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Planar Polarized Protrusions Break the Symmetry of EGFR Signaling during *Drosophila* Bract Cell Fate Induction

Ying Peng,[1,3] Chun Han,[2] and Jeffery D. Axelrod[1,*]
[1]Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, USA
[2]Howard Hughes Medical Institute, Department of Physiology and Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA 94148, USA
[3]Present address: Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905, USA
*Correspondence: jaxelrod@stanford.edu
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SUMMARY

Secreted signaling molecules typically float in the outer leaflet of the plasma membrane or freely diffuse away from the signaling cell, suggesting that a signal should be sensed equally by all neighboring cells. However, we demonstrate that Spitz (Spi)-mediated epidermal growth factor receptor (EGFR) signaling is spatially biased to selectively determine the induction of a single bract cell on the proximal side of each mechanosensory organ on the *Drosophila* leg. Dynamic and oriented cellular protrusions emanating from the socket cell, the source of Spi, robustly favor the Spi/EGFR signaling response in a particular cell among equally competent neighbors. We propose that these protrusive structures enhance signaling by increasing contact between the signaling and responding cells. The planar polarized direction of the protrusions determines the direction of the signaling outcome. This asymmetric cell signaling serves as a developmental mechanism to generate spatially patterned cell fates.

INTRODUCTION

At least two mechanisms that tightly regulate spatial specificity of cell fate induction by intercellular signaling (Wolpert, 2007) are commonly described. First, ''competence'' allows only cells arising from a given development linage at a particular time and location to be capable of adopting the cell fate in response to the signal. Second, ''combinatorial signaling'' requires that cell fate decisions depend on the combination of two or more concurrent signal inputs from distinct sources. Spatial specificity of signaling might also be achieved by a third mechanism involving directional presentation or delivery of signaling molecules by the signaling cell to a particular neighbor. The plausibility of this scenario is questionable if one assumes free diffusability of signaling molecules that would tend to produce a symmetrical response. However, in recent years, a large number of signaling molecules have been found to be either covalently modified by a lipid moiety (Amanai and Jiang, 2001; Chamoun et al., 2001; Chen et al., 2004; Micchelli et al., 2002; Miura et al., 2006; Steinhauer and Treisman, 2009; Takada et al., 2006; Willert et al., 2003) or shown to bind to extracellular lipid-protein particles (Panáková et al., 2005; Vyas et al., 2008). These modifications could lead to a significant restraint on their mobility, potentially facilitating more spatially restricted signal presentation (Chuang and Kornberg, 2000; Miura et al., 2006; Tanaka et al., 2005; Vyas et al., 2008; Willert et al., 2003). Here, we show that such a signaling molecule, the *Drosophila* TGF-α homolog, Spitz, induces a cell fate in a single cell among equivalent neighbors, in a defined position, and we identify special adaptations of the signaling cell that facilitate such a directional signaling event.

Drosophila bract cells can be distinguished by a thick, pigmented trichome (a bract), and on the distal leg segments (femur, tibia, and tarsus) are found exclusively on the proximal side of mechanosensory bristles (Hannah-Alava, 1958) (Figure 1A). The four cells of the mature mechanosensory organ (bristle, socket, sheath, and neuron) arise from asymmetric divisions of the sensory organ precursor (SOP) cell (Jan and Jan, 2001). The bract cell is not lineally related but is induced from the surrounding epithelium by cells within the SOP lineage (García-Bellido, 1966; Tobler, 1966; Tokunaga, 1962). The specific cell within the sensory organ that produces the inductive signal was not easily discerned, as disrupting development of either the socket cell or shaft cell produced bractless organs (Tobler, 1969; Tobler et al., 1973). Subsequent investigations demonstrated that two signaling pathways are involved in bract cell fate induction: epidermal growth factor receptor (EGFR) signaling promotes bract cell fate (del Alamo et al., 2002; Held, 2002), while Notch activation suppresses it (del Alamo et al., 2002). Although the role of Notch was suggested to be limited to excluding bract formation around chemosensory organs (Held, 2002; Layalle et al., 2004), this interpretation is subject to caveats, leaving open the possibility that EGF and Notch pathways act combinatorially to produce spatially biased bract cell induction.

The planar cell polarity (PCP) pathway, while not directly involved in bract cell induction, is required to determine the direction of the bract cell induction event (Held et al., 1986a). In wild-type, a single bract is induced strictly on the proximal side of the mechanosensory organ. In contrast, in PCP mutants, bracts are frequently induced in an incorrect position relative to

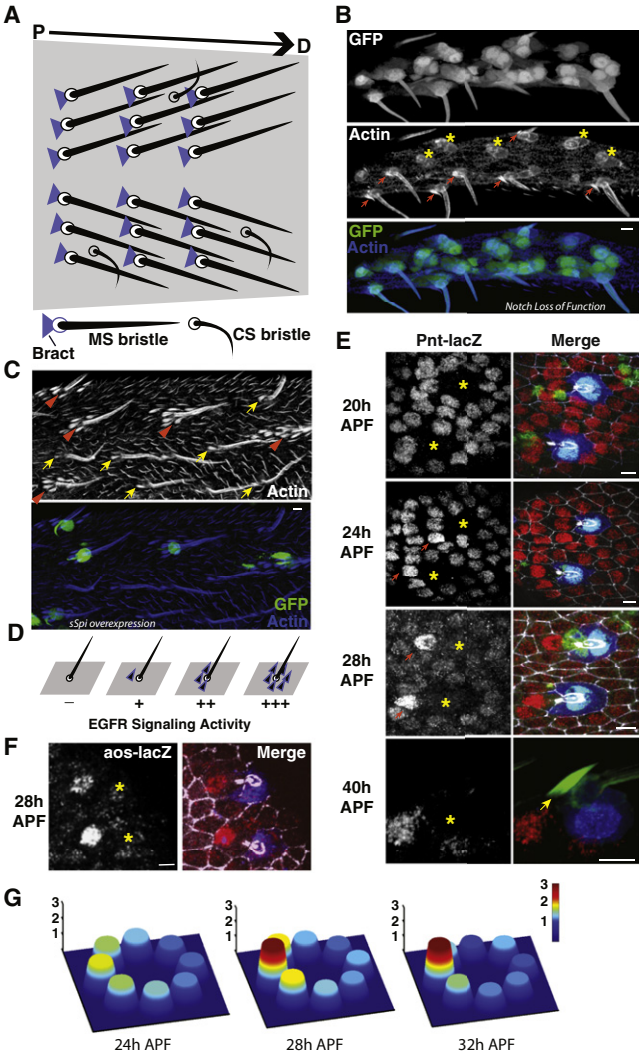


Figure 1. Proximal Induction of Bract Cell Fate Depends on Asymmetric EGFR Signaling around Sensory Organs

(A) Diagram of the *Drosophila* leg epithilium, modeled after a figure by Lewis Held (Held, 2002). Single bract cells (blue triangles) are specifically associated with the proximal side of each mechanosensory bristle (dark straight lines) but not chemosensory bristles (black curved lines). The proximal-distal axis of the tissue is from left to right in this and all subsequent panels.

(B) Green fluorescent protein (GFP) marks SOPs with Notch activity rescued in a *Notch* loss of function background. Note that high Notch activity causes some SOPs to produce double sockets/lack of shafts (asterisks). SOPs that develop normally have a single bract associated with the proximal side of the bristles (red arrows).

(C) Overexpression of sSpi-GFP in sensory organs results in a circle of bract cells (arrowheads, upper panel) around the overexpressing organs. Neighboring wild-type sensory organs have single bracts (arrows).

(D) Summary diagram of bract cell fates around sensory organs with varying levels of EGF/Spi signaling.

