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Subdivision of the *Drosophila* wing imaginal disc by EGFR-mediated signaling

Myriam Zecca and Gary Struhl

Department of Genetics and Development, Howard Hughes Medical Institute, Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA
*Author for correspondence (e-mail: struhl@cuccfa.ccc.columbia.edu)

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SUMMARY

Growth and patterning of the *Drosophila* wing imaginal disc depends on its subdivision into dorsoventral (DV) compartments and limb (wing) and body wall (notum) primordia. We present evidence that both the DV and wing-notum subdivisions are specified by activation of the *Drosophila* Epidermal Growth Factor Receptor (EGFR). We show that EGFR signaling is necessary and sufficient to activate *apterous* (*ap*) expression, thereby segregating the wing disc into D (*ap*-ON) and V (*ap*-OFF) compartments. Similarly, we demonstrate that EGFR signaling directs the expression of Iroquois Complex (Iro-C) genes in prospective notum cells, rendering them distinct from, and immiscible with, neighboring wing cells. However, EGFR signaling acts only early in development to heritably activate *ap*, whereas it is required persistently during subsequent development to maintain Iro-C gene expression. Hence, as the disc grows, the DV compartment boundary can shift ventrally, beyond the range of the instructive EGFR signal(s), in contrast to the notum-wing boundary, which continues to be defined by EGFR input.

Key words: EGFR signaling, Iroquois Complex genes, *apterous*, Wing imaginal disc, Compartments, Selector genes, Pattern formation, *Drosophila*

INTRODUCTION

Most animals are composed of distinct body parts, with each part itself subdivided into further well-defined territories. For example, insects are composed of a metameric series of segments, many of which bear limbs which are outgrowths from the body wall. Moreover, both the body wall and limbs are further partitioned, e.g. into anteroposterior (AP) and dorsoventral (DV) compartments. The modular nature of animal form thus poses the questions of how and why such domains are generated during development.

The segregation of the *Drosophila* wing into AP and DV compartments has provided a valuable paradigm (reviewed by Blair, 1995; Lawrence and Struhl, 1996). The wing derives from a larval imaginal disc which is composed of two populations of founder cells, the anterior (A) and posterior (P) compartments, which are established early in development (Garcia-Bellido et al., 1973; Garcia-Bellido et al., 1976; Lawrence and Morata, 1977). As the wing disc grows during larval life, it undergoes a further subdivision into dorsal (D) and ventral (V) compartments (Garcia-Bellido et al., 1973; Garcia-Bellido et al., 1976). Both sets of compartments are controlled by selector genes which are heritably activated in the founder cells of one compartment (e.g. D), thereby distinguishing these cells from the founders of the other compartment (V) (Morata and Lawrence, 1975; Lawrence and Morata, 1976; Diaz-Benjumea and Cohen, 1993; Blair et al., 1994). The difference in selector gene activity then programs cells in adjacent compartments not to intermix (Morata and Lawrence, 1975; Blair et al., 1994; Blair and Ralston, 1997; Rodriguez and Basler, 1997; Micchelli and Blair, 1999; Rauskolb et al., 1999; Dahmann and Basler, 2000), but instead to interact along the common boundary between them (Basler and Struhl, 1994; Williams et al., 1994; Diaz-Benjumea and Cohen, 1995; Zecca et al., 1995; Fleming et al., 1997; Panin et al., 1997), leading to the induction of morphogens which organize growth and patterning of both compartments (Lecuit et al., 1996; Nellen et al., 1996; Zecca et al., 1996; Neumann and Cohen, 1997). Thus, compartmental segregations do not function merely to partition tissues; they also generate the signals that organize how tissues develop.

When compartments were first discovered, cell lineage analyses suggested the possibility of further binary segregations of the wing disc into wing (limb) and notum (body wall) compartments, and into proximal (hinge) and distal (blade) compartments within the wing (Garcia-Bellido et al., 1973; Garcia-Bellido et al., 1976). Several transcription factors have since been shown to be expressed in discrete subdomains that give rise to some of these putative compartments (reviewed by Mann and Morata, 2000). For example, the *pannier* (*pnr*) and Iroquois Complex (Iro-C) genes are expressed in neighboring medial and lateral domains, which together comprise the prospective notum (Diez del Corral et al., 1999; Calleja et al., 2000); *homothorax* (*hth*) and *teashirt* (*tsh*) are expressed in the presumptive wing hinge (Casares and Mann, 2000); and *Distalless* and *vestigial* (*vg*) are expressed within

the primordium of the wing blade (Williams et al., 1991; Carroll et al., 1994). In addition, gain- and loss-of-function experiments have provided evidence that at least some of these transcription factors specify the regional character of the cells in which they are expressed, consistent with their functioning as selector genes within the proposed wing-notum and hinge-blade compartments (Kim et al., 1996; Diez del Corral et al., 1999; Azpiazu and Morata, 2000; Calleja et al., 2000; Casares and Mann, 2000).

Despite these apparent similarities, it remains uncertain whether these putative compartments are equivalent to the classical AP and DV compartments. Unlike the AP and DV compartments, these territories do not appear to have precisely defined boundaries, whether assayed by cell lineage experiments or by the expression of their corresponding selector-like genes. Moreover, the approximate boundaries between these domains do not appear to serve as inductive interfaces that generate signals organizing growth or patterning in surrounding tissues. Thus, we do not understand how these regions are defined and maintained; nor do we understand what roles they serve.

Recently, Wang et al. (Wang et al., 2000) have presented evidence that signals transduced by the Epidermal Growth Factor Receptor (EGFR) organize both the DV and wing-notum subdivisions of the wing imaginal disc. We confirm and extend these findings, and then focus on distinguishing how EGFR signaling governs these two types of subdivisions. We show that EGFR signaling acts during a discrete early stage of wing disc development to induce *ap* expression in dorsally situated cells, and that the descendents of cells throughout the disc then inherit their state of *ap* expression ('on' or 'off') without further reference to EGFR signaling. By contrast, EGFR signaling appears to be required continuously to maintain Iro-C gene expression, allocating cells to the notum primordium on an ongoing basis according to their position and creating an affinity barrier that segregates them from neighboring cells in the wing primordium. These findings indicate that the DV and wing-notum segregations represent different types of developmental partitions and provide a foundation for a further analysis of the nature, source and mode of action of the instructive EGFR signal(s) (Zecca and Struhl, 2002).

MATERIALS AND METHODS

Generation of marked clones of mutant cells

Marked clones of cells homozygous for the $Egfr^{IK35}$ (*Egfr*$^{-}$) (Dominguez et al., 1998), $Egfr^{tsla}$ ($Egfr^{ts}$) (Kumar et al., 1998) or ras^{x7b} (*ras*$^{-}$) (Halfar et al., 2001) mutations were generated using the FLP/FRT technique to induce mitotic clones (Golic, 1991; Xu and Rubin, 1993). Clones were induced by heat shock (37°C, 60 minutes) in larvae staged 24-48, 48-72, 72-96 and 96-120 hours after egg laying (AEL), which correspond approximately to the first, second, early third and mid-third larval instars during normal development (at 25°C). In some experiments, the *Minute* technique (Morata and Ripoll, 1975) was used to increase the viability and growth potential of mutant cells. The larvae in such experiments were heterozygous for either the *Minute(2)IK* or $Minute(3)w^{124}$ mutations. The *Minute(2)IK* mutation causes only modest developmental delays (around 24-48 hours over the course of development from egg laying to adult emergence) so that larvae during a given timed interval (e.g. 72-96 hours AEL) are at a slightly earlier development stage (e.g. late second to early third instar) relative to non-*Minute* larvae. Finally, we note that both the $Egfr^{+}$ and ras^{+} gene products are likely to perdure at least one or a few cell generations in clones of mutant cells generated by mitotic recombination, providing transient rescue of the mutant genotype for at least 12-24 hours after clone induction.

