

# Zebrafish *dou yan* Mutation Causes Patterning Defects and Extensive Cell Death in the Retina

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The size of an organ is largely determined by the number of cells it contains, which in turn is regulated by two opposing processes, cell proliferation and cell death, however, it is generally not clear how cell proliferation and cell death are coordinated during development. Here, we characterize the zebrafish *dou yan*<sup>mi234</sup> mutation that results in a dramatic reduction of retinal size and a disruption of retinal differentiation and lamination. The retinal size reduction is caused by increased retinal cell death in a non-cell-autonomous manner during early development. The phenotypic defect in *dou yan*<sup>mi234</sup> arises coincident with the onset of retinal neurogenesis and differentiation. Interestingly, unlike many other small eye mutations, the mutation does not increase the level of cell death in the brain, suggesting that the brain and retina use different mechanisms to maintain cell survival. Identification and further study of the *dou yan* gene will enhance our understanding of the molecular mechanisms regulating retinal cellular homeostasis, i.e., the balance between cell proliferation and cell death. *Developmental Dynamics* 236: 1295–1306, 2007. © 2007 Wiley-Liss, Inc.

**Key words:** retina; *dou yan*; retinal lamination; cell death; small eye

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## INTRODUCTION

The development of multicellular organisms from a fertilized, one-cell egg requires constant and dynamic coordination between cell proliferation, cell differentiation, and cell death. Cell proliferation and differentiation provide the building blocks for ontogenesis, whereas programmed cell death eliminates inappropriate cells and plays an indispensable role in sculpting tissues and organs (Jacobson et al., 1997). The balance between cell proliferation and cell death determines the total number of cells in a given tissue or organ, such as the vertebrate retina, and consequently its

size. The size of the vertebrate retina appears to be largely regulated by intrinsic factors, as demonstrated by the preservation of the small eye size of *Ambystoma punctatum* even when transplanted to a large and fast-growing *Ambystoma tigrinum* host, and vice versa (Stone, 1930). The intrinsic mechanisms that regulate the balance between cell proliferation and death during retinal development are unknown. Cell proliferation and death occur both before and during retinal neurogenesis in all vertebrates (Potts et al., 1982; Sengelaub et al., 1986; Robinson, 1988; Linden et al., 1999; Biehler et al., 2001; Cole and Ross,

2001; Vecino et al., 2004; Donovan and Dyer, 2005), suggesting that early developmental stages are critical in regulating the size of the differentiated retina.

In the past, genetic and molecular analyses of animal models and human patients have revealed several genes that play a role in the control of retinal size. Some of these genes are involved in cell proliferation, such as *Rb*, *Rab11-FIP4*, *Mcm5*, and *XOpx2* (Friend et al., 1987; Zuber et al., 1999; Ryu et al., 2005; Muto et al., 2006). Other genes affect cell differentiation, such as *shh*, *pitx3*, and *dzd* (Neumann and Nusslein-Volhard, 2000; Wang

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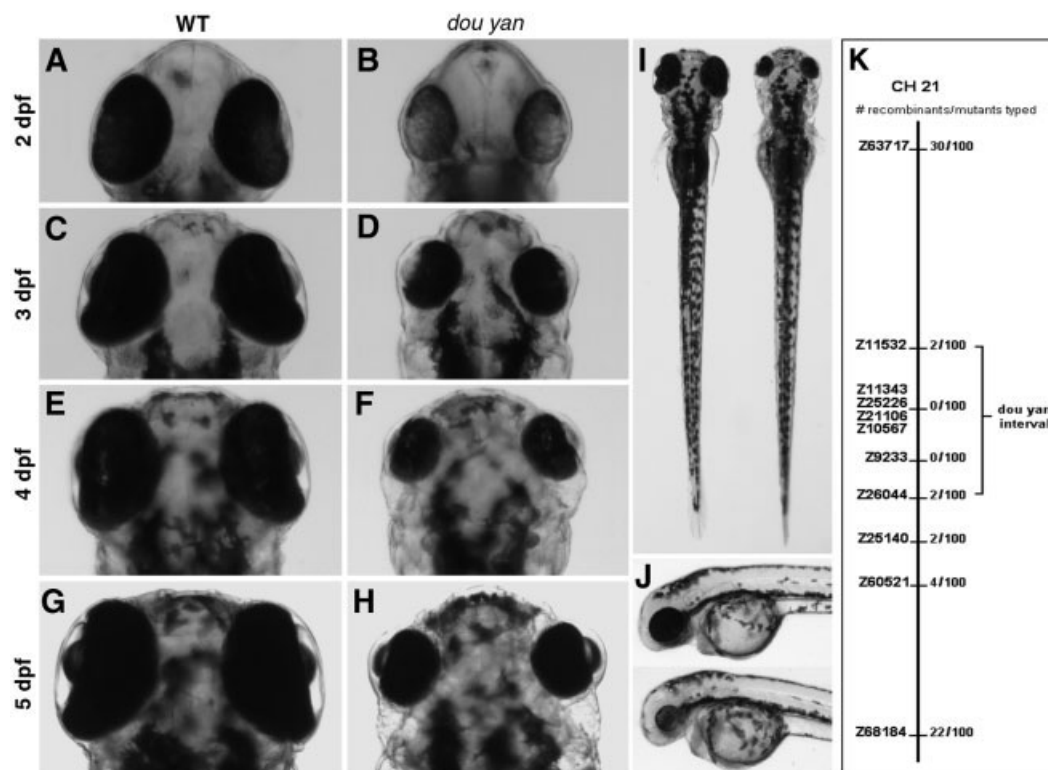
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**Fig. 1.** The zebrafish *dou yan*<sup>mi234</sup> mutation causes reduction in eye size. **A–H:** The small eye phenotype in the mutants is evident at 2 days postfertilization (dpf). The mutant eyes remain smaller than the wild-type eyes at subsequent developmental stages. **I:** The *dou yan*<sup>mi234</sup> mutation does not lead to reduction of body size in mutants. In a dorsal view at 4 dpf, the body of this mutant fish (right) is slightly longer than that of a wild-type sibling (left). **J:** A lateral view of a wild-type (top) and mutant fish (bottom) at 3 dpf shows no paracardiac edema in *dou yan* mutants. **K:** A bulk segregant mapping analysis revealed that the *dou yan* gene localizes to a genomic region that is flanked by markers Z11532 and Z26044 on chromosome 21.

et al., 2005; Shi et al., 2005; Perkins et al., 2005). Still others are necessary for the survival of retinal cells at early development stages, such as *mikre oko*, *oval*, and *elipsa*, or for the survival of retinal cells in adults, such as *pde6b* and *TULPs* (Pittler and Baehr, 1991; Hagstrom et al., 1998; Reme et al., 1998; Doerre and Malicki, 2001, 2002). While the list of genetic disorders that affect retinal size continues to grow, it is unclear how the functions of these genes are coordinated to achieve retinal cellular homeostasis.

Here, we report the isolation of the zebrafish *dou yan*<sup>mi234</sup> mutant line. Phenotypic characterization of the line indicates that the mutation reduces eye size and disrupts retinal lamination during embryonic development. We show that the reduction in eye size is the result of extensive retinal cell death rather than reduced cell proliferation. Of interest, the *dou yan* mutation does not increase cell death in the brain, even though the retina is originally derived from an anterior region of the brain neuroepi-

thelium. We conclude that the product of the *dou yan* gene is essential specifically for retinal cell survival.

