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Genetics of photoreceptor development and function in zebrafish

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ABSTRACT The vertebrate photoreceptor is a cell of unique morphology and function. It is an exquisite light detector, both sensitive and adaptable. Several unusual morphological features facilitate photoreceptor function. Signal detection is accomplished by a specialized apical structure, the outer segment. There, the capture of light produces fluctuations in cell membrane potential, which are then transmitted to the downstream circuitry of the retina via a rare type of synaptic junction, the ribbon synapse. The development, maintenance and function of the vertebrate photoreceptor cell have been studied mainly in four model organisms, ranging from an amphibian to man. A teleost fish, the zebrafish, is an important recent addition to this group. Genetic screens in zebrafish have identified an impressive collection of photoreceptor cell mutants, including the absence or malformation of specific morphological features as well as functional abnormalities. These mutant strains are currently studied using both molecular and embryological tools and provide important insights into photoreceptor biology.

KEY WORDS: *zebrafish, photoreceptor, outer segment, cell polarity, vision*

Introduction

The vertebrate photoreceptor cell is an extraordinary biological sensor that functions both in intense sunlight and in the dim illumination of the night. In near-darkness, rod photoreceptor cells detect single photons. Cone photoreceptors, on the other hand, remain responsive to changes of light intensity even at an illumination that is eleven orders of magnitude higher (reviewed in Pugh and Lamb, 2000). The detection of light is accomplished by photoreceptor outer segments, elongated stacks of hundreds of membrane folds that in some species store more than 10^9 visual pigment molecules. The activation of visual pigments, opsins, by light leads to a complex chain of events that involve numerous signal transducers, enzymes, and ion channels of the outer segment. Ultimately, photopigment activation leads to changes in photoreceptor membrane potential that are passed through an unusual type of synapse, the so-called ribbon synapse, to the interneurons of the inner retina. The photoreceptor is a cell of unusual morphology and remarkable function. How does it form in the course of embryogenesis?

Several important regulators of photoreceptor cell fate have been recently identified. Two homeobox transcription factors, *Otx2* and *Crx*, control the earliest stages of photoreceptor development. In the absence of *Otx2* function, photoreceptor cells are missing while amacrine cell numbers increase (Nishida *et al.,* 2003). *Crx*, a downstream target of *Otx2*, acts somewhat later and is necessary for early steps of photoreceptor differentiation (Furukawa *et al.,* 1999). Photoreceptor cells form in *Crx*-deficient animals, but they express dramatically reduced amounts of visual pigments as well as other components of the phototransduction cascade, and subsequently degenerate (Furukawa *et al.,* 1999). While *Otx2* and *Crx* play a general role in photoreceptor cell fate acquisition and differentiation, three other transcription factors, *TRβ2*, *Nrl*, and *Nr2e3*, regulate the specification photoreceptor types. The loss of *TRβ2*, a thyroid hormone receptor, results in an increase of short wavelength cones at the expense of middle wavelength cells in mice (Ng *et al.,* 2001). Murine loss of function mutations in *Nrl*, and *Nr2e3*, on the other hand, result in the overproduction of short wavelength cones at the expense of rods (Haider *et al.,* 2001, Mears *et al.,* 2001, Milam *et al.,* 2002).

In addition to these advances in the understanding of early photoreceptor development, several genes have been shown to function in the differentiation of the unique photoreceptor morphology. Rod outer segments do not differentiate in the absence of rod opsin expression (Lem *et al.,* 1999), and two tetraspanins, peripherin and ROM1, are thought to function in the assembly of

Abbreviations used in this paper: CC, connecting cilium; hpf, hours post fertilization; IS, inner segment; OLM, outer limiting membrane; OS, outer segment; ST, synaptic terminus; TEM, transmission electron microscopy.

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the outer segment membrane folds (Clarke *et al.,* 2000, Connell *et al.,* 1991, Travis *et al.,* 1991). Despite these accomplishments, much remains to be learned both about early steps of photoreceptor cell fate acquisition and even more about photoreceptor differentiation. The zebrafish model offers an excellent opportunity to advance the understanding of these areas.

Zebrafish photoreceptor cells

The zebrafish is well suited to study vertebrate embryogenesis (reviewed in Driever *et al.,* 1994, Malicki, 2000, Thisse and Zon, 2002). Its embryos and larvae are largely transparent and develop externally, making it easy to monitor many developmental processes. Another key asset of the zebrafish model is its rapid embryogenesis. The development of most organs has begun by 24 hours postfertilization (hpf) and many organ systems are fully functional within the first 5 days of development. The retina is not an exception: during approximately two days, from 28 to 80 hpf, the optic cup transforms from a single neuroepithelial sheet into a functional multilayered structure which in behavioral tests is capable of responding to light (Easter and Nicola, 1996). Finally, large numbers of zebrafish embryos can be easily produced allowing the application of high-throughput approaches to the study of embryogenesis.

It is a fortuitous circumstance that gross anatomical features of the vertebrate retina have remained largely unchanged in the course of evolution. Similar to the mammalian eye, the zebrafish retina consists of seven major cell classes, and its photoreceptor cells display the same gross morphological characteristics as mouse or human cells. The differentiation of zebrafish photoreceptors, starting from cell cycle exit, and culminating in the formation of outer segments and synaptic ribbons, occurs in a short period of less than 20 hours. Birth dating studies indicate that zebrafish photoreceptors first exit the cell cycle between 43 and 48 hpf, and develop their characteristic elongated morphology by 48 hpf (Hu and Easter, 1999, Larison and Bremiller, 1990, Nawrocki, 1985). The expression of rod opsin becomes detectable around 50 hpf, and blue and red opsins appear shortly thereafter at 52 hpf (Raymond *et al.,* 1995). Zebrafish photoreceptors express at least six opsin genes: blue, red, rod, ultraviolet, and two types of green (Vihtelic *et al.,* 1999). With the exception of the two green opsin polypeptides, which are thought to be co-expressed in the same cells, each of the opsin gene products appears to be present in a distinct photoreceptor type: blue opsin in long single cones, UV opsin in short single cones, red and green opsins in the two members of the double cone pair, and rhodopsin in rods (Vihtelic *et al.,* 1999). The rudiments of photoreceptor outer segments appear shortly after the onset of opsin expression at 54 hpf, and the synaptic ribbons are discernible in the ventral patch by 62 hpf (Schmitt and Dowling, 1999). In addition to opsin expression, different types of zebrafish photoreceptor cells can be identified based on morphological features such as the shape or the location of their outer segments (Branchek and Bremiller, 1984). The UV cones are the first to develop unique morphology and become distinguishable on histological sections between 3 and 4 days postfertilization (dpf). By 12 dpf, all zebrafish photoreceptor types are morphologically distinct (Branchek and Bremiller, 1984).

