

Two Zebrafish Mutants, *ebony* and *ivory*, Uncover Benefits of Neighborhood on Photoreceptor Survival

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ABSTRACT: Zebrafish offer a tractable system for the study of retinal development and degeneration, to provide insights into human retinal degeneration. We have begun to dissect the question of neighborhood effects on photoreceptor differentiation and survival through the isolation and characterization of mutants with retinal degeneration. We describe two mutants, *ebony* and *ivory*, isolated through a behavioral screen for blind mutants induced by ethyl nitrosourea mutagenesis. Chimeric analysis was conducted to attempt to res-

cue the photoreceptor degeneration. In *ebony*, the photoreceptor cell death was both cell autonomous and nonautonomous in nature, whilst the photoreceptor cell death was strikingly nonautonomous in *ivory*. The rescue at a distance is in keeping with a putative diffusible survival factor. We propose a density-dependent nonautonomous neighborhood effect to explain these findings.

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INTRODUCTION

Zebrafish are particularly amenable to the study of photoreceptor differentiation and survival (Malicki, 1999), complementing the well-studied mouse and rat models. The zebrafish eye is large and develops rapidly, and vision can be easily assayed by physiology and behavior. Zebrafish also have rich color vision, with a corresponding cone-dense retina, an important consideration given the clinical dominance of the cone photoreceptor degeneration in humans. This gives a distinct advantage over the rodent models.

Indeed, the evolution of rodents to a nocturnally tuned retina is reflected genetically. For example, the retinal guanylate cyclase-activating protein, GCAP3, is expressed solely in cones in humans and zebrafish, but appears to have been lost in rodents (Imanishi et al., 2002).

Various forward-genetic screens have uncovered potential zebrafish models of retinal degeneration. In the original Tübingen and Boston large-scale screens for developmental mutants (Driever et al., 1996; Haffter et al., 1996), fish with obvious morphological abnormalities before day 5 postfertilization (5 dpf) were identified. Although photoreceptor survival, as such, was not a focus of these screens, among the hundreds of mutants isolated, there were about 50 mutants with developmental abnormalities of the eye, such as an alteration in eye shape, size, or pigmentation (Malicki et al., 1996). Some of those phenotypes were degenerative in nature. Other retinal degeneration mutants with morphologically normal eyes were later revealed based on their impaired visual re-

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sponses using behavioral analysis (Neuhauss et al., 1999). Since then, smaller, but more focused, behavioral and morphological screens have revealed additional retinal degeneration mutants (Brockerhoff et al., 1997, 1998; Fadool et al., 1997; Li and Dowling, 1997; Link et al., 2000; Brockerhoff, 2001; Doerre and Malicki, 2002).

Some of the mutant phenotypes resemble diseases of the human retina. For instance, *eli* and *flr* mutants (Drummond et al., 1998; Neuhauss et al., 1999; Doerre and Malicki, 2002) have a combination of photoreceptor and kidney defect, reminiscent of the human Senior-Loken and Bardet-Biedl syndromes (OMIM 266900 and 209900). Another mutation, *nrc*, has been mapped to chromosome 10 in the zebrafish genome. Its phenotype is strikingly similar to Duchenne/Becker muscular dystrophy (Allwardt et al., 2001; Van Epps et al., 2001). Eye and brain patterning defects seen in *glo*, *nok*, and *ome* mutants may have their correlates in the human Walker-Warburg, oculocerebro-renal, and muscle-eye-brain syndromes, respectively (OMIM 236670, 309000, and 253280). The *nok* gene has recently been shown to encode a membrane-associated guanylate kinase-family scaffold protein (Wei and Malicki, 2002). The *lak* mutation, which disrupts a basic helix-loop-helix transcription factor of the Atonal family and results in the absence of retinal ganglion cells (Kay et al., 2001), may underlie congenital optic nerve aplasia (OMIM 165550). It is likely that zebrafish genetics will help to unravel some of the pathological mechanisms for these diseases, as it has already done for diseases of blood and the heart (Dooley and Zon, 2000; Shin and Fishman, 2002).

A common phenomenon in human retinal degenerations is the degeneration of a second photoreceptor class following the degeneration of the primary cell class. For example, in many so-called rod degenerations, a later degeneration of cones may be seen (John et al., 2000). Similar findings have been reported in various rodent models (Mohand-Said et al., 2000) and have led to the notion that interphotoreceptor signaling is critical for photoreceptor survival. The responsible factors, however, have not been identified. Their identification is important because they would suggest a novel therapeutic route to delay photoreceptor death in retinal degeneration. Zebrafish are an ideal system to dissect intercellular signaling mechanisms on account of their amenability to chimera analysis (Ho and Kane, 1990). Studies have already shown both cell autonomous and noncell autonomous effects involved in retinal patterning (Malicki, 1999; Link et al., 2000). Here we address the question of photoreceptor neighborhood effects in *ebony* (*eby*) and *ivory* (*ivy*), two zebrafish mutants with unique forms of retinal degen-

eration. We characterize the distinct mechanisms of photoreceptor loss in these two models by chimera analysis to answer the question as to what type of signaling is involved in photoreceptor differentiation and survival and to propose a density-dependent non-autonomous neighborhood effect.

