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# Hedgehog activates the EGF receptor pathway during *Drosophila* head development

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## SUMMARY

**The Hedgehog (Hh) and Epidermal growth factor receptor (EGFR) signaling pathways play critical roles in pattern formation and cell proliferation in invertebrates and vertebrates. In this study, we demonstrate a direct link between these two pathways in *Drosophila melanogaster*. Hh and EGFR signaling are each required for the formation of a specific region of the head of the adult fruitfly. We show that *hh* and *vein (vn)*, which encodes a ligand of the *Drosophila* EGFR (Schnepp, B., Grumbling, G., Donaldson, T. and Simcox, A. (1996) *Genes Dev.* 10, 2302-13), are expressed in adjacent domains within the imaginal primordium of this region. Using loss- and gain-of-function approaches, we demonstrate that Hh activates *vn* expression. We also show that Hh activation of *vn* is mediated through the gene *cubitus interruptus (ci)* and that this activation requires the C-terminal region of the Ci protein. Finally, we demonstrate that *wingless (wg)* represses *vn* expression, thereby limiting the domain of EGFR signaling.**

Key words: *hedgehog*, *vein*, Epidermal growth factor receptor, *Drosophila*, Head development

## INTRODUCTION

Communication among cells within a developmental field is mediated through signal transduction pathways. Although individual signaling cascades have been studied in detail, the mechanisms through which they interact remain poorly understood. Such interactions can occur between pathways that act either sequentially or in parallel. In the first case, the signal received by a cell through a particular cascade activates or blocks signaling by a downstream pathway. In the case of parallel signaling, two pathways can interact directly, or converge at the level of common signal transducers.

One of the most important pathways controlling development is triggered by the Hh protein. Hh mediates both short- and long-range signaling, and is capable in specific tissues of activating the TGFβ, Wnt and FGF pathways (reviewed in Ingham, 1995; Perrimon, 1995; Altaba, 1997). In both invertebrates and vertebrates, the multiple functions of Hh signaling are reflected in the tissue-specific patterns in which *hh* and *hh*-related genes are expressed.

A second critical developmental signal is transduced through the EGFR. The EGFR is a member of the erbB family of tyrosine kinase receptors, which control both cell proliferation and cell fate. Signals transmitted through the *Drosophila* EGFR (DER) specify both axes of the oocyte and are required for the development of the embryonic ventral midline, the adult eye and wing, and other tissues (reviewed in Freeman, 1997; Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997). Recent studies have shown that the EGFR cascade interacts with both the Wg and Decapentaplegic (Dpp) pathways in *Drosophila*. During embryogenesis, competition between EGFR and Wg signaling determines cell fate within the ventral epidermis (O'Keefe et al., 1997; Szuts et al., 1997), while the EGFR and Dpp pathways interact during endoderm specification (Szuts et al., 1998) and tracheal development (Wappner et al., 1997).

The DER protein has three known activating ligands. The first is Gurken, which functions only in the germline (Neuman-Silberberg and Schupbach, 1993). The second is Spitz, which is ubiquitously expressed but must be processed to be active (Rutledge et al., 1992; Freeman, 1994; Schweitzer et al., 1995; Golembo et al., 1996b; Gabay et al., 1997). The third ligand, Vn, is a secreted neuregulin-like molecule expressed in a tissue-specific fashion during embryonic and imaginal development (Schnepp et al., 1996; Simcox et al., 1996; Simcox, 1997; Yarnitzky et al., 1997; Szuts et al., 1998).

In this study, we demonstrate that Hh induces EGFR signaling during *Drosophila* head development. We show that eliminating *vn* expression deletes a subset of the dorsal head structures dependent on Hh for their formation. We also demonstrate that Hh induces *vn* expression within the imaginal primordium of the dorsal head and analyze the role of the transcription factor Ci in *vn* induction. Finally, we show that

the lateral boundary of the region of EGFR activation is set by Wg, which prevents *vn* expression. These results extend our understanding of how signaling pathways interact during developmental pattern formation.

## MATERIALS AND METHODS

### *Drosophila* stocks

The *wg-lacZ*, $hh^{ts2}$ and *hh-lacZ* strains have been described previously (Kassis et al., 1992; Ma et al., 1993). The $wg^{IL114}$ allele (referred to here as $wg^{ts}$) produces a protein that is essentially functional at 17°C but not secreted at 25°C (Baker, 1988; Bejsovec and Martinez-Arias, 1991; Gonzalez et al., 1991). The *vn-lacZ* strain contains an 11 kb fragment upstream of the *vn* transcription start site cloned into the vector pCaSpeR hs43lacZ (A. Simcox, personal communication). The UAS-DN-DER strain (29-77; 29-8) contains insertions of a DN-DER gene (lacking the intracellular domain) on both the second and third chromosomes (Buff et al., 1998). For additional information regarding mutant alleles, see Lindsley and Zimm (1992).

