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

Corinne Maurel-Zaffran and Jessica E. Treisman*

Skirball Institute of Biomolecular Medicine and Department of Cell Biology, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA
*Author for correspondence (e-mail: treisman@saturn.med.nyu.edu)

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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 activated 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 of chromatin 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, and so far, the most upstream is *wg* expressed at the dorsal margin of the eye disc.

The expression of *wg* is restricted to the more anterior regions of the lateral margins by the activity of Dpp present at the posterior margin (Wiersdorff et al., 1996); however, little is known about which positive regulators are responsible for its expression in the eye disc. In this paper we report that *pannier* (*pnr*), which encodes a transcription factor homologous to vertebrate GATA-1 (Ramain et al., 1993), activates the dorsal expression of *wg*. *pnr* and the *iro-C* genes act as prepattern genes in the presumptive notal region of the wing disc, where they are required to delimit the expression of the proneural genes *achaete* (*ac*) and *scute* (*sc*) (Diez del Corral et al., 1999; Garcia-Garcia et al., 1999; Gomez-Skarmeta et al., 1996; Kehl et al., 1998; Leyns et al., 1996; Ramain et al., 1993). Pnr also acts as a regulator of *wg* in the notum (Calleja et al., 1996; Garcia-Garcia et al., 1999). The expression of *pnr* at the dorsal margin of the eye disc led us to investigate its function during eye development using mosaic analysis and ectopic expression. We show that the boundary between cells expressing and not expressing *pnr* promotes eye growth and organizes ommatidial polarity. We find that *pnr* acts upstream of *wg* by positively regulating its dorsal expression. Consequently, *pnr* is also required to activate dorsal *mirr* expression and repress ventral *fng* expression, leading to 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^{VX6} (Heitzler et al., 1996), ush^{2} (Nüsslein-Volhard et al., 1984), and wg^{CX2} (Baker, 1987). Transgenic flies used were UAS-pnr^{D4} (Haenlin et al., 1997), UAS-*ush* (Cubadda et al., 1997), UAS-*ara* (Gomez-Skarmeta et al., 1996), UAS-*wg* (Azpiazu et al., 1996), UAS-*fluΔarm* (Zecca et al., 1996), UAS-*FLP* (Duffy et al., 1998), UAS-N^{intra} (Doherty et al., 1996), *eyFLP1* (a generous gift from B. Dickson), *pnr*-GAL4 (Calleja et al., 1996), and *ey*-GAL4 (Hazelett et al., 1998). The *lacZ* reporters were wg^{P} (Kassis et al., 1992), mrr^{cre2} (Hartenstein and Jan, 1992), iro^{rF209} (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^{VX6} mutant clones in the eye, FRT82, pnr^{VX6}/TM6B males were crossed with *eyFLP1*;FRT82, *P(arm-lacZ)*/TM6B females. To generate clones in a *Minute* background, the same males were crossed with *eyFLP1*;FRT82, *P(arm-lacZ)*, *M(3)96C*/ TM6B females. To generate wg^{CX2} or ush^{2} mutant clones in the eye in a *Minute* background, FRT40,wg^{CX2} or ush^{2}/SM6;TM6B males were crossed with *eyFLP1*;FRT40, *M(2)24F, P(arm-lacZ)*/CyO females. To rescue pnr^{VX6} 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^{VX6}/ 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 PEM (0.1 M Pipes, pH 7.0, 2 mM $MgSO_4$, 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 $MgCl_2$, 3 mM $K_4[FeII(CN)_6]$, 3 mM $K_3[FeIII(CN)_6]$, 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 Biochemichals) 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,

