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In Vivo Time-Lapse Imaging of Cell Divisions during Neurogenesis in the Developing Zebrafish Retina

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Summary

Two-photon excitation microscopy was used to reconstruct cell divisions in living zebrafish embryonic retinas. Contrary to proposed models for vertebrate asymmetric divisions, no apico-basal cell divisions take place in the zebrafish retina during the generation of postmitotic neurons. However, a surprising shift in the orientation of cell division from central-peripheral to circumferential occurs within the plane of the ventricular surface. In the *sonic you (syu)* and *lakritz (lak)* mutants, the shift from central-peripheral to circumferential divisions is absent or delayed, correlating with the delay in neuronal differentiation and neurogenesis in these mutants. The reconstructions here show that mitotic cells always remain in contact with the opposite basal surface by means of a thin basal process that can be inherited asymmetrically.

Introduction

The development of the zebrafish eye occurs in two phases. The first is a series of morphogenetic changes, starting as an evagination from the neural keel at around 11 hpf (hours postfertilization) and finishing with the formation of the optic cup at around 24 hpf (Schmitt and Dowling, 1994). During this early period of morphogenetic change, the level of cell proliferation is low, and the length of the cell cycle is transiently increased while the volume of the retina remains almost constant. Following the morphogenetic changes that transform the solid disc-like mass continuous with the neural keel into a cup-shaped and more spherical optic vesicle, an abrupt transition to increased proliferation and shorter cell cycle lengths occurs at around 33 hpf when the retina is ready to grow in volume (Li et al., 2000a, 2000b).

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At the same time, the retina starts its transformation from a morphologically homogeneous neuroepithelium into a tissue consisting of six neuronal and one glial cell types organized into cell type-specific laminae.

Asymmetric cell division is thought to be one mechanism by which the diversity of cell fates evident in the nervous system could be generated. In asymmetric cell divisions, the unequal segregation of cell fate determinants results in one daughter cell becoming postmitotic while the other continues to proliferate. During development of the *Drosophila* central nervous system, neuroblasts divide along the apico-basal axis, rather than along a planar axis, to give rise to a ganglion mother cell basally and a neuroblast apically. The apico-basal orientation of division allows cell fate determinants, such as the protein Numb, that are segregated along the apico-basal axis within the cytoplasm of the neuroblast to be distributed differentially to the two daughter cells, which results in different cell fates for the daughter cells (Doe and Bowerman, 2001; Lu et al., 2000). Studies of neurogenesis in the vertebrate central nervous system have shown a minority of cell divisions taking place apico-basally, with the majority orientated in the plane of the tissue. Apico-basal divisions have been seen in DiI-labeled ferret cortical slices by time-lapse imaging while fixed tissue studies of chick and rat retina and chick and mouse cortex have also shown cells dividing apico-basally (Chenn and McConnell, 1995; Cayouette et al., 2001; Silva et al., 2002; Wakamatsu et al., 1999; Zhong et al., 1996). Evidence that these apico-basal divisions are asymmetric comes from observations of asymmetric expression of vertebrate homologs of Numb or Notch, a protein that affects cell fate and is potentially antagonized by Numb, in apico-basally dividing neural progenitor cells (Cayouette et al., 2001; Chenn and McConnell, 1995; Johansson et al., 1999; Silva et al., 2002; Wakamatsu et al., 1999; Zhong et al., 1996). Apico-basal divisions may have been seen in sections of the zebrafish retina (Horne-Badovinac et al., 2001), but it has not yet been shown that these division are asymmetric. Some data, however, suggest that planar divisions can also be asymmetric. First, there is a precedent for asymmetric divisions taking place in a planar axis in the *Drosophila* peripheral nervous system (Gho and Schweisguth, 1998). Second, the number of apico-basal divisions observed in the ferret cortex (Chenn and McConnell, 1995), rat retina (Cayouette et al., 2001), and zebrafish retina (Horne-Badovinac et al., 2001) appear to be too low to account for the number of neurons that are born around the same time. Thus, some proportion of planar divisions need to be asymmetric. Interestingly, a recent study of the chick retina found no correlation between relatively rare apico-basal divisions and the expression of an antigen expressed early in differentiating retinal ganglion cells (Silva et al., 2002).

Neurogenesis in each cellular layer of the zebrafish retina is initiated in a wave-like manner starting in an area known as the ventro-nasal patch, adjacent to the choroid fissure (Hu and Easter, 1999). This neurogenic wave progresses around the retina circumferentially in

a ventral to nasal to dorsal to temporal direction. It has been demonstrated using markers of differentiating cells (Laessing and Stuermer, 1996; Masai et al., 2000; Neumann and Nuesslein-Volhard, 2000; Roegiers et al., 2001) and BrdU pulse labeling (Hu and Easter, 1999). Given that the wave is involved in neurogenesis, it may therefore also be involved in regulating or initiating neurogenic cell divisions. If so, are orientations or patterns of cell division correlated with these waves or affected in zebrafish mutants in which neurogenesis is delayed or this wave is disrupted?

Using two-photon excitation of fluorescent probes, 3D representations of almost the entire zebrafish retina can be obtained over a long period of time due to the lower levels of photodamage that normally result from laser scanning microscopy (Piston, 1999). An advantage for the study of morphogenesis is that measurements of the specimen can be taken in the knowledge that no artifact will have been introduced from fixing, staining, or mounting the specimen. Furthermore, because time-lapse data is being used, relative changes in morphogenesis can be observed in single specimens. Shorter-term changes in morphology during cell division can also be studied. Of particular interest is the issue of the basal process by which neuroepithelial progenitor cells are attached to the basal side of the retina. During the cell cycle in the vertebrate retina, the nucleus undergoes interkinetic nuclear migration, which involves movement of the nucleus within a neuroepithelial cell that spans the entire retina or ventricular zone. At mitosis, the nucleus always migrates to the apical side of the tissue where the cell divides. Recent time-lapse studies of cortical glial cells showed that the basal process seemed to remain in place during mitosis of radial glial cells (Miyata et al., 2001; Noctor et al., 2001). This may relate to reports of cultured neuroblasts in the developing superior cervical ganglion of the rat that can divide after having elaborated axonal processes (Wolf et al., 1996). In such cases, the process persists and is inherited by one of the daughter cells. By imaging retinal progenitor cells in vivo, we show that the basal process does persist during mitosis. This also raises questions about the fate of the basal process at mitosis. We have tried to determine whether one daughter cell or both daughter cells can inherit the basal process, and whether any pattern of inheritance of the basal process might bear any relation to asymmetric cell division.

Results

There Are No Apico-Basal Divisions in the Zebrafish Retina

Zebrafish embryos were stained with Bodipy-FL-C_5-Ceramide, a fluorescent dye that is distributed in the interstitial space between cells. Imaging by confocal laser scanning microscopy allowed the outline of all retinal cells to be visualized over time. Dividing cells could be clearly identified (Figure 1), but labeling of every cell in the retina made tracking the morphology and position of individual cells extremely difficult. Nonetheless, analysis of images of dividing cells provided two avenues for further investigation. First, no divisions were noticed with a cleavage plane parallel to the ventricular surface. Second, mitotic cells were found that seemed to retain a process to the basal side of the retina. In order to label fewer cells so that they could be visualized more clearly, we imaged zebrafish embryos injected with DNA encoding GFP-based fusion proteins. H2B-GFP, a fusion protein of Human Histone 2B and eGFP, localizes to the chromatin in nuclei (Kanda et al., 1998). When injected into zebrafish embryos, it is an extremely effective label for studying cell divisions, as changes in chromatin arrangement during mitosis can be seen easily in time-lapse movies. Embryos were imaged in 10 to 15 min intervals for a minimum of 10 hr starting at 27.5 hpf up to 45 hpf, a time period encompassing the beginning of neurogenesis in the retina (28 hpf) and the birth of all retinal ganglion cells as well as most inner nuclear layer cells in the central retina (Hu and Easter, 1999). Each time point consisted of a volume that was reconstructed and could be rotated around any axis (see Movie S1 in the Supplemental Data at http://www.neuron.org/cgi/content/full/37/4/597/DC1). The angle of orientation was initially analyzed with respect to the apico-basal axis and the retinal or apical surface (Figure 2). An orientation of 0° represents a division that occurred parallel to the tangent plane of the apical surface at the point of division, while an orientation of 90° is characteristic of a division orthogonal to this plane, i.e., an apico-basal cell division. Surprisingly, no cell divisions occurred at orientations greater than 40° at any of the time points analyzed (Figure 3A). Seventy percent of the observed cell divisions ($n = 131$) were orientated by 10° or less toward the retinal surface. The rest were orientated by larger angles. The first cells to become postmitotic in the zebrafish retina are ganglion cells born around 28 hpf, with the peak rate of neurogenesis between 33 hpf and 48 hpf (Nawrocki, 1985). The distribution of orientations of cell divisions, however, remained the same, regardless of time, as revealed by cumulative plots of orientations at different time periods (Figure 3B). If some postmitotic cells in the zebrafish retina are indeed born of asymmetric cell divisions, the lack of apico-basal divisions would indicate that these asymmetries occur in a near planar orientation.

