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Transcriptional regulation of *atonal* required for *Drosophila* larval eye development by concerted action of Eyes absent, Sine oculis and Hedgehog signaling independent of Fused kinase and Cubitus interruptus

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SUMMARY

Bolwig's organ is the larval light-sensing system consisting of 12 photoreceptors and its development requires *atonal* activity. Here, we showed that Bolwig's organ formation and *atonal* expression are controlled by the concerted function of *hedgehog*, *eyes absent* and *sine oculis*. Bolwig's organ primordium was first detected as a cluster of about 14 Atonal-positive cells at the posterior edge of the ocular segment in embryos and hence, *atonal* expression may define the region from which a few Atonal-positive founder cells (future primary photoreceptor cells) are generated by lateral specification. In Bolwig's organ development, neural differentiation precedes photoreceptor specification, since Elav, a neuron-specific antigen, whose expression is under the control of *atonal*, is expressed in virtually all early-Atonal-positive cells prior to the establishment of founder cells. Neither Atonal expression nor Bolwig's organ formation occurred in the absence of *hedgehog*, *eyes absent* or *sine oculis* activity. Genetic and histochemical analyses indicated that (1) responsible Hedgehog signals derive from the ocular segment, (2) Eyes absent and Sine oculis act downstream of or in parallel with Hedgehog signaling and (3) the Hedgehog signaling pathway required for Bolwig's organ development is a new type and lacks Fused kinase and Cubitus interruptus as downstream components.

Key words: *Drosophila*, Larval photoreceptor, Bolwig's organ, *hedgehog*, *atonal*, *sine oculis*, *eyes absent*, *smoothened*, *patched*, *fused*, *cubitus interruptus*, Transcriptonal regulation

INTRODUCTION

Although morphologically quite different, vertebrate and insect visual systems may share in common a regulatory network of genes encoding eye- or neuronal-cell-specific transcription factors such as Twin of eyeless (Toy; Czerny et al., 1999), Eyeless (Ey; Halder et al., 1995), Sine oculis (So; Cheyette et al., 1994), Eyes absent (Eya; Bonini et al., 1993; Pignoni et al., 1997) and Dachshund (Dac; Mardon et al., 1994). Vertebrates possess homologues for each of these 'early eye genes' of *Drosophila*, capable of inducing ectopic eye formation at various positions of the *Drosophila* body upon misexpression (e.g., Halder et al., 1995). As with *ey* and *toy*, the mammalian counterpart, *Pax6* (Quiring et al., 1994), is capable not only of rescuing *Drosophila ey* mutations but also of generating ectopic compound eyes in *Drosophila* (Halder et al., 1995). Thus, clarification of relationships of 'early eye genes' and other genes involved in eye development is of particular importance.

The embryonic visual system in *Drosophila* may be useful in the study of molecular interactions responsible for early events of visual-system formation, in consideration of its simple structure consisting of Bolwig's organ (the larval eye) and optic lobe primordium, both derived from a small head-ectodermal region expressing *so* (Green et al., 1993; Daniel et al., 1999). Bolwig's organ formation and optic lobe development require *so* (Cheyette et al., 1994), *atonal* (*ato*; Jarman et al., 1993) and *tailless* (*tll*; Pignoni et al., 1990). Recent analysis of *tll* (Daniel et al., 1999) indicated that normal *tll* expression is confined to putative optic lobe primordium and *tll* is capable of driving cells to optic lobe fate as opposed to Bolwig's organ fate.

Daniel et al. (1999) propose a two-step differentiation model of Bolwig's organ formation. The first step is the formation of three *ato*-expressing founder cells, later to become first clustered larval eye photoreceptors. In the second step, cells surrounding founders are incorporated into the larval eye as secondary photoreceptor precursors and this involves Spitz (Spi) and unknown signals, produced by and emanating from Bolwig's organ founder cells. This model is reminiscent of that of ommatidium formation of adult eyes in third instar larvae, in which *ato*-expressing R8 is the first photoreceptor established and Spi signals, initially emanating from R8, induce neighboring cells to become photoreceptor precursors (Freeman, 1996).

hedgehog is a segment polarity gene in *Drosophila* and

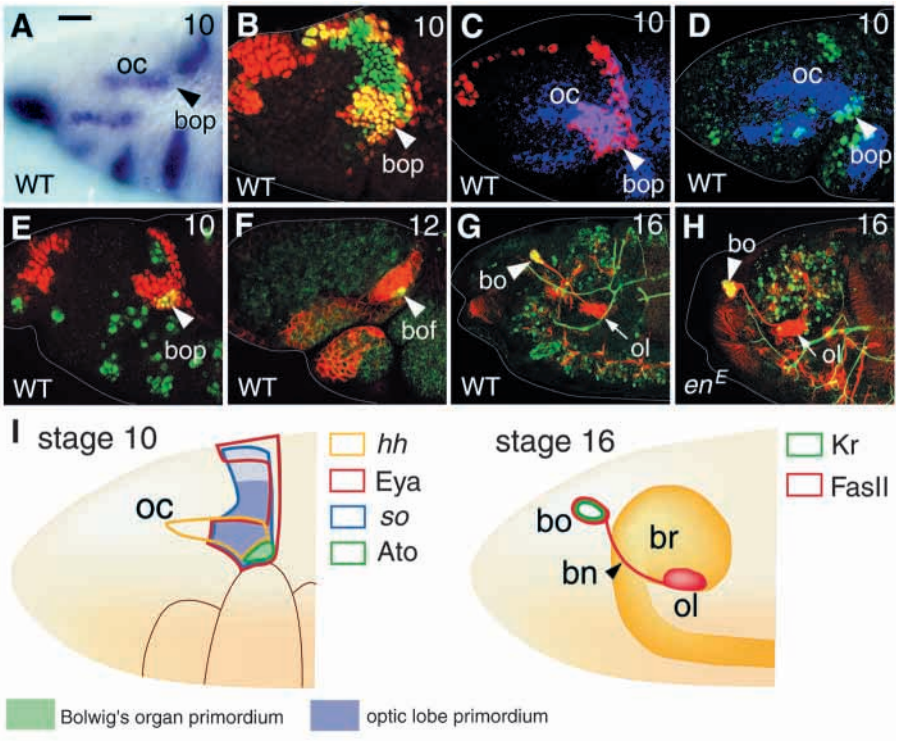
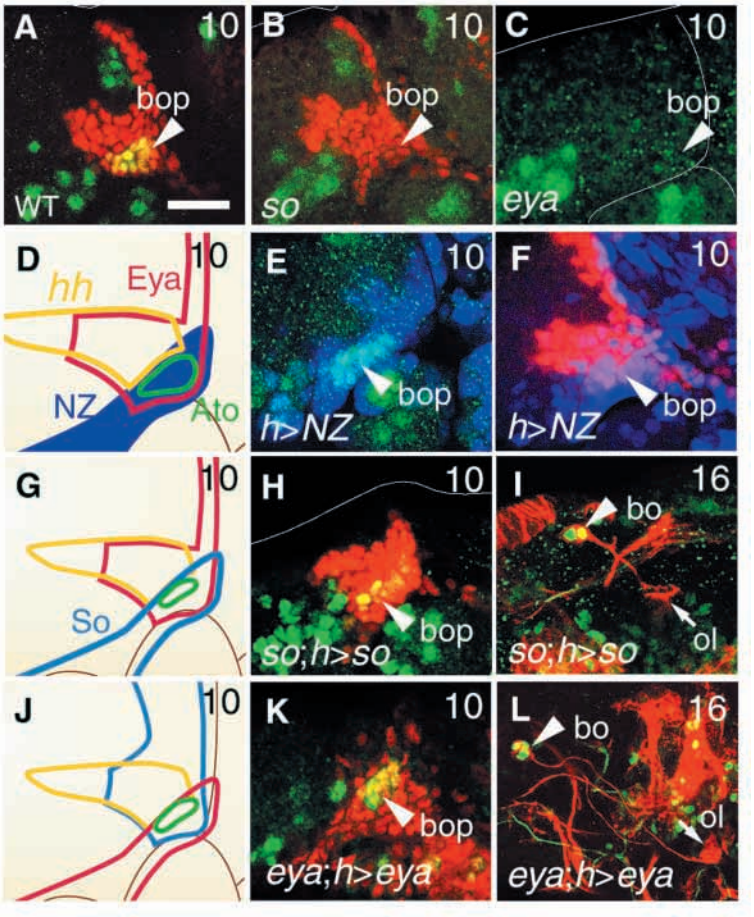


