

# Sprouty: how does the branch manager work?

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## Summary

Since the discovery of the prototypical Sprouty (Spry) protein in *Drosophila*, there has been an effort to determine how these novel modulators of the Ras/MAP-kinase pathway function. A clue to their mechanism of action comes from the several highly conserved sequences within all the currently known Spry isoforms: an ~110-residue cysteine-rich sequence in the C-terminal half that directs Spry proteins to a concentration of signaling proteins at the plasma membrane; a small motif surrounding a tyrosine residue (Y55 in human Spry2) that is responsible for interaction with other proteins. In cultured mammalian cells, hSpry2 inhibits epidermal growth factor receptor (EGFR) endocytosis and subsequently sustains the

activation of MAP kinase but negatively regulates the same pathway following stimulation of fibroblast growth factor receptors (FGFRs). Current evidence indicates that Cbl is a key protein that interacts directly with Spry2 following activation of receptor tyrosine kinases (RTKs). It appears to be the ability of Cbl to interact as an E3 ubiquitin ligase on specific target proteins and as a docking protein in other contexts that dictates the differential effects Spry2 has on the Ras/MAP-kinase pathway following EGFR and FGFR activation.

Key words: Sprouty, Fibroblast growth factor, Receptor tyrosine kinase, Epidermal growth factor receptor, MAP kinase

## Introduction

The fruitfly has been an extremely useful developmental model as well as a discovery resource for many signaling proteins. Hacohen et al. first discovered the Sprouty (dSpry) protein while investigating genes involved in tracheal branching in *Drosophila* (Hacohen et al., 1998). In the fruitfly the *branchless* gene encodes a fibroblast growth factor (FGF) homologue (Bnl) that plays a key role in patterning the early branching events (Sutherland et al., 1996). In tracheal development Bnl is required for branching and is expressed in discrete clusters of cells surrounding the tracheal sacs at each position where a new branch will bud. The secreted growth factor activates *breathless* FGFR (Btl), a receptor tyrosine kinase (RTK) expressed on all tracheal cells (Glazer and Shilo, 1991; Lee et al., 1996), and guides tracheal cell migrations during primary branch formation.

Bnl has a secondary role in patterning: it induces later programs of branching in cells near the ends of the primary branches and in this way contributes to the apical bias in secondary branching. dSpry<sup>-/-</sup> mutants cause excessive branching from the stalks of primary branches, close to the tips where secondary branches normally divide. Furthermore, the excessive branching appears to result from overactivity of the Bnl pathway. Molecular evidence presented by Hacohen et al. indicated that *spry* encodes a novel inhibitor that limits the range of Bnl signaling and thus restricts secondary budding to apical positions closest to the FGF signaling centers (Hacohen et al., 1998).

The dSpry open reading frame gave a predicted 591-residue (63 kDa) polypeptide whose most striking feature was a 124-residue C-terminal, cysteine-rich region (22 cysteine residues), which is flanked by cysteine-free regions lacking any

recognizable protein-protein interaction domain. A databank search subsequently identified three human homologues that are separate gene products, designated hSpry1, hSpry2 and hSpry3. hSpry2 is a 315-residue (35 kDa) polypeptide that also contains a cysteine-rich domain, which shows 51% identity to the similar domain in dSpry (Hacohen et al., 1998). Subsequently, four full-length murine Spry proteins (mSpry1-mSpry4) were cloned and sequenced (Tefft et al., 1999; de Maximy et al., 1999). At least one short sequence in the N-termini of all Spry proteins, as well as several isolated sequences or residues distributed throughout the protein family, is also conserved (Fig. 1). The conserved cysteine-rich domain has also been found in another family of proteins, termed Sprouty-related proteins with an EVH-1 domain (Spreads) (Wakioka et al., 2001). The Spred proteins also possess both an N-terminal enabled/yasodilator-stimulated phosphoprotein (VASP) homology domain (EVH1) and a c-Kit-binding domain (KBD) (Fig. 2), which suggests that the two families have different cellular functions with respect to their interaction with likely binding partners. EVH1 domains bind polyproline peptides with the consensus FPPPP, whereas the KBD was defined by Wakioka et al. as a common domain on Spred 1 and Spred 2 necessary for binding to the c-Kit RTK (Wakioka et al., 2001; Niebuhr et al., 1997; Renfranz and Beckerle, 2002).

