pannier acts upstream of wingless to direct dorsal eye disc development in *Drosophila*

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SUMMARY

The dorsoventral midline of the *Drosophila* eye disc is a source of signals that stimulate growth of the eye disc, define the point at which differentiation initiates, and direct ommatidial rotation in opposite directions in the two halves of the eye disc. This boundary region seems to be established by the genes of the *iroquois* complex, which are expressed in the dorsal half of the disc and inhibit *fringe* expression there. Fringe controls the activation of Notch and the expression of its ligands, with the result that Notch is activated only at the *fringe* expression boundary at the midline. The secreted protein Wingless activates the dorsal expression of the *iroquois* genes. We show here that *pannier*, which encodes a GATA family transcription factor expressed at the dorsal margin of the eye disc from embryonic stages on, acts upstream of *wingless* to control *mirror* and *fringe* expression and establish the dorsoventral boundary. Loss of *pannier* function leads to the formation of an ectopic eye field and the reorganization of ommatidial polarity, and ubiquitous *pannier* expression can abolish the eye field. Pannier is thus the most upstream element yet described in dorsoventral patterning of the eye disc.

Key words: *pannier*, *Equator*, Planar polarity, *wingless*, *iroquois*, *fringe*, Notch, *Drosophila*

INTRODUCTION

Developmental fields are frequently organized by a boundary formed between two differently determined regions, which subsequently serves as a source of patterning molecules (Vincent, 1998). The best studied example is the anterior-posterior compartment boundary of the *Drosophila* wing disc, which is formed at the border between posterior cells expressing the homeodomain protein Engrailed (En), and anterior cells that do not express En (Tabata et al., 1995). En controls the expression of Hedgehog (Hh) protein, which is secreted from posterior cells and activates the expression of Decapentaplegic (Dpp) on the anterior side of the boundary (Basler and Struhl, 1994; Tabata and Kornberg, 1994; Zecca et al., 1995). Secreted Dpp then acts over a long range to pattern the entire wing disc (Lecuit et al., 1996; Nellen et al., 1996). In the *Drosophila* eye disc, the morphogenetic furrow, where *dpp* is expressed under the control of Hh secreted by posterior retinal cells, resembles the wing anterior-posterior compartment boundary, but is a moving border that only transiently separates anterior and posterior regions of the disc (Heberlein and Moses, 1995). However, the dorsoventral midline of the eye disc has been shown to act as a lineage restriction boundary and a patterning center, which both promotes growth of the eye field and organizes its polarity (Cavodeassi et al., 1999; Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Zheng et al., 1995).

The adult compound eye of *Drosophila* comprises approximately 800 ommatidial units, each composed of eight photoreceptors organized in a characteristic and asymmetric trapezoid, as well as cone and pigment accessory cells. Two chiral forms of these ommatidial clusters are arranged in a mirror-symmetric pattern around the dorsoventral midline, also known as the equator (Ready et al., 1976). This global mirror symmetry originates during the third larval instar. The ommatidial preclusters are initially symmetric, but as they mature, cell movements introduce asymmetry within each precluster. In addition, preclusters on the dorsal and ventral sides of the disc undergo a 90° rotation in opposite directions (Ready et al., 1976; Tomlinson, 1985). The direction of this rotation appears to be controlled by tissue polarity genes including *wingless* (*wg*; Ma and Moses, 1995; Wehrli and Tomlinson, 1998), *frizzled* (*fz*; Zheng et al., 1995), *Notch* (*N*; Cooper and Bray, 1999; Fanto and Mlodzik, 1999), *prickle/spiny legs* (Gubb, 1993), *strabismus* (Wolff and Rubin, 1998) and *RhoA* (Strutt et al., 1997), while its extent depends on the genes *nemo* and *roulette* (Choi and Benzer, 1994).