(E) EGFR signaling response assayed via Pnt-LacZ becomes asymmetric around sensory organs during the course of bract cell induction. Left panels: Pnt-lacZ. Asterisks label the position of sensory organs. Right panels: merge of cut (green, labeling all cells in the SOP linage), Pnt-LacZ (red), Su(H) (blue, cell body of socket cells), and E-Cadherin (gray, adherens junctions). In the 40-hr-APF-merged image, the green channel displays F-actin, showing a characteristic bract structure (arrow) growing from the cell with high Pnt-lacZ activity.

(F) EGFR signaling response assayed via Aos-LacZ reporter (left panel) is asymmetric around sensory organs during bract cell fate induction. Image captured at 28 hr APF, comparable to the third panel in (E). Asterisks label positions of sensory organs. Right panel: merge of Aos-LacZ (red), Su(H) (Blue), and E-Cadherin (gray).

(G) Quantifications of EGFR signaling asymmetry via Pnt-LacZ through the time course of bract cell fate induction. Both the height and peak color (see color bar) of each cell indicate the relative strength of signaling. Scale bars, 5 μm.

the mechanosensory organ while continuing to respect the one bract per sensory organ rule. This observation led del Alamo and colleagues to hypothesize that polarization of the SOP cell polarizes epidermal growth factor (EGF)/Spi signaling to determine the position of the bract (del Alamo et al., 2002). The PCP signaling pathway has been intensively studied in recent years in various model organisms and has been found to organize a large variety of tissues along their planar axis (Vladar et al., 2009). However, it is unknown how PCP influences directionality of Spitz-mediated bract induction.

This study describes a PCP-controlled lamellipodia-like protrusive cellular structure that potentiates the directional delivery of an EGF signal mediating a cell fate induction event. We identify the socket cell as the source of the inductive EGF/Spi signal. EGF/Spi induces the bract cell fate selectively in a proximal neighbor among equivalent neighboring epithelial cells. Socket cells produce planar polarized and highly dynamic protrusions from their basolateral surfaces, and the direction of those processes determines the direction of enhanced EFG/Spi signaling. We propose that the protrusions potentiate delivery of the EGF/Spi signal by increasing contact between the signaling and responding cell, resulting in the selective enhancement of signaling efficiency in a specified direction.

RESULTS

Spatial Bias of Bract Cell Induction Is due to Asymmetry of EGFR Signaling Levels

Loss- and gain-of-function experiments have shown that EGF signaling by Spitz (Spi) induces bract cell fate (del Alamo et al., 2002; Held, 2002). Previous studies in which widespread ectopic Ras activation was produced demonstrated that almost all leg epithelial cells have the potential to adopt the bract cell fate (del Alamo et al., 2002; Held, 2002). However, manipulations that modestly increased the magnitude of Spi/EGFR signaling from the sensory organs resulted in induction of extra bracts only on the proximal side (del Alamo et al., 2002; Held, 2002). To address whether the cells neighboring the sensory organ on all sides are competent to become bract cells, we further increased the strength of the Spi/EGF signal from the sensory organs using Neu-Gal4 to drive sSpi-GFP (secreted Spitz ligand) and observed induction of bract cell fate in all the surrounding cells, although, in some cases, at a greater distance on the proximal side (Figures 1C and 1D). Therefore, all neighbors of the sensory organ are competent for bract fate induction by responding to a Spi/EGF signal from the SOP.

We then investigated the possibility that spatial bias of bract induction might result from the combination of symmetric activation and asymmetric inhibitory activity from an independent

signaling pathway. Notch signaling has previously been shown to suppress bract cell fate (del Alamo et al., 2002). It was proposed that the only role for Notch was suppression of bract cell induction near chemosensory bristles where bracts do not normally form (Held, 1990; Layalle et al., 2004). However, a potential contribution of Notch signaling to biasing bract cell fate induction could not be assessed during the overlapping periods of competence for bract formation (12–30 hr after puparium formation [APF]; Held, 2002), and Notch-dependent asymmetric divisions of SOP cells (roughly 17–21 hr APF; Gho et al., 1999). Failure of asymmetric SOP cell division itself could also cause an absence of the associated bract cell (Held, 1990). To circumvent this complication, we inactivated Notch from 12 hr APF onward while restoring Notch activity selectively in the sensory organs. We observed normal development of the majority of mechanosensory organs, and each of those correctly induced a single proximal bract (Figure 1B). Furthermore, single bract cells were induced on the proximal side of chemosensory bristles (data not shown). These results provide compelling evidence that Notch signaling does not contribute to the spatial bias of bract cell induction, although it does prevent chemosensory organs from inducing bracts (Held, 1990; Layalle et al., 2004).

To directly visualize whether EGFR signaling itself might be asymmetric, we took advantage of a LacZ reporter line that indicates the expression of Argos (Aos), a bona fide EGFR signaling target (Golembo et al., 1996). We observed high Aos-LacZ reporter activity selectively in the proximal neighbors of the sensory organs, presumably the prospective bract cells (Figure 1F; data not shown). We further characterized the development of asymmetry using a Pnt-LacZ reporter line (Figures 1E and 1G) that reflects the expression of Pnt2, a known EGFR/MAPK pathway target (Brunner et al., 1994; Gabay et al., 1996; Klaes et al., 1994; Scholz et al., 1993). The Pnt-LacZ reporter has the advantage that its activity is suppressed within the sensory organ lineage (Frankfort and Mardon, 2004) (Figure 1E, asterisks). In the cells surrounding sensory organs, Pnt-LacZ is modestly elevated by 20 hr APF, but there is no apparent difference in levels among those neighbors. By 24 hr APF, Pnt-LacZ activity begins to show obvious spatial bias around many sensory organs, favoring the proximal neighbors. This asymmetry is gradually consolidated, such that by 28 hr APF, there are one or two cells on the proximal side of each sensory organ that show substantially elevated Pnt-LacZ expression. Consolidated Pnt-LacZ expression faithfully indicates the Bract fate, since it persists even after the bract cell is morphologically differentiated (Figure 1E). Recently, asymmetry of Aos and Pnt expressions has been independently confirmed (Mou et al., 2012). The proximal most neighbor of the sensory organ accumulates, on average, approximately three times the reporter activity of the distal most neighbor (Figure 1G). Our analysis, therefore, shows that during bract cell fate induction, there is a low-level signaling response in all surrounding cells and a gradual but robust enhancement of EGF signaling activation specifically in the proximal neighbors. Since neither competency nor combinational signaling with Notch is responsible for the spatial bias of cell fate, we postulate that the asymmetry of EGFR signaling is directly responsible for the selective induction of a bract cell on the proximal side of the sensory organ.

The Socket Cell Is the Spitz Signal-Producing Cell Responsible for Inducing Bract Cell Fate

To understand how the EGF signaling response might be biased toward the proximal side, we determined which cell in the SOP lineage is responsible for sending the Spi/EGF signal (Figure 2A). The SOP cell itself cannot induce bract cell fate, because bract cells are not induced when asymmetric SOP cell divisions are disrupted (Held, 1990). This is consistent with our observation that asymmetry of the EGF signaling response, as reported by Pnt-LacZ and Aos-LacZ, is first detected around 24 hr APF (Figure 1D). By then, the four granddaughter cells of the SOP have been born, suggesting that correct fate determination of one or more of them is necessary. We therefore focused on this 24 hr APF time point to identify the signaling cell. Rhomboid (Rho), an intramembrane serine protease, is the key regulator mediating proteolytic activation of the Spi precursor, and its restricted expression usually identifies signal-producing cells (Freeman et al., 1992a). Using a Rho-LacZ enhancer trap line (Freeman et al., 1992a), Rho was found to be specifically expressed in leg SOPs as early as 8 hr APF (del Alamo et al., 2002). At 24 hr APF, when the asymmetry of signaling is first detected, we consistently observed Rho-LacZ in the socket cell and the shaft cell, the two outer cells arising from pIIa, (Figure 2B). Conversely, the two inner cells (sheath and neuron) had no detectable Rho-LacZ activity, ruling them out as signal-producing cells. This is confirmed by the observation that organs consisting of only a sheath and neuron, resulting from overexpression of Tribbles (Fichelson and Gho, 2004), fail to induce a bract cell (data not shown).

