

## Mutations affecting development of the zebrafish retina

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### SUMMARY

In a large scale screen for genetic defects in zebrafish embryogenesis we identified 49 mutations affecting development of the retina. Based on analysis of living embryos as well as histological sections, we grouped the isolated mutations into six phenotypic categories. (1) Mutations in three loci result in a loss of wild-type laminar pattern of the neural retina. (2) Defects in four loci lead to an abnormal specification of the eye anlagen. Only one eye frequently forms in this class of mutants. (3) Seven loci predominantly affect development of the outer retinal layers. Mutants in this category display cell loss mainly in the photoreceptor cell layer. (4) Nine mutations cause retardation of eye growth without any other obvious abnormalities in

the retina. (5) A group of twelve mutations is characterized by nonspecific retinal degeneration. (6) Four mutations display retinal degeneration associated with a pigmentation defect. Finally, two mutations, one with absence of the ventral retina and one with an eye-specific pigmentation defect, are not classified in any of the above groups. The identified mutations affect numerous aspects of eye development, including: specification of the eye anlage, growth rate of the optic cup, establishment of retinal stratification, specification or differentiation of retinal neurons and formation of the dorsoventral axis in the developing eye.

Key words: retina, neurogenesis, zebrafish

### INTRODUCTION

Neurons of the vertebrate central nervous system, including the retina, originate from an initially uniform sheet of pseudostratified neuroepithelium (Jacobson, 1991). In the neural retina seven major cell types, including six types of neurons and the Muller glia, combine in three cellular layers to form a powerful and versatile sensory structure (Dowling, 1987). Several problems of general significance are associated with the development of the vertebrate retina. How is the unique identity of the retina specified within the neuroepithelial sheet of the neural tube? How are the identities of the six retinal neurons and one glial cell type acquired during the process of development? What cues guide individual cell types to occupy specific positions within the stratified structure of the retina? These questions are not unique to the retina and are equally relevant to many regions of the vertebrate brain.

For several reasons the zebrafish retina is particularly suited for studies of neuronal specification and patterning in the vertebrate central nervous system. In teleost embryos the eye is relatively large and easily accessible. The cellular architecture of the retina is relatively simple and well characterized (Dowling, 1987). Subtypes of retinal neurons are easily recognizable by their position and morphology. Molecular markers specific to many retinal neurons are available (Larison and Bremiller, 1990; Trevarrow et al., 1990; Raymond et al.,

1993; Sandell et al., 1994). Importantly, in the central region of the zebrafish retina, the vast majority of neurons are born and organized in distinct laminae by 60 hours postfertilization (hpf). Taken together, these characteristics make the development of the zebrafish retina particularly amenable to genetic analysis.

Early development of the zebrafish eye has been previously described in detail (Schmitt and Dowling, 1994). The optic lobe can be first distinguished at the 6 somite stage. The pigmented epithelium and the neural retina become distinct starting at the 11-12 somite stage. Invagination of the optic lobe is initiated at the 14 somite stage and is accompanied by the formation of the lens rudiment. By the end of somitogenesis (30 somites; 24 hpf) the lens is spherical and has detached from the epidermis. The optic cup consists of two distinct layers: a thick layer of columnar pseudostratified neuroepithelium from which the neural retina will form, and a thin layer of flat pigmented epithelial cells. The first pigment granules appear in the pigmented epithelium at approximately 24 hpf.

As in other vertebrates, ganglion cells are the first neurons to be born in the zebrafish retina (Nawrocki, 1985; reviewed by Altshuler et al., 1991). Birth-dating studies indicate that the first postmitotic neurons appear between 29 and 34 hpf (Nawrocki, 1985). At 36 hpf none of the neuronal cell layers are clearly distinguishable (Fig. 1A). By 60 hpf the vast majority of neurons in the central retina have already been born

(Nawrocki, 1985) and are organized into three nuclear layers separated by two plexiform layers (Fig. 1B). The stratification of the retina becomes progressively more distinct at later stages of development (Fig. 1C,D).

The photoreceptor cell layer of the zebrafish retina contains five photoreceptor types: rods, short single cones, long single cones, and long and short members of the double cone pair (Branchek and Bremiller, 1984). Initially, different photoreceptor types are not distinguishable by morphological criteria. Short single cones can be first distinguished from other photoreceptor cells at about 4 days postfertilization (dpf) (Fig. 1D). By 12 dpf all photoreceptor types can be distinguished on the basis of morphological criteria (Branchek and Bremiller, 1984).

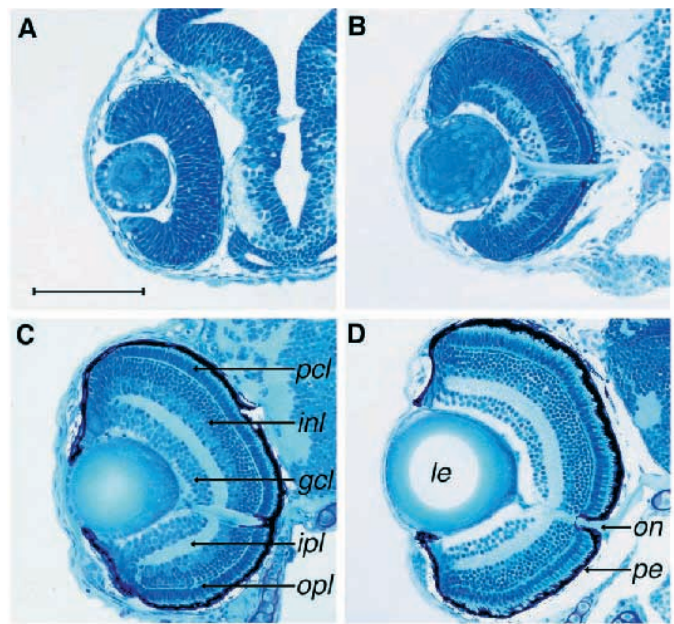
Genetic analysis has been exceptionally successful in dissecting the development of invertebrate sensory organs. In the *Drosophila* eye, genes involved in the specification of the eye imaginal discs (Halder et al., 1995), progression of the morphogenetic furrow (Haberlein et al., 1993; Ma et al., 1993; Brown et al., 1995) and specification of photoreceptor cell fates (Tomlinson et al., 1988; Baker et al., 1990; reviewed in Zipursky and Rubin, 1994) have been identified. It is not clear however, to what extent the genetic circuitry utilized in *Drosophila* is conserved in the development of the vertebrate eye. Several genes known to play important roles in *Drosophila* eye development have vertebrate homologs with expression patterns in developing eyes (Della et al., 1993; Fjose et al., 1993; Quiring et al., 1994). The function of most of these genes has not yet been determined. Thus far, the strongest point of similarity between insects and vertebrates is specification of the eye primordium. The vertebrate gene *Pax-6* (*Small eye*) and its fruit fly homologue *eyeless* have similar loss of function phenotypes causing reduction or absence of eyes in mice and fruit flies, respectively (Hill et al., 1991; Quiring et al., 1994). Amazingly, both *eyeless* and *Pax-6* are sufficient to induce ectopic specification of eyes in the developing fly (Halder et al., 1995).

In a large scale mutagenesis screen in zebrafish (Driever et al., 1996), we attempted to identify recessive, zygotic loci involved in several aspects of vertebrate eye development. Our search led to isolation of 49 mutations falling into the following six phenotypic categories: neuronal patterning defect; cyclopia; defect of the outer retina; growth retardation; nonspecific retinal degeneration; and retinal degeneration associated with a pigmentation defect. In this work we report the initial genetic and phenotypic characterization of the isolated mutants.

