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Dorsal eye selector *pannier* (*pn*) suppresses the eye fate to define dorsal margin of the *Drosophila* eye

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Abstract

Axial patterning is crucial for organogenesis. During *Drosophila* eye development, dorso-ventral (DV) axis determination is the first lineage restriction event. The eye primordium begins with a default ventral fate, on which the dorsal eye fate is established by expression of the GATA-1 transcription factor *pannier (pnr)*. Earlier, it was suggested that loss of *pnr* function induces enlargement in the dorsal eye due to ectopic equator formation. Interestingly, we found that in addition to regulating DV patterning, *pnr* suppresses the eye fate by downregulating the core retinal determination genes *eyes absent (eya)*, *sine oculis (so)* and *dacshund (dac)* to define the dorsal eye margin. We found that *pnr* acts downstream of Ey and affect the retinal determination pathway by suppressing *eya*. Further analysis of the "eye suppression" function of *pnr* revealed that this function is likely mediated through suppression of the homeotic gene *teashirt (tsh)* and is independent of *homothorax (hth)*, a negative regulator of eye. Pnr expression is restricted to the peripodial membrane on the dorsal eye margin, which gives rise to head structures around the eye, and *pnr* is not expressed in the eye disc proper that forms the retina. Thus, *pnr* has dual function, during early developmental stages *pnr* is involved in axial patterning whereas later it promotes the head specific fate. These studies will help in understanding the developmental regulation of boundary formation of the eye field on the dorsal eye margin.
INTRODUCTION

Axial patterning is required for the transition of a single sheet of cells into a three-dimensional organ. Axial patterning involves generation of two different cell populations or compartments. Compartments are the fundamental units of patterning that are generated by localized expression of transcription factors called selectors. The selectors, when expressed in a group of cells, can confer compartment-specific properties to these cells (Curtiss et al., 2002; Mann and Carroll, 2002). Signaling between the cells of two compartments is crucial for the patterning, growth and differentiation of a developing field (Blair, 2001). The developing eye of the fruit fly, Drosophila melanogaster, has been extensively used to study patterning and growth. The compound eye of the adult fly develops from an epithelial bi-layer called the eye-antennal imaginal disc that is derived from the embryonic ectoderm (reviewed by Cohen, 1993, Held, 2002). The imaginal disc is a sac-like structure present inside the larva, which is the product of two different layers: the peripodial membrane (PM) and the disc proper (DP). The Drosophila retina develops from the DP while the PM of the eye-antennal imaginal disc contributes to the adult head structures (Milner et al., 1983; Haynie and Bryant, 1986; Atkins and Mardon, 2009). Morphogenesis during animal development involves signaling between different layers of the tissue (Furuta and Hogan, 1998; Obara-Ishihara et al., 1999). Similarly, signaling between the PM and the DP of the eye disc is essential for Dorso-ventral (DV) axis establishment and patterning (Cho et al., 2000; Gibson and Schubiger, 2000; Atkins and Mardon, 2009).

The adult eye is a highly precise hexagonal array of ~800 ommatidial clusters or unit eyes (Ready et al., 1976; Wolff and Ready, 1993). The ommatidia are arranged in two chiral forms, which are in a mirror image asymmetry along the DV midline called the equator. The
equator demarcates the boundary between the dorsal and the ventral eye, and is the site for upregulation of Notch (N) signaling, which triggers cell proliferation and differentiation (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Singh et al., 2005b). Although the mirror image asymmetry is generated during the third instar stage of larval eye development, the subdivision of the eye into dorsal and ventral compartments takes place even earlier by domain specific expression and function of DV patterning genes (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Cavodeassi et al., 1999; Maurel-Zaffran and Treisman, 2000; Singh et al., 2005b). In antenna, wing and leg imaginal discs, DV boundary formation takes place after the Antero-Posterior (AP) lineage restriction is generated (Blair, 2001; Garcia-Bellido and Santamaria, 1972; Milan and Cohen, 2003; Morata and Lawrence, 1975; Tabata et al., 1995). However, in the eye disc, the AP pattern is established dynamically in the third larval instar stage (after DV lineage is established) when the morphogenetic furrow (MF) is initiated (Singh et al., 2005b). The MF is a wave of retinal differentiation, which progresses anteriorly resulting in the transformation of undifferentiated retinal precursor cells (anterior to MF) into differentiated photoreceptor neurons (posterior to MF) of the eye (Ready et al., 1976; Wolff and Ready, 1993; Heberlein and Moses, 1995; Lee and Treisman, 2001). Therefore, the DV lineage, which is established at late first instar or early second instar larval stages, is the first lineage restriction event in the eye, and is crucial for the growth and differentiation of the eye (Singh and Choi, 2003; Singh et al., 2005b).

During genesis of eye, the entire early eye imaginal primordium initiates from the default ventral fate, which depends on the functions of Lobe (L) and Serrate (Ser) genes (Chern and Choi, 2002; Singh and Choi, 2003; Singh et al., 2005a, b; 2006). The onset of expression of the dorsal selector gene pnr, a member of the GATA-1 family of transcription
factors, at the dorsal margin of early second instar larval eye discs establishes the DV lineage in the eye (Maurel-Zaffran and Treisman, 2000; Singh and Choi, 2003; Singh et al., 2005a). It has been shown that \textit{pnr} (Maurel-Zaffran and Treisman, 2000) and members of the \textit{Iro-C} homeodomain genes \textit{viz., araucan (ara)}, \textit{caupolican (caup)} (Cavodeassi et al., 1999;) and \textit{mirror (mirr)} (Kehl et al., 1998; McNeil et al., 1997) are expressed in the dorsal region of the prospective eye (Dominguez and de Celis, 1998; McNeil et al., 1997) and act as the dorsal eye fate selectors. \textit{pnr}, the most upstream gene known in the dorsal eye gene hierarchy, regulates the expression of downstream \textit{Iro-C} genes through Wingless (Wg) signaling (Heberlein et al., 1998; Maurel-Zaffran and Treisman, 2000). Wg, which encodes a secreted protein, is expressed along the antero-lateral margins of the third instar eye-antennal imaginal disc (Baker, 1988), and prevents ectopic initiation of retinal differentiation from these positions (Ma and Moses, 1995; Treisman and Rubin, 1995). Wg signaling promotes the growth of cells in the eye-antennal disc and is sufficient to maintain cells in an undifferentiated state such that these cells continue to express anterior head specific markers (Lee and Treisman, 2001). In the dorsal eye, Wg promotes expression of \textit{Iro-C} genes during early eye development. The dorsal eye genes and the genes involved in ventral eye development act antagonistically to each other (Singh et al., 2005a, b). These genetic interactions define a signaling pathway that contributes toward the positioning of the equator (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Maurel-Zaffran and Treisman, 2000). Thus, \textit{pnr} is known to specify dorsal eye fate. However, the role of \textit{pnr} during retinal differentiation of the eye is not known. Thus, it is important to discern the role of DV patterning gene, \textit{pnr}, during later stages of eye development.

Interestingly, loss-of-function of DV patterning genes manifest defects in the eye growth and patterning but the mechanism by which the DV patterning genes contribute to
retinal determination is unknown. It is known that eye specification, and determination depends on a core of retinal determination (hereafter, RD) genes. These RD genes include PAX-6 homolog eyeless (ey), twin of eyeless (toy), eyes absent (eya), sine oculis (so) dachshund (dac), optix (opt), and eyegone (eyg) (Pappu & Mardon, 2004; Dominguez and Casares, 2005; Kumar, 2009). Ey is one of the early expressed gene which is required for eye field specification and is reported to induce expression ofeya and so to promote the eye growth and specification (for review Pappu and Mardon, 2004; Silver and Rebay, 2005; Kumar, 2009). The multiple feedback and cross regulatory interactions among RD genes lead to formation of the eye. Loss-of-function of RD genes results in the loss of eye field whereas ectopic expression of RD genes results in the induction of ectopic eyes (Halder et al., 1995; Pappu & Mardon, 2004; Silver and Rebay; 2005; Kumar, 2009). Several genes other than RD genes contribute towards eye development. A homeotic gene, tsh, which encodes a C2H2 Zinc finger transcription factor with three widely spaced Zinc finger domains (Fasano et al., 1991), has been suggested to act upstream of eya, so and dac during eye development (Pan and Rubin, 1998; Kumar, 2009). Interestingly, tsh also exhibits asymmetric DV response in the eye (Singh et al., 2002). Misexpression of tsh suppresses the eye fate in the ventral eye and promotes ectopic dorsal eye enlargement (Singh et al., 2002). It has been shown that tsh collaborates with the genes that express in a domain specific manner to exhibit DV asymmetric response in the developing eye disc (Singh et al., 2004). Interestingly, in the dorsal eye, the gain-of-function phenotype of teashirt (tsh) is similar to the loss-of-function phenotype of pnr. However, the mechanism of their interaction during eye development is not fully understood. In the ventral eye, tsh suppresses the eye by induction of a Meis class of homeotic gene, homothorax (hth) (Reichkof et al., 1997; Singh et al., 2002). Tsh has been shown to physically bind Hth anterior to the eye field (Bessa et al., 2002). Hth, is known to act
as a negative regulator of eye development. Loss-of-function of *hth* results in induction of the ventral eye or enlargement of the ventral eye domain (Pai et al., 1998). However, loss-of-function of *hth* in the dorsal eye has no effect even though *hth* is expressed in the dorsal eye (Pichaud & Casares, 2000; Jaw et al., 2000). Further, *tsh* does not affect *hth* expression in the dorsal eye whereas *tsh* acts upstream of *hth* in the ventral eye (Singh et al., 2002). Therefore, the mechanism of *hth* regulation in the dorsal eye remains unknown. Interestingly, the mechanism of genetic regulation of dorsal eye field growth is not very clear.

