Development 130, 3053-3062
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doi:10.1242/dev.00534

Mechanism of *hedgehog* signaling during *Drosophila* eye development

Kartik S. Pappu[1], Rui Chen[2], Brooke W. Middlebrooks[3], Catherine Woo[4], Ulrike Heberlein[5] and Graeme Mardon[1,2,3,6,7,*]

[1]Program in Developmental Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
[2]Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
[3]Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
[4]Biomedical Initiatives, University-wide AIDS Research Program, University of California Office of the President, 300 Lakeside Drive, 6th Floor, Oakland, CA 94612, USA
[5]Department of Anatomy, University of California San Francisco, Box 0452, 513 Parnassus, San Francisco, CA 94143-0452, USA
[6]Department of Ophthalmology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
[7]Department of Neuroscience, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
*Author for correspondence (e-mail: gmardon@bcm.tmc.edu)

Accepted 27 March 2003

SUMMARY

Although Hedgehog (Hh) signaling is essential for morphogenesis of the *Drosophila* eye, its exact link to the network of tissue-specific genes that regulate retinal determination has remained elusive. In this report, we demonstrate that the retinal determination gene *eyes absent* (*eya*) is the crucial link between the Hedgehog signaling pathway and photoreceptor differentiation. Specifically, we show that the mechanism by which Hh signaling controls initiation of photoreceptor differentiation is to alleviate repression of *eya* and *decapentaplegic* (*dpp*) expression by the zinc-finger transcription factor Cubitus interruptus (Ci^{rep}). Furthermore, our results suggest that stabilized, full length Ci (Ci^{act}) plays little or no role in *Drosophila* eye development. Moreover, while the effects of Hh are primarily concentration dependent in other tissues, *hh* signaling in the eye acts as a binary switch to initiate retinal morphogenesis by inducing expression of the tissue-specific factor Eya.

Key words: *eyes absent*, *hedgehog*, *Drosophila*, Retinal determination, *cubitus interruptus*, Photoreceptor, Morphogenetic furrow

INTRODUCTION

Members of the Hedgehog family of secreted signaling proteins play crucial roles throughout development (recently reviewed by Ingham and McMahon, 2001). Much of our understanding of the Hedgehog signaling pathway comes from studies on the *Drosophila* ortholog *hedgehog* (*hh*) (Ingham and McMahon, 2001). *Drosophila hh* plays important roles in patterning the anteroposterior embryonic axis, wing, leg, eye, gut, trachea and gonads, and in the development of the optic lamina (Ingham and McMahon, 2001). This rather global requirement for *hh* signaling leads to obvious questions about how specific responses are achieved within the receptive cells. For example, in addition to pattern generation, *hh* signaling is required for cell proliferation (Duman-Scheel et al., 2002; Fan and Khavari, 1999), cell survival (Ahlgren and Bronner-Fraser, 1999; Miao et al., 1997) and cell fate specification (Treier et al., 2001). Despite extensive research, few tissue-specific targets of *hh* signaling have been uncovered to date in *Drosophila*. Many of the effects of *hh* signaling, instead, seem to be mediated by induction of other, widely expressed, secreted signaling molecules, including *decapentaplegic* (*dpp*), *wingless* (*wg*) and the epidermal growth factor receptor ligand *vein* (*vn*) (reviewed by Ingham and McMahon, 2001). Dpp belongs to the transforming growth factor β (TGFβ) superfamily of secreted signaling molecules and has multiple crucial roles throughout *Drosophila* development (Gelbart, 1989; Spencer et al., 1982). We have previously demonstrated that *dpp* functions reiteratively in a network to control retinal cell fate determination (Chen et al., 1999). Specifically, *dpp* signaling appears to synergistically feed into a regulatory network that consists of four genes that encode nuclear proteins: *eyeless* (*ey*), *eyes absent* (*eya*), *sine oculis* (*so*), and *dachshund* (*dac*). Several studies suggest that these four genes act in a network to regulate retinal determination. First, each gene is necessary for eye development and loss-of-function mutations in these genes lead to reduced or no eye phenotypes (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994). Second, with the exception of *so*, each gene is sufficient to induce ectopic eye development (Bonini et al., 1997; Halder et al., 1995; Shen and Mardon, 1997). Finally, the proteins encoded by these genes appear to form complexes to regulate the expression of each other and potential downstream targets (Chen et al., 1997; Halder et al., 1998; Pignoni et al., 1997a). In this study, we have revisited the relationship between *hh*, *dpp* and the retinal determination network during *Drosophila* eye development.

The adult *Drosophila* eye contains between 750 and 800

ommatidia organized in a precise hexagonal array. Eight photoreceptors and 12 accessory cells, including four cone cells, six pigment cells and one mechanosensory bristle, comprise each ommatidium (Wolff and Ready, 1993). The adult eye develops from an epithelial monolayer called the eye imaginal disc, which is derived from a few cells set aside during late embryogenesis (Garcia-Bellido and Merriam, 1969). Photoreceptor differentiation is initiated in early third instar larvae at the posterior margin of the eye disc and proceeds anteriorly following a synchronous wave of cellular changes termed the morphogenetic furrow (MF) (Ready et al., 1976). Alterations in cell shape, cell cycle and patterns of gene expression occur within the MF, and these changes ultimately generate differentiated photoreceptors that are left in its wake (Wolff and Ready, 1991). Therefore, a crucial event during *Drosophila* eye development is the initiation of the MF.

Many lines of evidence suggest that *hh* signaling is required for the initiation of the morphogenetic furrow. First, *hh* is expressed at the posterior margin of the eye imaginal disc prior to photoreceptor differentiation and in all cells posterior to the MF during its progression (Borod and Heberlein, 1998). Second, loss of *hh* function blocks initiation of the MF and impedes its progression (Borod and Heberlein, 1998). Third, posterior margin clones of a null allele of *smoothened* (*smo*), the cell-autonomous receptor of *hh* signaling, lack differentiated photoreceptors (Curtiss and Mlodzik, 2000; Greenwood and Struhl, 1999). Fourth, loss-of-function clones of *protein kinase A* (*pka*), an intracellular negative regulator of *hh* signaling, result in ectopic activation of the *hh* signaling pathway and precocious photoreceptor differentiation (Chanut and Heberlein, 1995; Dominguez, 1999; Pan and Rubin, 1995; Strutt et al., 1995). Similarly, several studies indicate that loss of *dpp* signaling in the eye imaginal disc also blocks initiation of photoreceptor differentiation. First, *dpp* is also expressed in the posterior margin of the eye disc prior to initiation of photoreceptor differentiation (Borod and Heberlein, 1998; Chanut and Heberlein, 1997b). Second, loss-of-function posterior margin clones of *mothers against decapentaplegic* (*mad*), a nuclear effector of *dpp* signaling, lack photoreceptor differentiation (Wiersdorff et al., 1996). Third, the MF fails to initiate from ventral regions of eye discs from flies that mutant for a hypomorphic allele of *dpp* (Chanut and Heberlein, 1997a; Chanut and Heberlein, 1997b; Treisman and Rubin, 1995). Finally, ectopic expression of *dpp* leads to ectopic induction of the MF from the anterior margin of the eye imaginal disc (Chanut and Heberlein, 1997b; Pignoni et al., 1997a). These phenotypic similarities, coupled with the requirement for *hh* to activate and maintain *dpp* expression (Borod and Heberlein, 1998; Burke and Basler, 1996), suggest that *dpp* may be the sole target of *hh* signaling during *Drosophila* eye development.

