Introduction

Communication between cells during development provides much of the necessary information for constructing specialized tissues (reviewed by Gerhart, 1999). Despite the diversity of cell types that are specified, only a small number of conserved signalling pathways are used throughout development to instruct cell fate. The activation of these signalling cascades results in different outcomes in different tissues; the same signal is interpreted differently depending on the state of the cell receiving it (reviewed by Freeman, 1997; Shilo, 2003; Tan and Kim, 1999). Although most analyses have focussed on the direct role of signalling pathways on determining cell fate, the full developmental functions of signalling pathways are rarely well understood: individual signalling pathways can even have multiple functions within the same tissue.

The Drosophila embryonic ventral epidermis has served as a tractable tissue for the genetic analysis of patterning, and remains one of the rare instances where the developmental programs used to pattern fields of cells have been studied from early through to late stages (Alexandre et al., 1999; DiNardo et al., 1994; Hatini and DiNardo, 2001; O’Keefe et al., 1997; Payre et al., 1999; Szüts et al., 1997). Here, two cell types are specified: epidermal cells that secrete short, thick hair-like structures called denticles (used by the larvae for traction), and smooth-cuticle cells, which secrete a protective cuticle that lacks denticles. Denticles occur in belts composed of several rows of denticle-secreting cells in the anterior half of each parasegment, which alternate with smooth cuticle regions that make up the posterior of each parasegment. This segmental pattern is repeated along the anterior-posterior axis (see Fig. 1A). Although deceptively simple, this pattern is quite intricate and precise; each of the six rows of denticle-secreting cells in the abdominal parasegments produce denticles of distinct polarity and morphology, while many denticle belts have segment-specific characteristics (Szüts et al., 1997; Wiellette and McGinnis, 1999).

Although denticle morphologies result from the juxtaposition of several different signalling domains (Alexandre et al., 1999; Hatini and DiNardo, 2001; Sanson, 2001; Wiellette and McGinnis, 1999), the basic cell fate pattern of alternating denticle belt and smooth cuticle stripes is established by antagonism between the epidermal growth factor receptor (EGFR) and Wingless signalling pathways (O’Keefe et al., 1997; Payre et al., 1999; Szüts et al., 1997). In 15. Multiple factors stimulate EGFR signalling to promote smooth-cuticle cell survival: in addition to the spitz group genes, Rhomboid-3/roughoid, but not Rhomboid-2 or -4, and the neuregulin-like ligand Vein also function in survival signalling. Pointed mutants display the lowest frequency of fusions, suggesting that EGFR signalling may inhibit apoptosis primarily at the post-translational level. All ventral epidermal cells therefore require some level of EGFR signalling; high levels specify the denticle fate, while lower levels maintain smooth-cuticle cell survival. This strategy might guard against developmental errors, and may be conserved in mammalian epidermal patterning.

Key words: EGF receptor, Denticle belt fusion, spitz group, Rhomboid, Epidermis, Intramembrane proteolysis, Apoptosis, Cuticle, Pointed
Materials and methods

Stocks used and genetic schemes

The alleles analysed were rho1PAS5, rho1PAS5 (Freeman et al., 1992), ru/rho1TM43 (Mayer and Nusslein-Volhard, 1988), spkA14 (Mayer and Nusslein-Volhard, 1988), S218 (Heberlein and Rubin, 1991), sim2 (Thomas et al., 1988), pm4A88 (Scholz et al., 1993), rho3PlpLb (Wasserman et al., 2000) and wnt1057 (Spradling et al., 1999). We analysed both rho1PAS5 and rho1PAS5 nulls initially for denticle belt fusions to be sure that the lower penetrance was not due to stock differences; rho1PAS5 was then used throughout, except in Fig. 4C,D. The arm-gal4 (Sanson et al., 1996), wg-gal4 (Pfeiffer et al., 2000) and prd-gal4 (T'offe et al., 1995) embryonic drivers were used to express UAS-DN-EGFR 1-7 (strong line) (Freeman, 1996), UAS-DN-EGFR 29-77-1 (weak line used in Fig. 5C) (Buff et al., 1998), UAS-torD- DER (Reichman-Fried et al., 1994), UAS-rasV12 (Fortini et al., 1992), UAS-rasN17 (Fortini et al., 1992) and UAS-DN-rap (Brand and Perrimon, 1994). The H99 deletion (White et al., 1994) was recombined onto the ru/rho1TM43 chromosome to assess the role of apoptosis in denticle belt fusions. Further information regarding these stocks can be found at Flybase (http://flybase.bio.indiana.edu).

