Mechanism of hedgehog signaling during Drosophila eye development

Kartik S. Pappu¹, Rui Chen², Brooke W. Middlebrooks³, Catherine Woo⁴, Ulrike Heberlein⁵ and Graeme Mardon¹,²,³,⁵,⁶,⁷,*

¹Program in Developmental Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
²Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
³Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
⁴Biomedical Initiatives, University-wide AIDS Research Program, University of California Office of the President, 300 Lakeside Drive, 6th Floor, Oakland, CA 94612, USA
⁵Department of Anatomy, University of California San Francisco, Box 0452, 513 Parnassus, San Francisco, CA 94143-0452, USA
⁶Department of Ophthalmology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
⁷Department of Neurosciences, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

*Author for correspondence (e-mail: gmardon@bcm.tmc.edu)

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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 (Ci⁵⁶). Furthermore, our results suggest that stabilized, full length Ci (Ci⁵⁶) 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<sup>rep</sup>. Additionally, loss-of-function analyses suggest that the full length, activated Ci<sup>act</sup> 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<sup>16+</sup> mutation is a genetic null and was provided by Gary Struhl (Chen and Struhl, 1998). The hh<sup>P30</sup> 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-GALA 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<sup>16+</sup>- 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<sup>16+</sup>- clones, a variation of the MARCM technique was employed (Lee and Luo, 1999). Generally, y w hs-FLP; smo<sup>16+</sup> FRT40A/CyO; UAS-gene(s)/TM6B, Tb females were crossed to w; M(2)24F arm-lacZ/tub-GAL80 FRT40A/Bc Elp; ey-GALA 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 recombinated the M(2)53<sup>3</sup> 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[c11] hsp70-GFP arm-lacZ M(2)53/FRT42; ci<sup>1</sup> y<sup>w</sup>

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
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\textsuperscript{acs}), 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\textsuperscript{cpp}) (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\textsuperscript{cpp}. In the posterior compartment, however, the absence of Ci\textsuperscript{cpp} 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\textsuperscript{cpp} 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\textsuperscript{cpp}. 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\textsuperscript{lacZ} reporter (hh\textsuperscript{P3055}) 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.
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^116-^, 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
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. 3).
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 penetration (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 anterior 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<sup>rep</sup>, which normally represses eya expression.

**eya, 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<sup>rep</sup>. 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<sup>rep</sup> and converts it into an activated form, Ci<sup>act</sup>, in the anterior compartment of the wing disc. Ci<sup>act</sup> 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<sup>rep</sup>.

**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).
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 CiREP.

The adult Drosophila eye develops normally in the absence of ci

The data from our ectopic expression analyses in the wing disc suggest that CiREP has a major role in blocking eya expression in areas that are not exposed to Hh signaling. However, CiACT 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 Hb independent. By contrast, Ci_{rep} persists in the absence of smo function and thus 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\textsuperscript{rep}. Furthermore, our data demonstrate 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\textsuperscript{act} into Ci\textsuperscript{rep}, thus allowing initiation of dpp expression. Once dpp expression is established, the absence of Ci\textsuperscript{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 Nusslein-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.

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