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The zebrafish *young* mutation acts non-cell-autonomously to uncouple differentiation from specification for all retinal cells

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SUMMARY

Embryos from mutagenized zebrafish were screened for disruptions in retinal lamination to identify factors involved in vertebrate retinal cell specification and differentiation. Two alleles of a recessive mutation, *young*, were isolated in which final differentiation and normal lamination of retinal cells were blocked. Early aspects of retinogenesis including the specification of cells along the inner optic cup as retinal tissue, polarity of the retinal neuroepithelium, and confinement of cell divisions to the apical pigmented epithelial boarder were normal in *young* mutants. BrdU incorporation experiments showed that the initiation and pattern of cell cycle withdrawal across the retina was comparable to wild-type siblings; however, this process took longer in the mutant. Analysis of early markers for cell type differentiation revealed that each of the major classes of retinal neurons, as well as non-neural Müller glial cells, are specified in *young* embryos. However, the retinal cells fail to elaborate morphological specializations, and analysis of late cell-type-specific markers suggests that the retinal cells were inhibited from fully differentiating. Other regions of the nervous system showed no obvious defects in *young* mutants. Mosaic analysis demonstrated that the *young* mutation acts non-cell-autonomously within the retina, as final morphological and molecular differentiation was rescued when genetically mutant cells were transplanted into wild-type hosts. Conversely, differentiation was prevented in wild-type cells when placed in *young* mutant retinas. Mosaic experiments also suggest that *young* functions at or near the cell surface and is not freely diffusible. We conclude that the *young* mutation disrupts the post-specification development of all retinal neurons and glia cells.

Key words: Retina, Zebrafish, Lamination, Neuron, Cell differentiation

INTRODUCTION

During vertebrate retinal development a morphologically homogenous, pseudostratified neuroepithelium gives rise to a highly organized, laminated structure. The mature retina consists of three cellular (nuclear) and two synaptic (plexiform) layers. Six major retinal cell classes are located within three nuclear layers (reviewed by Dowling, 1987). The innermost layer, the ganglion cell layer (GCL), consists primarily of ganglion cells, whereas the middle or inner nuclear layer (INL) is composed primarily of amacrine, bipolar, Müller (glial), and horizontal cells. The outer nuclear layer (ONL), closest to the pigmented epithelium, is made up of rod and cone photoreceptor cell bodies.

Retinal lamination is initiated by terminal cell cycle withdrawal of multipotent precursor cells, and their subsequent migration to appropriate axial positions. Following cellular specification, differentiation and functional maturation occur. Specification – the commitment to become one particular cell type, can be assessed by the expression of cell-type-specific markers. Differentiation and maturation are marked by changes in cellular morphology and the accumulation of the molecular machinery required for the cell's specialized functions. We have taken advantage of zebrafish genetics to study retinal development and the relationship between retinal cell type specification and differentiation.

A general model for retinal cell type specification in vertebrates has emerged from data of several laboratories (reviewed in Cepko et al., 1996; Harris, 1997). The results from cellular birth-dating, lineage analyses, and in vitro studies, suggest that the six major cell types are determined by changes in local signaling microenvironments, as well as restrictions in the competence of progenitor cells to produce different retinal sub-types. [^{3}H]thymidine labeling experiments indicate that specific retinal cell types are generated in a temporally conserved but overlapping order (Sidman, 1961; Hollyfield, 1972; Young, 1985). In most species including zebrafish, ganglion cells, cones, and amacrine cells are the first types generated (Nawrocki, 1985; Schmidt and Dowling, 1996 and 1999). However, predictions of a specific cell type cannot be made based on birth-order alone because cell type genesis is overlapping. Furthermore, specification of retinal cell types is

independent of lineage: cell progeny tracings have revealed clones composed of all classes of retinal cells (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988). Retinal lineage analyses have also demonstrated that specification often, if not always, occurs at or following the terminal mitosis, as two-cell clones were found fated to different retinal cell types (Turner and Cepko, 1987; Turner et al., 1990). These studies underline the importance of differential extrinsic influences and the establishment of microenvironments for dictating cell fate.

Other investigations have demonstrated the role of intrinsic and cell autonomous influences on cellular specification, particularly in a precursor cell's ability to respond to the local environment. Evidence for intrinsic influences derive primarily from heterochronic culture studies and cell labeling experiments. Retinal progenitors cultured from different aged embryos display distinct responses to other cells (Watanabe and Raff, 1990 and Austin et al., 1995, Belecky-Adams et al., 1996) or to soluble factors (Lillien and Cepko, 1992; Kelley et al., 1995), suggesting that the interpretation of specific signaling cues changes with development. In addition, subsets of progenitors distinguished in vivo with the VC1.1 antibody, showed biases in the fates of their progeny (Alexiades and Cepko, 1997). During early periods of retinal development, VC1.1-positive cells produce primarily amacrine and horizontal cells, while VC1.1-negative progenitors give rise primarily to cone photoreceptors. This work demonstrated that distinct groups of mitotic progenitors with intrinsic developmental biases exist in the developing retina.

Following cellular specification, newly fated cells initiate differentiation programs that result in morphological changes and molecular specializations. How retinal differentiation and the establishment of lamination are coordinated remains largely unknown. However, several factors important for differentiation of particular cell classes within the retina have been identified. For example, the homeobox transcription factor Crx has recently been shown to participate in the differentiation program of photoreceptor cells (Furukawa et al., 1997). The POU domain factor, Brn-3b is required for the differentiation of specified ganglion cells (Gan et al., 1999). A third nuclear factor, Chx10/Vsx facilitates bipolar cell differentiation (Burnmeister et al., 1996).

To identify additional factors that participate in differentiation processes, we are screening zebrafish for mutations that disrupt retinal lamination. Formation of a well laminated retina requires that cell type specification and execution of differentiation programs occur normally. Mutations that affect lamination will serve as tools to address questions pertaining to the regulation and relationships between cell-type specification, differentiation, and laminar pattern formation. In this manuscript we describe one recessive mutation *young*. The retinas of *young* (*yng*) mutants appear immature and are void of lamination and morphological differentiation of specific cell types. Results from experiments addressing neuroepithelium integrity, cell cycle withdrawal, and cellular specification, differentiation, and patterning within *young* mutants are described. Lastly, results from chimeric analyses are presented, lending insight to the cellular autonomy of the *young* gene product and revealing intraclonal cell-cell interactions.

MATERIALS AND METHODS

Mutagenesis and screen for retinal lamination

The mutagenesis protocol used was a modification of the method of Solnica-Krezel et al. (1994) and was performed as described by Fadool et al. (1997). To identify defects in retinal lamination, embryos resulting from F_2 incrosses were treated at 18 hours post fertilization (hpf) with 0.2 mM 1-phenyl-2-thiourea (PTU). PTU inhibits melanin synthesis and enabled morphological observation of retinal lamination at 4 days post fertilization (dpf), which would otherwise be obscured by the pigment epithelium. A minimum of 12 embryos from each pairwise cross was screened using differential interference contrast (Nomarski) microscopy at a final magnification of 200×. For screening, embryos were transferred from Petri dishes to depressed-well glass slides containing a small amount of fish water to keep movement of the embryos to a minimum. This procedure did not affect the survival of screened embryos. Clutches that showed 1/4 of the embryos with defects in retinal lamination were identified and the parents recrossed to confirm the phenotype. The parents were outcrossed with wild-type AB fish and the mutant line propagated by successive AB outcrossing. The *young* mutations a8 and a50 show similar and stable phenotypes with complete penetrance in all generations screened to date.

