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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 *fng* 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

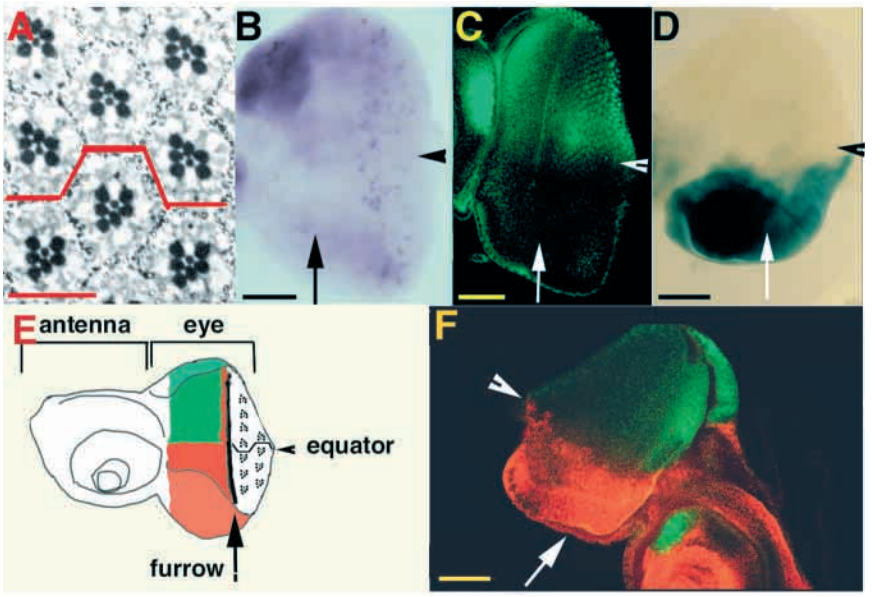

Fig. 1. The *Drosophila* eye is divided into dorsal and ventral mirror image fields of ommatidia. In all the following panels except for F, anterior is to the left and dorsal up. Arrows indicate the approximate position of the furrow. Arrowheads indicate approximate position of the mid-point of the eye. Scale bar: (A), 25 μm; (B,D,F), 45 μm. (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-galatosidase (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 border 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

