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Dual role for Hedgehog in the regulation of the proneural gene *atonal* during ommatidia development

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SUMMARY

The differentiation of cells in the *Drosophila* eye is precisely coordinated in time and space. Each ommatidium is founded by a photoreceptor (R)8 cell and these founder cells are added in consecutive rows. Within a row, the nascent R8 cells appear in precise locations that lie out of register with the R8 cells in the previous row. The bHLH protein Atonal determines the development of the R8 cells. The expression of *atonal* is induced shortly before the selection of a new row of R8 cells and is initially detected in a stripe. Subsequently *atonal* expression resolves into regularly spaced clusters (proneural clusters) that prefigure the positions of the future R8 cells. The serial induction of *atonal* expression, and hence the increase in the number of rows of R8 cells, requires Hedgehog function. Here it is shown that, in addition to this role, Hedgehog signalling is also required to repress *atonal* expression between the nascent proneural clusters. This repression has not been previously described and appears to be critical for the positioning of Atonal proneural clusters and, therefore, the R8 cells. The two temporal responses to Hedgehog are due to direct stimulation of the responding cells by Hedgehog itself.

Key words: *Drosophila*, Eye, Atonal, Hedgehog, Ommatidia

INTRODUCTION

The differentiation of cells in the *Drosophila* eye begins at the posteriormost edge of the third instar eye disc where the first row of ommatidia are initiated (Treisman and Heberlein, 1998; Wolff and Ready, 1993); from here a morphogenetic furrow begins its steady spread across the disc epithelium from posterior to anterior (Tomlinson and Ready, 1987). The passage of the furrow is accompanied by a row-on-row addition of photoreceptor (R)8 cells (Tomlinson and Ready, 1987). Each R8 cell initiates an ommatidium and then the other cells are specified around it in a strict and regular sequence (Tomlinson and Ready, 1987). Thus, the furrow sweeps across the disc epithelium and, in its wake, spaced clusters of cells mature into adult ommatidia (Wolff and Ready, 1993).

It has been proposed that R8 cells in one row are patterned by secreted signals emanating from the differentiating cells in the previous row (Baker et al., 1996; Baker and Yu, 1997; Baker and Zitron, 1995; Held, 1991); such signals would be inhibitory and would regulate both the number and positions of the nascent R8 cells. In the model, a cell would become an R8 cell (1) if the morphogenetic furrow had reached it and (2) if it lay outside the inhibitory field of any preexisting R8 cell. This mechanism would ensure that all R8 cells arise in the interstices of the inhibitory fields of the previous row, leading to a hexagonal array.

The Atonal (Ato) protein is a bHLH factor that specifies the R8 cells (Jarman et al., 1994, 1995); it is expressed in a complex pattern. In the early developing eye disc (at the time the furrow initiates), expression of *ato* is first seen in four spaced clusters of cells localised anterior to a strip of *hedgehog* (*hh*) expression in the eye margin (Domínguez and Hafen, 1997). It is clear that the Hh signalling molecule induces this early expression of *ato* (Borod and Heberlein, 1998; Domínguez and Hafen, 1997), and there is some evidence that it does so directly (i.e. not via secondary secreted signal). Firstly, the expression of *ato* and the initiation of the furrow is blocked when *hh* expression is lost from the marginal cells (Borod and Heberlein, 1998; Domínguez and Hafen, 1997). Secondly, activation of the Hh signalling pathway in a clone of cells in the anterior unpatterned region activates expression of *ato* autonomously in the clone (Domínguez and Hafen, 1997).

During furrow progression, the expression of *ato* and *hh* are separated (see Fig. 1A), suggesting that Hh acts at a distance to induce *ato*. Such an induction would require a long-range action of Hh, acting either directly or indirectly, for example through a relay of secondary secreted signal(s). There is one piece of evidence supporting this. Within clones of cells mutant for *smoothened*, Hh reception is blocked yet the furrow and the clusters of ommatidia form normally; however, just in the centre of the clones there is some delay in progression of the furrow (Strutt and Mlodzik, 1997). The authors argue that the failure to receive Hh is rescued at the perimeter of the clone by a secondary signal that is normally necessary for movement of the furrow (Strutt and Mlodzik, 1997). Since the expression of *ato* was not analysed in this study, it is yet unknown how

ato is regulated by *hh* signalling during the process of furrow propagation.

Hh induces *ato* in more than just the cells that ultimately become R8 cells. Therefore, the initial expression of *ato* must be refined first into groups of cells and then restricted to the presumptive R8 cell. This gradual refinement of *ato* can be seen in a single disc preparation (Fig. 1A). At any time point during this process, the most anterior (and advanced) strip of cells expresses *ato* and, behind this strip, there is first a regular array of (proneural) clusters of cells and, further behind, three consecutive rows of Ato-positive R8 cells, spaced in a regular hexagonal array (Dokucu et al., 1996; Jarman et al., 1994, 1995). It is widely believed that this later refinement of *ato* to clusters and R8 cells is a Hh-independent process. Furthermore, it has been suggested that cells in and behind the furrow are insensitive to Hh signals and that refinement is almost entirely controlled by lateral inhibition mediated by the Notch signalling (Baker and Yu, 1997). However, conclusive evidence for such a proposal has not yet been reported. There are other alternative possibilities to account for the regulation of *ato* expression anterior and behind the furrow. One of them is that Hh could have a dual function, activating *ato* at low levels (that is far from the source of Hh) and repressing it at high levels (close to the source). Hh is a secreted molecule that presumably diffuses from posterior cells, where it is made, across the furrow to form a gradient of activity. It is probable that the repression of *ato* may only occur above a certain threshold of Hh. Every time a new row of ommatidial clusters forms, the activation of *hh* expression by these clusters would shift the peak of the Hh gradient anteriorly. As the Hh signal strengthens anterior to the peak, cells there will be exposed to high levels and therefore begin to refine *ato* expression leading to separated single R8s; refinement of *ato* to the R8s would require a higher threshold level than activation. As the Hh gradient migrates anteriorly, the two Hh-mediated responses would follow, one behind the other.

This hypothesis is tested in a series of experiments that examine cells that have lost or gained Hh signalling. The immediate consequences on the pattern of *ato* expression and of R8 determination in time and space are reported. In the absence of Hh reception, analysed using clones of *smoothened* mutant cells, anterior cells do not activate the *ato* gene. This result shows that, in a first step, Hh induces the expression of *ato* in a stripe of cells anterior to the morphogenetic furrow directly. In the wild type, this early expression of *ato* soon becomes uneven, with the highest Ato levels prefiguring the positions of the ommatidial clusters. This later process also depends on Hh, for *smoothened* mutant cells do not repress *ato* and the pattern of *ato* expression therefore fails to resolve into clusters. It therefore seems that in a second step of *ato* expression, Hh is required to repress the expression of *ato* between the nascent proneural clusters. Following a gain of *hh* expression (a membrane-tethered form of Hh was used) *ato* is immediately activated and then later, as the Hh signalling strengthened in the cells, *ato* becomes repressed. The dual role of Hh appears crucial to building precision and geometry into the adult retina. A discussion follows as to how Hh might work; whether it acts directly or through intermediaries.

