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Planar cell polarity: the orientation of larval denticles in *Drosophila* appears to depend on gradients of Dachsous and Fat

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SUMMARY

The larval ventral belts of *Drosophila* consist of six to seven rows of denticles that are oriented, some pointing forwards, some backwards. We present evidence that denticle orientation is determined almost entirely by Dachsous and Fat, one of two planar cell polarity systems. If we change the distribution of Dachsous we can alter the polarity of denticles. We suggest that the orientation of the individual denticle rows, in both the anterior compartment (which mostly point backwards) and the posterior compartment (which point forwards), is determined by the opposing slopes of a Dachsous/Fat gradient. We show, by altering the concentration gradients of Dachsous during development, that we can change the polarity of the denticles made by larval cells as they progress between the first and third larval instars without mitosis.

KEY WORDS: Van Gogh (Strabismus), Dachsous, Fat, Frizzled, Gradients, Starry night (Flamingo), *Drosophila*

INTRODUCTION

Planar cell polarity (PCP) is likely to be a property of all or most epithelial cells, even though they do not always evince it (Lawrence et al., 2007). Some cells reveal their polarity by indicators; examples are oriented hairs in insects and polarised stereocilia in mammals. The mechanisms responsible for PCP appear to be universal as they depend on genes that are conserved in many organisms (Klein and Mlodzik, 2005). *Drosophila* has been particularly well studied and there is evidence that there are (at least) two genetic systems responsible: first, the 'Stan system', which depends crucially on Flamingo (also known as Starry night or Stan), Frizzled (Fz) and Van Gogh (Vang); and second, the 'Ds/Ft system, which incorporates Fat (Ft), Dachsous (Ds) and Four-jointed (Fj) (Lawrence et al., 2007). Most studies with *Drosophila* have used the hairs on the wing or abdomen or the ommatidia of adults, but here we turn to the larvae. The cuticular denticles are found on the ventral surface of larvae in metameric stripes and provide traction for movement (Dixit et al., 2008). The denticle rows have differing characteristics (Alexandre et al., 1999) and are oriented in specific ways (Walters et al., 2006). We now ask how the mechanisms of PCP determine the polarity of these larval denticles.

In the cuticle of the newly hatched larva there are six imperfectly defined rows, of which rows 2, 3, 5 and 6 point backwards and rows 1 and 4 point forwards (DiNardo et al., 1994). In later larval stages, a seventh row (row 0) is added anterior to row 1 (Struhl et al., 1997a) and this also points forwards. Rows 0 and 1 are made by cells belonging to the posterior (P) compartment, whereas the remaining rows are formed by cells within the front half of each anterior (A) compartment (DiNardo et al., 1994).

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Denticle formation has been described in the embryo (Dickinson and Thatcher, 1997; Price et al., 2006; Walters et al., 2006; Dilks and DiNardo, 2010): preceding the outgrowth of denticles there are apical accumulations of actin, which form along the rear of the cell. At first, all six rows of actin protrusions appear to point backwards but, later in embryonic development, the denticles of rows 1 and 4 turn or reshape to point forwards, a process that is still undescribed. The rows of mature denticles are not evenly spaced and they may form on one edge of the cell, initially at the posterior edge (Dilks and DiNardo, 2010). Thus, there are at least two indicators of polarity: the polarity of the denticles themselves and their formation at a particular edge of the cell.

In the adult abdomen, the two systems of PCP can act independently to alter the polarity of cuticular hairs and each makes a contribution to hair orientation in the wild type (Casal et al., 2006). But, in larvae lacking genes of the Stan system, the polarity of denticles appears normal, although occasional slight errors in rows 1 and 2 have been reported by others (Price et al., 2006; Walters et al., 2006). In larvae that lack either Fat or Ds, the denticles are largely depolarised. Removing both the Ds/Ft and the Stan systems gives third stage larvae with denticles that are more disturbed than when only the Ds/Ft system is inactivated, arguing that although the Ds/Ft system is largely responsible for denticle orientation and is adequate to establish normal polarity, there is nevertheless some contribution from the Stan system (Casal et al., 2006).

There is evidence from the adult that the slopes of the Ds/Ft gradient decline in opposing directions in the A and the P compartments (Casal et al., 2002). Here we suggest that the orientation of rows 0, 1, 2, 3, 5 and 6 are direct readouts of these slopes. We presume that row 4 becomes repolarised by an additional step (Dilks and DiNardo, 2010).

