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Wingless and Hedgehog pattern *Drosophila* denticle belts by regulating the production of short-range signals

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SUMMARY

The secreted proteins Wingless and Hedgehog are essential to the elaboration of the denticle pattern in the epidermis of *Drosophila* embryos. We show that signaling by Wingless and Hedgehog regulates the expression of *veinlet (rhomboid)* and *Serrate*, two genes expressed in prospective denticle belts. Thus, *Serrate* and *veinlet (rhom)* partake in the last layer of the segmentation cascade. Ultimately, Wingless, Hedgehog, Veinlet (an indirect activator of the Egfr) and Serrate (an activator of Notch) are expressed in non-overlapping narrow stripes. The interface between any two stripes allows a reliable prediction of individual denticle types and polarity suggesting that contact-dependent signaling modulates individual cell fates. Attributes of a morphogen can be ascribed to Hedgehog in this system. However, no single morphogen organises the whole denticle pattern.

Key words: *Drosophila*, Denticle pattern, Segment polarity, Hedgehog, Wingless, Egfr, Notch

INTRODUCTION

The embryonic epidermis of *Drosophila* provides a unique system for studying pattern formation at single cell resolution. At the end of embryonic development, the ventral epidermis is decorated by a segmentally repeated denticle pattern (DiNardo et al., 1994). One segmental unit is about 11 cells wide. It comprises 6 rows of cells that secrete denticles while the remaining cells make bald cuticle. Patterning occurs progressively from the deployment of coarsely expressed gap genes to the more refined segment polarity genes (reviewed by Ingham, 1988). Segment polarity genes are known to regulate each other's expression. Most familiar is the non-cell autonomous feedback loop that links the expression of *wingless* and *engrailed* (or *hedgehog*) (DiNardo et al., 1988; Martinez-Arias et al., 1988). In addition, since they are at the bottom of the segmentation cascade, segment polarity gene products are believed to contribute to the specification of individual pattern elements (Bejsovec and Wieschaus, 1993). So far, only the bald fate is well understood: it arises because *shavenbaby* is repressed in response to Wingless signaling (Payre et al., 1999). Individual denticles (numbered 1 to 6) are distinguishable by their shape, size and polarity (Fig. 1A). The precision and resolution of this pattern imply sophisticated mechanisms of positional information. What are those mechanisms and how are they regulated by segment polarity genes?

Studies of the wing imaginal disks of *Drosophila* have led to a general model of pattern formation (reviewed by Lawrence and Struhl, 1996). In essence, at the interface between the anterior and posterior compartments, a morphogen, the TGF-β homolog Dpp, is produced and spreads across the entire disk to specify different cell fates at different thresholds of activity. The boundary separating the anterior and posterior compartments (the compartment boundary) is a clonal boundary established during embryogenesis (Garcia-Bellido et al., 1973; Vincent and O'Farrell, 1992). In the embryo, this boundary is called the parasegment border (Martinez-Arias and Lawrence, 1985) and is marked by the expression of *wingless* at the anterior and *engrailed* (along with *hedgehog*) at the posterior. By analogy with the disk system, this cellular interface could be a source of morphogen (possibly Hedgehog or Wingless itself) that organizes the whole segmental pattern. In support for such a notion, in the absence of both signals, segmental organization and denticle diversity are seemingly lost (Bejsovec and Wieschaus, 1993). However, this loss of segmental patterning could equally be explained if *wingless* (*wg)* and *hedgehog* (*hh)* were at the top of a genetic hierarchy leading to ever more refined expression of short-range signals (Martinez-Arias et al., 1988). Indeed, additional signaling pathways such as those of Epidermal growth factor receptor (Egfr) and Notch are activated in the epidermis of the late embryo.

Patterned activation of Egfr in the embryonic epidermis is primarily modulated by *veinlet*, also known as *rhomboid*. A current hypothesis is that Veinlet *(rhom*), a 7-transmembrane protein, regulates the cleavage of Spitz (a TGF-α homolog) and turns it into a soluble active ligand of Egfr (Schweitzer et al., 1995). In the ventral ectoderm of late embryos, *veinlet (rhom)* is expressed in stripes at the posterior of the *engrailed* domain, a region due to make denticles (Bier et al., 1990; O'Keefe et

al., 1997; Szüts et al., 1997). Since Veinlet *(rhom)* activates Spitz and since Spitz is expressed everywhere (Rutledge et al., 1992), the stripes of *veinlet (rhom)* expression are believed to be a source of active Spitz. Consistent with a role for Egfr signaling in denticle formation, *veinlet (rhom)* contributes to the formation of denticle types 1-4 (Wiellette and McGinnis, 1999) and Spitz has even been proposed to specify different denticle types in a concentration-dependent manner (Szüts et al., 1997). The role of Notch signaling in denticle patterning was suggested more recently by the finding that mutants in *Serrate*, a gene that encodes a Notch ligand, have denticle defects (Wiellette and McGinnis, 1999). Moreover, *Serrate* is also expressed within prospective denticle belts, in a pattern that does not overlap with that of *veinlet (rhom)* expression (Wiellette and McGinnis, 1999). Since both *veinlet (rhom)* and *Serrate* are involved in denticle patterning, we have investigated the regulation of their expression by Hedgehog and Wingless, the two signals originating from the parasegment boundary.

It is already known that Wingless signaling represses *veinlet (rhom)* expression (Sanson et al., 1999; Gritzan et al., 1999). We now find that Hedgehog and Serrate both contribute positively to *veinlet (rhom)* expression while *Serrate* is negatively regulated by both Hedgehog and Wingless. Thus, at the time when cuticular fates are specified, 4 molecules activating 4 signaling pathways (Wingless, Hedgehog, Egfr and Notch) are expressed in a precise spatial pattern within an 8-cell-wide band. We argue that juxtacrine (contact-dependent) interactions mediated by these pathways can account for all denticle types. Nevertheless, we find that, in this system, Hedgehog has differential quantitative effects across three cell diameters. Wingless too acts over several cell diameters, although not in a concentration-dependent manner within the realm of our assays.