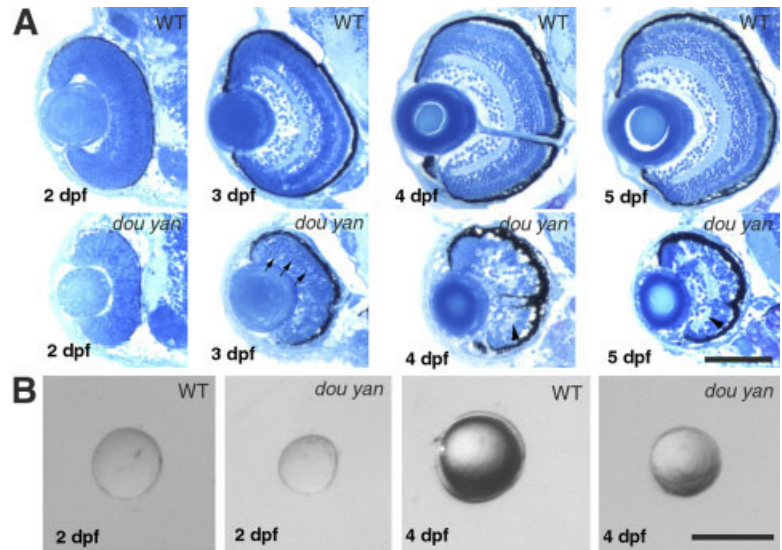
## RESULTS

### *dou yan*<sup>mi234</sup> Mutation Causes a Small Eye Phenotype

We isolated a recessive zebrafish mutant line that displays dramatic reduction in eye size in homozygous embryos. The genetic locus is named *dou yan* (meaning “beady eyes”), and the allele is *mi234* (a serial number of the mutation isolated from a Michigan mutagenesis screen). A bulk segregant mapping analysis revealed that the *dou yan* gene is located between markers Z11532 and Z26044 on chromosome 21 (Fig. 1K). Before 28 hours postfertilization (hpf), the eye size phenotype is not distinguishable between wild-type and *dou yan*<sup>mi234</sup> mutant embryos (data not shown). At 36 hpf, the eyes of the mutant embryos start to appear smaller than

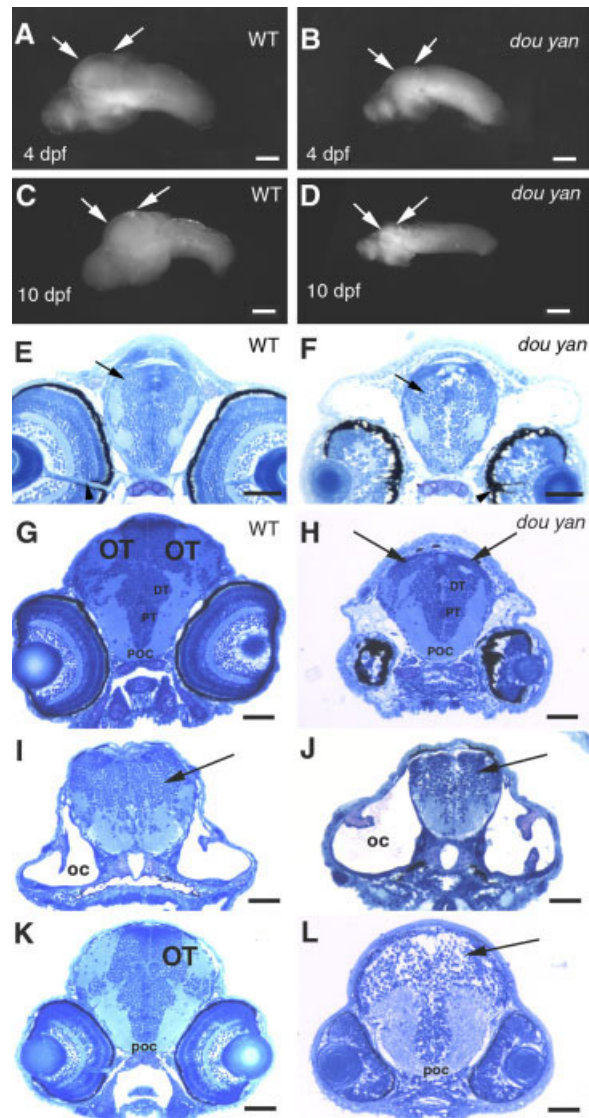
their wild-type siblings. However, genotyping by the size of the eyes is only approximately 80% accurate at this stage, likely due to developmental variations among individual embryos. By 48 hpf, the small eye phenotype becomes evident in 100% of the mutants. At this stage, the diameter of the mutant eyes along the anterior–posterior (AP) axis is approximately 70% of the diameter of wild-type eyes (Fig. 1A,B). The size difference between mutant and wild-type eyes increases over time. By 5 days postfertilization (dpf), the mutant eyes are only approximately 50% of the diameter of the wild-type eyes along the AP axis (Fig. 1A–H). Histological analysis indicates that the small eye phenotype can be primarily attributed to the small size of the mutant retinas (Fig. 2A). In addition, we observed approximately 6% and 23% reduction in lens diameter at 48 hpf and 4 dpf, respectively (Fig. 2B). Comparing to the 30% and 50% reduction in the eye size as a whole at the same stages, lens reduction is a secondary cause of the eye

reduction. Because the retinal pigment epithelium (RPE) is only a single layer of flat cells, the RPE does not influence the diameter of the eye, even though it also shrinks and maintains a tight contact with the retina in the mutants (Fig. 2A). Because retinal neurogenesis starts at approximately



**Fig. 2.** The *dou yan*<sup>mi234</sup> mutation disrupts cytoarchitecture of the retina and reduces the retinal size more severely than it does to the lens. **A:** Histological analyses sections of wild-type and mutant retinas at 2, 3, 4, and 5 days post-fertilization (dpf) reveal that retinal lamination is severely disrupted in the mutant eyes. Arrows indicate the darkly stained pyknotic nuclei in the mutant retina. Arrowheads indicate the disrupted plexiform layers. **B:** The isolated lenses of mutant embryos are 6% and 30% smaller than their counterparts of wild-type embryos at 48 hours postfertilization (hpf) and 4 dpf, respectively. Scale bars = 100  $\mu$ m.

**Fig. 3.** The *dou yan*<sup>mi234</sup> mutation preferentially affects the development of the optic tectum more than it does the other regions of the brain. **A,B:** Lateral views of the isolated wild-type (A) and mutant (B) brains at 4 days postfertilization (dpf) confirm that most regions of the mutant brain are only slightly smaller than those of the wild-type brain. However, the optic tectum is drastically reduced in the mutant embryos (arrows). **C,D:** The arrows indicate the optic tectum regions of brains isolated from wild-type (C) and surviving mutants (D) at 10 dpf. **E,F:** Cross-sections of the heads of wild-type (E) and mutant (F) embryos at the optic nerve region indicate similar forebrain size at 4 dpf, with the dorsal-ventral diameter of the mutant forebrains (arrows) being approximately 94% of that of the wild-type brains. The overall patterning of the gray (cell bodies and nuclei) and white (neuronal processes) matter is similar between the mutant and the wild-type embryos. Arrowheads indicate the optic nerves, which serve as a reference point for comparable section planes. **G,H:** The cross-sections of 4 dpf wild-type (G) and mutant (H) heads through the mid-brain region reveal that the optic tectum (OT) is almost missing in the comparable region in the mutant embryos (arrows, H). However, the posterior commissure (POC), posterior tuberculum (PT), and the dorsal thalamus (DT) are comparable between mutant and wild-type embryos. **I,J:** The cellular patterning of the hindbrain mutant (J) is similar to that of the wild-type brain (I) at 4 dpf. Arrows indicate the hindbrains. The otic capsules (OC) were selected as a reference point for comparable section planes. **K,L:** At 3 dpf, cross-sections through the midbrain revealed the optic tectum in wild-type embryos (K). However, no optic tectum-like structure is evident in the mutant embryos (L) at the corresponding region (arrow). The posterior commissure (POC) is selected as a reference point for matching comparison. Scale bars = 100  $\mu$ m in A,B,C–G, 50  $\mu$ m in E,F,G–L.



**Fig. 3.**

28 hpf with the differentiation of the first retinal ganglion cells (Hu and Easter, 1999), the timing of the onset of the small eye phenotype suggests that the *dou yan*<sup>mi234</sup> mutation does not affect the genes that are involved in specification of the eye field, formation of the optic primordium, or early morphogenesis of the eye.

