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Initial state of the *Drosophila* eye before dorsoventral specification is equivalent to ventral

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Summary

Dorsoventral (DV) patterning is crucial for eye development in invertebrates and higher animals. DV lineage restriction is the primary event in undifferentiated early eye primordia of *Drosophila*. In *Drosophila* eye disc, a dorsal-specific GATA family transcription factor *pannier* (*pnr*) controls *Iroquois-Complex* (*Iro-C*) genes to establish the dorsal eye fate whereas *Lobe* (*L*), which is involved in controlling a Notch ligand *Serrate* (*Ser*), is specifically required for ventral growth. However, fate of eye disc cells before the onset of dorsal expression of *pnr* and *Iro-C* is not known. We show that *L/Ser* are expressed in entire early eye disc before the expression of *pnr* and *Iro-C* is initiated in late first instar dorsal eye margin cells. Our evidence suggests that during embryogenesis *pnr* activity is not essential for eye development. We present evidence that loss of *L* or *Ser* function prior to initiation of *pnr* expression results in elimination of the entire eye, whereas after the onset of *pnr* expression it results only in preferential loss of ventral half of eye. We demonstrate that dorsal eye disc cells also become *L* or *Ser* dependent when they are ventralized by removal of *pnr* or *Iro-C* gene function. Therefore, we propose that early state of the eye prior to DV lineage restriction is equivalent to ventral and requires *L* and *Ser* gene function.

Key words: *Drosophila*, Dorsoventral eye patterning, *Lobe*, *Serrate*, *pannier*, *Iro-C*

Introduction

Development of a field requires generation of lineage restriction boundary, which results in two differently determined cell populations called compartments (Garcia-Bellido et al., 1973). Compartments are the fundamental units of patterning generated by localized expression of transcription factors, which are called selectors as they can confer the compartment-specific properties to the group of cells in which they are expressed (Curtiss et al., 2002; Mann and Carroll, 2002). Activity of these selector genes generate lineage restriction boundary and control signaling at the boundary (Blair, 1995; Wu and Rao, 1999). Signaling between the cells of two compartments contributes to growth and differentiation of an undifferentiated field to its adult counterpart (Blair, 2001).

The development of an imaginal disc into an adult structure requires generation of anteroposterior (AP) and dorsoventral (DV) lineage restrictions. In antenna, wing and leg imaginal discs, early-arising AP boundary is the first lineage restriction event. This is followed by DV boundary generation midway through the growth phase of the disc, which further subdivides these discs into dorsal and ventral compartments (Blair, 1995; Blair, 2001; Diaz-Benjumea and Cohen, 1993; Garcia-Bellido and Santamaria, 1972; Milan and Cohen, 2003; Morata and Lawrence, 1975; Tabata et al., 1995). By contrast, the eye disc does not show a strict anterior versus posterior lineage restriction (Morata and Lawrence, 1978). AP pattern in the eye disc is established dynamically as the morphogenetic furrow (MF), a wave of differentiation, progresses anteriorly, resulting in the distinction of the AP domains. In fact, anterior and posterior domains correspond to undifferentiated (anterior to MF) and differentiated regions (posterior to MF) of eye (Ready et al., 1976; Wolff and Ready, 1993), rather than the compartments of different cell lineages separated by strict lineage restriction boundary. Therefore, the eye disc remains at anterior undifferentiated ground state until the early third larval instar, when MF is initiated to generate the AP pattern (Heberlein and Moses, 1995; Lee and Treisman, 2001). However, unlike the AP axis, DV lineage restriction and domain-specific gene expression of DV patterning genes takes place very early during the eye disc development (Baker, 1978; Cho and Choi, 1998; Dominguez and de Celis, 1998). Consequently, DV lineage restriction, which is secondary event in other imaginal discs becomes the first lineage restriction event in eye disc and is crucial for its growth and differentiation.

Eye disc develops into the adult compound eye, which is a highly precise hexagonal array of 800 ommatidia (Ready et al., 1976; Wolff and Ready, 1993). Two chiral forms of these ommatidial clusters are arranged in mirror image symmetry along the DV midline called equator to form dorsal and ventral eye. Although the mirror image symmetry is generated during third instar of development but the subdivision of eye into dorsal and ventral lineage territories takes place even earlier (Baker, 1978; Cavodeassi et al., 1999; Cho and Choi, 1998; Dominguez and de Celis, 1998; Maurel-Zaffran and Treisman,
gene function from the dorsal eye cells can revert the dorsal eye fate to the ventral, which behaves in a similar fashion to the early eye disc in terms of its sensitivity to L/Ser activity. We also show that early pnr expression during embryogenesis has little or no functional contribution to DV patterning of eye. Therefore, we propose that early eye disc has ventral-equivalent state, even before the onset of the dorsal selector genes expression, which results in DV lineage restriction event.

