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Zebrafish: A Model System for the Study of Eye Genetics

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Abstract

Over the last decade, the use of the zebrafish as a genetic model has moved beyond the proof-of-concept for the analysis of vertebrate embryonic development to demonstrated utility as a mainstream model organism for the understanding of human disease. The initial identification of a variety of zebrafish mutations affecting the eye and retina, and the subsequent cloning of mutated genes have revealed cellular, molecular and physiological processes fundamental to visual system development. With the increasing development of genetic manipulations, sophisticated techniques for phenotypic characterization, behavioral approaches and screening strategies, the identification of novel genes or novel gene functions will have important implications for our understanding of human eye diseases, pathogenesis, and treatment.

Keywords

zebrafish genetics; retina; lens; development; disease

1. Introduction

The zebrafish (*Danio rerio*; *Brachydanio rerio* in older literature) has become a powerful model system to study genetic mechanisms of vertebrate development and disease. Much of the current success can be traced back to the pioneering work of George Streisinger and colleagues at the University of Oregon. Like many of his peers who had acclaimed research programs on phage genetics, Streisinger sought a eukaryotic system to expand further the known roles of genes in biological processes. Whereas Seymour Benzer focused his efforts on *Drosophila* and Sydney Brenner (Brenner, 1974) adopted the nematode worm, Streisinger, a fish hobbyist, turned his efforts towards the zebrafish (Streisinger *et al.*, 1981; Chakrabarti *et al.*, 1983; Walker and Streisinger, 1983; Grunwald and Streisinger, 1992).

Streisinger first recognized many of the oft-cited advantages for the use of zebrafish as a genetic model (Mullins and Nusslein-Volhard, 1993; Driever *et al.*, 1994; Solnica-Krezel *et al.*, 1994). Zebrafish, small freshwater teleosts, are easily adapted to the laboratory setting and can be maintained in a relatively small space. The fish typically reach sexual maturity in 3 to 4 months, and a breeding pair of fish can produce >200 fertilized eggs per mating. Fertilization is external, and the egg and embryo are transparent, facilitating visual identification of

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morphogenetic movements and organogenesis with a standard dissecting microscope. Development is rapid; by 24 hours post-fertilization (hpf) all of the major organ systems have formed and spontaneous muscle flexures soon begin. Prior to 48 hpf the first behavioral responses can be observed, and by 3 days post-fertilization (dpf) a free swimming larva that actively feeds upon small prey has emerged. Many of the methods in use today, including gamma ray and chemical mutagenesis, haploid screens and diploidization, transgenesis and forward and reverse genetic approaches, have underpinned its rapid success for experimental and genetic manipulations of the visual system.

2. Mutagenesis

Forward genetic screens represent an unbiased approach to uncover novel genes or novel gene functions. An organism is mutagenized with a chemical, radiation or a DNA mutagen, and the appearance of an interesting phenotype is sought in subsequent generations. The *mutated* gene leading to the phenotype is isolated, cloned and sequenced. Not only can the function of the mutated gene be elucidated by this method, but also fundamental cellular or behavioral processes can be studied in the absence of the specific gene product. Following the pioneering work at the University of Oregon, two laboratories developed methods for efficient and large-scale chemical mutagenesis of zebrafish for the expressed purpose of identifying recessive mutations affecting embryonic development (Mullins and Nusslein-Volhard, 1993; Driever *et al.*, 1994; Solnica-Krezel *et al.*, 1994). Both screens used the alkylating agent N-ethyl-N-nitrosourea (ENU) to induce point mutations in zebrafish spermatogonia. The effectiveness of ENU mutagenesis typically generates more than one mutant phenotype per genome. Recessive mutations are then recovered in a traditional third-generation screen. The high rate of mutagenesis combined with morphological analysis, enabled the isolation of thousands of mutations affecting hundreds of loci essential to development of the vertebrate embryo, including the eye and visual system (Brockerhoff *et al.*, 1995; Baier *et al.*, 1996; Driever *et al.*, 1996; Haffter *et al.*, 1996; Malicki *et al.*, 1996). These methods subsequently have been adopted for several highly focused screens to identify specifically mutations affecting the development and function of the retina (Fadool, *et al.*, 1997; Li and Dowling, 1997; Vihtelic, *et al.*, 2001; Perkins *et al.*, 2002; Wehman *et al.*, 2005). Recent progress on the genome assembly, enhanced mapping techniques, finer resolution of the genetic map, and assignment of cloned genes to genetic loci, have led to the cloning of an increasing number of ENU-induced mutations including those involved in the visual system by positional or candidate gene approach. Comparative studies provide evidence of a genome wide duplication in the lineage of ray-finned fishes. Estimates suggest that for at least twenty percent of human genes the zebrafish has preserved two orthologues (reviewed in Postlethwait, *et al.*, 2004). Though the initial assumptions held that the presence of two functional copies of a gene would mask the effects of mutations in one or the other, many of the zebrafish co-orthologues demonstrate subfunctionalization of the roles of the ancestral gene. That is, the spatial or temporal pattern of expression of both copies is necessary to fulfill the function of the orthologous gene. This has had the serendipitous benefit of enabling the genetic dissection of each subfunction independently.

2.1 Insertional Mutagenesis

Another major advance in zebrafish forward genetic screens occurred with the application of a pseudotype retrovirus vector for insertional mutagenesis, enhancer trap and gene trap screens (Lin *et al.*, 1994; Gaiano *et al.*, 1996; Golling *et al.*, 2002; Amsterdam *et al.*, 2004). First developed as a vector for gene therapy and genetic studies, the engineered virus can infect a wide range of organisms and efficiently integrate into the genome. In zebrafish, transformation rates are approaching 100%, with most founders transmitting on average 10 proviral inserts to progeny. One in 80 inserts results in an embryonic lethal mutation, and in a large-scale screen

hundreds of insertional mutations were recovered over a several year period, although only a fraction of these resulted in specific developmental phenotypes. It is estimated that the 315 saved mutants reflect 25% of the genes essential for the development of many different embryonic structures and organs including the eye (Allende *et al.*, 1996; Becker *et al.*, 1998; Amsterdam *et al.*, 2004; Gross *et al.*, 2005). Comparisons to other species, namely *Saccharomyces cerevisiae* and *Caenorhabditis elegans*, revealed that 77% and 72% respectively of the essential fish genes are evolutionarily essential in the other species. One clear advantage of insertional mutagenesis is that the proviral insert acts as a molecular tag that facilitates the rapid cloning of the mutated gene as compared to the laborious effort required for positional cloning of ENU-induced mutations (Gaiano *et al.*, 1996). However, the use of the retrovirus techniques in modestly sized screens or to isolate specific phenotypes may be limited. Other transgenesis methods based upon transposon technologies have been developed to identify gene regulatory sequences making possible enhancer trap and gene traps, though the mutagenesis frequency of these remains relatively low.

2.2 Reverse Genetics

Although embryonic stem cells and targeted mutagenesis by homologous recombination as routinely performed in mice have not yet been developed for zebrafish, alternative reverse genetic and gene knock-down methods in zebrafish have enabled the analysis of phenotypes of known genes. Targeting induced local lesions in genomes (TILLING), a method originally developed for plant mutagenesis screens, has been used successfully to identify genetic lesions in specific genes of interest (McCallum *et al.*, 2000; Wienholds *et al.*, 2002, 2003; Henikoff *et al.*, 2004; Till *et al.*, 2004). Although providing the opportunity to screen for mutations in virtually any gene, TILLING and similar approaches are very labor intensive, require large-scale ENU mutagenesis combined with PCR-based assays to identify fish carrying lesions in the genes of interest, followed by the recovery of the alleles in subsequent generations. The early reports also provide evidence that many of the mutations are silent or that the amino acid substitutions do not alter the function of the gene product. However, the ability to target exons of known functional importance makes this a very promising method to isolate alleles with alterations similar to human disease genes or with potentially known alterations in function. In all of these examples, the mutated genes provide starting points to identify upstream and downstream components of the genetic pathway.