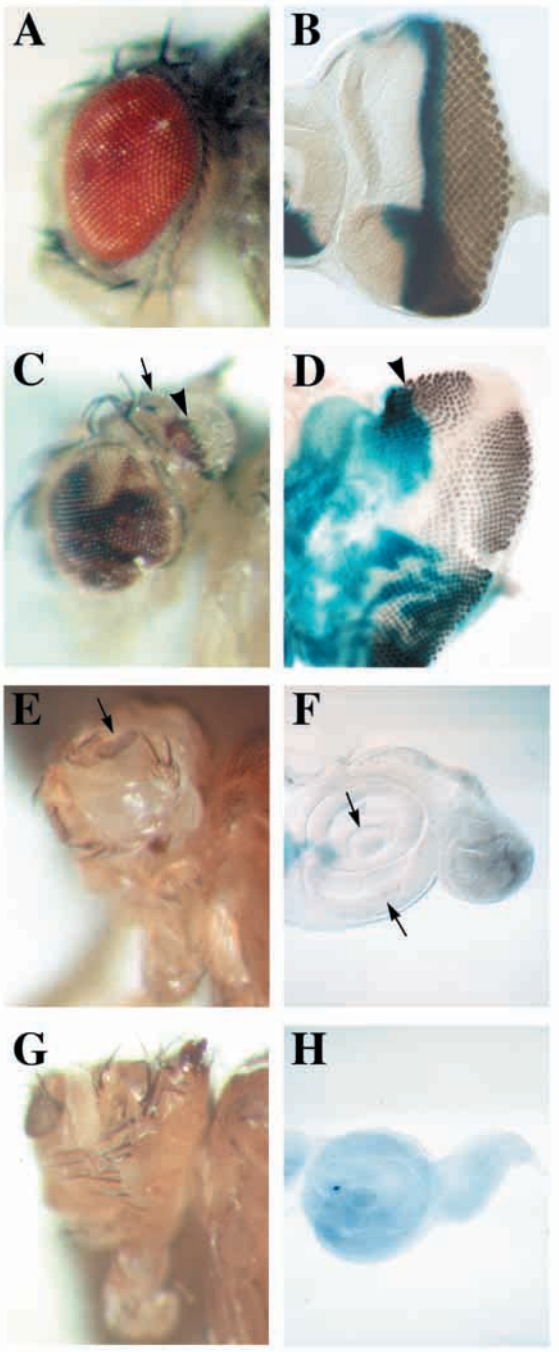

Fig. 1. Phenotypes produced by gain or loss of *pnr* function in the eye. (A,C,E,G) are adult heads and (B,D,F,H) are eye imaginal discs from late third instar larvae stained with anti-Elav antibody to label differentiated photoreceptors (brown) and doubly labeled with X-gal to show *dpp-lacZ* expression 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.

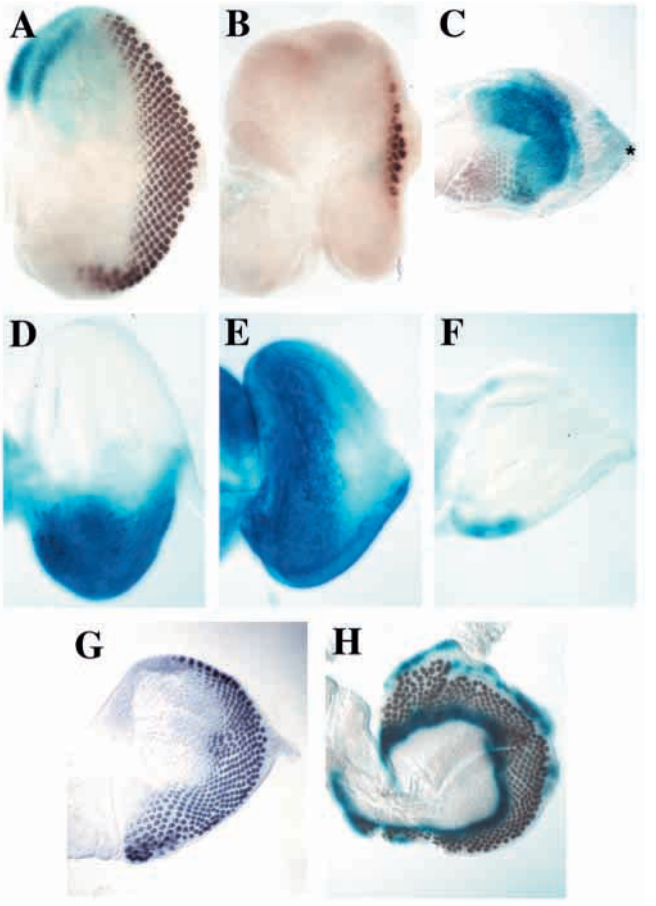

Fig. 2. *pnr* acts upstream of *mrr* and *fng*. (A-H) Eye discs from third instar larval eye discs doubly labeled with anti-Elav (brown) and X-gal (blue) in (A-C,H), stained with X-gal alone (D-F) or with anti-Elav alone (G). (A) Wild-type expression of the *mrr-lacZ* enhancer trap is restricted to the 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.

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,I). We chose to investigate the role of its expression in the eye disc.

To address this, we used the FLP-FRT system to generate clones of cells mutant for pnr^{VX6}, 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. We frequently also observed a duplication of the antenna (Fig. 1C,E,F), probably reflecting the function of *pnr* expressed dorsally in the antennal disc (Heitzler et al., 1996).