Cuticle analysis

Embryos were collected from cages containing 25 or more females for 24 hours at 25°C onto apple juice plates containing fresh yeast. If a stock containing a lethal mutation was used (such as the spitz group mutations), the double balancer lethal progeny were eliminated by using males that resulted from an outcross to wild type. Embryos were dechorionated using 50% bleach and mounted in Hoyer's mountant/cartic acid (1:1) (Wieschaus and Nusslein-Volhard, 1998).

RNA interference

Achieving a robust phenocopy using RNA interference (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999) was dependent on maximising penetrance while reducing nonspecific embryo lethality. High concentrations of dsRNA were essential to maximise penetrance. RNA was transynthetised in 50 μl in vitro transcription reactions (using Promega's Ribomax method) with 5 μg of linearized pBluescript containing rhomboid genes as templates. Transcription was allowed to continue for 4 hours at 37°C, which resulted in the production of ~100 μg of RNA for each strand. 1U DNsase per μg template was added and incubated at 37°C for 1 hour, and the RNA was purified using RNeasy (Qiagen). The yield and integrity of the RNA was examined by formaldehyde denaturing agarose gel electrophoresis. Equivalent quantities of each strand were mixed, boiled for 5 minutes and allowed to cool to room temperature overnight. The dsRNA was precipitated with sodium acetate/ethanol, and resuspended in 0.1× PBS at 1-2 μg/μl prior to use. The variable that had the most significant effect on increasing embryo survival was injecting the embryos through the chorion. This enhanced survival since the embryos are much heartier in this state, and the chorion also keeps them from leaking after injection. Survival also relied on using uncrowded, well fed, young flies as older flies laid fewer eggs with significantly decreased hatching rates (with a concomitant increase in the number of unfertilised eggs). Embryos were washed off plates and aligned along the length of the slide while wet. The chorions of embryos that were dried for 5 minutes in silica gel containers became immobilized onto the slide surface (no glue required). Embryos were covered with Voltalafs 10S oil, which quickly rendered the chorion transparent, allowing embryo staging. Embryos were injected laterally, not posteriorly, as this was found to increase survival by 5-10%.

The injected embryos were incubated on slides at room temperature in a level humidified chamber. Hatching rate was assessed by counting the number of unhatched embryos after 2 days, with both positive (lethal gene) and negative (buffer) controls included in each set of injections. Overall, a typical hatching rate of 80% was achieved with...
injecting buffer, which was very consistent (approx. ±5%). Unhatched embryos were mounted for cuticle analysis.

**Embryo stainings**

RNA expression patterns of wg-gal4 and prd-gal4 (driving rhomboid-2 and -4 as probe targets, respectively) were visualized using digoxigenin-labelled antisense RNA probes prepared from 1-2 µg linearized DNA templates using Boehringer Mannheim reagents. Probes were fragmented in 40 mM NaHCO$_3$, 60 mM Na$_2$CO$_3$ pH 10.2 for 135 minutes, and hybridisation and detection were performed according to standard protocols.

Embryos were stained with anti-Engrailed (4D9) and anti-GFP using standard protocols, and mounted in Vectorshied. TUNEL labelling was performed after antibody detection by permeabilizing embryos in 0.5% Triton X-100, 0.1 M sodium citrate for 30 minutes at 70°C, rinsing in PBS + 0.5% Triton X-100, and incubating with TUNEL reaction components (Roche) at 37°C for 2.5 hours. Confocal images were collected using a MRC Radiance 2001 confocal microscope.

