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Sprouty: a common antagonist of FGF and EGF signaling pathways in *Drosophila*

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SUMMARY

Extracellular factors such as FGF and EGF control various aspects of morphogenesis, patterning and cellular proliferation in both invertebrates and vertebrates. In most systems, it is primarily the distribution of these factors that controls the differential behavior of the responding cells. Here we describe the role of Sprouty in eye development. Sprouty is an extracellular protein that has been shown to antagonize FGF signaling during tracheal branching in *Drosophila*. It is a novel type of protein with a highly conserved cysteine-rich region. In addition to the embryonic tracheal system, *sprouty* is also expressed in other tissues including the developing eye imaginal disc, embryonic chordotonal organ precursors and the midline glia. In each of these tissues, EGF receptor signaling is known to participate in the control of the correct number of neurons or glia. We show that, in all three tissues, the loss of *sprouty* results in supernumerary neurons or glia, respectively. Furthermore, overexpression of *sprouty* in wing veins and ovarian follicle cells, two other tissues where EGF signaling is required for patterning, results in phenotypes that resemble the loss-of-function phenotypes of *Egf receptor*. These results suggest that Sprouty acts as an antagonist of EGF as well as FGF signaling pathways. These receptor tyrosine kinase-mediated pathways may share not only intracellular signaling components but also extracellular factors that modulate the strength of the signal.

Key words: Eye development, Chordotonal organ, ras signaling, Sprouty, FGF, EGF, *Drosophila*

INTRODUCTION

FGF and EGF are extracellular signaling factors that control various aspects of morphogenesis, patterning and cellular proliferation in both invertebrates and vertebrates. These ligands act through high-affinity transmembrane receptors with an intracellular tyrosine kinase moiety. In *Drosophila*, a single EGF receptor homolog, encoded by the *Egfr* gene, and two FGF receptor homologs, encoded by *breathless* and *heartless*, have been identified (Beiman et al., 1996; Gisselbrecht et al., 1996; Glazer and Shilo, 1991; Klämbt et al., 1992; Livneh et al., 1985). Upon reception of the extracellular signals, these receptor tyrosine kinases (RTKs) activate the ras/MAPK signaling pathway. Many of the molecular components of this signaling pathway, such as ras GTPase, raf kinase and MAPK are shared among different RTKs. Thus information from multiple extracellular signals are interpreted using the same molecular cassette (reviewed by Schlessinger, 1993).

While the intracellular tyrosine kinase domain is conserved, the extracellular domains of the RTKs are specialized for their diverse inputs. Of these three receptors, the EGF receptor is the most complex, as it is responsive to three tissue-specific activating ligands, Spitz, Gurken and Vein (reviewed by Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997). Spitz acts in the ventral ectoderm, the midline of the central nervous system (CNS), the chordotonal organs and imaginal discs. Gurken is the key determinant in patterning the ovarian follicle cells. The third ligand, Vein, functions during wing vein formation and in the attachment of the embryonic muscles. Unlike Spitz and Gurken, which are members of the TGFα family, Vein shows similarity to neuregulins (Neuman-Silberberg and Schüpbach, 1993; Rutledge et al., 1992; Schnepp et al., 1996). The only known ligand for the FGF receptors is the Branchless FGF, which acts through Breathless during tracheal branching (Sutherland et al., 1996). In most of these systems, the distribution of the activating ligands, which is governed mainly by their synthesis, processing and diffusion, is the primary factor controlling the differential behavior of the responding cells. In addition, the EGF signaling pathway is subject to negative feedback regulation. The production of the EGF-like antagonist Argos is dependent on EGFR signaling (Schweitzer et al., 1995; Golembo et al., 1996)

and a hyperactivation of EGFR results in a downregulation of receptor expression (Sturtevant et al., 1994). Elucidating the mechanisms that regulate RTK signaling is key to the understanding of how extracellular signals achieve precise cellular responses.

Recently, we have reported that Sprouty (SPRY) acts as a novel extracellular antagonist of FGF signaling during tracheal development (Hacohen et al., 1998). Similar to Argos, which is induced by EGFR signaling (Golembo et al., 1996), *spry* expression is induced by the FGF pathway that it inhibits. Here we show that *spry* is also expressed in other developmental systems such as the eye imaginal disc, the embryonic chordotonal organ precursors and the midline glia. In all of these systems, EGF receptor signaling is known to participate in the control of the correct number of neurons or glia. We examine *spry* function in several of these tissues, focusing on its regulation of EGF-induced neuronal differentiation in the eye.

The *Drosophila* compound eye is a stereotyped array of 800 unit eyes or ommatidia, each of which comprises an invariant number of cells; 8 photoreceptor neurons (named R1 through R8) and 12 non-neuronal accessory cells. The photoreceptor cells are recruited by a stereotyped sequence of inductive interactions, mediated by two RTKs, EGFR and Sevenless (SEV). EGFR signaling is required for the recruitment or maintenance of all ommatidial cells (Freeman, 1996; Kumar et al., 1998; Xu and Rubin, 1993), whereas SEV is required specifically for the neuronal specification of the R7 cell (Tomlinson and Ready, 1986). Activation of EGFR by its ligand Spitz (Freeman, 1994; Tio et al., 1994) or SEV by BOSS (Van Vactor et al., 1991) activates the ras/MAPK pathway, which ultimately impinges on a number of nuclear targets such as the Pointed Ets-domain transcription factor (Brunner et al., 1994; O'Neill et al., 1994) and results in the initiation of neuronal development (for review see Dickson, 1995; Zipursky and Rubin, 1994).

In addition to the presumptive photoreceptor neurons, two cell types within an ommatidium can be induced to become neurons upon ectopic activation of the RTK signaling pathways: the lens-secreting non-neuronal cone cells and the mystery cells, two cells transiently associated with the ommatidial precluster (Tomlinson and Ready, 1987; Wolff and Ready, 1991a). Ectopic activation of the ras/MAPK pathway in these cells is sufficient to elicit their differentiation as neurons (Basler et al., 1991; Dickson et al., 1992; Fortini et al., 1992). In normal development, ectopic activation of the pathway is prevented by the action of several negative regulators of RTK signaling. *Gap1* encodes a GTPase activating protein that is thought to act by decreasing the levels of activated Ras1 in the eye, whereas *yan* encodes for an Ets-domain protein that interferes with Pointed signaling (Gaul et al., 1992; Lai and Rubin, 1992). Mutations in either of these loci causes the cone cells and mystery cells to differentiate as photoreceptor neurons. Absence of a third factor, Argos, a secreted antagonist of EGF signaling, results in a transformation of mystery cells into outer photoreceptor cells and in the recruitment of extra cone and pigment cells, but does not affect the development of the presumptive cone cells (Freeman et al., 1992b; Kretzschmar et al., 1992; Okano et al., 1992).

Here we show that mutation in *spry* causes a transformation of non-neuronal cone cells into R7 cells and of mystery cells into outer photoreceptor cells, a phenotype identical to the one seen with hyperactivation of EGFR signaling. SPRY can also antagonize EGFR signaling in other tissues such as the midline glia, the chordotonal organs, the wing and the ovarian follicle cells. Since SPRY acts as an antagonist of FGFR-mediated signaling during tracheal development, the EGFR and FGFR signaling pathways appear to share an unexpected degree of conservation in the extracellular compartment.

