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Bristles induce bracts via the EGFR pathway on *Drosophila* legs

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Abstract

A long-standing mystery in *Drosophila* has been: how do certain bristles induce adjacent cells to make bracts (a type of thick hair) on their proximal side? The apparent answer, based on loss- and gain-of-function studies, is that they emit a signal that neighbors then transduce via the epidermal growth factor receptor pathway. Suppressing this pathway removes bracts, while hyperactivating it evokes bracts indiscriminately on distal leg segments. Misexpression of the diffusible ligand Spitz (but not its membrane-bound precursor) elicits extra bracts at normal sites. What remains unclear is how a secreted signal can have effects in one specific direction.

Keywords: *Drosophila*; Pattern formation; Imaginal discs; Legs; Bristles; Bracts; Signaling pathway; Epidermal growth factor receptor; Induction

1. Introduction

Metazoan development entails extensive intercellular communication. One of the most versatile communication channels is the epidermal growth factor receptor (EGFR) signaling pathway (Bogdan and Klämbt, 2001; Hackel et al., 1999). In the fruit fly *Drosophila melanogaster*, this pathway mediates various patterning decisions at the organ level (e.g. eye vs. antenna (Kumar and Moses, 2001a,b) and notum vs. wing (Wang et al., 2000; Zecca and Struhl, 2002a,b)) and cell level (e.g. chordotonal organs vs. epidermis (zur Lage et al., 1997; zur Lage and Jarman, 1999) and photoreceptors vs. other cell types (Freeman, 1996, 1998)). Evidence presented here indicates that it also implements the induction of bracts.

Bracts are cuticular protrusions that resemble wing hairs ('trichomes') (Mitchell et al., 1990; Mitchell and Petersen, 1989) insofar as they are secreted by single cells (Reed et al., 1975; Walt and Tobler, 1978), but they are considerably thicker and darker (Hannah-Alava, 1958). Their function, if any, is unknown.

What makes bracts intriguing from a developmental standpoint is that they are only found in association with bristles (Poodry, 1980). They arise next to mechanosensory (MS) bristles on the distal segments of the legs (femur, tibia, and tarsal segments) (Hannah-Alava, 1958) and the proximal costa of the wings (Bryant, 1975; Cifuentes and García-Bellido, 1997; Peyer and Hadorn, 1965). Why they are lacking from other bristles is not known.

The spatial correlation of bracts with bristles suggests a causal link (García-Bellido, 1972; Held and Bryant, 1984; Postlethwait and Schneiderman, 1973), but the link cannot involve cell lineage because the bract cell does not belong to the bristle organ clone. Each MS bristle comes from a sensory organ precursor (SOP) that undergoes three mitoses to produce five descendants: a shaft cell, socket cell, neuron, sheath cell, and glial cell (Gho et al., 1999; Reddy and Rodrigues, 1999). Cell lineage studies have shown that the bract cell arises separately from this clone (Held, 1979a; Lawrence et al., 1979; Tokunaga, 1962).

Given the lack of a pedigree link, the presumption has been that bracts are induced by one or more SOP descendants (Held and Bryant, 1984; Poodry, 1980). Indeed, bracts fail to develop whenever either the shaft or socket cell is suppressed genetically (Held, 1990; Poodry et al., 1973; Tobler et al., 1973) or pharmacologically (Tobler, 1969; Tobler and Maier, 1970; Walt and Tobler, 1978), and they fail to develop independently of bristles when epithelial cells are dissociated and reaggregated (Tobler, 1966).

Ever since a clonal affiliation was ruled out in 1962 (Tokunaga, 1962), the abiding riddle has been *how* bristles inform neighboring cells to make bracts. The Decapentaplegic, Hedgehog, Notch, and Wingless pathways seem irrelevant (Held, 1993; Held and Heup, 1996; Held et al., 1994; Poodry et al., 1973; Shellenbarger and Mohler, 1978; Struhl et al., 1993), except that chemosensory (CS) bristles can acquire bracts when the Notch pathway malfunctions

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(Held, 1990). In 1997, Simcox reported that mutations in *pointed* (a gene in the EGFR pathway) cause enlarged bracts (Simcox, 1997). The present investigation was undertaken to test the role of the EGFR pathway.

2. Results

The tibia (Ti) and basitarsus (Ba) of the second leg were the main subjects for analysis. These segments have ten (Ti) or eight (Ba) longitudinal rows of MS bristles (Hannah-Alava, 1958; Hollingsworth, 1964), all of which normally possess bracts (Fig. 1). They also have a few CS bristles that lack bracts (~8 on Ti and ~5 on Ba). MS bristles can be distinguished from CS bristles by their shapes (straight vs. curved and thick vs. thin) even when their bracts are artificially suppressed.

In wild-type males (Oregon R strain) the second legs have an average of 139 MS bristles on the Ti and 74 MS bristles on the Ba ($N = 10$ legs). Data below report the percentages of MS bristles that possess bracts. Thus, a wild-type fly is '100%Ti and 100%Ba', while a fly lacking bracts would be '0%Ti and 0%Ba'. Unless stated otherwise, percentages were calculated from ten male second legs, and stages denote hours after pupariation (h AP) at a standard temperature of 25°C.

2.1. Loss-of-function phenotypes

For ommatidia of the fly eye, Heberlein et al. (1993) showed the involvement of the EGFR pathway via dosage-sensitive interactions between loss-of-function (LOF) alleles of *Star* and *Ras1*. If the EGFR pathway were instrumental in bract development, then those same alleles might be expected to also manifest dosage effects on the frequencies of bracts. Indeed, they do.

