EGF receptor signalling protects smooth-cuticle cells from apoptosis during *Drosophila* ventral epidermis development

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Summary

Patterning of the *Drosophila* ventral epidermis is a tractable model for understanding the role of signalling pathways in development. Interplay between Wingless and EGFR signalling determines the segmentally repeated pattern of alternating denticle belts and smooth cuticle: *spitz* group genes, which encode factors that stimulate EGFR signalling, induce the denticle fate, while Wingless signalling antagonizes the effect of EGFR signalling, allowing cells to adopt the smooth-cuticle fate. Medial fusion of denticle belts is also a hallmark of *spitz* group genes, yet its underlying cause is unknown. We have studied this phenotype and discovered a new function for EGFR signalling in epidermal patterning. Smooth-cuticle cells, which are receiving Wingless signalling, are nevertheless dependent on EGFR signalling for survival. Reducing EGFR signalling results in apoptosis of smooth-cuticle cells between stages 12 and 14, bringing adjacent denticle regions together to result in denticle belt fusions by stage 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

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

fact, mutations in most of the factors involved in these signalling pathways were isolated based on defects in epidermal patterning (Jurgens et al., 1984). These signalling pathways antagonise each other at multiple points (Alexandre et al., 1999; Szüts et al., 1997), but ultimately converge on the selector gene *shavenbaby/ovo* (Payre et al., 1999), encoding a transcription factor that specifies the denticle fate. EGFR signalling directly activates *shavenbaby* transcription, resulting in the denticle fate, while Wingless signalling represses it, thus specifying smooth cuticle cells. This basic strategy has wider implications since it may be conserved in mammalian hair patterning (Payre et al., 1999).

The '*spitz* group' genes including *rhomboid-1*, *Star* and *spitz* that initiate EGFR signalling were originally identified and grouped according to their defects in cuticle patterning (Mayer and Nusslein-Volhard, 1988; Nusslein-Volhard et al., 1984). Mechanistic analyses have established that Spitz is the primary ligand of EGFR signalling during embryogenesis, but is produced in all cells in an inert transmembrane form (Rutledge et al., 1992). Signalling is activated when and where it is needed by the membrane proteins Star and Rhomboid-1 (reviewed by Shilo, 2003). Star is an export factor required for Spitz exit from the ER (Lee et al., 2001; Tsruya et al., 2002), while Rhomboid-1 is the protease responsible for Spitz activation (Urban and Freeman, 2003; Urban et al., 2001). Rhomboid-1 is expressed in three rows of cells in the future denticle regions (Alexandre et al., 1999; Sanson et al., 1999), which constitute the site of Spitz processing during ventral epidermal patterning and induce the denticle cell fate in these and neighbouring cells.

The cuticle phenotype of several *spitz* group genes indicates that the role of EGFR signalling in epidermal patterning is more complex. In addition to the predictable defects in denticle specification, mutation of many *spitz* group genes also results in denticle belt fusions (Mayer and Nusslein-Volhard, 1988; Nusslein-Volhard et al., 1984). This is perhaps the most striking and distinguishing cuticle phenotype of the *spitz* group genes, and results in the variable fusion of adjacent denticle belts in their central regions at the expense of a region that is normally smooth cuticle (Mayer and Nusslein-Volhard, 1988) (see Fig. 1A). Since EGFR signalling is believed to be involved only in specifying denticle fate and to have no role in smooth-cuticle cells (O'Keefe et al., 1997; Payre et al., 1999; Szüts et al., 1997), it is unclear why this EGFR signalling defect causes a phenotype in smooth-cuticle regions.

We have investigated the additional roles of EGFR signalling during epidermal development by studying the denticle fusion phenotype. Although high levels of EGFR signalling specify the denticle fate, lower levels of signalling are required in smooth-cuticle cells for survival. Reduction of EGFR signalling in *spitz* group mutant embryos causes smooth-cuticle cells to die, resulting in fusions of adjacent denticle belts. Rhomboid-3/Roughoid, but not Rhomboid-2 or -4, and the soluble ligand Vein cooperate with canonical *spitz* group genes in stimulating this survival signalling. These analyses now specifically demonstrate an unrecognized survival function for EGFR signalling during epidermal patterning, and illustrate one way in which different rhomboid proteases are deployed to fulfil the requirements of EGFR signalling during development.