MATERIALS AND METHODS

Genetics

We identified five EMS-induced alleles of *spry* in a genetic screen for dominant suppressors of the *ro-svp* eye phenotype (Kramer et al., 1995). Misexpression of *svp* in R2/R5 by the *ro-svp* transgene causes misspecification of these cells, resulting in compromised neuronal induction of R1/R6. *spry* dominantly suppresses this phenotype by rescuing the neuronal differentiation of R1/R6, without restoring the misspecification of R2/R5 (data not shown). The screen was carried out essentially as described previously for suppressors of the *sev-svp* phenotype (Kramer et al., 1995), except that mutagenized *st e* males were crossed to *CyO*, 2 × P[*ro-svp*]/*Sp* virgin females. 50,000 F_1 progeny were screened and *Su*(*ro-svp*) on the third chromosome were balanced over *TM3*, *ry*, 2 × P[*ro-svp*]. Mutations on the third chromosome were mapped meiotically using the *rucuca* chromosome (*ru*, *h*, *th*, *st*, *cu*, *sr*, *e*, *ca*). Deficiency mapping of *spry* was carried out with Df(3L)HR119, Df(3L)HR232, Df(3L)HR370 (Wohlwill and Bonner, 1991), Df(3L)A466 (Kulkarni et al., 1994) and Df(3L)1226 (gift of S. Paine-Saunders and J. Fristrom). Df(3L)HR119 (63C1; 63D3), Df(3L)HR232 (63C6; 63E) and Df(3L)1226 (63C6; 63E1) failed to complement the lethality of *spry* EMS alleles, whereas Df(3L)HR370 (63A1; 63D1) and Df(3L)A466 (63D1-2; 64B1-2) complemented it. The eye phenotypes of various *spry* alleles in *trans* to each other were indistinguishable from those over a deficiency of the locus, suggesting that all *spry* alleles tested were amorphs or strong hypomorphs (Table 1).

$spry^{254}$ clones and clones doubly mutant for $spry^{254}$ and a strong hypomorphic allele of *sina* ($sina^2$; Carthew and Rubin, 1990) or a null allele of *argos* ($argos^{257}$; Okabe et al., 1996) were generated using the FRT technique (Xu and Rubin, 1993). $spry^{\Delta 5}$ clones in a sev^- background were generated in animals of the genotype w^{1118}, sev^{d2}, P[hs-FLP]; $spry^{\Delta 5}$, P[FRT]80B/P[w^+]70C, P[FRT]80B. Control animals were raised without heat shock. $Gap1^{B2}$ and yan^1 are described in Gaul et al. (1992), and Lai and Rubin (1992), respectively. $pointed^{8B}$ is an amorph (Klämbt, 1993) and $Ras1^{e2F}/Ras1^{C33}$ is a combination of *Ras1* hypomorphic alleles that survives to the third instar larval stage (our unpublished observation). $breathless^{\Delta 10}$, $heartless^{80}$ and $branchless^{P1}$ are amorphs or strong hypomorphs (Klämbt et al., 1992; Shishido et al., 1997; Sutherland et al., 1996). For ectopic expression studies, *UAS-spry* was placed under the control of *elav-GAL4* C155 (Lin and Goodman, 1994), *sevE-GAL4* K25 (Brunner et al., 1994), *en-GAL4* (gift of A. Brand and N. Perrimon), CY2 (Queenan et al., 1997) and BH1 (Schüpbach and Wieschaus, 1998). *sevE-*$Ras1^{N17}$, *UAS-spry* and *GMR-argos* lines are described in Allard et al. (1996), Hacohen et al. (1998) and Sawamoto et al. (1998), respectively.

Histology and histochemistry

Fixation and sectioning of adult heads and antibody staining of imaginal discs were performed as described (Tomlinson and Ready, 1987), except that, in some instances, the peripodial membrane was not removed from imaginal discs. SPRY protein was detected using two polyclonal rabbit antisera; 26A was raised against full-length

Table 1. Comparison of recessive phenotypes of different *spry* allelic combinations

Genotype	Average no. of outer PRCs	Average no. of R7 cells	Ommatidia scored
$spry^{\Delta5}/spry^{\Delta5}$	6.09	3.09	120
$spry^{\Delta5}$/Df(3L)1226	6.09	2.71	82
$spry^{226}$/Df(3L)1226	6.17	3.26	115
$spry^{\Delta5}/spry^{226}$	5.96	3.04	190
$spry^{F7}/spry^{226}$	6.04	3.25	114
$spry^{254}$ clone	6.14	3.08	126
sev^{d2}, $spry^{254}$ clone	6.15	2.96	96

Retinular phenotypes of animals of the indicated genotypes were examined in apical tangential sections and average numbers of outer PRCs and R7 cells per ommatidium calculated for each genotype. For the wild type, the average number of outer PRCs would be 6.0 and the average number of R7 cells would be 1.0.

SPRY protein and 32C against a peptide extending from amino acid 63 through 462 (Hacohen et al., 1998). An affinity-purified antibody against BarH1/BarH2 proteins (Higashijima et al., 1992), a monoclonal antibody against ELAV protein and monoclonal antibody 22C10 (Fujita et al., 1982) were gifts of the Saigo, Rubin and Goodman laboratories, respectively. Monoclonal antibodies against β-galactosidase were purchased from Promega. The expression pattern of enhancer trap insertion 9143 was detected using anti-β-galactosidase mAb 40-1a (obtained from Developmental Studies Hybridoma Bank) and the Vectastain elite kit (Vector laboratories). Embryo stainings were carried out according to Patel (1994).

The following *lacZ* enhancer trap lines were used as cell-type-specific markers; X81 and AE127 are enhancer trap insertion in the *rhomboid* gene (Freeman et al., 1992a) and the *svp* gene (Mlodzik et al., 1990), respectively. P82 expresses *lacZ* in R3, R4 and R7 (Kramer et al., 1995), H214 is an enhancer trap insertion that expresses β-galactosidase at high levels only in the R7 cell (Mlodzik et al., 1992) and AA142 is an enhancer trap insertion with expression in the midline glia (Klämbt et al., 1991).

In situ hybridizations to eye imaginal discs and embryos were performed essentially as described by O'Neill and Bier (1994), and Lehmann and Tautz (1994). RNA probes were transcribed from a 1 kb and a 2.2 kb *Eco*RI fragment of the longest *spry* cDNA cloned into $BSSK^+$ (Hacohen et al., 1998) using the Boehringer Mannheim kit according to the instructions of the manufacturer.

RESULTS

spry is required to prevent neuronal induction of non-neuronal cells in the retina

Animals homozygous for any of the EMS-induced alleles of *spry* or $spry^{\Delta5}$ died as pharate adults. The rare escapers had eyes that were similar in size to wild-type eyes but had a disorganized exterior. A majority of the ommatidia in these animals contained supernumerary photoreceptor neurons, which by their morphology were R7 cells. In addition, some of the extra photoreceptors resembled outer photoreceptor neurons (Fig. 1C; Table 1). Examination of the early stages of neuronal development in the eye imaginal disc with molecular markers revealed that the supernumerary photoreceptors originated from non-neuronal cone cells and mystery cells that had assumed R7 and R3/R4 fates, respectively (Fig. 2). Neuronal markers were inappropriately activated in cone and mystery cells at the same time in development as in the normal photoreceptors, implying that the defect in the mutant occurs at the normal time of photoreceptor induction. Thus, *spry* functions in the eye imaginal disc to prevent neuronal induction of these non-neuronal cells.

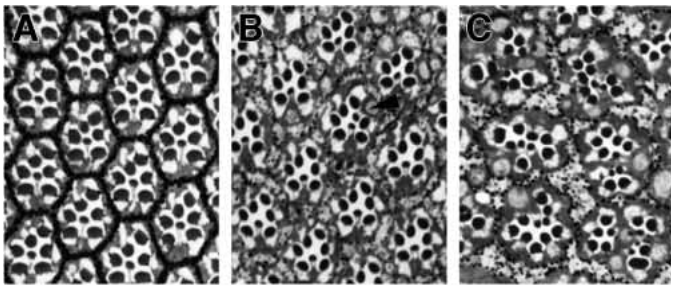


Fig. 1. The eye phenotype of *spry* loss-of-function alleles. (A) A tangential section of a wild-type eye at the apical level shows a regular array of ommatidia. Each ommatidium contains eight photoreceptor neurons (R1-8), which can be identified by the size and position of their darkly stained light-gathering structure (rhabdomeres). Based on their morphology, the photoreceptor cells (PRCs) can be subdivided into three classes. The outer PRCs (R1 through R6) have large rhabdomeres that extend through the entire depth of the retina and are arranged in a trapezoidal array. R7 and R8 have small rhabdomeres that project into the center of the ommatidium. In this apical section, only the rhabdomere of the R7 cell is visible. R8 is underlying the R7 cell in a more basal position. (B) *spry* loss-of-function alleles had a weak dominant phenotype, shown here is an apical tangential section of a $spry^{226}$/+ eye. In eyes of animals heterozygous for a *spry* EMS allele, on average 3.4% of ommatidia had an extra R7 cell (arrowhead) with some variation between different alleles ($spry^{226}$: 7.2%, $spry^{G5}$: 5.5%, $spry^{F7}$: 1.7%, $spry^{254}$: 1.2%, $spry^{211}$: 1.0%). $spry^{\Delta5}$ showed the weakest dominant phenotype; 0.8% of ommatidia had an extra outer PRC. (C) An apical tangential section of an eye of an animal homozygous for $spry^{\Delta5}$, a putative amorph of *spry.* Most ommatidia contained between one and four extra R7-like photoreceptor cells, and 27% of ommatidia had one or two extra outer photoreceptor cells. In addition, a small percentage of ommatidia had less than the normal number of photoreceptor cells.