Using a combination of loss- and gain-of-function genetics, we demonstrate that the major role of Hh signaling during *Drosophila* eye development is to alleviate the repression of *dpp* and *eya* by Ci^{rep}. Additionally, loss-of-function analyses suggest that the full length, activated Ci^{act} plays little or no role in *Drosophila* eye development. Based on these results, we conclude that *eya* is the critical tissue-specific target of Hh signaling during the initiation of normal photoreceptor differentiation in *Drosophila*. Furthermore, our results suggest that Hh does not function as a classical morphogen during the initiation of retinal morphogenesis (Freeman and Gurdon, 2002). Instead, we propose that Hh signaling acts as a binary switch to initiate photoreceptor differentiation during *Drosophila* eye development.

MATERIALS AND METHODS

Drosophila genetics

All *Drosophila* crosses were carried out at 25°C on standard media. The smo^{d16} mutation is a genetic null and was provided by Gary Struhl (Chen and Struhl, 1998). The hh^{P30} line is a *lacZ* enhancer trap in the *hh* locus (Lee et al., 1992). The *30A-GAL4*, *UAS-ey*, *UAS-eya* and *UAS-so* flies have been described previously (Brand and Perrimon, 1993; Pignoni et al., 1997a). *UAS-eya* and *UAS-so* stocks were provided by Francesca Pignoni and Larry Zipursky. *ey-GAL4* flies were provided by Nancy Bonini. Flies containing the *tub-GAL80* insertion on chromosome 2 were obtained from Liqun Luo (Lee and Luo, 1999). All other stocks were obtained from the Bloomington stock center. Flies containing multiple transgenes were generated by meiotic recombination using eye color as an initial selection. Polymerase chain reaction (PCR) with gene specific primers was used to confirm genotypes. Ectopic expression followed by antibody staining (where possible) was used to confirm expression of individual genes from recombinant chromosomes.

Clonal analysis

To induce large clones of $smo^{d16-/-}$ in the eye, we used the FLP-mediated mitotic recombination system in a Minute background (Xu and Rubin, 1993). Mutant clones from such discs are marked by the lack of a β-galactosidase reporter. To reintroduce single gene or multi-gene combinations into $smo^{d16-/-}$ clones, a variation of the MARCM technique was employed (Lee and Luo, 1999). Generally, *y w hs-FLP; smo^{d16} FRT40A/CyO; UAS-gene(s)/TM6B, Tb* females were crossed to *w*; *M(2)24F arm-lacZ tub-GAL80 FRT40A/Bc Elp; ey-GAL4* males. Half the non-*Bc*, non-*Tb* larvae contained negatively marked (lack of β-galactosidase expression) clones. Additionally, within these clones, GAL4 repression by GAL80 is lost and the transgene(s) of interest is expressed. A minimum of 10 eye discs containing large *smo* clones were analyzed for each genotype and yielded consistent results.

Larvae containing marked *ci* mutant clones were generated as described previously (Methot and Basler, 1999). In order to induce large mutant clones, we recombined the $M(2)53^{1}$ mutation onto the *ci* genomic rescue chromosome described previously (Methot and Basler, 1999). Additionally, we recombined an *arm-lacZ* transgene onto the same genomic rescue chromosome to unambiguously mark mutant cells in both larval discs and adult sections. The genotype of the animals is: *y w hs-FLP; FRT42 P{ci1} hsp70-GFP arm-lacZ $M(2)53^{1}$/FRT42; ci^{94}/ci^{94}*.

Adult animals containing clones were identified by the yellow mutant phenotype, the mosaic eye color and the presence of wing phenotypes. Adult eyes were fixed, embedded and sectioned as described previously (Tomlinson and Ready, 1987).

Immunohistochemistry

The following primary antibodies were used in this study: rat anti-Elav (1:600), rabbit anti-β-galactosidase (1:1000; Cappel), mouse Anti-Eya, 10H6 (1:200), guinea pig Anti-Senseless (1:800) (Frankfort et al., 2001). Conjugated goat anti-mouse, rat, rabbit and guinea pig fluorescent secondary antibodies were ALEXA 488 (Molecular Probes), Cy3 (Jackson Immunochemicals) or Cy5 (Jackson Immunochemicals), all at 1:600 dilution. HRP-conjugated goat anti-mouse antibodies were used as previously described (Chen et al., 1999). Discs were then processed as described previously (Frankfort et al., 2001). Fluorescent images were captured with a Zeiss LSM 510 confocal microscope. All other images were captured on a Zeiss

Axioplan microscope with Nomarski optics. All images were processed with Adobe Photoshop software.

RESULTS

Synergistic activation of ectopic photoreceptor differentiation by co-expression of *ey* and *hh*

The GAL4 line *30A* drives expression of UAS transgenes in a ring around the wing pouch, a region that will become the adult wing blade (Brand and Perrimon, 1993). Misexpression of *ey* in the wing disc using the *30A-GAL4* driver can induce photoreceptor differentiation only in regions where endogenous Hh and Dpp signaling are both active (Chen et al., 1999). One interpretation of this result is that *ey* can activate photoreceptor differentiation only in regions where Hh signaling can induce *dpp* expression, such as the anteroposterior (A/P) compartment boundary (Basler and Struhl, 1994; Methot and Basler, 1999). If *dpp* is the sole target of Hh signaling during *Drosophila* eye development, then misexpression of *dpp* and *ey* together using the *30A-GAL4* driver should be sufficient to induce photoreceptor differentiation in a ring around the wing pouch. Surprisingly, misexpression of *ey* and *dpp* using the *30A-GAL4* line induces Eya expression and photoreceptor differentiation only in the posterior compartment of the wing disc (Fig. 1A,D) (Chen et al., 1999). This suggests that some other factor that regulates *ey*-mediated photoreceptor differentiation must differ in its function in the anterior and posterior compartments. One obvious candidate for this factor is *hh* (Chen et al., 1999).

hh is expressed only in the posterior compartment of the wing disc, while targets of Hh are activated only at the AP compartment boundary where Hh signaling stabilizes full-length Cubitus interruptus (Ci^{act}), the nuclear effector of Hh signaling (reviewed by Vervoort, 2000). In the anterior compartment away from the AP boundary, the Hh signal is not received and target gene expression is repressed by a 75 kDa proteolytically cleaved form of Ci (Ci^{rep}) (Aza-Blanc et al., 1997; Methot and Basler, 1999). Misexpression of Hh in the anterior compartment induces expression of target genes such as *dpp*. In the posterior compartment, expression of *ci* and *dpp* are repressed by the homeotic selector protein Engrailed (En) (Alexandre et al., 1996) and the Hh signal is not transduced.