### Immunostaining and X-gal staining

Eye-antennal discs and adult heads were prepared and processed for antibody or X-gal staining as described (Royet and Finkelstein, 1996). In situ hybridization was performed as described, with modifications for the use of RNA probes (Tautz and Pfeifle, 1989; Gao et al., 1996). Combined in situ hybridization and X-gal staining was performed as described (Su et al., 1998). Eye-antennal discs and adult heads were visualized using a Zeiss Axioskop equipped with Nomarski optics. Anti-dp-ERK antibodies were purchased from Sigma.

### Generation of mutant clones and induction of ectopic gene expression

Homozygous *vn* clones were made by γ-irradiation by Wurst and colleagues, using the *vn* allele RG436, as described previously (Wurst et al., 1984), In the experimental irradiations, eggs were collected from females with the genotype *y; Dp(1:3)* $sc^{J4}$,$y^{+}$ *M(3)* $i^{55}$*/TM1* that had been mated to males with the genotype *y; mwh RG436 e/TM3*,$y^{+}$ *Sb e Ser.* Clones on the dorsal head were visualized by their *mwh* phenotype. In this region, the *mwh* mutation causes a dramatic decrease in hair length but no obvious appearance of multiple hair elements (A. A., unpublished observations).

To induce ectopic *ci* expression, flies containing the C591-GAL4 driver were crossed to flies containing either full-length Ci (Ci155), an N-terminal fragment that encodes a nuclear repressor form of Ci (Ci76; Aza-Blanc et al., 1997), or a C-terminal fragment that includes the zinc finger domain (CiZn/C; Hepker et al., 1997), each under UAS control. Ectopic expression of DN-DER was induced by crossing C591-GAL4 flies to UAS-DN-DER flies (see above). Clones of ectopic *hh* expression were generated using the Flp-out technique as described previously (Royet and Finkelstein, 1997). Ectopic *hh* expression in the eye primordium was produced by crossing *dpp*-GAL4 flies (Staehling-Hampton et al., 1994) to UAS-*hh* flies (provided by N. Perrimon). The eye-specific *dpp* control region lies 3′ to the *dpp* gene and has been previously described (Blackman et al., 1991).

## RESULTS

The dorsal head capsule, which lies between the compound eyes, contains three morphologically distinct domains (Fig. 1A). The medial domain includes the ocelli and their associated bristles, which lie on the triangular ocellar cuticle. The mediolateral region contains the frons cuticle, which consists of a series of closely spaced parallel ridges. The lateral region is occupied by the orbital cuticle, which contains a stereotypical pattern of bristles.

The head capsule forms primarily from the two eye-antennal imaginal discs. Each half of the dorsal head derives from a