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 *ey*FLP, this results in an eye composed almost entirely of homozygous mutant tissue. Again, we examined adult eyes and eye discs from flies containing very large *pnr* mutant clones. In some cases, we observed a dramatic loss of the eye and an absence of differentiating photoreceptors in the eye disc, resulting from the loss of all *pnr* function (Fig. 1E,F). In other cases eye overgrowth was observed (data not shown); probably these eye discs retained some *pnr*-expressing cells, allowing the establishment of an ectopic *pnr* expression boundary. Only a small percentage of adults with large *pnr* clones were recovered; most animals died as late pupae and their heads were sometimes entirely missing, probably due to loss of all tissues deriving from the eye-antennal disc. A similar phenotype has been reported for some hypomorphic combinations of *pnr* alleles (Heitzler et al., 1996).

In the notum, the activity of Pnr as a transcriptional activator is inhibited by binding to the zinc finger protein U-shaped (Ush), which is expressed in an adjacent domain (Cubadda et al., 1997; Haenlin et al., 1997). Ush does not appear to be required in the eye disc, as clones mutant for *ush* developed normally even when they were very large (data not shown). However, ectopically expressed *ush* was able to inhibit the function of Pnr in the eye disc; expression of *ush* with a *pnr*-GAL4 driver resulted in phenotypes similar to those induced by *pnr* mutant clones (data not shown). Thus Pnr is likely to act by activating the transcription of target genes in the eye as well as in the notum.

We next eliminated the *pnr* boundary using the opposite approach, by inducing ubiquitous *pnr* expression using the UAS/GAL4 system (Brand and Perrimon, 1993). We used an *ey*-GAL4 driver (Hazelett et al., 1998) to ectopically express *pnr* throughout the early eye disc. We used a form of Pnr (Pnr^{D4}) that is resistant to inhibition by Ush, but appears to behave like wild-type Pnr in the absence of Ush function (Haenlin et al., 1997). As a consequence, we observed a complete loss of the eye (Fig. 1G,H), confirming the importance of the border of *pnr* expression.

From these experiments, we conclude that the dorsally restricted expression of *pnr* is critical for eye development. A boundary between *pnr*-expressing cells and *pnr*-non-expressing cells appears to be necessary to induce growth and differentiation of the eye field.

pannier acts upstream of *mirror* and *fringe* in the pathway leading to Notch activation at the dorsoventral midline

Recently, several studies have established that N activation along the dorsoventral midline of the eye disc is critical for eye growth as well as for positioning the equator. This local activation is the consequence of the ventrally restricted expression of *fng*, which is negatively controlled by the *iro-C* homeobox genes expressed in the dorsal half of the eye disc (Papayannopoulos et al., 1998; Cho and Choi, 1998; Dominguez and de Celis, 1998). Either loss of *fng* function, or ubiquitous expression of *fng*, *caup* or *mirr*, abolishes eye growth. The *iro-C* genes appear to act redundantly, as both *ara* and *caup* must be removed from clones of cells to promote the formation of ectopic dorsal eyes similar to those we report for *pnr* (Cavodeassi et al., 1999; McNeill et al., 1997; F. Pichaud and 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 *iro-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^{D4} 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^{D4} (Fig. 2F).

It thus appears that *pnr* acts upstream of the *iro-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*, *iro-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 *fng*, 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^{intra} (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^{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^{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^{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 β-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 *fng*, 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

