Egfr/Ras pathway mediates interactions between peripodial and disc proper cells in *Drosophila* wing discs

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Summary

All imaginal discs in *Drosophila* are made up of a layer of columnar epithelium or the disc proper and a layer of squamous epithelium called the peripodial membrane. Although the developmental and molecular events in columnar epithelium or the disc proper are well understood, the peripodial membrane has gained attention only recently. Using the technique of lineage tracing, we show that peripodial and disc proper cells arise from a common set of precursors cells in the embryo, and that these cells diverge in the early larval stages. However, peripodial and disc proper cells maintain a spatial relationship even after the separation of their lineages. The peripodial membrane plays a significant role during the regional subdivision of the wing disc into presumptive wing, notum and hinge. The Egfr/Ras pathway mediates this function of the peripodial membrane. These results on signaling between squamous and columnar epithelia are particularly significant in the context of in vitro studies using human cell lines that suggest a role for the Egfr/Ras pathway in metastasis and tumour progression.

Key words: *Drosophila*, Wing, Peripodial membrane, Ultrabithorax, Egfr/Ras pathway, Notum

Introduction

All imaginal discs in *Drosophila* are made up of two layers of epithelial cells. A layer of columnar epithelium representing the disc proper (DP) differentiates into adult tissues. Overlying the DP is a layer of squamous epithelium called the peripodial membrane (PM). Recent reports suggest that the PM is required for proper patterning of the DP during eye development (Gibson and Schubiger, 2000; Cho et al., 2000). Downregulation of signaling molecules such as Hh (Cho et al., 2000) and Serrate (Ser) (Gibson and Schubiger, 2000) in the PM alone is sufficient to affect eye development. This function has been shown to be dependent on microtubule extensions from the peripodial to the DP. It has been proposed that PM supplies inductive signals to DP through these cellular processes for disc patterning events.

Surgical ablation experiments suggest that the wing disc DP is capable of differentiating into wing blade without the PM, although the PM is required for proper development of the wing margin (Gibson and Schubiger, 2000). Those experiments did not address the role of PM in patterning events, as the PM was removed from late third instar larval discs. Subsequently, Gibson et al. (Gibson et al., 2002) showed that survival of PM cells requires Decapentaplegic (Dpp) signaling from the DP. More importantly, inhibition of Dpp signaling in the PM affected the growth of the entire disc (Gibson et al., 2002), suggesting a crucial role for the PM in wing development.

Although the PM has not been specifically studied for its role in wing development, it has been observed that several wing-patterning genes are expressed in PM cells. *puckered* (*puc*), a negative regulator of the JNK pathway, and *hemipterous* (*hep*), which encodes *Drosophila* JNK-Kinase, are expressed in cells at the medial edge of the wing disc PM (Agnes et al., 1999). The *hep* mutants show loss of Puc in the PM and, perhaps as a consequence, are defective in thorax closure. In addition, *engrailed* (*en*), *decapentaplegic* (*dpp*), *patched* (*ptc*), *combsgap*, Capichua (Cic), *teashirt* (*tsh*), Broad complex genes, *E74A*, *DHR3*, *hep* and *Ultrabithorax* (*Ubx*) are expressed in the PM of wing imaginal discs (White and Wilcox, 1985; Brower, 1987; Boyd et al., 1991; Emery et al., 1994; Lam et al., 1997; Panin et al., 1997; Agnes et al., 1999; Svendsen et al., 2000; Roch et al., 2002; Wu and Cohen, 2002). We have made use of GAL4 drivers derived from some of these genes (particularly *Ubx*-GAL4) to study the nature of interactions between the PM and DP, and in lineage tracing experiments to study the possible lineage relationship between the two.

Our results suggest the following.

(1) PM and DP cells arise from common precursor cells that form the embryonic wing imaginal primordium. These lineages get separated before the onset of the rapid proliferative phase of imaginal cells.

(2) Peripodial and disc proper cells maintain a spatial relationship even after their lineages diverge.

(3) Ras is required for the viability of PM cells.

(4) The PM plays a role in wing *vs* notum/hinge decision during early stages of wing development.

(5) The Egfr/Ras pathway mediates this function of the peripodial membrane.

Materials and methods

Genetics

Balancing mutations, making recombinant chromosomes and

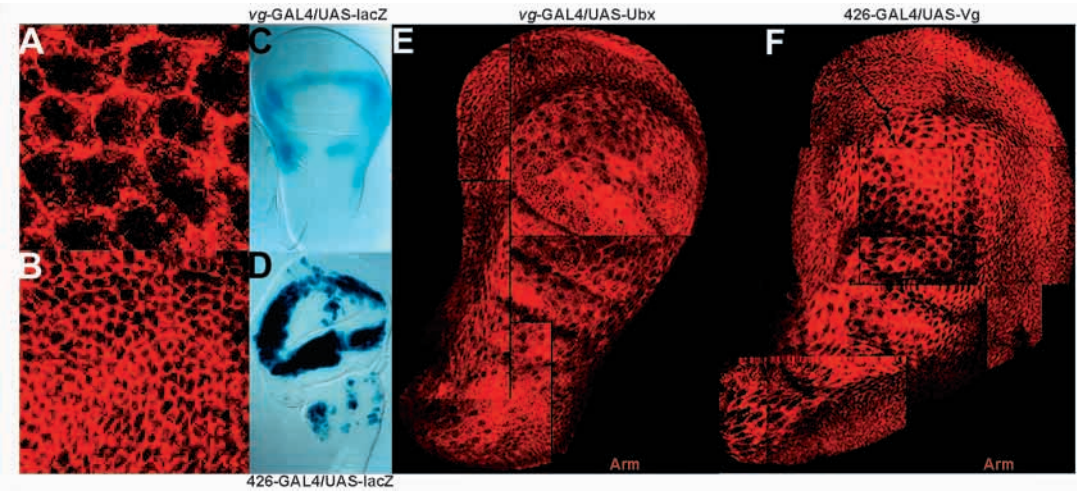

Fig. 1. Counting peripodial membrane cells. Wing discs of different genotypes were stained for Armadillo and large peripodial cells were counted on a laser-scanning confocal microscope at 60× magnification. (A) A single optical section of only peripodial membrane. Such optical sections were used to obtain a relative count of peripodial cells in different genetic backgrounds. (B) A single optical section of disc proper. (C,D) *vg*-GAL4 (C) and 426-GAL4 (D) expression patterns as seen with UAS-lacZ. (E,F) *vg*-GAL4/UAS-Ubx (E) and 426-GAL4/UAS-Vg (F) wing discs reconstructed from several optical scans showing large peripodial and small disc proper cells.

combinations of different mutations and/or markers were according to standard genetic techniques. FLP-FRT method (Xu and Rubin, 1993) was used for generating mitotic clones of *Ras*. *Ras*^{1} allele was recombined with P[FRT]82 πMyc and P[FRT]82 *Ubi*-GFP to generate P[FRT] *Ras*^{1} and P[FRT] *Ubi*-GFP *Ras*^{1} stocks, respectively. Clones were generated with the help of hsFLP using either *Ubi*-GFP or *forked* as clonal markers.

en- and *ptc*-GAL4 (Brand and Perrimon, 1993), *ap*- and *pnr*-GAL4 (Calleja et al., 1996), *AgiR*-GAL4 (Gibson et al., 2002), *vg*-GAL4 (Simmonds et al., 1995), and *tsh*-GAL4 [personal communication to FlyBase (Calleja, 1996.10.16)] drivers have been previously reported. 426-GAL4, *odd*-GAL4 (an insertion in *odd-paired* locus) and *coro*-GAL4 (insertion in *Drosophila* homologue of *coronin*) drivers were identified in the laboratory in a GAL4-enhancer trap screen. UAS lines used are UAS-Argos [personal communication to FlyBase (Michelson, 1999.8.9)], UAS-P35 (Hay et al., 1994), UAS-Ubx (Castelli-Gair et al., 1994), UAS-Vg (Kim et al., 1996) and dominant-negative forms of Serrate (UAS-DN-Ser) (Sun and Artavanis-Tsakonas, 1997), Glued (UAS-DN-Glu) (Allen et al., 1999), *Drosophila* Ras (UAS-Dras^{N17}) (Lee et al., 1996), human RAS (UAS-ras^{N17}) (Lee et al., 1996), Raf (UAS-DN Raf$^{3.1}$) (Martin-Blanco et al., 1999) and DER (UAS-DN-DER) (Golembo et al., 1996).