The Orientation of Cell Division Shifts from a Central-Peripheral to a Circumferential Axis over Time

The orientation of divisions parallel to the apical surface was characterized with respect to the central-peripheral axis. Considering the retina as a thickened shell of an ellipsoid body, a central-peripheral axis would be equivalent to lines of longitude or great circles drawn on its surface (green in Figure 4A) with the center of the lens as one of the poles and the optic nerve head as the opposite pole. In a 2D projection of the ellipsoid as seen from the optic nerve head pole, as is done when making flat-mount preparations of the retina, these lines would look like radial lines emanating from the center. Perpendicular to the central-peripheral axis is a circumferential axis equivalent to lines of latitude on a globe (red in Figure 4A). In our analysis, an orientation of 0° represents a cell division along the central-peripheral axis, while an orientation of 90° represents a division along the circumferential axis (Figures 4B–4E). Figure 5A depicts

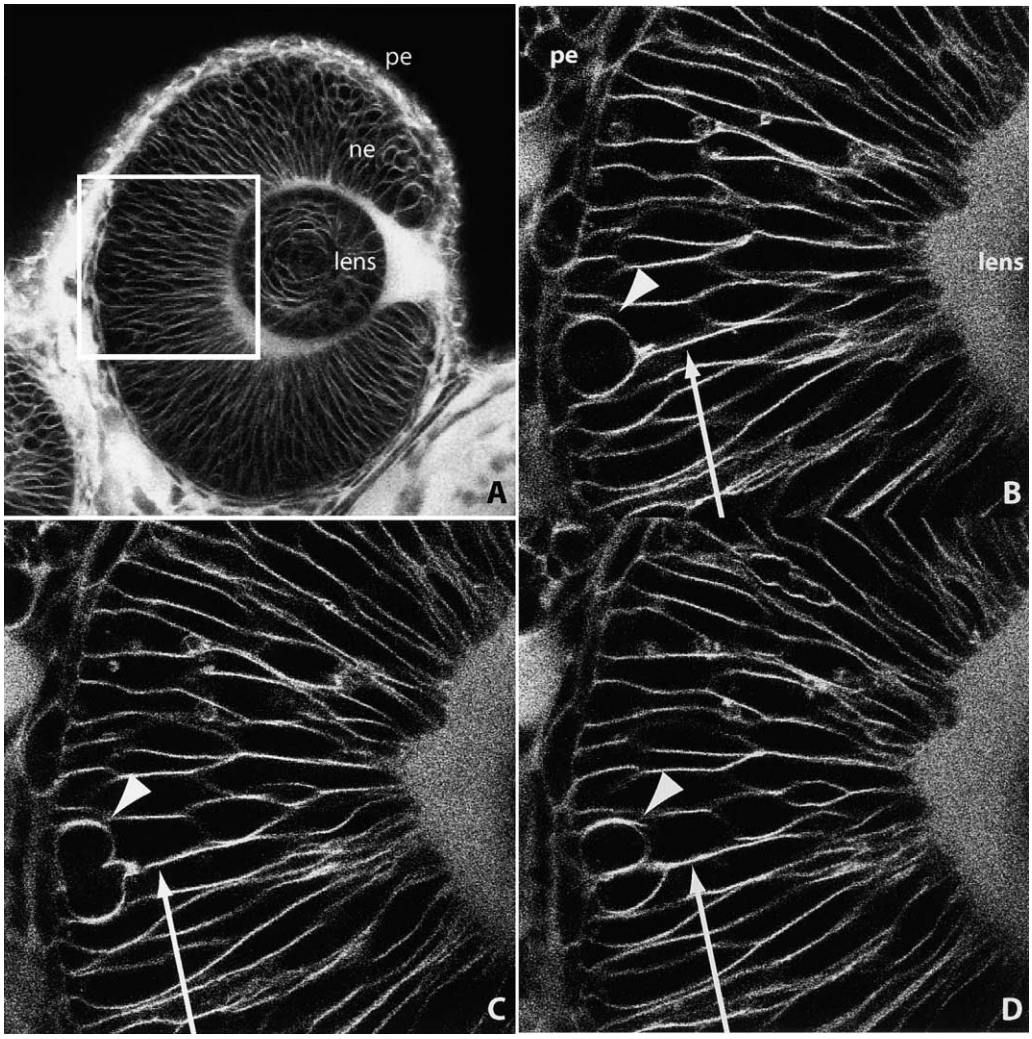

Figure 1. Time-Lapse Analysis of Cell Division in the Zebrafish Embryonic Retina

This specimen, stained with Bodipy-FL-C_5-Ceramide, shows the outline of all cells in the retinal neuroepithelium (ne). The ventricular or apical surface is adjacent to the retinal pigment epithelium (pe), and the basal surface is adjacent to the lens (le).

(A) Low-magnification view of the entire retina using confocal microscopy. A white box frames an area equivalent to that shown in (B)–(D).

(B–D) The frames are confocal sections 5 min apart at the same position in the same specimen. A cell division can be seen clearly (arrowhead). The arrow points to a possible basal process, inherited asymmetrically by one daughter cell, as indicated by the intense signal at the area where it emanates from the dividing cell.

the cumulative distribution of orientations of division in relation to these two axes and its change over time. Before 30 hpf, when neurogenesis is just beginning in the retina, 68% of cell divisions (13/19) were orientated at $<45°$. However, after 40 hpf, by which time all retinal ganglion cells have been born and inner nuclear layer cells have started to become postmitotic (Hu and Easter, 1999), only 18% (2/11) of cells divided at $<45°$. In fact, all remaining cells from this time period divided at an orientation greater than 60° from the central-peripheral axis (Figure 6). The distribution of orientations before 30 hpf was significantly different from the distribution after 40 hpf (Figure 5A). The proportion of cell divisions occurring along the circumferential axis, therefore, increases over time at the expense of central-peripheral axis divisions. Two possibilities may explain this change. First, the retina undergoes morphogenetic changes including an abrupt increase in volume from 33 hpf (Li et al., 2000b). Different orientations of cell divisions may contribute differently to the growth of the retina in the appropriate proportions for each dimension during this time. Alternatively, the increase in circumferential cell divisions in the retina may reflect an increase in the number of asymmetric cell divisions, which would provide a basis for the increase in rate of neurogenesis that occurs over the time period studied.