**Results**

**Characteristics of the denticle-belt fusion phenotype**

To define further the role of EGFR signalling in epidermal patterning, we first examined the denticle belt fusion phenotype of individual spitz, Star and rhomboid-1 mutants. The penetrance of the fusion phenotype is the proportion of embryos having at least one fusion. In both Star and spitz null mutants, 83-94% of embryos contained at least one fusion, although most displayed only between one to three fusions per embryo (Fig. 1A). In contrast, removing Rhomboid-1 activity with well-defined null mutations resulted in only 30% of mutant embryos displaying this phenotype. Removing (Pointed), a transcription factor that transduces EGFR signalling, with the null allele pntD$_{88}$ resulted in only 23% fusions. Loss of function of the EGFR itself results in a severe embryonic lethality phenotype that does not produce a cuticle that can be analysed for cuticle patterning (Price et al., 1989; Schejter and Shilo, 1989). However, expression of a dominant-negative form of the EGFR (DN-EGFR) (Freeman, 1996) throughout the epidermis using arm-gal4 resulted in denticle belt fusions, further confirming that this phenotype was indeed the result of reduced EGFR signalling. Although fusions were observed between all possible parasegments in spitz group mutant embryos, the phenotype occurred much more frequently between T3-A1, A4-5, and A5-6 denticle belts (Fig. 1B).

The lower frequency of denticle belt fusions in rhomboid-1 null mutants, and the variability and overall low number of fusions per embryo in all spitz group mutants suggested that other EGFR signalling components might also be involved in epidermal patterning. We therefore investigated the role of other possible rhomboids and EGFR ligands in this process.

**Rhomboid-1 and -3 cooperate in suppressing denticle-belt fusions**

Since Spitz, Star and Rhomboid are all obligate components of the EGFR signal activation pathway (Lee et al., 2001; Mayer and Nusslein-Volhard, 1988; Tsruya et al., 2002; Urban and Freeman, 2003), the lower penetrance of denticle belt fusions in rhomboid-1 mutant embryos suggested that another rhomboid protease might be acting with Rhomboid-1 in
Rhomboid-1 (Urban and Freeman, 2002; Urban et al., 2002). We therefore examined the physiological role of these proteases in embryogenesis using RNA interference (Fire et al., 1998; Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999).

Microinjection of rhomboid-1 dsRNA resulted in dose-dependent embryonic lethality with all embryos recapitulating the characteristic rhomboid-1 epidermal phenotype, including missing row one denticles, reversal of row four denticle polarity, and denticle belt fusions (Fig. 2A). Although this effect was very reproducible, it required high concentrations of dsRNA, possibly because rhomboid proteases are such efficient enzymes (Urban et al., 2001; Urban et al., 2002). However, injection of dsRNA corresponding to rhomboid-2, -3 and -4 had no detectable effect on survival rates or phenotypes of the injected embryos (Fig. 2B). This is consistent with genetic analysis of rhomboid-2 and -3 since their null mutants have been recently isolated and do not display embryonic phenotypes (Schulz et al., 2002; Wasserman et al., 2000).

To address whether combinations of rhomboid proteases were involved in suppressing the fusion phenotype of rhomboid-1 mutants, we assessed the effect of removing multiple rhomboids simultaneously. This was achieved by injecting embryos mutant for rhomboid-1, rhomboid-3 and rhomboid-3 rhomboid-1 with the necessary mixtures of the other dsRNAs to produce all rhomboid mutant combinations. Injection of rhomboid-3 embryos with dsRNA corresponding to rhomboid-2 and -4 or both did not result in embryonic lethality (Fig. 2C). Similarly, injection of rhomboid-2 and -4 dsRNA into rhomboid-1 or rhomboid-3 rhomboid-1 double embryos did not enhance their cuticle phenotypes (not shown). However, injection of rhomboid-1 dsRNA into rhomboid-3 mutant embryos resulted in a dramatically increased frequency of denticle belt fusions (compare white and grey bars in Fig. 2D). This analysis suggested a role for rhomboid-3/roughoid in epidermal patterning, and in suppressing the penetrance of the null rhomboid-1 fusion phenotype.

