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The EGF receptor and N signalling pathways act antagonistically in *Drosophila* mesothorax bristle patterning

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SUMMARY

An early step in the development of the large mesothoracic bristles (macrochaetae) of *Drosophila* is the expression of the proneural genes of the *achaete-scute* complex (AS-C) in small groups of cells (proneural clusters) of the wing imaginal disc. This is followed by a much increased accumulation of AS-C proneural proteins in the cell that will give rise to the sensory organ, the SMC (sensory organ mother cell). This accumulation is driven by *cis*-regulatory sequences, SMC-specific enhancers, that permit self-stimulation of the *achaete*, *scute* and *asense* proneural genes. Negative interactions among the cells of the cluster, triggered by the proneural proteins and mediated by the Notch receptor (lateral inhibition), block this accumulation in most cluster cells, thereby limiting the number of SMCs. Here we show that the proneural proteins trigger, in addition, positive interactions among cells of the cluster that are mediated by the Epidermal growth factor receptor (EGFR) and the Ras/Raf pathway. These interactions, which we denominate 'lateral co-operation', are essential for macrochaetae SMC emergence. Activation of the EGFR/Ras pathway appears to promote proneural gene self-stimulation mediated by the SMC-specific enhancers. Excess EGFR signalling can overrule lateral inhibition and allow adjacent cells to become SMCs and sensory organs. Thus, the EGFR and Notch pathways act antagonistically in notum macrochaetae determination.

Key words: EGF receptor, Ras, Notch, Sense organ, Proneural genes, *Drosophila melanogaster*

INTRODUCTION

During development, the epidermis of *Drosophila* generates over one thousand bristles and other types of sensory organs (SOs) (Lindsley and Zimm, 1992). Many of these bristles appear at stereotyped positions, such as the conspicuous large bristles (macrochaetae) that arise on the head and the dorsal mesothorax (notum). This arrangement of macrochaetae provides a classical model to study pattern formation (Lindsley and Zimm, 1992). Each macrochaetae derives from a single SO mother cell (SMC) which undergoes two differential divisions (Bodmer et al., 1989; Hartenstein and Posakony, 1989). The four progeny cells subsequently differentiate into the components of the SO. During the third instar larva and early pupa stages, SMCs appear in precise positions of the imaginal discs, the larval epithelia that will give rise to a large part of the adult epidermis (Cubas et al., 1991; Huang et al., 1991). Thus, the accurate position of macrochaetae is largely due to the emergence of their corresponding SMCs at specific sites of the imaginal discs.

Formation of this pattern of SMCs require the participation of genes collectively known as the proneural genes and of cell to cell signalling systems. Proneural genes confer on cells the ability to become SMCs. Two of them, *achaete* (*ac*) and *scute* (*sc*), members of the *ac-sc* complex (AS-C) (Campuzano and Modolell, 1992), are the most important for the development of macrochaetae. They encode transcriptional regulators of the basic region-helix-loop-helix (bHLH) family (Garrell and Campuzano, 1991; Jan and Jan, 1993) and probably commit cells into becoming SMCs by activating downstream genes that participate in the neural differentiation program. *ac* and *sc* are coexpressed in relatively small groups of cells, the proneural clusters, which prefigure the pattern of macrochaetae (Cubas et al., 1991; Skeath and Carroll, 1991). A fixed number of SMCs arise from each cluster, usually one or two. In the imaginal wing disc, a typical cluster that gives rise to one bristle may consist of 20-30 cells, but the SMC is selected from a smaller subgroup of cells that accumulate higher levels of Ac-Sc proteins than their neighbours (the proneural field, Cubas et al., 1991; Cubas and Modolell, 1992; Skeath and Carroll, 1991). This subgroup and the SMC, which accumulates the highest levels of Ac-Sc, always occupy the same position within the cluster. The SMC also accumulates Asense, another bHLH protein encoded in the AS-C (Brand et al., 1993; Domínguez and Campuzano, 1993; Jarman et al., 1993). Recently, an enhancer that mediates the increased accumulation of proneural protein in the SMC has been characterized (Culí and Modolell, 1998). It promotes proneural gene self-stimulation specifically in this cell and this activation is an early and essential step of SMC commitment. Additional,

as yet uncharacterized factors are required for the action of this enhancer. Thus, SMC commitment is at present a poorly understood process.

The Notch (N) cell to cell signalling pathway prevents additional cells of a proneural cluster from becoming SMCs, and therefore the development of many macrochaetae from a single cluster. Indeed, in the absence of N signalling many proneural cluster cells become SMCs (Artavanis-Tsakonas et al., 1995; review). It is currently thought that the more proneural protein a cell accumulates, the stronger is its ability to signal and the less inhibited it will be by their neighbours. Thus, in a proneural cluster, the cells of the proneural field (which have the highest levels of proneural protein) tend to escape from the inhibition. When a cell does so, it becomes an SMC, it signals maximally and prevents its neighbours from acquiring the same fate (lateral inhibition; Heitzler and Simpson, 1991; Simpson, 1990; Simpson, 1997). The reception of this strong signal maintains the SMC-specific enhancer in these neighbouring cells in an 'off' state (Culí and Modolell, 1998).

The Epidermal growth factor receptor (EGFR) signalling pathway has also been implicated in macrochaetae development (Clifford and Schüpbach, 1989). EGFR signalling is transduced by the Ras/Raf/MAP kinase cascade. It participates in diverse processes of *Drosophila* development, like embryonic ventral ectoderm fate, head development, wing and haltere development, notum differentiation, ommatidial cell recruitment and differentiation, induction of dorsal follicle cell fate in the ovary, etc. (Freeman, 1998; Schweitzer and Shilo, 1997; reviews). How activation of the same Ras/Raf/MAP kinase pathway causes cells to adopt different fates is a very active area of research, specifically motivated by the fact that misactivation of the pathway in humans is associated with many kinds of tumours (Li and Perrimon, 1997; Moghal and Sternberg, 1999; reviews). Contrary to the inhibitory signals mediated by the N pathway, in *Drosophila* EGFR signalling seems to promote macrochaetae development. Indeed, hypomorphic mutations at the *Egfr* gene were found to remove several notum macrochaetae with different frequencies (Clifford and Schüpbach, 1989), although some macrochaetae were sometimes duplicated. Absence and duplications of macrochaetae have also been observed in nota mosaic for hypomorphic *Egfr* alleles (Díaz-Benjumea and García-Bellido, 1990). Microchaetae develop normally in these clones, although with a higher density probably due to the reduced size of the mutant cells. Moreover, clones of cells with amorphic *Egfr* alleles in the tergites can autonomously develop bristles and attract and incorporate neighbouring wild-type bristles. These results suggest that different groups of bristles have distinct *Egfr* function requirements.

We have analysed the role of EGFR signalling in the determination of the notum macrochaetae. While distinct proneural clusters show different requirements in the level of EGFR signalling for wild-type levels of *ac-sc* expression, SMCs generally fail to be determined in the absence of this signal. Our data indicate that reception of the EGFR signal is necessary for the triggering of the self-stimulatory loop of *sc* that is characteristic of and a requisite for SMC determination. We also show that the levels of EGFR signalling have to be regulated, as excess signalling leads to too many cells from each proneural cluster becoming SMCs. This regulation may be accomplished in part by the N-mediated interactions that occur among cells of the proneural cluster.

