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Drosophila Atonal controls photoreceptor R8-specific properties and modulates both Receptor Tyrosine Kinase and Hedgehog signalling

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SUMMARY

During *Drosophila* eye development, the proneural gene *atonal* specifies founding R8 photoreceptors of individual ommatidia, evenly spaced relative to one another in a pattern that prefigures ommatidial organisation in the mature compound eye. Beyond providing neural competence, however, it has remained unclear to what extent *atonal* controls specific R8 properties. We show here that reduced Atonal function gives rise to R8 photoreceptors that are functionally compromised: both recruitment and axon pathfinding defects are evident. Conversely, prolonged Atonal expression in R8 photoreceptors induces defects in inductive recruitment as a consequence of hyperactive EGFR signalling. Surprisingly, such prolonged expression also results in R8 pattern formation defects in a process associated with both Hedgehog and Receptor Tyrosine Kinase signalling. Our results strongly suggest that Atonal regulates signalling and other properties of R8 precursors.

Key words: *Drosophila melanogaster*, Proneural, *atonal*, Photoreceptor, *hedgehog*, Receptor Tyrosine Kinase (RTK)

INTRODUCTION

The *Drosophila melanogaster* adult compound eye is composed of approximately eight hundred facets, or ommatidia, arranged in a highly stereotypical hexagonal pattern (for reviews see Ready, 1989; Cagan, 1993). This pattern is established during the third larval instar in a process marked by the morphogenetic furrow (MF), a wave of apical cell constriction that traverses the unpatterned eye imaginal disc epithelium from posterior to anterior over a period of approximately 2 days (Heberlein and Moses, 1995; Treisman and Heberlein, 1998). As the MF moves, rows of evenly spaced cells are selected to become the 'founding' R8 photoreceptors, so-called because they initiate recruitment of the remaining cells of each ommatidium (Tomlinson and Ready, 1987; Freeman, 1996; Cagan, 1997). Since each R8 cell marks the position in which an ommatidium will form, pattern formation in the mature adult eye is determined during the larval stage by the position of R8 precursors relative to one another in the eye imaginal disc.

An essential component of R8 precursor selection is the basic-helix-loop-helix transcription factor encoded by the proneural gene *atonal* (*ato*) (Jarman et al., 1994). *ato* is required in the eye for the specification of R8 photoreceptors and elsewhere for the specification of chordotonal sense organs and a subset of olfactory organs. In common with other proneural genes, *ato* expression endows cells with neural competence. In the eye imaginal disc, *ato* is activated in an essentially homogeneous stripe along the anterior edge of the MF in response to *hedgehog* (*hh*) signalling from differentiating cells posterior to the MF (reviewed in Treisman and Heberlein, 1998). As the MF progresses, R8 spacing is decided by a complex and as yet partly understood process that restricts neural competence and *ato* expression to isolated R8 precursors (Jarman et al., 1994, 1995; Baker et al., 1996; Dokucu et al., 1996). Initially, the *ato* stripe resolves to evenly spaced clusters of approximately 12 cells, the 'intermediate clusters' (Jarman et al., 1995), and then to 2-3 cells, the 'R8 equivalence groups' (Dokucu et al., 1996). Ultimately, expression of *ato* in each R8 equivalence group resolves to a single cell, the R8 precursor, after which *ato* is downregulated (Fig. 1H). The restriction of neural competence to R8 precursors during eye development is reminiscent of the selection of sense organ precursors (SOPs) from neurally competent 'proneural clusters' in the peripheral nervous system (PNS) (Ghysen and Dambly-Chaudière, 1989; Ghysen et al., 1993). In both the eye and the PNS, the resolution of proneural expression depends on a common process, lateral inhibition. Thus, when mutated, genes such as *Notch (N), Delta (Dl)* and *scabrous* (*sca*) result in R8 hyperplasia (Cagan and Ready, 1989; Cubas et al., 1991; Parks and Muskavitch, 1993; Campos-Ortega, 1994; Baker et al., 1995; Baker and Zitron, 1995). *ato* resolution in the eye also has some distinct features, such as a requirement for *rough (ro)*: in *ro* mutants most of the cells of the R8 equivalence group adopt an R8 fate (Dokucu et al., 1996).

Whilst many studies of *ato* and other proneural genes have concentrated on their neural competence function, less

attention has been paid to the possibility that these genes also determine specific neural properties of the different precursor subtypes they specify. Indeed, the progressive determination model of neural precursor selection proposed that selection (requiring proneural genes) is divorced from neural subtype specification (requiring subtype selector genes) (Ghysen and Dambly-Chaudière, 1989; Jan and Jan, 1993). This view may hold true for the proneural genes of the *achaete-scute* complex (AS-C) because they specify the precursors of a variety of cell types from equivalence groups in a range of neural and non-neural developmental contexts (e.g. Carmena et al., 1995). There are good reasons, however, to think that *ato* controls specific neural functions in addition to providing general neural competence (see also Jarman et al., 1993; Chien et al., 1996). First, *ato* is required for a few distinct subsets of sense organs and, in contrast to the AS-C, has no apparent function outside the nervous system. Second, *ato* seems to control specific functions of chordotonal sense organ precursors to make them distinct from the precursors of external sense organs specified by the AS-C (zur Lage et al., 1997; Jarman and Ahmed, 1998; zur Lage and Jarman, 1999). In the eye too, there is potential for similar *ato* functions: *ato* expression remains in R8 cells some 6-8 hours after their initial appearance, and so could control properties that make R8 photoreceptors distinct from the other photoreceptors (R1-R7) both physiologically and developmentally. For example, R8 acts as an organiser, triggering inductive recruitment of other cell types in the ommatidium through Receptor Tyrosine Kinase (RTK) signalling pathways (Tomlinson and Ready, 1987; Basler and Hafen, 1991; Freeman, 1996).

So far, mutant analysis has only revealed *ato's* role in the selection of R8 cells, because these cells are completely absent in the described *ato* mutants (Fig. 1E,F); hence it has been impossible to analyse whether *ato* determines R8 properties after their selection. In such circumstances, gain-of-function studies can prove informative for uncovering additional gene functions, as shown by misexpression analysis of *ato* in chordotonal organ development (Jarman and Ahmed, 1998). In this report, we present evidence that *ato* indeed controls specific R8 properties. Firstly, we show in a hypomorphic *ato* mutant (ato^2) that reducing *ato* function specifically in selected R8 precursors renders them functionally compromised in photoreceptor recruitment and axon pathfinding. Secondly, we show by targeted misexpression that prolonged *ato* function within R8 cells increases inductive recruitment as a consequence of hyperactive EGFR signalling. Surprisingly, this misexpression also interferes with R8 precursor selection and pattern formation, apparently via Hh and RTK signalling. Interestingly, *ato* appears to modulate Hh signalling rather than control the overall level of Hh expression. We conclude that, in addition to endowing cells with neural competence, *ato* also controls some of the key functions of R8 precursors.