Next, we directly investigated the signaling roles of socket and shaft cells by making labeled *spi* mutant clones within the SOP lineage using a SOP-Flpase strain (see Experimental Procedures) and correlating bract induction with the capacity for Spi signaling in each cell type (Figure 2C; Figure S1 available online). In the case of two-cell mutant clones, bract induction is normal when the inner cells (sheath and neuron) lack Spi, confirming that they and their immediate mother cell, pIIIb (also mutant for *spi*), is dispensable for inducing the bract fate. In contrast, bracts are not induced when two-cell clones of outer cells (shaft and socket) lack Spi, consistent with the observed Rho expression in these cells. Dramatically different roles for socket and shaft cells were revealed when we analyzed single-cell mutant clones: most socket cell clones failed to induce bract cells, while *spi* clones within the shaft cell had no effect on bract cell induction (Figure 2C). Although we cannot rule out a possible signaling contribution from pIIa, the immediate precursor to both the socket and shaft cell, pIIa itself is not sufficient, as pIIa retains signaling capacity in single socket cell *spi* clones that do not induce bracts. We therefore conclude that the socket cell is the key signal-producing cell for inducing the bract cell fate.

By blocking development of either the shaft cell (via *Hairless* or *shaven* mutants; Tobler et al., 1973) or socket cell (via applying mitomycin; Tobler, 1969), Tobler found that bract cells are not induced without either of these two cell types. Our genetic mosaic analysis distinguished the different signaling roles of those two cell types while keeping stereotypical cellular composition and structural integrity of the sensory organs intact. Nonetheless, Tobler's observations suggest a requirement for the shaft cell as well, which, taken in the context of our results,

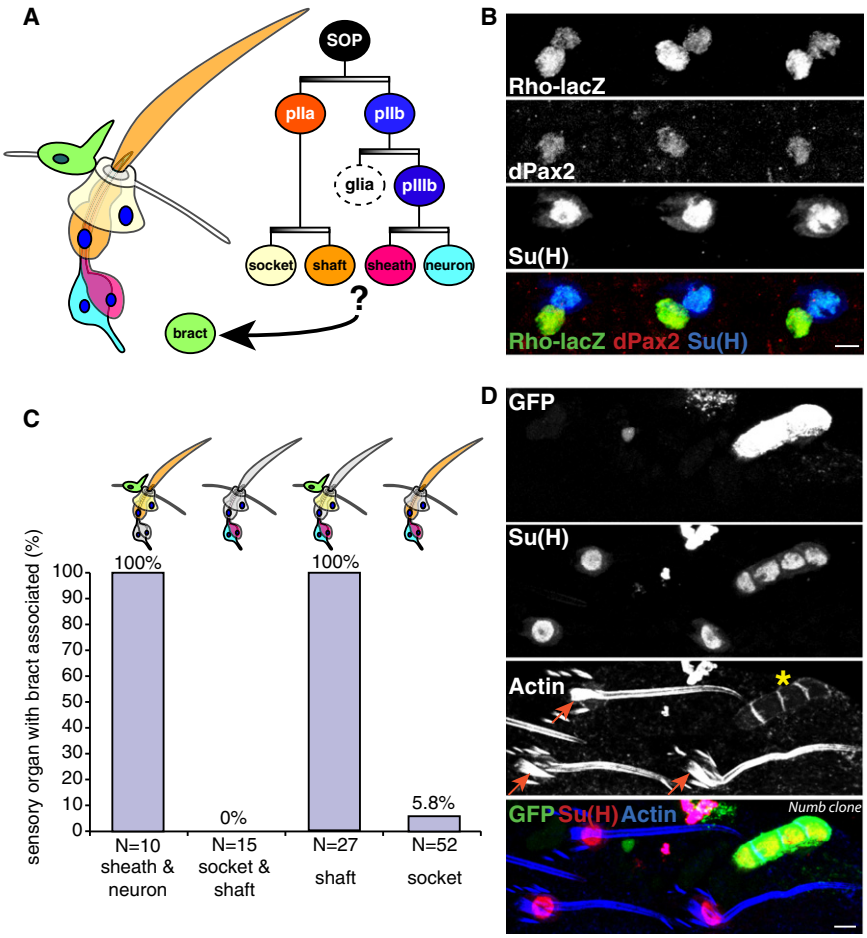


Figure 2. Within the SOP Lineage, the Socket Cell Is the Key Signaling Cell for Inducing Bract Cell Fate

(A) Schematic of sensory organ with associated bract cell. Asymmetric divisions (darker side of graded bars indicates higher Notch activity) generate four mature daughter cells with distinct fates.

(B) Expression of Rhomboid (Rho-lacZ) is restricted to the two outer cells within each mature sensory organ: the shaft, expressing dPax2, and the socket, expressing Su(H). Three adjacent sensory organs on a 24 hr APF leg are shown.

(C) Percentage of mosaic *spi* mutant sensory organs with associated bract cells. The mutant cell type(s) is indicated. See Figure S1 for representative images.

(D) On a 40 hr APF leg, a GFP positive *numb*^{15} mutant clone with four socket cells positive for Su(H) (asterisk), induces no bract; a single proximally associated bract is associated with each wild-type sensory organ (arrows). Scale bars, 5 μm.

may be to facilitate the signaling function of the socket cell. A facilitating role for the shaft cell is further confirmed by the observation that sensory organs comprising only socket cells (produced in *numb* mutant clones; Guo et al., 1996) fail to induce bract cells (Figure 2D). This failure is likely not due to inability of the *numb* mutant socket cells to generate active ligand, since Rho-LacZ reporter levels within these mutant sensory organs are equivalent to those in neighboring wild-type socket cells (Figure 3B). Apparently, socket cells within sensory organs lacking other key cell types (most likely shaft cells) are defective in some essential attribute required for effective signaling.

Socket Cells Extend Planar Polarized Protrusions toward Proximal Neighbors

The nature of the defect became apparent when we compared wild-type sensory organs with those comprising only socket cells by expressing APC2-GFP to reveal cytoskeletal structures (see Experimental Procedures for details). At 24h APF, we observed prominent cable-like protrusions projecting proximally from almost every wild-type sensory organ (Figure 3A). The protrusions typically reach one to two cells away from the sensory organ, contact two to three of the proximal neighbors at any given time, and are highly dynamic (Movies S1 and S2). Labeling only a single cell using a socket-cell-specific Gal4 driver (Barolo et al., 2000), we demonstrated that the protrusions originate from the socket cell (Figure 3C; Movie S2). The protrusions contain actin cables, labeled by APC2-GFP as well as other F-actin markers (data not shown), and are also visible with Cherry-Jupiter, a known microtubule reporter (Cabernard and Doe, 2009). These lamellipodia-like protrusive structures project from the basolateral surface on the proximal side of the socket cells (Figure 3C; Movie S1), are less than 1 μm in thickness, and appear to contact or envelop the basolateral surface of several neighboring epithelial cells (Movie S2). Importantly, when we used APC2-GFP to label sensory organs consisting of only socket cells, no similar protrusions were observed (Figure 3B). While this difference indicates that the protrusions might depend on the presence of one or more of the other sensory organ cell types, it also suggests that the lack of proximal protrusions might explain why sensory organs comprising only four socket cells fail to induce bracts.