Star^{5671}/+ heterozygotes have a missing-bract phenotype (31%Ti and 89%Ba), which is aggravated slightly in deficiency heterozygotes such as *Df(2L)ast4*/+ (17%Ti and 78%Ba; Fig. 2b). In contrast, *Ras1*^{e1B}/+ heterozygotes look nearly wild-type (96%Ti and 100%Ba). The double heterozygote shows synergistic effects: *Star*^{5671}/+; *Ras1*^{e1B}/+ flies have fewer bracts than either heterozygote alone (2%Ti and 60%Ba).

In each of the above genotypes, the Ti was more strongly affected than the Ba. This disparity was seen in other contexts as well (see subsequently). Another trend in differential sensitivity was found among the basitarsal bristle rows: dorsal bristles tend to lose bracts more readily than ventral ones (Fig. 3).

2.2. Gain-of-function phenotypes

If all epidermal cells are competent to make bracts in response to EGFR stimulation, then it should be possible to fool them into 'thinking' that they have 'heard' a signal (when in fact they have not) by activating the pathway downstream of the receptor. For this purpose, a constitutively active *Ras1* transgene was used under the control of a heat shock promoter. When *hs-Ras1*$^{*M11.2}$ males were heat-shocked at any time from 5 to 27 h AP, their legs acquired extra bracts. On the Ti, these excess bracts are patchily distributed. On the Ba, the bracts are also patchy (mainly found near bristles) for shocks between 11 and 27 h AP (Fig. 2e), but earlier shocks (5–10 h AP) typically yield a confluent lawn of unpigmented bracts (Fig. 2d).

Since Star acts upstream of the EGF receptor (Hsiung et al., 2001; Klämbt, 2002; Lee et al., 2001; Tsruya et al.,

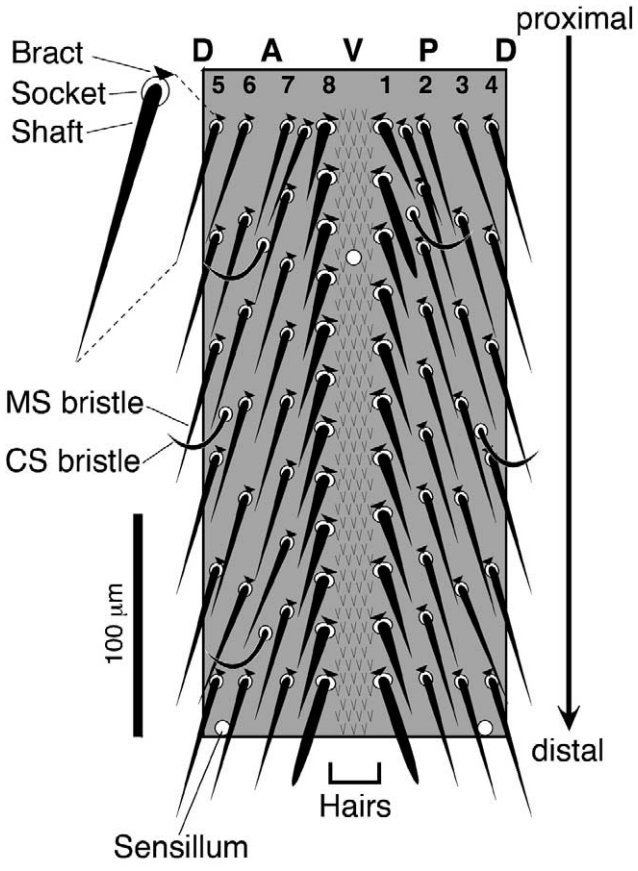

Fig. 1. Basitarsus (a.k.a. first tarsal segment) of the second leg. The basitarsus is a cylindrical leg segment just below the tibia. In this panoramic map of an actual basitarsus of a wild-type male (right leg), the segment has been imaginarily slit along its dorsal midline, pried open, and flattened to display all its bristles (D, dorsal; A, anterior; V, ventral; P, posterior). The shafts of mechanosensory (MS) bristles are thick and straight, while those of chemosensory (CS) bristles are thin and curved. MS bristles have a bract (triangle) on the proximal side of the socket, whereas CS bristles lack bracts. Most MS bristles occupy eight longitudinal rows (numbered at top). Among the rows, bristle lengths and intervals generally increase from ventral to dorsal while the fewer CS bristles reside between the bristle rows. Three stretch-sensitive sensilla campaniformia (white circles) (Zacharuk, 1985) are also found at certain sites. Along the ventral midline is a lawn of hairs (V-shaped protrusions) that resemble bracts, except that bracts are thicker and darkly pigmented. The remaining cells of the basitarsal epidermis, which has ~2000 cells in all (Held, 1979b), make smooth cuticle (gray background). Segment width was slightly exaggerated here (by 1.25×) to avoid bristle overlaps (length is accurate).