Fig. 1. Expression of *hh*, *so*-β-gal, Eya, Ato, Kr and FasII in developing wild-type (A-G) and mutant (H) embryonic heads. The following abbreviations are used in Figs 1-8; bn, Bolwig's nerve; bo, Bolwig's organ; bof, Bolwig's organ founder cells; bop, BOP; br, Brain; oc, ocular segment; ol, optic lobe; WT, wild type. Numbers at right corners indicate embryonic stages. If necessary, genetic backgrounds are shown at left corners. When Gal4-driven UAS transgenes are included, both Gal4 drivers and UAS genes are given on the right side of a semicolon using a symbol (>), which separates the Gal4-driver used and UAS-gene(s). For example, (*so*; *h>hh*) means the expression of UAS-*hh* driven by *h*-Gal4 on a *so* mutant background. Arrowheads in A-E indicate BOP positions. (A) *hh* RNA expression at stage 10. (B) Stage 10 so^{7}-trap-line head expressing Eya (red) and *so*-β-gal (green). The arrowhead indicates *so* and *eya* expressed in putative BOP. (C) *hh* RNA (blue) and Eya (red) expression at stage 10. (D) *hh* RNA (blue) and Ato (green) expression at stage 10. BOP abuts the ocular *hh* domain. (E) Eya (red) and Ato (green) expression at stage 10. Yellow signals show Ato-positive BOP to express Eya. (F) FasII (red) and Ato (green) expression at stage 12. The arrowhead indicates the restricted expression of Ato in a few founder cells. (G,H) The formation of Bolwig's organ (arrowhead) and optic lobe (arrow) at stage 16 in wild type (G) and en^{E} mutants (H); FasII expression is shown in red and Kr expression in green. (I) The expression of Bolwig's organ related genes at stages 10 and 16 are schematically shown. Scale bar in A, 20 μm.



encodes a secretory protein required for the formation and/or specification of neural and non-neural cells (reviewed by Hammerschmidt et al., 1997; Ingham, 1998). Hh signaling is essential for adult eye formation. *hh* is expressed at the posterior margin of eye discs shortly before the onset of photoreceptor formation and its absence results in the failure of compound eye formation and *ato* expression in the eye initiation area (Dominguez and Hafen, 1997). *hh* may also be involved in larval eye formation, since *ptc* mutants lacking a putative *hh* receptor generate Bolwig's organs with supernumerary photoreceptors (Schmucker et al., 1994).

Here, we showed that Bolwig's organ formation is governed by *ato*, the expression of which is under the

Fig. 2. Requirements of *so* and *eya* for Bolwig's organ formation. All panels except the schematic drawings (D,G,J) and stage 16 embryos (I,L) show Ato (green) and Eya (red) expression in BOP or putative BOP at stage10. In I,L, Kr expression and FasII expression are colored in green and red, respectively. (A) Wild type. About 14 cells express Ato strongly or weakly. (B) Stage 10 so^{3} mutants lacking Ato expression. The arrowhead shows the absence of Ato signals at putative BOP. (C) Stage 10 eya^{clift1} embryos lacking Ato expression. (D-F) Expression of UAS-*NZ* driven by *h*-GAL4. β-gal expression at stage 10, mimicking *h* expression, is colored in blue. (D) Schematic diagram of gene expression shown in E and F. (G-I) Rescue of so^{3} mutant phenotypes by UAS-*so* driven by *h*-GAL4. Arrowheads, BOP or Bolwig's organ. (J-L) Rescue of eya^{clift1} mutant phenotypes by UAS-*eya*. Scale bar in A, 20 μm (A-C,G-K); 16 μm (D-F); 25 μm (L).

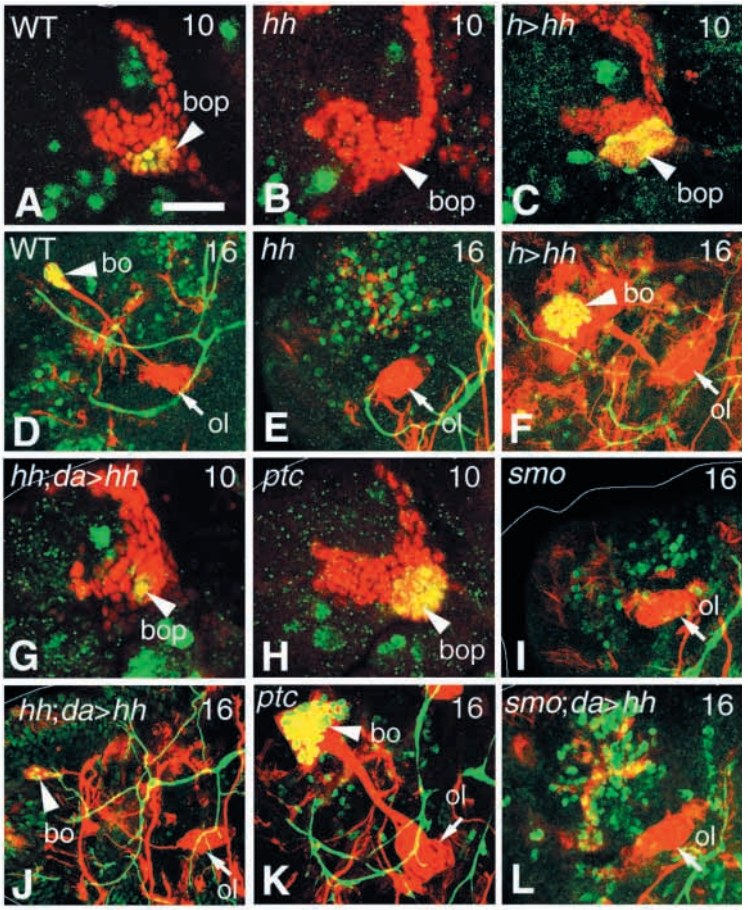
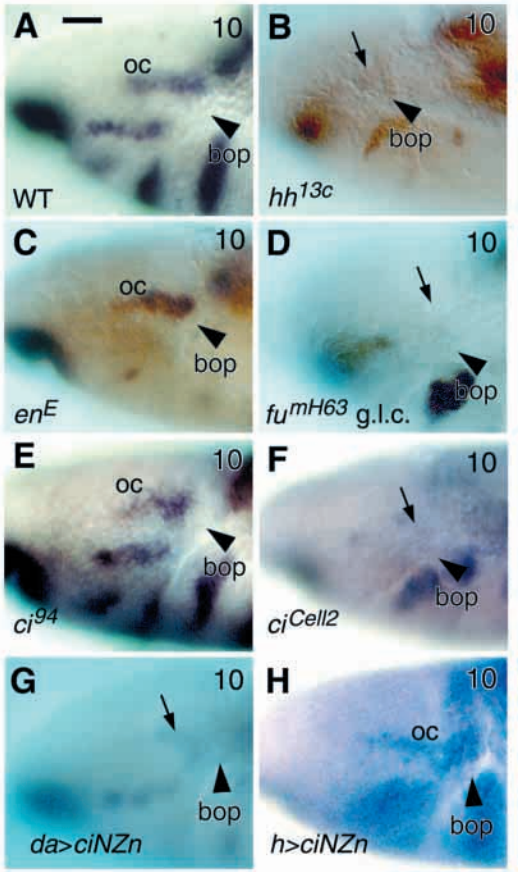