Ubx-GAL4 driver was generated using previously reported transposon-swapping strategy (Sepp and Auld, 1999). Casares et al. (Casares et al., 1997) have reported a *Ubx*-lacZ insertion (cytology: 89D6-9 and carried *ry*$^{+}$ marker), which reflects near-complete pattern of *Ubx* expression. In wing imaginal discs, it is expressed only in PM. We used *ptc*-GAL4 (cytology: 44D5-E1 and carried *w*$^{+}$ marker) as the donor GAL4-P element. Generation of *Ubx*-GAL4 strain was confirmed by testing the strain for the absence of *lacZ* by X-gal staining and for the presence of GAL4 by crossing to UAS-GFP. Lineage-tracing technique is essentially as described by Weigmann and Cohen (Weigmann and Cohen, 1999). *lacZ* clones were generated by crossing Actin5C>stop>lacZ to UAS-FLP;*Ubx*-GAL4 at 25°C. Third larval instar wing discs were stained for *lacZ* with anti-β-galactosidase.

Histology

X-gal staining and immunohistochemical staining were essentially as described by Ghysen and O'Kane (Ghysen and O'Kane, 1989) and Patel et al. (Patel et al., 1989). The *lacZ* reporter gene constructs used are *lio-lacZ* (Bolwig et al., 1995) and *aos-lacZ* (Freeman et al., 1992). The primary antibodies used are anti-Arm (Riggleman et al., 1990); anti β-galactosidase (Sigma, St Louis, MO), anti-Ci (Motzny and Holmgren, 1995); anti-Cut (Blochlinger et al., 1993); anti-En (Patel et al., 1989); anti-Ubx (White and Wilcox, 1984); and anti-Wg (Brook and Cohen, 1996). Anti-Arm and anti-Wg antibodies were obtained from the Development Studies Hybridoma Bank (University of Iowa, IA). Confocal microscopy was carried out on Meridian Ultima. The adult appendages were processed for microscopy as described before (Shashidhara et al., 1999).

Results

Peripodial membrane responds to changes in growth-patterns of disc proper

We first examined if PM cells respond to changes in cell proliferation patterns in the DP. In the wild type third instar wing disc, which contains ~50,000 columnar epithelial cells (Cohen, 1993), there are 400-450 squamous epithelial cells (smaller medial edge cells are not included in our estimate). Reduction in the size of DP because of mis-expression of Ubx in DV boundary cells (using *vg*-GAL4 and UAS-Ubx) (Shashidhara et al., 1999) resulted in a decrease in the number of peripodial cells (to less than 250) (Fig. 1E). Conversely, overgrowth induced by the overexpression of Vestigial (Vg) in the DP alone using 426-GAL4 (Fig. 1F), resulted in an increase in the number of peripodial cells (to more than 550). In both the experiments described above, growth pattern changes in the DP were induced at late stages, much after the formation of the PM, suggesting continuous interactions between the two layers.

PM-specific GAL4 drivers for studies on wing disc peripodial membrane

In addition to the genes mentioned above, we have observed that *lionette* (*lio*)-*lacZ*, *odd-paired* (*odd*)-GAL4 and *coronin* (*coro*)-GAL4 enhancer trap lines are expressed either exclusively or predominantly in the wing disc PM (Fig. 2A-C). En, which marks the posterior compartment of the DP, is expressed in the PM cells that overlay both anterior and posterior compartments (Fig. 2D).

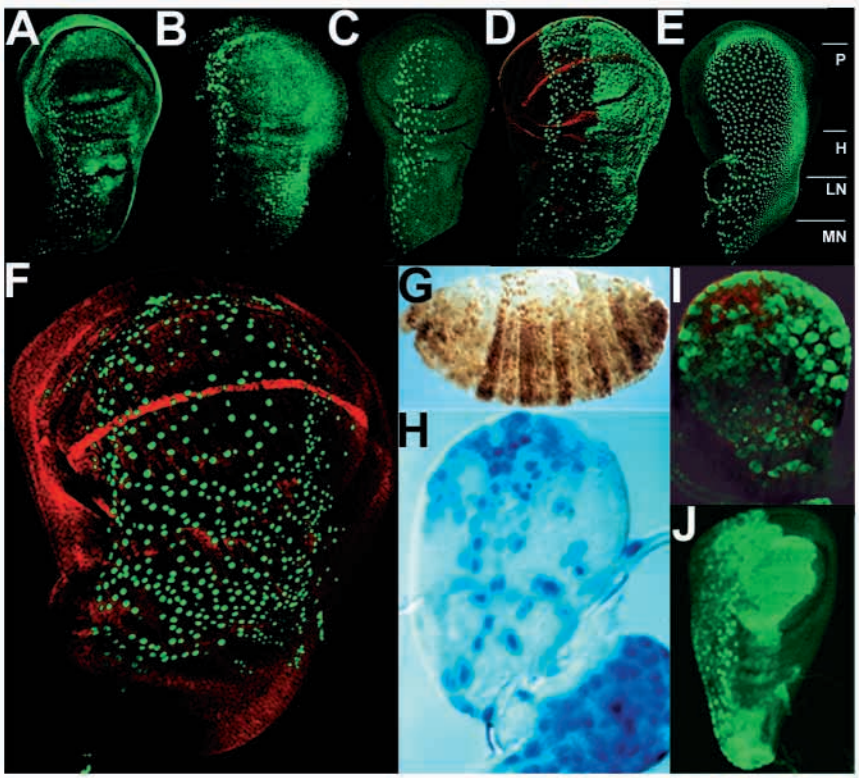

Fig. 2. Genes/enhancer-trap lines expressed in the wing disc peripodial membrane. (A-D) *lio*-lac*Z* (A), *odd*-GAL4/UAS-nuclear lac*Z* (B), *coro*-GAL4/UAS-nuclear lac*Z* (C) and *en*-GAL4/UAS-nuclear lac*Z* (D) wing discs stained with anti-β-galactosidase antibodies. (E) Wild-type wing disc stained with anti-Ubx antibodies. *coro*-GAL4 and Ubx are not expressed in the DP, whereas only Ubx is expressed in all PM cells. E also shows spatial domains of the DP. P, pouch; H, hinge, LN, lateral notum; MN, medial notum. (F-J) *Ubx*-GAL4 expression patterns during different stages of development. The expression of *Ubx*-GAL4 was detected by either anti-β-gal antibody staining (F,G,I,J) or X-gal staining (H). (F) In the wing disc, *Ubx*-GAL4 is expressed (green) only in the peripodial membrane. The disc proper is stained with anti-Wg antibody (red). (G) Stage 15 embryo showing lacZ expression in T2, T3 and in all abdominal segments. (H,I) Mid- to late second instar wing discs, showing PM-specific expression of *Ubx*-GAL4. In I, the disc proper is stained with anti-Wg antibody (red), which is expressed in the anterior-ventral quadrant of second instar discs. (J) *Ubx*-GAL4 expression pattern in the haltere disc. It is expressed in the disc proper as well as in the peripodial membrane. This GAL4 driver is predominantly expressed in the entire anterior compartment of the disc.

Ubx is the only gene known that is expressed in all cells of the wing disc PM (Fig. 2E) and is not expressed anywhere in the DP. We, therefore, generated a *Ubx*-GAL4 driver by swapping previously reported *Ubx-lacZ* insertion with *ptc*-GAL4 enhancer P-element (see Materials and methods). We confirmed that both embryonic and post-embryonic expression patterns of this *Ubx*-GAL4 driver are identical to the expression patterns of *Ubx-lacZ* strain. In third instar wing discs, *Ubx*-GAL4 is expressed in the entire PM and no expression is detected in the DP (Fig. 2F). We also followed its expression pattern in different stages of wing disc development (Fig. 2H,I). The wing imaginal primordium originates as a small cluster of 25 cells during mid-embryogenesis (Cohen, 1993). These cells remain quiescent in the embryo, but at the end of the first larval instar stage, they proliferate rapidly in response to ecdysone. Patterning events take place during this proliferation phase. *Ubx*-GAL4 is expressed in the wing imaginal primordium in stage 15 embryos (Fig. 2G). At mid to late second instar stage, when Wingless (Wg) is expressed in the anteroventral quadrant, we observed that *Ubx*-GAL4 expression is restricted to the PM (Fig. 2I). As expected, it is expressed in both DP and PM cells of haltere discs, although only in the anterior compartment (Fig. 2J).

Peripodial membrane and disc proper cells are clonally related

The anterior-most expression domain of *Ubx*-GAL4 is parasegment 4 (Fig. 2G). However, in the third instar wing disc, it is expressed only in the PM (Fig. 2F). We made use of this dynamic expression pattern of *Ubx*-GAL4 to determine the lineage relationship between PM and DP cells. We used a lineage-tagging method described by Weigmann and Cohen (Weigmann and Cohen, 1999) for this purpose. We generated Act5C>*lacZ*-expressing clones by crossing UAS-flp; *Ubx*-GAL4 to Act5C>stop>*lacZ*.

