IDENTIFICATION AND FUNCTIONAL ANALYSIS OF ZEBRAFISH ORTHOLOGS OF *ROUNDABOUT* GENES

DISSERATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the
Graduate School of The Ohio State University

By

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* * * * *

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ABSTRACT

Cell motility and migration are integral to the complex mechanics of animal development, growth and maintenance. The precise and stereotypical orchestration of cell motility is controlled by a number of molecules, both within cells and in the extracellular environment. The phenomenon of axonal growth cone motility during neural circuit formation is a special case of cell movement, and has been studied extensively over the past several years. A number of receptor-ligand interactions have been described that regulate this process and one example involves the Roundabout (Robo) receptor and its extracellular, secreted ligand Slit. Both of these molecules are evolutionarily conserved from worms to humans and have been shown to play important roles in axon guidance. To gain a better understanding of Robo orthologs in vertebrates, we chose zebrafish as our model system for the many experimental advantages it offers. A PCR-based strategy was employed to find zebrafish orthologs of Robo. Two orthologs (robo1 and robo3) were identified whose expression patterns in the nervous system and non-neural tissues suggested a role in axon guidance and cell motility. Further characterization of robo3 unveiled the presence of two distinct isoforms (robo3a and robo3b). The two isoforms exhibited spatially and temporally dynamic gene expression patterns. Functional analysis of robo3 isoforms using an antisense gene ‘knock-down’ strategy suggested that robo3b functions during gastrulation, whereas robo3a is required for motor axon pathfinding. This study uncovers a novel function of the Robo receptor family in cell movements during gastrulation, complementing an earlier study that suggests a role of zebrafish Slit orthologs in gastrulation. The implication of Robo-Slit signaling in
cell movements during gastrulation underscores the conservation of molecular mechanisms in a
variety of cell motility phenomena.
to the countless embryonic danios

whose lives were sacrificed in pursuit of knowledge
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<td>horizontal myoseptum</td>
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<td>heat shock promoter</td>
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<td>Wiskott-Aldrich Syndrome Protein</td>
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CHAPTER 1

INTRODUCTION

Metazoan life forms are typified by the presence of a multitude of cell types and cell differentiation gives rise to these diverse cell identities. But the mere presence of multiple cell types cannot ensure the development of an organism. Morphogenesis, by way of changes in cell shape and position, facilitates the establishment of a primitive body plan in the nascent embryo upon which tissue organization and organogenesis are dependent. In other words, both cell differentiation and morphogenesis have very tightly integrated and complementary roles in the shaping of an embryo. While cell differentiation involves subcellular molecular events, morphogenesis involves a combination of cellular and subcellular molecular events manifesting in the transformation of cell shape and movement of cells in the embryo (Trinkaus, 1984).

A variety of cell movements can be seen in the life histories of many multicellular life forms. Cell movements during embryonic development are a universal feature amongst metazoans; regeneration of lost body parts, wound healing and tissue repair are accomplished due to an important role played by cell movements; cells in the immune system move towards the site of inflammation in order to defend the organism from foreign bodies. Cell motility during two embryonic events will be the focus of my discussion, namely, movements seen in relatively unspecialized cells during vertebrate gastrulation and motility seen at the tip of a growing neurite, the cellular extension of the differentiated neuron. The underlying theme would be to illustrate the
common mechanisms governing all motility events, despite their overt morphological and functional differences during development.

Descriptive studies using histological sections of embryos were mostly done to elucidate the organization and formation of gastrulae during the 19th century. It was Friedrich Kopsch (Berlin) who introduced a new approach to understanding gastrulation; he used serial timelapse photographs to show the first evidence for morphogenetic cell movements during gastrulation in frogs and axolotls (see a historical review by Beetschen, 2001). Subsequent studies to further the knowledge of gastrulation were also done in amphibian embryos. Despite the visible distinctions in all vertebrate embryos at blastula stages (prior to gastrulation), the principal movements involved in gastrulation are conserved between amphibians and other vertebrates. The three precisely orchestrated gastrulation movements are (a) epiboly, (b), internalization of mesendoderm, and (c) convergence and extension (C&E) (Figure 1.1). Epiboly movements begin during the blastula stage. Intercalation of deep cells and superficial cells causes spreading and thinning of the tissue and result in the mixing of blastoderm cells. In the teleost (bony fish), zebrafish, the end of epiboly occurs when the blastoderm covers the yolk completely (Kimmel et al., 1995; Myers 2001). The three germ layers are formed when mesendodermal cells move beneath the overlying ectoderm by way of cell internalization. Subsequently convergence, which is the mediolateral narrowing of the germ layers, and extension, which are the movements elongating the germ layers anteroposteriorly, define the dorsoventral and rostrocaudal axes of the developing embryo, respectively.

C&E can occur due to a variety of cell behaviors. Analyzing cell movement in frog and teleost fish embryos has given us important insights into the common themes and differences in the mechanics of C&E (Keller 2000; Myers et al., 2002; Trinkaus, 1992; Trinkaus 1998). In Xenopus embryos, C&E are coupled in mesodermal cell migrations and they are interdependent. Mesodermal cells, which are mediolaterally elongated, gain bipolar protrusive activity and drive
Figure 1.1: Cell movements during gastrulation.

Cartoon showing the three types of cell movements in a teleost gastrula. (A) Epiboly ensures the even spreading of cells from animal to vegetal pole over the yolk. (B) Involution or internalization of the mesendoderm leads to the germ layer formation. (C,D) Concerted movement of cells converging towards the dorsal midline and extending along the rostro-caudal aspect of the gastrula results in the laying of the primary body axes. (adapted from Solnica-Krezel and Cooper, 2002).
convergent-extension by mediolateral cell intercalations - simultaneous narrowing and elongation of sheets of cells. Ectodermal cells gain monopolar protrusive activity and converge by dorsally directed intercalations whereas cells in the dorsal midline show random protrusive behavior. Monopolar, medially protrusive activity in the neural ectodermal cells is possibly induced by midline signals (Keller, 2002; Elul and Keller, 2000). In teleost embryos, C&E movements have similarities as well as differences with those in the frog. C&E in the mesoderm of teleost fish occur due to movement of individual cells or small groups and not as sheets (Myers et al., 2002; Concha and Adams, 1998; Trinkaus, 1992). Teleosts also differ from frogs in another aspect. While convergence and extension in frog embryos are interdependent and simultaneous (consequently the use of the term convergent-extension, CE), they seem to be separable in teleost embryos. Analysis of cell behavior in *Fundulus*, another teleost fish, by labelling individual gastrulating cells and zebrafish mutants provided the evidence for uncoupling of convergence and extension movements in fish embryos (Trinkaus, 1992; Glickman et al., 2003). In gastrulating teleost embryos, three domains of cell migration have been described. Mesodermal cells in the ventral region of the gastrula do not converge dorsally but spread over the yolk, while individual cells in the lateral region converge dorsally via directed migration with increasing speeds. In the dorsal domain, cells extend without much convergence (Trinkaus, 1992; Trinkaus, 1998, Myers et al., 2002; Sepich et al., 2000). Observations from these studies suggest that, in teleost gastrulae, a gradient of signal(s) emanating from the dorsal aspect of the embryo is important to cause directed migration of individual cells thus effecting convergence. Complementing these suggestions, dynamic morphologies with filopodial extensions are seen in neurectodermal and mesendodermal cells during C&E (Heisenberg and Tada, 2002). In essence, active cell motility and directed migration occur in cell populations and individual cells during C&E.
Once the embryonic germ layers are defined and the basic body plan established, differentiation of neurons and the subsequent growth of neurites (axons and dendrites) occurs. Extension of axons and dendrites from the soma of neurons is an obvious, yet special case of cell motility. It is not cell motility in the strict sense because the cell body remains stationary. Nevertheless, since the essential feature of all cell movements is the extension of the leading edge, neurite outgrowth can be considered as an interesting example. The phenomenon of axonal outgrowth and axon guidance to precise targets has historical importance. Ramón y Santiago Cajal predicted and described axonal outgrowth to precise targets from his meticulous observations of histological sections. Ross Harrison provided the first, elegant experimental evidence for the ability of neurons to extend axons by themselves. He employed the then infantile technique of tissue culture (Trinkaus, 1984). Cajal predicted that long-range chemoattractants selectively guide axons to their appropriate targets. With the application of more analytical experimental approaches in the 1930s and 40s, the chemical selectivity concepts for axon outgrowth were supplanted by a predominantly mechanical interpretation. Subsequent work by Roger Sperry and his colleagues led to the resurgence of explanations based on chemoaffinity mechanisms in the orderly growth of nerve fiber patterns and connections (Sperry, 1963; Meyer, 1998). Sperry’s chemoaffinity hypothesis has clearly laid the foundations for our current investigations and understanding of the molecular mechanisms underlying axon guidance.

Both invertebrate and vertebrate models have contributed significantly to the understanding of the cellular behaviors underlying axon guidance. In the *Drosophila* CNS as well as vertebrate central nervous systems, the midline structures play an important role in guiding axon growth. In the ventral nerve cord of *Drosophila*, a subset of neurons extends ipsilateral axons while the majority of neurons extend axons contralaterally, across the midline to make final contacts. While the contralaterally projecting axons are initially attracted towards the midline,
they are repelled by the same midline after crossing. Studies on motor and commissural interneuron axon guidance in the PNS of invertebrates, as well as vertebrates have revealed the role of intermediate targets in shaping the final trajectory of axon tracts towards their final targets (Goodman and Tessier-Lavigne, 1996). Growing axons show stereotypical behaviors of stalling, turning and branching at different intermediate targets suggesting that important decisions are being made by the leading edge of the growing axon, which is termed the growth cone. It is pertinent to mention that growth cone tips, as well as gastrulating cells are made of filopodia, which are the principal structures sensing the microenvironment (Heisenberg and Tada, 2002).

Whether it is a migrating cell in a gastrulating embryo, motility of the growth cone or changes in cell shape, the fundamental process controlling them is rearrangement of the actin cytoskeleton. Formation of membrane protrusions, which is the first step in cell locomotion or cell motility, occurs due to formation of polarized groups of actin filaments. The actin cytoskeleton is relatively inert and its remodelling, which manifests as polymerization and depolymerization of actin filaments, are due to activation of signaling cascades at the plasma membrane. These signaling cascades are initiated at the plasma membrane upon the receipt of extracellular stimuli (Carlier et al., 2003). Large protein scaffolds assemble in a dynamic way and connect the extracellular signals and the growing actin filaments. Cellular factors such as coflin (or ADF - actin depolymerizing factor) and capping proteins facilitate active treadmilling of actin filaments. Profilin is an actin monomer binding protein that regulates actin polymerization in response to extracellular cues. In contrast, the Arp2/3 complex is a key factor involved in the generation of new filaments. Members of the WASP (Wiskott-Aldrich Syndrome Protein) family are cellular activators of the Arp2/3 complex. The WASP family proteins mediate a variety of signaling pathways involving receptor tyrosine kinases and G-proteins to the Arp2/3 complex and thus the actin cytoskeleton (Pantaloni et al., 2001; Figure 1.2).
Factors such as Pak and Rock are regulated by GTPases which in turn affect the activity of Actin Depolymerizing Factor (ADF). In contrast to ADF regulated depolymerization, Profilin influences polymerization at the leading edges via Ena/VASP proteins. Arp2/3 complex on the other hand nucleates branch points for actin polymerization. Arp2/3 complex is activated by the N-WASP proteins. (adapted from Meyers and Feldman, 2002.)
Extracellular cues bind to membrane receptors causing the downstream activation of small G-proteins belonging to the Rho family, which in turn, control the assembly of actin filaments. Primarily there are three subfamilies in the Rho family typified by Rho, Rac and Cdc42. Although the members of these subfamilies have been implicated in signaling mechanisms linked to cell morphology, cell division, cell survival and nuclear gene regulation, the analysis of these proteins has persistently showed their direct role in actin organization and dynamics (Nobes and Hall, 1995; Tapon and Hall, 1997; Giniger, 2002). Rho, Rac and Cdc42 have been demonstrated to affect three different actin-based cytoskeletal structures. Activation of Rho leads to the formation of stress fibers in cells which become contracted while Rac mediates actin polymerization leading to the formation of lamellipodia and membrane ruffles. The third class, Cdc42, causes the formation of filopodia (microspikes) at the edges of lamellipodia. Extracellular signals and membrane receptors are linked to the activation states of rho GTPases by regulator proteins, which are either GTPase Activating Proteins (GAPs) or Guanine-nucleotide Exchange Factors (GEFs) (Meyer and Feldman, 2002). Several downstream effectors of Rho GTPases like Pak, Rho kinase and actin associated proteins belonging to the Ena/VASP family also play a role in actin filament elongation and in turn cause cell motility.

Over the past decade several extracellular molecules and their receptors have been identified which initiate signal transduction cascades culminating in the remodelling of actin cytoskeleton. Genetic and biochemical analysis of axon guidance have contributed immensely to the discovery of these molecules. Broadly, the mechanisms causing axon outgrowth can either be attractive or repulsive. Attractive cues in the environment cause the axons to grow towards their source while repulsive cues either inhibit or repel axon outgrowth. Further, both attractive and repulsive mechanisms can act either in short range (contact-mediated) or in long range (chemotaxis via gradients of secreted signals). Both attractive and repulsive environmental signals (ligands) are
‘sensed’ and interpreted by the extending tips of axons by help of cell surface proteins (receptors). In addition to receptor-ligand pairs there are cell adhesion and extracellular matrix molecules that also modulate axon outgrowth and guidance. This discussion will focus mainly on receptor-ligand interactions. Several such ligands and receptors that play pivotal roles in axon guidance have been identified in the past decade by employing biochemical and genetic tools. Not surprisingly, many of these molecules are evolutionarily conserved across species. Amongst the many molecules identified, netrins, semaphorins, ephrins and slits are the best-studied ligands. Netrins were the first diffusible molecules to be identified for their role in chemoattraction of commissural axons in the vertebrate central nervous system. Netrin-1 is expressed by the ventral midline structure, the floor plate, and creates a chemotropic gradient, which attracts the commissural axons in the embryonic spinal cord (Kennedy et al., 1994; Serafini et al., 1994). Netrins act through the UNC40/DCC receptors to mediate the attractive response in growing axons. Interestingly, Netrins can also elicit a repulsive guidance response. Axon repulsion is mediated by the either UNC5 alone or in combination in with the UNC40/DCC receptors. There are other reported cases where the internal physiological state of the cell dictates whether attraction or repulsion would ensue. Ca^{2+} levels in the cell can change the responsiveness of a growth cone from being attracted to getting repulsed to a gradient of extracellular netrin (Caroni, 1998; Hong et al., 2000; Ming et al., 1997).