The Distribution of Orientation of Cell Divisions also Shifts over Time in the Developing Rat Retina

We performed a similar analysis for planar orientations of retinal progenitor cell divisions using fixed rat retinas from three different time points in development (E16, P0, and P4). During E16 and P0, there was no difference

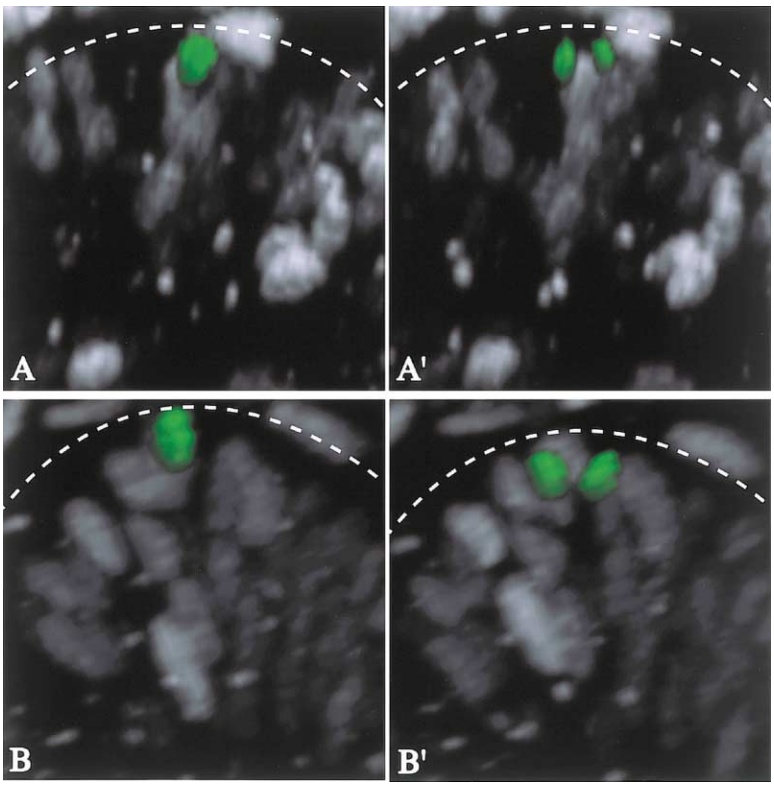

Figure 2. Retinal Neuroepithelial Cells Divide Parallel to the Retinal Apical Surface

Three-dimensional reconstructions of parts of retinas from zebrafish injected with H2B-GFP DNA are shown 10′ (A–A′; B–B′) apart. Nuclei of dividing cells are highlighted in green. White dashed lines represent the apical surface of the retina. The orientation of cell division is measured as the orientation between a line joining the center of the daughter nuclei and a tangent to the closest part of the apical surface.

between central-peripheral and circumferential divisions (52.5% versus 47.5% and 55% versus 45%, respectively). However, during P4, 73% of cells divided circumferentially, while only 26% of cells divided along the central-peripheral axis (Figure 5B). The distribution of orientations of cell divisions during P4 is similar in tendency to the distribution of cell division orientations in zebrafish aged between 35 and 45 hpf. In the rat retina, rods, Müller cells, and bipolar cells are predominantly born after P0 (Young, 1985), while in zebrafish, cells from the inner nuclear layer including Müller cells and bipolar cells are born between 38 and 48 hpf. The correspondence in shifting proportions of cell division orientation between zebrafish and rat models implies that the change toward circumferential divisions is important in the development of the vertebrate retina.

Orientation of Division in *syu* and *lak* Mutants

We considered the possibility that circumferential divisions represent asymmetric divisions, as this is the axis along which the neurogenic waves of differentiative divisions in the zebrafish retina spread (Hu and Easter, 1999). The signaling molecule Sonic Hedgehog (Shh) and the transcription factor Ath5 are both expressed in a spatio-temporal pattern characteristic of the neurogenic wave (Masai et al., 2000; Neumann and Nuesslein-Volhard, 2000). Both Shh and Ath5 have a role in initiating retinal ganglion cell differentiation (Kay et al., 2001; Neumann and Nuesslein-Volhard, 2000). A correlation between the spread of Shh or Ath5 expression, representing neurogenic activity, along the circumferential axis of the retina and the increase in circumferentially orientated cell divisions would provide further evidence for such divisions tending to be asymmetric. In the *sonic you (syu)* mutant, Shh expression is reduced and the wave of expression does not spread, resulting in a lack of normal retinal ganglion cell differentiation. To check if the distribution of orientations of cell divisions was changed due to the disruption of the wave of differentiation, H2B-GFP was injected into *syu* mutants, and orientations of cell divisions in the retina analyzed as described above. In terms of the apico-basal orientation of cell divisions, the majority of divisions occurred parallel to the retinal epithelial surface, as in the wild-type. The gradual shift in proportion from central-peripheral to circumferential divisions is absent or delayed in *syu* (Figure 5C). The difference in the temporal change of proportions of cell division orientations between wild-type and *syu* embryos is consistent with the hypothesis that circumferential cell divisions tend to be asymmetric and that the lack of Shh secretion delays the expected increase in asymmetric neurogenic divisions. However, we cannot rule out the hypothesis that the failure of the retinal ganglion cell layer to form properly affects the morphogenesis of the growing retina. Indeed, the retina of *syu* mutants is less organized and slightly smaller than the wild-type retina. It is also possible that in *syu* there is simply a delay in growth relative to the wild-type retina, and that this delay causes a consequent delay in the change of proportions of cell division orientations observed.

Lakritz (lak) is a mutant for the transcription factor Ath5, and retinal ganglion cells are not generated in this line. Instead, neurogenesis begins with the generation of inner nuclear layer cells at a time comparable with the generation of inner nuclear layer cells in the wild-

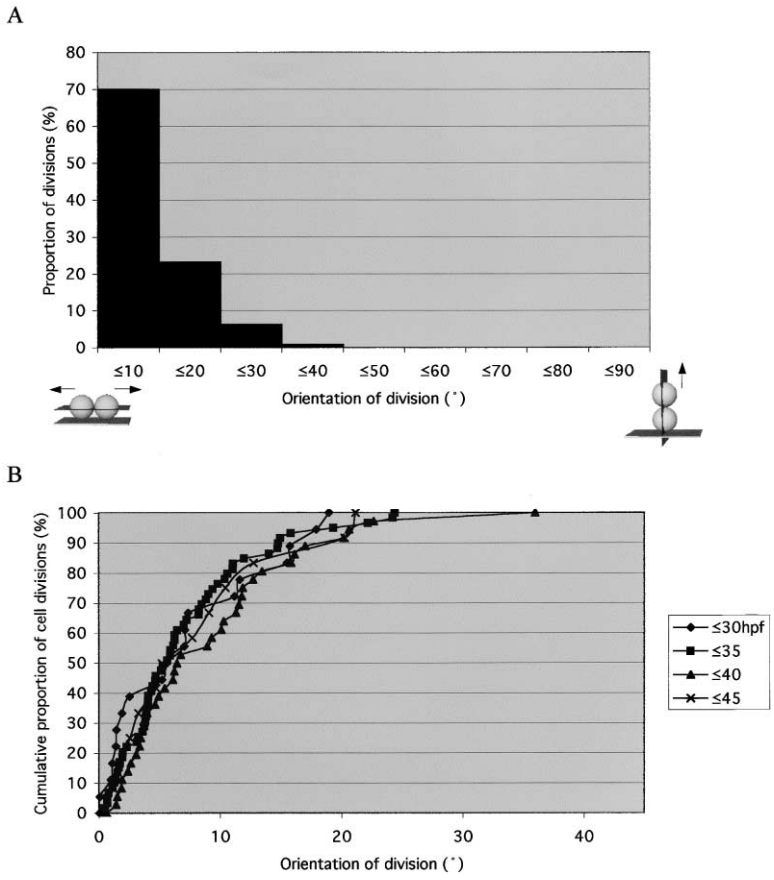

Figure 3. Orientation of Cell Division with Respect to the Apico-Basal Axis and Retinal Surface

(A) An angle of 0° represents a cell division parallel to the retinal surface. An angle of 90° represents a cell dividing along the apico-basal axis. All divisions observed tended toward the retinal surface, with most divisions almost parallel to the surface. No apico-basal divisions were observed.

(B) Cumulative plot of orientations with respect to the apical retinal surface of all observed cell divisions (n = 131) over four time periods. There is no significant change in distribution of orientations between the time points.

type. Despite this, the *lak* retina retains normal morphology and order and is not markedly different in size compared to wild-type. The *lak* pigmentation defect used to identify mutants is expressed at 4 dpf (Kelsh et al., 1996), which is too late for in vivo imaging starting at 24 hpf. To surmount this problem, *lak* embryos were identified by PCR and fixed in paraformaldehyde. Nuclei were visualized at specific time points (30 hpf, 40 hpf, and 55 hpf) by Hoechst staining to measure the orientation of anaphase nuclei. Similar to *syu, lak* showed no change in the distribution of orientations of division over time (Figure 5E), while wild-type siblings showed a significant change toward circumferential divisions (Figure 5D). The lack of change in distribution of orientations of cell division seen in *syu* and *lak* strengthens the hypothesis that this change in trend of orientation of division plays a role in neurogenesis in the vertebrate retina.

