% RNA probe in hybridization buffer at 70°C overnight. The following day, they were equilibrated in 2X saline-sodium citrate (SSC)(175.3g NaCl, 88.2g Na₃Citrate mixed in 10L H₂O), rehydrated in PBT, and incubated in 1:1000 anti-DIG (Roche) for 2 hours at room temperature. Embryos were placed in NBT-BCIP (nitro blue tetrazolium, chloride-5-bromo-4-chloro-3-indolyl phosphate) (Thermo Scientific) to visualize probe binding. Embryos were treated with 100% EtOH to remove background staining and imaged in PBT at 50X with a dissecting scope. All *in situ* experiments were performed at least once with a minimum of 10 embryos per age. A no-probe experiment was performed as a control (Supplemental Data, Figure 2).

MORPHOLINOS

Antisense oligonucleotide morpholinos (Gene Tools) were designed to target an exon/intron boundary within the Ras/GAP domain of *plexinA1a* mRNA and the exon 2/intron 2 boundary of

plexinA2 mRNA (Table 2). Morpholinos were injected into rx3:GFP (early eye fields marked with green fluorescent protein) transgenic embryos at the one cell stage. Optimal knockdown was achieved at 4.0 nanograms and 2.0 nanograms of the *plexinA1a* and *plexinA2* morpholino respectively. In the double knockdown experiment, MOs were injected independently at 0.8 nanograms as well as together at the same doses. Control and morphant embryos were fixed overnight in PFA and stored in MeOH or PBT as previously described. Embryos were imaged at 40X on a Nikon Eclipse confocal microscope to compare morphant and control embryos. Eye area was measured on images taken at 20X on an Olympus IX71 fluorescent microscope using the Spot software (Diagnostic Instruments, Inc.). Embryo death was quantified at 18 hpf as a percent of the total embryos collected.

Gene	Target site
<i>plexinA1a</i>	5' ACCAGTCGTTTGCTACCGTACCTCC 3'
<i>plexinA2</i>	5' AAAAGCGATGTCTTTCTCACCTTCC 3'

Table 2. Sites for antisense morpholino binding

PHOSPHO-HISTONE H3 STAINING

Control and morphant embryos were taken off PBT and immunostained with a 1:1000 serine-10 phospho-histone H3 (pHH3) antibody (Cell Signaling Technologies) to detect proliferating cells. This antibody binds to condensed chromosomes during M phase of the cell cycle. Embryos were confocal imaged at 40X. Eye proliferation was assessed from whole mount fluorescent pictures by comparing the eye area with the amount of pHH3+ cells within the GFP+ eye field.

CRISPR/CAS9 MUTAGENESIS

Target sites for mutation within each gene were identified using the CHOPCHOP website (<https://chopchop.rc.fas.harvard.edu/>). Targets were designed to be approximately 20 base pairs in length and end with “NGG”, which comprises the PAM sequence (Table 3). Total sequences include the target oligo which acts as a guide template. Primers were also designed with CHOPCHOP to be a minimum of 20 base pairs away from the target site and produce a PCR product of 100-160 base pairs for identification of correct oligos (Table 4).

Gene	Target site
<i>plexinA1a</i>	5' AGCGCTGAGCCTCCAGCGTG 3'
<i>plexinA2</i>	5' GACATCAACCAGCCCCTTGG 3'
<i>sema6a</i>	5' GACCTGGAAGTCCAGACAGG 3'

Table 3. Sites for targeted CRISPR mutation

	Forward Primer	Reverse Primer
<i>plexinA1a</i>	5' GTGGAGAAGGCTTTGACTCTGT 3'	5' CCCTAAACCAGTCGTTTGCTAC 3'
<i>plexinA2</i>	5' CCTTTTCACCCTCCTACTGATC 3'	5' CCTTTTTCAGTCGTCCACTCTT 3'
<i>sema6a</i>	5' ATATGAAGCTGAGCTGTGCTGA 3'	5' GTTATGACACTCGTCCTGCAAA 3'

Table 4. Primers for CRISPR verification

Protocol to generate CRISPR/Cas9 guide RNAs was adapted from Gagnon and colleagues (2014). CRISPR target oligos were annealed to the constant oligo and the DNA was precipitated in EtOH overnight. Oligo constructs were run on a 2% agarose gel to determine that they were the desired size of 120 base pairs. RNA was synthesized from these DNA constructs using the Sp6 mMessage mMachine Kit (Ambion, Inc.) and again precipitated in sodium acetate and EtOH overnight.

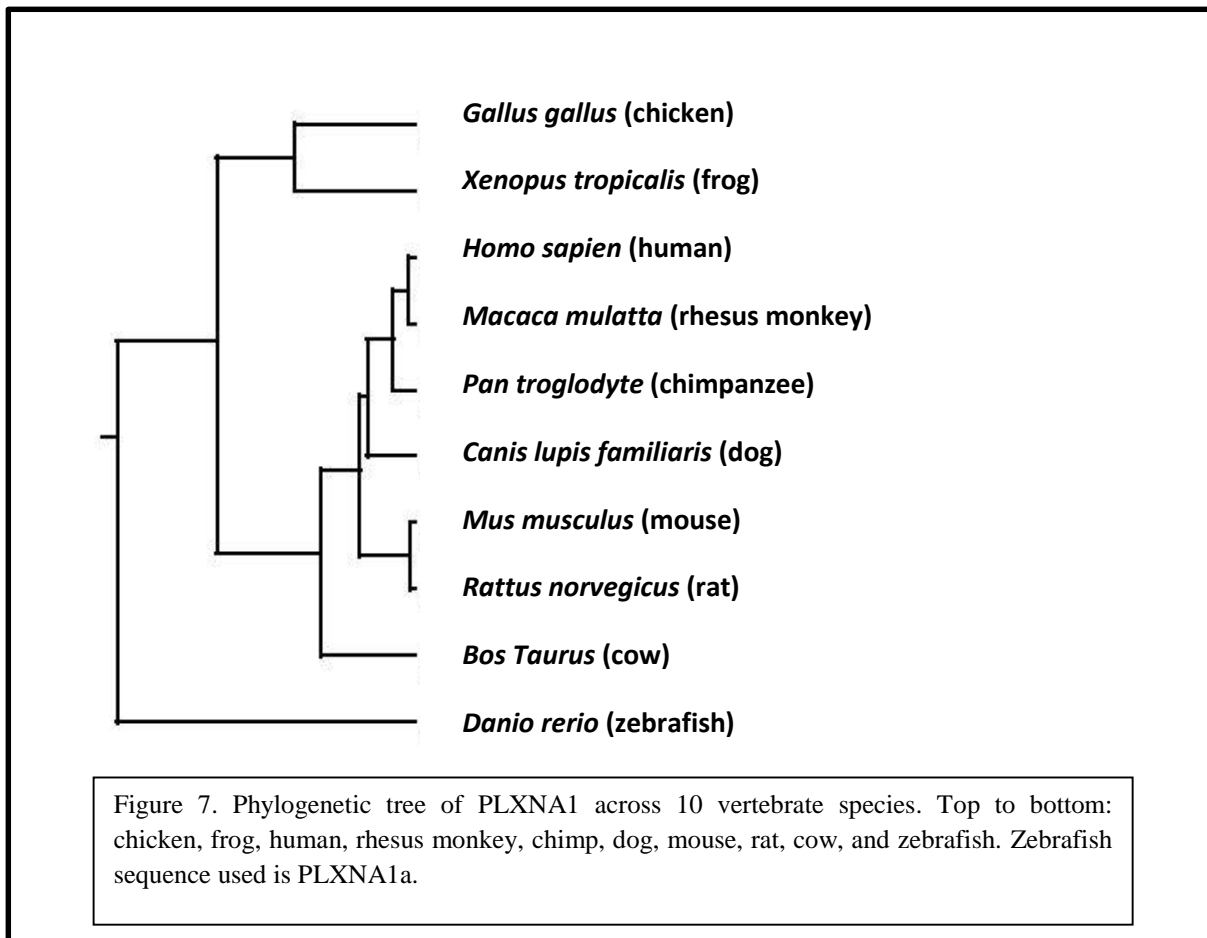
Chapter 3: Results

Phylogenetics and sequencing of *plexinA1a*, *plexinA1b*, and *plexinA2*

As mentioned previously, plexins are highly conserved molecules across vertebrate species, as demonstrated by a preliminary phylogenetic tree (Figure 7). The zebrafish plexinA family members that we are particularly interested in show high sequence homology. *plexinA1a* and *plexinA1b* are found in close proximity on chromosome 23 while *plexinA2* is located on a different chromosome. This distance in the genome indicates that they may have a shared ancestral origin and could potentially be gene duplications within the zebrafish genome. At the protein level, PLXNA1a and PLXNA2 are 64% identical and 78% similar (Figure 8). This indicates that the protein products of these genes likely have similar structure and function.

Additionally, the functional domains of the PLXNA2 and PLXNA1a receptors show the same or increased homology as compared to the full protein. The sema domain where the receptor binds its specific semaphorin ligand and the Ras/GAP domain, which initiates downstream signaling cascades within the cell, are 54% and 81% identical respectively for these two plexins (Figure 9A & B). The similarity between the sema domains specifically of PLXNA1a and PLXNA2 suggests that they may be interacting with the same semaphorin partner in the early eye tissue (Figure 9A). Co-expression of both plexins along with SEMA6A in the same early neuronal regions may signal that this semaphorin has multiple binding partners that can interact and cause different or similar downstream signaling events (Ebert et al., 2014). This is supported by the similarity between the Ras/GAP domains, which determine the intracellular binding partners of the receptor (Figure 9B).