Semaphorins belong to another class of ligands and have a role in axon growth cone collapse, repulsion, or restricting the trajectory of axons. These molecules act through the Neuropilin/Plexin receptor complex and can either be secreted or membrane tethered. In contrast to netrins and semaphorins, ephrins are exclusively membrane bound ligands and act via the Eph family of receptors (Himanen and Nikolov, 2003). A unique characteristic feature of the Eph-ephrin signaling is that it is bi-directional and affects the cells expressing the ligand and the receptor. Eph-ephrin signaling has been shown to play an important role in guiding axons in the retinotectal
system of vertebrates. Most of these molecules are evolutionarily conserved and have been shown
to play significant roles not only in axon guidance but also in motility behaviors of other cell types.

Our lab is interested in the Roundabout (Robo) proteins, which form yet another family
of receptor molecules. The roundabout gene was first identified in a genetic screen performed in
Drosophila to find mutations involved in controlling the midline crossing of axons in the ventral
nerve cord (Seeger et al., 1993). The ventral nerve cord in the developing embryos of Drosophila
have a stereotypical ladder-like structure with longitudinal axon bundles on either side of the
midline connected by two commissures in each hemisegment. Some neurons send ipsilateral
projections, which stay on the same side of the midline, while most axons make contralateral
projections across the midline and never recross to the side they began their journey. But in robo
mutant embryos axons that usually made ipsilateral trajectories frequently crossed the midline
while commissural axons recrossed the midline. Based on this phenotype it was predicted that
Robo protein acts as a ‘gatekeeper’ molecule controlling the trajectories of axons across the
midline. Subsequent studies revealed that Robo is a receptor protein expressed by neurons and
localized on growth cone surfaces (Kidd et al., 1998). Robo is a single-pass transmembrane protien
and belongs to the Immunoglobulin superfamily. The core protein has 5 Ig repeats and 3
fibronectin type III (FNIII) repeats in the extracellular domain, a transmembrane domain and a
cytoplasmic domain with several conserved motifs including some proline rich repeats. Robo
molecules form an evolutionarily conserved family of receptor proteins, members of which are
found in the C.elegans, Fugu, Xenopus, mouse, rat and human genomes.

Multiple Robo molecules have been found in Drosophila and all vertebrate models studied
thus far. Drosophila Robo and Robo2 are important for midline guidance; while robo mutant
embryos show defects in midline crossing the defects are less severe than those in slit mutant
embryos. On the other hand robo/robo2 double mutants exhibit defects identical to those in slit
mutant embryos (Simpson et al., 2000b). This study shows the distinct function of Robo and Robo2 in guiding axons at the midline. In addition, Robo, Robo2 and Robo3 function combinatorially in determining the lateral positions of axon tracts in the CNS (Simpson et al., 2000a). Vertebrate Robo molecules have also been shown to play a role in axon pathfinding. The zebrafish mutant astray exhibits errors in retinal axon guidance wherein axons show anteroposterior pathfinding defects, cross the midline excessively and exhibit defasciculations. 

astray corresponds to the Robo2 ortholog (Fricke et al., 2001). Robo orthologs in Humans also play a role in axon guidance. Robo1 and Robo2 can show homophilic adhesion and also interact with each other. They stimulate axon outgrowth in neurons growing in culture (Hivert et al., 2002).

In Drosophila, regulation of Robo protein is under the control of another molecule called Commissureless (Comm). comm was identified in the same mutagenesis screen that revealed Robo. comm mutant embryos exhibit an apparently complete loss of commissural axons in the CNS suggesting that no contralateral projections were made. This phenotype in the ventral nerve cord is complementary to that in robo mutant embryos. In addition to these complementary phenotypes between robo and comm mutant embryos, double mutants exhibited defects similar to those in robo mutant embryos suggesting that they might be a part of the same pathway and Comm could be downstream of Robo (Seeger et al., 1993). Subsequent studies demonstrated that Comm can regulate Robo post-translationally by clearing it from the cell surface via endocytosis (Choi, 2003; Keleman et al., 2002). Comm has been found only in insects; no homologous sequence has been found in any of the vertebrate genomes. This suggests that regulation of vertebrate Robo molecules might have a different mechanism.

Robo receptors bind to the secreted ligands belonging to the Slit family. Slits have been identified and studied both by genetic and biochemical analysis in both invertebrates and vertebrates (Brose et al., 1999; Brose and Tessier-Lavigne, 2000; Fernandis and Ganju, 2001; Zinn
and Sun, 1999). Slits are large extracellular, secreted glycoproteins containing leucine rich repeats (LRR) and EGF repeats. Slit has been shown to play an important role in guiding axons via a repulsive mechanism in the Drosophila CNS. Midline glia secrete the Slit protein and create a gradient of repulsive cues. Interactions between Robo and Slit proteins initiate a signaling cascade that controls both the contralateral projections of axons and longitudinal fascicles in the ventral nerve cord. Biochemical studies in vertebrates have demonstrated the role of Slits in the branching and elongation of sensory axons (Wang, 1999), in addition to their role as a chemorepellent in the olfactory bulb (Li, 1999). In essence, Slits exhibit a variety of functions during axonogenesis.

Multiple Slit variants have been described in vertebrates. Genetic analysis in Drosophila indicated that Ableson (Abl) kinase and Enabled (Ena) are involved in Robo-Slit signaling. Biochemical studies revealed that the conserved motifs in the Robo cytoplasmic domain bind to Ena and Abl (Bashaw et al., 2000; Wong et al., 2002). Another study has indicated that a family of GTPase-activating proteins (GAP), the srGAPs, function in Robo-Slit signaling (Wong et al., 2001). SrGAPs have domains that can interact with Robo as well as Rho GTPases. Slits were shown to increase the interaction of srGAP with Robo and Cdc42 but decrease the interaction with RhoA (Wong et al., 2001). Other biochemical studies have suggested the presence of modulators of Robo-Slit signaling. For example, Glypican is a heparan sulfate proteoglycan and has been shown to be important for Robo-Slit interactions. Removal of cell surface proteoglycan leads to a decrease in the affinity of Slit for Robo receptor. Upon heparinase treatment, migrating neurons and growing axons to lose responsiveness to Slit mediated chemopulsion (Hu, 2001). Another molecule, the chemokine Stromal Derived Factor 1 (SDF-1) that has been implicated in leukocyte migration is a modulator of Slit-mediated repulsion. SDF-1, acting via its receptor CXCR4, reduces the Slit2-mediated repulsion in axons of cultured retinal
ganglion cells (Chalasani et al., 2003). While these molecules have been implicated in Robo-Slit interactions, a precise pathway has not been established as yet.

Since the underlying cellular and subcellular behaviors in all metazoan cell motility phenomena have some common elements, it is very likely that signaling pathways controlling axon guidance also control cell movements in gastrulating embryos. Since extracellular cues and membrane receptors are critical for the initiation of cytoplasmic signaling cascades leading to actin cytoskeletal remodelling, one might suggest that some axon guidance receptors and their ligands play important roles during gastrulation. A study suggesting the involvement of Eph signaling in zebrafish gastrulation was the first example showing evidence for this suggestion (Oates et al., 1999). Oates and co-workers injected soluble forms of human EphA3 and ephrinA-5 mRNA, which acted as dominant negative inhibitors. Overexpression of these dominant negative forms resulted in defective somite development and hindbrain organization. These developmental defects are consistent with cell movement defects during gastrulation. Substantiating these data, zebrafish EphA3 RNA expression is seen from very early stages when the embryo is undergoing gastrulation (Oates et al., 1999). These two pieces of data strongly suggest a role for Eph signaling in regulating cell movements during gastrulation (Figure 1.3).

In another study, Yeo and co-workers (2001) reported the identification and role of zebrafish orthologs of slit genes, slit2 and slit3. Both orthologs are expressed in floor plate cells, similar to what has been reported in other vertebrates, in addition to other regions in the developing embryo. Floor plate is the midline structure in the vertebrate spinal cord and has been shown to be very important for controlling axon growth towards and at the midline (Colamarino and Tessier-Lavigne, 1995). Floor plate can direct axon growth by expressing short-range cues as well secreting long-range cues. The presence of zebrafish slit RNA in the floor plate cells correlates to its possible role in guiding axon growth by creating a chemorepulsive gradient from the midline.
This idea remains to be experimentally verified. While expression in the midline is consistent with
the suggested role of Slit orthologs in axon guidance, slit2 mRNA was also seen in the anterior
eural plate and anterior mesoderm around 70% epiboly when gastrulating cell migrations are
occurring. This observation immediately raises the possibility of Slit function during cell
migrations during gastrulations. In agreement with this possibility, overexpression of slit2 mRNA
in zebrafish embryos lead to defects in convergence and extension cell movements visualized by
defective patterns of marker gene expression (Yeo et al., 2001). Transgenic zebrafish with slit2
expression under the control of the zebrafish hsp70 promoter were produced and tested to confirm
the results obtained by mRNA injections. This study provides significant evidence for a novel role
for Slits in cell migratory events in early vertebrate development (Figure 1.3).

Of the numerous mutations that have been isolated in two large-scale mutagenesis screens
performed in 1996, a subset of mutants exhibited defects in cellular rearrangements during
gastrulation (Solnica-Krezel et al., 1996). Some of them have been well characterized and found to
be components of the non-canonical Wnt signaling pathway. silberblick (slb) and pipetail (ppt)
mutants are defective in Wnt11 and Wnt5a respectively. slb mutants show defects in C&E
movements during gastrulation, while ppt mutants manifest defects in tail formation (Heisenberg
et al., 2000; Rauch et al., 1997). trilobite (tri), another mutation encodes the Strabismus (Stbm)/
Van Gogh (Vang) gene, has been associated with planar cell polarity (PCP) in Drosophila and
PCP and Wnt signaling pathway in vertebrates. tri mutants also show defects in C&E movements
during gastrulation, and seems to function in regulating mediolateral polarity that causes
intercalation and dorsally directed migration of cells (Jessen et al., 2002). Yet another mutation,
knypek (kny) is an important player in controlling cell polarity and migration during gastrulation.
It encodes a heparan sulfate proteoglycan, belonging to the glypican family (Topczewski et al.,
2001). Double mutants for kny;tri showed that both these genes interact (Henry et al., 2000). All
Figure 1.3: Signaling pathways implicated in the control of cell migrations during gastrulation. A combination of mutational analysis and gene overexpression studies in zebrafish and Xenopus provided evidence for the involvement of the non-canonical Wnt signaling pathway in convergence & extension during gastrulation. Wnt11/$slb$ and Wnt5/$ppt$ are ligands which interact with the Fz receptor, probably via the glypican, $kny$. Downstream components of this pathway include Dsh, Rho and Rho-dependent kinase (Rok2) (See review by Solnica-Krezel and Cooper, 2002). Misexpression studies using dominant negative forms of an Eph receptor and an ephrin in zebrafish suggested the role of Eph signalling in gastrulating cell movements (Oates et al., 1999). Overexpression of Slit resulted in cell movement defects during zebrafish gastrulation (Yeo et al., 2001). All pathways are suggested to converge on GTPases and effect the polymerization of actin and thus cell motility. (adapted from Solnica-Krezel and Cooper, 2002).
the above-mentioned genes are suggested components of the PCP non-canonical Wnt signaling pathway and they affect cell motility without influencing cell specification. Other components of the non-canonical Wnt signaling pathway are the Frizzled receptor, its effector Dishevelled and downstream components including small GTPases (Solnica-Krezel and Cooper, 2002). Some aspects of Rho signaling in mammalian cell culture and Drosophila PCP pathways are mediated by the Rho-associated kinase 2 (Rok2) (Winter et al., 2001). Overexpression of dominant negative Rho-associated kinase 2 (Rok3) in zebrafish embryos effects convergence extension movements (Marlow et al., 2002). These studies indicate that downstream targets of the non-canonical Wnt signaling pathway also can affect the remodelling of the actin cytoskeleton similar to the suggested Robo-Slit pathway (Figure 1.3).

The ability to visualize and experimentally manipulate developmental events from the time a zygote forms is a very powerful attribute of zebrafish as a developmental model system. External fertilization and development make this possible. Optical transparency of zebrafish embryos during development allows easy and detailed observation of morphogenesis under the microscope using differential interference contrast (DIC) or Nomarski optics. In addition zebrafish also offer many more advantages in studying developmental genetic mechanisms. Embryonic development is very rapid with formation of basic organ primordia by 24 hours after fertilization. A short generation time and large clutch size from pairwise matings enable researchers to perform large-scale mutagenesis screens to identify genes involved in various processes. Morphological and cellular observations can be supplemented with genetic studies, enabling a better understanding of developmental events. Complementing forward genetic analysis, reverse genetic strategies allow the study of molecular genetic function in greater detail.

My work uses the many advantages offered by the zebrafish system in elucidating the function of Roundabout molecules during early development. I describe the identification of Robo...
orthologs in zebrafish and provide evidence for the role of Robo molecules in cell movements
during gastrulation and motor axon guidance. The approach I took in understanding the function
of Robo molecules during zebrafish development points to the power of reverse genetic strategies in
unveiling functions of genes effecting events in early embryogenesis.
CHAPTER 2

IDENTIFICATION AND CHARACTERIZATION OF \textit{ROUNDABOUT} ORTHOLOGS IN ZEBRAFISH

2.1 Introduction

The complex circuitry of the nervous system is generated by the migration of axonal growth cones of neurons to appropriate target regions followed by the formation of specific synaptic contacts. In order to reach their targets, axons are guided by a variety of molecular cues in their environment. These cues can be either attractive or repulsive and can function as long-range, secreted signals or as short-range, membrane-bound guidance cues. A host of receptors and ligands that mediate attractive and repulsive responses of growth cones have been identified (Tessier-Lavigne and Goodman, 1996). One such pair of axon guidance receptors and ligands is the Roundabout family of repulsive axon guidance receptors and their ligands, the Slit family of extracellular proteins (see Flanagan and Van Vactor, 1998 and Zinn and Sun, 1999 for reviews).