Histology

Embryos were dechorionated and fixed overnight at 4°C in either 4% paraformaldehyde in PBS, pH 7.0 or 2.5% glutaraldehyde/1% paraformaldehyde in phosphate-buffered sucrose, pH 7.4. The next morning, embryos were dehydrated and infiltrated with Epon/Araldite (Schmitt and Dowling, 1996). Transverse sections, 1-2 μm thick, were heat mounted on gelatin-coated glass slides and stained with 1% Methylene Blue in 1% borax.

Antibodies and immunohistochemistry

Embryos were fixed in 4% paraformaldehyde/PBS as described for histology. Following fixation, embryos were washed in PBS and processed for either cryosectioned immunohistochemistry or whole-mount immunohistochemistry. For immunohistochemistry of cryosections embryos were infiltrated with 15% sucrose, 30% sucrose, and 100% Tissue-Tek OCT (Miles Inc.) sequentially for 1 hour each at 25°C. 15-20 wild-type and mutant embryos, were oriented in freezing molds and stored at –80°C until sectioning. 20 μm sections were cut on a cryostat, mounted on gelatin-coated glass slides, and dried for 2-3 hours at 25°C. Slide edges were lined with a hydrophobic marker (PAP pen) and washed with PBS before blocking for 1 hour in 3% normal donkey serum. Blocking solution was replaced with the primary antibody (diluted as described below) and incubated overnight at 4°C. Following 1 hour incubations with fluorescently conjugated secondary $F[ab]_2$ antibody fragments (Jackson Labs), immunolocalized antigens were visualized using confocal microscopy. 5-12 μm optical sections were captured and stored digitally. Whole-mount immunohistochemistry was performed as described by Schmitt and Dowling (1996). The listed antigens and cell types were localized by the following antibodies diluted in blocking solution: mAb 16A11 (5 μg/ml), Hu neuronal RNA-binding protein (Marusich et al., 1994; provided by the Oregon Monoclonal Bank). 39.4D5 (1:10), Islet 1 protein (Ericson et al., 1992; provided by the Developmental Studies Hybridoma Bank). Zn8 (1:6), retinal ganglion cells and their axons (Trevarrow et al., 1990; provided by the Oregon Monoclonal Bank). 7A11 (1:2), ganglion cells and their axons (Chang, 1994). 5E11 (1:20), amacrine cells and their processes (Fadool et al., 1999). HC-II.7 (1:20), horizontal cells and their processes (Young and Dowling, 1984). Zpr1 (Fret43) (1:4), red-green double cones (Larison and Bremiller, 1990; provided by the Oregon Monoclonal Bank). 1D1 (1:1), rod photoreceptors (Fadool et al., 1999). Anti-carbonic anhydrase II (1:400), (Peterson et al., 1997). 3A10 (1:1), neurofilament associated protein (Serafini et al., 1996;

provided by the Developmental Studies Hybridoma Bank). Tu-30 (1:1), gamma-tubulin in centrioles (Nováková et al., 1996). Anti-PCNA (1:100), proliferating cell nuclear antigen (Chemicon MAB4076). Anti-tyrosine hydroxylase (1:100), interplexiform cells (Chemicon MAB318). Anti-GABA, amacrine cells, (Chemicon AB131). Anti-protein kinase C (1:800), bipolar cells, (Chemicon AB1610). Anti-muscarinic AChR2 (Chemicon MAB367).

RNA probes and in situ hybridization

Localization of mRNA in situ was performed as described by Jowett and Lettice (1994) using digoxigenin-labeled antisense RNA probes. Following alkaline phosphatase reaction, embryos were dehydrated and infiltrated with Epon/Araldite and sectioned at 5 μm. Some sections were counterstained with 1% Fast Green. Probe synthesis constructs for the listed genes were generous gifts from the following researchers: *vsx1* and *vsx2* (Passini et al., 1997), Nisson Schechter (State Univ. of New York at Stony Brook, City, State). *rx1* and *rx2* (Mathers et al., 1997), Peter Mathers (West Virginia Univ. School of Medicine, Morgantown, West Virginia) and Milan Jamrich (Baylor College of Medicine, Houston, Texas). *pax2.1, pax2.2* and *pax8* (Pfeffer et al., 1998), Peter Pfeffer (Research Institute of Molecular Pathology, Vienna, Austria).

BrdU labeling

BrdU labeling was performed essentially as described by Hu and Easter (1999). Briefly, mitotic cells were identified at various stages by injecting anesthetized embryos into the yolk with the following DNA incorporation mix: 10 mM 5-bromo-2′-deoxyuridine, 1 mM fluorodeoxyuridine in 1% phenol red (Larison and BreMiller, 1990). Injections were made at least 12 hours prior to the time when the embryos were fixed. As the cell cycle period for zebrafish retinoblasts is less than 10 hours (Hu and Easter, 1999), unlabeled nuclei likely represent postmitotic retinal cells. Following overnight fixation in 4% paraformaldehyde/PBS, embryos were embedded in cryoprotectant and frozen sectioned at 15 μm. Dried sections were washed in PBS and incubated in 2 N HCl at 37ºC for 20 minutes to relax the chromatin and facilitate immunodetection of incorporated BrdU. Acid-treated sections were washed extensively in PBS and processed for double-immunolabeling using a rat anti-BrdU antibody (Harlan Sera Labs MAS-250, diluted 1:1000 in blocking solution) and mouse anti-HNK1 epitope (zn-12, Metcalfe et al., 1990; provided by the Oregon Monoclonal Bank). BrdU was visualized with Cy3-conjugated donkey anti-rat (Jackson ImmunoResearch, 712-165-153) and zn-12 was detected with FITC-conjugated donkey anti-mouse (Jackson ImmunoResearch, 715-095-151).

Cell type quantitation

Cells immunoreactive for specific markers were counted following labeling in transverse oriented, 10 μm cryosections. Immunoreactive cells were visualized with fluorescently conjugated secondary antibodies. Total cells were marked with the DNA dye Hoechst 33258 (Sigma). For each estimation, all immunoreactive cells in central-most retinal sections (as judged by maximum lens diameter) from 5 different eyes were averaged.

Mosaic analysis

Mosaic retinas were created by blastomere transplantation (Ho and Kane, 1990). Clutches of embryos from pairwise *young* heterozygous matings were divided into two groups designated donors and hosts. Donors were dechorionated and injected at the 1-8 cell stage with a lineage tracing label (1:9 mix of Texas Red to biotin-conjugated 10 kDa dextran (Molecular Probes) at a total concentration of 5% w/v in 0.2 M KCl and filtered through 0.22 μm microfuge spin columns). At the 1000 cell stage, 8-40 donor cells were transplanted to the animal pole of dechorionated host blastocysts, the region fated for eye and forebrain (Kimmel et al., 1995). Donor embryos were phenotyped at 3 dpf and host embryos were fixed in 4% paraformaldehyde either 3, 5, or 8 dpf. In this fashion all genotypic mosaic combinations were created and analyzed. Donor cells in host embryos were labeled by whole-mount immunohistochemistry using streptavidin-HRP (Jackson Immunoreasearch Labs) or assessed following cryosectioning. Cryosections were used to investigate co-localization of cell-type specific markers to donor cells. In these experiments primary antibodies were incubated overnight prior to labeling with FITC-conjugated secondary and streptavidin-Cy3. Images were viewed and captured using confocal microscopy.

Clonal composition analysis

Mutant and wild-type donor cells that were transplanted into wild-type hosts were scored as small clones (<10 cells) and mid-sized clones (10-25 cells). Cells in clones larger than 26 cells were not scored as differentiation was often inhibited when the donors were derived from *young* embryos (see Results). Clones were defined as isolated columns of labeled cells further away than 20 cell bodies from another labeled column. Clones were assessed in 3.5 dpf chimeras processed using whole-mount immunohistochemistry and sectioned at 5 μm in the transverse orientation. Only clones found in central sections, between the poles of the lens were counted. One clone per eye was scored in a given chimera. Cells were scored as photoreceptors, horizontal cells, bipolar/Müller glia (we were not able to distinguish reliably between these two cell classes at this stage of development), amacrine cells, and ganglion cells. Classifications were assigned based on morphology and laminar position and in some experiments confirmed by cell-type-specific marker expression. Shifts in clonal compositions between wild-type and donor cells were assessed statistically through Chi-square analysis.