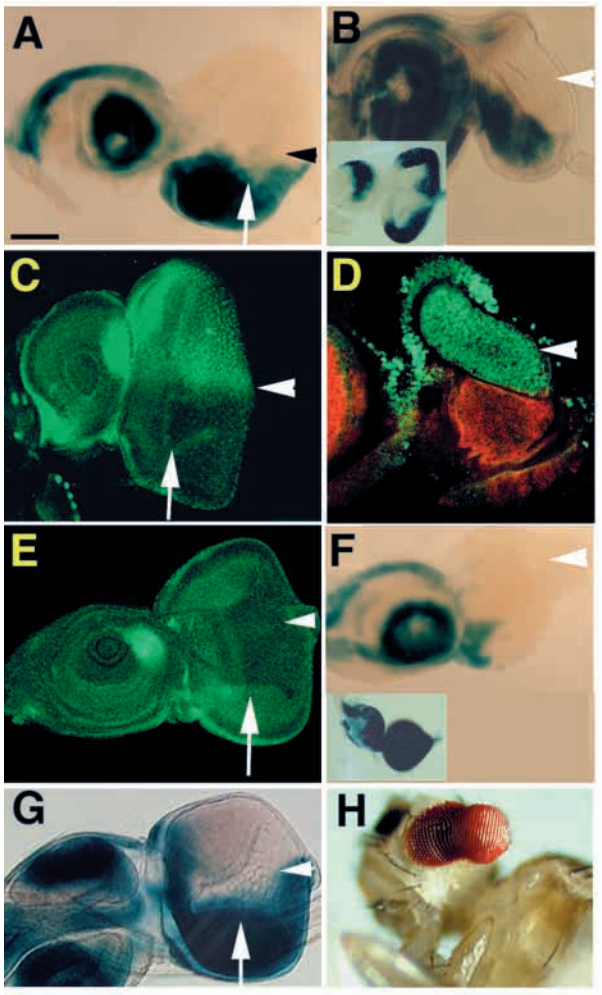

Fig. 2. Mrr represses *fng-lacZ* expression in the eye. For all the panels in this figure, anterior is to the left and dorsal up. Arrows indicate the approximate position of the furrow. Arrowheads indicate the approximate position of the mid-point of the eye. Scale bar: 45 μm. (A) Staining of β-gal activities in an eye disc derived from a *fng-lacZ*/+ larvae shows that the *fng-lacZ* expression (blue) is restricted to the ventral portion of the eye disc. Note that expression of *fng-lacZ* is also present in the antennal portion of the disc. (B) Staining for β-gal activity in an eye disc derived from a *dpp*-Gal4 × UAS-*wg* larva shows that the expression of *fng-lacZ* is further restricted ventrally**.** Inset shows expression pattern of *dpp*-Gal4 driver line crossed to UAS-*lacZ*. (C) Staining of Mrr protein in a wild-type eye disc shows that expression of Mrr is restricted to the dorsal part of the eye as well as a group of cells in the antennal portion of the disc. (D) Co-staining of Mrr protein and β-gal protein in an eye disc derived form a *dpp*-Gal4 × UAS-*wg* larva shows that the domain of *fng-lacZ* (red) expression is diminished in the ventral portion of the disc while expression of Mrr (green) is both intensified and expanded ventrally. Note that expression of Mrr and *fng-lacZ* remain non-overlapping and a physical groove is frequently seen at the novel boundary between the *mirr*-expressing and non-expressing cells (also see Heberlein et al., 1997). (E) Staining of Mrr protein in an eye disc derived from a $mirr^{X52}/mirr^{D3}$ larva shows that expression of Mrr (green) is greatly reduced in the eye portion of the disc but is not affected in the antennal portion of the disc. (F) Staining of β-gal activities in an eye disc derived from an *eyeles*s-Gal4 X UAS-*mirr* larva shows that expression of *fng-lacZ* (blue) is greatly diminished when *mirr* is ectopically expressed in the entire eye disc (also see Choi and Cho,1998). Note that although the size of the eye portion of the disc is reduced to about 2/3 of the wild-type size, the reduction in size cannot fully account for the reduction in the expression domain of the *fng-lacZ*. Inset shows staining pattern of *eyeless*-Gal4 in a cross to UAS-*lacZ*. (G) Staining for β-gal activity of an eye disc derived from a $mirr^{X52}/mirr^{D3}$ larva shows that expression of *fng-lacZ* (blue) is ectopically present in the dorsal anterior region of the eye. Note that duplicated antenna are frequently observed in these discs. (H) A $mirr^{X52}/mirr^{D3}$ adult that displayed a dramatic protrusion in the dorsal posterior region of eye. Note that the position of the furrow is not specified in B, D and F, since ommatidial differentiation is impaired in these discs.

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*; $mirr^{e48}$/TM3 and UAS-$mirr^{12}$ were generated in this lab. *w*; FRT80B, $mirr^{e48}$/TM3 was kindly provided by Andrew Tomlinson. $mirr^{D3}$/TM3, *dpp*-Gal4 and UAS-*wg* were obtained from Bloomington stock center. *w*; FRT80B, fng^{13}/TM3 and *fng-lacZ* (*35UZ-1*) were generously provided by Ken Irvine. *w*; FRT80B, $mirr^{e48}$,fng^{13}/TM3 double mutant was generated by recombination. *eyeless*-GAL4 was kindly provided by Uwe Walldorf. *w*; FRT 80B and *y,w*, hsFLP122; FRT80B, [w^{+}]70C were kindly provided by Naoto Ito. *w*; $mirr^{X52}$/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 determined by counting 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-Mrr serum

A 948 bp PCR fragment of *mirr* cDNA was cloned into the pGEX-KG vector (Pharmacia) to produce GST-Mrr fusion protein that contained the carboxy-terminal region of Mrr. 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-Mrr antibodies and 1/100 diluted rabbit anti β-galactosidase 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-galactosidase and anti-Mrr 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-galactosidase 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 Cho 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 *mirr* 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