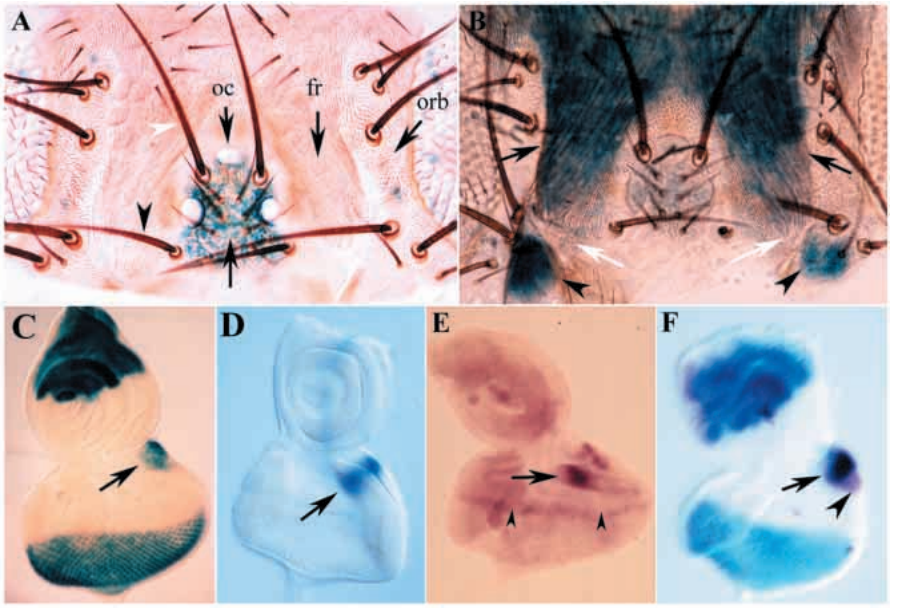


**Fig. 1.** *hh* and *vn* are expressed in adjacent domains during head development. (A,B) Dorsal views of the head capsules of adult flies; (C-F) third instar eye-antennal imaginal discs. Anterior is up in all panels. (A) Head of a *hh-lacZ* fly stained with X-gal. *hh* is expressed in the interocellar cuticle (arrow). The normal appearance of the ocellar region (oc), frons (fr), and orbital region (orb) can be seen. The two ocellar bristles, which lie near the medial ocellus, are evident (white arrowhead indicates one of them) as are the two postvertical bristles (black arrowhead). The interocellar cuticle also contains 6-8 microchaetes (the interocellar bristles). (B) Head of a *vn-lacZ* fly stained with X-gal. *vn* is expressed in the ridged frons cuticle (arrows) and in a spot within the posterior orbital cuticle (arrowheads). Note the gap between these two expression domains (white arrows). (C) *hh-lacZ* eye-antennal disc stained with X-gal. *hh* is expressed in cells that give rise to the interocellar cuticle (Royet and Finkelstein, 1996). Note that the *hh* expression domain is immediately adjacent to the antennal anlage. (D) *vn-lacZ* disc. *vn* is also expressed in the dorsal head primordium (arrow). Note that *vn* expression is not immediately next to the antennal anlage, but lies posterior to that of *hh* (compare to C). (E) Wild-type disc labeled with a digoxigenin-labeled *vn* probe. In situ hybridization reveals that, in addition to being expressed in the dorsal head primordium (arrow), *vn* is expressed at low levels in the morphogenetic furrow (arrowheads). This expression is only detectable when the disc is stained for long time periods and is stronger in late third instar discs. It is not detectable using the *vn-lacZ* line used in D. (F) *hh-lacZ* disc stained with X-gal and labeled with a digoxigenin-labeled *vn* probe. Double-labeling confirms that *vn* expression (arrowhead) is posteriorly adjacent to that of *hh* (arrow).

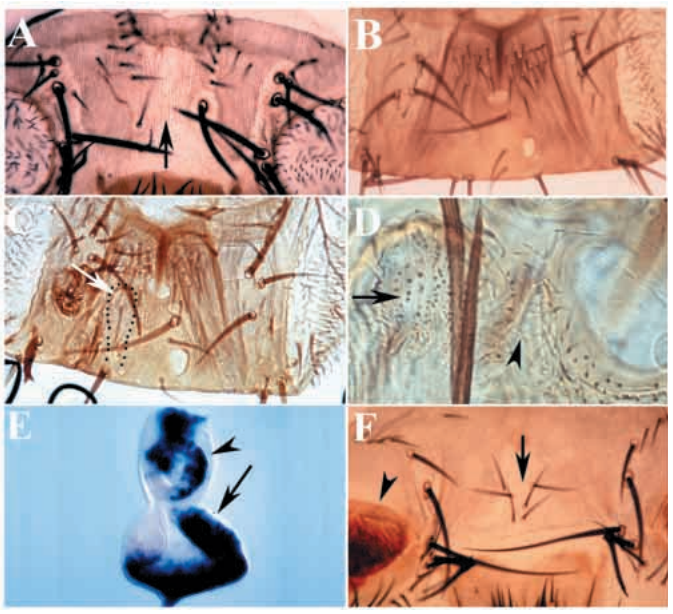


**Fig. 2.** Loss of EGFR signaling deletes a subset of Hh-dependent head structures. (A-D,F) Dorsal views of the head capsules of flies of the genotypes indicated; (E) an eye-antennal disc stained with X-gal. (A) Head of a $hh^{ts2}$ / $hh^{ts2}$ fly raised at the restrictive temperature (30°C) during the third larval instar. Loss of Hh function eliminates the entire medial domain of the dorsal head, including the ocelli, interocellar cuticle, and the ocellar, postvertical, and interocellar bristles (compare to Fig. 1A). This region is replaced by ridged frons cuticle (arrow). (B) Homozygous *vn* clone. Loss of *vn* deletes one of the ocelli and a subset of the bristles flanking the ocelli (compare to Fig. 1A). Although limited invasion of frons into the ocellar region sometimes occurs (data not shown), most of the interocellar cuticle and varying numbers of interocellar bristles are generally retained. Note that since each eye-antennal disc gives rise to one lateral ocellus and half of the medial ocellus, clones induced during imaginal development can remove only one lateral ocellus. (C) Homozygous *vn* clone. In addition to eliminating ocelli, loss of *vn* expression occasionally causes irregularities in the dorsal head cuticle (arrow; shown at higher magnification in panel D). The approximate boundaries of the clone, visualized by its *mwh* phenotype (see Materials and Methods), are shown. (D) The homozygous *vn* clone in C, rotated slightly clockwise and shown at higher magnification. The *mwh* trichomes within the clone are extremely short (arrow), as compared to hairs outside the clone (arrowhead). The medial ocellus is irregular in shape, which we believe results from the loss or reduction of the half of this ocellus contributed by the eye-antennal disc containing the clone. Since the *mwh* marker is difficult to detect in parts of this region, we cannot be certain about whether *vn* acts in a cell-autonomous or nonautonomous fashion in this primordium. (E) The C591-GAL4 driver induces *lacZ* expression across the entire dorsal head primordium (arrow) as well as in the anlagen of the antenna (arrowhead) and compound eye. (F) Head of a C591-GAL4 / UAS-DN-DER fly. Ectopic DN-DER expression, like the loss of *vn*, eliminates the ocelli but not the interocellar cuticle (arrow indicates the interocellar bristles, which lie on this cuticle). Note that the compound eye is reduced in size by the loss of EGFR signaling (arrowhead).

