A Mutation of Early Photoreceptor Development, *mikre oko*, Reveals Cell–Cell Interactions Involved in the Survival and Differentiation of Zebrafish Photoreceptors

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To gain insight into mechanisms involved in photoreceptor development, we characterized a zebrafish mutation in the *mikre oko* locus that produces early loss of photoreceptor cells. *mikre oko* photoreceptors lose their elongated morphology at the time of wild-type outer segment formation and undergo cell death within a few days. To investigate whether this phenotype involves cell–cell interaction defects, we performed analysis of genetically mosaic animals. Interactions of *mikre oko* photoreceptors with wild-type cells rescue several aspects of the mutant phenotype. When placed in a wild-type environment, *mikre oko* photoreceptor cells retain elongated morphology and survive longer. Moreover, although mutant *mikre oko* photoreceptor outer segments develop only infrequently and are usually

disorganized, *mikre oko* cone and rod cells in mosaic retinas develop robust outer segments that closely resemble the wild type. In contrast to the outer segments, the proximal regions of *mikre oko* photoreceptor cells, including their inner segments, the nuclear regions, and the synaptic termini, retain the mutant appearance. *mikre oko* outer segment rescue is not mediated by interactions with the retinal pigment epithelium. These studies demonstrate that the differentiation of outer segments is surprisingly independent from the more proximal photoreceptor cell features and that outer segment development includes retinal pigment epithelium-independent cell–cell interactions.

Key words: retina; photoreceptor; outer segment; cell–cell signaling; genetics; zebrafish

The vertebrate neural retina is an evolutionarily highly conserved sensory organ that develops from a single neuroepithelial sheet into a complex, laminar structure comprising primarily six neuronal and one glial cell type (Ramon y Cajal, 1893; Rodieck, 1973; Dowling, 1987). Within the outer retina are found the photoreceptors whose outer segments contain visual pigments and other components of the signal transduction mechanism responsible for the detection of light. In outer segments, the visual signal is converted into an electrical impulse, which in turn is processed by horizontal, bipolar, and amacrine cells of the inner nuclear layer (INL), and relayed to the brain by the neurons of the ganglion cell layer (GCL). This diverse collection of neurons is produced during zebrafish embryogenesis in a narrow window of ~2 d, between ~28 and ~72 hr postfertilization (hpf). In the zebrafish retina, as in all vertebrates, the ganglion cells are the first to differentiate, becoming postmitotic starting at 28 hpf. INL cells follow, starting at 38 hpf, and the first photoreceptors become postmitotic at ~43 hpf (Nawrocki, 1985; Burrill et al., 1995; Hu and Easter, 1999). By 72 hpf, almost all neurons in the central retina are postmitotic, and lamination is well developed (Schmitt and Dowling, 1999). Neurogenesis also continues at

later stages but is confined to a relatively small group of cells at the retinal margin and in the outer retina (Marcus et al., 1999). The appearance of optokinetic and visual startle responses is the final indication that the central retina is functional by ~80 hpf (Easter and Nicola, 1996).

The genetic mechanisms that are involved in vertebrate photoreceptor development are largely unknown. Current models propose that both environmental cues and the ability of pluripotent progenitor cells to respond to them change over time, resulting in an ordered appearance of different retinal cell types (Cepko et al., 1996). A number of transcription factors, hormones, and growth factors are found within the developing retina, some of which have been shown to be involved in photoreceptor specification (for review, see Cepko et al., 1996; Freund et al., 1996; Harris, 1997; Livesey and Cepko, 2001). For instance, retinoic acid (RA) is asymmetrically distributed within the developing vertebrate retina and thought to play a role in dorsoventral patterning. Additionally, RA may be involved in photoreceptor specification, because the application of exogenous RA in both zebrafish and rat promotes rod development at the expense of cones and amacrine cells (Hyatt et al., 1996; Kelley et al., 1999). Tissue culture studies implicate CNTF, LIF, FGFs, and sonic hedgehog among others, in directing photoreceptor specification and differentiation (Ezzeddine et al., 1997; Levine et al., 1997; Neophytou et al., 1997; McFarlane et al., 1998). Several transcription factors have also been suggested to regulate vertebrate photoreceptor development. These include *crx*, *nrl*, and *not really finished* (*nrf*) (Rehmtulla et al., 1996; Chen et al., 1997; Furukawa et al., 1997; Becker et al., 1998). Although evidence for the involvement of some of these factors in photoreceptor development is compelling, it is fair to state that we are only beginning to understand the genetic mechanisms that are involved in the specification and the differentiation of this cell type.

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Cell–cell interactions have been demonstrated to play important roles in CNS development. Analysis of genetically mosaic animals containing intermingled wild-type and mutant cell clones provides a productive approach to search for evidence of cell–cell signaling events. One of the first studies of this type in the retina demonstrated that the photoreceptor degeneration phenotype in the RCS rat originates in the retinal pigment epithelium (RPE) (Mullen and LaVail, 1976). More recently, studies of retinal mosaic for a dominant rod opsin mutation (substitution of Pro347 to Ser) and a recessive *retinal degeneration slow* (*rds*) defect revealed other, RPE-independent types of cell–cell interactions involved in the survival of photoreceptor cells (Huang et al., 1993; Kedzierski et al., 1998). Because both rod opsin and peripherin genes are specifically expressed in photoreceptor cells, these experiments suggest that cell–cell interactions occur within the photoreceptor cell layer (PRCL). This is supported further by the studies of human photoreceptor degeneration. Although rod opsin is specifically expressed in rod photoreceptor cells, mutations in this gene in the human retina lead to a loss of both rods and cones (Dryja et al., 1990; Li et al., 1994). Mosaic analysis has also been applied to the search for cell–cell interactions in the zebrafish (Ho and Kane, 1990; Halpern et al., 1993; Malicki and Driever, 1999). Given the abundance of photoreceptor mutants, mosaic studies in this species show a great potential to further identify cell–cell interactions in the development of vertebrate photoreceptors.

The zebrafish has been recently established as a genetic model of vertebrate eye development (for review, see Malicki, 2000a,b). Genetic screens in zebrafish have identified numerous loci that are involved in photoreceptor development (Malicki et al., 1996; Fadool et al., 1997; Becker et al., 1998; Brockerhoff et al., 1998; Neuhauss et al., 1999). To gain insight into cell–cell interactions that are involved in the development of this cell type, we chose to study one of the earliest defects of photoreceptor development thus far identified in zebrafish, *mikre oko* (*mok*). *mok* mutant fish are characterized by an early loss of the characteristic elongated photoreceptor morphology that coincides with the first stages of outer segment formation. This phenotype suggests that *mikre oko* plays a role in the early steps of photoreceptor development. *mikre oko* mutant animals display almost complete lack of photoreceptor outer segments and synaptic termini. To investigate whether this mutant phenotype involves defective cell–cell interaction events, we constructed genetically mosaic animals. Here, we show that cell–cell interactions that are involved in the photoreceptor development are far more potent than previously expected, influencing both the survival and the differentiation of mutant cells. Remarkably, they are able to entirely rescue the development of photoreceptor outer segments in a mutant that almost completely lacks these structures. The rescue of the outer segment phenotype is not mediated by the retinal pigment epithelium. Although the development of outer segments is frequently rescued to its wild-type form, the more proximal elements of photoreceptor morphology, i.e., the inner segment, the nuclear region, and the synaptic terminus, retain a mutant phenotype. These observations demonstrate that the differentiation of photoreceptor outer segments is surprisingly independent from the more proximal features of photoreceptor structure and involves RPE-independent cell–cell interactions.