MATERIALS AND METHODS

Fly stocks

In(1)sc$^{10.1}$ and *N*^{ts} have been described previously (Lindsley and Zimm, 1992). A temperature sensitive condition for the *Egfr* gene was obtained using the heteroallelic combination *Egfr*^{tsla}/*Egfr*^{CO}, where *Egfr*^{CO} is a deficiency of the locus (Kumar et al., 1998). Larvae were raised at 18°C and incubated at 30°C for 12-15 hours before dissection. Lines carrying the transgenes *UAS-ras*^{V12} (Karim and Rubin, 1998), *UAS-argos* (Freeman, 1994), *UAS-raf*$^{DN2.1}$ (Martín-Blanco et al., 1999), *UAS-Egfr* and *UAS-Egfr*^{DN} (Buff et al., 1998) and *UAS-Spitz*^{soluble} (Schweitzer et al., 1995b) were used in combination with the Gal4 drivers C253 (Culí and Modolell, 1998), C765 (Gómez-Skarmeta et al., 1996), *pnr*^{MD237} (Heitzler et al., 1996), 179b (Brand and Perrimon, 1993), *ap-Gal4* (Calleja et al., 1996), *sca-Gal4* (Nakao and Campos-Ortega, 1996) and *dpp*^{disk}*-Gal4* (Staehling-Hampton et al., 1994). Both *sca-Gal4* and *253-Gal4* drive expression in proneural clusters (our unpublished data, and Culí, 1998), as they are presumably activated by *ac-sc* (Culí, 1998; Mlodzik et al., 1990). To generate clones of cells expressing the Gal4 protein and the green fluorescent protein (GFP) marker, females of the genotype *yFLP122*; P{Act5C>y+>Gal4}, *UAS-GFP* (Ito et al., 1997) were crossed with males harbouring the required UAS line. Larvae (36-60 hours after egg laying; AEL) were incubated at 36°C for 2-6 minutes and raised at 25°C until dissection. The SMC-specific *lacZ* reporter transgenes used were *neuralized* (*neu*)*-lacZ* A101.IF3 (Huang et al., 1991) and *SRV-lacZ* (Culí and Modolell, 1998).

Histochemistry

lacZ expression was analysed in wing imaginal discs by X-gal staining (Gomez-Skarmeta et al., 1995). Anti-Sc and anti-Sens (Nolo et al., 2000) antibody staining was performed as described by Cubas et al. (Cubas et al., 1991) for conventional microscopy or using lissamine rhodamine-conjugated anti-rabbit and CY5-conjugated anti-guinea pig secondary antibodies for confocal microscopy.

In situ hybridization to detect *rho/ve* mRNA was performed as described by González-Crespo and Levine (González-Crespo and Levine, 1993) using an antisense DIG-labeled RNA probe.

RESULTS

Macrochaetae development requires EGFR-mediated signalling

Weak hypomorphic *Egfr* alleles cause the partial removal of several notum macrochaetae (Clifford and Schüpbach, 1989). The effect of stronger loss-of-function *Egfr* mutations has not been determined since these mutations drastically reduce the size of the imaginal wing discs and cause lethality. Moreover, clones of cells homozygous for amorphic or nearly amorphic *Egfr* mutations do not survive in the prospective notum (Díaz-Benjumea and García-Bellido, 1990; and our unpublished results). Consequently, we have reexamined these findings using the temperature sensitive combination *Egfr*^{tsla}/*Egfr*^{CO} (Kumar et al., 1998; Table 1). At a permissive temperature (18°C), three bristles (ASA, PSA and PPA) were often missing and the anterior postalar (APA) and anterior dorsocentral (ADC) were frequently duplicated. When late third instar larvae were placed at a non-permissive temperature (30°C) for 15 hours (pupation took place during this interval) and

completed development at 18°C, the presence of all notum macrochaetae was affected to different extents, excepting the scutellars and the APA, a bristle that was sometimes duplicated (Table 1). Stronger phenotypes were obtained by overexpressing a dominant negative form of EGFR (*UAS-Egfr^{DN}*) with either the drivers *sca-Gal4* (expressed in proneural clusters; Mlodzik et al., 1990) or *ap-Gal4* (expressed in the dorsal compartment of the disc; Table 1). With *ap-Gal4* at 29°C most notum macrochaetae were removed, although microchaetae were unaffected. *UAS-aos*, which encodes the Argos protein (an EGFR inhibitory ligand; Schweitzer et al., 1995a), driven in proneural clusters by *C253-Gal4* suppressed both macro and microchaetae and only a few bristle sockets remained (Fig. 1H). These results suggest that EGFR signalling is essential for bristle development. Consistent with this conclusion, increasing the levels of the wild-type receptor (*ap-Gal4/UAS-Egfr*) promoted development of extra macrochaetae, but only in the vicinity of the extant ones (Fig. 1I). This suggests that the excess signalling causes extra macrochaetae precursors to arise within the extant proneural clusters.

We next examined which stage(s) of macrochaetae development is/are affected by the decreased function of EGFR. One of the earliest events, the establishment of the proneural clusters, was analyzed by examining the accumulation of Sc protein. Heat-treated *Egfr^{tsla}/Egfr^{CO}* discs showed that the proneural clusters located at the central region of the prospective notum, the dorsal radius and the pleura had reduced levels of Sc, while those at the scutellar and ANP clusters were increased (compare Fig. 1A and B). These effects, which were not observed in discs from non heat-treated larvae (not shown), suggest different requirements for *Egfr* function to accomplish wild-type levels of *sc* expression in different regions of the imaginal disc. Note, however, that for many proneural clusters the modified levels of Sc protein still permitted the development of the corresponding macrochaetae with near wild-type frequency (Table 1).

The next step in bristle development, SMC emergence from proneural clusters, was also sensitive to the loss of EGFR signalling. Accumulation of the Aos inhibitory ligand in proneural clusters (*C253-Gal4/UAS-aos*) was almost not enough to modify the Sc protein levels (Fig. 1C, compare with 1A). However, SMCs, distinguishable by their enhanced accumulation of Sc, were not identifiable. Their absence was verified by the lack of expression of the SMC-specific marker *neu-lacZ* (A101.IF3 enhancer trap line; Huang et al., 1991; Fig. 1F). Consistent with these findings, in the heat-treated *Egfr^{tsla}/Egfr^{CO}* discs, expression of *neu-lacZ* was clearly reduced and/or delayed (Fig. 1E). These results suggest that EGFR signalling is generally required for SMC emergence, although the necessary levels may vary for different SMCs. This may explain the suppression of essentially all notum bristles by Aos overexpression (Fig. 1H).

EGFR signalling is mediated by the activation of the Ras/Raf signal transduction pathway (Freeman, 1998; Schweitzer and Shilo, 1997; reviews). We verified that bristle development also required the activation of this pathway by overexpressing a dominant negative form of Raf (*UAS-raf$^{DN2.1}$*). Relatively mild but ubiquitous expression in the notum (*C765-Gal4* driver) eliminated (95-100%) the ASA and PSA macrochaetae and promoted (45% of heminota) the generation of one extra DC. Relatively late overexpression in proneural clusters (*C253-Gal4* driver) only eliminated the APA (83%) and occasionally generated an extra DC (7% of heminota). With the *pnr^{MD237}-Gal4* driver, which promotes early expression at the dorsal-most part of the presumptive notum, the DC proneural cluster was sharply reduced and the DC bristles were absent (100% of ADC and 91% of PDC, 33

