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 *atona*-positive cells at the posterior edge of the ocular segment in embryos and hence, *atonal* expression may define the region from which a few *atona*-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*, *atona*, *sine oculis*, eyes absent, smoothened, patched, fused, cubitus interruptus, Transcriptional 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), *atona* (*ato*; Jarman et al., 1993) and *tailless* (*ttl*; Pignoni et al., 1990). Recent analysis of *ttl* (Daniel et al., 1999) indicated that normal *ttl* expression is confined to putative optic lobe primordium and *ttl* 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
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
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^{tig1} (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-ci^{NZn} and UAS-ci^{Znc} (Hepker et al., 1997); daughterless (da)-GAL4 (GAL4-daG32; Wodarz et al., 1995); hairy (h)-GAL4 (h^{123}; 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}. smo^{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.
expression was initially noted in about 14 cells within the Eya domain overlapping the so-β-gal domain (Fig. 1.E.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. 1.F). 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. 2.B,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. 2.D-F). When UAS-so was driven by h-GAL4 in so mutants, ato expression in putative BOP was partially recovered (Fig. 2.G,H). Bolwig’s organ containing several Kr-positive neurons along with a small optic lobe were generated in most embryos at stage 16 (Fig. 2.I). 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. 2.J-L).

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

hh is required to initiate eye formation in third-instar larval eye discs (Domínguez 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. 1.C) or Ato (Fig. 1.D). The results are summarized in Fig. 2.I. 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 hh118 mutant embryos. As shown in Fig. 3.B,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 hh13c 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 independent of Fu and Ci.

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of initial Ato-positive cells</th>
<th>Number of BO neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>++a</td>
<td>++b</td>
</tr>
<tr>
<td>hh13c</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>h&gt;hh</td>
<td>++c</td>
<td>++c</td>
</tr>
<tr>
<td>da&gt;hh</td>
<td>++c</td>
<td>++c</td>
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<tr>
<td>hh13c; h&gt;hh</td>
<td>4d</td>
<td>4d</td>
</tr>
<tr>
<td>hh13c; da&gt;hh</td>
<td>4d</td>
<td>4d</td>
</tr>
<tr>
<td>ptc7M9</td>
<td>++c</td>
<td>++c</td>
</tr>
<tr>
<td>so4</td>
<td>–</td>
<td>–</td>
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<td>so4; h&gt;hh</td>
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<td>4d</td>
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<td>eya0011; h&gt;eya</td>
<td>4d</td>
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a, about 14 cells; b, 12 cells; c, 20-40 cells; d, 2-8 cells.

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.

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.

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. ci514 has been identified as a true null allele of ci (Methot and
Basler, 1999). In ci94 flies, neither the activator nor repressor forms of Ci is produced. To our surprise, both atr expression in BOP and Bolwig’s organ formation occurred normally in ci94 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 ciD+Rev+101A (Fig. 6B,F). atr 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 atr 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 ci94 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. ciCell2 is a ci mutation that gives rise only to the repressor form (Methot and Basler, 1999) and in ciCell2, the ci repressor is misexpressed in the ocular segment hh domain (Fig. 6L,M). There was neither atr expression in putative BOP (Fig. 6C), Bolwig’s organ formation (Fig. 6G) nor hh expression in the ocular segment for ciCell2. This is in agreement with the findings that ciCell2 embryos trans-heterozygous for UAS-hh and da-GAL4. Both early atr 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 ciCell2 (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).
Elav signals are very weak, if any (arrowhead). Scale bar in A, 15 μm (A-F); 20 μm (G,H).

expression in the ocular segment (Fig. 4F) in c\text{cell2} embryos. Similar defects were induced by ubiquitous misexpression of Ci\text{NZn} (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-ci\text{NZn} 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 Ci\text{NZn} (Fig. 4H) and ago expression and Bolwig’s organ formation were apparent in BOP irrespective of Ci\text{NZn} misexpression (Fig. 6K).

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

Fig. 7. Elav expression is under the control of early-Ago in BOP. Green, Ago; 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). Ago expression begins before the onset of Elav expression at mid stage 10. At early stage 11, virtually all BOP cells co-express Ago and Elav. At stage 12, Ago expression is restricted to three founder cells but Elav expression persists in all BOP cells. (D,E,F) ptc\text{M59} 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, Ago and Elav are co-expressed in almost all BOP cells. (F) Ago 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) Ago\text{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).

so and eya are epistatic to hh
so, eya and hh regulate ago expression and any single loss of these genes results in that of ago 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. 11).

**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 ato1 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 3GJ), 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 ocellar-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 posteroverentral 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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