Materials and methods
Stocks
Stock used were y w; FRT82 pnr<sup>16</sup>/TM6B, a null allele of pnr (Heitzler et al., 1996); y w hsFLP<sup>122</sup>; in<sup>DM3</sup> FRT80 /TM6B (Diez del Corral et al., 1999); y w; mirr<sup>B1-12</sup>/TM6B (Choi et al., 1996); y w eyFLP (Newhouse et al., 2000); y w hsFLP<sup>122</sup> (Strobl and Basler, 1993); UAS-Ser<sup>DN</sup> (Hukriede et al., 1997); y w: FRT42D, L<sup>rev6-3</sup>/CyO and UAS-L (Chern and Choi, 2002); ey-GALA (Hazellett et al., 1998); pnr-GALA (Calleja et al., 1996); UAS-Ush (Fossett et al., 2001) and P/UAS-GFP<sup>S65T/T10</sup> (B. J. Dickson, unpublished). These stocks are described in FlyBase (http://flybase.bio.indiana.edu). We have used GAL4/UAS system for targeted misexpression (Brand and Perrimon, 1993). GAL4/UAS crosses were carried out at 18°C, 25°C and 29°C, to sample the effect of different induction level.

Generation of loss-of-function clones
Loss-of-function clones were generated using the FLP/FRT system of mitotic recombination (Xu and Rubin, 1993). To generate loss-of-function clones of L in eye, eyFLP; FRT42 ubi-<i>U</i>F were crossed to FRT42D L<sup>rev6-3</sup> males. For the generation of heat-shock FLP-mediated clones of L, hsFLP<sup>122</sup>; FRT42 ubi-<i>U</i>F females were crossed to FRT42D, L<sup>rev6-3</sup> males. Eggs were collected for 2 hours and a single heat shock was administered for 1 hour at 37°C. All larvae were transferred to 25°C for recovery and further development.

To generate the loss-of-function clones of pnr<sup>16</sup>, y w;+/+ FRT82 pnr<sup>16</sup>/TM6B, males were crossed to eyFLP; FRT82 ubi-<i>U</i>F females. Iro-C loss-of-function clones were generated by crossing hsFLP<sup>122</sup>; FRT80, in<sup>DM3</sup>/TM6Tb males with the eyFLP, FRT80, ubi-<i>U</i>F females.

As pnr and Iro-C genes play important roles in different developing fields during development, we wanted to generate the flies that have only the eyes homozgous for the pnr or Iro-C mutation and in the same mutant eye disc overexpress another gene of interest. These flies were generated by using the EGUF (eyeless-GALA UAS-FLP) system (Stowers and Schwarz, 1999). EGUF system has been generated by combining the GAL4/UAS system (Brand and Perrimon, 1993) and the FLP recombinase system (Xu and Rubin, 1993) via the UAS-FLP transgene (Duffy et al., 1998). The ey-GALA drives UAS-FLP recombinase only in the eye and wild-type cells (heterozygous and +/+ twin spot cells) are selectively eliminated by GMR<sup>hid</sup> later during differentiation (Stowers and Schwarz, 1999). As the clones are generated earlier by ey-GALA and the wild-type cells are killed later by GMR<sup>hid</sup>, the discs get time to grow.

Temperature shift regimen
Eggs were collected for the genotype, ey-GALA; UAS-Ser<sup>DN</sup> (ey<sup>+</sup>/Ser<sup>DN</sup>) from a synchronous culture for 2 hours. Each egg collection was divided into several batches. These independent batches were reared at 18°C except for a single shift to 29°C in a 12 hour time window. This single 12 hour heat shock of each sample was performed during different periods of development spanning from t=0 hour AEL (after egg laying) to the late third larval instar. These cultures after the 12 hour exposure to 29°C were returned to 18°C for the later part of development until the discs were dissected and stained or till the adult flies emerged (superscript DN indicates dominant negative).
Another temperature shift regimen was carried out for ey-GAL4; UAS-Ush (ey-Ush) in a similar way except the time windows of exposure to restrictive temperature were different (see Fig. 2A for details).