PLXNA1a also shares high homology with PLXNA1b (Figure 10). We were unable to use the entire protein sequence for PLXNA1b because the full genetic sequence was not available. Therefore, we aligned the proteins from the C-terminal end to investigate their similarity. Due to the close genomic proximity of these two homologs, it is likely that they are duplicated genes. A full alignment of the current amino acid sequences for all three plexin receptors to compare domains and predicted phosphorylation sites and important residues was also performed (Supplemental Data, Figure 1).



```
PLXNA1a KNHDHTDQREVDKRGSKMVSEIYLTRLLATKGTLQKFVDDL FETIFSTAHRGSALPLAIKY
KNH+H DQ+E DRGSKMVSEIYLTRLLATKGTLQKFVDDL FETIFSTAHRGSALPLAIKY
PLXNA2 KNHEHGDQKEGDRGSKMVSEIYLTRLLATKGTLQKFVDDL FETIFSTAHRGSALPLAIKY

MFDLDEQADRHMISDPDVRHTWKSNCPLRFWVWVIKNPQVFVDIHKNSITDACLSVA
MFDLDEQAD+H I DP VRHTWKSNCPLRFWVWVIKNPQVFVDIHK+SITDACLSVA
MFDLDEQADKHNIHDPHVRHTWKSNCPLRFWVWVIKNPQVFVDIHKSSITDACLSVA

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QTFMDCSTSEHRLKGDSPSNKLLYAKDIPSYKSWVERYYSDISKMPAISDQDMNAYLAE

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QSR+H+N+FN+MS+L EI+SYI KY +EI+SALE+D+ +R+QRL KLEQV+ M+L S
QSRMHMNEFNTMSSLSEIYSYIGKYTEEIVSALEQDDGARKQRLAYKLEQVAFMSLES
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Figure 8. PLXNA1a and PLXNA2 share high sequence homology. Partial c-terminus alignment of *Danio rerio* PLXNA1a (top) and PLXNA2 (bottom). At the amino acid level, the total sequences are 64% identical and 78% similar.

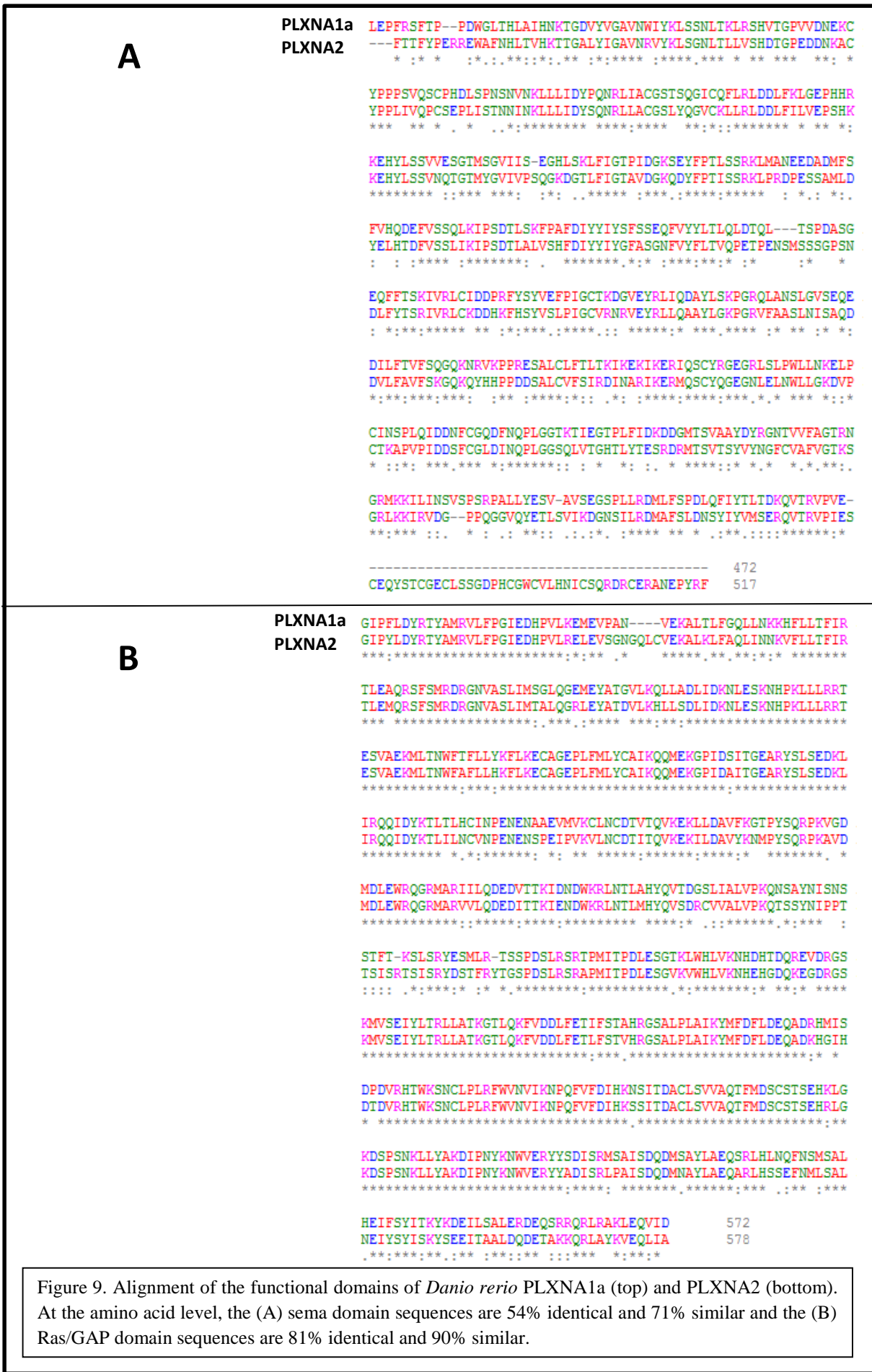


Figure 9. Alignment of the functional domains of *Danio rerio* PLXNA1a (top) and PLXNA2 (bottom). At the amino acid level, the (A) sema domain sequences are 54% identical and 71% similar and the (B) Ras/GAP domain sequences are 81% identical and 90% similar.

The role of PLXNA1 in visual system development of *Danio rerio*

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+ DYRA+SP+ FTFV+PYF RV P+ GP+SSGGTRITI G+HLNAGS+V++ IG + C F+
VADYRALSPRAF+TFVTFYFARVQPAQGPLSSGGTRITIEGNHLNAGSSVAVNIGRHPCHF 308

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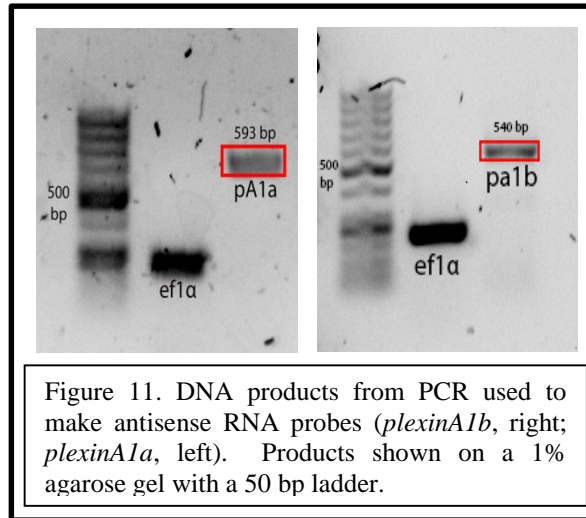
FNSMSALHEIFSYITKYKDEILSALERDEQSRQRRLRAKLEQVIDTMALPS 1885
FNSMSALHEI+SYITKYKDEILSAL++DE SRRQRLR+KLEQVIDTMAL S
FNSMSALHEIYSYITKYKDEILSALQKDELSSRRQRLRSKLEQVIDTMALTS 1199

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Figure 10. Alignment of *Danio rerio* PLXNA1a (top) and PLXNA1b (bottom). At the amino acid level, sequences are 86% identical and 93% similar.

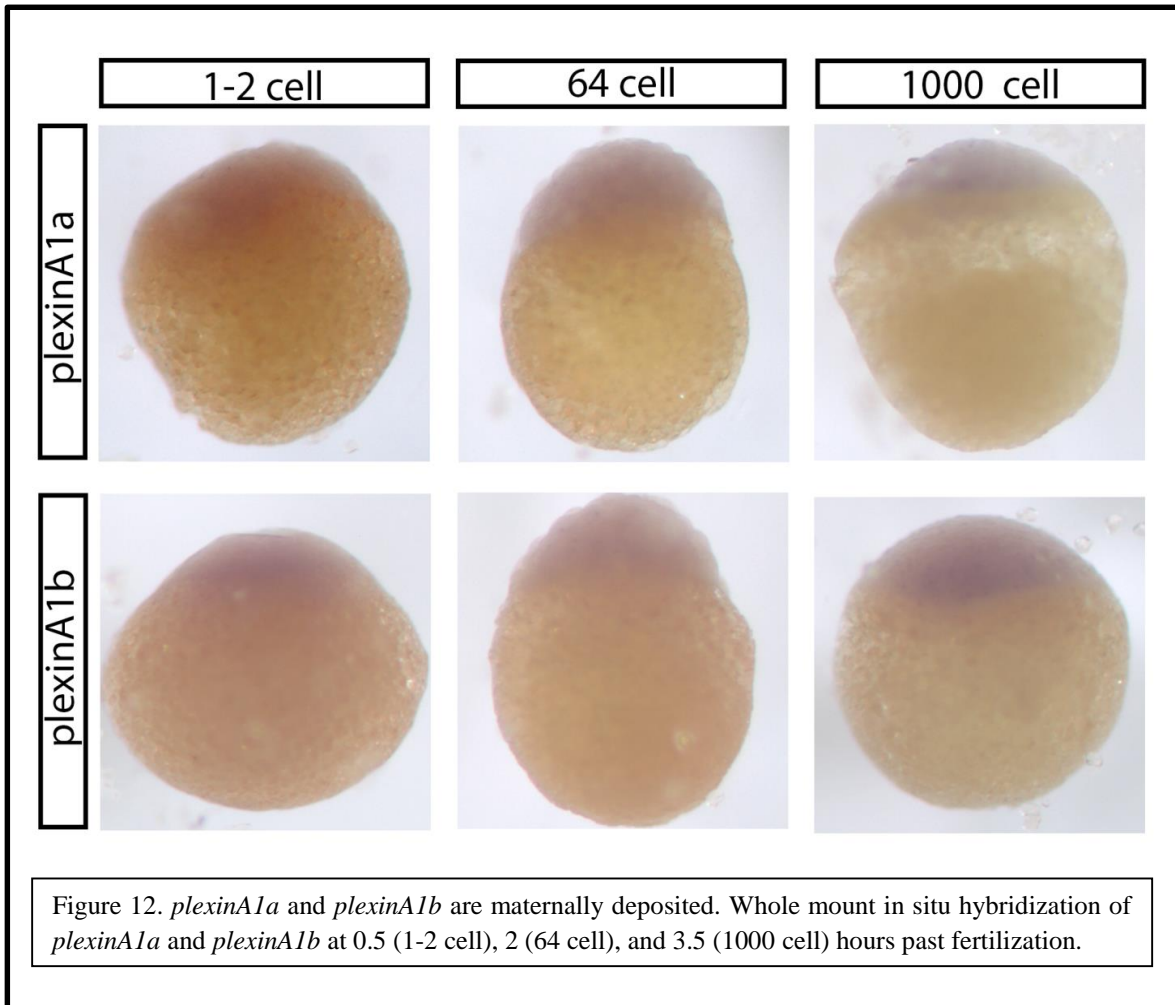
Expression profiles of *plexinA1a* and *plexinA1b*

The first avenue of investigation we explored is the spatial and temporal mRNA expression patterns of the *plexinA1a* and *plexinA1b* receptors in the developing zebrafish embryo, specifically in the eye. This was accomplished by *in situ* hybridization to detect mRNA transcripts with complementary antisense probes that were amplified from



primers designed to the gene sequence by PCR (Figure 11). Preliminary data have indicated whole-embryo expression patterns but more investigation was necessary to localize expression to specific tissues. Results of the *in situ* hybridization would lend insight into when and where we could expect a phenotype with *plexinA1* knockdown. Additionally, it would help us begin to understand the interaction between these plexin receptors, if one exists.

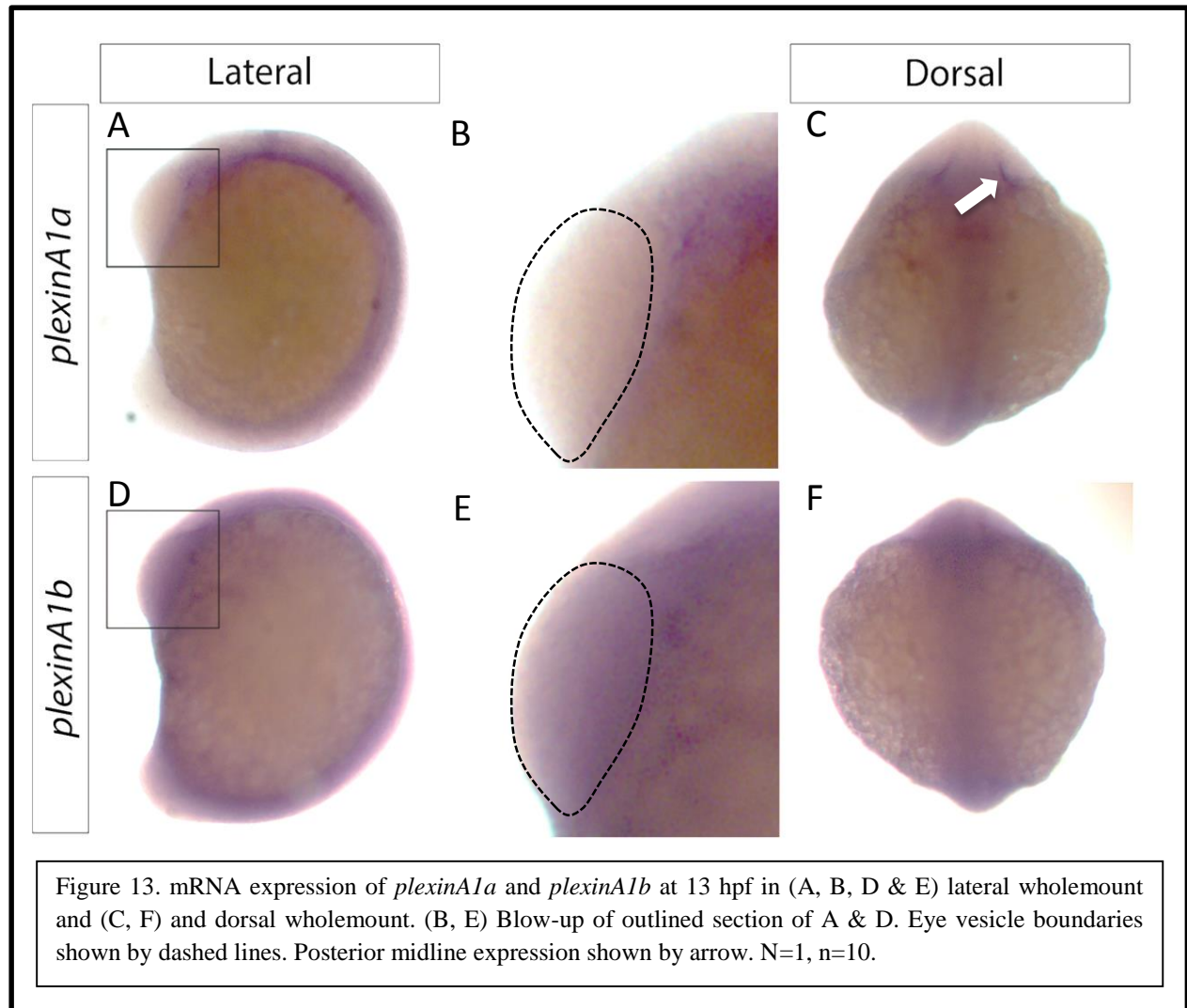
