EXPRESSION AND FUNCTION OF THE BASIC HELIX-LOOP-HELIX TRANSCRIPTION FACTOR NEUROD IN THE TELEOST RETINA

by

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“The most beautiful experience we can have is the mysterious…the fundamental emotion which stands at the cradle of true art and true science.”

- Albert Einstein

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DEDICATION

To my parents, Teresa and Władysław Ochociński

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CHAPTER I

INTRODUCTION

The Vertebrate Retina

An ancient proverb states that the eyes are a window to the soul. A more modern application of this statement is that the retina is a window to the brain. As a readily accessible part of the central nervous system, the retina is an ideal model tissue to study neural development and function. Any perturbations to its architecture are readily apparent and relatively easily interpreted. In addition, both the development and structure of the vertebrate retina is highly conserved across species. The vertebrate retina is a light sensing nervous tissue, which lines the back of the eye and is responsible for processing visual input and sending it to the brain via the optic nerve. The retina is a highly ordered and evolutionarily conserved laminar structure composed of six neuronal cell types and one glial cell type (Fig.1). It is divided into three major layers; ganglion cells and displaced amacrine cells comprise the ganglion cell layer, the amacrine and horizontal cells are located in the inner nuclear layer, and cone and rod photoreceptors reside in the outer nuclear layer. Only one glial cell type spans the retina and is named the Müller glia. Light is detected by the photoreceptors and is transduced as electrical impulses through interneurons to the ganglion cells. The signal is then sent through the optic nerve to the visual cortex of the brain where it is processed and interpreted as visual perception.

Vertebrate Retina Development

Development of the eye and retina is also highly conserved among vertebrates. Cells of the optic primordia differentiate into the optic stalk, neural retina, the retinal pigment epithelium, the iris epithelium, and the ciliary epithelium. In turn, the optic primordium sends signals to the surface ectoderm and instructs parts of the ectoderm to differentiate into the cornea and lens. The remaining structures such as the choroid, sclera, and extra ocular muscles are derived from a combination of neural crest and head mesoderm (reviewed in Chow and Lang, 2001).

The retina develops from multipotent progenitor cells in the optic vesicle that express a combination of eye-field transcription factors, such as Rx, Pax6, Six3 and Chx10 (vsx2 in goldfish and zebrafish) (Macdonald et al., 1995; Rojaz-Munoz et al., 2005; Loosli et al., 1998; Loosli et al., 1999; Loosli et al., 2003; Nornes et al., 1998; Seo et al., 1998; Wargelius et al., 2003; Chuang et al., 1999; Chuang and Raymond, 2001; Chuang and Raymond 2002; Zuber et al., 2003). These multipotent progenitors undergo multiple divisions and generate all the neurons and glia in the retina (Holt et al., 1988; Wetts and Frazer, 1988; Wetts et al., 1989; Fekete et al., 1994). The retinal cells develop in a specific order which is largely conserved across species. In the mammalian and avian retina, neurons and glia differentiate sequentially in overlapping gradients (Cepko, 1996; Adler, 2000; Livesey and Cepko, 2001). The early stage of retinogenesis produces ganglion cells, cone photoreceptors, and horizontal cells, whereas amacrine, rod photoreceptors, bipolar cells, and Müller glia are produced during the late stage

of retinogenesis. Whilst the basic differentiation process is conserved in the teleost retina, the retinal neurons are formed in three waves of differentiation beginning with the ganglion cells, interneurons, and photoreceptors and Müller glia differentiating last (Hu and Easter, 1999; Easter, 2000; Li et al., 2000; Easter and Malicki, 2002). It is still unclear whether this developmental order is due to intrinsic or extrinsic factors. Intrinsic factors in retinal progenitors may change over time restricting their competence to generate particular cell types. Alternatively, extrinsic factors from the environment may restrict progenitor cells to specific fates. Currently, a model is emerging which incorporates both intrinsic and extrinsic factors in retinogenesis (Reh and Kljavin, 1989; Reh and Cagan, 1994; Rappaport and Dorsky, 1998; Livesey and Cepko, 2001; Moore et al., 2002; James et al., 2003; James et al., 2004; Van Raay and Vetter, 2004; Amato et al., 2004 a,b; Bernardos et al., 2005; Cayouette et al., 2006; Locker et al., 2006).

Development of the Zebrafish Retina

The zebrafish retina develops in three distinct stages. The first stage is characterized by the specification of the eye fields and early eye morphogenesis. The neural epithelium, which will become the retina, is specified by the expression of transcription factors, including zic1, Six3a, Pax6, and rx1-3 (Macdonald et al., 1995; Rojaz-Munoz et al., 2005; Loosli et al., 1998; Loosli et al., 1999; Loosli et al., 2003; Nornes et al., 1998; Seo et al., 1998; Wargelius et al., 2003; Chuang et al., 1999; Chuang and Raymond, 2001; Chuang and Raymond 2002). This early eye field forms at the end of gastrulation, and by 12

hours post fertilization (hpf) it is split into two optic primordia by Nodal and Hedgehog signals secreted from the ventral midline (Masai et al., 2000; Stenkamp et al., 2002; Stenkamp and Frey, 2003; Stadler et al., 2004). Precursor cells of the ventral diencephalon displace the optic primordia, which extend laterally toward the ectoderm, and by 13hpf, the posterior parts of the optic primordia separate from the neural keel. The optic cup is formed by 24hpf as the primordia invaginate and the anterior portion of the neural keel rotates ventrally (Schmitt and Dowling, 1994; Schmitt and Dowling, 1999; Li et al., 2000; reviewed by Hitchcock and Raymond, 2004). As the zebrafish brain rotates more ventrally, the eye rotates concomitantly, and the choroid fissure, originally in the posterior-ventral corner and now located at the ventral optic cup, demarcates the nasal and temporal regions (Schmitt and Dowling, 1994; Li et al., 2000; Hitchcock and Raymond, 2004).

The second stage of retinal development includes cellular differentiation and the formation of retinal laminae. Cellular differentiation begins in a region adjacent to the optic stalk and spreads as a circumferential wave across the retina from ventronasal to dorsal to ventrotemporal. Ventronasal retina is the location of a small patch of retina that in teleosts differentiates precociously (Kljavin, 1987; Schmitt and Dowling, 1994; Burrill and Easter, 1995; Raymond et al., 1995; Schmitt and Dowling, 1999; Hu and Easter, 1999). Ganglion cells are the first cells to differentiate, and the ganglion cell layer forms between 28hpf and 38hpf (Burrill and Easter, 1995; Hu and Easter, 1999). Cells in the inner nuclear layer differentiate between 38hpf and 48hpf. By 48hpf, all the retinal laminae can

be distinguished, although lamination in the dorsal-most retina is markedly less advanced and is not complete until ~76hpf (Schmitt and Dowling, 1999; Li et al., 2000). Cells in the outer nuclear layer are the last to differentiate, between 48hpf and 96hpf, but do so without the neurogenic wave characteristic of the two inner layers (Hu and Easter, 1999; Li et al., 2000). Cone photoreceptors differentiate first and in order of their spectral type, beginning with red/green cones, UV, and blue cones (Raymond and Barthel, 2004). Excluding the ventral patch (Kljavin, 1987; Easter and Malicki, 2002; also see above), rod photoreceptors are the last cell type to differentiate. The genesis and differentiation of retinal cells in distinct layers over time is controlled by several mechanisms that establish the timing of withdrawal from the cell cycle, cell polarity, migration, adhesion, and cell fate (Malicki and Driever, 1999; Link et al., 2000; Neumann and Nuesslein-Volhard, 2000; Pujic and Malicki, 2001; Stadler et al., 2004; Shkumatava et al., 2004; Shkumatava et al., 2005; Jensen and Westerfield, 2004; Kay et al., 2005; Gross et al., 2005; Omori and Malicki, 2006; Baye and Link, 2007a,b).

The third stage begins once the initial cellular differentiation and lamination is complete. The retina then grows throughout the life of the animal through a combination of retinal stretch and persistent neurogenesis. The persistent neurogenesis is a feature the retina of the zebrafish shares with amphibians, birds, and other fish species (see next section).

Persistent Neurogenesis in the Retinas of Amphibians, Birds, and Fish

In amphibians, birds, and fish, after the initial process of retinogenesis is complete, a remnant of retinal stem cells persists at the retinal margin in a region

called the ciliary marginal zone (CMZ). New neurons and glia are added to the periphery throughout the lifetime of these animals (Fig.2; Hitchcock et al., 1996; Belecky-Adams et al., 1997; Levine et al., 1997; Passini et al., 1997; Sullivan et al., 1997; Huang and Sato, 1998; Perron et al., 1998; Ohnuma et al., 2002; Wargelius et al., 2003; Harris and Perron, 1998; Perron and Harris, 2000; Kubota et al., 2002; Amato et al., 2004a; Wehman et al., 2005; Raymond et al., 2006). The first evidence of persistent neurogenesis from the ciliary margin of the vertebrate retina was demonstrated in post-embryonic amphibians (Hollyfield, 1968; Straznicky and Gaze, 1971). H^3-thymidine was used to mark mitotically active cells, and this revealed labeled cells at the retinal margin, which were incorporated into more central retina over time, suggesting that newly generated retinal cells originate from the retinal margin.

Only recently was it demonstrated that post-hatch birds have a ciliary marginal zone in some ways similar to that of amphibians and fish. The first evidence, though not generally accepted, of a proliferative zone at the retinal margin of the post-hatch chick retina came from a study using H^3-thymidine to label mitotically-active cells in the post-hatch bird retina (Morris et al., 1976). Subsequent studies using BrdU to label mitotically active cells in the retina of hatched chicks and adult birds confirmed and extended this original observation (Fischer and Reh, 2000; Kubota et al., 2002). The BrdU-positive cells, however, were found mostly in the inner nuclear layer, the location of amacrine, bipolar, and glial cells, suggesting that the progenitor cells in the avian CMZ are more

restricted in their lineage and do not have the full multipotential characteristics of the cells in the amphibian CMZ.

As in amphibians and birds, a similar germinal zone has been identified in the fish retina (Fig.3). The first demonstration of persistent neurogenesis in the fish retina came from cell counts, comparing the retinas of young and old fish. The greater number of retinal cells in old fish was attributed to the addition of cells from the ciliary marginal zone, which contained numerous mitotic figures and cells with a neuroepithelial morphology (Müller, 1952; Lyall, 1957a, b). Direct evidence of a proliferating marginal zone came from H^3-thymidine and BrdU labeling experiments (Johns, 1977; Meyer, 1978; Hagedorn and Fernald, 1992; Julian et al., 1998; Marcus et al., 1999; Olson et al., 1999). The CMZ in the fish retina generates all cell types except for rod photoreceptors, which are produced from another source of stem cells in the central retina (see next section).

The CMZ is equivalent to the early retinal neuroepithelium, and this is revealed by the evolutionary conservation of developmental genes expressed in the retinal progenitors in the CMZ (Hitchcock et al., 1996; Belecky-Adams et al., 1997; Levine et al., 1997; Passini et al., 1997; Sullivan et al., 1997; Huang and Sato, 1998; Perron et al., 1998; Ohnuma et al., 2002; Wargelius et al., 2003; Raymond et al., 2006). The CMZ is composed of distinct progenitor populations, and based on patterns of gene expression the CMZ can be subdivided into at least four zones, with the most peripheral CMZ cells expressing genes found earliest in retinal development and cells located more centrally expressing genes found in later stages of development (Perron et al., 1998; Casarosa et al., 2005;

Raymond et al., 2006). The first zone includes the least determined, putative stem cells, which are located closest to the iris and express several stem cell markers, including Six3, Pax6, and rx1. These stem cells give rise to the second zone of pluripotent retinoblasts, which continue to express the early genes and begin to express markers of later stage progenitors, including Notch, Delta, Ash1, and Ash3. The pluripotent retinoblasts in turn produce more restricted retinoblasts located in the third zone that express all the previous genes and begin to express Ath3, Ath5, Otx2, and NeuroD. These retinoblasts generate daughter cells in the fourth zone that express all of the previous genes, with the exception of Ash3, but these cells are no longer dividing and begin to differentiate as they are incorporated into the central retina. The gene expression pattern of the progenitor populations in the CMZ suggests that retinal stem cells and their progeny continuously recapitulate in space the embryonic generation of the retina over developmental time.

Several soluble growth factors have been identified that effect the proliferation of the postnatal retinal progenitor cells in the CMZ. In the post-hatch bird, insulin, insulin-like growth factor-I, and epidermal growth factor enhance proliferation in the CMZ (Reh and Levine, 1998; Fischer and Reh, 2000). Wnt signaling also plays a role in regulating the proliferation of progenitor cells in the CMZ of the chick (Kubo et al., 2003, Kubo et al., 2005; Cho and Cepko, 2006). Furthermore, administering exogenous growth factors extends the neurogenic potential of the CMZ in the bird, suggesting that the limited potential of these cells (see above) is due to extrinsic factors and not the intrinsic qualities of the

stem cells or their progeny (Fischer et al., 2002). In fish, retinal growth is regulated by the growth hormone - insulin-like growth factor-1 axis (GH/IGF-I axis; Hitchcock et al., 2001; Otteson et al., 2002). Changes in growth hormone levels have significant effect on body size, including the eye (Duan, 1998), and components of the GH/IGF-I axis have been identified in the retina (Otteson et al., 2002). Exogenous IGF-I promotes the proliferation of cells in the CMZ and rod precursors (Mack and Fernald, 1993; Boucher and Hitchcock, 1998). Similar results were observed following intraperitoneal injection of recombinant GH (Otteson et al., 2002).

Persistent Neurogenesis in Central Retina: The Rod Photoreceptor Lineage

In addition to the CMZ, a component of retinal growth in fish is retinal stretch (Lyall, 1957a, b; Johns, 1977; Johns and Easter, 1977; Johns, 1982; Powers et al., 1988), which results in a decrease in the density of most retinal cells, except for rod photoreceptors whose density increases slightly with the expanding retina (Powers et al., 1988). To compensate for the decrease in density and to maintain visual sensitivity, rod photoreceptors are insinuated into the existing central retina from a second population of stem cells residing in the inner nuclear layer of the differentiated retina (Meyer, 1978; Johns and Fernald, 1981; Johns, 1982; Hagedorn and Fernald, 1992).

The continual addition of rods as the retina expands was inferred from earlier studies where an increase in rod photoreceptors was observed (Lyall et al., 1957b). The origin of these rod photoreceptors was determined in subsequent studies that identified a second population of dividing cells in the

outer nuclear layer, which specifically generate rod photoreceptors (Johns and Fernald, 1981; Johns, 1982; Hagedorn and Fernald, 1992). The mitotically active cells in the outer nuclear layer were termed rod precursors, and rod progenitors were subsequently identified in the inner nuclear layer of the differentiated retina to give rise to these rod precursors in the outer nuclear layer (Raymond and Rivlin, 1987). Thus, mitotically active cells in the inner nuclear layer, rod progenitors, migrate in radial columns along Müller glia processes from the inner nuclear layer to the outer nuclear layer, where they are called rod precursors and give rise to rod photoreceptors (Raymond and Rivlin, 1987; Mack and Fernald, 1997; Hoke and Fernald, 1997). This process of rod genesis occurs throughout the retina in juvenile and adult fish (Julian et al., 1998; Otteson et al., 2001)

Collectively, studies have led to a consensus model of rod photoreceptor genesis and identification of a rod photoreceptor lineage (Fig.3). Stationary stem cells in the inner nuclear layer, which express the early developmental gene Pax6 (Otteson et al., 2001), give rise to migrating rod progenitors. These rod progenitors and rod precursors do not express Pax6, but instead express the basic helix-loop-helix (bHLH) transcription factor NeuroD (Hitchcock and Kakuk-Atkins, 2004; Hitchcock et al., 2004), which is expressed downstream of Pax6 during retinal development (Perron et al., 1998) and implicated in rod photoreceptor genesis in the retina (Morrow et al., 1999). Rod progenitors migrate from the inner nuclear layer to the outer nuclear layer where they divide and differentiate into rod photoreceptors (Johns and Fernald, 1981; Raymond and Rivlin, 1987; Hoke and Fernald, 1997; Julian et al., 1998; Otteson et al.,

2001; Otteson and Hitchcock, 2003; Hitchcock and Kakuk-Atkins, 2004; Ochocinska and Hitchcock, 2007). Recent evidence suggests that, in contrast to the earlier lineage model (Otteson et al., 2001), Müller glia are the stem cells of the rod lineage (Raymond et al., 2006; Bernardos et al., 2007; see also Vihtelic and Hyde, 2000; Wu et al., 2001; Yurco and Cameron, 2005; Fausett et al., 2006).

Basic Helix-Loop-Helix Transcription Factors

Cell fate determination and differentiation are dictated by cascades of basic helix-loop-helix (bHLH) genes (Gowan et al., 2001; Vetter and Brown, 2001; Hatakeyama et al., 2001; Akagi et al., 2004; Van Raay and Vetter, 2004; Yan et al., 2005; Wang and Harris, 2005; Hevner et al., 2006; Sugimori et al., 2007). bHLH transcription factors belong to a class of proneural regulatory proteins that act as a molecular link connecting withdrawal from the cell cycle, cell fate determination, and differentiation (Bertrand et al., 2002; Chae et al., 2004; Yan et al., 2005; Sugimori et al., 2007). Studies in invertebrate and vertebrate models have shown that a relatively small number of proneural genes encode the bHLH class of transcription factors (Fig.4). The structural similarity of bHLH proneural genes suggests that they may have conserved function and biochemical properties. Determining the mechanisms by which these proneural regulators function is important to understanding the process of neurogenesis.

bHLH transcription factors possess several common properties: First, each bHLH factor shares a common structural motif composed of a basic region and two alpha helices separated by a loop (Bertrand et al., 2000). The basic

domain functions in DNA-binding, whereas the two alpha helices mediate dimerization (Fig.5). bHLH genes can be grouped into distinct families based on family-specific residues in the bHLH domain (Fig.6). Second, all known bHLH genes bind to an E-box element of their target gene consisting of the common hexamer CANNTG. Third, some bHLH transcription factors function as transcriptional activators, with the exception of Olig2 (Novitch et al., 2001), and heterodimerize with E proteins, E12 and E47. To activate transcription bHLH proteins interact with coactivators, CREB-binding protein (CBP) and p300, which facilitate gene transcription by serving as mediators between the DNA-binding site of bHLH transcription factors and the RNA polymerase II transcriptional machinery (Massari and Murre, 2000). Fourth, all bHLH transcription factors are inhibited by common repressor genes. Since the process of dimerization is required for DNA binding, factors which inhibit this dimerization process act as repressors of proneural genes. HLH proteins which lack the basic DNA binding domain, such as the Id protein family, act as inhibitors of differentiation by competing with bHLH proneural proteins for E protein binding (Massari and Murre, 2000; Yokota, 2001). The Hes family is another group of proneural gene inhibitors (Van Doren et al., 1994; Fisher and Caudy, 1998; Davis and Turner, 2001, Kageyama et al., 2005), which repress transcription by recruiting the Groucho/TLE/Grg corepressor to the bHLH promoter. In addition, Hes proteins sequester E proteins and prevent the heterodimerization step of bHLH proteins (Davis and Turner, 2001).

The proneural function of bHLH transcription factors involves several mechanisms. First, bHLH proteins use the Notch signaling pathway to restrict proneural activity to single progenitor cells. Notch is not activated in a differentiating neuron and RBP-J represses Hes1 and Hes5 expression (Kageyama et al., 2005): In the absence of inhibitors, neurogenic bHLH activators induce the expression of Hes6 and inhibit Hes1 function thereby reinforcing the neurogenic process through a positive feedback loop.

Second, bHLH proneural genes may directly regulate the cell cycle. Neuronal differentiation of bHLH transfected cells is preceded by elevated expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and cell cycle withdrawal (Farah et al., 2000). Misexpression of Ngn2 in the neural tube leads to premature cell cycle exit and neuronal differentiation in neuroepithelial cells (Novitch et al., 2001). The bHLH proteins can therefore link neuronal differentiation to withdrawal from the cell cycle, possibly by activating the expression of cyclin-dependent kinase inhibitors, such as $p27^{Kip1}$ (Farah et al., 2000).

Third, gain and loss-of-function studies have shown that bHLH proteins are involved in the development of specific neuronal lineages and inhibition of gliogenesis in the CNS. Several proneural genes have been implicated in commitment of multipotent progenitors to a neuronal lineage and inhibition of glial cell fate. bHLH transcription factors from both the Achaete-Scute family and the Neurogenin family promote the neuronal versus glial cell fate decision in the CNS (Sun et al., 2001; Morrow et al., 1999; Tomita et al., 2000).

In the developing retina, proneural bHLH genes have distinct expression patterns and serve as important regulators of retinal neurogenesis. bHLH transcription factors play an important role in the generation of all cell types in the retina. A bHLH transcription factor cascade is involved in regulating retinal cell differentiation and the function of these transcription factors appears to be conserved in all vertebrates. Retinal ganglion cells are specified by the bHLH factor Ath5, the vertebrate orthologue of the Drosophila atonal gene. Specifically, Ath5 is required for generating ganglion cells from multipotent progenitors, cell cycle progression, and ganglion cell differentiation (Kanekar et al., 1997, Brown et al., 2001; Kay et al., 2001; Wang et al., 2001; Le et al., 2006). In the inner nuclear layer, horizontal and amacrine cell fates are determined by the bHLH factor Ptf1a (Fujitani et al., 2006; Nakhai et al., 2007). Amacrine and cone bipolar subtypes are specified by the bHLH factor Bhlhb5 in mouse retina (Bramblett et al., 2004; Feng et al., 2006). In addition to its function downstream of retinogenic factors to specify bipolar and amacrine subtypes, Bhlhb5 is also required for rod bipolar cell maturation (Bramblett et al., 2004). The bHLH genes Mash1 and Math3 and the homeobox gene Chx10 are also essential for generation of bipolar cells (Hatakeyama, 2001).

In addition to neurons, the retina also contains one glial cell type, which is also regulated by bHLH transcription factors. The bHLH factor cNSCL2 has been shown to regulate Müller glia and misexpression of cNSCL2 results in atrophy of Müller glia and death of photoreceptor cells in chick retina (Li et al., 2001). In addition, Hes5 is specifically expressed by differentiating Müller glial cells and

misexpression of Hes5 with recombinant retrovirus significantly increases the population of glial cells at the expense of neurons. Hes5-deficient retina show a significant decrease of Müller glial cell number without affecting cell survival. These results indicate that Hes5 modulates glial cell fate specification in the retina consistent with the role of Hes genes in inhibiting proneural genes (Hojo et al., 2000).

NeuroD

NeuroD is a relative of the Drosophila *atonal* gene and a member of the family of basic helix-loop-helix transcription factors. The properties of NeuroD were first demonstrated in Xenopus embryos, where ectopic expression converts epithelial cells into neurons (Lee et al., 1995), and, *in vitro*, where transfection of P19 cells induces cell cycle withdrawal and the expression of neuronal proteins (Farah et al., 2000; see also Cho et al., 2001). In the brain and non-nervous tissues, NeuroD has specific functions based on the mitotic state of the cell. In mitotically active cells, NeuroD regulates proliferation (Miyata et al., 1999; Nibu et al., 2001; Manglapus et al., 2004; Schonhoff et al., 2004; Lawoko-Kerali et al., 2004) and exit from the cell cycle (Mutoh et al., 1998), and its absence results in proliferation defects. In postmitotic cells, NeuroD is required for survival and its absence results in cell death during differentiation (Miyata et al., 1999; Schwab et al., 2000; Lee et al., 2000; Cai et al., 2000; Liu et al., 2000a,b; Kim et al., 2001; Nibu et al., 2001). NeuroD retains these functions in persistently mitotic tissues in adult animals (Schonhoff et al., 2004; Naya et al., 1997; Mutoh et al., 1998; Kim et al., 2001; Bedard and Parent, 2004). It appears that NeuroD function can be

divided into common categories that involve cell cycle regulation, cell fate specification, and cell survival. More importantly, NeuroD may serve as the common link that connects all these processes.

The goal of my thesis research is to characterize the expression and function of NeuroD in the photoreceptor lineages of the developing zebrafish retina and to utilize genetic strategies to test the hypothesis that NeuroD plays a fundamental role in photoreceptor genesis. Chapter 2 describes the temporal and spatial pattern of *neuroD* expression during development of the retina. Chapter 3 characterizes the function of NeuroD in the retina *in vivo* using a gain-of-function approach. Chapter 4 investigates *in vivo* effects of the loss of NeuroD function. Together, these studies show the dynamic expression of *neuroD* in rod and cone photoreceptor lineages, and suggest its fundamental role in photoreceptor genesis and cone maturation. Conditional gain- and loss-of function experiments reveal that NeuroD functions as a regulator of mitotic activity and promotes cell cycle exit and photoreceptor differentiation.

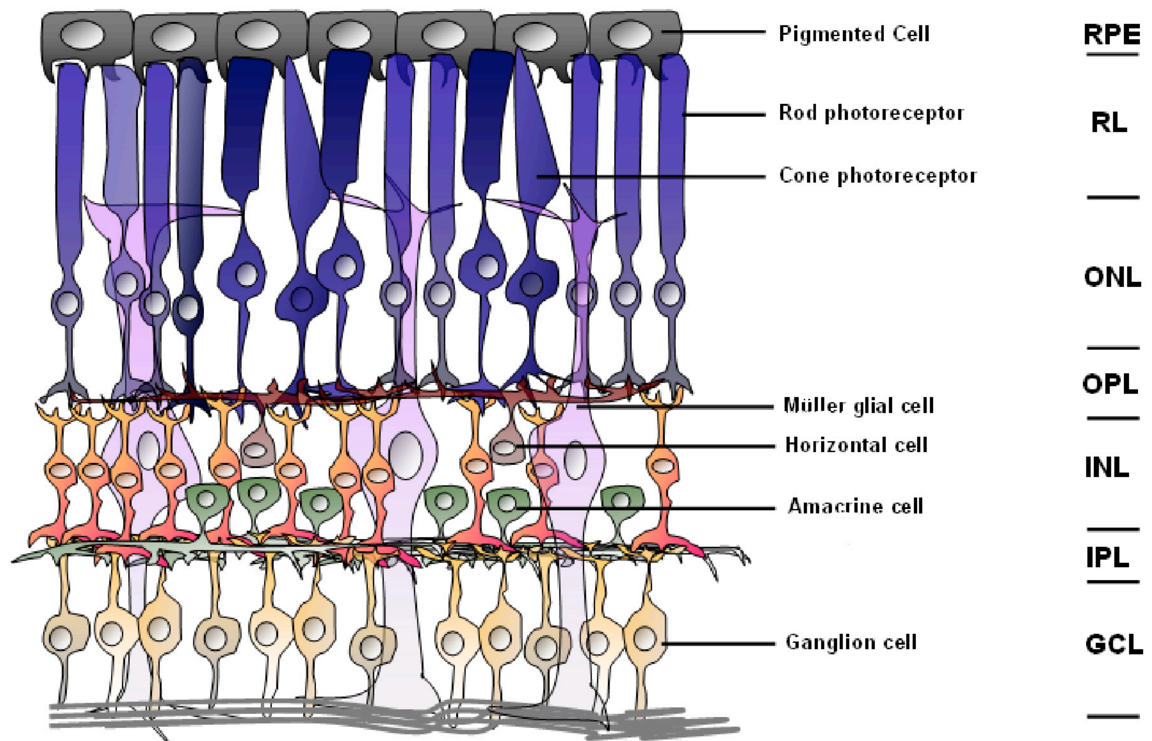

Fig. I-1: The vertebrate retina.

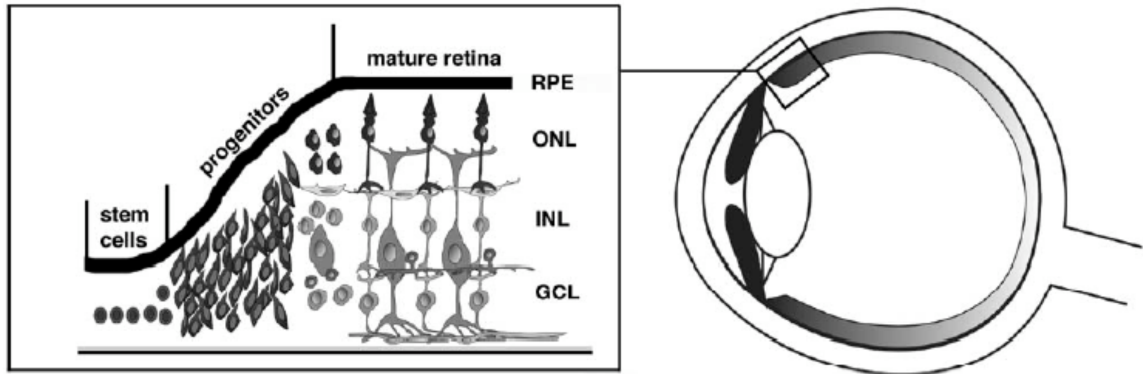

Fig. I-2: Diagram of the circumferential germinal zone in the retina of amphibians, birds, and teleosts.

Amphibian, teleost, and bird eyes continue to grow throughout life and new neurons are generated in concentric rings at the retinal margin to compensate for the expanding eye. This growth is coordinated with the continuous addition of new neurons at the retinal margin in a region called the ciliary marginal zone (CMZ). This is the location of stem cells which generate new annuli of neurons throughout the lifespan of the animal. GCL–ganglion cell layer, INL-inner nuclear layer, ONL-outer nuclear layer, RPE-retinal pigmented epithelium.

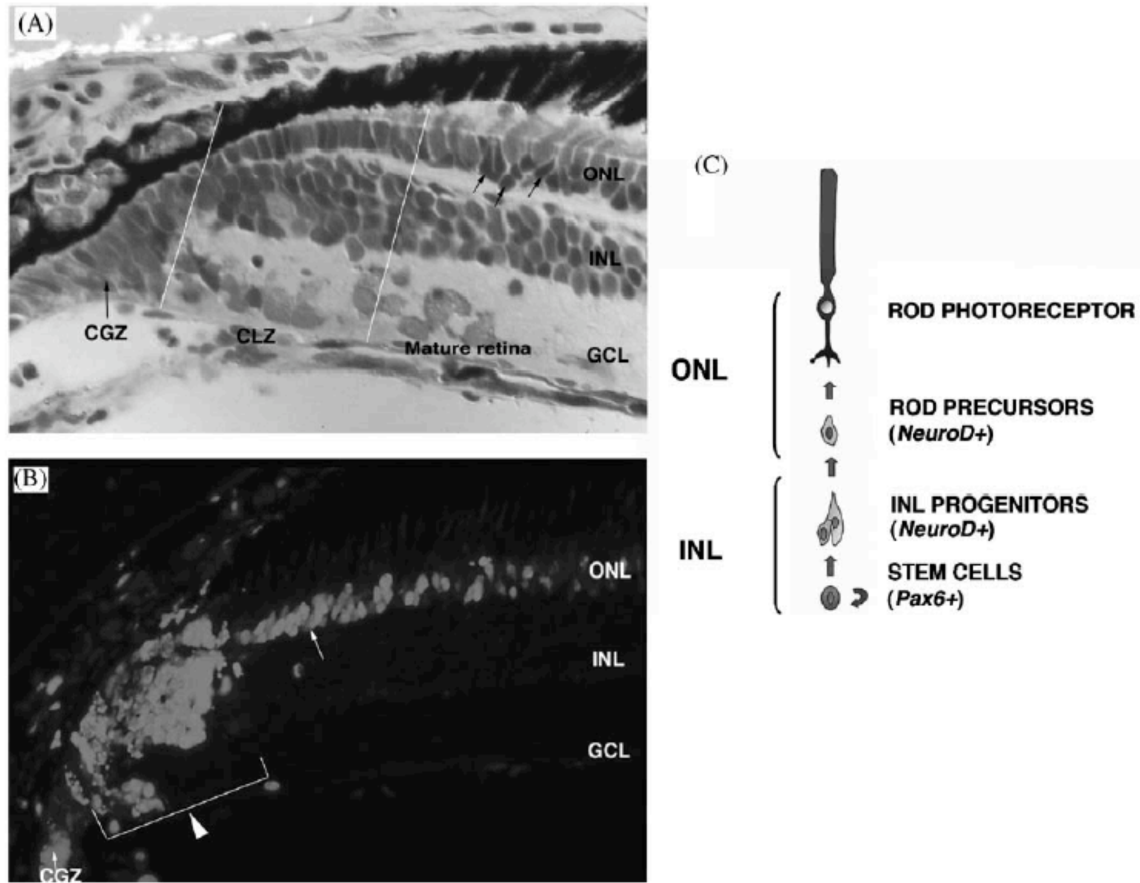

Fig. I-3: Persistent neurogenesis in the teleost retina.