primordium in the disc immediately adjacent to the anlage of the compound eye (Haynie and Bryant, 1986). During the pupal stage, the two discs fuse at what will form the midline of the dorsal head capsule.

### *hh* and *vn* are expressed in adjacent regions of the dorsal head primordium

*hh* is expressed within the medial domain of the dorsal head capsule (Royet and Finkelstein, 1996). Specifically, it is expressed in the interocellar cuticle, which contains the small interocellar bristles (Fig. 1A). Using a *vn-lacZ* strain (see Materials and Methods), we found that *vn* is also expressed on the dorsal head (Fig. 1B). *vn* expression lies primarily within the mediolateral frons cuticle, near but not immediately adjacent to the region of *hh* transcription.

We also compared the regions of *hh* and *vn* expression in the dorsal head primordium of the eye-antennal disc. Consistent with its expression on the adult head capsule, *hh* is expressed in the region of this primordium that lies between the precursor cells of the ocelli (Lee et al., 1992; Royet and Finkelstein, 1996; Fig. 1C). *vn* is expressed in the wing and haltere discs (Simcox et al., 1996), but its expression in the eye-antennal disc has not been described.

Using both the *vn-lacZ* strain and in situ hybridization with a *vn* probe, we found that *vn* is also expressed in the dorsal head primordium (Fig. 1D,E). As on the adult head, *vn* expression lies near that of *hh*. Double-labeling showed that the domains of *hh* and *vn* expression are immediately adjacent to each other (Fig. 1F). In situ hybridization revealed that *vn* is also expressed at low levels in the morphogenetic furrow (Fig. 1E).

### Eliminating EGFR signaling deletes Hh-dependent head structures

Eliminating Hh function during head development results in the deletion of the entire medial domain, including the interocellar cuticle and bristles, and the ocelli and their associated bristles (Ma et al., 1993; Royet and Finkelstein, 1996; Fig. 2A). This region is replaced by frons cuticle, which is normally confined to the mediolateral region of the head capsule. Ectopic *hh* expression generates ectopic medial structures at more lateral positions (Royet and Finkelstein, 1996). Hh is therefore necessary for the specification of the medial domain and sufficient to direct more lateral regions of the dorsal head towards a medial fate.

A previous study showed that particular combinations of EGFR alleles cause a reduction in the size of the ocelli and the loss of the two ocellar bristles, which flank the medial ocellus (Clifford and Schupbach, 1989). Since *vn* is expressed within the dorsal head primordium, we determined the effects of eliminating either *vn* expression or EGFR-mediated signaling on head development.

Examination of *vn* mutant clones (see Materials and Methods) showed that Vn is required for the development of some, but not all, of the Hh-dependent medial head structures (Fig. 2B-D). The ocelli and ocellar bristles are deleted and the postvertical bristles, which lie near the lateral ocelli, are also lost. However, most of the interocellar cuticle is retained, indicating that the *vn* dorsal head phenotype is less global than that caused by loss of Hh function.

Since *vn* encodes a ligand for DER, we also examined the effects of eliminating EGFR-mediated signaling on head development. To do so, we used the GAL4/UAS system (Brand and Perrimon, 1993) to express a dominant negative form of the EGFR (DN-DER; Buff et al., 1998) across the entire dorsal head primordium (Fig. 2E,F). DN-DER expression eliminates