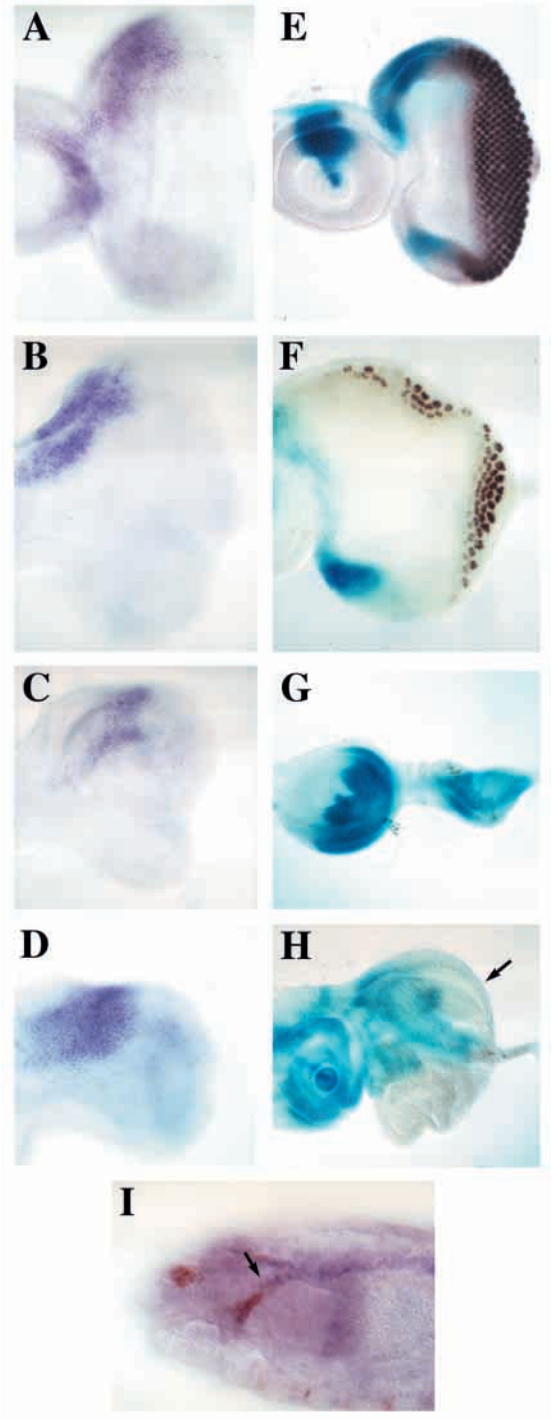

Fig. 3. *pnr* regulates the dorsal expression of *wg*. (A-D) *pnr* mRNA expression in third instar eye discs. (A) Wild type; (B) wg^{CX2} clones in a *Minute* background; (C) UAS-*wg*/*ey*-GAL4; (D) UAS-*fluΔarm*/*ey*-GAL4. In all these genetic backgrounds *pnr* RNA expression remains restricted to the dorsal margin of the disc. (E-H) Eye discs stained with anti-Elav (brown) and X-gal (blue). Eye discs carry the enhancer trap *wg-lacZ* (E-G), or the clonal marker *arm-lacZ* (H). (E) Wild-type expression of *wg-lacZ* enhancer trap. (F) The dorsal expression of *wg-lacZ* is not detected in pnr^{VX6} clones produced in a *Minute* background. (G) *wg-lacZ* is expressed throughout the eye disc and expanded in the antennal disc in larvae of the genotype UAS-pnr^{D4}/*ey*-GAL4. (H) *ey*-GAL4, UAS-*FLP*;FRT82, *P(arm-lacZ)*/UAS-*wg*, FRT82, pnr^{VX6}. Clones of cells mutant for *pnr* are marked by the absence of *arm-lacZ* staining; the arrow points to a large dorsal clone of *pnr* in which ectopic eye formation has been suppressed by overexpression of *wg*. (I) Lateral view of an *ey-lacZ* embryo stained with anti-β-galactosidase (brown) to label the eye disc primordium and hybridized to a probe for *pnr* RNA (purple). *pnr* expression is detected in the dorsalmost cells expressing *ey* (arrow).

mutant clones induced a polarity reversal, suggest that the effect of *pnr* on polarity is mediated by non-autonomous components. Indeed, the ommatidial repolarizations seen in pnr^{VX6} clones are very similar to those induced by clones mutant for downstream components of the *wg* signaling pathway (Wehrli and Tomlinson, 1998). The loss of *wg* expression caused by removing *pnr* would be expected to alter the polarity of ommatidia receiving insufficient levels of Wg. As Wg is a secreted protein that is thought to act by regulating the expression of a second non-autonomous signal, it is not surprising that the effects of loss of *pnr* are not autonomous.

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 *iro-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;