A reduction of organ size is apparent only in the eyes from an external examination. The body size of the *dou yan*<sup>mi234</sup> mutants is comparable to that of wild-type, and on average, the mutants are actually 2–3% longer than their wild-type siblings at 4 dpf (Fig. 1I). In addition, unlike other retinal patterning mutants such as *nok*, where the body axis is curled and a paracardiac edema develops as early as 36 hpf (Malicki et al., 1996), the body axis of the *dou yan*<sup>mi234</sup> mutant is as straight as the wild-type fish (Fig. 1I), with no development of paracardiac edema (Fig. 1J). However, abdominal edema and paraocular edema start to develop at 5 dpf (data not shown). The *dou yan*<sup>mi234</sup> mutant embryos are viable up to approximately 10 days. It is uncertain what the cause of their eventual death is. Together, our observations suggest that the *dou yan* gene is essential for maintaining the proper size of the retina during early development.

### *dou yan*<sup>mi234</sup> Mutation Preferentially Affects Retinal Structure

To determine whether the *dou yan*<sup>mi234</sup> mutation also affects the cellular architecture of the retina, we examined JB4 plastic sections of mutant and wild-type embryos between 2 dpf and 5 dpf and found that retinal lamination is disrupted by the mutation. In wild-type retinas, retinal lamination develops between 2 dpf and 5 dpf (Fig. 2, top panel). As they differentiate, retinal neurons are segregated into three distinct nuclear laminae according to their identity, and the plexiform layers containing neuronal processes separate the layers containing cell bodies. In the mutant retinas, the plexiform layers are segregated into islands of tangled neuronal processes (Fig. 2, arrowheads). In addition, many darkly stained pyknotic nuclei are observed in the mutant retinas,

especially at 3 dpf (Fig. 2, arrows). In contrast, by examination of the isolated 4 dpf brains, we found that the size of the mutant brains is similar to that of the wild-type brains, except for an apparent reduction of the optic tectum (Fig. 3A,B). At 10 dpf, the mutant brain of surviving fish is similar to the mutant brain at 4 dpf, although slightly smaller (Fig. 3C,D). Histological analysis confirmed that the cellular architecture, such as the pattern of gray and white matter, of the forebrain and hindbrain of the mutant is similar to that of wild-type (Fig. 3E,F,I,J). The cross-sections of 4 dpf wild-type and mutant through the midbrain region confirm that the optic tectum is almost missing in the mutant embryos (Fig. 3G,H). However, in the same cross-sections, other brain structures, such as the posterior commissure, posterior tuberculum, and the dorsal thalamus are present and appear to be healthy in the mutant embryos (Fig. 3G,H). We then analyzed the midbrain region at an early stage, 3 dpf. It revealed that the cells in the potential optic tectum region do

not organize properly as in wild-type (Fig. 3K,L). We infer that the overall function of the nervous system, except for the visual system, is not dramatically disrupted in the mutants. This inference is supported by the observation of a normal touch response in mutant embryos (data not shown). Thus, we conclude that the *dou yan*<sup>mi234</sup> mutation specifically disrupts the development of the retina and the optic tectum, or at least the effect is much more profound in the visual system than elsewhere in the nervous system.

### *dou yan*<sup>mi234</sup> Mutation Causes Extensive Cell Death in the Retina but Not in the Brain

Because pyknotic nuclei are a hallmark of dead cells, the observation of pyknotic nuclei in the mutant retinas suggests that many retinal cells are dying prematurely during the early stages of retinal neurogenesis and differentiation (Fig. 2, arrows). To confirm this prediction, we performed acridine orange vital staining of living

**Fig. 4.** The *dou yan*<sup>mi234</sup> mutation causes extensive cell death in the retina but not in the brain or lens. **A–F:** Staining with acridine orange (AO, green), which preferentially stains the nuclei of dead or dying cells, indicates extensive cell death in the central regions of the mutant retinas (D–F, arrows) compared with the wild-type retinas (A–C). The cell nuclei are stained with TO-PRO-3 (red). Cell nuclei with an elongated shape are found at the marginal zone of the mutant retina (D–F, arrowheads), similar to the germinal zone of proliferating cells found at the retinal margin in wild-type retinas at this stage (A–C). In the lenses (asterisks), no increase in cell death was observed in *dou yan*. However, nonspecific acridine orange staining is sometimes observed in non-nuclear regions in the lens. **G,H:** Cell nuclei that are heavily stained with acridine orange are mainly found in the mutant retina (arrows) but not in the brain (asterisks). Scale bars = 20  $\mu\text{m}$  in A–F, 50  $\mu\text{m}$  in G,H.

**Fig. 5.** The *dou yan*<sup>mi234</sup> mutation causes non-cell-autonomous retinal cell death. **A–C:** Clones of dextran-labeled wild-type donor cells (red) in the wild-type retina form vertical columns of cells with a differentiated morphology that span all three retinal nuclear layers (arrows). The cell nuclei were stained with YO-PRO-1 (green); C is the merged image of A and B. **D–F:** Extensive punctate staining of donor cells (red, arrowheads in E) was observed in the mutant retinas, indicating that many wild-type donor cells were already dead at the time of fixation. Some donor cells appeared intact in the mutant retina, but they had an elongated morphology typical of undifferentiated retinal progenitors (E, arrow). Pyknotic nuclei heavily stained with YO-PRO-1 are indicated with arrows in D. **G:** A histogram illustrates the percentages of dead wild-type donor clones in four host conditions: in wild-type brains, in wild-type retinas, in mutant brains, and in mutant retinas. The total number of clones counted in each condition is indicated in parentheses. Comparison of the percentages of dead donor cell clones with the  $\chi^2$  test indicates that the increase in dead wild-type donor clones in the mutant retina, but not the brain, is statistically significant. **H–J:** Healthy-appearing *dou yan* mutant donor cells (red, arrows) develop properly in the wild-type retina and can localize to all three retinal cellular layers. The cell nuclei are counterstained with YO-PRO-1 (green). **K–M:** Large amounts of punctate staining foci derived from mutant donor cells (arrowheads) were observed in mutant host retinas, indicating that many of the donor cells were already dead at the time of fixation. **N:** A histogram illustrates the percentages of dead clones of mutant donor cells in four host conditions: in wild-type brains, in wild-type retinas, in mutant brains, and in mutant retinas. The total numbers of clones counted in each condition are indicated in parentheses. Comparison of the percentages of dead donor cell clones with the  $\chi^2$  test indicates that statistically more mutant donor cells survived in the wild-type retinas than in the mutant retinas at the time of examination. Scale bars = 10  $\mu\text{m}$ .

fish at 3 dpf. Acridine orange preferentially stains the cell nuclei of dying and dead cells (McCall and Peterson, 2004). Numerous round and condensed nuclei showing strong acridine orange staining were found in the mutant retinas (Fig. 4D–F, arrows) but not in the wild-type retinas (Fig. 4A). These pyknotic nuclei are concentrated in central regions of retina and are scattered throughout the thickness of the retinal tissue. However, in the proliferative germinal zone at the ciliary margin of the mutant retinas, most cell nuclei display a normal, elongated morphology (Fig. 4D–F, arrowheads). At 3 dpf, this region contains undifferentiated, proliferating cells, while the central retina predominantly consists of differentiating cells, suggesting that the extensive retinal cell death caused by *dou yan*<sup>mi234</sup> primarily affects postmitotic cells. This inference is consistent with the observation that retinal size is not affected and cell death is not observed in *dou yan* mutants until after 28 hpf,

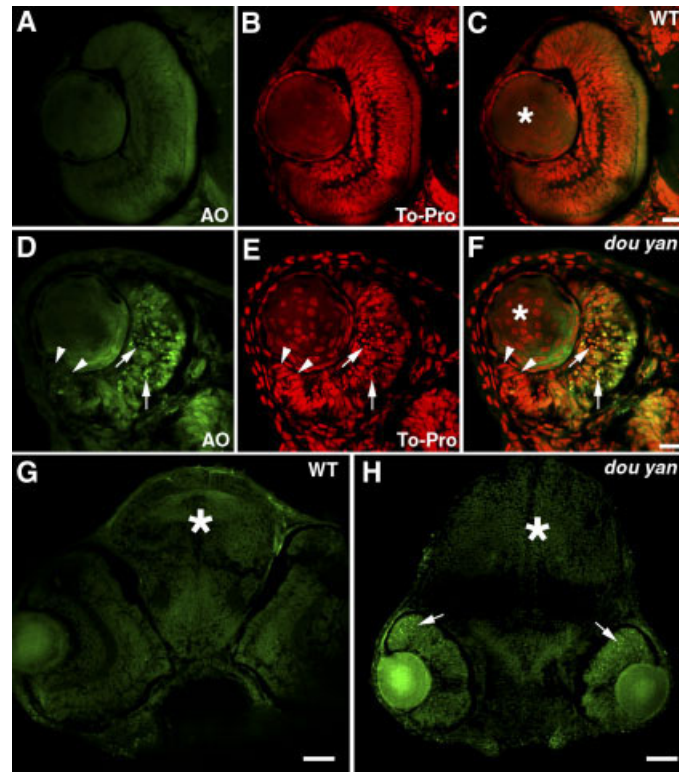


Fig. 4.