Indeed, the temporal and spatial characteristics of the protrusions suggest that they may be responsible for breaking the symmetry of EGFR signaling. First, they are observed as early as 20 hr APF, before signaling asymmetry is readily apparent (Figure 1E), and persist for the next approximately 10 hr, during which time EGFR signaling asymmetry becomes robust (Movie S3). Second, the protrusions are sufficiently dynamic that, with our current frame rate of live imaging (1 frame every 2 min), we typically observed the protrusions to explore different neighborhoods between adjacent frames, yet they rarely drastically change their overall direction of projection (Figure 4D; Movie S3). Since the presence of these protrusive structures could potentially increase the contact between the signaling and recipient cells, as a surrogate measure of such enhanced contact, we quantified the area of the protrusions as a function of direction. Using a movie recorded from 24 to 27 hr APF, we confirmed

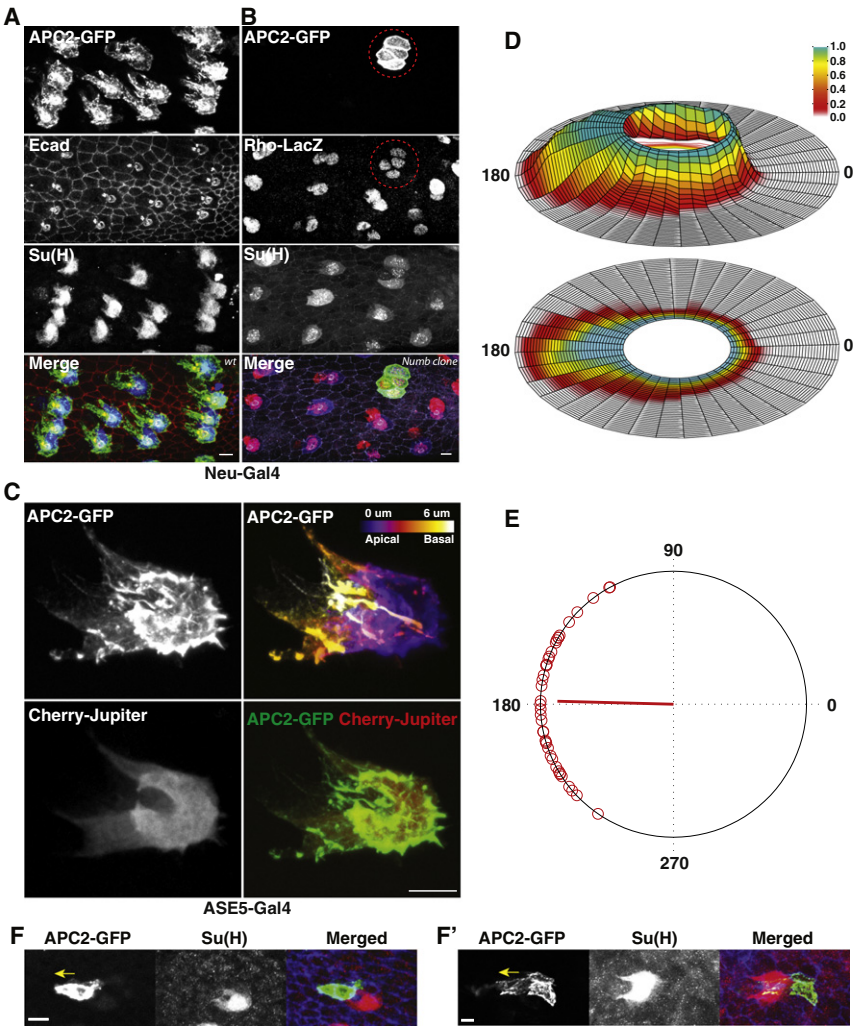


Figure 3. Socket Cells Produce Proximally Oriented Planar Polarized Protrusions

(A) Planar polarized protrusions on a 24 hr APF leg (portion of femur shown) are revealed by expression of APC2-GFP driven by Neu-Gal4. E-Cad and Su(H) label adherens junctions and socket cell bodies, respectively. All protrusions (as identified by GFP-positive cables extending beyond socket cell body) project proximally.

(B) A *numb*^{15} mutant sensory organ (dashed circles), labeled with APC2-GFP, does not exhibit protrusions but expresses approximately wild-type levels Rhomboid-LacZ.

(C) Planar polarized protrusions originate from socket cells. Protrusions are visualized by expression of APC2-GFP (top panels) or Cherry-Jupiter (bottom panels) driven by a socket-cell-specific ASE5-Gal4 driver. The depth of the protrusions seen by APC2-GFP within the epidermis is color coded in the top right panel (see color bar). Bottom right panel shows an overlay of the maximal projections from GFP and Cherry. Protrusions are not induced as a consequence of the EGF signaling response. See also Figure S2.

(D) Probability plot of protrusions from a single socket cell reaching a given distance from the cell body in any given sector. Data are derived from a three-hour movie, starting from 24hAPF, of a socket cell labeled with ASE5-Gal4 UAS-APC2-GFP. The distance is normalized against the size of socket cell body (unit circle in the center). Height (top panel) and color (top and bottom panels) indicate the probability that the protrusions reach each area in a single frame of the movie.

(E) Distributions of protrusion directions for wild-type sensory organs (n = 40, from 24 hr APF fixed tarsal segments). Each red circle on the polar plot represents the mean angle of protrusions from an individual sensory organ. The mean vector is indicated by the red line from the center of the plot.

(F and F') Small clones of cells expressing APC2-GFP reveal proximal protrusions from most or all leg epithelial cells at 24 hr APF. Protrusions in epithelial cells on the proximal (F) or distal (F') side of the sensory organ, socket cell labeled by Su(H), point proximally (yellow arrows). Scale bars, 5 μm.

that the socket cell protrusions overwhelmingly prefer to explore the cell's proximal neighborhood, such that roughly two thirds of the overall potential contact via these protrusions falls within a ±30° angle from its mean orientation (Figure 3D). Fifty percent of the time, protrusions extend in the proximal direction to a distance of 9.49 μm beyond the range of the cell body, but only 1.33 μm in the distal direction. The proximal bias of protrusions among different sensory organs is also very robust (Figures 3A and 3E); the overall orientation of protrusions from almost all sensory organs examined at 24 hr APF falls within a neighborhood of roughly ± 60° from the proximal direction, with the mean orientation of the population indistinguishable from the proximal direction and a circular SD of 29.3° (Figure 3E). Importantly, the protrusions are not induced as a consequence of the Spi/EGFR signaling response, because the protrusions are present in sensory organs in which the EGFR pathway has been suppressed by either overexpression of the inhibitor, Argos, or removal the ligand, Spitz (Figure S2).

By expressing the APC2-GFP reporter in small clones, we found that, in addition to the socket cells, epithelial cells also produce proximally directed basolateral protrusions, including those immediately proximal or distal to the sensory organs (Figures 3F and 3F′).

Orientation of Socket Cell Protrusions and EGFR Signaling Asymmetry Are Controlled by the Planar Cell Polarity Pathway

The observation of proximal basolateral protrusions on both developing leg epithelial cells and socket cells suggested that they might be a relatively general manifestation of epithelial PCP. We investigated how the orientation of protrusions is controlled, focusing on socket cell protrusions since these appear to be associated with bract fate induction (Figures 2D and 3B). The PCP pathway was previously shown to affect the orientation of bract cells relative to the mechanosensory bristles (del Alamo et al., 2002; Held et al., 1986b). To address whether