Hacohen et al. showed that dSpry localizes to the plasma membrane and that a proportion of the protein can be released into the extracellular environment and compete with FGF ligand for binding to its receptor (Hacohen et al., 1998). There has subsequently been no evidence to support this mode of dSpry or mammalian Spry action. dSpry is expressed in developing embryos in the tracheal system, the midline glia

mSpry2	MEARAQSG-NGSQPLLQTAHDSGR---QRGEPDRDALTQQVHVLSDQIR--AIRNTNEYTEGPTVVPRPGLKPAPRPSTQHKHE	80
hSpry2	MEARAQSG-NGSQPLLQTPRDGGR---QRGEPDRDALTQQVHVLSDQIR--AIRNTNEYTEGPTVVPRPGLKPAPRPSTQHKHE	80
mSpry1	MDSPSQHGSHTSLVVIQPPAVEGR---QRLDYD-RD--TQPATILSLDQIK--AIRGSNEYTEGPTSVARRPAPRTAPRPEKQERTH	78
mSpry4	MEPPVPQS---SFPVNPSSVMVQP---LLDSRAPHSRLQHPILTILPIDQMK--TSHVENDYIDNPSLAPATGPK--RP--RGGPP	73
mSpry3	MDATVID-----ELQQILPIEQLR--STHASNDYVERPPAPCKQALS-SPSLIVQTHKS	51
dSpry	SGSGSVSGSSSFTRRRPPAPVR[ 21 ]QSAEPASNALGQPASPVTLAQPRPESERLTNEYVDTPLQHATRSQLHPAGQQDNGQTTT	226
mSpry2	RLHGLPEHRQPPRLQPSQVHSS-RAPLSRSISTVSSGSRST-RTSTSSSSSEQRLLGSPFSHGPA---AADGIIRVQPKS-ELKP	160
hSpry2	RLHGLPEHRQPPRLQHSQVHSSARAPLSRSISTVSSGSRST-RTSTSSSSSEQRLLGSSFSGGP---VADGIIRVQPKS-ELKP	160
mSpry1	EIIPANVNSSYHRPASHPGNARGSVLSRSTSTGSAASSG-----SSSSVSEQGLLGRSPPTPIPIGHRSDRVIRTQPK-QLLV	157
mSpry4	ELAPTARCD---QDITHHWIS---FSGRSPSSV-----SSSSTSSDQRLLDHMAPP-PVAEQASPRAVRLQPKVVHCKP	141
mSpry3	DWSLATMPTALP-RSISQCHQLQP--LPQHLSQSSISS-----MSQSTTASDGRLLASITPS-P----SGQSIIRTQPGAGHPK	124
dSpry	HLLLLLPQRNQHLLHQHQ[ 28 ]ARLATTQATSVGSDHTDGLLHSHLQNSTTKPPASKQPALPRL[ 13 ]QPIITKQTPATQKE	345
mSpry2	GD--VK-----PLSK--DDLGLHAYRLEDCKKCKEECTYPRPLPSDWICDKQCLCSAQNVIDYGTVMCVKGLFYHCSNDDDED--	235
hSpry2	GE--LK-----PLSK--EDLGLHAYRLEDCKKCKEECTYPRPLPSDWICDKQCLCSAQNVIDYGTVMCVKGLFYHCSNDDDED--	235
mSpry1	ED--LK-----ASLK--EDPTQHKFIEQCGCKCKGECTAPRALPSCACDRQCLCSAESMVEYGTMLLVKGI FYHCSNDDDGG-	233
mSpry4	LD--LK-----GPTAP--PELDKHFLLCEACGKCKECCASPRTPLPSCWVCNQECLCSAQTLVNYGTMLLVQGI FYHCSNDDDED-	218
mSpry3	VDGALK---GEAEQSVGHSSDHLFIIEECGRKCKVPTAVRPLPSCWMCNQRCLCSAESLLDYGTMLCVKGLFYHCSNDDDED--	204
dSpry	RMHALE[ 12 ]GPLVMAGDPSLLNPVCPRCGRRCQEQQSPPLPQTWVCNKTCCLCSAESVIDYASCLCAKALFYHCSNDDDED[ 9 ]	445
mSpry2	NCAINPCSCSQSHCCTRWSAMGVMLFLPCLWCYLPKAGLKLKCGCYDRVNRPGCRCK-NSNTVCCVPTVPPRN--FEKPT-	315
hSpry2	NCAINPCSCSQSHCCTRWSAMGVMLFLPCLWCYLPKAGLKLKCGCYDRVNRPGCRCK-NSNTVCCVPTVPPRN--FEKPT-	315
mSpry1	SYSDNPCSCSQSHCCSRYLKMGALSLCLPCLLCYLPKAGLKLKCRGCDWTHRPGCRCK-NSNTVYCKLESCPSRA--QGKLS-	313
mSpry4	SCADHPSCSGSNCCAFWSFMGALSVVLPCLLCYLPKAGLKLKCRGCDWTHRPGCRCK-HTNSVIKAAASGDTKTSRSDKPF-	300
mSpry3	NCAIEPCSCGPPSSCFIRWAAMSLIFLFLPCLCCYLPTRGQLHMCQQGYDSLRRPGCRCKRHTNTVCRKISSSSSFPKAEKSV	288
dSpry	PCVINPCSCGPKYKRTQVWGLGALSIFLFLPCLWFWPMRGMKLCCKCYGRFAGRGRC[ 66 ]RSILRKGDLTPKRLDSSPDY	591