We examined a number of wing discs (n=42), some expressing *lacZ* only in the PM (n=20) and others in both PM and the DP. We did not observe any disc that expressed *lacZ* only in DP cells. Because in wing discs that expressed *lacZ* in both PM and DP there was no direct way of determining if *lacZ*-expressing cells correspond to a single clone, we characterized the nature of clones in the following way. All clones that expressed *lacZ* in both PM and DP were always large (Fig. 3A-D), whereas the size of PM-only clones varied from just two cells to more than 100 cells (Fig. 3E-H). In all wing discs that showed *lacZ* expression in both PM and DP cells, the ratio between *lacZ*-expressing PM and DP cells remained at around 1:80, similar to the ratio between PM to DP cells for the entire disc. Moreover, in a wing disc, the number of clusters of *lacZ*-expressing PM cells was always equal to or more than the number of clusters of *lacZ*-expressing DP cells. These observations suggest that PM and DP cells share a common lineage in the embryonic disc primordium and they become separated later during development. The similar ratio of PM to DP cells in clones and in the whole disc suggests that the proliferation pattern of PM cells is similar to that of DP cells. Wing imaginal primordium divides every 10-12 hours during three larval instars before the wing disc everts and starts differentiating (Cohen, 1993). We observed large PM-only clones comprising >128 cells (Fig. 3G,H). As such clones would have undergone seven or eight cell divisions, they must have been generated in the early first larval instar stage. Thus, it is likely that PM and DP cells are separated before the onset

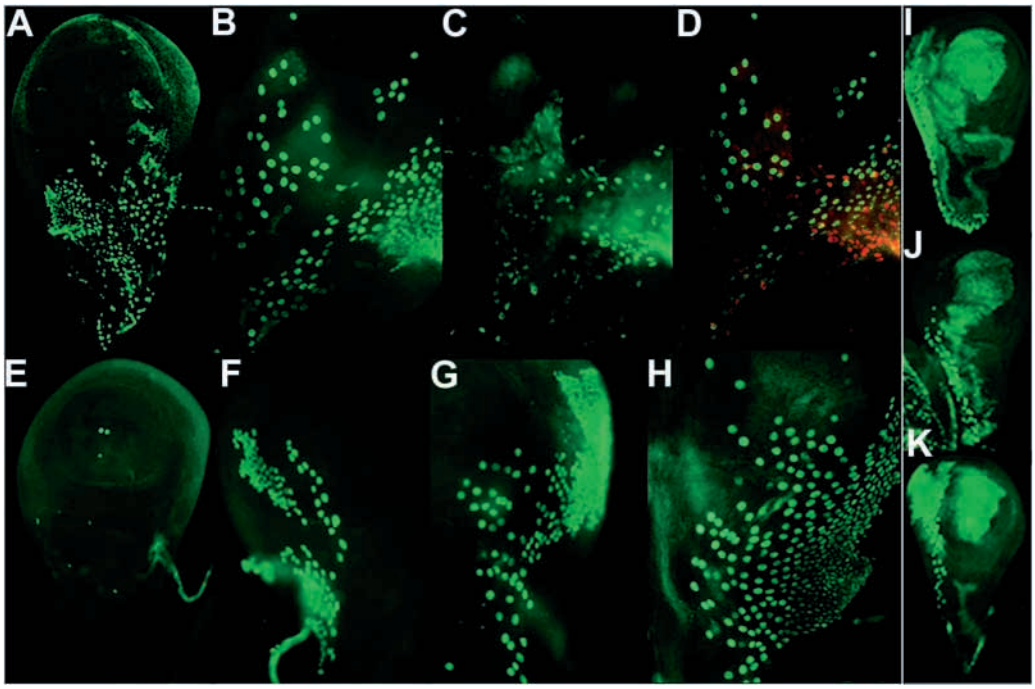

Fig. 3. Peripodial and disc proper cells are clonally related. Actin5C>*lacZ* clones were induced by *Ubx*-GAL4/UAS-FLP. All discs were stained with anti-β-galactosidase antibodies. (A) A wing disc showing clones in both peripodial membrane and disc proper. After confocal imaging, the optical sections were reconstructed using 3D imaging software, which would distinctly show both large peripodial and small disc proper cells. (B-D) Optical sections of a part of the same wing disc showing PM cells (B) and DP cells (C) at higher magnification. The optical section in C focuses on only a few DP cells in the field. The two optical sections were differently colour-coded and merged together to show spatial relationship of PM (green) and DP (red) clones (D). (E-H) Peripodial-only clones. Size of peripodial-only clones varied from just two cells (E) to >128 cells (H). In F-H, there appear to be more than one clone, as medial edge cells are also stained. We did not observe any disc with clone/s only in the disc proper. (I-K) Haltere discs showing both PM and DP clones. In some discs, the relative size of DP clones is much larger than those in wing discs (I-J). For example, in I, DP clone/s cover the entire anterior compartment.

or at the beginning of proliferation phase of the disc primordium. Because we observed large PM-only clones in virtually all parts of the wing disc, we also infer that *Ubx*-GAL4 continues to be expressed in all PM-progenitor cells and is switched-off in DP cells as soon as the lineages are separated.

In contrast to wing discs, the nature of DP and PM clones were very different in haltere discs, wherein *Ubx*-GAL4 is expressed both in PM and DP cells. All haltere discs ($n \geq 120$) had both PM and DP clones (perhaps owing to high levels of FLP at all stages of development). We did not observe any DP-only or PM-only clones. In addition, DP clones were much larger (Fig. 3I-K) and the ratio between DP and PM cells in such clones were much higher than for the entire haltere disc.

Interestingly, in wing discs that showed *lacZ* expression in both PM and DP cells, the clones were always within the same subdivision of the wing disc. For example, for every cluster of *lacZ*-expressing DP cells in the notum there was a cluster of *lacZ*-expressing PM cells overlaying the notum (Fig. 3D). A similar correlation was observed for the wing pouch. This suggests that clonally related PM and DP cells maintain a spatial relationship, which may be indicative of a role for PM in wing patterning.

Ras is required for the viability of PM cells

Ras/Egfr signaling is required for the survival of notum cells. A temperature sensitive allelic combination of *Egfr* ($Egfr^{tsla}/Egfr^{f24}$) shows severe reduction of notum (Wang et al., 2000) and mitotic clones of *Egfr* and *Ras* mutations do not survive in the notum (Zecca and Struhl, 2002). We generated loss-of-function mitotic clones of *Ras* to examine if it is required for the viability of PM cells. In one set of experiments, Ras^{-}/Ras^{-} clones were marked with GFP and in the other set Ras^{+}/Ras^{+} cells were marked with GFP. Consistent with the earlier reports, Ras^{-} clones (induced at 48 to 72 hours AEL) were generally viable in the pouch region but not in the notum (data not shown). We observed that in the PM too, Ras^{-}/Ras^{-} clones were associated with reduced cell viability (Fig. 4A). Interestingly, unlike in the DP (wherein Ras^{-} clones show differential viability in the notum and the pouch), Ras^{-} clones showed reduced viability in all parts of the PM.

Vn/Egfr signaling is also known to specify notum by antagonizing wing development and by activating notum-specifying genes (Baonza et al., 2000; Wang et al., 2000). Expression of dominant-negative forms of *Drosophila* Raf (UAS-DN-Raf) or Ras (UAS-$Dras1^{N17}$ or DN-Ras) using *en*-GAL4 induces notum/hinge-to-wing transformations (Baonza et al., 2000). In contrast to the above report, we did not observe any notum/hinge-to-wing transformation when we overexpressed DN-Ras using a DP-specific GAL4 driver, such as *ap*-GAL4. Instead, it caused cell death and consequent loss of notum (Fig. 4E), a phenotype consistent with the behaviour of mitotic clones of *Egfr* and *Ras* mutations. A possible reason could be higher levels of DN-Ras expressed from *ap*-GAL4 driver causing cell lethality that overrides its effect on notum specification. To examine this possibility, we co-expressed DN-Ras and the anti-apoptotic protein P35 (Hay et al., 1994). We observed significant rescue of lethality associated with DN-Ras, but still did not observe any notum/hinge-to-wing transformation (Fig. 4F). An alternative possibility is that *ap*-GAL4 driven expression of DN-Ras is not early enough to cause reversal of the notum fate. This possibility can be ruled out as downregulation of negative

