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Establishing primordia in the *Drosophila* eye-antennal imaginal disc: the roles of *decapentaplegic, wingless* and *hedgehog*

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SUMMARY

The eye-antennal imaginal discs of *Drosophila melanogaster* form the head capsule of the adult fly. Unlike the limb primordia, each eye-antennal disc gives rise to morphologically and functionally distinct structures. As a result, these discs provide an excellent model system for determining how the fates of primordia are specified during development.

In this study, we investigated how the adjacent primordia of the compound eye and dorsal head vertex are specified. We show that the genes *wingless (wg)* and *orthodenticle (otd)* are expressed throughout the entire second instar eye-antennal disc, conferring a default fate of dorsal vertex cuticle. Activation of *decapentaplegic (dpp)* expression in the posterior eye disc eliminates *wg* and *otd* expression, thereby permitting eye differentiation. We also demonstrate that *otd* is activated by *wg* in the vertex primordium. Finally, we show that early activation of *dpp* depends on *hedgehog (hh*) expression in the eye anlage prior to morphogenetic furrow formation.

Key words: *decapentaplegic*, *wingless*, *hedgehog*, *orthodenticle*, *Drosophila*, imaginal disc

INTRODUCTION

A critical question in development is how regional identity is established. The limb imaginal discs of *Drosophila melanogaster* have provided an important model for studies of regional specification (reviewed in Cohen, 1993**).** The best understood mechanism for patterning the limb primordia involves compartment formation mediated by selector genes. In this process, cells acquire compartment specific identity by expressing genes in a cell-lineage-restricted manner (Garcia-Bellido et al., 1973). Within the wing and leg discs, for example, *engrailed* and *invected* are expressed in all the cells of the posterior compartment where they are required to specify posterior cells fate (Sanicola et al., 1995; Zecca et al., 1995; Tabata et al., 1995; Morata and Lawrence, 1975; Kornberg et al., 1985), while the LIM/homeobox gene *apterous* specifies dorsoventral identity (Diaz-Benjumea and Cohen, 1993; Blair et al., 1994). A different mechanism governs dorsoventral subdivision of the leg discs. In each leg primordium, dorsoventral territories are established by mutual repression mediated by the secreted DPP and WG proteins (Brook and Cohen, 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Morimura et al., 1996; Penton and Hoffman, 1996). This repression, which does not require compartmentalization, results in dorsal *dpp* expression and ventral *wg* expression. If each gene product specifies a distinct differentiation program, the result is regional specification in the absence of selector gene activity.

The mechanisms described above specify subdomains of individual structures, the appendages of the adult fly. To understand how the primordia of different domains are established, we have focused on the patterning of the eye-antennal imaginal discs. These discs are particularly interesting because, unlike the limb discs, they each produce different adult structures (Haynie and Bryant, 1986; reviewed in Jurgens and Hartenstein, 1993). Each disc is subdivided into primordia which give rise to the antennae, compound eyes and specific regions of the head capsule.

How are the adjacent primordia within a single eye-antennal disc distinguished? Clonal analysis indicates that, apart from late anteroposterior subdivision of the antennal anlage, compartments do not form within the eye-antennal discs (Morata and Lawrence, 1979). Therefore, selector gene-mediated compartmentalization is not likely to underlie regionalization outside of the antennal primordium. A second possible explanation involves the embryonic origin of these discs. Unlike the limb discs, which derive from single trunk segments, each eye-antennal disc arises from multiple embryonic head segments (Younossi-Hartenstein et al., 1993). Divisions between segment primordia within the disc, which have not been clearly defined, could contribute to certain aspects of regional specification. Finally, the early roles of the *dpp*, *wg* and *hh* gene products in patterning the early eye-antennal disc are not well understood.

Most molecular studies of the eye-antennal disc have focused not on early patterning, but on later events in disc differentiation. Of particular interest has been the progression of the morphogenetic furrow, which traverses the disc epithelium beginning in the third instar larval stage and leaves differentiated retinal cells in its wake (Ready et al., 1976; Tomlinson and Ready, 1987). *dpp*, *wg* and *hh* are involved in regulating furrow progression. Mutant analysis and ectopic expression experiments suggest that *hh* expression in differentiating photoreceptor cells induces both

dpp expression in anteriorly adjacent cells and progression of the furrow (Heberlein, 1993; Ma et al., 1993). Although the role of *dpp* in furrow progression may be relatively minor (Burke and Basler, 1996), its early expression along the posterior and lateral margins of the eye-antennal disc is necessary for furrow initiation (Blackman et al., 1991; Treisman and Rubin, 1995; Burke and Basler, 1996). *dpp*-mediated furrow formation is antagonized by *wg*, which is expressed along the ventral and dorsal margins of the eye primordium (Ma and Moses, 1995; Treisman and Rubin, 1995; Wiersdorff et al., 1996).

Here, we investigated how, before morphogenetic furrow formation, the early eye primordium is distinguished from the adjacent primordium of the dorsal head capsule (also called the head vertex). We show that, in addition to its later role in furrow formation, *dpp* prevents dorsal head fate in the eye primordium. This early function of *dpp* is mediated by its repression of both *wg* and the homeobox gene *orthodenticle (otd)*, which normally collaborate to specify vertex identity. We also show that *otd* is a *wg* target gene in the vertex primordium. Finally, we present evidence that this early role of *dpp* in regional specification depends upon *hh* expression in the early eye primordium.