Although rhomboid-1 and -3 are within 80 kb of each other on chromosome 3L and as such are too close to be practically recombined to produce a double mutant, we had previously determined that one rhomboid-1 mutation (7M43) was originally generated on a chromosome that contained a rhomboid-3 mutation (Wasserman et al., 2000). Analysis of these rhomboid-3 rhomboid-1 double mutant embryos revealed that the frequency of fusions was more than double compared to rhomboid-1 alone (Fig. 1A), confirming the RNAi analysis. Although this rhomboid-3 rhomboid-1 double mutation did not fully recapitulate the severity of the Star and spitz mutants, this rhomboid-3 allele (roughoid1) causes a reduction rather than a loss of Rhomboid-3 activity (Wasserman et al., 2000). It should be noted that the fusion phenotype of this widely used rhomboid-1 stock (7M43) has been attributed to Rhomboid-1 alone as the significance of the roughoid mutation was unknown (Mayer and Nusslein-Volhard, 1988).
The only aspect of embryonic development for which we detected cooperation between Rhomboid-3 and Rhomboid-1 was in the formation of denticle belt fusions. In all other contexts examined, including ventral narrowing, which is diagnostic of a defect in ventrolateral specification (Fig. 3A,B), and other aspects of denticle determination (Fig. 3C), the rhomboid-1 mutation alone was fully penetrant.

Multiple ligands activate EGFR signalling to suppress denticle belt fusions

It is striking that even in the most severely affected spitz group mutant embryos less than half of their denticle belts are fused (Fig. 4A,B); this phenotype is much more severe when EGFR activity is reduced by expressing dominant-negative EGFR at high levels, which results in essentially all denticle belts being fused (see prd-gal4 driven embryo in Fig. 5B, but note that this driver only expresses in alternate parasegments). This implied that either some signalling occurs through the EGFR independent of ligand stimulation, or that even in Star; rhomboid-3 rhomboid-1 triple mutants (which display severity of fusions similar to individual spitz group single mutants, not shown) another ligand is causing EGFR stimulation.

To distinguish between these possibilities, we analysed the effect of removing Vein, a soluble neuregulin-like protein that is the only EGFR ligand thought to be independent of Rhomboid-1 and Star (Schnepp et al., 1996). Since spitz; vein double mutants are too severely affected for analysis of denticle patterning (Schnepp et al., 1996), we generated a rhomboid-1 vein double mutant (because rhomboid-1 mutants produced weaker denticle belt fusion phenotypes). Under these conditions about one third of rhomboid-1 vein mutant embryos could be analysed for denticle phenotypes, the rest being too severely affected (Fig. 4C).

Intriguingly, although the proportion of embryos displaying at least one fusion was not significantly increased compared to rhomboid-1 alone, these embryos displayed an increase in the frequency of multiple fusions per embryo (Fig. 4D). This suggested that the lack of complete fusions in spitz group mutant embryos is due to EGFR stimulation by Vein. Indeed, Vein is known to be expressed in the ventral epidermis at the time epidermal fates are being specified (Schnepp et al., 1996), although it has not previously been described to have a role in epidermal patterning.

Since the denticle belt fusion phenotype caused by EGFR loss could be accounted for by removing multiple ligands or their activators, this analysis indicates that ligand-independent EGFR activation may not occur physiologically, at least not during epidermal patterning.

