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Progression of the morphogenetic furrow in the *Drosophila* eye: the roles of Hedgehog, Decapentaplegic and the Raf pathway

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SUMMARY

During *Drosophila* eye development, Hedgehog (Hh) protein secreted by maturing photoreceptors directs a wave of differentiation that sweeps anteriorly across the retinal primordium. The crest of this wave is marked by the morphogenetic furrow, a visible indentation that demarcates the boundary between developing photoreceptors located posteriorly and undifferentiated cells located anteriorly. Here, we present evidence that Hh controls progression of the furrow by inducing the expression of two downstream signals. The first signal, Decapentaplegic (Dpp), acts at long range on undifferentiated cells anterior to the furrow, causing them to enter a 'pre-proneural' state marked by upregulated expression of the transcription factor Hairy. Acquisition of the pre-proneural state appears essential for all prospective retinal cells to enter the proneural pathway and differentiate as photoreceptors. The second signal, presently unknown, acts at short range and is transduced via activation of the Serine-Threonine kinase Raf. Activation of Raf is both necessary and sufficient to cause pre-proneural cells to become proneural, a transition marked by downregulation of Hairy and upregulation of the proneural activator, Atonal (Ato), which initiates differentiation of the R8 photoreceptor. The R8 photoreceptor then organizes the recruitment of the remaining photoreceptors (R1-R7) through additional rounds of Raf activation in neighboring pre-proneural cells. Finally, we show that Dpp signaling is not essential for establishing either the pre-proneural or proneural states, or for progression of the furrow. Instead, Dpp signaling appears to increase the rate of furrow progression by accelerating the transition to the pre-proneural state. In the abnormal situation in which Dpp signaling is blocked, Hh signaling can induce undifferentiated cells to become pre-proneural but does so less efficiently than Dpp, resulting in a retarded rate of furrow progression and the formation of a rudimentary eye.

Key words: *Drosophila*, Hedgehog, Decapentaplegic, Atonal, Morphogenetic furrow, Eye

INTRODUCTION

The adult retina of *Drosophila* is composed of a crystalline array of repeating units, ommatidia, which are themselves highly organised structures containing a precise, stereotyped pattern of photoreceptors and support cells (Ready et al., 1976). Ommatidia first appear in dorsal-ventral columns at the posterior edge of the retinal primordium. As these units mature they induce columns of undifferentiated cells located anteriorly to initiate ommatidial development. The first cell of each nascent ommatidium to differentiate as a photoreceptor is the R8 cell, which subsequently recruits other photoreceptors into the growing cluster (Tomlinson and Ready, 1987). Immediately anterior to the most recently formed column of R8 cells is a physical indentation, the morphogenetic furrow, that runs parallel to the ommatidial columns. As the eye develops, the furrow progresses anteriorly, ahead of the photoreceptors, marking the crest of a wave of retinal differentiation that sweeps across the eye. The rate of progression of this wave of differentiation determines the size of the eye, while the orderly recruitment of differentiating cells behind the wave generates the pattern of the retina.

The movement of the morphogenetic furrow, and concomitant photoreceptor differentiation, is driven by the extracellular signaling molecule Hedgehog (Hh). Hh is secreted by differentiated photoreceptors located many cell diameter lengths posterior to the furrow. Loss of Hh severely retards, or halts, furrow progression (Heberlein et al., 1993; Ma et al., 1993; Treisman and Heberlein, 1998). Conversely, ectopic activation of the Hh transduction pathway, produced either by directly expressing Hh (Heberlein et al., 1995) or activating downstream components of the pathway (Dominguez and Hafen, 1997; Ma and Moses, 1995; Pan and Rubin, 1995; Strutt et al., 1995; Wehrli and Tomlinson, 1995), triggers ectopic photoreceptor differentiation and the formation of an ectopic furrow ahead of the endogenous furrow.

Although Hh is essential for orchestrating furrow progression, the mechanisms involved have remained

uncertain. Of particular interest are the results of experiments in which Hh signal transduction is blocked in clones of cells by the elimination of Smoothened (Smo), an essential component of the Hh signal transduction pathway (Alcedo et al., 1996; Chen and Struhl, 1996; van den Heuvel and Ingham, 1996). Although clones of cells lacking Smo function cannot transduce Hh, they nevertheless remain capable of forming wild-type ommatidia (Strutt and Mlodzik, 1997; Domínguez, 1999), presumably in response to signals induced by Hh in neighboring, wild-type tissue.

Here we show that Hh controls furrow progression as well as the initiation of photoreceptor differentiation by inducing at least two other signals. The first signal is Decapentaplegic (Dpp), which is expressed within and ahead of the morphogenetic furrow (Blackman et al., 1991). We show that Dpp acts at long range from Hh secreting cells to shift cells in front of the furrow from a naive, undifferentiated state to a discrete 'pre-proneural' state. This pre-proneural state is marked by the upregulation of a proneural repressor, Hairy, which together with a related repressor, Extramacrochaetae (Emc), blocks the further transition of these cells to the proneural state.

In addition, we find that Hh induces a second signal, as yet unknown, which operates at short range to shift pre-proneural cells to the proneural state. This transition is marked by the downregulation of Hairy and the expression of Atonal (Ato), a proneural activator that is responsible for initiating differentiation of the R8 photoreceptor, the founding cell of the ommatidium (Jarman et al., 1994). This second signal is transduced by activation of the Serine-Threonine kinase Raf and we show that Raf activity is both necessary and sufficient to induce pre-proneural cells to express Ato, become proneural, and differentiate as R8 photoreceptors.

Finally, we present evidence that acquisition of the pre-proneural state is essential for any cell in the retinal primordium to enter the proneural pathway and differentiate as a photoreceptor in response to Raf activation. Although Dpp signaling is sufficient to establish the pre-proneural state, it is not essential. Instead, Hh itself can induce the pre-proneural state in the absence of Dpp signaling, albeit at shorter range. Under these circumstances, the morphogenetic furrow still progresses, but at an abnormally slow rate, resulting in a rudimentary eye. These findings lead us to propose that the primary role of Dpp signaling in the eye is to accelerate the transition of undifferentiated cells to the pre-proneural state. This acceleration is responsible for the normally rapid rate of furrow progression, and hence for size of the eye.

MATERIALS AND METHODS

Mutations

The following null or amorphic alleles for *smo*, *tkv*, *raf* and *DER* were used: *smo*^{3} (Chen and Struhl, 1996), *tkv*^{strII} (Nellen et al., 1994), *Egfr*^{top18A} (Price et al., 1989) and *raf*^{EA75} (Melnick et al., 1993).

Transgenes

The following previously described transgenes were used: *arm-lacZ* (inserted on 2L or 2R; Vincent et al., 1994), *arm-Gal4* (Sanson et al., 1996), *Actin5C>raf*$^{+}$*>nuc-lacZ* (Struhl and Basler, 1993), *dpp-lacZ3.0* (Blackman et al., 1991), *FRT 39* (Chou and Perrimon, 1992), *FRT42* (Xu and Rubin, 1993), *hs-flp* (inserted on chromosomes 1 or 3; Struhl and Basler, 1993), and *UAS-tkv*^{Q253D}, *UAS>CD2 y*$^{+}$*>tkv*^{Q253D} and *UAS>CD2 y*$^{+}$*>dpp* (Nellen et al., 1996).

Tubulinα1> f^{+}*y*$^{+}$*>raf**, an activated form of the *raf* gene, *raf**, was generated by fusing the coding sequence for the N-terminal myristylation signal of *Drosophila* Src (MGNKCCSKRQDTMA) to that of a constitutively active form of Human Raf (22W pΔraf, a 305 amino acid N-terminal truncation of human Raf (Stanton et al., 1989; Casanova et al., 1994). The *raf** gene was then inserted into a P-element transformation vector (*C20NXT* with the *ry* gene deleted), downstream from the *Tubulinα1* promoter, and interrupted by an 'FRT cassette' containing the *forked*$^{+}$ *and yellow*$^{+}$ genes (Zecca et al., 1995).

Activating and removing gene activity

Clones of *tkv*, *smo*, *DER* and *smo tkv* mutant cells were generated by FRT-mediated recombination (Golic and Lindquist, 1989; Xu and Rubin, 1993). To express Raf*, Dpp and Tkv^{Q253D}, or to remove Raf function, various permutations of the 'flp-out' technique (Struhl and Basler, 1993) were utilized as indicated below. To create clones by mitotic recombination, first or second instar larvae were heat shocked for 60 minutes at 37°C. To catalyse uniform expression from 'flp-out' transgenes, larvae were heat shocked for 60 minutes at 37°C. To produce rare, isolated clones by the flp-out technique, first or second instar larvae were heat shocked for 30 minutes at 34°C.

Experimental genotypes

Activating Raf: *hsp70-flp/+* or *Y; Tubulin α1> f*$^{+}$*y*$^{+}$*>raf*/+*.
Removing Raf: *raf*^{EA75} *hsp70-flp; Actin5C>raf*$^{+}$*>nuc-lacZ/+*.
Activating Dpp: *hsp70-flp/+* or *Y; arm-Gal4/UAS>CD2 y*$^{+}$*>dpp*.
Activating Tkv: *hsp70-flp/+* or *Y; arm-Gal4/UAS>CD2 y*$^{+}$*>tkv*^{Q253D}*/+*.
Removing Tkv: *hsp70-flp; tkv*^{strII} *FRT39E/arm-lacZ FRT39E*.
Removing Smo: *hsp70-flp; smo*^{3} *FRT39E/arm-lacZ FRT39E*.
Removing both Tkv and Smo: *hsp70-flp; smo*^{3} *tkv*^{strII} *FRT39E/arm-lacZ FRT39E*.
Removing DER: *hsp70-flp; FRT42 Egfr*^{top18A}*/FRT42 arm-lacZ KP135* (*KP135* is a P-element insertion that causes a Minute phenotype; Hart et al., 1993).