MATERIALS AND METHODS

Fly strains and clonal analysis

The wild-type strains used in this study were *Oregon-R* or *yw*. Reporter genes used were *dpp-lac Z* BS3.0 (Blackman et al., 1991) and hh^{P30}-*lacZ* (Lee et al., 1992). The $dpp^{d\text{-}blk}$ allele (Spencer et al., 1982) is a 5 kb deletion of sequences 3′ to the *dpp* transcribed region (Blackman et al., 1991). The mutations $In(2L)dpp^{e12}$ and $Df(2L)dpp^{d14}$ do not affect *dpp* embryonic function, but disrupt enhancers required for imaginal expression (St. Johnston et al., 1990; Blackman et al., 1991). The $Mad^{1\text{-}2}$ allele is described in Wiersdorff et al. (1996) and the *so*, *dsh* and *zw3* alleles used were so^1, dsh^{VA153} (a null allele; Flybase) and $sgg\text{-}zw3^{D127}$ (a null allele; Ruel et al., 1993) . Balancer chromosomes and other mutations are described in Lindsley and Zimm (1992).

Clonal analysis was performed using the FLP/FRT system (Xu and Rubin, 1993), using *P[miniW+; armadillo-lacZ]* as a clonal marker (Vincent et al., 1994).

zw3 and *dsh* mutant clones were induced in larvae of the genotypes *w, sgg-zw3*^{D127} *FRT18A/armadillo-lacZ FRT18A; hsp70-flp38/+* and dsh^{VA153} *FRT18A/armadillo-lacZ FRT18; hsp70-flp38/+*, respectively. Clones were generated by a 1 hour heat shock at 37°C during the first or second larval instar, and dissected and stained in late third instar discs.

Flies homozygous for the temperature-sensitive allele hh^{ts2} were raised at 17°C until the end of the first instar stage, then shifted to 29°C. After 24-36 hours, larvae were dissected and eye-antennal discs labeled.

Histochemistry, immunohistochemistry and analysis of head morphology

Larvae were grown in uncrowded conditions to ensure optimal disc morphology. Discs were dissected (attached to the larval mouthhooks) in PBS and fixed for 20 minutes at room temperature in 4% paraformaldehyde/PBS saturated with heptane. They were washed briefly in methanol once, 3× 5 minutes in PBT (PBS + 0.1% Tween-20) and incubated overnight at 4°C with the indicated primary antibodies preadsorbed against fixed embryos. Antisera used were rat polyclonal OTD antiserum (Wieschaus et al., 1992) used at a 1:500 dilution, rabbit polyclonal WG antiserum (van den Heuvel et al., 1993) used at a 1:100 dilution, and a mouse monoclonal antibody to β-galactosidase (Cappel) used at a 1:500 dilution. Following incubation in primary antibody, discs were washed 3× 1 hour in PBT and incubated for 3 hours at room temperature with biotinylated or fluorescently labeled secondary antibodies (Cappel). For immunocytochemistry, after 3× 1 hour washes in PBT, discs were treated for 1 hour with biotinylated horseradish peroxidase-avidin solution (Vector laboratories, Elite ABC Kit) diluted 1:50 in PBT, and washed again for 3× 45 minutes in PBT. Staining was visualized by incubating discs in 0.5 mg/ml diaminobenzidine in PBT in the presence of 0.04% H_2O_2. Discs were mounted in 80% glycerol in PBS and viewed under Nomarski optics using a Zeiss Axioskop microscope. For immunofluorescence, discs were washed 3× 1 hour at room temperature, and then mounted in 75% glycerol with 50 mg/ml n-propyl gallate and observed with a scanning confocal microscope (BioRad).

For X-gal staining, discs were dissected in cold PBS, fixed in 1% glutaraldehyde for 20 minutes, and washed 3× 10 minutes in PBT. Discs were then incubated in prewarmed staining solution plus 0.2% X-gal.

To analyse dorsal head structures, heads were severed with a razor blade and mounted in 30% glycerol in ethanol.

RESULTS

On the *Drosophila* head capsule, the compound eyes are separated dorsally by the head vertex, laterally by the shingle cuticle and ventrally by the gena (Fig. 1A; Haynie and Bryant, 1986). The vertex includes three morphologically distinct domains: (1) ocellar cuticle, containing the three ocelli and surrounding sensory bristles, (2) frons cuticle, consisting of a series of closely spaced parallel ridges flanking the ocellar region, and (3) orbital cuticle, which resembles ocellar cuticle and is also marked by a precise pattern of macrochaetes (Fig. 1A,B). In the vicinity of the ocelli, the compound eye is adjacent to the orbital cuticle, while closer to the antennae, the eye lies immediately next to the frons.

dpp and *wg* /*otd* are expressed in adjacent domains of the early third instar eye-antennal disc

Mutations that decrease *dpp* expression in the eye primordia lead to the formation of severely reduced eyes (Masucci et al., 1990; St. Johnston et al., 1990; Blackman et al., 1991). Similarly, the loss of *otd* or *wg* function in the vertex primordia causes the elimination of dorsal head structures (Wieschaus et al., 1992; Royet and Finkelstein, 1996). To understand the respective roles of these genes in regional specification, we first compared their expression patterns in the developing eye-antennal disc.

Through disc transplantion experiments, the anlagen of the primordia of adult head structures have been mapped on the third instar eye-antennal disc (Fig. 2A; Haynie and Bryant, 1986). The eye primordium occupies most of the posterior half of the disc (the ‘eye disc’). The head vertex forms from the dorsomedial region of the disc, while the antenna develops from the anterior half of the disc (the ‘antennal disc’).