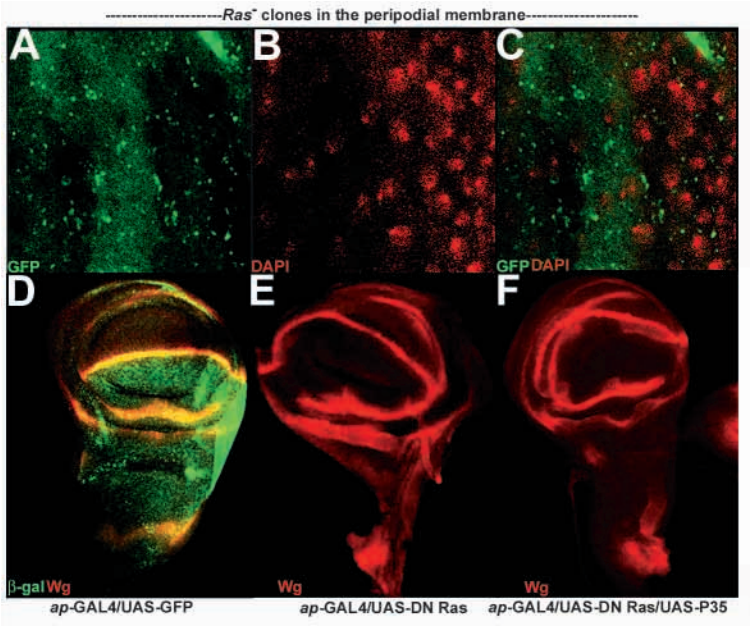

Fig. 4. Ras is required for the viability of PM cells. (A-C) hsFLP; P[FRT] *Ras1* Ubi-GFP /P[FRT]82B f^+ wing disc with Ras^1/Ras^1 mitotic clones in the PM. Ras^1/Ras^1 cells are marked with GFP (A). The disc is also stained for DAPI (B). C is the merge of A and B. Note that the clones are degenerating (note speckled pattern of GFP expression) and are occupied by surrounding normal cells. The regions without GFP expression represent Ras^+/Ras^+ cells (twin of Ras^1/Ras^1 clones), which survive normally. (D) *ap*-GAL4/UAS-nuclear *lacZ* wing disc stained for β-galactosidase and Wg expression. *ap*-GAL4 is expressed in the entire dorsal compartment. (E) *ap*-GAL4/UAS-$Dras1^{N17}$ wing disc stained for Wg expression. Note severe reduction in the notum size. (F) *ap*-GAL4/UAS-$Dras1^{N17}$; UAS-P35. DN-Ras-induced reduction in notum is partially rescued by P35.

regulators of Wg using *ap*-GAL4 driver causes notum-to-wing transformations (Collins and Treisman, 2000).

As *en*-GAL4 is expressed in both PM and DP cells (Fig. 2D), it is possible that the reported notum/hinge-to-wing transformation (caused by the overexpression of DN-Raf or DN-Ras using *en*-GAL4 driver) is mediated through PM. A parallel phenomenon has been demonstrated for eye discs. Overexpression of Fringed (Fng) results in identical phenotypes when expressed using either a PM-specific GAL4 (*c311*-GAL4) or a GAL4 driver (*ey*-GAL4), which is expressed in both PM and DP (Gibson and Schubiger, 2000).

Downregulation of the Egfr pathway in PM alone is sufficient to induce fate transformations in the disc proper

We re-examined the role of the Egfr pathway in specifying notum/hinge fate. We could not make use of Ras^- clones for this purpose, as Ras^- clones induced at early stages of development (coinciding with the time at which the Egfr pathway is required for both survival and specification of the notum) were always lethal. Because clones were induced during the rapid proliferation stage, they were generated always in large numbers, invariably in both PM and DP. Furthermore, owing to lethality associated with Ras^- clones in both PM and the notum, it was often difficult to confirm if we had generated PM-only clones, which made analysis of the effect of removal of *Ras* from PM cells inconclusive.

To circumvent the problem of direct genetic analysis of Ras function in PM, we used various GAL4 drivers and dominant-negative forms of DER, Ras and Raf or wild-type Argos (Aos; a negative regulator of the Egfr pathway) to downregulate the Egfr pathway in PM and/or DP cells. First, we overexpressed these proteins using the PM-specific GAL4 driver *Ubx*-GAL4. Overexpression of all the four proteins in the wing disc PM resulted in wing duplication in the posterior compartment (Fig. 5B-G). All the flies, which showed distinct pattern duplication, also had partial

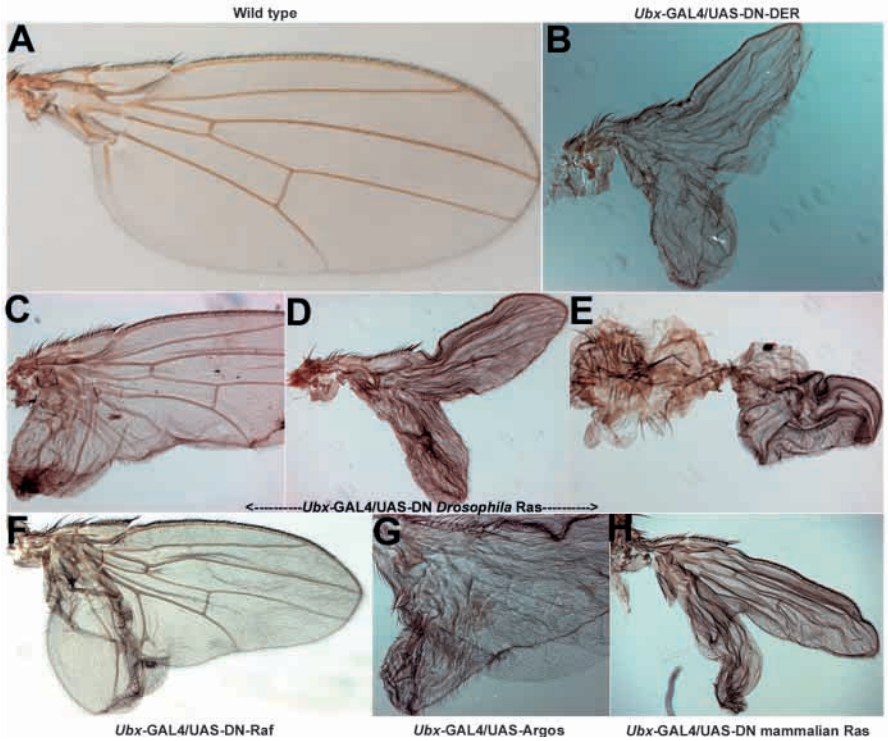

Fig. 5. Reduction in Egfr activity in the PM is sufficient to cause pattern duplications in the DP. (A) Wild-type wing blade. (B-H) Adult wing blades of *Ubx*-GAL4/UAS-DN-DER (expressed from two copies of the transgene; B), *Ubx*-GAL4/UAS-$Dras1^{N17}$ (C-E), *Ubx*-GAL4/UAS-DN-Raf (F), *Ubx*-GAL4/UAS-Aos (G) and *Ubx*-GAL4/UAS-ras^{N17} (dominant-negative form of mammalian Ras; H) flies. Note pattern duplications (notum-to-wing transformation) in the wing blades and anterior-specific margin bristles in the transformed region. Associated reduction in notum tissue is shown in E. In all wing blades, anterior is towards the top.

loss of notum and/or hinge (Fig. 5E). We also observed several pharate adults, which died within the pupal case, with severe loss of notum tissue. In such pharate adults, wing blade development was also severely affected, probably owing to defective notum development (data not shown). Ectopic expression of a dominant-negative form of the mammalian Ras (UAS-ras^{N17}) too showed the same phenotype when crossed to *Ubx*-GAL4 (Fig. 5H). Wing discs overexpressing DN-DER, DN-Ras, DN-Raf or Aos in the PM showed altered morphology, particularly overgrowth in the posterior hinge and mesonotum. When stained for Wg, they indicated a new pouch complete with the DV boundary (Fig. 6A-C). Double staining with Ci and Wg (Fig. 6D), and En and Wg (Fig. 6E) indicated that pattern duplication is associated with loss of notum, confirming notum/hinge-to-wing transformations. When stained for Ubx and Arm, transformed discs showed intact PM (Fig. 6F-I), which rules out loss of PM identity in the transformed wing discs. Interestingly, *Ras*$^{-}$ clones generated during larval stages do not survive in the notum (Zecca and Struhl, 2002) or in the PM (Fig. 4A). The overexpression of DN-Ras, however, causes cell lethality in the notum (Fig. 4E), but not in the PM (Fig. 6F-I). This observation suggests that notum cells are more sensitive to loss of Egfr pathway than are PM cells.