METHODS

Mutant Strains

The *ivy*^{tm271} mutation (Kelsh et al., 1996) was obtained from the Tuebingen stock center. The *eby*^{s3556} mutation was discovered in a two-generation screen of ethyl nitroso-urea induced mutations (H. Baier, unpublished observation). ENU mutagenesis was carried out using previously described procedures (Mullins et al., 1994). Mutants were propagated by outcrossing carriers and by reidentification in subsequent generations. Fish breeding and husbandry were done according to standard protocols.

Assessment of the Optokinetic Response

A 1 cm diameter glass dish filled with 3% methyl cellulose was placed on a stationary platform surrounded by a rotating striped drum. The drum diameter was 5 cm and consisted of eight black and white stripes, each subtending an angle of 23°. The fish were oriented with forceps so that each was upright, close to the periphery of the dish and facing the stripes head on. The striped drum was then rotated at speeds varying from three to eight revolutions per minute in both a clockwise and counter-clockwise direction. The fish eye movements were viewed with a dissecting microscope positioned over the apparatus. The fish were illuminated with optic fiber lights.

Plastic Sections

Embryos were fixed in 4% glutaraldehyde for 4 h at room temperature or overnight at 4°C. The fix was washed off with 3 × 10 min changes of 0.1 M Pipes buffer, pH 7.2. They were then postfixed in osmium fix for 1 h (2.5 mL 4% osmium tetroxide, 5 mL 0.2 M Pipes buffer, 100 µL of calcium chloride, 2.5 mL dH₂O). The postfix was washed off with 3 × 10 min changes of maleate buffer, pH 5.3, prior to being placed in bulk stain for 1 h. They were then dehydrated and transferred into a 50:50 Spurr resin:propylene oxide mix, then pure resin. Sections (1 µm) were cut and stained with a methylene blue stain prepared from 1% methylene blue, 1% borax, and 1% azure II (Sigma), mixed together with dH₂O and then filtered.

TUNEL Staining

Embryos were fixed in 4% PFA for 2 h at 4°C, washed with PBS, equilibrated with 30% sucrose, frozen in OCT, then

cut into 12 μm sections. The embryos were then postfixed for 5 min in a 50:50 methanol:acetic acid mix at -20°C . Samples were then processed using the Apoptag S7110 kit (Intergen Company, NY).

Immunohistochemistry

Sections were prepared as above. These were air dried for 2 h prior to 3×15 min washes in PBT. The samples were then blocked for 30 min in a PBT/5% goat serum solution. They were then incubated for 60–90 min at room temperature, or overnight at 4°C , in primary antibody diluted in PBT plus 5% goat serum. The primary antibody was then washed off with 3×10 min changes of PBT. The sections were then incubated for 60 min in darkness in secondary antibody (Cy3 or Alexa 488; Molecular Probes) diluted in PBT plus 5% goat serum. Zpr1 (rod), zpr2 (retinal pigment epithelium, RPE), and zpr3 (double cone) antibodies were used at 1:200 dilution, zna 1 (ganglion cells), SV (plexiform layers), and CA (Müller cells, a gift from Dr Paul Linser) antibodies at 1:100. Secondary antibodies were diluted to 1:500.

Chimera Analysis

Single cell embryos were labeled with lysine-fixable miniruby biotin and tetramethyl rhodamine dextran, relative molecular weight 10,000 (Molecular Probes D-3312), in 200 mM KCl, 10 mM HEPES, and 2 mM sodium azide, using a picospritzer (General Valve) and microdriver connected to a 100 μL Hamilton syringe. The system was filled with embryo compatible mineral oil (Sigma). The optimal time for transplantation was from the sphere stage to 50% epiboly. Donor and host embryos were of identical stages. The embryo was first manually dechorionated. The embryo was pipetted onto a cover slip such that the drop of embryo medium containing the embryo was nestling against a line of methyl cellulose. A donor and a host embryo were gently positioned with forceps, so the yolks lay against the methyl cellulose. Cells were then aspirated from the vertex of the donor, the site of the presumptive eye field, and transferred to the vertex of the host. The cover slip was then transferred to a Petri dish and embryo medium added. Donor-host pairs were kept isolated and were checked for the development of a suitable mosaic pattern using a dissecting fluorescent microscope. At a suitable stage, their optokinetic response was assessed, prior to cryosectioning and staining with either a photoreceptor specific antibody or a biotin detection kit. Biotin-labeled dextran was detected by means of an immunoperoxidase procedure using the Vectastain ABC kit (Vectorlabs).

Image Processing

The same settings and gains were used for taking images from both wild-type and mutant embryos on the same slide. Images were taken either using a Nikon microscope using Improvion software or with a Leica laser-scanning confo-

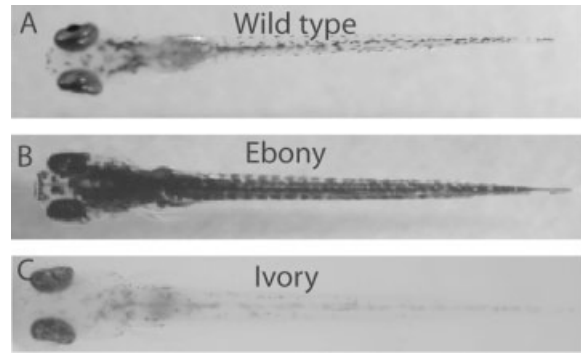


Figure 1 Appearance of mutants (A, B, and C) are dorsal views of day 5 wild-type, *ebony*, and *ivory* mutant embryos, respectively.

cal microscope. The Improvion images were saved as an eight-bit gray file and then converted into an RGB format in Adobe Photoshop to allow the varying brightness to be represented in color. All images were orientated and resized in Adobe Photoshop. Figures were prepared using Adobe Illustrator.