spry functions as a dosage-dependent inhibitor of neuronal induction. In the heterozygous condition, all *spry* alleles examined contained 1-7% of ommatidia with an extra R7 cell or an occasional gain or loss of an outer photoreceptor cell (Fig. 1B; Table 2). Conversely, increased levels of SPRY in the developing eye disc inhibited the induction of normal photoreceptor cells. Animals that expressed *UAS-spry* under either the *sev-GAL4* or the *elav-GAL4* driver had small disorganized eyes. The majority of ommatidia lacked one or more outer photoreceptor cells of the R3/R4/R1/R6 subtype (Fig. 3A-C). The R7 cell was missing in 11±4% of *sevE-GAL4/UAS-spry*, and in 18±6% of *elav-GAL4/UAS-spry* ommatidia. Development of the R2/R5 and R8 photoreceptors

Table 2. Dominant interactions between *spry* and the intracellular negative regulators of ras signaling

Genotype	Ommatidia with extra R7 (%)
$spry^{226}$/+	6.3±2.2
yan^{1}/+	0.4±0.4
$Gap1^{B2}$/+	<0.2
yan^{1}/+; $spry^{226}$/+;	61±21
$Gap1^{B2}$/+; $spry^{226}$/+	43±3

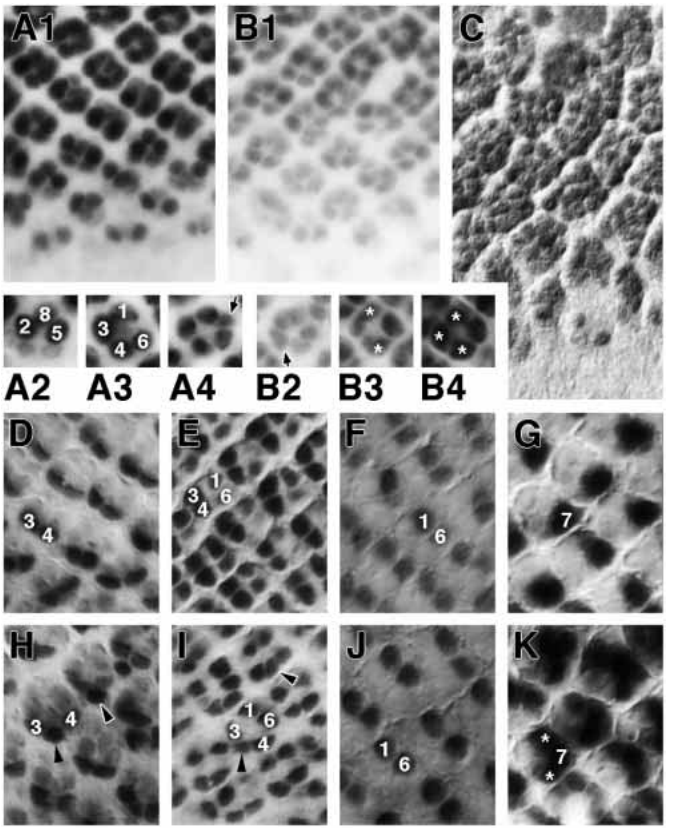


Fig. 2. *spry* prevents non-neuronal cells from acquiring neuronal cell fates. (A,B) Expression of the ELAV antigen in wild-type (A) and *spry*$^{-}$ third instar larval eye discs (B). In the wild-type disc, ELAV protein is expressed in the photoreceptor neurons in the order in which they differentiate (A). ELAV expression is first detected in R2/R5 in row 1, followed by R8 in row 2 (A2), R3/R4 in row 4, then R1/R6 in row 6 (A3), and lastly R7 in row 10 (A4, arrow). (B) In *spry* discs, cells in the position of the mystery cells express ELAV starting in row 5 (B2, arrow). Starting in row 8, cells in the positions of cone cell precursors express ELAV (B3, B4, asterisks). (C) Massive neuronal differentiation occurs in a *spry*^{254}, *argos*^{257} mutant clone. This panel shows part of a large *Minute*$^{+}$ clone, labeled with a *ub-GFP* marker. The movement of the morphogenetic furrow was accelerated in the mutant region (not shown). (D-K) Expression patterns of molecular markers specific for subsets of photoreceptor neurons in imaginal discs of wild-type (D-G) or *spry*$^{-}$ (H-K) third instar larvae. An R3/R4-specific molecular marker (enhancer trap line P82, D) and a R3/R4/R1/R6-specific molecular marker (enhancer trap line AE127, E) are ectopically expressed in some of the mystery cells in *spry*$^{-}$ discs (H,I, arrowheads). This is in contrast to a R1/R6-specific marker, the Bar-antigen (F), expression of which is unaltered in *spry*$^{-}$ discs (J). Enhancer trap line H214 is expressed at high levels only in the R7 cell in wild-type discs (G). In *spry*$^{-}$ discs, H214 is ectopically expressed in non-neuronal cells in the positions of cone cell precursors (K, asterisks). Heteroallelic combinations of EMS alleles of *spry* were used as *spry*$^{-}$ animals.

was unaffected (Fig. 3D,E). The mild haploinsufficient phenotype of *spry* was dominantly enhanced by loss-of-function mutations in two other negative regulators of photoreceptor induction, *Gap1* and *yan* (Table 2), suggesting that *spry* acts on the same pathway as these genes to inhibit neuronal induction, as it does during secondary tracheal branch induction in the embryo (Hacohen et al., 1998).