The Roundabout (Robo) repulsive axon guidance receptor was first identified in \textit{Drosophila} in a systematic mutagenesis screen for genes involved in axon guidance (Seeger \textit{et al.}, 1993; Kidd \textit{et al.}, 1998a). In robo mutant embryos, axons that normally do not cross the \textit{Drosophila} CNS midline inappropriately cross this repulsive boundary, often multiple times. Expression of the Robo protein on the surface of axons allows them to respond to the midline chemorepellent Slit (Kidd \textit{et al.} 1999; Battye \textit{et al.}, 1999). The level of Robo protein on the surface of growth cones is tightly regulated. By greatly decreasing the amount of Robo on the
surface of commissural growth cones, these growth cones are able to extend across the CNS midline (Kidd et al. 1998b). Robo family members have now been identified in C. elegans, rat, mouse, Xenopus, and human (Kidd et al., 1998a; Zallen et al., 1998; Li et al., 1999; Yuan et al., 1999a). Robo receptors form an evolutionarily conserved sub-family of the immunoglobulin (Ig) superfamily and are characterized by the presence of five Ig repeats and three fibronectin-type III repeats in the extracellular domain, a transmembrane domain, and a cytoplasmic domain with several conserved motifs that play important roles in Robo-mediated signaling (Figure 2.1; Kidd et al., 1998a; Zallen et al., 1998; Bashaw et al., 2000). Furthermore, multiple robo genes have been described in Drosophila, rat, mouse and humans, raising the possibility of potential redundancy and diversity in robo gene function; the three robo genes in Drosophila have been shown to play combinatorial roles in specifying the longitudinal tracts in the CNS (Rajagopalan et al., 2000a; Rajagopalan et al. 2000b, Simpson et al., 2000a).

A number of studies have addressed the function of Slit family members during vertebrate neural development (Brose et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Wu et al., 1999; Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000; Zou et al., 2000), studies directly addressing the role of vertebrate Robo receptors have been less extensive. We sought to use zebrafish as a model system to address the function of Robo proteins in a vertebrate. Zebrafish is an ideal system for deciphering the mechanisms of axon guidance (Bernhardt, 1999; Beattie, 2000). Axonogenesis in zebrafish is initiated at approximately 18 hours of development by a subset of interneurons, sensory neurons and motoneurons. Due to the ability to identify and analyze individual neurons during early stages of development, particularly in the hindbrain and spinal cord, their axonal morphology and development have been well characterized. Thus, the zebrafish nervous system is an excellent in vivo model for elucidating the function of axon guidance molecules. As a first step towards the analysis of Robo axon guidance receptor function in
zebrafish, we initiated experiments to identify Robo orthologues. The cloning, mapping, and characterization of spatial patterns of gene expression for two zebrafish robo genes is presented in this report.

2.2 Results and Discussion

2.2.1 Cloning and sequence of zebrafish Robo orthologs

The Robo family of axon guidance receptors is evolutionarily conserved from *C. elegans* to humans (Kidd *et al.*, 1998a; Zallen *et al.*, 1998). Using sequence conservation amongst previously characterized members of this gene family, several degenerate primers were designed against conserved regions of the extracellular domain using the CODEHOP program (Rose *et al.*, 1998). Degenerate primers from the first and fifth Ig domains were used successfully to amplify a 1200 bp fragment from first-strand cDNA generated from zebrafish embryonic RNA. DNA sequence analysis of this fragment indicated that it exhibited high similarity to vertebrate Robo sequences.

This cloned PCR fragment was then used to screen a 33-36 h zebrafish cDNA library. Multiple cDNA clones that hybridized with this 1200 bp PCR product were recovered. DNA sequence analysis and restriction enzyme digests of these various clones indicated that they could be divided into two classes, representing two distinct robo genes. The largest clone of each class (4 kb and 3 kb) was sequenced in its entirety. Sequence analysis of the 4 kb clone indicated that it included the 5’ untranslated region through a limited portion of the cytoplasmic domain of one zebrafish robo gene. The 3 kb clone encoded sequences from the first Ig domain through the transmembrane domain and into the cytoplasmic domain of a second zebrafish robo gene.

Northern blot analysis using the 4 kb and 3 kb clones indicated that the full-length transcript sizes for these genes were approximately 5500 and 7500 nucleotides, respectively (Figure 2.2). We named the clone corresponding to the longer transcript size, 7500 nucleotides, *robo1*, and the clone corresponding to the shorter transcript size, 5500 nucleotides, *robo3* (see below for details.
of nomenclature). In order to obtain full-length clones, we performed 3’ RACE PCR for robo3 and both 5’ and 3’ RACE PCR for robo1. Full-length sequences for both robo1 and robo3 have been obtained, since the addition of 5’ and 3’ RACE products to the original cDNA clone lengths corresponds to the transcript size as predicted by Northern blot analysis.

The ORFs encoded by robo1 and robo3 were 1675 and 1419 amino acids respectively (Figure 2.3). Both genes encode the characteristic motifs of Robo family members; five Ig repeats and three FN type III repeats in the extracellular domain, a transmembrane domain and cytoplasmic domain with several conserved motifs as described by Kidd, et al. (1998a). A phylogenetic analysis of vertebrate Robo molecules based on alignments of the amino acids sequences using the ClustalW program suggested that the gene encoding the 1675 aa protein was most closely related to human ROBO1, rat Robo1, and mouse Dutt1, so we named it robo1 (Figure 2.6). Since the gene encoding the 1419 aa protein was somewhat diverged from both human ROBO2 and rat Robo2, we named it robo3. The zebrafish robo orthologs robo1 and robo3 share 50% amino acid identity and 63% similarity over their entire length. This sequence conservation was greatest in the extracellular and transmembrane domains; conservation was much more limited in the cytoplasmic domain. Furthermore, a third zebrafish Robo ortholog has been isolated which is more similar to Human ROBO2 and rat Robo2 (Lee et al., 2001). Zebrafish robo2 shares 52% identity and 66% similarity with robo1 while sharing 48% identity and 62% similarity with robo3.

Previous studies have identified conserved motifs in the cytoplasmic domains of Robo family members from C. elegans, Drosophila, rat and human that are likely to play key roles in Robo-mediated repulsive signaling (Kidd et al., 1998a; Zallen et al., 1998). Motifs containing proline-rich sequences could serve as binding sites for cytoplasmic adapter proteins and conserved tyrosine, serine and threonine residues could be targets for phosphorylation. Recent experimental studies have substantiated these suggestions based on the identification of these motifs (Bashaw et
Conserved cytoplasmic sequence 2 (CC2; LPPPP) was found to be a binding site for *Drosophila* Enabled (Bashaw *et al.*, 2000). A mutant form of *Drosophila* Robo1 that interferes with Enabled binding is partially defective in Robo-mediated repulsive signaling. This Enabled binding motif is conserved in both zebrafish *robo1* and *robo3* (Figure 2.3). CC3 (PPPPVPPP) has been shown to bind to the SH3 domain of the Abelson tyrosine kinase (Bashaw *et al.*, 2000). This motif is also conserved in both these zebrafish Robo family members (Figure 2.3). Bashaw and colleagues (2000) also demonstrated that CC1 (PTPYATT) is a target for phosphorylation by the Ableson kinase. Phosphorylation of this tyrosine seems to attenuate Robo-mediated repulsive signaling, since mutating this tyrosine leads to a hyperactive Robo receptor. This motif is conserved in both zebrafish Robo proteins as are two additional tyrosine residues that have been shown to be phosphorylated by the Ableson kinase *in vitro* (see Figure 2.3).

With the addition of the two zebrafish amino acid sequences, several additional motifs that are conserved amongst vertebrate Robo family members became apparent (Figure 2.6). The conservation of these sequences is striking, although similar sequences are not obvious in the invertebrate members of this gene family. Interestingly, the second motif contains a conserved tyrosine, along with multiple conserved serines and threonines, and may be a target for phosphorylation. The potential role of these conserved motifs will require experimental studies analogous to the work done in *Drosophila* on motifs CC0, CC1, CC2 and CC3.

### 2.2.2 Zebrafish *robo* RNA Expression

Using whole mount RNA in situ hybridization, we analyzed expression of *robo3* and *robo1* during the first three days of development.

**robo3 expression in the somites**

*robo3* is expressed diffusely as early as shield stage (See Chapter 3), however, a distinct expression pattern becomes evident at 12 h. A dorsal view of a 12 h embryo reveals a discreet row
of mesodermal cells expressing *robo3* on either side of the midline; a location suggestive of adaxial cells (Fig. 3; Devoto et al., 1996; Thisse et al., 1993). By 14 h *robo3* RNA is expressed in the paraxial mesoderm (Figure 2.7, 2.8). At this time, and even more dramatically at 16 h, expression is restricted to the posterior portion of mid-trunk (Figure 2.8, asterisks) and caudal somites. In rostral trunk segments, *robo3* expression is uniform (data not shown). This pattern persists until approximately 20 h. By 24 h, *robo3* somite expression begins to diminish in the rostral trunk (Figure 2.8C) with expression persisting in tail somites. After 24 h, *robo3* expression in somites is lost altogether (Figure 2.8D). The restricted and robust expression of *robo3* in the posterior half of somites between 14-20 h may indicate a role for Robo in anterior-posterior somite patterning. Zebrafish motor axons extend along the anterior region of developing myotomes starting at approximately 18 h (Eisen et al., 1986; Bernhardt et al., 1998); a time when *robo3* is expressed at high levels in the posterior region of myotomes. Thus, *robo* expression in myotomes could affect motor axon guidance. Interestingly zebrafish *slit3* mRNA expression was seen in motor neurons, in the ventral spinal cord (Yeo et al., 2001) and expression of these *robo* and *slit* genes is complementary in mouse somites suggesting a role for them in motor axon guidance and neural crest migration (Yuan et al., 1999b). These genes may be playing a similar role in zebrafish.

**robo3** expression in the spinal cord

Since *robo* genes have been shown to function in axon guidance, we examined their expression in the developing nervous system. The first cells expressing *robo3* in the spinal cord were seen at 14 h in the anterior trunk corresponding to the first approximately four spinal cord hemisegments (Figure 2.8A). At 16 h, more spinal neurons expressed *robo3* and were present in the first 8 spinal cord hemisegments (Figure 2.8B). This pattern persisted and by 24 h *robo3* expressing spinal neurons were observed along the entire rostrocaudal axis (Figure 2.8C). *robo3* expression in the spinal cord was diffuse during the second day of development (Figure 2.8D) and
no robo3 expression was seen in the spinal cord during the third day of development (data not shown).

Cross sections through the trunk of 20 h embryos revealed that some robo3 expressing neurons were situated slightly dorsal within the spinal cord. To determine whether these cells were the dorsally located Rohon-Beard sensory neurons, we performed robo3 RNA in situ hybridization followed by immunohistochemistry to reveal Rohon-Beard neurons (zn12 antibody; Metcalfe et al., 1990). At 20 h, we found that robo3 expression in the spinal cord did not co-localize with zn12 expression indicating that these cells were not Rohon-Beard neurons (Figure 2.9A). robo3 positive cells were also present in the ventral spinal cord next to the floor plate, consistent with the position of motor neurons (Figure 2.9B). Thus, robo3 appears to be expressed in both interneurons and motoneurons. Although both of these neuronal cell types are present segmentally within spinal cord hemisegments (Eisen et al., 1986; Bernhardt et al., 1990), we did not observe an obvious segmental pattern of robo3 expression in the spinal cord. This finding may indicate that robo3 expression is dynamic within the spinal cord, thus not revealing a reiterated pattern.

Spinal interneurons initiate axonogenesis at approximately 18 h. Subsets of spinal interneurons have axons that cross the spinal cord midline and thus are commissural neurons, whereas others extend axons ipsilaterally (Bernhardt et al., 1990). robo3 expression within interneurons is consistent with it playing a role in axon guidance decisions at the spinal cord midline. Zebrafish motoneurons do not cross the midline, but extend out of the spinal cord and innervate axial muscle. The Robo ligand, mammalian Slit2, repels motor axons in vitro suggesting a role for these molecules in motor axon guidance (Brose et al., 1999). Moreover, studies in Drosophila demonstrated that axons of the robo expressing RP2 motoneurons retracted filopodia upon contacting slit expressing cells (Murray and Whittington, 1999). Thus, robo3 expression in
zebrafish motoneurons may contribute to neuromuscular specificity by functioning to keep motor axons in the appropriate myotome regions.

**robo3 expression in the hindbrain**

The zebrafish hindbrain is comprised of seven rhombomeres containing identified reticulospinal interneurons that project axons either contralaterally or ipsilaterally within the spinal cord (Metcalfe *et al.*, 1986; Mendelson, 1986). We first see faint *robo3* expression at 14 h in discreet hindbrain cells (Figure 2.10A). By 16 h there is at least one pair of cells expressing *robo3* in each rhombomere (Figure 2.10B). The number of cells expressing *robo3* in the hindbrain increases between 16 h to 24 h (Figure 2.10C, D). This increase in expression continues until 36 h and then begins to decrease in intensity during the third day of development.

Like spinal interneurons, axons of reticulospinal interneurons either remain ipsilateral or cross the midline. The location of *robo3* expressing cells in the hindbrain is consistent with the location of reticulospinal neurons and the timing of expression is coincident with the period of axonogenesis. The increasing number of *robo3* expressing reticulospinal neurons might indicate that either there is an earlier requirement of *robo3* in some cells compared to others or that as neurons differentiate and begin axonogenesis they begin to express *robo3*. Both these possibilities have yet to be tested.

**Late expression of robo3**

We also observed *robo3* expression in both neuronal and non-neuronal tissues starting on the second day of development. *robo3* expression was present in specific brain regions at 24 h and persisted through 72 h, the latest time analyzed. At 48 h expression was detected in the tectal cells (Figure 2.11A, B), cerebellum (Figure 2.11A), retinal photoreceptor cell layer (Figure 2.11B) and the diencephalon (data not shown). Starting at 36 h and observed through at least 72 h, *robo3* expression was detected in distal cells of the pectoral fin bud (Figure 2.11C). *robo3* expression was
also observed in the caudal fin mesenchyme starting at 48 h with a greater number of cells expressing at 60 h (Figure 2.11D) and persisting through 72 h, the last time point analyzed.

In mouse, *robo2* and *robo1* are expressed in the retinal ganglia cell whereas *slit1* and *slit2* genes are expressed in areas that repel retinal axons suggesting a role for these genes in retinal ganglia axon guidance (Niclou *et al*., 2000; Ringstedt *et al*., 2000). In zebrafish, however, we see expression of *robo3* in the cell layer containing photoreceptor cells, which do not project axons into the tectum. Furthermore, we see *robo3* (and *robo1*, see below) expression in tectal cells. It is likely that zebrafish *robo* genes play a role in guiding the intertectal commissural projections in response to some cue from the tectal roofplate.

**robo1 RNA expression**

Unlike *robo3*, distinct patterns of *robo1* expression were not observed until approximately 24 h. At this time, *robo1* was expressed in ventral somites (Figure 2.12A); a pattern which began to diminish at 36 h and was undetectable after 48 h. Hindbrain and spinal cord expression was weak and only observed at 24 h in a limited number of cells (Figure 2.12B, C). Distinct expression domains in the forebrain and midbrain were observed at 24 h with the pattern becoming more robust by 48 h (Figure 2.13A). Unlike *robo3*, *robo1* expression was not detected in the retina, but was present in the supraoptic tract, hypothalamus and tectal cells at 48 h. Also similar to *robo3*, *robo1* is expressed in distal cells of the pectoral fin buds (Figure 2.13B). *robo1* was also expressed between 24 and 48 h in the lateral line primordia, a placoidly derived migratory structure that deposits neuromasts along the trunk (Figure 2.13C).