The Basal Process Persists during Mitosis

To follow the basal process through the division of retinal neuroepithelial cells, a membrane-targeted eGFP construct (GAP-GFP) was used to visualize cell membranes. These time-resolved experiments revealed that the basal process does indeed persist during cell division. Out of 81 cell divisions observed between 27 and 48 hpf, 77 cells had a basal process that clearly persisted throughout the division (see Movie S2 in Supplemental Data at http://www.neuron.org/cgi/content/full/37/4/597/DC1). In the other four cases, the presence or absence of the basal process could not be determined due to low signal intensity. Dividing cells also had an "apex," a slight protrusion that the basal process emanated from, which could be seen in all 81 cases. There were no clear cases where the basal process was seen to retract during cell division. The most likely possibility, therefore, is that all dividing cells in the zebrafish retina retain a basal process. Most of the cytoplasm seems to end up in the mitotic cell body at the apical side so that the basal process itself becomes extremely thin. If labeled neighbors do not surround the dividing cell, the basal process can be seen clearly in 3D reconstructions of the volume (Figure 6A). If the cell is surrounded by labeled neighbors, however, the persistence of the membrane is indicated by a higher intensity at the expected position along the cell membrane of adjacent cells. After metaphase, the basal process becomes more visible again as cytoplasm moves back into it. The position of the basal process at this time is almost identical to that of the process prior to cell division, indicating that the basal process persisted and retained its structure during the division. In many cases, the time elapsed between mitosis and the process being refilled with cytoplasm is very short, on the order of 15 min. Occasionally, as the nucleus and cytoplasm of a cell shift to the apical side in preparation for cell division, a small volume of cytoplasm will fail to move from the basal process into the soma. It becomes trapped in a bubble-like swelling of the basal process at some distance from the main body of cytoplasm (see Movie S3 in Supplemental Data). After cell division, the stray cyto-

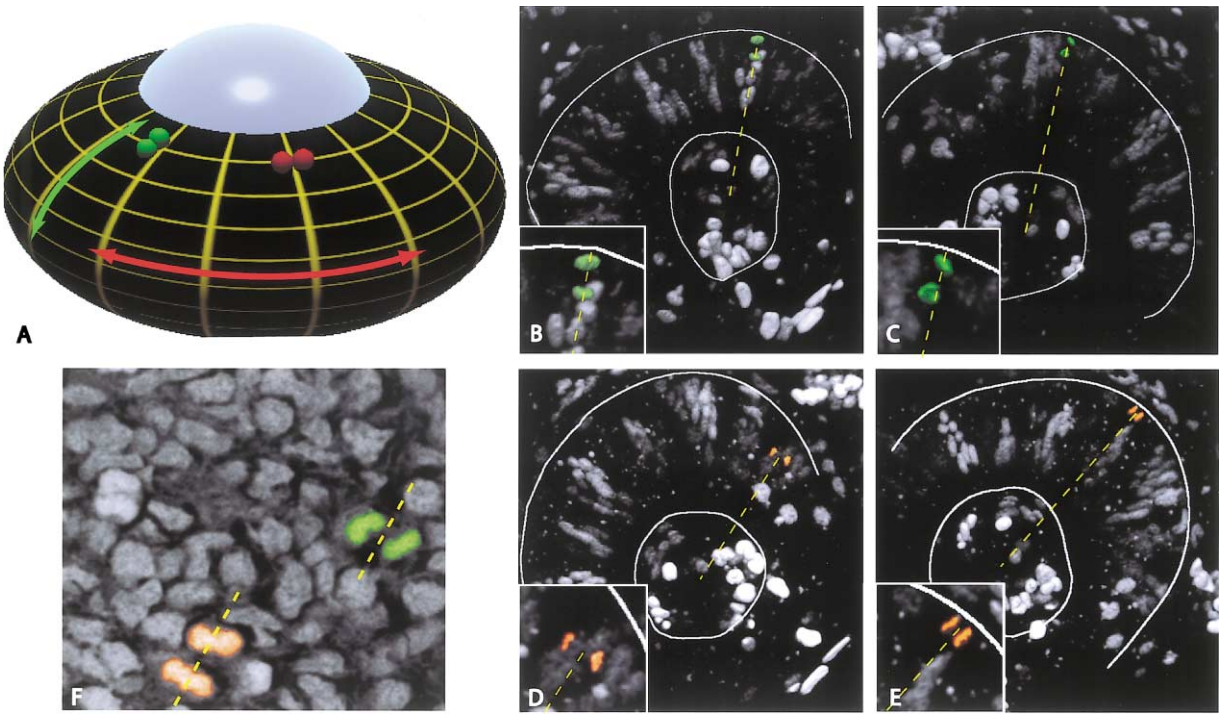

Figure 4. Analysis of Cell Divisions Orientated Parallel to the Apical Surface

(A) Three-dimensional representation of the zebrafish retina as an ellipsoid body covered by a grid formed from two axes, the central-peripheral axis (green) and the circumferential axis (red). Also shown are cells dividing along both the axes. The orientations of cell divisions were measured with respect to the central-peripheral axis.

(B–E) Zebrafish. Examples of central-peripheral divisions (0°) highlighted in green and circumferential cell divisions (90°) highlighted in red. The central-peripheral axis is represented by the yellow dashed line. The retina itself is outlined in white. The highlighted cells are magnified in the insets.

(F) Rat. Example of a central-peripheral division (0°) highlighted in green and a circumferential cell division (90°) highlighted in red. The yellow broken lines represent the circumferential axis. Cells were highlighted in this and further figures for emphasis using Adobe Photoshop or Volocity.

plasm always joins one of the two daughter cells produced, confirming the existence of a continuous connection between at least one daughter cell and the stray cytoplasm and, thus, the persistence of the basal process (Figure 6B).

Inheritance of the Basal Process after Cell Division

By time-lapse analysis, we noticed that, in some cases, the basal process seemed to be inherited by one of the two daughter cells, similar to the report of asymmetric inheritance of the basal process in mouse radial glial cells (Miyata et al., 2001). We asked two further questions. First, how exactly is the basal process of a dividing cell inherited by its daughter cells? And second, if only one of the two daughter cells inherited the basal process, was this event correlated in any way to any other asymmetry in such a division? Of the 77 cells whose basal process could be seen, 54 (70%) divided such that only one daughter cell clearly inherited the basal process. For each of these cases, we found that the basal process or the apex that it emanated from was already positioned slightly off-center with respect to the basal pole of the cell prior to mitosis (Figure 6C). By rotating a rendering of the prophase cell around its apico-basal axis, the position of the process was determined in relation to the central-peripheral and circumferential axes of the retina. A basal process positioned off-center in one axis results in an asymmetry of inheritance if the cleavage plane of division is in the other axis. The position of the basal process could be consistently used (71.5% of the time for 54 cells) to predict which daughter cell, if not both, would inherit the process given a certain orientation of division. This shows that the basal process does not have to split two ways nor is it always inherited by just one daughter cell. In fact, its position at prophase and the orientation of division will determine how it is inherited. Within 15 or 20 min of mitosis, the daughter cell that did not inherit the process usually rejoined its sibling's basal process with its basal apex lying adjacent to it. Retinas labeled with GAP-GFP were then analyzed for the orientation of cell division along with the asymmetric inheritance of the process (Table 1) as well as for temporal differences in the proportion of cells whose basal process is asymmetrically inherited. Asymmetric inheritance of the basal process and the planar orientation of cell division were found not to correlate with each other ($p = 0.29$) and are, therefore, independent of each other. Similarly, the proportions of cell divisions that led to asymmetric inheritance of the basal process did not change significantly between 26 and 48 hpf, indicating