Imaginal disc staining

Standard protocols for immunofluoresence (Struhl and Basler, 1993) were followed using mouse αCD2 (OX34; Serotec), rabbit αβgal (Cappel), mouse αβgal (Promega), mouse α22C10 (Zipursky et al., 1984), rabbit αato (Jarman et al., 1994), rat αhairy (Howard and Struhl, 1990), rat αCi (Motzny and Holmgren, 1995), mouse αhairy (Carroll and Whyte, 1989) and mouse αelav antisera (O'Neill et al., 1994).

RESULTS

Hh regulates Atonal and Hairy expression and induces photoreceptor differentiation through the action of other signaling molecules

Hh, secreted by maturing photoreceptor cells, is normally responsible for inducing cells within and ahead of the morphogenetic furrow to initiate photoreceptor differentiation (reviewed in Treisman and Heberlein, 1998). Nevertheless, cells which lack Smoothened (Smo) function, and hence the ability to transduce Hh, can form normal ommatidia (Strutt and Mlodzik, 1997; Domínguez, 1999). These findings suggest that Hh can induce photoreceptor differentiation in Smo-deficient cells through the induction of other signaling molecules in neighboring wild-type tissue. As a first step towards identifying such secondary signals and analyzing their roles, we have

examined the consequences of creating clones of cells homozygous for *smo*^{3}, an amorphic mutation, on two early markers of retinal development, the expression of Ato and Hairy, which are expressed in adjacent dorso-ventral stripes within and anterior to the morphogenetic furrow.

Ato expression has two prominent phases in the developing eye (Dokucu et al., 1996; Sun et al., 1998; Fig. 1A,B; see also Fig. 7A). In the first phase, Ato is expressed uniformly in a narrow dorso-ventral swath of cells, which demarcates the anterior edge of the furrow. This uniform swath then breaks up into small clusters of Ato expressing cells and resolves into the second phase, a spaced pattern of single Ato expressing cells (the future R8 photoreceptor cells). The first phase of Ato expression is severely reduced or absent in clones of *smo*^{3} cells, similar to large clones that lack Hh (Domínguez and Hafen, 1997). However, the second phase of expression still occurs, even though it is displaced posteriorly indicating that it is delayed (Fig. 1A,B). This displacement is more severe in the middle of the clone than along the dorsal and ventral borders, producing a crescent shaped distortion of the line of spaced single cells that express Ato. We conclude that cells within *smo*$^{-}$ mutant clones can be induced to express Ato even though they cannot receive Hh, provided that they are located near to wild-type cells across the clone border.

Equivalent effects were observed for Hairy expression. Hairy is normally expressed at peak levels in a dorso-ventral stripe positioned immediately anterior to the Ato stripe, but is abruptly downregulated in more posteriorly situated cells (Carroll and Whyte, 1989; Brown et al., 1995; Fig. 1C,D; see also Fig. 7A). Clones of *smo*^{3} cells have only a modest effect on Hairy expression anterior to the furrow, causing a slight, but consistent, posterior displacement of the anterior edge of the stripe. However, they are associated with a pronounced failure to repress Hairy expression in some, but not all, posteriorly situated *smo*^{3} cells (Fig. 1C). As in the case of Ato expression, the exceptional mutant cells which retain the normal downregulation of Hairy are those positioned close to the lateral and posterior borders of the clones. Just within the lateral border, we typically observe a line of cells, one or two cell diameter lengths wide, where Hairy expression is repressed (e.g. Fig. 1D). Along the posterior border, the zone of mutant cells in which Hairy expression is repressed is usually wider.

We interpret these results to indicate (1) that Hh normally induces cells to express a secondary signal (or signals), which can activate Ato expression and repress Hairy expression; (2) that this signal acts non-autonomously, allowing it to move from wild-type cells where it is induced by Hh to nearby *smo*^{3} cells where it regulates Ato and Hairy expression; and (3) that the range of this signal is short, restricting its action to only one or two cells across the lateral borders of *smo*^{3} mutant clones. A somewhat greater range of action is apparent along the posterior borders of such clones, perhaps because the adjacent wild-type cells were induced by Hh to send this signal at an earlier time than those along the lateral (more anterior) borders of the clone, allowing the signal more time to accumulate to higher levels and to move deeper into mutant tissue.

To examine how the posterior displacements in Ato and Hairy regulation in *smo*^{3} clones influence subsequent ommatidial development, we examined the expression of the protein Elav, a marker of photoreceptor differentiation (Robinow and White, 1988). As shown in Fig. 2, clones of *smo*^{3} cells are capable of differentiating as photoreceptors, in agreement with previous findings (Strutt and Mlodzik, 1997; Domínguez, 1999). However, there is a significant delay. In wild-type tissue, Elav expression initiates immediately posterior to the morphogenetic furrow with the specification of the R8 cell and continues as other photoreceptors are recruited into the ommatidial cluster (Fig. 2A). In clones of *smo*^{3} cells, there is a clear posterior displacement in the onset of photoreceptor differentiation in mutant cells (Fig. 2A,B): photoreceptor differentiation is first seen at the posterior, and occasionally lateral, edges of the clone, correlating with the effects of neighboring wild-type tissue on Hairy and Ato expression and indicating a general delay in photoreceptor differentiation. However, as seen in more posteriorly situated clones (Fig. 2C), most or all of the *smo*^{3} tissue eventually differentiates as normally patterned ommatidia.

Thus, Hh signal transduction is not autonomously required for presumptive eye cells to express Ato, downregulate Hairy, or differentiate as photoreceptors. This is in contrast to the general requirement for Hh signaling revealed by experiments in which Hh signaling is blocked throughout the entire disc using temperature-sensitive *hh* mutations (Ma et al., 1993). In the latter case, loss of Hh signaling causes a rapid and complete block in photoreceptor differentiation and furrow progression. As we demonstrate below, Hh signaling appears to induce at least two secondary signals that are essential for the normal recruitment of undifferentiated cells to form the R8 photoreceptors. One of them appears to be the short-range signal that can induce Ato expression and repress Hairy in clones of *smo*$^{-}$ cells. The second, Decapentaplegic (Dpp), appears to act at longer range to prime cells to receive this short range signal.

Dpp, induced by Hh, acts on cells anterior to the furrow to upregulate Hairy expression and regulate the rate of furrow progression

One candidate for a secondary signal, which acts downstream of Hh in the developing retina, is the TGF-β homologue Dpp. Dpp is induced by Hh just anterior to the morphogenetic furrow (Heberlein et al., 1993). Moreover, experiments in other discs have established that Dpp can act at long range from its source to mediate the organizing activity of Hh on more anteriorly situated tissue (Zecca et al., 1995; Lecuit et al., 1996; Nellen et al., 1996). However, previous studies have shown that Dpp signaling is not essential for either photoreceptor differentiation or propagation of the furrow once photoreceptor differentiation initiates at the posterior edge of the eye primordium (Burke and Basler, 1996; Wiersdorff et al., 1996; Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997). These findings challenge the notion that Dpp mediates the organizing activity of Hh in front of the furrow.

To test whether Dpp has such a organizing role we performed two kinds of experiments. In the first, we ectopically expressed Dpp, or activated Thickveins (Tkv), a type I TGFβ receptor required for all known Dpp activities (Ruberte et al., 1995), anterior to the furrow. In the second, we blocked Dpp expression or Tkv activity. The results of these experiments indicate that Dpp signaling is both necessary and sufficient to upregulate Hairy expression anterior to the furrow and to