RESULTS

ebony Mutants Combine Blindness with Progressive Paralysis

The *ebony* mutant was isolated based on absence of all visual responses in our behavioral assays (H. Baier, unpublished observation). The mutation is recessive and completely penetrant. Homozygous mutants have the normal complement of pigment cells, but are darker than their wild-type siblings due to a failure to adjust the distribution of their melanin pigment to ambient light levels [Fig. 1(A,B)]. The *ebony* mutant has expanded melanophores (the melanin containing granules), and so appears darker. This visually mediated background adaptation is a retina-dependent neuroendocrine response. Its lack is a telltale sign of blindness (Neuhauss et al., 1999).

The underlying reason for their blindness is apparent on sectioning the retina. The *ebony* retina is devoid of almost all photoreceptors [Fig. 2(A,B)]. Centrally, the outer nuclear layer is absent. This is the layer that normally contains the photoreceptors. Peripherally, close to the ciliary marginal zone, however, elongated cells resembling immature photoreceptors can be detected in Nissl stains and with specific antibodies raised against rods or cones [Fig. 2(C,D)]. The ciliary marginal zone is the site where photoreceptor stem cells normally reside. Otherwise, the eye is of roughly normal size, and all retinal layers (except for the outer nuclear layer) are present.

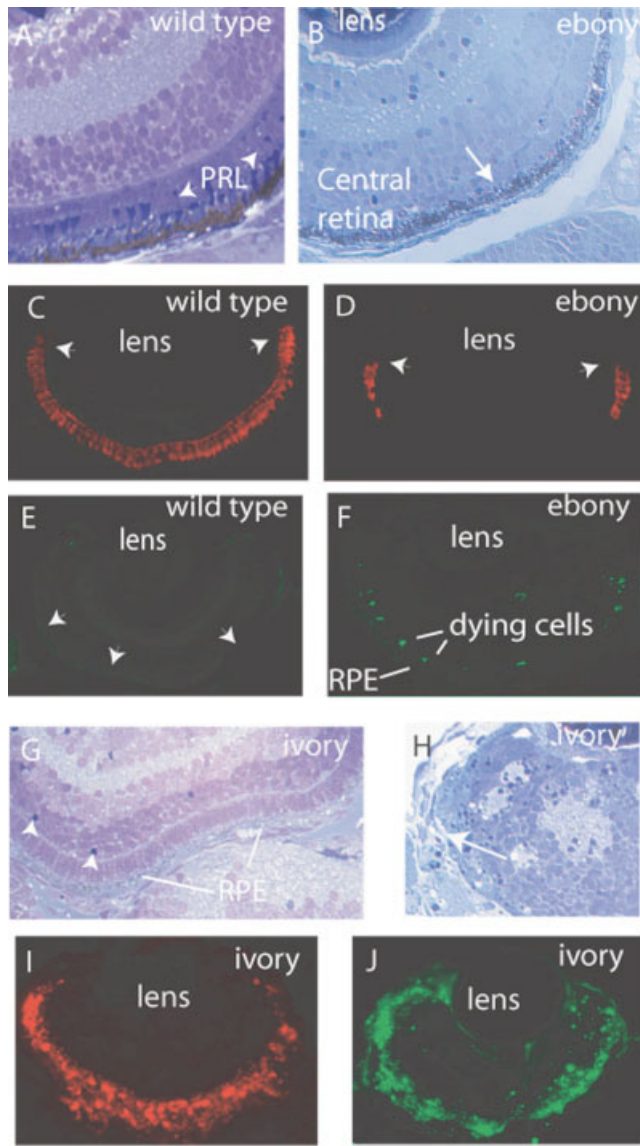


Figure 2

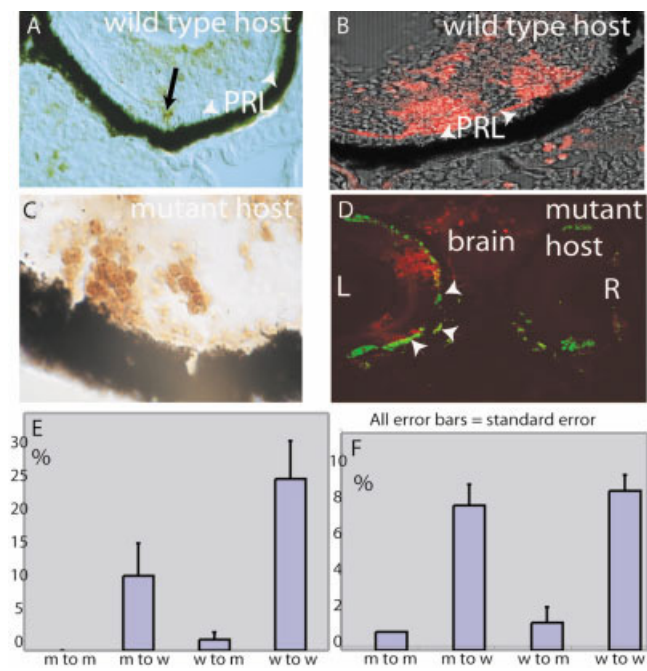


Figure 3

The loss of photoreceptors matches the behavioral phenotype: while the wild-type optokinetic response matures quickly, from weak and variable beginnings on day 3 after fertilization (3 dpf), to a robust and easily scorable behavior on day 6, *eby* mutants show no detectable optokinetic response at any stage of development (tested between 3 and 10 dpf).

