FRS2-dependent SRC activation is required for fibroblast growth factor receptor-induced phosphorylation of Sprouty and suppression of ERK activity

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Summary

Activation of signalling by fibroblast growth factor receptor leads to phosphorylation of the signalling attenuator human Sprouty 2 (hSpry2) on residue Y55. This event requires the presence of the signalling adaptor fibroblast growth factor receptor substrate 2 (FRS2). The phosphorylation of hSpry2 is therefore mediated by an intermediate kinase. Using a SRC family kinase-specific inhibitor and mutant cells, we show that hSpry2 is a direct substrate for SRC family kinases, including SRC itself. Activation of SRC via fibroblast growth factor signalling is dependent upon FRS2 and fibroblast growth factor receptor kinase activity. SRC forms a complex with hSpry2 and this interaction is enhanced by hSpry2 phosphorylation. Phosphorylation of hSpry2 is required for hSpry2 to inhibit activation of the extracellular signal-regulated kinase pathway. These results show that recruitment of SRC to FRS2 leads to activation of signal attenuation pathways.

Key words: FGFR, FRS2, Sprouty, SRC, ERK

Introduction

A major question in growth factor action is how signalling dynamics are controlled by the components of the activated receptor complex (Yamada et al., 2004). Fibroblast growth factor receptors (FGFRs) are signal transduction receptors that direct diverse cellular processes including cell growth, survival differentiation, migration and tissue morphogenesis (Mason, 1994; Wilkie et al., 1995). FGFRs are receptor tyrosine kinases (RTKs) that form an activated multi-component signalling complex as a result of FGF ligand-binding, receptor dimerisation and inter-molecular tyrosine phosphorylation (Weiss and Schlessinger, 1998; Schlessinger, 2000). The exact architecture(s) of the FGFR signalling complex is not fully defined. It is known that inter-molecular phosphorylation of FGFRs creates recognition sites for proteins, which contain sequence-specific phosphotyrosine binding domains. These can interact directly with the receptor – such as PLCγ (Mohammadi et al., 1991) – or indirectly via receptor-mediated tyrosine phosphorylation of adaptor proteins such as FGF receptor substrate 2/SNT1 (FRS2, also known as Suc-associated neurotrophic factor 1). Association of substrates with the phosphorylated form of FRS2 leads to activation of multiple signalling pathways (Hadari et al., 1998; Kusakabe et al., 2001; Ong et al., 2001; Stoletov et al., 2002; Wong et al., 2002a).

FRS2 is a lipid-anchored adaptor protein that serves as the primary link between FGFR activation and Ras-MAP kinase signalling (Lin et al., 1998; Xu et al., 1998; Dhalluin et al., 2000; Ong et al., 2000). FRS2 comprises a receptor recognition domain of the phosphotyrosine binding (PTB) class (Ong et al., 2000) – which constitutively associates with the juxta-membrane region of the FGFR – and an effector domain which contains multiple tyrosine and serine phosphorylation sites which direct the association of further components of the signalling complex (Xu et al., 1998; Ong et al., 2000). Tyrosine-phosphorylation of FRS2 induced by FGFR activation promotes robust activation of the Ras-MAP kinase (ERK1/2) pathway (Xu and Goldfarb, 2001). The sequence within the FGFR1 juxta-membrane region required for specific interaction with FRS2 was initially identified as KSIPLRRQVTVS (amino acids 419-430 in human FRS2) (Ong et al., 2000). It was revealed that the inclusion or elimination – via alternative splicing – of two amino acids in this region (V427 and T428 in FGFR1) dictates the formation of the FRS2-dependent signalling complex (Burgar et al., 2002) and subsequent activation of the Ras-MAP kinase pathway (Twigg et al., 1998). Exploitation of these VT+ and VT– FGFR splice variants provides a powerful method to determine the role of FRS2 in FGFR-mediated signalling processes and the identity of FRS2 partners.

In some growth factor receptor systems, activation of receptor kinases results in subsequent activation of non-receptor protein tyrosine kinases typified by SRC family, SYK family and Janus kinases. The SRC family kinases (SFKs) encompass several related members: SRC, FYN and YES are ubiquitously expressed; the expression of HCK, FGR, LYN

and BLK is tissue-restricted (Courtneidge, 2002). SRC is maintained in an inactive form by an interaction between phosphorylated Y527 in the C-terminal region and its N-terminal SRC homology domain 2 (SH2) domain. Disrupting this interaction causes SRC activation. This is coincident with phosphorylation of Y416 in the activation loop of SRC (Bjorge et al., 2000; Harrison, 2003). RTK-mediated SRC activation was first observed following treatment of fibroblasts with platelet-derived growth factor (Ralston and Bishop, 1985; Gould and Hunter, 1988). Subsequently, SFKs were shown to associate with activated platelet derived growth factor (PDGF) receptors (Kypta et al., 1990). SFKs have also been implicated in signalling by other RTKs, including epidermal growth factor receptor, colony-stimulating factor receptor, nerve growth factor receptor and hepatocyte growth factor receptor (Thomas and Brugge, 1997; Abram and Courtneidge, 2000). SFKs are potential substrates for activated FGFRs (Zhan et al., 1994) and it has been reported that FGFR activation may play either a stimulatory or inhibitory regulatory role on SRC kinase activity in a cell-type specific manner (Landgren et al., 1995; Klint and Claesson-Welsh, 1999). However, neither the mechanism by which SFKs interact with activated FGFRs (Landgren et al., 1995) or the physiological substrates for FGFR-activated SFKs have been defined. Here, we identify the protein human Sprouty 2 (hSpry2) as a physiological substrate for FGFR-activated SFKs.