A: The retinal margin in a juvenile goldfish. Note the columnar epithelial cells that form the CGZ. The white lines bracket the CLZ, which lacks rod photoreceptors in the ONL Central to this is the mature retina, which contains both cones and rods (arrows) in the ONL. B: The retinal margin from a fish exposed to BrdU and allowed to survive for 30 days. The white bracket marks BrdU-labeled neurons that were labeled with BrdU and born during the survival time. The arrow in the ONL identifies rod photoreceptors that were also generated during this time. C: A schematic of a model of the rod-photoreceptor lineage in the fish retina, GCL-ganglion cell layer, INL-inner nuclear layer, ONL-outer nuclear layer, CGZ-circumferential germinal zone, CLZ-circumferential larval zone.

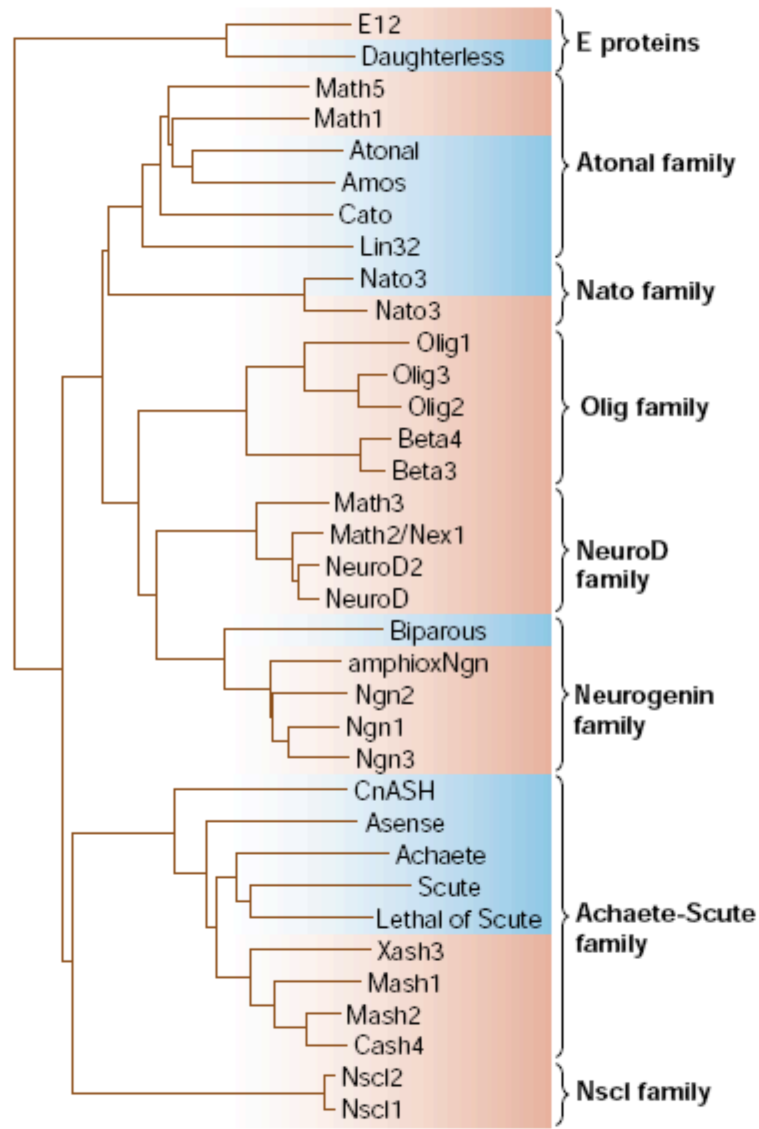

Fig. I-4: The basic helix-loop-helix family.

A dendrogram of basic helix-loop-helix (bHLH) sequences from invertebrates (blue) and vertebrates (red). The bHLH domain sequences have been grouped based on sequence homology.

Reproduced from Bertrand et al., 2002.

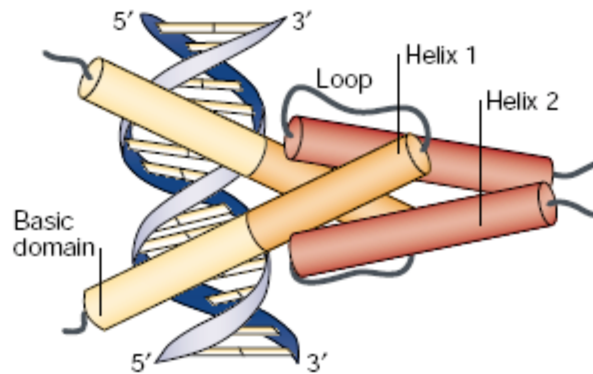

Fig. I-5: Structure of the basic helix-loop-helix proteins.

All bHLH factors share a common structural motif composed of a basic region and two alpha helices separated by a loop. The basic domain functions in DNA-binding while the two alpha helices mediate dimerization. Reproduced from Bertrand et al., 2002.

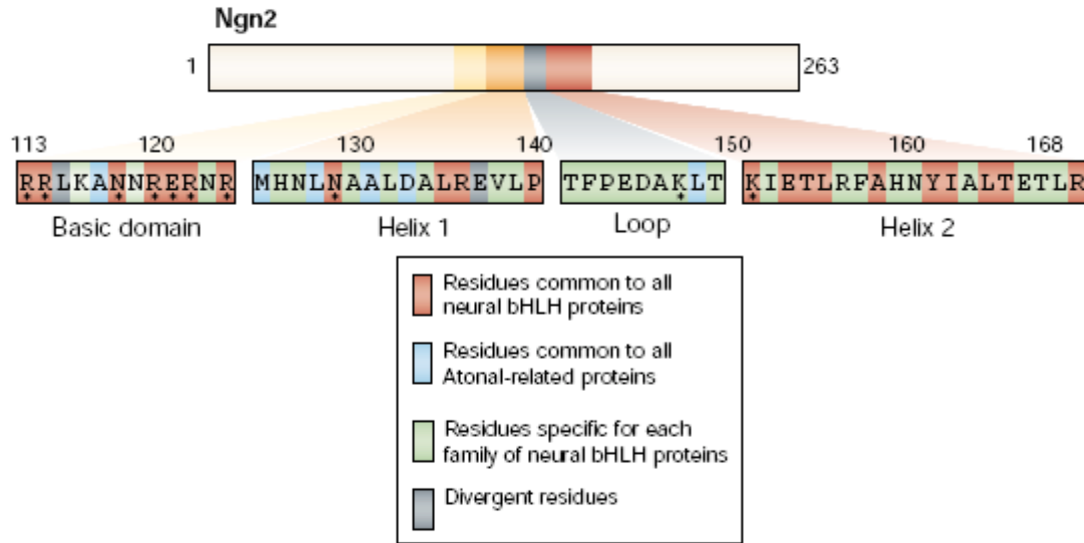

Fig.I-6: Sequence conservation of basic helix-loop-helix proteins.

bHLH genes can be grouped into distinct families based on family-specific residues in the bHLH domain. Reproduced from Bertrand et al., 2002.

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CHAPTER II

NEUROD EXPRESSION PATTERN

This chapter describes the cellular pattern of *neuroD* expression during embryonic and larval retinal development of the zebrafish.

Introduction

Several studies have shown NeuroD to be instrumental in neuronal development and cell cycle regulation (Lee et al., 1995; Farah et al., 2000; Cho et al., 2001; Chae et al., 2004). NeuroD may play similar, conserved roles in the developing retina, but the cellular details appear to be species-specific. In the mouse retina, *neuroD* is expressed in multipotent retinal progenitors, regulates neuronal versus glial cell fate and is determinative for amacrine cells (Morrow et al., 1999; Ahmad et al., 1998; Acharya et al., 1997; Moore et al., 2002; Inoue et al., 2002; Pennesi et al., 2003). Further, *neuroD*-null mice show age-related degeneration of rod photoreceptors (Pennesi et al., 2003), indicating that, in mammals, NeuroD regulates the survival of a subset of postmitotic retinal cells. In the chick, *neuroD* is also expressed in multipotent progenitors, but is determinative for cone and rod photoreceptors only (Yan and Wang, 1998; Yan and Wang, 2000; Yan and Wang, 2004; see also Fischer et al., 2004). In adult goldfish *neuroD* is not expressed in multipotent progenitors, but is expressed in mitotic cells that give rise exclusively to rod photoreceptors. In addition, *neuroD*

is transiently expressed in newly postmitotic cone photoreceptors (Hitchcock and Kakuk-Atkins, 2004). Rod photoreceptor genesis has been previously characterized and a consensus model of the rod photoreceptor lineage has been described (Raymond and Rivlin, 1987; Mack and Fernald, 1997; Julian et al., 1998; Otteson et al., 2001). In the central retina, stem cells in the inner nuclear layer give rise to rod progenitor cells which move from the inner nuclear layer to the outer nuclear where they are called rod precursor cells. These cells divide a few times and give rise to rod photoreceptors. Recent findings revealed that both rod progenitors and rod precursors express *neuroD*, which suggests that in teleosts NeuroD might play a specific role in generating this one cell type (Hitchcock and Kakuk-Atkins, 2004). Thus, in homeothermic vertebrates, such as the mouse and chick, NeuroD appears to play a role in cell fate determination, whereas in the adult teleost retina it may play a more restricted role in generating rod photoreceptors. Whether early photoreceptor genesis in teleosts fits the avian and mammalian models remains to be determined.

The zebrafish has recently become a prominent system in developmental biology, amenable to approaches that investigate gene regulation and function. Furthermore, zebrafish retinal development has been extensively studied, and *neuroD* is expressed in the retina (Korzh et al., 1998; Masai et al., 2000; Mueller and Wullimann, 2002). The function of NeuroD in the teleost retina has not yet been experimentally investigated, and the spatial and temporal expression pattern of this gene during embryonic and larval development of the retina is not yet known.

Materials and Methods

Wild-type zebrafish were used to generate embryos. Breeders were maintained on a 14-h light/10h-dark daily cycle, and embryos were collected following light onset. Embryos were maintained in embryo rearing solution (ERS; Westerfield, 2000) for the first 12 hours and then placed into ERS containing 0.2 mM 1-phenyl-2-thiourea (PTU; Sigma, St. Louis, MO) to prevent melanin pigmentation. Embryos were raised at 28.5°C, staged in hours post fertilization (hpf) according to Westerfield (2000), and analyzed between 25hpf and 96hpf. Protocols for animal husbandry and sacrifice were approved by the University Committee for Use and Care of Animals (UCUCA) at the University of Michigan and conform to NIH guidelines.

Histology

Embryos were dechorionated with watchmakers' forceps, if necessary, and fixed in 4% (w/v) paraformaldehyde for 1 hour at room temperature. Different protocols were followed, for specimens processed as whole-mounts or histological sections. For whole-mount *in situ* hybridization, fixation was followed by two 5-minute washes in 0.1M phosphate buffered saline (PBS; pH 7.2) and two 5-minute washes in 100% methanol. The embryos were then placed in a 100% methanol solution and stored at -20ºC for at least 30-min before proceeding with the *in situ* hybridization protocol. For sectioned animals, fixation was followed by infiltration in 20% sucrose in PBS overnight. The next day, animals were washed in 2:1 (20% sucrose: OCT medium) for 30 minutes and frozen in Tissue-Tek®

Optimal Cutting Temperature (O.C.T.) medium (Sakura Finetek U.S.A., Inc., Torrance, CA). Sections were cut in the frontal plane on a cryostat at 5μm.

In Situ **Hybridization**

In situ hybridization on whole embryos was performed according to Westerfield (2000) in 1.5ml eppendorf tubes. Embryos stored in 100% methanol at –20˚C were returned to room temperature, rehydrated, fixed in 4% paraformaldehyde, permeabilized with 0.1 M proteinase K, fixed a second time in 4% paraformaldehyde, treated with acetic anhydride, washed in PBS with 1% Tween, and pre-hybridized in hybridization buffer for 1-2 hours. The pre-hybridization solution was removed and 200ng of probe in 80μl of hybridization solution was pipetted onto embryos and hybridized overnight at 55°C. The next day, the embryos were washed and probes were detected using alkaline-phosphatase-conjugated antibody against digoxygenin and the subsequent colorimetric reaction with 4-nitroblue-tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche Molecular Biochemicals, Indianapolis, IN). The color reaction was allowed to proceed for approximately 60 minutes and stopped with PBS. The embryos were then transferred to single concavity slides (Tri-Ess Sciences, Inc., Burbank, CA) and coverslipped for inspection and photography. Animals were sacrificed at 25, 31, 38, and 48hpf. Ten animals were processed per time point.

In situ hybridization with single probes on sections was performed as previously described (Hitchcock et al., 2001). Briefly, full-length *neuroD* cDNA (Korzh et al., 1998) was linearized and DIG-labeled riboprobes were synthesized

with an RNA labeling kit (Roche Diagnostic Corp., Indianapolis, IN). Following prehybridization two hundred nanograms of probe in 80µl of hybridization solution was pipetted onto each slide, coverslipped, and hybridized overnight at 55°C. The next day, the sections were washed and digoxygenin was immunolabeled using an alkaline-phosphatase-conjugated antibody and visualized with NBT/BCIP. The slides were then coverslipped for microscopy or combined with several immunocytochemistry protocols described below. Animals were sacrificed at 25, 31, 38, 48, 60, 76, and 96hpf, as well as at 3 months. Ten animals were analyzed per time point. *NeuroD* sense probes served as negative controls for all *in situ* hybridization protocols. Following hybridization with *neuroD* sense probes, no staining was observed (data not shown).

Double *in situ* hybridization on sections was performed with probes for *neuroD* and *red opsin*, or *Crx*. The protocol was modified from the one described above (see also Hitchcock and Kakuk-Atkins, 2004). Fluorescein-labeled riboprobes for *red opsin* and *Crx* were synthesized and two hundred nanograms of probe in 80µl of hybridization solution was pipetted onto each slide. Combinations of probes for *neuroD* and *red opsin*, or *neuroD* and *Crx* were diluted in buffer and hybridized simultaneously on the sections. After post-hybridization washes, sections were immunostained with antibodies against digoxygenin conjugated to alkaline phosphatase and antibodies against fluorescein conjugated to peroxidase. The sections were rinsed and *neuroD* probes were visualized with fast red (Roche Diagnostic Corp., Indianapolis, IN) as the substrate. After further rinses, *red opsin*, or *Crx* probes were visualized by

using the Tyramide Signal Amplification Kit (Perkin Elmer, Norwalk, CT) with streptavidin-Alexafluor 488 (Molecular Probes, Eugene, OR).

BrdU labeling

Bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) was used to label mitotically active cells. Embryos were exposed to BrdU for 15 minutes by soaking in 5mM BrdU and 15% DMSO in embryo rearing solution (protocol adapted from Steve Devoto, University of Oregon; http://zebra.biol.sc.edu/methods/brdu.html) and sacrificed immediately following BrdU exposure. Ten animals were analyzed per time point. Additional animals were exposed to BrdU for 15 minutes at 56hpf and sacrificed 8 hours or 40 hours later. After BrdU exposure, these animals were housed in 10mM thymidine in embryo rearing solution to prevent continuous labeling of cells with accumulated systemic BrdU. Ten animals were analyzed.

Immunocytochemistry

All immunocytochemistry protocols were performed as previously described (Hitchcock et al., 1996). Omitting primary antibodies served as negative controls. In the absence of primary antibodies, no staining was observed (data not shown). Amacrine cells were labeled using the cell type-specific monoclonal antibody against rat syntaxin (Monoclonal Anti-Syntaxin Clone HPC-1; Sigma; catalog #S0664) and a monoclonal antibody against the human neuronal protein HuC/D (Formerly 16A11; Molecular Probes, Eugene, OR; catalog # A21271; Kay et al., 2001), diluted 1:200, and a rabbit polyclonal antibody against mouse Pax6, diluted 1:1000 (COVANCE®, Berkeley, CA; lot #14811801; Hitchcock et al., 1996). The syntaxin antibody was raised in mouse against a synaptosomal

plasma membrane fraction from adult rat hippocampus and on western blot binds a 35kD protein. The Hu antibody was raised in mouse against the synthetic peptide QAQRFRLDNLLN, corresponding to amino acids 240-251 of human HuD peptide, and on western blot binds 36kD, 40kD, and 42kD proteins corresponding to ELAVL2, ELAVL3, and ELAVL4. The pax6 antibody was raised in rabbit against the synthetic peptide REEKLRNQRRQASNTPSHI, corresponding to amino acids 281-299 of mouse Pax6, and on western blot binds a 47kD protein and can be blocked with Pax6 peptide. Cone photoreceptors were labeled using the mouse monoclonal antibody zpr-1 (The Zebrafish International Resource Center, Eugene, OR; catalog #092502), and diluted 1:200. The zpr-1 (formerly Fret43; Larison and Bremiller, 1990), and zpr-3 (see below), antibody was raised in mouse against a mix of antigens from 2-day-old whole zebrafish embryos followed by selection for tissue/cell specific binding of antibodies. The zpr1 antibody labels an unidentified epitope on red/green cones. Rod photoreceptors were labeled using the mouse monoclonal antibody zpr-3 (The Zebrafish International Resource Center, Eugene, OR; catalog #011604), and diluted 1:200. The zpr-3 (formerly Fret11; Schmitt and Dowling, 1996) antibody labels an unidentified epitope on rod photoreceptors. On Western blots, Fret 11 binds a 38-kD protein and displays a banding pattern typical of antibodies possessing specificity for opsin proteins, including rhodopsin. BrdU was detected using a monoclonal antibody against BrdU (Becton Dickinson Immunocytochemistry Systems, San Jose, CA; catalog #347580) diluted 1:200. For double immunocytochemistry experiments combining zpr-1 or zpr-3 and

BrdU, the tissue was processed for zpr-1 or zpr-3 antibody staining first, fixed a second time in 4% paraformaldehyde in phosphate buffer, and then processed for BrdU immunocytochemistry using a rat monoclonal antibody (abcam®, Cambridge, MA) diluted 1:200. All secondary antibodies conjugated to fluorescent labels were diluted 1:200.

Photography

Histological sections and whole-mounts were photographed with a Nikon DMX 1200 digital camera. Digital overlays and figures were assembled in Adobe Photoshop 7.0.

Results

Expression pattern of neuroD in embryonic and larval retinas

NeuroD is first expressed at 31 hpf in a small cluster of cells in the ventronasal retina (Fig.1 A, B). This location corresponds to a small patch of retina that in teleosts differentiates precociously (Schmitt and Dowling, 1999; Hu and Easter, 1999). At this stage, however, both the ventral patch and the remainder of the retina are an undifferentiated neuroepithelium, consisting largely of mitotically active cells. The *neuroD*-expressing cells in the precocious ventral patch are proliferative and are labeled with BrdU following a brief exposure (data not shown).

Between 31hpf and 48hpf, *neuroD* expression expands from the ventronasal patch throughout the retinal neuroepithelium, which in a frontal plane appears to spread from ventral to dorsal (Fig.1 C). At this stage, all the *neuroD*-expressing cells are mitotically active (data not shown). By 48hpf, retinal laminae can be distinguished, and at this time *neuroD* expression segregates into the nascent inner and outer nuclear layers (Fig.1 D; see also Mueller and Wullimann, 2002), although the lamination in the dorsal-most retina is markedly less advanced, and *neuroD* expression there is reminiscent of that seen at earlier stages. The circumferential germinal zone at the retinal margin is first recognizable at 48hpf, and it is noteworthy that at no time is *neuroD* expressed in these cells (Fig.1, D-G).

Between 48hpf and 76hpf, *neuroD* continues to be expressed in both the inner and outer nuclear layers. By 60hpf, *neuroD*-expressing cells appear as

clusters in the inner nuclear layer and as a relatively thick row of cells in the outer nuclear layer (Fig.1 E). This is also the stage when a population of large, presumptive amacrine cells that express *neuroD* first appears in the inner nuclear layer (Fig.1 E and F, see also Fig.3). By 76hpf lamination is complete (Schmitt and Dowling, 1999; Li et al., 2000). At this stage, *neuroD* expressing-cells continue to be present in both the inner and outer nuclear layers, although the *neuroD*-expressing cells in the inner nuclear layer are less dense than at 60hpf (compare Fig.1 E and F).

Between 76hpf and 96hpf the vast majority of cells within the outer nuclear layer cease expressing *neuroD*, and outer nuclear layer expression becomes restricted to the annulus of immature retina adjacent to the circumferential germinal zone (Fig.1 G, H). A few mitotically-active, *neuroD*-expressing cells are still present within the outer nuclear layer. Within the inner nuclear layer two populations of *neuroD*-expressing cells persist, presumptive neurons and rare, mitotic cells. At 96hpf (Fig.1 G) the cellular pattern of *neuroD* expression achieves an adult-like pattern and remains unchanged from this stage forward (Fig.2; see also Hitchcock and Kakuk-Atkins, 2004).

NeuroD is expressed in both post-mitotic and mitotic cell populations

Amacrine cells

Three amacrine cell markers were used to establish the identity of the post-mitotic cell types that express *neuroD*. Beginning at 76hpf *neuroD* is expressed in a subset of cells in the inner nuclear layer that, by virtue of their morphology and location, are identified as presumptive amacrine cells (Fig.1 F, arrow heads;

see also insets in Fig.3 A and C). These cells have large somata, reside in the inner half of the inner nuclear layer and are uniformly spaced across the retinal section. To confirm the identity of these cells, *in situ* hybridization for *neuroD* expression was combined with immunocytochemistry using antibodies against markers of amacrine cells, syntaxin, pax6, and HuC/D. Antibodies against syntaxin label the cytoplasm of amacrine cells, whereas antibodies against the transcription factor *pax6* label nuclei (Fig.3; see also Hitchcock et al., 1996). The antibody against HuC/D labels the cytoplasm of a subset of amacrine in the retina (see also Kay et al., 2001). All three markers, when combined with *in situ* hybridization using probes for *neuroD,* show that the large, *neuroD*-expressing cells in the inner nuclear layer are amacrine cells (Fig.3). The majority of the *neuroD*-expressing cells, with the morphology described above, co-label with syntaxin, pax6, or HuC/D.

Nascent cone photoreceptors

Except for the ventronasal patch (Kljavin, 1987; also see above), cone photoreceptors are the first cell type to differentiate as neurogenesis progresses within the outer nuclear layer (Branchek and Bremiller, 1984; Raymond and Barthel, 2004), and in the adult retina *neuroD* is not expressed by rods (Fig.2; Hitchcock and Kakuk-Atkins, 2004). This suggests that between 48hpf and 96hpf *neuroD* expression within the outer nuclear layer is restricted to differentiating cone photoreceptors and mitotically-active photoreceptor progenitors. To confirm that *neuroD* is expressed by nascent cone photoreceptors, sections from retinas at 76hpf were processed for double *in situ* hybridization for *neuroD* and *red*

opsin, a marker for one of the first cone types to differentiate (Branchek and Bremiller, 1984; Larison and Bremiller, 1990). This revealed that the vast majority of *neuroD*-expressing cells in the outer nuclear layer are cones (Fig.4; arrow in Fig.4 A, B, and C). Further, there appears to be a discrete boundary between cells in the outer nuclear layer which express *neuroD* only and those that express both *neuroD* and *red opsin* (arrowhead in Fig.4 A, B, and C). This double labeling approach clearly demarcates the region of cone genesis and maturation, which lies between the circumferential germinal zone and the first *neuroD*/*red opsin*-expressing cones (Fig.4 C).

Cells of the rod lineage

We used BrdU combined with double-labeling methods to determine the identity of the mitotic cells that express *neuroD*. Following the initial waves of neurogenesis and lamination, mitotically active cells remain within both the inner and outer nuclear layers. Based on data from studies of retinas from adult teleosts, it is inferred that in the larval retina these dividing cells are members of the rod lineage. Rod genesis in adult teleosts has been extensively described (see review by Hitchcock and Raymond, 2004), and the rod lineage consists of dividing cells in both the inner and outer nuclear layers. *NeuroD* is expressed by a population of cells in the inner nuclear layer not labeled with amacrine cell markers. These cells are characterized by a neuroepithelial morphology (Fig.5; arrow in c1), resembling the morphology of the *neuroD*-expressing cells found in the 38hpf embryonic retina (compare arrowhead in Fig.1 C and arrow in Fig.5 c1), and they are proliferative as evidenced by their labeling with BrdU (Fig.5 C;

arrow in c1 and c2). Between 48hpf and 76hpf, *neuroD* is also expressed in proliferating cells in the outer nuclear layer. These cells can be labeled with BrdU, and are interspersed among the orderly arrangement of cone nuclei (Fig.5 C; arrow in c3-c4).

To confirm the identity of these mitotically-active cells retinas were exposed to BrdU at 56hpf, sacrificed at 96hpf, and double labeled with antibodies to BrdU and zpr-3, a marker of rod photoreceptors. This revealed that at 56hpf mitotically active cells in the laminated retina give rise to rod photoreceptors (Fig.6). It should be noted, however, that only a minority of cells labeled with BrdU at 56hpf express zpr-3 at 96hpf. We interpret this to show that not all cells of the rod lineage become fully differentiated during the interval we examined and therefore remain labeled with BrdU but do not yet express zpr-3. These observations suggest that, as in the adult teleost retina, *neuroD* is expressed in the rod photoreceptor lineage in the developing teleost retina.

Cone progenitors

We also evaluated *neuroD* expression among cells that give rise to cone photoreceptors. Although visual inspection of the outer nuclear layer, based on morphology and markers of differentiated cells, suggests that cone genesis in the central retina ceases by 76hpf (see Fig.1 F and Fig.4), we cannot exclude the possibility that the outer nuclear layer contains mitotically active cells which express *neuroD* and give rise to cones. To determine whether or not cones are generated in central retina during this time, animals were exposed to BrdU at 56hpf and sacrificed at 64hpf. The retinas were then double-labeled with

antibodies against BrdU and zpr-1, a marker of cone photoreceptors. These experiments showed that cones are still being generated in central retina at this time (Fig.7), which raises the possibility that some BrdU-positive, *neuroD*-expressing cells may give rise to cones.

To determine more directly whether or not *neuroD*-expressing cells generate cones, we examined the junction between the circumferential germinal zone and the nascent outer nuclear layer, a site where dividing cells merge with the all-cone outer nuclear layer. We surmise that by virtue of their position, the dividing cells here serve as cone progenitors (arrow in Fig.8 A, B; see also Stenkamp et al., 1997). To determine whether or not these cone progenitors express *neuroD*, animals were exposed to BrdU at 96hpf and processed for *in situ* hybridization using *neuroD* probes and BrdU immunohistochemistry. This revealed that cells, which express *neuroD* and are contiguous with the all-cone outer nuclear layer, are mitotically active (Fig.8 C, D). In addition to relying on position to identify cone progenitors, we performed double *in situ* hybridization using *neuroD* and *red opsin* probes combined with BrdU immunohistochemistry to identify stages of cone differentiation and *neuroD* expression (Fig.8 E-H). Mitotically active cells at the periphery begin expressing *neuroD* and this expression is maintained in immature cones. As the cones mature and begin expressing *red opsin*, there is a small region of overlap of *neuroD* and *red opsin* expression. However, left of this transition point *neuroD* expression is downregulated in the more mature *red opsin* expressing cells. Our observations suggest that *neuroD* is expressed in a small subset of cells within the

circumferential germinal zone, perhaps during their ultimate or penultimate mitosis, which serve exclusively as cone progenitors. By extension, because the retina periphery recapitulates development, we infer that in the developing central retina some dividing cells that express *neuroD* there also give rise to cones. This analysis revealed that *neuroD* expression marks stages of cone development.

NeuroD and the developmental regulatory gene Crx

It is interesting to note that along with *neuroD*, only one other developmental regulatory gene, cone-rod homeobox (Crx), has been shown to be expressed among photoreceptor progenitors in the zebrafish retina (Shen and Raymond, 2004). This coincidence of cellular expression suggests that neuroD and Crx may interact genetically, which can be tested experimentally. To confirm whether *neuroD* and *Crx* are expressed in the same cells, sections from retinas at 76hpf were processed for double *in situ* hybridization for *neuroD* and *Crx*. This revealed that the vast majority of *neuroD*-expressing cells in the outer nuclear layer also express *Crx* (Fig.9). Further, whereas *neuroD* and *Crx* are co-expressed in the outer nuclear layer, there is no co-localization of *Crx* and *neuroD* expression in the inner nuclear layer. Crx expression is restricted to the outer part of the inner nuclear layer while *neuroD* expression is localized to the middle and inner part of the inner nuclear layer (Fig.9, E-H).

Discussion

The cellular expression of *neuroD* in the teleost retina is dynamic and spans early neurogenesis, the maturation of cone photoreceptors, and the acquisition of the adult pattern, which in zebrafish is present by at least 96hpf. The current study further suggests that *neuroD* is expressed in two separate lineages that give rise to rod and cone photoreceptors, respectively, and is transiently expressed in differentiated cones, suggesting this gene may play a role in the maturation of this cell type. The expression data further indicate that *neuroD* is co-expressed with *Crx* in cells in the developing photoreceptor layer, raising the possibility that, as in mammals (Akagi et al., 2005), these transcriptional regulators interact genetically.

During early retinogenesis (31-48hpf) *neuroD* is expressed in cells of the proliferative neuroepithelium. Although variable in age of onset (see Korzh et al., 1998; Masai et al., 2000), *neuroD* expression is first observed in ventronasal retina. Numerous studies have shown that in teleosts the ventronasal patch of retina develops precociously (Raymond et al., 1995; Schmitt and Dowling, 1999; Hu and Easter, 1999), and the localized onset of *neuroD* expression in this patch presages local, precocious differentiation. In the larval and adult retinas, *neuroD* is expressed in cells of the rod lineage (Hitchcock and Kakuk-Atkins, 2004; present results), and the expression of *neuroD* in the ventronasal patch suggests that the photoreceptors generated there originate from the same cellular lineage that generates rods at later developmental and adult stages (see Discussion in Raymond et al., 1995).

As retinogenesis progresses, the expression of *neuroD* expands from the precocious patch throughout the remaining retinal neuroepithelium. The neuroepithelial cells that express *neuroD* at this stage may correspond to photoreceptor progenitors, which give rise to the cells that establish the future outer nuclear layer. Thus, after the differentiation of cells in the ventral patch, mitotically active cells that express *neuroD* may be destined to become either cone or rod photoreceptors. The expanding *neuroD* expression across the developing neuroepithelium may also mark the formation of the lineages that give rise to rods and cones in central retina.

As neurogenesis continues and laminae appear (48-96hpf), *neuroD*-expression segregates to the inner and outer nuclear layers, marking four different cell types, two post-mitotic and two mitotic. The two post-mitotic cell types that express *neuroD* are amacrine cells and nascent cone photoreceptors. In the inner nuclear layer, *neuroD* is expressed in a small subset of postmitotic amacrine cells. This was speculated upon previously (Hitchcock and Kakuk-Atkins, 2004) and confirmed here using a double-labeling approach. However, even though all amacrine antibodies used have been well characterized in the literature and label subsets of mature amacrine cells, in other neural tissues these antibodies label neural progenitors. We cannot exclude the possibility that some of the double-labeled cells with amacrine morphology may correspond to progenitor cells (see below). In the outer nuclear layer, *neuroD* is expressed by nascent cone photoreceptors. This is demonstrated directly via double *in situ* hybridization at 76hpf, which reveals that most *neuroD*-expressing cells in the

outer nuclear layer at this time are cone photoreceptors. As the retina matures, the nascent cones in central retina down regulate *neuroD* expression, and by 96hpf *neuroD* is absent from central cones. Consistent with this temporal pattern, from 96hpf onward expression is maintained in each cohort of immature cones at the periphery. This indicates that *neuroD* expression in cones is transient, lasting merely hours, and suggests this gene may play a role in regulating an aspect of early cone maturation.

It is well established that in the adult teleosts rod photoreceptors are generated by a lineage of proliferative cells that originates in the inner nuclear layer (Julian et al., 1998; Otteson et al., 2001), and these cells express *neuroD* (Hitchcock and Kakuk-Atkins, 2004). At 56hpf in the zebrafish, a time when neurogenesis is largely complete in the inner retina but photoreceptor genesis is still ongoing (Hu and Easter, 1999), the inner nuclear layer of the zebrafish retina contains proliferative cells that express *neuroD*. We infer that these cells are rod progenitors, which give rise to the first generation of central rod photoreceptors (see also, Raymond and Rivlin, 1987). This was confirmed here by labeling cells with BrdU and double immunostaining with antibodies against BrdU and zpr-3 (a marker of rod photoreceptors), which demonstrated the presence of BrdU-labeled rods.

The double-labeling approach also established that in central retina at 56hpf cones are also being born. In teleosts, in general, cone genesis precedes rod genesis (Johns, 1982), but there is an interval where in central retina of the zebrafish these two cell types are generated contemporaneously (Raymond et

al., 1995; Schmitt and Dowling, 1999; see also Larison and Bremiller, 1990; present results). Our data show that, during this interval, there are numerous BrdU-positive cells in the retina that express *neuroD*. Based on these observations, we speculated that some dividing, *neuroD*-positive cells might also give rise to cone photoreceptors. Because at 56hpf one does not know if a dividing cell in central retina will give rise to a cone or rod, we examined the germinative margin for evidence of dividing cells that both express *neuroD* and occupy a location that indicates they serve as cone progenitors. We found such cells in the outer nuclear layer lying at the interface between the circumferential germinal zone and newly postmitotic cones. From this evidence we conclude that there exists a narrow annulus of proliferative, *neuroD*-expressing cells at the germinative margin that give rise exclusively to cone photoreceptors, and that these cells express *neuroD* at the time of their ultimate or penultimate division. Further, and by extension, we conclude that some dividing cells in central retina that express *neuroD* must also give rise to cones, including those cones first generated in the ventronasal patch.