The more common strategy to determine the function of a known gene is by decreasing the level of its expression through injection of modified antisense oligonucleotides (morpholinos) to block translation of the desired mRNA or post-transcriptional processing of the nascent RNA (Nasevicius and Ekker, 2000). More recently, peptide nucleic acids have been evaluated as an alternative to morpholinos. Peptide nucleic acids demonstrate stringent hybridization properties, are resistant to most peptidases and nucleases (Urtishak *et al.*, 2003; Wickstrom *et al.*, 2004a, b). Although not truly genetic methods, these knock-down approaches allow investigators to test the role of a specific gene in a given process, confirm that a mutant phenotype can be phenocopied by blocking expression of the suspected gene, or used to inhibit functions of several genes simultaneously without the time-constraints imposed by interbreeding of heterozygous carriers of different mutations. Morpholino-modified oligonucleotides are designed to be complementary to the translation initiation sequence or putative intron – exon splice-sites of the target gene. When injected into the one-cell-stage embryo, the modified oligonucleotides hybridize to the target sequence and inhibit translation of the mRNA or splicing of the preRNA respectively. These approaches have been used successfully in several studies to address specifically early patterning and cellular differentiation in the neural retina, although several limitations have been identified (Malicki, 2000; Gregg *et al.*, 2003; Tsujikawa and Malicki, 2004a; Van Epps *et al.*, 2004). For example, morpholinos typically inhibit protein expression for only 2 to 3 days and continued proliferation

may effectively decrease the cellular concentration of the morpholino. Interestingly, peptide nucleic acids targeted to the *dharma* (*bozozok*) gene effectively phenocopied the genetic mutation while morpholinos did not. Most recently, the potential use of short interfering RNAs (siRNAs) has been demonstrated in zebrafish, offering yet a third possibility for altering the expression of the target gene (Boonanuntanasarn *et al.*, 2003; Dodd *et al.*, 2004). Taken together, the knock-down strategies provide necessary and viable alternatives to genetic methods for investigating developmental processes by manipulating specific gene products.

3. Eye anatomy

The anatomy, histology, circuitry and biochemistry of the eye are strikingly conserved among most classes of vertebrate. Not surprising, therefore, were the observations that development of the eye also proceeds in very similar manners. The eye develops from no less than three distinct embryological tissues, neuroectoderm which gives rise to the neural retina, pigmented epithelium, optic stalk and ciliary margin; skin ectoderm, which is induced to form the lens and subsequently the cornea; and head mesenchyme of neural crest cell origin that minimally forms connective tissue of the cornea and sclera. The highly conserved mechanisms of development and function have contributed significantly to our overall understanding of visual processes and led to rapid advances in understanding the basic mechanisms of diseases.

3.1 Retinal Anatomy

Like most classes of extant vertebrates, the zebrafish retina is composed of seven major cell types derived from the neural ectoderm, six neurons and a single glial cell, the Müller cell (Figure 1A-C). However, this simplified view grossly understates the true diversity of neuronal types that contribute to the complex circuitry of the vertebrate retina (Kolb *et al.*, 2001; Masland, 2001). The major classes of interneurons, the horizontal, bipolar and amacrine cells can also be subdivided into numerous subpopulations based upon morphological, immunohistochemical and physiological profiles. Using a recently developed method to label cells randomly with lipophilic dyes, referred to as DiOlistics, several groups have classified the retinal interneurons based upon morphology, synaptic location and terminal arborization (Gan *et al.*, 2000; Connaughton *et al.*, 2004). The ability of the lipophilic dyes to freely diffuse within the plasma membrane of the labeled neurons provides a method reminiscent of the Golgi staining so elegantly utilized by Cajal. From DiOlistic, fluorescent images and physiological recordings, horizontal cells were classified into three or perhaps four subtypes, H1/H2, H3 and H4 (Song *et al.*, 2007), and whole-cell recording demonstrates many properties similar to other teleost horizontal cells (McMahon, 1994). Amacrine cells were categorized into seven morphological subtypes, and though these morphological classifications are in basic agreement with previous reports using immunolabelling and metabolic signatures, a greater diversity of the amacrine cell subtypes will likely be identified using additional methods (Marc and Cameron, 2001; Yazulla and Studholme, 2001). Bipolar cells have been best characterized in zebrafish using a combination of physiological recordings followed by backfilling with a fluorescent dye or DiOlistic labeling (Connaughton and Nelson, 2000). The majority of the bipolar cells demonstrate ON or OFF responses consistent with their terminals being in sublamina b of the inner plexiform layer or sublamina a respectively. The remaining is multistratified with terminals in both sublaminae. Ganglion cells have been characterized by fluorescent labeling in flat mounted retinas in combination with confocal image reconstruction (Mangrum *et al.*, 2002). Eleven morphological ganglion cell subtypes have been identified including wide-field and narrow-field ganglion cells as well as unistratified, multistratified and diffusely branching types.

Unlike rodent models, the zebrafish is diurnal and its retina contains a large number of diverse cone subtypes in addition to rods (Branchek, 1984; Branchek and Bremiller, 1984; Larison and Bremiller, 1990; Raymond *et al.*, 1993; Raymond *et al.*, 1995; Schmitt and Dowling, 1996).

The cones are subdivided into four classes based upon spectral sensitivity and morphology (Raymond *et al.*, 1993; Robinson *et al.*, 1993). The cone photoreceptors are tiered within the outer nuclear layer with the red- and green-sensitive cones paired as distinct long double cones (Figure 1E). The red cone is the slightly longer principal member, whereas the green cone is the shorter accessory member. The long single cones are blue-sensitive while the short single cones are the ultraviolet (uv)-sensitive cones. The rod cell bodies are located vitread to the cone nuclei, and in the light-adapted retina, the thin rod inner and outer segments project beyond the cones to interdigitate with the apical microvilli of the pigment epithelium (Burnside, 2001).

3.2 Mosaic Organization

The well-characterized laminar organization of the retina is complemented by the nonrandom or mosaic organization of the neuronal populations within each of the layers (Wässle and Riemann, 1978; Cameron and Carney, 2000; Rockhill *et al.*, 2000; Fadool, 2003). Gaps in the distribution of cells or random clustering would result in under-representation or over-sampling of information in those regions of the visual field. In the fish retina, this arrangement is most evident in the outer nuclear layer where the position of each cone subtype is precisely arranged relative to the others (Fadool, 2003; Robinson *et al.*, 1993) resulting in a highly ordered crystalline-like mosaic. In adult zebrafish, the mosaic is composed of columns of alternating blue- and UV-sensitive single cones that alternate in turn with columns of red- and green-sensitive double cones. The parallel columns are aligned such that in a horizontal row, the green-sensitive members of the double cones flank the short single cones, whereas the long single cones flank the red-sensitive member of the double cone. In the larval retina, the basic rules governing photoreceptor cell patterns are observed, with green photoreceptors alternating with red cones and blue cones alternating with UV cones; however, the mosaic pattern is far less precise than that of the adult (Figure 1D & F).

Although the necessity for a mosaic organization is well recognized, little is known about the mechanisms underlying this organization. The conservation between the fly and vertebrates of numerous aspects of neurogenesis has lead to the speculation that lateral inhibition plays a role in mosaic formation in the vertebrate retina analogous to the role of lateral inhibition during specification and patterning of photoreceptor cells in the *Drosophila* ommatidia (Cagan and Ready, 1989; Baker *et al.*, 1990; Raymond *et al.*, 1995; Schmitt and Dowling, 1996). In the retina, lateral inhibition plays a role in the specification of early versus late neuronal cell types and has been suggested to have a second role in mosaic organization of the photoreceptor cells through maintenance of radial patterning (Ahmad *et al.*, 1997; Austin *et al.*, 1995; Bernardos, *et al.*, 2005; Waid and McLoon, 1998). Yet, there are conflicting views from mammals on the role of lateral cellular migration and pruning of dendritic processes during formation of the mosaics of interneurons and ganglion cells (Cook and Chalupa, 2000; Eglen *et al.*, 2000; Galli-Resta, 2002; Lin *et al.*, 2004; Novelli *et al.*, 2004). With the genetic tools afforded by the zebrafish, in combination with its highly ordered arrangements of neurons, it is possible to distinguish between these hypotheses and dissect the gene networks involved in the mosaic patterning and subtype specification of vertebrate neurons in the same manner that the crystalline arrangement of *Drosophila* photoreceptors provided a model of cell-to-cell interaction dependent neural development.

4. Eye development

During zebrafish development, eye and lens morphogenesis, retinal histology and the expression of transcription factors exhibit a great deal of consistency with other vertebrates. During neurulation, expression of the transcription factors *Six3a* and *Pax6* in the anterior neural plate specify the ocular tissues (Loosli *et al.*, 1998, 1999, 2003; Nornes *et al.*, 1998; Seo *et al.*, 1998; Wargelius *et al.*, 2003). Through subsequent morphogenetic movements and

inductive interactions, the zebrafish eyes develop from bilateral paddle-shaped masses of cells that evaginate from the forebrain (Schmitt and Dowling, 1994). Disruption of the *chokh/rx3* genes results in a failure of the retinal progenitor cells to evaginate leading to an eyeless phenotype (Loosli *et al.*, 2003). Twenty-two hours post fertilization, invagination of the central portions of this eye-mass and formation of the optic lumen contribute to the formation of an optic cup (Table I; (Schmitt and Dowling, 1994, 1999)). The inner layer continues to proliferate and produces the neural retina, whereas the outer layer gives rise to the retinal pigment epithelium (RPE), likely through the action of *mitf* expression (Lister *et al.*, 2001). The positioning of the optic stalk is regulated by the expression of the *Pax2* and *Pax6* genes (Macdonald *et al.*, 1995).