The subdivision of the eye disc into dorsal and ventral territories takes place prior to ommatidial differentiation and is essential to define the site at which differentiation will initiate and to promote the growth of the eye field. The critical outcome of this subdivision appears to be the activation of the Notch (*N*) receptor in cells at the dorsoventral midline. This is due to the restricted expression of *fringe* (*fng*) in the ventral half of the eye disc (Papayannopoulos et al., 1998; Cho and Choi, 1998;
Dominguez and de Celis, 1998). *fng* encodes a secreted molecule that mediates the interactions between N and its ligands Delta (Dl) and Serrate (Ser), promoting the response of N to Dl and inhibiting its response to Ser (Irvine and Wieschaus, 1994; Panin et al., 1997). In the wing disc, *fng* is coexpressed with *Ser* in the dorsal compartment, while *Dl* is expressed in the ventral compartment; this results in N activation only at the dorsoventral compartment boundary (Kim et al., 1995; Panin et al., 1997). In the eye disc these interactions are very similar, except that *fng* and *Ser* are expressed ventrally and *Dl* dorsally (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). Either loss of *fng* activity or misexpression of *fng* throughout the disc causes a failure of the eye disc to grow and differentiate (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998).

This failure of eye development can be rescued by a constitutively activating form of N, which leads to overgrowth of the eye field (Dominguez and de Celis, 1998).

*fng* expression is restricted to the ventral half of the eye disc, apparently due to dorsal repression by genes of the *iroquois* Complex (*iro-C*) (Cavodeassi et al., 1999; Cho and Choi, 1998; Dominguez and de Celis, 1998). The *iro-C* contains three genes, *araucan (ara)*, *caupolican (caup)* and *mirror (mirr)*, encoding highly related homeoproteins that are expressed in the dorsal half of the eye (Cho and Choi, 1998; Dominguez and de Celis, 1998; Gomez-Skarmeta et al., 1996; McNeill et al., 1997). Ectopic expression of either *caup* or *mirr* has been shown to repress *fng* expression (Cho and Choi, 1998; Dominguez and de Celis, 1998), while loss of *iro-C* function leads to ectopic *fng* expression (Cavodeassi et al., 1999). Furthermore, it has been shown that the dorsal expression of *iro-C* genes requires the secreted proteins Wingless (Wg) and Hh (Cavodeassi et al., 1999; Heberlein et al., 1998). Early Wg expression appears to be restricted to the dorsal side of the eye disc (Cavodeassi et al., 1999), although it is also present at the ventral margin of the disc later in development (Baker, 1988). The JAK/STAT pathway ligand Unpaired (Upd), present at the midline of the posterior margin (Zeidler et al., 1999), and genes of the *Polycomb* group ofchromatin repressors (Netter et al., 1998) have been implicated in the ventral repression of *mirr*. These genes thus define a signaling pathway that functions to position the equator. The most downstream component of this cascade is the activation of N at the dorsoventral midline. These results implicate Pnr as the most upstream component of the signaling cascade that leads to the normal position of the equator.

The presence of *pnr* in the dorsalmost cells of the embryonic eye disc primordium suggests that dorsoventral patterning of the embryo directly establishes the dorsoventral axis of the eye disc.

**MATERIALS AND METHODS**

**Fly strains**

Alleles used were *pnr*<sup>VX6</sup> (Heitzler et al., 1996), *ush<sup>2</sup>* (Nüsslein-Volhard et al., 1984), and *wg<sup>CX2</sup>* (Baker, 1987). Transgenic flies were used were UAS-*pnr<sup>D4</sup>* (Haenlin et al., 1997), UAS-*ush* (Cubadda et al., 1997), UAS-*ara* (Gomez-Skarmeta et al., 1996), UAS-*wg* (Azpiazu et al., 1996), UAS-*flu*-*arm* (Zecca et al., 1996), UAS-*FLP* (Duffy et al., 1998), UAS-*N<sup>mos</sup>* (Doherty et al., 1996), *ey*-*FLP*1 (a generous gift from B. Dickson), *pnr*-*GAL4* (Calleja et al., 1996), and *ey*-*GAL4* (Hazelett et al., 1998). The lac-Z reporters were *w<sup>gl</sup>* (Kassis et al., 1992), *mrr<sup>2</sup>* (Hartenstein and Jan, 1992), *irp<sup>2</sup>* (Gomez-Skarmeta et al., 1996), *ey-*lacZ (Quiring et al., 1994) and the *35UZ-1 P* insertion in *fng* (Irvine and Wieschaus, 1994).