The specific genotypes for each experiments are as follows. *ras*$^{-}$ clones in a wild-type background: *y w hsp70-flp; mirr-lacZ FRT82* ras^{x7b}*/FRT82 hsp70-flu-GFP* (used for mosaic analysis; see below); and *y w hsp70-flp; tsh-lacZ/+; FRT82* ras^{x7b}*/FRT82 hsp70-flu-GFP.* *ras*$^{-}$ clones in a *Minute* background: *y w hsp70-flp; FRT82* ras^{x7b}*/FRT82* $M(3)w^{124}$ *hsp70-CD2* (or *hsp70-flu-GFP*). *Egfr*$^{-}$ and $Egfr^{ts}$ clones in a *Minute* background: *y w hsp70-flp; FRT42 Egfr*$^{-}$ (or $Egfr^{ts}$*)/FRT42 hsp70-flu-GFP M(2)IK;* vg^{Q}*-lacZ/mirr-lacZ.*

Mosaic analysis

ras^{-} clones (Fig. 1D, Fig. 2B)

ras^{-} clones were induced 24-48 hours AEL and recognized by loss of GFP expression, while the wild-type twin spots, which carry two copies of the *hsp70-flu-GFP* transgene were recognized by strong GFP expression. All wild-type twin spots within a disc were counted whether or not they were associated with a *ras*$^{-}$ clone. *ras*$^{-}$ clones, when present, were usually small, so the associated twin spot was assigned to one of the four areas (Fig. 2B), based on the location of the mutant clone. Wild-type twin spots without an associated *ras*$^{-}$ clone typically extended into two or more areas; such clones were assigned to the area that contained the largest region of the twin spot. Survival rate of *ras*$^{-}$ clones within a given area was calculated as the percentage of the total number of wild-type twin spots recovered within that area.

$Egfr^{ts}$ clones (Fig. 6A)

Clones were induced 24-48 hours AEL and kept at the temperatures indicated in the figure thereafter.

Egfr^{-} clones (Fig. 6B,C)

Clones were induced during the indicated timed intervals. To assess the relative size of clones in different compartments in Fig. 6C, we first used the polygon function of the Lasersharp 2000 program (BioRad) to measure the area of marked wing cells associated with each clone. The measured areas of all of the clones for a given batch of discs (defined by the time of clone induction) were then summed to give a total area of mutant tissue. Finally, the areas of clones within a given compartment were summed and the resulting totals expressed in Fig. 6C as the percentage of the total area of mutant tissue in the same batch of discs.

Ectopic expression studies

Marked clones of cells ectopically expressing a given protein were generated using a combination of the FLP-out and Gal4/UAS techniques (Golic, 1991; Brand and Perrimon, 1993; Zecca et al., 1996). Clones ectopically expressing $EGFR^{\lambda}$ or Rho were induced by heat shocking *hsp70-flp* larvae carrying a *Tubα1>flu-GFP,y*$^{+}$*>Gal4* transgene in combination with either the *UAS-*$EGFR^{\lambda}$ (Queenan et al., 1997) or *UAS-Rho* (Golembo et al., 1996) transgenes, respectively, and recognized by the loss of GFP expression. The *Tubα1>flu-GFP,y*$^{+}$*>Gal4* transgene is similar to the *Act5C>CD2>Gal4* transgene (Pignoni and Zipursky, 1997), except that the *Act5C* promotor was replaced by the *Tubα1* promoter and the CD2-coding sequence was replaced by the coding sequence for flu-tagged GFP (flu-GFP) followed by the y^{+} gene. Clones ectopically expressing either Ras^{V12} or Spi* were generated by heat shocking *hsp70-flp UAS-GFPnls* larvae carrying a *Tubα1>Gal80, y*$^{+}$*>Gal4* transgene together with either a *UAS-*Ras^{V12} (Karim and Rubin, 1998) or *UAS-Spi** (Schweitzer et al., 1995) transgene, and recognized by the gain of GFP expression. The *Tubα1>Gal80, y*$^{+}$*>Gal4* transgene is similar to the *Tubα1>flu-GFP,y*$^{+}$*>Gal4* transgene, except that the *flu-GFP* was replaced by the coding sequence for the yeast Gal80 protein, which

represses transcriptional activation by the Gal4 protein (Ma and Ptashne, 1987; Lee and Luo, 1999).

To generate clones during the first instar, larvae were heat shocked 24-48 hours AEL for 30 minutes at 36°C. To generate clones during second instar, larvae were heat shocked 48-72 hours AEL for 30 minutes at 35°C. For the Ras^{V12} experiments, we note that some Gal80 gene product perdures for at least two to three cell generations after excision of the *>Gal80, y*$^{+}$>Flp-out cassette, delaying the expression of the *UAS-Ras*^{V12} transgene for at least 24 hours after clone induction.

lacZ reporter lines

The following *lacZ*-reporter lines were used: *ap-lacZ* (Cohen et al., 1992), *mirr-lacZ* (McNeill et al., 1997), *tsh-lacZ* (*tsh*^{1}) (Fasano et al., 1991) and *vg*^{Q}*-lacZ* (Kim et al., 1996).

Antibody staining

The following antisera were used: anti-Ap (Lundgren et al., 1995), anti-Ara (anti-Caup) (Diez del Corral et al., 1999), anti-Tsh (Roder et al., 1992), anti-Vg (Williams et al., 1991) and anti-Wg (Brook and Cohen, 1996). Imaginal discs from wandering third instar larvae were fixed and stained following standard protocols.

RESULTS

The subdivision of the wing imaginal disc into AP and DV compartments, as well as prospective body wall (notum) and limb (wing) territories is shown in Fig. 1A. Each of these subdivisions is marked by the expression of particular regulatory genes, such as the selector gene *engrailed* (*en*) in the P compartment (green), the selector gene *apterous* (*ap*) in the D compartment (blue), and the genes of the Iroquois Complex (Iro-C) [*mirror* (*mirr*), *auracan* (*ara*) and *caupalican* (*caup*)] in the lateral notum (represented by *mirr-lacZ*; red). In mature third instar wing discs, the Iro-C genes are expressed not only within the prospective lateral notum, but in additional locations, including a thin stripe of cells that extends ventrally along the edge of the disc (Fig. 1A,B), as well as in specific subpopulations of cells in the prospective wing blade (Gomez-Skarmeta et al., 1996). We address the role of EGFR signaling in controlling notum development and Iro-C gene expression therein, and then focus on the role of EGFR signaling in inducing *ap* expression and establishing the DV compartments.

