Expression and Function of Netrin and its Receptors in Sea Urchin Embryos: Implications for Neural and Ectoderm Development

by

Andrew Juurinen
BSc, University of Waterloo, 2003

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE
in the Department of Biochemistry

Supervisory Committee

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Abstract

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Functional and temporal-spatial studies of Netrin and its receptors have been reported in several species including, *M. musculus, D. melanogaster* and *C. elegans.* These studies indicate that Netrins are a family of evolutionarily conserved, secreted proteins that function to elicit the extension and turning responses of axons. Here, I describe the sequences for *netrin* and its receptors, *unc5* and *neogenin*, in *Strongylocentrotus purpuratus* and show that the larval nervous system is patterned predictably with respect to cell body and axon location, early in its development. These findings led to a tentative hypothesis that Sp-Netrin functions to guide axonal growth in the larval nervous system. Quantitative PCR indicates that *Sp-netrin* and *Sp-unc5* are expressed prior to neurogenesis, whereas Sp-neogenin is expressed close to the stage at which neurons differentiate. A polyclonal antibody to Sp-Netrin and *in situ* hybridizations reveal that Sp-Netrin is initially expressed in the vegetal plate, the archenteron and the protein is present on the basal surface of the oral ectoderm in early prism stage embryos. Suppression of Netrin expression, with a morpholino antisense oligonucleotide, results in loss of neurons, loss of ciliary band cells and loss of the oral ectoderm markers, Chordin and Goosecoid. These findings suggest that Netrin is

responsible for maintaining or differentiating oral and ciliary band ectoderm, which is necessary for neural specification or differentiation. Further study of this model is necessary to determine if Sp-Netrin retains a role in axon guidance.

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List of Abbreviations

<	less than
>	greater than
≥	greater than or equal to
%	percent
°C	degrees Celsius
α	alpha
β	beta
µg	microgram
µL	microlitre
µm	micrometre
µM	micromolar
A	anus
AP	animal plate
AR	archenteron
ATA	3-amino-1,2,4-triazole
AVM	anterior ventral mechnosensory
BLAST	basic local alignment search tool
BMP	bone morphogenetic protein
BSA	bovine serum albumin
bp	base pairs
C-terminal	carboxy-terminal
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CSFFSB	chelating sepharose fast flow start buffer
CN	central neuron
CNS	central nervous system
DCC	deleted in colorectal cancer

DIC	differential interference contrast
DNA	deoxyribonucleic acid
dNTP	deoxynucleotidetriphosphate
ECM	extracellular matrix
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
EST	expressed sequence tag
FAK	focal adhesion kinase
FNIII	fibronectin type III
FSW	filtered sea water
hpf	hours post fertilization
G	gravity
HEK	human embryonic kidney
Ig	immunoglobulin
IgC2	immunoglobulin C-2 type
IPTG	isopropyl β-D-1-thiogalactopyranoside
KCl	potassium chloride
kDa	kiloDalton
L	litre
LamNT	laminin N terminal
LCB	lateral ciliary band
LN	lateral neuron
LB	Luria-Bertani broth
M	mouth
MASO	morpholino antisense oligonucleotide
mg	milligram
mL	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
N	amino-terminal
NCBI	National Centre for Biotechnology Information
OD	optical density

OE	oral ectoderm
ORF	open reading frame
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with Tween20
PBSW	phosphate buffered sea water
PCR	polymerase chain reaction
Pfam	protein family
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
PMSF	phenylmethylsulfonyl fluoride
PO	post-oral
PVM	posterior ventral mechonsensory
QPCR	quantitative polymerase chain reaction
RGM	repulsive guidance molecule
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
Sfrp	secreted frizzled related protein
SMART	simple modular architecture research tool
TBS	tris buffered saline
TGFβ	transforming growth factor beta
TSP1	thrombospondin type 1
x	times
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
UNC	uncoordinated
UTR	untranslated region
VTCB	ventral transverse ciliary band

Acknowledgments

I deeply appreciate the opportunity Dr. Robert Burke gave me to study under his supervision. I had many exciting and wonderful experiences, learning and working, because of his guidance. I would also like to thank my committee members, Dr. Bob Chow and Dr. Perry Howard for their advice and support during my studies here at the University of Victoria.

I would like to thank Diana Wang and Nahida el Warry for giving me great technical advice and laboratory support and Stu Trenholm for his initial work on Sp-Netrin. I would like to thank all the past and present members of the lab: Elizabeth Brothers, Nick Church, Kate MacDonald, Navraj Chima, Nathan West, Jocelyn Milburn, Christina van Netten-Thomas, Oliver Krupke, Claire Wright and Mireille Potentier. I wouldn't have accomplished as much without your help. A special thank you to my parents and grandparents for their support. It was wonderful knowing that you had my back if times got tough.

Finally, I would like to thank my girlfriend, Megan Lailey, for listening to me talk at length about all of my science related problems even though you didn't know what I was talking about most of the time. I couldn't have finished this thesis without your loving support.

Chapter 1 - Introduction

1.1 Netrin and its receptors

Santiago Ramón y Cajal first documented that commisural axons project toward the ventral midline of the embryonic spinal cord. From this, he proposed that the floor plate cells were secreting a diffusible cue that acted as a chemoattractant to guide these axons (Ramón y Cajal., 1909; Kennedy, 2000). Much later, these observations were substantiated when Ishii and others began to describe the gene family, *netrin*, and its role as a secreted, axonal guidance molecule (Ishii et al., 1992; Serafini et al., 1996). The function of Netrin is derived from studies in several species. Loss of function evidence in *D. melanogaster* for example, reveals defects in commissural axon guidance (Harris et al. 1996), whereas gain of function evidence, obtained by the ectopic expression of Netrin, produces a similar, defective axonal guidance phenotype (Keino-Masu et al., 1996) . Similarly, Netrin perturbation in *C. elegans* results in the misguidance of pioneer axons migrating dorsally and ventrally and ectopic Netrin expression results in a similar phenotype (Ishii et al. 1992). While this evidence indicates that Netrin is responsible for the outgrowth and patterning of axons, it does not reveal the more specific function of Netrin, that being, as a chemoattractant or chemorepellent to turn axons toward or away from Netrin containing regions. Evidence for this was provided by Colamrino and Tessier-Lavigne (1995), when they revealed that floor plate or heterologous cells engineered to secrete Netrin, will repel trochlear motor axons *in vitro*. Axonal turning has also been demonstrated more convincingly with "open book" preparations. In these preparations, isolated spinal cord explants are cut in half at the dorsal midline and are cultured with cells engineered to secrete Netrin on one side of the explant. Results from

these experiments show that commissural axons turn toward the Netrin secreting cells (Liu et al., 2007).

Localizations of both *netrin* mRNA and protein have been described in several species. In chick for example, antibodies generated against Netrin and *netrin* specific *in situ* hybidizations reveal a gradient of *netrin* emminating from the embryonic floor plate (Kennedy et al., 1994; Kennedy et al., 2006; MacLennan et al., 1997), just as Ramón y Cajal had predicted almost a century earlier. Similarly, in *C. elegans* and *D. melanogaster*, Netrin is expressed at the ventral midline (Harris et al., 1996; Wadsworth et al., 1996). Overall, these data support a model where *netrin* homologues are expressed at the midline and function as axonal guidance cues that attract or repel subsets of axons.

More recently, additional roles for Netrin have been discovered. For example, Netrin has been implicated in providing an adhesive function in non-neural morphogenesis since the loss of Netrin in mice is reported to produce loose cells in the terminal end buds of mammary glands (Srinivasan et al., 2003). These investigators also used an *in vitro* assay to show that Neogenin (a Netrin receptor) expressing L1 cells aggregate in a Netrin dependent manner, thereby providing further evidence that Netrin has an adhesive function. Moreover, Yebra et al. (2003) report an adhesive interaction between Netrin and integrins. They report that function blocking antibodies to α6β4 integrin inhibit cell attachment to Netrin-1, indicating that the epithelial α6β4 integrin functions as a Netrin receptor. Cell migration is also implicated in this study, since they report that function blocking antibodies to α6β4 and α3β1 integrins inhibit the migration of CFPAC-1 cells on a Netrin coated membrane (Yebra et al., 2003). Schwarting et al., 2004 provide additional evidence for Netrin mediated cell migration since they report that

the loss of Netrin results in defects in neuron migration during development in mice. Several experiments have indicated that Netrin functions in the regulation of vascular morphogenesis (Larrivée et al., 2007; Lu et al., 2004; Park et al., 2004; Wilson et al., 2006). Both proangiogenic and antiangiogenic effects of Netrin-1 are reported. For example, Wilson et al., (2006) report that overexpression of Netrin-1 enables limb revascularization following femoral artery ligation, whereas endothelial tip cells of blood vessels treated with Netrin-1 induce filopodial retraction in an Unc5b (a netrin receptor) dependent manner (Lu et al., 2004). A review by Freitas et al. (2008) suggests that receptors for Netrin could be responsible for the proangiogenic (Park et al., 2004; Wilson et al., 2006) and anti-angiogenic (Larrivée et al., 2007; Lu et al., 2004) phenotypes but further research is needed to deduce the mechanisms that are responsible for these differing effects. Netrin has also been implicated in tumorigenesis as an anti-apoptotic survival factor (Mazelin et al., 2004). The apoptotic role for Netrin was reported by Mehlen et al. (1998), by revealing that DCC (a netrin receptor) induces apoptosis in the absence of Netrin-1 but blocks apoptosis when engaged to the Netrin-1 ligand. Mazelin et al., (2004) expand the implications of this finding by suggesting that apoptotic cell survival regulation is responsible for intestinal tumor development. They show that, in addition to enhancing early stage tumor development, overexpression of Netrin-1 enhances the adenoma to adenoma-carcinoma transition. Thus, since Netrin is implicated to have multiple functions that include, axonal guidance, cell adhesion, cell migration, angiogenesis and tumorigenesis, rigorous testing will be required to ascertain the full extent of Netrin's influence in development.

Orthologues of Netrin consist of a C-terminal region termed domain C that is similar to the C-terminal region of the thioester-containing α-macroglobulin protein superfamily complement components C3, C4 and C5 (Ishii et al., 1992). Several functions have been proposed for this domain. Rajasekharan and Kennedy (2009) suggest that since domain C binds heparin with a high affinity, it may be reponsible for presenting secreted Netrins on cell surfaces and for keeping Netrins in the ECM through the binding of heparin sulfate proteoglycans. In addition, Lopez-Rios et al. (2008) report that secreted frizzled related proteins (Sfrp) contain a $Sfrp_{NTR}$ domain, which is homologous to domain C, that antagonizes the activity of Wnt ligands at the neural plate. Moreover, deletions of domain C in Netrin, result in mild axonal guidance defects (Rajasekharan and Kennedy, 2009), but overall, the definitive function of this domain remains poorly understood. The N-terminal region consists of domains homologous to the laminin subunit proteins and were thereby termed VI and V (Yurchenco and Wadsworth, 2004). Domain VI is globular whereas domain V is composed of three epidermal growth factor (EGF) repeats and both domains bind to DCC and Unc5 receptors (Geisbrecht et al., 2003; Rajasekharan and Kennedy, 2009). Orthologues have an approximate molecular mass of 70kDA (Gillespie et al., 2005).

The bifunctionality of netrin, is due in part to the receptors that bind Netrin. Keino-Masu et al. (1996) found that HEK 293 cells expressing a recombinant Deleted in Colorectal Cancer (*DCC*), result in significant binding of netrin-1. Their experiments also showed that *DCC* protein is expressed on commissural axons. The function of *DCC* has been determined in several organisms. Keino-Masu et al. (1996), perturbed *DCC* by the addition of a *DCC* function blocking monoclonal antibody to spinal cord explants. This

resulted in a reduction in commissural axon outgrowth. De la Torre et al. (1997) showed that in *Xenopus,* retinal ganglion cell growth cones turn toward an *in vitro* source of Netrin, but this effect can be blocked by *DCC* neutralizing antibodies. In *C. elegans,* Unc-40 (a *DCC* orthologue) mutants resulted in PVM and AVM neurons that do not fully extend to the Unc-6 (a Netrin orthologue) expressing, ventral nerve cord, whereas a majority of positive and wild type controls reach the ventral nerve cord. To rescue this phenotype, *unc-40* mutants were modified with a mec-7 promoter designed to direct the expression of Unc-40 in AVM and PVM neurons. A majority of these neurons reach the ventral nerve cord, thereby demonstrating that Unc-40 functions cell autonomously (Chan et al., 1996). Thus, *DCC* appears to be a functionally conserved, neurally expressed receptor that is responsible for the cell autonomous, chemoattractive, axonal guidance response to Netrin expression.

Neogenin is a homologue of *DCC,* shares 50% amino acid identity, and binds Netrin with a similar affinity (Wilson and Key, 2007). Unlike *DCC*, Neogenin expression is weak in the early developing CNS but intensifies as neurogenesis proceeds and is found in many non-neural mesodermal derivatives (Gad et al., 1997) and is reported to be expressed in a gradient across the chick retina (Wilson an Key, 2007). Although the neural-related functional properties of Neogenin are not as thoroughly studied as they are in *DCC*, *in vivo* studies have revealed similarities between *DCC* and Neogenin perturbed neural phenotypes. For example, knockdowns using a morpholino antisense oligonucleotides (MASO) specific for *neogenin*, have revealed that Neogenin expression is necessary for dorsoventral axon guidance in *Xenopus.* In addition, a study from Rajagopalan et al. (2004) indicates that Neogenin may mediate a chemorepulsive

response in axons to the Neogenin binding partner, RGMa by revealing that temporal axons avoid chick-RGM expression *in vitro,* in a Neogenin dependent manner. Taken together, these studies suggest a neural guidance role for Neogenin, but further gain-of-function and loss-of-function evidence is needed to more precisely define its role in development. *Neogenin* has been implicated to have additional functions. Knockout mice, for example, do not have a detectable axon guidance phenotype, but are instead, perinatally lethal, suggesting an essential role in early development (Srinivasan et al., 2003). In addition to axon guidance, Neogenin has been implicated in neuronal differentiation, apoptosis, iron homeostasis, cell adhesion and tissue morphogenesis (Wilson and Key, 2007). Considering the implications of these studies, there is a need to clarify the possible roles for Neogenin in development and axonal guidance.

Extracellularly, *DCC* and Neogenin contain four Ig domains and six fibronectin type III (FNIII) domains. Intracellularly, they contain three highly conserved domains termed P1, P2 and P3. In *DCC*, the P3 domain binds Focal adhesion kinase (*FAK*) and Phosphatidylinositol Transfer Protein-alpha and the P1 domain has a demonstrated binding affinity for a subdomain of Unc5 - the repulsive cue receptor. P3 is also responsible for homodimerization, which is necessary for axon attraction in *DCC* (Xie et al., 2006). In both Neogenin and *DCC*, Netrin appears to bind FNIII domains, whereas *RGMa* binds the FNIII domains of Neogenin but not *DCC* (Rajagopalan et al., 2004; Wilson and Key, 2006). Some of these binding partners are illustrated in Figure 1 - a diagram that illustrates the Netrin axonal guidance signaling pathways discussed in this section.

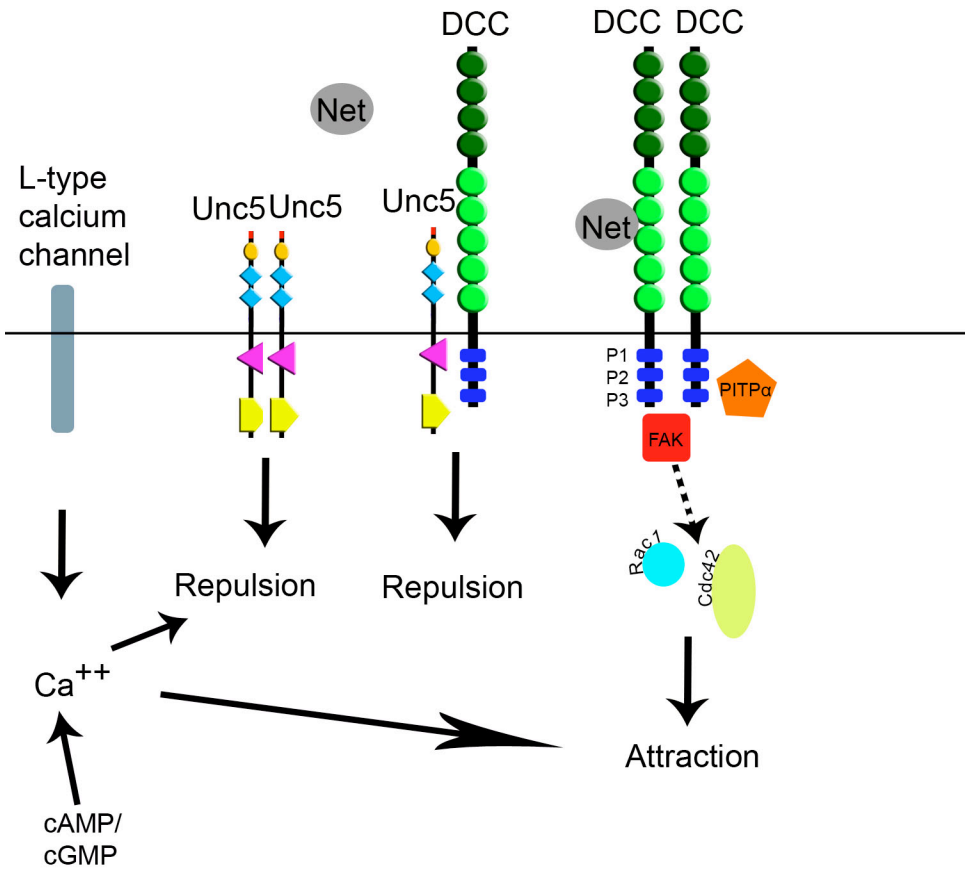


Figure 1. Netrin Receptor Complexes.

Netrin induces the homodimerization of *DCC* resulting in axon attraction. Attractive signaling is mediated through the binding of *FAK* and *PITP*α, and other proteins (not shown). In contrast, Netrin induced homodimerization of Unc-5 or heterodimerization of *DCC* and Unc5 result in a repulsive axonal response. Changes in calcium concentration are mediated by changes in cAMP/cGMP ratios, resulting in attractive and repulsive responses.

Pull down assays using human Netrin protein reveal another Netrin receptor termed *Unc5* (Geisbrecht et al., 2003). *Unc5* was shown to localize to axons using antisera generated against *Unc5* protein (Keleman and Dickson, 2001). Early *Unc5* functional analysis in *C. elegans* reveals that ectopic expression of *Unc5* in touch

receptor neurons results in axons projecting dorsally, uncharacteristically away from sources of Netrin (Hamelin et al., 1993), whereas loss of *Unc5* results in axon migration defects (Hedgecock et al., 1990). Later studies employing a chimeric DCC/Unc5 receptor, in which the cytoplasmic domains of DCC and Unc5 were fused, reveal that the cytoplasmic domain association of DCC and Unc5 is sufficient in converting Netrin-induced axon attraction to repulsion (Hong et al., 1999). Keleman and Dickson (2001) later revealed that, in *D. melanogaster*, ectopic expression of *Unc5* can elicit short or long range axon repulsion from the midline, whereas long range repulsion requires *Unc5* and *Netrin* but does not require *Frazzled* (a *DCC* homologue). Like *DCC*, *Unc5* is also reported to act cell autonomously. Experiments by Labrador et al., (2005) using *D. melanogaster* show that *unc5* mutants result in motor nerves that do not repel from epidermal stripes of Netrin expression. However, if *Unc5* is neuronally expressed in these *Unc5* mutants, the repulsive phenotype is rescued. Taken together, these studies suggest a model where *Unc5* is responsible for a cell autonomous, chemorepulsive axonal guidance response to Netrin expression, whether expressed alone or with *DCC*.

Extracellularly, Unc5 proteins contain one or two Ig domains and two thrombospondin type domains. A single pass transmembrane domain is followed by several intracellular domains. These include, a ZU5 domain, a *DCC* binding motif domain, and a death domain (Wang et al., 2009). Overall, these experiments demonstrate functions for *netrin*, *DCC*, *neogenin* and *unc5* in axon guidance. That is, the Netrin ligand, which is often found at the midline, acts as as a bifunctional axonal guidance cue that is chemoattractive for axons expressing *DCC* and chemorepulsive for axons

expressing *Unc5* alone or *Unc5* and *DCC*, while Neogenin has been implicated as a possible mediator for axon repulsion.

Other factors have been identified in the Netrin signal transduction pathways that contribute to axon pathfinding. For example, blocking the influx or release of intracellular stores of Ca^{++} has revealed that this is sufficient to convert attraction to repulsion of Netrin mediated axonal growth (Hong et al., 2000). Growth cones expressing *DCC* or *DCC*/*Unc5* have been shown to alter their response to Netrin when treated with varying ratios of cAMP/cGMP analogs (Nishiyama et al., 2003). Furthermore, Nishiyama et al. propose that a Netrin/*DCC* mediated attractive response occurs when an influx of Ca^{++} moves through L-type Ca^{++} channels only when a high ratio of cAMP/cGMP is present, while a low ratio of cAMP/cGMP results in repulsion and an inhibition of Ca^{++} influx. Increases of cytosolic Ca^{++} through a treatment with ryanodine, have revealed an upregulation of downstream effectors of the netrin signal transduction pathway, *Rac* and *Cdc42* (Jin et al., 2005). Mutant forms of *Cdc42* have shown to perturb attraction of isolated Xenopus spinal cord neurons in cell culture (Yuan et al., 2003). Thus, neural activity and Ca^{++} regulators appear to modulate downstream responses of axons to Netrin.

1.2 The Larval Nervous System of *S. purpuratus*

The feeding, swimming and responsive behaviours documented by Strathman et al., (1971) and Mackie et al., (1969) were among the first evidence to indicate that a nervous system may be exist in echinoplutei. The principal effectors are the muscles of the esophagus, the mouth, arms and ciliated cells of the ciliary band. Cilia reverse the direction of beat as food particles approach and coordinated reversals change the

direction of swimming (Strathman, 1975). Subsequent research, has characterized the echinopluteus nervous system by electron microscopy, immunohistochemistry and genomic approaches (Beet et al., 2001; Burke, 1978; Bisgrove and Burke, 1987; Nakajima et al. 2004; Yaguchi et al. 2000; Burke et al., 2006).

Burke, (1978) was the first to thoroughly describe nerve cells located along margins of the ciliary band that extend neurite tracts running along the length of the ciliary band in *Strongylocentrotus purpuratus* using electron microscopy. Later, this work was supported with immunohistochemistry, when it was revealed that serotonin and synaptotagmin expressing neuroblasts first appear in the animal plate whereas synaptotagmin expressing cells appear later in the presumptive ciliary band of the late gastrula (Bisgrove and Burke, 1986; Nakajima et al., 2004; Yaguchi et al., 2000) (Fig. 2). Beer et al. (2001) and Nakajima et al. (2004) confirmed that these serotonin and synaptotagmin expressing neuroblasts extend projections to form tracts of neurites that are associated with the larval ciliary band. Clusters of neurons form four separate ganglia. The apical ganglion forms at the most apical part of the embryo, consisting of 10-12 synaptotagmin expressing neurons and 4-6 bilaterally positioned serotonergic neurons. A pair of lateral ganglia form on either sides of the embryo that project neurites to the posterior part of the larva and to adjacent ciliary band associated neurons (Nakajima et al., 2004). Echinopluteus larvae also contain an oral ganglion that contains cross-reactive dopaminergic and serotonergic columnar shaped cells at the lower lip of the mouth (Bisgrove and Burke, 1987).

Considering that later stage echinoplutei form neurons that develop predictably and given that other deuterostomes that exhibit predictable neurital patterning have

axonal guidance molecules, it seems plausible that the patterning of neurites in the *S. purpuratus* nervous system is also determined by axonal guidance molecules. In fact, Burke et al., (2006) identified orthologues for several deuterostome axonal guidance genes using sequence data produced from the sea urchin genome project (Sea urchin genome sequencing consortium, 2006). Burke et al., (2006) list predictions for orthologues of *semaphorin* and plexins, *slit* and its receptor *robo* and the B-type *eph* receptor and *ephrin* ligand and *netrin*. Thus, to determine if these axonal guidance molecules are patterning the nervous system, it is necessary to dissect out the functions and spatio-temporal distributions for each of these genes.

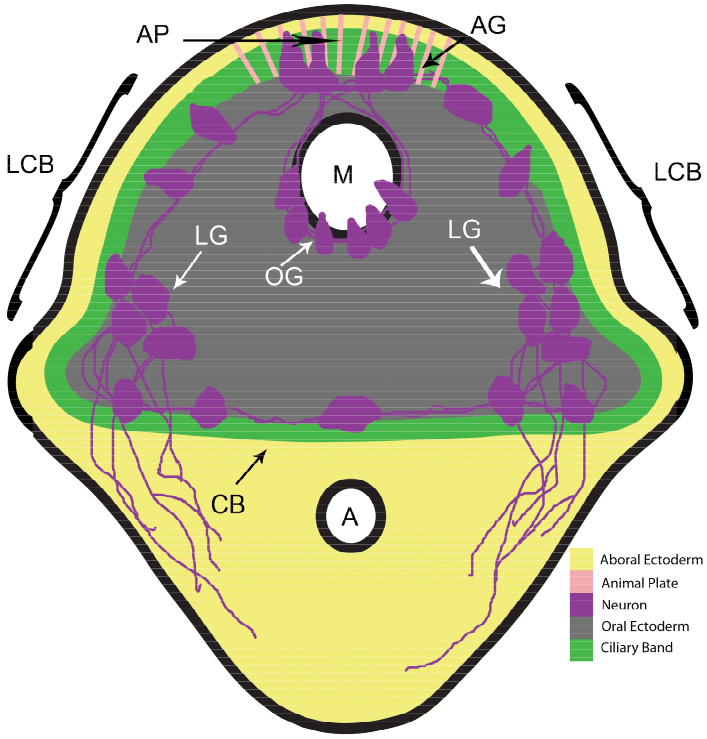


Figure 2. Schematic representation of the late pluteus nervous system (96 hpf). Post oral cells increase in number and extended neurites to other neural cells along the ventral transverse ciliary band (VTCB) and the LCB. More lateral cells appear, forming lateral ganglions (LG) on both sides of the embryo and connect with projections to other neural cells in the CB. Eight or nine apical neurons interconnect to form the apical

ganglion (AG). The oral ganglion (OG) forms around the lower portion of the mouth (M). Anus (A)

1.1 Neural and ectoderm specification

Recent evidence reveals that neural development is dependent on ectodermal specification in echinoderms (Yaguchi et al., in press, Bradham et al., 2009). Thus, it will be useful to review some of the proteins responsible for these processes. There are four major regions of embryonic ectoderm in early pluteus stage embryos. These regions are (ventral) oral ectoderm, (dorsal) aboral ectoderm, ciliary band and the animal plate. Recently, research has focused on the specification of each type of ectoderm (Lapraz et al., 2009, Duboc et al., 2004; Duboc et al., 2008; Yaguchi et al., submitted). A review of the key regulatory proteins involved in neural and ectodermal specification will be useful in understanding the phenotypes that are presented in this report.

By blocking vegetal signalling and investigating the expression patterns of proteins that specify oral ectoderm, aboral ectoderm and ciliary band ectoderm, Yaguchi et al., (2006) reported that the signalling pathways that specify and restrict the expansion of the animal plate are dependent on vegetal canonical *wnt* and function to eliminate a suppressor of *nodal* expression. Further studies are needed however, to characterize the signalling events leading to animal plate ectoderm specification.

P38 is one of the earliest known expressed proteins in the specification of the oral-aboral axis (Bradham et al., 2009). It is uniformly activated early in development and is inactivated breifly in the future aboral side of blastula stage embryos. If *p38* is inhibited, embryos have a small animal plate, become aboralized, have an expanded ciliary band, cease to express Nodal (a well characterized initiator of oral signalling

(Duboc et al., 2004; Duboc et al., 2005; Flowers et al., 2004)) and block most neural development, indicating that *p38* is not necessary for restricting the size of the animal plate but is necessary for restricting the size of the ciliary band and differentiating neurons and oral ectoderm (Bradham et al., 2009; Bradham and McClay, 2006).

Nodal has a pivotal function in regulating the formation of the oral-aboral axis in sea urchin embryos. If *nodal* expression is perturbed, oral and aboral ectoderm are improperly specified, the ciliary band domain expands and neural patterning alters and is mostly associated with the ciliary band. In contrast, overexpression of Nodal results in an extension of expression of oral ectoderm markers, such as *goosecoid*, *antivin*, and *BMP2/4,* all around the embryo as well as a restriction of the ciliary band and neurons to the animal plate (Duboc et al., 2004; Yaguchi et al., submitted). Transcription of *nodal* is activated in the presumptive oral ectoderm at the 30 cell stage (Duboc et al., 2004). Thus, Nodal functions early in development to specify oral ectoderm and positions neurons and the ciliary band.

Further downstream in the oral/aboral/neural specification pathway is *BMP2/4*. *BMP2/4*, which is dependent on Nodal expression, is typically characterized as a promoter of aboral ectoderm, since embryos injected with *BMP2/4* MASO inhibit aboral ectoderm specification (Lapraz et al., 2009; Duboc et al., 2004). These embryos also display altered ciliary band and neural patterning (Yaguchi et al., submitted). Conversely, injection of *BMP2/4* mRNA results in embryos with aboral character with no differentiation of ciliary band or neurons (Duboc et al., 2004; Yaguchi et al., submitted). Thus, *BMP2/4* can function to differentiate aboral ectoderm and can pattern or differentiate neurons and ciliary band.

Chordin is expressed at the hatched blastula stage (Bradham et al., 2009) and is traditionally known as an antagonist to BMP2/4 signaling (Oelgeschläger et al., 2003), functioning to pattern dorsal-ventral axis specification, although differences have been reported in *L. variegatus* and *S. purpuratus* with respect to the oral-aboral phenotypes observed in Chordin perturbed embryos (Bradham et al., 2009; Lapraz et al., 2009). Perturbation of Chordin in the echinoderm, *Lytechinus variegatus*, results in loss of ciliary band cells and loss of synaptotagmin expressing cells, whereas overexpression of Chordin results in the aberrant establishment of the ciliary band and excessive and disorganized neurons (Bradham et al., 2009). Thus, by acting as an antagonist to BMP2/4, Chordin patterns dorsal-ventral axis specification and, when compared to BMP2/4, has reciprocal effects on ciliary band and neurons.

Like Chordin, Lefty is also secreted by oral ectoderm. Lefty is expressed at the 128 cell stage after the expression of Nodal. If Lefty is overexpressed, embryos exhibit the same phenotype as *nodal* MASO injected embryos: they do not specify oral ectoderm and neurons differentiate along a thickened ciliary band. In contrast, when Lefty function is perturbed, embryos exhibit the same phenotype as Nodal mRNA injected embryos: most of the ectoderm is converted into oral ectoderm by the ectopic expression of Nodal and neurons and markers for ciliary band are found in the animal plate (Duboc et al., 2008). Duboc et al. (2008) suggest a model where Lefty, which depends on nodal expression, functions as a long range feedback inhibitor that restricts Nodal to the oral ectoderm.

Citing some of the results that I have mentioned in this section, and additional supportive evidence involving the injection of constitutively active or dominant negative

smad1/5, and Alk3/6 MASO, Yaguchi et al., (submitted) report how the patterning of oral-aboral ectoderm regulates the formation of the ciliary band and neuron differentiation: They demonstrate that the oral boundary of the ciliary band is positioned by Nodal signaling and this, in turn, is positioned by Lefty. Both margins of the ciliary band are affected by BMP2/4 signaling, which is in turn, positioned by Chordin. In addition, Yaguchi et al., (submitted) report that Hnf6 (a marker of ciliary band) is not sufficient to correctly pattern the ciliary band neurons and in the absence of a correctly positioned ciliary band, through Nodal and BMP2/4 signaling, neurons do not form interconnecting neurite tracts. Thus, since many other signaling molecules such as Tbx2/3, Dri, NK1 and FoxA have been implicated in the regulation of ectoderm and since the ectodermal gene regulatory network is still incomplete, (Su et al., 2009), further investigation of ectodermal regulatory proteins will likely be required to determine the full extent of their effects on the positioning and differentiation of the ciliary band and ciliary band neurons.