Fig. 3. Requirements of Hh signaling including a putative Ptc/Smo receptor complex for Bolwig's organ formation. (A-C,G,H) Stage 10 embryonic heads including BOP were stained for Ato (green) and Eya (red). (D-F,I-L) Stage 16 embryos stained for Kr (green) and FasII (red). (A,D) Wild type. (B,E) hh^{13c} mutants lacking both *ato* expression (B) and Bolwig's organ formation (E). (C,F) UAS-*hh*/+; *h*-GAL4/+ embryos. The numbers of early Ato-positive cells and Bolwig's organ neurons significantly increased (compare C,F with A,D). (G,J) hh^{13c} embryos transheterozygous for UAS-*hh* and *da*-GAL4. Note that *ato* expression in BOP and Bolwig's organ formation are partially rescued by *hh* ubiquitous expression (arrowheads). (H,K) ptc^{7M59} embryos. Early Ato-positive cells and Bolwig's organ cells increased. (I) The absence of Bolwig's organ formation from smo^{2} germline clones. Arrow, optic lobe. (L) smo^{2} germline clones expressing *da*-GAL4-driven UAS-*hh*. That no Bolwig's organ is formed indicates that *smo* acts downstream of *hh*. Scale bar in A, 20 μm (A-E,G-I,L); 27 μm (F,J,K).

control of *eya*, *so* and *hh*. Hh signaling involved in Bolwig's organ formation is a new type that lacks Fused kinase (Fu; Thérond et al., 1993) and Cubitus interruptus (Ci; Eaton and Kornberg, 1990; Orenic et al., 1990), components of the typical Hh signaling pathway (reviewed by Ingham, 1998). Epistasis analysis indicated that Eya and So act downstream of or in parallel with Hh signaling. We also found evidence that in Bolwig's organ development, neural differentiation precedes photoreceptor specification.



MATERIALS AND METHODS

Fly strains

Canton S was used as a wild type. Mutant strains, UAS lines and GAL4 lines used were: hh^{13c}; ptc^{7M59}; wg^{CX4}; en^{E}; so^{3}; eya^{clift1} (Pignoni et al., 1997); ato^{1}; ci^{94} and ci^{Cell2} (Methot and Basler, 1999); $ci^{D+rev9A-101A}$; C(4)RM; UAS-*hh* (H. Kobayashi and K. S., unpublished data); UAS-*eya* and UAS-*so* (Pignoni et al., 1997); UAS-*NZ* (UAS-*lacZ*); UAS-*ciNZn* and UAS-*ciZnC* (Hepker et al., 1997); *daughterless* (*da*)-GAL4 ($GAL4^{daG32}$; Wodarz et al., 1995); *hairy* (*h*)-GAL4 (h^{1J3}; Brand and Perrimon, 1993). See FlyBase for fly strains whose sources are not indicated. Germline clones were generated using fu^{mH63} (Thérond et al., 1996) and smo^{2}. so^{7} is a *PlacZ* insertion line (Cheyette et al., 1994). Stage 10 and 16 embryos homozygous for *ci* were identified by weak anti-Wg antibody staining in the ventral ectoderm and segmentation defects, respectively. Male embryos lacking *fu* activity were identified by anti-Sxl antibody staining. Germline clones were generated according to Ohlmeyer and Kalderon

Fig. 4. Self-regulation of ocular-segment *hh* expression. *hh* RNA expression at stage 10 is shown. Arrows and arrowheads, respectively, show the presumed positions of the ocular segment and BOP.(A) Wild type. (B)hh^{13c}. Note the absence of ocular-segment *hh* expression. (C) *hh* expression in en^{E} embryos. Note the normal ocular *hh* expression. (D) The absence of ocular *hh* expression of fu^{mH63} germline clones (g.l.c.). (E) The presence of the ocular *hh* domain in ci^{94}. (F) ci^{Cell2}. Little ocular-*hh* is expressed (arrow). (G,H) Ocular-segment *hh* expression is lost in embryos transheterozygous for UAS-*ciNZn* driven by *da*-GAL4 (G), but not by *h*-GAL4 (H). Scale bar, 20 μm.

(1997). *nullo 4* embryos were selected from the offspring of C(4)RM flies. If necessary, UAS-*hh*, *da*-GAL4 or *h*-GAL4 was introduced into mutant chromosomes by recombination. Lethal mutations on second and third chromosomes, respectively, were balanced by *11en-CyO* and *TM3 ftz-lacZ* to identify homozygotes. Embryonic stages are given according to Campos-Ortega and Hartenstein (1985).

Antibody staining and in situ hybridization

Embryos were stained with antibody according to the method of Shishido et al. (1997). Primary antibodies used were: anti-Ci (AbN; 1:1000; from T. Kornberg); anti-Eya (1:1000; Bonini et al., 1993); anti-Ato (1:1000; from Y. N. Jan); anti-Fasciclin II (FasII; 1:10; Grenningloh et al., 1991). anti-Kr (1:500; Gaul et al., 1987); anti-Wg (mAb4D4 from DSHB; 1:20); anti-Elav (Rat-Elav-7E8A10 from DSHB; 1:3); anti-β-gal (rabbit polyclonal (Cappel); mouse monoclonal (Promega)); anti-Sxl (M18 from DSHB; 1:10). Secondary antibodies used were: biotin-conjugated anti-rabbit and anti-mouse (Vector), Fluoroscein-conjugated Avidin (Pierce), Cy3-conjugated anti-mouse (Amersham) and anti-rat (Biological Detection Systems), and Cy5-conjugated anti-mouse (Amersham) antibodies. TSA indirect amplification kit (Renaissance) was used if necessary. In situ hybridization was carried out using digoxigenin-labeled *hh* RNA as a probe (see Shishido et al., 1997). AP-conjugated anti-digoxigenin antibody (1:100; Boehringer Mannheim), BCIP/NBT kit (Vector) or HNPP-detection kit (Boehringer Mannheim) were also used. Simultaneous staining with antibody and in situ hybridization was done according to the method of Goto and Hayashi (1997).