MATERIALS AND METHODS

Animals. Zebrafish (*Danio rerio*) were kept on a 14/10 hr light/dark cycle according to standard procedures (Westerfield, 1994). Embryos were

collected from pairwise matings and raised at 28.5°C. Mutations under investigation were maintained in either AB, Tübingen, India, or WIK genetic backgrounds. To determine their genotype, embryos were inspected for morphological defects under a dissecting microscope at 3–5 days postfertilization (dpf) as previously described (Malicki et al., 1996).

Histology. Mutant and wild-type siblings were collected at appropriate time points, fixed in 4% (w/v) paraformaldehyde in PBST (PBS, 0.1% Tween 20) for 2 hr at room temperature or overnight at 4°C, washed in PBST for 10 min, and dehydrated in 10 min washes with a graded ethanol series (30, 50, 75, 85, and 95%, and two times 100%). After overnight infiltration in JB-4 resin (Polysciences, Warrington, PA), embryos were embedded according to manufacturer's directions, sectioned at 3 μ m, and dried on a hotplate. Sections were collected on Superfrost Plus slides (Fisher Scientific, Houston, TX), stained with methylene blue-azure II for 10 sec (Humphrey and Pittman, 1974), rinsed in water for 10 min, and mounted in Permount (Fisher Scientific). Sections were analyzed with a Zeiss Axioscope microscope using differential interference (DIC) optics, and images were recorded using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI) and Photoshop software (Adobe Systems, San Jose, CA).

Electron microscopy. Embryos were fixed in 4% (w/v) paraformaldehyde/2% glutaraldehyde in 75 mM phosphate buffer, pH 7.2, for 2 hr on ice, decapitated with a razor blade, and post-fixed with 2% osmium tetroxide in 50 mM phosphate buffer, pH 7.2. After being washed in 50 mM maleate buffer, pH 5.9, for 15 min on ice, specimens were stained with 2% uranyl acetate in maleate buffer for 15 min on ice. Then, specimens were dehydrated in 10 min steps through a graded ethanol series as above, infiltrated in a graded Epon–propylene oxide series for several hours to overnight, and embedded in Epon (Polysciences). After thin sectioning, sections were transferred to formvar-coated grids (Fisher Scientific) and poststained with lead citrate. Analysis was performed using Phillips EM410 and CM10 electron microscopes.

Immunohistochemistry. Embryos were fixed in 4% (w/v) paraformaldehyde, washed in PBS, infiltrated in 30% sucrose in PBS, and embedded using TBS freezing medium (Triangle Biomedical Sciences, Durham, NC). Cryosections (20 μ m) were heated to 80°C for 2 min, air-dried for 60 min, washed in PBS, and blocked for 1 hr with 10% goat serum in PBD (0.5% Triton X-100, 0.1% Tween 20, 1% DMSO in PBS). Sections were incubated in primary antibody in block solution for 2–5 hr at room temperature or overnight at 4°C. The following antibodies and dilutions were used: Fret 43 (1:100; University of Oregon Stock Center, Eugene, OR), Ret 11 (1:200; University of Oregon Stock Center), Zn8 (1:25; University of Oregon Stock Center), anti-rhodopsin (1:1000), anti-UV opsin (1:1000), anti-green opsin (1:500), anti-red and anti-blue opsin (1:200; gifts of Tom Vihitelic and David Hyde, University of Notre Dame), and anti-carbonic anhydrase II (1:250; gift from Paul Linser, Whitney Laboratory, St. Augustine, FL). After being washed three times in PBD, sections were exposed to the appropriate combinations of FITC-, Cy3-, or Cy5-conjugated secondary antibodies (1:500; Jackson ImmunoResearch, West Grove, PA) for 1 hr, washed three times in PBD, mounted in 50% glycerol, 2% propyl gallate (Sigma, St. Louis, MO) in 0.2 M Tris-HCl, pH 8.0, and analyzed using a Leica TCS 4D confocal microscope. Z-series of mosaic clones (1 μ m) were recorded and analyzed using Scanware 5.1 (Leica, Nussloch, Germany) and Photoshop software.

Quantitation of cell survival. Embryos were fixed in 4% (w/v) paraformaldehyde in PBST for 2 hr at room temperature or overnight at 4°C and decapitated. Embryonic heads were washed in PBST and water for 10 min each and treated with prechilled acetone for 5 min at –20°C. Subsequently, embryos were washed once in water and then in PBST for 5 min each, incubated with 200 μ g/ml collagenase (C0773; Sigma) in PBST for 30 min at room temperature, fixed again (10 min, 4% PFA at room temperature), and washed three times for 5 min each in PBST. After being blocked with 10% goat serum in PBST for 1 hr at room temperature, heads were incubated overnight at 4°C using opsin or Fret 43 antisera at the dilutions specified above. Washing and secondary antibody detection were performed as described above with the appropriate fluorophores. To evaluate the distribution and intensity of staining, specimens were dehydrated, infiltrated, embedded in JB-4 resin, and sectioned at 3 μ m as described above, with the following modifications: the percentage of JB-4 catalyst that was added was decreased to 0.7%, and sections were air-dried and shielded from light to protect fluorophore stability. Sections were immersed for 15 min in 0.2 μ g/ml Hoechst 33258 (Molecular Probes, Eugene, OR) in PBD, washed three times for 5 min each with water, and mounted in glycerol–propyl gallate as

Figure 1. Histological analysis of *mikre oko* development. Nomarski images of transverse sections through central retinas at or near the optic nerve at indicated time points. Retinas in C–F have been treated with PTU to inhibit pigment granule formation in the RPE. A, C, Wild-type retinas display a continuous, uniform layer of elongated photoreceptor cells at both 3 and 5 dpf (arrowheads). B, *mok* retinas at 3 dpf lack a distinct PRCL. Although elongated cells are sometimes seen in the central retina (arrowhead), cells along the periphery appear rounded or dead (arrows). Horizontal cells are not distinguishable. D, By 5 dpf, all surviving *mok* PRCL cells have adopted a rounded morphology (arrows). E, F, Enlargements of the central retina seen in C and D, close to the optic nerve. E, In wild-type retinas, tangentially elongated horizontal cells, radially elongated photoreceptor cell bodies, and the outer segments (arrows) are clearly visible. F, In *mok* retinas, on the other hand, few elongated nuclei (arrowheads) and no outer segment outlines are apparent. In A–D, dorsal is upward, RPE is right. In E and F, dorsal is left, RPE is upward. * indicates the optic nerve. rpe, Retinal pigment epithelium; prcl, photoreceptor cell layer; inl, inner nuclear layer; opl, outer plexiform layer; ipl, inner plexiform layer; gcl, ganglion cell layer; d, dpf. Scale bar: A–D, 100 μm; E, F, 20 μm.

described above. Alternatively, frozen sections were stained with Fret 43 and anti-opsin antibodies as described above. Nuclear staining was observed on a Zeiss Axioscope microscope equipped with a mercury arc lamp and recorded using a SPOT digital camera as described above. Photoreceptor-specific staining was analyzed by confocal microscopy as described above.

Mosaic analysis. Blastomere transplantations were performed as previously described (Ho and Kane, 1990; Westerfield, 1994; Malicki and Driever, 1999). Donor embryos were injected at the two to eight cell stage with a 2.5% mixture of biotin- and Texas red-conjugated dextrans in a 9:1 ratio (Molecular Probes). At late blastula stage, 5–50 cells were removed from a labeled donor with a glass pipette and transferred to an unlabeled host. Incorporation of donor cells into the retinal neuroepithelial layer was scored at 24 hr by immunofluorescence, and positive donor and host embryos were analyzed at time points ranging from 3 to 7 dpf.

