**mirror** controls planar polarity and equator formation through repression of *fringe* expression and through control of cell affinities

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**SUMMARY**

The *Drosophila* eye is divided into dorsal and ventral mirror image fields that are separated by a sharp boundary known as the equator. We have previously demonstrated that Mirror, a homeodomain-containing putative transcription factor with a dorsal-specific expression pattern in the eye, induces the formation of the equator at the boundary between *mirror*-expressing and non-expressing cells. Here, we provide evidence that suggests *mirror* regulates equator formation by two mechanisms. First, *mirror* defines the location of the equator by creating a boundary of *fringe* expression at the mid-point of the eye. We show that *mirror* creates this boundary by repressing *fringe* expression in the dorsal half of the eye. Significantly, a boundary of *mirror* expression cannot induce the formation of an equator unless a boundary of *fringe* expression is formed simultaneously. Second, *mirror* acts to sharpen the equator by reducing the mixing of dorsal and ventral cells at the equator. In support of this model, we show that clones of cells lacking *mirror* function tend not to mix with surrounding *mirror*-expressing cells. The tendency of *mirror*-expressing and non-expressing cells to avoid mixing with each other is not determined by their differences in *fringe* expression. Thus *mirror* acts to regulate equator formation by both physically separating the dorsal cells from ventral cells, and restricting the formation of a *fringe* expression boundary to the border where the dorsal and ventral cells meet.

Key words: Polarity, Border, Eye development, *Drosophila melanogaster*

**INTRODUCTION**

Studying the processes by which boundaries between fields of cells are initiated and maintained is important in understanding the development of multi-cellular organisms. Once formed, such boundaries often serve as signaling centers for subsequent growth and patterning (Blair, 1995). Restrictions in cell lineage and prevention of cell-cell mixing are mechanisms commonly used to generate boundaries. Selector genes such as *engrailed* have been suggested to play an important role in lineage restriction, and in conferring differential cell affinities during boundary formation between different compartments of a tissue (reviewed by Lawrence and Struhl, 1996). Recent studies of the vertebrate hindbrain (Xu et al., 1999) and the *Drosophila* wing (Blair and Ralston, 1997; Rodriguez and Basler, 1997) have demonstrated that cell-cell signaling also plays an important role in altering cell affinities across boundaries that separate fields of cells. However, the exact mechanisms that establish differential cell affinities during the process of boundary formation are still not well understood.

The dorsal/ventral (D/V) border of the *Drosophila* eye is an example of an extremely precise border (reviewed by Reifegerste and Moses, 1999). The adult eye is composed of nearly 800 units of photoreceptor clusters known as ommatidia, which are divided into a dorsal and a ventral field. The polarity of an ommatidium in the dorsal half of the eye is a mirror image of that in the ventral half of the eye (Fig. 1A). The boundary where the dorsal and ventral fields of ommatidia meet is known as the equator. The equator is a highly regular structure that bisects the eye, rarely deviating more than one ommatidial width as it crosses the eye from posterior to anterior. In a wild-type eye, ommatidia of dorsal polarity are never found in the ventral field, nor are ommatidia of ventral polarity found in the dorsal field. Both the precision in the division of dorsal and ventral fields and the regularity in the path of the equator suggest that equator formation is a highly regulated process. Strict restriction in cell lineage has been discounted as a mechanism for forming the equator, since occasionally a single clone of cells can give rise to both dorsal and ventral ommatidia (Ready et al., 1976).

The process of equator formation begins in the eye imaginal disc. The eye imaginal disc is the larval precursor of the adult eye and consists of a single sheet of epithelium. Differentiation initiates at the posterior margin of the eye disc during the 3rd instar larval stage of development and moves forward as a wave, which is marked by an indentation in the epithelium known as the morphogenetic furrow. As the furrow moves forward, it leaves behind ommatidial preclusters that are...
Fig. 1. The Drosophila eye is divided into dorsal and ventral mirror image fields of ommatidia. In all the following panels except for F, anterior is to the left and dorsal up. Arrows indicate the approximate position of the furrow. Arrowheads indicate approximate position of the mid-point of the eye. (A) A section through the equatorial region of an adult eye shows that the ommatidia located dorsal to the equator are mirror images of those located ventral to the equator. The equator is highlighted in red. An ommatidium is the hexagonal unit that consists of 7 visible rhabdomeres and surrounding pigment cells. (B) In situ analysis of mirr in a wild-type eye disc shows that expression of mirr transcript (purple) is restricted to the dorsal half of the eye anterior to the morphogenetic furrow. (C) Staining of Mrr protein in a wild-type eye disc shows that expression of Mrr protein (green) is restricted to the dorsal region of the eye. However unlike the transcript, protein expression persists in a subset of cells located posterior to the furrow. (D) Staining for β-galactosidase (β-gal) activity in an eye disc derived from a fng-lacZ/+ larvae shows that the β-gal expression (blue) is restricted to the ventral portion of the eye. fng-lacZ is a P-element containing white and lac-Z inserted near the fng locus which is expressed in a ventral-specific pattern in all stages of the eye disc development. (E) A diagram of an eye disc. The eye disc is composed of an eye portion and an antennal portion. Cells in front of the furrow in the eye disc are undifferentiated and express several molecular markers. Dorsal cells express mirr, as well as ara and caup (green). Ventral cells express fng (orange). fng transcript can also be detected transiently in a thin band of cells adjacent to the furrow in the dorsal half of the eye during the 3rd instar larval stage of development. Posterior to the furrow, dorsal and ventral ommatidia rotate in opposite directions, resulting in the formation of an equator. (F) Co-staining of Mrr protein (green) and β-gal protein (red) in an eye disc derived from a fng-lacZ/+ larvae shows that the expression of Mrr and fng-lacZ are complementary. Note that in this figure, anterior is to the right and dorsal up.