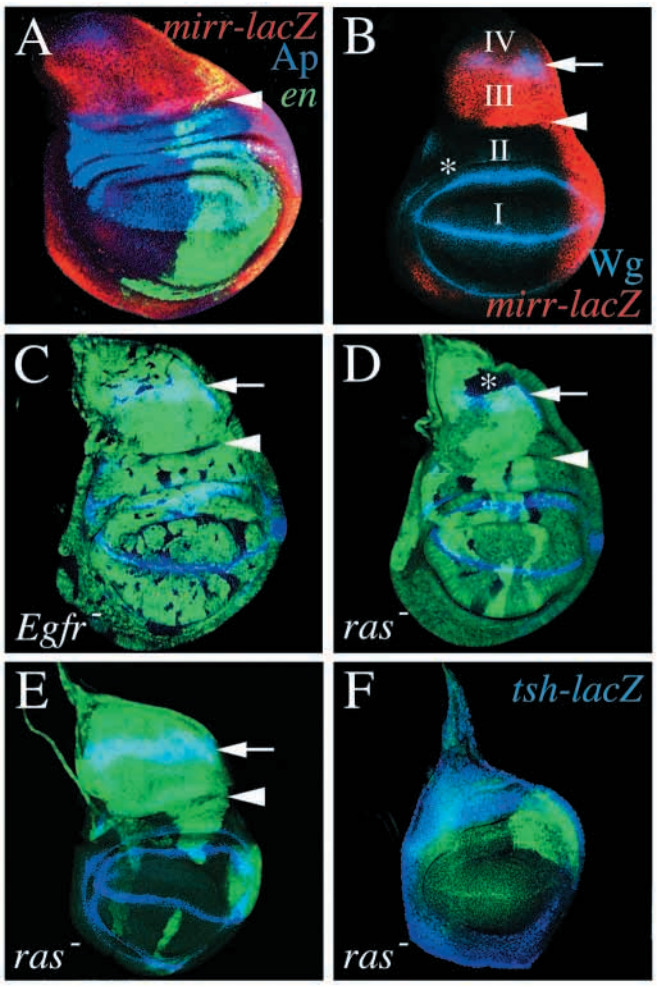

Fig. 1. EGFR/Ras signaling is required for notum development. (A,B) Organization of the mature wing imaginal disc. (A) Subdivision of a wild-type wing imaginal disc into AP compartments (as marked by *en-Gal4* driven GFP expression in posterior cells; green), DV compartments (as marked by the expression of Ap in dorsal cells; blue) and notum-wing primordium (as marked by elevated expression of *mirr-lacZ* in prospective notum cells; red). The notum-wing boundary is marked by an arrowhead; *mirr-lacZ* is also expressed in prospective pleural cells in the mature wing disc (to the right and below the arrowhead). (B) *mirr-lacZ* expression (red) relative to Wg expression (blue) in a wild-type wing imaginal disc. Overlapping expression appears in pink. Note that *mirr-lacZ* expression is generally restricted to the prospective lateral notum (region III), which is demarcated dorsally by a stripe of Wg expression (arrows in B-E) and ventrally by a characteristic deep fold (arrowheads in A-E). However, as noted in the text, the Iro-C genes are also expressed in additional domains in mature, third instar wing discs, including in a stripe of cells extending ventrally along the edge of the disc. The prospective medial notum (region IV) is located dorsal to this stripe of Wg expression, and the prospective wing blade (region I) is encircled by two closely associated rings of Wg expression (only one ring is visible here, asterisk), distinguishing it from the surrounding prospective wing hinge (region II). Wg is also expressed along the DV compartment boundary, bisecting the wing blade primordium into D and V halves. (C-F) *Egfr*$^{-}$ (C) and *ras*$^{-}$ (D-F) clones marked by the absence of either GFP expression (C-E, green) or CD2 expression (F, green). Wg expression is shown in blue (C-E) and faint green (F); *tsh-lacZ* expression, a marker for prospective wing hinge, is shown in blue (F). (C) Distribution of *Egfr*$^{-}$ clones induced during late second/early third larval instar using the *Minute* technique. Note that clones are present throughout the disc, except in an area corresponding to the prospective lateral notum (between the arrow and arrowhead). (D) Distribution of *ras*$^{-}$ clones generated in a non-*Minute* background during first larval instar. *ras*$^{+}$ twin spots are marked by bright GFP expression. *ras*$^{-}$ clones generally failed to survive in the prospective lateral notum (see also Fig. 2B). Note the presence of a large *ras*$^{-}$ clone in the prospective medial notum (asterisk), which abuts the prospective lateral notum (defined by the stripe of Wg expression), which straddles the boundary between the two domains. (E) Disc with at least two large *ras*$^{-}$ clones generated during first larval instar using the *Minute* technique. Note that the mutant clones populate most of the prospective wing blade and wing hinge and show a normal pattern of Wg expression; however, the clones do not contribute to the notum, which also appears to develop normally and shows normal Wg expression. (F) Disc with large *ras*$^{-}$ clones generated during first larval instar using the *Minute* technique. The notum is ablated and the disc is composed largely of prospective wing hinge tissue (marked by high levels of *tsh-lacZ* expression; blue) and wing blade tissue (encircled by a thin stripe of Wg expression and bisected by an additional stripe of Wg expression along the DV compartment boundary (faint green).

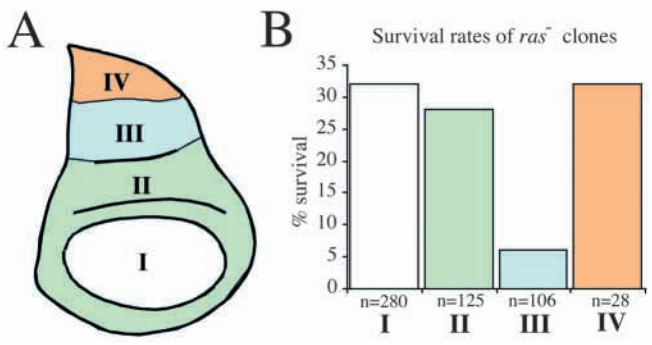

Fig. 2. Survival of *ras*– clones in the prospective wing blade, wing hinge, lateral notum and medial notum. (A) Subdomains of the wing imaginal disc: (I) presumptive wing blade, (II) presumptive wing hinge, (III) presumptive lateral notum and (IV) presumptive medial notum (see also Fig. 1B). (B) Histogram showing the survival rates of *ras*– clones in each of the four territories in the mature third instar wing disc, following clone induction during the first larval instar (see Materials and Methods). *ras*– clones generally survive less well than their *ras*+ twin clones. However, survival in the presumptive lateral notum was severely reduced relative to survival in other regions of the wing disc. Bars represent the percentage of *ras*+ clones associated with a *ras*– twin clone within a given disc area. *n*, total number of *ras*+ clones (with or without a paired *ras*– clone). Clones were induced at 24-48 hours after egg laying (AEL).

EGFR/Ras signaling is required for notum development

To assess the requirement for signals transduced by the EGFR during normal wing disc development, we examined the behavior of clones of cells that are homozygous for null or temperature-sensitive mutations of the *Egfr* gene (referred to subsequently as *Egfr*– or *Egfr*^{ts}), or for a loss of function mutation of the *ras* gene (*ras*–), which encodes the Ras GTPase, a conserved downstream effector of the EGFR signal transduction pathway (Diaz-Benjumea and Hafen, 1994). Clones of mutant cells were generated during different stages of larval development and their size, shape and distribution assayed in each of the four distinct primordia that make up the mature wing disc: the prospective wing blade (I), wing hinge (II), lateral notum (III) and medial notum (IV) (see Materials and Methods; see Fig. 1B, Fig. 2A). In general, loss of EGFR activity caused more penetrant and severe effects than the loss of Ras activity, possibly reflecting a shorter perdurance of EGFR function relative to that of Ras following loss of the wild-type gene, or a restricted requirement for Ras in mediating some, but not all, downstream outputs of EGFR activation. *ras*– clones, in particular, were more viable than *Egfr* mutant clones, allowing us to use the twin spot method of clonal analysis and to generate mutant clones of large size using the *Minute* technique (see Materials and Methods). However, aside from this difference, the effects of *Egfr* and *ras* mutant clones on Iro-C gene expression were the same. In these, and subsequent experiments, mutant clones were marked either by the presence or absence of the reporter proteins GFP or CD2, shown in green in the figures (see legends for details).

Egfr– clones induced in the wing disc during the first and second instars do not survive to the late third instar, apparently because of defects in cell proliferation and/or viability (Diaz-Benjumea and Garcia-Bellido, 1990; Diaz-Benjumea and

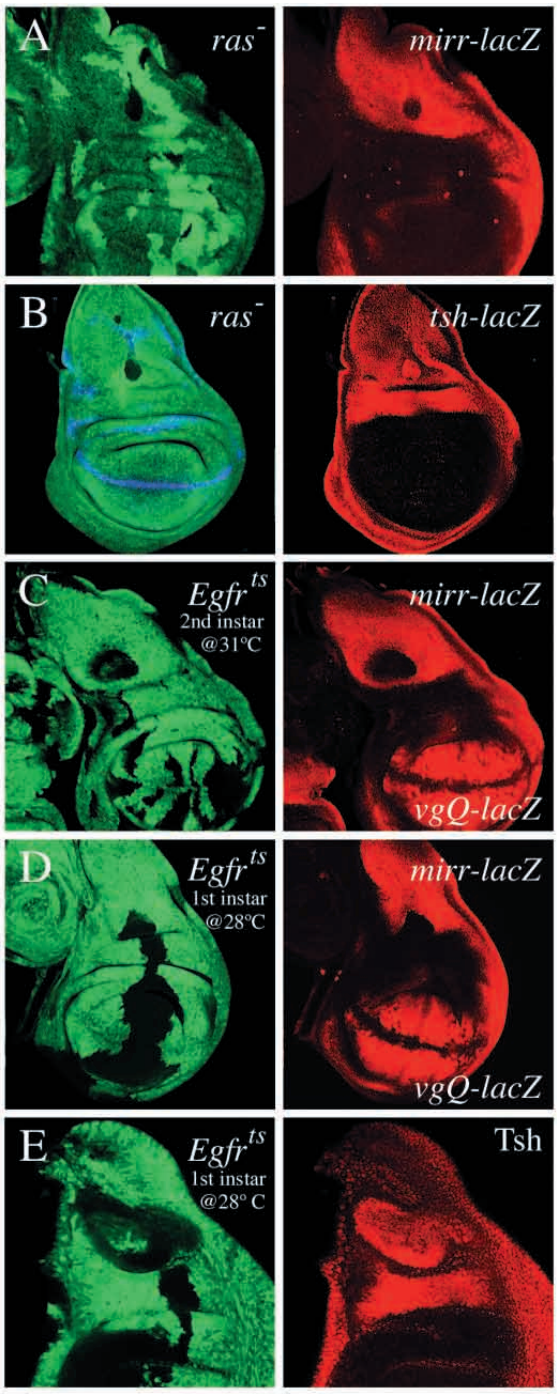

Fig. 3. EGFR/Ras signaling is required for Iro-C expression. (A-E) *ras*– (A-B) and *Egfr*^{ts} (C-E) clones, marked by the absence of GFP (green), fail autonomously to express *mirr-lacZ* (A,C,D, red) and autonomously upregulate expression of *tsh-lacZ* (B, red) or endogenous Tsh protein (E, red). Wing blade specific *vgQ-lacZ* expression is also seen (C,D, red). *Egfr*^{ts} clones were generated using the *Minute* technique. Note the round shape of mutant clones (A-C,E) located within the prospective lateral notum when compared with the irregular shape of the clones located elsewhere in the disc. (A,B) *ras*– clones generated during first larval instar. (C) *Egfr*^{ts} clones generated during second larval instar and raised at the severely restrictive temperature of 31°C. (D-E) *Egfr*^{ts} clones generated during first larval instar and raised at intermediate restrictive temperature of 28°C.