Interestingly, both *plexinA1a* and *plexinA1b* show mRNA expression even at very early developmental stages (Figure 12). These transcripts are expressed in the animal pole of the embryo as early as a half hour after the egg has been fertilized. Transcriptional and translational machinery is not yet active this early in development so the presence of these mRNAs indicates that they are maternally deposited in the egg. This means that they were made from the mother's genome and then deposited into the oocyte (Abrams & Mullins, 2009). Many maternally deposited genes have important roles in initiation of critical early developmental events and it is possible that PLXNA1 may play an important role during these early stages.

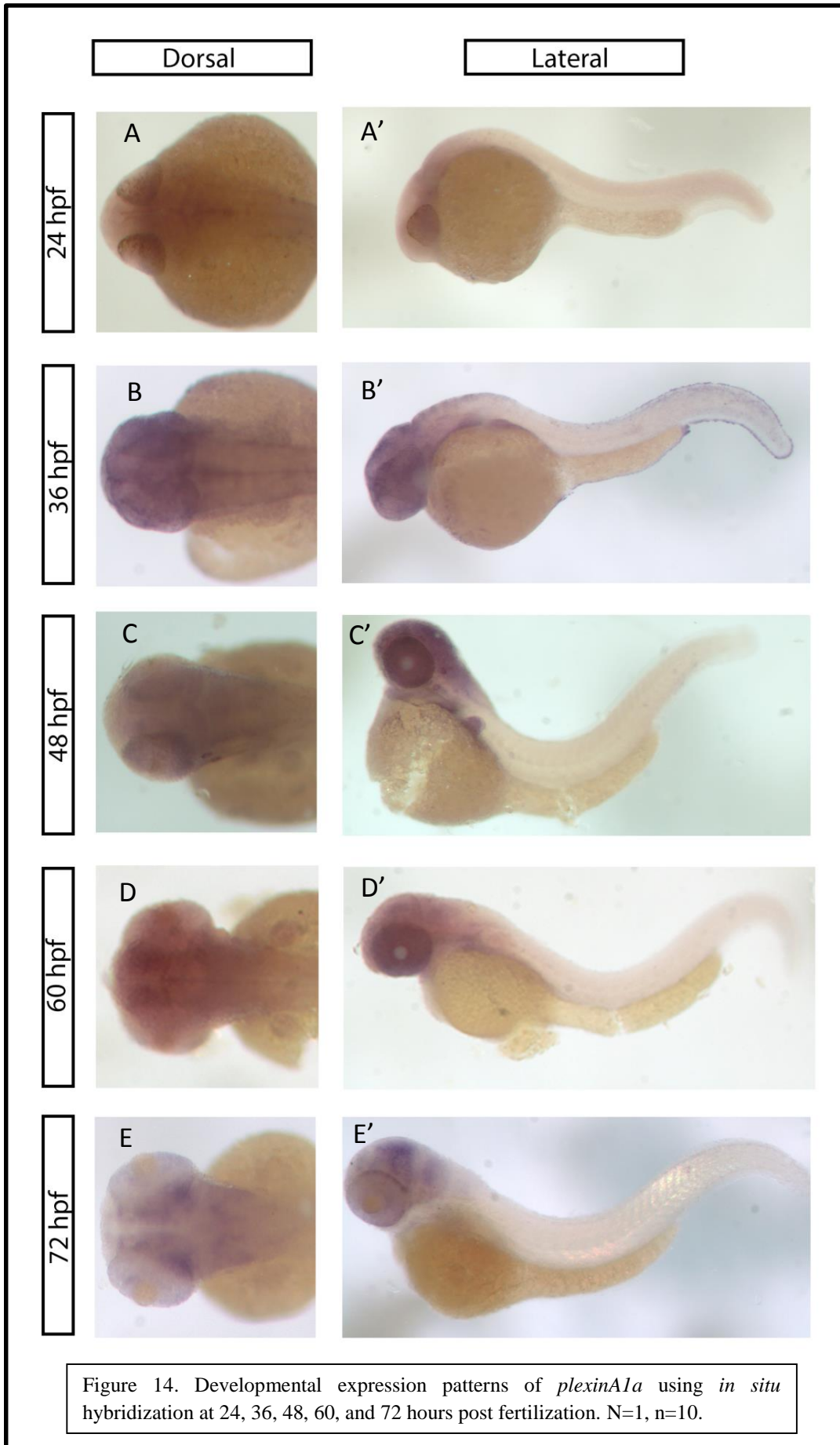


When examined at the 8 somite (13 hpf) stage, *plexinA1a* and *plexinA1b* have already begun to show divergent expression patterns (Figure 12). While the transcripts for both are present in the anterior neural regions of head mesenchyme and presumptive brain tissue, *plexinA1b* is found in high concentration in the ventral domain of the early eye vesicle (Figure 13D, E). This is very similar to the expression patterns of *plexinA2* at this stage that have already been described (Figure 3) (Ebert et al., 2014). In contrast, *plexinA1a* is not seen in the eye vesicle at all (Figure 13A, B). Instead, it appears at the posterior midline of the head mesenchyme (Figure 13C, arrow). This suggests that PLXNA1a may be acting as a boundary cue for the early eye tissue.

To further investigate the potential roles of these two plexin receptors, we performed *in situ* staining on embryos up to 72 hpf. As previously discussed, the eye is essentially done developing and fully functional at this stage. *plexinA1a* has a diffuse neuronal expression early in development but is concentrated in the anterior neural regions of the embryo. Strong retinal expression is present from 24 hpf until 48 hpf (Figure 14A-C') and is not present at later time points. The highest expression levels are seen in the medial periphery of the retina at 48 hpf (Figure 14C) which resembles the posterior midline expression pattern seen at 13 hpf (Figure 13C). Expression levels are higher in the developing brain areas and at 72 hpf, concentrated expression is also seen in the lateral regions of the optic tectum (Figure 14E, E'). These patterns of expression suggest that *plexinA1a* is involved in patterning and formation of the eye.

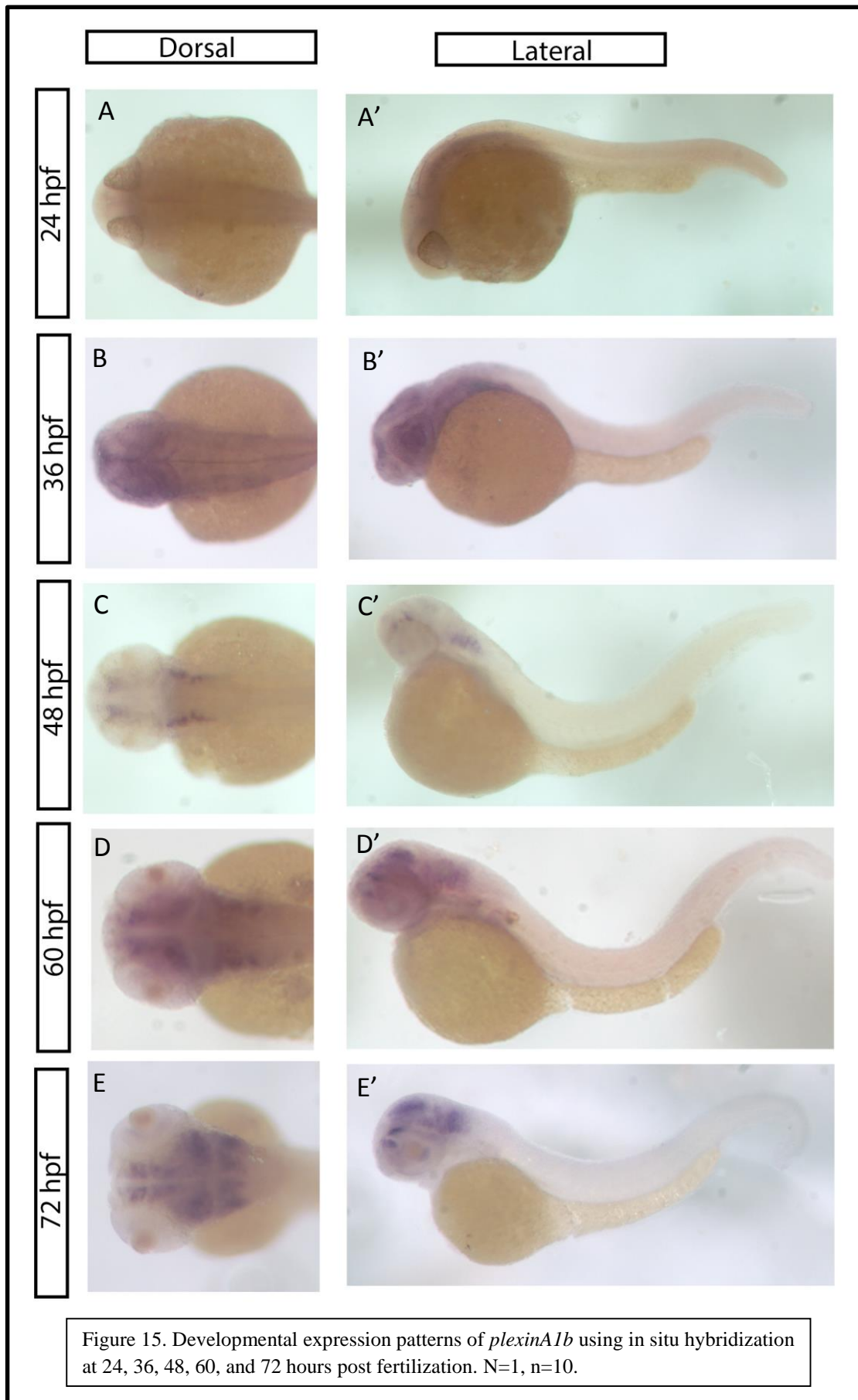
plexinA1b also is expressed in diffuse neuronal regions at early developmental stages and its expression patterns seem to mirror *plexinA1a* (Figure 15A, B & Figure 14A, B). Interestingly, there is almost no expression seen in the retina after 36 hpf. At 60 and 72 hpf both plexins share a similar expression in the brain (Figure 14D, E & Figure 15D, E). *plexinA1b* appears to be





localized to the optic tectum in particular but is more widespread in this region than *plexinA1a* (Figure 15D, E).

This distinct but overlapping expression of these two plexins over time can be more clearly appreciated in a side-by-side comparison (Figure 16). At 48 hpf, only *plexinA1a* is present in the retina. At 72 hpf, the brain expression of each plexin receptor has become very specific although they do overlap in some regions. This difference in expression patterns between these two plexin homologs suggests that they play different roles in development but they may have compensatory or interacting functions in the tissues where they are co-expressed.



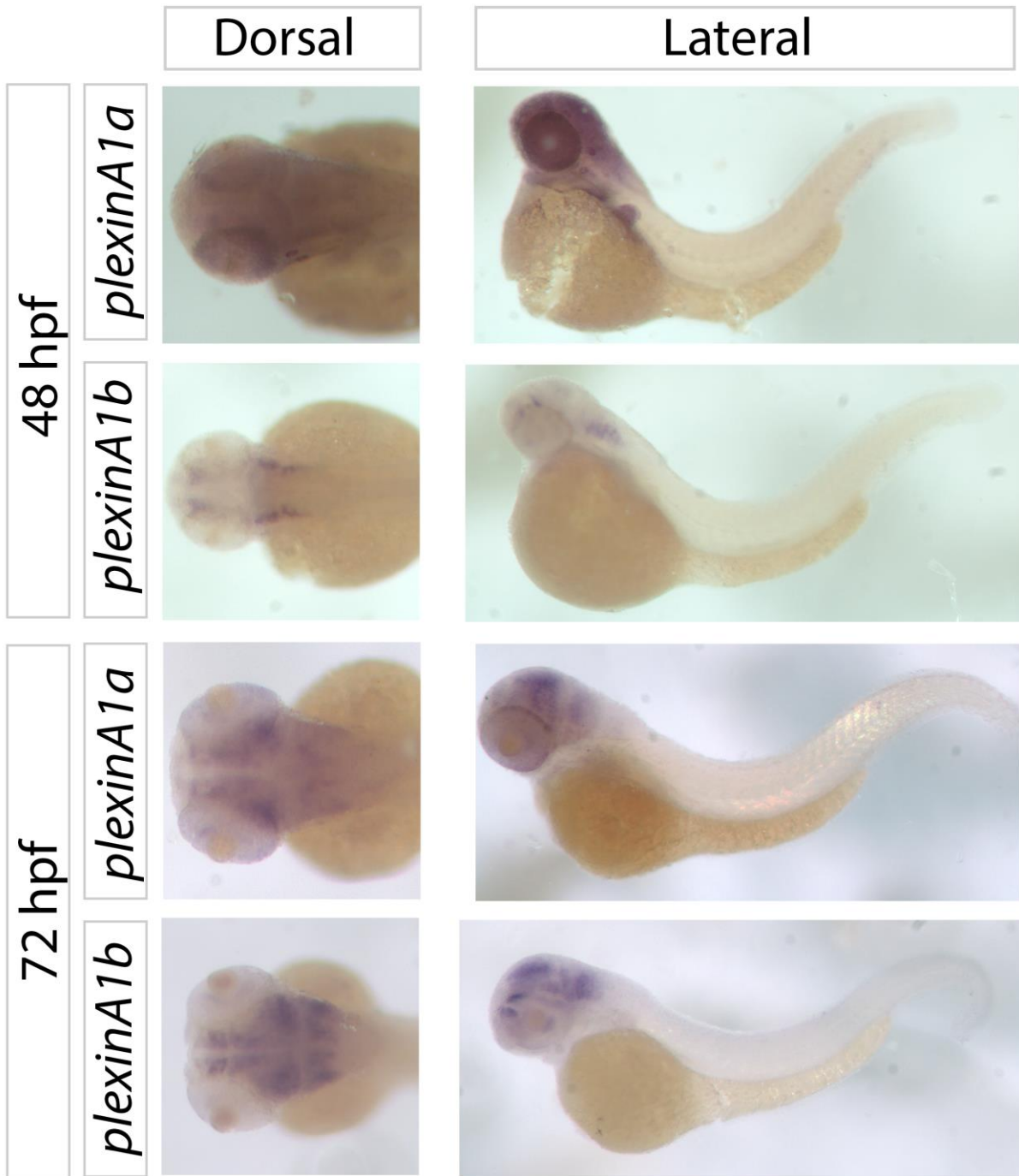
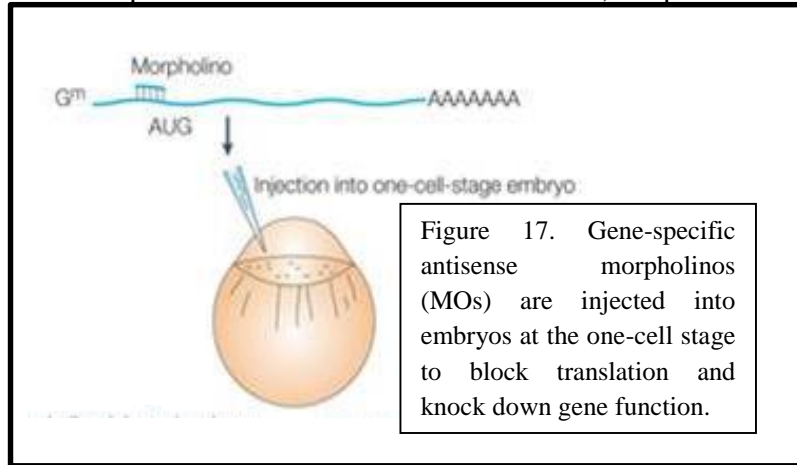