MATERIALS AND METHODS

Generation of mitotic recombination clones

Mitotic clones were induced by Flp-mediated mitotic recombination (Xu and Rubin, 1993). In all cases, the *flp* gene was activated at second instar larvae by heat shocking the larvae for 1 hour at 37°C. Third instar eye discs were dissected for histochemistry. The genotype of the larvae in Fig. 4C is *ptc*^{IIw}*FRT*^{42}/ *arm-lacZ FRT*^{42}; in Figs 2E, 4D, 5A, 6, 7 are *smo*^{3}*FRT*^{40}/ *arm-lacZ FRT*^{40}; in Fig. 2F is *hh*^{AC}/*FRT*^{82B} *Minute*; Fig. 2G is *hh*^{AC}/*FRT*^{82B}*arm-lacZ*; Fig. 5B is *pka*$^{B3\text{-}DCO}$ *FRT*^{40}/*arm-lacZ FRT*^{40} and in Fig. 5C is *smo*^{3} *pka*$^{B3\text{-}DCO}$ *FRT*^{39E}/*hsp-70-CD2 FRT*^{39E}.

Ectopic expression experiments

To generate clones cells expressing the membrane-tethered form of Hh and the full-length Ci protein, the GAL4/UAS system were combined with the Flip-out technique (Struhl and Basler, 1993). The genotype of the larva carrying misexpressing clones as referred in the text are as following:

Hh-CD2 clones: flies carrying *hsp-70-flp; abx/ubx>f*$^{+}$*>Gal4-LacZ* and *UAS.Hh-CD2* (heat shocked for 5 minutes at 38°C).

Act>Ci clones: flies carrying *hsp-70-flp; actin>CD2>GAL4* and *UAS.Ci* (heat shocked for 20 minutes at 38°C). The activation of *ato* expression in the *Act>Ci* clones occurred at low penetrance and was seen more frequently in the dorsal part of the disc.

Fly stocks

Mutations, insertions and transgenes used are described as the following: *ptc* ^{IIw}, *smo*^{3} are amorphic alleles and *pka*$^{B3\text{-}DCO}$, is a strong hypomorphic allele of *pka* (Chen and Struhl, 1996; and references therein). *hh*^{AC} is an amorphic allele of *hh* gene (Ma et al., 1993). *hh*^{1}, is a hypomorphic allele of the *hh* gene that causes a ‘stop-furrow’ phenotype (Heberlein et al., 1993). *hh*^{P30}, an enhancer trap insertion at the *hh* locus and its expression in the eye disc is described in (Ma et al., 1993). *ci*^{W} is a weak gain-of-function mutation and *fu*^{62} is a viable hypomorphic allele of the *fused* gene (Lindsley and Zimm, 1992). *UAS.Hh-CD2* (Strigini and Cohen, 1997) and a *UAS.Ci* (Domínguez et al., 1996). *Act>CD2>Gal4* (a gift from Y. Hiromi) and *abx/ubx>f*$^{+}$*>GAL4-LacZ* (de Celis and Bray, 1997).

Antibody staining

The following antibodies used were: a rabbit polyclonal anti-Ato (gift from Y. N. Jan), a rabbit and mouse anti-β-galactosidase (Cappel), a rat monoclonal anti-Ci (a gift from P. Holmgren), a mouse rat-CD2 (Serotech), a mouse anti-Scabrous, mouse anti-Boss, a rabbit Spalt (a gift from R. Barrios) and mouse mAb323 (a gift from S. Bray). Secondary antibodies, either an FITC- or a Texas-Red-conjugated, were from Jackson Inc. Whole-mount in situ hybridisation and antibody staining were done as described (Ma et al., 1993).

All confocal images were taken on a Biorad MRC 1024 confocal microscope system. Images were then processed and arranged using Photoshop (Adobe).

Histology

Adult eyes were processed for sectioning and analysis as described previously (Ma et al., 1993).

RESULTS

Spatial relationship between *ato*, *ci* and *hh*

The initial expression of *ato* in the eye discs (Fig. 1B,D,E) has been reported to occur in a strip of cells anterior to the morphogenetic furrow (Dokucu et al., 1996; Jarman et al., 1994, 1995). Fig. 1D demonstrates that the levels of Ato within this stripe vary, with enhanced Ato expression corresponding to the approximate position of proneural clusters. Behind the furrow, the only cells that express *ato* are the future R8 cells. In mature R8 cells, the expression of *ato* is repressed (Jarman et al., 1994). When the *ato* and *hh*

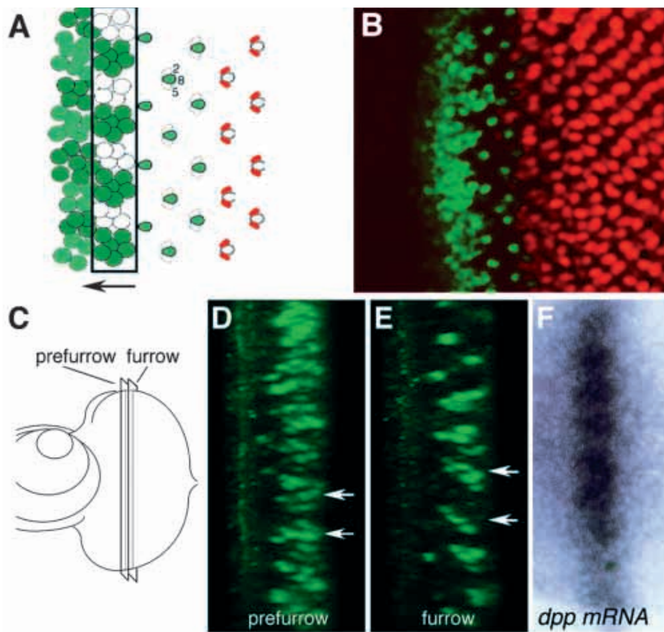

Fig. 1. The temporal and spatial relationship between the *ato* and *hh* genes. (A,C) Schematic representations and (B,D,E) confocal images of eye discs double- (B) and single- (D,E) labelled for Ato (green) and β-galactosidase (red) in the hh^{P30} line. In this and all the subsequent figures, posterior is to the right and the furrow moves towards the left and late third instar larval eye discs are shown. The clustered expression of *ato* coincides with the furrow (framed box in A). An example of vertical scans of Ato expression at the position of the continuous stripe (D) and the clusters in the furrow (E) as represented in C. Note that groups of highest (arrows, D) levels of Ato and the proneural clusters (arrows, E) are out of register. The vertical scan rules out that the heterogeneous Ato distribution is an artefact of the packing of nuclei in that anterior region. (F) Another Hh target, Dpp (Heberlein et al., 1993), is also expressed unevenly in the furrow, with maxima and minima of *mRNA* accumulation, suggesting that the front of Hh signal is uneven and that the prepattern of *ato* is regulated by Hh itself.

expressions are compared (Fig. 1B) it appears that the refinement of *ato* expression occurs in cells close to the *hh*-expressing cells, whereas the continuous stripe of *ato*, which is believed to be induced by Hh, is 5-7 ommatidial rows distant from the first row of *hh*-expressing cells (Fig. 1B). This observation suggests that Hh acts at a distance to induce *ato*. Such a long-range action of Hh could be direct or indirectly through a relay of a secondary signal, as suggested in Strutt and Mlodzik (1997).

