Wg and Egfr signalling antagonise the development of the peripodial epithelium in *Drosophila* wing discs

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Summary

Imaginal discs contain a population of cells, known as peripodial epithelium, that differ morphologically and genetically from the rest of imaginal cells. The peripodial epithelium has a small contribution to the adult epidermis, though it is essential for the eversion of the discs during metamorphosis. The genetic mechanisms that control the identity and cellular morphology of the peripodial epithelia are poorly understood. In this report, we investigate the mechanisms that pattern the peripodial side of the wing imaginal disc during early larval development. At this time, the activities of the Wingless (Wg) and Epidermal growth factor receptor (Egfr) signalling pathways specify the prospective wing and notum fields, respectively. We show that peripodial epithelium specification occurs in the absence of Wingless and Egfr signalling. The ectopic activity in the peripodial epithelium of any of these signalling pathways transforms the shape of peripodial cells from squamous to columnar and resets their gene expression profile. Furthermore, peripodial cells where Wingless signalling is ectopically active acquire hinge identity, while ectopic Egfr activation results in notum specification. These findings suggest that suppression of Wg and Egfr activities is an early step in the development of the peripodial epithelium of the wing discs.

Key words: Peripodial epithelium, *Drosophila*, Squamous cells, Imaginal discs, Wing disc, Patterning, Wingless, Egfr

Introduction

The development of *Drosophila* imaginal discs, from which most of the adult epidermis derives, is a classical model for the study of pattern formation. Small groups of cells of the embryonic ectoderm are genetically specified in stereotyped positions to constitute the primordia of imaginal discs (Cohen, 1993). Later on, during larval development, the primordia invaginate and become flat epithelial sacs with two opposing faces enclosing the disc lumen (Cohen, 1993). In mature imaginal discs, both sides present easily noticeable differences in cell morphology and cell density (Fig. 1A), maybe with the exception of genital discs (Littlefield and Bryant, 1979). One side contains most of the cells of the disc in the form of a columnar monolayered epithelium, while the other face (peripodial side) consists of squamous cells, in the peripodial epithelium (PE), surrounded by cubic cells (Fig. 1A) (Auerbach, 1936; Waddington, 1940). The PE is required, together with the cells of the stalk, for the eversion of the discs during metamorphosis (Fristrom and Fristrom, 1993; Milner et al., 1984) (J.C.P.-P., E. Martín-Blanco and A. García-Bellido, unpublished), and their subsequent closure (Agnes et al., 1999; Martin-Blanco et al., 2000; Usui and Simpson, 2000; Zeitlinger and Bohmann, 1999), but has a small contribution to the adult cuticle (J.C.P.-P., E. Martín-Blanco and A. García-Bellido, unpublished). Cubic cells produce adult pleural structures (Bryant, 1978; Sprey and Oldenhave, 1974). The columnar and cubic cells, therefore, are imaginal cells in the strictest sense, as they eventually give rise to all the adult epidermal structures except for the abdomen (Bryant, 1978).

Cell proliferation in the imaginal discs during larval development is coordinated with progressive, genetically determined territorial segregation. Pattern formation and territorial segregation in imaginal discs have been extensively studied, but little attention has been drawn to the development of the peripodial side of the discs. Cell lineage analyses in the PE of the wing imaginal disc show preferential growth territories associated to gene expression domains, although the anteroposterior border is the only clonal restriction described so far in the PE (Resino et al., 2002). Recent reports suggested that several signalling proteins produced in the PE of the disc, such as Wingless (Wg), Hedgehog (Hh) and Decapentaplegic (Dpp), might be necessary for the proliferation and patterning of imaginal discs, either released by cell contact through translumenal extensions (Cho et al., 2000; Gibson and Schubiger, 2000) or secreted into the disc lumen (Gibson et al., 2002). These findings underline the relevance of exploring the development of the PE in order to understand how the size and pattern of the whole wing disc is established.

At the end of the third instar, the peripodial side of the wing imaginal disc consists of several cell types with different morphology (Fig. 1A) and gene expression profiles (Fig. 1D,F). The central territories in the peripodial side of the wing imaginal disc consist of around 400 squamous cells that constitute its PE. *Ultrabithorax* (*Ubx*) (Fig. 1D) and *puckered* (*puc*), among other genes (Gibson and Schubiger, 2001), are

expressed in the PE territory. *Ubx*, although expressed in all posterior cells of the embryonic primordium of the wing imaginal disc, is later restricted to its peripodial side (Brower, 1987). The cubic distal cells show differential expression of *zinc finger homeodomain 2* (*zfh-2*) (Fig. 1F) and *dachsous* (*ds*) (not shown), while proximal cubic cells express the genes of the *iroquois complex* (*iro-C*) (Fig. 1F). These genes expressed in cubic cells are also expressed in the wing-notum side of the disc, where their contribution to the patterning of the disc has been studied. The expression of *zfh-2* depends on Wg signalling (Whitworth and Russell, 2003) and is required, together with *ds*, for wing hinge specification (Clark et al., 1995; Whitworth and Russell, 2003). The *iro-C* genes, however, specify different territories in the notum (Diez del Corral et al., 1999) and their expression there depends on Epidermal growth factor receptor (Egfr) signalling (Wang et al., 2000; Zecca and Struhl, 2002).

The complementary and mutually exclusive activities of the Wg and Egfr signalling pathways are responsible for the subdivision of the wing-notum side of the wing disc into proximal (notum) and distal (wing and hinge) territories early during the second larval instar (Baonza et al., 2000; Klein, 2001; Wang et al., 2000; Zecca and Struhl, 2002). These signalling pathways are activated, respectively, by the diffusible ligands Wg (Ng et al., 1996; Williams et al., 1993) and Vein (Vn) (Simcox et al., 1996). At this stage, the expression domain of *wg* is restricted to a sector of anterior distal cells (Williams et al., 1993), while the expression domain of *vn* is restricted to a central line of proximal cells (Simcox et al., 1996). Loss of function of the Wg pathway prevents the development of distal structures, thus allowing the expansion of Egfr activity to distal territories. Conversely, ectopic Wg activity represses Egfr signalling in the notum, causing wing duplications (Baonza et al., 2000), though loss of Egfr function does not allow expansion of Wg signalling (Wang et al., 2000). Published reports only evaluate the activity of both signalling pathways in the wing-notum side of the disc, despite the diffusive ability of Wg and Vn. The wing field is later subdivided into wing blade and wing hinge territories, by the combined action of Wg signalling and Vestigial (wing blade) (Baena-Lopez and Garcia-Bellido, 2003; Klein and Martínez-Arias, 1999) or Wg signalling alone (wing hinge) (Baena-Lopez and Garcia-Bellido, 2003; Klein, 2001; Whitworth and Russell, 2003).