Immunohistochemistry

Eye-antenna discs were stained following the standard protocol (Singh et al., 2002). Antibodies used were mouse anti-L (1:100) (Chern and Choi, 2002); rabbit anti-β-galactosidase (1:200) (Cappel); chicken anti-GFP (1:200) (Upstate biotechnology); rabbit anti-Ey (1:500) (a gift from Uwe Wallidorf); rat anti-Elav (1:100); mouse 22C10 (1:20); mouse anti-Wg (1:20) (Developmental Studies Hybridoma Bank). Secondary antibodies (Jackson Laboratories) were goat anti-rat IgG conjugated with Cy5 (1:200); donkey anti-rabbit IgG conjugated to FITC (1:200); or donkey anti-chicken IgG conjugated to FITC. pnr expression was detected using pnrGAL4>UAS-GFP, which has been commonly used to detect pnr expression, as seen in wing and eye discs (Calleja et al., 2000; Pichaud and Casares, 2000). Immunofluorescent images were analyzed by using Zeiss LSM laser confocal microscope.

Results

Lobe and Ser are expressed earlier than dorsal selectors in eye

To check how and when the DV fates are established in eye, we examined the onset of expression of dorsal eye selector genes and L/Ser during larval development. In the first instar eye disc, L is expressed ubiquitously, whereas pnr expression is not seen (Fig. 1A; arrows, A'). Expression of pnr has been seen in the embryonic eye primordia (Maurel-Zaffran and Treisman, 2000). But in the early first instar eye disc, pnr expression was shut off or downregulated to undetectable level. In late first- or early second-instar disc, pnr expression is initiated in a small group of cells in the dorsal eye close to its anterior tip (Fig. 1B, arrow; B'), whereas L is expressed in entire disc. This suggests that pnr has a very dynamic expression during eye development.

In late second instar, pnr expression extends to the dorsal margin of eye (Fig. 1C, C'). In third instar disc, pnr is expressed in a wedge of cells on the dorsal margin of the eye, whereas L expression does not change (data not shown). Furthermore, expression of pnr is restricted only to the group of cells in the lateral margin of the dorsal eye as previously reported (Maurel-Zaffran and Treisman, 2000). Interestingly, we found that pnr is expressed in the dorsal peripodial cells of the eye disc throughout most larval stages, but could not definitively determine whether pnr+ cells are in the peripodial membrane in the first instar disc.

Expression of mirr, an Iro-C member, is not initiated in early first instar eye disc (Fig. 1D, arrowhead) whereas Ser is expressed in entire disc (data not shown). In early second instar, mirr is restricted to the dorsal eye (Fig. 1E, E', F'), whereas Ser is also preferentially expressed in ventral with a weaker expression in dorsal eye disc (data not shown) (Cho and Choi, 1998). mirr expression stays in dorsal region of third instar eye disc (data not shown) (McNeill et al., 1997). mirr is expressed in much broader dorsal domain in comparison to pnr as it is controlled by secreted Wg, which acts downstream to pnr (Maurel-Zaffran and Treisman, 2000). The expression of ara using antibody against Ara protein was similar to mirr (data not shown).

Pnr activity is not essential for DV patterning during embryogenesis

We could not detect pnr in the early first instar eye disc (Fig. 1A), despite its expression in embryo (Maurel-Zaffran and Treisman, 2000). The significance of disappearance of pnr expression between embryogenesis to late first instar larva is not yet clear. We performed a functional test to determine whether pnr is active in the eye primordium in the embryo. We misexpressed U-shaped (Ush) using ey-GAL4 during embryonic development to block pnr transcriptional activity. Ush, which is normally not expressed in eye (Maurel-Zaffran and Treisman, 2000; Fossett et al., 2001), encodes a zinc-finger protein that dimerizes with Pnr and acts as a negative regulator of pnr transcriptional activity (Haenlin et al., 1997). The aim was to determine if pnr has any role in DV patterning of eye during embryogenesis. We used temperature-shift approach in three different conditions as shown in Fig. 2A. First, we maintained the cultures at 29°C all along the development, which served as control and resulted in no eye (Fig. 2B, C) to a small eye phenotype (Fig. 2D) in almost 80% (51/64) of the adult flies scored, also seen by Fossett et al. (Fossett et al., 2001).
29°C  18°C
Embryo  Larva  Pupa