**Mosaic analysis**

To generate *pnr*<sup>VX6</sup> mutant clones in the eye, FRT82, *pnr*<sup>VX6</sup>/TM6B males were crossed with *ey*-*FLP*1;FRT82, *P(arm-lacZ)*/TM6B females. To generate clones in a Minute background, the same males were crossed with *ey*-*FLP*1;FRT82, *P(arm-lacZ)*, *M(3)96C* TM6B females. To generate *wg*<sup>CX2</sup> or *ush<sup>2</sup>* mutant clones in the eye in a Minute background, FRT40, *wg*<sup>CX2</sup> or *ush<sup>2</sup>*/SM6;TM6B males were crossed with *ey*-*FLP*1;FRT40, *M(2)24F*, *P(arm-lacZ)*/CyO females. To rescue *pnr*<sup>VX6</sup> clones by overexpressing *wg* or *ara*, *ey-*GAL4, UAS-*FLP*;FRT82, *P(arm-lacZ)/SM6*;TM6B females were crossed to UAS-*wg* or UAS-*ara*, FRT82, *pnr*<sup>VX6</sup>/TM6B males.

**Immunohistochemistry and histology**

Eye imaginal discs were dissected from third instar larvae into 0.1 M phosphate buffer, pH 7.2, fixed for 30 minutes on ice in 4% formaldehyde in PEMS (0.1 M Pipes, pH 7.0, 2 mM MgSO<sub>4</sub>, 1 mM EGTA) and washed in 0.1 M phosphate buffer, 0.2% Triton X-100. Eye imaginal discs were stained with rat anti-Elav (Robinow and White, 1991; diluted 1:5) as described by Hazelett et al. (1998), followed by X-gal staining at 37°C in staining buffer (0.2% X-gal, 10 mM phosphate buffer, pH 7.2, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 3 mM K<sub>3</sub>[Fe(III)CN]<sub>6</sub> 3 mM K<sub>3</sub>[Fe(II)CN]<sub>6</sub>, 0.2% Triton X-100) to reveal the pattern of *arm*-lacZ expression. Tangential sections of adult eyes were prepared as described by Tomlinson and Ready (1987).

**In situ hybridization**

In situ hybridization to whole-mount eye-antennal imaginal discs was performed using an antisense RNA probe labeled with digoxigenin-UTP (Roche Molecular Biochemicals) and synthesized from a 2 kb fragment of genomic DNA containing the GATA domain of *pnr* (a gift from M. Frasch). Eye-antennal discs were dissected in PBS, fixed for 20 minutes on ice in 4% formaldehyde in PBS, and fixed again at room temperature for 20 minutes in 4% formaldehyde, 0.1% sodium deoxycholate in PBT (PBS + 0.1% Tween 20). After five washes in PBT, they were dehydrated through an ethanol series and stored, if required, in 70% ethanol/PBT at ~20°C. On the day of hybridization, discs were rehydrated through an ethanol series and washed three times in PBT. They were then fixed in 10% formaldehyde in PBT for 20 minutes and rinsed five times in PBT. Prehybridization, hybridization,
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anti-DIG incubation and signal detection were performed as described for in situ hybridization to whole-mount embryos by Ronchi et al. (1993). In situ hybridization to embryos was also performed as described by Ronchi et al. (1993) and was followed by dehydration, rehydration and staining with anti-β-galactosidase (Cappel; 1:5000).

RESULTS

The dorsal restriction of pannier expression is required for normal eye growth

The pnr gene is expressed in the dorsalmost embryonic cells, in a domain of the notum surrounding the dorsal midline, and at the dorsal anterior margin of the eye disc (Heitzler et al., 1996; Ramain et al., 1993; Winick et al., 1993; see Fig. 3A,J). We chose to investigate the role of its expression in the eye disc.

