Chapter I

INTRODUCTION

The Nervous system

> "Our nervous system developed for one sole purpose, to maintain our lives and satisfy our needs. All our reflexes serve this purpose. This makes us utterly egotistic. With rare exceptions people are really interested in one thing only: themselves. Everybody, by necessity, is the center of his own universe."-- Albert Szent-Györgyi

The nervous system is a network of specialized cells that allows an organism to be aware of itself and its environment and allows the organism to interact with its environment. The nervous system is traditionally broken up into 2 systems: the central nervous system (CNS) and the peripheral nervous system (PNS). The peripheral nervous system is further subdivided into the autonomic nervous system (ANS) and the somatic nervous system (SNS) . The ANS, often referred to as the visceral nervous system, receives sensory information from the internal body organs and acts as a control center by which the body can maintain homeostasis. The ANS generally controls the body's involuntary functions such as heart rate, digestion, respiration rate, perspiration, urination, sexual arousal and pupil size (Tortora and Anagnostakos, 1990).

The SNS, also called the voluntary nervous system, is the second component of the peripheral nervous system. The SNS controls all voluntary body movements through innervation of skeletal muscle and reception of external stimuli such as skin and sense organs (Tortora and Anagnostakos, 1990). This allows the body to physically respond to changes in the environment. Although, both branches of the peripheral nervous system are important in maintaining homeostasis and responding to environmental changes, the PNS alone cannot elicit a response. The PNS requires that its sensory information be wired through the CNS in order to illicit a motor response.

Our ability to integrate sensory information and respond to stimuli is a direct result of the control center of the body, the CNS. The CNS consists of the brain and spinal cord, and is responsible for regulating organ function, higher thought, and body movements (Fig. 1). The CNS functions as a relay station, where afferent information is passed through the spinal cord into the brain where the sensory information is processed and an appropriate efferent response is elicited.

Development of the Central Nervous System

Neurulation

After gastrulation, an embryo has a defined body axis as well as three defined germ layers: endoderm, mesoderm and ectoderm. Shortly after the specification of the three germ layers, the notochord, a mesodermal rod like structure, which defines the body axis in early embryos, begins secretion of the signaling

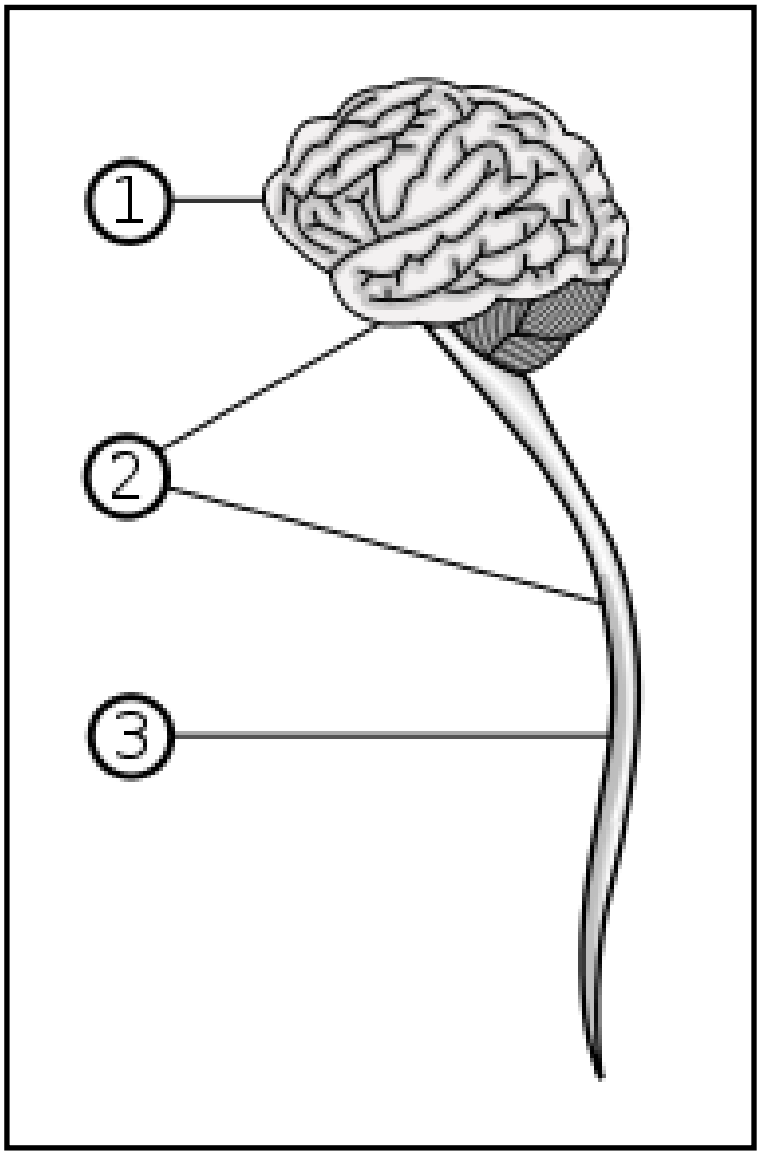


Figure 1. The Central nervous system. The central nervous system (CNS) is one of the two principal divisions of the body's nervous system. Consisting of the brain(1) and the spinal cord(3), the CNS(2) is the control center for the entire nervous system.

molecule Sonic hedgehog (Shh). Shh an antagonist of the growth factor bone morphogenic protein (BMP) restricts BMP expression from the overlying ectoderm to form the neural ectoderm or neural plate. This neural plate undergoes a series of precise morphogenic movements in which the neural plate converges and intercalates to form the hollow neural tube. The process by which this occurs is termed neurulation. If these morphogenic movements are not precise, neural tube defects like spina bifida can occur. Spina bifida is a congenital birth defect that happens when the neural tube fails to close and portions of the spinal cord reside outside the vertebral column (Kondo et al., 2009).

Although the end result of neurulation may be the same, the process by which various organisms undergo neurulation may be different. For example, frog, rabbit, mouse and chick undergo a t.wofold neurulation process characterized as primary and secondary neurulation, whereas neurulation in some vertebrates like zebrafish has been characterized only as secondary neurulation (Copp et al., 2000; Copp et al., 2003). During primary neurulation, the neural plate invaginates, while the lateral ends of the neural plate converge towards each other. As the lateral ends of the neural plate meet they downregulate expression of E-cadherin, an epithelial cell adhesion marker and begin expressing N-cadherin and N-CAM, neural cell adhesion molecules (Copp et al., 2003; Hong and Brewster, 2006; Lowery and Sive, 2004). After the neural tube fuses, the neural crest cells migrate away from the dorsal neural tube and the non neural ectoderm slides over the neural tube and fuses. Secondary

neurulation refers to the process in which the neural plate ectodermn condenses to form a neural tube, instead of the neural plate rolling. (Copp et al., 2003; Lowery and Sive, 2004).

Unlike in birds and mammals, zebrafish neurulation initially begins with the formation of the neural keel a solid rod like structure that was initially thought to be a mass of mesenchymal cells. The lumen or central canal forms through a secondary process known as cavitation. During cavitation, the ventral cells of the neural keel downregulate cell adhesion markers and begin to separate in a ventral to dorsa fashion creating a central canal throughout the length of the CNS (Lowery and Sive, 2004; Schmitz et al., 1993). Adding to the confusion, in zebrafish the neuroectoderm does not express any of the classical polarized epithelial cell markers such as zona occludins 1 (ZO-1) or occludins (Aaku-Saraste et al., 1996; Geldmacher-Voss et al., 2003). Despite the lack of epithelial cell markers, lineage tracing and time-lapse imaging provides evidence that these columnar neural plate cells move and behave like an epithelium (Papan and Campos-Ortega, 1994). Hence, zebrafish neurulation may have more in common with primary neurulation than was previously thought.

Dorsal-ventral patterning of the Central Nervous System

No matter how an organism constructs its neural tube, the end result is a neural tube consisting of naïve neuroepithelial cells that require specific genetic instructions in order for them to differentiate and populate the CNS. Because the CNS is composed of the brain and spinal cord, both of which have their own

specific mechanisms of dorsoventral patterning, I will focus on dorsoventral patterning of the spinal cord as it is better understood and more relevant to my research.

Dorsoventral patterning of the spinal cord is the process by which signaling molecules and transcription factors pattern the spinal cord so that cell specification and cell distribution occurs within spatially restricted domains. After neurulation, the primitive neural tube is induced by high levels of Shh secreted by the notochord to form the floor plate. The floor plate is a group of non neuronal ventral midline cells, that express and secrete the morphogen Shh. Concomitant with Shh secretion, the dorsal midline cells, begin expressing and secreting the TGFβ growth factor, Bone morphogenic protein (BMP) (Altman and Bayer, 1984; Placzek et al., 1991; van Straaten and Hekking, 1991; van Straaten et al., 1985; Yamada et al., 1991) (Fig. 2). BMP and Shh are distributed in opposing gradients, which establishes different concentrations of the two morphogens along the dorsal ventral axis of the spinal cord (Altman and Bayer, 1984; Jessell, 2000; Lee and Jessell, 1999; Mekki-Dauriac et al., 2002). This differential concentration of the two morphogens creates discrete domains where specific transcription factors are activated based on the amount of Shh and BMP present. This allows for specific cell types to be specified within certain areas along the dorsal ventral axis of the spinal cord (Rowitch, 2004) (Fig. 2). Loss and gain of function experiments revealed that these domains can regulate their adjacent domains and that disruption of domain specific transcription factors or signaling molecules leads to an expansion or a loss of an adjacent domain. Therefore to

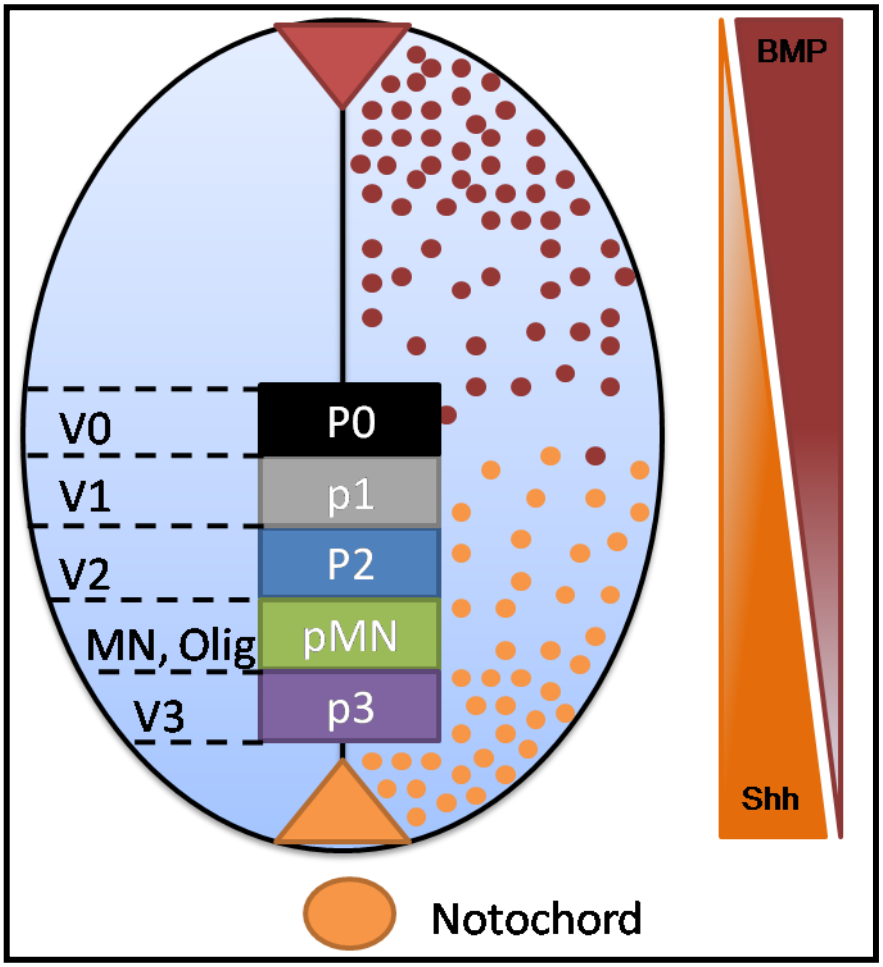


Figure 2. Dorsal-ventral patterning of the spinal cord. Opposing concentration gradients of Sonic hedgehog (Shh), and Bone Mophogenic Protein (BMP) create distinct and discrete domains within the spinal cord. These domains express unique combinations of homeobox domain proteins, that give rise to distinct neural cell types.

maintain normal number and distribution of neural cells it is important that the spinal cord is patterned correctly along its dorsal ventral axis.

Ventral spinal cord domains

The ventral spinal cord is composed of five distinct precursor domains: p0, p1, p2, pMN and p3 (Fig. 2). Each of these domains is patterned by the concentration of Shh they receive. Just lateral to the ventral midline floor plate cells is the p3 domain. The homeobox domain protein Nkx2.2 is expressed in this domain and restricts *pax6* expression dorsally. In the p3 domain, *Nkx2.2* expression specifies v3 interneurons, perineurial glia and a subset of oligodendrocytes (Briscoe et al., 1999; Kim et al., 2008a; Kim et al., 2008b; Kucenas et al., 2008). Dorsally adjacent to the p3 domain is the progenitor motor neuron domain (pMN), which gives rise to motor neurons, adult proliferative precursors and most oligodendrocytes. The pMN domain expresses the basic helix loop helix transcription factor Olig2, which has been shown to be important in both motor neuron and oligodendrocyte specification (Park et al., 2002; Rowitch, 2004). Just dorsal to the pMN domain are the p2, p1 and p0 domains. These domains give rise mainly to interneurons with each domain specifying a specific subtype of interneurons (Batista et al., 2008; Burrill et al., 1997; Ericson et al., 1996; Ericson et al., 1997) (Fig. 2).

Major Cell types of the Central Nervous System

The central nervous system consists of neurons and glial cells. Neurons comprise about half the volume of the CNS and glial cells account for the rest. Together these two populations of cells function to maintain CNS homeostasis by receiving signals from throughout the body, interpreting these signals and then coordinating the body's response.

Neurons

In 1923, Santiago Ramon y Cajal, the father of modern neuroscience, described the idea of neurons as the fundamental unit of the nervous system, which he called the neuron theory. While much has changed in the field of neuroscience, his neuron theory still remains accurate (Lopez-Munoz et al., 2006). Neurons are a major class of cells in the nervous system. In vertebrates, neurons are found in the brain, the spinal cord and in the nerves and ganglia of the peripheral nervous system. They have excitable membranes, which allow them to generate and propagate electrical impulses. There are three types of neurons in the vertebrate body: sensory neurons, interneurons and motor neurons. Although the size and functions of a neuron may differ, their overall morphology remains the same. They all have an axon, a cell body and dendrites. The axon is the part of the neuron that responsible for conducting and propagating nerve electrical impulses. A cell body or soma contains the nucleus and other organelles. The dendrites are the branched projections of the cell body that receives and transmits electrical impulses from other neurons

Motor neurons

Our ability to move our body within our environment is due directly to the functions of motor neurons. Motor neurons are born in the CNS but project their axons into the PNS, where they innervate muscle directly. Zebrafish undergo a two stage neurogenesis. Primary neurogenesis starts at around 9-10 hpf when the primary motor neurons are formed (Kimmel et al., 1994; Myers et al., 1986). At about 16 hpf the first secondary motor neurons appear (Myers et al., 1986). These two classes of motor neurons differ in the size, shape and position in the spinal cord. The early born primary motor neurons have larger somata and axons, and are usually found more dorsal to the secondary motor neurons (Myers et al., 1986). The zebrafish primary motor neurons are divided into four subtypes: CaP, RoP, MiP and VaP. Three of these primary motor neuron subtypes are named for their positions within the hemisegment: CaP-caudal primary motor neuron, RoP- rostral primary motor neuron and MiP- middle primary motor neuron (Bernhardt et al., 1990; Hutson and Chien, 2002; Westerfield et al., 1986). The fourth primary motor neuron type, variable primary motor neurons (VaP), appears transiently and is normally eliminated via apoptosis (Westerfield et al., 1986).

In the pMN domain the transcription factor Olig2 and the LIM homeobox genes *islet1* and *islet2* are important in specifying primary motor neurons (Hutchinson and Eisen, 2006; Park et al., 2002; Takebayashi et al., 2002). Loss of function experiments, using anti-sense morpholino oligoneucleotides, showed

that reducing *olig2* expression in the spinal cord produced fewer primary motor neurons. Conversely, RNA overexpression of *olig2* increased the number of primary motor neurons in the spinal cord (Park et al., 2002). Similar loss of function experiments showed that in the absence of *islet1*, primary motor neuron numbers were reduced (Hutchinson and Eisen, 2006).

Hedgehog signaling has also been shown to play an important role in specifying motor neurons. Using the *smoothened* mutants (*smu*), which lack functional Hedgehog signaling protein Smoothened and are devoid of Hedgehog signaling, primary motor neurons are reduced and secondary motor neurons are absent (Chen et al., 2001; Lewis and Eisen, 2001). Evidence suggests that the relatively few primary motor neurons that are specified in *smu* mutants are the result of lingering activity of the maternal Smoothened protein, because embryos which completely lacked Hedgehog signaling were devoid of primary motor neurons (Lewis and Eisen, 2001).

Sensory Neurons

Sensory neurons are nerve cells within the nervous system responsible for converting external stimuli from the organism's environment into internal electrical impulses. Sensory nerves take in and communicate information about temperature, pressure, pain and position. Some sensory neurons respond to tactile stimuli and can activate motor neurons in order to achieve muscle contraction. Such connections between sensory and motor neurons underlie motor reflex loops and several forms of involuntary behavior, including pain

avoidance. In humans, such reflex circuits are commonly located in the spinal cord.

Rohon-Beard Neurons

For animals that develop outside their mothers the ability to respond to light and other external stimuli early during development is important. Rohon-Beard (RB) neurons are the first sensory neurons to develop in anamniote vertebrates and they control embryonic and early larval senses and movements (Clarke et al., 1984a; Williams et al., 2000). They develop along the dorsal spinal cord, beginning in the hindbrain and terminating in the caudal spinal cord. RB neurons are known by their large cell body as well as their characteristic morphology. Each RB neuron extends axons rostrally towards the hindbrain and caudally with the spinal cord (Bernhardt et al., 1990; Metcalfe et al., 1990). They also extend a peripheral axon that exits the spinal cord, and terminates its free nerve ending in the skin. As the sensory neurons of the dorsal root ganglion develop they functionally replace the RB neurons, and as a consequence the RB neurons undergo apoptosis (Williams et al., 2000). However, some organisms like the newt and lamprey maintain their RB neurons into adulthood (Clarke et al., 1984b; Nakao and Ishizawa, 1987).

Glia

In 1856, German pathologist, Rudolph Virchow observed a non neuronal component to the nervous system. He called this non neuronal nervous tissue glia, which when translated means putty or glue. He hypothesized that glia

functioned as a sort of connective tissue that provided rigidity and support to the nervous system (Webster and Astrom, 2009). For most of the 20th century glia were described as providing physical and structural support for neurons, and although the supportive function ascribed to glia was correct, scientists now believe glia to be more dynamic than once thought. Glial cells insulate neurons, maintain homeostasis, remove cellular debris from the nervous system, and serve as adult stem cells (Lledo et al., 2008; Streit, 2000; Streit et al., 1988; Webster and Astrom, 2009).

Radial Glia

Radial glia are a type of glia that are defined by two characteristics. Firstly, they extend a radial process from their cell bodies which make contact with the pial surface, and secondly, like most glia they express glial fibrillary acid protein (GFAP). They are derived from polarized neuroepithelial cells early in development, and like neuroepithelial cells they are also polarized, having apical-basal cell polarity (Temple, 2001). Just prior to the end of embryonic development, a portion of radial glia tranform into astrocytes in the adult mammalian CNS (Cepko et al., 1993; Halliday and Cepko, 1992; Noctor et al., 2001; Walsh and Cepko, 1993). Historically radial glia were thought to have a supportive role in CNS development, in which they serve as a scaffold for migrating neurons. However, emerging data argues that radial glia are neurogenic, gliogenic and give rise to adult neural stem cells in the mammalian and zebrafish CNS (Anthony et al., 2004; Malatesta et al., 2000; Merkle et al., 2004; Park et al., 2007).

Using retroviral labeling of radial glia in embryonic mice, it has been shown *in vivo* that radial glia can divide asymmetrically producing neurons (Ever and Gaiano, 2005; Fishell and Kriegstein, 2003; Gotz et al., 2002). In the mouse striatum, radial glia derived from neuroepithelial cells give rise to adult stem cells (Gates et al., 1995; Mao et al., 2001; Sundholm-Peters et al., 2004). These adult stem cells proliferate in the adult brain and give rise to neurons in the olfactory bulb and the hippocampus (Ihrie and Alvarez-Buylla, 2008). Lineage tracing experiments show that these striatial radial glia can give rise to astrocytes, oligodendrocytes and many neuronal subtypes. In the zebrafish spinal cord, the *olig2*$^{+}$ expressing radial glia display many of the characteristics of post embryonic stem cells. They appear to divide asymmetrically and give rise to oligodendrocytes but not neurons (Merkle et al., 2004; Park et al., 2007).

Oligodendrocytes

Oligodendrocytes are the myelinating cells of the CNS and they function to increase the rate of which an electrical impulse can travel along an axon in the central nervous system. Oligodendrocytes achieve this by wrapping their plasma membranes around an axon and producing a lipid rich material known as myelin (Simons and Trotter, 2007). Myelin insulates the axon and allows for electrical impulses to jump between areas of myelination, which speeds up the rate of which the electrical impulse travels. This jumping of electrical impulses is technically referred to as saltatory conduction. As an embryo develops the need for myelination increases, as these electrical impulses are now required to travel longer distances.

Most oligodendrocytes are born in the ventral spinal cord, in the pMN domain after primary motor neurons are specified. Loss and gain of function experiments have shown that the *Olig* family of genes are required for specification of oligodendrocytes. In mice, loss of *Olig* gene function resulted in a dramatic reduction in the number of oligodendrocytes in the spinal cord (Lu et al., 2002; Park and Moon, 2002; Zhou and Anderson, 2002). Conversely, overexpression of *olig2* lead to an increase in oligodendrocytes (Park et al., 2002; Park and Moon, 2002; Zhou and Anderson, 2002). The continued expression of *olig2* may be critical for oligodendrocyte development, because unlike neurons that downregulate *olig2* expression shortly after specification, OPCs express *olig2* during all stages of oligodendrocyte development.

Specification of spinal cord OPCs is also regulated by Delta-Notch signaling. Delta-Notch is a highly conserved cell to cell signaling pathway that has been shown to be important in several cell regulatory processes like cell differentiation. In mutants lacking Delta-Notch signaling, excess motor neurons are specified at the expense of OPCs. In these mutants, the amount of cell division is reduced when compared to wild type suggesting that in the absence of Delta-Notch signaling, the proliferative OPCs exit the cell cycle and prematurely differentiate. In addition to controlling cell cycle exit Delta-Notch signaling regulates the levels of *olig2* expression in the spinal cord. Regulation of *olig2* expression is critical for specification of OPCs. In Delta-Notch signaling mutants, *olig2* expression is absent from the developing CNS, however, *ngn1*, which specifies motor neurons in the pMN domain, is increased (Yang et al., 2006).

These data argue that Delta-Notch signaling is required to maintain pMN precursors so that they are able to produce OPCs later in development.

In the zebrafish spinal cord, OPCs are specified at 36 hpf when they begin expressing the transcription factor and OPC specification marker Sox10 . At this stage OPCs are immature and have a bipolar morphology. At around 50 hpf spinal cord OPCs begin migrating out of the ventral *olig2* domain. As they migrate OPCs mature and change their morphology. Their cell bodies are no longer rounded and take on a more elongated appearance. They also extend more filopodial-like processes and begin searching out their target axons. At 72 hpf the OPCs mature to oligodendrocytes and begin wrapping their target neurons. Shortly after wrapping oligodendrocytes begin myelinating axons. This stage is marked by their expression of the myelin genes myelin basic protein (*mbp*) and proteolipid protein (*plp/dm20*).

Pathogenesis

In the absence of properly functioning oligodendrocytes, conduction of electrical signals throughout the CNS is impaired leading to loss of motor skills, sensations and cognition. Multiple sclerosis, Devic's disease and optic neuritis are some CNS demyelinating diseases in which oligodendrocytes lose their ability to wrap axons. Demyelinating diseases refers to any disease or condition that results in damage to the myelin sheets. The term demyelinating disease refers to the condition rather than the cause, as there are several demyelinating disease, many of which have their own etiology.

Multiple sclerosis is an autoimmune disease, characterized by the inflammatory demyelination of neurons (Hewagama and Richardson, 2009). In multiple sclerosis, the body recognizes myelin basic protein and proteolipid protein as foreign and mounts an immune response against the myelin sheath leading to the formation of lesions or plaques (Hewagama and Richardson, 2009; Korn, 2008; Weiner, 2009). Multiple sclerosis affects women more than men with onset of the disease presenting between 15 and 50 years of age(Korn, 2008). Although this disease was first indentified over a century ago, the etiology of the disease still remains unclear. The prevailing hypothesis is that genetically susceptible individuals get infected with a virus. This hypothesis proposes that the body mounts a response to the viral antigens, which are similar to myelin protein genes (Hewagama and Richardson, 2009; Korn, 2008; Weiner, 2009). The inability of the body's immune cells to distinguish between viral antigens and self myelin proteins lead to an inflammatory attack on the oligodendrocytes. Evidence suggests that the body tries to remyelinate axons but is unsuccessful. Therefore, by understanding the mechanisms that regulates oligodendrocyte specification and differentiation scientist are hopeful that they will be able to provide effective therapeutic strategies.

Stem Cells

Stem cells are classically defined as a type of undifferentiated cells that can self-renew and give rise to many different cell types (Fig. 3). They are believed to function as a sort of internal repair system, dividing essentially without limit to replenish diseased or dying cells for the life of the organism. In addition, they

function to maintain the normal turnover of regenerative organs, such as blood, skin or intestinal tissues. Stem cells have the ability to go through numerous cycles of cell division while maintaining an undifferentiated or naive state. Secondly, stem cells are classified based on their potency (Alison and Islam, 2009; Alison et al., 2002). Potency refers to the differentiation potential of the stem cell, or the capacity of the stem cell to differentiate into specialized cell types. Most stem cells are classified as pluripotent or multipotent. Pluripotent stem cells have the ability to differentiate into nearly all cell types, whereas multipotent stem cells have a more limited cell fate potential (Alison and Islam, 2009; Alison et al., 2002). Multipotent stem cells can differentiate into a number of cell types, but only those cell types associated with a particular organ system.

Currently, scientists have categorized stem cells into two broadly defined groups: embryonic stem cells and adult stem cells. Embryonic stem cells (ES cells) are stem cells derived from the inner cell mass of an early stage of mammalian embryo known as a blastocyst. ES cells are pluripotent, as they are able to differentiate into all derivatives of the three primary germ layers. Because they are pluripotent, scientists are hopeful that ES cells may be used for tissue transplantation in the future.

Adult stem cells are undifferentiated cells, found throughout the body after embryonic development, that replenish dying cells and regenerate damaged tissues. Scientific interest in adult stem cells has centered on their ability to self-renew indefinitely and generate all the cell types of the organ from which they

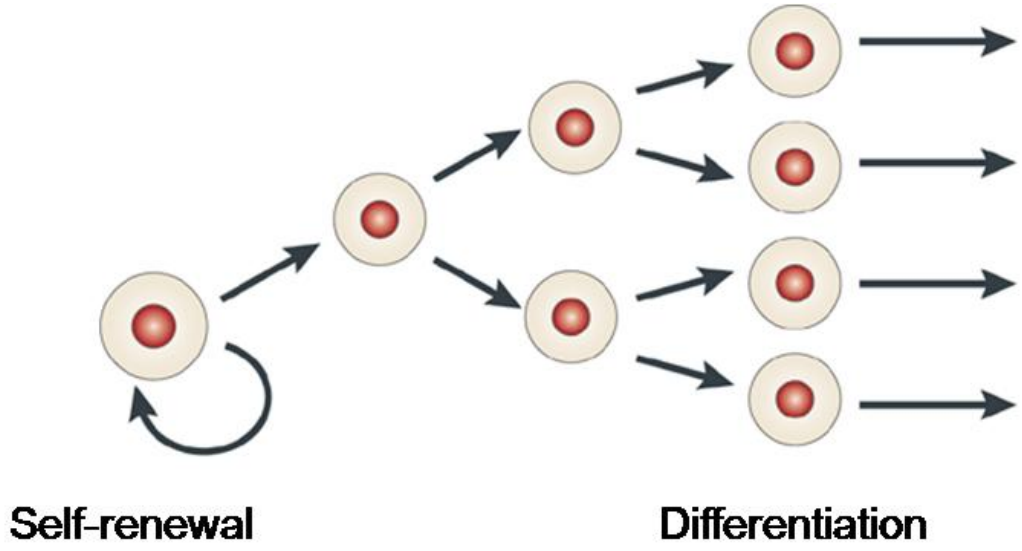


Figure 3. Stem Cells have the ability to self-renew and differentiate. Stem cells divide asymmetrically. Asymmetric cell division produces 2 daughter cells with different properties. Stem cells divide asymmetrically to give rise to two distinct daughter cells: a copy of themselves and a cell that is programmed to differentiate into another cell type.

originate. Adult stem cells are found within discrete tissue pockets called niches. These stem cell niches provide support and maintenance of the adult stem cell by regulating their proliferation and differentiation (Walker et al., 2009). The adult stem cells in these niches are believed to be specified early in development and proliferate undifferentiated until they receive the proper signals to initiate differentiation (Walker et al., 2009). Currently, the mechanisms that specify these adult stem cells early in development are not clearly understood.

At the end of neurulation, the mammalian CNS is made up of a pseudostratified epithelium where bipolar neuroepithial cells span the entire thickness of the neural tube. Neuroepithelial cells represents a population of embryonic precursor cells that give rise to all cell types of the developing CNS. These neural precursors proliferate to increase their numbers, and undergo a characteristic movement of the nucleus (interkinetic nuclear migration) between the basal and the apical surface (Baye and Link, 2008; Sauer, 1936). The position of the nucleus is determined by its progression through the cell cycle. At mitosis, the nucleus resides at the apical surface near the ventricle. During G1 the nucleus begins moving towards the basal surface and reaches its most basal location in S phase. During G2, the neuroepithelial nucleus begins its migration back to the apical membrane (Baye and Link, 2008). Because, proliferation of these neuroepithelial cells is not synchronized, it is possible for these cells to be in different phases of the cell cycle at any given time. This asynchronous proliferation results in the epithelium being pseudostratified (Sauer, 1936). Although interkinetic nuclear migration has been established in the mammalian

CNS, at present no evidence argues for this occurring in the zebrafish brain and spinal cord. However, there is evidence for interkinetic nuclear migration occurring in retinal development (Baye and Link, 2007; Baye and Link, 2008).

After several rounds of cell divisions, lineage tracing experiments demonstrated that many neuroepithelial cells are bi-potent progenitors that give rise first to neurons and then to radial glial cells (Williams and Price, 1995). Already at this early stage some neuroepithelial cells give rise exclusively to neurons or glial cells implying a certain degree of lineage heterogeneity (McCarthy et al., 2001; Williams and Price, 1995). Eventually as development continues, radial glial cells become the predominant precursor population after the onset of neurogenesis. In the mouse cortex, the transition of neuroepithelial to radial glial cells appears to occur around embryonic day E10. This transition is characterized by expression of astroglial markers such as GLAST and BLBP and an alteration in tight junctional coupling (Aaku-Saraste et al., 1996; Malatesta et al., 2003). The basic-helix–loop–helix (bHLH) Hes transcription factors are important for the transition of neuroepithelial cells to radial glia. These downstream targets of Notch signaling are crucial for the transition of neuroepithelial cells to radial glia, as mice deficient in Hes1 and Hes5 show normal neuroepithelial cells at E8 but impaired radial glial cell differentiation at E9.5 (Hatakeyama et al., 2004). Therefore it seems that Notch signaling mediated by Hes transcription factors is not required by neuroepithelial cells or may be occurring via a molecular mechanisms independent of Hes-mediated transcription prior to E9, because no defects are obvious in the Hes1, 3, 5 triple

mutants at this stage (Hatakeyama et al., 2004). It is only at the onset of neurogenesis, when radial glial cells appear, that Hes-mediated transcription becomes essential for radial glial maintenance. Likewise in zebrafish, conditional inhibition of Notch signaling after the onset of neurogenesis resulted a reduction in the number of radial glia (Kim et al., 2008b). These data suggest, that although neuroepithial cells and radial glia may have similar morphological characteristics, there are molecular differences between neuroepithelial and radial glial cells.