In this article, we analyse the mechanisms of genetic specification of the wing disc PE. We show that the PE is a third developmental field set in the early development of the wing disc, different from the wing and notum fields, with distinct genetic requirements. We also show that Wg and Egfr signalling pathways are active up to the border between cubic and squamous cells in the peripodial side of the wing disc. Correct development of the PE does not require the activity of these signalling cascades. Furthermore, Wg or Egfr signalling transform the morphology and genetic specification of squamous cells into those characteristic of territories in the wing-notum side.

Materials and methods

Fly strains

We have used the following fly strains: *UAS-E-cadherin^{intra5}* (Sanson et al., 1996), *UAS-wingless^{G}* (Klein and Arias, 1998), *UAS-Ras^{V12}* (constitutive activator of the Egfr pathway) (Karim and Rubin, 1998), *UAS-DNRaf$^{3.1}$* (=UAS-phl.KMRaf3.1) (dominant negative effect on the Egfr pathway) (Martin-Blanco et al., 1999), *UAS-vein* (Schnepp et al., 1998), *UAS-armadillo Δα* (constitutive activator of the Wg pathway, unable to target adherens junctions) (White et al., 1998), *UAS-vestigial^{Z}* (Paumard-Rigal et al., 1998) and *apterous-Gal4* (Calleja et al., 1996). The *zfh-2^{MS209} GAL4* line (Capdevila and Guerrero, 1994) expresses GAL4 in the same pattern as the gene *zfh-2* (Whitworth and Russell, 2003).

Clonal analysis

Gain-of-function clones were generated by the FLP/FRT technique (Chou and Perrimon, 1992). All the *UAS* fly strains were crossed with *y w hsp70-flp; Act FRT y^{+} FRT Gal4 UAS-GFP* (Ito et al., 1997) and heat shocked for 7 minutes at 37°C at 24-48 or 48-72 hours after egg laying (AEL).

Wg gain-of-function clones were also generated by the MARCM technique (Fig. 3B,E,G) (Lee and Luo, 1999). *y w hsp70-flp Tubulinα1-Gal4 UAS-GFPnls; UAS-wg^{G}; Tubulinα1-Gal80 FRT2A/ FRT2A* (Struhl and Greenwald, 2001) larvae were heat shocked at 37°C for 15 minutes at 48-72 hours AEL.

No less than 20 clones were examined for each genotype.

Immunohistochemistry

Dissected larvae were fixed for 20 minutes in a 4% paraformaldehyde solution in PBT (PBS/0.1% Tween 20) and immunostained with mouse anti-Ubx (provided by Ernesto Sanchez-Herrero), rat anti-Iro-C (provided by Juan Modolell), rat anti-Zfh-2 (provided by Martha Lundell), mouse anti-Armadillo (Arm) (Hybridoma Bank), mouse anti-Discs large 1 (Dlg1) (Hybridoma Bank), mouse anti-Wg (Hybridoma Bank), rabbit anti-Vg (provided by Sean Carroll), rabbit anti-Distalless (Dll) (provided by Sean Carroll), rat anti-Ds (provided by Michael Simon), mouse anti-Nubbin (Nub) (provided by S. Cohen), rat anti-Tailup (Tup) (provided by Jim Skeath) or guinea pig anti-Eyegone (Eyg) (provided by Natalia Azpiazu) antibodies in PBT-BSA (PBT/0.3% BSA). Alexa Fluor-488-, -546- (Molecular Probes) or Cy5 (Jackson InmunoResearch)-conjugated secondary antibodies were used to detect primary antibodies. F-actin staining was performed with Texas Red-coupled phalloidin (Sigma). Cell nuclei were stained for 20 minutes in a 1 mM To-Pro-3 iodide (Molecular Probes) solution in PBT. Imaginal discs were mounted in Vectashield (Vector Laboratories, Inc).

Microscopy and image treatment

Images were acquired in a BioRad 2000 confocal microscope and treated with the Metaview (Universal Imaging) and Photoshop 7.0 (Adobe Corp) image programs.

Results

Early development of the peripodial side of the wing imaginal disc

At the beginning of the second larval instar, all cells of the wing imaginal disc show columnar morphology (Fig. 1B). Soon after, in mid second instar, some proximal cells at the disc peripodial side start to change their shapes from columnar to squamous (Fig. 1C). In the third instar disc, squamous cells fill most of the peripodial side, constituting its PE (Fig. 1D). The differences in cell shape, however, are preceded by differences in gene expression between the two sides of the disc (Fig. 1B). In mid second instar, the squamous cells, as well as surrounding cubic cells in the peripodial side of the disc, express high levels of *Ubx* (Fig. 1C). Later on, in the third instar disc, high levels of *Ubx* are maintained only in squamous cells, while posterior cubic cells show a decreasing gradient of