MATERIALS AND METHODS

Fly stocks

109-68Gal4 is an enhancer trap insertion on the second chromosome (Chien et al., 1996; Jarman and Ahmed, 1998). In all experiments, UAS-*ato1* was used (Jarman and Ahmed, 1998). To generate the balanced *109-68Gal4*, UAS-*ato* ($ato^{109\text{-}68}$/ *CyO*) line, the *109-68Gal4* and UAS-*ato* elements were recombined onto the same chromosome. hh^{AC} is an amorphic allele of the *hh* gene, and hh^{P30} is an enhancer trap insertion at the *hh* locus (Ma et al., 1993). ptc^5 (=tuf^5), ro^1, sev^{d2} (=sev^{14}), phl^{C110} (=phl^1), $Egfr^{f3}$ and Dl^{9P} are all described in Lindsley and Zimm (1992). *ptc*, *ro*, *Egfr* and *Dl* alleles were all obtained from the Bloomington Stock Center. phl^{C110}, sev^{d2} and $Ras1^{e2f}$ alleles were kindly provided by E. Hafen, and UAS-*hh* and hh^{P30} by D. Strutt. ato^1 is either a strong hypomorph or an amorphic allele of *ato* (Jarman et al., 1995). Enhancer trap lines marking photoreceptor subtype identity were XA12 for R7 (Mlodzik et al., 1990) and BBO2 for R8 (Hart et al., 1990) and were obtained from L. Zipursky. Crosses with *109-68Gal4* and $ato^{109\text{-}68}$ were performed at 29°C to increase Gal4 activity (the severity of the phenotype is reduced at lower temperatures). All other crosses were performed at 25°C. Stocks were maintained on standard fly medium.

ato^2 was isolated in an X-ray mutagenesis screen (E. H. G., Y. N. Jan, A. P. J., unpublished). Segregation analysis revealed an X-linked lethal mutation associated with the ato^2 allele. Cytogenetic analysis of polytene chromosome squashes revealed the basis for this as a translocation of a large portion of the X chromosome into the *ato* region of the third chromosome (84F). This translocation is associated with complex genetics that makes manipulation difficult. It is homozygous lethal, and male sterile as a heterozygote due to compulsory simultaneous segregation of the deleted X chromosome.

Histology

Scanning electron microscopy (SEM) and sectioning of the adult retina were performed according to standard procedures (see the Carthew group website: http://info.pitt.edu/~carthew/manual/Manual.html). All scanning electron micrographs were taken at 150× magnification. For immunohistochemistry, eye-antennal imaginal discs were dissected from wandering 3rd instar larvae and fixed in 3.7% formaldehyde (15 minutes). Incubations with primary and secondary antibodies were performed according to standard procedures. Light microscope images were taken on an Olympus Provis microscope. Confocal images were taken on an Leica TCS-NT microscope, and scanning electron micrographs were taken on a Cambridge Stereoscan 250. All images were adjusted and composed using Adobe Photoshop 3.0.

Antibodies

The following antibodies were used: mouse anti-β-galactosidase (1/1000; Promega), rabbit anti-β-galactosidase (1:10,000; Cappel), affinity-purified rabbit anti-Ato (1/2000), mouse anti-Bride of sevenless (Boss, 1/200; provided by S. L. Zipursky), rat anti-Ci (1/10; provided by R. Holmgren), and mouse mAb22C10 (1/200; Developmental Biology Hybridoma Bank, Iowa, USA). Secondary antibodies (used at a concentration of 1/1000) were obtained from Jackson Laboratories.

RESULTS

Evidence from a loss-of-function mutation that Ato regulates recruitment

The previously described *ato* mutant alleles show a complete failure to select R8 precursors (Jarman et al., 1994, 1995). Consequently, it has not been possible to determine whether *ato* has additional roles in determining the characteristics of R8 cells after their selection. We have therefore examined a weaker allele, ato^2. ato^2/ato^1 flies have severely reduced eyes, which nevertheless contain ommatidia (approximately 200-300 in males, 5-15 in females), indicating that *ato* function and R8 selection are not entirely abolished (Fig. 1C,D). Sequencing revealed no mutations within the ato^2 coding region. Instead,