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**Fig. 1.** Phenotypes produced by gain or loss of pnr function in the eye. (A,C,E,G) are adult heads and (B,D,F,H) are eye imaginal discs from late third instar larvae stained with anti-Elav antibody to label differentiated photoreceptors (brown) and doubly labeled with X-gal to show dpp-lacZ expression in (B) and the clonal marker arm-lacZ in (D,F) (blue). (A,B) Wild type. (C,D) pnr VX6 clones. Clones are visualized by the absence of red pigmentation in (C,E) and by the absence of blue X-gal staining in (D,F). Arrowheads show the boundary between cells that express pnr and those that do not express pnr in the ectopic eye field. The arrow shows an ectopic antenna. (E,F) pnr VX6 clones in a Minute background. In these animals, eyes are entirely missing. The arrow in E shows an ectopic antenna; arrows in F show the centers of two antennal imaginal discs, of which one is ectopic. (G,H) Adult head and eye-antennal disc from UAS-pnr D4/ey-GAL4 flies show the absence of eye development.

**Fig. 2.** pnr acts upstream of mrr and fng. (A-H) Eye discs from third instar larval eye discs doubly labeled with anti-Elav (brown) and X-gal (blue) in (A-C,H), stained with X-gal alone (D-F) or with anti-Elav alone (G). (A) Wild-type expression of the mrr-lacZ enhancer trap is restricted to the dorsal half of the eye disc. This dorsal expression is no longer detected in pnr VX6 clones produced in a Minute background (B), while it is expanded ventrally in UAS-pnr D4/ey-GAL4 larvae (C). (D) Wild-type expression of the fng-lacZ enhancer trap is restricted to the ventral half of the eye disc. (E) In pnr mutant clones in a Minute background fng-lacZ expression is derepressed dorsally. (F) In UAS-pnr D4/ey-GAL4 eye discs, fng-lacZ expression is strongly reduced. (G) Flies carrying ey-GAL4, UAS-pnr D4 and UAS-N intra show restored photoreceptor differentiation. (H) Flies carrying ey-GAL4, UAS-wg and UAS-N intra show photoreceptor differentiation initiating all around the margins.
To address this, we used the FLP-FRT system to generate clones of cells mutant for pnr\textsuperscript{D\textregistered}, a null allele caused by a deletion of all but 9 amino acids of the coding region (Ramain et al., 1993; Heitzler et al., 1996; Xu and Rubin, 1993). Mutant clones were produced in the eye disc using the yeast FLP recombinase expressed under the control of the eye-specific enhancer of eyeless (ey; Quiring et al., 1994; B. Dickson, unpublished data).

As expected, considering the dorsal expression of pnr, only clones produced at the dorsal margin of the eye disc gave rise to a phenotype. In such discs an ectopic field of differentiating photoreceptors appeared anterior to the main eye field (Fig. 1D). In adult flies this resulted in the formation of an ectopic eye field in the dorsal head cuticle, which could be either separate from or fused with the normal eye (Fig. 1C and data not shown). Interestingly, these ectopic eye fields did not arise exclusively from the pnr mutant cells within the clone itself, but also contained a domain of wild-type cells (marked by red pigment in Fig. 1C and by blue X-gal staining in Fig. 1D). These observations suggested that the new boundary of pnr expression present at the edge of the clone could be responsible for the induction of this new eye field.

To test the hypothesis that the boundary of pnr expression, rather than the absence of pnr, could be important for promoting eye growth, we removed all pnr function in the eye. For this purpose, we placed a Minute mutation, causing slow growth and recessive cell lethality (Morata and Ripoll, 1975), on the wild-type chromosome. In combination with F. Casares, personal communication). The similar effects we observed for gain or loss of pnr function suggested strongly that pnr might act in the same pathway as the ara-C and fng. To confirm this and to order pnr with respect to these genes, we looked at the expression of mirr and fng in eye discs mutant for pnr or misexpressing pnr. To follow mirr and fng expression, we used enhancer-trap insertions in these genes. In eye discs in which pnr function had been removed by producing mutant clones in a Minute background, we found that mirr expression was greatly reduced (Fig. 2B), whereas fng was derepressed dorsally (Fig. 2E). In eye discs expressing pnr\textsuperscript{D\textregistered} ubiquitously from an ey-GAL4 driver, mirr expression was expanded ventrally, shifting the point of morphogenetic furrow initiation to the ventral side (Fig. 2C). The enhancer trap insertion disrupts one copy of the mirr gene, perhaps explaining the weaker effect on photoreceptor differentiation than we observed when we overexpressed pnr in the absence of mirr-lacZ (Fig. 1H). pnr overexpression had the same effect on the expression of another enhancer trap insertion, rF209, thought to reflect the expression of ara and caup (Gomez-Skarmeta et al., 1996; data not shown). fng expression was dramatically reduced in discs overexpressing pnr\textsuperscript{D\textregistered} (Fig. 2F).

