Sprouty is a general inhibitor of receptor tyrosine kinase signaling

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SUMMARY

Sprouty was originally identified as an inhibitor of *Drosophila* FGF receptor signaling during tracheal development. By following the capacity of ectopic Sprouty to abolish the pattern of activated MAP kinase in embryos, we show that Sprouty can inhibit other receptor tyrosine kinase (RTK) signaling pathways, namely the Heartless FGF receptor and the EGF receptor. Similarly, in wing imaginal discs, ectopic Sprouty abolishes activated MAP kinase induced by the EGF receptor pathway. Sprouty expression is induced by the EGFR pathway in some, but not all, tissues in which EGFR is activated, most notably in follicle cells of the ovary, the wing imaginal disc and the eye disc. In the ovary, induction of *sprouty* expression follows the pattern of EGFR activation in the follicle cells. Generation of homozygous *sprouty* mutant follicle-cell clones demonstrates an essential role for Sprouty in restricting EGFR activation throughout oogenesis. At the stage when dorso-ventral polarity of the follicle cells is established, Sprouty limits the ventral expansion of the activating Gurken signal. Later, when dorsal appendage fates are determined, reduction of signaling by Sprouty facilitates the induction of inter-appendage cell fates. The capacity of Sprouty to reduce or eliminate accumulation of activated MAP kinase indicates that in vivo it intersects with the pathway upstream to MAP kinase. The ability of ectopic Sprouty to rescue lethality caused by activated Raf suggests that it may impinge upon the pathway by interacting with Raf or downstream to it.

Key words: Sprouty, EGFR, Breathless, Receptor tyrosine kinase, MAP kinase, *Drosophila melanogaster*, Oogenesis

INTRODUCTION

Growth and differentiation during embryonic and post-embryonic development are governed by highly regulated signals that are present in defined positions in space and time. In many cases, such signals pattern a field of cells, providing distinct levels of stimulation to different cells. Such signals cannot be controlled solely by the precise presentation of the signal for two cardinal reasons. First, the absolute levels of the signal, or those of the responding components, are subjected to fluctuations, as can be expected for any biological system. In the absence of additional regulatory mechanisms, such fluctuations may render the system incapable of producing reproducible patterns. Second, several signaling pathways induce an enzymatic activity at the receptor level or downstream to it. Receptor tyrosine kinases and the cytoplasmic cascade of kinases they trigger, or TGFβ serine/threonine kinase receptors are examples. In these cases, the time window of signaling should be tightly regulated, since sustained low levels of enzymatic activity triggered by corresponding amounts of activating ligands, may be additive and lead to the same consequences as high levels of signaling.

Regulation of morphogenetic pathways requires, in addition to the inducing signals, a parallel set of inhibitory mechanisms, in order to achieve an accurate and reproducible response. A variety of inhibitory modules have been uncovered in recent years. One class can be regarded as constitutive inhibitors. These raise the threshold for signaling, and allow the system to respond only to higher levels of induction. Such mechanisms include the inhibitory ETS-domain protein Yan, which competes with other ETS-domain proteins (Lai and Rubin, 1992), the inhibitory protein Groucho, which raises the threshold required for transcriptional activation by the Torso or Wnt pathways (Paroush et al., 1997; Cavallo et al., 1998), or the POU-domain protein Nubbin, which raises the threshold for Notch signaling in the wing disc (Neumann and Cohen, 1998). Other inhibitors are not uniformly distributed, thus they not only reduce, but actually alter the pattern of response to the activating morphogen. For example, in *Xenopus* embryos BMP4 and its inhibitors (Chordin, Noggin and Follistatin) are expressed on opposite poles of the embryo (Sasai et al., 1995; Zimmerman et al., 1996; Hemmati-Brivanlou et al., 1994). Expression of these inhibitors is not affected by the pathway which they repress.