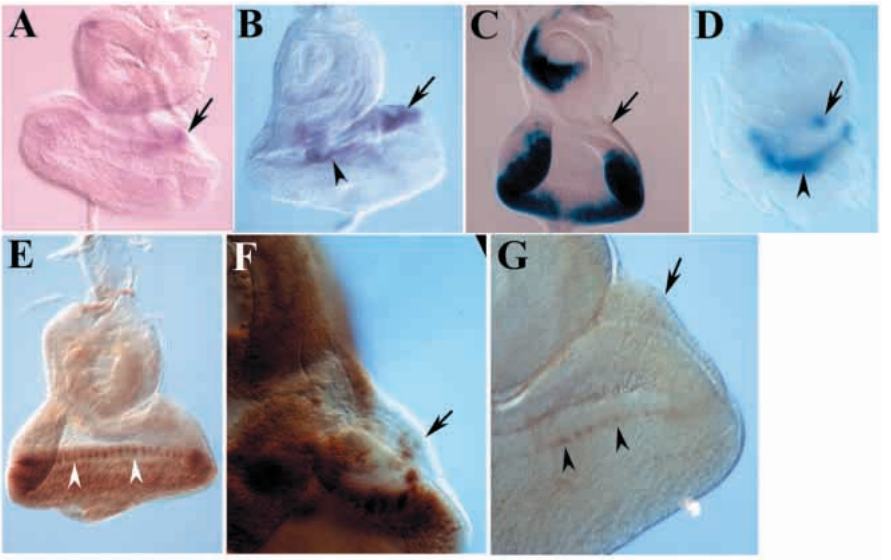


**Fig. 3.** Hh activates *vn* expression. (A-C) Eye-antennal discs of the indicated genotypes labeled with either a digoxigenin *vn* probe (A,B) or X-gal (C). (A) $hh^{ts2}/hh^{ts2}$ disc. Eliminating Hh function during the third larval instar strongly reduces or eliminates *vn* expression in the dorsal head primordium (arrow). *vn* expression in the morphogenetic furrow is also lost (data not shown), although this expression would not be seen in this disc, since it is detectable only after long staining periods in late third instar discs. (B) Ectopic *hh* clone. Flp recombinase-induced *hh* expression induces ectopic *vn* expression in the interior region of the disc (arrowhead). Arrow indicates normal *vn* expression in the dorsal head primordium. (C) *dpp*- GAL4/ UAS-*lacZ* disc. The *dpp* enhancer drives expression along the posterior and lateral margins of the eye disc, and in a sector of the antennal anlage. (D) *dpp*-GAL4/ UAS-*hh* disc. Ectopic *hh* expression in the eye primordium induces a band of ectopic *vn* expression (arrowhead) anterior to the region where *hh* is induced. Cross was performed in the background of a *vn-lacZ* chromosome (see Fig. 1D). Note that the morphology of the disc is severly disrupted by ectopic *hh* expression. (E) wild-type disc. Labeling with anti-dp-ERK antibodies reveals expression in the morphogenetic furrow (arrowheads) and weaker expression in ommatidia posterior to the furrow. (F) wild-type disc. When anti-dp-ERK staining is allowed to develop for long time periods, weaker expression is detectable in cells in the medial region of the dorsal head primordium (arrow). (G) $hh^{ts2}$ / $hh^{ts2}$ disc. Eliminating Hh function (as in A) eliminates or strongly reduces staining in the furrow (arrowheads) and the dorsal head primordium. Staining was developed for approximately the same time period as in F.

the same structures deleted in *vn* clones, suggesting that *vn* is primarily responsible for activating EGFR signaling in this region. As was the case for Vn, the interocellar cuticle is retained in the absence of EGFR signaling (Fig. 2F).

## Hh activates *vn* expression

Since the *hh* mutant phenotype is more extensive than either the *vn* or EGFR phenotypes, we tested whether Hh acts upstream of the EGFR pathway. Using a temperature-sensitive *hh* allele ($hh^{ts2}$; Ma et al., 1993), we eliminated Hh function during the third instar larval stage. Loss of Hh eliminated or strongly reduced *vn* expression in both the dorsal head primordium and the morphogenetic furrow (Fig. 3A).

To determine whether Hh can induce *vn* expression outside the dorsal head primordium, we induced ectopic *hh* expression using the Flp recombinase technique (Struhl and Basler, 1993). We found that Hh is capable of activating *vn* in other regions of the eye disc (Fig. 3B). We also used a disc-specific enhancer from the *dpp* gene to induce ectopic *hh* expression using the GAL4/UAS system (see Materials and Methods). This enhancer drives reporter gene expression at the posterior and lateral margins of the third instar eye disc as well as in a portion of the antennal anlage (Fig. 3C). Ectopic *hh* expression induced by this enhancer severely disrupted eye-antennal disc morphology (Fig. 3D). It also induced a band of ectopic *vn* expression anterior to the region of *hh* transcription. Combined with the previous results, these experiments demonstrate that Hh is necessary for *vn* expression in the dorsal head primordium, and sufficient to induce ectopic *vn* expression in other regions of the disc.

To test whether Hh is also required to activate EGFR-mediated signaling, we used a monoclonal antibody that specifically recognizes the active, dually phosphorylated form of mitogen-activated protein kinase (dp-ERK; Gabay et al., 1997). It had been shown previously that dp-ERK is expressed at high levels in the morphogenetic furrow, and at lower levels in ommatidia posterior to the furrow (Gabay et al., 1997; Fig. 3E). We found that when the anti-dp-ERK signal is allowed to develop for longer periods, weaker expression appears in cells within the dorsal head primordium (Fig. 3F). Eliminating Hh function reduced or eliminated dp-ERK expression both in these cells and in the furrow (Fig. 3G).