MATERIALS AND METHODS

Denticles and epidermal stainings

Third stage larvae were collected and heated in water at 60°C for 20 minutes, then transferred to hot fixative (1:4 glycerol:acetic acid) for 20 minutes. For study of the denticle patterns, they were sliced horizontally with a razor

blade, internal contents removed and the ventral cuticle mounted in Hoyers. Orientation of the denticles was easily read one by one and row by row down the microscope. Cuticles were photographed under slight differential interference contrast using a Nikon D-300 camera and Nikon Camera Control Pro. Images were processed with Helicon Focus Pro and Adobe Photoshop CS4. For Fig. 1, pieces of third instar cuticles with the attached epidermis were fixed in 4% formaldehyde (Polysciences, Eppelheim, Germany) in PBT (PBS containing 0.1% Triton X-100) for 20 minutes. After several washes in PBT, they were incubated with anti-Fasciclin 3 antibodies (Developmental Studies Hybridoma Bank, IA, USA) overnight at 4°C, washed again, incubated with secondary antibodies (Jackson ImmunoResearch, Stratech, Newmarket, UK) and mounted in Fluormount (SouthernBiotech, AL, USA) for examination using a Leica SP5 microscope.

Experimental genotypes

The following are the relevant mutant alleles and insertions used in this work (Tweedie et al., 2009):

ds⁻: ds^{UA071};
ft⁻: ft^{15} and ft^{12};
UAS.ft: $ft^{Scer\UAS.cMa}$;
UAS.ds: $ds^{Scer\UAS.cTa}$;
UAS.ectoDs: $ds^{ecto.Scer\UAS}$;
UAS.fz: $fz^{Scer\UAS.cZa}$ and $fz^{Scer\UAS.cSa}$;
arm.Gal4: $Scer\GAL4^{arm.PS}$;
ptc.Gal4: $Scer\Gal4^{ptc-559.1}$;
en.Gal4: $Scer\GAL4^{en-e16E}$;
tub.Gal80^{ts}: $Scer\GAL80^{ts.\alpha Tub84B}$; and
UAS.GFP::act: $Act5C^{Scer\UAS.T:Avic\GFP}$.

Flies were reared in standard medium at 24°C unless otherwise stated; the experimental genotypes are as follows:

arm.Gal4 UAS.ds larvae: *w; arm.Gal4/UAS.ds*;
arm.Gal4 UAS.ft larvae: *w; arm.Gal4/UAS.ft*;
en.Gal4 UAS.ft larvae: *w; en.Gal4/+; UAS.ft/+*;
en.Gal4 UAS.ds larvae: *w; en.Gal4/+; UAS.ds/+*;
ptc.Gal4 UAS.ft larvae: *w;* ff^{d1} *ptc.Gal4/+; UAS.ft/+*;
ptc.Gal4 UAS.ds larvae: *w;* ff^{d1} *ptc.Gal4/+; UAS.ds/+*;
ds⁻ larvae: *w/y hs.FLP; ds*⁻ *ck FRT40A/ds*⁻ $Pka\text{-}C1^{E95}$ *FRT40A*;
ft⁻ larvae: *y; ft*⁻ *FRT39*;
ds⁻ *ft*⁻ larvae: *y w hs.FLP; ds*⁻ *ft*⁻ *FRT39*;
ptc.Gal4 UAS.fz in ft⁻ larvae: *y w hs.FLP; ft*⁻ *FRT42D pwn sha/ft*⁻ *ptc.Gal4; UAS.fz/+*;
en.Gal4 UAS.GFP::act larvae: *w; en.Gal4 UAS.GFP::act/CyO*; and
ptc.Gal4 UAS.GFP::act larvae: *w; ptc.Gal4/UAS.GFP::act*.

For temperature shift experiments, embryos of genotype *y w hs.FLP;* ff^{d1} *ptc.Gal4/FRT42D pwn sha; tub.Gal80*^{ts}*/UAS.ectoDs* were grown at 17°C until soon after hatching at 48-72 hours after egg laying (AEL) and then shifted to 29°C. Similarly, embryos kept at 29°C were shifted to 17°C soon after hatching (24-48 hours AEL) and kept at that temperature until the third stage.