A more elaborate type of inhibition represents a direct response to the activating pathway. These inhibitors share the common feature of being transcriptionally induced by the pathway they eventually inhibit. The utility of such a mechanism is the capacity to respond to varying levels of stimuli. Such negative feedback mechanisms may adjust the final response to a desired level, irrespective of variations in the original signal, thus generating a robust response...
mechanism. Inducible inhibitors include the inhibitory SMAD proteins, which attenuate the response to TGFβ signaling (Nakao et al., 1997; Tsuneizumi et al., 1997). The phosphatases Erp and Puckered were shown to be induced by serum response and the JUNK pathway, respectively (Noguchi et al., 1993; Martin-Blanco et al., 1998). The induction of patched expression was shown to be a crucial negative feedback response to the stimulation by Hedgehog (Hh), thus acting as a sink for the ligand and limiting the domains influenced by Hh signaling (Chen and Struhl, 1996).

Two inducible inhibitors were identified for the EGF receptor pathway in Drosophila. Argos, a secreted protein containing an EGF-like domain (Freeman et al., 1992), inhibits stimulation of the receptor by the activating ligands Spitz and Gurken (Schweitzer et al., 1995a; Wasserman and Freeman, 1998). Argos has a high threshold for induction by the EGF receptor pathway, and is induced only in cells where maximal activation takes place (Golembo et al., 1996; Queenan et al., 1997). In some contexts Argos exerts the inhibitory effect over a long range (Golembo et al., 1996; Freeman et al., 1992), while in others the effect is more confined to the site of synthesis (Wasserman and Freeman, 1998). Another inducible inhibitor of the same pathway is Kekkon, a membrane-spanning protein that is capable of interacting with the EGF receptor and blocking its activity (Ghiglione et al., 1999).

Finally, Sprouty was identified as an inhibitor of the Breathless FGF receptor in Drosophila (Hacohen et al., 1998). Sprouty is a novel protein with multiple cysteine residues, and has several homologues in vertebrates (de Maximy et al., 1999; Tefft et al., 1999). Sprouty expression is induced in the cells receiving maximal activation of the FGF receptor by the activating ligand Branchless. These cells assume a terminal cell fate whereas adjacent cells are inhibited from doing so.

Several observations indicate that Sprouty may not be a restricted inhibitor of the FGF receptor pathway. It was shown that during eye development the Ras pathway, triggered by the EGF receptor, inhibits apoptosis by transcriptional and post-translational modification of the Head involution defective (HID) protein. In a screen for modifiers of HID overexpression in the eye, Gap1 mutations were isolated. Interestingly, the sprouty locus was also identified, suggesting that during eye development, Sprouty may normally inhibit the EGF receptor pathway (Bergmann et al., 1998).

In this work, the capacity of Sprouty to inhibit the EGF receptor pathway was examined. We show that in several (but not all) tissues where EGF receptor is required, such as the wing imaginal disc and the follicle cells of the ovary, sprouty expression is induced by the pathway. Overexpression of Sprouty in these tissues gives rise to phenotypes resembling a reduction or elimination of EGF receptor activity. Absence of normal Sprouty activity in the ovary results in hyperactivation of the EGF receptor pathway at several phases in egg chamber development. Sprouty intersects with the EGF receptor pathway upstream to MAP kinase, since overexpression of Sprouty leads to elimination of activated MAP kinase. Epistasis tests with activated elements along the Ras pathway...
demonstrate that Sprouty intersects with the pathway downstream to the receptor, and possibly downstream to, or at Raf. The capacity of Sprouty to impinge on the pathway at a stage shared by all receptor tyrosine kinases, explains how Sprouty can inhibit signaling by different RTKs, and highlights it as a general inhibitor of the pathway.

**MATERIALS AND METHODS**

**Fly strains**

The following Gal4 driver lines were used for misexpression: *mato4-Gal4-VP16* (obtained from D. St Johnston), *MS1096-Gal4*, *dpp-Gal4* (obtained from S. Cohen), *71B-Gal4*, *T155-Gal4* (obtained from T. Schüpbach) and *55B-Gal4* (obtained from A. Brand). The *UAS* lines used include *UAS-spry4.1* (on the 2nd chromosome, obtained from M. Krasnow), *UAS-λop* (obtained from T. Schüpbach), *UAS-λhil* (obtained from A. Michelson), *UAS-Raf activated* (obtained from N. Perrimon), *UAS-lacZ-GFP* (obtained from S. Hayashi) and *UAS-DN DER*, *UAS-spry*.* UAS-spry* were generated, and identified by PCR reactions on genomic DNA.