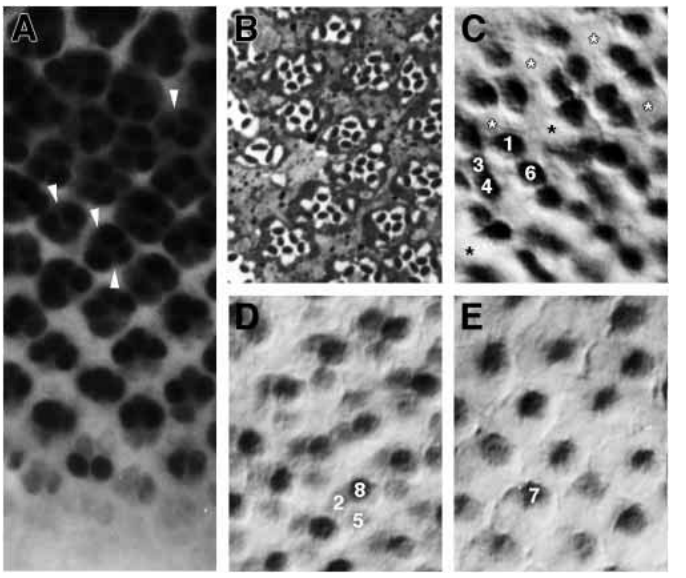


Fig. 3. Overexpression of *spry* results in loss of photoreceptor neurons. (A,C-E) Expression of molecular markers in eye imaginal discs of *sevE-GAL4/UAS-spry* third instar larvae. (B) Section of an *sevE-GAL4/UAS-spry* adult eye. A majority of the ommatidia lacked 1-2 outer PRCs (average number of outer PRCs: 4.73±0.13) and less frequently the R7 cell (average number of R7 cells: 0.95±0.05). Similar results were obtained with *elav-GAL4/UAS-spry* (outer PRCs: 4.38±0.24; R7 cells: 0.88±0.06). (A) The expression pattern of the ELAV antigen in *sevE-GAL4/UAS-spry* imaginal discs revealed a normal number of clusters with the appropriate spacing. While ELAV expression in R2/R5 and R8 was unaffected in *sevE-GAL4/UAS-spry* eye discs, we observe loss of ELAV expression from one or two cells of the R3/R4/R1/R6 cell group in a majority of clusters (indicated by white arrowheads). (C) An R3/R4/R1/R6-specific marker (AE127) was lost from one cell of the R3/R4 cell pair in 23% of ommatidia (indicated by black asterisks), from one cell of the R1/R6 cell pair in 18% (white asterisks), and from two or more cells per cluster in 10% of ommatidia (refer to Fig. 2E for a wild-type control). (D) Expression of X81 in R2/R5 and R8 was unaltered in *sevE-GAL4/UAS-spry* eye discs. Note however the disorganized appearance of clusters. (E) Expression of the R7-specific marker H214 was unaffected in *sevE-GAL4/UAS-spry* eye imaginal discs (refer to Fig. 2G for a wild-type control). Similar results were obtained for the expression of molecular markers in *elav-GAL4/UAS-spry* eye imaginal discs (data not shown).

spry antagonizes neuronal induction by the EGFR pathway

Two RTK signaling pathways are required for neuronal induction of photoreceptor cells, the EGFR pathway and the SEV pathway. While SEV signaling is required only for R7 development, EGFR signaling is necessary for the specification and maintenance of all photoreceptor neurons. Overexpression of *spry* results in a loss of mainly outer photoreceptor cells, which is also seen with compromised EGFR signaling (Freeman, 1996; Kumar et al., 1998). Conversely, expression of an activated form of EGFR (Queenan et al., 1997) causes excess neuronal differentiation, which is reminiscent of the *spry* loss-of-function phenotype (data not shown). These results are consistent with the idea that *spry* antagonizes EGFR signaling.

Since hyperactivation of SEV signaling also produces excess

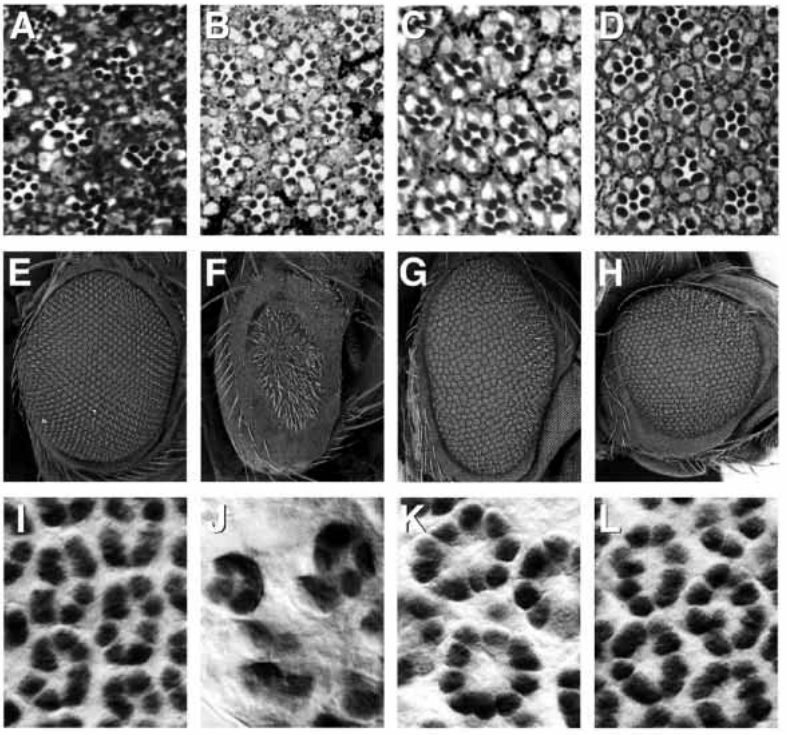


Fig. 4. Epistatic relationship of *spry* with components of the ras signaling pathway. (A,B) Apical tangential sections of a sev^{d2}; $spry^{254}$ clone in a sev^{d2} background (A) and of a $spry^{254}$, $sina^{2}$ mutant clone (B). Clones were marked by the absence of a functional *white* gene. (A) Extra R7 cells in $spry^{254}$ mutant ommatidia are independent of *sev* gene function. (B) In contrast, extra R7 cells in $spry^{254}$ mutant ommatidia require *sina* function. Out of 97 $spry^{254}$, $sina^{2}$ ommatidia scored, none contained an R7 cell. The small central rhabdomeres visible in B were shown to be apically displaced R8 cells by serial sectioning. (C,D) Apical tangential eye sections of *sevE-*$Ras1^{N17}$/+ (C) and *sevE-*$Ras1^{N17}$/+; $spry^{F7}$/$spry^{226}$ animals (D). In the eyes of animals expressing one copy of *sevE-*$Ras1^{N17}$, a dominant negative ras, the R7 cell is lost in approximately half of the ommatidia (C). *sevE-*$Ras1^{N17}$ effectively suppresses the appearance of extra R7 cells in *sevE-*$Ras1^{N17}$/+; $spry^{F7}$/$spry^{226}$ eyes; the average number of R7 cells per ommatidium was decreased to 0.95±0.04 (D; compare Fig. 1C for $spry^{-}$ phenotype). (E-L) *spry* suppresses the effect of overexpression of Argos. (E-H) Scanning electron micrographs of adult retinae. (I-L) ELAV expression in the pupal eye. In animals carrying two copies of the *GMR-argos* transgene, photoreceptor neurons undergo apoptosis, resulting in small eyes with fewer photoreceptor neurons per ommatidium (F,J), compared to the wild type (E,I). The eyes of animals homozygous for $spry^{\Delta 5}$ are normal in size, but have additional photoreceptor neurons (G,K). The eye phenotype of *GMR-argos*; $spry^{\Delta 5}$ animals is similar to the one of $spry^{\Delta 5}$ animals; the eye size is normal and extra photoreceptor neurons are present in most ommatidia (H,L). These animals carry two copies of the *GMR-argos* transgene and are homozygous for $spry^{\Delta 5}$.

photoreceptor neurons, we tested whether *spry* acted by antagonizing the SEV RTK. If the extra R7 photoreceptors in *spry* mutants resulted from overactivity of SEV, then the affected cells should require *sev* for their development. However, we found that removing *sev* had little or no effect on R7 development in the *spry* mutant background: the average number of R7 cells was 3.08 for $spry^{-}$ ommatidia and 2.96 for sev^{-}, $spry^{-}$ ommatidia (Fig. 4A; Table 1). Thus in *spry* mutants, both the normal R7 cell and the cone cells complete neuronal differentiation in the absence of SEV, indicating that SPRY antagonizes a RTK other than SEV, or that SPRY exerts its effect downstream or parallel to SEV RTK.