The Hh-signalling pathway activates gene expression through the Zn-finger transcription factor, Cubitus interruptus (Ci). Ci protein is related to the vertebrate Gli families and the transcriptional activator form correlates with high levels of the full-length Ci protein, which are induced by Hh (reviewed in Ruiz i Altaba, 1997). In the eye disc, the Ci protein is expressed dynamically (Motzny and Holmgren, 1995; Strutt et al., 1996) and (Fig. 2A-C) with the highest levels of Ci protein overlapping with Ato expression (Fig. 2D). Accordingly, misexpression of high levels of Ci in clones of cells (*Act>Ci* clones, Methods) showed that Ci is able to induce Ato (Fig. 3A,B). A weak gain-of-function mutation, ci^W, also causes an anterior expansion of the stripe of *ato* that results in formation of extra R8 cells (Fig. 3D,F).

The Ci accumulation in cells ahead of the furrow depends on Hh, for cells lacking *smo* activity have low uniform levels of Ci (Strutt and Mlodzik, 1997; Fig. 2E). Loss of Hh reception more posteriorly (Fig. 2E,G-I) results in the failure to downregulate Ci levels and consequently mutant cells have inappropriately high Ci protein levels when compared to wild-type neighbours, indicating that Hh stimulates (at long-range) and inhibits (at short-range) Ci accumulation.

Hedgehog can both activate and repress the expression of the *ato* gene

The regulation of *ato* by the Hh-signalling pathway was studied further by generating clones of marked cells expressing a membrane-tethered Hh protein tagged with CD2 (Hh-CD2) (Strigini and Cohen, 1997). Clones were induced using the GAL4/UAS system combined with the Flip-out technique (Methods) and discs were labelled for Ato at two different time points, 24-36 hours and 72-84 hours, after clone induction.

Following a gain of *hh* expression (in the 24-36 hours *Hh-CD2* clones), *ato* was immediately activated (arrows in Fig. 4A), and then later, in the 72 hours *Hh-CD2* cells, *ato* became repressed (Fig. 4B). This sequence of events in these anterior *Hh-CD2* clones parallels the events in the endogenous furrow (see Fig. 1A). Furthermore, once Ato expression is refined in these anterior clones, the normal cascade of ommatidial development is triggered and an ectopic furrow propagates outwards from the clone. When anterior clones are close to the endogenous furrow, it accelerates around them (Fig. 4B), consistent with previous descriptions of clones of cells expressing a wild-type form of Hh (*Tubulin-α1>hh* clones; Heberlein et al., 1995). In these *Tubulin-α1>hh* clones, formation of ectopic furrows and *ato* induction was restricted to anterior cells close to the endogenous furrow, suggesting a competent zone around the furrow. The GAL4/UAS system yields higher levels of gene expression and, in such conditions, there are no such restrictions in the anterior region.

Small *Hh-CD2* clones consisting of 2-6 cells were also associated with cell-autonomous repression of the endogenous *ato* gene (arrowheads in Fig. 4A), indicating that membrane-tethered Hh is also able to repress *ato*. A similar phenomenon by Wingless has been proposed for the regulation of *labial* in the midgut (Bienz, 1997), where low levels of Wingless stimulate and high levels repress. Does a similar phenomenon occurs in the regulation of *ato* in the eye? To this end, different levels of membrane-tethered Hh were misexpressed using different GAL4 drivers and activation was found always to be the immediate consequence of increased Hh (not shown). Furthermore, when ptc^{IIw} activity were removed in clones of mutant cells – a condition that is believed to correspond to maximal Hh activation, the *ato* gene was also activated cell-autonomously in the anterior region (Fig. 4C). Thus, the two developmental responses appear to depend on the time of exposure rather than the dose of Hh.

Smoothened acts directly to both activate and repress the *ato* gene

The repression is linked and follows on an initial activation of *ato* in the *Hh-CD2* or ptc^{IIw} anterior clones, but is uncoupled from activation in the small *Hh-CD2* clones around the furrow

(arrowheads in Fig. 4A), suggesting that activation and repression could be regulated independently of each other. To address this issue directly, cells that have lost Hh signalling were examined for immediate effects on the pattern of *ato* expression and R8 determination. Hh acts via a receptor, Ptc, that binds to a seven-transmembrane protein Smo (Ruiz i Altaba, 1997). *smo*^{3}, an amorphic allele of the *smo* gene, blocks the reception of Hh signalling in other systems (Alcedo et al., 1996; Chen and Struhl, 1996; van den Heuvel and Ingham, 1996). Small *smo*^{3} clones were studied as to their effect on the expression of *ato*.

smo^{3} mutant clones cells anterior to the furrow do not activate the *ato* gene (arrowheads Fig. 4D), indicating that Hh is absolutely required to induce this expression of *ato*. In the wild type, this initial expression of *ato* soon becomes refined to evenly spaced clusters. This later refinement also appears to depend on Hh, since *smo*^{3} mutant cells derepress *ato* in between the nascent *ato* clusters and, consequently, this pattern of *ato* expression fails to resolve into clusters or isolated cells (arrows in Fig. 4D).

Clones of *smo*^{3} cells spanning the furrow (asterisk in Fig. 4D) showed that *ato* derepression can occur in these same cells that never initially expressed *ato*, consistent with the idea that repression and the initial activation are regulated independently of each other.