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

Genotype	*mirr,fng*	*mirr*	*fng*
No. of clones sectioned	41	48	31
No. of clones containing ommatidia with polarity changes at the 'equatorial' border	5 (1 to 2 ommatidia with polarity changes per clone)	25 (5 to 9 ommatidia with polarity changes per clone)	2 (1 ommatidium with polarity change per clone)

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 examine, 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

Genotype	*fng*	*mirr*	*mirr,fng*
No. of clones sectioned	31	48	41
No. of clones that displayed polarity changes at the 'polar' border	12 (1 to 3 ommatidia with polarity changes per clone)	0 (4 to 9 ommatidia with polarity changes per clone)	11

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.

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^{+}/fng^{-} boundaries created at the polar borders of *fng* dorsal clones as the furrow passed through the clones.

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.

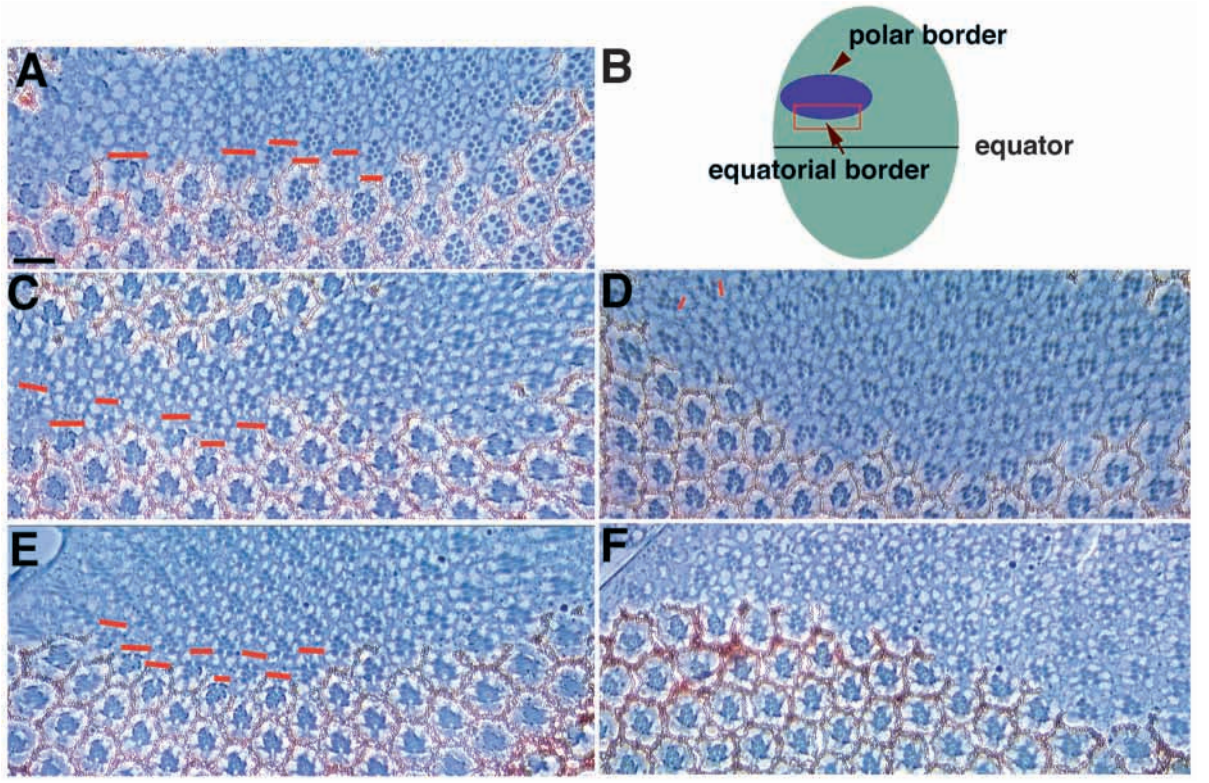

Fig. 3. Removal of *mirr* from a clone of cells in the dorsal region of the eye causes an ectopic equator to form at the equatorial border of the clone. The formation of such an ectopic equator can be suppressed when *fng* is prevented from being ectopically activated within the *mirr* dorsal clone. 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 *mirr* 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. *mirr* 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 *mirr,fng* 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 *mirr,fng* 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 *mirr* dorsal clones. (F) Section of a *mirr,fng* double mutant dorsal clone in which no ectopic equator is present at the clonal boundary.