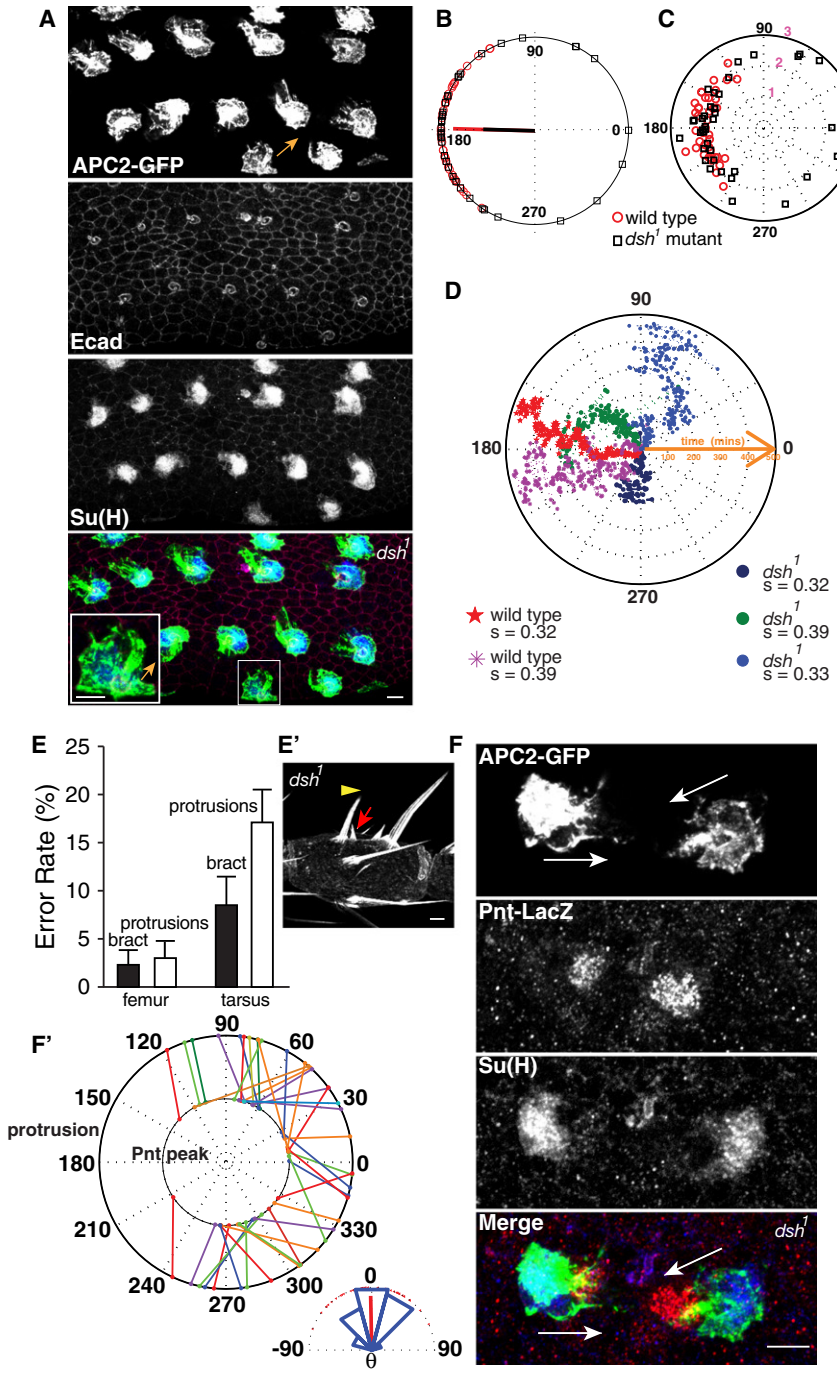


Figure 4. Misorientation of Polarized Protrusions Leads to Mistakes of Bract Cell Orientation

(A) Planar polarized protrusions on a 24 hr APF *dsh*^{1} mutant leg (portion of femur shown) revealed by Neu-Gal4 driven expression of APC2-GFP (top panels). E-Cad and Su(H) label adherens junctions and socket cell bodies, respectively. Most protrusions project proximally as in wild-type tissue (Figure 3A), while misorientation of protrusions (arrow) is occasionally observed (magnified view shown in inset). See also Figure S3.

(B) Distributions of protrusion directions in *dsh*^{1} mutant sensory organs (n = 37, from 24 hr APF fixed tarsal segments) and comparison with wild-type sensory organs (n = 40). Black squares (*dsh*^{1} mutant) and red circles (wild-type) on the unit circle represent the mean angle of protrusions from individual sensory organs. Mean vectors for *dsh*^{1} and wild-type cells are represented by black and red lines, respectively.

(C) Comparison of protrusion area and orientation among individual *dsh*^{1} (black squares) and wild-type (red circles) sensory organs. The position of each sensory organ on the plot is determined by the overall orientation of protrusion (angle) and the ratio, area of cell body + protrusion/area of cell body (radius).

(D) Persistence of protrusion orientation during the course of bract cell fate induction. Each color represents data points from a different movie. The circular SD(s) and genotype of each cell are shown.

(E) Correlation of protrusion error rate and bract orientation mistakes—see example in (E')—in femur and tarsal leg segments of *dsh*^{1} mutant flies. Error bars show SD (n = 14 femurs; n = 17 tarsi).

(E') The orientation of mispositioned bract (red arrow) can be independent of the direction of associated shaft (yellow arrowhead). Portion of a *dsh*^{1} mutant tarsus is shown.

See also Figure S3.

(F) Two oppositely oriented sensory organs on a 28 hr APF *dsh*^{1} mutant leg, showing orientation of protrusions (APC2-GFP) correlates with the direction of peak EGFR signaling (Pnt-LacZ). Arrows indicate protrusion directions.

(F') Correlations between protrusion directions and the direction of EGF signaling asymmetry (n = 40, from 28 hr APF misoriented tarsal SOPs of *dsh*^{1} mutant). Protrusion orientation of each SOP and direction of adjacent cell showing peak pnt-LacZ activity are represented by dots with the same color on the outer and inner circles respectively and linked by a solid line. The angular distribution of the difference between these two angles for each sensory organ is shown in the inset. Scale bars, 5 μm.

the direction of socket cell protrusions determines the direction of bract cell induction, we first assessed the correlation between protrusion direction and the direction of bract cells relative to the sensory organs in several PCP mutants.

In *dsh*^{1} mutant legs at 24 hr APF, a majority of sensory organs project protrusions indistinguishable in morphology and orientation compared to their wild-type counterparts (Figure 4A). Improperly positioned bract cells and misdirected protrusions (defined as a deviation of ≥90° from the proximal direction) are observed at similar, low, frequencies in the femur (2.3 ± 1.4% and 3.0 ± 1.6%, respectively) and at similar but higher frequencies in the tarsi (8.5 ± 2.9% and 17.1 ± 3.3%, respectively) (Figures 4A, 4E, and S3). Nearly identical observations were made in *pk-sple*^{13} legs (data not shown). It is important to note that we have never observed these errors in wild-type legs and that the maximum possible error rate is 50% if directed randomly.

Thus, there is an approximate correlation between the frequency of incorrectly positioned bracts and misoriented protrusions in PCP mutants, suggesting that the direction of protrusions may determine the position at which the bract is induced.

To further characterize the protrusion orientation defects in the tarsal segments of *dsh*^{1} mutant legs compared to wild-type, we plotted the angle of protrusions from individual sensory organs (Figure 4B). As expected, the means are similarly proximal for both *dsh*^{1} and wild-type. However, the distribution of their orientations is substantially more dispersed in *dsh*^{1} (circular SD = 54.4°; circular kurtosis = 0.36) compared to wild-type (circular SD = 29.3°, circular kurtosis = 0.55), with a significant portion of outliers protruding toward the distal side. Importantly, disrupting the core PCP pathway does not significantly change other geometric and dynamic properties of the protrusions. For example, protrusions from mutant sensory organs have roughly equivalent areas compared to wild-type (132 versus 141 μm^2) (Figure 4C). Similarly, individual mutant protrusions do not substantially change their overall orientations over time, regardless of how far they deviate from the proximal direction (Figure 4D; Movie S3).