The finding that *robo1* expression is restricted suggests that its function is more limited. In contrast to *robo3*, *robo1* expression in the hindbrain and spinal cord was late relative to axonogenesis suggesting that *robo3* may play a more important role in axon guidance. *robo1*
expression in fin bud and lateral line primordia suggests a role for \textit{robo1} in tissue growth and cell movements.

2.2.3 Radiation Hybrid Mapping

To reveal the location of \textit{robo1} and \textit{robo3} on the genetic map of the zebrafish genome, we used the LN54 radiation hybrid (RH) panel (Hukriede \textit{et al.}, 1999). \textit{robo1} mapped to LG15 and \textit{robo3} mapped to LG10. These results were confirmed on the T-51 RH panel (Dr. Len Zon’s lab). The zebrafish mutant, \textit{astray} which has a genetic lesion in the \textit{robo2} gene is mapped on to LG15, in close proximity to \textit{robo1} if not immediately adjacent. It is possible that zebrafish \textit{robo1} and \textit{robo2} are paralogous genes resulting from a duplication event followed by modifications.

2.3 Materials and methods

2.3.1 Zebrafish embryos

Zebrafish embryos were collected and allowed to develop between 27 and 29°C and staged as described by Westerfield (1995). To facilitate visualization of RNA in situ hybridization in embryos older than 36 h, 0.2 mM phenylthiourea was added to the fish water at approximately 22 h.

2.3.2 Cloning of Robo orthologs from zebrafish

Consensus-degenerate hybrid primers (CODEHOP) for Robo were designed using the BLOCKS program (Rose \textit{et al.}, 1998). Forward primer (AF), CCGCCACCTTCAACTGCMARGYNGARGG, was designed to a conserved motif in the first Ig repeat and the reverse primer (HR), CAGGTCCCTGCTGGATGAYNGGNGGNGG, was designed to a conserved motif in the fifth Ig repeat. Screening for \textit{robo} clones was done on a 33-36 h post fertilization zebrafish cDNA library (a kind gift of Dr Kai Zinn). 5' and 3' RACE were performed
in order to obtain full-length sequences using the Marathon cDNA Amplification kit and AdvanTaq enzyme (Clontech).

### 2.3.3 Northern Blot Analysis

Total RNA was extracted from 24-36 h embryos and run on a denaturing formaldehyde gel prior to transferring the RNA onto a nitrocellulose membrane. Partial cDNA clones obtained from screening the library were used to synthesize P$^{32}$ labelled sequences to probe the membrane. The signal was detected by storage phosphor screen autoradiography (Phospho Imager, Molecular Dynamics).

### 2.3.4 In situ hybridizations and immunohistochemistry

Fragments from the 5’ end of *robo1* and *robo3* clones were used to synthesize digoxigenin (DIG)-labeled antisense riboprobes for in situ hybridizations as described in Thisse *et al.* (1993). *krox-20* riboprobe was used as an indicator of rhombomeres 3 and 5 (Oxtoby and Jowett, 1993) and was detected using Fast Red TR/Naphthol AS-MX Tablets (Sigma). Whole-mount in situ hybridizations and antibody staining were performed as described by Xu *et al.* (1994). zn12 immunohistochemistry with an Oregon Green conjugated secondary antibody (Molecular Probes) was used to identify the sensory Rohon-Beard neurons (Metcalf *et al*., 1990).

### 2.3.5 Mapping zebrafish *robo* orthologs

LN54 RH panel used to map *robo1* and *robo3* was a kind gift of Dr Marc Ekker. PCR amplification was done using primers from the 3’ UTR regions of *robo1* and *robo3* cDNA sequences. RH mapping of *robo1* and *robo3* was also done on the T51 panel by Dr Zon’s laboratory on request.
Figure 2.1: Schematic representation of the Robo family of proteins.

The evolutionarily conserved Robo family has type 1 transmembrane proteins with 5 Ig repeats and 3 fibronectin type III repeats in the extracellular domain, a transmembrane domain, and a cytoplasmic domain with conserved motifs.
Figure 2.2: Northern Blot for robo1 and robo3.
Total RNA from 24-36h embryos run on a 1% formaldehyde gel and probed with labelled robo1 and robo3 DNA fragments. The left lane shows a signal corresponding to an approximately 6 kb robo3 band while the right lane shows a signal corresponding to a 7 kb robo1 band.
Figure 2.3: Amino acid sequence alignment of zebrafish Robo1 and Robo3 with rat Robo1.
Figure 2.4: Amino acid sequence alignment, continued.
Figure 2.5: Amino acid sequence alignment, continued.

ClustalX generated amino acid sequence alignment of zebrafish Robo1 (GenBank accession number AF304130), zebrafish Robo3 (AF304131), and rat Robo1 (AF041082) using the Gonnet 250 Protein weight matrix with a Gap Opening Penalty of 15, Gap Extension Penalty of 0.30 and Delay Divergent Sequence being 30%. Residue numbers are indicated on the right. The extent of the five Ig domains, three FN type III repeats, transmembrane (TM) domain, and previously reported conserved cytoplasmic domain motifs (CC0, CC1, CC2, CC3; Kidd et al., 1998a; Bashaw et al., 2000) are highlighted.
Figure 2.6: Phylogenetic tree of vertebrate Robo family members.

(A) Phylogenetic tree (obtained by Neighbor-Joining Method) of vertebrate Robo family members based on a ClustalW generated alignment of mouse Dutt1 (Y17793), rat Robo1 (AF041082), human ROBO1 (AF040090), zebrafish Robo1 (AF304130), human Robo2 (AF040091), rat Robo2 (AF182037), zebrafish Robo3 (AF304131), and mouse Rig-1 (AF060570). (B) Sequence alignment of several regions within the cytoplasmic domain that are highly conserved and specific to vertebrate Robo family members. Numbers to the right indicate amino acid number.
Figure 2.7: Early expression of robo3 RNA in adaxial and paraxial mesoderm.

(A) Lateral view of a 12 h embryo (anterior to the top) showing a diffuse pattern of robo3 RNA expression. (C) Dorsal view (anterior to the top) of the same embryo reveals a single row of cells expressing robo3 RNA on either side of the midline consistent with the location of adaxial cells (arrow heads). (B) A lateral view of a 14 h embryo showing robo3 expression in the somites and dorsal view of the same embryo (D) showing robo3 expression in the somites and presumed adaxial cells. krox-20 in red (B) denotes hindbrain rhombomeres 3 and 5. Scale bar: C, D = 38 µm.
Figure 2.8: Trunk expression of robo3 during the first two days of development.

robo3 expression is seen both in the somites and a subset of spinal neurons during the first day of development. Somite expression of robo3 at (A) 14 h and (B) 16 h is restricted to the posterior portion of somites (asterisks in B). Somite expression is dramatically decreased in 24 h embryos (C) and absent at later times (48 h; D). Arrowheads point to the neuronal expression of robo3 RNA in (A) 14 h, (B) 16 h, and (C) 24 h embryos. Staining in the spinal cord becomes diffuse after 48 h (D). In these and all subsequent lateral views, anterior is to the left and dorsal is to the top. Scale bar: A,B = 40 µm; C,D = 50 µm.
Figure 2.9: *robo3* is expressed in spinal interneurons and motoneurons.

(A) Cross section through the trunk of an 18 h embryo stained with the zn12 antibody (green) and *robo3* RNA expression (blue). Sensory Rohon-Beard cells (asterisks) do not express *robo3* whereas spinal interneurons (white arrowheads) express *robo3* RNA. (B) An 18 h embryo showing *robo3* expression in ventrally located cells consistent with the position of motoneurons (arrowheads). Scale bar = 25 µm.
Figure 2.10: Hindbrain neurons express robo3.

Faint expression of robo3 RNA is first seen at 14 h (A) in a few hindbrain cells (arrowheads). The number of hindbrain cells expressing robo3 increases as development proceeds; (B) 16 h, (C) 18 h, and (D) 24 h. Hindbrain rhombomeres 3 and 5 are visualized by krox-20 staining (A-C, red). Dorsal views with anterior to the left. Scale bar = 45 µm.
Figure 2.11: Late expression of robo3 RNA.
Dorsal view (anterior to the left) of robo3 expression in the brain at 48 h. Arrowheads indicate tectal cells and asterisks denote cerebellar expression. (B) Cross section through a 48 h embryo at the level of the eyes. Arrowheads indicate the photoreceptor cell layer and the arrow indicates expression in the tectum. (C) robo3 RNA expression at 48 h is also seen in the pectoral fin bud. (D) Lateral view of a 60 h embryo showing robo3 expression in the fin mesenchyme. Scale bar: A = 50 µm, B = 87 µm, C = 15µm, D = 25 µm.
Figure 2.12: *robo1* RNA expression at 24 h.

(A) Lateral view showing *robo1* expression in ventral somites (arrowheads). (B) Dorsal view (anterior to the left) showing *robo1* expression in hindbrain cells (arrowheads). (C) Trunk cross section showing robo1 expression in spinal neurons (arrowheads). ov, otic vesicle. Scale bar: A, B =50 µm; C=25 µm.
Figure 2.13: Late expression of robo1 RNA.

(A) Dorsal view of the head (anterior to the left) of a 48 h embryo. White arrowheads denote tectal expression. (B) Lateral view of a pectoral fin bud at 48 h expressing robo1 (arrowheads). (C) Lateral view of the trunk at 36 h showing robo1 expression in the migrating lateral line primordium. Scale bar: A= 16 μm; B= 100 μm; C= 50 μm.
CHAPTER 3

ROLE OF ZEBRAFISH ROBO3 ISOFORMS DURING EMBRYONIC DEVELOPMENT

3.1 Introduction

Evolutionary conservation of molecules and molecular machinery involved in various biological processes has become almost axiomatic in recent times. In addition, many signal transduction pathways in multicellular eukaryotic organisms have been shown to have pleiotropic effects due to a variety of roles during growth and development. Many examples can be found in the conservation of molecules involved in axon guidance. Many axon guidance receptors and ligands are evolutionarily conserved across species and play pivotal roles in controlling the growth of axons as well as function in other developmental processes involving cell motility and migration (Bagri and Tessier-Lavigne, 2002; De Castro, 2003; Fernandis and Ganju, 2001; Wilkinson, 2001).

Mechanisms of cell motility are very ancient and many key molecular players are conserved from protozoa to vertebrates. Accordingly, mechanisms involved in axonal growth cone motility are similar to those involved in directional cell motility in other cellular and developmental contexts. Both processes are achieved as a result of membrane protrusions and the subsequent movement of cell contents (Pollard and Borisy, 2003). Membrane protrusions can manifest either as filopodia or as lamellipodia, which are formed by actin filaments. While the actin polymerization machine is intrinsically inert, signaling cascades mediated by extracellular signals
and a host of cellular proteins trigger assembly and disassembly of the actin cytoskeleton. These cascades drive cells and axonal growth cones to move (Pollard, 2003; Pollard and Borisy, 2003).

Several extracellular signals and their receptors that modulate actin polymerization and deopolymerization have been identified, and are being actively investigated in the context of axon guidance during embryonic development (Dickson, 2002). In contrast, our understanding of molecular mechanisms governing cell motility during earlier embryonic development, specifically gastrulation, is relatively rudimentary.

Cell biological studies using amphibian and teleost embryos have led to an extensive knowledge of cell movements during gastrulation. During vertebrate gastrulation, the concerted dorsal convergence and anteroposterior extension movements of prospective mesendodermal cells beneath the ectoderm give rise to the three germ layers and subsequently to the basic architecture of the embryo. While convergent-extension is predominantly due to mediolateral cell intercalation in the amphibian gastrula (Keller et al., 2000), C&E in the dorsal and lateral aspects of zebrafish gastrulae are driven by distinct behaviors of individual cells. While dorsal cells undergo extension with little convergence due to mediolateral cell intercalations, lateral cells migrate dorsally at increasing speeds either as individuals or as small groups without much cell intercalation (Concha and Adams, 1998; Kimmel et al., 1994; Sepich et al., 2000; Trinkaus et al., 1992). Cell labelling studies done in Fundulus (Trinkaus, 1998; Trinkaus et al., 1992) suggested that directed migration of converging cells in the lateral mesoderm towards the dorsal midline during gastrulation can be satisfactorily explained by chemotaxis governed by a gradient of molecular cues originating in the dorsal midline.

Of the many molecules involved in axon guidance, proteins belonging to the Slit family of secreted ligands have been shown to have multiple functions during development. They are important repellent cues governing the midline crossing behavior of axons (Battye et al., 1999;
Brose et al., 1999; Harris and Holt, 1999; Kidd et al., 1999; Li et al., 1999; Zinn and Sun, 1999) and also play important roles in axon branching and mesoderm migration in Drosophila (Brose and Tessier-Lavigne, 2000; Kramer et al., 2001; Wang et al., 1999). Slits have been shown to initiate a signaling cascade via the Robo family of receptor proteins (Brose et al., 1999) mediating chemorepulsion. Zebraslfish slit orthologs, slit2 and slit3, have been identified and their mRNA expression was observed in the axial mesoderm during mid-blastula and gastrula stages in addition to dynamic expression patterns during later developmental stages. Overexpression of slit2 mRNA in zebrafish embryos resulted in convergent-extension cell movement defects in the mesoderm (Yeo et al., 2001). These defects are similar to those found in zebrafish mutants slb and kny (Heisenberg and Tada, 2002; Heisenberg et al., 2000; Marlow et al., 1998), which show aberrant cell movements during gastrulation. Overexpression of Slit2 in zebrafish embryos caused a broadening of the neural anlage, broadening and shortening of the chordamesoderm, and positioning of the prechordal mesoderm posteriorly. These data are suggestive of defective cell movements in both the ectodermal and mesodermal layers (Solnica-Krezel and Cooper, 2002). While Robo-Slit interactions during axon guidance are well documented, it is not evident whether Slits signal through the Robo receptors during gastrulation. In addition it is not known whether Slit regulates attractive or repulsive mechanisms in cells undergoing gastrulation in embryos (Solnica-Krezel and Cooper, 2002).