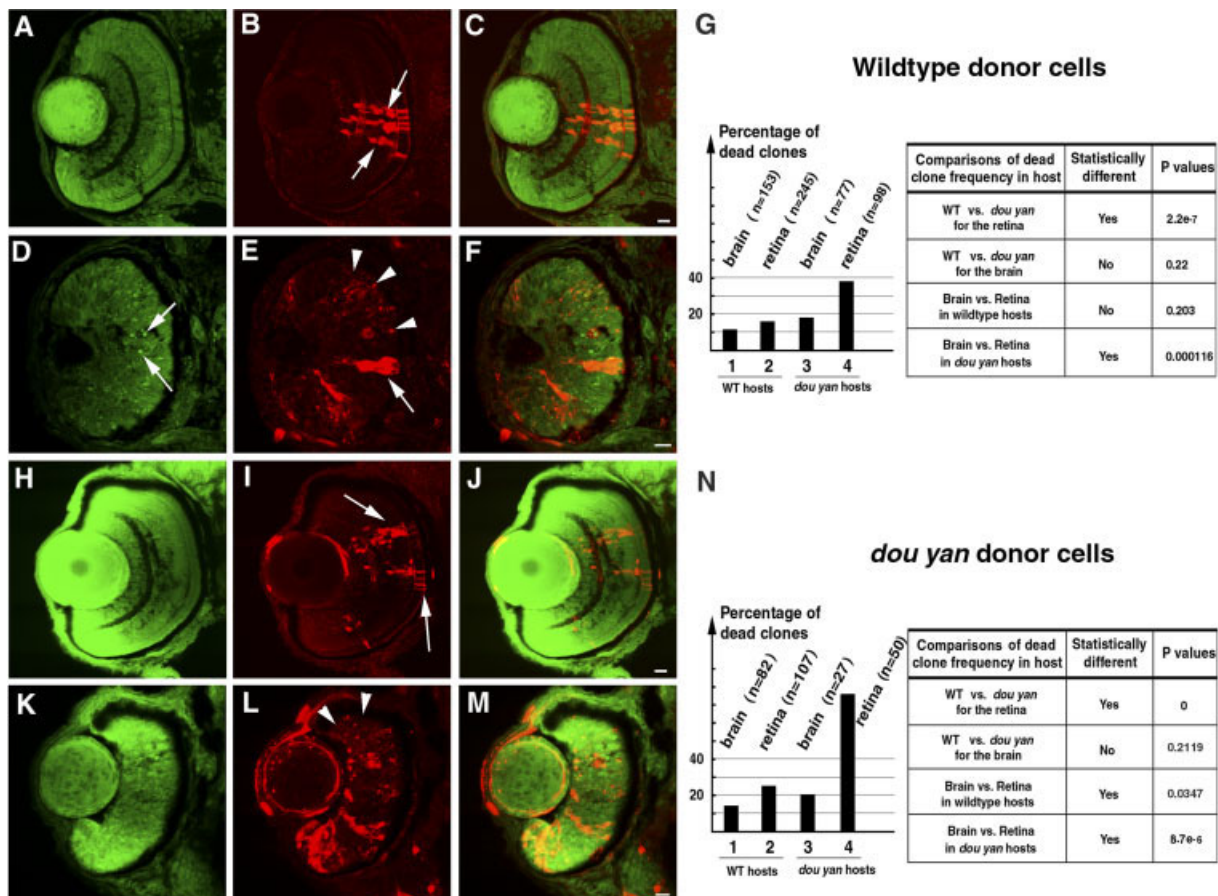


Fig. 5.

when retinal cells begin to differentiate (Hu and Easter, 1999). In contrast, the brains of mutant fish display the same level of cell death found in the wild-type brains (Fig. 4G,H). We also did not observe an elevation in cell death in the mutant lenses by acridine orange nuclear staining (Fig. 4A–F). In the mutant embryos, the RPE tightly juxtaposes the retina, and also displays a reduction in size. However, due to the monolayer structure and the low density of the cell nuclei in the RPE, our observation of acridine orange staining could not yield conclusive evidence regarding whether the level of cell death in the RPE is increased by the mutation. We conclude that the *dou yan* mutation causes extensive cell death in the retina but not in the brain or lens.

### ***dou yan*<sup>mi234</sup> Mutation Causes Retinal Cell Death in a Non-Cell-Autonomous Manner**

Cell death can result either from signals within the cell or from external environmental triggers. To determine whether the increased cell death observed in *dou yan*<sup>mi234</sup> mutant retinas is cell-autonomous or non-cell-autonomous, we created genetic mosaic embryos by performing transplantation analysis with biotinylated dextran-labeled donor cells. In living cells, the dextran label diffuses throughout the cytoplasm of donor cells and highlights their morphology (Fig. 5B, arrows). However, when donor cells die, the dextran molecules become concentrated in cell debris and appear as small, brightly stained foci (Fig. 5E, arrowheads). Consequently, the staining pattern reflects donor cell viability in the host retinas. To quantify viability of wild-type donor cells after transplantation, we counted the numbers of living and dead clones in both the brains and the retinas of wild-type and mutant hosts. The results indicate that wild-type donor cells transplanted into mutant retinas die at significantly higher rates than wild-type cells transplanted into wild-type retinas (Fig. 5G,H). We cannot exclude the possibility that some closely spaced dead clones might have been mistakenly scored as one clone, so our counts of dead clones might actually

underestimate the actual numbers. Of interest, the *dou yan*<sup>mi234</sup> mutation does not increase the level of cell death in the brain as much as in the retina (Fig. 5G,H). Furthermore, the reciprocal experiment of transplanting mutant cells into wild-type embryos revealed that the wild-type retinal environment promotes the survival of mutant cells (Fig. 5H–N). We thus conclude that the *dou yan*<sup>mi234</sup> mutation causes retinal cell death in a non-cell-autonomous manner. These observations indicate that the mutation specifically alters the microenvironment in the retina and reduces retinal cell survival.

### ***dou yan*<sup>mi234</sup> Mutation Does Not Inhibit Cell Proliferation in the Retina**

To determine whether or not cell proliferation is also affected by the *dou yan*<sup>mi234</sup> mutation and consequently contributes to the small eye phenotype, we analyzed the level of active cell proliferation. We stained the retinas with anti-phospho-Histone H3 (pH3) antibody, which specifically stains cell nuclei in M-phase (Fig. 6A–F). We calculated the average number of positive cells per 3,600  $\mu\text{m}^2$  of retinal section, and we found no significant difference between the relative numbers of pH3-positive cells in mutant retinas vs. the wild-type retinas at 40, 48, and 72 hpf (Fig. 6G, with *P* values of 0.7129, 0.3729, and 0.6734, respectively). Bromodeoxyuridine (BrdU) labeling at 49 to 51.5 hpf verified the existence of active DNA replication in mutant and wild-type retinas (Fig. 6H,I). Thus, the *dou yan*<sup>mi234</sup> mutation appears not to grossly affect cell proliferation dynamics in the zebrafish retina. This conclusion is consistent with the observation that the mutant retinas do not appear smaller before 28 hpf when the retinal primordium consists entirely of proliferating progenitor cells.