Sprouty was first identified as an antagonist of FGF-dependent signalling pathways required for the correct apical branching of *Drosophila melanogaster* airways (Hacohen et al., 1998) and was confirmed as an intracellular protein capable of both positively and negatively modulating RTK signalling pathways in later studies (Christofori, 2003; Li et al., 2003). Homologues of Sprouty have been identified in *Xenopus laevis*, chicken, mice and humans (Hacohen et al., 1998; de Maximy et al., 1999; Minowada et al., 1999; Tefft et al., 1999; Chambers and Mason, 2000), implying an evolutionarily conserved function. Four *sprouty* genes have been identified in mammalian genomes (de Maximy et al., 1999; Minowada et al., 1999; Tefft et al., 1999; Guy et al., 2003). Sprouty proteins comprise an N-terminal effector region containing a single tyrosine residue (Y55 in hSpry2), which is tyrosine-phosphorylated following either FGFR or epidermal growth factor receptor (EGFR) activation (Egan et al., 2002; Hanafusa et al., 2002; Wong et al., 2002b; Fong et al., 2003; Hall et al., 2003; Rubin et al., 2003) and a C-terminal domain, which anchors the protein to the internal face of the plasma membrane (Hacohen et al., 1998; Casci et al., 1999; Lim et al., 2000; Impagnatiello et al., 2001). The exact function of Sprouty in different RTK signalling systems is controversial. However, it has been reported in a variety of species that FGFR signalling via the Ras-MAP kinase – and other undefined pathways – is attenuated in the presence of Sprouty protein (Minowada et al., 1999; Tefft et al., 1999; Impagnatiello et al., 2001; Lee et al., 2001; Maileux et al., 2001; Nutt et al., 2001). A key issue, therefore, is the mechanism that connects FGFR activation to Sprouty phosphorylation and the role of this event in Sprouty function.

That a SRC-like kinase was required for tyrosine-phosphorylation of Sprouty2 by growth factors has been suggested in a very recent study, using both pharmacological inhibitors and a dominant-negative SRC construct (Mason et al., 2004). In this study, we demonstrate that hSpry2 is not a direct substrate for activated FGFRs but requires activated FGFR kinase, association of FRS2 with FGFRs and the consequential activation of SFKs. We also show that hSpry2 is a direct physiological substrate for activated SRC and that phosphorylation of hSpry2 on Y55 by SRC is necessary for hSpry2-mediated attenuation of activated ERK1/2. These findings reveal that activation of SRC via FRS2 is a key element of the FGFR signalling machine and that activation of SRC is required to attenuate signalling via the ERK1/2 pathway.

Materials and Methods

Plasmid constructs

Full-length hSpry2 cDNA was obtained from a human foetus-derived expressed sequence tag (EST) sequence by PCR (primers: 5′-CCC-GGATCCGTCGACATGGAGGCCAGAGCTCAGAGTGG-3′ and 5′-GGCCCTCGAGCTATGTTGGTTTTTCAAAG-3′) and sub-cloned into pEF-BOS-ires-Topaz mammalian expression vector (Burgar et al., 2002) at *Bam*HI-*Xho*I sites. A point mutation in hSpry2 (Y55 to A55) was introduced using the QuickChange technique (Stratagene) (primers: 5′-CGAAACACCAATGAGGCGACAGAG-GGGCCTAC-3′ 5′-GTAGGCCCCTCTGTCGCCTCATTGGTGTTT-CG-3′). A sequence encoding the N-terminal region of hSpry2 (amino acids 1-172) was generated by PCR (primers: 5′-CCCGGATCC-ATGGAGGCCAGAGCTCAGAGTGG-3′ 5′-AGTGAATTCGCCC-AAATCTTCCTTGCTCAGTG-3′) and sub-cloned into pGEX3C vector (Hudson et al., 1996; Barton et al., 1999) at *Bam*HI-*Eco*RI sites, generating a GST fusion protein. This vector contains an HRV3C protease recognition site between the GST and sequence of interest. Recombinant murine FGFR1s constructs: constitutively active FcFGFR1/FA/VT+, constitutively active FcFGFR1/FA/VT–, kinase Dead FcFGFR1/FD, and truncated FcFGFR1/FT have previously been described (Burgar et al., 2002). Full-length human FGFR2 IIIc cDNA (see Anderson et al., 1998) was sub-cloned into pcDNA3 (Invitrogen, Paisley, UK) at *Bam*HI-*Xba*I sites. Mammalian expression vectors containing wild-type *Src* or wild-type *Fyn* were kindly provided by Margaret C. Frame (Cancer Research UK Beaston Laboratories, Glasgow, UK).

Antibodies

A polyclonal antibody against the N-terminal region of hSpry2 was generated by the Binding Site Ltd (Birmingham, UK), which also supplied anti-sheep IgG HRP secondary antibody. Monoclonal anti-phospho-tyrosine antibodies 4G10 and PY20 were purchased from Upstate Biotechnology (Lake Placid, NY, USA) and ICN Biomedicals (Aurora, OH, USA), respectively. Anti-human IgG Fc HRP was purchased from Pierce [Rockford, IL, USA). Polyclonal antibody against the C-terminal domain of FGFR2 (Bek (c-17)] and polyclonal antibody against SRC tyrosine kinases [Src (SRC2)] were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-SRC (mab327) antibody was purchased from Oncogene Research Products (San Diego, CA, USA). Polyclonal antibody against active MAPK was purchased from Promega (Madison, WI, USA). Polyclonal antibody against p44/42 MAP kinase and polyclonal antibody against phospho-SRC (pY416) were purchased from Cell Signalling (Beverly, MA, USA). Anti-mouse IgG HRP and anti-rabbit IgG HRP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Cell culture and transfections

Human embryonic kidney epithelial 293T cells were cultured at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)

(Invitrogen, Paisley, UK) supplemented with 2 mM L-glutamine (Invitrogen), 0.1 mg/ml streptomycin, 0.2 units/ml penicillin 1 mM sodium pyruvate (all Sigma, UK) and 10% foetal calf serum (v/v) (Labtech International). Mouse embryonic fibroblast-derived NIH3T3 cells were cultured at 37°C, 5% CO_2 in DMEM supplemented with 2 mM L-Glutamine, 0.1 mg/ml streptomycin, 0.2 units/ml penicillin and 10% donor calf serum (Sigma). The SYF cell line derived from *Src*$^{-/-}$ *Yes*$^{-/-}$ *Fyn*$^{-/-}$ mouse embryos was purchased from ATCC (American Type Culture Collection). SYF cell lines stably expressing either wild-type *Src* (wtSrc), an active mutant (Y527F), or a kinase dead mutant (K295R) were generously provided by Jonathan A. Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA) (Cary et al., 2002). SYF wild-type counterpart control cell line derived from mouse embryo fibroblast was generously provided by Margaret C. Frame (Cancer Research UK Beaston Laboratories, Glasgow, UK). All SYF related cell lines were cultured at 37°C, 5% CO_2 in DMEM supplemented with 4 mM L-glutamine, 1.5 g/l sodium bicarbonate (Invitrogen), 0.1 mg/ml streptomycin, 0.2 units/ml penicillin and 10% foetal bovine serum (Sigma). 293T cell transfection was carried out using the standard calcium phosphate method as described previously (Burgar et al., 2002). Transfection of NIH3T3 cells and SYF cells was performed using lipofactamine 2000 (Invitrogen) following the manufacturer's instructions.