Ubx-GAL4 is expressed in the embryonic imaginal primordium that gives rise to both the DP and the PM. Is the observed phenotype, therefore, due to downregulation of the Egfr pathway in the imaginal primordium? Elaborate temperature shift experiments using a temperature-sensitive allele of *Egfr* (*Egfr*^{tsla}) have shown that DER signaling is required for notum specification between 55 and 72 hours after egg-laying (Baonza et al., 2000). Loss of Egfr signaling prior to 55 hours does not induce transformations. Our lineage analysis suggests that *Ubx*-GAL4 ceases to express in DP cells in the first instar larval stage itself. Therefore, observed notum/hinge-to-wing transformations cannot be attributed to the *Ubx*-GAL4 driven expression of DN-DER, DN-Ras, DN-Raf or Aos in the imaginal primordium. Additional evidence for this comes from the observation that overexpression of DN-Ras using *ap*-GAL4 driver causes cell lethality in the notum (Fig. 4E). Therefore, if in our experiments the Egfr pathway was downregulated in the DP in first or early second-instar stage (prior to the observed *Ubx*-GAL4 expression in the mid-second instar wing disc; Fig. 2I), we would have observed reduced notum and/or hinge rather than notum/hinge-to-wing transformations. Although we did observe reduced notum, it was always associated with expanded pouch (Fig. 6A-E), suggesting that reduction in the notum and/or hinge size is due to notum/hinge-to-wing transformations.

We further examined the specificity of the observed phenotype using a number of GAL4 drivers, which express either in the DP alone or in both DP and the PM. They are 426-, *pnr*-, *ap*- (all express only in the DP), *tsh* and *AgiR*- (express in both PM and DP) GAL4 drivers. 426-GAL4 driver is expressed only in the presumptive hinge (Fig. 7A) and *pnr*-GAL4 (Fig. 7D) is expressed only in the notum. However, both are late-expressing GAL4 drivers, beginning from early third instar stage. *ap*-GAL4 is an early expressing (second instar) driver and is expressed in the entire dorsal compartment. Overexpression of DN-Ras and Aos using 426- and *pnr*-GAL4 drivers did not show any phenotype (Fig. 7B,C,E,F). Their overexpression using *ap*-GAL4 driver resulted in severe reduction of the notum (Fig. 7H,I). Thus, although the effects on the viability of notum/hinge cells are consistent with the clonal analysis of Zecca and Struhl (Zecca and Struhl, 2002), we did not observe any cell fate transformations. *tsh*-GAL4 and *AgiR*-GAL4 drivers are expressed in the notum and hinge and also in the PM (Fig. 7J,M). Overexpression of DN-Ras and Aos using these drivers caused loss of notum, severe with *tsh*-GAL4 [which is expressed in the entire notum (Fig. 7K,L)] and partial with *AgiR*-GAL4 [which is expressed only in a subset of lateral notum cells (Fig. 7N,O)].

Thus, our results suggest that downregulation of the Egfr

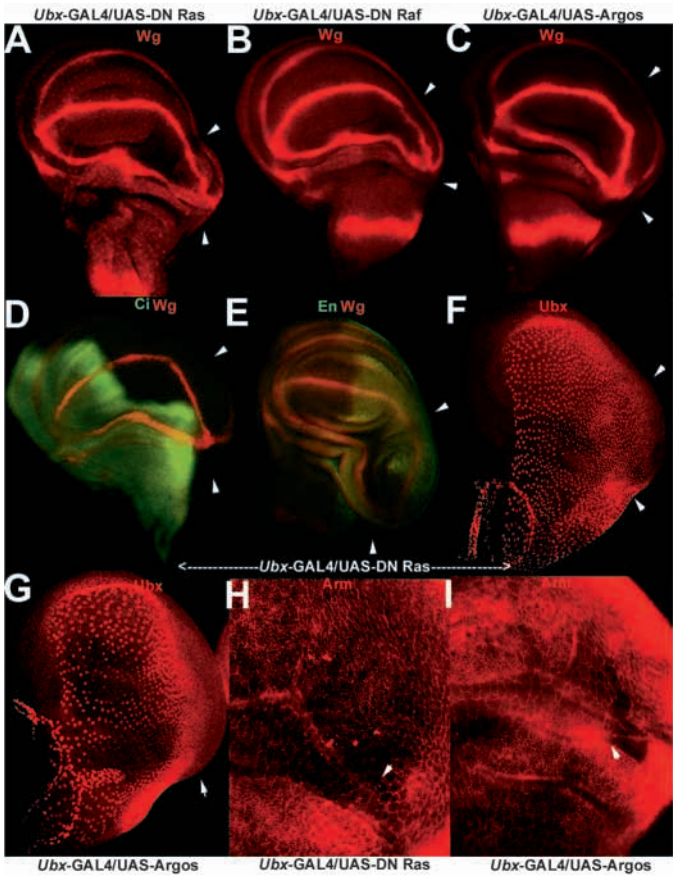

Fig. 6. Pattern duplications observed in flies over-expressing DN-DER, DN-RAS, DN-Raf and Aos in the PM reflect notum/hinge-to-wing transformations in wing discs. (A-C) *Ubx*-GAL4/UAS-Dras1^{N17} (A), *Ubx*-GAL4/UAS-DN-Raf (B) and *Ubx*-GAL4/UAS-Aos (C) wing discs stained for Wg. Note mesonotum-to-wing blade transformation as indicated by the over-growth and extension of the DV boundary to the mesonotum region (arrowheads). Larvae with such high degree of notum-to-wing transformation are invariably early pupal lethal. (D,E) *Ubx*-GAL4/UAS-Dras1^{N17} wing discs double stained with Ci and Wg (D), and En and Wg (E) to show pattern duplications (arrowheads). (F,G) *Ubx*-GAL4/UAS-Dras1^{N17} (F) and *Ubx*-GAL4/UAS-Aos (G) wing discs stained for Ubx. Note that Ubx expression in the PM is not altered in these discs. The figure also suggests that the identity of PM cells is not changed. (H,I) *Ubx*-GAL4/UAS-Dras1^{N17} (H) and *Ubx*-GAL4/UAS-Aos (I) wing discs stained for Arm. Only transformed part of discs are shown at higher magnification. Note that PM cells in these experiments have retained their normal morphology (arrowheads).