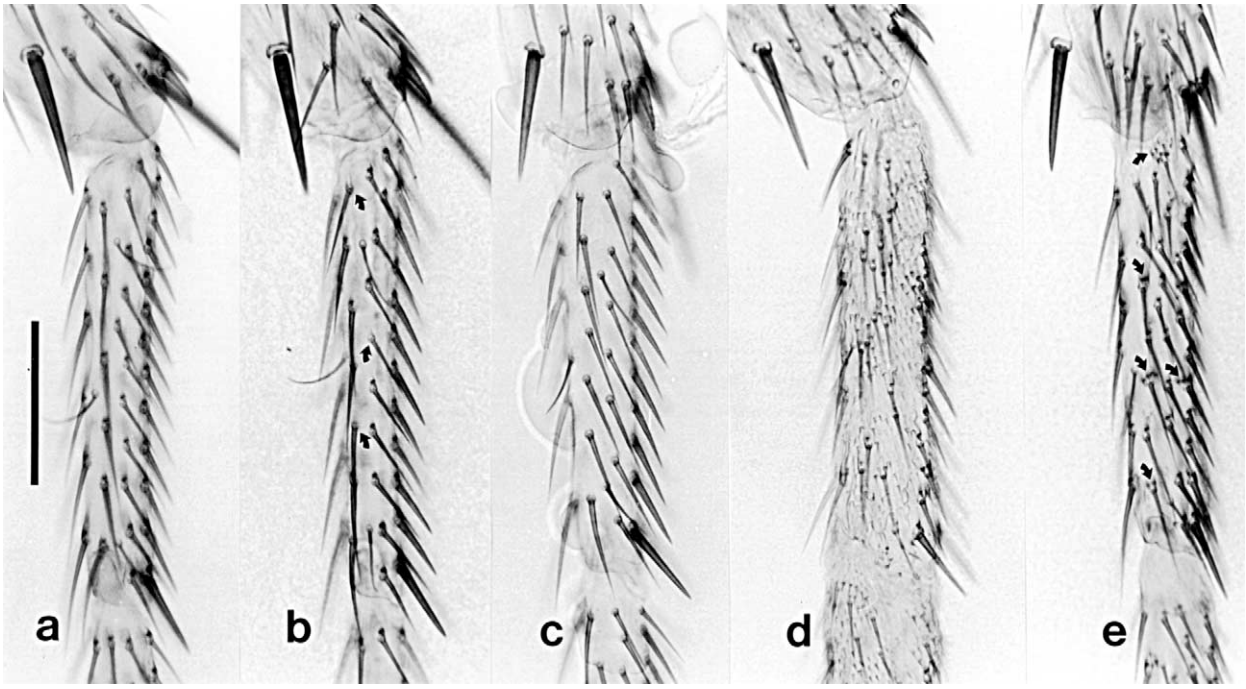

Fig. 2. Effects of EGFR pathway manipulations on bract development. All panels show the anterior face of a male second-leg basitarsus, with row 5 along the left edge and row 8 along the right edge. In each case, the proximal end of the segment is near the top. The large bristles near the distal end of the tibia are the pre-apical bristle (in focus at left) and the apical bristle (out of focus at right). Curved bristles are chemosensory. Photographs are at the same magnification; bar length = 100 μm. (a) Wild type. Bracts are the tiny, dark, triangular structures above the sockets of the straight (mechanosensory) bristles. (b) *Df(2L)ast4*/+ fly with only one dose of the *Star* gene, instead of the normal two. Arrows indicate MS bristles that are missing bracts. (c) $Egfr^{ts1a}/Egfr^{CO}$ male that was shifted to the restrictive temperature at pupariation. All bracts are missing. (d) *hs-Ras1*$^{*M11.2}$ fly heat-shocked at 5 h AP. Most of the epidermal cells have made unpigmented bracts, instead of smooth cuticle. Some bristles are missing, and the remaining bristles are disorganized. The tarsus has failed to shrink to its normal diameter (which happens at ~12–24 h AP in wild-type pupae), and the segment boundary between the basitarsus and the next tarsal segment (T2) has failed to form. (e) Fly of the same genotype as (d) heat-shocked at 24 h AP. Most extra bracts are now pigmented but less common and distributed mainly near bristle sites. Note the patch near the proximal end of the segment and the arcs of bracts above certain bristles more distally (arrows). Additional phenotypes (not shown) observed in the heat-shocked *hs-Ras1*$^{*M11.2}$ flies included (1) disorganized transverse rows (seen in other genotypes also, especially *sca* > *Egfr*), (2) misaligned sex combs, (3) extra sensilla near most of the sensilla nests on the leg, and (4) an absence of joint invaginations.

2002), the missing-bract defect of $Star^{5671}$/+; $Ras1^{e1B}$/+ heterozygotes should be rescueable by hyperactivating *Ras1*. When the *hs-Ras1*$^{*M11.2}$ transgene was introduced and the resulting $Star^{5671}$/*hs-Ras1*$^{*M11.2}$; $Ras1^{e1B}$/+ pupae were heat-shocked during the extra-bract sensitive period (24 h AP), a partial rescue was indeed observed. The shocked flies have significantly more bracts (30%Ti and 59%Ba) than their unshocked control siblings (0%Ti and 40%Ba).

2.3. Temporal requirement for the EGF receptor

The recent availability of a temperature-sensitive LOF allele for the *Egfr* gene ($Egfr^{ts1a}$) (Kumar et al., 1998) makes it possible to define the sensitive period when the Egfr protein is needed for signal transduction. In the upshift series, $Egfr^{ts1a}/Egfr^{CO}$ mutants ($Egfr^{CO}$ is a null allele) were raised at the permissive temperature of 18°C and then shifted to the restrictive temperature of 29°C at different times for the duration of development. In the downshift series, flies of the same genotype were raised at 18°C (to bypass earlier lethal periods), transferred to 29°C at pupariation (before the sensitive period for bract induction begins) and shifted back to 18°C at different times.

Flies raised continuously at 18°C had a wild-type pattern of bracts (99%Ti and 100%Ba), while those kept at 29°C during the pupal period lacked all bracts (0%Ti and 0%Ba). For the Ti, the 50% midpoint for bract removal is 17 h AP for upshifts and 28 h AP for downshifts (Fig. 4). These times are the 25°C equivalents, computed as described in Section 4. The sensitive period for the Ti would thus be defined as 17–28 h AP. For the Ba, this period begins 4 h earlier (13–28 h AP).

Basitarsal bristle rows are heterogeneous in their time-courses (Fig. 5). Relative to the bracts of the ventral rows, the bracts of the dorsal rows acquire immunity to upshifts (Egfr inactivation) later but lose their ability to be rescued by downshifts (restoration of Egfr function) earlier.