After embryogenesis, these radial glial cells are destined to one of two fates. The cell fate decision seems to be predicated on the type of organism the radial glial cells inhabit. In lower vertebrates like reptiles, birds, fish and amphibians, where neurogenesis persists in a rather widespread fashion in the adult brain, radial glial cells with access to the ventricle can produce neurons, oligodendrocytes, and astrocytes, or persist into adulthood (Adolf et al., 2006; Alvarez-Buylla, 1990; Alvarez-Buylla and Nottebohm, 1988; Chapouton et al., 2006; Jia and Halpern, 1998; Stevenson and Yoon, 1981). However, in the mammalian CNS, where adult neurogenesis occurs in discrete pockets, radial glial cells within these pockets transition to stem cells with astrocyte characteristics (Alvarez-Buylla and Lim, 2004). These astrocyte like adult stem cells are the source of new neurons in the adult mammalian brain (Alvarez-Buylla et al., 2001; Alvarez-Buylla and Lim, 2004).

Adult neurogenesis occurs in the subventricular zone (SVZ), of the forebrain lateral ventricles, and in the subgranular zone (SGZ), of the dentate gyrus, within the hippocampus (Doetsch and Hen, 2005). The SVZ is a layer of

dividing cells that are found along the lateral walls of the lateral ventricle. Neurons born in the SVZ are initially specified as neuroblasts that feed into the rostral migratory stream leading to the olfactory bulb, where they differentiate into two kinds of inhibitory neurons: granule and periglomerular cells (Doetsch and Hen, 2005). In the subgranular zone of the hippocampus, adult neural stem cells generate neurons and glia that contribute to the granular layer of the dentate gyrus (Cameron et al., 1993; Namba et al., 2005). It has been hypothesized that the new neurons generated in the hippocampus may play a critical role in learning and memory processing. As individuals age, their learning and memory capacity is reduced. It has been proposed that this reduction is due to a decrease of adult neurogenesis in the subgranular zone of the hippocampus (Taupin and Gage, 2002).

Stem Cell Maintenance

Scientists hypothesize that early in development, adult stem cell precursors are deposited into their stem cell niche (Slack, 2008). This niche provides them with support that allows them to slowly divide and remain undifferentiated until required. There are two strategies by which stem cells can self renew, while at the same time producing cells that differentiate: symmetric and asymmetric division. Stem cells can divide symmetrically to generate two similar daughter cells and expand the stem cell pool or asymmetrically to self-renew and generate differentiating daughter cells. The proper balance between symmetric and asymmetric division is critical for the generation and subsequent repair of tissues.

Asymmetric cell division is a process by which a cell divides to generate two daughter cells that are molecularly different at birth. Such divisions have already been shown as a means to generate cellular diversity during development and it provides an attractive strategy as to how stem cells can maintain the balance of the competing needs of self-renewal and differentiation (Morrison and Kimble, 2006). Neural stem cells have the potential to divide symmetrically, producing two identical daughter cells, or asymmetrically, producing two different daughter cells. Neural stem cells may also undergo symmetric differentiative divisions, depleting the stem cell pool and producing two developmentally restricted precursors or post-mitotic progeny. Proliferative symmetric divisions serve to rapidly increase a progenitor pool, whereas asymmetric divisions are important for differentiation, as they generate daughter cells that can differ in size and cell fate (Morrison and Kimble, 2006). Vertebrate neuroepithelial cells, which are considered to be neural stem cells, initially undergo several rounds of proliferative symmetric divisions to increase their number. Later, during neurogenesis, asymmetric divisions are observed in the ventricular zone of the developing cortex, when neuroepithelial cells directly generate neurons and self-renew (Gotz and Huttner, 2005). Differentiative symmetric divisions were also observed in the subventricular zone of the developing cortex, where intermediate progenitors can give rise to two post-mitotic cells (Gotz and Huttner, 2005; Noctor et al., 2004). Control of stem cell division and differentiation is thought to be controlled by cell polarity and asymmetric cell division.

Cell polarity

Cell polarity, which is fundamental to many aspects of cell and developmental biology, is involved in the processes of differentiation, proliferation and morphogenesis in both unicellular and multicellular organisms. There are several different planes of polarization that are necessary for proper development, but I will only discuss two: apical-basal polarity and planar cell polarity, as they both play an important role in CNS development. Planar cell polarity (PCP) helps to organize cells within the plane of the epithelium. The PCP pathway was first recognized in *Drosophila melanogaster*, where it regulates organization of ommatidia in the compound eye and hairs and bristles on wings and legs (Adler, 2002). Within the cell, PCP pathway regulates localization of signaling complexes that regulate asymmetric organization of the actin cytoskeleton. Key members of the PCP pathway include transmembrane receptor Frizzled (Fz) and its downstream effector Dishevelled (Dsh), which regulates the actin cytoskeleton, and the Jun kinase pathway. Other important PCP pathway proteins include the transmembrane protein Van Gogh/Strabismus (Vang/Stbm) and its binding partner Prickle (Pk) (Fig. 4). Together they function to regulate localization and activity of the Fz/Dsh protein complex (Klein and Mlodzik, 2005; Komiya and Habas, 2008) (Fig. 4).

The PCP pathway is also required in vertebrates. It functions in the regulation of body hair orientation, polarization of the sensory epithelia within the inner ear, and organized cellular movements during convergent extension, a

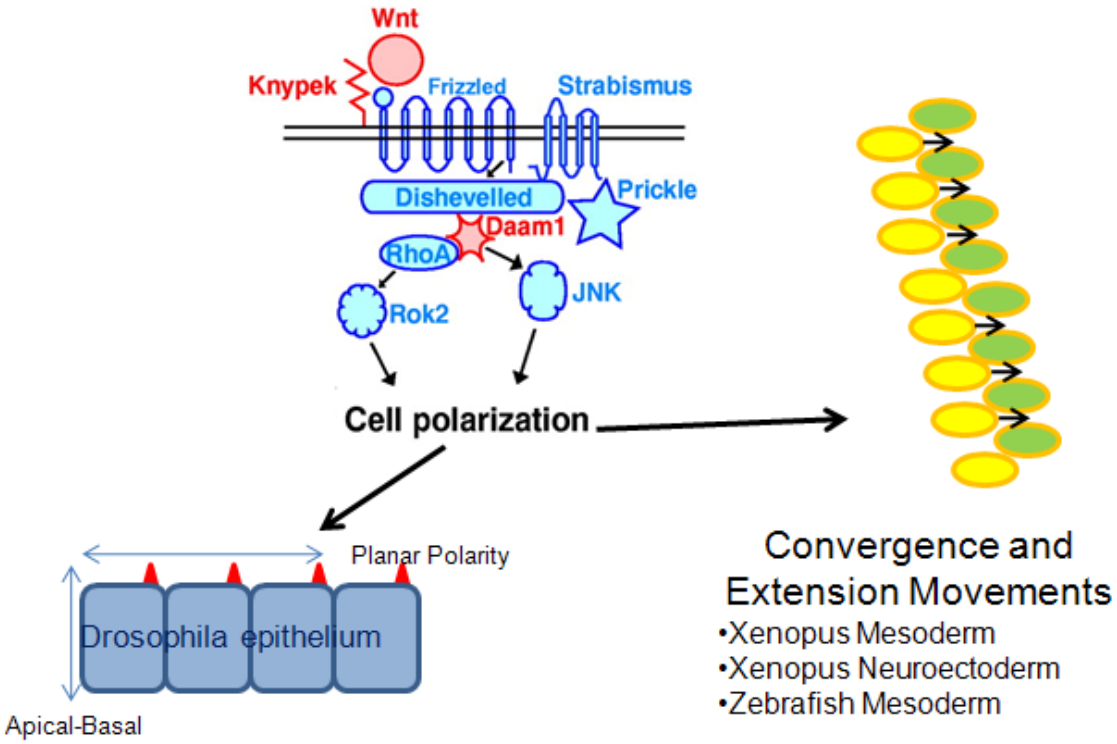


Figure 4. The Planar Cell Polarity Pathway. Evolutionarily conserved planar cell polarity (PCP) signaling pathway regulates diverse cellular behaviors during invertebrate and vertebrate development. The *D. melanogaster* PCP pathway mediates cell polarity in the plane of epithelia. In vertebrates, the PCP pathway controls distinct convergence and extension movements. In X. *laevis* mesoderm it controls mediolateral intercalation as well as dorsally directed neuroectoderm intercalation and in zebrafish, the PCP pathway controls lateral mesoderm anterior and dorsally directed migration.

process critical in gastrulation and neural tube formation (Jessen et al., 2002; Ueno and Greene, 2003)(Fig. 4). During convergent extension, cells intercalate in a polarized fashion, bring lateral structures closer together, and at the same time elongate the anterior-posterior body axis (Fig. 4). Many vertebrate mutants of the key genes involved in PCP pathway display convergent extension defects resulting in abnormal gastrulation and neural tube development (Copp et al., 2003; Jessen et al., 2002). In mammalian and Xenopus embryos, this often results in neural tube closure defects like spina bifida, and in zebrafish embryos, the loss of function of the critical PCP pathway gene s*tbm* leads to defects in convergent extension resulting in a dramatic shortening of the body axis (Jessen et al., 2002; Park and Moon, 2002).

Apical-Basal Polarity

Apical-basal polarity is a fundamental property of all eukaryotic cells. As an organism develops, regulation of cell polarity is essential for the spatial and temporal regulation of cell and tissue morphogenesis. For example, polarization of epithelial cells is an essential component in their ability to form an epithelial barrier as well as many other critical cellular functions. Cell polarity is also involved in the regulation of cell identity. During asymmetric cell division, cell fate identity relies on the polarized distribution and unequal segregation of cell fate determinants at mitosis. The polarized distribution of key regulatory molecules not only underlies the generation of differentiated cell types in developing tissues but also controls the initiation of the anterior posterior body axis in flies and worms (Suzuki and Ohno, 2006). Finally, cell polarity is a key aspect of cell

differentiation, underlying cell migration and morphogenesis. As scientists have continued to work with polarity proteins, a signaling complex of three proteins, Par3, Par6 and atypical protein kinase C (aPKC), has emerged as a central player in the mechanisms that regulate apical-basal cell polarity in the different cell types of various organisms.

The first apical-basal polarity genes were discovered in *C. elegans*, during a genetic screen for maternal-effect mutations affecting the unequal partitioning of polar granules to the posterior cell during the asymmetric division of the one-cell embryo (Kemphues et al., 1988). Two of the genes discovered, *par-3* and *par-6*, encode Post synaptic density protein /Discs-large/ZO1 (PDZ) domain proteins that co-localize at the anterior pole of the embryo (Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999). A third protein, aPKC, was later shown to bind Par3 and to co-localize with Par3 and Par6, leading to the proposal that the three proteins form a complex (Izumi et al., 1998).

In *C. elegans* the Par complex of proteins functions to establish anterior posterior polarity and regulates the first asymmetric division after fertilization. Upon fertilization, the microtubule-nucleating activity of the sperm asters acts to exclude the Par3/Par6/aPKC complex from the posterior cortex of the cell (Pellettieri and Seydoux, 2002; Sadler and Shakes, 2000). In *Drosophila* Par-6, aPKC and Par-3 proteins also form a complex that is known to regulate several developmental processes. For example, the Par3/Par6/aPKC complex regulates apical–basal polarity of epithelial cells in the embryo, polarized migration of border cells in the egg chamber and establishment of asymmetry in mitotic

neuroblasts (Wodarz, 2002). In Drosophila, these three proteins appear to also co-localize together, suggesting that, like their *C. elegans* homologues, the Drosophila proteins Par-6 and aPKC, together with Par3, act as a signaling complex (Wodarz, 2001; Wodarz, 2002; Wodarz et al., 2000). In vertebrates, homologues of Par6, aPKC (aPKCl and aPKCz) and of Par3 (ASIP) have also been identified. Together, these three proteins were shown to co-localize at tight junctions. These three proteins have also been shown to be important in the establishment and maintenance of apical–basal polarity in epithelia, as studies in cultured epithelial cells indicate that the Par3/Par6/aPKC complex promotes the formation of epithelial tight junctions. Lastly, Par6 and aPKC have also been described in vitro as regulators of cell polarity in migrating primary rat astrocytes. In these cells, Par3 does not appear to co-localize with Par6 and aPKC and is therefore not part of the complex (Etienne-Manneville and Hall, 2001).

In the embryonic Drosophila CNS, the Par-aPKC complex has been shown to be important in regulating cell fate through asymmetric cell divisions. Neuroblasts, a population of Drosophila CNS precursor cells, delaminate from the neural ectoderm and are polarized expressing aPKC, Par3, and Par6 in the apical membrane (Doe, 2008; Rolls et al., 2003; Rolls and Doe, 2004) (Fig. 5). The Par-aPKC complex proteins that direct neuroblast polarity are expressed and localized in epithelial cells, where they act to establish apico-basal polarity. The neuroblast inherits these proteins prior to delamination (Rolls et al., 2003). After neuroblasts delaminate, their mitotic spindle rotates 90°, so that subsequent

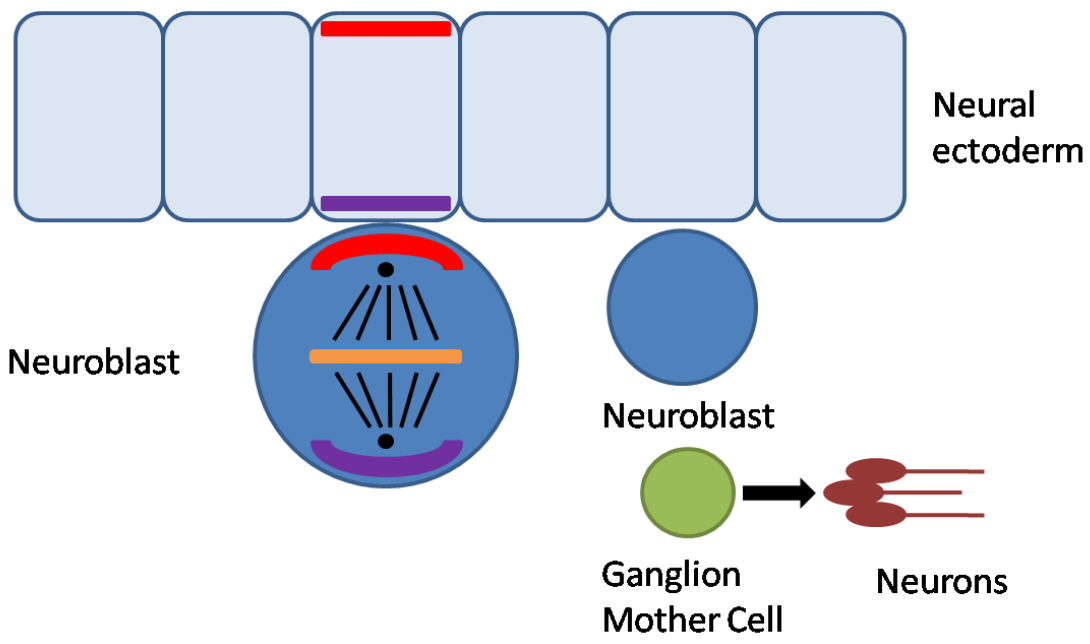


Figure 5. The Par-aPKC complex regulates asymmetric neuroblast divisions in the *Drosophila* CNS. After delaminating from the neuroepithelium, the neuroblast localizes aPKC, Par-6 and Par-3 to its apical membrane (red). This evolutionarily conserved apical complex is known as the aPKC-Par complex and is responsible for segregating proteins like Numb, Miranda and Prospero to the basal side of the cell (purple). This polarized neuroblast divides asymmetrically to give rise to another neuroblast and a ganglion mother cell (green). The ganglion mother cell differentiates and gives rise to neurons.

divisions are oriented along the apico-basal axis of the embryo (Kaltschmidt and Brand, 2002; Kaltschmidt et al., 2000). Neuroblasts divide asymmetrically and these unequal divisions produce larger apical daughter cells that retain the stem-cell-like properties of a neuroblast and smaller, fate-committed basal daughter cells called ganglion mother cells (GMCs) that produce neurons (Lee et al., 2006a; Lee et al., 2006b; Rolls et al., 2003). Spindle rotation and the subsequent asymmetric segregation of cell-fate determinants to the basal GMC are dependent on the Par-aPKC complex at the apical cortex of the neuroblast.

In the Drosophila neuroblast, the Par-aPKC complex binds Inscuteable (Insc) through Par3, and Insc, in turn, recruits Partner of Inscuteable (Pins) and Locomotion defective (Loco), which are two GDP-dissociation inhibitors (GDIs) that interact with the heterotrimeric G protein subunit, Gαi. (Kraut et al., 1996; Yu and David, 2004; Yu et al., 2002). Through loss of function and over expression experiments, Gαi and Pins have been shown to be important proteins in regulating spindle orientation and asymmetric cell divisions (Nipper et al., 2007).

The apical complex is important in directing cell-fate determinants to the basal cortex. The homeodomain protein Prospero (Pros) and *pros* RNA, as well as the phosphotyrosine-binding domain protein Numb, are localized as basal crescents and segregate to the basal GMC (Betschinger et al., 2003; Doe et al., 1991; Uemura et al., 1989; Vaessin et al., 1991). Of all the proteins localized to the basal side of the neuroblast, only Pros appears to act as a cell-fate determinant in the GMC, whereas Numb plays a role later in development, when the GMC divides, to discriminate between the sibling neurons (Buescher et al.,

1998; Spana and Doe, 1995). Asymmetric segregation of Pros protein and *pros* RNA is mediated by the adaptor protein Miranda (Mira). Once Pros is segregated to the GMC, Mira is degraded, thereby releasing Pros from the cortex (Ikeshima-Kataoka et al., 1997; Matsuzaki et al., 1998). Pros can then enter the nucleus, where it has been thought to specify GMC identity by promoting the expression of GMC-specific genes and repressing the expression of neuroblast-specific genes (Choksi et al., 2006).

Development of zebrafish as a model System.

Danio rerio, commonly referred to as the zebrafish, is a relatively young scientific model organism. Its rise as a model organism began at the University of Oregon, Eugene, when George Streisinger recognized the need for a vertebrate model in which to study developmental genetic mutations. In the late 1960s Streisinger began working with this tropical freshwater fish as he saw its potential to "study features of the organization and embryological development of the vertebrate nervous system through the use of mutant strains" (Grunwald and Eisen, 2002). While working with zebrafish he recognized that they were relatively easy to maintain in a laboratory and that its fecundity would be beneficial genetically as a single female can produce over a hundred embryos in a single clutch. Zebrafish develop externally from their mothers and during early development, embryos are transparent. These two characteristics make zebrafish an excellent model for live imaging. Zebrafish also develop rapidly as a fertilized embryo can develop into a free swimming larva within 72 hours.

In 1993, two large scale forward genetic screens began in Tübingen, Germany and Boston, Massachusetts. In a forward genetic screen a physical trait or phenotype is first identified and then through genetic mapping, the mutated gene is positionally cloned. In the relatively short time of three years, the results from these screens were published in a landmark December issue of Development in 1996. So important and numerous were the results that the entire issue was dedicated to the screen. From this screen approximately 4,000 embryonic mutant phenotypes were recovered and identified, one of which, *heart and soul (has$^{-/-}$),* is the basis for my thesis. The results from this screen solidified zebrafish as a viable vertebrate genetic model, as it showed that many of the known molecular mechanisms were conserved in zebrafish.

CHAPTER II

THE APICAL POLARITY PROTEIN PRKCI IS NECESSARY FOR MAINTENANCE OF SPINAL CORD PRECURSORS IN ZEBRAFISH

Abstract

During spinal cord development, precursor cells transition from proliferative divisions to differentiative divisions. Traditionally proliferative divisions, which increase cell numbers, are thought to be symmetric, whereas differentiative divisions are thought to occur both by symmetric and asymmetric divisions. Currently, the mechanisms that control this differentiative cell division fate remain to be defined. However, studies conducted using atypical protein kinase C (aPKC) suggests that aPKC has a conserved function in controlling cell division orientation. In this study, we look at the role aPKC may play in maintaining precursor division in the zebrafish spinal cord. Through time-lapse imaging and loss of function studies we were able to show that aPKC does regulate precursor division in the zebrafish spinal cord, and in its absence excess oligodendrocyte precursor cells (OPCs) are specified at the possible expense of adult precursors.

Introduction

In the developing central nervous system of vertebrates, regulation of cell division influences the balance of neural precursors, neurons and glia. During early stages of neural development, precursors undergo symmetric, proliferative divisions to expand the precursor population. Later, precursors exhibit

asymmetric, self-renewing divisions to produce one precursor and one differentiated cell or symmetric, differentiative divisions to produce two progeny fated to exit the cell cycle. Therefore, mechanisms that regulate the number and type of symmetric versus asymmetric divisions influence brain size and cell composition.

In *Drosophila*, asymmetric division of embryonic neuroblasts to produce progeny having different fates is regulated by cell polarity and orientation of cell cleavage. An evolutionarily conserved complex of proteins consisting of Par3/Bazooka, Par6 and atypical Protein Kinase C (aPKC) is localized to the apical membrane of epithelial neuroectodermal cells and thereby to the apical membrane of neuroblasts as they delaminate from the ectoderm (Petronczki and Knoblich, 2001; Schober et al., 1999; Wodarz, 2001; Wodarz, 2002; Wodarz et al., 2000). Par complex proteins act through the tumor suppressor Lethal giant larvae to exclude other proteins, including the cell fate determinants Prospero and Numb, from apical membrane, limiting their localization to basal membrane (Betschinger et al., 2003; Plant et al., 2003; Yamanaka et al., 2003). As neuroblasts delaminate from the epithelium, Inscuteable and a cassette of heterotrimeric G protein signaling factors are recruited to the apical membrane and orient the mitotic spindle perpendicular to the plane of the epithelium, resulting in cleavage that is orthogonal to the axis of apicobasal polarity (Kraut and Campos-Ortega, 1996; Kraut et al., 1996; Schaefer et al., 2000; Yu et al., 2000). Consquently, Prospero and Numb are segregated to the basal progeny cell, which becomes a Ganglion Mother Cell (GMC) fated to divide once to

produce two neurons. The apical progeny cell, lacking Propero and Numb, remains as a neuroblast (Wodarz and Huttner, 2003).

Regulation of the plane in which neuroepithelial precursors divide by apicobasal polarity cues in the CNS of vertebrate embryos could provide an effective means for regulating symmetric versus asymmetric divisions. Consistent with this possibility, some investigations have described analyses of fixed tissue and live cell imaging that reveal both planar division (division in the plane of the epithelium, also known as vertical cleavage) and orthogonal division (division perpendicular to the plane of the epithelium, also known as horizontal cleavage) (Chenn and McConnell, 1995; Haydar et al., 2003; Sanada and Tsai, 2005). Additionally, Par complex proteins are localized to the apical membrane of neuroepithelial cells (Afonso and Henrique, 2006; Geldmacher-Voss et al., 2003; Imai et al., 2006; Kovac et al., 2007; Manabe et al., 2002; von Trotha et al., 2006), similar to localization of homologous proteins in the *Drosophila* neuroepithelium. Several other studies, however, indicate that most divisions in the vertebrate neuroectoderm are planar during both proliferative and neurogenic phases of neural development (Geldmacher-Voss et al., 2003; Kosodo et al., 2004; Lyons et al., 2003; Morin et al., 2007; Noctor et al., 2008). Therefore, the relationship between cell division pattern and cell fate in the vertebrate CNS remains unclear.

In the spinal cord, the ventral pMN precursor domain, defined by expression of the transcription factor-encoding gene *Olig2*, gives rise first to motor neurons and later to oligodendrocyte progenitor cells (OPCs), which

migrate throughout the spinal cord, divide and differentiate as myelinating oligodendrocytes (Adam et al., 2000; Masahira et al., 2006; Park et al., 2004; Takebayashi et al., 2000; Zhou et al., 2000). Our analysis of the clonal progeny of *olig2*$^{+}$ neural plate cells in zebrafish implied the existence of asymmetric divisions that give rise to motor neurons, OPCs and some ventral interneurons (Park et al., 2004). Near the end of embryogenesis remaining *olig2*$^{+}$ precursors adopt radial glial characteristics and are maintained as slowly dividing OPC precursors into adulthood (Park et al., 2007). We found that Notch signaling is required continuously during development to maintain *olig2*$^{+}$ precursors and regulate the numbers of precursors specified for motor neurons and oligodendroctye fates (Kim et al., 2008a; Shin et al., 2003) but we are still uncertain about the exact mechanisms that maintain and specify *olig2*$^{+}$ precursors and the potential role of cell polarity.

In this study we investigated the role of apical cell polarity in spinal cord precursor maintenance and specification using the *heart and soul (has)* mutation, which disrupts function of aPKCλ (Horne-Badovinac et al., 2001). Time-lapse imaging revealed that in *has* mutant embryos neuroepithelial cells gradually switch from planar to oblique divisions. Concomitant with this switch in cell division pattern is a loss of apical character, loss of neuroepithelial precursors and formation of excess neurons and OPCs. We conclude that planar cell division, directed by apically localized aPKCλ, is required for maintenance of neuroepithelial precursors.

Experimental Procedures

Fish Husbandry

Embryos were produced by pair-wise mating and kept at 28.5^{0}C in egg water or embryo medium. Embryos were staged to hours post fertilization (hpf) or days post fertilization (dpf) according to established zebrafish guidelines (Kimmel et al., 1995). Embryos that were to be used for live imaging, immunocytochemistry, or in situ hybridization were treated in 0.003% phenylthiourea (PTU) in egg water to block pigmentation. The experiments conducted in this paper used the following strains of zebrafish: AB, *has*^{m567} (Stainier et al., 1996), *Tg(olig2:egfp)* (Shin et al., 2003), and *Tg(h2afv:gfp)* (Pauls et al., 2001).

Immunocytochemistry

Embryos and larvae were fixed in 4% antibody fix (4% paraformaldehyde, 8% sucrose, 1x PBS) overnight at 4^{0}C. After fixing, the embryos were embedded in 1.5% agar/ 5% sucrose blocks and placed in 30% sucrose/PBS solution to equilibrate overnight. The blocks were then frozen over 2-methylbutane chilled by liquid nitrogen. We collected 10-12 □ m sections on superfrost microscope slides using a cryostat microtome. The sections were rehydrated in 1x PBS for 30 min. and then blocked in 2% BSA/sheep serum in 1x PBS for 30 min. before incubating with primary antibody overnight at 4^{o}C. For fluorescent detection of antibody labeling, we used Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647 goat anti-mouse or goat anti-rabbit conjugates (1:500, Molecular Probes). The

primary antibodies used included rabbit anti-aPKC (#sc-216, 1:200, Santa Cruz Biotechnology, Inc.), mouse anti-Islet (clone # 39.4D5, 1:1,000, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA), rabbit anti-phospho-Histone-H3 (# 06570, 1:1000, Upstate Biotechnology, Charlottesville, VA), rabbit anti-Sox10 (1:1,000) (Park et al., 2005), mouse anti-ZO-1 (#33-9100, 1:200, Invitrogen) and mouse anti-ZRF-1 (1:500, University of Oregon Monoclonal Antibody Facility).

In situ hybridization

Embryos and larvae were fixed in 4% paraformaldehyde overnight at 4^0C and stored in methanol at -20^0C. After linearizing plasmids with the appropriate restriction enzymes, anti-sense cRNA was transcribed using Roche DIG-labeling reagents and T3, T7 or SP6 RNA polymerases (New England Biolabs). *cldnk* (Noctor et al., 2001) was identified in a microarray screen for oligodendrocytes-specific genes and will be described elsewhere. *sox19b* was originally named *sox31* (Girard et al., 2001). After processing embryos for *in situ* RNA hybridization embryos were embedded in agar and sectioned as described above. Sections were rehydrated in 1X PBS for 30 min. then covered with 75% glycerol. Images were obtained using a Retiga EXI camera attached to a Zeiss Axiovert 200 microscope equipped with Openlab software.

In vivo time-lapse imaging

Embryos were raised in egg water containing PTU and at the appropriate stages manually dechorionated using watchmaker forceps. Embryos were anesthetized

using 3-aminobenzoic acid ethyl ester (Tricaine) and mounted laterally or dorsally in 35 mm glass bottom petri dishes containing 0.8% low-melting temperature agarose. Confocal time-lapse movies were obtain by using a 40X oil immersion objective mounted on a Zeiss Axiovert 200 microscope equipped with a PerkinElmer spinning disk confocal system. Z-stack images were obtained every 3-5 min and compiled into a Quicktime movie using Volocity software (Improvision). Widefield time-lapse movies were obtained using a 40X objective on a Zeiss Axiovert 200 microscope equipped with a Retiga EXI camera. Z-stacked images were obtained every 10-12 min. Embryos were maintained at 28.5^{0}C using a heated stage chamber during imaging.

Angle of division measurements

PTU-treated *Tg(h2afv:gfp)* and *Tg(h2afv:gfp);has*$^{-/-}$ embryos were mounted dorsally at 27 hpf in low melting point agarose. Z-stack images were obtained at the level of the central canal every 3-5 minutes. These images were then compiled into a Quicktime time-lapse movie using Volocity. Movies were analyzed using Openlab software. Going frame by frame, we tracked dividing cells at the central canal into telophase and then drew a line parallel to the separated chromatids. With the central canal as a reference, we measured the angle between the line and the central canal, using a program called Screen Protractor (Iconico.com).

Results

PrkCi is Required for Maintenance of Apical Polarity and Adherens Junctions in the Spinal Cord Neuroepithelium

We initiated an analysis of zebrafish spinal cord neuroepithelial polarity by labeling transverse sections with an antibody that recognizes a carboxyl terminal epitope common to PrkCi and Protein kinase C, zeta (PrkCz) (Cui et al., 2007; Horne-Badovinac et al., 2001). At 24 hours post fertilization (hpf) and continuing through 48 hpf PrkCi/z proteins were localized to apical cell membranes contacting the spinal cord medial septum and central canal (Fig. 1A,B). By 72 hpf, when most spinal cord cell divisions have ceased (Park et al., 2007), PrkCi/z proteins were diminished at the medium septum, but retained around the central canal (Fig. 1C). Zonula Occludins-1 (ZO-1) antibody, which recognizes a protein associated with apical neuroepithelial adherens junctions (Aaku-Saraste et al., 1996; Hurd et al., 2003; Manabe et al., 2002), revealed a similar pattern of localization (Fig. 1G-I).

In the *Drosophila* CNS, apical localization of Par/aPKC complexes is dependent on aPKC function (Wodarz et al., 2000). Consistent with this, targeted mutation of *Prkci* in mice results in loss of neuroepithelial adherens junctions within the neocortex (Imai et al., 2006). To investigate whether the apical polarity of zebrafish neuroepithelial cells similarly requires PrkCi function, we examined embryos homozygous for the *m567* allele of *heart and soul (has)*, which express a truncated, inactive PrkCi protein (Horne-Badovinac et al., 2001). We first assessed the amount and localization of PrkC proteins. Because the *has*^{m567}

allele eliminates the antibody epitope from PrkCi, any labeling evident in mutant embryos represents PrkCz and maternally expressed PrkCi (Horne-Badovinac et al., 2001). At 24 hpf, PrkC localization in *has*$^{-/-}$ embryos was indistinguishable from wild-type embryos (Fig. 1D). However, by 48 hpf, *has*$^{-/-}$ embryos had diminished levels of PrkC. Particularly, anti-PrkC labeling was nearly absent from the medial septum and revealed a smaller central canal (Fig. 1E). Additionally, PrkC was absent in many sections (data not shown), raising the possibility that the central canal is discontinuous along the length of the spinal cord in mutant embryos. At 72 hpf, diminished levels of PrkC were similarly localized to a smaller, apparently discontinuous central canal (Fig. 1F).

ZO-1 immunocytochemistry revealed a localization pattern identical to that of PrkC in *has*$^{-/-}$ embryos. At 24 hpf, ZO-1 appeared normal in mutant embryos (Fig. 1J), indicating that in the absence of PrkCi, PrkCz is sufficient to localize adherens junction proteins. By contrast, at 48 and 72 hpf ZO-1 was mostly absent from the medial septum and outlined a small, discontinuous central canal (Fig. 1K,L). Taken together, these data indicate that apical polarity of spinal neuroepithelial cells is initially normal in the absence of zygotically encoded PrkCi function but that apical polarity, adherens junctions and central canal integrity gradually degrade.