## MATERIALS AND METHODS

### Genetic analysis

Mutations were induced using N-ethyl-N-nitrosourea (ENU) as described previously (Solnica-Krezel et al., 1994). All mutants were initially identified in the progeny of crosses between individuals of the F<sub>2</sub> generation bred from mutagenized G<sub>0</sub> males (Driever et al., 1996). All crosses which led to the identification of mutant phenotypes were repeated. For further analysis, heterozygous carriers of mutant alleles were outcrossed to wild-type AB (Chakrabarti et al., 1983), Tubingen (Mullins et al., 1994) or India (Knapik et al., 1996) strains. Mutant heterozygotes were identified in the progeny of outcrosses by sibling mating.



**Fig. 1.** Development of wild-type zebrafish eye. (A) At 36 hpf retinal cells do not form distinct layers. (B) Three nuclear and two plexiform layers are already visible at 60 hpf. (C) At 72 hpf the retinal stratification is well developed. gcl, ganglion cell layer; inl, inner nuclear layer; ipl, inner plexiform layer; opl, outer plexiform layer; pcl, photoreceptor cell layer. This basic laminar pattern is preserved into adulthood. Within the inner nuclear layer, cells adjacent to its inner rim stain less intensely. These are presumably the amacrine neurons. (D) The short single cones can be distinguished in the photoreceptor cell layer at 5 dpf. Their lightly staining outer segments intercalate between the surrounding cells. le, lens; on, optic nerve; pe, pigmented epithelium. In all panels ventral is down. All sections are transverse. Scale bar, 100  $\mu$ m.

Complementation tests were performed between heterozygous carriers of mutations falling into the same phenotypic category. Both mutants in the 'miscellaneous' category were tested with the neuronal patterning group. At least 30 embryos, often from two independent crosses, were scored for the mutant phenotype. For mutations with no assigned locus name (Table 1), complementation testing was not performed.

### Phenotypic analysis

Embryos were maintained at 28°C in egg water (Westerfield, 1994) with addition of methylene blue (Sigma, Inc.) at the concentration of 1 mg/l. Staging was performed as described previously (Kimmel et al., 1995). For observations, embryos were anesthetized with a 0.02% solution of 3-aminobenzoic acid methyl ester (Sigma, Inc.) and embedded in 3% methylcellulose in egg water. Living embryos were initially observed and photographed under a dissecting microscope. Detailed observations were conducted using an Axiophot microscope and Nomarski optics (Zeiss, Inc.).

For histological analysis, embryos were fixed in PFA/GA/Acrolein fix (Kuwada et al., 1990), embedded in JB-4 resin (Polysciences, Inc.) and sectioned at 3  $\mu$ m. For each mutant strain 3-6 embryos were sectioned in a single block. Sections were collected on slides, stained with methylene blue-azure II (Humphrey and Pittman, 1974) for 10 seconds, rinsed in distilled water for 10 minutes, dried and mounted with Permount (Fisher Scientific, Inc.). Depending upon the onset of phenotype, mutant retinæ were sectioned at either 3 or 5 dpf. In most cases a wild-type sibling of the mutant individuals was embedded for sectioning in the same block.

**Table 1. Mutations affecting development of the zebrafish retina**

Locus name	Alleles	Eye phenotype	Other phenotypes	References
<b>Group I: Neuronal patterning defect</b>				
<i>oko meduzy (ome)</i>	<i>m98; m289; m298; m320</i>	Eye pigmentation, retina disorganized	Brain, circulation	b
<i>glass onion (glo)</i>	<i>m117</i>	Eye pigmentation, retina disorganized	Brain, circulation, tail	a
<i>nagie oko (nok)</i>	<i>m227; m520</i>	Eye pigmentation, retina disorganized	Brain, circulation	b
<b>Group II: Cyclopia</b>				
<i>cyclops (cyc)</i>	<i>m101; m122; m294</i>	Eye anlage fused	Brain, spinal chord	f
<i>one eyed pinhead (oep)</i>	<i>m134</i>	Eye anlage fused	Brain, spinal chord	b, c
<i>bozozok (boz)</i>	<i>m168</i>	Eye anlage fused	Brain, spinal chord	b, c
<i>uncle freddy (unf)</i>	<i>m768</i>	Eye anlage fused	Brain, spinal chord	b, c
<b>Group III: Defect of the outer retina</b>				
<i>brudas (bru)</i>	<i>m148</i>	Reduced eye size, PRCL absent (3 dpf)	Touch response, pigmentation	
<i>sinusoida (sid)</i>	<i>m604</i>	Reduced eye size, PRCL discontinuous	Brain	
<i>mikre oko (mok)</i>	<i>m632</i>	Reduced eye size, PRCL absent (5 dpf)	None	
<i>elipsa (eli)</i>	<i>m649</i>	Oval eye shape, PRCL absent (5 dpf)	Pronephros	
<i>krenty (krt)</i>	<i>m699</i>	Reduced eye size, PRCL discontinuous	Brain	
<i>discontinuous (dis)</i>	<i>m704</i>	Reduced eye size, PRCL discontinuous	Brain	
<i>niezerka (nie)</i>	<i>m743</i>	Reduced eye size, PRCL absent (5 dpf)	None	
<b>Group IV: Growth retardation</b>				
<i>out of sight (out)</i>	<i>m233; m306; m390</i>	Reduced eye size (2 dpf)	Brain, pigmentation	
<i>spy eye (spy)</i>	<i>m319</i>	Reduced eye size (5 dpf)	None	
<i>cleopatra (cle)</i>	<i>m474</i>	Reduced eye size (5 dpf)	None	
<i>visionary (vis)</i>	<i>m630</i>	Reduced eye size (5 dpf)	None	
<i>podgladacz (pod)</i>	<i>m689</i>	Reduced eye size (5 dpf)	None	
-	<i>m489</i>	Reduced eye size (5 dpf)	None	
-	<i>m771</i>	Reduced eye size (5 dpf)	None	
<b>Group V: Nonspecific retinal degeneration</b>				
<i>pyry (pyr)</i>	<i>m124</i>	Reduced eye size (3 dpf)	Brain	
<i>turbulent (tub)</i>	<i>m125</i>	Reduced eye size (3 dpf)	Brain	
<i>zimny (zny)</i>	<i>m419</i>	Reduced eye size (2 dpf)	Brain	
<i>lichee (che)</i>	<i>m599</i>	Reduced eye size (2 dpf)	Brain	
<i>ziemniok (zem)</i>	<i>m709</i>	Reduced eye size (3 dpf)	Brain	
-	<i>m210</i>	Reduced eye size (3 dpf)	Brain	
-	<i>m220</i>	Reduced eye size (5 dpf)	Brain	
-	<i>m267</i>	Reduced eye size (3 dpf)	Brain	
-	<i>m283</i>	Reduced eye size (5 dpf)	Brain	
-	<i>m303</i>	Reduced eye size (3 dpf)	Brain	
-	<i>m393</i>	Reduced eye size (5 dpf)	Brain	
-	<i>m394</i>	Reduced eye size (5 dpf)	Brain	
<b>Group VI: Retinal degeneration associated with pigmentation defect</b>				
<i>piegus (pgu)</i>	<i>m286; m300</i>	Reduced eye size (3 dpf)	Pigmentation, ear	
<i>punktata (pkt)</i>	<i>m288</i>	Reduced eye size (3 dpf)	Pigmentation, brain, ear	
<i>mizerny (miz)</i>	<i>m293</i>	Reduced eye size (3 dpf)	Pigmentation, brain, ear	
<b>Group VII: Miscellaneous</b>				
<i>heart and soul (has)</i>	<i>m129; m567; m781</i>	Eye pigmentation, retinal degeneration	Brain, blood, circulation	b, d
<i>pandora (pan)</i>	<i>m313</i>	Ventral retina absent	Brain, ear, tail, pigmentation	a, d, e

Other phenotypic aspects of mutants presented in this table are described by: a, Abdelilah et al. (1996); b, Schier et al. (1996); c, Solnica-Krezel et al. (1996); d, Stainier et al. (1996); e, Malicki et al. (1996); f, Hatta et al. (1991).