Denticle quantitation

The orientation of the denticles in the A4 segment of four to five larvae was recorded one by one and row by row under a 25× objective with phase contrast. One half of the denticles pointing sideways was assigned to each class, unless there was only one such denticle, which was then assigned to the class representing the most disfavourable hypothesis.

RESULTS AND DISCUSSION

What is the contribution of the Stan and Ds/Ft systems to the cuticular pattern in *Drosophila* larvae?

Our results support the hypothesis that the Ds/Ft system largely determines denticle polarity, with only a minor contribution from the Stan system.

We could detect no effect of mutants in the Stan system (*Vang*⁻, *stan*⁻, *dsh*⁻, *fz*⁻ and *pk*⁻) in third stage larva (Price et al., 2006) (our unpublished data). Work on adults suggests that a more sensitive assay for the effects of the Stan system in larvae would be to overexpress Fz in patches of cells and look for polarity changes near the edges of the patch (Adler et al., 1997). Denticles in the A compartment normally point down the putative Fz gradient, that is backwards from its high point in row 2 cells at the extreme front of the A compartment (Lawrence et al., 2004). Thus, driving extra Fz at this high point [*ptc* is expressed in row 2 (Fig. 1) (Struhl et al., 1997a)] should reinforce the normal Fz gradient and therefore not affect the polarity of denticles in the anterior of the A compartment. However, the higher peak of Fz in row 2 cells should produce a reversed Fz gradient anterior to that peak – in adults, such a peak reverses the polarity of cells at the back of the P compartment (Lawrence et al., 2004). However, no effect was seen in the larva on row 1 cells (not shown). Alternatively, driving Vang or Stan in the P compartment (with *en.Gal4*) might be expected to reverse the polarity at the front of the A compartment (Lawrence et al., 2004); however, these two experiments have no effect on any rows of denticles in the larva (not shown).

By contrast with Stan system mutants, knocking out the Ds/Ft system has strong effects on polarity; *ds*⁻, *ft*⁻ and *ds*⁻ *ft*⁻ larvae were largely depolarised and could not be reliably distinguished when screened blind, although row 5 and 6 tended to be less disturbed in *ds*⁻ larvae (Fig. 2). This similarity of phenotypes is expected from work on adults (Casal et al., 2006): we have proposed that Ds and Ft are equally essential to build PCP because they work together as transheterodimers (Ma et al., 2003; Matakatsu and Blair, 2004; Casal et al., 2006). Note that, in addition to effects on the orientation of the denticles, the rows partially lost their distinctive characteristics (Fig. 2).

In the adult, manipulating the Stan system has more substantial effects in flies lacking the Ds/Ft system than in wild type (Casal et al., 2006). Therefore, we drove *fz* with *ptc.Gal4* in *ds*⁻ and *ft*⁻ larvae but saw no effect on polarity (see Fig. S1 in the

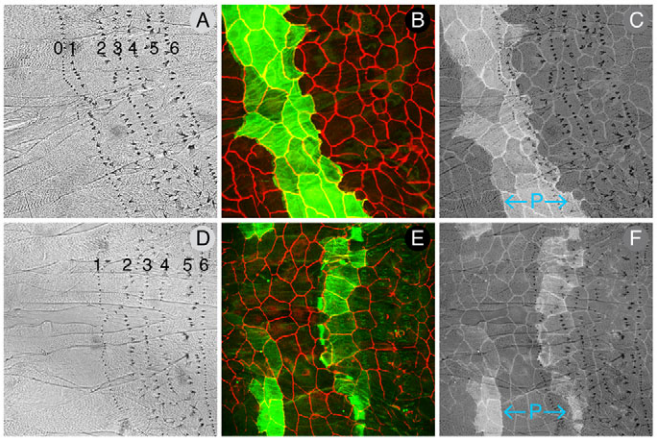

Fig. 1. Specificity of the drivers. (**A-C**) When driven by *en.Gal4*, *UAS.GFP::act* (green) is found in the cells that make row 0 and 1 denticles. (**D-F**) When driven by *ptc.Gal4*, *UAS.GFP::act* (green) is found in the cells that make row 2 denticles (occasionally, cells that make row 3 denticles show weak expression). Cell boundaries are marked by Fasciclin 3 (red). Note that the registration shown between denticles and cells in the merges (C,F) is imperfect owing to the very different planes of focus; we estimate that it is accurate to about half of one cell diameter. The *Gal4/UAS* method gives variable levels of expression, perhaps explaining the discontinuity in the row of *ptc* expression at the back of the anterior (A) compartment. The extent of the posterior (P) compartment is indicated.