4.1 Lens Development

As in all vertebrate studied to date, the lens placode of zebrafish is induced to form from the overlying skin ectoderm though here exist subtle differences in the morphogenetic processes between mammals, chick and zebrafish. In zebrafish, the lens placode delaminates from the surface ectoderm to form a solid sphere of cells rather than through invagination and formation of a lens vesicle (Figure 2A; (Schmitt and Dowling, 1994; Soules and Link, 2005; Zhau, *et al.*, 2006)). Subsequently, the mass of cells detaches from the ectoderm by apoptosis of the intervening cells. Like other species, the anterior lens epithelium proliferates, giving rise to the elongating cells of the transition zone that differentiate into the crystalline fiber cells at the lens core. Corneal formation has also been well described and follows similar patterns of morphogenesis of the ectoderm, migration of neural crest cells that contribute to the endodermal layer, and protein expression patterns very similar to that observed in other vertebrates though surprisingly, the formation of the fully mature, laminar collagen architecture is quite protracted, appearing some thirty days post fertilization, and well after the zebrafish become visually active. The significance of these subtle differences for visual function has yet to be determined.

4.2 Neurogenesis

Returning to the retina, birthdating studies have described an orderly process of retinal neurogenesis. Similar to that observed in other species, the first cells to exit the cell cycle differentiate into ganglion cells. Neurogenesis then follows in an approximate inner to outer retinal order (Hu and Easter, 1999). The first postmitotic cells and differentiation of ganglion cells are identifiable between 28 hpf and 32 hpf, in the ventral patch, a region of precocious neural development in the ventral-nasal retina (Kljavin, 1987; Schmitt and Dowling, 1994, 1996; Burrill and Easter, 1995; Hu and Easter, 1999). Differentiation then spreads dorsally around to the ventral temporal retina in a wave-like manner reminiscent of the movement of the morphogenetic furrow in *Drosophila* (Schmitt and Dowling, 1996). Also similar to *Drosophila*, the wave of differentiation is associated with a wave of *sonic hedgehog* expression by the differentiating cells (Neumann and Nusslein-Volhard, 2000; Shkumatava *et al.*, 2004). The similarities do not end there. The specification of ganglion cells requires the expression of the basic helix-loop-helix transcription factor *atonal5*, a homologue of the *Drosophila* gene *atonal*, a gene required for the specification of the R8 photoreceptor. *Atonal5* is required for ganglion cell specification in many vertebrate species (Brown *et al.*, 1998; Kay *et al.*, 2001; Liu *et al.*, 2001). In the absence of *ath5* in the *lakritz* mutant, neuroblasts fail to be specified as ganglion cells and remain in the cell cycle giving rise to later born cell types (Kay *et al.*, 2001). However, expression of *Sonic hedgehog* by amacrine cells appears to mediate specification of the other retinal neurons (Shkumatava *et al.*, 2004). As we have alluded to, the differentiation of the ganglion cells is followed very closely by the differentiation of amacrine cells, interneurons and retinal lamination. By 48 hpf, lamination has spread across most of the retina (Schmitt and Dowling, 1999). Cytochemically, Müller glia are amongst the last to express the mature phenotype in many species including zebrafish, and their

differentiation is promoted by signaling through the notch pathway (Bernados, et al., 2005; Peterson *et al.*, 2001a; Scheer, *et al.*, 2001). Although cells of the inner nuclear layer are postmitotic by 48 hpf, glutamine synthetase and carbonic anhydrase, two markers of functional Müller glia cells are not detectable until approximately 72 and 96 hpf, respectively, and their expression can be blocked by the pharmacological inhibition of the notch pathway (Bernados, et al., 2005) consistent with the role of neural glial interactions in Muller cell maturation (Linser and Moscona, 1979 & 1983).

4.3 Photoreceptor Development

In the outer retina, rhodopsin and red-cone opsin are initially detected in the ventral patch by 50 hpf, preceding the expression of the other cone opsins (Kljavin, 1987; Larison and Bremiller, 1990; Raymond *et al.*, 1995; Schmitt and Dowling, 1996). The expression of the cone opsins also spreads in a wave-like manner into the dorsal and temporal retina. Interestingly, the UV-sensitive cones are the first cones to mature in zebrafish, whereas the red – green double cones are the last to mature. Between 72 to 96 hpf, most major classes of cells can be identified in the central retina by morphological or cytochemical criteria. During this period of differentiation, the first behavioral responses can be elicited, coincident with the appearance of the outer segments and synaptic ribbons (Table I; Easter and Nicola, 1996, 1997; Schmitt and Dowling, 1996, 1999). In teleosts, some data suggest that differentiation of rods follows a developmental program distinct from that of the cones (Johns, 1982; Raymond and Rivlin, 1987; Otteson *et al.*, 2001; Otteson and Hitchcock, 2003). In zebrafish larvae, the regular spread of cone opsin expression into the dorsal and temporal retina is in contrast to the sporadically distribution of rod opsin across the retina (Raymond *et al.*, 1995; Schmitt and Dowling, 1996; Fadool, 2003). The first detectable rod responses by ERG appear between 15 to 21 dpf (Saszik *et al.*, 1999; Bilotta *et al.*, 2001).

5. Genetic Screens

The zebrafish has proven a powerful tool for the genetic analysis of visual system development and function. The large-scale genetic screens, and many other smaller screens, have recovered numerous loci with discrete functions in cellular specification and morphogenesis, retinal lamination, axonal guidance and photoreceptor cell function (Brockerhoff *et al.*, 1995; Allende *et al.*, 1996; Baier *et al.*, 1996; Karlstrom *et al.*, 1996; Malicki *et al.*, 1996; Trowe *et al.*, 1996; Fadool *et al.*, 1997; Li and Dowling, 1997; Neuhauss *et al.*, 1999; Doerre and Malicki, 2001, 2002; Vihtelic *et al.*, 2001; Holzschuh *et al.*, 2003; Jensen and Westerfield, 2004). Three types of primary assays have been utilized to identify mutations affecting the visual system – morphology, visually evoked behaviors, and gene expression based on either transgene expression or *in situ* labeling, with additional screens having been proposed or initiated.

5.1 Morphological Screens

The clarity of the early embryo and relatively large size of the eye and lens make screening for morphological defects relatively straightforward. In the large-scale screen in Boston, mutations at 36 loci affecting various aspects of eye development were identified (Malicki *et al.*, 1996). These were classified into seven categories based on phenotype, including alterations in retinal patterning, photoreceptor cell survival, eye shape and size, and pigmentation. In an ongoing, multifaceted screen for morphological and behavioral defects of the visual system, 17 mutations leading to morphological defects were initially reported, including defects of the anterior chamber, altered retinal lamination and several demonstrating diminished cell proliferation at the retinal margin (Brockerhoff *et al.*, 1995; Fadool *et al.*, 1997; Li and Dowling, 1997; Kainz *et al.*, 2003). Though many of the morphological mutants fell into several of the categories described in the large-scale screen, the latter demonstrated that a single lab could successfully conduct a highly focused multifaceted screen directed at a single organ

system (Fadool *et al.*, 1997). In total, the morphological screens have provided significant inroads into the genetic pathways or cellular functions essential to fundamental processes of retinal development. For example, from several different labs mutations have been isolated resulting in altered retinal lamination and formation of photoreceptor rosettes but otherwise with normal neuronal differentiation (Malicki *et al.*, 1996, 2003; Jensen *et al.*, 2001; Masai *et al.*, 2003). In the mutant embryos, mitotic activity was not restricted to the apical margin but rather was distributed across the width of the neuroepithelium. Subsequent cloning revealed that several of the mutations disrupted genes involved in epithelial junctional complexes, demonstrating the importance of maintaining epithelial polarity for the appropriate radial arrangement of neuroprogenitors and lamination of the retina (Jensen and Westerfield, 2004; Malicki, *et al.*, 2003; Wei and Malicki, 2002; Wei *et al.*, 2004).

