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Role of the EGF receptor pathway in growth and patterning of the *Drosophila* wing through the regulation of *vestigial*

Raghavendra Nagaraj[1], Amanda T. Pickup[1], Robert Howes[2], Kevin Moses[3], Matthew Freeman[2] and Utpal Banerjee[1,*]

[1]Department of Molecular, Cell and Developmental Biology, Department of Biological Chemistry and Molecular Biology Institute, 405 Hilgard Ave, UCLA, Los Angeles, CA 90095, USA
[2]MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK
[3]Department of Cell Biology, Emory University School of Medicine, 1648 Pierce Drive, Atlanta, GA 30322, USA
*Author for correspondence (e-mail: banerjee@mbi.ucla.edu)

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SUMMARY

Growth and patterning of the *Drosophila* wing disc depends on the coordinated expression of the key regulatory gene *vestigial* both in the Dorsal-Ventral (D/V) boundary cells and in the wing pouch. We propose that a short-range signal originating from the core of the D/V boundary cells is responsible for activating EGFR in a zone of organizing cells on the edges of the D/V boundary. Using loss-of-function mutations and ectopic expression studies, we show that EGFR signaling is essential for *vestigial* transcription in these cells and for making them competent to undergo subsequent *vestigial*-mediated proliferation within the wing pouch.

Key words: *Drosophila*, Wing, D/V boundary, *EGFR* signaling, *Notch* signaling, *spitz*, *vestigial*

INTRODUCTION

The *Drosophila* wing develops as an outgrowth of the pouch region of the larval wing imaginal disc (Fig. 1A). The pouch is compartmentalized by Anterior-Posterior (A/P) and Dorso-Ventral (D/V) boundaries (reviewed by Blair, 1995; Cohen, 1996). The D/V boundary functions as an organizer that is set up in the late second instar larval stage. Localized activation of the Notch receptor along this boundary leads to transcriptional regulation of target genes, which play crucial roles in the subsequent growth and patterning of the wing disc (Jack et al., 1991; Rulifson and Blair, 1995; Kim et al., 1995; Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996).

At the second instar larval stage the dorsal compartment of the wing pouch is distinguished by its expression of Apterous, a LIM domain-containing transcription factor (Cohen et al., 1992). It is unclear how Apterous expression is restricted to this compartment of the disc, but the function of this protein is crucial for imparting dorsal identity to cells in which it is expressed (Diaz-Benjumea and Cohen, 1993). Apterous up-regulates the expression of several genes important for the proper patterning of the disc including Serrate, a ligand for Notch (Fleming et al., 1990; Thomas et al., 1991) and Fringe, a molecule that influences the response of cells to Notch signaling in a ligand-dependent manner (Panin et al., 1997). Serrate functions to activate Notch signaling in cells in the ventral compartment abutting the D/V boundary. Delta, another ligand for Notch, is expressed at high levels in the ventral compartment and functions to activate Notch signaling in cells of the dorsal compartment (Doherty et al., 1996). The combined action of Delta and Serrate results in symmetrical activation of Notch at the D/V boundary. This step is a prerequisite for these cells to adopt an organizer function, which is important for the global patterning of the pouch.

Notch signaling in cells of the D/V boundary results in the transcriptional activation of several genes including *vestigial (vg), wingless (wg), cut (ct)* and members of the *Enhancer of split* complex, *E(Spl)* (Kim et al., 1995; Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; de Celis et al., 1996). *vg* encodes a nuclear protein which functions as an important regulator of growth and patterning of the wing pouch cells (Williams et al., 1991). In *vg* mutants, cells of the wing pouch fail to proliferate and ectopic expression of Vg in other imaginal discs causes them to adopt wing pouch identity (Kim et al., 1996). Expression of Vg along the D/V boundary is mediated directly by the Notch signal and is a result of the activation of a specific enhancer element called the boundary enhancer (BE) (Williams et al., 1994). This enhancer contains a binding site for Suppressor of Hairless, Su(H), which functions as a transcription factor downstream of Notch (Kim et al., 1996). Away from the boundaries, Vg expression in the pouch is controlled non-autonomously by the TGF-β homolog Dpp, and utilizes another distinct *vg* enhancer named the quadrant enhancer, QE (Kim et al., 1997). Dpp is expressed along the A/P axis and functions as a morphogen that diffuses across the wing pouch activating downstream genes (Nellen et al., 1996; Lecuit et al., 1996).

The cells of the D/V boundary function as an organizer and control the growth and proliferation of the wing pouch cells away from the boundary in a non-autonomous manner. Kim et al. (1996) have proposed that in addition to the Dpp signal initiated along the A/P axis, a second diffusible morphogen made at the D/V boundary is necessary to activate Vg expression in the pouch. One secreted molecule expressed along the D/V boundary is Wg, which is required for neuronal differentiation along the D/V boundary and also for the expression of Vg in the pouch cells (Zecca et al., 1996; Neumann and Cohen, 1997). Thus, Wg is a candidate for the D/V boundary signal (Kim et al., 1996). Wg function is also essential for the development of sensory bristles along the margin and for sharpening its own expression along the D/V boundary through its interaction with the Notch pathway (Phillips and Whittle, 1993; Rulifson et al., 1996).

The Epidermal Growth Factor Receptor (EGFR) signaling pathway functions in many stages of *Drosophila* development (reviewed in Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997). During embryogenesis, the patterning of the ventral epidermis of the embryo requires a graded activation of EGFR signaling (Raz and Shilo, 1993; Schweitzer et al., 1995). Spitz, a ligand for EGFR, is processed in the midline cells of the embryo and diffuses to the cells of the ventral epidermis and activates the EGFR pathway, which imparts differential fates to these cells (Golembo et al., 1996). During imaginal disc development, hypomorphic allelic combinations of *EGFR* were shown to cause patterning defects in eye and wing imaginal discs (Clifford and Schupbach, 1989; Diaz-Benjumea and Garcia-Bellido, 1990). In eye discs, clones of loss-of-function mutations in EGFR result in several defects including reduced cell proliferation and increased cell death (Xu and Rubin, 1993; Dominguez et al., 1998). Additionally, the EGFR pathway also functions in cell fate specification of neuronal as well as non-neuronal cells in the eye imaginal disc (Freeman, 1996; Tio and Moses, 1997). In the wing disc, loss of EGFR function in early mitotic clones and also in certain heteroallelic combinations of components of the EGFR pathway shows lack of cell proliferation (Diaz-Benjumea and Garcia-Bellido, 1990; Simcox, 1997). However, loss-of-function clones of EGFR generated during later (early pupal) stages of wing development do survive and show defects in wing vein specification (Sturtevant and Bier, 1995; Diaz-Benjumea and Hafen, 1994). Thus, EGFR function is required both for cell proliferation and cell fate specification in the eye as well as in the pupal wing disc.

In this paper we focus on the role of the EGFR pathway in the specification of the D/V boundary during the development of the wing. Our results suggest that EGFR signaling is critical for the expression of Vg in the pouch region. We propose a model in which EGFR function along the D/V boundary specifies the fate of adjacent cells that become competent to express Vg and proliferate to populate the pouch.

MATERIALS AND METHODS

Drosophila stocks

S^{X104E}, $pnt^{\Delta 88}$, vg^{1} fly stocks were obtained from the Bloomington *Drosophila* stock center. vg^{83b27}, *vg-BE-lacZ*, *vg-QE-lacZ* stocks were obtained from S. B. Carroll (Williams et al., 1991, 1994; Kim et al., 1996), UAS-*sspi*, from B.-Z. Shilo (Schweitzer et al., 1995), pnt^{1230} from C. Klambt, *dpp*-Gal4 from M. Hoffmann and *A9*-Gal4 flies were obtained from M. O'Connor.