Donor-derived cells were detected using either HRP staining or immunofluorescence. For HRP detection, donor and host embryos were fixed in 4% (w/v) paraformaldehyde in PBST for 2 hr at room temperature or overnight at 4°C, washed in PBST and water for 10 min each, and treated with prechilled –20°C acetone for 5 min. Subsequently, embryos were washed once in water and PBST for 5 min each and incubated with 100 μg/ml collagenase in PBST for 30 min at room temperature. After a 5 min wash in PBST and blocking with 10% goat serum in PBST for 1 hr at room temperature, dextran tracer was detected using 2% (v/v) avidin–biotin HRP complex in block solution for 1 hr at room temperature, according to manufacturers' instructions (Vector Laboratories, Burlingame, CA). Signal was visualized using diaminobenzidine (Sigma) as a substrate. Stained embryos were mounted in JB-4 resin, sectioned, and analyzed by light microscopy as above.

Alternatively, host embryos were cryosectioned and processed for Fret43, Ret11, or anti-carbonic anhydrase immunofluorescence as described above, with the additional modification that dextran tracer signal was detected using Cy3-conjugated streptavidin (1:500; Jackson ImmunoResearch) during the secondary antibody step. Confocal analysis of mosaic clones was performed as above.

RESULTS

mikre oko affects early stages of photoreceptor development

In the vertebrate eye, photoreceptor cells form a layer of radially elongated cells that are adjacent to the retinal pigment epithelium and separated from the inner nuclear layer by a synaptic outer plexiform layer (OPL). The photoreceptor cell layer is readily apparent in histological sections of wild-type zebrafish retinas at 3 and 5 dpf (Fig. 1A,C,E). Histological analysis of *mikre oko* retinas demonstrates a severe defect of the PRCL already at 3 dpf. Fewer elongated cells are seen in the periphery of the retina at 3 dpf (Fig. 1B), as compared with a wild-type PRCL at equivalent stage. At 3 dpf, there is still some evidence of a discontinuous OPL, and a number of morphologically distinguishable photoreceptor cells are present in the center of the retina of most animals (Fig. 1B, arrowhead; data not shown). Although most of the inner retina appears relatively normal, morphologically distinguishable horizontal cells are largely absent. Additionally, there appears to

Figure 2. Pattern and time course of *mikre oko* photoreceptor loss. Confocal images of transverse retinal sections near the optic nerve immunostained with Fret 43 antibody for double cones (green) and with anti-carbonic anhydrase for Mueller glia (red) at indicated time points. *A, C, E, G*, Wild-type retinas exhibit a uniform photoreceptor staining throughout the PRCL at all stages. Mueller glia extend radial processes spanning the entire thickness of the retina. *B*, The *mok* PRCL already displays photoreceptor loss in the periphery at 3 dpf (arrows), whereas some elongated cells persist in the central PRCL (arrowhead). Mueller glia also appear fewer in number, and their processes are absent or disorganized. *D, F*, Photoreceptor loss increases in severity (arrows), and all cells adopt a rounded shape by 4 dpf. Glial staining is decreased. *H*, By 7 dpf, Fret 43 immunoreactivity frequently is absent in *mok*, and few carbonic anhydrase-positive cells can be found. Dorsal is upward, and RPE is right in all panels. * indicates optic nerve. *d*, dpf. Scale bar, 100 μ m.

Table 1. Cell survival in *mikre oko* retinas

	Nuclear counts				Photoreceptor-specific markers				
	GCL	INL	PRCL	Total	Fret43	Blue	Rhod	UV	Total
7dpf <i>wt</i>	170 $\pm$ 14	542 $\pm$ 15	169 $\pm$ 4	881 $\pm$ 30	80.1 $\pm$ 1.4	34.4 $\pm$ 2.1	41.6 $\pm$ 2.1	43.2 $\pm$ 2.4	199.3 $\pm$ 8.0
7dpf <i>mok</i>	154 $\pm$ 11	416 $\pm$ 12	0	570 $\pm$ 21	0.6 $\pm$ 0.4	2.2 $\pm$ 0.7	2.6 $\pm$ 0.8	0.2 $\pm$ 0.2	5.6 $\pm$ 2.1

Quantitation of retinal cell type survival. Nuclear counts: cells were visualized in transverse sections by Hoechst nuclear staining and identified by laminar position and nuclear morphology at 7 dpf. Significant cell loss is observed in the *mikre oko* PRCL and INL, whereas the GCL appears relatively normal. Total, Total number of retinal cells counted; GCL, number of cells in ganglion cell layer; INL, number of cells in inner nuclear layer; PRCL, number of cells in PRCL as scored by nuclear morphology. Photoreceptor-specific markers: photoreceptor cell survival was assessed in transverse sections with molecular markers specific to photoreceptor subtypes. Severe cell loss appears to be fairly evenly distributed among all photoreceptor types. Total, Total number of photoreceptor cells detected by immunostaining with Fret 43, anti-blue opsin, anti-rod opsin, and anti-UV opsin antibodies (see Materials and Methods). Average of five or more counts is provided for each data point.

be an increased number of densely staining pyknotic nuclei throughout both the INL and the outer retina (data not shown). By 5 dpf, the central retina of *mok* no longer contains any morphologically distinguishable photoreceptors (Fig. 1*D,F*). Although no morphological defects outside the retina are obvious in *mok*, mutant larvae do not survive past 10 dpf.

To precisely determine the timing of photoreceptor loss, a time course of immunohistochemical staining was performed with Fret 43, an antibody specific for double cones, the most prevalent photoreceptor subtype in early larval retinas (Larison and Bremiller, 1990). In wild-type retina, the distribution of red and green double cones is uniform throughout the wild-type PRCL from 3 to 7 dpf (Fig. 2*A,C,E,G*). In contrast, staining in *mok* is already less consistent beginning at 3 dpf; notable specifically is the loss of signal in the peripheral retina (Fig. 2*B*, arrows). Subsequently, at 4–5 dpf, photoreceptor loss progresses as indicated by a decrease in the number of Fret 43-positive cells (Fig.

2). The remaining Fret 43-positive cells take on a rounded, abnormal morphology (Fig. 2*D,F*). By 7 dpf, Fret 43 immunoreactivity in *mok* is almost completely absent (Fig. 2*H*).

To evaluate whether cell loss occurs at the same rate for all types of photoreceptor cells, the percentage of opsin- and Fret 43-expressing cells remaining in mutant retinas at 7 dpf was assayed by immunohistochemistry. At 7 dpf, the number of wild-type photoreceptor cells accounts for $\sim$ 19% of the total number of retinal cells (169 of 881, $n = 5$) (Table 1). Based on antibody staining, $\sim$ 40% (80 of 199) of these photoreceptor cells are Fret 43-positive, 22% (43 of 199) are UV opsin-positive, 17% (34 of 199) are blue-opsin positive, and 21% (42 of 199) are rod opsin-positive (Fig. 3, Table 1). In contrast to the wild type, in *mok* retinas at this stage, the PRCL is no longer morphologically distinguishable, and on average only 3% (6 of 199) (Table 1) of cells are positive for photoreceptor-specific markers. Cell loss of different photoreceptor categories exhibits small differences only.