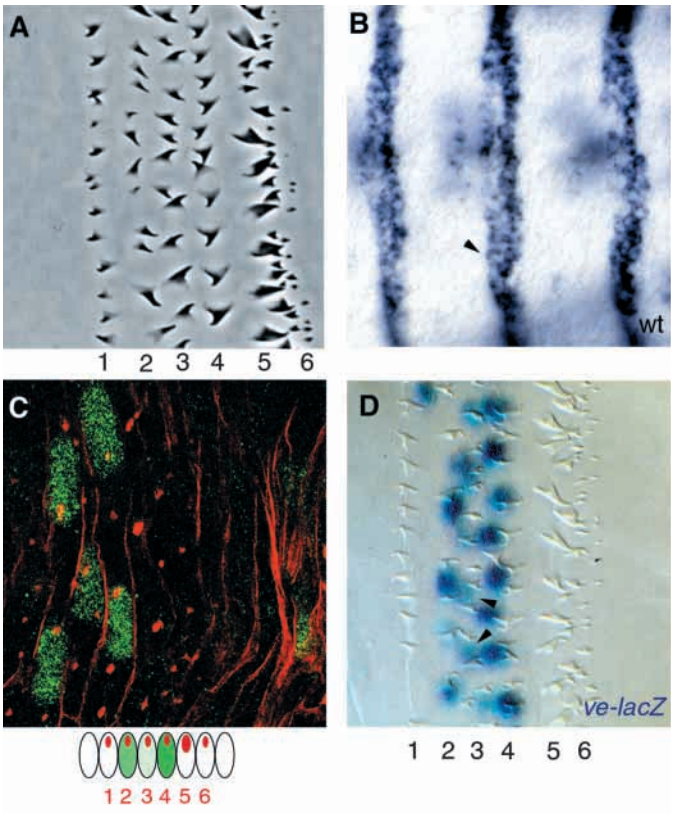


Fig. 1. Wild-type denticle pattern and fine structure of *veinlet (rhom)* expression. (A) Denticle belt from the third abdominal segment. Note the stereotypical shape and polarity of denticles in individual rows, numbered 1 to 6. (B) Expression of *veinlet (rhom)* at stage 13 as revealed by in situ hybridisation. (C) Embryo carrying a *veinlet-nuclear lacZ* enhancer trap gene triple stained for actin and Fasciclin 3 in red and anti-β-galactosidase in green. Actin bundles form in the middle of the apical surface. At the anterior (left) one row of bundles can be seen in β-galactosidase-negative cells while the second row of bundles corresponds to the most anterior *veinlet (rhom)*-expressing cells. Fat actin bundles (anticipating row 5 denticles) can be recognised. These are just posterior to (outside of) the *veinlet (rhom)* expression domain. Note the cells' elongated aspect along the dorsoventral axis. (D) First instar *veinlet-nuclear lacZ* larva stained with X-gal. The domain of *veinlet (rhom)* expression corresponds to denticle types 2 to 4 with weak expressing cells underlying type 3 denticles (arrowheads).

MATERIALS AND METHODS

Drosophila stocks

wg^{cx4} (Baker, 1988), hh^{AC} (Lee et al., 1992), ptc^{9} (Hooper and Scott, 1989), $Df(2R)en^{E}$ (Tabata et al., 1995) and ser^{RX106} (obtained from Sarah Bray, Department of Anatomy, Cambridge) are presumed null alleles (see Flybase at http://gin.ebi.ac.uk:7081/). The $ser^{RX106}hh^{AC}$ recombinant was created by standard genetic methods and the $wg^{cx4}Df(2R)en^{E}$ recombinant was obtained from Peter Lawrence (LMB, Cambridge).

The following transgenic stocks were used: *armadillo-Gal4*^{11} (Sanson et al., 1996), *engrailed-Gal4* (gift from Andrea Brand, Wellcome Institute, Cambridge, UK), *tubulin-Gal4* (Pignoni and Zipursky, 1997), UAS-*Ci* (Alexandre et al., 1996), UAS-*CiN/Zn*, which we call UAS-*Ci[repressor]* (Hepker et al., 1997), UAS-N^{intra} (gift from M. Haenlin, CNRS Toulouse), *veinlet-lacZ* (line X81; Freeman et al., 1992), UAS-arm^{S10} (Pai et al., 1997).

Genotypes of analysed embryos are as follow: *tubulin-Gal4*/UAS-*Ci* (Fig. 2B); wg^{cx4}/wg^{cx4}; *tubulin-Gal4*/UAS-*Ci* (Fig. 2C); *armadillo-Gal4*^{11}/UAS-N^{intra} (Fig. 3B and 3C); $hh^{AC}ser^{RX106}/hh^{AC}ser^{RX106}$ (Fig. 3D); *armadillo-Gal4*^{11}/UAS-*Ci* (Fig. 4B); *armadillo-Gal4*^{11}/UAS-arm^{S10} (Fig. 4C); hh^{AC}/hh^{AC} (Fig. 4D), hh^{AC}*ftz-lacZ*/hh^{AC}*ftz-lacZ* (Fig. 4E); wg^{cx4}/wg^{cx4}; *ftz-lacZ engrailed-Gal4*/UAS-*Ci[repressor]* (Fig. 5A-C); wg^{cx4}/wg^{cx4} (Fig. 6A,B); ptc^{9}/ptc^{9} (Fig. 6C,D); $wg^{cx4}Df(2R)en^{E}/wg^{cx4}Df(2R)en^{E}$ (Fig. 6E,F).

The *ftz-LacZ* transgene encodes a nuclear localised GFP-β-galactosidase fusion protein under the control of the complete *fushi tarazu* promoter. Details of the construction can be obtained upon request.

Embryo preparations

Except when anti-actin was used, immunofluorescence was done according to standard protocols (e.g. Vincent and O'Farrell, 1992) using Alexa^{TM} fluorescent conjugates (Alexa 488 and Alexa 592, Molecular probes). For anti-actin staining (Fig. 1C), embryos were manually devitellinised to avoid methanol treatment, which destabilises the cytoskeleton. Antibodies used were rabbit anti-β-galactosidase (Cappell), mouse anti-β-galactosidase (Promega), rabbit anti-Engrailed (gift from C. H. Girdham and P. H. O'Farrell, UCSF), mouse anti-Fasciclin 3 (mAb 7G10 from Developmental Studies Hybridoma Bank), mouse anti-actin (mAb JLA20 from Developmental Studies Hybridoma Bank).

For RNA single and double in situ hybridisation, embryos were fixed and hybridised with digoxigenin- or fluorescein-labelled single-

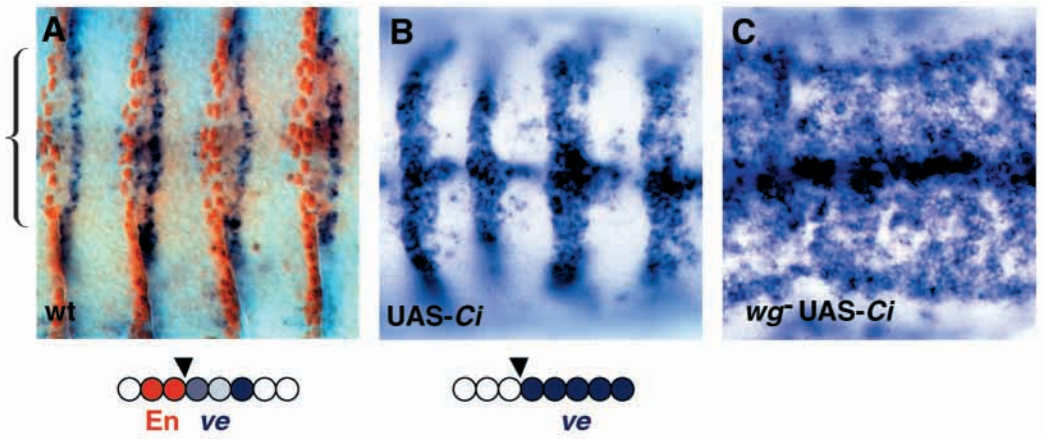


Fig. 2. Hedgehog signaling activates *veinlet (rhom)* expression. (A) Wild-type stage 13 embryos stained with anti-Engrailed in ochre and a *veinlet (rhom)* RNA probe in purple. The weak-staining anterior *veinlet (rhom)* substripe abuts the domain of Engrailed expression, the source of secreted Hedgehog. The segment boundary (marked with an arrowhead in the cartoon) lies at the interface between these cell rows. Bracket indicates the ventralmost cells, whose behavior is the object of this paper. (B) Pattern of *veinlet (rhom)* expression in a stage 13 embryo expressing Ci uniformly (driven by the *tubulin-Gal4* driver). The domain of *veinlet (rhom)* expression is widened. Anterior edge of the expanded *veinlet (rhom)* domain still corresponds to the segmental grooves (arrowhead in diagram) implying that widening is towards the posterior. (C) Overexpression of Ci in a *wingless* mutant. This results in widespread *veinlet (rhom)* expression.

stranded RNA probe as described by Jowett (1997) except that fixed embryos were kept in 100% methanol and no proteinase K treatment was performed. The following cDNAs were used: *Serrate* (gift from E. Knust, Düsseldorf University, Germany), *veinlet (rhom)* (gift from J. F. de Celis, Cambridge University), *engrailed* (Poole et al., 1985) and *hedgehog* (gift from M. van den Heuvel, Oxford University).