morphologically identical in the dorsal and ventral halves of the eye. Opposite polarities between dorsal and ventral ommatidia are seen later when preclusters situated in the dorsal and ventral part of the disc rotate 90 degrees in opposite directions. The process by which dorsal and ventral ommatidial preclusters choose the direction to rotate is thought to be regulated by signal(s) emanating from the D/V boundary created early in development (reviewed by Blair, 1999; Reifegerste and Moses, 1999). The formation of the D/V boundary is thought to initiate before or during the 1st instar larval stage of development, since dorsal and ventral cells already express distinct sets of molecular markers at that stage (Brodsky and Steller, 1996; Sun et al., 1995; Papayannopoulos et al., 1998).

Two genes that have been shown to be specifically involved in regulating equator formation are fringe (fng) and mirror (mirr). fng encodes a glycosyltransferase-like molecule that is expressed in the ventral half of the developing eye disc (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). Juxtaposition of fng-expressing and non-expressing cells by the removal of fng function from a clone of cells in the ventral region of the eye results in the formation of an ectopic equator at the clonal border (Papayannopoulos et al., 1998; Dominguez and de Celis, 1998; Cho and Choi, 1998). Interaction between fng-expressing and non-expressing cells at the fng-expressing/non-expressing boundary (fng+/fng−) is thought to initiate equator formation by inducing Notch activation restricted to the border (Papayannopoulos et al, 1998; Dominguez and de Celis, 1998; Cho and Choi, 1998). However, the mechanism by which Notch activation leads to equator formation is not well understood. mirr encodes an Iroquois (IRO) family homeodomain-containing protein that is expressed specifically in the dorsal half of the developing eye disc (McNeill et al., 1997). Juxtaposition of mirr-expressing and non-expressing cells has also been shown to be important in regulating equator formation. Inducing an ectopic mirr-expressing/non-expressing boundary (mirr+/mirr−) by removing mirr function from a clone of cells in the dorsal region of the eye causes an ectopic equator to form at the equatorial border of the clone (McNeill et al., 1997). In addition, disrupting the mirr+/mirr− boundary by removing mirr function from a clone of cells that traverse the D/V boundary causes the equator to alter its path. Recent studies of mirr and fng show that ectopic expression of mirr in the ventral region of the eye can repress fng expression, whereas ectopic expression of fng in the dorsal region of the eye fails to repress mirr expression (Cho and Choi, 1998). These results have led to the hypothesis that equator formation is induced by a fng+/fng− boundary created at the mid-point of the eye and that mirr contributes to formation of the fng+/fng− boundary by restricting fng expression to the ventral half of the eye (reviewed by Blair, 1999).

Several issues remain to be addressed to confirm the validity of this hypothesis. First, although ectopic expression of mirr can repress fng expression, it has not yet been shown that mirr is necessary to restrict fng expression to the ventral half of the eye. It is important to note that ectopic expression of caupolican (caup) has also been shown to be able to repress fng expression (Dominguez and de Celis, 1998). Caup is another Iroquois family transcription factor (Gomez-Skarmeta et al., 1996) highly related to Mrr and is similarly expressed in a dorsal-specific pattern in the eye. This raises the possibility
that caup is the true repressor of fng in the dorsal region of the eye, and that ectopic expression of mirr in the ventral region of the eye represses fng by mimicking caup function. In addition, although an ectopic fng+/fng− boundary is capable of inducing an ectopic equator, the ectopic equator is often irregular in shape compared to the highly ordered wild-type equator. This observation suggests that events other than the generation of a fng+/fng− boundary at the D/V border may contribute to the regularity exhibited by the wild-type equator.