Materials and methods

Stocks used and genetic schemes

The alleles analysed were $rho1^{P\Delta 38}$, $rho1^{P\Delta 5}$ (Freeman et al., 1992), $ru^{1}rho1^{7M43}$ (Mayer and Nusslein-Volhard, 1988), spi^{A14} (Mayer and Nusslein-Volhard, 1988), S^{218} (Heberlein and Rubin, 1991), sim^{2} (Thomas et al., 1988), $pnt^{\Delta 88}$ (Scholz et al., 1993), $rho3^{PLLb}$ (Wasserman et al., 2000) and vn^{10567} (Spradling et al., 1999). We analysed both $rho1^{P\Delta 5}$ and $rho1^{P\Delta 38}$ nulls initially for denticle belt fusions to be sure that the lower penetrance was not due to stock differences; $rho1^{P\Delta 38}$ 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* (Yoffe 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-raf* (Brand and Perrimon, 1994). The *H99* deletion (White et al., 1994) was recombined onto the $ru^{1}rho1^{7M43}$ 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:lactic 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 synthesised 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 DNaseI 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 Voltalefs 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_2CO_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 Vectashield. 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 $pnt^{\Delta 88}$ 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 epidermal patterning. Previous biochemical analysis suggested that at least three other rhomboid proteases could substitute for