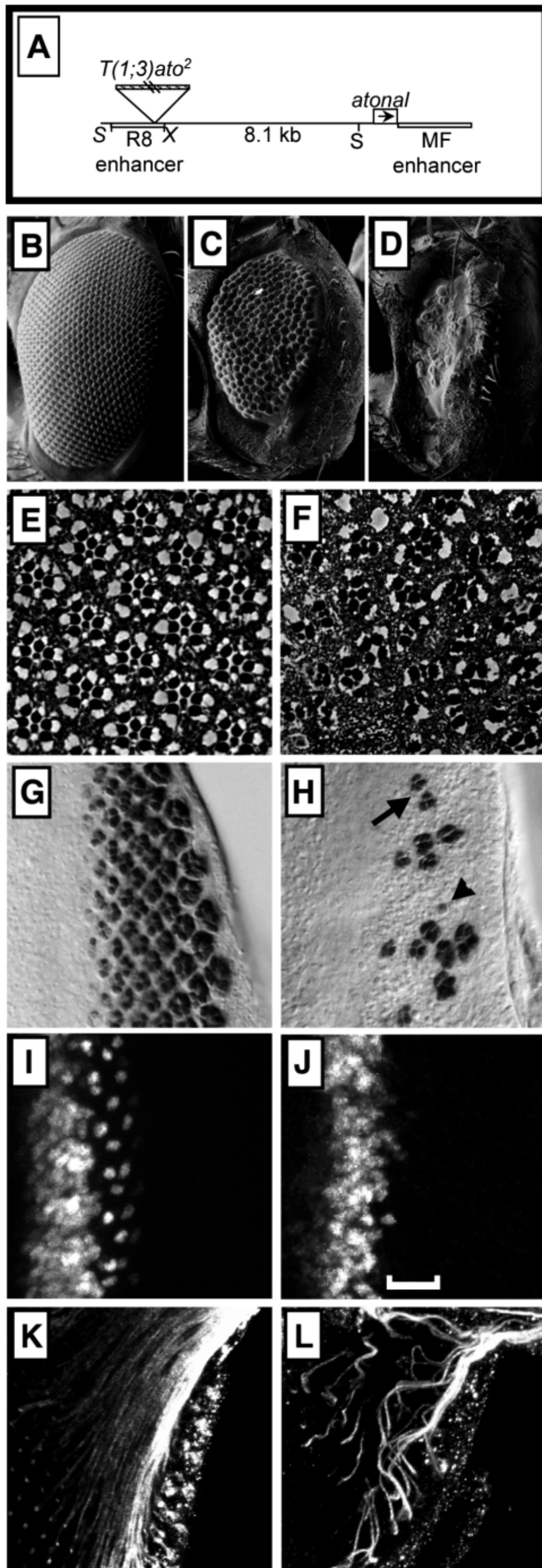

Fig. 1. R8 photoreceptors are developmentally compromised in the *ato*^{2} mutant. (A) Diagram of the *ato*^{2} allele showing the site of insertion upstream of the *ato* ORF that is responsible for separating the gene from its R8-specific enhancer element (enhancer elements mapped by Sun et al., 1998). (B-D) Scanning electron microscopy of the adult compound eye. (B) Wild type. (C) *ato*^{2}/*ato*^{1} male. (D) *ato*^{2}/*ato*^{1} female. (E,F) Tangential sections through the adult compound eye. (E) Wild type. (F) *ato*^{2}/*ato*^{1} male. The ommatidia frequently lack the correct complement of photoreceptors, indicative of a recruitment defect. (G,H) Photoreceptors detected in 3rd instar larval eye imaginal discs by antibodies to the nuclear protein, Elav. (G) Wild type, showing progressive recruitment from left to right. (H) *ato*^{2}/*ato*^{1} female, showing variable clusters of photoreceptors containing from 1 (arrowhead) to 8 cells. There is a frequent occurrence of 3-cell clusters (arrows), suggesting a frequent blockage of recruitment after R2,5 formation.
(I-L) Immunohistochemical detection of Ato (I,J) and 22C10 (K,L) in eye imaginal discs. (I,K) Wild type. (J,L) *ato*^{2}/*ato*^{1} male. Ato is conspicuously absent in the region where selected R8 precursors are located (bracket in J). In addition, photoreceptor axons navigate to the optic stalk poorly in *ato*^{2}/*ato*^{1}, indicative of a defect in axon pathfinding (L). The axon pathfinding defect is more severe in females. Anterior is to the left in all figures.

molecular characterisation showed that an insertion had occurred between 7 and 8 kb upstream of the *ato* gene (unpublished data and Fig. 1A). Thus, *ato*^{2} is a regulatory mutation in which an insertion has resulted in the separation of an eye-specific 5′ enhancer element from the *ato* gene (Sun et al., 1998).

If *ato* has no function beyond R8 selection, one would expect that ommatidial assembly can proceed normally around the few R8 precursors that are formed in this mutant. This is not the case: ommatidia that differentiate in *ato*^{2}/*ato*^{1} mostly contain fewer photoreceptors than wild type (Fig. 1F), indicative of a recruitment defect. Detection of photoreceptor recruitment in imaginal discs confirmed this and showed that photoreceptor cluster sizes were variably reduced, containing from 1-8 photoreceptors (Fig. 1H). This suggests that recruitment may be blocked at any stage. Consistent with this, analysis of photoreceptor subtype markers showed that any or all recruited photoreceptors could be affected (data not shown) (although later cell types may be more frequently lost than earlier types). Thus, it appears that the R8 precursors that form in *ato*^{2}/*ato*^{1} flies are functionally compromised. Significantly, the defects in recruitment correlate with the absence of *ato* expression from selected R8 precursors in *ato*^{2}/*ato*^{1} (Fig. 1J), as expected from the loss of an R8-specific enhancer. The loss of these cells suggests that although R8 selection can occur, *ato* expression is needed in these cells to regulate recruitment.

In addition to the recruitment defect, staining *ato*^{2}/*ato*^{1} eye discs with a cytoplasmic neural marker revealed that photoreceptor axon bundles frequently navigate incorrectly to the optic stalk (Fig. 1L). However, those axons that reach the optic stalk are able to continue to their target sites within the optic lobe (data not shown). Since photoreceptor axon pathfinding is thought to be guided by the R8 axon, the guidance defects evident in *ato*^{2}/*ato*^{1} are perhaps indicative of a lack of pathfinding properties in some R8 cells. Clearly, these findings are consistent with the idea that *ato* controls some of the neural properties of R8 photoreceptors.

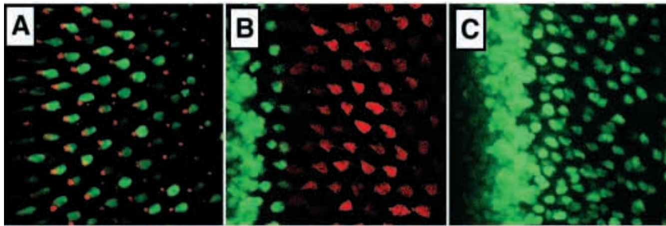

Fig. 2. Misexpression of *ato* in differentiating R8 photoreceptors. Confocal microscopy for immunohistochemical detection in 3rd instar larval eye imaginal discs. (A) 109-68*Gal4*/UAS-*lacZ* showing β-galactosidase (green) in differentiating R8 photoreceptors compared with the R8 marker, Boss (red). (B,C) Detection of Ato (green) and β-galactosidase (red). (B) 109-68*Gal4*/UAS-*lacZ*. Wild-type *ato* is downregulated in the β-galactosidase-expressing R8 neural precursors shortly after selection. (C) 109-68*Gal4*/UAS-*ato* (abbreviated as $ato^{109\text{-}68}$). Ato is detected in more posterior regions of the disc in differentiating R8 cells.

Prolonged expression of *ato* in differentiating R8 photoreceptors induces EGFR-dependent recruitment defects

If *ato* determines R8 recruitment properties then experimentally increasing *ato* expression within R8 precursors may result in excessive recruitment. To test this, we used a *Gal4* enhancer trap line that in the eye drives expression of *Gal4* solely in patterned, differentiating R8 photoreceptors (109-68*Gal4*, Fig. 2A,B). Thus, *ato* expression is reinforced and prolonged in R8 precursors at the time endogenous expression is normally downregulated (Fig. 2C). Hence, any effects on eye development should be the result of an alteration in R8 cell behaviour after selection.