In the current study we describe two isoforms of the Robo3 ortholog in zebrafish and report the role of one of the isoforms in gastrulation. This study provides further evidence for multiple roles played by Robo family members during zebrafish embryogenesis, and in particular a novel role in cell movements during gastrulation.
3.2 Results

3.2.1 Isoforms of zebrafish robo3 ortholog

We have described the identification and initial characterization of two zebrafish roundabout (robo) orthologs, robo1 and robo3 (Challa et al., 2001). Further analysis of the robo3 ortholog by 5' RACE PCR using total RNA from 24-36 h old embryos and subsequent subcloning of the PCR amplified product revealed that there are at least two distinct species. Sequencing of the two subclones provided evidence for the existence of two isoforms of robo3. The two isoforms were identical in the sequence encoding the core protein but diverged at the 5' end, which included the 5' UTR and sequence encoding a short stretch of amino acids corresponding to the signal sequence; we named them robo3a and robo3b. While robo3b (Genebank Accession No. AF304131) sequence was the same as the one described earlier by us, robo3a sequence was identical to that described by Lee and co-workers (Lee et al., 2001; Genebank Accession No. AF337036). To further confirm the presence of both isoforms during embryogenesis, we performed RT-PCR experiments using isoform specific forward primers and common reverse primers. Bands of the expected size for robo3a and robo3b were generated in the experiments, confirming the initial results (Figure 3.1).

The divergent sequences of robo3a and robo3b are 460 bp and 510 bp, respectively. robo3a has a relatively longer 5'UTR and a shorter signal sequence in comparison with a shorter 5' UTR and longer signal sequence of robo3b. robo3a and robo3b have 21 and 52 amino acids, respectively, at the N' terminus which are unique. Human Robo1 protein has been shown to have two isoforms which also differ only at the N-terminus; Human Robo1a (Genebank Accession No. NP_002932) with 57 amino acids and Robo1b (Genebank Accession No. NP_598334 ) with 19 amino acids which are unique in the putative signal sequence (Dallol et al., 2002).

Since we observed divergence in the putative signal peptide encoding sequences, we analyzed the hydrophobicity index of these sequences (Figure 3.2). The hydrophobicity index of
the two isoforms using the Kyte-Doolittle plot showed that Robo3a has a sharp hydrophobic peak at the N terminus, a characteristic feature of most eukaryotic signal peptides, while Robo3b lacks a highly hydrophobic peak. It is interesting to note that Mouse Rig-1 (GeneBank Accession No. AF060570), the protein that most closely resembles zebrafish Robo3 protein, also lacks a very hydrophobic peak in the signal peptide. To substantiate the hydrophobicity indices, signal peptide predictions were also made using Neural Networks (NN) and hidden Markov models (HMM) trained on eukaryotic sequences (Nielsen et al., 1997; Nielsen and Krogh, 1998). Both methods predicted that Zebrafish Robo3a sequence had a signal peptide and the most likely cleavage site was between positions 22 and 23 (SQG-SR). The HMM method predicted the signal peptide probability to be 0.953 (Figure 3.3). On the other hand, the Zebrafish Robo3b sequence was predicted to be a non-secretory protein based on the low signal peptide probability of 0.10 (Figure 3.4). Similarly, Mouse Rig-1 was also predicted to be non-secretory proteins based on a very low signal peptide probability score of 0.001 (Figure 3.5). This data indicates that the lack of a signal peptide is not unique to zebrafish Robo3b. On the other hand, SignalP predictions using NN and HMM methods on the two isoforms of Human Robo1 indicate that both of them have signal peptides (data not shown).

Since Neural Network and HMM predictions did not show a signal peptide in the Robo3b sequence, we asked whether the protein still gets to the cell surface as Robo proteins are characteristically receptor molecules. To analyze the cellular localization of the two Robo3 isoforms, myc-epitopes were added to the cytoplasmic terminus of the full-length constructs, and they were used to transiently transfect 293T cells followed by immunocytochemistry to detect the myc epitope. Using confocal microscopy we found that there were no differences in the localization patterns of both the isoforms in the transfected cells (Figure 3.6). Both Robo3a and Robo3b proteins were seen associated with the cell surface, consistent with their suggested receptor
function. This data indicates that despite the contrasting differences in signal peptide regions of
Robo3a and Robo3b retain their ability to associate with the cell membrane.

3.2.2 Expression of robo3 isoforms during early embryogenesis

Since the core protein coding regions of both isoforms were identical and their cellular
localization seemed indistinct, we wanted to test whether the two isoforms were differentially
expressed during embryogenesis. Antisense DIG labeled riboprobes against robo3 isoforms were
synthesized using the respective unique sequences and whole mount in situ RNA hybridization
experiments were performed.

Beginning at 14 h, both isoforms showed distinct tissue expression. robo3a was found in
the somitic tissues while robo3b RNA was seen specifically in hindbrain and spinal neurons (Figure
3.7). This isoform-specific, distinct pattern of RNA expression was observed until about 24 h.
Soon afterwards, robo3a and robo3b RNA expression patterns remained broadly distinct, although
there was overlap in the domains of expression in the embryo.

robo3a was clearly expressed in the posterior aspect of each somite (Figure 3.7A, C).
This expression began to disappear by about 24 h with only the caudal-most somites showing some
robo3a expression. While the somitic expression was disappearing, a number of neurons in the
spinal cord and hindbrain regions began to express robo3a during the second day of development.
robo3a expressing neurons in the ventral spinal cord corresponded to the location of motor neurons
and possibly some interneurons, as seen in lateral and cross sections (Figure 3.7E and G).
Expression in these ventral spinal neurons was very transient and was not observed after 48 h.
robo3a RNA is seen in the brain regions for a longer time during development, until about 60 h,
but the domains are very restricted and the levels of RNA seem to be low with the signal fainter
than that of robo3b (Figure 3.8A, C). robo3a RNA is also seen in pectoral fin bud during the
second day of development (data not shown).
robo3b RNA expression is observed in individual spinal neurons corresponding to interneurons. Cross sections of trunk indicated that none of the ventrally located neurons, corresponding to motor neurons, expressed robo3b. Many hindbrain neurons and cells in other parts of the brain also show high levels of robo3b RNA until about 60 h (Figure 3.8B,D). robo3b RNA is also found expressing in the pectoral fin bud (data not shown). robo3b RNA is persistent as late at 6 days in a distinct group of cells in the diencephalic region (Figure 3.9).

The sum of the expression patterns of both robo3a and robo3b correspond very closely to the expression patterns observed earlier using an antisense riboprobe generated against the sequence common to both the isoforms (Challa et al., 2001). This is more obvious at earlier stages when the robo3a expression is restricted to the somitic tissue while robo3b expression is restricted to neuronal cells.

3.2.3 Early expression of robo3 isoforms

While robo3 isoforms have distinct expression patterns at later developmental stages, they are also expressed globally during earlier development. In situ hybridization experiments using isoform specific antisense riboprobes indicated the presence of robo3a and robo3b mRNA as early as the 2-cell stage (Figure 3.10a) indicating the maternal loading of these transcripts. To confirm the maternal contribution of robo3 isoforms, RT-PCR experiments were performed using total RNA obtained from embryos at different early stages with isoform specific forward primers (Figure 3.10). Since zygotic transcription in zebrafish embryos is thought to begin at the 512-cell stage, RNA present in 2-16-cell stage embryos would therefore be the result of maternal contribution. Amplification products of both robo3 isoforms were observed from maternal RNA, early zygotic RNA (512-1000 cell stage) and RNA obtained from 10 h embryos. RT-PCR using RNA from 24 h embryos was our positive control since expression in distinct cells is obvious at that time. Thus, the RT-PCR results support our in situ hybridization experiment results (Figure
The specificity of amplification was controlled by nested PCR. The first strand cDNA synthesis reaction and the PCR were performed with non-overlapping reverse primers to decrease the possibility of non-specific amplification.

RT-PCR experiments were also performed to test the expression of other known robo orthologs, robo1 and robo2, at the developmental time points when robo3 isoforms are expressed (Figure 3.10b). We found that both robo1 and robo2 are also expressed along with robo3a and robo3b. We did observe that the levels of amplification were not uniform at the various time points tested. There is a reduction in the level of robo3a amplification during early zygotic stages and a reduction in the level of robo2 and robo3b amplifications at 10 h. Since we do not have adequate information on the regulation of protein expression and the half-lives of the proteins at these early time points, it is difficult to extrapolate the activity of Robo proteins based only on their RNA levels.

While we observed robo3a and robo3b mRNA expression ubiquitously in the embryo until about 12 h, an in situ hybridization screen (Thisse et al., 2001) revealed that probe cb503, which corresponded to the cytoplasmic sequence of robo3, specifically labels cells in the anterior neural plate at around 70% epiboly stage during gastrulation (ZFIN ID: ZDB-XPAT-030521-38). This is very likely due to the use of a more sensitive technique that allows the visualization of specific domains of RNA expression in embryos at early stages.

To complement our observations on RNA expression of robo3 isoforms during gastrulation, we performed Western Blot analysis to test Robo3 protein expression. We generated anti-Robo3 antiserum (rabbit polyclonal) against a 225 amino acid fragment in the Robo3 cytoplasmic domain. The amino acid sequence was His-tagged and the purified fusion protein fragment was used as the antigen. Since the antiserum was against a common cytoplasmic region, we could not distinguish the two Robo3 isoforms in this analysis. The efficiency of Robo3
antiserum was tested against \textit{in vitro} translated myc-epitope tagged Robo3a and Robo3b proteins. The bands recognized by anti-myc antibodies on a Western Blot correlated to the bands recognized by the anti-Robo3 antiserum.

Western Blot analysis using zebrafish protein extracts from different developmental stages showed that Robo3 protein expression begins during gastrulation, albeit at a low level (between 50\% - 80\% epiboly; data not shown) consistent with the RNA expression data (Figure 3.10). Beginning at 10 h, Robo3 protein is expressed robustly (Figure 3.11) and continues at similar levels even at later stages, which is consistent with the RNA expression (Figure 3.7).

\subsection*{3.2.4 Gene ‘knock-down’ studies using antisense oligonucleotides}

To test the function of \textit{robo3} isoforms during zebrafish embryonic development we employed sequence specific antisense morpholino oligonucleotides (MOs) to ‘knock-down’ gene function (Ekker, 2000; Nasevicius and Ekker, 2000; Summerton, 1999). Antisense MOs were designed against sequences just upstream of the initiation codon, for both \textit{robo3a} and \textit{robo3b}. To assess the efficacy of these MOs in inhibiting gene expression, presumably by inhibiting translation, we performed coupled \textit{in vitro} transcription + translation reactions in the presence of increasing concentrations of MOs (Figure 3.12a). Myc-epitope tagged plasmid DNA constructs were used as templates in the reaction and the translated protein was detected on a Western Blot using anti-myc antibody. In the absence of any MO, we found the protein of expected size recognized by the anti-myc antibody. As the concentration increased from 200nM to 20\mu M, there was a corresponding decrease in the amount of protein detected. At 20\mu M MO concentration almost no protein was detected. As a initial test of specificity, \textit{robo3b} specific MO was added to \textit{robo3a} \textit{in vitro} reaction and vice versa (Figure 3.12). Even at a significantly higher concentration (40\mu M) the respective proteins were detected, supporting the specificity of MO binding to the transcript in a sequence specific manner.
To further confirm the specificity of MO activity, we used 4 base pair (bp) mismatch antisense MO oligos. The 4 mismatches were spread throughout the 20 base sequence. If the binding is highly sequence specific, the MO oligos cannot bind to a sequence with any mismatches. Figure 3.12b shows evidence for the inability of the mismatch MO oligos, at the same concentration, to inhibit protein synthesis \textit{in vitro}.

To test the knock-down of \textit{robo3} isoform expression during development, Robo3 protein levels in MO injected embryos were detected by Western Blotting. Since we detected transcripts of both isoforms during gastrulation and the antibodies we generated cannot distinguish the two isoforms, a combination of \textit{robo3a} and \textit{robo3b} MOs were injected to knockdown expression of both the isoforms. Whole embryo protein extracts were obtained at 11 h and subjected to PAGE and Western Blot analysis using anti-Robo3 antiserum (Figure 3.12(c)). A significant reduction in the protein levels was seen in the MO injected embryos when compared to the protein levels seen in control MO injected embryos. Actin levels in protein extracts from \textit{robo3a}+\textit{robo3b} MO injected embryos and mismatch MO injected embryos were determined to ensure that the reduction in Robo3 protein is not due to differences in the amount of protein loaded.

\textbf{3.2.5 Knock-down of \textit{robo3b} causes defects in convergence of neural ectoderm}

\textit{robo3b} specific MOs were injected into 1-4 cell stage embryos and observed at different time points during development, under the light microscope. Around 24 h, \textit{robo3b} injected embryos showed defects in the trunk formation, which varied in severity (Figure 3.15). In the mildly affected embryos a consistent curving of the tail was observed. Moderately affected embryos had their tails ‘trapped’ in the yolk, consequently giving rise to trunk defects. At higher MO concentration injections, severe reductions in trunk formation were observed. While the development of head structures was apparently normal, in comparison to the obvious defects in the
trunk, we observed very subtle abnormalities in the patterning of hindbrain and forebrain structures. Similar defects were not evident in robo3b_4mm MO, robo3a MO and robo3a_4mm MO injected embryos.

In order to test whether the severe defects observed around 24 h were due to earlier defects we observed robo3b MO injected embryos during gastrulation. Beginning at around 70% epiboly, elongation of the embryos along the AP axis was observed. With the progression of gastrulation, the elongation became more obvious. In contrast, wild type embryos remained predominantly spherical (Figure 3.15). Embryo lengths along the AP axis observed at about 11 h in wild type, robo3b MO and robo3b_4mm MO injected embryos were significantly different (P = 1.894E-35, ANOVA; Table 3.2a). Further analysis using paired Student’s T-test showed that robo3b MO injected embryos were significantly longer along the AP axis when compared to wild type and control MO injected embryos (Figure 3.15).

The moderate and severe phenotypes seen in robo3b MO injected embryos resembled zebrafish mutants that exhibit gastrulation defects. Since Robo proteins are receptors known to modulate actin cytoskeleton remodelling, we hypothesized that the defects seen in robo3b MO injected embryos are due to abnormal cell migrations during gastrulation, especially C&E (Solnica-Krezel et al., 1996; Hammerschmidt et al., 1996; Marlow et al., 1998). In order to test this hypothesis we performed in situ hybridization experiments on robo3b MO injected embryos around 11 h, with markers of axial mesoderm (ntl) and posterior neural ectoderm (Krox-20). The gene expression domain of axial mesodermal marker, ntl, was indistinct from that in wild-type embryos. On the other hand, the neural ectoderm marker, Krox-20, was expanded and was found to be significantly broader than the domain in either wild type embryos or the 4 bp mismatch MO injected embryos. This suggested that there are defects in convergence cell movements. Embryos injected with robo3a MO and its 4bp mismatch MO did not show comparable expansions in the
Krox-20 expression domain suggesting that this defect is specific to robo3b (Figure 3.17). It is also important to note that robo3a MOs did not show similar defects in trunk formation as seen with robo3b MO at 24 h.