Mutants with no locus name were not complementation tested.

PRCL, photoreceptor cell layer; dpf, days postfertilization.

## RESULTS

### A screen for mutations affecting eye development

As part of a large scale mutagenesis screen we searched for zygotic recessive mutations affecting development of the zebrafish eye (Driever et al., 1996). We paid particular attention to several aspects of eye development: morphogenesis of the optic cup; appearance of the pigmented epithelium; the size and shape of the eye. Relevant aspects of development were evaluated at 1, 2, 3 and 5 dpf.

Not all mutations affecting development of the eye were subjected to a detailed analysis. For example, at 5 dpf, a small eye phenotype was frequently associated with an overall reduction of brain size and a delay in the development of

branchial arches and pectoral fins. Similarly, at earlier stages, mutant embryos frequently displayed a severe reduction of eye and brain size. These phenotypes appeared to reflect a general delay of development or non-region-specific neuronal degeneration. Mutations leading to such phenotypes were not analyzed further.

For all mutations, retinæ were sectioned to assess the fate of individual neural cell types. Based on observation of living embryos and analysis of histological sections we grouped the isolated mutations into six phenotypic categories: neuronal patterning defect; cyclopia; defect of the outer retina; growth retardation; nonspecific retinal degeneration; retinal degeneration associated with a pigmentation defect (Table 1). Complementation tests were performed for mutations classified in the

same groups. Below we discuss the phenotypic characteristics of mutants in each of the identified categories.

### Group I: mutants with a neuronal patterning defect

In wild-type zebrafish pigmentation appears at 24 hpf both in the pigmented epithelium and melanophores. The mutants *oko meduzy* (*ome*)<sup>m98</sup>, *glass onion* (*glo*)<sup>m117</sup> and *nagie oko* (*nok*)<sup>m227</sup> exhibit an eye specific pigmentation defect (Table 1). The eye phenotype of these mutations becomes visible shortly after the onset of pigmentation. Wild-type eye pigmentation is uniform (Fig. 2A,F). In contrast, eye pigmentation of the mutant animals is patchy or absent (Fig. 2B,C,E,G). Fig. 2 shows the mutant phenotypes in this category at 36 hpf. Melanophores have a normal appearance in the mutant embryos.

We inspected retinæ of the mutant embryos at 3 dpf on histological sections. At this stage, wild-type retinal neurons form an easily recognizable pattern of nuclear and plexiform layers (Fig. 1C). In all three mutants the laminar arrangement of retinal neurons is disorganized (Fig. 3A-C). Instead of forming layers, the plexiform matter is distributed in patches. Although photoreceptor cells have a distinct, elongated shape, rudiments of the photoreceptor cell layer were never observed in any mutant retina. We evaluated whether the amount of cell death is abnormal in *ome*<sup>m98</sup> embryos. Cell corpses are easily recognizable on histological sections by their condensed, round appearance. In the *ome*<sup>m98</sup> retina at 48 hpf the amount of cell death does not exceed the wild-type level. By 3 dpf the number of cell corpses in the mutant retinæ appears to be approximately 4 times higher than in the wild type (data not shown). These observations suggest that the patterning defect in *ome*<sup>m98</sup> is not a consequence of an extensive degeneration of retinal neurons. All mutations in this group produce fully penetrant phenotypes in the retina.

Mutations in this category also lead to an abnormal brain shape (Schier et al., 1996), curved body axis and frequently reduced or absent circulation. The phenotypes caused by *ome*<sup>m98</sup> and *nok*<sup>m227</sup> are very similar to each other. The *glo*<sup>m117</sup> phenotype is more pleiotropic, causing a much stronger brain defect and abnormal tail development (Fig. 2B). In *ome*<sup>m98</sup> and *nok*<sup>m227</sup>, overall brain patterning appears to be normal on histological sections, whereas the brain pattern of *glo*<sup>m117</sup> is disorganized (data not shown).

### Group II: mutants with cyclopia

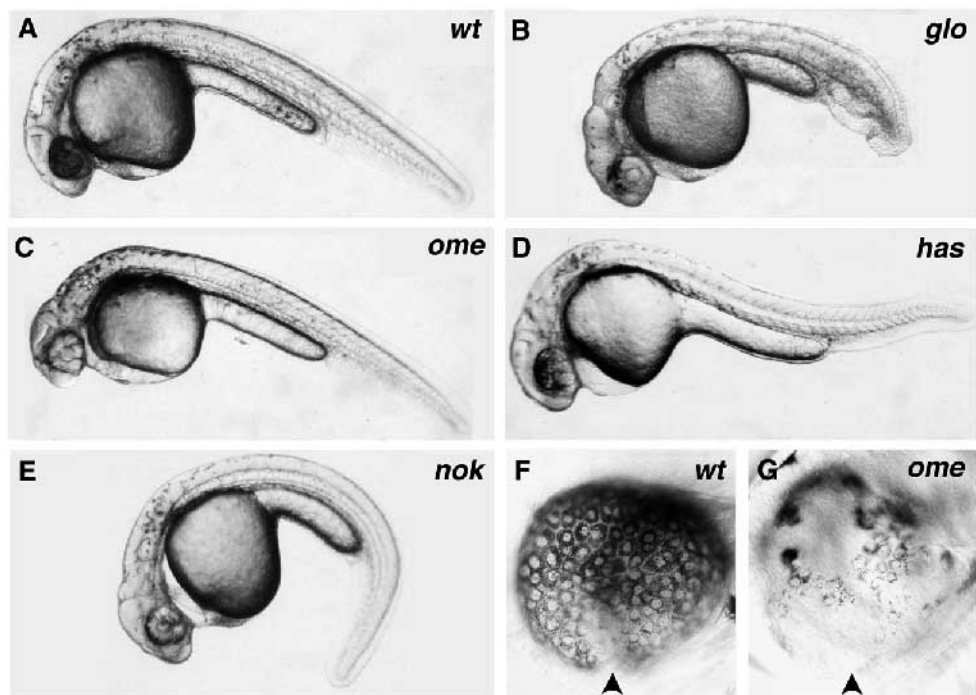
Mutations in four loci lead to a single-eye phenotype (cyclopia) (Table 1). The *cyclops* (*cyc*) locus (Hatta et al., 1991) has been previously shown to be involved in pat-

terning of the ventral brain (Hatta, 1992). The three other loci producing cyclopic phenotypes are *bozozok* (*boz*)<sup>m168</sup>, *one eyed pinhead* (*oep*)<sup>m134</sup> and *uncle freddy* (*unf*)<sup>m768</sup>. The degree of eye fusion is variable in these mutants. In the most severe cases of the *boz*<sup>m168</sup> phenotype, the cyclopic eye is very small or even absent. In less affected individuals two eyes form closely next to each other (Fig. 3G). In order to assess whether fusion of the eye anlage affects patterning of retinal neurons, we prepared histological sections through the eyes of *cyc*<sup>b16</sup> (Hatta et al., 1991), *oep*<sup>m134</sup> and *boz*<sup>m168</sup> mutants at 4 dpf. Although retinal development is somewhat delayed in the cyclopic mutants, neuronal lamination forms at least to some degree (Fig. 3E-G). Patterning abnormalities are present in some mutant individuals and are particularly evident in the mutant *boz*<sup>m168</sup>. On histological sections, abnormal patterning of the retina is accompanied by extensive cell death.