109-68*Gal4*/UAS-*ato* flies (hereafter abbreviated as $ato^{109\text{-}68}$) have rough eyes (Fig. 3C), with many ommatidia containing inappropriate complements of photoreceptors and support cells (Fig. 3F,G). Cell-fate markers showed that all cell types can be affected in $ato^{109\text{-}68}$, and there could be both an increase and decrease in any specific cell type. We show below that these result from a combination of recruitment and R8 spacing defects. Moreover, this effect is not replicated by identical misexpression of other proneural genes (including *scute* and the closely related *amos* gene; Goulding et al., 2000), showing that it is highly specific to *ato* and unlikely to result from mimicry of or interference with a related bHLH protein.

The effect of *ato* misexpression is non-autonomous since the presence of supernumerary cells is not always correlated with ectopic Ato expression within them (data not shown). Since recruitment requires EGFR activity (Freeman, 1996), we tested genetically whether R8 precursors in $ato^{109\text{-}68}$ induce excessive photoreceptor recruitment via upregulated RTK signalling. This appears to be the case. Mutations reducing the efficiency of RTK signal transduction can suppress ectopic photoreceptor formation in $ato^{109\text{-}68}$. For example, the gene that encodes the *Drosophila* homologue of mammalian Raf-1 serine/threonine kinase (*polehole* (*phl*) Ambrosio et al., 1989) (Fig. 4B) and *daughter of sevenless (dos)* (Herbst et al., 1996; Raabe et al., 1996) (data not shown) gives strong suppression, whereas *Ras* mutants suppressed $ato^{109\text{-}68}$ somewhat less effectively (Fig. 4C). Reducing the dosage of *EGFR* itself also resulted in suppression

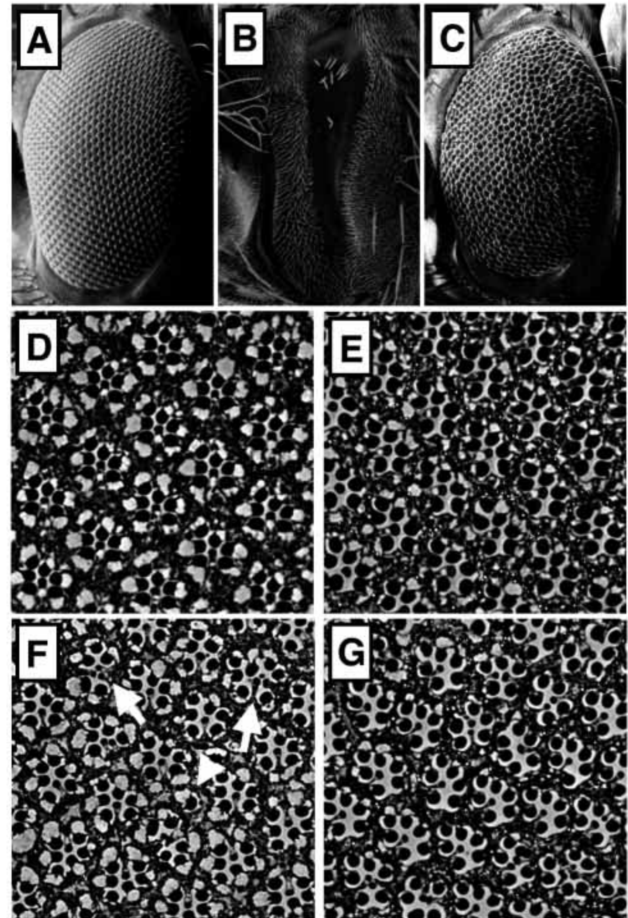

Fig. 3. $ato^{109\text{-}68}$ results in a rough eye phenotype as a consequence of defects in recruitment and pattern formation. (A-C) Scanning electron microscopy of the adult compound eye. (A) Wild type. (B) ato^{1}/ato^{1}. Since ommatidial founder cells (R8 photoreceptors) are not selected in this mutant, the compound eye is absent. (C) $ato^{109\text{-}68}/+$. As a consequence of abnormal ommatidial packing, the $ato^{109\text{-}68}$ compound eye is rough, although regions of hexagonal packing are still apparent. (D-G) Tangential sections through the adult compound eye at the level of the R7 (D,F) and R8 (E,G) photoreceptors. (D,E) Wild type. (F,G) $ato^{109\text{-}68}/+$. The ommatidia are no longer arranged in straight rows, and ectopic photoreceptors infrequently differentiate in the interommatidial spaces (arrowhead in F). There are also frequent defects in recruitment (arrows in F), with many of the ommatidia containing ectopic photoreceptors with morphology characteristic of R7 or R8.

of the recruitment defects in $ato^{109\text{-}68}$ (Fig. 4D). Thus, prolonged Ato induces recruitment defects via RTK signalling.

$ato^{109\text{-}68}$-induced EGFR bypasses Sev signalling in R7 formation

Among the recruitment defects in $ato^{109\text{-}68}$, analysis of sections (Fig. 3F) and enhancer trap elements expressed in specific subsets of developing photoreceptors revealed the pronounced occurrence of supernumerary R7 photoreceptors. R7 formation requires both EGFR and Sev signalling, but it is known that experimentally induced hyperactive EGFR can rescue R7 formation in the absence of *sev* (Freeman, 1996).