We hypothesized that the inability of *ey* and *dpp* misexpression to activate photoreceptor differentiation in the anterior compartment of the wing disc is due to the repression of Hh target genes by Ci^{rep}. In the posterior compartment, however, the absence of Ci^{rep} allows *ey*- and *dpp*-mediated retinal differentiation. This model predicts that misexpression of *ey* and *hh* together in the *30A-GAL4* pattern would prevent production of Ci^{rep} and induce photoreceptor differentiation, but only in the anterior compartment. Indeed, *ey* and *hh* misexpression induces robust Eya expression and photoreceptor differentiation specifically in the anterior compartment (Fig. 1B,E). In addition, we find that misexpression of *ey*, *dpp* and *hh* together with the *30A-GAL4* driver leads to Eya activation and photoreceptor differentiation in both compartments of the wing disc (Fig. 1C,F). These results demonstrate that *dpp* alone cannot bypass the requirement for Hh signaling to induce Eya expression during *ey*-mediated ectopic photoreceptor differentiation in the anterior compartment of the wing disc. The induction of robust Eya expression in the anterior compartment of the wing disc upon co-expression of *ey* and *hh* led us to hypothesize that *eya* is normally repressed by Ci^{rep}. Furthermore, this hypothesis predicts that expression of *ey*, *dpp* and *eya* together should bypass the requirement for Hh signaling and induce photoreceptors in both compartments of the wing disc.

eya and *dpp* can bypass the requirement for Hh signaling in the wing disc

30A-GAL4 driven expression of *dpp*, *ey* and *eya* can induce Dac expression and photoreceptor differentiation in both compartments of the wing disc (Fig. 2A,D). This effect becomes more penetrant when *dpp*, *ey*, *eya* and *so* are co-expressed (Fig. 2B,E). However, the effects of *dpp*, *ey*, *eya* and *so* misexpression are not due to induction of *hh* because a *hh-lacZ* reporter (hh^{P30}) is not activated in the anterior compartment (Fig. 2G-I). Finally, expression of *ey*, *hh*, *eya* and *so* can induce Dac expression and photoreceptor differentiation

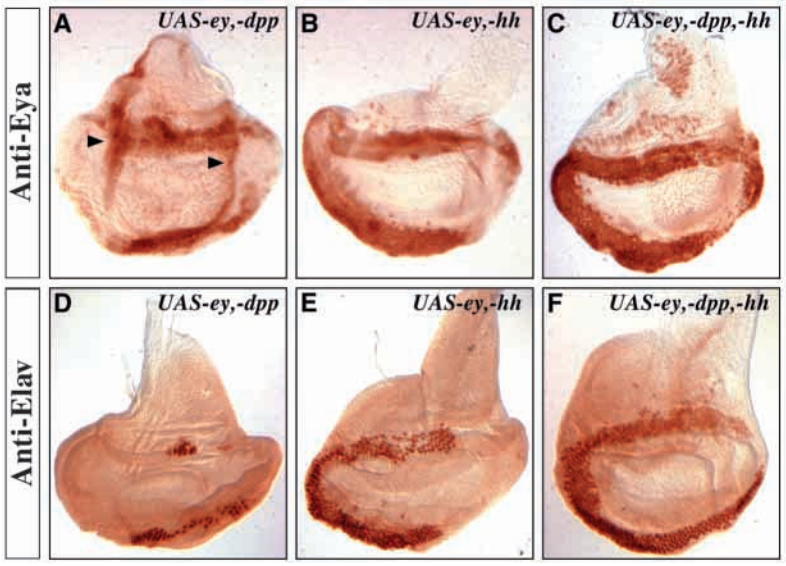

Fig. 1. *hh* functions synergistically with *ey* to induce Eya expression and photoreceptor differentiation. (A-F) All panels show wing discs (anterior towards the left and dorsal upwards) with different combinations of UAS-transgenes driven by the *30A-GAL4* driver. The expansion of the wing disc in all panels is a result of overexpression of either *dpp* or *hh*, and reflects the capacity of these genes to induce growth and proliferation of imaginal disc cells. (A,D) *ey* and *dpp* co-expression in a ring around the wing pouch induces Eya expression (A) and photoreceptor differentiation, but only in the posterior compartment. Endogenous Eya expression in the wing disc appears as vertical stripes of staining in the anterior and posterior compartment (arrowheads in A). (B,E) When *ey* and *hh* are co-expressed, Eya is expressed (B) and photoreceptors differentiate, but only in the anterior compartment. (C,F) Overexpression of *ey*, *dpp* and *hh* together induces Eya expression (C) and photoreceptors in both compartments of the disc pouch. (D-F) Photoreceptor differentiation is visualized by an antibody to the pan-neuronal protein Elav.