FGF2 stimulation

Either 24 or 48 hours after transfection, growth media was aspirated and the cells were washed carefully with PBS. The media was then replaced with serum-free media. Following serum-deprivation for 5 hours, cells were incubated with 100 ng/ml FGF2 (for details, see Anderson et al., 1998) and 10 μg/ml heparin (Celsus labs, Cincinnati, OH) at 37°C, 5% CO_2 for the indicated times. To terminate stimulation, the media was removed and the cells were washed with cold PBS and kept on ice before lysis.

SU6656 inhibition

SU6656 (Calbiochem, Nottingham, UK) was dissolved in DMSO and stored at –20°C. Either 24 or 48 hours after transfection, the cells were washed carefully with PBS and changed to serum-free media. Cells were serum-deprived for 5 hours and then incubated with SU6656 (or DMSO as control) at indicated concentrations for 1 hour at 37°C, 5% CO_2 prior to FGF2 stimulation.

Cell lysis, immunoprecipitation (IP), and western blot analysis (IB)

Cell lysis, immunoprecipitation and western blots were essentially performed as described previously (Burgar et al., 2002). Cells were lysed in a Triton X-100-based lysis buffer [10 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$, 5 mM EGTA pH 8.0, 0.5% Triton X-100 (w/v), 1 mM Na_3VO_4, 50 mM NaF and 1 tablet of protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) per 10 ml of buffer]. For analysis of recombinant murine FcFGFR1/FA/VT+, FcFGFR1/FA/VT–, kinase Dead FGFR1, and truncated FGFR1, protein A-sepharose fast flow slurries (Amersham Biosciences, Inc., UK) were directly added to aliquots of cleared cell lysates and incubated overnight at 4°C. For the immunoprecipitation of hSpry2, FGFR2 and SRC, aliquots of cleared lysates were incubated with 1 μg of corresponding antibodies for 1 hour at 4°C prior to the addition of 60 μl 50% washed-protein A or G-sepharose fast flow slurries (Amersham Biosciences). Following overnight incubation at 4°C and washing with PBS, bound proteins were eluted with SDS sample buffer [125 mM Tris-HCl pH 6.8, 20% glycerol (w/v), 4% SDS (w/v), 0.1% bromophenol blue (w/v), 10% β-mercaptoethanol (w/v)] and subjected to SDS PAGE. Anti-phosphotyrosine immunoblotting always utilised a cocktail of PY20 and 4G10.

In-vitro peptide binding assay

Two 18-amino-acid-long peptides of hSpry2 which included Y55 (RAIRNTNEYTEGPTVVPR) in both its phosphorylated and non-phosphorylated form were synthesised (Alta Bioscience, Birmingham, UK). Both peptides were biotin-acetylated at the N-terminus and dissolved in DMSO to a concentration of 10 μg/μl. Peptides were stored at –20°C. Biotin-acetylated peptides (600 μg) were immobilised on 150 μl 50% UltraLink immobilised NeutrAvidin slurry (immobilised on 3M Emphaze™ Biosupport Medium AB 1) (Pierce, Rockford, IL) by a 1-hour incubation at RT. 293T cells (8×10^7) were lysed and cleared cell lysates collected by centrifugation (20,800 ***g*** for 30 minutes). Cleared cell lysates were then added to the peptide-immobilised NeutrAvidin Medium AB 1. After an overnight incubation at 4°C, the media was washed thoroughly with PBS and then SDS sample buffer was added before boiling (100°C for 5 minutes). Peptide-associated proteins were separated on SDS-PAGE gel for subsequent western blot analysis.

Purification of N-terminal hSpry2 and in vitro western blot kinase assay

A GST-fused N-terminal region of hSpry2 was expressed in *E. coli* strain BL21. Protein expression, purification and 3C protease cleavage were performed as described previously (Hudson et al., 1996; Burgar et al., 2002; Underhill-Day et al., 2003). SDS-PAGE and subsequent staining with Coomassie Blue confirmed the purity of this protein. This N-terminal region of hSpry2 was then utilised as a substrate for 'in-vitro kinase assays' as described previously (Ulrich et al., 2003). Kinase reactions were performed in a final volume of 20 μl kinase reaction buffer (25 mM Tris-HCl pH 7.2, 31.25 mM $MgCl_2$, 21.25 mM $MnCl_2$, 1.5 mM EGTA, 262.5 μM Na_3VO_4, 0.7 mM DTT, 4 mM MOPS (pH 7.2) and 5 mM β-glycerol phosphate) containing 5 μg of the N-terminal region of hSpry2, in the presence or absence of either 100 μM ATP or 200 ng active SRC (Upstate, Lake Placid, NY). Reactions were incubated for 10 minutes at 30°C and stopped by adding SDS sample buffer and by boiling for 5 minutes. Proteins were resolved by SDS-PAGE (12%) and transferred to PVDF membrane (Millipore, Watford, UK) before probing with an anti-phosphotyrosine cocktail and a hSpry2 antibody.

Results

Activated FGFR phosphorylates hSpry2 in an FRS2-dependent manner

We began this study by examining the relationship between activated FGFRs and phosphorylation of hSpry2. We employed a version (and mutant derivates) of mouse FGFR1, in which the extracellular domain was replaced by the Fc component of human immunoglobulin (Burgar et al., 2002). This form of FGFR1 exhibits constitutive tyrosine kinase activity via covalent dimerisation of the Fc moiety (Fig. 1A, lane 3). Coexpression of FcFGFR1/FA/VT+ and hSpry2 in transfected 293T cells results in tyrosine phosphorylation of hSpry2 (Fig. 1A, lane 3). No tyrosine-phosphorylation of hSpry2 was observed when coexpressed with either a 'kinase dead' version of FGFR1 (FcFGFR1/FD) or a truncated FGFR1 (FcFGFR1/FT) in which the kinase and juxta-membrane domains had been deleted (Fig. 1A, lane 4 and 5, respectively) despite comparative expression of all receptor constructs and hSpry2 (Fig. 1A, IB:Fc and IB:hSpry2, respectively). We also determined that there was no basal phosphorylation of either endogenous hSpry2 or transfected hSpry2 by the endogenous unstimulated FGFR population (Fig. 1A, lane 1 and 2,

respectively). This experiment shows that activation of FGFR1 kinase results in phosphorylation of hSpry2.