During the early third instar stage (70-80 hours after egg laying [AEL]), *dpp* is expressed in a horseshoe-shaped domain along the ventral, posterior and dorsal periphery of the eye disc (Fig. 2B; Masucci et al., 1990). Dorsal *dpp* expression does not extend as far anteriorly as ventral expression, but instead ends at the vertex primordium. At this stage, *otd* expression covers the vertex primordium and extends along the edge of the antennal disc (Fig. 2C; Wieschaus et al., 1992; Royet and Finkelstein, 1995). The posterior boundary of *otd* expression in the vertex anlage approximately coincides with the anterior boundary of the *dpp* domain (Fig. 2D).

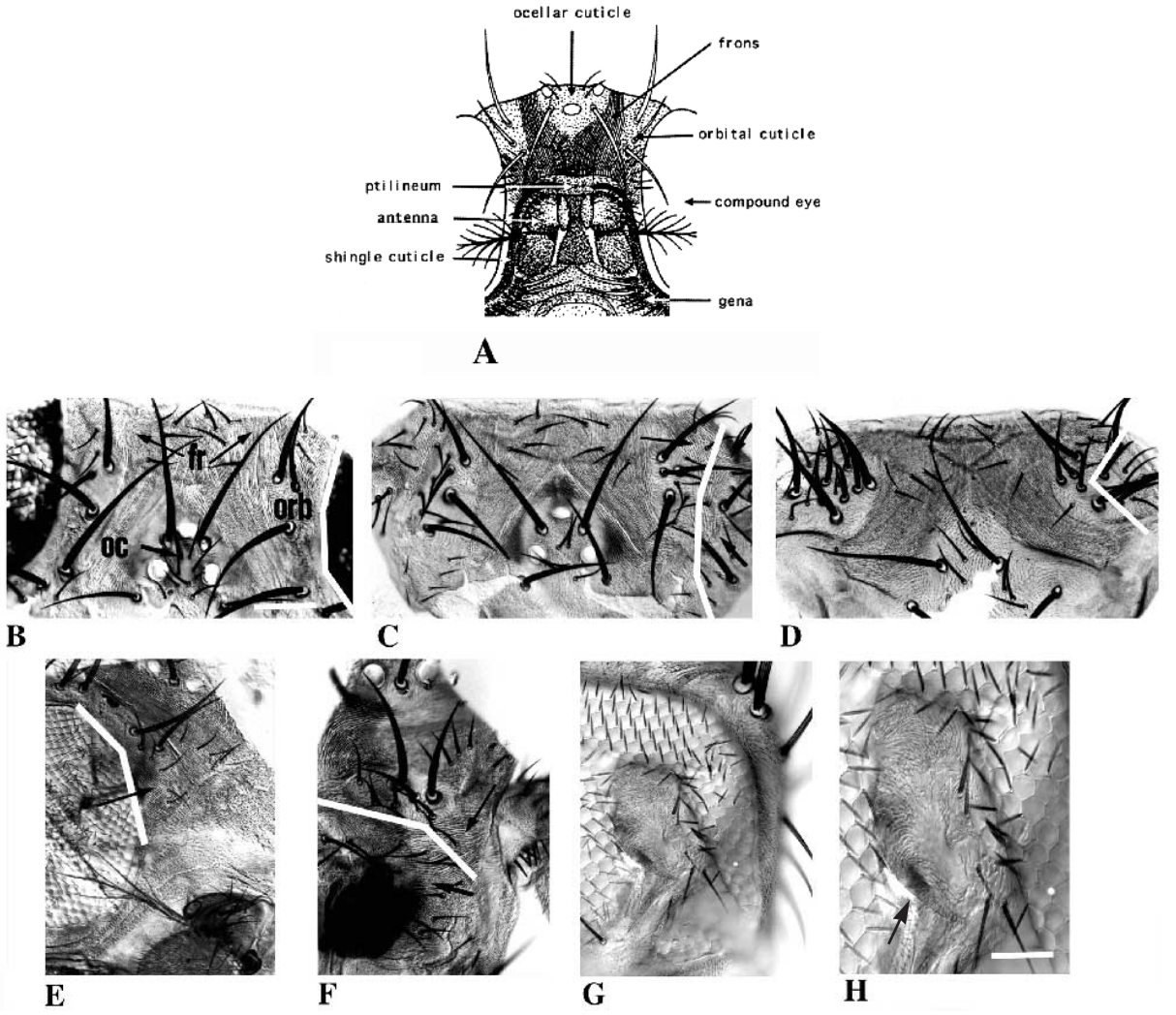

Fig. 1. *dpp* prevents the formation of dorsal head cuticle in the eye. (A) Schematic of the wild-type *Drosophila* head capsule, while (B-F) show the head capsules of wild-type (B,E), homozygous $dpp^{d\text{-}blk}$ (C,F) and *so* (D) flies. White lines mark the position of the boundary between the compound eye and dorsal head in a wild-type fly. (A) Dorsally, the compound eyes are separated by the vertex of the head, which includes the medial ocellar cuticle and the flanking ridged frons cuticle. Anteriorly, the frons is adjacent to the eye, while posteriorly, it is separated from the eye by a narrow strip of orbital cuticle (Note: the term vertex is sometimes used only to refer to the apex of the dorsal head; e.g. Ferris, 1950). Anterior is up. (B) The vertex. Shows the ocellar cuticle (oc) and frons (fr), separated from the eye by orbital cuticle (orb). (C) In a $dpp^{d\text{-}blk}$ head, the eyes are absent or greatly reduced. Frons is now present on both sides of the orbital cuticle (arrow), replacing the anterodorsal region of each eye. (D) In a *so* head, the eyes are absent but not replaced by frons. Ectopic micro- and macrochaetes are visible. (E) Frontolateral view of a wild-type head, showing the small amount of frons normally present near the anterior eye (arrow). (F) Frontolateral view of a $dpp^{d\text{-}blk}$ head, showing ectopic frons extending into the region normally occupied by the eye (lower arrow). (G,H) $Mad^{1\text{-}2}$ mutant clone in part of the eye. Ommatidia are replaced by frons cuticle, whose characteristic ridged morphology is seen in a closeup view (arrow in H). Scale bars: B-F (shown in B), 100 μm; H, 30 μm.