1.2 Project Overview

S. purpuratus is an intriguing model organism in which to study neural guidance for several reasons. First, the genome for *Strongylocentrotus purpuratus* (Sea urchin genome sequencing consortium, 2006), yielded gene predictions for neural guidance regulators, Netrin, *DCC* and *Unc5*. Secondly, although urchin morphology appears very different from the chordate body plan, many aspects of early development (cleavage, gastrulation) are similar. In fact, among the sequenced genomes of bilaterians, urchins have a very large number of orthologues with humans and mice (repectively, 7077 and 7021, 1:1 orthologues) (Materna et al., 2006). This places echinoderms in an

advantageous phylogenetic position to learn about genes that have been added, retained or altered during evolution, since echinoderms lie between the chordate branch of the deuterostomes and non-deuterostomes (Sea Urchin Genome Sequencing Consortium, 2006). Moreover, embryos are transparent and thousands of embryos can be fertilized together to develop synchronously. There are robust methods for knocking down and overexpressing genes, which make urchin embryos useful models of deuterostome development.

Since orthologues of Netrin, *DCC*/Neogenin and *Unc5* function in guiding axons in other organisms, and *S. purpuratus* appear to have a predetermined neurital pattern, it seemed logical to investigate whether Netrin, *DCC*/Neogenin and *Unc5* act in neurite guidance in *S. purpuratus*. I initially hypothesized that the functions and distribution of ligand and receptors act to pattern the larval nervous system. More specifically, neurital patterning is likely brought about because Netrin is expressed by non-neural tissues and functions as a chemoattractant or chemorepellent to guide neurites, whereas *DCC/ Neogenin* and *Unc5* are expressed by neurons and function to attract or repel neurites from a source of Netrin. Clearly, spatial and temporal data, as well as functional analyses are needed to test this hypothesis. As of yet, little research has focused on neurite guidance in echinoderms; albeit, a recent paper by Katow (2008) sheds light on the spatio-temporal distribution and function of Netrin in the echinoderm, *Hemicentrotus pulcherrimus.*

To carry out this analysis in *S. purpuratus*, sequence information is needed for *Sp-netrin*, *Sp-neogenin*/*dcc* and *Sp-unc5*. Genomic sequence and gene predictions for netrin, neogenin/*DCC* and *Unc5* are available from the whole genome sequencing effort for *S.*

purpuratus (http://www.spbase.org/SpBase/). Additional gene predictions are made available through, NCBI (Strongylocentrotus purpuratus genome version 2.1, (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=7668). Since, genomic predictions were only available for these genes, sequences of cDNAs were determined. Phylogenetic analyses of the protein sequences allowed direct comparisons with proteins in other taxa. Published data on the development of the larval nervous system is not sufficiently detailed. To determine how and if the *early* larval nervous system is patterned in *S. purpuratus*, and thus, could serve as a system that could test my hypothesis, an analysis of the early larval nervous system was undertaken in 56-72hpf embryos using the pan-neural marker, synaptotagmin.

An analysis of netrin transcript abundance by quantitative PCR (QPCR) was carried out to provide a basis for comparison for *in situ* hybridization and immunohistochemistry and to provide insight to gene function. Transcript abundance was also measured for Sp-Netrin receptors, Sp-*neogenin* and Sp-*unc5*. Based on their tentative role in neural guidance in *S. purpuratus,* I hypothesized *netrin*, *neogenin* and *unc5* transcript proteins would be expressed close to when the first neurons begin to appear in the embryo.

Localizations of *netrin* transcript and protein were completed by *in situ* hybridization and immunohistochemistry. I expected these data to correlate temporally with data generated from QPCR and reveal Netrin is present at the midline, based on the conserved functions of Netrin reported in other bilaterians. I expected Netrin to be present on the basal surface of ectodermal cells, since neurites project along these surfaces.

The final objective of this study was to determine the function of Netrin in *S. purpuratus*. This was accomplished through a knockdown of *Sp-netrin* expression by injecting embryos with a *Sp-netrin* specific MASO. Since Netrin acts as a neural guidance cue in other bilaterians, I hypothesized that Sp-Netrin perturbation would result in the miss-patterning of neurites. Knockdowns result in a loss of neurons and reveals a novel function for Netrin. In *S. purpuratus*, Netrin appears to function indirectly as a regulator of neural differentiation.

Chapter 2 - Materials and Methods

2.1 Gamete Collection and Fertilizations

Strongylocentrotus purpuratus adults were collected from Sooke, British Columbia and housed in 11-13°C seawater. Gametes were collected by intercoelomic injections of 0.55M KCl. Sperm was collected above water to maintain viability (up to four days at 4 °C) and eggs were collected and stored in sea water (up to 2 days). Eggs were rinsed three times with filtered sea water (FSW) to remove the jelly coat. For fertilizations, sperm was diluted 1000 fold in FSW and added to beakers containing a monolayer of eggs. 50mg/L Streptomycin sulphate was added to some cultures to reduce bacterial growth. Embryos were cultured at 11-13 °C and were fed a mixture of algae cells (3000 cells/ ml) to cultures older than 96 hours.

2.2 Analysis of Larval Neural Development

2.2.1 Whole Mount Immunohistochemistry

Embryos that were incubated in mouse-anti-synaptotagmin (1e11, 1:200), rat-anti-Hnf-6 (Hnf-6, 1:500) or rat-anti-Netrin primary antibodies (Net, 1:200) were fixed for 15 min in ice cold 100% methanol. Embryos that were incubated in guinea pig-anti-Goosecoid (Gsc (1:100)) and rat-anti-Chordin (Chd 1:250) primary antibodies, were fixed for 15 min in 4% paraformaldehyde in PBSW (0.8mM Na2HPO4–12H2O, 0.15mM KH2PO4, 420mM NaCl, 0.27mM KCl, pH 7.4) + 160mM $CaCl_2$. After washing twice in PBSW or PBS (0.8mM Na2HPO4–12H2O, 0.15mM KH2PO4, 14mM NaCl, 0.27mM KCl, pH 7.4), embryos were blocked in 5% lamb serum in PBS-T (1xPBS + 0.1% Tween-20) for 30 min. Primary antibody was added directly to the blocking solution or diluted in 5% lab serum in PBS and incubated overnight (4˚C).

Embryos were then rinsed three times in PBSW or PBS then incubated in secondary antibody for 2 hours at room temperature and diluted in 5% lab serum in PBS. For anti-1e11, the secondary antibody was goat-anti-mouse (Alexa Fluor 568, Invitrogen Molecular Probes, Catalogue No: A-11031 (1:1200) or Alexa Fluor 635, Invitrogen Molecular Probes, Catalogue No: A-31575 (1:800) For Hnf-6 and Chd, the secondary antibody was goat-anti-rat (Alexa Fluor 488, Invitrogen Molecular Probes, Catalogue No: A-11066 (1:400)). For Gsc, the secondary antibody was goat-anti-guinea pig (Alexa Fluor 568, Invitrogen Molecular Probes, Catalogue No: A11075 (1:800)). For Netrin, the secondary antibody was goat-anti-rat (Alexa Fluor 568, Invitrogen Molecular Probes, Catalogue No: A-11077 (1:1200)) . Embryos were rinsed three times with PBSW or PBS.

For some preparations, PEM-FX Buffer (100mM PIPES, 5mM EGTA, 2mM $MgCl_2$, 0.2% Triton, X-100) (1:9 (37% formaldehyde) was used for 15 min. After washing twice in PBSW, embryos were blocked in 5% lamb serum in PBS-T for 30 min. Primary antibody was added directly to the blocking solution and incubated overnight (4˚C). Embryos were then rinsed three times in PBSW then incubated in secondary antibody for 2 hours at room temperature and diluted in PBSW. Secondary antibody for 1e11 is as listed above and the secondary antibody used for Hnf-6 was goat-anti-rat (Alexa Fluor 488, Invitrogen Molecular Probes, Catalogue No: A-11066 (1:400)). Embryos were finally rinsed three times with PBSW.

Embryos were imaged with a Zeiss LSM7000 or a Leica CTR6000 fluorescence microscope. Images were rotated, cropped and adjusted for brightness and/or contrast using Adobe Photoshop CS3. Before fixation some embryos were scored for their ability

to swim. The number of neural cells and the direction of neurites were quantified in some embryos. Statistical comparisons between these embryos were completed with an unpaired t-test with Welch's correction. A Fisher's exact test was used to compare injected embryos analyzed for expression of Hnf6, Goosecoid and Chordin. All data was analyzed in GraphPad Prism 4.03.

2.3 Generation of Confirmed Gene Sequences

2.3.1 Primer design

Primers for Sp-*unc5*, Sp-*neogenin* and Sp-*netrin* were designed with GeneRunner© (ver. 3.01) from genomic sequences (NCBI - http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=7668, Baylor College of Medicine (http://annotation.hgsc.bcm.tmc.edu/Urchin or http://genboree.org/java-bin/PurpleUrchin, SpBase - http://www.spbase.org/SpBase/). All primers were purchased from AlphaDNA (Montreal, QC). Primer sequences are in Appendix I.

2.3.2 RNA isolation and RT-PCR:

Small modifications were made to the total RNA isolation protocol by Ransick (2004). Unfertilized egg, 24 hpf, 48 hpf, 72 hpf and 96 hpf embryos were used to prepare cDNA for use in QPCR. A standard, 20 000 embryos were collected for use in QPCR, whereas a variable number of embryos were used for cDNA production. The embryos were pelleted by centrifugation (800 x g), sea water was removed and was replaced with 500 µL Trizol (Invitrogen, Catalogue No. 15596-026) when used for QPCR or 200 µL Trizol when used for standard cDNA production. Samples were then vortexed and incubated with 10%(w/v) chloroform (5 min, room temperature) then centrifuged (16 000 x g) for 15 min (4°C). The aqueous layer was incubated overnight (-20°C) with an equal

volume of isopropanol and 1µl glycogen. Samples were centrifuged (16 000 x g) for 15 min (4°C) and the pellet was rinsed three times with 75% ethanol and left to dry. RNA was resuspended with 10µL of nuclease free water when used for full-length cDNA determination and 30µL when used for QPCR.

Components of a RT-PCR reaction included 300ng of random hexamer primers (Invitrogen, Catalogue No. 48109011), 1µL of 10mM dNTPs (Amersham Biosciences, Catalogue NO: 27-2035-01), 1-5µg of total RNA when used for full lenth cDNA determination or 4µL of total RNA when used for QPCR, and nuclease free water to a total volume of 12µL. Samples were heated for 5 min (65˚C) then placed on ice for 2 min. After cooling, 4 µL of 5X First Strand Buffer (Invitrogen, Catalogue No: 18064-022), 2 µL of 0.1M DTT (Invitrogen, Catalogue No: 18064- 022), and 1 µL of RNase inhibitor (Invitrogen, Catalogue No: 15518-012) were added to the sample. RT-PCR reactions were completed in a MyCycler thermocycler (Bio-Rad, Catalogue No: 170-9703). Samples were heated for 2 min (25˚C), then 1µL of SuperScript II (Invitrogen, Catalogue No: 18064- 022) was added. To complete the reaction, samples were incubated 10 min at 25˚C, 60 min at 42˚C and 15 min at 70˚C. The resulting cDNA was frozen at -80˚C until needed.

2.3.3 PCR

Full-length cDNA sequences for *Sp-unc5*, *Sp_neogenin-1* and *Sp_netrin* were generated from cDNA from ~72 hpf embryos. All PCR reactions were completed in a MyCycler thermocycler (Bio-

Rad, Catalogue No: 170-9703). PCR reactions used the following components (in a 50µL reaction): 5 µL of 10X Ex Taq Buffer, 4µL of 25mM dNTP, 2.5 µL of varying concentrations of cDNA, 0.5 µL of 20mM forward primer, 0.5 µL of 20mM reverse primer, 0.25 µL of 5U/ µL Takara *Ex Taq* DNA Polymerase (Fisher Scientific, Catalogue No: TAK RR001A) and 37.25 µL nuclease free water. Reaction volumes were scaled up as needed. The following basic PCR program was used for amplification: 94˚C for 2 min (denaturation), 94˚C for 30 sec (denaturation), 60˚C for 30 sec (annealing), 72˚C for 1 min (35X) (elongation); 72˚C for 7 min (elongation). However, elongation steps varied according to the size of the expected product. A rule of 1-min elongation per 1 Kb of product was used.

2.3.4 Cloning and Sequencing

PCR products were separated in 1.5% (w/v) agarose gels (EMD Biosciences, (Catalogue No. 9012-36-6) with gel electrophoresis and individual bands excised and extracted using a QiaQuick Gel Extraction Kit (Qiagen, Catalogue No. 28704) according to the manufacturer's protocols. The pGEM-T Easy vector ligation kit (Promega, Catalogue No: A1360) was used for ligating the PCR products into the pGEM-T easy vector system according to the manufacturer's instructions. After ligation, DH5α cells (Invitrogen, 12297016) or JM109 cells (Promega, Catalogue No. L2001) were transformed by incubating 50µL of cells for 30 min on ice with 3µL of the ligation mixture. The transformation mixture was heat shocked for 45 sec at 37˚C and cooled for 2 min on ice. Cells were incubated (37˚C) for 1hr with 250µL of SOC medium. LB agar plates containing 100 µg/mL ampicillin were prepared by spreading 40 µL of 5 mM stock

X-gal on the plates. Transformation mixtures were spread on plates and incubated overnight (37˚C). White colonies were picked from the surface, grown while shaking in 10ml LB media containing 0.1% (v/v) ampicillin overnight (37˚C). PCR primers were used to screen cultures for plasmids containing a cloned insert. 1µL if culture was used in the reaction mixture as template with the original primer sets.

Using either a QIAprep Spin Miniprep Kit (Qiagen, Catalogue No: 27106) or a GeneJET Plasmid Miniprep Kit (Fermentas, Catalogue No: K0503), plasmids were recovered from positive PCR screened clones. NotI (NEB, Item No. R3189S) was used in restriction fragment analysis to confirm insert size. Digestion reactions used 1 µg of plasmid and followed manufacturer's instructions. Digestion products were seperated by electrophoresis on a 1.5% (w/v) agarose gel. All plasmids were sequenced by the Centre for Biomedical Research at the University of Victoria.

2.4 Phylogenetic Analysis

To prepare a phylogenetic tree, full-length protein sequences of Sp-Netrin, Sp-Neogenin-1 and Sp-Unc5 were used to query the NCBI database using the BLASTp search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). BLASTp parameters were as follows: Expect threshold – 10, Word size 3, Matrix - BLOSUM62, Gap Costs – Existence: 11, Extension: 1, conditional composition score matrix adjustment. The full-length, confirmed sequences were chosen from the list of BLASTp results that have E-values $<10^{-3}$, have over a 20% amino acid identity after an alignment of all sequences and are phylogenetically diverse. Sequences were aligned in MEGA 4.0 using a ClustalW

alignment algorithm (Appendix III). A Neighbour-Joining method was used to construct a bootstrap consensus tree inferred from 2000 replicates.

2.5 QPCR

2.5.1 Primer Design

QPCR Primers for *Sp-netrin*, *Sp-neogenin-1* and *Sp-un5* were designed in GeneRunner© Version 3.01 using full-length cDNA sequences for design and were sequence confirmed (Appendix IV). The primers were designed for the following amplicons: *Sp-netrin* primers amplify a 144 bp sequence, *Sp-unc5* primers amplify a 143 bp sequence and *Sp-neogenin* primers amplify a 158 bp sequence. *Sp-ubiquitin* primers were designed to amplify a 147 bp seqeunce.

2.5.2 RNA isolation and RT-PCR

cDNA was tested for genomic DNA content by amplifying with TBPint primers designed by Javier Tello (University of Victoria). TBPint primers amplify an intron/exon boundary region of the Tata binding protein in *S. purpuratus*. If an 800 bp band was amplified it indicated genomic DNA was present in the cDNA mixture. If a 283bp amplified, this indicated that no introns were present in the cDNA mixture and it was used for QPCR.

2.5.3 QPCR

The following components were used in QPCR reactions:

Table 1 QPCR Components

Component	Volume (μL)
Sybr Green Master Mix	7.5

Forward Primer (5µM)	0.15
Reverse Primer (5µM)	0.15
cDNA	4

QPCR runs were completed in a Stratagene Mx3005P (Agilent Technologies, Catalogue No. 401449) and an Eppendorf Mastercycler Realplex. The following program was used to perform the QPCR reaction: 95˚C for 2 min (1x), (95˚C for 15 sec, 60˚C for 15 sec and 72˚C for 20 sec (40x)), 20 minute melting curve. Data was analyzed with a Stratagene Mx3005P or Eppendorf Mastercycler Realplex software and subsequently OpenOffice.org Calc and GraphPad Prism 4.03. Reactions were setup in 96-well plates (Eppendorf, Catalogue No: 951022055) and 8-strip tubes (Axygen Scientific, Item No. PCR-2CP-RT-C, PCR-0208-A), . iQ Sybr Green Master Mix (Bio-Rad, Catalogue No: 170-8885) was used as a fluorescent marker. Sp_Ubiquitin has shown to be present in relatively constant amounts during development (Wei et al., 2006). and was used as a reference gene for *Sp-netrin*, *Sp-neogenin* and *Sp-unc5*.

Primer efficiencies of all primer pairs were calculated by using 10-fold serial dilutions of cDNA as template. A plot of the Ct values on the y-axis and the log of the cDNA concentration on the x-axis was used to determine the line of best fit. Primer efficiency was calculated based on the formula, efficiency = $10^{(-1/\text{slope})}$-1. This plot was also used to determine ΔCt values, using the formula, $\Delta Ct = Ct_{gene} - Ct_{ubiquitin}$. To determine if primers were amplifying at similar efficiencies, ΔCt values were plotted against the serial dilutions of cDNA. Slopes calculated from the line of best fit confirmed this, as slopes were all under 0.7. Relative expression for each gene at egg, 24 hpf, 48

hpf, 72 hpf and 96 hpf was calculated by first determining the ΔCt value of each time point for each gene, then calculating the ΔΔCt value from the following formula, $\Delta Ct_{\text{time point A}} - \Delta Ct_{\text{time point B}}$. Two experimental trials were completed, each using 3 reactions per timepoint. Efficiency corrected calculations used the following formula to determine the final expression levels using the formula, $X^{-\Delta\Delta Ct}$ (where X = primer efficiency $_{\text{gene}}$ + primer efficiency $_{\text{ubiquitin}}$).

2.6 Whole mount *in situ* Hybridization

2.6.1 Preparation of *Sp-netrin in situ* Hybridization Probe

Nucleotide blast searches of Sp-Netrin revealed no paralogues. The probe used for *in situ* hybridization included nucleotides, 1- 1827. Sense and anti-sense probes were produced by performing a restriction digest with Sac II (NEB, Catalogue No. R0157S) for the sense probe and a restriction digest with Spe I for the anti-sense probe according to manufacturer's instructions (NEB, Catalogue No. R0157S) Products of the restriction digest were separated by electrophoresis on a 1.5% agarose gel to confirm that the plasmids had linearized and were extracted from the gel using a Qiagen quick gel extraction kit (Qiagen, Catalogue No. 28704). Digoxigenin-labeled RNA probes were transcribed using a Roche DIG-labeling mix (Catalogue No. 1175033910), employing the Sp6 promoter for the SacII digested construct and the T7 promoter for the SpeI digested construct according to manufacturer's instructions. A sample of the reaction mixture was separated by electrophoresis on a 1.5% agarose gel to confirm the presence of an RNA product.

2.6.2 Whole Mount i*n situ* Hybridization

Whole mount *in situ* hybridizations were completed as in C. Arenas-Mena et al. (2000) with modifications by T. Minokawa and Diana Wang. Embryos were cultured to 24 hpf, 48 hpf, 72 hpf then fixed with 4% paraformaldehyde, 32.5% artificial sea water, 32.5mM MOPS (pH7) and 162.5mM NaCl, overnight (4˚C). Embryos were washed in MOPS Buffer (0.1M MOPS (pH7), 0.5M NaCl, 0.1% Tween-20 and stored in 70% ethanol at -20˚C for up to 4 weeks. Embryos were washed in MOPS Buffer three times for 15 min each. Pre-hybridization of embryos took place for 3 hours at 50˚C in fresh Hybridization buffer (70% formamide, 0.1M MPOS (pH7), 0.5M NaCl, 0.1% Tween-20, 1mg/ml BSA) then underwent hybridization for 7 days at 50˚C in Hybridization buffer containing probe. Probes were removed by washing 5 times with MOPS buffer at room temperature then incubated in hybridization buffer for 3 h (50˚C). Embryos were washed again 3 times in MOPS buffer at room temperature. Samples were blocked in 10mg/ml BSA in MOPS Buffer for 20 min at room temperature then in 10% goat serum with 1mg/ml BSA in MOPS buffer for 30 min (37˚C). Incubation of alkaline phosphatase conjugated Fab fragments (Roche Molecular Biochemicals, Catalogue No. 1093274910) with a 1:500 dilution in 1% goat serum, 0.1mg/ml BSA in MOPS buffer was performed overnight at room temperature. Antibody was removed by washing the embryos in MOPS buffer four times for 2 hours then overnight for 1 final wash at room temperature. Staining was performed by first incubating the embryos in 50mM $MgCl_2$, 1mM Levamisole in 100mM Tris/NaCl (pH9.5) twice for 30 min at room temperature then incubating in staining solution (10% dimethyl formamide, NBT and BCIP in 10%

formamide, 0.1M Tris (pH9.5), 50mM $MgCl_2$, 0.1M NaCl and 1mM Levamisole.) Imaging of the embryos took place on a Leica CTR6000 microscope or a Zeiss Universal.

2.7 Antibody Production

2.7.1 Primer Design

Primers were synthesized that were designed to amplify the three laminin domains and the C345C domain of netrin. (See Appendix I for sequences). The forward primer contained a restriction enzyme cut site for Sac I (NEB, Catalogue No. R0156S). The reverse primer contained a restriction enzyme cut site for Not1 (NEB, Catalogue No. R0189S).

2.7.2 Cloning and Sequencing

Using the full-length netrin/pGEM-T easy construct as template, nucleotides 807-1821 was amplified and cloned into a pGEM-T easy vector as in section 2.3.4. DH5α cells were transformed with the vector and the plasmid was prepared for digestion. Approximately 5µg of the Netrin/pGEM-T easy construct and 2µg of pET-28b were digested with Not1 and Sac1 restriction enzymes as per manufacturer's directions. Both digests were run on a 1.5% (w/v) gel to confirm that the netrin insert had been removed (1.1 kb band) from the pGEM-T easy vector and if the pET-28b vector had linearized (5.4 kb band). Plasmid and insert were isolated then ligated (4 h at 37˚C) by mixing 61.2ng of the netrin insert with 100ng of the pET-28b vector (3:1 molar ratio), 5µL of 2x Ligation buffer (Catalogue No:A1360, Part No. C671A) and 1µL of T4 DNA Ligase (Catalogue No:A1360, Part No. M180A). The ligated plasmid was isolated, screened and sent for

sequencing as in section 2.3.4. After sequence confirmation, the vector was transformed into BL21 cells for protein production.

2.7.3 Protein Production

BL21 cells that were transformed with the netrin/pET28-b construct were cultured in 5ml vials (37˚C) for 12-16 hours. Flasks that contained 300ml to 600ml of terrific broth were then inoculated with 5 ml of log phage cells and incubated until they reached an OD of 0.6. IPTG was added to the cultures (final concentration of 1mM). Cultures were incubated overnight at 37˚C, then centrifuged and the supernatant was removed. Pelleted cells were then resuspended in 1X BugBuster Protein Extraction Reagent. (Novagen, Catalogue No. 70921) and were shaken for 10 min at room temperature to lyse cells. Cell lysates were then centrifuged for 5 min at 16000 x g. The pellets were resuspended in Chelating Sepharose Fast Flow Start Buffer (CSFFSB) (20mM Na_2HPO_4, 0.5M NaCl, 10mM imidazole, 8M Urea). To decrease the viscosity of the solution, the solution was passed through an 18 guage syringe needle followed by a 26 guage needle, then centrifuged. The solution was then run through a 0.45μm filter to remove debris.

2.7.4 Protein Isolation

A chelating sepharose fast flow (GE Healthcare Life Sciences, Product No: 17-0575-02) gel was washed and charged with 0.1 M $NiSO_4$, according to manufacturer's instructions. Approximately 2 ml of gel was decanted into a column, connected to a peristaltic pump. The gel was rinsed for 5 min with CSFFSB. The protein solution was

cycled for 30 min through the column to maximize binding to the histidine-tagged protein. The gel was rinsed with 15 ml of CSFFSB and the flow-through was collected. Increasing concentrations of imidazole (10 mM, 40 mM, 65 mM, 75 mM, 100 mM, 125 mM, 140 mM, 250 mM, 500 mM) were used to elute bound proteins. Fractions were collected in 10ml aliquots.

Sample aliquots were separated by PAGE on a 12% polyacrylamide gel and rinsed with Gelcode blue stain reagent (Thermoscientific, Catalogue No. 24592) to visualize the eluted proteins. Fractions that contained a band corresponding to the histidine tagged Netrin, were concentrated with YM10 ultrafiltration membrane (Millipore, Catalogue No. 13622) according to manufacturer's instructions The concentrated protein was electrophoresed on a 12.5% polyacrylamide gel, transfered to a nitrocellulose membrane, and blocked for 1hr (room temperature) with TBS (50 mM Tris, 150 mM NaCl, pH 7.6) + 5% milk powder. An anti - 6 x histidine primary monoclonal antibody diluted 1/500 in TBS + 0.1% Tween + 5% milk was incubated with the membrane (room temperature) while shaking for 1hr, the membrane was rinsed for 15 min three times with TBS + 0.1% Tween. Following these rinses, the gel was incubated for 1hr with a goat-anti-mouse IRDye 800 secondary antibody (Rockland Inc., Catalogue No: 610132121) diluted 1/20000 in TBS + 0.1% Tween + 5% milk. The membrane was rinsed again for 15 min three times with TBS + 0.1% Tween. Membranes were imaged with a LiCor Laser Scanning Fluorescent imaging system.

Concentrated protein was electrophoresed on a 12.5% polyacrylamide gel, cut out, diced and immersed in a gel elution buffer (50mM Tris-HCl, 150mM NaCl, 0.1mM

EDTA, pH7.5) overnight at 30˚C. Gel elution buffer was removed and transferred to a dialysis cassette (Pierce, Catalogue No. 66370) and buffer exchanged with PBS overnight at 4˚C. A small aliquot was electrophoresed on a 12.5% polyacrylamide gel and stained with Gelcode blue stain reagent (Thermo Scientific, Catalogue No. 24590) to determine the protein concentration.

2.7.5 Antibody Production

Prior to injection of rats for antibody production, a small amount of blood was collected from the two rats and prepared as pre-immune serum. Antigen prepared in section 2.7.4 was mixed in a 1:1 ratio with Freund's complete adjuvant and sonicated. Two rats were injected with 100 μg (in 200 μl) of antigen. Rats were given booster injections once every 3-4 weeks and animals were bled 10 days after each booster injection to assess antibody titre. Rats were sacrificed after the titre was deemed appropriate. Serum was isolated by heating blood (1 hour at 37˚C), incubating the blood at 4˚C overnight then removing serum from the clotted blood.

2.7.6 Antibody Validation

2.7.6.1 Immunoblot Analysis

A 0.5ml pellet of 72hpf embryos were collected after centrifugation then transfered to 500ml RIPA buffer (150mM NaCl, 50mM Tris-HCl (pH7.4), 1% Triton Z-100, 1% Sodium Deoxychloic Acid, 0.1% SDS, 1X protease inhibitor cocktail (Roche Molecular Biochemicals, Catalogue No. 04 693 124 001), 5μg/ml leupeptin and 1mM PMSF. Embryos were sheared by passing the solution through two syringes connected by a 26-guage needle. This solution was mixed in a 1:1 ratio with 2x Laemmli Buffer and separated on a 12.5% polyacrylamide gel, transferred to a nitrocellulose membrane and

blocked with TBS + 0.1% Tween for 1hr (room temperature). Expressed protein was also mixed with 2X Laemmli Buffer, separated and blocked in a similar manner. Serum collected in section 2.7.5 and normal rat serum was diluted 1:200 in TBS + 0.1% Tween + 5% milk and incubated with the membrane at 4˚C with shaking. The membrane was rinsed for 15 min three times with TBS + 0.1% Tween, incubated for 1hr with a goat-anti-rat IRDYE 700 secondary antibody (Rockland Inc., Catalogue No: 612130120) diluted 1/20000 in TBS + 0.1% Tween + 5% milk. The membrane was rinsed again for 15 min, three times with TBS + 0.1% Tween. Fluorescent bands were visualized on on a Li-Cor fluorescent imaging system.

2.8 Knockdown of Gene Expression

2.8.1 Microinjection

Eggs were rinsed three times with FSW and filtered 5-12 times through a 100µm filter to remove the jelly coat. Microinjection dishes were prepared by coating a 1mm wide strip of 1% (w/v) protamine sulphate and allowed to sit for 1 min. Dishes were filled with distilled water, allowed to sit for 5 min, then allowed to dry. Plates were filled with 1mM ATA in FSW. Eggs were placed by mouth pipette along the row of 1% (w/v) protamine sulphate and allowed to adhere to the dish surface. Needles used in injection (World Precision Instrument, Inc., Catalogue No: TW100F-4) were made by heating the glass needles then pulling using a micropipette puller (Sutter Instrument Co. Flaming/Brown Micropipette puller model P-97). Pulled needles were then loaded with 300µM Sp_Netrin MASO or the standard control MASO in 22.5% glycerol. The solutions were then filtered through a 0.2µm RNase-free filter. The Sp-Netrin MASO

sequence and standard control MASO are listed in Appendix I. Adherent eggs were fertilized by applying 10μL of a 1/100 dilution of sperm directly on to the eggs. Embryos were pressure injected with by using a Picospritzer II (General Valve Corporation), set for continuous flow and injector and MMN-1 (Narishige) manipulator. After injection, embryos were rinsed with FSW to dilute the ATA.

Chapter 3 – Results

3.1 DNA sequences and predicted protein domain structure

3.1.1 Sp-Netrin

To determine the full-length cDNA sequence, a Glean3_04245 prediction from Baylor College of Medicine (BCM) was used to provide sequence for primer design (Fig. 3 a). Two primers amplified an 1827 bp *Sp-netrin* sequence (Fig. 3c,d). An ORF predicts a 69.4kDa protein with 608 amino acids (Fig. 3 e). SMART analysis (http://smart.embl-heidelberg.de/) predicts a protein domain structure that is consistent with homologues of netrin - one laminin N terminal domain (Lam NT), three laminin-type epidermal growth factor-like domains (EGF Lam) and a C345C domain. A signal peptide protein sequence is predicted (P = 0.999) by Signal P 3.0 (Bendsten et al., 2004) and is likely to be cleaved between amino acids 21 and 22 (P = 0.872).

3.1.2 Sp-Unc5

To determine the full length, cDNA sequence for *Sp-unc5,* a Glean3_ 10776 prediction from BCM, a *Paracentrotus lividus* EST - SP0ACLEB18YG07RM1 sequence from the Max Planck institute for molecular genetics (http://www.molgen.mpg.de/~ag_seaurchin/) and a scaffold sequence_v2_22300:30136 obtained from spbase.org were used for primer design (Fig. 4 a). Three overlapping amplicons were generated to construct the full-length, 3111bp *Sp-unc5 sequence* (Fig. 4 c,d). An ORF predicts a 114.2kDa protein with 1037 amino acids (Fig. 4 e). SMART analysis predicts one immunoglobulin C-2 type domain (IgC2), two thrombospondin type 1 repeats (TSP1), a transmembrane domain, a ZU5 domain and a death domain. SMART

also reveals that this domain structure is consistent with homologues of *unc5*. Signal P 3.0 predicts a signal peptide protein sequence (P = 0.963). The signal peptide is predicted to be cleaved between amino acids 30 and 31 (P = 0.932).