Figure 16. Comparison of expression patterns of *plexinA1a* and *plexinA1b* in the retina at 48 hpf and in the optic tectum at 72 hpf. N=1, n=10.

Functional role of *plexinA1a* and *plexinA1b*

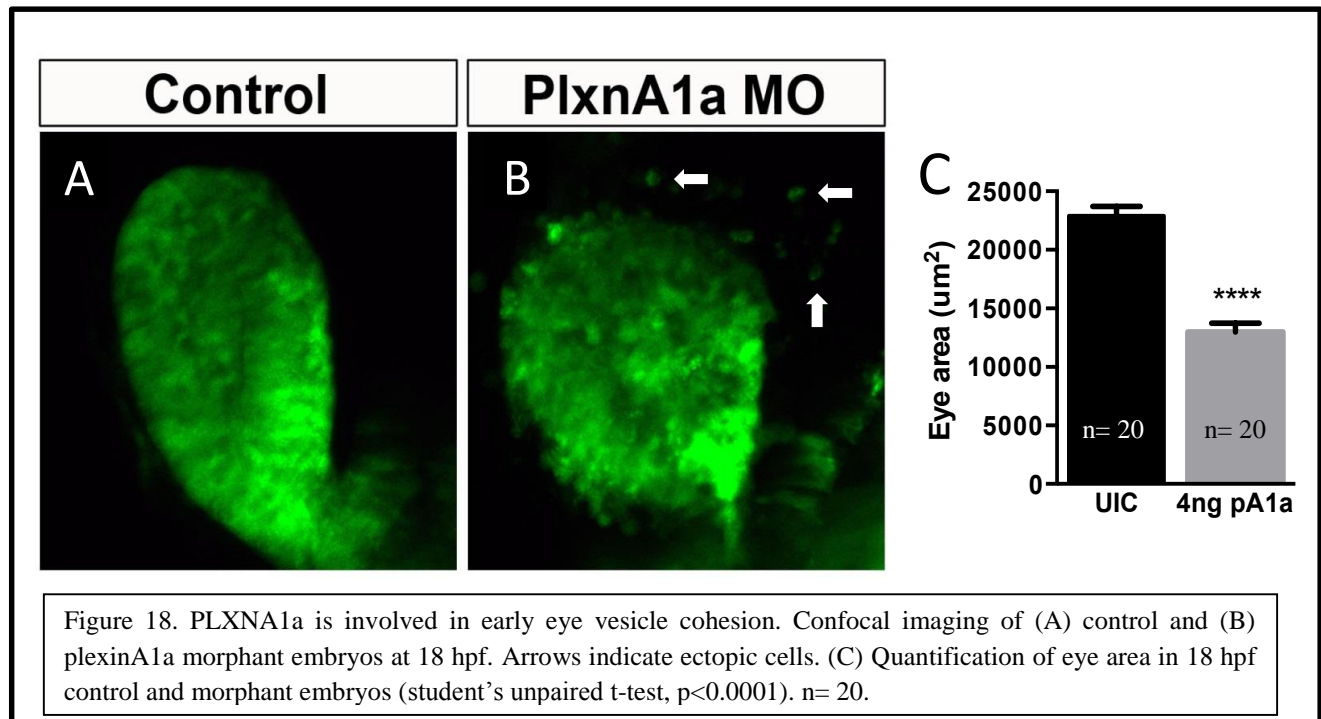
Unfortunately, after using multiple approaches, we were unable to find the N-terminal sequence for PLXNA1b in the genomic sequence of chromosome 23. Therefore, we pursued

PLXNA1a only in further experimental paradigms. To assess a functional role for *plexinA1a* in the early visual system, antisense oligonucleotide morpholinos



(MOs) were used to knock-down protein translation of *plexinA1a* (Figure 17). As previously mentioned, these MOs were designed to target a specific intron/exon boundary within the Ras/GAP domain of the mRNA, interfering with the correct splicing needed to make the final protein product. We expect that binding of this morpholino will create a truncated protein product. These MOs were injected at the one-cell stage into the rx3:GFP transgenic line, which expresses green fluorescence protein in the eye precursor cells (Rembold et al., 2006).

Knocking down PLXNA1a caused significantly smaller early eye fields in morphants as compared to controls (student's unpaired t-test, $p < 0.0001$) (Figure 18). This phenotype was observed as early as the 10-12 somite stage (approximately 14-16 hpf). Additionally, the shape of the eye field was altered, resulting in a more rounded eye field that did not separate as well from the midline presumptive brain tissue as opposed to a well-defined, elongated elliptical field. This decrease in eye size of morphants as compared to controls was observed still at 5 days post fertilization (dpf) as well (student's unpaired t-test, $p < 0.05$, data not shown). Abnormal ectopic cells were also observed in 95% of morphant embryos (Figure 18B, arrows). In a separate

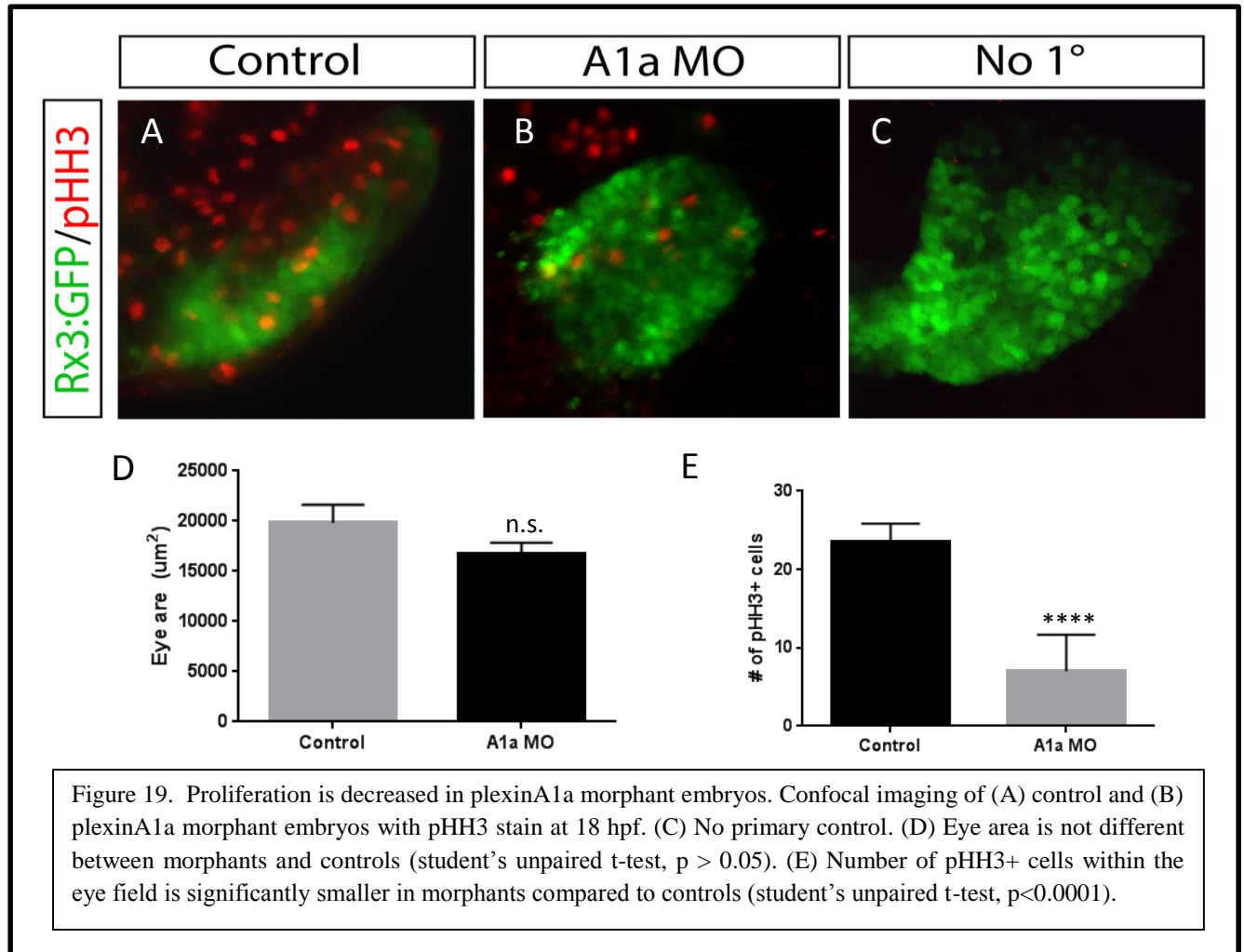


experiment, the *plexinA1a* morpholino was also co-injected with a p53 MO at 4.0 nanograms and no difference in phenotype was seen, indicating that the morpholino was functioning as expected and not activating non-specific cell-death cascades (Robu et al., 2007) (Supplemental Data Figure 3).

Morphant embryos were immunostained with a pHH3 antibody, which labels cells undergoing mitosis to observe proliferation in the early eye. Less staining was seen within the GFP+ eye field in morphants than controls indicating that there is a significant loss of proliferation. Eye area did not differ significantly in this case (student's unpaired t-test, $p > 0.05$) so the decrease in pHH3 labeling (student's unpaired t-test, $p < 0.0001$) cannot be accounted for by a decrease in total eye size (Figure 19).

Under a bright field microscope, the morphants embryos show a lot of cell death (qualitatively observed by darkened tissue color) in the head region and some have significant developmental defects. Of those that survived the first few days, a few interesting non-eye phenotypes were observed. At 5 and 7 dpf, some morphant embryos exhibited cardiac edema or swelling of the heart, a curved body axis, and a protruding jaw (Supplemental Data Figure 4). These three phenotypes are similar to those seen for knockdowns of PLXNA2 and suggest that loss of PLXNA1a may have some widespread effects on general aspects of development. There were also a few observed cases of coloboma, in which the retina does not fully fuse around the lens. These are interesting to us because they may be related to initial defects in the formation of the optic cup as it migrated laterally from the midline.

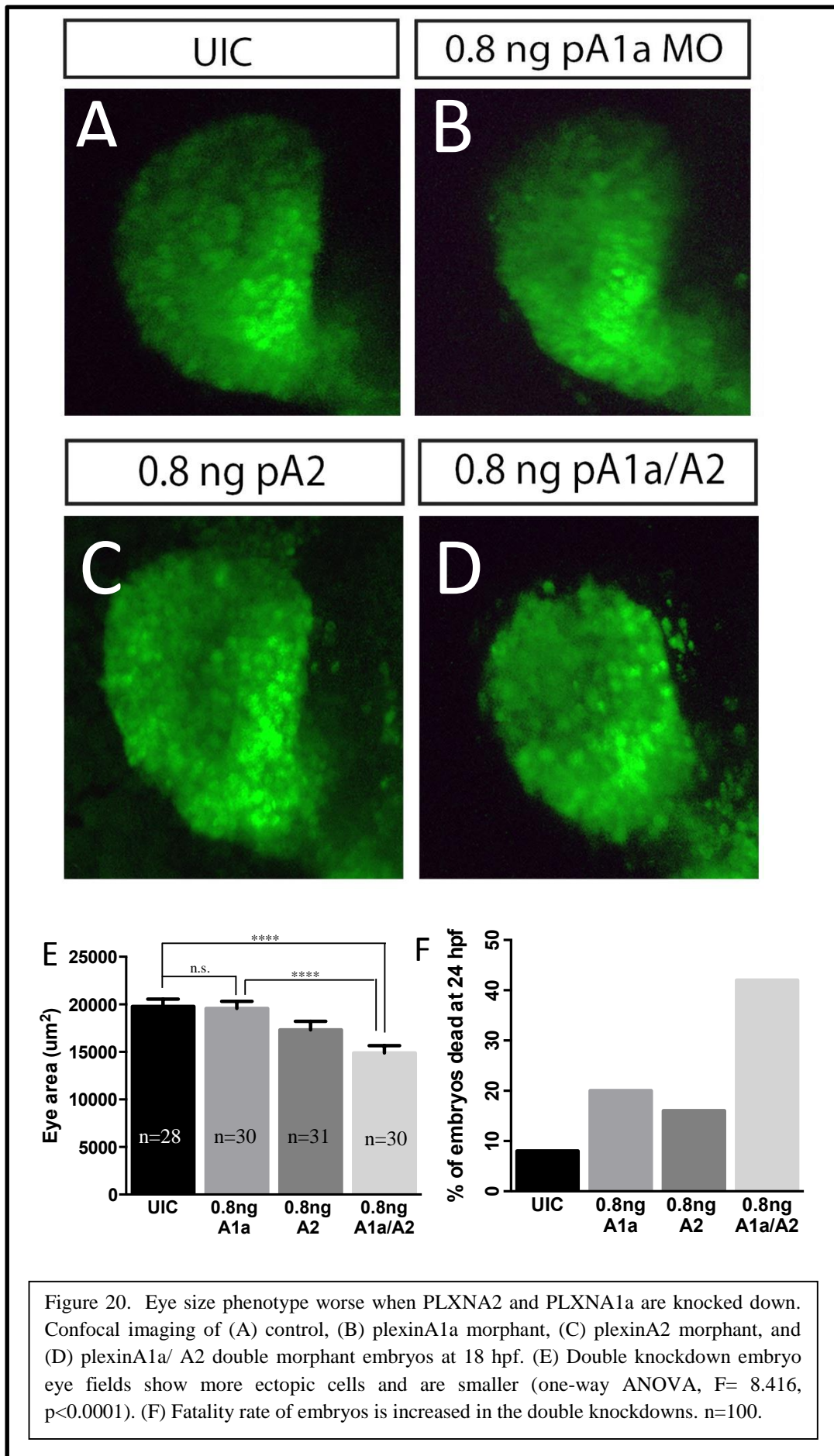