RESULTS

Lamination is disrupted in *young* mutants

Two alleles of a recessive mutation, *young*, that disrupts retinal lamination have been isolated. The mutations *young* a8 and *young* a50 fail to complement each other and are of equal phenotypic strength. *Young* mutants are first identified at 32 hpf by a delay in pigmentation. By 60 hpf, affected embryos are clearly distinct from wild-type siblings owing to slightly smaller eyes, a mild cardiac edema, and often a slight laterally or dorsally flexed tail (Fig. 1A). Embryos appear less active but do respond vigorously to touch. The hearts of *young* embryos are developmentally impaired: atrial and ventricular chambers can be distinguished, but these compartments are dysmorphic and more tube-like than the well defined and tightly looped chambers of wild-type fish. Although *young* hearts beat slower, blood readily circulates throughout the head and to the tip of the tail. By 6 dpf, the cardiac edema has expanded, stretching the heart to the point where it arrests and the *young* embryos die. The subsequent analyses described in this paper will detail the ocular phenotype and the function of *young* with respect to the retina.

We have followed retinal development in normal and *young* embryos by assessing mRNA and proteins that are expressed in specific cell types at defined stages and through histology by observing changes in cellular morphology. For example, neuroepithelial cells in the optic cup that are fated as retinal tissue express transcripts for *retinal homeobox 2* (*rx2*, Mathers et al., 1997) and *visual system homeobox 2* (*vsx2*, Passini et al., 1997). After retinal cell types are specified these transcripts are downregulated, although *rx2* and *vsx2* are later re-expressed in photoreceptor and bipolar cells, respectively. Histologically,

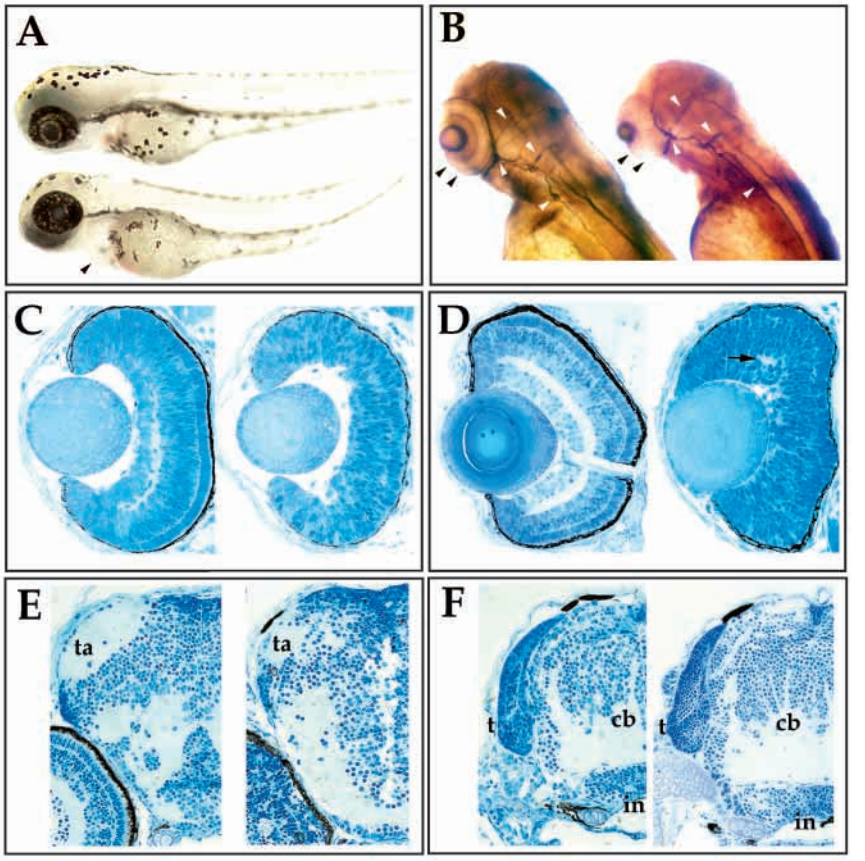

Fig. 1. Morphological characterization of the *young* mutation. (A) Lateral view of wild-type (upper) and *young* (lower) sibling embryos at 60 hpf. Mutant embryos are characterized by swelling of the pericardium (arrowhead) and a slight curvature of the tail. (B) 3A10 immunoreactivity in wild-type (left) and *young* (right) siblings at 72 hpf. Mutant embryos lack retinal plexiform staining (black arrowheads), but contain 3A10-positive axonal tracts in the brain and spinal cord (white arrowheads). (C) Transverse sections of 50 hpf wild-type (left) and *young* (right) retina. (D) Transverse sections of 72 hpf wild-type (left) and *young* (right) retina. Visible lamination is absent in the *young* retina, although small patches of plexiform regions can form (arrow). (E) Transverse sections through the forebrain of wild-type (left) and *young* (right) embryos at 96 hpf. Tectal arborization fields (ta) form in both. (F) Transverse sections through the hindbrain of wild-type (left) and *young* (right) embryos at 96 hpf. The tectum (t), cerebellum (cb), infundibulum (in) have formed in both the mutant and in wild-type fish.

these retinoblasts lose their psuedostratified morphology and become more rounded. Following specification, each cell type expresses an array of markers that changes from early stages to later stages of development. At early stages, many of the markers are shared among different cell classes. Islet1, a LIM homeodomain transcription factor, and Hu, an RNA-binding protein, serve as examples as each are expressed in early differentiating ganglion and other cell classes (Korzh et al., 1993; Marusich et al., 1994). Later, proteins such the zn8 antigen (ganglion cells) or the 5E11 antigen (amacrine cells) are expressed in single cell classes. Morphologically, ganglion cells can be identified by their large cell bodies, lightly staining nuclei, and characteristic axons (Schmitt and Dowling, 1999). Other cell types in the zebrafish retina show pronounced morphological changes during development which facilitates their identification (Schmitt and Dowling, 1996 and 1999). The morphological criteria and molecular markers used for this study are shown in Fig. 7 and Table 2.

While mutations that affect retinal lamination were first identified morphologically in living embryos using Nomarski optics, retinal defects in *young* embryos are more clearly highlighted following whole-mount immunostaining against the neurofilament associated antigen 3A10. Such staining demonstrates a lack of retinal lamination in *young* embryos at a time when the antibody strongly labels plexiform layers in wild-type siblings (Fig. 1B, black arrows). The 3A10 antibody also recognizes major neural tracts in the CNS including those of the cranial sensory ganglia, forebrain and midbrain commissural neurons, hindbrain reticulo-spinal neurons, and spinal projection neurons (Jiang et al., 1996). Each of these tracts is present in *young* embryos, suggesting that the CNS, except for the retina, is relatively unaffected by the *young* mutation (Fig. 1B, white arrows).