### Group III: mutants with defects of the outer retina

Mutations at seven loci predominantly affect development of the outer retinal layers (Table 1). With the exception of *elipsa* (*eli*)<sup>m649</sup>, mutants in this category exhibit reduced eye size at 5 dpf in comparison to their wild-type siblings (Fig. 4). In the lateral view the eye diameter of the mutant larvae is reduced by 20% to 30% at this stage. The eye of *eli*<sup>m649</sup> has an abnormal, oval shape at this stage (Fig. 4D). Histological sections through the mutant retinæ reveal three types of photoreceptor deficit in this group.

The first type is present in *mikre oko* (*mok*)<sup>m632</sup> and *nieszkerka*



**Fig. 2.** Phenotypes of mutants characterized by eye specific pigmentation defect. (A) At 36 hpf pigmentation is apparent both in the eye and skin of wild-type embryos. In (B) *glass onion* (*glo*)<sup>m117</sup>, (C) *oko meduzy* (*ome*)<sup>m98</sup>, (D) *heart and soul* (*has*)<sup>m129</sup> and (E) *nagie oko* (*nok*)<sup>m227</sup>, we observed abnormal differentiation of the pigmented epithelium. Melanocytes of these mutants have normal appearance although their development may be somewhat delayed. (F) Wild-type and (G) *ome*<sup>m98</sup> pigmented epithelium in living embryos. The eye pigmentation is uniform in the wild type and patchy in the mutant. Arrowheads indicate position of the choroid fissure. In all panels dorsal is up and anterior left.

(*nie*)<sup>m743</sup>. In these two mutants the photoreceptor cell layer is absent at 5 dpf while the ganglion and the inner plexiform layers appear to be relatively normal (Fig. 5B,G). Some deficits may also be present in the inner nuclear layer. At 3 dpf some photoreceptor cells can be found in the central retina in both mutants. Fig. 5A shows the appearance of the *mok*<sup>m632</sup> retina at 3 dpf. Many cells in the photoreceptor layer do not have the elongated appearance characteristic of wild-type photoreceptors. The brain shape of both mutants appears to be unaffected (Fig. 4B,C). Numerous cell corpses, predominantly in the photoreceptor cell layer, are present in the retinae of both mutants at 3 dpf.

In 5 dpf retinae of *elipsa* (*eli*)<sup>m649</sup> and *brudas* (*bru*)<sup>m148</sup>, most photoreceptors are missing. In contrast to *mok*<sup>m632</sup> and *nie*<sup>m743</sup>, however, some morphologically normal cells are present in the periphery of the photoreceptor layer (arrowheads in Fig. 5D,H). The onset of the *eli*<sup>m649</sup> phenotype is late relative to other mutations in this group. At 3 dpf the photoreceptor cell layer of *eli*<sup>m649</sup> is relatively normal (Fig. 5C). Excessive cell death is noticeable mainly in the center of the retina (arrowhead in Fig. 5C). The retinal phenotype of *bru*<sup>m148</sup> is much more evident at this stage (Fig. 5H). Most photoreceptors in the *bru*<sup>m148</sup> retina are missing at 3 dpf. Similar to *eli*<sup>m649</sup> at 5 dpf, some photoreceptors persist in the periphery of the *bru*<sup>m148</sup> retina (arrowhead in Fig. 5H). For both loci the retinal abnormalities are associated with other defects. Starting during day 3 of development, *bru*<sup>m148</sup> mutants are characterized by a reduced touch response and a darker pigmentation. *eli*<sup>m649</sup> mutants have curled body axis and pronephric cysts (Iain Drummond, personal communication).

In *krenty* (*krt*)<sup>m699</sup>, *sinusoida* (*sid*)<sup>m604</sup> and *discontinuous* (*dis*)<sup>m704</sup>, the photoreceptor cell layer is characterized by discontinuities (arrowhead in Fig. 5E,F) which are already distinct at 3 dpf. The pattern of photoreceptor deficiencies persists unchanged till 5 dpf (Fig. 5F). In *krt*<sup>m699</sup>, gaps in the photoreceptor cell layer seem to be associated with abnormalities of the inner nuclear layer. On histological sections of wild-type retinae, a subset of cells in the inner nuclear layer (presumptive amacrine cells) is less intensely stained (Fig. 1C). In *krt*<sup>m699</sup> these weakly staining cells are more numerous in the vicinity of the photoreceptor-deficient areas (arrowhead in Fig. 5E). The *krt*<sup>m699</sup> phenotype is variable. In the least affected individuals the photoreceptor cell layer is normal; in the most severe cases the *krt*<sup>m699</sup> phenotype is associated with extensive cell death. The phenotypes of all three mutants involve a slight decrease in brain size, which in *krt*<sup>m699</sup> and *sid*<sup>m604</sup> is already present during day 4 of development. In *dis*<sup>m704</sup> the brain defect is not obvious until day 5.

#### Group IV: mutants with retardation of eye growth

The nine mutations in this category are characterized by reduced eye size (Table 1). Histological sections reveal that all neuronal laminae are present in these mutants at all inspected stages (Fig. 6C, and data not shown), and cell death in excess of wild-type levels is not observed. The most severe phenotype in this group is present in *out of sight* (*out*)<sup>m233</sup> mutants (Fig. 6A,B). Three alleles were found at this locus. The *out*<sup>m233</sup> phenotype is distinguishable during day 2 of development. The other two alleles, *out*<sup>m306</sup> and *out*<sup>m390</sup> are weaker and do not produce any obvious phenotype until 5 dpf. Pigmentation appears to be darker in *out*<sup>m233</sup> than in its wild-type siblings at

5 dpf. From a dorsal view the brain appears narrower in some *out*<sup>m233</sup> individuals. Other organs have no obvious mutant phenotype.

#### Group V: mutants with nonspecific retinal degeneration phenotype

Mutants in this class (Table 1) are characterized by reduced eye size and brain shape defects (Fig. 6D,E,G,H). Histological sections reveal extensive cell death in their retinae. Cell corpses appear as small, round, intensely staining particles (Fig. 6F). Cell death is not localized to any particular cell layer in the retinae of these mutants. The shape and localization of plexiform layers is frequently abnormal and variable (Fig. 6F,I). These patterning defects are most likely due to cell death. In *ziemniok* (*zem*)<sup>m709</sup> the photoreceptor cells appear to be more affected by cell death than other neurons (not shown). All mutations in this category lead to slightly reduced brain size starting at 2 to 3 dpf. The brain abnormalities are most evident in the forebrain and midbrain.