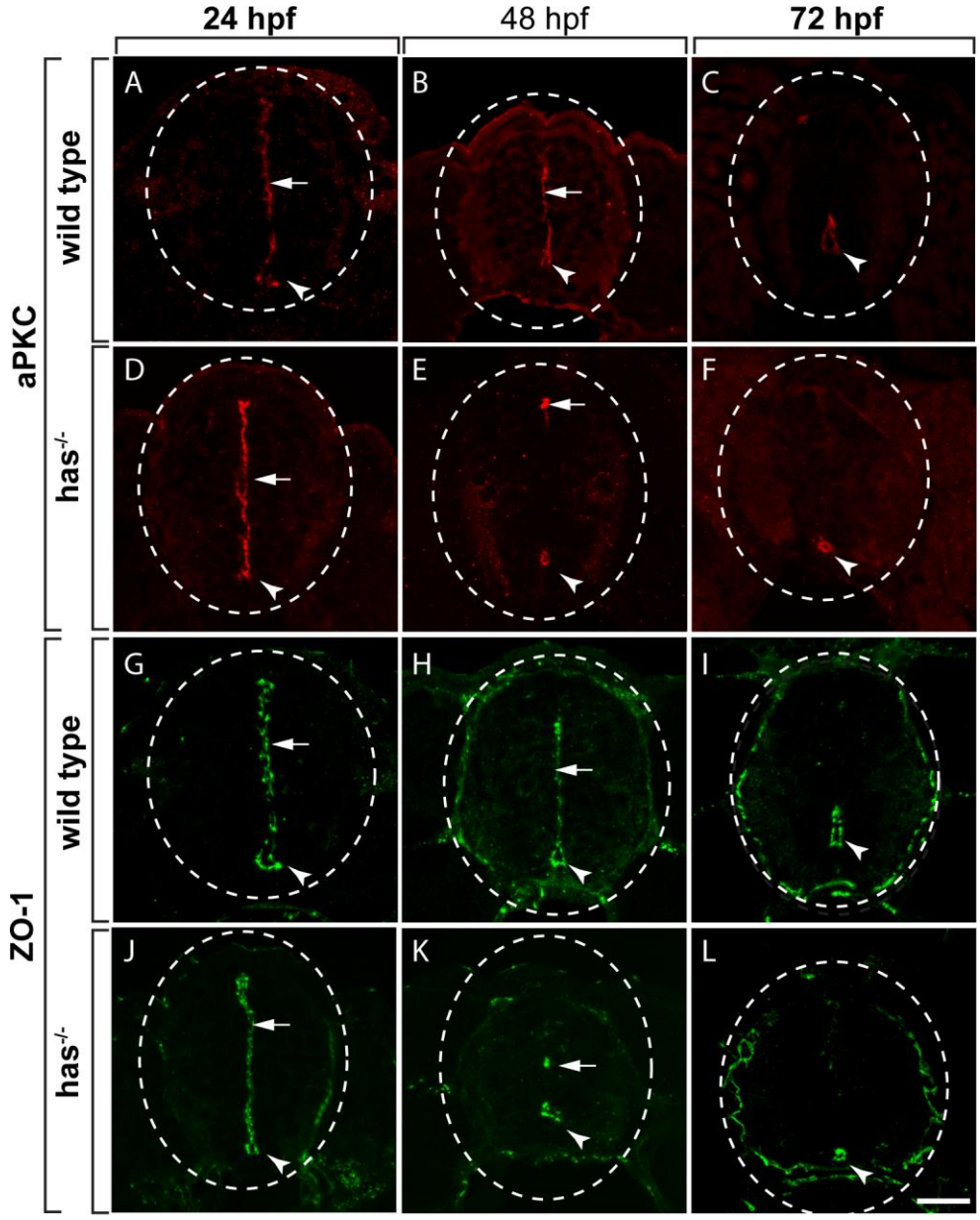


Fig. 1. Zebrafish spinal cord cells have apical polarity, which requires PrkCi function. All panels show transverse sections through trunk spinal cord, dorsal up. Dashed circle marks the perimeter of the spinal cord. Arrowheads and arrows indicate central canal and medial septum, respectively. **A-F:** Sections labeled with anti-PrkCi/z antibody. PrkC is localized to the medial septum and central canal of wild-type embryos at 24 and 48 hpf (A,B). At 72 hpf, PrkC is absent from the medial septum but remains around the central canal of wild-type larvae (C). At 24 hpf, PrkC localization is normal in *has*$^{-/-}$ embryos (D). However, by 48 hpf very little PrkC is evident at the medial septum and, although PrkC remains around the central canal, the central canal is reduced in size (E) or entirely absent (not shown). Sections of 72 hpf *has*$^{-/-}$ larvae similarly reveal PrkC localization around an abnormally small and discontinuous central canal (F). The apical protein ZO-1 has a similar localization pattern to PrkC in wild-type and *has*$^{-/-}$ embryos and larvae (G-L). Scale bar = 20 μM.

PrkCi is Required to Maintain Planar Divisions of Spinal Cord Precursors

In zebrafish, cell divisions within the medial neural plate are oriented in the mediolateral plane of the neuroepithelium (Concha and Adams, 1998; Geldmacher-Voss et al., 2003). As the neural plate condenses to form the neural keel and then neural rod, cell divisions remain perpendicular to the anteroposterior axis and become orthogonal to the plane of the neuroepithelium (Geldmacher-Voss et al., 2003). Upon formation of the neural tube, cell divisions rotate 90^0 so that they occur within the plane of the neuroepithelium (Geldmacher-Voss et al., 2003; Lyons et al., 2003). As a prelude to our investigation of neural cell polarity and fate, we performed our own analysis of cell division orientation, but focused on a later period of development than previous studies. To mark dividing cells we used the transgenic reporter *Tg(h2afv:gfp),* which expresses EGFP fused to a histone protein (Pauls et al., 2001). We imaged embryos from a dorsal view, focusing on cells that line the central canal starting at 27 hpf and continuing until 33 hpf. This corresponds to the period during which most spinal cord neurogenesis is completed and precedes OPC formation (Park et al., 2002; Shin et al., 2003). Consistent with previous reports of cell division patterns in both zebrafish and chick neural tube (Geldmacher-Voss et al., 2003; Lyons et al., 2003; Morin et al., 2007), divisions of cells bordering the central canal were always planar, parallel to the central canal (Fig. 2A,C,E).

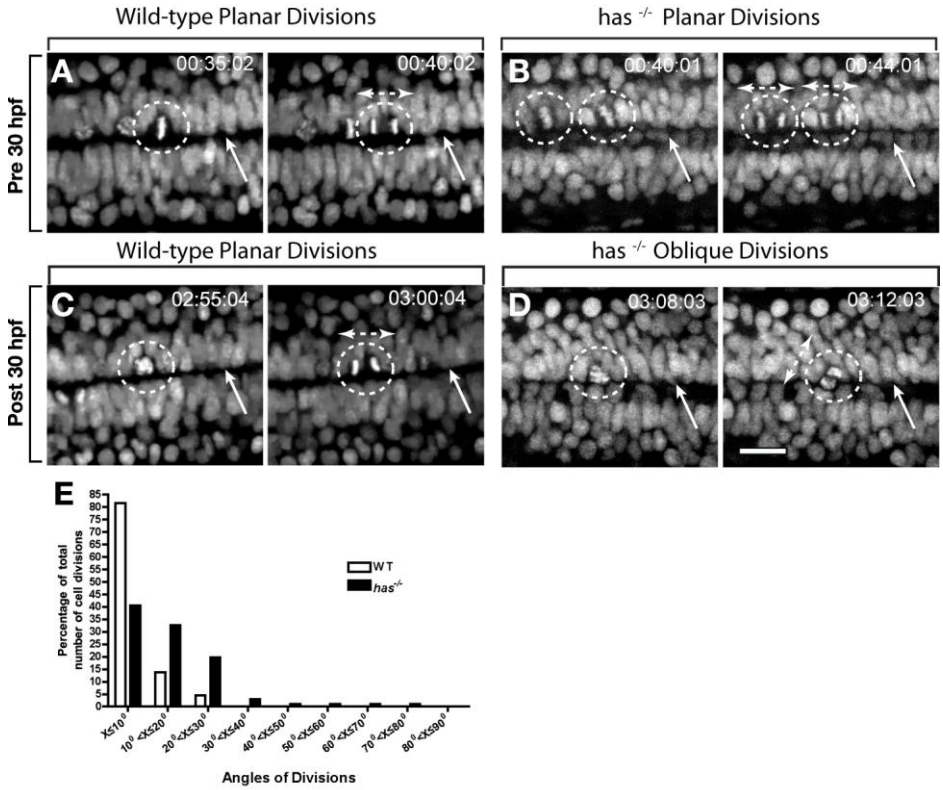


Fig. 2. PrkCi maintains planar divisions of cells that divide along the central canal. A-D: Frames captured from time-lapse movies, from a dorsal view, of wild-type and *has*$^{-/-}$ embryos carrying the *Tg(h2afv:egfp)* transgene. Numbers in upper right corners indicate time elapsed from beginning of imaging at 27 hpf. Dashed circles outline dividing cells, arrows point to the central canal and bi-directional arrows indicate orientation of the mitotic spindle and angle of division. A,B: In both wild-type and *has*$^{-/-}$ embryos, divisions that occur before 30 hpf are planar. The central canal is less distinct in *has*$^{-/-}$ embryos than in wild type. C,D: Divisions after 30 hpf remain planar in wild-type embryos (C) but become oblique in *has*$^{-/-}$ embryos (D). The central canal is indistinct and appears to have been replaced by cells. E,F: Quantification of angles of division in wild-type (E) and *has*$^{-/-}$ embryos (F). In wild type most division planes are within 150 of the plane of the epithelium, indicated by the central canal. *has*$^{-/-}$ embryos have numerous divisions greater than 150. Scale bar = 24 μM.

Time-lapse imaging revealed the orientation of cell divisions changes with time in *has* mutant embryos. Prior to 30 hpf, cells bordering the central canal divide similarly to those in wild-type (Fig. 2B). Beginning at about 30 hpf, however, most divisions were oblique to the plane of the epithelium, with some nearly perpendicular (Fig. 2D,E). At the same time, the central canal became less distinct, with the space occupied by spinal cord cells (Fig. 2D). Therefore, loss of PrkCi function results in disruption of planar division and a breakdown of the neuroepithelium.

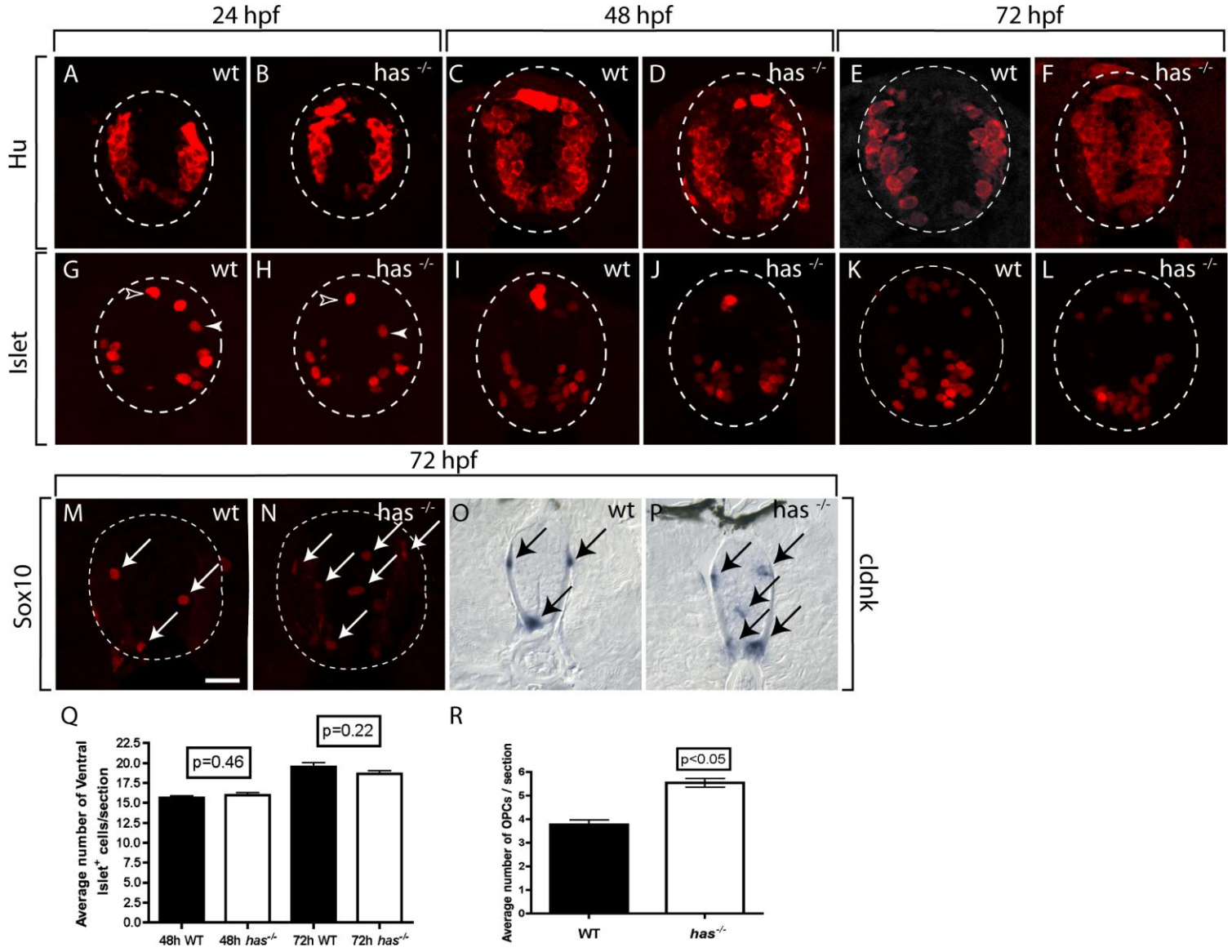
Loss of PrkCi Function Causes Formation of Excess OPCs Without Affecting Motor Neuron Formation

The above data reveal that PrkCi is required for maintenance of apical polarity and planar divisions of spinal cord precursors. Because cell polarity and division pattern often influence cell fate, we assessed formation of neurons and glia using molecular markers. We first labeled transverse sections of wild-type and *has*$^{-/-}$ spinal cords with anti-Hu antibody, which marks all newly formed neurons (Marusich et al., 1994). No apparent differences in the distribution of Hu+ neurons was evident between wild-type and mutant embryos at 24, 48 and 72 hpf (Fig. 3A-F). To quantify the number of neurons, we labeled sections of 72 hpf wild-type and mutant larvae with anti-Hu antibody and a nuclear stain and determined the proportion of spinal cord cells that had neuronal identity. This revealed little difference in the number of neurons formed by larval stage (data not shown). This phenotype is similar to that of embryos deficient for Notch signaling in which precursors prematurely exit the cell cycle and differentiate as

neurons except that in the latter excess neurons are evident at the earliest stages of neurogenesis (Appel and Eisen, 1998; Appel et al., 2001; Itoh et al., 2003; Shin et al., 2003). Therefore, we examined Isl^{+} motor neurons, Rohon-Beard sensory neurons and interneurons, which are mostly formed by about 48 hpf. No obvious differences in the number and distribution of Isl^{+} neurons was apparent between wild type and mutant through 72 hpf (Fig. 3G-L) and quantification of Isl^{+} motor neurons revealed no statistically signficant differences at 48 and 72 hpf (Fig. 3Q). We also examined Isl+ motor neurons, Rohon-Beard sensory neurons and interneurons, which are mostly formed by about 48 hpf. No obvious differences in the number and distribution of Isl+ neurons was apparent between wild type and mutant through 72 hpf (Fig. 3G-L) and quantification of Isl+ motor neurons revealed no statistically significant differences at 48 and 72 hpf (Fig. 3Q). Therefore, formation of the earliest-formed spinal cord neurons is not apparently affected by absence of PrkCi function, consistent with our observation that PrkCz localization, apical polarity and cell division pattern appear normal during early neurogenesis.

To investigate formation of OPCs, we labeled sections with anti-Sox10 antibody, which serves as a specific marker of OPCs and differentiating oligodendroctyes (Kuhlbrodt et al., 1998; Park et al., 2005). At 48 hpf, soon after specification of spinal cord OPCs begins, the number and distributon of OPCs was indistinguishable between wild-type and *has* mutant embryos (data not shown). However, by 72 hpf, both the number and distribution of OPCs was altered in $has^{-/-}$ spinal cords relative to wild type (Fig. 3M,N). In particular, mutant

Fig. 3. **Loss of PrkCi function produces excess OPCs.** All images are of transverse sections through trunk spinal cord, dorsal up. Outlined circle marks the perimeter of the spinal cord. **A-F**: The number and distribution of neurons, marked by anti-Hu labeling, are similar in wild-type and *has*$^{-/-}$ embryos at 24, 48 and 72 hpf (A-F). G-L: Isl^{+} motor neurons (brackets), interneurons (solid arrowheads) and Rohon-Beard sensory neurons (open arrowheads) are similar in wild-type and *has*$^{-/-}$ embryos and larvae through 72 hpf. M,N: Anti-Sox10 labeling reveals excess OPCs in 72 hpf *has*$^{-/-}$ larva (N) compared to wild type (M). O,P: *cldnk* RNA expression marking differentiating oligodendrocytes. In wild-type, *cldnk*$^{+}$ cells (arrows) are at the pial surface in dorsal and ventral spinal cord (O). *has*$^{-/-}$ larvae have excess *cldnk*$^{+}$ cells and some occupy ectopic positions in medial spinal cord (P). Q: Quantification of spinal cord Isl^{+} motor neurons in the spinal cord between wild-type and *has*$^{-/-}$ embryos at 48 and 72 hpf. R: Quantification of spinal cord Sox10^{+} OPCs. Error bars represent s.e.m. Statistical significance was determined using Student's *t*-test. Scale bar = 20 μM.

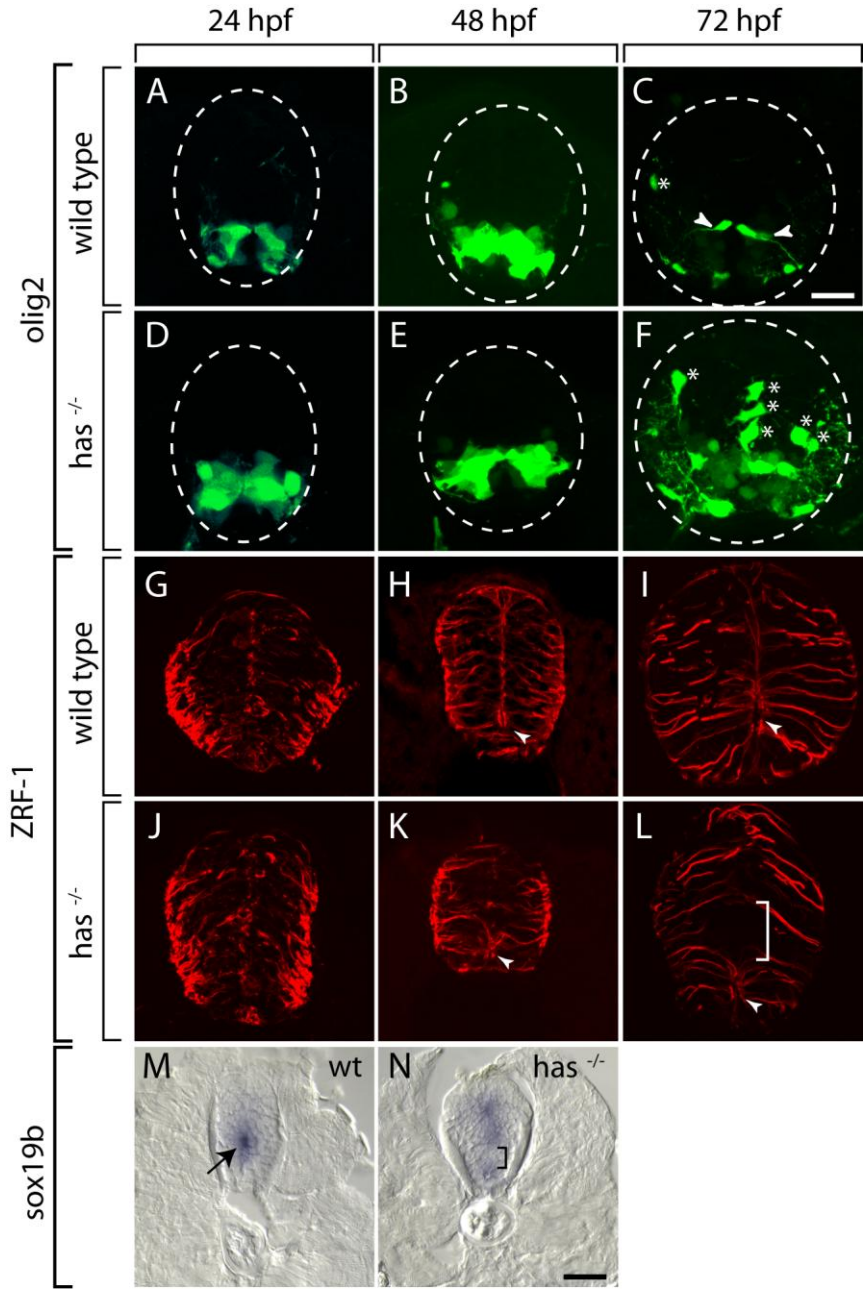

24 hpf
48 hpf
72 hpf
Hu
Islet
Sox10
cldnk
A wt
B has -/-
C wt
D has -/-
E wt
F has -/-
G wt
H has -/-
I wt
J has -/-
K wt
L has -/-
M wt
N has -/-
O wt
P has -/-
Q
Average number of Ventral Islet+ cells/section
p=0.46
p=0.22
48h WT
48h has-/-
72h WT
72h has-/-
R
Average number of OPCs / section
p<0.05
WT
has-/-

larvae had 1.4-fold more OPCs than wild type (Fig. 3R) and some OPCs were abnormally positioned within medial regions of the spinal cord (Fig. 3N). We also performed in situ RNA hybridization to detect expression of *cldnk*, which specifically marks differentiating oligodendrocytes (N. Takada and B. Appel, unpublished data). This similarly revealed an excess of oligodendrocytes in *has*$^{-/-}$ spinal cords relative to wild type (Fig. 3O,P). Additionally, *cldnk*$^{+}$ cells sometimes occupied medial spinal cord, where they are normally not found. These data indicate that PrkCi function limits both the formation and differentiation of oligodendrocyte lineage cells.

PrkCi Function and Apical Polarity are Required to Maintain Spinal Cord Precursors

In zebrafish, slowly-dividing *olig2*$^{+}$ spinal cord precursors that have radial glial characteristics and continuously give rise to new OPCs are maintained through late embryonic stage into adulthood (Park et al., 2007). One possible explanation for the excess OPC phenotype of *has* mutant larvae, therefore, is that, in the absence of PrkCi function, *olig2*$^{+}$ cells lose precursor characteristics and develop as oligodendroctyes. Consistent with this, whereas *olig2*$^{+}$ radial fibers are evident by 72 hpf in transverse sections of wild-type *Tg(olig2:egfp)* larvae (Fig. 4A-C), similar processes were rare in *has*$^{-/-}$*;Tg(olig2:egfp)* larvae (Fig. 4D-E). We next labeled sections with anti-Zrf-1 antibody, which marks spinal cord radial glia (Metcalfe et al., 1990). Through 48 hpf, Zrf-1^{+} fibers were similar in wild-type and *has* mutant embryos (Fig. 4G,H,J,K). However, by 72 hpf, whereas GFAP^{+} radial glia were distributed uniformly throughout the spinal cord of wild-type larvae (Fig.

Fig. 4. PrkCi is required to maintain ventral spinal cord cells with precursor characteristics. All images are of transverse sections through trunk spinal cord, dorsal up. Outlined circle marks the perimeter of the spinal cord. **A-F:** EGFP expression driven by the *Tg(olig2:egfp)* promoter. In wild-type embryos, EGFP marks motor neurons and pMN precursors through 48 hpf (A,B). By 72 hpf, EGFP+ fibers (arrowheads), marking precursors that persist into larval stage, become evident. EGFP expression appears normal in *has–/–* embryos at 24 and 48 hpf (D,E). At 72 hpf, few EGFP+ radial fibers are evident (F). Asterisks mark dorsally migrated OPCs. **G-L:** Zrf-1 immunocytochemistry to label radial glial fibers. In wild-type embryos and larvae, radial fibers are distributed uniformly through the spinal cord (G-I). In ventral spinal cord, the apical membrane of some radial glia surround the central canal (arrowheads). In *has* mutants (J-L), radial fibers initially appear normal but by 72 hpf a gap appears in ventral spinal cord (bracket) and the central canal is reduced or absent. **M,N:** *sox19b* RNA expression. At 72 hpf in wild type, *sox19b* expression marks cells near the central canal (arrow) (M). At 72 hpf *sox19b* is not expressed at its normal position in *has–/–* larva (bracket). Instead, ventral and dorsal spinal cord cells express *sox19b.* Scale bar = 20 μM.


24 hpf
48 hpf
72 hpf
olig2
wild type
has -/-
ZRF-1
wild type
has -/-
sox19b
A
B
C
D
E
F
G
H
I
J
K
L
M
wt
N
has -/-

4I), radial glia were consistently absent from the spinal cord just dorsal to remnants of the central canal (Fig. 4L). We next used in situ RNA hybridization to detect expression of *sox19b,* which marks cells of the medial spinal cord that are likely dividing precursors (Fig. 4M-O). Similar to Zrf-1, *sox19b* expression appeared normal through 48 hpf in *has* mutant embryos (Fig. 4P,Q). By 72 hpf, *sox19b* expression is limited to cells near the central canal in wild-type larvae (Fig. 4O). By contrast, *sox19b* expression was absent from a comparable position in *has* mutant larvae but was maintained in more dorsal and ventral cells (Fig. 4R). Taken together, these data indicate that loss of PrkCi function results in loss of spinal cord precursors near the central canal during late embryogenesis.

To further investigate the relationship between apical polarity and spinal cord precursor division, we labeled embryos and larvae carrying the *Tg(olig2:egfp)* transgene with antibody specific to Phospho-Histone H3 (anti-PH3), which labels cells in M phase. At 2 dpf, numerous *olig2*$^{+}$ PH3^{+} cells were evident along the central canal of wild type embryos (Fig. 5A). By 3 dpf, the number of PH3^{+} cells was substantially decreased (Fig. 5B), consistent with our previous data showing a significant decline in the number of dividing cells in the spinal cord between 2 and 3 dpf (Park et al., 2007). In *has* mutant embryos, the central canal, outlined by *olig2*$^{+}$ cells, was discontinuous with remaining pockets of central canal surrounded by rosettes of *olig2*$^{+}$ cells (Fig. 5C,D). Notably, PH3^{+} cells were evident, but appeared only within the rosettes. As in wild type, 3 dpf

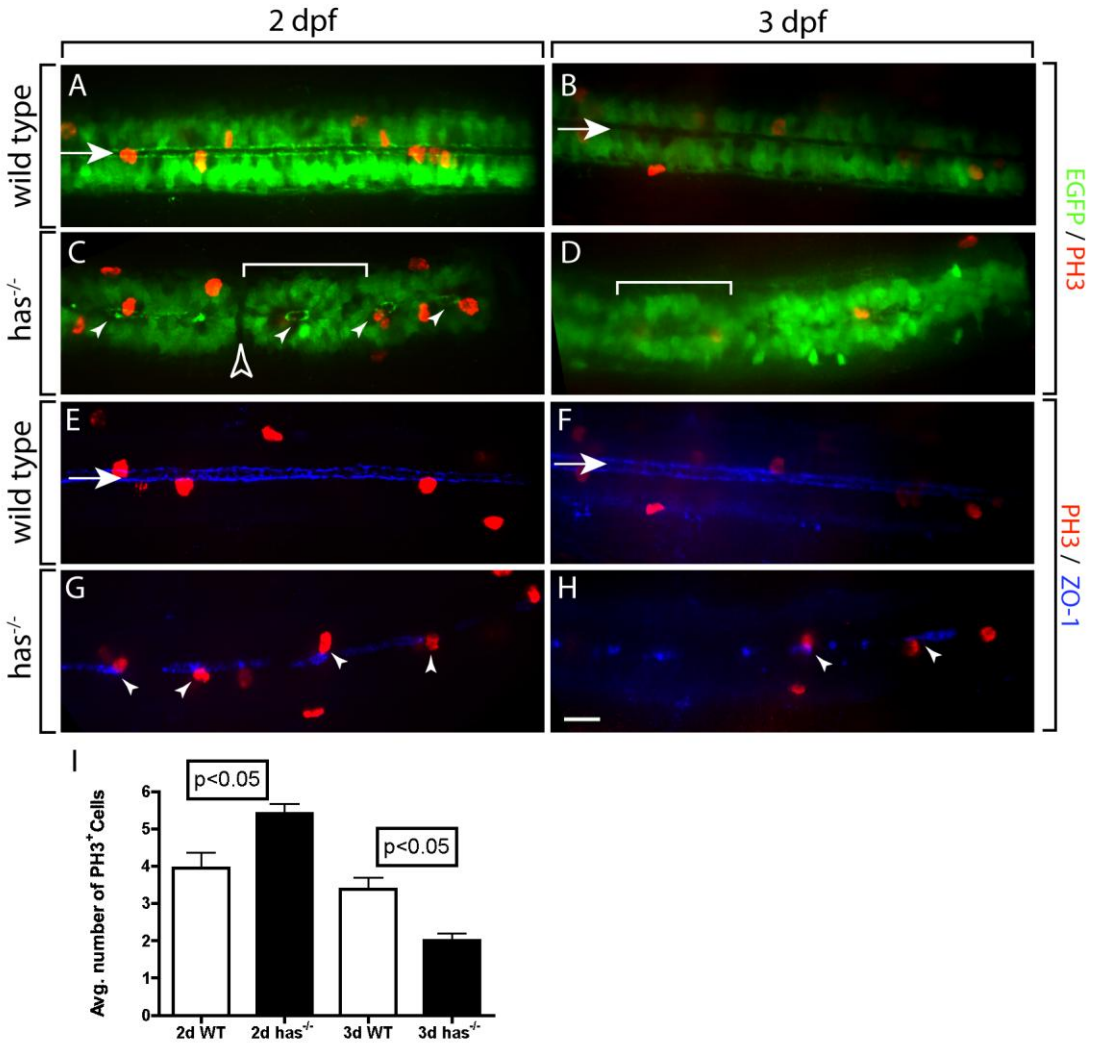


Fig. 5. Loss of PrkCi function causes a transient excess and then deficit of dividing spinal cord precursors. A-D: Images, from dorsal view and focused on the trunk spinal cord, of wild-type and *has*$^{-/-}$ embryos and larvae carrying the *Tg(olig2:egfp)* reporter and labeled with anti-PH3 antibody to mark M phase cells. In wild type most mitotic cells (arrowheads) are adjacent to the central canal (arrow). More cells divide at 2 dpf (A) than at 3 dpf (B). In *has*$^{-/-}$ embryos (C,D) many EGFP^{+} cells form rosettes (brackets) surrounding discontinuous portions of central canal (asterisks). PH3^{+} are usually only found within rosettes. **E-H:** Images, from dorsal view and focused on the trunk spinal cord, of wild-type and *has*$^{-/-}$ embryos and larvae labeled with anti-PH3 antibody (red) and anti-ZO-1 antibody (blue) to mark apical membrane. In wild type (E,F), ZO-1 localization outlines a continuous central canal closely associated with PH3^{+} mitotic cells. In *has*$^{-/-}$ embryos and larvae (G,H) ZO-1 labeling is discontinuous but mitotic cells are nearly always associated with remaining apical membrane. **I:** Quantification of PH3^{+} cells at 2 and 3 dpf over a 288 µm length of trunk spinal cord. Error bars represent s.e.m. Statistical significance was determined using Student's *t*-test.

has mutant larvae had fewer $PH3^+$ cells than at 2 dpf. We also use ZO-1 labeling to examine the distribution of dividing cells. As expected, $PH3^+$ cells in wild type had apical polarity and were associated with the central canal (Fig. 5E,F). 2 dpf *has* mutant embryos had patches of apical membrane, consistent with the discontinuous nature of the central canal, and $PH3^+$ cells were nearly always associated with ZO-1 labeling (Fig. 5G). We noticed that the density of $PH3^+$ cells seemed to be greater in mutant embryos than in wild type. In fact, whereas wild-type embryos had 3.9 $PH3^+$ cells within a 288 μm length of trunk spinal cord, mutant embryos had 5.4 $PH3^+$ cells over the same distance (Fig. 5I). At 3 dpf, the gaps in ZO-1 labeling of mutant larvae were larger and more frequent than at 2 dpf (Fig. 5H) and mutant larvae had fewer M phase cells (Fig. 5I). Therefore, $has^{-/-}$ mutant embryos have a transient increase and then deficit of dividing spinal cord precursors.