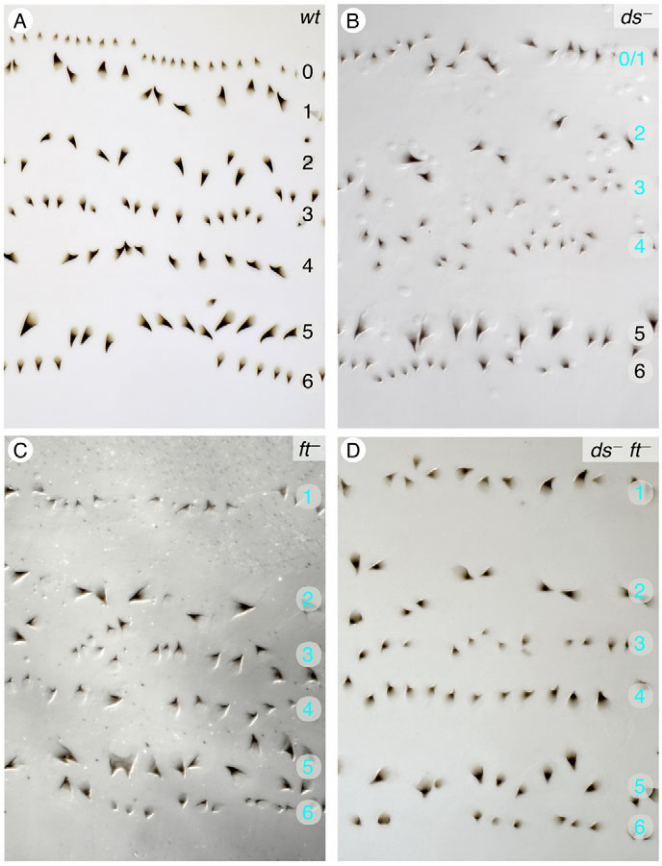

Fig. 2. *Drosophila* larval denticles. In this and subsequent figures, comparable parts of the seven ventral denticle belts are shown, the numbers indicating whether the belt is normal (black), depolarised (blue) or reversed (red); an exclamation mark (see Figs 4, 5) indicates some other abnormality in the row. Owing to variability of the denticle belts we cannot show every feature that we describe in the text in these single images. Genotypes are indicated. Note that even the wild type (**A**) departs from the ideal pattern described in textbooks and *ds*⁻ (**B**), *ft*⁻ (**C**) and *ds*⁻ *ft*⁻ (**D**) larvae show considerable depolarisation and some loss of denticle row individuality.