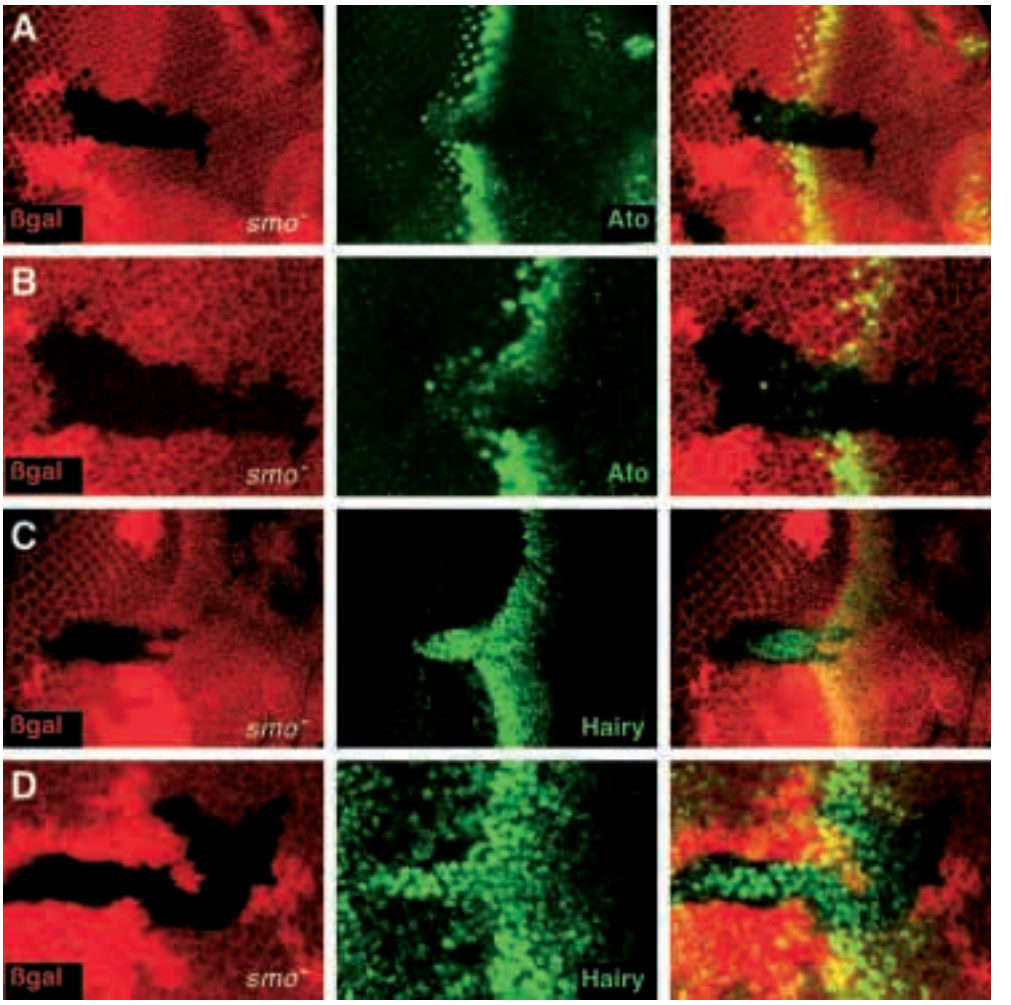

Fig. 1. Ato and Hairy expression in clones of cells that cannot transduce Hh owing to the loss of Smo activity. The expression of either Ato (A,B) or Hairy (C,D) is shown in green in eye disks containing *smo*⁻ clones marked by the absence of β-galactosidase (red). Here, and in the remaining figures, posterior is to the left and the morphogenetic furrow is in the process of sweeping from left (maturing photoreceptors) to right (undifferentiated cells). In *smo*⁻ clones, the first phase of Ato expression, a uniform stripe within the furrow, is greatly reduced or absent, and the second phase, spaced expression in single presumptive R8 cells, appears to be delayed relative to neighboring wild-type cells. (B) is a higher magnification view of A. (C,D) The expression of Hairy ahead of the furrow appears normal in *smo*⁻ cells. However mutant cells located posteriorly still express Hairy at high levels, where it would normally be downregulated, except in cells along the posterior and lateral borders of the clone where Hairy expression is repressed. D is shown at higher magnification than C.

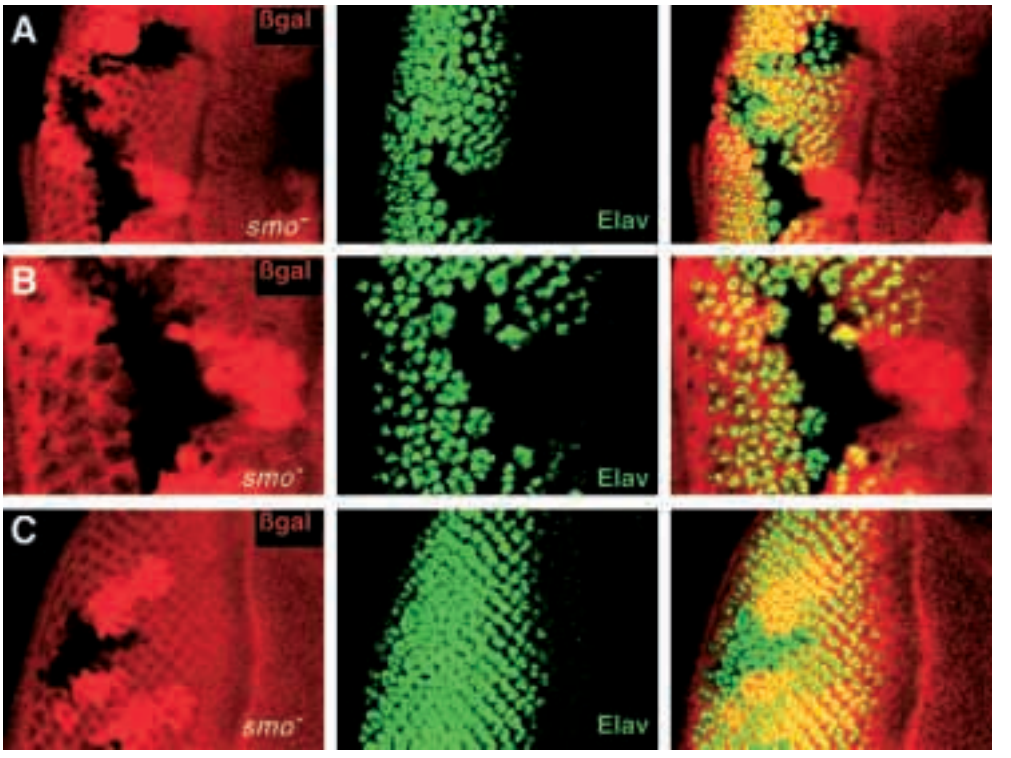

Fig. 2. Loss of Hh signal transduction delays, but does not block, photoreceptor differentiation. Photoreceptor differentiation, as visualised by Elav expression, is shown in green in eye disks containing *smo*⁻ clones marked by the absence of β-galactosidase (red) as in Fig. 1. (A) Photoreceptor differentiation occurs in *smo*⁻ clones, but is delayed relative to neighboring wild-type tissue, appearing first in more posterior and lateral portions of clones. (B) is a higher magnification view of A. (C) Such clones eventually form a normally spaced pattern of mature ommatidia, which appears indistinguishable from that of surrounding wild-type tissue.

maintain the normal rate of furrow progression, but that it is neither necessary nor sufficient to activate Ato expression and initiate photoreceptor differentiation in more posterior cells.

Ectopic Dpp signaling

Dpp was constitutively expressed in most cells of the eye primordium, including those ahead of the furrow, using a combination of the Flp-out and Gal4/UAS techniques (Nellen et al., 1996; see Materials and Methods). Normally, Hairy is expressed at high level in a stripe of cells positioned just anterior to the furrow, its level of expression fading in progressively more anterior cells to form a gradient (e.g. Fig. 3A,C; see also Fig. 7A). However, in eye discs in which Dpp expression is induced in most cells, Hairy is expressed at uniformly high levels in presumptive eye cells anterior to the furrow (Fig. 3B,D). In contrast, there appears to be little if any alteration in the behavior of cells within or behind the furrow: both the expression of Ato as well as the pattern of neural differentiation (assayed by staining with the monoclonal antibody MAb22C10; Zipursky et al., 1984) appear normal.

Ectopic activation of Tkv

A constitutively active form of Tkv, Tkv^{Q253D}, was expressed, as above, except using a modification of the Flp-out/Gal4 technique to generate isolated clones of tkv^{Q253D} expressing cells marked by the loss of expression of the reporter protein CD2 (Nellen et al., 1996; see Materials and Methods). We find that clones of $UAS>tkv^{Q253D}$ cells express uniformly high levels of Hairy when they are located ahead of the furrow, even in regions where the level of Hairy expression is normally low or undetectable (Fig. 3E). Moreover, this high-level Hairy expression appears to be autonomous to the clone, indicating that Hairy is normally upregulated only in anterior eye cells in which the Dpp signal transduction pathway is active. Clones of $UAS>tkv^{Q253D}$ cells located within or behind the furrow appear to develop normally in terms of Hairy expression and photoreceptor differentiation (Fig. 3F; data not shown), except that $UAS>tkv^{Q253D}$ cells in the vicinity of the furrow are associated with a slight anterior displacement in the stripe of Hairy expression (Fig. 3F). This anterior displacement suggests that the furrow has accelerated as it progressed through the clone. These results, as well as the results obtained with ectopic expression of Dpp, suggest that Dpp signaling anterior to the furrow normally induces high levels of Hairy expression, but is not sufficient to initiate photoreceptor differentiation. They also suggest that Dpp signaling positively regulates the rate of furrow progression.

