Development 133, 1133-1142 (2006) doi:10.1242/dev.02255

Sprouty proteins are in vivo targets of Corkscrew/SHP-2 tyrosine phosphatases

Lesley A. Jarvis[1,*,†], Stephanie J. Toering[1,*,‡], Michael A. Simon[2], Mark A. Krasnow[1,¶] and Rachel K. Smith-Bolton[2,*,§]

Drosophila Corkscrew protein and its vertebrate ortholog SHP-2 (now known as Ptpn11) positively modulate receptor tyrosine kinase (RTK) signaling during development, but how these tyrosine phosphatases promote tyrosine kinase signaling is not well understood. Sprouty proteins are tyrosine-phosphorylated RTK feedback inhibitors, but their regulation and mechanism of action are also poorly understood. Here, we show that Corkscrew/SHP-2 proteins control Sprouty phosphorylation and function. Genetic experiments demonstrate that Corkscrew/SHP-2 and Sprouty proteins have opposite effects on RTK-mediated developmental events in *Drosophila* and an RTK signaling process in cultured mammalian cells, and the genes display dose-sensitive genetic interactions. In cultured cells, inactivation of SHP-2 increases phosphorylation on the critical tyrosine of Sprouty 1. SHP-2 associates in a complex with Sprouty 1 in cultured cells and in vitro, and a purified SHP-2 protein dephosphorylates the critical tyrosine of Sprouty 1. Substrate-trapping forms of Corkscrew bind Sprouty in cultured *Drosophila* cells and the developing eye. These results identify Sprouty proteins as in vivo targets of Corkscrew/SHP-2 tyrosine phosphatases and show how Corkscrew/SHP-2 proteins can promote RTK signaling by inactivating a feedback inhibitor. We propose that this double-negative feedback circuit shapes the output profile of RTK signaling events.

KEY WORDS: Sprouty (Spry), Corkscrew (Csw), Ptpn11 (SHP-2), Tyrosine phosphatase, Receptor tyrosine kinase (RTK) signaling, *Drosophila*

INTRODUCTION

RTK signaling pathways regulate many cellular and developmental events, including respiratory system branching and photoreceptor differentiation in *Drosophila*. The proper magnitude and duration of signaling is crucial for such events, because altering these parameters by constitutively activating an RTK pathway or altering its kinetics can lead to different outcomes (Marshall, 1995). Indeed, many RTK pathways are equipped with positively acting regulators and negative feedback loops, the balance of which dictates the cellular response (Perrimon and McMahon, 1999). Two of the best known but least well understood classes of regulators are the Corkscrew/SHP-2 family of tyrosine phosphatases that promote RTK signaling and the Sprouty family of RTK feedback inhibitors, whose genetic and molecular interactions we explore here.

corkscrew was identified by its requirement for development of the terminal regions of the embryo. It encodes an SH2 domain-containing protein tyrosine phosphatase that functions downstream of, and promotes signaling through, the RTK Torso (Perkins et al., 1992). A vertebrate homolog, SHP-2 (now known as Ptpn11), was found to promote PDGFR signaling (Freeman et al., 1992; Bennett et al., 1994). Further work demonstrated that Csw and SHP-2 promotes signaling downstream of many RTKs in a variety of systems (Perkins et al., 1996) (reviewed by Feng, 1999), and that this activity requires their tyrosine phosphate-binding SH2 domains and tyrosine phosphatase activity (Allard et al., 1998; Deb et al., 1998).

How can tyrosine phosphatases promote tyrosine kinase signaling? To account for their positive effect on signaling, Csw and SHP-2 were postulated to dephosphorylate either a positive RTK effector that is inactivated by phosphorylation or a negative regulator that is activated by phosphorylation (Stein-Gerlach et al., 1998; Huyer and Alexander, 1999). This initiated searches for Csw/SHP-2 substrates by genetic screens (Herbst et al., 1996; Firth et al., 2000), biochemical screens using substrate-trapping forms of the phosphatases (Herbst et al., 1996; Agazie and Hayman, 2003a), and tests of candidate proteins. These identified several signal transduction scaffolding proteins, including DOS, GAB1, GAB2, SHPS1 and IRS1 (Qu, 2002). However, dephosphorylation of these substrates by Csw/SHP-2 inhibits RTK signaling, so they cannot account for the positive effects of Csw/SHP-2.

Two other types of substrates may account for some positive effects of Csw/SHP-2 on RTK signaling. One is the phosphotyrosines on Torso, PDGFR and EGFR that mediate binding and signal inhibition by RasGAP (Cleghon et al., 1998; Ekman et al., 2002; Agazie and Hayman, 2003b). The other is the CSK-binding proteins Paxillin and PAG/Cbp, dephosphorylation of which prevent CSK-mediated inactivation of Src kinase activity and its positive effect on RTK signaling (Ren et al., 2004; Zhang et al., 2004). However, neither of these is likely to be a general target and other crucial substrates remain to be identified.

Several observations make Sprouty proteins appealing candidates for Csw/SHP-2 substrates. First, although Sprouty (Spry) was identified by its role as an Fgf feedback inhibitor in *Drosophila* tracheal (respiratory) development (Hacohen et al., 1998), Spry and its four mammalian homologs, Spry1-Spry4, are now known to function more broadly as RTK feedback inhibitors that can regulate a variety of RTK pathways including FGF, EGF, VEGF and PDGF pathways (Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999;

[1]Department of Biochemistry and Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305-5307 USA. [2]Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020 USA.

*These authors contributed equally to this work
[†]Present address: Department of Radiation Oncology, Stanford University School of Medicine, Stanford, CA 94305-5307, USA
[‡]Present address: Biology Department, Wartburg College, Waverly, IA 50677, USA
[§]Present address: Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA
[¶]Author for correspondence (e-mail: krasnow@cmgm.stanford.edu)

Accepted 16 December 2005

Impagnatiello et al., 2001; Lee et al., 2001; Nutt et al., 2001), all of which are also regulated by Csw/SHP-2 (Perkins et al., 1996; Feng, 1999). Both Spry and SHP-2 influence RTK signaling kinetics (Saxton et al., 1997; Dikic and Giordano, 2003). Second, although the mechanism by which Spry proteins modulate signaling is not understood, like Csw/SHP-2 they localize to the membrane following signaling and associate with RTK signaling complexes (Allard et al., 1996; Herbst et al., 1999; Qu, 2000; Hanafusa et al., 2002). Third, cell culture studies of Spry1 and Spry2 demonstrate that they are phosphorylated on a conserved tyrosine in response to RTK signaling, and the modification is required for Spry function (Hanafusa et al., 2002; Hall et al., 2003; Rubin et al., 2003; Li et al., 2004; Mason et al., 2004).

Here, we investigate the relationship between Csw/SHP-2 and Sprouty in RTK pathways in vivo and in vitro. We establish a close functional relationship by manipulating gene activity during RTK signaling in *Drosophila* development and in cultured mammalian cells. We use the cell culture system to demonstrate that SHP-2 controls phosphorylation on the essential tyrosine of Spry1. Biochemical experiments show that SHP-2 associates in a complex with Spry1 and can dephosphorylate the essential tyrosine. Substrate-trapping forms of Csw bind Spry in cultured *Drosophila* cells and during development. The results demonstrate that Spry proteins are targets of Csw/SHP-2 tyrosine phosphatases in *Drosophila* and vertebrate RTK pathways, and explain how Csw/SHP-2 can enhance RTK signaling by inactivating a feedback inhibitor. While this manuscript was in preparation, complementary work showing that SHP-2 can reduce tyrosine phosphorylation on a Spry protein in PC12 cells was reported (Hanafusa et al., 2004).

MATERIALS AND METHODS

Drosophila stocks and histology

spry$^{\Delta 5}$ null allele was used (Hacohen et al., 1998). *csw* transgenes under *sevenless* enhancer control were used for Csw misexpression during eye development: *SE-csw*^{G547E}, *SE-csw*^{C583S}, *SE-csw*^{src90}, *SE-csw*^{G547Esrc90} and *SE-csw*^{C583Ssrc90} (Allard et al., 1996; Allard et al., 1998). We refer to *csw*^{src90} as *myr-csw*. Protein misexpression with GAL4/UAS system (Brand and Perrimon, 1993) used *sev-GAL4* (Brand and Perrimon, 1993), *btl-GAL4* (Shiga et al., 1996), UAS-GFP (Jarecki et al., 1999), UAS-*csw*^{src90} (Johnson Hamlet and Perkins, 2001) and UAS-*spry* (Hacohen et al., 1998). UAS-*spry*^{Y201F} was made by introducing a TAT>TTT mutation in pUAST-*spry* (Hacohen et al., 1998) and establishing transgenic lines by P element transformation; homozygous viable insertions on chromosome II were used (insertions 13, 15, 37). Crosses were carried out at 25°C.

Tracheae were visualized by fluorescence microscopy of heat-killed (65°C for 5 seconds) third instar larvae carrying FRT2A and viable insertions of *btl-GAL4* and UAS-*GFP* on chromosome II. Sections (2 μm) through adult eyes were prepared as described (Tomlinson and Ready, 1987).

Cell culture and transfections

HEK293 cells (ATCC #CRL-1573) were grown in a humidified chamber containing 5% CO_2 at 37°C in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Invitrogen). For transfections, 2×10^5 log phase cells were cultured in 35 mm plates and transfected with 0.5 μg of each expression plasmid using Fugene 6 lipid transfection reagent (Invitrogen). Total DNA per transfection was 1 or 2 μg depending on number of plasmids used; empty vector was used to keep total DNA constant.