3.1.3. Sp-Neogenin

To determine the full-length sequence for *Sp-neogenin*, two predictions were used for primer design. These were, Glean3_25975 from BCM and hmm182407 from the National Centre for Biotechnology Information (NCBI) (Fig. 5 a). Nine overlapping amplicons were generated to construct two (5550bp, 2313bp) cDNA sequences (Fig. 5 c,d). The *Sp-neogenin-1* ORF terminates in a stop codon and predicts a 200.3 kDa protein with 1850 amino acids (Fig. 5 e). SMART and Pfam analysis of Sp-Neogenin-1 predicts, 2 immunoglobulin domains (IG), 2 C-2 type immunoglobulin domains (IGC2), 5 fibronectin type 3 domains (FN3) and a PFAM Neogenin_C domain. Although SMART reveals that this domain structure is similar to homologues of Neogenin, Neogenin homologues typically consist of 6 fibronectin type 3 domains rather than the 5 that are predicted for Sp-Neogenin-1. *Sp-neogenin-2* consists of the first 3 of 9 amplicons that were used to construct the *Sp-neogenin-1* cDNA. However, a sequence region from the 3' end of *Sp-neogenin-2* is 58% dissimilar to *Sp-neogenin-1*. Specifically, the 3' end of the final exon from the amplicon, D2309FR, is inconsistent with the amplicon, D718FR. However, cDNA sequence from D2309FR is consistent with sequence from amplicons, D4FR and D718FR. Specifically, D2309FR overlaps on its 5' end with D4FR and the second last exon of D2309FR overlaps with the first exon of D718FR. 5' sequence from the final exon of D2309FR is also consistent with the second exon of D718FR. Although,

the 3' end from the final exon of D2309FR is not consistent with the amplicons generated here or the Glean3-25975 and HMM182407 predictions, this sequence is consistent with adjacent contig sequence that was used to generate annotations for *Sp-Neogenin*. SMART analysis of the 771 amino acid *Sp-neogenin-2* ORF, reveals a domain structure that consists of the first 4 immunoglobulin domains and the first 3 fibronectin domains of Sp-Neogenin-2. However, the domain structure of *Sp-neogenin-2* is incomplete since its 3' end does not contain a stop codon. Signal P 3.0 does not predict a signal peptide protein sequence for either protein (P = 0.065).

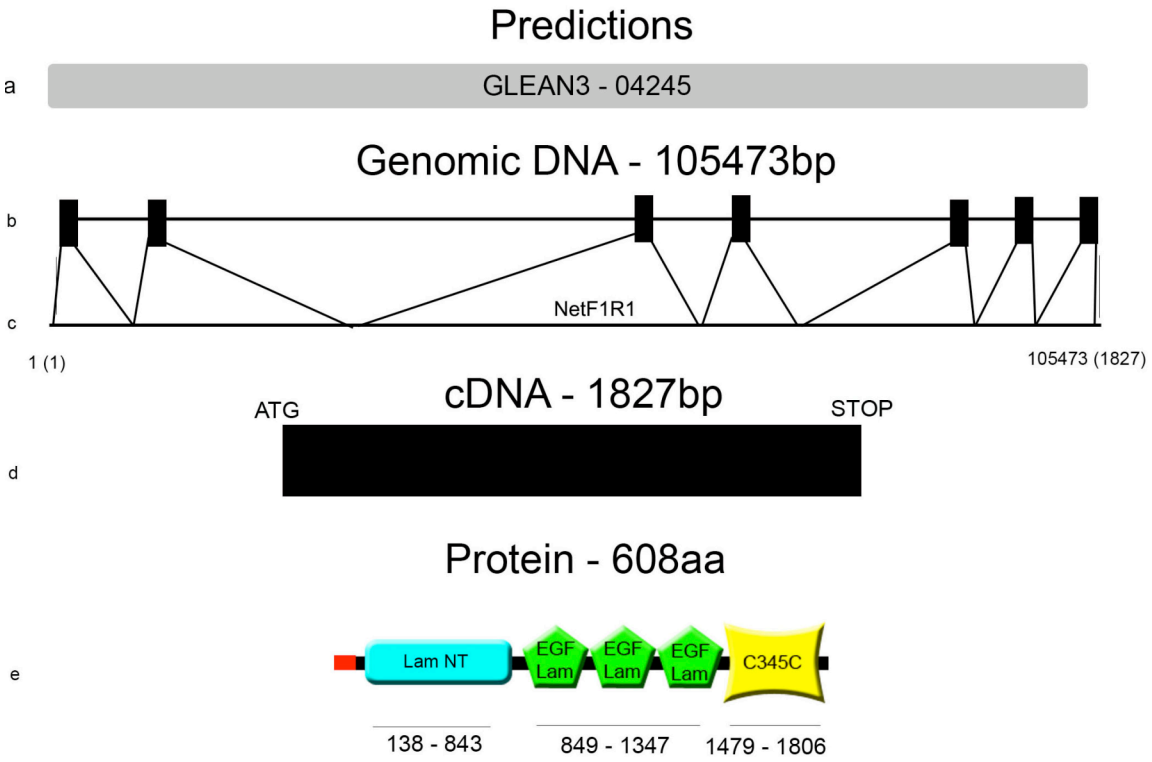


Figure 3. Schematic of gene predictions, genomic DNA, amplified regions, cDNA and SMART protein predictions for Sp-Netrin

a) Predictions - a Glean3 prediction used to generate primer sequence. b) Genomic DNA - black bars represent exons. c) Amplicon generated by PCR - numbers in parentheses

indicate cDNA base pair position. Numbers without parentheses represent genomic DNA base pair position. d) cDNA – full length obtained from one amplicon. e) Protein - underlined regions indicate base pair position with respect to protein domain structure.

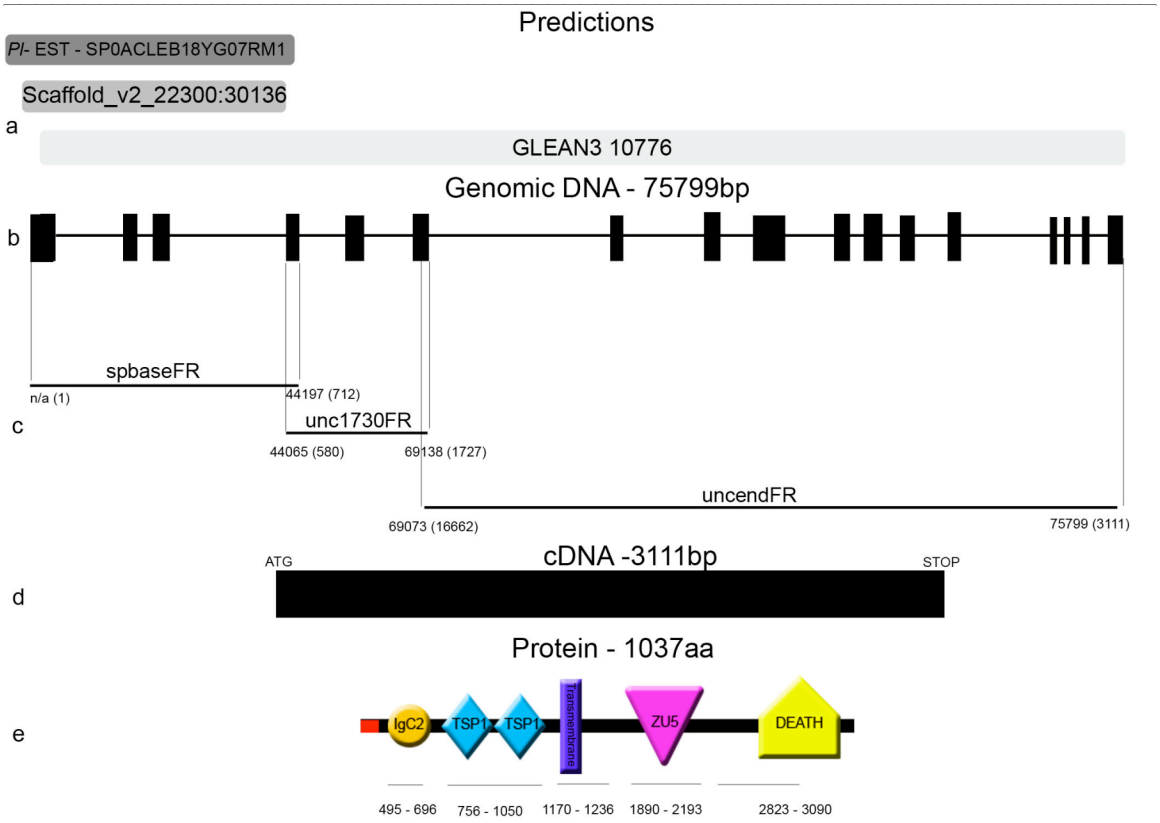


Figure 4. Schematic of gene predictions, genomic DNA, amplified regions, cDNA and SMART protein predictions for *Sp-Unc5*.

a) Predictions - a Glean3 prediction, a *Paracentrotus lividus* EST and a scaffold sequence were used to generate primer sequence. b) Genomic DNA - black bars represent exons. c) Overlapping amplicons generated by PCR - numbers in parentheses indicate cDNA base pair position. Numbers without parentheses represent genomic DNA base pair position. d) cDNA – virtually constructed from overlapping amplified DNA. e) Protein - underlined regions indicate base pair position with respect to protein domain structure.

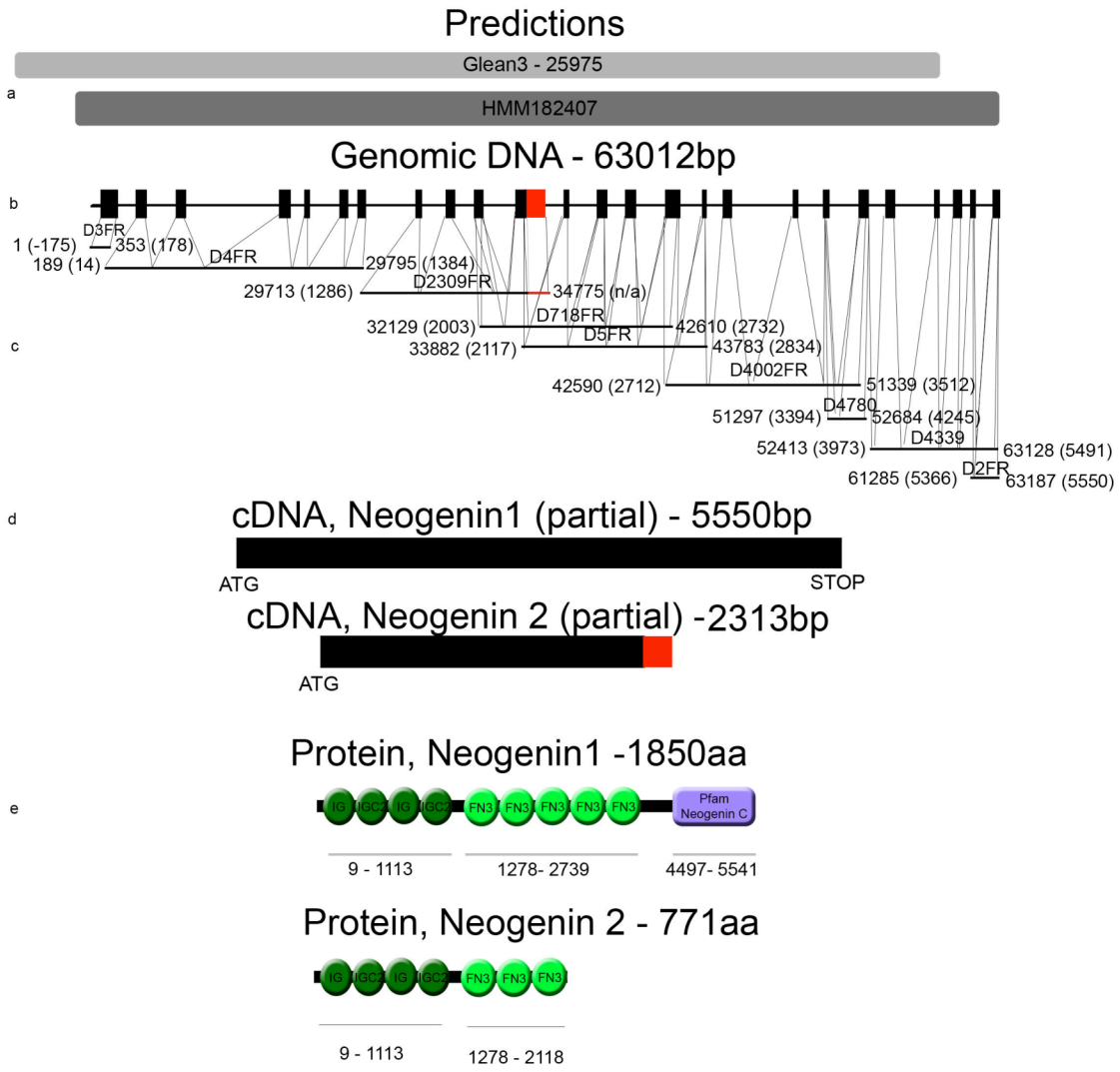


Figure 5. Schematic of gene predictions, genomic DNA, amplified regions, cDNA and hybridized SMART and PFAM protein predictions for *Sp-neogenin*

a) Predictions - Glean3 and HMM predictions used to generate primer sequence. b) Genomic DNA - black bars represent exons, red bars represent exons that were amplified with an alternatively spliced region of the gene. c) Overlapping amplicons generated by PCR - numbers in parentheses indicate cDNA base pair position. Numbers without parentheses represent genomic DNA base pair position. d) cDNA - Two alternatively spliced products that are virtually constructed from overlapping amplified DNA. e)

Protein - underlined regions indicate base pair position with respect to protein domain structure.

3.2 Phylogenetic Analysis

3.2.1 Sp-Netrin

BLASTp searches with Sp-Netrin sequence identify Netrin homologues from several model organisms and these were used to produce a Neighbour-Joining tree (Fig. 6). The phylogenetic tree reveals that netrin homologues from chordates group together (*H. sapiens, M. musculus, D. rerio*), Netrin homologues from echinoderms group together (*S. purpuratus, H. pulcherrimus*), the hemichordate Netrin homologue from *S. kowalevskii* groups closest with the echinoderms and Netrin homologues from the arthropod, *D. melanogaster* and the Nematode, *C. elegans* are outgroups. Some segments of the Clustal W alignment (Appendix III, bolded examples) reveal small regions of greater similarity between Hs-Netrin and Dm-Netrin than Sp-Netrin and Hs-Netrin. Nonetheless, these groupings are consistent with the current deuterostome phylogenies (Cameron et al., 2000)

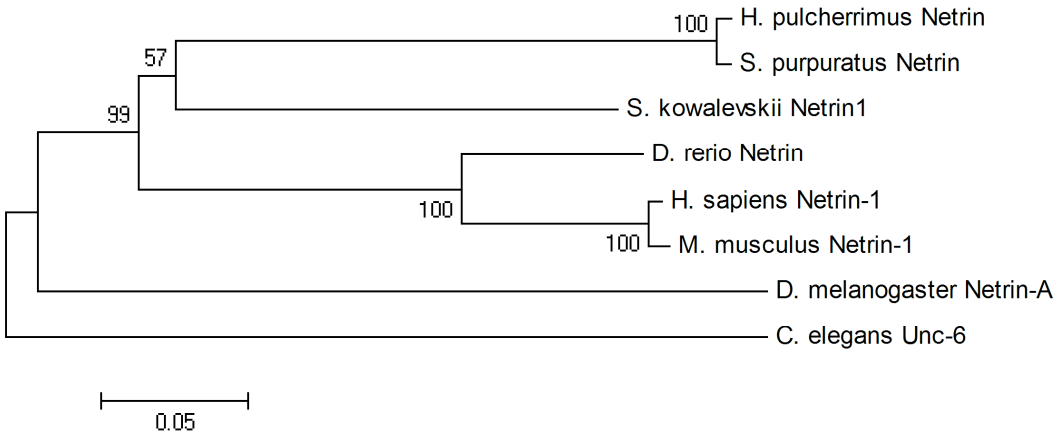


Figure 6. Neighbour joining tree of Sp-Netrin protein sequence.
Bootstrap values were inferred from 2000 replicates and are reported at nodes as percentages. Unlabeled nodes indicate a sequence that MEGA 4.0 software labels as an outgroup. Branch lengths are proportional to the amino acid changes per site in the aligned sequences. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 767 positions in the final dataset.

3.2.2 Sp-Unc5

Homologues of Unc5 from several model organisms show highest similarity to full length Sp-Unc5, when the Blastp search tool is used to query the NCBI database and 5 of these were chosen for phylogenetic analysis (Fig. 7). The phylogenetic tree reveals that: 1) Unc5 homologues from chordates group with *H. sapiens* and *M. musculus* and closely with the early vertebrate, *D. rerio* 2) An Unc5 homologue from the hemichordate *S. kowalevskii* groups closest with the chordates 3) Sp-Unc5 appears more closely related to chordate and hemichordate Unc5 homologues than the Nematode Unc5 homologue.

However Sp-Unc5 is treated as an outgroup. 4) The Nematode Unc5 homologue from *C. elegans* is the least similar and is the least similar outgroup. Since echinoderms and hemichordates are the most closely related of these phyla (Cameron et al., 2000), this grouping of Sp-Neogenin is not consistent with the phylogenies for the groups. Thus, Sp-Unc5 is more divergent than hemichordate Sk-Unc5, but is more similar to the other taxa than the distantly related Ce-Unc5c.

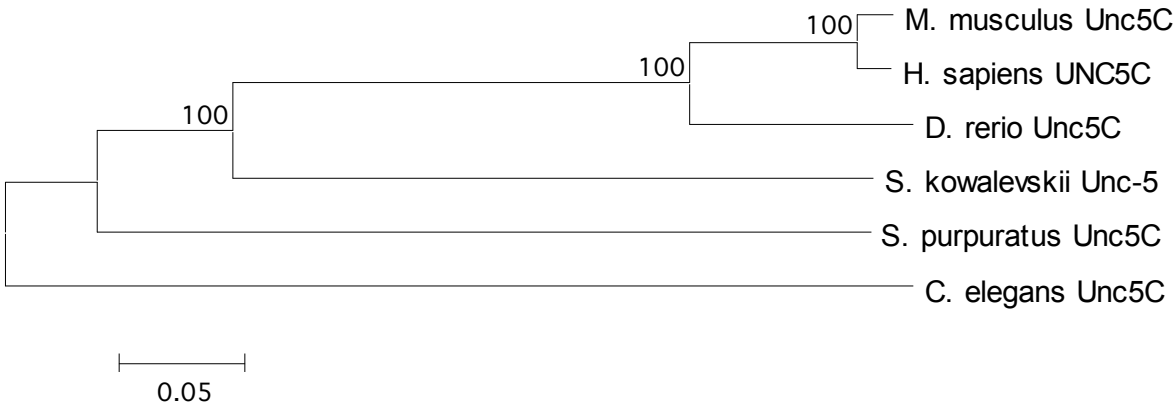


Figure 7. Neighbour joining tree of Sp-Unc5 protein sequence.
Bootstrap values were inferred from 2000 replicates and are reported at nodes as percentages. Unlabeled nodes indicate a sequence that MEGA 4.0 software labels as an outgroup.. Branch lengths are proportional to the amino acid changes per site in the aligned sequences. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 500 positions in the final dataset.

3.2.3 Sp-Neogenin

Homologues of Neogenin from several model organisms are most similar to Sp-Neogenin1, when its full-length protein sequence is used to query the NCBI database

using the BLASTp search tool. Organisms chosen for the Neighbour-Joining tree grouped into four phyla (Fig. 8). The phylogenetic tree reveals that Neogenin homologues from chordates group together (*H. sapiens, M. musculus, D. rerio*), the hemichordate Neogenin homologue groups closest to the chordates (*S. kowalevskii*), the echinoderm, Sp-Neogenin-1 is most similar to both the hemichordate and chordate sequences and a Neogenin homologue from the arthropod *D. melanogaster* is more distantly related and is treated as an outgroup. The Neogenin homologue from the Nematode, *C. elegans* is least similar to all the other sequences and is also considered an outgroup. Since echinoderms are closely related to hemichordates and chordates respectively, this grouping of Sp-Neogenin is consistent with the current deuterostome phylogenies (Cameron et al., 2000).

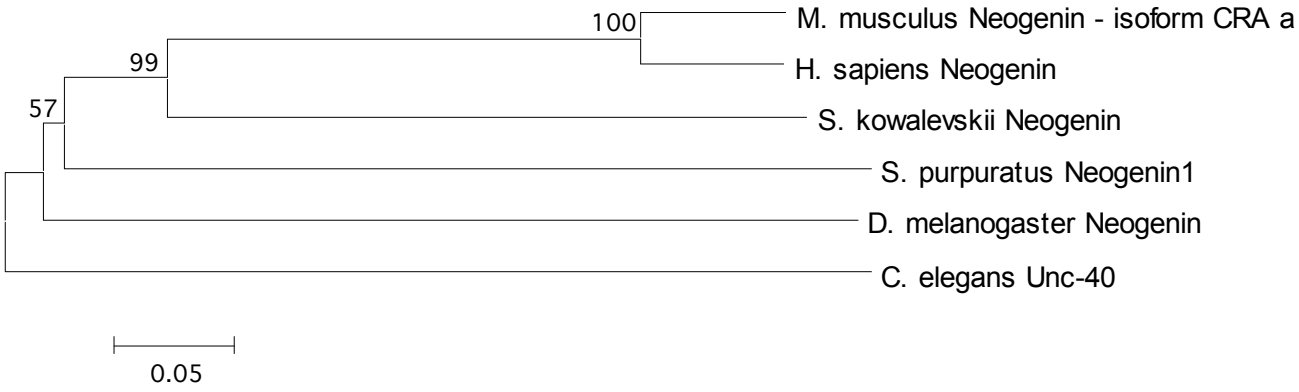


Figure 8. Neighbour joining tree of Sp-Neogenin protein sequence.
Bootstrap values were inferred from 2000 replicates and are reported at nodes as percentages. Unlabeled nodes indicate a sequence that MEGA 4.0 software labels as an outgroup. Branch lengths are proportional to the amino acid changes per site in the aligned sequences. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were

a total of 339 positions in the final dataset.

3.3 Analysis of Larval Neural Development

Echinoderms form a patterned nervous system. While the echinoderm larval nervous system has been well characterized in late pluteus stage embryos (Burke, 1978; Burke et al., 2006, Bisgrove and Burke, 1987; Beer et al., 2001; Yaguchi and Katow, 2003; Nakajima et al., 2004) it has not been extensively described in embryos undergoing neurogenesis. To determine if the larval nervous system forms in a predictable manner, an analysis of early neural cell development was undertaken using the synaptotagmin marker, 1e11. Although some variation exists between individual embryos, most early pluteus stage embryos show the first signs of neurogenesis around 48 hpf with the appearance of, from 2 to 4 synaptotagmin and serotonin positive cells in the thickened animal plate (AP) that project neurites toward the midline (Fig. 9) (Bisgrove and Burke, 1987). Around 54hpf, two, synaptotagmin expressing cells appear in bilaterally symmetric positions in the oral ectoderm just above the developing ventral transverse ciliary band (VTCB). These I term post-oral (PO) neurons. Neurites from PO neurons project toward the lateral ciliary band (LCB) and the VTCB. PO neuronal development may vary from this typical model (Fig. 10b, 11). For example, a single central neuron (C) may appear at the midline and project neurites laterally, parallel to the VTCB. Alternatively, in a small proportion of embryos, 5 or more C and PO neurons may appear centrally spaced along the VTCB. In this case, cells farther from the midline project

toward both the LCB and VTCB whereas cells closer to the midline project neurites that connect to other neurons along the VTCB. Two to four cells, which I term lateral neurons (LN) differentiate beneath the edge of the LCB and extend axons into the LCB and posteriorly beneath the aboral ectoderm. The number of neurites from LNs varies (Fig. 10, 12). For example, one or more neurites will project toward the LCB whereas none or several neurites may project away from the LCB.

As embryos develop further, more neurons appear along the VTCB and extend neurites to other neurons along the VTCB and LCB (Fig. 9, 10). Lateral ganglia develop on both sides of the embryo as more lateral cells appear and interconnect with other LNs and neural cells in the CB. An apical ganglion develops to contain eight or nine apical neurons that interconnect in the thickened animal plate . An oral ganglion also forms around the lower portion of the mouth (Bisgrove and Burke, 1987). Although there are small variances in larval nervous system development, the model systems presented here (Fig. 2, 9) represents the stereotypic nervous system for larval development.

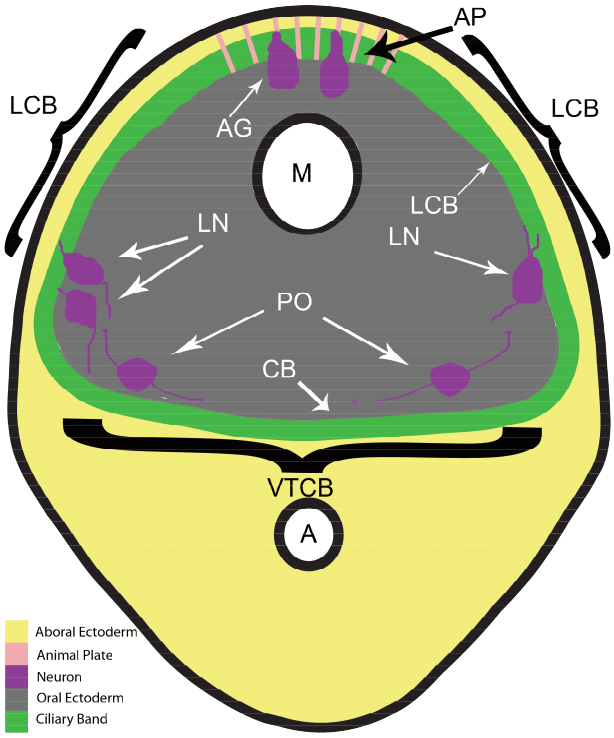


Figure 9. Schematic representation of the early pluteus nervous system.

In an early pluteus (56hpf), post oral cells (PO) appear on the oral ectoderm (grey) and project neurites anteriorly and medially. Lateral neurons (LN) differentiate beneath the ciliary band (CB), close to the edge of the lateral ciliary band (LCB) and extend axons into the CB and posteriorly beneath the aboral ectoderm (yellow). Two to four apical ganglion cells (AG) appear in the thickened animal plate ectoderm (AP) and extend axons toward the midline.

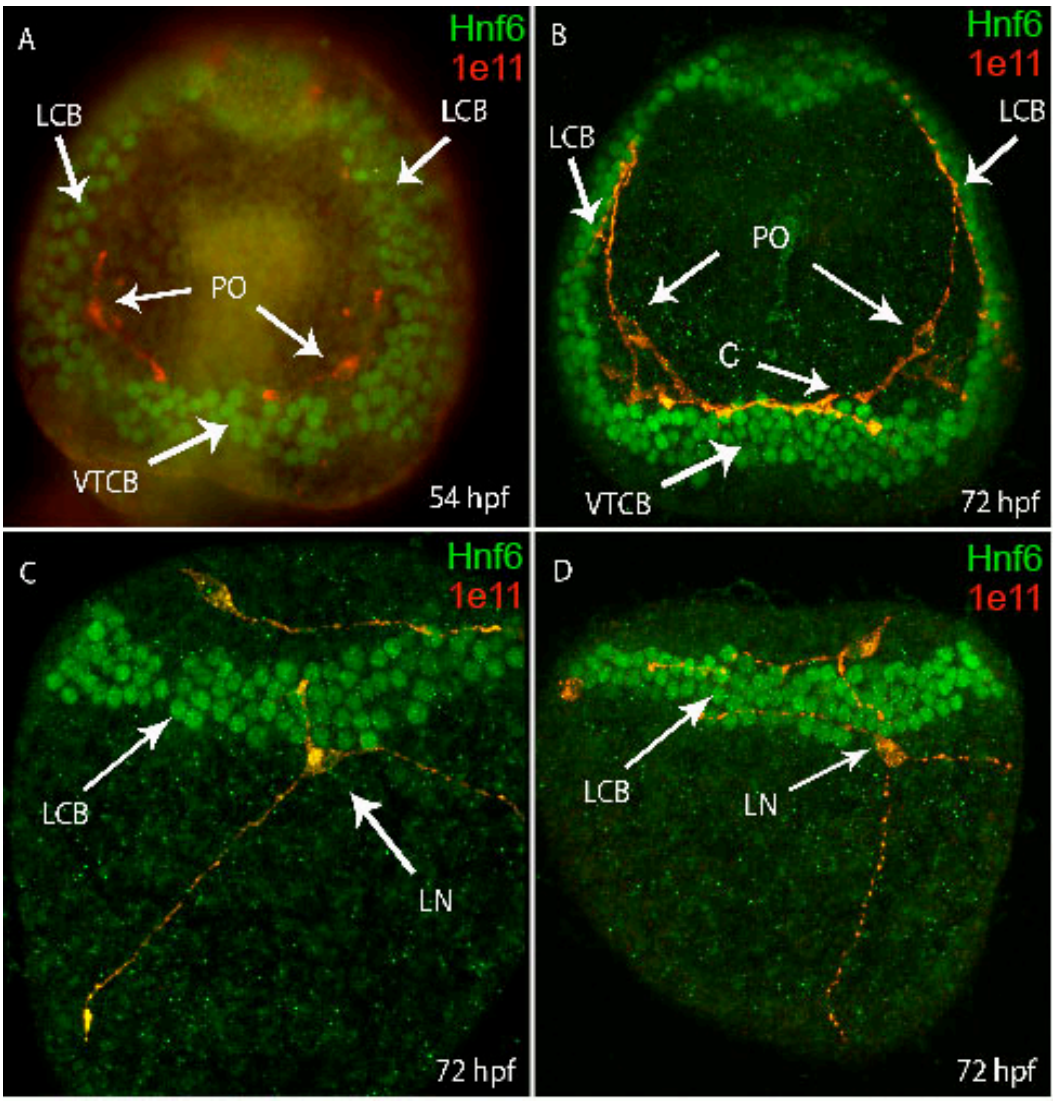


Figure 10. Pluteus stage nervous system as revealed by anti-synaptotagmin (1e11) with ciliary band (Hnf6).

A - (54 hpf) A frontal view showing two PO neurons in the oral ectoderm that project neurites toward the LCB and the VTCB. B - (72 hpf) Frontal view of an embryo with two PO neurons and one central neuron that have extended neurites along the CB forming a single tract of axons. C - (72 hpf) A lateral view showing a LN at the aboral side of the LCB that projects 1 neurite toward the LCB and 2 neurites away from the LCB. D - (72 hpf) A lateral view showing a LN projecting two neurites toward the LCB and two neurites away from the ciliary band.

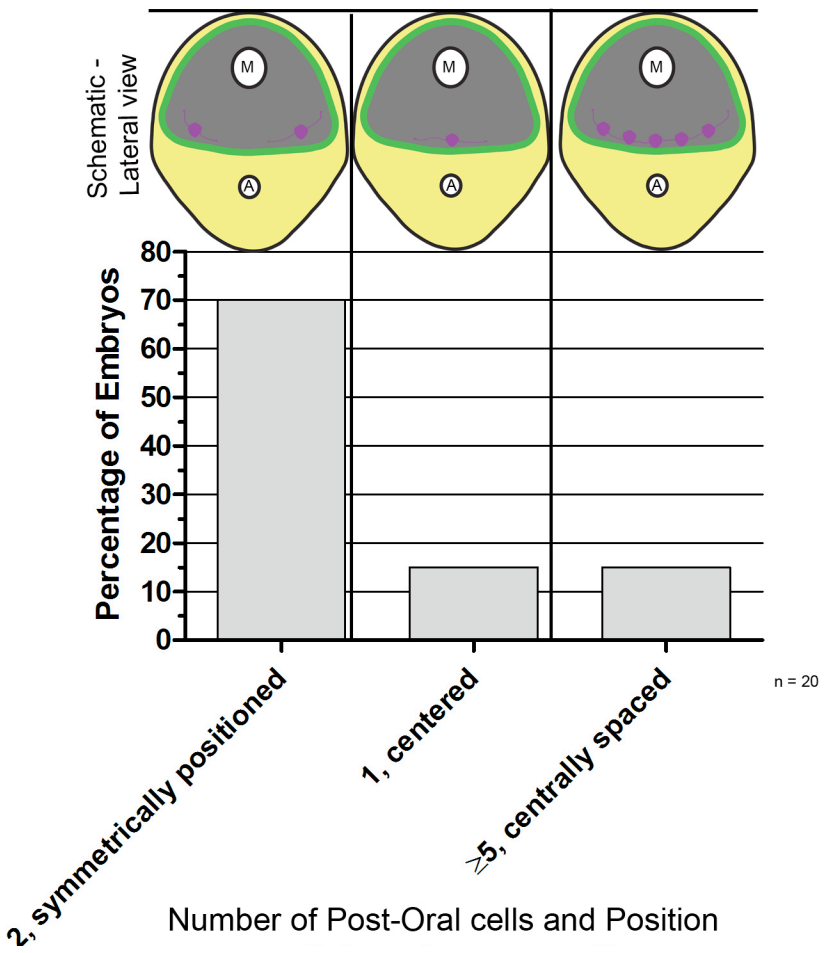


Figure 11. Post-Oral (PO) cell positioning and direction of neurite growth in 56hpf embryos.