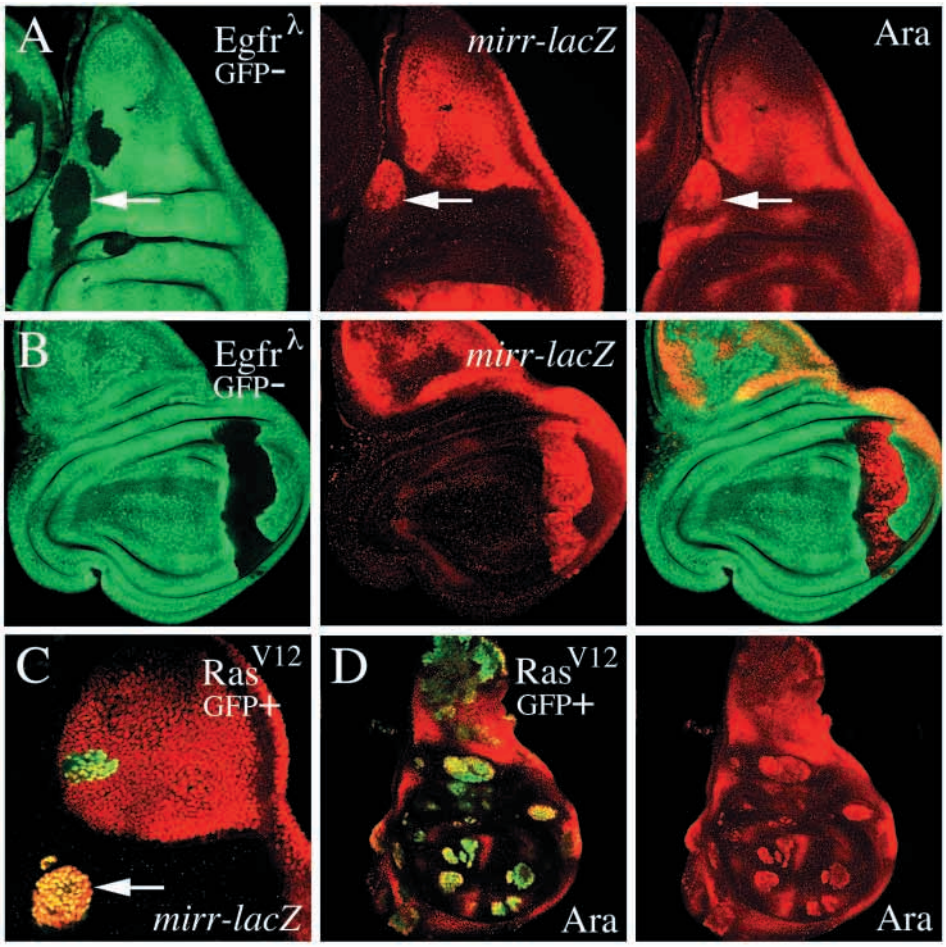

Fig. 4. EGFR/Ras signaling is sufficient to activate Iro-C expression. (A-D) Ectopic *mirr-lacZ* and Ara/Caup expression (red; arrows) associated with clones ectopically expressing either $EGFR^{\lambda}$ (A,B) or Ras^{V12} (C,D). Clones are marked either by loss (A,B) or gain (C,D) of GFP expression. Cells co-expressing GFP and *mirr-lacZ* appear yellow. $EGFR^{\lambda}$-expressing clones are associated with ectopic *mirr-lacZ* and Ara/Caup expression in some regions of the prospective wing hinge, but not others (A; see also Fig. 5); they can also induce *mirr-lacZ* expression in the prospective wing blade (B). Ras^{V12}-expressing clones activate *mirr-lacZ* (C) and Ara/Caup (D) expression in a more penetrant and strictly autonomous fashion within the wing hinge and blade (but not within the medial notum), as indicated by the exact correspondence between the clone marker (GFP, green) and *mirr-lacZ* or Ara/Caup (red) expression (overlapped expression appears in yellow). Note that Ara/Caup is normally expressed in localized patches of anterior compartment cells within the prospective wing blade; these appear red instead of yellow in D (left-hand panel).

Hafen, 1994). To increase the likelihood that mutant clones might survive, we used the *Minute* technique (Morata and Ripoll, 1975) to give $Egfr^{-}$ cells a growth advantage relative to surrounding $Egfr^{+}$ cells (see Materials and Methods). Under these circumstances, we find that $Egfr^{-}$ clones induced during the first or early second instar contributed only to the prospective wing blade, whereas clones induced during the late second or early third instar could also populate the prospective wing hinge and medial notum domains (Fig. 1C). However, $Egfr^{-}$ clones were invariably excluded from the prospective lateral notum. Similar results were obtained for clones of cells homozygous for the $Egfr^{ts}$ mutation, which reduces but does not eliminate EGFR activity at the non-permissive temperature (30-31°C), except that the clones tended to be larger than their $Egfr^{-}$ counterparts. $Egfr^{ts}$ clones induced after the mid-second instar could also contribute to the prospective lateral notum, albeit rarely. However, these clones were abnormally round in shape, suggesting they developed abnormally (Fig. 3C and not shown; see below).

Unlike $Egfr^{-}$ clones, ras^{-} clones induced during the first or second larval instar can survive without the benefit of the *Minute* technique (Prober and Edgar, 2000). Under these conditions, mitotic recombination generates 'twin spots' composed of genetically marked ras^{-} and ras^{+} sister clones, which descend from the same mother cell. As shown in Fig. 1D and Fig. 2, twin spots could be recovered in the prospective wing blade domain, wing hinge and medial notum domains. However, only single ras^{+} spots were generally observed in the prospective lateral notum domain (Fig. 2), indicating that their ras^{-} sister spots failed to survive in this domain; the few ras^{-} sister spots obtained in this domain appeared abnormal (Fig. 3A,B) and are considered further below. Similar results were obtained when ras^{-} cells were generated during the first larval instar using the *Minute* technique. Such ras^{-} clones could form large, and apparently normal, regions of the prospective wing blade and wing hinge (Fig. 1E). Nevertheless, they appeared to be excluded from the presumptive notum territory (Fig. 1E). Strikingly, some of the discs obtained under these conditions appeared to lack most or all prospective notal tissue and to consist predominantly of prospective wing blade and hinge tissue (Fig. 1F).

In summary, $Egfr^{-}$, $Egfr^{ts}$ and ras^{-} clones can contribute to the prospective wing blade, wing hinge and medial notum. However, all three classes of mutant clones generally failed to populate the prospective lateral notum, indicating that EGFR signaling is essential for the normal development of this region of the wing disc.