Our data raise the possibility that excess OPCs arise in $has^{-/-}$ mutant embryos due to misallocation of spinal cord precursors to an oligodendrocyte fate. To test this, we performed time-lapse imaging of wild-type and *has* mutant embryos carrying the *Tg(olig2:egfp)* transgene. Over a 10 hour period, approximately 1.5 fold more OPCs migrated from ventral spinal cord into dorsal spinal cord in *has* mutant embryos relative to wild type (Fig. 6A-C). Migrating OPCs did not divide more frequently in *has* mutant embryos than in wild type. Therefore, excess OPCs arise in *has* mutant embryos from specification of excess pMN precursors for oligodendrocyte fate.

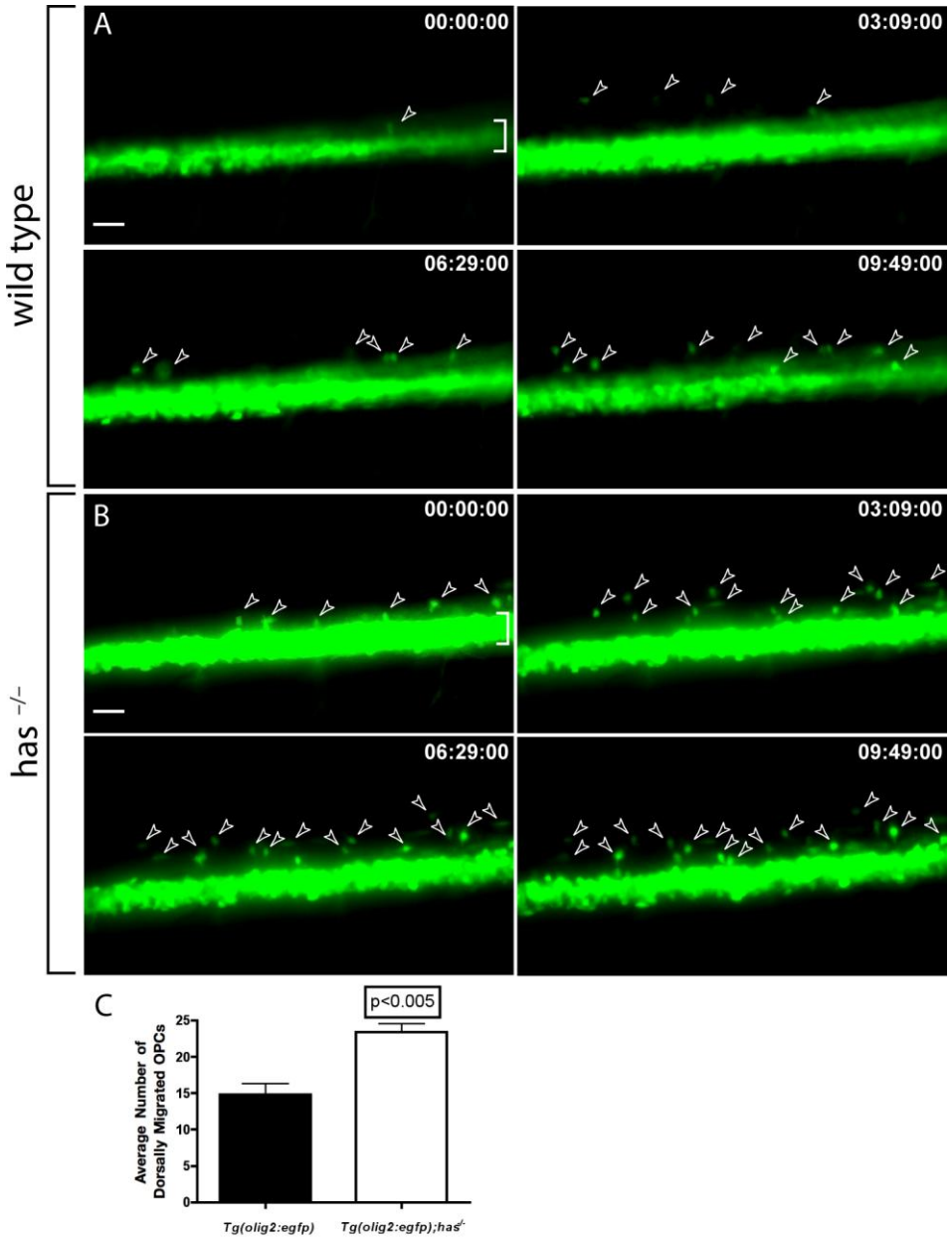


Fig. 6. Loss of PrkCi function causes formation of excess OPCs from pMN precursors. Panels show frames captured from time-lapse videos of wild-type and *has*$^{-/-}$ embryos carrying the *Tg(olig2:egfp)* reporter. Dorsal is up and time elapsed from beginning of imaging at 50 hpf is indicated in upper right corners of panels. Brackets mark pMN precursor domain in ventral spinal cord. **A:** Wild-type embryo. Arrowheads mark OPCs migrating dorsally from the ventral pMN precursor domain. **B:** *has*$^{-/-}$ embryo. More OPCs (arrowheads) emerge from the pMN domain than in wild type over a similar period of time. **C:** Quantification of dorsally migrating OPCs. Data were obtained from time-lapse movies of 6 wild-type and 5 *has*$^{-/-}$ embryos. Error bars represent s.e.m. Statistical significance was determined using Student's *t*-test.

Discussion

During development, neural precursors divide both to produce new precursors and to give rise to differentiating neurons and glial cells. In vertebrates, most precursors transform into differentiated cell types by early postnatal stages but, at least in some regions of the CNS, others gain characteristics of stem cells (Gotz and Huttner, 2005; Merkle and Alvarez-Buylla, 2006). Therefore, the mechanisms that determine whether precursors undergo self-renewing or differentiative divisions play key roles in neural development and homeostasis.

Extensive investigations of the development of the *Drosophila* nervous system have uncovered mechanisms for regulation of cell division and cell fate that provide a compelling model for vertebrate neural development (Doe, 2008). The nervous system of the fly embryo is produced from a sheet of ventral neuroectoderm. Divisions of neuroectodermal cells occur within the plane of the epithelium and the resulting progeny cells thereby remain within it. By virtue of a lateral inhibition mechanism mediated by Notch signaling, a select number of neuroectodermal cells are specified as neuroblasts, which begin to delaminate from the epithelium in the basal direction (Egger et al., 2008). Par complex proteins, which are localized to the apical membrane of neuroectodermal cells, remain with apical membrane of the neuroblasts as they delaminate (Yu et al., 2006). The apical localization of Par complex proteins serves to both restrict proteins that specify Ganglion Mother Cell (GMC) fate to basal membrane and, by organizing a complex of proteins including Inscuteable, Pins and heterotrimeric G proteins, to direct a 90^0 rotation of the mitotic spindle (Egger et al., 2008; Wu et al., 2008; Yu et al., 2006). Subsequent division of the neuroblast

occurs perpendicular to the plane of the epithelium resulting in distribution of GMC fate determinants to the basal progeny cell but not to the apical cell, which remains as a neuroblast. In embryos the GMCs then generally divide once to produce a pair of differentiated neurons.

In the neural tube of vertebrate embryos, the mitotic apparatus of dividing neuroepithelial cells is apically positioned so that divisions occur close to the lumen (Hollyday, 2001). As cells exit the mitotic cycle, they lose contact with the apical, lumenal surface and migrate basally where they differentiate as neurons or glia. Following the *Drosophila* example, divisions within the plane of the epithelium might then produce progeny that remain as neuroepithelial precursors whereas divisions that are perpendicular could be asymmetric, producing one apically positioned precursor cell and one basally positioned differentiated cell. Consistent with this, imaging of living cells in slices of ferret brain revealed both planar and perpendicular divisions as well as some intermediate divisions (Chenn and McConnell, 1995). Imaging of slices from embryonic mouse cortex revealed similar mitotic patterns accompanied by rapid migration of basal progeny produced by perpendicular divisions (Haydar et al., 2003). However, several other studies now indicate that the majority of precursor divisions in the neural tube and retina of vertebrate embryos are planar (Das et al., 2003; Geldmacher-Voss et al., 2003; Konno et al., 2008; Kosodo et al., 2004; Lyons et al., 2003; Morin et al., 2007; Noctor et al., 2008). Our time-lapse imaging of zebrafish spinal cord precursors revealed a complete absence of perpendicular divisions, even during a period when many neurons and OPCs are formed.

Therefore, the simple model in which asymmetric neural cell fates result from perpendicular divisions seemingly cannot account for the many neurons and glia that arise during development. Careful examination of neural precursor divisions revealed that some result in unequal segregation of apical membrane to progeny cells, which correlated with asymmetric fate (Konno et al., 2008; Kosodo et al., 2004). Whether slight differences in the amount of apical membrane produces asymmetric fates via asymmetric distribution of fate determinants and whether these differences can fully account for the decision to remain as a precursor or differentiate remain unknown.

Maintenance of adherens junctions is associated with maintenance of some stem cell cell populations (Fuchs et al., 2004; Song et al., 2002) and could provide a mechanism by which asymmetric segregation of apical membrane to progeny cells influences fate. Vertebrate embryos localize Par complex proteins to apical adherens junctions of the neuroepithelium (Afonso and Henrique, 2006; Geldmacher-Voss et al., 2003; Imai et al., 2006; Kovac et al., 2007; Manabe et al., 2002; von Trotha et al., 2006) and these are lost from neuroepithelial cells as they differentiate. Therefore, Par complex and adherens junction associated proteins might be neccesary for maintaining proliferative neuropithelial precursors. This idea has been tested by examining mice deficient for *Prkci* function in Nestin^{+} neural precursors (Imai et al., 2006). Neuroeptihelial character and adherens junctions were lost from mutant mice but, surprisingly, the total number of dividing cells and neurons were normal at E15.5 suggesting that, at least in this instance, *Prkci* is not necessary for neurogenesis.

Similar conclusions have been made from some studies testing the functions of vertebrate proteins homologous to fly proteins that orient the mitotic spindle. In both chick and mouse, disruption of LGN, a G protein regulator homologous to *Drosophila* Pins, during early periods of neurogenesis randomized the normally planar orientation of neuroepithelial cell divisions without overtly altering neurogenesis but did cause a basal displacement of dividing cells (Konno et al., 2008; Morin et al., 2007). By contrast, another study showed a higher frequency of non-planar divisions at slightly later stages of neurogenesis and found that blocking G$\beta\lambda$ signaling and function of the Pins homolog AGS3 increases the number of planar divisions that produce two postmitotic neurons (Sanada and Tsai, 2005).

Our own investigation now reveals that loss of PrkCi function and adherens junctions in zebrafish results in the loss of substantial numbers of spinal cord precursors during late embryogenesis, with concomitant increases in OPCs. In wild type embryos, ZO-1 labeling reveals adherens junctions at the medial septum and surrounding the central canal of the spinal cord during neurogenic stages. ZO-1 is gradually lost from the medial septum as neurogenesis subsides at late embryonic stage but persists around the central canal, an active site of cell division, into juvenile stage (Park et al., 2007). In *has* mutant embryos, ZO-1 localization initially appeared normal, probably due to Prkcz function. Accordingly, neural precursor divisions resembled wild type, remaining within the plane of the neuroepithelium, and neurons were formed in apparently normal number. However, ZO-1 disappeared prematurely from the

medial septum and only marked a reduced, apparently discontinous central canal at late embryonic and early larval stages. At about the same time, divisions of many precursors at the level of the central canal shifted from planar to oblique and the central canal appeared to be invaded by cells. These observations indidate that PrkCi maintains the neuroepithelial character of the spinal cord, consistent with the proposed role of PrkCi in the mouse neocortex (Imai et al., 2006). In *has* mutant embryos, spinal cord cells formed rosettes around remnants of central canal and these contained mitotically active cells. After a transient increase in the number of mitotic cells at a late embryonic stage, the number of dividing cells fell below normal and radial glia, revealed by an *olig2* transgenic reporter and Zrf-1 labeling, were lost from the region of the central canal by early larval stage. Additionally, by larval stage the central region of the spinal cord, normally occupied by radial glial cells bodies, was populated by neurons and mutants had a slight but statistically significant increase in the number of OPCs formed from ventral spinal cord *olig2*$^{+}$ cells. We conclude that PrkCi is necessary to maintain spinal cord precursors and that in its absence dividing cells exit the cell cycle and differentiate as neurons and glia.

CHAPTER III

CDC42 SIGNALING REGULATES MIGRATION OF THE *NKX2.2A*$^{+}$ SUBSET OF ZEBRAFISH OPCS

Introduction

The myelinating cells of the CNS, oligodendrocytes, wrap and insulate axons to allow for rapid propagation of electrical impulses over long distances. Oligodendrocytes are specified in the pMN domain of the ventral spinal cord. After specification, oligodendrocytes must migrate to their target axons located throughout the CNS. Although oligodendrocytes function so intimately with neurons, they often are not specified near their interacting neuron and may have to travel long distances to myelinate an axon. The mechanisms that control oligodendrocyte migration remain to be clearly defined.

Oligodendrocytes are initially specified as an immature oligodendrocyte progenitors cells (OPCs). These OPCs migrate out of the pMN domain to occupy positions in the dorsal and ventral spinal cord. As they migrate, OPCs mature and eventually become oligodendrocytes. Part of this maturation process involves changing their morphology as well as regulating expression of different transcription factors. When an OPC is fully mature, the oligodendrocyte ceases migration and begins ensheathing axons and at a later point begins producing myelin. It is becoming increasingly clear that several mechanisms may be involved in oligodendrocyte migration.

Currently, our understanding of OPC migration is very limited and most work focuses on in vitro migration. In 2006, Kirby et al performed in vivo studies in which they investigated OPC migration and wrapping. From these experiments we learned that OPC migration is a dynamic and highly variable process that may involve the constant remodeling of the actin cytoskeleton. We also learned that migrating OPCs extend and retract highly protrusive filopodial like processes that appear to sense their surrounding environment and direct OPC migration. Because the Rho family of GTPase is important in regulating filopodial formation and OPC migration appears to receive migratory information from these filopodial like processes, we hypothesized that members of the Rho family of proteins played a role in OPC migration.

The Rho family of small GTPases is made up of about 20 intracellular signaling molecules, many of which have been shown to be important in regulating the actin cytoskeleton. These Rho family GTPases function by shuttling between a GTP-bound active form and a GDP-bound inactive form. The shuttling between these two states is controlled by two sets of proteins, guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Heasman and Ridley, 2008) (Fig. 1). In their GTP bound active form Rho GTPases can interact with and activate downstream effectors leading to regulation of intracellular processes such as vesicle transport, cell cycle progression, microtubule rearrangements and gene expression. Rho-GTPases can also lead to stimulation of certain processes like migration, neuronal development, cell adhesion and cell division (Heasman and Ridley, 2008).

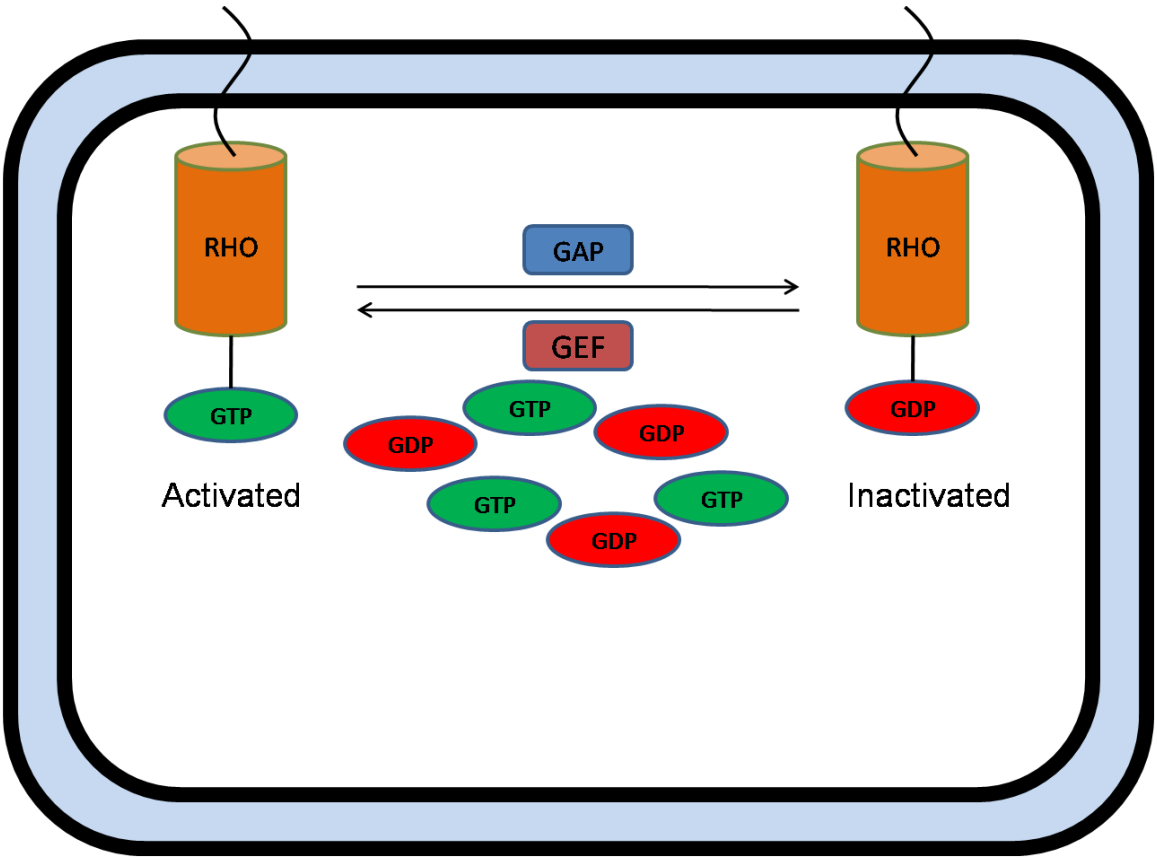


Figure 1. Small Rho-GTPase Activation. A cartoon schematic showing the activation of the small Rho-GTPases Cdc42. Cdc42 is activated when guanine exchange factors (GEFs) promote the release of GDP and the binding of GTP. Conversely, GTPase-activating proteins (GAPs) inactivates Cdc42, by activating the intrinsic GTPase activity of Cdc42.

Cdc42 is an evolutionarily conserved small Rho family GTPase that functions in regulating cell polarity and the remodeling of the actin cytoskeleton. It was originally detected in the yeast *Saccharomyces cerevisiae* as a mutation that caused defects in budding and cell polarity (Adams et al., 1990; Johnson and Pringle, 1990). Since then Cdc42 has been shown to function in a variety of cellular processes like cell migration, cell polarity and cellular actin dynamics (Etienne-Manneville et al., 2005; Hall, 2005; Yang and Zheng, 2007).

In many cell types Cdc42 is important in regulating apical polarity by recruiting members of the apical polarity complex, Par6/PrkCi ,to the apical membrane by binding to the semi-CRIB domain of Par6 (Garrard et al., 2003). Although the partitioning defective protein, Par3, function is part of the evolutionarily conserved Par/PrkCi complex, its role in Cdc42 signaling remains unclear (Atwood et al., 2007; Welchman et al., 2007). In vitro cell migration assays on cultured astrocytes show a need for Cdc42 in cell migration and filopodial formation, as astrocytes fail to migrate in its absence (Etienne-Manneville et al., 2005). In addition, highly filopodial process like axon growth cone projection and neurite extension require Cdc42 for proper pathfinding and extension (Kim et al., 2002). Because Cdc42 seems to be required for migration and filopodial extensions in highly migratory cells, we hypothesized that Cdc42 be required for OPC migration *in vivo*. The following experiments were designed to test this hypothesis.

Experimental Procedures

Fish Husbandry

Embryos were produced by pair-wise mating and kept at 28.5^{o}C in egg water or embryo medium. Embryos were staged to hours post fertilization (hpf) or days post fertilization (dpf) according to established zebrafish guidelines (Kimmel et al., 1995). Embryos that were to be used for live imaging, immunohistochemistry, or in situ hybridization were treated in 0.003% phenylthiourea (PTU) in egg water to block pigmentation. The experiments conducted in this chapter used the following strains of zebrafish were used: AB, *Tg(nkx2.2a:megfp)* (Kirby et al., 2006), *Tg(sox10(7.2):mrfp)* (Kucenas et al., 2008) and *Tg(hsp70:cdc42(N17))*.

Immunocytochemistry

Embryos and larvae were fixed in 4% antibody fix (4% paraformaldehyde, 8% sucrose, 1x PBS) overnight at 4^{o}C. After fixing, the embryos were embedded in 1.5% agar/ 5% sucrose blocks and placed in 30% sucrose/PBS solution to equilibrate overnight. The blocks were then frozen over 2-methylbutane chilled by liquid nitrogen. We collected 10-12 μm sections on superfrost microscope slides using a cryostat microtome. The sections were rehydrated in 1x PBS for 30 min. and then blocked in 2% BSA/sheep serum in 1x PBS for 30 min. before incubating with primary antibody overnight at 4^{o}C. For fluorescent detection of antibody labeling, we used Alexa Fluor 488, Alexa Fluor 568 goat anti-mouse or goat anti-rabbit conjugates (1:500, Molecular Probes). The primary antibodies

used included rabbit anti-PrkCi (#sc-216, 1:200, Santa Cruz Biotechnology, Inc.), mouse anti-Islet (clone # 39.4D5-s, 1:1,000, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA), rabbit anti-Sox10 (1:1,000) (Park et al., 2005), and mouse anti-Myc (1:200, Fisher) and mouse anti-ZRF-1 (1:500, DSHB).

In vivo time-lapse imaging

Embryos were raised in egg water containing PTU and at the appropriate stages manually dechorionated using watchmaker forceps. Embryos were anesthetized using 3-aminobenzoic acid ethyl ester (Tricaine) and mounted laterally or dorsally in 35 mm glass bottom petri dishes containing 0.8% low-melting temperature agarose. Confocal time-lapse movies were obtain by using a 40X oil immersion objective mounted on a Zeiss Axiovert 200 microscope equipped with a PerkinElmer spinning disk confocal system. Z-stack images were obtained every 15 min and compiled into a Quicktime movie using Volocity software (Improvision).

Heat-shock induction

To induce expression of dominant negative Cdc42(N17), *Tg(hsp70:cdc42(N17)myc)* heterozygous fish were crossed either with *Tg(nkx2.2:megfp)* or *Tg(sox10(7.2):mrfp)* fish and the resultant embryos raised in egg water containing PTU at 28.5°C. Beakers containing PTU egg water were allowed to come to temperature in a water bath set at 40°C. Embryos younger than 36 hpf were heat shocked in their chorions. Embryos were heat shocked for

30 min and then transferred to a 28.5° incubator to come back down to temperature. Transgenic embryos identified by Myc expression and morphology were selected for analysis.

Results

Loss of PrkCi function disrupts OPC distribution in the spinal cord.

In situ hybridizations using *claudink (cldk)* anti-sense riboprobe revealed that OPCs in *has*$^{-/-}$ embryos, which lack functional PrkCi, have OPCs that are ectopically located in the medial spinal cord (Fig. 2 B). In wildtype embryos, OPCs were absent from the medial septum. (Fig.2 A). Similarly, antibody labeling using the OPC specification marker Sox10, also revealed ectopic OPCs (Fig. 2 C,D). Zebrafish OPC migration is a highly dynamic process in which OPCs extend and retract their filopodial like processes until they encounter a target axon to wrap and myelinate. In the absence of PrkCi a subset of OPCs may lose their ability to migrate normally. To investigate this further, we decided to look at signaling pathways in which PrkCi may function. Because OPC migration is such a dynamic process, we hypothesized that OPC migration requires the constant remodeling and reorganization of the actin cytoskeleton so that the migrating OPC can achieve polarized directed migration. To investigate this hypothesis, we focused on the small rho GTPase Cdc42, which has been shown to have an evolutionarily conserved role in cytoskeletal remodeling.

Phenotypic characterization of dominant negative Cdc42(N17) embryos.

Because the Rho-GTPase Cdc42 is important in several developmental processes, it was important for us to have temporal control of Cdc42 signaling. To achieve this, we disrupted Cdc42 using a transgenic zebrafish line that expresses a dominant negative form of Cdc42 (Cdc42(N17)) under the control of the heat shock promoter. Dominant negative Cdc42(N17) has a threonine to asparagine substitution on residue 17, which abolishes its affinity for GTP and lowers its affinity for GDP (Luo et al., 1994). When expressed at high levels, Cdc42(N17) binds GEFs thereby preventing them from activitating endogenous Cdc42 (Luo et al., 1994). Heatshocking wild-type embryos at 24, 36 and 48 hpf had no affect on their morphology at 3 dpf (Fig. 3 A). By contrast, *Tg(hsp70:cdc42(N17*)) embryos that were heatshocked at 24, 36 and 48 hpf and analyzed at 3 dpf had dorsally curved tails, small eyes, cardiac edema, and some forebrain and hindbrain necrosis (Fig. 3 B). This necrosis did not extend to the rest of the CNS because the spinal cord was necrosis free (Fig. 3 B). The *Tg(hsp70:cdc42(N17)myc)* transgenic line was designed so that a Myc epitope tag is expressed at the C-terminal end of the dominant negative Cdc42(N17) protein. Therefore, we performed antibody labeling on embryos that were heatshocked to determine the expression of the Cdc42(N17) protein in the spinal cord using an antibody against Myc. Cdc42(N17)$^{+}$ embryos heat shocked at 24 hpf and fixed 4 hours later showed Myc expression in all cells of the spinal cord and the surrounding

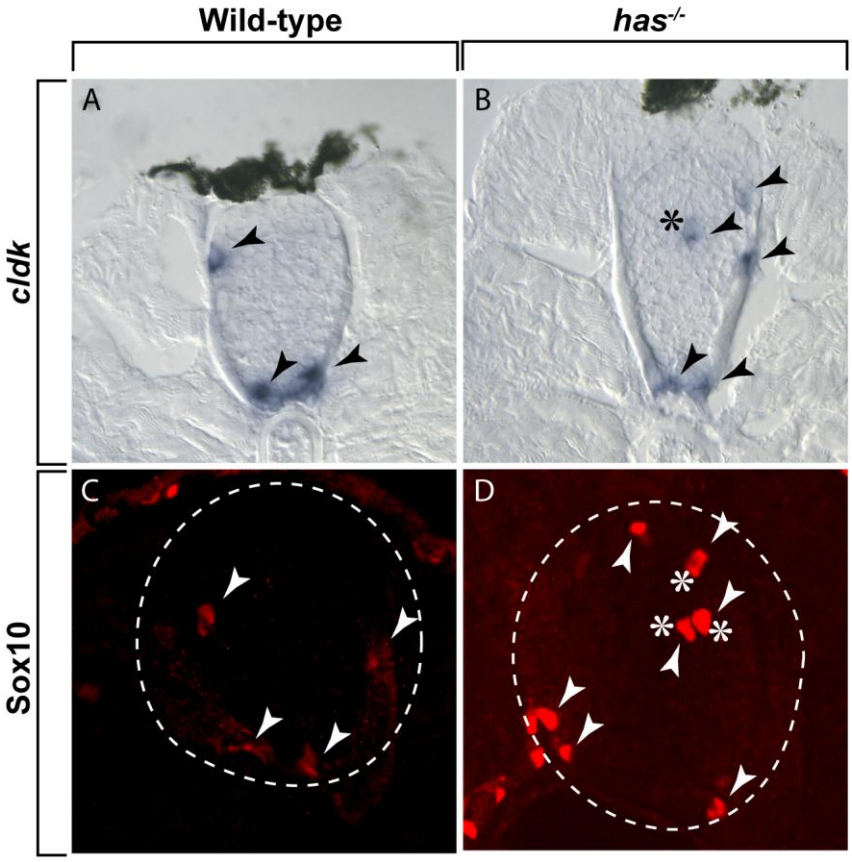


Figure 2. PrkCi may control oligodendrocyte progenitor cell migration. Dashed circle demarcates the spinal cord border. Arrowheads indicate the OPCs, and asterisks denote ectopically positions OPCs. **A,B:** In situ hybridization using *cldnk* riboprobe shows an increase in the number of OPCs, as well ectopic OPCs in the medial septum. **C,D:** Sections labeled with anti-Sox10 antibody. Sox10 labeling also showed ectopic OPCs.

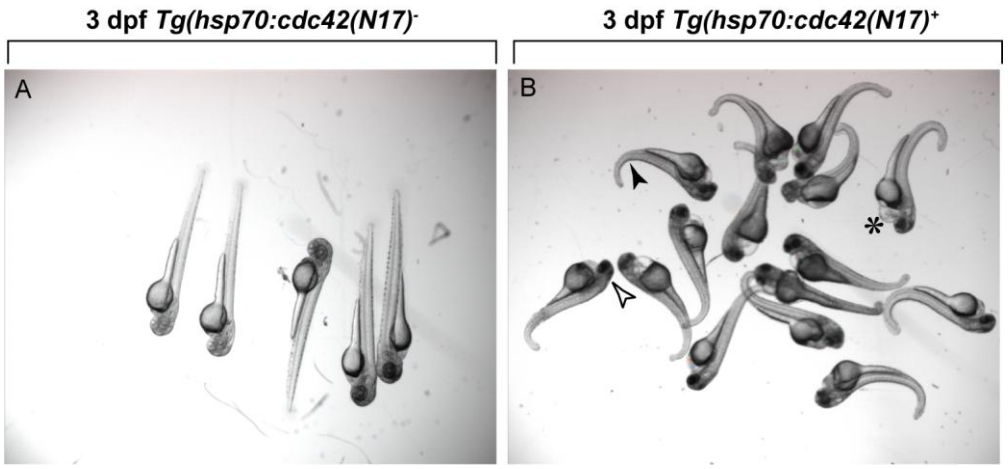


Figure 3. Expression of Cdc42(N17) perturbs embryo morphology.
A. Tg(hsp70:Cdc42(N17))$^{-}$ embryos maintain a wildtype morphology after heatshocking at 24, 48. and 72 hpf. B. Tg(hsp70:Cdc42(N17))$^{+}$ embryos have reduced eyes, dorsally curved tails, cardiac edema and some head necrosis after heatshocking at 24, 48 and 72 hpf.

muscle (data not shown). Similar expression was observed in Cdc42(N17)$^{+}$ embryos that were heatshocked at 48 and 72 hpf (data not shown). Cdc42(N17)$^{-}$ embryos heat shocked at similar time points lacked Myc expression in the spinal cord at all time points. Myc labeling throughout the embryo demonstrated that heat shocking *Tg(hsp70:cdc42(N17))* embryos drove expression of the Cdc42(N17)-Myc fusion protein. This suggests that Cdc42 function is being inhibited; however biochemical analysis would be needed to determine that conclusively.

Disruption of Cdc42 function affects OPC migration but not specification. We have shown that the loss of PrkCi function results in the formation of excess OPCs and loss of neural precursors. Cdc42 plays an important role in stabilizing PrkCi to the apical membrane. Because apical polarity proteins are dependent on each other for proper localization to the apical membrane, disruption in Cdc42 may affect apical protein localization. Therefore, we reasoned that expression of dominant negative Cdc42 protein might produce a phenotype similar to that of loss of PrkCi function. To test this we performed antibody labeling on 3 dpf Cdc42(N17)$^{-}$ and Cdc42(N17)$^{+}$ embryos that were heat shocked at 24, 48 and 72 hpf. The 3 dpf time point was chosen because in *has*$^{-/-}$ embryos we observed the biggest phenotypical differences at 3 dpf. Using an antibody against PrkC we observed that PrkC is localized around the central canal at 3 dpf in Cdc42(N17)$^{-}$ embryos (Fig. 4A). In heatshocked Cdc42(N17)$^{+}$ embryos we observed that PrkC is also localized around the central canal. However in Cdc42(N17)$^{+}$ embryos we

observed residual localization of PrkC in medial septum (Fig. 4B). In wildtype embryos PrkC is localized the medial septum until 48 hpf when we begin to observe gaps in PrkC localization. By 72 hpf there is no localization of PrkC in the medial septum.