These observations and our inferences from the data suggest one of two possibilities. First, in central retina rod and cone lineages emerge separately from the neuroepithelium and separately generate the two photoreceptor types, or, second, during the interval when both cell types are generated, rods and cones share a common lineage or progenitor. The fact that in the teleost retina, from early larval development through adulthood, rods and cones are generated from spatially separate progenitors argues in favor of the first possibility, that from the

outset rods and cones are generated from separate cell lineages. In contrast, direct evidence from both birds and mammals suggests that rods and cones can share a common progenitor (Yan and Wang, 1998; Yan and Wang 2000; Yan and Wang, 2004; Mears et al., 2001). Whether or not early photoreceptor genesis in teleosts fits the avian and mammalian model remains to be determined.

It has been suggested that cones and rods are produced simultaneously in the ventral patch while the remainder of photoreceptors are formed during the final wave of terminal mitoses in the outer nuclear layer (Raymond and Barthel, 2004). It is interesting to note that another developmental gene Cone-rod homeobox (*Crx*) is also expressed by mitotically active progenitors in the outer nuclear layer (Shen and Raymond, 2004). These data suggest that *Crx* may be expressed in late-stage photoreceptor progenitors as they exit the cell cycle. *Crx* regulates differentiation and survival of retinal photoreceptors and may also play a role in promoting differentiation of retinal progenitors. Several studies demonstrate that bHLH factors function in concert with homeodomain genes during the patterning of the retina (Hatakeyama et al., 2001; Akagi et al., 2004). Our current data reveal that *neuroD* and *Crx* are expressed in the same cells in the outer nuclear layer. This suggests that in zebrafish neuroD may interact with Crx during the genesis of photoreceptors, perhaps as progenitors exit the cell cycle, and that a similar genetic signaling cascade is present in both homeothermic vertebrates and teleosts.

Finally, the data from our study allow us to expand current models of photoreceptor genesis in the mature retina of teleost fish (Raymond and Rivlin, 1987; Otteson et al., 2001; Otteson and Hitchcock, 2003). We show that the rod lineage and associated *neuroD* expression, first described in adult goldfish (Hitchcock and Kakuk-Atkins, 2004), is present in the embryonic retina as early as 56hpf in zebrafish and perhaps earlier (Fig.10 A). In addition, there is a narrow annulus of dividing cells at the germinative margin that express *neuroD* and serve exclusively as cone progenitors (Fig.10 B). We suggest that as these cells express *neuroD* they divide a limited number of times, perhaps only once, and then continue to express this gene for the first few hours of maturation. Our results also suggest that, by virtue of their spatial separation, in the teleost retina one can separately assay the function of *neuroD* in the lineages of cells that give rise to rods (centrally) and cones (at the germinative margin), respectively.

There is an expanding body of evidence pointing to a common role for neuroD in persistently mitotic cellular lineages, linking cell cycle withdrawal with terminal differentiation. The existence of a mitotic lineage generating a single cell type, such as the rod photoreceptor lineage, is not unique and is present in other persistently mitotic regions in the adult central nervous system, including cells in the subventricular zone and rostral migratory stream, dentate gyrus, and cerebellum (Miyata et al., 1999; Schwab et al., 2000; Pleasure et al., 2000; Lee et al., 2000; Liu et al., 2001a; Bedard and Parent, 2004; Manglapus et al., 2004; Hevner et al., 2006; see also Naya et al., 1997; Mutoh et al., 1998; Cai et al., 2000; Nibu et al., 2001; Liu et al., 2001b; Kim et al., 2001; Schonhoff et al., 2004;

Lawoko-Kerali et al., 2004). In each of these regions, *neuroD* is expressed in late stage progenitors, perhaps during their ultimate or penultimate mitosis, and appears to be essential for terminal differentiation. Current evidence thus suggests a common function of NeuroD in cell lineages and determining this function in the rod and cone lineages in the zebrafish retina may shed light on the overarching role that NeuroD is playing in persistently mitotic tissues of adult vertebrates.

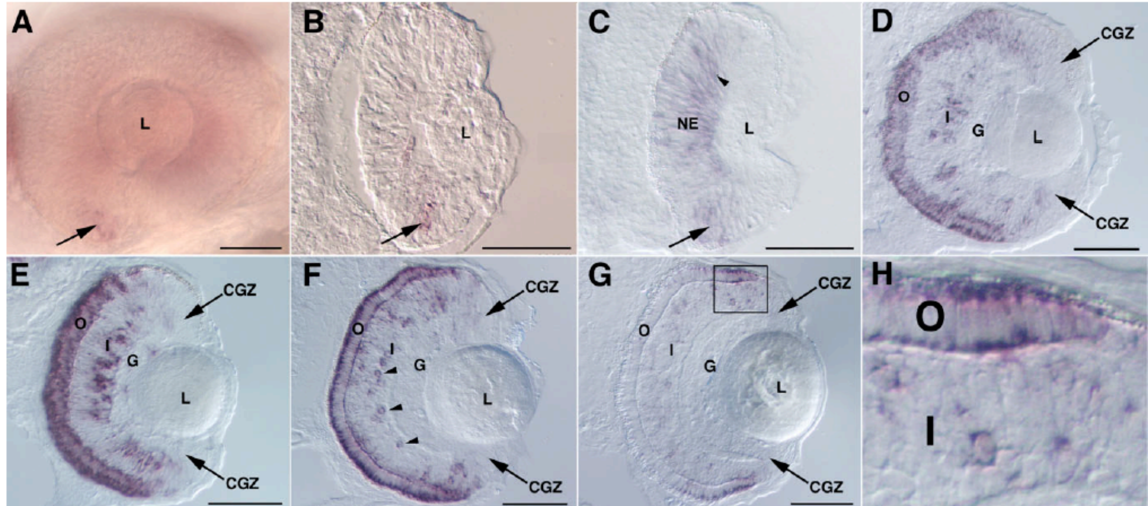

Fig. II-1: Cellular pattern of *neuroD* expression in the developing retina of the zebrafish.

A: *neuroD* expression at 31 hours post fertilization (hpf) (arrow). B: Transverse section of a 31hpf retina showing cells expressing *neuroD* (arrow). C: *neuroD* expression at 38hpf spanning the neuroepithelium and persisting in the ventral retina (arrow), arrowhead indicates an individual *neuroD*-expressing cell with neuroepithelial morphology. D: *neuroD* expression at 48hpf. E: *neuroD* expression at 60hpf. F: *neuroD* expression at 76hpf, arrowheads indicate *neuroD*-expressing amacrine cells G: *neuroD* expression at 96hpf and in the retina margin (see inset H). Note that *neuroD* is not expressed in the circumferential germinal zone (arrows in 1D-1G). Scale bar equals 50µm; O = outer nuclear layer; I = inner nuclear layer; G = ganglion cell layer; L = lens; CGZ = circumferential germinal zone; NE = neuroepithelium.

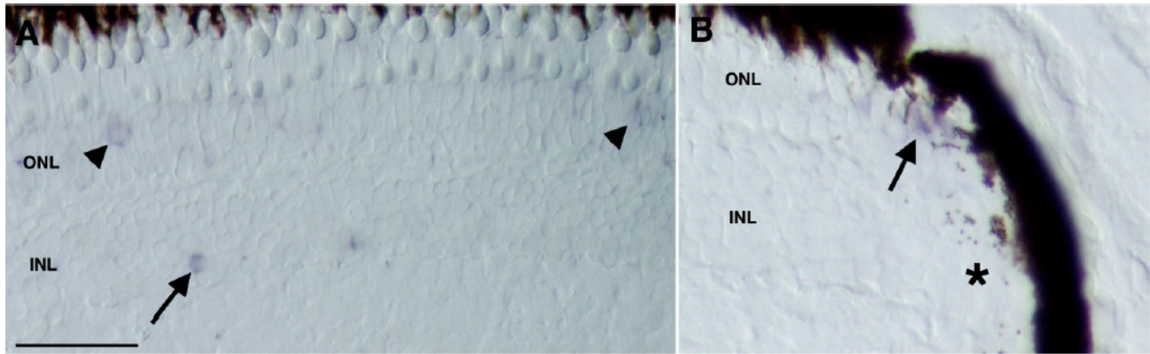

Fig. II-2: Cellular pattern of *neuroD* expression in the adult zebrafish.

A: *neuroD* expression in central retina showing a labeled amacrine cell (arrow), and presumptive cells of the rod lineage (arrow heads). B: *neuroD* expression at the retinal margin showing labeled nascent cone photoreceptors (arrow). Scale bar equals 50μm; ONL = outer nuclear layer; INL = inner nuclear layer; * = circumferential germinal zone.

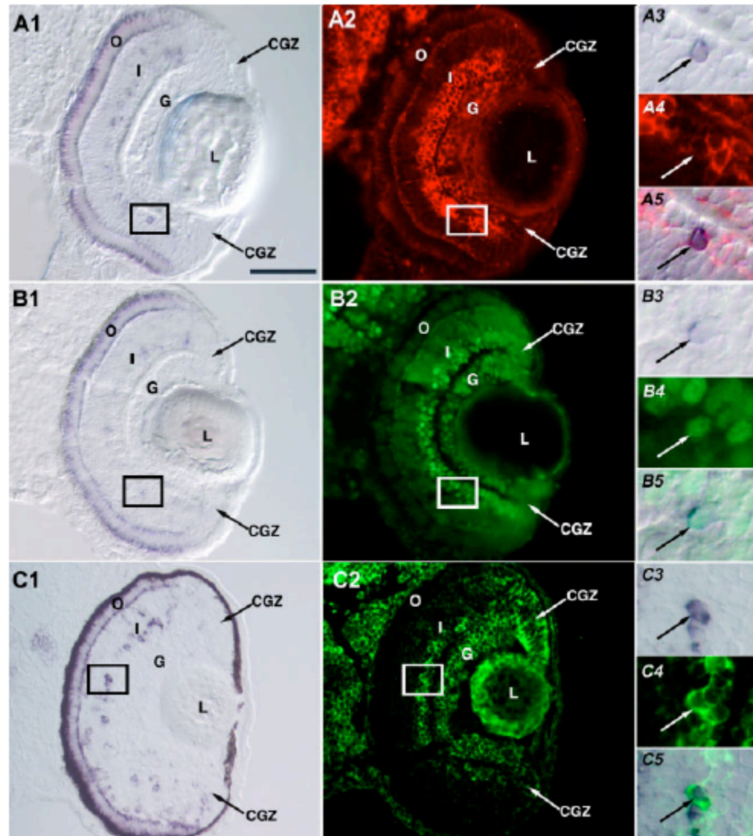

Fig. II-3: *neuroD* is expressed in a subset of amacrine cells in the inner nuclear layer.

A1 and A2: At 76 hours post fertilization (hpf) *neuroD*-expressing cells in the inner nuclear layer (INL) co-label with antibodies against syntaxin (Panels A). A3: The arrow depicts an individual *neuroD*-expressing cell in the INL. A4: The arrow depicts the same cell as in A3, labeled with syntaxin. A5: Overlay of *neuroD* and syntaxin shown in A3 and A4. The arrow depicts the same cell shown in A3 and A4, which expresses both *neuroD* and is labeled with syntaxin. B1 and B2: *neuroD*-expressing cells in the INL co-label with antibodies against pax6 (Panels B). B3: The arrow depicts an individual *neuroD*-expressing cell in the INL. B4: The arrow depicts the same cell as in B3, labeled with pax6. B5: Overlay of *neuroD* and pax6 shown in B3 and B4. The arrow depicts the same cell shown in B3 and B4, which expresses both *neuroD* and is labeled with pax6. C1 and C2: *neuroD*-expressing cells in the INL co-label with antibodies against HuC/D (Panels C). C3: The arrow depicts an individual *neuroD*-expressing cell in the INL. C4: The arrow depicts the same cell as in C3, labeled with HuC/D. C5: Overlay of *neuroD* and HuC/D shown in C3 and C4. The arrow depicts the same cell shown in C3 and C4, which expresses both *neuroD* and is labeled with HuC/D. Scale bar equals 50μm; O = outer nuclear layer; I = inner nuclear layer; G = ganglion cell layer; L = lens; CGZ = circumferential germinal zone.

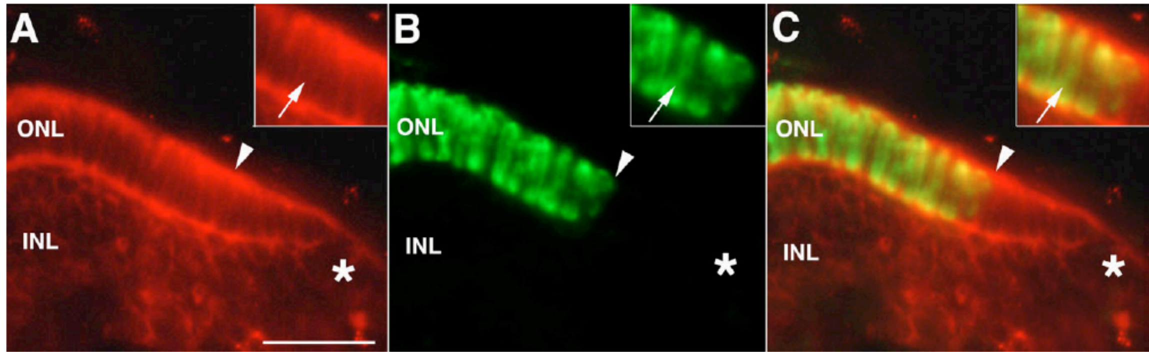

Fig. II-4: *neuroD* is expressed in nascent cone photoreceptors.

A: Retina at 76 hours post fertilization (hpf) labeled with a *neuroD* probe. Cells throughout the outer nuclear layer (ONL) express *neuroD* and the arrow depicts an individual *neuroD*-expressing cell (see inset in C). B: Cells in the ONL labeled with a probe for *red opsin*. The arrow depicts an individual *red opsin*-expressing cell (see inset in B). C: Overlay of *neuroD* and *red opsin in situ* shown in A and B. Cells in the ONL co-express *neuroD* and *red opsin*. The arrow depicts the same cell shown in A and B which expresses both *neuroD* and *red-opsin* (see inset in C). The arrowhead in all figures depicts the transition between cones in the ONL expressing *neuroD* only and those that express both *neuroD* and *red opsin*. The asterix indicates the CGZ. Scale bar equals 50µm; ONL = outer nuclear layer; INL = inner nuclear layer; CGZ = circumferential germinal zone.

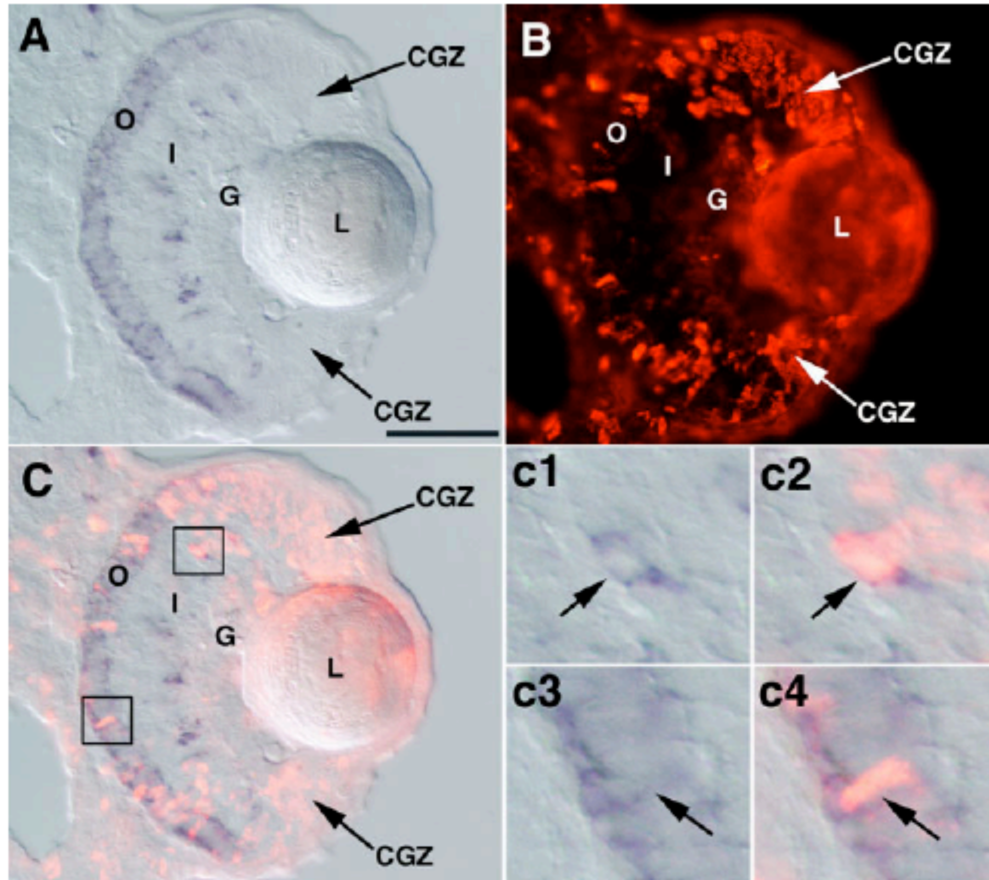

Fig. II-5: *neuroD* is expressed in cells of the rod photoreceptor lineage.

A and B: 76 hours post fertilization (hpf) retina showing *neuroD in situ* (A) and BrdU immunohistochemistry (ICC) (B), respectively. C: Overlay of neuroD *in situ* and BrdU ICC. c1 and c2: Rod progenitors in the inner nuclear layer (INL) are characterized by neuroepithelial morphology and BrdU incorporation (see arrow in c2). c3 and c4: Rod precursors in the outer nuclear layer (ONL) are characterized by BrdU incorporation and morphology (see arrow in c4). Note the absence of *neuroD* expression in the mitotically active multipotent progenitors in the circumferential germinal zone (CGZ) (arrows in C). Scale bar equals 50µm; hpf = hours post fertilization; O = outer nuclear layer; I = inner nuclear layer; G = ganglion cell layer; L = lens; CGZ = circumferential germinal zone.

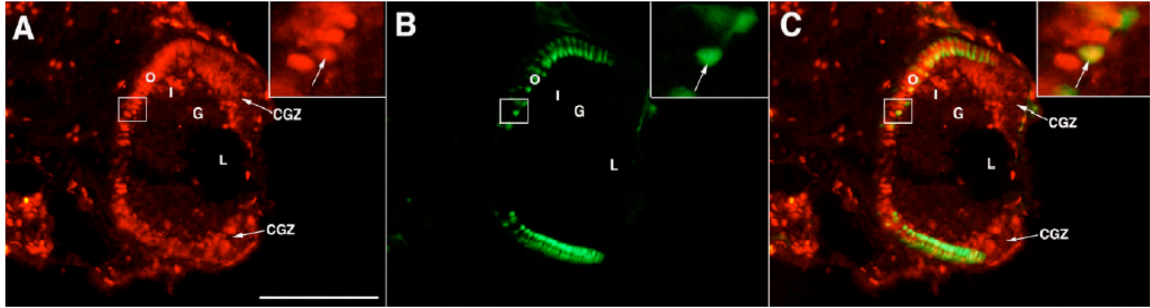

Fig. II-6: BrdU-labeled cells in the outer nuclear layer give rise to rod photoreceptors.

A and B: 96 hours post fertilization (hpf) retina exposed to BrdU at 55hpf and sacrificed at 96hpf showing BrdU labeling (A) and zpr-3 staining (B), respectively. Inset in both A and B depicts an individual cell in the outer nuclear layer (ONL) labeled with BrdU and zpr-3, respectively. C: Overlay of BrdU and zpr-3 immunohistochemistry shown in A and B. Inset in C shows one BrdU positive cells in the ONL co-labeled with BrdU and zpr-3 (arrow). Note that the majority of the cells in the ONL are not co-labeled with BrdU and zpr-3. Scale bar equals 50µm; hpf = hours post fertilization; O = outer nuclear layer; I= inner nuclear layer; G = ganglion cell layer; L = lens; CGZ = circumferential germinal zone.

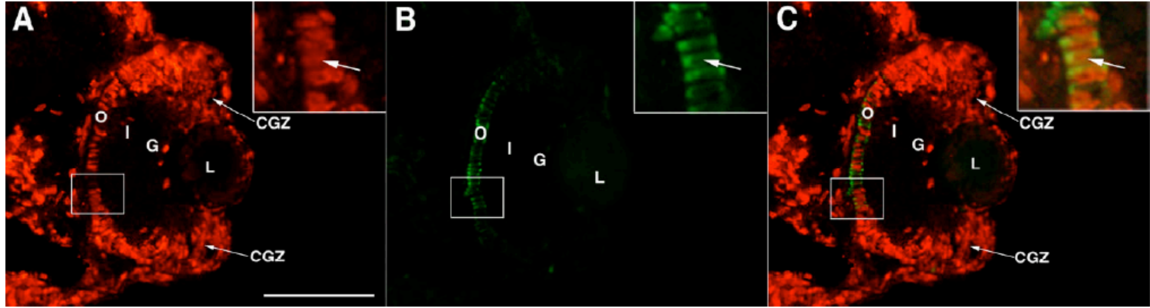

Fig. II-7: BrdU labels cone progenitors in the outer nuclear layer.

A and B: 64 hours post fertilization (hpf) retina exposed to BrdU at 56hpf and sacrificed at 64hpf showing BrdU labeling (A) and zpr-1 staining (B), respectively. Inset in both A and B depicts an individual cell in the outer nuclear layer (ONL) labeled with BrdU and zpr-1, respectively. C: Overlay of BrdU and zpr-1 immunohistochemistry shown in A and B. Inset in C shows one BrdU positive cells in the ONL co-labeled with BrdU and zpr-1 (arrow). Scale bar equals 50µm; hpf = hours post fertilization; O = outer nuclear layer; I= inner nuclear layer; G = ganglion cell layer; L = lens; CGZ = circumferential germinal zone.

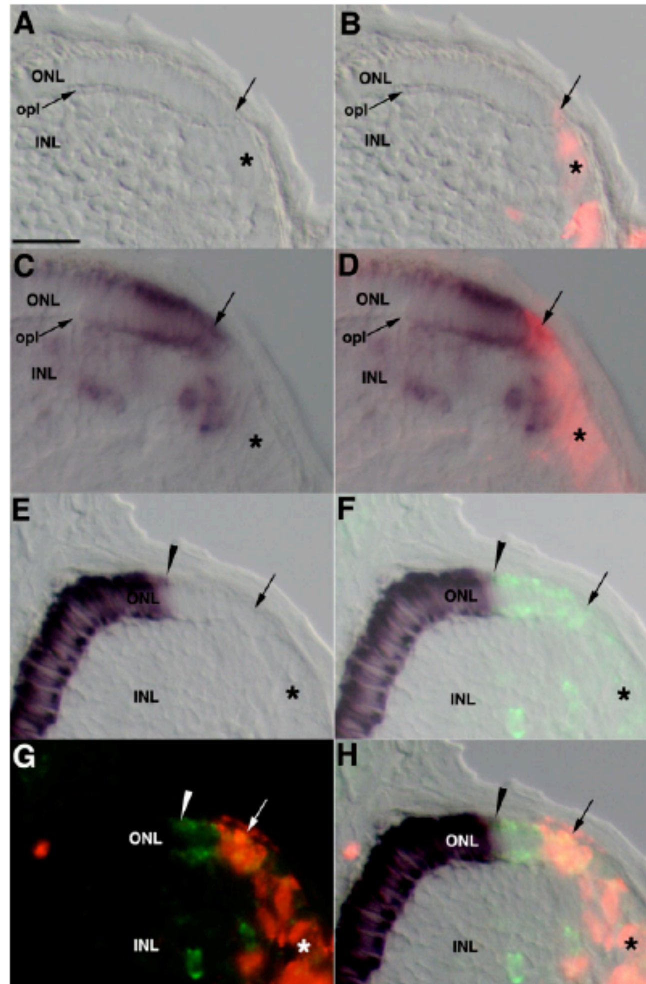

Fig. II-8: *neuroD* is expressed in cone progenitors.

A and B: Brightfield image of a 96 hours post fertilization (hpf) retina (A) and overlay with BrdU immunohistochemistry (B), respectively. Note the labeled BrdU positive cells directly above the outer plexiform layer (arrow). C and D: 96hpf retina showing *neuroD in situ* (C) and overlay with BrdU immunohistochemistry (D), respectively. Arrows in both C and D depict an individual cell, which is double labeled with *neuroD* and BrdU. *NeuroD* expression marks stages of cone development. E and F: 96hpf retina showing *red opsin in situ* (E) and overlay with *neuroD in situ* (F), respectively. Arrowheads in both E and F depict an individual cell, which co-expresses *red opsin* and *neuroD*. Note that left of this transition point, marked by the arrowhead, neuroD expression is downregulated in the more mature red opsin expressing cells (see also C and D). G and H: The same retina as in E and F, showing *neuroD* expression (G, green) and BrdU positive cells (G, red), and (H) overlay with both *red opsin* and *neuroD in situs* from E and F. Arrows in E-H depict an individual cell, which expresses *neuroD* and is BrdU positive. The asterix indicates the CGZ. Scale bar equals 50µm; ONL = outer nuclear layer; INL = inner nuclear layer; CGZ = circumferential germinal zone.

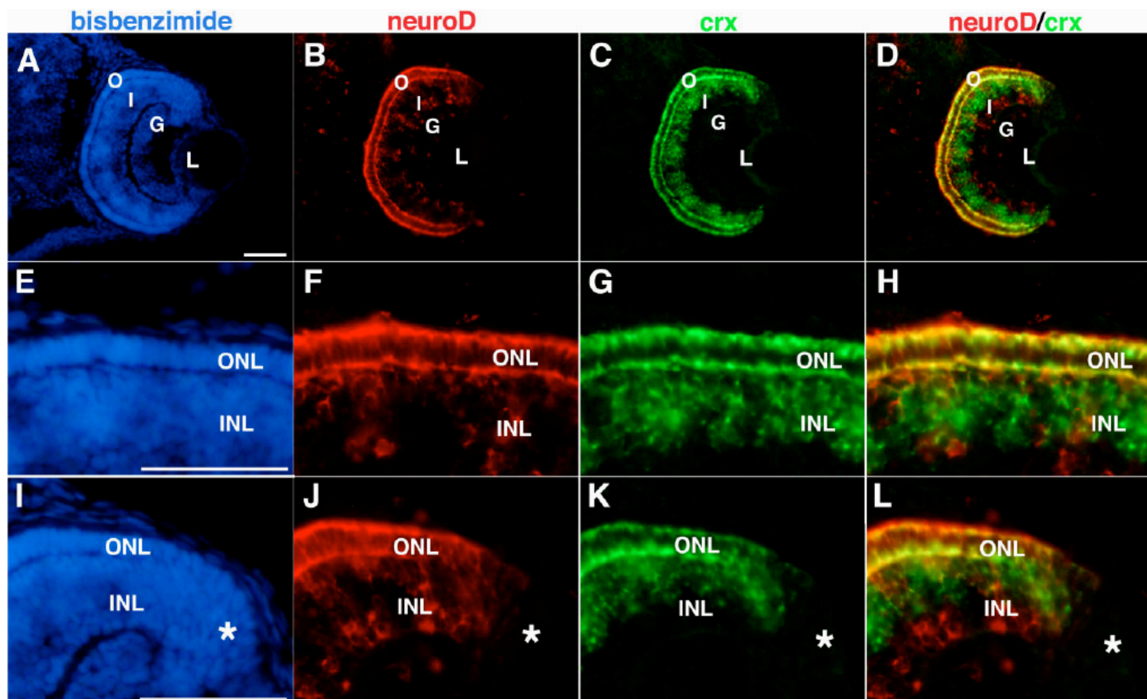

Fig. II-9: *neuroD* and *Crx* are co-expressed in nascent cone photoreceptors.

A, E, and I: Retina at 76 hours post fertilization (hpf) labeled with bisbenzimide. B, F, and J: Retina at 76hpf labeled with a neuroD probe, Cells throughout the ONL express *neuroD*. C, G, and K: Cells in the ONL labeled with a probe for *Crx*. Cells throughout the ONL express *Crx*. D, H, and L: Overlay of *neuroD* and *Crx in situ* shown in B, F, J and C, G, K, respectively. Cells in the ONL co-express *neuroD* and *Crx*. The asterix indicates the CGZ. Scale bar equals 50μm; ONL = outer nuclear layer; INL = inner nuclear layer; CGZ = circumferential germinal zone.

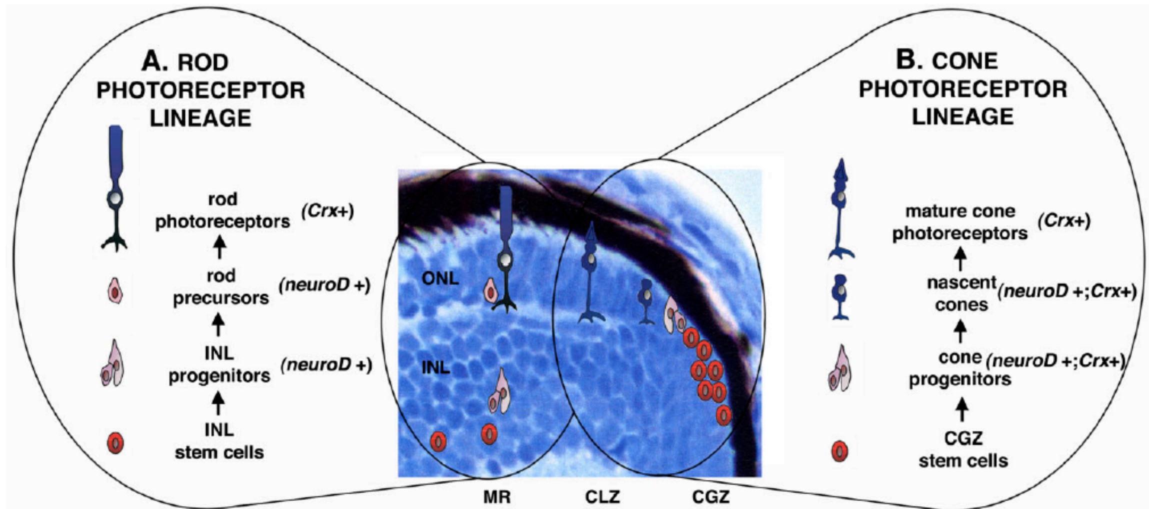

Fig. II-10: Lineage model illustrating rod and cone genesis in the teleost retina.

A: Lineage model of rod genesis. Inner nuclear layer (INL) stem cells give rise to *neuroD*-expressing INL progenitors, which traverse to the outer nuclear layer and give rise to *neuroD*-expressing rod precursors, which give rise to rod photoreceptors that do not express *neuroD* but do express *Crx*. B: Lineage model of cone genesis. Circumferential germinal zone stem cells give rise to *neuroD* and *Crx*-expressing cone progenitors, which give rise to *neuroD* and *Crx*-expressing nascent cone photoreceptor, which give rise to mature cone photoreceptors that do not express *neuroD* but continue to express *Crx*. ONL = outer nuclear layer; INL = inner nuclear layer; GCL = ganglion cell layer; MR = mature retina; CLZ = circumferential larval zone; CGZ = circumferential germinal zone.

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CHAPTER III

GAIN-OF-FUNCTION

This chapter characterizes the function of NeuroD in the retina *in vivo* by utilizing a line of zebrafish line transgenic for Hsp:nrd-EGFP, which allows for conditional gain-of-function following application of a heat shock.

Introduction

The function of basic helix-loop-helix transcription factors has been extensively studied in the retina (Livesey and Cepko, 2001; Van Raay and Vetter, 2004; Yan et al., 2005; Wang and Harris, 2005). There is evidence that members of the bHLH class of proneural regulatory proteins act as a molecular link connecting withdrawal from the cell cycle, cell fate determination, and differentiation (Bertrand et al., 2002; Chae et al., 2004; Yan et al., 2005; Sugimori et al., 2007). However, the mechanisms by which bHLH proteins link cell cycle withdrawal to differentiation remain largely unknown. Due to structural similarity of bHLH proneural proteins, elucidating the role of a particular bHLH transcription factor may help determine the mechanisms of bHLH function in the complex process of neurogenesis. NeuroD is a basic helix-loop-helix (bHLH) transcription factor critical for determining neuronal cell fate and regulating withdrawal from the cell cycle (Lee et al., 1995; Farah et al., 2000). In the mammalian retina, NeuroD regulates neuron versus glial cell fate (Morrow et al., 1999) and amacrine cell

genesis (Ahmad et al., 1998; Acharya et al.,1997; Moore et al., 2002; Inoue et al., 2002) and in chick is determinative for photoreceptors (Yan and Wang, 1998; Yan and Wang, 2000; Yan and Wang, 2004). In the retinas of adult teleosts, *neuroD* is constitutively expressed in a subset of amacrine cells and in the lineages of cells that give rise to rod and cone photoreceptors (Otteson and Hitchcock, 2003; Hitchcock et al., 2004; Hitchcock and Kakuk-Atkins, 2004; Ochocinska and Hitchcock, 2007; Chapter 2).