Several unique features characterize photoreceptor morphology. The most obvious one is the presence of the outer segment (Fig. 1 A,B,C). Outer segments are elongated stacks of membrane folds that harbor the components of the phototransduction apparatus, including opsins. Similar to other vertebrate species, rod and cone outer segments display distinct morphologies. In fact, the names of these cell subpopulations reflect outer segment shapes: conical in cones, and rod-like in rods. The spatial arrangement of cell membrane is another distinguishing feature of rod and cone outer segments. While cone outer segments consist mainly of numerous cell membrane infoldings that run parallel to each other and open to the extracellular environment, the rod outer segment membrane forms an array of sealed pouches, flattened and stacked on top of each other (Rodieck, 1973). The outer segment membrane connects to the cell body via a narrow stalk, which tightly surrounds a primary cilium (the so-called connecting cilium, Fig. 1 A,D). The basal body of this cilium localizes to the apical-most region of the inner segment. At least four areas can be distinguished basal to the connecting cilium in the photoreceptor soma, based on the content of its cytoplasm: the mitochondria-rich ellipsoid, the contractile myoid, the perinuclear area, and the synaptic terminus (Dowling, 1987, Rodieck, 1973). The photoreceptor cell surface features another important subdivision. Similar to epithelial cells, a belt of cell junctions partitions the photoreceptor cell membrane into apical and baso-lateral domains (Fig. 1 A,B,C,E,F). For historical reasons, the junctional region is termed the outer limiting membrane (Rodieck, 1973). Its position varies relative to the cell nucleus depending on the developmental stage and photoreceptor type. In the adult zebrafish retina, rod nuclei are located basal to the outer limiting membrane, while most cone nuclei are localized apical to it (Branchek and Bremiller, 1984, Raymond *et al.,* 1993). The apical processes of Muller glia contribute to cell junctions of the outer limiting membrane and terminate in its region (Fig. 1F).

The proximal-most photoreceptor cell feature is the synaptic terminus (Fig. 1 A,G). In zebrafish cone photoreceptors, it features a single invagination, which accommodates a tight bundle of bipolar and horizontal cell dendrites (Allwardt *et al.,* 2001). These associate with each other forming so-called triads. Within a triad, two horizontal cell processes surround bipolar cell dendrites. The presynaptic membranes of photoreceptor synapses are associated with specialized structures termed synaptic ribbons. The function of these is thought to assure a graded and continuous neurotransmitter release (Juusola *et al.,* 1996). Interspersed between ribbon synapses, zebrafish pedicles also contain so-called basal contacts, another type of cell junction that displays at least some synaptic characteristics (Allwardt *et al.,* 2001). Consistent with behavioral studies of retinal function, the synaptic apparatus of zebrafish cone photoreceptor cells is well differentiated by 75 hpf.

Similar to other cell classes, zebrafish photoreceptors are generated in a complex spatial-temporal pattern. The first cells to differentiate are located in a small area of the eye cup nasal to the choroid fissure, the so-called ventral patch (Raymond *et al.,* 1995). This is followed by a gradual appearance of photoreceptor cells in progressively more dorsal regions. The retina of an early zebrafish larva is dominated by cones, which start to form a regular array as early as 60 hpf (Larison and Bremiller, 1990). Rods are initially present at a low density and do not appear to be organized in any specific way. Between 10 and 21 dpf, as their density increases, rows of rods become noticeable in the retina. In the adult, the rows of cones and the rows of rods alternate (Fadool, 2003, Larison and