As a preliminary examination of the possible behavioral effects of loss of PLXNA1a, a tap test was done at 5 dpf. This measures the unconditioned startle reflex of the animals to a

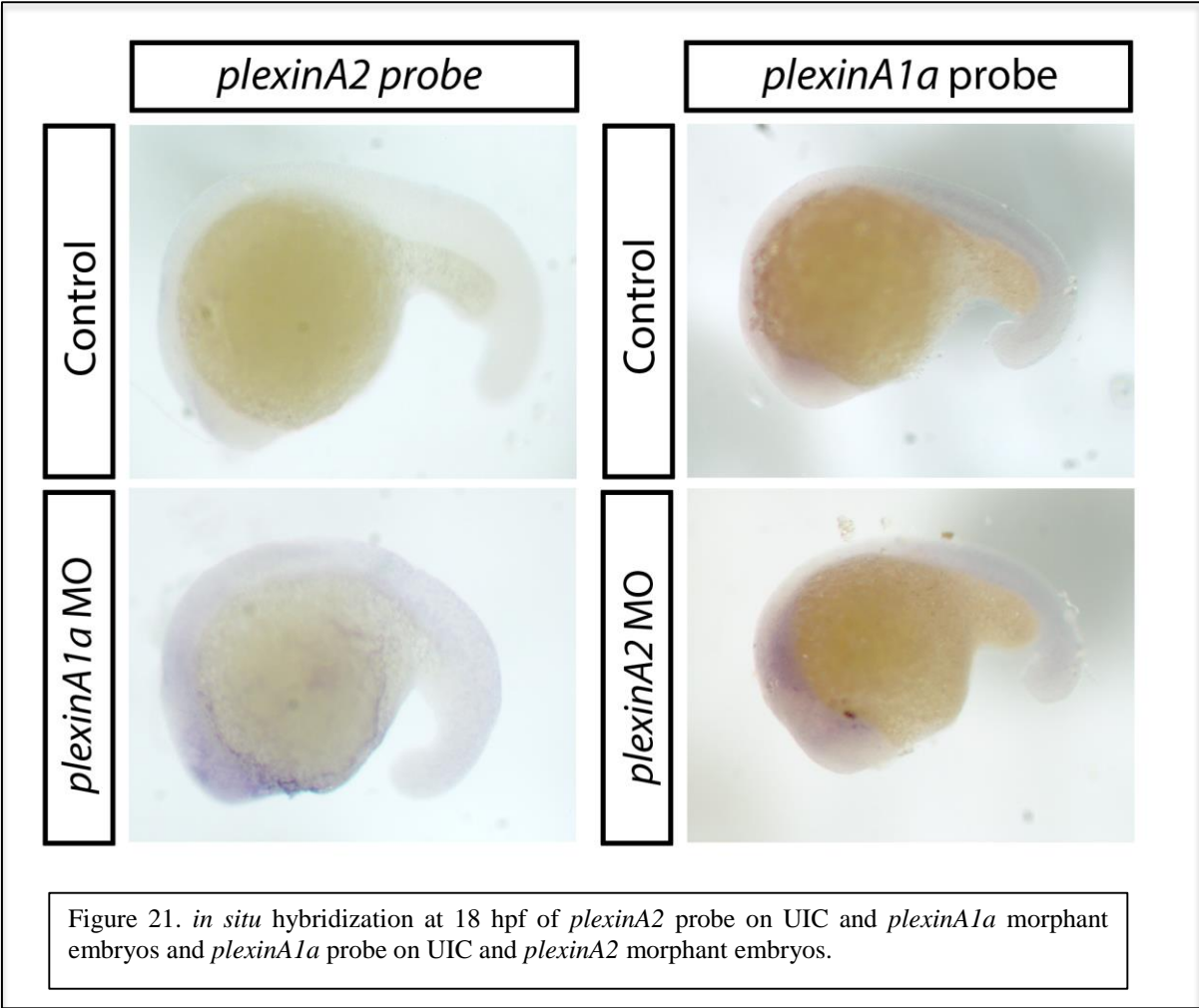


stimulus. Normal fish will swim in short straight bursts at each application of the stimulus (Bailey, Oliveri, & Levin, 2013). During this test, *plexinA1a* morphants showed less movement in general and some individual fish exhibited difficulty with balance and motor coordination needed to initiate movement (data not shown). These deficits are likely not related to visual problems but they speak to the occurrence of general developmental abnormalities associated with these knockdown experiments.

To assess the possible interaction of PLXNA1a and PLXNA2 in formation of the early eye, reduced concentrations of each morpholino that produced only a slight phenotype were individually injected. When these low-dose morpholinos were combined, a phenotype was seen similar in strength to that observed at the optimal dose for each morpholino, indicating a synergistic loss-of-function effect (Figure 20)(one-way ANOVA, $F= 8.416$, $p<0.0001$). Eyes in either single knockdown morphant groups were not significantly different from controls or from the opposite morphant group. The smallest eyes were seen in double morphants and were significantly different from controls at 18 hpf (student's unpaired t-test, $p<0.0001$). Double morphants had significantly smaller eyes than the *plexinA1a* morphants (student's unpaired t-test, $p<0.0001$) but not the *plexinA2* morphants (Figure 20E). This is likely a result of the increased dose sensitivity of the *plexinA2* morpholino.

Additionally, preliminary *in situ* data suggests that the knockdown of PLXNA2 upregulates the mRNA expression of PLXNA1a and vice versa (Figure 21). This experiment was done under the same time frame for both control and morphant embryos in order to show more clearly the differences in staining. Therefore, it may not match the robust staining profile seen in previous experiments.

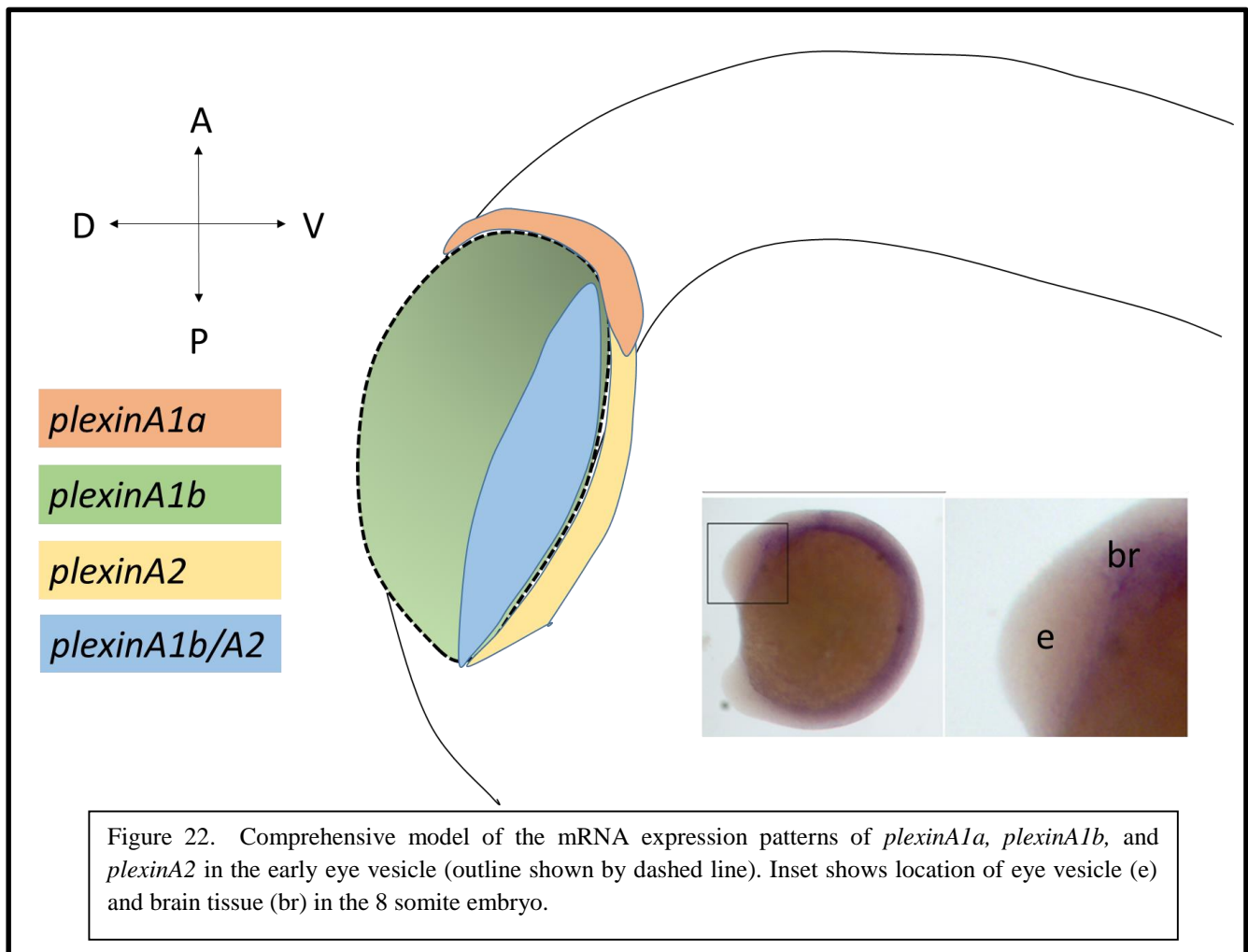




Chapter 4: Discussion

Plexins are highly conserved across vertebrate species and have been implicated in many developmental neuronal processes. The interaction between semaphorins and plexins is well known in the context of axon guidance through growth cone remodeling in response to the local environment. A novel role of PLXNA2 and SEMA6A was recently discovered in the formation, cohesion and proliferation of the early eye. We predicted that due to their similar expression pattern to *plexinA2* in the early eye and surrounding neuronal tissues that *plexinA1a* and *plexinA1b* may also be involved in these processes.

The expression data we gathered suggested that the *plexinA1a* and *plexinA1b* receptors are not expressed in the same domains as *plexinA2* during early eye development. While *plexinA2* is concentrated in the ventral domain and surrounding head mesenchyme of the eye vesicle, *plexinA1a* is not found in the eye vesicle at all and is concentrated at the posterior midline in the bordering head mesenchyme. The current model suggests that PLXNA2 is acting as a repulsive cue within the ventral domain to direct cohesion and proliferation of the eye. It is possible that PLXNA1a is acting through a similar mechanism at the posterior boundary (Figure 22). If both of these receptors interact with SEMA6A found on neuronal precursors migrating into the eye vesicle, they may be mediating the cohesion and patterning of the eye through this repulsion in overlapping regions. The downstream signaling molecules that are involved in this process is an ongoing avenue of investigation in the Ebert lab.