## Hh induction of *vn* expression is mediated through *ci*

The *ci* gene encodes a critical component of the Hh pathway (reviewed in Altaba, 1997). The *ci* gene product is a zinc finger-containing protein (Ci155), that acts as an activator of gene expression in vitro (Alexandre et al., 1996). In the anterior compartment of the wing disc, Ci155 is cleaved to form an N-terminal protein (Ci75) that represses Hh target genes in vivo (Aza-Blanc et al., 1997). Hh protein produced by posterior compartment cells inhibits the cleavage of Ci in immediately adjacent anterior compartment cells. By preventing the formation of the Ci75 repressor, Hh permits the expression of downstream target genes.

In the eye-antennal disc, *ci* is expressed in a domain complementary to that of *hh* (Eaton and Kornberg, 1990; Motzny and Holmgren, 1995), with the ocelli originating near the boundary between *ci* and *hh* expression. To determine if *ci* regulates *vn*, we used the GAL4/UAS system to express specific forms of the Ci protein across the entire dorsal head primordium.

Expression of an N-terminal fragment of Ci with repressor activity (see Materials and Methods) reduced or eliminated *vn* expression in the dorsal head analage (Fig. 4A). The consequence of this expression was a head phenotype almost identical to the *hh* mutant phenotype (compare Figs 4B and

2A). Expression of the Ci155 activator, on the contrary, increased the intensity and extent of *vn* expression (Fig. 4C) and caused the ocelli to increase in size and fuse (Fig. 4D).

These results suggested that Ci155 normally plays a positive role in regulating *vn* expression. It had been shown previously that a C-terminal fragment of Ci, which includes the zinc finger domain, is sufficient to activate Ci target genes in the wing disc (Alexandre et al., 1996). To determine whether this is true of Ci regulation of *vn*, we expressed this fragment (CiZn/C) across the dorsal head primordium. As was the case for Ci155, CiZn/C expression increased *vn* expression and enlarged the ocelli (Fig. 4E,F). This suggests that the C-terminal region of Ci mediates these functions during dorsal head development.

### Wg signaling represses *vn*

*wg* is broadly expressed throughout the early eye-antennal disc, where it confers a default state of head cuticle (Ma and Moses, 1995; Treisman and Rubin, 1995; Royet and Finkelstein, 1997). Later, *wg* expression becomes restricted to the primordia of the orbital cuticle and ptilinium, and to a portion of the antennal anlage (Baker, 1988; Royet and Finkelstein, 1996). Just as *hh* expression is medially adjacent to that of *vn* on the adult head capsule, *wg* expression abuts *vn* in the frons both laterally and anteriorly (compare Figs 5A and 1B). Loss of Wg signaling causes the deletion of both the frons and orbital cuticles (Royet and Finkelstein, 1996).

To determine whether Wg participates in *vn* regulation, we used a temperature-sensitive allele to eliminate Wg function during second instar development. In contrast to Hh, we found that Wg negatively regulates *vn* (Fig. 5B). Loss of Wg activity during this time window expanded the domain of *vn* expression in the dorsal head primordium and induced ectopic *vn* expression in other regions of the eye-antennal disc.