We propose that mirr controls equator formation by two separate mechanisms. First, mirr acts to define the location of the equator by restricting fng expression to the ventral half of the eye. We show that removal of mirr in the dorsal region of the eye results in ectopic expression of fng. In addition, the mirr+/mirr− boundary at the equatorial border of a mirr dorsal clone fails to induce an ectopic equator if fng cannot be activated within the clone to generate an ectopic fng+/fng− boundary. Second, mirr acts to sharpen the path of the equator by reducing cell–cell mixing between dorsal and ventral cells. Analysis of clone shapes of wild-type, mirr, mirr/fng and fng dorsal clones suggests that mirr-expressing and non-expressing cells tend not to mix. Importantly, the tendency of mirr-expressing and non-expressing cells to avoid mixing is not determined by their differences in fng expression. mirr therefore acts to define the location of the equator by restricting expression of fng to the ventral half of the eye, and in parallel acts to increase the sharpness of the equator by reducing cell mixing between dorsal and ventral cells.

**MATERIALS AND METHODS**

**Stocks**

All stocks were maintained under standard culture conditions. w; mirr358/TM3 and UAS-mirr12 were generated in this lab. w; FRT80B, mirr358/TM3 was kindly provided by Andrew Tomlinson. mirr12/TM3, dpp-Gal4 and UAS-wg were obtained from Bloomington stock center. w; FRT80B, fng13/TM3 and fng-lacZ (35UZ-1) were generously provided by Ken Irvine. w; FRT80B, mirr358/fng13/TM3 double mutant was generated by recombination. eyeless-GAL4 was kindly provided by Uwe Walldorf. w; FRT80B and y,w, hsFLP122; FRT80B, [w7]70C were kindly provided by Naoto Ito. w; mirr532/TM3 was kindly provided by Michael Brodsky.

**Clonal analysis**

Mitotic clones were generated by the standard FLP/recombinase technique (Xu and Rubin, 1993). Animals were heat-shocked for 2 hours at 37 °C during the 1st instar larval stage to induce mitotic clones. Eyes containing homozygous mutant clones, marked by the absence of pigment granules due to loss of white gene function, were embedded in resin and sectioned using standard histological methods.
(Tomlinson and Ready, 1987). Sections of clones were examined by standard microscopy.

To quantify the differences in clone shape between wild-type, mirr, fng and mirr-fng clones, images of individual clones were taken from a Sony CCD camera in order to determine the A/L^2 ratio (L: circumference of the clone, A: area of the clone) for each individual clone. The rationale for using the A/L^2 formula was as follows: for a given number of cells in a clone (A), the stronger the tendency of these cells to avoid mixing with cells located outside of the clone, the smaller the circumference (L) would be relative to the area (A) (see also Lawrence et al., 1999). The boundary of a clone was defined by scoring the pigment cells at the border of the clone. The area (A) of a clone was determined by counting the number of ommatidia included in the clone. The circumference (L) of a clone was the number of ommatidia that lay at the border of the clone. The distributions of A/L^2 for individual sets of clones were first determined and then compared to each other using the data analysis tool (t-test and histogram) of Microsoft Excel.

**Generation of the anti-Mirr serum**

A 948 bp PCR fragment of mirr cDNA was cloned into the pGEX-KG vector (Pharmacia) to produce GST-Mirr fusion protein that contained the carboxy-terminal region of Mirr. The fusion protein was then expressed and purified from *Escherichia coli* with glutathione affinity chromatography and injected into rats for antibody production.

**Immunohistochemistry**

Eye discs were dissected in cold phosphate buffer, fixed in 2% formaldehyde solution (EM grade, Polysciences) for 30 minutes on ice, and rinsed 20 minutes in PBS-DT (PBS + 0.3% deoxycholate + 0.3% Triton X-100). Fixed discs were then incubated in PBS-DT containing 1/2000 diluted rat anti-Mirr antibodies and 1/100 diluted rabbit anti β-glucuronidase antibodies (Cappel) overnight at 4°C and then washed 5× 20 minutes in PBS-DT + 0.1 M NaCl at room temperature. FITC and Cy3-conjugated secondary antibodies were used for fluorescence labeling of anti-β-glucuronidase and anti-Mirr antibodies for 5 hours at room temperature. Labeled discs were washed 5× 20 minutes in PBS-DT + 0.1 M NaCl at room temperature before mounting in Vectashield (Vector) and examination by confocal microscopy. Staining for β-glucuronidase activity was as previously described (McNeill, 1997).

**RESULTS**

**mirr represses fng expression in the dorsal region of the eye**

We have previously shown that expression of mirr transcript is restricted to the dorsal cells in front of the morphogenetic furrow (Fig. 1B). In order to examine the expression of Mirr protein in the eye discs, an antibody against Mirr was generated to stain eye discs for the presence of Mirr. The expression of Mirr protein was restricted to the dorsal cells in the eyes (Fig. 1C). However, in contrast to mirr transcript, Mirr protein persisted in a subset of dorsal cells long after the morphogenetic furrow had passed and photoreceptors have differentiated.