The importance of chromatin remodeling complexes in neurogenesis was demonstrated by several mutations. The *young* (*yng*) mutant embryos display a variety of defects including a failure of retinal cell differentiation (Link *et al.*, 2000). Surprisingly, the normal expression pattern of *ath5* in *yng* mutant larvae was observed as was the wave of expression of the signaling molecule *sonic hedgehog*. However, mitogen-activated protein kinase (MAPK) and *Brn3.2*, a transcription factor necessary for ganglion cell differentiation, were severely hindered in expression (Gregg *et al.*, 2003; DeCarvalho and Fadool, unpublished observations). Positional cloning of the *yng* mutation identified an essential role for the *brahma-related* (*brg1*) chromatin-remodeling complex in mediating retinal cell differentiation (Gregg *et al.*, 2003). *Brg1* is a helicase associated with a large megadalton chromatin-remodeling complex implicated in development, cell proliferation and tumorigenesis. The mutation was partially rescued by injection of a bacterial artificial chromosome (BAC) encompassing the entire *brg1* gene sequence and was phenocopied with morpholinos confirming that the *brg1* is the gene disrupted in *yng* mutant embryos. A similar retinal phenotype display a general lack of differentiation was also observed in embryos mutant for *baf53* (*hi550*), a factor known to interact with Brg1, supporting a novel role for this chromatin remodeling complex at the juncture between specification and differentiation of cells in the vertebrate retina (Gregg *et al.*, 2003; Amsterdam *et al.*, 2004). Adaptation of protocols for the microdissection of the neural retina and retinal pigmented epithelium from zebrafish embryos and RNA amplification methods have enabled microarray analysis of changes in gene expression during eye development (Leung, *et al.*, 2005; Leung, *et al.*, 2007). The comparison of wildtype and *yng* mutant tissues revealed several pathways and gene groups were deregulated when *brg1* was mutated including those involved in neurite outgrowth, cell-cycle regulation, cell signaling and transcriptional cascades (Leung, *et al.*, in preparation). The importance of chromatin remodeling for retinal neurogenesis was further supported by the isolation of a mutant allele for the gene encoding histone deacetylase 1 (*Hdac1*), which also showed a lack of retinal neurogenesis, but in sharp contrast to *yng*, the neural epithelial cells in the *Hdac1* mutant remained mitotically active and expression of the proneural transcription factor *ath5* was markedly reduced (Stadler, *et al.*, 2005; Yamaguchi, *et al.*, 2005). These data suggest a model whereby *hdac* and the *brg1* chromatin remodeling complexes act sequentially. First, *Hdac1* is required at the juncture leading from a mitotic progenitor cell to the specification of a neuronal fate, followed by *brg1*-mediated transition from cellular specification to neuronal differentiation. Interestingly, mutation of *snf2h*, an ATPase gene of a different chromatin-remodeling complex, did not affect retinal cell differentiation. Taken together, these provide compelling evidence for tissue-specific and stage-specific roles for different chromatin-remodeling complexes. In support of this conclusion, expression in the mouse of several different chromatin remodeling complex factors also demonstrated neural-specific expression patterns during development (Olave *et al.*, 2002; Seo *et al.*, 2005). However, the targeted disruption of *Brg1* in the mouse led to lethality in the preimplantation embryo, precluding analysis of a potential role in neural development (Bultman *et al.*, 2000). This latter result raises an important issue; if *brg1* is essential for early stages of murine development, then how did

the zebrafish embryos survive? In zebrafish, transcripts for many genes involved in early development such as *brg1* are expressed maternally (Gregg *et al.*, 2003; Dosch *et al.*, 2004; Wagner *et al.*, 2004). These maternal stores likely permit embryos to progress through the early stages of development thereby uncovering the novel function of the gene in eye development. For more detailed descriptions of the nature of other genes and their roles in the early stages of retina development, the reader is referred to several recent publications on the topic (Malicki, 2000; Malicki *et al.*, 2002). We shall focus our discussion on the many advantages offered by the zebrafish as a behavioral model to uncover novel gene functions or reveal fundamental processes in retinal physiology, health and disease.

5.2. Optokinetic Reflex

The development of behavioral assays to detect visual system deficits in zebrafish may hold the greatest potential to contribute to our understanding of retinal function and visual system processing. Zebrafish larvae and adults are highly visual animals. The first visually evoked startle responses are observed 3 dpf. By 4 dpf, many larvae demonstrate an optokinetic reflex (OKR) in response to moving objects, and by 5 dpf, >95% of zebrafish larvae display smooth pursuit and saccade eye movements in response to illuminated rotating stripes (Brockerhoff *et al.*, 1995; Easter and Nicola, 1996, 1997). The basic function of the OKR is to keep an object stably positioned on the retina while moving through the environment. The robustness of the OKR, the ability to screen young larvae and the potential to vary the assay to detect multiple types of visual system defects led Brockerhoff and colleagues (Brockerhoff *et al.*, 1995) to use the OKR as a robust method to identify recessive mutations affecting the visual system in otherwise normal appearing larvae (Brockerhoff *et al.*, 1995, 2003; Taylor *et al.*, 2004). The assay is rapid, the responses from several larvae can be obtained simultaneously and an entire clutch can be assayed in minutes. To conduct the assay, larvae are immobilized in a petri dish containing methylcellulose and placed on a stationary pedestal in the middle of a rotating drum covered with stripes. Rotating the drum elicits eye movements in the direction of the rotation of the stripes. Although the rate of isolating mutations affecting the OKR in otherwise normal appearing larvae is several fold less than the frequency of morphological mutants, the benefits are apparent. By varying the stimulus from bright to dim white light or using a long-wavelength illumination, subtle defects affecting specific aspects of photoreceptor function, single photoreceptor cell types, synaptic activity or biochemical pathways have been isolated (Brockerhoff *et al.*, 1995, 1997, 2003; Allwardt *et al.*, 2001; Kainz *et al.*, 2003). One potential drawback of any behavioral screen is isolating the origin of the defect to the region of the CNS of interest, in our case, the retina. Therefore, recording of the electroretinogram (ERG) is routinely applied as a secondary screen to distinguish between a retinal defect versus alterations in midbrain nuclei or other structures necessary for the OKR such as the extraocular muscles or the neuromuscular junction. The ERG, however, provides information mainly about the outer retina, but single unit recordings can be made from zebrafish ganglion cells, providing information about inner retinal function (Emran *et al.*, in preparation). Once an interesting defect is confirmed as retinal in origin, positional cloning and a candidate gene approach are used to identify the mutated gene. The assay was also used to screen a collection of 450 mutants previously identified by morphological criteria, of which a total of 25 displayed visual system impairment (Neuhauss *et al.*, 1999). And others continue to refine the paradigm to evaluate motion detection, color discrimination and higher order processes.

The potential of the assay to identify larvae with subtle defects is well illustrated by the identification of a red-blind mutant, *partial optokinetic response b (pob)* (Brockerhoff *et al.*, 1995, 1997). In most respects, *pob* mutant larvae are morphologically indistinguishable from their wild-type siblings, and they demonstrate robust eye movements in response to moving black and white stripes illuminated with white light. However, they do not move their eyes when the stripes are illuminated with red light. This difference in response to red versus white

light strongly suggested a retinal rather than a central deficit. In the secondary screen, the ERG confirmed the retinal nature of the defect; *pob* mutant larvae showed markedly attenuated responses to red light compared to wild-type larvae (Figure 3). *In situ* hybridization for the cone opsins and cell counts demonstrated a selective loss of the red cones in the retina of the *pob* mutant larvae although the probe for the red-cone opsin labeled small cone profiles near the margin suggesting the red cones initially begin to differentiate and then die (Brockerhoff *et al.*, 1997). Surprisingly, cloning of the locus demonstrated that *pob* encodes a widely distributed 30kDa protein of unknown function (Taylor *et al.*, 2005), but based upon highly conserved sequence homology, the authors proposed that the protein product plays a role in protein sorting and/or trafficking essential to red cone function.

Two other mutations, *nrc* and *nof* illustrate the value of the forward genetic screen as a means to develop greater understanding of cellular physiology through the analysis of retinal function in the absence of specific genes products (Brockerhoff *et al.*, 1995, 2003). Neither *nrc* nor *nof* mutant larvae demonstrate an OKR, and the ERGs are consistent with photoreceptor-specific defects. Curiously, the ERG of *nrc* mutant larvae demonstrated an odd oscillatory wave, similar to that observed in individuals affected by Duchenne's muscular dystrophy. Ultrastructural analysis of the cone terminals of *nrc* mutant larvae showed a lack of proper invaginating synapse development, with free floating ribbons and fewer synaptic vesicles than observed in wild-type larvae, consistent with the altered ERG (Figure 4; (Allwardt *et al.*, 2001)). However, some presumed synapses (basal contacts) on OFF-bipolar cells dendrites were observed along the base of the photoreceptor terminals and no alterations of the non-invaginated ribbon synapses in the inner plexiform layer were observed. A subsequent study showed that ganglion cell activity can be recorded from *nrc* eyes, but most cells respond only at light offset (OFF cells). In wild-type larval zebrafish eyes, 90% of the ganglion cells respond to both the onset and offset of light (ON-OFF cells) or only to light onset (ON-cells); only 10% are OFF-cells (Emran *et al.*, in preparation). Positional cloning of the *nrc* locus revealed a premature stop codon in synaptojanin1, a phosphatidyl phosphatase previously implicated in clathrin-mediated endocytosis and actin cytoskeleton rearrangements (Van Epps *et al.*, 2004). Although synaptojanin1 had previously been cloned in mammalian models, identification of the *nrc* mutation revealed a novel role for phosphatide metabolism in cone photoreceptor synapse formation and function.