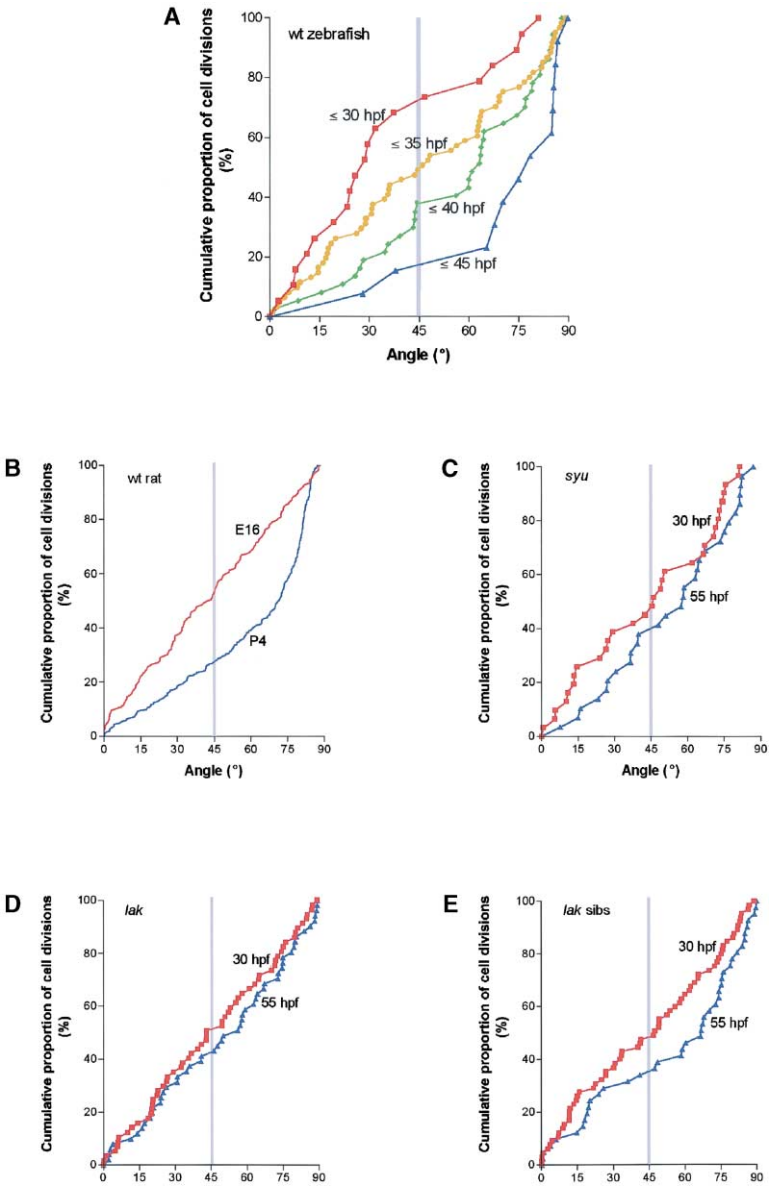

Figure 5. Cumulative Distributions of Orientations of Cell Divisions Parallel to the Retinal Surface over Time

The gray lines represent 45° thresholds.

(A) In zebrafish, earlier distributions contain a greater proportion of central-peripheral (<45°) divisions, while later distributions contain more circumferential (>45°) divisions. The ≤30 hpf and ≤ 45 hpf distributions differ significantly as determined by the Kolmogorov-Smirnov test ($p < 0.01$, $n = 19, 61, 37, 11$).

(B) In the rat retina, the distribution of orientations of cell divisions at P4 tends toward circumferential divisions compared to the distributions at E16 or P0 ($n = 158, 283$).

(C and D) In *sonic you* (C) ($n = 31, 29$) and *lak* (D) ($n = 57, 51$) embryos, there is no significant difference between the distributions of cell division orientations over time.

(E) The distribution of cell division orientations differs significantly between 30 and 55 hpf in the wild-type siblings of *lakritz* embryos ($p = 0.03$, $n = 65, 41$).

that inheritance of the basal process is not regulated by the same processes that regulate the temporal changes in cell division orientation.

Discussion

Direct visualization of the zebrafish retina in vivo has allowed us to avoid the issues involved with maintaining structural and physiological integrity of specimens while analyzing the movement and changes in morphology of neural progenitor cells. A model of a cell dividing in the zebrafish retina that summarizes much of this data is shown in Figure 7. Time-lapse analysis has revealed changes in cell division patterns that correlate temporally to an expected transition from symmetric to asymmetric divisions (Cayouette et al., 2001; Chenn and McConnell, 1995) and clearly confirmed the persistence and occasional asymmetric inheritance of the basal process of neuroepithelial cells during cell division.

Orientation of Cell Division

We analyzed the orientation of cell division with respect to the apico-basal axis and the ventricular surface of the retina by imaging zebrafish with their nuclei labeled by the transient expression of H2B-GFP. The current model for asymmetric cell division in the vertebrate cortex and retina holds that cell divisions occurring along the apico-basal axis are asymmetric, with the basal daughter cell becoming a postmitotic neuron or a Müller glial cell. Evidence for this comes from time-lapse studies of dividing cells in the neuroepithelium of the ferret cortex (Chenn and McConnell, 1995) as well as antibody staining of Numb, an asymmetrically segregated cytoplasmic cell fate determinant, in chick, mouse, and rat

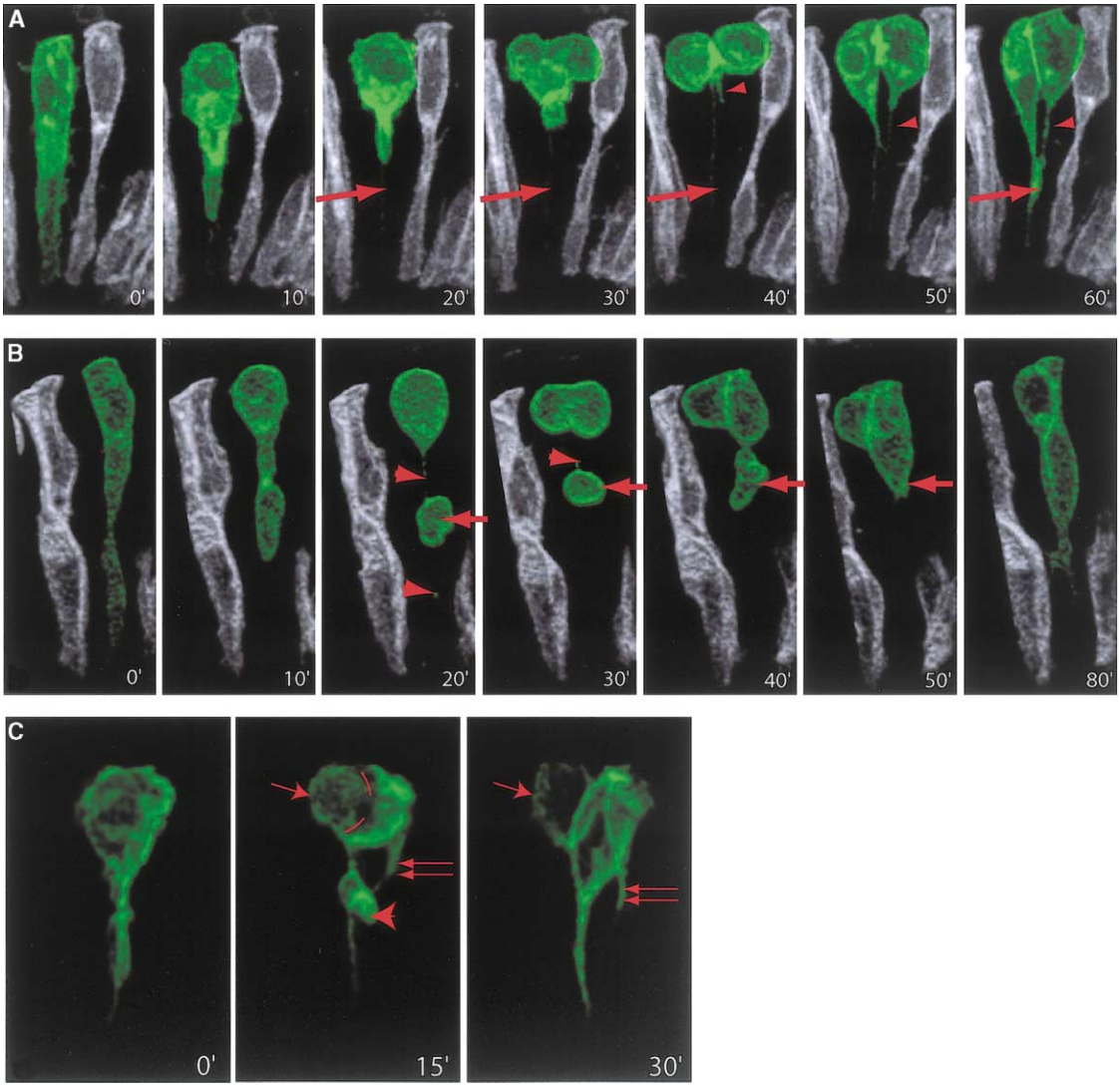

Figure 6. Persistence of the Basal Process during Cell Division In Vivo

The highlighted cell is labeled with GAP-GFP to label the membranes.