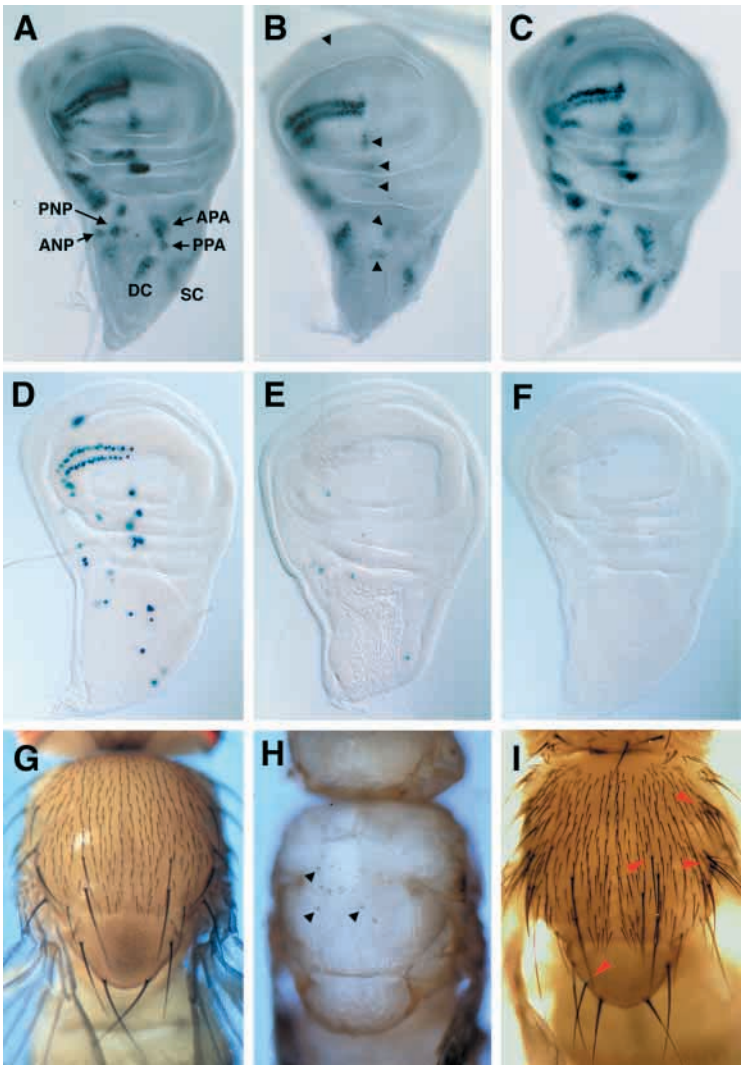

Fig. 1. Inhibition of EGFR activity impairs SMC and macrochaetae emergence. (A-C) Sc protein accumulation in late third instar wing discs of (A) wild-type, (B) *Egfr^{tsla}/Egfr^{CO}* (incubated at 30°C for 12 hours before dissection) and (C) *C253-Gal4/UAS-aos* larvae. Arrowheads in B point to some of the proneural clusters with decreased Sc accumulation. Ventral is to the top and anterior to the left. ANP and PNP, anterior and posterior notopleural; APA and PPA, anterior and posterior postalar; DC, dorsocentral; SC, scutellar, clusters. (D-F) Discs carrying the *neu-lacZ* (A101.IF3) enhancer-trap insertion in the same genetic backgrounds as A-C, respectively, and stained for β-galactosidase accumulation. (G-I) Nota of a wild-type, a *C253-Gal4/UAS-aos* and a *ap-Gal4/UAS-Egfr* fly. In H, all bristles are missing but some bristle sockets, corresponding to microchaetae, remain (arrowheads). Note in I, ectopic bristles near, but not adjacent to, extant ones (arrowheads).

Table 1. Presence of notum macrochaetae in *Egfr^{tsla}/Egfr^{CO}*, *Egfr^{DN}/sca-Gal4* and *Egfr^{DN}/ap-Gal4* flies

	Egfr^{tsla}/Egfr^{CO}		*sca-Gal/Egfr^{DN}*	*ap-Gal4/Egfr^{DN}**	
Macrochaetae	18°C	18°C + 30°C‡	29°C	17°C	25°C
ANP	1	0.9	0.1	0.9	1
PNP	1	0.7	0.7	0.1	0
PS	0.9	0.8	0.5	0.5	0.1
ASA	0.6	0.1	0.6	0	0
PSA	0	0	0	0	0
APA	1.4	1.2	0.3	0	0
PPA	0.7	0.2	0.7	0	0
ADC	1.4	0.8	0.1	0.1	0
PDC	1	0.9	0.3	0.4	0
ASC	1.1	1	0.8	0.9	0.2
PSC	1	1	1	1	0.8

Results are averages for 12 (first three columns) or 20 heminota. Abbreviations for macrochaetae are according to Lindsley and Zimm, 1992. Flies were cultured at the indicated temperatures. Notum microchaetae were not overtly affected under any of these conditions.

*These flies were a gift from J. F. de Celis.

‡In this case, development took place at 18°C until late third instar, when larvae were transfered to 30°C for 15 hours. Pupation occurred during the 15 hours interval and development was completed at 18°C.

thoraces examined, not shown). A generalized and stronger expression of *UAS-raf$^{DN2.1}$* (*179b-Gal4*) was lethal and the few pharate adults recovered lacked 50-80% of notum macrochaetae, although in approximately half of these cases the socket of the missing bristle was present. This socket phenotype, as well as that shown in Fig. 1H, suggests that the Ras/Raf signalling pathway is also required for the late process of bristle differentiation.

We also examined the effect of *UAS-raf$^{DN2.1}$* on the establishment of proneural clusters and SMC emergence by analyzing Sc accumulation in clones of cells overexpressing this transgene. In the SC and PNP clusters, this accumulation was not overtly modified (Fig. 2A,B). In contrast, cells within the APA, DC or vein L3 proneural clusters that expressed *UAS-raf$^{DN2.1}$* autonomously lost or had reduced expression of *sc* (Fig. 2C,D). These results confirm that cells of proneural clusters near the central region of the prospective notum require the EGFR signal to optimally express *sc*, while those located further away, like the NP and SC clusters, do not show this requirement (Fig. 1A,B). SMCs, recognized by their increased accumulation of Sc or their *senseless* (*sens*) expression (Nolo et al., 2000) were found only outside the *UAS-raf$^{DN2.1}$*-overexpressing clones. Moreover, these cells can emerge in abnormal positions when the clones occupied the sites where SMCs normally appear (Fig. 2E-H).

Activation of the Ras pathway induces ectopic *sc* expression, SMCs and macrochaetae

Overactivity of EGFR signalling was mimicked by overexpressing a constitutively activated form of Ras by means of the *UAS-ras1^{V12}* transgene (Karim and Rubin, 1998). With either *ap-Gal4*, *pnr^{MD237}-Gal4* and *dpp^{disk}-Gal4*, which drive expression in subregions of the wing disc, or *179b-Gal4* and *C765-Gal4*, which promote ubiquitous expression, *sc* was ectopically activated (Fig. 3A,B and results not shown). In the notum territory, high levels of ectopic *sc* expression occurred mostly in single cells. Many of them were SMCs, as they expressed *lacZ* under the control of an *sc* SMC-specific enhancer (Culí and Modolell, 1998) (Fig. 3D,E). We could not determine whether these SMCs gave rise to bristles since the overexpression of activated Ras was lethal.

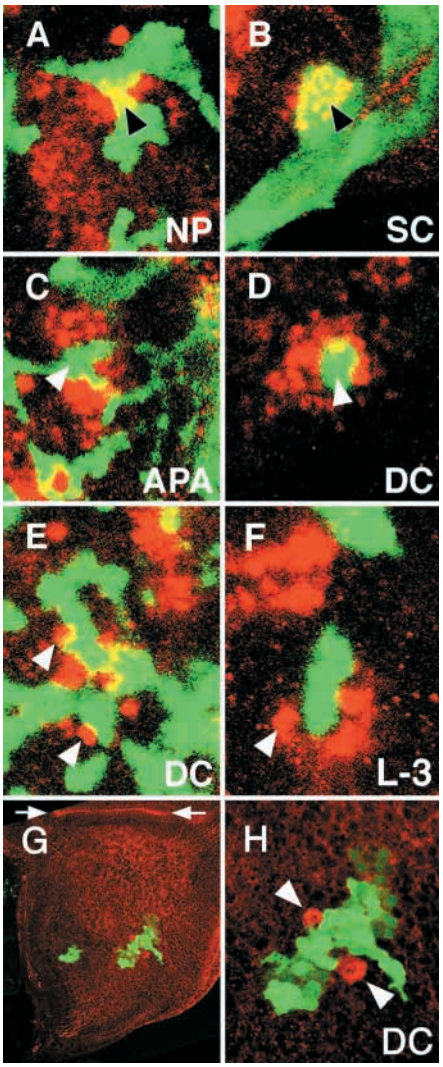

Fig. 2. Cells overexpressing a dominant negative form of Raf may only accumulate Sc at reduced levels and do not become SMCs. Clones of cells that expressed *UAS-raf$^{DN2.1}$*, and *UAS-GFP*, which was used as a marker (green), were generated. Discs were stained with anti-Sc antibody (red) and examined using confocal microscopy. (A,B) Accumulation of Sc in notopleural (A) and scutellar cluster (B) cells, was not overtly affected (strong yellow, arrowheads). (C,D) Cells of the anterior postalar (C) and dorsocentral (D) clusters that express *UAS-raf$^{DN2.1}$* (green, arrowheads) accumulated reduced amounts of Sc. (E,F) At the DC (E) and L-3 (F) clusters, cells accumulating large amounts of Sc, presumably SMCs (arrowheads), appeared outside normal positions, which were occupied by *UAS-raf$^{DN2.1}$*-expressing cells. In wild-type clusters, SMCs emerge near the centre (L-3 cluster) and at the dorsal-most part (DC cluster; Cubas et al., 1991). (G) Low and (H) high magnification of a prospective notum showing DC SMCs, revealed by anti-Sens antibody (red, arrowheads), adjacent to an *UAS-raf$^{DN2.1}$*-expressing clone (green). One or both SMCs were outside normal positions since DC SMCs always define a line parallel to the notum/hinge fold (between arrows; Cubas et al., 1991). Nuclei of other SMCs were apparent in different focal planes.