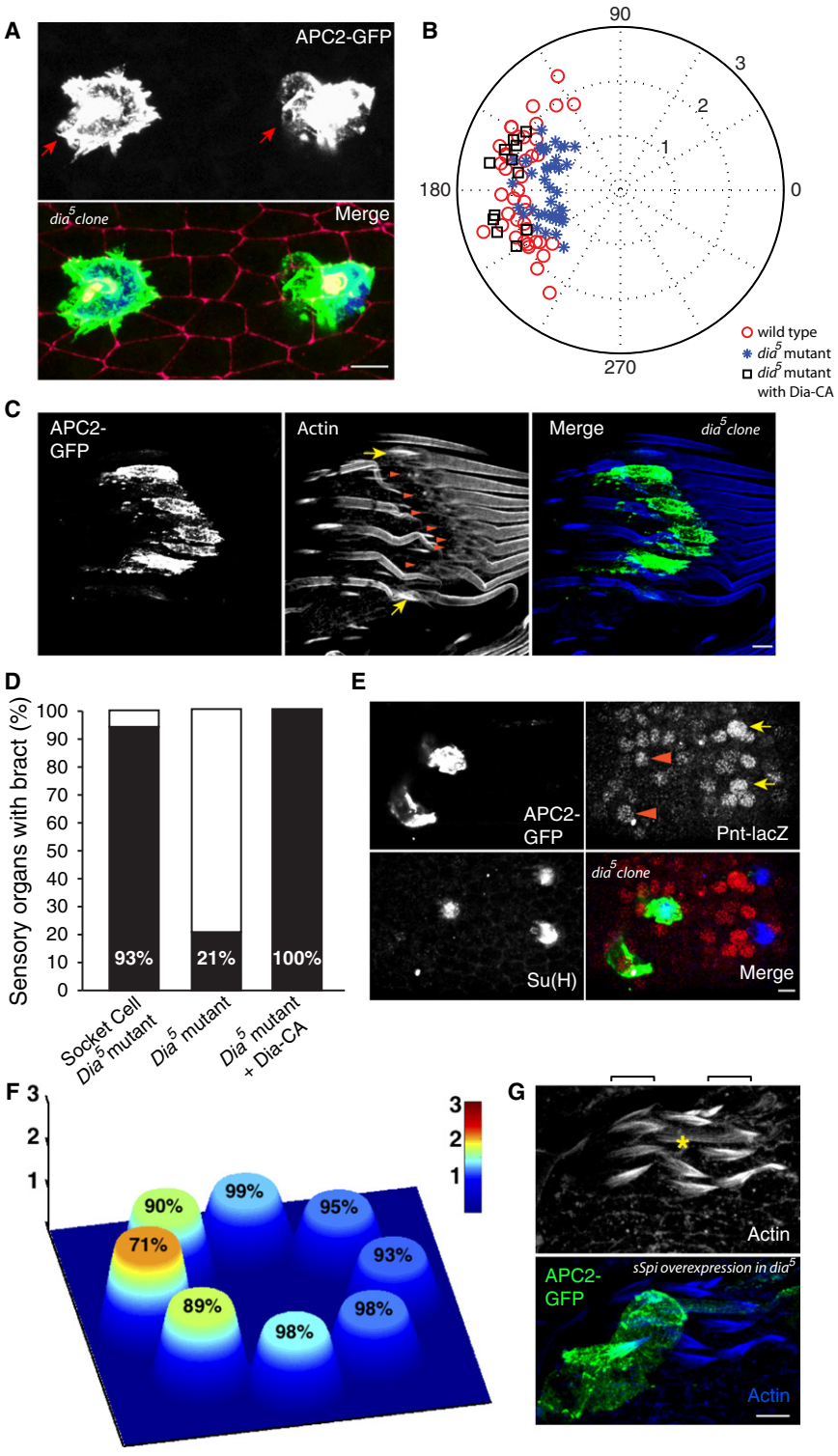
Since the aforementioned data show that a reasonable estimate of the overall orientation of any protrusion over the period of bract fate induction can be obtained from a measurement made at any particular time point, we simultaneously assayed protrusion orientation and EGF signaling responses using Pnt-LacZ, in 28 hr APF *dsh*^{1} legs. If the direction of protrusions determines the ultimate position of bract induction, we would expect to observe a tight correlation between the orientation of protrusions and the consolidating EGFR signaling asymmetry at this time. Indeed, among samples in which the protrusions are misoriented by over 60°, we found that peak LacZ reporter activity points in approximately the same direction (± 45°) as protrusion orientation in around 90% of cases, and none differed by more than 75° (Figures 4F and 4F′). Indeed, their distributions strongly correlate, with a circular correlation coefficient of 0.90 and a p value of 1.6e-07. This correlation is surprisingly strong, given the dynamic nature of the protrusions (Figure 4D; Movie S3). It is essential to note that the direction of bract cell induction does not necessarily reflect the orientation of the sensory organ as a whole. In some cases, the tilt of the shaft is altered to correspond to bract cell direction, but, in many other cases, bract direction is abnormal (distal or lateral) while the tilt of the shaft is not correspondingly affected (Figure 4E′; Figure S3B′). Thus, the orientation of bract induction is tightly correlated to the direction of socket cell protrusions but not to the overall orientation of the bristle. We conclude that the PCP pathway regulates the orientation of the socket cell protrusions, and this, in turn, determines the orientation of EGFR signaling asymmetry and, thus, bract cell positioning.

Protrusions Potentiate EGFR Signaling to Facilitate Achieving the Signaling Threshold for Bract Cell Fate Induction

While the correlation between the direction of protrusion extension and activation of signaling suggests that the protrusions are responsible for signaling asymmetry, we wished to suppress protrusions directly to confirm their role in signaling. We found that, when the activity of the formin Diaphanous (Dia; Castrillon and Wasserman, 1994) was compromised during sensory organ development by inducing large mutant clones of the hypomorphic allele *dia*^{5} in leg epidermis, the four cells within the mutant SOP lineage developed in correct number and position, but the socket cell protrusions were significantly suppressed and disorganized (Figure 5A). Compared to wild-type, the APC2-GFP-positive F-actin cables from mutant sensory organs usually extend only a short distance beyond the cell cortex. This phenotype is reminiscent of the shortened protrusions from *dia*^{5} mutant leading-edge cells during dorsal closure in the fly embryo (Homem and Peifer, 2009) and is consistent with the well-established role of Dia in promoting actin filament assembly and bundling at the tips of filopodia (Block et al., 2008; Schirenbeck et al., 2005; Yang et al., 2007). The size of mutant protrusions is diminished compared to wild-type, covering, on average, 55% less area at 24 hr APF (mean area of 68 versus 141 μm^2 for wild-type) (Figure 5B). Nevertheless, compromising Dia function does not abolish these protrusions, nor does it dramatically change the overall proximal orientation of the residual protrusive structures (Figure 5B).

We next asked whether SOPs with compromised protrusions are impaired in their ability to induce bract cells. When we made small *dia*^{5} mutant clones encompassing socket cells, using SOP-Flpase, a modest fraction of sensory organs (6 of 83 clones examined) did not induce an associated bract cell (Figure 5D; Figure S4). We hypothesized that the low penetrance might result from the perdurance of residual wild-type protein that small *dia*^{5} clones inherit from their progenitor cells. To test this interpretation, we examined large *dia*^{5} clones generated by hs-FLP at larval stages and found a highly penetrant absence of bract cell induction within the clones (53 of 67 mutant SOPs examined) (Figures 5C and 5D). To rule out the possibility that the absence of bract cells within large *dia* clones might be due to absence of Dia in the prospective bract cell, we rescued *dia* selectively in the SOP linage using a constitutively active Dia construct, which lacks the C-terminal autoinhibitory DAD domain (Homem and Peifer, 2008, 2009; Wallar et al., 2006). Such rescue restored both protrusions (mean area of 158 versus 67 μm^2 in unrescued clones and 141 μm^2 in wild-type; Figure 5B; Figure S5) and associated bract cells (17 of 17 SOP examined) (Figure 5C; Figure S5). Thus, epithelial cells do not need Dia to become bract cells, and the loss of bract induction in large *dia* mutant clones can be directly attributed to the mutant SOPs.