(A) The time-lapse series shows the cell dividing, while the basal process (arrows) remains in place during the entire event. In preparation for division, the nucleus and all the cytoplasm move to the apical side of the retina. After division ($t = 40'$), the cell on the left seems to have inherited the basal process while the cell on the right seems to be growing a new process alongside its sibling (arrowheads).

(B) Evidence for the basal process during cell division. The highlighted retinal cell expresses GAP-GFP. As the cell divides, some cytoplasm is left behind (arrow) in the basal process at M phase ($t = 20'$), while the rest is being resorbed into the cell body. The basal process itself is difficult to visualize; however, the stray cytoplasm rejoins one of the daughter cells after division, so it is likely to have remained connected via the basal process (arrowheads) during cell division. In addition, the basal process was inherited by the right-hand cell, as this cell is the only one to merge with the stray cytoplasm.

(C) Asymmetric inheritance of the basal process. A single neuroepithelial cell divides into two unequal siblings. The dashed lines denote the cleavage plane. The larger, brighter cell inherits the basal process, while the smaller, dimmer one (arrow) does not. Even before mitosis, the basal process can be seen to "belong" to the half of the cell that will inherit it. The arrow points to the smaller sibling. Ectopic cytoplasm (arrowhead) remains in the basal process during division and rejoins the cytoplasm of the larger sibling. The double arrow points to the basal process of a third irrelevant cell.

progenitor cells (Cayouette et al., 2001; Wakamatsu et al., 1999; Zhong et al., 1996), and Notch, a potential target for Numb in mouse, rat, and ferret progenitor cells (Chenn and McConnell, 1995; Johansson et al., 1999; Zhong et al., 1997). We were, therefore, quite surprised that no apico-basal divisions take place in the zebrafish retina. Our results are also in contrast to previous analysis of sections of zebrafish retina that had shown the presence of apico-basal divisions (Horne-Badovinac et al., 2001). Could these differences be due to the possibility that we simply missed the apico-basal division? In the rat retina, minimally 3% of divisions were found to be apico-basal. From the data presented here, the chance that no apico-basal divisions are found if 3% are expected is less than 0.05 (chi-squared test). The likelihood that none are found when 10%–20% of divi-

Table 1. The Number of Observed and Expected Cell Divisions that Are Oriented along a Central-Peripheral or Circumferential Axis and which Resulted in Symmetric or Asymmetric Inheritance of the Basal Process

		Axis	
		Central-Peripheral	Circumferential
Observed Data			
Inheritance	Asymmetric	22.0	15.0
	Symmetric	18.0	22.00
Expected Data			
Inheritance	Asymmetric	19.73	17.27
	Symmetric	20.27	17.73

The chi-squared test for independence between the orientation of cell division and the mode of inheritance of the basal process shows that the two factors are independent of each other ($p = 0.29$).

sions are expected to be apico-basal, the peak proportion in rat (Cayouette et al., 2001), is much lower still ($p < 0.0001$). The 4%–7% of divisions seen as apico-basal in single sections of the zebrafish retina (Horne-Badovinac et al., 2001) yielded similar small measures of likelihood of missing such divisions in our analysis. It is possible that the visualization of division orientation in retinal sections may be misleading if dividing cells span more than one section or are dividing parallel to the uneven ventricular surface. Indeed, we saw such "apparent" apico-basal divisions when we examined single sections of our own data, but these always resolved into near planar division when the retinas were fully reconstructed in three dimensions.

In the study of apico-basal divisions in the rat retina, the authors found that m-Numb was expressed in the apical daughter of an apico-basal division, while Notch was expressed throughout the progenitor cell and therefore in both daughter cells (Cayouette et al., 2001). The proposed model is that m-Numb inhibits Notch in the apical cell but lack of inhibition in the basal cell allows Notch to promote the differentiation of the basal cell into a Müller glial cell. This would be consistent with the low frequency of apico-basal divisions in the rat retina being responsible for only one cell type. The present study covers part of the time, until 45 hpf, when Müller glial cells, which lie in the inner nuclear layer, are expected to be born (38 to 48 hpf) (Hu and Easter, 1999). Assuming that about 5%–10% of cells will become Müller cells (McFarlane et al., 1998; Ohnuma et al., 1999), one would expect the same proportion of recorded divisions to be apico-basal according to the proposed model. Since 50 divisions were recorded in this time period, five of these should give rise to Müller glial cells, yet we noticed no apico-basal divisions during this period. The probability of observing none when the expectation is five is 1.8%; therefore, it is unlikely that in the zebrafish retina apico-basal divisions are responsible for generating Müller cells.

Asymmetric divisions may also be manifest in a different way from apico-basal divisions. If cells in the neuroepithelium possess planar polarity, cytoplasmic cell fate determinants could segregate asymmetrically along the planar axes, parallel to the ventricular surface. In the *Drosophila* peripheral nervous system, asymmetric divisions have been shown to take place in the plane of the neuroepithelium along the anterior-posterior axis. The first division of the sensory organ precursor lineage is an asymmetric division of the pI cell in the a-p axis, with the polarity of pI mediated by Frizzled signaling. pIIa, one of pI's daughters, also divides along the a-p axis although its polarity is controlled by a different mechanism (Bellaiche et al., 2001; Gho and Schweisguth, 1998). Given the presence of homologous proteins in vertebrates of many of the proteins involved in planar cell polarity in *Drosophila* (Huttner and Brand, 1997; Knoblich, 2001; Lu et al., 2000), similar mechanisms may mediate a planar polarity in zebrafish retinal cells. Furthermore, c-Numb has been shown to be occasionally asymmetrically segregated in planar divisions of the chick retina (Silva et al., 2002). We were unsuccessful in labeling zebrafish Numb: we tried with several anti-

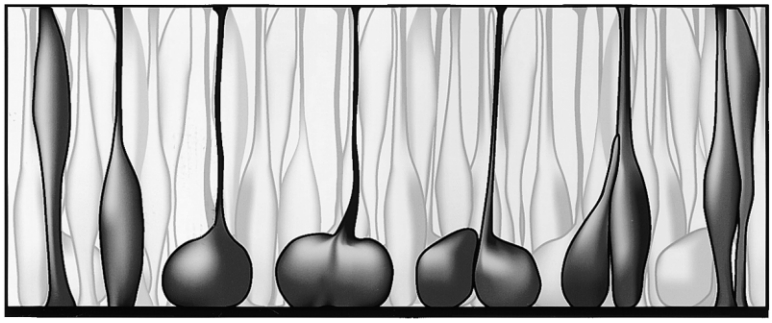

Figure 7. Sequence of Events for Some Cell Divisions in the Developing Zebrafish Retina

Prior to division, a cell's nucleus and cytoplasm collects at the apical surface of the retina, allowing the cell to round up for mitosis. However, a connection is maintained with the basal surface of the retina through a basal process. At division, the slightly asymmetric position of the basal process permits one of the daughter cells to inherit it and maintain the position at which this cell continues its progression through the cell cycle or at which this cell migrates into the retina following differentiation. Its sibling may grow a new basal process alongside the first cell, thus maintaining a spatial relationship between the siblings in a radial column.

bodies that work in other species, and we tried misexpressing the *Drosophila* partner-of-numb-GFP fusion protein. Unfortunately, none of these approaches gave us a specific signal. The potential for a planar polarity in the neuroepithelial cells led us to analyze the orientation of cell divisions within the planar surface. Two orthogonal axes that run along the retinal surface were defined—a central-peripheral axis and a circumferential axis. We found, in both zebrafish and rat, that the proportion of cells dividing in the circumferential axis increased over time at the expense of divisions orientated with the central-peripheral axis. Time-lapse imaging of mitotic spindles in the ventricular zone of rat cortical slices showed that although the spindles spin around just prior to M-phase, they spend most of their time in two orthogonal orientations parallel to the ventricular surface, finally stabilizing in one of the two orientations for mitosis (Adams, 1996). This would suggest that the orientation of cell divisions during cortical neurogenesis is regulated within the plane of the ventricular zone. In the retina, the change in trend of division orientation over time could be due to two different phenomena: it could be part of the morphological changes of growth that the retina goes through, including an associated increase in the volume of space in which the cells can divide, or it could reflect the transition from symmetric to asymmetric divisions that would be expected with increasing levels of terminal neurogenesis.