Heat pulse protocol

For temperature-shift experiments involving $Notch^{ts}$ and $EGFR^{ts}/top^{1}$, late second and early third instar larvae were collected and heat pulsed at 30°C for 36 hours. Wing discs were dissected and stained with X-gal or α-Vg antibody.

Immunocytochemistry

Wing discs were dissected in PBS and fixed with 4% formaldehyde in PBS for 20 minutes at room temperature. The discs were then permeabilized in PBST (PBS + 0.1% Triton X-100), incubated in primary antibody (α-Vg, diluted 1:200, α-Wg, 1:10 and α-β-gal, 1:50) overnight, washed in PBST five times and incubated with appropriate secondary antibody for 3 hours at room temperature. The color reaction used DAB with 0.2% nickel chloride. Discs were mounted in 100% glycerol.

Spitz clones

The following stocks were used to generate *spi* clones: *y w* $HSflp^{12}$*; stc* spi^{A14} *FRT40A/CyO* and *y w* $HSflp^{12}$*; M(2)24F FRT40A/CyO*.

spi^{A14} behaves as an amorphic allele. Clones were induced with a single heat shock pulse (38°C) of 1 hour at 24-48 hours after egg laying. Clones were marked with *stc*, a recessive trichome marker (Jiang and Struhl, 1996). The clone boundaries shown in Fig. 2G,H are approximate as individual cells cannot be unambiguously scored with *stc*. The spi^{+} tissue is M^{-}/M^{+} and thus has a growth disadvantage compared to the spi^{-}/M^{+} tissue, allowing recovery of large clones.

RESULTS

In the wing imaginal disc, Vg expression is seen uniformly throughout the wing pouch and in selected regions of the notum (Fig. 1B). The expression of *vg* in the pouch is regulated by the activity of two distinct enhancer elements within this locus (Kim et al., 1996). The second intron of *vg* contains the boundary enhancer (BE) element that activates *vg* along the D/V and A/P boundaries (Fig. 1C; Williams et al., 1994). The fourth intron of the *vg* gene harbors the quadrant enhancer (QE) that drives *vg* expression in four quadrants of the wing pouch (Kim et al., 1996) but not along the D/V boundary (Fig. 1D). The observed uniform expression of Vg (Fig. 1B) thus results from a combination of these two expression patterns (Fig. 1C,D). In a temperature-sensitive mutant of *Notch*, $Notch^{ts}$, raised at the non-permissive temperature during wing development, the expression of Vg is down-regulated along the boundaries as well as in the pouch (Fig. 1E). Therefore, activation of Notch along the D/V boundary is not only essential for Vg expression at this boundary, but is also important for its expression in the pouch. In fact, Kim et al. (1996) have demonstrated that in the vg^{83b27} mutant, where the BE element is mutated, Vg expression is not only lost along the D/V boundary but also non-cell-autonomously in the pouch (Kim et al., 1996). In Fig. 1F, we show that QE reporter expression is eliminated in the vg^{83b27} mutant, suggesting that a functional BE element is essential for QE expression. This implies that the establishment of D/V boundary identity is essential for the expression of Vg in the pouch region.

EGFR activity promotes growth and expression of Vg in the pouch

Previous studies have suggested that EGFR function is required for the proliferation of wing disc cells (Clifford and

Schupbach, 1989; Diaz-Benjumea and Garcia-Bellido, 1990). We wanted to determine if this Receptor Tyrosine Kinase (RTK) pathway also plays a role in the morphogenesis of the wing disc and if the ligand for the EGF receptor, Spitz (Spi) functions as a signaling molecule in the developing wing disc, as it does in the eye disc and the embryo (Golembo et al., 1996; Freeman, 1996; Tio and Moses, 1997). Third instar wing discs stained with an antibody directed against the N-terminal portion of the Spi protein (Tio and Moses, 1997) show a strong expression of Spi along the D/V boundary and weaker expression throughout the disc (Fig. 2A). This elevated protein level at the D/V boundary is likely to reflect post-transcriptional control, since work from several laboratories has shown that *spi* RNA is expressed ubiquitously at low levels throughout the wing disc (Sturtevant et al., 1993; Simcox et al., 1996; Schweitzer and Shilo, 1997). Also, a *spitz* enhancer trap (Fig. 2B) shows uniform expression throughout the wing disc. Unfortunately, analysis of the regulation of Spi by the Notch pathway will have to await the generation of a new antibody since the mouse polyclonal antibody against Spi is now exhausted. In this paper, we have focused instead on the events downstream of EGFR activation.

The growth-promoting activity of the Notch pathway has been demonstrated by ectopically activating Notch along the A/P boundary (Diaz-Benjumea and Cohen, 1995; Kim et al., 1995; Couso et al., 1995). To determine if this is mediated by EGFR, we used the *dpp*-Gal4 driver to ectopically express an activated, secreted form of Spi (sSpi; Schweitzer et al., 1995) that would activate EGFR along this boundary. This results in an extensive outgrowth of the wing pouch (Fig. 2C). When these discs are stained with an α-Vg antibody, the overproliferating cells are found to express Vg (Fig. 2D). These results can either imply that Vg expression is activated by the EGFR pathway leading to cell proliferation or that EGFR activation results in random proliferation of cells within the pouch, which then secondarily express the Vg protein. To distinguish between these possibilities, we utilized the vg^1 mutant allele in which Vg expression is reduced but not eliminated. In *dpp*-Gal4/UAS-*sspi*; vg^1/vg^1 flies there is no expansion of the wing pouch (compare Fig. 2E with C). We conclude that activation of EGFR leads to expression of Vg, which functions downstream of or parallel to the EGFR pathway for the proper proliferation of cells in the pouch.

Attempts to generate early mutant clones of EGFR in the wing pouch have not been successful (Diaz-Benjumea and Garcia-Bellido, 1990). Previous studies of wing development have described a later role of the EGFR pathway and its ligand Vein in the proper patterning of the wing veins (Sturtevant and Bier, 1995; Schnepp et al., 1997). To address the early role of EGFR in wing patterning, we used four alternative strategies involving loss-of-function mutations in components of the EGFR pathway.

First, a temperature-sensitive allele of the *EGFR* gene ($EGFR^{ts}$) was used to inactivate the pathway. The heteroallelic combination $EGFR^{ts}/EGFR^{top1}$ gives rise to a null phenotype at the non-permissive temperature (Kumar et al., 1998) and shows decreased Vg expression in the wing pouch (compare Figs 1B, 2F). The expression of Vg in the folds outside the pouch region is unaffected and is therefore not responsive to EGFR signaling. In these discs, the notum is significantly reduced in size, consistent with the fact that the alternative EGFR ligand, Vein, is expressed in the third instar notum (Simcox et al., 1996). When stained with an antibody against Wg, these discs show normal Wg expression along the D/V boundary (not shown). Thus, the small wing pouch phenotype and loss of Vg expression that result from loss of EGFR signaling are not indirectly caused by an elimination of the D/V boundary.