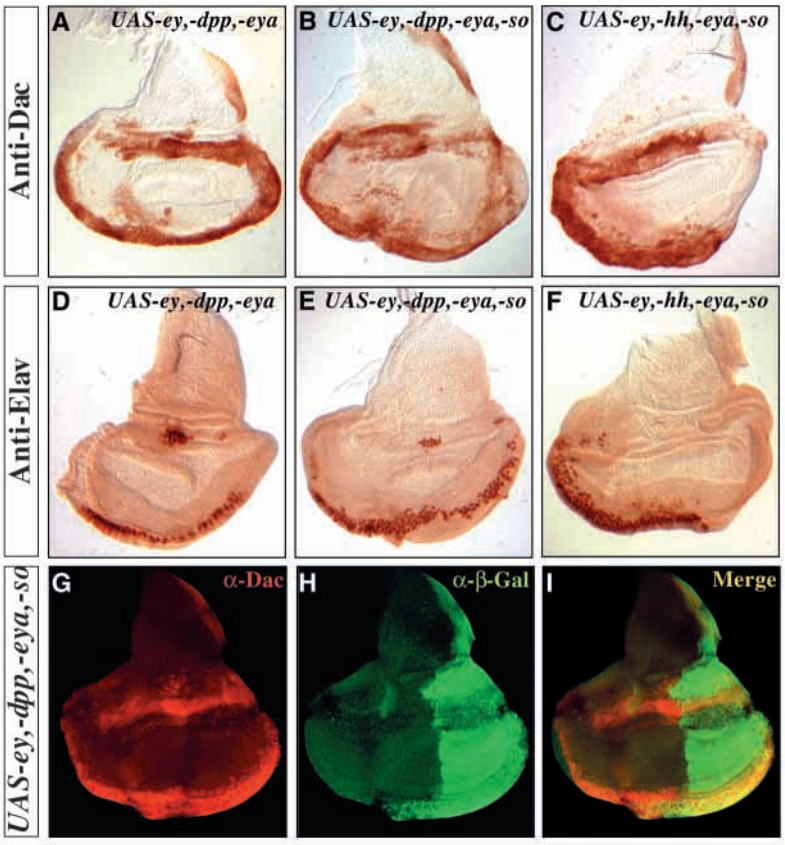

Fig. 2. *eya* and *dpp* can bypass the requirement for *hh* to induce ectopic photoreceptor differentiation in the wing disc. (A-I) All panels show late third instar wing discs (anterior towards the left and dorsal upwards) with different combinations of UAS-transgenes driven by the *30A-GAL4* driver. (A,C,D,F) Misexpression of *ey*, *dpp* and *eya* (A,D), but not *ey*, *hh*, *eya* and *so* (C,F), in the wing disc induces Dac expression (A,C) and photoreceptor differentiation in the anterior and posterior compartments. (B,E) This effect is more penetrant when *ey*, *dpp*, *eya* and *so* are misexpressed.
(G-I) Misexpression of *ey*, *dpp*, *eya* and *so* together in the wing disc in the presence of a *lacZ* enhancer trap in the *hh* locus (hh^{P30}). The same wing disc stained with anti-Dac (red, G), anti-β-galactosidase (green, H) or a merge of the two channels (I) are shown. Dac expression is induced in a ring around the wing pouch (D) but *hh* expression (H) is restricted to the posterior compartment. (D-F) Photoreceptor differentiation is visualized by an antibody to the pan-neuronal protein Elav.

only in the anterior compartment of the wing disc, confirming that *dpp* is essential in this process (Fig. 2C,F). Thus, *eya* and *dpp* together are sufficient to bypass the requirement for Hh signaling in the wing disc. Moreover, these results suggest that in addition to *dpp*, *hh* is also required for *eya* expression during normal retinal development, most probably by blocking Ci^{rep}. Two models are consistent with the ability of *dpp* and *ey* to induce photoreceptor differentiation in the posterior compartment of the wing disc where Hh signaling is not normally active. First, co-expression of *dpp* and *ey* may lead to the misexpression of *ci* in the posterior compartment of the wing disc, thereby allowing Hh signaling to occur. This appears unlikely because no Ci induction is detected in the posterior compartment in response to *ey* and *dpp* expression (data not shown). We favor an alternate model in which *ey* and *dpp* together may be sufficient to induce Eya expression and photoreceptor differentiation in the posterior compartment of the wing disc in the absence of Ci^{rep}. If true, this model predicts that loss of *ci* function in the eye should have no effect on Eya expression and photoreceptor differentiation.

Differential requirements for Hh pathway components during *Drosophila* eye development

We induced *ci* mutant clones in the eye in a *Minute* background (see Materials and Methods). Large *ci*-null mutant clones do not block Eya expression, MF initiation, progression or photoreceptor differentiation in the eye disc (Fig. 3A,B). Furthermore, rhabdomere organization, and spacing appear to be normal in adult sections of eyes containing *ci* mutant clones (Fig. 3C). However, wing discs and adult wings containing *ci* mutant clones develop anterior compartment abnormalities, suggesting that the effects of loss of *ci* are different in the wing and the eye during *Drosophila* development (Fig. 3D,G). Additionally, these results further support the model that the primary function of Hh signaling during retinal determination is to alleviate the repression of *eya* by Ci^{rep}. Consistent with this model, *ey-Gal4* driven misexpression of Ci^{rep} in the eye drastically reduces *eya* expression and photoreceptor differentiation (data not shown). Furthermore, this model predicts that loss of *hh* signaling in the eye blocks Eya expression.

In the eye imaginal disc, posterior margin clones of $smo^{d16-/-}$, a null allele of the cell autonomous receptor of the Hh signal, do not express Eya and lack photoreceptor differentiation (Fig. 4A-G) (Curtiss and Mlodzik, 2000). Progression of the morphogenetic furrow is delayed within *smo* clones that do not encompass any part of the posterior margin (Fig. 4D,G) (Greenwood and Struhl, 1999). In all eye discs with internal *smo* clones, photoreceptor differentiation can spread into mutant tissue, recruiting up to two rows of photoreceptor clusters (arrowhead in Fig. 4D). All discs with large posterior margin mutant clones have residual Eya expression in internal areas that lie close to wild-type tissue (white arrow in Fig. 4D). These results suggest that although Hh signaling is required for Eya expression at the posterior margin of the eye disc, Eya expression in more anterior areas may be subject to different regulatory control. Furthermore, these results confirm previous findings that in the absence of Hh signaling, photoreceptor differentiation can spread into *smo* mutant tissue only if the process of photoreceptor differentiation has already initiated at the posterior margin (Curtiss and Mlodzik, 2000). Finally, *dpp*-mediated induction