Figure 3. Early differentiation of individual *mikre oko* photoreceptor types. *A–D*, Confocal images of transverse retinal sections immunostained with anti-rhodopsin antibody (green) and anti-carbonic anhydrase antibody to Mueller glia (red) at 60 hpf (*A, B*) and 72 hpf (*C, D*). Zn-8 antibody to ganglion cells and optic nerve (green) was included as an internal control to monitor the stage of early embryos. *A, C*, In wild-type retinas, rhodopsin localizes mostly to the photoreceptor outer segments (arrowheads). *B, D*, In *mok*, rhodopsin is mislocalized throughout the PRCL (arrowheads), even appearing near the INL. Also, note the reduction of glial staining (red in *B*). *E–H*, Bright-field images of transverse retinal sections showing *in situ* hybridization signal obtained with *rod opsin* (*E, F*) or *red opsin* (*G, H*) probes at 72 hpf. *E, G*, Wild-type PRCL displays an even distribution of *opsin* expression in elongated photoreceptor cells (arrowheads). *F, H*, The mutant PRCL displays discontinuities, and *opsin*-expressing cells have an abnormal, rounded morphology (arrowheads). *I–L*, Confocal images of transverse retinal sections (ventral retina only) immunostained with anti-blue opsin antibody (green) and Fret 43 (red) (*I, J*), or with anti-UV opsin antibody (green) and anti-rod opsin antibody (red) (*K, L*). *I, K*, Wild-type rod and cone visual pigments localize to the apical (outer segment) portion of the PRCL (arrowheads), whereas Fret 43 stains the entire bodies of double cones. *J, L*, The *mok* PRCL displays discontinuities, defective photoreceptor morphology, and abnormal distribution of opsin immunoreactivity (arrowheads). In all panels, dorsal is upward, lens is left. * indicates the optic nerve. Scale bar: *A–H*, 100 μ m; *I–L*, 20 μ m.

The Fret 43-positive double cones and UV cones appear to deteriorate at a somewhat faster rate than rods and blue cones (Table 1). In parallel to photoreceptor loss, 7 dpf *mok* retinas suffer a 35% decrease in total cell number relative to the wild type (Table 1). This is more than the 20% decrease that would be expected if only the normal photoreceptor population were absent, and these differences are likely caused by INL abnormalities (see below). The time course and pattern of photoreceptor loss observed with Fret 43 antibody has been confirmed with blue, red, UV, and rod opsin *in situ* probes (Fig. 3*E–H*). Thus, it appears that both rods and cones are initially formed. However, their differentiation becomes aberrant early in development as evidenced by the abnormal shape of opsin- and Fret 43-positive cells, as well as their decreased number. The rate of photoreceptor loss is approximately the same for all photoreceptor types including both rods and cones, suggesting that the primary defects are not confined to a single photoreceptor type or to a subset of photoreceptor types.

The subcellular distribution of photoreceptor visual pigments in *mok* was examined with antisera specific to opsin genes. Mutant immunostaining patterns differed noticeably from wild-type retinas. At 60 and 72 hpf, wild-type rod opsin is concentrated at the apical ends of photoreceptors in developing outer segments (Fig. 3*A, C*). In *mok* retinas, on the other hand, already at 72 hpf, rod opsin staining is concentrated in foci frequently mislocalized relative to the apical photoreceptor termini (Fig. 3*D*). Similar distribution patterns are observed for blue, UV, red, and green opsins (Fig. 3*J–L*; data not shown). Simultaneous mislocalization of visual pigments in rods and several cone types is another indication that the *mok* mutation affects mechanisms common to the whole photoreceptor cell class and not to individual photoreceptor types.

***mikre oko* affects the formation of both photoreceptor outer segments and synaptic termini**

To gain a better understanding which aspects of photoreceptor morphogenesis are affected in *mikre oko*, electron microscopic analysis was performed at 3 and 5 dpf, revealing a variety of structural defects. In wild-type zebrafish retina, the majority of inner and outer segments develop between 60 and 72 hpf (Branchek and Bremiller, 1984) (Fig. 4*A*). The *mok* phenotype is already visible by 72 hpf, before or concomitant with wild-type outer segment formation. In some, mostly peripheral, regions of the retina, photoreceptor nuclei lose their typical elongated morphology and assume a rounded appearance (Fig. 4*B, right side*). Neither inner nor outer segments of photoreceptor cells are observed in these regions. In other, mostly central, regions of the retina (Fig. 4*B, left side*), photoreceptor cells retain their elongated morphology and, on rare occasions, form rudimentary outer segments (Fig. 4, compare *D* to a wild-type outer segment in *C*). Furthermore, there is a high amount of cell death throughout the outer retina (Fig. 4*B*), and the photoreceptor synaptic termini appear to be poorly differentiated (Fig. 4, compare *F* to wild type in *E*). In sections of wild-type retinas at 3 dpf, on average, three outer segments were found per 10 μ m of RPE (51 outer segments were found in an area of 188 μ m sampled from two retinas), whereas only ~0.2 aberrant outer segments (7% of the wild-type number) were present per 10 μ m of *mok* RPE (three outer segments were found in an area of 171 μ m sampled from four retinas). Later in development, although outer segments continue to grow in the wild type, their number decreases in *mok* retinas. In 5 dpf wild-type retinas, on average five outer segments were found per 10 μ m of RPE (154 outer segments were found in an area of 308 μ m sampled from two retinas) (Fig. 4*G*). In contrast, only 0.04 (<1% of wild-type number) of usually

Figure 4. Ultrastructure of wild-type and *mikre oko* photoreceptor cells. *A*, Electron micrograph of a wild-type retina at 3 dpf reveals elongated nuclei in the PRCL, prominent outer segments adjacent to the RPE (arrowheads), and ellipsoid assemblies of mitochondria in the inner segments in between (asterisks). *B*, At 3 dpf, *mok* retinas have few to no outer segments (arrowheads) or ellipsoids (asterisks). The photoreceptor nuclei display elongated morphology toward the central retina (left), but become increasingly disorganized toward the periphery (right). Additionally, numerous cell corpses are present (*p*). *C*, *D*, Enlargements of the PRCL apical region at 3 dpf. *C*, In wild-type outer segments, a stack of membranous discs is clearly discernible. *D*, *mok* outer segments are found much less frequently, are smaller, and appear disorganized. *E*, *F*, Enlargements of the PRCL synaptic region at 3 dpf. *E*, Wild-type photoreceptor termini form characteristic invaginations occupied by postsynaptic processes of bipolar and horizontal cells. A synaptic ribbon is indicated (arrowhead). *F*, *mok* synaptic termini are poorly differentiated, and no synaptic ribbons are observed. *G*, *H*, Enlargements of PRCL apical region at 5 dpf. *G*, Wild-type photoreceptors form well differentiated inner (asterisks) and outer segments (arrowheads). *H*, *mok* outer segments, when present, are severely disorganized, and inner segments are not obvious. Arrowheads indicate outer segments. Asterisks indicate inner segments. RPE is upward in all panels. Scale bars, 1 μ m.

aberrant outer segments are present per 10 μ m of *mok* RPE (two outer segments were found in an area of 503 μ m in four retinas) (Fig. 4*H*). Almost complete absence of outer segments and complete absence of normal outer segments is a striking phenotype only seldom observed at early stages of photoreceptor development in vertebrate mutants. In most mutant strains characterized so far, defects appear after the outer segments are differentiated (Bok and Hall, 1971; Blanks et al., 1982; White et al., 1993; Liou et al., 1998; Redmond et al., 1998; Hagstrom et al., 1999; Weng et al., 1999).