Since the domain of fng expression is reciprocal to that of mirr expression (Fig. 1C,D,F and also see Papayannopoulos et al., 1998), we were interested in determining if mirr represses fng expression. We first examined fng expression under conditions in which mirr expression was expanded ventrally into the region where fng was normally expressed. It has been shown that expression of a mirr-lacZ enhancer trap is up-regulated and shifted ventrally when wingless (wg) is overexpressed at the dorsal and ventral margins of the discs using the GAL4-UAS system (Heberlein et al., 1998). We used this same approach to overexpress wg at the dorsal and ventral margins of the eye discs and examined the effect on Mirr expression (Fig. 2D). The intensity of Mirr expression was increased and the domain of Mirr expression was altered in these discs (compare Fig. 2C to 2D). We used a fng-lacZ enhancer trap to monitor fng expression in these discs and found that the domain of fng-lacZ expression was diminished (compare Fig. 2A to 2B) and remained non-overlapping with that of Mirr expression (Fig. 2D). This result is consistent with the idea that Mirr inhibits fng expression. Interestingly, a sharp indentation of the epithelium was consistently observed at the novel border where mirr-expressing and non-expressing cells were juxtaposed in these discs. This observation raised the possibility that a physical barrier exists that prevents cell-cell mixing at the novel mirr^+/mirr^- boundary.

We then tested directly if ectopic expression of mirr in the ventral region of the disc could repress fng expression. The GAL4-UAS system was used to express mirr ubiquitously in the eye discs. The expression of fng-lacZ was significantly reduced in these discs (compare Fig. 2A to 2F; see also Choi and Choi, 1998). Thus, ectopic expression of mirr in the ventral region of the eye represses fng expression.

Since ectopic expression of caup can also inhibit fng in the eye (Dominguez and de Celis, 1998), we tested if mirr is specifically required to repress fng expression in the dorsal part of the eye. In order to do so, we examined fng-lacZ expression in mutant eye discs. Animals homozygous for strong loss-of-function mirr alleles die during embryogenesis. However, the allelic combination of two lethal mirr alleles, mirr\(^{X52}\) and mirr\(^{D3}\), was found to allow a few animals to survive until stages when eye discs could be examined. The expression of Mirr protein in the mirr\(^{X52}\)/mirr\(^{D3}\) discs was greatly reduced (compare Fig. 2C to 2E). The expression of fng-lacZ was then examined in these discs and found to be present ectopically in the dorsal region of the discs (Fig. 2G). Furthermore, the ectopic expression of fng-lacZ was most frequently observed in the dorsal anterior region of the discs. Other combinations of mirr alleles also resulted in ectopic expression of fng-lacZ (data not shown). Together these data suggest that mirr represses fng expression but that this repression is limited to the dorsal anterior region of the eye. Interestingly, we found that occasionally a few mirr\(^{X52}/\)mirr\(^{D3}\) flies survived until adulthood and displayed a protrusion in the dorsal posterior region of the eye (Fig. 2H). Such protrusion is reminiscent of a group of cells that are attempting to sort out due to their altered cell affinities.

**Ectopic equator formation requires a fng^+/fng^- border**

We have previously reported that removal of mirr function from a clone of cells in the dorsal region of the eye can cause an ectopic equator to form at the equatorial border of the clone (see Fig. 3B for definition of equatorial versus polar border in this work). The generation of the ectopic equators appeared to be restricted to the anterior clones since no ectopic equator was ever detected at the borders of posterior clones. Interestingly, after examining a large number of anterior clones, we found that the formation of an ectopic equator was restricted to the
segments of clonal borders that were located within the anterior one third of the eye, even when the borders extended more posteriorly (Fig. 3A,C,E). A Drosophila eye typically consists of 32-34 vertical columns of ommatidia. We never detected any ommatidia of ventral polarity at a mirr mutant clonal border beyond column 12 counted from the anterior edge. Since the ectopic expression of fng-lacZ in mirr mutant discs was also restricted to the dorsal anterior region of the eye, this data is consistent with the hypothesis that ectopic fng expression in mirr mutant clones is required to induce ectopic equator formation at the equatorial borders of clones.

We then asked directly if the formation of the ectopic equators at the equatorial borders of mirr dorsal clones is dependent on ectopic fng expression within the clones. To test this possibility, we examined the equatorial borders of mirr mutant dorsal clones in which fng cannot be produced. mirr and fng mutations were recombined onto the same chromosome arm in order to produce clones in which the functions of fng and mirr were removed simultaneously. We examined the equatorial borders of the mirr,fng double mutant dorsal clones and found that the occurrence of ectopic equator formation was dramatically reduced (see Table 1 and also compare Fig. 3A,C,E to 3D,F). Therefore ectopic expression of fng within the mirr mutant clones is required to induce ectopic equators at the equatorial borders of the clones. Thus one way in which mirr regulates equator formation is by repressing fng expression in the dorsal region of the eye.