After 4 dpf, *eby* mutants also show a locomotor deficit: they move little and lie on the bottom of the dish on their sides, although they respond to a touch on the head by turning 180° and swimming away. This escape response appears normal. The circulation and heart beat also appear normal. By day 7 the mutants appear completely paralyzed; their touch response has regressed to a wiggle. On day 9 they become increasingly opisthotonic, with a bent tail, and a day later they die.

ivy Mutants Combine Gradual Loss of Vision with Hypopigmentation

The recessive *ivy* mutant was first described by Kelsh et al. (1996) as a mutant with reduced body pigmentation [Fig. 1(C)]. In a behavioral rescreen, *ivy* was later found to be visually impaired and to show signs of photoreceptor degeneration in Nissl-stained sections (Neuhauss et al., 1999). On plastic sections, the *ivy* mutant retina appears initially normal until 4 dpf, except for a less densely pigmented RPE and occasional densely staining small rounded nuclei in all

retinal layers, characteristic of apoptotic cells [Fig. 2(G)]. By 5 dpf, the RPE appears swollen with gaps appearing, indicative of degeneration. The photoreceptor layer is still visible, but is also beginning to degenerate. By 7 dpf, it is difficult to discern any photoreceptors [Fig. 2(H)]. This is accompanied by an increase in cell death in the inner retina. The time course of photoreceptor disappearance is correlated with loss of visual responsiveness, as measured by the optokinetic assay. At 4 dpf, a clear optokinetic response is seen, which becomes weaker over the next 2 days and is absent from 7 dpf larvae. Loss of vision is not accelerated by raising the larvae in bright light, nor is it slowed down by raising them in the dark. Some *ivy* mutants inflate their swimbladders, but all die at around 10 dpf.

Photoreceptors Degenerate by Apoptosis in *eby* and *ivy* Mutants

The loss of photoreceptors was confirmed in both mutants, using antibodies against double cones [Fig. 2(C,D,I)] and rods (data not shown). It is gradual in *ivy* mutants and more pervasive in *eby*. Occasionally, in 5 dpf *eby* mutants, positively staining cells are seen in the central retina, although they are lacking the characteristic rectangular shape of photoreceptors (data not shown). By day 7, no central staining is seen in *eby* mutants, whereas the peripheral photoreceptors appear of normal morphology [Fig. 2(D)]. Photoreceptors appear normal in 5 dpf *ivy* mutants, but have

Figure 2 Histological analysis: (A) day 7 wild-type; (B) day 7 *eby* mutant. Both 40X Nissl stained sections. The lens is superior, the photoreceptor layer (PRL) inferior. The long arrow indicates where the photoreceptors disappear from the *eby* photoreceptor layer towards the central retina. (C) Day 7 wild-type; (D) day 7 *eby* mutant. Both 25X. Red: double cone antibody. The arrowheads point to the ciliary marginal zones. (E) Day 8 wild-type; (F) day 8 *eby* mutant. TUNEL assay, dying cells shown as green. Both 25X. Arrowheads indicate the position of the wild-type photoreceptor layer. (G) Day 2 *ivy* mutant (40X). Arrowheads indicate occasional pyknotic nuclei in the inner retina. (H) Day 7 *ivy* mutant, Nissl stain, 40X. (I) Day 8 *ivy* mutant, double cone immunohistochemistry (red). (J) Day 8 *ivy* mutant. TUNEL assay, dying cells shown as green.

Figure 3 Chimera analysis of *eby*. In (A) mutant cells have been transplanted into a wild-type embryo, here viewed at day 6 (40X). The mutant donor cells have been labeled with biotin-conjugated dextran and then stained using a DAB kit (here appearing brown). Occasional donor mutant cells form normal photoreceptors (arrow). However, a more common finding is an absence of clone extension into the photoreceptor layer, such as shown in (B), in which the donor cells have been labeled with an immunofluorescent marker and appear red in the image. In (C) wild-type cells (appearing brown) have been transplanted into a mutant embryo (100X; DAB staining). Normal photoreceptors are not seen. However, (D), which shows a coronal section through both eyes of a single embryo (10X), in which the majority of the donor cells have ended up in the left eye, suggests a partial rescue, as more green staining, from a double cone counterstain, is seen in the left eye than in the right. (E,F) m, mutant; w, wild type. (E) shows the percentage of donor cells forming normal double cone photoreceptors, from those transplants analyzed with immunohistochemistry (X axis). Column 1, $n = 2$; Column 2, $n = 13$; Column 3, $n = 39$; Column 4, $n = 11$.