Cell-cell interactions are involved in the development of elongated morphology of photoreceptor cells

The mutant photoreceptor phenotypes could be caused by intrinsic defects in the photoreceptor cells themselves, or by defective cell–cell or cell–extracellular matrix interactions. To distinguish between these possibilities, genetically mosaic animals were constructed using the blastomere transplantation technique. In this method, cells are removed from a tracer-labeled donor embryo and placed into unlabeled hosts (Ho and Kane, 1990; Halpern et al., 1993; Malicki and Driever, 1999). Wild-type or mutant embryos can be used as donors or hosts. Descendants of donor cells retain the tracer and can be visualized via immunohistochemistry. The resulting embryos contain a mosaic of genotypically different cells that can be analyzed at later stages for photorecep-

tor development. A cell-autonomous phenotype in the PRCL would be evidenced through the formation of normal photoreceptors from wild-type cells transplanted into a mutant retina and the failure of mutant cells to form photoreceptors in a wild-type PRCL. Conversely, a cell-nonautonomous phenotype would be apparent by the failure of wild-type cells to form photoreceptors in a mutant PRCL, as well as the rescued development of mutant photoreceptors in a wild-type environment.

Blastomere transplantations were performed to produce four classes of mosaics: wild-type embryos containing wild-type donor cell clones, wild-type embryos containing mutant donor cell clones, mutant embryos containing wild-type donor clones, and mutant embryos containing mutant donor clones. In these experiments, donor cells were labeled using biotinylated dextran and detected using avidin–HRP conjugate. Representative clones are shown in Figure 5, *A*, *C*, *E*, and *G*. Transplants between genotypically wild-type embryos produce retinal clones in which donor cells contribute to all cell layers of 7 dpf retina (Fig. 5*A*). Approximately 20% of donor cells contribute to PRCL cells (152 of 783 cells in 17 independent clones) and develop elongated morphology characteristic of photoreceptor cells. Similarly, mutant *mikre oko* cells transplanted into a wild-type host contribute to all layers of the retina, and ~9% of them (44 of 472 cells in 17 independent clones) form morphologically distinguishable, elon-

Figure 5. Morphology and survival of photoreceptor cells in *mikre oko* mosaic animals. Examples of four possible genotypic combinations of mosaic retinas at 7 dpf are visualized with two different detection methods. *A, C, E, G*, Bright-field images of mosaic retinas processed with HRP-avidin to detect donor-derived cells (brown). *B, D, F, H*, Confocal images of mosaic retinas immunostained with a mix of all anti-opsin antibodies (green) and Cy3-streptavidin to detect tracer (red). *A, B*, Wild-type donor cells contribute to all retinal layers in wild-type hosts, including the PRCL (arrows). *C, D*, Elongated morphology of *mok* photoreceptor cells is rescued in wild-type environment (arrows). *E*, Wild-type cells do not form elongated photoreceptors next to the RPE of *mok* host embryos. *F*, Wild-type donor cells express opsins in *mok* retinas (arrows). *G, H*, Mutant *mok* cells do not form morphologically distinguishable photoreceptors in a mutant background and, with rare exceptions, do not express opsin genes (arrows). Insets in the bottom left of *B* and *D* show twofold magnifications of the areas enclosed in boxes. The approximate position of the outer plexiform layer is indicated with dashes. Note that *mikre oko* photoreceptor cells in the wild-type environment extend from the outer plexiform layer to the opsin-positive outer segment layer. Donor-host genotypes are indicated at the bottom of each panel. Dorsal is upward, and RPE is right in all panels. *m*, Mutant; *w*, wild type. Scale bar, 10 μ m.

gated photoreceptors surviving at least until 7 dpf (Fig. 5C). In contrast, 0% (0 of 102 cells in five independent clones) of wild-type cells developing in mutant PRCL display elongated morphology at this time (Fig. 5E). Similarly, as expected on the basis of histological studies, mutant donor cells in a mutant environment do not develop elongated morphology (0 of 103 cells in eight independent clones at 5 and 7 dpf) (Figs. 5G, 6F). Thus, the *mok* photoreceptor morphology phenotype displays a strong cell-nonautonomous component. Because the percentage of mutant *mok* photoreceptor cells in clones developing in a wild-type environment is lower than that found in wild-type to wild-type transplants, the *mok* phenotype appears to combine cell-autonomous components as well. In a wild-type PRCL environment, numerous *mok* mutant photoreceptors retain an elongated morphology until at least 7 dpf, more than twice as long as photoreceptors in unmanipulated *mok* PRCL, which completely lose elongated morphology by 4 dpf (compare Fig. 2D to 5C,D). These results indicate that the development of elongated photoreceptor morphology involves cell-cell interactions and that these interactions are disrupted in *mok*.

Cell-cell interactions are involved in the survival of photoreceptor cells

The survival of photoreceptor cells in mosaic retinas was quantitated as a ratio of photoreceptors to the total number of cells in donor clones. Because in mutant retinas photoreceptor cells lose elongated morphology and cannot be distinguished by morphological criteria, we stained cryosections of mosaic retinas with a

mix of antibodies directed to all five zebrafish opsins to determine the photoreceptor survival rate in mutant eyes. When wild-type clones of donor cells develop in wild-type host retinas, ~21% form photoreceptors (in 18 clones inspected, 134 of 653 cells formed photoreceptors at 7 dpf) (Fig. 5B, Table 2). These results are in agreement with the previous studies in which we used HRP-avidin to detect donor-derived photoreceptor cells as well as with the estimates based on Hoechst-stained zebrafish retinas (Table 1). As expected, mutant *mikre oko* photoreceptors developing in the mutant environment survive at a low rate of ~8.2% (47 of 576 cells in 18 clones) (Fig. 5H, Table 2). To investigate whether the survival of mutant cells depends on interactions with the surrounding tissue, in the same experiments we determined the survival rates of mutant photoreceptor cells in wild-type environment. Approximately 22% (83 photoreceptors of 380 cells total, 16 independent clones) of *mok* mutant donor cells form photoreceptors in wild-type hosts, consistent with the results obtained in previous experiments (Fig. 5D, Table 2). This represents a dramatic increase compared with the survival rate of the mutant cells developing in the mutant environment. These results demonstrate that *mok* photoreceptor survival is enhanced by interactions with a wild-type environment, indicating that the *mok* gene is directly or indirectly involved in cell-cell interactions that promote photoreceptor cell survival. Surprisingly, in contrast to the loss of the elongated morphology, this cell-nonautonomous effect is not obvious in wild-type to mutant transplants. Approximately 16% of wild-type cells express opsin