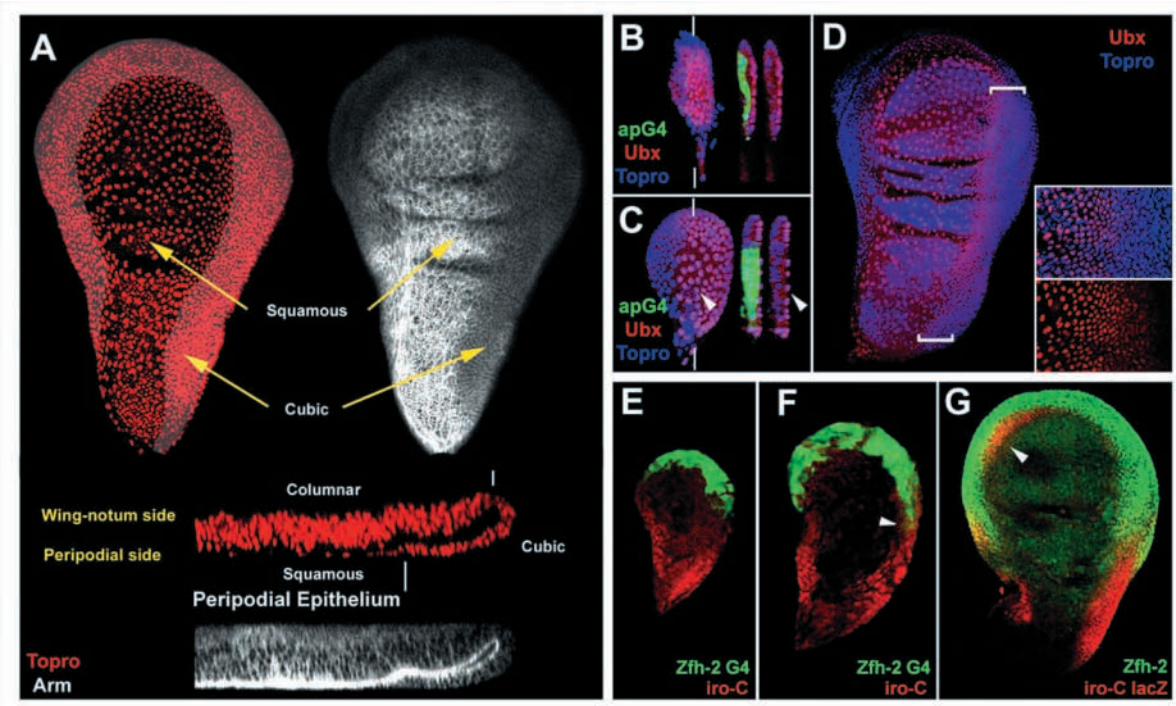

Fig. 1. Development of the peripodial side of the wing disc. (A) PE structure in a third instar wing disc. Characteristic low-density nuclear distribution and squamous cell morphology are noticed in the PE. Nuclei are stained with the DNA dye TO-PRO-3 (red) and Arm localization in the *zonula adherens* (white) reveals cell shape. A white shadow delimits the surrounding cubic cells. The different cell types are described in a transverse section of a disc. (B-D) *Ubx* expression in the developing PE. In mid second instar (B), major differences in cell shape throughout the disc are not observed. In late second instar (C), squamous cells are seen in a proximal territory of the peripodial side of the disc (arrowheads). At this time, the expression of *Ubx* is still in poor correlation with squamous morphology. GFP expression in the wing-notum side of second instar discs, driven by *ap*-GAL4, allows comparison of cell shape between the two sides of the disc in the transversal sections. In third instar discs (D), *Ubx* is expressed in squamous and some cubic cells. Notice the decreasing gradient of *Ubx* expression in cubic cells (brackets and inset at higher magnification). (E-G) *zfh-2* (green) and *iro-C* (red) expression domains in developing wing discs. In early mid second instar (E), *zfh-2*^{MS209} *GAL4* (see Materials and methods) and *iro-C* are expressed in proximal and distal cubic cells, respectively. Note that in early third instar discs (F) *zfh-2* and *iro-C* domains now overlap in cubic posterior cells (arrowhead). During late larval development (G), *iro-C-lacZ* expression appears in some distal cubic cells (arrowhead). Discs are oriented anterior towards the left and distal upwards in all panels and figures.

Ubx expression (Fig. 1D). The expression of *Ubx*, although it is a good genetic marker of the wing disc PE, is not required for PE development, as loss-of-function clones of *Ubx* are normal in size and shape and do not modify peripodial cell morphology (L. de Navas and E. Sanchez-Herrero, personal communication).

Proximodistal (PD) territorial segregation in the wing-notum side of the wing disc proceeds simultaneously to the morphological changes of peripodial cells, and it is driven by the complementary and mutually exclusive activities of the Wg and Egfr signalling pathways (Baonza et al., 2000; Klein, 2001; Wang et al., 2000; Zecca and Struhl, 2002). In the second instar, cubic cells express *zfh-2* and *iro-C* in distal and proximal domains, respectively (Fig. 1E). The early expression pattern of these genes, which is dependent on Wg and Egfr signalling (Baonza et al., 2000; Klein, 2001; Wang et al., 2000; Whitworth and Russell, 2003; Zecca and Struhl, 2002) suggests that the mechanism of PD segregation is also active in the peripodial side of the disc, although their expression pattern later evolves to overlap in some regions (Fig. 1F,G). In this context, proximal cubic cells would be part of the notum developmental field, whereas distal cubic cells would be part of the wing developmental field. Interestingly, Zfh-2 and Iro-C are not detected in the PE. Also, the limit of expression of Zfh-2 and Iro-C is sharp and coincident with the limit between cubic and squamous cells.

Wg and Egfr signalling activities are not required for PE development

The absence of expression of Wg and Egfr signalling downstream genes suggests that the activity of both signalling pathways might be repressed in the prospective PE from embryonic development or early larval stages in order to generate a difference between the wing and notum fields and the PE of the disc. To evaluate the role of Wg and Egfr signalling in the development of the peripodial side of the wing imaginal disc, we generated loss-of-function clones for both signalling pathways at 24-48 and 48-72 hours after egg laying (AEL).

Clones expressing the intracellular domain of E-cadherin (E-Cadh^{intra5}) autonomously lack Wg signalling because of sequestering of Armadillo (Arm) (Sanson et al., 1996), the transcriptional effector of Wg signalling, consistent with the cytoplasmic accumulation of Arm we observe (Fig. 2A). E-Cadh^{intra5} overexpression early in the prospective wing field abolishes its development, causing notum duplications and a complete lack of Wg function (Sanson et al., 1996; Sharma and Chopra, 1976). These clones do not survive or are smaller in