**Fig. 1.** Conservation of key functional amino acids. When the various Spry proteins are aligned by the CLUSTAL program there are a number of sequences and single residues that are 100% conserved. The SpryTD and the Y55 conserved sequences are highlighted in green and orange, respectively. The triangle above the line denotes the conserved basic residue deemed necessary for binding to PtdIns(4,5)P<sub>2</sub>. Other than these regions that have been assigned crucial functions in Spry action there are several other isolated regions that are similarly conserved (depicted in blue or purple). Notably several of these are serine residues, which raises the question whether serine phosphorylation is important for Spry function.

and the dorsal vessel, all places where Bnl and Btl are known to function. It is also expressed in a small subset of VNC neurons, oenocytes and the eye imaginal disc. Its expression is induced by the Bnl pathway, thus making the mode of repression a classical feedback mechanism. Tefft et al. demonstrated that the control of branching by dSpry in the *Drosophila* tracheal system has a parallel in mammals (Tefft et al., 1999). Using an antisense oligonucleotide strategy they demonstrated that inhibition of mSpry2 expression in E11.5 murine embryonic cells produces a 72% increase in murine lung branching. Northern blotting analysis showed that mSpry2 is also expressed in the adult lung, as well as the heart, brain, skeletal muscle and kidney.

Another hallmark paper showed that in *Drosophila*, at least, the role of Spry is not confined to modulation of tissue branching initiated by FGF. Casci et al. identified dSpry in a genetic screen as an inhibitor of EGF receptor (EGFR) signaling (Casci et al., 1999). EGFR triggers cell recruitment in the eye, and the authors showed that in eyes lacking dSpry there is an excess of photoreceptors, cone cells and pigment cells. Furthermore, they demonstrated in genetic screens that signaling from other receptor tyrosine kinases, Torso and Sevenless, is also inhibited by dSpry. Studies by Kramer et al. reinforced these observations, demonstrating that

overexpression of dSpry in wing veins and ovarian follicle cells, two systems where EGFR activation is required for patterning, results in a phenotype that resembles that of EGFR loss-of-function mutants (Kramer et al., 1999). Casci et al. further demonstrated that dSpry localizes to the inside surface of the plasma membrane and intercepts signaling at a point between the receptor and activation of Ras. This study implicated Spry as a likely generic inhibitor of the Ras/MAP-kinase signaling pathway. Subsequent work from most labs indicates that Spry proteins act intracellularly. Casci et al. employed an in vitro candidate approach and revealed that dSpry binds to Drk, the homologue of mammalian Grb2, and Gap1, an inositol-phospholipid-binding GTPase-activating protein (GAP) that has activity against Ras (Casci et al., 1999). It was therefore postulated that dSpry might sequester vital components of the Ras/MAP-kinase pathway. The authors further showed that the binding to Drk and Gap1 is mediated by the non-conserved N-terminal region of dSpry, whereas the conserved cysteine-rich domain is responsible for localizing the protein to the membrane. It thus appeared that the Sprys have a conserved, C-terminal domain responsible for targeting and a divergent protein-interacting domain through which the different isoforms recruit a range of proteins to fulfill their physiological functions.

Genetic analysis in *Drosophila* reinforced these observations allowing Reich et al. to demonstrate that ectopic expression of dSpry abolishes the pattern of activated MAP kinase observed in embryos and specifically in another FGFR system involving heartless (another FGFR1 homologue), as well as EGF-controlled systems, such as wing imaginal discs (Reich et al., 1999). dSpry expression is induced by activation of the EGFR pathway in some but not all cells. The responsive cells include follicle cells of the ovary, the wing imaginal disc and the eye disc, and these studies deduced that dSpry intersects the Ras/MAP-kinase pathway at or downstream of the Raf kinase. This represented the third postulated mode of dSpry action. It was therefore possible that dSpry might have multiple or different intersection points for different factors; alternatively some unifying evidence remained to be discovered.

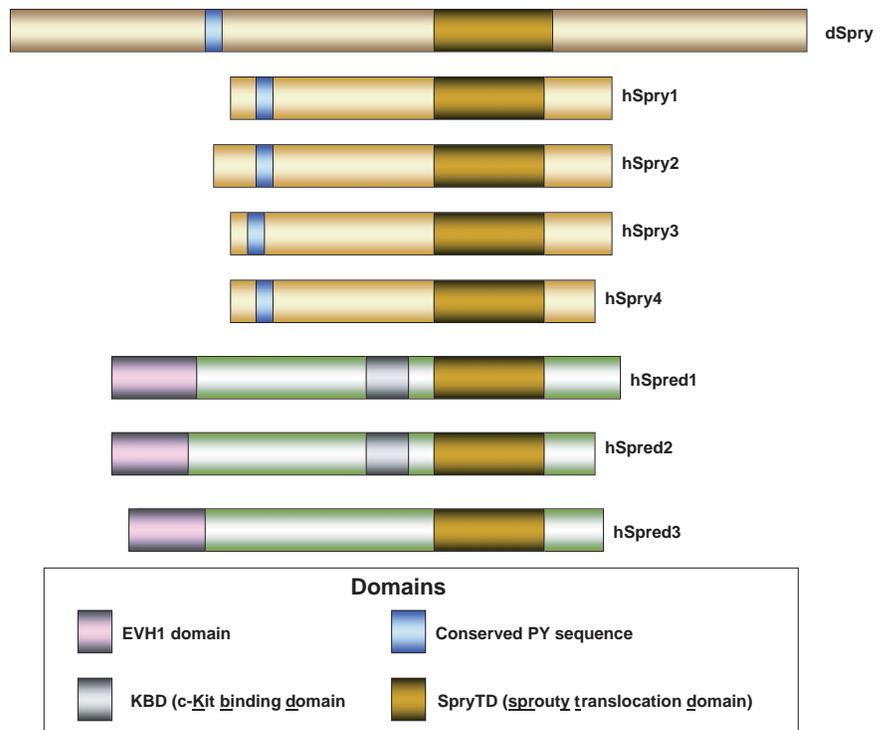
In another non-mammalian species, *Xenopus*, Nutt et al. demonstrated that the expression of xSpry2 is induced by the FGF/Ras/MAP-kinase pathway (Nutt et al., 2001). xSpry2 inhibits mesoderm induction and results in truncation of the anterior-posterior axis. Although blocking the Ras/MAP-kinase pathway inhibits mesoderm induction, xSpry2 does not seem to act on this level but, rather, inhibits FGF-dependent calcium signaling.