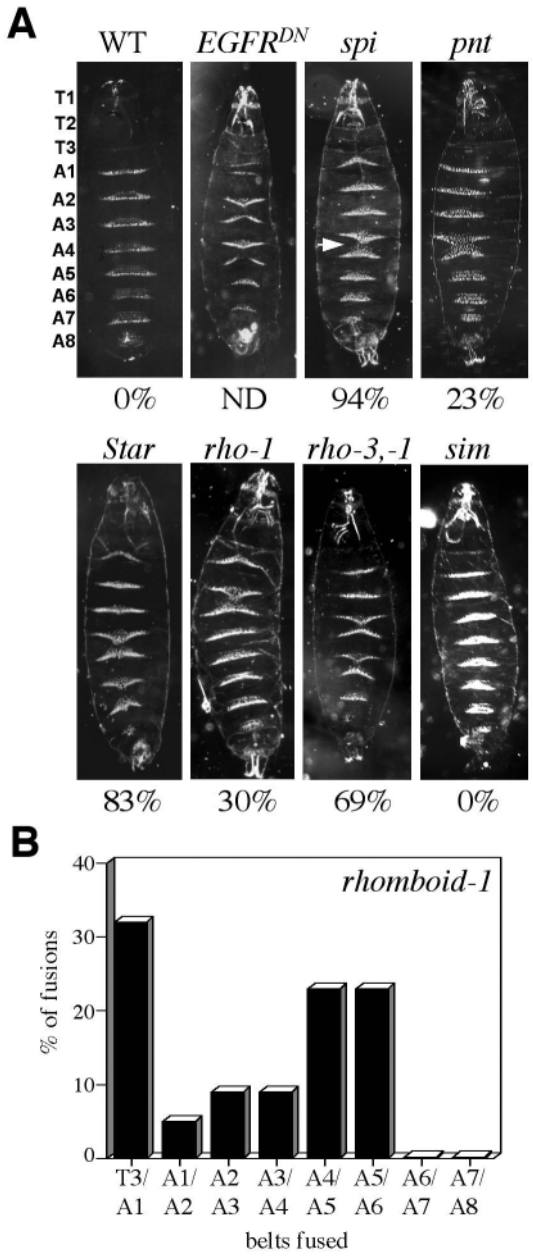


Fig. 1. Analysis of the denticle belt fusion phenotype (for example, see arrow in A) of *spitz* group mutants. (A) Penetrance (percentage of embryos having at least one fusion) of the denticle belt fusion phenotype of null mutations in different *spitz* group genes. The denticle belts of each parasegment are labelled to the right of the wild-type cuticle for reference. Expression of a dominant-negative form of the EGFR throughout the epidermis resulted in denticle belt fusions, confirming that this phenotype was indeed the result of reduced EGFR signalling. Note that *single minded*, while being a member of the *spitz* group, is not an EGFR component: consistent with this, mutants do not show denticle fusions. (B) Distribution of denticle belt fusions observed in each parasegment (analysis of a *rhomboid-1* mutant is shown but other *spitz* group genes showed similar spectra). Denticle belt fusions were observed in all parasegments, but occurred most frequently between the T3 and A1, A4 and 5, and A5 and 6 denticle belts.

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).

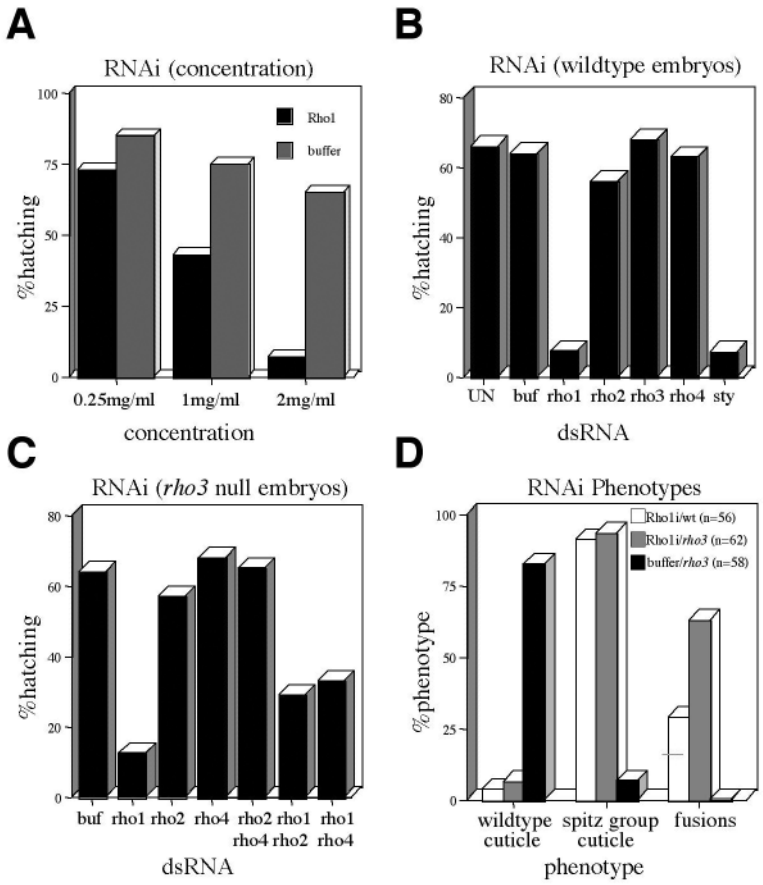
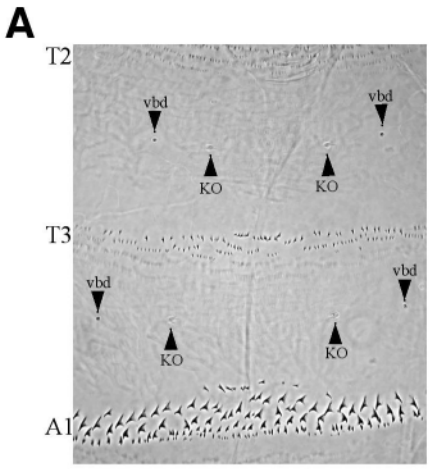