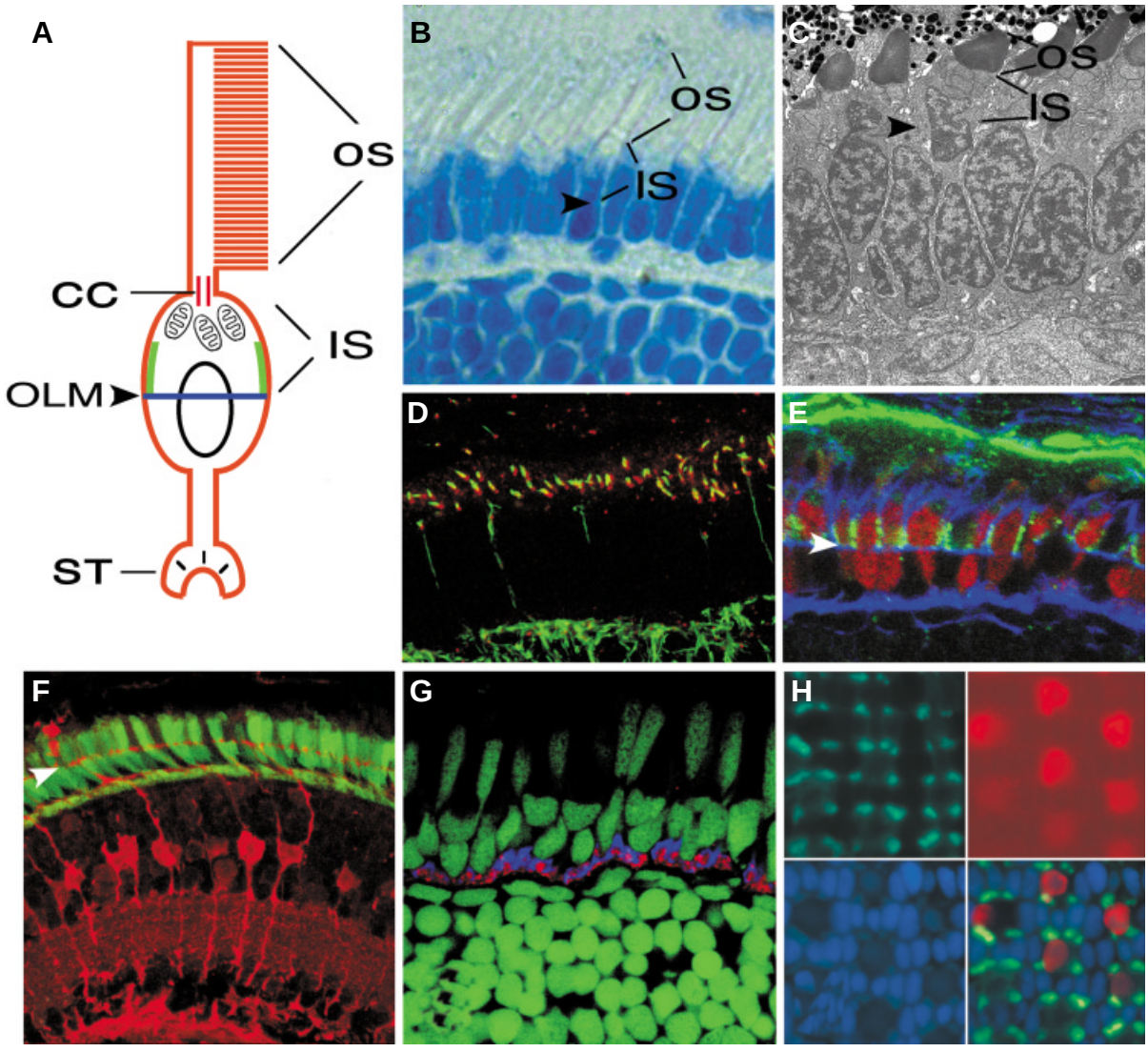

Fig. 1. The photoreceptor cell. (A) *A schematic representation of the vertebrate photoreceptor cell. The photosensitive part of the photoreceptor cell, the outer segment, is a tall stack of membrane folds that connects to the rest of the photoreceptor body via a primary cilium, known as the connecting cilium. The basal end of the connecting cilium is anchored in the inner segment, the apical-most part of the photoreceptor perikaryal region, characterized by the presence of numerous mitochondria. The basal-most region of the photoreceptor cell features the synaptic terminus, which contains specialized synaptic junctions, called ribbon synapses. Similar to epithelial cells from which photoreceptors originate, the photoreceptor cell body is subdivided by a belt of cell junctions (OLM) into apical and baso-lateral domains. Distinct molecular properties of these membrane domains are evident in the distribution of several polypeptides, such as the* nok *or* has *gene products (green lines).* **(B)** *At 5 dpf, photoreceptor cells of the zebrafish larva feature long outer segments and are functional by electrophysiological criteria. In this panel, photoreceptor cells and their outer segments are visualized by methylene blue-staining of a plastic section. The retina shown in this panel was treated with PTU, a chemical that inhibits pigmentation revealing the presence of outer segments.* **(C)** *Inner segments are characterized by the presence of numerous mitochondria. They are evident on this TEM image of a section through the photoreceptor cell layer at 3 dpf.* **(D)** *Photoreceptor connecting cilia are visualized with anti-acetylated tubulin antibodies (green). The IFT52 polypeptide, a component of the intraflagellar transport particle, is particularly highly concentrated at the base of cilia. Its presence is revealed by antibody staining (red).* **(E)** *Double cones of the zebrafish retina are visualized by antibody staining (red). Similar to other photoreceptor types, their surface is subdivided into apical and baso-lateral domains by a belt of adherens junctions, here visualized with fluorophore-conjugated phalloidin (blue). Antibody staining reveals that the Nagie oko polypeptide localizes next to adherens junctions in a narrow apical region of the photoreceptor surface (green).* **(F)** *The processes of Muller glia, here revealed by staining with anti-carbonic anhydrase antibody (red), extend into the photoreceptor cell layer and contribute to the junctions of the outer limiting membrane that subdivide the surface of photoreceptor cells (green).* **(G)** *Photoreceptor synapses are visualized with antibodies against syntaxin 3 (red) and SV2 (blue).* **(H)** *In the plane of the photoreceptor cell layer, cells form a regular pattern. Clockwise, starting from the lower left image: photoreceptor nuclei stained with DAPI (blue), rod opsin-GFP transgene expression (green), anti-UV-opsin antibody staining of short single cones (red), and a merged image of the previous three. CC, connecting cilium; IS, inner segment; OLM, outer limiting membrane; OS, outer segment, ST, synaptic terminus. In B through G, retinal pigment epithelium is up. Arrowheads indicate the approximate position of the outer limiting membrane. Panel H provided courtesy of Jim Fadool.*

Bremiller, 1990). Within each row, cones display a recurrent order: long single cone, double cone, blue cone, double cone ... etc. Rods localize to both sides of each cone-cone interface. Consequently, each cone is surrounded by four rods that appear to occupy corners of a square (Fig. 1H) (Fadool, 2003). This precise spatial arrangement of rods and cones is referred to as the photoreceptor mosaic. As the number of rods increases in the adult further, in some areas of the retina the relatively sparse and regular spacing of rods becomes much more crowded so that the rows of cones become separated by continuous streams of densely packed rod photoreceptor cells (Fadool, 2003, Larison and Bremiller, 1990).

Photoreceptors also exist outside the eye. Although the pineal photoreceptors are not as precisely organized as the retinal ones, they display a number of features characteristic of their retinal counterparts. Their outer segments, for example, are attached to the cell body via the connecting cilium, and are surrounded by calycal processes. The pineal photoreceptor outer segments are cone-shaped and their cell membrane forms infoldings instead of free-floating discs, suggesting that they are related to cones (Allwardt *et al.,* 2001). Despite these cone-like features, the pineal photoreceptors express both red cone opsin and exo-rhodopsin, a visual pigment that arose by a duplication of an ancesteral rod opsin gene (Asaoka *et al.,* 2002, Mano *et al.,* 1999, Robinson *et al.,* 1995). In contrast to morphology, the type of visual pigment that they express suggests a relatedness of pineal photoreceptors to rods. Similar to cells in the retina, the synaptic termini of pineal photoreceptors also contain ribbons. In contrast to retina, however, pineal synapses contact two postsynaptic processes forming a configuration that resembles the dyad arrangement of retinal bipolar cells (Allwardt and Dowling, 2001). It is not clear whether the pineal eye represents a primitive stage during eye evolution or has evolved largely independently (Pichaud and Desplan, 2002). The analysis of pineal photoreceptor cells may offer insights into this interesting issue.