In *nof* mutant larvae, ERG recordings also suggested a photoreceptor origin to the visual deficit (Brockerhoff *et al.*, 2003). Positional cloning identified a premature stop codon in the alpha subunit of cone transducin in *nof* mutant larvae, and the behavioral effect could be phenocopied by morpholinos. The study demonstrated first of all that transducin is not essential for normal cone development. In the absence of any obvious ultrastructural changes in the photoreceptor cells, whole-cell electrical recording was used to investigate the cellular physiology of cones in the absence of transducin-mediated phototransduction. The dark currents for *nof* and wild-type cones differed by less than 30%, and as anticipated, no light-induced changes in current were detected with moderate intensity stimulation. However, photoresponses could be elicited in cones isolated from *nof* mutant when stimulated with a step increase in bright light that bleached a few per cent of the visual pigment per second. The response demonstrated a slow onset, on the order of ~1 second compared to 0.1 to 0.2 seconds for wild-type cones, and the low response amplitude suggested a mechanism different from the canonical transducin-mediated phototransduction. The response of *nof* cones was attenuated by preloading the cones with the membrane permeant form of the Ca^{2+} chelator BAPTA (1,2-bis (*o*-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid), suggesting a role for Ca^{2+} in the observed currents. Imaging the responses with the fluorescent Ca^{2+} indicator Fluo-4 provided proof that the observed currents in *nof* cones were mediated by a transducin-independent increase in cytosolic Ca^{2+} following light stimulation. Calcium changes have been recorded in other photoreceptors exposed to light; however, the role of the observed changes in photoreceptor cell physiology

is not fully understood (Matthews and Fain, 2001, 2002). Further biochemical and physiological studies should help resolve the source of the Ca^{2+} pool and elucidate the role of the Ca^{2+} release in mediating the changes in whole-cell current in wild-type and mutant photoreceptors.

Behavioural analysis of mutant larvae has also revealed aspects of the circuitry underlying the elicited eye movements. The *belladonna* mutation (*bel*), so named for a pigmentation defect of the eye resulting in the appearance of a dilated pupil, also was found to display a misrouting of ganglion cell axons (Karlstrom *et al.*, 1996; Trowe *et al.*, 1996; Neuhauss *et al.*, 1999; Rick *et al.*, 2000). Whereas in wild-type larvae contralateral projections from ganglion cells to the tectum are the norm, in *bel* mutants ganglion cell axons project to the ipsilateral tectum (Figure 5). The phenotype in *bel* mutant larvae ranges from relatively mild, displaying few altered projections, to fully penetrant with only ipsilateral projections. Analysis of the OKR in *bel* mutant larvae revealed two interesting properties. First and foremost, in response to the moving stripes, the eyes of the mutant larvae moved in the direction opposite to the direction of the stimulus; for example, in response to stripes sweeping across the right eye in a nasal to temporal direction, the eye moved in a temporal to nasal direction. Second, for *bel* mutant larvae demonstrating reverse eye movements, eye velocity was independent of stimulus velocity. The movement of the stripes initiated eye movement but did not influence the rate of the pursuit, and, in contrast to wild-type larvae, the amplitude of the movement of the stimulated eye was less than the amplitude of movement of the opposite eye. Although the optic tectum does not mediate the OKR in zebrafish, the level of misrouting to the tectum correlated well with the altered behavior and may therefore reflect the degree of misrouting of ganglion cell projects to other nuclei, including the pretectal nuclei thought to be involved in mediating the OKR (Roeser and Baier, 2003). Cloning of the mutant loci identified a point mutation in the gene encoding the lim domain transcription factor Lhx2 (Seth, *et al.*, 2006); neither gene function nor expression data provide an immediate clue to the molecular mechanism leading to the behavioral mutant phenotype. Rather, based upon careful scrutiny of the behavioral deficit (Huang, *et al.*, 2006; Rick, *et al.*, 2000) it was proposed that: in the wild-type fish, visual stimulation of one eye drives movement of that eye through projections to a contralateral OKR-mediated nucleus and integrator nucleus that ultimately controls the ipsilateral motor nuclei and the ocular muscles of the stimulated eye (Figure 5; (Rick *et al.*, 2000)). In this model, the neural basis of the altered behavior in *bel* mutants can be attributed to the singular defect in the projection of ganglion cell axons to the ipsilateral OKR-mediated nucleus. The ipsilateral projections innervate the ipsilateral OKR-mediated nucleus and integrator nucleus, but the output neurons from the integrator nucleus still cross the midline and subsequently drive the motor nucleus of the unstimulated eye.

6. Models of human disease

Initially, much interest in the zebrafish centered on the advantages of the model for vertebrate development, however, it has become an increasingly important model of studying mechanisms of various human diseases including those affecting the visual system (for relevant list of genes see Table 1S in Amsterdam and Hopkins, 2006). Heritable diseases are among the leading causes of blindness in developed countries. *Retinitis pigmentosa* (RP) and allied dystrophies represent a heterogeneous collection of diseases that affect the function and survival of the photoreceptor cells of the retina, in many cases leaving the second-order neurons intact (Berson *et al.*, 2002; Rivolta *et al.*, 2002). Patients suffering from RP lose their peripheral vision in adolescence or as young adults and become completely blind between 30 and 60 years of age. Approximately 40% of the cases of RP demonstrate an autosomal dominant form of inheritance. By comparison, Leber congenital amaurosis has a much lower incidence with an autosomal recessive mode of inheritance and typically presents at birth. Since the seminal identification in 1989 of the first locus associated with an inherited photoreceptor cell

degeneration, and during the following year (McWilliam *et al.*, 1989; Dryja *et al.*, 1990a, b), the subsequent determination of mutations in the *rhodopsin* (*RHO*) gene responsible for autosomal dominant RP, over 150 loci and 100 genes have been associated with photoreceptor cell dystrophies (A comprehensive and updated list can be found at Retnet (<http://www.sph.uth.tmc.edu/Retnet/disease.htm>)). It is not surprising that many of the initial discoveries were genes associated with the unique processes of photoreceptor cells, such as phototransduction, photoreceptor cell structure or cellular interactions unique to the photoreceptor cells and the retinal pigment epithelium (RPE). For example, mutations in *RHO* are the single most prevalent alterations leading to RP (Berson *et al.*, 2002; Rivolta *et al.*, 2002). But these findings led to new questions, such as how mutations in genes exclusively expressed by rod photoreceptors result in the progressive yet irreversible loss of cones (Papermaster, 1995). Unforeseen were the significant numbers of human mutations in genes with very diverse functions and widespread patterns of expression that led to loss of vision. These genes include mitochondrial-specific factors, RNA splicing components or metabolic proteins. It has been postulated that the effects may be associated with the unique metabolic and structural features of the photoreceptor cells, which render them hypersensitive to mutations in these additional genes; however, this requires additional support. Similarly, the genetic basis of numerous heritable diseases affecting the anterior segment and inner retina, such as dominant cataracts, aniridia, glaucoma, etc. have been identified or linked to some forms of the diseases. The number of loci or genes associated with anterior segment dysgenesis is approaching fifty with many yet to be identified.

6.1 Photoreceptor dystrophies

Several recessive mutations affecting the visual system underscore the power of the zebrafish as a genetic model of human congenital defects. The *noa* locus was identified based on the absence of the OKR (Brockerhoff *et al.*, 1995). Recent cloning of the *noa* gene product demonstrated a deficiency for dihydrolipoamide S-acetyltransferase, and the neurological phenotype of *noa* mutant fish displays several characteristics similar to pyruvate dehydrogenase deficiency (Taylor *et al.*, 2004). Furthermore, rescue of the severe effects of the mutation was accomplished by dietary supplementation, providing a novel model for understanding this human disease. Similarly, two genes essential for the function of photoreceptor cells and associated with retinal dystrophy have recently been identified. The mutation in the zebrafish orthologue of the human *choroideremia* gene, which encodes the Rab escort protein-1 and is responsible for a human disease marked by slow-onset degeneration of rod photoreceptors and retinal pigment epithelial cells, was recently identified (Starr *et al.*, 2004). Subsequent analysis suggested that the gene function is necessary in the RPE, and the photoreceptor deficit in secondary to this alteration (Krock, *et al.*, 2007). Mutations in intraflagellar transport protein loci, known to be associated with proper function of the connecting cilium in photoreceptor cells have also been recovered. Mutation in the *oval* gene, which encodes an intraflagellar transport protein, leads to photoreceptor cell dysgenesis as well as defects in other sensory receptor cells (Tsujikawa and Malicki, 2004). This provides just one example of a number of genetic models to study the basic cell biology of intraflagellar transport in vertebrate cells as well as providing additional models for diseases of the visual system.