It thus appears that pnr acts upstream of the ara-C genes, activating their expression dorsally. Consistent with this, we found that ubiquitous expression of ara abolished photoreceptor differentiation, and that removal of pnr function either by making pnr mutant clones or by misexpressing ush did not restore photoreceptor formation (data not shown). If pnr were downstream of ara, blocking its function should have induced ectopic eye development even in the presence of ara. In the absence of pnr, ara-C expression is lost and fng is thus ectopically activated. We expect that this would lead to an ectopic region of N activation at the new boundary of fng.
expression, promoting morphogenetic furrow initiation from an ectopic position. Overexpression of pnr leads to expansion of iro-C expression and loss of fnig, which should prevent N activation. As predicted, we could rescue the loss of eye development caused by ectopic pnr by co-expressing a constitutively activated form of N, $N^{\text{Act}}$ (Fig. 2G).

**pannier is required to activate the dorsal expression of wingless**

The results above show that Pnr acts upstream of the iro-C genes to regulate dorsal eye development. Another molecule that has been shown to act upstream of the iro-C in this context is Wg (Cavodeassi et al., 1999; Heberlein et al., 1998). wg is required to inhibit the initiation of the morphogenetic furrow at the lateral margins of the eye disc, preventing ectopic eye differentiation there (Ma and Moses, 1995; Treisman and Rubin, 1995). The dorsal ectopic eyes induced by removing pnr function thus suggested that the functions of pnr and wg may be related. Consistent with this idea, the block in morphogenetic furrow initiation caused by expressing wg throughout the eye disc (Hazelett et al., 1998), like the block caused by expressing $pnr^{\text{D4}}$, could be rescued by co-expressing an activated form of $N$ (Fig. 2H). pnr and wg may thus act in the same cascade to prevent eye differentiation.

We used in situ hybridization to show that pnr mRNA was restricted to the dorsal margin of the eye disc, anterior to and overlapping the morphogenetic furrow. Its domain of expression was broader than was previously reported (Ramain et al., 1993; Fig. 3A), though not as broad as the expression of lacZ driven by a GAL4 insertion in the pnr locus (Heitzler et al., 1996). wg is expressed at the dorsal and ventral edges of the eye disc with stronger expression dorsally (Baker, 1988), and its dorsal domain of expression resembles that of pnr (compare Fig. 3A with E).

To test the epistatic relationship between wg and pnr, we looked at pnr mRNA expression in eye discs from which we had removed wg function by producing clones of a null allele ($wg^{\text{cx2}}$) in a Minute background, as described above for pnr. Adult flies carrying such clones show a transformation of the dorsal head cuticle into ectopic eye tissue, as well as missing antennae (data not shown). Eye-antennal discs carrying such clones were identified by a severe reduction in the size of the antennal disc. In these eye discs pnr mRNA expression was wild type (Fig. 3B), showing that wg is not required for pnr expression. Overexpression of either Wg or an activated form of Armadillo (Arm), a downstream component of the Wg pathway (Peifer and Wieschaus, 1990; Zecca et al., 1996), using the ey-GAL4 driver also had no effect on pnr expression (Fig. 3C,D). Thus, wg is neither necessary nor sufficient for pnr expression.

We next tested whether pnr might be responsible for the dorsal expression of wg. Indeed, when pnr mutant clones were produced in a Minute background, dorsal wg expression was lost (Fig. 3F). Conversely, when we overexpressed $pnr^{\text{D4}}$, although the size of the eye disc was dramatically reduced, we observed a derepression of wg expression in both the eye and the antennal discs (Fig. 3G; compare wg expression in the antennal disc in Fig. 3E). We conclude that pnr indeed activates wg expression at the dorsal margin.