Finally, we investigated whether failure of bract cell induction within the large *dia* clones is due to compromised EGF signaling when the protrusions are suppressed. We observed that Pnt-LacZ asymmetry began to develop around mutant sensory organs at roughly the same time (24 hr APF) as in wild-type (data not shown), but we detected a quantitative difference in EGF signaling around mutant and wild-type sensory organs (Figures 5E and 5F). At 28 hr APF, while Pnt-LacZ activity was substantially higher in one or two proximal neighbors around wild-type sensory organs, the extent of asymmetry around mutant sensory organs was decreased significantly compared to wild-type (~70% of the wild-type gradient; compare Figure 5F to Figure 1G, middle panel). The difference was largely due to reduced levels on the proximal side of the sensory organ, while distal levels remained similar (Figure 5F). We interpret this to mean that the mutant socket cells produced a Spi signal, but that in the absence of intact protrusions, it was not selectively



28h APF *Dia*^{5} clone

Figure 5. Suppression of Planar Polarized Protrusions Results in Decreased EGFR Signaling Asymmetry and the Failure of Bract Cell Induction

(A) Top: Planar polarized protrusions (red arrows) from *dia*^{5} mutant sensory organs (labeled by APC2-GFP) are less extensive than wild-type (cf. Figure 3A). Bottom: An overlay of APC2-GFP (green); E-Cad (red; adherens junctions) and Su(H) (blue; socket cell bodies). Portion of an ~24 hr APF femur segment is shown. See also Figure S4.

(B) Comparison of protrusion area and orientation from individual sensory organs between *dia*^{5} mutant (blue asterisks), *dia*^{5} mutant rescued with Dia-CA (black squares), and wild-type (red circles). Position of each sensory organ on the plot is determined by the overall orientation of protrusion (angle) and the ratio, area of cell body + protrusion/area of cell body (radius).

(C) Bracts (revealed by F-actin) are not induced (arrowheads) adjacent to *dia*^{5} mutant sensory organs in a large *dia*^{5} clone (expressing APC2-GFP). Note that adjacent wild-type sensory organs have associated bracts (arrows). Portion of an ~40 hr APF leg is shown.

(D) Percentage of *dia*^{5} mutant mechanosensory organs with associated bract cells (black portion of the bars) with three different manipulations: small socket cell clones generated with SOP-Flpase, large clones generated with hs-Flpase, and large clones within which Dia is rescued in SOP cells. See Figure S4 for representative images.

(E) Comparison of EGFR signaling asymmetry (Pnt-lacZ) around *dia*^{5} mutant sensory organs (labeled with APC2-GFP) and adjacent wild-type sensory organs (GFP negative, Su(H) positive). Note the peak reporter activities proximal to mutant sensory organs (arrowheads) are significantly lower than wild-type (arrows).

(F) Quantification of EGFR signaling asymmetry around *dia*^{5} mutant sensory organs at 28 hr APF (comparable with the panel for 28 hr APF in Figure 1E). Percentages on top of each cell indicate the ratio of signal intensity compared with the corresponding cell around wild-type sensory organs.

(G) Rescue of missing bract phenotype in large *dia*^{5} clones by expressing sSpi-GFP within mutant sensory organs. Note ectopic bracts (seen by F-actin staining at 40 APF) are induced around the shaft of sensory organ expressing sSpi (asterisk). This is similar to the result of sSpi-GFP overexpression in wild-type sensory organs, except that extra bracts at a greater distance on the proximal side are not observed (cf. Figure 1C). Scale bar, 5 μm.

and efficiently delivered to the proximal neighbors. Hence, a minimal threshold for bract cell induction was frequently not reached. Consistent with this, when expressed with a socket-cell-specific driver, we observed the presence of sSpi-GFP within wild-type socket cell protrusions (Movie S4), proving that active ligand can be delivered to the protrusions. This interpretation was further confirmed by the observation that increasing the amount of secreted Spi within *dia* mutant sensory organs rescued bract cell induction and even induced ectopic bract cells uniformly around mutant SOPs (Figure 5G). Thus,

the protrusions appear to facilitate achieving a critical threshold of EGF signaling in a specific subset of SOP neighbors, and the subthreshold signaling level when the protrusions are impaired can be overcome with overwhelming amounts of free ligand.

These results also strongly argue that it is asymmetry of the socket cell, but not that of the prospective bract cell, that contributes to the polarized response. Protrusions from the proximal neighbor point away from the socket cell, while those from the distal neighbor point toward the socket cell (Figures 3F and 3F′). If polarity of the receiving cells controls signal directionality, one would therefore have to hypothesize that EGF signal receptivity is anticorrelated to the protrusion direction. Although conceivable, this would not explain the reduction of signaling with manipulations that specifically affect the signaling cell (by perturbation of Dia selectively in the socket cell or by making a socket only sensory organ). This difference is also seen in the altered range of bract induction when sensory organs expressing ectopic sSpi are made mutant for *dia*. Therefore, the only parsimonious conclusion is that the socket cell protrusion potentiates the EGF/Spi signal and its orientation determines the direction of signaling.

DISCUSSION

This study has demonstrated that a signaling cell can use a secreted molecule to initiate cell fate induction in a selected cell among equivalent competent neighbors. We refer to this mechanism as "asymmetric cell signaling." The robust asymmetry of EGF/Spi signaling we describe is the biological basis for the spatial bias of bract cell fate induction on the *Drosophila* leg. Cellular protrusions projected by the socket cell to its proximal neighbors generate the spatially biased signaling outcome. The PCP pathway provides directional information that aligns the direction of this inductive signal with the tissue axes.

Production and Polarization of Socket Cell Protrusions

Prior studies demonstrated that both the socket and the shaft cell are required for bract cell induction but failed to identify which is responsible for sending the inductive signal (Tobler, 1969; Tobler et al., 1973). Identification of Spi as the inductive signal (del Alamo et al., 2002) and development of sensory organ specific genetic mosaic analysis (this study) have allowed us to demonstrate that Spi from the socket cell alone is necessary and sufficient for bract cell induction. As it expresses Rhomboid, the shaft cell is likely also generating activated Spi ligand, but mutant clones of Spi affecting only this cell do not reveal an obvious developmental process for which it is required.

However, consistent with the previously identified requirement for the shaft cell (Tobler et al., 1973), we found that sensory organs made of only socket cells do not induce a bract cell. The socket cells in these sensory organs fail to elaborate protrusions, suggesting that the shaft cell facilitates extension of socket cell protrusions. We envision that these two cells undergo interdependent morphogenesis during development (Le Borgne et al., 2002) and that this interaction might be related to production of the protrusions. An alternate interpretation is that the presence of multiple socket cells suppresses the protrusions.

How does the socket cell sense polarity information? We speculate that directional information is inherited from the PCP-dependent orientation of the SOP division through the stereotypically oriented cell divisions that give rise to the sensory organ (Gho and Schweisguth, 1998; Le Borgne et al., 2002). We note that the disruption of protrusion orientation and the disruption of bract position in core PCP mutants are incomplete. This may be due to the architecture of the PCP signaling pathway (Axelrod, 2009) or to additional mechanisms contributing to orientation of SOP progenitors, socket cell polarization, and bract induction. Nevertheless, the misorientation of socket cell protrusions in PCP mutants has proven to be an invaluable tool in demonstrating that the protrusions are responsible for the asymmetry of EGF/Spi signaling.

Breaking Symmetry: How Asymmetric Cell Signaling Is Achieved

The finding that the responsive epithelial cells surrounding the sensory organ are polarized raises the possibility that competence to become a bract and orientation of the protrusions are each independently under PCP control. In such a scenario, one would predict that, upon loss of the PCP signal, each would be randomly misdirected; therefore, one would also have to explain why, in cases when induction is incorrectly oriented, these independent processes are coordinately misdirected. The possibility that PCP mutants cause incorrect bract location by changing competence in the epithelial cells is more definitively ruled out by manipulations that affect just the SOP.

Two sets of data, considered together, make a strong argument that socket cell protrusions enhance the efficacy of EGF/Spi signaling in a specified direction to produce asymmetric cell signaling. First, in PCP mutants, we find a tight correlation between orientation of protrusions and the position of enhanced EGFR signaling and bract induction. Second, we find that diminishing protrusions by mutating *dia* interferes with bract induction. Given the competence of all surrounding epithelial cells, these two observations argue strongly that the protrusion is responsible for facilitating the induction event.