*pnr* plays an important role in the dorsal eye development. Onset of *pnr* expression in the dorsal eye margin is associated with DV lineage restriction (Maurel-Zaffran and Treisman, 2000; Cavodeassi et al., 2000; Singh et al., 2005b). The loss-of-function phenotypes of *pnr* result in ectopic eye enlargement of the dorsal eye. Here we report that in addition to its earlier reported role of dorsal selector during axial (DV) patterning, *pnr* plays an important role in defining the dorsal eye margin by regulating retinal determination. We have found that gain of function of *pnr* suppresses the retinal determination whereas the loss of *pnr* results in the ectopic induction of retinal determination genes. Our data suggests that *pnr* suppresses the retinal determination by downregulation of homeotic gene *tsh*, and is independent of *hth*. Interestingly, *pnr* is expressed only in the peripodial membrane and not in the disc proper, which gives rise to the retina. Thus, a late function of *pnr* is to block retinal determination in the peripodial membrane to define the dorsal eye margin.
MATERIALS AND METHODS

Stocks

Fly stocks used in this study are described in Flybase (http://flybase.bio.indiana.edu). We used y, w, eyFLP (Newsome et al., 2000), y, w; FRT82B pnr<sup>Vx6</sup>/CyO, (Heitzler et al. 1996), UAS-pnr<sup>D4</sup> (Haenlin et al. 1997), UAS-pnr<sup>ENR</sup> (Klinedinst and Bodmer 2003), FRT82D hth<sup>P2</sup> (Noro et al., 2006), UAS-ara (Gomez-Skarmeta et al. 1996), UAS-hth<sup>I2</sup> (Pai et al., 1998), UAS-hth-ENR (Inbal et al., 2001), y, w; tsh<sup>B</sup>/CyO (Fasano et al., 1991); UAS-tsh (Gallet et al., 1998), UAS-ds tsh (Bessa and Casares, 2005), y w; tsh<sup>A8</sup> (Sun et al., 1995); y, w; UAS-NLS-GFP<sup>S65T</sup> (Ito et al., 1997) UAS-wg (Azpiazu and Morata, 1998), pnr-Gal4, UAS-GFP (Singh et al., 2005a), ey-Gal4 (Hazelett et al., 1998), bi-Gal4 (Calleja et al., 1996). We have used Gal4/UAS system for targeted misexpression studies (Brand and Perrimon, 1993). All Gal4/UAS crosses were done at 18° C, 25° C and 29° C, unless specified, to sample different induction levels.

Genetic Mosaic Analysis

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 pnr in the eye, eyFLP; FRT82B Ubi-GFP females were crossed to y, w; FRT82B pnr<sup>Vx6</sup> males. Gain-of-function clones of pnr were generated using hs-FLP method where y, w, hsFLP<sup>122</sup>; P(Act>y<sup>+</sup>)<sup><sub>Gal4</sub></sup> 25 P(UAS-GFP<sup>S65T</sup>)/CyO (Struhl and Basler, 1993) flies were crossed to UAS-pnr<sup>D4</sup> flies.

Immunohistochemistry

Eye-antennal imaginal discs were dissected from wandering third instar larvae and stained following the standard protocol (Singh et al., 2002). Antibodies used were mouse and rabbit
anti-β galactosidase (1:200) (Cappel); chicken anti-GFP (1:200) (Upstate biotechnology), rat anti-Elav (1:100); mouse anti-WG (1:50) (Developmental Studies Hybridoma Bank); rabbit anti-Dlg (a gift from K. Cho), rabbit anti-Ey (a gift from Uwe Walldorf and Patrick Callaerts), anti-Hth (a gift from H. Sun and R. Mann) mouse anti-So (1:100), mouse anti-Dac (1:100), mouse anti-Eya (1:100) (Developmental Studies Hybridoma Bank), Rat anti-Tsh (1:50) (Gallet et al., 1998). Secondary antibodies (Jackson Laboratories) were goat anti-rat IgG conjugated with Cy5 (1:200), donkey anti-rabbit IgG conjugated to Cy3 (1:250), donkey anti-rabbit IgG conjugated to FITC, donkey anti-mouse IgG conjugated to Cy3 (1:200). Pnr expression was detected using pnr-Gal4> UAS-GFP (Pichaud and Casares, 2000; Singh and Choi, 2003; Singh et al., 2005a). Immunofluorescent images were analyzed using the Olympus Fluoview 1000 Laser Scanning Confocal Microscope.

**Scanning Electron Microscopy (SEM)**

The flies were prepared for scanning electron microscopy by dehydration through a series of increasing concentrations of acetone. Dehydrated flies were then stored in 1:1 mixture of acetone and Hexa Methyl Di Silazane (HMDS, Electron Microscopy Sciences), and then stored in 100% HMDS. The flies were allowed to air dry in HMDS. Dehydrated flies were mounted on a carbon conductive tape on EM stubs. Fly samples were coated with gold using a Denton vacuum sputter coater and analyzed using a Hitachi S-4800 High Resolution Scanning Electron Microscope (HRSEM).
RESULTS

Pnr expression is restricted to the peripodial membrane (PM) of the dorsal eye margin

In the *Drosophila* embryo, *pnr* is expressed in the dorsal most embryonic cells in a domain of presumptive notum surrounding the dorsal midline, and at the dorsal anterior margin of the eye disc (Heitzler et al., 1996; Ramain et al., 1993; Maurel-Zaffran and Treisman, 2000). Pnr is not expressed in the first instar larval eye-antennal imaginal disc (Fig. 1A). In the eye-antennal disc, *pnr* expression begins either in the late first instar stage or in the early second instar stage (Singh and Choi, 2003). In the early second instar eye-antennal imaginal disc, *pnr* expression begins in 5-7 cells in the dorsal margin of antenna and head region of the eye-antennal disc (Fig. 1A). During mid- to late- second instar of larval eye development, *pnr* begins to express in 30-35 cells (Fig. 1B). During the late second instar stage of larval eye development, *pnr* expression spreads to 80-100 cells in the dorsal eye domain (Fig. 1C). Interestingly, *pnr* expression is not seen in any disc proper cells, which differentiate to retinal photoreceptor cells in the eye (Fig. 1C’). Furthermore, *pnr* expression is restricted to the peripodial membrane on the dorsal eye margin (Pereira et al., 2006). Rarely a few disc proper cells (anterior to the morphogenetic furrow) at the border with peripodial membrane in the eye disc show *pnr* expression (Fig. 1C”). As the larva progresses into its third instar stage, *pnr* extends throughout the dorsal-most region of the head and antenna. The *pnr* expression in the third instar eye-antennal imaginal evolves into 4-5 rows of cells on the dorsal eye margin (Fig. 1D). Pnr expression overlaps with the MF but does not coincide with retinal cells as it is expressed only in the peripodial membrane.

Wg expression, which acts downstream to Pnr, is localized to the dorsal as well as the ventral eye margins in the disc proper cells (Fig. 1B-E). Wg is also expressed throughout all
larval stages in the PM (Cho et al., 2000). Wg is expressed in both the dorsal and ventral compartments, but expression in the dorsal is constant throughout all larval stages in the peripodial membrane. Wg is controlled by pnr in the peripodial membrane only; its disc proper expression is not influenced by any identified gene as of yet. In the first instar and early second instar eye disc, hth is expressed in the entire eye disc (Fig. 1B; Singh et al., 2002; Bessa et al., 2002). The expression of hth in the disc proper (DP) begins to retract in late second instar (Fig. 1C') whereas hth is expressed in the entire peripodial membrane (PM). In late third instar stage, hth expression stays anterior to the MF in the late third instar stage (Fig. 1D).