Drosophila S2 cells were maintained at 22°C in M3 insect medium (Sigma) or Schneider's medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum. Stably transfected S2 cell lines expressing Csw, Csw^{C583S} and Breathless with C-terminal FLAG epitope (Herbst et al., 1996; Toering, 2003) were maintained in medium containing 200 μg/ml hygromycin.

Expression plasmids

Expression plasmids for HEK293 cells were: pRc/CMV/SHP-2 and pRc/CMV/SHP-2^{C459S} (Paul Khavari, Stanford) with CMV promoter driving expression; pEFBOS/mFGFR3 and pEFBOS/mFGFR3^{K644E} (thanatophoric dysplasia type II mutation) (Su et al., 1997) with human EF-1α promoter; pMO/IRES/FGFR1c (David Ornitz, Washington University) with MoLTR; and pBJ5/ERK2-HA (Gerald Crabtree, Stanford) with SRα promoter. pTA/HA-Spry1, with CMV promoter driving expression of mouse Spry1 with N-terminal HA epitope (YPYDVPDYA), was constructed by PCR amplification of Spry1 cDNA (Minowada et al., 1999) using a forward primer encoding initiator methionine, HA epitope and residues 2-6 of Spry1, and insertion into pTA vector (Invitrogen). Spry1^{Y53F} (TAC>TTC) and Spry1^{Y89F} (TAC>TTC) mutations were introduced into pTA/HA-Spry1 by site-directed mutagenesis. pCDNA/mFL-Spry1 was constructed as above except FLAG epitope (DYKDDDDK) and pCDNA vector (Invitrogen) were used. pCDNA/SHP-2-V5 was constructed in similar manner by amplifying SHP-2 sequence in pRc/CMV/SHP-2 with reverse primer encoding V5 epitope (GKPIPNPLLGLDST). Coding sequences of constructs were verified by DNA sequencing.

Antisera production

Rabbit antisera were raised against an N-terminal peptide of *Drosophila* Sprouty (residues 19-37, LPRVHRPRAPEPTLSGVDH) and against a C-terminal peptide (574-591, RKGDLTPEKRLLDSSPDY) (Biosynthesis, Lewisville, TX). Sera were affinity purified on columns containing the immobilized peptide and used at 1:1000 dilution (immunoprecipitation) or 1:2000-1:5000 (immunoblots). Rabbit anti-pY53 mouse Spry1 antiserum was raised against phosphopeptide GCGSNEpYTEGPSVARRPAPR that includes Spry1 residues 49-66. Eight week post-immunization bleed was used at 1:2000.

Erk2 assay

Twenty-four hours after transfection of HEK293 cells with pBJ5/ERK2-HA, human recombinant bFGF (Invitrogen) was added to the medium to 25 ng/ml. At times indicated after bFGF addition, cells were rinsed with PBS and lysed with 0.4 ml RIPA buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease inhibitors (Complete-Mini, Roche) and 0.2 mM sodium vanadate to inhibit tyrosine phosphatases. HA-ERK2 was immunoprecipitated by incubating lysate at 4°C with 10 μg anti-HA mAb (Roche). After 3 hours, 35 μl protein A agarose (Sigma) was added and incubation continued for 1 hour. Immunoprecipitate was washed three times at 4°C with 1 ml NP40 wash buffer (1% NP40, 1 mM EDTA) and once with 1 ml TNE (10 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA), boiled in Laemmli loading buffer and resolved on 10% SDS-PAGE gel. Gel was probed with anti-dpERK mAb (Sigma), HRP-conjugated secondary antibody and HRP chemistry (Enhanced Chemiluminescence, Amersham). dpERK levels were quantitated by scanning densitometry of fluorograms. Blots were reprobed with anti-HA to detect total ERK2-HA. Expression of other proteins was monitored by SDS-PAGE and immunoblotting of aliquots of cell lysates.

Spry phosphorylation analysis

HEK293 cells were transfected with pTA/HA-Spry1, pMO/IRES/FGFR1c and the plasmids noted. Twenty-four hours later, bFGF was added to medium at 200 ng/ml. After 30 minutes, cells were lysed and HA-Spry1 was immunoprecipitated by incubation with 35 μl anti-HA agarose (Santa Cruz Biotechnology) at 4°C for 4 hours. Immunoprecipitates were washed and separated by SDS-PAGE. Immunoblots were probed with anti-phosphotyrosine mAb (Upstate Biotechnology) and HRP immunochemistry. Same conditions were used for analysis of Spry1 Y53 phosphorylation, except cells were treated with bFGF for times noted, HA-Spry1 was immunoprecipitated with anti-HA and immunoblots were probed with anti-pY53.

To identify Spry1 phosphotyrosines, 2×10^6 HEK293 cells were transfected with pTA/HA-Spry1 and pMO/IRES/FGFR1c. Forty-eight hours later, bFGF was added to 200 ng/ml. After 30 minutes, cells were lysed, HA-Spry1 was immunoprecipitated with anti-HA, resolved by SDS-PAGE, and stained with Coomassie Blue. HA-Spry1 band was excised, treated with

trypsin and proteolytic fragments were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and identified using Mascot MS/MS Ions Search (Mass Spectrometry Lab, Stanford, CA).

To analyze tyrosine phosphorylation of *Drosophila* Spry, 5×10^6 exponentially growing S2 cells expressing Breathless-FLAG were treated for 15 minutes with 0.1 mM pervanadate, then washed and lysed. Endogenous Spry was immunoprecipitated with anti-Spry C-terminal antiserum, resolved by SDS-PAGE and probed on immunoblots with anti-phosphotyrosine.

Coimmunoprecipitation assays

HEK293 cells were transfected with plasmids expressing SHP-2-V5, HA-Spry1 and FGFR3 or FGFR3^{K644E} to activate the FGF pathway. After 48 hours, cells were lysed with 0.4 ml NP40 lysis buffer (150 mM NaCl, 1% NP40, 50 mM Tris pH 8) containing protease inhibitors. SHP-2-V5 was immunoprecipitated with anti-V5 mAb (Invitrogen), washed and resolved by SDS-PAGE. HA-Spry1 in immunoprecipitate was detected on immunoblots probed with rabbit anti-HA (Santa Cruz Biotechnology).

To assay Csw-Spry association in stably transfected S2 cells expressing Csw or Csw^{C583S}, ~2×10^7 exponentially growing cells in M3 medium were harvested by centrifugation and lysed in 1 ml NP-40 lysis buffer containing protease inhibitors. Csw was immunoprecipitated at 4°C for 2-4 hours with 1 μl anti-Csw-CT (Allard et al., 1996) and 50% (v/v) protein G Sepharose beads (Sigma). Beads were washed three times with lysis buffer, boiled in Laemmli loading buffer and proteins resolved on 8% SDS-PAGE gels. Endogenous Spry that co-immunoprecipitated was detected on immunoblots probed with anti-Spry C-terminal. Spry immunoprecipitation was carried out as above using anti-Spry and protein A beads; co-immunoprecipitated Csw was detected with anti-Csw-CT.

For *Drosophila* imaginal discs, 100 pairs of third instar eye-antennal discs of each genotype were dissected into ice-cold PBS (Fig. 5E) or snap frozen and stored at –80°C, homogenized as above and cleared by centrifugation. Csw immunoprecipitation and subsequent analysis were as above.

GST pulldown assay

GST-SHP-2 fusion proteins were expressed in *E. coli* and purified as described (O'Reilly et al., 2000). To prepare lysates containing Spry1, HEK293 cells were transfected with pTA/HA-Spry1 and pEFBOS/FGFR3^{K644E} to induce HA-Spry1 phosphorylation. Cells were lysed in NP40 lysis buffer with protease inhibitors. Lysate was mixed with 100 μl of 50% (v/v) glutathione-Sepharose beads coated with a GST-fusion protein and incubated at 4°C. After three hours, beads were washed three times with NP40 wash and once with TNE. Proteins were separated by SDS-PAGE, and immunoblots were probed with anti-HA.

SHP-2 phosphatase assay

Phosphatase assay (O'Reilly et al., 2000) used purified GST-SHP-2 proteins. To prepare phosphorylated HA-Spry1 substrate, transfected HEK293 cells expressing FGFR1c and HA-Spry1 were treated with bFGF at 200 ng/ml and 0.1 mM pervanadate for 30 minutes. Cells were lysed in NP40 lysis buffer and HA-Spry1 was immunoprecipitated with anti-HA. Immunoprecipitates were washed with NP40 wash buffer and TNE, and resuspended in phosphatase buffer (25 mM HEPES 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM DTT). Phosphatase reactions (25 μl) containing phosphatase buffer, 50 ng GST-SHP-2 fusion protein, and substrate immunoprecipitated from 2×10^5 cells, were incubated at 37°C. After 30 minutes, Laemmli sample buffer was added, and products were resolved by SDS-PAGE. Immunoblots were probed with anti-phosphotyrosine, anti-pY53 or anti-HA. ERK2-HA substrate was prepared from pBJ5/ERK2-HA-transfected cells and analyzed on immunoblots with anti-dpERK.

RESULTS

Sprouty and Corkscrew/SHP-2 have opposing roles in RTK pathways in vivo

We compared the effects of Spry and Csw/SHP-2 on RTK signaling processes in two different *Drosophila* tissues and a human cell line. In the *Drosophila* tracheal system, Spry negatively regulates the Branchless (Bnl) FGF pathway that controls branch sprouting. In *spry*$^-$ mutants, this RTK pathway is overactive and extra cells become terminal cells and form ectopic branches (Hacohen et al., 1998). *csw*$^-$ mutations also cause tracheal defects among other embryonic defects (Perkins et al., 1996; Firth et al., 2000), and we found that *csw*^{G547E}, a dominant-negative allele (Allard et al., 1996) (Fig. 1A), enhanced the penetrance of ganglionic branch outgrowth defects in *bnl* heterozygous embryos (data not shown), as does partial inactivation of other Bnl pathway genes (Sutherland et al., 1996). Furthermore, expression of myr-Csw, an activated membrane-localized form of Csw containing the c-Src myristylation site (Allard et al., 1996), in the developing tracheal system using a *btl-GAL4* driver induced ectopic terminal cells and branches, just like *spry* null mutations (Fig. 1B-E). These results imply that Csw and Spry have opposite effects on the Bnl RTK pathway.