For double labelling with an antibody and an RNA probe, the RNA in situ was performed first with the hybridization step performed at 58°C. After histochemical staining, embryos were fixed in 4% formaldehyde for 30 minutes and standard immunostaining was subsequently performed.

β-galactosidase activity was detected in cuticle preparations as described previously by Sanson et al. (1999).

For visualisation of the cuticle pattern, 24-hour-old embryos were mounted in Hoyer's and photographed in DIC or phase-contrast microscopy.

RESULTS

Regulation of *veinlet (rhom)* expression

Experiments in this paper are concerned with gene expression and pattern formation in the ventralmost region of the *Drosophila* ectoderm. In the wild type, segmental expression of *veinlet (rhom)* commences at late stage 11 and is clearly established at stage 12 (not shown). We find that the structure of *veinlet (rhom)* stripes is subtler than previously described (Bier et al., 1990; Szüts et al., 1997; Wiellette and McGinnis, 1999). In situ hybridization with a *veinlet (rhom)* probe reveals expression in twinned substripes: a strong substripe at the posterior and a weaker one at the anterior (Fig. 1B). The substripes are separated by a single row of cells that do not stain. These 'middle cells' do (or did) nevertheless express *veinlet (rhom)* at low level since, in *veinlet-lacZ* embryos, they contain weak β-galactosidase activity (see below). To map more precisely the fine structure of the *veinlet (rhom)* domain onto specific cell fates, we used the finding that actin bundles serve as a template for denticles and thus anticipate the mature denticle pattern (Dickinson and Thatcher, 1997; Edwards et al., 1997). *veinlet-lacZ* embryos were triple stained with anti-actin to locate prospective denticles, anti-Fasciclin 3 to visualize cell boundaries and anti-β-galactosidase to detect *veinlet (rhom)*

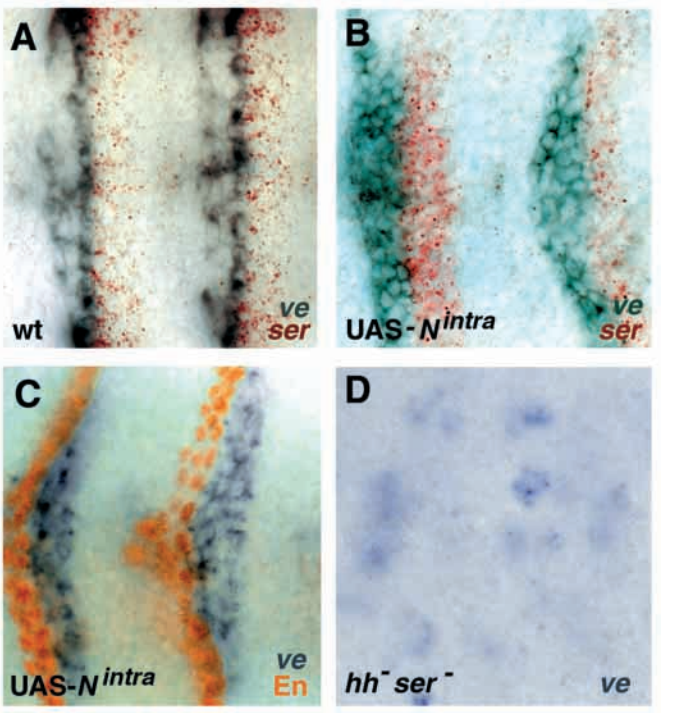


Fig. 3. Notch signaling activates *veinlet (rhom)* expression. (A) Wild-type stage 13 embryos stained with a *veinlet (rhom)* RNA probe in black and a *Serrate* probe in red. The *Serrate* domain is at the posterior of the *veinlet (rhom)* domain. (B) Ubiquitous activation of the Notch pathway from uniform expression of N^{intra} leads to an increase of *veinlet (rhom)* expression. *Serrate* expression is seemingly unchanged and is still at the posterior of the increased *veinlet (rhom)* domain. (C) Embryos of the same genotype as in B, stained with anti-Engrailed in ochre and a *veinlet (rhom)* RNA probe in purple. This shows that *veinlet (rhom)* expression is still bounded by Engrailed when the Notch pathway is overactivated. In both B and C, the domain of *veinlet (rhom)* expression contain more cells despite being bounded by Engrailed and *Serrate* as in the wild-type. Maybe an extra cell division has occurred in this domain. (D) Lack of *veinlet (rhom)* expression in a $hh^{-}ser^{-}$ double mutant. No expression is detectable in the ectoderm although there may be weak expression in underlying tissues.

expression (Fig. 1C). From such preparations, we find that the second row of denticles (counting from the anterior) is secreted by the most anterior cells of each *veinlet (rhom)* stripe while the most posterior *veinlet (rhom)*-expressing cells make the fourth row of actin bundles. Squeezed between these two rows are the middle cells, which produce the third row of actin bundles. These weakly staining cells can still be detected at hatching and are seen to underlie type 3 denticles as expected (arrowheads in Fig. 1D). We now address how the twinned structure of the *veinlet (rhom)* stripes arises.

The anterior *veinlet (rhom)* substripe abuts the posterior compartment, the domain of *engrailed* expression and the source of Hedgehog in each segment (Fig. 2A). This component of *veinlet (rhom)* expression could therefore be activated by Hedgehog signaling. We found that, indeed, ectopic activation of the Hedgehog pathway by overexpression of the transcription factor Cubitus interruptus (Ci) leads to additional *veinlet (rhom)* expression. Embryos carrying the ubiquitous *armadillo-Gal4* driver and the UAS-*Ci* responder have expanded domains of *veinlet (rhom)* expression (Fig. 2B). Expansion is towards the posterior since the expanded stripes are still bounded by segmental grooves at the anterior. The domain where *veinlet (rhom)* is not activated corresponds to the *engrailed*-expressing cells and the zone of Wingless influence. This suggests that Engrailed itself could possibly repress *veinlet (rhom)*. Repression by Wingless signaling has already been shown (Sanson et al., 1999; Gritzan et al., 1999). As expected, therefore, uniform Ci expression in a *wingless* mutant leads to widespread *veinlet (rhom)* expression (Fig. 2C). This confirms that activation of the Hedgehog pathway is sufficient to induce *veinlet (rhom)* expression except where the Wingless pathway is active (and also where Engrailed is present). However, *veinlet (rhom)* expression is still detected in the ectoderm of *hedgehog* mutants (Sanson et al., 1999) suggesting that an additional signal operates during normal development.