Fig. 2. Analysis of rhomboid function during embryogenesis using RNA interference. (A) High concentrations of rhomboid-1 dsRNA were important in maximizing penetrance, with 2 mg/ml dsRNA yielding most penetrant phenocopies. A-C show the percentage of injected embryos that hatched. Essentially all rhomboid-1-injected embryos that did not hatch had missing row 1 denticles, polarity reversal of row 4 denticles, and occasional belt fusions characteristic of the *spitz* group denticle phenotype. (B) Although dsRNA corresponding to both *rhomboid-1* and *sprouty* caused lethality with strong phenotypic effects, none of the remaining rhomboids yielded any discernible phenotypes when inactivated in wild-type embryos. UN, uninjected embryos; buf, those injected with buffer only. (C) Injection of dsRNAs corresponding to *rhomboid-2* and *-4* into *rhomboid-3* null embryos did not produce lethality, or discernible modification of the denticle phenotype resulting from co-injecting rhomboid-1 dsRNA. Note that co-injecting rhomboid-1 dsRNA with other dsRNAs resulted in decreased lethality because it reduced the concentration of rhomboid-1 dsRNA. Injection of *rhomboid-1*, or *rhomboid-3 rhomboid-1* double mutant embryos resulted in 25% lethality because of the lethal phenotype of mutated *rhomboid-1* (present in 25% of embryos derived from a cross between heterozygous parents), but no enhancement of the *spitz* group denticle phenotype was evident when rhomboid-2 and -4 dsRNA was injected (not shown). (D) Phenotypic analysis of wild-type embryos injected with rhomboid-1 dsRNA (white bars), *rhomboid-3* null embryos injected with rhomboid-1 dsRNA (grey bars), and *rhomboid-3* null embryos injected with only buffer (black bars). Only embryos injected with buffer displayed wild-type cuticles, while embryos injected with rhomboid-1 dsRNA displayed *spitz* group cuticle phenotypes. Strikingly, injection of *rhomboid-3* null embryos with rhomboid-1 dsRNA produced a marked increase in denticle belt fusions compared to wild-type embryos injected with rhomboid-1 dsRNA.

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 (*roughoid*^{1}) causes a reduction rather then 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).



B

		WT	*rho-3*	*rho-1*	*ru[1]rho-1*
T1 KO	mean	25.98	26.26		
	n	53	46		
	SE	00.28	00.33		
T2 KO	mean	34.15	35.10		
	n	54	48		
	SE	00.33	00.79		
T3 KO	mean	51.74	51.96		
	n	53	50		
	SE	00.50	00.46		
T1 vbd	mean	62.90	62.07	44.27	47.69
	n	48	43	11	13
	SE	00.58	00.72	00.87	02.29
T2 vbd	mean	71.35	70.41	51.74	61.86
	n	52	46	27	29
	SE	00.63	00.76	00.72	02.55
T3 vbd	mean	93.06	92.78	71.00	83.04
	n	53	46	19	26
	SE	00.96	00.73	00.75	02.42

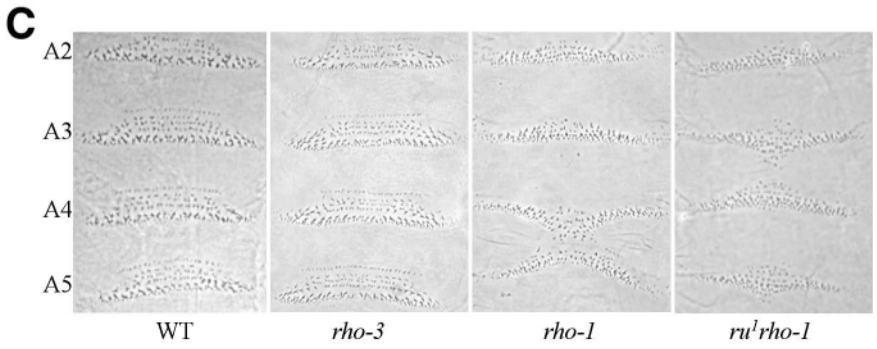


Fig. 3. Ventral narrowing of *rhomboid* mutants. (A) The ventral cuticle of thoracic parasegments from a wild-type embryo is shown for reference. The Keilin's organs (KO) and ventral black dots (vbd) are indicated with arrowheads, and the denticle belts are labelled to the left of the image. (B) The distance between KOs and vbds was measured in arbitrary units. This analysis served as a measure of ventral narrowing and thus of ventrolateral specification (Mayer and Nusslein-Volhard, 1988). Mutation of *rhomboid-3* alone caused no ventral narrowing, nor did it enhance the narrowing phenotype of *rhomboid-1*. Note that *rhomboid-1* nulls often lack KOs and as such embryos could not be scored for this measurement (n is the number of embryos analysed; SE is standard error). (C) No additional defects in abdominal denticle specification were evident in *rhomboid-3*, or *rhomboid-3 rhomboid-1* double mutant embryos compared to wild-type and *rhomboid-1* mutants, respectively.