RESULTS

Atonal protein expression in future Bolwig's organ cells

At stage 10 in embryogenesis, the ventral half of the *so* expression region, where Eya is expressed (Fig. 1B,I; Bonini et al., 1998), reorganizes into a placode that gives rise to the optic lobe and Bolwig's organ (Green et al., 1993; Cheyette et al., 1994; Daniel et al., 1999). Near mid stage 11, Bolwig's organ is recognizable as a dome-shaped protrusion in the ventral region of the placode (Daniel et al., 1999). During stage 13, the optic lobe invaginates and leaves the ectodermal layer, while Bolwig's organ temporarily remains in the ectoderm (Green et al., 1993). Optic lobe and Bolwig's organ neurons exhibit strong FasII signals (Fig. 1F,G; Schmucker et al., 1997). All twelve Bolwig's organ neurons specifically express Krüppel (Kr; Gaul et al., 1987) at late embryonic developmental stages (Schmucker et al., 1992), thus making it possible to easily discriminate Bolwig's organ from the optic lobe at stages 13-16 (see Fig. 1G,I).

Ato, a basic helix-loop-helix transcription factor, is essential for compound eye development (Jarman et al., 1994). In the eye disc, Ato is expressed in cells in a stripe just in front of the morphogenetic furrow and R8, the first photoreceptor acquiring neural fate. Daniel et al. (1999) found similar *ato* expression in the future Bolwig's organ region. Experiments using in situ hybridization revealed *ato* RNA to be expressed in stage 12 head in several small cell clusters, one of which is a group of a few cells belonging to Bolwig's organ primordium. Initial *ato* RNA expression was also demonstrated to occur weakly in 6-8 cells in future Bolwig's organ at stage 11.

We independently found similar dynamic change in *ato* expression by anti-Ato antibody staining. Ato protein expression was initially noted in about 14 cells within the Eya expression domain overlapping the *so*-β-gal domain (Fig. 1E,I) at mid stage 10. This early expression was restricted to a few cells situated within the Bolwig's organ dome at early stage 12 (late *ato* expression; Fig. 1F). Late Ato protein expression disappeared by the end of stage 12. At mid stage 11, when the Bolwig's organ dome-like protrusion is apparent, nearly all cells within the dome were Ato-positive, while cells surrounding the dome were Ato-negative. The size of the Bolwig's organ dome was noted to increase on genetic backgrounds which increase the number of Ato-positive cells and to disappear on those abolishing Ato signals (see below). It may thus follow that early *ato* expression is an important determinant of the size of Bolwig's organ. We hereafter refer to a cell cluster showing early Ato protein expression as Bolwig's organ primordium (BOP).

Requirements of *so* and *eya* for *ato* expression in BOP

As shown in Fig. 1B,E,I, Ato, Eya and So (*so*-β-gal) are co-expressed in BOP at stage 10 and no Bolwig's organ is formed in *eya* (Daniel et al., 1999) or *so* mutants (Cheyette et al., 1994). This poses the question as to whether *ato* expression in BOP requires *eya* or *so* activity. Neither *eya* nor *so* mutants exhibited *ato* expression in putative BOP during stages 10-12, while other *ato* expression was noted to be virtually normal (Fig. 2B,C), indicating that early and late *ato* expression in putative BOP requires *eya* and *so* activity. Note that So and Eya must form a complex with each other to be activated (Pignoni et al., 1997).

That *ato* expression in BOP may require *so* and *eya* activity was further confirmed by misexpression experiments using the GAL4/UAS system. As a GAL4 driver, *h*-GAL4 was used, which activates target genes from late stage 9 onwards. The head *h* stripe includes BOP (Fig. 2D-F). When UAS-*so* was driven by *h*-GAL4 in *so* mutants, *ato* expression in putative BOP was partially recovered (Fig. 2G,H); Bolwig's organ containing several Kr-positive neurons along with a small optic lobe were generated in most embryos at stage 16 (Fig. 2I). Previous experiments showed optic lobe formation to require *so* and *eya* activity (Cheyette et al., 1994; Daniel et al., 1999). Similar incomplete rescue of *ato* expression and the formation of Kr-positive Bolwig's organ neurons were also observed when UAS-*eya* was driven by *h*-GAL4 in *eya* mutants (Fig. 2J-L).

Requirements of *hh* for *ato* expression in BOP

hh is required to initiate eye formation in third-instar larval eye discs (Dominguez and Hafen, 1997). We thus examined the relationship between *hh* and *ato* expression at stage 10. Embryos were stained for *hh* RNA and Eya (Fig. 1C) or Ato (Fig. 1D). The results are summarized in Fig. 1I. The area of *ato* expression (BOP) was immediately adjacent to the posterior edge of the ocular-segment *hh* stripe. *hh* expression in the ocular segment disappeared by stage 12. Thus, *hh* expression in the ocular segment may be related temporally and spatially to early *ato* expression in BOP.

To determine whether *hh* is required for BOP *ato* expression and subsequent Bolwig's organ formation, *ato* and *Kr* expression were examined in hh^{13c} mutant embryos. As shown in Fig. 3B,E, neither *ato* nor *Kr* expression could be detected in putative BOP at stages 10-12 and putative photoreceptors at

stage 16, respectively, while the expression of Eya and FasII was virtually normal, indicating that *hh* is essential for the expression of early and late Ato in BOP along with Bolwig's organ formation, but not for optic lobe formation.

Unlike *hh* stripes in trunk and other head regions, *hh* expression in the ocular segment occurs independently of *en* and *wg* activity (Fig. 4C; Gallitano-Mendel and Finkelstein, 1997). In these mutants, the optic lobe and Bolwig's organ formation was essentially normal (Fig. 1H), thus indicating that *hh* in the ocular segment is quite likely responsible for *ato* expression in BOP and Bolwig's organ formation.

To further confirm the above possibility, *hh* was misexpressed under the control of *h*-GAL4 or *da*-GAL4 drivers. *h*-GAL4 induces *hh* misexpression in the head ectoderm ventral to the authentic ocular-segment *hh* stripe (see Fig. 2D), while *da*-GAL4 drives ubiquitous *hh* expression in the ectoderm which is initially weak at stage 9 and subsequently strong from early stage 10 onwards (Wodarz et al., 1995). In either case, not only the early *ato* expression area but also the number of Bolwig's organ neurons increased 2-3 fold when *hh* was misexpressed on a wild-type background (Fig. 3C,F and Table 1). Similar *hh*-misexpression-dependent enhancement of early *ato* expression in BOP and increase in Bolwig's organ neurons were observed for other genetic backgrounds such as *so* mutants with *h*-GAL4-driven UAS-*so* (Table 1).