As a second approach, we ectopically expressed a dominant negative version of EGFR ($EGFR^{DN}$; Freeman, 1996) using the

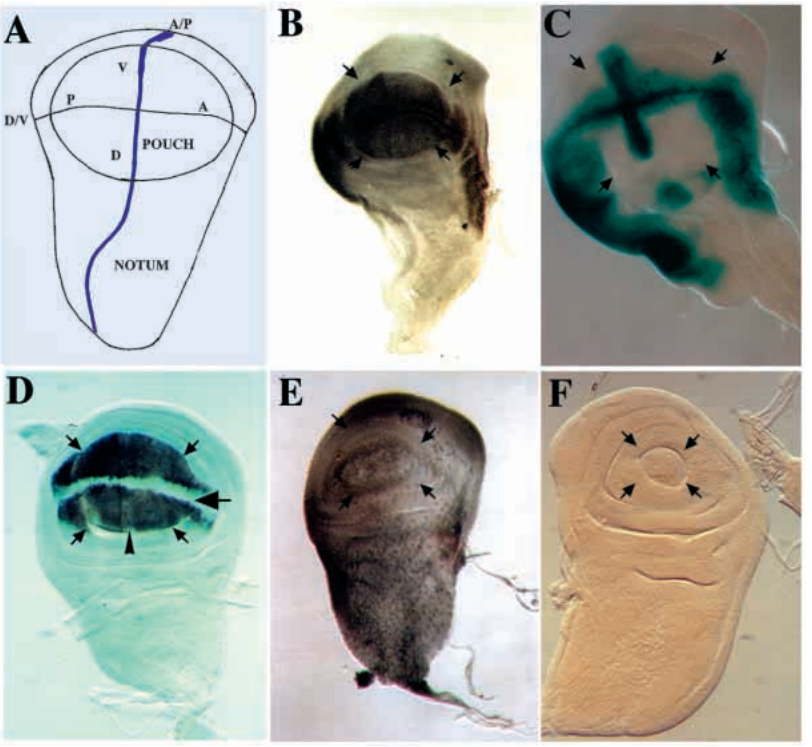

Fig. 1. Regulation of *vg* along the D/V boundary and in the wing pouch. Arrows demarcate boundaries of the pouch. (A) A third larval instar wing disc is shown schematically. Compartment boundaries, wing pouch and notum regions are indicated. A/P, Anterior-Posterior compartment boundary; D/V, Dorsal-Ventral compartment boundary. (B) A wild-type wing disc stained with α-Vg antibody shows that Vg protein is expressed throughout the pouch and in some areas of the notum. (C) The *vestigial* boundary enhancer reporter construct, BE-*lacZ*, is expressed along the D/V and A/P boundaries of the pouch. Additional staining for *lacZ* activity is seen along the edges of the disc and in the notum. (D) The *vestigial* quadrant enhancer reporter construct, QE-*lacZ*, is expressed in the four quadrants of the pouch, but is repressed strongly along the D/V boundary (large arrow) and weakly along the A/P axis (arrowhead). (E) $Notch^{ts}/Notch^{55E11}$ disc stained with α-Vg antibody shows a reduction in Vg expression along the D/V boundary and also in the pouch compared with B. This leads to a reduction in the pouch size. $Notch^{55E11}$ is a null allele for the *Notch* locus. (F) vg^{83b27}/vg^{83b27}, QE-*lacZ* disc stained for β-galactosidase activity. No staining is evident, suggesting that QE expression is eliminated in this genetic background. vg^{83b27} is a *vg* mutant allele that only affects the boundary enhancer.

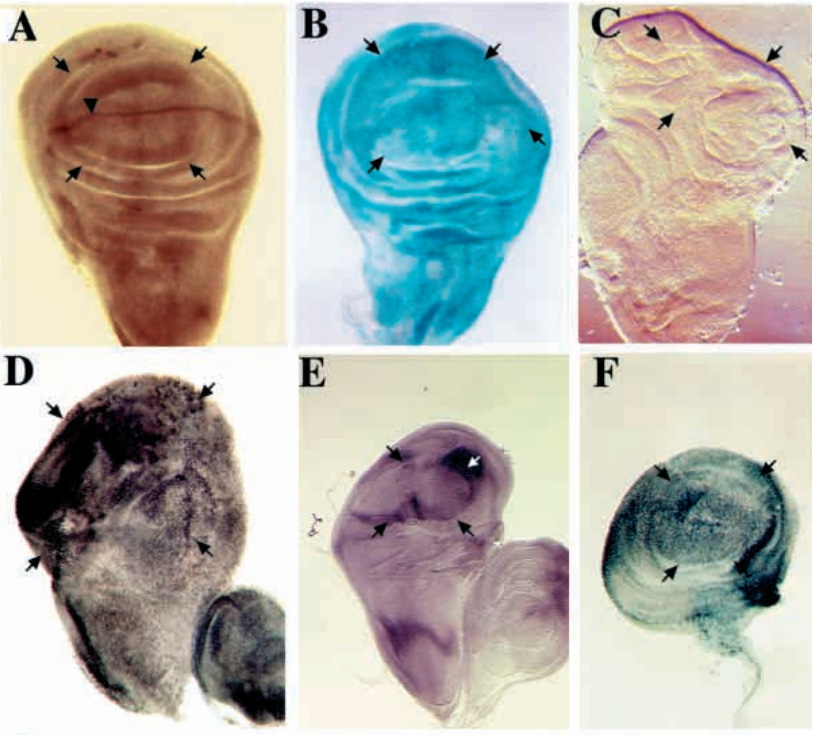

Fig. 2. Regulation of wing pouch growth and *vg* expression by the EGFR pathway. Arrows mark boundaries of the wing pouch. (A) A wild-type wing disc stained with α-Spi antibody shows strong staining in 2-3 rows of cells along the D/V boundary (arrowhead) and weaker staining throughout the disc. (B) A wing disc from the *spi* enhancer trap, spi^{SCP1}, stained for β-galactosidase activity shows ubiquitous *spi* transcription throughout the wing disc. (C) *dpp*-Gal4; UAS-*sspi*. Activation of EGFR along the A/P boundary causes the wing pouch to be expanded to a size larger than in wild type. (D) *dpp*-Gal4; UAS-*sspi* wing disc stained with α-Vg antibody. The same overgrowth as in C is evident and the proliferating cells are found to express Vg. (E) *dpp*-Gal4; UAS-*sspi*, vg^1/vg^1 wing disc stained with α-Wg antibody. Partial loss of *vg* function suppresses sSpi-mediated ectopic growth of the wing pouch seen in C. (F) $EGFR^{ts}/EGFR^{top1}$ wing disc stained with α-Vg antibody. Vg expression is significantly reduced in the pouch cells (compare with Fig. 1B).

UAS/Gal4 system (Brand and Perrimon, 1993). Beginning in early third instar stages, the *A9*-Gal4 element causes expression of a reporter gene mostly in the dorsal compartment of the wing pouch and at lower levels in the ventral compartment (Fig. 3A). *A9*-Gal4; UAS-$EGFR^{DN}$ wing discs show a dramatic reduction in the dorsal compartment of the wing pouch (Fig. 3B). This is not a secondary consequence of a perturbation in D/V boundary specification since the expression pattern of Wg along the D/V boundary is maintained. Rather this effect is mediated through the control of *vg*, since the expression of BE (compare Fig. 3C with D) and QE enhancers (compare Fig. 3E with F) are dramatically reduced.

As a third approach to attenuate EGFR signaling during development we used a hypomorphic allele of *pointed* (*pnt*), which encodes an ETS domain transcription factor that functions as a downstream member of the EGFR pathway (O'Neill et al., 1994; Klambt, 1993). pnt^{1230}/pnt^{1230} wing discs show reduced pouch size compared with wild type (Fig. 3G), again supporting the conclusion from previous experiments that the EGFR pathway is necessary for the growth of the wing pouch.