In the developing eye, both *csw* and *spry* regulate EGFR and/or Sevenless RTK pathways that specify the pattern and fates of photoreceptors and neighboring cells (Allard et al., 1996; Allard et al., 1998; Casci et al., 1999; Kramer et al., 1999). Indeed, chromosomal deficiencies encompassing *spry* were identified in a screen for suppressors of the eye and wing vein phenotypes caused by a *csw* weak loss-of-function allele (Firth et al., 2000), indicating that these genes have opposing roles during RTK signaling in the developing eye and wing. Consistent with this, removing one copy of *spry* enhanced the phenotype of extra R7 photoreceptors caused by eye-specific expression of myr-Csw (Allard et al., 1996), increasing the number of R7 cells per ommatidium from 2.1±0.06 (mean ± s.e.m.) to 3.0±0.07 (Fig. 1F). Likewise, removing one copy of *spry* suppressed the loss of photoreceptor phenotype caused by eye-specific expression of dominant negative Csw^{G547E} (Allard et al., 1998), increasing the number of outer photoreceptors per ommatidium from 3.3±0.05 to 4.1±0.03 (Fig. 1G). Reducing *spry* dose did not suppress the effect of another dominant-negative Csw, Csw^{C583S} (Fig. 1H; 5.1±0.03 for *spry*$^+$ versus 4.8±0.03 for *spry*$^+$/*spry*$^{\Delta5}$). Csw^{C583S} is a substrate-trapping form of the enzyme that, unlike Csw^{G547E}, can bind its substrates, although it does not hydrolyze or release them (Herbst et al., 1996). We consider the implications of this result in the Discussion.

To compare effects of Spry and Csw/SHP-2 on RTK signal transduction, we assessed their effects on MAPK activation induced by FGF signaling in human embryonic kidney 293 (HEK293) cells, using an antiserum specific for diphosphorylated (activated) MAPK (dpERK). Transient transfection of a Spry1 expression plasmid reduced activation of MAPK in response to basic FGF (bFGF), as did transfection of a plasmid expressing dominant negative SHP-2^{C459S} (Fig. 1I). When both Spry1 and SHP-2^{C459S} were expressed, MAPK activation was almost completely abolished. Spry1 and SHP-2 both predominantly affected the duration of MAPK activation in response to bFGF, rather than the magnitude of the response, consistent with the idea that they regulate the same step in signal transduction (Fig. 1J). Thus, in all three systems investigated, Spry and Csw/SHP-2 proteins regulated the same RTK signaling processes but in opposite directions.

SHP-2 negatively regulates tyrosine phosphorylation on Spry1

To determine the regulatory relationship between Csw/SHP-2 and Spry during RTK signaling, the effect of SHP-2 on tyrosine phosphorylation of Spry1 was analyzed in HEK293 cells. FGF signaling induces tyrosine phosphorylation of Spry proteins in several cell lines including HEK293 cells (Hanafusa et al., 2002; Tefft et al., 2002) (Fig. 2B, lanes 1,4). If Spry1 is a substrate of

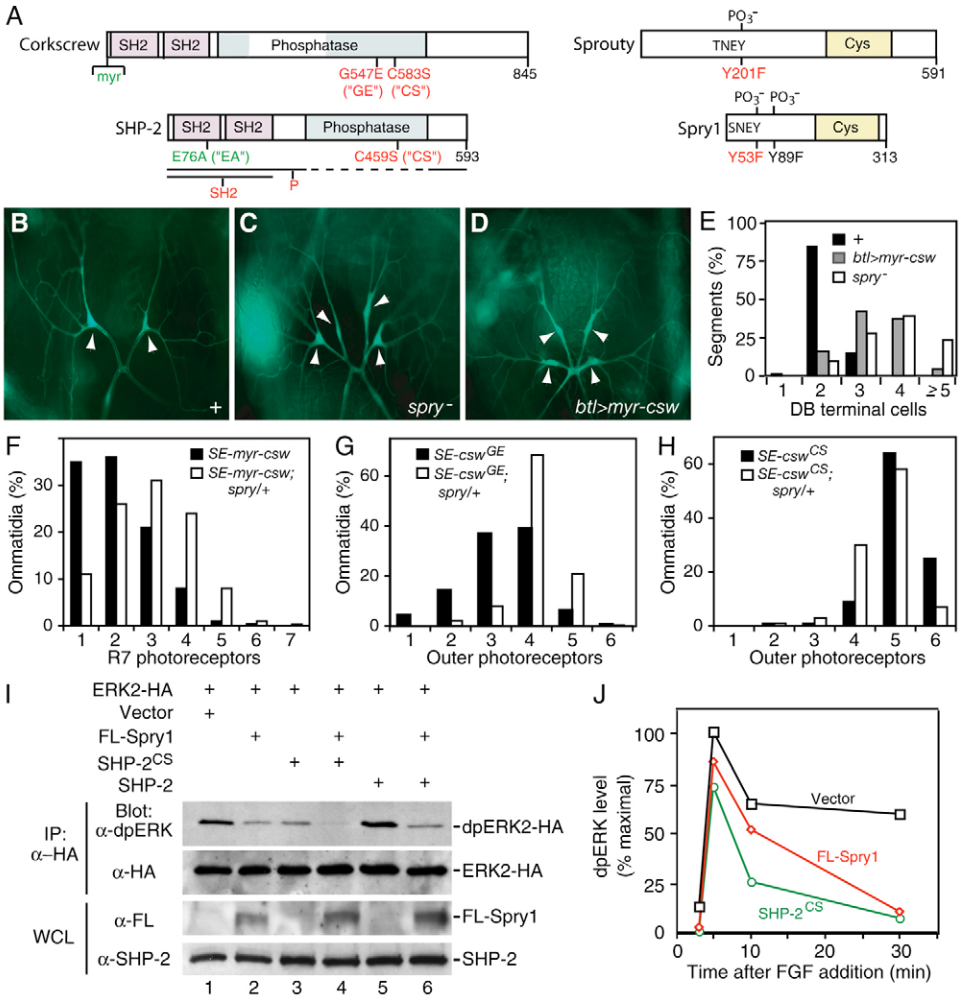

Fig. 1. Spry and Csw/SHP-2 have opposing roles during RTK signaling. (**A**) Csw, SHP-2 and Spry structures. Open bars, wild-type proteins. SH2 and phosphatase domains of Csw and SHP-2, and the T/SNEY amino acid motif and cysteine-rich domain of Spry proteins are indicated. White gap in Csw phosphatase domain indicates a non-conserved insertion. Mutant forms are indicated below bars: activating mutations (green), inactivating and dominant-negative mutations (red), and neutral mutations (black). myr, N-terminal 90 residues of Src64 including myristylation site. PO_3^-, phosphotyrosine. (B-E) Effects of *spry* loss of function and *csw* gain of function on tracheal development. (**B**) Fluorescence micrograph of ends of two dorsal branches (DB) from a control *spry*$^+$ third instar *w*; *btl-GAL4, UAS-GFP* larva expressing GFP throughout tracheal system. Dorsal view, anterior upwards. Terminal cells (arrowheads) extend branches anteriorly and laterally. (**C**) Same view of third instar *w*; *btl-GAL4,* UAS-*GFP*; *spry*$^{\Delta 5}$ larva showing extra terminal cells. (**D**) Same view of third instar *y,w*; UAS-*myr-csw*/*btl-GAL4,* UAS-*GFP* larva that expresses myristylated (activated) Csw throughout developing tracheal system. Extra terminal cells are present as in C. (**E**) Number of DB terminal cells per segment in genotypes shown in B-D. Mean values (±s.e.m.): *spry*$^+$ (2.2±0.04, *n*=123 segments), *spry*$^{\Delta 5}$ (3.8±0.1, *n*=133), *btl>myr-csw* (3.3±0.06, *n*=210). (F-H) Effect of *spry* dose on *csw* gain- and loss-of-function phenotypes in eye development. (**F**) Number of R7 cells per ommatidium in *SE-myr-csw*/+ flies expressing *myr-csw* in developing eyes (black bars, *n*=293 ommatidia), and in *SE-myr-csw*/+; *spry*$^{\Delta 5}$/+ flies (white bars, *n*=324). Wild type has one R7 cell per ommatidium. *myr-csw* effect increased when *spry* dose was reduced. (**G**) Number of outer photoreceptors per ommatidium in *SE-csw*^{G547E}/+ flies expressing dominant-negative Csw^{G547E} in developing eyes (black bars, *n*=292) and in *SE-csw*^{G547E}/+; *spry*$^{\Delta 5}$/+ flies (white bars, *n*=329). Wild type has six outer photoreceptors per ommatidium. The Csw^{G547E} effect was suppressed when *spry* dose was reduced. (**H**) Number of outer photoreceptors per ommatidium in *SE-csw*^{C583S}/+ flies expressing dominant-negative, substrate-trapping Csw^{C583S} protein in developing eyes (filled bars, *n*=582), and in *SE-csw*^{C583S}/*spry*$^{\Delta 5}$ flies (open bars, *n*=604). Csw^{C583S} effect was not suppressed when *spry* dose was reduced. (I,J) Effect of SHP-2 and Spry1 on FGF-induced phosphorylation of ERK2 in HEK293 cells. (**I**) HEK293 cells were transfected with plasmid expressing ERK2 with HA epitope (ERK2-HA) and empty vector or vector expressing Spry1 with FLAG epitope (FL-Spry1), dominant-negative SHP-2^{C459S} or wild-type SHP-2 as indicated. After transfection, bFGF was added for 30 minutes to activate FGF pathway. (Top panels) ERK2-HA immunoprecipitated (IP) from cell lysates with anti-HA antiserum and analyzed on immunoblots with anti-dpERK to show diphosphorylated (active) ERK2-HA or with anti-HA to show total ERK2-HA. (Bottom panels) Immunoblots of whole cell lysates (WCL) probed with anti-FLAG to detect FL-Spry1 or anti-SHP-2 to detect endogenous SHP-2 and SHP-2 from transfected plasmids. Similar results were obtained in three experiments. (**J**) Effect of Spry1 and dominant negative SHP-2^{C459S} on kinetics of ERK2 activation by FGF. As in I, except ERK2 analysis was carried out at times indicated after FGF addition. Similar results were obtained in two experiments.