mirr controls cell mixing

We noticed another previously unrecognized phenotype of *mirr* dorsal clones after examining a large number of clones. We found that shapes of *mirr* 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 *mirr* 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 *mirr* 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 (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Lawrence et al., 1999). A round clone shape indicates that cells located inside of the clone tend to minimize contacts with cells located outside of the clone. Therefore, the observation that a significantly higher number of *mirr* dorsal clones had a round shape compared to wild-type clones suggests that *mirr*-expressing and non-expressing cells tend not to mix.

To examine more carefully whether *mirr* was affecting cell-cell mixing at the $mirr^{+}/mirr^{-}$ boundary, we quantified and compared the relative roundness of wild-type dorsal and *mirr* 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, *mirr* dorsal n=44, *mirr,fng* dorsal n=43, wild-type ventral n=29, *fng* ventral n=37, *mirr* ventral n=32 and *mirr,fng* ventral = 21 clones). We then compared the distributions of A/L^2 ratios calculated for *mirr* dorsal and wild-type dorsal clones, and found that the shapes of *mirr* dorsal clones were significantly rounder than those of wild-type dorsal clones ($P \ll 0.001$) (Fig.

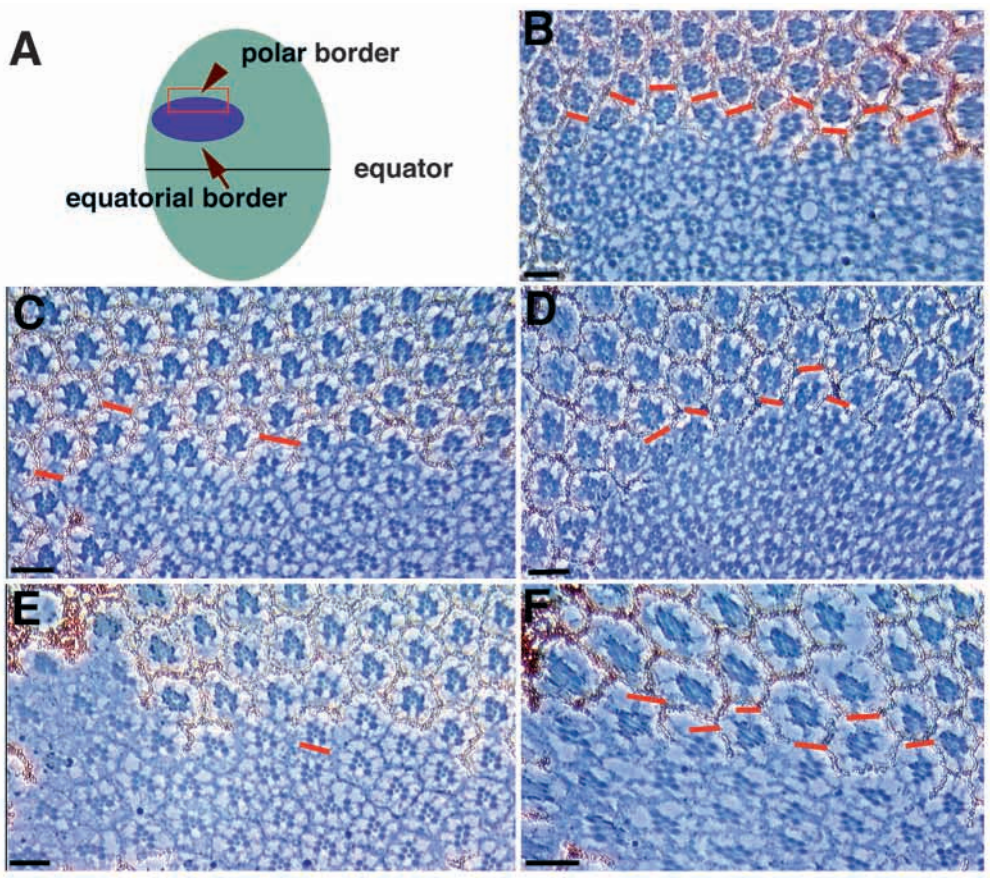

Fig. 4. Removal of *fng* from a clone of cells in the dorsal region of the eye causes ectopic mini-equator formation at the polar border of the clone. Removal of *mirr* function from the *fng* clone dramatically increases the length of such an ectopic mini-equator. For all the panels in this figure, anterior is to the left and dorsal up. Scale bar: 25 μm for panels except A. (A) A diagram showing region of clones examined