2.4. Targeted misexpression studies

To confirm the role of the EGFR pathway, attempts were made to activate or repress the pathway by the *Gal4-UAS* method ('driver > slave') of Brand and Perrimon (Brand

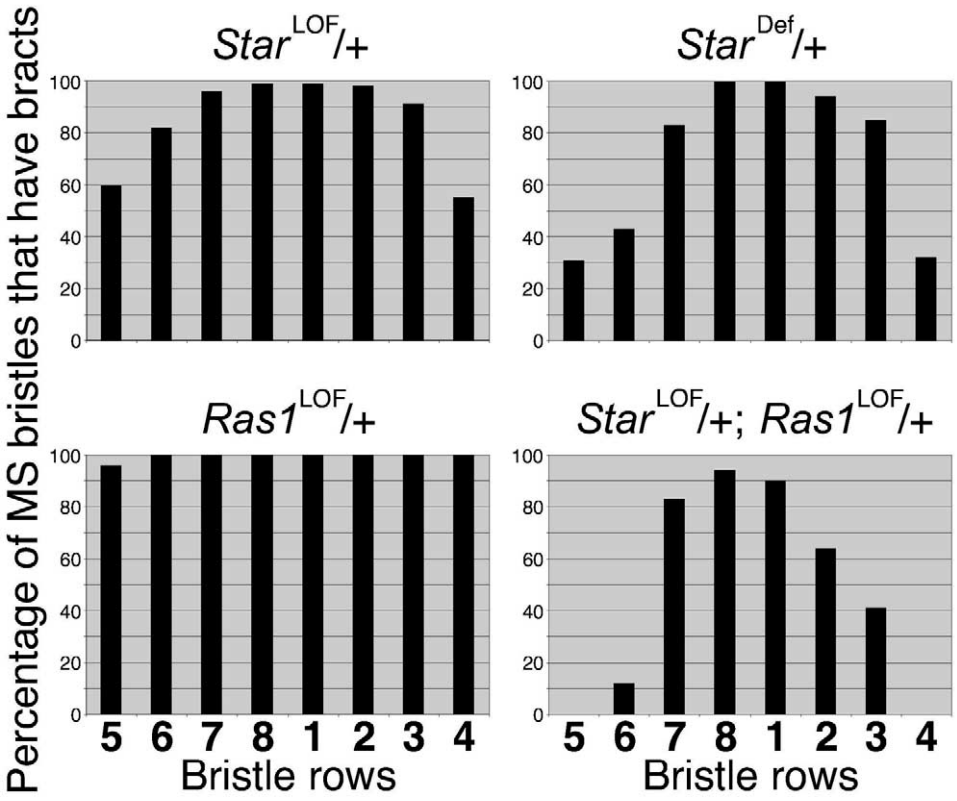

Fig. 3. Dosage effects of EGFR pathway genes on bract development. Histograms indicate the frequencies of bracts on second-leg basitarsi (average from $N = 10$ legs per panel) as a function of bristle row. Heterozygosity for a LOF allele of *Star* (*Star*^{5671}*/CyO*, upper left) reduces the number of bracts in the dorsal rows (3–6), and the reduction is exacerbated by heterozygosity for a *Star* deficiency (*Df(2L)ast4/+*, upper right). Heterozygosity for a LOF allele of *Ras1* (*Ras1*^{e1B}*/TM3*, lower left) has little effect, but in combination with heterozygosity for *Star*^{LOF} (lower right) a synergistic loss is seen in the dorsal rows, and appreciable effects now appear in the ventral rows.

and Perrimon, 1993). Two types of *Gal4* drivers were used: *scabrous-Gal4* (*sca-Gal4*) is expressed in bristle SOPs and in the proneural clusters (PNCs) whence they arise (Mlodzik et al., 1990), whereas *Distal-less-Gal4* (*Dll-Gal4*) is expressed throughout the tarsus and distal Ti (Gorfinkiel et al., 1997).

In the first series of experiments, the *UAS* slaves encoded ligands: *UAS-mSpi*, *UAS-sSpi*, and *UAS-argos*. Spitz (Spi) is a ligand that activates Egfr in various tissues (Freeman, 1994; Golembo et al., 1996; Rutledge et al., 1992; Schweitzer et al., 1995b; Tio et al., 1994; Yarnitzky et al., 1998). It is synthesized as a membrane-bound precursor (mSpi) that must be cleaved and released by the action of Star and Rhomboid in order to activate Egfr (Bang and Kintner, 2000; Hsiung et al., 2001; Klämbt, 2002; Lee et al., 2001; Tsruya et al., 2002; Urban et al., 2001). In contrast, the *UAS-sSpi* transgene was engineered to encode only the extracellular part (Schweitzer et al., 1995b), thus allowing sSpi to be secreted directly without cleavage. Argos is a diffusible inhibitor of Egfr (Howes et al., 1998; Jin et al., 2000; Schweitzer et al., 1995a) that also does not require cleavage for its secretion (Freeman et al., 1992).

Misexpressing mSpi (via *sca* > *mSpi* or *Dll* > *mSpi*) had no detectable effect on bracts, while misexpressing sSpi caused some extra bracts at normal sites. For *sca* > *sSpi* the number of bristles with extra bracts averaged 1.5 per Ti, 7 per Ba, and 25 per leg overall ($N = 10$ female legs), while for *Dll* > *sSpi* there were 1.4 per Ti, 12 per Ba, and 29 per leg overall ($N = 10$ female legs). Within each sample, the frequencies varied from 4–36 or 1–63 multiply bracted bristles per leg, respectively. Most of the affected bristles have two adjacent bracts of normal size, while a few (one or two bristles per leg, respectively) have three adjacent bracts in a proximal arc (data not shown). In contrast, misexpressing Argos reduces the number of bracts to 27%Ti and 85%Ba for *sca* > *argos* and 26%Ti and 30%Ba for *Dll* > *argos* ($N = 10$ female legs in each case). Both types of flies had fewer bracts in dorsal vs. ventral rows of the Ba. For *sca* > *argos*, 27% (row 4) and 44% (row 5) of the dorsal bristles had bracts vs. 85–100% for the remaining rows, and on *Dll* > *argos* basitarsi the frequencies were 5% (row 4 and row 5) vs. 22–44% elsewhere. *Dll* > *argos* legs also lacked claws and apodemes.