SHP-2 (Fig. 2A, arrow 2), then inhibiting SHP-2 should increase tyrosine phosphorylation on Spry1, even though it reduces signaling overall. However, if SHP-2 functions upstream of Spry1 phosphorylation (arrow 1), then inhibiting SHP-2 should decrease Spry1 phosphorylation. If SHP-2 acts downstream or parallel to Spry1 (arrow 3), no change in Spry1 phosphorylation is expected.

To assess the effect of SHP-2 inhibition on Spry1, HEK293 cells were co-transfected with plasmids expressing dominant-negative SHP-2 and Spry1 with a hemagglutinin epitope (HA-Spry1). Tyrosine phosphorylation of HA-Spry1 was assayed following FGF induction. Expression of SHP-2^{C459S} increased phosphorylation on Spry1 (Fig. 2B, lanes 1,2,4,5), as did expression of another dominant-negative SHP-2, SHP-2$^{\Delta P}$ (data not shown). Thus, SHP-2 negatively regulates tyrosine phosphorylation on Spry1.

Overexpression of wild-type SHP-2 reduced Spry1 tyrosine phosphorylation, consistent with this model (Fig. 2B, lanes 3,6). However, the effect was small and variable, presumably because SHP-2 levels are not limiting and its phosphatase activity is controlled by binding via its SH2 domain to scaffolding proteins as in other contexts (Barford and Neel, 1998).

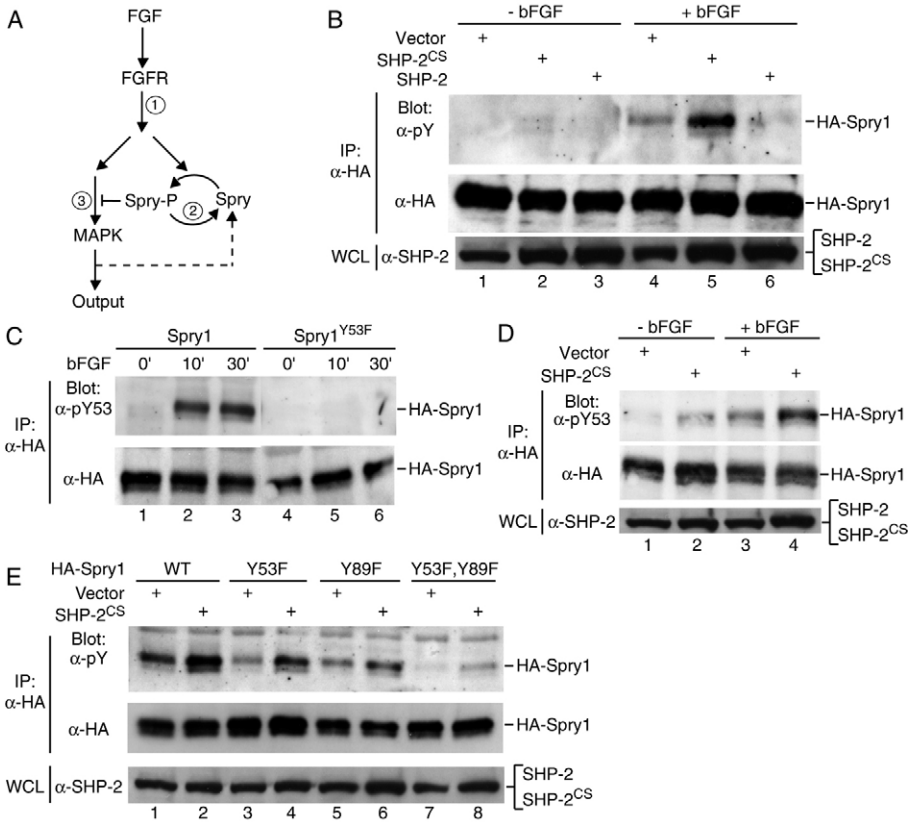

Fig. 2. SHP-2 regulates Spry phosphorylation. (**A**) FGF signaling pathway with Spry feedback loop. FGF activates Spry feedback by inducing *spry* expression (broken line) and stimulating tyrosine phosphorylation of Spry to generate Spry-P. Circled numbers indicate steps by which SHP-2/Csw could promote to increase signal output. (**B**) Effect of SHP-2 on Spry1 tyrosine phosphorylation. HEK293 cells were transfected with expression constructs for HA-Spry1, FGFR1c and either empty vector or expression constructs for dominant-negative SHP-2^{C459S} or wild-type SHP-2 as indicated. Transfected cells were left untreated (lanes 1-3) or treated with bFGF (lanes 4-6). (Top) Immunoprecipitated HA-Spry1 analyzed on immunoblot with anti-phosphotyrosine antiserum. SHP-2^{C459S} increased HA-Spry1 phosphorylation (lane 5) and SHP-2 reduced it (lane 6). (Middle) Immunoblot reprobed with anti-HA to show total HA-Spry1. (Bottom) Immunoblot of whole cell lysates probed with anti-SHP-2. Similar results were obtained in two experiments. (**C**) Specificity of phospho-specific Spry1 antiserum. HEK293 cells were transfected with expression plasmids for FGFR1c and HA-Spry1 or HA-Spry1^{Y53F} as indicated. Transfected cells were treated with bFGF for times indicated, and HA-Spry1 was immunoprecipitated and analyzed on immunoblots probed with α-pY53 antiserum (top) or anti-HA to show total HA-Spry1 (bottom). (**D**) Effect of SHP-2^{C459S} on Spry1 Y53 phosphorylation. HEK293 cells were transfected with plasmids expressing FGFR1c and HA-Spry1, and empty vector or vector expressing dominant-negative SHP-2^{C459S} as indicated. Transfected cells were left untreated (lanes 1,2) or treated with bFGF for 60 minutes (lanes 3,4). (Top) Immunoblot of immunoprecipitated HA-Spry1 probed with α-pY53. SHP-2^{C459S} increased phosphorylation on pY53. (Middle) Control immunoblot probed with anti-HA. (Bottom) Immunoblot of whole cell lysates probed with anti-SHP-2. Similar results were obtained in two experiments. (**E**) Effect of SHP-2^{C459S} on other Spry1 phosphotyrosines. HEK293 cells were transfected with plasmids expressing FGFR1c and either empty vector or vector expressing dominant-negative SHP-2^{C459S} as indicated, and plasmids expressing HA-Spry1 (WT, lanes 1,2), HA-Spry1^{Y53F} (lanes 3, 4), HA-Spry1^{Y89F} (lanes 5, 6), or HA-Spry1$^{Y53F, Y89F}$ (lanes 7, 8). FGF treatment and subsequent analysis with anti-phosphotyrosine antiserum was as in lanes 4 and 5 (of B). SHP-2^{C459S} influenced tyrosine phosphorylation on HA-Spry1 when Y53 was altered (lanes 3,4), indicating that SHP-2 also affects other tyrosine(s), notably Y89 (lanes 5-8). Similar results were obtained in three experiments.

There are five conserved tyrosines in vertebrate Sprouty proteins, one of which (Y53 in Spry1 and Spry4, Y55 in Spry2) is crucial for Spry1 and 2 activity in PC-12 cells (Hanafusa et al., 2002) and for Spry2 and Spry4 in HEK293 cells (Sasaki et al., 2001). Y53 is also crucial for Spry1 activity in HEK293 cells, because changing it to phenylalanine (Spry1^{Y53F}) abolished its ability to block bFGF activation of MAPK (data not shown). To determine whether phosphorylation on this critical tyrosine residue is regulated by SHP-2, a phospho-specific antiserum was generated that recognizes the pY53 form of Spry1 (Fig. 2C). Expression of dominant-negative SHP-2^{C459S} increased phosphorylation on Y53, both in the absence and presence of added FGF (Fig. 2D). Thus, SHP-2 regulates phosphorylation of this crucial Spry1 tyrosine.

To determine whether SHP-2 regulates phosphorylation of other Spry1 tyrosines, the effects of dominant negative SHP-2 on Spry1^{Y53F} were examined using a general anti-phosphotyrosine antibody. The Y53F mutation did not completely eliminate tyrosine phosphorylation of Spry1, and the residual phosphorylation increased in the presence of dominant negative SHP-2 (Fig. 2E, lanes 1-4). Thus, there is at least one other Spry1 phosphotyrosine regulated by SHP-2.