**Loss-of-function clones of pnr show four different phenotypes:**

We employed the genetic mosaic approach to generate loss-of-function clones of pnr in the developing eye-antennal imaginal disc (Xu and Rubin, 1993). We used the pnr<sup>yx6</sup> mutant, a null allele which has a deletion of all but 9 amino acids of the coding region (Ramain et al., 1993; Heitzler et al., 1996), to generate the genetic mosaic clones. Mutant clones were generated in the eye using the FLP/FRT system where Flippase is under the control of an eye-specific enhancer of eyeless, (ey), (Quiring et al., 1994). Since pnr is expressed in the dorsal eye, the loss-of-function clones of pnr that are located only in the dorsal eye margin exhibit phenotypes. The loss-of-function clonal phenotypes of pnr can be classified into four different categories:

**(I) Non-autonomous dorsal eye enlargement:** Loss-of-function clones of pnr in the dorsal eye result in an ectopic eye field or enlargement of the existing eye field comprising of differentiating photoreceptor neurons. These eye field enlargements or ectopic eye fields can even extend anterior to the furrow only on the dorsal eye margin (Fig. 2A; marked by white
dotted line). Further, these eye enlargement phenotypes are non-autonomous, which include both mutant cells (lack GFP reporter, marked by dotted boundary in Fig. 2A) as well as the adjoining wild-type cells (GFP positive). In the adult eyes, the *pnr* clones were marked by absence of the mini-*white* reporter gene, which is involved in the pigment uptake in the eye (Sun et al., 1995). These clones resulted in either enlargement of the pre-existing dorsal eye field or generation of a *de novo* ectopic eye field in the dorsal head cuticle (Fig. 2B; marked by black dotted line). These ectopic eye fields did not arise exclusively within the *pnr* mutant clones, but also contained a domain of the wild-type cells (marked by dark red pigment). The phenotype of these clones resembled the loss-of-function clone phenotypes of *pnr* described earlier (Maurel-Zaffran and Treisman, 2000; Singh and Choi, 2003). These phenotypes were explained to be due to generation of a *de novo* equator between the *pnr*− and *pnr*+ cells. The frequency of these clones is around ~8.3% of the total *pnr* loss-of-function clones (Table 1). Strikingly, we observe only the bigger size clones in this category.

(II) **Autonomous dorsal eye enlargement**: Loss-of-function clones of *pnr* in this category showed an ectopic field of differentiating photoreceptors anterior to the morphogenetic furrow in the dorsal eye domain. Unlike the clones of previous category, the ectopic eye field in these clones was autonomous (restricted within the *pnr* loss-of-function clones) (Fig. 2C, clonal boundary marked by white dotted line). In the adult flies, these clones resulted in the formation of an ectopic eye field in the dorsal head cuticle anterior to the eye field (Fig. 2D, black dotted line). The ectopic eye field in the clones of this category was devoid of any wild-type *pnr*+ cells, clearly suggesting that *pnr* loss-of-function led to the generation of ectopic eyes. The frequency of these clones was nearly 12.6%, which comprises of both the smaller (7.0%) as well as the bigger (5.6%) clones (Table 1). Some of these clones were accompanied by cuticle enlargement in the head.
(III) Absence of dorsal eye enlargement: Unlike the previous two categories of \textit{pnr} clones (Fig. 2A-D), loss-of-function clones in this category does not result in any ectopic dorsal eye enlargement (Fig. 2E). Interestingly, even though these clones span both the anterior as well as the posterior regions of the morphogenetic furrow in the dorsal eye-antennal imaginal disc but did not result in any ectopic eyes in the eye disc (Fig. 2E) as well as in adult flies (Fig. 2F). The frequency of these clones is nearly 75.3% (Table 1). We found that these clones unlike the clones from the previous two categories, are restricted only to the disc proper in the dorsal eye; a domain where \textit{pnr} is normally not expressed (Fig. 1).

(IV) Antennal duplication: Loss-of-function clones of \textit{pnr} in the antenna region of the eye-antennal imaginal disc, results in the duplication of antennal field (Fig. 2G). Interestingly, most of these antennal duplications were accompanied with ectopic eye enlargements (Fig. 2H). However, in some of these clones only antennal duplication were observed. These clones led to duplication of the ventral head structures such as antenna and maxillary palps in the dorsal head (Fig. 2H; Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh et al., 2005b). The frequency of these clones was ~3.6% (Table 1). Thus, our analysis of loss-of-function clones suggests that \textit{pnr} may be involved in the suppression of eye fate.

\textbf{Pnr suppresses the eye fate in the dorsal eye}

To test the role of \textit{pnr} in eye fate determination, we used the target system of Gal4/UAS to misexpress \textit{pnr} in the eye (Brand and Perrimon, 1993). We used a \textit{UAS-\textit{pnr}^{D4}} construct that behaves like wild-type \textit{pnr} in the absence of \textit{U-shaped} (\textit{ush}) function (Haenlin et al., 1997; Maurel-Zaffran and Treisman, 2000; Fossett et al., 2001; Singh & Choi, 2003). \textit{Ush} encodes a zinc finger protein that dimerizes with Pnr and acts as a negative regulator of \textit{pnr} transcriptional activity (Haenlin et al., 1997). Since \textit{ush} is not expressed in the eye-antennal
imaginal disc, the *UAS-pnr*\(^D4\) construct behaves in a wild-type fashion in the eye (Maurel-Zaffran and Treisman, 2000; Fossett et al., 2001). We used an *ey*-Gal4 driver that drives the expression of UAS-GFP transgene in the entire eye disc, which comprises of differentiating retinal neurons (posterior to MF, marked by white arrowhead) as well as the region forming the prospective head cuticle (anterior to MF) (Fig. 3A; Singh et al., 2005a). Misexpression of *pnr* in the entire eye disc using *ey*-Gal4 (*ey>*pnr\(^D4\)) results in the complete loss of eye field as evident from the absence of neuronal marker Elav whereas the size of antennal field is not affected (Fig. 3C). The misexpression of *pnr* in the entire eye (*ey>*pnr\(^D4\)) results in the adult flies with highly reduced eye field or what we refer to as the "no-eye" phenotypes (Fig. 3D) as compared to the wild-type eyes (Fig.3B). To test if there is any domain specific response of *pnr* misexpression, we employed *bi*-Gal4 driver, which drives the expression of UAS- GFP transgene (*bi>*GFP) on both dorsal and ventral margins of the developing eye-antennal imaginal disc (Fig 3E; Calleja et al., 1996; Singh et al., 2002; 2004). Misexpression of *pnr* using *bi*-Gal4 (*bi>*pnr\(^D4\)) suppressed the eye fate on both dorsal and ventral eye margins as evident from the absence of Elav expression (Fig. 3F; white arrows). This suggests that *pnr* upon misexpression suppresses the eye fate, irrespective of dorsal or ventral domains.

Random gain-of-function clones of *pnr* in the eye using UAS-*pnr*\(^D4\) (marked by GFP reporter) caused suppression of photoreceptors as evident from the absence of Elav positive cells in the eye disc (Fig.3G, G’) as well as in the adult eye (Fig. 3H). In addition to the small eye phenotypes seen in the gain-of-function clone of *pnr*, we observed necrosis as evident from presence of dark spots in the adult eye (Fig. 3H). These results suggest *pnr* can suppress the eye fate. The frequency of *pnr* gain-of-function clones was extremely low in the eye disc as well as the adults probably due to issues with cell survival.
We then disrupted Pnr function using dominant negative pnr (UAS-pnr\textsuperscript{ENR}) (Fu et al., 1998; Klinedinst and Bodmer, 2003) where the construct contains the repressor domain from the Engrailed transcription factor (EnR, amino acid 2-298) (Jaynes and O’Farrell, 1991) and the two N-terminal zinc-finger domains from pnr (amino acids 153-293) (Ramain et al., 1993). Disrupting pnr function in the entire eye-antennal imaginal disc all along during eye development by misexpression of pnr\textsuperscript{ENR} (ey> pnr\textsuperscript{ENR}) resulted in a small group of Elav positive retinal cells in the eye field (Fig. 3G), and a highly reduced eye in the adult fly (Fig. 3H). However, there was no affect on the developing antennal field (Fig. 3G, H). This suggests that during early eye development when DV patterning is being established pnr function is crucial for eye development. However, misexpression of pnr\textsuperscript{ENR} on both dorsal and ventral eye margins using bi-Gal4 (bi>pnr\textsuperscript{ENR}) caused enlargement of only the dorsal eye (Fig. 3H, arrow). In bi>pnr\textsuperscript{ENR} background, pnr function was abolished only in a few subset of cells within the endogenous pnr expression domain in the peripodial membrane of the dorsal eye. These dorsal eye enlargement phenotypes further substantiated the possibility that pnr may suppress retinal determination in the dorsal eye margin.