Figure 6. Outer segment rescue in *mikre oko* photoreceptors at 3, 5, and 7 dpf. PTU treatment inhibits the appearance of pigmentation and permits the observation of outer segment morphology. *A*, Wild-type donor-derived cells form elongated photoreceptors in a 3 dpf wild-type host PRCL, extending outer segment rudiments (arrowheads) toward the RPE (bracket). *B*, *mok* donor cells form elongated photoreceptors in a 3 dpf wild-type host PRCL. Although their inner segment regions appear abnormally thin, the outer segments of these cells closely resemble the wild type. *Inset* shows an enlargement of a *mok* photoreceptor cell in a wild-type retina. The horizontal arrow indicates an abnormal constriction in the inner segment region of this cell. Such constrictions are not seen in wild-type cells. *C*, At 5 dpf, wild-type donor cells display distinct outer segments (arrowheads) and robust inner segments. *D*, *mok* donor cells elaborate both cone (thin arrowhead) and rod (wide arrowhead) outer segments. The inner segment regions of rescued *mok* photoreceptor cells are frequently abnormally thin. This is evident in the case of the rod cell indicated with a wide arrowhead. *E*, *F*, Neither wild-type nor *mok* donor cells form elongated photoreceptors at 5 dpf when placed in mutant environment, and there is no evidence of outer segments. *G*, *H*, *I*, Enlargements of HRP-stained donor photoreceptors in PTU-treated retinas at 5 dpf. *G*, Wild-type photoreceptors in wild-type environment display robust inner and outer segments (arrowheads) and well developed synaptic pedicles (vertical arrow). Both rod (*H*) and cone (*I*) *mok* outer segments are rescued in wild-type environment (arrowheads), whereas more proximal cell regions deteriorate and no pedicles are distinguishable (vertical arrows). *Panel I* is a composite of two images taken in different planes of focus. Horizontal arrows indicate abnormal constrictions in the inner segment region. In wild-type cells, such constrictions are found only between the nucleus and the synaptic terminus. *J*, *K*, Confocal reconstructions of donor photoreceptors at 7 dpf. *J*, Tracer-containing wild-type donor cells (red) in a wild-type host PRCL. Outer segments (arrowheads) and a pedicle (vertical arrow) are indicated. *K*, *mok* donor cells adopt an elongated shape (red), yet their inner segment regions (horizontal arrow) and synaptic termini (vertical arrows) are grossly abnormal. RPE is upward in all panels. *m*, Mutant; *w*, wild type. Scale bar: *A–F*, 8 μ m; *G–K*, 4 μ m.

in a mutant environment (54 of 330 cells, 12 independent clones) (Fig. 5*F*, Table 2). One interpretation of the increased survival of wild-type versus mutant cells in a mutant environment is that wild-type cells are providing a rescuing cell-nonautonomous component through interactions with each other. More likely, this observation indicates that although *mok*-dependent cell–cell interactions are sufficient to promote the survival of mutant photoreceptor cells, they are not necessary for the survival of wild-type cells.

RPE-independent cell–cell interactions are involved in the development of photoreceptor outer segments

The close physical association of photoreceptors to the RPE obscures photoreceptor outer segments. To study inner and outer segment development in mosaic animals, after blastomere transplantations, host animals were raised in the presence of 1-phenyl-

2-thiourea (PTU), a compound that inhibits pigment accumulation in the RPE (Westerfield, 1994; Malicki, 1999). Interestingly, the majority of rescued *mikre oko* photoreceptors are capable of developing outer segments within the context of a wild-type PRCL (Fig. 6*B,D,H,I*; arrowheads indicate outer segments). These well formed *mok* outer segments are virtually indistinguishable from the outer segments seen in wild-type donor photoreceptors in wild-type hosts (Fig. 6*C,G*, arrowheads). Because rod and cone outer segments are characterized by distinct morphologies, we have been able to determine that both are rescued (Fig. 6*D,H,I*; wide arrowheads indicate rod outer segments, thin arrowheads indicate cones). The rescue of the outer segment morphology is remarkable, given that at 3 dpf, *mok* photoreceptors already display severe outer segment defects. At 5 dpf, <1% of outer segments are present in *mok* compared with the wild

Table 2. Survival of photoreceptors in mosaic retinas

	Experiment 1	Experiment 2	Experiment 3	Total	<i>n</i>
+ → +	84/454 (18.5%)	13/72 (18.1%)	51/195 (26.2%)	134/653 (20.5%)	18
m → +	24/140 (17.1%)	19/102 (18.6%)	41/150 (27.3%)	83/380 (21.8%)	16
+ → m	6/90 (6.7%)	16/96 (16.7%)	33/161 (20.5%)	54/330 (16.4%)	12
m → m	7/212 (3.3%)	4/102 (3.9%)	36/262 (13.7%)	47/576 (8.2%)	18

Cross sections through mosaic retinas were processed for dextran tracer and opsin staining at 7 dpf. Mosaic genotype is indicated in the left-most column. Three separate experiments are presented. In wild-type hosts, the fraction of donor cells displaying elongated photoreceptor morphology and/or opsin expression in the PRCL is provided, because of the difficulty of associating a particular opsin-expressing outer segment with a tracer containing donor cell. In mutant hosts, because no elongated morphology is evident, the ratio of donor cells displaying opsin expression to all cells was scored. The same ratio is also expressed as a percentage in parentheses. Although there was some variability among independent experiments, the relative ratios of survival are consistent. In all experiments, mutant cells in a wild-type environment survive at least twice as well as in mutant to mutant controls. A cumulative total for all three experiments is provided. *n*, Number of clones analyzed.

Table 3. Photoreceptor morphology in mosaic retinas

	Day 3 phenotype			Day 5 phenotype		
	ISN	OS	<i>n</i>	ISN	OS	<i>n</i>
+ → +	62/67 (92.5%)	60/67 (89.6%)	6	64/68 (94.1%)	65/68 (95.6%)	14
m → +	7/46 (15.2%)	26/46 (56.5%)	8	10/83 (12.0%)	50/83 (60.2%)	16
+ → m	N/A	N/A	—	0/75 (0%)	0/75 (0%)	9
m → m	N/A	N/A	—	0/45 (0%)	0/45 (0%)	7

Cross sections of PTU-treated, HRP-stained retinas were analyzed at 3 and 5 dpf. Mosaic genotype is indicated in the left-most column. Top row indicates the stage examined. The ratio of donor cells displaying normal inner segment/nuclear region (ISN) or outer segment (OS) morphology to all cells examined is provided. The same ratio is also expressed as a percentage. ISN was judged normal if its width was greater than or equal to 20% of cell body length. At 3 dpf, the presence of OS was estimated based solely on the pointed shape of their apical termini. At 5 dpf, OS were considered normal if their length exceeded 25% of the total cell body length. A significantly higher number of normal *mok* outer segments were formed in a wild-type than in a mutant background. χ^2 analysis returned a value of $p < 0.0001$ that this rescue could be attributable to chance alone. *n*, Number of clones analyzed; N/A, not analyzed.

type as evidenced by electron microscopy (Fig. 4). Additionally, although 60% of mutant cells in a wild-type environment display outer segments at 5 dpf, such structures are completely absent by gross morphological inspection in a mutant host environment (Fig. 6*F*, Table 3). The cell-nonautonomous behavior of the *mok* outer segment phenotype is supported further by the observation that wild-type cell clones in a mutant environment do not develop outer segments either (Fig. 6*E*, Table 3). Unexpectedly, although outer segments are largely wild-type in appearance, inner segments, nuclear regions, and synaptic termini of *mok* photoreceptors transplanted into a wild-type PRCL display grossly abnormal morphology (Fig. 6, compare *D*, *H*, *I*, and *K* with wild type in *C*, *G*, and *J*; synaptic termini indicated with vertical arrows). We estimated the percentage of cells with abnormal morphological features. Defects are already obvious at 3 dpf because ~85% of cells display abnormal shape (Fig. 6*B*, Table 3). At 5 dpf, ~88% of rescued photoreceptor cells exhibit shape defects (Fig. 6*D*, *H*, *I*, abnormal constrictions in the inner segment region are indicated by horizontal arrows; compare with the wild type in *C* and *G*, Table 3). This situation persists at least until 7 dpf (Fig. 6*K*). Thus, from 3 to 7 dpf, although their outer segments become progressively more robust, most rescued photoreceptor cells display an abnormally thin and long connection between the outer segment and the nuclear region, indicating that outer segments differentiate in the absence of properly formed inner segments and nuclear regions. These results imply that *mok* functions in a cell-nonautonomous manner in outer segment formation, yet has a cell-autonomous component in the development or maintenance of the inner segment, the nuclear region, and the synaptic terminus. The differentiation and maintenance of outer segments

are surprisingly independent of more proximal photoreceptor cell features.