#### Group VI: mutants with retinal degeneration associated with a pigmentation defect

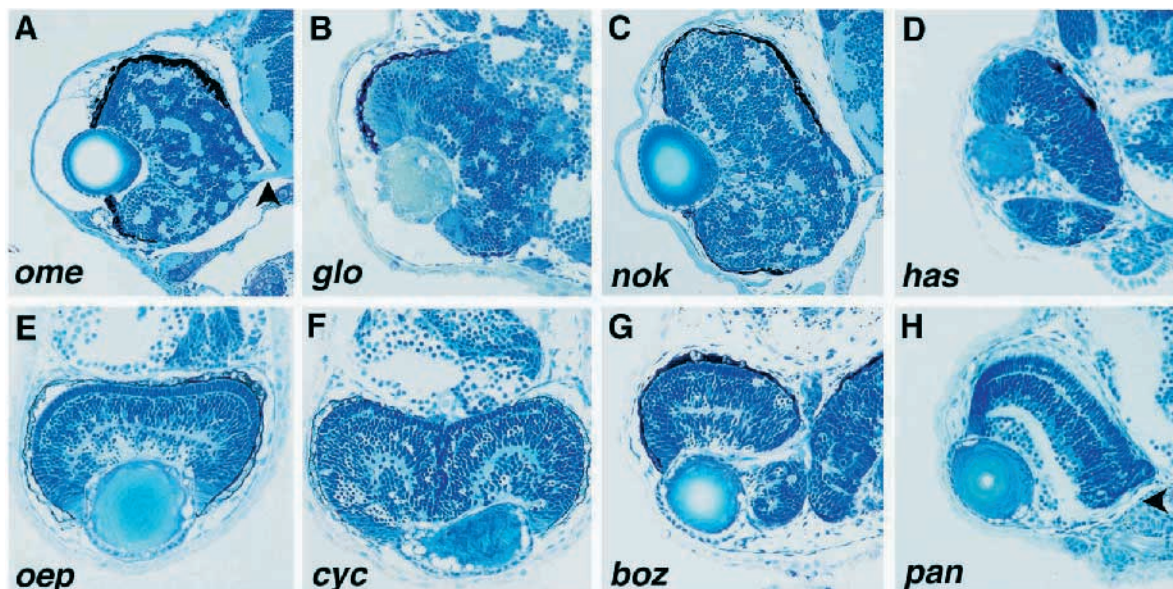
Reduction of eye size and increased amount of cell death in the retina are associated with a general loss of pigmentation in this group of four mutations belonging to three complementation groups (Fig. 7). The pigmentation defect becomes distinct during day 2 of development and the eye size defect during day 4. In all mutants the majority of melanocytes are condensed and round at 5 dpf (Fig. 7B,E,H). Histological sections through the mutant retinae reveal excessive cell death (Fig. 7). The highest concentration of cell corpses is present near the marginal zone of the *piegus* (*pgu*)<sup>m286</sup> retina (arrow in Fig. 7K). In contrast, cell corpses in the *mizerny* (*miz*)<sup>m293</sup> retina are evenly distributed along the neuronal laminae (Fig. 7L). In the most severely affected mutant individuals, gaps appear in the photoreceptor cell layer of *punktata* (*pkt*)<sup>m288</sup> and *miz*<sup>m293</sup>. In *pkt*<sup>m288</sup> at 5 dpf photoreceptors have abnormal appearance and are frequently reduced in number (Fig. 7F). Growth of *pkt*<sup>m288</sup> and *miz*<sup>m293</sup> is slightly retarded at 5 dpf and both mutants have somewhat abnormal brain shape. Ear otoliths are smaller in all mutants belonging to this group (Malicki et al., 1996).

#### Group VII: miscellaneous mutants

The mutation *pandora* (*pan*)<sup>m313</sup> produces a very pleiotropic phenotype. At 24 hpf *pan*<sup>m313</sup> embryos are characterized by abnormal tail, brain and otic vesicle (not shown). Later in development *pan*<sup>m313</sup> mutant embryos are smaller than their wild-type siblings, do not develop body pigmentation, and display heart and circulation defects. At 1 dpf, eyes of mutant embryos appear to have abnormal morphology in the area of the optic stalk. Pigmentation develops only in the dorsal eye (not shown). Sections through the mutant retinae reveal a variable degree of cell death. At 3 dpf the retinal neurons frequently do not form a wild-type, laminar pattern. In the least affected embryos the retinal stratification develops normally (Fig. 3H). In wild-type embryos, the exit point of the optic nerve is located somewhat ventral to the mediolateral axis of the retina (Fig. 1). Interestingly, at 3 dpf in *pan*<sup>m313</sup> the portion of the retina ventral to the optic nerve (arrowhead in Fig. 3H) is missing.

The second locus in the miscellaneous category is *heart and*





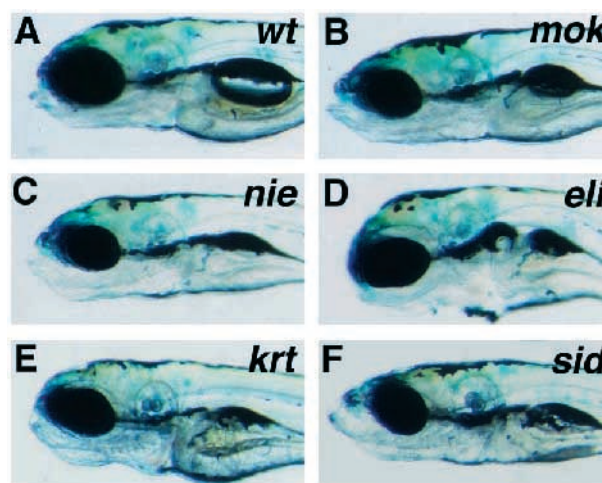
**Fig. 3.** Transverse sections of mutant retinæ at 3 dpf. The phenotypes of (A) *oko meduzy (ome)<sup>m98</sup>*, (B) *glass onion (glo)<sup>m117</sup>* and (C) *nagie oko (nok)<sup>m227</sup>* mutants are similar to each other. In the retinæ of these mutants the plexiform matter forms patches instead of laminae. *ome<sup>m98</sup>* and *nok<sup>m227</sup>* form a well differentiated optic nerves (arrowhead in A). (D) Retina of *heart and soul (has)<sup>m129</sup>* mutant at 60 hpf. Although the optic nerve is present, the retina of *has<sup>m129</sup>* does not develop stratification. (E) *one eyed pinhead (oep)<sup>m134</sup>*, (F) *cyclops (cyc)<sup>b16</sup>* and (G) *bozozok (boz)<sup>m168</sup>* develop cyclopia. In these mutants retinal stratification develops to a limited degree. Abnormalities of the neuronal patterning are usually associated with presence of numerous cell corpses. (H) Some *pandora (pan)<sup>m313</sup>* mutants develop normal neuronal lamination. The portion of the retina ventral to the optic nerve (arrowhead in H) is absent in *pan<sup>m313</sup>*. In all panels ventral is down.

*soul (has)* (Fig. 2D). Similar to mutants in the neuronal patterning class, *has<sup>m129</sup>* has an eye-specific pigmentation defect, abnormal brain shape and a defective heart. *has<sup>m129</sup>* embryos become retarded during day 3 of development. Plexiform layers do not develop in the *has<sup>m129</sup>* retina and we observed numerous cell corpses on sections prepared from 60 hpf embryos (Fig. 3D). Due to the delay in development, it is difficult to assess whether retinal neurons in *has<sup>m129</sup>* are capable of forming normal lamination. Both mutations in this category complement Group I loci.

## DISCUSSION

In a large scale mutagenesis screen we analyzed progeny of over 10,000 crosses between siblings from 1,808 F2 generation families bred from ENU mutagenized males (Driever et al., 1996). We recovered 49 recessive mutations with defects in the retina of the zebrafish eye. Several characteristics of zebrafish made this approach possible. High fecundity allows the efficient search for recessive phenotypes in a single clutch of embryos. Extraterine development enables easy visual inspection of the developing embryos. Small size and relative hardiness allow for maintenance of a large number of lines at a relatively low cost. These characteristics will also prove helpful in further genetic and phenotypic characterization of the identified loci.