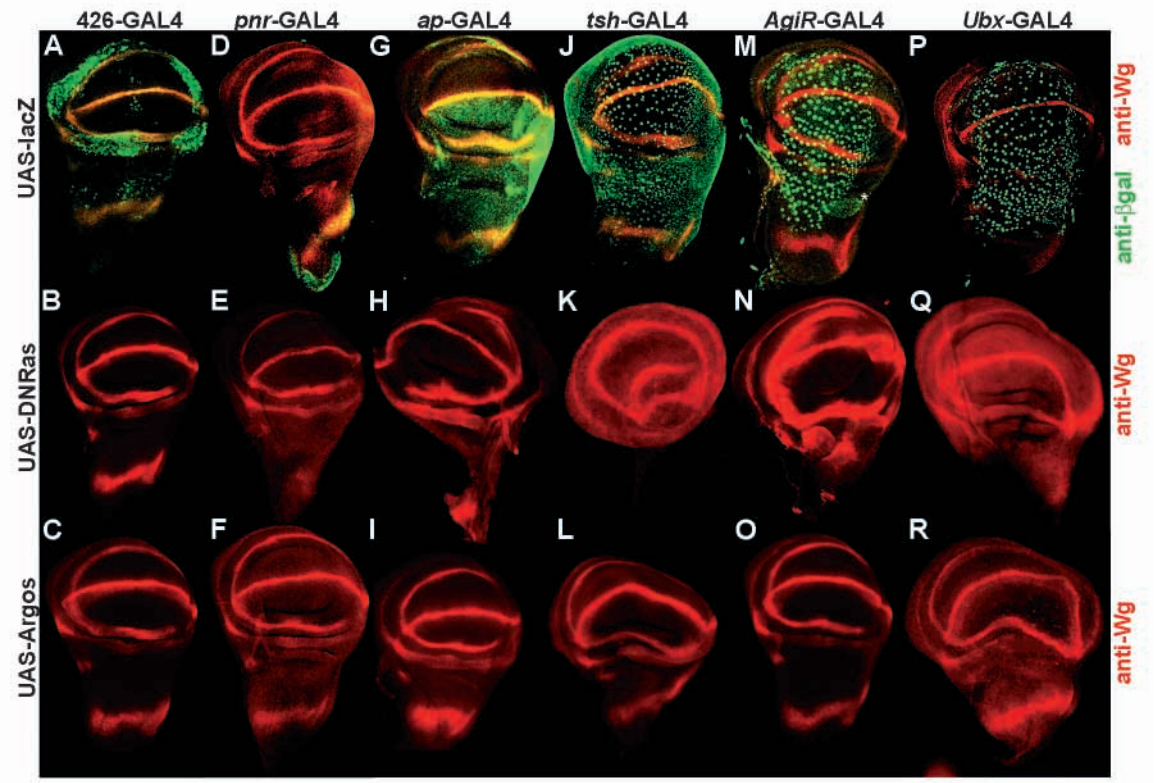

Fig. 7. Notum-to-wing transformation observed in *Ubx*-GAL4/UAS-Dras1^{N17} or *Ubx*-GAL4/UAS-Aos was due to their expression in the PM and not in the DP. All wing discs shown in this figure stained for Wg expression. Top panel shows the expression patterns of different GAL4 drivers (visualized with UAS-nuclear *lacZ*) used in this study. Middle panel shows the effect of overexpression of DN-Ras using those GAL4 drivers and the lower panel shows the effect of overexpression of DN-Aos. 426-GAL4 is expressed only in the presumptive hinge (A), whereas *pnr*-GAL4 in the presumptive lateral notum (D). Overexpression of DN-Ras (B,E) and Aos (C,F) using these two GAL4 drivers does not affect wing development, reflecting late (early to mid-third instar larval stages) activation of 426- and *pnr*-GAL4 expression. *ap*-GAL4 is expressed in the dorsal pouch and in the entire notum (G). Overexpression of DN-Ras (H) or Aos (I) using this GAL4 driver causes severe reduction in notum tissue; however, no transformation is observed. (J-M) *tsh*- (J) and *AgiR*-GAL4 (M) drivers are expressed in both DP and PM cells. In the DP, *tsh*-GAL4 is expressed in the hinge and in the presumptive lateral notum (J), whereas *AgiR*-GAL4 is expressed in the posterior mesonotum (asterisk in M). Overexpression of DN-Ras (K) and Aos (L) using *tsh*-GAL4 caused severe loss of notum tissue, and no pattern duplication is observed. DN-Ras overexpression using *AgiR*-GAL4 resulted in loss of notum tissue (N), whereas no phenotype was observed with Aos (O). (P-R) *Ubx*-GAL4 expression pattern (P) and the effect of overexpression of DN-Ras (Q) and Aos (R) using this GAL4 driver is shown again (for details, see Fig. 6) for comparison.

pathway in the PM alone is sufficient to induce fate transformations in the disc proper.

Dynamic expression of Aos in the peripodial membrane

Our results described above suggest a role for the Egfr pathway in the PM to specify notum/hinge identity. Whenever the Egfr pathway is activated, it induces the expression of Aos, which in turn acts as a negative regulator, thus keeping the pathway under tight feedback regulation (Golembo et al., 1996). Thus, expression of Aos marks the activity of the Egfr pathway. If the Egfr pathway active in the PM at any stage during development, it would be reflected in Aos expression pattern. We examined the expression pattern of *aos-lacZ*, which reflects endogenous Aos expression pattern (Freeman et al., 1992). In third instar wing imaginal discs, Aos is expressed both in the pouch and the notum (Fig. 8A). Double staining with Ubx indicated that Aos is not expressed in PM cells (Fig. 8B). Large cells that express Aos in the notum are adepithelial cells (myoblasts), which are marked by Cut (Ct) expression (Fig. 8C,D). We then examined if Aos-*lacZ* is expressed in earlier stages, which may reflect activation of Egfr during wing disc patterning. In early second instar wing discs, Aos is expressed in PM cells overlaying the posterior notum (Fig. 8E). Double staining with Ct suggested that the large cells expressing Aos are not myoblasts (Fig. 8E). Ct is expressed only in the anterior compartment and Aos is expressed only in the posterior compartment. Expression of Aos in the PM spreads in the mid- and late-second instar larval stages (Fig. 8F,G). Double staining with Ubx confirmed that Aos is expressed in PM cells, although its expression is mostly restricted to the PM cells overlaying dorso-posterior mesonotum (Fig. 8K). Only in the early third instar wing discs is Aos expression first seen in the pouch (Fig. 8L).

Thus, Aos expression in PM cells coincides (Fig. 8E-G) with the time at which Egfr pathway specifies notum development (Baonza et al., 2000) and its expression in the wing pouch coincides (Fig. 8L) with the stage at which the Egfr pathway is required to specify vein and intervein development (Diaz-Benjumea and Hafen, 1994). We therefore conclude that Egfr

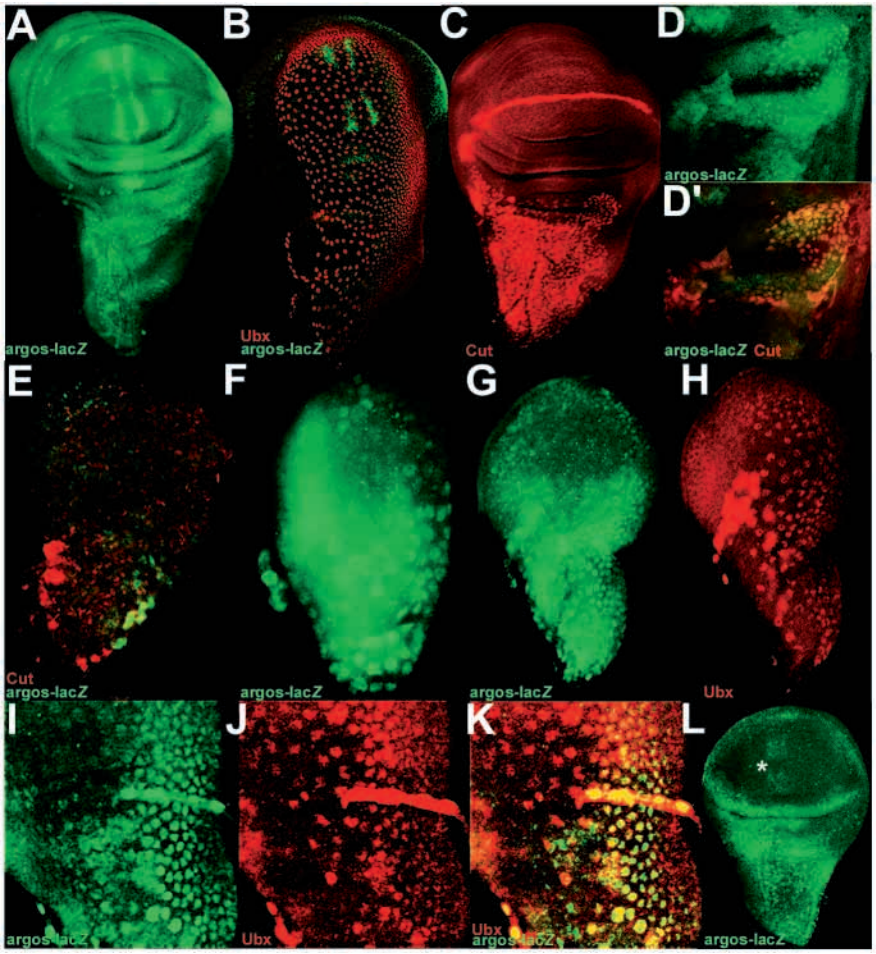

Fig. 8. Dynamic expression pattern of Aos in the wing imaginal disc. (A-B) Third instar *aos-lacZ* wing imaginal disc stained for β-galactosidase expression (green) and Ubx (red; shown only in B). Aos is expressed in the presumptive veins and in the notum. Note that *aos-lacZ* and Ubx are not co-localized, suggesting that at this stage of development Aos is expressed only DP cells. Large cells in that notum that express Aos are probably adepithelial cells. (C) Third instar wild type wing disc stained for Ct expression. In the notum Ct is expressed in the myoblasts. (D and D′) Part of the notum of *aos-lacZ* wing imaginal disc stained for β-galactosidase expression (green) and Ct (red; shown only in D′). Note co-localization of Aos and Ct in the myoblasts. (E,F) Early second instar *aos-lacZ* wing imaginal discs stained for β-galactosidase expression (green). Aos is expressed in large PM cells overlaying the notum as well as the pouch. Disc in E is also stained for Ct (red), which marks myoblasts. At this stage during development, myoblasts are localized to the anterior compartment, whereas Aos-expressing cells are in the posterior compartment. (G) Late second instar *aos-lacZ* wing imaginal disc. (H) Late second instar wild-type wing imaginal disc stained for Ubx expression. Note that Aos and Ubx share a similar expression pattern. (I-K) Late second instar *aos-lacZ* wing imaginal disc stained for both β-galactosidase (green; I) and Ubx (red; J) expression. K is the merge of I and K. Note co-localization of Aos and Ubx in PM cells. (L) Early third instar *aos-lacZ* disc. Aos expression in the pouch (asterisk) begins at this stage during development.

function is required in the PM to specify notum and hinge development, and to suppress wing blade development.

Discussion

Drosophila genetics offer several tools to study complex developmental events. One such developmental event is the wing disc patterning and subsequent development and differentiation of the wing and the notum. Although wing development is by and large one of the well studied developmental systems in *Drosophila*, the role of PM in wing development is poorly understood. Recently, Gibson et al. (Gibson et al., 2002) have shown that viability of wing disc PM cells depend on Dpp signal coming from DP cells. Although reduced viability of PM cells has an effect on the wing disc morphology, it is not known if the PM has any specific role during wing development. We have generated *Ubx*-GAL4 driver, which in wing discs is expressed only in the PM. Using this GAL4 driver in lineage-tracing experiments, we have observed that PM and DP cells arise from common embryonic imaginal primordium. Their lineages get separated before the onset of rapid proliferative phase of imaginal cells. However, further investigation is needed to identify precisely those cells of the imaginal primordium that give rise to PM cells and the molecular mechanism involved in the specification of the PM.