To identify additional Spry1 phosphotyrosines, HA-Spry1 was purified from FGF-stimulated HEK293 cells and analyzed by tandem mass spectrometry. This confirmed phosphorylation of Y53 and identified Y89 as a second phosphorylation site. Mutation of Y89 to phenylalanine (Spry1^{Y89F}) reduced Spry1 tyrosine phosphorylation in response to FGF (Fig. 2E, lanes 1,5), implying that Y89 is also a major phosphorylation site. However, Spry1^{Y89F} inhibited MAPK activation by FGF signaling in the HEK293 cell assay, demonstrating that phosphorylation of Y89 is not essential for this activity (data not shown). Although dominant-negative SHP-2 increased tyrosine phosphorylation on both Spry1^{Y53F} and Spry1^{Y89F} (Fig. 2E, lanes 3-6), there was little tyrosine phosphorylation and only a small effect of dominant-negative SHP-2 on the Spry1$^{Y53F/Y89F}$ double mutant (Fig. 2E, lanes 7,8). Thus, tyrosines 53 and 89 are the major tyrosine phosphorylation sites on Spry1 and both are regulated by SHP-2.

SHP-2 and Spry1 associate in a complex

If Spry1 is a SHP-2 substrate, the proteins must associate at least transiently in vivo. To test this, HA-Spry1 and SHP-2 with a V5 epitope (SHP-2-V5) were co-expressed in HEK293 cells. SHP-2-

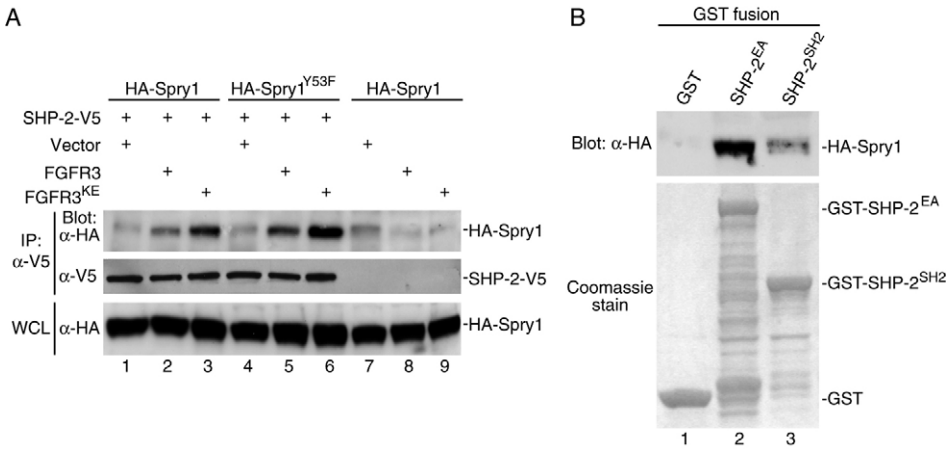

Fig. 3. SHP-2 forms a complex with Spry1. (**A**) Co-immunoprecipitation analysis. HEK293 cells were transfected with plasmids expressing HA-Spry1 (lanes 1-3, 7-9) or HA-Spry1^{Y53F} (lanes 4-6), plasmid expressing SHP-2-V5 (lanes 1-6), and empty vector (lanes 1, 4, 7) or vector expressing FGFR3 (lanes 2, 5, 8) or constitutively-active FGFR3^{K644E} (lanes 3, 6, 9) to increase FGF pathway activity. SHP-2-V5 was immunoprecipitated from cell lysates. (Top) Immunoblot of immunoprecipitates probed with anti-HA to detect co-immunoprecipitated HA-Spry1. (Middle) Blot reprobed with anti-V5 showing SHP-2-V5 in immunoprecipitates. (Bottom) Immunoblot of whole cell lysates probed with anti-HA to show HA-Spry1 expression. Similar results were obtained in three experiments. (**B**) GST pull-down analysis of SHP-2 interaction with Spry1. HEK293 cells were transfected with plasmids expressing HA-Spry1 and FGFR3^{K644E} to increase HA-Spry1 phosphorylation. Cell lysates were incubated with beads coated with purified GST (lane 1), or GST fused to constitutively active SHP-2^{E76A} (lane 2) or to truncated SHP-2 containing only the SH2 domains (lane 3). (Top) Immunoblot of proteins bound to beads, probed with anti-HA. HA-Spry1 bound to GST-SHP-2^{E76A} (lane 2) and, less well, to GST-SHP-2^{SH2} (lane 3). Similar results were obtained in three experiments. (Bottom) Coomassie Blue-stained SDS-PAGE gel of purified GST fusion proteins. Positions of full-length proteins are indicated; lower molecular weight forms are presumably breakdown products.

V5 was immunoprecipitated from cell extracts, and HA-Spry1 that co-immunoprecipitated was detected on immunoblots. Little HA-Spry1 was detected in immunoprecipitates from unstimulated cells (Fig. 3A, lane 1), comparable with that observed in controls lacking SHP-2-V5 (lanes 7-9). However, when FGF signaling was activated by overexpression of FGFR3, an association between the two proteins was detected, and more was observed when constitutively active FGFR3^{K644E} (Su et al., 1997) was expressed (lanes 2,3). Similar results were obtained when the antibody treatments were reversed (data not shown). Thus, FGF signaling induces formation of a complex containing SHP-2 and Spry1. Complex formation does not require the major Spry1 tyrosine phosphorylation sites, because it was not affected by Spry1^{Y53F} or Spry1$^{Y53F/Y89F}$ mutations (Fig. 3A, lanes 4-6 and data not shown).

Interaction between SHP-2 and Spry1 was confirmed by GST pull-down experiments using a full-length, constitutively-active SHP-2 (SHP-2^{E76A}) GST fusion protein (O'Reilly et al., 2000). When purified SHP-2^{E76A}-GST attached to beads was incubated with extracts of HEK293 cells expressing HA-Spry1, HA-Spry1 bound to the beads, whereas little bound to control beads coated with GST (Fig. 3B). HA-Spry1 also bound to beads coated with a GST-fusion containing only the SH2 domains of SHP-2 (SHP-2^{SH2}), but with reduced efficiency. Thus, SHP-2 and Spry1 associate in a complex, and the interaction is mediated at least in part by SHP-2 SH2 domains.

SHP-2 dephosphorylates Spry1

To test whether SHP-2 can dephosphorylate Spry1, purified SHP-2^{E76A}-GST was incubated with HA-Spry1 protein isolated from FGF-stimulated HEK293 cells. The SHP-2 E76A mutation prevents auto-inhibition of the phosphatase domain, alleviating the need for factors that might be necessary in vivo to relieve auto-inhibition (O'Reilly et al., 2000). SHP-2^{E76A}-GST eliminated tyrosine phosphorylation on HA-Spry1, including the crucial phosphotyrosine (Y53), whereas control proteins lacking the phosphatase domain (SHP-2^{SH2}, SHP-2$^{\Delta P}$) and GST alone had no

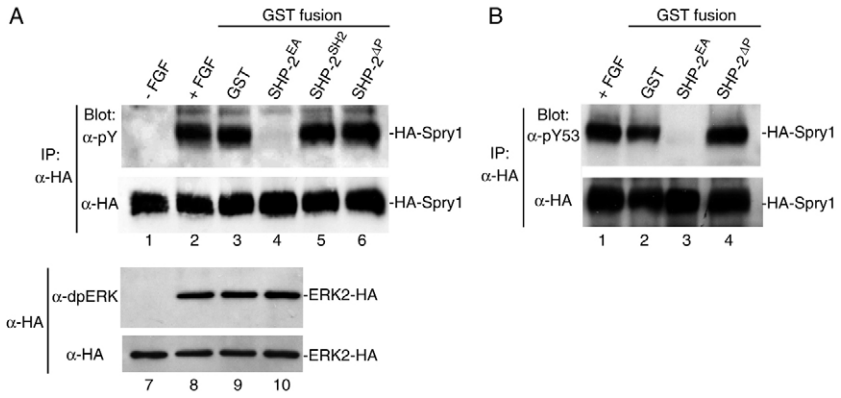

Fig. 4. SHP-2 dephosphorylates Spry1 in vitro. (**A**) (Top) Phosphorylated HA-Spry1 isolated from transfected HEK293 cells treated with bFGF was incubated alone (lane 2) or with purified GST (lane 3), GST-SHP-2^{E76A} (lane 4), GST-SHP-2^{SH2} (lane 5) or GST-SHP-2$^{\Delta P}$ (lane 6). Lane 1, HA-Spry1 before bFGF addition. Upper blot, immunoblot of products probed with anti-phosphotyrosine to detect phosphorylated HA-Spry1. GST-SHP-2^{E76A} dephosphorylated HA-Spry1 (lane 4). Lower blot, immunoblot probed with anti-HA to detect all HA-Spry1. Similar results were obtained in three experiments. (Bottom) Tyrosine phosphorylated ERK2-HA was prepared and tested as a SHP-2 substrate as above. Upper blot, immunoblot of ERK2-HA incubated alone (lane 8), with GST (lane 9) or with GST-SHP-2^{E76A} (lane 10), probed with anti-dpERK to detect phosphorylated ERK2-HA. Tyrosine phosphorylation site detected by anti-dpERK (Y185) was not affected by GST-SHP-2^{E76A}. Lower blot, same blot reprobed with anti-HA to detect all ERK2-HA. Lane 7, ERK2-HA before bFGF addition. ERK2 and Spry1 have another phosphorylation site near the phosphotyrosine: S50 for Spry1 (data not shown) and T183 for ERK2. Similar results were obtained in two experiments. (**B**) Experiment as in A (top), except upper blot was probed with anti-pY53.