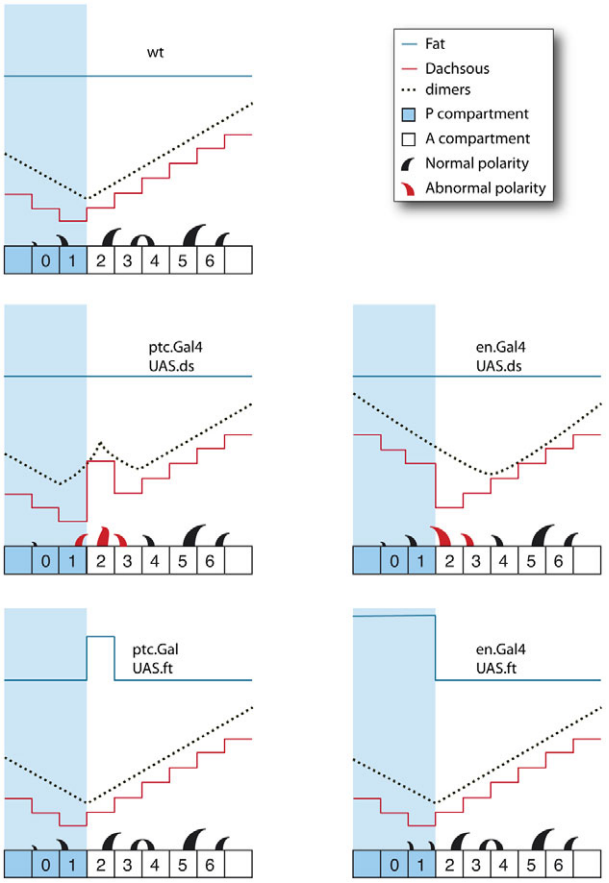

Fig. 3. Model of denticle polarity in the *Drosophila* larva. Gradients of Ds and transheterodimer concentrations are indicated (and are due in part to Four-jointed, not shown). The denticles of all rows, except row 4, point up the imagined gradients of heterodimers. Ft is considered to be ungraded. When *ptc.Gal4* drives *ds* we see both cell-autonomous (row 2) and non-cell-autonomous effects (rows 1 and 3). In the case of *en.Gal4*, we see only non-cell-autonomous effects (rows 2 and 3). Overexpressing *ft* has no consequences, except some slight effects on rows 0 and 1 within the P compartment. For the experimental and theoretical background to this model, see Casal et al. (Casal et al., 2006). wt, wild type.

supplementary material), even though one might expect a steepened gradient of Fz to reorganise the disrupted row 2 and make them point backwards.

However, inactivating the Stan system in *ds*⁻ or *ft*⁻ mutant larvae increases the depolarisation of the denticles (see Casal et al., 2006); this might argue that there is input from the Stan system into the wild type, which is masked by the Ds/Ft system.

These results are straightforward and argue that the polarity of all larval denticle rows, except row 4, depends on the Ds/Ft gradient and not much, if at all, on the most-studied PCP pathway, the Stan system.

The denticle orientation can be changed by altering the slopes of the Ds/Ft system gradient in the larva

To test the existing description of the wild type (Struhl et al., 1997b), we have driven GFP::actin in the P compartment with *en.Gal4* and the result confirms that rows 0 and 1 are P compartment in provenance, whereas rows 2-6 are made by A cells (Fig. 1). The following experiments test the model that denticle polarity in the larva depends on the Ds/Ft system in a similar manner to that in the adult (Casal et al., 2006).

arm.Gal4/UAS.ds (or *UAS.ft*)

Generalised overexpression of *ds* caused a disruption of row structure and disturbance in the polarity of the denticles, most strongly in the A compartment (not shown). By contrast, generalised overexpression of *ft* had little effect. One could conjecture from this finding that the primary determinant of polarity is the gradient of Ds (and not Ft), a conclusion that fits well with the results we have obtained and the observation that in different PCP systems Ds has been reported to be graded, but Ft evenly expressed (reviewed by Strutt, 2009).

en.Gal4 UAS.ds

Overexpression of *ds* in the P compartment will increase the amount of Ds in cells making row 0 and 1. This should mean that the cells at the front of the A compartment (row 2) will now have