One such additional regulator of *veinlet (rhom)* expression is Serrate (Wiellette and McGinnis, 1999), a protein expressed behind each *veinlet (rhom)* domain right against the posterior substripe (Fig. 3A). In the absence of *Serrate*, *veinlet (rhom)* stripes become thinner with expression being lost at the posterior (Wiellette and McGinnis, 1999). Since Serrate is a known ligand of Notch, we asked whether ectopic activation of the Notch pathway is sufficient to activate *veinlet (rhom)* expression. We expressed N^{intra} (a ligand-independent form of Notch, Lieber et al., 1993; Struhl et al., 1993) uniformly and assayed *veinlet (rhom)* expression. Expression is increased, especially in the middle cells and twinned stripes are no longer resolved (compare Fig. 3B,C with A). However, the domain of *veinlet (rhom)* expression is still bounded by Engrailed at the anterior and *Serrate* at the posterior (Fig. 3B,C). Again, we presume that *veinlet (rhom)* is not activated everywhere because of the presence of antagonistic signals such as Wingless and possibly other factors (see below). Nevertheless, the Notch pathway has a positive effect on *veinlet (rhom)* expression implying that Notch may contribute to normal *veinlet (rhom)* expression. Most likely, expression at the posterior of each *veinlet (rhom)* substripe is activated by Serrate produced in posterior adjacent cells (see also Wiellette and McGinnis, 1999). Since expression of *Serrate* is detected earlier than that of *veinlet (rhom)*, around early stage 11 (not shown), it appears that Serrate defines the posterior edge of *veinlet (rhom)* stripes. Note that *veinlet (rhom)* is not expressed in *Serrate*-expressing cells and that, therefore, activation of *veinlet (rhom)* by Serrate is strictly non-cell autonomous.

In *hedgehog* mutants, *Serrate* expression is still present and even expanded (Wiellette and McGinnis, 1999 and see below) and this could account for the remaining *veinlet (rhom)* expression in these embryos. If this were the case, one would expect that *veinlet (rhom)* would not be expressed in the absence of both *hedgehog* and *Serrate*. We therefore assayed *veinlet (rhom)* expression in $hh^{-}ser^{-}$ double mutants. No expression is detectable in the epidermis (Fig. 3D), confirming that Hedgehog and Serrate both contribute to the activation of *veinlet (rhom)* expression, Hedgehog at the anterior and Serrate at the posterior. Thus, *veinlet (rhom)* expression is positively regulated in a position-dependent manner by two distinct signals.

Regulation of *Serrate* expression

As we show above, Serrate contributes to *veinlet (rhom)* regulation. Since we would like to obtain a complete picture of how Wingless and Hedgehog (the two proteins secreted at the parasegment border) regulate *veinlet (rhom)* expression, we decided to investigate the effect of these two signaling pathways on *Serrate* expression. In the ventral ectoderm, *Serrate* is expressed in stripes (2- to 3-cell wide) located at the posterior of each *veinlet (rhom)* domain (Fig. 3A). Overactivation of either the Hedgehog or the Wingless pathway (by overexpressing the downstream effectors Ci or Armadillo) completely abrogates this expression (Fig. 4A-C). This result shows that, at least in an overexpression assay, signaling by Hedgehog or Wingless represses *Serrate* expression. We now test the validity of these regulatory relationships in mutant embryos.

Consistent with the repressive activity of overactivated Hedgehog signaling, *Serrate* expression is expanded in *hedgehog* mutants and only thin bands of non-expression remain (Wiellette and McGinnis, 1999; Gritzan et al., 1999). Double staining shows that these thin bands express *veinlet (rhom)* (Fig. 4D). Thus *veinlet (rhom)* expression flanks the expanded domain of *Serrate* expression and is most certainly activated by Serrate since $hh^{-}ser^{-}$ double mutants no longer express *veinlet (rhom)* (see above).

We mapped the expansion of *serrate* expression in *hedgehog* mutants using *ftz-lacZ* as a permanent marker of the parasegment boundary, which lines the anterior edge of the posterior compartment. With this landmark, we can see that, in hh^{-}, *Serrate* expression expands posteriorward all the way to the parasegment boundary (Fig. 4E). Expression of *Serrate* also expands anteriorly since only a thin band of non-expressing cells remain. We conclude therefore that both the anterior and the posterior edge of the *Serrate* domain are affected in *hedgehog* mutants. The cells that do not express *Serrate* (and express *veinlet*) in hh^{-} correspond to those that expressed Engrailed previously (in hh^{-} embryos, Engrailed decays progressively; Bejsovec and Wieschaus, 1993) and, therefore, Engrailed expression may have a repressive effect on the *Serrate* promoter.

In *wingless* mutants, *Serrate* expression also expands, again consistent with the repressive effect of Wingless. Expansion occurs to a lesser extent than in *hedgehog* mutants (Wiellette

and McGinnis, 1999; Gritzan et al., 1999). Using the same *ftz-lacZ* transgene, we find that in *wg*⁻ embryos, the repressed state is maintained in stripes that include the domain of extinct Engrailed expression and flanking cells (Fig. 4F). These flanking cells are those that were exposed to Hedgehog during early development (*hedgehog* is briefly expressed in *wingless* mutants, Lee et al., 1992) and this may explain why they do not express *Serrate* (see Discussion).

Since expression of *wingless* and *hedgehog* is mutually dependent, the expansion of *Serrate* expression in either mutant could be the compounded outcome of losing both activities. (The overexpression experiments suggest that both do repress *Serrate*). Therefore, the relative contributions of each mutation in *Serrate* deregulation are not easily distinguished. We suggest that posterior expansion of *Serrate* expression in *hh*⁻ is due indirectly to the loss of Wingless expression because Hedgehog is not believed to act beyond the *wingless*-expressing cells (Fietz et al., 1995). Therefore, the range of Wingless defines the posterior edge of each *Serrate* stripe. Conversely, Wingless signaling is not operative at the posterior of Engrailed stripes (Sanson et al., 1999) and, therefore, it must be Hedgehog that prevents *Serrate* expression from creeping anteriorward. In other words, Hedgehog appears to specify the anterior limit of the *Serrate* domain. This implies that Hedgehog acts over three cell diameters, slightly more than is expected from its known range of action in embryos. This issue will be addressed in the Discussion.

Overall, it appears that *Serrate* expression is primarily regulated negatively and that its pattern is delimited by Wingless, Hedgehog and Engrailed.

Correlating gene expression with denticle patterns

In *Serrate* mutants, *veinlet (rhom)* expression wanes at the posterior of each stripe (Wiellette and McGinnis, 1999). By analogy, one might predict that, in *hedgehog* mutants, *veinlet (rhom)* expression would decay at the anterior of each stripe since Hedgehog activates the anterior *veinlet (rhom)* substripe. However, the phenotype of *hedgehog* mutants is not so straightforward because of the loss of *wingless* expression in these embryos. As shown above, loss of *wingless* results in the expansion of the *Serrate* domain and this in turn, leads to the activation of *veinlet (rhom)* expression in ectopic cells (see below). In order to assess the proximal role of Hedgehog, we sought a way to block its expression around stage 11 when the denticle pattern begins to be specified. This was achieved by expressing a truncated form of Ci (CiN/Zn, Hepker et al., 1997), which represses the expression of *hedgehog* (Fig. 5A). We hence refer to the UAS-*CiN/Zn* transgene as UAS-*Ci[repressor]*. In embryos of the genotype *engrailed-Gal4* UAS-*Ci[repressor]* (which we call *Ci[repressor]* embryos), *hedgehog* is expressed normally until early stage 11 and then fades away. In these embryos, *veinlet (rhom)* expression decays at the anterior of each stripe as predicted and, in its place, *Serrate* expression appears. The latter observation confirms the negative effect of Hedgehog on *Serrate* expression. In addition, *veinlet (rhom)* expression is upregulated in the middle cells. This, we think, is because the middle cells are now in contact with *Serrate*-expressing cells and have a fully activated Notch pathway. The final outcome is that *veinlet (rhom)* is expressed in 2-cell-wide stripes at uniformly high intensity (Fig. 5B,C).