In a majority of early neurogenic embryos, PO neurons occur in bilaterally symmetric positions with neurites that project toward the LCB and VTCB. In a much smaller fraction of embryos, a single cell appears at the midline with neurites projecting along the VTCB or 5 or more cells appear centrally spaced above the VTCB. For the latter instance, cells farther from the midline projected toward both the LCB and VTCB while cells closer to the midline projected along towards other neurons along the VTCB. M – mouth, S – stomach, A – anus.

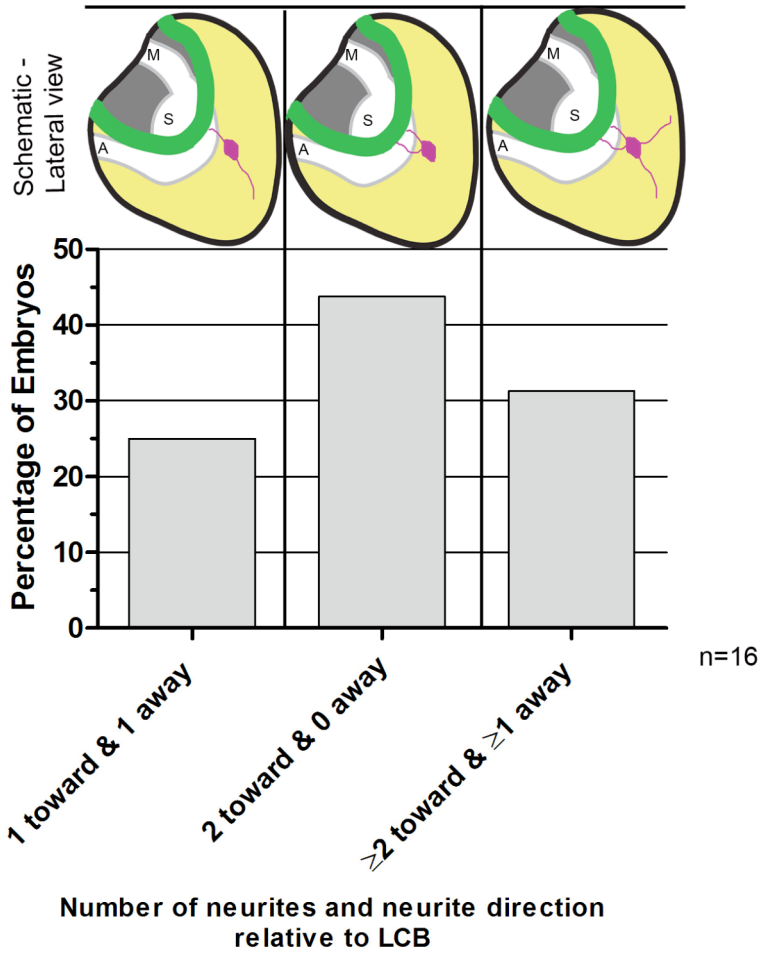


Figure 12. Variability of LC neurites with respect to number of projections and direction of projection in 56hpf embryos.

Lateral cells projected neurites toward or away from the LCB. Roughly equal proportions of early neurogenic embryos have projections in all three categories. Roughly 2/3 of

embryos had one LC appearing while 1/3 of embryos had two LCs appearing at 56hpf. M – mouth, S – stomach, A – anus.

3.4 QPCR

To determine the temporal expression of *Sp-netrin, Sp-unc5* and *Sp-neogenin*, I used QPCR to measure relative abundance of mRNA (Fig. 13). Using this method, a Ct value for one gene at a given timepoint is measured relative to Sp-Ubiquitin and compared to another timepoint, relative to Ubiquitin. Primer efficiencies for primer pairs are as follows: *Sp-netrin* primers – 1.85, *Sp-unc5* primers 1.78, *Sp-Neogenin* primers – 2.00. Sequenced amplicons, and melting curves are found in Appendix IV. *Sp-unc5* is present as maternal transcript, while *Sp-netrin* and *Sp-neogenin* transcripts are not present in the unfertilized egg. *Sp-netrin* and *Sp-unc5* are the only transcripts present at a blastula stage (24hpf). When compared to the relative expression level at 24hpf, *Sp-netrin* expression increases 9 fold at gastrula stage (48hpf), stays relatively level at pluteus stage (72hpf) then increases a further 3 fold at 96hpf. *Sp-unc5* transcript decreases dramatically at blastula stage, then increases expression 27 fold at gastrula stage when compared to levels of the blastula. At 72hpf, relative expression decreases nearly in half. At 96hpf relative expression increases again 36 fold compared to levels at 24hpf. *Sp-neogenin* is first present at gastrula stage and remains relatively level in expression until a 4 fold increase in expression at 96hpf.

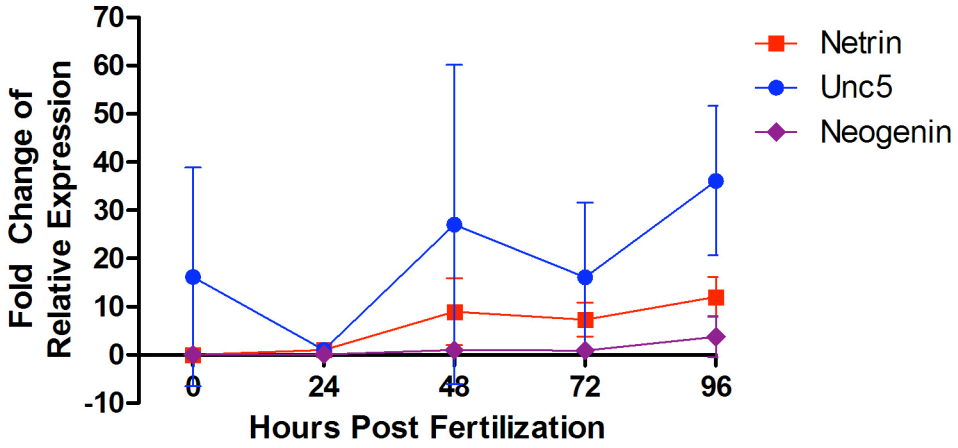


Figure 13. Fold change of relative expression levels for *Sp-netrin, Sp-unc5* and *Sp-neogenin* during the first 96 hours of development.

Fold changes for *Sp-netrin* and *Sp-unc5* were calculated from a base value of 1, that was assigned for expression in 24 hour embryos, whereas fold changes for *Sp-neogenin* were calculated from a base value of 1 that was assigned for expression in 48 hpf embryos. For *Sp-netrin*, abundance of transcript is nil in the egg and increases after 24 hours. For *Sp-unc5* transcript is present in the egg, decreases in abudance at 24hpf then increases at 48 hpf. For *Sp-neogenin*, abundance of transcript is nil in the egg and 24 hpf. At 48 hpf, RNA is transcribed and at 96 hpf, transcript increases in abundance. Error bars are generated from 2 experimental trials.

A chip based oligonucleotide hybridization assay of six developmental stages by Wei et al., (2006) reports embryonic expression patterns of the Glean3 predictions for *Sp-netrin, Sp-unc5* and *Sp-neogenin* (Appenidix II). Wei et al. indicate that low levels of Glean3-04245 (*Sp-netrin*) transcript are present at 2hpf and 15hpf. At 24hpf, abundance increases and stays relatively constant until 72hpf. Analysis of Glean3-10776 (*Sp-unc5*) reveals that relatively high levels of transcript are present at 2hpf then, drop to negligible levels at 15hpf and 30hpf. Levels increase at 48hpf and 72hpf. Analysis of Glean3 -

25975 (*Sp-neogenin*) indicate that no transcript is present at 2hpf, and negligible levels are present at 30hpf whereas at 48hpf and 72hpf, levels increase.

These data indicate that all genes are expressed during neurogenesis and that *Sp-netrin* and *Sp-unc5* transcripts are present in the embryo prior to the appearance of neurons. All transcripts are in highest abundance at 96 hpf.

3.5 *In situ* hybridizations

Anti-sense and sense *in situ* hybridization probes for *Sp-netrin* mRNA were hybridized to embryos at the blastula, gastrula and pluteus stages of development to determine the location of the *Sp-netrin* transcript (Fig. 14). At blastula stage (24 hpf), transcript is detected in the vegetal plate (Fig. 14 A, B). During gastrulation (48 hpf), localization of the transcript expands to the oral field and the archenteron (Fig. 14 D, E). Pluteus stage embryos (72 hpf) have expression in the gut, animal plate and in the oral field. In addition, a concentrated region of expression appears to correspond to a portion of the oral ectoderm between the mouth and the VTCB (Fig. 14 G, H). Negative control staining is negligible (Fig. 14 C, F, I) and positive control staining with an Hnf-6 anti-sense probe revealed staining in the ciliary band. (Fig. 14 J). These data indicate that the expression of *Sp-netrin* correlates with temporal expression patterns found with QPCR data and reveals the tissues expressing this gene.

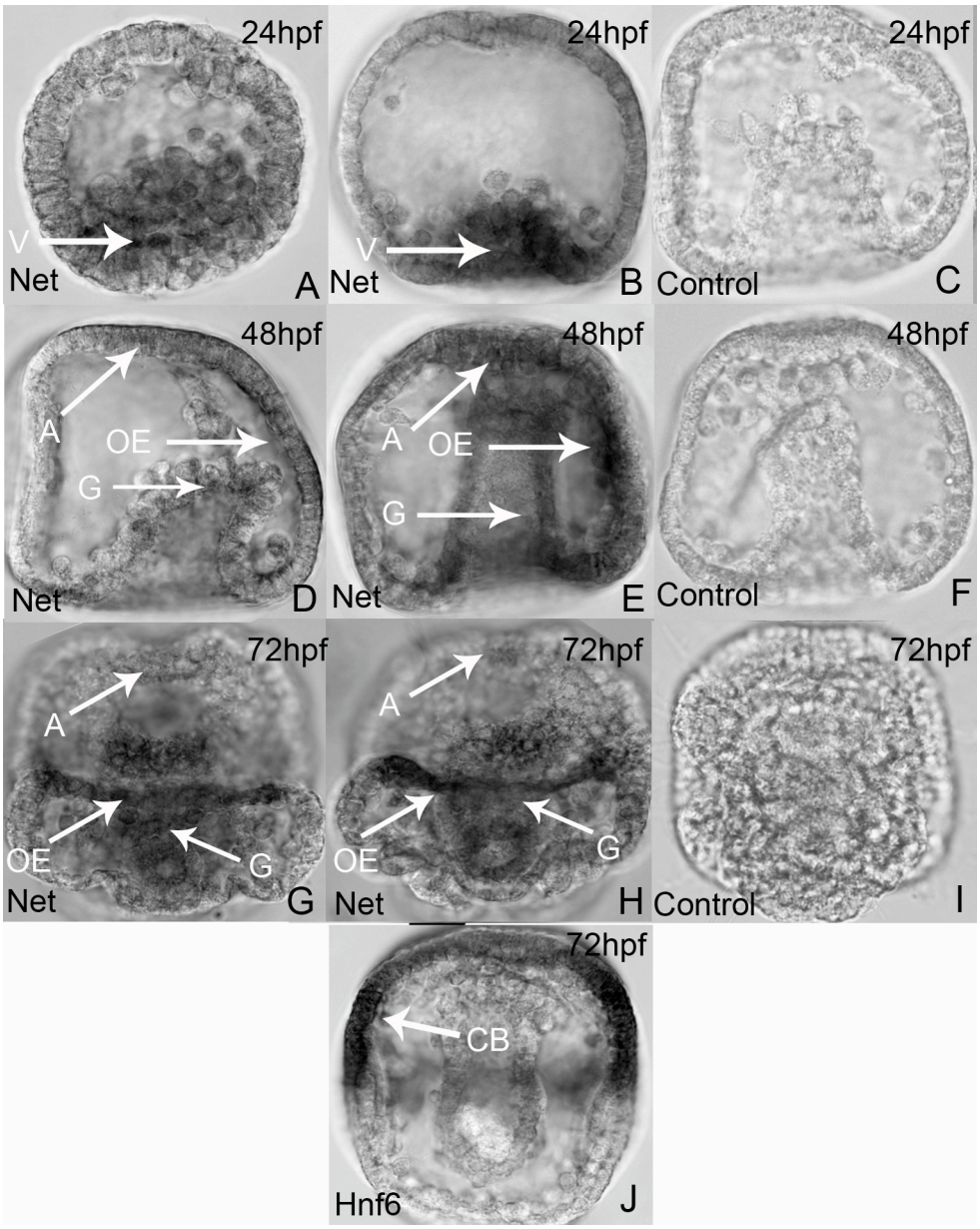


Figure 14. *In situ* RNA hybridizations of *Sp-netrin* probes on early developmental stages.

Dark staining regions correspond to bound probe. A,B - 24 hpf embryos reveal anti-sense probe bound in the vegetal plate. D,E - 48 hpf embryos reveal staining in the gut and oral ectoderm. G, H - 72 hpf embryos reveal staining in the gut, animal plate and oral ectoderm. C,F,I - Staining in negative controls was negligible. J - staining with an Hnf-6 anti-sense probe revealed staining on the ciliary band. V- vegetal plate, A- animal plate ectoderm, G – gut, OE – oral ectoderm, CB – ciliary band.

3.6 Antibody Validation

To determine the specificity of the netrin antibody, immunoblots of expressed and native protein, preimmune serum immunofluorescence controls and netrin knockdowns were completed. An immunoblot blot of unpurified, bacterially expressed Sp-Netrin protein probed with the netrin antiserum shows the antiserum recognizes the 40kDA protein fragment and likely other bacterially expressed peptides (Fig 15). A considerable number of immunoblots were attempted to get the Sp-Netrin antibody to recognize native protein (68kDA). No bands were recognized by the netrin antiserum and positive controls were successful (not shown). Immunofluorescence preparations of 24 hpf, 48 hpf, 72 hpf and 96 hpf embryos using pre-immune antiserum served as a negative control for the Sp-Netrin antiserum. These reveal no staining (not shown). The specificity of the Sp-Netrin antibody was tested by knocking down Sp-Netrin and then immunostaining with the Sp-Netrin antiserum. This results in a loss of fluorescence (Fig. 23, 24), thereby indicating that a loss of Sp-Netrin is sufficient to inhibit the binding of the antibody. Details of these results are under knockdown validation, section 3.9. Moreover, the spatial localization pattern of *netrin* mRNA, determined from *in situ* hybridizations (Fig. 14), correlate with the distribution of Sp-Netrin protein, determined from immunofluorescence data (Fig. 16, 17). Overall, these data reveal that the Sp-Netrin antiserum specifically recognizes Netrin protein, but further work will be needed to confirm that the antiserum recognizes a protein of the correct size.

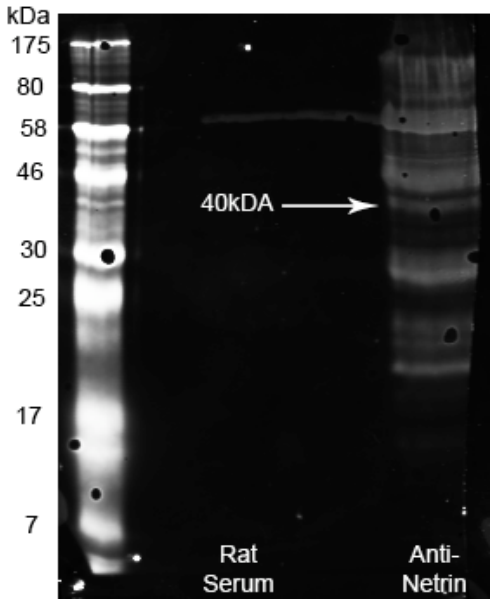


Figure 15. Western blot of unpurified, expressed protein blotted with Sp-Netrin antiserum.

Netrin antiserum recognizes the bacterially expressed netrin protein fragment (40kDA) and other bacterially expressed proteins. The control rat serum does not recognize any of the bacterially expressed proteins.

3.7 Netrin protein distribution

An antiserum generated against Sp-Netrin was produced in rat to specifically determine the cellular localizations of the Sp-Netrin protein during development. Immunofluorescence preparations reveal that in blastula and gastrula stage embryos, the Sp-Netrin signal is weak (Fig. 16 A, B) but is localized to the basal surface of the vegetal plate. During gastrulation, the antibody weakly localizes to the tip of the archenteron, the basal surface of the oral ectoderm and the basal surface of the thickened animal plate. In pluteus stage embryos (72 hpf) there is a much stronger signal to noise ratio (Fig. 16 C, D, F, G). Here, expression of Sp-Netrin is restricted to the basal surface of the oral

ectoderm, the animal plate and the lower portion of the archenteron. When using anti-synaptotagmin as a secondary marker, it becomes apparent that the majority of netrin, which is located in the oral ectoderm, is surrounded by developing neurons (Fig. 16 E, F, G). Netrin expression in the basal lamina of the oral ectoderm is shown quantitatively by a signal intensity plot of oral ectoderm vs aboral ectoderm (Fig. 17). These data indicate that the expression of Sp-Netrin correlates with temporal expression patterns found with QPCR data, the localization patterns from *in situ* hybridizations and reveals the tissues expressing Netrin protein.

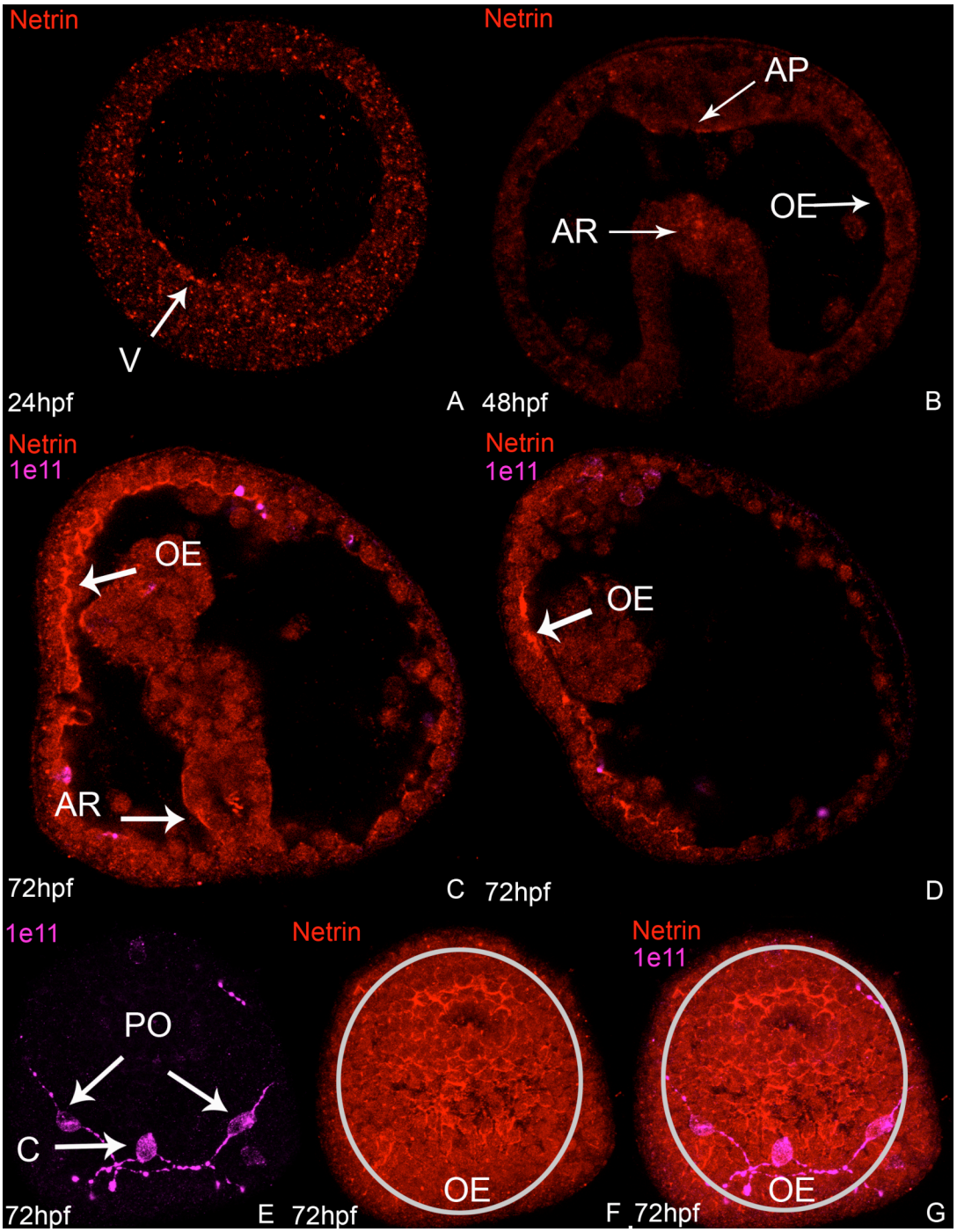


Figure 16. Immunolocalizations of anti-Netrin on early developmental stages.
A - Optical section of hatched blastula. Netrin is expressed at the basal surface of the vegetal plate (V). B - Optical section of gastrula in lateral view. The tip of the archenteron (AR), the basal surface of the oral ectoderm (OE) the thickened animal plate (AP) express Netrin. C - Optical section of pluteus in lateral view. Expression of Sp-

Netrin is restricted to the basal surface of the oral ectoderm, the animal plate and the lower portion of the archenteron. D - Optical section of pluteus in lateral view. Expression on the basal surface of oral ectoderm. E - Z-stack of pluteus with frontal view reveals the distribution of synaptotagmin expressing PO and C neurons. F - Z-stack projection of pluteus stage embryo from Fig. 13 E. Netrin expression is localized primarily in the oral ectoderm (OE). G - Combined image of E and F shows that netrin expression is highest in the oral field, where synaptotagmin expressing cells are absent.

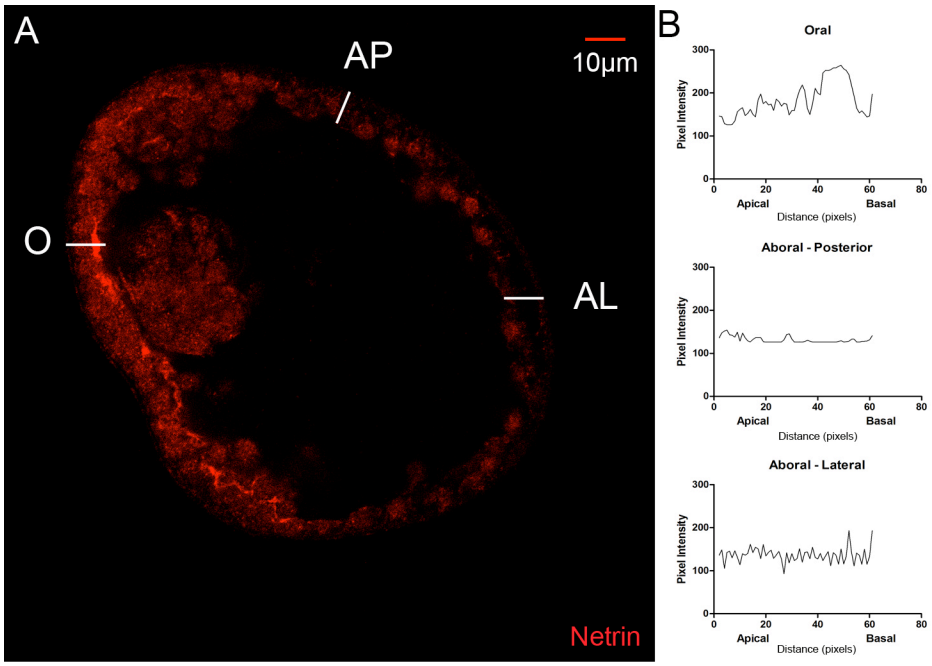


Figure 17. Distribution of signal intensity from immunolocalizations of anti-Netrin. A - an optical slice of a 72 hpf embryo in lateral view. Lines indicate regions that were analyzed for signal intensity for oral (O), aboral - posterior (AP) and aboral lateral (AL) ectoderm. B - profile plots indicate that signal intensity is higher in the oral ectoderm than in aboral ectoderm.

3.8 Netrin Knockdown

3.8.1 Neural specification and embryo morphology.

To determine the effect of knocking down Sp-Netrin on early development, eggs were injected with a *Sp-netrin* specific, translation blocking MASO. Analysis of 66 hpf *Sp-netrin* MASO injected embryos reveals that a majority of these embryos do not swim (90%, n = 21), whereas a minority of standard control injected embryos show a defect in swimming ability (26%, n = 27). At 60 hpf and 72 hpf, standard control injected embryos appear prism like (Fig. 18 B; Fig. 19 F), due to the flattened appearance of the oral ectoderm and the posteriorly elongated, aboral ectoderm. In contrast, *Sp-netrin* MASO injected embryos appear rounded in shape, with no discernible flattened oral surface or elongated aboral ectoderm (Fig. 18 A; Fig. 19 D, E). At 96 hpf and 120 hpf, *Sp-netrin* knockdown results in the appearance of a contracted oral end and no oral or aboral arms. At 96 hpf embryos are rounded whereas 120 hpf embryos have an extended aboral ectoderm (Fig. 18 C; Fig. 19 G, H). Standard control injected embryos develop normally by 96 hpf and 120 hpf, with a flattened, wide oral ectoderm surface, oral and aboral arms, and an elongated aboral ectoderm (Fig. 18 D; Fig. 19 I).

Immunofluorescence using the anti-synaptotagmin marker, 1e11, reveals that most neurons have not differentiated. In all 60 hpf embryos and most 72 hpf *Sp-netrin* MASO injected embryos, no neurons can be detected with 1e11 (Fig. 19 A, B, D). In a small proportion of 72 hpf embryos, 1 or 2 weakly expressing 1e11 positive cells can be detected (Fig. 19 E). In 120hpf *Sp-netrin* MASO injected embryos, some neurons can be detected in the contracted anterior end of the embryo (Fig. 19 G, H). When 1e11 positive

cells are counted in *Sp-netrin* MASO injected embryos and standard control injected embryos, an unpaired t-test reveals that netrin MASO injected 72hpf and 120 hpf embryos have significantly fewer number of neurons than the control (72hpf, $p < 0.0001$; 120 hpf, $p < 0.0001$) (Fig. 20). Taken together, these results show that a loss of netrin results in a change in embryo shape, defects in swimming ability and a loss of neurons.

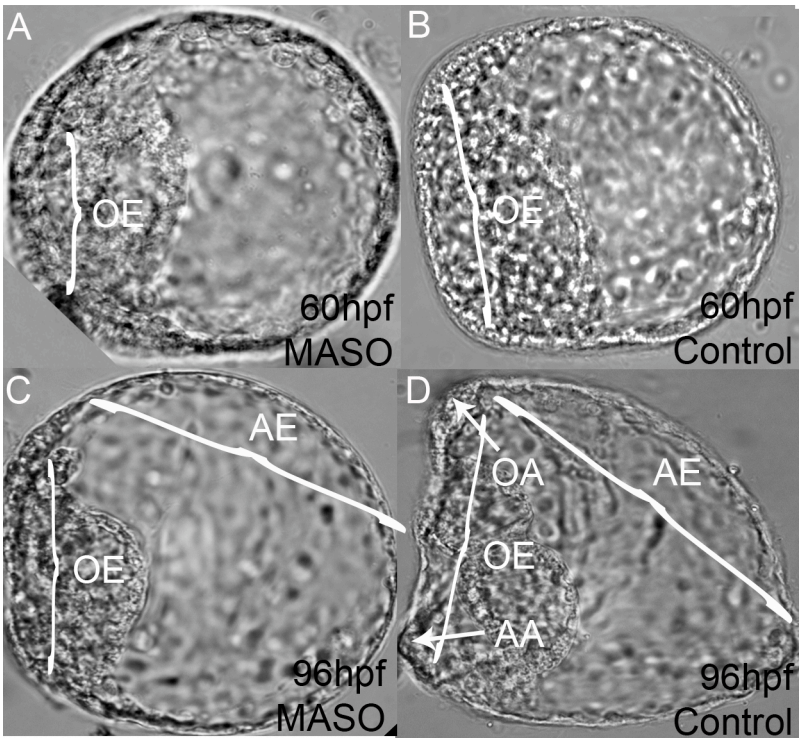


Figure 18. DIC images of embryos injected with *Sp-netin* MASO and standard control MASO at 60 hpf and 96 hpf.

A - The lateral view of a *Sp-netrin* MASO injected embryo at 60 hpf revealing a rounded shaped embryo. B - The lateral view of a standard control MASO injected embryo at 60 hpf showing a flattened oral ectoderm and the elongated aboral ectoderm. C - The lateral view of a *Sp-netrin* MASO injected embryo at 96 hpf revealing a contracted oral ectoderm and rounded aboral ectoderm. D - The lateral view of a standard control injected embryo at 96 hpf showing a wide and flattened oral ectoderm, oral and anal arm

buds and an elongated aboral ectoderm. OE – oral ectoderm, AE – aboral ectoderm, OA – oral arm, AA – anal arm.

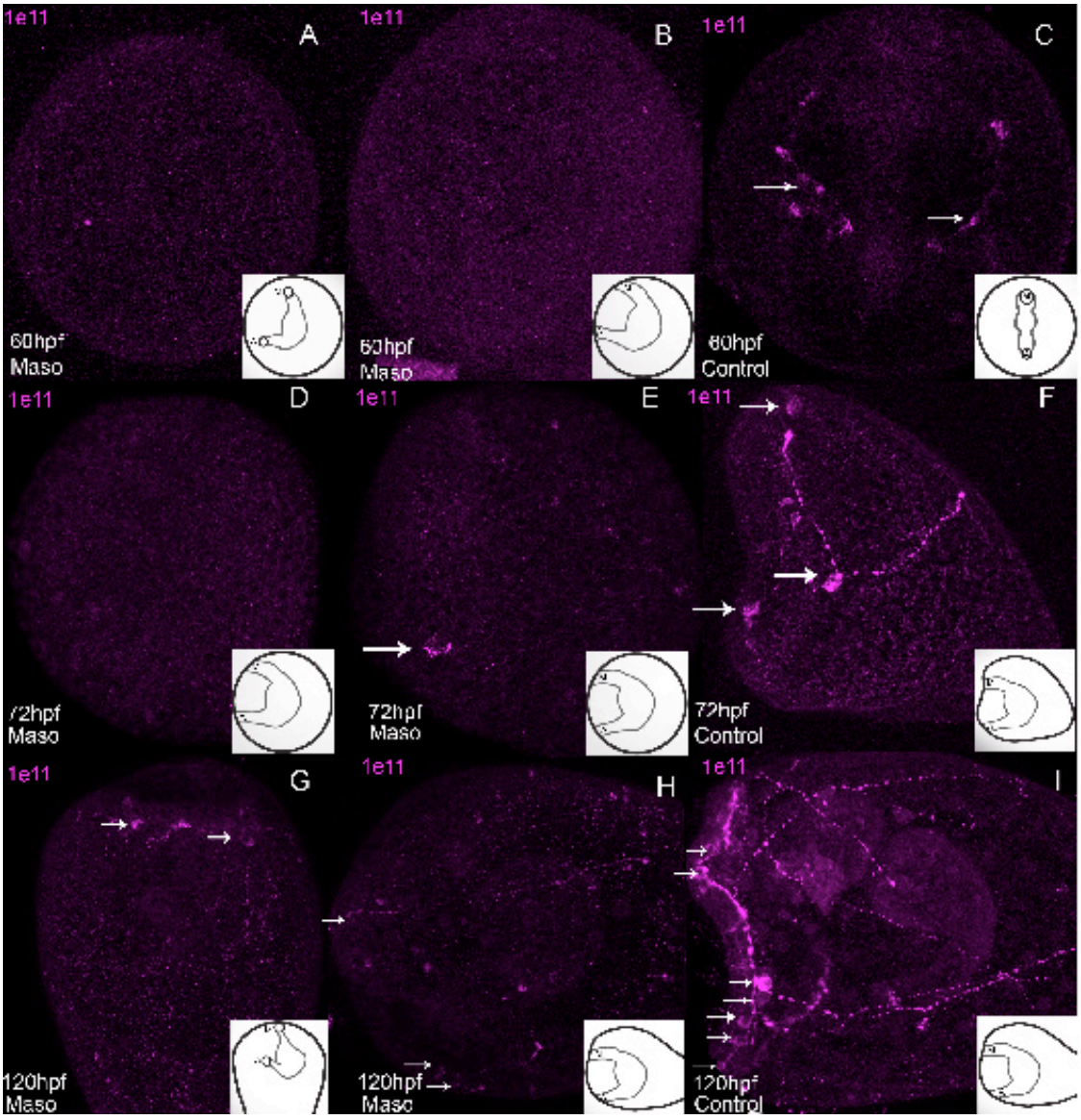


Figure 19. Immunolocalization of anti-synaptotagmin (1e11) in 60 hpf, 72hpf and 120hpf embryos after Sp-Netrin knockdown.