A new requirement for EGFR signalling in smooth-cuticle cells

EGFR signalling is known to stimulate cells to adopt the denticle fate, while Wingless signalling antagonises EGFR signalling, allowing cells to adopt the smooth cuticle fate (O’Keefe et al., 1997; Payre et al., 1999; Sanson et al., 1999; Szüts et al., 1997). Contrary to these established roles, the denticle belt fusion phenotype in spitz group mutant embryos was manifest in smooth cuticle domains, where Wingless signalling is high and there is no known function for EGFR signalling. To test whether EGFR signalling is indeed specifically required in these cells, we blocked EGFR signalling by expressing a dominant negative form of the receptor (Freeman, 1996) in all
The penetrance of the fusion phenotype compared to that of double mutant dramatically increased the expressivity (but not rhomboid-1 vein analysed for ventral epidermal patterning. (D) A partial denticle belt fusions. We examined the physiological negative EGFR (DN-EGFR) in these cells also resulted in cuticle cells (Pfeiffer et al., 2000), expressing dominant-\text{wg-gal4} driver cuticle cells in each parasegment using the 1995), or in one or two of the five posterior rows of smooth cuticle cells of alternating parasegments (Fig. 5B). Domínguez et al., 1998; Campos-Ortega and Hartenstein, 1997), this domain denticle belts (Fig. 5B). Strikingly, removal of EGFR signalling in only smooth cuticle cells expressing \text{prd-gal4} driver (Moline et al., 1999; Yoffe et al., 1995), or in one or two of the five posterior rows of smooth cuticle cells in each parasegment using the \text{wg-gal4} driver (Pfeiffer et al., 2000). The expression patterns of these drivers have been characterized previously, and are shown for reference in Fig. 5A.

Strikingly, removal of EGFR signalling in only smooth cuticle cells using \text{prd-gal4} resulted in strong denticle belt fusions in essentially all \textit{paired} domain denticle belts (Fig. 5B). Although \text{wg-gal4} expresses in only one or two rows of smooth cuticle cells (Pfeiffer et al., 2000), expressing dominant-negative EGFR (DN-EGFR) in these cells also resulted in partial denticle belt fusions. We examined the physiological significance and specificity of the fusions caused by reducing EGFR signalling in smooth-cuticle cells by testing genetic interactions between \textit{spitz} group mutant embryos and perturbing EGFR signalling using these transgenes. The fusions in \textit{rhomboid-3 rhomboid-1} double mutant embryos were completely rescued in \textit{paired} domains by the expression of activated forms of EGFR (TorD-EGFR) or Ras (RasV12) (Fig. 5C). Conversely, reducing EGFR signalling by expressing a weak line of DN-EGFR or dominant negative forms of Ras (RasN17) or Raf (DN-Raf) all enhanced the fusions of \textit{rhomboid-3 rhomboid-1} double mutant embryos (Fig. 5C). Note that these transgenes are weak and did not result in phenotypes when expressed by themselves in wild-type embryos using \textit{prd-gal4}. These observations strongly indicate that smooth-cuticle cells, which are receiving the Wingless signal to antagonise the denticle-inducing effects of EGFR signalling, are nevertheless specifically dependent on EGFR signalling for their normal development.

Interestingly, cells near the midline appear particularly sensitive to reduced EGFR signalling since denticle belt fusions of \textit{spitz} group mutant embryos have an hourglass shape and vary in thickness at the point of fusion (Fig. 5D). But from this observation it was not clear whether all ventral cells have some requirement for EGFR signalling. We addressed this further by expressing the strong DN-EGFR transgene along the entire width of the smooth-cuticle parasegment using \textit{prd-gal4} (see Fig. 5A and Fig. 6B for expression pattern). This resulted in fusion of no more than the central two thirds of each denticle belt (Fig. 5D), suggesting that only the ventral epidermal cells, but not ventrolateral cells, are sensitive to reduced EGFR signalling.

**Denticle belt fusions result from cell death in the absence of EGFR signalling**

Careful physical analysis of the denticle belt fusion phenotype revealed a likely cause: in most cases the areas adjacent to the fused midline had folds, and in more extreme cases it was possible to identify holes in the middle of the fused area (Fig. 6A). This suggested that the midline fusion formed because of loss of smooth-cuticle cells in the midline, bringing the adjacent denticle cells together, and resulting in folding of the extra smooth-cuticle on either side of the midline. Missing smooth-cuticle cells could result either from a defect in their proliferation or survival, and EGFR signalling has been linked to regulation of both cell cycle progression and apoptosis in other developmental contexts (Bergmann et al., 1998; Domínguez et al., 1998; Kurada and White, 1998).