EGFR/Ras signaling is required for normal Iro-C gene expression in the prospective lateral notum

Iro-C genes are initially expressed throughout the notum primordium, and then become restricted to a discrete lateral domain therein (Gomez-Skarmeta et al., 1996; Kehl et al., 1998; Diez del Corral et al., 1999). Like clones of $Egfr^{-}$ and $Egfr^{ts}$ cells, clones of Iro-C^{-} cells generated during the first

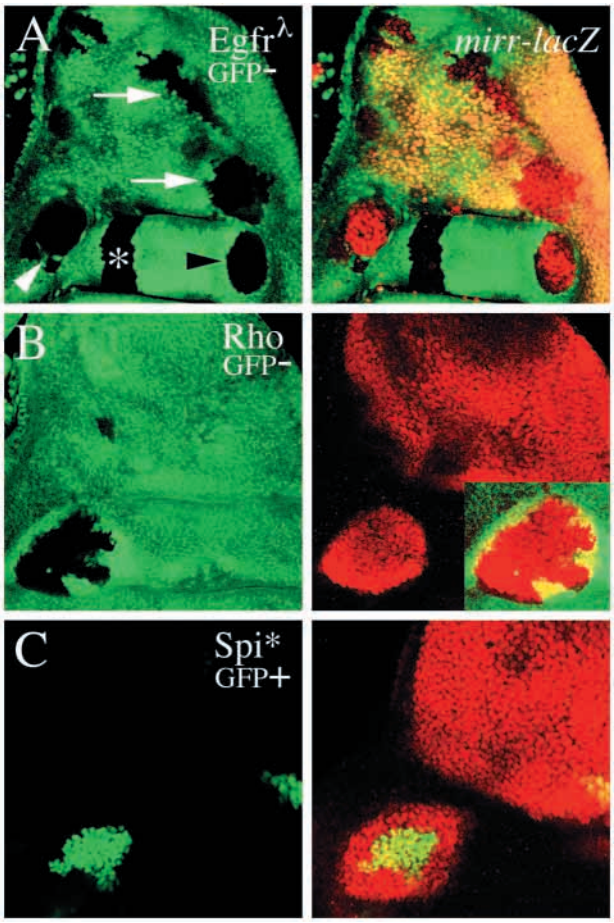

Fig. 5. EGFR signaling controls cell-affinity. (A-C) Wild-type wing imaginal discs containing clones of cells ectopically expressing either EGFR$^{\lambda}$ (A), Rho (B) or Spi* (C), monitored for *mirr-lacZ* expression (red). Clones were induced during the first larval instar and are marked either by loss (A,B) or gain (C) of GFP expression (green). (A) EGFR$^{\lambda}$-expressing clones located within prospective notum (arrows) intermix freely with surrounding cells, as indicated by their 'wiggly' borders. Some clones within the hinge ectopically express *mirr-lacZ* and these tend to adopt a circular shape and sort out from surrounding wild-type cells (black arrowhead). Other clones do not express *mirr-lacZ* and these intermix with surrounding cells (asterisk). Finally, the sorting out of high *mirr-lacZ*-expressing from neighboring cells can occur within the same clone (white arrowhead). (B,C) Clones of Rho- or Spi*-expressing cells located within the prospective wing hinge form 'wiggly' borders and induce circular patches of *mirr-lacZ*-expressing cells that tend to sort out from surrounding cells that do not express *mirr-lacZ*. The inset in the left panel of B shows the overlay between GFP and *mirr-lacZ* expression in the vicinity of the clone.

larval instar fail to contribute to the notum, and clones induced later are excluded from the prospective lateral notum or develop abnormally in this region (Diez del Corral et al., 1999). Hence, *Egfr*$^{-}$, *Egfr*^{ts} and *ras*$^{-}$ clones may fail to populate the prospective lateral notum because they are unable to express Iro-C genes.

To test this possibility, we examined the expression of a *mirr-lacZ* reporter gene in rare *ras*$^{-}$ and *Egfr*^{ts} clones that survived in the prospective notum. We find that *mirr-lacZ* expression is absent in both classes of clones (Fig. 3A,C). However, cells within these clones express high levels of the *teashirt* (*tsh*) gene, which encodes a transcription factor whose expression is normally elevated in the prospective wing hinge (Fig. 3B,E). Thus, the failure of the mutant cells to express *mirr-lacZ* does not appear to reflect impaired cell viability or survival; instead, these cells develop inappropriately as prospective wing hinge cells. We also examined *Egfr*^{ts} clones maintained at the less restrictive temperature of 28°C rather than 31°C after clone induction. Under these conditions, large clones could be recovered within the prospective lateral notum, even when induced during the first instar. However, these clones showed cell-autonomous reductions of *mirr-lacZ* expression, as well as elevated levels of Tsh protein (Fig. 3D,E).

Thus, *Egfr*^{ts} and *ras*$^{-}$ clones that survive within the prospective lateral notum express *mirr* either poorly or not at all and adopt hinge-like characteristics. By contrast, mutant clones that populated other regions of the disc appeared to show normal expression of *wg* and other regional control genes, such as *vg*, *hth* and *tsh* (Fig. 1, Fig. 3, data not shown). We conclude that there is an absolute requirement for EGFR/Ras activity to maintain Iro-C gene expression during wing disc development.

EGFR/Ras activation can induce Iro-C gene expression

To determine if EGFR/Ras activity is sufficient to induce Iro-C gene expression, we generated genetically marked clones of cells that express constitutively active forms of EGFR (EGFR$^{\lambda}$) (Queenan et al., 1997) and Ras (Ras^{V12}) (Trahey and McCormick, 1987) under Gal4/UAS control and assayed for *mirr-lacZ* and Ara/Caup expression (Materials and Methods). As shown in Fig. 4A, we find that some EGFR$^{\lambda}$-expressing clones are associated with ectopic expression of *mirr-lacZ*, as well as Ara/Caup protein, although this ectopic expression is generally limited to particular subregions of the prospective wing hinge. In addition, we find that some EGFR$^{\lambda}$-expressing clones in the wing blade domain induce ectopic *mirr-lacZ* expression (Fig. 4B) and reduce expression of Vestigial (Vg; not shown), a transcription factor that distinguishes prospective wing blade cells (Kim et al., 1996). In contrast to the spatially restricted and incompletely penetrant effects of EGFR$^{\lambda}$ expression, clones of Ras^{V12}-expressing cells located in the prospective wing hinge region autonomously expressed ectopic *mirr-lacZ* and Ara/Caup protein (Fig. 4C,D). In addition, clones of Ras^{V12}-expressing cells located within the prospective wing blade are frequently associated with moderate levels of ectopic *mirr-lacZ* and Ara/Caup protein, consistent with a partial transformation of these cells into presumptive notum (Fig. 4D).

Thus, forced activation of the EGFR/Ras transduction pathway in the prospective wing hinge and wing blade can induce ectopic Iro-C gene expression. We suggest that exogenous Ras^{V12} expression is more effective at activating the Ras transduction pathway than is exogenous EGFR$^{\lambda}$ expression, accounting for the more consistent and dramatic effects of Ras^{V12}-expressing clones. Because ectopic Ras^{V12} expression activates Iro-C gene expression in a cell autonomous fashion, we infer that the normal pattern of Iro-C expression reflects a direct response of cells to EGFR-mediated signaling, rather than to secondary signals induced in response to activation of the EGFR pathway.