The following fly lines were used for analysis in mutant backgrounds or to follow *sprouty* gene expression: *LacZ52083* (obtained from M. Scott and M. Krasnow), *spry*Δ5 representing a null allele of the locus (obtained from M. Krasnow), *grkHK36*, *grkWG41*, and *fs(1)K10* (obtained from T. Schüpbach).

To generate *spry* clones in the ovary the FRT, *spry*Δ5 chromosome (obtained from M. Krasnow) was used, over the 80B FRT, [hs-neo] chromosome. In one set of experiments, the clones were induced by the *HS-flp* X chromosome. Clones were induced by incubation of third instar larvae at 38°C for 30 minutes, on 2 subsequent days. Alternatively, the *E22c-Gal4* line was used (2nd chromosome) to drive expression of (*UAS-flp1.D*)JD1 expression, and the generation of ectopic inter-appendage cell fates.

**Antibody staining**

Anti-βGal antibodies (Cappel) were used according to standard procedures. The following protocol was used for anti-dpERK staining. Antibodies were purchased from Sigma (M8159). Discs were dissected in 0.1 M NaPO4 (pH 7.2), and fixed for 45 minutes on ice in freshly prepared PLP (2% paraformaldehyde, 0.01 M NaIO4, 0.1 M NaPO4).

**Fig. 2.** Expression of *sprouty* in the ovary is induced by EGFR. (A) *LacZ52083* enhancer trap line in the *sprouty* locus is expressed in an identical pattern to EGFR-target genes in the ovary such as kekkon. Expression begins at stage 7 in the posterior follicle cells (arrow). It continues broadly during stage 9 when the follicle cells migrate posteriorly over the oocyte, and is restricted to the dorsal-anterior follicle cells at stage 10. (B) In homozygous *gurken* mutant females carrying two *grk* alleles, expression of *sprouty* is significantly reduced. After extended incubations with X-Gal, only marginal expression is detected, presumably reflecting the fact that this mutant combination is not null. (C) In *fsl1K10* mutant females where Gurken activates EGFR around the anterior circumference of the egg, a similar expansion of *sprouty* expression is observed.

(2nd chromosome) specifically in follicle cells, in region 2 of the gerarium (Duffy et al., 1998).

**Fig. 3.** Sprouty represses EGFR signaling during oogenesis. (A) Wild-type egg. Note the two dorsal appendages and the inter-appendage space between them. (B) Ectopic induction of *UAS-spry* by the *T155-Gal4* driver expressed in the anterior follicle cells ventralizes the egg, as can be deduced from the longer shape of the egg and the reduced and fused dorsal appendages (arrow). (C) Generation of homozygous *spry* clones by induction of the *HS-flp* recombinase generates different aberrations in egg morphology, depending upon the size and position of the clones. The most severe phenotype, generated presumably by a large clone, represents a dorsalized egg, with dorsal appendage material around the anterior circumference. This indicates that normally repression by Sprouty is essential at stage 9, when dorso-ventral polarity is established in the course of follicle cell migration over the oocyte. (D) Generation of *spry* clones by targeting expression of *flp* to the follicle cells with the *E22c-Gal4* driver, leads to the accumulation of extra dorsal appendage material (arrow). This phenotype appears to reflect a function for Sprouty inhibition during the establishment of the dorsal appendages at stage 10. (E) In rare cases, split dorsal appendages are formed (arrows), most likely as a result of the formation of small *spry* clones at the position where the dorsal appendages are formed. This may lead to a local induction of *argos* expression, and the generation of ectopic inter-appendage cell fates.
0.075 M lysine, 0.037 M NaPO₄, pH 7.2). Discs were washed in 0.1 M NaPO₄, 0.1% saponin for 15 minutes, and incubated overnight at 4°C with anti-dpERK antibody (in the presence of 5% normal goat serum, in 0.1 M NaPO₄, 0.1% saponin). Discs were washed three times in 0.1 M NaPO₄, 0.1% saponin, incubated for 2 hours at room temperature with the secondary antibody (HRP-conjugated goat anti-mouse, Jackson Laboratories), and washed similarly. Following addition of biotinyl Tyramide (from the TSA-amplification kit, NEN) amplification was continued according to instructions, using PTW (1x PBS, 0.1% Tween 20) for all incubations, dilutions and washes. In embryos, staining was according to Gabay et al. (1997a), followed by TSA-indirect amplification using PTW as above.