I previously determined the spatial and temporal expression pattern of NeuroD in the embryonic and larval zebrafish retina (Chapter 2). The expression of *neuroD* begins in a cluster of cells in the early retina, presaging the location of the first born rod and cone photoreceptors. At later stages *neuroD* expression was established more definitively and is localized to cells of the rod and cone photoreceptor lineages. There is also transient expression of *neuroD* in nascent cone photoreceptors, which is turned off in mature cones that begin to express opsin. These findings suggest that NeuroD functions to regulate rod and cone genesis and maturation of cone photoreceptors. In contrast to other vertebrate retinas, in the teleost retina *neuroD* is not expressed in multipotent progenitors, indicating that in teleosts NeuroD does not play a role in early cell fate restriction. This makes the zebrafish retina a unique system to study the role of NeuroD in the lineages of cells that give rise exclusively to photoreceptors.

Based on previous studies of NeuroD function and the cellular pattern of NeuroD expression described in Chapter 2, I hypothesize that NeuroD promotes cell cycle withdrawal of photoreceptor progenitors and photoreceptor genesis. To

test this hypothesis, I created a line of zebrafish transgenic for heat shock 70/4:neuroD-EGFP (Hsp:nrd-EGFP) for conditional gain-of-function experiments. This reverse genetic approach allows for temporal control of the induction of NeuroD fusion protein. Following induced expression of NeuroD, the effect on proliferation and photoreceptor genesis was evaluated in the retinas of transgenic and wild type control animals.

Materials and Methods

Experimental Animals

Zebrafish were maintained at 28.5°C on a 14/10 hr light/dark cycle in a zebrafish modular (ZMOD) breeding facility. Embryos were collected after natural spawns, developed at 28.5°C, and staged by hours post fertilization (hpf) as described previously (Kimmel et al., 1995).

The Hsp70/4:nrd-EGFP expression construct.

The *pHsp70/4:nrd-EGFP* construct was made by inserting the open reading frame of 1 kb zebrafish *nrd*, obtained from full-length *neuroD* cDNA (Korzh et al., 1998), between the *SalI* and *SacII* restriction sites in the *pHsp70/4:EGFP* vector (provided by John Kuwada, University of Michigan). The *SalI* and *SacII* restriction sites were added to the nrd open reading frame by PCR. Primers were designed using the Primer3 program (web site address: http://primer3.sourceforge.net/) and used to add SalI and SacII restriction sites to flank the nrd open reading frame. In addition, the primer used to generate the SacII restriction site was also

designed to exclude the nrd stop codon. The insert was sequenced to rule out potential errors in the PCR using the following three primers:

Hsp70/4-left: 5'-CAATGAACAGACGGGCATTT-3'

neuroD-left: 5'-GGGGTCCCAAGAAGAAGAAG-3'

neuroD-right: 5'-TAAGGGGTCCGTCAAATGAG-3'

***Hsp70/4:nrd-EGFP transgenic zebrafish lines*.**

Plasmid DNA was isolated for injection with the Qiagen Maxi Kit, linearized with the SacII restriction enzyme upstream of the Hsp70/4 promoter and diluted to 50ng/μl in 1x Danieau buffer (Nasevicius and Ekker, 2000) that contained 0.25% phenol red. Micropipettes for DNA injections were pulled from thin-walled, fiber-filled glass tubing (1 mm outer diameter) with a Flaming Brown Micropipette puller and back filled by capillary action with the DNA solution. Recently fertilized embryos (one to four cell stage) were placed in an embryo injection chamber, which was mounted onto a compound microscope (Olympus, BMHJ, Japan). Embryos were viewed at 4x magnification, and 2ng of DNA was injected by insertion of the micropipette tip into blastomeres from the animal pole. DNA was injected into embryos with several pressure pulses (40 psi, 40 msec) delivered by a Picospritzer (General Valve Corporation, Picospritzer II, Fairfield, NJ). The volume of the microinjected DNA as indicated by the phenol red was one-fifth of the volume of cytoplasm.

Injected embryos were raised to sexual maturity and crossed in a pair-wise manner to identify founder fish. Cohorts of F1 embryos from the pair-wise crosses were heat-shocked at 24hpf and assayed for EGFP fluorescence at

48hpf to identify founders. PCR was also used to confirm these findings. Genomic DNA was extracted from pools of 150–200 2 day old F1 embryos from the pair-wise crosses using the Bio-Rad Aquapure Genomic DNA Isolation Kit (Hercules, CA). PCR reactions were performed with primers from the enhanced green fluorescent protein (EGFP) sequence to yield a 452 bp product:

5'-CGTCCATGCCGAGAGTGATC-3' and 5'-TCAA-GTCCGCCATGCCCGAA-3'

PCR reactions were performed on ~100–200 ng of DNA in 1x PCR buffer (Invitrogen, Carlsbad, CA). PCR reactions consisted of denaturing step of 4 min at 95°C and 30 cycles of 30 sec at 95°C, 2 min at 55°C, and 2 min at 72°C. followed by a final step of 72°C for 10min, and held at 4°C.

After pairs were identified, the male and female were crossed with wild-type fish to identify the founder fish. The F1 embryos from the founder were heat-shocked for 1 hr in a 37°C water bath and induction of the fusion protein was assayed by examination on a fluorescence microscope. Since the heat shock protein is known to be constitutively expressed in the lens by 72hpf (Blechinger et al., 2002), subsequent cohorts of F1 embryos from identified founders were not heat-shocked but were screened at 72hpf for green lenses and raised to sexual maturity. Animals with green lenses were identified as transgenic fish. The F1 transgenic fish were bred with wild type fish to generate F2 heterozygous progeny. The F2 progeny were raised to sexual maturity and pair-wise crosses of these heterozygous animals resulted in F3 progeny with a 1:2:1 ratio of wild type/heterozygous/homozygous embryos. In all experiments described here homozygous embryos were used.

Zebrafish zNrd antibody and Western Blots

A zebrafish NeuroD (zNrd) antibody was generated by ZYMED Laboratories (Invitrogen). The C-terminal, nonconserved region of zebrafish NeuroD, excluding the bHLH domain was used to produce peptide antibodies against NeuroD. The zNrd antibody was raised in rabbit against the synthetic peptide C HSHHERVMNAQLNAIFHDS-COOH, corresponding to amino acids 332-350 of zebrafish NeuroD. Two rabbits were injected with the synthetic peptide and serum containing NeuroD peptide antibodies was affinity purified. Affinity purified peptide antibodies were assayed through Western analysis and competition with synthetic peptides.

NeuroD-EGFP from heat-shocked transgenic embryos was detected by Western blotting. Pools of 150–200 2 day old F3 embryos from heterozygous F2 crosses were heat-shocked at 24hpf and processed at 48hpf. Proteins were extracted by lysing the embryos with protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany). Protein concentration was quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL) and a Perkin-Elmer Lamba Bio 20 spectrophotometer. Proteins were separated in a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Sigma-Aldrich, St. Louis, MO). The membrane was blocked in blocking buffer overnight and probed with the polyclonal anti-Nrd antibody (Zymed; see above) at 1:1000, and anti-GFP antibody (Chemokine) at 1:10,000 dilution. Horseradish peroxidase-coupled anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO) was used as a secondary antibody.

Chemiluminescence (ECL detection system; Amersham Biosciences, Arlington Heights, IL) was used to detect the immunoreactivity signal.

Antibody specificity was further demonstrated by antigen peptide blocking. Briefly, 1µl of NeuroD antibody was reacted with 50µg excess peptide. 2µl of NeuroD antibody was placed in 100µl saline/PBS. Two tubes were prepared; one tube with antigen/peptide solution and another with the same volume of saline/PBS but without peptide/antigen. The tubes were incubated at 37°C for 2 hrs and another 2 hrs at 4°C. The tubes were centrifuged for 15 min at 4°C in a microfuge (12000rpm) to pellet any immune complexes. The supernatant was removed from both tubes and added to two subsequent tubes plus PBS-Tween with BSA for a final volume of 2ml. Both solutions (with and without antigen/peptide) were then used for Western blotting as per protocol (see above).

Southern Blots

To estimate the copy number of the integrated plasmid in the three Hsp70/4:nrd-EGFP lines, genomic DNA from fins of F3 fish was digested with *SalI*, which cuts at a unique site on the plasmid, and the DNA was subjected to Southern blot analysis. The intensity of labeling of the endogenous NeuroD band was compared with labeling of the Hsp70/4:nrd-EGFP integrated plasmid.

BrdU labeling

Bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) was used to label mitotically active cells. Embryos were exposed to BrdU for 20 minutes by soaking in 5mM BrdU and 15% DMSO in embryo rearing solution as previously described

(Ochocinska and Hitchcock, 2007). Twelve Hsp:nrd-EGFP transgenic animals and twelve control animals were analysed.

Immunohistochemistry and TUNEL assay

All immunocytochemistry protocols were performed as previously described (Hitchcock et al., 1996). Omitting primary antibodies served as negative controls. In the absence of primary antibodies, no staining was observed. Ganglion cells were labeled using the mouse monoclonal antibody zn-12 (The Zebrafish International Resource Center, Eugene, OR; catalog #072103), which labels early born neurons in the zebrafish and ganglion cells in the retina. Amacrine cells were labeled using the cell type-specific monoclonal antibody against rat syntaxin (Monoclonal Anti-Syntaxin Clone HPC-1; Sigma; catalog #S0664). Müller glia cells were labeled using a monoclonal antibody against glutamine synthetase (GS; Chemicon, Temecula, CA, catalog #MAB305), diluted 1:500. Cone photoreceptors were labeled using the mouse monoclonal antibody zpr-1, formerly Fret43, (Larison and Bremiller, 1990; The Zebrafish International Resource Center, Eugene, OR; catalog #092502), which labels an unidentified epitope on red/green cones. Rod photoreceptors were labeled using the mouse monoclonal antibody zpr-3 (The Zebrafish International Resource Center, Eugene, OR; catalog #011604; formerly Fret11; Schmitt and Dowling, 1996), which labels an unidentified epitope on rod photoreceptors. BrdU was detected using a monoclonal antibody against BrdU (Becton Dickinson Immunocytochemistry Systems, San Jose, CA; catalog #347580). For double immunocytochemistry experiments combining zn12, HPC1, GS, zpr-1 or zpr-3

and BrdU, the tissue was processed for zn12, HPC1, GS, zpr-1 or zpr-3 antibody staining first, fixed a second time in 4% paraformaldehyde in phosphate buffer, and then processed for BrdU immunocytochemistry using a rat monoclonal antibody (Abcam®, Cambridge, MA). All primary antibodies, unless otherwise noted, and secondary antibodies conjugated to fluorescent labels were diluted 1:200. For animals heat-shocked at 24hpf and analyzed at 48hpf, twelve transgenic and twelve control animals were analyzed. For animals heat-shocked at 48hpf and analyzed at 72hpf, five transgenic and five control animals were analyzed. An In Situ Cell Death Detection Kit, TMR red (Roche) was also used to detect apoptotic cells.

***In situ* hybridization**

In situ hybridization on whole embryos was performed according to Westerfield (2000) in 1.5ml eppendorf tubes. *In situ* hybridization was performed using the following DIG-labeled riboprobes: neuroD and Islet1. Embryos were fixed in 4% paraformaldehyde, dehydrated in a Methanol series and stored in 100% methanol at –20˚C. Embryos were returned to room temperature, rehydrated, fixed in 4% paraformaldehyde, permeabilized with 0.1 M proteinase K, fixed a second time in 4% paraformaldehyde, treated with acetic anhydride, washed in PBS with 1% Tween, and pre-hybridized in hybridization buffer for 1-2 hours. The pre-hybridization solution was removed and 200ng of probe in 80µl of hybridization solution was pipetted onto embryos and hybridized overnight at 55°C. The next day, the embryos were washed and probes were detected using alkaline-phosphatase-conjugated antibody against digoxygenin and the

subsequent colorimetric reaction with 4-nitroblue-tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche Molecular Biochemicals, Indianapolis, IN). The color reaction was allowed to proceed for approximately 60 minutes and stopped with PBS. The embryos were then transferred to single concavity slides (Tri-Ess Sciences, Inc., Burbank, CA) and coverslipped for inspection and photography. For each probe, pools of 25–50 transgenic and control animals were analyzed.

In situ hybridization with single probes on sections was performed as previously described (Hitchcock et al., 2001). *In situ* hybridization was performed using the following DIG-labeled riboprobes: neuroD, CyclinD1, CyclinB, CyclinE, and p27. Briefly, full-length cDNAs were linearized and DIG-labeled riboprobes were synthesized with an RNA labeling kit (Roche Diagnostic Corp., Indianapolis, IN). Following prehybridization two hundred nanograms of probe in 80μl of hybridization solution was pipetted onto each slide, coverslipped, and hybridized overnight at 55°C. The next day, the sections were washed and digoxygenin was immunolabeled using an alkaline-phosphatase-conjugated antibody and visualized with NBT/BCIP. Twelve transgenic and twelve control animals were analyzed.

Cell Counts

Transgenic and control animals were heat-shocked at 24hpf and sacrificed at 48hpf. 12 transgenic and 12 control animals were used, and one section was counted per animal. Retinas were labeled with phospo-histone H3. Labeled cells were counted in the entire retina and divided by retinal area. Another set of 12

transgenic and 12 control animals were used for TUNEL cell counts. One section was counted per animal and labeled cells were counted in the entire retina.

Transgenic and control animals were exposed to BrdU and heat-shocked at 48hpf and sacrificed at 72hpf. Five transgenic and five control animals were used, and three sections containing the optic nerve were counted per animal. Cell counts were limited to a region demarcated by BrdU+ cells just central to the CGZ. Retinas were labeled with a panel of cell-type specific markers, zn12, HPC-1, zpr-1, and zpr3. The dorsal and ventral portions of the retina were analyzed separately. Labeled cells were counted in the demarcated region, and ratios of photoreceptors to other neurons and glia, as well as ratios of glia to other neurons were calculated. The ratio was calculated as the average number of cells counted in the three sections of one cell type divided by the average number of cells counted in the same three sections of another cell type. Ratios obtained for controls were compared to ratios obtained in retinas of transgenic animals. The standard error was calculated as the square root of the variance of the ratio of means (Cochran, 1977). To compare ratios between retinas of transgenic and control animals, the pooled two-sample t procedure was used to determine the t statistic (Moore and McCabe, 1998).

Photography

Histological sections and whole-mounts were photographed with a Nikon DMX 1200 digital camera. Digital overlays and figures were assembled in Adobe Photoshop 7.0.

Results

The Hsp70/4:nrd-EGFP expression construct is transcribed in vitro and functional in vivo.

The Hsp70/4:nrdEGFP construct was tested in HEK293 cells *in vitro* and in transient transgenic embryos *in vivo*. To determine if the Hsp:nrd-EGFP construct is transcribed and whether it is targeted to the nucleus *in vitro*, HEK293 cells were transfected with either empty Hsp:EGFP vector or Hsp:nrd-EGFP vector containing NeuroD (Fig.1A). HEK293 cells transfected with the Hsp:EGFP vector contain EGFP protein in the cytoplasm but not in the nucleus (EGFP+ cytoplasm; Fig.1 A and B). In contrast, HEK293 cells transfected with the Hsp:nrd-EGFP vector contain EGFP exclusively in the nucleus (EGFP+ nucleus; Fig.1C and D). These data suggest that whereas both EGFP protein and NeuroD-EGFP fusion protein are transcribed in HEK293 cells, the addition of NeuroD results in targeting of the fusion protein to the nucleus.

Transient transgenic embryos were used to determine if the Hsp:nrd-EGFP construct is transcribed and whether it is targeted to the nucleus *in vivo*. Wild type embryos were injected with the Hsp:nrd-EGFP construct at the 1-2 cell stage and heat-shocked at 24hpf. Animals were then evaluated for EGFP fluorescence at 12 and 24 hours post heat shock (hphs). There was a mosaic of EGFP-expressing cells throughout these embryos at 12 and 24hphs (Fig.2A and B). Transverse sections of transient transgenic embryos at 24hphs show EGFP+ nuclei in the brain (Fig.2C and D). These data show that the Hsp:nrd-EGFP

construct is transcribed *in vivo*, and that the NeuroD fusion protein is targeted to the nucleus.

To determine if the Hsp:nrd-EGFP construct induces ectopic expression of NeuroD, *in situ* hybridization within transient transgenic embryos, using a *neuroD* riboprobe was performed on wholemounts and on sections. NeuroD *in situs* on wholemounts of transient transgenic embryos show ectopic expression of *neuroD*, from head to tail (Fig.3A-D). In transverse sections, ectopic expression of *neuroD* is also detected in clusters of cells throughout the brain and retina (Fig.3E). These data offer further proof that the Hsp:nrd-EGFP is transcribed *in vivo*.

***Hsp70/4:nrd-EGFP transgenic zebrafish lines*.**

After the Hsp70/4:nrd-EGFP construct was validated, it was used to generate stable transgenic lines (see Halloran et al., 2000). Over 150 embryos were injected, successfully raised to adulthood, and screened for germline transmission. Based on the results of the screen, three transgenic lines were established. Southern blotting of digested DNA from each of the lines exhibited a strong hybridization band at 12kb compared to a 5kb band of endogenous NeuroD. The presence of a prominent single 12kb band in the transgenic lines demonstrates that multiple copies of the Hsp70/4:nrd-EGFP plasmid had been integrated into a single site (Fig.4). A measurement of optical density shows that the number of copies is equivalent to 50 copies per haploid genome. This is within the range previously described for other transgenic lines (Xiao et al., 2003). Of the three lines, only one featured robust fluorescence in the retina. The

second line had only a few fluorescent cells in the retina. The third line did not have EGFP positive cells in the retina but did have positive cells in the olfactory placode. The transgenic line with the most robust fluorescence in the entire embryo including the retina was used for the remainder of the experiments.

To determine if the NeuroD fusion protein is inducible in the Hsp:nrd-EGFP transgenic zebrafish line, transgenic embryos and wild type controls from the same clutch were heat-shocked at 15hpf, 24hpf, or 48hpf and evaluated for the presence EGFP+ cells at 32hpf, 48hpf, or 72hpf, respectively. Compared to heat-shocked wild type animals from the same clutch, which do not have EGFP fluorescent cells, EGFP+ cells were present throughout the entire embryo (Fig.5A-F). Specifically, robust EGFP fluorescence can be seen in transgenic animals heat-shocked at 15hpf and photographed at 32hpf (Fig.5A and B), heat-shocked at 25hpf and photographed at 48hpf (Fig.5C and D), and heat-shocked at 48hpf and photographed at 76hpf (Fig.5E and F). These data demonstrate that the NeuroD fusion protein is inducible in the Hsp:nrd-EGFP transgenic line.

To determine if the NeuroD fusion protein is stable *in vivo*, a NeuroD zebrafish antibody (zNrd) was generated and used to detect NeuroD fusion protein from the Hsp:nrd-EGFP transgenic line (Fig.6). The zNrd antibody was affinity purified and assayed through competition with synthetic peptides and Western analysis (Fig.6A and B). The zNrd antibody was generated in two rabbits (ZYMED Laboratories; Invitrogen), and the affinity purified antibody from rabbit #1 was used for the remainder of the experiments (Fig.6A; lane 5). The pre-immune serum from rabbit #1 does not detect any bands in protein lysates

from adult zebrafish brain compared with pre-immune serum from rabbit #2 (Fig.6A; lane 5; compare lane 4 with lane 3). On Western blots of protein obtained from adult zebrafish brain, the zNrd antibody binds 30kD and 36kD proteins (Fig.6A and B). In a competition assay, these protein bands can be blocked with NeuroD peptide (Fig.6B). This suggests that the zNrd antibody specifically binds endogenous NeuroD protein.

After the zNrd antibody was characterized, it was used to detect NeuroD fusion protein in the Hsp:nrd-EGFP transgenic line. Protein was extracted from a cohort of 150 embryos from a heterozygous cross heat-shocked at 24hpf and sacrificed at 48hpf. The zNrd antibody binds 30kD and 80kD proteins on western blots (Fig.6C). The 30kD band corresponds to the endogenous NeuroD protein; however, the 36kD band observed in adult brain is not detected in the embryo (compare Fig.6B and C). The 80kD band corresponds to the NeuroD-EGFP fusion protein, which can also be detected using an EGFP antibody. Since the zNrd and EGFP antibodies detect the same 80kD band (Fig.6C; compare lane 2 with lane 1), this suggests that the NeuroD fusion protein can be detected on Western blot using the zNrd antibody (Fig.6C) and that the NeuroD fusion protein is stable *in vivo*.

To determine if the NeuroD fusion protein is functional *in vivo*, Hsp:nrd-EGFP transgenic embryos were examined for the expression of *neuroD* or *islet-1*, a putative downstream target of NeuroD (Wang et al., 2000). Transgenic and wild type embryos from the same clutch were heat-shocked at 15hpf and assayed at 25hpf for *neuroD* and *islet-1* expression (Fig.7A and B). Compared to

controls, there was ectopic expression of both *neuroD* (Fig.7C and D) and its downstream target *islet-1* (Fig.7E and F). These data show that the NeuroD fusion protein is transcribed, as neuroD message can be detected, and that the fusion protein can induce ectopic expression of its downstream target *islet-1*. This demonstrates that the NeuroD fusion protein is functional *in vivo*.

NeuroD promotes cell cycle exit *in vivo*

NeuroD has been shown to promote cell cycle withdrawal in other systems (Lee et al., 1995; Farah et al., 2000). Zebrafish transgenic for Hsp:nrd-EGFP were used to test the hypothesis that NeuroD promotes cell cycle withdrawal among photoreceptor progenitors in the retina. Approximately 12 hours following heat shock animals from a single heterozygous cross were sorted based on EGFP fluorescence into wild-type (no EGFP+ cells), heterozygous (few EGFP+ cells), and homozygous groups (many EGFP+ throughout the embryo including the retina). Only the wild-type and homozygous, here termed transgenic, groups were used for these experiments. The heterozygous animals were discarded. After sorting, wild-type and transgenic animals were exposed to a brief systemic pulse of BrdU at 48hpf by immersion in a 5mM solution of BrdU in 15% DMSO for 20 minutes and sacrificed immediately afterward.

The retinas of transgenic fish contained many EGFP fluorescent cells throughout the retina (Fig.8A). The control retinas (Fig.8D) contained many mitotically-active, BrdU-positive cells in the circumferential germinal zone, the outer nuclear layer, and portions of the inner nuclear layer. In contrast, the retinas of transgenic fish contained many fewer BrdU+ cells (Fig.8B). This

difference is especially striking for the CGZ, which, in wild-type retina, contains many BrdU-positive cells and, in retinas of transgenic animals, contains none or few BrdU-positive cells (compare with Fig.8B and Fig.8D; arrows). Only a few BrdU positive cells can be seen in the transgenic retina at 48hpf, and these cells do not co-localize with EGFP (Fig.8C). The number of mitotically active cells was quantified using another marker of proliferation, phospho-histone H3. The data show significantly fewer ph3 labeled cells in transgenic retinas following heat shock compared to controls (Fig.9A). Taken together, the BrdU and phospho-histone H3 data suggest that NeuroD fusion protein promotes cell cycle exit in the embryonic zebrafish retina.

An alternative explanation for the data, however, is that the transgenic cells do not exit from the cell cycle but rather undergo apoptosis. To test for this, apoptotic cells in the retinas of transgenic and wild type fish were labeled using the TUNEL assay. Cell counts show that compared to controls there is no increase in TUNEL+ cells in the retinas of transgenic fish and, in fact, there are significantly fewer TUNEL+ cells in the retinas of transgenic fish (Fig.9B). These data show that the presence of the NeuroD fusion protein does not result in increased cell death. This suggests that the decrease in BrdU+ and pH3+ cells is a consequence of cells withdrawing from the cell cycle and not cell death.

A panel of probes for cell cycle regulatory proteins including CyclinD1, CyclinB, CyclinE, and the cyclin inhibitors p27 and p57 was used to investigate the potential mechanism by which NeuroD promotes cell cycle exit. Animals were heat-shocked at 24hpf and assayed by *in situ* hybridization at 48hpf. The retinas

of transgenic and control animals were processed on the same slides with a panel of cyclin probes. Compared to controls, the retinas of transgenic animals contain many fewer cells that express CyclinD, CyclinB, and CyclinE (Fig.10A-F). In control retinas, CyclinD1 is expressed in cells in the CGZ, and this expression is absent in the retinas of transgenic animals (Fig.10A and B). In controls, CyclinB is expressed throughout the retina, but the level of expression, as indicated by the intensity of riboporobe staining, is higher in cells found in the circumferential germinal zone (Fig.10C; arrows). In contrast, in the retinas of transgenic animals, CyclinB expression is significantly reduced in the retinas of transgenic animals, including in cells in the CGZ (Fig.10C and D; arrows). In control animals, CyclinE is expressed in the CGZ and in scattered cells in the remainder of the retina (Fig.10E). In contrast, in the retinas of transgenic animals, CyclinE expression is absent (Fig.10F; arrows). The retinas of transgenic and control animals were also processed with probes for cyclin inhibitors. In controls, p27 is expressed throughout the retina (Fig.10G). In the retinas of transgenic animals, the level of p27 expression is significantly increased, as indicated by the intensity of riboporobe staining, and p27 riboprobe label is especially intense in the CGZ (Fig. 10G-J; arrows). In controls, low level of p57 expression is present throughout the retina except for the peripheral portion of the CGZ where p57 is not expressed (Fig.10 I; arrows). In contrast, in the retinas of transgenic animals, p57 is expressed in the CGZ (Fig.10J; arrows), and the level of p57 expression, as indicated by the intensity of p57 riboprobe staining, is increased throughout the retina (compare Fig.10 I and Fig.10 J). These data suggests that the

presence of NeuroD fusion protein results in a decrease in the expression of CyclinD, CyclinB, and CyclinE and an increase in the expression of p27 and p57. Based on these data, I conclude that NeuroD promotes cell cycle withdrawal by regulating the expression of cyclin inhibitors and cyclins required for cell cycle progression.

NeuroD promotes photoreceptor genesis and inhibits gliogenesis in the late but not early stage of retinal neurogenesis.

bHLH transcription factors, including NeuroD, have been shown to be sufficient to induce neuronal determination and differentiation (Lee et al., 1995; Farah et al., 2000). To test the hypothesis that NeuroD promotes photoreceptor genesis, transgenic and control embryos were heat-shocked at 24hpf and assayed at 48hpf using a panel of cell-type specific neuronal markers, including zn12 (ganglion cells), HPC-1 (amacrine cells), zpr-1 (cone photoreceptors), and zpr-3 (rod photoreceptors). The data show that cells which withdraw from the cell cycle in retinas of transgenic animals between 24-48hpf do not express markers of differentiated cells (Fig.11E-H). This demonstrates that, between 24-48hpf, the NeuroD fusion protein is not sufficient to promote photoreceptor or neuronal genesis *in vivo*.

One explanation for these data is that between 24-48hpf the retinal environment is not permissive for photoreceptor genesis, which could explain why the NeuroD fusion protein is not sufficient to promote photoreceptor genesis at this time. To surmount this potential problem, zebrafish were exposed to BrdU and heat-shocked at 48hpf, a time when the retinal environment is permissive

(Raymond et al., 1995). NeuroD fusion protein was strongly induced in the CGZ following heat shock at 48hpf (Fig.12; arrows), but EGFP fluorescence was not detectable in the retina by conventional fluorescence microscopy at 72hpf (Fig.13). Therefore, BrdU was used to track the cohort of cells within the CGZ at 48hpf, and the fates of these cells were assayed at 72hpf using the panel of cell type specific markers described above, plus glutamine synthetase (GS) for Müller glia. Further, because there is a dorsal and ventral asymmetry in the zebrafish retina at 72hpf (Hyatt et al., 1996; Schmitt and Dowling, 1999), the dorsal and ventral portions of the retina were analyzed separately. Cell counts were limited to a region demarcated by BrdU-labeled cells just central to the CGZ (Fig.14A and B; the white lines indicate the left and right boundary for the dorsal and ventral retina regions evaluated, respectively). Cell labeled with cell-type specific markers were counted, and ratios of photoreceptors to other neurons and glia, as well as ratios of glia to other neurons were calculated (Fig.14C-H; see also Table 1 for original tabulated cell counts). Three major observations emerged from this experiment, corresponding to cone photoreceptor ratios, rod photoreceptor ratios, and Müller glia ratios. First, in the dorsal retina of transgenic animals, there is no significant difference in the proportion of cone photoreceptors to other cell types (Fig.14C). However, data show that there is a higher proportion of cone photoreceptors to Müller glia and a lower proportion of cone photoreceptors to amacrine cells (Fig.14D). Second, the data show a significantly higher proportion of rod photoreceptors compared to all other cell types in the dorsal retina (Fig.14E). In the ventral retina, the data also show a higher proportion of rod

photoreceptors to Müller glia (Fig.14F). Third, in dorsal retina of transgenic animals, the proportion of Müller glia is significantly lower compared to rod photoreceptors (Fig.14G). In ventral retina of transgenic animals, the proportion of Müller glia is significantly lower compared to all other cell types (Fig.14H). Taken together, these data show that among the cells born at the CGZ, there was a higher proportion of rod and cone photoreceptors and a concomitantly lower proportion of Müller glia in the retinas of transgenic animals. These results suggest that induced expression of NeuroD in retinal progenitors of the CGZ is sufficient to generate photoreceptors between 48-72hpf. The data further suggest that the NeuroD fusion protein inhibits gliogenesis.

Discussion

The present study used a line of zebrafish transgenic for Hsp70/4:nrd-EGFP for conditional gain-of-function experiments to test the hypothesis that NeuroD promotes cell cycle withdrawal of photoreceptor progenitors and promotes photoreceptor genesis in the zebrafish retina. Proliferation and photoreceptor genesis, the two components of the hypothesis, were examined separately. The Hsp70/4:nrd-EGFP line of zebrafish allowed for temporal control of induced expression of NeuroD. Proliferation was examined using BrdU and phospho-histone H3 labeling, and potential mechanisms were investigated utilizing probes for cell cycle regulatory proteins. Photoreceptor genesis was characterized using a panel of cell type-specific antibodies. Results indicate that NeuroD promotes cell cycle withdrawal, and at 48hpf is sufficient to promote photoreceptor genesis

and inhibit gliogenesis. These experiments are the first *in vivo* functional assay of cell cycle regulation by NeuroD in the vertebrate retina.

Previous *in vitro* studies have shown that NeuroD promotes cell cycle withdrawal (Farah et al., 2000; Ohnuma et al., 2001; Ohnuma et al., 2002). In my study, two approaches were used to determine the effect of NeuroD on the cell cycle *in vivo*. The first used cell cycle markers, BrdU and phospho-histone H3, to compare proliferation in retinas in transgenic and control animals. The second approach investigated the potential mechanism of cell cycle regulation using probes for cell cycle regulatory proteins, CyclinD1, CyclinB, CyclinE, p27, and p57, to evaluate induced NeuroD on the expression of cell cycle regulatory proteins. The conditional gain-of-function experiments reveal that in the zebrafish retina NeuroD promotes cell cycle exit. Following heat shock retinas in transgenic animals showed robust EGFP fluorescence throughout the retina, indicating the presence of NeuroD fusion protein. In addition, compared with wild type controls, there was a significant decrease in the number of BrdU+ and phospho-histone H3+ cells. This was especially striking in the circumferential germinal zone, which, in the transgenic retinas, was almost devoid of BrdU labeled cells. These results suggest that NeuroD plays a key role in promoting cell cycle withdrawal.

To investigate the mechanism through which NeuroD promotes cell cycle withdrawal in the retina, probes for cell cycle regulatory proteins, CyclinD1, CyclinB, CyclinE, p27, and p57, were used to evaluate induced NeuroD on the expression of cell cycle regulatory proteins. Several studies indicate that NeuroD promotes cell cycle withdrawal by modulating the expression of cell cycle

regulatory proteins. Cells transfected with NeuroD show elevated expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and cell cycle withdrawal (Farah et al., 2000). In enteroendocrine cells, NeuroD induces cell cycle arrest with a concomitant increase in p21 expression, an inhibitor of cyclin-dependent kinases (Naya et al., 1997; Mutoh et al., 1998; Schonhoff et al., 2004). In the mouse retina, it has been shown that p27 can regulate cell cycle withdrawal of late progenitor cells (Levine et al., 2000; Dyer and Cepko, 2001a,b). In the present study, induced expression of NeuroD leads to the upregulation of *p27* and *p57* and concomitant downregulation of *CyclinD1*, *CyclinB* and *CyclinE*. Thus, in the zebrafish retina, consistent with the previous studies, NeuroD may promote cell cycle withdrawal by modulating the expression of cell cycle regulatory proteins.