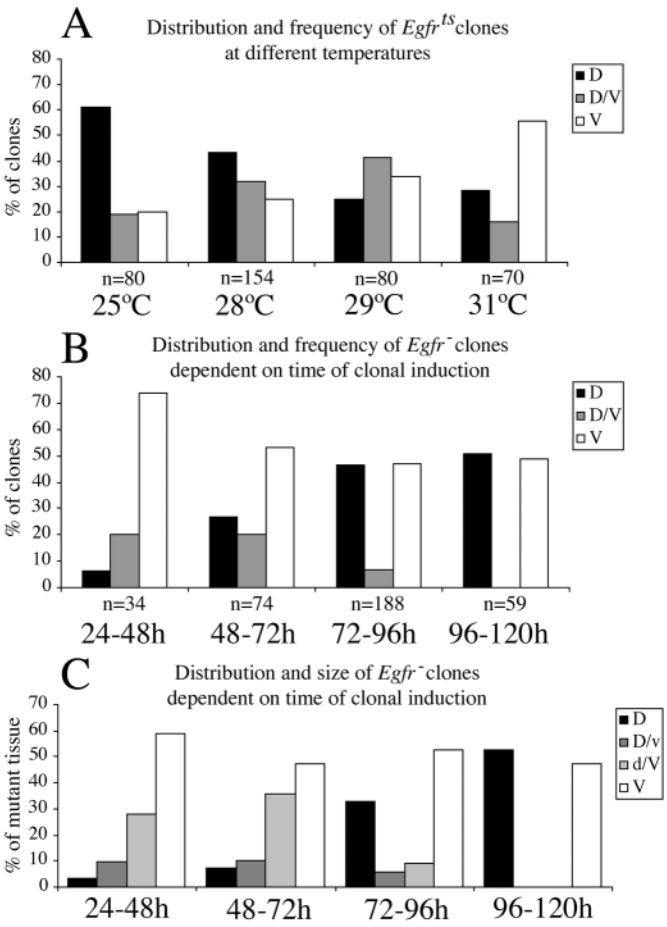

Fig. 6. Distribution, size and frequency of *Egfr* mutant clones in the D and V compartments. (A) Frequency of $Egfr^{ts}$ clones that populate the D (black) or V (white) compartment, or both compartments (D/V, gray) as a function of temperature. Clones were induced during the first larval instar using the *Minute* technique, and larvae were kept at the indicated restrictive temperatures thereafter. *n*, total number of clones scored for each temperature condition. The percentage of $Egfr^{ts}$ clones in the D compartment declines progressively as the temperature increases; conversely, the percentage of these clones rises in the V compartment. More clones are found within the D compartment than in the V compartment at the permissive temperature, reflecting the larger pool of cells from which the D compartment will arise. All clones within the wing disc were scored in this experiment. (B) Frequency of $Egfr^{-}$ clones that populate the D (black) or V (white) compartment, or both compartments (D/V, gray), as a function of the time of clone induction [indicated by hours (h) AEL]. The *Minute* technique was used; *n*, total number of clones scored for each time interval. $Egfr^{-}$ clones generated before the DV compartments are established during the second larval instar (approximately 48-72 hours AEL) preferentially populate the ventral compartment; clones induced thereafter frequent both compartments equally. Only clones populating the presumptive wing blade were scored in this experiment. (C) Relative sizes of $Egfr^{-}$ clones shown in B (see Materials and Methods for quantitation of size); for clones that populated both compartments, the sizes of the mutant territories in the D and V compartments were scored separately and are designated, respectively as D/v (dark gray bars) and d/V (light gray bars). $Egfr^{-}$ clones induced before the DV compartments contribute poorly to the D compartment, probably because most of the cells fail to activate *ap* and sort into the V compartment or out of the disc epithelium; clones induced thereafter contribute equally well to each compartment.

Formation of sharp borders of Iro-C gene expression in response to localized EGFR signaling

The lateral border delimiting Iro-C expression in the wing disc is relatively straight and sharp (e.g. Fig. 1B, Fig. 4C), raising the question of how such a well-defined border can be established and maintained in response to EGFR signaling. Several lines of evidence indicate that this border is not a compartmental boundary. In particular, the border of Iro-C expression is not absolutely sharp; instead, the level of Iro-C expression declines progressively from peak levels to undetectable levels over a range of a few cell diameters. Moreover, we and others (Diez del Corral et al., 1999) have observed that clones of marked cells show little if any tendency to respect the Iro-C expression border in mature wing discs. An alternative possibility is that the induction of Iro-C gene activity by EGFR signaling causes Iro-C-expressing cells to assort with each other rather than with neighboring non-expressing cells, creating an abrupt affinity barrier that sharpens and straightens the boundary between the notum and wing primordia. The properties of $EGFR^{\lambda}$-expressing clones in the notum and wing hinge primordia provide evidence for such a mechanism.

Clones of $EGFR^{\lambda}$-expressing cells appear to develop normally within the endogenous Iro-C expression domain. In particular, they form ‘wiggly’ borders with surrounding cells, suggesting that they are able to intermix freely with these cells (arrows in Fig. 5A). By contrast, clones that arise in regions of the wing hinge primordium just across the normal Iro-C expression border show a more complex behavior. Some of these clones express high levels of *mirr-lacZ* and form circular patches, indicating that *mirr-lacZ*-expressing cells cannot mix with surrounding non-expressing cells (black arrowhead, Fig. 5A), while other clones express little or no *mirr-lacZ* and form irregularly shaped patches, indicating that cells within the clone can interdigitate with surrounding cells (asterisk, Fig. 5A). More strikingly, the sorting out of *mirr-lacZ*-expressing cells from non-expressing cells can occur within a single clone of $EGFR^{\lambda}$-expressing cells (white arrowhead, Fig. 5A).

We also examined the consequences of creating an ectopic source of EGFR signaling in the prospective notum and wing hinge by generating clones of cells that express active forms of the EGFR ligand Spitz (Spi). The *spi* gene is normally expressed in all cells during wing disc development, yielding a membrane bound, but inactive, form of Spi protein (Rutledge et al., 1992; Sturtevant et al., 1993). This inert form of Spi is then processed to generate the active, secreted form of the ligand, an event that requires the spatially restricted activity of the transmembrane protein Rhomboid (Rho) (Schweitzer et al., 1995; Golembo et al., 1996; Bang and Kintner, 2000). Rho is not expressed until relatively late in wing development, and neither Spi nor Rho activity is required for the normal segregation of the DV compartments or the wing-notum primordia (Simcox, 1997). Hence, we generated ectopic sources of active Spi in early wing discs by making clones of cells that express the coding sequence either for Rho or for a truncated, constitutively active form of Spi, Spi* (Schweitzer et al., 1995). Clones of either Rho- or Spi*-expressing cells located within the notum primordium express similar levels of *mirr-lacZ* to surrounding wild-type cells and have ‘wiggly’ borders (not shown). When such clones are located in the neighboring wing hinge primordium they ectopically express *mirr-lacZ* and induce a halo of surrounding wild-type cells to

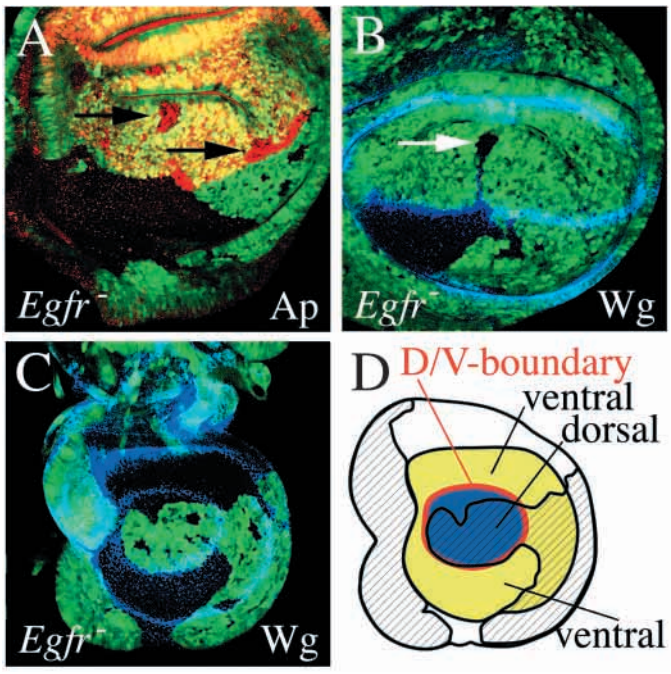

Fig. 7. Early EGFR/Ras-mediated signaling is required for establishing the D compartment. (A,B) Examples of large *Egfr*⁻ clones that preferentially populate the ventral compartment. The clones were generated during first larval instar using the *Minute* technique and are marked by the absence of GFP (green). Ap protein is shown in red (A), Wg protein in blue (B,C). Black arrows in A indicate small mutant clones located within the dorsal compartment. The white arrow in B indicates a small cluster of mutant cells that appear to have been left behind in the D compartment when the remainder of the clone sorted into the V compartment. Note that cells within this cluster do not express Wg, indicating that they are of D, rather than V type. (C,D) Example (C) and schematic representation (D) of a disc containing a dorsally situated clone of *Egfr*⁻ cells that has developed as an ectopic V compartment. The wing blade primordium (all cells located within the outer ring of Wg expression) is shown in color: V compartment cells are represented in yellow, and D compartment cells in blue. *Egfr*⁺ cells are indicated by hatching. The dorsoventral compartment boundary, which correlates with the inner ring of Wg expression, is outlined in red.

express *mirr-lacZ*. As shown in Fig. 5B,C, the borders of such clones are contained within the domains of ectopic *mirr-lacZ* expression and are 'wiggly'. By contrast, the domains of ectopic *mirr-lacZ* expression are round in shape, have smooth borders and tend to sort out from the surrounding cells.

Taken together, these results suggest a mechanism by which the boundary of Iro-C gene expression is defined by a graded EGFR signal that spreads from a localized source and activates Iro-C gene transcription when its concentration exceeds a given threshold. Iro-C gene function, in turn, then regulates cell affinity, causing Iro-C-expressing cells to assort with each other rather than with surrounding non-expressing cells, thus sharpening and straightening the boundary of the Iro-C expression domain.