In the second series of experiments, the *UAS* slaves encoded various versions of Egfr itself: *UAS-Egfr* (wild-type product), *UAS-Egfr*$^{*top4.2}$ (constitutively activated form), and *UAS-Egfr*^{DN} (dominant-negative form). Misexpressing the normal Egfr was expected to cause extra bracts,

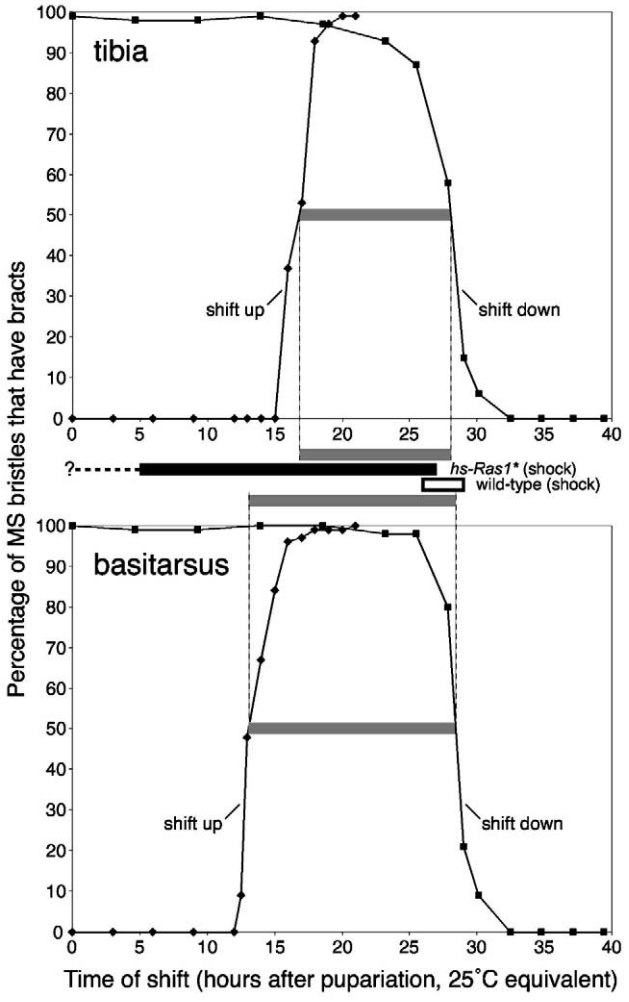

Fig. 4. Effects of temperature shifts on temperature-sensitive $Egfr^{ts1a}/Egfr^{CO}$ mutants. The percentage of MS bristles that have bracts is plotted for the tibia (above) and basitarsus (below) as a function of age (equivalent hours at 25°C) for upshifts (18 to 29°C) vs. downshifts (29 to 18°C). Gray bars indicate the sensitive period, as defined by the 50% midpoints. For comparison, the black bar marks the sensitive period when heat shocks induce extra bracts in *hs-Ras1*$^{M11.2}$ pupae, and the unfilled bar indicates the period when basitarsal bracts can be removed by heat-shocking wild-type pupae (Held, 1990). $N = 6$ legs per time point, except that $N = 4$ legs for upshifts at 14, 16, and 21 h AP and downshifts at 27.8, 37.1, and 39.4 h AP. (See Section 4 for normalization of ages to 25°C and other details.)

but in fact it eliminated bracts (0%Ti and 0%Ba for *sca* > *Egfr* and 10%Ti and 0%Ba for *Dll* > *Egfr*; $N = 10$ female legs in each case). Also surprising were the findings that (1) misexpressing the activated receptor had no detectable effect on bracts with either driver and (2) misexpressing the DN form via *Dll-Gal4* ($N = 2$ male legs) likewise had no effect. These negative results cannot be ascribed to impotence of the transgenes, since other defects were obvious. To wit, tarsal segments 2–4 were shortened and fused in *Dll* > $Egfr^{DN}$ (and *Dll* > *Egfr*) legs, and the entire tarsus was reduced to a bump on the end of a swollen Ti in *Dll* > $Egfr^{*top4.2}$ legs (data not shown). No *sca* > $Egfr^{DN}$ flies survived to the adult stage.

An apparently unrelated phenotype was observed on the distal Ti – a transformation of the huge 'apical' bristle (but never the pre-apical bristle) into a CS bristle of ordinary size. This homeotic replacement (not shown) was seen in 4/10 *Dll* > *argos* legs, 3/10 *sca* > *argos* legs, 3/10 *Dll* > *sSpi* legs, 1/10 *sca* > *sSpi* legs, 1/10 *Dll* > *Egfr* legs, and 1/10 *sca* > *Egfr* legs.

3. Discussion

3.1. The role of the EGFR pathway

Evidently, the EGFR pathway is necessary and sufficient for bract induction. Its necessity is shown by the ability of pathway suppression to remove bracts, and its sufficiency is shown by the ability of pathway hyperactivity to cause extra bracts or to restore bracts to defective mutants. Suppression was enforced by: (1) heterozygosity for the LOF $Star^{5671}$ allele, (2) haploidy for *Star* in a deficiency heterozygote, (3) dosage interactions between $Star^{LOF}$ and $Ras1^{LOF}$, (4) exposure of $Egfr^{ts1a}$ mutants to restrictive temperature, and (5) misexpression of the Egfr inhibitor Argos via *sca* > *argos* and *Dll* > *argos*. Hyperactivation was achieved by: (1) exposure of *hs-Ras1*$^{*M11.2}$ pupae to heat shocks and (2) misexpression of Spi via *sca* > *sSpi* and *Dll* > *sSpi*. An independent study by del Álamo et al. (2002) used different approaches to reach the same conclusion (i.e. that Egfr mediates induction).