Rough is a retinal-specific transcriptional repressor acting downstream of Hedgehog

The derepression of *ato* in *smo*^{3} clones suggests a role for Hh in and behind the furrow to regulate gene expression. In agreement with this, the expression of the homeodomain protein Rough (Heberlein et al., 1994; Kimmel et al., 1990) in and behind the furrow was found to be regulated by Hh (Fig. 5A-C). The *rough* expression in the furrow was abolished or strongly reduced in *smo*^{3} clones (Fig. 5A) and expressed ectopically in anterior cells within *pka*$^{B3\text{-}DC0}$ clones (Fig. 5B). The loss of the *rough* furrow expression was rescued by providing receptor-independent activation of Hh signalling (by removing *pka*) in the *smo*^{3} cells (Fig. 5C), demonstrating that Hh induces *rough* expression. In the wild type, *rough* and *ato* have complementary expression patterns (Dokucu et al., 1996) and it has been proposed that Rough represses *ato*. Accordingly, modulation of *ato* expression in the anterior *smo*^{3} *pka*$^{B3\text{-}DCO}$ clones was associated with gain of *rough* expression (Fig. 5C). A gain-of-function *rough* mutation, *ro*^{DOM}, results in a premature stop of furrow progression (Heberlein et al., 1993) and this effect can be corrected by reducing doses of *hh* (Treisman et al., 1997), consistent with the positive regulation of *rough* by Hh. Thus, the regulation and function of *rough* gene suggest that its product acts downstream of Hh to refine *ato* expression.

The expression of *rough* is also eliminated in clones of cells lacking the *Drosophila* Epidermal Growth Factor receptor (*EGFR*) activity (Domínguez et al., 1998), suggesting that the EGFR might mediate the Hh-dependent activation of *rough*. In the *EGFR*$^{-}$ clones, there is clearly an excess of single-out Ato-positive cells, a phenotype that resembles the effect of loss of *rough* itself; however, unlike the effects of removing *smo* activity, most *EGFR*$^{-}$ cells do not block the restriction of *ato* to isolated cells, indicating that EGFR mediates only some functions of Hh.

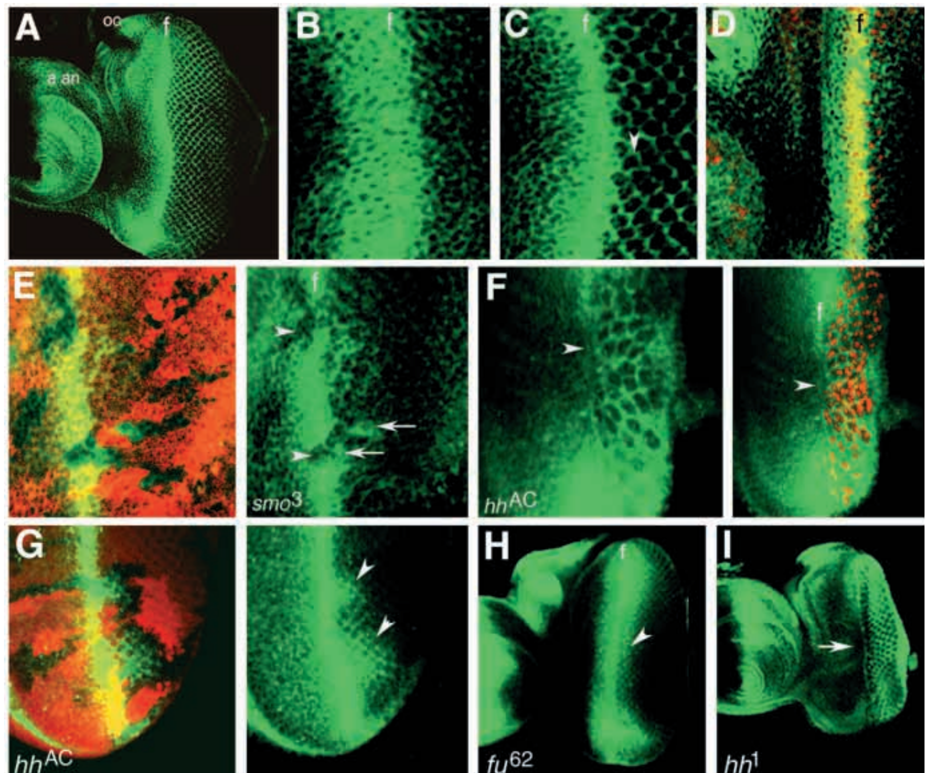

Fig. 2. The regulation of the pattern of Ci distribution by Hh. (A) In the eye field, Ci is present in almost all cells, except in the dorsal head region around the occelli (oc). (B,C) High magnification views of the region around the furrow focused at the basal (B) or at the apical (C) levels. The highest levels of Ci coincides within the furrow (f) and overlaps with the expression of Ato (red), denoted by the yellow coincident staining (D). The posterior edge of the high Ci is sinusoidal and Ci levels decrease abruptly to a homogeneous low level behind the furrow. (E) *smo*^{3} clones spanning the furrow exhibits low Ci levels (arrowheads) in the region ahead of the furrow and higher than normal levels in and behind the furrow (arrows). (F) A *hh*^{AC} clone induced in a *Minute* background and not marked. The Ci labelling in the unrecruited cells illustrates the disorganisation of ommatidial clusters formed in the absence of *hh* (compared with Fig. 2C). Ahead of the disorganised clusters, Ci fails to accumulate (arrowhead). A lower magnification of the disc and showing Spalt expression in differentiating cells (red) and Ci (green) is shown in the right panel. (G) Eye disc containing *hh*^{AC} clones marked by the absence of β-galactosidase (red). Note the inappropriate upregulation of Ci in the centre of posterior *hh*^{AC} mutant clone (arrowheads). (H) Similarly, cells unable to transduce the Hh signal, in the *fu*^{62} mutant discs, have elevated Ci levels (arrowhead) posterior to the furrow. (I) The loss-of-function *hh*^{1} mutation lacks *hh* expression in the photoreceptor cells from mid third instar. In the late third instar *hh*^{1} mutant eye disc, Ci fails to accumulate anterior to the furrow (arrow) and its expression becomes slightly higher behind the furrow in the cells surrounding the developing clusters. a an, anterior antenna; f, furrow.