Defects in hh^{13c} were partially rescued by *hh* expression driven by *da*-GAL4 or *h*-GAL4 (Fig. 3G,J and Table 1); the numbers of early Ato-positive cells and Kr-positive Bolwig's organ neurons were each 4-8. Early and late *ato* expressions in BOP and Bolwig's organ neuron formation are thus clearly shown to be positively regulated by Hh signaling.

Requirements of *ptc* and *smo* for *ato* expression in BOP and Bolwig's organ formation

A typical Hh pathway includes two transmembrane proteins, Ptc and Smo, as downstream components (reviewed by Alcedo and Noll, 1997). Ptc is a putative receptor of Hh and prevents Smo from transducing signals in the interior of cells. This Ptc repression of Smo is eliminated with the binding of Hh to Ptc (Chen and Struhl, 1998), and accordingly, phenotypes of *ptc* and *smo* mutants, respectively, are very similar, if not identical, to those of gain- and loss-of-function mutants of *hh*.

Fig. 3H,K shows the phenotypes of *ptc* mutants to resemble those of embryos overexpressing *hh*, which provide expanded BOP expressing Ato at stage 10 and significantly increased Bolwig's organ neurons (see Fig. 3C,F). The same has been noted for *ptc* mutants by Schmucker et al. (1994). Thus, as with other Hh signaling systems, Ptc may serve as a receptor in Hh signaling required for *ato* expression in BOP and Bolwig's organ formation.

We also made a germline clone lacking *smo* activity. Bolwig's organ and *ato* expression in putative BOP were absent from *smo* mutant embryos without loss of the optic lobe (Fig. 3I). Thus, phenotypes are apparently quite similar to those of *hh* mutants, suggesting Smo involvement in Hh signaling required for either Bolwig's organ development or the initiation and/or maintenance of ocular-segment *hh* expression or both. To determine which is the case, *hh* RNA expression in hh^{13c} and smo^{2} mutants was examined. No ocular *hh* expression was found in hh^{13c} and smo^{2} mutant embryos at stage 10 (Fig. 4B), indicating that, in the ocular segment, *hh* expression at least requires its own signaling including Hh and Smo. Should *smo* not be involved in Hh signaling for Bolwig's organ development, ubiquitous *hh* misexpression would probably rescue the defects in *ato* expression in BOP and Bolwig's organ neuron formation in *smo* mutants. This possibility was examined by forced expression of *hh* in embryos lacking *smo* activity maternally and zygotically. In contrast to the *hh* mutant background (Fig. 3G,J), neither BOP *ato* expression nor Bolwig's organ formation was rescued on a *smo* mutant background (Fig. 3L). Thus, we conclude that, as with other Hh signaling pathways, *smo* is involved in the Hh signaling pathway required for *ato* expression in BOP and Bolwig's organ formation.

Table 1. Proportional correlation between numbers of Ato-positive cells and BO neurons

Genotype	Number of initial Ato-positive cells	Number of BO neurons
Wild type	++[a]	++[b]
hh^{13c}	–	–
h>hh	+++[c]	+++[c]
da>hh	+++[c]	+++[c]
hh^{13c}; *h>hh*	+[d]	+[d]
hh^{13c}; *da>hh*	+[d]	+[d]
ptc^{7M59}	+++[c]	+++[c]
so^{3}	–	–
so^{3}; *h>hh*	–	–
so^{3}; *h>so*	+[d]	+[d]
so^{3}; *h>hh, so*	+++[c]	+++[c]
eya^{clift1}	–	–
eya^{clift1}; *h>eya*	+[d]	+[d]
eya^{clift1}; *h>hh*	–	–

a, about 14 cells; b, 12 cells; c, 20-40 cells; d, 2-8 cells.

Dispensability of Fused for BOP *ato* expression and Bolwig's organ formation

Fused (Fu) kinase is considered to form a complex with Costal-2 (Cos2; Robbins et al., 1997; Sisson et al., 1997), Suppressor of fused (Su(fu); Monnier et al., 1998) and Ci, and mediates Hh signaling (reviewed by Ingham, 1998). We made a germline clone lacking *fu* activity. As with *smo* embryos, *fu* embryos failed to express Ato in putative BOP and to generate Bolwig's organ but not the optic lobe (Fig. 5A,B). As noted for *smo* mutants, *hh* expression in the ocular segment was abolished in *fu* embryos (Fig. 4D). In contrast to *smo* mutants, appreciable *ato* expression in putative BOP and Bolwig's organ formation with 2-6 Kr-positive neurons could be seen subsequently to forced expression of *hh* by *da*-GAL4 in embryos lacking *fu* activity maternally and zygotically (Fig. 5C,D). Thus it was concluded that Fu was unnecessary for the Hh signaling pathway required for BOP *ato* expression and Bolwig's organ formation. The failure of Bolwig's organ formation in *fu* mutants is likely to be due to *fu*-mutation-dependent loss of *hh* activity in the ocular segment.

Absence of Ci from Hh signaling pathway for Bolwig's organ development

Ci is considered as a transcription factor that activates *hh* target genes in response to Hh signaling. Ci was thus examined for its role in *ato* expression in BOP and Bolwig's organ formation. ci^{94} has been identified as a true null allele of *ci* (Methot and

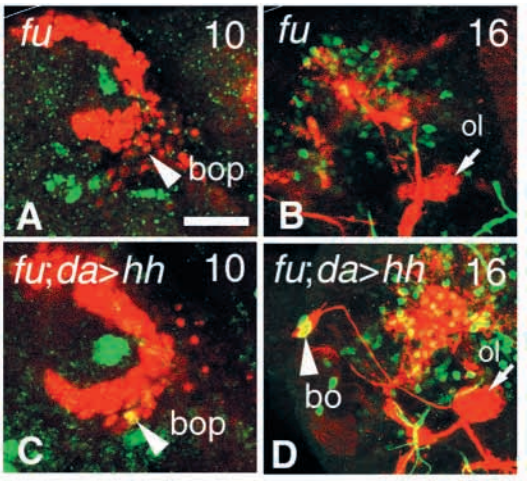


Fig. 5. Fu is dispensable for Bolwig's organ formation. (A,C) Ato (green) and Eya (red) expression in BOP at stage10. (B,D) Kr (green) and FasII (red) expression at stage 16. (A,B) fu^{mH63} germline clones. Not only Ato expression (arrowhead; A) but also Bolwig's organ formation (B) are abolished. (C,D) fu^{mH63} germline clones transheterozygous for UAS-*hh* and *da*-GAL4. There is partial rescue of early Ato expression and Bolwig's organ formation (arrowheads). Arrows, optic lobe. Scale bar, 20 μm.