To distinguish between these alternatives, we marked future smooth-cuticle cells expressing DN-EGFR in \textit{paired} domains by co-expressing GFP, and analysed the fate of these cells at different stages of embryogenesis. Importantly, expression of DN-EGFR results in strongly penetrant fusions in \textit{paired} domains, and this is the only cuticle phenotype of these embryos (Fig. 6G). No defects could be observed in \textit{paired} domains of stage 10/11 embryos expressing DN-EGFR compared to those expressing only GFP (Fig. 6B). Since epidermal cells do not proliferate significantly after the initial series of three mitoses following syncitial development, with the final division occurring around stage 10 when ventrolateral fates are being specified (Bodmer et al., 1989; Campos-Ortega and Hartenstein, 1997), this
observation indicated that reduced proliferation is not the cause of the fusion phenotype.

Conversely, expressing DN-EGFR in future smooth-cuticle cells resulted in dramatically increased apoptosis in paired domains as visualized by TUNEL labelling (Fig. 6C,D). Elevated levels of apoptosis were first evident at stages 10/11, became strong at stage 12 (Fig. 6C), and persisted in regions expressing DN-EGFR in and around the midline during stages 13-14 (Fig. 6D). Strikingly, following these late stages of apoptosis, denticle belt fusions first became evident as curvatures of Engrailed-expressing cell stripes in ventrolateral regions of paired domains and the absence of Engrailed-marked cells in the midline, around stage 15 before any epidermal differentiation occurs (Fig. 6E,F). Note that since Engrailed marks parasegment boundaries rather than epidermal cell fate, curvature of Engrailed cell stripes directly confirms that the fusion phenotype results from pulling of denticle cells into smooth regions rather than fate change of smooth cells into denticle cells. Collectively, these observations indicate that denticle belt fusions result from apoptosis of future smooth-cuticle cells as a consequence of reduced EGFR signalling during stages 13 and 14, resulting in fusion of adjacent denticle belt regions at stage 15.

In support of this model, elevated levels of apoptosis were also prominent in the midline regions of stage 13/14 spitz null embryos, and less so earlier in stages 10-12 (Fig. 7A,B). Removing the three main apoptosis-activating genes using the H99 deletion (Foley and Cooley, 1998; White et al., 1994) also partly rescued denticle belt fusions in rhomboid-3 rhomboid-1 double mutant embryos (Fig. 7C). The absence of any other recognizable smooth cuticle phenotypes in these mutant embryos partially blocked for apoptosis suggests that EGFR signalling does not have any additional roles in smooth cuticle patterning (Fig. 7D). These analyses indicate that EGFR signalling provides an important survival function in the developing ventral epidermis: ventral smooth-cuticle cells, which are receiving Wingless signalling to antagonise the effect of EGFR signalling on the denticle fate, are nevertheless dependent on EGFR signalling for survival.