Finally, a loss of function in genes belonging to the *EGFR* pathway interact genetically with *vg*. A single copy loss of *vg* (Fig. 4A), or any of the components of the *EGFR* pathway (not shown), has no phenotypic effect on the adult wing, but a fly lacking a single copy of *vg* and one copy of either *spi* (Fig. 4B), *Star* (Fig. 4C) or *pnt* (Fig. 4D) shows notching at the distal edge of its wing margin. Thus, mutations in several different genes belonging to the *EGFR* pathway show dosage-sensitive interactions with *vg*. The interaction between *spi* and *vg* is not allele-specific since two independent alleles of *spi*, spi^{SC1} and spi^{SC2}, both of which are considered nulls (Tio and Moses, 1994), interact with vg^{CX1} as well as with two deletions for the *vg* locus. The mutant phenotype observed in these trans-heterozygous flies is similar to that seen for *Notch* mutants (Fig. 4E), suggesting a functional relationship between the EGFR and Notch pathways in the patterning of the wing. Consistent with this idea, the wing margin phenotype of *Notch* can be dominantly suppressed by a gain-of-function mutation in *Dsor1* (*MEK*) (Fig. 4F), which functions downstream of *EGFR* (Tsuda et al., 1993).

While the expression pattern of Spi in the wing disc (Fig. 2A) and the dosage-dependent interaction between *vg* and *spi* (Fig. 4B) suggest that Spi is the ligand that activates EGFR, clones of null alleles of *spi* showed no phenotype in the adult wing. Two different alleles of *spi* were used in these experiments and large clones crossing the D/V boundary were generated using the Minute technique (Fig. 4G,H). This result contrasts with the lack-of-proliferation phenotype seen in clones of EGFR (Diaz-Benjumea and Garcia-Bellido, 1990) and also with the loss-of-function result of Fig. 4B. Possible explanations for the differences in results obtained from these experiments are discussed later.

EGFR signaling activates the boundary enhancer and represses the quadrant enhancer of *vg*

In Fig. 2, we have shown that activation of EGFR pathway can promote the expression of Vg. However, it was not clear from these experiments which of the two enhancers of *vg* is involved in this regulation. Therefore, the expression pattern from each of the two enhancers was determined in a *dpp*-Gal4;UAS-*sspi* background. We found that when expressed along the A/P boundary, sSpi causes a complete loss of QE expression in the wing pouch tissue, even though these cells express high levels of Vg protein (compare Figs 5A, 2D). In contrast, the expression of BE is expanded from the boundary to the rest of the wing pouch (Fig. 5B). In the pupal stages, EGFR signaling has been

shown to function in patterning veins. We monitored the expression of the BE-*lacZ* and QE-*lacZ* reporter lines in pupal wing discs and found that BE is activated in vein primordia (Fig. 5G) while QE is seen in intervein regions (Fig. 5H).

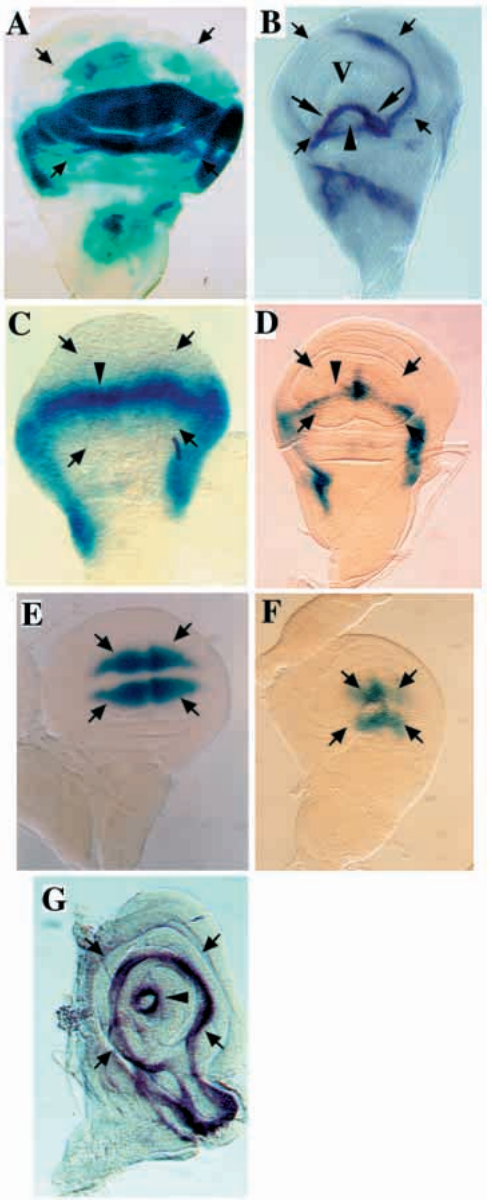

Fig. 3. Further evidence of regulation of wing pouch growth and *vg* expression by the EGFR pathway. (A) *A9*-Gal4; UAS-*lacZ* stained for β-galactosidase activity. Strong expression is evident in the dorsal compartment of the pouch. (B) *A9*-Gal4, UAS-*EGFR*^{DN} wing disc stained with an α-Wg antibody. The dorsal compartment (arrowhead) of the pouch is significantly smaller than the ventral compartment (V). Wg is still expressed along the D/V boundary (long arrows). (C) Wild-type wing disc showing the expression of the BE-*lacZ* expression along the D/V boundary (arrowhead). (D) BE-*lac*Z expression in *A9*-Gal4, UAS-*EGFR*^{DN}. Note the reduction in the expression along the D/V boundary (arrowhead). (E) Wild-type expression of QE-*lacZ* in third instar wing disc. (F) Expression of QE in *A9*-Gal4, UAS-*EGFR*^{DN} wing disc is reduced as compared to wild type (compare E with F). (G) *pnt*^{1230}/*pnt*^{1230} wing disc stained with α-Wg antibody. The pouch is reduced in size and the D/V boundary expression is present, but distorted (arrowhead).

If the Notch pathway were responsible for this EGFR activity, one would predict that activation of Notch should give a phenotype similar to that seen with sSpi. This was indeed found to be the case. When Dl, a ligand for Notch, is misexpressed along the A/P boundary, it causes activation of Notch primarily in the dorsal compartment (Doherty et al., 1996), resulting in proliferation of cells in this compartment. We found that misexpression of Dl along the A/P boundary results in the expression of BE throughout the dorsal region of the pouch (Fig. 5C), while QE expression is reduced in the dorsal compartment (Fig. 5D). Similar results were also obtained in the ventral compartment using the other Notch ligand, Serrate (Fig. 5E). As a loss of function complement to the above experiment, when *Notch*^{ts} larvae are maintained at the non-permissive temperature, QE expression is greatly reduced in all of the pouch regions but is also no longer repressed along the D/V boundary (Fig. 5F). Thus activation of either the Notch or EGFR pathway will result in a positive regulation of the boundary enhancer element and a negative regulation of the quadrant enhancer element of the *vg* gene in the same cell.

Distinct functions of EGFR and Wingless pathways

The above studies show that the EGFR pathway can activate expression of Vg and is required for the proliferation of cells in the pouch. Another pathway that plays an important role in wing development and functions along the D/V boundary involves Wg (Fig. 6A). In certain experiments, the function of these two pathways seem similar. For example, loss of either EGFR (Fig. 2F) or Wg signaling (Blair, 1994; Zecca et al., 1996; Neumann and Cohen, 1997) causes loss of some Vg expression and a reduced pouch size. However, other experiments highlight the differences in their functions. In sharp contrast to the results presented in Fig. 2, when Wg is misexpressed along the A/P boundary, there is no ectopic growth of the pouch (Fig. 6B). Instead, Wg expression along the A/P boundary can result in a duplication of the pouch due to a change in the fate of the notum to that of the wing pouch (Fig. 6C). When expressed along the A/P boundary, sSpi eliminates QE expression (Fig. 4A), but no such effect was evident when Wg was misexpressed along the A/P boundary (Fig. 6D). An alternative way to activate the *wg* pathway is to use a truncated and constitutively activated form of the downstream effector molecule Armadillo (Pai et al., 1997). When this protein is expressed using either the *dpp*-Gal4 (along the A/P boundary) or the highly expressing*T80*-Gal4 (ubiquitous) driver line, it causes the conversion of notum to pouch tissue, but no ectopic growth in the pouch or change in QE expression is seen (Fig. 6E,F). With the T80 driver line, the growth of the notum is very striking, but even under these extreme conditions, no ectopic growth of the pouch is evident (Fig. 6F). Thus, unlike activation of Notch and EGFR, activation of the Wg pathway using similar experimental paradigms does not induce non-cell-autonomous proliferation in the wing pouch cells. Our experiments have not ruled out a possible synergistic interaction between Wg and EGFR pathway in the control of Vg expression, but unlike EGFR activation, Wg on its own is not able to induce high enough levels of Vg expression to cause cell proliferation. Wg does, however, have several important functions in the patterning of the wing. These include distinguishing the identity of the

pouch cells from those of the notum, specifying the bristles along the anterior margin and refining the D/V boundary (Phillips and Whittle, 1993; Couso et al., 1994; Ng et al., 1996; Rulifson et al., 1996; Micchelli et al., 1997).