Basler, 1999). In ci^{94} flies, neither the activator nor repressor forms of Ci is produced. To our surprise, both *ato* expression in BOP and Bolwig's organ formation occurred normally in ci^{94} embryos (Fig. 6A,E). This is not due to allelic effects of *ci*, since similar results were obtained for two other *ci* mutants, *nullo 4* and $ci^{D+Rev+101A}$ (Fig. 6B,F). *ato* expression in BOP and the formation of Bolwig's organ with several Kr-positive neurons apparently come about in *nullo 4* embryos whose fourth chromosome, where *ci* is located, is entirely lost. CiZnC, the activated form of Ci (Hepker et al., 1997), was also misexpressed on a wild-type background by *h*-GAL4 or *da*-GAL4 drivers without significant change in the number of Kr-positive neurons or Ato-positive cells in BOP. Ci may thus not be required for *ato* expression in BOP or Bolwig's organ development, at least as a transcription activator.

hh expression in the ocular segment requires its own signaling. The above finding may thus also demonstrate the dispensability of Ci in Hh signaling for ocular-segment *hh* expression. *hh* transcription was almost entirely normal in the ci^{94} ocular-segment (Fig. 4E). The presence of Bolwig's organ neurons in *nullo 4* embryos may also indicate that not only *ci*, but also other chromosome 4 genes such as *pangolin* (*pan*; Brunner et al., 1997), *ey* and *toy* have little, if any, positive role in Bolwig's organ development.

The activator form of Ci does not participate in Bolwig's organ formation but this does not necessarily mean no involvement of the repressor form of Ci in Bolwig's organ development. ci^{Cell2} is a *ci* mutation that gives rise only to the repressor form (Methot and Basler, 1999) and in ci^{Cell2}, the *ci* repressor is misexpressed in the ocular segment *hh* domain (Fig. 6L,M). There was neither *ato* expression in putative BOP (Fig. 6C), Bolwig's organ formation (Fig. 6G) nor *hh*

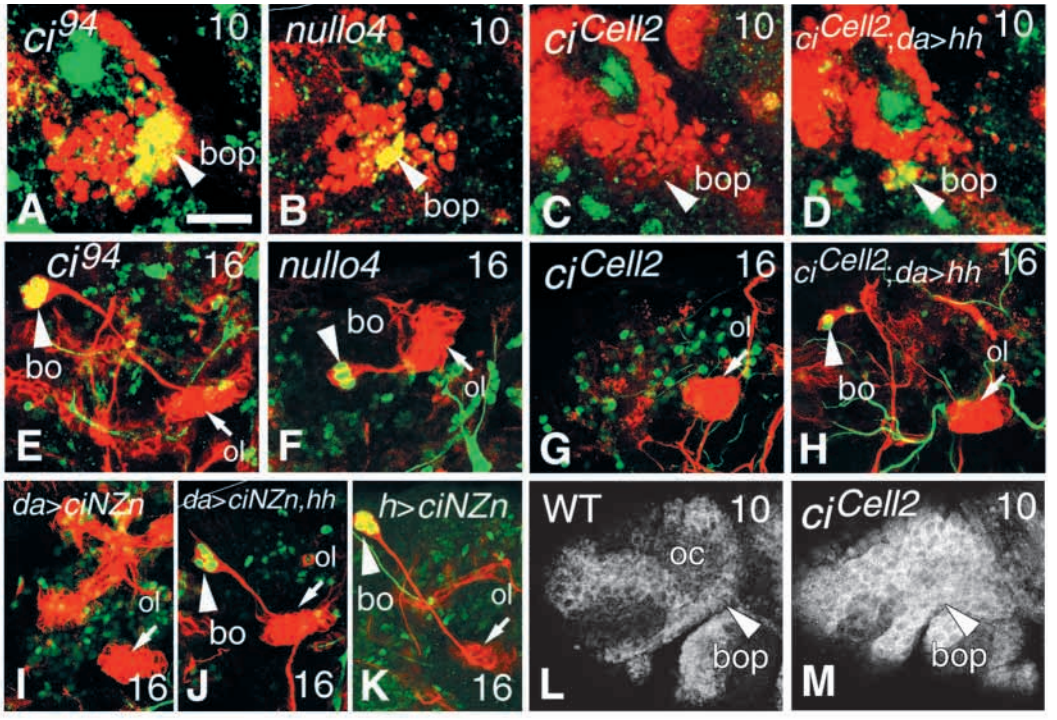


Fig. 6. Ci is dispensable for Bolwig's organ formation. (A-D) Stage-10 BOP stained for Ato (green) and Eya (red; nuclear signals). In A-D, embryos were further stained for Wg (red; membrane signals) to identify *ci* homozygotes. E-K show stage 16 Kr (green) and FasII (red) expression. (L,M) Stage 10 embryos stained with anti-Ci antibody. (A,E) ci^{94}. Ato expression and Bolwig's organ formation appear normal, indicating that the activator form of *ci* is dispensable for Bolwig's organ formation. (B,F) *nullo 4* embryos. That Ato is expressed (B; arrowhead) and Bolwig's organ is formed (F; arrowhead) may indicate that *ci* (activator form), *toy* and *ey* are dispensable for larval eye formation. (C,G,M) On a ci^{Cell2} background, Ci is expressed throughout the head and neither Ato expression (C) nor Bolwig's organ formation (G) occurs. (D,H) ci^{Cell2} embryos trans-heterozygous for UAS-*hh* and *da*-GAL4. Both early *ato* expression and Bolwig's organ formation occurred. (I) Bolwig's organ formation was suppressed by UAS-*ciNZn* expression driven by *da*-GAL4. (J) UAS-*ciNZn*, UAS-*hh*/+; *da*-GAL4/+ embryos. Bolwig's organ defects due to UAS-*ciNZn* expression driven by *da*-GAL4 were rescued by ubiquitous *hh* misexpression (arrowhead). (K) UAS-*ciNZn*/+; *h*-GAL4/+ embryos. In contrast to UAS-*ciNZn*/+; *da*-GAL4/+ embryos, Bolwig's organ formation was normal (arrowhead), indicating that it is *hh* expression but not Bolwig's organ formation that is repressed by CiNZn. (L,M) Distribution of Ci in wild-type (L) and ci^{Cell2} (M) embryos. Note that Ci expression is repressed in the ocular segment where *hh* is expressed. Scale bar in A, 20 μm (A-K); 35 μm (L,M).