Collective evidence strongly indicates that Spry isoforms play a role, probably in opposition to FGF signaling pathways, in modeling different varieties of branching tissue during development. Lung, kidney, prostate, blood vessels and breast ducts all seem likely to come under the modulating influence of induced Spry proteins. The question can therefore be asked: how does the branch manager work?

Note that a number of studies have attempted to address the mode of action of Sprys and there has been some diversity in the observations made. Here we emphasize those that appear to have been substantiated by more than one laboratory.

### Unraveling the mechanism of action of mammalian Spry proteins

Minowada et al. demonstrated that the inhibitory effects of dSpry on RTK signaling were reiterated in mammalian systems (Minowada et al., 1999). They showed in chick and mouse that overexpressed Spry antagonizes FGF signaling, specifically in the chick limb, where Spry inhibits chondrocyte differentiation and produces phenotypes that resemble those induced by FGFR3 mutations. An interesting study, based on a differential display analysis of genes involved in patterning the mid/hindbrain of the chick neural tube, identified Spry2 (Chambers et al., 2000; Chambers and Mason, 2000). These researchers found a close correlation of Spry2 expression with known sites of FGF activity but little correlation with expression patterns of members of the EGF family. This was the first indication that in mammals Spry2 might be closely



**Fig. 2.** A representation of the proteins that contain the conserved SpryTD together with associated domains.

associated with the FGF signaling system rather than a generic RTK/MAP-kinase downregulator. Whether this applies to the other Spry isoforms remains to be seen.

### Target of the conserved cysteine-rich domain

Evidence from studies in *Drosophila* has clearly demonstrated that the C-terminal, cysteine-rich domain of dSpry localizes it to the inner surface of the plasma membrane. Early evidence from mammalian cells indicated that Spry1 and Spry2 are localized to the membrane through palmitoylation and association with caveolin (Impagnatiello et al., 2001). However, we have been unable to observe any pronounced association with caveolin, and our initial study on mammalian Spry proteins surprisingly revealed that in unstimulated cells all the Spry proteins, when expressed ectopically in COS-1 or 293T cells, are cytosolic. Upon stimulation by either FGF or EGF, Spry proteins rapidly translocate to a peripheral membrane location, specifically to ruffles (Lim et al., 2000). The data derived from ectopic expression studies was supported by evidence showing that endogenous hSpry-1 in HUVEC cells similarly translocates from the cytosol to the membrane periphery following activation by FGF (Impagnatiello et al., 2001). Tefft et al. have also demonstrated that mSpry2 translocates to plasma membranes when mouse lung epithelial cells are stimulated with FGF10 (Tefft et al., 2002). The Spry translocation domain (SpryTD) comprises a particularly well-conserved sequence of the cysteine-rich sequence from residues 178-282 of hSpry2. Activated Rac, previously shown to be necessary for ruffle formation (Ridley et al., 1992), also induces the translocation of Spry proteins,

independently of growth factor stimulation (Lim et al., 2002). The target of the SpryTD appears to be downstream of activated Rac and is either cryptic in the quiescent state or synthesized rapidly as a result of RTK stimulation. Alternatively the SpryTD might be cryptic in the quiescent state, as are the C1/C2 domains of various PKC enzymes in which other parts of the protein (pseudosubstrate sequences) inhibit activation through intramolecular binding (Newton, 1995). This is considered to be unlikely, however, because both the C-terminal half of Spry and a smaller derived sequence are capable of translocation when cells are stimulated by FGF or EGF. It was deemed that the cellular target is probably produced rapidly upon RTK stimulation, and indeed recent ectopic expression studies indicate that the SpryTD, in a similar manner to a diverse array of previously characterized domains, targets PtdIns(4,5) $P_2$  (Lim et al., 2002). In common with other domains that bind to inositol phospholipids, a basic residue appears to be involved in binding of the lipid head group. An R252D point mutation in hSpry2 abrogates FGF-stimulated translocation, resulting in inability of the protein to inhibit the Ras/MAP-kinase pathway (Lim et al., 2002). Membrane location or translocation thus appears necessary for the inhibitory effects of Spry proteins.