SINA is a nuclear factor required for the development of the R7, R1 and R6 cells and acts downstream of the ras/MAPK cascade (Carthew and Rubin, 1990). In contrast to sev^{-}, $spry^{-}$ ommatidia, no R7 neurons were formed in $spry^{-}$, $sina^{-}$ double mutant ommatidia (Fig. 4B). The formation of extra R7 neurons in *spry* mutants was also efficiently suppressed by expression of a dominant negative form of Ras1 from a *sevE-*$Ras1^{N17}$ transgene (Fig. 4C,D). Thus, the R7 cells arising in the absence of *spry* were independent of *sev* function, but dependent on normal downstream functions in the RTK signaling pathways. Taken together these data suggest that *spry* acts as an antagonist of the EGFR pathway in the eye.

spry is expressed near EGF signaling centers during neuronal induction

The expression pattern of *spry* in developing eye imaginal discs during the time of the neuronal inductions was determined by RNA in situ hybridization. Induction of the eight photoreceptor cells in each ommatidium occurs in the 5 or 6 rows posterior to the morphogenetic furrow in the third instar larval eye imaginal disc. *spry* mRNA was found in a stripe of cells corresponding to rows 1 through 5 posterior to the furrow. In addition, there was a second stripe of *spry*-expressing cells in more mature ommatidial preclusters corresponding to rows 8 through 11 (Fig. 5A). The low resolution of in situ hybridization experiments precluded identification of the spry-expressing cells in these regions. However, a *spry lacZ* enhancer trap line mimics expression of the gene in other tissues (Hacohen et al., 1998) in addition to the eye imaginal disc, and expression of the reporter can be resolved at cellular resolution. The *spry* reporter was expressed in all photoreceptor neurons, with particularly high levels in photoreceptors R2, R5 and R7. These three cells directly contact the R8 cell, which is the first photoreceptor specified and, along with R2 and R5, presumably constitutes the EGF signaling center. These three cells are the initial source of Spitz (Freeman, 1994; Tio et al., 1994) and they specifically express the *rhomboid* and *Star* genes required to generate the active EGFR ligand (reviewed in Freeman, 1997). Lower levels of expression of the *spry* reporter were detected in photoreceptor cells R3/R4/R1/R6 which are located slightly further from the R8 cell (Fig. 5G,H,J), and no expression was detected in the mystery or cone cells located just beyond them. At slightly later stages in ommatidial development, corresponding to rows 8 and beyond behind the furrow, *spry* expression was detected at low levels in cone cells, and it was present at higher levels in cone cells at the pupal stage (Fig. 5J,K). The *spry* reporter was not expressed in *Ras1* mutant eye discs, or in clones mutant for *pointed* (Fig. 5L,M). Thus, as in the embryonic trachea (Hacohen et al., 1998), *spry* is expressed near an RTK

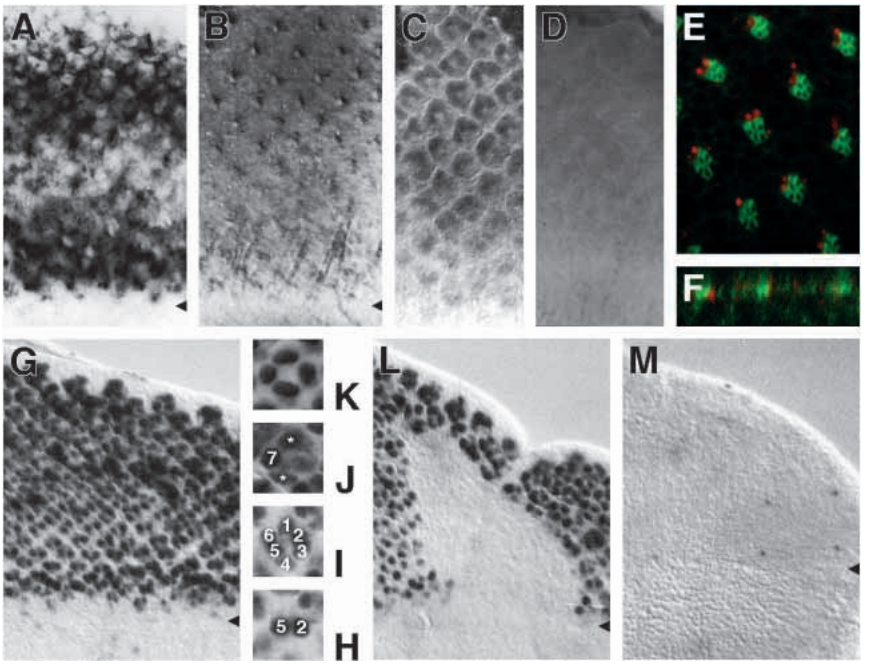


Fig. 5. *spry* expression pattern in the eye imaginal disc. In situ hybridization of a third instar wild-type eye disc with a *SPRY* probe (A) show *SPRY* mRNA expression in two waves corresponding to approximately rows 1 through 5 and to rows 8 through 11 posterior to the MF (arrowhead). Similar results were obtained with probes transcribed from two distinct fragments of the longest *spry* cDNA (1.0 kb fragment shown). Sense transcripts of either fragment did not hybridize to wild-type discs (data not shown). (B) The expression pattern of SPRY protein in eye discs of third instar larvae was determined using a polyclonal rabbit antiserum raised against full-length SPRY protein (Hacohen et al., 1998). SPRY protein is expressed at low levels in rows 1 through 3 posterior to the MF, and more strongly in rows 8 through 16 in apically localized clusters that coincide with the position of ommatidial clusters. In $spry^{226}/spry^{G5}$ eye discs, the SPRY antigen is present but is localized in a more diffuse pattern (C). Similar results were obtained with transheterozygote combinations of other *spry* EMS alleles (data not shown). In $spry^{\Delta 5}$ eye discs, SPRY antigen expression is below detection levels (D). Similar results were obtained with an antiserum directed against the N-terminal portion of SPRY protein (data not shown). (E) Confocal double labeling for SPRY (in red) and the apical membrane marker Armadillo (Peifer and Wieschaus, 1990; in green) detected the SPRY antigen in the vicinity of the neuronal cells of the ommatidial precluster that are outlined by the ARM protein. (F) In a Z-section (18 optical sections of 0.5 μm each), SPRY was detected in the apical region of the eye imaginal disc in the same focal plane as ARM protein. (G) Enhancer trap line 9143 shows β-galactosidase expression in all PRCs and in the cone cells. 9143 is expressed strongly in R2/R5 and at lower levels in R8 in about six rows posterior to the morphogenetic furrow (H). Low levels of β-galactosidase expression were detected in R3, R4, R1 and R6 (I). Starting in row 8, high levels of β-galactosidase expression were seen in the R7 cell and moderate levels in the cone cells (J). In addition, we detected β-galactosidase expression in glia in the basal region of the eye imaginal disc (data not shown). In the pupal eye disc, 9143 is strongly expressed in the cone cells (K). (L,M) Expression of enhancer trap line 9143 is abolished in a $pointed^{8B}$ mutant clone (L) or in a $Ras1^{e2F}/Ras1^{C33}$ mutant eye disc (M).

signaling center and is dependent on the RTK pathway for its expression.

SPRY protein was found to be localized in a punctate pattern near the apical region of ommatidial clusters. These appear to be intracellular vesicles in cells neighboring photoreceptor neurons (Fig. 5B,E,F). They might be secretory vesicles in cells that synthesize the protein or uptake vesicles that receive it. No SPRY immunoreactivity was detected in $spry^{\Delta 5}$ imaginal discs, confirming the specificity of the antisera (Fig. 5D). In five EMS-induced mutants that are predicted to produce C-terminally truncated SPRY proteins (Hacohen et al., 1998), the SPRY antigen was found throughout the cytoplasm (Fig. 5C), demonstrating that the C-terminal region of SPRY is required for apical localization or secretion of the protein.

spry mutations cause phenotypes in the embryonic central and peripheral nervous system similar to those seen with increased EGFR signaling

In addition to the eye imaginal disc, *spry* is expressed in several other developing tissues known to require EGFR for cell fate induction or tissue patterning. For example, *spry* is expressed in the anlage of the embryonic chordotonal organs, neural sensory structures that require EGFR signaling for their proper development (Lage et al., 1997; Okabe and Okano, 1997). *spry* RNA is expressed in cells that become chordotonal organ precursors (COPs), as well as cells that surround the COPs (Fig. 6C). In *spry* mutants, between 26% and 37% of hemisegments contained an extra chordotonal organ in the lateral cluster (Fig. 6D,E). This is the same phenotype seen in mutants for negative regulators of EGFR signaling, such as *argos*, *Gap1* and *yan* (Okabe and Okano, 1997; Okabe et al., 1996). Overexpression of *spry* resulted in the reduction in the number of chordotonal organs (Fig. 6F), similar to the effect of reduced EGF signaling. In contrast, mutations in all of the known FGF ligands and receptors (*breathless*, *heartless*, *branchless*) all had a normal number of chordotonal organs (data not shown). Thus it is unlikely that the *spry* phenotype is due to hyperactivation of FGF signaling in developing chordotonal organs.

spry is also expressed in several developing midline glial cells in each segment of the embryonic CNS (Hacohen et al., 1998). Both FGF and EGF pathways are required for development of the midline glia cells, the former for proper cell migration and the latter for cell survival (Dong and Jacobs, 1997; Klämbt et al., 1992; Scholz et al., 1997). We found that $spry^{-}$ embryos have extra midline glial cells (Fig. 6A,B), as do embryos in which EGFR signaling is hyperactivated by mutation in the *argos* gene (Scholz et al., 1997). These results suggest that *spry* antagonizes EGFR signaling in the developing midline glia and the chordotonal organs as well as in the eye.