29°C  18°C

Fig. 2. Pnr is not essential for DV patterning of eye during embryogenesis. Ush was misexpressed in eye by ey-GAL4: UAS-Ush (ey>Ush) and cultures were shifted to 29°C during different stages of development. (A) Three different restrictive temperature conditions used. (B,D-F) Eye discs were stained for Wg or L (blue), Elav (red) and Ey (green). (B-D) Cultures maintained at 29°C throughout development served as controls and resulted in elimination of entire eye field (B) in eye disc and (C) in adult eye. (D) Some weaker phenotypes of very small eye marked by Elav-positive cells were also observed. (E) Maintenance at 18°C during embryonic development and then shift to 29°C for subsequent development also resulted in complete elimination of eye field. (F,G) When cultures were shifted to 29°C during embryonic development to block pnr activity and later allowed to develop at 18°C, they did not show any eye suppression phenotype (F) in the eye disc and (G) in adult eye.

Lobe mutations suppress ventral eye development

As L and Ser are expressed in eye disc earlier than dorsal eye genes, we checked for the role of L and Ser during various stages of eye development. L mutant shows a selective loss of ventral eye (Fig. 3A) (Chern and Choi, 2002). We generated loss-of-function clones of L in the eye during different time windows using L\textsuperscript{rev6-3} (hereafter L\textsuperscript{−}), a null allele of L (Chern and Choi, 2002). These phenotypes can be broadly divided into two groups. First, ey-FLP was used (Newsome et al., 2000) to generate loss-of-function clones of L exclusively in the eye (Xu and Rubin, 1993). These clones showed asymmetric response in the dorsal and the ventral eye. Loss-of-function clones of L in the dorsal eye did not affect the ommatidial development (Fig. 3B, arrow, B′) but the ventral eye disc clones inhibited the ommatidial development (Fig. 3B, arrowhead, B′) and corresponding phenotypes were also observed in adult eye (Fig. 3C). In the adult eye, presence of ommatidia in the wild-type twin spot cells (L\textsuperscript{+}/L\textsuperscript{+}) for a ventral (L\textsuperscript{−}/L\textsuperscript{−}) clone suggested that L is required for the ventral eye development (Fig. 3C, arrow). We checked the fate of the cells in the loss-of-function clones of L by staining the eye discs with antibody against Pax6 homolog protein Eyeless (Ey). Ey marks the undifferentiated cells anterior to the morphogenetic furrow in the third instar disc (Halder et al., 1998). We found that in the ventral clones where the eye fate is blocked, ectopic Ey induction was seen behind the MF (Fig. 3D,D′ arrowhead). This suggested that in the absence of L gene function the ventral eye cells remain undifferentiated. As expected, dorsal eye clones where retinal differentiation was not blocked, did not show any ectopic Ey induction (data not shown).

Second, loss-of-function clones of L generated in the eye of early first instar larva using the heat shock FLP source (Struhl and Basler, 1993) could completely eliminate the eye fate, whereas the antennal development in the same disc was not compromised (Fig. 3E,E′). We obtained more consistent results with heat-shock FLP because of controlled induction of FLP during short time windows. Clones with eyFLP were probably induced stochastically during any time from embryogenesis onwards, which might cause more variable phenotypes. Earlier it has been shown that loss-of-function of L can selectively eliminate the ventral eye fate (Chern and Choi, 2002) but the removal of entire eye within the early loss-of-function clones was a surprise. It suggested that very early during development entire eye may be ventral in fate. Alternatively, it can also be interpreted that loss-of-function clones of L results in partial loss of Ser, which is also under the control of fng (Papayannopoulos et al., 1998). In this case, loss of Ser may prevent the correct establishment of the DV organizing center and hence affecting the growth of the entire disc. But this possibility can be ruled out as it has been shown that L mutant clones cause little effect on Ser expression near the DV border, although it results in strong reduction of Ser in other ventral region (Chern and Choi, 2002). Furthermore, we did not see the similar phenotypes of loss of entire eye or selective reduction of ventral eye when we generated loss-of-function clones of fng alone in different time windows. fng is preferentially expressed in ventral domain of early eye disc and its loss-of-function clones showed ommatidial polarity defects (Cho and Choi, 1998; Papayannopoulos et al., 1998) rather than complete elimination of ventral eye. On the contrary, loss-of-function clones of fng generated ectopic equator (Cho and
Choi, 1998) and in rare cases (14/159) 5-10% of adult flies scored showed enlargement rather than loss of the ventral eye pattern (A.S. and K.-W.C., unpublished). These results suggest that Ser function in the early eye disc is independent of fng regulation. Because all the phenotypes of fng loss-of-function clones are manifested in terms of effect on polarity suggest that fng functions after the early DV lineage restriction is established in the eye.