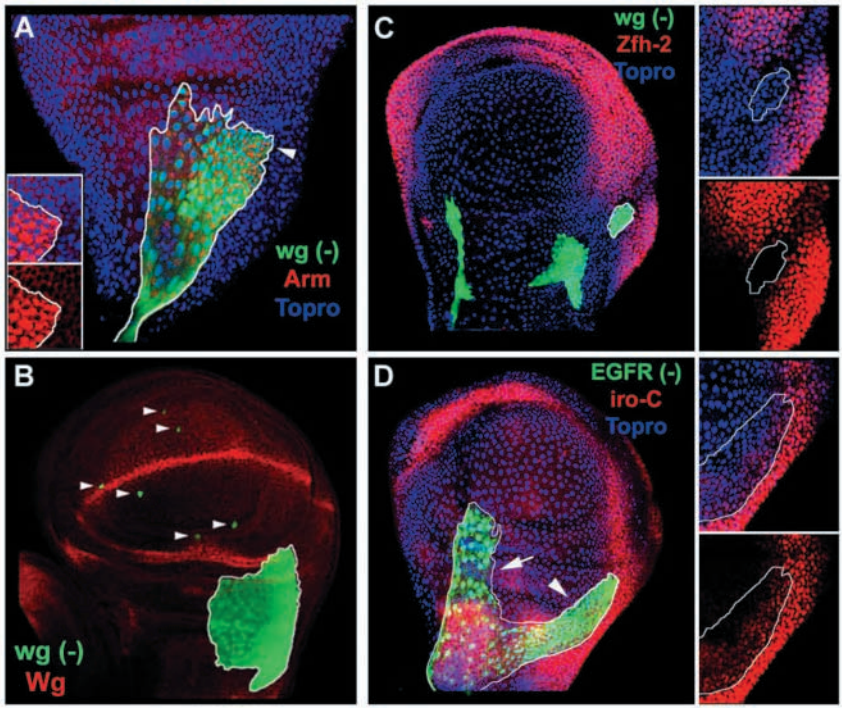

Fig. 2. Wg and Egfr signalling pathways are not required for the development of the PE. (A-C) Clones lacking Wg signalling because of E-Cadh^{intra5} overexpression (green). These clones accumulate the Wg signalling transcriptional effector Arm (red) in the cytoplasms (A, 24-48 hours AEL). The arrowhead indicates the region of the clone magnified in the insets. (B) E-Cadh^{intra5} overexpression reduces the proliferation and survival of wing-notum cells (arrowheads), but have no effect in peripodial cells (outlined clone). Wg is detected in red. Clones generated 24-48 hours AEL. (C) These clones also eliminate the expression of *zfh-2* (red) in cubic cells (inset at higher magnification of the outlined clone). Clones generated 48-72 hours AEL. (D) Clones lacking Egfr signalling because of overexpression of DN-Raf$^{3.1}$ (green, 24-48 h AEL) reduce *iro-C* expression (red) in cubic cells (inset at higher magnification of the region pointed by the arrowhead). The same clone does not affect the proliferation and survival of squamous cells (arrow).

size than controls in the wing-notum side of the disc, but are normal in size in the PE (Fig. 2B). In addition, these clones eliminate the expression of *zfh-2* in distal cubic cells of the peripodial side (Fig. 2C).

To analyse the requirement of Egfr signalling, we repressed Egfr activity in clones of cells by overexpressing a dominant-negative form of the Raf protein (DNRaf$^{3.1}$) (Martin-Blanco et al., 1999), which is required for the intracellular transduction of the signal. The expression of DNRaf$^{3.1}$ in early larval development reduces notum territories (Baonza et al., 2000; Wang et al., 2000; Zecca and Struhl, 2002) and causes wing duplications (Baonza et al., 2000). We found that clones lacking Egfr signalling have a normal size in the PE, but show reduced expression of *iro-C* in cubic cells (Fig. 2D).

The above results show that the activities of the Wg and Egfr signalling pathways are required for the normal development of cubic cells, but not for the proliferation, survival and genetic specification of the PE. Finally, we do not observe modifications in cell morphology when E-Cadh^{intra5} and DN-Raf$^{3.1}$ are co-expressed in cubic cells (not shown). This indicates that the development of the PE has specific genetic requirements that preclude an expansion of the peripodial field to adjacent wing and notum territories in the absence of Wg and Egfr activities.

Ectopic Wg signalling transforms the cell morphology and fate of the PE into those of the wing hinge

To further evaluate the role of Wg signalling in the development of the peripodial side of the wing disc, we generated *wg* expressing clones at 24-48 and 48-72 hours AEL. In these clones, the shape of PE cells changes from squamous into the columnar morphology characteristic of cells in the wing-notum side of the disc (Fig. 3A,B,E). The transformation is not only cell autonomous but affects cells non-autonomously within a range of 7-15 cellular diameters away from the clone (Fig. 3B). The severity of the morphological change decreases with distance to the clone, which suggests a dependence on Wg concentration (Fig. 3B). By contrast, autonomous activation of Wg signalling in Arm$\Delta\alpha$ overexpressing clones only results in autonomous transformations of the cellular morphology and genetic identity of PE cells (Fig. 3D). The increase in cell density induced by both Wg and Arm$\Delta\alpha$ in the PE may be due not only to the change in cell shape, but also to a higher rate of proliferation, evident when the size of these clones is compared with controls.

In addition to cell morphology changes, ectopic Wg in the PE induces autonomous and non-autonomous expression of characteristic wing hinge genetic markers such as *ds* (Fig. 3E) and *zfh-2* (Fig. 3F), and represses *Ubx* in the PE (Fig. 3C). Wg-expressing clones never induce the expression of wing blade or notum markers such as *vestigial* (*vg*), *distalless* (*dll*), *iro-C* and *eyegone* (*eyg*) (not shown). These data show that ectopic Wg signalling transforms the PE cells into cells of the wing hinge. Interestingly, ectopic expression of Wg induces autonomous expression of *nubbin* (*nub*), a transcription factor expressed in the proximal hinge and wing blade. However, in contrast to the strictly nuclear wild-type localization of Nub, these clones show high levels of Nub in the cytoplasm of the transformed cells (Fig. 3G). This result points to an incomplete transformation of the PE into wing hinge, which suggests that the PE is refractory to this transformation. The ability of ectopic Wg to transform the PE into wing hinge is independent of the developmental time when the clones are generated, suggesting that repression of Wg activity may be essential for the correct development of the PE. Clones expressing Wg also induce *zfh-2* expression in proximal cubic cells (Fig. 3F) and repress the expression of *iro-C* (not shown), which again suggests that the mechanism that drives PD segregation in the wing-notum side is also active in cubic cells.