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 mutants 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

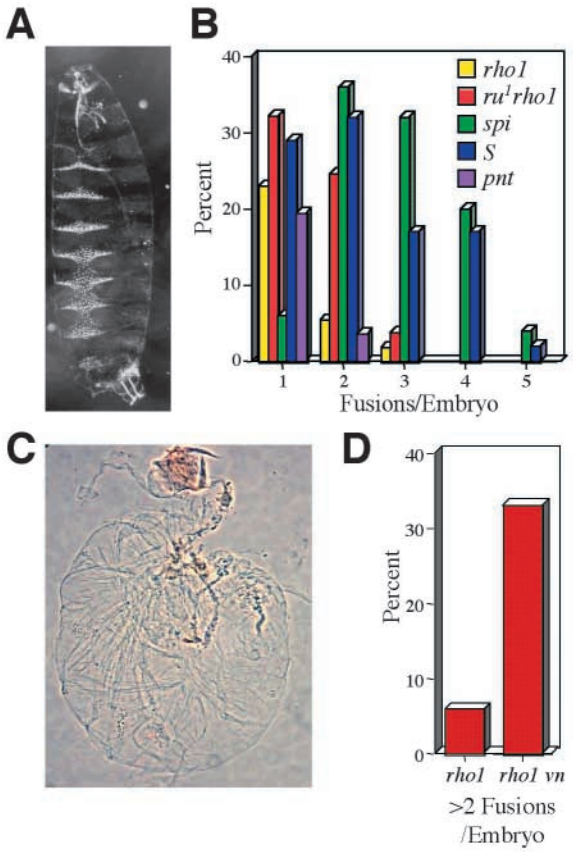


Fig. 4. Expressivity of the denticle belt fusion phenotype in different *spitz* group mutants. Expressivity reflects the severity of the denticle belt fusion phenotype, and is measured as the number of fusions per embryo. Note that one denticle belt fusion results from the midline joining of two denticle belts; as such, a total of ten fusions are possible in a single embryo since there are a total of 11 (three thoracic and eight abdominal) denticle belts. (A) An example of a *spitz* null embryo displaying four denticle belt fusions. (B) The expressivity of various *spitz* group genes was analysed, which revealed that most mutant embryos contained between one and three fusions, but never more than five of the 10 possible fusions. (C) *rhomboid-1 vein* double mutant embryos are more severely affected than *spitz* group embryos, and only one in three could be analysed for ventral epidermal patterning. (D) A *rhomboid-1 vein* double mutant dramatically increased the expressivity (but not penetrance) of the fusion phenotype compared to that of *rhomboid-1* alone.

five rows of smooth cuticle cells of alternating parasegments using the *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 *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 *prd-gal4* resulted in strong denticle belt fusions in essentially all *paired* domain denticle belts (Fig. 5B). Although *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 *spitz* group mutant embryos and perturbing EGFR signalling using these transgenes. The fusions in *rhomboid-3 rhomboid-1* double mutant embryos were completely rescued in *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 *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 *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 *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 *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 *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 *paired* domains, and this is the only cuticle phenotype of these embryos (Fig. 6G). No defects could be observed in *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