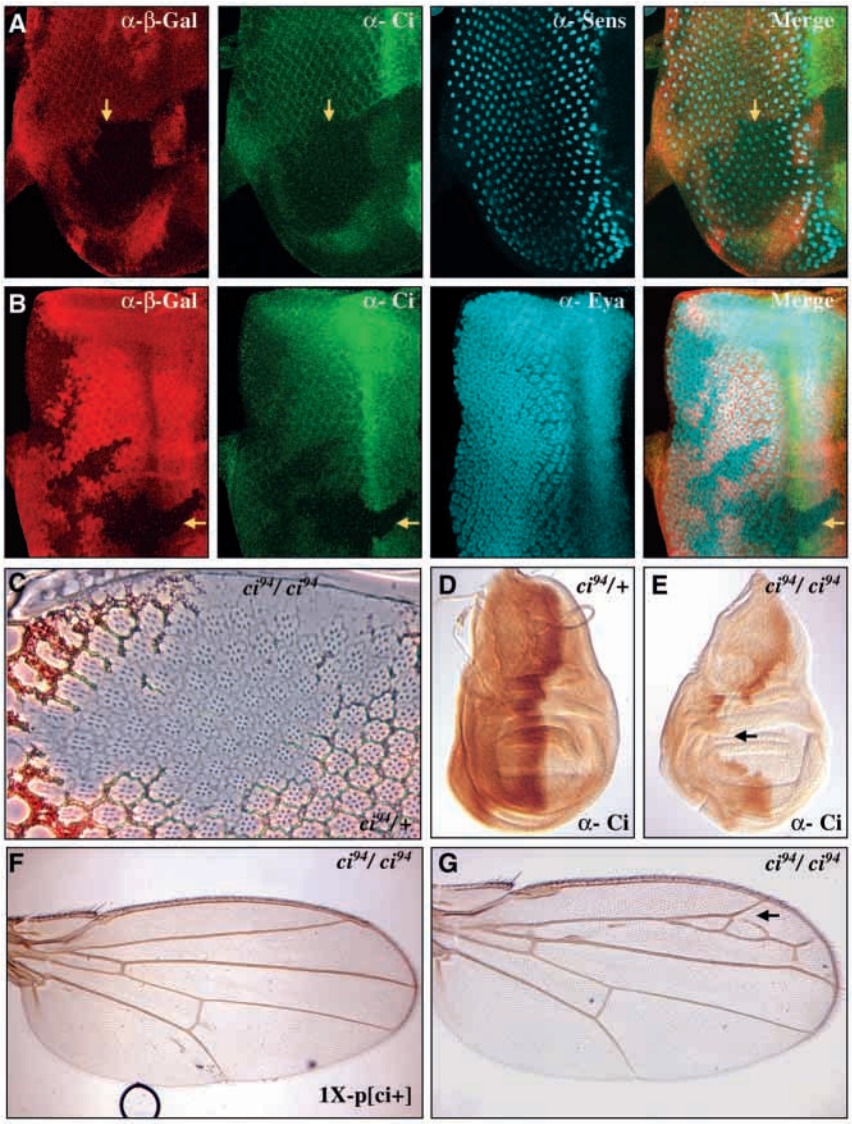

Fig. 3. *ci* mutant clones in the eye disc do not block Eya expression, MF initiation, progression or photoreceptor differentiation. (A,B) Each set of four panels in A and B show the same eye disc containing *ci* mutant clones stained with (A) anti-β-galactosidase, anti-Ci, anti-Senseless and a merge of all three channels or (B) anti-β-galactosidase, anti-Ci, anti-Eya and a merge. The yellow arrows in A and B mark clonal areas. No obvious disruption in Eya or Senseless staining (A) is observed in *ci*-null mutant clones. (C) Thin plastic sections of adult eyes containing *ci* mutant tissue. Mutant clones are negatively marked by the lack of pigment granules. Photoreceptor differentiation is normal even in very large clones of *ci* mutant tissue. (D,F) Animals heterozygous for ci^{94} or homozygous for ci^{94}, but rescued by one copy of the P[ci^{+}] transgene, have normal Ci staining in the wings disc (D) and adult wings (F). (E,G) By contrast, induction of mutant clones in the anterior compartment of the wing disc leads to loss of Ci in the wing disc (arrow, E) and disruption of pattern in the anterior adult wing disc (arrow in G).

of an ectopic MF anterior to the endogenous furrow (Pignoni and Zipursky, 1997b) is lost when *smo* clones encompass these regions (data not shown; discussed further below). This suggests that *dpp*-induced ectopic MFs depend on Hh signaling. The lack of Eya in posterior margin *smo* clones, coupled with the observation that *ey*, *dpp*, and *eya* can bypass Hh signaling to induce photoreceptor differentiation in the wing disc, led us to hypothesize that *eya* may be the critical target of Hh signaling during normal photoreceptor differentiation.

eya is the crucial target of *hh* signaling during *Drosophila* eye development

We used a modified version of the mosaic analysis with repressible cell marker (MARCM) technique (Lee and Luo, 1999) to induce clones lacking *smo* in the eye disc that also express the UAS-transgene(s) of choice driven by *ey-GAL4* specifically in the mutant tissue (see Materials and Methods). This method allows us to determine which combination of genes is sufficient to restore photoreceptor differentiation in the absence of *smo* function in the eye. We used three distinct assays for analyzing rescue of photoreceptor differentiation within *smo* mutant clones. First, we assessed which combinations of *dpp*, *eya* and *so* expression are sufficient to rescue photoreceptor differentiation in posterior margin *smo* clones. Second, we examined rescue of delayed furrow progression within internal *smo* clones. Finally, we tested if these genes could restore *dpp*-mediated induction of ectopic MFs within anterior *smo* mutant clones. All experiments were conducted without the addition of *ey* because Ey expression persists in *smo* clones posterior to the morphogenetic furrow (Lee and Treisman, 2001).

Restoration of either *dpp* or *eya* expression alone in posterior margin *smo* clones does not rescue photoreceptor differentiation within the clone (Fig. 5A,C). Similarly, delayed progression of the MF in interior *smo* clones is not rescued by the expression of *dpp* or *eya* alone (Fig. 5B,D). Furthermore, co-expression of *eya* and *so* also does not rescue either initiation or progression of photoreceptor differentiation within *smo* clones (data not shown). By contrast, expression of *dpp* and *eya* together restores photoreceptor differentiation in posterior margin *smo* clones with complete penetrance (Fig.