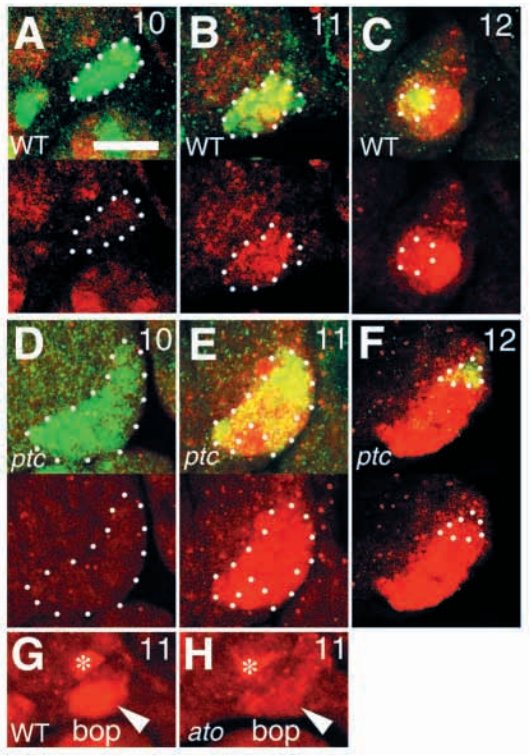


Fig. 7. Elav expression is under the control of early-Ato in BOP. Green, Ato; red, Elav. (A-F) Upper half of panels show merged images, while lower half of panels, show Elav signals only. (A-C) Wild type at mid stage 10 (A), early stage 11 (B) and stage 12 (C). Ato expression begins before the onset of Elav expression at mid stage 10. At early stage 11, virtually all BOP cells co-express Ato and Elav. At stage 12, Ato expression is restricted to three founder cells but Elav expression persists in all BOP cells. (D,E,F) ptc^{7M59} embryos at mid stage 10 (D), stage 11 (E) and stage 12 (F). (E) Although the BOP area in *ptc* mutants is much larger than that of wild type, Ato and Elav are co-expressed in almost all BOP cells. (F) Ato expression is restricted to putative founder cells at stage 12. (G) Wild type at mid stage 11. Strong Elav expression is seen (arrowhead). Asterisks indicate unspecified neurons taken as an internal control. (H) ato^{1} embryos at the same stage as in G. BOP Elav signals are very weak, if any (arrowhead). Scale bar in A, 15 μm (A-F); 20 μm (G,H).

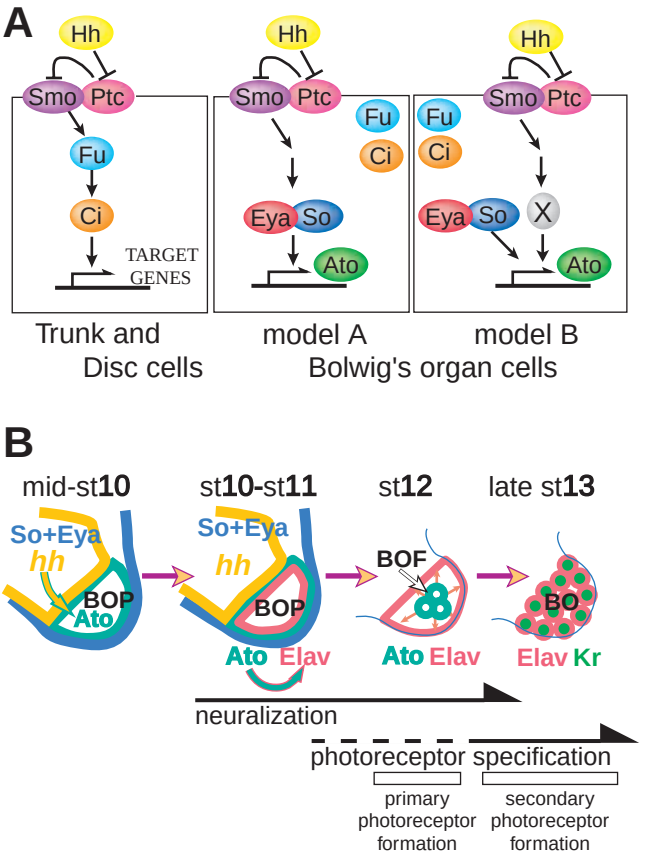


Fig. 8. (A) Possible models of Hh signaling pathway required for Bolwig's organ formation. Left panel, a typical Hh signaling pathway considered to be used for trunk segment and imaginal disc development. In this, Ci and Fu act as downstream factors. Center and right panels, two possible Hh signaling models for Bolwig's organ development including early *ato* expression. In both, Ci and Fu are dispensable. In model A, Eya and So act downstream of Hh, while, in model B, Eya and So act in parallel with X, an unidentified transcriptional activator of Hh signaling. (B) Time course of Ato and Elav expression and Bolwig's organ development. Early (mid-stage-10) *ato* expression is initiated by the concerted action of Hh signaling, Eya and So. Neural differentiation begins at late stage 10 to early stage 11, when virtually all BOP cells co-express Elav and Ato. *ato* expression is restricted to three founder cells during stage 12 and disappears by the end of stage 12. In contrast, Elav expression persists throughout Bolwig's organ development. Photoreceptor-specific markers begin to be expressed in putative founder cells at late stage 11. Secondary photoreceptor formation may require Spi and other recruitment signals (thin orange arrows) emanating from founder cells (primary photoreceptors) as described by Daniel et al. (1999).

expression in the ocular segment (Fig. 4F) in ci^{Cell2} embryos. Similar defects were induced by ubiquitous misexpression of CiNZn (repressor form of Ci; Hepker et al., 1997) (Figs 4G, 6I). These defects were rescued considerably by ubiquitous misexpression of *hh* (Fig. 6D,H,J), and thus Bolwig's organ development defects due to the repressor form of Ci may be considered to result only from reduction in *hh* expression in the ocular segment. To further confirm this, UAS-*ciNZn* was driven by *h*-GAL4 on a wild-type background. As expected, *hh* expression was almost completely normal in the putative ocular-segment *hh* expression domain that does not express CiNZn (Fig. 4H) and *ato* expression and Bolwig's organ formation were apparent in BOP irrespective of CiNZn misexpression (Fig. 6K).

Ci is thus shown not to be involved in the Hh signaling pathway essential for *ato* expression in BOP and Bolwig's organ formation.

so and *eya* are epistatic to *hh*

so, *eya* and *hh* regulate *ato* expression and any single loss of these genes results in that of *ato* expression. Anyone of these three genes appears regulated independently of the other two, judging from the findings that *hh* RNA expression at stage 10 is almost normal in *eya* and *so* mutants, Eya expression at stage 10 is normal in *so* mutants and vice versa, and Eya and *so* RNA expression is normal for the most part in *hh* mutants (Figs 2B, 3B and data not shown). To determine the relationship of Hh signaling to Eya or So, epistasis analysis was carried out by

misexpressing Hh in *so* or *eya* mutant embryos by *h*-GAL4. Neither BOP *ato* expression nor Bolwig's organ formation was induced in *so* and *eya* mutants (Table 1), indicating that *so* and *eya* is epistatic to *hh*. *so* and *eya* would thus appear to act cell-autonomously downstream of or parallel to the Hh signaling pathway for Bolwig's organ development. Consistent with this, *ato* expression was always evident only in a particular set of cells within a region simultaneously expressing Eya and So (see Fig. 1I).

Requirements of *ato* activity for *elav* expression in early BOP cells

Elav is a neuron-specific antigen (Robinow and White, 1991). Our results, summarized in Table 1, show that the number of Ato-expressing BOP cells at stage 10 is positively correlated to the number of Bolwig's organ photoreceptor neurons at stage 16. Thus, we examined the expression of Elav in wild-type BOP during stages 10-16. To our surprise, *elav* expression began in virtually all Ato-positive BOP cells slightly after the onset of early *ato* expression (Fig. 7A-C), indicating that in BOP cells, neuralization may occur prior to the formation of founder cells and hence photoreceptor specification.