We compared observed changes in retinal dimensions to expected changes in retinal dimensions that were estimated from the changes in proportions of orientations of cell division within an equatorial, rectangular patch of the retina. Given the greater proportion of circumferential divisions seen at later time points, we expected a greater increase in the circumferential dimension of the retina compared to the central-peripheral dimension (6.8% versus 1.7%). In fact, we found that at later time points, the retinal surface increased less in the circumferential axis than it did in the central-peripheral dimension (3.5% versus 7.6%). These figures do not support the hypothesis that orientation of cell division drives the shape change of the eye. If circumferential divisions are not driving morphogenesis, perhaps they relate to asymmetric cell divisions that throw off differentiating daughters. The neurogenic wave could be the basis for a signal spreading along the circumferential axis that provides planar polarity to dividing cells. The present data sets do not allow us to assess whether reorientation occurs in the ventral-nasal patch before the dorsal patch as might be expected if this were the case. However, a few cell fate determining genes, such as *ath5* and *sonic hedgehog*, have been identified that are expressed in the same pattern as the neurogenic wave and are involved in the differentiation of retinal ganglion cells (Kay et al., 2001; Masai et al., 2000; Neumann and Nuesslein-Volhard, 2000). Since the wave itself spreads along the circumferential axis of the retina, one could speculate that the later increase in proportion of divisions orientated along the circumferential axis is related to the advent of the neurogenic wave and hence to the differentiation of cells. More circumferentially orientated divisions could be asymmetric in nature compared to central-peripheral axis divisions, with one cell being exposed to a higher concentration of a signaling protein that propagates the neurogenic wave. In *syu* zebrafish embryos, the expression of Shh is reduced, affecting the production of retinal ganglion cells but not of cells in the inner nuclear layer or photoreceptor layer. In *lak* embryos, ath5 expression is abolished, leading to a complete loss of retinal ganglion cell generation. The increase in circumferential divisions is either absent or delayed in these mutants. This difference may correspond to the absence or delay of asymmetric cell divisions that would normally give rise to retinal ganglion cells. The model that circumferential divisions are likely to be asymmetric is thus strongly supported by correlational evidence, but direct evidence is lacking.

The Basal Process

Neuroepithelial cells span the entire width of the retina. In order to divide into two cells, the nucleus and most of the cytoplasm of a cell will migrate to the apical surface of the retina and round up for mitosis. From reconstructions of cells from E13 mouse retinal sections visualized by electron microscopy, Hinds and Hinds were able to build a sequence of morphological stages that a neuroepithelial cell would presumably pass through as it differentiated into a retinal ganglion cell (Hinds and Hinds, 1974). They found one instance of a cell in mitosis that no longer had a process connecting it to the basal side of the retina. They concluded that the basal process must retract or degenerate during mitosis and grow back as the cell moved into interphase or differentiated. However, recent time-lapse studies in embryonic mouse cerebral slices have shown that the basal process persists during the mitosis of radial glial cells (Miyata et al., 2001; Noctor et al., 2001). Our time-lapse analysis of membrane-labeled cells has also shown that, in all clear cases, the basal process of neural progenitor cells persists through mitosis. The persistence of the basal process during mitosis brings up the interesting question of asymmetry of inheritance of the basal process. In the cases where the basal process was clearly inherited by only one daughter cell, it was also found asymmetrically positioned on the basal surface of the mitotic cell. Generally, the daughter that did not inherit the basal process would either promptly grow its own process along its sibling's process, or simply join the sibling's process. We could not distinguish between these two alternatives by laser scanning microscopy. The fact that the noninheriting cell joined its sibling implies either some sort of recognition between the two siblings or a persisting physical link between the two. The importance of such a link becomes evident when one considers the structure of the retina as a collection of radial units similar to those in the cortex (Dowling, 1987; Rakic, 1995). Cells of a clone can be physically kept together by the persisting basal process of at least one cell acting as a scaffold for other cells in the clone. This would result in the radial pattern of cells seen when single progenitor cells are marked with lineage tracers (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990).

It has been suggested that basal processes become the axons of retinal ganglion cells or dendrites of horizontal cell, while cells that do not have a basal process after division become photoreceptor cells or amacrine

cells (Hinds and Hinds, 1974, 1979). The basal process extends from the neuroepithelial cell from a structure seemingly analogous to the axon hillock. In terms of cell differentiation and the basal process, two hypotheses present themselves: as the cell differentiates, its basal process must start retracting from the basal surface. Alternatively, the daughter cell that does not inherit the basal process could start to differentiate without having to grow its basal process all the way to the basal side of the retina. Interestingly, Miyata et al. (2001) suggest that the neuronal progeny of radial glial cells tend to inherit the basal process. This would allow migration of the neuronal cell body to its correct lamina as well as establish the basis for the cell's axon. Our cell tracing experiments showed a division in which the daughter cell that did not inherit the basal process at mitosis was found to divide again 18 hr later, excluding an equation between noninheritance of the basal process and terminal cell differentiation.

Asymmetric inheritance of the basal process was found to be independent of orientation of cell division. If asymmetric inheritance of the basal process is a physical sign of asymmetric division, this would support a hypothesis where the planar orientation of division is not an indicator of asymmetry, but rather plays a role in retinal morphogenesis. The only certainty is that the two factors, basal process inheritance and planar orientation of division, are not both involved in asymmetric cell division, since they do not correlate with each other. Future studies using transgenic zebrafish that express fluorescent proteins in specific cell types and zebrafish mutants in which neural cell proliferation, morphogenesis, and differentiation are disrupted will certainly shed light on these issues.

Experimental Procedures

Fish

Fish were maintained at 26.5°C. Embryos were raised at 28.5°C and staged in hours postfertilization (hpf). Embryos used for imaging were of wild-type strains (Ab, wik) or homozygous for the *sdy*^{tk20} mutation. Wild-type embryos were treated with 0.003% PTU (Sigma) from 11 to 24 hpf to delay pigment formation in the eye. *Sdy* embryos fail to develop melanophores and do not require PTU treatment to prevent pigmentation. Because PTU treatment is less effective in the retina after about 48 hpf, *sdy* embryos are better suited to long-term imaging. Embryos carrying the *sonic you* (*syu*^{t4}) mutation (a gift from S. Wilson, UCL) and the *lakritz* (*lak*^{th241}) mutation (a gift from H. Baier, UCSF) were used in experiments to study the role of the neurogenic wave (see Introduction). Retinal ganglion cells expressing GAP-GFP, a membrane-tagged version of eGFP, were visualized using transgenic fish that express the GAP-GFP gene under the control of the zebrafish Brn3c promoter (a gift from T. Roeser, UCSF). *Syu*, *lak*, and Brn3c-GAP-GFP fish were also treated with PTU for imaging experiments.

Bodipy-Ceramide Labeling

Bodipy-FL-C_5-Ceramide (D-3521, Molecular Probes) stock solutions were made using DMSO to a concentration of 6.25 μg/μl. Prior to labeling, a 5 μl aliquot was further dissolved in 150 μl of embryo medium containing 0.01 M HEPES buffer. 15 to 20 embryos were bathed in the solution in darkness at 28.5°C for 4 to 8 hr. The embryos were rinsed thoroughly in embryo medium and dechorionated prior to mounting as described below.

Plasmids

A number of plasmids were used as in vivo markers of cell membranes or nuclei for studies of cell division. GAP-GFP, which consists of eGFP preceded by a palmitoylation signal from GAP43, was subcloned into pCS2+ for use as a cell membrane marker. H2B-GFP (BD Pharmingen) was used to label cell nuclei. H2B-GFP is a fusion protein of human Histone 2B and eGFP driven by an EF-1α promoter in the mammalian vector, pBOS. GAP-GFP or H2B-GFP were injected as supercoiled plasmids into cells of 1–8 cell stage embryos at concentrations of around 10 ng/μl in 0.25% Phenol Red.