Overexpression of *spry* in the developing wing and ovary mimics *Egfr* mutant phenotypes

We investigated whether SPRY can antagonize EGFR

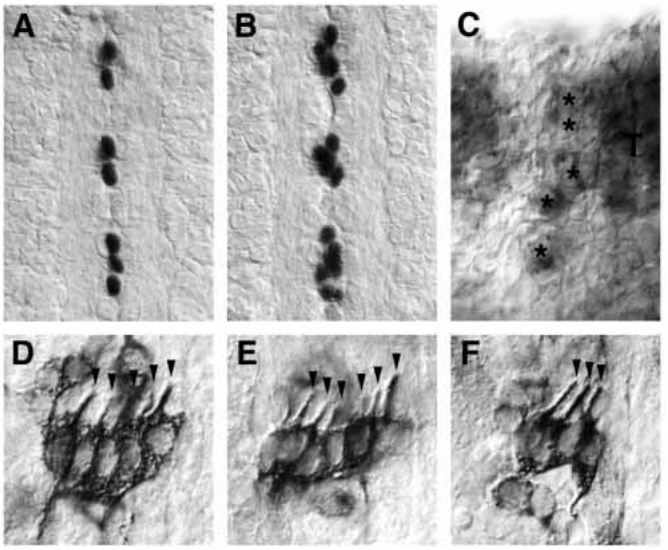


Fig. 6. *spry* phenotype in the midline glia and the chordotonal organ. (A,B) Midline glia of wild-type (A) and $spry^{\Delta 5}$ embryos (B), visualized using enhancer trap line AA142. In segments T1 to A7, wild-type embryos had 2.98±0.56 midline glia per segment (n=49), whereas *spry* mutant embryos had 4.74±0.74 (n=35). (C) Expression of *SPRY* mRNA in the region that forms chordotonal organ precursors (COPs). The position of five COPs that have already delaminated are indicated by asterisks. These cells express SPRY, but are out of the plane of focus. *SPRY* mRNA is present in a group of cells that surround these five COPs. Three additional COPs will delaminate from this group of cells. *SPRY* mRNA can be detected in the tracheal pit (T) at stage 11 and is expressed in the region of primary branches at stage 12 on, as previously described (Hacokhen et al., 1998). (D-F) The effect of *spry* loss- and gain-of-function on chordotonal organ development. Embryos were stained with mAb 22C10, which recognizes all peripheral neurons. In wild-type embryos, the lateral cluster contains five chordotonal organs with one neuron each (D). In $spry^{\Delta 5}$ embryos 26% of hemisegments (n=68) contain an extra chordotonal organ in the lateral cluster (E). In $spry^{254}$ embryos, this phenotype was seen in 37% of hemisegments. Overexpression of *spry* in *en-GAL4/UAS-spry* embryos causes a reduction of the number of chordotonal organs per hemisegment (F). The positions of the neurons of the lateral chordotonal organ are indicated by arrowheads.

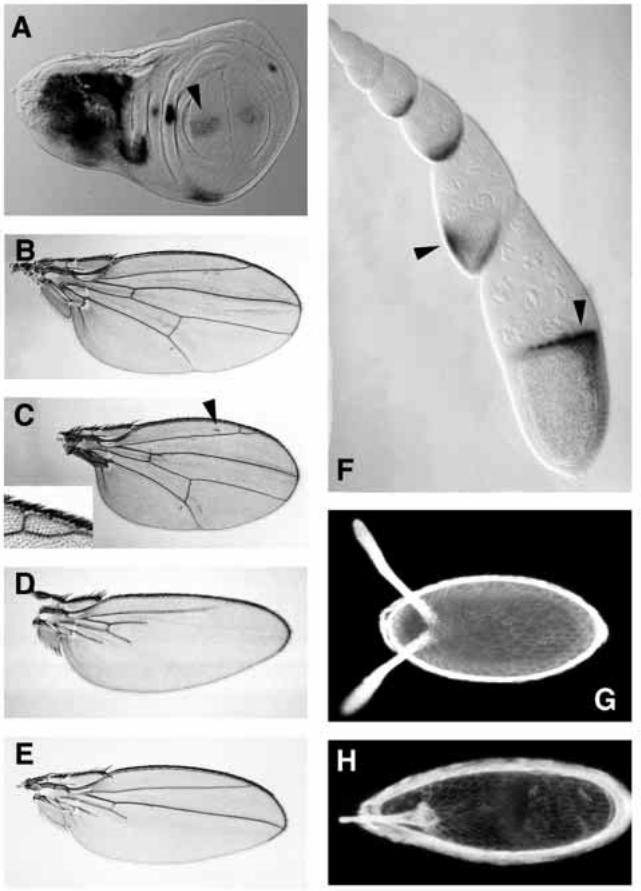
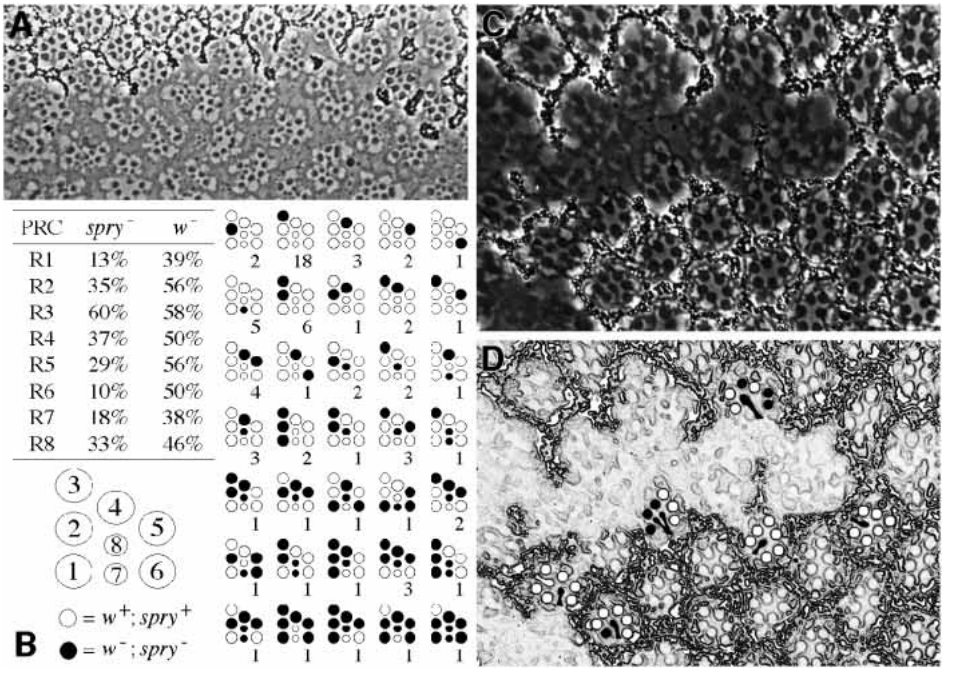