Removal of fng in the dorsal region of the eye causes an ectopic mini-equator to form at the polar border of the clone

We discovered a previously unrecognized phenotype of fng mutant clones located in the dorsal region of the eye during our characterization of the phenotypes of mirr, mirr,fng and fng mutant dorsal clones. Although previous studies did not report any phenotype associated with fng clones located in the dorsal region of the eye (Cho and Choi, 1998), we found that one to three ommatidia of ventral polarity were often generated at the polar boundaries of fng dorsal clones (see Table 2 and also see Fig. 4C and 4E). We termed the ectopic juxtaposition of the dorsal and ventral ommatidia at the polar boundary of a fng dorsal clone a ‘mini-equator’ because of its short length. Although the expression of fng transcript is restricted to the ventral region of the eye during the early stages of eye development, it is later transiently present in a narrow band of cells associated with the furrow in the dorsal region of the eye (Papayannopoulos et al, 1998; Dominguez and de Celis, 1998; Cho and Choi, 1998; also see Fig. 1E). Thus the formation of the ectopic mini-equators may be in part due to the transient fng/+ boundaries created at the polar borders of fng dorsal clones as the furrow passed through the clones.

**Table 1. Prevention of ectopic fng expression within a mirr dorsal clone suppresses ectopic equator formation at the ‘equatorial’ border of the clone**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>mirr,fng</th>
<th>mirr</th>
<th>fng</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of clones sectioned</td>
<td>41</td>
<td>48</td>
<td>31</td>
</tr>
<tr>
<td>No. of clones containing ommatidia with polarity changes at the ‘equatorial’ border</td>
<td>5</td>
<td>25</td>
<td>2</td>
</tr>
</tbody>
</table>

25 out of 48 mirr dorsal clones formed ectopic equators (or ommatidia of ventral polarity) at their equatorial borders whereas only 5 out of 41 mirr,fng double mutant clones displayed ommatidia of ventral polarity at their equatorial borders. The suppression is also obvious in the reduction of length of the ectopic equators formed at the mirr,fng clonal boundaries. Only 1 to 3 ommatidia of ventral polarity were still observed at the equatorial borders of the 5 mirr,fng clones, regardless of the size of the clones. In contrast, a significantly higher number of ventral ommatidia were detected at the borders of the 25 mirr clones. All clones examined were dorsal anterior clones. Out of 31 fng dorsal clones we examined, 2 of the clones contained 1 ommatidium of ventral polarity at the equatorial border of the fng dorsal clones. Note that there was no major difference in the size between the two sets of clones.

**Table 2. Removing fng in a clone of cells in the dorsal part of the eye can lead to ectopic mini-equator formation at the ‘polar’ border of the clone. Removing expression of mirr within the fng dorsal clone causes a dramatic increase in the length of the ectopic equator formed at the ‘polar’ border of a fng clone**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>fng</th>
<th>mirr</th>
<th>mirr,fng</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of clones sectioned</td>
<td>31</td>
<td>48</td>
<td>41</td>
</tr>
<tr>
<td>No. of clones containing polarity changes at the ‘polar’ border</td>
<td>12</td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
</table>

12 out of 31 fng dorsal clones formed an ectopic “mini-equator” at their polar borders. These ectopic equators are very short in length, and often consist of only 1 to 3 ommatidia with polarity changes at the border. In contrast, at the polar borders of 11 out of 41 mirr,fng double mutant clones, a significantly longer equator was detected, that often consisted of 4 to 9 ommatidia of ventral polarity. No ectopic equator could be found at the polar borders of the 48 mirr clones examined.

*mirr acts independently of repression of fng to enhance equator formation*

If mirr regulates equator formation strictly by repressing fng expression, removal of mirr function from fng mutant dorsal clones should not affect the phenotype of mini-equator formation associated with the clones. Interestingly, we found that when mirr function was removed from fng mutant dorsal clones, the length of the polar mini-equators increased significantly (compare Fig. 4C,E to 4B,D,F and also see Table 2). Since removal of mirr function could not induce ectopic fng in mirr,fng mutant clones, the expansion of the mini-equator must be induced by a mechanism independent of mirr-mediated repression of fng. This is the first evidence to suggest that mirr has a role besides repressing fng in regulating equator formation.
mrr controls cell mixing

We noticed another previously unrecognized phenotype of mrr dorsal clones after examining a large number of clones. We found that shapes of mrr dorsal clones tended to be rounder compared to those of wild-type dorsal clones (compare Fig. 5A-C to 5D-F). 8% of the 427 wild-type clones examined had a round shape while 92% of the clones displayed shapes that appeared to be elongated in the A/P axis or were irregular. In contrast, 38% of 261 mrr dorsal clones had a shape that belonged to the ‘round’ class. No dramatic differences in the distribution of clone shapes were observed between wild-type ventral clones and mrr ventral clones or between wild-type ventral clones and fng ventral clones (compare Fig. 6A-C to 6D-F). It has been suggested that the shape of a clone can be used as an indicator of the degree of cell-cell mixing between cells located inside and outside of the clone. Therefore, the observation that a significantly higher number of mrr dorsal clones had a round shape compared to wild-type clones suggests that mrr-expressing and non-expressing cells tend not to mix.