We have observed that PM and DP cells maintain a spatial relationship even after the separation of their lineages. Thus, notum cells are clonally related to PM cells overlaying the notum and pouch cells are clonally related to PM cells overlaying the pouch. Morphologically, PM cells over the pouch and the notum form two distinct groups. In the notum region, PM cells are densely populated and they send out microtubule extensions to DP cells through the disc lumen (Gibson and Schubiger, 2000; Cho et al., 2000). Over the wing pouch, PM cells appear to be less densely arranged and are bigger and flatter. The expression patterns of various gene markers further divide the PM into distinct spatial domains (compare the expression patterns of *lio*-lacZ, *odd*-GAL4, 409-GAL4 and *AgiR*-GAL4). These spatial domains may represent specific functional domains. Taken together, these observations point to a more developmental function for the wing disc PM, rather than just the provision of structural support to the growing DP. As there is a large difference (1:80) in the number of clonally related PM and DP cells, the PM may influence the growth and/or patterning of a relatively larger region of the wing disc rather than individual DP cells.

A significant finding of our study is the role of the PM in wing/notum/hinge decision. The wing disc is initially divided into anterior and posterior compartments by virtue of En expression only in a subset of disc cells. Later, it is subdivided into three distinct groups of cells, wing, notum and the hinge.

This is marked by the expression of Wg in the presumptive wing region (Ng et al., 1996), Pnr in the presumptive notum (Calleja et al., 2000) and Tsh in the presumptive hinge (Casares and Mann, 2000; Wu and Cohen, 2002). PM cells over the notum and the pouch may provide positional cues for notum/hinge-wing decision. We have observed that the Egfr pathway functions in the PM to specify notum-specific genes and/or to inhibit wing-specific genes. Mis-expression of DN-DER, DN-Ras, DN-Raf or Aos in the PM was enough to induce notum/hinge-to-wing transformations. The dynamic expression pattern of Aos marks the spatial and temporal pattern of Egfr activation. In the second instar larval stage, when wing-notum decision is made, Aos is expressed specifically in those PM cells that overlay the posterior notum. Once the wing-notum decision is made, Aos expression recedes from PM cells and it starts expressing in the notum-associated myoblasts and in the pouch. Although at this stage we cannot rule out the possibility that the Egfr pathway is required in both PM and DP cells to specify notum fate, our results suggest that the Egfr pathway mediates interactions between PM and DP cells during the notum/hinge specification.

All the observations on notum/hinge-to-wing transformations in this report and elsewhere (Wang et al., 2000; Baonza et al., 2000) are restricted to the posterior compartment. However, ectopic expression of Wg can cause notum-to-wing transformation in both the anterior and posterior compartments (Ng et al., 1996). En is expressed in large number of PM cells that overlay part of the anterior compartment. Ubx, which is expressed only in the posterior compartment of T2 (parasegment 5), is expressed in all PM cells. In addition, overexpression of Hh in PM cells does not induce ectopic *dpp-lacZ* expression in those cells (P.K., unpublished observations). These observations suggest posterior identity of all PM cells. Is this the reason for observed notum/hinge-to-wing transformations only in the posterior compartment? If the answer is yes, how is the notum specified in the anterior compartment? Further investigation is needed to determine the compartmentalization within the PM and compartment-specific interactions between PM and DP.

Interestingly, the only other function so far attributed to the wing disc PM is dorsal closure of the notum (Agnes et al., 1999). Does the PM have a role in patterning other regions of the wing disc? At later stages during wing patterning, the Egfr signaling is required to specify *apterous* (*ap*) expression in the dorsal compartment, and thereby to specify dorsoventral axis formation (Wang et al., 2000). In addition, it is required to specify the vein and intervein development (Diaz-Benjumea and Hafen, 1994). The Egfr pathway is also implicated in signaling from the dorsoventral organizer (Nagaraj et al., 1999). However, we did not see any phenotype in the wing pouch following the expression of DN-DER, DN-Ras, DN-Raf or Aos in PM cells. It is possible that the Egfr pathway is functional from DP itself to specify Ap expression. Alternatively, as the Egfr pathway plays a more permissive role than instructive role in specifying Ap expression (Zecca and Struhl, 2002), lowering of its activity in the PM alone may not be sufficient to affect DV patterning events. After the specification of dorsal and ventral compartments, *fng* and *Ser* interact to activate Notch (N) in the DV boundary. Ser is known to express and function in the PM of eye imaginal discs (Gibson and Schubiger, 2000; Cho et al., 2000). Ectopic expression of a dominant-negative form of Ser (DN-Ser) in the eye disc PM affects ommatidial patterning (Gibson and Schubiger, 2000). Ectopic expression of DN-Ser in the wing disc PM using *Ubx*-GAL4 did not affect wing patterning (data not shown). These results suggest differences in the function of the PM in eye and wing discs.

Precise genetic ablation of PM cells during wing patterning may provide insights, if the PM has a role in patterning other regions of the wing disc. We did try to ablate wing disc PM cells genetically using UAS-FLP, *Ubx*-GAL4 and UAS-FLPout-Ricin (Hidalgo et al., 1995). However, in all our experiments animals were invariably early larval lethal, presumably owing to Ricin expression in other tissues. To circumvent this problem, one would need more precise GAL4 driver that, while being specific to the PM in wing discs, is not expressed in other tissues. Nevertheless, our results on signaling from DP to regulate growth properties of PM cells and the Egfr signaling from PM to DP cells to specify notum establish bi-directional signaling between the two epithelial layers.

Although our results suggest a role for the PM in specifying notum development, further investigation is required to determine how the Egfr pathway is activated in PM cells. Does DP play a role in activating the Egfr pathway in PM cells, which in turn specifies notum development in the DP? This would be analogous to oocyte-follicle cell interactions, wherein Gurken expressed in the oocyte is responsible for the activation of Egfr pathway in the follicle cells. Subsequently, follicle cells signal to the oocyte, which results in the re-organization of the cytoskeleton of the latter. Identification of the ligand for Egfr in PM cells and the signal that goes from PM to DP may lead us to the mechanism by which it is activated in PM cells. Because only PM cells over the notum send out microtubule extensions, any molecule that signals to DP to specify notum development may depend on these processes. Such microtubule extensions have also been observed in the eye disc and are shown to be required for proper signaling from PM to DP. Overexpression of a dominant-negative form of glued (DN-Glu), a component of microtubule-binding motor complex proteins in the eye disc PM causes delay in the progression of the morphogenetic furrow (Gibson and Schubiger, 2000). However, we did not see any effect of expressing DN-Glu in the wing disc PM using *Ubx*-GAL4 driver (P.K., unpublished observations). The role, if any, of microtubule extensions in the wing disc would probably be independent of the motor complex. Further investigation is needed to identify signal molecules involved in PM to DP interactions.

Identification of signal transduction pathways that mediate interactions between different types of epithelial sheets during normal development may provide us with clues to understand development of tissues and pathological situations leading to metastasis. In vitro studies using human cell lines suggest a role for Egfr/Ras signaling in cell motility and tumour invasion (Krueger et al., 2001; Mahoney et al., 2002; Lotz et al., 2003). We have reported that the Egfr pathway mediates signaling between squamous and columnar epithelia. Further studies on the interaction between PM and DP may help identifying several key factors mediating cancer progression.

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References

Agnes, F., Suzanne, M. and Noselli, S. (1999). The *Drosophila* JNK pathway controls the morphogenesis of imaginal discs during metamorphosis. *Development* **126**, 5453-5462.

Allen, M. J., Shan, X., Caruccio, P., Froggett, S. J., Moffat, K. G. and Murphey, R. K. (1999). Targeted expression of truncated *Glued* disrupts giant fiber synapse formation in *Drosophila. J. Neurosci.* **19**, 9374-9384.

Baonza, A., Roch, F. and Martin-Blanco, E. (2000). DER signaling restricts the boundaries of the wing field during *Drosophila* development. *Proc. Natl. Acad. Sci. USA* **97**, 7331-7335.

Blochlinger, K., Jan, L. Y. and Jan, Y. N. (1993). Postembryonic patterns of expression of *cut*, a locus regulating sensory organ identity in *Drosophila*. *Development* **117**, 441-450.

Bolwig, G. M., del Vecchio, M., Hannon, G. and Tully, T. (1995). Molecular cloning of *linotte* in *Drosophila*: a novel gene that functions in adults during associative learning. *Neuron* **15**, 829-842.