Genetics of photoreceptor development in zebrafish

Several characteristics of the zebrafish model make it suitable for genetic experiments, particularly mutagenesis screens (reviewed in Malicki, 2000). Large numbers of zebrafish can be maintained in a fairly small laboratory space, their generation time is relatively short, and a single female can produce a large number of progeny. The power of zebrafish genetics is best illustrated by the results of two mutagenesis approaches, chemical and retroviral, that have been successfully applied on a large-scale to isolate thousands of mutant lines (Driever *et al.,* 1996, Golling *et al.,* 2002, Haffter *et al.,* 1996).

Fortuitously, photoreceptor mutants in zebrafish appear to be relatively easy to isolate. This is most likely the case because photoreceptor cells are more bulky compared to other retinal neurons, and consequently their loss or malformation is easily detectable as a reduction of eye size. While mutations in over 15 loci are known to produce cell loss predominantly in the photoreceptor cell layer (Table 1), fewer than 5 mutations have been described to result in similar defects within all the remaining retinal strata. In all zebrafish mutants of photoreceptor development identified so far, photoreceptor cells are initially formed, although they frequently develop severe abnormalities already during early steps of differentiation. The severity and the time of onset vary greatly in zebrafish photoreceptor mutants. One of the earliest defects of differentiation is observed in the mutant *mikre oko* (*mok*) which displays abnormal morphology as early as 60 hpf (Doerre and Malicki, 2002, and unpublished results). On the opposite end of the spectrum, two mutants, *night blindness a* (*nba*) and *night blindness d* (*nbd*), produce late-onset dominant photoreceptor defects, by 4 months and by 2 years of age, respectively (Li and Dowling, 1997, Maaswinkel *et al.,* 2003). The importance of late-onset phenotypes lies in their relevance to age-related retinal diseases, a very frequent group of disorders in the human population.

Several mutations appear to affect specific features of photoreceptor cell morphology. Defects in three loci, *oval* (*ovl*), *elipsa* (*eli*), and *fleer* (*fli*), block outer segment formation. In mutant animals, photoreceptor inner segments as well as the rest of the photoreceptor cell somata appear normal (Doerre and Malicki, 2002, Tsujikawa and Malicki, 2004). A decrease of the outer segment size has also been reported in the mutant *vestigial outer segments* (*vos*). Also in this strain of animals, the rest of the photoreceptor cell body is normal (Mohideen *et al.,* 2003). By contrast, defects of the *mok* and the *niezerka* (*nie*) loci affect both outer segments and the morphology of more proximal photoreceptor cell features (Doerre and Malicki, 2002). Nearly all *mok* photoreceptors lack outer segments and their somata frequently do not differentiate an elongated morphology characteristic of this cell class (Doerre and Malicki, 2001). Interestingly, in genetically mosaic retinae, *mok* mutant cells that are surrounded by wild-type tissue, differentiate robust outer segments while their perikaryal regions and synaptic termini remain grossly abnormal (Doerre and Malicki, 2001). The cell-nonautonomous component of the *mok* phenotype is thus confined to the outer segment. Finally, mutations in at least one gene, *no optokinetic response c* (*nrc*), predominantly affect the synaptic region while outer segments and perinuclear areas appear normal. Few postsynaptic processes invaginate into the *nrc* synaptic termini and the synaptic ribbons do not properly attach to the presynaptic membrane (Allwardt *et al.,* 2001). The analysis of these mutants has revealed that genetically independent mechanisms regulate several photoreceptor features, such as the outer segment or the synaptic terminus. So far, the identity of most

TABLE 1 (opposite)

MUTATIONS AFFECTING ZEBRAFISH PHOTORECEPTOR CELLS

The following criteria were used to define the phenotypic categories included in this table. Mutant loci that produce obvious defects in histological (HIS) or electron microscopic (EM) analysis by 3 dpf were classified as affecting **differentiation**. Mutants that show normal photoreceptor development at 3 dpf and then start to degenerate are classified as **survival**. In some cases we were unable to assign a mutant locus to differentiation or survival categories due to the lack of relevant data. These mutants were classified as **differentiation/survival**. Mutants that do not display a phenotype in histological or electron microscopic analyses at all stages examined, are classified as **functional**. As *nab* and *nbd* display a particularly late survival defect, they were classified as **survival (late phenotype)**. Mutations that predominantly affect the organization of cells, are classified as **patterning**. Genetic defects that cause cell degeneration throughout the retina are not listed, even though they may also affect photoreceptor cells.

Mutants of any given category may also contain additional, as yet uncharacterized defects in other aspects of retinal biology. Further studies of these mutants may thus alter their classification. As complementation tests have not been exhaustively performed, it is possible that mutations of single locus are currently represented by two different entries. Entries are sorted alphabetically by "general category" and "phenotype". Abbreviations: CNS, central nervous system; dpf, days postfertilization; EM, electron microscopy; ERG, electroretinograms; HIS, Histology; IS, inner segment; LG, linkage group; MA, mosaic analysis; mpf, months postfertilization; OPL, outer plexiform layer; OS, outer segment; PRC, photoreceptor cell; RPE, retinal pigmented epithelium; ST, synaptic terminus.