**Pnr downregulates the retinal determination genes to suppress the eye**

To address the role of pnr in retinal determination, we checked the expression of members of the retinal determination (RD) gene pathway in the loss-of-function clones of pnr in eye-antennal imaginal disc. The loss-of-function clones of pnr (marked by absence of the GFP reporter) that exhibit enlargement of the dorsal eye showed no ectopic induction of Ey (Fig. 4A, A', A“). It has been shown that Ey is expressed in undifferentiated retinal precursor cells early in eye development and after the onset of photoreceptor differentiation; Ey continues to express in the undifferentiated retinal precursor cells anterior to the MF (Quiring et al., 1994;
Lee and Treisman, 2001; Singh et al., 2002; Bessa et al., 2002). Although Ey expression was not affected, the loss-of-function clones of \textit{pnr} which caused ectopic dorsal eye enlargements showed ectopic induction of Eya (Fig. 4B, B’, B’’), which acts downstream to Ey. The loss-of-function clones of \textit{pnr} in the ventral domain of the eye did not exhibit any affect on wild-type Eya expression. During the late first instar stage, \textit{eya} begins expression in the eye region of the disc. A short time after \textit{eya} expression, \textit{so}, \textit{dac}, and \textit{eyg} are expressed in the late second instar stage in the region posterior to the MF (Bonini et al. 1993; Cheyette et al. 1994; Jang et al. 2003; Kenyon et al., 2003; Mardon et al., 1994). After the MF begins expression of \textit{eya} and \textit{so} are restricted to the area within and posterior to the MF (Bonini et al., 1993; Cheyette et al., 1994). The expression of \textit{dac} is restricted to the MF in the area that directly precedes the MF and continues in R1, R6 and R7 for a few columns posterior to the MF and sharply disappears after that domain (Mardon et al., 1994; Tavsanli et al, 2004). The loss-of-function clones of \textit{pnr} in the dorsal eye showed ectopic induction of So (Fig. 4C, C’, C’’; white arrows) and Dac (Fig. 4D, D’, D’’; white arrows). Dac is expressed downstream to \textit{eya} and \textit{so} (Chen et al., 1997). The ventral eye clones did not exhibit any effect on the expression of RD genes. These results suggest that \textit{ey}, a gene expressed in undifferentiated cells, is not induced in ectopic dorsal eye enlargement whereas the downstream RD genes like \textit{eya}, \textit{so}, \textit{dac} that are expressed in differentiating photoreceptor neurons are upregulated in the \textit{pnr} loss-of-function clones. The \textit{pnr} loss-of-function clones which only result in antennal duplications does not affect RD genes expression (data not shown). These results suggest that \textit{pnr} may act downstream of \textit{ey} to suppress retinal determination on the dorsal eye margin. Furthermore, since \textit{pnr} is expressed in the peripodial membrane on the dorsal eye margin, these loss-of-function clonal phenotypes of \textit{pnr} suggests that \textit{pnr} blocks retinal determination in the
peripodial membrane of the dorsal eye margin. Thus, \textit{pnr} may promote head specific fate by blocking retinal determination in the peripodial membrane.

We tested this hypothesis by checking RD gene expression in \textit{bi>pnr}^{D4} background where \textit{pnr} misexpression suppresses the eye both on dorsal and ventral eye margins (Fig. 3F). Interestingly, \textit{Ey} was present on both dorsal and ventral margins (Fig. 4 E, E'; white arrowheads). However, the expression of other RD genes like \textit{Eya} (Fig. 4 F, F'; white arrowheads), \textit{So} (Fig. 4 G, G'; white arrowheads), and \textit{Dac} (Fig. 4 I, I'; white arrowheads) was down regulated on both the dorsal as well as the ventral eye margins of highly reduced eye-antennal imaginal disc. \textit{Dac} expression anterior to the MF is not affected (Fig. 4 I, I'). These results strongly suggest that \textit{pnr} suppresses retinal determination by blocking \textit{Eya}, \textit{So}, and \textit{Dac} expression.

\textbf{Pnr suppresses eye by induction of its downstream target Wg.}

In order to test if, the misexpressed \textit{pnr} is functional in the eye disc; we tested the levels of Wg as a functional read out of \textit{pnr} in the eye. \textit{Pnr} acts upstream to the signaling molecule Wg, that suppresses the eye fate (Ma and Moses, 1995; Treisman and Rubin, 1995; Lee and Treisman, 2001). Wg is expressed on the antero-lateral margin of both dorsal and ventral eye (Fig. 1D). Misexpression of \textit{pnr} in the eye disc (\textit{ey>pnr}^{D4}) results in the suppression of eye (Fig. 3B). We found that misexpression of \textit{pnr} on both the dorsal and the ventral margin (\textit{bi>pnr}^{D4}) of eye disc results in robust induction of Wg along with a strong suppression of the eye on both the dorsal as well as the ventral margins (Fig. 5A, A'). \textit{bi>pnr}^{D4} showed similar eye suppression phenotypes on both the dorsal and ventral eye margins in the adult eye (Fig. 5B). This phenotype is similar to the misexpression of Wg on both dorsal and ventral margins (\textit{bi>wg}) that results in suppression of the eye on both dorsal as well as ventral
Targeted misexpression of \( pnr \) using (\( ey>pnr^{D4} \)) results in a “no-eye” phenotype by induction of Wg in the entire eye (Fig. 5C). These phenotypes are comparable to the ectopic induction of Wg in the eye (\( ey>wg \)) (data not shown, Singh et al., 2002). Interestingly, the ectopic eye induction and Wg downregulation did not cover the entire loss-of-function clone of \( pnr \) in the dorsal eye (Fig. 5D, marked by the white dotted line). But the Wg expression was present within the clone juxtaposed to the wild-type \( wg \) expression domain in the head region (Fig. 5D). This Wg expression phenotype can be explained as rescue of Wg within clone from the wild-type cells due to the secretory nature of Wg. The loss-of-function clones of \( pnr \) in the disc proper (DP) did not result in ectopic eye enlargements and showed no affect on Wg expression (Fig. 5F, F’). In some of the larger loss-of-function clones of \( pnr \), which extend from the dorsal eye margin into the antennal field, we observed a surprising result where the entire clone did not show ectopic eye enlargement. The area of the clone, which did not have eye enlargement, showed strong Wg expression even though the expression domain of this Wg was not close to wild-type expression domain of Wg. This result suggests that \( pnr \) is not the sole regulator of \( wg \) expression in the dorsal eye. Since \( wg \) acts in a feedback loop with \( hth \) in the ventral eye (Pichaud and Casares, 2000; Singh et al., 2002), there is a possibility of the role of \( hth \) in \( wg \) regulation in the dorsal eye.

**Pnr eye suppression function in the dorsal eye margin is independent of \( hth \)**

In order to understand the mechanism by which \( pnr \) suppresses the eye fate, we tested whether loss of \( pnr \) function induces \( hth \). Misexpression of \( hth \) on both dorsal and ventral eye margins (\( bi>hth \)) results in suppression of the eye on both dorsal and ventral eye margins (Fig. 6A, A’, arrows) as also seen in \( bi>pnr^{D4} \) eye-antennal discs (Fig. 3F). Therefore, we
tested levels of Hth in bi\(\text{>pnr}^{D4}\) eye-antennal disc. Interestingly, Hth was induced both on the dorsal as well as the ventral eye margins (Fig. 6B, B', arrows). This raises the possibility that \textit{pnr} may suppress the eye development by inducing downstream \textit{hth}. Therefore, if \textit{hth} is downstream to \textit{pnr} in the dorsal eye, then loss-of-function of \textit{hth} must be similar to \textit{pnr} loss-of-function phenotypes. We generated loss-of-function clones of \textit{hth} in the eye disc. We found that the loss-of-function clones of \textit{hth} induced at any time during larval development autonomously induced ectopic eyes only in the ventral head capsule (Fig. 6C, C'; marked by white dotted line, Pai et al., 1998; Pichaud and Casares, 2000). However, \textit{hth} loss-of-function clones did not show any ectopic dorsal eye enlargements as seen in the \textit{pnr} loss-of-function clones (Fig. 2). Thus, unlike gain-of-function of \textit{hth} that corresponds to the gain-of-function of \textit{pnr} (Fig. 3), the loss-of-function of \textit{hth} does not match \textit{pnr}- loss-of-function clonal phenotypes. This result rules out the possibility of \textit{pnr} acting upstream of \textit{hth} in the dorsal eye. We studied the expression of Hth in the \textit{pnr} loss-of-function clones and found that Hth expression was not affected in loss-of-function clones of \textit{pnr} showing ectopic eye enlargements (Fig. 6D, D'). Hth marks the undifferentiated retinal precursor cells anterior to the furrow (Fig. 1; Pai et al., 1998; Bessa et al., 2002). In \textit{pnr} loss-of-function clones, where no ectopic eye enlargements were seen, Hth expression was not affected (Fig. 6E, E'). Interestingly, Hth expression was induced in \textit{pnr} loss-of-function clones where the duplication of antennal region took place (Fig. 6F, F'). The duplication of antennal field represents the ventral structures in head capsule (Casares and Mann, 1998). Since \textit{hth} is expressed in the proximal domains of antennal field there is ectopic induction of \textit{hth} when duplication of the antennal field and cuticle enlargement occurs. Thus, our results suggest that \textit{pnr} does not directly affect the \textit{hth} expression in the dorsal eye.