We next investigated the requirement for FRS2 in the FGFR1-mediated phosphorylation of hSpry2. For this purpose we used the VT– form of the FcFGFR1/FA construct (Burgar et al., 2002) which, while retaining full kinase activity, does not exhibit constitutive association with FRS2 and cannot execute FRS2-dependent functions. As we expected, FcFGFR1/VT+ and FcFGFR1/VT– were constitutively active in the absence of FGF2 stimulation (data not shown). However, FcFGFR1-dependent tyrosine phosphorylation of hSpry2 is substantially attenuated when co-transfected with FcFGFR1/VT– (Fig. 1B, compare lanes 3 and 5). This suggests that FGFR1-mediated phosphorylation of hSpry2 is mainly indirect and depends upon the association of FRS2 with FGFR1.

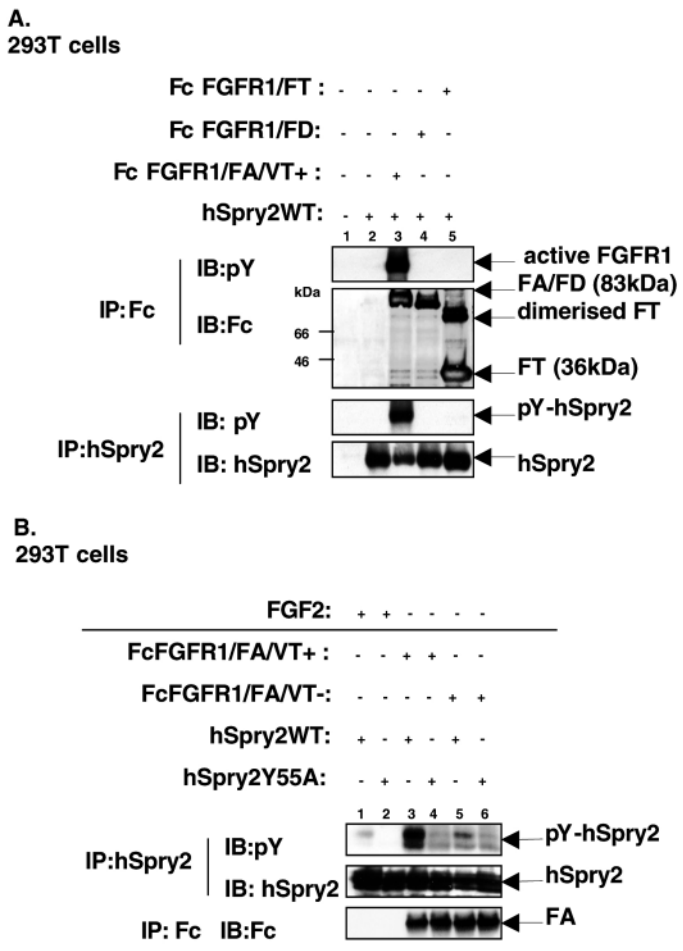

Fig. 1. Activated FGFR phosphorylates hSpry2 Y55 in an FRS2-dependent manner. (A) hSpry2 was tyrosine-phosphorylated downstream of the active FGFR. Wild-type hSpry2 (hSpry2WT) was co-transfected into 293T cells with either constitutively active FGFR1 (FcFGFR1/FA/VT+), kinase dead FGFR1 (FcFGFR1/FD) or truncated FcFGFR1 (FcFGFR1/FT). FGFR constructs were immunoprecipitated by protein A (IP:Fc) and either immunoblotted with anti-IgG Fc or anti-phosphotyrosine antibodies. Similarly, hSpry2 was immunoprecipitated and immunoblotted with either anti-hSpry2 antibody or anti-phosphotyrosine antibodies (IP:hSpry2). (B) Selective phosphorylation of hSpry2 on Y55 by the FGFR1/VT+ splice isoform (FcFGFR1/FA/VT+). 293T cells were either stimulated with FGF2 (30 minutes) or transfected with either FcFGFR1/FA/VT+ or VT– isoforms in the presence of either hSpry2WT or hSpry2Y55A. hSpry2 was immunoprecipitated and immunoblotted with anti-hSpry2 antibody and anti-phosphotyrosine antibodies (IP:hSpry2). FGFR constructs were immunoprecipitated by protein A and immunoblotted with anti-IgG Fc (IP:Fc).

The identity of the hSpry2 tyrosine residue phosphorylated by FcFGFR1/FA in an FRS2-dependent manner was tested by examining the phosphorylation of a mutant form of hSpry2, in which Y55 was replaced by alanine (hSpry2Y55A). This mutant is not phosphorylated in response to either FcFGFR1/FA/VT+ or FcFGFR1/FA/VT– (Fig. 1B, lanes 4 and 6) despite equivalent expression levels (Fig. 1B, bottom and centre panel, respectively). This is not a peculiarity of the FcFGFR1/FA construct because activation of endogenous FGFRs by FGF2 results in phosphorylation of hSpry2WT but not hSpry2Y55A (Fig. 1B, lanes 1 and 2, respectively).

Taken together, these results reveal that hSpry2 is phosphorylated on Y55 by a tyrosine kinase which is recruited by a mechanism that depends upon activation of FGFR kinase activity and FRS2. This kinase is not FGFR itself.

SU6656 inhibits FGFR-mediated tyrosine phosphorylation of hSpry2

Evidence from *Xenopus* FRS2 interactions with SRC-like kinase Laloo (Kusakabe et al., 2001) and in vitro association evidence from SRC and bacterially expressed GST-FGFR1 fusion proteins (Zhan et al., 1994) implicates SFKs as candidate kinases in the FRS2-dependent phosphorylation of hSpry2 Y55. We coexpressed FGFR2 and hSpry2WT, and examined the tyrosine phosphorylation of hSpry2WT in response to FGF2-activated FGFRs in the absence or presence of SU6656 (a selective SFK inhibitor) (see Blake et al., 2000) (Fig. 2A, lanes 2 and 3-6, respectively). FGFR activation-dependent tyrosine phosphorylation of hSpry2WT exhibits a dose-dependent inhibition when cells are pre-treated with SU6656 over a concentration range of 0.5-20 μM (Fig. 2A, lanes 2-6). This correlates with SRC activity, as shown by anti-SRC pY416 antibody (Fig. 2A, second lower panel), with an apparent IC_{50} of 2 μM (data not shown). A dose-dependent inhibition of Spry2 phosphorylation by SU6656 was also observed by Mason et al. (Mason et al., 2004). These data indicate that SFKs could be responsible for the phosphorylation of hSpry2.