What cells in the environment are responsible for the rescue of the *mok* outer segment phenotype? Mosaic analysis of *RCS* rat retina as well as other embryological studies suggests that the RPE may be involved in this process (Hollyfield and Witkovsky, 1974; Mullen and LaVail, 1976). To test the hypothesis that *mok* outer segments are rescued by interactions with wild-type RPE cells, we generated mosaic animals containing clones of mutant RPE cells overlying a wild-type PRCL or clones of wild-type RPE cells overlying a mutant PRCL and analyzed them at 5 dpf. In these experiments, mutant RPE cells do not display obvious adverse effects on the outer segments of neighboring wild-type photoreceptor cells (four clones inspected) (Fig. 7*B*). Similarly, wild-type RPE cells alone are unable to rescue the outer segment phenotype of *mok* photoreceptor cells (five clones inspected) (Fig. 7*C*). These results strongly suggest that cell-nonautonomy of the *mok* outer segment phenotype is not mediated by the RPE.

Mueller glia and horizontal cells are affected in *mikre oko* retina

Horizontal cells are clearly distinguishable in sections of wild-type retinas as a layer of elongated cells at the ventricular edge of the inner nuclear layer oriented tangentially to the curvature of the outer plexiform layer (Fig. 1*A,C,E*). These cells are not apparent or are severely reduced in *mikre oko* at 3 dpf (Fig. 1*B,D,F*). Because presently there are no reliable molecular markers to establish horizontal cell identity in embryonic zebrafish, it is uncertain whether this INL phenotype reflects a loss of the normally elongated horizontal cell morphology or a true absence

Figure 7. Effect of RPE cells on photoreceptor development in mosaic *mikre oko* retinas at 5 dpf. *A, B*, In a wild-type host, *mok* donor-derived RPE cells (*B*) have no deleterious impact on outer segment morphology (arrowheads) in adjacent photoreceptor cells when compared with wild-type donor-derived RPE cells (*A*, arrowheads). *C*, Wild-type donor RPE cells do not have a rescuing effect on *mok* putative photoreceptors, which retain a rounded morphology even when positioned directly adjacent to a wild-type cell. *D*, Similarly, putative mutant photoreceptor cells in a *mok* host retina remain rounded when positioned adjacent to a *mok* RPE donor cell. Donor–host genotypes are indicated at the bottom of each panel. RPE is upward in all panels. *m*, Mutant; *w*, wild type. Scale bar, 10 μ m.

of these cells. The morphological phenotype of horizontal cells appears to be cell-nonautonomous because mutant cells in wild-type environment display a normal shape and are present at a normal frequency relative to other cell types. At 7 dpf, in wild-type clones in a wild-type environment, ~8% of cells develop as horizontal neurons (34 of 412 cells, 12 clones). A similar fraction of horizontal cells, 9% (22 of 238 in 8 clones), is present in *mok* clones developing in wild-type environment (Fig. 7*A,B*).

Mueller cells are the major glial component of the retina, forming extensions toward the inner and outer limiting membranes (Dowling, 1987). In zebrafish, the morphology of Mueller glia can be visualized by immunohistochemical staining for carbonic anhydrase (Peterson et al., 2001). In wild-type retinas, such staining clearly reveals cell bodies localized to the INL as well as fine, radially extending processes (Fig. 2*A,C,E,G*). In *mok* retinas on the other hand, both the number of Mueller glial cells and their morphology appear severely compromised already at 3 dpf; carbonic anhydrase-positive cell bodies and radial extensions are significantly fewer or absent in mutant retinas (Fig. 2*B,D,F,H*). Because of a relative scarcity of this cell type, we have not been able to systematically investigate whether the *mok* glial phenotype is cell-autonomous. One has to keep in mind, however, that both horizontal cells and Mueller glia are physically associated with photoreceptors and may be a source of important cell–cell interactions.

DISCUSSION

mikre oko mutation affects early aspects of photoreceptor differentiation

In the zebrafish retina, a layer of photoreceptor cells first becomes morphologically distinguishable between 40 and 50 hpf (Schmitt and Dowling, 1999). Outside of a small ventral region,

the ventral patch, the photoreceptor cell outer segments appear between 60 and 72 hpf. *mikre oko* affects the development of zebrafish photoreceptor cells at the time of first outer segment appearance. Both Fret 43 staining and electron microscopy indicate that already by 72 hpf, many photoreceptor cells lose elongated morphology. These cells do not develop the inner and outer segments and eventually undergo cell death. Within the next 24 hr, the majority of photoreceptor cells display the same phenotype. Thus, in most of *mok* photoreceptor cells, the outer segments either do not develop or are almost entirely lost during the first 12 hr of their development.

A loss of outer segments shortly after their appearance has only rarely been observed in vertebrate mutants of photoreceptor development. Several reports of mutations affecting zebrafish eye development describe the occurrence of irregular or deformed outer segments (Malicki et al., 1996; Fadool et al., 1997; Becker et al., 1998). The most severe of these mutations, *brudas* (*bru*), *elipsa* (*eli*), *fleur* (*flr*), *krenty* (*krt*), *niezerka* (*nie*), and *not really finished* (*nrf*), produce a phenotype by 3 dpf (Malicki et al., 1996; Becker et al., 1998; Drummond et al., 1998). With the exception of *nrf*, however, a thorough analysis of their phenotypes has not yet been reported. The *nrf* photoreceptor phenotype appears to be less severe than *mok* because, as evidenced by electron microscopic analysis, substantial numbers of relatively intact *nrf* outer segments persist in the central retina at least until 5 dpf (Becker et al., 1998). In *mok*, on the other hand, <1% of cells retain outer segments until this stage of development. The remaining outer segments are smaller and severely disorganized. Complementation testing revealed that *nrf* and *mok* affect independent loci. Electron microscopic analysis of *niezerka*, *elipsa*, and *brudas* indicates that, in these mutants, too, the photoreceptors are less severely affected compared with *mok* (our unpublished observa-

tions). Thus, among the zebrafish mutants, *mok* appears to produce a particularly early photoreceptor phenotype.

In other vertebrate model systems, early defects in outer segment development have been observed only infrequently. In the mouse, in which outer segments appear between postnatal day 9 and 10 (White et al., 1993), spontaneous mutations and targeted gene knockouts usually cause a slow degeneration of outer segments, which in many cases can continue for months (Blanks et al., 1982; White et al., 1993; Sidman et al., 1997; Redmond et al., 1998; Weng et al., 1999).

***mikre oko* affects mechanisms common to the development of all photoreceptor types**

The zebrafish retina features five types of photoreceptor cells, each characterized by a specific morphology, spectral sensitivity, and visual pigment expression (for review, see Malicki, 1999). Given the close proximity of these cell types, a defect in one of them could trigger an eventual loss of the entire PRCL. Is this the case in *mikre oko* retina? This possibility appears unlikely because all the aspects of photoreceptor loss compared so far are very similar for all photoreceptor types. Thus, at early stages of development, defects of opsin distribution are present in all photoreceptor cells to similar degree. This observation has also been confirmed by *in situ* hybridization experiments with opsin probes. Similarly, the estimates of cell survival of individual photoreceptor types at 7 dpf do not reveal striking differences. Finally, mosaic analysis indicates that both rod and cone outer segments can be rescued by wild-type environment. These observations argue that the *mok* gene plays a role in genetic mechanisms common to all photoreceptor types.