### **Distinct Classes of Retinal Cells Are Specified in *dou yan***

We next investigated whether or not the *dou yan*<sup>mi234</sup> mutation affects retinal cell specification. We performed immunohistochemical analysis with

cell type-specific markers to determine whether red/green double cones, interplexiform cells, rods, Muller cells, ganglion cells, and lin7-positive bipolar cells differentiated in the mutant retinas. We found that all of these cell-specific markers appeared in *dou yan* mutant retinas, although the labeled cells were greatly reduced in number compared with wild-type retinas at the same developmental stages (Fig. 7A–T). We did not find any cells that simultaneously expressed two markers that are normally expressed in different retinal cell classes, but the morphologies of the labeled cells were markedly different from their wild-type counterparts. For example, photoreceptors in the mutants did not have the typical elongated shape (Fig. 7B,F), and Muller cells did not extend processes to the apical and basal ends of the retina (Fig. 7K,O). We next performed a similar analysis with embryos at 78 hpf. We found that a considerable number of ganglion cells are specified in the mutants at 78 hpf. However, many late-appearing cell types such as photoreceptors are not specified in most of the embryos at this stage (Fig. 8). Of interest, the Lin7 is distributed throughout the mutant retinas, with an elevated expression level in the ganglion cell patch (Fig. 8F). This broader expression pattern of Lin7 resembles its expression at around 48 hpf in wild-type (Wei et al., 2006a). These results suggest that the cell differentiation process is delayed in the mutant retinas. Taken together, we conclude that, although cell specification apparently occurs in *dou yan* mutant retinas, the level of cell specification is reduced and cell differentiation is abnormal. Further investigation is needed to determine to what extent the extensive retinal cell death and delayed cell differentiation process contribute to the reduction in the amount of cells for each distinct retinal cell type.

### ***dou yan*<sup>mi234</sup> Mutation Does Not Affect Retinal Epithelial Polarity**

Several retinal patterning mutations with phenotypes similar to *dou yan* (e.g., lack of retinal lamination) have been identified in earlier studies (Horne-Badovinac et al., 2001; Wei

and Malicki, 2002; Malicki et al., 2003; Jensen and Westerfield, 2004), and all of these mutations disrupt epithelial polarity of the retina at early stages of development. To determine whether retinal epithelial polarity is also affected by the *dou yan*<sup>mi234</sup> mutation, we compared the distribution pattern of the apical polarity markers ZO-1, adherens junction-associating actin bundles, and M-phase nuclei in wild-type and mutant retinas. Because the mutant embryos cannot be phenotyped before 28 hpf, we first examined 48 hpf embryos. As shown in Figure 9, wild-type and mutant retinas do not differ in terms of the localization of these apical markers. To examine apical epithelial polarity at earlier stages, we randomly chose 20 embryos at 27 hpf from an incross of *dou yan* heterozygous parents (from which we would expect five, or one quarter, of the embryos to be homozygous mutant), and we found no differ-

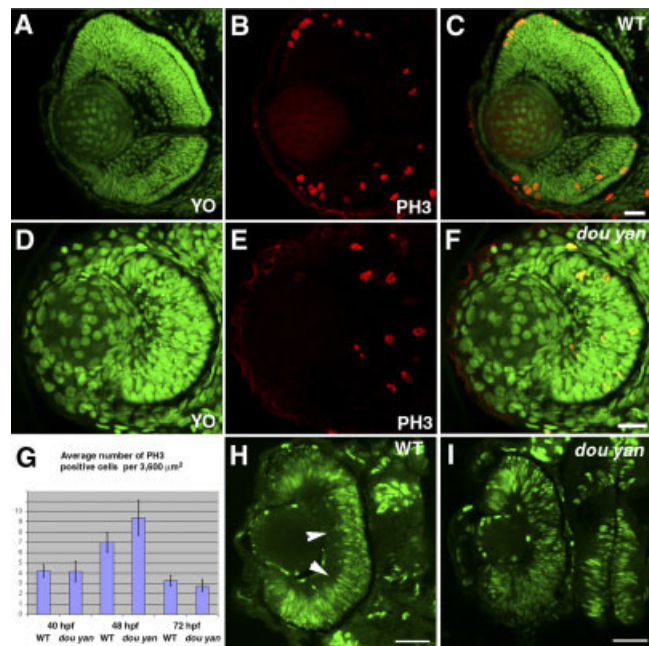


Fig. 6.

**Fig. 6.** The *dou yan*<sup>mi234</sup> mutation does not disrupt cell proliferation in the retina. **A-F:** M-phase nuclei, visualized by anti-phospho-Histone 3 antibody (PH3, red), are observed in both wild-type (A-C) and mutant retinas (D-F) at 3 days postfertilization (dpf). All nuclei are stained with YO-PRO-1 iodide (YO, green). C is the merged image of A and B. F is the merged image of D and E. **G:** The average number of PH3-positive cells per 3,600 μm<sup>2</sup> in mutant retinas is similar to wild-type retinas at 40 hours postfertilization (hpf), 48 hpf, and 72 hpf. The error bars denote the standard error of the mean. **H:** Bromodeoxyuridine (BrdU) labeling of the wild-type retina between 49 hpf and 51.5 hpf reveals proliferating cells in most regions of the retina. Arrowheads indicate BrdU-negative basal regions in the wild-type retina. **I:** BrdU labeling of *dou yan* mutants between 49 hpf and 51.5 hpf confirms that many retinal cells are actively synthesizing DNA. Scale bars = 20 μm.