RESULTS

Ectopic Sprouty reduces dpERK in the embryo

To test the possibility that Sprouty can inhibit signaling by several RTKs, ectopic expression of Sprouty was induced by the maternal Gal4 driver, mated4-Gal4-VP16 (shown to induce expression of UAS-lacZ starting at pre-cellularized embryos; not shown), and the resulting cuticles were followed. A range of phenotypes were observed, resembling in the most extreme cases faint little ball (Egfr) severe or intermediate alleles (Fig. 1A,B). Similar cuticle phenotypes were obtained when the maternal driver was used to express UAS-argos (not shown).

The effect of Sprouty overexpression in embryos can be more accurately assessed by following the pattern of activated, double phosphorylated MAP kinase (dpERK). In Drosophila embryos, the major patterns of dpERK were attributed to the activation of four RTKs: Torso, EGFR, Heartless and Breathless (Gabay et al., 1997a,b). If Sprouty inhibits signaling by several RTKs, we expect to see a reduction in the respective patterns of dpERK.

dpERK pattern was monitored in embryos where Sprouty expression is induced by the maternal Gal4 driver. Although all embryos express Gal4, the effects were variable. Some embryos exhibited normal dpERK patterns, but in a significant fraction of the embryos (approx. 50%), a reduction or complete elimination of dpERK was observed. This reduction was not observed in a parallel staining in which the same Gal4 driver was used to induce expression of UAS-lacZ. The capacity of ectopic Sprouty to reduce the levels of dpERK demonstrates that its inhibitory effect converges with the signaling pathway upstream to the activation of MAP kinase by MEK. Furthermore, the identity of the inhibited RTK pathways could be directly inferred from the missing dpERK patterns.

Since normal dpERK induced by Torso is transient, we could not reliably record a reduction in this pattern following ectopic Sprouty expression. At stage 8, a prominent pattern of Heartless-induced dpERK is normally observed in the migrating mesodermal cells. Following ectopic expression of Sprouty, only a small number of mesodermal cells display dpERK (Fig. 1C,D). At stage 10 two distinct EGFR-induced patterns are found in normal embryos, at the ventral ectoderm and tracheal placodes. Both aspects are reduced or eliminated following ectopic expression of Sprouty in embryos (Fig. 1E,F). Finally, at stage 11 EGFR-induced dpERK is observed in the ventral ectoderm and chordotonal organs, while the tracheal pits display dpERK induced by Breathless. Again, all of these aspects are diminished in embryos expressing ectopic Sprouty (Fig. 1G,H). Similar induction of UAS-argos gave rise to a reduction only in the EGFR-induced dpERK patterns.

By monitoring directly the activation of normal RTK signaling, we can thus conclude that Sprouty inhibits signaling of three pathways. Furthermore, this assay demonstrates that in vivo Sprouty impinges upon these signaling pathways upstream to MAP kinase.

Sprouty in the ovary

The EGF receptor pathway was shown to determine both anterior-posterior and dorso-ventral polarity in the follicle cells of the ovary (reviewed in Ray and Schüpbach, 1996). To examine the possible involvement of Sprouty in these processes, expression of sprouty was followed by examining the sprouty LacZ²683 enhancer trap line in the locus, representing a pattern corresponding to that of sprouty mRNA (Hacohen et al., 1998). Expression can be detected from stage 7 of oogenesis, in the posterior follicle cells (Fig. 2A). During stage 9, when the follicle cells migrate posteriorly over the oocyte nucleus, expression is observed in the dorsal and lateral cells, and is excluded from the ventral cells (not shown). Finally, once migration of the follicle cells is complete, sprouty is expressed in the dorsal-anterior corner of the egg chamber (Fig. 2A). This expression profile is reminiscent of the kekkon gene, shown to be a target gene of the pathway (Musacchio and Perrimon, 1996; Sapir et al., 1998). Like kekkon, sprouty expression is significantly reduced in gurken mutant egg chambers (Fig. 2B), and is expanded as an anterior ring in egg chambers of fsl(K10 mutants (Fig. 2C), where gurken RNA is broadly distributed (Roth and Schüpbach, 1994). sprouty is therefore a target gene of the EGF receptor pathway in the follicle cells. In situ RNA hybridization of sprouty in the ovary revealed an expression pattern similar to that of the LacZ²683 enhancer trap (Peri et al., 1999).