At the same stage of disc development, *wg* is expressed in two regions of the eye disc (Fig. 2A,E). One corresponds to the future gena and the other to the head vertex. In the early vertex primordium, *wg* and *otd* expression approximately colocalize (not shown), with the posterior boundaries of both expression domains lying immediately adjacent to the *dpp* domain.

dpp prevents dorsal head development in the eye primordium

The double-labeling described above showed that *wg* and *otd* are coexpressed in the vertex primordium, while *dpp* is expressed in the adjacent primordium of the compound eye. This juxtaposition of expression suggested that these genes could interact to establish the boundary between the two primordia. To test this hypothesis, we examined the phenotype of flies homozygous for the $dpp^{d\text{-}blk}$ allele. This mutation reduces *dpp* activity in the eye primordium and permits morphogenetic furrow movement only in the central region of the eye disc (Masucci et al., 1990). The result is a greatly reduced compound eye composed of only a few residual ommatidia (compare Fig. 1E and F).

Closer examination of $dpp^{d\text{-}blk}$ flies revealed that the eyes are replaced largely by frons cuticle, which normally appears only on the dorsal head (compare Fig. 1C,F with B,E). This ectopic frons lies between the orbital cuticle and the remaining ommatidia as well as more anteriorly, between the shingle cuticle and the ommatidia. To determine whether the loss of

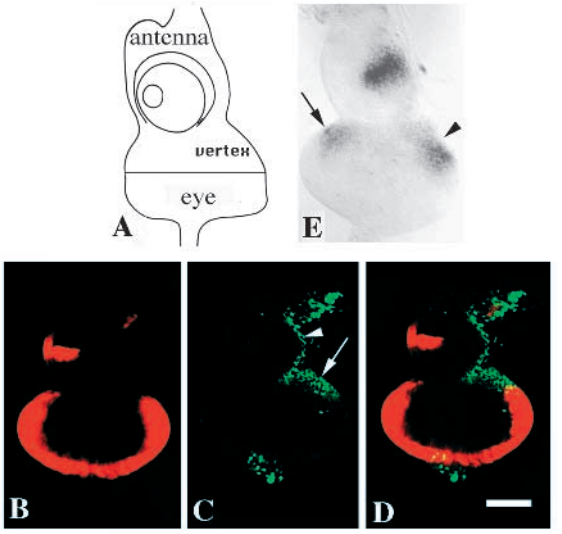

Fig. 2. Expression domains of *dpp*, *otd* and *wg*. (A) Schematic of a third instar eye-antennal disc, showing the primordia of the antenna, vertex and compound eye (see Haynie and Bryant, 1986 for a more detailed fate map). In the eye primordium, dorsal is to the right and posterior towards the bottom. (B-D) Double staining of a *dpp-lacZ* early third instar disc with antibodies against β-galactosidase (red) and OTD (green). D is an overlay of B and C. (B) *dpp* is expressed in a horseshoe-shaped region along the margins of the eye primordium as well as in a sector of the antennal anlage. (C,D) *otd* is expressed in the vertex primordium (arrow) and along the margin of the antennal primordium (arrowhead). The posterior limit of *otd* expression coincides with the anterior limit of the *dpp* domain (D). Staining in the posterior region of the eye disc is in the optic stalk and is not detected by in situ hybridization with an *otd* probe (J. Royet, unpublished results). (E) *wg* expression in the early third instar disc. *wg* is expressed in the antennal anlage and in the primordia of the vertex (arrowhead) and gena (arrow). Scale bars: B-E (shown in D), 30 μm. In all panels, anterior is up and dorsal is right.