Boyd, L., O'Toole, E. and Thummel, C. S. (1991). Patterns of E74A RNA and protein expression at the onset of metamorphosis in *Drosophila*. *Development* **112**, 981-995.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brook, W. J. and Cohen, S. M. (1996). Antagonistic interactions between *wingless* and *decapentaplegic* responsible for dorsal-ventral pattern in the *Drosophila* leg. *Science* **273**, 1373-1377.

Brower, D. L. (1987). *Ultrabithorax* gene expression in *Drosophila* imaginal discs and larval nervous system. *Development* **101**, 83-92.

Calleja, M., Moreno, E., Pelaz, S. and Morata, G. (1996). Visualization of gene expression in living adult *Drosophila*. *Science* **274**, 252-255.

Calleja, M., Herranz, H., Estella, C., Casal, J., Lawrence, P., Simpson, P. and Morata, G. (2000). Generation of medial and lateral dorsal body domains by the pannier gene of *Drosophila*. *Development* **127**, 3971-3980.

Casares, F. and Mann, R. S. (2000). A dual role for homothorax in inhibiting wing blade development and specifying proximal wing identities in *Drosophila*. *Development* **127**, 1499-1508.

Casares, F., Bender, W., Merriam, J. and Sanchez-Herrero, E. (1997). Interactions of *Drosophila Ultrabithorax* regulatory regions with native and foreign promoters. *Genetics* **145**, 126-137.

Castelli-Gair, J., Greig, S., Micklem, G. and Akam, M. (1994). Dissecting the temporal requirements for homeotic gene function. *Development* **120**, 1983-1995.

Cho, K.-O., Chern, J., Izaddoost, S. and Choi, K.-W. (2000). Novel signaling from the peripodial membrane is essential for eye disc patterning in *Drosophila*. *Cell* **103**, 331-342.

Cohen, S. M. (1993). Imaginal disc development. In *The Development of* Drosophila melanogaster, pp. 747-841. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Collins, R. T. and Treisman, J. E. (2000). Osa-containing Brahma chromatin remodeling complexes are required for the repression of Wingless target genes. *Genes Dev.* **15**, 3140-3152.

Diaz-Benjumea, F. J. and Hafen, E. (1994). The Sevenless signalling cassette mediates *Drosophila* EGF receptor function during epidermal development. *Development* **120**, 569-578.

Emery, I. F., Bedian, V. and Guild, G. M. (1994). Differential expression of Broad-Complex transcription factors may forecast tissue-specific developmental fates during *Drosophila* metamorphosis. *Development* **120**, 3275-3287.

Freeman, M., Klambt, C., Goodman, C. S. and Rubin, G. M. (1992). The *argos* gene encodes a diffusible factor that regulates cell fate decisions in the *Drosophila* eye. *Cell* **69**, 963-975.

Ghysen, A. and O'Kane, C. (1989). Neural enhancer-like elements as specific cell markers in *Drosophila*. *Development* **105**, 35-52.

Gibson, M. C. and Schubiger, G. (2000). Peripodial cells regulate proliferation and patterning of *Drosophila* imaginal discs. *Cell* **103**, 343-350.

Gibson, M. C., Lehman, D. A. and Schubiger, G. (2002). Lumenal transmission of Decapentaplegic in *Drosophila* imaginal discs. *Dev. Cell* **3**, 451-460.

Golembo, M., Schweitzer, R., Freeman, M. and Shilo, B. (1996). *Argos* transcription is induced by the *Drosophila* EGF receptor pathway to form an inhibitory feedback loop. *Development Suppl.* **122**, 223-230.

Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* **120**, 2121-2129.

Hidalgo, A., Urban, J. and Brand, A. H. (1995). Targeted ablation of glia disrupts axon tract formation in the *Drosophila* CNS. *Development* **121**, 3703-3712.

Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, K., Magee, J. and Carroll, S. B. (1996). Integration of positional signals and regulation of wing formation and identity by *Drosophila* vestigial gene. *Nature* **382**, 133-138.

Krueger, J. S., Keshamouni, V. G., Atanaskova, N. and Reddy, K. B. (2001). Temporal and quantitative regulation of mitogen-activated protein kinase (MAPK) modulates cell motility and invasion. *Oncogene* **20**, 4209-4218.

Lam, G. T., Jiang, C. and Thummel, C. S. (1997). Coordination of larval and prepupal gene expression by the DHR3 orphan receptor during *Drosophila* metamorphosis. *Development* **124**, 1757-1769.

Lee, T., Feig, L. and Montell, D. J. (1996). Two distinct roles for Ras in a developmentally regulated cell migration *Development* **122**, 409-418.

Lotz, M., Wang, H. H., Cance, W., Matthews, J. and Pories, S. (2003). Epidermal growth factor stimulation can substitute for c-Src overexpression in promoting breast carcinoma invasion. *J. Surg. Res.* **109**, 123-129.

Mahoney, M. G., Simpson, A., Jost, M., Noe, M., Kari, C., Pepe, D., Choi, Y. W., Uitto, J. and Rodeck, U. (2002). Metastasis-associated protein (MTA)1 enhances migration, invasion, and anchorage-independent survival of immortalized human keratinocytes. *Oncogene* **21**, 2161-2170.

Martin-Blanco, E., Roch, F., Noll, E., Baonza, A., Duffy, J. B. and Perrimon, N. (1999). A temporal switch in DER signaling controls the specification and differentiation of veins and interveins in the *Drosophila* wing. *Development* **126**, 5739-5747.

Motzny, C. K. and Holmgren, R. (1995). The *Drosophila* cubitus interruptus protein and its role in the *wingless* and *hedgehog* signal transduction pathways. *Mech. Dev.* **52**, 137-150.

Nagaraj, R., Pickup, A. T, Howes, R., Moses, K., Freeman, M. and Banerjee, U. (1999). Role of EGF receptor pathway in the growth and patterning of the *Drosophila* wing through the regulation of *vestigial*. *Development* **126**, 975-985.

Ng, M., Diaz-Benjumea, F. J., Vincent, J. P., Wu, J. and Cohen, S. M. (1996). Specification of the wing by localized expression of wingless protein. *Nature* **381**, 316-318.

Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-912.

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S. (1989). Expression of engrailed proteins in arthropods, annelids and chordates. *Cell* **58**, 955-968.

Riggleman, B., Schedl, P. and Wieschaus, E. (1990). Spatial expression of the *Drosophila* segment polarity gene *armadillo* is posttranscriptionally regulated by Wingless. *Cell* **63**, 549-560.

Roch, F., Jimenez, G. and Casanova, J. (2002). EGFR signalling inhibits Capicua-dependent repressionduring specification of *Drosophila* wing veins. *Development* **129**, 993-1002.

Sepp, K. J. and Auld, V. J. (1999). Conversion of lacZ enhancer trap lines to GAL4 lines using targeted transposition in *Drosophila melanogaster*. *Genetics* **151**, 1093-1101.

Shashidhara, L. S., Agrawal, N., Bajpai, R., Bharathi, V. and Sinha, P. (1999). Negative regulation of dorsoventral signaling by the homeotic gene *Ultrabithorax* during haltere development in *Drosophila*. *Dev. Biol.* **212**, 491-502.

Simmonds, A. J., Brook, W. J., Cohen, S. M. and Bell, J. B. (1995). Distinguishable functions for *engrailed* and *invected* in anterior-posterior patterning in the *Drosophila* wing. *Nature* **37**, 424-427.

Sun, X. and Artavanis-Tsakonas, S. (1997). Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in *Drosophila*. *Development* **124**, 3439-3448.

Svendsen, P. C., Marshall, S. D. G., Kyba, M. and Brook, W. J. (2000). The combgap locus encodes a zinc-finger protein that regulates cubitus interruptus during limb development in *Drosophila* melanogaster. *Development* **127**, 4083-4093.

Wang, S.-H., Simcox, A. and Campbell, G. (2000). Dual role for *Drosophila* epidermal growth factor receptor signaling in early wing disc development. *Genes Dev.* **14**, 2271-2276.

Weigmann, K. and Cohen, S. M. (1999). Lineage tracing cell born in different domains along the P/D axis of the developing *Drosophila* leg. *Development* **126**, 3823-3830.

White, R. and Wilcox, M. (1984). Protein products of the Bithorax complex in *Drosophila*. *Cell* **39**, 163-171.

White, R. and Wilcox, M. (1985). Distribution of Ultrabithorax proteins in *Drosophila*. *EMBO J.* **4**, 2035-2043.

Wu, J. and Cohen, S. M. (2002). Repression of Teashirt marks the initiation of wing development. *Development* **129**, 2411-2418.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.

Zecca, M. and Struhl, G. (2002). Subdivision of the *Drosophila* wing imaginal disc by EGFR-mediated signaling. *Development* **129**, 1357-1368.