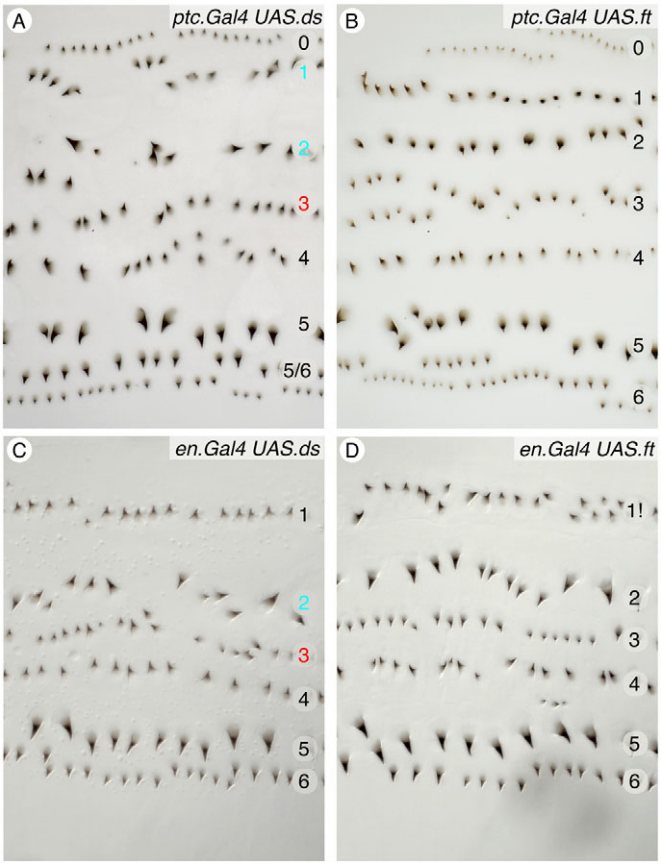

Fig. 4. Effects of overexpressing *ds* and *ft* on denticle polarity. (**A-D**) Compare with Fig. 3, which shows the four outcomes diagrammatically. The loss of row 0 shown is rare in *en.Gal4 UAS.ds* (C) but common in *en.Gal4 UAS.ft* (D). For quantitation, see Table S1 in the supplementary material. For every row indicated by blue and red numbers (also in Fig. 5) there is a highly significant difference from the wild type ($\chi^2 >> \chi^2_{\alpha=0.001}$), whereas values in black are not significantly different ($\chi^2 < \chi^2_{\alpha=0.05}$). The effects we describe are thus consistent and conspicuous.

a high level of Ds anterior to them, and, if it is correct that denticles point up the Ds gradient, they should now point forwards (instead of backwards as in wild type; Fig. 3). In fact, row 2 was depolarised without showing a clear bias and row 3 was reversed to now point forwards (Fig. 4 and see Table S1 in the supplementary material). This effect on row 3 indicates that there has been propagation of a polarity change to the next cell (there can be no direct effect on row 3 as Ds is upregulated in only the P compartment and cells of row 3 are two cell rows away from P cells). Such propagation is commonplace in the adult and we have put forward one model to explain it (Casal et al., 2006).

ptc.Gal4 UAS.ds

The driver *ptc.Gal4* makes an excess of Ds in the cells of row 2 of the third stage larva (Fig. 1) (Struhl et al., 1997a). Consistent with this, *ptc.Gal4 UAS.ds* depolarised row 2, perhaps because it is at a peak of Ds concentration and both its neighbouring cells (rows 1 and 3) are lower. Both rows 1 and 3 pointed up towards the neighbouring cells of row 2 (which presumably have a higher concentration of Ds) and, in both, their polarity was reversed from that of the wild type. Rows 4-6 were unaffected. Row 0, as is normal, pointed forwards (Figs 3, 4 and see Table S1 in the supplementary material). These changes in polarity were not accompanied by any gross changes in denticle shape or size, suggesting that they retain their proper identity (see Fig. S2 in the supplementary material).

en.Gal4 UAS.ft

Overexpression of *ft* in the P compartment is expected to have no non-cell-autonomous effects on denticle polarity because of its lack of effect on the existing gradient slopes (Fig. 3), but might have autonomous effects within the P compartment. Indeed, in adults, overexpressing *ft* in the P compartment causes whorly polarity in the P compartment of the wing and tergites (Matakatsu and Blair, 2004) (our unpublished data). However, in larvae there was only a small consequence in that, in some cases, row 1 cells made clusters instead of rows of normally oriented denticles (Figs 3, 4 and see Table S1 in the supplementary material).

ptc.Gal4 UAS.ft

Overexpression of *ft* under *ptc.Gal4* control is not expected to alter the gradient slopes (Fig. 3) in the region of the denticles, and no polarity changes were observed (Fig. 4 and see Table S1 in the supplementary material).

Our model, based on experiments in the adult, is that polarity is determined by Ds/Ft transheterodimers forming bridges from cell to cell that make concentration gradients with opposing slopes in the A and P compartments (Casal et al., 2002; Casal et al., 2006). Under this model, the polarity of an individual cell depends on the distribution of these dimers on its surface, a distribution that itself is affected by the numbers and activity of Ds and/or Ft molecules in neighbouring cells. In the adult we have presented some evidence that a Ds activity gradient might peak at or near the A/P (parasegmental) border and decline anteriorly and posteriorly from this peak. Thus, with respect to this Ds gradient in the larva, we could state that all denticle rows in both P and A compartments (except row 4) point up the Ds slope (Fig. 3). However, if a patch of cells is made with a large amount of extra Ds, this would alter the distribution of heterodimers such that cells at both sides of the patch will tend to point in towards it: of course, only on one side of the patch will the denticle polarity be altered from the normal (Fig. 3). All the experiments reported above support the model and argue that larval denticle polarity, like the adult hairs and bristles, is a readout of the Ds/Ft activity gradient. It is not known how Row 4 reads polarity cues and why it points in the anterior direction, but recent evidence (Dilks and DiNardo, 2010) argues that row 4 is polarised independently.