To examine the capacity of Sprouty to inhibit the EGF receptor pathway in the ovary, we expressed sprouty in an anterior ring of follicle cells, by the T135-Gal4 driver (Queenan et al., 1997). Under these conditions, a single reduced and fused dorsal appendage was observed, instead of the two dorsal appendages (Fig. 3A,B). The eggs were also more elongated, indicating that the follicle cells were ventralized. Similar phenotypes are observed when the activity of the EGF receptor pathway is reduced in the ovary, in gurk or top mutant backgrounds (Schüpbach, 1987).

Is the normal expression of sprouty in follicle cells significant for modulating activity of the EGF receptor pathway? Follicle cell clones homozygous for a sprouty null mutation were generated at a high frequency, using either heat shocks to induce expression of Flp to mediate FRT recombination, or by targeting its expression to the follicle stem cells. Since more than one follicle cell precursor populates each egg chamber, the expected size and distribution of the clones may vary, and hence the resulting phenotypes.

The phenotypes observed are consistent with an inhibitory role for Sprouty in two distinct stages of dorso-ventral patterning. The ventral follicle cell fates are thought to be induced during stage 9, as they migrate posteriorly over the oocyte, and receive the dorsalizing Gurken signal from the oocyte (Sapir et al., 1998). In a few egg chambers where presumably large and early sprouty homozygous clones were induced, a pronounced dorsalized phenotype was observed.
Sprouty inhibits RTK signaling

Sprouty used to monitor expression in the wing disc. This pattern of expression suggests that margin of the third instar wing disc (Fig. 4C). Expression of is induced by the EGF receptor pathway. Following the completion of follicle cell migration at stage 10, induction of rhomboid expression in the dorsal anterior follicle cells elevates EGFR signaling by providing a second activating ligand, secreted Spitz (Sapir et al., 1998; Wasserman and Freeman, 1998). This leads to a localized expression of Argos in the dorsalmost cells, generating a local reduction in signaling in the domain that will form the inter-appendage region (Queenan et al., 1997; Wasserman and Freeman, 1998).

When sprouty homozygous follicle cell clones were induced by the E22c Gal4 driver, defective eggs (44/296) showing expansion of dorsal appendage material laterally, and into the dorsal inter-appendage region were observed (Fig. 3D). This phenotype is consistent with an increased activity of the EGF receptor pathway at the later stages, when the identity of the dorsal appendages and inter-appendage region is determined, and is likely to reflect the generation of smaller clones of homozygous sprouty mutant follicle cells. Thus, Sprouty is also essential for reducing EGFR signaling at the stages when the dorsal appendage domains are defined. Finally, some of the egg chambers (4/296) displayed duplication of the dorsal appendages (Fig. 3E). The possible interpretation of this phenotype will be discussed below.

Sprouty in the wing disc

In wing imaginal discs the EGF receptor pathway participates in the regulation of cell proliferation and determination of vein/intervein territories. The position of future veins is marked by the expression of rhomboid, which dictates the sites of maximal EGF receptor activation and subsequent accumulation of dpERK (Sturtevant et al., 1993; Gabay et al., 1997a; Fig. 4A). Expression of a dominant-negative EGF receptor in the wing pouch abolishes the appearance of dpERK in the future wing veins (Fig. 4B). Under these conditions a smaller wing disc is formed due to reduced cell proliferation. Induction of secreted Spitz by the same driver gave rise to a dramatic expansion of dpERK (not shown).