Schematics in the lower right corner of each frame indicate the orientation of each embryo, where M indicates mouth and A indicates anus. Arrows indicate visible nerve cell bodies. The frontal (A) and lateral (B) view of early pluteus stage embryos (60 hpf) injected with a Sp-netrin MASO revealing an absence of neural cells. C - The frontal view of an early pluteus embryo injected with a standard control MASO, showing early

PO cell development. D, E - The lateral view of an early pluteus stage embryo injected with a Sp-netrin MASO reveals a loss of neural cells and a rounded shaped embryo. F - The lateral view of a pluteus stage embryo injected with a standard control shows typical development of the LG and an embryo with a prism like morphology. The frontal (G) and lateral (H) view of a late pluteus stage embryo injected with a Sp-netrin MASO reveals a loss of neural cells and the appearance of a contracted anterior end. I - The lateral view of a late stage pluteus embryo injected with a standard control MASO shows a tract of axons along the CB, neurites that project toward the posterior end of the embryo and the appearance of oral and anal arms.

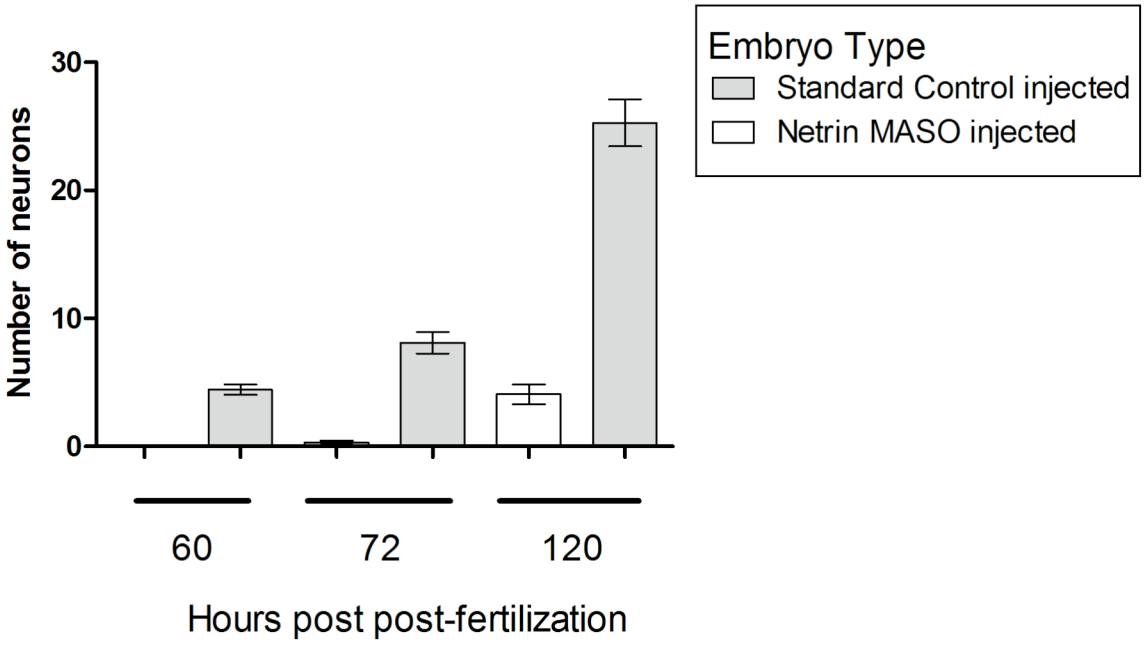


Figure 20. Knockdown of Sp-Netrin results in a loss of neurons in 60 hpf, 72hpf and 120hpf embryos.

Neurons were not detected in Sp-netrin MASO injected embryos at 60 hpf, whereas standard control injected embryos developed several neurons. An unpaired t-test comparing both Sp-netrin (n=10) and standard control MASO injected embryos (n=10) at

72hpf, results in a p value of < 0.0001, thereby revealing that there are significantly fewer neurons present in Sp-Netrin perturbed embryos. At 120hpf, netrin (n=12) and standard control MASO (n=11) injected embryos also give a p value of <0.0001, indicating that there are a significantly fewer neurons in Sp-netrin MASO injected embryos.

3.8.2 Ciliary band specification or patterning

To determine if the change in shape of Sp-Netrin perturbed embryos correlated with a defect in ciliary band formation, embryos injected with *Sp-netrin* MASO and standard control MASO were scored for the presence or absence of the ciliary band marker, Hnf-6. A Fisher's exact test reveals a statistically significant loss of Hnf-6 expression, in *Sp-netrin* MASO injected embryos since none of these embryos expressed Hnf-6 (n=8) (Fig. 21 A, B) and all control injected embryos expressed the Hnf-6 marker (n=10) in 72 hpf embryos ($p < 0.0001$) (Fig. 21 C). Similarly, Sp-Netrin perturbed embryos that were allowed to develop to 120 hpf also revealed an absence of Hnf-6 expression in all embryos (n=2) (Fig. 21 D, E), whereas, control injected embryos that were developed to 120hpf, did not show any ciliary band abnormalities, as indicated by the presence of Hnf-6 expression in all embryos (n=6) (Fig. 21 F). A Fisher's exact test comparing the embryos injected with Sp-Netrin MASO and control MASO reveals that the loss of ciliary band in 120hpf Sp-Netrin MASO injected embryos is also statistically significant ($p = 0.0357$) thereby indicating that Netrin is necessary for expression of the ciliary band marker, Hnf-6.

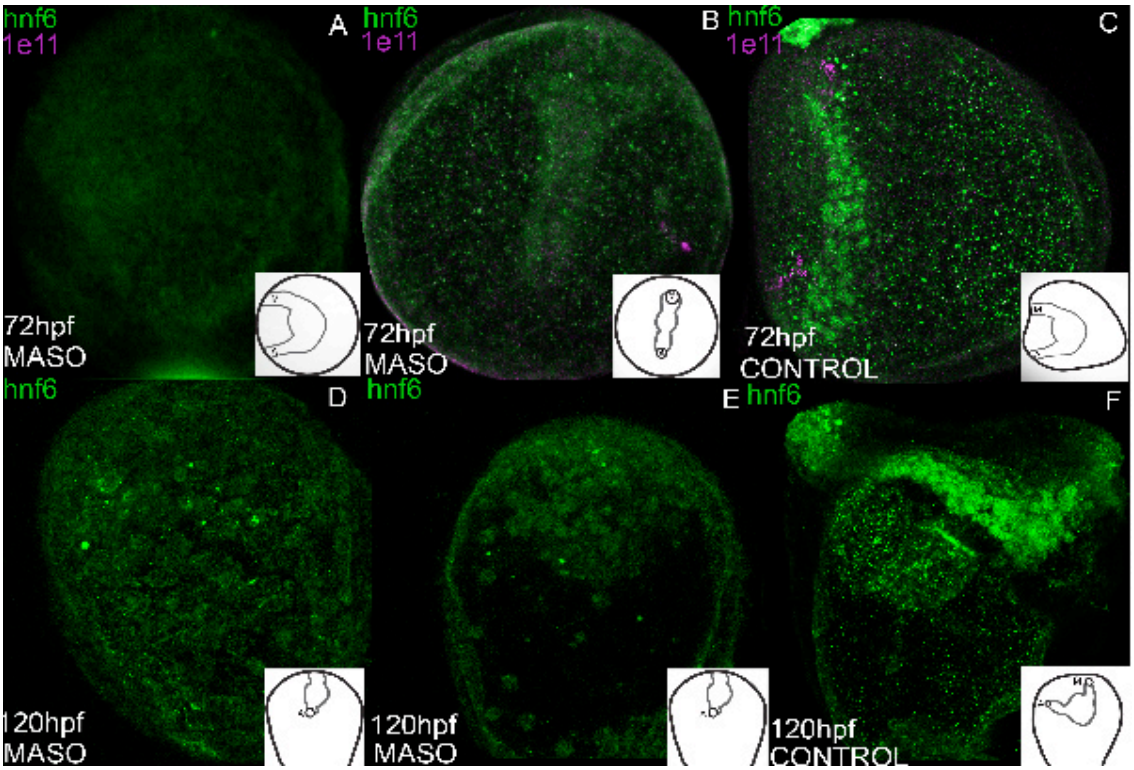


Figure 21. Immunolocalizations of Hnf-6 in 72hpf and 120hpf embryos after Sp-Netrin knockdown.

Schematics in the lower right corner of each frame indicate the orientation of each embryo, where M indicates mouth and A indicates anus. The lateral (A) and frontal (B) view of a pluteus stage embryo injected with Sp-netrin MASO results in a loss of a properly formed ciliary band as indicated by the ciliary band maker Hnf-6. (C) The lateral view of a early pluteus stage embryo injected with a standard control MASO results in CB that divides the oral and aboral ectoderm. (D, E) The frontal view of late stage pluteus embryos injected with Sp-netrin MASO results in a loss of a properly formed CB. (F) The lateral-frontal view of a 120hpf embryo injected with a standard control MASO results in a normal CB phenotype similar to C.

3.8.2 Oral ectoderm specification and patterning

To determine if the change in shape of Sp-Netrin perturbed embryos correlated with a change in oral ectoderm signaling, embryos injected with *Sp-netrin* MASO and

standard control MASO were scored for the presence or the (complete or partial) loss of the orally expressed, aboral gene repressor, Goosecoid (*Gsc*) and the orally expressed Chordin, a secreted BMP inhibitor (Bradham et al., 2009). A comparison revealed that pluteus stage embryos (72hpf) injected with *Sp-netrin* MASO, all resulted in a reduction (Fig. 22 A) or complete loss (Fig. 22 B) of *Gsc* expression in the oral ectoderm (n=5), whereas all control injected embryos revealed strong *Gsc* expression in the oral ectoderm (n=6) (Fig. 22 C). A comparison of these two groups, using a Fisher's exact test, reveals that this is a significant loss of *Gsc* in Sp-Netrin perturbed embryos ($p = 0.0022$).

Sp-netrin MASO injected embryos resulted in the absence of Chordin expression in all 72 hpf embryos (n=3) (Fig. 22 D, E) whereas all control injected embryos revealed the presence of Chordin expression (n=6) (Fig. 22, F). A comparison of these two groups, using a Fisher's exact test, reveals that this is a significant loss of Chordin expression in Netrin perturbed embryos ($p = 0.0119$). These results reveal that Sp-Netrin perturbation results in loss of the oral markers, Chordin and *Gsc*.

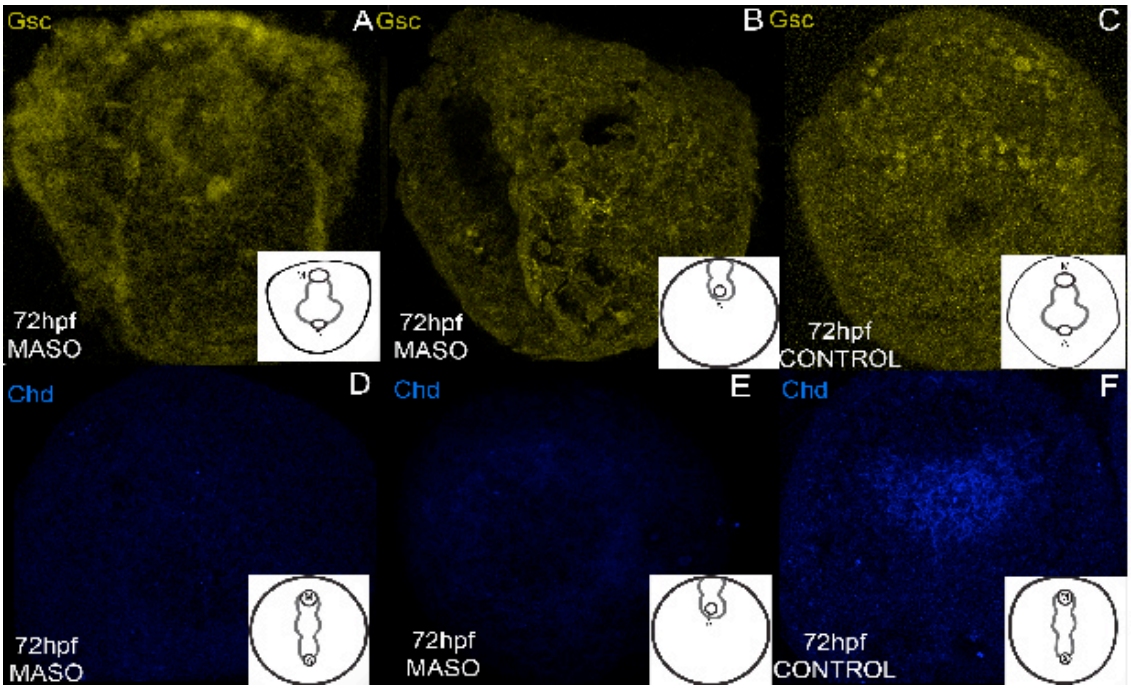


Figure 22. Immunolocalizations of Goosecoid and Chordin in 72hpf embryos after Sp-Netrin knockdown.

Schematics in the lower right corner of each frame indicate the orientation of each embryo, where M indicates mouth and A indicates anus. (A) The frontal and ventral-posterior (B) views of pluteus stage embryos injected with netrin MASO result in a reduction and complete loss of the oral ectoderm marker Goosecoid (Gsc), respectively. (C) The frontal view of a early pluteus stage embryo injected with a standard control MASO reveals Gsc signal in the oral ectoderm. (D,E) Early pluteus stage embryos injected with netrin MASO result in a loss of the oral ectoderm marker Chordin. (F) An early stage pluteus embryo injected with a standard control MASO reveals the expression of Chordin in the oral ectoderm.

3.8.5 Netrin Knockdown Validation

To validate the specificity of both the *Sp-netrin* MASO and the Netrin antibody, *Sp-netrin* MASO and standard control MASO were injected into embryos, embryos were developed to 72 hpf and scored for the appearance or loss of Sp-Netrin protein expression with the *Sp-netrin* antiserum. Results indicate that in all *Sp-netrin* MASO

injected embryos (n=3), there is a reduction in bound antibody (Fig. 23 B), embryos are rounded and do not express 1e11 (Fig. 23 A, C). All control injected embryos are prism-like, express 1e11 and recognize the Sp-Netrin-specific antiserum (n=4) (Fig. 23 D, E, F). A Fisher's exact test comparing *Sp-netrin* MASO and control MASO injected embryos reveal that the loss of Sp-Netrin signal is statistically significant ($p = 0.0286$) in *Sp-netrin* MASO injected embryos. To further validate these findings, netrin signal intensity was plotted in *Sp-netrin* MASO and control MASO injected and uninjected embryos. A scoring of the maximum pixel intensity in *Sp-netrin* MASO injected embryos was compared to control injected MASO and uninjected embryos and was found to be significantly lower than these groups ($P<0.05$) (Fig. 24). Maximum signal intensity of injected control and uninjected control embryos were not significantly different ($P>0.05$) . From these data, I conclude that the Sp-Netrin antiserum specifically recognizes Sp-Netrin and *Sp-netrin* MASO reduces expression of the Sp-Netrin protein.

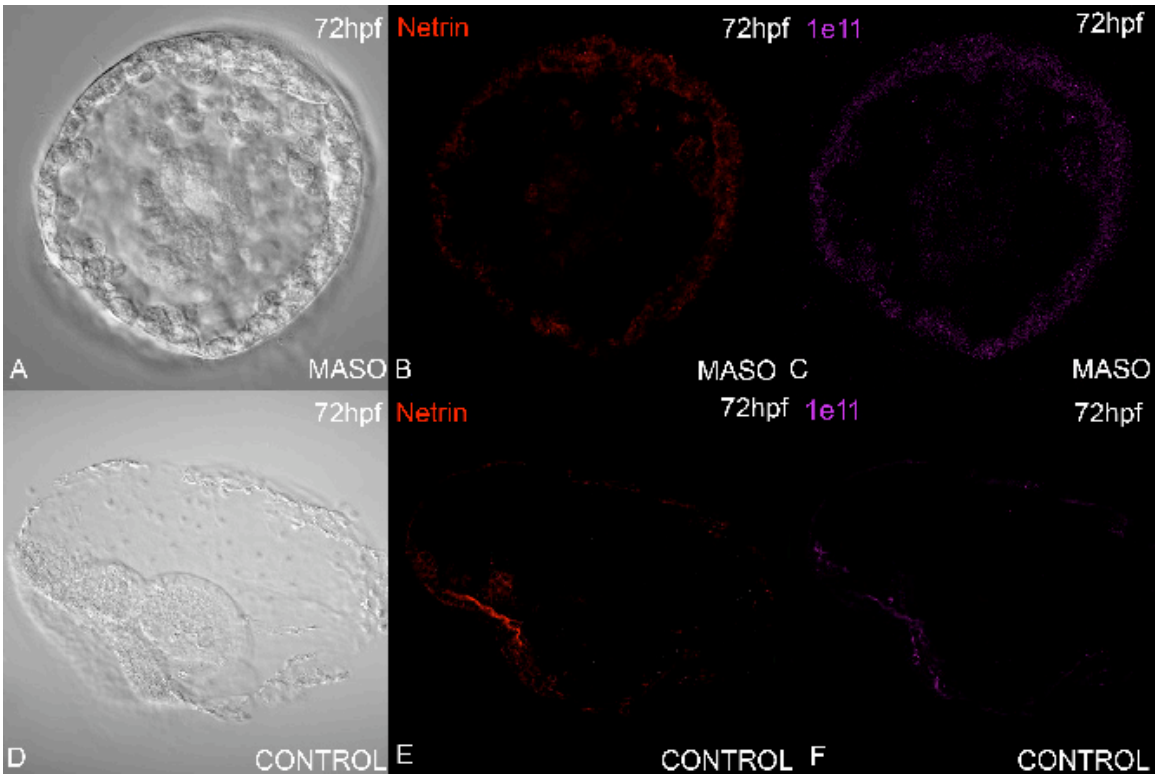


Figure 23. Immunolocalizations of anti-Netrin after Sp-Netrin knockdown.
A. A DIC image of a pluteus embryo injected with Sp-netrin MASO. B,C - Lack of Sp-Netrin expression or expression of 1e11. D - A DIC image of a pluteus stage embryo injected with a standard control MASO revealing a normal prism shaped embryo. E, F - expression of Sp-Netrin in the oral ectoderm and expression of 1e11.

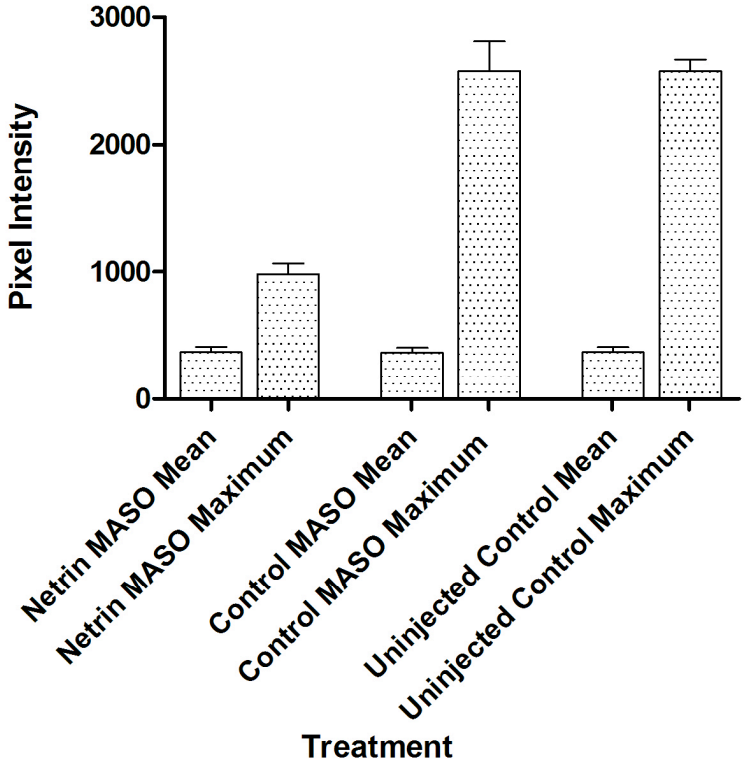


Figure 24. Signal intensity of anti-netrin after injection of netrin MASO or standard control MASO.

One way ANOVA analysis reveals that 72hpf embryos that were injected with netrin MASO (3 embryos, 15 measurements) have significantly lower anti-Netrin maximum pixel intensities in oral ectoderm than both control MASO injected embryos (4 embryos, 15 measurements) and uninjected embryos (4 embryos, 13 measurements) ($P < 0.05$).

Chapter 4 - Discussion

4.1 Sequencing and Phylogenetic Analysis

Given that gene prediction programs are prone to error, (Guigó et al., 2006), it is important to determine the cDNA structure of the genes of interest, using the gene predictions from the *S. purpuratus* genome sequencing project (www.spbase.org ; Sea Urchin Genome Sequencing Consortium et al., 2006). The *Sp-netrin* cDNA sequence is accurately predicted by Glean3_04245 and results in a ORF that is consistent with the size of vertebrate Netrin homologues (Kennedy et al., 2006) and the echinoderm Netrin homologue from *Hemicentrotus pulcherimus* (Katow et al., 2009). In addition, the predicted protein domain architectures for Sp-Netrin are consistent with Netrin homologues (Livesey, 1999). Neighbour-joining methods were used to reconstruct phylogenetic trees from evolutionary distance data for Sp-Netrin, Sp-Unc5 and Sp-Neogenin. Groupings produced from the neighbour-joining tree of Sp-Netrin and Netrin orthologues are consistent with current deuterostome phylogenies (Cameron et al., 2000). Although some regions of the Sp-Netrin sequence have diverged from chordate sequences, these analyses confirm that *Sp-netrin* is a legitimate *netrin* homologue.

Pl-EST-SP0ACLEB18YG07RM1 and Scaffold_v2_22300:30136 were needed to supplement sequence data from the Glean3-10776 prediction in order to complete a full-length cDNA for *Sp-unc5*. An ORF is consistent with the size of *unc5* homologues (Keleman and Dickson, 2001**;** Leung-Hagesteijn et al., 1992) and predicts a protein with canonical domain architectures of unc5 homologues (Livesey, 1999). The neighbour-joining tree of Sp-Unc5 and Unc5 orthologues indicate that Sp-Unc5 is more similar to chordate and hemichordate Unc5 homologues than the more distantly related homologue

from *C. elegans*. Considering these analyses, I conclude that Sp-unc5 is a true *unc5* homologue.

Sequence from HMM182407 was needed to supplement the GLEAN3-25975 gene prediction, in order to complete the full length Sp-neogenin cDNA. An ORF produces a 1850 amino acid protein that is slightly larger than some neogenin homologues (Chick - 1442 amino acids, Mouse - 1465) (Conrad et al., 2010; Vielmetter et al., 1994). Homologues contain 6 FN3 domains and a transmembrane domain (Wilson and Key, 2007), whereas Sp-Neogenin-1 contains 5 FN3 domains and lacks a transmembrane domain. Neogenin-2 is incomplete in the 3' direction, and appears to be an alternatively spliced variant. Affinity assays have shown that binding partners of Neogenin (Netrin and RGM) will bind to an unspecified region of the FN3 repeats (Rajagopalan et al., 2004). Therefore it remains possible that Sp-Neogenin-1 or Sp-Neogenin-2 may retain some of the functions associated with these binding partners. However, Sp-Neogenin-1 and Sp-Neogenin-2 do not contain a transmembrane domain, thus, it is possible that the role of Neogenin in *S. purpuratus* is different than what has been observed in other organisms.

Sp-neogenin sequence is constructed from predictions that specify the related paralogue, *DCC* (Deleted in Colorectal Cancer). Phylogenetic analysis reveals that this gene product is most similar to homologues of Neogenin rather than DCC. The neighbour joining tree of Sp-Neogenin-1 and Neogenin orthologues indicate that Sp-Neogenin is consistent with the current deuterostome phylogenies (Cameron et al, 2000). Overall, these analyses show that *Sp-neogenin* is a homologue of *neogenin*

Errors in *Sp-neogenin*, *Sp-netrin* and *Sp-unc5* sequence, due to the incorporation of genomic DNA are unlikely since all amplicons had at least one intron/exon boundary (with the exception of the first and last amplicons of Sp-Neogenin). Our confirmed sequences with a transcription start site have permitted the synthesis of translation blocking MASOs for *Sp-netrin* and *Sp-unc5*. However, since sequence obtained for *Sp-neogenin* did not contain a likely signal peptide, and thus a likely transcription initiation site, an *Sp-neogenin* specific MASO that targets the transcription initiation site was not designed (Summerton, 1999). Alternatively, a MASO that targets an intron/exon boundary may be used to evaluate the functions of specific domains within Sp-Neogenin.

4.2 Nervous System Development

To determine if the early larval nervous system of *S. purpuratus* develops in a stereotypic manner, and can be used as to testable system to determine Sp-Netrin's role in neurite guidance, I completed an analysis of early nervous system development. This data expands upon the analysis of synaptotagmin expression by Nakajima et al. (2004), which provided only a brief analysis of synaptotagmin expression early in neurogenesis.

My results show that there are several stereotypic aspects of early neural development. Specifically, the location of LN, PO and C neurons and the orientation of neurites projecting from these cells are predictable. The small amount of variability in the number of LN, PO, and C neurons may be attributed to differences in the rate of development among these embryos. Variability was also observed in the number of neurites that neurons produce, however the neurites extend only within the ciliary band, where they form bundles, or beneath the aboral ectoderm, where they do not form bundles, and do not project beneath oral ectoderm (Yaguchi et al., submitted). These

analyses show that the patterning of early neural development can be anticipated and does not develop in a manner akin to the nerve net observed in members of the Cnidarian phylum (Milijkovic-Licina et al., 2004). Analyses of nervous system development in later stage pluteus embryos (Beer et al., 2001; Bisgrove and Burke, 1987; Nakajima et al., 2004; Yaguchi et al., 2000) support this finding and aid my interpretations of neural phenotypes in Netrin knockdown experiments. The observations I made on early neural development indicate that ciliary band neurons appear in specific locations, and project neurites in stereotypical patterns.

4.3 Temporal Expression

To ascertain the relative abundances of *Sp-netrin, Sp-unc5* and *Sp-neogenin* mRNA during development, QPCR was used. Analysis of this data shows that *Sp-neogenin* is transcribed around the same time as the first appearance of neurons (48hpf). Interestingly, *Sp-netrin* and *Sp-unc5* are transcribed well before the first appearance of neurons (24hpf and unfertilized egg). Although it is possible that some transcripts may undergo delayed translation (Linder et al., 1995), the presence of transcript well prior to neurogenesis suggests that *Sp-unc5* and *Sp-netrin* may function in a non-neural process. During larval development, transcript abundance increases for all genes around the time of neurogenesis (48hpf) and increases as the number of neurons increases, consistent with a role in neurogeneisis.

The temporal expression patterns I present agree in large part with the analysis of embryonic expression by Wei et al. (2006) (Section 3.4 and Appendix II). Exceptions include: 1) Sp-Netrin expression by QPCR indicate a several fold change in abundance

from 24hpf to 48hpf, whereas Wei et al. (2006) report little variation during this period. 2) Glean3-10776 (Sp-unc5) expression determined by Wei et al. indicates that expression increases at 72hpf. However, my QPCR data indicates that these levels drop slightly. The differences observed between these two data sets may be resolved if more trials of QPCR are completed, producing smaller standard deviations. Reverse transcriptase PCR data published from, *Hemicentrotus pulcherrimus* (Katow, 2008), correlates qualitatively with my QPCR data; it reveals that Hp-netrin transcript is also produced in the blastula through pluteus stages. Overall, these analyses provide a consistent increase of late embryonic temporal expression for these genes.

4.4 Localization of Netrin

Several lines of evidence suggest that the antiserum prepared against Sp-Netrin is specific. The immunoblot in which the bacterially expressed protein is immunoreactive when probed with the anti-serum suggest that antibodies in the serum recognize the protein fragment used as the antigen. In addition, the locations of *Sp-netrin* mRNA and Netrin protein were determined by i*n situ* hybridizations and immunolocalizations on several stages of *S. purpuratus* development. Cells and tissues such as the basal surface of oral ectoderm, gut and the vegetal and animal plate bind the labelled RNA probes are the same cells and tissues that are immunoreactive in immunofluroescence preparations. Indeed, these methods indicate that *Sp-netrin* mRNA and Sp-Netrin protein is expressed in the vegetal plate of blastula stage embryos. *Sp-netrin* transcript and protein are found in the oral field, animal plate and the archenteron in gastrulating embryos whereas Sp-Netrin protein is found in the same tissues, yet restricted to the base of the archenteron.

Pluteus stage embryos have *Sp-netrin* transcript in the gut epithelium, animal plate and the oral field, between the mouth and the ciliary band. Correspondingly, Sp-Netrin protein is present in all these tissues. It is noteworthy that the protein appears most concentrated in the basal lamina of the oral field since neurites extend on and around this tissue. Temporal patterns of expression determined from QPCR results also correlate with the temporal patterns of expression found from *in situ* hybridizations and immunolocalizations. The Sp-Netrin preimmune serum does not recognize any native proteins in an immunoblot or *in vivo*, indicating that the Sp-Netrin antiserum does not spuriously bind proteins in the embryo. Finally, a knockdown of Sp-Netrin using a *Sp-netrin* specific translation blocking MASO reduced fluorescence to background levels, indicating that the Sp-Netrin antiserum requires the expression of Sp-Netrin to bind to the embryo.

However, evidence validating that the antiserum is recognizing a protein of the predicted size remains inconclusive, since several immunoblots failed to bind a band of the predicted size (68 kDa). The antiserum does not recognize any proteins in blots prepared with sample of embryo lysates, which suggests Sp-Netrin protein is not solubilized, or recognition is compromised by preparation of the samples. Additional methods of solubilization should be attempted for future trials.

Hp-Netrin specific immunohistochemistry data by Katow (2008) reveals some differences in Netrin immunolocalization. Hp-Netrin expression is expanded in blastula and gastrula stage embryos to the entire basal surface of cells and localizes to the animal plate, gut and contrastingly, the basal surface of the aboral ectoderm and ciliary band of

pluteus stage embryos. These differences could simply highlight species differences in *Netrin* expression or could indicate that one of these antibodies has a greater specificity. A trial determining the localization patterns of the Hp-Netrin antiserum in *S. purpuratus* and the Sp-Netrin antiserum in *H. pulcherrimus*, could reveal species differences or differences in antiserum specificity.

There are several curious aspects about the localization patterns I report. Netrin expression, occurs much earlier than the expression of neuron specific markers in late blastula stage embryos and is reported in the vegetal plate, which gives rise to mesoderm, and endoderm - tissues that do not give rise to neurons. This may hint at a non- neural role for Sp-Netrin. Also, in pluteus stage embryos, the majority of Sp-Netrin is centrally expressed in the oral field, where neurites are absent. Neurites that lie outside this central region may be responding to this cue in a chemorepulsive manner, thereby forming a central region void of neurites. A characterization of antibodies generated against Sp-Unc5 and Sp-Neogenin, as well as Sp-Unc5 and Sp-Neogenin knockdowns and overexpression analyses will be needed to determine if this conjecture is true. In addition, Sp-Netrin expression is located in the middle of the oral ectoderm, which is similar to the midline pattern of Netrin expression observed in other species (Harris et al., 1996; Wadsworth et al., 1996). Overall, these analyses support the contention that the Sp-Netrin antiserum and *in situ* hybridizations are specific and that the distribution of Netrin suggests it may function in a non-neural processes.

4.5 Netrin Knockdowns

4.5.1 Sp-Netrin and the differentiation of neurons and ciliary band.

To determine the function of Sp-Netrin, a knockdown using a *Sp-netrin* specific MASO was used. Early development in *Sp-netrin* MASO injected embryos appears unperturbed and the earliest sign of abnormalities appears as a deficit in swimming ability. However, the most striking aspect of the effects of *Sp-netrin* MASO injection is the almost total absence of neurons and the loss of the ciliary band. The inability to swim is likely tied to the loss of an organized ciliary band or neurons, since the ciliary band is the swimming organ and the larval nervous system is thought to be responsible for coordinated ciliary activity (Strathmann, 1975). An analysis of swimming ability prior to ciliary band formation (24 hpf) would be useful in determining if Sp-Netrin affects non-ciliary band cilia.