Based on the literature and my observations in this study, a parsimonious interpretation of the results is that NeuroD upregulates the expression of p27 and p57, which, in turn, leads to the downregulation of the expression of cyclins D, B and E, which are required for cell cycle progression. This suggests that cyclin inhibitors p27 and p57 may be downstream targets of NeuroD. Several lines of evidence support this speculation. Cell cycle exit of NeuroD-transfected cells is preceded by elevated expression of the cyclin-dependent kinase inhibitor p27Kip1 and cell cycle withdrawal (Farah et al., 2000). NeuroD null mice show reduced p21 expression, with increased expression of cell proliferation markers, suggesting that NeuroD regulates cell cycle progression though p21 (Schonhoff et al., 2004). Another bHLH transcription factor MyoD has been shown to synergize with p27 to promote cell cycle withdrawal of muscle progenitors

(Vernon and Philpott, 2003). Taken together, based on its expression pattern in photoreceptor progenitors (Chapter II) and the data presented here, NeuroD may act on a network of cell cycle regulatory genes, which are modulated by cyclins and cyclin inhibitors, to promote cell cycle exit of photoreceptor progenitors in the zebrafish retina.

The Hsp:nrd-EGFP transgenic line was also used to test the hypothesis that NeuroD promotes photoreceptor genesis. In other systems NeuroD is sufficient to promote neurogenesis (Lee et al., 1995; Lee et al., 1997; Farah et al., 2000). In the central and peripheral nervous systems, NeuroD is expressed transiently in a subset of neurons at the time of their terminal differentiation (Lee et al., 1995). Ectopic expression of NeuroD in Xenopus embryos causes premature differentiation of neuronal precursors in the embryo and ectopic differentiation of neurons in the retina (Lee et al., 1995; Moore et al., 2002; Logan et al., 2005). NeuroD can also convert presumptive epidermal cells into neurons (Lee et al., 1995; Lee et al., 1997). In the present study, although NeuroD is sufficient to promote cell cycle withdrawal in the retina at 24hpf, it is not sufficient to promote photoreceptor genesis. The transgenic cells that withdraw from the cell cycle do not precociously express markers of differentiated cells. In contrast, at 48hpf, NeuroD is sufficient to promote photoreceptor genesis, and, in addition, inhibit gliogenesis. These results suggest that NeuroD is sufficient to promote photoreceptor genesis in a retina that is permissive for this process to occur.

The significant increase in the proportion of photoreceptors, and the concomitant decrease in the proportion of Müller glia, in transgenic fish are

consistent with the role of endogenous NeuroD, and bHLH transcription factors in general, as promoters of neurogenesis and inhibitors of gliogenesis (Sun et al., 2001; Morrow et al., 1999; Tomita et al., 2000). In the retinas of NeuroD-null mice there is a three to fourfold increase in Müller glia, and forced expression of NeuroD in progenitors promotes neurogenesis and blocks gliogenesis (Morrow et al., 1999). In chick, retinal cells transfected with NeuroD never express glutamine synthetase, suggesting that NeuroD suppresses glial differentiation (Fischer et al., 2004). In the mammalian cerebral cortex, a panel of bHLH transcription factors was investigated, and a subset of the bHLH genes, including NeuroD, was capable of promoting the choice of neuronal versus glial cell fate (Cai et al., 2000).

The data from my present study support the hypothesis that NeuroD promotes photoreceptor genesis in the retina. However, the process of genesis involves everything from division, cell fate specification, withdrawal from the cell cycle, and differentiation. It is important to parse out the exact function of NeuroD in this process. So far I have offered evidence that NeuroD promotes cell cycle withdrawal, but whether NeuroD is determinative for photoreceptors or only promotes their differentiation is still not resolved. There are lines of evidence that support both scenarios. In the chick retina, NeuroD is determinative for photoreceptors, and misexpression of NeuroD through replication-competent transformation-deficient retroviruses results in retina with three instead of two layers of photoreceptor cells (Yan and Wang, 1998), and loss-of-function assays result in absence of photoreceptors (Yan and Wang, 2004). In addition,

misexpression of NeuroD in monolayer cultures of retinal pigment epithelium yields *de novo* production of photoreceptor cells only (Yan and Wang, 1998; Yan and Wang, 2000), which express visinin and a number of photoreceptor genes. These findings suggest that NeuroD promotes photoreceptor cell production in the chick retina and may function in both determination and differentiation of photoreceptors. In my present study, I induce expression of NeuroD fusion protein in the CGZ, which contains cells that don't normally express this gene (see Chapter2). The data show that among cells born at the CGZ, induced expression of NeuroD results in an increased proportion of photoreceptors and decreased proportion of Müller glia, suggesting that NeuroD functions in photoreceptor determination. In addition, fewer Müller glia are generated suggesting that NeuroD also inhibits gliogenesis and that cells destined to the glia cell fate now become photoreceptors. Thus, one interpretation is that NeuroD functions in the determination step of the photoreceptor genesis process.

In contrast to chick and zebrafish retina, in mouse and rat retinas NeuroD is expressed in postmitotic photoreceptor precursors in early as well as late neurogenesis suggesting that NeuroD plays an important role in the terminal differentiation of photoreceptors (Ahmad et al., 1999; Akagi et al., 2005; Cheng et al., 2006). Along with other key transcriptional regulators Nrl, Crx, and Nr2E3, NeuroD forms part of a large regulatory complex which promotes rod photoreceptor differentiation in the mouse retina (Mears et al., 2001; Cheng et al., 2006; Oh et al., 2007). Overexpression of NeuroD during late neurogenesis promotes premature differentiation of late-born neurons, including rod

photoreceptors, and NeuroD specifically interacts with the E-box element in the proximal promoter of the phenotype-specific gene, opsin (Ahmad et al., 1999). These findings suggest that NeuroD promotes differentiation of photoreceptors. Thus an alternative interpretation of results from the present study is that NeuroD may function in the terminal differentiation step of photoreceptor genesis. The induction of NeuroD may simply promote premature differentiation of photoreceptor cells leading to the increased proportions of photoreceptors observed in the retinas of transgenic animals.

In summary, conditional induction of NeuroD expression reveals that NeuroD promotes cell cycle withdrawal, potentially by inducing the expression of cyclin inhibitors p27 and p57, and promotes photoreceptor genesis and inhibits gliogenesis. This suggests that in mitotically active cells of the rod and cone lineages, NeuroD normally functions as the molecular link that ties cell cycle withdrawal with determination and or differentiation.

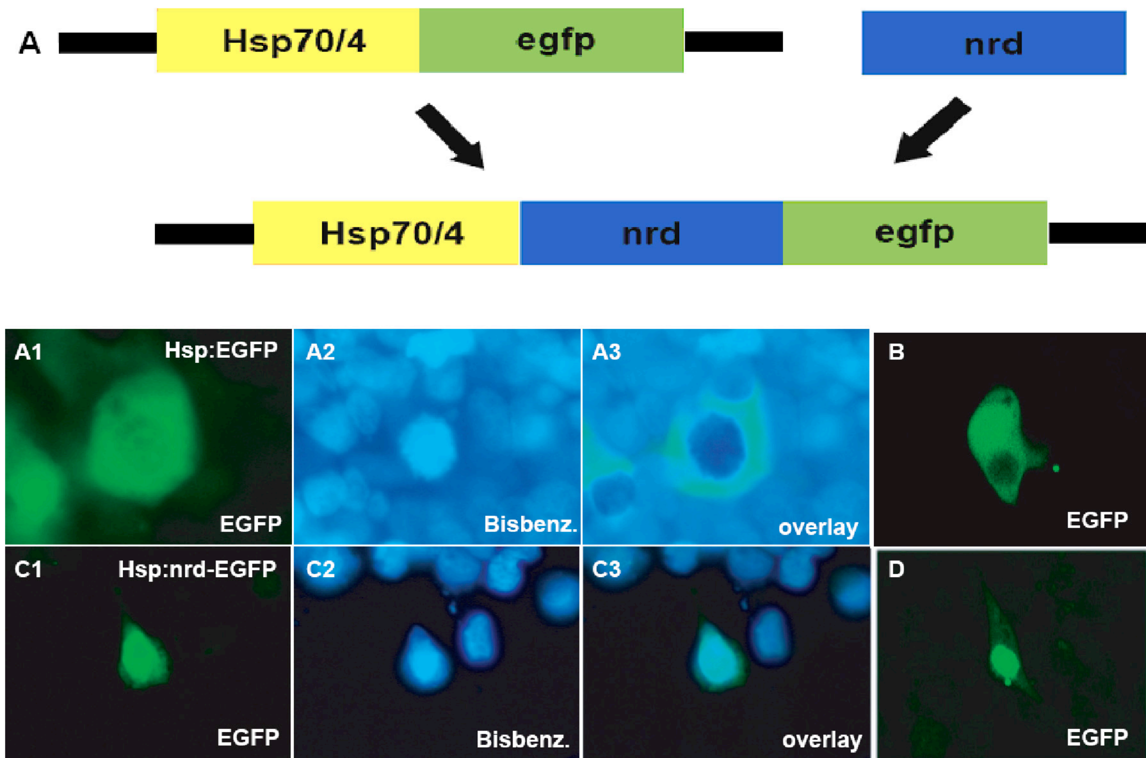


Fig. III-1: The Hsp:nrd-EGFP construct is transcribed *in vitro*.

A: The Hsp70/4-EGFP construct and 1kb coding region of the zebrafish nrd. The Hsp70/4:nrd-EGFP construct was assembled by subcloning the 1kb coding region of the zebrafish nrd gene into the Hsp70/4:EGFP vector. A1-A3 and B: The empty Hsp:EGFP construct is localized in the cytoplasm of HEK293 cells. C1-C3 and D: The Hsp:nrd-EGFP construct is selectively targeted to the nucleus and transcribed (EGFP+ nucleus).

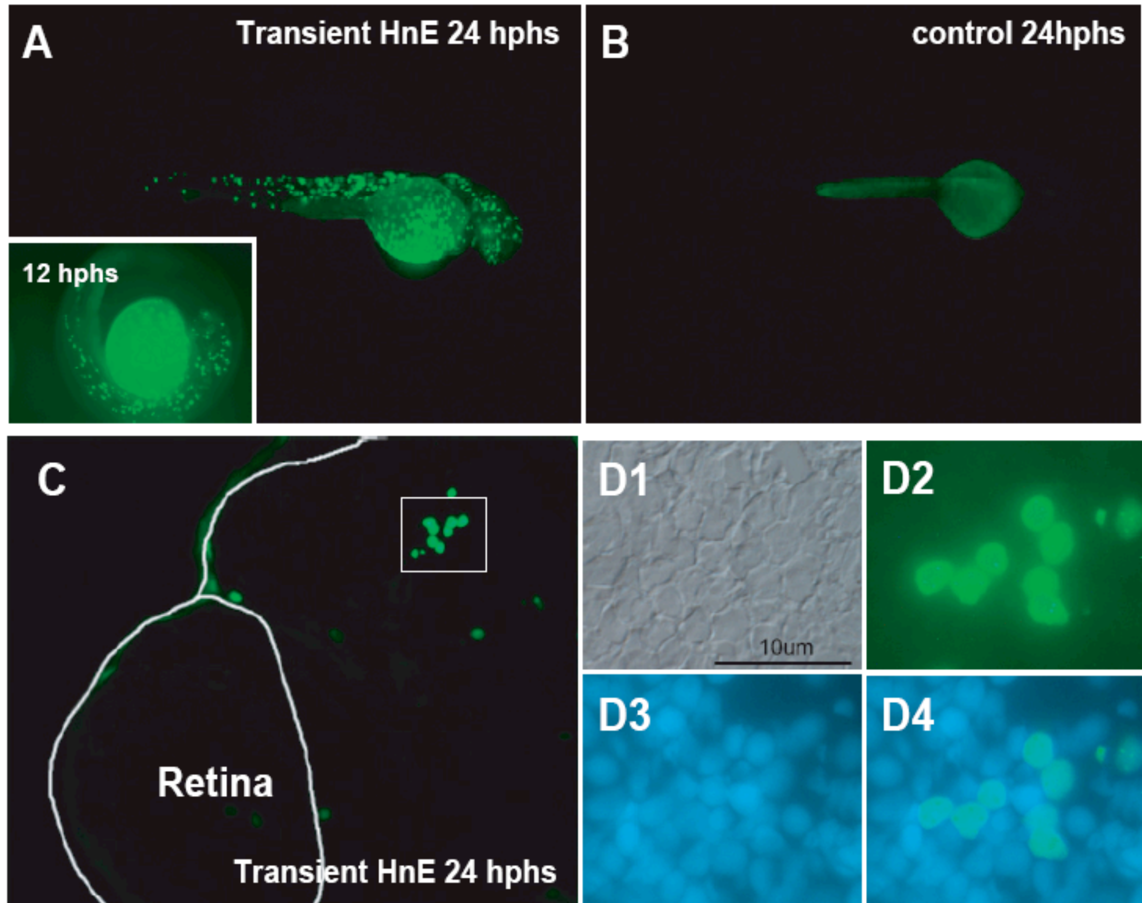


Fig. III-2: The Hsp:nrd-EGFP construct is transcribed *in vivo*.

A: Transient transgenic embryo heat-shocked at 24hpf showing mosaic EGFP+ cells 24 hours post heat shock (hphs) and at 12hphs (inset). B: Control embryo 24hphs. C-D: Nuclear localization of the Hsp:nrd-EGFP construct in a transient transgenic embryo. hphs-hours post heat shock

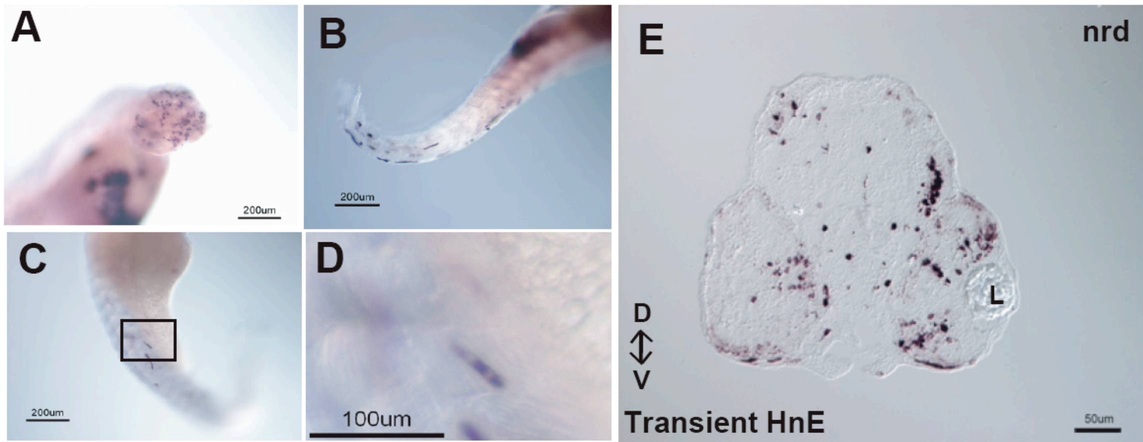


Fig. III-3: The Hsp:nrd-EGFP construct induces ectopic *neuroD* expression.

A-D: NeuroD *in situs* showing ectopic NeuroD expression in wholemounts from head to tail. E: Transverse section of Hsp:nrd-EGFP injected embryo 72hphs (96hpf).

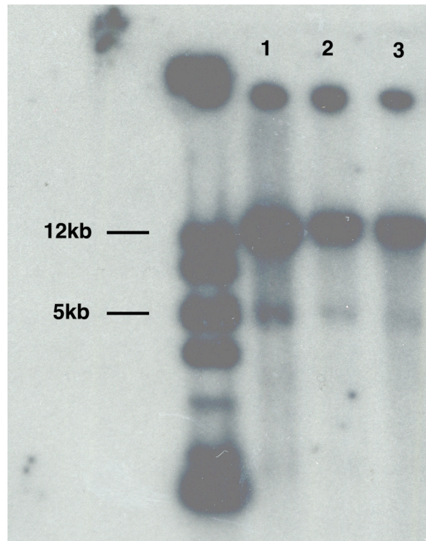


Fig. III-4: Southern analysis of zebrafish lines transgenic for Hsp:nrd-EGFP.

The first lane is the marker lane and lanes labeled 1-3 correspond to the three Hsp:nrd-EGFP transgenic lines. Lane 3 is from the line that was chosen based on robust fluorescence throughout the embryo and retina. Note the endogenous 5kb NeuroD band and the intensely labeled 12kb band depicting the transgene.

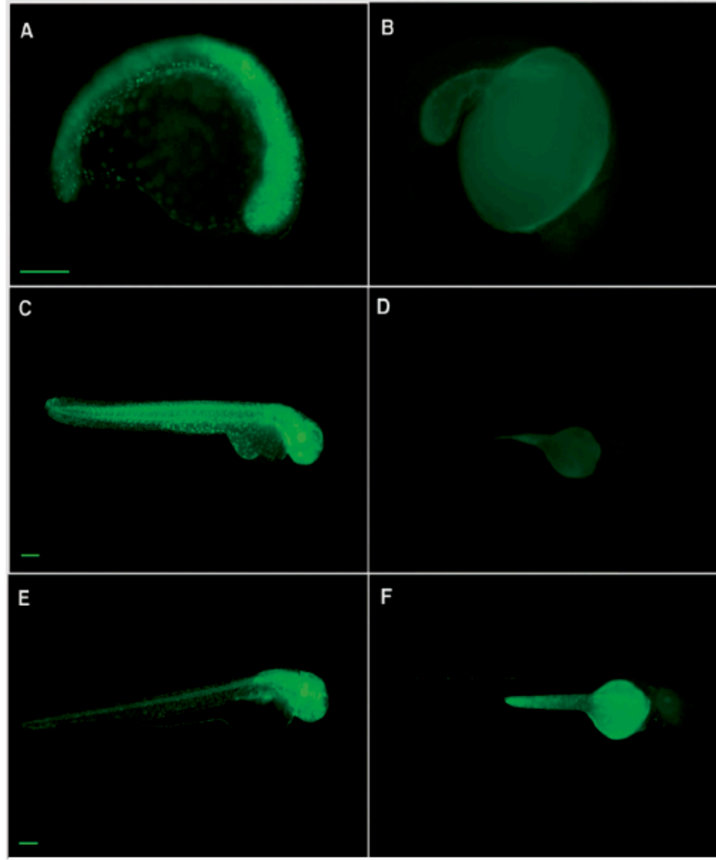


Fig. III-5: The NeuroD fusion protein is inducible in the Hsp:nrd-EGFP transgenic zebrafish line.

A and B: Transgenic embryo (A) and wild type control (B) heat-shocked at 15hpf and photographed at 32hpf. C and D: Transgenic embryo (C) and wild type control (D) heat-shocked at 25hpf and photographed at 48hpf. E and F: Transgenic embryo (E) and wild type control (F) heat-shocked at 48hpf and photographed at 76hpf.

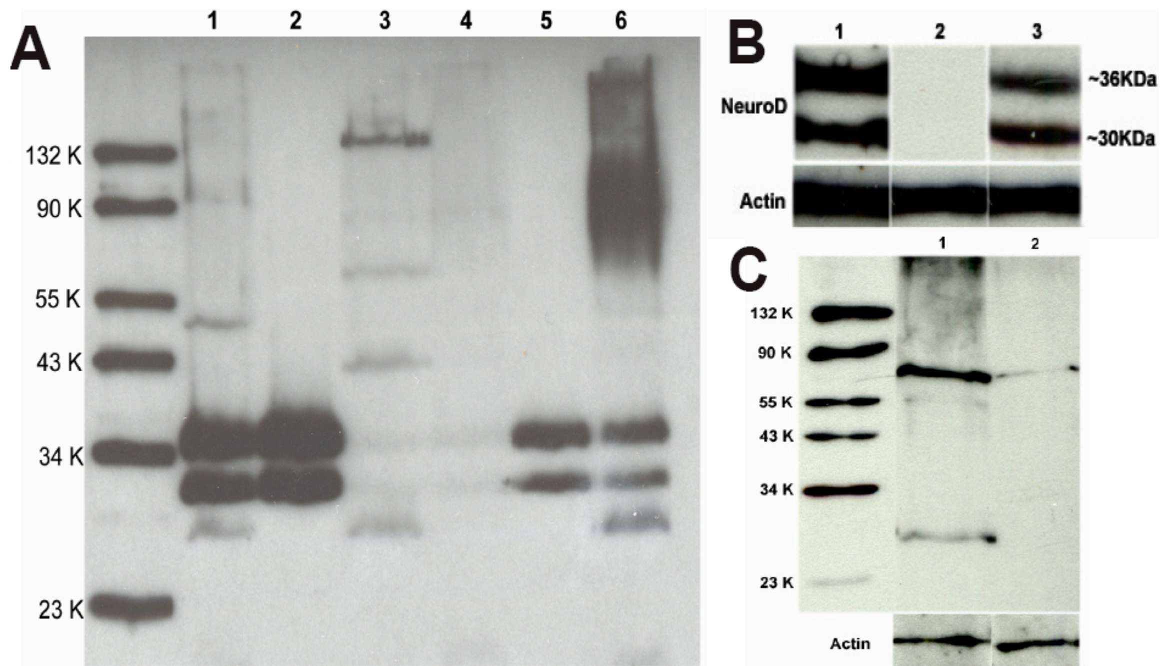


Fig. III-6: Western analysis of the zebrafish NeuroD antibody (Ab zNrd).

A: Pre-affinity purified (1, 6), Affinity purified (2, 5) and pre-immune serum (3, 4) immunoreactivity with adult zebrafish brain. Lanes 1-3 correspond to rabbit #2 and lanes 4-6 correspond to rabbit #1. B: Peptide competition of Ab zNrd. Uncompeted (1), competed (2), and (3) blot 2 stripped and reprobed with Ab zNrd. C: Ab zNrd binds endogenous NeuroD and fusion protein in HnE transgenic line. 24HS48hpf embryos labeled with Ab zNrd (1) and EGFP antibody (2).

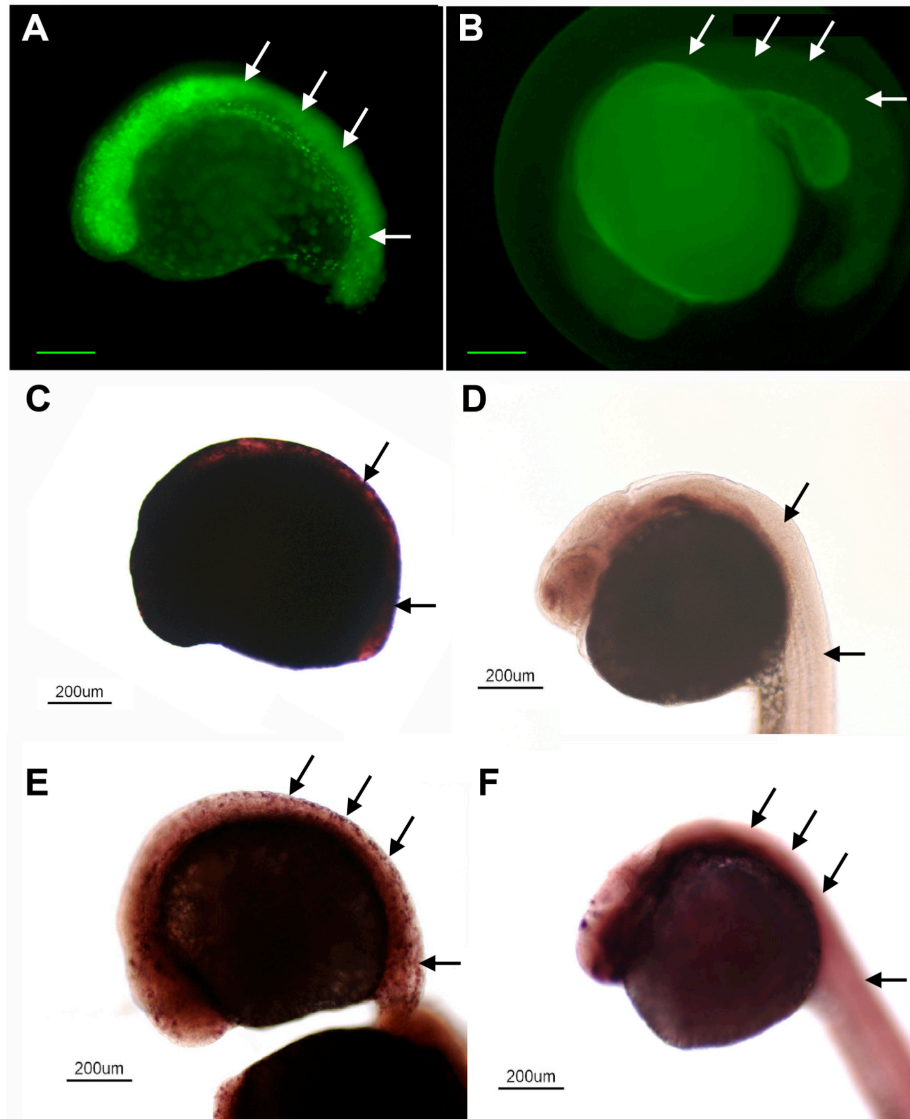


Fig. III-7: The NeuroD fusion protein is functional *in vivo*.

A and B: transgenic and wild type embryos from the same clutch heat-shocked at 15hpf and assayed at 25hpf. C and D: neuroD expression in transgenic and wild type control embryos, respectively. Note the ubiquitous expression of EGFP fluorescence throughout the transgenic embryo (C) compared to the control (D). E and F: Islet-1 expression in transgenic and wild-type control embryos. Note the ectopic expression of both neuroD (C, arrows) and its downstream target islet-1 (E, arrows) in the Hsp:nrd-EGFP transgenic embryos compared to controls (D and F, respectively).

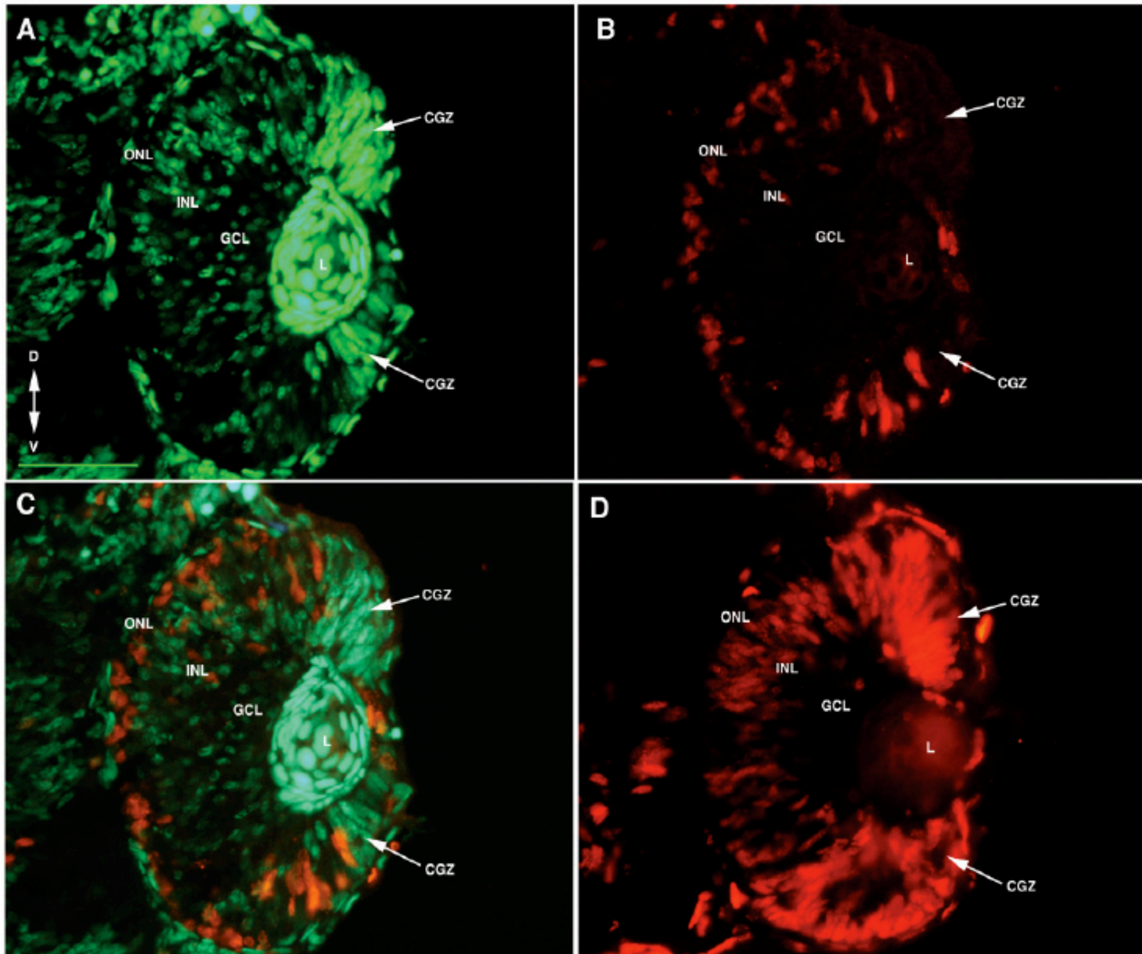


Fig. III-8: NeuroD promotes cell cycle exit *in vivo*.

A and B: EGFP expression (A) and BrdU labeling (B) in the retina of a transgenic embryo heat-shocked at 24hpf and sacrificed at 48hpf; Note the absence of BrdU labeling in the circumferential germinal zone (CGZ). C: Overlay of A and B. D: BrdU labeling in an age-matched heat-shocked wild-type control (compare with B).

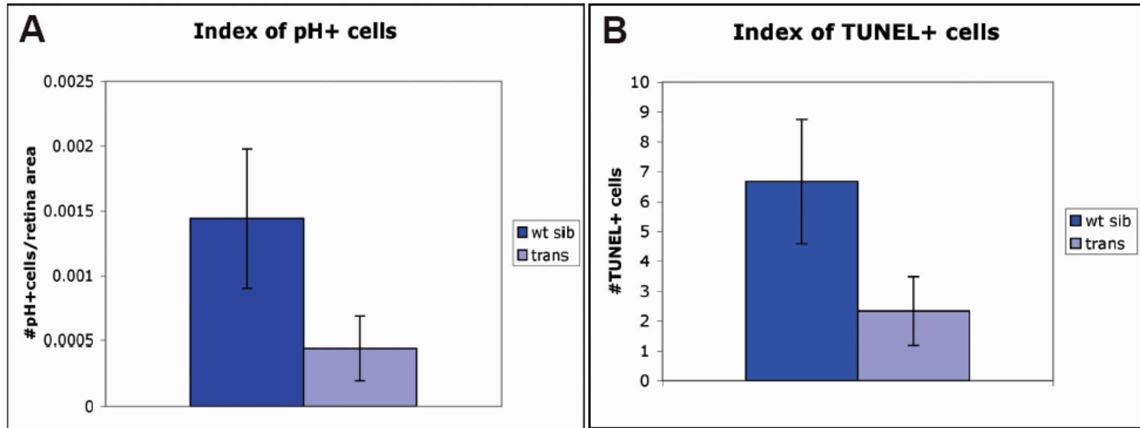


Fig. III-9: NeuroD promotes cell cycle exit *in vivo* and the presence of the NeuroD fusion protein does not result in cell death.

A: Bar graph showing tabulated pH3 data for 12 transgenic and 12 wild type retinas. Note the significant decrease in the number of mitotically active cells in the transgenic compared to control retinas. B: Bar graph showing tabulated TUNEL data for 12 transgenic and 12 wild type retinas. Note the low levels of TUNEL+ cells in both wild type and transgenic retinas, plus a significant decrease in TUNEL+ cells in the transgenic compared to control retina.