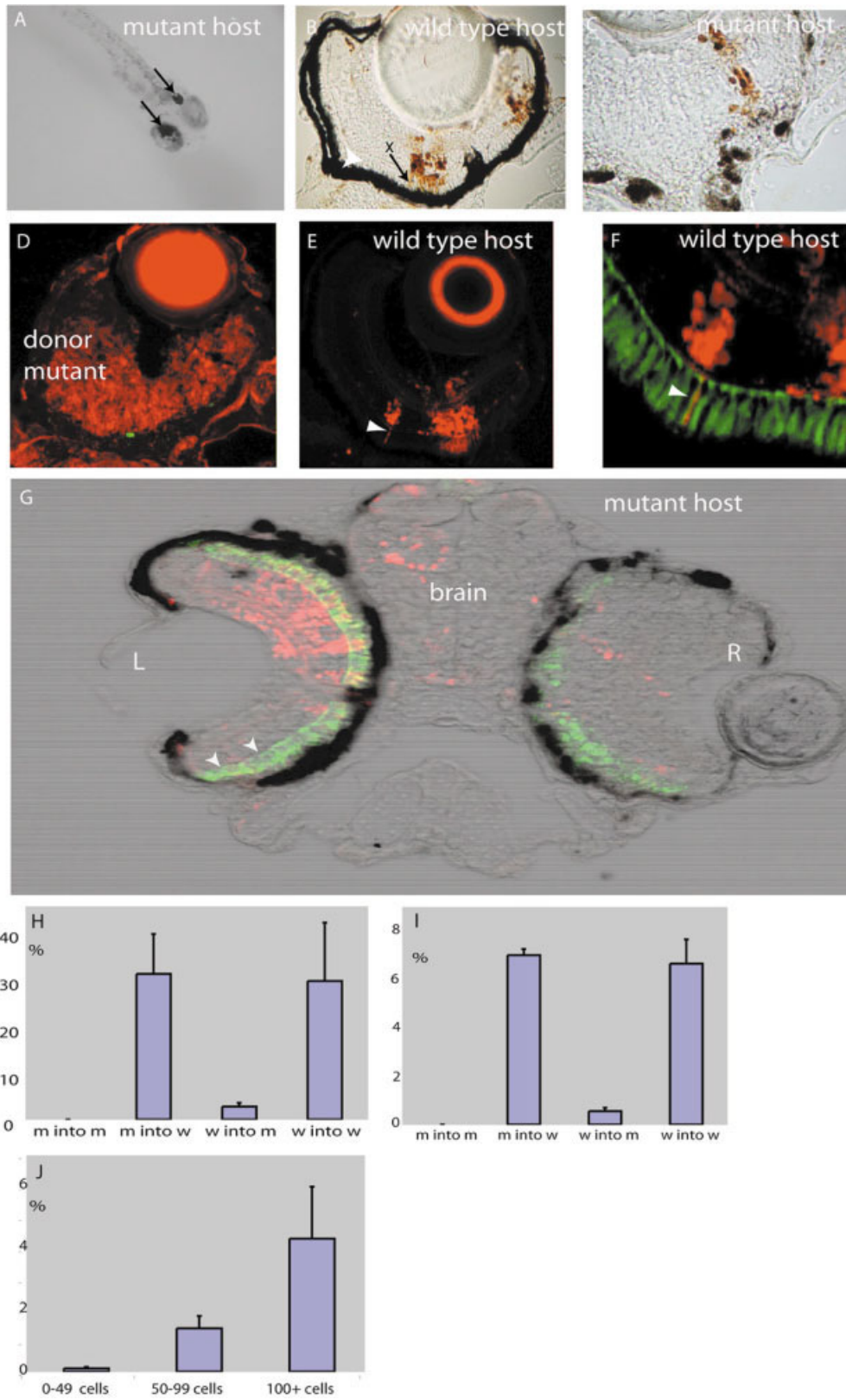


Figure 4

clearly degenerated 2 days later [Fig. 2(I)], with those remaining having lost their characteristic shape.

The nature and time course of photoreceptor cell death were assessed with the TUNEL method [Fig. 2(E,F,J); dying cells appear green]. Cell death occurs by apoptosis (TUNEL+) and increases over time. Apoptotic cells are also seen in the inner retina of both mutants at 7 dpf. Surprisingly, despite histological signs of RPE degeneration in *ivy* [Fig. 2(H)], the *zpr2* antibody used as an RPE marker appears unaffected (data not shown). It is unclear if the degeneration of the inner retina is a secondary consequence of photoreceptor loss, or if it is intrinsic to the cells situated there. Müller glial cells, which have been implicated in photoreceptor survival, are intact in both mutants, as are dopaminergic amacrine cells (data not shown).

Photoreceptor Cell Death Is Both Cell-Autonomous and Nonautonomous in *eby* Mutants

We created chimeric larvae composed of mutant and wild-type cells to determine whether *eby* acts in a cell-autonomous or nonautonomous fashion. When cells from an *eby* mutant donor were transplanted at the blastula stage into a wild-type host, only very rarely did the mutant clones contain photoreceptors [Fig. 3(A)]. The typical finding was a clone of mutant cells that extended up to, but did not include, the photoreceptor cell layer [Fig. 3(B)]. This finding suggests that *eby* acts photoreceptor-autonomously. When wild-type cells are transplanted into a mutant environment, the number of photoreceptors they form

is markedly reduced from the number that would be formed in a wild-type environment [Fig. 3(C)]. Figure 3(D) shows a coronal section through both eyes of a single mutant embryo. Most of the donor wild-type cells have ended up in the right eye. Although morphologically normal photoreceptors have not formed, there is more expression of the cone marker in the right eye than in the left eye, suggesting a small degree of rescue has taken place. This finding, together with the death of wild-type cells in a mutant host, suggests that there is also a nonautonomous component to the *eby* mechanism of action.