At least these possibilities may explain the loss of neurons in *Sp-netrin* MASO injected embryos. One possibility is that Sp-Netrin may be acting directly as a neurotrophic factor. This is possible since Neogenin and Unc5 are known as dependence receptors (Mehlen and Bredesen, 2004; Wilson and Key, 2007). Dependence receptors cause apoptosis in neurons when not bound to the Netrin ligand, but inhibit this effect when presented with the Netrin ligand. A second possibility is that Sp-Netrin is instead necessary for neuron specification or differentiation, since in control embryos, LN, PO and C neurons are first immunoreactive at approximately 56 hours and Sp-Netrin perturbed embryos at ≥60 hpf show no or relatively few synaptotagmin expressing neurons, or even dead, fragmented cells that are expressing synaptotagmin. In order to

provide further evidence of this, it may be necessary to employ either a TUNEL assay or DAPI staining to reveal if the small number of neurons that appear in Sp-Netrin MASO injected embryos are succumbing to apoptosis and yielding the reduced neuron phenotype or are specified and fail to differentiate. In the latter case, developing antibodies to an early neural marker such as, Nestin (Glean3-24601) or β-III-Tubulin (Glean3-00062) may help distinguish if neurons become specified.

Since Sp-Netrin perturbation results in morphological changes to the ectoderm, such as contraction of the apical end in 120 hpf embryos and a rounded shape in 72 hpf embryos, it is possible that changes in the expression of non-autonomous neuronal differentiation factors, as a consequence of defects in ectoderm, may be responsible for the loss of neuron phenotype. Several of the components responsible for ectodermal specification and regulation have been identified: Nodal and BMP2/4 are known to be responsible for specifying oral and aboral ectoderm during cleavage stages (Duboc, 2004, Lapraz et al., 2009). Chordin, Hnf-6 and Goosecoid, are regulated by this signalling and are differentiation markers for oral and ciliary band ectoderm (Duboc et a., 2004; ;Otim et al., 2004; Angerer et al., 2001).

Given that Nodal perturbed and BMP2/4 perturbed embryos result in severely radialized embryos (Duboc et al., 2004), as a result of a loss of ectoderm specification, and Sp-Netrin perturbed embryos result in polarized embryos, Sp-Netrin is likely specifying, but rather, differentiating or maintaining oral and ciliary band ectoderm, since Sp-Netrin perturbation results in a loss of oral and ciliary band markers. Although Netrin has been previously identified as a negatively regulated downstream effecter of BMP2/4

expression in hemichordates (Lowe et al., 2006), an analysis of Nodal and BMP2/4 expression in Sp-Netrin perturbed embryos will be necessary to confirm that Sp-Netrin is downstream of these, ectoderm specifying, TGFβ signalling proteins.

My assertion that the neural phenotype of Sp-Netrin perturbed embryos is a result of defects in ectoderm signaling, is supported by evidence reported by Yaguchi et al., (submitted). They propose a model where the ciliary band is necessary for the normal development of neurons and their bundled neurites. Yaguchi et al. (submitted) provide numerous examples demonstrating that if the ciliary band is displaced, neurons differentiate at the new site and if the ciliary band is missing ciliary band neurons do not differentiate. Yaguchi et al. (submitted) report similar but distinct phenotypes when embryos are injected with BMP2/4 mRNA. These experiments reveal similarities since embryos do not develop synaptotagmin expressing cells, ciliary band cells and do not express Gsc (Yaguchi et al., submitted) but remain distinct since they become severely radialized (Lapraz et al., 2009). An opposing version of the Sp-Netrin phenotype can be acquired through injection of a MASO specific for the urchin BMP2/4 receptor, Alk3/6. These embryos reveal a thickened ciliary band in which synaptotagmin expressing cells are displaced throughout (Yaguchi et al., submitted). Given this and additional evidence (see introduction), Yaguchi et al. (submitted) reveal a region free of TGFβ signalling is necessary, for neurons to differentiate. Since Sp-Netrin perturbed embryos lack normal synaptotagmin expression and are also missing a ciliary band, it is logical to assume their phenotype is consistent with this model. Thus, if Sp-Netrin is a maintenance factor necessary for the expression of ciliary band ectoderm, and the ciliary band is lost as a

consequence of Sp-Netrin perturbation, the loss of neurons may be an indirect consequence of this.

Similar knockdown experiments were completed by Katow (2008) and serve as a useful comparison to the work presented here. Knockdowns of Hp-Netrin using a 20μM concentration of Hp-Netrin specific MASO result in severe inhibition of morphogenesis and may confirm the abnormal ectodermal phenotype reported herein. Katow (2008) also reports that lower concentrations of Netrin MASO result in serotonergic neurons with neurite extensions that were extremely inhibited. Although Katow's reported knockdown data is limited, it seems to correlate with the data presented here. Further studies involving Sp-Netrin perturbation should investigate serotonin expression in the apical ganglion to determine if these effects are common to echinoplutei.

4.5.2 Netrin and the effects of Chordin and Goosecoid

Chordin and Goosecoid are regulated by TGFβ signalling and are differentiation markers for oral ectoderm that were evaluated in *Sp-netrin* MASO injected embryos. My analysis of Chordin, reveals that Sp-Netrin perturbation causes a loss of Chordin expression. Thus, revealing that Chordin may be downstream from Sp-Netrin. An analysis of Chordin function in echinoderms was previously completed by Bradham et al. (2009) and Lapraz et al., (2009) and gives insight to the Sp-Netrin perturbed phenotype. Similar to my observations, Bradham et al. (2009) report that embryos injected with Chordin mRNA or Chordin MASO do not develop oral or anal arms. Additionally, through a series of perturbation analyses and functional assays, Bradham et al. (2009) ultimately reveal that Chordin expression promotes the development of synaptotagmin

positive neurons and promotes patterning of the ciliary band. Their analysis places Chordin upstream of BMP and downstream from p38 and Nodal but not from Gsc. Since Chordin expression is lost in Sp-Netrin perturbed embryos (my results), and like Sp-Netrin perturbation, Chordin perturbation results in a loss of ciliary band cells, loss of synaptotagmin expressing cells and a loss of oral and anal arms, it is possible that this phenotype in Sp-Netrin perturbed embryos is the result of the loss of Chordin signalling.

Bradham et al. (2009) and Lapraz offer contrasting evidence regarding Chordin's role in oral/aboral specification. Bradham et al. (2009) report that Chordin MASO and Chordin mRNA injected embryos did not effect the expression patterns of the orally expressed regulators, Gsc, FoxA, and the aborally-expressed Tbx2/3 suggesting that Chordin is not required for oral/aboral specification. However, Lapraz et al. (2009) report an expansion of the Tbx2/3 expression domain in Chordin MASO injected embryos and a loss of expression of Tbx2/3 in Chordin mRNA injected embryos. Moreover, knockdown of Chordin resulted in the expansion of staining for phospho-smad whereas phospho-smad staining was dramatically reduced or eliminated in *chordin* mRNA injected embryos, indicating that Chordin is responsible for oral aboral specification. Like Sp-Netrin injected embryos, Lapraz et al. (2009) report rounded embryos but also report radialized embryos and embryos that develop a luge-like shape. Until these differences are resolved, the changes observed in oral/aboral signalling as a consequence of the loss of Chordin in *Sp-netrin* MASO injected embryos, will remain ambiguous.

An analysis of Goosecoid in Sp-Netrin MASO injected embryos demonstrated that Sp-Netrin perturbation causes a loss of Goosecoid expression, thereby revealing that Goosecoid is likely downstream of Sp-Netrin. The loss of Gsc in Sp-Netrin perturbed

embryos is likely to affect oral signalling patterns since gain and loss of function experiments in *Lytechinus variegatus* embryos reveal that Gsc represses aboral ectoderm specification and promotes oral ectoderm differentiation in the echinoderm (Angerer et al. 2001). Analysis of the Gsc MASO phenotype by Angerer et al. (2001) also reveals that these embryos appear radialized and do not properly form a gut. Although most of the Sp-Netrin MASO injected embryos appeared to have a properly formed gut, the gut phenotype reported by Angerer et al. (2001) will need to be confirmed in Sp-Netrin perturbed embryos in the future, as a proper analysis of gut formation was not completed in this project.

Interestingly, QPCR measurements reveal that *Gsc* expression decreases if Hnf-6 is perturbed (Otim et al. 2004). Conversely, embryos that are injected with *gsc* MASO, reveal a decrease or complete loss of Hnf-6 expression (Bradham et al., 2009;Yaguchi et al., 2006). Thus, the loss of *Gsc* and the loss of Chordin may be responsible for the loss of ciliary band cells in *Sp-netrin* MASO injected embryos, or the loss of Hnf-6 may have decreased Gsc expression.

LvGsc specific MASO and Gsc mRNA injections have no effect on Chordin expression thereby suggesting that Chordin expression could be independent of Gsc regulation in *S. purpuratus* (Bradham et al., 2009). These experiments also revealed that neuron differentiation is independent of Gsc expression in L. *variegatus*, since gain or loss of Gsc expression results in the presence of serotonin and synaptotagmin expressing cells. This contrasts from experiments from Yaguchi et al., (2006) which reveal that expression of Gsc mRNA in *S. purpuratus,* results in a loss of serotonergic neurons. Thus, the effects caused by the loss of Gsc in Sp-Netrin perturbed *S. purpuratus* embryos

remains inconclusive with respect to neural differentiation and will need to be examined in future studies.

Evidence for non-neural roles for Netrin is not unprecedented since homologues of Netrin have been reported to have various non-neural roles, such as apoptosis, angiogenesis and tumorigenesis (Larrivée et al., 2007; Lu et al., 2004; Mazelin et al., 2004; Mehlen et al., 1998; Wilson et al., 2006). Indeed, evidence supporting non-neural adhesive (Yebra et al., 2003) and migratory (Schwarting et al., 2004; Yebra et al., 2003) functions of Netrin may be an indicator of the mechanism by which Sp-Netrin is maintaining the oral and ciliary band phenotypes since a disruption in adhesion or migration in the oral ectoderm could disrupt signalling in the ectoderm. Since α6β4 and α3β1 integrins are implicated in mediating the adhesive and migratory properties of Netrin (Yebra et al., 2003) and integrins are implicated in interacting with regulators of TGFβ signalling such as Chordin (Larrain et al., 2003), it may be worthwhile investigating if function blocking antibodies to integrins result in similar phenotypes.

4.6 Conclusions

This project provides a wide-ranging data set that advances our understanding of the echinoderm larval nervous system and the formation of larval ectoderm. A detailed description of how the larval nervous system forms and a model for stereotypic neural development is given. Sequencing *Sp-netrin*, *Sp-neogenin* and *Sp-unc5* broadens our knowledge of these genes and has allowed for a comparison of these molecules to other species. We now know when these genes are expressed and where and when Sp-Netrin protein is expressed. My guiding hypothesis was that *Sp-netrin* is responsible for neurite

guidance. However, my analysis yielded surprising results and supports a model where Netrin affects oral ectoderm, ciliary band and neural development. This project has raised some pressing questions regarding the role of Sp-Netrin in neural guidance, ectoderm and neuron development.

What proteins are responsible for regulating Sp-Netrin? What other proteins are downstream from Sp-Netrin? The answers to these questions may reveal in more detail how ectoderm is dependent on Sp-Netrin. Examination of the Sp-Netrin MASO phenotype should explore possible upstream regulators, Nodal and BMP2/4 to determine if their expression patterns remain consistent after Sp-Netrin perturbation. Another possible upstream regulator could be p38. Bradham and McClay (2006, 2007) have placed Gsc, like Chordin, downstream of both p38 and Nodal. P38 has been reported to be required for neurite growth and chemoattractive response to Netrin-1 in commissural axons of the rodent spinal cord (Forcet et al., 2002). In echinoderms, p38 may also be responsible for neural specification since inhibition of p38 blocked synaptotagmin expression (Bradham et al., 2009). If these proteins are indeed upstream of Sp-Netrin, knockdowns of these proteins should affect its expression pattern. Investigation of downstream regulators should include other markers for oral ectoderm (antivin), ciliary band (*eph* and *actin*) and aboral ectoderm (*Tbx2/3* and *29D*) to determine the extent of Sp-Netrin's effects on these tissues.

The loss of neurons due to Sp-Netrin perturbation, indicates that neuronal differentiation or specification is disrupted. Sp-Netrin knockdown experiments that employ early neuron differentiation markers can address how these neurons are lost.

However, neuroblast markers need to be identified and antibodies need to be produced in *S. purpuratus* prior to this analysis in order to explore this question.

Future studies should attempt to co-inject *Sp-netrin* MASO with *Sp-netrin* mRNA to confirm that the disruption of oral signalling by Sp-Netrin perturbation can be rescued by Sp-Netrin expression. Moreover, if Sp-Netrin is functioning as a chemoattractant or chemorepellent to guide neurites, a rescue of oral signalling may result in the misspatterning of neurites, since Sp-Netrin expression would no longer be concentrated in specific regions of the embryo. Alternatively, future studies that attempt to determine the role of *Sp-netrin* in neurite guidance should employ cell turning assays using embryonic cell cultures. Of course, immunolocalizations and injections of *Sp-unc5* and *Sp-neogenin*, MASO and mRNA should also be completed in future work to evaluate their locations and respective functions during development.

The findings presented here provide many research opportunities that can give insight into gene regulatory systems for ectoderm, neural specification and neurite guidance. In light of these findings, building on this knowledge will require a detailed understanding of the processes of differentiation and morphogenesis, but holds great promise for a researcher willing to undertake this challenge.

Bibliography

Angerer, L. M., Oleksyn, D. W., Levine, A. M., Li, X., Klein, W. H., & Angerer, R. C. (2001). Sea urchin goosecoid function links fate specification along the animal-vegetal and oral-aboral embryonic axes. *Development, 128*(22), 4393-4404.

Beer, A., Moss, C., & Thorndyke, M. (2001). Development of serotonin-like and SALMFamide-like immunoreactivity in the nervous system of the sea urchin psammechinus miliaris. *The Biological Bulletin, 200*(3), 268-280.

Bisgrove, B., & Burke, R. (1987). Development of the nervous system of the pluteus larva of strongylocentrotus droebachiensis. *Cell and Tissue Research, 248*(2), 335-343.

Bradham, C. A., & McClay, D. R. (2006). p38 MAPK is essential for secondary axis specification and patterning in sea urchin embryos. *Development, 133*(1), 21-32.

Bradham, C. A., & McClay, D. R. (2007). Secondary axis specification in sea urchin embryos. *Signal Transduction, 7*(2), 181-186.

Bradham, C. A., Oikonomou, C., Kühn, A., Core, A. B., Modell, J. W., McClay, D. R., et al. (2009). Chordin is required for neural but not axial development in sea urchin embryos. *Developmental Biology, 328*(2), 221-233.

Burke, R. D., Angerer, L. M., Elphick, M. R., Humphrey, G. W., Yaguchi, S., Kiyama, T., et al. (2006). A genomic view of the sea urchin nervous system. *Developmental Biology, 300*(1), 434.

Burke, R. D. (1978). The structure of the nervous system of the pluteus larva of Strongylocentrotus purpuratus. *Cell and Tissue Research, 191*(2), 233-247.

Cameron, C. B., Garey, J. R., & Swalla, B. J. (2000). Evolution of the chordate body plan: New insights from phylogenetic analyses of deuterostome phyla. *Proceedings of the National Academy of Sciences of the United States of America, 97*(9), 4469-4474.

Chan, S. S. -., Zheng, H., Su, M. -., Wilk, R., Killeen, M. T., Hedgecock, E. M., et al. (1996). UNC-40, a C. elegans homolog of DCC (deleted in colorectal cancer), is required in motile cells responding to UNC-6 netrin cues. *Cell, 87*(2), 187-195.

Conrad, S., Stimpfle, F., Montazeri, S., Oldekamp, J., Seid, K., Alvarez-Bolado, G., et al. (2010). RGMb controls aggregation and migration of neogenin-positive cells in vitro and in vivo. *Mollecular and Cellular Neuroscience, 43*(2), 222-231.

Duboc, V., Lapraz, F., Besnardeau, L., & Lepage, T. (2008). Lefty acts as an essential modulator of nodal activity during sea urchin oral–aboral axis formation. *Developmental Biology, 320*(1), 49-59.

Duboc, V., Röttinger, E., Besnardeau, L., & Lepage, T. (2004). Nodal and BMP2/4 signaling organizes the oral-aboral axis of the sea urchin embryo. *Developmental Cell, 6*(3), 397.

Duboc, V., Röttinger, E., Lapraz, F., Besnardeau, L., & Lepage, T. (2005). Left-right asymmetry in the sea urchin embryo is regulated by nodal signaling on the right side. *Developmental Cell, 9*(1), 147.

Flowers, V. L., Courteau, G. R., Poustka, A. J., Weng, W., & Venuti, J. M. (2004). Nodal/activin signaling establishes oral-aboral polarity in the early sea urchin embryo. *Developmental Dynamics, 231*(4), 727-740.

Forcet, C., Stein, E., Pays, L., Corset, V., Llambi, F., Tessier-Lavigne, M., et al. (2002). Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer-dependent MAPK activation. *Nature, 417*(6887), 443-447.

Freitas, C., Larrivée, B., & Eichmann, A. (2008). Netrins and UNC5 receptors in angiogenesis. *Angiogenesis, 11*(1), 23-29.

Gad, J. M., Keeling, S. L., Wilks, A. F., Tan, S., & Cooper, H. M. (1997). The expression patterns of guidance receptors, DCC and neogenin, are spatially and temporally distinct throughout mouse embryogenesis. *Developmental Biology, 192*(2), 258.

Geisbrecht, B. V., Dowd, K. A., Barfield, R. W., Longo, P. A., & Leahy, D. J. (2003). Netrin binds discrete subdomains of DCC and UNC5 and mediates interactions between DCC and heparin. *Journal of Biological Chemistry, 278*(35), 32561-32568.

Gillespie, L. N., Marzella, P. L., Clark, G. M., & Crook, J. M. (2005). Netrin-1 as a guidance molecule in the postnatal rat cochlea. *Hearing Research, 199*(1-2), 117-123.

Guigo, R., Flicek, P., Abril, J., Reymond, A., Lagarde, J., Denoeud, F., et al. (2006). EGASP: The human ENCODE genome annotation assessment project. *Genome Biology, 7*(Suppl 1), S2.

Hamelin, M., Zhou, Y., Su, M., Scott, I. M., & Culotti, J. G. (1993). Expression of the UNC-5 guidance receptor in the touch neurons of C. elegans steers their axons dorsally. *Nature, 364*(6435), 327-330.

Harris, R., Sabatelli, L. M., & Seeger, M. A. (1996). Guidance cues at the drosophila CNS midline: Identification and characterization of two drosophila Netrin/UNC-6 homologs [Abstract]. *Neuron, 17*(2) 217-228.

Hedgecock, E. M., Culotti, J. G., & Hall, D. H. (1990). The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans. *Neuron, 4*(1), 61-85.

Hong, K., Hinck, L., Nishiyama, M., Poo, M., Tessier-Lavigne, M., & Stein, E. (1999). A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. *Cell, 97*(7), 927-941.

Hong, K., Nishiyama, M., Henley, J., Tessier-Lavigne, M., & Poo, M. (2000). Calcium signalling in the guidance of nerve growth by netrin-1. *Nature, 403*(6765), 93-98.

Ishii, N., Wadsworth, W. G., Stern, B. D., Culotti, J. G., & Hedgecock, E. M. (1992). UNC-6, a laminin-related protein, guides cell and pioneer axon migrations in C. elegans. *Neuron, 9*(5), 873-881.

Jin, M., Guan, C., Jiang, Y., Chen, G., Zhao, C., Cui, K., et al. (2005). Ca2+-dependent regulation of rho GTPases triggers turning of nerve growth cones. *Journal of Neuroscience, 25*(9), 2338-2347.

Katow, H. (2008). Spatio-temporal expression of a netrin homolog in the sea urchin hemicentrotus pulcherrimus (HpNetrin) during serotonergic axon extension. *The International Journal of Developmental Biology, 52*(8), 1077-1088.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E. D., Chan, S. S., Culotti, J. G., et al. (1996). Deleted in colorectal cancer (DCC) encodes a netrin receptor. *Cell, 87*(2), 175-185.

Keleman, K., & Dickson, B. J. (2001). Short- and long-range repulsion by the drosophila Unc5 netrin receptor [Abstract]. *Neuron, 32*(4) 605-617.

Kennedy, T. E. (2000). Cellular mechanisms of netrin function: Long-range and short-range actions. *Biochemistry and Cell Biology, 78*(5), 569-575.

Kennedy, T. E., Serafini, T., de la Torre, J. R., & Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell, 78*(3), 425-435.

Kennedy, T. E., Wang, H., Marshall, W., & Tessier-Lavigne, M. (2006). Axon guidance by diffusible chemoattractants: A gradient of netrin protein in the developing spinal cord. *Journal of Neuroscience, 26*(34), 8866-8874.

Labrador, J. P., O'Keefe, D., Yoshikawa, S., McKinnon, R. D., Thomas, J. B., & Bashaw, G. J. (2005). The homeobox transcription factor even-skipped regulates netrin-receptor expression to control dorsal motor-axon projections in drosophila. *Current Biology, 15*(15), 1413-1419.

Lapraz, F., Besnardeau, L., & Lepage, T. (2009). Patterning of the dorsal-ventral axis in echinoderms: Insights into the evolution of the BMP-chordin signaling network. *PLoS Biology, 7*(11), e1000248.

Larraín, J., Brown, C., & Robertis, E. M. D. (2003). Integrin-[alpha]3 mediates binding of chordin to the cell surface andpromotes its endocytosis. *EMBO Reports, 4*(8), 813–818.

Larrivée, B., Freitas, C., Trombe, M., Lv, X., DeLafarge, B., Yuan, L., et al. (2007). Activation of the UNC5B receptor by netrin-1 inhibits sprouting angiogenesis. *Genes & Development, 21*(19), 2433-2447.

Leung-Hagesteijn, C., Spence, A. M., Stern, B. D., Zhou, Y., Su, M., Hedgecock, E. M., et al. (1992). UNC-5, a transmembrane protein with immunoglobulin and thrombospondin type 1 domains, guides cell and pioneer axon migrations in C. elegans. *Cell, 71*(2), 289.

Linder, B., Bammer, S., & Heinlein, U. A. O. (1995). Delayed translation and posttranslational processing of cyritestin, an integral transmembrane protein of the mouse acrosome. *Experimental Cell Research, 221*(1), 66-72.

Liu GF, Li WQ, Gao X, Li XL, Jurgensen C, Park HT, et al. (2007). p130(CAS) is required for netrin signaling and commissural axon guidance. *Journal of Neuroscience, 27*(4)

Livesey, F. J. (1999). Netrins and netrin receptors. *Cellular and Molecular Life Sciences, 56*(1), 62-68.

Lopez-Rios, J., Esteve, P., Ruiz, J., & Bovolenta, P. (2008). The netrin-related domain of Sfrp1 interacts with wnt ligands and antagonizes their activity in the anterior neural plate. *Neural Development, 3*(1), 19.

Lowe, C. J., Terasaki, M., Wu, M., Freeman, R. M., Jr., Runft, L., Kwan, K., et al. (2006). Dorsoventral patterning in hemichordates: Insights into early chordate evolution. *PLoS Biology, 4*(9), e291.

Lu, X., le Noble, F., Yuan, L., Jiang, Q., de Lafarge, B., Sugiyama, D., et al. (2004). The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. *Nature, 432*(7014), 179-186.

Mackie, G. O., Spencer, A. N., & Strathmann, R. (1969). Electrical activity associated with ciliary reversal in an echinoderm larva. *Nature, 223*(5213), 1384-1385.

MacLennan, A. J., McLaurin, D. L., Marks, L., Vinson, E. N., Pfeifer, M., Szulc, S. V., et al. (1997). Immunohistochemical localization of netrin-1 in the embryonic chick nervous system. *Journal of Neuroscience, 17*(14), 5466-5479.

Materna, S. C., Berney, K., & Cameron, R. A. (2006). The S. purpuratus genome: A comparative perspective. *Developmental Biology, 300*(1), 485-495.

Mazelin, L., Bernet, A., Bonod-Bidaud, C., Pays, L., Arnaud, S., Gespach, C., et al. (2004). Netrin-1 controls colorectal tumorigenesis by regulating apoptosis. *Nature, 431*(7004), 80-84.

Mehlen, P., & Bredesen, D. E. (2004). The dependence receptor hypothesis. *Apoptosis, 9*(1), 37-49.

Mehlen, P., Rabizadeh, S., Snipas, S. J., Assa-Munt, N., Salvesen, G. S., & Bredesen, D. E. (1998). The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis. *Nature, 395*(6704), 801-804.

Miljkovic-Licina, M., Gauchat, D., & Galliot, B. (2004). Neuronal evolution: Analysis of regulatory genes in a first-evolved nervous system, the hydra nervous system. *Biosystems, 76*(1-3), 75-87.

Nakajima, Y., Kaneko, H., Murray, G., & Burke, R. D. (2004). Divergent patterns of neural development in larval echinoids and asteroids. *Evolution & Development, 6*(2), 95-104.

Nishiyama, M., Hoshino, A., Tsai, L., Henley, J. R., Goshima, Y., Tessier-Lavigne, M., et al. (2003). Cyclic AMP/GMP-dependent modulation of Ca2+ channels sets the polarity of nerve growth-cone turning. *Nature, 423*(6943), 990-995.

Oelgeschläger, M., Kuroda, H., Reversade, B., & Robertis, E. M. D. (2003). Chordin is required for the spemann organizer transplantation phenomenon in xenopus embryos. *Developmental Cell, 4*(2), 219.

Otim, O., Amore, G., Minokawa, T., McClay, D. R., & Davidson, E. H. (2004). SpHnf6, a transcription factor that executes multiple functions in sea urchin embryogenesis. *Developmental Biology, 273*(2), 226.

Park, K. W., Crouse, D., Lee, M., Karnik, S. K., Sorensen, L. K., Murphy, K. J., et al. (2004). The axonal attractant netrin-1 is an angiogenic factor. *Proceedings of the National Academy of Sciences of the United States of America, 101*(46), 16210-16215.

Rajagopalan, S., Deitinghoff, L., Davis, D., Conrad, S., Skutella, T., Chedotal, A., et al. (2004). Neogenin mediates the action of repulsive guidance molecule. *Nature Cell Biology, 6*(8), 756-762.

Rajasekharan, S., & Kennedy, T. (2009). The netrin protein family. *Genome Biology, 10*(9), 239.

Ramón y Cajal, S. (1909). *Histologie du systeme nerveux de l'homme et des vertebres,* Madrid*:* Consejo Superior de investigaciones Cientificas.

Ramón y Cajal, S. (1999). *Texture of the nervous system of man and the vertebrates*. Vienna/New York: Springer.

Ransick, A. (2004). Detection of mRNA by in situ hybridization and RT-PCR. *Methods in Cell Biology, 74*, 601-620.

Ransick, A., Rast, J. P., Minokawa, T., Calestani, C., & Davidson, E. H. (2002). New early zygotic regulators expressed in endomesoderm of sea urchin embryos discovered by differential array hybridization. *Developmental Biology, 246*(1), 132-147.

Schwarting, G. A., Raitcheva, D., Bless, E. P., Ackerman, S. L., & Tobet, S. (2004). Netrin 1-mediated chemoattraction regulates the migratory pathway of LHRH neurons. *European Journal of Neuroscience, 19*(1), 11-20.

Sea Urchin Genome Sequencing Consortium, Sodergren, E., Weinstock, G. M., Davidson, E. H., Cameron, R. A., Gibbs, R. A., et al. (2006). The genome of the sea urchin strongylocentrotus purpuratus. *Science, 314*(5801), 941-952.

Serafini, T., Colamarino, S. A., Leonardo, E. D., Wang, H., Beddington, R., Skarnes, W. C. et al. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system [Abstract]. *Cell, 87*(6) 1001-1014.

Srinivasan, K., Strickland, P., Valdes, A., Shin, G. C., & Hinck, L. (2003). Netrin-1/Neogenin interaction stabilizes multipotent progenitor cap cells during mammary gland morphogenesis. *Developmental Cell, 4*(3), 371.

Strathmann, R. (1975). Larval feeding in echinoderms. *Integrative and Comparative Biology, 15*(3), 717-730.

Strathmann, R. R. (1971). The feeding behavior of planktotrophic echinoderm larvae: Mechanisms, regulation, and rates of suspensionfeeding. *Journal of Experimental Marine Biology and Ecology, 6*(2), 109-160.

Su, Y., Li, E., Geiss, G. K., Longabaugh, W. J. R., Krämer, A., & Davidson, E. H. (2009). A perturbation model of the gene regulatory network for oral and aboral ectoderm specification in the sea urchin embryo. *Developmental Biology, 329*(2), 410-421.

Summerton, J. (1999). Morpholino antisense oligomers: The case for an RNase H-independent structural type. *Biochimica Et Biophysica Acta (BBA) - Gene Structure and Expression, 1489*(1), 141.

Torre, J. R. d. l., Höpker, V. H., Ming, G., Poo, M., Tessier-Lavigne, M., Hemmati-Brivanlou, A., et al. (1997). Turning of retinal growth cones in a netrin-1 gradient mediated by the netrin receptor DCC. *Neuron, 19*(6), 1211.

Vielmetter, J., Kayyem, J. F., Roman, J. M., & Dreyer, W. J. (1994). Neogenin, an avian cell surface protein expressed during terminal neuronal differentiation, is closely related to the human tumor suppressor molecule deleted in colorectal cancer. *The Journal of Cell Biology, 127*(6), 2009-2020.

Wadsworth, W. G., Bhatt, H., & Hedgecock, E. M. (1996). Neuroglia and pioneer neurons express UNC-6 to provide global and local netrin cues for guiding migrations in C. elegans. *Neuron, 16*(1), 35-46.

Wang, R., Wei, Z., Jin, H., Wu, H., Yu, C., Wen, W. et al. (2009). Autoinhibition of UNC5b revealed by the cytoplasmic domain structure of the receptor [Abstract]. *Mollecular Cell, 33*(6) 692-703.

Wei, Z., Angerer, R. C., & Angerer, L. M. (2006). A database of mRNA expression patterns for the sea urchin embryo. *Developmental Biology, 300*(1), 476.

Wilson, B. D., Ii, M., Park, K. W., Suli, A., Sorensen, L. K., Larrieu-Lahargue, F., et al. (2006). Netrins promote developmental and therapeutic angiogenesis. *Science, 313*(5787), 640-644.

Wilson, N. H., & Key, B. (2006). Neogenin interacts with RGMa and netrin-1 to guide axons within the embryonic vertebrate forebrain. *Developmental Biology, 296*(2), 485.

Wilson, N. H., & Key, B. (2007). Neogenin: One receptor, many functions. *The International Journal of Biochemistry & Cell Biology, 39*(5), 874-878.

Xie, Y., Hong, Y., Ma, X., Ren, X., Ackerman, S., Mei, L., et al. (2006). DCC-dependent phospholipase C signaling in netrin-1-induced neurite elongation. *Journal of Biological Chemistry, 281*(5), 2605-2611.

Yaguchi, S., Kanoh, K., Amemiya, S., & Katow, H. (2000). Initial analysis of immunochemical cell surface properties, location and formation of the serotonergic apical ganglion in sea urchin embryos. *Development Growth & Differentiation, 42*(5), 479-488.

Yaguchi, S., Yaguchi, J., Angerer, L. M., Angerer, R. C., & Burke, R. D. (submitted). TGFβ signaling positions the ciliary band and patterns neurons in the sea urchin embryo.

Yaguchi, S., Yaguchi, J., & Burke, R. D. (2006). Specification of ectoderm restricts the size of the animal plate and patterns neurogenesis in sea urchin embryos. *Development, 133*(12), 2337-2346.

Yebra, M., Montgomery, A. M. P., Diaferia, G. R., Kaido, T., Silletti, S., Perez, B., et al. (2003). Recognition of the neural chemoattractant netrin-1 by integrins α6β4 and α3β1 regulates epithelial cell adhesion and migration. *Developmental Cell, 5*(5), 695-707.

Yuan, X., Jin, M., Xu, X., Song, Y., Wu, C., Poo, M., et al. (2003). Signalling and crosstalk of rho GTPases in mediating axon guidance. *Nature Cell Biology, 5*(1), 38-45.

Yurchenco, P. D., & Wadsworth, W. G. (2004). Assembly and tissue functions of early embryonic laminins and netrins. *Current Opinion in Cell Biology, 16*(5), 572-579.