A determinative function of EGFR at the D/V boundary

The location of a cell with respect to the D/V boundary dictates whether BE or QE expression will be activated in that cell. In Fig. 7A, we have schematically represented the developing wing disc, dividing the pouch into three regions. The first is a narrow band of cells at the core of the D/V boundary that express Vg under the direct control of the Notch pathway (Fig. 1C). Flanking the boundary cells in the dorsal and ventral compartment is a strip of cells that stain strongly with an antibody that recognizes the activated form of MAP kinase (Fig. 7B). We have designated this region as the competence zone (CZ; Fig. 7A). This activation of MAPK in the CZ region can be seen in mid-third instar discs, prior to MAPK activation in presumptive vein cells (Gabay et al., 1997). The activation of MAPK in the CZ cells is Notch-dependent, since it is eliminated in a *Notch*^{ts} background at the non-permissive temperature (Fig. 7C). Also, activation of the Notch pathway along the A/P axis causes ectopic expression of activated MAPK. Thus Notch function in the D/V boundary is important for MAPK activation in the CZ cells. Taken together, results presented in this paper suggest that the restricted pattern of activated MAPK staining in the CZ region is a result of the activation of EGFR.

To provide further evidence for a specialized requirement of the EGFR pathway in the cells flanking the D/V boundary, we used a BE-Gal4 driver line to express EGFR^{DN} in the boundary region including the presumptive CZ cells (Fig. 7E), but not in the rest of the pouch. We monitored the expression of BE-*lacZ* and QE-*lacZ* expression in this background after pulsing the larvae at 29°C for 36 hours, and our results suggest that the expression of not only the BE enhancer, whose expression pattern corresponds to the expression pattern of EGFR^{DN}, but also the expression of QE in rest of the pouch, is eliminated (Fig. 7F,G). The wing phenotype of the BE-Gal4/UAS-*EGFR*^{DN} adult flies is shown in Fig. 7I,J. A significant reduction in wing size and some notching of the distal margin can be seen, but the veins and marginal bristles form normally (Fig. 7I). When grown at 29°C this phenotype is enhanced and the adult wing is exceedingly small (Fig. 7J). Once again the marginal bristles are Wg-dependent and develop normally. This result further substantiates our hypothesis that a localized loss of EGFR function in the boundary area has a profound non-autonomous effect on the size of the entire pouch.

DISCUSSION

Loss of function clones of EGFR fail to proliferate, suggesting that its function is critical in the growth of the wing pouch (Diaz-Benjumea and Garcia-Bellido, 1990). In this paper we have determined that EGFR signal functions in the growth as well as the patterning of the wing pouch cells. Using a temperature-sensitive allele of EGFR, we have shown that loss

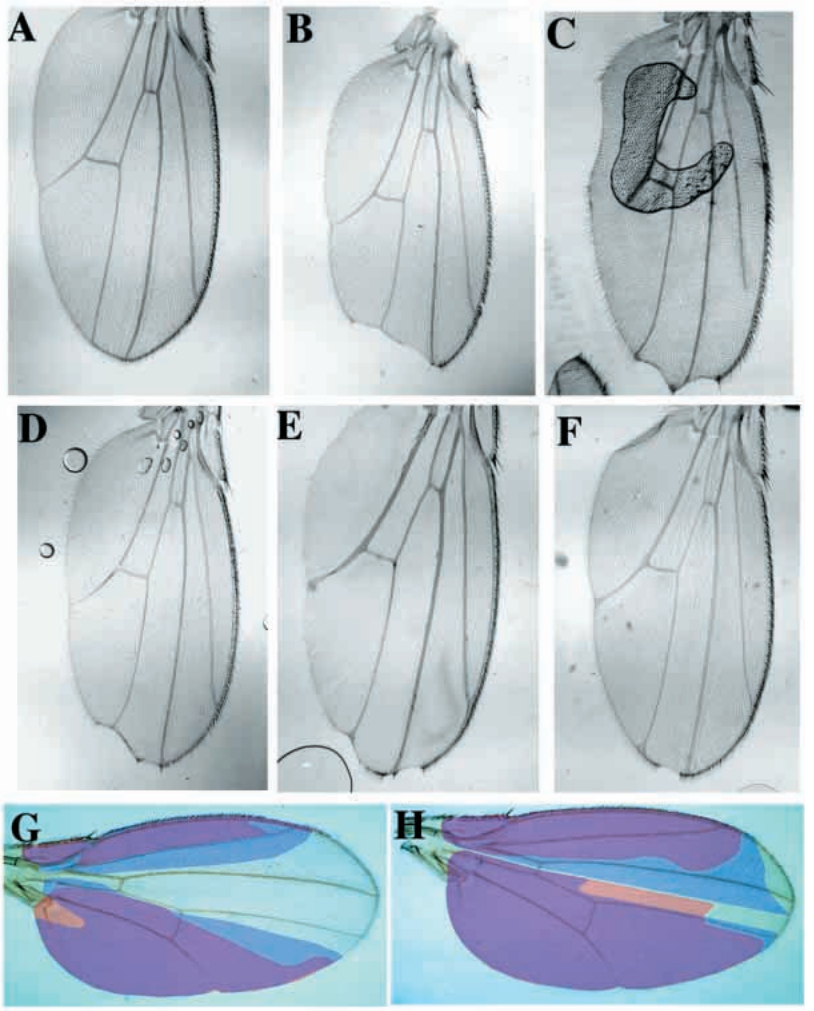

Fig. 4. Genetic interaction of *vg* with members of the *EGFR* pathway and *spi* clones in the wing. (A) *vg*^{CX1}/+. The adult wing is wild type in appearance. The same is true for *spi*^{SC1}/+ or *S*^{X104E}/+ or *pnt*$^{\Delta 88}$/+ wings (not shown). The *vg* allele, *vg*^{CX1}, is synonymous with Df(2R)CX1, which is a deletion for the *vg* locus. *spi*^{SC1}, *S*^{X104E} and *pnt*$^{\Delta 88}$ are genetic nulls for their respective loci. (B) *vg*^{CX1},+/+,*spi*^{SC1}. Notching of the wing margin indicates that *spitz* shows a dosage-sensitive interaction with *vestigial*. (C) *vg*^{CX1},+/+,*S*^{X104E}. Distal margins are notched. (D) *vg*^{CX1}/+; *pnt*$^{\Delta 88}$/+. These wings show notching of the margins although the *vg* and *pnt* heterozygotes on their own are wild type. (E) *Notch*^{55E11}/+ flies show a mild dominant notching of their distal wing margin. (F) *Notch*^{55E11},+/+,*Dsor1*^{Su1}. The gain-of-function allele of *Dsor1* (*MEK*) suppresses the *Notch* wing margin defect. (G,H) Two examples of large *spi* null clones scored in the adult wing. The dorsal aspect of the clone is colored blue and the ventral aspect light red. Overlap of the clone regions on the two surfaces of the wing is shown as purple in these photographs. The clones were generated early since they cross the D/V boundary. The large size of the clones results from the use of the Minute technique (see Materials and methods). The clones show no obvious phenotypes.