To confirm that the function of pnr in the eye is mediated by wg, we attempted to rescue the phenotype of pnr mutant clones by overexpressing wg. We used in combination the FLP-FRT system to produce pnr clones and the UAS/GAL4 system to ectopically express wg throughout the eye disc using ey-GAL4 as the driver (see Materials and Methods). Misexpressing wg with ey-GAL4 in a wild-type background prevents photoreceptor differentiation (Hazelett et al., 1998). If wg acts downstream of pnr, loss of pnr should not alter the effect of wg overexpression, while if wg produces its effects by misregulating pnr, removal of pnr function should restore normal photoreceptor differentiation. As predicted, even large dorsal clones of pnr mutant cells, which would be likely to produce an ectopic eye field in a wild-type background (Fig. 1D), showed no photoreceptor differentiation in discs overexpressing wg (Fig. 3H). Taken together, these results allowed us to conclude that pnr acts upstream of wg by activating its expression at the dorsal margin.

It is not known what regulates the dorsal expression of pnr in the eye disc. pnr is expressed in a dorsal stripe along the length of the embryo, where its expression seems to be established by embryonic dorsal-ventral patterning genes (Heitzler et al., 1996; Winick et al., 1993). Its later dorsal expression in the dorsal imaginal discs could simply reflect maintenance of expression in the derivatives of embryonically expressing cells. To test this, we looked for an overlap between the embryonic stripe of pnr and the eye disc primordium, which we identified by its expression of $\beta$-galactosidase driven by the ey promoter (Quiring et al., 1994). Indeed, we found that the dorsalmost cells of the eye primordium contained pnr RNA in late embryos (Fig. 3I). We have also observed pnr expression at the dorsal margin of second instar eye discs (data not shown), suggesting that its expression is continuous. Dorsal-ventral patterning of the eye disc may thus be a direct consequence of the establishment of the dorsal-ventral axis in the embryo.

**Dorsal clones mutant for pnr cause ommatidial repolarization**

Both wg, acting at the dorsal and ventral margins of the eye disc, and fnig, controlling N activation at the equator, have been shown to regulate the polarity signals that direct ommatidial rotation (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Reifegerste et al., 1997; Wehrli and Tomlinson, 1998). We therefore tested whether pnr was also involved in the control of ommatidial polarity. We analyzed ommatidial polarity in tangential sections of adult eyes containing pnr mutant clones. Consistent with the dorsal expression of pnr, ventral clones and dorsal clones lying close to the equator did not show any polarity defects (Fig. 4B,B'). Only the largest and most dorsal clones, up to eight ommatidial rows from the equator, were abnormal. Ommatidial clusters in the equatorial regions of these clones adopted a ventral polarity and chirality, and more dorsally, the formation of an ectopic equator was observed (Fig. 4C,C',D,D'). This new equator formed within the clone rather than at its boundary, and the polarity inversion did not strictly follow the borders of the clone. On one hand, some mutant clusters near the margins of the clone showed normal dorsal polarity; on the other, wild-type ommatidia adjacent to a mutant clone sometimes showed chirality changes (Fig. 4C,C',D,D').

These findings, together with the observation that only large
DISCUSSION

A pathway for dorsal specification of the eye disc

We have demonstrated that pnr acts to specify the dorsal margin of the eye disc to form head cuticle rather than initiating photoreceptor development. The same effect has previously been reported for wg (Ma and Moses, 1995; Treisman and Rubin, 1995). The genes of the irl-C were originally implicated in dorsal eye development by gain-of-function studies (Cho and Choi, 1998; Dominguez and de Celis, 1998). The phenotype of removal of all three genes has recently been shown to resemble that of pnr (Cavodeassi et al., 1999; F. Pichaud and F. Casares, personal communication), although loss of the single gene mirr has a much weaker phenotype (McNeill et al., 1997). Our results show that pnr acts upstream of these components; the expression patterns of wg, the wg target mirr and the mirr target fng are altered in conditions in which pnr is removed or misexpressed, while pnr expression is unaffected by changes in Wg activity. In addition, the pnr mutant phenotype can be suppressed by expression of wg from an exogenous promoter. Finally, pnr is present in the dorsal eye disc primordium at late embryonic stages, suggesting that it reflects a very early step in dorsoventral patterning of the eye disc. The downstream response to this cascade is the activation of N at the dorsoventral midline (Fig. 5).