Absence of Dpp signaling

The dpp^{blk} mutation is associated with a deletion of *cis*-acting regulatory sequences that are essential for Hh-dependent transcription of *dpp* in the eye

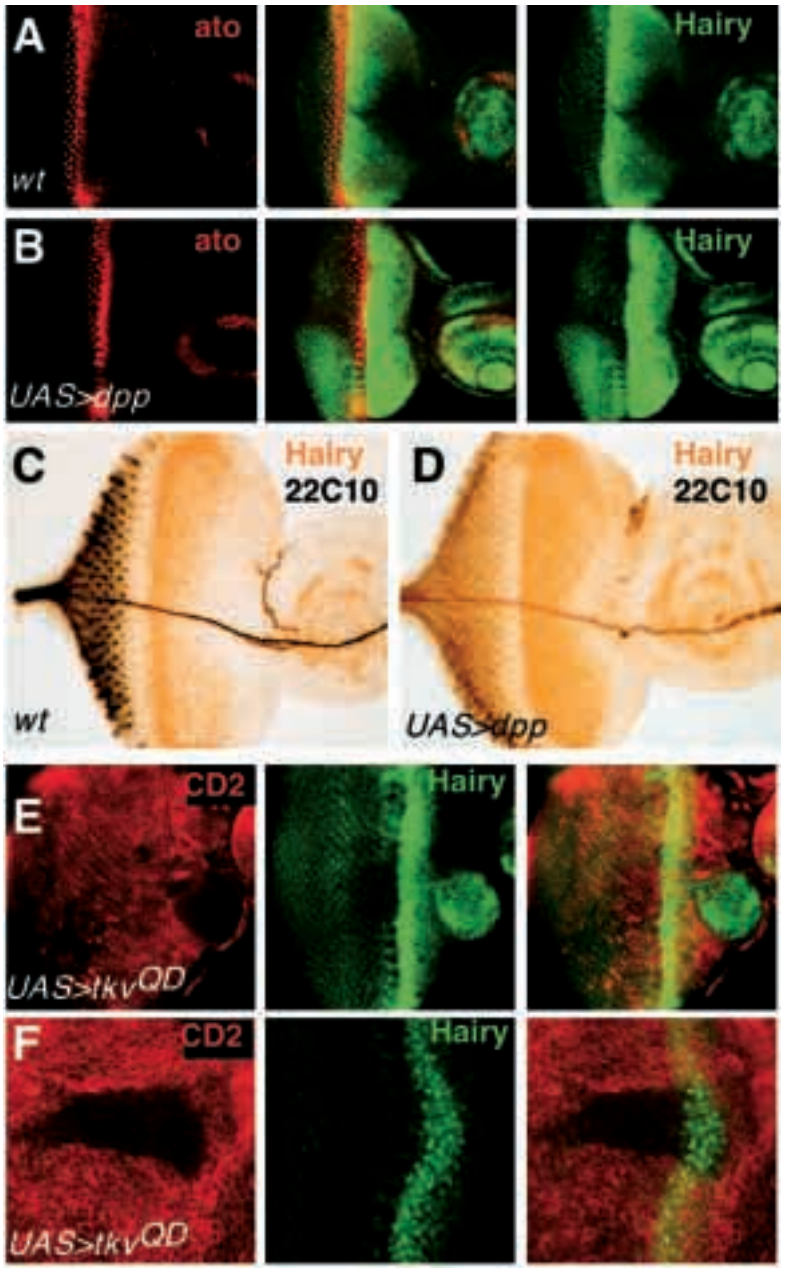

Fig. 3. Ectopic Dpp signaling induces upregulation of Hairy expression anterior to the furrow. Wild-type eye disks (A,C), or disks ectopically expressing Dpp (B,D) or Tkv^{Q253D}, an activated Dpp receptor (E,F), visualized for Hairy (green in A,B,E,F; brown in C,D), Ato (red in A,B), and mAb 22C10 (black in C,D), a marker of neural differentiation. Ectopic Dpp expression (B,D) is provided indiscriminately throughout the disc using a severe heat shock applied 12-15 hours before staining, whereas ectopic expression of Tkv^{Q253D} (E,F) is provided in isolated clones of cells induced 72 hours prior to staining and marked by the absence of CD2 staining (red). In wild-type disks Hairy is expressed at high levels immediately anterior to the morphogenetic furrow with expression tapering off in a graded fashion further anteriorly (A,C). Indiscriminate Dpp expression (B,D) causes Hairy to be expressed uniformly at peak levels throughout the anterior portion of the disk. However no change is seen in either Ato or mAb 22C10 expression. (E,F) Ectopic expression of Tkv^{Q253D} in clones located anterior to the furrow causes the cell-autonomous upregulation of Hairy expression (E). No effect on Hairy expression is seen in clones that lie behind the furrow (F). However in these clones there appears to be a slight increase in the rate of furrow progression, as seen by the modest anterior bulge in the furrow visualized by Hairy expression. (F) is shown at a higher magnification than E.

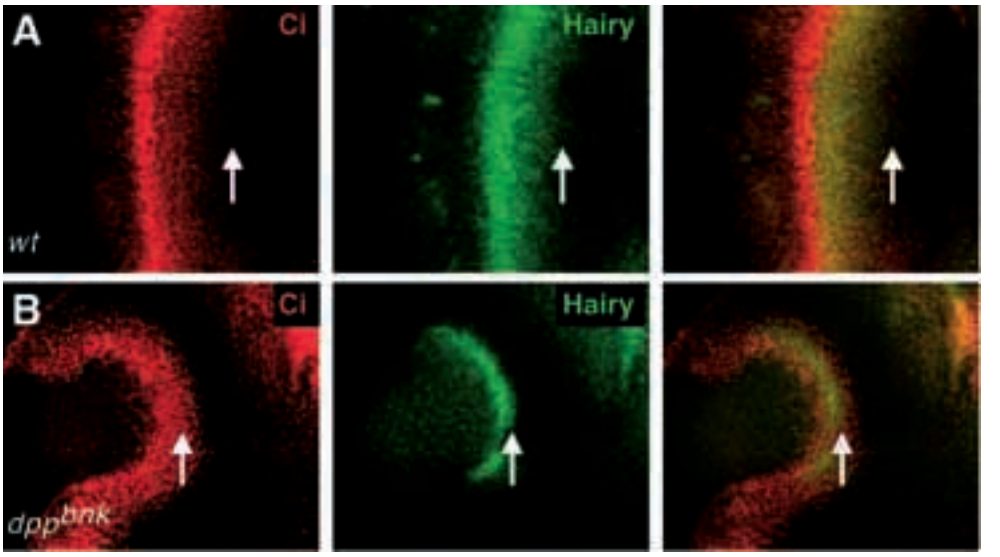

Fig. 4. Upregulation of Hairy expression does not occur anterior to the furrow in *dpp*^{blk} eye discs. Wild-type (A) and *dpp*^{blk} (B) disks stained for Ci (red) and Hairy (green) expression. Arrows indicate the anterior extent of Hairy expression in each disk. In wild type (A), the furrow is marked by peak levels of expression of Ci, which is upregulated in response to Hh secreted by maturing photoreceptors behind the furrow. The stripe of peak Hairy expression is located just anterior to that of Ci, with Hairy expression declining in a graded fashion more anteriorly. In *dpp*^{blk} (B), Hairy is not expressed anterior to the furrow (marked by peak Ci expression), indicating that its upregulation anterior to the furrow in wild-type discs depends on Dpp signaling. Conversely, Hairy is expressed at a high level within the stripe of maximal Ci expression, behind the leading edge of the furrow, a situation which does not normally occur in wild-type discs. This abnormal, posterior expression of Hairy also occurs when Dpp signal transduction is blocked (see Fig. 5) and is dependent on Hh signaling (see Fig. 6 and text). We note that the stripe of peak Hairy expression overlaps that of Ci only in the middle portion of the furrow in *dpp*^{blk} discs (B). We attribute the lack of Hairy expression at the dorsal and ventral extremes of the Ci stripe to Wingless (Wg), which represses photoreceptor differentiation at the dorsal and ventral margins of the eye primordium (Ma and Moses, 1995; Treisman and Rubin, 1995).