Because disruption of PrkCi in the spinal cord resulted in a loss of neural precursors, we decided to looked at the patterning and location of the radial glia in the spinal cord. ZRF-1 labeling in the spinal cord of 3 dpf heat shocked Cdc42(N17)$^{-}$ and heat shocked Cdc42(N17)$^{+}$ embryos showed the characteristic radial glia morphology in which cell body makes contact with the apical membrane of the spinal cord and the radial process makes contact with the pial surface (Fig. 4 C,D). In Cdc42(N17)$^{+}$ embryos the radial glia still extend from the apical to the pial surface, however, their radial fibers appear more disorganized but there is no apparent loss of radial glia in the medial spinal cord similar to *has*$^{-/-}$ embryos (Fig. 4 D). To determine if specification of neuronal subtypes were affected, we next looked at the number of Islet^{+} cells in the absence of normal Cdc42 function. At 3 dpf Islet antibody labeling showed no difference in the number of ventral Islet^{+} cells between wild-type and *has*$^{-/-}$ spinal cords(Fig. 4 E,F). Similar to the PrkCi loss of function data, there was also no difference in the number of ventral Islet^{+} cells in heat shocked Cdc42(N17)$^{+}$ when compared to Cdc42(N17)$^{-}$ embryos (Fig. 4 I).

To investigate OPC specification, we performed immunocytochemistry using anti-Sox10 antibody to mark OPCs. This revealed no statistical significance differences in the number of Sox10^{+} cells in Cdc42(N17)$^{-}$ and Cdc42(N17)$^{+}$

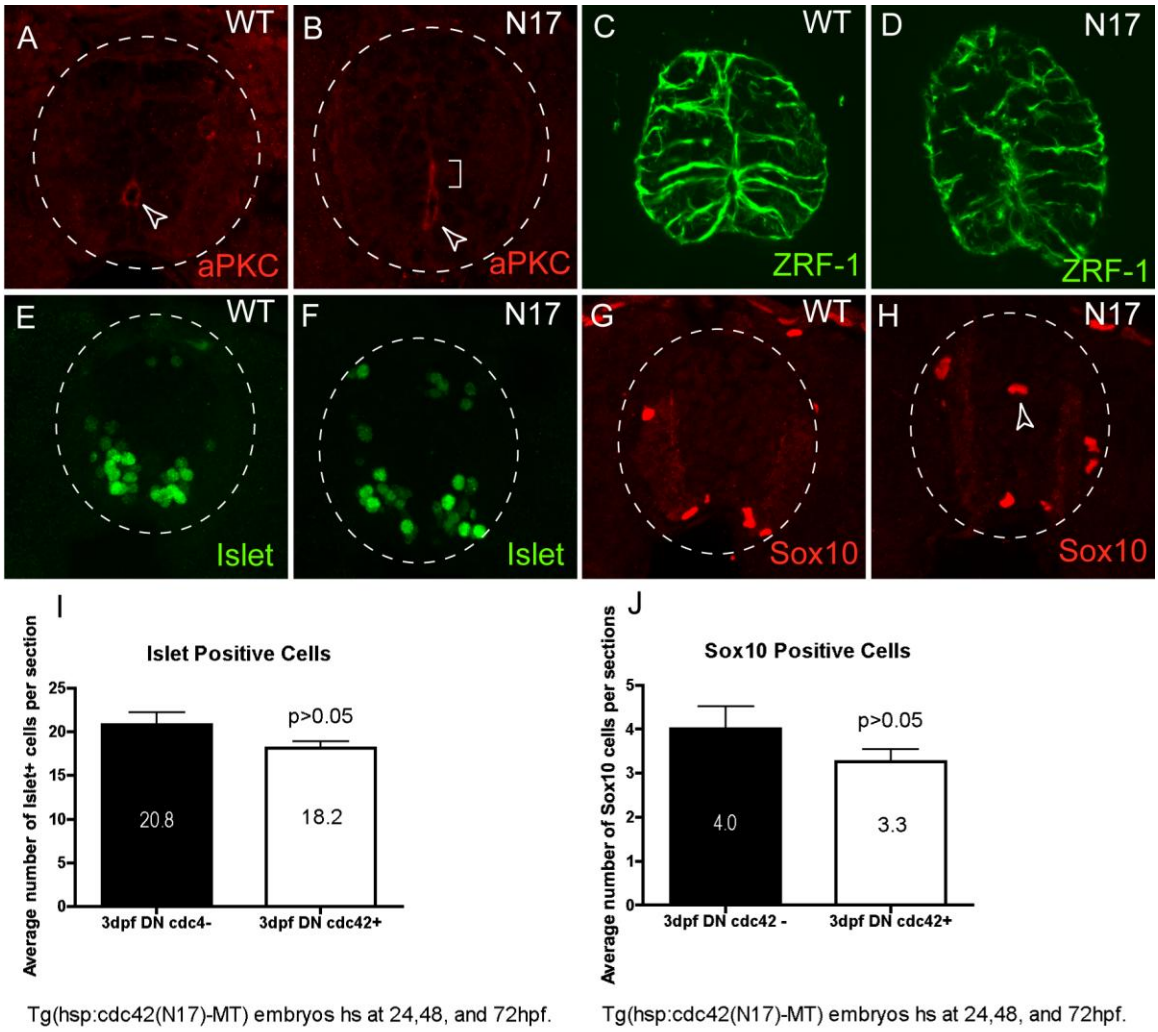


Figure 4. **Loss of Cdc42 function produces ectopic OPCs but does not affect apical polarity.** All panels show transverse sections through trunk spinal cord. Dashed circle demarcates the perimeter of the spinal cord. **A,B**: Sections labeled with anti-PrkCi antibody. PrkCi is localized to the central canal of the spinal cord of wild-type embryos at 3 dpf (open arrowhead) (A). In *Tg(hsp70:Cdc42(N17)myc)*$^{+}$ embryos heatshocked at 24, 48 and 72 hpf PrkCi is localized to the central canal (open arrowhead) and a small portion of the medial septum (bracket) (B). **C,D:** Anti-ZRF-1 labeling revealed normal number and distribution of radial glia in the spinal cord at 3dpf. In the Cdc(N17)$^{+}$ embryos the radial processes of the radial glia were less organized (D). **E,F:** Anti-Islet antibody labeling revealed normal numbers of motor neurons and Rohon-Beard sensory neurons were similar in wild-type and CdcN17)$^{+}$ embryos at 3 dpf. **G,H:** Anti-Sox10 antibody labeling reveal no difference in the number of Sox10^{+} cells in wild-type and Cdc42(N17)$^{+}$ spinal cords at 3dpf. Cdc42(N17)$^{+}$ embryos did however, have ectoicly positioned OPCs in their spinal cord at 3dpf (open arrowhead)(H). Quantification of spinal cord Islet+ cells. Quantification of spinal cord Sox10+ OPCs. Error bars represent s.e.m. Statiscal significance was determined using Student's t-test. Scale bar = 20μm

spinal cords at 3dpf (Fig. 4 G,H J). Although the number of OPCs was unaffected by expression of dominant negative Cdc42, the positions of the OPCs were disrupted. OPCs were ectopically positioned in the medial spinal cords of *Tg(hsp70l:Cdc42(N17)* embryos following heat shock (Fig. 4H), similar to the abnormal positions of OPCs in *has*$^{-/-}$ embryos.

***nkx2.2a*$^{+}$ OPCs fail to migrate properly when Cdc42 signaling is disrupted.** Sox10 antibody labeling revealed ectopic OPCs in the absence of functional Cdc42. One interpretation of these data is that Cdc42 is required for the proper migration of OPCs. To test this, we decided to look at OPC migration in the absence of Cdc42 using time-lapse microscopy. To achieve this goal, we heat shocked *Tg(nkx2.2a:megfp)* embryos at 24, 36 and 48 hpf and began imaging laterally mounted embryos at 50 hpf for a period of 24 h. The *Tg(nkx2.2:megfp)* zebrafish line allows us to observe migration of OPCs and perineurial glia in the zebrafish spinal cord (Kirby et al., 2006; Kucenas et al., 2008).

During the time-lapse movies we observed that shortly after 50 hpf *nkx2.2a*$^{+}$ OPCs begin to extend and retract membrane processes into the dorsal spinal cord (Fig. 5). Eventually the OPC cell bodies followed their projections and migrated out of the ventral *nkx2.2* domain. As the time-lapse continues, OPCs were observed migrating throughout the spinal cord in the general direction of the dorsal longitudinal fascicle (DLF) (Fig. 5). This process is repeated over several hours as more OPCs migrate and populate the dorsal spinal cord. Just prior to 72

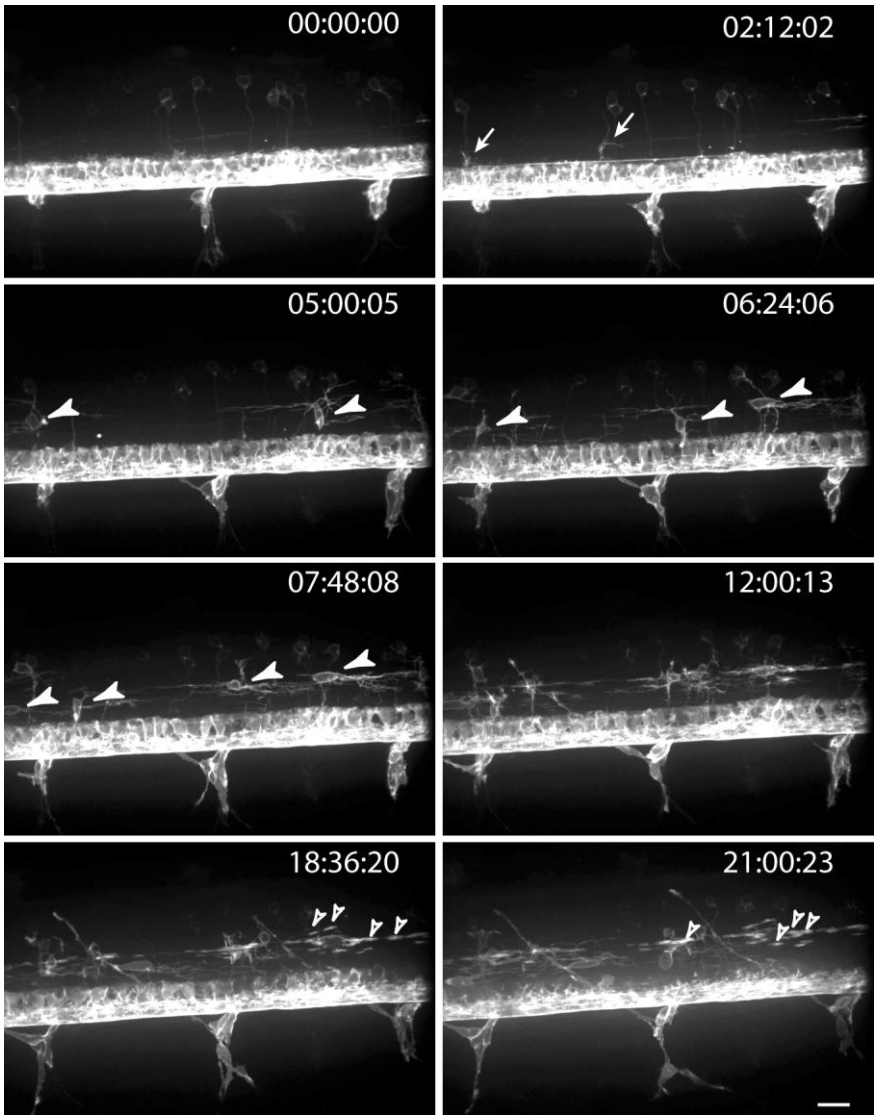


Figure 5. ***nkx2.2*$^{+}$ OPC migration in the zebrafish spinal cord.** Frames captured from time-lapse movies, from a lateral view, of *Tg(nkx2.2a:megfp)* embryos. Numbers in upper right corners indicate time elapsed from beginning of imaging at 50 hpf. *nkx2.2a*$^{+}$ OPCs send fine filopodial like processes out of the ventral *nkx2.2a* domain (arrows). Eventually the *nkx2.2a*$^{+}$ OPCs migrate out of the ventral *nkx2.2a* domain and take up positions within the dorsal spinal cord (closed arrowheads).

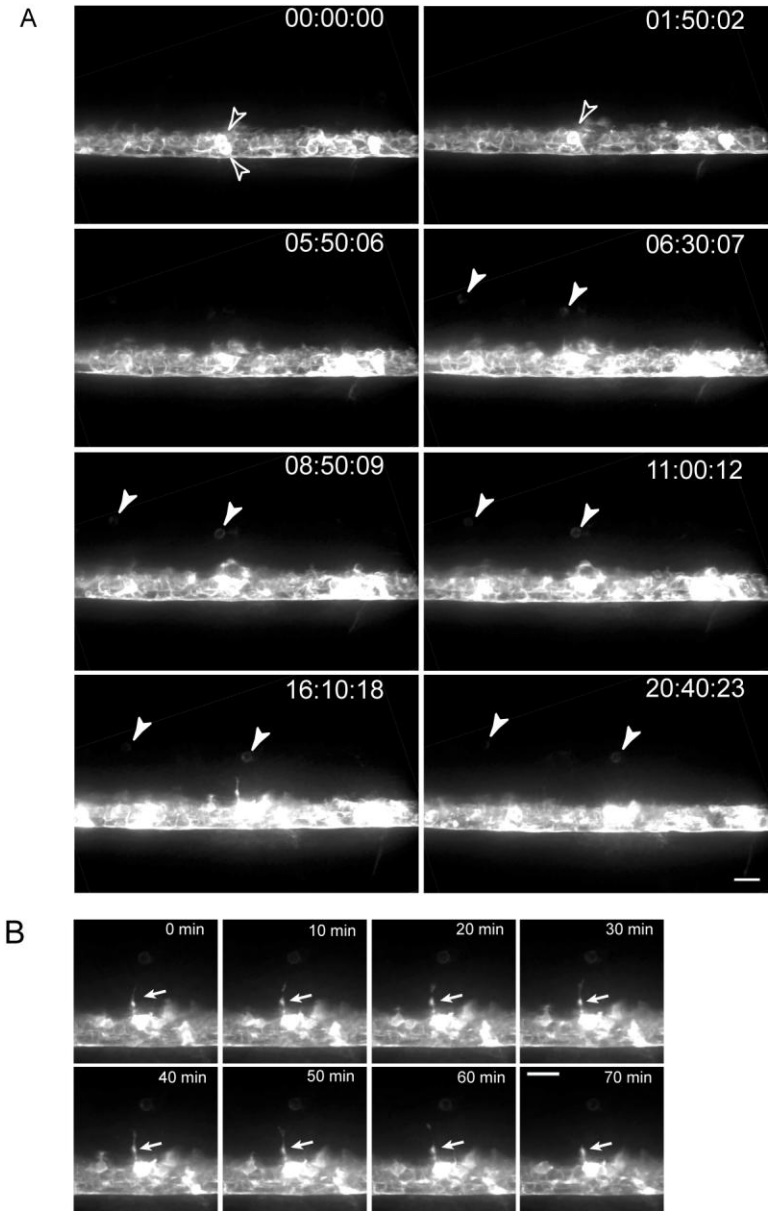


Figure 6. ***nkx2.2*$^{+}$ OPC migration is perturbed in *Tg(hsp:Cdc42(N17))*$^{+}$ embryos.** Frames captured from time-lapse movies, from a lateral view, of *Tg(nkx2.2a:megfp);(hsp:Cdc42(N17))*$^{+}$ embryos. Numbers in upper right corners indicate time elapsed from beginning of imaging at 50 hpf. (A) Unlike in wild-type embryos, the *nkx2.2*$^{+}$ OPCs in *Tg(hsp:Cdc42(N17))*$^{+}$ embryos fail to extend any fine filopdial like processes out the ventral *nkx2.2a*$^{+}$ domain. As the time-lapse imaging begins, rounded *nkx2.2a*$^{+}$ cell are observed the ventral *nkx2.2a*$^{+}$ domain (open arrowhead). As the movie progresses, rounded *nkx2.2a*$^{+}$ cells can bee seen migrating through the dorsal spinal cord. These cells fail to extend any filopodial like processes and are never observed wrapping axons. (B) As the time-lapse imaging continues, filopodilial processes are observed coming out of the ventral spinal cord.

hpf some of the *nkx2.2a*$^{+}$ cells stop migrating and begin to wrap the axons of the DLF (Fig. 5). In *Tg(nkx2.2a:megfp;(hsp70:Cdc42(N17)myc))*$^{+}$ embryos heat shocked at 24, 36 and 48 hpf a different pattern of migration was observed. In the *nkx2.2* domain, rounded *nkx2.2a*$^{+}$ cells were seen moving throughout the domain (Fig. 6 A). As time elapsed these rounded *nkx2.2a*$^{+}$ cells, which lack any filopodial like projections, migrated out of the *nkx2.2* domain to occupy positions in the dorsal spinal cord (Fig. 6 A). As the time-lapse continued, we observed that the number of dorsally migrated *nkx2.2a*$^{+}$ OPCs was much less in the Cdc42 (N17)$^{+}$ embryos than in control embryos. In addition, as the experiment progressed, we observed much more activity of the *nkx2.2a*$^{+}$ cells in the *nkx2.2a* domain. Their morphology changed from being rounded and took on an oblong shape, similar to the morphology of *nkx2.2a*$^{+}$ observed in control embryos. Eventually highly protrusive *nkx2.2a*$^{+}$OPCs can be seen migrating out of the ventral *nkx2.2a* domain (Fig. 6 B). This suggests that when Cdc42 signaling is blocked, Nkx2.2a+ OPCs are unable to pathfind out of the ventral *nkx2.2a* domain.

Sox10^{+} OPCs maintain normal morphology and migration in the absence of Cdc42 signaling.

In the ventral spinal cord, the *nkx2.2* and *olig2* domains can give rise to OPCs. In the mouse and chick spinal cord, both populations of OPCs eventually begin to express both *Nkx2.2* and *Olig2*. However, in the zebrafish spinal cord there are two populations of OPCs: *nkx2.2a*$^{-}$*olig2*$^{+}$ and *nkx2.2a*$^{+}$*olig2*$^{+}$ OPCs, although both populations express the OPCs marker Sox10. From my time-lapse experiments,

we determined that the number of *nkx2.2a*$^{+}$ OPCs were reduced, however antibody labeling showed no difference in the average number of Sox10^{+} OPCs in the spinal cord between heat shocked control and Cdc42(N17)$^{+}$ embryos (Fig. 4 G,H,J). Because *nkx2.2a*$^{+}$ OPCs represent a portion of the Sox10 expressing OPCs in the spinal cord, we decided to look at the morphology and migration of all the OPCs in the spinal cord using *Tg(sox10(7.2):mrfp)* transgenic line. After heatshocking *Tg(sox10(7.2):mrfp)* embryos at 24, 36 and 48 hpf we laterally mounted embryos and began imaging at 50 hpf.

At the start of the time-lapse, Sox10^{+} cells are seen in the ventral part of the spinal cord where they are specified, however as the time-lapse continues Sox10^{+} OPCs can be seen extending and retracting filopodial like projections as they prepare to migrate out of the ventral domain. Shortly after 50 hpf the Sox10^{+} OPCs begin to take a dorsal migratory path, generally in the direction of the filopdial protrusions (Fig. 7). In addition to their dorsal migration, these Sox10^{+} OPCs move laterally searching out a position in which to settle. Just prior to 72 hpf Sox10^{+} OPCs stop migrating and can be seen wrapping target axons in the dorsal spinal cord (Fig. 7).

Time-lapse movies of *Tg(sox10(7.2):mrfp);(hsp70:Cdc42(N17)myc))*$^{+}$ embryos heat shocked at 24, 36 and 48 hpf showed a similar phenotype to control embryos. Similar to control embryo, Sox10^{+} OPCs began migrating out of the ventral spinal cord shortly after 50 hpf in *Tg(sox10(7.2):mrfp); (hsp70:Cdc42(N17)myc))*$^{+}$ embryos (Fig. 8). OPCs appeared to have a

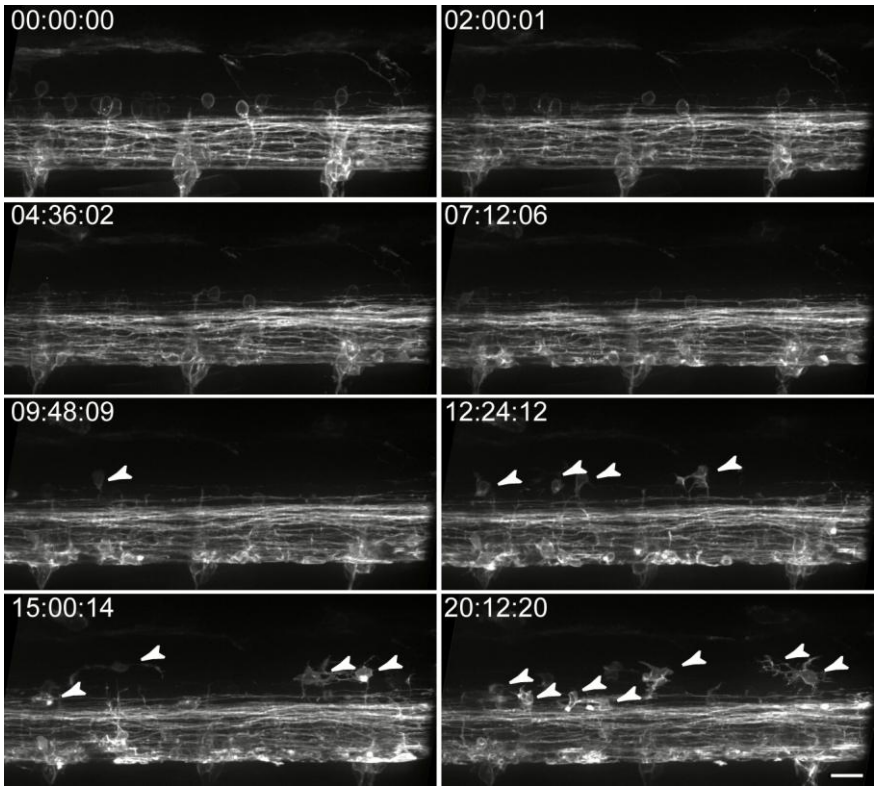


Figure 7. **Sox10^{+} OPC migration in the zebrafish spinal cord.** Frames captured from time-lapse movies, from a lateral view, of *Tg(sox10(7.2):mrfp)* embryos. Numbers in upper right corners indicate time elapsed from beginning of imaging at 50 hpf. Sox10^{+} OPCs send fine filopodial like processes out of the ventral spinal cord. Eventually the Sox10^{+} OPCs migrate out of the ventral domain and take up positions within the dorsal spinal cord (arrowheads).

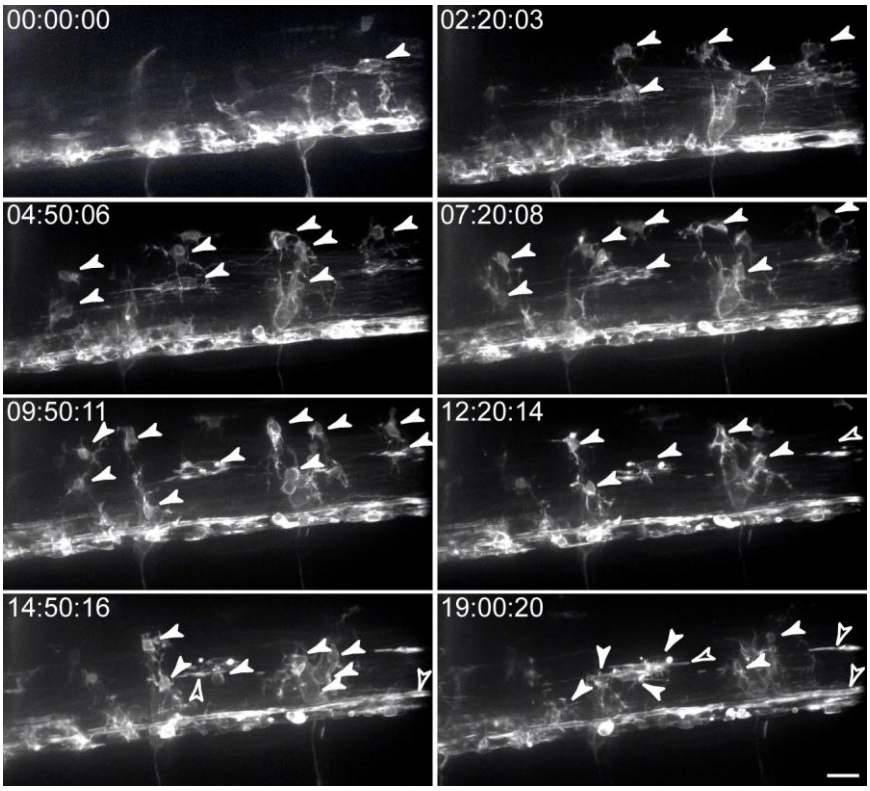


Figure 8. **Sox10^{+} OPCs migrate normally in the absence of Cdc42 function.** Frames captured from time-lapse movies, from a lateral view, of *Tg(sox10(7.2):mrfp);(hsp70:cdc42(n17))*$^{+}$ embryos. Numbers in upper right corners indicate time elapsed from beginning of imaging at 50 hpf. Sox10^{+} OPCs send fine filopodial like processes out of the ventral spinal cord similar to control embryos. Eventually the Sox10^{+} OPCs migrate out of the ventral domain and take up positions within the dorsal spinal cord (arrowheads) and begin to wrap axons (open arrowheads).

morphology similar to control embryos and appeared to exhibit normal behavior patterns as the OPCs extended and retracted their filopodial like projections before settling into position to begin wrapping axons at around 72 hpf (Fig. 8). In addition, no Sox10^{+} OPCs displayed a rounded morphology similar to what was observed in heat shocked *Tg(nkx2.2a:megfp;(hsp70:Cdc42(N17)myc))*$^{+}$ embryos, strongly suggesting that the *nkx2.2a*$^{+}$ cells may not be specified as OPCs in the absence of Cdc42 (Fig. 8).

Discussion

Conduction of electrical impulses along the axons requires proper and uniform myelination of axons. Early in development, oligodendrocytes are specified in ventral spinal cord and are often required to migrate out of the ventral spinal cord to meet target axons. Disruption of OPC migration means that axons are not myelinated and that electrical impulses are not transmitted efficiently leading to disruption of motor and cognitive skills. As more research is done, it is becoming clear that no single pathway or secreted factor may control OPC migration. In fact OPC migration may be a concerted dance in which OPCs have the ability to respond to the various signals they encounter.

Prior to migration, a cell becomes polarized, by reorganizing its cellular architecture, such that the microtubule-organizing center (MTOC), microtubules and Golgi apparatus are positioned in front of the nucleus. This process is called polarization and is dependent on Cdc42 and the Par complex proteins. RNA interference of Cdc42 in the one cell *Caenorhabditis elegans* embryos resulted in

embryos that could not localize Par6, Par3 and PrkCi to the apical side of the embryo. The embryos fail to become polarized and resemble par6, par3 and PrkCi mutant embryos (Gotta et al., 2001). In addition, Cdc42 is critical for the recruitment and activation of the Par complex proteins to initial cell-cell contacts, resulting in local activation of PAR polarity complex, which is essential for the formation of tight junctions, maturation of the apical junctional complex and establishment of cell polarity (Ohno, 2001; Yamanaka et al., 2001). From our data, we observed that in the absence of the apical polarity Par complex protein PrkCi, OPCs are found in ectopic positions in the medial spinal cord. Similarly, when Cdc42 signaling was disrupted OPCs failed to migrate properly and were found in ectopic locations within the medial spinal cord. However, unlike PrkCi mutants, embryos expressing the dominant negative form of Cdc42 did not have mislocalization of Par complex proteins, excess OPCs or loss of precursors. In addition the number and morphology of Islet^{+} cells were comparable to wildtype and and heat shocked controls. This suggests that Cdc42 may regulate OPC migration by signaling through PrkCi. However, unlike in single cell *C. elegans* embryos and polarized epithelia, Cdc42 may function with PrkCi in a pathway that does not affect cellular polarity.

As OPCs migrate, they extend fine filopdial like process that appear to sense the environment and direct the OPC. Total internal reflection (TIRF) microscopy showed that Cdc42, Par6 and PrkCi are expressed in the leading edge of cells during polarized migration (Etienne-Manneville and Hall, 2001). Inhibition of Cdc42 by microinjection of a dominant negative at leading edge in

migrating astrocytes resulted in a failure of the MTOC to become polarized. Cdc42 has an evolutionarily conserved role in regulating the formation of filopdia. In vitro time-lapse microscopy imaging of cultured cells lines shows that Cdc42 is important for filopodia formation, as quiescent cell lines began to initiate filopdia formation, shortly after the addition of exogenous Cdc42 (Etienne-Manneville and Hall, 2001; Kozma et al., 1995; Nobes and Hall, 1995). Conversely, when cultured cells were microinjected with the dominant negative, cells were unable to form filopdia (Etienne-Manneville and Hall, 2001; Kozma et al., 1995; Nobes and Hall, 1995). Our results show that when Cdc42 function is impaired in Tg(nkx2.2a:megfp);(hsp:Cdc42(N17)) embryos, the highly migratory and filopodial *nkx2.2a*$^{+}$ OPCs fail to migrate and appear to drift randomly. In addition, these *nkx2.2a*$^{+}$ OPCs have a rounded morphology and fail to extend any filopodial like protrusions. This argues that Cdc42 may be required for formation of filopodial like protrusions in the *nkx2.2a*$^{+}$ OPCs. The randomness of the migration of the *nkx2.2a*$^{+}$ OPCs suggests that without their filopodial like protrusions the migratory *nkx2.2a*$^{+}$ OPCs are incapable of sensing and responding to their environment. Because, filopdia have been ascribed to function in a sensory and exploratory capacity (Hall, 2005). Interestingly, as the time-lapse experiments continued, the ventral *nkx2.2a*$^{+}$ lost their rounded morphologies, and cells began to extend more filopodial like processes into the dorsal spinal cord. Eventually, some *nkx2.2a*$^{+}$ OPCs even migrated out of the ventral *nkx2.2* domain and occupied positions in the dorsal spinal cord. Reporter protein expression levels have been shown to spike 2-4 hours after heat shock

induction and that these expression levels gradually decrease so that by 12 hour post heat shock induction reporter protein expression levels are modestly higher than control embryos (Adam et al., 2000). Therefore, one explanation for the increase in filopodial activity and migration of the *nkx2.2a*$^{+}$ OPCs is that as time elapses, the levels of Cdc42(N17) protein decrease and eventually endogenous Cdc42 protein is able to out compete the dominant negative.

In the ventral spinal cord, OPCs are specified in the pMN and p3 domain. OPCs specified in the pMN domain express the basic helix loop helix transcription factor *Olig2*, whereas OPCs specified in the p3 domain express the transcription factor *Nkx2.2* (Rowitch, 2004). Cell specified in these domains receive differential concentrations of the morphogens *shh* and *bmp* and therefore up or down regulate different combinations of genes. In the chick spinal cord, antibody labeling experiements show that most migratory *Nkx2.2*$^{+}$ OPCs become *Olig2*$^{+}$ shortly after beginning migration. However, in the mouse spinal cord, OPCs express both *Nkx2.2* and *Olig2* prior to initiating migration. In the zebrafish spinal cord, the relationship between *nkx2.2* and *olig2* becomes more complicated. In zebrafish, there are two distinct populations of OPCs: Nkx2.2a^{+}Olig2^{+} and Nkx2.2^{-}Olig2^{+}, both of which express the OPC specification marker Sox10.

Our Sox10 antibody data showed no statistical difference in the number of OPCs in the spinal cord at 3 dpf. However, our time-lapse movies and whole mount imaging data showed fewer dorsally migrated *nkx2.2a*$^{+}$ cells in the spinal cord of 3 dpf *Tg(nkx2.2:megfp)(hsp70:Cdc42(N17))* embryos as compared to

control embryos. One explanation for these results is that in the absence of the *nkx2.2a*$^{+}$ OPCs, the *nkx2.2a*$^{-}$ population of OPCs may compensate for the deficiency. This idea of replacement/compensation is not unheard as laser ablation experiments show that when OPCs are lost, the remaining OPCs can divide and migrate to replace lost OPCs (Kirby et al., 2006). To investigate this possibility, we performed time-lapse imaging on heat shocked *Tg(sox10(7.2):mrfp)* and *Tg(sox10(7.2):mrfp);(hsp70:Cdc42(N17)*$^{+}$ embryos. From these experiments, we learned that the Sox10^{+} OPCs in *Tg(sox10(7.2):mrfp);(hsp70:Cdc42(N17)*$^{+}$ embryos appear to migrate normally and OPC behaviors were similar to control time-lapse movies. In the Cdc42(N17)$^{+}$ embryos the OPCs extend and retract their fine filopodial like process as they move in and out of the dorsal spinal cord and eventually begin wrapping axons.

Cdc42 has a conserved role in regulating cell polarity and actin cytoskeleton dynamics across many species. By perturbing Cdc42 function I was able to demonstrate that Cdc42 may have a novel role in regulating the migration of the *nkx2.2a*$^{+}$ subset of OPCs in the zebrafish spinal cord. Although the mechanistic basis for this defect in *nkx2.2a*$^{+}$ OPC migration phenotype is unknown, I have provided an excellent starting point for those types of experiments to progress.