### Inhibition of the mammalian Ras/MAP-kinase pathway

FGF and VEGF induce phosphorylation and activation of MAP kinase in vascular endothelial cells. This can be inhibited by co-expression of mSpry4, which appears to act upstream of Ras, because co-expression of constitutively active L6I Ras rescues the inhibition (Lee et al., 2001). Impagnatiello et al. have reported that overexpressed mSpry1 and mSpry2 inhibit FGF- and VEGF-induced proliferation and differentiation by repressing pathways leading to MAP-kinase activation (Impagnatiello et al., 2001); however, they observed that although EGF-induced proliferation of endothelial cells is also inhibited by mSpry1 and mSpry2, the activation of MAP kinase in this case is unaffected.

Sasaki et al. have observed similar differences in the inhibitory effects that the mammalian Spry proteins have on the EGF and FGF pathways. They demonstrated that EGF, FGF and phorbol ester rapidly induce Spry2 and Spry4 in cultured cells. Overexpression of hSpry2 or hSpry4 inhibited FGF-induced MAP-kinase activation but did not affect EGF- or PDBu-induced MAP-kinase activation. They also generated dominant negative point mutants of hSpry2 (Y55 hSpry2) and hSpry4 (Y53 hSpry4). Whereas wild-type hSpry4 inhibits FGF-induced differentiation in PC12 cells, Y53 hSpry4 enhances differentiation. Previously the extension of neurites in PC12 cells has been linked to the strength and duration of a MAP-kinase signal (Marshall, 1995). Two further reports have reinforced the idea that Spry1 or Spry2 inhibits FGF-induced Ras/MAP-kinase activation (revealing incidentally that they do not affect either the p38 or JNK MAP-kinase pathways or the Akt pathway) (Gross et al., 2001; Yusoff et al., 2002). The former placed the Spry proteins before Ras, while the latter put them at the level of Raf. In the former study it was demonstrated that expression of hSpry2 results in formation of less Ras-GTP (Gross et al., 2001). This was not reiterated in the study of Yusoff et al., which showed that when hSpry2 was

similarly expressed in cells it inhibited activation of MAP kinase that was stimulated by the constitutively active V12 Ras mutant (Yusoff et al., 2002). In another study, Leeksa et al. showed that the ubiquitously expressed hSpry4 suppresses insulin- and EGFR-receptor-induced MAP-kinase signaling probably at the level of or upstream of Ras (Leeksa et al., 2002).

The work discussed above indicates that Spry1, Spry2 and Spry4 inhibit FGF-induced Ras/MAP-kinase activation in a relatively specific manner and that in mammals there seems to be some deviation from what is observed in *Drosophila* with respect to the effects of Spry proteins on EGF-induced signaling. Subsequent publications have provided some insight into the apparent differences between the effects of Spry on EGF- and FGF-activated signaling, demonstrating that Spry2 interacts with the adaptor c-Cbl (Egan et al., 2002; Wong, E. S. et al., 2002), which is necessary for the ubiquitylation and endocytosis of EGFR (Waterman and Yarden, 2001). This might provide one mechanism by which inhibition is effected (see below).

### Binding partners for Spry proteins

dSpry binds to Drk (the *Drosophila* homologue of Grb2) and Gap-1 [a PtdIns-binding RasGAP (Casci et al., 1999), not to be confused with the 120 kDa, tyrosine-phosphorylated RasGAP]. Several groups have reported that in mammalian systems the various Spry isoforms bind to Grb2, but the binding to Gap-1 is debatable. Tefft et al. showed that mSpry binding to Gap-1 is diminished when FGFR is activated (Tefft et al., 2002), but we have failed to confirm that mammalian or indeed *Drosophila* Spry proteins bind to Gap-1 (Q. Chen and G. R. Guy, unpublished). One would expect that binding partners of the Spry proteins in mammals are key components or modulators of the mainstream Ras/MAP-kinase pathway.

### Binding partners in FGF signaling

Tefft et al., working on the inhibitory effects of mSpry2 on FGF10-activated MAP kinase, demonstrated that a plethora of upstream MAP-kinase pathway components interact either directly or indirectly with mSpry2 (Tefft et al., 2002). In precipitation experiments, it was demonstrated that, when the lysates from FGF-stimulated cells were compared with parallel lysates from untreated cells, there was increased co-precipitation of FGFR substrate 2 (FRS2) and Raf1, with a concomitant decrease in co-precipitation of Shp2 and Gap-1. None of these interactions was extensively analyzed or shown to involve direct binding. Other groups (Casci et al., 1999; Yusoff et al., 2002), have found no interaction between Raf-1 and either dSpry or hSpry2. Hanafusa et al. have demonstrated that ectopic expression of mSpry2 decreases association of Grb2 with FRS2 and Shp2 following FGFR activation (Hanafusa et al., 2002). Such a result suggests that mSpry2 affects the FRS2/Shp2/Grb2 SH2-mediated interactions that are the main FGFR-directed route to MAP-kinase activation (Wong, A. et al., 2002; Lax et al., 2002). Again, the evidence is suggestive only and there was no demonstration of mechanism, direct binding or characterization of binding motifs.