in following sections (see Fig. 3B for details). (C,E) Sections through the polar borders of *fng* dorsal clones shows that 1 to 3 ommatidia of ventral polarity (indicated by the red bars) are found occasionally at the polar borders of *fng* dorsal clones. (B,D,F) Sections through the polar borders of *mirr,fng* double mutant clones show that a dramatic increase in the number of ommatidia with polarity changes (indicated by the red bars) is found when *mirr* function is removed from the *fng* clones.

7A). 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 *mirr* clonal borders was caused by the ectopic *fng* expression within the *mirr* dorsal clones, we compared the distribution of A/L^2 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/L^2 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

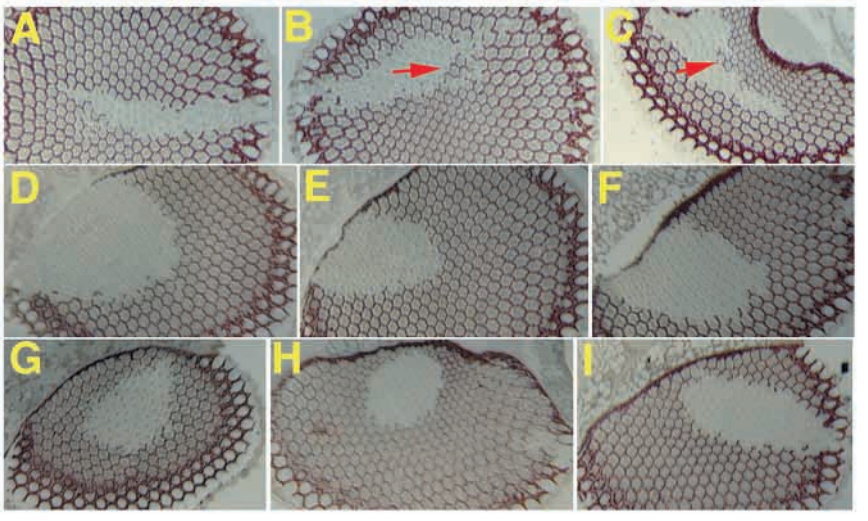

Fig. 5. Shapes of *mirr* and *mirr,fng* dorsal clones are rounder than wild-type dorsal clones. (A-C) Low-magnification views of sections of wild-type dorsal clones. Note that the shapes of wild-type dorsal clones are generally not round and tend to be elongated in the anterior/posterior (A/P) axis. A clone can be distinguished from the rest of the eye by its lack of pigment granules (reddish brown). Note that boundaries of these clones are not very smooth and islands of pigment cells (arrow) can often be found within the clones. (D-F) Low-magnification views of sections of *mirr* dorsal clones. Note that the shapes of these clones are generally rounder when compared to wild-type clones in A-C. Also the clonal borders of these clones appear to be smoother than wild-type clones. (G-I) Low-magnification views of sections of *mirr,fng* double mutant dorsal clones. Note that shapes of these clones are also significantly rounder and the clonal borders appear smoother compared to wild-type dorsal clones.