EGFR signaling is required for DV compartmentalization

In the course of analyzing the role of EGFR signaling in maintaining the wing-notum subdivision, we obtained evidence that EGFR signaling was also responsible for establishing the DV compartment segregation via the activation of *ap* expression; related and complementary findings have recently been reported by Wang et al. (Wang et al., 2000).

The DV compartmental segregation occurs during the second larval instar (Cohen et al., 1992; Diaz-Benjumea and Cohen, 1993; Williams et al., 1993; Blair et al., 1994). Clones of $Egfr^{ts}$ cells generated before this stage, using the *Minute* technique, developed normally under permissive conditions (25°C), contributing to either, or both, the D and V compartments (Fig. 6A). However, $Egfr^{ts}$ clones obtained at the moderately restrictive temperature of 28°C show an enhanced tendency to populate the V compartment, and this effect is progressively more pronounced at the more severely restrictive temperature of 29°C and 31°C (Fig. 6A). Finally, *Egfr*⁻ clones showed an extreme preference for the V compartment, populating it exclusively or disproportionally (Fig. 6B,C), only occasionally leaving one or a few small patches of *Egfr*⁻ cells behind in the D compartment (Fig. 7A,B). A few discs were also recovered that showed an aberrant DV subdivision of the prospective wing blade in which dorsally located *Egfr*⁻ clones appear to contribute to an ectopic V compartment (Fig. 7C,D). In contrast to these early induced clones, *Egfr*⁻ and $Egfr^{ts}$ clones induced after the DV compartmental segregation were invariably restricted to either the D or V compartment and appeared to populate and survive equally well in each compartment of the prospective wing blade (Fig. 6B,C).

We interpret the unusual properties of early induced *Egfr* mutant clones as evidence that the EGFR normally transduces a dorsally localized signal that allocates cells to the nascent D compartment. We suppose that dorsally situated cells that lack EGFR activity cannot respond to this signal, and hence become committed to the V state by default. In rare cases, the descendents of such cells may form an ectopic V compartment (Fig. 7C,D). In general, however, we suggest that they sort out of the epithelium or into the neighboring V compartment (Fig. 7A,B), accounting for the shortfall of early induced mutant clones that survive in the D compartment (Fig. 6A,B). We note that sufficient EGFR function may perdure in some of the descendents of the mutant cells induced during the first larval instar to allow these descendents to transduce the signal and choose, correctly, to enter the D compartment. Such 'rescued' descendents may give rise to the small patches of mutant tissue that remain behind in the D compartment and appear to develop normally (Fig. 7A,B). Early induced *ras*⁻ clones also survive and develop normally in the D compartment (data not shown), a result we similarly attribute to perdurance, in this case of wild-type Ras activity, after removal of the *ras*⁺ gene.

Early EGFR/Ras activation can induce *ap* expression and establish ectopic D compartments

To assess whether early EGFR activation is sufficient to induce *ap* expression and commit cells to the D compartment fate, we examined the consequences of ectopically activating EGFR or Ras in ventrally situated cells during the first larval instar. We first generated clones of cells that express $EGFR^{\lambda}$, the constitutively active form of EGFR and assayed them for *ap-lacZ* expression. Most $EGFR^{\lambda}$-expressing clones that were obtained in the ventral region of the disc appeared to behave in the same way as normal V compartment clones in that they

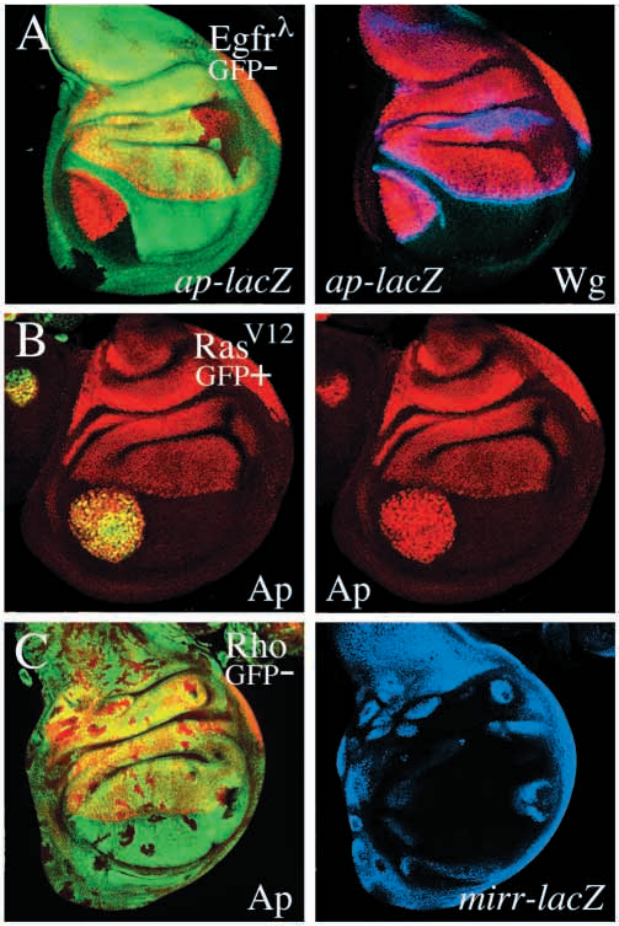

Fig. 8. Early activation of the EGFR/Ras transduction pathway is sufficient to generate an ectopic D compartment. (A-C) Wing imaginal discs containing clones of cells ectopically expressing either EGFR$^{\lambda}$ (A), Ras^{V12} (B) or Rho (C), monitored for *ap-lacZ* (A, red), Ap (B,C, red), Wg (A, blue) or *mirr-lacZ* (C, blue) expression. Clones were induced during first (A,B) or late second/early third larval instar (C), and are marked either by absence (A,C) or presence (B) of GFP expression. Cells expressing both Ap (or *ap-lacZ*) and GFP appear yellow. (A) EGFR$^{\lambda}$-expressing cells can form an ectopic D compartment within the V compartment. Note that only some cells within the clone express *ap-lacZ* and form the ectopic D compartment, and that the ectopic D compartment is encircled by a stripe of Wg-expressing cells that flank the ectopic DV boundary. (B) Early-induced Ras^{V12}-expressing clones autonomously express *ap* and form an ectopic D compartment. (C) Late-induced Rho-expressing clones fail to induce ectopic Ap expression, but still retain the ability of inducing ectopic *mirr-lacZ* expression within the presumptive wing hinge.

did not express *ap-lacZ*. However, some ventrally situated clones included a discrete subset of cells that ectopically expressed *ap-lacZ* and induced cells along the *ap*^{ON}-*ap*^{OFF} interface to express Wg (Fig. 8A), as expected if the *ap-lacZ*-expressing cells formed an ectopic D compartment. We infer that these subpopulations of ectopic *ap-lacZ*-expressing cells derive from cells in which the level of ectopic EGFR$^{\lambda}$ activity exceeded a crucial threshold necessary to activate *ap* expression. Hence, we conclude that early, ectopic EGFR activation is sufficient to activate ap expression and recruit cells to become D compartment founders.

We next induced clones of cells that express Ras^{V12}, the constitutively active form of Ras. Consistent with our finding that Ras^{V12} is a more potent activator of the EGFR transduction pathway than EGFR$^{\lambda}$ (see above), we observed that most ventral clones that initiated Ras^{V12} expression during the second instar (~70% or 24/36) were associated with ectopic and cell-autonomous *ap-lacZ* expression in all cells within the clone, confirming that ectopic activation of the EGFR/Ras pathway can induce *ap* gene expression (Fig. 8B; Ras^{V12} expression initiates around 24 hours after clone induction, see Materials and Methods). However, clones that initiated Ras^{V12} expression during the late second or early third larval instar were only rarely associated with ectopic *ap-lacZ* expression (~10% or 5/37), even though most such clones (~80% or 70/88) located within the prospective wing hinge induce *mirr-lacZ* expression (not shown). Similar findings were also obtained for clones of cells that express Rho. In particular, clones of cells that initiate Rho expression during the late second or early third instar generally induced ectopic *mirr-lacZ* expression, but not ectopic *ap* expression (Fig. 8C).

Thus, ectopic activation of the EGFR/Ras pathway appears to induce ectopic *ap* expression during a discrete early period of wing disc development, but not thereafter. By contrast, Iro-C gene expression remains responsive to EGFR/Ras activation during subsequent wing disc development.