Importantly, *Ci[repressor]* embryos display a denticle pattern unlike any previously seen in mutants (Fig. 5C). In the middle of each belt, one clearly recognises a mirror-image 5, 4, 4, 5 pattern. This is flanked by small denticles pointing away from the belt. As seen with a *veinlet-lacZ* transgene, the type 4 denticles in these embryos correspond to the domain of *veinlet (rhom)* expression. Thus the ectopic *Serrate*/*veinlet (rhom)* interface corresponds to the formation of denticle types 5/4 at the anterior of each belt while types 4/5 still form at the normal *veinlet (rhom)*/*Serrate* interface. Note that this interface (whether endogenous or ectopic) is characterized by a polarity reversal. The identity of the small anterior-pointing denticles at the anterior of each belt is not readily apparent. On the basis of size and morphology, they could be either of type 1 or type 6. We suggest that they are of type 1 because, like type 1 denticles in the wild type, they express Engrailed. This was shown by mapping Engrailed expression in *Ci[repressor]* larvae with a *lacZ* reporter (not shown). Finally, it seems natural to assign the type 6 label to the small denticles at the posterior of *Ci[repressor]* denticle belts since gene expression there is the same as in the wild type.

In the previous paragraph, we argued that a *veinlet (rhom)*/*Serrate* interface corresponds to denticle types 4/5 with its attendant polarity reversal while Engrailed expression corresponds to type 1 (as suggested by Dougan and DiNardo, 1992; Lawrence et al., 1996). These correlations apply to both *Ci[repressor]* and wild-type embryos. Further inspection of the wild-type situation suggests additional relationships. First, the interface between cells expressing *hedgehog* (and *engrailed*) and *veinlet (rhom)* corresponds to types 1 and 2 and is also accompanied by a polarity reversal. Second, type 3 denticles are made in the middle of the *veinlet (rhom)* domain and may correspond to normal activation of the Egfr pathway without influence from another pathway. Finally, activation of the Wingless pathway is always associated with the absence of denticles (Bejsovec and Martinez Arias, 1991; Sanson et al., 1999; Payre et al., 1999).

We now ask if the relationships outlined above have any predictive value in various mutant conditions. We start with *wingless* mutants because they have been described as making only type 5 denticles (Bejsovec and Wieschaus, 1993) and yet the striped expression pattern of *veinlet (rhom)* and *Serrate* in these embryos (Fig. 6A) suggests that more pattern elements should form. We re-examined *wg*⁻ cuticle preparation at high magnification and found reproducible, ordered arrays of denticles of more than one type (see also Gritzan et al., 1999). In particular, types 4 and 5 are recognisable by their size, shape and polarity (Fig. 6B). This arrangement is expected from the *veinlet (rhom)*/*Serrate* interfaces seen at earlier embryonic stages (Fig. 6A). Since *hedgehog* (and *engrailed*) are no longer expressed in *wingless* mutants, no *hedgehog* (*engrailed*)/*veinlet (rhom)* interfaces exist and, consistent with this, denticles types 1 and 2 are not recognisable.

We now turn to a second mutation. The phenotype of *patched* mutants has been described as a mirror-image duplication with denticle types 1, 2, 3, 2, 1 in sequence (Martinez-Arias et al., 1988). Although this is broadly accurate, we find the middle denticles difficult to type. What is clear is the presence of 1/2 and 2/1 interfaces (Fig. 6D). Those are easy to interpret in light of the expression of all the relevant genes. First, no *Serrate* is expressed in these embryos

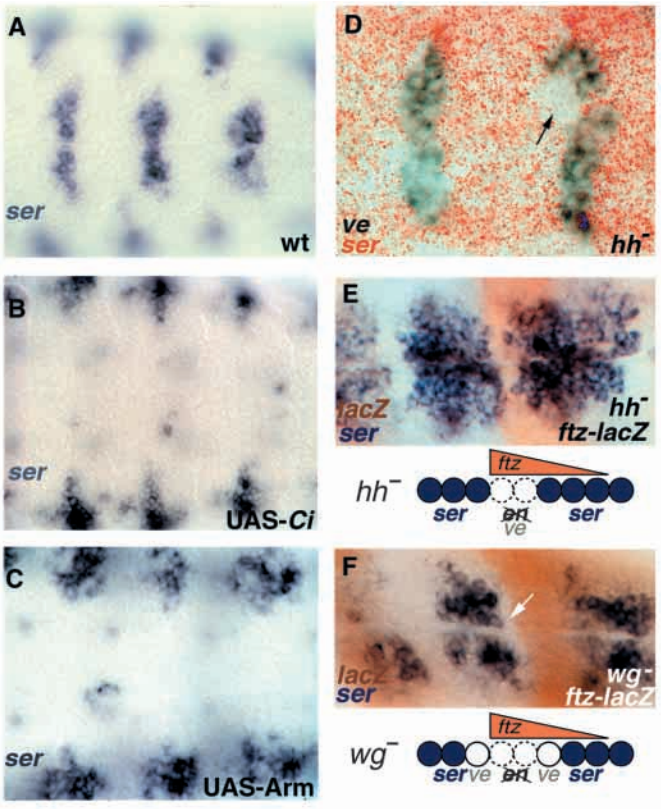


Fig. 4. Expression of *Serrate* is negatively regulated by Wingless, Hedgehog and possibly Engrailed. (A) Expression of *Serrate* in the wild-type at stage 11 (detected with an RNA probe). In the ventral region, *Serrate* is expressed in segmental stripes. Lateral expression (at the edge of the picture) corresponds to the tracheal pits. (B) Overactivation of the Hedgehog pathway by uniform expression of Ci (with *armadillo-Gal4*) eradicates ventral expression. Expression in the tracheal pits is unaffected. (C) Repression of *Serrate* is also seen when the Wingless pathway is uniformly activated as in embryos carrying *armadillo-Gal4* and UAS-*Armadillo*^{S10} (an activated form of Armadillo; Pai et al., 1997). (D) Stage 13 *hh*$^{-}$ embryos stained with a *Serrate* RNA probe in red and a *veinlet (rhom)* probe in purple. *Serrate* expression is clearly expanded compared to the wild-type and *veinlet (rhom)* expression nearly fills the region where *Serrate* is not expressed. Occasional islands where neither gene is expressed are always located at the anterior of the *veinlet (rhom)* stripe (arrow). They may reflect a long lasting repressive influence from Wingless expressed early. For the sake of completeness, we mention that extensive cell death occurs in segment polarity mutants (Pazdera et al., 1998) and this may contribute to the ultimate expression pattern. (E) Expression of β-galactosidase (brown) and *Serrate* (purple) in a stage 12 *hedgehog* mutant embryo carrying a *ftz-lacZ* transgene. The posterior edge of the expanded *Serrate* domain aligns with the anterior edge of the *ftz-lacZ* domain. This is known to correspond to the parasegment boundary and the anterior edge of the Engrailed domain in alternate segments (Lawrence and Johnston, 1989). Schematic representation of this result is shown in the diagram. F: Expression of β-galactosidase (brown) and *Serrate* (purple) in a stage 12 *wingless* mutant embryo carrying a *ftz-lacZ* transgene. The posterior edge of the expanded *Serrate* domain just falls short of the anterior edge of the *ftz-lacZ* domain (white arrow). This suggests the presence of residual repressive activity originating from the posterior compartment. Diagram shows schematically the expression of all relevant genes: *veinlet (rhom)* expression flanks the domain of extinct Engrailed expression (Sanson et al., 1999) and *Serrate* expansion is more limited than in a *hedgehog* mutant (Wiellette and McGinnis, 1999).