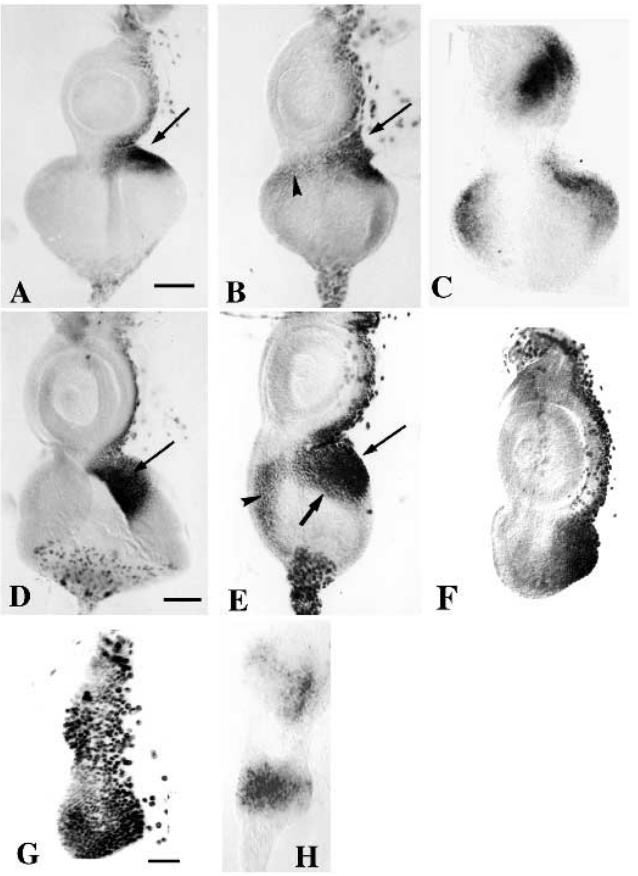

Fig. 3. *dpp* suppresses *otd* and *wg* expression in the eye primordium. (A,B) *otd* expression (arrows) in early third instar wild-type (A) and $dpp^{d\text{-}blk}$ (B) eye-antennal discs. In $dpp^{d\text{-}blk}$ discs, *otd* expression expands ventrally (arrowhead). (C) *wg* expression in an early third instar $dpp^{d\text{-}blk}$ disc. Both ventral and dorsal *wg* expression expand posteriorly (compare to Fig. 2E). (D-F) *otd* expression in late third instar wild-type (D), $dpp^{d\text{-}blk}$ (E) and *so* (F) discs. In $dpp^{d\text{-}blk}$ discs, *otd* vertex expression expands posteriorly (lower arrow) and ventrally (arrowhead) into the eye primordium. This expansion does not occur in *so* discs. (G,H) OTD (G) and WG (H) protein expression in $In(2L)dpp^{e12}/Df(2L)dpp^{d1}$ mutant discs. This transheterozygous combination of *dpp* alleles provides *dpp* embryonic function but eliminates imaginal expression. Resulting eye-antennal discs are reduced in size, particularly in the eye anlage. In these discs, *otd* is expressed throughout the eye primordium and of almost all the cells of the antennal anlage (compare to Fig. 3A). *wg* is also expressed throughout the eye primordium (compare to Fig. 2E). Scale bars: A-C (shown in A), 30 μm; D-F (shown in D), 50 μm; G,H (shown in H), 20 μm. In all panels, anterior is up and dorsal is right.

ommatidia is always associated with ectopic dorsal head cuticle, we examined flies carrying other mutations that prevent eye formation. In *sine oculis* (Fig. 1D) or *eyes absent* (not shown) flies, the eyes are completely lost but are not replaced by ectopic frons. This suggests that dorsal head cuticle does not result simply from loss of the eyes, but is caused instead by the loss of *dpp* function.

To determine whether other mutations affecting the *dpp* pathway cause a similar change in regional identity, we examined the effect of *Mothers against dpp (Mad)* mutant clones on eye development. The *Mad* gene product is required for the reception of the *dpp* signal (Raftery et al., 1995; Sekelsky et al., 1995) and *Mad* clones in the eye field have been reported to give rise to head cuticle (Wiersdorff et al., 1996). We found that *Mad* clones induced in first instar larvae cause a transformation of ommatidia into frons (Fig. 1G,H).

dpp prevents *wg* and *otd* expression in the eye primordium

On the head vertex, the formation of frons cuticle requires both *otd* and *wg* function (Wieschaus et al., 1992; Royet and Finkelstein, 1995). To determine whether the ectopic frons seen in $dpp^{d\text{-}blk}$ flies is associated with ectopic *otd* and *wg* expression, we examined their expression in $dpp^{d\text{-}blk}$ eye-antennal discs. In mid-third instar (80-90 AEL) $dpp^{d\text{-}blk}$ discs, OTD protein is not limited to the vertex anlage but instead is expressed in a band extending across the entire eye disc along the anterior margin of the eye primordium (compare Fig. 3A and B). In late third instar discs, the *otd* expression domain expands posteriorly into the eye anlage (compare Fig. 3D and E). Since existing fate maps of the eye-antennal disc are not precise, ectopic *otd* expression cannot be mapped precisely with respect to the primordia of head structures. However, the *otd* domain expands towards the anlagen of the shingle cuticle and the compound eye, consistent with the location of ectopic frons cuticle on $dpp^{d\text{-}blk}$ mutant heads. No

such ectopic *otd* expression can be detected in an *so* disc (Fig. 3F). It has previously been reported that *wg* expression also expands in late third instar $dpp^{d\text{-}blk}$ mutant discs (Wiersdorff et al., 1996; Chanut and Heberlein, 1997). In the early third instar disc, we found that *wg* expression in both the head vertex and gena primordia expands significantly towards the posterior edge of the eye disc (compare Figs 3C and 2E).

Although the $dpp^{d\text{-}blk}$ mutation severely reduces eye development, several observations suggest that it does not totally eliminate *dpp* function in the eye primordium (Treisman and Rubin, 1995). $dpp^{d\text{-}blk}$ eye-antennal discs are indistinguishable from wild-type discs until the third instar larval stage, suggesting that the mutation does not affect early development of this primordium. To determine the effect of a more severe reduction in *dpp* expression, we used a *trans*heterozygous combination of alleles ($In(2L)dpp^{e12}/Df(2L)dpp^{d1}$) that almost completely eliminates *dpp* imaginal function and fails to produce adult flies (St. Johnston et al., 1990; Blackman et al., 1991; Diaz-Benjumea et al., 1994). In these mutant discs, we observed a dramatic expansion of the *otd* expression domain, such that almost all cells of the eye and antennal primordia express *otd* (Fig. 3G). In the eye primordium, approximately three times as many cells express OTD protein as in wild-type discs at an equivalent stage. We observed a similar expansion of the *wg* domain (Fig. 3H). This indicates that, in both these primordia, *dpp* acts to establish domains free from *otd* and *wg* expression.