Considering a possible transmission of Wg signalling

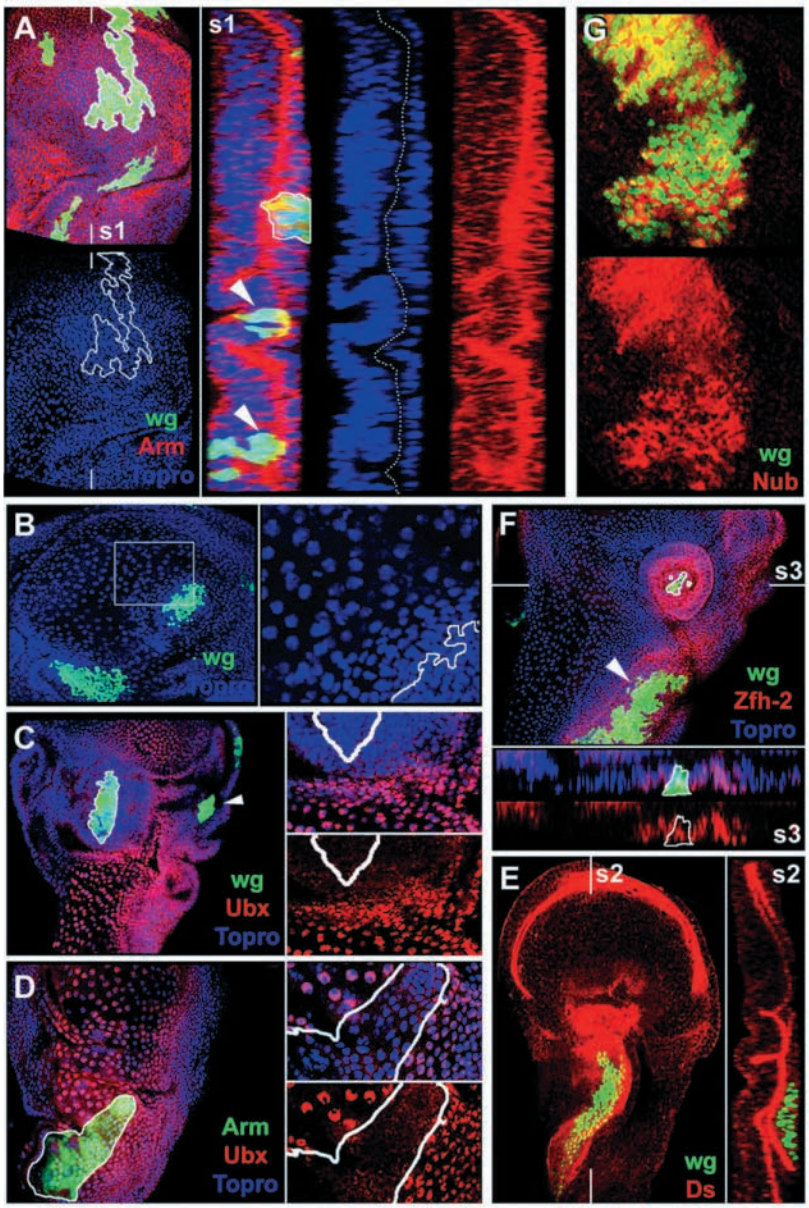

Fig. 3. Ectopic Wg signalling transforms the identity and cell morphology of the PE into wing hinge. (A) *wg*-expressing clones (green, 48-72 hours AEL) transform both cell autonomously and non-autonomously squamous peripodial cells into columnar cells, as seen both in a surface view and a longitudinal section of the outlined clone. Arm (red) and TO-PRO-3 (blue) staining reveal cell shape and tissue structure (apical membranes of both sides of the disc separated by a dotted line in the section s1). The arrowheads in the section indicate clones in the wing-notum side that do not transform the shape of apposed squamous cells. (B) The transformations extend 7-15 cell diameters away from the clone (green, 48-72 hours AEL). The severity of the transformation decreases the further the affected cell is from the clone (see inset). (C) These clones (48-72 hours AEL) eliminate *Ubx* expression (red) in peripodial transformed cells (outlined clone and inset) and cubic cells (arrowhead). (D) By contrast, in Armα overexpression clones (24-48 hours AEL) both the transformation in shape and the elimination of *Ubx* expression are only cell autonomous (inset). *wg* expressing clones (green) induce expression of hinge markers in the peripodial side of the wing disc, such as *ds* (E), *zfh-2* (F) and *nub* (G). (E) In these clones *ds* (red) is induced in cells both inside and outside the clone (see longitudinal section s2). Clone generated 24-48 hours AEL. (F) Clones in squamous (outlined clone) and proximal cubic cells (arrowhead) induce cell autonomous (see transversal section s3) and non-autonomous expression of *zfh-2* (red). Clone generated 24-48 hours AEL. (G) Ectopic Wg (48-72 hours AEL), however, induces only autonomous expression of *nub* (red), which is expressed in the wild-type wing blade and proximal hinge. Notice that the expression of Nub induced is mostly cytoplasmic.

through the lumen of imaginal discs (Cho et al., 2000; Gibson et al., 2002; Gibson and Schubiger, 2000; Gibson and Schubiger, 2001), we evaluated whether ectopic expression of Wg in one side of the wing disc affects the opposite side. Ectopic Wg in cells of the wing-notum side does not change the specification (*Ubx* expression) or morphology of squamous cells (not shown). Changes in the reverse direction do not take place either (not shown). This shows that Wg signalling is not transmitted through the lumen of the wing imaginal disc. Finally, the ectopic expression of Wg transforms peripodial cell morphology in the wing, haltere and leg imaginal discs, but does not change the squamousity in the PE of the eye-antenna imaginal disc (not shown).

Ectopic Wg and Vg transform the cell morphology and fate of the PE into those of the wing blade

Cells expressing Wg and Vg in the wing-notum side of the disc acquire the identity of wing blade cells (Baena-Lopez and Garcia-Bellido, 2003; Klein and Martínez-Arias, 1999). We asked whether PE cells, besides the ability to be transformed into hinge, can acquire the more distal fate of the wing blade. Ectopic expression clones of Vg in the PE do not modify the identity or the morphology of squamous cells (not shown), which reinforces the idea that PE cells lack Wg activity. Peripodial cells co-expressing Wg and Vg, by contrast, acquire the identity of the wing blade, as suggested by the cell-autonomous induction of the wing genetic marker *dll* (Fig. 4C). Wg-Vg co-expression changes the shape of squamous cells into columnar (Fig. 4A,B). The effect of Wg-Vg clones on cell morphology is not only autonomous, expanding more cell diameters away than in clones expressing Wg only. This region of non-autonomous transformation around the clones expresses exclusively wing-hinge genetic markers, such as *zfh-2* (Fig. 4B) or *nub* (Fig. 4A,C). In contrast to clones overexpressing Wg, the autonomous and non-autonomous expression of *nub* induced by Wg-Vg clones is always nuclear (Fig. 4A,C), an indication that Vg might contribute to the translocation or maintenance of Nub into the nucleus.

The induction of genetic markers induced by Wg-Vg co-expression depends on the developmental time when the clones are generated, contrary to what happens in clones