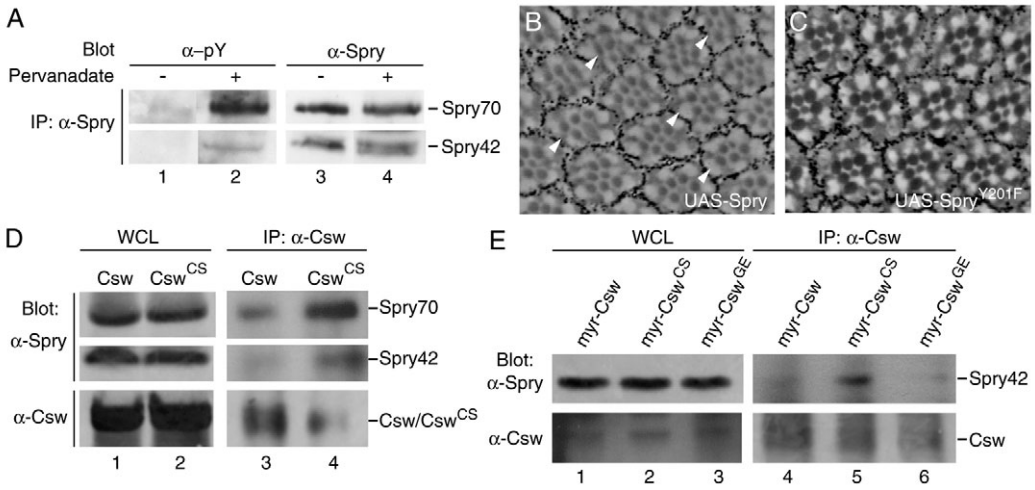

Fig. 5. *Drosophila* Spry is phosphorylated and binds a substrate-trapping form of Csw. (**A**) Tyrosine phosphorylation of Spry. S2 cells expressing Breathless-FLAG were mock-treated (lanes 1, 3) or treated with 0.1 mM pervanadate, a tyrosine phosphatase inhibitor (lanes 2, 4) and endogenous Spry was immunoprecipitated from cell extracts and analyzed on immunoblots. (Lanes 1, 2) Immunoblot probed with anti-phosphotyrosine to detect phosphorylated Spry. (Lanes 3, 4) Immunoblot probed with anti-Spry to detect all Spry (~70 kDa isoform, Spry70; doublet of ~42 kDa isoforms, Spry42). There is tyrosine phosphorylation of Spry70 and a Spry42 isoform (lane 2). Similar results were obtained in three experiments. (**B**,**C**) Requirement of Spry Y201. (B) Section through eye of *w; sev-GAL4*/UAS-*spry* fly that expresses Spry under control of *sev-GAL4* in developing eye. Ommatidia are disorganized and some (arrowheads) are missing photoreceptors. (C) Similar section of *w; sev-GAL4*/UAS-*spry*^{Y201F} that expresses Spry^{Y201F} under control of *sev-GAL4*. Ommatidia appear normal. (**D**) Binding of Spry to substrate-trapping Csw in S2 cells. Whole cell lysates were prepared from transfected S2 cells expressing Csw (lanes 1, 3) or Csw^{C583S}, a substrate-trapping form of the enzyme (lanes 2, 4). Aliquots of lysates were directly resolved by SDS-PAGE (lanes 1, 2) or first immunoprecipitated with anti-Csw (lanes 3, 4). Immunoblots were probed with anti-Spry or anti-Csw as indicated. More Spry co-immunoprecipated with Csw^{C583S} (lane 4) than with Csw (lane 3). Similar results were obtained in three experiments. (**E**) Binding of Spry to substrate-trapping Csw in imaginal discs. Eye-antennal discs dissected from third instar transgenic larvae expressing myr-Csw, myr-Csw^{C583S} or myr-Csw^{G547E}, a dominant-negative Csw that does not function as a substrate trap, were homogenized and directly resolved by SDS-PAGE (lanes 1-3) or immunoprecipitated with anti-Csw and then resolved by SDS-PAGE (lanes 4-6). Immunoblots were probed with anti-Spry or anti-Csw as indicated. Positions of Spry42 and Csw are shown; Spry70 is variably detected in eye disc lysates. More Spry42 co-immunoprecipitated with myristylated Csw^{C583S} than with myristylated Csw or Csw^{G547E}. A similar result obtained in a repeat experiment, except in this case Spry70 isoform predominated in co-immunoprecipitate.

effect (Fig. 4A,B). Under identical conditions, SHP-2^{E76A}-GST did not dephosphorylate ERK2 phosphotyrosine (Fig. 4A, lower panel). Thus, Spry1 is a substrate of SHP-2 in vitro.

Drosophila Spry binds a substrate-trapping form of Csw in vivo

The mammalian cell culture and biochemical experiments demonstrate that SHP-2 negatively regulates tyrosine phosphorylation on Spry1, that the proteins associate in a signal-induced complex, and that SHP-2 selectively dephosphorylates Spry1 in vitro. Genetic interactions between *csw* and *sprouty* in tracheal and eye development suggest that a similar regulatory relationship might exist for the *Drosophila* proteins.

To determine if *Drosophila* Spry is tyrosine phosphorylated, endogenous Spry in Breathless-expressing S2 cells was immunoprecipitated and probed with anti-phosphotyrosine antiserum (Fig. 5A). Spry isoforms of 70 kDa and 42 kDa are present in S2 cells (Toering, 2003). Tyrosine phosphorylation of the 70 kDa species was detected at low levels in untreated cells, and phosphorylation of both forms was apparent when cells were treated with the phosphatase inhibitor pervanadate, implying that Spry phosphorylation is regulated by an endogenous phosphatase.

The tyrosine crucial for vertebrate Spry function is conserved in *Drosophila* Spry (Hanafusa et al., 2002). To determine if the conserved tyrosine (Y201) is important for function, transgenes encoding wild-type Spry (UAS-*spry*) or mutant Spry with the tyrosine substituted with phenylalanine (UAS-*spry*^{Y201F}) were expressed using *sev-GAL4* driver, and their effects on eye development examined. Spry expression caused misrotation and disorganization of ommatidia, missing photoreceptors in 5% of ommatidia, and external roughening of the eye (Fig. 5B and data not shown). Spry^{Y201F} expression had little or no effect. In two out of three UAS-*spry*^{Y201F} insertions analyzed, ommatidia had normal organization and no missing photoreceptors or eye roughening (Fig. 5C). The same was true of the third insertion, except it caused a low frequency of photoreceptor loss (0.4% of ommatidia). Thus, the conserved tyrosine is crucial for biological activity of *Drosophila* Spry.

The above results and the genetic interactions between *csw* and *spry* suggest that Csw might regulate Spry directly by dephosphorylation. In vivo substrates of Csw/SHP-2 can be identified with substrate-trapping forms of the enzymes, such as Csw^{C583S}, which bind but do not dephosphorylate or release their substrates (Herbst et al., 1996; Agazie and Hayman, 2003a). To determine if Csw^{C583S} can trap endogenous Spry in S2 cells, wild-type Csw and Csw^{C583S} were expressed in S2 cells and the amount of Spry that bound to each was determined by co-immunoprecipitation (Fig. 5D). A small amount of both Spry isoforms bound wild-type Csw (lane 3). Substantially more of each isoform bound Csw^{C583S} (lane 4). This implies that Spry is a direct target of Csw in S2 cells.

To test for a substrate-trapping interaction between Csw^{C583S} and Spry during development, binding was analyzed in a similar manner in extracts of eye-antennal imaginal discs dissected from transgenic larvae expressing modified Csw proteins (myr-Csw, myr-Csw^{C583S}

or myr-Csw^{G547E}). Myristylated forms of Csw were used to facilitate membrane localization of the enzyme and detection of interaction with substrates (Allard et al., 1996). There was low but detectable interaction between myr-Csw and endogenous Spry (Fig. 5E, lane 4). Stronger interaction was observed with myr-Csw^{C583S} (lane 5). The enhanced interaction was not simply due to stronger interaction between the SH2 domains of the mutant Csw and a scaffolding protein such as Dos that cannot be dephosphorylated by it. If so, other catalytically inactive forms of Csw, such as myr-Csw^{G547E}, should show the same enhanced interaction, which was not observed (lane 6). We conclude that the enhanced interaction between Spry and myr-Csw^{C583S} is a substrate-trapping effect, providing strong evidence that Spry is a substrate of Csw during eye development.

DISCUSSION

Since discovery over a decade ago of the key roles of Csw/SHP-2 tyrosine phosphatases in promoting RTK signaling, identification of their crucial substrates has remained an important goal. Our results identify Spry proteins as in vivo substrates of Csw/SHP-2, and show how Csw/SHP-2 can promote RTK signaling by dephosphorylating and inactivating these RTK feedback inhibitors.

Four lines of evidence support the conclusion that Csw/SHP-2 inactivate Spry proteins by direct binding and dephosphorylation. First, genetic experiments in developing *Drosophila* eye and trachea and HEK293 cells demonstrated that Csw/SHP-2 and Spry act in the same RTK signaling events but in opposite directions. Indeed, manipulating their activity in opposite directions caused similar *Drosophila* phenotypes and similar effects on MAPK activation in HEK293 cells, and reducing *spry* dose suppressed the *csw* loss-of-function phenotype in the eye and enhanced the gain-of-function phenotype, supporting the idea that they regulate the same step in signaling. Second, molecular epistasis experiments in HEK293 cells demonstrated that SHP-2 functions upstream of, and negatively regulates, phosphorylation of the critical tyrosine residue (Y53) of Spry1. Third, biochemical studies of extracts of HEK293 cells, *Drosophila* S2 cells, and eye discs demonstrated that Csw/SHP-2 proteins associate in complexes with Spry proteins. Interaction was enhanced in S2 cells and eye discs when a substrate-trapping Csw was used. Interaction involves more than just binding of Csw/SHP-2 to the crucial tyrosine, because complex formation was observed with SHP-2 mutants lacking the phosphatase domain and with a Spry mutant lacking the tyrosine. Finally, purified SHP-2 selectively dephosphorylated Spry1 in vitro. These data support the conclusion that Spry proteins are direct targets of Csw/SHP-2 in all three systems examined.