LOCUS NAME (ABBREVIATION)	***GENE*/ LG**	**GENERAL CATEGORY**	**PHOTORECEPTOR PHENOTYPE**	**OTHER PHENOTYPES**	**ALLELES**	**REFERENCE**
brudas (bru)	?/? ?/LG9	differentiation	HIS: PRC loss, central (3 dpf) EM: gross morphology abnormal, no OS, few IS MA: autonomous	pigmentation, touch response	m148 tw212d s3556	(Doerre and Malicki, 2002, Goldsmith *et al.,* 2003, Malicki *et al.,* 1996)
elipsa (eli)	?/?	differentiation	HIS: PRC loss, central (3 dpf) EM: no OS formation ERG: no response MA: autonomous	pronephros, body axis curved	m649 tp49d	(Bahadori *et al.,* 2003, Doerre and Malicki, 2002, Malicki *et al.,* 1996)
fleer (flr)		differentiation	HIS: PRC loss, central (3 dpf) EM: no OS formation MA: autonomous	pronephros, body axis curved	m477	(Doerre and Malicki, 2002)
oval (ovl)	*IFT88*/ LG9	differentiation	HIS: PRC loss, central (3 dpf) EM: no OS formation ERG: no response MA: autonomous	pronephros, body axis curved	tz288b	(Bahadori *et al.,* 2003, Doerre and Malicki, 2002)
discontinuous (dis)	?/?	differentiation	HIS: PRC loss, patchy (3 dpf)	brain	m704	(Malicki *et al.,* 1996)
krenty (krt)	?/?	differentiation	HIS: PRC loss, patchy (3 dpf)	brain	m704	(Malicki *et al.,* 1996)
sinusoida (sid)	?/?	differentiation	HIS: PRC loss, patchy (3 dpf)	brain	m704	(Malicki *et al.,* 1996)
mikre oko (mok)	?/?	differentiation	HIS: PRC loss, peripheral (3 dpf) EM: gross morphology abnormal, few OS MA: nonautonomous	none	m632	(Doerre and Malicki, 2001, Malicki *et al.,* 1996)
niezerka (nie)	?/?	differentiation	HIS: PRC loss, peripheral (3 dpf) EM: OS and IS defect MA: nonautonomous	brain slightly smaller	m743	(Doerre and Malicki, 2002, Malicki *et al.,* 1996)
not really finished (nrf)	*nrf1*/ LG4	differentiation	HIS: PRC loss, peripheral (3 dpf) EM: OS malformation	brain	hi39a	(Becker *et al.,* 1998)
bleached (blc)	?/?	differentiation/ survival	HIS: PRC loss (5 dpf) ERG: no response	pigmentation, RPE defect	th204b ts23c ty89	(Neuhauss *et al.,* 2003)
punktata (pkt)	?/?	differentiation/ survival	HIS: PRC loss (5 dpf)	pigmentation	m288	(Malicki *et al.,* 1996)
flathead (fla)	?/?	differentiation/ survival	HIS: PRC loss (6 dpf) ERG: abnormal	brain, jaw, branchial arches	ta53c tf21c	(Neuhauss *et al.,* 1999)
fade out (fad)	?/?	differentiation/ survival	HIS: PRC loss, (6 dpf) ERG: abnormal	pigmentation,RPE defect	tc7b t14 tk224 tm63c tp94c	(Neuhauss *et al.,* 1999)
fading vision (fdv)	?/?	differentiation/ survival	HIS: PRC loss, (6 dpf) ERG: abnormal	pigmentation, RPE defect	th236a	(Neuhauss *et al.,* 1999)
sunbleached (sbl)	?/?	differentiation/ survival	HIS: PRC loss, (6 dpf) ERG: abnormal	pigmentation. RPE defect	to4	(Neuhauss *et al.,* 1999)
photoreceptors absent (pca)	?/?	differentiation/ survival	HIS: PRC loss, central (5 dpf)	none reported	a2	(Fadool *et al.,* 1997)
sleepy (sly)	*lamc1*/ LG2	differentiation/ survival (?)	HIS: shorter OS (larve)	brain, notochord	ts33a m446 m86 tp16 te333	(Karlstrom *et al.,* 1996, Neuhauss *et al.,* 1999, Odenthal *et al.,* 1996)
no optokinetic response c (nrc)	?/ LG10	differentiation/ survival	HIS: thin OPL (6 dpf) EM: ST defect ERG: b-wave delayed and reduced	none	a14	(Allwardt *et al.,* 2001)
no optokinetic response b (nrb)	?/?	function	HIS: no defect ERG: delayed and reduced b-wave, a-wave larger	none	a13	(Brockerhoff *et al.,* 1998)
no optokinetic response f (nof)	*gnat2* LG8	function	HIS: no defect EM: no defect	none	w21	(Brockerhoff *et al.,* 2003)
mosaic eyes (moe)	*epb41l5* /LG9	patterning	HIS: all retinal cell layers, including photoreceptors, disorganized MA: nonautonomous	brain, blood circulation	b476 b781	(Jensen *et al.,* 2001; Jensen and Westerfield, 2004)
n-cadherin (ncad), formerly *glass onion, parachute*	*cdh2*/ LG20	patterning	HIS: all retinal cell layers, including photoreceptors, disorganized MA: partially nonautonomous	brain, somites, notochord, heart, tail	m117 fr7 rw95 tm101…	(Malicki *et al.,* 2003, Malicki *et al.,* 1996, Masai *et al.,* 2003)
nagie oko (nok)	*mpp*/ LG17	patterning	HIS: all retinal cell layers, including photoreceptors, disorganized MA: nonautonomous	brain, blood circulation	m227 m520 jj2	(Malicki *et al.,* 1996, Wei and Malicki, 2002)
oko meduzy (ome)	?/?	patterning	HIS: all retinal cell layers, including photoreceptors, disorganized MA: nonautonomous	brain, blood circulation	m289 m298 m320 m98	(Malicki and Driever, 1999, Malicki *et al.,* 1996)
heart and soul (has)	*prkci (aPKC)*/ LG2	patterning	HIS: all retinal cell layers, including photoreceptors, disorganized or degenerating	brain, heart, blood circulation	m129 m567 m781	(Horne-Badovinac *et al.,* 2001, Malicki *et al.,* 1996, Peterson *et al.,* 2001)
partial optokinetic response b (pob)	?/?	survival	HIS: loss of red cones (5 dpf) ERG: a-wave enhanced, b-wave delayed	brain	a1	(Brockerhoff *et al.,* 1997)
ivory (ivy)	?/ LG20	survival	HIS: PRC loss, (6 dpf) MA: nonautonomous	pigmentation, RPE defect	tm271a tp30	(Goldsmith *et al.,* 2003)
vestigial outer segments (vos)	?/ LG23	survival	HIS: PRC loss, peripheral (5 dpf) EM: OS defect	RPE defect	kc18	(Mohideen *et al.,* 2003)
night blindness a (nba)	?/?	survival (late phenotype)	HIS: cell death in PRC (heterozygotes, 4 mpf) nonspecific cell death (homozygotes, 2 dpf) ERG: delayed and reduced b-wave (heterozygotes)	brain (homozygotes)	da10	(Li and Dowling, 1997) (Maaswinkel
night blindness d (nbd)	?/?	survival (late phenotype)	HIS: fewer OS (2 years) (heterozygote) nonspecific cell death (homozygotes, 2 dpf)	brain (homozygetes)	da54	*et al.,* 2003)

zebrafish genes involved in the development of photoreceptor morphology remains unknown. The cloning of these loci, the next obvious step of analysis, will provide insight into the molecular nature of these mechanisms.