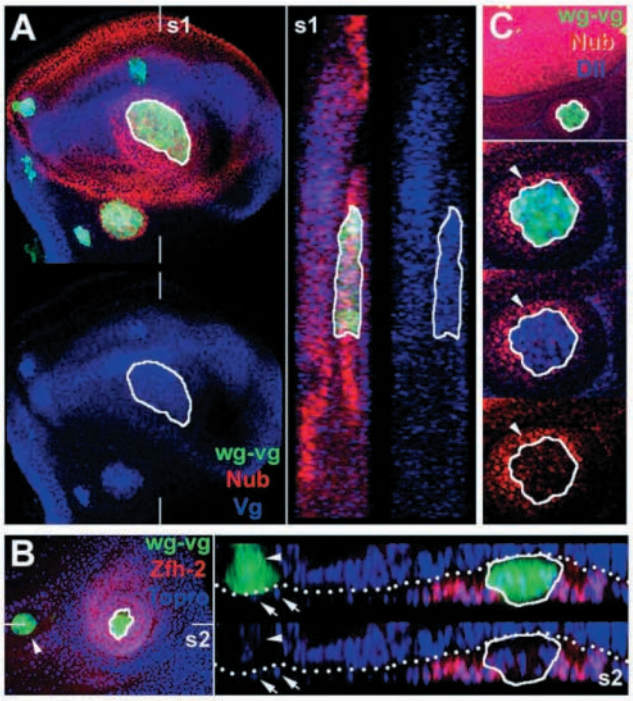

Fig. 4. Ectopic co-expression of *wg* and *vg* transforms the PE into wing blade. (A) w*g-vg* expressing clones (green, 24-48 hours AEL) transform squamous cells into columnar wing blade cells (see longitudinal section s1 through the outlined clone). Vg (blue) is present only in cells of the clone, while *nub* (red) is expressed both cell autonomously and non-autonomously. (B) w*g-vg* expressing clones generated 24-48 hours AEL induce the expression of the hinge marker *zfh-2* in an exclusively non-autonomous way (see transverse section s2). The arrowheads indicate a clone in the wing-notum side that neither induces *zfh-2* expression in apposed peripodial cells nor affects their morphology. Note that the clone in the peripodial side does not induce *zfh-2* expression in the wing-notum side either. Arrows indicate untransformed peripodial nuclei. (C) Clones generated 48-72 hours AEL promote autonomous expression of the wing pouch genetic marker *dll*. In contrast to earlier clones, the induced expression of *nub* is exclusively non-autonomous (insets at higher magnification).

overexpressing Wg, and also Ras^{V12} (see below). Clones generated in early larval development (24-48 hours AEL) express *nub* autonomously and non-autonomously (Fig. 4A), while it is expressed only non-autonomously in clones generated later (48-72 hours AEL) (Fig. 4C). *zfh-2* induction, in turn, is exclusively non-autonomous in early clones (Fig. 4B), while in clones generated late in larval development it is both autonomous and non-autonomous (not shown). These results suggest that early Wg-Vg clones are more able to reproduce the developmental program of the wing field than clones induced later, an indication that the PE might acquire a progressive genetic specification during larval development, preventing a more complete transformation in later clones.

Ectopic Egfr signalling transforms the cell morphology and fate of the PE into those of the notum

The role of Egfr signalling in the development of the peripodial side of the wing imaginal disc was studied by generating clones expressing Vn, a diffusible Egfr ligand (Simcox et al., 1996), at 24-48 hours AEL. Eight out of 25 peripodial clones expressing Vn showed peripodial cells transformed from squamous into columnar (Fig. 5A), although not all the cells of the clones were affected to the same extent. The transformation also affected non-autonomously some cells outside these eight clones, though, as described for the autonomous transformation, its penetrance is not complete (Fig. 5A). Accordingly, *Ubx* expression is repressed in some peripodial cells (Fig. 5A). It should be noticed that Vn overexpression is also unable to induce completely penetrant wing-to-notum transformations when expressed in the prospective wing field (Wang et al., 2000). A fully penetrant transformation, however, is achieved when a constitutively active form of the Ras protein (Ras^{V12}) (Karim and Rubin, 1998) is expressed in peripodial cells (Fig. 5B-D). Clones expressing Ras^{V12} change the squamous morphology of PE cells both autonomously and non-autonomously (Fig. 5B,C), which suggests that Egfr activation induces Vn expression, a positive loop also observed in the expression of Vn in the notum (Wang et al., 2000). The increase in cell density observed in Ras^{V12} clones may be associated to an excess in cell proliferation.

The morphological transformation detected in Ras^{V12} clones is only associated with autonomous changes in gene expression. The expression of *Ubx* is only reduced in cells of the clone (Fig. 5C) and that of *iro-C* only autonomously induced (Fig. 5D). The expression of *wg* and wing blade (*vg*, *dll*), wing hinge (*ds*, *zfh-2)* or late notum [*tailup* (*tup*), *eyg*] genetic markers is not induced in these clones (not shown). These results, taken together, suggest that expression of Ras^{V12} transforms the PE cells into cells of the notum field. Similar to clones expressing Wg, the transformations induced by the expression of Ras^{V12} are independent of the developmental time when the clones are generated, suggesting that Egfr signalling in PE cells must be repressed throughout larval development in order to achieve correct specification. Ras^{V12} clones reduce the expression of *zfh-2* in distal cubic cells (not shown) and induce the expression of *iro-C* (Fig. 5E), which supports again that the same mechanism driving wing-notum segregation is active in cubic cells. Similar to Wg-overexpressing clones, Vn expression or autonomous Egfr signalling in the wing-notum side of the disc does not affect the specification or shape of squamous cells (Fig. 5C,D). Translumenal effects of Egfr signalling when clones were induced in the PE were not observed either (Fig. 5B,D). Expression of Ras^{V12} transforms peripodial cell morphology in wing, leg and haltere discs, but not in the eye-antennal disc (not shown), confirmation that the genetic requirements for the development of the PE are not shared by all imaginal discs.

Discussion

Territorial specification and pattern formation in imaginal discs have been the subject of extensive research, but the development of the peripodial side of the discs has been often neglected in these studies. In the present work, we have studied the genetic specification of the PE in the wing imaginal disc, addressing the mechanisms that pattern the peripodial side of the disc and control the morphology of its cells.