One genetic result did not readily fit with the model that Csw functions by inactivating Spry by dephosphorylation. Whereas reduction of *spry* dose suppressed the eye phenotype of a hypomorphic *csw* allele (Firth et al., 2000) and dominant-negative *Csw^{G547E}*, consistent with the model, it did not suppress the milder phenotype of dominant-negative *Csw^{C583S}* (Fig. 1G,H). This catalytically inactive, substrate trapping form of Csw has unusual properties: it behaves in a dominant-negative fashion, interfering with wild-type Csw function, but also retains some wild-type Csw function because it partially rescues other dominant-negative and hypomorphic *csw* alleles (Allard et al., 1998). This residual activity of Csw^{C583S} is proposed to result from its ability to partially mimic the effect of dephosphorylating a substrate by binding to it tightly (Allard et al., 1998). Spry binds Csw^{C583S} and could be such a substrate (Fig. 5D,E). If so, this could explain the lack of suppression of Csw^{C583S} phenotype by reduction in *spry* dose: decreasing *spry* levels would not reduce *spry* function under conditions in which it is already trapped in an inactive or partially inactive form by Csw^{C583S}.

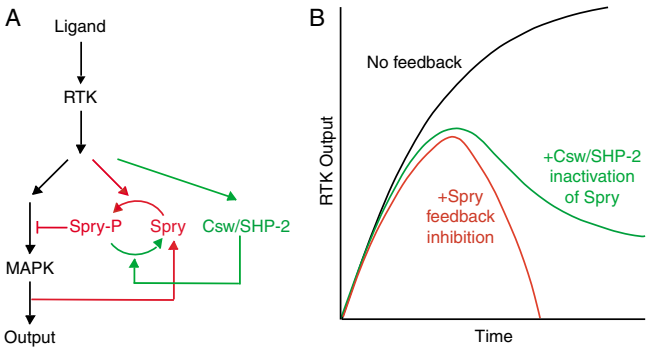

Fig. 6. Csw/SHP-2-Spry feedback circuit shapes RTK signaling profile. (**A**) Ligand binding to RTK activates RAS/MAPK cascade, Spry negative-feedback loop (red), and Csw/SHP-2 tyrosine phosphatase (green). Csw/SHP-2 dephosphorylates Spry, inactivating it. This creates a double-negative regulatory circuit in which Csw/SHP-2 increases RTK output by inactivating a feedback inhibitor. (**B**) Conceptualized plots of RTK kinetics when no feedback (black), Spry feedback (red) or the complete Csw/SHP-2–Spry circuit (green) is operative.

Implications of the Csw/SHP-2–Spry feedback circuit on RTK signaling profiles

Csw/SHP-2 binding and dephosphorylation of Spry creates an interesting regulatory circuit downstream of RTKs (Fig. 6A). Both components of the circuit are induced and activated following receptor activation, Csw/SHP-2 by SH2 domain interactions with phosphotyrosines, and Spry proteins by transcriptional induction of their genes and tyrosine phosphorylation of the proteins. One induced component (Spry) is a signaling inhibitor, the other (Csw/SHP-2) is a signaling promoter that acts by inactivating the inhibitor.

Why does a signaling pathway induce both a feedback inhibitor and a protein that inactivates it? One possibility is that this double-negative circuit provides a mechanism for rapidly resetting the signaling system: the inhibitor terminates signaling and the deactivator restores the inhibitor to its original (inactive) state, readying the cell for another round of signaling. This may be important when cells experience successive waves of signaling, such as the waves of EGFR and Sevenless signaling in eye development (Freeman, 1996).

Another possibility is that the double-negative circuit allows precise control of the signal output profile (Fig. 6B). In the absence of feedback, the response to a signal is simple and sustained, increasing monotonically until reaching saturation (black curve). If a basic negative-feedback system is operative, the magnitude and duration of the response are limited, generating a parabolic response profile (red curve). However, if the pathway contains both a feedback inhibitor (Spry) and an inducible component (Csw/SHP-2) that deactivates it, this creates more complex output profiles, such as the irregularly shaped curve observed for MAPK activation following FGFR activation in HEK293 cells (Fig. 1J; Fig. 6B, green curve). By altering activity of individual feedback components, other complex profiles can be generated (Fig. 1J). If cells can distinguish different profiles, as some cells distinguish different calcium oscillations (Lewis, 2003), this could lead to different outcomes. The shape of the RTK response profile could be as

important to outcome as the magnitude and duration of the response. In a similar way, differential induction of individual components of a double-negative feedback circuit can transform simple signaling gradients into complex spatial patterns of signal output.

The Csw/SHP-2–Sprouty circuit does not operate in all RTK signaling processes

Although our results imply that the Csw/SHP-2-Spry circuit operates in a variety of RTK signaling processes, it is unlikely to operate in all such events. Csw and SHP-2 are widely expressed and required in many and perhaps all RTK signaling processes, whereas Spry genes are expressed in more limited domains during development and appear to function in only a subset of such processes. For example, expression of *spry* genes in *Drosophila* and mouse embryos is largely confined to FGF signaling centers (Hacohen et al., 1998; Minowada et al., 1999).

In RTK signaling processes where Spry proteins are not expressed, Csw/SHP-2 must stimulate signaling by dephosphorylating other substrates, such as the autophosphorylation site on EGFR and Torso/PDGFRs that recruits RasGAP (Cleghon et al., 1998; Ekman et al., 2002; Agazie and Hayman, 2003b) or sites on Src kinase regulators (Ren et al., 2004; Zhang et al., 2004). In some pathways, such as the Torso pathway, more than one Csw/SHP-2 substrate is likely to be present and regulated by the enzyme (Cleghon et al., 1998; Casci et al., 1999). The specific substrates present in each cell should alter signaling kinetics in different ways, creating a rich diversity of output profiles. It will be interesting to determine the extent and importance of this diversity, and whether alterations in signal output profile and Spry feedback inhibition contribute to pathogenesis of human diseases associated with misregulation of SHP-2 activity (Tartaglia et al., 2001; Musante et al., 2003).

Work was supported by National Institutes of Health grants (to M.A.S. and M.A.K.), National Science Foundation Predoctoral Fellowships (to R.K.S.-B. and S.J.T.) and National Cancer Institute Training Grant (to L.A.J.). M.A.K. is an Investigator of Howard Hughes Medical Institute. R.K.S.-B. conceived the project. All authors conceived and designed experiments. L.A.J., R.K.S.-B. and S.J.T. performed experiments. All authors analyzed data and wrote the paper.

References

Agazie, Y. M. and Hayman, M. J. (2003a). Development of an efficient 'substrate trapping' mutant of SHP2, and identification of EGFR, Gab1 and three other proteins as target substrates. *J. Biol. Chem.* **11**, 11.

Agazie, Y. M. and Hayman, M. J. (2003b). Molecular mechanism for a role of SHP2 in epidermal growth factor receptor signaling. *Mol. Cell. Biol.* **23**, 7875-7886.

Allard, J. D., Chang, H. C., Herbst, R., McNeill, H. and Simon, M. A. (1996). The SH2-containing tyrosine phosphatase corkscrew is required during signaling by sevenless, Ras1 and Raf. *Development* **122**, 1137-1146.

Allard, J. D., Herbst, R., Carroll, P. M. and Simon, M. A. (1998). Mutational analysis of the SRC homology 2 domain protein-tyrosine phosphatase Corkscrew. *J. Biol. Chem.* **273**, 13129-13135.

Barford, D. and Neel, B. G. (1998). Revealing mechanisms for SH2 domain mediated regulation of the protein tyrosine phosphatase SHP-2. *Structure* **6**, 249-254.

Bennett, A. M., Tang, T. L., Sugimoto, S., Walsh, C. T. and Neel, B. G. (1994). Protein-tyrosine-phosphatase SHPTP2 couples platelet-derived growth factor receptor beta to Ras. *Proc. Natl. Acad. Sci. USA* **91**, 7335-7339.

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.

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

Cleghon, V., Feldmann, P., Ghiglione, C., Copeland, T. D., Perrimon, N., Hughes, D. A. and Morrison, D. K. (1998). Opposing actions of CSW and RasGAP modulate the strength of Torso RTK signaling in the Drosophila terminal pathway. *Mol. Cell* **2**, 719-727.

Deb, T. B., Wong, L., Salomon, D. S., Zhou, G., Dixon, J. E., Gutkind, J. S., Thompson, S. A. and Johnson, G. R. (1998). A common requirement for the catalytic activity and both SH2 domains of SHP-2 in mitogen-activated protein (MAP) kinase activation by the ErbB family of receptors. A specific role for SHP-2 in map, but not c-Jun amino-terminal kinase activation. *J. Biol. Chem.* **273**, 16643-16646.

Dikic, I. and Giordano, S. (2003). Negative receptor signalling. *Curr. Opin. Cell Biol.* **15**, 128-135.

Ekman, S., Kallin, A., Engstrom, U., Heldin, C. H. and Ronnstrand, L. (2002). SHP-2 is involved in heterodimer specific loss of phosphorylation of Tyr771 in the PDGF beta-receptor. *Oncogene* **21**, 1870-1875.

Feng, G. S. (1999). Shp-2 tyrosine phosphatase: signaling one cell or many. *Exp. Cell Res.* **253**, 47-54.