Ser is required for early eye field development

Ser is known to be the downstream target of genes which affect ventral eye development, such as fng (Irvine, 1999; Papayannopoulos et al., 1998) and L (Chern and Choi, 2002). SerDN, a dominant-negative allele encoding a truncated form of Ser, is capable of antagonizing wild-type Ser functions (Hukriede et al., 1997). It consists of extracellular domain but lacks the transmembrane domain of Ser. SerDN was used to generate loss-of-function phenotype of Ser (Chern and Choi, 2002; Hukriede et al., 1997; Kumar and Moses, 2001). We used the temperature-dependent expression of the GAL4 enhancer trap (Brand and Perrimon, 1993), to determine the phenocritical period of SerDN overexpression (ey > SerDN) in the eye (Kumar and Moses, 2001). The rationale was to check the period when the Ser function is crucial for DV eye field development. Basically, the phenotypes scored in the eye disc can be grossly classified into three major categories as summarized in Fig. 4A. First category showed complete elimination of eye field to a very small eye. Second category included the eye discs with preferential elimination of the ventral eye pattern. The third category comprised the discs where there were two antennal fields also seen by Kumar and Moses (Kumar and Moses, 2001). These discs were also accompanied by suppression of eye field. The split of the two antennal fields along with suppression of eye suggests that Ser also plays a role in patterning of antennal field.

In the early time window of 12-72 hours of development, misexpression of SerDN caused complete elimination of the eye field (Fig. 4B,C). These discs had a few Ey-expressing undifferentiated cells of anterior eye, whereas the antennal development was not at all affected in these discs (Fig. 4B). In the same 12- to 72-hour time window, some extremely small eye discs with a few photoreceptors were also seen (Fig. 4D). Misexpression of SerDN at 72-96 hours of development caused significant reduction in frequency of no-eye phenotype from near 100% to 60% (Fig. 4A) along with an increase in frequency of selective eye suppression in the ventral eye from near 0 to ~40%; (Fig. 4A,E). During 96-168 hours of development, concomitant with the presence of pnr-expressing dorsal cells, there is a sharp increase in frequency of eyes showing preferential loss of ventral eye pattern (Fig. 4A,F) when compared with no-eye phenotypes. We found that removal of L/Ser gene function during early eye development can completely abolish the entire eye field, whereas later during development these eye inhibition phenotypes become restricted to only the ventral eye. These time-dependent effects of SerDN further substantiated our view that the fate of early eye disc prior to the emergence of pnr+ cells is most probably ventral equivalent.

Loss-of-function of dorsal selectors change dorsal eye fate to early ventral-equivalent state

Lack of sensitivity of dorsal cells to L/Ser led us to check for the role of dorsal selectors in early DV patterning of eye. We generated loss-of-function clones of pnr in the eye using pnrVX6, a null allele generated by a deletion of all but nine amino acids of the coding region (Heitzler et al., 1996). As previously described (Maurel-Zaffran and Treisman, 2000), loss-of-function clones of pnr changed the dorsal eye fate to ventral, which resulted in dorsal eye enlargements or ectopic eye caused by generation of new boundary of the pnr expressing- and non-expressing cells (data not shown) (Maurel-Zaffran and Treisman, 2000). Loss-of-function clones of pnr in the ventral eye had no effect as pnr is expressed only in the dorsal eye (Maurel-Zaffran and Treisman, 2000).