A handful of upstream MAP-kinase pathway proteins have

thus been implicated in binding directly or indirectly to Spry isoforms during FGFR signaling but a unifying mechanism has yet to emerge. In the case of EGFR signaling, however, strong evidence indicates that interaction with Cbl family proteins has a central role in Spry function, as we discuss below.

### The role of Cbl family proteins

Spry proteins might be variously palmitoylated or serine/threonine phosphorylated (Impagnatiello et al., 2001), but there is currently no evidence that these modifications have any profound effect on Spry function. A number of reports detail the tyrosine phosphorylation of Spry proteins and its potential impact on their function. Tefft et al. first reported the tyrosine phosphorylation of a Spry protein (Tefft et al., 2002) and subsequent papers showed that tyrosine phosphorylation of Spry proteins is not only an observable biochemical event but necessary for physiological function (Sasaki et al., 2001; Hanafusa et al., 2002; Rubin et al., 2003) (C. W. Fong, H. F. Leong, E. S. M. Wong, J. Lim, P. Yusoff and G. R. Guy, unpublished). Several groups almost in parallel identified a conserved tyrosine residue in the Spry isoforms that is the target of unspecified tyrosine kinases and lies in a conserved NXYXXXP motif (Fig. 3). The phosphorylated tyrosine residue will bind directly to the SH2 domain of Cbl (which has binding characteristics more resembling a PTB domain). The first study identifying hSpry2-associated proteins was by Wong et al. (Wong, E. S. et al., 2001). c-Cbl (and Cbl-b) was demonstrated to constitutively associate with hSpry2. Binding was shown to be direct; specifically a region in the N-terminal end of hSpry2 was shown to bind to the RING finger domain of c-Cbl. This binding would have the potential to impact on the ability of c-Cbl to ubiquitlate its physiological targets. Additional evidence, however, involving c-Cbl (see later in review) provides a more profound explanation for the inhibition of EGFR endocytosis induced by hSpry2. Binding

of c-Cbl has several downstream effects (Tsygankov et al., 2001). Rubin et al. have shown that hSpry2 can be ubiquitlated through the association with c-Cbl and this can lead to the downregulation of the Spry isoform (Rubin et al., 2003). Fong et al. further demonstrated that when the three highly conserved residues (Fig. 3) are individually mutated to alanine residues, c-Cbl binding is inhibited and this correlates well with the ability of hSpry2 to function as a MAP-kinase inhibitor (C. W. Fong, H. F. Leong, E. S. M. Wong, J. Lim, P. Yusoff and G. R. Guy, unpublished). Thus phosphorylation seems to be necessary for hSpry2 function, but it also requires the integrity of the highly conserved flanking sequences. Work from several labs has detailed how expression of hSpry2 causes an abrogation of c-Cbl-directed EGFR ubiquitlation and subsequent receptor endocytosis (Egan et al., 2002; Wong, E. S. et al., 2002).

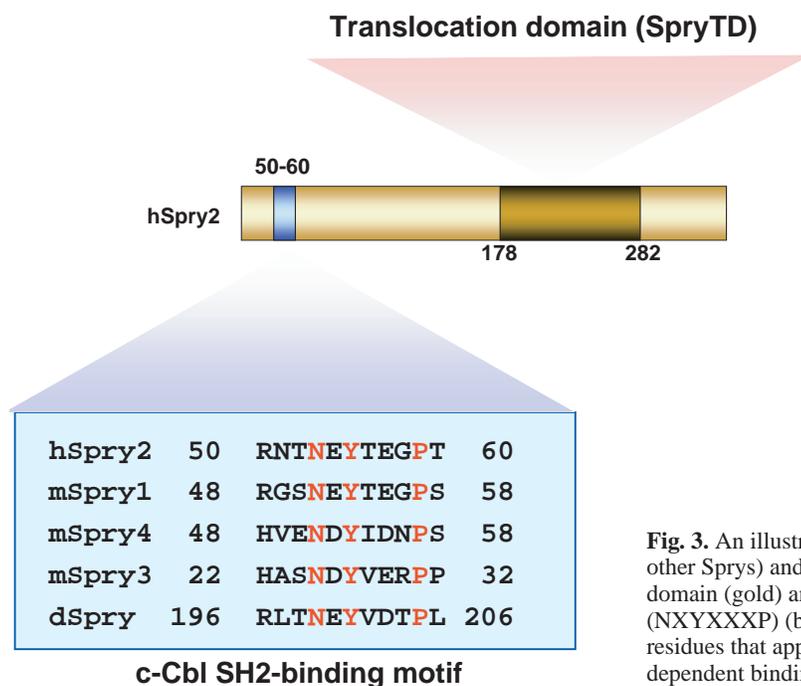
### Mechanism of action of hSpry 2

Currently it is too early to put forward a complete model for the inhibition of EGF- and FGF-induced MAP-kinase activation by hSpry2. Nevertheless two conserved sequences appear to be necessary for all the observed actions of Spry2 (see Fig. 3): the SpryTD and the sequence around the phosphorylated tyrosine residue that binds c-Cbl.