Histological analysis at successive developmental stages confirmed a defect in retinal organization. In transverse sections through the eye, *young* mutants were indistinguishable from normal siblings until 36 hpf, when the inner plexiform layer in wild-type fish first becomes defined (Schmitt and Dowling, 1994). By 50 hpf, both the inner plexiform layer and outer plexiform layers, as well as the optic nerve are present (Larison and Bremiller, 1990; Schmitt and Dowling, 1999; Fig. 1C). In *young* mutants at 50 hpf, no plexiform layers are visible (Fig. 1C). By 96 hpf in wild-type embryos, retinal lamina are well-defined and cells have undergone morphological differentiation (Schmitt and Dowling, 1999; Fig. 1D). However, neither lamination nor morphological differentiation occur to any great extent in *young* siblings at this or later times. Occasionally, small plexiform-like areas are seen in *young* embryos (Fig. 1D, arrow). Starting at 80 hpf, pyknotic cells can be found scattered throughout the retina of *young* embryos. Histological analysis also revealed that in addition to the presence of principal axon tracts in the CNS of *young* mutants, the major structures of brain develop relatively normally. For example the arborization fields of the tectum and telencephalon are visible (Fig. 1E). In the tectal arborization fields of *young* embryos, cell bodies are more prevalent than in wild-type embryos. This slight disorganization may be due to the absence of ganglion cell axons which is important for proper tectal organization maintenance (Bagnoli et al., 1989). More caudally, the cerebellum has begun to laminate and the tectum

and infundibulum have formed in both wild-type and mutant embryos (Fig. 1F).

Neuroepithelial cells are normal in the *young* retina

To address whether the cells within the optic cup of *young* embryos are specified as neural retina, we analyzed transcripts for *rx2* and *vsx2*, both of which mark proliferating retinoblasts. All embryos resulting from matings of identified *young* heterozygotes showed high levels of expression for both genes at 24 hpf. At 32 hpf, when the *young* phenotype is discernible, expression for *rx2* and *vsx2* was maintained within optic cup cells of both wild-type and mutant siblings, suggesting that mutant neuroblasts are fated as retinal cells (Fig. 2A and data not shown). In addition, no differences in mRNA expression were detected for *pax2* or *pax8*, markers of the optic stalk and ventral retina, lending further support that early developmental pathways are not disturbed in the *young* retina (data not shown).

Neuroepithelial cells, including those of the nascent retina, show an apical to basal polarity in their organization. Among other features, certain intracellular organelles are localized in discrete zones along the apical to basal axis. For example, the centrioles remain at the apical pole (Bacallao et al., 1989) as the nucleus migrates from the apical to basal surface during the cell cycle (Sauer, 1935). Disruption of retinal neuroepithelial cell polarity has been described for the *oko meduzy* mutation, which later displays patterning defects in the retina (Malicki and Driever, 1999). Gamma-tubulin is one of the major proteins found in the centriole and this microtubule subunit isoform is specific to the sub-cellular structure (Stearns and Kirschner, 1994). Monoclonal antibodies specific to gamma-tubulin show that neuroepithelial cell polarity is normal in mutants as the centrioles localize to the apical portion of cells in both wild-type and *young* retina (Fig. 2B, arrowheads). In addition, metaphase nuclei, visualized with Hoechst DNA dye, localized to the apical mitotic zone in all progeny of *young* heterozygous pairs. These results show that *young* neuroepithelial cells behave normally: optic cup cells show appropriate polarity, become fated to neural retina, and cell divisions are confined to the apical mitotic zone.

The pattern of cell cycle withdrawal is not altered in the *young* retina

Following expansion of the retinoblast pool, but prior to differentiation and lamina formation, neuroepithelial precursor cells must stop dividing. Cell cycle withdrawal initiates at 28 hpf in the inner portion of the ventronasal retina. Retinoblasts continue to exit the cell cycle in a wave-like pattern that sweeps from this ventronasal patch through the dorsal retina, and back to the ventrotemporal region (Hu and Easter, 1999). With respect to radial position, newly born cells migrate to their final level in an inside to outside fashion. Following cell cycle withdrawal, retinal neurons begin to express the zn-12 antigen roughly 12 hours after their birthdate. To address whether the *young* mutation affects the timing, pattern, or ability to withdraw from the cell cycle, we assessed BrdU-incorporation and initiation of zn-12 expression in the manner of Hu and Easter (1999). Saturating pulses of BrdU (greater than 12

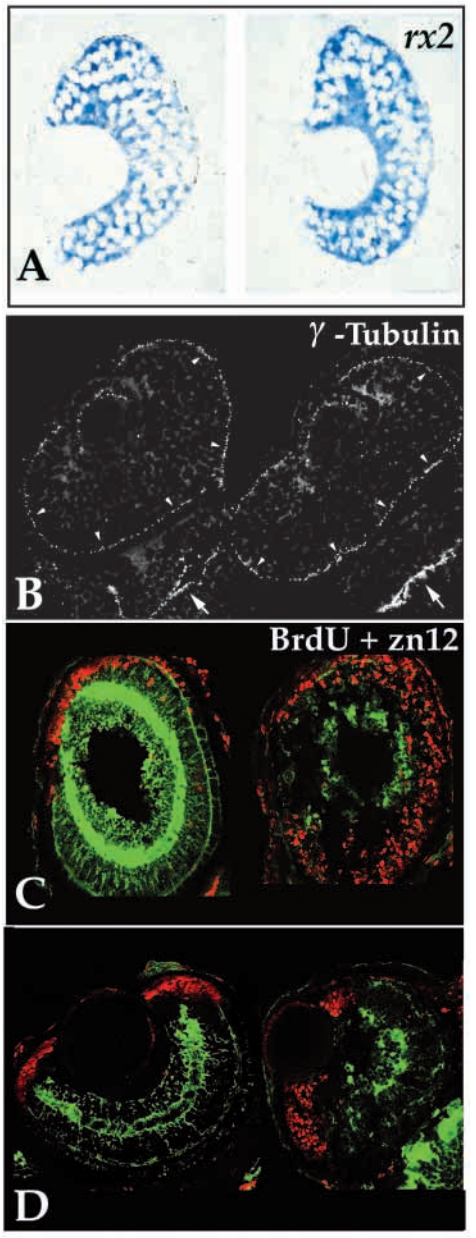

Fig. 2. Analysis of the retinal neuroepithelium in *young* mutants. (A) Transverse retinal sections of 32 hpf wild-type (left) and *young* (right) embryos probed for *rx2* transcript expression. No differences in expression patterns or levels were detected between wild-type and mutants at 32 hpf or earlier. (B) Immunoreactivity for γ-tubulin localizing the centrioles in the retina (arrowheads) and brain (arrows) of two embryos at 24 hpf. Centrioles were found to localize to apical positions in all embryos resulting from *young* heterozygous matings. At 24 hpf, mutant and wild-type embryos could not be consistently distinguished. (C) BrdU incorporation (red) from 62 hpf until 74 hpf when embryos were fixed for assessment of zn-12 expression. Sagittal sections, anterior up, dorsal right, of wild type (left) and *young* (right). In sagittal section the wave of retinal cell birthdating can be viewed in the ventral to dorsal sweep as well as from inside to outside. Note that cell proliferation has nearly ceased by 62 hpf in the wild type, while significant numbers of mutant cells destined for the outer retina, particularly in the dorsal region, continue to divide. (D). BrdU incorporation (74 hpf injection, 96 hpf fixation; red label) and zn-12 immunoreactivity (green label) in transverse sections reveal that only marginal zone cells are proliferating. *Young* retinoblasts (right) have exited the cell cycle similar to those in wild-type siblings (left).

hours) at 30 hpf demonstrated that *young* retinoblasts within the ventral region start to exit the cell cycle similar to wild-type retinoblasts. By 42 hpf in wild-type retina, only cells destined for the outer layers are synthesizing DNA. At this time in the *young* retina, a significantly higher proportion of cells are dividing. Differences in the number of dividing cells were also observed with injections at 62 hpf, but the pattern of withdrawal, from inside to out and ventronasal to dorsotemporal (as viewed in sagittal sections), was the same in both *young* and wild types (Fig. 2C). The expression of zn-12 also progressed in a comparable manner, although the number of cells to do so in the *young* retina was reduced. By 74 hpf only cells at the peripheral margins (when viewed in transverse sections) incorporated BrdU in either mutant or wild-type retinas (Fig. 2D). In zebrafish, as in other fish and some amphibians, these marginal zone cells divide as multipotent stem cells throughout the life of the organism. This experiment shows that the initiation and pattern of retinogenesis is normal in the *young* retina. However, retinogenesis progresses more slowly in the mutant.