We have seen that before the onset of dorsal selector gene function, the entire eye disc is sensitive to L/Ser activity. We wanted to check if the eye disc ventralized by eliminating the dorsal selector gene function again becomes sensitive to
L/Ser activity as seen in early eye disc. Interestingly, we found that if L levels are increased continuously above the wild-type levels by using ey-GAL4 (ey>L), it selectively eliminates the ventral eye pattern (Fig. 5A,B arrows; Table 1) (J. J. Chern, PhD Thesis, Baylor College of Medicine, 2003). This suggests that optimum levels of L are required for ventral eye growth and development. We used this property of L as an assay system to check if the eye discs when mutated for dorsal selector gene function can revert back to ventral, which is sensitive to levels of L gene function. We used the EGUF system (Stowers and Schwarz, 1999) to generate eye disc where all the cells other than those mutant for pnr were ablated using GMR>hid. The rationale of using this approach is that GMR>hid kills the cells later during eye differentiation, therefore these mosaic eye discs could grow. Eye disc mutant for pnr gene function showed dorsal overgrowths in disc (Fig. 5C) and in adult eyes (Fig. 5D, Table 1). By contrast, when L was overexpressed continuously in eye using ey-GAL4 driver (ey>L), pnr mutant discs resulted in very small eye (Fig. 5E,F, arrow and arrowhead; Table 1). The small eye phenotype was different from either of the two controls used; ey>L alone causes ventral-specific eye loss (Fig. 5A,B), whereas EGUF clones of pnr results in dorsally enlarged eye (Fig. 5C,D). Therefore, these results suggest that small eye phenotype was generated because of suppression of eye by overexpression of L on both dorsal (which has changed to ventral) and ventral eye margins. Furthermore, we also analysed the fate of cells left in the small eyes generated by EGUF clones and overexpression of L by sectioning the adult eyes. The polarity of most of the ommatidia left in these eyes was dorsal along with a few ventral or with a polarity defect (data not shown). We also checked the sensitivity of the pnr mutant discs to Ser.

Table 1. Summary of phenotypes shown by EGUF clones of pnr and Iro-C

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Wild type controls (no EGUF)</th>
<th>EGUF pnr&lt;sup&gt;DN&lt;/sup&gt; clones</th>
<th>EGUF Iro-C&lt;sup&gt;DN&lt;/sup&gt; clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type controls (no GAL4/UAS)</td>
<td>Wild-type eye</td>
<td>Dorsal eye enlargements</td>
<td>Dorsal eye enlargements</td>
</tr>
<tr>
<td>UAS-L; ey-GAL4 (ey&gt;L)</td>
<td>Twenty percent of flies show ventral eye-specific reduction*</td>
<td>Nearly 20% of the flies show small eye due to reduction of eye on both dorsal and ventral eye margins</td>
<td>Nearly 20% of the flies show small eye due to reduction of eye on both dorsal and ventral eye margins</td>
</tr>
<tr>
<td>ey-GAL4; UAS-Se&lt;sup&gt;DN&lt;/sup&gt; (ey&gt;Ser&lt;sup&gt;DN&lt;/sup&gt;) at 25°C</td>
<td>Fifty percent of flies show complete loss of eye to very small eye</td>
<td>Ninety-nine percent of flies show complete loss of eye</td>
<td>Ninety-nine percent of flies show complete loss of eye</td>
</tr>
</tbody>
</table>

Percentages have been calculated based on eye disc and adult eye phenotypes independently and results presented are averages of both. Minimum sample size for each experiment was 20 imaginal discs and 100 adult flies.

*There is low penetrance in ey-GAL4; UAS-L phenotype in eye.
Fig. 5. Loss-of-function of pnr and Iro-C changes dorsal eye sensitivity to ventral. (A,B) Overexpression of L by ey>L causes selective ventral eye suppression in disc (A, arrow) and adult (B, arrow). Loss-of-function clones of pnr were generated in eye using EGUF approach, which resulted in dorsal eye enlargement (C) in disc and (D) in adult. In the ventralized disc with EGUF pnr clones in dorsal when L (ey>L) was overexpressed resulted in small eye because of suppression of eye fate on both dorsal and ventral margin of (E) disc (arrows) and in (F) adult eye (arrowhead). Misexpression of SerDN (ey>SerDN) in the ventralized disc with EGUF pnr clones completely abolished the entire eye fate (G) in disc and in (H) adult eye. Loss-of-function clones of Iro-C show dorsal eye enlargement in (I) disc. (J) EGUF Iro-C clones in eye disc also result in dorsal eye enlargements. (K,L) In the ventralized eye disc mutant for Iro-C, overexpression of ey>L (K) or ey>SerDN (L) results in suppression of eye on both DV margins and complete removal of the eye fate, respectively.

activity. Misexpression of SerDN continuously during development in the same pnr mutant discs at 25°C completely abolished the eye fate in nearly 99% of discs (Fig. 5H, Table 1), and corresponding phenotypes were also seen in the unhatched pupae that were dissected out to check their phenotypes (data not shown). These results suggest that removal of pnr gene function in the eye disc changes the dorsal eye fate to ventral, which makes the entire disc sensitive to ey>L or SerDN as observed in early eye disc.