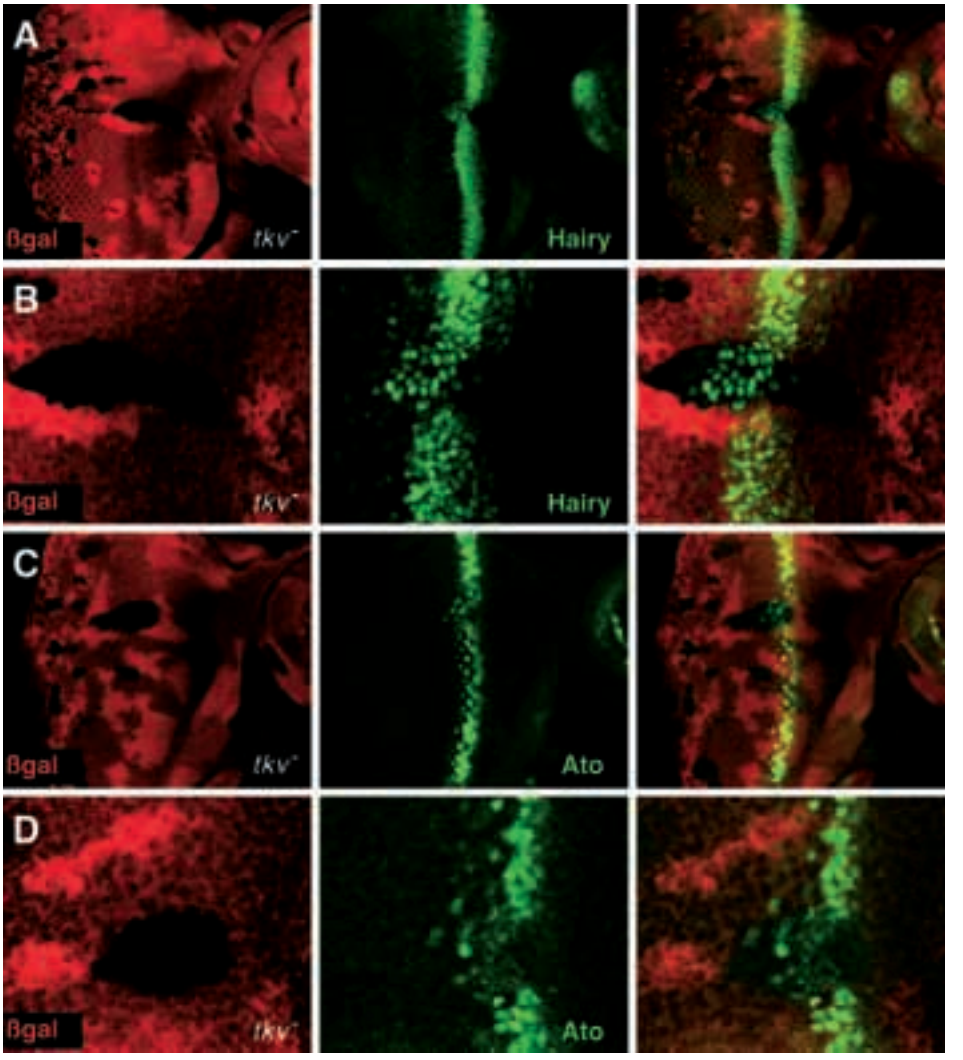

Fig. 5. Ato and Hairy expression in clones of cells that cannot transduce Dpp owing to the loss of Tkv activity. The expression of either Hairy (A,B) or Ato (C,D) is shown in green in eye disks containing *tkv*$^{-}$ clones marked by the absence of β-galactosidase (red). (A,B) The normal upregulation of Hairy expression does not occur in *tkv*$^{-}$ cells. However, Hairy is expressed in *tkv*$^{-}$ cells located at more posterior positions, next to neighboring, wild-type cells that are located behind the furrow and no longer express Hairy. (B) is a higher magnification view of A. (C) Ato expression is also displaced posteriorly in clones of *tkv*$^{-}$ cells compared with neighbouring wild-type cells. (D) A higher magnification view of a *tkv*$^{-}$ mutant clone shows that the initial phase of high, uniform expression of Ato is greatly reduced or absent in the clone. Clones were induced 72 hours prior to staining.

(Masucci et al., 1990). As a result, Dpp signaling in the eye disc is abolished or severely reduced anterior to the furrow, and the resulting eye is greatly reduced in size in both the dorsal-ventral and antero-posterior axis. We compared Hairy expression in wild-type and *dpp^{blk}* disks, using the upregulation of Cubitus interruptis (Ci), a protein that is stabilized in response to Hh signaling (reviewed in Ingham, 1998), as a marker of the position at which the furrow should normally form (Fig. 4).

In wild-type eye discs, Ci accumulates to peak levels in a dorso-ventral stripe of cells just posterior to the stripe of peak Hairy expression (Fig. 4A), consistent with our finding that Hairy expression is repressed in response to Hh signaling within the furrow (Fig. 1C,D), but is activated by Dpp signaling anterior to the furrow (Fig. 3). In contrast, the stripe of maximal Hairy expression is displaced posteriorly in *dpp^{blk}* discs relative to the stripe of maximal Ci expression (Fig. 4B). Moreover, the furrow appears to have moved only a small distance from the posterior edge of the presumptive eye primordium, even in eye discs from mature third instar larvae, consistent with the 'small eye' phenotype observed in the adult.

These results indicate that Dpp signaling is normally required to activate high level Hairy expression in a stripe positioned just anterior to the furrow. They also indicate that Dpp signaling is necessary to sustain the normal rate of furrow progression. Finally, they suggest that Dpp signaling influences the response of cells to peak levels of Hh signal transduction: Hairy expression is downregulated in these cells in wild-type discs, but not in *dpp^{blk}* discs.

Absence of Tkv activity

Tkv function was eliminated in clones of marked cells using Flp-mediated mitotic recombination (Golic, 1991; see Materials and Methods). As shown in Fig. 5, clones of cells homozygous for *tkv^{strII}*, a null allele (Nellen et al., 1994), are associated with a severe reduction in Hairy expression anterior to the normal position of the furrow. In addition there appears to be a posterior displacement of Hairy expression, so that the gene is upregulated in neighboring, posteriorly situated cells where it would normally be downregulated. Based on these results, as well as those obtained using the *dpp^{blk}* allele, we infer that there are at least two factors in the eye capable of upregulating Hairy expression. The first is Dpp, which is normally required for the upregulation of Hairy anterior to the furrow. The second is a factor that can induce Hairy expression within and behind the furrow, but which does so only in the abnormal situation in which Dpp signaling is blocked. As we show below, this second factor is Hh itself.

Ato expression is also altered in *tkv*$^{-}$ clones (Fig. 5C,D). In particular, the first phase of uniform, high level Ato expression is absent or greatly reduced, correlating with the displaced, posterior expression of Hairy. However, the second phase of spaced, single cell expression of Ato in the presumptive R8 cells is apparent within the mutant clones, although it is displaced posteriorly relative to spaced single cells expression of Ato in neighboring, wild-type tissue (Fig. 4C,D). The posterior displacements we observe in both Ato and Hairy expression indicate that the rate of furrow progression is retarded in mutant cells, corroborating previous findings that *tkv* mutant clones cause delays in subsequent steps of photoreceptor differentiation (Burke and Basler, 1996). Thus,

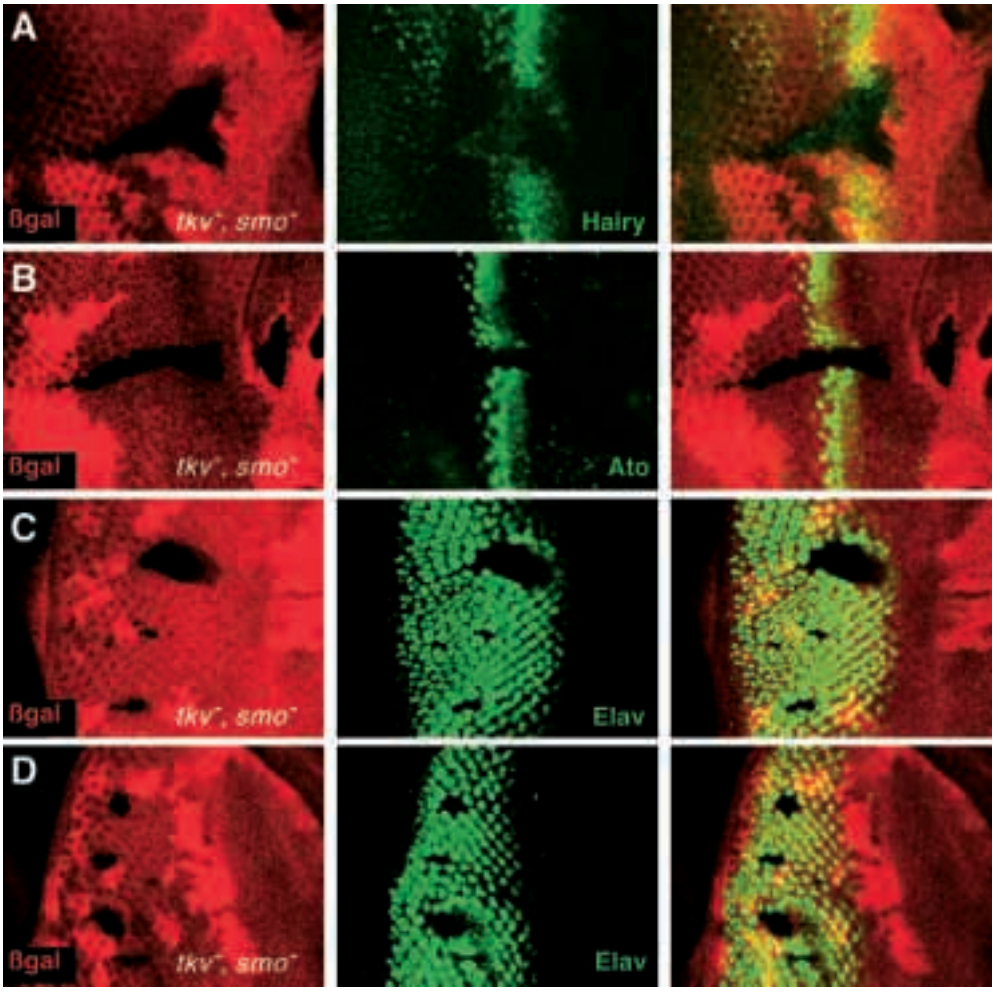

Fig. 6. *smo*$^{-}$ *tkv*$^{-}$ cells fail to express Hairy and Ato, and cannot differentiate as photoreceptors. Eye disks containing *smo*$^{-}$ *tkv*$^{-}$ double mutant clones stained for the expression of Hairy (A), Ato (B) or Elav (C,D) in green and marked by the absence of β-galactosidase (red). Hairy expression is not upregulated in clones of *smo*$^{-}$ *tkv*$^{-}$ cells (A), and we cannot detect expression of Ato (B). Similarly, *smo*$^{-}$ *tkv*$^{-}$ cells fail to differentiate as photoreceptors, as indicated by the complete absence of Elav expressing mutant cells, even several ommatidial columns posterior to the furrow (C,D). Clones were induced 96 hours prior to staining.

our results with both the *dpp^{blk}* mutation and *tkv*$^{-}$ mutant clones show that Dpp signaling is not essential for Ato expression or for photoreceptor differentiation. However, Dpp signaling does appear to be necessary to prime cells anterior to the furrow so that they are incorporated into the furrow at the normal rate.

Simultaneous loss of Hh and Dpp signal transduction blocks Hairy upregulation, Ato expression and photoreceptor differentiation

In the absence of Dpp signaling, Hairy upregulation and Ato expression are retained, but are displaced posteriorly so that they now occur in cells that are likely to be exposed to peak levels of Hh (Figs 4, 5). To determine whether this delayed expression is induced directly by Hh signaling or is due to another signaling molecule induced in response to Hh, we generated clones of cells lacking the activities of both Smo as well as Tkv (see Materials and Methods). We find that *smo^{3} tkv^{strII}* clones fail to express high levels of Hairy in a cell autonomous fashion (Fig. 6A). Similarly, *smo^{3} tkv^{strII}* clones autonomously fail to express Ato (Fig. 6B). These results indicate that in the absence of Dpp signaling, undifferentiated eye cells must receive Hh in order to upregulate Hairy and express Ato.