\textbf{Pnr suppresses the eye by downregulating tsh}
We employed a candidate gene approach and looked for the genes which might affect the dorsal eye patterning. Homeotic gene teashirt (tsh) shows an asymmetric response on the dorsal and ventral eye margins. In the early eye, tsh is expressed in the entire disc (Singh et al., 2002; Bessa et al., 2002). In the second instar eye-antennal imaginal disc, Tsh expression begins to retract anteriorly (Fig. 7A), and in the third instar eye disc Tsh is expressed in the retinal precursor cells anterior to the MF (Fig. 7A, B; Bessa et al., 2002; Singh et al., 2002). We tested expression of tsh in the loss-of-function clones of pnr in the dorsal eye. We found that pnr loss-of-function clones, which exhibit dorsal eye enlargement also exhibit ectopic induction of Tsh (Fig. 7C, C’) as well as the tsh reporter y, w; tshA8 (Fig. 7C, C”). It suggests that pnr might suppress tsh expression at the transcription level. In order to test whether pnr suppresses the eye by downregulating tsh, we generated pnr loss-of-function clones where tsh levels were reduced to 50% using a heterozygous combination of tsh8, a null allele of tsh (Fasano et al., 1991). Interestingly, in these pnr loss-of-function clones where tsh function was reduced to half, we did not see any ectopic eye enlargement (Fig. 7D). Interestingly, although the dorsal clones did induce overgrowths/ enlargement, these clones did not show any ectopic Elav expression. However, these clones exhibited strong Ey expression (Fig. 7 D’, D”). The adult eye phenotype of these clones is similar to the eye disc phenotype of lack of any dorsal eye enlargements (Fig. 7E). To further test our hypothesis that pnr affects tsh expression at the transcription level, we misexpressed pnr on the dorsal and ventral eye margins (bi>pnrD4) and checked the expression of the tsh reporter. We found that tsh expression was downregulated on both the dorsal and ventral eye margins (Fig. 7 F, arrows). Since endogenous expression of pnr is restricted to the dorsal eye margin, our results suggest that pnr suppresses the tsh expression in the dorsal eye. We further tested pnr and tsh interaction, by misexpressing pnr on both dorsal and ventral eye margins.
in a \( tsh^{g/+} \) heterozygous background (\( bi>pnr^{D4}; tsh^{g/+} \)). In this \( tsh \) heterozygous background, misexpression of \( pnr \) strongly enhances the eye suppression phenotype on both the dorsal and the ventral eye margins (Fig. 7G) as compared to \( bi>pnr^{D4} \) alone (Fig. 7F). These results suggest that \( pnr \) may suppress \( tsh \) at the dorsal eye margin. Misexpression of \( tsh \) on the dorsal and the ventral eye margin (\( bi>tsh \)) results in dorsal eye enlargement and ventral eye suppression (Fig. 7H; Singh et al., 2002). This phenotype is complementary to the \( pnr \) loss-of-function phenotype. Misexpression of \( tsh \) RNAi using \( bi \)-Gal4 results in complementary to \( bi>tsh \) phenotype in the dorsal eye (data not shown). We therefore tested whether misexpression of \( tsh \) can rescue the \( pnr \) misexpression phenotype. Misexpression of both \( pnr \) and \( tsh \) (\( bi>tsh+pnr^{D4} \)) resulted in the lethality as early as the first instar larval stage. We therefore, misexpressed \( ara \), a downstream target of \( pnr \) and a member of Iro-C complex, with \( tsh \) on the dorsal and ventral eye margins (\( bi>tsh+ara \)), which resulted in the enlargement of the eye on the dorsal eye margin (Fig. 7C; Singh et al., 2004). We further tested the hypothesis that \( pnr \) down regulates \( tsh \) in the dorsal eye to suppress eye fate. Misexpression of \( tsh \) in the dorsal eye margin using a \( pnr \)-Gal4 driver (\( pnr>tsh \)) resulted in the enlargement of eye on the dorsal margin (Fig. 7J). Lastly, we tested whether reducing \( pnr \) levels affects the \( bi>tsh \) phenotype. In heterozygous \( pnr \) background we misexpressed \( tsh \) (\( bi>tsh; pnr^{ax6}/+ \)) and found that it results in the dorsal eye enlargements (Fig. 7K). These eye enlargements were similar to that of the \( bi>tsh \) alone (Fig. 7H). This suggests that \( tsh \) acts downstream to \( pnr \) and therefore levels of \( tsh \) are crucial for the dorsal eye enlargement phenotype. Thus, \( pnr \) suppresses the eye development on the dorsal eye margin by suppressing \( tsh \).
DISCUSSION

We have addressed a basic question pertaining to regulation of patterning, growth and differentiation of the developing eye field. Our results provide an important insight into the role of *pnr*, a gene known to confer dorsal eye identity during axial patterning of the eye (Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh and Choi, 2003). We and others have shown that the onset of *pnr* expression during early eye development results in the generation of dorsal lineage in the eye. It results in the formation of a DV boundary (equator), which triggers N signaling at the border of the dorsal and ventral compartments to initiate growth and differentiation (Cho and Choi, 1998; Dominguez and deCelis, 1998, Papayannopoulous et al., 1998; Singh et al., 2005b).

Earlier, we have tested the spatial as well as temporal requirement for the genes controlling ventral eye growth and development (Singh and Choi, 2003). During early eye development, prior to the onset of *pnr* expression in the dorsal eye, entire early eye primordium is ventral in fate (Singh and Choi, 2003). Removal of function of genes controlling ventral eye development prior to the onset of *pnr* expression, result in complete elimination of the eye field whereas later when *pnr* starts expressing, the eye suppression phenotype gets restricted only to the ventral eye (Singh and Choi, 2003; Singh et al., 2005a). These studies suggested that *pnr* plays an important role in dorso-ventral (axial) patterning. However, the role of dorsal selector *pnr* in retinal determination was unknown.

**Pnr suppresses the eye fate**

Loss-of-function clones of *pnr* in the dorsal eye exhibit eye enlargement (Fig. 2 A; Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh and Choi, 2003). It was
suggested that when \( pnr \) function was abolished in the dorsal eye using loss-of-function clones, it results in the change of dorsal eye fate to ventral. This results in generation of a \textit{de novo} equator, the border between dorsal and ventral half of the eye, which triggers ectopic N signaling to promote growth and cell proliferation. The same premise was used to explain the gain-of-function phenotype of \( pnr \) in the eye. Misexpression of \( pnr \) in the entire eye (\( ey>pnr^{D4} \)) generates a completely dorsalized eye field as \( pnr \) acts as the dorsal fate selector. The fully dorsalized eye lack DV polarity (equator), which results in the complete loss of eye field due to lack of N upregulation (Fig. 3C, D; Maurel-Zaffran and Treisman, 2000). Here, we addressed another possibility to see if \( pnr \) suppresses the eye fate upon misexpression in the entire eye as evident from the 'no-eye" phenotype.

To test if \( pnr \) suppresses the eye fate, we misexpressed \( pnr \) both in the dorsal and ventral (DV) eye margins of the eye using a \( bi\)-Gal4 driver (\( bi>pnr^{D4} \)) (Fig. 3F). The rationale was if \( pnr \) is only required to assign the dorsal eye fate, in that case \( pnr \) misexpression (\( bi>pnr^{D4} \)) will assign a dorsal fate on the margin of ventral eye. Thus, by this logic, it would result in generation of a \textit{de novo} equator on the ventral eye margin, which should manifest as eye enlargements in the ventral eye. The argument was based on the similar premise that was employed to explain that loss-of-function clones of \( pnr \) in the dorsal eye generated a new equator and led to the dorsal eye enlargement. We did not observe any ventral eye enlargements in \( bi>pnr^{D4} \) eye disc (Fig. 3F). Instead we saw suppression of the eye on both the dorsal as well as ventral margins (Fig. 3F). The suppression of eye fate on both the dorsal and the ventral margins suggests that \( pnr \), irrespective of the domain where it is expressed, can suppress the eye fate.