The sprouty enhancer trap line was used to monitor expression in the wing disc. sprouty is expressed in the future veins and margin of the third instar wing disc (Fig. 4C). This pattern of expression suggests that sprouty is induced by the EGF receptor pathway. Expression of sprouty was monitored in wing discs in which activity of the EGF receptor pathway was compromised, by ectopic expression of a dominant-negative receptor. sprouty expression in the wing pouch was abolished, and retained only in notum region where the driver is not expressed (Fig. 4D). To demonstrate that EGFR activation is sufficient for induction of sprouty expression, the EGF receptor pathway was hyperactivated by expression of secreted Spitz, the active form of the most potent ligand of the EGF receptor (Schweitzer et al., 1995b). Indeed, the wing pouch is expanded due to increased cell proliferation, and ubiquitous sprouty expression was observed (Fig. 4E). Thus, in the wing disc activation of the EGF receptor pathway is necessary and sufficient for expression of sprouty in the future wing veins.

To test the capacity of Sprouty to reduce EGFR pathway activation in the wing, the consequences of ectopic Sprouty expression were monitored. Depending upon the expression pattern of the Gal4 driver used, reduction or elimination of the respective wing veins was observed (Fig. 5B,D). A parallel reduction in the pattern of dpERK in the region corresponding to the induction of Sprouty was monitored (Fig. 5C,E).

Most sprouty homozygous flies develop to the pupal stages. The wing morphologies of rare surviving adults could be followed, but only marginal defects of small patches of extra vein material were seen (not shown). In sprouty mutant wing discs stained with anti-dpERK antibodies, the pattern was indistinguishable from wild type discs (not shown). The possibility of functional redundancy between Sprouty and other EGFR inhibitors, which are expressed in the same pattern in the wing disc, will be discussed below.
all extracellular sequences of the EGF receptor, Sprouty repression does not involve interaction with the extracellular domain of the receptor. A similar test was carried out with the activated FGF receptor, \( \lambda \text{Heartless} \). Thickening of L5 and induction of an extra vein anterior to L2 was observed (Fig. 6C). Coexpression of Sprouty reduces these defects significantly (Fig. 6D). This result again confirms the capacity of Sprouty to inhibit signaling by different RTKs.

We next examined components that are downstream to the receptor. Expression of activated Raf by the 71B-Gal4 driver was lethal, but coexpression of Sprouty rescues this lethality, and the few surviving flies display a wing phenotype similar to those induced by Sprouty expression alone (Fig. 6E,F). Interaction between Sprouty and activated Raf was also tested in the ovary. Expression of activated Raf by the 55B-Gal4 follicle cell driver leads to expanded dorsal appendages (Brand and Perrimon, 1994; Sapir et al., 1998). Expression of Sprouty alone, or Sprouty and activated Raf by the same driver, gave rise to normal eggs (not shown). These experiments indicate that Sprouty may inhibit the pathway downstream to, or at Raf, while the dpERK stainings demonstrated that Sprouty impinges on the pathway at, or upstream, to MEK.

**DISCUSSION**

**Sprouty expression is induced by the FGF and EGF receptor pathways**

Sprouty was initially identified by its expression in the leading cells of each migrating tracheal branch (Hacohen et al., 1998). Other positions in which expression was noted did not correlate with known sites of FGF receptor activation, for example, the oenocytes (Hacohen et al., 1998) and the ectodermal muscle attachment cells (not shown). Sprouty can inhibit other RTKs, such as the EGF receptor, by intersecting with common intracellular signaling components. Transcriptional induction of *sprouty* can thus be regarded as a more universal inhibitory response to RTK signaling. Induction of *sprouty* expression is not an obligatory consequence of signaling by RTKs at all times. For example, in several tissues in which the EGF receptor is activated, most notably the ventral ectoderm (stages 6/7 and 9/10) (Gabay et al., 1997a), no expression of *sprouty* is detected. *sprouty* expression is not induced in response to activation of other RTK pathways (such as Torso or Heartless). *sprouty* expression appears to be dictated by additional factors such as tissue context, and timing of induction. *sprouty* transcription may also be triggered by some RTKs and not by others, depending perhaps on the adaptor molecules utilized and parallel signaling pathways they induce. In the tracheal system *sprouty* expression was shown to be triggered by the transcriptional activator Pointed P1 (Hacohen et al., 1998), but in the ventral ectoderm, induction of Pointed P1 by the EGF receptor pathway, does not lead to the induction of *sprouty*. Thus, by employing more complex regulatory circuits, it may be possible to couple *sprouty* induction to signaling by some RTKs but not others, and only in certain contexts.