We note that the loss of both Hh and Dpp signal transduction not only prevents Ato expression, which is required for R8 photoreceptor differentiation, but also results in a general block in photoreceptor differentiation. First, we find that there is a complete and autonomous absence of Elav expression in *smo^{3} tkv^{strII}* clones (Fig. 6C,D). Second, such clones fail to contribute any photoreceptors in the adult eye (G. S. and A. Tomlinson; data not shown). These findings contrast with those obtained from the analysis of clones of *ato* mutant cells (Jarman et al., 1994). Although cells deficient for Ato activity cannot differentiate as R8 photoreceptors, they can be recruited by wild-type R8 cells to make any or all of the remaining photoreceptors in an ommatidial cluster. Thus, presumptive eye cells must receive either Hh or Dpp in order to be able to enter the proneural pathway and differentiate as photoreceptors (see Discussion).

The Serine-Threonine kinase Raf transduces a second signal required for photoreceptor differentiation

Our analysis indicates that Hh orchestrates Hairy and Ato expression as well as furrow progression in a manner that depends on Dpp signaling ahead of the furrow. Yet Dpp signaling is not sufficient to activate Ato or to initiate photoreceptor differentiation (Figs 3, 4). Conversely, cells devoid of Smo, and hence unable to receive Hh, can still be induced by neighboring wild-type cells to express Ato and make photoreceptors (Figs 1, 2). These findings argue for an additional signal involved in mediating the organizing activity of Hh on photoreceptor differentiation and furrow progression. The signal transduction pathway downstream of receptor tyrosine kinases involving Ras and Raf has been shown to have multiple roles during the formation of the *Drosophila* eye, most notably in mediating signals by the Sevenless and Epidermal Growth Factor receptors (reviewed in Wassarman et al., 1995; Freeman, 1997). Further, misexpression of activated forms of Ras, or the EGF receptor, anterior to the furrow can initiate photoreceptor differentiation (Domínguez et al., 1998; Hazelett et al., 1998). We therefore asked whether this signaling pathway might also be utilized in the induction of Ato.

To constitutively activate the Raf transduction pathway in the eye disc, we expressed a myristylated and truncated form of human Raf, which we call Raf*, in clones of cells using the Flp-out technique (Materials and Methods). Under conditions that cause expression of constitutively active Raf* in most cells, we observe that the anterior stripe of peak Ato expression expands several cell diameter lengths forward, to fill the region just anterior to the position of the furrow where Hairy is normally expressed at peak level (compare Fig. 7A,B). However, the anterior limit of peak Ato expression does not extend past that of Hairy expression. Examination of Hairy under the same conditions reveals that the normally high levels of expression just anterior to the furrow are diminished (Fig. 7B). This reduction in Hairy expression, alone, is not likely to account for the expansion of Ato expression, because Ato expression is not altered in eye discs lacking *hairy* activity (Brown et al., 1995). However the combined loss of both Hairy and the related repressor protein Emc causes ectopic Ato expression anterior to the furrow (Brown et al., 1995). In the presence of indiscriminate Raf* activity, we find that Emc expression, like that of Hairy, appears to be diminished anterior to the furrow (data not shown). Hence, we can attribute the expansion of Ato expression to the concomitant reduction in both Hairy and Emc expression.

Constitutive expression of Raf* also induces ectopic photoreceptor differentiation anterior to the furrow, as assayed by the expression of the neuronal antigen 22C10 (Fig. 7C). Similar to the expansion of Ato induced by Raf* (Fig. 7B), ectopic photoreceptor differentiation is restricted to a dorso-ventral stripe of cells just anterior to the normal position of the furrow. Not all the cells within this column differentiate as photoreceptors. Rather, there is a saltatory pattern of differentiation, and we attribute this to the process of lateral inhibition which normally restricts the number of proneural cells that differentiate as photoreceptors (Cagan and Ready, 1989; Baker et al., 1996; Baker and Yu, 1997; Spencer et al., 1998; Chen and Chien, 1999).

Similar results are obtained under conditions that create small, rare clones of cells expressing Raf* (see Materials and Methods). In this case, we observe small, isolated clusters of photoreceptors, located anterior to the endogenous furrow and surrounded by what appears to be an ectopic furrow (marked in this experiment by the expression of a *dpp-lacZ* reporter gene; Fig. 7D). As we observed for neural differentiation induced by widespread expression of Raf* (Fig. 7C), small clones of Raf* expressing cells initiate ectopic photoreceptor development only when they are close to the furrow.

We interpret these findings as follows. During normal development, Dpp signaling anterior to the furrow creates a pool of cells which are primed to enter the proneural pathway, but blocked from doing so by the expression of high levels of Hairy and Emc. We refer to these cells as being 'pre-proneural'. Release from this block requires an additional signal which is induced at short range by Hh signaling and transduced by activation of the Raf pathway.

Raf, but not the *Drosophila* EGF receptor, is required for Ato expression and photoreceptor differentiation

The results seen with activated Raf suggest that endogenous Raf may normally regulate Ato expression. Therefore, Ato expression was examined in clones of cells that lack Raf

function. Clones of *raf*$^{-}$ cells marked by the expression of a nuclear-localized form of β-galactosidase were generated using the Flp-out technique (see Materials and Methods). *Raf*$^{-}$ cells do not express Ato (Fig. 7F-H). Moreover, the absence of Ato expression is cell-autonomous, indicating that Raf is normally required to transduce a signal that is essential for Ato expression.

Raf normally mediates signals from receptor tyrosine kinases (reviewed in Schlessinger and Bar-Sagi, 1994; Marshall, 1995). Two candidate receptor tyrosine kinases, which could activate Raf ahead of the furrow, are the *Drosophila* EGF receptor (DER) and one of the *Drosophila* FGF receptor homologues, Heartless (Htl). DER is expressed at high levels ahead of the furrow (Zak and Shilo, 1992), and is required for photoreceptor differentiation (Xu and Rubin, 1993). Similarly, two ligands for DER, Spitz and Vein, are active within the furrow and regulate photoreceptor differentiation (Tio et al., 1994; Tio and Moses, 1997; Spencer et al., 1998). However, we find that Ato is expressed in *DER*$^{-}$ clones, indicating that DER is not essential for the Raf-dependent activation of Ato expression (Fig. 7E).

Htl is also expressed in the morphogenetic furrow (Shishido et al., 1993; data not shown). Similar to DER, however, removal of Htl has no effect on Ato expression (data not shown). It is possible that both DER and Htl can receive the Ato inducing signal, accounting for the ability of cells lacking one or the other function to initiate Ato expression. Alternatively, Raf may transduce a signal that is received by another receptor.

DISCUSSION

The organizing activity of Hh in most ectodermal derivatives of *Drosophila* depends on their subdivision into anterior (A) and posterior (P) compartments (reviewed in Lawrence and Struhl, 1996). This subdivision is governed by the selective activity of the transcription factor Engrailed (En) in P compartment cells, which directs them to express Hh. Conversely, the absence of En activity in A cells renders them responsive to Hh and immiscible with P cells, creating a stable, inductive interface between P and A cells. Hh signaling across this interface directly controls the development of nearby A compartment cells (Mullor et al., 1996; Strigini and Cohen, 1997; Struhl et al., 1997a,b) and induces secondary signals such as Dpp and Wg, which act at longer range to organize growth and patterning throughout the segment (Basler and Struhl, 1994; Tabata and Kornberg, 1994).

The development of the *Drosophila* eye is a notable exception to this general paradigm for Hh signaling. Here, Hh signaling is not linked to a fixed, compartmental segregation between Hh-sending and Hh-receiving cells. Instead, the entire eye arises from cells within the A compartment, and special mechanisms exist, first, to create a small subpopulation of Hh expressing cells at the posterior edge of the eye primordium (Pignoni and Zipursky, 1997), and second, to recruit additional cells to enter and expand the population of Hh secreting cells anteriorly across the primordium (Heberlein and Moses, 1995; Treisman and Heberlein, 1998). Recruitment depends on the addition of a novel regulatory loop between Hh reception and Hh signaling that is not found in other ectodermal derivatives. In essence, presumptive eye cells that receive an Hh signal are induced to become Hh secreting cells, thereby creating a 'rolling' Hh signaling interface. This novel regulatory loop is also linked to neural differentiation. The first cells in the eye to secrete Hh appear to be the first mature photoreceptors. Hh emanating from these cells then induces anteriorly situated, undifferentiated cells to develop into Hh-secreting photoreceptors, thus linking the rolling Hh interface with the wave of retinal differentiation which progresses across the eye primordium.

Here, we show that normal progression of the Hh-signaling interface across the retinal primordium requires at least two additional signals, both induced by Hh (Fig. 8). The first signal, Dpp, appears to act at long range on anteriorly situated cells to prime them to be responsive to a second, proneural inducing signal. We term such primed cells as 'pre-proneural', a state which correlates with the upregulation of Hairy. The second signal, as yet unknown, appears to act at short range and to induce pre-proneural cells to enter the proneural pathway. Transition to the proneural state depends on activation of the Raf signal transduction pathway and correlates with the upregulation of Ato, a proneural activator. Finally, we find that in the absence of Dpp signaling, undifferentiated cells can still be induced by Hh to adopt the pre-proneural state. However, this transition occurs at shorter range and appears to be less efficient, reducing the rate of furrow progression and the size of the eye. Thus, we infer that Dpp signaling is not required for progression of the furrow, per se, but rather for the normal rate of progression.