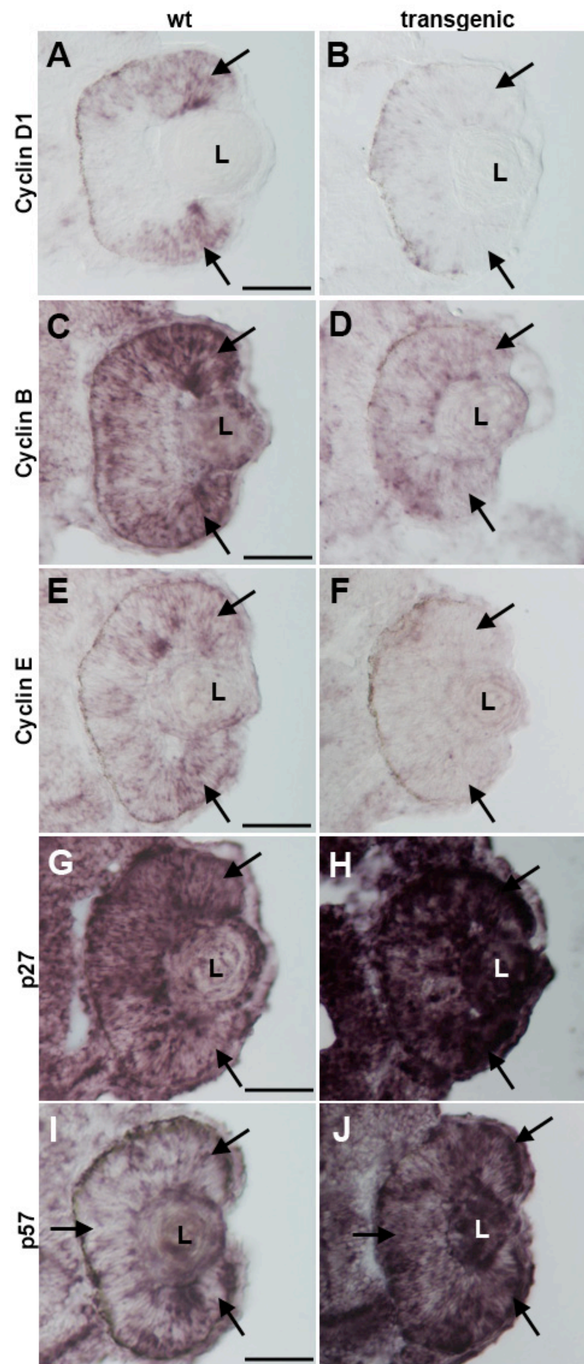


Fig. III-10: NeuroD inhibits cyclin expression and promotes the expression of cyclin inhibitors.

A-F: The expression of CyclinD, CyclinD, and CyclinE is downregulated in transgenic retinas compared to controls. G-J: The expression of cyclin inhibitors p27 and p57 is upregulated in transgenic retinas compared to controls. Animals were heat-shocked at 24hpf and assayed at 48hpf.

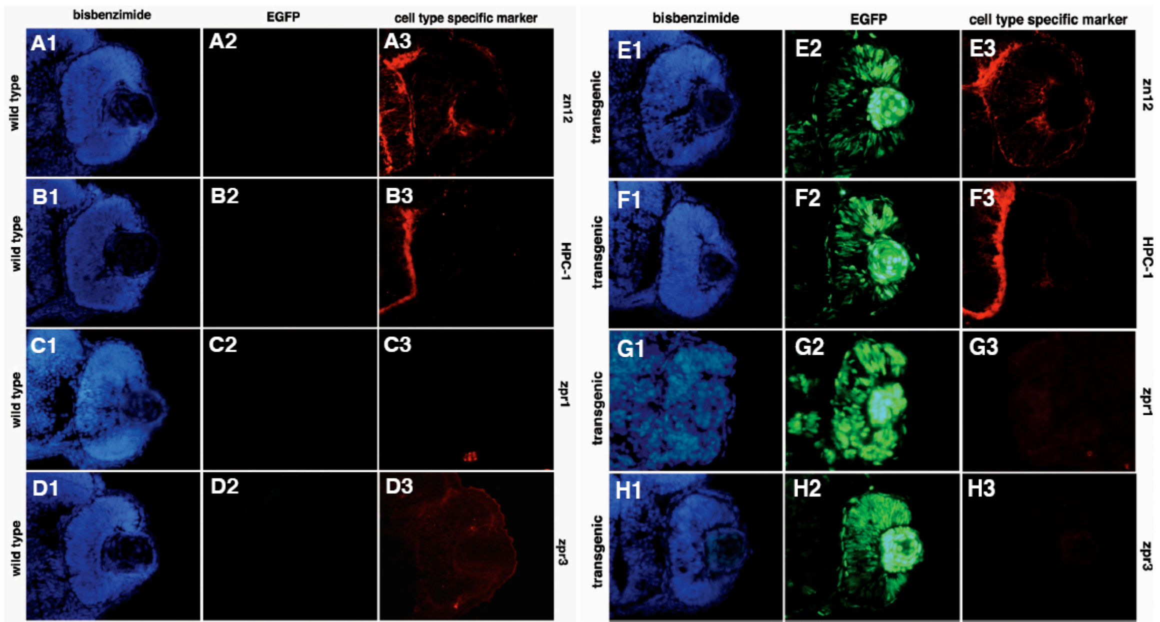


Fig. III-11: Transgenic cells that withdraw from the cell cycle do not precociously express markers of differentiated cells in the early retina.

A-D and E-H: wild type and transgenic retina, respectively, heat-shocked at 24hpf and assayed at 48hpf for zn-12 (ganglion cell marker), HPC-1 (syntaxin, ganglion and amacrine cell marker, zpr-1 (cone marker), and zpr3 (rod marker) staining). Note the absence of neuronal marker staining in transgenic retinas (E-H).

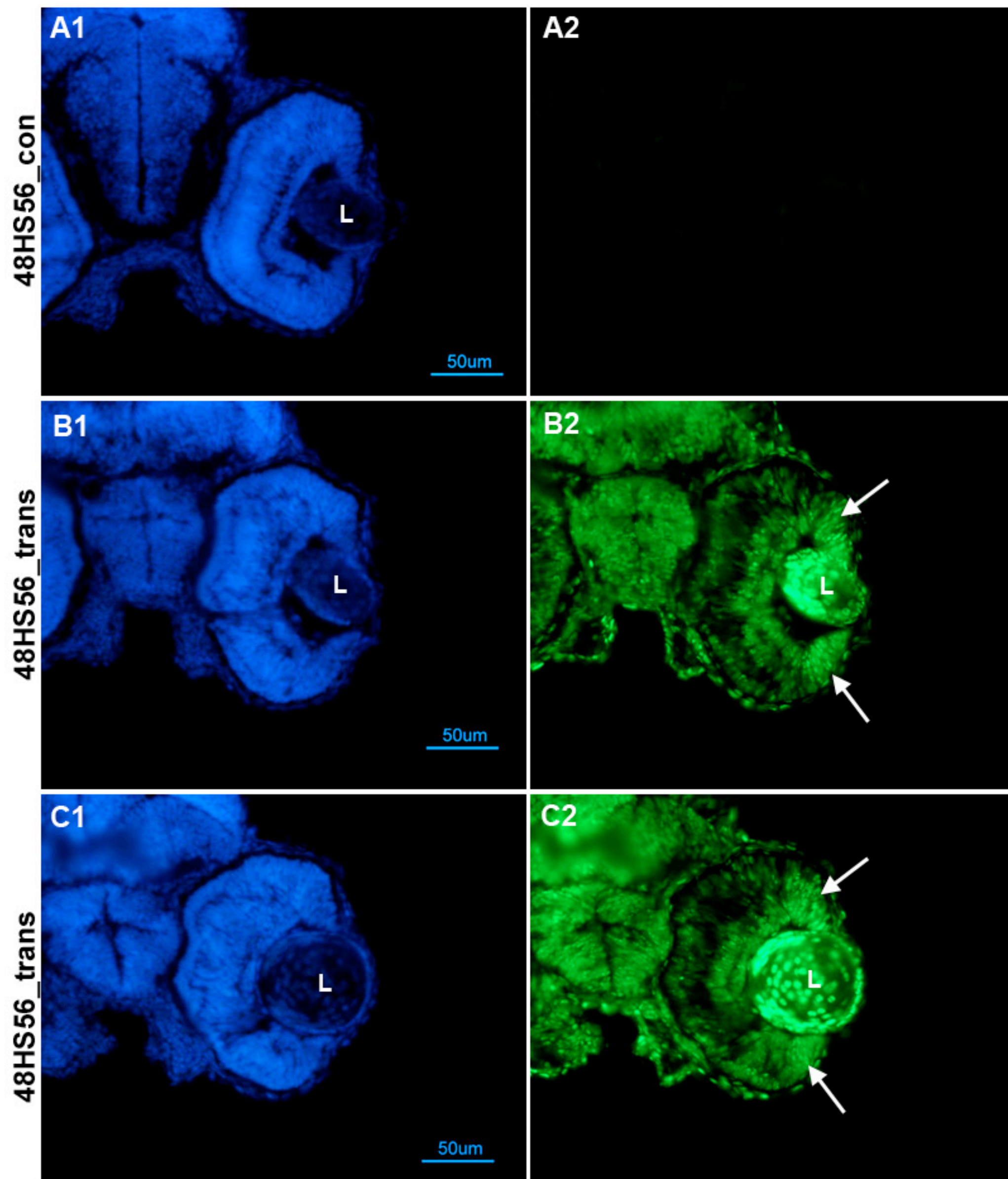


Fig. III-12: The NeuroD fusion protein is induced following heat shock at 48 hours post fertilization.

A, B, and C: transgenic and wild type embryos from the same clutch heat-shocked at 48hpf and assayed at 56hpf. A: Control embryo showing absence of EGFP fluorescence. B and C: The majority of the cells in the retina and brain are EGFP positive.

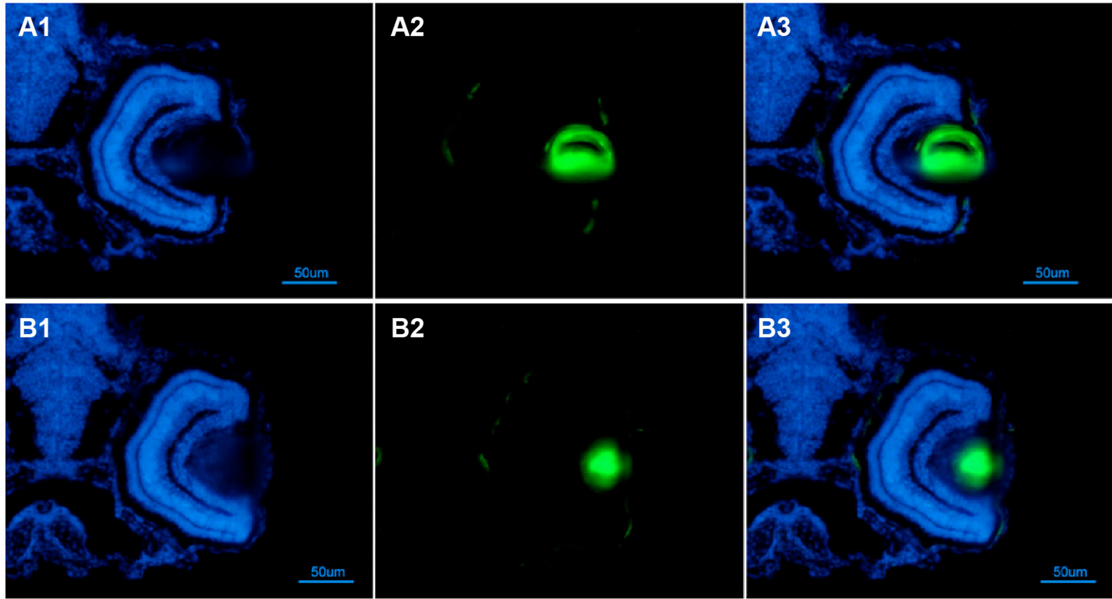


Fig. III-13: EGFP fluorescence is not detected by fluorescence microscopy in embryos heat-shocked at 48 hours post fertilization and sacrificed at 72 hours post fertilization.

A and B: Transgenic embryos heat-shocked at 48hpf and assayed at 72hpf. EGFP fluorescence is not detectable by conventional fluorescence microscopy by 72hpf. Note the presence of EGFP positive cells in the lens, where the fusion protein is constitutively active.

Table 1: Cell Counts in Dorsal and Ventral Retina of 48BrHS72hpf Hsp:nrd-EGFP zebrafish cohorts

A. Dorsal Retina - wild type

48BrHS72	control	Animal1	Animal2	Animal3	Animal4	Animal5
Marker	Section	Dorsal cell #	Dorsal cell #	Dorsal cell #	Dorsal cell #	Dorsal cell #
zn12	1	20	22	18	15	13
	2	17	19	14	14	14
	3	18	20	14	10	15
HPC-1	1	18	21	12	14	14
	2	16	22	16	11	18
	3	15	15	12	15	20
zpr-1	1	7	4	1	10	10
	2	5	4	7	9	7
	3	2	7	3	7	6
zpr-3	1	2	3	1	0	2
	2	4	9	0	0	4
	3	7	10	2	0	5
GS	1	5	3	3	2	3
	2	1	0	2	1	2
	3	1	1	3	2	2

B. Dorsal Retina - transgenic

48BrHS72	transgenic	Animal1	Animal2	Animal3	Animal4	Animal5
Marker	Section	Dorsal cell #	Dorsal cell #	Dorsal cell #	Dorsal cell #	Dorsal cell #
zn12	1	35	10	21	16	12
	2	14	15	15	12	20
	3	21	20	18	11	12
HPC-1	1	23	18	13	17	12
	2	29	19	19	18	16
	3	23	20	17	16	17
zpr-1	1	11	5	14	8	8
	2	9	9	14	5	5
	3	4	6	9	4	6
zpr-3	1	8	10	10	6	7
	2	10	7	8	8	3
	3	3	7	6	6	7
GS	1	3	3	2	2	4
	2	3	1	2	2	3
	3	0	1	2	3	2

C. Ventral Retina - wild type

48BrHS72	control	Animal1	Animal2	Animal3	Animal4	Animal5
Marker	Section	Ventral cell#	Ventral cell#	Ventral cell#	Ventral cell#	Ventral cell#
zn12	1	12	15	19	17	11
	2	12	17	15	12	10
	3	14	12	20	12	12
HPC-1	1	12	19	15	13	15
	2	13	20	20	15	15
	3	12	16	10	14	16
zpr-1	1	12	6	7	11	13
	2	9	12	10	13	14
	3	5	6	10	10	9
zpr-3	1	10	12	7	8	9
	2	15	7	3	7	11
	3	14	8	10	9	10
GS	1	3	3	2	1	2
	2	4	4	3	3	2
	3	4	4	2	3	3

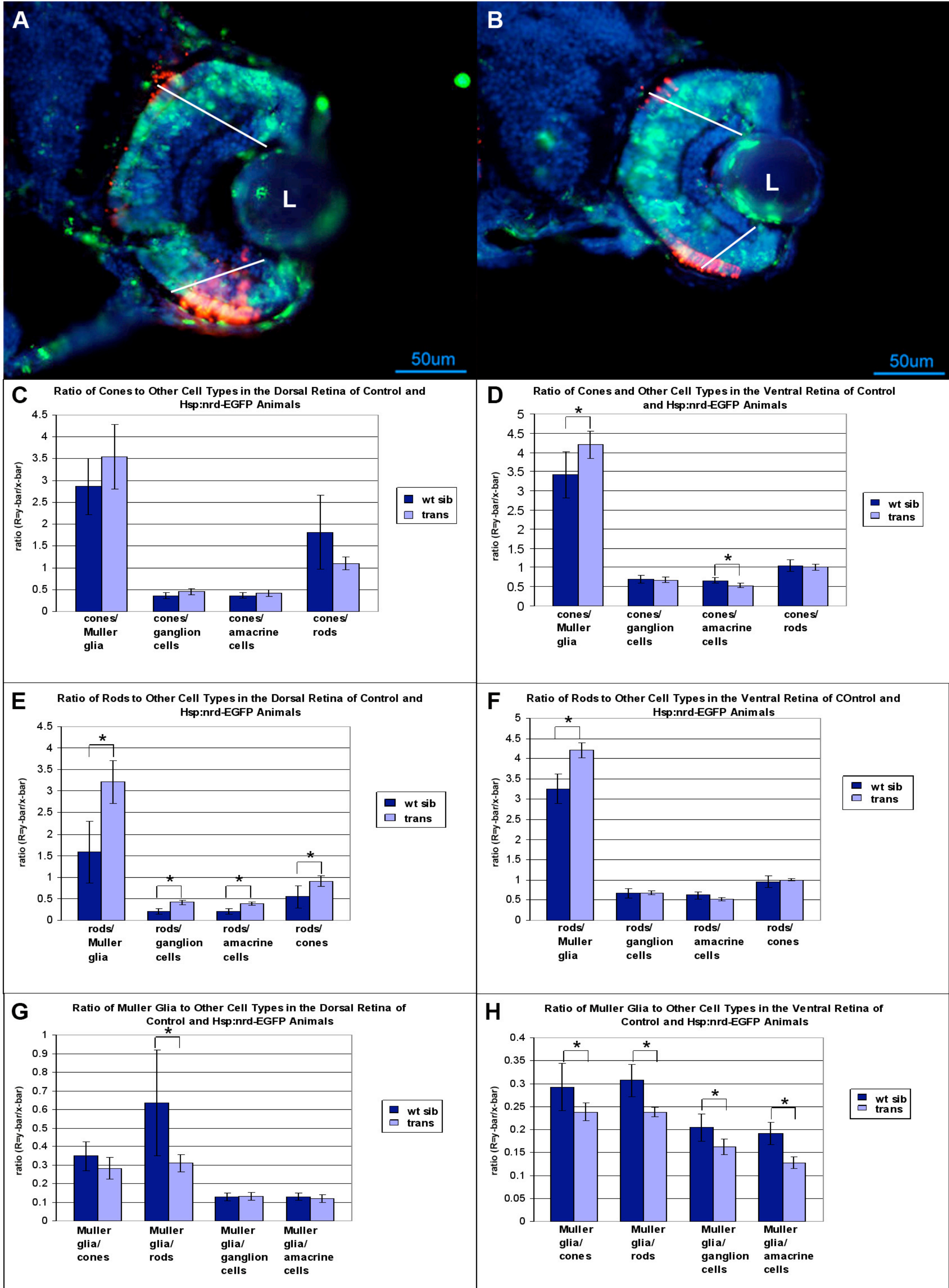
D. Ventral Retina - transgenic

48BrHS72	transgenic	Animal1	Animal2	Animal3	Animal4	Animal5
Marker	Section	Ventral cell#	Ventral cell#	Ventral cell#	Ventral cell#	Ventral cell#
zn12	1	21	8	27	14	10
	2	8	9	10	18	15
	3	18	19	15	14	10
HPC-1	1	24	12	16	21	15
	2	27	15	20	14	14
	3	20	17	22	18	20
zpr-1	1	7	6	12	7	11
	2	11	10	11	8	14
	3	9	10	13	10	8
zpr-3	1	8	12	12	11	10
	2	9	8	9	10	7
	3	15	10	8	7	11
GS	1	3	2	2	1	3
	2	2	3	3	2	3
	3	2	3	2	3	1

Table1: Cell Counts in Dorsal and Ventral Retina of 48BrHS72hpf Hsp:nrd-EGFP zebrafish cohorts.

Fig. III-14: The NeuroD fusion protein promotes photoreceptor genesis and inhibits gliogenesis.

A and B: 72hpf wild type and transgenic retinas, respectively, labeled with BrdU and heat-shocked at 48hpf showing the area in ventral and dorsal retina that was used to count cells generated from the CGZ at 48hpf (here is an example of retinas labeled with zpr3 (rod marker)). C-G and D-H: Graphs showing the tabulated ratios of cone, rods, and Muller glia to other cell types in control and transgenic retinas in the dorsal and ventral retina, respectively. Cell type specific markers used include zn-12 (ganglion cell marker), HPC-1 (syntaxin, ganglion and amacrine cell marker, zpr-1 (cone marker), and zpr3 (rod marker) staining). Note the significant increase in the proportion of rod photoreceptors and the significant decrease in the proportion of Muller glia cells to other cell types in the transgenic compared to control retinas. The asterisks (*) indicate a statistically significant difference as calculated by the pooled two-sample t-test procedure ($p<0.05$; Moore and McCabe, 1998; Cochran, 1977)



A
B
L
L
50um
50um
C
Ratio of Cones to Other Cell Types in the Dorsal Retina of Control and Hsp:nrd-EGFP Animals
ratio (R=y-bar/x-bar)
wt sib
trans
cones/ Muller glia
cones/ ganglion cells
cones/ amacrine cells
cones/ rods
D
Ratio of Cones and Other Cell Types in the Ventral Retina of Control and Hsp:nrd-EGFP Animals
ratio (R=y-bar/x-bar)
wt sib
trans
cones/ Muller glia
cones/ ganglion cells
cones/ amacrine cells
cones/ rods
E
Ratio of Rods to Other Cell Types in the Dorsal Retina of Control and Hsp:nrd-EGFP Animals
ratio (R=y-bar/x-bar)
wt sib
trans
rods/ Muller glia
rods/ ganglion cells
rods/ amacrine cells
rods/ cones
F
Ratio of Rods to Other Cell Types in the Ventral Retina of COntrol and Hsp:nrd-EGFP Animals
ratio (R=y-bar/x-bar)
wt sib
trans
rods/ Muller glia
rods/ ganglion cells
rods/ amacrine cells
rods/ cones
G
Ratio of Muller Glia to Other Cell Types in the Dorsal Retina of Control and Hsp:nrd-EGFP Animals
ratio (R=y-bar/x-bar)
wt sib
trans
Muller glia/ cones
Muller glia/ rods
Muller glia/ ganglion cells
Muller glia/ amacrine cells
H
Ratio of Muller Glia to Other Cell Types in the Ventral Retina of Control and Hsp:nrd-EGFP Animals
ratio (R=y-bar/x-bar)
wt sib
trans
Muller glia/ cones
Muller glia/ rods
Muller glia/ ganglion cells
Muller glia/ amacrine cells

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CHAPTER IV

LOSS-OF-FUNCTION

This chapter extends the studies described in Chapter II and Chapter III by using Morpholino oligonucleotides to block translation of the NeuroD protein.

Introduction

NeuroD is a member of the family of basic helix-loop-helix (bHLH) transcription factors that shares properties of other bHLH transcription factors (Livesey and Cepko, 2001; Van Raay and Vetter, 2004; Yan et al., 2005; Wang and Harris, 2005; Chapter III). In other systems, NeuroD is involved in regulating withdrawal from the cell cycle and neuronal differentiation (Lee et al., 1995; Farah et al., 2000). In the retina, NeuroD regulates neuron versus glial cell fate (Morrow et al., 1999) and promotes amacrine cell genesis (Ahmad et al., 1998; Acharya et al., 1997; Moore et al., 2002; Inoue et al., 2002), and in chick and in zebrafish promotes photoreceptor genesis (Yan and Wang, 1998; Yan and Wang, 2004; Chapter III).

In larval and adult teleosts NeuroD is expressed in proliferating cells of rod and cone photoreceptor lineages and in differentiating cone photoreceptors (Otteson and Hitchcock, 2003; Hitchcock et al., 2004; Hitchcock and Kakuk-Atkins, 2004; Chapter II). I previously used lines of zebrafish transgenic for Heat shock-NeuroD-EGFP (Hsp:nrd-EGFP) for conditional gain-of-function

experiments to test the hypothesis that NeuroD promotes cell cycle withdrawal and photoreceptor genesis. Results from these studies showed that NeuroD promotes cell cycle withdrawal in the retina and is sufficient to promote photoreceptor genesis. These findings combined with the cellular pattern of expression suggest that NeuroD functions to modulate the mitotic activity of cells in the rod and cone photoreceptor lineages and to promote photoreceptor genesis. As an additional test of this hypothesis, I used NeuroD morpholino oligonucleotides in loss-of-function experiments.

Materials and Methods

Experimental Animals

Zebrafish were maintained at 28.5°C on a 14/10 hr light/dark cycle in a zebrafish modular (ZMOD) breeding facility. Embryos were collected after natural spawns, developed at 28.5°C, and staged by HPF as described previously (Kimmel et al., 1995). Wild-type zebrafish were used for this study.

Morpholinos and microinjections

Morpholinos (MOs) were synthesized by Gene Tools, LLC (Cowallis, OR). The nrd MO1 was complementary to the initiation codon (AUG) of the zebrafish nrd mRNA sequence (GenBank accession number: AF036148). A 5-bp mismatch morpholino was used as a control. The sequences of 25-mer MOs used were as follows:

nrd MO1: 5'-TGACTTCGTCATGTCGGAACTCTAG-3'

5-mis-nrdMO1: 5' TGAgTTgGTCATcTCGcAACTgTAG-3'

The MOs were diluted in 1x Danieau buffer (Nasevicius and Ekker, 2000) at 1mg/ml. Embryos were injected with 5nl of MOs at 2- to 8-cell stages. Morphant retinas were compared to uninjected and mis-match injected retinas at 72 hours post fertilization using BrdU incorporation and a series of cell type specific markers.

BrdU labeling

Bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) was used to label mitotically active cells. Embryos were exposed to BrdU for 20 minutes by soaking in 5mM BrdU and 15% DMSO in embryo rearing solution as previously described (Ochocinska and Hitchcock, 2007). Twelve morphant and twelve control animals were analyzed.

Immunohistochemistry

All immunocytochemistry protocols were performed as previously described (Hitchcock et al., 1996). Omitting primary antibodies served as negative controls. In the absence of primary antibodies, no staining was observed (data not shown). Ganglion cells were labeled using the mouse monoclonal antibody zn-12 (The Zebrafish International Resource Center, Eugene, OR; catalog #072103), which labels early born neurons in the zebrafish and ganglion cells in the retina. Amacrine cells were labeled using the cell type-specific monoclonal antibody against rat syntaxin (Monoclonal Anti-Syntaxin Clone HPC-1; Sigma; catalog #S0664). Cone photoreceptors were labeled using the mouse monoclonal antibody zpr-1 (The Zebrafish International Resource Center, Eugene, OR; catalog #092502), which labels an unidentified epitope on red/green cones. Rod

photoreceptors were labeled using the mouse monoclonal antibody zpr-3 (The Zebrafish International Resource Center, Eugene, OR; catalog #011604). BrdU was detected using a monoclonal antibody against BrdU (Becton Dickinson Immunocytochemistry Systems, San Jose, CA; catalog #347580) diluted 1:200. Proliferating cells were also labeled with a monoclonal antibody against Proliferating Cell Nuclear Antigen (PCNA). All primary antibodies and secondary antibodies conjugated to fluorescent labels were diluted 1:200. For each label, twelve morphant and twelve control animals were analyzed.

In situ hybridization

In situ hybridization with single probes on sections was performed as previously described (Hitchcock et al., 2001). *In situ* hybridization using CyclinD1 and p27 DIG-labeled riboprobes was performed on wild type and morphant retinas. Briefly, full-length cDNAs were linearized and DIG-labeled riboprobes were synthesized with an RNA labeling kit (Roche Diagnostic Corp., Indianapolis, IN). Following prehybridization two hundred nanograms of probe in 80μl of hybridization solution was pipetted onto each slide, coverslipped, and hybridized overnight at 55°C. The next day, the sections were washed and digoxygenin was immunolabeled using an alkaline-phosphatase-conjugated antibody and visualized with NBT/BCIP. The slides were then coverslipped for microscopy or combined with several immunocytochemistry protocols described below. Following hybridization with *neuroD* sense probes, no staining was observed (data not shown). For each probe, twelve morphant and twelve control animals were analyzed.

Results

Nrd *atg* morpholinos: effects on development and specificity.

The presence of morpholino oligonucleotides may have non-specific effects on embryonic development (Corey and Abrams, 2001). To determine if the NeuroD morpholino affects general development, embryos from a wild-type cross were divided into three groups. The first experimental group was injected with a 25-bp *atg*-morpholino (*atg*-MO) targeted against the translation start site of NeuroD. The second control group was injected with mismatch morpholinos (MM-MO), which contained a 5-bp mismatch in the 25-mer *atg* sequence. The third control group consisted of uninjected, wild-type embryos. The morphology of the embryos was then compared among the three groups. Gross examination at 72hpf indicated there was no apparent difference in morphology between the uninjected controls, embryos injected with the MM-MO, or embryos injected with the *atg*-MO (Fig.1A-C). In all three groups, embryos developed normally, and the size of the body and eyes was comparable. These data suggest that the NeuroD morpholino oligonucleotides used here do not affect the general development of zebrafish embryos.

The specificity of the NeuroD morpholino was tested in Western blots and in the Hsp:nrd-EGFP transgenic line (see Chapter III). The zNrd antibody (see Chapter III) was used on Western blots of protein lysates from 72hpf morphant and control embryos. The zNrd antibody binds to NeuroD in uninjected controls, and this 30kD band is significantly reduced in the NeuroD morphants (Fig.1D). This shows that the *atg*-MO prevents translation of NeuroD protein *in vivo*. As a

second, independent test of specificity, *atg*-MO and MM-MO controls were injected into embryos from the Hsp-nrd-EGFP line (see Chapter 3), and EGFP expression was assayed following heat shock. A clutch of embryos from a heterozygous cross was divided into two groups at the 1-2 cell stage. One group was injected with *atg*-MO and the other group was injected with the MM-MO control. All injected embryos were heat-shocked at 24hpf and assayed at 48hpf for induction of the NeuroD-EGFP fusion protein. At 48hpf, embryos injected with the MM-MO showed robust EGFP fluorescence (Fig.2A), whereas embryos injected with the *atg*-MO showed an absence of EGFP fluorescence or only a few EGFP+ cells (Fig.2B). These data show that the presence of the *atg*-morpholino results in a significant decrease in EGFP fluorescence (Fig.2A and B; though, see **'Specific rescue of morphant retinas'**), demonstrating that, in embryos at 24hpf, the *atg*-MO prevents translation of the NeuroD fusion protein *in vivo*.

NeuroD is required for photoreceptor progenitors to exit the cell cycle.

As an additional test of the hypothesis that in photoreceptor progenitors NeuroD promotes cell cycle withdrawal, the effect of NeuroD protein knock-down on the cell cycle was analyzed. BrdU incorporation and Proliferating Cell Nuclear Antigen (PCNA) labeling was used to determine whether or not NeuroD knock-down alters proliferation. In contrast to the gain-of-function experiments (Chapter III) where NeuroD was induced in all cells, in these loss-of-function experiments, NeuroD is only removed from cells that normally express this protein. Since NeuroD is expressed in mitotically active cells of the rod and cone lineages,

these studies can directly test the consequence of removing NeuroD in these cells.

Embryos from a wild-type cross were divided into three groups. The first control group was not injected, the second group was injected with mismatch morpholino control (MM-MO), and the third group was injected with the *atg*-morpholino (*atg*-MO). At 72hpf all three groups were exposed to a brief pulse of BrdU by immersion in a solution of 5mM BrdU in 15% DMSO for 20 minutes and sacrificed immediately afterwards. In both control groups, BrdU+ cells were present in the circumferential germinal zone and absent in the inner and outer nuclear layer (Fig.3A and B; arrows). Similar to controls, the retinas of *atg*-morphants contained BrdU+ cells in the CGZ (Fig.3C; arrows), however, in striking contrast to controls, BrdU+ cells were present throughout the outer nuclear layer (Fig.3C; arrows) and individual cells and clusters of cells were present in the inner nuclear layer (Fig.3C; arrow heads). These data show that in the absence of NeuroD, cells in both the inner and the outer nuclear layers continue to proliferate.

Proliferating Cell Nuclear Antigen (PCNA), another marker which labels a larger proportion of mitotically active cells during the cell cycle than BrdU, was used at 72hpf to label retinas of uninjected, MM-MO and *atg*-MO injected animals. In both control groups, PCNA+ cells were present in the CGZ, but labeled a larger cohort of cells than with BrdU (Fig.3A and B; arrows; compare with Fig.2A and B). In morphant retinas injected with *atg*-MO, as for BrdU+ cells, there were PCNA+ cells throughout the outer and inner nuclear layers (Fig.4C).

To investigate the potential mechanism by which NeuroD promotes cell cycle withdrawal, morphant retinas were analyzed with probes for cell cycle regulatory proteins CyclinD1 and p27. At 72hpf, retinas of morphants and uninjected controls were labeled with CyclinD and p27 riboprobes. In the wild-type control retina, CyclinD is expressed in the CGZ (Fig.5A1), whereas in the morphant retina CyclinD is also expressed in portions of the inner and outer nuclear layers (Fig.5A2; arrows). These data suggest that the absence of NeuroD protein results in the upregulation of CyclinD expression in the outer nuclear layer. There is no clear difference observed in p27 expression between morphant and control retinas (Fig.5B1 and B2).

NeuroD morphant retinas fail to express markers of differentiated photoreceptors.

NeuroD, along with other bHLH transcription factors, has been shown to be sufficient to induce neuronal differentiation (Lee et al., 1995; Farah et al., 2000). Even though the BrdU data suggests that, in the morphant retina, most cells in the outer nuclear are dividing, there may be some cells that have exited the cell cycle. If this is the case, knock down of NeuroD in these cells may affect their differentiation. To test the hypothesis that NeuroD promotes photoreceptor genesis, morphant and wild type retinas were assayed at 72hpf using a panel of cell-type specific markers. The data show that the gaglion cell layer and inner nuclear layer develop normally in wild-type and morphant retinas (Fig.6A-B and E-F). However, retinas in NeuroD morphants do not express markers of differentiated photoreceptors (Fig.6C-D and G-H). This suggests that, in the

absence of NeuroD protein, any postmitotic cells present in the outer nuclear layer in the morphant retina fail to differentiate into photoreceptors.