The pattern of photoreceptor degeneration varies in zebrafish mutants. In some mutant strains, photoreceptor loss is first visible in the central portion of the retina, in others it first appears in the retinal periphery (Table 1). Finally, in yet another group of mutants photoreceptor abnormalities affect patches of the photoreceptor cell layer (Malicki *et al.,* 1996). The significance of these different patterns is not clear. Photoreceptor loss that starts in the center of the retina may reflect the temporal order of photoreceptor differentiation: photoreceptor progenitors first exit the cell cycle and differentiate in the early larval retina by 43 hpf (Nawrocki, 1985). Although nearly all cells are postmitotic in the central retina by 72 hpf, neurogenesis continues in the retinal periphery (Marcus *et al.,* 1999). This process persists throughout the lifetime of the organism and consequently the age of photoreceptor cells gradually decreases from the center towards the periphery. The central-to-peripheral sequence of photoreceptor loss may thus simply reflect the fact that defects accumulate first in the oldest, centrally located photoreceptors. The opposite sequence of photoreceptor loss, that starts in the periphery, could be explained by a competition of newly generated photoreceptor cells for a factor that is in a limited supply (Doerre and Malicki, 2002). If this hypothesis is true, the phenotypes of these mutants should display in cell-nonautonomous features. This is, in fact, the case: *mok* and *nie*, two mutants characterized by a peripheral photoreceptor loss, display cell-nonautonomous phenotypes in mosaic experiments.

Mosaic analysis is an experimental tool that allows one to evaluate whether abnormalities in a particular cell population are due to an intrinsic defect or to a deficiency in cell's environment. To evaluate whether a deficiency of an extracellular factor is responsible for a mutant phenotype, one generates retinae that contain a mixture of mutant and wild-type cells in varying proportions. In zebrafish, this is accomplished by blastomere transplantations (Ho and Kane, 1990, Malicki, 1999, Pujic and Malicki, 2001). Studies of several zebrafish photoreceptor mutants using this approach revealed both cell-autonomous and cell-nonautonomous components (Doerre and Malicki, 2001, Doerre and Malicki, 2002, Goldsmith *et al.,* 2003). In some cases, mutant cells survive longer when they exist as small clones surrounded by wild-type tissue. This is true for photoreceptors of mutants *mok*, *nie*, and *ivory*(*ivy*) (Doerre and Malicki, 2002, Goldsmith *et al.,* 2003). By contrast, the wild-type environment has little or no effect on photoreceptor survival in *ovl*, *eli*, *flr*, and *brudas* (*bru*). A particularly strong cell-nonautonomous phenotype has been observed in *ivy* mutants: clones of wild-type cells rescue the morphology of *ivy* photoreceptors across several cell diameters, suggesting that the *ivy* mutation causes a loss of a long-range diffusible factor (Goldsmith *et al.,* 2003). Molecular characterization of this and other extracellular factors that affect zebrafish photoreceptor survival may have a considerable practical importance in the treatment of human eye disease.

Genetic analysis also provides clues to the relatedness of pineal and retinal photoreceptor cells. Pineal photoreceptor phenotypes were analyzed in two mutants, *nie* and *nrc*. In *nie* animals, photoreceptors degenerate both in the pineal gland and in the retina early in development, indicating that these two cell populations share genetic circuitry that regulates their differentiation (Allwardt and Dowling, 2001, Doerre and Malicki, 2002). In contrast to that, the *nrc* mutation affects ribbon synapses of retinal photoreceptors only. The ribbon synapses of both pineal photoreceptors and retinal bipolar cells are intact in this mutant (Allwardt and Dowling, 2001). This observation, as well as morphological similarities, suggest that pineal photoreceptor synapses may be related to those of bipolar cells.

Outer segment formation

Outer segments contain the light detection apparatus of photoreceptor cells, including visual pigments and other components of the phototransduction cascade. The opsin polypeptide is the most abundant component of the outer segment membranes: a salamander rod outer segment contains ca. $3x10^9$ rhodopsin molecules and in the absence of rhodopsin expression the outer segments of mouse rod photoreceptors do not form at all (Lem *et al.,* 1999, Pugh and Lamb, 2000). Other components of the phototransduction apparatus, although also abundant, are usually at least an order of magnitude less concentrated. The outer segment membranes are continuously replaced: the distal-most membrane folds are removed while the new ones are added at the outer segment base (Young, 1967). It has been shown that rodent photoreceptor cells renew their outer segments every 10 days (Young, 1967). This rapid turnover of the outer segment membranes requires a continuous transport of massive amounts of proteins to the apical region of the photoreceptor cell and into the outer segment. How is this accomplished?

As outer segments are devoid of protein synthesis, their protein components have to be transported from the cell body. Although other modes of transport cannot be entirely excluded (reviewed in Sung and Tai, 2000), the most likely route of protein movement into the outer segment is via the connecting cilium, a primary cilium of 9 + 0 microtubule configuration (Rodieck, 1973, Rosenbaum *et al.,* 1999). Protein transport along cilia, a process studied most extensively in *Chlamydomonas* and *C. elegans*, is thought to be mediated by so-called intraflagellar transport (IFT) particles. Proteins required in cilia and their derivatives, such as photoreceptor outer segments, are presumed to form complexes with IFT particles at the connecting cilium base. Subsequently, they are thought to associate with kinesin motor proteins that mediate their anterograde transport along the ciliary axoneme. This mode of transport finds support in the observation that kinesin mutants in *Chlamydomonas* and *C. elegans* display cilia defects (reviewed in Scholey, 2003, Signor *et al.,* 1999, Walther *et al.,* 1994). Kinesin may also play a similar role in photoreceptor cells: kinesin-II-deficient mouse photoreceptors show ectopic opsin and arrestin accumulation in inner segments, suggesting that kinesin-mediated transport is necessary for proper outer segment formation or maintenance (Marszalek *et al.,* 2000).