Although we cannot completely rule out possible contributions of bract induction by cryptic polarized features other than protrusions that might be affected in PCP mutants, the orientation of bracts can be uncoupled from the orientation of the SOP as a whole (Held et al., 1986b; see, for examples, Figure 4E′ and Figure S3B′). By contrast, the observation that we cannot experimentally dissociate protrusion polarity, the socket-shaft vector, and the subsequent position of bract induction (Figure 4F′; data not shown), even in cases where other structural features of the sensory organ are not correspondingly aligned, argues in favor of a biologically relevant causal relationship. Furthermore, such potential cryptic, polarized features of sensory organs are highly unlikely to be similarly diminished with impaired Dia function. We cannot completely rule out the possibility that compromising Dia function could affect EGF signaling by unknown mechanisms other than perturbing protrusions. However, we view other possibilities as unlikely for the following reasons. First, we selected a hypomorphic allele, dia^5 for these experiments, and sensory organs with compromised Dia are grossly normal in cell morphology and are still capable of making protrusions, albeit smaller ones. Second, the signaling defect in dia^5 mutant sensory organs is overcome by overexpressing sSpi, indicating that Spi can still be secreted from dia^5 mutant cells, but with little

or no directional bias, as indicated by the presence of uniformly induced ectopic bracts. Finally, and most compellingly, compared to wild-type sensory organs, in *dia*^{5} mutants, the cells proximal to mutant sensory organs show the most significant drop in EGFR signaling strength, while the reduction in distal neighbors is negligible. This difference argues strongly against any general, nonpolarized process as the cause of signaling defects within *dia*^{5} clones. In the extreme case of absent protrusions, observed in the four-socket-cell sensory organs, symmetric weak activation of EGF signaling results in failure of bract induction. The correlation between protrusion direction and stronger signaling, and the reduction in elevated signaling specifically in the direction of protrusions when protrusions are diminished, therefore strongly suggest that the protrusions are responsible for generating EGFR signaling asymmetry by potentiating signaling in a specified direction.

Although we cannot readily visualize the dynamic distribution of activated ligand, based on prior work (Miura et al., 2006), we know that EGF/Spi is palmitoylated, and upon secretion, either remains tethered to the membrane or, if freed from the cell, is expected to have limited mobility. In either case, the observed asymmetric signaling response suggests the necessity of a regulated mode of ligand delivery. Since the protrusions have been demonstrated to potentiate the signal, we hypothesize that they do so either by creating direct contact between signaling and responding cells or by locally increasing the concentration of a poorly diffusing ligand.

Signal Integration

The levels of Pnt-LacZ or Aos-LacZ we used as assays of EGFR signaling activity reflect the cumulative history of EGFR signaling, in effect integrating signaling activity over time. We hypothesize that EGFR pathway activity fluctuates as a function of protrusion contact, with the cumulative duration of signal activation read out as increasing levels of Pnt-LacZ or Aos-LacZ. Direct visualization and measurement of this process will require the development of live, real-time probes of pathway activity and gene expression. We propose that a cumulative threshold is reached at which a bistable switch fixes the cell fate decision.

We also postulate that signal integration might be involved in limiting suprathreshold EGF/Spi response to a single cell. Our time-lapse analysis (Figure 3D) is consistent with the notion that integration over time produces an increasingly regular peak signal in the most proximal cell, with weaker signal on either side. In addition, or alternatively, Argos, a potent EGF inhibitor usually expressed from cells responding to high levels of EGF/Spi signaling (Figure 1F), could provide a negative feedback that limits the response (Golembo et al., 1996). Mutation of *Argos* frequently produces one or two extra proximal bracts (del Alamo et al., 2002). Whether or how much these mechanisms might contribute to limiting the response to a single cell is unknown, and it is also possible that an as yet unrecognized lateral inhibition mechanism also contributes.

EXPERIMENTAL PROCEDURES

Fly Genetics

Fly culture and crosses were performed according to standard procedures and cultures raised at room temperature unless otherwise stated. To generate the SOP-Flpase strain, a derivative of the 1.0 kb BamHI-*Xho*I enhancer element from the *E(spl)mα* gene with five Su(H) binding sites mutated (Castro et al., 2005) was cloned upstream of the *flipase* coding region. Transgenic flies were generated by phiC31 integrase mediated transformation. To generate mitotic clones using hs-Flpase, flies were heat shocked for ~2 hr at 37°C during late second/early third instar stage. Newly enclosed pupae were heat shocked at 37°C for 5 min to induce single cell flip-on clones.

Immunohistochemistry

Pupae collected at the white prepupal stage and aged for precise times, as indicated, were dissected in PBS and fixed in PBS containing 4% formaldehyde for 30 min at room temperature. After fixation, pupal legs were dissected from the cuticle and stained following standard procedures. The primary antibodies used were: anti-beta-gal (Promega, 1:200), anti-ECAD2 (DSHB, 1:100), anti-dPax2 (a gift from Marcus Noll, 1:50), anti-Su(H) (Santa Cruz Biotechnology, 1:1,000), and anti-Cut (DSHB, 1:100).

Due to the difficulty of removing cuticles from legs younger than 20 hr APF, we only focused on developmental stages from this point on. To visualize bracts and bristles, legs staged between 36 hr and 48 hr APF were incubated with 1% Alexa Dye-conjugated phalloidin (Molecular Probes) for 1 hr before imaging.

Live Imaging of Pupal Legs

After carefully removing the pupal case of ~24 hr APF pupae, "naked" pupae were mounted on a translucent hydrophobic membrane dish (VIVA Science), with the ventral side of the animal abutting the membrane. The dish was moistened with drops of water and inverted for live imaging using an upright Leica SP5 confocal microscope with an oil-immersion objective lens directly above the membrane.

Visualization of Protrusions

Cable-like proximal protrusions from sensory organs in the pupal legs are obvious when UAS-APC2-GFP (*Drosophila* Adenomatous polyposis coli homolog 2; APC2; McCartney et al., 1999; Webb et al., 2009) is expressed within all SOP cells under control of the Neu-Gal4 driver or a socket-cell-specific driver ASE5-Gal4 (Barolo et al., 2000). For fixed samples, ~10 μm Z-axis-stacked projections were generated from the surface of the epidermis in order to image the full extent of protrusions. A second reporter, Cherry-Jupiter (Karpova et al., 2006), was also used to visualize the protrusions. The GFP signal in protrusions imaged using ASE5-Gal4 was weaker than with Neu-Gal4, perhaps reflecting weaker activity of the ASE5-Gal4 driver, and it labels an increasing portion of socket cells through 36 hr APF. For this reason, the Neur-Gal4 driver was preferred for MARCM-based clonal analysis due to its strong labeling from every sensory organ from all stages. For live imaging using ASE5-Gal4, UAS-APC2-GFP reporter, a Histone2Av-mRFP transgene was used occasionally in the background to indicate the position of the socket cell nucleus as well as those of neighboring cells. To visualize protrusions from other epithelial cells, a flip-on Act-Gal4 driver line is used to express UAS-APC2-GFP reporter in randomly generated single-cell Flp-activated clones.

Temperature Shift Schemes

Flies with *Notch*^{ts1} in the background were kept at 19°C. For Notch inactivation during bract development, pupae were shifted to 29°C from the equivalent of 12 hr APF until sacrificed around 40 hr APF.

For mosaic analysis using *dia*^{5} mutants, flies were raised at room temperature with the exception of a heat shock pulse to induce clones. Because of the temperature-sensitive nature of the *dia*^{5} allele (Homem and Peifer, 2008), mosaic animals were moved to 29°C from the wandering third instar stage until the time of sacrifice. The age at which pupae were sacrificed was calculated by accounting for the faster rate of development at 29°C.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, four movies, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2012.07.016.

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