CHAPTER IV

NOTCH SIGNALING MAY BE REQUIRED FOR THE MAINTENANCE OF THE POSTEMBRYONIC PRECURSORS IN THE SPINAL CORD

Introduction

Because the likelihood of recovery from a neurodegenerative disease or a major CNS insult is extremely low, scientists in the field came to the erroneous conclusion that “adult” or postembryonic neurogenesis does not occur in the mammalian brain. This fallacy was dispelled by work done by the Altman lab, in which they used thymidine analogs to show that constitutive proliferation does occur in the hippocampus and subventricular zone of the adult mammalian brain (Altman, 1962; Altman, 1963; Emsley et al., 2005; Temple, 2001). However, this work was slow to gain acceptance, because many in the scientific community did not believe the data. The subventricular zone of the lateral ventricle and the subgranular zone of the hippocampus are the two best characterized areas of adult proliferation and neurogenesis in mammals (Emsley et al., 2005). However, postembryonic proliferative cells have been isolated and cultured from the optic nerve, corpus callosum, spinal cord and hypothalamus, as well as other regions of the brain. Cells derived from the subventricular zone and the subgranular zone are highly migratory and often travel long distances to reach their targets. The proliferative cells of the subventricular zone enter the rostral migratory stream and travel to the olfactory bulb where they differentiate to

become interneurons (Aguirre and Gallo, 2004; Doetsch and Hen, 2005; Lim et al., 1997). The proliferative cells of the subgranular zone travel a shorter distance as they differentiate to become hippocampal granule neurons (Aguirre and Gallo, 2004; Doetsch and Hen, 2005; Lim et al., 1997). From these experiments, we now understand that the CNS is not a static organ system and is capable of neural regeneration. These postembryonic changes may coincide with distinct periods of development. In many organisms, the transition from embryonic to postembryonic stages is followed by dramatic changes in body plan and morphology. These dramatic transitions are generally referred to as metamorphosis. However, many organism, especially mammal do not undergo dramatic metamorphological changes to their morphology.

Zebrafish, for example, do not undergo dramatic changes in their body plan and morphology while undertaking metamorphosis. So slight are the changes that some argue that metamorphosis does not occur and that these slight morphological changes are just transition phases (Balon, 1990; Brown, 1997; Youson, 1988). However, some point to the changes in zebrafish pigmentation, scales and fin shape at discrete times in development to suggest that zebrafish undergo some form of metamorphosis (Brown, 1997; Parichy, 2003; Quigley and Parichy, 2002). Besides expression levels of various thyroid hormones, very little is known about the changes that are occurring in the zebrafish embryo as it transitions from embryonic stages to the postembryonic stages. Many questions remain to be answered for example are there any cellular or morphological changes occurring with the specific organ system that

can be attributed to changes in gene expression during this stage of development? If so, do these changes in gene expression and morphology represent metamorphological changes.

In embryonic development, there are several genes and signaling pathways that play important roles in regulating and patterning the various tissues of the developing embryo. One such signaling pathway, Notch, has been shown to be required for many aspects of development, such as nervous system development. Notch signaling, which get its name from the notched wing phenotype described in *Drosophila* mutants, is an evolutionarily conserved pathway and has been described to function in many organisms (Artavanis-Tsakonas et al., 1999). Notch signaling is generally ascribed to important in regulating binary cell fate decisions through lateral inhibition. Lateral inhibition is the process by which cells all with the same developmental potential, differentiate into 2 different populations of cells. In lateral inhibition, cell that achieve one cell fate begin to up-regulate Notch which prohibits neighboring cells from achieving a similar fate, creating cellular diversity within a population (Artavanis-Tsakonas et al., 1999).

The Notch family of receptors is composed of a type1 single pass transmembrane receptor protein. The Notch receptor generally consists of a large extracellular domain (NECD) and a smaller intracellular domain (NICD) (Ehebauer et al., 2006). Activation of Notch signaling requires binding of a Notch ligand, which activates a cascade of proteolytic cleavages. The Notch ligands are

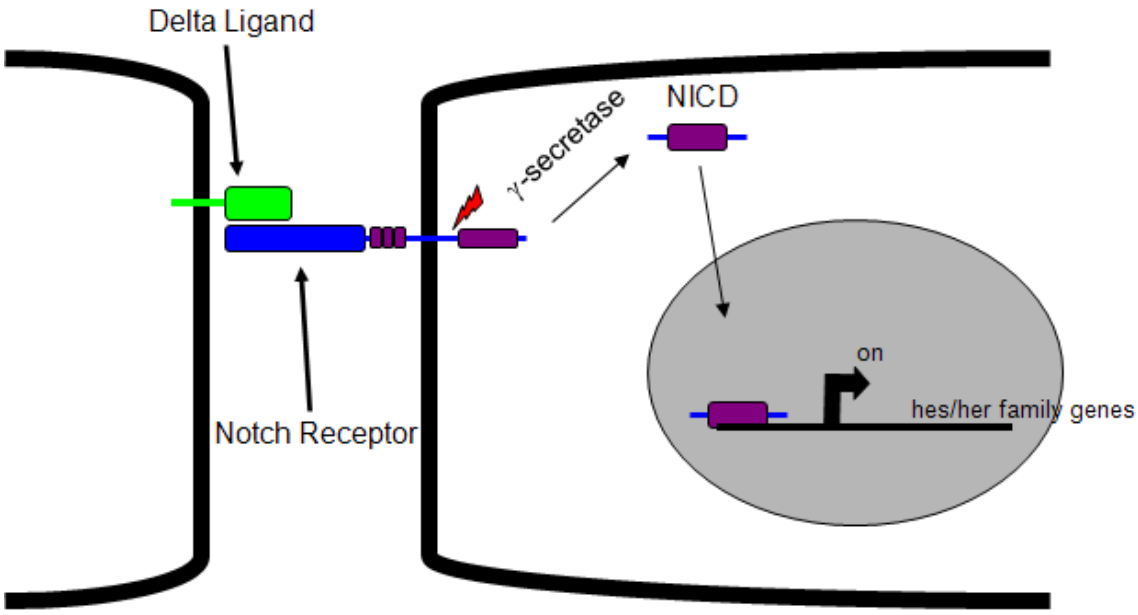


Figure 1. The Notch signaling pathway. A cartoon schematic showing the Notch signaling pathway. Binding of the Notch ligand to the Notch receptor triggers g-secretase cleavage of the intracellular domain of the Notch receptor (NICD). NICD then translocates to the nucleus where it activate Notch target genes.

also type1 single pass transmembrane proteins and are members of the DSL (Delta/Serrate/Lag-2) family of proteins (Ehebauer et al., 2006). Because both the receptor and the ligand are both transmembrane proteins, activation of Notch signaling requires cell-to-cell contact. When the Notch ligand binds to the Notch receptor, the Notch receptor is cleaved through γ-secretase activity, which results in the release of the NICD. The NICD then translocates to the nucleus where it can alter gene expression of Notch target genes (Ehebauer et al., 2006) (Fig. 1). Much of what is known about Notch signaling occurs in the early embryo, where Notch signaling is regulating early cell fate decisions. Recent insights and discoveries into adult neurogenesis and adult stem cells show that proliferative and differentiative divisions are occurring in the postembryonic and adult CNS (Amrein and Lipp, 2009; Zupanc, 2008). Still, questions as to the mechanisms that regulate these postembryonic populations remain to be answered. Are the same mechanisms that were important in regulating cell proliferation and differentiation of the precursor population in the embryonic CNS important in regulating these precursor cell populationin the postembryonic or adult CNS?

Experimental Procedures

Fish Husbandry

Embryos were produced by pair-wise mating and kept at 28.5^{o}C in egg water or embryo medium. Embryos were staged to hours post fertilization (hpf) or days post fertilization (dpf) according to established zebrafish guidelines (Kimmel et

al., 1995). Embryos that were to be used for live imaging, immunohistochemistry, or in situ hybridization were treated in 0.003% phenylthiourea (PTU) in egg water to block pigmentation. The experiments conducted in this chapter used the AB strain.

Immunocytochemistry

Embryos and larvae were fixed in 4% antibody fix (4% paraformaldehyde, 8% sucrose, 1x PBS) overnight at 4^{o}C. After fixing, the embryos were embedded in 1.5% agar/ 5% sucrose blocks and placed in 30% sucrose/PBS solution to equilibrate overnight. The blocks were then frozen over 2-methylbutane chilled by liquid nitrogen. We collected 10-12 μm sections on superfrost microscope slides using a cryostat microtome. The sections were rehydrated in 1x PBS for 30 min. and then blocked in 2% BSA/sheep serum in 1x PBS for 30 min. before incubating with primary antibody overnight at 4^{o}C. For fluorescent detection of antibody labeling, we used Alexa Fluor 488, Alexa Fluor 568 goat anti-mouse or goat anti-rabbit conjugates (1:500, Molecular Probes). The primary antibodies used included rabbit anti-Sox10 (1:1,000) (Park et al., 2005) mouse anti-HuC (1:100, Molecular Probes) mouse anti-PCNA (1:200, #ab15497 Abcam)and mouse anti-ZRF-1 (1:500, DSHB).

Antigen Retrieval

All Immunohistochemical labeling with PCNA antibody required this step to increase better binding of the antibody to the antigen. Sections of 15 and 17 dpf

embryos were transferred to a Coplin jar containing 10 mM sodium citrate buffer (pH 8.5). The Coplin jar was then placed in a 100°C water bath for 30 minutes. After 30 minutes the Coplin jar was removed and allowed to come to room temperature on the bench. When sections were cooled immunohistochemical labeling was done as described above.

In situ hybridization on sections

Embryos and larvae were fixed in 4% paraformaldehyde overnight at 4^{o}C. After linearizing plasmids with the appropriate restriction enzymes, anti-sense cRNA was transcribed using Roche DIG-labeling reagents and T3, T7 or SP6 RNA polymerases (New England Biolabs). For the experiments contained within this paper the following anti-sense RNA probes were used: *notch1a* and *dld*. After fixing, the embryos were embedded in 1.5% agar/ 5% sucrose RNase free blocks and placed in 30% sucrose/PBS RNase free solution to equilibrate overnight. The blocks were then frozen over 2-methylbutane chilled by liquid nitrogen. We collected 10-12 μm sections on superfrost microscope slides using a cryostat microtome. Sections were rehydrated in 1X PBS for 30 min. then covered with 75% glycerol. Images were obtained using a Retiga EXI camera attached to a Olympus Novius EX70 microscope equipped with Openlab software.

DAPT Treatment

To block Notch signaling, DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl]-S-phenylglycine-t-butyl ester; Calbiochem, La Jolla, CA) treatments were

performed as previously described (Geling et al., 2002). DAPT was reconstituted with DMSO to make a stock concentration of 10 μM. For experiments, aliquots were diluted to 100 M in EM. Larval fish were placed in the DAPT solution at 15 and 17 dpf and incubated overnight at 28.5°C. Control embryos were incubated in an equivalent concentration (1%) of DMSO in EM and incubated overnight.

Results

The rate of cell division in the spinal cord fluctuates during development.

Until recently postembryonic neurogenesis was thought to be absent in the CNS. Studies performed on the adult mammalian and avian brains show that it does occur and that it may be a way of regenerating neurons in the adult brain. The spinal cord, like the brain, is often thought of as a fully differentiated tissue with little regenerative capacity. I wanted to determine if the spinal cord contained pockets of cells that could proliferate leading to neurogenesis in a juvenile to adult fish, similar to what is observed in the adult brain of other models. To do this we employed a strategy that would label mitotic cells. During mitosis a cell must replicate its DNA in a semi conservative fashion. Using the existing strands of DNA as a template, the appropriate complementary base is added creating two copies of the genome. To determine the rate of cell division we used the cell's replication machinery to label cells with BrdU, a thymine analog, at stages ranging from 1 dpf to 30 dpf.

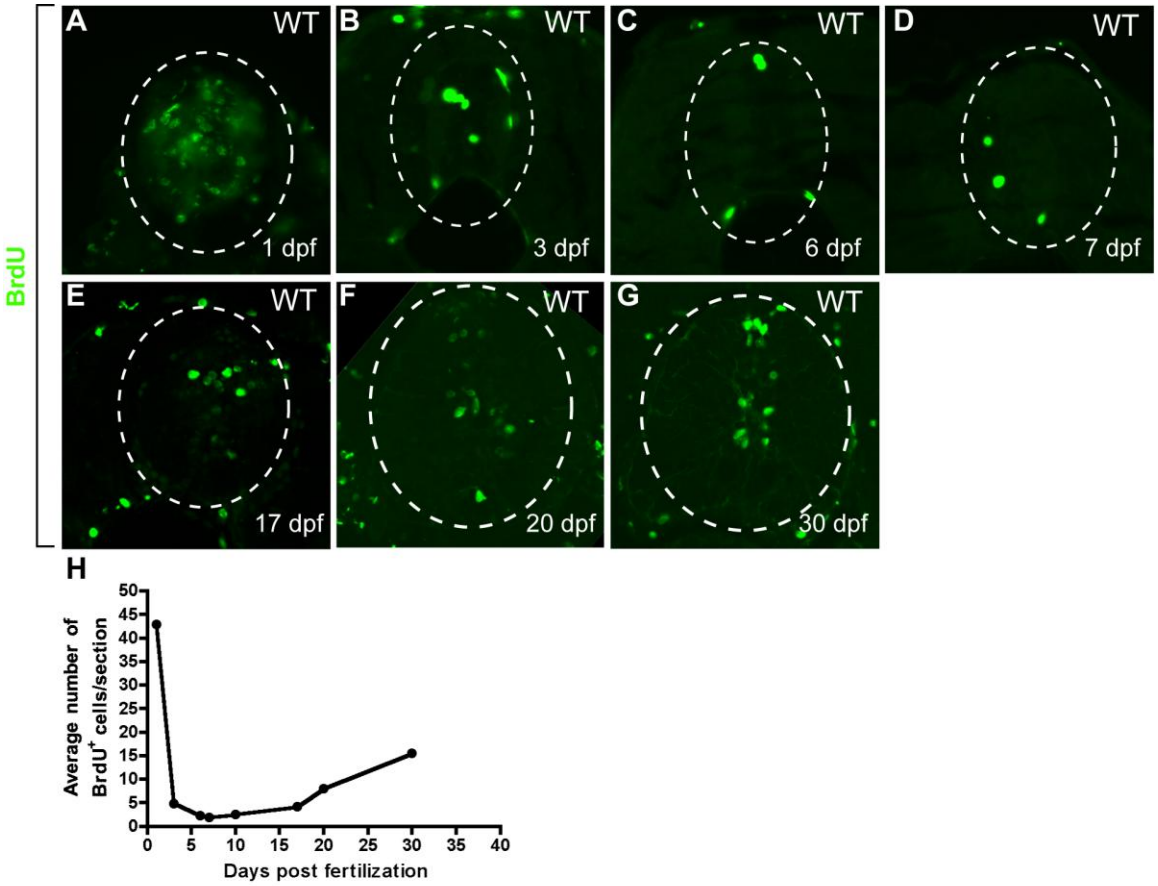


Figure 2. Dividing cells of the zebrafish spinal cord. All panels show transverse section through trunk spinal cord. Dashed circle denotes the spinal cord boundary. **A-G.** Embryos, larvae and juveniles treated with BrdU. Sections labeled with anti-BrdU antibody. **H.** Graph showing the rate of cell divisions in the zebrafish spinal cord.

At 1 dpf cell division in the spinal cord occurred rapidly and many cells were $BrdU^+$. On average there were approximately 42.9 $Brdu^+$ cells per section (Fig. 2A, H). At 3, 6 and 7 dpf cell divisions decreased dramatically and on average the number of $BrdU^+$ cells per section was 4.9, 2.3 and 1.9 respectively (Fig. 2 B,C,D,H). At 10 dpf the number of cell divisions in the spinal cord began to increase and the average number of $BrdU^+$ cells per section was 2.5 (Fig. 2 H). At 17, 20 and 30 dpf the increase in spinal cord cell divisions continued with an average of 4.2, 8 and 15.5 cells per section respectively (Fig. 2 E,F,G,H). Interestingly, early in development, cell division occur randomly throughout the spinal cord; however, as the embryo develops and transitions into a juvenile, cell divisions appear to be restricted to the ventricular zone of the spinal cord.

Post-embryonic cell divisions may give rise to radial glia and OPCs but not neurons.

Adult neurogenesis occurs in distinct niches within the adult mammalian brain. In these niches the adult stem cells differentiate and under in vivo conditions give rise only to neurons (Alvarez-Buylla and Lim, 2004). And although under extreme exogenous conditions the fate of the adult neural stem cells can be altered, the default cell fate are hippocampal neurons or olfactory bulb neurons. (Hack et al., 2005; Jackson et al., 2006; Jessberger et al., 2008). As previously described, most of the postembryonic cell divisions appear to be limited to the ventricular zone of the spinal cord. We were interested in determining the fate of these dividing ventricular zone cells. To achieve this we pulsed postembryonic juvenile embryos with the S-phase marker BrdU. I used BrdU as a lineage tracer,

because DNA replicates semi conservatively so any incorporated BrdU would be passed on in successive divisions.

Using Zrf-1 antibody which labels the radial fibers and cell membranes of the radial glia, we observed $BrdU^+$ cells along the ventricle of the spinal cord at 17 dpf. These $BrdU^+$ cells were outlined with the Zrf-1 positive membranes (Fig. 3 A) (arrowheads). Similar labeling was observed at 20 and 30 dpf suggesting that $BrdU^+$ cells were giving rise to rise to radial glia or that the radial glia were dividing giving rise to other cell types. Next I wanted to determine if the BrdU positive cell were giving rise to any other cell type. Using an antibody to Sox10, an OPC specification marker, I observed BrdU and Sox10 double labeled cells at 30dpf. Next I wanted to determine if these postembryonic proliferative cells gave rise to neurons as is the norm in the adult mammalian brain. Using the pan neuronal marker HuC, I was unable to identify any HuC and BrdU double labeled cells in the spinal cord at 15 and 17 dpf. This suggested that the proliferative cells in the spinal cord do not give rise to neurons, unlike what is observed in the adult mammalian brain (Alvarez-Buylla et al., 2001; Alvarez-Buylla and Lim, 2004; Alvarez-Buylla and Lois, 1995).

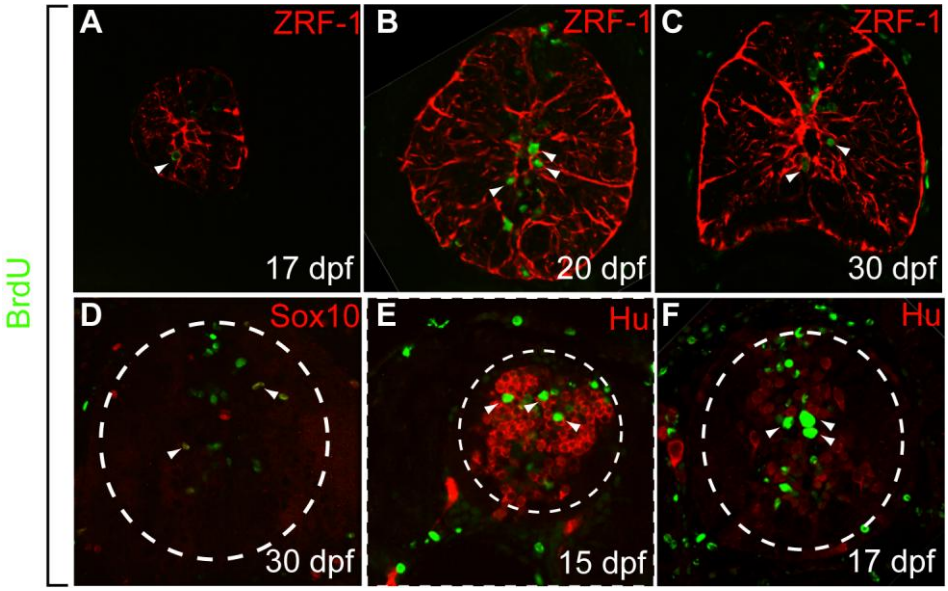


Figure 3. Radial glia and OPCs arise from post embryonic proliferative cells. All panels show transverse sections through trunk spinal cord. All panels are labeled with anti-BrdU antibody after being treated with BrdU. Dashed circle demarcates the perimeter of the spinal cord. **A-C:** Sections labeled with anti-ZRF-1 antibody. ZRF-1 labels the cell membranes of radial glia. Arrowheads indicate radial glia labeled with ZRF-1 and Brdu. **D:** Sections labeled with anti-Sox10 antibody, which labels the OPCs. Arrowheads indicate OPCs labeled with Sox10 and Brdu. **E,F:** Sections labeled with anti-Hu antibody. Hu labels all post mitotic neurons. Arrowheads indicate $BrdU^{+}$ cells that do not colocalize with BrdU. All post mitotic neurons were devoid of BrdU staining. Scale bar = 20μm.

Notch signaling may regulate postembryonic proliferation in the zebrafish spinal cord.

As was described earlier, divisions within the spinal cord transition from a rapidly dividing period to a quiescent period, and back to a period of rapid division. During the rapidly dividing periods, cells were exiting the cell cycle and differentiating. I wanted to determine what mechanisms behind this transformation. Loss of function and ablation studies have shown that Notch signaling is important in regulating binary cell fate decision as well as regulating differentiation through lateral inhibition (Shin et al., 2007). During neural development, Notch signaling maintains precursor cells in the undifferentiated state and in its absence, precursor cells exited the cell cycle and differentiate. In zebrafish, blocking Notch signaling resulted in early born neurons at the expense of the undifferentiated precursors. I wanted to determine if expression of Notch signaling proteins played a role in regulating postembryonic proliferation. To do this, I looked at expression of various *delta* and *notch* genes at various embryonic and postembryonic stages.

Of the many *delta* and *notch* genes we investigated, only 2 genes had an interesting pattern of expression: *deltad* and *notch1a*. *deltad* (*dld)* expression at 5 dpf is largely absent and found in random punctate expression throughout the spinal cord. At 10 dpf *dld* is still randomly expressed throughout the spinal cord similar to its expression at 5 dpf. At 20 and 30 dpf *dld* expression increases and is expressed strongly in the ventricular zone of the spinal cord (Fig 4 A-D). *notch1a* expression at 5 dpf is expressed in a diffuse pattern around the spinal cord. At 10 dpf a similar diffuse pattern of *notch1a* expression is observed,

however at 20 and 30 dpf expression of *notch1a* increases and is restricted to the cells of the ventricular zone (Fig 4 E-H). Both the timing and pattern of expression of *dld* and *notch1a* coincided with the timing and pattern of cell division in the postembryonic spinal cord.

Inhibiting Notch signaling decreases postembryonic cell division.

After observing that increases in the levels of *dld* and *notch1a* gene expression coincided with increases in the number of postembryonic cell divisions, I wanted to determine if Notch signaling affected cell divisions in the postembryonic spinal cord. I tested this by knocking down Notch signaling using the chemical inhibitor N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT). DAPT is a gamma secretase inhibitor that blocks Notch signaling by inhibiting the cleavage and nuclear translocation of the Notch intracellular domain (NICD). Earlier, I determined that postembryonic proliferation in the zebrafish spinal cord begins to increase at around 15 dpf. Therefore, I reasoned that if Notch signaling was important for increasing the rate of cell divisions in the postembryonic zebrafish spinal cord, then blocking it at this time would have the most dramatic result. At 15 dpf the average number of $PCNA^{+}$ cells in the spinal cord averaged 4 cells per section in the DMSO treated controls and 1.8 cells per section in the DMSO treated postembryonic larvae (Fig. 5 A,B,C). Two days later, at 17 dpf, an average of 3.1 PCNA positive cells per section were found in the DMSO control and 1.9 cells per section in the DAPT treated postembryonic embryos. These

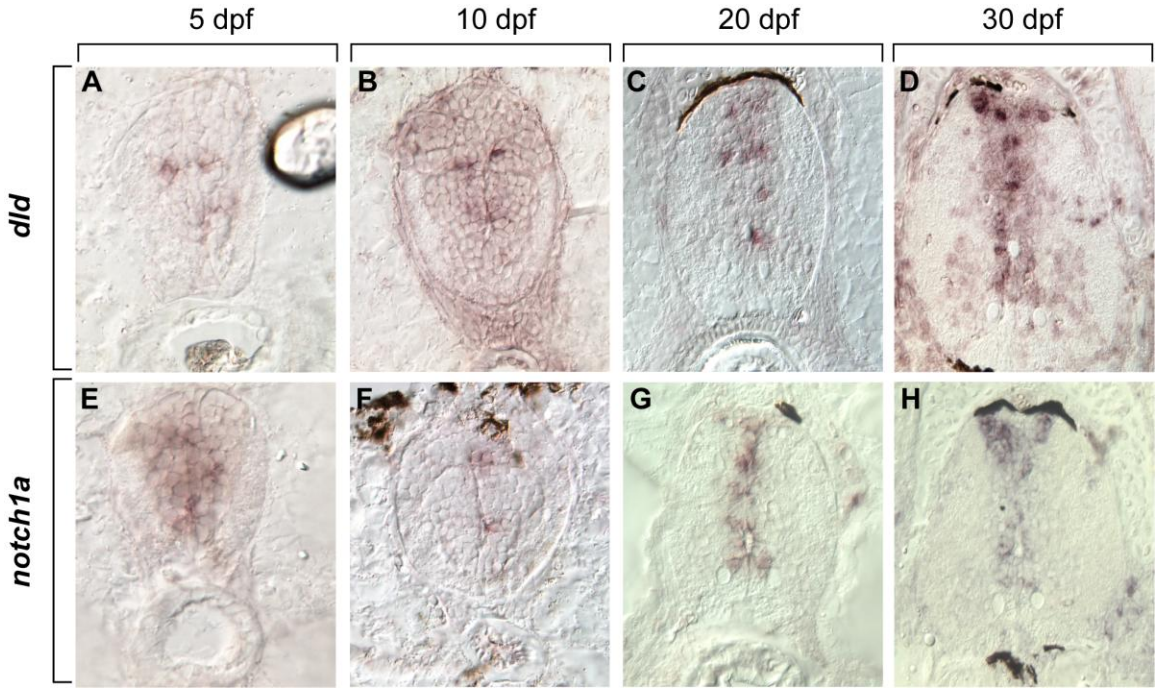


Figure 4. *dld* and *notch1a* RNA expression is up regulated during postembryonic stages. All panels show transverse sections through trunk spinal cord at 5,10, 20 and 30 dpf. **A-D:** In situ hybridization using *dld* riboprobe shows an increase in RNA expression in the cells of the medial septum during late postembryonic periods. *dld* RNA expression is diffuse at 5 and 10 dpf however by 20 and 30 dpf *dld* RNA expression is present in the cell lining the proliferative zone of the spinal cord. **E-H:** In situ hybridization using *notch1a* riboprobe shows an increase in gene expression in the cells of the medial septum during late postembryonic periods. Similar to dld RNA expression, notch1a RNA is absent from the spinal cord at 5 and 10 dpf but is highly expressed in the cells of the proliferative zone at 20 and 30 dpf. Scale bar = 20μm

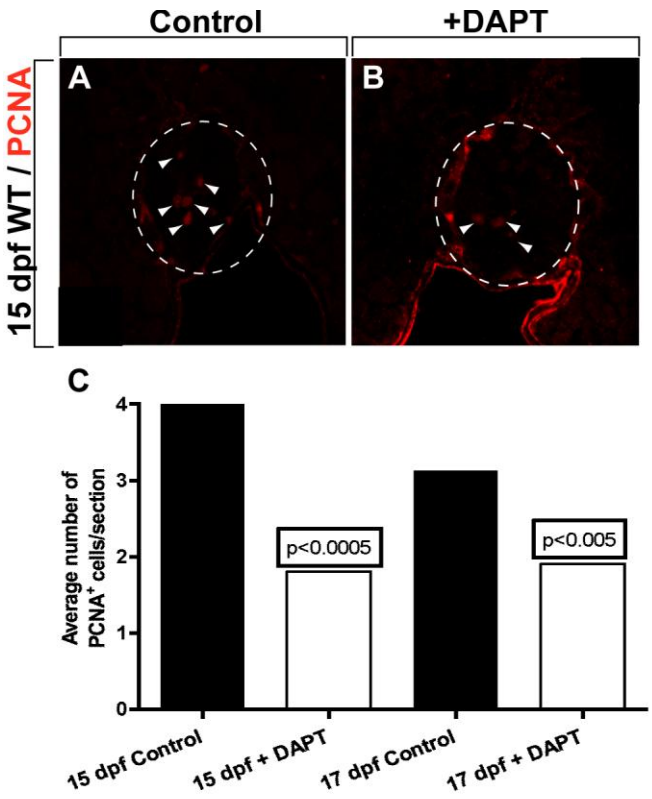


Figure 5. Delta-Notch signaling regulates postembryonic proliferation in the spinal cord. All panels show transverse sections through trunk spinal cord at 15 dpf. **A,B:**Sections labeled with an anti-PCNA antibody. Embryos treated with DAPT, a gamma secretase inhibitor that block Delta-Notch signaling, have reduced numbers of proliferative cells. **C.** Quantification of spinal cord $PCNA^{+}$ proliferative cells between control and DAPT treated embryos. Statistical significance was determined using Student's t-test. Scale bar = 20μm

data argues strongly for a role in Delta-Notch signaling in regulating postembryonic proliferation in the zebrafish spinal cord.

Discussion

As an organism ages, injured and dying cells are replaced as needed. Proper maintenance of this homeostasis allows for the organ systems in the body to function without any noticeable interruption. This was thought to be true of most organ systems except for the nervous system. Ramon y Cajal argued that the adult nervous system was composed entirely of terminally differentiated cells that ceased proliferating after embryogenesis (Lopez-Munoz et al., 2006). In his theory, the cells comprising the nervous system were all specified shortly after birth, and replacement of dying or injured cells did not occur via postembryonic neurogenesis (Lopez-Munoz et al., 2006). Although evidence for postembryonic neurogenesis was found in the reptilian brain as early as 1950, it was not until Altman and colleagues published a series of papers reporting [H3]-thymidine evidence for new neurons in various regions of adult rats, including the dentate gyrus of the hippocampus, neocortex and olfactory bulb that the long held dogma of no postembryonic proliferation in the nervous system was debunked (Altman, 1962; Altman, 1963; Altman and Das, 1965; Alvarez-Buylla and Lois, 1995). At present, very little is known about the molecular mechanisms controlling postembryonic proliferation and regulating the transition of precursor cells from a quiescent state to an actively proliferating state. In addition, cell fate determination of these proliferative cells is not well defined. Here we identified a population of post embryonic proliferative cells in the zebrafish spinal cord, and

investigated the role of the Delta-Notch signaling pathway in regulating these post embryonic proliferative cells.

Since the 1960's, the discovery of postembryonic neurogenesis in the adult mammalian brain, numerous studies have documented widespread neurogenesis in non-mammals, such as fish, amphibians, reptiles and birds (Alvarez-Buylla and Nottebohm, 1988; Chapouton et al., 2006; Grandel et al., 2006; Kaslin et al., 2008; Lopez-Garcia et al., 1988; Raymond and Easter, 1983). Our data shows that in addition to areas of proliferation in the adult brain, there are areas of postembryonic proliferation, in zebrafish spinal cord. Understanding the mechanisms that control postembryonic proliferation in the spinal cord is important, as studies looking at cell specification and differentiation in the brain and spinal cord, shows that these two areas regulate cell specification and differentiation differently.

One of the key features of zebrafish as a model system is its rapid external development. A fertilized zebrafish embryo can develop from a single cell embryo to a free swimming larva within 72 hours (Kimmel et al., 1995). This rapid development allowed us to investigate rates of proliferation in the zebrafish spinal cord over several stages of development. Through BrdU incorporation experiments, we found that at 1 dpf the spinal cord is undergoing a period of widespread rapid cell divisions. This rapid rate of cell divisions is followed by a dramatic decrease in cell divisions at 2 and 3 dpf, towards the end of embryogenesis. At around 15 dpf there is a dramatic increase in the rate of cell

division in the spinal cord. The increase in number of postembryonic proliferative cell divisions

In the mammalian brain, postembryonic proliferative cells give rise to neurons and oligodendrocytes. These newborn cells integrate themselves into the CNS to form functional circuits. To determine the fate of these proliferative postembryonic spinal cord cells in zebrafish, we used BrdU as a lineage tracer. Our data showed that the dividing cells of the postembryonic spinal cord gave rise to oligodendrocytes and radial glia. However, contrary to what is thought to occur, our data showed that no new neurons are specified from the postembryonic proliferative cells.