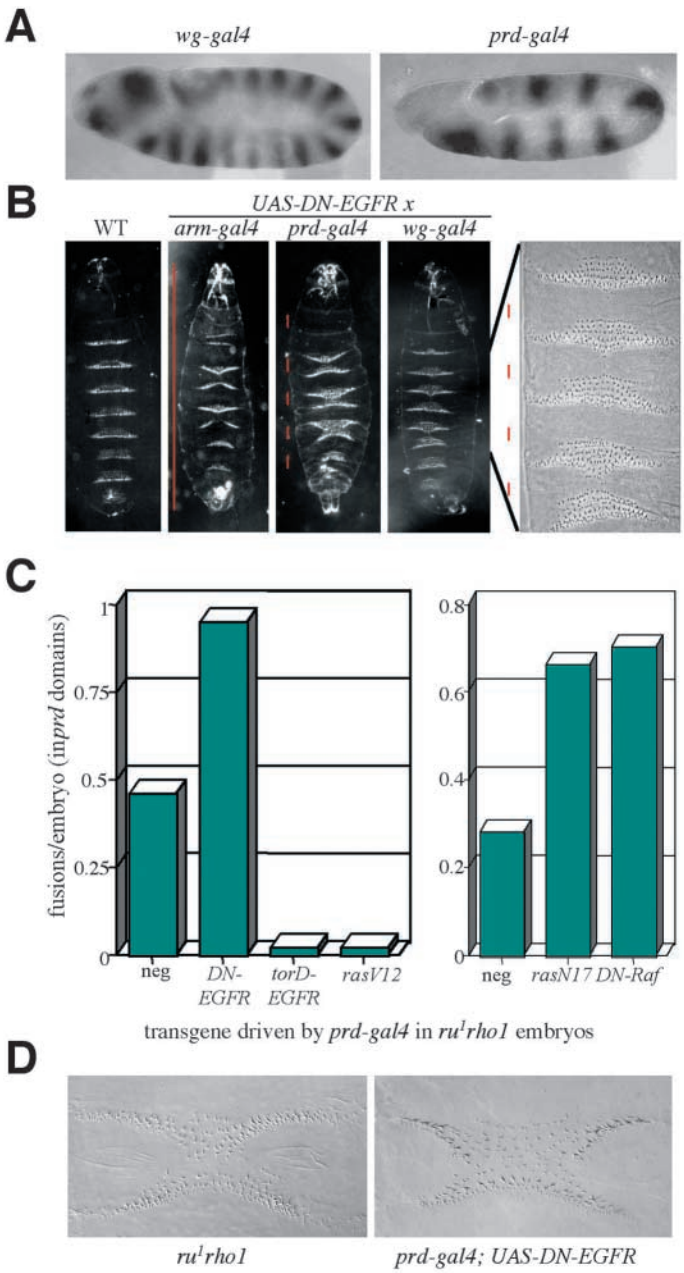


Fig. 5. A requirement for EGFR signalling in smooth-cuticle cells. (A) *wg-gal4* and *prd-gal4* drivers were used to analyse the fusion phenotype; their expression patterns were visualized using RNA in situ hybridisation. (B) DN-EGFR was expressed in smooth-cuticle cells in the regions of the embryos indicated by the red lines using *arm-gal4*, *prd-gal4* and *wg-gal4* drivers. Note that the *prd-gal4* and *wg-gal4* drivers are expressed at higher levels per cell than the *arm-gal4* driver. Expression of DN-EGFR in alternating smooth-cuticle domains resulted in strong denticle belt fusions in essentially all *paired* domains, and never in non-*paired* parasegments. The *wg-gal4* driver expressed only in two cell rows in each stripe, but incomplete fusions were evident (far right). (C) The specificity of activating and inhibiting EGFR signalling on the fusion phenotype was tested by modifying the fusion phenotype of *rhomboid-3 rhomboid-1* double mutant (ru^1rho1^{7M43}) embryos. Note that the analysis was performed only in *paired* domains (alternating smooth-cuticle stripes), and that these weak *EGFR*, *ras* and *raf* transgenes caused no phenotypes when expressed by themselves in wild-type embryos using *prd-gal4*. Transgenes were expressed at 25°C (graph on left), except *rasN17* and *DN-raf*, which were expressed at 29°C (graph on right). Note that at 29°C fewer denticle belt fusions occurred in *rhomboid-3 rhomboid-1* double mutant embryos. (D) Although the width of the medial fusion varied in *spitz* group embryos, expressing DN-EGFR laterally in smooth-cuticle cells along the entire circumference of the parasegment (see A above) resulted in fusions that were never wider than the middle two thirds of denticle belts.