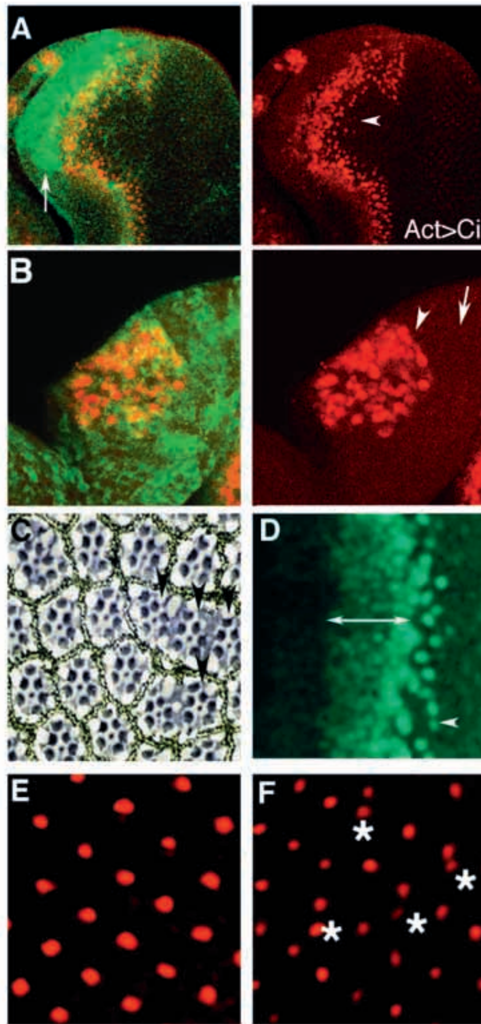

Fig. 3. Gain of Ci induces precocious *ato* and promotes R8 determination. (A,B) Eye discs containing *Act>Ci* clone resulted in an dramatically accelerated furrow in the dorsal part of the eye (arrowhead, A) and misexpression of *ato* (red) at the margin (arrowhead, B). Note that some *Act>Ci* cells (arrow, B) do not induce *ato*. The clones are identified by the increased expression of Ci (green and arrow, A) driven by the *actin* promoter. ci^W flies have at low penetrance rough eyes. (C) A section through such a rough ci^W eye. Note the presence of ommatidia arranged in a linear pattern (arrowheads) instead of the normal hexagonal one. More rarely, ci^W ommatidia lack photoreceptors or present mirror-image duplication of the ommatidia (not shown). (D) The anterior expression of Ato is expanded (double arrow) in the ci^W eye discs and extra R8 Ato-positive cells (arrowhead) form at ectopic positions. Portions of eye discs stained with anti-Boss to visualise the array of R8 cells in the wild-type (E) and ci^W (F) discs. The extra R8 cells (asterisks, F) disrupt locally the hexagonal order of R8 cells.

Ommatidial differentiation in *smo* clones

The complex expression pattern of *ato* may be essential for its normal function. For example, the restricted expression in the proneural clusters may provide a bias for the positioning of the R8 cells (Lee et al., 1996; Baker and Zitron, 1995). Furthermore, the refinement of *ato* to single cells has been proposed to be obligatory for the selection of isolated R8 precursors (Baker and Zitron, 1995; Baker et al., 1996). The expression pattern of *ato* never resolves into single cells in the smo^3 clones (Fig. 4D) and, therefore, it is surprising that the ommatidial formation is relatively normal (Strutt and Mlodzik, 1997).

The formation of these R8 precursors has been studied by looking at the expression of *scabrous* in the smo^3 clones. In the wild-type eye discs, the Scabrous protein is first seen in clusters of cells ahead of the furrow and later is activated in the emerging R8 precursors (Baker et al., 1990; Mlodzik et al., 1990; Lee et al., 1996). In the smo^3 clones, the clustered expression of *scabrous* is abolished (Fig. 6A), consistent with this expression being induced by Ato (Jarman et al., 1995). The expression of *scabrous* in the R8 precursors is not affected (Fig. 6A,B) and this expression is used to visualise directly the singling out of R8 cells within the smo^3 tissue. Assessed by *scabrous* expression, the timing of emergence of the mutant R8 precursors is normal (Fig. 6A,B) and, like the wild-type precursors, they appear isolated (Fig. 6A,B) but the spacing is wider, suggesting a reduction in the number of R8 precursors forming in the smo^3 tissue and the spatial organisation is very disrupted (see below).

The isolated Scabrous-positive (R8) precursors may be selected via a lateral inhibition process involving the Notch pathway. To confirm the idea that Notch activation is unaffected by the loss of smo^3, the expression of the bHLH genes of the Enhancer of split complex [E(spl)], which are direct targets of the Notch signalling (Jennings et al., 1994), was studied. The *E(spl)* expression ahead of the furrow is affected (not shown), consistent with this expression being dependent on Ato (Dokucu et al., 1996). In the furrow and behind it (and within the smo^3 tissue), *E(spl)* is in spaced clusters (Fig. 6C), indicating that Notch pathway is activated there. The activation of Notch can account for the formation of isolated R8 precursors. This result also shows that the refinement of *ato* expression is not obligatory for the singling out of R8 cells by Notch-mediated lateral inhibition.

Spatial organisation of R8 cells in the absence of Hh signalling

As noted by Strutt and Mlodzik (1997), neuronal differentiation is dramatically delayed in the *smo* tissue (about 3-5 ommatidial rows delayed, Fig. 7A-C). However, a closer examination of ommatidial differentiation at the border of smo^3/smo^+ cells shows that the effect is autonomous (see example in Fig. 7C). Ommatidia at the perimeter and the centre of the clone suffer the same delay in differentiation and this effect cannot be completely explained by a similar delay in the singling out of R8 precursors (see Figs 6A,B, 7F). Thus, the loss of smo^3 may directly affect when cells begin to differentiate as photoreceptor neurones.

Another phenotype seen in the smo^3 clones is disarray of the rows of ommatidial clusters. This is particularly apparent at the posterior borders of smo^3/smo^+ tissue (Fig. 7A-C). In the wild-type tissue, within a row the nascent R8 cells lie out of register with the extant R8 cells in the previous row (see scheme in Fig. 1A). In the smo^3 tissue, the R8 cells in the nascent rows (anterior to the wild-type tissue) appear either in register or out of register to the R8 cells in the previous row. This disorganisation of the rows of ommatidia is observed across the *smo* clones, resulting in flaws during the packing of ommatidial clusters that can be seen in the adult eyes