We discovered that there are two PLXNA1 genes in the zebrafish that encode extremely similar proteins and were likely replicates that arose during the evolution and duplication of the genome. However, it is interesting to note that their expression patterns are not identical, which indicates that their function may have diverged over time. Both *plexinA1a* and *plexinA1b* are expressed in the optic tectum at later developmental stages but in different domains. This differential expression patterns may be directing retinal ganglion cells within this brain region as they are migrating from the retina. Preliminary data also suggests that *plexinA1b* is present in the later developing eye where *plexinA1a* is absent. Again, this points to a potential separation of function for these two copies of PLXNA1.

Additionally, we observed that *plexinA1b* is diffusely expressed in the early eye vesicle with a greater concentration at the ventral side. This expression pattern overlaps with *plexinA2* in the ventral domain. The function of PLXNA1b warrants further exploration because it has such a similar expression pattern to PLXNA2 at early stages of eye development. It may also have a conserved function to PLXNA1a because they are so similar.

The loss of cohesion and proliferation phenotype of the early eye seen originally in the *plexinA2* morphants was also observed in the *plexinA1a* morphants, leading us to believe that these protein receptors are involved in the same or similar developmental mechanisms. This theory is further supported by the synergistic phenotypic profile of the double morphants as compared to single morphants. The mRNA up-regulation seen in the inverse *in situ* experiment suggests that they may function in a compensatory circuit, by which the expression of one plexin is directly or indirectly dependent on the other. When one becomes depleted or knocked down, the other will increase expression to compensate for the loss. This observed change in mRNA expression should be quantified with quantitative RT-PCR. Both plexin receptors must be active

in a normal cellular environment though, or the individual phenotypes from the single knockdown experiments would not have been observed.

While this evidence is compelling, it is still unclear as to the exact mechanism by which these two plexin receptors promote cohesion of the early eye. They may be activating the same intracellular cascades to cause the phenotype we observed. Microarray analysis of *plexinA2* and *sema6A* morphants has identified 58 genes that change expression significantly in response to knockdown of either this plexin receptor or the specific semaphorin ligand (Ebert lab unpublished data). These affected genes are likely to be mediating this phenotype within the downstream signaling cascades. It may be interesting to explore a second microarray between *plexinA2* and *plexinA1a* to identify common molecules of interest and also determine whether they are both equally responsible for maintenance of cohesion.

Further research is needed to determine whether PLXNA1a also binds the SEMA6A ligand as PLXNA2 does or whether it has a different signaling partner. Thus, investigation into the expression patterns of different semaphorins in the early eye may be helpful. Additionally, experiments looking into which specific boundaries of the eye vesicle are being affected by this loss of cohesion in the absence of one or more of the plexin receptors and whether this lines up with the *in situ* expression data would be very interesting and could lend further support to the model that is being developed.