Loss-of-function clones of Iro-C mutation were generated in the eye using IroDfM3, a deficiency for all three members, i.e. ara, caup and mirr (Diez del Corral et al., 1999). These clones also showed enlargement in the dorsal eye (Fig. 5I) (Cavodeassi et al., 1999), a phenotype similar to that seen in the pnr loss-of-function clones, whereas in the ventral eye there was no effect of these clones (data not shown) (Cavodeassi et al., 1999). Eye discs mutant for Iro-C gene function generated by EGUF approach resulted in enlarged disc (Fig. 5J), as seen in loss-of-function clones in Fig. 5I. Overexpression of L (ey>L) in eye disc with Iro-C EGUF clones resulted in small eyes with suppression of the eye on both dorsal and ventral margins (Fig. 5K, Table 1), but the phenotypes were not as severe as seen in pnr. Misexpression of SerDN in Iro-C mutant eye discs completely abolished the eye fate (Fig. 5L). These results suggest that when the eye fate changes from dorsal to ventral in response to removing dorsal selector gene function, the eye fate reverts back from the dorsal to its default ventral state. Therefore, the entire eye disc responds to L/Ser activity in a similar fashion to that seen in the early eye disc before the onset of expression of dorsal eye selector genes.

Discussion

We have addressed a basic question of how patterning and growth of early eye primordium are regulated. Our results provide an important insight into the role of genes controlling ventral eye growth. Previously, L/Ser were thought to be required for ventral eye growth after the DV lineage restriction boundary was established, which corresponds to the onset of expression of dorsal eye selectors. Our results clearly suggest that L/Ser are required much earlier for the growth of the entire early eye disc, even before the DV patterning is established. In contrast to the function of dorsal selector genes in eye patterning, L and Ser have been shown to play a distinct role in controlling ventral-specific growth of eye disc.

Temporal requirement of genes controlling ventral eye development

It has been shown that loss-of-function phenotypes of L or Ser are restricted to the ventral eye (Chern and Choi, 2002). We checked the spatial as well as temporal requirement of these genes in the ventral eye pattern formation. We found that extent of loss of ventral eye pattern in loss-of-function clones of L/Ser varied along the temporal scale. During early eye disc development, prior to onset of pnr expression in dorsal eye, removal of L or Ser function resulted in complete elimination of the eye field, whereas later when dorsal eye selector genes starts expressing the eye suppression phenotype becomes restricted only to the ventral eye (Figs 2-5). Therefore, DV lineage border in the eye can also be interpreted as the border between the cells sensitive and insensitive to the L/Ser gene function.
Initial state of eye is ventral equivalent

The eye antennal disc has the most complex origin in the embryo. The eye disc is initiated from a small group of ~70 precursor cells on each side contributed by six different head segments of the embryo (Jurgens and Hartenstein, 1993). These embryonic precursors do not physically separate from the surrounding larval primordia and are therefore difficult to discern morphologically.

Once the cells for the eye-antennal disc are committed, these discs proliferate and undergo differentiation into an adult eye, which requires generation of DV lineage restriction in eye. There are possibly three different ways by which genesis of eye discs proliferate and undergo differentiation into an adult eye, which requires generation of DV lineage restriction in eye. During early first instar development, the pnr+ cells emerge and initiate the expression of downstream Iro-C genes, which results in DV specification of eye. Establishment of DV lineage in eye restricts the L/Ser requirement to the ventral cells only. pnr+ and Iro-C+ cells become independent of L/Ser requirement. Therefore, the initial state prior to DV specification is probably equivalent to ventral eye in nature.