\textbf{Pnr suppresses the Retinal Determination (RD) genes function}
Since pnr suppresses the eye fate, it is possible that it may be involved in regulation of expression of genes of the core retinal determination machinery. Loss-of-function of pnr in the dorsal eye clones results in the eye enlargements as evident from Elav positive cells but it does not induce ectopic Ey (Fig. 4A). Ey expression evolves during eye development and is localized anterior to the morphogenetic furrow in retinal precursor cells and is downregulated and degraded posterior to the furrow in differentiation retinal neurons (Quiring et al., 1994; Halder et al., 1995; 1998; Baonza and Freeman, 2002; Kango-Singh et al., 2003; Lee and Treisman, 2001). We found that in the loss-of-function clones of pnr in the eye, the expression of retinal determination pathway members like Eya, So and Dac, which act downstream to Ey, was ectopically induced (Fig. 4B-D). In the converse situation, where pnr was misexpressed on both dorsal and ventral eye margins (bi>pnr\textsuperscript{D4}), we observed ectopic induction of Ey on both the dorsal and the ventral margins (Fig. 4E) whereas the expression of Eya (Fig. 4F), So (Fig. 4G) and Dac (Fig. 4H) were suppressed. Thus, misexpression of pnr in the eye prevents the photoreceptor differentiation irrespective of the dorsal or ventral domain. Based on these results we can propose that pnr suppresses the eye fate on the dorsal eye margin by downregulating RD genes like eya, so and dac (Fig. 8).

Since ey is responsible for the specification of the eye field and marks the retinal precursor cells, it suggests that pnr does not affect the eye field formation or specification. In fact pnr affects expression of RD genes eya, so and dac (Fig. 4), which acts downstream to ey, and are involved in retinal determination (Kango-Singh et al., 2003; Pappu and Mardon, 2004; Silver and Rebay, 2005; Kumar, 2009). Our results suggest that pnr suppress retinal determination genes. Since endogenous expression of pnr is restricted to the peripodial membrane of the dorsal eye margin (Fig.1), it suggests that pnr may be involved in suppression of retinal determination on the dorsal peripodial membrane. Thus, our results
suggest that \textit{pnr} generally acts at the stage when photoreceptor differentiation is initiated with the formation of the morphogenetic furrow (MF) and promotes the dorsal head cuticle fate by suppressing retinal differentiation (Fig. 8).

**Dual function of \textit{pnr} during eye development**

Based on our new findings and other previously published results, we propose that \textit{pnr} may be required for two different functions during eye development: (1) axis determination during DV patterning and (2) suppression of the retinal determination process to define the dorsal eye field margin. These functions of \textit{pnr} appear to be temporally controlled as DV axis determination takes place in late first- or early second- instar of eye development (Singh et al., 2003; Singh et al., 2005b) while suppression of the eye fate is evident in late second instar of larval development.

The axis determination function of \textit{pnr} is required in the earlier time window. This is further validated by the loss-of-function clones of \textit{pnr} of first category, which are bigger and exhibits non-autonomous dorsal eye enlargement phenotypes (Fig. 2A, B). These dorsal eye enlargements that are spanning both wild-type and \textit{pnr} mutant cells in the eye disc conforms to the notion that when \textit{pnr} is lost in a group of cells during early development, it fails to confer dorsal identity over the default ventral state. As a consequence de novo equator is generated which results in the ectopic dorsal eye enlargements (Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh et al., 2005b). Since these clones are always bigger (Table 1), suggesting that they might be formed earlier. The late function of \textit{pnr} in suppression of retinal determination is validated both by the gain-of-function studies (Fig. 3) as well as the loss-of-function clones of second category, which are both bigger as well as smaller in size and are autonomous in nature (Fig. 2C, D; Table 1). These clones
have ectopic dorsal eyes, which are restricted within the clones, thereby suggesting that absence of *pnr* function promotes ectopic eye formation in the dorsal eye margin. Thus, during the early second instar of development, before the onset of retinal differentiation, *pnr* is required for defining the dorsal lineage by inducing Wg and members of the Iro-C complex (Maurel-Zaffran and Treisman, 2000; Singh et al., 2005b). However, later during the late second- instar stage of eye development, when the morphogenetic furrow (MF) is initiated, *pnr* suppresses the photoreceptor differentiation at the dorsal eye margin. The endogenous expression of *pnr* in only the peripodial membrane of the dorsal eye margin further confirms this notion (Fig. 1; Pereira et al., 2006). Lack of phenotypes in *pnr* clones which are restricted to the disc proper (DP) alone verifies *pnr* localization (Fig. 2 E, F). Thus, *pnr* defines the boundary between the eye field and the head cuticle on the dorsal margin. An interesting question will be to identify which gene is responsible for defining the ventral eye margin. In the ventral eye where *pnr* is not expressed, *hth* is known to suppress the eye fate (Pai et al., 1998; Pichaud and Casares, 2000). There is a strong possibility that *hth* may be involved in defining the boundary of eye field on the ventral eye margin between the disc proper and peripodial membrane.

**Pnr induces Wg to suppress the eye development independent of hth**

Since *pnr* induces Wg, it is expected that *pnr* may suppress the eye by induction of Wg (Fig. 8). Even though Wg signaling is responsible for suppression of photoreceptor differentiation on both dorsal and ventral eye margins (Ma and Moses, 1995; Treisman and Rubin, 1995; Lee and Treisman, 2001; Baonza and Freeman, 2002), its regulation is different on both dorsal and ventral eye margins (Pichaud and Casares, 2000; Maurel-Zaffran and Treisman, 2000). In the ventral eye, *wg* is involved in a feedback loop with *hth* to suppress the eye fate
In the dorsal eye, Pnr induces Wg signaling, which in turn induces the members of Iro-C complex, and ultimately these signaling interactions define the dorsal eye fate. Interestingly, it seems Pnr is not the sole regulator of Wg in the dorsal eye (Fig. 5). Since loss-of-function of \( hth \) does not exhibit the phenotypes similar to the loss-of-function of \( pnr \) (Fig. 6) or \( wg \) (Treisman and Rubin, 1995; Ma and Moses, 1995), it is expected that the positive feedback loop regulation of \( wg \) and \( hth \) as seen in the ventral eye margin does not hold true on the dorsal eye margin. We found that \( hth \) is not affected in the \( pnr \) clones that exhibit dorsal eye enlargements. Furthermore, when we made clones of \( hth \) in \( pnr \) heterozygous condition, we did not see any dorsal eye enlargements suggesting that \( hth \) and \( pnr \) do not interact. Thus, like others, our results also verified that \( hth \) is not involved in \( pnr \) mediated eye suppression on the dorsal eye margin (Fig. 8; Pichaud and Casares, 2000).

**Eye suppression function of \( pnr \) is mediated through the suppression of \( tsh \)**

It is known that the gain of function of \( tsh \) in the dorsal eye results in ectopic eye enlargement whereas gain of function of \( tsh \) in the ventral eye result in suppression of ventral eye (Singh et al., 2002; 2004). The dorsal eye enlargements seen in \( tsh \) gain-of-function is a phenotype similar to \( pnr \) loss-of-function in the dorsal eye. In \( pnr \) loss-of-function clones, we found that \( tsh \) was ectopically induced (Fig. 7C). Furthermore, when \( pnr \) loss-of-function clones were generated in a heterozygous background of the \( tsh \) null allele \( tsh^8/CyO \) (Fasano et al., 1991), the dorsal eye enlargement phenotype was dramatically suppressed and no longer observed. Interestingly, we found that dorsal enlargements were there but were not accompanied with ectopic eyes as evident from absence of Elav expression (Fig. 7D). All these dorsal enlargements were showing strong Ey expression. Among 500 flies counted we found only
two flies that showed subtle dorsal eye enlargements. Interestingly, we also found that in \textit{pnr} loss-of-function clones the mini-\textit{white} reporter gene under \textit{tsh} was ectopically induced (data not shown). The loss-of-function phenotypes of \textit{pnr} were more pronounced when \textit{tsh} was misexpressed in the clones. Thus, \textit{pnr} expressed in the dorsal peripodial membrane may suppress \textit{tsh} in the dorsal eye to suppress the eye fate (Fig. 8). Interestingly, \textit{tsh} is known to act upstream of \textit{eya}, so and \textit{dac} (Pan and Rubin, 1998). Thus, the dorsal eye enlargement observed in the \textit{pnr} mutant is due to ectopic induction of \textit{tsh} in the dorsal eye, which in turn can induce the RD genes (Fig. 8).