Finally, although they are useful tool for genetic manipulation, morpholinos can present issues such as off-target effects (Robu et al., 2007) so this research is being continued using other avenues of genetic mutant generation known as clustered regularly interspaced short palindromic repeats (CRISPRs) (Hwang et al., 2013). CRISPRs are a natural system of acquired immunity

found in bacteria and archaea, similar to the RNA interference mechanism in eukaryotes (Koonin et al., 2009). They function in concert with Cas9 proteins, which function as endonucleases that degrade invading DNA. In recent years, they have been adapted for use as a specific genome-editing tool to introduce mutations in genes of interest both *in vitro* and *in vivo* (Hruscha et al., 2013; Hwang et al., 2013) (Figure 23). CRISPR technology allows us to perform site-directed mutagenesis to create specific point mutations in our gene of interest at the transcriptional level to create genetic mutants. These mutants will be immensely helpful in future research in cleaner experimentation procedures as the validity of morpholinos as research tools has recently been called into question (Kok et al., 2015).

In conclusion, we have identified two plexin homologs, *plexinA1a* and *plexinA1b*, that share similar sequence homology to a previously investigated receptor, *plexinA2*. These three receptors share overlapping expression patterns in certain regions of the developing eye vesicle. Knockdown of either *plexinA1a* or *plexinA2* results in disruption of cohesion and proliferation of the early eye, indicating that these two receptors are mediating a similar signaling pathway that promotes these effects.

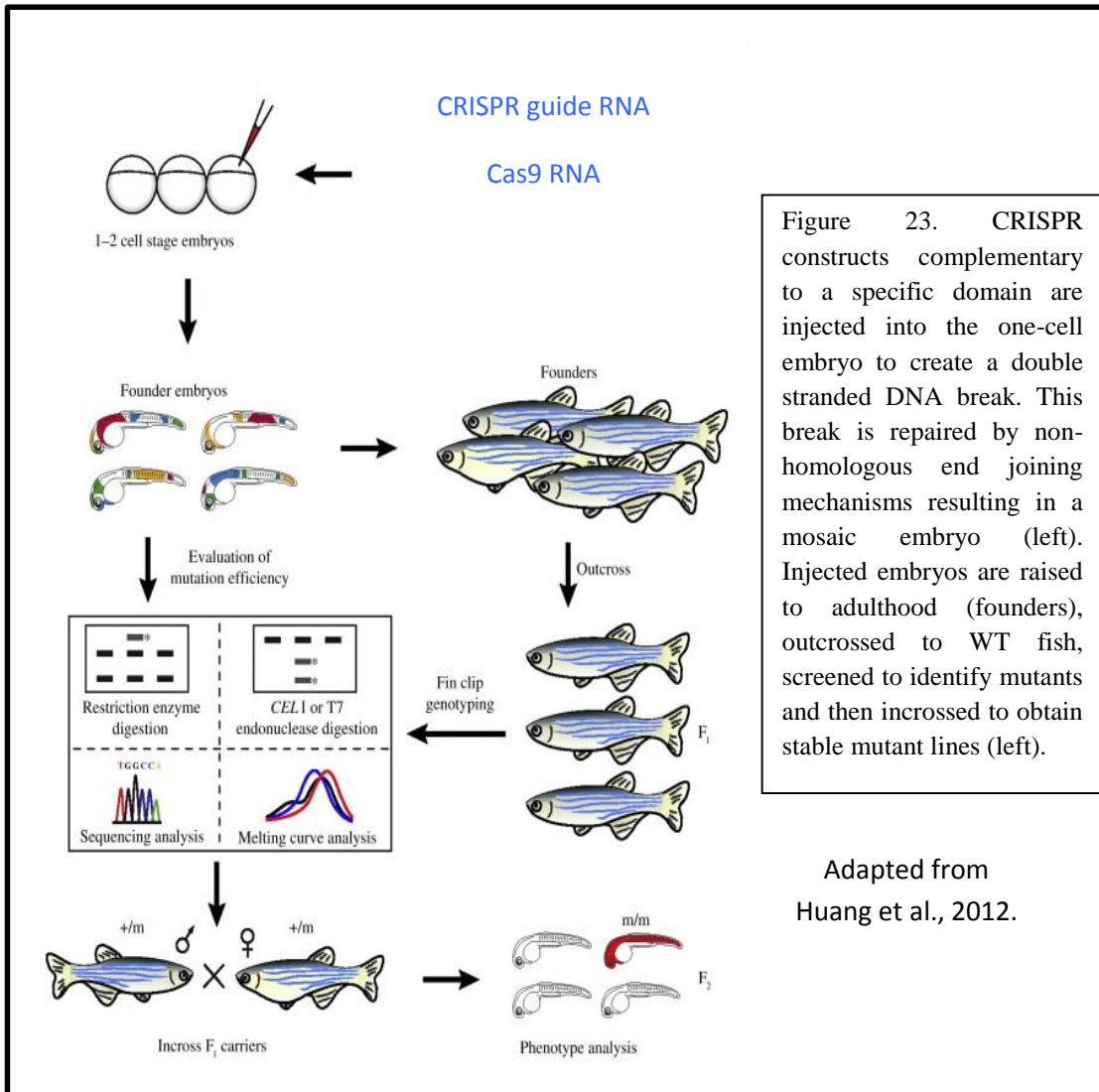


Figure 23. CRISPR constructs complementary to a specific domain are injected into the one-cell embryo to create a double stranded DNA break. This break is repaired by non-homologous end joining mechanisms resulting in a mosaic embryo (left). Injected embryos are raised to adulthood (founders), outcrossed to WT fish, screened to identify mutants and then incrossed to obtain stable mutant lines (left).

Adapted from Huang et al., 2012.

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The role of PLXNA1 in visual system development of *Danio rerio*

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Supplemental Data

Figure 1. Full alignment of PLXNA1a (top line), PLXNA11b (middle line), and PLXNA2 (bottom line) including predicted **sema**, **transmembrane**, and **RasGAP** domains. **Phosphorylation sites**, **acetylation sites**, and **ubiquitination sites** are also identified. **Primer binding sites** and **MO binding sites** have also been highlighted.

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-----MWPPQGPQCIIISPKTVVLLGLLT
-----
MGGQQDSQRTLKQSPGQGFTLSFTCWLLVL-ILPV

ICNSPHHSEGALEPFRSFT--PPDWGLTHLAIHNKTGDVYVGAVNWIYKLSNNLTKLRSHVTGPVVDN
-----
VCCE-----QSFTTFYPERREWAFFNHLTVHKTTGALYIGAVNRVYKLSGNLTLVSHDTGPEDDN

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SSVVESGTMGVIIIS-EGHLSKLFIGTPIDGKSEYFPTLSSRKLMANEEDADMFSFVHQDEFVSSQLK
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The role of PLXNA1 in visual system development of *Danio rerio*

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NYEGSDISDLVDLSVWVWNGNFIIDNPYNIQAHLYKCYALRESCGLCLKADPSFECGWCVQDRKCSLR
DYEGNDISDLVDLSVWVWNGDFVIDNPYNIQAHLYKCFAMRDSCGMCLKADPRFDCGWCVQEKKCSLR