3.2.6 Knock-down of Robo3a causes defects in motor axon projections

robo3a specific MOs were injected into 1-4 cell stage embryos and allowed to develop at 28 °C. The development in these MO injected embryos was predominantly normal, without any obvious or consistent phenotypic defects. We did observe occasional defects like curved tails, which were also apparent in control MO injected embryos (data not shown).

Past studies have indicated that rostro-caudal patterning of the somite potentially plays a role in guiding motor axons (Keynes and Stern, 1984), which can be explained by differential expression of guidance molecules within the somitic mesoderm (Koblar et al., 1999). Since the earliest distinct expression of robo3a RNA was in the posterior aspect of the developing somites (between 14h and 18 h), and motor axons extend out of the spinal cord approximately at 18 h (Myers et al., 1986), we hypothesized that the restricted expression of robo3a might influence the stereotypical projections of motor axons.

Embryos injected with robo3a MO and robo3a_4mm MO were allowed to develop until about 36 h. They were fixed and assayed for the pattern of motor axon projections using the znp-1 antibody, which labels motor axons and some sensory axons (Trevarrow et al., 1990; Melancon et al., 1997). The ventral projections of CaP motor neurons are along the ventromedial aspect of each myotome (Myers et al., 1986). This projection was observed in robo3a MO injected embryos. MO injected embryos had consistent and significantly higher number of defects in CaP axonal projections, in comparison to embryos injected with robo3a_4mm MO. Motor axon trajectories were truncated anywhere between the horizontal myoseptum (HM) and the proximal ventral myotome (PVM), with a greater incidence of truncation at the HM. An average of 3.5 truncations
were observed in every lateral side examined (with a range of 0-10) in comparison to an average of 0.14 truncations observed in the control MO injected embryos (with a range of 0-1). About 10 nerves in the mid-trunk region were examined in each MO injected embryo. Due to the variability of truncations per lateral side observed, the deviation from the average number is very large. The number of CaP motor axon truncations in robo3a MO injected embryos is significantly higher than that in control MO injected embryos (P=0.01). In addition to truncations, premature branching was also observed in robo3a MO injected embryos. An average of 2.5 branching defects were observed per lateral side observed in robo3a MO injected embryos (with a range of 0-6) in comparison to no branching defects in the control MO injected embryos. As in the case of truncation defects, due to the large variability of observations, the deviation from the mean is very high and the differences are statistically significant (P=0.01). Branching was observed at various positions along the dorso-ventral aspect of the trunk, with a greater incidence of premature branching at the distal ventral myotome. These data suggest that absence of Robo3a protein have an effect on pathfinding of motor axons.

3.3 Discussion

3.3.1 robo3 ortholog has two isoforms

We report the identification of two isoforms of the zebrafish robo3 ortholog, robo3a and robo3b, which are identical in the core protein-coding region and differ only in a region at the 5’ end of the sequence. While multiple Robo orthologs have been identified in many species, no study has addressed the role of different isoforms of Robo proteins during development. Since their domains of expression are clearly distinct, one can postulate that there would be tissue specific transcriptional control of the Robo3 locus. Sequence comparison and phylogenetic analyses using known vertebrate robo orthologs revealed that zebrafish Robo3 isoforms are most closely related to
Mouse Rig-1 (Figure 2.6). This is true despite the lack of the first conserved cytoplasmic domain (CC0) in Rig-1 and the presence of all conserved cytoplasmic domains in zebrafish Robo3 isoforms.

The only differences between robo3a and robo3b sequences lie in the 5’ end with variable UTR regions and putative signal peptide encoding regions. Predictions using Neural Networks and HMMs showed that Robo3a has a characteristic signal peptide at the N-terminus, validating the fact that it is targeted to the plasma membrane. In contrast, Robo3b sequence did not show any characteristic features of a signal peptide at the N-terminus. The absence of any obvious signal peptide sequence even in the Mouse Rig-1 protein sequence suggested that there are other members of the Robo family with similar properties. The lack of a signal peptide in zebrafish Robo3b and Mouse Rig-1 could possibly have functional implications, which can be unveiled only upon further characterization of these genes. Transient expression of the two zebrafish Robo3 isoforms in mammalian cells showed no difference in their cellular localization. Both Robo3a and Robo3b seemed to be associated with the membrane.

3.3.2 robo3 isoforms show dynamic spatio-temporal expression patterns

Our study shows clear spatial and temporal differences in the expression patterns of robo3a and robo3b. robo3b RNA expression in hindbrain and spinal neurons beginning at 14 h, and continuing during the second day of development, is suggestive of its role in controlling axon guidance. robo3b is principally expressed in neuronal tissues, with the exception of pectoral fin buds during the second day of development. In contrast, it is interesting to see that robo3a RNA is seen predominantly in the posterior aspect of the somitic mesoderm between 14 h and 24 h. What developmental mechanism would employ a receptor protein in the posterior region of the somite? Especially, in the wake of the finding that zebrafish slit2 mRNA, a known member of the family of ligands for Robo proteins, is expressed in the anterior margin of the somite at 16 h. Analyzing the
temporal patterns of \textit{slit2} mRNA expression in the somites, between 16 h and 24 h will enable us to further evaluate potential interactions between \textit{robo3a} and \textit{slit2} in the somite.

Around the time when the somitic expression of \textit{robo3a} disappears, neurons in the medial and ventral regions of the spinal cord begin to express \textit{robo3a} at a low level, as seen in RNA in situ hybridization experiments. Interestingly, zebrafish \textit{slit3} mRNA is seen in ventral spinal neurons around the same time (Yeo \textit{et al.}, 2001). If the same cells express \textit{robo3a} and \textit{slit3}, it raises the possibility of Slit3 having an autocrine function via Robo3a. The dynamic spatial and temporal expression patterns of \textit{robo3a} in the developing embryo suggests that the protein has multiple functions.

In addition to the distinct patterns of \textit{robo3} isoform expression after 14 h, we show that they are also expressed at earlier time points during development. Our studies show that \textit{robo3a} and \textit{robo3b} are maternally expressed and are ubiquitously present in the embryo during gastrulation. The observed ubiquitous presence of \textit{robo3} isoforms during gastrulation could be due to a lack of resolution in detecting the signal in specific domains of the embryo. This seems likely since another study (Thisse \textit{et al.}, 2001) shows that a probe (cb503) which has sequence identity to a region in the cytoplasmic domain of \textit{robo3} isoforms, is clearly expressed in the anterior neural plate during gastrulation. Based on the distinct patterns of expression at later time points we can extrapolate that \textit{robo3a} and \textit{robo3b} might have distinct domains of expression even during gastrulation. Another correlation between the expression patterns of \textit{robo3} isoforms and \textit{slit2} is apparent here. Yeo \textit{et al.} (2001) show evidence for the expression of \textit{slit2} in the anterior margin of the neural plate. In addition, overexpression of \textit{slit2} causes defects in C&E movements during gastrulation (Yeo \textit{et al.}, 2001), and our study suggests a role of \textit{robo3} in gastrulation. All these observations strongly suggest that Robo-Slit interactions play an important role during gastrulation.
3.3.3 Morpholino mediated gene ‘knock-down’ to understand Robo3 function

In order to understand the function of robo3 isoforms during zebrafish development we employed a gene ‘knock-down’ strategy using antisense technology. Antisense morpholino oligonucleotides with their modified backbones have been shown to be effective in ‘knocking-down’ gene expression (Ekker, 2000; Nasevicius and Ekker, 2000). This strategy has been exceptionally successful in a large number of reverse genetic studies done in zebrafish (see Ekker and Larson, 2001). Despite the advantages of this strategy, care should be taken by incorporating proper controls to validate any results. One way is to use two independent, non-overlapping antisense oligonucleotides showing the same effects during development. Recently, studies have shown that even a 4 bp mismatch in the oligonucleotide sequence can dramatically reduce the efficacy of binding to the transcript and thus do not cause gene ‘knock-down’ (Lee et al., 2002; Yamashita et al., 2002). We chose to use the 4 bp mismatch oligonucleotides as controls in our experiments.

The efficacy and specificity of morpholino oligos were first tested in an in vitro assay. Coupled transcription and translation of myc epitope-tagged, recombinant Robo3 isoforms in rabbit reticulocyte lysate system was performed either in the absence, or in the presence of increasing concentrations of the antisense morpholino oligonucleotides. The in vitro synthesized proteins were detected using anti-myc antibodies on Western Blots. These in vitro assays showed that the morpholinos were efficiently knocking-down gene expression and the 4 bp mismatch morpholinos were unable to do the same. Protein levels in MO injected embryos confirmed the observations made from in vitro experiments. Any non-specific effects should be common to both the morpholino and its 4 bp mismatch counterpart so that any phenotypic consequences observed with the morpholino and not with the 4 bp mismatch morpholino can be taken as specific effects due to gene ‘knock-down’.
3.3.4 Role of Robo3b in cell movements during gastrulation

Knockdown of robo3b resulted in phenotypes which include grossly defective trunk formation and subtle defects in the head regions. Distinct expression of robo3b in hindbrain and spinal neurons cannot account for such dramatic phenotypic consequences. In contrast, expression during gastrula stages does suggest a possible role in controlling cell movements. Defects in cell movements at these early stages in development can lead to gross abnormalities later on, as seen at 24 h.

robo3b morpholino injected embryos become elongated during gastrulation. Despite the elongation, the width of ntl expression domain is no different than wild-type embryos. This demonstrates that the specification of the axial mesoderm is not affected. It is important to note that dorsal cells contributing to the axial mesoderm in zebrafish are known to undergo extension, with little convergence (Keller et al., 2000; Myers et al., 2002; Wallingford et al., 2002). In contrast, broadening of the Krox20 domain suggests defects in convergence cell movements in the neural ectoderm. Cells in the lateral mesoderm and neural ectoderm have been suggested to exhibit directional migration as individuals or clusters of cells in teleost fish (Trinkaus, 1998; Trinkaus et al., 1992; Wallingford et al., 2002), and their migration patterns can be satisfactorily explained by the presence of a gradient of signal(s) emanating from the dorsal midline (Trinkaus, 1998; Trinkaus et al., 1992). The expression of slit2 in the dorsal midline and anterior margin of the anterior neural plate (Yeo et al., 2001), could potentially create a gradient to which the robo3 expressing cells respond. This is an attractive model to address the question of directional migration of individual cells towards the dorsal midline. The role of Slit and Robo in mesodermal migration during Drosophila myogenesis sets a precedent to this model (Kramer et al., 2001).

Furthermore, we show that all known robo3 orthologs in zebrafish, robo1, robo2 and robo3 isoforms, are expressed both maternally and during gastrulation (Figure 3.10 ). It is likely that
these Robo proteins interact in order to initiate a signaling cascade. Some supporting evidence comes from a recent study in cell culture that shows the ability of extracellular domains of Human Robo1 and Robo2 to interact with each other (Hivert et al., 2002). The possibility that different Robo orthologs can interact gives rise to an additional dimension in cell migration during gastrulation.

### 3.3.5 Role of Robo3a in motor axon guidance

Despite being expressed maternally and ubiquitously during early embryonic stages, *robo3a* seems to have little effect on early development. This could be due to some redundancy with *robo3b*. The striking and consistent defect found in *robo3a* knockdown embryos was in motor axons. Over 90% of *robo3a* MO injected embryos showed obvious truncations in motor axons projections. This is significantly higher in comparison to only about 20% of embryos showing defects in control MO injected embryos. At first glance, it is hard to reconcile the fact that motor neurons, which do not express *robo3a* when they first send out axons, fail to undergo proper and complete axonogenesis. But the expression of *robo3a* in the posterior aspect of the developing somites provides a possible clue to understanding this phenotype. In vertebrates, spinal motor axons project along the rostral half of the somite, while being excluded from the caudal half. In zebrafish, the CaP motor axons project along the anterior myotome. Keynes and Stern (1984) showed that factors present in the avian somite determine the segmental arrangement of motor axons. Molecules implicated to play roles in axon guidance have been shown to have differential expression in the somites which might indicate their potential role in patterning spinal motor axon projections (Koblar et al., 2000; Krull and Koblar, 2000; Tzarfati-Majar et al., 2001). Rostro-caudal patterning of the somite can indirectly influence the control of motor axon guidance. Members of the Ephrin and their Eph receptor families have been shown to be required for segmentation and differentiation of the somites in zebrafish (Durbin et al., 1998). Consequently,
defects in somite patterning can lead to defects in motor axon trajectories (Gray et al., 2000; Gray et al., in preparation). In this context, complementary expression of ligands and their receptors in different somitic regions might be suggestive of their interactions that influence myotome patterning and subsequent effects on motor axon outgrowth. Preliminary evidence shows that zebrafish slit2 is expressed in the anterior margin of the somites (at 16 h) and robos3a is seen in the posterior aspect of the somites around the same time. Their interactions could lead to signaling which in turn might be controlling motor axon projections. Knocking down robos3a leads to CaP motor axon truncations and premature branching, which suggests Robo3a protein function somehow facilitates the proper extension and branching of these axons. This could potentially involve the function of slit2. But detailed analysis have to be done to test this hypothesis.

In summary, we show evidence for multiple roles played by Robo3 isoforms during zebrafish development. We suggest that zebrafish robos isoforms function in cell movements in gastrulation and in patterning motor axon pathways in the peripheral nervous system.

3.4 Materials and Methods

3.4.1 Zebrafish and embryo care

Zebrafish embryos were collected and allowed to develop between 27 and 29°C and staged as described by Westerfield (1995). To facilitate visualization of RNA in situ hybridization in embryos older than 36 h 0.2 mM phenylthiourea, which blocks melanin biosynthesis, was added to the fish water at approximately 22 h.

3.4.2 RNA extraction, 5’ RACE PCR and subcloning

24-36h *AB embryos were frozen in liquid nitrogen and stored at -80°C. RNeasy kit (QIAGEN) was used to extract total RNA using the protocol provided by the manufacturer. First strand cDNA was synthesized using the GeneRacer Kit (Invitrogen) to enrich the pool with intact 5’ ends of sequences. The first round of PCR was performed using the GeneRacer 5’ RACE
forward primer and robo3 gene specific reverse primer (5’ TACGACTGACGGCCTCGCCCAAGT 3’). Nested PCR was performed using the PCR product from first round PCR as the template, a 5’ nested forward primer and a robo3 specific nested reverse primer (5’ CTCCACCATGGGGGTCCGCTCC 3’). The PCR product was cloned into the pCR TOPO4 plasmid using the TOPO TA Cloning kit. Two clones with distinct insert sizes were identified and sequenced. A unique BamHI site was found at the point where the divergence of the two sequences ended. Inserts obtained from BamHI and EcoRI double digestions were subcloned into pBlueScript SK.