To examine more carefully whether mrr was affecting cell-cell mixing at the mrr+/-mrr- boundary, we quantified and compared the relative roundness of wild-type dorsal and mrr dorsal clones. The A/L^2 (L: circumference, A: area) ratio was calculated for individual clones as an indicator of their roundness (Lawrence et al., 1999 and also see Materials and Methods). In order to determine both the circumference and area of individual clones in units of ommatidium, thin sections of clones were examined (wild-type dorsal n=39, fng dorsal n=40, mrr dorsal n=44, mrrfng dorsal n=43, wild-type ventral n=29, fng ventral n=37, mrr ventral n=32 and mrrfng ventral = 21 clones). We then compared the distributions of A/L^2 ratios calculated for mrr dorsal and wild-type dorsal clones, and found that the shapes of mrr dorsal clones were significantly rounder than those of wild-type dorsal clones (P≤0.001) (Fig.

Fig. 3. Removal of mrr from a clone of cells in the dorsal region of the eye causes an ectopic equator to form at the equatorial border of the clone. The formation of such an ectopic equator can be suppressed when fng is prevented from being ectopically activated within the mrr dorsal clone. For all the panels in this figure, anterior is to the left and dorsal up. Scale bar: 25 μm for all panels except B. (A,C,E) Sections of mrr dorsal clones show that formation of ectopic equators is restricted to clonal boundaries located within the anterior of the eye even though the boundaries extend further posteriorly. The ectopic equator is indicated by the red bars. mrr mutant clones can be distinguished from the wild-type region of the eye by their lack of the pigment granules. The anterior margin of the eye starts at the left edges of these panels. (B) A diagram showing a clone of cells (blue) in which specific genes are removed can be distinguished from the rest of the eye (green) by their lack of pigment granules. The ‘polar border’ (arrowhead) is the clonal border that faces the dorsal pole of the eye and the ‘equatorial border’ (arrow) is the clonal border that faces the equator of the eye. The equatorial border of the clone is boxed in red in this diagram, indicating that a high-magnification view of the boxed segment of real clones will be examined in this figure. (D) Section of a mrrfng double mutant clone in which only 2 ommatidia of ventral polarity (as indicated by the red bars) are present near the equatorial border of the clone. See Table 1 for quantification of the suppression. Defects in photoreceptor development can be seen in mrrfng dorsal clones and are presumably caused by the absence of fng function in the clones. Similar defects are also present in fng dorsal clones but are never found in mrr dorsal clones. (F) Section of a mrrfng double mutant dorsal clone in which no ectopic equator is present at the clonal boundary.
Importantly, there were no significant differences in shapes observed between mirr ventral clones and wild-type ventral clones.

To examine the possibility that the reduction in cell-cell mixing between mirr-expressing and non-expressing cells at the borders of fng ventral clones was caused by the ectopic fng expression within the mirr dorsal clones, we compared the distribution of A/L² ratios of mirr,fng dorsal clones to that of wild-type dorsal clones. The shapes of mirr,fng clones were found to be still significantly rounder than those of wild-type clones (P < 0.001) (compare Fig. 5G-I with 5 A-C), suggesting that the reduction of cell-cell mixing at the mirr clonal borders was not caused by the ectopic expression of fng in the clones. Consistent with this observation, no significant reduction in cell-cell mixing was observed between fng-expressing and non-expressing cells at the borders of fng ventral clones since the distribution of A/L² ratios of fng ventral clones was not significantly different from that of wild-type ventral clones. Therefore, differences in fng expression do not account for the tendency of mirr-expressing cells to avoid mixing with non-expressing cells.

**DISCUSSION**

The Drosophila eye is divided precisely into dorsal and ventral
mirror image fields that are separated by a sharp boundary known as the equator. We have provided evidence here that suggests mirr acts to regulate the formation of the equator by two different mechanisms. First, mirr creates a fng+/fng− boundary at the mid-point of the eye by repressing fng expression in the dorsal half of the eye. Second, mirr sharpens the equator formed at the mid-point of the eye by reducing cell-cell mixing between dorsal and ventral cells.