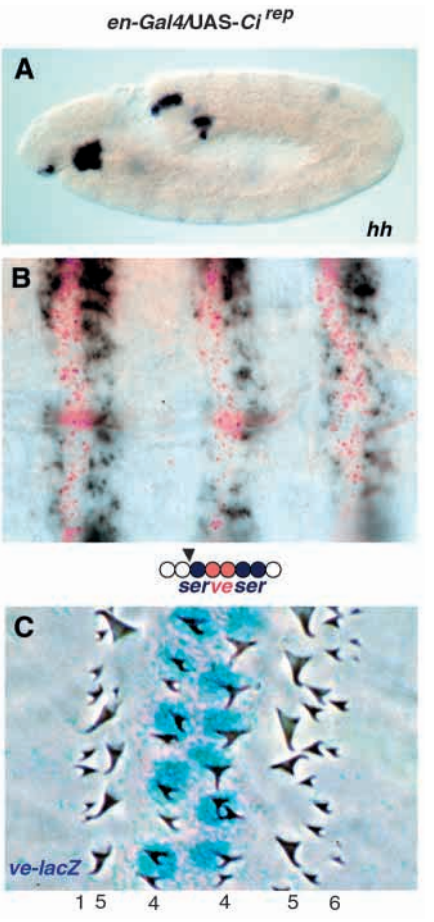


Fig. 5. Late removal of *hedgehog* expression leads to a novel denticle pattern. (A) Expression of *hedgehog* in a stage 11 Ci[repressor] embryo (*engrailed-Gal4* UAS-*Ci[repressor*). Expression is no longer detectable in the segmented ectoderm. This is consistent with the finding that Ci is a repressor of *hedgehog* expression in imaginal disks (Dominguez et al., 1996). Expression of *engrailed* is normal in such embryos (not shown). (B) Expression of *veinlet* (red) and *Serrate* (black) in an embryo of the same genotype as in A. The domain of *veinlet (rhom)* expression is reduced by one cell at the anterior and this is replaced by ectopic *Serrate* expression. (C) Denticle pattern of a *Ci[repressor]* larva (as in A,B) carrying a *veinlet-nuclear lacZ* enhancer trap gene. Staining with X-gal shows that the *veinlet (rhom)*-expressing cells give rise to type 4 denticles. Note that *veinlet (rhom)* expression is uniform in the two rows of expressing cells. Polarity reversals are clearly seen at the *veinlet (rhom)*/*Serrate* interfaces (ectopic and endogenous). This denticle phenotype is partially rescued by adding back extra Hedgehog from a transgene (not shown). This suggests that loss of *hedgehog* transcription is the principal cause of the phenotype but we cannot exclude the possibility that Ci[repressor] represses an additional factor.

(Wiellette and McGinnis, 1999). This is expected since loss of *patched* function leads to ubiquitous activation of the Hedgehog pathway (Ingham et al., 1991) and this, as we have shown, leads to repression of *Serrate*. As a consequence, no *Serrate*/*veinlet (rhom)* interface exist and therefore, no denticle types 4/5 form. However, ectopic Engrailed stripes appear (Martinez-Arias et al., 1988) and hence new *hedgehog* (*engrailed*)/*veinlet (rhom)* interfaces are created (Fig. 6C) and ectopic 2/1 types form at the posterior of each belt.

Finally, we look at *wg*$^{-}$*en*$^{-}$ double mutants. Although these embryos are lacking much pattern (Lawrence et al., 1996), remaining whorls of polarity reversals are readily seen (Fig. 6F). These whorls are anticipated by holes in the otherwise uniform expression of *Serrate* (Fig. 6E). We suggest that these holes follow, at least in part, from a long-lasting repressive effect of Hedgehog, which is probably expressed fleetingly in these mutants (Bejsovec and Wieschaus, 1993). In these mutants, expression of *veinlet (rhom)* is in rings that are

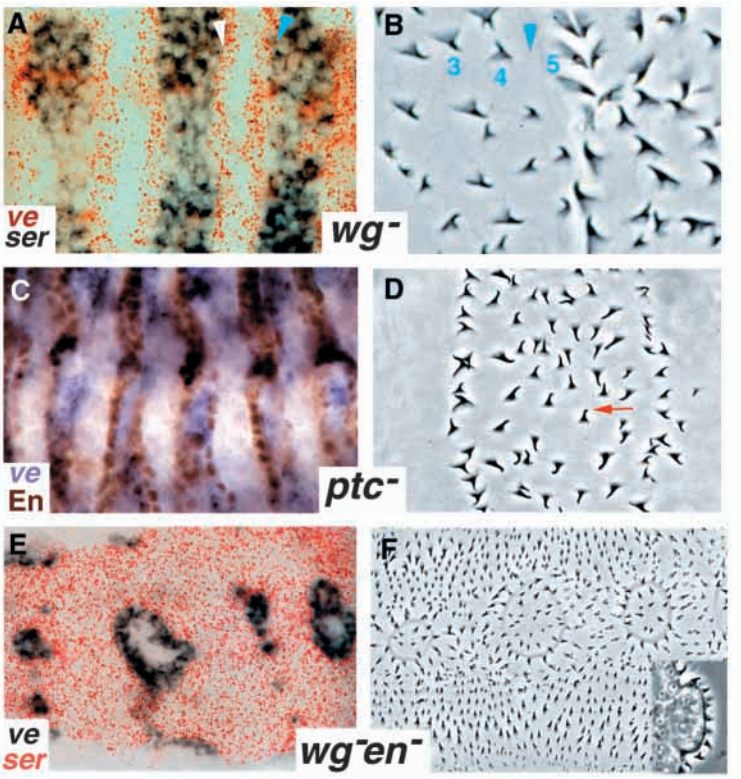
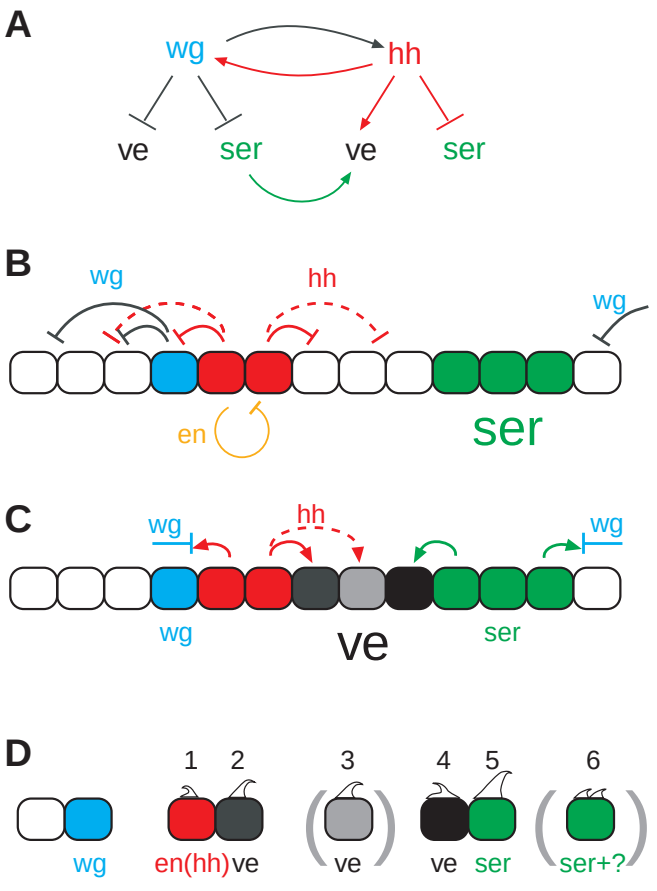