**Functional conservation of dorsal selector Pnr**

The \textit{Drosophila} eye is similar to the vertebrate eye in several features (Sanes and Zipursky, 2010) like: (i) the morphogenetic furrow in the fly eye is analogous to the wave of neurogenesis in the vertebrate eye (Neumann and Nuesslein-Volhard, 2000; Hartenstein and Reh, 2002), (ii) Like \textit{Drosophila}, in higher vertebrates dorsal eye genes like \textit{Bmp4} and \textit{Tbx5} act as `dorsal selectors' and restrict the expression of ventral eye genes \textit{Vax2} and \textit{Pax2} (Koshiba-Takeuchi et al., 2000; Peters and Cepko, 2002). These DV expression domains or developmental compartments (Peters, 2002) lead to formation of DV lineage restriction as seen in the \textit{Drosophila} eye (Singh and Choi, 2003; Singh et al., 2005a), (iii) The DV lineage in the vertebrate eye also develops from a ventral-equivalent initial state (for review see Singh et al., 2005b). The dorsal genes \textit{pnr} and \textit{iro-C} are highly conserved across the species, and are involved in organogenesis and neural development (Gomez-Skarmaeta and Modolell, 2002; Singh et al., 2005b). Therefore, it would be interesting to see whether the dorsal selectors in the vertebrate eye play a role in defining the boundary of the eye by suppressing retinal differentiation.
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FIGURE LEGEND

Fig.1. Pnr expression is restricted to the peripodial membrane (PM) of the dorsal eye margin. (A) $pnr$ expression ($pnr$ Gal4 drive UAS-GFP, Singh and Choi, 2003; Singh et al., 2005) is absent in the first instar eye-antennal imaginal disc whereas Wg (red) is expressed in the entire eye disc. Note that $pnr$ expression in the brain at this stage is seen. (B) In the second instar eye-antennal imaginal disc, $pnr$ expression (green) is initiated in 15-20 cells on the dorsal eye margin and Wg (red) is expressed laterally on both dorsal and ventral eye margins. At this stage, Hth (blue) expression is present in the entire eye disc. (C, C',C") In the early third instar eye-antennal disc, $pnr$ expression in the dorsal eye margin is restricted only to the peripodial membrane (PM) whereas Hth (blue) is also expressed in peripodial membrane (PM) of the eye-antennal disc. (C') $pnr$ (green) expression at this stage is absent in the disc proper (DP). Hth (blue) expression begins to retract with the initiation of MF and stays anterior to the furrow (Pai et al., 1998; Bessa et al., 2002; Singh et al., 2002). (C") Pnr expression is restricted to the peripodial membrane (PM) specific cells on the dorsal eye margin. (D) In the late third instar eye-antennal imaginal disc, $pnr$ (green) expression is restricted to the dorsal eye margin whereas Wg (red) expression is restricted to the dorsal and ventral eye margins. Hth (Blue) is expressed in rings in the proximal region of antenna and expressed both in the dorsal and ventral part of the disc proper anterior to the furrow. Dashed lines indicate the approximate midline, the border between D (Dorsal) and V (ventral) eye. All the eye-antennal imaginal discs and the adult eyes are organized as Dorsal (D) up and the ventral (V) down. Markers for immunostaining are shown in color labels. (AN: Antenna)
Fig.2. Loss-of-function of *pnr* exhibits a range of eye enlargements and antennal duplications in the dorsal eye. (A, B) Loss-of-function clones of *pnr* in the dorsal eye margin {marked by the absence of GFP (green) in the eye-antennal imaginal disc and absence of the mini-white reporter (red) in the adult eye} results in a non-autonomous ectopic eye enlargement as seen in the eye-antennal imaginal disc and in the adult eye. The ectopic eye enlargements are not restricted within the clone. However, they extend both in the wild-type as well as in the *pnr* mutant cells of the eye-antennal disc. Note that the dorsal clone boundary is marked by white dotted line in the eye disc and by black dotted line in the adult eye. (C, D) Loss-of-function of *pnr* in the dorsal eye results in an autonomous ectopic dorsal eye anterior to the normal eye field. These ectopic eyes are restricted to within the clones. Note that not all the cells of the *pnr* loss-of-function clone differentiate to the photoreceptors. (E, F) Loss-of-function clones of *pnr* in the dorsal eye have no effect on the eye field as seen in the eye disc and the adult eye. All these clones were restricted to the disc proper. (G, H) Loss-of-function clones of *pnr* in the antenna results in duplication of the antennal field as seen in (G) the eye-antennal disc and (H) the adult head. (H) Scanning electron microscopy (SEM) of the adult head showing antennal duplication and dorsal eye enlargement (Magnification X180). Note that only a few *pnr* loss-of-function clones show both dorsal eye enlargements along with the antennal duplication.

Fig.3. *Pnr* suppresses the eye fate. (A) Eye-antennal imaginal disc showing domain of expression of GFP reporter (green) under the *ey* Gal4 (*ey>GFP*). Note that the *ey* Gal4 drives the expression of GFP reporter in the entire eye-antennal imaginal disc (both anterior as well as posterior to the morphogenetic furrow (MF) marked by white arrowhead). (B) Wild-type adult eye. (C, D) Misexpression of *pnr* in the eye using the *ey*-Gal4 driver (*ey>pnr^{D4}* ) results...
in the suppression of eye fate and leads to a “no-eye” phenotype in the (C) eye-antennal disc (Elav, a pan-neural marker, which marks the photoreceptors) as well as in the (D) adult eye. The white dotted line in 3C marks the possible outline of the eye disc. There is no effect on the antennal field both in the eye-antennal imaginal disc as well as the adult head. (E) Another Gal4 driver, bi-Gal4 drives expression of a GFP reporter (bi>GFP) both on the dorsal and the ventral eye disc margin. (F) Misexpression of pnr using bi-Gal4 (bi>pnr\textsuperscript{D4}) results in the suppression of eye fate on both the dorsal and the ventral eye margin as evident from the loss of Elav expression (white arrows). (G, G', H) Gain-of-function clones of pnr (marked by GFP, white arrowhead) generated by random “flp-out” approach in the eye using the heat shock-FLP showed the suppression of eye as evident from the absence of (G') Elav in the eye disc as well as (H) in the adult eye. Note that the eye suppression in the pnr heat shock "flp out" clones was seen only in the larger clones. Further, necrosis (black spots) is also seen in the adult eye upon misexpression of pnr in the eye. (I, J) Blocking pnr function in the entire eye using pnr\textsuperscript{ENR} construct (ey>pnr\textsuperscript{ENR}) results in a “small eye” phenotype as seen (I) in the eye imaginal disc as well as (J) in the adult eye. (K) However, blocking pnr function both on the dorsal and the ventral eye disc margin (bi>pnr\textsuperscript{ENR}) results in the dorsal eye enlargement whereas there was no effect on the ventral eye margin. This data suggests that pnr suppresses eye on the dorsal eye margin.

Fig.4. Pnr suppresses the expression of retinal differentiation genes in the eye. (A, A', A'') Loss-of-function clones of pnr in the eye exhibit dorsal eye enlargement by (A'') ectopic Elav expression. In these clones where dorsal eye enlargement is seen, (A') the expression of retinal precursor marker Ey is restricted anterior to the furrow (white arrow). The dorsal eye enlargement, marked by Elav (blue) is the outcome of the pnr loss-of-function clone. Note that
Ey is absent in the differentiating photoreceptors. Therefore, Ey is not seen in these clones. (B-D) Loss-of-function clones of pnr showing an ectopic dorsal eye phenotype with ectopic induction of retinal determination genes like (B, B', B") Eya (white arrow), (C, C', C") So (white arrow), and (D, D', D") Dac (white arrow). Note that these retinal determination genes, which act downstream to Ey, and unlike Ey are expressed in the differentiating photoreceptor neurons. (E- H) Gain-of-function of pnr in the eye suppresses the retinal determination genes. (E, E') Misexpression of pnr on dorsal and ventral eye margins by using a bi-Gal4 driver (bi>pnrD4), results in strong upregulation of Ey on both dorsal and ventral eye margins (marked by a white arrowhead). (F-H) However, misexpression of pnr (bi>pnrD4) suppresses the downstream retinal differentiation genes (F, F') Eya, (G) So, (H) Dac on both dorsal and ventral margins (marked by arrow heads). The anterior Dac expression (anterior to furrow) went all the way down to the posterior margin in bi>pnrD4 misexpression. Ey marks retinal precursor cells and is required for the specification of eye field. Our results suggest that pnr may not affect early eye specification function of Ey, whereas pnr suppresses the retinal determination genes likeeya, so and dac, which acts downstream to Ey.