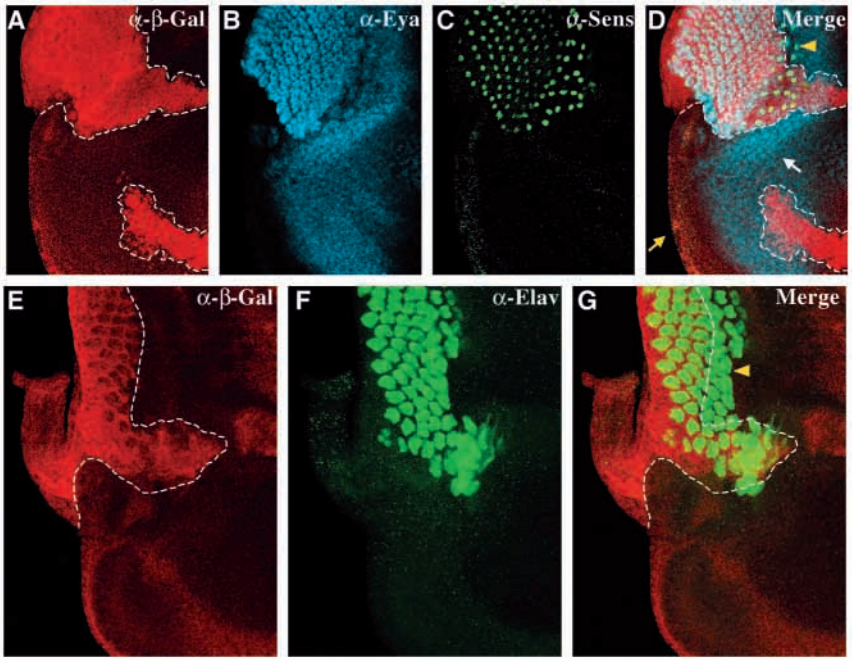

Fig. 4. Posterior margin *smo* mutant clones block Eya expression and photoreceptor differentiation. (A-G) The same eye disc stained with anti-β-galactosidase (red in A,D,E,G), anti-Eya (cyan in B,D), anti-Elav (green in F,G) and anti-Senseless, an R8 photoreceptor-specific marker (green in C,D). The clone boundaries are marked with broken white lines (A,D,E,G). (C,D,F,G) *smo* clones are negatively marked by the absence of β-galactosidase and lack expression Senseless (C,D) or the pan-neuronal marker Elav (F,G). (B,D) Loss of Eya expression in posterior margin clones (B; yellow arrow in D) and a reduction of Eya expression internally in large *smo* clones (white arrow in D). (D,G) Furrow progression is delayed in internal *smo* clones, but up to two rows of photoreceptor clusters differentiate in mutant tissue (yellow arrowheads in D,G).

5E). Similarly, furrow progression through internal *smo* clones expressing both *dpp* and *eya* is normal (Fig. 5F). Anterior margin *smo* clones expressing both *dpp* and *eya* also differentiate clusters of photoreceptors but with incomplete penetrance (data not shown). These results demonstrate that *dpp* is not the sole target of Hh signaling in the eye and that *eya* is the crucial tissue-specific Hh target during retinal morphogenesis. Our analysis in the wing disc suggests that overexpression of a combination of *ey*, *dpp*, *eya* and *so* is most effective in bypassing the requirement for Hh signaling during ectopic photoreceptor differentiation (Fig. 2C). We tested whether co-expression of *dpp*, *eya* and *so* in *smo* clones was also more effective in inducing photoreceptor differentiation. Although posterior margin *smo* clones are rescued with similar efficiency as the combination of *dpp* and *eya*, ectopic anterior furrows are induced with high frequency when *dpp*, *eya* and *so* are expressed in *smo* clones (Fig. 5E-H). This result is consistent with the synergistic effects of *eya* and *so* co-expression during ectopic photoreceptor differentiation (Pignoni et al., 1997a). Finally, expression of *dpp* and *so* in *smo* mutant clones does not rescue photoreceptor differentiation, further demonstrating that *eya* is the specific downstream target of Hh signaling during the initiation of the MF (data not shown).

DISCUSSION

The morphogenesis of adult structures requires cellular integration of signaling inputs from global growth and patterning factors with developmental cues provided by tissue-specific factors. Signaling by the secreted growth factor Hh plays important roles in coordinating the growth and patterning of almost all tissues in *Drosophila*. In the *Drosophila* eye, loss of Hh signaling blocks the initiation of photoreceptor morphogenesis. However, the basis for this phenotypic outcome is poorly understood. In this study, we demonstrate that the retinal determination gene *eya* is a crucial eye-specific target of Hh signaling. Furthermore, our results demonstrate that the major role of Hh signaling during the initiation of photoreceptor differentiation is to prevent the production of Ci^{rep}, which normally represses *eya* expression.

ey, *dpp* and *eya* can bypass the requirement for Hh signaling to initiate ectopic retinal morphogenesis

Misexpression of *ey* in the wing disc causes ectopic photoreceptor differentiation only in regions where both *dpp* and *hh* signaling are normally active. The simplest explanation for this effect invokes a linear regulatory hierarchy where *hh* induces *dpp*, which in turn cooperates with *ey* to initiate retinal morphogenesis. While, misexpression of *ey* and *dpp* together does indeed lead to synergistic photoreceptor differentiation, this occurs only in the posterior compartment of the wing disc. Notably, Hh signaling is not transduced in the posterior compartment of the wing disc due to the repression of *ci* by En (Alexandre et al., 1996). Furthermore, *dpp* and *ey* expression does not induce Ci expression in the posterior compartment of the wing disc. Thus, we conclude that *dpp* and *ey* can induce Eya expression and photoreceptor differentiation in the posterior compartment of the wing disc in the absence of Hh signaling and Ci^{rep}. Misexpression of *hh* and *ey* induces robust *eya* expression and photoreceptor differentiation in the wing disc, but only in the anterior compartment. This result is consistent with a model in which Hh signaling normally blocks the production of Ci^{rep} and converts it into an activated form, Ci^{act}, in the anterior compartment of the wing disc. Ci^{act} can induce *dpp* expression in the anterior compartment (Alexandre et al., 1996) and *dpp* can in turn cooperate with *ey* to induce robust Eya expression and photoreceptor differentiation. Consistent with this model, co-expression of *hh*, *dpp* and *ey* leads to Eya expression and photoreceptor differentiation in both compartments of the wing disc. Taken together, these results suggest that, in the wing disc, *ey* and *dpp* can activate *eya* expression only in absence of Ci^{rep}.