Interestingly, mutations in several zebrafish loci produce the absence of photoreceptor outer segments. One of the best-characterized genes in this category is *ova1* (*ovl*). While inner segments of the *ovl* photoreceptor cells appear intact, their outer segments are entirely missing, indicating a defect in a mechanism that is essential for outer segment formation (Doerre and Malicki, 2002). Cloning of the *ovl* locus revealed that it encodes the zebrafish homolog of IFT88, one of the IFT complex components

(Tsujikawa and Malicki, 2004). The *ovl*^{tz288b} mutant allele contains a nonsense codon in the first one-third of the polypeptide, resulting in a complete loss of IFT88 function (Tsujikawa and Malicki, 2004). Does the *ovl* gene play a role in retinal ciliogenesis? Prior to the onset of neurogenesis and photoreceptor differentiation, the *ovl* cilia appear normal. This changes later in development: by 3 dpf, the *ovl*^{tz288b} mutant photoreceptors lack connecting cilia, although their basal bodies localize to correct positions in the apical portion of the inner segment. The lack of cilia is accompanied by the absence of outer segment membrane stacks, although occasional arrays of several membranes that run parallel to each other are found on the lateral surface of photoreceptor inner segments. These may represent outer segment membrane folds that form ectopically in the absence of intraflagellar transport.

Defects of *ovl* outer segment morphogenesis are followed by photoreceptor degeneration that proceeds following the central-to-peripheral pattern discussed above. Why do *ovl* photoreceptors die? A cause for cell death is suggested by the observation that in the absence of outer segment formation, opsins are detected throughout the entire photoreceptor cell membrane (Tsujikawa and Malicki, 2004). Cell culture experiments suggest that light-induced activation of ectopically localized visual pigments leads to cell death (Alfinito and Townes-Anderson, 2002). Abnormal rod opsin localization followed by photoreceptor cell death is also observed in a transgenic animal model of retinitis pigmentosa (Li *et al.,* 1998). Two experiments demonstrate that this may also be the case in *ovl* animals. First, blocking rod opsin expression with anti-rod opsin morpholino oligonucleotides specifically prolongs the survival of rod photoreceptors in *ovl* retinae. Second, fish reared in constant darkness display a weaker photoreceptor loss (Tsujikawa and Malicki, 2004). These studies suggest the following scenario: in the absence of outer segment formation, rod opsin is misrouted into other cellular compartments, where its activity interferes with intracellular signaling pathways, eventually resulting in cell death. Although this may not be the only mechanism that leads to *ovl* photoreceptor loss, it is likely to be an important contributor to cell death in this and related outer segment mutants.

The IFT particle consists of two protein complexes, termed A and B, each composed of several polypeptides (reviewed in Rosenbaum and Witman, 2002, Scholey, 2003). Ovl/IFT88 is one of over 10 components that form complex B. Are the functions of other complex A and B genes similar to *ovl*? As mutant alleles of other IFT loci have not been described in zebrafish so far, their function has been assayed using an antisense knockdown approach. Blocking the expression of complex B components, IFT52 or IFT57, also results in photoreceptor degeneration, suggesting that their function in the retina is similar to *ovl*. On the contrary, the knockdown of a complex A component, IFT140, does not affect photoreceptor development in assays that have been performed so far. These observations suggest that complex B is essential for photoreceptor differentiation whereas complex A may play only an ancillary role.

In addition to photoreceptors, *ovl* plays important roles in two other classes of sensory cells: auditory hair cells, and olfactory sensory neurons (Tsujikawa and Malicki, 2004). In both of these cell classes, *ovl* is necessary for the maintenance of cilia rather than for their formation. Similar to the visual system, *ovl* function is also required for the survival of sensory cells in the auditory and olfactory organs. In addition to *ovl*, defects in two other zebrafish loci produce closely related outer segment phenotypes. Mutations of the genes *elipsa* (*eli*) and *fleer* (*fli*) result in outer segment abnormalities accompanied by pronephric cysts and curved body axis: in both mutants outer segments appear entirely absent while inner segments retain normal morphology (Doerre and Malicki, 2002, Malicki *et al.,* 1996). As cilia are known to play essential roles both in kidney development and in embryonic patterning, these phenotypes are also likely to be associated with ciliary malfunction"(Huangfu *et al.,* 2003, McGrath *et al.,* 2003, Pazour *et al.,* 2000). Thus *eli* and *flr* may encode intraflagellar transport-related genes, or components of other, as yet unknown, mechanisms that are associated with the formation of cilia.

Cell polarity

Cell surface polarity is an important feature of photoreceptor cells. Similar to epithelial cells, the photoreceptor surface is subdivided by a belt of cell junctions into apical and basolateral domains (Fig. 1A). Studies in *Drosophila* indicate that genetic regulators of photoreceptor polarity are related to genes that control polarity in epithelial cells. First, the products of epithelial polarity genes, Crumbs, Stardust, DmPar-6 and others, localize to the apical membrane of fly photoreceptor cells (Hong *et al.,* 2003, Nam and Choi, 2003). Second, the loss of *crumbs*, *stardust,* or *bazooka* function result in abnormal photoreceptor polarity (Hong *et al.,* 2003, Pellikka *et al.,* 2002, Wodarz *et al.,* 1995). Finally, *crumbs* overexpression results in a 4-fold expansion of the photoreceptor cell apical membrane. The analysis of the vertebrate eye also lends credence to the idea that the regulators of photoreceptor polarity are related to epithelial polarity pathways: homologs of *Drosophila* epidermal polarity genes are expressed in vertebrate photoreceptors and also display polarized distributions (Fig. 1 A,E).