Specification of major cell types occurs, but retinal differentiation is inhibited in *young* mutants

Following cell cycle withdrawal, retinoblasts begin expressing cell-type-specific markers. To determine whether cells in the *young* retina are fated to particular cell types, we assessed expression of molecular markers for the various retinal cell classes. In *young* mutants, retinoblasts can become specified to particular cell classes, but full differentiation is inhibited (Fig. 3; Table 2). Furthermore, proper laminar patterning varies among the different classes of retinal cells. For example, cone photoreceptors and Müller cells show significant numbers of ectopically positioned cells, whereas amacrine and ganglion cells are confined to the inner half of the retina.

Ganglion and amacrine cells

Marker analysis and morphological criteria showed that ganglion and amacrine cells were specified in *young* mutant retinas and were relatively well patterned, but complete differentiation did not occur. Islet-1 and the Hu antigen, were expressed early in ganglion and amacrine cells of the *young* retina and localized in a comparable radial position to wild-type siblings. Disrupted differentiation is shown by the reduced expression of zn-8 and 5E11, two late-markers that are specific for ganglion and amacrine cells, respectively, and by the absence of dendrites and an organized inner plexiform layer (Fig. 3A-C). The expression of the muscarinic acetylcholine receptor 2, found on a majority of ganglion cells in wild types, was also never established in the *young* retina (Table 1). In addition to reduced expression of late ganglion cell markers, the optic nerves in the *young* retina were undetectable in 8 of the 14 mutant retina serially sectioned for histology. When

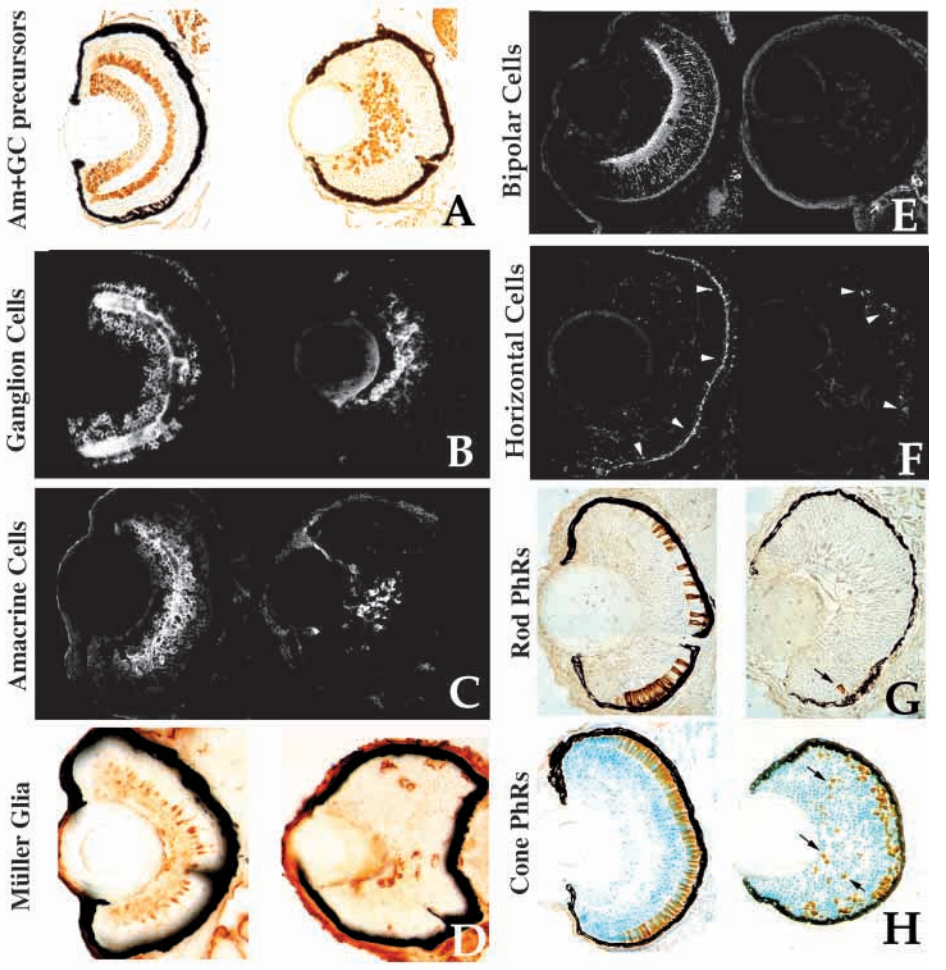

Fig. 3. Retinal cell type marker analysis in *young* mutants. Central, transverse retinal sections of wild-type (left) and *young* mutant (right) siblings assessed for cell-type-specific markers (see Text and Table 1 for details). (A) Hu antigen (ganglion and amacrine cells and their precursors), 3 dpf. (B) 7A11 antigen (ganglion cells and their axons), 3 dpf. (C) 5E11 antigen (amacrine cells and their processes), 3 dpf. (D) Carbonic anhydrase II immunoreactivity (Müller glial cells), 4 dpf. Carbonic anhydrase II is also expressed in non-ocular ectoderm. (E) Protein kinase C immunoreactivity (bipolar cells and processes), 4 dpf. (F) HC-II.7 immunoreactivity (arrowheads) (horizontal cells and their processes), 3.5 dpf. (G) ID1 antigen (rod photoreceptors), 4 dpf. Arrow indicates rod cell in the ventral patch of a mutant. (H) Zn-1 antigen (red and green cone photoreceptors], 4 dpf. Arrows indicate ectopic, mispatterned photoreceptors in the *young* retina.

Table 1. Quantitation of precursor differentiation and cell type maturation for ganglion and bipolar cells

	WT	*young* (%WT)
Precursors		
Hu antigen (ganglion cells)	179.4±19.3	59.2±16.1 (33%)
Islt1 (bipolar cells)	218.4±19.2	98.8±13.4 (45%)
Hoechst (total cells, 60 hpf)	625.2±63.2	487.6±75.0 (78%)
Mature cells		
mAChR 2 (ganglion cells)	137.8±17.9	No expression in the retina
PKCα (bipolar cells)	187.8±23.5	No expression in the retina
Hoechst (total cells, 96 hpf)	729.8±54.4	519.2±63 (71%)

Data represents average ± s.e.m. for the total or the number of immunoreactive cells in a 10 μm transverse, central retina section (n=5).

Table 2. Summary of *young* retinal cell type marker analysis

Cell class	Morphological differentiation	Marker expression	Ectopic retinal location
GC	No	Hu (+), Islet 1 (+) 7A11 (+), Zn8 (+) mAChR 2 (–)	No
Am	No	Hu (++), 5E11 (+), GABA (–), Tyrosine hydrox (–)	No
BiP	No	Islet 1 (+), vsx2 (–/+), PKCα (–)	No
M. Glia	No	Carbonic anhydrase (+)	Yes
Hz	No	Islet 1 (+), HC-II.7 (–/+)	No
Rod	No	1D1 (–/+), rhodopsin (–/+)	No
Cone	No	Fret43 (+), cone opsins (+)	Yes

(–), expression not detected in *young* mutant retina.
(–/+), expression detected, but estimated <10% of wild type.
(+), expression estimated 10%-50% of wild type.
GC, ganglion cells; Am, amacrine cells; BiP, bipolar cells; M. Glia, Müller glial cells; Hz, horizontal cells.

observed, the optic nerves were thin and lacked organized fasciculation. Like other retinal cell classes, amacrine cells can be sub-typed based on multiple criteria (see MacNeil and Masland, 1998). Using markers for the neurotransmitter systems dopamine and GABA, we found no evidence for amacrine cell sub-types in *young* embryos (Table 2; Fig. 6).