**Intersection of Sprouty with RTK signaling pathways**

Identification of Sprouty as an inhibitor of both FGF receptor and EGF receptor pathways raises the issue of the mechanism

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**Fig. 5.** Ectopic Sprouty inhibits vein formation. (A) Wild-type wing. (B) Ectopic expression of Sprouty driven by dpp-Gal4 in a stripe corresponding to the position of L3, gives rise to discontinuity in this vein in the adult wing (arrow). (C) Examination of the wing discs of the same cross shows elimination of dpERK in the region of Sprouty expression (arrow). (D) Broader expression of Sprouty by the MS1096-Gal4 driver eliminates the distal regions of L2, L3 and L4 (arrows). (E) In the same cross, dpERK in the wing disc is eliminated in the region of expression (arrow).
Sprouty inhibits RTK signaling by which Sprouty exerts its effects. Since Sprouty is a novel molecule, its biological properties are still obscure. *sprouty* mutant clones were shown to exert a non-autonomous phenotype in tracheal development (Hacohen et al., 1998). It is not known, however, if Sprouty itself is the signal transmitted from one cell to the other, or if the activity of Sprouty in the leading tip cells affects their capacity to signal to the adjacent cell through a different signaling module. If Sprouty is a secreted protein, one would expect its inhibitory signal to be relayed to the cytoplasm, by a hitherto unknown pathway. Conversely, if Sprouty functions in the cells in which it is produced, it may impinge directly on the cytoplasmic signaling pathways of RTKs. Recent evidence suggests that Sprouty may be an intracellular protein. Sprouty interacts in vitro with Drk/Grb2 and GAP proteins, but the possible functional significance of this interaction has not been determined (Casci et al., 1999).

We have narrowed down the possible intersection(s) of Sprouty with RTK signaling pathways. The capacity of Sprouty to suppress activated λTop and λHtl constructs demonstrates that it intersects with the pathway downstream to the receptor. We showed that ectopic Sprouty eliminates the normal accumulation of dpERK in the embryo and wing discs, demonstrating that the intersection point is at the MAP kinase kinase (MEK) stage, or upstream to it. Due to lethality resulting from ectopic expression of activated Ras or Raf, it was difficult to determine the intersection point more precisely. However, the capacity of ectopic Sprouty to rescue lethality induced by activated Raf suggests that it can inhibit Raf itself or components downstream to Raf. Similarly, in the ovary Sprouty expression was also capable of eliminating phenotypes induced by activated Raf.

This fairly crude analysis leaves Raf or MEK as the possible points of intersection. It is interesting to note that the activity of KSR, a cytoplasmic serine/threonine kinase, was shown to impinge on RTK signaling by facilitating signaling through Raf and MEK (Therrien et al., 1996), utilizing a mechanism which is still not understood. It is thus possible that Sprouty signaling exerts its inhibitory effect by compromising the activity of KSR. Another possible point of intersection is the CNK protein (Therrien et al., 1998), a multi-domain protein that binds Raf and is required for Ras signaling.

Casci et al. (1999) demonstrated that Sprouty interacts with Drk and GAP, which are upstream to Raf. However, it is possible that the interactions observed between Sprouty and Drk or GAP are not physiologically significant. Alternatively, Sprouty may intersect the pathway at several junctions, functioning as an ‘inhibitory scaffold’ protein.