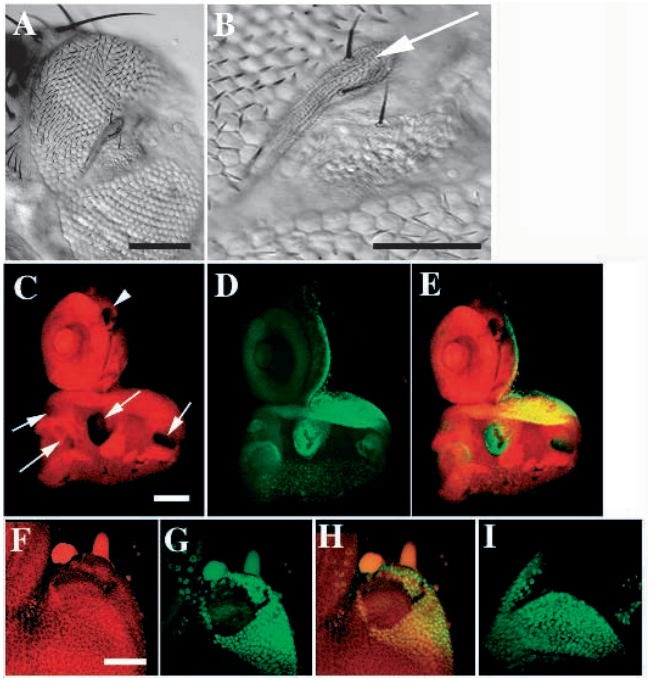

Fig. 4. Activation of the *wg* pathway in the eye primordium induces both ectopic frons cuticle and *otd* expression. (A,B) A *zw3* clone in the eye causes the replacement of ommatidia by ectopic cuticle. Higher magnification (B) shows the typical ridged morphology of frons cuticle(arrow). (C-I) *zw3* (C-E) or *dsh* (F-I) mutant clones were induced in first or second instar larvae. Third instar eye-antennal discs were labeled with antibodies to β-galactosidase (red) or OTD (green). (E) An overlay of C and D, and (H) an overlay of F and G. Mutant clones are visualized by the lack of β-galactosidase staining. In *zw3* clones in the eye primordium (arrows in C), *otd* is ectopically expressed in a cell autonomous fashion (D). In the antennal anlage, a *zw3* clone (arrowhead in C) does not express *otd* (D). (H) In a *dsh* clone in the vertex anlage, endogenous *otd* expression is lost compared to a wild-type disc (I). Scale bars: A, 100 μm; B, 40 μm; C-E (shown in C), 50 μm; F-I (shown in I), 40 μm. In C-I, anterior is up and dorsal is right.

Ectopic activation of the *wg* pathway in the eye primordium induces *otd* expression and vertex formation

Ectopic *wg* and *otd* expression caused by the loss of *dpp* can be explained in two ways. First, *wg* and *otd* could simply be markers of dorsal head cuticle and their expanded expression domains a secondary consequence of the enlarged head vertex of $dpp^{d\text{-}blk}$ mutant flies. Alternatively, as suggested by the important roles of *wg* and *otd* in vertex formation, their ectopic expression could instruct the formation of dorsal head structures in the eye primordium.

To distinguish between these possibilities, we determined the phenotypic consequence of activating the *wg* pathway in the eye anlage. To do so, we generated clones mutant for *zeste-white 3 (zw3,* also known as *shaggy)* in first or second instar larvae and observed their effect on eye differentiaion. Loss of *zw3* function results in constitutively activated *wg* signaling (Siegfried et al., 1992; Siegfried and Perrimon, 1994). A previous study showed that in *zw3* clones, ommatidia are replaced by dorsal head cuticle (Heslip et al., 1997). Closer examination revealed that these clones, like *Mad* clones, contain frons cuticle (Fig. 4A,B), which is normally *wg*-dependent on the dorsal head. This demonstrates that *wg* activation is sufficient to respecify cell fate in the eye primordium.

In the vertex primordium, both *wg* and *otd* are necessary for frons formation (Royet and Finkelstein, 1996). We therefore tested whether *otd* is ectopically expressed in *zw3* mutant clones. We found that, in the third instar eye disc, such clones express OTD protein in a cell autonomous fashion (Fig. 4C-E). This suggests that *otd* expression in the vertex primordium is normally activated or maintained by *wg*. To test this, we made clones in this primordium homozygous mutant for the *dishevelled (dsh)* gene, which is required for the reception of the *wg* signal (Klingensmith et al., 1994; Theisen et al., 1994). In these clones, endogenous *otd* expression was lost (Fig. 4F-I). Combined, these results strongly suggest that *otd* is a *wg* target gene in vertex formation.

wg and *otd* are expressed throughout the eye primordium of the second instar eye-antennal disc

The results described above show that *dpp* acts to exclude *wg* (and consequently *otd*) expression from the eye primordium. They raise the question of whether *wg* and *otd* are initially expressed throughout this primordium and only later excluded by the activation of *dpp*. Alternatively, the two genes might never be expressed in the eye anlage during normal development.

To address this issue, we examined *wg* and *otd* expression in second instar eye-antennal discs (Fig. 5). We found that, at a stage when the disc consists of fewer than 50 cells (50 hours AEL; Fig. 5A), WG protein expression is present in almost all the cells of the eye primordium (Fig. 5B,C). OTD protein is also evident in virtually all the cells of the eye disc, as well as in the antennal primordium (Fig. 5D).

Early *dpp* expression in the eye primordium is *hh*-dependent

How is *dpp* expression initiated in the early eye primordium?