Photoreceptors

Curiously, rod and cone photoreceptors, displayed a somewhat different developmental behavior in *young* embryos. Markers for both the rods and cones were detected at 2 dpf in the ventral patch, the initial site of retinal differentiation in normal embryos (Kljavin, 1987; Branchek and BreMiller, 1984; Schmitt and Dowling, 1996). By 3 dpf, about half of the cells that stain with cone markers were found correctly patterned in the distal retina, while the other half were incorrectly located in more vitreal positions. By 4 dpf, the ratio of normally patterned cone cells to those ectopically positioned had increased (Fig. 3H). Morphologically, cells labelled with cone markers appeared rounded and without polarity or outer segments. Rod photoreceptor markers were detected in only 2-3 cells per eye within the ventral patch from 3 dpf onward. The few cells labeled with rod markers were often elongated, more reminiscent of a normal morphology (Fig. 3G).

Bipolar, horizontal and Müller cells

Bipolar cells were initially identified using *vsx2* RNA probes or Islet1 antibodies. Vsx2 undergoes a dynamic expression pattern during zebrafish retinogenesis (Passini et al., 1997). It is first expressed throughout the neuroepithelium but is then restricted to the marginal zone and to cells surrounding the optic nerve. By 72 hpf in the wild-type retina, marginal zone expression has faded and *vsx2* probes decorate bipolar cells in the outer portion of the inner nuclear layer. In *young* embryos at 72 hpf, marginal zone expression was present but only a few *vsx2*-positive cells were detected where bipolar cells normally reside (data not shown). Islet1 expression was more robust in the middle region of the mutant retina (Table 1). Protein kinase C is also expressed in differentiated bipolar cells. Protein kinase C immunoreactivity in wild-type retinas was first detected at 4 dpf in bipolar cells bodies and in their processes in the inner and outer plexiform layers. In *young* retinas, this late marker for bipolar cells was not expressed, consistent with a failure of the bipolar cell to fully differentiate (Fig. 3E). On the other hand, PKC immunoreactivity was detected in forebrain neurons, providing further evidence that non-retinal nervous tissue in *young* embryos is relatively normal (Fig. 3E).

Horizontal cells were marked by their immunoreactivity to the HC-II.7 monoclonal antibody (Young and Dowling, 1984). This antibody stains horizontal cell bodies and their processes. In wild-type retinas at 3.5 dpf, a layer of horizontal cells can be seen extending across the retina just proximal to the outer plexiform layer. In the retinas of mutant siblings, only an occasional HC-II.7-immunoreactive cell was observed in the outer retina (Fig. 3F, arrowheads). Furthermore, these horizontal cells of the mutant retina appeared rounded and immature, lacking the flattened morphology of wild-type horizontal cells.

Müller cells are among the last cell types to be specified in the retina. These non-neuronal glial cells were identified by carbonic anhydrase II immunoreactivity and are well represented in the *young* retina as compared to some of the other cell types such as rods and bipolar cells. In the mutant, glia cell patterning was disrupted. The Müller cell bodies, normally found in the middle to outer portion of the inner nuclear layer, were scattered from the vitreal edge to the outermost portion of the retina (Fig. 3D). In addition, the processes, which in wild-type retinas span the apical to basal width, were randomly directed.

To address whether differentiation was inhibited to the same extent in early born cells versus late born cells, we counted cells expressing either a precursor-cell marker or a maturation marker for an early born (ganglion) and a late born (bipolar) cell type (Table 1). These data suggests that early born and late born cells are inhibited from differentiating in an equivalent manner and argues against an extended developmental delay in mediating the retinal phenotype in *young* embryos. Expression patterns for all of the cell types are summarized in Table 2. This analysis suggests that the *young* gene product is not

required for retinal cell specification as members of each of the major cell classes were found, but proper *young* function is essential for morphological and molecular maturation of all retinal cell types.

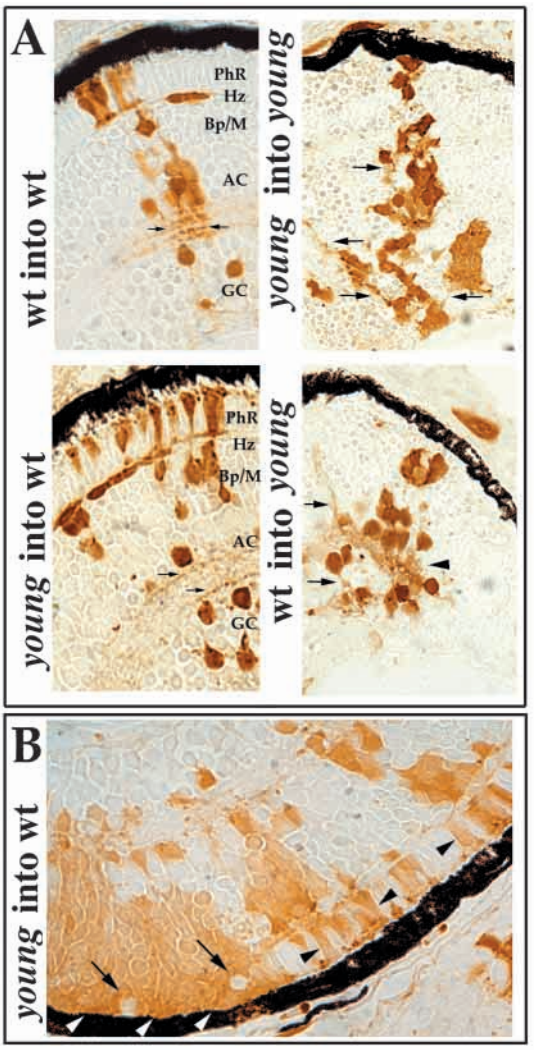

Fig. 4. Morphological analysis of retinal cell differentiation in chimeric eyes. Chimeras were created through blastomere transplantation. Donor cells are labeled brown. (A) Donor clones in wild-type hosts (left two panels in A) that were derived from either wild-type (upper left) or mutant embryos (lower left). Photoreceptors (PhR), horizontal cells (Hz), bipolar and Müller cells (Bp/M), amacrine cells (AC), and GC (ganglion cells) each differentiate in wild-type environments and can be identified morphologically. Note the organization of sub-lamina of donor cell processes in the inner plexiform layer (arrows). Donor clones in *young* mutant hosts (right two panels in A) that were derived from either wild-type (lower right) or mutant embryos (upper right) are prevented from differentiating. Some neuronal processes can extend from cells of either genotype, but these axons and dendrites fail to form an organized plexiform layer (arrows). (B) Large patches of *young* cells in wild-type hosts show local inhibition of morphological differentiation (white arrowheads), whereas smaller clones develop normally (black arrowheads). Local inhibition is conferred on genetically wild-type (host) cells when completely surrounded by mutant cells (arrows).

young function is required non-cell-autonomously within the retina

Mosaic analysis was performed to test the cellular autonomy of the *young* mutation with respect to morphological differentiation in the retina. Genetically mosaic eyes in either *young* or wild-type embryos were generated by transplantation of blastula stage cells (Ho and Kane, 1990) and assessed for cell autonomy by the criteria put forward by Rossant and Spence (1998). The contribution of donor cells to the chimeric tissue was controlled by varying the number of transplanted blastula cells. Small numbers of donor blastula cells (5-10), transplanted to eye-fated regions in the host, resulted in isolated clones where lamination and differentiation could be assessed. All genotypic combinations of mosaics were created and analyzed. Wild-type cells transplanted into wild-type embryos gave rise to clones in which all of the major cell types were present and had differentiated (Fig. 4A, top left). *young* cells transplanted into *young* embryos did not show significant differentiation. However, radial migrations away from the apical ventricular zone did occur in the mutant as labeled cells were found throughout the thickness of the retinas. Some axogenesis and process formation, although limited and disorganized, was observed in these transplanted cells (Fig. 4A, top right). When *young* mutant cells were transplanted into wild-type embryos, morphological differentiation of each of the major retinal cell types was observed, and distinct strata within the inner plexiform layer, indicative of the presence of subtypes of amacrine and ganglion cells, were seen (arrows, Fig. 4A, bottom left). Conversely, when genetically normal cells were transplanted into mutant hosts, the cells were inhibited from differentiating although some disorganized plexiform pockets were established by these cells (arrowhead, Fig. 4A, bottom, right). These experiments strongly suggest that *young* functions non-cell-autonomously with respect to differentiation in the retina.