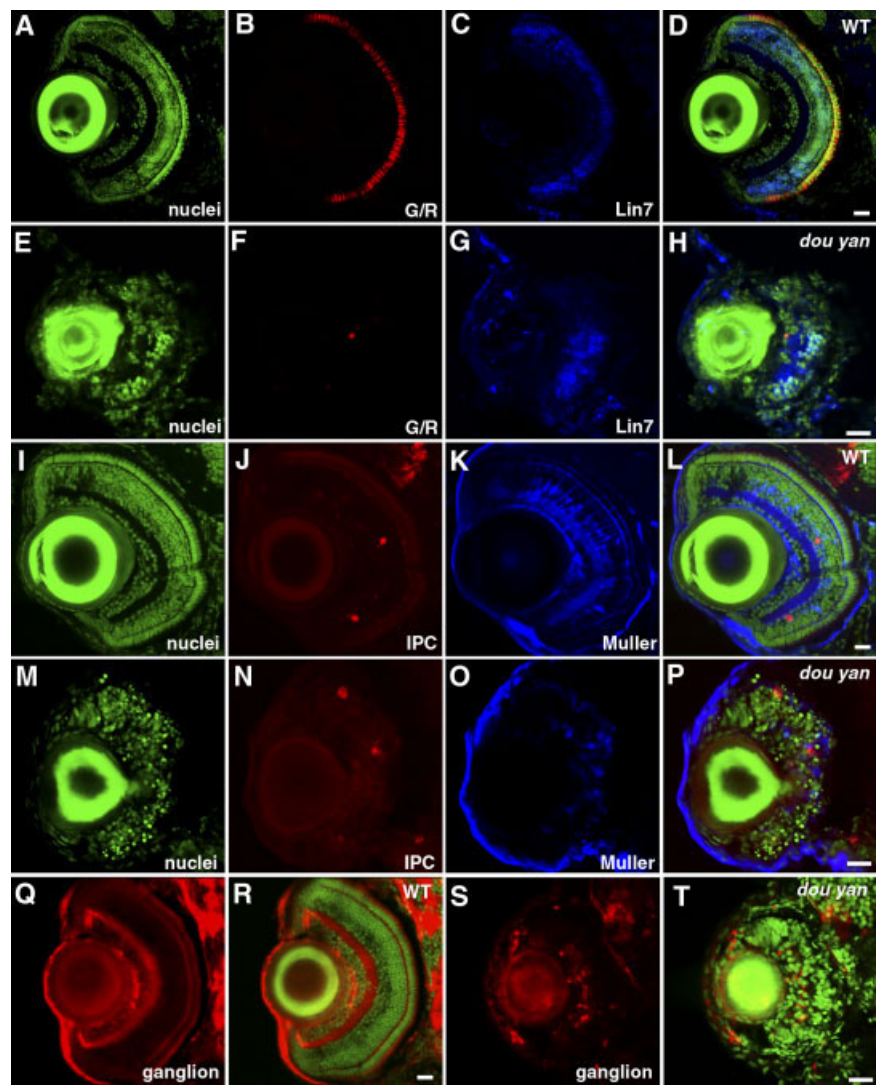
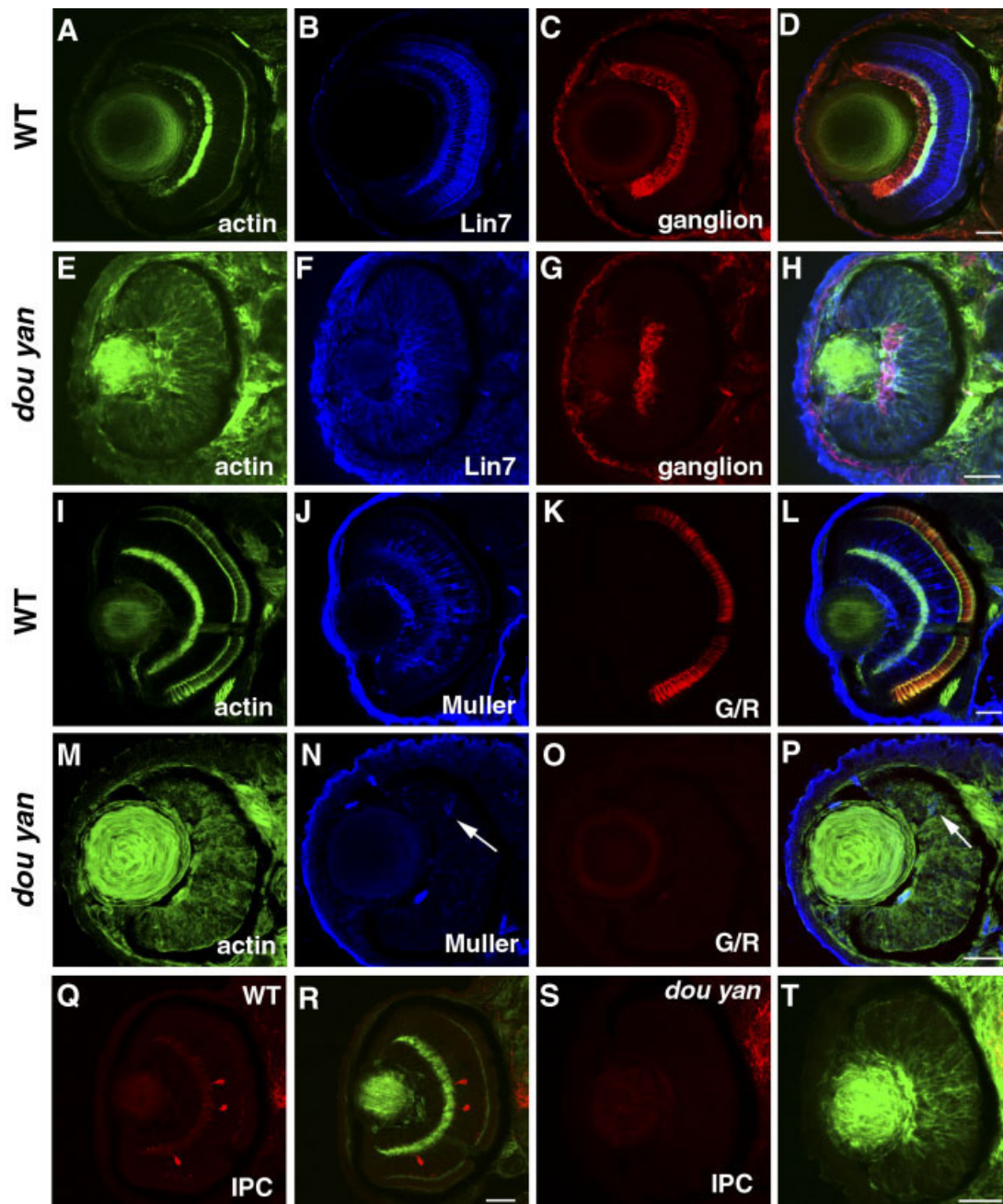


Fig. 7.

**Fig. 7.** Distinct types of retinal cells are specified in *dou yan*<sup>mi234</sup> mutants, but they fail to differentiate properly. **A-H:** At 5 days postfertilization (dpf), green/red double cones (G/R, B and F) and Lin7-positive bipolar cells (Lin7, C and G) are observed in both mutant (E-H) and wild-type (A-D) retinas. YO-PRO staining was used to visualize the entire population of cell nuclei (nuclei, A,E). D and H are merged images. **I-P:** Interplexiform cells (IPC, J,N) and Muller cells (Muller, K and O) are specified in both wild-type (I-L) and mutant retinas (M-P) at 5 dpf. Cell nuclei were visualized by YO-PRO staining (I,M). L and P are merged images. **Q-T:** Zn8 (red) -expressing ganglion cells are specified in both wild-type (Q,R) and mutant (S,T) retinas. Cell nuclei were visualized with YO-PRO staining (green). Scale bars = 20 μm.



**Fig. 8.** Retinal cell specification is delayed by the *dou yan* mutation. **A–D:** At 78 hours postfertilization (hpf) in wild-type retinas, Lin7 expression (blue) is concentrated in the bipolar cell body region and the plexiform layers. The ganglion cells stained with zn8 antibody (red) localize to the basal region of the retinas. The actin distribution as revealed by phalloidin staining highlights the plexiform layers. **E–H:** In *dou yan* mutant retina at 78 hpf, Lin7 (blue) is expressed in the entire retina with increased accumulation in the basal retinal region. This expression pattern is similar to Lin7 expression in wild-type retinas at 48 hpf (Wei et al., 2006a). A patch of retinal cells that express zn8 antigen is noticeable at the basal region of the retina in the mutants. **I–L:** Muller cells (blue) and green/red double cones (G/R, red) are properly differentiated in the wild-type retinas at 78 hpf. **M–P:** In most of the mutant retinas at 78 hpf, only a few cells display positive expression of Muller cell marker carbonic anhydrase (arrows, blue) and the double cone marker Zpr1 was not detectable. **Q–T:** Interplexiform layer cells (IPC, red) are present in wild-type retinas (Q,R) but not in the mutant retina (S,T) at 78 hpf. Scale bars = 20  $\mu\text{m}$ .

ences in the localization or distribution of apical markers among the 20 embryos (data not shown). Thus, the *dou yan*<sup>mi234</sup> mutation does not appear to affect the polarity of the retinal or brain epithelia at early stages of development.