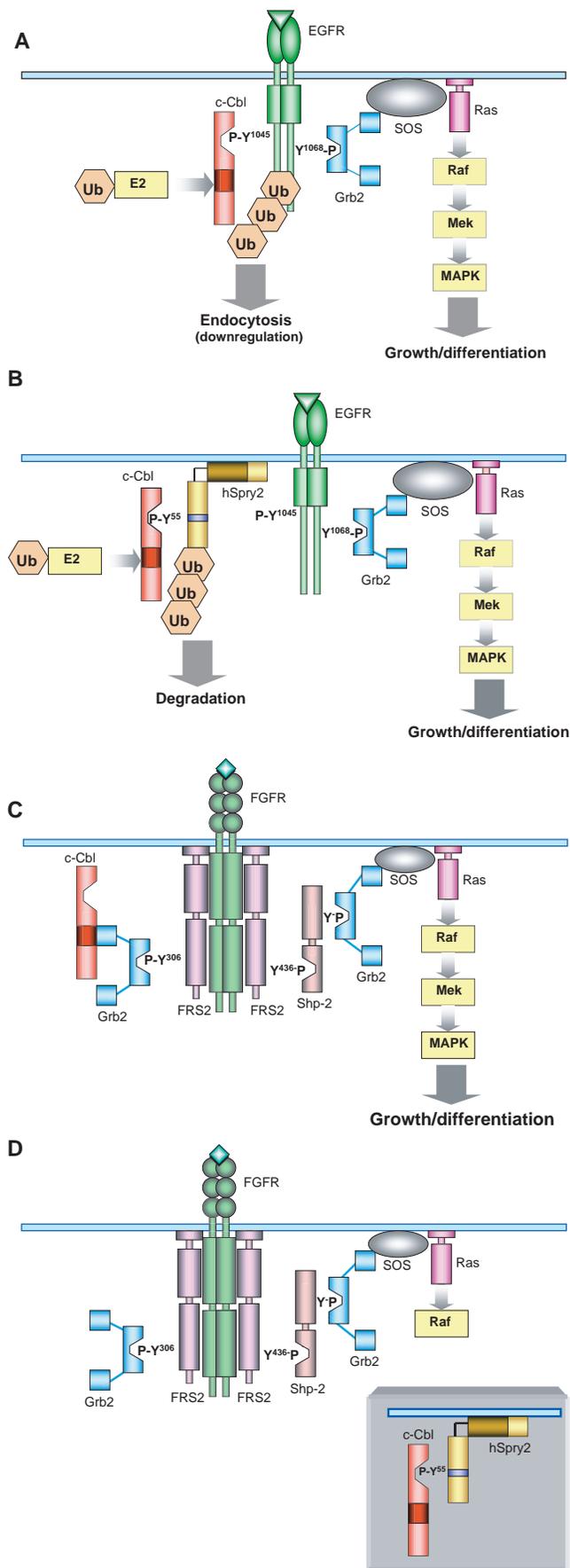
We propose the following hypothesis for the inhibition of EGFR endocytosis (Fig. 4A,B). In this model, hSpry2 binds constitutively to c-Cbl (an N-terminal sequence in hSpry2 binds to the RING finger of c-Cbl). Upon stimulation of RTKs, the conserved tyrosine residue becomes phosphorylated and binds directly to the SH2 domain of c-Cbl, which normally binds at a site within a similar motif on the activated EGFR (Y1045). Following binding to EGFR, c-Cbl assumes its role as an E3 ubiquitin ligase and ubiquitlates the EGFR, targeting it for destruction by the endocytotic machinery of the cell (Fig. 4A). Thus hSpry2, which has a similar sequence around the Y55 residue, binds to the site where receptors are highly concentrated in membrane ruffles and acts as a decoy, 'luring' c-Cbl away from the EGFR by direct competition. This would thus inhibit EGFR endocytosis and consequently the downstream pathways, such as the Ras/MAP-kinase pathway, would remain in an activated state (Fig. 4B). The FGFR isoforms do not have a similar targeting sequence for c-Cbl and consequently are immune to this form of binding competition and subsequent attenuation of 'normal' downregulatory events (Fig. 4C,D).

### Perspectives

Yigzaw et al. reported that hSpry2 inhibited both proliferation and migration in cultured mammalian cells (Yigzaw et al., 2001).



**Fig. 3.** An illustration of two functionally important domains on hSpry2 (and other Sprys) and the functions associated with them. The SpryTD (178-282) domain (gold) and the conserved tyrosine phosphorylation sequence (NXYXXXP) (blue). The residues highlighted in red are highly conserved residues that appear to be necessary for the tyrosine phosphorylation-dependent binding of c-Cbl to Spry proteins.