6.2 Age-related Degenerations

It is anticipated that in the forthcoming years, many more candidates for disease-causing genes will be identified in zebrafish genetic screens. Unfortunately, the majority of the published mutations affecting the zebrafish are larval lethal or require extraordinary measures to maintain the mutant fish beyond larval stages (Brockerhoff *et al.*, 1995; Malicki *et al.*, 1996; Fadool *et al.*, 1997). Therefore, a systematic screen of adult and late-larval-stage zebrafish for both dominant and recessive mutations is necessary to generate more representative models of

retinal dystrophies. The visually mediated escape response was developed as an assay to quantify visual sensitivity as a potential tool to detect retinal dystrophies in adult zebrafish (Li and Dowling, 1997). To assay the escape response, free-swimming adult zebrafish are placed in a small circular dish. On a rotating drum located outside of the dish is a single black segment, simulating a threatening object. Upon encountering the rotating black segment, the zebrafish takes refuge behind a single post located in the middle of the dish. The direction of the rotation can be altered, and the intensity of illumination can be easily controlled with neutral density filters. Using this simple paradigm, visual threshold, circadian control and dark adaptation were evaluated in a screen of 245 adult F1 generation zebrafish of mutagenized adults (Figure 6). Seven dominant mutations (*night blind a*, *nba*; *night blind b*, *nbb*, etc . . .) affecting visual sensitivity (Li and Dowling, 1997) were identified. In subsequent generations, the onsets of the dominant phenotypes were found to vary from several months to greater than two years suggesting a situation similar to late-onset retinal dystrophies in humans. The severity of phenotypes also varied. Fish heterozygous for the *nba* mutation demonstrate slow progressive photoreceptor cell degeneration with loss of both rods and cones in patches across the retina, with a corresponding alteration in the ERG. By comparison, *nbc* heterozygous fish demonstrated changes ranging from the slow progressive loss of rod and cone photoreceptor outer segments, to others demonstrating only alterations in rods, and still others displaying no obvious morphological phenotype (Maaswinkel *et al.*, 2003). However, the ERGs of *nbc* mutant and wild-type fish were similar, making the origin of the behavioral deficit unknown. Unexpectedly, larvae homozygous for either mutation displayed severe and widespread neural degeneration, suggesting the affected genes are not photoreceptor cell specific (Li and Dowling, 1997; Maaswinkel *et al.*, 2003). Interestingly, in a subsequent off-the-shelf screen, several heterozygous adults for previously identified recessive mutations displayed an adult phenotype – that is, the fish were night-blind (Darland and Li, personal communication).

6.3 Rod Degeneration

Unexpectedly, the genetic ablation of rods in the zebrafish retina does not result in the cone deficit frequently observed in human RP and murine models of rod loss (Morris *et al.*, 2005). A transgenic line of zebrafish expressing the membrane variant of cyan fluorescent protein (mCFP) under the regulatory control of the *Xenopus* opsin promoter showed the unexpected yet complete loss of mature rods in the larvae and the adult retina shortly after expression of the reporter gene. However, histological examination revealed that the patterning and arrangement of the cone photoreceptors was indistinguishable from wildtype siblings. Consistent with the histological data, ERG thresholds and spectral sensitivity curves of adult fish demonstrated no significant alteration of cone responses in the absence of the rods. Morris *et al.* conjectured that the rod death resulted from the high level of expression of the palmytalated form of CFP leading to altered cellular metabolism, but the maintenance of cone function is quite unexpected. Surprisingly, a significant increase in the number of mitotic rod progenitor cells was observed in the outer nuclear layer of the transgenic fish. It has been shown in humans afflicted by RP, that as few as 25% of the original number of rods is sufficient to protect the cones from complete loss (Cideciyan, *et al.*, 1998). Our working model is that the increased numbers of the rod progenitors in the transgenic line of zebrafish is one potential mechanism underlying the maintenance of cone function.

6.4. Stem Cells and Photoreceptor Regeneration

Interest in the rod progenitor cells of the teleost retina is not unique. Neurogenesis in the adult central nervous systems (CNS) of nonmammalian vertebrates has provided valuable models to investigate the basic molecular and cellular processes underlying neurogenesis and guiding differentiation. Many teleosts and amphibians continue to grow throughout their life, and the increase in body mass is matched by an increase in the size of the eye and the area of the retina (Fernald, 1990; Johns and Fernald, 1981; Marcus *et al.*, 1999; Ottensson and Hitchcock,

2003). For example, in goldfish, a fourfold increase in body length is associated with a six fold increase in retinal area. The increase in retinal area is due in part to the addition of new neurons, including cone photoreceptors, at the retinal margin. As the fish grows, new neurons including photoreceptor cells are added at the retinal margin by a population of mitotic progenitor cells located within the circumferential mitotic zone. The mitotic cells possess properties of stem cells, maintaining a balance between self-renewal and the generation of multipotent neuroblasts that differentiate into all classes of neurons and glia. This annular growth has been compared to the addition of new growth rings on a tree with older cells located more centrally and younger cells residing closer to the margin. However, unlike the latter, the newly generated neurons integrate seamlessly with the adjoining retina. The recent isolation of retinal stem cells from the ciliary margin of rodents and identification of persistent neurogenesis at the margin of the hatchling chick retina suggests a conserved system although further experimental evidence is needed (Ahmad *et al.*, 2000; Fischer and Reh, 2000; Haruta *et al.*, 2001; Tropepe *et al.*, 2000).

During post-embryonic growth, the retina is gradually stretched within the expanding optic cup with a comparative thinning of the retinal layers and increased spacing between the cone nuclei (Fernald, 1990). Visual acuity is maintained by the increase in the size of the retinal image proportional to the increase in the size of the eye. Visual sensitivity is maintained by generation of new rods in the central retina from a second population of mitotic cells referred to as rod progenitor cells. Elucidating the origin and developmental potential of these proliferative cells is an area of considerable investigation (Johns and Fernald, 1981; Johns, 1982; Otteson and Hitchcock, 2003; Ottenson, *et al.*, 2001). In the initial reports identifying the origin of the rods, injection of tritiated thymidine in the larval goldfish or cichlid resulted in the labeling of the CGZ as expected but also revealed proliferating cells distributed across the ONL (Johns and Fernald, 1981; Johns, 1982). Following survival times of several weeks or months, the label became restricted to nuclei of differentiated rods, suggesting that the mitotic cells were the direct precursors of the rods. Multiple injections in the larval goldfish labeled groups of radially arranged cells, referred to as neurogenic clusters that appeared to migrate along Muller glia from the INL to the ONL. Their lower frequency of labeling compared to the CGZ or precursors in the ONL suggested a more slowly dividing population of cells. The author proposed that the clusters of proliferating cells of the INL migrated to the outer retina and became the source of the rod precursors in the ONL.

Recent experimental evidence suggests that a subset of the Muller glia constitute an INL population of progenitor cells in fish and other vertebrates (Bernardos, *et al.*, 2007; Fausett and Goldman, 2006; Fimble, *et al.*, 2007; Fischer and Reh, 2001 & 2003; Ooto, *et al.*, 2004; Yurco and Cameron, 2005). In teleosts including zebrafish, mechanical damage, chemical toxicity, and phototoxicity stimulated an increase in proliferation of cells in the INL and ONL (Fausett and Goldman, 2006; Vihtelic and Hyde, 2000; Vihtelic, *et al.*, 2006; Wu *et al.*, 2001). Taking advantage of transgenic lines and cell specific labeling, recent analysis revealed that initially many of the proliferating cells had gene expression profiles consistent with Muller glia (Bernardos, *et al.*, 2007; Fausett and Goldman, 2006; Yurco and Cameron, 2005). The dividing cells appeared to migrate to the ONL before differentiating into rods and cones. Older literature suggested that damage to the photoreceptor cell layer was necessary to stimulate the regenerative response. However, toxic injury to neurons of the inner retina stimulated proliferation of the INL cells and replacement of the lesioned retina indicating a greater plasticity in the response than previously concluded (Fimble, *et al.*, 2007). Changes in the microenvironment appear to recapitulate developmental pathways that play important roles in redirecting the progenitors into different cell fates (Raymond, *et al.*, 2006). The genesis of rods and photoreceptor cell regeneration in the teleost provides a model to systematically investigate the potential and the limitations of neural stem cells for replacement of lost photoreceptor cells. The regulated proliferation of rod precursors, the predictable pattern of migration in the ONL,

and the differentiation into functional photoreceptor are precisely the demands required of stem cell therapies for the treatment of the many retinal degenerations. Yet, while great progress has been made in isolating and testing the potential of mammalian neural stem cells, rod progenitor cells have not been propagated in vitro, hampering our ability to manipulate the cells, examine their developmental potential, or attempt to specifically engineer their fate. The recent development of transgenic lines that demonstrate expression of fluorescent reporter genes in cells of this lineage and genetic models displaying loss of either rod or cone photoreceptors show much promise in hastening these efforts.