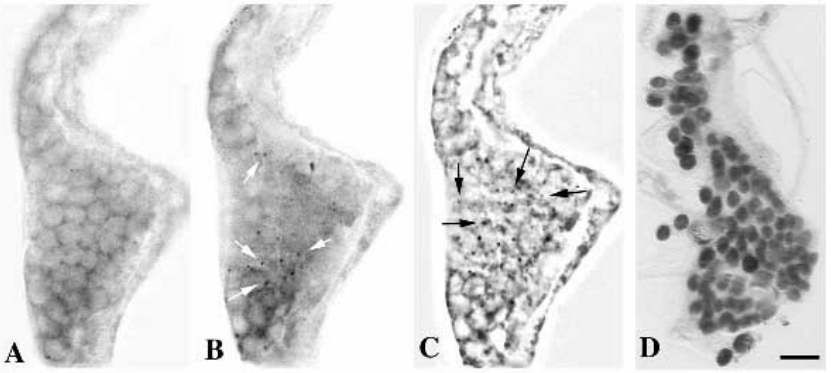

Fig. 5. *wg* and *otd* are ubiquitously expressed throughout the second instar eye-antennal disc. (A-C) Early second instar, wild-type eye-antennal discs (approximately 50 hours AEL) labeled with antibodies to WG. (A) At this stage, the disc consists of approximately 45 cells. In the focal plane shown, cells can be visualized but not WG protein expression. (B) The typical punctate appearance of WG expression can be seen in the posterior and anterior part of the eye (arrows). The small size of the disc and the distribution of WG-containing vesicles across different planes of focus makes it difficult to visualize the full extent of staining in this Nomarski image. (C) A phase contrast image reveals that almost all the cells of the disc contain WG-containing vesicles arrows. (D) Early second instar, wild-type eye-antennal disc labeled with antibodies to OTD. Every cell of the disc expresses OTD protein. Scale bars: A-D (shown in D), 5 μm. In all panels, anterior is up and dorsal is right.

In the morphogenetic furrow and limb discs, *dpp* is activated by *hh*, but *dpp* expression in the early eye disc is thought to be *hh*-independent (Heberlein, 1993; Ma et al., 1993; Basler and Struhl, 1994; Lee et al., 1994; Tabata and Kornberg, 1994). To test this assumption, we used a temperature-sensitive allele (Ma et al., 1993) to eliminate *hh* function before furrow initiation. We found that loss of *hh* activity during the second instar larval stage eliminated *dpp* expression along the posterior and lateral margins of the eye disc and in the antennal primordium (Fig. 6A). This loss of *dpp* expression was again associated with a dramatic expansion of the *otd* expression domain (Fig. 6B). *wg* expression also expands into the eye primodium (Fig. 6C).

This dependence of early *dpp* expression on *hh* was surprising, because in situ hybridization experiments have failed to detect *hh* expression in the eye primordium prior to retinal differentiation (Lee et al., 1992; Ma et al., 1993). Using a *hh-lacZ* reporter strain that reproduces endogenous *hh* expression (Lee et al., 1992), we examined *hh* expression in the early third instar disc. In addition to the previously reported *hh* expression domains in the antenna and vertex primordia, we detected *lacZ* expression at the posterior margin of the eye disc (Fig. 6D). This expression was also present in second instar discs, well before morphogenetic furrow initiation (not shown).

DISCUSSION

Early *dpp* expression creates the eye primordium by eliminating *wg* expression

The role of *dpp* in the patterning and differentiation of the eye primordium has previously been described (see Introduction). Here, we have shown that *dpp* is also required to specify the fate of this primordium. Early in disc development, both *wg* and *otd* are expressed throughout the eye anlage. We propose that, by repressing *wg* expression, *dpp* permits the development of the compound eye. In the absence of *dpp*, the eye is transformed into its default fate, dorsal head cuticle (frons).

After complementary domains of *dpp* (eye) and *wg* (vertex) expression are established, they may be maintained by mutual repression. A recent study showed that *dsh* mutant clones in the vertex primordium develop into ectopic ommatidia on the dorsal head (Heslip et al., 1997). This led these authors to propose *dpp/wg* mutual repression similar to that which subdivides the leg disc. In addition, Ma and colleagues used a temperature-sensitive *wg* allele to show that the elimination of *wg* function causes expansion of the eyes at the expense of dorsal head cuticle (Ma and Moses, 1995). It has not yet been demonstrated, however, that loss of *wg* activity in the vertex primordium leads to ectopic *dpp* expression. In addition, our results suggest that *wg* expression in the vertex primordium does not depend on *hh*, which differs from the situation in the leg primordia where *wg* and *dpp* expression require constant *hh* signalling.

In the eye-antennal disc, *dpp/wg* interactions, rather than subdividing a single appendage, distinguish the primordia of two very different structures, the compound eye and dorsal head capsule. As noted earlier, *wg* is also expressed in the pri-

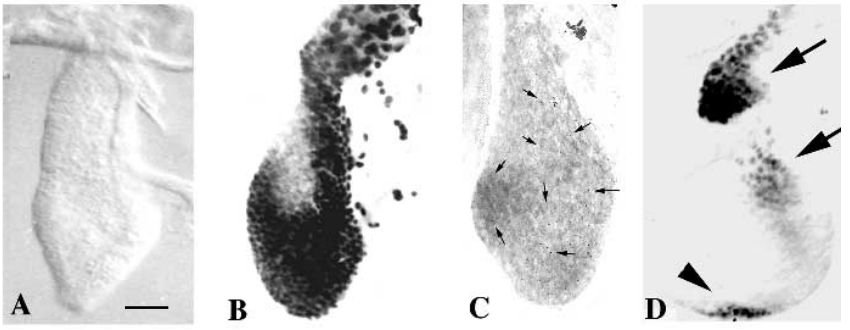

Fig. 6. Early *dpp* expression in the eye primordium depends on *hh*. (A-C) *hh* mutant discs. hh^{ts2}*; dpp-lacZ*/+ embryos were raised at the permissive temperature (17°C) until the end of the first instar stage and shifted to the restrictive temperature (29°C) until the early third instar. (A) *dpp-lacZ* expression is completely eliminated by the loss of *hh* (compare to Fig. 2B). Loss of *hh* expression, like the absence of *dpp*, causes a reduction in the size of the eye-antennal disc. (B) OTD protein expression expands throughout the eye primordium. Expression in the antennal anlage also expands, as in discs lacking *dpp* but do not reach the most ventral part of the antenna (Fig. 3G). (C) WG protein expression expands both posteriorly and towards the center of the disc (arrows). WG protein is never detected in the posterior part and center of a wild-type early third instar eye disc (Fig. 2E). (D) *hh-lacZ* expression. In addition to expression in the antennal and vertex anlagen (arrows), expression is present at the posterior edge of the wild-type eye primordium (arrowhead). Scale bars: A-D (shown in A), 20 μm. In all panels, anterior is up and dorsal is right