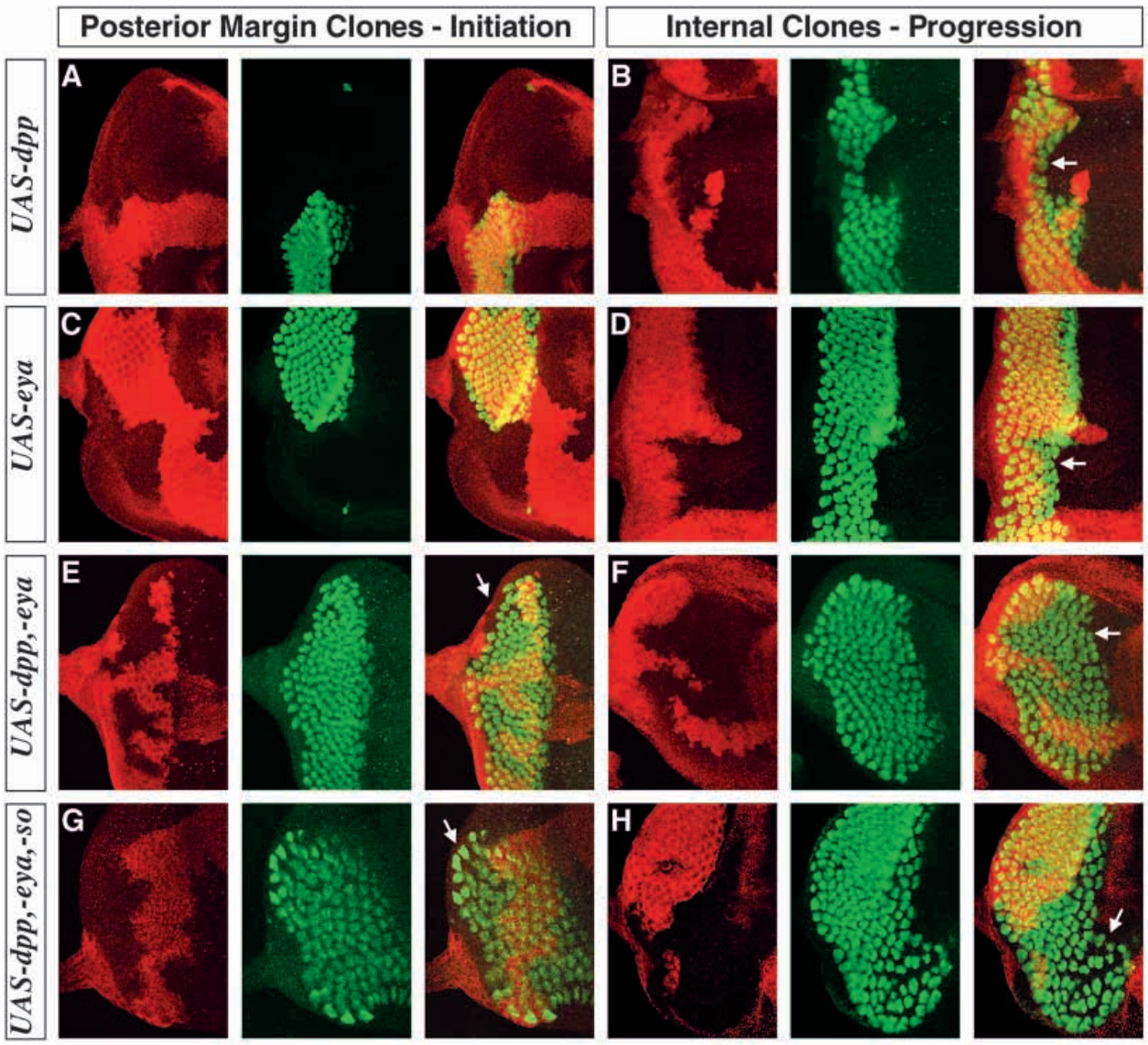

Fig. 5. Co-expression of *dpp* and *eya* rescues photoreceptor differentiation and furrow progression in *smo* mutant clones. (A-H) Each set of three panels in A-H depicts the same disc containing large *smo* clones stained for anti β-galactosidase (red), Anti-Elav (green) or a merge of the two. The *ey-GAL4* driver was used to induce transgene expression in all cases. (A-D) Neither *dpp* (A,C) nor *eya* (B,D) expression alone can rescue the loss of photoreceptor differentiation in posterior margin *smo* clones (A,C) or the slowing of furrow progression in internal *smo* clones (arrows in B,D). (E,F) Posterior margin *smo* clones expressing both *dpp* and *eya* differentiate photoreceptors as visualized by Elav immunoreactivity (arrow in E). Furrow progression is not delayed in internal *smo* clones expressing *dpp* and *eya* (arrow in F). (G,H) Similarly, co-expression of *dpp*, *eya* and *so* also rescues photoreceptor differentiation and furrow progression in *smo* clones, often inducing ectopic furrows from anterior *smo* clonal areas (arrows in G,H).

Co-expression of *dpp*, *ey* and *eya* using the *30A-Gal4* driver induces photoreceptor differentiation in both wing compartments, albeit with low penetrance. This effect becomes stronger and more penetrant when *dpp*, *ey*, *eya* and *so* are misexpressed in a ring around the wing pouch. These results demonstrate that providing *ey*, *dpp* and *eya* from an exogenous source is sufficient to bypass the requirement for Hh signaling during initiation of ectopic photoreceptor differentiation. In addition, these results implicate *eya* as a key target for Hh signaling during the initiation of normal retinal morphogenesis, most likely by blocking Ci^{rep}.

The adult *Drosophila* eye develops normally in the absence of *ci*

The data from our ectopic expression analyses in the wing disc suggest that Ci^{rep} has a major role in blocking *eya* expression in areas that are not exposed to Hh signaling. However, Ci^{act} also plays an important role in patterning the anterior compartment of the wing disc (Methot and Basler, 1999). For example, adult wings that contain *ci* mutant clones develop with defects in the anterior compartment (Methot and Basler, 1999) (this paper). In the *Drosophila* eye disc, *ci* is expressed uniformly but Ci protein expression follows a dynamic pattern. It has been proposed that in regions anterior to the furrow Ci

is subject to PKA-dependent phosphorylation and SCF^{Slimb}-dependent processing into Ci^{rep} (Ou et al., 2002). Cells in the MF, however, receive and transduce the Hh signal and prevent the proteolytic processing of Ci, therefore blocking production of Ci^{rep}. Furthermore, it has been proposed that cells that are posterior to the MF do not accumulate Ci^{rep} in a PKA-dependent manner. Instead, these cells use a *smo*- and *cullin3*-dependent proteolytic process leading to the complete degradation of Ci (Ou et al., 2002). Therefore, the role for Ci in the eye appears to be limited only to cells that are part of, and anterior to, the MF. However, these studies do not establish separate functional roles for Ci^{act} and Ci^{rep} in the developing eye.

Surprisingly, Eya expression and photoreceptor differentiation are not perturbed in *Drosophila* eye discs that contain large *ci*-null mutant clones. Similarly, adult eyes containing large *ci* mutant clones appear normal both externally (data not shown) and in internal sections. These results, coupled with our ectopic expression analysis in the wing disc, suggest that Ci^{act} plays little or no role during normal photoreceptor differentiation. Furthermore, these results support a model in which the major role for Hh signaling during the initiation of photoreceptor differentiation is to prevent the production of Ci^{rep}.

Interestingly, *ci*-null mutant clones that span the furrow do not hasten furrow progression. Although ectopic activation of the Hh pathway is sufficient to induce precocious furrow advancement and photoreceptor differentiation (Heberlein et al., 1993; Pan and Rubin, 1995; Strutt et al., 1995), loss of Ci is not. A likely explanation for this apparent contradiction may be found in the distinction between loss- and gain-of-function experiments. Specifically, although Ci^{act} normally plays little or no role in eye development, ectopic production of Ci^{act} is sufficient to induce precocious furrow advancement. Intriguingly, vertebrate homologs of *Drosophila ci* have evolved to carry out either activator (*Gli1* and *Gli2*) or repressor (*Gli3* and perhaps *Gli2*) functions independently (Ingham and McMahon, 2001). Our findings demonstrate that in the absence of gene duplication, tissue-specific separation of these functions has also occurred in *Drosophila*.