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, $mirr^{P1}$, 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 $mirr^{P1}$ eyes, since ommatidia of ventral polarity can occasionally be found in the dorsal half of the eye. In contrast, disruption in the fidelity in 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 ($mirr^{X52}/mirr^{D3}$, $mirr^{e48}/mirr^{D3}$) 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;

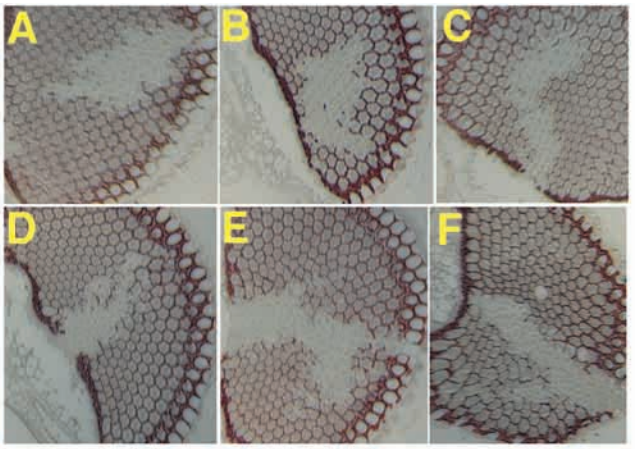

Fig. 6. Shapes of *fng* ventral clones tend not to be round and do not differ significantly from those of wild-type ventral clones. (A-C) Low magnification views of sections of wild-type ventral clones. Note that the shapes of these clones are less round compared to the *mirr* dorsal clones. Also note that the clonal borders of these clones are not very smooth. (D-F) Low magnification views of sections of *fng* ventral clones. Note that the shapes and borders of these clones do not differ significantly from those of wild-type ventral clones.

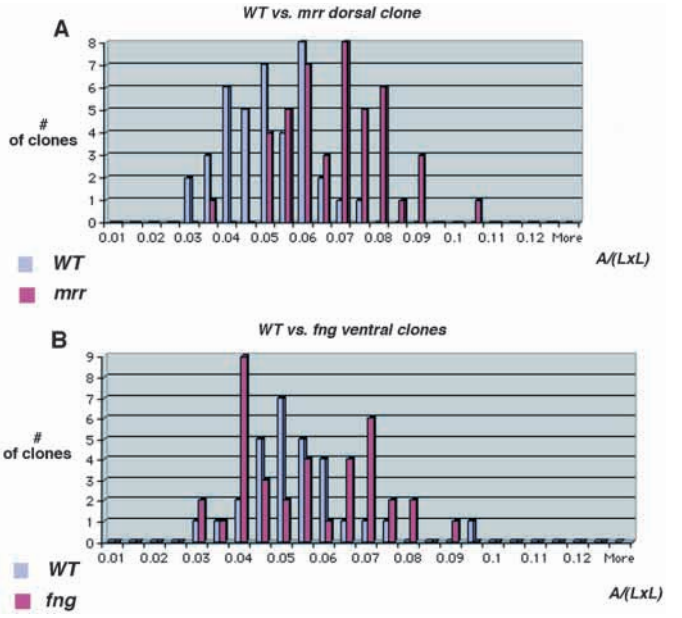

Fig. 7. Shapes of *mirr* dorsal clones are significantly rounder compared to those of wild-type dorsal clones whereas shapes of *fng* ventral clones do not differ significantly from those of wild-type ventral clones. (A) The distribution of clone-shape index (A/L^2) of roundness calculated for 39 wild-type 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/L^2) of 29 wild-type ventral clones (blue columns) is not significantly different from that of 37 *fng* ventral clones (red columns).

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

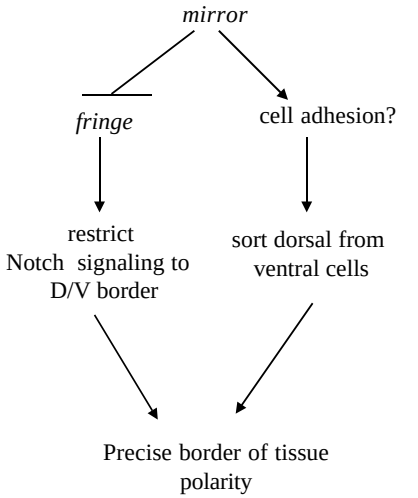

Fig. 8. *mirr* acts to control equator formation through two independent, complementary pathways.

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 (Micchelli and Blair, 1999; Rauskolb et al., 1999). In addition, ectopically expressing *fng* in a clone of cells in the ventral part of the dics 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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