Large mosaic patches, created by transplanting large numbers of blastula cells (up to 40 cells), suggests that the *young* mutation affects a protein that functions at or near the cell surface. Large islands of *young* clones within wild-type host environments showed differentiation at the edges, but not at the center of the mutant clusters (Fig. 4B, white arrowheads). This local inhibition was also imposed on genetically wild-type cells which were surrounded by mutant cells (Fig. 4B, arrows). Within the same retina, smaller mutant clones – with greater access to the wild-type environment – did not show local inhibition (Fig. 4B, black arrowheads). These results suggest that the affected protein(s) in *young* mutants function at or near the cell surface and also demonstrate that the retinal phenotype is a primary defect and not secondary to another affected developmental process, such as heart differentiation. That is, local inhibition of retinal differentiation in large *young* clones was induced in otherwise normal embryos.

young clonal compositions are biased to late born cell types

A number of *young* clones in wild-type hosts showed an unusually high number of cells differentiating in the outer portion of the inner nuclear layer, the normal position of bipolar and Müller cell nuclei. Three examples of such clones are shown in Fig. 5A (compare to wild-type clones in Fig. 4A). To assess whether there were differences in cell type

Table 3. Cell type compositions in small and mid sized clones

		Clone size			
		Small (<10 cells)		Mid-size (11-25 cells)	
Cell type	Lamina	Wt	*young*	Wt	*young*
PhRs	ONL	0.42	0.42	0.28	0.21
Hz	oINL	0.05	0.07	0.06	0.07
BP+M.Glia	oINL	0.18	0.17	0.25	0.41
Am	iINL	0.14	0.15	0.22	0.19
GC	GCL	0.20	0.19	0.19	0.12
χ^2 significance		P=0.91		P=0.000001	
No. clones analyzed		22	22	16	16

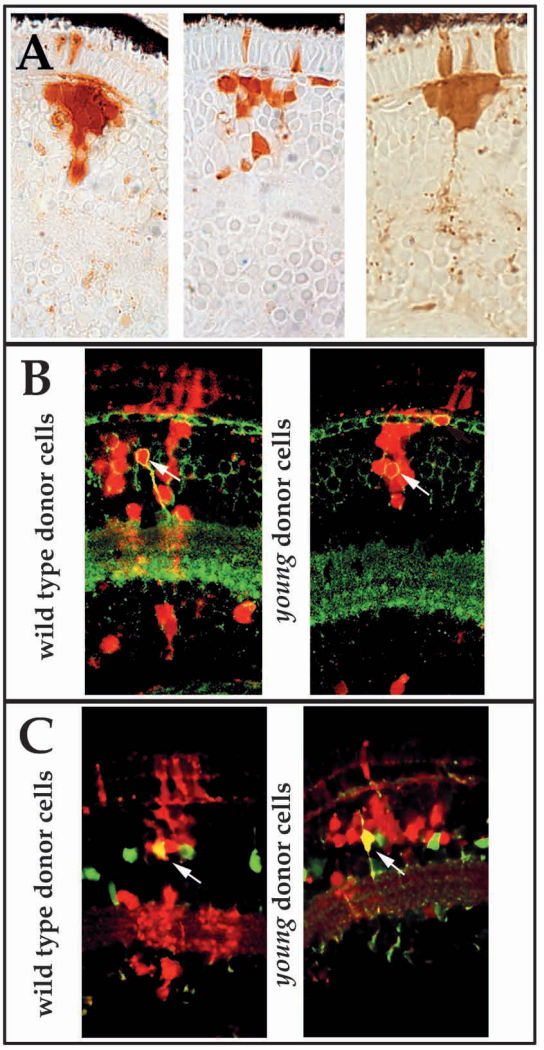

Fig. 5. Some *young* clones in wild-type hosts show a bias for cells of the outer portion of the inner nuclear layer. (A) Examples of three such clones at 3.5 dpf. (compare to clones of 4A). (B) Protein kinase C immunoreactivity marking bipolar cells and their processes in the inner plexiform layer and in close apposition to horizontal cells and photoreceptor terminals in the outer plexiform layer (green) in wild-type (left) and *young* (right) derived clones (red) at 8 dpf. (C) Carbonic anhydrase II immunoreactivity marking Müller glial cells in wild-type (left) and *young* (right) derived clones (red) at 5 dpf. Co-localization of molecular markers with *young* donor cells (yellow cells indicated with arrows) demonstrates that biased mutant clones contain both bipolar and Müller glial cells.

composition of *young* verses wild-type clones, we counted the number of cells for each retinal cell class in either mutant or wild-type clones from cells transplanted into normal host environments. Comparisons were made with mid-sized (10-25 cells) or small (<10 cells) clones. The cells in the center of larger *young* clones (>25 cells) failed to differentiate as described above, precluding clonal composition analysis. Mid-sized clones showed a significant bias towards the late born cell types – bipolar and Müller cells – at the expense of the earlier born cell types – ganglion, amacrine, and photoreceptor cells (Table 3). Because bipolar cell and Müller cell nuclei occupy the same laminar position and their morphology is similar at early developmental stages, we tested whether one of these cell types was excluded in mid-sized *young* clones. However, both protein kinase C and carbonic anhydrase II immunoreactivity were detected in *young* donor clones (Fig. 5B and C). In contrast to mid-sized clones, small *young* clones did not show a bias for retinal cell class (data not shown).

Molecular maturation of *young* cells is restored by contact with wild-type cells

To address the extent of differentiation of mutant retinal cells transplanted into a wild-type environment, we studied the expression of two late markers (tyrosine hydroxylase and GABA immunoreactivity) within transplanted *young* clones.

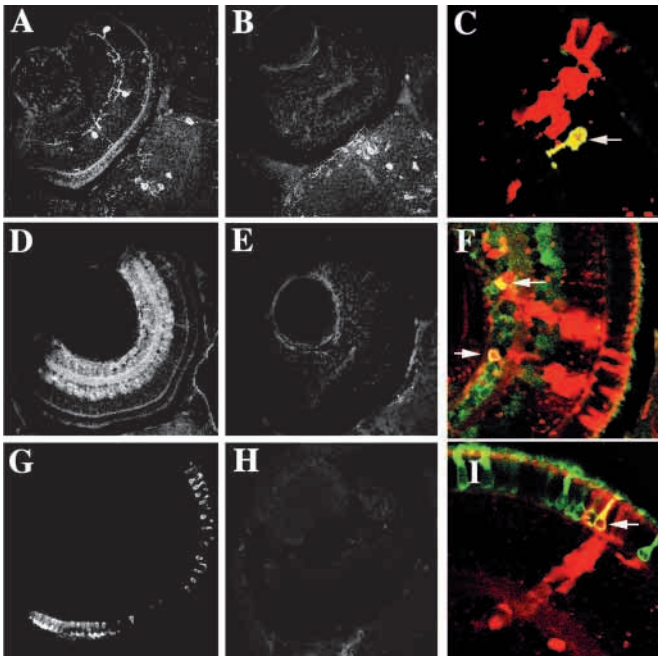

Fig. 6. Rescue of expression of molecular markers in cells derived from *young* embryos when transplanted into wild-type retinas. (A-C) Tyrosine hydroxylase immunoreactiviy (interplexiform cells) in wild-type (A), *young* (B) and chimeric (C) eyes. In chimeric eyes, *young* donor cells (red) can express tyrosine hydroxylase (yellow cell indicated with arrow). (D-F) GABA immunoreactivity (amacrine cells, primarily) in wild-type (D), *young* (E) and chimeric (F) eyes. In chimeric eyes, *young* donor cells (red) can express GABA (yellow cells indicated with arrows). (G, H, I) 1D1 (rod photoreceptors) in wild-type (G), young (H), and chimeric (I) eyes. In chimeric eyes, *young* donor cells (red) can express 1D1 (yellow cell indicated with arrow).