**mirr acts to define the equator in part by repressing fng in the dorsal half of the eye**

Several lines of evidence suggest that mirr promotes equator formation by generating a fng+/fng− boundary through repressing fng expression. First, removing mirr function from the dorsal half of the eye where mirr is normally expressed resulted in ectopic fng expression. Second, blocking this ectopic fng expression by removing fng simultaneously with mirr greatly reduced the formation of an ectopic equator at the equatorial border of mirr clones. Since the mirr+/mirr− boundary requires a fng+/fng− boundary simultaneously created in order to induce ectopic equator formation, mirr acts to define the equator, at least in part, through the generation of a fng+/fng− boundary.

However it is important to note that in a few cases, one or two ommatidia of ventral polarity persist at the equatorial border of the mirr/fng mutant clones. There are at least two possible explanations for such a phenomenon. First, in 2 out of the 31 fng dorsal clones examined, 1 ommatidium of ventral polarity can be found at the equatorial borders of the clones (see Table 1). Thus the ventral ommatidia observed at the equatorial border of the few mirr/fng dorsal clones may simply be due to removal of fng in the clones. Alternatively, mirr could act through other molecules in addition to repressing fng in regulating equator formation. Thus preventing fng expression alone within the mirr mutant clones is not sufficient to completely prevent a mirr+/mirr− boundary in forming an equator at the mirr clonal border.

Another important point in our analysis is that the induction of both the ectopic fng expression in the mirr mutant discs and the ectopic equators at the equatorial borders of mirr dorsal clones was restricted to the dorsal anterior region of the eye. Other Mrr-like transcription factors, such as Araucan (Ara) or Caup, which are both expressed in the dorsal half of the eye, may repress fng in the posterior region of the eye. Consistent with this idea, ectopic expression of caup has been shown to be capable of repressing fng in the eye (Dominguez and de Celis, 1998). It is not known how mirr (or ara and caup) acts to repress fng expression. However, because Mirr is a putative transcription factor, it is possible that Mirr directly regulates the transcription of fng.

**mirr acts to enhance equator formation initiated by a fng+/fng− boundary**

We have shown that an ectopic mini-equator that was one or two ommatidia in width could be frequently detected at the polar border of fng mutant dorsal clones. One possible explanation for this observation is that the mini-equators are induced by the transient fng+/fng− boundaries created at the boundaries of the clone as the morphogenetic furrow passes through a fng mutant dorsal clone. The formation of such a transient fng+/fng− border could then lead to a weak activation of Notch that subsequently causes the formation of an ectopic ‘mini-equator. Surprisingly, we found that removal of mirr function from the fng dorsal clones can greatly increase the length of these ectopic mini-equators. This suggests that mirr could enhance equator formation by a mechanism independent of repressing fng expression. Thus, one function of mirr is to create a difference in some property other than fng expression between mirr-expressing and non-expressing cells. Such mirr-generated differences could then act to enhance the ability of a fng+/fng− boundary to induce a stronger activation of Notch.

We do not understand why a mirr dorsal clone causes an ectopic equator to form strictly at the equatorial border of the clone while a mirr/fng or fng dorsal clone creates an equator at the polar border of the clone. However, equatorial or polar border specific ectopic equators can also be seen in clones in which functions of components in the Wg or JAK/STAT signaling pathway have been altered. Such border-specific effects are hypothesized to be caused by an alteration of an unknown factor X that is emanating from the equator and graded toward the poles (Wehrli and Tomlinson, 1998; Zeidler et al., 1999a; Zheng et al., 1995). Recent data suggests that there is redundancy in the system, and there may be a factor Y (Zeidler et al., 1999b). Thus one possibility for the border specific effects observed in mirr and mirr/fng dorsal clones might due to their differential impact on X and Y activity in these clones.

**mirr acts to sharpen the D/V boundary**

The sharpness displayed by the path of the wild-type equator is a poorly understood aspect of equator formation. Several previous observations have suggested that mirr is involved in controlling the sharpness of the equator. For example, the eyes of flies homozygous for a hypomorphic mirr allele, mirrP1, have equators that are less sharp compared to the wild-type equator (McNeill et al., 1997). The irregularities displayed in these equators include either three consecutive steps in a single direction or single steps of three ommatidial widths. In contrast, in wild-type eyes, the equator moves up and down one ommatidial width as it traverses the eye, and rarely if ever moves more than two ommatidial units at a time. Disruptions in the fidelity of D/V division also occur in mirrP1 eyes, since ommatidia of ventral polarity can occasionally be found in the dorsal half of the eye. In contrast, disruption in the fidelity of the D/V division is never observed in wild-type eyes. These observations suggested that a reduction in mirr expression causes a reduction in the sharpness of the equator.