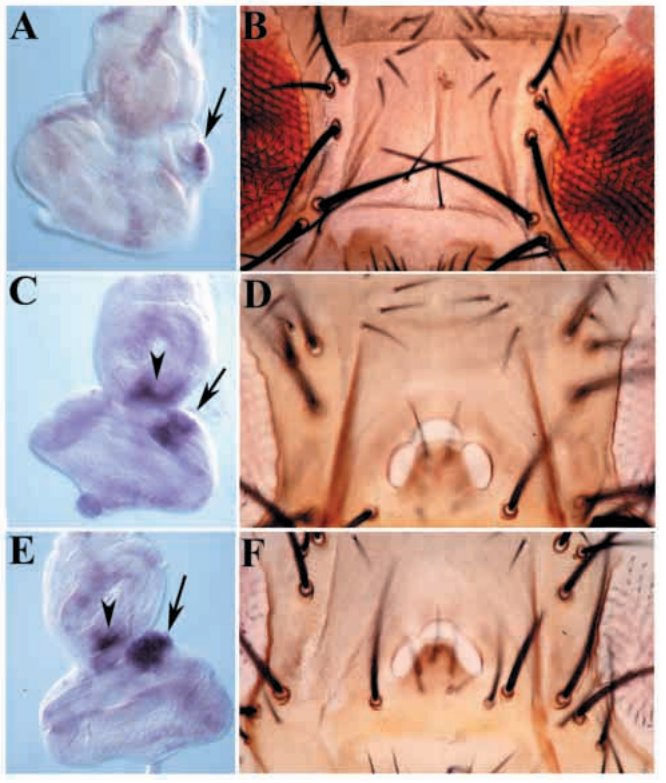


**Fig. 4.** Regulation of *vn* expression by Ci. The C591 GAL4 driver (see Fig. 2E) was used to express various forms of the Ci protein across the dorsal head primordium. (A,C, E) Eye-antennal discs labeled with a digoxigenin *vn* probe; (B,D,F) adult head capsules of the corresponding genotypes. (A,B) Ectopic expression of an N-terminal fragment of Ci (Ci76) reduces *vn* expression (A) and eliminates the ocelli (B). Just as when Hh function is eliminated, ectopic frons cuticle appears in the medial region (compare to Fig. 2A). (C,D) Ectopic expression of Ci155 increases both *vn* expression (C) and the size of the ocelli (D). Note that *vn* expression increases in intensity and extent in the dorsal head primordium (arrow), and also appears ectopically in the antennal anlage (arrowhead). (E,F) Ectopic expression of a C-terminal fragment of Ci (CiZn/C) also increases *vn* expression (E) and the size of the ocelli (F). The effects of CiZn/C are generally somewhat stronger than those of Ci155 (Y. L. and A. A., unpublished results).

## DISCUSSION

### Hh/EGFR interactions and head development

We have shown that, during *Drosophila* head development, Hh activates EGFR signaling by inducing the tissue-specific expression of a particular DER ligand. Specifically, *hh* expression in the interocellar region of the eye-antennal disc induces *vn* in the adjacent primordium of the frons. Vn is not required for frons development, but instead appears to act non-autonomously to induce the formation of the ocelli, which originate near the boundary separating *hh* and *vn* expression in the third instar disc. Several recent studies dissected the functions of the EGFR and its ligands in patterning the eye primordium (Dominguez et al., 1998; Kumar et al., 1998; Spencer et al., 1998). In the developing eye, Vn is required early for cell proliferation and later for the specification of the R8 photoreceptor (Spencer et al., 1998). However, activation of EGFR signaling in the retina appears to require active collaboration between the Vn and Rho ligands.

How does Hh induce *vn* expression? The simplest

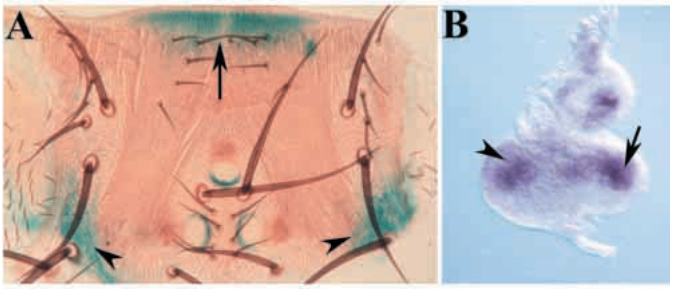


**Fig. 5.** Wg signaling prevents *vn* expression. (A) Adult head capsule from a *wg-lacZ* strain stained with X-gal; (B) eye-antennal disc labeled with a digoxigenin *vn* probe. (A) *wg* expression is complementary to that of *vn*. *wg* is expressed in a region of the orbital cuticle where *vn* is expression is absent (arrowheads, compare to Fig. 1B) and in the ptilinium (arrow) where *vn* is also not expressed. The staining in the periphery of the ocelli does not appear until late in development, since it is not present in third instar or early pupal discs. (B) $wg^{ts}$ disc. Ectopic *vn* expression appears in discs raised at the restrictive temperature during the second larval instar. *vn* expression expands in the dorsal head primordium (arrow) and appears on the opposite side of the disc (arrowhead), in the primordium of the shingle cuticle, which flanks the antennae on the adult head capsule.

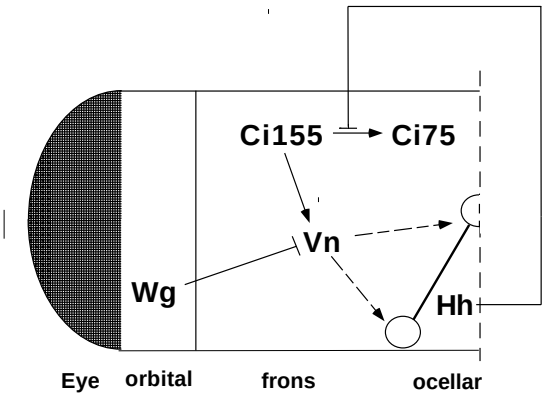


**Fig. 6.** Proposed interactions between the Hh, EGFR and Wg pathways: a simplified schematic of half of the laterally symmetric head capsule. Our results indicate that Hh protein produced by cells in the interocellar region causes the activation of *vn* expression in the frons cuticle. We propose that, as in the wing disc, Hh prevents the formation of the Ci75 repressor in the frons primordium, thereby allowing the localized expression of Ci155. Ci 155 activates *vn* expression, which is necessary for the formation of the ocelli and their associated bristles. Wg protein produced in the posterior orbital region blocks *vn* expression, thus limiting the domain of EGFR activation.

interpretation of our results is that Hh signals through Ci. Although our experiments show that Ci 155 is capable of promoting ocelli development, it will be important to determine whether eliminating Ci expression (in *ci* clones) prevents ocelli formation and the activation of *vn* expression. If clonal analysis proves that Ci is required for *vn* induction, it will be critical to test whether Ci binds directly to *vn* regulatory regions or acts through intermediary proteins.