Specific rescue of NeuroD morphant retinas

As another test of specificity of NeuroD ATG morpholino and as a test of the hypothesis that NeuroD promotes cell cycle withdrawal among photoreceptor progenitors, a rescue experiment was devised using the Hsp70/4:nrd-EGFP transgenic line (Fig.7; see also Chapter 3 for description of transgenic line). *atg*-MOs were injected into 1-2 cell stage Hsp70/4:nrd-EGFP embryos, which were heat-shocked at 48hpf and at 72hpf were separated based on EGFP fluorescence. In contrast to embryos from the Hsp:nrd-EGFP line injected with atg-MOs heat-shocked at 24hpf and evaluated at 48hpf (see above and Fig.2), it was possible to sort embryos according to fluorescence at 72hpf (Fig.7). These embryos were exposed to BrdU at 72hpf, and BrdU incorporation was compared between transgenic and wild type retinas. Wild-type embryos show BrdU+ cells in the CGZ, the outer nuclear layer and inner nuclear layer (Fig.7A and B), consistent with the morphant phenotype. In contrast, the retinas of transgenic embryos have significantly fewer BrdU+ cells (Fig.7C and D). This demonstrates that inducing NeuroD fusion protein can partially rescue the morphant phenotype and induces cell cycle withdrawal specifically among photoreceptor progenitor cells.

A second rescue experiment was performed to determine whether the morphant retinas recover from the effect of NeuroD loss-of-function. Embryos from a wild-type cross were injected with *atg*-MOs at the 1-2 cell stage. A subset

of these embryos was tested at 72hpf (see previous section and Fig.8A-D), and another subset was set aside to be evaluated at 7 days post fertilization (dpf) using cell-type specific markers for cone and rod photoreceptors. In contrast to 72hpf, by 7dpf photoreceptors in NeuroD morphants express protein found on mature cone and rod photoreceptors (Fig.8E-F).This demonstrates that between 72 hpf and 7dpf, as the effect of the morpholinos is attenuated, the cells that at 72hpf are either mitotically active, or don't express markers of differentiated cells, now exit the cell cycle and express markers of differentiated cone and rod photoreceptors. Thus, the progenitor cells of the rod and cone progenitor lineages cells, which normally require NeuroD protein, upon re-expression of NeuroD protein exit the cell cycle and differentiate.

Discussion

The present study used a loss-of-function approach which is complementary to the gain-of-function studies described in Chapter III. To test the hypothesis that NeuroD promotes cell cycle withdrawal among photoreceptor progenitors and promotes photoreceptor genesis, morpholino oligonucleotides were used to block translation of NeuroD protein. The loss-of-function experiments described in this chapter reveal that, in the absence of NeuroD, cells of the rod and cone photoreceptor lineages that normally express NeuroD continue to proliferate and fail to exit the cell cycle. In addition, whereas the inner nuclear layer markers are expressed, cells in the outer nuclear layer fail to express markers of differentiated photoreceptors. Thus the complementary results from both the gain and loss-of-

function suggest that NeuroD is required for the cell cycle exit of rod and cone photoreceptor lineages.

NeuroD promotes cell cycle withdrawal in other systems (Lee et al., 1995; Farah et al., 2000). I have previously shown that NeuroD promotes cell cycle withdrawal in the retina (Chapter III). As an additional test of the hypothesis that in photoreceptor progenitors NeuroD promotes cell cycle withdrawal, the effect of NeuroD protein knock-down on the cell cycle was analyzed. In the absence of NeuroD protein, cells, which normally express NeuroD in both the inner and outer nuclear layer, are labeled with BrdU and PCNA. The mitotically active cells in the outer nuclear layer correspond to photoreceptor progenitor cells. In addition, cells in the inner nuclear layer labeled with BrdU are scattered and sometimes appear in clusters extending towards the outer nuclear layer. Based on location, morphology, distribution, and the cellular pattern of *neuroD* expression, these cells, which continue to proliferate in the absence of NeuroD protein, are cells of the rod photoreceptor lineage, as described in embryonic, larval and adult teleosts (Otteson et al., 2001; Hitchcock and Kakuk-Atkins, 2004; see also Chapter 2). In addition, cells in the outer nuclear layer in morphant retinas fail to express markers of differentiated photoreceptors. This demonstrates that, in the absence of NeuroD, photoreceptor progenitors continue to proliferate and fail to differentiate.

The morphant phenotype described here is consistent with NeuroD loss-of-function results in the chick retina (Yan and Wang, 2004). Regions of retina infected with NeuroD repression construct show absence of photoreceptor

marker staining and presence of Chx10+ cells in the outer nuclear layer, a marker of progenitor cells. As the authors note, the Chx10+ cells may be progenitor cells unable to proceed to differentiation as photoreceptor cells due to NeuroD repression. The present study is consistent with this and shows that NeuroD in the zebrafish retina is required for cell cycle exit of photoreceptor progenitors. In addition, by 7dpf photoreceptors in NeuroD morphants can be labeled with markers of cone and rod photoreceptors, suggesting that between 72 hpf and 7dpf as the effect of the morpholinos is attenuated, mitotically active cells exit the cell cycle and generate photoreceptors.

To investigate the mechanism through which NeuroD promotes cell cycle withdrawal in the retina, probes for cell cycle regulatory proteins, CyclinD1 and p27 were used to evaluate the absence of NeuroD on the expression of cell cycle regulatory proteins. NeuroD null mice show reduced expression of the p27, an inhibitor of cyclin-dependent kinases, combined with increased expression of cell proliferation markers (Schonhoff et al., 2004). In the present study, at 72hpf CyclinD1 expression is increased in the morphant retinas and includes portions of the outer and inner nuclear layers. The absence of NeuroD protein does not appear to affect the expression of the cyclin-inhibitor p27. However, there are high levels of p27 expression at this time and it may be difficult to detect subtle changes in this expression. Alternatively CyclinD1 expression at this time may be regulated by other cyclin inhibitors, such as p57. Taken together, the gain-of-function data presented in Chapter III and the loss-of-function data suggest that

NeuroD may function to coordinate cell cycle arrest with the upregulation of cyclin inhibitors and concomitant downregulation of cyclin expression.

In contrast to the gain-of-function experiments (Chapter III), where NeuroD was expressed in all cells, in the loss-of-function experiments, NeuroD was only removed from cells that normally express this protein. Since NeuroD is expressed in mitotically active cells of the rod and cone lineages, these studies could directly test the consequence of specifically removing NeuroD from the cells that normally require NeuroD. As another test of the hypothesis that NeuroD promotes cell cycle withdrawal of rod and cone photoreceptor progenitors, a rescue experiment was devised using the Hsp70/4:nrd-EGFP transgenic line (Chapter 3). Wild-type embryos injected with *atg*-morpholino show BrdU+ cells in the CGZ, the outer nuclear layer and inner nuclear layer, consistent with the morphant phenotype. In contrast, the retinas of transgenic embryos injected with *atg*-morpholino, following heat shock and induction of NeuroD fusion protein, have significantly fewer BrdU+ cells. This demonstrates that inducing NeuroD fusion protein can partially rescue the morphant phenotype and promotes cell cycle withdrawal specifically among photoreceptor progenitor cells.

Earlier work has speculated that based on the cellular expression pattern in nascent cones, NeuroD may regulate early aspects of cone maturation (Chapter 2). NeuroD is expressed in immature cones, and this expression is turned off in mature cones which begin to express opsins (Chapter 2). This suggests that, in addition to its role in cell cycle withdrawal of cone progenitor cells, NeuroD may also regulate early aspects of their maturation. NeuroD

morphant retinas fail to express markers of differentiated cone photoreceptors, but after the effect of the morpholino is attenuated, cells in the outer nuclear can be labeled with cone photoreceptor markers. One potential explanation is that, in the absence of NeuroD, cone photoreceptors remain in an immature state and NeuroD is required for their differentiation. This is consistent with the presence of cone photoreceptor differentiation once the effect of the NeuroD morpholino is removed. As the levels of NeuroD protein return to normal, the nascent cones begin to mature and express markers of differentiated cone photoreceptors.

Several lines of evidence support this and show that NeuroD plays a role in the maturation of other cell types in the brain. NeuroD may play a role in the maturation of olfactory and taste bud cells (Bedard and Parent, 2004; Suzuki et al., 2002). NeuroD is expressed in newborn cells in the granular and glomerular layers of the human olfactory bulb, and in immature taste buds. NeuroD is also expressed strongly in immature neurons, but not in mature neurons, in cultured cortical neurons, and expression of NeuroD decreases with increasing days in culture, as neurons become mature (Katayama et al., 1997); In the cerebellum (Miyata et al., 1999), the olfactory bulb (Lee et al., 2000), and the olfactory epithelium (Nibu et al., 1999), where active neurogenesis occurs even in the adult stage, high levels of NeuroD expression persist during postnatal development. In the dentate granule cell layer, where neurons continue to be added at later ages, NeuroD continues to be expressed and is at its highest levels in the most immature neurons (Pleasure et al., 2000). This implies that NeuroD regulates the expression of genes involved in the maturation of granule

cells and their integration into the existing neuronal network. Likewise, in the zebrafish retina NeuroD may promote the expression of genes involved in the maturation of cone photoreceptors.

Taken together, based on the cellular pattern of neuroD expression (Chapter II) combined with gain-of-function (Chapter III) data, the present results confirm that NeuroD plays a direct role in promoting the cell cycle withdrawal of photoreceptor progenitors, as well as a potential role in cone photoreceptor maturation. In the absence of NeuroD, photoreceptor progenitor cells remain mitotically active. There is a concomitant increase in markers of mitotically active cells as assayed by BrdU incorporation and PCNA labeling and an increase CyclinD1 expression. In addition, morphant retinas fail to express markers of differentiated photoreceptors. This indicates that NeuroD is required for cell cycle withdrawal of photoreceptor progenitor cells, and may also function in maturation of cone photoreceptors, which, in the absence of NeuroD protein, fail to differentiate. Thus a model is emerging for NeuroD function in the retina. The cellular pattern of *neuroD* expression and the complementary results from gain-of-function and loss-of-function experiments suggest that NeuroD promotes cell cycle withdrawal of photoreceptor progenitors, promotes photoreceptor genesis, and may function in maturation of cone photoreceptors. In addition, NeuroD functions as the molecular link that ties cell cycle withdrawal with determination and or differentiation.

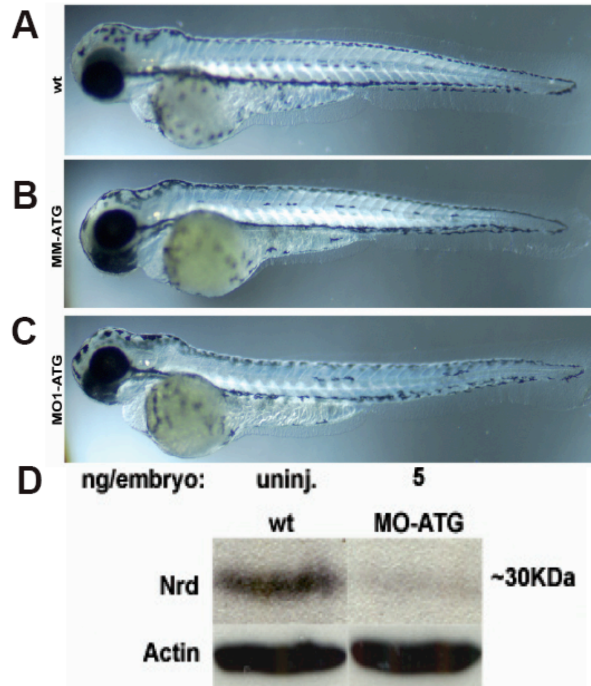

Fig. IV-1: NeuroD morpholino oligonucleotides (MOs): effect on development and Western analysis.

A, B, and C: Wild type (uninjected), mismatch-ATG morpholino injected, and MO-ATG injected morphants at 72hpf. D: Western blot showing the knock-down of NeuroD protein in 72hpf morphants compared to uninjected controls.

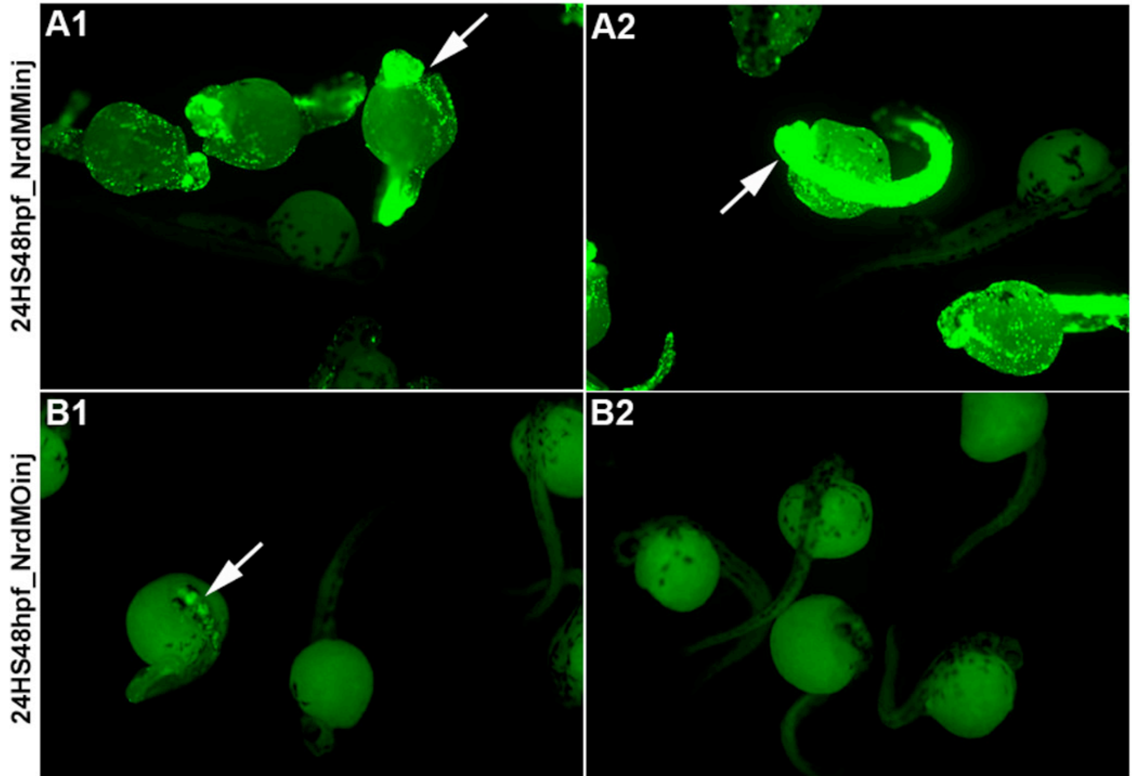

Fig. IV-2: NeuroD morpholino oligonucleotides (MOs) inhibit induction of NeuroD fusion protein in the Hsp:nrd-EGFP transgenic line.

A1 and 2: One half of a cohort of Hsp:nrd-EGFP embryos from a heterozygous cross injected with mismatch-ATG morpholino control, heat-shocked at 24hpf and photographed at 48hpf. Note the presence of EGFP+ cells throughout the embryo (arrows). B1 and 2: The other half of the cohort of Hsp:nrd-EGFP embryos from the heterozygous cross injected with MO-ATG morpholino, heat-shocked at 24hpf and photographed at 48hpf. Note the absence of EGFP+ cells in the embryos or a significant decrease in EGFP fluorescence (arrow).

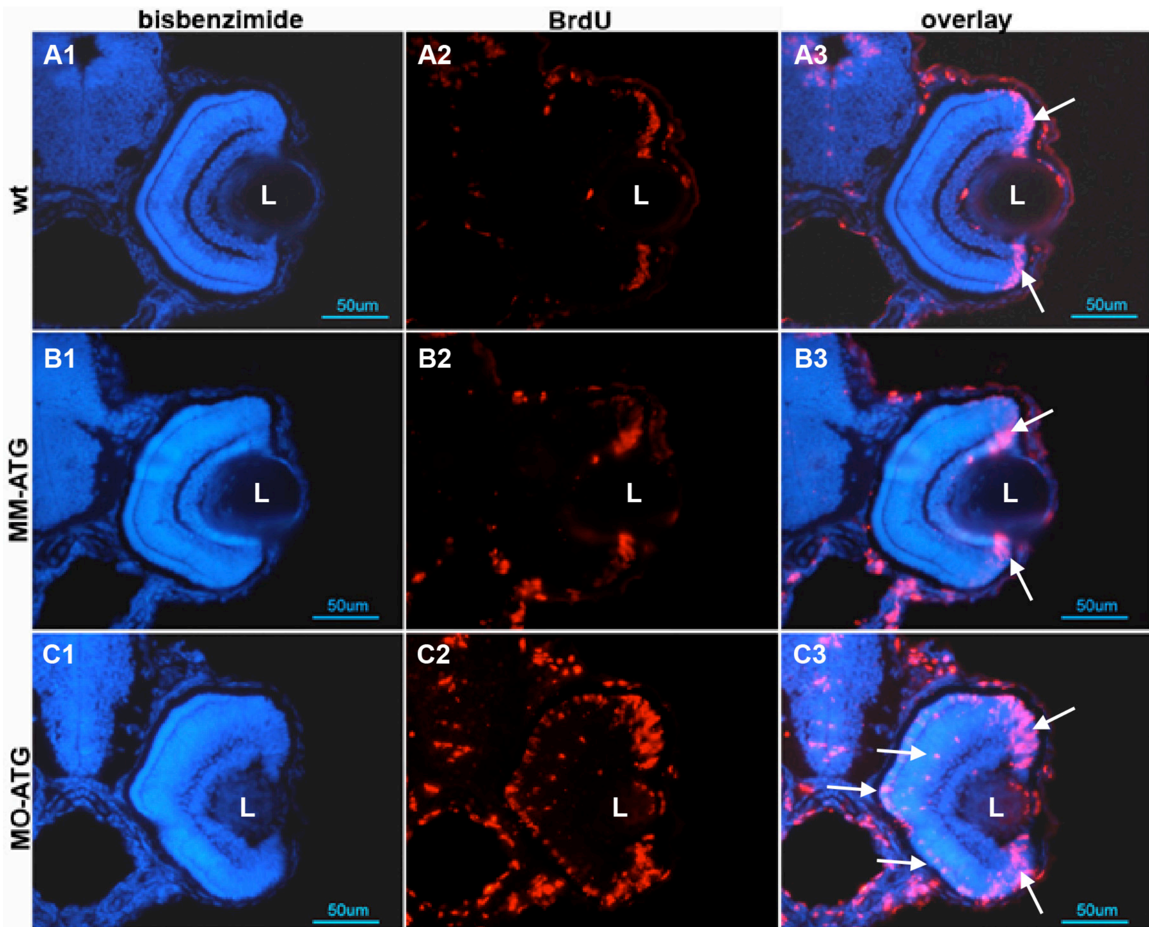

Fig. IV-3: NeuroD is required for cell cycle exit of photoreceptor progenitors.

A, B, and C: uninjected (wt), MM-ATG and MO-ATG morphant sections through the brain and retina assayed for BrdU incorporation at 72hpf. Note the persistence of mitotically active cells in the outer nuclear layer of the morphant retinas compared to controls.

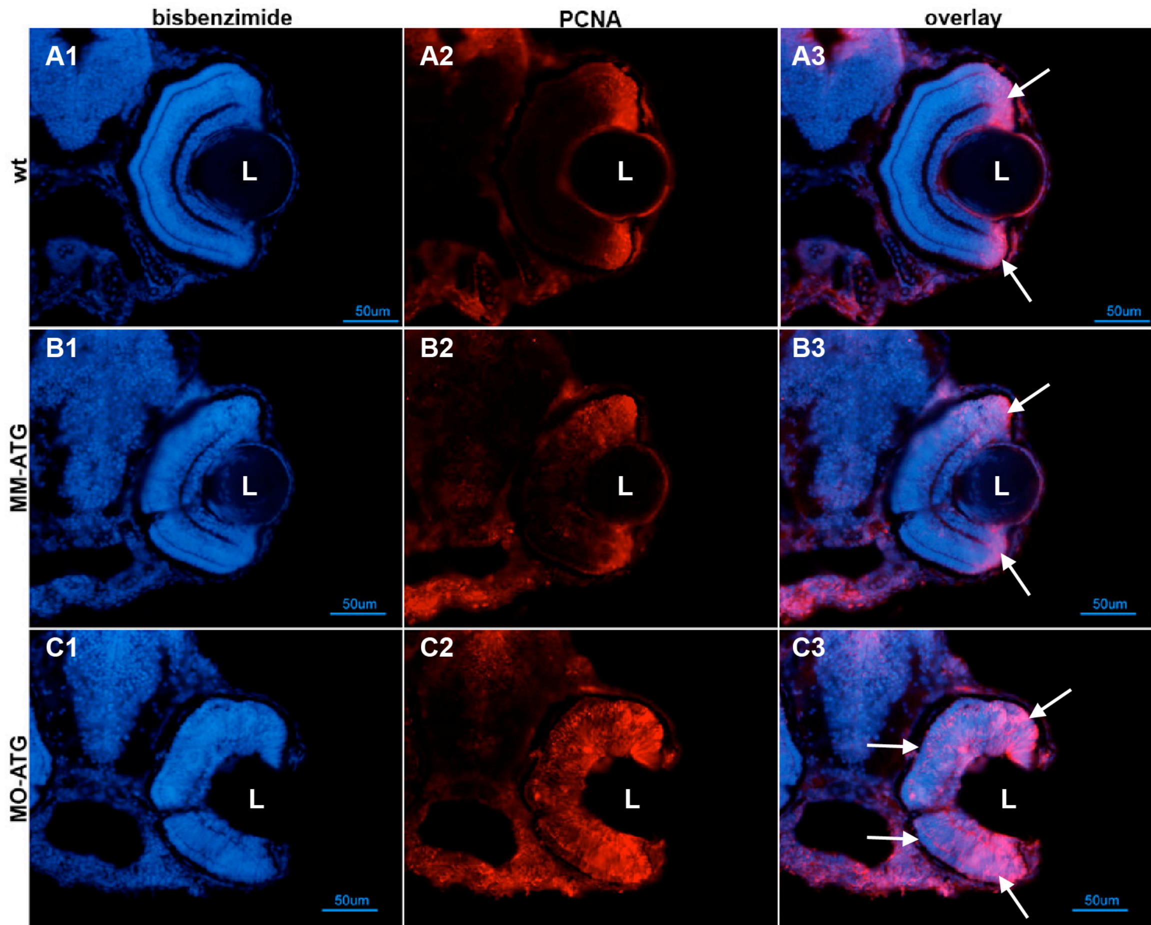

Fig. IV-4: NeuroD is required for cell cycle exit of photoreceptor progenitors.

A, B, and C: uninjected (wt), MM-ATG and MO-ATG morphant sections through the brain and retina assayed PCNA labeling at 72hpf. Note the persistence of mitotically active cells in the outer and inner nuclear layers of the morphant retinas compared to controls.

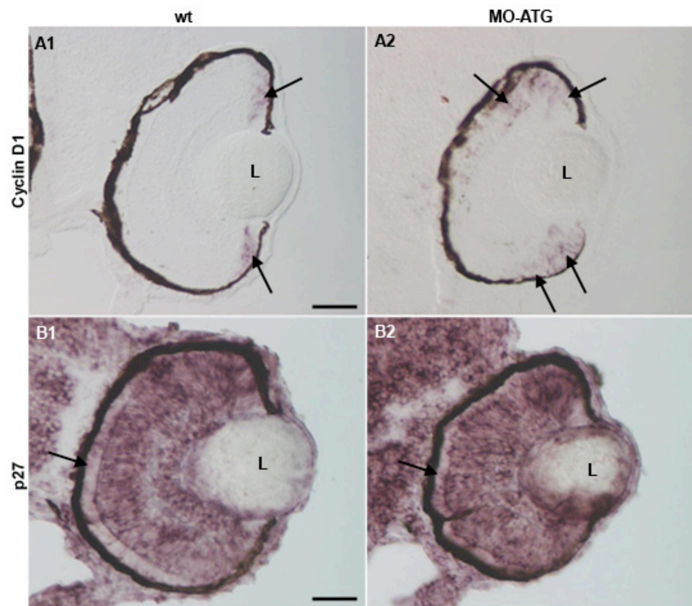

Fig. IV-5: Cyclin D1 is upregulated in the absence of NeuroD.

A and B: The expression of CyclinD is upregulated in morphant retinas compared to controls. C and D: The expression of cyclin inhibitors p27 remains unchanged in morphant retinas compared to controls.

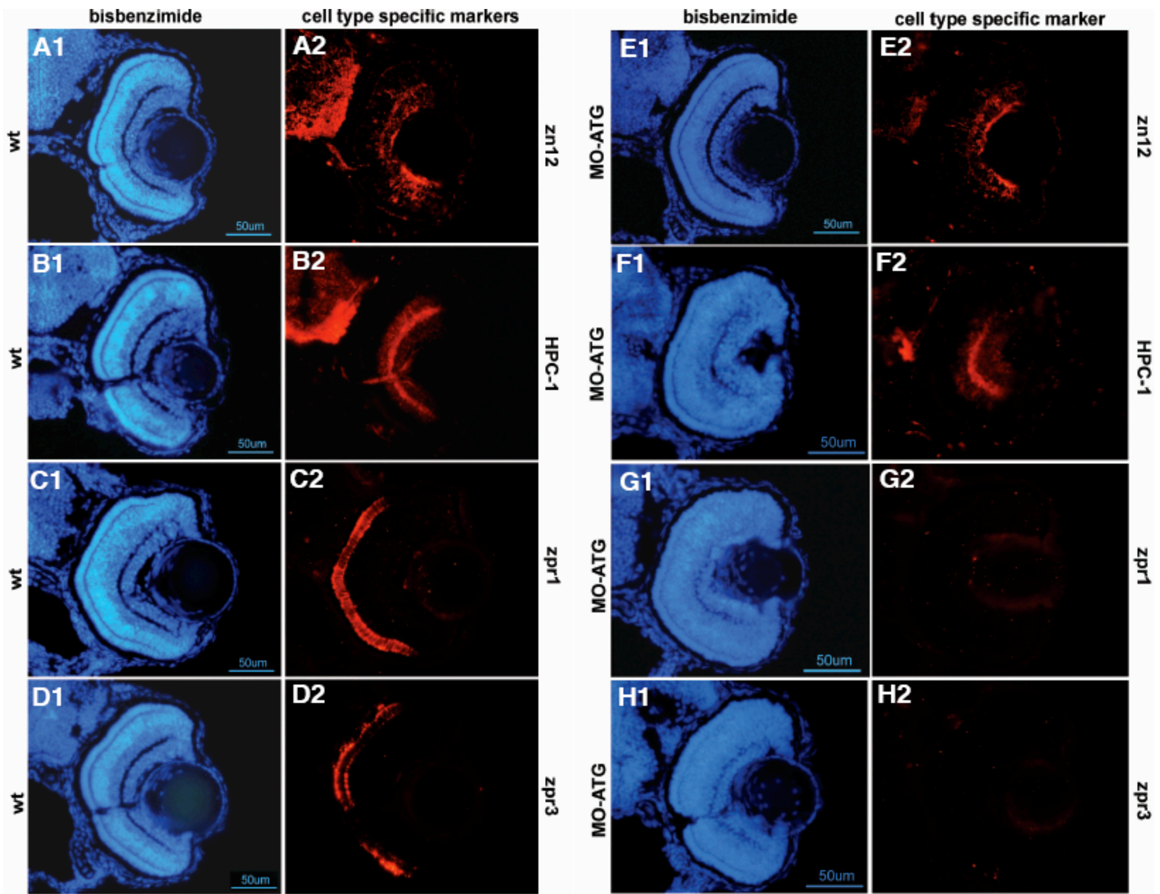

Fig. IV-6: NeuroD morphant retinas fail to express markers of differentiated photoreceptors.

A-D and E-H: wild type and NeuroD morphant retina, respectively, assayed at 72hpf for zn-12 (ganglion cell marker), HPC-1 (syntaxin, ganglion and amacrine cell marker, zpr-1 (cone marker), and zpr3 (rod marker) staining. Note the absence of photoreceptor marker staining in morphant retinas (G, H) compared to controls (C, D).

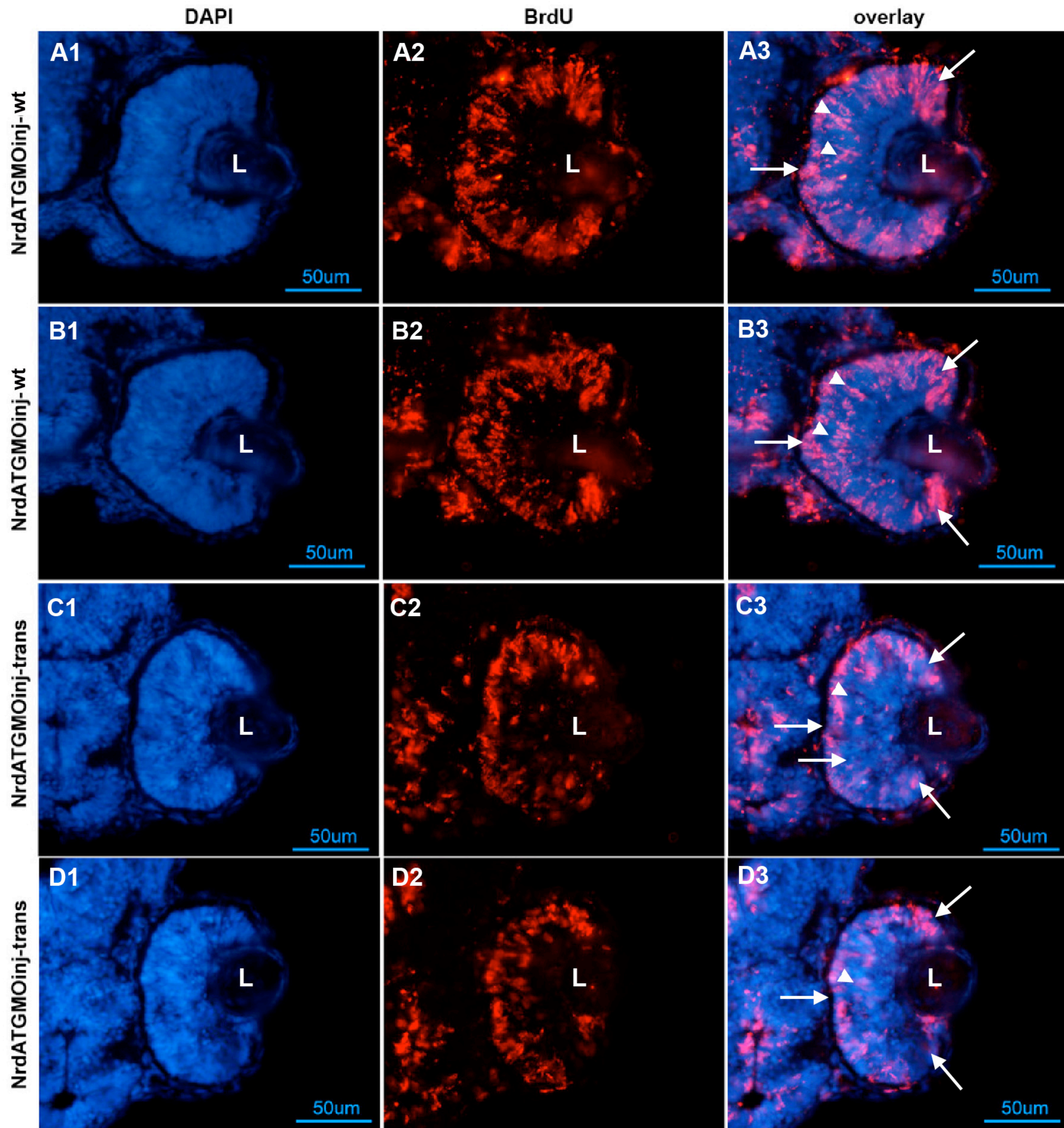

Fig. IV-7: The NeuroD fusion protein rescues the NeuroD morphant phenotype.

A and B: Hsp:nrd-EGFP control retinas from embryos injected with Nrd-ATG morpholino at the 1-2 cell stage and assayed for BrdU incorporation at 72hpf. C and D: Hsp:nrd-EGFP transgenic retinas from embryos injected with Nrd-ATG morpholino at the 1-2 cell stage and assayed for BrdU incorporation at 72hpf.