Second, change of dorsal eye fate to ventral upon removal of dorsal selectors. It has been observed that the mutants, which affect ventral eye development, show two major phenotypes in eye: either there is no or very small eye, or there is a preferential loss of ventral eye based on the time they affect their function but none of the mutants for dorsal eye selectors show phenotypes of loss of only dorsal eye. Conversely, loss-of-function clones of pnr or Iro-C causes dorsal eye enlargement or ectopic eye formation rather than loss of only dorsal eye clonal tissue (Fig. 4) (Maurel-Zaffran and Treisman, 2000; Cavodeassi et al., 1999). This phenotype is probably due to generation of ectopic boundary of pnr-expressing and non-expressing cells (rather than absence of pnr), which could be important for promoting eye growth (Maurel-Zaffran and Treisman, 2000). Overexpression of Ush or Fog proteins in eye discs results in loss of pnr activity, causing complete elimination of eye development (Fossett et al., 2001). By removing pnr activity at different time points we found that pnr activity in embryo and early first instar is not essential for eye disc development (Fig. 2). Later, pnr becomes essential for DV patterning consistent with its strong expression in dorsal margin of eye disc after early first instar stage.

Dorsal selectors and Lobe/Ser affect the eye development at two different tiers

In contrast to enlargements or ectopic eyes induced by loss-of-function clones of dorsal selectors (Cavodeassi et al., 1999; Maurel-Zaffran and Treisman, 2000), the loss-of-function clones of L or Ser always resulted in the elimination of the eye fate. L/Ser are primarily required for the maintenance and development of ventral or ventral-equivalent state of the eye, whereas dorsal genes establish the DV border. This suggests that dorsal genes and L/Ser, although involved in a common goal of generation of DV lineage in eye, probably affect eye development at two different tiers.

Fng, another essential component of DV patterning in eye, is expressed preferentially in the ventral domain of early eye disc and is required for restriction of N signaling to the DV border (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). Although fng is known to act upstream of Ser in the wing and eye discs (Irvine, 1999), there is also an apparent difference between the two genes. Unlike L/Ser, the main function of fng seems to affect DV ommatidial polarity but not the growth (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). This suggests that fng may be selectively required for DV patterning after dorsal selectors initiate domain specification. This may be the reason why phenotypes of loss-of-function clones of fng are different from those of L and Ser in the eye. It has been observed that the pattern of fng expression is not altered in L mutants, and vice versa, supporting the independent functions of these two genes in controlling DV border formation and growth of ventral domain (data not shown).

Functional conservation of dorsal selector Pnr

The function of Pnr in organizing the DV pattern from an initial ventral-equivalent state raises an interesting question of whether similar patterning processes occur in other developing tissues and organs. Interestingly, Pnr is expressed in a broad dorsal domain in early embryos, but later refined...
in a longitudinal dorsal domain extending along the thoracic and abdominal segments. During this stage, Pnr has an instructive and selector-like function, determining the identity of the medial dorsal structures (Calleja et al., 2000). It has been shown that loss of pnr eliminates the dorsomedial pattern in the larval cuticle whereas the dorsolateral pattern extends dorsally without cell loss (Herranz and Morata, 2001). This suggests that DV pattern in the larval cuticle is established with the onset of Pnr expression in the dorsomedial domain, and ventral may be the initial fate of epidermal cells.

The compound eye of *Drosophila* shares some similarities with the vertebrate eye (Hartenstein and Reh, 2002). Like *Drosophila*, in higher vertebrates dorsal eye genes (e.g. *Bmp4* and *Tbx5*) also act as ‘dorsal selectors’ and restrict the expression of genes involved in ventral eye development (e.g. *Vax* and *Pax2*) to the ventral eye (Koshiba-Takeuchi et al., 2000; Peters and Cepko, 2002). These DV expression domains correspond to developmental compartments (Peters, 2002) and thereby generate DV lineage restrictions in a way similar to *Drosophila* eye. Furthermore, conservation is also seen at the level of genes and probably their functions. For example, *Ser* has a vertebrate homolog *Jagl*, the loss of function of which shows a strong eye reduction phenotype (Xue et al., 1999). Other dorsal eye genes, such as *pnr* and *Iro-C*, are also highly conserved. *Iro-C* genes are involved in neural development in vertebrates (Gomez-Skarmeta and Modolell, 2002). There is conservation even in the eye patterning mechanism because the wave of neurogenesis in the vertebrate eye is analogous to the morphogenetic furrow in the fly eye (Holt and Harris, 1993; Neumann and Nusslein-Volhard, 2000; Peters, 2002). Therefore, it would be interesting to see whether the DV lineage in the vertebrate eye also develops from a ventral-equivalent initial state. It has been observed that DV patterning regulates the connectivity of retinal ganglion cells to their targets in brain (Peters, 2002). Therefore, the study of DV patterning in vertebrate eye holds immense potential.

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