3.4.3 RT PCR of zebrafish robo orthologs

Total RNA from different embryonic stages (2-16 cells, 512-1000 cell, 10 h, 22-24 h) was extracted using the Get pureRNA kit (Dojindo Molecular Technologies, Inc., Gaithersburg, MD). First strand synthesis was done using gene specific primers; robo1: 5’ CCGTTGACTGACAACCTTCACGC 3’ and 5’ CCGCTGGGATGGTCTGATGCTG 3’, robo2: 5’ CGACTGTGGGCTGCTGGGGTTGA 3’, robo3: 5’ CTGTGAGGGCGGCTGACTGGG 3’ and 5’ TACGACTGACGGCCTCGCCCAAGT 3’. First strand cDNA from each embryonic stage was used as the template in PCR with the following primer sets. robo1: 5’ CCAGGACGTCTCACAGGAG 3’ as the forward primer and 5’ GGCGGCGGGATTAACCGGAGG 3’ as the reverse primer; robo2: 5’ GCAGCAAACCTGATGAGGGGCGG 3’ as the forward primer and 5’ CGTACCTCCCGGCGGGGATTTGC 3’ as the reverse primer; either 5’ CTGTGAGGGCGGCTGACTGGG 3’ or 5’ TACGACTGACGGCCTCGCCCAAGT 3’ as common reverse primers for both robo3a or robo3b and 5’ GCCCGTGACATGAACGTTGGAG 3’ and 5’ CCTACAGGAGAGAGGGAAGAAG 3’ as the forward primers for robo3a and robo3b respectively.
3.4.4 Robo3 antibodies

DNA sequence encoding a cytoplasmic protein fragment (225 amino acids) corresponding to a sequence just downstream of the conserved CC2 region and including the conserved CC3 region was cloned into pRSET C vector (Invitrogen) using BglII and NcoI sites to construct a 6X His tagged version of the protein fragment. *E.coli* BL21 cells were transformed and induced to overexpress the fusion protein. The fusion protein was purified under denaturing conditions (8M Urea) with Ni NTA resin (QIAGEN) as suggested by the manufacturer. The purified fusion protein was used to raise rabbit polyclonal antiserum (Cocalico Biologicals, Pennsylvania). The antiserum was concentrated using Amicon Ultra-15 Centrifugal Filter Units (Millipore, Bedford MA).

3.4.5 Protein extraction from zebrafish embryos and Western Blotting

Zebrafish embryos at different stages were obtained and dechorinated either manually or by a brief pronase treatment. Dechorinated embryos were homogenized in lysis buffer (10mM HEPES pH 7.5, 100 mM NaCl, 1mM EGTA, 25ug/mL Aprotinin, 25ug/mL Leupeptin) using a 18-20 guage needle alone or after crude homogenization with pestle at 4°C. The homogenized embryos were centrifuged at 750g for 5-10 minutes. The pellet was dissolved in 2X sample buffer, boiled and used in PAGE and Western Blotting. PAGE and Western Blotting were done according to standard protocols and the ECL system was used to detect the antibody signal. Anti-Robo3 antiserum (primary antibody source) was used at a dilution of 1:1000.

3.4.6 RNA in situ hybridization

The divergent sequences between *robo3a* and *robo3b* were cloned into pBlueScript SK. *robo3a, robo3b, ntl* and *Krox-20* digoxigenin (DIG)-labeled antisense riboprobes (Roche Biochemicals) were generated as described in Thisse *et al.*, 1993.
3.4.7 robo3 plasmid DNA constructs

A short stretch of sequence encoding the terminal 15 amino acids from the cytoplasmic end of robo3 was removed using the unique NruI site and fused with the myc epitope in frame. robo3a:Myc and robo3b:Myc sequences were subcloned into pBlueScript SK and pCS2+ for in vitro and in vivo studies.

3.4.8 293T cell culture and transfections

Transient transfections of 293T cells were done using the Calcium Phosphate protocol as described by Asubel et al (Short Protocols in Molecular Biology 4th Ed.). robo3a:Myc and robo3b:Myc constructs subcloned into the pCS2+ vector, which contains an upstream CMV IE94 promoter to drive the expression of the gene sequences, were used in to transfect the cells. Cells were visualized using confocal microscopy (BioRad).

3.4.9 Coupled in vitro transcription and translation

pBSK.robo3a:MT and pBSK.robo3b:MT constructs were used as templates in coupled transcription/translation reactions (TnT Coupled Reticulocyte Lysate System, Promega Corporation). T7 RNA Polymerase was used to initiate the transcription of sense strands. Varying concentrations of specific morpholino oligonucleotides designed to target robo3a and robo3b were added to the coupled transcription/translation reactions to test the efficacy and specificity of translation inhibition.

3.4.10 Morpholino knock down of gene expression

robo3a and robo3b antisense morpholino oligonucleotide sequences: robo3a: 5’
CCCTAAAAGCGCTACAATCCACCTG 3’; robo3b: 5’ CTCTTCTTTAGCAGCGGAGGGACG 3’. 4mM stock solutions of the morpholino oligos were made in water and 0.4 mM (3.3 ng/nl) working concentration was used while injecting the morpholinos into wild type embryos. 1-3 nl of
morpholinos were injected into 1-4 cell stage embryos and allowed to grow at 28.5°C in embryo medium with Penicillin and Streptomycin.
Figure 3.1: RT-PCR indicating the presence of two robo3 isoforms.

Total RNA extracted from 24 h embryos was used as template and first strand cDNA was primed with an oligonucleotide corresponding to the end of the first Ig domain. Isoform specific forward primers in the 5’ UTR along with a common reverse primer corresponding to the beginning sequence of the first Ig domain were used in the PCR. The amplification products indicated that robo3a (lane 1; ≈ 650 bp) and robo3b (lane 2; ≈ 600 bp) are distinct isoforms. Lane ‘L’ is loaded with 1 kb plus ladder indicating DNA molecular weight standards.
Figure 3.2: Hydropathy (Kyte-Doolittle) plots for the first 500 amino acids of Zebrafish Robo3a and Robo3b, and Mouse Rig-1 proteins.
Neural Networks method assigns higher values than cutoff values for raw cleavage site score \(C=0.65;\) cutoff=0.33, combined cleavage site score \(S=0.643;\) cutoff=0.32 and signal peptide score \(Y=0.94;\) cutoff=0.82 for Robo3a. HMM predicts Robo3a sequence to contain a signal peptide at a probability of 0.953 and a maximum cleavage site probability of 0.6 between positions 22 and 23 (SQG-SR).

**Figure 3.3 : Signal Peptide Prediction for Robo3a protein.**

Neural Networks method assigns higher values than cutoff values for raw cleavage site score \(C=0.65;\) cutoff=0.33, combined cleavage site score \(S=0.643;\) cutoff=0.32 and signal peptide score \(Y=0.94;\) cutoff=0.82 for Robo3a. HMM predicts Robo3a sequence to contain a signal peptide at a probability of 0.953 and a maximum cleavage site probability of 0.6 between positions 22 and 23 (SQG-SR).
Figure 3.4: *Signal Peptide Prediction for Robo3b protein.*

Neural Networks method assigns lower values than cutoff scores for raw cleavage site score (C=0.125; cutoff=0.33) and combined cleavage site score (Y=0.22; cutoff=0.32) but a slightly higher value than cutoff for the signal peptide score (S=0.895; cutoff=0.82) for Robo3b. The most likely cleavage site is predicted to be between positions 16 and 17 (LFT-DT). HMM predicts Robo3b to be a non-secretory protein with a signal peptide probability of 0.01 and a maximum cleavage site probability of 0.004 between the positions 21 and 22 (RD-A).
Neural Networks method assigns lower scores than cutoff scores for raw cleavage site (C=0.123; cutoff=0.33), combined cleavage site score (Y=0.07; cutoff=0.32) and signal peptide score (S=0.223; cutoff=0.82) for Mouse Rig-1. HMM predicts Mouse Rig-1 to be a non-secretory protein with a signal peptide probability of 0.001 and a maximum cleavage site probability of 0.001 between positions 32 and 33 (SPG-SR).
Figure 3.6: Protein localization of Robo3 isoforms in transiently transfected mammalian cells. Myc-tagged robo3 isoforms driven by CMV promoter were transfected into 293T cells and protein expression was detected by immunofluorescence using anti-Myc antibodies. Top panels show Robo3a protein localization and bottom panels show Robo3b localization associated with the cell surface. (A, C are at 20X magnification; B, D are at 40X magnification)
Figure 3.7: RNA expression patterns of robo3a and robo3b during the first day of development. Whole mount RNA in situ hybridization using isoform specific probes showed differential expression of robo3a (A, C, E and G) and robo3b (B, D, F and H) in the developing embryo. A,B,C and D: Lateral views of embryos at 16 h. robo3a RNA is seen in the somites along the trunk (A), and at higher magnification (C) can be seen in the posterior aspect of the somites. In contrast, robo3b is not expressed in the somites (B) but seen in distinct spinal neurons in the trunk (D,F). At 26 h, robo3a is seen diffusely in the ventral spinal cord and robo3b is seen in distinct spinal neurons in the dorsal and medial aspects (E,F). Cross sections of the trunk at 26 h, show that robo3a is seen in cells in medial and ventral spinal cord (G) while robo3b is seen expressed in dorsal and medial cells (H), confirming the observation from lateral views (E,F). Anterior is to the left in A-F and dorsal is to the top in G and H.
Figure 3.8: RNA expression patterns of robo3a and robo3b at 48 h post fertilization.

Lateral (A,C) and dorsal views of the head regions showing whole-mount RNA in situ hybridizations for robo3a and robo3b at 48 h. A,B show the diffuse expression of robo3a in all the regions of the brain while C,D show distinct and robust expression of robo3b in all the regions of the brain. Expression of robo3b in the diencephalic regions (arrowheads in C and D) is obvious, along with a few distinct cell groups in the telencephalon. Overlapping regions of expression in the hindbrain region can be seen for robo3a and robo3b (B and C).
Figure 3.9: RNA expression pattern of robo3b in a 6 day old embryo. robo3b expression is seen as late as 6 days in the brain. The prominent region of the brain expressing the RNA is in the diencephalic region (arrowhead).
(a) RNA in situ hybridization using robo3a and robo3b specific probes on 2 cell stage embryos

(b) RT-PCR amplification of robo orthologs during early development

Figure 3.10: RNA expression of robo orthologs.
(a) Whole-mount RNA in situ hybridization experiments on 2-cell stage embryos show the expression of robo3a and robo3b RNA. (b) RT-PCR experiments using total RNA extracted from different developmental stages (M=maternal, 2-16 cell stage; Z=early zygotic, 512-1000 cell stage; 10= 10 hours and 24= 24 hours) shows that robo1, robo2, robo3a and robo3b are expressed at all stages. The expression levels, as indicated by PCR amplifications, are relatively uniform for robo1, decreased expression at 10 h is observed for robo2 and robo3b, and decreased expression is found at early zygotic stage for robo3a.
Figure 3.11: Western Blot analysis on Robo3 proteins.

In vitro translated, myc-epitope tagged Robo3a and Robo3b proteins were identified by using Anti-myc antibody (Lanes 1 and 2). The same bands were recognized by anti-Robo3 antiserum (Lane 3, Robo3b:myc protein). Robo3 proteins from zebrafish embryo extracts, corresponding to the same size as that of in vitro translated proteins, were also identified by anti-Robo3 antiserum (Lane 4-7). Robo3 proteins were observed as early as 100% epiboly (Lane 4) and continued to be expressed at 11 h, 12 h, and 16-18 h (Lanes 5, 6, and 7 respectively).
(a) Efficacy of robo3 morpholino (MO) mediated gene knock-down

(b) Specificity of robo3 morpholino (MO) mediated gene knock-down

(c) Knock-down of Robo3 protein in morpholino injected embryos

Figure 3.12: Analysis of morpholino efficacy and specificity in knocking down gene expression. Western Blot analysis using anti-myc (a,b) and anti-Robo3(c) antibodies. (a) Coupled in vitro transcription + translation in rabbit reticulocyte lysates, using myc-tagged robo3 isoforms as templates, in the absence of any antisense morpholino (MO) oligonucleotides (Lane 1) or increasing concentrations of the MO from 5nM to 20µM (Lanes 2-5). At 20µM MO concentrations, neither of the Robo3 isoforms are detectable. robo3b specific MO was added at 40µM concentration to robo3a reaction mixture (Lane 6, top row) and vice versa (Lane 6, bottom row). (b) Coupled in vitro transcription + translation reactions for robo3a (Lanes 1-3) and robo3b (Lanes 4-6) in the absence of MO (Lanes 1 and 4), presence of 20µM MO (Lanes 2 and 5) and 20µM 4 base pair (bp) mismatch MO (Lanes 3 and 6). (c) Total protein extracted from 11 h embryos injected with either 0.6mM robo3a+robo3b MOs (Lane 1) or robo3a_4mm+robo3b_4mmMOs (Lane 2). Anti-actin antibody (lower panels) was used as a protein loading control.
Figure 3.13: Severity of phenotypes in robo3b MO injected embryos at 26-28 h.