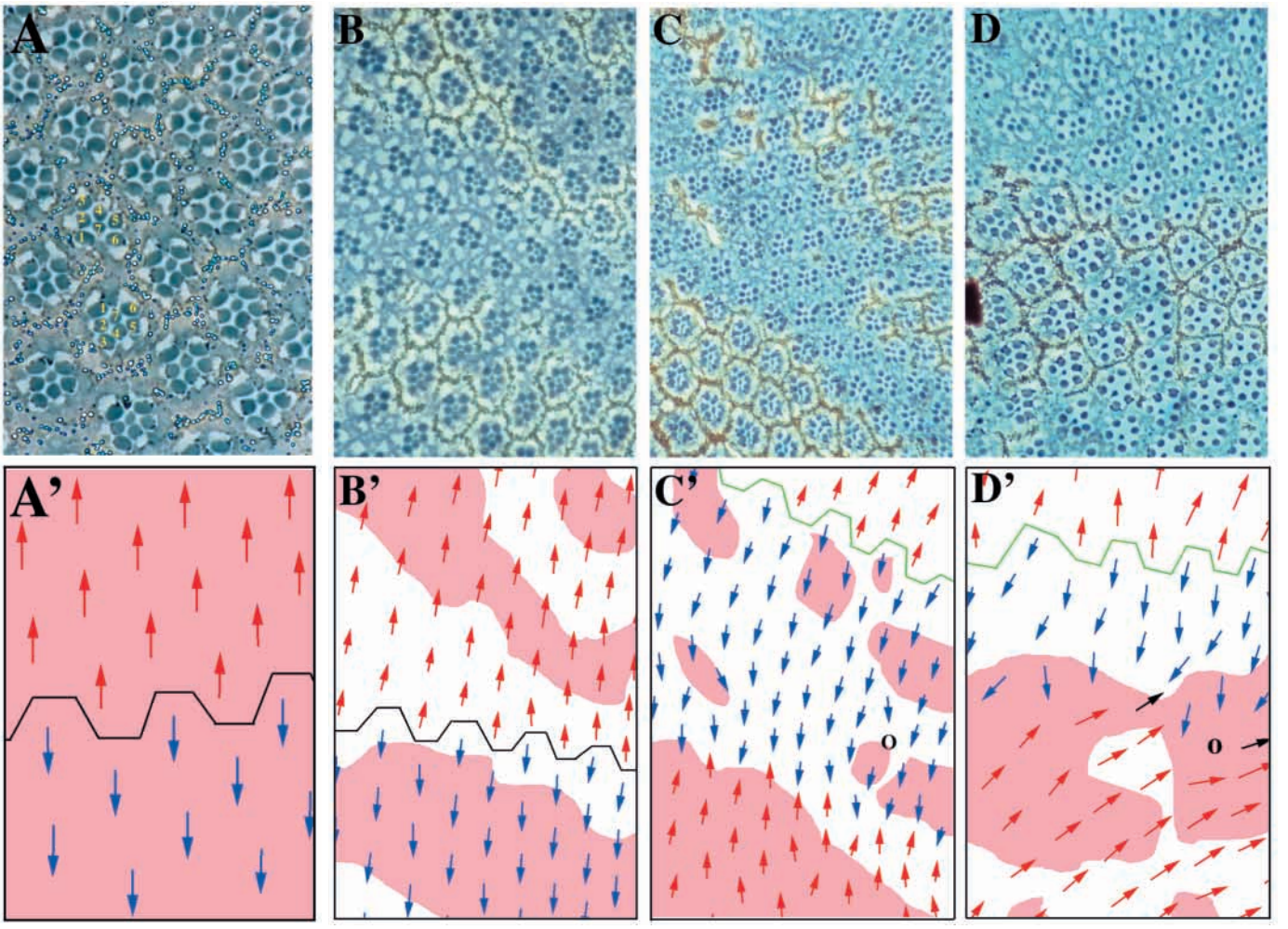

Fig. 4. Dorsal *pnr* mutant clones induce inversion of ommatidial polarity. (A-D) Tangential sections through a wild-type eye (A) or eyes containing pnr^{VX6} clones (B-D). Clones are visualized by the absence of pigmentation. Yellow numbers in (A) indicate the position of the R1 to R7 rhabdomeres in 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 omatidial polarity. (C,C′,D,D′) Large dorsal *pnr* clones showing repolarization at their equatorial sides and formation of ectopic equators (in green).

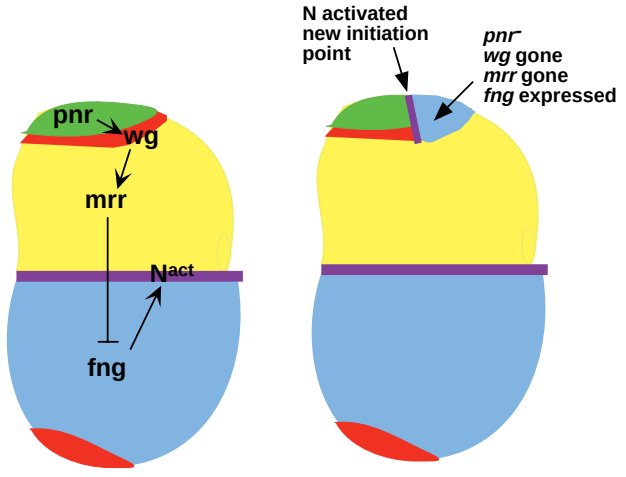

Fig. 5. Model for specification of the dorsoventral boundary. (Left) The positive (arrows) and negative (perpendicular line) genetic interactions between *pnr* (green), *wg* (red), *mrr* (yellow), *fng* (blue) and N activation (purple). (Right) The result of removing *pnr* function from a clone of cells. 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 dorsalmost 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 dorsalmost 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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