LYEGMRISELPVDFSVWVWNGNFIIDNPENIKVHLYKCGAQRDSCGVCLKAERKFQCGWCSMEGRCTLR

QECAQ---PENTW MHATAGNSRCTHPRITKLSPETGPRQGGTRLTISGENLGLQFKDIQNGVRIGKVS
QECAP---PESSWMNPSAGNSRCAHPKINKLFPETGPRQGGTRLTITGENLGLQFRDIMTGVRIGKVC
QHCPMTNPHYTSRWLHLASTNVKCTNPRITEVTPVAGPPEGTRVTIRGVNLGLSFSDMVNNVQVAGVQ

CQPIEEYISAEQIVCLADATGYRVQEAHVEVCVRDCLQDYRAVSPKVFTFVSPYFTRVVPSPYGPIS
CPIEEYVSAERIVCLLDATGYRVQEAQVEVCVRDCVADYRALS PRAFTFVTPYFARVQPAQGPLS
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GGTRITIIYGSHLNAGSAVSIKIGLNTCQFERRSAREIVCVTPAGLGSGG-TPVMVDINAELRNPEVK
GGTRITIEGNHLNAGSSAVNIGRHPCHFVKRSKEIVCVTPAGVNAVSTPVMVDIDSAELRNPEVK
GGTKVTIMGENLGAGSSVTVLFGNQTCEFYERTMTEIVCYAPS LTGVGSVQISVSVDRQVR-DILA

FNYTDDPTILKIEPDWSIASGGTPLTVTGVLNATVREPKMRAKYGTISSVNNCTVLNNTVMVCAVPSV
FNYTDDPTVLKIDPDWSIASGGTLLTISGTNLATIKEPKIRAKYGAESFHNTVFNNVSVMLAPS
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AGSERGFSEVGNAPDEIGFIMDHVQMLLVV-NESFSFYDPVFEAFGPA-MLELKPSPILKGNLI
ADSERGFAETGSGPDEIGFYMDNVHALVVV-NESFSYDPDPIFEPLSPTGILELKPTSPLILKGNLI
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PAAPGNSRLNYTVFIGETPCVLTSETQLLCEWPNLTGQHKTIVIRAGGFEYSPTGLQIYSDSLLTLPA
PSAAGGVKLNNTVFIGETPCSVTVSESQLLCEPPNLTGQYKIMVQVGLHVS PGSVNILSDSLLTLPA

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IIGIGGGGGLLLLVIIVLIIAYKRKSRDADRTLKRLQMQMDNLESRVALECKEAFELQTDIHELTDQ
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LDGSGIPFLDYRTYAMRVLFPGIEDHPVLKEMEVPAN---VEKALTLFGQLLNKKHFLTFTIRTL
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LDRAGIPFLDYRTYAMRVLFPGIEDHPVRELEVSGNGQLCVEKALKLFAQLINNKVFLTFTIRTL

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QRSFSMRDRGNVASLIMTALQGEYATGVLKQLLSDLIDKNLESKNHPKLLRRTESVAEKMLTNWF
QRSFSMRDRGNVASLIMTALQGRLEYATDVLKHLSDLIDKNLESKNHPKLLRRTESVAEKMLTNWF

TFLLYKFLKECAGEPLFMYCAIKQQMEKGPIDSITGEARYSLSEDKLIHQIDYKTLTLHCINPENE
TFLLYKFLKECAGEPLFMYCAMKQQMEKGPIDAITGEARYSLSEDKLIHQIDYKTLTLHCINPENE
AFLHKFLKECAGEPLFMYCAIKQQMEKGPIDAITGEARYSLSEDKLIHQIDYKTLTLHCINPENE

NAAEVMVKCLNCDTITQVKEKLLDAVFKGTPYSQRPKVGDMDLEWRQGRMARIILQDEDVTTKIDNDW
NGPEVTVKCLNCDTITQVKEKLLDAVYKGPYSQRPKAGDMDLEWRQGRMARIILQDEDVTTKIDNDW
NSPEIPVKVLNCDTITQVKEKILLDAVYKNMPYSQRPKAVDMDLEWRQGRMARVVLODEDITTKIENDW

KRLNTLAHYQVTDGSLIALVPKQNSAYNINSSSTF-TKSLSRYSMLRT-SSPDSLRSRTPMITPDL

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KRLNSLAHYQVTDG**S**VIALVPKQNSA**Y**NISNS**S**T**F**-TKSL**S**RY**E**SMLRTASSPDSLRSRTPMI**T**PDLE
KRLNTLMHYQVSDRCVVALVPKQ**T**SS**Y**NIP**P**T**S**ISRTSISRYDSTFRY**T**GS**P**DSLRSRAPMITPDLE

SGTKLWHLVKNHDHTDQREVD**R**GSKMVSEIYL**R**LLATKGT**L**QKFVDDLFETIFSTAH**R**GSALPLAIK
SGTKLWHLVKNHDHADQREGDRGSKMVSEIYL**R**LLATKGT**L**QKFVDDLFETIFSTAH**R**GSALPLAIK
SGVKVWHLVKNHEHG**D**QKEGDRGSKMVSEIYL**R**LLATKGT**L**QKFVDDLFET**L**FSTV**H**RGSALPLAIK

YMFDFLDEQADRH**M**ISDPDVRHTWKS**N**CLPLRFWV**N**VIK**N**PQFVFDI**H**KNSITDACL**S**VVAQ**T**FMDSC
YMFDFLDEQADKHQITDS**D**VRHTWKS**N**CLPLRFWV**N**VIK**N**PQFVFDI**H**KNSITDACL**S**VVAQ**T**FMDSC
YMFDFLDEQADKHGIHDTDVRHTWKS**N**CLPLRFWV**N**VIK**N**PQFVFDI**H**KSSITDACL**S**VVAQ**T**FMDSC

STSEHKL**G**KD**S**PSN**K**LLYAKDIPNYKNW**V**ERY**Y**SDISRMSA**I**SDQDMSAYLAEQ**S**RLHL**N**QFN**S**MSAL
STSEHKL**G**KD**S**PSN**K**LLYAKDIPNYKNW**V**ERY**Y**SDIAR**M**PA**I**SDQDMSAYLAEQ**S**RLHL**S**QFN**S**MSAL
STSEH**R**L**G**KD**S**PSN**K**LL**Y**AKDIP**N**YKNW**V**ERY**Y**ADIS**R**LP**A**ISDQDM**N**AYLAEQ**A**RL**S**S**E**FN**M**LSAL

HEIFSYITKYKDE**I**LSALERDEQ**S**RRQ**R**LRAKLEQVIDTMALPS
HEIYSYITKYKDE**I**LSALQKDEL**S**RRQ**R**LRSKLEQVIDTMALTS
NEIYSYISK**Y**SE**E**IT**A**ALDQ**E**TAK**Q**RLAYKVEQLIAAMSLES

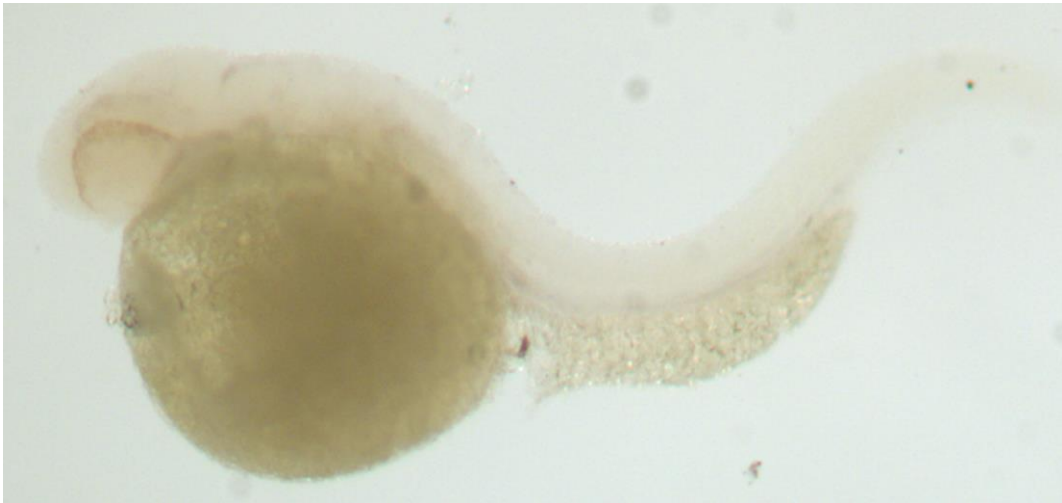


Figure 2. No probe control for *in situ* experiments.