Injecting 6-9ng MO per embryo results in three classes of phenotypes based on severity. Most severely affected embryos have greatly reduced trunk tissues. Defects in head structures are also visible but all regions of the head are distinguishable, unlike those in the trunk (A). Moderately affected embryos have reduced trunk tissue with frequent observations of the tail/posterior trunk inserted in the yolk (B). Mildly affected embryos exhibit curved tails (C). 70% of the MO injected embryos showed severe to moderate phenotypes (n=300).
Figure 3.14: Morphology of embryos at 11 h.

robo3b MO injected embryos are oblong in shape in comparison to the spherical shape of wild type embryos.
### (a) Summary

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### (b) ANOVA

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<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>1964.86</td>
<td>2</td>
<td>982.43</td>
<td>28.63</td>
<td>3.5E-10</td>
<td>3.11 (95%)</td>
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<tr>
<td>Within Groups</td>
<td>2848.36</td>
<td>83</td>
<td>34.32</td>
<td>4.79-4.98 (99%)</td>
<td>4.79-4.98 (99%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4813.22</td>
<td>85</td>
<td>34.32</td>
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<td></td>
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### (c) Values for Paired Student t Test

<table>
<thead>
<tr>
<th>t values</th>
<th>wildtype</th>
<th>robo3b MO</th>
</tr>
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<tbody>
<tr>
<td>robo3b MO</td>
<td>6.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.00 - 1.98 [95%]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.66 - 2.617 [99%]</td>
<td></td>
</tr>
<tr>
<td>robo3b_4mm MO</td>
<td>0.863</td>
<td>5.288</td>
</tr>
<tr>
<td></td>
<td>2.021 [95%]</td>
<td>2.00 - 1.98 [95%]</td>
</tr>
<tr>
<td></td>
<td>2.704 [99%]</td>
<td>2.66 - 2.617 [99%]</td>
</tr>
</tbody>
</table>

**Table 3.1:** Statistics for the length of embryos along the AP axis.  
F value obtained from Analysis of Variance, 28.63, is greater than the critical F values of 3.11 at p=0.05 and 4.79-4.98 at p=0.01, so the variation in the population is not stochastic. t values obtained from paired statistics show that increased length of robo3b MO injected embryos in comparison to wildtype embryo lengths (6.23) and robo3b_4mm MO injected embryo lengths (5.288) are beyond the acceptance regions at 95% (2.00-1.98) and 99% (2.66-2.62) confidence levels indicating that the differences in the length of embryos is significant. In contrast, the t value obtained for paired statistics of wild type and robo3b_4mm MO injected embryos (0.863) is well within the acceptance region at 95% (2.021) and 99% (2.704) confidence levels indicating that the differences in the length of embryos are not significant.
Figure 3.15: Morphometric analysis of wild type and robo3b morpholino injected embryos at 11 h.
Figure 3.16: Knock-down of robo3b causes expansion of the Kro20 domain. Kro20 and ntl (arrowhead in A) double RNA in situ hybridization in MO injected embryos at 11 h. 6-9 ng of MO or 4 bp mismatch MO were injected into each embryo. 4 bp mismatch MO injected embryos did not exhibit any significant defects in the width of Kro20 domain (A; dotted arrowheads indicate rhombomeres 3 and 5) but robo3b MO injected embryos exhibited expansion of the Kro20 domain (B, C and D). (double headed arrows in A indicate the extent of Kro20 expression).
<table>
<thead>
<tr>
<th>Groups</th>
<th>Count (n)</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
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<td>Wildtype</td>
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<td>448</td>
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<td>528</td>
<td>22.96</td>
<td>9.32</td>
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<tr>
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<td>584</td>
<td>25.39</td>
<td>16.07</td>
</tr>
<tr>
<td>robo3b MO</td>
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<td>2028</td>
<td>40.56</td>
<td>65.64</td>
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<td>robo3b_4mm MO</td>
<td>28</td>
<td>657</td>
<td>23.46</td>
<td>16.11</td>
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</table>

(a) Summary

<table>
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<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>10319.36</td>
<td>4</td>
<td>2579.84</td>
<td>80.57</td>
<td>1.894E-35</td>
<td>2.44 (95%)</td>
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<tr>
<td>Within Groups</td>
<td>4514.81</td>
<td>141</td>
<td>32.01993</td>
<td>3.41-3.48 (99%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14834.17</td>
<td>145</td>
<td></td>
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</tbody>
</table>

(b) ANOVA

<table>
<thead>
<tr>
<th>t values</th>
<th>robo3a MO</th>
<th>robo3b MO</th>
</tr>
</thead>
<tbody>
<tr>
<td>robo3a_4mm MO</td>
<td>1.618</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.704 - 2.66 [99%]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.021 - 2.00 [95%]</td>
<td></td>
</tr>
<tr>
<td>robo3b_4mm MO</td>
<td>10.612</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.66 - 2.617 [99%]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.00 - 1.98 [95%]</td>
<td></td>
</tr>
</tbody>
</table>

(c) Values for Paired Student t Test

Table 3.2 : Statistics for the width of Krox20 domain at 11 h.

F value obtained from Analysis of Variance, 80.57, is greater than the critical F values of 2.44 at p=0.05 and 3.41-3.48 at p=0.01, so the variation in the population is not stochastic. t values were obtained from paired statistics of experimental and 4 bp mismatch control MOs for robo3a (1.618) and robo3b (10.612). t value for robo3a MOs lies within the acceptance region at 95% (2.021-2.00) and 99% (2.704-2.66) confidence levels indicating that the variation is not significant. In contrast, t value for robo3b MOs does not lie in the acceptance region either at 95% (2.00-1.98) or 99% (2.66-2.617) confidence levels.
Figure 3.17 : Analysis of Krox20 expression domain in wildtype and morpholino injected embryos
Figure 3.18: Znp1 antibody staining on wildtype and robo3a MO injected embryos.
Lateral views of uninjected (A) and robo3a MO injected embryos (B,C and D) at 36 h showing the trajectory of ventrally projecting motor axons. All axons reach the ventral aspect of the somites in wild type embryos (A) while motor axon truncations were seen in robo3a MO injected embryos (arrowheads in B and C). Embryos in B and D were morphologically wild type while the embryo in D had visible trunk defects.
CHAPTER 4

DISCUSSION

Zebrafish has emerged as a very powerful genetic model system enabling us to understand and elucidate complex events in vertebrate development and disease. The ability to perform large-scale mutagenesis screens has facilitated the identification and study of several important and interesting genes and their mechanism of action. In addition to being amenable to forward genetic analysis, reverse genetic strategies have become popular tools for understanding the function of evolutionarily conserved and novel genes in the zebrafish genome. Reverse genetics approaches are becoming very relevant as the genome sequencing of zebrafish is nearing completion. Reverse genetics approaches facilitate better understanding of pleiotropic effects caused by evolutionarily conserved genes and gene families. This dissertation describes the identification and characterization of orthologs of the evolutionarily conserved Roundabout (Robo) gene family. Analysis of the functional role of one of the orthologs using an antisense gene knock-down strategy during zebrafish development is discussed.

Robo proteins are receptors for the Slit family of secreted ligands. Robo proteins act as ‘gatekeeper’ molecules in controlling the crossing behavior of commissural and ipsilateral axons across the Drosophila midline (Kidd et al., 1998; Kidd et al., 1999). Multiple Robo genes have been found in Drosophila and they coordinate to pattern the lateral axon tracts in the Drosophila CNS (Rajagopalan et al., 2000a; Rajagopalan et al., 2000b). One of the Robo family orthologs in
zebrafish, robo2/astray, controls the motility behavior of retinal axons as they project towards their contralateral tectal targets (Hutson and Chien, 2002; Fricke et al., 2001). In essence, Robo proteins, via their likely interactions with Slit ligands, modulate motility of axonal growth cones thus contributing to the proper ‘wiring’ of nervous system.

Robo-Slit interactions initiate a cascade of signaling events with several cytoplasmic players that eventually lead to the remodelling of the actin cytoskeleton (Wong et al., 2002). Remodelling of the actin cytoskeleton is the basis of most cell motility behaviors in animal cells. Therefore, the role of axon guidance molecules in other cell motility phenomena is conceivable. Indeed, Slit proteins and Robo-Slit interactions have been shown to play roles in cell migratory events other than axon growth cone motility (De Castro, 2003; Kramer et al., 2001; Brose et al., 2000).

Gastrulation encompasses a complex set of cell movements that are orchestrated to create the basic body structure of the developing embryo. An earlier study suggested the role of Slit proteins during zebrafish gastrulation (Yeo et al., 2001). Our study shows evidence for the role of zebrafish Robo family members during the same process. In addition to their role in gastrulation, this study also unveils a possible role of Robo proteins in zebrafish motor axon guidance. This raises many more questions with regards to the specific role of Robos and Slits during these developmental processes.

Directional migration of cells towards the dorsal midline by chemotaxis during teleost gastrulation has been well described (Trinkaus, 1998). But the cues which direct the migration, although hypothesized, have not yet been identified. With the observations from our study and by Yeo et al. (2001) it is likely that Robo and Slit molecules are part of the chemotactic machinery directing the migration of cells towards the midline. Further studies addressing the physical interactions between Robo and Slit molecules during gastrulation need to be carried out. These
studies also will be able to address the mechanism by which Robo and Slit molecules cause the directional migration. The mechanisms mediated by Robo-Slit interactions become important since, like many other axon guidance molecules, they can play roles in both attractive and repulsive guidance behaviors. The possible interactions between multiple Robo orthologs during gastrulation add another dimension to this scenario. The elucidation of Robo-Slit functions during gastrulation can be furthered by concurrently studying the downstream signaling events and in other cell motility behaviors, e.g. axon growth cone motility or primordial germ cell migration.

Although some suggestions about the function of Robos in non-neuronal cells are available, their dynamic expression in a range of tissues in vertebrates cannot yet be adequately explained in a functional context. It is very plausible that Robo signaling can have effects distinct from those that have been described in the growth cones of extending neurites. For example, the complementary expression patterns of Robo and Slit orthologs in the somitic mesoderm in both zebrafish and mice are very intriguing (Chapter 3; Yuan et al., 1999). Extensive cell movements are involved in the formation of the limb/fin buds. Expression patterns of Robo orthologs in limb/fin buds also raise interesting questions with regards to their role in limb formation by affecting cell movements.

Although several examples point to the role of Robo and Slit molecules in axon repulsion, very little is known about the exact mechanism by which repulsion is caused. In this context, a number of other molecules and their interactions have to be placed in the signaling cascade. Glypican-1, a cell surface heparan sulfate proteoglycan, interacts with Slit proteins and enhances binding affinity with Robo receptors. This interaction is essential for the repulsive activity of Slit. These studies were done in cell culture assays and thus it remains to be seen how the interaction between the three molecules affect various developmental processes (Hu, 2001; Liang et al., 1999; Ronca et al., 2001). Whether a glypican is involved in enhancing Robo-Slit interactions during
gastrulation and even in axon guidance is yet to be tested. It is interesting to note that the zebrafish mutation knypek (kny), which causes defects in C&E cell movements during gastrulation, is also a heparan sulfate proteoglycan belonging to the glypican family (Topczewski et al., 2001). Although a genetic interaction between kny and the non-canonical Wnt signaling pathway has been demonstrated, the possibility of kny’s role in Robo-Slit interactions can also be tested.

Stromal Derive Factor-1 (SDF-1), a chemokine, and its receptor are involved in leukocyte migration. Robo and Slit interact with SDF-1 and its receptor to inhibit leukocyte chemotaxis (Wu et al., 2001). SDF-1 is also required in zebrafish primordial germ cell (PGC) migration and control of lateral line primordium. Not only do these molecules play a role in cell migration, but a recent study by Chalasani and co-workers (2002) showed that SDF-1 has modulatory effects on axonal responsiveness to various guidance cues. SDF-1, via its receptor CXCR4, reduces the repellent activity of Slit-2 in axons of cultured retinal ganglion cells. It is not clear whether this SDF-1 mediated reduction in Slit-2 repellent activity is via the Robo receptors.

Understanding the complexity of cross talk between molecules in the extracellular environment and membrane-associated proteins will enable us to reveal the specificity of mechanisms causing attraction or repulsion in migrating cells. This will facilitate the delineation of the molecular toolkit which could either make a cell move towards or away from an environmental cue. Understanding the conservation and differences of molecular mechanisms will also enable us to address the origins and evolution of animal cell motility.

Our functional analysis employing an antisense gene knock-down strategy has revealed novel functions for Robo orthologs during embryonic development. Due to the presence of maternal transcripts of Robo3 isoforms in zebrafish, a classical zygotic loss-of-function mutagenesis screen would not be able to unravel the early requirement of this gene product. In contrast, the requirement of the gene product at later time points in development cannot be studied very well
using the antisense strategy. Therefore, both the forward and reverse genetic strategies truly complement each other in understanding the role of genes functioning at early and late embryonic development. An upcoming reverse genetic strategy - TILLING (Targeting Induced Local Lesions IN Genomes) - will aid better understanding of gene function by its ability to uncover an allelic series of gene specific induced point mutations (McCallum *et al.*, 2000; Wienholds *et al.*, 2002).


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APPENDIX A

ZEBRAFISH DEVELOPMENTAL STAGING

<table>
<thead>
<tr>
<th>Period</th>
<th>Time Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zygote Period</td>
<td>0-3/4 h</td>
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<tr>
<td>Cleavage Period</td>
<td>0.7- 2.2 h</td>
</tr>
<tr>
<td>2-cell stage</td>
<td>3/4 h</td>
</tr>
<tr>
<td>4-cell stage</td>
<td>1 h</td>
</tr>
<tr>
<td>8-cell stage</td>
<td>1 1/4 h</td>
</tr>
<tr>
<td>16-cell stage</td>
<td>1 1/2 h</td>
</tr>
<tr>
<td>32-cell stage</td>
<td>1 3/4 h</td>
</tr>
<tr>
<td>64-cell stage</td>
<td>2 h</td>
</tr>
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<td>Blastula Period</td>
<td>2 1/4 - 5 1/4 h</td>
</tr>
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<td>128-cell stage</td>
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</tr>
<tr>
<td>256-cell stage</td>
<td>2 3/4 h</td>
</tr>
<tr>
<td>512-cell stage</td>
<td>2 3/4 h</td>
</tr>
<tr>
<td>1-k-cell stage</td>
<td>3 h</td>
</tr>
<tr>
<td>High stage</td>
<td>3 1/3 h</td>
</tr>
<tr>
<td>Sphere stage</td>
<td>4 h</td>
</tr>
<tr>
<td>Dome stage</td>
<td>4 1/3 h</td>
</tr>
<tr>
<td>30%-epiboly stage</td>
<td>4 2/3 h</td>
</tr>
<tr>
<td>Gastrula Period</td>
<td>5 1/4 - 10 1/3h</td>
</tr>
<tr>
<td>50%-epiboly stage</td>
<td>5 1/4 h</td>
</tr>
<tr>
<td>Germ-ring stage</td>
<td>5 2/3 h</td>
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<tr>
<td>Shield stage</td>
<td>6 h</td>
</tr>
<tr>
<td>75%-epiboly stage</td>
<td>8 h</td>
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<tr>
<td>90%-epiboly stage</td>
<td>9 h</td>
</tr>
<tr>
<td>Bud stage</td>
<td>10 h</td>
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<tr>
<td>Segmentation Period</td>
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<tr>
<td>1-somite stage</td>
<td>10 1/3 h</td>
</tr>
<tr>
<td>5-somite stage</td>
<td>11 2/3 h</td>
</tr>
<tr>
<td>14-somite stage</td>
<td>16 h</td>
</tr>
<tr>
<td>20-somite stage</td>
<td>19 h</td>
</tr>
<tr>
<td>26-somite stage</td>
<td>22 h</td>
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Table A.1: Embryonic Stages.

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<td>Prim-15 stage</td>
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<td>High-pec stage</td>
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<td>Pec-fin stage</td>
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