6.5 Anterior Chamber Defects

Several groups have included or developed assays with the specific goal of identifying and characterizing mutations affecting the anterior chamber (Link, *et al.*, 2004; McMahon, *et al.*, 2004; Vihtelic, *et al.*, 2001; Semina *et al.*, 2007; DeCarvalho, *et al.*, in preparation). Forming a backdrop for this work, the earlier cited publications provided important comparative analyses on the morphogenesis, microscopic anatomy and gene expression patterns of the developing lens, cornea and anterior chamber (Soules and Link, 2005; Zhau, *et al.*, 2006). However, many of the mutations initially identified revealed novel functions for known genes in lens development and maintenance but also displayed pleiotropy and embryonic lethality limiting their utility as models of human disease. To improve on this strategy, Link and colleagues have adapted novel tools for measuring subtle differences of intraocular pressure in larval and adult zebrafish to specifically develop models of glaucoma and related diseases (Link, *et al.*, 2004; McMahon, *et al.*, 2004). This provides the opportunity to correlate measurements of intraocular pressure to changes in ganglion cell number and optic nerve morphology. In a separate avenue, free swimming zebrafish larvae and third generation adults of mutagenized founders have been screened for subtle morphological defects in the anterior chamber or altered patterning of the photoreceptor cells (Morris and Fadool, 2005). The premise of the screen is that these late onset phenotypes would serve as much needed models of human anterior defects, retinal dystrophies and congenital defects. In this proof of concept, numerous mutations that display lens cataracts, ectopic lens or vacuolization of the lens epithelium have been isolated. In most instances, the homozygous mutants larvae develop normally and can be reared to breeding stages, suggesting the defects are limited mostly to the lens. A single example best demonstrates the value of these new resources. Mutation of the *ufo* locus was first identified by a ruptured lens capsule and ectopic lens first appearing 5–6 dpf. Prior to this, the larvae display a normal OKR. Curiously, the mutant adults, though blind, display a late onset vestibular deficit (DeCarvalho, *et al.*, in preparation). Upon startling, rather than swimming in a straight line, the mutant adults swim in concentric circles, a behavior frequently observed in fish with altered vestibular function (Nicholson, *et al.*, 1998). Genetic linkage analysis suggested alteration in a component of the epithelial basement. Combined, the lens phenotype, vestibular defect and linkage analysis provide strong evidence that this line represents a novel model for the sensory defects associated with Alport syndrome (Hudson, *et al.*, 2003). It must still be determined if the mutant zebrafish also display hemeurea or proteinuria, the most common and clinically significant symptoms of Alport syndrome in humans. Taken as a whole, the genetic screens of zebrafish and the continued development of novel screening strategies and analysis methods provide a rich resource for investigating fundamental processes of visual system development and physiology.

7. Chemical screens

The combination of external fertilization and clarity of the embryo that has propelled zebrafish as a genetic model of vertebrate development likewise enables chemical screens to identify agents that specifically alter retinal development and nervous system function. In one of the early chemical screens, Hyatt and colleagues looked for compounds that altered development

of the eyes and discovered a novel role for retinoic acid (RA) in visual system development (Hyatt *et al.*, 1992). Retinoic acid is a potent morphogen and its importance during neural development is well documented (Hyatt and Dowling, 1997; Maden and Holder, 1992; Ross *et al.*, 2000). At high concentrations it displays teratogenic effects, and its absence can lead to visual impairment among other congenital defects. Application of RA during early neurulation of zebrafish resulted in an apparent duplication of the neural retina (Hyatt *et al.*, 1992). The treated embryos had an expanded ventral retina, producing two concave surfaces that in some cases were associated with duplicated lenses. Endogenous RA is synthesized from retinaldehyde by a dehydrogenase. In the zebrafish and mouse, expression of a specific dehydrogenase in the ventral retina potentially leads to a gradient of RA across the neural retina suggesting a necessary function during patterning of the dorsal-ventral axis (Marsh-Armstrong *et al.*, 1994; Hyatt *et al.*, 1996b).

Subsequently it was demonstrated that inhibition of the dehydrogenase leads to a lack of ventral retinal structures in a stage-specific manner. The application of RA at later stages of development promoted rod differentiation while it inhibited cone maturation consistent with other models on the role of RA in photoreceptor cell development (Hyatt *et al.*, 1996a; Levine *et al.*, 2000).

7.1 Chemical Genetics

These studies highlighted the potential use of the zebrafish for large-scale chemical genetic screens for small molecules that perturb specific aspects of organogenesis, pattern formation and neural genesis (Peterson *et al.*, 2000, 2001b). However, unlike the example of the RA pathway screen, the chemical screens do not necessarily target a known biochemical pathway; rather, like forward genetic screens, they take an unbiased approach to identify compounds that when applied to developing vertebrate embryos yield a specific developmental phenotype. Similar to the design of cell culture assays, zebrafish embryos are arrayed into microtitre plates and exposed to chemical agents by adding the dissolved compounds into embryo medium. In this way, tens of thousands of small molecules previously arrayed into microtitre dishes can be systematically screened for effects upon discrete aspects of development. The *in vivo* model has several clear advantages over other culture assays. First, cellular and tissue interactions not present *in vitro* are maintained *in vivo* thus expanding the assay to detect alterations in tissue induction, cell migration and morphogenesis. Second, compounds may be applied to the embryos at any stage of development, thereby revealing the timing of gene action and limiting the pitfalls associated with the loss of function mutations displaying an earlier developmental phenotype that may otherwise obscure later functions of the gene. The ability to screen large numbers of compounds led to a novel application of the chemical genetic screen to identify compounds with the potential to suppress the lethal phenotype of a genetic mutation (Peterson *et al.*, 2004). Two of the 5000 compounds tested rescued the embryonic vascular defect associated with the mutation *gridlock* (*grl* affects *hey2*), and following the early treatment, the rescued mutants remained viable into adulthood. Given the number of identified and potential loci that when mutated result in vision deficit in humans, it has been proposed that many mutations ultimately lead to photoreceptor cell death through a small number of common pathways. Thus the potential exists for retinal-specific mutations in zebrafish to be models for identifying novel therapeutic agents to circumvent a pathway disrupted by a mutation or stimulate a compensatory pathway to alleviate a congenital defect even in the absence of identifying the mutated gene.

8. Future Directions

Even with the wealth of information gained by the analysis of the existing mutations in zebrafish, additional novel screens are necessary to reveal mutations not detected by current assays. Just as the OKR offered a clear advantage over morphological screens for detecting

some types of visual deficits in otherwise normal larvae, other well thought out assays can uncover additional phenotypes. For example, the OKR requires that a fish detect movement. Mutant *nrc* fish fail to show an OKR, but do respond behaviorally to the offset of a prolonged light stimulus (Emran *et al.*, in preparation). It is also likely that mutations specifically affecting rods were not detected in the previous larval screens. Based upon the ERG and visual-evoked behaviors, rod function usually cannot be detected prior to 21 dpf (Saszik *et al.*, 1999; Bilotta *et al.*, 2001) whereas most screens are conducted at 5 dpf. To detect changes in the rods, alternative approaches needed to be developed. One such method was the development of transgenic lines of zebrafish demonstrating rod-specific expression of a protein chimera between the enhanced green fluorescent protein and the C-terminal sequence of opsin as a reporter (Perkins *et al.*, 2002). This permits screening live zebrafish larvae for changes in the number and spacing of rod photoreceptors as well as the vectorial sorting of opsin to the outer segment. We and others have demonstrated the utility of *in situ* antibody labeling or histology-based screens to detect changes in specific cells of the neural retina (Morris and Fadool, 2005). Although somewhat more labor intensive than a transgenic screen, the latter offers the potential to label with multiple probes to thereby detect simultaneously changes in numerous cell types. With the growing number of transgenic lines demonstrating retinal-specific expression of fluorescent reporter genes, the development of more sophisticated behavioral assays and the rapidly advancing cloning techniques, the analysis of new or existing mutations should continue to uncover a wealth of information on the development and physiology of the retina.