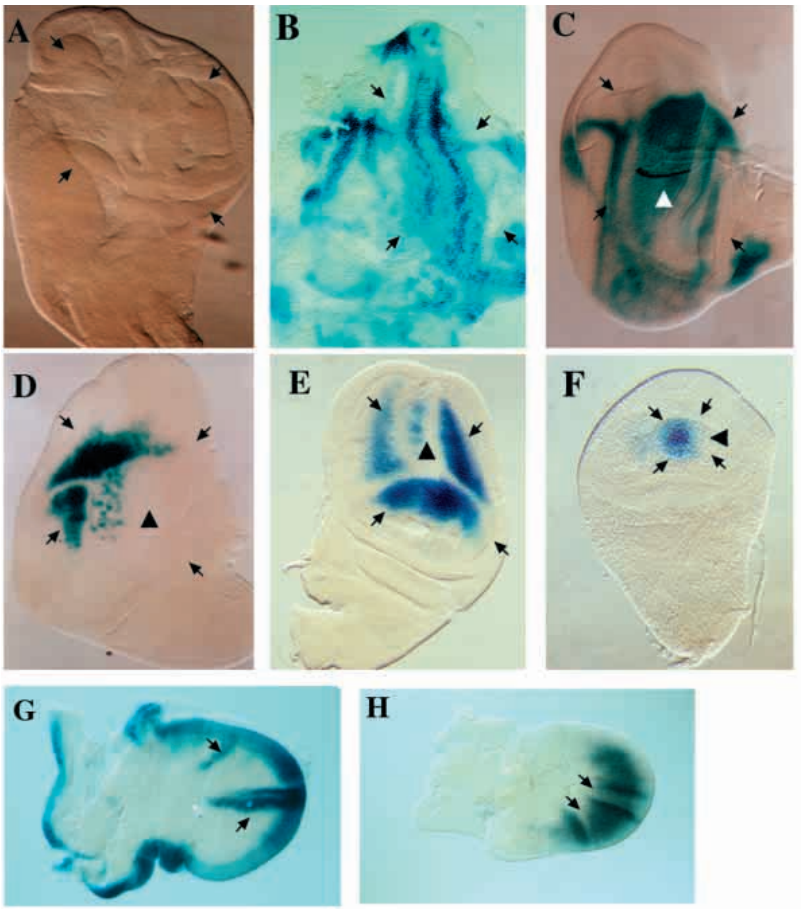

Fig. 5. Control of *vg* boundary enhancer (BE) and quadrant enhancer (QE) expression by the EGFR and Notch pathways. All wing discs have been stained for β-galactosidase activity. Arrows mark the boundaries of the wing pouch. (A) *dpp*-Gal4; UAS-*sspi*/QE-*lac*Z. Discs in which sSpi is misexpressed along the A/P axis show no staining for β-galactosidase activity, indicating a complete repression of QE expression even though the pouch is expanded and expresses high levels of Vg protein (Fig. 2D). (B) *dpp*-Gal4; UAS-*sspi*/BE-*lac*Z. Misexpression of sSpi along the A/P boundary causes an expansion of BE expression from the boundaries to the entire wing pouch. (C) *dpp*-Gal4; UAS-*Dl*/BE-*lac*Z. Misexpression of Dl leads to a non-cell autonomous activation of BE expression within the dorsal compartment of the pouch (arrowhead). (D) *dpp*-Gal4; UAS-*Dl*/QE-*lac*Z. The same Dl misexpression as in C causes a non-cell-autonomous suppression of QE-*lac*Z expression in parts of the dorsal compartment (arrowhead). (E) *dpp*-Gal4; UAS-*Ser*/QE-*lac*Z. Overexpression of Ser leads to a reduction of QE-lacZ expression in the ventral compartment of the wing pouch (arrowhead). (F) *Notch*^{ts}/Y; QE-*lac*Z. Loss of Notch function in discs pulsed at the non-permissive temperature causes de-repression of QE-*lac*Z along the D/V boundary (arrowhead). (G) BE-*lac*Z stained for β-galactosidase activity in pupal wing disc. The expression due to BE is seen in the vein primordia (arrows). (H) Expression of QE-*lac*Z in pupal wing disc. The strong expression is observed in the intervein regions while the expression in the vein regions is low (arrows).

of EGFR function during larval development results in a reduction of the wing pouch and an attenuation of the expression of Vg in the pouch cells. This suggests that the growth-promoting function of EGFR in the pouch cells is mediated by regulation of Vg expression. Consistent with this notion is our observation that ectopic activation of EGFR results in ectopic expression of Vg and also that a loss of function mutation in *vg* completely suppresses the growth promoted by ectopic activation of EGFR.

Activation of the EGFR pathway in cells adjacent to the D/V boundary leads to the localized activation of MAPK in thin strips of cells flanking the D/V boundary. We call these regions of MAPK activation the competence zone (CZ; Fig. 7A). The activation of MAPK in this region is also dependent on a functional Notch signal at the D/V boundary. The fact that EGFR signaling is operative in this zone is also supported by the earlier finding that *argos* and *rhomboid* are also expressed in this region (Sturtevant and Bier, 1995; Gabay et al., 1997). Also consistent with our hypothesis that Notch signal is essential for the activation of the EGFR in the CZ region, it has been reported that loss of Notch results in the loss of *rho* expression along the D/V boundary even as the expression of *rho* in the vein regions is greatly expanded upon loss of the Notch signal (Sturtevant and Bier, 1995). Localized Rhomboid expression has been implicated in EGFR signaling (Golembo et al., 1996) and could therefore account for the localized induction of EGFR activation at the D/V boundary. Most importantly, our results show that a localized inactivation of the EGFR signal exclusively at the D/V boundary results in dramatic loss of Vg in the remainder of the pouch. Thus, localized activation of the Ras pathway in cells flanking the D/V boundary is important for the patterning of the entire pouch. Previous work has suggested that loss of Notch function at the D/V boundary has a non-cell-autonomous effect on the expression of Vg in the pouch and the proliferation of cells in rest of the pouch region. Our results suggest that this effect is mediated through the EGFR pathway. We hypothesize that high levels of EGFR signaling are required in these cells for providing them with competence to express Vg and therefore proliferate.

In addition to the loss-of-function results, ectopic activation of Notch and EGFR causes virtually identical phenotypes. In both cases proliferation of cells away from the actual region of the ectopic activation is seen. Also in both cases, proliferation is Vg-dependent, and involves positive regulation of the boundary enhancer and negative regulation of the quadrant enhancer element of the *vg* gene. The involvement of EGFR in this process is supported by the recent finding that an activated version of Ras causes extensive proliferation of cells in the pouch (Karim and Rubin, 1998).