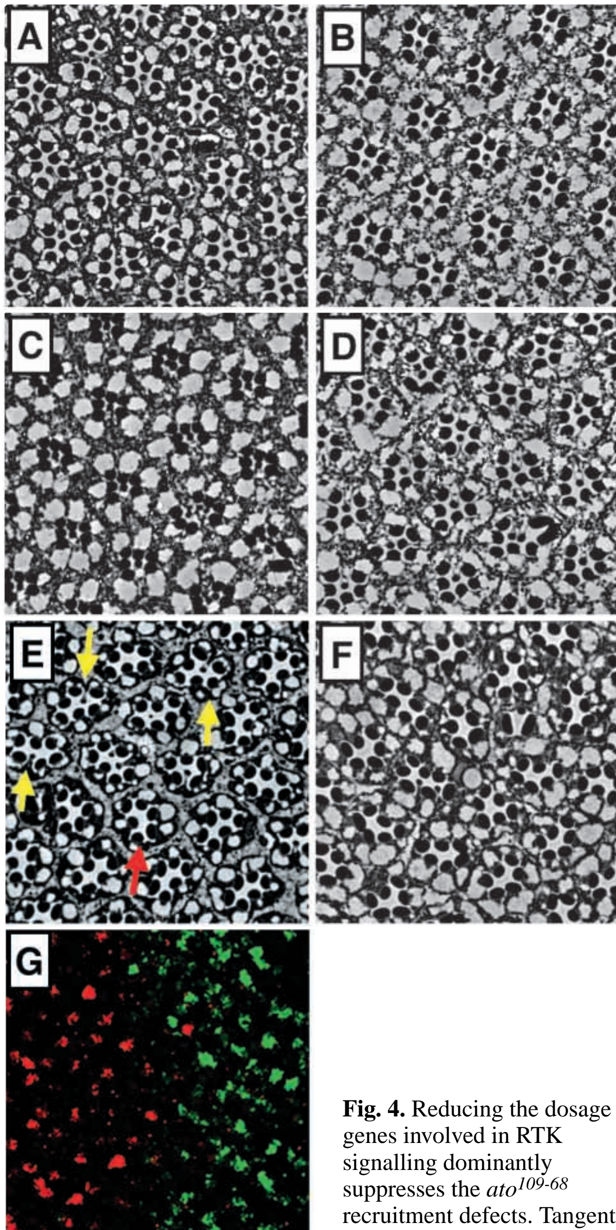

Fig. 4. Reducing the dosage of genes involved in RTK signalling dominantly suppresses the *ato*$^{109\text{-}68}$ recruitment defects. Tangential sections through the adult compound eye at the level of the R7 photoreceptors. (A) *ato*$^{109\text{-}68}$/+. (B) *phl*^{C110}/+; *ato*$^{109\text{-}68}$/+, showing strong suppression of all defects associated with *ato*$^{109\text{-}68}$. (C) *ato*$^{109\text{-}68}$/+; *Ras1*^{e2f}/+, showing weak suppression the *ato*$^{109\text{-}68}$ rough eye. (D) *ato*$^{109\text{-}68}$/*Egfr*^{f3}. Notice that although defects in recruitment are suppressed when the dose of *Egfr* is reduced, there is still evidence of abnormal pattern formation. (E,F) Ectopic R7 photoreceptor formation in *ato*$^{109\text{-}68}$ is a consequence of hyperactive EGFR, rather than abnormal Sev signalling. (E). *sev*^{d2}/*sev*^{d2}; *ato*$^{109\text{-}68}$/+. In the absence of Sev activity, the ommatidia normally lack R7 photoreceptors (as marked by the red arrow). In the presence of *ato*$^{109\text{-}68}$, however, many R7 photoreceptors still differentiate (yellow arrows). (F) *sev*^{d2}/*sev*^{d2}; *ato*$^{109\text{-}68}$/ *Egfr*^{f3}. The *ato*$^{109\text{-}68}$-dependent rescue of R7 formation is strongly suppressed when the dosage of *Egfr* is reduced. (G) *sev*^{d2}/*sev*^{d2}; *ato*$^{109\text{-}68}$/+, with R7-specific enhancer trap line, XA12. Confocal microscopy of 3rd instar larval eye imaginal discs showing β-galactosidase (green) in R7 photoreceptors compared with misexpressed Ato protein (red). There is significant rescue of R7 formation (normally there is no expression of this enhancer trap in *sev*^{d2}/*sev*^{d2} discs).

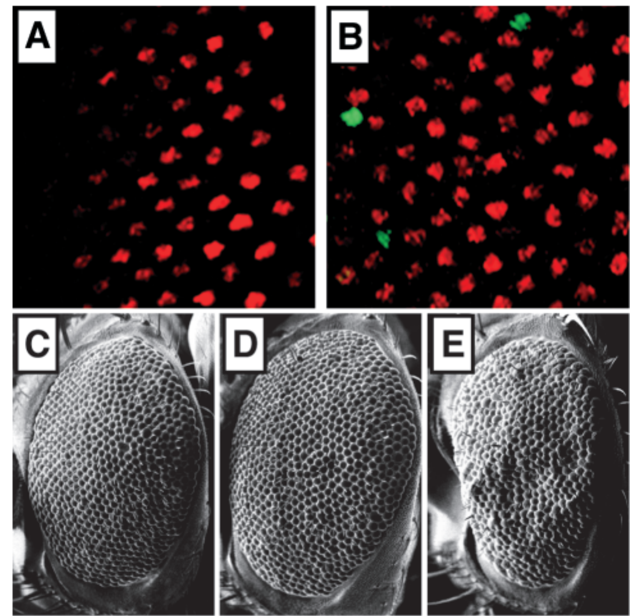

Fig. 5. *ato*$^{109\text{-}68}$ disrupts processes regulating R8 patterning and selection. (A,B) The pattern of differentiating R8 photoreceptors visualised using an R8-specific enhancer trap element (BBO2) (αAto, green). (A) Wild type. (B) *ato*$^{109\text{-}68}$/+, showing incorrectly patterned R8 photoreceptors. (C-D) Scanning electron microscopy of the adult compound eye. (C) *ato*$^{109\text{-}68}$/+. (D) *ato*$^{109\text{-}68}$/+; *ro*^{1}/+. (E) *ato*$^{109\text{-}68}$/+; *Dl*^{9P}/+. The *ato*$^{109\text{-}68}$ rough eye is enhanced in both cases. The enhancement is stronger in D, perhaps because reducing the dose of *Dl* would affect the 'proneural cluster' stage (i.e. 12-16 cells), whereas *ro* acts later during selection of the R8 precursor from cells of the R8 equivalence group (i.e. 2-3 cells).

Therefore, we investigated whether excessive R7 formation in *ato*$^{109\text{-}68}$ was a consequence of either upregulated EGFR or Sev signalling. The former seems to be the case, since significant R7 recruitment still occurred in the absence of *sev* activity (which normally completely abolishes R7 formation). Thus in *sev*^{d2}; *ato*$^{109\text{-}68}$ flies, 16±2% of ommatidia contained an R7-like cell as judged by morphology in eye sections (Fig. 4E). R7 formation was confirmed by R7-specific enhancer trap expression in eye discs (Fig. 4G). Furthermore, reducing *EGFR* gene dosage suppresses the *ato*$^{109\text{-}68}$-dependent rescue of R7 recruitment in *sev* eyes (Fig. 4F; 6±1% of ommatidia contained an R7 like cell). Thus, EGFR signalling is hyperactive in *ato*$^{109\text{-}68}$ and, at the appropriate time in development, can bypass the requirement for *sev* during R7 recruitment. We have not ruled out the possibility that Sev signalling is also hyperactivated, although the suppression of recruitment defects by *EGFR* gene dosage reduction suggests it is not.