These results are quantitated in graphs in Figure 3(E,F). Mutant cells transplanted into a mutant embryo do not form any photoreceptors (first column). Approximately 26% of wild-type cells transplanted into a wild-type embryo form photoreceptors (fourth column). Approximately 11% of mutant cells, when transplanted into a wild-type environment, form double cone photoreceptors. Similar results are obtained when transplants are analyzed using DAB staining (results not shown). Figure 3(F) shows the percentage of host cells forming normal photoreceptors, in those sections with a clone of donor cells, again analyzed with immunofluorescence (*x* axis). The percentage of host cells forming double cone photoreceptors is not altered by the presence of wild-type donor cells. Virtually no photoreceptors remain in a mutant embryo, with the presence of mutant donor cells again not making any difference to this. The presence of donor wild-type cells does not lead to a change in fate of mutant host cells.

Figure 4 Chimera analysis of *ivy*. (A) Wild-type cells transplanted into a mutant embryo form patches of pigmentation of normal intensity (arrows). (B) Donor mutant cells, labeled with a biotin dextran, transplanted into a wild-type retina. White arrowhead shows photoreceptor layer. Donor cells can be seen in the photoreceptor layer (black arrow). (C) Wild-type cells transplanted into a mutant host. There are a few wild-type cells lying adjacent to the pigment epithelium, where photoreceptors would normally be expected to lie. No cells with the characteristic shape of photoreceptors are seen. (D) Eight dpf mutant donor embryo labeled with a fluorescent dextran (appearing red). The pigmentation is patchy and the photoreceptors have degenerated (double cone counterstain in green). (E) shows the result of transplanting cells from this mutant embryo into a wild-type embryo, here shown on day 8. Double cones are shown in green. Donor mutant cells (red) can be seen in the photoreceptor layer. A single cell with the characteristic elongated shape of a photoreceptor is clearly seen (arrowhead). (F) is a composite image of the same section, with the green double cone counterstain. Double stained cells now appear yellow. In (G) wild-type donor cells (red) have been transplanted into a mutant host (image at 10X). The embryo is 8 dpf. Most of the donor cells have ended up in the left eye. Here, these donor cells not only form photoreceptors, but mutant cells are being rescued at a distance (arrows). In contrast, the small number of wild-type cells in the right eye is not forming photoreceptors [all images are at 25X, except (C) at 40X]. These results are quantitated in graphs (H–J). m, mutant; w, wild-type. Graphs (H,I): Column 1, *n* = 2; Column 2, *n* = 36; Column 3, *n* = 11; Column 4, *n* = 3; Graph (J): Column 1, *n* = 14; Column 2, *n* = 15; Column 3, *n* = 4.

Photoreceptor Cell Death Is Nonautonomous in *ivy* Mutants

Wild-type cells are seen forming patches of normal pigmentation when transplanted into a mutant embryo [Fig. 4(A)]. With regards to photoreceptor survival, *ivy* mutant cells transplanted at the blastula stage into a wild-type host form normal photoreceptors [Fig. 4(B,F)], demonstrating that *ivy* acts nonautonomously. The observed rescue by a wild-type environment is seen with isolated mutant photoreceptors, as well as with mutant cells that are not in contact with wild-type cells, but are surrounded by other (presumably clonally related) mutant cells [Fig. 4(B)]. The latter finding suggests that a rescuing factor from the wild-type cells acts at a distance. Conversely, small numbers of wild-type cells transplanted into a mutant environment do not form normal photoreceptors [Fig. 4(C)]. However, if the number of wild-type donor cells is sufficiently large then self-rescue is observed, together with rescue of neighboring mutant cells [Fig. 4(G)].

Figure 4(H) shows the percentage of donor cells forming normal double cone photoreceptors, from those transplants analyzed with immunohistochemistry (x axis). Mutant cells transplanted into a mutant embryo do not form any photoreceptors (first column). Just under 30% of wild-type cells transplanted into a wild-type embryo form photoreceptors (fourth column). A similar number is seen when mutant cells are transplanted into a wild-type embryo, that is, the cells are behaving appropriate to their environment. Similar results are obtained when transplants are analyzed using DAB staining (results not shown). In contrast, far fewer wild-type cells form photoreceptors when in a mutant. Figure 4(I) shows the percentage of host cells forming normal photoreceptors, in those sections with a clone of donor cells, analyzed with immunofluorescence (x axis). About 7% of host cells form double cone photoreceptors in the presence of wild-type donor cells. Normally, no photoreceptors remain in a mutant embryo, with the presence of mutant donor cells again not making any difference to this. However, the presence of donor wild-type cells does lead to a change in fate of host cells, with some mutant host cells now forming photoreceptors. We find a clear correlation of the amount of rescue with size of the donor clone [Fig. 4(J)]. Figure 4(J) shows the percentage of host retinal cells that are double cones, in a mutant host, (in a section that has a clone of donor cells), analyzed in three bins corresponding to the absolute number of wild-type donor cells (x axis). The results show that rescue of the mutant cells occurs when a critical number of wild-type cells are transplanted.