Fig. 7. *spry* overexpression in the wing and in follicle cells. (A) *spry* enhancer trap line 9143 expression in the wing imaginal disc. *spry* is expressed in the prospective notum, proximal wing and the central region of the wing blade (arrowhead). A similar expression pattern was seen with *SPRY* mRNA (data not shown). (B-E) The effect of *spry* loss- and gain-of-function on wing vein patterning. (B) Wild-type wing. (C) *spry* mutant wings often have extra wing vein material (arrowhead), especially in the distal region of L2 (inset). Animals overexpressing *spry* (BH1/*UAS-spry*) lack most wing veins (D). In addition the size of the wing is decreased. *en-GAL4/UAS-spry* animals lack veins in the posterior compartment (E). (F) *spry* enhancer trap expression in the ovarian follicle cells. *spry* is expressed first in the posterior follicle cells, then in the dorsal-anterior follicle cells (arrowheads). (G,H) The effect of *spry* misexpression in the follicle cells. In wild-type embryos, two dorsal appendages arise from the dorsolateral follicle cells (G). Overexpression of *spry* in all follicle cells using *GAL4*-line CY2 causes a ventralization of the egg shell, resulting in eggs with a fused thin dorsal appendage (H). The embryos developing from these eggs are likewise weakly ventralized.

signaling in two other tissues where EGFR signaling is known to operate, the developing wing and ovary. In the wing imaginal disc, *spry* enhancer trap line 9143 is expressed in the presumptive notum, in clusters of cells that give rise to sensory organs in the proximal wing and in the central region of the wing blade (Fig. 7A). $spry^{-}$ mutant wings showed minor vein defects, with occasional extra vein material (Fig. 7C). When *spry* was overexpressed in the wing imaginal disc, wing veins failed to form in the region of *spry* overexpression (Fig. 7D,E). In addition, there was a reduction in the size of the wing blade, indicating a reduction in the rate of cell proliferation. These phenotypes are similar to the ones seen with loss of EGFR function (Clifford and Schüpbach, 1989) or in the absence of the EGFR ligand Vein (Simcox et al., 1996).

In the developing ovary, the *spry* enhancer trap line is expressed first in the posterior-most follicle cells, and then in the dorsal anterior follicle cells overlying the oocyte nucleus (Fig. 7F). These are precisely the regions where EGFR is activated by its ligand Gurken (Gonzalez-Reyes et al., 1995; Roth et al., 1995), suggesting that *spry* expression is induced by EGF signaling. When *spry* was ectopically expressed in all follicle cells, the resultant eggs had a ventralized egg shell and the embryos that developed from the eggs were likewise ventralized (Fig. 7G,H; data not shown). The *spry* overexpression phenotype mimics the loss of EGFR function in the ovary (Schüpbach, 1987), indicating that SPRY antagonizes EGFR signaling in the follicle cells.



PRC	*spry*⁻	*w*⁻
R1	13%	39%
R2	35%	56%
R3	60%	58%
R4	37%	50%
R5	29%	56%
R6	10%	50%
R7	18%	38%
R8	33%	46%

2	18	3	2	1
5	6	1	2	1
4	1	2	2	1
3	2	1	3	1
1	1	1	1	2
1	1	1	3	1
1	1	1	1	1

Fig. 8. Genetic mosaic analysis of *spry* in the eye imaginal disc. (A) Section of a mosaic retina containing a $spry^{254}$ clone, marked with the *white* mutation. Note that the $spry^-$ mutant phenotype is not rescued at the boundary of the mosaic clone. (B) Results of a mosaic analysis on $spry^{254}$ mutant clones. The genotypes of individual PRCs were identified by the presence (w^+, $spry^+$) or absence (w^-, $spry^-$) of pigment granules visible as black dots at the base of rhabdomeres. A total number of 79 mosaic ommatidia of wild-type construction were scored. The table shows the frequencies of w^-, $spry^-$ PRCs in ommatidia mosaic for *spry* and *w* (first column, $spry^-$), and of w^-, $spry^+$ PRCs in control clones mosaic for *white* (second column, w^-). The genotypes of the 79 mosaic ommatidia are depicted graphically. Photoreceptor cells R1, R6 and R7 showed an increased requirement for *spry* function in mosaic ommatidia of wild-type construction. Note however that, in two ommatidia, R1, R6, and R7 were $spry^-$ and, in an additional five ommatidia, two of these three cells were $spry^-$. (C,D) Section (C) and tracing (D) of a sev^{d2}, $spry^{\Delta 5}$ mosaic clone induced in a sev^{d2} eye. Ommatidia with one or more R7 cells and the normal number of outer PRCs were scored. w^+, $spry^+$ PRCs are outlined in white and w^-, $spry^-$ PRCs in black. Of 140 R7 cells examined, 137 (97.9%) were w^-, $spry^-$ and 3 (2.1%) were w^+, $spry^+$. Of 99 ommatidia scored, 3 (3.0%) had a $spry^+$ R7 cell. In the eyes of control animals of the same genotype raised without heat shock, 0.2% of ommatidia had an extra R7 cell. The frequency of $spry^+$ R7 cells seen in the mosaic analysis and the frequency of R7 cells seen in the control were distinct with $P<0.01$ (Students *t*-test). 16 ommatidia were wild-type in appearance, i.e. one R7 cell projected into the center of the ommatidium in the correct position relative to the R8 cell. The frequency of $spry^+$ PRCs in these ommatidia (R1, 56%; R2, 87%; R3, 75%; R4, 75%; R5, 75%; R6, 50%; R7, 0%; R8, 87%) was comparable to the frequency of $spry^+$ PRCs (R1-R6, 66%; R8, 71%) in ommatidia with multiple R7 cells.

SPRY acts in parallel to Argos in antagonizing the EGFR pathway

Many of the processes that are controlled by EGFR signaling are antagonized by the extracellular factor Argos (reviewed in Schweitzer and Shilo, 1997). We therefore tested the genetic relationship between *spry* and *argos*. Overexpression of Argos using a *GMR-argos* transgene causes apoptosis of photoreceptor neurons and a reduced eye structure (Sawamoto et al., 1998). By contrast, *GMR-argos*; $spry^-$ animals have normal-sized eyes that contain excess photoreceptor neurons, similar to *spry* mutants (Fig. 4E-L). Since the effects of overexpression of Argos are ameliorated in the absence of *spry* function, SPRY acts either downstream or parallel to Argos. If Argos activity was mediated solely by SPRY, we would expect that removal of Argos would not affect the *spry* mutant phenotype. However, $spry^-$, $argos^-$ double mutant ommatidia exhibit massive neuronal differentiation with each ommatidium containing more than a dozen neurons (Fig. 2C). Thus *argos* and *spry* are unlikely to act in series, but have parallel and partially redundant functions in antagonizing EGFR signaling.

spry can function mainly autonomously to prevent cone cells from becoming R7 cells

As SPRY appears to function as an intercellular signal in the developing tracheal system, we asked whether SPRY could act at a distance in the developing eye. Initially, we examined ommatidia located at the boundary of $spry^-$ clones in the eye. The ommatidial phenotype changed abruptly at the boundary (Fig. 8A), indicating that SPRY does not act over long distances.

To determine which cells require $spry^+$ function for the construction of phenotypically wild-type ommatidia, we carried out a mosaic analysis on $spry^-$ clones, using a $white^+$ transgene to mark $spry^+$ photoreceptor neurons. Since there are no lineage restrictions during ommatidial assembly (Lawrence and Green, 1979; Ready et al., 1976; Wolff and Ready, 1991b), some ommatidia at the clone border are composed of both $spry^+$ and $spry^-$ cells. We found that any photoreceptor cell can be genotypically $spry^-$ in normally constructed ommatidia, indicating that there is no absolute requirement for $spry^+$ function in any of the photoreceptor neurons (Fig. 8B). However, all photoreceptor cells except R3 had a decreased probability of being $spry^-$. This suggests that there is redundancy among photoreceptor cells in the requirement for $spry^+$ function, and/or that there is a requirement for *spry* in non-neuronal cells that are related to the photoreceptor cells, such as the cone cells.