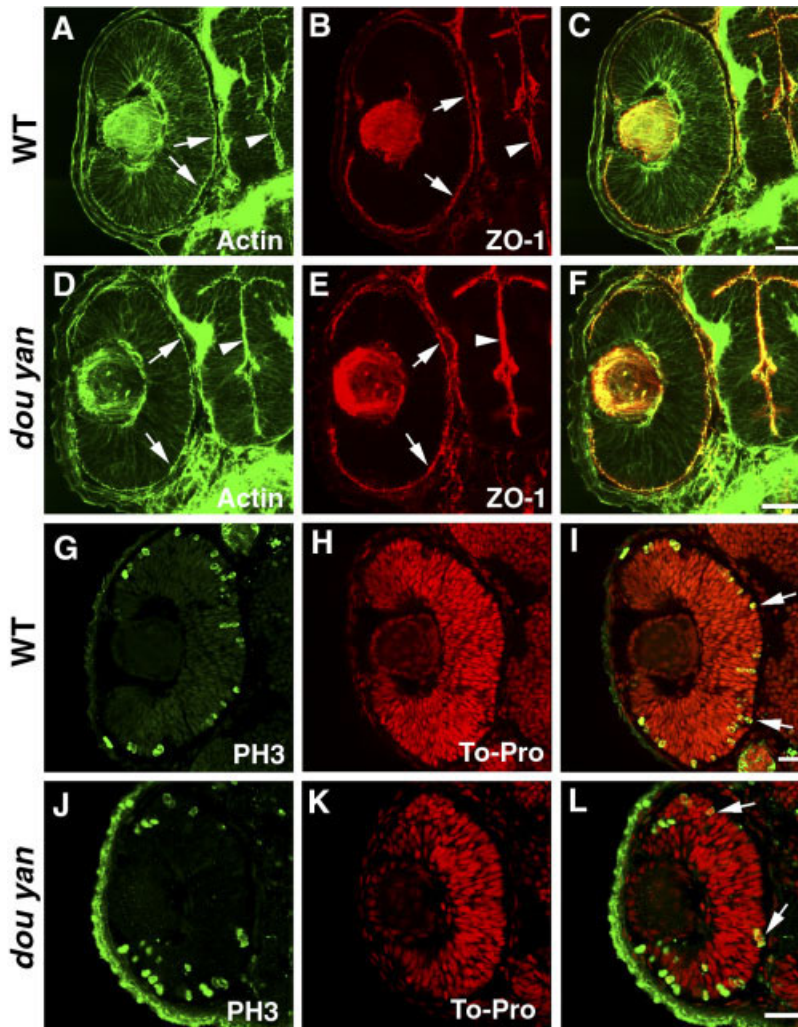
## DISCUSSION

We have isolated and characterized a zebrafish mutation, *dou yan*<sup>mi234</sup>, whose primary phenotypic characteristic is failure of the eyes to grow after the onset of retinal neurogenesis. The

mutation also disrupts retinal lamination and causes extensive retinal cell death.

A small eye phenotype can emerge at a variety of developmental stages, depending on the genes that are affected. Loss-of-function of transcrip-





**Fig. 9.** The *dou yan*<sup>mi234</sup> mutation does not affect apical epithelial polarity. **A–C:** Apical localization of adherens junctions in the retina (arrows) and brain (arrowheads) of the 48 hours postfertilization (hpf) wild-type embryos is visualized by the staining patterns of ZO-1 (red) and adherens junction-associated actin bundles (A, green, arrows). **D–F:** ZO-1 (red) and adherens junction-associated actin bundles (green) localize properly to the apical surface of the retina (arrows) and brain (arrowheads) in *dou yan*<sup>mi234</sup> embryos at 48 hpf. **G–L:** In 48 hpf retinas, the M-phase nuclei as visualized with anti-phosphorylated-Histone H3 antibody (PH3, green) localize to the apical regions of the retinas of mutant (J–L) and wild-type (G–I) embryos. Cell nuclei were labeled with TO-PRO (red). Scale bars = 20  $\mu$ m.

tion factors that are required for early specification and morphogenesis of the eye, such as Pax6, Six3, Rx1, Lhx2, Tll, and Optx2, can lead to early onset of eyeless or small eye phenotypes that appear before retinal neurogenesis begins (Hill et al., 1991; Perron et al., 1998; Zuber et al., 2003). On the other hand, defects in genes that are required for the survival of differentiated retinal cells would be predicted to interfere with retinal growth and may even cause shrinkage of the eye. In zebrafish, early morphogenesis of the eyecup is completed before 24 hpf

and the first differentiated retinal ganglion cells appear at approximately 28 hpf (Schmitt and Dowling, 1994; Hu and Easter, 1999). Because the *dou yan*<sup>mi234</sup> small eye phenotype becomes evident only after the onset of retinal differentiation, we suggest that the product of the *dou yan* gene is required for the differentiation and/or survival of retinal cells. This suggestion is consistent with the finding that cell proliferation in the undifferentiated retina is not affected in *dou yan* mutants, and the germinal zone at the ciliary margin of the mutant embryonic retina

appears normal and contains proliferating retinal progenitor cells at least up to 3 dpf.

Retinal cell death might target either the entire retinal cell population or affect only certain classes of retinal cells. In human retinitis pigmentosa patients, the genetic defects initially affect only photoreceptors and lead to their degeneration (Wang et al., 2001). In zebrafish, the *mikre oko*, *oval*, *pob*, and *fleer* mutations cause cell death in photoreceptors (Doerre and Malicki, 2001, 2002; Taylor et al., 2005) and the *chiorny* mutation causes death in certain subtypes of amacrine cells (Avanesov et al., 2005). Unlike these mutations, the *dou yan* mutation leads to massive cell death throughout the central retina, suggesting that the survival of all retinal cell classes is affected. We also showed that the cell survival defect in the *dou yan* mutation is non-cell-autonomous, so the mutation likely disrupts an aspect of the microenvironment in the retina that promotes cell survival, perhaps a secreted neurotrophic factor.

Previous mutagenesis screens and reverse genetic approaches have identified several genes that are important in controlling the size of zebrafish eye: *Rab11-FIP4*, *Mcm5*, *PITX3*, *small heart*, *no tectal neuron*, and *dazed* (Matsuda and Mishina, 2004; Yuan and Joseph, 2004; Babb et al., 2005; Perkins et al., 2005; Shi et al., 2005; Ryu et al., 2005; Muto et al., 2006). These genes map to chromosome regions different from the one in which the *dou yan* gene resides. Furthermore, in addition to the small eye phenotype, malfunction of these gene products also causes other defects. For example, disruption of *Rab11-FIP4* and *Mcm5* genes leads to extensive cell death not only in the retina but also in the brain (Ryu et al., 2005; Muto et al., 2006); the *small heart* mutation causes malformation of the heart and a curled body axis (Yuan and Joseph, 2004); disruption of the *PITX3* gene function, which is required for lens development, likely has an indirect effect on retinal development as a consequence of disrupted interactions between lens and retina (Yamamoto and Jeffery, 2000; Shi et al., 2005); in *no tectal neuron*, the small eye phenotype does not appear

until after 48 hpf, much later than that in *dou yan*<sup>mi234</sup> (Matsuda and Mishina, 2004); and the *dazed* small eye mutant has normal retinal lamination (Perkins et al., 2005). These phenotypic differences suggest that the *dou yan* gene regulates the size of the eyes through a pathway different from the pathways that concern other identified genes.

In addition to a small eye, the *dou yan*<sup>mi234</sup> mutation also causes a defect in retinal lamination. Five other zebrafish genes that are necessary for retinal lamination have been identified: *nok*, *moe*, *N-cad*, *has*, and *yng* (Horne-Badovinac et al., 2001; Wei and Malicki, 2002; Malicki et al., 2003; Gregg et al., 2003; Jensen and Westerfield, 2004). The first four mutations affect four different epithelial apical–basal polarity genes, which are expressed in the retina and in other tissues as well. Consequently, developmental defects in these mutants are not restricted to the retina, as manifested by a curled body axis, circulation defects and malformation of the digestive system (Horne-Badovinac et al., 2003). While the *yng* mutation causes retinal patterning defects, it does not disrupt retinal epithelial polarity (Link et al., 2000). In contrast, the *dou yan*<sup>mi234</sup> mutant phenotype is restricted to the visual system: morphogenesis of the body is unaffected and embryos exhibit a normal touch response. Furthermore, the extensive retinal cell death observed in *dou yan*<sup>mi234</sup> is not seen in *nok*, *moe*, *N-cad*, *has*, and *yng* mutants. And finally, unlike these other retinal patterning mutations, the *dou yan*<sup>mi234</sup> mutation does not affect the apical–basal polarity of the retinal and brain epithelia. These phenotypic differences indicate that the loss of retinal lamination in *dou yan*<sup>mi234</sup> mutants is mechanistically distinct from an apical–basal epithelial polarity-related patterning defect.