The rescuing factor is probably derived from photoreceptors themselves, rather than from the RPE, because, in our chimeras, photoreceptors are occasionally seen dying beneath pigmented, presumably normal RPE, and healthy photoreceptors are occasionally seen directly adjacent to depigmented, presumably mutant RPE. Additionally, mutant RPE does not seem to be rescued by the presence of a large clone of adjacent wild-type photoreceptors. Nevertheless, it is possible that the rescuing factor is derived from the RPE, or alternatively from inner retinal cells, in particular Müller cells, although Müller cell morphology appeared normal in mutant retinæ.

Underlying Genes Map to Different Linkage Groups

The *ebony* gene has been mapped to linkage group 3 and the *ivory* gene to linkage group 20. Fine mapping is currently being undertaken.

DISCUSSION

Mutations in the zebrafish genes *eby* and *ivy* result in distinct forms of RD. As with human RDs (Chang et al., 1993), photoreceptor apoptosis is evident in both mutants. Apoptosis is rarely seen during normal zebrafish retinal development and is extremely scarce beyond 72 h (Li et al., 2000; Biehlmaier et al., 2001), so the massive cell death seen in these mutants is particularly striking. The *eby* gene product acts predominantly photoreceptor-autonomously, whilst the *ivy* gene product acts in an extremely nonautonomous fashion and is shared with cells involved in pigmentation in both the body and the RPE. The two mutants are different from other zebrafish RD mutants described to date. The degeneration in our mutants is restricted to photoreceptors and to few other cell types, unlike the extensive and early cell death seen in most of the earlier found mutants (Malicki et al., 1996; Fadool et al., 1997; Brockerhoff et al., 1998; Neuhauss et al., 1999; Daly and Sandell, 2000). The *itf* mutation (Vihtelic and Hyde, 2002) results in decreased RPE pigmentation and swollen, vacuolated areas where the RPE contacts the photoreceptor outer segments, somewhat similar to *ivy*. However, unlike *ivy*, there is not a general defect in pigmentation, and photoreceptor cell death is also not observed.

In *eby* mutants, photoreceptor death occurs quickly after differentiation. Loss of photoreceptors is therefore complete in the central retina (where the oldest retinal cells reside). The ONL is absent here and cone markers are not expressed. A very small number of photoreceptor-like cells are present in the periphery of

the eye, where new cells are continually added from a pool of stem cells in the marginal zone to support the life-long growth of the eye. These cells have the typical elongated shape of photoreceptors and express the cone marker *zpr3*. At no point in development, however, can these cells support measurable optokinetic behavior. Although we did not attempt to follow the fate of individual cells in the margin of the *eby* eye, the dynamic picture that emerges from our snapshots is one of a quick turnover of photoreceptors with short life expectancy. Death may trail the last mitosis by only a few hours. Photoreceptor degeneration is largely, but not completely, cell-autonomous in *eby* mutants. A wild-type environment rescues mutant cells only to a slight degree; most of the mutant photoreceptors die off quickly. Wild-type cells also largely degenerate when transplanted into an *eby* mutant host. This experiment needs to be viewed in the context of the results obtained from *ivy* chimeras, where zebrafish photoreceptors are extremely interdependent, requiring a critical number of healthy bystanders to survive (see below). Apoptosis may occur if cells fail to differentiate properly (Parsons et al., 2002). It is therefore possible that some late step of photoreceptor differentiation is affected by the *eby* mutation. Alternatively, the cells may develop normally, but function inadequately. Numerous forms of human RD are due to mutations of components of the phototransduction cascade. The *eby* gene may encode for a gene involved in function or a late differentiation step of photoreceptors.

In *ivy* mutants, the onset of photoreceptor degeneration is more gradual than for *eby* and is delayed into early larval stages. Optokinetic behavior is still detectable in young larvae, but fades in older larvae. Loss of photoreceptors seems to roughly coincide with degeneration of the RPE (although the RPE is hypopigmented early on, long before cell death is observed) and precedes degeneration of the inner retina. There are reports of a trophic interdependence of RPE and photoreceptors (Hewitt et al., 1990). Thus, given that the RPE and other pigment cells in the body are pale in *ivy* mutants, we investigated whether photoreceptors die because they lack support from the RPE. However, in our chimeras composed of wild-type and *ivy* cells, we observed that the degeneration of the photoreceptors was not directly related to the presence or absence of adjacent preserved RPE. Dying photoreceptors were seen next to healthy-looking, black RPE, and normal photoreceptors were found close to pale, mutant RPE. The *ivy* mutation, therefore, appears to eliminate a factor that is essential for both melanin formation and RPE survival, but this factor is also important for photoreceptor survival independent of its RPE function.

When *ivy* mutant cells are transplanted into a wild-type host, then genotypically mutant photoreceptors are nonautonomously rescued by the presence of neighboring wild-type photoreceptors. Conversely, wild-type donor cells grafted into a mutant retina do not form healthy photoreceptors if the donor clones are sparse. However, if the clone of wild-type cells is sufficiently large, not only do the wild-type cells maintain themselves, they also preserve mutant host cells surrounding them. This threshold effect suggests that the *ivy*-dependent signaling pathway requires a critical number of healthy photoreceptors. The rescue effect works over a distance of several cell diameters and may extend to the entire mutant retina given a large enough wild-type clone size. These observations are strong evidence for the existence of a photoreceptor-derived, secreted factor, which diffuses, or is relayed, over a long range, and whose absence leads to retinal degeneration. The *ivy* gene is predicted to encode the factor itself, or a protein necessary for its production.