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

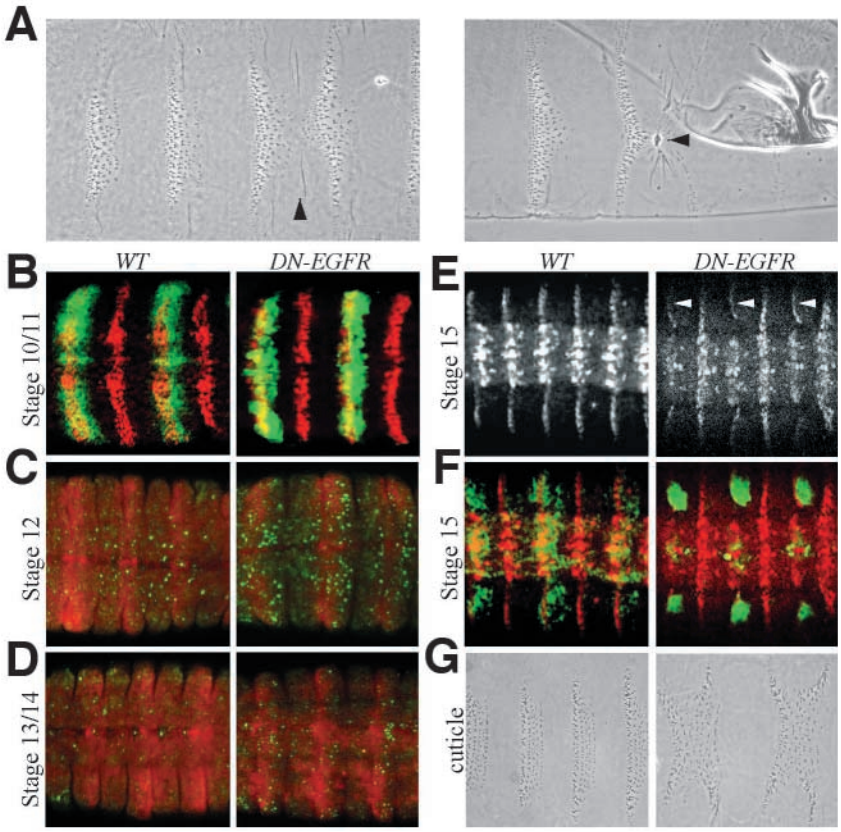


Fig. 6. Phenotypic analysis of denticle belt fusions during embryogenesis. (A) The denticle belt fusion phenotype resulted in folds around the surrounding fused areas (left panel, arrowhead), and in rare events a hole in the cuticle could be seen at the centre of the fusion (right panel, arrowhead). (B) No defects could be observed in *paired* domains of stage 11/12 embryos expressing DN-EGFR compared to wild-type embryos: *paired* domains were marked by co-expression of GFP (green) and Engrailed was used as a segmental marker (in red). In these and subsequent images anterior is to the right. (C,D) Apoptosis (detected by TUNEL labelling in green) was elevated predominantly in *paired* domains (in red) of ventral epidermal cells of stage 12 embryos (C), and persisted in stage 13 and 14 embryos (D) expressing DN-EGFR (right panels) compared with wild-type embryos (left panels). (E,F) By stage 15, fusions became evident as curvature of Engrailed stripes laterally (arrowheads) and missing stripes medially only in *paired* domains of embryos expressing DN-EGFR (F shows a merged image of E with *paired* domain marked by co-expression of GFP in green, and Engrailed in red). (G) The cuticle phenotype of embryos expressing DN-EGFR in *paired* domains compared to those expressing GFP alone: the only cuticle phenotype of the DN-EGFR-expressing embryos was strong denticle belt fusions in alternating parasegments (*paired* domains).