Complete inhibition of hSpry2WT phosphorylation is only observed at 20 μM SU6656. We therefore investigated the possibility that non-selective inhibition of other kinases, such as FGFRs, occurs. The ligand-dependent or independent tyrosine phosphorylation state of FGFR2 is unaffected in the presence of 20 μM SU6656 (Fig. 2B, lanes 3 and 4, respectively). Elevated levels of FGFR activation and tyrosine phosphorylation of downstream signalling molecules are observed in this system which may be owing to an autocrine signalling mechanism in 293T cells (M. K. Hajihosseini, L.M.W. and X.L., unpublished). Tyrosine-phosphorylation of proteins that co-immunoprecipitated with FGFR2 is decreased compared with FGFR2 not exposed to SU6656 (Fig. 2B, upper panel). This result suggests that SU6656-sensitive SFKs may play a broad role in mediation of FGFR signalling. One or more SFKs might be directly regulated by FGFR activation in an FRS2-dependent fashion resulting in subsequent tyrosine-phosphorylation of hSpry2WT.

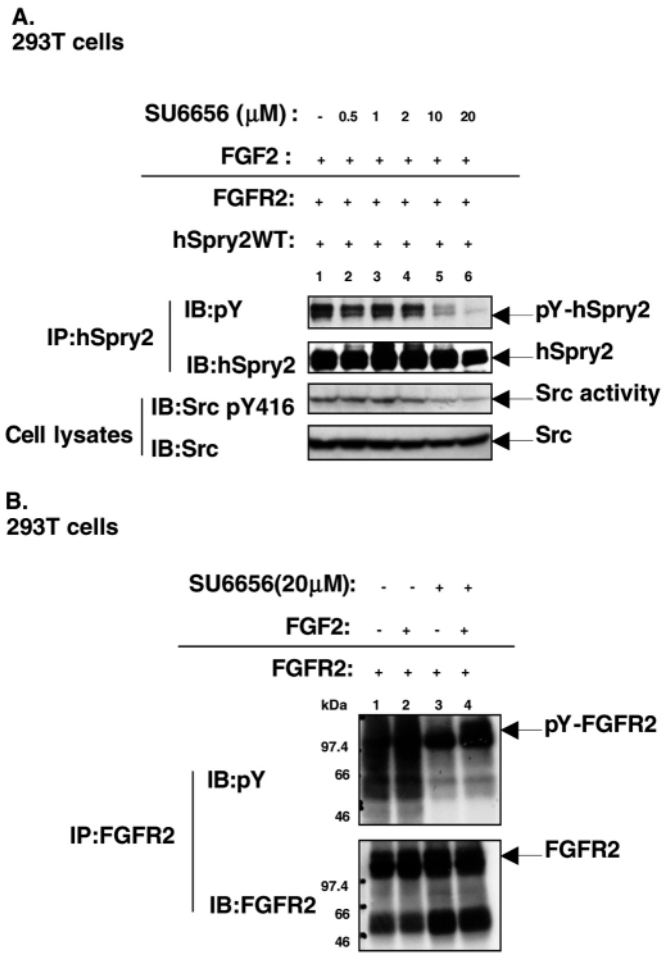

Fig. 2. SU6656 inhibits activated FGFR-mediated tyrosine phosphorylation of hSpry2. (A) Inhibition of activated FGFR-mediated tyrosine phosphorylation of hSpry2 by SU6656. hSpry2 was co-transfected with wild-type FGFR2 into 293T cells. Cells were pre-treated with various concentrations of SU6656 for 1 hour before FGF2 stimulation (30 minutes). hSpry2 was immunoprecipitated and then immunoblotted with anti-hSpry2 antibody or anti-phosphotyrosine antibodies (IP:hSpry2). Antibodies against SFKs (anti-SRC2) and active SFKs (anti-SRC pY416) were used to examine SFK expression and activity. (B) Effect of 20uM SU6656 on overexpressed FGFR2-induced tyrosine phosphorylation profile. 293T cells were transfected with FGFR2 and were either pre-treated with 20μM SU6656 or DMSO (control) for 1 hour prior to FGF2 stimulation for 30 minutes. FGFR2 was immunoprecipitated (IP:FGFR2) and immunoblotted with both and anti-phosphotyrosine antibody anti-FGFR2 antibodies (IB:pY and IB:FGFR2, respectively).

SFKs are required and sufficient for phosphorylation of hSpry2

We utilised the SYF cell line (*Src*/*Yes*/*Fyn* null) and derivatives thereof (Cary et al., 2002) to genetically define the requirement for SFKs in hSpry2 Y55 phosphorylation. SYF cells that stably express either wild-type SRC (SYF/wtSRC) or a constitutively active SRC (SYF/Y527F) were transfected with hSpry2WT and compared to similarly transfected cells stimulated with FGF2. Constitutively active SRC kinase results in elevated tyrosine-phosphorylation of hSpry2WT, independently of FGF2-mediated FGFR activation (Fig. 3A, lanes 3 and 4). Conversely, hSpry2WT phosphorylation in response to wild-type SRC activation is FGF2-dependent, revealing a physiological requirement for FGFR activation (Fig. 3A, lanes 1 and 2). SRC activity, revealed by anti-SRC pY416, correlates directly with the tyrosine-phosphorylation of hSpry2. This shows that hSpry2 is an FGF-dependent SRC tyrosine kinase substrate. We cannot exclude the possibility that other SFK(s) are involved in the FGF-dependent tyrosine phosphorylation of hSpry2.

We therefore predicted that, in the SFK-deficient SYF cell line, phosphorylation of hSpry2WT would be blocked in response to FGF2-mediated receptor activation. SRC/YES/FYN deficiency in SYF cells was confirmed by immunoblotting with an anti-SRC2 antibody which is immunoreactive against SFKs. Residual immunoreactivity may represent non-specific antibody binding or indicate the expression of one or more SFKs which are not SRC, YES or FYN. Using NIH3T3 cells as a positive control cell line, both cell lines were stimulated with FGF2 in the absence or presence of transfected hSpry2WT. Only NIH3T3 cells transfected with hSpry2WT exhibited FGFR ligand-dependent tyrosine phosphorylation of hSpry2 (Fig. 3B, lane 2). No phosphorylation is observed in the SYF null background (Fig. 3B, lane 4). Inhibition of ERK1/2 activation is only detected under conditions that result in hSpry2 tyrosine phosphorylation (Fig. 3B, lane 2), which is in sharp contrast to the complete absence of hSpry2 phosphorylation in SYF cells and an unaltered ERK activity (Fig. 3B, lane 4). These data confirm that one or more of SRC, FYN and YES tyrosine kinases phosphorylate hSpry2WT on Y55 in response to FGFR activation.