The first differences at the morphological level between cells

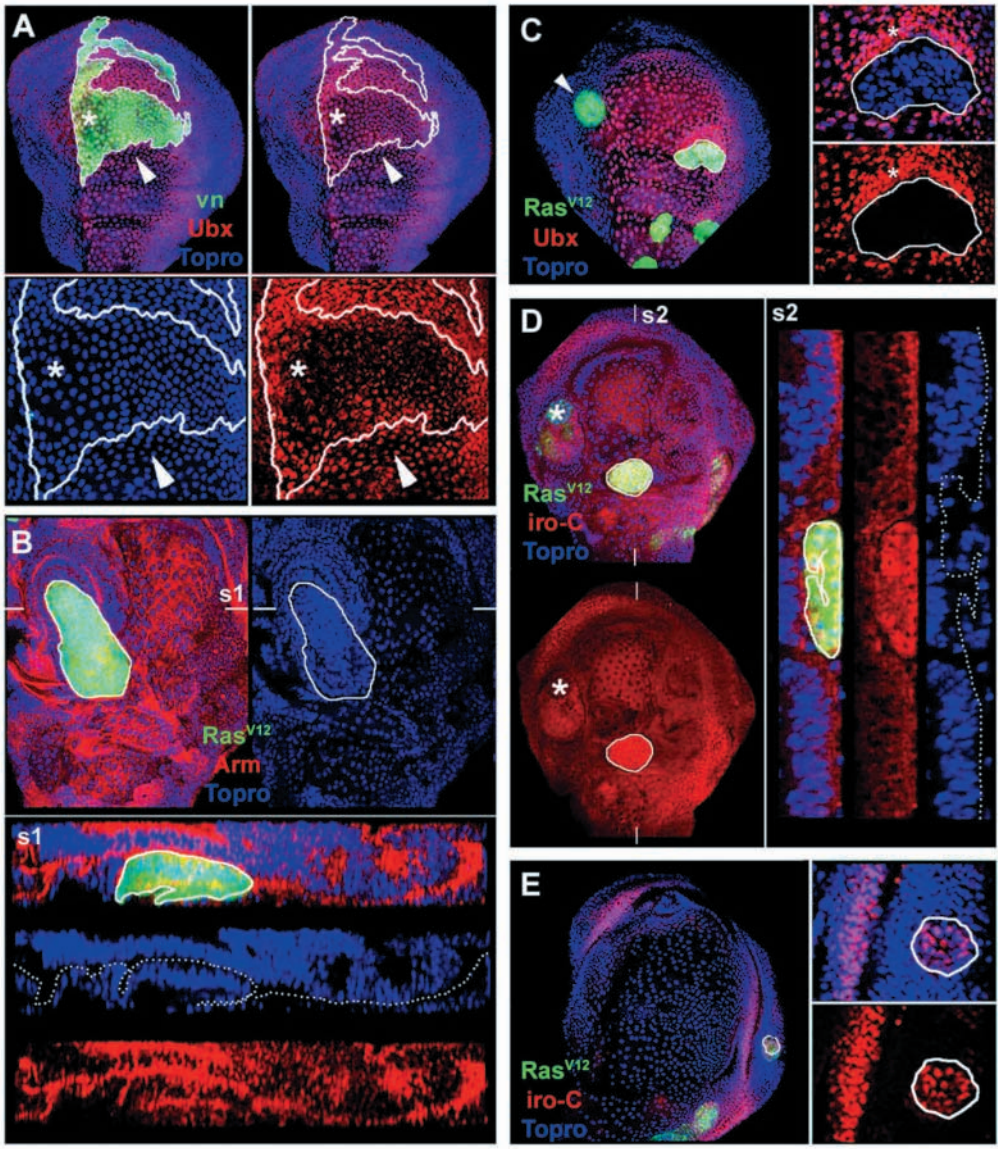

Fig. 5. Ectopic Egfr signalling transforms the identity and cell morphology of the PE into notum. (A) *vn*-expressing clones (green) transform the cell morphology of peripodial cells in a incompletely penetrant way (asterisk in a region of the clone not transformed). The transformation affects cells outside the clone (arrowhead). *Ubx* expression is repressed in some regions of the clone. (B) Expression of the constitutive Egfr activator Ras^{V12} (clones in green) transforms both cell autonomously and non-autonomously squamous peripodial cells into columnar cells, as seen both in a surface view and a longitudinal section (s1). Arm (red) and TO-PRO-3 (blue) staining reveal cell shape and tissue structure (apical membranes of both sides of the disc separated by a broken line). (C) Ras^{V12}-expressing clones eliminate *Ubx* expression (red) in transformed cells inside, but not outside the clone (inset at higher magnification of the outlined clone). An asterisk marks the region of non-autonomous transformation, not fully penetrant around the clone. The arrowhead indicates a clone in the wing-notum side that does not affect apposed squamous cells. (D) *iro-C* is induced autonomously by Ras^{V12} expression in peripodial cells (longitudinal section s2 of the outlined clone). Asterisk indicates a clone in the wing-notum side of the disc that does not affect apposed squamous cells. (E) Ras^{V12} induces expression of *iro-C* in distal cubic cells (inset at higher magnification of the outlined clone). The clones in all panels were generated 48-72 hours AEL.

of the peripodial and wing-notum side of the wing disc arise during the second larval instar, though a previous genetic heterogeneity may already exist (discussed below). The phenotype of clones lacking Wg and Egfr signalling, as well as the expression patterns of downstream genes (*zfh-2* and *iro-C*) in wild-type discs, indicate that the activity of both signalling cascades is required in the peripodial side of the disc only for normal development of cubic cells surrounding the PE, but not in the PE itself. Ectopic activation in the PE of the Wg or Egfr pathways causes squamous cells to adopt the columnar morphology characteristic of the cells of the wing-notum side (Fig. 6A-C). This transformation does not involve changes in the apicobasal polarity of the cell, as seen by normal localization of Arm (Fig. 3A, Fig. 5B), F-actin and Discs large 1 (Dlg1) (not shown). The transformation induced by ectopic Wg signalling affects exclusively the cells of the clone when this pathway is activated by the expression of the autonomous activator Arm$\Delta\alpha$. However, the transformations are both cell autonomous and non-autonomous when ectopic activity is induced by the overexpression of the diffusible ligands Wg and Vn, though this latter in a non-fully penetrant way. The transformations induced by Ras^{V12} were also both autonomous and non-autonomous, which may be explained by the fact that these clones could also induce the expression of Vn. The above observations suggest an involvement of both signalling pathways in the control of cell morphology in the imaginal disc.

In addition to the change in cell morphology of the PE cells, their genetic identity is also modified by these clones, as deduced from the loss of the PE marker *Ubx* in the transformed territories. The fate of the territories transformed by either expression of Wg or Egfr activity corresponds to wing hinge and notum, respectively, as suggested by the genetic markers expressed in these clones (Fig. 6A-C). Ectopic Wg signalling induces the expression of *zfh-2*, *ds* and *nub*. The expression of *nub* induced in these cells is localized mostly in the cytoplasm,