Fig. 6. Correlation between gene expression and denticle pattern in three mutant situations. (A) Expression of *Serrate* (black) and *veinlet* (red) in a stage 13 *wingless* mutant. An endogenous *Serrate*/*veinlet (rhom)* interface is marked by a blue arrowhead. Expression of *Serrate* has expanded posteriorly and induced an ectopic *veinlet (rhom)* stripe there. Ectopic activation of *veinlet (rhom)* occurs because Wingless is no longer present to counter the induction of *veinlet (rhom)* by Serrate. An ectopic interface (white arrowhead) forms as a result. (B) High magnification view of a *wg*⁻ cuticle pattern. Different types of ordered denticle types can be seen. The presumed position of an endogenous *veinlet (rhom)*/*Serrate* interface is shown with a blue arrowhead as in A. (C) Expression of Engrailed (brown) and *veinlet* (purple) in a stage 13 *patched* mutant. Segmental grooves form at both the endogenous and the ectopic *engrailed* (*hedgehog*)/*veinlet (rhom*) interfaces. Both grooves are deeper than wild-type grooves. Many cells flanking the grooves are below the plane of focus and cannot be seen as a result. (D) Cuticle pattern of a *patched* mutant. The *engrailed* (*hedgehog*)/*veinlet (rhom*) interfaces correspond to polarity reversal at the anterior and the posterior of each belt. Although these reversals are not always perfect, they are completely penetrant. Cells in the middle of each belt make unusually shaped denticles (notice the round tip, red arrow). This (and also the deep grooves) may be a consequence of the overactivation of the Egfr pathway since *veinlet (rhom*) expression is stronger in *patched* mutants than in wild type siblings. (E) Expression of *Serrate* (red) and *veinlet* (black) in a *wg*⁻*en*⁻ double mutant. Expression of *Serrate* is widespread except in segmentally repeated holes. These holes are lined with *veinlet (rhom*)-expressing cells. (F) Cuticle preparation of a *wg*⁻*en*⁻ double mutant. Segmentally repeated denticle whorls are visible. Inset shows a whorl in side view, demonstrating the 'bulging out' effect.



inscribed within the *Serrate* holes (Fig. 6E). This is to be expected since Serrate activates *veinlet (rhom)* expression non-autonomously. Note that, although one would intuitively assume that the rings of *veinlet (rhom)* expression are induced by an activity originating from within (such as factor X in Gritzan et al., 1999), it is the surrounding cells that produce the activator. As seen in Fig. 6F, the circular *veinlet (rhom)*/*Serrate* interfaces lead to local polarity reversal. We would like to suggest that type 5 denticles surround each circle while type 4 denticles line its inner face and type 3 fill the interior in response to Egfr signaling. However, although the shape of the various denticles is broadly consistent with this suggestion, we acknowledge that there is a subjective element to denticle typing. Note that, in *wg*⁻*en*⁻ embryos, most denticles point towards the midline. This could be due to influences from the dorsoventral patterning system. We note in passing that denticle whorls bulge out from the epithelium (Fig.

Fig. 7. From Wingless and Hedgehog to the denticle pattern. (A) Summary of the regulatory interactions between *hedgehog*, *wingless*, *Serrate* and *veinlet (rhom*). (B) Spatial regulation of *Serrate* expression by Wingless and Hedgehog signaling and Engrailed. Dotted lines emanating from the Hedgehog cells reflect an early action of Hedgehog whose range may require cell movement and proliferation as described in the text. (C) Spatial regulation of *veinlet (rhom*) expression by Wingless, Hedgehog and Serrate signaling. Dotted arrows emanating from the Hedgehog cells indicate weak Hedgehog action beyond one cell diameter. (D) Apparent correlations between gene expression and specific cuticular structures. Further experiments are needed to confirm the correlations in parentheses.

6F, inset). This may reflect local changes in cell adhesion at *veinlet (rhom)*/*Serrate* interfaces. We are currently investigating this further.

DISCUSSION

A final layer in the segmentation cascade

In this paper, we show that *veinlet (rhom)* and *Serrate* are segmentation genes acting downstream of segment polarity genes and thus form an additional layer in the segmentation cascade initiated by gap and pair-rule genes. We have worked out how the localized expression of Wingless and Hedgehog at the parasegment boundary leads to expression of *Serrate* and *veinlet (rhom)* at specific positions within the segmental pattern (Fig. 7A-C). Both signaling pathways repress *Serrate* expression. Since both pathways are believed to activate transcription, we imagine that they activate the expression of a repressor of *Serrate*. In addition, *Serrate* may also be negatively regulated by the transcriptional repressor Engrailed. In contrast to *Serrate*, *veinlet (rhom)* is regulated both positively and negatively: it is repressed by Wingless (Sanson et al., 1999; Gritzan et al., 1999) and activated by Hedgehog (Fig. 2).

In addition to this vertical flow of information, regulatory interactions also exist between *veinlet (rhom)* and *Serrate*. At the least, Serrate activates *veinlet (rhom)* expression by way of the Notch pathway (Fig. 3). This effect is purely non-cell autonomous. In contrast, Serrate appears to repress *veinlet (rhom)* in a cell autonomous manner (indeed, in cells where it is expressed, Serrate represses the Notch pathway; Micchelli et al., 1997). However, it is also possible that whichever mechanism activates *Serrate* expression also represses *veinlet (rhom)* expression. This would explain why the expression of *Serrate* and *veinlet (rhom)* is always mutually exclusive.