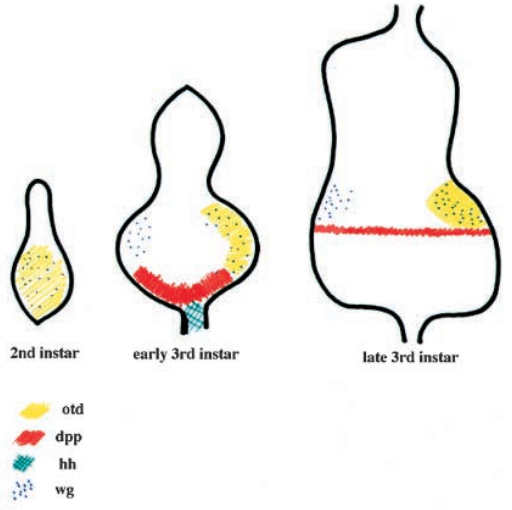

Fig. 7. Establishing adjacent primordia in the eye-antennal disc. *wg* and *otd* are initially expressed throughout the eye primordium of the eye-antennal disc. During the early third larval instar, *hh* expression in the posterior eye anlage activates *dpp* expression along the margins of the eye disc. *dpp* in turn eliminates *wg* (and consequently *otd*) expression from the eye region. This permits eye differentiation (i.e. morphogenetic furrow progression), as well as formation of the adjacent structures of the vertex and gena. As described in the text, the loss of *hh* or *dpp* function causes the eye primordium to be replaced by dorsal head cuticle.

mordium of the gena, which lies in the ventral region of the eye disc. A similar process of mutual repression may specify the position and size of this primordium.

wg activates *otd* in the dorsal head primordium

In the early vertex primordium, *hh*, *wg* and *otd* expression approximately coincide (Royet and Finkelstein, 1996). This raises the question of the regulatory relationships among these genes. We showed previously that ectopic *hh* expression activates ectopic *otd* and *wg* expression in a specific region of the eye-antennal disc (Royet and Finkelstein, 1996; J. Royet, unpublished results). However, the results described above using a temperature-sensitive *hh* allele suggest that endogenous *wg* and *otd* expression in the vertex primordium do not require *hh*.

We have also demonstrated that *wg* is epistatic to *otd* in the pathway of dorsal head formation. As we have shown, ectopic activation of the *wg* pathway induces *otd* expression in the eye primordium in a cell autonomous fashion. In addition, inhibition of the *wg* pathway in the vertex anlage eliminates *otd* expression. Combined, these observations strongly suggest that *otd* is a *wg* target gene in the vertex anlage.

Loss of *dpp* activity induces ectopic *wg* and *otd* expression in both the eye and antennal primordia. In the antennal disc, however, *zw3* mutant clones do not express *otd* (J. Royet, unpublished observations). This suggests that the activation of the *wg* pathway may be necessary but not sufficient for *otd* induction. It also indicates that the eye-antennal disc contains underlying patterning information independent of *dpp* and *wg* signaling.

Early *hh* expression is required to define the eye primordium

Previous studies demonstrated a requirement for *hh* in morphogenetic furrow progression and ommatidial differentiation. We propose that, in addition to this later function, *hh* plays an early role in the global patterning of the eye-antennal disc. In the absence of *hh* function, *dpp* is not expressed in the posterior eye disc. As a result, both *wg* and *otd* are ectopically expressed, causing an eye-to-vertex transformation. Consistent with this function, we found that *hh* is expressed at the posterior margin of the eye anlage significantly before furrow initiation. Since we detected this expression using a *hh-lacZ* reporter strain, we cannot be sure of the time window during which HH protein is normally expressed. It is possible that *hh* expression in this region occurs early in eye-antennal disc development, and that the expression we see just before furrow initiation results from perdurance of the β-galactosidase protein.

We propose that *wg* and *otd* expression in the eye-antennal discs are inherited from the embryo, where the two genes are expressed in segments from which these discs are derived. The almost ubiquitous expression of the two genes programs the early disc to the vertex fate. Later, *hh* expression in the posterior region of the future eye disc induces *dpp* expression along the margins of the eye primordium. *dpp* represses *wg*, permitting the formation of the eye primordium (see schematic in Fig. 7).

In vertebrates, homologues of the genes analyzed in this study are expressed in the developing head and brain (reviewed in Rubenstein et al., 1994; Tickle, 1995). Have the genetic regulatory relationships demonstrated here been evolutionarily conserved? The link between *wg* and *otd* expression is one example of a regulatory interaction that may have been retained in vertebrate head development. Itoh and Solkol recently showed that injection of *Xenopus dsh* or *Wnt8* mRNA induces *Otx2* expression, resembling the induction of *otd* by the *wg* pathway that we have observed (Itoh and Sokol, 1997). Paradoxically, however, microinjection of the *zw3* homologue glycogen synthase kinase also induces *Otx2* in frogs (Itoh et al., 1995). Further studies will be required to elucidate which elements of the signaling cascade described here have been conserved in higher animals.

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