Activated Ras expressed within proneural clusters (*C253-Gal4/UAS-ras1^{V12}*) did not overtly affect Sc accumulation in the clusters (Fig. 3C). However, extra SMCs did appear within the clusters, since the *neu-lacZ* SMC-specific marker often revealed several neighbouring *neu-lacZ*-positive cells (Fig. 3F,G) and several macrochaetae were generated near the extant ones (Fig. 4E). This again indicated that EGFR signalling promotes SMC determination.

When the expression of *UAS-ras1^{V12}* was restricted to clones of cells, it was clear that *sc* activation was cell-autonomous, although the levels of expression varied from site to site within the disc (Fig. 4B). At the central part of the prospective notum and pleura (not shown) activation was maximal, while it could not be detected in areas like the proximal-most notum. Moreover, the accumulation of Sc also varied among the cells of a clone, and many of the ones with the highest levels were probably SMCs, since they expressed the *SRV-lacZ* SMC-specific marker (Fig. 4C,D). Moreover, flies in which few clones of *UAS-ras1^{V12}*-expressing cells were induced (2 versus 6 minute induction) survived to adulthood and showed clusters of adjacent macrochaetae (Fig. 4F,G). Taken together, these results indicate that reception of the Ras-mediated signal can induce accumulation of Ac-Sc and SMC commitment. As this can occur in adjacent cells (Figs 3F,G, 4C,F,G) excess Ras signalling appears to overrule lateral inhibition promoted by N signalling (Heitzler and Simpson, 1991; Simpson, 1990; Simpson, 1997).

The Ras/Raf signalling cassette is downstream of several receptor tyrosine kinases (Perrimon, 1994). Hence, we verified that the *sc* activation observed by overexpressing Ras1^{V12} could also be accomplished by activating EGFR itself. Expression in cell clones of the soluble form of Spitz, an activating ligand of EGFR (Golembo et al., 1996; Schweitzer et al., 1995b), promoted ectopic expression of *sc* in cells both within and outside of the clones (Fig. 4A), as expected of a diffusible ligand. The effect was maximal within and near the soluble Spitz-producing cells and, again, in the more central regions of the prospective notum.

rho/ve is activated in proneural clusters

The transmembrane protein Rhomboid/veinlet (Rho/ve) is known to activate EGFR signalling (reviewed by Wasserman and Freeman, 1997) by presenting and helping solubilize the ubiquitous, membrane-bound form of Spitz (Bang and Kintner, 2000). In the wing imaginal disc, *rho/ve* is strongly expressed in the presumptive wing veins, the wing margin, part of the dorsal radius and the nascent trachea (Sturtevant et al., 1993), but expression at the prospective notum has not been appropriately described. Although weak, *rho/ve* mRNA accumulation was detected in most proneural clusters, the DC showing the highest levels (Fig. 5A). Expression was not detectable in all the cells constituting each proneural cluster and the clusters in which expression occurred varied from disc to disc. This is compatible with a dynamic and short-lived expression. *rho/ve* transcription was dependent on *ac-sc*, as it was undetectable in *In(1)sc$^{10.1}$* discs, which lack Ac and Sc proneural proteins (Fig. 5B). This suggests that the proneural proteins activate *rho/ve* in proneural clusters and this should help promote EGFR signalling within at least part of their cells. Note however, that a low level of EGFR activation must also occur in a rather generalized way in the prospective notum, since cell proliferation is impaired in the complete absence of EGFR activity (Clifford and Schüpbach, 1989; Simcox et al., 1996).

We attempted to monitor in wild-type nota the activity of the EGFR pathway by examining the accumulation of the doubly phosphorylated mitogen-activated protein (MAP) kinase (dp-ERK; Gabay et al., 1997). However, the low levels of dp-ERK in the presumptive notum precluded consistent detection with currently available antibodies.

Antagonistic activities of the EGFR and N signalling pathways

The previous results indicate that EGFR signalling occurs among the cells of proneural clusters and that it promotes SMC emergence. In contrast, Dl-N signalling among cells of a

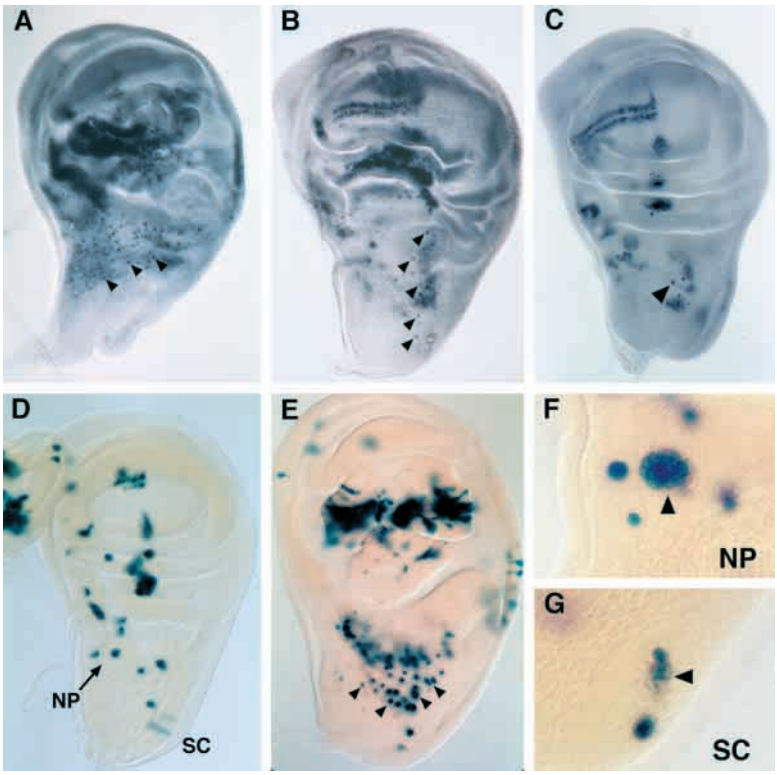

Fig. 3. Expression of a constitutively activated form of Ras promotes *sc* expression and emergence of SMCs. (A-C) Sc accumulation in late third instar wing discs that express *UAS-ras1^{V12}* under the control of *ap-Gal4* (A), *dpp^{disk}Gal4* (B) or *C253-Gal4* (C). Arrowheads indicate some of the cells that ectopically accumulate Sc in prospective nota. Cell overproliferation induced by activated Ras (Karim and Rubin, 1998) distorted the wing pouch and hinge regions of the discs in A and B. (D,E) Discs carrying the *SRV-lacZ* transgene in an otherwise wild-type (D) or *ap-Gal4/UAS-ras1^{V12}* (E) genetic background, respectively. Note the large number of ectopic SMCs induced by Ras1^{V12} (arrowheads). (F,G) High magnification views of the notopleural (F) and scutellar (G) regions, of a disc that has accumulated Ras1^{V12} in proneural clusters (*C253-Gal4/UAS-ras1^{V12}*). Note the ectopic expression of the SMC-specific marker A101.IF3 *neu-lacZ* (arrowheads; compare with Fig. 1D).