Our results unambiguously show the involvement of EGFR in Vg control and growth. However, the question of the ligand involved in the activation of EGFR along the D/V boundary remains unresolved. The data on the role of Spitz in this process are contradictory. On the one hand, Simcox (1997) has shown that hypomorphic alleles of *spi* do not show growth defects in the pouch cells and our results show that clones of null alleles of *spi* do not have wing defects. On the other hand,

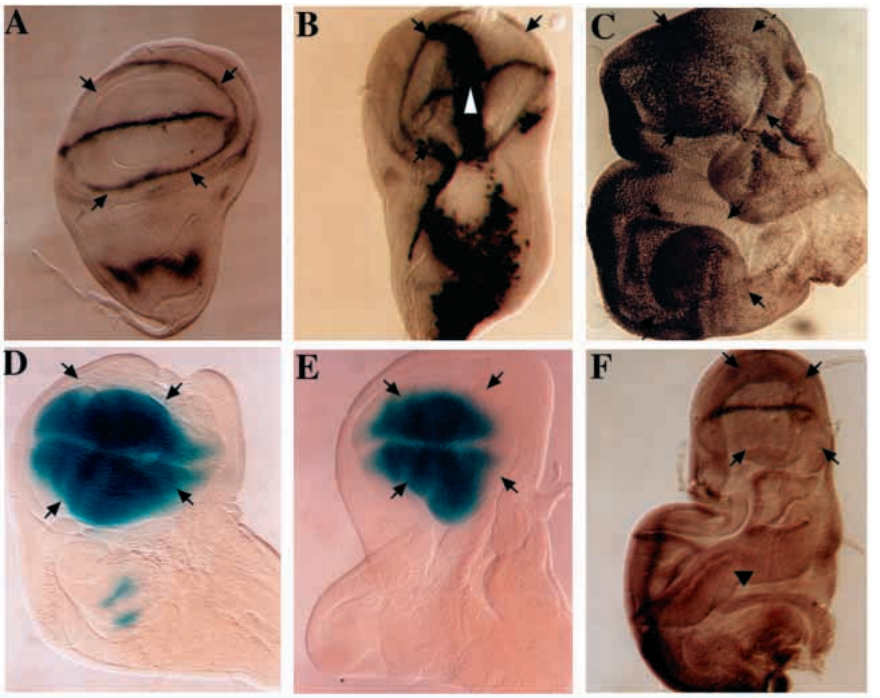

Fig. 6. Ectopic Wg does not alter growth of the wing pouch. Wing discs were stained with α-Wg antibody (A,B,F), with α-Vg antibody (C) or for β-galactosidase activity (D,E). Arrows mark the edges of the wing pouch. (A) Wild type. Wg protein is expressed in 2-3 rows of cells along the D/V boundary. Wg expression also marks the edges of the wing pouch. (B) *dpp*-Gal4; UAS-*wg*. In spite of strong ectopic Wg expression along the A/P boundary (arrowhead), no expansion of the pouch is evident. (C) *dpp*-Gal4; UAS-*wg*. In this disc, high levels of misexpression of Wg along the A/P boundary resulted in a duplicated pouch. (D) *dpp*-Gal4; UAS-*wg*/QE-*lacZ*. Overexpression of Wg along the A/P boundary does not affect QE expression. (E) UAS-$Arm^{\Delta N}$/*dpp*-Gal4; QE-*lacZ*. In this genetic background, $Arm^{\Delta N}$ causes constitutive activation of the Wg pathway along the A/P boundary. As in D, this causes no visible repression of QE expression. (F) *T80*-Gal4; UAS-$Arm^{\Delta N}$. Ubiquitous activation of the Wg pathway throughout the wing disc causes a dramatic expansion of the notum (arrowhead), but no additional growth of the wing pouch.

Spi is expressed at high levels at the D/V boundary, and we also observe strong interactions between *spi* and *vg* mutations. Similarly, *spi* clones did not show any vein defects, yet Sturtevant et al. (1993) have shown that *spi* interacts genetically with components involved in wing vein patterning. These differences are puzzling and perhaps reflect the complexity of EGFR activation in the tissue. Multiple ligands could be involved with strict redundancies. Alternatively, it is possible that a ligand such as Vein, which is expressed in the early third instar wing disc, functions with Spi and provides a longer range activation pattern. Further experiments are needed to resolve the ligand involved in the activation of EGFR in this system, but our results are consistent with the D/V boundary being a source of this signal.

Wg is also expressed along the D/V boundary (Couso et al., 1994; Diaz-Benjumea and Cohen, 1995) and loss-of-function mutations in *wg* or in components of the Wg pathway result in wing margin defects (Blair, 1994) Additionally, recent studies suggest that Wg can affect the expression of Vg in the pouch cells (Zecca et al., 1996; Neumann and Cohen, 1997). However, in contrast to EGFR activation, ectopic expression of Wg did not result in ectopic growth of the pouch cells and did not cause repression of the expression of the quadrant enhancer of *vg*. It is unlikely that on its own Wg is responsible for the proliferation of cells in the pouch. However, it is possible that EGFR and Wg pathways collaborate in making the cells in the pouch competent to proliferate. EGFR has a general proliferative role in the pouch, but our results show that the function of EGFR in the CZ cells is likely to be determinative for these cells and has a non-autonomous effect on the expansion of the rest of the pouch. Although they arise from cells within the CZ region, the marginal bristles develop normally when EGFR function is disrupted, suggesting that cell viablity and Wg function are not altered. We conclude that ligands for EGFR and Wg both function as short-range diffusible signals at the D/V boundary. The EGFR pathway acts as a specification signal for the cells that will enter multiple cell-division cycles and populate the pouch, just as the Wg signal will affect the fate of neighboring cells that will give rise to the bristles along the margin.

In CZ cells, *vg* expression is controlled by the boundary enhancer. Kim et al. (1996) have shown that mutation of the single Su(H) binding site on this enhancer results in the loss of BE expression along the D/V boundary. While this demonstrates a direct role of the Notch signal in the control of Vg, this result does not establish that the Su(H) function is sufficent for the activation of the BE enhancer at the D/V boundary. Cells of the D/V boundary receive and interpret a complex series of signals that help in its refinement. For example, it has been shown recently that the POU domain protein Nubbin sets a threshold for Notch-regulated gene activity (Neumann and Cohen, 1998). When Notch activity is eliminated, cells belonging to the boundary lose their identity and function as pouch cells, expressing Vg now through the quadrant enhancer.

Blocking EGFR signaling in the CZ region not only causes a loss of BE expression along the D/V boundary, but also a loss of QE expression in the rest of the pouch. QE expression is controlled directly by the Dpp pathway (Kim et al., 1997) and initiates at the intersection of the A/P and D/V boundaries during late 2nd instar larval stages, expanding with the pouch (Kim et al., 1996). We propose that the activation of the EGFR pathway in the CZ cells is a determinative step which allows CZ cell progeny to respond to Dpp and activate *vestigial*. In this model, the short-range diffusion of an EGFR ligand provides competence to a set of cells that eventually undergo several rounds of cell division and populate the pouch. The cells that result from these mitoses are responsive to the Dpp signal (Fig. 7K).