Firth, L., Manchester, J., Lorenzen, J. A., Baron, M. and Perkins, L. A. (2000). Identification of genomic regions that interact with a viable allele of the Drosophila protein tyrosine phosphatase corkscrew. *Genetics* **156**, 733-748.

Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. *Cell* **87**, 651-660.

Freeman, R. M., Plutzky, J. and Neel, B. G. (1992). Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of Drosophila corkscrew. *Proc. Natl. Acad. Sci. USA* **89**, 11239-11243.

Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y. and Krasnow, M. A. (1998). sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. *Cell* **92**, 253-263.

Hall, A. B., Jura, N., DaSilva, J., Jang, Y. J., Gong, D. and Bar-Sagi, D. (2003). hSpry2 Is Targeted to the Ubiquitin-Dependent Proteasome Pathway by c-Cbl. *Curr. Biol.* **13**, 308-314.

Hanafusa, H., Torii, S., Yasunaga, T. and Nishida, E. (2002). Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. *Nat. Cell Biol.* **4**, 850-858.

Hanafusa, H., Torii, S., Yasunaga, T., Matsumoto, K. and Nishida, E. (2004). Shp2, an SH2-containing protein-tyrosine phosphatase, positively regulates receptor tyrosine kinase signaling by dephosphorylating and inactivating the inhibitor Sprouty. *J. Biol. Chem.* **279**, 22992-22995.

Herbst, R., Carroll, P. M., Allard, J. D., Schilling, J., Raabe, T. and Simon, M. A. (1996). Daughter of sevenless is a substrate of the phosphotyrosine phosphatase Corkscrew and functions during sevenless signaling. *Cell* **85**, 899-909.

Herbst, R., Zhang, X., Qin, J. and Simon, M. A. (1999). Recruitment of the protein tyrosine phosphatase CSW by DOS is an essential step during signaling by the sevenless receptor tyrosine kinase. *EMBO J.* **18**, 6950-6961.

Huyer, G. and Alexander, D. R. (1999). Immune signalling: SHP-2 docks at multiple ports. *Curr. Biol.* **9**, R129-R132.

Impagnatiello, M. A., Weitzer, S., Gannon, G., Compagni, A., Cotten, M. and Christofori, G. (2001). Mammalian sprouty-1 and –2 are membrane-anchored phosphoprotein inhibitors of growth factor signaling in endothelial cells. *J. Cell Biol.* **152**, 1087-1098.

Jarecki, J., Johnson, E. and Krasnow, M. A. (1999). Oxygen regulation of airway branching in Drosophila is mediated by branchless FGF. *Cell* **99**, 211-220.

Johnson Hamlet, M. R. and Perkins, L. A. (2001). Analysis of corkscrew signaling in the Drosophila epidermal growth factor receptor pathway during myogenesis. *Genetics* **159**, 1073-1087.

Kramer, S., Okabe, M., Hacohen, N., Krasnow, M. A. and Hiromi, Y. (1999). Sprouty: a common antagonist of FGF and EGF signaling pathways in Drosophila. *Development* **126**, 2515-2525.

Lee, S. H., Schloss, D. J., Jarvis, L. A., Krasnow, M. A. and Swain, J. L. (2001). Inhibition of angiogenesis by a mouse sprouty protein. *J. Biol. Chem.* **276**, 4128-4133.

Lewis, R. S. (2003). Calcium oscillations in T-cells: mechanisms and consequences for gene expression. *Biochem. Soc. Trans.* **31**, 925-929.

Li, X., Brunton, V. G., Burgar, H. R., Wheldon, L. M. and Heath, J. K. (2004). FRS2-dependent SRC activation is required for fibroblast growth factor receptor-induced phosphorylation of Sprouty and suppression of ERK activity. *J. Cell Sci.* **117**, 6007-6017.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179-185.

Mason, J. M., Morrison, D. J., Bassit, B., Dimri, M., Band, H., Licht, J. D. and Gross, I. (2004). Tyrosine phosphorylation of Sprouty proteins regulates their ability to inhibit growth factor signaling: a dual feedback loop. *Mol. Biol. Cell* **15**, 2176-2188.

Minowada, G., Jarvis, L. A., Chi, C. L., Neubuser, A., Sun, X., Hacohen, N., Krasnow, M. A. and Martin, G. R. (1999). Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. *Development* **126**, 4465-4475.

Musante, L., Kehl, H. G., Majewski, F., Meinecke, P., Schweiger, S., Gillessen-Kaesbach, G., Wieczorek, D., Hinkel, G. K., Tinschert, S., Hoeltzenbein, M. et al. (2003). Spectrum of mutations in PTPN11 and genotype-phenotype correlation in 96 patients with Noonan syndrome and five

patients with cardio-facio-cutaneous syndrome. *Eur. J. Hum. Genet.* **11**, 201-206.
Nutt, S. L., Dingwell, K. S., Holt, C. E. and Amaya, E. (2001). Xenopus Sprouty2 inhibits FGF-mediated gastrulation movements but does not affect mesoderm induction and patterning. *Genes Dev.* **15**, 1152-1166.
O'Reilly, A. M., Pluskey, S., Shoelson, S. E. and Neel, B. G. (2000). Activated mutants of SHP-2 preferentially induce elongation of Xenopus animal caps. *Mol. Cell. Biol.* **20**, 299-311.
Perkins, L. A., Larsen, I. and Perrimon, N. (1992). corkscrew encodes a putative protein tyrosine phosphatase that functions to transduce the terminal signal from the receptor tyrosine kinase torso. *Cell* **70**, 225-236.
Perkins, L. A., Johnson, M. R., Melnick, M. B. and Perrimon, N. (1996). The nonreceptor protein tyrosine phosphatase corkscrew functions in multiple receptor tyrosine kinase pathways in Drosophila. *Dev. Biol.* **180**, 63-81.
Perrimon, N. and McMahon, A. P. (1999). Negative feedback mechanisms and their roles during pattern formation. *Cell* **97**, 13-16.
Qu, C. K. (2000). The SHP-2 tyrosine phosphatase: signaling mechanisms and biological functions. *Cell Res.* **10**, 279-288.
Qu, C. K. (2002). Role of the SHP-2 tyrosine phosphatase in cytokine-induced signaling and cellular response. *Biochim. Biophys. Acta* **1592**, 297-301.
Reich, A., Sapir, A. and Shilo, B. (1999). Sprouty is a general inhibitor of receptor tyrosine kinase signaling. *Development* **126**, 4139-4147.
Ren, Y., Meng, S., Mei, L., Zhao, Z. J., Jove, R. and Wu, J. (2004). Roles of Gab1 and SHP2 in paxillin tyrosine dephosphorylation and Src activation in response to epidermal growth factor. *J. Biol. Chem.* **279**, 8497-8505.
Rubin, C., Litvak, V., Medvedovsky, H., Zwang, Y., Lev, S. and Yarden, Y. (2003). Sprouty fine-tunes EGF signaling through interlinked positive and negative feedback loops. *Curr. Biol.* **13**, 297-307.
Sasaki, A., Taketomi, T., Wakioka, T., Kato, R. and Yoshimura, A. (2001). Identification of a dominant negative mutant of Sprouty that potentiates fibroblast growth factor- but not epidermal growth factor-induced ERK activation. *J. Biol. Chem.* **276**, 36804-36808.
Saxton, T. M., Henkemeyer, M., Gasca, S., Shen, R., Rossi, D. J., Shalaby, F., Feng, G. S. and Pawson, T. (1997). Abnormal mesoderm patterning in mouse embryos mutant for the SH2 tyrosine phosphatase Shp-2. *EMBO J.* **16**, 2352-2364.
Shiga, Y., Tanaka-Matakatsu, M. and Hayashi, S. (1996). A nuclear GFP/β-galactosidase fusion protein as a marker for morphogenesis in living Drosophila. *Dev. Growth Differ.* **38**, 99-106.
Stein-Gerlach, M., Wallasch, C. and Ullrich, A. (1998). SHP-2, SH2-containing protein tyrosine phosphatase-2. *Int. J. Biochem. Cell Biol.* **30**, 559-566.
Su, W. C., Kitagawa, M., Xue, N., Xie, B., Garofalo, S., Cho, J., Deng, C., Horton, W. A. and Fu, X. Y. (1997). Activation of Stat1 by mutant fibroblast growth-factor receptor in thanatophoric dysplasia type II dwarfism. *Nature* **386**, 288-292.
Sutherland, D., Samakovlis, C. and Krasnow, M. A. (1996). branchless encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell* **87**, 1091-1101.
Tartaglia, M., Mehler, E. L., Goldberg, R., Zampino, G., Brunner, H. G., Kremer, H., van der Burgt, I., Crosby, A. H., Ion, A., Jeffery, S. et al. (2001). Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. *Nat. Genet.* **29**, 465-468.
Tefft, D., Lee, M., Smith, S., Crowe, D. L., Bellusci, S. and Warburton, D. (2002). mSprouty2 inhibits FGF10-activated MAP kinase by differentially binding to upstream target proteins. *Am. J. Physiol. Lung Cell Mol. Physiol.* **283**, L700-706.
Toering, S. (2003). *Genetic and Biochemical Analysis of Drosophila Sprouty.* Stanford, CA: Stanford University Press.
Tomlinson, A. and Ready, D. F. (1987). Neuronal differentiation in the *Drosophila* ommatidium. *Dev. Biol.* **120**, 366-376.
Zhang, S. Q., Yang, W., Kontaridis, M. I., Bivona, T. G., Wen, G., Araki, T., Luo, J., Thompson, J. A., Schraven, B. L., Philips, M. R. et al. (2004). Shp2 regulates SRC family kinase activity and Ras/Erk activation by controlling Csk recruitment. *Mol. Cell* **13**, 341-355.