SYF cells were transfected with hSpry2 (Fig. 3C, lane 1) or co-transfected with either wild-type SRC or FYN (Fig. 3C, lanes 3 and 4, respectively) and stimulated with FGF2 to identify which SRC family kinases were involved in FGF2-induced hSpry2WT tyrosine phosphorylation. An SYF control cell line was used that expressed SRC, YES and FYN at endogenous levels (Fig. 3C, lane 2). The results demonstrate that expression of either SRC or FYN in SYF cells restores FGF-mediated tyrosine-phosphorylation of hSpry2 (Fig. 3C, lanes 3 and 4, respectively). Endogenous SFK expression or transfection of SRC or FYN was also sufficient to restore inhibition of ERK1/2 activity in SYF cells (Fig. 3C, lanes 2, 3 and 4, respectively). hSpry2WT tyrosine phosphorylation correlated with SFK activity as revealed by immunoblotting with anti-SRC pY416 antibody – which defines SFK activity – and anti-SRC2 – which defines SFK expression. We noticed that cells expressing SRC/YES/FYN at endogenous levels had a similar effect on the level of ERK1/2 inhibition, coincident with a decreased hSpry2 phosphorylation (Fig. 3C, lane 2). Minimal phosphorylation of hSpry2 in the presence of multiple SFKs may be sufficient to enable hSpry2-mediated inhibition of ERK. Over-expression of either SRC or FYN can reproduce the inhibitory effect of one or more SFKs in SYF cell line.

These results together demonstrate that FGF-induced SRC tyrosine kinase activity is essential and sufficient for an hSpry2-mediated inhibition of ERK1/2 activation. The FGFR ligand-independent phosphorylation of hSpry2WT by constitutively active SRC also shows that SRC activation can modulate multiple receptor pathways by the phosphorylation of hSpry2WT and subsequent inhibition of ERK1/2.

SRC-mediated phosphorylation of hSpry2 on Y55 is required for inhibition of ERK1/2

Having revealed that hSpry2 was a selective substrate for specific SFKs, we investigated the role of hSpry2 tyrosine phosphorylation in ERK inhibition. Several reports claim that phosphorylation of Y55 in Sprouty2 is required for ERK1/2 inhibition (Sasaki et al., 2001; Hanafusa et al., 2002). Either hSpry2WT or hSpry2Y55A were transfected into NIH3T3 cells and FGFR activation was initiated by stimulation with FGF2 (Fig. 4A, lanes 4-6). Endogenous FGFR2 was immunoprecipitated and immunoblotted with anti-FGFR2 or, as an index of activation, anti-phosphotyrosine antibodies (Fig. 4A). These results demonstrate that neither hSpry2WT nor hSpry2Y55A were tyrosine phosphorylated in the absence of FGF2 stimulation (Fig. 4A, lanes 2 and 3, respectively). By contrast, hSpry2WT was tyrosine phosphorylated following FGF2-dependent FGFR activation (Fig. 4A, lane 5). Phosphorylation is completely ablated in the hSpry2Y55A mutant (Fig. 4A, lane 6), showing that hSpry2 Y55 is phosphorylated by endogenous FGFR signalling pathways.

SYF cells stably expressing wild-type SRC or a constitutively active mutant (Fig. 4B, lanes 1-3 and 4-6, respectively) were transfected with hSpry2WT or hSpry2Y55A to confirm that Y55 was the target for SFK-mediated tyrosine-phosphorylation. After FGF2 stimulation, hSpry2WT exhibited tyrosine phosphorylation when either wtSRC or SRC Y527F is stably expressed in SYF cells (Fig. 4B, lanes 2 and 5, respectively). Importantly, neither wtSRC nor SRC Y527F is able to phosphorylate hSpry2Y55A (Fig. 4B, lanes 3 and 6, respectively). Expression of hSpry2WT coincided with SRC-mediated inhibition of ERK activity that was completely abolished by hSpry2Y55A (Fig. 4B, compare lanes 2 and 5 with 3 and 6). These data show that Y55 is the principal (and possibly only) FGF2-induced SFK target site for phosphorylation on hSpry2 and is essential for the SRC-mediated inhibition of ERK1/2 activity.

hSpry2 is a direct substrate for SRC kinase

The foregoing data do not eliminate a role for an intermediate kinase in the FGFR1/FRS2-SRC/hSpry2 cascade. To establish that hSpry2 is a direct substrate for SRC kinase we performed an in vitro phosphorylation assay using purified SRC and the