The inability of *sca* > *mSpi* and *Dll* > *mSpi* to affect bracts may be due to the fact that Star is essential to convert mSpi into its active form (Bang and Kintner, 2000; Klämbt, 2002; Lee et al., 2001; Tsruya et al., 2002), but Star is present in stoichiometrically limiting amounts (Hsiung et al., 2001) – as is obvious from the sensitivity of bracts to *Star* dosage. The failure of activated (*Egfr**) or dominant-negative ($Egfr^{DN}$) *Egfr* to affect bracts is baffling, given the drastic effects of these same agents on tarsal morphology – a useful 'internal control' for their potency.

Also perplexing is that overexpressing the wild-type Egfr causes missing bracts, rather than extra bracts. However, it is important to realize that both *Gal4* drivers cause expression of the *UAS* transgenes not only in the cells surrounding the bristle SOP, but also in the SOP itself where excess Egfr may interfere with production or secretion of the ligand needed for bract induction (Wong and Chan, 2001).

Based on the extra-bract phenotypes of *sca* > *sSpi* and *Dll* > *sSpi*, the inductive ligand in wild-type flies could be sSpi itself (see del Álamo et al., 2002 for further evidence). If so, then it is hard to understand why their effects are so mild (≤30 bristles per leg with extra bracts) compared with those of *hs-Ras1*$^{*M11.2}$. The weakness could be due to (1) low output of sSpi relative to the burst of Ras1 from the heat-shock promoter or (2) the presence of inhibitors like Argos, which would not affect Ras1 because Ras1 acts downstream of Egfr (Bogdan and Klämbt, 2001; Hackel et al., 1999; Karim and Rubin, 1998).

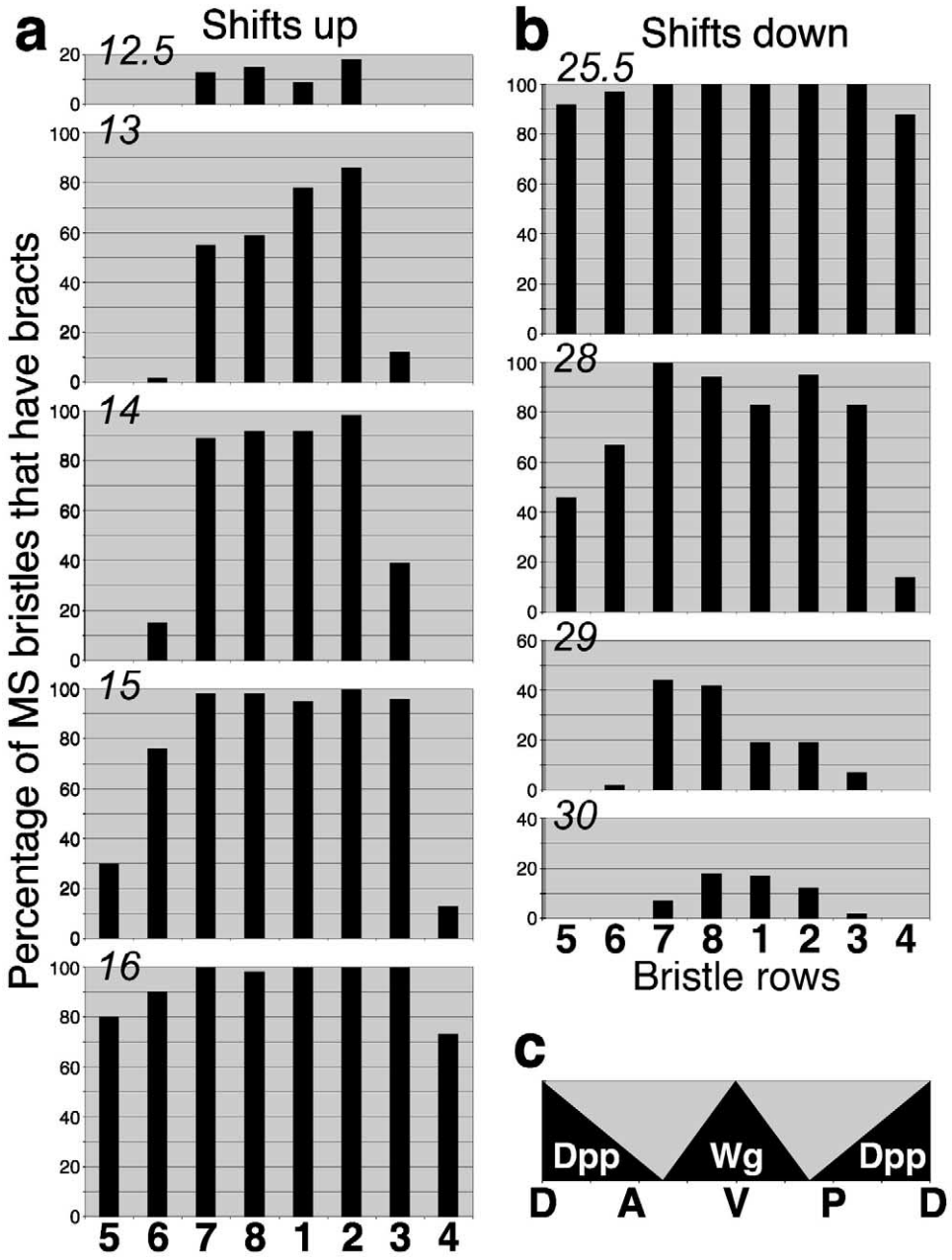

Fig. 5. Variation among basitarsal bristle rows in the timecourse of temperature sensitivity. Effects of temperature shifts on $Egfr^{ts1a}/Egfr^{CO}$ mutants are shown during the sigmoid phases that bracket the sensitive period (see Fig. 4). The percentage of MS bristles that have bracts is plotted for upshifts (a) and downshifts (b) as a function of age (numbers in italics are equivalent hours at 25°C) and bristle row. In both series (upshift and downshift) the bracts of the dorsal rows disappear more readily than those in the ventral rows. (c) These trends imply that dorsal cells need a stronger Egfr signal than ventral cells. The thresholds for EGFR pathway activation could theoretically be modulated by the two morphogens that control the dorsal–ventral axis. Decapentaplegic (Dpp) is synthesized at the dorsal midline and diffuses ventrally, while Wingless (Wg) is synthesized at the ventral midline and diffuses dorsally (Held, 1995). Their respective zones of influence (bounded by rows 7 and 2) were inferred from their LOF phenotypes (Held and Heup, 1996; Held et al., 1994).