Several of the observations reported here suggest that one role of mirr in sharpening the equator is to create a difference in cell affinities between dorsal and ventral cells. First, in our clonal analysis, we noted that cells that resided inside a mirr mutant dorsal clone tended to minimize interactions with the surrounding mirr-expressing cells, resulting in a rounded clone shape. In addition, the border where mirr-expressing and non-expressing cells juxtapose appears to be significantly smoother compared to the border where no difference in mirr exists across the border. Second, mirr mutants (mirrX52/mirrD3, mirre48/mirrD3) that occasionally survived until adulthood displayed a dramatic dorsal protrusion from the surface of their eyes. Such a protrusion is suggestive of a group of cells attempting to sort out from the epithelium due to differences in cell affinities (Lawrence et al., 1999; Steinberg, 1963;
Steinberg and Takeichi, 1994). Third, when mirr was overexpressed in dorsal regions of the eye through overexpression of wg, a visible indentation of the epithelium could be observed at the novel boundary formed between mirr-expressing and non-expressing cells. This suggests that increasing the differences in mirr expression between dorsal and ventral cells caused them to further minimize contacts with each other, forming a physical groove between them. Interestingly such a groove is transiently seen in wild-type discs at the mid-point of the eye (where mirr-expressing and non-expressing cells meet) before ommatidial differentiation occurs (Ready et al., 1976). Although a strict lineage restriction does not exist in the eye, it has long been known that clones only cross the mid-point of the eye at a low frequency (Becker, 1966, Campos-Ortega, 1980; Dominiguez and de Celis, 1998). The equatorial groove has been speculated to play a role in this partial restriction (Ready et al., 1976). Together these data suggest that a physical boundary might exist between dorsal (mirr+) and ventral (mirr−) cells, and that this boundary is generated at least in part by mirr modulating cell affinities.

It is important to note that although statistical analysis showed that the distribution of clone shapes of mirr dorsal clones was significantly different from that of wild-type dorsal clones, individual mirr clones did not always have a regular oval shape or a sharp clonal boundary. This is in contrast to the consistently sharp wild-type equator. One possible explanation for this difference may be that since the allele used in this study was not a null allele, the residual amount of mirr function in a mirr dorsal clone reduced the difference in affinities between cells located inside and outside of the clone. Alternatively, ara and caup may act in conjunction with mirr to reduce cell-cell mixing between dorsal and ventral cells at the wild-type equator. A recent analysis of the role of IRO-C in the developing wing disc suggests that this regulation of cell affinities may not be an eye-specific event. The border of clones in which ara, caup and mirr were simultaneously removed in the notum region of the developing wing disc also appears to be significantly smoother than that of wild-type clones (Diez del Corral et al, 1999).

One important aspect of our findings is that the segregation of dorsal and ventral cells appeared to be a process that is independent of the difference in their expression of fng. The shapes of mirr,fng dorsal clones remained significantly rounder compared to those of wild-type dorsal clones whereas shapes of fng ventral clones do not differ significantly from those of wild-type ventral clones. (A) The distribution of clone-shape index (A/L2) of roundness calculated for 39 wild-type dorsal clones (blue columns) is significantly smaller than that of 44 mirr dorsal clones (red columns). The higher the value in the index, the rounder the shape. (B) The distribution of clone-shape index (A/L2) of 29 wild-type ventral clones (blue columns) is not significantly different from that of 37 fng ventral clones (red columns).
than those of wild-type clones, thus the ectopic expression of fng within mirr mutant cells was not likely to be the cause for the reduction of cell-cell mixing between mirr-expressing and non-expressing cells. In addition, the shapes of fng mutant ventral clones were irregular and were not significantly different from those of wild-type ventral clones, thus the difference in fng expression between dorsal and ventral cells is unlikely to be the cause for the sharpness of the wild-type equator. Such a finding is in contrast to fng’s role in D/V border formation in the developing wing disc. In wing discs, removal of fng function in a clone of cells in the dorsal half of the wing disc, where fng is normally expressed, results in a very round clone with a smooth clonal border (Michelli and Blair, 1999; Rauskolb et al., 1999). In addition, ectopically expressing fng in a clone of cells in the ventral part of the discs where fng is typically absent also results in round clones with a very smooth border. It has been suggested that fng might have a role in controlling cell adhesion in the developing wing disc. Although we cannot rule out the possibility that fng is also important in regulating cell adhesion in the eye disc, our data strongly suggests that additional components regulated by mirr must be involved. One possible model is that mirr might be regulating some adhesion molecules that are differentially expressed between dorsal and ventral cells.

We conclude that mirr regulates equator formation in the eye by two independent yet complementary pathways (see Fig. 8). mirr acts to sort the dorsal cells from ventral cells by reducing cell-cell mixing at the boundary where the dorsal and ventral cells juxtapose. In addition, it restricts the activation of Notch signaling to the point where the dorsal and ventral cells meet by repressing fng in the dorsal cells. These two functions of mirr lead to a co-ordination of morphology and signaling in the process of equator formation.

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