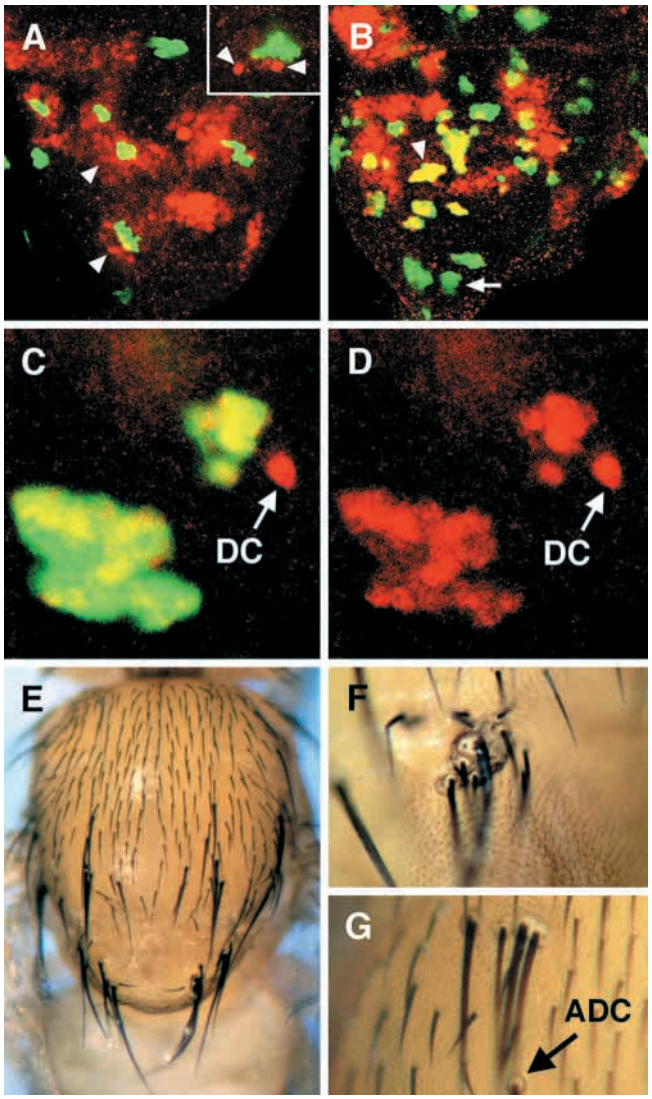

Fig. 4. Reception of Ras signalling promotes *sc* transcription and the acquisition of the SMC fate. (A) Clones of cells (green, *UAS-GFP* marker) overexpressing *UAS-sSpitz* in the presumptive notum can ectopically activate *sc* (red) in expressing and surrounding cells (arrowheads). Inset, *UAS-sSpitz*-expressing clone located in the posterior compartment of the wing pouch that induced strong expression of Sc and presumably the SMC fate (arrowheads) in neighbouring cells. (B) Notum clones (green, *UAS-GFP* marker) that overexpress *UAS-ras1*^{V12} autonomously induce ectopic *sc* expression (red) most effectively near the dorsocentral region (arrowhead) and poorly or not at all in regions like the dorsal-most notum (arrow). (C,D) Many cells of similar clones (green, shown at higher magnification) near the dorsocentral region accumulated β-galactosidase due to activation of the *SRV-lacZ* transgene (red channel, shown separately in D). (E) Notum of a fly in which *UAS-ras1*^{V12} was expressed in proneural clusters, as indicated in Fig. 3C, displays ectopic macrochaetae near extant ones (compare with Fig. 1G). (F,G) Clusters of bristles on the nota of flies with *UAS-ras1*^{V12}-expressing clones in the scutellum (F) and near the dorsocentral area (G).

cluster antagonizes SMC emergence (Artavanis-Tsakonas et al., 1995; review). This is accomplished by the activation of the bHLH genes of the E(spl)-C, which inhibit proneural gene expression directed by SMC-specific enhancers (Culí and Modolell, 1998). We have explored the presence of interactions between these pathways by manipulating one of them and monitoring the activity of the other. Depression of N signalling by using a N^{ts} allele at a non-permissive temperature (Shellenbarger and Mohler, 1978) strongly enhanced the accumulation of *rho/ve* mRNA in proneural clusters (Fig. 5C). A large part of this accumulation may be due to the enhanced levels of proneural protein in the extra SMCs that arise within proneural clusters under depleted N signalling (Fig. 5D,E). A self-stimulatory loop of the EGFR pathway, as shown in other systems (Martín-Blanco et al., 1999; Wasserman and Freeman, 1998), may also contribute to the increased expression of *rho/ve*. In any case, these data suggest that the reduction of N activity enhances EGFR signalling.

In a reciprocal experiment, we found that a large decrease in EGFR activity (*C253-Gal4/UAS-aos*) did not significantly modify the levels of *E(spl)-m8* mRNA in proneural clusters (not shown). This suggests that the EGFR pathway does not affect N signalling. We also found that under conditions of sharply reduced N signalling (overexpression of a dominant negative form of the ligand Dl in proneural clusters), EGFR signalling was still required for macrochaetae development, since in this genetic background the overexpression of *UAS-aos* eliminated these sensory organs (Fig. 5F,G). This is consistent with the N and EGFR pathways acting antagonistically and in parallel on the SMC-specific enhancers (see Discussion).

DISCUSSION

The development of the *Drosophila* mesothoracic macrochaetae requires the activity of the EGFR pathway. We have analyzed this requirement and found that EGFR signalling is involved in at least three stages of the development of these sensory organs, namely, formation of proneural clusters, emergence of SMCs from these clusters, and SO differentiation. This last aspect is suggested by the 'sockets without bristle' phenotype observed in flies in which EGFR activity is impaired by the expression of the inhibitory ligand Aos or the dominant negative form of Raf. We have not further studied this role of EGFR.

Proneural clusters show different requirements for EGFR signalling

The earliest stage in macrochaetae development is the formation of the proneural clusters of *ac-sc* expression. We find that accumulation of Sc in cells of proneural clusters located at the more central positions of the wing disc decreases upon reduction of the level of EGFR signalling. The effect is cell-autonomous, which indicates that reception of the signal is important for cells to express *sc* properly. In contrast, more marginally located clusters, like the notopleural or scutellar, were unmodified or slightly enhanced under conditions of insufficient EGFR signalling. It is known that expression of *ac-sc* in different proneural clusters depends on separate, functionally independent enhancers which are thought to respond to local, specific combinations of transcription factors (prepattern) (Gómez-Skarmeta et al., 1995). The different, spatially restricted effects of the insufficiency of EGFR function may thus be due to interference in the deployment or

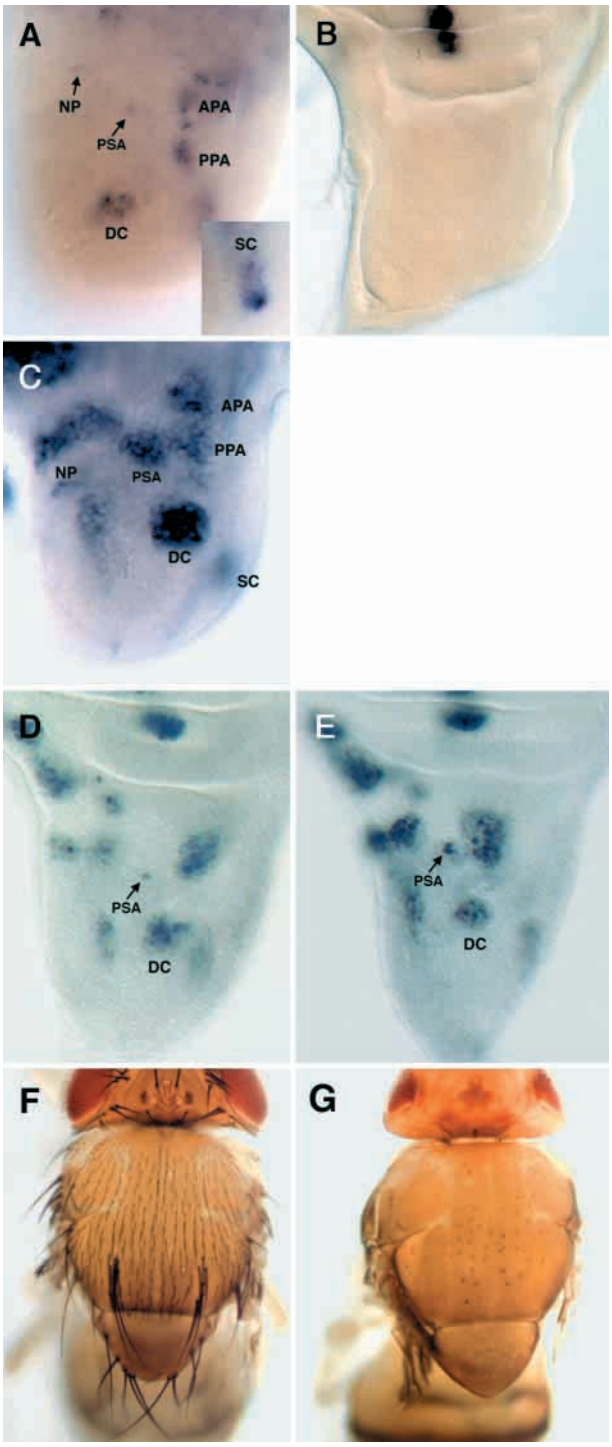

Fig. 5. *rho/ve* expression occurs in proneural clusters and is repressed by N signalling. (A-C) Late third instar wing discs were hybridized with a DIG-labeled *rho/ve* probe. Prospective nota are shown. (A) *rho/ve* mRNA is detectable in proneural clusters. Inset shows scutellar cluster. Abbreviations as in Fig. 1A. (B) *rho/ve* is not expressed in the notum region of discs that lack proneural clusters (*In(1)sc*$^{10.1}$). (C) *rho/ve* expression is strongly increased in proneural clusters of discs of *N*^{ts} larvae incubated at 30°C for 12 hours. (D,E) Presumptive notum of a wild-type (D) and a *N*^{ts} (E) larva incubated at 30°C for 12 hours, stained with anti-Sc antibody. (F,G) Inhibition of N signalling (*C253Gal4/UAS-Dl*^{DN}) promotes development of many macrochaetae from proneural clusters (F, compare with Fig. 1G), but in this genetic background, *UAS-aos* still blocked macrochaetae development (G).