The role of wg in directing dorsal development is unexpected because wg is also expressed at the ventral anterior margin of the eye disc, although at a lower level than at the dorsal margin (Baker, 1988); this expression must have an upstream regulator other than pnr. However, the effects of loss of wg are more robust on the dorsal than the ventral side of the eye disc (Ma and Moses, 1995; Treisman and Rubin, 1995), and misexpression of wg symmetrically at both lateral margins dorsalizes the eye disc (Heberlein et al., 1998). These observations may be explained by the finding that at early stages wg is limited to the dorsal side of the eye disc and may exert its dorsalizing effect at this time (Cavodeassi et al., 1999). It is interesting that clones of cells mutant for components of the dpp pathway, such as Mothers against dpp (Mad), often induce an ectopic morphogenetic furrow initiating at the ventral margin adjacent to them (Burke and Basler, 1996; Hazelett et al., 1998;
**Fig. 4.** Dorsal *pnr* mutant clones induce inversion of ommatidial polarity. (A-D) Tangential sections through a wild-type eye (A) or eyes containing *pnr*V66 clones (B-D). Clones are visualized by the absence of pigmentation. Yellow numbers in (A) indicate the position of the R1 to R7 rhabdomeres in dorsal and ventral ommatidia. Lower panels (A’-D’) are diagrams corresponding to the sections in A-D. The positions of the mutant clones are indicated in white, normal equators are represented by black lines, ectopic equators are in green, red and blue arrows indicate dorsal and ventral clusters, respectively, black arrows indicate ommatidia with reversed anterior-posterior polarity, and O are ommatidia with too few R cells. (B,B’) Small dorsal clones and clones at the equator of *pnr* do not affect ommatidial polarity. (C,C’,D,D’) Large dorsal *pnr* clones showing repolarization at their equatorial sides and formation of ectopic equators (in green).

**Fig. 5.** Model for specification of the dorsoventral boundary. (Left) The positive (arrows) and negative (perpendicular line) genetic interactions between *pnr* (green), *wg* (red), *mrr* (yellow), *fng* (blue) and N activation (purple). (Right) The result of removing *pnr* function from a clone of cells. The downstream targets of *pnr* are not expressed, resulting in expression of *fng* and activation of N at the dorsoventral midline of an ectopic eye field.
Wiersdorff et al., 1996). Mad mutant cells at the posterior margin have been shown to ectopically express wg (Wiersdorff et al., 1996); this ectopic wg might activate the iro-C genes to induce a new dorsoventral boundary. Indeed, ectopic furrows are very rarely induced by clones doubly mutant for both Mad and wg (J.E.T., unpublished data).

It is possible that another factor regulated by pnr cooperates with wg to regulate the iro-C genes. Since the concentration of Wg is likely to be very low near the midline of the eye disc, the sharp boundary of iro-C expression at the equator (Cavodeassi et al., 1999; Cho and Choi, 1998; Dominguez and de Celis, 1998; McNeill et al., 1997) is difficult to explain without invoking other regulators. The existence of another factor downstream of Pnr could also explain why diffusion of Wg from adjacent cells does not rescue the ectopic differentiation phenotype of pnr mutant clones. Hh may be a possible candidate for this factor (Cavodeassi et al., 1999). It is not clear whether a counterpart to pnr exists on the ventral side of the eye disc; no ventrally restricted molecules other than fng and Ser, which is a downstream target of fng (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998) have yet been described. One possible candidate is the homothorax (hth) homeobox gene, which is expressed at the anterior margin but seems to function to repress photoreceptor differentiation specifically on the ventral side (Pai et al., 1998). However, the effects of loss of hth are cell-autonomous, suggesting that it does not function in dorsoventral boundary induction.