To determine whether *spry* is required in the cone cells that are transformed into R7 neurons, we performed a mosaic analysis on $spry^-$ clones in a sev^- background. Since sev^- ommatidia lack the endogenous R7 cell, all R7 cells developing in the mutant clone must arise as a consequence of the absence of *spry* function. In 15 retinae containing sev^-, $spry^-$ mutant clones, 99 mosaic ommatidia with six outer photoreceptor cells and one or more R7 cells were scored (Fig. 8C,D). A vast

majority of the R7 cells (97.9%) were *spry*$^{-}$, indicating that the requirement for *spry*$^{+}$ function is mainly cell autonomous in the cells that differentiate as R7. However, three of the 140 R7 cells (2.1%) were *spry*$^{+}$ in genotype. This percentage is significantly higher than the 0.2% expected from the dominant effect of *spry* in the same heterozygous genotype but in which homozygous clones were not induced. (see Methods). Thus, *spry* can also function non-autonomously to prevent cone cells from differentiating as R7 photoreceptor neurons.

DISCUSSION

SPRY can antagonize EGF as well as FGF signaling pathways

SPRY functions as an antagonist of the Branchless FGF signaling pathway in embryonic tracheal development (Hacohen et al., 1998). We have provided several lines of evidence that, in several other tissues, SPRY functions as an antagonist of EGFR signaling. First, *spry* loss-of-function mutations cause phenotypes in the developing adult eye (extra photoreceptor neurons), the larval peripheral nervous system (extra chordotonal organs) and embryonic CNS (extra midline glial cells) that closely match those caused by hyperactivation of EGF signaling pathways. Second, overexpression of *spry* closely mimics the effect of EGFR pathway mutations in the developing eye as well as in the developing wing and ovary. Third, *spry* is expressed at the same time and in close proximity to the EGF signaling pathways that it appears to antagonize in these tissues. In contrast, no FGF ligand or receptor has been implicated in the processes affected in these tissues. Although not all FGF pathway genes have been examined in each tissue, we examined the three extant ligand and receptor genes (*breathless*, *heartless*, *branchless*) in chordotonal organ development and found that none of the three had any detectable role. Furthermore, in the midline glial cells, where the Breathless FGF receptor is known to function, it is required for a glial cell migration process that is distinct from the glial cell survival pathway affected by *spry* and EGFR pathway mutations. Although we cannot exclude the possibility that *spry* antagonizes the EGF pathway indirectly via an effect on an unknown FGF signaling pathway, the simplest interpretation of our results is that SPRY functions as an antagonist of EGF and FGF signaling pathways in different tissues.

One of the interesting aspects of *spry* function in the developing trachea is that expression of the gene is induced by the signaling pathway that it inhibits (Hacohen et al., 1998). This negative feedback circuit serves to restrict the range of FGF signaling, so that only the cells located close to the FGF signaling center are induced by the FGF pathway. In the tissues examined here, there appears to be a similar coupling of *spry* expression and the signaling pathway that it antagonizes. In the developing eye, *spry* is expressed at the highest levels in photoreceptor cells located closest to the EGF signaling center and at lower levels in the photoreceptors located farther away, and loss-of-function mutations in EGF signaling pathway components result in the absence of *spry* expression. In the developing ovary, *spry* is expressed in the follicle cells directly adjacent to the Gurken EGF signaling centers in the oocyte, suggesting that, in this tissue too, EGF signaling induces expression of the SPRY inhibitor. In the developing eye, absence of *spry* appears to result in overactivity of the EGF pathway and neuronal induction of non-neuronal cells (mystery and cone cells) that are located beyond the normal range of the EGF inductive signal.

In all three systems in which we have shown a requirement for *spry*, the loss of *spry* function results in supernumerary neurons or glia, respectively. While the midline glia normally show some variability in the number of glial cells that escape cell death (Sonnenfeld and Jacobs, 1995), the number of photoreceptor cells per ommatidium and the number of chordotonal organs per hemisegment is invariant. This invariance is achieved, at least in part, through the tight control of induction and its response. Since *spry* appears to be expressed as a primary response to the signal, a reduction in the inducing signal would likely result in reduced expression of the antagonist SPRY. This feedback loop would buffer the inductive response against small changes in the levels of the inducing signal.

Argos is another extracellular antagonist of EGF signaling. Like *spry*, its expression is induced by the EGF pathway that it inhibits (Golembo et al., 1996). The two antagonists are expressed in similar patterns in several developing tissues including the eye, the midline glia and the ovary, suggesting that they often function together to restrict EGF signaling activity. In fact, we have shown that *spry* and *argos* exhibit a synergistic phenotype in the eye. Despite these commonalities, there are a number of significant distinctions between the two antagonists. First, in mosaic retinae, *argos*$^{+}$ cells can rescue the *argos*$^{-}$ mutant phenotype over distances spanning several ommatidia, implying that Argos can act at long range (Freeman et al., 1992b). In contrast, SPRY action appears to be limited to the cells that express the protein or its close neighbors. Secondly, Argos appears to affect only EGF signaling, whereas our data indicate that SPRY can antagonize EGF and FGF signaling in different contexts. Thirdly, while Argos shows some limited structural similarity to EGF ligands and other proteins with EGF-like motifs, SPRY contains a novel cysteine-rich sequence motif, suggesting that the mode of action of these two proteins are distinct.

spry function in the eye has both autonomous and non-autonomous aspects

Genetic analysis of *spry* function in the trachea has shown that *spry* functions non-cell-autonomously to block FGF induction of tracheal branching: *spry* function is required in branching cells to prevent its non-branching neighbors from likewise elaborating secondary branches (Hacohen et al., 1998). Interestingly, our analysis of *spry* function in the eye indicated that *spry* acts mainly autonomously. We found that in most mosaic ommatidia cone cells needed to be *spry*$^{-}$ in order to aberrantly differentiate as R7 photoreceptor neurons although, in a few exceptional cases, *spry*$^{+}$ cells differentiated as R7 cells indicating that the nearby *spry*$^{-}$ cells had influenced their development. It is possible that the autonomous action of SPRY occurs inside the *spry*-expressing cells, for example while SPRY is in transit to the plasma membrane. However, given the results obtained in the tracheal system, we propose that in both tissues SPRY acts as a secreted antagonist of RTK signaling but, in the eye, it has mainly autocrine effects while in the tracheal system its effects are predominantly paracrine. The differences might be due to differences in the extracellular environments of the two tissues, which might affect release or

diffusion of the protein. Alternatively, these differences might be due to differences in the pathways responsible for SPRY reception or signal transduction in the two tissues (see below), or to differences in the pathways (EGF versus FGF) that are inhibited by SPRY action.

Implications for SPRY protein mechanism of action

Our results have important implications for the mechanism of SPRY action. Previously, we presented three possible models of how SPRY might antagonize the Branchless FGF pathway in the developing tracheal system. One was by direct binding or blockage of the FGF ligand. Another was by binding or blockage of the FGF receptor Breathless. A third model postulated a separate SPRY receptor on the receiving cells that antagonized the FGF pathway downstream of the FGF receptor in the receiving cells. Our data indicating that SPRY antagonizes both FGF and EGF pathways supports the third model. Because the structures of the two types of ligands (EGF and FGF) and the extracellular portions of their receptors do not show any striking sequence similarities, this argues against the first two models, which invoke direct interaction between SPRY protein with FGF or EGF ligands or receptors. However, the intracellular portions of the EGF and FGF receptors and the downstream signal transduction pathways show significant similarities. Thus, it is easy to imagine how SPRY interaction with its own receptor on a receiving cell could lead to inhibition of a common downstream step in the FGF and EGF signaling cascades. If SPRY acts through its own receptor, rather than by directly antagonizing the FGF or EGF receptors, then it is also easy to see how differences in autocrine versus paracrine activity of SPRY in different tissues could arise by differences in expression or activity of its receptor.

In summary, we have described that SPRY is a novel antagonist of EGFR- as well as FGFR-mediated signaling. These RTK signaling pathways therefore share not only intracellular signaling components such as the ras GTPase and MAPK, but also the extracellular antagonist SPRY. The ability of SPRY to regulate the activities of these two RTKs may provide a new way to allow refinement and cross-talk between these important developmental signaling pathways.

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Note added in proof

While this paper was in review, the following article appeared that proposes an alternative mode of action for Sprouty. Results of our mosaic analysis in the eye is also consistent with their interpretation.

Casci, T., Vinos, J. and Freeman, M. (1999). Sprouty, an intracellular inhibitor of Ras signaling. *Cell* **96**, 655-665.