Because Notch signaling plays an important role in maintaining the proliferative precursor population, we hypothesized that they may also play a role during the postembryonic stages. (Shin et al., 2007). Our *in situ* data shows that *notch1a* and *dld* transcripts are absent from the spinal cord during the quiescent postembryonic period. However, during the proliferative postembryonic periods, cells along the medial septum began expressing *notch1a* and *dld* transcripts. The temporal and spatial expression of both genes suggested a role for Notch signaling in during postembryonic proliferation. Using DAPT, we found that larvae, in which Notch signaling was disrupted, had fewer postembryonic proliferative cells in their spinal cords. By inhibiting Notch signaling at the onset of postembryonic proliferation, we were able to determine that Notch signaling does play a role in maintaining the postembryonic proliferative precursors in the zebrafish spinal cord.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Development of the spinal cord from a single layer of neuroepithelium is a complex process that involves the coordination of several processes including cell migration, cell specification and cell differentiation. Proper patterning of the spinal cord requires that these processes are spatially and temporally regulated, so that genes are turned on or off when and where required. Any disruption in these processes can affect the overall morphology of the spinal cord. In this thesis I show that in the absence of *prkci*, an apical polarity gene, zebrafish spinal cord precursor cells fail to maintain parallel precursor divisions. As a consequence, OPCs are specified in excess at the expense of the precursor population. In addition, I show that Cdc42 plays a role in the migration of the *Nkx2.2a*$^{+}$ OPC population in the zebrafish spinal cord. Lastly, I argue for the role of Notch signaling in maintaining the postembryonic proliferative cells in the spinal cord.

The apical polarity protein PrkCi is necessary for maintenance of spinal cord precursors in zebrafish.

Oligodendrocytes, the myelinating cells of the vertebrate CNS, play an important role in transmitting electrical impulses along the axons. In their absence, cognitive and motor functions are impaired. Demyelinating diseases affect more than two million people worldwide, and represent a significant world health

burden. By understanding the mechanisms that control how these cells develop, scientist are hopeful that they can prevent or cure demyelinating diseases.

I showed using the zebrafish spinal cord as a model that PrkCi, the zebrafish homologue to aPKC is important in specifying OPCs in normal numbers, by maintaining parallel precursor divisions. Through various loss of function experiments, aPKC has been shown to regulate cell specification in *Drosophila* and *C. elegans*. In this thesis, I show that in the zebrafish spinal cord, PrkCi has a similar function, as it regulates specification of zebrafish OPCs. In addition, aPKC regulates establishment and maintenance of apical-basal polarity in polarized epithelia in *Drosophila*, *C. elegans* and mammals. This role for aPKC is conserved in zebrafish, because in the absence of functional PrkCi apical basal polarity is disrupted in the zebrafish spinal cord. By performing antibody labeling experiments, using an antibody against PrkC, I showed that both PrkCi and PrkCz are expressed in the apical membranes of the spinal cord cells at the medial septum and around the central canal. In *has*$^{-/-}$ embryos I demonstrated that PrkCz was unable to compensate for the function of PrkCi. Localization of apical markers, like ZO-1, was diminished at the medial septum and around the central canal, showing that apical polarity is disrupted in *has* mutants. Although the Par-aPKC complex is an evolutionary conserved complex, accumulating evidence has also revealed diversity and complexity in the means by which different cells utilize the PAR-aPKC complex to establish polarity.

aPKC regulates asymmetric cell divisions in the *Drosophila* CNS. When aPKC is present, these asymmetric cell divisions occur perpendicular to plain of

the overlying epithelia and produces a nueroblast and a GMC. However, in the absence of aPKC, orientation of these divisions are randomized and produces 2 ganglion mother cells of equal potential. In C. elegans, aPKC-Par complex controls the asymmetric division of the fertilized 1- cell embryo. Disruption of apkc-par complex genes results in a fertilized embryo that fails to divide asymmetrically and produces two daughter cells of equal size and possible equal potential (Watts et al., 1996). In this thesis, I showed that in the zebrafish spinal cord, PrkCi regulates precursor cell divisions orientation, but in a different manner than in *Drosophila* and *C. elegans*. Time-lapse imaging experiments allowed me to show that PrkCi maintained parallel divisions in the presumptive precursor cells around the central canal. Using *Tg(h2afv:egfp) embryos*, I observed that the dividing cells at the central canal surface divided parallel to the plane of the central canal. Similar time-lapse experiments, using *Tg(h2afv:egfp);has*$^{-/-}$ embryos, which lack expression of functional PrkCi, showed that in the absence of PrkCi, division around the central canal could occur parallel or non parallel. This is unique, as stem cells and their resultant precursor cells are generally thought to undergo asymmetric proliferative divisions, in which they give rise to another precursor cell and a differentiated cell. These asymmetric proliferative divisions are thought to occur perpendicular to the plane of the epithelia. I also showed that initiation of the non-parallel divisions occurred around 30 hpf, which occurs just prior to OPC specification. This randomization of cell divisions may lead to a loss of precursor cell maintenance and an increase in differentiated cell types.

In addition, through loss of function experiment, I determined, that as a consequence of these non-parallel cell divisions at the central canal, a significant excess of OPCs were found in the spinal cord of *has*$^{-/-}$ embryos. However, early born cells like motor neurons and Rohon Beard cells were unaffected. This is different than what occurs in the Drosophila CNS; because when aPKC is absent from the Drosophila CNS, the neuroblasts divide symmetrically and give rise to excess GMCs, which go on to differentiate into excess neurons. As a consequence of dividing symmetrically, the number of neuroblasts is reduced. In situ hybridizations, antibody labeling and whole mount imaging, demonstrated that in the absence of PrkCi, precursor cell population was reduced, and that the reduction in precursor cells give rise to the excess OPCs.

Through time-lapse imaging, of *Tg(olig2:egfp)* and *Tg(olig2:egfp);has*$^{-/-}$ embryos, we provided evidence that the excess OPCs in *has*$^{-/-}$ embryos were due to increased specification of OPCs. By using time-lapse imaging, we were able to discern whether the excess OPCs in the *has*$^{-/-}$ spinal cords were due to increased cell proliferation in the dorsal spinal cord or due to increased OPC specification in the ventral *olig2* domain. In the time-lapse movies, I saw that in *has*$^{-/-}$ embryos, excess OPCs were specified in the ventral *olig2* domain and these excess OPCs migrated out into the dorsal spinal cord. It also showed that the excess OPCs were not because of increased OPC proliferation in the dorsal spinal cord.

In summary, I have shown that in the absence of PrkCi precursor cells fail to maintain parallel divisions, and excess OPCs are specified at the expense of

the precursor cells; however many outstanding questions remained to be answered. For example, does angle of divisions predict cell fate? In the *Drosophila* CNS, neuroblasts divide asymmetrically giving rise to two daughter cells of different cell fates. In this thesis, I was unable to show any direct evidence that established a connection between angle of division and cell fate. This was largely due to the lack of available tools. Because our *Tg(h2afv:egfp)* line labeled all the cell nuclei, it was impossible for us to determine the cell fates of a newly divided daughter cells once they migrated away from the central canal. One approach around this obstacle would be to inject either *h2afv:egfp* DNA or RNA at low concentrations into single cell transgenic embryos. Possible transgenic embryos for injection include: *Tg(sox10(7.2):mrfp)*, *Tg(olig2:dsred)* and *Tg(islet1:dsred)*. My goal with this experiment would be to create a transient transgenic that has mosaic expression of GFP in a few cells at the central canal. By labeling only a few cells it would allow us to track newly divided daughter cells as they mature and turn on cell specification genes like *islet, sox10* and *olig2*.

PrkCi has been shown to be part of the evolutionarily conserved Par-aPKC complex that regulates cell polarity and asymmetric divisions. In this thesis, I was unable to show definitively that phenotypes observed in the absence of PrkCi were direct results of disruption in the Par-aPKC complex. As members of the Par-aPKC complex are dependent on each other for proper localization to the apical membrane, disrupting one gene should affect the formation and function of the complex. In future experiments, I would suggest knocking down *par-3* and *par-6* expression by injection of morpholino antisense

oligonucleotides. If the phenotypes observed in PrkCi deficient embryos were the result of a loss of Par-aPKC complex signaling, disruption of other complex members should produce a similar phenotype. Morpholinos that disrupt Par-3 expression in zebrafish exist and the zebrafish homologue to par-6 has already been cloned, therefore disruption of *par-3* and *par-6* should be simple to achieve (Munson et al., 2008; Wei et al., 2004).

Although, I was able to show an overall reduction in precursor cells, I was unable to provide quantitative data for this reduction. To rectify this, I would perform whole-mount immunohistochemisty on *Tg(olig2:egfp)* and *Tg(olig2:egfp)has*$^{-/-}$ embryos using an antibody against ZRF-1, which labels the radial glia and the postembryonic proliferative precursors. Then the number of double labeled postembryonic proliferative cells could be counted and analyzed.

Cdc42 signaling regulates migration of the *nkx2.2a*$^{+}$ subset of zebrafish OPCs.

Cell migration is an essential process during many phases of development and adult life. It drives embryonic morphogenesis and contributes to tissue repair and regeneration (Keller, 2005; Luster et al., 2005). It also orchestrates disease progression in cancer, mental retardation, atherosclerosis, and arthritis (Adiguzel et al., 2009; Emma, 2009; Yamaguchi et al., 2005). Understanding the mechanism by which cells migrate may lead to the development of novel therapeutic strategies that can aide in disease prevention or cures. OPCs are a population of glial cells that migrate toward their target axons. Failure of OPC and axons to connect can lead to diseases that impair cognitive and motor

function. Advances in transgenesis and the transparent nature of zebrafish have made it possible to study the migration of migratory cells like OPCs *in vivo*.

Cdc42 has a conserved role in regulating cell polarity and the actin cytoskeleton in many eukaryotic organisms. Cdc42 has been shown to have a role in yeast budding, epithelial polarity, migratory polarity and fate specification during cell division. Cdc42-knockout mice are embryonic lethal and die before embryonic day 7.5 (Chen et al., 2000). Because Cdc42 plays such an important role in many early developmental pathways, other approaches to disrupting Cdc42 function were needed. For example, in many cell types, both constitutively active Cdc42 and dominant-negative Cdc42 have been shown to effective in disrupting Cdc42 function by affecting the formation of highly dynamic finger-like actin-rich protrusions known as filopodia (Gupton and Gertler, 2007). Through heat shocking experiments I observed that expression of dominant negative Cdc42(N17)-Myc in zebrafish produces embryos with dorsally curved tails, cardiac edema and with no apparent necrosis in the spinal cord. Heat shocked wildtype embryos appear normal and did appear to exhibit any abnormal phenotypes as a result of heatshocking. Myc labeling confirmed the expression of the Cdc42(N17)-Myc fusion protein throughout the entire embryo. This shows that heatshocking may be an effective way of inhibiting Cdc42 signaling.

Cell polarity is fundamental to many cellular processes, including migration, differentiation and morphogenesis. Cdc42 seems to function primarily through the polarity protein Par-6 and thereby with Par-3 and atypical protein kinase C (aPKC) isoforms to induce polarity in several different animal models

(Etienne-Manneville, 2004; Goldstein and Macara, 2007). Cdc42 and the aPKC-Par complex play important roles in the formation and maintenance of polarity in epithelial cells. Epithelial cells have a polarized morphology that is characterized by an asymmetrical distribution of proteins, which form distinct apical and basal domains. These distinct apical and basal domains allow for directed transport across the epithelial layer while at the same time acting as a barrier to certain small solutes and ions. Constitutively active and dominant-negative Cdc42 affect the polarized trafficking of proteins to the apical and basal domains (Etienne-Manneville, 2004; Macara, 2004). The localization of PAR6–aPKC to the apical domain is dependent on Cdc42 and is required for targeting of apical proteins (Wu et al., 2006). Through loss of function experiments, aPKC-Par complex proteins fail to maintain their apical epithelial localization in the absence of Cdc42, eventually leading to a breakdown of the epithelia (Wu et al., 2006). In the zebrafish spinal cord, blocking Cdc42 signaling produced ectopically positioned OPCs similar to what is seen in the absence of PrkCi. However, unlike what was observed in *has* mutants, blocking of Cdc42 signaling did not affect apical polarity, as the localization of apical polarity proteins PrkC and ZO-1 was normal. In addition the number of $Sox10^{+}$ OPCs was similar between control and $Cdc42(N17)^{+}$ embryos. This also suggests that Cdc42 and PrkCi may be functioning together, but through a pathway that does not affect apical polarity. This is not a unique role for Cdc42 as studies have shown that Cdc42 can regulate cell polarity by either not interacting with the aPKC-Par complex or by

interacting with selective members of the aPKC-Par complex(Etienne-Manneville et al., 2005; Gomes et al., 2005).

Timelapse imaging of heat shocked *Tg(nkx2.2a:megfp)(hsp:cdc42(n17)-myc)* embryos showed that the Nkx2.2a^{+} OPCs fail to migrate properly in the absence of Cdc42 signaling. However, timelapse imaging of *Tg(sox10(7.2):mrfp)(hsp:cdc42(n17)-myc)* under the same conditions, revealed that the Sox10^{+} OPCs migrate normally and in normal numbers. This suggests that the migration of various populations of OPCs may be regulated differently.

Our antibody and time-lapse data argues for a role of Cdc42 in the migration of the Nkx2.2a^{+} OPCs. However, our data also shows that in the absence of Cdc42 function, the number of Sox10^{+} OPCs in the spinal cord is comparable to wild type. In addition, our time-lapse data on *Tg(sox10(7.2):mfrp)(hsp:cdc42(N17)-myc)*$^{+}$ embryos shows that the Sox10^{+} OPCs migrate normally out of the ventral spinal cord and in normal numbers. This suggests that Cdc42 regulates migration of the Nkx2.2a^{+} Olig2^{+} Sox10^{+} OPCs and that the Nkx2.2a^{-} Olig2^{+} Sox10^{+} OPC can compensate for the Nkx2.2a^{+} Olig2^{+} Sox10^{+} OPCs. To test this hypothesis, I propose doing time lapse imaging on heatshocked *Tg(sox10(7.2):mfrp)(nkx2.2a:megfp)* and *Tg(sox10(7.2):mfrp)(hsp:cdc42(N17)-myc)*$^{+}$ embryos. If the Nkx2.2a^{-} Olig2^{+} Sox10^{+} OPCs are compensating for the Nkx2.2a^{+} Olig2^{+} Sox10^{+} OPCs in the absence of normal Cdc42 function, you would expect to see only Nkx2.2a^{-} Olig2^{+} Sox10^{+} OPCs.

Taken together these data show that Cdc42 may be important in either the specification and/or migration of the *nkx2.2a*$^{+}$ subset of OPCs. This could potentially be a novel function for Cdc42 in oligodendrocytes. Current studies show that Cdc42 plays an important role in many aspects of glial cell migration and development. For example, Cdc42 mutant mice have myelin sheaths that are thinner and more disorganized. This wrapping deficiency appears to be the result of a defect in removing cytoplasm as the oligodendrocyte plasma membranes wrap around the axon (Thurnherr2006). In addition, Cdc42 has been shown to be important in Schwann cell proliferation and myelination as well as rat astrocyte migration.(Benninger2007etienne-manville2006).

From our experiments in this thesis, I showed that heat shocked *Tg(hsp:cdc42(N17)-myc)* embryos had distinct morphological phenotypes, and expressed the dominant negative fusion protein Cdc42(N17)-Myc in all cells of the spinal cord. Although I identified the presence of the dominant negative I never tested the efficacy of the dominant negative. Heatshocking of *Tg(hsp:cdc42(N17)-myc)* embryos leads to the expression of a dominant negative Cdc42 that contains a asparagine to threonine substitution mutation at amino acid 17 (Heasman and Ridley, 2008). The dominant negative theoretically blocks endogenous Cdc42 signaling by out competing endogenous Cdc42 for binding to GEFs. The N17 mutation allows the binding of GEFs but does not allow the subsequent activation of Rho GTPase signaling. To test the efficacy of the dominant negative, I propose performing GEF activity assays. After heatshocking *Tg(hsp:cdc42(N17)-myc)* embryos, lysates can be made of control

and Cdc42(N17) embryos to test for the ability of the GEF to activate the release of GDP.

One caveat to our experiments is that heat shocking expresses a dominant negative that binds and sequesters GEFs. Several small Rho-GTPases can use the same GEF for activation of signaling (Heasman and Ridley, 2008). By sequestering GEF activity, I may be blocking more than one small Rho-GTPase. Therefore the results interpreted may not be due solely to the effects of inhibition of Cdc42 signaling.

Notch signaling may be required for the maintenance of the postembryonic precursors in the spinal cord.

Until recently, the CNS has long been thought of as a static organ system incapable of regeneration. This CNS plasticity appears to be evolutionarily conserved as many areas of adult neurogenesis or postembryonic proliferation have been identified across species. In the mammalian CNS, newborn postembryonic cells migrate to various brain regions, replace older ones and connect to existing circuits. Evidence suggests that this replacement is related to acquisition of new information. Therefore, postembryonic proliferation and replacement can be seen as a form of brain plasticity that enables organisms to adjust to environmental changes like learning and memory. In this thesis I identified a population of postembryonic proliferative precursors in the zebrafish spinal cord that appears to be maintained through Notch signaling. In addition, these precursors appear to be giving rise to radial glia and oligodendrocytes but not neurons.

By performing BrdU incorporation experiments, I was able to identify areas of postembryonic cell proliferation along the medial septum in the zebrafish spinal cord. There are two distinct periods of cell division. The first occurs during embryogenesis and is followed by a quiescent transition period during early larval stages. The second stage occurs at about 15 dpf where I observed an increase in cell divisions during the later larval stages. Cell proliferation in the larval stages is maintained into adulthood.

Using BrdU as a lineage tracer, I was able to identify that these postembryonic cells give rise to OPCs and possibly radial glia. However, at all time points examined, I was unable to detect any neurons that expressed the pan neuronal marker Hu and the cell proliferation marker Brdu, suggesting that these postembryonic proliferative cells do not give rise to neurons in the postembryonic spinal cord.

Because Notch signaling has been shown to be important in maintaining precursor cells, I looked at expression of notch genes at various stages in development. *In situ* hybrizations revealed that the temporal and spatial gene expression pattern of *notch1a* and *dld* were similar to the temporal and spatial pattern of cell division. To investigate this, I blocked Notch signaling using the chemical inhibitor DAPT. Antibody labeling, using an antibody PCNA as a cell division marker, revealed that in the absence of Notch signaling, the number of postembryonic proliferative cells was decreased. One pitfall to this experiment is that DAPT blocks all g-secretase activity within the embryo, which makes it impossible to know for sure if the affects are solely to a loss of Notch signaling.

To address this, I would use a transgenic line that expresses a dominant negative form of suppressor of hairless under the control of the heat shock promoter. This would make it possible for us to block Notch signaling in a time dependant fashion.

REFERENCES CITED

Aaku-Saraste, E., Hellwig, A. and Huttner, W. B. (1996). Loss of occludin and functional tight junctions, but not ZO-1, during neural tube closure--remodeling of the neuroepithelium prior to neurogenesis. *Dev Biol* **180**, 664-79.

Adam, A., Bartfai, R., Lele, Z., Krone, P. H. and Orban, L. (2000). Heat-inducible expression of a reporter gene detected by transient assay in zebrafish. *Exp Cell Res* **256**, 282-90.

Adams, A. E., Johnson, D. I., Longnecker, R. M., Sloat, B. F. and Pringle, J. R. (1990). CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast Saccharomyces cerevisiae. *J Cell Biol* **111**, 131-42.

Adiguzel, E., Ahmad, P. J., Franco, C. and Bendeck, M. P. (2009). Collagens in the progression and complications of atherosclerosis. *Vascular Medicine* **14**, 73-89.

Adler, P. N. (2002). Planar signaling and morphogenesis in Drosophila. *Dev Cell* **2**, 525-35.

Adolf, B., Chapouton, P., Lam, C. S., Topp, S., Tannh‰user, B., Str‰hle, U., Gˆtz, M. and Bally-Cuif, L. (2006). Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. *Developmental Biology* **295**, 278-293.

Afonso, C. and Henrique, D. (2006). PAR3 acts as a molecular organizer to define the apical domain of chick neuroepithelial cells. *J Cell Sci* **119**, 4293-304.

Aguirre, A. and Gallo, V. (2004). Postnatal neurogenesis and gliogenesis in the olfactory bulb from NG2-expressing progenitors of the subventricular zone. *J Neurosci* **24**, 10530-41.

Alison, M. R. and Islam, S. (2009). Attributes of adult stem cells. *J Pathol* **217**, 144-60.

Alison, M. R., Poulsom, R., Forbes, S. and Wright, N. A. (2002). An introduction to stem cells. *J Pathol* **197**, 419-23.

Altman, J. (1962). Are new neurons formed in the brains of adult mammals? *Science* **135**, 1127-8.

Altman, J. (1963). Autoradiographic investigation of cell proliferation in the brains of rats and cats. *Anat Rec* **145**, 573-91.

Altman, J. and Bayer, S. A. (1984). The development of the rat spinal cord. *Adv Anat Embryol Cell Biol* **85**, 1-164.

Altman, J. and Das, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol* **124**, 319-35.

Alvarez-Buylla, A. (1990). Mechanism of neurogenesis in adult avian brain. *Experientia* **46**, 948-55.

Alvarez-Buylla, A., Garcia-Verdugo, J. M. and Tramontin, A. D. (2001). A unified hypothesis on the lineage of neural stem cells. *Nat Rev Neurosci* **2**, 287-93.

Alvarez-Buylla, A. and Lim, D. A. (2004). For the Long Run: Maintaining Germinal Niches in the Adult Brain. **41**, 683-686.

Alvarez-Buylla, A. and Lois, C. (1995). Neuronal stem cells in the brain of adult vertebrates. *Stem Cells* **13**, 263-72.

Alvarez-Buylla, A. and Nottebohm, F. (1988). Migration of young neurons in adult avian brain. *Nature* **335**, 353-4.

Amrein, I. and Lipp, H. P. (2009). Adult hippocampal neurogenesis of mammals: evolution and life history. *Biol Lett* **5**, 141-4.

Anthony, T. E., Klein, C., Fishell, G. and Heintz, N. (2004). Radial glia serve as neuronal progenitors in all regions of the central nervous system. *Neuron* **41**, 881-90.

Appel, B. and Eisen, J. S. (1998). Regulation of neuronal specification in the zebrafish spinal cord by Delta function. *Development* **125**, 371-80.

Appel, B., Givan, L. A. and Eisen, J. S. (2001). Delta-Notch signaling and lateral inhibition in zebrafish spinal cord development. *BMC Dev Biol* **1**, 13.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch Signaling: Cell Fate Control and Signal Integration in Development. *Science* **284**, 770-776.

Atwood, S. X., Chabu, C., Penkert, R. R., Doe, C. Q. and Prehoda, K. E. (2007). Cdc42 acts downstream of Bazooka to regulate neuroblast polarity through Par-6 aPKC. *J Cell Sci* **120**, 3200-6.

Balon, E. K. (1990). Epigenesis of an epigeneticist : the development of some alternative concepts on the early ontogeny and evolution of fishes. *Guelph Ichthyology Reviews* **1**, 1-48.

Batista, M. F., Jacobstein, J. and Lewis, K. E. (2008). Zebrafish V2 cells develop into excitatory CiD and Notch signalling dependent inhibitory VeLD interneurons. *Developmental Biology* **322**, 263-275.

Baye, L. M. and Link, B. A. (2007). Interkinetic nuclear migration and the selection of neurogenic cell divisions during vertebrate retinogenesis. *J Neurosci* **27**, 10143-52.

Baye, L. M. and Link, B. A. (2008). Nuclear migration during retinal development. *Brain Research* **1192**, 29-36.

Bernhardt, R. R., Chitnis, A. B., Lindamer, L. and Kuwada, J. Y. (1990). Identification of spinal neurons in the embryonic and larval zebrafish. *J Comp Neurol* **302**, 603-16.

Betschinger, J., Mechtler, K. and Knoblich, J. A. (2003). The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. *Nature* **422**, 326-30.

Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T. M., Rubenstein, J. L. R. and Ericson, J. (1999). Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* **398**, 622-627.

Brown, L. D. (1997). The metamorphosis: conversion in historical context. *Bull N Y Acad Med* **74**, 238-47.

Buescher, M., Yeo, S. L., Udolph, G., Zavortink, M., Yang, X., Tear, G. and Chia, W. (1998). Binary sibling neuronal cell fate decisions in the Drosophila embryonic central nervous system are nonstochastic and require inscuteable-mediated asymmetry of ganglion mother cells. *Genes Dev* **12**, 1858-70.

Burrill, J. D., Moran, L., Goulding, M. D. and Saueressig, H. (1997). PAX2 is expressed in multiple spinal cord interneurons, including a population of EN1+ interneurons that require PAX6 for their development. *Development* **124**, 4493-503.

Cameron, H. A., Woolley, C. S., McEwen, B. S. and Gould, E. (1993). Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience* **56**, 337-44.

Cepko, C. L., Ryder, E. F., Austin, C. P., Walsh, C. and Fekete, D. M. (1993). Lineage analysis using retrovirus vectors. *Methods Enzymol* **225**, 933-60.

Chapouton, P., Adolf, B., Leucht, C., Tannhauser, B., Ryu, S., Driever, W. and Bally-Cuif, L. (2006). her5 expression reveals a pool of neural stem cells in the adult zebrafish midbrain. *Development* **133**, 4293-303.

Chen, F., Ma, L., Parrini, M. C., Mao, X., Lopez, M., Wu, C., Marks, P. W., Davidson, L., Kwiatkowski, D. J., Kirchhausen, T. et al. (2000). Cdc42 is required for PIP2-induced actin polymerization and early development but not for cell viability. *Current Biology* **10**, 758-765.

Chen, W., Burgess, S. and Hopkins, N. (2001). Analysis of the zebrafish smoothened mutant reveals conserved and divergent functions of hedgehog activity. *Development* **128**, 2385-2396.

Chenn, A. and McConnell, S. K. (1995). Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. *Cell* **82**, 631-41.

Choksi, S. P., Southall, T. D., Bossing, T., Edoff, K., de Wit, E., Fischer, B. E., van Steensel, B., Micklem, G. and Brand, A. H. (2006). Prospero Acts as a Binary Switch between Self-Renewal and Differentiation in Drosophila†Neural†Stem Cells. *Developmental Cell* **11**, 775-789.

Clarke, J. D., Hayes, B. P., Hunt, S. P. and Roberts, A. (1984a). Sensory physiology, anatomy and immunohistochemistry of Rohon-Beard neurones in embryos of Xenopus laevis. *J Physiol* **348**, 511-25.

Clarke, J. D., Hayes, B. P., Hunt, S. P. and Roberts, A. (1984b). Sensory physiology, anatomy and immunohistochemistry of Rohon-Beard neurones in embryos of Xenopus laevis. *J Physiol* **348**, 511-525.

Concha, M. L. and Adams, R. J. (1998). Oriented cell divisions and cellular morphogenesis in the zebrafish gastrula and neurula: a time-lapse analysis. *Development* **125**, 983-94.

Copp, A., Cogram, P., Fleming, A., Gerrelli, D., Henderson, D., Hynes, A., Kolatsi-Joannou, M., Murdoch, J. and Ybot-Gonzalez, P. (2000). Neurulation and neural tube closure defects. *Methods Mol Biol* **136**, 135-60.

Copp, A. J., Greene, N. D. and Murdoch, J. N. (2003). The genetic basis of mammalian neurulation. *Nat Rev Genet* **4**, 784-93.

Cui, S., Otten, C., Rohr, S., Abdelilah-Seyfried, S. and Link, B. A. (2007). Analysis of aPKClambda and aPKCzeta reveals multiple and redundant functions during vertebrate retinogenesis. *Mol Cell Neurosci* **34**, 431-44.

Das, T., Payer, B., Cayouette, M. and Harris, W. A. (2003). In vivo time-lapse imaging of cell divisions during neurogenesis in the developing zebrafish retina. *Neuron* **37**, 597-609.

Doe, C. Q. (2008). Neural stem cells: balancing self-renewal with differentiation. *Development* **135**, 1575-87.

Doe, C. Q., Chu-LaGraff, Q., Wright, D. M. and Scott, M. P. (1991). The prospero gene specifies cell fates in the drosophila central nervous system. *Cell* **65**, 451-464.

Doetsch, F. and Hen, R. (2005). Young and excitable: the function of new neurons in the adult mammalian brain. *Curr Opin Neurobiol* **15**, 121-8.

Egger, B., Chell, J. M. and Brand, A. H. (2008). Insights into neural stem cell biology from flies. *Philos Trans R Soc Lond B Biol Sci* **363**, 39-56.

Ehebauer, M., Hayward, P. and Martinez-Arias, A. (2006). Notch Signaling Pathway. *Sci. STKE* **2006**, cm7-.

Emma, J. B. (2009). Involvement of the cytoskeletal elements in articular cartilage homeostasis and pathology. *International Journal of Experimental Pathology* **90**, 1-15.

Emsley, J. G., Mitchell, B. D., Kempermann, G. and Macklis, J. D. (2005). Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. *Prog Neurobiol* **75**, 321-41.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-73.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-80.

Etemad-Moghadam, B., Guo, S. and Kemphues, K. J. (1995). Asymmetrically distributed PAR-3 protein contributes to cell polarity and spindle alignment in early C. elegans embryos. *Cell* **83**, 743-752.

Etienne-Manneville, S. (2004). Cdc42 - the centre of polarity. *J Cell Sci* **117**, 1291-1300.

Etienne-Manneville, S. and Hall, A. (2001). Integrin-Mediated Activation of Cdc42 Controls Cell Polarity in Migrating Astrocytes through PKC[zeta]. *Cell* **106**, 489-498.

Etienne-Manneville, S., Manneville, J.-B., Nicholls, S., Ferenczi, M. A. and Hall, A. (2005). Cdc42 and Par6-PKC{zeta} regulate the spatially localized association of Dlg1 and APC to control cell polarization. *J. Cell Biol.* **170**, 895-901.

Ever, L. and Gaiano, N. (2005). Radial 'glial' progenitors: neurogenesis and signaling. *Curr Opin Neurobiol* **15**, 29-33.

Fishell, G. and Kriegstein, A. R. (2003). Neurons from radial glia: the consequences of asymmetric inheritance. *Curr Opin Neurobiol* **13**, 34-41.

Fuchs, E., Tumbar, T. and Guasch, G. (2004). Socializing with the neighbors: stem cells and their niche. *Cell* **116**, 769-78.

Garrard, S. M., Capaldo, C. T., Gao, L., Rosen, M. K., Macara, I. G. and Tomchick, D. R. (2003). Structure of Cdc42 in a complex with the GTPase-binding domain of the cell polarity protein, Par6. *Embo J* **22**, 1125-33.

Gates, M. A., Thomas, L. B., Howard, E. M., Laywell, E. D., Sajin, B., Faissner, A., Gotz, B., Silver, J. and Steindler, D. A. (1995). Cell and molecular analysis of the developing and adult mouse subventricular zone of the cerebral hemispheres. *J Comp Neurol* **361**, 249-66.

Geldmacher-Voss, B., Reugels, A. M., Pauls, S. and Campos-Ortega, J. A. (2003). A 90{degrees} rotation of the mitotic spindle changes the orientation of mitoses of zebrafish neuroepithelial cells. *Development* **130**, 3767-3780.

Geling, A., Steiner, H., Willem, M., Bally-Cuif, L. and Haass, C. (2002). A gamma-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. *EMBO Rep* **3**, 688-94.

Girard, F., Cremazy, F., Berta, P. and Renucci, A. (2001). Expression pattern of the Sox31 gene during zebrafish embryonic development. *Mech Dev* **100**, 71-3.

Goldstein, B. and Macara, I. G. (2007). The PAR Proteins: Fundamental Players in Animal Cell Polarization. *Developmental Cell* **13**, 609-622.

Gomes, E. R., Jani, S. and Gundersen, G. G. (2005). Nuclear Movement Regulated by Cdc42, MRCK, Myosin, and Actin Flow Establishes MTOC Polarization in Migrating Cells. *Cell* **121**, 451-463.

Gotta, M., Abraham, M. C. and Ahringer, J. (2001). CDC-42 controls early cell polarity and spindle orientation in C. elegans. *Current Biology* **11**, 482-488.

Gotz, M., Hartfuss, E. and Malatesta, P. (2002). Radial glial cells as neuronal precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice. *Brain Res Bull* **57**, 777-88.

Gotz, M. and Huttner, W. B. (2005). The cell biology of neurogenesis. *Nat Rev Mol Cell Biol* **6**, 777-88.