At least two genes are likely to play a role in zebrafish vertebrate photoreceptor polarity: *nagie oko* (*nok*) and *heart and soul* (*has*), homologs of fruit fly loci involved in the polarity of embryonic epithelia, *stardust* and *DaPKC* respectively (Horne-Badovinac *et al.,* 2001, Wei and Malicki, 2002). The *nok* gene product is a MAGUK-family scaffolding factor while *has* encodes an atypical protein kinase C. Both Nok and Has polypeptides localize apical to cell junctions in retinal neuroepithelium and in the photoreceptor cell layer (Fig. 1E). More recently it has been shown that the *pard3a* gene, a zebrafish homolog of the fly locus *bazooka*, also localizes to the vicinity of the outer limiting membrane (Wei *et al.,* 2004). In addition to zebrafish genes, CRB1, a mouse homolog of Drosophila *crumbs*, localizes to photoreceptor cell surface area immediately apical to cell junctions (Pellikka *et al.,* 2002). Thus all apically distributed cell polarity regulators that have been characterized in the vertebrate retina so far localize to analogous cell membrane domains in photoreceptors and in epithelia. It has yet to be investigated whether baso-lateral determinants of epithelial polarity, such as *discs large* or *scribble*, also display baso-lateral distribution in photoreceptor cells.

What is the role of cell polarity determinants in photoreceptor development? Genetic studies in zebrafish indicate that *nok* and *has* functions are closely related to these of their *Drosophila* homologs. Mutations in these loci cause a baso-lateral displacement of apical structures in the retinal neuroepithelium (Horne-Badovinac *et al.,* 2001, Wei and Malicki, 2002). Do they also function in the polarity of

photoreceptor cells? This question is not straightforward to address because the retinae of *has* and *nok* mutant animals are dramatically disorganized following the differentiation of individual cell identities: mutant photoreceptors are scattered throughout the entire retina and do not form a distinct layer (Wei and Malicki, 2002). The differentiation of ectopic photoreceptors in *nok* and related mutants has not been thoroughly investigated so far. It is thus not clear whether the distribution of polarity markers is affected in these cells. Similarly, it has not been investigated whether they differentiate outer segments. The disorganized distribution of photoreceptor cells in *nok* retinae suggests, however, that *nok* is necessary for the integrity of cell junctions of the outer limiting membrane, a crucial element of photoreceptor cell polarity. As cell polarity is most likely essential for both photoreceptor structure and function, the zebrafish mutants are important assets in the study of photoreceptor biology.

Genetic analysis of photoreceptor function

The phototransduction cascade, the central component of photoreceptor function, is another area that is being studied using genetic approaches in zebrafish. Defects of the phototransduction cascade components are likely to produce slow photoreceptor loss and thus are difficult to detect using morphological criteria in zebrafish. Consequently, behavioral screens may be the best genetic approach to identify such defects. As visual system function is ultimately the outcome of a long sequence of developmental and physiological processes, behavioral screens are capable of detecting a particularly broad range of defects, including those in photoreceptor physiology. George Streisinger and his students, the early proponents of the zebrafish model, have already appreciated the potential of behavioral screens and demonstrated that the optokinetic response (OKR) can be used to identify mutations of the visual system (Clark, 1981). This line of investigation has been recently revived, leading to the isolation of several new mutant lines (Table 1). Some of these, such as *noa*^{m631}, *nrb*^{a13} and *nof*^{w21}, do not display obvious morphological or histological defects in the retina and thus appear to affect retinal function rather than development (Brockerhoff *et al.,* 1998, Brockerhoff *et al.,* 2003). Consistent with this conclusion, electroretinograms of these mutant strains frequently display defects. In *nrb*^{a13} mutant animals, for example, the a-wave is larger, suggesting photoreceptor malfunction (Brockerhoff *et al.,* 1998). Recent cloning of the first locus in this group, *nof*, demonstrated that it encodes the α subunit of cone transducin. Cone photoreceptors of *nof*^{w21} mutants are insensitive to low or moderate intensity of light. Interestingly, even in the absence of functional transducin, mutant cones do respond to bright light. These responses are thought to be mediated by transducin-independent calcium release from intracellular storage compartments (Brockerhoff *et al.,* 2003). In addition to development, the zebrafish retina is thus a suitable system to analyze the molecular components of photoreceptor physiology.

Future directions

Many aspects of photoreceptor development and function remain a mystery. Although the nature of several regulatory factors that are involved in early photoreceptor development is already known, the understanding of this process is far from complete. The genetic basis of photoreceptor diversity has also been investigated partially only. The retinae of many vertebrates, including the zebrafish, contain several types of cones but the mechanisms that produce this diversity are not known. In addition to the early steps of photoreceptor specification, the formation of the complex photoreceptor morphology is an extraordinary process that will need to be investigated in depth. It remains to be determined, for example, what molecular mechanisms regulate the formation of the remarkably regular array of hundreds of membrane folds that form the outer segment. Although several genes are already known to function in outer segment assembly, much remains to be done before this process is fully understood. The differentiation of the synaptic terminus and its complex synaptic apparatus is even more of an uncharted territory. Finally, an intriguing question that sill needs to be addressed is the genetic basis of the patterning process that produces the regular arrangement of photoreceptor mosaic in zebrafish and in other species.

One way to uncover the regulators of photoreceptor specification and morphogenesis is to continue genetic screens. A key feature of the screen-based forward genetic approach is that it is unbiased by molecular considerations and frequently leads to the discovery of unexpected new players in a developmental process. This approach has been remarkably successful in the zebrafish model so far, and is likely to provide valuable insights in the future. A promising screening tool that has recently become available in zebrafish are transgenic lines that express the fluorescent marker, GFP, in photoreceptor cells. At least three such lines have been generated. In all cases, GFP expression is driven by upstream regulatory sequences of either zebrafish or *Xenopus* rod opsin genes. In one line, the GFP polypeptide is fused to the 44 C-terminal amino acids of rod opsin and is transported into photoreceptor outer segments. These three lines can be used in genetic screens to monitor subtle aspects of photoreceptor cell development such as the density of rod photoreceptor cells, their distribution across the retinal surface, their morphology, and even the transport of the visual pigment into rod outer segments. Although GFP transgene-aided screens involve the same amount of effort as morphology-based experiments, they allow one to monitor many subtle aspects of photoreceptor position and structure with unprecedented sensitivity.

Not too long ago, the positional cloning of zebrafish mutant genes presented a daunting challenge. Owing to the progress of the zebrafish genome project, the cloning of chemically-induced mutant alleles has become much easier. The challenge of future genetic analysis is to devise new ways to detect defects in specific aspects of photoreceptor development or function. New screening approaches combined with genetic and genomic tools of analysis will assure that the zebrafish retina will continue to be a rich source of interesting insights into the mechanisms of photoreceptor development and function.

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