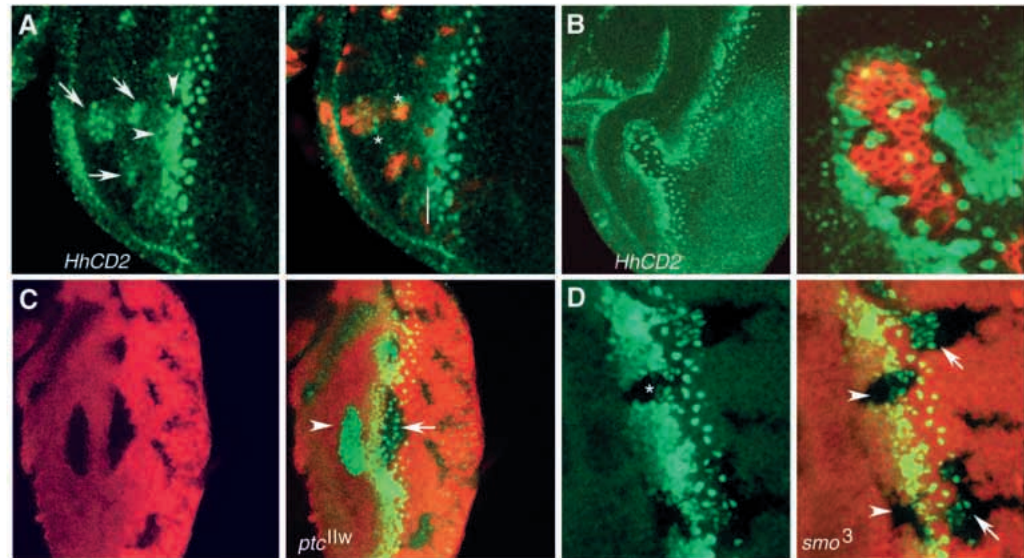

Fig. 4. Effects of gain and loss of Hh signalling on *ato* gene expression. (A,B) Eye disc carrying *Hh-CD2* clones labelled for Ato (green) and CD2 (red) 24 hours (A) and 72 hours (B) after clone induction. (A) Anterior *Hh-CD2* cells (arrows) and immediately adjacent cells (asterisks) activate *ato*. *Hh-CD2* clones located within the furrow lack autonomously Ato (arrowheads). The furrow is marked by the white line. (B) The initial uniform *ato* expression has declined at this stage and only isolated Ato-positive precursors, arranged irregularly, are seen within the *Hh-CD2* cells. The right panel shows a high magnification view of the clone marked by the presence of *CD2* (red) expression. The furrow has accelerated around the clone (denoted by the strip of wild-type *ato* cells). (C,D) Ato expression (green) in eye discs containing ptc^{IIw} (C) and smo^3 (D) clones marked by the absence of β-galactosidase (red). Single and superimposed images are shown next to each other. (C) *ato* is cell autonomously activated in anterior ptc^{IIw} clones (arrowhead). ptc^{IIw} clones closer to the furrow have already initiated repression and isolated Ato-positive cells are seen arranged irregularly (arrow). (D) smo^3 cells fail to activate *ato* anterior to the furrow (arrowhead) and exhibit ectopic *ato* when located in and just posterior to the furrow (arrows). The smo^3 clone marked by an asterisk (left panel) bisects the furrow and illustrates the temporal sequence of events with the smo^3 cells. The expression of *ato* ends in the posterior region at roughly the same time in the smo^3 cells and in the wild-type mature R8 precursors. Note that *ato* expression is at a lower level than that observed in smo^+ cells.

(arrowheads in Fig. 7D). Defects in the positioning of ommatidial clusters are also seen in large clones of *hh* null clones (hh^{AC}) that do not extend the margin of the eye disc (Fig. 2F), and in a weak gain-of-function ci^W mutants (Fig. 3C,F), where the Hh signalling pathway is constitutively activated. Unlike other mutants causing defects in the spacing and the number of R8 cells per cluster, the disarray of ommatidia seen in smo^3, hh^{AC} or ci^W eyes is not related to defects in the singling out of R8 precursors, suggesting that the Hh signalling pathway might control directly this aspect of eye development.

DISCUSSION

ato is expressed in a complex pattern before and during the progression of the furrow (Jarman et al., 1994, 1995). It is thought that Hh, which is secreted by cells behind the furrow, spreads forward to induce *ato* expression in the furrow (reviewed in Treisman and Heberlein, 1998). The response is limited to a narrow band ahead of the furrow, most probably because cells are only competent to respond to Hh for a short

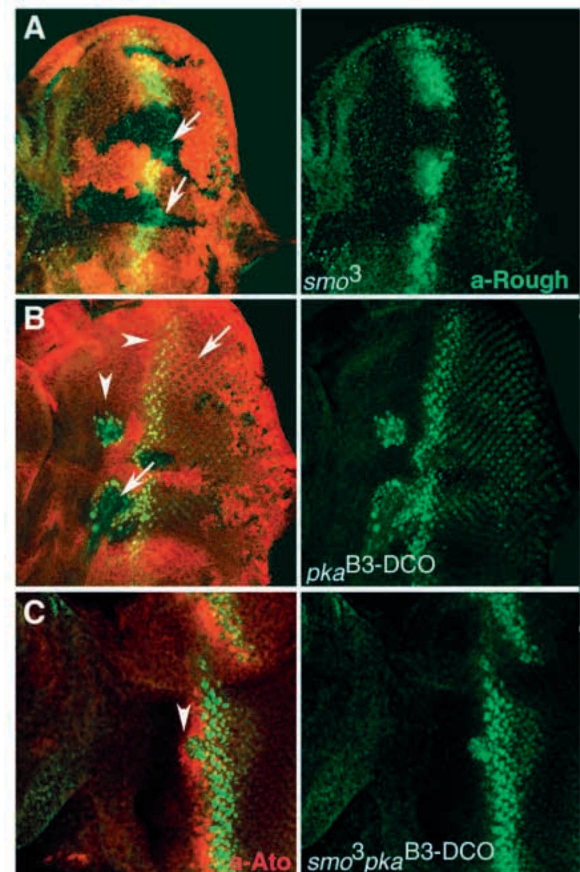

Fig. 5. Regulation of *rough* expression by Hh. (A,B) The mutant tissue is marked by the absence of β-galactosidase expression (red). (A) *rough* is expressed widely in and behind the furrow and this expression is abolished or strongly reduced (arrows) within the smo^3 cells. *rough* is also expressed at a lower levels in the outer photoreceptors R2-R5 (Heberlein et al., 1994; Kimmel et al., 1990). This expression is unimpeded in the smo^3 clones. (B) *rough* expression in a mosaic *pka* $^{B3\text{-}DCO}$ discs. Arrowheads point to strong (furrow) expression of *rough* in the endogenous and ectopic domain. Arrows point to the weaker expression in photoreceptors cells. (C) The earlier and stronger *rough* (green) expression in the furrow (arrowhead) is rescued in smo^3 *pka* $^{B3\text{-}DCO}$ cells and is complementary to ectopic Ato (red) expression.

time, afterwards becoming refractory (Baker and Yu, 1997). However, this is not sufficient to explain the pattern of *ato*: there is a gap equivalent to several ommatidial rows between the source of Hh and those cells containing Ato protein. Here this gap is shown to stem from repression of *ato* by the highest concentrations of Hh and the results of experiments on the directness of this repression are reported.

First, *ato* expression in cells that have gained *hh* was examined. Misexpression of *hh-CD2* can either activate (when clones are lying anteriorly) or repress (when they lie adjacent to the furrow) the expression of *ato*. Repression of *ato* is autonomous to the *hh-CD2* cells, suggesting that Hh may repress *ato* directly. These observations suggest that Hh is secreted near the advancing furrow: close to the source *ato* expression is inhibited, further away it is induced. If *hh-CD2* is misexpressed, naive cells begin to express *ato* prematurely and this ectopic *ato* initiates precocious ommatidial formation. However, slightly later (and within the region of influence of the endogenous *hh*), misexpression of *hh-CD2* results in the premature repression of *ato*; thus cells experiencing the extra Hh exhibit no *ato* expression while the wild-type neighbours just begin to express *ato*. This model was then tested by manipulating the reception of the Hh signal using in vivo assays, and genetic evidence showed that Hh is required for both promoting and inhibiting *ato* expression.