Tyrosine hydroxylase (TH) marks dopaminergic interplexiform cells in the retina (Dowling and Ehinger, 1978) and cells in the forebrain (Ekstrome et al., 1992; Guo et al., 1999). In *young* embryos, dopaminergic forebrain neurons can be found, but retinal TH-immunoreactivity was never observed (Fig. 6B). *Young* retinal cells developing in a wild-type environment do express TH and the labelled cells show appropriate positioning and morphology of interplexiform cells (Fig. 6C). Additional evidence for rescue of *young* cells was gained using antibodies for the neurotransmitter GABA and its synthetic enzyme GAD, which are found in most amacrine cells (Sandell et al., 1994; Fig. 6D). GABA and GAD, like dopamine, are not expressed in the retina of *young* embryos, but their expression can be induced in *young* cells when they are in proximity to wild-type cells (Fig. 6E and 6F, arrows). Finally, rod cells typically do not differentiate in *young* embryos, and when they do, they are restricted to the ventral patch. Mosaic experiments showed 1D1 expression, a rod marker, in mutant photoreceptor cells located within the dorsal and central regions of wild-type retinas (Fig. 6G-I).

DISCUSSION

We have isolated and characterized two zebrafish mutations, *young a8* and *young a50*, in which final cellular differentiation during retinal development is blocked. These mutations were induced independently in separate ENU-mutagenized parental lines, and based on non-complementation tests, similarity of phenotypes, and Mendelian inheritance, we conclude they are allelic and affect a single gene. In *young* embryos, initial specification of all the major retinal cell classes occurs, but final differentiation and maturation for each is inhibited (Fig. 7). In addition, appropriate retinal positioning is abnormal for some cell types including Müller cells and cone photoreceptors, but relatively normal for others such as ganglion, amacrine, and rod photoreceptor cells. Genetic mosaic studies show that *young* functions non-cell-autonomously with respect to retinal development. Differentiation was rescued in mutant cells through contact with wild-type cells as mature morphological phenotypes were achieved for all retinal cell classes. At the molecular level, expression of late markers was recovered for all five of the cell types tested. Border analysis in large mosaic patches suggest that the *young* mutation affects the ability of endogenous retinal cells to promote differentiation in adjacent retinal cells. Cumulatively, these studies with *young* mutations provide evidence that cell type specification can be uncoupled from cellular maturation for all retinal cell classes.

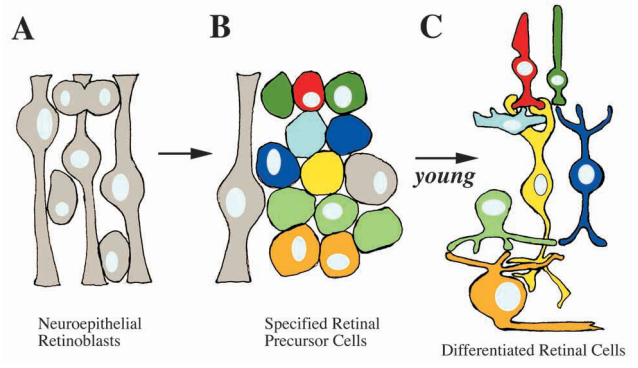

Fig. 7. Retinal development in normal and *young* mutant zebrafish. (A) Dividing neuroepithelial retinoblasts (long grey cells) and newly postmitotic, migrating precursor cells (rounded grey cells) are initially unspecified to a particular retinal cell class. (B) Following cell cycle withdrawal, retinal precursor cells become specified to one cell class and reside in the appropriate laminar position. (C) Morphological differences between retinal cell classes develop in normal zebrafish, but not in *young* mutant embryos, consistent with an essential role of the *young* gene product in final cellular differentiation of all cell classes in the zebrafish retina. Specified precursor cells and differentiated retinal cells are represented with the following colors: ganglion cells (orange); amacrine cells (light green); Müller cells (yellow); bipolar cells (dark blue); horizontal cells (light blue); cone photoreceptors (red); rod photoreceptors (dark green).

Intraclonal cell-cell interactions

Although specification for each of the major retinal cell types occurs with mutations in the *young* gene, alterations in cell type proportions generated within mutant mosaic clones suggest intraclonal cell-cell interactions may influence cell fate decisions. We propose *young* mutations shift cell type proportions within mid-sized clones indirectly, and not by directly regulating specification nor the competence for cell fate inductions. A direct role in governing cell fate is less likely as all cell types are specified in the retina of *young* mutants and no shifts in proportions were observed with small clones in mosaic studies.

In one model for an indirect action of the *young* gene product, mid-sized or large groups of mutant cells within chimeric retina are prevented from differentiating (or not induced) until sufficient access to wild-type cells is achieved. Sufficient access to wild-type cells, and thus differentiation, are delayed in mid-sized clones, while access and differentiation are prevented in larger clones (except for cells at a border to wild-type cells). Delays in differentiation for cells in mid-sized clones may effectively reduce cell fate directing signals contributed by early-born cells within the clone. Alternatively the delay in differentiation may enable a cell's intrinsic mechanisms to specify a late-fate as a function of the time at which the cell within the mutant clone was allowed to start to differentiate.

Relationship between specification and differentiation

In accord with research on retinal cell class specification, studies addressing retinal cell differentiation suggest the involvement of both intrinsic and extrinsic mechanisms. For example, rod photoreceptor cell differentiation has been shown to be modulated extrinsically by laminin (Hunter et al., 1992), taurine (Altshuler et al., 1993), retinoic acid (Kelly et al., 1994; Hyatt et al., 1996), and intrinsically by the transcription factors Nrl (Kumar et al., 1996) and Crx (Chen et al., 1997; Furukawa et al., 1997). Support for extrinsic and intrinsic regulation of ganglion cell differentiation has come from experiments on the secreted factor neuregulin (Zhao and Lemke, 1998) and the nuclear factor Brn-3b (Xiang, 1998; Gan et al., 1999), respectively. Examples for both modes of regulation exist for most of the cell types in the retina. However, these studies all suggest that retinal cell differentiation occurs relatively

autonomously: experimental manipulations affect only one or a few cell types without changes in the differentiation of other cell types.

The identification of a mutation that separates specification from differentiation for all retinal cell types suggests that the ability, or competence, of specified retinal cells to fully differentiate (regardless of cell class) is under regulatory control. The non-cell-autonomous nature of the *young* mutation suggests that this competence is regulated, at least in part, by cell-cell interactions. Once the competence to differentiate into a particular cell type is achieved, intrinsic factors may carry out the maturation process. This is consistent with the observation that retinal differentiation proceeds in a wave from an initial group of differentiated cells (Mann, 1928; Young, 1985). In zebrafish, differentiation commences in the ventronasal region and then rapidly proceeds to dorsal and temporal regions (Klajavin, 1987; Larison and BreMiller, 1990; Schmitt and Dowling, 1996; Hu and Easter, 1999). The *young* gene product may represent a key regulator for coordinated differentiation within the retina.

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