Threshold effects of Hh signaling during *Drosophila* eye development

We propose that Hh signaling acts as a binary switch during *Drosophila* eye development to control the timing of initiation of photoreceptor differentiation. Specifically, our data suggest that during early larval development Ci^{rep} normally inhibits retinal morphogenesis by blocking *eya* and *dpp* expression. Hh signaling in late second instar larvae blocks production of Ci^{rep}, which in turn allows *dpp* and *eya* expression, MF initiation, progression and photoreceptor differentiation. Rather than regulating the differentiation of multiple cell types in a concentration-dependent manner, our data suggest that Hh signaling acts as a molecular switch that is sufficient to initiate *dpp* and *eya* expression and retinal morphogenesis. This model also explains the seemingly contradictory phenotypes of loss of *smo* (blocks MF initiation) and loss of *ci* (no effect) during *Drosophila* eye development. Loss of *ci* creates a permissive environment for *eya* and *dpp* expression and photoreceptor differentiation, rendering eye development Hh independent. By contrast, Ci^{rep} persists in the absence of *smo* function and thus

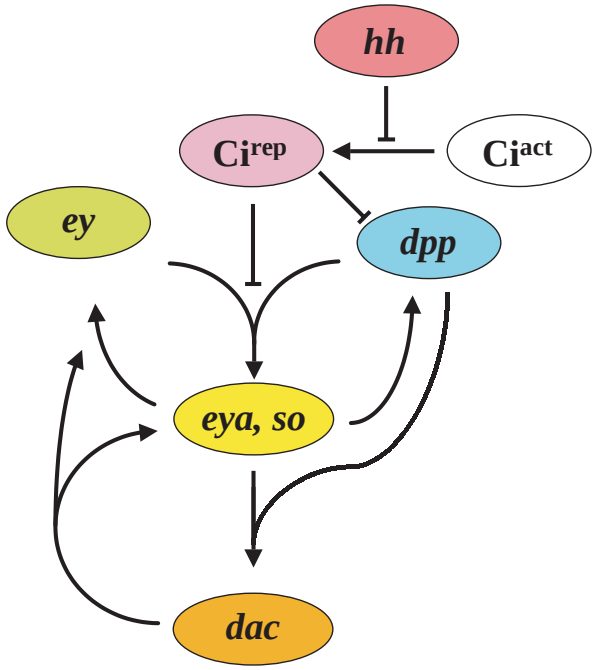

Fig. 6. A model for the genetic network that controls retinal determination in *Drosophila*. *hh* is required for both *dpp* and *eya* expression during photoreceptor differentiation (see text for additional details).

photoreceptor morphogenesis does not occur in *smo* clones. As *ci* null mutant clones in the eye develop normally, other Hh-independent mechanisms must also act to control the initiation of retinal morphogenesis in *Drosophila*.

Recent studies analyzing the role of the Hh signaling pathway in organizing dorsoventral pattern in the developing vertebrate neural tube present a useful comparison with the developing *Drosophila* eye. Specifically, Gli3, a homolog of *Drosophila* Ci, acts as a transcriptional repressor in patterning the intermediate region of the developing spinal cord (Persson et al., 2002). Normal patterning of the ventral spinal cord requires the establishment of a gradient of Hh signaling (strongest ventrally), which acts in part by preventing the repressive activity of Gli3 (Wijgerde et al., 2002). Furthermore, this gradient specifies multiple, distinct cell fates, depending on the distance from the source of Hh (Ingham and McMahon, 2001). In the absence of Hh signaling, Gli3 repression expands ventrally and inappropriately blocks certain ventral spinal cord cell fates (Wijgerde et al., 2002). Moreover, in *Smo Gli3* double mutant mice, these ventral cell fates are restored. These results suggest that blocking production of the Gli3 repressor is a key step in spinal cord development and closely parallels work presented in this study. However, in contrast to the *Drosophila* eye (where Hh acts as a binary switch), the actions of Hh signaling during the patterning of the vertebrate spinal cord are concentration dependent.

Co-expression of *dpp* and *eya* can rescue *smo* mutant clones

Posterior margin *smo* mutant clones lack Eya expression and photoreceptor differentiation (Curtiss and Mlodzik, 2000) (this

paper). We attribute the lack of *eya* expression in these cells to their inability to block the production of Ci^{rep}. Furthermore, our data demonstrates that co-expression of *dpp* and *eya* in these posterior *smo* mutant clones rescues photoreceptor differentiation. In addition, *dpp* and *eya* co-expression is sufficient to rescue delayed furrow progression in *smo* clones. However, the precise temporal and spatial order of photoreceptor recruitment may not be rescued in these clones. Thus, the requirement for Hh signaling in the eye can be circumvented by the expression of the downstream targets *dpp* and *eya*. These results demonstrate that *eya* is a crucial eye-specific target of Hh signaling during the initiation of retinal differentiation and has led to a new model for the initiation of retinal morphogenesis (Fig. 6). In this model, Hh signaling blocks the proteolytic degradation of Ci^{act} into Ci^{rep}, thus allowing initiation of *dpp* expression. Once *dpp* expression is established, the absence of Ci^{rep} allows *dpp* to act in parallel with *ey* to initiate *eya* expression, which in turn leads to *so* expression. Furthermore, *dpp* cooperates with *eya* and *so* to initiate the expression of *dac* and extensive feedback regulation among these genes leads to consolidation of retinal cell fates.

Many recent studies demonstrate that vertebrate Hh homologs also play important roles in promoting patterning, proliferation, and differentiation of many cells types within the developing eye (Ingham and McMahon, 2001). Furthermore, Shh patterns the zebrafish retina in a wave-like fashion reminiscent of the MF in *Drosophila*, suggesting that certain elements of insect retinal morphogenesis are conserved during vertebrate retinal determination (Neumann and Nuesslein-Volhard, 2000). Vertebrate homologs of *ey*, *eya*, *so* and *dac* have also been identified and some of these genes have been implicated in promoting normal vertebrate eye development (reviewed by Hanson, 2001). Thus, it is likely that mechanisms similar to those shown in this study exist throughout phylogeny, integrating patterning signals with tissue-specific factors to bestow unique cell fates.

We thank Liqun Luo, Gary Struhl, Francesca Pignoni, Nancy Bonini, Larry Zipursky, Hugo Bellen, the Bloomington stock center, and the Developmental Studies Hybridoma Bank for providing fly stocks and antibodies. We are grateful to Nicholas Baker, who alerted us to the possibility that Hh functions in the eye by relieving Ci repression. We thank Rajendra Kumar, Paul Overbeek, George Halder, Benjamin Frankfort, Richard Davis, Edwin Ostrin and Beril Tavsanli for discussions and comments on the manuscript. Research in the laboratory of G.M. is supported by funds from the National Eye Institute (R01 EY11232-01), the Retina Research Foundation and an Ophthalmology Core Grant from the National Eye Institute (EY02520).

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