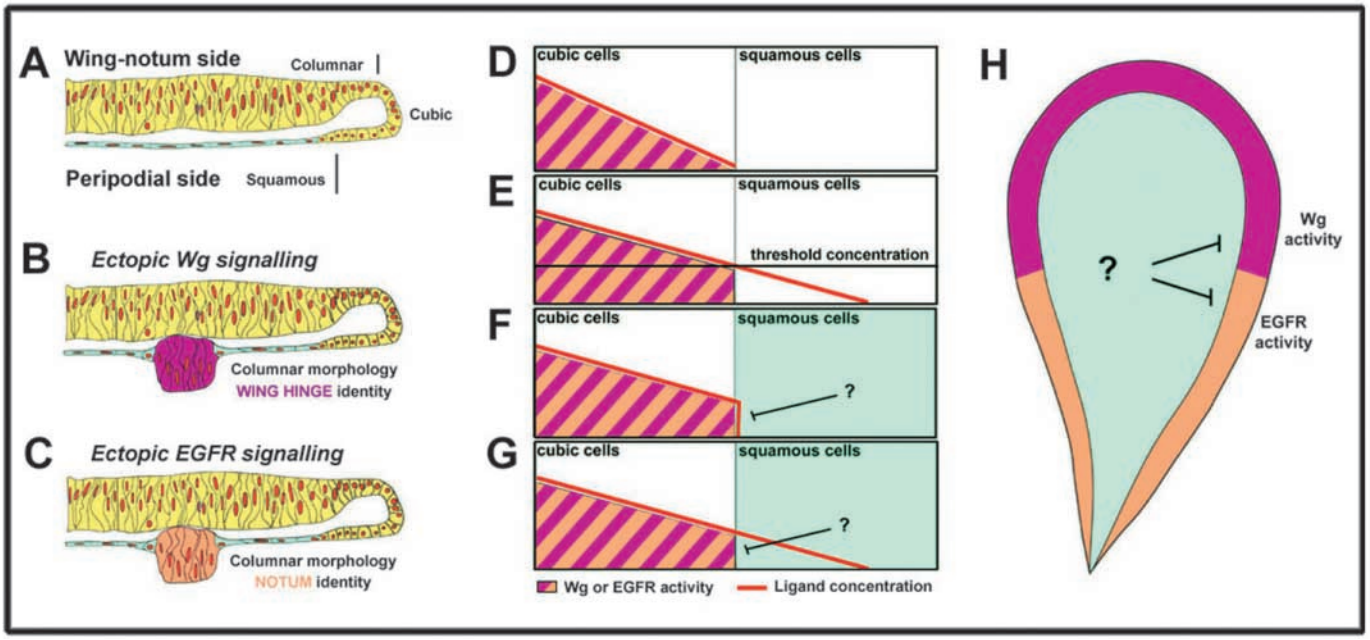

Fig. 6. (A-C) Schematic models of the effects of Wg and Egfr activities in the PE. (A) The squamous morphology and genetic specification of peripodial cells (A) is transformed by ectopic Wg (B) or Egfr (C) signalling. (D-G) Different mechanisms that could account for the absence of Wg and Egfr signalling in the PE (see Discussion). The decay in the concentration of the ligands from their sources in the wing-notum side could lower this concentration down to zero (D) or below a hypothetical activation threshold (E). Alternatively, peripodial cells could be refractory to Wg and Egfr activities because of repression of the signals downstream of the receptor level (F) or decreased diffusive ability of the ligands in peripodial cells (G). These two latter possibilities imply a previous genetic heterogeneity that sets the limits of the peripodial developmental field from early larval stages and implements suppression of Wg and Egfr signalling (H).

contrary to normal expression in hinge territories, which may indicate that the transformation is not complete. This suggests that the PE could be in some way reluctant to suffer this transformation or, alternatively, that additional factors are required for the specification of the wing hinge. Ectopic Egfr signalling changes the fate of peripodial cells towards a notum fate, as inferred from the induction of *iro-C*, but not of wing hinge (*zfh-2* and *nub*) or wing blade (*vg* and *dll*) genetic markers. The lack of induction of other more restricted notum genetic markers, such as *eyg* and *tup*, restricts the putative new fate of these cells to that of the subset of *iro-C*-expressing territories in the notum developmental field. Alternatively, the transformation could be incomplete. Unfortunately, it was not possible to obtain adult clones in order to accomplish a deeper study of the transformed fates.

The ability of Wg and Egfr ectopic signalling to change the fate of peripodial cells is independent of the developmental time when the clones are generated. However the PE must be subject to a progressive genetic specification as larval development proceeds. This is evidenced by the fact that Wg-Vg expression clones generated early are able to reproduce the developmental program of the wing field to a greater extent than the clones induced later, which implies that the PE is less competent to adopt this fate later in development.

Taking together the above results, we conclude that the PE constitutes a developmental field in early wing imaginal discs, different from the wing and notum fields. This peripodial field does not require the activity of the Wg and Egfr signalling pathways. Moreover, ectopic Wg and Egfr signalling are able to expand the wing and notum fields at the expense of the PE. So, it seems that reduced levels of Wg and Egfr signalling are a prerequisite for the development of the peripodial field. The reduced levels of Wg and Egfr signalling in the PE cannot be explained by absence of the receptors or any other elements required for the transduction of the Wg and Egfr signals, given the ability of the Wg and Vn ligands to induce transformations. Two possible mechanisms could account for the absence of Wg and Egfr activity in the PE. (1) Normal diffusion of the Wg and Vn ligands is unable to activate the pathway in the PE (Fig. 6D). (2) The normal decay of concentration of the ligands from their sources in the wing-notum side could lower this concentration down to zero (Fig. 6D) or below a hypothetical threshold (Fig. 6E). These possibilities, however, are challenged by the fact that Wg and Egfr signalling do not define the limit of the peripodial field, as the PE does not expand when we simultaneously eliminate the activities of the Wg and Egfr pathways in cubic cells. This suggests that a pre-existing genetic heterogeneity sets the limits of the prospective peripodial field. Although our data do not reveal the mechanisms that suppress Wg and Egfr signalling in the PE, they seem to favour a second model in which the PE is refractory to these signals (Fig. 6F-H). This could be due to a barrier to the diffusion of the ligands in the PE (Fig. 6F) or to repression of the signal downstream of the receptor level (Fig. 6G). Both a barrier to diffusion of the ligands and downstream repression could be overcome if the amount of ligand or downstream signalling is experimentally elevated. Suppression of the Wg and Egfr signalling pathways in late embryonic development, in addition, is a necessary step for

the specification of the wing disc primordium and its segregation from the leg disc (Kubota et al., 2000; Kubota et al., 2003). It seems possible, therefore, that the repression of these signalling pathways, inherited from the embryo, is later restricted to the peripodial side of the disc; alternatively, the repression of Wg and Egfr signalling in the PE might arise later as a non-related event. Furthermore, the early repression of these signals in the wing and notum fields could be, from an evolutionary perspective, a suitable mechanism for the generation of a peripodial field in ancestral uninvaginated discs (Svácha, 1992; Truman and Riddiford, 1999).

Some recent reports have focused on a role of the PE of the eye and wing discs in the patterning of the other side of the disc (Cho et al., 2000; Gibson et al., 2002; Gibson and Schubiger, 2000), showing the ability of Dpp and Hh proteins expressed in the PE to affect the development of the other side of the disc. Our results, however, rule out translumenal communication of the Wg and Egfr signals. Translumenal communication of intercellular signals, therefore, is not the simple outcome of the apposition of the two sides of the disc or general secretion of ligands into the disc lumen. On the contrary, transmission of a signal through the disc lumen would require specific mechanisms for every different signalling pathway.

Finally, the comparative analysis among the peripodial epithelia of different imaginal discs shows that the development of each PE has characteristic genetic requirements, at least after their specification, as evidenced by the inability of Wg and Ras^{V12} clones to transform the morphology of peripodial cells in the eye-antenna disc. The possibility of a common mechanism for the generation of the peripodial field in all imaginal discs, however, still remains.

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