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

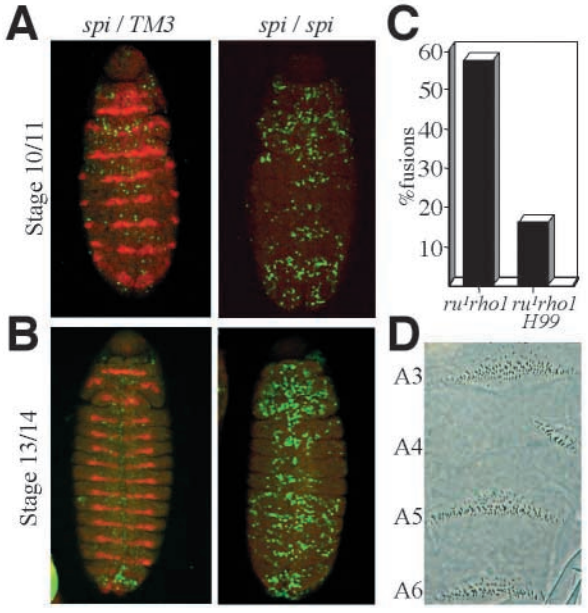


Fig. 7. Epidermal cell apoptosis in *spitz* group mutants causes denticle belt fusions. (A-D) Apoptosis of ventral epidermal cells was greatly elevated in *spitz* null embryos compared to wild-type or balanced embryos carrying one null copy of *spitz*. The ventral surface is shown for each embryo, with anterior being up in all images. Homozygous *spitz* null embryos were marked by the absence of Engrailed/β-galactosidase staining (in red), and apoptotic cells were detected by TUNEL labelling (in green). Although epidermal apoptosis was elevated as early as stage 10/11 (A), apoptosis in stage 13/14 *spitz* null embryos was much stronger, particularly in medial regions (B). Note that these *spitz* null embryos display the strongest fusion phenotype of any *spitz* group mutants (Fig. 4A,B). (C) Removing the three main apoptosis activators (*grim, reaper, hid*) using the *H99* deletion (White et al., 1994) suppressed the fusion phenotype of *rhomboid-3 rhomboid-1* double mutant embryos, but did not eliminate it completely. It should be noted that some apoptosis has been observed in the absence of these genes (Foley and Cooley, 1998). (D) No additional phenotypes were detected in smooth-cuticle cells of *rhomboid-3 rhomboid-1* double mutant embryos partially blocked for apoptosis by virtue of the *H99* deletion. Unexpectedly, the entire A4 denticle belt was frequently missing in these embryos.

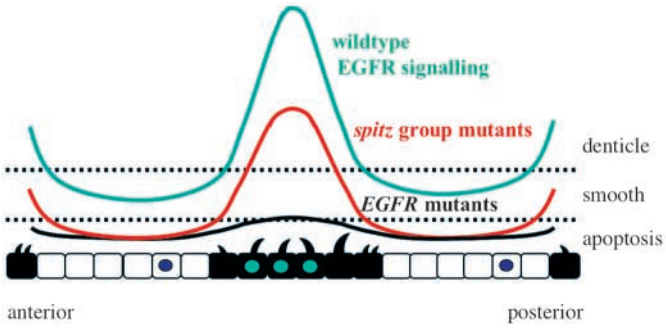


Fig. 8. A refined model for the roles of EGFR signalling in embryonic epidermal patterning (see Discussion). A lateral cross section of a part of the ventral epidermis is depicted with smooth-cuticle cells in white and denticle-secreting cells and denticles in black. The *rhomboid-1*-expressing cells that act as the source of cleaved Spitz are depicted with green nuclei, while cells that are the source of the Wingless signal have blue nuclei. Arbitrary levels of EGFR signalling versus two thresholds for the observed phenotypes are shown above the cells. Mutation of *spitz* group genes (red curve) results in apoptosis of smooth-cuticle cells when the levels of EGFR signalling fall below a threshold, and this is manifest as a denticle belt fusion. Reducing signalling further (for example, by removing the EGFR, black curve) results in denticle belt fusions and failure of denticle fate specification (Szüts et al., 1997).

low-level stimulation of the EGFR by cooperation between the ligands Vein and Spitz, which is activated by Rhomboid-1, Rhomboid-3 and Star.

Developmental basis of the denticle belt fusion phenotype

The ventral epidermis is patterned in multiple stages during 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 (Golembo 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 (Fig. 8). 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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