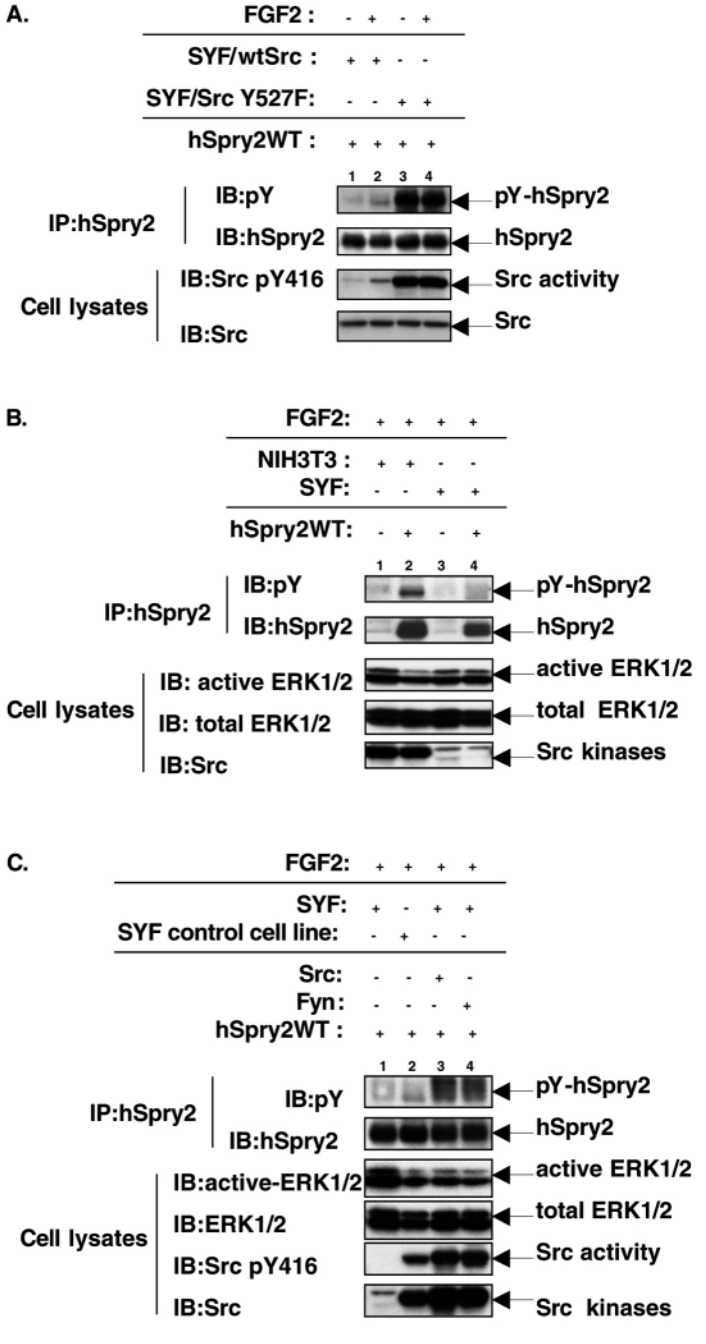

Fig. 3. hSpry2 is a substrate for activated SRC kinase induced by FGF stimulation. (A) Tyrosine-phosphorylation of hSpry2 in SYF/wtSRC cells was dependent on FGF2 stimulation. Wild-type hSpry2 was transfected into SYF cells stably expressing wild-type SRC (SYF/wtSrc) or SYF cells stably expressing kinase active SRC (SYF/Src Y527F) and cells were incubated in the presence or absence of FGF2 (30 minutes). hSpry2 was immunoprecipitated and immunoblotted with either anti-phosphotyrosine antibodies (IP:hSpry2) or anti-hSpry2 antibody (IB:pY and IB:hSpry2, respectively). Antibodies against SFKs (anti-SRC2) and active SFKs (anti-SRC pY416) were used to confirm SFK expression and activity. (B) SRC/FYN/YES deficiency resulted in loss of tyrosine-phosphorylation of hSpry2. SYF cells (deficient in SRC/FYN/YES tyrosine kinases) and NIH3T3 fibroblasts were stimulated with FGF2 (30 minutes) in either the presence or absence of transfected hSpry2WT. hSpry2 was immunoprecipitated and immunoblotted with either anti-phosphotyrosine antibodies or anti-hSpry2 antibody. Anti-SRC (SRC2) antibody was used to check SFK expression in the cells. Cell lysates were immunoblotted with anti-active-ERK1/2 to assess the activation of endogenous ERK1/2 and reprobed with anti-ERK1/2 to confirm comparable protein loading. (C) SRC and FYN restored tyrosine phosphorylation of hSpry2 in SYF cells. Wild-type hSpry2 was co-expressed with either SRC or FYN into SYF cells. SYF or SYF control cells transfected with hSpry2WT were stimulated with FGF2 (30 minutes). hSpry2 was immunoprecipitated and immunoblotted with either anti-hSpry2 antibody or anti-phosphotyrosine antibodies. Antibodies against SFKs (anti-SRC2) and active SFKs (anti-SRC pY416) were used to confirm SFK expression and activity. Cell lysates were immunoblotted with anti-active-ERK1/2 to assess the activation of endogenous ERK1/2 and reprobed with anti-ERK1/2 to confirm comparable protein loading.

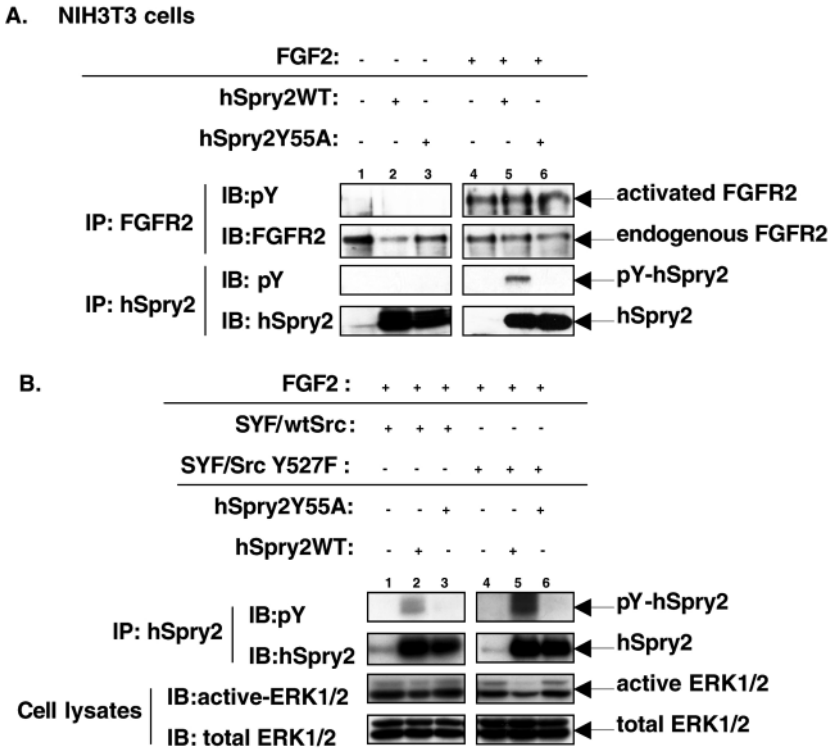

Fig. 4. SRC-dependent phosphorylation of hSpry2 on Y55 is required for hSpry2-mediated inhibition of MAP Kinase. (A) Phosphorylation of hSpry2 on Y55 was an FGFR activation-dependent event. NIH3T3 cells were transfected with either hSpry2WT or hSpry2Y55A and then incubated in the absence or presence of FGF2 (10 minutes). hSpry2 was immunoprecipitated and immunoblotted with either anti-hSpry2 antibody or anti-phosphotyrosine antibodies. Endogenously expressed FGFR2 was immunoprecipitated and immunoblotted with either anti-phosphotyrosine antibodies or anti-FGFR2 antibody (Bek c-17). (B) SRC-dependent phosphorylation of hSpry2 is absent in hSpry2Y55A. hSpry2WT or hSpry2Y55A were transfected into SYF cells stably expressing either wild-type SRC (SYF/wtSrc) or a constitutively active SRC (SYF/Src Y527F) before stimulation with FGF2 (30 minutes). hSpry2 was immunoprecipitated and immunoblotted with either anti-hSpry2 antibody or anti-phosphotyrosine antibodies. Cell lysates were immunoblotted with anti-active-ERK1/2 to assess the activation of endogenous ERK1/2 and reprobed with anti-ERK1/2 to confirm comparable protein loading.