The screening procedure used in this study was aimed at the isolation of relatively obvious morphological abnormalities in the developing eye. Minor deficiencies of eye size or shape were probably not recorded. During day 5 of the screening protocol we were searching for abnormal size or shape of the

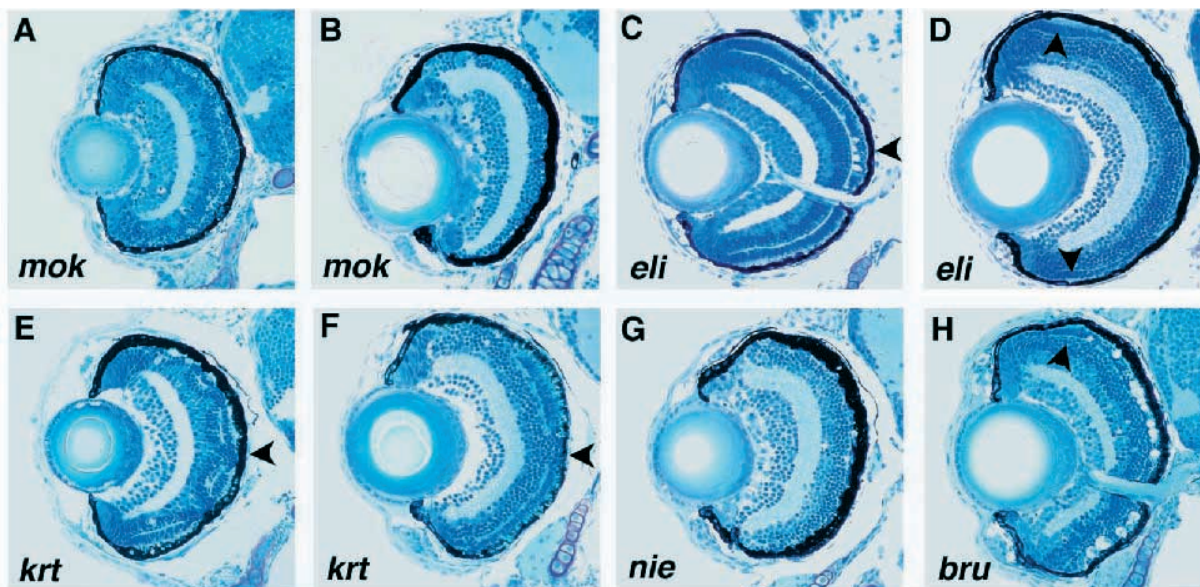


**Fig. 4.** Head and trunk phenotypes of mutations predominantly affecting development of the outer retina. (A) Wild type. (B) *mikre oko (mok)<sup>m632</sup>*. (C) *niezerka (nie)<sup>m743</sup>*. (D) *elipsa (eli)<sup>m649</sup>*. (E) *krenty (krt)<sup>m699</sup>*. (F) *sinusoida (sid)<sup>m604</sup>*. Mutants in B, C, E and F have smaller eyes than the wild type. Eyes of *eli<sup>m649</sup>* have abnormal oval shape (D). The brain shape defect is most pronounced in *krt<sup>m699</sup>*. In all panels anterior is to the left and dorsal is up. All phenotypes were photographed at 5 dpf.

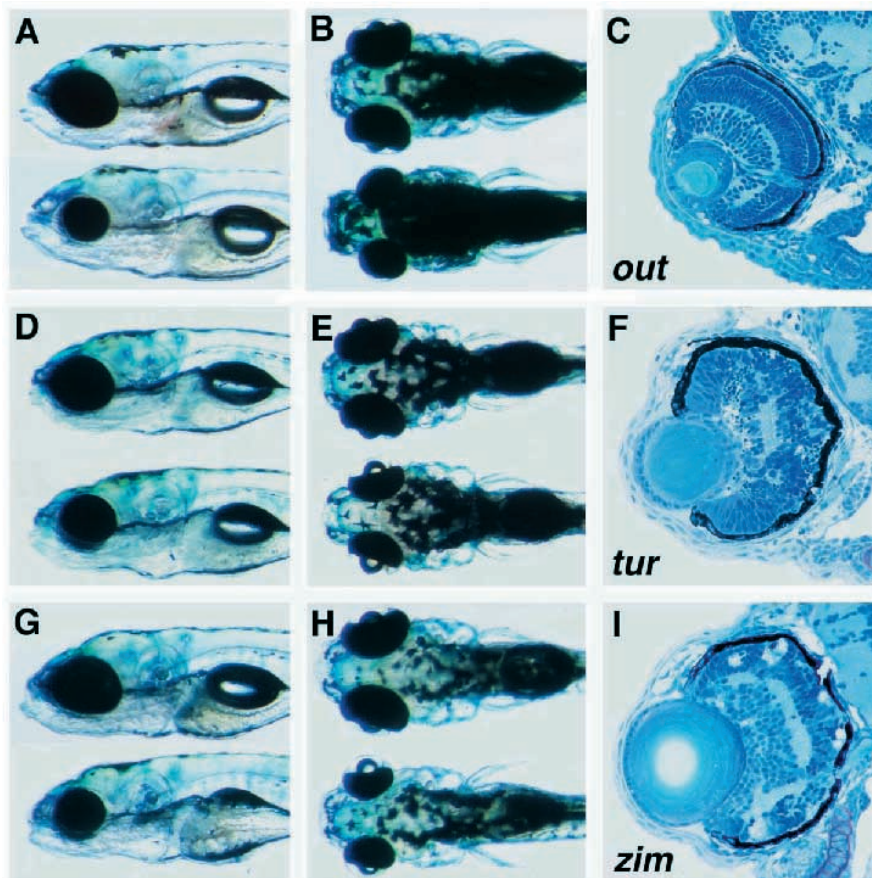
eye. This effort resulted in the isolation of mutations which produce rather drastic deficiencies, mainly in the outer cell layers of the retina. Mutations resulting in minor changes in cell count or survival rates of retinal neurons would not have been isolated in this screen.

Genetic analysis is subject to two general constraints. These





**Fig. 5.** Phenotypes of mutations predominantly affecting development of the outer retina analyzed on histological sections (A) In *mikre oko* (*mok*)<sup>m632</sup>, a poorly differentiated photoreceptor cell layer is present in the central retina at 3 dpf. The presumptive photoreceptor cells frequently do not display the wild-type, elongated shape. (B) At 5 dpf, the photoreceptor cell layer is absent in *mok*<sup>m632</sup>. (C) The *elipsa* (*eli*)<sup>m649</sup> retina at 3 dpf. Excessive cell death is most obvious in the central portion of the photoreceptor cell layer (arrowhead in C). (D) At 5 dpf most of the photoreceptor cells in *eli*<sup>m649</sup> are absent. Some cells survive in the periphery of the photoreceptor cell layer (arrowheads in D). (E) The *krenty* (*krt*)<sup>m699</sup> retina at 3 dpf. The photoreceptor cell layer is discontinuous (arrowhead in E). Gaps in the array of photoreceptor cells appear to be filled with cells originating from the inner nuclear layer. This pattern persists till 5 dpf (F). (G) Retina of *niederzanka* (*nie*)<sup>m743</sup> at 5 dpf. The photoreceptor cell layer is missing. (H) *brudas* (*bru*)<sup>m148</sup> retina at 3 dpf. As in *eli*<sup>m649</sup> some morphologically normal photoreceptors are present in the periphery (arrowhead in H). All sections are transverse. In all panels ventral is down.