function of particular factors expressed in the affected area. Interestingly, the expression of the homeobox genes of the *iroquois* complex, necessary for the expression of *ac-sc* in many notum proneural clusters (Leyns et al., 1996), is especially sensitive to the expression of the Vein EGFR ligand in the central region of the notum (Wang et al., 2000). Alternatively, since EGFR function is a well known requisite for growth and patterning of imaginal discs (Clifford and Schüpbach, 1989; Díaz-Benjumea and García-Bellido, 1990; Díaz-Benjumea and Hafen, 1994; Nagaraj et al., 1999; Simcox et al., 1996; Sturtevant et al., 1993; Wang et al., 2000), the reduced expression of *sc* may be due to a more general impairment of the patterning of the central area of the disc.

EGFR activity is necessary for SMC emergence

Our data support a key role for EGFR signalling in the emergence of the notum macrochaetae SMCs from proneural clusters. Indeed, expression of the EGFR inhibitory ligand Aos exclusively in proneural clusters, a condition that permits essentially wild-type Sc accumulation in these clusters, almost completely suppressed the appearance of SMCs and SOs. SMC emergence was also impaired in discs from heat-treated *Egfr*^{tsla}/*Egfr*^{CO} larvae and in clones of cells expressing *UAS-raf*$^{DN2.1}$. Moreover, when the cells that accumulated Raf$^{DN2.1}$ occupied positions where SMCs normally appear, wild-type neighbouring cells could give rise to displaced SMCs. This phenomenon is reminiscent of and in accordance with the observation, made with mosaic individuals, that when the position of a dorsocentral bristle is in *ac*$^{-}$ territory, this bristle does not develop, but a nearby *ac*$^{+}$ cell can give rise to a dorsocentral bristle displaced from its wild-type position (Stern, 1954). The cell-autonomous effect of Raf$^{DN2.1}$ indicates that reception of the EGFR signal, mediated by the Ras/Raf/MAP kinase cassette, is essential for notum macrochaetae SMC determination. This was further substantiated by the cell autonomous induction of SMCs and bristles in clones of cells overexpressing a constitutively activated form of Ras. Taken together, these results indicate that reception of the EGFR signal promotes *sc* expression and SMC determination.

We found that in the notum anlagen the expression of *rho/ve* occurred mainly in proneural clusters and that this expression was dependent on *ac-sc*. Rho/ve facilitates the processing of Spitz, an activating ligand of EGFR (Bang and Kintner, 2000). We also found that the soluble, active form of Spitz promoted ectopic *sc* expression and SMC emergence. Hence, these data suggest that, in proneural clusters, Ac-Sc promote expression of *rho/ve*, which by activating Spitz, would stimulate EGFR signalling in the cells of the cluster (Fig. 6). (The Vein EGFR ligand probably does not specifically act in proneural clusters, as many of these lie outside of its expression domain; Simcox et al., 1996; F. Cavodeassi, personal communication.) We thus propose that EGFR mediates a mutual positive signalling among cells of the proneural cluster, which promotes SMC emergence by probably reinforcing *ac-sc* expression. We call this positive signalling lateral cooperation. Evidently, this does

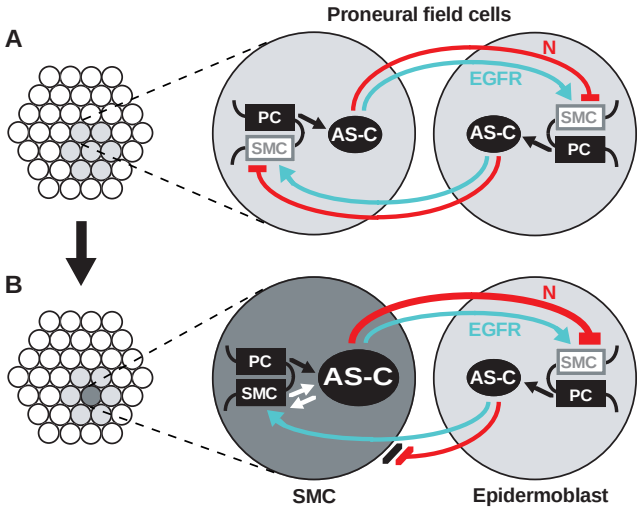

Fig. 6. Model of EGFR- and N-mediated interactions among cells of a proneural field. (A) Before SMC emergence, the expression of *ac* and *sc* in proneural clusters (at left) is driven by enhancers ('PC' boxes in magnified 'cells') that respond to combinations of factors heterogeneously distributed in the imaginal epithelium. This expression is not homogeneous and cells that accumulate more proneural protein (grey) constitute the proneural field (Cubas and Modolell, 1992). The AS-C proteins activate, by a mechanism that could be mediated by Rho/Ve and Spitz, the EGFR pathway in neighbouring cells (blue arrows) and possibly, in an autocrine way, in the same cell. This pathway tends to turn on the SMC-specific enhancers (grey boxes). However, this is prevented by the activation of the Dl/N pathway by the same AS-C products. N signalling, by means of the E(spl) bHLH proteins, blocks functioning of the SMC enhancers (red lines; Artavanis-Tsakonas et al., 1995; Simpson, 1997; reviews; Culí and Modolell, 1998). Activation of the Dl/N pathway may also help repress *rho/ve* expression and consequently down-regulate EGFR signalling. The N inhibitory interactions dominate over the EGFR SMC-promoting activity and cells are kept in an uncommited state. However, the cells with higher levels of AS-C proteins signal via Dl more strongly and, at the same time, they become more resistant to the signals from neighbouring cells (Simpson, 1997). Eventually (B), one cell will be sufficiently resistant to the inhibition (black bar), so that the positive signals it receives via EGFR will be able to promote the functioning of the SMC enhancers. These permit the self-stimulation of proneural genes (black SMC box), the amount of proneural protein increases sharply (dark grey), and the cell becomes an SMC. The SMC signals via Dl very strongly to their neighbours and these are effectively blocked from becoming SMCs (epidermoblast).

not exclude an autocrine activation of the EGFR pathway in the cells that express AS-C proteins, but we favor the lateral cooperation hypothesis since it is well established in other systems that the EGFR pathway is used mainly for intercellular communication (Freeman, 1998; Schweitzer and Shilo, 1997; reviews). As discussed below, this signalling should facilitate the acquisition of the SMC state by one or a few cells of a proneural cluster.

The SMC state is associated with greatly increased levels of proneural protein (Brand et al., 1993; Cubas et al., 1991; Culí and Modolell, 1998; Domínguez and Campuzano, 1993; Jarman et al., 1993; Skeath and Carroll, 1991). These are accomplished by the self-stimulation of *ac*, *sc* and *ase* mediated by AS-C enhancers that activate these genes specifically in the cells that become SMCs (Culí and Modolell, 1998). As we have shown that $Ras1^{V12}$ elicits the expression of both *sc* and *SRV-lacZ*, we propose that, in the extant proneural clusters, the SMC-specific enhancers are targets of EGFR signalling. Unidentified effector(s) of the EGFR/Ras pathway should facilitate the self-stimulation of the proneural genes mediated by the SMC-specific enhancers by, possibly, binding to these enhancers. Conclusive evidence in support of this role requires the identification of the signalling effector(s) and of their interaction with the enhancer. Interestingly, overexpression of the effector Pointed P1 promotes development of many extra macrochaetae on the notum (J. Culí, unpublished) and we have detected putative Ets-domain binding sites in the *sc* and *ase* SMC enhancers (GTGGAAAT and ACGGAAAC, respectively, Culí and Modolell, 1998).