In Vivo Imaging

Injected embryos were screened at 24 hpf under a fluorescent dissecting stereo microscope (Leica MZ FLIII) for those with strong eGFP expression in the retina. Chosen embryos were mounted in 0.2% agarose at pH 7.4, containing 0.04% MS-222 and 0.01 M HEPES onto a no. 0 coverslip that served as the bottom of a 35 mm petri dish. Embryos were also orientated such that the lateral side of the eye was closest to the coverslip. Acetate rings were used as spacers and another no. 0 coverslip was placed on top. The petri dish was placed in a heated stage at 28.5°C on an inverted microscope (Leica DMIRBE).

eGFP fluorescence in the specimens was imaged using a Leica TCS SP two-photon laser scanning microscope. A picosecond-pulsed Ti:Sapphire laser (Spectra Physics Tsunami, 5W) was tuned to a wavelength between 850 and 885 nm and focused onto the specimen using a Leica 63×, 1.2 NA water immersion objective with a long working distance (0.225 mm). Emission between 500 and 550 nm was detected using a nondescanned transmitted light detector. For long-term time lapses, optical sections 1 μm apart were taken through a volume of the retina up to 120 μm in depth and Kallmann averaged 2 to 6 times. Time points were between 10 and 30 min apart. For higher-resolution imaging, 0.4 μm optical sections were taken through volumes about 15 μm deep every 3 to 4 min. Image data was acquired and stored as TIF files using Leica TCS NT or Leica LCS software.

Visualization

To visualize the acquired data as time-resolved volumes, images were processed on a Macintosh computer using Object Image (http://simon.bio.uva.nl/object-image.html), a program based on NIH Image (http://rsb.info.nih.gov/nih-image/) or Volocity (Improvision, UK). NIH Image macros for 4D visualization (LOCI, University of Wisconsin-Madison) were adapted to produce maximum intensity projections (MIPs) for each volume (or time point) rotated through 360° around the *x* and *y* axes at 10° intervals. All projections in each data set were assembled into Quicktime movies using 4D Turnaround software and viewed using 4D Viewer (LOCI, University of Wisconsin-Madison). The software allows the user to view the MIPs generated from each time point, and therefore "rotate" the volume as well as step through the data in time. LOCI software is available at http://www.loci.wisc.edu/4d/native/4d.html. With Volocity, each time point was volume rendered and could be viewed from any angle while being viewed as part of a time-lapse series.

Imaging of lakritz *Mutants*

lakritz mutants could not be imaged in vivo as the phenotype of pigment aggregation, by which they were originally recognized (Kelsh et al., 1996), does not become apparent until 4 days postfertilization. Instead, mutant embryos were identified early by tissue genotyping and the heads of identified mutants fixed for imaging at specific time points. Embryos obtained from *lak* heterozygous matings were genotyped as previously described (Kay et al., 2001). Briefly, trunks of fish were digested with proteinase K, boiled, and diluted 1:5 in water. One microliter of lysate was used for PCR amplification using the primers 5′-CCGGAATTACATCCCAAGAAC and 5′-GGCCATGATGAGCTCAGAG in a 50 μl reaction (35 cycles, 55.6°C annealing temperature). Fifteen microliters of the 293 bp PCR product were digested with StuI and separated on a 2% agarose gel. In the case of wild-type fish, the product was cut into two fragments. In mutants the product remained uncut, and in heterozygous fish, all three bands were visible.

For the analysis of division planes in *lakritz* mutants, embryos were collected at 30, 40, and 55 hpf. Embryos were treated with 0.003% PTU from 11 hpf onward to inhibit pigmentation. After collection, the embryos were fixed for 5 hr in 4% PFA at 4°C. After washing in PBS, the trunks were separated from the heads for genotyping

(see above). The heads were then processed through a series of 25%, 50%, 75%, and 2× 100% Methanol in PBT (0.1% Tween-20 in PBS) and stored overnight in 100% Methanol (−20°C). After rehydrating through the same series of Methanol dilutions, the heads were washed in PBS and twice in distilled water. To permeabilize the tissue, acetone treatment (7 min, −20°C) was performed (Westerfield, 1995). Afterwards, heads were washed in water and twice in PBT. To visualize mitotic figures, DNA was stained overnight at 4°C with Hoechst (1 μg/ml in PBT) and then washed extensively to reduce background (PBT overnight at 4°C). The eyes were mounted on slides, and z-series (optical sections 1 μm apart) through entire eyes were collected by two-photon laser scanning microscopy as described above.

Definition of Retinal Axes

In order to analyze the orientation of cell divisions in the retina, we defined three orthogonal axes in the retina. The first is the apico-basal or radial axis, which is perpendicular to any given point on the surface of the retina and runs between its apical and basal sides. Orthogonal to the radial axis are two axes that run within the plane of the retinal surface itself. By considering the retina as an ellipsoid body much like a globe, with the lens at one pole and the optic nerve head at the other, we can define a central-peripheral axis analogous to lines of longitude or great circles on a globe and a circumferential axis analogous to lines of latitude running orthogonal to the central-peripheral axis (Figure 4A).

Analysis of Orientation of Cell Division in Zebrafish

In order to check if cells divided apico-basally or radially, we analyzed the orientation of cell division in the apico-basal direction with respect to the apical surface. To measure this orientation, maximum intensity projections of each time point of interest were rotated around the *x* or *y* axis into a position that revealed the closest relationship between the nuclei and the apical surface. The apical surface was marked as the contour formed by any labeled pigment epithelial cells. The difference in orientation between a line joining the centers of the two cells or nuclei (equivalent to the mitotic spindle axis) and an estimated tangent to the apical surface was measured in terms of an acute angle between 0° and 90°. An angle close to 0° represented a cell division parallel to the apical surface, while an angle closer to 90° represented an apico-basal division.

To measure the orientation of cell division within the apical or retinal surface, the midpoint of the "spindle axis" joining a pair of daughter cells was determined and a line was drawn from the center of the lens to the midpoint, to be taken as the 0° reference. Such a line is simply a projection of a central-peripheral line on the surface of the retina. The orientation of this line was compared to the orientation of the spindle axis and measured. An angle of 0° indicated a division in the central-peripheral axis while 90° meant a division in the circumferential axis. For daughter cell pairs that were aligned with the *z* axis of the volume, and therefore impossible to distinguish in the lateral view of the eye, the whole volume was rotated by 90° around the *x* or *y* axis. The measurement procedure was then followed as described above.

Analysis of Orientation of Cell Division in Rat

We studied the orientation of cell division in the retina of Sprague Dawley rats. Animals were killed at embryonic day 16 (E16), postnatal day 0 (P0), or P4. The eyes were removed and the retinas dissected free from surrounding tissue in cold HBSS. Retinas were flat-mounted (vitreous side down) on a microscope slide and fixed with 4% paraformaldehyde for 1 hr at room temperature. They were then rinsed three times in PBS and counterstained with propidium iodide (5 μg/ml in PBS + 25 U RNase A) to visualize nuclear morphology. They were examined with a laser scanning confocal microscope (BIO-RAD MRC 1024), and z-series in 1 μm steps were acquired in the central and peripheral regions of the retina.

To determine the orientation of cell divisions within the retinal surface, a line was drawn from the optic nerve head to the periphery of the retina (equivalent to the central-peripheral axis), and another line was drawn perpendicular to the first to serve as the 0° reference line. The angle between this reference line and the spindle axis was taken as the angle of division. An angle of division of 0° would correspond to a mitotic spindle aligned along the circumferential axis of the eye, whereas an angle of 90° would correspond to a mitotic spindle aligned along the central-peripheral axis of the eye. To be consistent with the fish data, however, we used the complementary angles to those of the rat results so that an angle of 0°, for example, became 90° (circumferential) and an angle of 90° became 0° (central-peripheral).

Analysis of the Basal Process

To visualize the basal process of a neuroepithelial cell during mitosis, zebrafish embryos were injected with GAP-GFP, which labels cell membranes, and their retinas were imaged as described above (see Visualization). A short time series of a volume of interest containing a dividing cell was rendered using Volocity (Improvision, UK). This allowed us to freely rotate the volume and attempt to determine whether the basal process could be seen. Looking at time points before and after mitosis also helped to identify the basal process. For basal processes that could be seen, we also described which area of the cell's surface the basal process emanated from, since a decentralized basal process could be inherited asymmetrically after mitosis depending on the cleavage plane of the cell. We defined its position in terms of the central-peripheral and circumferential axes mentioned above. On the basal aspect of the cell, the position of the process could be described in relation to the cell's pole as "lateral" or "central" in the circumferential axis and as "central" or "peripheral" in the central-peripheral axis.

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