**Fig. 6.** Examples of mutant phenotypes from the categories of Growth retardation and Nonspecific retinal degeneration. Mutant individuals (lower) are shown next to their wild-type siblings (upper). (A,B,C) *out of sight* (*out*)<sup>m233</sup>. (D,E,F) *turbulent* (*tub*)<sup>m125</sup>. (G,H,I) *zimny* (*ziny*)<sup>m419</sup>. (A,B) The eye of *out*<sup>m233</sup> is substantially reduced at 3 dpf. (C) All retinal laminae are present and cell death in excess of wild-type levels is not observed in *out*<sup>m233</sup> at this stage. At 5 dpf mutants *tub*<sup>m125</sup> (D,E) and *ziny*<sup>m419</sup> (G,H) are characterized by a reduced eye size and somewhat abnormal brain shape. Cell death is extensive in both *tub*<sup>m125</sup> (F) and *ziny*<sup>m419</sup> (I) retinæ at 3 dpf. Cell corpses appear as small, round, intensely staining particles (F). Retinal patterning defects are inconsistent in this group of mutants. A, D and G show lateral views; B, E and H dorsal views. The dorsal side is oriented up in panels showing lateral views or sections. In panels showing head phenotypes anterior is left. All sections are transverse.

are redundancy and pleiotropy of function at some genetic loci. The second constraint is particularly relevant in the analysis of eye development, which takes place relatively late in ontogenesis. Late functions of pleiotropic loci are frequently obscured by an early function. Numerous examples of this are known from invertebrates (Simon et al., 1991; Cheyette et al., 1994; Pan and Rubin, 1995). Clearly, for this reason, some pleiotropic loci important to retinal development may have been discarded as not sufficiently specific, or included in phenotypic categories not obviously related to eye development (Abdelilah et al., 1996; Schier et al., 1996; Solnica-Krezel et al., 1996). In invertebrates, late functions of such loci are routinely studied in mosaic animals (Simon et al., 1991; Xu and Rubin, 1993; Pan and Rubin, 1995). Mosaic analysis is well established for zebrafish (Ho and Kane, 1990) and will undoubtedly prove useful in future studies of selected mutants.

The degree of saturation achieved in a genetic screen can be estimated from the average number of alleles recovered at each locus. In the group of mutants affecting eye development we isolated an average of 1.4 alleles per locus. Approximately three quarters of the loci included in complementation testing are represented by a single allele (Table 1). This indicates a low degree of saturation and implies that many additional loci with function in eye development could be recovered by future morphological screening.

The mutants presented here are relevant to several aspects of central nervous system development. One of the major questions concerning formation of the retinal pattern is how particular cell types are able to find their final, proper positions within the neuronal layers. Three zebrafish mutants, *oko meduzy*<sup>m98</sup>, *nagie oko*<sup>m227</sup> and *glass onion*<sup>m117</sup>, cause loss of neuronal stratification in the retina. Presence of well developed plexiform patches and lack of extensive cell death in *ome*<sup>m98</sup> suggests that the retinal neurons in this mutant differentiate but are unable to form neuronal laminae. The phenotypes of *ome*<sup>m98</sup> and *nok*<sup>m227</sup> are very similar and largely confined to the retina, suggesting that these loci may belong to a single genetic pathway. On the other hand, *glo*<sup>m117</sup>, produces an earlier phenotype and affects the entire neural tube. The product of the *glo* locus is thus more likely to play a general role in the development of neuroepithelium.

Many structures of the vertebrate brain consist of several neural cell types organized into distinct laminae (Jacobson, 1991). Some interesting insights into the formation of neuronal lamination came from studies of mouse mutants (Caviness and Rakic, 1978). Studies of two loci have been particularly informative. The *reeler* (*rl*) mutation causes extensive disorganization of neuronal laminae in both cerebral and cerebellar cortex of the mouse. The *reeler* locus has been recently shown to encode an extracellular matrix protein with a homology to F-spondin (D'Arcangelo et al., 1995). Another mutation, *weaver* (*wv*), produces degeneration of granule cells prior to their migration into the appropriate cell layer in mouse cerebellum (Caviness and Rakic, 1978). This phenotype appears to be associated with a defect in a homotypic signaling event among precursors of the granule cells (Gao et al., 1992; Gao and Hatten, 1993). Further study of the neuronal patterning loci *ome*, *nok* and *glo* may lead to identification of novel patterning mechanisms relevant to the neuronal lamination of the retina and, possibly, other regions of the vertebrate brain.

Mutations producing defects in the outer retina are relevant

to the issue of cell fate acquisition in the central nervous system. Only preliminary information is available concerning factors involved in neuronal specification and differentiation in the vertebrate retina (Watanabe and Raff, 1990; Pittack et al., 1991; Altshuler et al., 1993; Kelley et al., 1994). The phenotypes of the mutants *mikre oko*<sup>m632</sup>, *niezerka*<sup>m743</sup>, *krenty*<sup>m699</sup>, *sinusoida*<sup>m604</sup> and some others may be associated with defective specification of the photoreceptor cells. Alternatively, they may involve deficiencies of factors necessary for photoreceptor survival. It seems likely that at least some mutants in this category also affect cells of the inner nuclear layer.

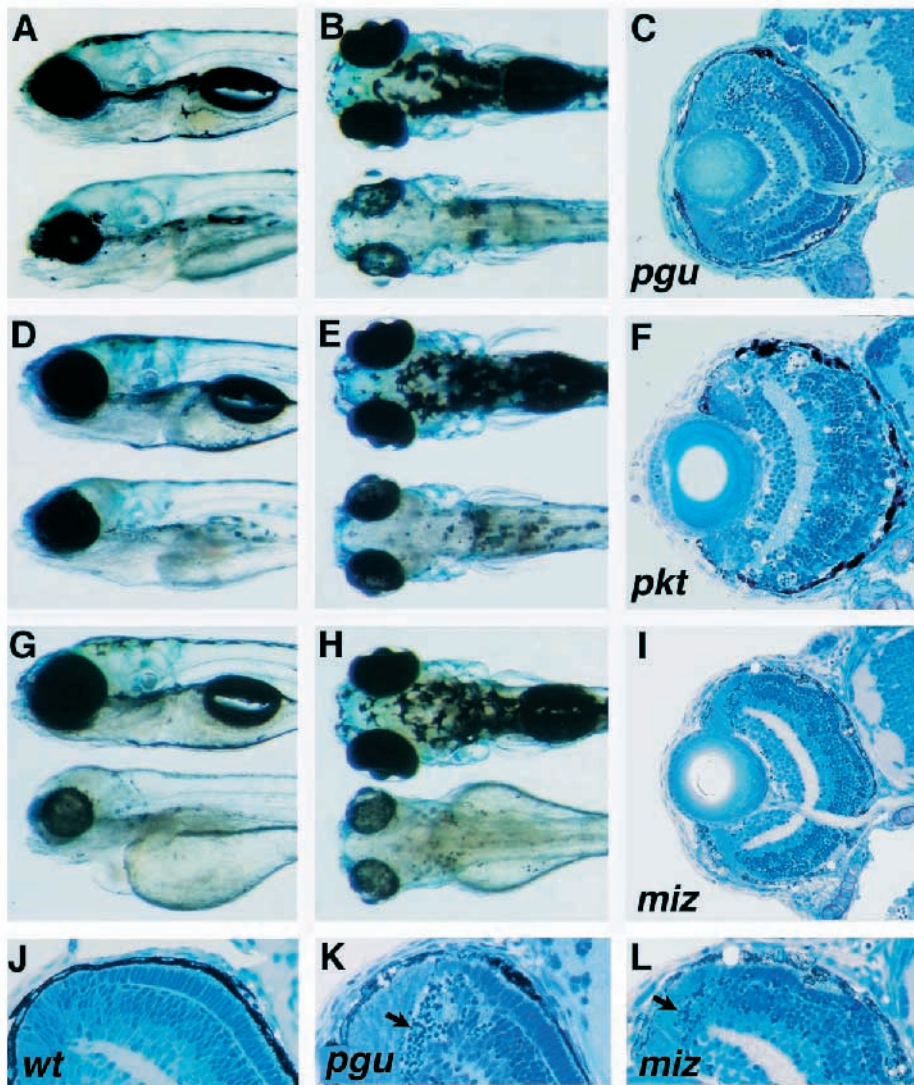
Interestingly, defects in the outer retina involve three different patterns of photoreceptor loss. In *mok*<sup>m632</sup> and *nie*<sup>m743</sup> the photoreceptor cells are missing in both the central and the peripheral retina. A different pattern is seen in *kr*<sup>m699</sup>, *sid*<sup>m604</sup> and *dis*<sup>m704</sup>. In these mutants, photoreceptor cells differentiate in patches along the ventricular margin of the retina. The mutations *elipsa*<sup>m649</sup> and *brudas*<sup>m148</sup> display the third pattern in which the defect is localized in the center of the retina, while some peripheral photoreceptors remain, at least temporarily, unaffected.