Polarity determination in the eye

Establishment of the dorsoventral midline of the eye is linked to the opposite polarities of ommatidia on each side of the midline, in a process involving a series of diffusible signals. Wg appears to act on both the dorsal and ventral sides of the eye disc to allow ommatidia to measure their distance from the polar regions (Ma and Moses, 1995; Wehrli and Tomlinson, 1998). Because cells mutant for intracellular components of the Wg signaling pathway show non-autonomous repolarizations of adjacent tissue, it has been proposed that Wg has this effect by regulating the levels of a second signal, factor X (Wehrli and Tomlinson, 1998). Although the JAK/STAT pathway ligand Upd is a polarity-altering signal secreted from the equator, its expression is not affected by Wg and it is therefore unlikely to be factor X (Zeidler et al., 1999). Factor X may act through the Frizzled (Fz) receptor and other Wnt pathway signaling components (Wehrli and Tomlinson, 1998; Zheng et al., 1995).

pnr has the effects on polarity that we would predict from its function as a positive regulator of wg expression. Small dorsal clones of cells mutant for pnr have no effect on polarity, probably because Wg produced by adjacent wild-type cells can diffuse in to rescue the clone. This also applies to the dorsoalmost regions of large pnr clones. The non-autonomous polarity inversions seen in wild-type tissue adjacent to pnr mutant clones may be caused by changes in the level of either Wg, factor X or Fng. Since pnr is only expressed at the dorsal margin of the eye disc, the border of repolarization is not related to the boundary of the pnr clone, which may extend beyond the region of Pnr function. Instead it should be related to the reduction in Wg activity, which would depend on the extent to which the pnr clone overlaps the wg domain. Although loss of pnr induces an ectopic morphogenetic furrow which is often perpendicular to the normal furrow, the midline of this ectopic eye field does not appear to act as an equator to organize the rotation of ommatidia (Chanut and Heberlein, 1995; Ma and Moses, 1995; Strutt and Mlodzik, 1995).

Regulation of wingless by pannier

pnr has been shown to encode a transcription factor of the GATA family (Ramain et al., 1993), and it is therefore possible that its positive regulation of wg expression may reflect direct transcriptional activation. Pnr has been best characterized as a transcriptional activator of the ac and sc genes in the notum, where it acts through defined GATA sites in the enhancer mediating ac-sc expression in the dorsocentral proneural cluster (Garcia-Garcia et al., 1999). In the dorsoalmost region of the notum, Pnr activity is inhibited by Ush, a zinc finger protein that dimerizes with Pnr to convert it from a transcriptional activator to a repressor (Cubadda et al., 1997; Garcia-Garcia et al., 1999; Haenlin et al., 1997). This mechanism is unlikely to operate in the eye because removal of ush function from very large regions of the eye disc has no effect. As misexpression of ush in the eye disc can induce pnr loss-of-function phenotypes, it is likely that ush is normally absent there rather than rendered non-functional.

wg also appears to be a target of pnr regulation in the notum, where it is transcribed in a stripe at the edge of the pnr domain (Calleja et al., 1996; Garcia-Garcia et al., 1999). This pattern seems to be a result of its transcriptional activation even by low levels of Pnr, but its repression by Pnr/Ush heterodimers within the Ush domain (Garcia-Garcia et al., 1999). The absence of ush function in the eye would explain the overlapping expression patterns we observe for pnr and wg. However, the action of Pnr on wg has not been proved to be direct in any system, and it is not known whether Pnr regulates the establishment or the maintenance of wg transcription. In the notum, the iro-C genes are expressed in a domain adjacent to the pnr domain, rather than the overlapping domain we see in the eye (Diez del Corral et al., 1999; Gomez-Skarmeta et al., 1996; Kehl et al., 1998; Leyns et al., 1996); iro-C function is also required for expression of the stripe of wg. However, in the wing pouch itself wg acts upstream of ara and caup to negatively regulate their expression at the wing margin (Gomez-Skarmeta and Modolell, 1996). Thus the regulatory relationship between these genes varies in different developmental contexts, suggesting that other factors contributing to their expression patterns remain to be identified.

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