Antagonism of EGFR and N signalling in SMC determination

EGFR-mediated lateral cooperation should tend to activate the SMC-specific enhancers in many cells of the proneural clusters (Fig. 6). This, however, is prevented by N signalling, which is activated by Ac and Sc in the cells of the cluster (Simpson, 1997, review). This signalling, by means of the bHLH proteins of the E(spl)-C (Artavanis-Tsakonas et al., 1995; review), blocks the *ac-sc-ase* self-stimulatory loop promoted by the SMC-specific enhancers (Culí and Modolell, 1998) (Fig. 6A). However, within a proneural cluster the cells of the proneural field accumulate greater amounts of Ac-Sc proteins (Cubas et al., 1991; Skeath and Carroll, 1991). As it has been hypothesized that cells that signal the most are the least inhibited by their neighbours, eventually, a cell of the proneural field will be released from the inhibitory loop and its levels of E(spl)-C bHLH protein will become minimal (Jennings et al., 1995). This cell will turn on the *ac-sc-ase* self-stimulation and become an SMC (Fig. 6B). The SMC signals maximally to its neighbours and prevents them from following the same fate (lateral inhibition).

Our results add to this scenario the requirement for EGFR-mediated signalling for one cell of the proneural field to turn on the *ac-sc-ase* self-stimulatory loops and become an SMC (Fig. 6). According to this model, Ac-Sc activate both the N- and EGFR-mediated signalling pathways, with their SMC-suppressing and SMC-promoting abilities, respectively, and both signalling systems appear to act on the same SMC-specific enhancers. Since an excess signalling by the N or the EGFR pathway will either prevent SMC determination or promote emergence of ectopic SMCs, the respective levels of signalling should balance each other so that only one SMC is determined at a time from each proneural cluster. How is this balance accomplished? This is at present unclear. The large enhancement of *rho/ve* mRNA in proneural clusters under conditions of insufficient N signalling suggests that this pathway may prevent the Rho/Ve-promoted activation of EGFR from rising to excessively high levels. In contrast, the insensitivity of the levels of E(spl)-m8 protein to the overexpression of *UAS-aos* in proneural clusters suggests that the EGFR pathway does not affect N signalling. Antagonistic interactions between the N and the EGFR pathways are found in other developing systems, as in the wing preveins (de Celis et al., 1997) and in the reiterative recruitment, from a long-

lived *atonal* proneural cluster, of the precursors of the 70-80 scolopidia of the femoral chordotonal organs (zur Lange and Jarman, 1999). In this later case, EGFR signalling promotes commitment of neural precursors and the Dl-N interaction prevents too many cells from being committed.

Only a subset of bristles requires EGFR signalling to develop?

In the presumptive notum, the inability of available antibodies to reliably detect dp-ERK and, in proneural clusters, the low levels of *rho/ve* mRNA (compared to those in the wing preveins; Sturtevant et al., 1993; Gómez-Skarmeta et al., 1996) suggest that low levels of EGFR activity are sufficient to ensure the emergence of the macrochaetae precursor cells. This may explain the failure of the *Egfr* hypomorphic alleles compatible with cell or adult viability to completely eliminate notum macrochaetae (Clifford and Schüpbach, 1989; Díaz-Benjumea and García-Bellido, 1990; and this paper). The notum microchaetae appear to be even more resistant to the lowering of EGFR signalling. Perhaps, they do not directly require it for development, similarly to the terguite bristles that can arise within *Egfr* amorphic clones (Díaz-Benjumea and García-Bellido, 1990). An essential difference between notum macrochaetae, on the one hand, and notum microchaetae and terguite bristles, on the other, is that the first appear in fixed positions while the others do not do so, being instead organized in density patterns. We speculate that EGFR signalling among the cells of the proneural field may make the selection of the SMC less ambiguous and, therefore, spatially more precise. A cell centrally located within this subset would receive the strongest signalling from their neighbours and would become a SMC in preference to more marginally located neighbours (Fig. 6). The observation that slight reduction in the level of EGFR signalling causes duplications of some notum macrochaetae (Clifford and Schüpbach, 1989; Díaz-Benjumea and García-Bellido, 1990; and Table 1), that is, it makes the decision of which cell becomes an SMC less precise and it allows two SMCs to arise from presumably the same proneural cluster, may be consistent with this interpretation.

The overexpression of *UAS-aos* in proneural clusters removes essentially all bristles, including those of the tergites (not shown). This may indicate that all SOs require some level of EGFR signalling to develop. However, the fact that in the tergite clones homozygous for amorphic $Egfr^{-}$ alleles still develop bristles (Díaz-Benjumea and García-Bellido, 1990) suggests that the Aos overexpression may be interferring with additional tyrosine kinase receptors that would be redundant with EGFR in the development of these bristles.

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REFERENCES

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signalling. *Science* **268**, 225-232.

Bang, A. G. and Kintner, C. (2000). Rhomboid and Star facilitate presentation and processing of the *Drosophila* TGF-α homolog Spitz. *Genes Dev.* **14**, 177-186.

Bodmer, R., Carretto, R. and Jan, Y. N. (1989). Neurogenesis of the peripheral nervous system in *Drosophila* embryos: DNA replication patterns and cell lineages. *Neuron* **3**, 21-32.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brand, M., Jarman, A. P., Jan, L. Y. and Jan, Y. N. (1993). *asense* is a *Drosophila* neural precursor gene and is capable of initiating sense organ formation. *Development* **119**, 1-17.

Buff, E., Carmena, A., Gisselbrecht, S., Jiménez, F. and Michelson, A. M. (1998). Signalling by the epidermal growth factor receptor is required for the specification and diversification of embryonic muscle progenitors. *Development* **125**, 2075-2086.

Calleja, M., Moreno, E., Pelaz, S. and Morata, G. (1996). Visualization of gene expression in living adult *Drosophila*. *Science* **274**, 252-255.

Campuzano, S. and Modolell, J. (1992). Patterning of the *Drosophila* nervous system: the *achaete-scute* gene complex. *Trends Genet.* **8**, 202-207.

Clifford, R. J. and Schüpbach, T. (1989). Coordinately and differentially mutable activities of *torpedo*, the *Drosophila melanogaster* homolog of the vertebrate EGF receptor gene. *Genetics* **123**, 771-787.

Cubas, P., de Celis, J. F., Campuzano, S. and Modolell, J. (1991). Proneural clusters of *achaete-scute* expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. *Genes Dev.* **5**, 996-1008.

Cubas, P. and Modolell, J. (1992). The *extramacrochaetae* gene provides information for sensory organ patterning. *EMBO J.* **11**, 3385-3393.

Culí, J. and Modolell, J. (1998). Proneural gene self-stimulation in neural precursors: an essential mechanism for sense organ development that is regulated by *Notch* signalling. *Genes Dev.* **12**, 2036-2047.

de Celis, J. F., Bray, S. and García-Bellido, A. (1997). Notch signalling regulates *veinlet* expression and establishes boundaries between veins and interveins in the *Drosophila* wing. *Development* **124**, 1919-1928.

Díaz-Benjumea, F. J. and García-Bellido, A. (1990). Behaviour of cells mutant for an EGF receptor homologue of *Drosophila* in genetic mosaics. *Proc. R. Soc. London* **242**, 36-44.

Díaz-Benjumea, F. J. and Hafen, E. (1994). The sevenless signalling cassette mediates *Drosophila* EGF receptor function during epidermal development. *Development* **120**, 569-578.

Domínguez, M. and Campuzano, S. (1993). *asense*, a member of the *Drosophila achaete-scute* complex, is a proneural and neural differentiation gene. *EMBO J.* **12**, 2049-2060.

Freeman, M. (1994). Misexpression of the *Drosophila* argos gene, a secreted regulator of cell determination. *Development* **120**, 2297-2304.

Freeman, M. (1998). Complexity of EGF receptor signalling revealed in *Drosophila*. *Curr. Opin. Genet. Dev.* **8**, 407-411.