Mutations in genes of the phototransduction cascade have been shown to cause loss of photoreceptor cells (Bowes et al., 1990; Dryja et al., 1990). In mice, mutations of this type lead to late onset phenotypes (Chang et al., 1993). In the marginal zone of the teleost retina neurons continue to be generated throughout the lifetime (Raymond, 1991). One would expect that mutations in the phototransduction cascade loci should primarily affect differentiated photoreceptor cells of the central retina while having little impact on newly born, peripheral cells. Two mutants, *eli*<sup>m649</sup> and *bru*<sup>m148</sup>, indeed display such a pattern. Unlike the phototransduction cascade mutants known in the mouse, both of them also produce strong phenotypes outside the visual system (Table 1).

A common human inherited retinopathy, retinitis pigmentosa, involves photoreceptor degeneration. The genetic causes of this disorder are very diverse. It is estimated that mutations in 50 to 100 loci are capable of causing this disorder (Dryja and Berson, 1995). Only a small fraction of them has been identified to date (Dryja et al., 1990; McLaughlin et al., 1993; Kajiwarra et al., 1994). Further studies of the genetic defects included in group III may shed light on other causes of this disease.

Growth of the retina appears to be a precisely regulated process. The factors that play a role in this regulation are currently poorly understood. The mutation *out of sight* (*out*)<sup>m233</sup> as well as others in its group may provide an entry point to the study of this issue. *eyeless-1,-2*, (*ey-1*, *ey-2*), *ocular retardation* (*or*), *fidget* (*fi*), *Small eye* (*Sey*) and a few other mouse mutants produce microphthalmia or anophthalmia (Green, 1990). Their phenotypes are different from *out*<sup>m233</sup> and related mutants. For example, *Sey* is also involved in development of the nasal pits (Hogan et al., 1986). The optic nerve of *or*<sup>J</sup> does not exit the optic cup (Silver and Robb, 1979). In addition to the eye defect, *fidget* mice do not form semicircular canals in the otic vesicle (Truslove, 1956). Such defects are not observed in *out*<sup>m233</sup> or related mutants. The *fi* retina is properly stratified. The reduction of eye size in this mutant has been shown to involve prolongation of the cell cycle in the retinal anlage (Konyukhov and Sazhina, 1976).





**Fig. 7.** Mutants involving retinal degeneration associated with a general pigmentation defect. Mutant individuals (lower) are shown next to their wild-type siblings (upper). (A,B,C) *piegus(pgu)<sup>m286</sup>*. (D,E,F) *punktata(pkt)<sup>m288</sup>*. (G,H,I) *mizerny(miz)<sup>m293</sup>*. All three mutants have abnormal melanocytes and reduced eye size. Sections through mutant retinæ at 3 dpf (C,I) and 5 dpf (F) reveal an excessive amount of cell death. (J,K,L) Higher magnification of the dorsal retina in the wild type (J), *pgu<sup>m286</sup>* (K) and *miz<sup>m293</sup>* (L). Cell corpses appear as small, round, intensely staining particles (arrows in K and L). In *pkt<sup>m288</sup>* and *miz<sup>m293</sup>* the photoreceptor cell layer has abnormal appearance (F,I). A, D and G show lateral views; B, E and H dorsal views. The dorsal side is oriented up in panels showing lateral views or sections. In panels showing head phenotypes anterior is left. All sections are transverse.

Mutations included in Group VI are reminiscent of the *microphthalmia (mi)* phenotype characterized in the mouse (Hertwig, 1942). Similarly to the zebrafish mutants *piegus<sup>m286</sup>*, *punktata<sup>m288</sup>* and *mizerny<sup>m293</sup>*, mouse mutations in the *mi* locus affect eye size, ocular pigmentation and differentiation of melanocytes. Molecular characterization of the mouse locus revealed that it encodes a putative transcription factor expressed in the pigmented epithelium (Hodgkinson et al., 1993). Given the phenotypic similarity, it is possible that one of the mutations included in this group affects the zebrafish homolog of the *microphthalmia* gene. The human auditory-pigmentary syndrome, Waardenburg syndrome type 2, is caused by mutations in the *MITF* gene, the homologue of *microphthalmia* (Tassabehji et al., 1994). Further studies of the zebrafish mutants that combine eye and pigmentation defects may prove valuable to understanding of this type of human disorders. The defect of the photoreceptor cell layer in *punktata<sup>m288</sup>* may have its primary causes in the pigmented epithelium, which appears to be abnormal in this mutant. The development of the photoreceptor outer segments is known to depend on interaction with the pigmented epithelium, (Hollyfield and Witkovsky, 1974). *pkt<sup>m288</sup>* and the related mutants

may thus reveal novel aspects of interaction between photoreceptor cells and the pigmented epithelium.

The mutation *pandora<sup>m313</sup>* appears to be relevant to yet another aspect of eye development. The dorsoventral polarity of the vertebrate eye is evident by both morphological and molecular criteria (Constantine-Paton et al., 1986; McCaffery et al., 1991; Schmitt and Dowling, 1994). *pan<sup>m313</sup>* causes absence of the ventral retina at 3 dpf. This phenotype suggests that the *pandora* locus may play a role in the specification of the dorsoventral axis in the zebrafish eye. Retinoic acid has recently been shown to influence the ventral cell fates in the zebrafish optic cup (Marsh-Armstrong et al., 1994). It is noteworthy that the eye defect produced by citral, an inhibitor of retinoic acid synthesis, is strikingly similar to the *pan<sup>m313</sup>* phenotype (Marsh-Armstrong et al., 1994).

Alternative screening methods for loci participating in the zebrafish eye development are conceivable. A screening procedure for retinal defects based on a functional test is available in zebrafish (Clark, 1981; Brockerhoff et al., 1995). It takes advantage of the optokinetic response, which in zebrafish appears early (4 dpf) in development (Clark, 1981). The assumption of this approach is that fish larvae with retinal

defects will not display a normal optokinetic response. A screen based on the optokinetic response can potentially detect mutants with minor developmental abnormalities, which do not result in obvious morphological changes of the eye. Similarly, mutations producing abnormal physiological characteristics of the retina can be recovered in such a screen (Brockerhoff et al., 1995). The disadvantage of screening procedures based on the optokinetic response is that they are substantially more laborious than screens based on morphological criteria.

Our studies demonstrate that zebrafish provide a powerful genetic system to study various aspects of vertebrate eye development, including: specification of the eye primordium, neuronal lamination, specification/differentiation of distinct neuronal classes and possibly the specification of axes. A detailed analysis of the identified mutants will enhance our understanding of vertebrate central nervous system development.

We thank Colleen Boggs, Jane Belak, Ioannis Batjakas, Heather Goldsboro, Lisa Anderson, Snorri Gunnarson and Pamela Cohen for technical help during the various stages of the screen. We are also grateful to Eliza Mountcastle-Shah for critical reading of the manuscript and Lisa Anderson for help with naming mutants. This work was supported in part by NIH RO1-HD29761 and a sponsored research agreement with Bristol Myers-Squibb (to W. D.). Further support in the form of fellowships came from HFSP and the Fullbright Program (to Z. R.), EMBO and Swiss National Fond (to A. S.), Helen Hay Whitney Foundation (to D. L. S. and D. Y. S.), and the Damon Runyon-Walter Winchell Cancer Research Fund (to J. M.).

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(Accepted 8 January 1996)