**Discussion**

**A new role for EGFR signalling in epidermal patterning**

The denticle belt fusion phenotype is one of the distinguishing features of the spitz group genes (Mayer and Nusslein-Volhard, 1988), yet its developmental basis has remained mysterious, since no function has been known for EGFR signalling in the smooth cuticle, which is the affected tissue. Our analysis of this phenotype has revealed its cause and uncovered a
previously unrecognised function for EGFR signalling in Drosophila epidermal development (Fig. 8). Spitz is the primary EGFR ligand in epidermal patterning, and is activated by proteolysis in three rows of rhomboid-1-expressing cells in the future denticle region (Fig. 8) (reviewed by Hatini and DiNardo, 2001; Sanson, 2001). As previously established, high EGFR signalling is required for cells to adopt the denticle fate (O’Keefe et al., 1997; Payre et al., 1999; Szüts et al., 1997), and other signalling pathways are used to elaborate the different denticle morphologies (Alexandre et al., 1999). The Wingless signal emanates from one posterior row of each parasegment and spreads anteriorly (Dubois et al., 2001; Sanson et al., 1999), suppressing the denticle fate and thus allowing cells to secrete a smooth cuticle. Our analysis now indicates that these future smooth-cuticle cells also require signalling through the EGFR for viability, and its absence results in apoptosis of future smooth-cuticle cells and thus denticle belt fusions. This survival signalling is mediated by...
and, as such, their denticle fate must have already been since denticle cells are being pulled into smooth cuticle regions. Finally, the fusion phenotype itself suggests that it forms late in development, with cell fate specification occurring late, through antagonism between EGFR and Wingless signalling around stages 12-14 (reviewed by DiNardo et al., 1994; Hatini and DiNardo, 2001; Sanson, 2001). Our direct phenotypic analysis indicates that EGFR signalling is required for smooth-cuticle cell survival during these fate specification stages and not earlier or later: epidermal cell apoptosis is greatly elevated in mutant embryos at stages 12-14, and the fusion phenotype first becomes apparent around stage 15 as curvature of Engrailed stripes.

This direct phenotypic analysis is also supported by several independent genetic observations. EGFR signalling is not required for survival in future smooth-cuticle cells early, when the ventrolateral fates are being specified (stage 10/11) since removing Rhomboid-1 expression at only this stage using the single-minded mutation never results in denticle belt fusions (Mayer and Nusslein-Volhard, 1988). Defects at this early stage also cause ventral narrowing in spitz group genes (Mayer and Nusslein-Volhard, 1988), and since rhomboid-3 does not enhance this phenotype, this suggests that it cooperates with rhomboid-1 only later in development. Vein acts independently of spitz group genes to suppress denticle belt fusions, and this cannot occur at stage 10/11 since at this early stage Vein expression is dependent on EGFR signalling through a positive feedback loop (Golembio et al., 1999; Wessells et al., 1999). Finally, the fusion phenotype itself suggests that it forms late since denticle cells are being pulled into smooth cuticle regions and, as such, their denticle fate must have already been determined and cannot be altered by receiving signals from these smooth domains.

Thus, two thresholds with different outcomes exist for EGFR signalling in patterning the ventral epidermis. The level of EGFR signalling that a cell receives is presumably dependent on its distance from the Spitz-processing cells; activated MAPK staining indicates that these rows of cells receive high levels of EGFR signalling (Payre et al., 1999). High levels of EGFR signalling are required to induce the denticle fate, while lower levels that reach smooth-cuticle cells are sufficient to suppress apoptosis. All ventral epidermal cells therefore require EGFR signalling, but the exact level, together with antagonism of shavenbaby transcription by Wingless signalling, determines the biological outcome. Importantly, these functions may be separate, as Wingless signalling is known to antagonise shavenbaby transcription to repress the denticle fate, but may not repress EGFR signalling itself in smooth-cuticle cells: activated MAPK staining suggests that some smooth-cuticle cells in the midline may also receive higher levels of EGFR signalling (see Payre et al., 1999).

These results indicate that cells only require EGFR signalling for their survival when they are starting to differentiate. A similar pattern was also observed in the developing eye imaginal disc where removing the EGFR resulted in cell death only once the morphogenetic furrow had passed (Domínguez et al., 1998). These observations raise the intriguing possibility that establishing a requirement for survival signals may be inherent in the differentiation program itself, perhaps for protecting against developmental errors. However, the observation that the requirement for survival signalling is restricted to the central region of the ventral epidermis implies that either this requirement is not ubiquitous, or that another signal is also involved.