Appendix I
Primer Sequences

Primer Name and Sequence (5' to 3')	**Annealing Temperature (ºC)**	**Amplicon Name (Refer to Figures 1,2,3**
Primers for *Sp-netrin* Sequencing		
NETF1: ATGGCTTTGTGGTGGTACG NETR1: CTAGCACTCATCCCGTCTC	60	NetF1R1
Primers for *Sp-unc5* Sequencing		
SPBASEF: ATGATACGGGGAAGTGC SPBASER: GATCTGTTGTTCTGTAAGTGG	58	spbaseFR
510F: CATTGACGACGAGAACCTC 1730R: CCATCATTGTCCTCCAAGTC	58	unc1730FR
UNC5ENDF: GCTGAATCCTCCAGAACCA UNC5ENDR: TCACATCCATGCAGCCAT	58	unc5endFR
Primers for *Sp-neogenin* Sequencing		
DCC3F: CATCTGTATGAAGGTAACATCG HMMMOD4452R1: GAGTACCTGCCATGATCTGTA	62	D3FR
DCC5F: ATGCTCTTGTGGTCAATGG DCC2309SR: AGAGCTGATATCGAAGTAGAGAG	61	D4FR
2309F: CTCTTGACCTGAGTGCCATC 3256R: GGATGTAACCCTCCACCAG	61	D2309FR
718F: GAGAGGGAATACCAGTCACC DCC2R: CCTATACCAGCACCATTGAC	60	D718FR
DCC5F: GACCGTTCACTGAGTGGATAG DCC4002S123: GCAATAGCCAGCCATAGC	60	D5FR
4002F: AGTCAATGGTGCTGGTATAGG 4879R: GCATCGTTTCCTTGGTATTC	60	D4002FR
4780F: CCAGTCTATAGAAGCAGCTAAC 5613R: GGTTGGTGATATCGCACTG	60	D4780FR
4339F: GGCAGCAACACCTCTACTG HMM6069R: GCATCATTTCAGCATTCAGA	58	D4339FR
DCC2F: CTCAACCTATGGCTGTCATCAC HMM6147R: TCAGTTTTGTAGTTCAGATTGTGTG	67	D2FR

Primers for QPCR		
TBPPINTF1: CAGGATGGAGGGCAACAGAGTC TBPPINTR1: GCATGGAGGGCAATTTTCTTCAGATC	60	n/a
QUNC5F1: CCAAGTCCGACCGATCAGATCC QUNC5R1: CCATGCAGCCATCTGTTTCTGG	60	n/a
QDCCF2: ACCCTCGGCAGCAATCATTCTG QDCCR2:CGGTCAATGGGTTTGGTGCAAG	60	n/a
QNETF1: TTCACCCGAAGCCCAGCACAAC QNETR1: TGCCATGTCTTGCCGAAGTCC	60	n/a
QUBIQF1: CACAGGCAAGACCATCACAC QUBIQR1: GAGAGAGTGCGACCATCCTC	60	n/a
Primers for Antibody Production		
NETIHCF1: GAGCTCTCTCTACTACGCTCTCTCG NETIHCF2: GCGGCCGCCTCATCCCGTCTCTGTTC	60	n/a

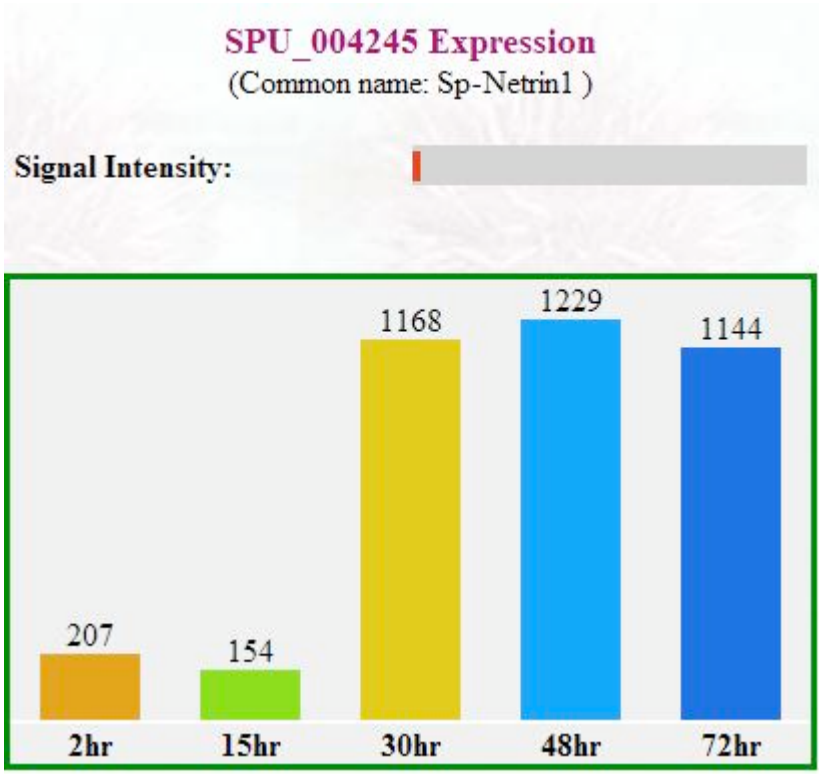
Sequence for Netrin MASO (5' to 3'):

TTACTATGCTCCGTCCTCCTTAGAC

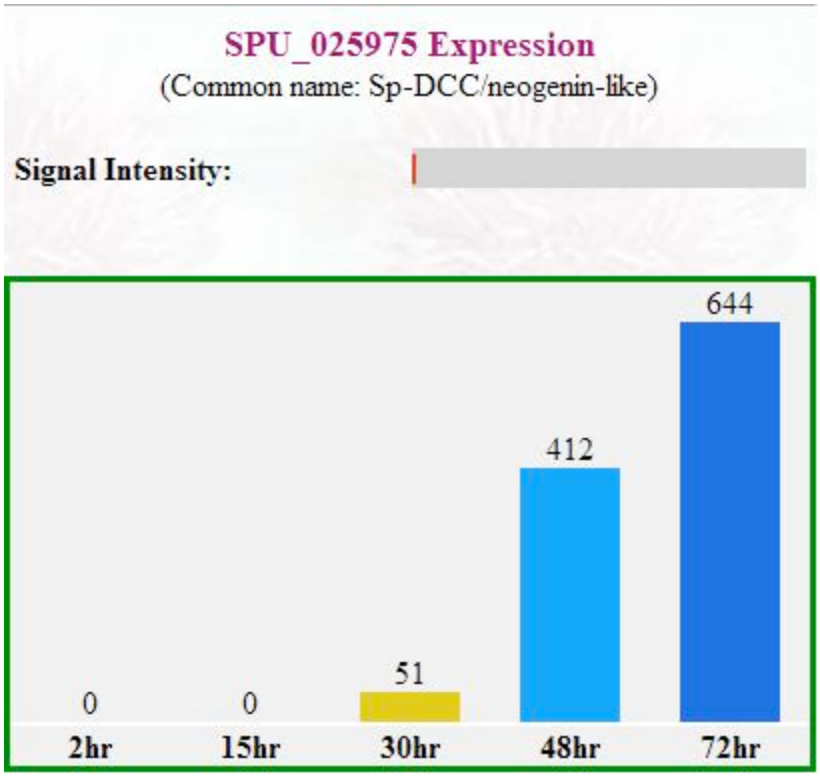
Sequence for Standard Control MASO (5' to 3'):

CCTCTTACCTCAGTTACAATTTATA

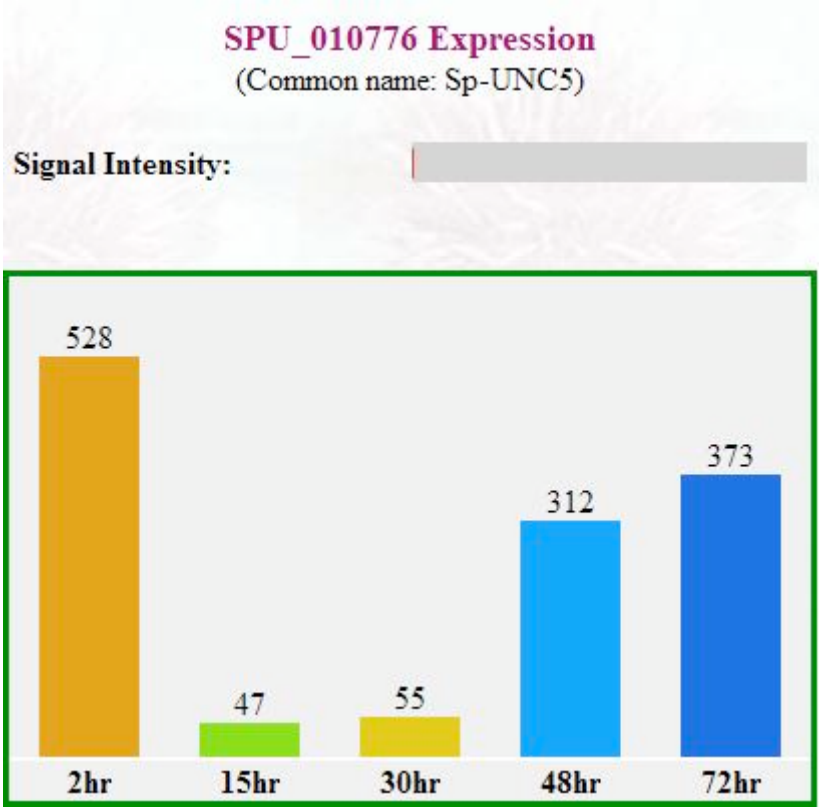
Appendix II
Temporal Expression Patterns



Chip hybridization data showing the temporal expression patterns of GLEAN3-004245 (Sp-Netrin) Signal intensities are indicated above each bar and are normalized to the amount of signal present at 48 hpf. Hours indicate hours post fertilization. Ubiquitin was used as control to standardize measurements. The overall signal for this gene is approximately 10% of the possible signal (see red bar). This data was obtained by producing five chip bound hybridization probes based on GLEAN3-004245 that were hybridized with fluorescently labelled mRNA and the average level of fluorescence was measured.



Chip hybridization data showing the temporal expression patterns of GLEAN3-025975 (Sp-Neogenin).
Signal intensities are indicated above each bar and are normalized to the amount of signal present at 48 hpf. Hours indicate hours post fertilization. Ubiquitin was used as control to standardize measurements. The overall signal for this gene is approximately 10% of the possible signal (see red bar). This data was obtained by producing five chip bound hybridization probes based on GLEAN3-025975 that were hybridized with fluorescently labelled mRNA and the average level of fluorescence was measured.



Chip hybridization data showing the temporal expression patterns of GLEAN3-010776 (Sp-Unc5).

Signal intensities are indicated above each bar and are normalized to the amount of signal present at 48 hpf. Hours indicate hours post fertilization. Ubiquitin was used as control to standardize measurements. The overall signal for this gene is approximately 10% of the possible signal (see red bar). This data was obtained by producing five chip bound hybridization probes based on GLEAN3-010776 that were hybridized with fluorescently labelled mRNA and the average level of fluorescence

Appendix III
Protein Alignments

Netrin Alignment

```
                          ....|....| ....|....| ....|....| ....|....|
                                   10         20         30         40
H. pulcherrimus Netrin    MQIVSLCLRR TEHSKMALWW YVVSVFVIMG FGSCWGARAG
S. kowalevskii Netrin1    -----MSYTP TSSRHLCLLL FVLAMFVVT- --------KG
D. rerio Netrin           ---------- ----MLR-VS DALVTLVTLC CV-LKGTVGG
H. sapiens Netrin-1       ---------- ----MMRAVW EALAALAAVA C--LVGAVRG
M. musculus Netrin-1      ---------- ----MMRAVW EALAALAAVA C--LVGAVRG
D. melanogaster Netrin    ---------- -MIRGILLLL LGTTRFSPIQ C------IFN
C. elegans Unc-6          ---------- ---MITSVLR YVLALYFCMG -------IAH
S. purpuratus Netrin      ---------- -----MALWW YVVSVFLIMG FGSCRGARSG

                          ....|....| ....|....| ....|....| ....|....|
                                   50         60         70         80
H. pulcherrimus Netrin    NSGISMFAAQ DSRRDPCYGE NNV PKRCEPD FVNAAFQAPV
S. kowalevskii Netrin1    YNGLSVM--Q QRPLDPCYDE NGN PKRCIPD FVNAAFGIPV
D. rerio Netrin           -YGMSMFAAQ TSPPDPCYDE NGH PRRCIPD FVNAAFGKEV
H. sapiens Netrin-1       GPGLSMFAGQ AAQPDPCSDE NGH PRRCIPD FVNAAFGKDV
M. musculus Netrin-1      -PGLSMFAGQ AAQPDPCSDE NGH PRRCIPD FVNAAFGKDV
D. melanogaster Netrin    DVYFKMFSQQ APPEDPCYNK AHE PRACIPD FVNAAYDAPV
C. elegans Unc-6          GAYFSQFSMR APDHDPCHDH TGR PVRCVPE FINAAFGKPV
S. purpuratus Netrin      NSGISMFAAQ DSRRDPCYGE NNV PKRCEPD FVNAAFQAPV

                          ....|....| ....|....| ....|....| ....|....|
                                   90        100        110        120
H. pulcherrimus Netrin    EASSTCGDP- PSRFCALSSG AEGERQRNCF ICDESHPKRR
S. kowalevskii Netrin1    KSSSTCGSP- PSRYCVMSTG ASGERSRICF VCDADHPKRR
D. rerio Netrin           RASSTCGKT- PSRYCVVTEK GD-ERHRNCH TCDASDPKKN
H. sapiens Netrin-1       RVSSTCGRP- PARYCVVSER GE-ERLRSCH LCNASDPKKA
M. musculus Netrin-1      RVSSTCGRP- PARYCVVSER GE-ERVRSCH LCNSSDPKKA
D. melanogaster Netrin    VASSTCGSSG AQRYCEYQ-- ---DHERSCH TCDMTDPLRS
C. elegans Unc-6          IASDTCGTNR PDKYCTVKEG PDGIIREQCD TCDARNHFQS
S. purpuratus Netrin      EASSTCGDP- PSRFCALSSG AEGERQRNCF ICDESHPKRR

                          ....|....| ....|....| ....|....| ....|....|
                                  130        140        150        160
H. pulcherrimus Netrin    HPASYLTDLH NPNNLTCWQS EPFTQSQHNV TLK LSLTKRF
S. kowalevskii Netrin1    HPPEFLTDLN NPNNLTCWQS EPYVQHPNNV TLV LSLEKKF
D. rerio Netrin           HPPAYLTDLN NPHNLTCWQS DNYLQYPQNV TLT LSLGKKF
H. sapiens Netrin-1       HPPAFLTDLN NPHNLTCWQS ENYLQFPHNV TLT LSLGKKF
M. musculus Netrin-1      HPPAFLTDLN NPHNLTCWQS ENYLQFPHNV TLT LSLGKKF
D. melanogaster Netrin    FPARSLTDLN NSNNVTCWRS EPVTGSGDNV TLT LSLGKKF
C. elegans Unc-6          HPASLLTDLN SIGNMTCWVS TPSL-SPQNV SLT LSLGKKF
S. purpuratus Netrin      HPASYLTDLH NPNNLTCWQS EPFTRSQHNV TLK LSLTKRF

                          ....|....| ....|....| ....|....| ....|....|
                                  170        180        190        200
H. pulcherrimus Netrin    EITYISMEFC --WLHPDSMV IFKSQDFGKT WQPYQYYSSQ
```

```
S. kowalevskii Netrin1   ELTYISLQFC --SARPDSMA IYKSQDYGKS WQPYQFYSSQ
D. rerio Netrin          EVTYVSLQFC --SPRPESMA IFKSMDYGKS WVPFQYYSTQ
H. sapiens Netrin-1      EVTYVSLQFC --SPRPESMA IYKSMDYGRT WVPFQFYSTQ
M. musculus Netrin-1     EVTYVSLQFC --SPRPESMA IYKSMDYGRT WVPFQFYSTQ
D. melanogaster Netrin   ELTYVILQLC PHAPRPDSMV IYKSTDHGLS WQPFQFFSSQ
C. elegans Unc-6         ELTYVSMHFC --SRLPDSMA LYKSADFGKT WTPFQFYSSE
S. purpuratus Netrin     EITYISMEFC --WLHPDSMV IFKSQDFGKT WQPYQYYSSQ

                         ....|....| ....|....| ....|....| ....|....|
                                 210        220        230        240
H. pulcherrimus Netrin   CRKTYGMPKN AVITKQNEQE PACTDIHSS- DLIAGSRVAF
S. kowalevskii Netrin1   CRKMYGISNR AAITKQNEQE ALCTDDHSSI EPMSGGRIAF
D. rerio Netrin          CRKMYNKPSK ATITKQNEQE AICTDSHTDM HPLSGGLIAF
H. sapiens Netrin-1      CRKMYNRPHR APITKQNEQE AVCTDSHTDM RPLSGGLIAF
M. musculus Netrin-1     CRKMYNRPHR APITKQNEQE AVCTDSHTDM RPLSGGLIAF
D. melanogaster Netrin   CRRLFGRPAR QSTGRHNEHE ARCSDVTRP- -LVS--RIAF
C. elegans Unc-6         CRRIFGRDPD VSITKSNEQE AVCTASHIM- -GPGGNRVAF
S. purpuratus Netrin     CRKTYGMPKN AVITKQNEQE PACTDIHSS- DLIAGSRVAF

                         ....|....| ....|....| ....|....| ....|....|
                                 250        260        270        280
H. pulcherrimus Netrin   STLEGRPSAY DFDNSPVLQD WVTATDIKIV FNKLP-----
S. kowalevskii Netrin1   STLKSRPSAY DFDNSPVLQD WVTATDIWGV FNRLN-----
D. rerio Netrin          STLDGRPSAH DFDNSPVLQD WVTATDIKVT FSRLH-----
H. sapiens Netrin-1      STLDGRPSAH DFDNSPVLQD WVTATDIRVA FSRLH-----
M. musculus Netrin-1     STLDGRPSAH DFDNSPVLQD WVTATDIRVA FSRLH-----
D. melanogaster Netrin   STLEGRPSSR DLDSSPVLQD WVTATDIRVV FHRLQRPDPQ
C. elegans Unc-6         PFLENRPSAQ NFENSPVLQD WVTATDIKVV FSRLSPDQ--
S. purpuratus Netrin     STLEGRPSAY GFDNSPVLQD WVTATDIKIV FNKLP-----

                         ....|....| ....|....| ....|....| ....|....|
                                 290        300        310        320
H. pulcherrimus Netrin   ---------- ---------- -----VLG-E NED----ENP
S. kowalevskii Netrin1   ---------- ---------- -----TYG-D EED----EIA
D. rerio Netrin          ---------- ---------- -----TFGDE NEDDS--ELA
H. sapiens Netrin-1      ---------- ---------- -----TFGDE NEDDS--ELA
M. musculus Netrin-1     ---------- ---------- -----TFGDE NEDDS--ELA
D. melanogaster Netrin   ALLSLEAGGA TDLASGKYSV PLANGPAGNN IEANLGGDVA
C. elegans Unc-6         ---------- ----AELYGL SNDVNSYGNE TDD----EVK
S. purpuratus Netrin     ---------- ---------- -----VLG-E NED----ENP

                         ....|....| ....|....| ....|....| ....|....|
                                 330        340        350        360
H. pulcherrimus Netrin   RE-S-LYYAL SDLSVGGRCK CNGHASSCGP GRDGEMECDC
S. kowalevskii Netrin1   RD-S-YYYSV SEFSVGGRCK CNGHASRCIK DRNGEMACEC
D. rerio Netrin          RD-S-YFYAV SDLQVGGRCK CNGHASRCVK DRDGNLVCEC
H. sapiens Netrin-1      RD-S-YFYAV SDLQVGGRCK CNGHAARCVR DRTDSLVCDC
M. musculus Netrin-1     RD-S-YYYAV SDLQVGGRCK CNGHAARCVR DRDDSLVCDC
D. melanogaster Netrin   TSGSGLHYAI SDFSVGGRCK CNGHASKCST DASGQLNCEC
C. elegans Unc-6         QR---YFYSM GELAVGGRCK CNGHASRCIF DKMGRYTCDC
S. purpuratus Netrin     RE-S-LYYAL SDLSVGGRCK CNGHASSCGP GRDGEMECDC
```

```
                          ....|....| ....|....| ....|....| ....|....|
                                  370        380        390        400
H. pulcherrimus Netrin    KHNTAGRECE LCKTFHYDRP WARATSKEAN ECIPCNCNLH
S. kowalevskii Netrin1    KHNTAGPECD QCKPFHYDRP WARATDEDAN ECVPCICNLH
D. rerio Netrin           KHNTAGPECD RCKPFHYDRP WQRATAREAN ECVACNCNLH
H. sapiens Netrin-1       RHNTAGPECD RCKPFHYDRP WQRATAREAN ECVACNCNLH
M. musculus Netrin-1      RHNTAGPECD RCKPFHYDRP WQRATAREAN ECVACNCNLH
D. melanogaster Netrin    SHNTAGRDCE RCKPFHFDRP WARATAKEAN ECKECNCNKH
C. elegans Unc-6          KHNTAGTECE MCKPFHYDRP WGRATANSAN SCVACNCNQH
S. purpuratus Netrin      KHNTAGRECE LCKTFHYDRP WARATSKEAN ECIPCNCNLH

                          ....|....| ....|....| ....|....| ....|....|
                                  410        420        430        440
H. pulcherrimus Netrin    ARKCRFNMEL YKLSGRKSGG VCLKCRHNTD GRYCHYCKEG
S. kowalevskii Netrin1    ARKCRFNMEL YNLSGRKSGG VCLNCRHNTA GRYCHYCEEG
D. rerio Netrin           ARRCRFNMEL YKLSGRKSGG VCLNCRHNTA GRHCHYCKEG
H. sapiens Netrin-1       ARRCRFNMEL YKLSGRKSGG VCLNCRHNTA GRHCHYCKEG
M. musculus Netrin-1      ARRCRFNMEL YKLSGRKSGG VCLNCRHNTA GRHCHYCKEG
D. melanogaster Netrin    ARQCRFNMEI FRLSQGVSGG VCQNCRHSTT GRNCHQCKEG
C. elegans Unc-6          AKRCRFDAEL FRLSGNRSGG VCLNCRHNTA GRNCHLCKPG
S. purpuratus Netrin      ARKCRFNMEL YKLSGRKSGG VCLKCRHNTD GRYCHYCKEG

                          ....|....| ....|....| ....|....| ....|....|
                                  450        460        470        480
H. pulcherrimus Netrin    YYRDQSKPIT HRKVCKACDC HPIGSLGSIC NQTTGQCPCK
S. kowalevskii Netrin1    YYRDQSKPIT HRKACKACEC HPVGAAGEIC NQTTGQCPCK
D. rerio Netrin           YYRDMSKPIS HRKACKACDC HPVGAAGKTC NQTTGQCPCK
H. sapiens Netrin-1       YYRDMGKPIT HRKACKACDC HPVGAAGKTC NQTTGQCPCK
M. musculus Netrin-1      FYRDMGKPIT HRKACKACDC HPVGAAGKTC NQTTGQCPCK
D. melanogaster Netrin    FYRDATKPLT HRKVCKACDC HPIGSSGKIC NSTSGQCPCK
C. elegans Unc-6          FVRDTSLPMT HRKACKSCGC HPVGSLGKSC NQSSGQCVCK
S. purpuratus Netrin      YYRDQSKPIT HRKVCKACDC HPIGSLGSIC NQTTGQCPCK

                          ....|....| ....|....| ....|....| ....|....|
                                  490        500        510        520
H. pulcherrimus Netrin    DGVTGLTCNR CSLGYQQSRS PVAPCIRIK- ----------
S. kowalevskii Netrin1    DGVTALTCNR CSPGYTQSRS PIAPCIKIP- ----------
D. rerio Netrin           DGVTGITCNR CANGYQQSRS PIAPCIKIP- ----------
H. sapiens Netrin-1       DGVTGITCNR CAKGYQQSRS PIAPCIKIP- ----------
M. musculus Netrin-1      DGVTGITCNR CAKGYQQSRS PIAPCIKIP- ----------
D. melanogaster Netrin    DGVTGLTCNR CARGYQQSRS HIAPCIKQPP RMINMLDTQN
C. elegans Unc-6          PGVTGTTCNR CAKGYQQSRS TVTPCIKIP- ----------
S. purpuratus Netrin      DGVTGLTCNR CSLGYQQSRS PVAPCIRIK- ----------

                          ....|....| ....|....| ....|....| ....|....|
                                  530        540        550        560
H. pulcherrimus Netrin    EHPRI---TS QPQ-----EQ RDEAAASSQT TDG-----DC
S. kowalevskii Netrin1    VEP------- -P-------- -ADFTTTPAT VNP-----GC
D. rerio Netrin           IAP------- -P-------- --TTTASSTE EPS-----DC
H. sapiens Netrin-1       VAP------- -P-------- --TTAASSVE EPE-----DC
M. musculus Netrin-1      VAP------- -P-------- --TTAASSVE EPE-----DC
D. melanogaster Netrin    TAPEPDAPES SPGSGGDRNG AAEWPPSLST IAPRAAGVKC
C. elegans Unc-6          TKA------- ---------- --DFIGSSHS EEQD----QC
```

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S. purpuratus Netrin      EHPRI---TS QPQ-----EQ RDEAAASSQT TDG-----DC

                          ....|....| ....|....| ....|....| ....|....|
                                  570        580        590        600
H. pulcherrimus Netrin    PRNCAGSTR- -KISQNKYCK KDYVLHAHVL SRE-------
S. kowalevskii Netrin1    PK-CKGSSR- -KVNMNKYCK KDYAVQANIL SRE-------
D. rerio Netrin           ESYCKASKGK LKINMKKYCK KDYAVQVHIL KAD-------
H. sapiens Netrin-1       DSYCKASKGK LKINMKKYCK KDYAVQIHIL KAD-------
M. musculus Netrin-1      DSYCKASKGK LKMNMKKYCR KDYAVQIHIL KAD-------
D. melanogaster Netrin    GK-CRVSTK- -RLNLNKFCK RDYAIMAKVI GRDTSSEAVS
C. elegans Unc-6          SK-CRIVPK- -RLNQKKFCK RDHAVQMVVV SRE-------
S. purpuratus Netrin      PRNCAGSTR- -KISQNKYCK KDYVLHAHVL SRE-------

                          ....|....| ....|....| ....|....| ....|....|
                                  610        620        630        640
H. pulcherrimus Netrin    ---------- ---------- ---------- -----MEDEW
S. kowalevskii Netrin1    ---------- ---------- ---------- -----QTTDW
D. rerio Netrin           ---------- ---------- ---------- -----KAGEW
H. sapiens Netrin-1       ---------- ---------- ---------- -----KAGDW
M. musculus Netrin-1      ---------- ---------- ---------- -----KAGDW
D. melanogaster Netrin    REVQRRAMDP DVADYEMDQV QPGSARSPIT GVYEFQAADY
C. elegans Unc-6          ---------- ---------- ---------- -----MVDGW
S. purpuratus Netrin      ---------- ---------- ---------- -----MEDEW

                          ....|....| ....|....| ....|....| ....|....|
                                  650        660        670        680
H. pulcherrimus Netrin    ---------- --VKFTVNVV TVYKR---GE Q---------
S. kowalevskii Netrin1    ---------- --VKFTINVM TVYKK---GQ N---------
D. rerio Netrin           ---------- --WKFTVNII SVYKQ---GE S---------
H. sapiens Netrin-1       ---------- --WKFTVNII SVYKQ---GT S---------
M. musculus Netrin-1      ---------- --WKFTVNII SVYKQ---GT S---------
D. melanogaster Netrin    PNPNPNPRGS EMERFDLQIQ AVFKRTRPGE SSGAGNVYGM
C. elegans Unc-6          ---------- --AKYKIVVE SVFKR---GT E---------
S. purpuratus Netrin      ---------- --VKFTVNIV TVYKR---GE Q---------

                          ....|....| ....|....| ....|....| ....|....|
                                  690        700        710        720
H. pulcherrimus Netrin    ---RIRRGNT FLYVRQRDLT CKCPKIRLRH RYLILGDEDD
S. kowalevskii Netrin1    ---KIRRGDQ ILYVHNRDLT CKCPKLRLGR KYLIIGN-DN
D. rerio Netrin           ---RIRRGDQ FLWVRAKDVA CKCPKIKSGK KYLLLGN-DE
H. sapiens Netrin-1       ---RIRRGDQ SLWIRSRDIA CKCPKIKPLK KYLLLGN-AE
M. musculus Netrin-1      ---RIRRGDQ SLWIRSRDIA CKCPKIKPLK KYLLLGN-AE
D. melanogaster Netrin    PNTTLKRGPM TWIIPTKDLE CRCPRIRVNR SYLILGRDSE
C. elegans Unc-6          ---NMQRGET SLWISPQGVI CKCPKLRVGR RYLLLGK-ND
S. purpuratus Netrin      ---RIRRGNT FLYVRQRDLT CKCPKIRLRH RYLILGDEDD

                          ....|....| ....|....| ....|....| ....|....|
                                  730        740        750        760
H. pulcherrimus Netrin    SSSKRDGIVV DNKSIVLKWR DEWDMRMRKF QREQRRDEC-
S. kowalevskii Netrin1    KNPNQNGLLA DHKSYVIPWR DDWNRRMRKF QRKARKS-C-
D. rerio Netrin           DSPGQSGMVA DKGSLVIQWR DTWARRLRKF QQREKKGKCK
H. sapiens Netrin-1       DSPDQSGIVA DKSSLVIQWR DTWARRLRKF QQREKKGKCK
```

```
M. musculus Netrin-1      DSPDQSGIVA DKSSLVIQWR DTWARRLRKF QQREKKGKCK
D. melanogaster Netrin    APPGYLGIGP --HSIVIEWK EDWYRRMKRF QRRART--CA
C. elegans Unc-6          SDHERDGLMV NPQTVLVEWE DDIMDKVLRF SKKDKLGQCP
S. purpuratus Netrin      SSSKRDGIVV DNKSIVLKWR DEWDMRMRKF QREQRRDEC-


                          ....|..