3.2. Temporal constraints on signaling

Extra bracts can be induced at any time from 5 to 27 h AP by heat-shocking *hs-Ras1*$^{*M11.2}$, and the starting time may be even earlier since the 0–5 h AP period is opaque due to the death of pupae shocked then. In contrast, the temperature-sensitive period (TSP) for $Egfr^{ts1a}$ occurs later: 13–28 h AP (Ba) and 17–28 h AP (Ti). Strangely, heat shocks to wild-type pupae only suppress bracts at 26–29 h AP (Held, 1990).

How can these disparities be reconciled? The $Egfr^{ts1a}$ allele is amazingly tight (100% wild-type vs. 100% null at low vs. high temperature) and fast-acting (reactive to pulses of $\leq$1 h) (Kumar et al., 1998), so it affords a precise probe. The start of Egfr's TSP, as canonically defined (Suzuki, 1970), is the rising sigmoid curve in each panel of Fig. 4.

This curve reflects the dawning of a bristle's ability to induce a bract even when the nascent bract's Egfr is disabled. In other words, the nascent bract has received enough Egfr input by this time to proceed on its own. Viewed thus, it is not surprising that extra bracts can be induced earlier by *hs-Ras1** since the dialog must have commenced earlier. The completion of Egfr's TSP is the falling sigmoid curve. This curve marks the last time when Egfr can be activated (by a downshift) and still let bracts arise. In other words, it reveals the end of the competence period for signaling. Hence, it makes sense that this time (28 h AP) nearly coincides with the end of Ras1's extra-bract sensitive period (27 h AP).

What about the narrower window (26–29 h AP) when heat shocks delete bracts from wild-type basitarsi (Held, 1990)? Those shocks were high enough (~40°C) to block transcription and translation (Mitchell and Petersen, 1982; Petersen and Young, 1989), but possibly not signaling per se. Thus, that window probably reveals the end of signaling, when the terminal effectors of the pathway (Pointed and Yan?) durably affect the transcription of EGFR target genes. Several lines of evidence indicate that one of those targets is the homeobox gene *Distal-less* (*Dll*) (Cohen et al., 1989): (1) whereas *Dll* is expressed broadly at earlier stages, it is expressed most strongly in the bract cells of adult legs (Campbell and Tomlinson, 1998); (2) Dll^{LOF} suppresses bracts (Campbell and Tomlinson, 1998; Sunkel and Whittle, 1987); (3) Dll^{null} clones lack bracts (Campbell and Tomlinson, 1998; Gorfinkiel et al., 1997), and (4) such mosaics reveal that *Dll* is needed in the responding (vs. the inducing) cells (L. Held, unpublished observations). Other implementing genes remain to be determined. One candidate ('*bractless*') maps to 2–51 at 35E6-36A7 (L. Held, unpublished observations).

3.3. Spatial constraints on signaling

Two spatial trends were found. One is the greater tendency for the Ti (vs. Ba) to lose bracts when EGFR signaling dwindles. The other is seen on the Ba itself: dorsal rows lose bracts more readily than ventral rows. Both trends exist in (1) $Star^{LOF}$ heterozygotes, (2) *Star* deficiency heterozygotes, (3) $Star^{LOF}$ $Ras1^{LOF}$ double heterozygotes (text and Fig. 3), and (4) *sca* > *argos* flies. *Dll* > *argos* flies displayed the latter trend but not the former, presumably because *Dll* is expressed more strongly distally (Campbell and Tomlinson, 1998; Cohen and Jürgens, 1989; Gorfinkiel et al., 1997; Wu and Cohen, 1999). Indeed, expression of *Dll* during larval life could be the key factor that enables a cell to make bracts in response to later EGFR input from a neighboring bristle cell. Its mode of action might resemble how the homeobox gene *Ultrabithorax* regulates hair development on different legs (Stern, 1998).

Interestingly, both of these trends can also be seen in the data from the temperature shifts. The fact that the Egfr TSP begins 4 h later for the Ti (Fig. 4) may reflect a higher threshold for signaling there. That is, tibial bract cells may need more EGFR stimulation (longer duration) than basitarsal bract cells before they can differentiate on their own. The fact that dorsal rows lag behind ventral rows (Fig. 5) can be explained similarly. The inferred difference in thresholds could also explain why dorsal bristle rows of wild-type basitarsi are sensitive to bract loss from heat shocks, while ventral bristle rows are virtually immune (i.e. retain their bracts regardless) (Held, 1990).

Why should cells in different places need different levels of EGFR input to become bracts? The reason for the proximal–distal axis (Ti vs. Ba) is unclear, but the dorsal–ventral discrepancy might stem from differential cross talk between EGFR and the other pathways that govern dorsal (Decapentaplegic) vs. ventral (Wingless) patterning (Fig. 5c) (Barinaga, 1995; Blumer and Johnson, 1994; Hackel et al., 1999; Moghal and Sternberg, 1999).

The patchiness of the $hs\text{-}Ras1{*}^{M11.2}$ extra-bract phenotype (Fig. 2e) suggests that different parts of a leg segment may become maximally competent at different times – not just in a patterned way along the dorsal–ventral axis, but stochastically throughout the epidermis as well.