Hh mediates its signalling activities via a heteromeric receptor complex, which includes the Smo protein and the receptor Ptc (reviewed in Ruiz i Altaba, 1997). Smo is required for transducing the Hh signal in the receiving cells, whereas Ptc is required to inhibit Smo activity in the absence of Hh. When *ptc* is removed in clones, the Hh signalling pathway is constitutively activated and the expression of *ato* is immediately induced. Examination of *ato* expression in these marked clones suggests that Hh has the ability to act autonomously in the receiving cells to induce *ato*. Hh activation also results in the induction of Rough, a previously identified negative regulator of *ato*. This result supports a second role of the Hh signalling pathway in inhibiting *ato*.

The *smo*^{3} data also supports the dual role of Hh: *smo*^{3} clones exhibit no expression of *ato* (when lying anterior to the furrow) but show ectopic expression of *ato* and absence of *rough* expression (when lying in the furrow). In the proposed model, the induction of Hh has two effects in the responding cells: (1) as an *ato* inducing signal, through the activation (by upregulation) of the Zn-finger transcription factor Ci, and (2) as an inhibitory signal, through activation of Rough, to inhibit *ato* expression in the cells in and behind the furrow. The two responses occur in a cell sequentially, as monitored by *ato* and *rough* expression in the wild-type pattern and by analysis of their expression in marked clones. The expression domains of *ato*, Ci protein and *rough* and their relationship with Hh supports the model. Ci and *rough* are activated and expressed, respectively, by Hh in restricted spatial domains across the furrow and their expression overlaps (in the case of Ci) or is complementary (in the case of *rough*) with *ato*, consistent with their role in promoting and inhibiting *ato* expression, respectively.

Regulation of *ato* expression in the absence of *hh* signalling

The *smo*^{3} data suggests how *ato* must be regulated by activation in the furrow. Sun et al. (1998) has recently reported that *ato* expression is controlled by two enhancer elements located 5′ or 3′ to the coding sequences. A 3′ enhancer directs initial expression in a stripe anterior to the furrow and a distinct 5′ enhancer drives expression in the proneural clusters and R8 cells within and posterior to the furrow. The 5′ enhancer, but not the 3′ enhancer, depends on endogenous *ato* function. The identification of the factors that activate the 5′ enhancer element will require refining the *ato* regulatory sequences followed by binding studies in vitro and in vivo. One of the factors binding to these *ato* promoters might be Ci. Preliminary results of loss of *ci* in mitotic clones are consistent with Ci acting as a positive transcriptional regulator of *ato* (M. D. and E. Hafen, unpublished results). During furrow progression, Ci is upregulated in the cells anterior to the furrow and in groups of cells in the furrow that coincide with cells expressing *ato*.

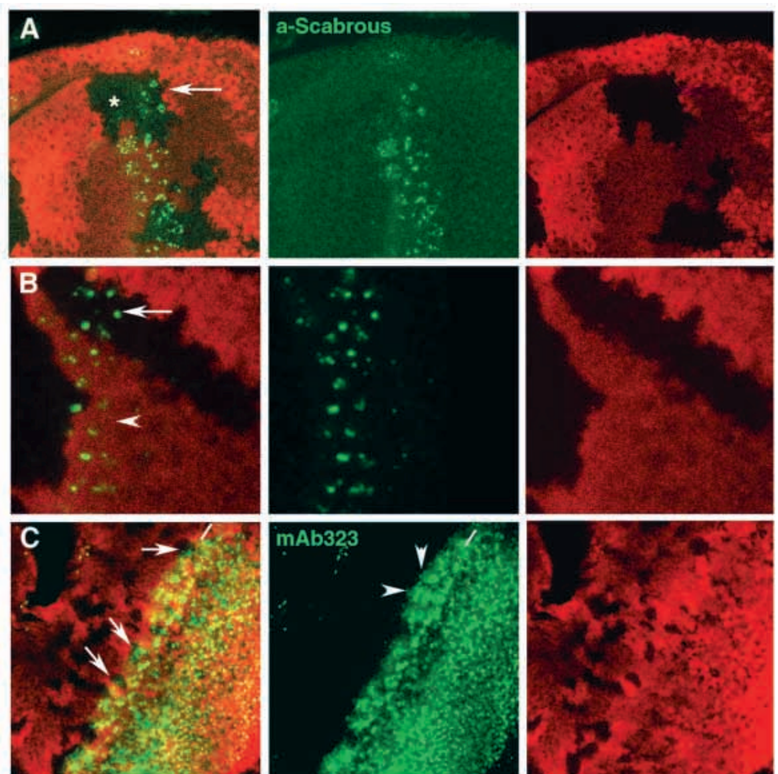

Fig. 6. Lateral inhibition operates in the absence of Hh reception. *smo*^{3} mutant discs stained with anti-Scabrous (green, A,B) and mAb 323 (green, C) and anti-β-galactosidase (red, A-C) to mark the mutant tissue. Single and superimposed images are shown. (A) The expression of *scabrous* in clusters of cells anterior to the furrow is blocked in the *smo*^{3} tissue (asterisk in A), in agreement with a requirement for Ato in the activation of *scabrous* (Jarman et al., 1995). The *smo*^{3} R8 precursors express Scabrous (arrows in A and B). Note that singling out of R8 cells is not delayed in the *smo*^{3} tissue. (C) *E(spl)* expression, using the mAb323 antibody (Jennings et al., 1994), is detected within *smo*^{3} cells (arrows, left panel) and it occurs in spaced clusters (arrowheads). The approximated position of the furrow is indicated by a white line.