Prolonged *ato* expression non-autonomously induces R8 patterning defects

In the above experiments, we used the 109-68*Gal4* driver to avoid directly altering *ato* patterning in the MF during R8 singling out, since this is known to disrupt R8 pattern formation (Dokucu et al., 1996; unpublished). Nevertheless, SEM, sections and disc stainings of *ato*$^{109\text{-}68}$ revealed

additional defects consistent with a disruption in R8 pattern formation, despite the fact that there is no evidence for *Gal4*-driven expression during the process of R8 selection (Fig. 2B). Ommatidial spacing was abnormal, and there appeared to be fused ommatidia (Fig. 3C and data not shown). In *ato*$^{109-68}$ discs, R8 cells are more dense, frequently juxtaposed (twinned), and unevenly spaced relative to one another, as shown by an R8-specific enhancer trap line (Fig. 5A,B) and the expression of endogenous *ato* itself (Fig. 2C). In addition, the normally regular refinement of *ato* expression is disrupted. These defects are reminiscent of R8 patterning mutations such as *sca* and *ro*, suggesting that mechanisms regulating endogenous *ato* expression during singling out are altered. This inference is supported by the observation that the *ato*$^{109-68}$ patterning defect is sensitive to changes in the dosage of genes involved in *ato* regulation and R8 singling out, including *ro* (Fig. 5D) and *Dl* (Fig. 5E), each as a result of the selection of an excess of R8 precursors (data not shown). As in mutants that disrupt R8 patterning, many of the later cell patterning defects appear to be a secondary consequence of the initial R8 patterning disruption.

Although R8 patterning depends on *ato* function, its disruption in *ato*$^{109-68}$ initially appears counterintuitive, since R8 cells should already be correctly patterned prior to the onset of ectopic *ato* expression within them. Therefore these defects do not reflect a direct interference with R8 patterning processes. Instead, they must reflect an alteration of R8 behaviour that is non-autonomously transmitted to these earlier R8 patterning events. Interestingly, the phenotype is opposite to that expected from increasing *ato* function in R8 during selection itself. In this case, one might expect an increase in lateral inhibition, and hence a decrease in R8 formation and density. The inference is that we are detecting a hitherto uncharacterised function of *ato* in patterned R8 cells.

Defects in R8 patterning are associated with RTK and Hedgehog signalling

The effect of *ato* misexpression in selected R8 cells must be communicated to the process of selecting new rows of R8 cells within the MF. Interestingly, mutations of Raf strongly suppressed the effect of *ato* misexpression both at the level of adult ommatidial patterning (Fig. 4B) and R8 patterning in discs (data not shown), apparently indicating that Raf-dependent signalling mediates both recruitment and R8 patterning defects. However, reducing the gene dosage of *EGFR* did not appear to suppress defects in pattern formation or R8 precursor selection, even though defects in recruitment were clearly suppressed (Fig. 4D). Therefore, it is possible that defects in pattern formation depend on hyperactivation of an RTK other than EGFR.

In wild type, differentiating photoreceptors secrete Hh, which diffuses anteriorly across the MF to activate *ato* (Domínguez and Hafen, 1997; Strutt and Mlodzik, 1997; Domínguez, 1999) in cells previously inhibited by the transcription factor encoded by *hairy* (*h*) (Brown et al., 1995). This drives *ato* activation, and further R8 selection across the disc (Heberlein et al., 1993; Ma et al., 1993; Treisman and Heberlein, 1998). Therefore, defects evident in *ato*$^{109-68}$ could be the result of a change in Hh signalling. Genetic interaction analysis strongly supports this possibility. Mutations of *hh* (Fig. 6A,B) and its target gene *cubitus interruptus* (*ci*) (data not shown) are strong dominant suppressors of the *ato*$^{109-68}$ rough eye and of R8 patterning defects (data not shown). Conversely, mutations of the inhibitory gene *patched* (*ptc*) were strong enhancers (Fig. 6C). Reducing the gene dose of *h*, which normally limits the Hh-induced anterior spread of the *ato* stripe (Brown et al., 1995), also enhanced the rough eye, albeit weakly (data not shown). Therefore, these genetic interactions are all consistent with an increase in extent and intensity of Hh signalling in *ato*$^{109-68}$.

We analysed *hh* expression using an enhancer trap element (*hh*^{P30}), which is reported to reflect faithfully endogenous *hh* activity (Ma et al., 1993). Surprisingly, in wild type little if any *hh*^{P30} expression was observed in R8, being instead largely restricted to photoreceptors R2 and R5 (Fig. 6D). Moreover, in *ato*$^{109-68}$; *hh*^{P30} discs, β-galactosidase and Ato expression were not coincident, even in cases of R8 twinning in which an R2 or R5 precursor had been switched to an R8 fate (Fig. 6E). In addition, the level of *hh*^{P30} expression in *ato*$^{109-68}$ was not, or at most only slightly, elevated in cells surrounding the R8s (Fig. 6E). Therefore, Ato is not a major activator of *hh* expression, either directly in R8 or indirectly in adjacent cells. On the contrary, *ato* may instead autonomously inhibit *hh* expression.

We next considered whether Hh activity is increased post-transcriptionally. The full-length form of Ci protein is stabilised by Hh signalling, and its abundance is therefore a reporter of Hh activity (Alexandre et al., 1996; Aza-Blanc et al., 1997). In wild type, this protein overlaps with Ato anterior to and within the MF (Fig. 6F) (Domínguez and Hafen, 1997; Domínguez, 1999). In *ato*$^{109-68}$, Ci expression was increased in abundance in the eye disc compared with its expression in the adjacent antennal disc (Fig. 6G). This increase was frequently small and hard to detect, but became very obvious in eye discs in which the *ato*$^{109-68}$ phenotype is enhanced by loss of one copy of *ptc*. In this case a clear increase in Ci level, and perhaps extent, was observed; such an increase was not apparent in *ptc* heterozygotes alone (data not shown).

hh upregulation does not itself result in patterning defects but enhances *ato*$^{109-68}$