The pre-proneural state

In our interpretation of Hh signaling and furrow progression, we propose that undifferentiated cells anterior to the furrow are first primed to become 'pre-proneural', and that acquisition of this distinct cell state is a prerequisite for their further progression to the proneural state, a transition that involves the activation of Raf and consequent upregulation of Ato. It is therefore relevant to ask what properties define pre-proneural cells and distinguish them from the other cells in the eye.

Although we have used the upregulation of Hairy to mark the pre-proneural state, acquisition of this state also correlates with other regulatory changes that occur anterior to the furrow, such as the concomitant upregulation of Daughterless (Da), a proneural activator (Brown et al., 1995, 1996; S. G., unpublished findings), the loss of Cyclin A expression, and the entry of cells into G_1 arrest (Thomas et al., 1994). We note that Hairy and Da, as well as Extramacrochaetae (Emc), a related basic helix-loop-helix transcription factor expressed anterior to the furrow, are likely to be determinants of the pre-proneural state. Hairy and Emc are transcriptional repressors that actively block the expression or activity of proneural activators including Ato and Da. Thus, acquisition of the pre-proneural state appears to involve the upregulation of proneural gene functions such as Da, as well as proneural repressors like Hairy which, together with Emc, holds these proneural activities in check.

This view is supported by the observation that the simultaneous loss of Hairy and Emc activity leads to the precocious differentiation of photoreceptors several cell diameter lengths anterior to the furrow (Brown et al., 1995). The ectopic differentiation thus revealed appears to coincide

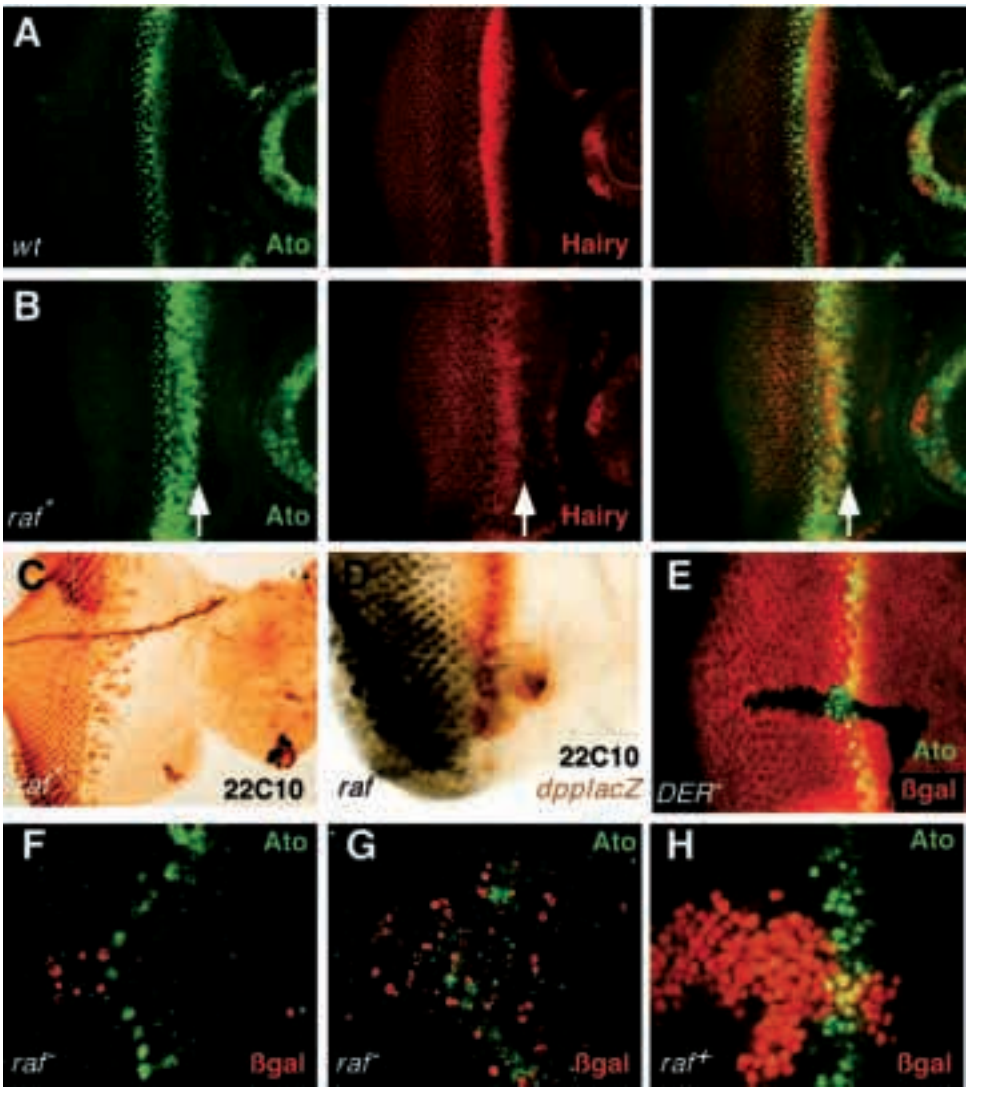

Fig. 7. Raf activity initiates Ato expression and neural differentiation during furrow progression. A wild-type disk (A) and a disk expressing Raf* in the majority of cells (B) stained for Ato (green) and Hairy (red) expression. The initial stripe of Ato expression normally abuts the stripe of peak Hairy expression (A); however, expression of constitutively active Raf* results in an anterior expansion of the stripe of Ato expression and a concomitant reduction of peak Hairy expression in the same cells. The discs in A and B were stained 9 hours following a severe heat shock (37°C for 1 hour). (C) An eye disk that was given a strong heat shock (37°C for 1 hour) to induce Raf* expression in the majority of cells and allowed to develop for 12 hours. Ectopic photoreceptor differentiation, marked by the expression of the neuronal antigen mAb 22C10 (brown), can be seen ahead of the morphogenetic furrow, in a restricted stripe of cells anterior to the furrow. (D) A disk given a mild heat shock (34°C for 30 minutes) to produce small clones of cells expressing Raf* and allowed to develop for an additional 72 hours. The disk has been stained for mAb 22C10 expression in black and for the reporter gene *dpp-lacZ* in brown. As in C, ectopic neural differentiation only occurs in the vicinity of the endogenous furrow, marked by expression of the *dpp-lacZ* transgene. (E) Ato expression (green) in *DER*$^{-}$ clones marked by the absence of β-galactosidase (red). Ato is still expressed in *DER*$^{-}$ clones, indicating that DER is not required for Ato induction. However, DER appears to play a role in the lateral inhibition mechanism, as the initial stripe of Ato expression does not appear to resolve into distinct R8 cell precursors in *DER*$^{-}$ clones. (F,G) Two focal planes of a single clone of *raf*$^{-}$ cells marked by the expression of a nuclear form of β-galactosidase (red) and stained for Ato expression (green). The clone straddles the furrow. Note that most of the nuclei of the *raf*$^{-}$ cells are located basally in the epithelium (G), while nuclei of cells that express Ato are positioned apically (F). None of the *raf*$^{-}$ cells express Ato. (H) A control, *raf*$^{+}$ clone similarly marked by β-galactosidase expression (red) and labelled for Ato (green), showing Ato expression in marked cells in the vicinity of the furrow. Clones were induced by a mild heat shock (34°C for 30 minutes) 96 hours prior to staining.

with that of the normal domain of peak Hairy and Emc expression. It is also located in the same position as the ectopic stripe of Ato expression caused by indiscriminate activation of Raf (e.g. Fig. 7). However, photoreceptor differentiation, or Ato expression, does not extend further anteriorly into the zone of mitotically active, undifferentiated cells in either case. Thus, pre-proneural cells can also be distinguished from more anteriorly situated, undifferentiated cells by their latent potential to activate Ato expression and differentiate as photoreceptors following either the loss of proneural repressors, or the activation of Raf.

We note that another attribute of the pre-proneural state is that it appears to be a general prerequisite for photoreceptor differentiation. In particular, we observe that cells which are devoid of both Smo and Tkv activity, and hence unable to make the transition to the pre-proneural state, appear unable to be recruited into developing ommatidia to make any of the photoreceptors. This result is in contrast to the finding that cells which retain either Smo or Tkv activity can be recruited to form any one, or all, of the eight photoreceptors (Burke and Basler, 1996; Strutt and Mlodzik, 1997; Domínguez, 1999; the present work). It also contrasts with previous findings showing that cells which lack Ato activity can form any photoreceptor except for R8 (Jarman et al., 1994).