3.4. Polarity constraints on signaling

Bract induction is the epitome of a private 'chat' between two cells, though more than one cell in the SOP clone may emit the signal. Its only rival is the famous tête-à-tête between the R8 photoreceptor precursor and a neighboring cell, whereby the latter is recruited to become an R7 photoreceptor.

How are other neighbors prevented from 'hearing' the signal (Bier, 1998) and thereby forming a ring of elements (bracts or R7s) around the 'speaker' cell, as is known to occur, for example, in the genesis of chordotonal organs (Okabe and Okano, 1997; zur Lage et al., 1997; zur Lage and Jarman, 1999) and oenocytes (Elstob et al., 2001; Gabay et al., 1997; Rusten et al., 2001)? For R7 induction, various transcription factors limit the ability of other neighbors to respond to the R8 signal (Held, 2002; Kumar and Moses, 1997). For bract induction the answer is less clear.

If a globally acting signal (e.g. emanating from segment boundaries) were enforcing the direction of bract induction, then bristles should always induce bracts on their proximal side, regardless of the orientation of the bristle itself (as lichens only grow on the shady side of trees). However, this is not the case. Misoriented bristles typically make bracts on the side of their socket opposite to the direction in which their shaft points (García-Bellido, 1972; Held et al., 1986).

The simplest way for a bristle cell to send its signal directionally (and to ensure that only one neighbor gets it) would be for it to present a membrane-bound ligand on part of its surface (see Bellaïche et al., 2001; Le Borgne et al., 2002; Winter et al., 2001). However, overexpressing mSpitz

(via *sca* > *mSpi* or *Dll* > *mSpi*) fails to evoke extra bracts, so this strategy seems unlikely.

Oddly, expressing secreted Spitz (via *sca* > *sSpi* or *Dll* > *sSpi*) elicits extra bracts only on the proximal (usual) side of bristle sockets, rather than in a ring as might have been expected a priori. Evidently, it is not the signal that is localized on one side of the emitting cell but rather the receptor that must be localized on one side of the receiving cell—or, indeed, perhaps on all epidermal cells. There are precedents for such polarization in the wing and eye, where Frizzled receptors localize on one specific face of each cell (Adler, 2002; Axelrod, 2001; Strutt, 2001; Strutt et al., 2002; Yang et al., 2002).

4. Experimental procedures

Genetically altered stocks of *Drosophila melanogaster* included *Egfr^{ts1a} cn bw/TSTL* (Kumar et al., 1998), *Egfr^{CO}/TSTL* (Clifford and Schüpbach, 1989), *UAS-Egfr* (Freeman, 1996), *UAS-Egfr*$^{top4.2}$* (Queenan et al., 1997), *UAS-Egfr^{DN}/In(2LR)Cy, Roi* (Freeman, 1996), *UAS-mSpi* (Schweitzer et al., 1995b), and *UAS-sSpi* (Schweitzer et al., 1995b) from Justin Kumar (Kumar and Moses, 2001b), *UAS-argos* from Amanda Simcox, *Star^{5671}/CyO* and *sev^{d2}; Ras1^{e1B}/TM3* from Todd Laverty (G. Rubin lab), *hs-Ras1*$^{M11.2}$* from Elizabeth Noll (N. Perrimon lab), *y w; sca-Gal4/CyO* from Susan Younger (Y.N. Jan lab), *Dll-Gal4/CyO; UAS-nls-GFP* from Konrad Basler via Grace Panganiban, and *Df(2L)ast4/SM1* from the Umeå Stock Center. Phenotypes similar to *Df(2L)ast4/+* were observed for *Df(2L)ast1*, *2*, *3*, *5*, and *6/+* heterozygotes (data not shown).

To calculate equivalent times at 25°C, ages of pupae raised at 18°C were divided by 2.0, and ages of pupae raised at 29°C were multiplied by 1.16 (Held, 1990). Heat shocks were administered by collecting white prepupae at hourly intervals from 25°C bottles, aging them on plastic petri dishes humidified at 25°C, and then floating the dishes on a 38°C water bath for 1 h. Temperature shifts were performed similarly using an 18°C incubator and a 29°C water bath. *Gal4-UAS* transgenic flies were raised at 25°C where possible, but several genotypes exhibited 100% prepupal lethality. To circumvent this problem, *Dll* > *Egfr*$^{top4.2}$* and *sca* > *Egfr*$^{top4.2}$* individuals were kept at 18°C until early-third instar (then put at 25°C), and *Dll* > *Egfr^{DN}* larvae had to be raised at 18°C until pupariation. Despite these measures, only one fly of the latter genotype reached maturity, and no *sca* > *Egfr^{DN}* adults (from $>10^3$ segregant eggs) were recovered under any circumstances.

Wherever flies failed to survive to eclosion (e.g. *Egfr^{ts1a}/Egfr^{CO}* upshifts), the legs of uneclosed pharate adults were used instead. No heat-shocked *hs-Ras1*$^{M11.2}$* pupae (0/713) eclosed regardless of when the shocks were administered (ages 0–36 h AP), and shocks at 0–4 h AP caused death before the pharate stage.

Fly legs were dissected in 70% ethanol, mounted in Faure's solution (Lee and Gerhart, 1973) between cover slips, and examined at 200 and 400× magnification under a compound microscope. Males were used routinely for observation, but females were used instead in some cases (see text) because males from certain crosses were homozygous for *yellow^{1}* (a sex-linked marker), which made scoring of bracts less precise. Remarkably, the vast majority (≥98.5%) of bristles in all series were clearly scoreable as either having or lacking a bract. In the remaining cases (≤1.5%), the bristle had an unpigmented hair intermediate in thickness between a bract and a trichome. Those marginal cases were tallied as bona fide bracts for calculation purposes.

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