Is a general increase in Hh activity sufficient to explain the patterning defects? Additional experiments suggest not. When *hh* expression levels were elevated by driving *hh* itself in R8 cells (109-68*Gal4*; UAS-*hh* flies), Ci protein was clearly increased (Fig. 6H), but the eye of pharate adults appeared essentially wild type (data not shown), and *ato* refinement was unperturbed (Fig. 6H,J). Furthermore, when *hh* was ectopically expressed in all cells posterior of the MF using a strong Gal4 driver (GMR*Gal4/*UAS-*hh*) there was again no obvious effect on eye patterning of pharate adults (although the GMR*Gal4* construct itself produces a slight rough eye, data not shown). These experiments suggest that, under these conditions, the global level of Hh or Ci expression does not affect patterning. Signficantly, however, when both *ato* and *hh* were ectopically expressed in R8 (109-68*Gal4*, UAS-*ato*; UAS-*hh*), the eye was extremely disorganised (data not shown) and *ato* expression was severely abnormal (Fig. 6I,K). Refinement of Ato to solitary R8 precursors rarely occurred, and the level of *ato*

expression within the MF fluctuated considerably. Thus, although increasing the level of *hh* expression alone does not induce defects in pattern formation, it strongly potentiates the effect of increased *ato*. These data together suggest that Ato modulates Hh signalling in a manner more subtle than regulating its overall intensity.

DISCUSSION

Using a combination of misexpression and loss-of-function genetic analyses, we have demonstrated that *ato* endows R8 cells with some key characteristics. The data indicate that in R8 *ato* modulates Hh signalling activity, initiates EGFR-dependent recruitment, and possibly regulates axon guidance. These findings support the concept that proneural bHLH transcription factors function not only to endow neural competence but also to control specific neural subtype properties. This has important implications not only for *Drosophila* neurogenesis, but also for the large number of vertebrate *ato* and AS-C homologues that have been identified. Indeed, recent studies indicate that at least some of the vertebrate *ato* homologues may also have subtype determining properties (e.g. Perez et al., 1999).

ato controls EGFR-mediated recruitment by R8

The hypothesis that *ato* determines characteristics within selected neural precursors was initially intimated from *ato* misexpression studies during chordotonal organ precursor selection. *ato* represses the homeobox gene *cut*, thereby diverting these precursors from an external sense organ fate and allowing them to adopt the alternative chordotonal organ fate (Jarman and Ahmed, 1998). Clearly, such aspects of *ato's* subtype-determining properties must be contingent on the developmental context (chordotonal versus R8 precursor). One property, however, appears to be common to both developmental contexts: EGFR-mediated local recruitment. Both photoreceptors (Freeman, 1996) and chordotonal precursors (Okabe and Okano, 1997; zur Lage et al., 1997; zur Lage and Jarman, 1999) are clustered as a consequence of EGFR-dependent recruitment. In the case of chordotonal recruitment, we proposed that *ato* directly controls the level of EGFR signalling (zur Lage et al., 1997; zur Lage and Jarman, 1999). In the eye, although it is clear that R8 initiates recruitment, it was previously unclear whether this property is directly controlled by *ato*. Our experiments suggest that it is. Prolonging *ato* activity in R8 results in excessive recruitment as a result of hyperactive EGFR signalling. Conversely, reducing *ato* activity specifically in selected R8 precursors (ato^2/ato^1 flies) results in ommatidia that contain fewer photoreceptors than wild type. We postulate that these R8 precursors are unable to trigger recruitment efficiently. It is instructive to compare this with the *Ellipse* (*Elp*) mutation: superficially, the reduction in R8 selection observed in *Elp* mutants (Baker and Rubin, 1992; Alexander et al., 1999) resembles that of ato^2. In contrast to ato^2/ato^1, however, the remaining ommatidia are of normal structure in *Elp* eyes, indicating that although fewer R8 precursors arise, they can function normally with respect to recruitment. Strikingly, whilst *ato* expression in selected R8 precursors is normal in *Elp* mutants (Jarman et al., 1995), it is not apparent in the R8 precursors in ato^2/ato^1, consistent with a role for Ato after R8 selection has occurred. In wild type, *ato* presumably triggers the initiation of signalling, since recruitment continues after *ato* is switched off. The appearance of ectopic R7 cells upon misexpression is, in this respect, an artefact of the delayed developmental timing of *ato* misexpression relative to wild type.

Effects of *ato* in R8 on future R8 patterning

Expression of *ato* in R8 photoreceptors induces defects in pattern formation at multiple stages of R8 singling out. Thus, ommatidial spacing is irregular as a result of aberrant spacing of the intermediate clusters, while twinning of R8 precursors is a consequence of a perturbation in fate refinement in the R8 equivalence group. Although *ato* is likely to be directly involved in these processes, the effect we have detected here is novel and unexpected, since we have misexpressed *ato* after these events have finished and so must primarily effect R8 behaviour subsequent to these processes. The effect of *ato* misexpression must therefore be communicated from the differentiating R8 cells to the MF. It is significant, therefore, that R8 patterning disruption in ato^{109-68} appears to be associated with both Hh- and Raf-dependent signalling. In both cases, increased signalling is associated with enhanced R8 formation. The Raf-dependent signalling is therefore distinct from the role of *ato* and EGFR signalling in intermediate cluster spacing (Domínguez et al., 1998; Spencer et al., 1998; Chen and Chien, 1999), which appears to be inhibitory via the activation of *ro* (Dokucu et al., 1996; Domínguez et al., 1998; Domínguez, 1999). Thus, we have detected a new *ato*- and Raf-dependent process that acts to promote subsequent R8 formation.

A weak gain-of-function *ci* mutation induces patterning defects similar to those induced by *ato* misexpression in R8 (Domínguez, 1999). This indicates that such defects in ato^{109-68} are feasibly explained by an effect on Hh signalling, yet it is unclear how perturbing Hh signalling (or Ci expression) disrupts pattern formation and R8 precursor selection. We found that simply increasing the amount of Hh (and consequently Ci) is not sufficient to disrupt R8 patterning. Moreover, it is clear that *hh* expression is not strongly regulated by *ato* either directly or indirectly. Of more importance may be local irregularities in Ci activation. An effect of this sort on pattern formation is consistent with the recent findings of Dominguez (1999). However, this study also proposed that Hh has two effects on *ato* expression: a long-range activation via *ci* and a short-range repression via *ro* in cells between the intermediate clusters (Dokucu et al., 1996; Domínguez, 1999). Our data are inconsistent with this model, since in ato^{109-68}, R8 cells are more densely spaced rather than less so, even though one would predict that short-range R8 inhibition should be exacerbated (given that *hh* suppresses the defects of *ato* misexpression). A possible explanation is that *ato* in R8 specifically modulates long-range Hh signalling alone.