**Fig. 5. pnr induces downstream target Wg to suppress the eye.** Wg is known to act as a negative suppressor of eye fate. Wg is expressed laterally both on the dorsal and the ventral eye margins (Fig. 1B). (A, A') Misexpression of pnr on both dorsal and ventral eye margin using bi-Gal4 results in the suppression of eye on both DV margins along with ectopic induction of Wg (marked by white arrows). (B) bi>pnrD4 results in the reduction of eye both on the dorsal and ventral eye margins. This phenotype is similar to bi>wg (Singh et al., 2002). (C) Misexpression of Wg in the entire eye using ey-Gal4 (ey>wg) results in “no-eye”. (D) Loss-of-function clones of pnr in the dorsal eye (marked by absence of GFP reporter and
white dotted line) result in the ectopic eye enlargement along with the suppression of Wg expression. (E, E’) Loss-of-function clones of *pnr* in the DP (marked by white dotted line) caused no effect on Wg expression as Pnr is not expressed in the DP. (E’) Higher magnification of the clone showing its location restricted to the DP. (F) Interestingly, some of the bigger loss-of-function clones of *pnr* (marked by white dotted line) exhibit the dorsal eye enlargement. However, this eye enlargement do not cover the entire clone. The part of the clone anterior to the eye enlargement show robust Wg expression. These clones show some overgrowth in the eye disc, which will form head specific structures, suggesting that within dorsal eye margin *pnr* is not the sole Wg regulator.

**Fig.6.** *pnr* suppresses the eye fate at dorsal eye margin independent of *hth*. *hth*, a Meis class of gene (Rieckhof et al., 1997), acts as a negative regulator of the eye (Pai et al., 1998). Hth expression is restricted anterior to furrow in 10-15 cell wide domain and in entire peripodial membrane (Fig.1). (A, A’) Misexpression of *hth* on both dorsal and ventral eye margin (*bi>*hth) results in the suppression of eye fate on both dorsal and ventral margin of the eye disc as evident from (A’) suppression of Elav (marked by white arrows). (B, B’) Misexpression of *pnr* on both dorsal and ventral eye margin (*bi>*pnr<sup>D4</sup>) results in suppression of eye on both dorsal and ventral eye margin (marked by white arrows), which is accompanied by induction of Wg (green) as well as Hth (red; white arrows). (C, C’) Loss-of-function clone of *hth* in the eye has DV asymmetric phenotypes. The loss-of-function clone of *hth* in the ventral eye results in the eye enlargement as evident from Elav expression (marked by white dotted line). Note that the dorsal eye clones do not exhibit any phenotype. (D- F) In loss-of-function clones of *pnr*, (D, D”) which result in dorsal eye enlargement (marked by white dotted line) or (E, E’) which do not exhibit dorsal eye enlargement (marked by white
Hth (red) expression stays anterior to the furrow as seen in the wild-type eye disc. Loss-of-function clones of pnr in the antennal disc which results in the duplication of antennal field exhibit ectopic Hth expression in the duplicated antennal disc (marked by white dotted line). Note that hth is expressed in the proximal region of the antennal disc.

Fig.7. Pnr suppresses the eye fate by downregulating teashirt (tsh) in the dorsal eye margin. Tsh, a Hox gene (Fasano et al., 1991), exhibits Dorso-ventral (DV) asymmetric function in the eye (Singh et al., 2002). pnr expression initiates in early second instar eye-antennal imaginal disc (Singh & Choi, 2003). (A) In the late second instar, pnr expression evolves and is restricted to 50-100 cells of the dorsal eye margin. At this stage when MF has just initiated, Tsh is expressed anterior to the furrow (MF). (B) In the third instar eye imaginal disc, pnr is expressed on the dorsal eye margin whereas tsh is expressed in the eye disc anterior to the furrow. (C- C”) Loss-of-function clone of pnr in the dorsal eye marked by the loss of GFP reporter (marked by white dotted line) exhibit (C’) ectopic localization of Tsh protein, and (C”) ectopic expression of tsh reporter (tsh\textsuperscript{A8}/CyO) in the dorsal eye. (D-D”) Loss-of-function clones of pnr (marked by loss of GFP), where tsh function is reduced to half using a heterozygous background of tsh null allele (tsh\textsuperscript{8}/+), (D’) exhibit outgrowth on the dorsal eye margin which is positive for Ey expression but there is no ectopic eye enlargement as evident from (D”) absence of neuronal marker Elav expression. The dorsal overgrowth exhibits robust expression of Ey, a marker for undifferentiated retinal precursor cells. (E) Loss-of-function clone of pnr in the tsh heterozygous background marked by the loss of mini-white reporter (red: clonal boundary marked by black dotted line) results in the absence of eye enlargement in the adult eye. These results suggest that pnr eye suppression function is mediated through down regulation of tsh. (F) Misexpression of pnr on the dorsal and the ventral eye margin,
bi>pnr\textsuperscript{D4}, results in the suppression of tsh reporter on both dorsal and ventral eye margin (white arrowhead) along with the suppression of eye as evident from Elav (blue) expression. Note that eye size is reduced on both margins. (G) Misexpression of pnr\textsuperscript{D4} both on dorsal and ventral eye margin in tsh heterozygous background (tsh\textsuperscript{8}/+; bi> pnr \textsuperscript{D4}) exhibits strong suppression of eye resulting in a highly reduced eye. Note that bi>pnr\textsuperscript{D4} alone (F) shows suppression of eye both on the dorsal and the ventral eye margin. However, the size of bi>tsh\textsuperscript{8}/+; pnr\textsuperscript{D4} eye imaginal disc size is extremely reduced as compared to bi>pnr\textsuperscript{D4} alone. (H) Misexpression of tsh on DV margin (bi>tsh) results in the suppression of eye on the ventral margin whereas eye enlargement in the dorsal eye (Singh et al., 2002). Misexpression of both tsh and pnr on DV margin results in early lethality. Therefore, we misexpressed pnr downstream target ara with tsh. (I) Misexpression of tsh with dorsal eye selector ara, a downstream target of pnr, on DV margin using bi-Gal4 (bi>tsh+ara) results in the enlargement on both dorsal and ventral eye margins. Misexpression of tsh and ara on DV margin results in strong dorsal eye enlargements. (J) Misexpression of tsh using pnr-Gal4 driver (pnr>tsh) results in the enlargement of the dorsal eye. (K) Misexpression of tsh in the heterozygous pnr background results in the dorsal eye enlargement. However, these eye enlargements are not bigger than what is seen in (H) bi>tsh or (J) pnr>tsh, suggesting that pnr acts upstream of tsh.

Fig. 8. Pnr suppresses the eye fate by downregulating tsh which results in suppression of retinal determination genes at the dorsal eye margin. GATA-1 transcription factor pnr, which is expressed in the peripodial membrane (PM) at the dorsal eye margin, suppresses the retinal determination. The suppression of retinal determination genes by pnr can be mediated by two possible ways: (i) pnr directly suppresses the retinal
determination genes to suppress the eye. *pnr* may act downstream to *ey* and suppress the downstream retinal determination target *eya* and other downstream genes *so* and *dac*. During eye development, *ey* is required for eye specification and other downstream targets are required for retinal determination. Our studies suggest that *pnr* acts on retinal determination process, which corresponds to the onset of *pnr* expression in the eye. (ii) Alternatively, *pnr* suppresses the eye by downregulating homeotic gene *teashirt (tsh)* in the dorsal eye. Interestingly, the *tsh* gain-of-function in the dorsal eye (Singh et al., 2002) is complementary to the loss-of-function of *pnr* in the dorsal eye. *tsh* is known to act upstream of *eya*, *so* and *dac* (Pan and Rubin, 1998). Thus, the dorsal eye enlargement observed in *pnr* mutant is due to ectopic induction of *tsh* in the dorsal eye, which in turn can induce the RD genes. Lastly, Pnr mediated suppression of the eye fate is independent of Meis class of homeotic gene, *homothorax (hth)* function.
Fig. 1

Oros et al., 2010

Oros et al., 2010 (DBIO-10-421)
Fig. 2

Oros et al., 2010

\[ \text{pnr}\{\text{GFP}\} \]

\[ \text{Elav} \]

\[ \text{pnr}\{\text{w}\} \]

\[ \text{Elav} \]
Fig. 3. Oros et al., 2010
Fig. 5

Oros et al., 2010

(DBIO-10-421)
Table 1. Classification of the loss-of-function phenotypes of *pannier* (*pnr*) clones in the eye and antenna.

<table>
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<tr>
<th>Phenotype</th>
<th>Large clones with dorsal eye enlargements</th>
<th>Small clones with dorsal eye enlargements</th>
<th>No dorsal eye enlargements</th>
<th>Antennal duplication</th>
<th>Total flies (with clones) counted</th>
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<tr>
<td>Clones with Non-autonomous dorsal eye enlargement</td>
<td>34 (8.3%)</td>
<td></td>
<td></td>
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<tr>
<td>Clones with autonomous dorsal eye enlargement Class II</td>
<td>23 (5.6%)</td>
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<td>No dorsal eye enlargement Class III</td>
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<td>308 (75.3%)</td>
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<td>308</td>
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<tr>
<td>Antennal Duplication Class IV</td>
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<td>(4)*</td>
<td>15 (3.6%)</td>
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<tr>
<td>Grand Total</td>
<td></td>
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* The flies in this category showed both antennal duplications dorsal eye enlargements of large and small size.