We interpret the absolute failure of *smo tkv* mutant cells to differentiate as photoreceptors as evidence for a two-step mechanism of photoreceptor differentiation in which cells are first induced by either Hh or Dpp to become pre-proneural, and then require activation of Raf to become proneural (Fig. 8). We further propose that this mechanism is general, beginning with recruitment of the R8 photoreceptor and applying to each subsequent round of Raf activation, which

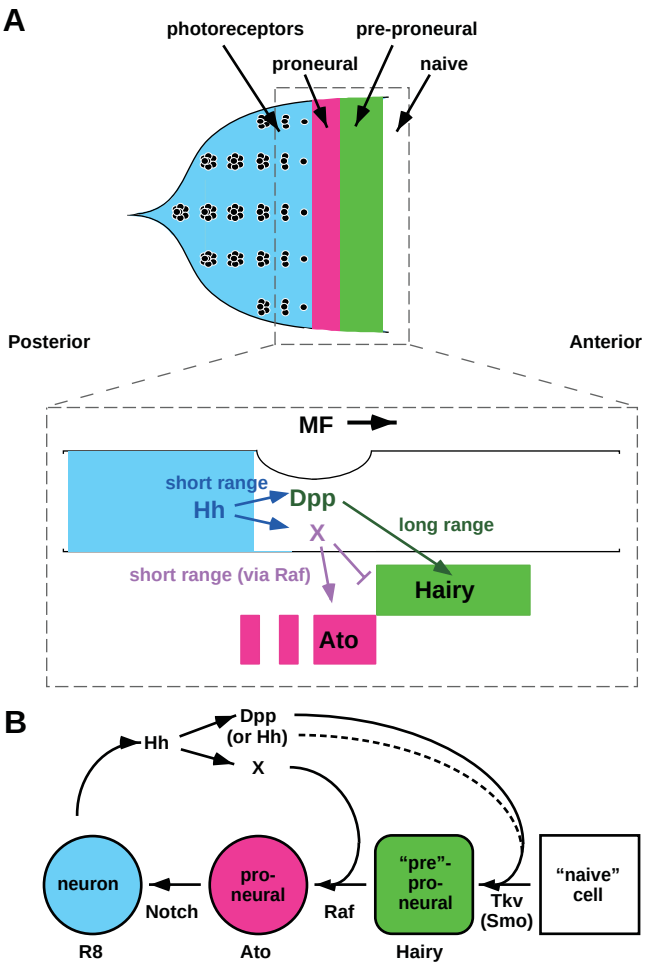

Fig. 8. Proposed roles of Hh, Dpp and Raf in initiating R8 photoreceptor differentiation and regulating progression of the morphogenetic furrow. (A) The proposed transitions in cell fate that occur during progression of the furrow are shown, as in the eye disc, with the furrow moving anteriorly from maturing R8 photoreceptors (left) towards undifferentiated tissue (right). Maturing photoreceptors secrete Hh, which induces a long-range signal, Dpp, and an unidentified short-range signal, X. Dpp induces undifferentiated cells to become 'pre-proneural', a state that correlates with the upregulation of both proneural activators, such as Daughterless, and pro-neural repressors, such as Hairy, which hold the activators in check. The presumed short-range signal, X, activates the Raf signal transduction pathway, inducing the subsequent transition to the proneural state. Acquisition of the proneural state correlates with the downregulation of Hairy and expression of Ato. Finally, expression of Ato is restricted to a spaced pattern of single cells by a process of lateral specification mediated by Notch and other signals regulating Raf. These Ato expressing cells differentiate as R8 photoreceptors while the remaining cells, which no longer express Ato, return to the pre-proneural state. The R8 cells then induce subsequent rounds of Raf activation in these pre-proneural cells, recruiting them to enter the ommatidia, differentiate as the R1-7 photoreceptors and secrete Hh, which drives progression of the furrow. In the absence of Dpp signal transduction, Hh can act directly to induce undifferentiated cells to adopt the pre-proneural state (dotted line), and these cells can in turn be induced by the short-range signal X to become proneural (B). However, under these conditions, either or both transitions in cell state occur less efficiently, so that the rate of furrow progression is severely retarded, resulting in an abnormally small eye.

adds the remaining photoreceptors that compose the mature ommatidium.

Evidence for a short-range secondary signal that induces differentiation of the R8 photoreceptor through induction of the Raf pathway

The finding that *smo* mutant cells can express Ato and form normally patterned ommatidia provided that they are near wild-type cells indicates that Hh can specify photoreceptor differentiation indirectly, through the induction of other signaling molecules. However, we have shown that Dpp, which is induced by Hh, does not have this capacity. Hence, we infer that some other signal induced by Hh, which we refer to as factor X (Fig. 8), is normally responsible for initiating the proneural state in pre-proneural cells.

Although we have not identified factor X, we have obtained evidence that it operates through the induction of the Raf transduction pathway. First, we find that ectopic activation of the Raf pathway can cause pre-proneural cells to ectopically express Ato and differentiate precociously as photoreceptors. Second, absence of Raf activity autonomously blocks the expression of Ato in these same cells. Our data also suggest that factor X has a relatively short range of action. For example, the ability of wild-type cells to block Hairy expression and induce Ato expression in neighboring *smo* mutant tissue appears tightly restricted, extending over only a few cell diameter (Fig. 1).

The most likely candidates for factor X are Spitz and Vein, ligands of the *Drosophila* EGF receptor (DER), which appear to be active within, or just posterior to, the furrow (Tio et al., 1994; Tio and Moses, 1997; Spencer et al., 1998). However, we have found that clones of cells that are devoid of DER activity nevertheless express Ato within the furrow with little if any posterior displacement relative to neighboring wild-type cells (Fig. 7E). We have obtained similar results for the FGF receptor Htl, which like DER is also expressed in the eye (Shishido et al., 1993; data not shown). This is in contrast to clones of cells devoid of Raf activity in which we have never observed Ato (e.g. Fig. 7F,G). These results suggest that if DER and Htl transduce factor X, they do so in a redundant fashion with each other, or with other receptors. Similarly, they also suggest that neither Spitz nor Vein correspond to factor X.

Transition to the proneural state

We envisage pre-proneural cells as metastable, having a latent proneural capacity that is actively held in check by proneural repressors such as Hairy and Emc. How does activation of Raf precipitate the transition to the proneural state? Because the simultaneous loss of both Hairy and Emc activities causes a similar expansion of Ato expression to that resulting from the expression of activated Raf, we suggest that Raf activation may normally induce transition to the proneural state by blocking the expression or activity of these repressors. Consistent with this possibility, Hairy contains potential phosphorylation sites for MAPK, a kinase downstream of Raf in the signaling pathway (Marshall, 1994). We note that Daughterless expression is also upregulated in the furrow and is necessary to maintain Ato expression (Brown et al., 1996). Moreover, Daughterless, like Hairy, contains phosphorylation sites for MAPK, raising the possibility that Raf activity may directly

potentiate proneural activators at the same time that it downregulates the activities of their repressors. Similar events may also occur in mammalian neural differentiation, as NGF-induced differentiation of the mammalian neuronal cell line PC12 is mediated by the phosphorylation of HES-1, a Hairy related protein (Strom et al., 1997).

Distinguishing the roles of Hh and Dpp signaling in furrow progression

When Dpp signaling is blocked by eliminating the signal itself (as in *dpp^{blk}* mutants; Fig. 4), or by eliminating Tkv, an essential component of the Dpp signal transduction pathway (Fig. 5), we find that undifferentiated cells can still be induced (1) to adopt the pre-proneural state (marked by Hairy expression), (2) to become proneural (marked by Ato expression) and (3) to differentiate as photoreceptors (marked by Elav and 22C10 staining and confirmed by the presence of mutant photoreceptors in the adult). Further, in this abnormal context, all three outputs depend on the direct action of Hh on the responding cells, as indicated by the cell-autonomous absence of Hairy and Ato expression in *smo tkv* mutant cells as well as the complete failure of these double mutant cells to differentiate into photoreceptors. Thus, Hh signaling, without any help from Dpp, can organize the normal progression of cell states observed during normal eye development.

Although Hh signaling can drive furrow progression independently of Dpp, the rate at which this progression occurs is abnormally slow. This is evident both in the marked posterior displacement of Hairy and Ato expression in *tkv* mutant clones, and in the severely retarded movement of the furrow in *dpp^{blk}* mutant discs. As a consequence, the mutant tissue forms too few ommatidia in the antero-posterior axis, contributing to the 'small eye' phenotype of *dpp^{blk}* mutant flies.

How might Dpp signaling regulate the rate of furrow progression? Dpp signaling could accelerate the conversion of undifferentiated cells into pre-proneural cells because it moves further anteriorly than Hh or because it is more potent than Hh in inducing this transition in cell state. Dpp signaling might also accelerate the transition of pre-proneural cells to proneural cells, even though it is not sufficient to induce this transition in the absence of Hh signaling or Raf activation. For example, Dpp signaling could enhance the ability of cells to respond to the short-range signal which induces Raf activity.

Parallels between Hh signaling in the limbs and eye

One way to view the small eye phenotype of *dpp^{blk}* eyes is that it represents an evolutionarily more primitive eye, dependent only on Hh signaling. From this perspective, the addition of Hh-induced Dpp signaling provides a significant enhancement of the organizing capacity of Hh through acceleration of the rate of furrow progression.

The situation in the eye may thus be analogous to that of Hh signaling in the limbs. Although Hh signaling can operate at short range in the limbs to govern patterning in A compartment cells close to the A/P compartment boundary (Jiang and Struhl, 1995; Mullor et al., 1997; Strigini and Cohen, 1997), the elaboration of limbs from the body wall appears to depend on the induction of Dpp and Wg as secondary signals (Campbell and Tomlinson, 1995). When these outputs are blocked, limbs do not form, or are rudimentary. The situation in the limb-bearing segments contrasts with that of the limb-less abdominal segments where Hh acts directly, rather than through Dpp and Wg, to organize most aspects of cell pattern in the A compartment (Struhl et al., 1997a,b). We suggest that the abdominal segments, like the eyes of *dpp^{blk}* flies, represent the evolutionarily more primitive condition, in which Hh signaling operates on its own. To organize larger, more complex structures such as the wild-type eye or the limbs, Hh signaling might need to operate through the induction of additional signaling molecules, such as Dpp and Wg.

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