Although the molecular basis of signal transduction appears to be similar in rods and cones, many components of the signal transduction machinery in these cell types are encoded by separate, although highly homologous, genes. This is true, for example, for the visual pigments themselves, transducin, cGMP phosphodiesterase, cGMP-gated Na/K channel, and arrestin (Lerea et al., 1986; Gillespie and Beavo, 1988; Lee et al., 1992; Bonigk et al., 1993). Mutations in these genes produce phenotypes specific to individual photoreceptor types. In the mouse, mutations in rod-specific genes cause a degeneration of rods first and only later of cones (Nir et al., 1989). Similarly, defects of cone-specific genes affect cones and leave rods intact (Biel et al., 1999). In contrast to the above examples, the *mok* mutation appears to affect all types of photoreceptor cells in the same way, arguing that the underlying gene plays a role in mechanisms common to all types of photoreceptor cells.

Cell-cell interactions influence survival and differentiation of photoreceptor cells

Cell-nonautonomy has been demonstrated in the case of several mutations affecting development of vertebrate photoreceptor cells. A mosaic mouse expressing a *rhodopsin* Pro344 to Ser mutant transgene in a subset of cells suffers uniform degeneration across the entire chimeric retina, not only in transgene-expressing photoreceptors but also in adjacent normal photoreceptors (Huang et al., 1993). Similarly, although *rd*s mutant photoreceptors can be rescued in a cell-autonomous fashion by a wild-type *rd*s transgene, the *rd*s mutation also displays a cell-nonautonomous component; in genetically mosaic retinas, the juxtaposition of *rd*s $-/-$ transgene-expressing photoreceptors with *rd*s $-/-$ transgene expression-negative cells results in death of both types of cells (Kedzierski et al., 1998). Additional support for the

cell-nonautonomy of photoreceptor loss comes from the observation that several forms of an inherited human retinopathy, retinitis pigmentosa, are caused by specific defects in rhodopsin, yet result in the degeneration of both rods and cones alike (Dryja and Li, 1995). Likewise, in a pig model of retinitis pigmentosa, the genetic rod-specific defect results in early and severe rod loss followed by degeneration of cones (Petters et al., 1997; Tso et al., 1997).

In all of the above experiments, the presence of mutant photoreceptor cells has been shown to cause a decreased survival of wild-type cells. Whether the opposite is true and the mutant cells survive longer when surrounded by wild-type cells has not been investigated. Our experiments demonstrate, for the first time, that mutant photoreceptor cells display increased survival when surrounded by wild-type cells. When placed in wild-type environment, *mok* photoreceptor cells survive until 7 dpf, and their survival rate is >2.5 times higher than that of mutant cells in mutant environment. These results are consistent with the observations in mammals indicating that photoreceptor survival involves cell-nonautonomous components. Previous investigations of cell-nonautonomous photoreceptor defects were limited, however, to the evaluation of cell survival (Huang et al., 1993; Kedzierski et al., 1998). Here, we demonstrate that cell-cell interactions in the retina rescue the overall elongated morphology of photoreceptor cells as well as the development of their outer segments.

Outer segments differentiate in the absence of normal inner segment and nuclear regions

An unexpected result of these experiments is that cell-cell interactions are sufficient to rescue the development of *mikre oko* outer segments. Although this effect is not completely penetrant, the size and shape of rescued rod and cone outer segments closely resemble the wild type. This is in sharp contrast to the mutant *mok* retinas in which only very few, abnormally shaped photoreceptor outer segments persist until 5 dpf. The rescued outer segments enlarge from 3 to 5 dpf in the absence of properly developed inner segments and nuclear regions, indicating that their development takes place largely independently from these cell features. This is surprising, given that the outer segment metabolism is very active and is presumed to depend on the continuous transport of polypeptides, including rhodopsin, from other cell regions (for review, see Sung and Tai, 2000). To our knowledge, normal outer segments have not been observed in the absence of well differentiated inner segment or nuclear regions in any of the photoreceptor mutants described previously. *mok* gene also appears to act at least partially cell-autonomously in the formation of photoreceptor synaptic termini because these structures are not rescued in mosaic animals. In mosaic retinas, *mok* photoreceptor cells exhibit bulbous dilations in the vicinity of the outer plexiform layer, instead of differentiating distinct nuclear regions and synaptic termini. These dilations connect through a long thin process to the outer segments. It should be noted that *mok* acts cell-nonautonomously in wild type to mutant transplants in that inner segments and synaptic termini are not apparent even in opsin-expressing cells, for reasons that remain unclear. Thus, our data show that the *mok* gene plays a partially cell-autonomous role in the proximal region of the photoreceptor cells and a cell-nonautonomous role in the outer segment formation.

The rescue of outer segment is RPE-independent

Both genetic and embryological studies provide evidence indicating that the retinal pigment epithelium plays an essential role in

the differentiation and maintenance of the photoreceptor outer segments. Surgical removal of the RPE results in an almost complete absence of photoreceptor outer segment development (Hollyfield and Witkovsky, 1974; Stiemke et al., 1994). Mutations in RPE65, a protein specifically expressed in the RPE, cause a loss of photoreceptor cells in mice and humans (Hamel et al., 1993; Gu et al., 1997; Redmond et al., 1998). Finally, the photoreceptor lethality in the *RCS* rat is rescued by wild-type RPE (Mullen and LaVail, 1976). Given the abundant evidence supporting the involvement of RPE in photoreceptor development and survival, we tested the role of RPE in the *mikre oko* phenotype. Mosaic analysis strongly suggests that RPE does not play a role in the rescue of *mok* outer segment phenotype. This is notable, given that the photoreceptor outer segments appear to be almost entirely surrounded by the RPE. The rescue of outer segments is thus mediated either via direct cell–cell interactions in the proximal region of the photoreceptor cell or by a diffusible factor secreted by cells other than RPE. To our knowledge, a role of RPE-independent cell–cell interactions in outer segment development has not been previously demonstrated.

***mikre oko* mutant phenotype is more pronounced in retinal periphery**

Analysis of zebrafish photoreceptor mutants revealed several patterns of photoreceptor loss (for review, see Malicki, 2000a,b). Mutants *mok*, *nie*, and *nrf* display stronger phenotypes in the retinal periphery, whereas *eli*, *bru*, *flr*, and *tz288b* exhibit more pronounced cell loss in the central photoreceptor cell layer. Other patterns of photoreceptor cell loss have also been observed (Malicki et al., 1996). Loss of cells originating in the center of the retina can be explained by the observation that central photoreceptor cells are born before the peripheral ones (Marcus et al., 1999). They are, therefore, more likely to accumulate defects first. What could explain a photoreceptor phenotype characterized by a more pronounced loss of peripheral cells? One possible explanation is that young photoreceptors compete for a factor that is in a limited supply in the mutant retina. The centrally located cells that differentiate first deplete the supply of this factor. Consequently, the later differentiating cells do not have access to it and suffer more severe defects. The presence of such a factor is consistent with cell-nonautonomy of the *mikre oko* phenotype. If the above hypothesis is true, at later stages of development, this hypothetical factor is not sufficient to promote survival of even centrally located cells. Alternatively, peripheral cell loss may be more pronounced because of nonuniform expression of the *mikre oko* gene. This is likely to be true if the *mok*^{m632} allele is hypomorphic. In addition to these two, other scenarios are also possible. The identification of factor(s) responsible for specific aspects of cell loss in *mikre oko* mutant animals may have to await the molecular cloning of the *mikre oko* gene.

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