Although the Ras/MAP-kinase pathway has been shown to be involved in cell proliferation, there is no compelling evidence it is involved in migration. Deletions of hSpry2 at residues 123-177 and 178-194 abrogate the inhibitory action of hSpry2 on migration. A subsequent publication (Yigzaw et al., 2003) demonstrated that the expression of hSpry2 increases the amount of soluble PTP1B, which correlates with the inhibition of cell migration but not proliferation. The impact of the Spry isoforms on other signaling pathways in mammalian cells clearly requires additional research.

There is a greater understanding of how Spry2 modulates EGFR endocytosis. How it regulates FGFR-induced MAP-kinase signaling and thus the basis of its developmental function in branching is much less clear. Sequence comparisons suggest that Spry proteins function by a similar mechanism in distantly related species and underscore the importance of the SpryTD and the N-terminal, short tyrosine-containing motif, which is 100% conserved from *Drosophila* to man. There are several serines that also appear to be conserved, which begs the question of whether these are phosphorylated in a physiological context.

If in mammalian systems Spry activity is confined mainly to FGFR pathways, a logical target for Spry would be the relatively specific FGFR or FRS2 docking protein. The latter is confined to the FGF and nerve growth factor (NGF) pathways, and is reported to act directly or indirectly with

**Fig. 4.** A suggested model for the hSpry2-induced abrogation of EGFR endocytosis. (A) Upon activation of EGFR, various tyrosine residues become phosphorylated on the cytosolic tail of the receptor and they become targets of both positive and negative regulatory proteins bearing intrinsic SH2 domains. Y1045 has the surrounding motif FLQRY(1045)SSDPT. The atypical SH2 domain on c-Cbl binds directly to Y1045. c-Cbl also functions as an E3 ubiquitin ligase where it 'tags' certain target proteins for ubiquitylation, endocytosis and destruction via the intracellular endosomal system. In this case, c-Cbl accepts ubiquitin from an E2 ligase to enzymatically polyubiquitylate the EGFR, the first step in the downregulation process. A positive signal, by which the Ras/MAP-kinase pathway gets activated, is initiated by the binding of the Grb2 SH2 domain to tyrosine residues exemplified by Y1068. (B) When hSpry2 is expressed in activated cells it translocates to the plasma membrane, ostensibly by binding to free PtdIns(4,5)P<sub>2</sub>, and becomes phosphorylated on Y55, which is imbedded in a motif similar to that around Y1045 on the EGFR. The two tyrosine-phosphorylated sites on EGFR and hSpry2 therefore compete for binding to the SH2 domain of c-Cbl. Instead of EGFR downregulation it appears that the interaction of c-Cbl and hSpry leads to ubiquitylation and subsequent downregulation of hSpry2. The net result of this is the failure of EGFRs to submit to endocytosis and destruction, thus allowing the Ras/MAP-kinase signal to be sustained. (C) In the case of FGFR signaling there is a similar balance between Ras/MAP-kinase activation and signal inhibition. FGFRs require the constitutively associated docker protein FRS2 to provide the appropriate tyrosine-phosphorylated sites. There are four sites for Grb2 SH2-binding, represented by Y306, and two Shp2 SH2-binding sites, represented by Y436. The Ras/MAP-kinase signal is channeled mainly through the Shp2 pathway, whereas the directly bound Grb2 has associated c-Cbl that is capable of ubiquitylating both FGFRs and FRS2. (D) When Spry is expressed it translocates and binds to c-Cbl as described in B. The next part of the model becomes descriptive rather than mechanistic, whereby the Ras/MAP-kinase signal is inhibited possibly as a corollary of c-Cbl associating via its SH2 domain to Y55 of Spry.

hSpry2 (Tefft et al., 2002; Hanafusa et al., 2002). However, FRS2 appears to be absent from *Drosophila* and as such would not fit into a universal scheme of Spry function. Currently it has been reasonably well established that phosphorylation of Y55 on hSpry2 is necessary for its inhibitory effect on the Ras/MAP-kinase pathway. It is likely the identity of the protein (or proteins) that binds to this site will provide the key to Spry function. It is plausible that proteins with SH2 or PTB domains that are key components of the Ras/MAP-kinase pathway are 'diverted' onto this site, which would take them away from functional complexes. Neither the SH2 domain of Grb2 nor Shp2 binds directly to the hSpry2 Y55 motif, because both have strict C-terminal adjunct residues in comparison to the NXYXXXP conserved sequence found in all Spry proteins. Currently c-Cbl is the only candidate for binding to hSpry2 Y55 and could fulfill the inhibitory function by one or more of the roles that have been assigned to this versatile protein (Clague and Urbe, 2001; Rao et al., 2002; Shtiegman and Yarden, 2003) (C. W. Fong, H. F. Leong, E. S. M. Wong, J. Lim, P. Yusoff and G. R. Guy, unpublished). How this actually works and what other proteins are involved will be the topic of further studies.

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