Gabay, L., Seger, R. and Shilo, B. Z. (1997). In situ activation pattern of *Drosophila* EGF receptor pathway during development. *Science* **277**, 1103-1106.

Garrell, J. and Campuzano, S. (1991). The helix-loop-helix domain: a common motif for bristles, muscles and sex. *BioEssays* **13**, 493-498.

Golembo, M., Raz, E. and Shilo, B. Z. (1996). The *Drosophila* embryonic midline is the site of Spitz processing, and induces activation of the EGF receptor in the ventral ectoderm. *Development* **122**, 3363-3370.

Gómez-Skarmeta, J. L., Diez del Corral, R., de la Calle-Mustienes, E., Ferrés-Marcó, D. and Modolell, J. (1996). *araucan* and *caupolican*, two members of the novel Iroquois complex, encode homeoproteins that control proneural and vein forming genes. *Cell* **85**, 95-105.

Gómez-Skarmeta, J. L., Rodríguez, I., Martínez, C., Culí, J., Ferrés-Marcó, M. D., Beamonte, D. and Modolell, J. (1995). Cis-regulation of *achaete* and *scute*: shared enhancer-like elements drive their coexpression in proneural clusters of the imaginal discs. *Genes Dev.* **9**, 1869-1882.

González-Crespo, S. and Levine, M. (1993). Interactions between *dorsal* and helix-loop-helix proteins initiate the differentiation of the embryonic mesoderm and neuroectoderm in *Drosophila*. *Genes Dev.* **7**, 1703-1713.

Hartenstein, V. and Posakony, J. W. (1989). Development of adult sensilla on the wing and notum of *Drosophila melanogaster*. *Development* **107**, 389-405.

Heitzler, P., Haenlin, M., Ramain, P., Calleja, M. and Simpson, P. (1996).

A genetic analysis of *pannier*, a gene necessary for viability of dorsal tissues and bristle postioning in *Drosophila*. *Genetics* **143**, 1271-1287.

Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of Drosophila. *Cell* **64**, 1083-1092.

Huang, F., Dambly-Chaudière, C. and Ghysen, A. (1991). The emergence of sense organs in the wing disc of *Drosophila*. *Development* **111**, 1087-1095.

Ito, K., Awano, W., Suzuki, K., Hiromi, Y. and Yamamoto, D. (1997). The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development* **124**, 761-771.

Jan, Y. N. and Jan, L. Y. (1993). HLH proteins, fly neurogenesis, and vertebrate myogenesis. *Cell* **75**, 827-830.

Jarman, A. P., Brand, M., Jan, L. Y. and Jan, Y. N. (1993). The regulation and function of the helix-loop-helix gene, *asense*, in *Drosophila* neural precursors. *Development* **119**, 19-29.

Jennings, B., de Celis, J., Delidakis, C., Preiss, A. and Bray, S. (1995). Role of Notch and achaete-scute complex in the expression of Enhancer of split bHLH proteins. *Development* **121**, 3745-3752.

Karim, F. D. and Rubin, G. M. (1998). Ectopic expression of activated Ras 1 induces hyperplastic growth and increased cell death in *Drosophila* imaginal tissues. *Development* **125**, 1-9.

Kumar, J. P., Tio, M., Hsiung, F., Akopyan, S., Gabay, L., Seger, R., Shilo, B. Z. and Moses, K. (1998). Dissecting the roles of the *Drosophila* EGF receptor in eye development and MAP kinase activation. *Development* **125**, 3875-3885.

Leyns, L., Gómez-Skarmeta, J. L. and Dambly-Chaudière, C. (1996). *iroquois*: a prepattern gene that controls the formation of bristles on the thorax of *Drosophila*. *Mech. Dev.* **59**, 63-72.

Li, W. and Perrimon, N. (1997). Specificity of receptor tyrosine kinase signalling pathways: lessons from *Drosophila*. *Genet. Eng.* **19**, 167-182.

Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of* Drosophila melanogaster. San Diego: Academic Press.

Martín-Blanco, E., Roch, F., E., N., Baonza, A., Duffy, J. B. and Perrimon, N. (1999). A temporal switch in DER signalling controls the specification and differentiation of veins and interveins in the *Drosophila* wing. *Development* **126**, 5739-5747.

Mlodzik, M., Baker, N. E. and Rubin, G. M. (1990). Isolation and expression of *scabrous*, a gene regulating neurogenesis in *Drosophila*. *Genes Dev.* **4**, 1848-1861.

Moghal, N. and Sternberg, P. W. (1999). Multiple positive and negative regulators of signalling by the EGF-receptor. *Curr. Opin. Cell Biol.* **11**, 190-196.

Nagaraj, R., Pickup, A. T., Howes, R., Moses, K., Freeman, M. and Banerjee, U. (1999). Role of the EGF receptor pathway in growth and patterning of the *Drosophila* wing through the regulation of *vestigial*. *Development* **126**, 975-985.

Nakao, K. and Campos-Ortega, J. A. (1996). Persistent expression of genes of the *Enhancer of Split* complex suppresses neural development in *Drosophila*. *Neuron* **16**, 275-286.

Nolo, R., Abbott, L. A. and Bellen, H. J. (2000). Sens, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. *Cell* **102**, 349-362.

Perrimon, N. (1994). Signalling pathways initiated by receptor protein tyrosine kinases in *Drosophila*. *Curr. Opin. Cell Biol.* **6**, 260-266.

Schweitzer, R., Howes, R., Smith, R., Shilo, B. Z. and Freeman, M. (1995a). Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos. *Nature* **376**, 699-702.

Schweitzer, R., Shaharabany, M., Seger, R. and Shilo, B. Z. (1995b). Secreted spitz triggers the DER signalling pathway and is a limiting component in embyonic ventral ectoderm determination. *Genes Dev.* **9**, 1518-1529.

Schweitzer, R. and Shilo, B. Z. (1997). A thousand and one roles for the *Drosophila* EGF receptor. *Trends Genet.* **13**, 191-196.

Shellenbarger, D. L. and Mohler, J. D. (1978). Temperature-sensitive periods and autonomy of pleiotropic effects of *l(1)N^{ts1}*, a conditional *Notch* lethal in *Drosophila*. *Dev. Biol.* **62**, 432-446.

Simcox, A. A., Grumbling, G., Schnepp, B., Bennington-Mathias, C., Hersperger, E. and Shearn, A. (1996). Molecular, phenotypic, and expression analysis of vein, a gene required for growth of the *Drosophila* wing disc. *Dev. Biol.* **177**, 475-489.

Simpson, P. (1990). Lateral inhibition and the development of the sensory bristles of the adult peripheral nervous system of *Drosophila*. *Development* **109**, 509-519.

Simpson, P. (1997). Notch signalling in development: on equivalence groups and asymmetric developmental potential. *Curr. Opin. Genet. Dev.* **7**, 537-542.

Skeath, J. B. and Carroll, S. B. (1991). Regulation of *achaete-scute* gene expression and sensory organ pattern formation in the *Drosophila* wing. *Genes Dev.* **5**, 984-995.

Staehling-Hampton, K., Jackson, P. D., Clark, M. J., Brand, A. H. and Hoffmann, F. M. (1994). Specificity of bone morphogenetic protein related factors: cell fate and gene expression changes in *Drosophila* embryos by *decapentaplegic* but not *60A*. *Cell Growth Differ.* **5**, 585-593.

Stern, C. (1954). Two or three bristles. *Am. Sci.* **42**, 213-247.

Sturtevant, M. A., Roark, M. and Bier, E. (1993). The *Drosophila rhomboid* gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signalling pathway. *Genes Dev.* **7**, 961-973.

Wang, S. H., Simcox, A. and Campbell, G. (2000). Dual role for epidermal growth factor receptor signaling in early wing disc development. *Genes Dev.* **14**, 2271-2276.

Wasserman, J. D. and Freeman, M. (1997). Control of EGF receptor activation in *Drosophila*. *Trends Cell Biol.* **7**, 431-436.

Wasserman, J. D. and Freeman, M. (1998). An autoregulatory cascade of EGF receptor signalling patterns the *Drosophila* egg. *Cell* **95**, 355-364.

zur Lange, P. and Jarman, A. P. (1999). Antagonism of EGFR and Notch signalling in the reiterative recruitment of *Drosophila* chordotonal sense organ precursors. *Development* **126**, 3149-3157.