In contrast to *ato* expression, *elav* expression persisted at least until stage 16. The co-expression of early Ato and Elav was detected similarly in *ptc* mutant embryos with expanded early-Ato-positive BOP (Fig. 7D-F). To clarify whether *ato* activity is required for *elav* expression, examination was made of *elav* expression in ato^{1} embryos. *elav* expression in the putative BOP of *ato* mutants was found to be very weak, if any (Fig. 7H). It may thus follow that Ato activity is essential for *elav* expression in BOP.

DISCUSSION

ato as a master gene for Bolwig's organ photoreceptor formation

A recent model (Daniel et al., 1999) has proposed that the larval eye is formed by a two-step mechanism: establishment of about three founder photoreceptor cells and recruitment of cells surrounding them as secondary photoreceptors. The present study showed that prior to the establishment of founder cells, virtually all BOP cells acquire neural fate. Fig. 8B schematically shows a view of timing of key events in Bolwig's organ development.

The earliest event of Bolwig's organ development may be *ato* expression at mid stage 10: this early *ato* expression defines the area of BOP. Early *ato* expression is regulated by the concerted action of Eya, So and Hh signals. During late stage 10 and early stage 11, Elav, a neuron-specific antigen, begins to be expressed in almost all BOP cells. This *elav* expression is likely to be regulated by Ato activity, since (1) BOP *elav* expression reduced extensively in *ato* mutants (Fig. 7H) and (2) the number of Elav-positive cells at stage 11 and Kr-positive Bolwig's organ neurons at stage 16 considerably increased upon *ato* misexpression (unpublished data). Our preliminary results also indicated that as with *ato* expression, *eya*, *so* and *hh* activity is essential for *elav* expression in BOP cells.

In contrast to *elav* expression, *ato* expression is restricted to three founder cells at stage 12 (Fig. 7C): this late *ato* expression disappeared by the end of stage 12. Photoreceptor specification of putative founder cells may start during stage 11, since our unpublished data showed that at late stage 11, 2-3 cells in a cluster start expressing Kr or Glass (Ellis et al., 1993), which are specific markers for larval photoreceptors. Cells expressing Kr and/or Glass increase during stages 12-13 and all 12 photoreceptors express both Kr and Glass by stage 16. Similarly, a peripheral nervous system-specific signal recognized by mAb22C10 appeared in a few BOP cells at stage 12 and became recognizable in all Bolwig's neurons by stage 16 (Schmucker et al., 1992). Late *ato* expression may also be essential for normal photoreceptor formation. In *ato* mutants, neither Kr-positive nor mAb22C10-positive cells could be seen in stage-16 future larval eyes. Daniel et al. (1999) have proposed that Spi and other unidentified signals emanating from founder cells are important for the survival and recruitment, respectively, of non-founder photoreceptor precursors.

As with BOP *ato* expression, *ato* expression in chordotonal organs and adult eyes occurs initially in a relatively wide area and then is restricted to a limited number of cells at later stages (Jarman et al., 1993, 1994). In these systems, late *ato* expression appears essential for the production of EGF signaling molecules such as Rhomboid (Freeman, 1996; Okabe and Okano, 1997). However, unlike early *ato* expression in BOP, early *ato* expression in these organs appears unrelated to *elav* expression at least in secondary neurons (Jarman et al., 1994; Okabe and Okano, 1997).

Novel Hh signaling that triggers Bolwig's organ development

Hh signaling in *Drosophila* has been extensively analyzed in embryonic trunk segments and imaginal discs, and many common downstream components have been identified (reviewed by Ingham, 1998). In both systems, Ci activates target genes in response to *hh* signal (Alexandre et al., 1996; Ohlmeyer and Kalderon, 1998; Methot and Basler, 1999). The pathway lying above Ci is thought to be bifurcated. Although the mechanism by which Smo passes signals to PKA or Fu remains unclear, PKA and Fu act under the direction of the putative Ptc/Smo receptor complex in parallel with each other. Ci is directly phosphorylated by PKA and cleaved to become a repressor (Chen et al., 1998), while Fu phosphorylates full-length Ci to make it a labile activator (Ohlmeyer and Kalderon, 1998). With these two pathways maintained in balance, it is possible for cells to acquire their fates during development.

Our results show that Bolwig's organ development is regulated through the concerted action of Eya, So and Hh signaling. Although these three factors are essential for *ato* expression at stage 10, the earliest event in Bolwig's organ development so far identified, whether they directly regulate other events of Bolwig's organ development remains to be clarified. Defects in stage-10 *ato* expression in BOP mutant for *eya*, *so* or *hh* were partially rescued by misexpression of the corresponding gene at late stage 9 and stage 10 (see Figs 2G-L and 3G,J), suggesting that *ato* is a direct target of the putative Eya/So complex and an activator downstream of Hh signaling involved in Bolwig's organ development.

Fig. 6L shows that Ci is expressed in BOP cells at stage 10. Fu is also ubiquitously expressed in the ectodermal head at stage 10 (Thérond et al., 1993, 1999). Figs 5 and 6 indicate that both Fu and Ci are not involved in Hh signaling for

Bolwig's organ development. Ci and Fu are components of Ci/Fu/Su(fu)/Cos2 complexes, required for Hh signal transduction in trunk and imaginal disc cells (reviewed by Ingham, 1998), and thus similar complexes would not be present in Hh signaling for Bolwig's organ development. Epistasis analysis indicated that Eya and So act either downstream of or in parallel with Hh/Ptc signaling. Should the latter be the case, Hh signal must activate an unknown transcription activator (X) to positively regulate *ato* (model B in Fig. 8A). To our knowledge, this is the first demonstration of Hh signaling independent of both Fu and Ci.

Fig. 4B,D,E may indicate that Hh signaling required for ocular-segment *hh* expression lacks Ci but not Fu, and this would imply the presence of another type of Hh signaling. The Hh signaling pathway required for *ptc* expression in cells posteroventral to Hh expression domains in trunk has recently been shown to lack Fu but not Ci (Thérond et al., 1999) and consequently there must be considerable diversity in the downstream pathway of Hh signaling in *Drosophila*.

toy and *ey* are dispensable for larval eye development

toy and *ey*, a master gene pair of *Drosophila* compound eye development (Halder et al., 1995; Czerny et al., 1999), are members of the Pax6 gene family, essential for the normal development of mammalian eyes (reviewed by Oliver and Gruss, 1997). Our results (Fig. 6B,F), however, showed that neither *toy* nor *ey* is required for *Drosophila* larval eye development.

Bolwig's organ development may be similar to the initiation of compound eye formation along the posterior eye-disc edge. Both systems may include *ato* as a proneural gene whose expression is regulated by Hh signaling. As with larval eye formation, compound eye formation is not initiated properly in *ato* mutants and *ato* expression is eliminated in *hh* mutants (Dominguez and Hafen, 1997). So, Eya and Hh are expressed along the posterior eye disc margin at the time when photoreceptors are initially formed in the second instar larvae (Bonini et al., 1993; Cheyette et al., 1994). Thus, as in the regulation of initial *ato* expression in larval eye development, *ato* expression at the initial stage of compound eye development may be positively regulated through the concerted action of Eya, So and Hh signaling.

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