The RTK and Hh signalling may be unrelated, or one may be mediated by the other. Given that R8 is not the major site of *hh* expression, *ato* must modulate Hh function non-autonomously. It is possible that the Raf-dependent signalling inferred above could mediate the effect of *ato* in R8 on Hh signalling. For example, close juxtaposition of Ato- and Hh-

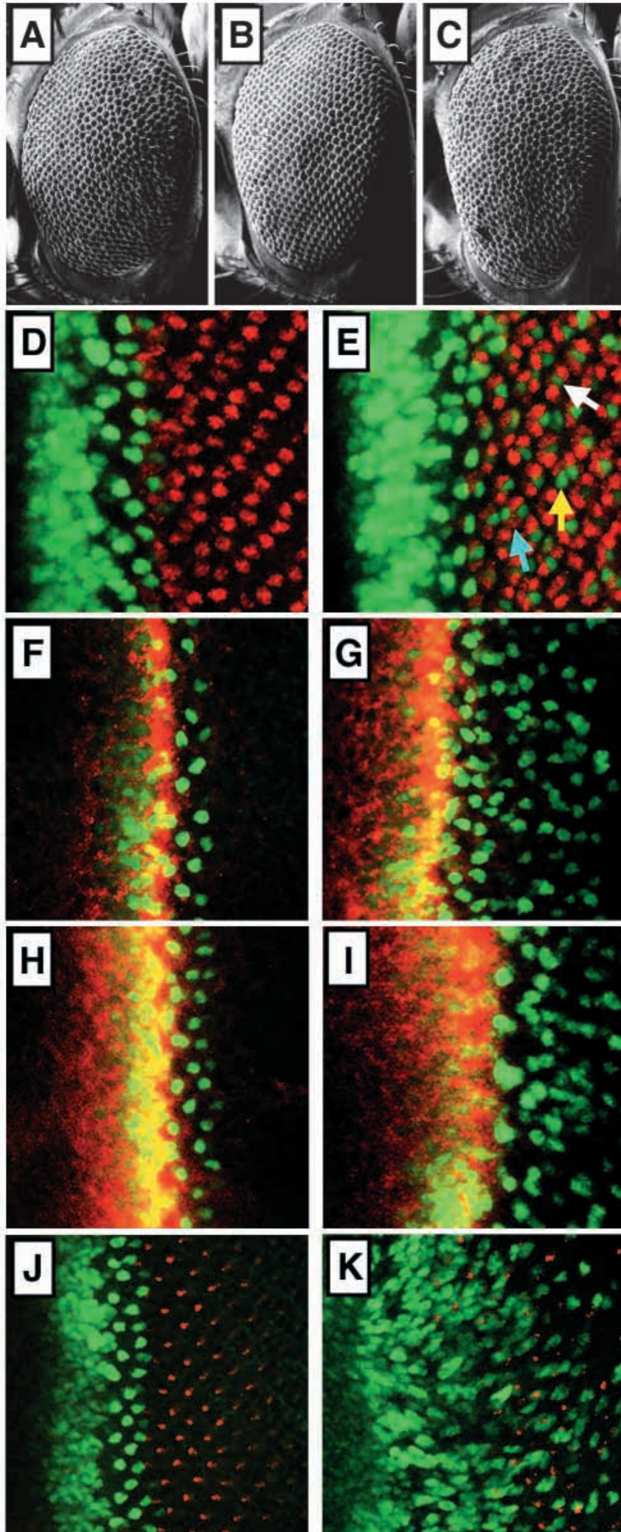

Fig. 6. Defects in pattern formation and R8 precursor selection in *ato*$^{109\text{-}68}$ are mediated by hyperactive Hh signalling. (A-C) Scanning electron microscopy of the adult compound eye. (A) *ato*$^{109\text{-}68}$/+. (B) *ato*$^{109\text{-}68}$/+*; hh*^{AC}/+. Reducing the dosage of *hh* clearly suppresses the *ato*$^{109\text{-}68}$ rough eye. (C) *ato*$^{109\text{-}68}$/*ptc*^{5}. Reducing the dosage of *ptc*, which limits precocious Hh signalling, enhances the *ato*$^{109\text{-}68}$ rough eye such that regions of hexagonal ommatidial packing are now rare. (D-K) Confocal microscopy of 3rd instar larval eye imaginal discs. (D,E) Immunohistochemical detection of Ato (green) and β-galactosidase (red) in the background of a *lacZ* reporter of endogenous *hh* expression (*hh*^{P30}). (D) *hh*^{P30}/+. (E) *ato*$^{109\text{-}68}$/+*; hh*^{P30}/+. Expression of *ato* and *hh* is mutually exclusive, with *ato* in R8 and *hh* in R2 and R5 (white arrow). Also, *hh* expression is abolished in cells transformed by *ato* in *ato*$^{109\text{-}68}$. For example, in one case *ato*, but not *hh*, is expressed in the R2 photoreceptor (yellow arrow), and in another, *ato* is expressed in the mystery cell between R2 and R5 (blue arrow).
(F-I) Immunohistochemical detection of Ato (green) and Ci (red). In all cases, Ci abundance in the eye disc was normalised by comparison with its abundance in the adjacent antennal disc.
(F) Wild type. (G) *ato*$^{109\text{-}68}$/+. The abundance of Ci is marginally higher in *ato*$^{109\text{-}68}$ relative to wild type. (H) *109-68Gal4*/+*;* UAS-*hh*/+ (i.e. without UAS-*ato*). Although the abundance of Ci is clearly increased, there is little or no evidence of defects in R8 patterning or selection. (I) *ato*$^{109\text{-}68}$/+*;* UAS-*hh*/+. When both UAS-*hh* and UAS-*ato* are present, pattern formation and R8 precursor selection are severely abnormal. (J,K) Detection of Ato (green) and Boss (red). (J) *109-68Gal4*/+*;* UAS-*hh*/+. (K) *ato*$^{109\text{-}68}$/+*;* UAS-*hh*/+. There is a dramatic failure to resolve Ato to spaced R8 cells.

expressing cells (R8 and R2,5, respectively) suggests a speculative mechanism in which Ato might control components that promote Hh diffusion (Bellaiche et al., 1998). Alternatively, Ato may regulate an as yet unidentified diffusible factor that mediates a cell's response to the Hh signal.

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