DISCUSSION

Insect segments develop in a modular fashion, undergoing a series of partitioning events that subdivide each segment into progressively smaller domains. We show that EGFR/Ras signaling controls the subdivision of the wing imaginal disc into body wall (notum) and limb (wing) primordia, and dorsal (D) and ventral (V) compartments, albeit by different mechanisms. We consider the source, identity and mode of action of the instructive EGFR signal(s) responsible for establishing these partitions in our accompanying paper (Zecca and Struhl, 2002).

The notum-wing subdivision: continuous maintenance of Iroquois Complex gene expression

Prospective notum cells are distinguished from wing cells by the activity of the Iroquois Complex (Iro-C) genes (Diez del Corral et al., 1999). Our results demonstrate (1) that activation of EGFR/Ras pathway is both necessary and sufficient to drive Iro-C gene expression in wing disc cells, and (2) that wing disc cells persistently monitor their level of EGFR/Ras input and are allocated to the wing or notum primordium on an ongoing basis, depending on the level of EGFR/Ras input they receive. This means that the wing-notum subdivision is not a stable compartmental partition between differently committed cell types, but rather a labile demarcation that reflects the current distribution of an instructive EGFR ligand.

Despite the provisional nature of the wing-notum segregation, the boundary between the two primordia is relatively straight and sharp. By manipulating EGFR/Ras signaling, we show that presumptive notum cells that lose the capacity to maintain Iro-C gene expression sort out of the notum primordium. Conversely, presumptive wing cells that ectopically activate the Iro-C genes sort out of the wing

primordium. Similar results have been obtained by altering Iro-C gene function directly, rather than through the manipulation of EGFR/Ras signaling (Diez del Corral et al., 1999). Taken together, these results suggest that Iro-C gene activity, under EGFR control, programs prospective notum cells to have a different affinity from prospective wing cells, thereby straightening and sharpening the boundary between the two primordia. Further support for such a mechanism comes from our experiments in which we generated clones of cells that ectopically express an activated form of Spi, an EGFR ligand, in the prospective wing hinge. All of the cells within these clones express the Iro-C genes and interdigitate freely with neighboring wild-type cells that are also induced to express the Iro-C genes. However, cells located further away do not receive sufficient Spi to activate Iro-C gene expression and these form a smooth boundary encircling the ectopic Iro-C-expressing cells.

Other non-compartmental partitioning events

The subdivision of the wing disc into wing and notum primordia resembles that of several other non-compartmental partitioning events that are correlated with the activation of other 'selector-like' genes such as *pnr*, *tsh*, *hth*, *vg*, *Dll*, *dac* and *ey* (reviewed by Mann and Morata, 2000). In most cases, the selector-like gene is expressed, or upregulated, in a relatively well-defined domain in response to known extracellular signals, such as Wingless (Wg) and Decapentaplegic (Dpp) (Zecca et al., 1996; Kim et al., 1997; Lecuit and Cohen, 1997; Neumann and Cohen, 1997; Casares and Mann, 2000), and in some cases (e.g. *Dll* in the leg disc and *pnr* in the notum), the activity of the selector-like gene is known to regulate cell affinity (Gorfinkiel et al., 1997; Campbell and Tomlinson, 1998; Wu and Cohen, 1999; Calleja et al., 2000). Thus, the wing-notum segregation may reflect a general mechanism for maintaining discrete regional primordia based on cell position rather than on cell ancestry.

The notum primordium, once established by the activation of Iro-C gene expression, is itself subdivided into distinct lateral and medial primordia by the localized activity of the *pnr* gene. *pnr* encodes a transcription factor that represses Iro-C gene expression and specifies medial as opposed to lateral notum differentiation (Calleja et al., 2000). *pnr* activity also causes medial cells to adopt a distinct affinity that prevents them from mixing with lateral cells (Calleja et al., 2000). It is tempting to speculate that *pnr* expression, like that of the Iro-C genes, is governed by EGFR signaling, e.g. being activated at a higher threshold concentration than the Iro-C genes, and hence in a smaller, more dorsally restricted domain. However, we find that cells do not require peak levels of EGFR/Ras activity to remain and develop normally within the medial primordium. Conversely, enhanced activation of the EGFR/Ras pathway does not appear to cause lateral cells to sort into the medial primordium or adopt medial characteristics (e.g. the loss of Iro-C gene expression). Instead, it seems that *pnr* expression and subdivision of the notum into medial and lateral domains may depend on other signals, such as Dpp (Sato and Saigo, 2000; Tomoyasu et al., 2000).

The relationship between *pnr* and the Iro-C gene expression in the notum is conserved in corresponding dorsolateral and dorsomedial regions of most of the adult segments, as well as in the embryonic and larval ectoderm (Calleja et al., 2000). Hence, it has been proposed that the deployment of these genes reflects a fundamental partitioning process reiterated in most or all body segments (Calleja et al., 2000). However, our analysis reveals significant differences in the way that the Iro-C genes are deployed in the wing disc compared with the eye-antenna disc, the only other context in which an equivalent analysis has been performed. First, during eye development, Iro-C gene expression is not governed by persistent signaling, in contrast to the wing disc. Instead, these genes are heritably activated early in eye development and behave as classical selector genes, performing a role that corresponds in most respects to that of *ap* in the wing (McNeill et al., 1997; Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Cavodeassi et al., 1999; Yang et al., 1999; Cavodeassi et al., 2000; Pichaud and Casares, 2000). Second, Iro-C gene expression is activated in the eye disc by Hedgehog and Wingless signaling, rather than by EGFR signaling (Cavodeassi et al., 1999). Thus, it appears that the Iro-C genes are activated by different signals and govern different types of partitioning events in these two contexts, raising the possibility that their deployment in other segments, and at other stages, may reflect similarly diverse inputs and developmental roles.

The DV compartmental segregation: heritable activation of *apterous*

As in the case of the Iro-C genes, we demonstrate that EGFR/Ras signaling is both necessary and sufficient to activate *ap* expression in early wing disc cells. Furthermore, we provide evidence that each wing disc cell chooses to express, or not to express, *ap* at this time, depending on its level of EGFR/Ras activation. However, in contrast to the Iro-C genes, the descendents of each cell then inherit this initial choice without further reference to EGFR/Ras signaling. The results of eliminating EGFR/Ras activity before the establishment of the DV compartments are particularly striking. Early loss of EGFR activity causes dorsally positioned cells within the disc to choose, incorrectly, to become V compartment founders. These cells and their descendents generally sort into the existing V compartment or out of the disc epithelium. In rare cases, they can form an ectopic V compartment within the D compartment. By contrast, later loss of EGFR activity has no effect on the DV compartmental segregation. These findings confirm and extend complementary results recently reported by Wang et al. (Wang et al., 2000), and establish that EGFR signaling is responsible for establishing the D and V compartments through the heritable activation of *ap*.

Although the Iro-C and *ap* genes are activated in overlapping dorsoproximal sectors of the early wing disc, the domain of *ap* expression expands relative to that of Iro-C gene expression during subsequent development, causing the DV boundary to be positioned up to 30 cell diameters ventral to the notum-wing boundary. We suggest that this shift occurs because *ap*-expressing cells no longer depend on EGFR/Ras input to continue to express *ap*. Hence, as *ap*-expressing cells within the notum primordium proliferate, some will move out of range of the instructive EGFR ligand, cease to express Iro-C genes and enter the wing primordium. In the accompanying paper (Zecca and Struhl, 2002), we provide evidence that this shift must occur in order for D and V compartment cells to interact to induce Wg and stimulate wing growth and differentiation.

Our results raise intriguing questions about the mechanism

of *ap* activation. For example, EGFR signaling induces *ap* expression only during a discrete window of opportunity during the second larval instar, even though EGFR signaling both precedes the initial activation of *ap* and continues thereafter. What makes the *ap* gene responsive to EGFR signaling only during this early window of opportunity? In addition, the state of *ap* gene expression during this period, whether 'on' or 'off', is inherited for the remainder of development. How are both states of expression rendered heritable? It is possible that a temporal signal, such as a flux of a unique combination of hormones (for example, ecdysone and juvenile hormone) or the unique prior history of signaling events in the early wing disc, might prime the *ap* locus for activation by EGFR signaling during this period. The state of expression chosen during this period might then be maintained subsequently by mechanisms involving positive autoregulation (for the 'on' state) or heritable silencing mediated by the Polycomb Group proteins (for the 'off' state). However, there is little evidence at present to support these speculations and the actual mechanisms remain unknown.

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