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Figure 1.

Histology of the zebrafish retina. (A–C) Fluorescent double immunolabelling of specific cell types in transverse section of the eye of a zebrafish larvae and DAPI (4', 6-diamidino-2-phenylindole) counterstaining reveal the archetypical laminar arrangement of the retina. (A) Labeling with the Zn-8 monoclonal antibody specific for ganglion cells (gc) and the red-cone opsin. (B) Labeling for the rod photoreceptors with the 1D1 antibody and Müller glia with anti-CAZ. (C) Labeling of amacrine cells (amc) with the 5E11 antibody and co-labeling for UV opsin. (D) Mosaic organization of the larval retina revealed by labeling red and green cones with the zrp1 antibody and DAPI counterstaining of nuclei. The red and green cones can be distinguished by the greater fluorescent labeling of the red cones, and the positions of the blue and ultraviolet cones can be discerned by labeling with DAPI. The identity of the cone subtypes are diagrammatically represented and color-coded. (E) Transverse section through the adult retina demonstrating EGFP fluorescence in rod photoreceptors (left panel in green) and counterstaining with DAPI (blue) to reveal the tiering of the cone nuclei (relative positions of the cone subtypes are diagrammatically represented). The rod cell bodies appear white due to double labeling for EGFP and DAPI. The rod outer segments are also immunolabeled for opsin. The plane of section in (F) is indicated by the arrow. (F) EGFP fluorescence of the rods, immunolabeling of the UV sensitive opsin, DAPI labeling of the cone nuclei in a tangential section of the adult retina and the merged image. Note the regular arrangement of the rod myoids around the outer segment of the UV-cone outer segments. (E & F from Fadool, 2003; reprinted with the permission of the publisher).

Figure 2.

Micrographs showing development of the zebrafish eye, cornea and lens. Histological sections at 24 hpf (A), 36 hpf (B), and 48 hpf (C). Neurogenesis and lamination of the retina progresses rapidly between 36 and 38 hpf. The lens vesicle detaches from the overlying ectoderm by 24 hpf and differentiation of the fiber cells also progresses rapidly between 36 and 48 hpf. The periocular mesenchyme is present at 24 hpf, but more prominent at 36 and 48 hpf (arrows). Hyaloid vasculature is indicated with asterisk. (D) Diagram of the embryonic zebrafish eye. Insets in (D) indicate corresponding histological sections through the 3 dpf lens transition zone (E), lens epithelium (F) and posterior lens (G). TEM micrographs (H,I) show developing lens epithelium (H), interdigitating lens fiber cells and establishment of rudimentary lens sutures (arrow, I). (Adapted from Soules and Link, 2005; reprinted with permission of the original author as defined by the publisher).

Figure 3.

Electretinograms (ERGs) from 6-day-old OKR+ (a) and *pob* mutant larvae (b). The responses were elicited with a short 0.01 s flashes of green (520 nm) light at the same intensity. In (a), only the b-wave is evident but both the a- and b-waves are present in (b). The vertical bars represent 50 μ V (a) or 100 μ V (b). Time markers = 0.1 ms. (c), Comparison of the spectral sensitivities of *pob* and normal sibling larvae. Spectral sensitivities were determined by ERG analysis. The inverse of the number of photons required to generate a threshold (20 μ V) b-wave response was calculated at each wavelength and normalized to the sensitivity of the normal larvae at 430 nm. Note the *pob* mutant larvae are about 2 log units less sensitive to red light. (From Brockerhoff *et al.*, 1997; reprinted with permission of the publisher).

Figure 4.

Electron micrographs of cone terminals in wild-type (A and B) and *nrc* mutant larva (C). (A) In the wild-type retina, bipolar and horizontal cell processes invaginate the pedicle in a tight bundle (*arrow*). Horizontal cell processes (*H*) are easily recognized by their large size, electron lucent cytoplasm and characteristic densities (*small arrowheads*). Synaptic ribbons (*R*) are associated with the presynaptic membrane via an arciform density (*curved arrow*). (B) Basal contacts (*B*) are found in wild-type cones between the ribbon synapses. *Inset*, Under high power, the basal contacts show dense cytoplasmic material on both sides of the junction. Synaptic vesicles (*V*) surround the synaptic ribbons (*R*). (C) In the *nrc* retina, synaptic ribbons (*R*) in most of the pedicles appear to be floating in the cytoplasm, unassociated with an arciform density and the presynaptic membrane. Few postsynaptic processes invaginate the pedicles. Many of these processes have small densities (*arrowheads*) suggesting they are horizontal cell processes. Basal contacts are made onto bipolar cells at the base of the pedicle (*B*). Synaptic vesicles (*V*) often clump and fail to distribute evenly in the pedicle. However, they surround synaptic ribbons as they do in wild-type pedicles (*small arrows*). Scale bar, 0.5 μm . (From Allwardt *et al.*, 2001; reprinted with permission of the publisher)

Figure 5.

Projection defect of retinal ganglion cells in *bel* mutant larvae revealed by injection of DiI (left eye, red) and DiO (right eye, green) into either eye. (A) Wild-type larvae have a complete contralateral projection with the optic nerves crossing at the chiasm. (B) *bel* mutant larva demonstrating complete ipsilateral projection with no formation of the optic chiasm. (C and D) Model of reversal in *bel* mutant. (C) In wild-type larvae, the visual stimulus is perceived in the stimulated eye (red) and transferred across the midline into the OKR-mediating nucleus (XN). This nucleus connects to an integrator nucleus (IN), which in turn controls the motor nucleus (MN) after crossing the midline. The IN also controls the movement of the unstimulated eye, albeit less robustly. (D) In *bel* mutant larvae the only defect is that the initial connections do not cross the midline but instead innervate the ipsilateral OKR-mediating nucleus. The result is the stimulated eye (red) drives the movement of the unstimulated eye. (Modified from Rick *et al.*, 2000; reprinted with permission of the publisher)

Figure 6.

(a) Dark adaptation curves for wild-type (circles) and two *nba* fish (triangles) determined by behavioral testing. The biphasic curve for the wild-type larva reflects cone dark adaptation (dashed line) and the second phase reflects rod adaptation (solid line). (b) Full-field ERGs of wild-type (left) and *nba* (right) fish to white light stimuli. a, a-wave; b, b-wave. Calibration bars (right lower) signify 0.2 s horizontally and 50 μ V vertically. (c) Histological sections showing the photoreceptor layer of 13-month-old wild-type (wt) and *nba* retina. Note the thinning of the rod outer segments (r) in the *nba* retina and the accumulation of lipid droplets in the PE (arrow). (c, cones; in, inner nuclear layer). (From Li and Dowling, 1997; reprinted

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Table 1
Comparison of retinal development in the zebrafish between 32 and 74 hpf with that of Retinotectal projections and behaviour^a

Hours post-fertilization	Outer retina		Inner retina		Ganglion cell layer	Other features	Retinotectal projections	Behaviour/retinal responses
	Photoreceptor	OPL	INL	IPL				
32					Differentiation in ventronasal patch	Pigmentation within pigment epithelium	Optic axons exit the retina	Weak touch response
36						Optic stalk attenuation commences	Optic axons reach optic chiasm	
40					Ganglion cell birth-days complete/differentiation in ventrotemporal region	Optic stalk attenuation complete	Optic axons reach tectum	Vigorous touch responses
50	Inner segment formation/opsin expression in ventronasal patch	Horizontal cell processes with zonulae adherentes	Differentiation of amacrine and horizontal cells	Amacrine cell processes/presumptive synaptic zones	Displaced amacrine cells in intraplexiform location	GABA and Trk immunoreactivity in optic nerve		
55	Outer segments in ventronasal patch							
60	Outer segments dorsal/nasal to optic nerve	Rod and cone synaptic terminals	Differentiation of bipolar cells	Bipolar cell processes/conventional synapses	Displaced amacrine cells in ganglion cell layer	GABA and Trk immunoreactivity in OPL, INL, IPL		Hatching begins
65		Synaptic ribbons (dyads and triads) in ventronasal patch					Arborization within nine fields in tectum	
70	Rhodopsin expression in dorsal retina	Synaptic ribbons in other regions		Bipolar cell ribbon synapses in ventronasal patch		Extraocular eye muscle maturation		First visual-evoked responses
72								
74	Specialized area in temporal retina first evident			Bipolar cell ribbon synapses in other regions/four sublaminae		Emmetropization of eye and lens	Arborization within tenth field in tectum	First electroretinographic responses Optokinetic responses

GABA, α -aminobutyric acid; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer.

^aSchmitt and Dowling (1999).