The polarity of a larval cell can be changed during development

Drosophila larvae have three instars. Is PCP set up de novo during each moult cycle or is the pattern irrevocably fixed during embryogenesis? Given that the polarity of some denticles is reversed by driving *UAS.ectoDs* with *ptc.Gal4*, we can now ask whether the polarity of cells, as expressed in the cuticle of the first stage larva, can be altered during subsequent moults. We used two separate temperature-sensitive components to ensure that ectopic expression of *UAS.ectoDs* is strong at 29°C but is blocked at 17°C. We showed that, at 17°C, there was no effect of driving *UAS.ectoDs*, and wild-type patterns were produced (Fig. 5A and see Table S1 in the supplementary material). However, when flies of the same genotype were grown at 29°C, almost the full effect of driving *UAS.ectoDs* was seen (Fig. 5C and see Table S1 in the supplementary material). We then shifted the temperature before the moult to second stage larva. Shifting down gave third stage larvae with a wild-type

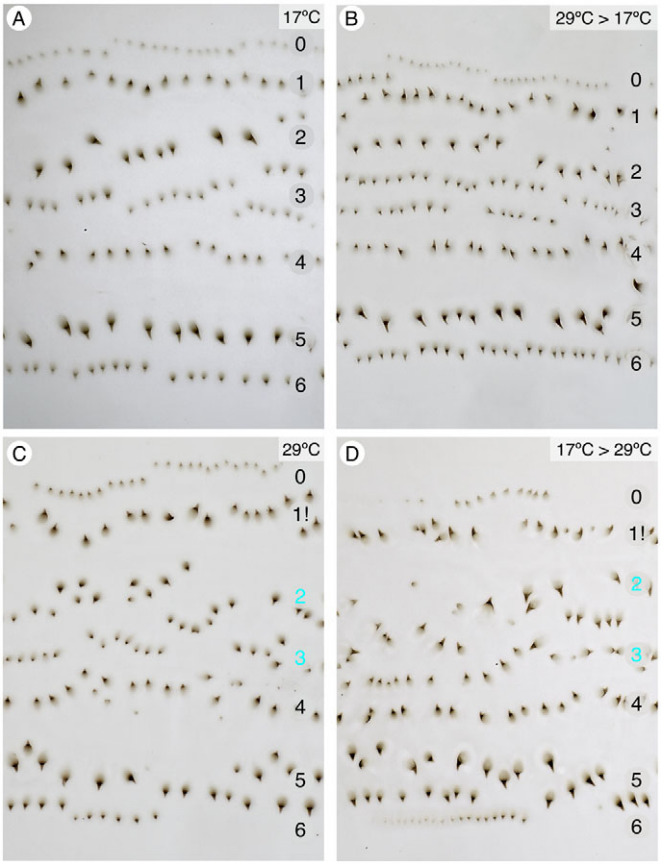

Fig. 5. Denticle polarity can be changed during development. (**A**,**B**) Similar phenotypes that are indistinguishable from the wild-type pattern are produced either by keeping the *ptc.Gal4 UAS.ds* larvae at 17°C (A) or shifting them from 29 to 17°C soon after hatching (B). (**C**,**D**) Driving *ds* with *ptc.Gal4* at 29°C produces a strong phenotype, when the temperature is kept at 29°C (C) or when the temperature is shifted from 17 to 29°C (D). For quantitation, see Table S1 in the supplementary material.

phenotype (Fig. 5B and see Table S1 in the supplementary material), and shifting up gave third stage larvae with a mutant phenotype (Fig. 5D and see Table S1 in the supplementary material).

Thus, even in the polyploid cells of the larva, polarity is not fixed and, if the Ds gradient landscape is changed during development to give new slopes, the polarity of the denticles responds in subsequent moults so that the denticles made by the same cells are now reversed in orientation. This is a new finding and supports the general hypothesis that PCP is not fixed but requires continuous input of information. We imagine that similar changes occur in adults when clones that change polarity (Gubb and Garcia-Bellido, 1982) are made during metamorphosis.

In summary, we have presented evidence that gradients of the Ds/Ft system are the main determinant of the PCP of larval denticles, and that this PCP is modifiable during development, even in polyploid larval cells.

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Competing interests statement
The authors declare no competing financial interests.

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