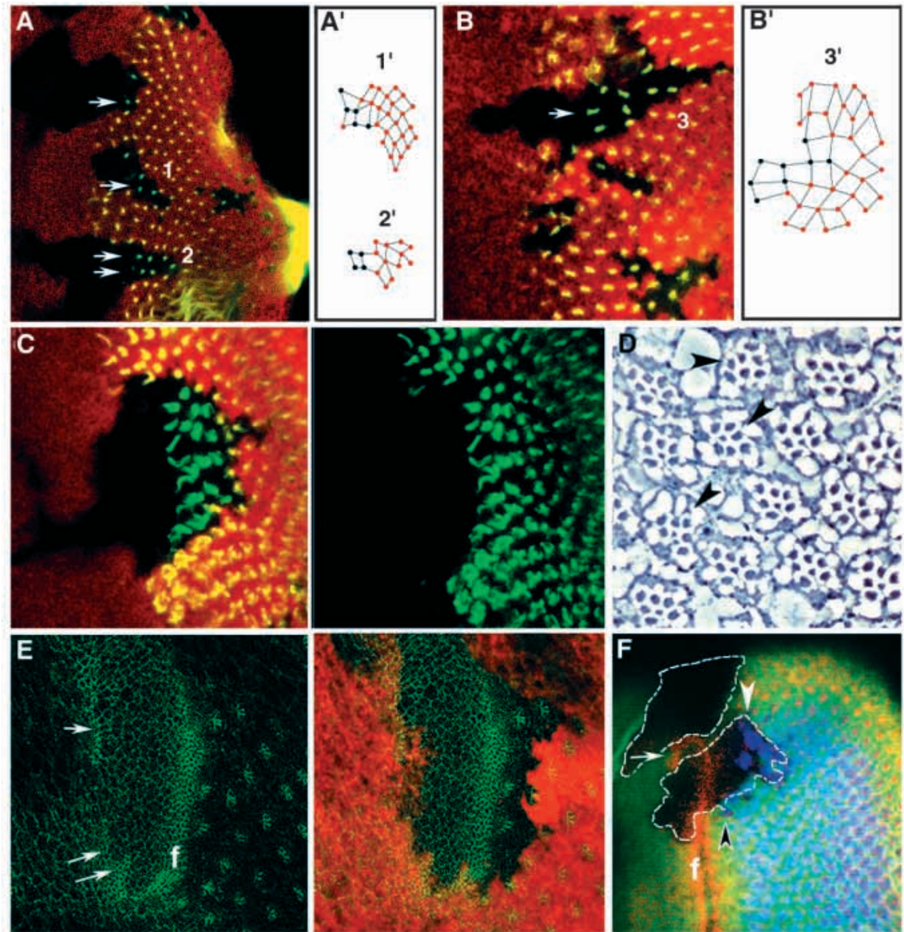

Fig. 7. Defects in the arrangement of ommatidia in the absence of *smo* activity. (A-C) Discs were stained with 22C10, a neuronal cell marker, and the images in A, B are at the R8 level. A′ and B′ are interpretations of the spatial organisation around the borders of three (1-3) clones in A,B. Red dots represent wild-type ommatidia and the black ones are *smo*^{3} ommatidia. Note also that the front of 22C10 expression is delayed about 3 to 5 ommatidial rows behind the differentiation of the photoreceptor R8 in adjacent wild-type ommatidia which develop normally (see examples in B,C). (D) Section through a mosaic *smo*^{3} adult eye. Arrowheads point to mispositoned ommatidia. (E,F) Arm staining (green, E and red, F) illustrates that changes in cell shape associated with morphogenesis are normal within the *smo*^{3} clones. Note that *smo*^{3} ommatidia are broadly spaced, suggesting a reduction in the number of ommatidia forming in the *smo* tissue. Apical constrictions similar to those in the endogenous furrow (f) are seen in the anterior border of *smo*$^+$/*smo*^{3} tissue (arrows, E,F). The *smo*^{3} tissue is marked by the absence of β-galactosidase (red, A-C,E and green, F). Elav expression (blue, F) is very delayed in the mutant tissue (white arrowhead) when compared to the wild type (black arrowhead).

These high levels of Ci are then later downregulated to a low level behind the furrow. Ci is thought to act as a transcriptional factor activating or repressing target genes in a concentration-dependent manner (Domínguez et al., 1996). The transcriptional activator form of Ci is thought to correlate with high levels of full-length Ci protein induced by Hh (reviewed in Ruiz i Altaba, 1998). This upregulation of Ci proteins by Hh is a conserved feature of Hh signalling in all systems. Therefore it is surprising that, in the eye, Ci is not upregulated near to the Hh source but only in cells far away. The analysis of Ci distribution in *smo*^{3}, *hh*^{AC} and viable *fused* alleles – where the reception and transduction of the Hh signal is blocked or very reduced – suggests that high levels of Hh protein may inhibit Ci protein levels. Probably this regulation is required to restrict the domain of Ci activation and therefore the cells competent to express *ato*. Thus, by combining a positive long-range inductive signal with short-range inhibition of Ci, Hh may act to pattern *ato* expression along the anteroposterior axis and refine the array of R8 cells.

Progression of the furrow in the absence of *smo* activity

The exact underlying mechanism of furrow progression is not yet fully understood but experiments that manipulate *hh* activity suggested that the process is controlled by Hh and its target proneural, antineural and cell cycle genes (reviewed in Treisman and Heberlein, 1998). Recent experiments by Strutt and Mlodzik (1997) have been interpreted to indicate that Hh acts indirectly in this process, probably by regulating the expression of an unknown secondary secreted signal, which would be necessary and sufficient to sustain furrow progression. The disruption of expression of this signal by the *smo*^{3} clones would be manifested as non-autonomous disruption in the pattern of gene expression and the furrow in more anterior regions. Several genes were tested here. In the case of *ato* and *ci*, their expressions in the furrow are altered in a way consistent with a dual (and probably direct) role of Hh in promoting and inhibiting its expression and activity, respectively. Expression of both *scabrous* and *E(spl)* appear to consist of two distinct domains: anterior to the furrow, an early expression domain in spaced clusters of cells that is Ato-dependent (Dokucu et al., 1996; Jarman et al., 1995). A second domain of expression, which begins in the furrow, is independent of Ato and the Hh pathway (this study). Accordingly, only the first domain of expression is affected by the *smo*^{3} clones. Together, these observations do not support a model of a secondary relay signal acting downstream of Hh, rather they suggest that other factors regulate gene expression and sustain furrow progression either independently or in combination with Hh signal.

Conclusions

Examination of gene expression and patterning defects in the cells that have lost and gained Hh signalling has revealed previously unsuspected genetic subdivisions of the process that controls the pattern of *ato* and hence R8 cells. Hh seems to

have a dual role in the establishment of the pattern of *ato* expression along the anteroposterior axis. Hh induces *ato* (at long range) in cells anterior to the furrow while inhibiting *ato* expression in nearby cells. The positive signal may be mediated through upregulation of Ci while the inhibitory signal may be through Rough activation. Second, the refinement of *ato* expression to proneural clusters or isolated cells is not obligatory for singling out of R8 cells mediated by the Scabrous and the Notch-mediated lateral specification. The refinement of *ato* by the Hh-mediated mechanism is important for the correct arrangement of R8 cells and hence of ommatidia, as monitored by defects in the larval eye disc and the adult eye. Therefore, by combining lateral inhibition with both positive and negative regulation by Hh, one can imagine how the hexagonal array of R8 cells can be patterned.

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