The regulatory interactions summarised above are sufficient to explain the spatial pattern of both *Serrate* (Fig. 7B) and *veinlet (rhom)* (Fig. 7C) expression. Non-autonomous repression of Serrate by Wingless and Hedgehog ensures that *Serrate* is expressed in stripes. As we have shown, the spread of Wingless towards the anterior defines the posterior edge of the domain of *Serrate* expression. Similarly, the anterior edge of the *Serrate* domain appears to be specified over three cell diameters by Hedgehog (Fig. 7B) slightly further than expected since Hedgehog is thought to act only over 1-2 cells in *Drosophila* embryos (Fietz et al., 1995). Expression of *veinlet (rhom)* is activated by two different signals, Hedgehog at the anterior and Serrate at the posterior. Although Hedgehog signaling is symmetrical, it does not activate *veinlet (rhom)* expression anteriorly (blocked red arrow in Fig. 7C) because there, Wingless represses *veinlet (rhom)* expression. Likewise, Serrate activates *veinlet (rhom)* expression but only on one side because of unilateral repression by Wingless (blocked green arrow in Fig. 7C).

These interactions display a clear temporal hierarchy. The secreted molecules Hedgehog and Wingless are expressed first and where they do not reach, *Serrate* expression is subsequently allowed. At stage 11, Hedgehog and Serrate activates *veinlet (rhom)* expression in separate cells. Ultimately, this chain of interactions results in detailed patterns of gene expression.

Correlating signaling pathways with denticle type

Mapping the expression pattern of various genes onto the denticle pattern suggests simple correlations, which are listed in the Results and summarised in Fig. 7D.

These correlations have allowed us to see pattern where it was previously thought there was none, as in *wingless* mutants (Fig. 6B). We now believe that *wingless* mutants make denticle type 3, 4 and 5 and not exclusively type 5 as suggested by Bejsovec and Wieschaus (1993). The correlations provide a guide to understand various phenotypes such as those of *patched* mutants and *wg*$^{-}$*en*$^{-}$ double mutants. In *wg*$^{-}$*en*$^{-}$ double mutants, the correlation between gene expression and denticle type/polarity is particularly evident. As we have shown, expression of *veinlet (rhom)* is in circles surrounded by *Serrate* expression and this correlates with polarity reversal in the cuticle. Non-uniform gene expression shows that these embryos have more pattern than previously noted (Lawrence et al., 1996). For such embryos to be truly unpatterned, they would have to express *Serrate* uniformly as well as not express *veinlet (rhom)*. This may occur in *wg*$^{-}$*en*$^{-}$*hh*$^{-}$ triple mutants since they may not contain any repressor of *Serrate*. We presume that the converse situation (*Serrate* 'off' and *veinlet (rhom)* 'on' everywhere) would also lead to unpatterned embryos. We predict that this situation would prevail in *wg*$^{-}$ *ptc*$^{-}$*en*$^{-}$ triple mutants.

Although the correlations have good predictive value (Fig. 6), they suffer from several limitations. First, denticle shape does not necessarily reflect an integer value. Indeed, unambiguous typing is not always possible and exact denticle shapes vary from segment to segment. Second, causal relationships between the activation of a particular signaling pathway and a given denticle type still remain to be investigated. We expect that the various signaling pathways control cytoskeletal behavior, which in turn affects denticle shape and cell polarity. Local polarity reversals indicate that individual cells are able to locate the source of a particular signal, suggesting that subcellular signaling complexes control the cytoskeleton directly. Third, we cannot exclude the involvement of additional regulators. In particular, it is possible that redundant regulators of the Notch and Egfr pathway contribute to the choice of denticle type. These could include Vein (another Egfr ligand; Schnepp et al., 1996), Delta (a Notch ligand; Fehon et al., 1990) or possibly Fringe. *vein* is not required for embryogenesis (Schnepp et al., 1996) suggesting that it does not play an important role if any. Possible contributions from Delta to denticle patterning are not readily assessed because of Delta's earlier action in neurogenesis (Lehmann et al., 1983). We are currently attempting to overcome this problem.

The role of morphogens in the denticle pattern

Our results show that no single morphogen organises the denticle pattern: patterning arises, at least initially, from the combined actions of Wingless and Hedgehog. We now discuss in turn to what extent either of these two factors have attributes of a morphogen in this system.

Wingless is clearly not involved in the specification of denticle types (or diversity) across each belt since it does not act in this region of the epidermis. If it did, *veinlet (rhom)* and *Serrate* would not be expressed because, as we have shown, they are both repressed by Wingless. Nevertheless, Wingless

acts at a distance, over 3- to 5-cell diameters to set the boundaries of the *Serrate* expression domain and thus establishes conditions for subsequent juxtacrine signaling. Long-range Wingless action is also required for the asymmetric action of Serrate: Serrate does not activate *veinlet (rhom)* expression posteriorly because of the presence of Wingless there, 3- to 5-cell diameters from the site of *wingless* transcription. In this sense, Wingless modulates, at a distance, the outcome of local signaling. In neither of these activities is there evidence for concentration-dependent signaling. However, one cannot formally exclude the possibility that the specification of type 6 denticle requires low-level Wingless. Furthermore, the suggestion that Wingless is not a morphogen in the embryonic epidermis is at odds with studies of the first thoracic segment where various levels of Wingless signalling lead to the specification of distinct cuticular structures (Lawrence et al., 1996). Re-assessment of these phenotypes with early molecular markers might tell whether or not Wingless acts directly in a concentration-dependent manner in the embryonic epidermis.

The situation with Hedgehog is clearer since it has qualitatively distinct effects over a narrow strip of cells (Fig. 7B,C). It activates *veinlet (rhom)* expression in adjoining posterior cells while its repressive effect on *Serrate* expression extends over three cell diameters. This suggests that, at high level, Hedgehog activates *veinlet (rhom)* (near the source) while at both low and high levels it repress *Serrate* expression (further away from the source). In this sense, Hedgehog qualifies as a morphogen (as originally suggested by Heemskerk and DiNardo, 1994). Whether differential responses at different distances from the Hedgehog source reflect true concentration dependence remains to be assessed. We note here that the repressive effect of Hedgehog on *Serrate* expression might take place early in development since, in *wingless* mutants, *hedgehog* expression decays around stage 10 (Lee et al., 1992) and yet *Serrate* expression is still confined at the anterior (Fig. 4F). We suggest that early Hedgehog has a repressive effect on *Serrate* expression that lasts at least until stage 11, when *veinlet (rhom)* expression commences. It is therefore conceivable that the 3-cell-wide domain where *Serrate* is repressed at stage 11 originates by cell proliferation from a single row of cells that abut the Hedgehog source at early embryonic stages. According to this scenario, the effects of Hedgehog on *Serrate* and *veinlet (rhom)* expression would both be occurring over one cell diameter. The apparent difference in range would reflect the difference in timing between these two effects and the intervening proliferation. We are currently testing this model by assessing the activity of a membrane-tethered form of Hedgehog.

To sum up, in the bald area of abdominal segments, one cell type forms in response to one signaling pathway while within denticle belts, a rich pattern of cell types arise from juxtacrine cell interactions initiated by the activation of distinct signaling pathways. Some of these pathways are controlled by the localised expression of segment polarity genes such as *wingless* and *hedgehog* while others are regulated by downstream genes like *veinlet (rhom)* and *Serrate*. Because *wingless* and *hedgehog* are expressed first, they are effectively at the top of the hierarchy and the knock-on effects of losing *hedgehog* or *wingless* function explain the “organiser activity” of the parasegment boundary. Interestingly, the denticle pattern arises from the combined action of Wingless and Hedgehog originating from the parasegment boundaries of adjacent segments and therefore, two parasegment boundaries are needed to provide the signals that pattern a single denticle belt.

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