The retinal patterning defect in *dou yan*<sup>mi234</sup> might be explained by extensive retinal cell death during early development, which could affect retinal lamination in one or more ways. First, cell death may constantly change the cellular environment in the retina, perhaps resulting in inconsistent migration and/or differentiation signals to the newly born retinal cells, leading

them to aberrant destinations. Second, premature death of retinal cells in *dou yan* mutants might affect the normal cell–cell interactions required for the stabilization of proper retinal laminar structure. Third, a lamination defect may occur because too few retinal neurons produce processes to form contiguous plexiform layers. These mechanisms are not mutually exclusive, and further work is needed to determine to what extent these or other factors contribute to the lamination defects observed in *dou yan*<sup>mi234</sup>.

Finally, it is noteworthy that a disruption of retinal lamination is not observed in all zebrafish mutants that have a small eye phenotype. Some mutations, such as *archie*, *round eye*, *out of sight*, *niezerka*, and *out of bounds*, produce a downsizing of the retina and cause no retinal lamination defects (Malicki et al., 1996; Fadool et al., 1997; Vihtelic and Hyde, 2002). These observations indicate that the retinal size control and cellular pattern formation can be regulated independently. However, the *dou yan* mutation apparently affects both processes. Like *dou yan*<sup>mi234</sup>, another small eye mutation, *barely started*, also affects retinal pattern formation (Vihtelic and Hyde, 2002). However, in *barely started* the nuclei of retinal cells are enlarged and irregular, which is not observed in *dou yan*. Further molecular characterization of these mutations will help us better understand the complex mechanisms by which retinal size and lamination is generated and maintained.

## EXPERIMENTAL PROCEDURES

### Mutagenesis Screen and Fish Care

Adult male zebrafish from an outbred wild-type colony (Mi fish line) were mutagenized with N-ethyl-N-nitrosurea (ENU, Sigma) according to established procedures (Mullins et al., 1994) followed by a three-generation breeding scheme to identify zygotic recessive mutations. The mutagenesis and screening was performed at the University of Michigan. The *dou yan*<sup>mi234</sup> mutation was identified morphologically by the small size of the eyes in F3 mutant embryos at 3 dpf.

Zebrafish embryos were raised at 28.5°C in E3 embryo rearing medium (Westerfield, 2000) until the desired developmental stages and were used for histological analysis. All procedures conformed to the University of Michigan and University of Pittsburgh standards for use and care of animals in research.

### Mapping Analysis of the *dou yan* Gene

To identify the chromosome region in which the *dou yan* gene resides, we used the Mutant Mapping Facility at the University of Louisville for a bulk segregant mapping analysis. Heterozygous map fish were obtained by crossing Mi heterozygous fish with wild-type TU fish. One hundred mutant embryos from crosses between a pair of heterozygous map fish were collected. DNA isolated from these mutant embryos and their parent fin clips were used for the analysis, following the standard procedure at the Mapping Facility in Louisville (<http://www.biochemistry.louisville.edu/zfmapping/index.html>).

### Histology and Immunohistochemistry

To determine the cellular pattern of the *dou yan* mutant retina, we fixed embryos in 4% paraformaldehyde in 1× phosphate buffered saline (PBS) at room temperature overnight then dehydrated and embedded the tissues in JB4 resin (Polysciences, Inc.) following the manufacturer's protocol. Embryos were sectioned at 4 μm thickness and stained with 1% methylene blue/1% azure II and observed and photographed with an AH2 Olympus microscope.

To determine whether retinal cells are specified normally in *dou yan* mutants, we used immunohistochemical analysis with cell-specific markers as described previously (Wei et al., 2006b). The following cell type-specific markers were used: zpr1 antibody for the Zpr1 antigen, which is specifically expressed in red/green double cones (ZFIN, 1:150 dilution); rabbit anti-Lin7 antibodies for Lin7-expressing cells (Wei et al., 2006a, 1:400 dilution); zn8 antibody against the Zn8 antigen that is expressed in retinal

ganglion cells (ZFIN, 1:100 dilution); rabbit anti-carbonic anhydrase for Muller glia (gift from Dr. Paul Linser; 1:400 dilution); anti-phosphorylated-Histone H3 antibody for M-phase nuclei (Upstate, catalog no. 06-570); anti-tyrosine hydroxylase for interplexiform cells (Chemicon; 1:100 dilution). The nuclei were stained with TO-PRO-3 iodide or YO-PRO-1 iodide (Molecular Probes, catalog no. T-3605 and catalog no. Y-3603; at 1:500 dilution). Actin was visualized with Alexa Fluor 488-conjugated phalloidin (Molecular Probes, catalog no. A-12397, 1:500 dilution). ZO-1 was visualized with monoclonal mouse anti-ZO-1 antibody (Zymed, 1:200 dilution).

### Acridine Orange Staining

To analyze cell death in the *dou yan* mutant retinas, we used acridine orange staining. Briefly, live fish embryos of both wild-type and mutant were stained with acridine orange (50  $\mu\text{g/ml}$  in E3 embryo rearing medium) at room temperature for 30 min. Stained embryos were washed three times with PBS at room temperature for 30 min each. Embryos were then fixed overnight with 4% paraformaldehyde at room temperature. Fixed embryos were infiltrated with 40% sucrose in  $1\times$  PBS and cryosectioned at 30  $\mu\text{m}$ , mounted in slow-fade mounting medium (Vector Laboratory), and observed under a Bio-Rad MRC1024 laser scanning confocal microscope.

### BrdU Labeling of the Retinas

The wild-type and mutant embryos were pulse labeled with 10 mM BrdU in E3 with 15% dimethyl sulfoxide at 28°C for 2.5 hr between 49 and 51.5 hpf. The embryos were then fixed with 4% paraformaldehyde at room temperature for 2 hr, followed by a DNA denaturation treatment with 2 N HCl at 37°C for 1 hr. The embryos were then processed for immunostaining as described above, with anti-BrdU antibody (Sigma catalog no. B2531).

### Quantification of Mitotic Cells

Phospho-Histone H3 (pH3) is a marker of M-phase mitotic cells (Ad-

ams et al., 2001). Because the wild-type retinas are larger and contain more total cells than the mutant retinas, we normalized the number of pH3-positive cells to retinal area in wild-type and mutant retinas. In brief, we counted pH3-positive retinal cells in five sections from five embryos each at 40, 48, and 72 hpf. The retinal regions counted were contoured and measured with MetaMorph v6.1, and we calculated the average number of pH3-positive cells per 3,600  $\mu\text{m}^2$  of retinal area. Statistical significance of the differences between mutant and wild-type was assessed with Student's *t*-test.

### Blastomere Transplantation and Mosaic Analysis

For genetic mosaic analysis, we labeled wild-type donor embryos by injecting 5% purified lysine fixable biotin-conjugated dextran (Molecular Probes; catalog no. BDA-10,000) at the one- and two-cell stages. The biotinylated dextran solution (in 0.2 M KCl) was purified by filtration with Microcon Centrifugal Filters (Millipore, catalog no. 42420). Labeled blastomeres were transplanted to wild-type and mutant embryos as described (Wei and Malicki, 2002). The host embryos were raised to 3 dpf and fixed in 4% paraformaldehyde at room temperature for 2 hr. Fixed embryos were cryosectioned at 30  $\mu\text{m}$  and stained with Cy3-conjugated avidin (Jackson ImmunoResearch) for donor cells and with YO-PRO-1 for the cell nuclei. The distribution and morphology of the donor cells in wild-type and *dou yan* mutant embryos was examined and photographed with a Bio-Rad MRC1024 laser scanning confocal microscope. The numbers of dead and live clones were counted according to the following criteria: (1) a group of vertically arranged, intact cells labeled with biotinylated dextran was counted as one live retinal clone; (2) in the brain, a group of spatially contiguous, intact labeled cells with no other labeled cells in the vicinity was counted as one clone; (3) a cluster of punctate biotinylated dextran staining, in which staining particles were less than one cell width apart was counted as one dead clone. Statistical

comparisons were assessed with the  $\chi^2$  test.

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