The cell-autonomy results are summarized for both mutants in Figure 5(A–D). Figure 5(D) schematically depicts a possible mechanism by which *ivy* exerts its density-dependent, nonautonomous effect. The interdependence of photoreceptors, revealed by *ivy*/wild-type chimeras, may explain the mixed autonomous/nonautonomous mechanism seen in the *eby* chimeras. Wild-type cells died in a mutant environment, because in our experiments the wild-type clones did not exceed the critical size. Mutant cells always died (because of the cell-autonomy of *eby*), albeit more slowly in a wild-type host. Thus, the nonautonomous component of *eby* may be attributable to absence of the *ivy* pathway in a photoreceptor-deficient retina. Although this appears to be the most parsimonious model, the existence of an additional secreted factor, dependent on *eby* function but independent of the *ivy* pathway, cannot be excluded at this point.

The observations of photoreceptor interdependence and community effects on survival are not without precedent from studies in mammals (Hewitt et al., 1990; Mohand-Said et al., 2000, 2001; Sahel et al., 2001). For example, transgenic mice expressing a dominant rhodopsin mutation undergo a photoreceptor degeneration that is slowed in wild-type/chimeric animals, and selective transplantation of rod photoreceptors may help maintain cone survival in the RD mouse (Mohand-Said et al., 2000), reaffirming interdependence of rods and cones. Indeed, if one photoreceptor subtype should degenerate due to a specific abnormality in that photoreceptor, a slower degeneration of the “normal” photoreceptor class may follow due to loss of some putative survival factor. One such factor is bFGF, which has been shown to slow the

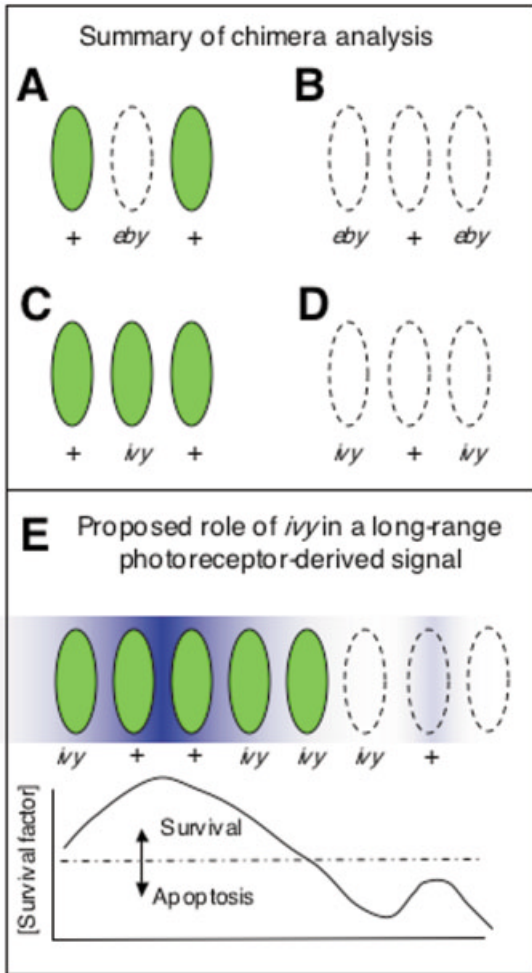


Figure 5 Summary of cell-transplantation experiments (A–D) and a model of *ivy* action (E). (A) *ebv* mutant cells die (hatched outline) when transplanted into a wild-type retina. Wild-type cells survive (green ovals). This indicates the photoreceptor-autonomy of *ebv*. (B) Wild-type cells die when transplanted into an *ebv* mutant retina. This indicates an additional nonautonomous effect of the *ebv* mutation. (C) Individual *ivy* cells survive in a wild-type environment. (D) Individual wild-type cells degenerate when surrounded by *ivy* cells. (C) and (D) show the nonautonomous action of *ivy*. Larger clones of wild-type cells, however, may support each other's survival in an otherwise *ivy* mutant retina (not shown here). (E) Possible involvement of *ivy* in a signaling mechanism between healthy photoreceptors. The signal is predicted to have several properties: it promotes survival, but only above a threshold concentration; it is released by photoreceptors; its effect is, therefore, dependent on the local density of healthy photoreceptors; and, lastly, it may act at a distance. The blue shading indicates the concentration of the survival factor in the example shown here. The concentration profile is depicted in the graph below. Healthy cells (green) are found in regions where the concentration exceeds a critical threshold (hatched/stippled line). These cells may include mutant bystanders. Cells away from such "self-supporting communities" degenerate, regardless of genotype (hatched). The *ivy* gene may encode the survival signal itself or a molecule required for its production.

progression of the retinal degeneration in the RCS rat (Faktorovich et al., 1990). Similarly, coculture of normal retinal explants with transgenic mutant explants decreases the degeneration on account of an unknown factor that can diffuse through a filter (Streichert et al., 1999). Our analysis has revealed the existence of community effects, essential for photoreceptor survival in zebrafish, which appear to be shared between all vertebrates.

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