**Biological functions of Sprouty**

The original biological function of Sprouty was identified in the trachea, where it inhibits cells adjacent to the leading tip cells from assuming a terminal cell fate (Hacohen et al., 1998). Identification of genetic interactions between the EGF receptor pathway and *sprouty* in eye development have suggested that *sprouty* plays a central role in restricting the activity of the EGF receptor pathway, as well (Bergmann et al., 1998; Kramer et al., 1999; Casci et al., 1999). In this work we have examined the effects of removing *sprouty* activity in the wing disc and ovary, tissues where its expression is induced by the EGF receptor. Surprisingly, in spite of *sprouty* expression in the wing vein precursors, very weak or no phenotypes are observed in its absence. It is important to note, however, that two other inhibitors of the EGF receptor pathway, Argos and Kekkon, are
expressed in exactly the same pattern (Sawamoto et al., 1994; Musacchio and Perrimon, 1996). It is possible that there is a redundancy between these inhibitors, such that the system can adjust to the absence of one of them. All three inhibitors are induced by the EGF receptor pathway. While during normal development each of the three may contribute to reduction of signaling, transcription of the other two inhibitors may be elevated upon removal of Sprouty. This adjustment could contribute to the functional redundancy and robustness of the system.

During oogenesis, removal of Sprouty gave rise to phenotypes consistent with hyperactivation of EGFR signaling in follicle cells. *sprouty* transcription has a low threshold for induction in follicle cells, and is observed in all cells in which activation of the EGF receptor pathway takes place. We can imagine that Sprouty reduces the signaling capacity of the EGF receptor uniformly, thus helping to preserve the graded effects of EGF receptor activation. This is especially pronounced when large clones of *sprouty* lead to dorsalized egg chambers, thus highlighting the normal role of Sprouty in preventing expansion of the dorsalizing Gurken activity to the ventral follicle cells. The functional distribution of Gurken activity is thus broader than previously visualized by following the distribution of *gurken* mRNA or protein. These results highlight the necessity to regulate and restrict not only the distribution of *gurken* mRNA and protein, but also the resulting EGFR activation by Gurken.

In contrast to the broad expression of *sprouty*, it was previously demonstrated that transcription of *argos* is induced only in the dorsal-anterior region, where maximal activation of the EGF receptor takes place (Queenan et al., 1997). This, in turn may lead to a local reduction of signaling, and to the generation of the inter-appendage region (Wasserman and Freeman, 1998). In *sprouty* mutant clones, EGFR activation in the inter-appendage region may rise above the threshold required to produce dorsal appendages. The most prevalent phenotype of *sprouty* clones we observed was the expansion of dorsal appendage cell fates to the inter-appendage region. This expansion takes place, in spite of the production of Argos in the same region.

In rare cases, a phenotype of duplicated dorsal appendages was observed following the generation of *sprouty* follicle cell clones (Fig. 3E). It is possible that in this case, small clones were generated, at positions which coincided with the region in which dorsal appendages are normally produced. This could lead to an elevated level of EGFR signaling and local Argos induction. The positions of Argos expression may in turn generate a region of inter-appendage fate, thus splitting the dorsal appendages.

It is interesting to note the different, non-redundant roles played by Sprouty and Argos in patterning the egg chamber. *argos* is induced only by maximal EGFR receptor activation. At the earlier stages, when EGFR activation levels are lower, no *argos* transcription is observed. At stage 10, when activation by Gurken synergizes with localized processing of Spitz mediated by Rhomboid (Sapir et al., 1998; Wasserman and Freeman, 1998), *argos* is transcribed, but only in a dorsalmost stripe of follicle cells. The localized induction of *argos* transcription coupled with a restricted diffusion of Argos in the ovary, thus serves to locally reduce the level of EGFR receptor activation, and generate the inter-appendage region (Wasserman and Freeman, 1998). *sprouty* transcription, on the other hand, has a low threshold for induction, and is observed in all follicle cells where EGF receptor activation takes place, at all stages. Sprouty thus serves to uniformly reduce the level of EGF receptor activation in the follicle cells. In contrast, in the wing imaginal disc *argos*, *sprouty* and *kekkon* are expressed in an identical pattern, and may thus have overlapping roles.

In conclusion, Sprouty emerges as an inhibitor of signaling by different receptor tyrosine kinases, intercepting common cytoplasmic elements. Highly regulated induction of *sprouty* expression by RTK signaling, recruits this inhibitor for distinct biological processes. In these cases, Sprouty restricts RTK signaling, thus contributing to generate the precise spatial and temporal patterns of response. Future work should reveal the detailed molecular mechanism by which Sprouty exerts its inhibitory effects.

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REFERENCES