EGFR survival signalling may be independent of Pointed

Pointed is an Ets domain-containing transcription factor that is responsible for transducing most known instances of EGFR signalling. Although it was previously clear that pointed mutant embryos rarely display denticle belt fusions (Mayer and Nusslein-Volhard, 1988), our analysis of a more recent null allele that removes both P1 and P2 transcripts demonstrates that even complete loss of pointed leads only to a very low frequency of denticle belt fusions. This is also consistent with the milder effects of pointed clones in the developing eye, and in particular the late onset of their apoptosis (Yang and Baker, 2003). These observations raise the possibility that EGFR-mediated survival signalling in general occurs primarily at a non-transcriptional level. Consistent with this model, EGFR signalling has been shown to reduce Hid protein stability, thus directly inhibiting apoptosis (Bergmann et al., 1998; Kurada and White, 1998).

The role of the rhomboid gene family in embryogenesis

Rhomboid exists as a seven-member family in Drosophila, and at least four of these are intramembrane serine proteases that can cleave all Drosophila membrane-tethered EGFR ligands and specifically activate EGFR signalling in vivo (Urban et al., 2002). Although the precise role of the rhomboid...
protease family in EGFR signalling and in other biological contexts has been unclear, mutations have now been isolated for both Rhomboid-2 and -3 (Schulz et al., 2002; Wasserman et al., 2000). Genetic analysis with null alleles has revealed that both act as tissue-specific activators of EGFR signalling much like Rhomboid-1. Rhomboid-2 is the only rhomboid known to be expressed early in gametogenesis (Guichard et al., 2000; Schulz et al., 2002), and is involved in sending EGFR signals from the germline to the soma to guide its encapsidation by somatic cells (Schulz et al., 2002). In this context, Rhomboid-2 appears to act alone. Rhomboid-3 displays strong expression in the developing eye imaginal disc, and is allelic to roughoid (Wasserman et al., 2000), one of the first Drosophila mutants described. Rhomboid-3 is the dominant rhomboid protease during eye development, but does not act alone: Rhomboid-3 cooperates with Rhomboid-1 in the developing eye.

Despite the power of these genetic approaches, it should be noted that rhomboid-1, -2 and -3 exist as a gene cluster on chromosome 3L and, as such, combined mutations are difficult to generate by recombination. Analysis of epidermal patterning using RNAi to overcome this limitation is the first implication of a rhomboid homologue function in embryogenesis. Interestingly, the rhomboid involved is Rhomboid-3, the rhomboid that was previously thought to be eye-specific (Wasserman et al., 2000). However, unlike in the developing eye where Rhomboid-3 has the dominant role, and removing Rhomboid-1 by itself has no effect (Freeman et al., 1992; Wasserman et al., 2000), the exact opposite is true in embryogenesis: Rhomboid-1 is the main protease in epidermal patterning while removing Rhomboid-3 alone did not result in detectable defects. This analysis suggests that different rhomboid proteases function predominantly to activate EGFR signalling in distinct tissues, but often act cooperatively or with a degree of redundancy.

A reciprocal survival signalling mechanism and its conservation

The requirement for high levels of signalling for fate specification and lower levels for viability in developing tissues may not be limited to the EGFR pathway. Intriguingly, analysis of cell death in wingless mutant embryos suggests that a reciprocal signalling function may also be required to maintain cell viability in denticle regions of the ventral epidermis: in conditions of reduced Wingless signalling, specifically during the stage of epidermal fate specification (but not earlier), cells corresponding to two denticle rows were observed to undergo apoptosis (Pazdera et al., 1998). Therefore, as with EGFR signalling, high levels of Wingless signalling induces the smooth-cuticle cell fate, while lower levels may be required for survival of a subset of denticle cells. Thus, the Wingless and EGFR signalling pathways may act antagonistically in specifying cell fate, while having complementary and reciprocal functions in maintaining cell viability in the developing epidermis of Drosophila. These survival functions may be conserved since EGFR signalling also has multiple roles in mammalian epidermal development (Jost et al., 2000), including maintaining cell survival (Rodeck et al., 1997), while some mammalian epidermal tumours are also specifically dependent on EGFR signalling for cell survival (Sibilia et al., 2000). Wnt signalling has also been linked to maintaining cell viability in certain developmental contexts (Tepera et al., 2003; You et al., 2002).

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