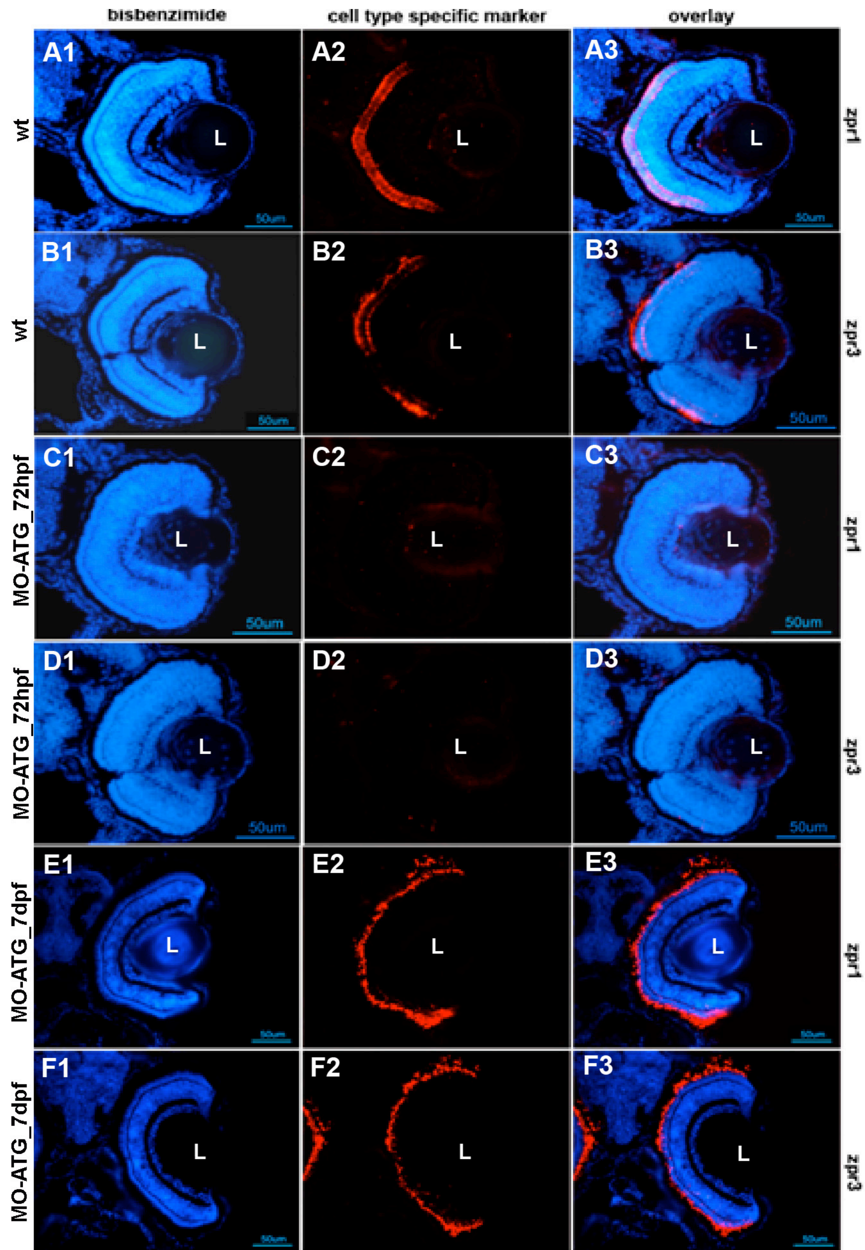

Fig. IV-8: Photoreceptors recover in NeuroD morphant retinas by 7 days post fertilization.

A-B and C-D: wild type and NeuroD morphant retinas, respectively, assayed at 72hpf for zpr-1 (cone marker) and zpr3 (rod marker) staining. E-F: NeuroD morphant retinas assayed at 7dpf for zpr1 and zpr3, respectively.

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CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

Summary of results

In summary, I took advantage of two complementary reverse genetic approaches to determine the function of NeuroD in the zebrafish retina, utilizing both gain and loss-of-function methods. In Chapter II, I showed that there is a dynamic expression pattern of *neuroD* in lineages of rod and cone photoreceptors, suggesting that NeuroD plays a fundamental role in photoreceptor genesis and cone maturation. For Chapter III, I created lines of zebrafish transgenic for HSP70/4:neuroD-EGFP for conditional gain-of-function experiments, and in Chapter IV, I used morpholino oligonucleotides to block translation of the NeuroD protein for loss-of-function experiments. The cellular pattern of *neuroD* expression and results from gain-of-function and loss-of-function experiments reveal that NeuroD promotes cell cycle withdrawal among photoreceptor progenitors, promotes photoreceptor genesis, and may play an additional role in early aspects of cone photoreceptor maturation.

Based on these expression and function data, combined with other studies (Otteson et al., 2001; Hitchcock and Kakuk-Atkins, 2005; Raymond et al., 2006; Bernardos et al., 2007), a model emerges, which incorporates the function

of NeuroD in adult rod and cone genesis (Fig.1A and B). For rods, *Pax6*-expressing Müller glia serve as stem cells, which give rise to *neuroD*-expressing INL progenitors, which traverse to the outer nuclear layer and give rise to *neuroD*- and *Crx*-expressing rod precursors, which give rise to rod photoreceptors that no longer express *neuroD*, but do express *Crx*. For cones, stem cells in the circumferential germinal zone (CGZ) give rise to *neuroD* and *Crx*-expressing cone progenitors, which give rise to nascent cone photoreceptors that express *neuroD* and *Crx*. As nascent cone photoreceptors mature, they no longer express *neuroD*, but continue to express *Crx*.

The current studies further suggest a potential mechanism through which NeuroD promotes cell cycle withdrawal of photoreceptor progenitor cells by regulating the expression of cell cycle regulatory proteins. The cell cycle of eukaryotic cells is composed of four phases, which include: 1) Mitosis (M) phase, when the nucleus and the cytoplasm divide, 2) DNA synthesis (S) phase, when DNA is replicated, 3) Gap 1 (G1) phases, when mitotically active cells respond to signals that promote cell cycle progression or withdrawal from the cell cycle (G0) and differentiation, and 4) Gap 2 (G2) phase between the DNA synthesis phase and Mitosis phase, when DNA replication is completed before the onset of cell division. The progression through all of the phases of the cell cycle is under the control of cyclin–CDK (cyclin-dependent kinase) complexes, and the activity of these cyclin:CDK complexes is regulated by CDK inhibitors, including the CIP/KiP family (Soprano and Giordano, 2003; Dehay and Kennedy, 2007). Whereas cyclin–CDK complexes positively drive progression of the cell cycle,

CDK inhibitors negatively regulate progression through the cell cycle by binding to and inactivating cyclin–CDKs (Dyer and Cepko, 2001a,b; Soprano and Giordano, 2003; Dehay and Kennedy, 2007).

The cyclin inhibitors are regulated at the transcriptional and post-translational levels and studies have shown that NeuroD upregulates the expression of CDK inhibitors p21, p27, and p57 (Naya et al., 1997; Mutoh et al., 1998; Farah et al., 2000; Schonhoff et al., 2004; Chapter III and Chapter IV). Based on the current data, I propose that NeuroD promotes cell cycle withdrawal of photoreceptor progenitor cells by induction of cyclin inhibitors p27 and p57. Induction of p27 and p57, in turn, inhibits cyclin expression and leads to cell cycle withdrawal of photoreceptor progenitor cells (Fig.1C).

NeuroD also promotes photoreceptor genesis and inhibits Müller glia genesis in the zebrafish retina (Fig.1D). Induction of NeuroD expression in cells in the CGZ, which do not normally express NeuroD, promotes the generation of photoreceptors and inhibits the generation of Müller glia. This is consistent with other studies of NeuroD, and bHLH transcription factors in general, as promoters of neurogenesis and inhibitors of gliogenesis (Sun et al., 2001; Morrow et al., 1999; Tomita et al., 2000).

NeuroD and photoreceptor genesis

Whereas in homeothermic vertebrates, such as the mouse and chick, NeuroD appears to play a role in cell fate determination, the teleost retina offers a unique opportunity to study the function of NeuroD specifically in lineages of cells that

give rise to rod and cone photoreceptors. The results in Chapters II-IV show that, in the teleost retina, *neuroD* is only expressed in mitotic cells that give rise exclusively to rod and cone photoreceptors, promotes cell cycle withdrawal of these photoreceptor progenitors, and may function in cone photoreceptor maturation. In contrast, in the mouse retina, *neuroD* is expressed in multipotent retinal progenitors, regulates neuronal versus glial cell fate, is determinative for amacrine cells, and is required for the survival of rod photoreceptors (Morrow et al., 1999; Ahmad et al., 1998; Acharya et al., 1997; Moore et al., 2002; Inoue et al., 2002; Pennesi et al., 2003). NeuroD is also expressed in multipotent progenitors in the chick retina, but is only determinative for cone and rod photoreceptors (Yan and Wang, 1998; Yan and Wang, 2000; Yan and Wang, 2004; Yan et al., 2005; see also Fischer et al., 2004).

The existence of a mitotic lineage generating a single cell type, such as the rod photoreceptor lineage, is not unique and is present in other persistently mitotic regions in the adult central nervous system, including cells in the subventricular zone, dentate gyrus, and cerebellum (Miyata et al., 1999; Schwab et al., 2000; Pleasure et al., 2000; Lee et al., 2000; Alvarez-Buylla and Garcia-Verdugo, 2002; Bedard and Parent, 2004; Hevner et al., 2006; see also Naya et al., 1997; Mutoh et al., 1998; Schonhoff et al., 2004). In each of these regions, *neuroD* is expressed in late stage progenitors, perhaps during their ultimate or penultimate mitosis, and appears to be essential for terminal differentiation. This suggests that NeuroD may have a common function in all of these cell lineages

and insight gained from the teleost retina may be applied to the role of NeuroD in other persistently mitotic tissues of adult vertebrates.

Future Directions

The results in Chapter II, III and IV demonstrate that in the teleost retina NeuroD promotes photoreceptor genesis. However, the precise role of NeuroD in photoreceptor genesis remains to be determined. The process of neurogenesis spans division, cell fate specification, withdrawal from the cell cycle, and differentiation. It is not possible at the present time to parse out the precise role of NeuroD in photoreceptor genesis, due to limitations in currently available methods. These limitations include: 1) absence of available markers that exclusively label mitotically-active photoreceptor progenitors or newly post-mitotic and undifferentiated photoreceptors, 2) lack of reliable methods to track individual cells in the retina, 3) no temporal control of morpholino knock-down. It will be important to address these issues in the future and the Hsp:nrd-EGFP construct, as well as the line of fish transgenic for Hsp:nrd-EGFP that I have created, can be useful tools in this process. I present several approaches below that can be utilized to surmount the potential limitations of both the gain and loss-of-function approaches.

A limitation of the current studies is that there are no available markers that exclusively label mitotically-active photoreceptor progenitors or newly post-mitotic, undifferentiated photoreceptors. While other markers such as cone-rod homeobox, Crx, and retinal homeobox, rx1, genes have been identified, they

label both mitotically-active progenitor cells and differentiated photoreceptors (Shen and Raymond, 2004; Raymond et al., 2006). Thus, it has not been possible to label or track individual photoreceptor progenitor cells to determine the precise function of NeuroD in these cells. In the gain-of-function experiments presented in Chapter III, NeuroD was induced in all cells. However, it is also possible in the Hsp:nrd-EGFP line to induce NeuroD expression in individual cells and this expression can be controlled in both space and time. Methods have been established for zebrafish lines transgenic for a heat shock fusion protein that can allow laser-induced gene expression in specific cells (Halloran et al., 2000). Targeted cells, after application of a sublethal laser microbeam appear to develop normally: progenitor cells divide and give rise to normal progeny, cells migrate normally, and neurons project axons that follow normal pathways (Halloran et al., 2000). It is thus possible, with the use of the sublethal laser microbeam, to induce NeuroD expression in individual cells. In addition, taking advantage of the accessibility and optical clarity of zebrafish embryos and time lapse imaging technology (Das et al., 2003; Baye and Link, 2007), it would be possible to examine in the retina the *in vivo* activity of NeuroD by laser-inducing specific cells and tracking their progeny or developmental process upon induction of NeuroD.

Another approach is to use transient transgenic embryos and evaluate the clones of cells generated in the retina. In Chapter III I show that, in the retina of animals with transiently transgenic for Hsp:nrd-EGFP, there is ectopic expression of *neuroD*. Furthermore, these cells appear as clusters throughout the retina.

This suggests that by inducing NeuroD fusion protein in the retina at 48hpf and assaying 24 or 48 hours later, clones of cells can be tracked and characterized. This is another approach to determine whether NeuroD functions in determination or differentiation of photoreceptors.

Since there are both intrinsic and extrinsic factors involved in photoreceptor genesis, it is important to study both the effect of transcription factors as well as the environment on photoreceptor genesis. To determine whether NeuroD functions in a cell-autonomous or cell-nonautonomous manner, cells from Hsp:nrd-EGFP embryos can be transplanted into wild type retinas. In addition, a method for zebrafish cell culture has been recently established (Vallone et al., 2007). It is possible to culture Hsp:nrd-EGFP retinal cells and determine the optimal factors required for photoreceptor genesis.

The limitation of the loss-of-function approach is that NeuroD is knocked down from the very beginning of retinal development and there is no temporal control knock-down of NeuroD. *In vivo* electroporation has recently been developed as a method for studying gene function at different developmental time points and in specific regions of the organism (Cerda et al., 2006; Hendricks and Jesuthasan, 2007). The focal application of current allows macromolecules to be efficiently introduced into a targeted region at any point in development (Cerda et al., 2006). Transfected cells in the zebrafish brain are amenable to *in vivo* time-lapse imaging and explants containing transfected neurons can be cultured for *in vitro* analysis. Furthermore, due to their optical clarity, embryos can be electroporated with NeuroD morpholinos and the dividing photoreceptor

progenitor cells can be tracked using *in vivo* time-lapse imaging (Das et al., 2003).

In the loss-of-function studies described in Chapter IV, cells continue to proliferate in the outer nuclear layer in the absence of NeuroD. However, it is not possible to determine whether NeuroD only functions in promoting the cell cycle withdrawal of cone photoreceptor progenitors or if it also plays a role in their maturation after they exit from the cell cycle. The electroporation experiments described above can be used to parse out the role of NeuroD in proliferation vs. maturation of cone photoreceptors. This approach can be used to effectively knock-down NeuroD after 48hpf, at a time when the majority of cone progenitors have exited the cell cycle, but do not yet express opsins. At this point in time it will be possible to test whether NeuroD plays a direct role in the maturation of cone photoreceptors. If these nascent cone photoreceptors, in the absence of NeuroD, fail to express markers of differentiated cones, then this will demonstrate that NeuroD does play a role in their maturation. Alternatively, if these nascent cone photoreceptors, in the absence of NeuroD, re-enter the cell cycle, this will demonstrate that NeuroD plays a role in maintaining nascent cones in a post-mitotic state and preventing cell cycle re-entry. There is evidence that differentiated cells must actively maintain their cell cycle in check and perturbations in gene expressions can initiate cell cycle re-entry (Herrup and Yang, 2007). Furthermore, In Chapter IV, I demonstrate that between 72hpf and 7dpf, the effect of morpholinos is attenuated and cells of the rod and cone photoreceptor lineages exit the cell cycle and differentiate. Time-lapse imaging

could also be used to track diving cells during this recovery period and determine if and when they generate photoreceptors.

Another approach that can complement the results from my loss-of-function data in Chapter 4 is the use of the newly developed ZeneMark® Retroviral Insertional Mutation Library. This library contains a comprehensive retroviral insertion library in zebrafish including catalogued mutations in many zebrafish genes. There is a ZeneMark® line currently available that has a mutation in the NeuroD coding region. It would be important to characterize the phenotype of this line and determine whether it phenocopies the morpholino-induced loss-of-function phenotype observed here. This line could also be used to identify other cell cycle regulatory proteins that are altered in the NeuroD mutant line.

NeuroD and downstream targets

Although the pro-neural function of NeuroD has been extensively characterized, the mechanism of its action, including downstream targets, remains largely unknown. In Chapter III, I describe the Hsp:nrd-EGFP transgenic line as well as the production of a polyclonal zebrafish NeuroD antibody (zNrd). Both the transgenic line and the zNrd antibody can be used in Chromatin Immunoprecipitation assays (ChiP) to determine DNA sequences, which bind to NeuroD *in vivo*. ChiP is a procedure used to determine whether a given protein binds to or is localized to a specific DNA sequence *in vivo*. This assay is based on the principle that DNA-bound proteins, such as transcription factors, in living

tissue can be cross-linked to the chromatin on which they are localized. Proteins are first immobilized on the chromatin and the chromatin is then fragmented, allowing for whole protein-DNA complexes to be immunoprecipitated using an antibody specific for the protein in question. The DNA obtained from the protein/DNA fraction that is labeled with the specific antibody, can then be purified. For candidate genes that are hypothesized to bind to NeuroD, the identity of the DNA fragments isolated in complex with the protein of interest can be determined by PCR. Alternatively, to identify unknown gene targets of NeuroD, a DNA microarray can be used (ChIP-on-chip or ChIP-chip). Based on its role in promoting cell cycle withdrawal of photoreceptor progenitors, NeuroD may interact with specific cell cycle regulatory proteins. Previous studies show that NeuroD promotes the upregulation of cyclin-dependent kinase inhibitors, such as p21 and p27 (Farah et al., 2000; Schonhoff et al., 2004). Here I report that NeuroD fusion protein also upregulates p27 and p57 in the retina (Chapter III). It would be important to determine if NeuroD interacts directly with p27 and p57 by utilizing the ChiP assay. Other potential downstream targets of NeuroD could also be identified using this method. Recent studies in Xenopus have identified several putative transcription targets of NeuroD (Logan et al., 2005). Other studies describe the interaction of bHLH transcription factors with chromatin during retinal development (Skowronska-Krawczyk et al., 2004). It will be important to determine in the zebrafish retina whether these mechanisms are conserved and if NeuroD interacts with homologous downstream targets.

NeuroD regulation

In addition to putative downstream targets, NeuroD itself is also a highly regulated molecule. Recent data has shown that NeuroD is regulated at both the post-transcriptional and post-tanslational level (Gaudilliere et al., 2004), and the consequence of NeuroD protein modification is context-dependent at both the molecular and functional levels (Dufton et al., 2005; Song et al., 1998; Khoo et al., 2003). The isolation and characterization of the zebrafish NeuroD gene promoter would be useful in determining elements involved in photoreceptor genesis and differentiation in the retina.

Characterizing the zebrafish NeuroD promoter would enable investigators to describe the structure and regulation of the zebrafish NeuroD gene. In addition, lines of zebrafish transgenic for NeuroD could be created to elucidate *in vivo* the complex expression pattern of NeuroD during retinogenesis. This data could also complement the expression data described in Chapter 2. To date, both the mouse and human NeuroD promoters have been characterized. The Mouse Beta2/NeuroD gene promoter was isolated and characterized (Xu and Murphy, 1998). The cloning and sequencing of the NeuroD promoter allowed for detailed investigation of the elements responsible for islet restricted expression and provided insight into the molecular mechanisms underlying pancreatic β–cell differentiation. The human NeuroD (BETA2/BHF1) gene was isolated and characterized (Miyachi et al., 1999). NeuroD transfection assays revealed that the E1 and E4 boxes are associated with autoactivation. Results suggest that NeuroD gene expression is positively regulated though the E box sequence, not

only by NeuroD itself but also by another E box binding protein (Ahmad et al., 1998).

Along with other key transcriptional regulators Nrl, Crx, and Nr2E3, NeuroD forms part of a large regulatory complex, which promotes rod photoreceptor differentiation in the mouse retina (Ahmad et al., 1998; Mears et al., 2001; Cheng et al., 2006; Oh et al., 2007). In the zebrafish retina, NeuroD is co-expressed with the cone-rod-homeobox transcription factor, Crx, in putative cone progenitors and nascent cone photoreceptors (Chapter III; see also Shen and Raymond, 2004), suggesting that in the zebrafish retina, like in other vertebrate retinas, similar genetic cascades regulate photoreceptor genesis and maturation. However, since *neuroD* is not expressed in multipotent progenitors in the teleost retina, it will be possible to study its function specifically in lineages of cells that give rise to photoreceptors.

The consequence of NeuroD protein modification is context-dependent at both the molecular and functional levels (Dufton et al., 2005). In Xenopus, mutation of serines 266 and 274 of NeuroD to alanines has a positive impact on ectopic neurogenesis, in contrast to its negative effects on mouse NeuroD activity in pancreatic beta cells (Khoo et al., 2003). The modification of the same residue in NeuroD can change its activity by invoking different molecular mechanisms in different cell types. The mutations also result in higher NeuroD protein accumulation, since phosphorylation may directly regulate protein stability by targeting it for ubiquitin-mediated degradation. A similar strategy is used to control the degradation of a related bHLH protein, MyoD (Song et al., 1998),

where phosphorylation of a single serine residue was found to catalyze ubiquitin-mediated targeting to the proteosome. In the zebrafish retina, this same strategy is used by GSK3β, which has been shown to phosphorylate NeuroD and inhibit its function in the early retina and this inhibition is removed during later stage of retinal development (Moore et al., 2002).

Growth hormone may be another factor that modulates the activity of NeuroD. Growth hormone acts through IGF-I in a signaling pathway to promote neurogenesis in the retina by regulating the mitotic activity of the resident populations of stem cells (Boucher and Hitchcock, 1998). NeuroD in photoreceptor progenitor cells may interact with the GH/IGF-I axis in modulating the pool of photoreceptor progenitor cells. Recent evidence has shown that cone photoreceptors are a source of Igf-1 that regulates rod progenitor proliferation in the retina (Zygar et al., 2005). It has been suggested that Igf-1 promotes the proliferation of rod progenitor cells and may be involved in a feedback loop during development. Growth hormone has been shown to inhibit another bHLH factor Ngn1 (Turnley et al., 2002). Since my data indicates that NeuroD promotes cell cycle withdrawal of rod photoreceptor progenitors, Igf-1 may regulate the action of NeuroD.

Studies have shown that chromatin modification plays a critical role in the regulation of cell-type-specific gene expression. An inhibitor of histone deacetylase (HDAC), valproic acid (VPA), promotes neurogenesis and inhibits gliogenesis through the induction of neurogenic transcription factors, including NeuroD (Hsie et al., 2004). Recent evidence, however, has shown that HDAC

activity is required for rod photoreceptor genesis (Chen and Cepko, 2007). HDAC inhibition in the retina results in cell death, reduction in proliferation, and a complete loss of rod photoreceptors and Müller glial cells, with a concomitant increase in bipolar cells. The precise role of NeuroD in this process and its interaction with HDAC remains to be determined.

In addition, NeuroD is the first identified transcription factor involved in neuronal development and survival whose activity is modulated by Huntingtin, Htt, a key protein associated with Huntington's disease (Marcora et al., 2003). Two proteins have been identified that interact with NeuroD, Huntingtin-associated protein 1 (HAP1) and mixed-lineage kinase 2 (MLK2) and Htt, together with HAP1, may function as a scaffold for the activation of NeuroD by MLK2. The zebrafish Huntingtin gene has been cloned (Karlovich et al., 1998) and its role on development has been recently investigated (Lumsden et al., 2007). It would be important to determine if in the zebrafish NeuroD interacts with Huntingtin to investigate the potential mechanisms involved in Huntington's disease.

NeuroD and cone photoreceptor maturation

Based on its expression pattern, I hypothesized that in the zebrafish retina NeuroD functions in the maturation of cone photoreceptors. NeuroD is expressed in cone progenitors and then remains transiently expressed in immature cones. This expression is turned off in mature cones which begin to express opsin. This is consistent with data that shows that NeuroD plays a role in the maturation of

numerous other cell types in the brain, including cells in the olfactory bulb and olfactory epithelium, taste bud cells, and granule cells in the dentate gyrus and cerebellum (Bedard and Parent, 2004; Suzuki et al., 2002; Katayama et al., 1997; Miyata et al., 1999; Lee et al., 2000; Nibu et al., 1999; Pleasure et al., 2000; Hevner et al., 2006). In all of these cases, NeuroD may regulate the expression of genes involved in the maturation of these cells and their integration into the existing neuronal network.

A clue to a possible mechanism for the role of NeuroD in cone photoreceptor maturation has recently come from work in a related bHLH transcription factor, NeuroD2 (Ince-Dunn et al., 2006). During cortical development both activity-dependent and genetically determined mechanisms are required to establish proper neuronal connectivity. The existence of a mechanism of activity-dependent transcription and synaptic modification has been postulated 60 years ago by Donal Hebb (1948). A recent study indicates that NeuroD2, a calcium-regulated transcription factor, plays a critical role in regulating activity-dependent maturation of glutamatergic synapses (Ince-Dunn et al., 2006; Molnar and Molnar, 2006). NeuroD was recently shown to be calcium regulated and requires phosphorylation of a key serine residue for activation (Wu et al., 1996; Gaudilliere et al., 2004). The fact that both NeuroD and NeuroD2 act as calcium-regulated transcription factors identifies this protein family as a major target of calcium signaling in neurons. Like its counterpart NeuroD2, NeuroD may also regulate the expression of factors that are required for synaptic maturation.

It is interesting to note that the photoreceptor ribbon synapse is a highly specialized glutamatergic synapse designed for the continuous flow of synaptic vesicles to the neurotransmitter release site (Dick et al., 2003). NeuroD may function in cone photoreceptor maturation by promoting synaptogenesis. Synaptic maturation at the photoreceptor terminals in zebrafish was examined with antibodies against synapse associated proteins (Biehlmaier et al., 2003) and similar methods can be used to evaluate cone photoreceptor maturation in NeuroD gain and loss-of-function studies.

NeuroD and cell migration

Another possible function of NeuroD in neurogenesis is neuronal migration. There is misdirected migration of inner ear sensory neurons in NeuroD null mice, suggesting that NeuroD not only regulates survival of inner ear sensory neurons but also plays a role in placing neurons in proper topological positions (Kim et al., 2001). A similar defect is found among NeuroD-null pancreatic endocrine cells which cluster near the ductal epithelium, rather than migrating and forming islets (Naya et al., 1997). NeuroD may control some factors involved in cell migration, however, such a developmental defect could also be due to incomplete differentiation resulting in arrested or defective migration. In Chapter IV, I describe that, in the absence of NeuroD protein, photoreceptor progenitor cells continue to proliferate in the inner and outer nuclear layer. Cells in the inner nuclear layer labeled with BrdU are scattered and sometimes appear in clusters extending towards the outer nuclear layer. In Chapter IV, I propose that these are

cells of the rod photoreceptor lineage (Otteson et al., 2001; Hitchcock and Kakuk-Atkins, 2004; Chapter 2), which continue to proliferate in the absence of NeuroD fusion protein. These cells normally migrate from the inner to the outer nuclear layer where they generate rod photoreceptors. In the morphant retina, they remain in the inner nuclear layer and may fail to migrate to the outer nuclear layer. NeuroD may thus play a role in the migration of rod precursors from the inner nuclear layer to the outer nuclear layer.

Photoreceptor genesis and disease

Retinal degenerative diseases result in irreversible and permanent vision loss. Extensive research has focused on understanding the mechanisms involved in stimulating photoreceptor genesis, repair, protection, and regeneration in the retina. *NeuroD*, a basic helix-loop-helix transcription factor, which is a member of a large family of proneural genes, has been implicated in cell cycle regulation, retinal cell genesis, and neuronal development, and misexpression of this gene in other systems has led to *de novo* photoreceptor genesis in the retina.

NeuroD may be one of the key transcription factors that can promote photoreceptor genesis to compensate for photoreceptor degenerative diseases in the human retina. Results reveal that NeuroD not only plays an important role in terminal differentiation of photoreceptors but also serves as a potential survival factor (Pennesi et al., 2003). Loss of NeuroD results in an age-related degeneration of both rods and cones. NeuroD is thus important for photoreceptor survival and maintenance. NeuroD transcripts and NeuroD immunoreactivity in the human retina are predominantly localized to the outer nuclear layer which

contains photoreceptors (Acharya et al., 1997). Based on the expression and localization analysis NeuroD may be involved in the differentiation as well as maintenance of the differentiated properties of photoreceptors.

There is mounting evidence pointing to a common role for NeuroD in persistently mitotic cellular lineages, linking cell cycle withdrawal with terminal differentiation. In all persistently mitotic regions in the adult central nervous system, *neuroD* is expressed in late stage progenitors and appears to be essential for terminal differentiation. (Miyata et al., 1999; Schwab et al., 2000; Pleasure et al., 2000; Lee et al., 2000; Bedard and Parent, 2004; Hevner et al., 2006; see also Naya et al., 1997; Mutoh et al., 1998; Schonhoff et al., 2004). Determining the mechanisms involved in persistent neurogenesis in the teleost retina will be instrumental in developing methods of stimulating photoreceptor genesis in the human retina following photoreceptor degenerative diseases.

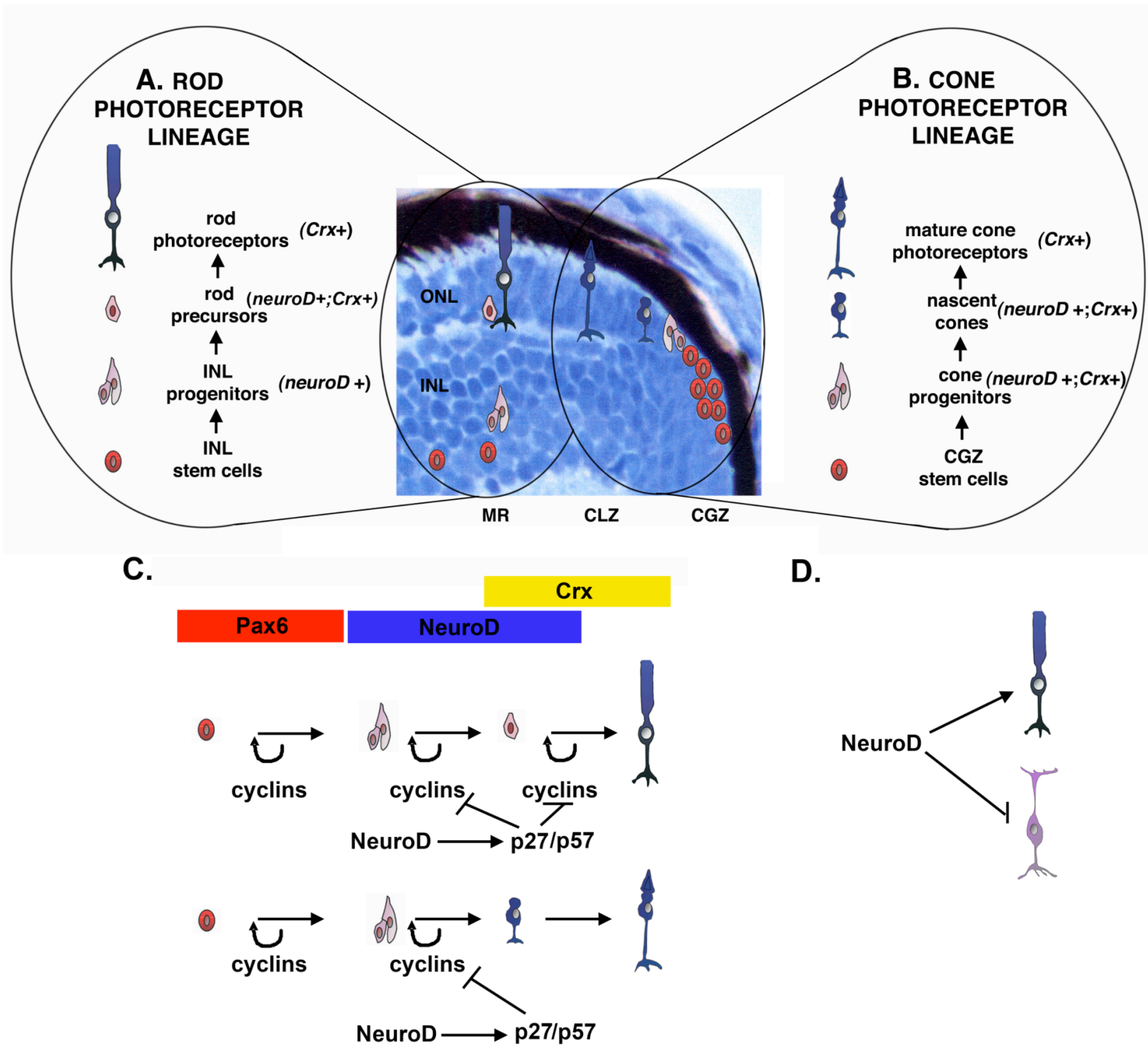

Fig. V-1: Model illustrating rod and cone genesis in the teleost retina and the function of NeuroD.

A: Lineage model of rod genesis. Inner nuclear layer (INL) stem cells give rise to *neuroD*-expressing INL progenitors, which traverse to the outer nuclear layer and give rise to *neuroD*-expressing rod precursors, which give rise to rod photoreceptors that do not express *neuroD* but do express *Crx*. B: Lineage model of cone genesis. Circumferential germinal zone stem cells give rise to *neuroD* and *Crx*-expressing cone progenitors, which give rise to *neuroD* and *Crx*-expressing nascent cone photoreceptor, which give rise to mature cone photoreceptors that do not express *neuroD* but continue to express *Crx*. C: NeuroD induces expression of p27 which inhibits cyclin expression and leads to cell cycle withdrawal of photoreceptor progenitor cells. D: NeuroD promotes photoreceptor genesis and inhibits Muller glia genesis in the zebrafish retina. ONL = outer nuclear layer; INL = inner nuclear layer; GCL = ganglion cell layer; MR = mature retina; CLZ = circumferential larval zone; CGZ = circumferential germinal zone.

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