Grandel, H., Kaslin, J., Ganz, J., Wenzel, I. and Brand, M. (2006). Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate. *Dev Biol* **295**, 263-77.

Grunwald, D. J. and Eisen, J. S. (2002). Headwaters of the zebrafish [mdash] emergence of a new model vertebrate. *Nat Rev Genet* **3**, 717-724.

Gupton, S. L. and Gertler, F. B. (2007). Filopodia: The Fingers That Do the Walking. *Sci. STKE* **2007**, re5-.

Hack, M. A., Saghatelyan, A., de Chevigny, A., Pfeifer, A., Ashery-Padan, R., Lledo, P.-M. and Gotz, M. (2005). Neuronal fate determinants of adult olfactory bulb neurogenesis. *Nat Neurosci* **8**, 865-872.

Hall, A. (2005). Rho GTPases and the control of cell behaviour. *Biochem Soc Trans* **33**, 891-5.

Halliday, A. L. and Cepko, C. L. (1992). Generation and migration of cells in the developing striatum. *Neuron* **9**, 15-26.

Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F. and Kageyama, R. (2004). Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. *Development* **131**, 5539-5550.

Haydar, T. F., Ang, E., Jr. and Rakic, P. (2003). Mitotic spindle rotation and mode of cell division in the developing telencephalon. *Proc Natl Acad Sci U S A* **100**, 2890-5.

Heasman, S. J. and Ridley, A. J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat Rev Mol Cell Biol* **9**, 690-701.

Hewagama, A. and Richardson, B. (2009). The genetics and epigenetics of autoimmune diseases. *J Autoimmun*.

Hollyday, M. (2001). Neurogenesis in the vertebrate neural tube. *Int J Dev Neurosci* **19**, 161-73.

Hong, E. and Brewster, R. (2006). N-cadherin is required for the polarized cell behaviors that drive neurulation in the zebrafish. *Development* **133**, 3895-3905.

Horne-Badovinac, S., Lin, D., Waldron, S., Schwarz, M., Mbamalu, G., Pawson, T., Jan, Y., Stainier, D. Y. and Abdelilah-Seyfried, S. (2001). Positional cloning of heart and soul reveals multiple roles for PKC lambda in zebrafish organogenesis. *Curr Biol* **11**, 1492-502.

Hung, T. J. and Kemphues, K. J. (1999). PAR-6 is a conserved PDZ domain-containing protein that colocalizes with PAR-3 in Caenorhabditis elegans embryos. *Development* **126**, 127-35.

Hurd, T. W., Gao, L., Roh, M. H., Macara, I. G. and Margolis, B. (2003). Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. *Nat Cell Biol* **5**, 137-42.

Hutchinson, S. A. and Eisen, J. S. (2006). Islet1 and Islet2 have equivalent abilities to promote motoneuron formation and to specify motoneuron subtype identity. *Development* **133**, 2137-2147.

Hutson, L. D. and Chien, C.-B. (2002). Wiring the zebrafish: axon guidance and synaptogenesis. *Current Opinion in Neurobiology* **12**, 87-92.

Ihrie, R. A. and Alvarez-Buylla, A. (2008). Cells in the astroglial lineage are neural stem cells. *Cell Tissue Res* **331**, 179-91.

Ikeshima-Kataoka, H., Skeath, J. B., Nabeshima, Y.-i., Doe, C. Q. and Matsuzaki, F. (1997). Miranda directs Prospero to a daughter cell during Drosophila asymmetric divisions. *Nature* **390**, 625-629.

Imai, F., Hirai, S.-i., Akimoto, K., Koyama, H., Miyata, T., Ogawa, M., Noguchi, S., Sasaoka, T., Noda, T. and Ohno, S. (2006). Inactivation of aPKC{lambda} results in the loss of adherens junctions in neuroepithelial cells without affecting neurogenesis in mouse neocortex. *Development* **133**, 1735-1744.

Itoh, M., Kim, C. H., Palardy, G., Oda, T., Jiang, Y. J., Maust, D., Yeo, S. Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L. et al. (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev Cell* **4**, 67-82.

Izumi, Y., Hirose, T., Tamai, Y., Hirai, S.-i., Nagashima, Y., Fujimoto, T., Tabuse, Y., Kemphues, K. J. and Ohno, S. (1998). An Atypical PKC Directly Associates and Colocalizes at the Epithelial Tight Junction with ASIP, a Mammalian Homologue of Caenorhabditis elegans Polarity Protein PAR-3. *J. Cell Biol.* **143**, 95-106.

Jackson, E. L., Garcia-Verdugo, J. M., Gil-Perotin, S., Roy, M., Quinones-Hinojosa, A., VandenBerg, S. and Alvarez-Buylla, A. (2006). PDGFR±-Positive B Cells Are Neural Stem Cells in the Adult SVZ that Form Glioma-like Growths in Response to Increased PDGF Signaling. **51**, 187-199.

Jessberger, S., Toni, N., Clemenson Jr, G. D., Ray, J. and Gage, F. H. (2008). Directed differentiation of hippocampal stem/progenitor cells in the adult brain. *Nat Neurosci* **11**, 888-893.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat Rev Genet* **1**, 20-9.

Jessen, J. R., Topczewski, J., Bingham, S., Sepich, D. S., Marlow, F., Chandrasekhar, A. and Solnica-Krezel, L. (2002). Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. *Nat Cell Biol* **4**, 610-5.

Jia, C. and Halpern, M. (1998). Neurogenesis and migration of receptor neurons in the vomeronasal sensory epithelium in the opossum, Monodelphis domestica. *J Comp Neurol* **400**, 287-97.

Johnson, D. I. and Pringle, J. R. (1990). Molecular characterization of CDC42, a Saccharomyces cerevisiae gene involved in the development of cell polarity. *J Cell Biol* **111**, 143-52.

Kaltschmidt, J. A. and Brand, A. H. (2002). Asymmetric cell division: microtubule dynamics and spindle asymmetry. *J Cell Sci* **115**, 2257-2264.

Kaltschmidt, J. A., Davidson, C. M., Brown, N. H. and Brand, A. H. (2000). Rotation and asymmetry of the mitotic spindle direct asymmetric cell division in the developing central nervous system. *Nat Cell Biol* **2**, 7-12.

Kaslin, J., Ganz, J. and Brand, M. (2008). Proliferation, neurogenesis and regeneration in the non-mammalian vertebrate brain. *Philos Trans R Soc Lond B Biol Sci* **363**, 101-22.

Keller, R. (2005). Cell migration during gastrulation. *Current Opinion in Cell Biology* **17**, 533-541.

Kemphues, K. J., Priess, J. R., Morton, D. G. and Cheng, N. (1988). Identification of genes required for cytoplasmic localization in early C. elegans embryos. *Cell* **52**, 311-320.

Kim, H., Kim, S., Chung, A. Y., Bae, Y. K., Hibi, M., Lim, C. S. and Park, H. C. (2008a). Notch-regulated perineurium development from zebrafish spinal cord. *Neurosci Lett* **448**, 240-4.

Kim, H., Shin, J., Kim, S., Poling, J., Park, H. C. and Appel, B. (2008b). Notch-regulated oligodendrocyte specification from radial glia in the spinal cord of zebrafish embryos. *Dev Dyn* **237**, 2081-9.

Kim, M. D., Kolodziej, P. and Chiba, A. (2002). Growth Cone Pathfinding and Filopodial Dynamics Are Mediated Separately by Cdc42 Activation. *J. Neurosci.* **22**, 1794-1806.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn* **203**, 253-310.

Kimmel, C. B., Warga, R. M. and Kane, D. A. (1994). Cell cycles and clonal strings during formation of the zebrafish central nervous system. *Development* **120**, 265-76.

Kirby, B. B., Takada, N., Latimer, A. J., Shin, J., Carney, T. J., Kelsh, R. N. and Appel, B. (2006). In vivo time-lapse imaging shows dynamic oligodendrocyte progenitor behavior during zebrafish development. *Nat Neurosci* **9**, 1506-11.

Klein, T. J. and Mlodzik, M. (2005). Planar cell polarization: an emerging model points in the right direction. *Annu Rev Cell Dev Biol* **21**, 155-76.

Komiya, Y. and Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis* **4**, 68-75.

Kondo, A., Kamihira, O. and Ozawa, H. (2009). Neural tube defects: prevalence, etiology and prevention. *Int J Urol* **16**, 49-57.

Konno, D., Shioi, G., Shitamukai, A., Mori, A., Kiyonari, H., Miyata, T. and Matsuzaki, F. (2008). Neuroepithelial progenitors undergo LGN-dependent planar divisions to maintain self-renewability during mammalian neurogenesis. *Nat Cell Biol* **10**, 93-101.

Korn, T. (2008). Pathophysiology of multiple sclerosis. *J Neurol* **255 Suppl 6**, 2-6.

Kosodo, Y., Roper, K., Haubensak, W., Marzesco, A. M., Corbeil, D. and Huttner, W. B. (2004). Asymmetric distribution of the apical plasma membrane during neurogenic divisions of mammalian neuroepithelial cells. *Embo J* **23**, 2314-24.

Kovac, J., Oster, H. and Leitges, M. (2007). Expression of the atypical protein kinase C (aPKC) isoforms iota/lambda and zeta during mouse embryogenesis. *Gene Expr Patterns* **7**, 187-96.

Kozma, R., Ahmed, S., Best, A. and Lim, L. (1995). The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol. Cell. Biol.* **15**, 1942-1952.

Kraut, R. and Campos-Ortega, J. A. (1996). inscuteable,A Neural Precursor Gene ofDrosophila,Encodes a Candidate for a Cytoskeleton Adaptor Protein. *Developmental Biology* **174**, 65-81.

Kraut, R., Chia, W., Jan, L. Y., Jan, Y. N. and Knoblich, J. A. (1996). Role of inscuteable in orienting asymmetric cell divisions in Drosophila. *Nature* **383**, 50-5.

Kucenas, S., Takada, N., Park, H. C., Woodruff, E., Broadie, K. and Appel, B. (2008). CNS-derived glia ensheath peripheral nerves and mediate motor root development. *Nat Neurosci* **11**, 143-51.

Kuhlbrodt, K., Herbarth, B., Sock, E., Hermans-Borgmeyer, I. and Wegner, M. (1998). Sox10, a novel transcriptional modulator in glial cells. *J Neurosci* **18**, 237-50.

Lee, C. Y., Andersen, R. O., Cabernard, C., Manning, L., Tran, K. D., Lanskey, M. J., Bashirullah, A. and Doe, C. Q. (2006a). Drosophila Aurora-A kinase inhibits neuroblast self-renewal by regulating aPKC/Numb cortical polarity and spindle orientation. *Genes Dev* **20**, 3464-74.

Lee, C. Y., Robinson, K. J. and Doe, C. Q. (2006b). Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. *Nature* **439**, 594-8.

Lee, K. J. and Jessell, T. M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. *Annu Rev Neurosci* **22**, 261-94.

Lewis, K. E. and Eisen, J. S. (2001). Hedgehog signaling is required for primary motoneuron induction in zebrafish. *Development* **128**, 3485-3495.

Lim, D. A., Fishell, G. J. and Alvarez-Buylla, A. (1997). Postnatal mouse subventricular zone neuronal precursors can migrate and differentiate within multiple levels of the developing neuraxis. *Proc Natl Acad Sci U S A* **94**, 14832-6.

Lledo, P.-M., Merkle, F. T. and Alvarez-Buylla, A. (2008). Origin and function of olfactory bulb interneuron diversity. *Trends in Neurosciences* **31**, 392-400.

Lopez-Garcia, C., Molowny, A., Garcia-Verdugo, J. M. and Ferrer, I. (1988). Delayed postnatal neurogenesis in the cerebral cortex of lizards. *Developmental Brain Research* **43**, 167-174.

Lopez-Munoz, F., Boya, J. and Alamo, C. (2006). Neuron theory, the cornerstone of neuroscience, on the centenary of the Nobel Prize award to Santiago Ramon y Cajal. *Brain Res Bull* **70**, 391-405.

Lowery, L. A. and Sive, H. (2004). Strategies of vertebrate neurulation and a re-evaluation of teleost neural tube formation. *Mech Dev* **121**, 1189-97.

Lu, Q. R., Sun, T., Zhu, Z., Ma, N., Garcia, M., Stiles, C. D. and Rowitch, D. H. (2002). Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. *Cell* **109**, 75-86.

Luo, L., Liao, Y. J., Jan, L. Y. and Jan, Y. N. (1994). Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev* **8**, 1787-802.

Luster, A. D., Alon, R. and von Andrian, U. H. (2005). Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol* **6**, 1182-1190.

Lyons, D. A., Guy, A. T. and Clarke, J. D. W. (2003). Monitoring neural progenitor fate through multiple rounds of division in an intact vertebrate brain. *Development* **130**, 3427-3436.

Macara, I. G. (2004). Parsing the Polarity Code. *Nat Rev Mol Cell Biol* **5**, 220-231.

Malatesta, P., Hack, M. A., Hartfuss, E., Kettenmann, H., Klinkert, W., Kirchhoff, F. and Gˆtz, M. (2003). Neuronal or Glial Progeny: Regional Differences in Radial Glia Fate. **37**, 751-764.

Malatesta, P., Hartfuss, E. and Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development* **127**, 5253-63.

Manabe, N., Hirai, S., Imai, F., Nakanishi, H., Takai, Y. and Ohno, S. (2002). Association of ASIP/mPAR-3 with adherens junctions of mouse neuroepithelial cells. *Dev Dyn* **225**, 61-9.

Mao, L., Lau, Y. S., Petroske, E. and Wang, J. Q. (2001). Profound astrogenesis in the striatum of adult mice following nigrostriatal dopaminergic lesion by repeated MPTP administration. *Brain Res Dev Brain Res* **131**, 57-65.

Marusich, M. F., Furneaux, H. M., Henion, P. D. and Weston, J. A. (1994). Hu neuronal proteins are expressed in proliferating neurogenic cells. *J Neurobiol* **25**, 143-55.

Masahira, N., Takebayashi, H., Ono, K., Watanabe, K., Ding, L., Furusho, M., Ogawa, Y., Nabeshima, Y.-i., Alvarez-Buylla, A., Shimizu, K. et al. (2006). Olig2-positive progenitors in the embryonic spinal cord give rise not only to motoneurons and oligodendrocytes, but also to a subset of astrocytes and ependymal cells. *Developmental Biology* **293**, 358-369.

Matsuzaki, F., Ohshiro, T., Ikeshima-Kataoka, H. and Izumi, H. (1998). miranda localizes staufen and prospero asymmetrically in mitotic neuroblasts and epithelial cells in early Drosophila embryogenesis. *Development* **125**, 4089-4098.

McCarthy, M., Turnbull, D. H., Walsh, C. A. and Fishell, G. (2001). Telencephalic Neural Progenitors Appear To Be Restricted to Regional and Glial Fates before the Onset of Neurogenesis. *J. Neurosci.* **21**, 6772-6781.

Mekki-Dauriac, S., Agius, E., Kan, P. and Cochard, P. (2002). Bone morphogenetic proteins negatively control oligodendrocyte precursor specification in the chick spinal cord. *Development* **129**, 5117-30.

Merkle, F. T. and Alvarez-Buylla, A. (2006). Neural stem cells in mammalian development. *Curr Opin Cell Biol* **18**, 704-9.

Merkle, F. T., Tramontin, A. D., GarcÃ-a-Verdugo, J. M. and Alvarez-Buylla, A. (2004). Radial glia give rise to adult neural stem cells in the subventricular zone. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 17528-17532.

Metcalfe, W. K., Myers, P. Z., Trevarrow, B., Bass, M. B. and Kimmel, C. B. (1990). Primary neurons that express the L2/HNK-1 carbohydrate during early development in the zebrafish. *Development* **110**, 491-504.

Morin, X., Jaouen, F. and Durbec, P. (2007). Control of planar divisions by the G-protein regulator LGN maintains progenitors in the chick neuroepithelium. *Nat Neurosci* **10**, 1440-8.

Morrison, S. J. and Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* **441**, 1068-1074.

Munson, C., Huisken, J., Bit-Avragim, N., Kuo, T., Dong, P. D., Ober, E. A., Verkade, H., Abdelilah-Seyfried, S. and Stainier, D. Y. R. (2008). Regulation of neurocoel morphogenesis by Pard6[gamma]b. *Developmental Biology* **324**, 41-54.

Myers, P. Z., Eisen, J. S. and Westerfield, M. (1986). Development and axonal outgrowth of identified motoneurons in the zebrafish. *J Neurosci* **6**, 2278-89.

Nakao, T. and Ishizawa, A. (1987). Development of the spinal nerves of the larval lamprey: IV. Spinal nerve roots of 21-mm larval and adult lampreys, with special reference to the relation of meninges with the root sheath and the perineurium. *J Comp Neurol* **256**, 386-99.

Namba, T., Mochizuki, H., Onodera, M., Mizuno, Y., Namiki, H. and Seki, T. (2005). The fate of neural progenitor cells expressing astrocytic and radial glial markers in the postnatal rat dentate gyrus. *Eur J Neurosci* **22**, 1928-41.

Nipper, R. W., Siller, K. H., Smith, N. R., Doe, C. Q. and Prehoda, K. E. (2007). Galphai generates multiple Pins activation states to link cortical polarity and spindle orientation in Drosophila neuroblasts. *Proc Natl Acad Sci U S A* **104**, 14306-11.

Nobes, C. D. and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62.

Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S. and Kriegstein, A. R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409**, 714-20.

Noctor, S. C., Martinez-Cerdeno, V., Ivic, L. and Kriegstein, A. R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat Neurosci* **7**, 136-44.

Noctor, S. C., Martinez-Cerdeno, V. and Kriegstein, A. R. (2008). Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. *J Comp Neurol* **508**, 28-44.

Ohno, S. (2001). Intercellular junctions and cellular polarity: the PAR-aPKC complex, a conserved core cassette playing fundamental roles in cell polarity. *Curr Opin Cell Biol* **13**, 641-8.

Papan, C. and Campos-Ortega, J. A. (1994). On the formation of the neural keel and neural tube in the zebrafishDanio (Brachydanio) rerio. *Development Genes and Evolution* **203**, 178-186.

Parichy, D. M. (2003). Pigment patterns: fish in stripes and spots. *Current Biology* **13**, R947-R950.

Park, H.-C., Boyce, J., Shin, J. and Appel, B. (2005). Oligodendrocyte Specification in Zebrafish Requires Notch-Regulated Cyclin-Dependent Kinase Inhibitor Function. *J. Neurosci.* **25**, 6836-6844.

Park, H. C., Mehta, A., Richardson, J. S. and Appel, B. (2002). olig2 is required for zebrafish primary motor neuron and oligodendrocyte development. *Dev Biol* **248**, 356-68.

Park, H. C., Shin, J. and Appel, B. (2004). Spatial and temporal regulation of ventral spinal cord precursor specification by Hedgehog signaling. *Development* **131**, 5959-69.

Park, H. C., Shin, J., Roberts, R. K. and Appel, B. (2007). An olig2 reporter gene marks oligodendrocyte precursors in the postembryonic spinal cord of zebrafish. *Dev Dyn* **236**, 3402-7.

Park, M. and Moon, R. T. (2002). The planar cell-polarity gene stbm regulates cell behaviour and cell fate in vertebrate embryos. *Nat Cell Biol* **4**, 20-5.

Pauls, S., Geldmacher-Voss, B. and Campos-Ortega, J. A. (2001). A zebrafish histone variant H2A.F/Z and a transgenic H2A.F/Z:GFP fusion protein for in vivo studies of embryonic development. *Dev Genes Evol* **211**, 603-10.

Pellettieri, J. and Seydoux, G. (2002). Anterior-Posterior Polarity in C. elegans and Drosophila--PARallels and Differences. *Science* **298**, 1946-1950.

Petronczki, M. and Knoblich, J. A. (2001). DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in Drosophila. *Nat Cell Biol* **3**, 43-9.

Placzek, M., Yamada, T., Tessier-Lavigne, M., Jessell, T. and Dodd, J. (1991). Control of dorsoventral pattern in vertebrate neural development: induction and polarizing properties of the floor plate. *Development* **Suppl 2**, 105-22.

Plant, P. J., Fawcett, J. P., Lin, D. C., Holdorf, A. D., Binns, K., Kulkarni, S. and Pawson, T. (2003). A polarity complex of mPar-6 and atypical PKC binds, phosphorylates and regulates mammalian Lgl. *Nat Cell Biol* **5**, 301-8.

Quigley, I. K. and Parichy, D. M. (2002). Pigment pattern formation in zebrafish: a model for developmental genetics and the evolution of form. *Microsc Res Tech* **58**, 442-55.

Raymond, P. A. and Easter, S. S., Jr. (1983). Postembryonic growth of the optic tectum in goldfish. I. Location of germinal cells and numbers of neurons produced. *J Neurosci* **3**, 1077-91.

Rolls, M. M., Albertson, R., Shih, H. P., Lee, C. Y. and Doe, C. Q. (2003). Drosophila aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. *J Cell Biol* **163**, 1089-98.

Rolls, M. M. and Doe, C. Q. (2004). Baz, Par-6 and aPKC are not required for axon or dendrite specification in Drosophila. *Nat Neurosci* **7**, 1293-5.

Rowitch, D. H. (2004). Glial specification in the vertebrate neural tube. *Nat Rev Neurosci* **5**, 409-419.

Sadler, P. L. and Shakes, D. C. (2000). Anucleate Caenorhabditis elegans sperm can crawl, fertilize oocytes and direct anterior-posterior polarization of the 1-cell embryo. *Development* **127**, 355-66.

Sanada, K. and Tsai, L. H. (2005). G protein betagamma subunits and AGS3 control spindle orientation and asymmetric cell fate of cerebral cortical progenitors. *Cell* **122**, 119-31.

Sauer, F. C. (1936). The interkinetic migration of embryonic epithelial nuclei. *Journal of Morphology* **60**, 1-11.

Schaefer, M., Shevchenko, A. and Knoblich, J. A. (2000). A protein complex containing Inscuteable and the Galpha-binding protein Pins orients asymmetric cell divisions in Drosophila. *Curr Biol* **10**, 353-62.

Schmitz, B., Papan, C. and Campos-Ortega, J. A. (1993). Neurulation in the anterior trunk region of the zebrafish Brachydanio rerio. *Development Genes and Evolution* **202**, 250-259.

Schober, M., Schaefer, M. and Knoblich, J. A. (1999). Bazooka recruits Inscuteable to orient asymmetric cell divisions in Drosophila neuroblasts. *Nature* **402**, 548-51.

Shin, J., Park, H. C., Topczewska, J. M., Mawdsley, D. J. and Appel, B. (2003). Neural cell fate analysis in zebrafish using olig2 BAC transgenics. *Methods Cell Sci* **25**, 7-14.

Shin, J., Poling, J., Park, H.-C. and Appel, B. (2007). Notch signaling regulates neural precursor allocation and binary neuronal fate decisions in zebrafish. *Development* **134**, 1911-1920.

Simons, M. and Trotter, J. (2007). Wrapping it up: the cell biology of myelination. *Current Opinion in Neurobiology* **17**, 533-540.

Slack, J. M. W. (2008). Origin of Stem Cells in Organogenesis. *Science* **322**, 1498-1501.

Song, X., Zhu, C. H., Doan, C. and Xie, T. (2002). Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. *Science* **296**, 1855-7.

Spana, E. P. and Doe, C. Q. (1995). The prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in Drosophila. *Development* **121**, 3187-3195.

Stainier, D. Y., Fouquet, B., Chen, J. N., Warren, K. S., Weinstein, B. M., Meiler, S. E., Mohideen, M. A., Neuhauss, S. C., Solnica-Krezel, L., Schier, A. F. et al. (1996). Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* **123**, 285-292.

Stevenson, J. A. and Yoon, M. G. (1981). Mitosis of radial glial cells in the optic tectum of adult goldfish. *J. Neurosci.* **1**, 862-875.

Streit, W. J. (2000). Microglial response to brain injury: a brief synopsis. *Toxicol Pathol* **28**, 28-30.

Streit, W. J., Graeber, M. B. and Kreutzberg, G. W. (1988). Functional plasticity of microglia: a review. *Glia* **1**, 301-7.

Sundholm-Peters, N. L., Yang, H. K., Goings, G. E., Walker, A. S. and Szele, F. G. (2004). Radial glia-like cells at the base of the lateral ventricles in adult mice. *J Neurocytol* **33**, 153-64.

Suzuki, A. and Ohno, S. (2006). The PAR-aPKC system: lessons in polarity. *J Cell Sci* **119**, 979-87.

Takebayashi, H., Nabeshima, Y., Yoshida, S., Chisaka, O., Ikenaka, K. and Nabeshima, Y. (2002). The basic helix-loop-helix factor olig2 is essential for the development of motoneuron and oligodendrocyte lineages. *Curr Biol* **12**, 1157-63.

Takebayashi, H., Yoshida, S., Sugimori, M., Kosako, H., Kominami, R., Nakafuku, M. and Nabeshima, Y. (2000). Dynamic expression of basic helix-loop-helix Olig family members: implication of Olig2 in neuron and oligodendrocyte differentiation and identification of a new member, Olig3. *Mech Dev* **99**, 143-8.

Taupin, P. and Gage, F. H. (2002). Adult neurogenesis and neural stem cells of the central nervous system in mammals. *J Neurosci Res* **69**, 745-9.

Temple, S. (2001). The development of neural stem cells. *Nature* **414**, 112-7.

Tortora, G. J. and Anagnostakos, N. P. (1990). Principles of anatomy and physiology. New York: Harper & Row.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L. Y. and Jan, Y. N. (1989). numb, a gene required in determination of cell fate during sensory organ formation in Drosophila embryos. *Cell* **58**, 349-360.

Ueno, N. and Greene, N. D. (2003). Planar cell polarity genes and neural tube closure. *Birth Defects Res C Embryo Today* **69**, 318-24.

Vaessin, H., Grell, E., Wolff, E., Bier, E., Jan, L. Y. and Jan, Y. N. (1991). prospero is expressed in neuronal precursors and encodes a nuclear protein that is involved in the control of axonal outgrowth in Drosophila. *Cell* **67**, 941-953.

van Straaten, H. W. and Hekking, J. W. (1991). Development of floor plate, neurons and axonal outgrowth pattern in the early spinal cord of the notochord-deficient chick embryo. *Anat Embryol (Berl)* **184**, 55-63.

van Straaten, H. W., Hekking, J. W., Thors, F., Wiertz-Hoessels, E. L. and Drukker, J. (1985). Induction of an additional floor plate in the neural tube. *Acta Morphol Neerl Scand* **23**, 91-7.

von Trotha, J. W., Campos-Ortega, J. A. and Reugels, A. M. (2006). Apical localization of ASIP/PAR-3:EGFP in zebrafish neuroepithelial cells involves the oligomerization domain CR1, the PDZ domains, and the C-terminal portion of the protein. *Dev Dyn* **235**, 967-77.

Walker, M. R., Patel, K. K. and Stappenbeck, T. S. (2009). The stem cell niche. *J Pathol* **217**, 169-80.

Walsh, C. and Cepko, C. L. (1993). Clonal dispersion in proliferative layers of developing cerebral cortex. *Nature* **362**, 632-5.

Watts, J. L., Etemad-Moghadam, B., Guo, S., Boyd, L., Draper, B. W., Mello, C. C., Priess, J. R. and Kemphues, K. J. (1996). par-6, a gene involved in the establishment of asymmetry in early C. elegans embryos, mediates the asymmetric localization of PAR-3. *Development* **122**, 3133-3140.

Webster, H. and Astrom, K. E. (2009). Gliogenesis: historical perspectives, 1839-1985. *Adv Anat Embryol Cell Biol* **202**, 1-109.

Wei, X., Cheng, Y., Luo, Y., Shi, X., Nelson, S. and Hyde, D. R. (2004). The zebrafish Pard3 ortholog is required for separation of the eye fields and retinal lamination. *Developmental Biology* **269**, 286-301.

Weiner, H. L. (2009). The challenge of multiple sclerosis: how do we cure a chronic heterogeneous disease? *Ann Neurol* **65**, 239-48.

Welchman, D. P., Mathies, L. D. and Ahringer, J. (2007). Similar requirements for CDC-42 and the PAR-3/PAR-6/PKC-3 complex in diverse cell types. *Dev Biol* **305**, 347-57.

Westerfield, M., McMurray, J. V. and Eisen, J. S. (1986). Identified motoneurons and their innervation of axial muscles in the zebrafish. *J. Neurosci.* **6**, 2267-2277.

Williams, B. P. and Price, J. (1995). Evidence for multiple precursor cell types in the embryonic rat cerebral cortex. *Neuron* **14**, 1181-8.

Williams, J. A., Barrios, A., Gatchalian, C., Rubin, L., Wilson, S. W. and Holder, N. (2000). Programmed Cell Death in Zebrafish Rohon Beard Neurons Is Influenced by TrkC1/NT-3 Signaling. *Developmental Biology* **226**, 220-230.

Wodarz, A. (2001). Cell polarity: no need to reinvent the wheel. *Curr Biol* **11**, R975-8.

Wodarz, A. (2002). Establishing cell polarity in development. *Nat Cell Biol* **4**, E39-44.

Wodarz, A. and Huttner, W. B. (2003). Asymmetric cell division during neurogenesis in Drosophila and vertebrates. *Mech Dev* **120**, 1297-309.

Wodarz, A., Ramrath, A., Grimm, A. and Knust, E. (2000). Drosophila atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J Cell Biol* **150**, 1361-74.

Wu, P. S., Egger, B. and Brand, A. H. (2008). Asymmetric stem cell division: lessons from Drosophila. *Semin Cell Dev Biol* **19**, 283-93.

Wu, X., Quondamatteo, F., Lefever, T., Czuchra, A., Meyer, H., Chrostek, A., Paus, R., Langbein, L. and Brakebusch, C. (2006). Cdc42 controls progenitor cell differentiation and beta-catenin turnover in skin. *Genes Dev* **20**, 571-85.

Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T. M. (1991). Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. *Cell* **64**, 635-47.

Yamaguchi, H., Wyckoff, J. and Condeelis, J. (2005). Cell migration in tumors. *Current Opinion in Cell Biology* **17**, 559-564.

Yamanaka, T., Horikoshi, Y., Sugiyama, Y., Ishiyama, C., Suzuki, A., Hirose, T., Iwamatsu, A., Shinohara, A. and Ohno, S. (2003). Mammalian Lgl forms a protein complex with PAR-6 and aPKC independently of PAR-3 to regulate epithelial cell polarity. *Curr Biol* **13**, 734-43.

Yamanaka, T., Horikoshi, Y., Suzuki, A., Sugiyama, Y., Kitamura, K., Maniwa, R., Nagai, Y., Yamashita, A., Hirose, T., Ishikawa, H. et al. (2001). PAR-6 regulates aPKC activity in a novel way and mediates cell-cell contact-induced formation of the epithelial junctional complex. *Genes Cells* **6**, 721-31.

Yang, L. and Zheng, Y. (2007). Cdc42: a signal coordinator in hematopoietic stem cell maintenance. *Cell Cycle* **6**, 1445-50.

Yang, X., Tomita, T., Wines-Samuelson, M., Beglopoulos, V., Tansey, M. G., Kopan, R. and Shen, J. (2006). Notch1 signaling influences v2 interneuron and motor neuron development in the spinal cord. *Dev Neurosci* **28**, 102-17.

Youson, J. H. (1988). First metamorphosis. San Diego: Academic Press.

Yu, F. and David, P. S. (2004). Analysis of the Roles of Pins and Heterotrimeric G Proteins in Asymmetric Division of Drosophila Neuroblasts. In *Methods in Enzymology*, vol. Volume 389, pp. 364-382: Academic Press.

Yu, F., Kuo, C. T. and Jan, Y. N. (2006). Drosophila neuroblast asymmetric cell division: recent advances and implications for stem cell biology. *Neuron* **51**, 13-20.

Yu, F., Morin, X., Cai, Y., Yang, X. and Chia, W. (2000). Analysis of partner of inscuteable, a novel player of Drosophila asymmetric divisions, reveals two distinct steps in inscuteable apical localization. *Cell* **100**, 399-409.

Yu, F., Ong, C. T., Chia, W. and Yang, X. (2002). Membrane Targeting and Asymmetric Localization of Drosophila Partner of Inscuteable Are Discrete Steps Controlled by Distinct Regions of the Protein. *Mol. Cell. Biol.* **22**, 4230-4240.

Zhou, Q. and Anderson, D. J. (2002). The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. *Cell* **109**, 61-73.

Zhou, Q., Wang, S. and Anderson, D. J. (2000). Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. *Neuron* **25**, 331-43.

Zupanc, G. K. (2008). Adult neurogenesis and neuronal regeneration in the brain of teleost fish. *J Physiol Paris* **102**, 357-73.