Repression of the QE element in the wild-type CZ region, later in the vein primordia, and also in experiments in which we ectopically activated the EGFR pathway, presumably results

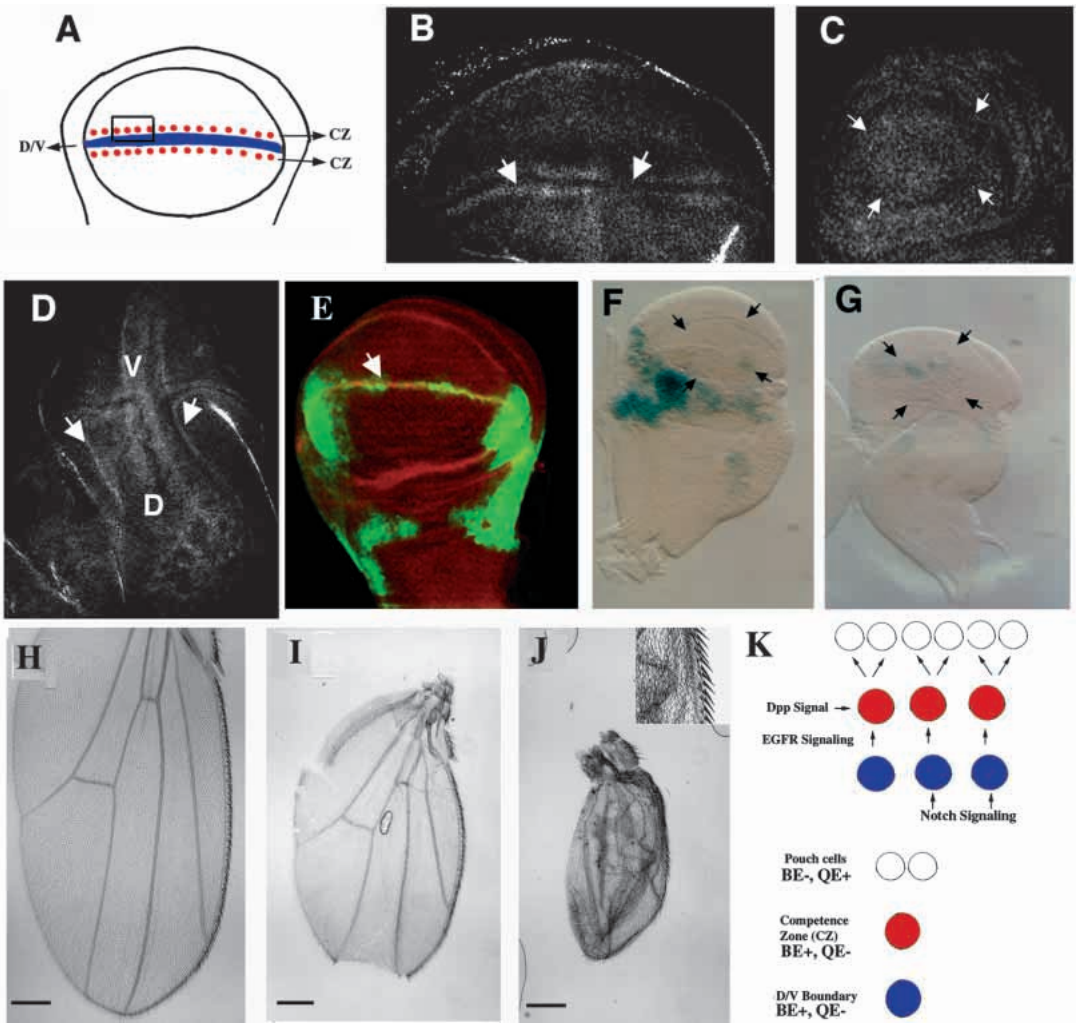

Fig. 7. A specialized requirement for EGFR signaling in the Competence Zone (CZ). (A) Schematic representation and definition of the CZ region. In the mid-third instar wing disc a strip 2-3 cells wide constitutes the core of the D/V boundary and is shown as a solid line. EGFR is activated in adjacent cells marked by red dots and designated the Competence Zone (CZ). (B-D) Mid-third instar wing discs stained with an antibody that recognizes the activated form of MAPK. (B) Wild type. Staining is observed on either side of the D/V boundary (marked by arrows). This indicates a higher level of MAPK activation in the CZ region than in the surrounding tissue. (C) N^{ts}/Y pulsed at the non-permissive temperature. MAPK activation in the CZ zone is lost. Arrows demarcate the reduced pouch. (D) *dpp*-Gal4; UAS-*Dl.* Misexpression of Dl along the A/P boundary results in the activation of Notch and consequent activation of MAPK along the A/P boundary. Arrows point to the endogenous D/V boundary; D, dorsal compartment; V, ventral compartment. (E) Wild-type third instar disc stained with α-Wg antibody (red) and observed for BE-Gal4/UAS-GFP expression (green). Within the pouch, GFP is expressed in a narrow band of cells at the D/V boundary (yellow; arrow). This indicates that the expression of $EGFR^{DN}$ in the next experiment (F) will also be limited to this narrow group of cells within the pouch. (F) BE-Gal4; UAS-$EGFR^{DN}$. The expression of BE, monitored with the reporter *lac*Z, is lost along the D/V boundary. Arrows demarcate the reduced pouch. (G) QE-*lac*Z expression in BE-Gal4;UAS-$EGFR^{DN}$ wing disc. Expression due to QE is non-autonomously lost in the pouch when EGFR function is eliminated in the CZ region. Arrows demarcate the reduced pouch. (H-J) Adult wings shown at same magnification. (H) Wild type. (I) BE-Gal4; UAS-$EGFR^{DN}$ wing from a fly raised at 25°C showing reduced wing size and notching of the distal margin. Veins and marginal bristles develop normally. (J) BE-Gal4;UAS-$EGFR^{DN}$ wing from a fly raised at 29°C showing a dramatic reduction in wing size, but normal marginal bristles (magnified in inset). (K) A model for EGFR function. Activation of EGFR in these cells leads to their specification as precursors of a pool of mitotically active cells that will populate the pouch. The determinative signal, received by the CZ cells from the EGFR pathway, allows their progeny to express Vg in response to the Dpp signal from the A/P boundary. Bars, 100 μm (H-J).

from an antagonistic interaction between the Dpp and EGFR pathways. Recent studies in mammalian cell lines (Kretzschmar et al., 1997) have suggested that activation of EGFR signaling in a cell that is simultaneously activated for the Dpp/TGF-β pathway results in a suppression of the response to the Dpp/TGF-β signal. This results from the EGFR downstream component MAPK phosphorylating and inactivating the TGF-β downstream component SMAD (Kretzschmar et al., 1997). In the context of wing development, activation of EGFR in the CZ regions could cause a down-regulation of the Dpp signal in a similar way, thus turning off QE. Antagonistic relationships between the Dpp and EGFR pathway have also been seen in the *Drosophila* tracheal system (Wappner et al., 1997).

The pattern of activation of EGFR in the wing disc shows many similarities to its role in the organization of the *Drosophila* embryo and eye disc. Notch signaling is needed in midline cells of the embryo for their proper differentiation into midline cells (Menne and Klambt, 1994). Processing of Spi in the midline cells and the subsequent local diffusion of the secreted protein activates the EGFR cascade and specifies the fate of the cells belonging to the flanking ventral epidermis (Golembo et al., 1996). By analogy, activation of EGFR at the D/V boundary leads to the specification of the adjacent CZ region as a separate group of cells, distinguishing them from the rest of the pouch. In the eye disc, activation of EGFR is needed for cell division, cell survival and cell-fate specification

of neighboring cells (Freeman 1994, 1996; Tio et al., 1994; Tio and Moses, 1997; Dominguez et al., 1998). Similarly in the wing, we propose that EGFR activation causes proliferation in the pouch as well as the specification of CZ cell fate.

Recent studies have suggested many intriguing similarities between wing development in flies and limb development in vertebrates (reviewed in Shubin et al., 1997). The Apical Ectodermal Ridge (AER) of the vertebrate limb seems to be analogous to the D/V boundary in the fly wing disc. Like the D/V boundary, loss of the AER results in a complete loss of limb development. As in the fly wing, the formation of the AER in the vertebrate limb depends on the function of Notch and Wg (Wnt). Additionally, the AER is known to express the RTK ligands FGF-4 and FGF-8 (Niswander et al., 1994). Our results provide evidence for the function of an RTK cascade in the specification of the presumptive wing margin. The function of an EGFR ligand at the D/V boundary in the regulation of growth and patterning of the wing suggests yet another similarity between the role of the AER in the vertebrate limb and the D/V boundary in *Drosophila* wing development.

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