Based on previous studies of the wing disc (Aza-Blanc et al., 1997), we propose that Hh also acts in the dorsal head primordium to prevent the cleavage of full-length Ci (Ci155) to the truncated repressor form Ci75 (Fig. 6). This would create a zone (i.e. the frons primordium) near the source of secreted Hh protein in which Ci155, but not Ci75 is present, and a more distal region (the orbital primordium) in which Ci75 is present. Consistent with the pattern of *vn* expression, we have shown that Ci155 is an activator and Ci75 a repressor of *vn*. To test this model, it will be necessary to determine exactly where the two forms of Ci are expressed in the dorsal head anlage. This is difficult however, because Ci protein levels are relatively low in this portion of the eye-antennal disc (Y. L. and A. A., unpublished results). It is also possible that the mechanism of Hh function is different in this primordium than in the wing anlage. Hh could modulate Ci expression and/or function in a different fashion than in the wing, or could induce *vn* expression through a novel signaling pathway. However, the effects of Ci155 and Ci75 on *vn* expression strongly suggest that Ci mediates Hh function in this region.

A second important question involves the relative positions of the domains of *hh* and *vn* expression. Although these domains are immediately adjacent to each other in the third instar disc, they are separated by a gap on the adult head capsule (compare Fig. 1A and B). It is possible that the *vn-lacZ* line used in our experiments does not fully reproduce the pattern of endogenous *vn* expression. Alternatively, *ci* may not be expressed within this gap, so that Hh cannot induce *vn* expression. Finally, an independent mechanism could exist that blocks *vn* expression within this region.

As described earlier, eliminating Hh function during third instar development causes the loss of both the ocelli and the interocellar region. In contrast, eliminating *vn* expression or blocking EGFR signaling primarily affects the ocelli. This suggests that, in addition to inducing the EGFR cascade, Hh activates a second pathway required for the formation of the interocellar region. It is possible that this pathway is mediated through the *engrailed (en)* gene, which is activated by Hh in this region of the disc (Royet and Finkelstein, 1996). Consistent with this idea, preliminary experiments indicate that *en* mutant clones cause the loss of the interocellar cuticle but not the ocelli (J. Royet, Q. Gao and R. F., unpublished results).

## Wg and dorsal head development

In the early larva, Wg is required for cell proliferation in all the imaginal discs (Simcox et al., 1989). Later in imaginal development, it plays a more specific role in the formation of particular adult structures. As mentioned earlier, for example, *wg* expression is necessary for the formation of both the frons and orbital cuticles (Royet and Finkelstein, 1996).

Our results show that one of the functions of Wg is to define the limit of the region of EGFR signaling by preventing *vn* expression. This is reminiscent of the functionally antagonistic interaction between Wg and EGFR signaling in the ventral epidermis of the embryo (O'Keefe et al., 1997; Szuts et al., 1997). However, the relationship between the Wg and EGFR pathways in dorsal head development is not reciprocal, since ectopic DN-DER expression across the dorsal head primordium does not affect *wg* expression (data not shown).

## Future directions

In order to understand the respective roles of the Hh and EGFR pathways in head development, it will be important to determine whether the phenotypes that we have described are caused by alterations in cell fate specification, cell proliferation or cell death. EGFR expression, for example, is required for cell proliferation during eye development (Xu and Rubin, 1993) and expression of the Vn ligand is necessary for early proliferation of the wing disc (Simcox et al., 1996). By using the temperature-sensitive DER allele $flb^{IF26}$(Clifford and Schupbach, 1992; Raz and Shilo, 1992), and monitoring cell proliferation and cell death, it will be possible to determine the function of EGFR signaling during different windows of head development.

A second question is whether the control of EGFR signaling by Hh occurs elsewhere during development. In this regard, it will be important to determine whether *vn* expression in the *Drosophila* wing disc or embryonic ventral epidermis is induced by the Hh pathway, and more specifically by Ci. It is also possible that, in specific tissues, Hh controls the localized processing of the activating DER ligand Spitz or the expression of the negatively acting ligand Argos (Freeman et al., 1992; Schweitzer et al., 1995; Golembo et al., 1996a). Finally, it will be critical to determine whether a similar link between these two important signaling pathways occurs during vertebrate development.

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