H. pulcherrimus Netrin    -------
S. kowalevskii Netrin1    -------
D. rerio Netrin           KA-----
H. sapiens Netrin-1       KA-----
M. musculus Netrin-1      KA-----
D. melanogaster Netrin    -------
C. elegans Unc-6          EITSHRY
S. purpuratus Netrin      ------.
```

Neogenin Alignment

```
                             ....|....| ....|....| ....|....| ....|....|
                                      10         20         30
40
S. purpuratus Neogenin1      --PRDALVVN GTGLTLNCSA QLSND----- VDIEWY-KNE
C. elegans Unc-40            -PRRNVTVME SSSHLLECSY VLAHERLVHD VRIEWK-RDG
D. melanogaster Neogenin     ---CAGTASI GRGG--KSKS NLPSS----- VSIRWRGPDG
M. musculus Neogenin         ---------- GSSVILNCSA YSEPS----- PNIEWK-KDG
H. sapiens Neogenin          -------SVR GSSVILNCSA YSEPS----- PKIEWK-KDG
S. kowalevskii Neogenin      LEPVDMVIQK GQPAILNCSA FGNPR----- PTVEWK-KDG


                             ....|....| ....|....| ....|....| ....|....|
                                      50         60         70
80
S. purpuratus Neogenin1      NYINVRNSQY YNLLPTGALQ IMAGTPAQQL T---GVYYCT
C. elegans Unc-40            VLLSERTSSR IKVMSNGSLW IESVSSAEEG TYQCAVHVTT
D. melanogaster Neogenin     QDLVIVGDTF RTQLKNGSLY ISSVEENRGL T---GAYQCL
M. musculus Neogenin         TFLNLESDDR RQLLPDGSLF ISNVVHSKHN KPDEGFYQCV
H. sapiens Neogenin          TFLNLVSDDR RQLLPDGSLF ISNVVHSKHN KPDEGYYQCV
S. kowalevskii Neogenin      LFLNLLGDTR RTILPSGALR INNVTHSRND RPDEGNYECV


                             ....|....| ....|....| ....|....| ....|....|
                                      90        100        110
120
S. purpuratus Neogenin1      SYVKALG--L IESRKAHVTL ATLGEIRS-P FGVTVYPGDT
C. elegans Unc-40            KSDQTSDTWT FLSRKATLRL ADLAKFELQA IDRTLAKGQP
D. melanogaster Neogenin     LTAEGVG--S ILSRPALVAI VRQPDLNQDF LETYLLPGQT
M. musculus Neogenin         ATVDNLG--T IVSRTAKLTV AGLPRFTSQP EPSSVYVGNS
H. sapiens Neogenin          ATVESLG--T IISRTAKLIV AGLPRFTSQP EPSSVYAGNN
S. kowalevskii Neogenin      ATVDNLG--T IVSRSAKLQV ADLGRIDEGP ESTSVYTGDT


                             ....|....| ....|....| ....|....| ....|....|
                                     130        140        150
160
S. purpuratus Neogenin1      ARFECEVNGT -----VPKTT TWLKDRQSID LTEERYASRY
```

```
C. elegans Unc-40           TAFHCLINSK -----PTPTA VWLHNDEPIV N-----GGEY
D. melanogaster Neogenin    AYFRCMLGEA NWQEGVKHSV QWLKDDLPLP LD----KLRM
M. musculus Neogenin        AILNCEVNAD -----LVPFV RWEQNRQPL- L----LDDRI
H. sapiens Neogenin         AILNCEVNAD -----LVPFV RWEQNRQPL- L----LDDRV
S. kowalevskii Neogenin     AIFKCVVTAV -----PGAVI RWQKNRVNLD LATN-VDERY

                            ....|....| ....|....| ....|....| ....|....|
                                    170        180        190
200
S. purpuratus Neogenin1     TLLP-SGALE IRDVREGDAG RYRCKVDSLL LPDQRRRSTD
C. elegans Unc-40           HILPVSNTLE ISSTQSRHEG TYRCTVEGA- --GKRRSSQT
D. melanogaster Neogenin    VVLP-NGALE IDEVGPSDRG SYQCNVTSG- --SSSRLSSK
M. musculus Neogenin        VKLP-SGTLV ISNATEGDGG LYRCIVESG- --GPPKFSDE
H. sapiens Neogenin         IKLP-SGMLV ISNATEGDGG LYRCVVESG- --GPPKYSDE
S. kowalevskii Neogenin     TELE-SGTLE IKNVKMSDEA DYRCRATNI- --EGMRRSEE

                            ....|....| ....|....| ....|....| ....|....|
                                    210        220        230
240
S. purpuratus Neogenin1     AELVVRSDPA P-AQRPEDLA FLVAPGPSQI EALIGSTITL
C. elegans Unc-40           ARLTVTTETV S--NELV--- FITTP--RLQ VVEQGDEFLL
D. melanogaster Neogenin    TNLNIKKPSD PGAENSVAPS FLVGP--SPK TVREGDTVTL
M. musculus Neogenin        AELKVLQDPE E-IVDLV--- FLMRP--SSM MKVTGQSAVL
H. sapiens Neogenin         VELKVLPDPE V-ISDLV--- FLKQP--SPL VRVIGQDVVL
S. kowalevskii Neogenin     ASLSIIFSGS M-VRRPE--- FASRP--QNA EVLEGGDVVL

                            ....|....| ....|....| ....|....| ....|....|
                                    250        260        270
280
S. purpuratus Neogenin1     EAATT-EP-A TLEWYKGEKK IN--MQGDHY RRLGQG-SLQ
C. elegans Unc-40           ECLVASLIRP QVRWLKDSRQ II--VDGVRI RRVGVS-SIL
D. melanogaster Neogenin    DCVANGVPKP QIKWLRNGMD LDFNDLDSRF SIVGTG-SLQ
M. musculus Neogenin        PCVVSGLPAP VVRWMKNEEV LD-TESSGRL VLLAGG-CLE
H. sapiens Neogenin         PCVASGLPTP TIKWMKNEEA LD-TESSERL VLLAGG-SLE
S. kowalevskii Neogenin     ECAVNANPRP TVTWWKDGQP ITPSRNPDYH YSYVGAYNLR

                            ....|....| ....|....| ....|....| ....|....|
                                    290        300        310
320
S. purpuratus Neogenin1     ITDLGEIDTG IYRVVAISIL DQSRVERGVT VVVQVPPEFL
C. elegans Unc-40           VSRASIEDTG LYTCRASN-- NDDSIDRAVS VEVRAPPRIT
D. melanogaster Neogenin    ISSAEDIDSG NYQCRASN-- TVDSLDAQAT VQVQEPPKFI
M. musculus Neogenin        ISDVTEDDAG TYFCIADN-- GNKTVEAQAE LTVQVPPGFL
H. sapiens Neogenin         ISDVTEDDAG TYFCIADN-- GNETIEAQAE LTVQAQPEFL
S. kowalevskii Neogenin     IKSATEQDTG TYICSASNVI TNTNIDARVE LLVQVTPRYE

                            ....|....| ....|....| ....|....| ....|....|
                                    330        340        350
360
S. purpuratus Neogenin1     EMPTNTYARL GSTRTLPC-T VYGIPTPSIQ WKSHGDDIDL
C. elegans Unc-40           TRPTTKVAVE TADVELECGT AAARPEARVN WYKNGE--AI
D. melanogaster Neogenin    KAPKDTTAHE KDEPELKC-D IWGKPKPVIR WLKNGD--LI
```

```
M. musculus Neogenin          KQPANIYAHE SMDIVFEC-E VTGKPTPTVK WVKNGD--VV
H. sapiens Neogenin           KQPTNIYAHE SMDIVFEC-E VTGKPTPTVK WVKNGD--MV
S. kowalevskii Neogenin       KEPTSLFAEV NSDINFEC-K VYGVPFPTIE WIKNGD--TI


                              ....|....| ....|....| ....|....| ....|....|
                                      370        380        390
400
S. purpuratus Neogenin1       VANGDFMSVD NQGLTIQDLT DQDSGIYQCL ASNRWG----
C. elegans Unc-40             IGSEYF-VIE PNRLRILGVV RADQAIYQCI AENDVG----
D. melanogaster Neogenin      TPNDYMQLVD GHNLKILGLL NSDAGMFQCV GTNAAGSVHA
M. musculus Neogenin          IPSDYFKIVK EHNLQVLGLV KSDEGFYQCI AENDVGNAQ-
H. sapiens Neogenin           IPSDYFKIVK EHNLQVLGLV KSDEGFYQCI AENDVG----
S. kowalevskii Neogenin       TPSDYFKIVE GTNLKILGLV KSDEGLYQCK ASNDIGNIQT


                              ....|....| ....|....| ....|....| ....|....
                                      410        420        430
S. purpuratus Neogenin1       ---------- ---------- ---------- ---------
C. elegans Unc-40             ---------- ---------- ---------- ---------
D. melanogaster Neogenin      AARLRVVPQG DS-------- ---------- ---------
M. musculus Neogenin          ---------- ---------- ---------- ---------
H. sapiens Neogenin           ---------- ---------- ---------- ---------
S. kowalevskii Neogenin       SAQLIIVDRD ESLPTMPVLI STTTTSPSPT AMVGTDTSP
```

Unc5 Alignment

```
                              ....|....| ....|....| ....|....| ....|....|
                                       10         20         30
40
S. kowalevskii Unc-5          ---------- ---------- ---------- ----------
D. rerio Unc5c                ---------- ---------- ---------- ----------
M. musculus Unc5C             LFLRTAWRWP DPASCLAGLS AVCASPGGVS LPRTPLPTMR
H. sapiens UNC5C              ---------- ---------- ---------- --------MR
C. elegans Unc5               ---------- ---------- ---------- ----------
S. purpuratus Unc5C           ---------- ---------- ---------- ----------


                              ....|....| ....|....| ....|....| ....|....|
                                       50         60         70
80
S. kowalevskii Unc-5          MGEHMSTSMA RLNVLSLYLL LIATIFLVLA SR-IARAQVP
D. rerio Unc5c                -----MCWRS GTASGSGARM MM-------S VRVTSSSAAA
M. musculus Unc5C             KGLRATAARC GLGLGYLLQM LVLPALALLS ASGTGSAAQD
H. sapiens UNC5C              KGLRATAARC GLGLGYLLQM LVLPALALLS ASGTGSAAQD
C. elegans Unc5               ---------- ---------- ---------- --------MD
S. purpuratus Unc5C           ----MIRGSA GLRIQEVTVV LV--LFGAIA LR-PVLGQLD
```

```
                          ....|....| ....|....| ....|....| ....|....|
                                   90        100        110
120
S. kowalevskii Unc-5      DQDYGAMGN- --PDEPIP-- EFIVEPEDGY IVKNADITLT
D. rerio Unc5c            DDDYGGTSD- ---------- ------HMAY -VKNK-VN-Y
M. musculus Unc5C         DEFFHELPET FPSDPPEPLP HFLIEPEEAY IVKNKPVNLY
H. sapiens UNC5C          DDFFHELPET FPSDPPEPLP HFLIEPEEAY IVKNKPVNLY
C. elegans Unc5           EITITTQPK- ---------- -------SGY VIRNKPLRLQ
S. purpuratus Unc5C       GQSAQDVPT- --TMTPLPPT QFFIVPQDSY ITKRS-VDLE


                          ....|....| ....|....| ....|....| ....|....|
                                  130        140        150
160
S. kowalevskii Unc-5      CVAAPAMHIY FKCNGDWVRG KHLVQQETLD EAG-NAQKEA
D. rerio Unc5c            CKATAT---- YKCNS-WVH- KHTV---RVD TSG--VVRAS
M. musculus Unc5C         CKASPATQIY FKCNSEWVHQ KDHVVDERVD ETSGLIVREV
H. sapiens UNC5C          CKASPATQIY FKCNSEWVHQ KDHIVDERVD ETSGLIVREV
C. elegans Unc5           CRANHATKIR YKCSSKWIDD SRIEKLIGTD STSGVGYIDA
S. purpuratus Unc5C       CKAGPSPNVY FICNDEKIAD GRTHSIGTFD EYY-EDIRHI


                          ....|....| ....|....| ....|....| ....|....|
                                  170        180        190
200
S. kowalevskii Unc-5      SIVLTRNEIE SFFGDEDYWC QCVAWGEGGS --VKSRKATI
D. rerio Unc5c            ----TR---- --VGD---WC -CVAWSSAGT --TKSRKAHV
M. musculus Unc5C         SIEISRQQVE ELFGPEDYWC QCVAWSSAGT --TKSRKAYV
H. sapiens UNC5C          SIEISRQQVE ELFGPEDYWC QCVAWSSAGT --TKSRKAYV
C. elegans Unc5           SVDISRIDVD TSGHVDAFQC QCYASGDDDQ DVVASDVATV
S. purpuratus Unc5C       ALSITKDEVQ EYFGDEDFWC VCEAAAVPQP --IRTEKAYI


                          ....|....| ....|....| ....|....| ....|....|
                                  210        220        230
240
S. kowalevskii Unc-5      VISFLKKAFE REPLGANIEI ESAFQMMCRP PEGNPLPDIY
D. rerio Unc5c            R-AY-RKT-- ----DGKVSV -------CR- --G---AAV-
M. musculus Unc5C         RIAYLRKTFE QEPLGKEVSL EQEVLLQCRP PEGIPVAEVE
H. sapiens UNC5C          RIAYLRKTFE QEPLGKEVSL EQEVLLQCRP PEGIPVAEVE
C. elegans Unc5           HLAYMRKHFL KSPVAQRVQE GTTLQLPCQA PESDPKAELT
S. purpuratus Unc5C       REAYLRKQFL QMPLDHSVPL HDKFHLLCRA PEGVPEPTIH


                          ....|....| ....|....| ....|....| ....|....|
                                  250        260        270
280
S. kowalevskii Unc-5      WEKDGRQIDV QEDRNYIITT DASLIINQAR LADTGNYTCV
D. rerio Unc5c            WKND------ ADDRNY---T DH----NKAR -SDTANYTCV
M. musculus Unc5C         WLKNEDIIDP AEDRNFYITI DHNLIIKQAR LSDTANYTCV
H. sapiens UNC5C          WLKNEDIIDP VEDRNFYIII DHNLIIKQAR LSDTANYTCV
C. elegans Unc5           WYKDG--VVV QPDANVIRAS DGSLIMSAAR LSDSGNYTCE
S. purpuratus Unc5C       WEMDGVPIDD ENLVHYVVTY DGTLIVNEAT LADNGNYTCV


                          ....|....| ....|....| ....|....| ....|....|
```

```
                                290        300        310
320
S. kowalevskii Unc-5        AQNMASKRKS DVAKVNVYVN ---GAWSTWS AWT-GCDSRC
D. rerio Unc5c              AKNVA-KRRS TTATV-VYVN ---GGWSTWT -WS-VCSSRC
M. musculus Unc5C           AKNIVAKRKS TTATVIVYVN ---GGWSTWT EWS-VCNSRC
H. sapiens UNC5C            AKNIVAKRKS TTATVIVYVN ---GGWSTWT EWS-VCNSRC
C. elegans Unc5             ATNVANSRKT DPVEVQIYVD ---GGWSEWS PWIGTCHVDC
S. purpuratus Unc5C         ATNVATYRTT DPARVIVYDN TNDGAWTIWT EWS-TCTGDC


                            ....|....| ....|....| ....|....| ....|....|
                                330        340        350
360
S. kowalevskii Unc-5        ------GKGI ---------Q RRTRTCTNPA PLNGGAPCPG
D. rerio Unc5c              ------GRG- ---------- KRTRSCTN-- -ANGGA-C-G
M. musculus Unc5C           ------GRGY ---------Q KRTRTCTNPA PLNGGAFCEG
H. sapiens UNC5C            ------GRGY ---------Q KRTRTCTNPA PLNGGAFCEG
C. elegans Unc5             PLLRQHAHRI RDPHDVLPHQ RRTRTCNNPA PLNDGEYCKG
S. purpuratus Unc5C         ------DGGT ---------R RRMRYCTNPA PLIDGANCVG


                            ....|....| ....|....| ....|....| ....|....|
                                370        380        390
400
S. kowalevskii Unc-5        SSSQTSPCTT LCPVDGS-WT SWNEWSNCSP ECNHIRSRTC
D. rerio Unc5c              ----AKACN- -C-VDG--WT -WSKWSTCG- TCTHWR-RRC
M. musculus Unc5C           QSVQKIACTT LCPVDGR-WT SWSKWSTCGT ECTHWRRREC
H. sapiens UNC5C            QSVQKIACTT LCPVDGR-WT PWSKWSTCGT ECTHWRRREC
C. elegans Unc5             EEEMTRSCKV PCKLDGG-WS SWSDWSACSS SCHRYRTRAC
S. purpuratus Unc5C         KAVQTEDCSI DCPAAAIGWS VWSMWSRCTD ECVQIRTRTC


                            ....|....| ....|....| ....|....| ....|....|
                                410        420        430
440
S. kowalevskii Unc-5        SNPRPQNSGK LCKGLDMESR NCTDGLCK-- --LV----GI
D. rerio Unc5c              S--AAKNGGK DC-G--TVSK NCTDG-CMS- ----------
M. musculus Unc5C           TAPAPKNGGK DCDGLVLQSK NCTDGLCMQ- --------GF
H. sapiens UNC5C            TAPAPKNGGK DCDGLVLQSK NCTDGLCMQ- --------SF
C. elegans Unc5             TVPPPMNGGQ PCFGDDLMTQ ECPAQLCT-- ----------
S. purpuratus Unc5C         T--------R QCSGEPQQRR NCSGGLCLSE PPLVVDDPGI


                            ....|....| ....|....| ....|....| ....|....|
                                450        460        470
480
S. kowalevskii Unc-5        EGAEHQESDL TEPKV--KKG SGDGIALYVG LIVACVVFML
D. rerio Unc5c              -SYKSSHTDK SNAHS----A STDDVA-YVG VAVMCVSVVA
M. musculus Unc5C           IYPISTEHRP QNEYGFSSAP DSDDVALYVG IVIAVTVCLA
H. sapiens UNC5C            IYPISTEQRT QNEYGFSSAP DSDDVALYVG IVIAVIVCLA
C. elegans Unc5             --ADSSRIVI SDTAVY---G SVASIFIVAS FILAILAMFC
S. purpuratus Unc5C         FSPSAGDTTS QNPAAGGKNG LSKQIPVYIG ISLAIVVLLL


                            ....|....| ....|....| ....|....| ....|....|
                                490        500        510
520
```

```
S. kowalevskii Unc-5        VCVFI-VYLI HRKVR-QAGV YIDINASDMT LPGACPNQIK
D. rerio Unc5c              V--------Y RKNHR----- ----DDSDM- ----------
M. musculus Unc5C           ITVVVALFVY RKNHR----- --DFES-DI- ----------
H. sapiens UNC5C            ISVVVALFVY RKNHR----- --DFES-DI- ----------
C. elegans Unc5             CKR-----GN SKKSK----- --PLKPQKM- ----------
S. purpuratus Unc5C         VFLFIAIYLV TKRKRGNSPS YTTTSTEDCN LAMLSAQPPD

                            ....|....| ....|....| ....|....| ....|....|
                                    530        540        550
560
S. kowalevskii Unc-5        GHKIGQDNAH MAMLSIQPDV TQSTCQLRNQ NLMMRNGDMD
D. rerio Unc5c              -----DSSAN GG---VN--- ---------- ---------K
M. musculus Unc5C           ---IDSSALN GGFQPVN--- ---------- --------IK
H. sapiens UNC5C            ---IDSSALN GGFQPVN--- ---------- --------IK
C. elegans Unc5             ----NSEKAG GIYYSEP--- ---------- ---------P
S. purpuratus Unc5C         ---ITTQTMH SSLRSNH--V ALSS---HNE KIPMSGTPTH

                            ....|....| ....|....| ....|....| ....|....|
                                    570        580        590
600
S. kowalevskii Unc-5        TTKLVMISPP VPPIYNIPNN KSKMSDSMTL SATTPSENKY
D. rerio Unc5c              TAR------- TADTADTSAA AYRG------ ---------V
M. musculus Unc5C           AARQDLLA-- VPPDLTSAAA MYRG------ --------PV
H. sapiens UNC5C            AARQDLLA-- VPPDLTSAAA MYRG------ --------PV
C. elegans Unc5             GVRRLLLEHQ HGTLLGEKIS SCSQ------ ---------Y
S. purpuratus Unc5C         PPHPDLTGGK IPHQLTVQFS PKKGGTITQL GCLGSSDISQ

                            ....|....| ....|....| ....|....| ....|....|
                                    610        620        630
640
S. kowalevskii Unc-5        ETIQPLSCPE CSVTPSQISP RSSFVSSCYS PVHSPDKELN
D. rerio Unc5c              YA-HDVA--- DKMTNS---- ---------- -----NKK--
M. musculus Unc5C           YALHDVS-DK IPMTNS---P I-------LD PL--PNLKIK
H. sapiens UNC5C            YALHDVS-DK IPMTNS---P I-------LD PL--PNLKIK
C. elegans Unc5             FEPPPLP-HS TTLRSG-KSA FSG-----YS STRNAGSRAA
S. purpuratus Unc5C         YSLNNGSLHS HPHSRS-LSP TKSHT---YV PLVGPGQEKH

                            ....|....| ....|....| ....|....| ....|....|
                                    650        660        670
680
S. kowalevskii Unc-5        VLP-LDDEAL KEKLDIIRET DLIGSSTDQS IKMAVETDDC
D. rerio Unc5c              VYN--SSGVT ---------- ----DDSDAS KRADGDTGRR
M. musculus Unc5C           VYN--SSGAV TPQD------ ----DLAEFS SKLSPQMTQS
H. sapiens UNC5C            VYN--TSGAV TPQD------ ----DLSEFT SKLSPQMTQS
C. elegans Unc5             LIQECSSSSS ---------- ----GSGGKR TMLRTSSSNC
S. purpuratus Unc5C         IYATLNPPEP PPQDPRYCQT RCYGDLEDND GYIMEEADEN

                            ....|....| ....|....| ....|....| ....|....|
                                    690        700        710
720
S. kowalevskii Unc-5        NSEKSFGTLK SLSDSSSRPM SMYDSDNLGN SMCSIASTTL
D. rerio Unc5c              ---------- ---------- -THTTT---- ----------
```

```
M. musculus Unc5C          LLEN-----E ALN------- -LKNQS---- ---------L
H. sapiens UNC5C           LLEN-----E ALS------- -LKNQS---- ---------L
C. elegans Unc5            SDDDNY---A TLY------D YMEDKS---- --------VL
S. purpuratus Unc5C        YMLQGHSSEE SLE-----PQ SMCDSD-PGR TLSGSALG-L

                           ....|....| ....|....| ....|....| ....|....|
                                   730        740        750
760
S. kowalevskii Unc-5       PTHIDPRCIA WGMMGHRGGR LTVPDTGVSV LIAEGALAKG
D. rerio Unc5c             MRCWD-SCTA -G--SNAGGH V---NSGVSV ---AGA---G
M. musculus Unc5C          ARQTDPSCTA FGTFNSLGGH LIIPNSGVSL LIPAGAIPQG
H. sapiens UNC5C           ARQTDPSCTA FGSFNSLGGH LIVPNSGVSL LIPAGAIPQG
C. elegans Unc5            GLDTS-QNIV AAQIDSNGAR LSLSKSGARL IVPELAVE-G
S. purpuratus Unc5C        PMHSD-YSIA TGHVGSRGGR LVLPDSGVSL MIPEGAIARG

                           ....|....| ....|....| ....|....| ....|....|
                                   770        780        790
800
S. kowalevskii Unc-5       QTEEIYIAVC REEKDRPKVK AGKETILSPV IMCGPTHL--
D. rerio Unc5c             RVY-MYVTVH RK-SMR--VD D---ATVS-V VSCGGS----
M. musculus Unc5C          RVYEMYVTVH RKENMRPPME D-SQTLLTPV VSCGPPGA--
H. sapiens UNC5C           RVYEMYVTVH RKETMR---- ---------- ----------
C. elegans Unc5            EKM-LYLAVS DTLTDQPHLK P-IESALSPV IVIGQCDVSM
S. purpuratus Unc5C        QTVEIYLAVS HELMDRPHIE H-NQTLLSPV ILCGPPSV--

                           ....|....| ....|....| ....|....| ....|....|
                                   810        820        830
840
S. kowalevskii Unc-5       -----NFKKP VVVQFPHCAH -VEGCNWVSS IYTCDSHPDE
D. rerio Unc5c             -------TR- --VTMHHCA- --ADSDWKSS ----------
M. musculus Unc5C          -----LLTRP VILTLHHCAD -PSTEDWKIQ LKNQAVQG--
H. sapiens UNC5C           ---------- ---------- ---------- ----------
C. elegans Unc5            SAHDNILRRP VVVSFRHCAS TFPRDNWQFT LYADEGS---
S. purpuratus Unc5C        -----VLAKS VVLVLPHCAQ -MSKNDWRHS VIASSTHPTD

                           ....|....| ....|....| ....|....| ....|....|
                                   850        860        870
880
S. kowalevskii Unc-5       PPNWEKVVTI GEETINTPLY CQLDESE--- -----CFLMV
D. rerio Unc5c             --GWD-VVVV G----NTTCY ----DAA--- -----CHTT-
M. musculus Unc5C          --QWEDVVVV GEENFTTPCY IQLDAEA--- -----CHILT
H. sapiens UNC5C           ---------- ---------- ---------- ----------
C. elegans Unc5            --GWQKAVTI GEENLNTNMF VQFEQPGKKN DGFGWCHVMT
S. purpuratus Unc5C        DKCWEKQTTV GEETINLQVF CQVDSHL--- -----SCIVT

                           ....|....| ....|....| ....|....| ....|....|
                                   890        900        910
920
S. kowalevskii Unc-5       DRLSRFALVG ESR-PGHEAC KILKLAAFAQ PLRSLA--DY
D. rerio Unc5c             ---GTYC--- ----TGSVSA ATTKRKA-G- --VSCT--VY
M. musculus Unc5C          ENLSTYALVG QS--TTKAAA KRLKLAIFGP LCCSSL--EY
H. sapiens UNC5C           ---------- ---------- ---------- ----------
```

```
C. elegans Unc5             YSLARLMLAG HPRRNSLSAA KRVHLAVFGP TEMSAYRRPF
S. purpuratus Unc5C         DQLGWYALSG ESR-PERQSA KRLKLLAFGP ALRSTL--DY


                            ....|....| ....|....| ....|....| ....|....|
                                    930        940        950
960
S. kowalevskii Unc-5        NIRVYVVDDT LEALEGVVQV EQRLGGHLLD KPKQLEFVDS
D. rerio Unc5c              N-RVYC-DDT -DA--KVM-- --KMGGK--D KSN----KDS
M. musculus Unc5C           SIRVYCLDDT QDALKEVLQL ERQMGGQLLE EPKALHFKGS
H. sapiens UNC5C            ---------- ---------- ---------- ----------
C. elegans Unc5             ELRVYCVPET GAAMESVWK- -QEDGSRLLC ESNDFILNEK
S. purpuratus Unc5C         HIKVYISEDT PDAIEHILNV ERRLGGQPLQ GGCQFDYHDN


                            ....|....| ....|....| ....|....| ....|....|
                                    970        980        990
1000
S. kowalevskii Unc-5        GASLCLTIDN IMTG-WRSKL SANYQEIPYY HIWSGNMNAL
D. rerio Unc5c              THNRSHDVHT -----WKSKA K--------Y SVWSGSRH--
M. musculus Unc5C           IHNLRLSIHD IAHSLWKSKL LAKYQEIPFY HIWSGSQRNL
H. sapiens UNC5C            ---------- ---------- ---------- ----------
C. elegans Unc5             G-NLCICIED VIPG-FSCDG PEVVEISETQ HRFV-AQNGL
S. purpuratus Unc5C         GNNLRLEIQN IVPG-WQCKL AKNCQEIPFY HVWSGTSNTL


                            ....|....| ....|....| ....|....| ....|....|
                                    1010       1020       1030
1040
S. kowalevskii Unc-5        HCAFTLERLD RQIQKIHCRI TVYQVSGGKE KQCLQILTNV
D. rerio Unc5c              HCCTRCSSTS -------CKC V--RVGG--- -------NTT
M. musculus Unc5C           HCTFTLERLS LNTVELVCKL CVRQVEG--E GQIFQLNCTV
H. sapiens UNC5C            ---------- ---------- ---------- ----------
C. elegans Unc5             HCSLKFRPKE INGSQFSTRV IVYQKAS--- STEPMVMEVS
S. purpuratus Unc5C         HCSFALVKTE PGIGPIGCRL ---EVTQGTY TTSMQVMERI

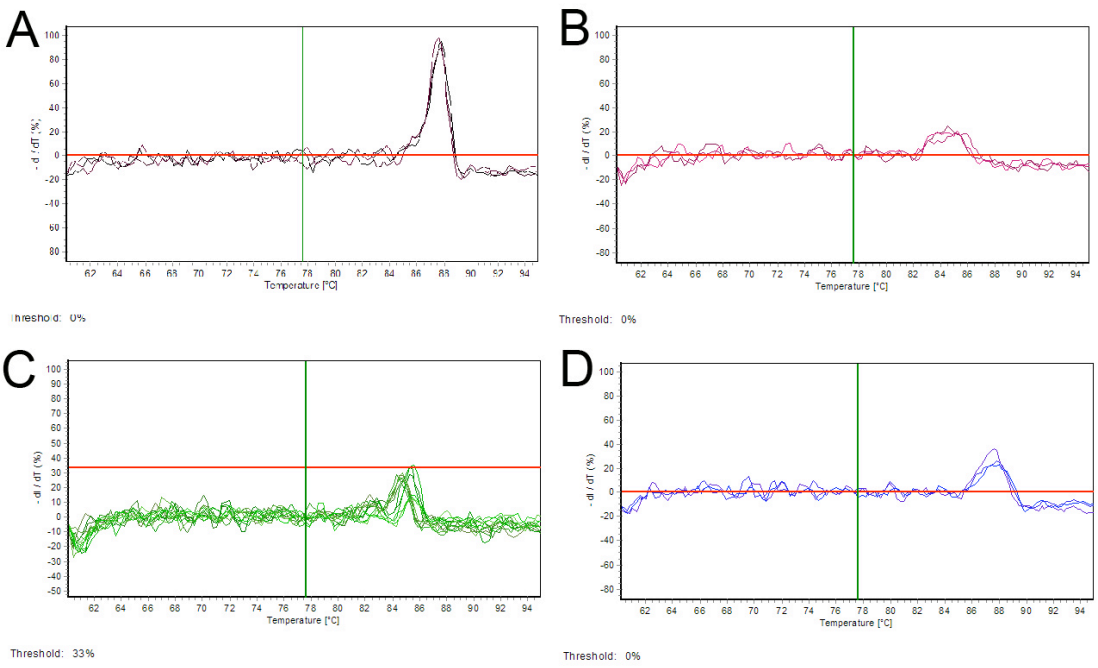

                            ....|....| ....|....| ....|....| ....|....|
                                    1050       1060       1070
1080
S. kowalevskii Unc-5        NETKAQFPPE MLYGLGRSRS STVTTNSTSG CSSMTVDPPS
D. rerio Unc5c              TD-------- ---------- ---------- -SCDTSDASN
M. musculus Unc5C           SE-------- --------EP TGIDLPLLDP ASTITTVTGP
H. sapiens UNC5C            ---------- ---------- ---------- ----------
C. elegans Unc5             NE-------- ---------P ELYDAT---- SEEREKGSVC
S. purpuratus Unc5C         ID-------- -----GVVVE QELRLN---G YSPNHMVPPQ


                            ....|....| ....|....| ....|....| ....|....|
                                    1090       1100       1110
1120
S. kowalevskii Unc-5        TVFRIPRPTR NKLCICLDRP MKKGNDWRLL ARKLKVDRYI
D. rerio Unc5c              TTVGN---AR SRKCGS-DA- -TRGNDWR-M AHKN--DRYN
M. musculus Unc5C           SAFSIPLPIR QKLCSSLDAP QTRGHDWRML AHKLNLDRYL
H. sapiens UNC5C            ---------- ---------- ---------- ----------
C. elegans Unc5             VEFRLPFGVK DELARLLDMP NESHSDWRGL AKKLHYDRYL
S. purpuratus Unc5C         NAFHLSKPVR TALCQCLDIP QPKGNDCKLL ATKLGVDGYL
```

```
                                ....|....| ....|....| ....|....| ....|....|
                                        1130       1140       1150
1160
S. kowalevskii Unc-5            NFFATKP--S PTEHILDLWE AR-CRGERAL SDLLRIFKEM
D. rerio Unc5c                  --YATKS--S -TG--VD-WA HD---GNN-- --RAAVMGRH
M. musculus Unc5C               NYFATKS--S PTGVILDLWE AQNFPDGN-L SMLAAVLEEM
H. sapiens UNC5C                ---------- ---------- ---------- ----------
C. elegans Unc5                 QFFASFPDCS PTSLLLDLWE ASSSGSARAV PDLLQTLRVM
S. purpuratus Unc5C             NFFGIKP--S PTDQILDMWE ARDQRGGRPH EVSRSIPVDG


                                ....|....| ....|....| ..
                                        1170       1180
S. kowalevskii Unc-5            GRNDVVDLIE KDVGSWI--- --
D. rerio Unc5c                  GAH------- ---------- --
M. musculus Unc5C               GRHETVVSLA AEGQYRSSDF S-
H. sapiens UNC5C                ---------- ---------- --
C. elegans Unc5                 GRPDAVMVLE RFLSAFPQIV SP
S. purpuratus Unc5C             PARRRHDSPE TDGCMDV--- --
```

Appendix IV – qPCR data



Dissociation curves of qPCR amplicons for Sp-ubiquitin (A), Sp-neogenin (B) and Sp-unc5 (C) and Sp-netrin (D). All graphs show the absence of secondary qPCR products.

qPCR Amplicons

Netrin qPCR amplicon:
TTCACCCGAAGCCAGCACAACGTCACACTGAAGCTATCCCTGACAAAGCGCT
TCGAGATCACCTACATCTCCATGGAGTTTTGCTGGCTTCACCCAGACTCCATG
GTCATCTTCAAGTCGCAGGACTTCGGCAAGACATGGCA

Un5 qPCR amplicon:
CCAAGTCCGACCGATCAGATCCTTGACATGTGGGAAGCGCGGGATCAGCGAG
GAGGGCGCCCTCATGAGGTTAGCAGGAGCATTCCAGTAGATGGGCCGGCACG
ACGCCGTCACGATAGTCCAGAAACAGATGGCTGCATGG

Neogenin qPCR amplicon:
ACCCTCGGCAGCAATCATTCTGAGTTTCCTGAGACCAGGATGCGTGAGAACT
CTGACTCTCAACAGTCTGCCGTGGGTTATCACGACGGTCAGATTGTGACCTTC
CCCGATGGTCAAATTGTGGCCAGTAGTGGACTTGCACCAAACCCATTGACCG

Ubiquitin qPCR amplicon:

CACAGGCAAGACCATCACACTCGAGGTCGAGCCAAGTGACTCCATCGAGAAC
GTAAAGGCCAAGATCCAGGACAAAGAAGGCATCCCTCCCGATCAGCAGCGT
CTTATCTTTGCTGGCAAGCAACTTGAGGATGGTCGCACTCTCTC