N-terminal region of hSpry2 (amino acids 1-172, containing Y55) as a substrate. The N-terminal hSpry2 region was purified as described previously (see Materials and methods). The results reveal that SRC directly phosphorylates hSpry2 Y55 (Fig. 5) in a reconstituted in vitro system using purified components.

Tyrosine-phosphorylated hSpry2 is physically associated with SRC

We have demonstrated that hSpry2 is tyrosine-phosphorylated by SFKs in both SYF cells and in vitro. We therefore examined whether there is a physical interaction between hSpry2WT and SFKs and the role of hSpry2 Y55 phosphorylation in the formation of such a complex. In SYF cells or SYF cells transfected with either wtSRC, SRC Y527F or a kinase dead SRC (SRC KD), hSpry2WT coimmunoprecipitated with the activated SRC Y527F in the repeated experiments (Fig. 6A, lane 3), and no association of hSpry2 and either wtSRC or SRC KD was observed (Fig. 6A, lanes 2 and 4, respectively). This reveals that tyrosine-phosphorylation of hSpry2 in the presence of constitutively activated SRC results in the formation of a stable complex between SRC and hSpry2.

We used two peptides (RAIRNTNEYTEGPTVVPR) derived from hSpry2WT that contained Y55 in its phosphorylated and non-phosphorylated form to examine the requirement for Y55 phosphorylation in the hSpry2/SRC association. Peptides were biotin-acetylated, immobilised on NeutrAvidin media and incubated with 293T whole-cell lysates. Peptide-bound proteins were isolated and an anti-phosphotyrosine immunoreactive band of 60 kDa was confirmed as a SFK by anti-SRC2 antibody. The association of SRC2-reactive SFKs is significantly enriched in the phosphorylated peptide fraction compared to the non-phosphorylated peptide (Fig. 6B).

These data reveal that the physical association between hSpry2 and SFKs is enhanced by phosphorylation of hSpry2 Y55. Thus, the phosphorylation of hSpry2 Y55 in response to FGF stimulation results in a stable physical complex, which includes SRC and hSpry2, and which is required for FGFR/FRS2-SRC/hSpry2-mediated suppression of ERK signalling.

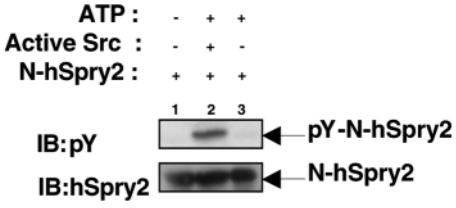

Fig. 5. hSpry2 is a direct substrate for SRC kinase. Five micrograms of the N-terminal region of hSpry2WT (amino acids 1-172) was used as a substrate for an in-vitro kinase assay. Reaction conditions included either the absence or presence of 100 μM ATP and/or 200ng of active SRC. Tyrosine-phosphorylation was confirmed using anti-phosphotyrosine antibodies. Equal substrate concentration was confirmed by anti-hSpry2 antibody.

Discussion

The importance of FGFR signalling dynamics in the regulation of downstream responses has been highlighted in both experimental (Hachonen et al., 1998; Minowada et al., 1999; Hajihosseini et al., 2004) and theoretical models (Yamada et al., 2004). The magnitude and duration of FGFR signalling is

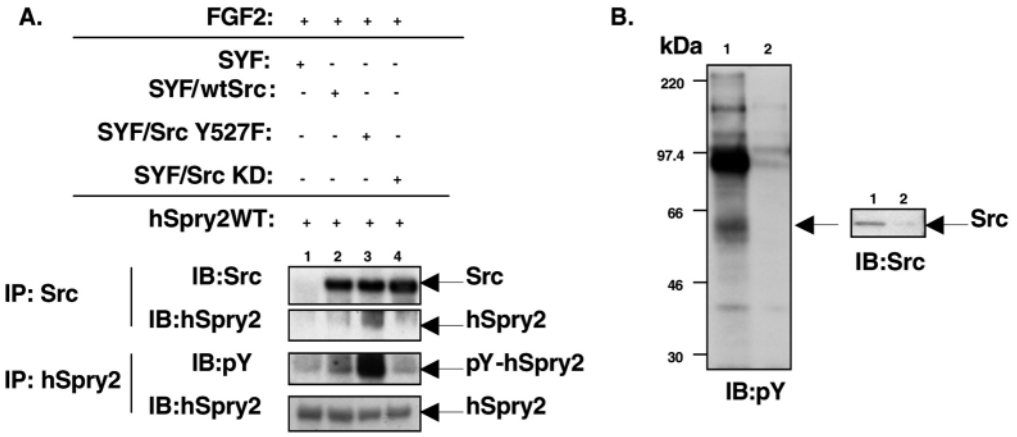

Fig. 6. Tyrosine-phosphorylated hSpry2 is associated with SRC. (A) hSpry2 was associated with SRC. hSpry2WT was transfected into SYF cells alone or SYF cells stably expressing wtSRC (SYF/wtSrc), kinase active SRC (SYF/Src Y527F) or a kinase dead SRC (SYF/Src KD). Cells were subsequently stimulated with FGF2 (30 minutes). SRC was immunoprecipitated with a monoclonal anti-SRCMab327 antibody and immunoblotted with anti-SRC (SRC2) antibody and hSpry2 association was examined using anti-hSpry2 antibody. Relative expression and phosphorylation of hSpry2 was confirmed with either anti-hSpry2 antibody or anti-phosphotyrosine antibodies. (B) Preferential pY55-mediated association of SRC with hSpry2. Peptides derived from hSpry2 that included Y55 in both its phosphorylated (lanes 1) and non-phosphorylated (lanes 2) form were used to isolate potential binding partners. Peptide-associated proteins were resolved on SDS-PAGE gel, and the presence of SFKs revealed utilizing anti-phosphotyrosine antibodies and anti-SFK (SRC2) antibody.

dictated by feed-forward processes – such as activation of the Ras-MAP kinase pathway – and counteracted by inhibitory processes that include the action of signal attenuators exemplified by the Sprouty family of proteins (Hanafusa et al., 2002; Yusoff et al., 2002). A key issue in defining FGFR signalling dynamics is to determine the molecular basis by which feed-forward processes are connected to the activity of signal attenuators. Here, we demonstrate that activation of FGFR signalling leads to activation of the attenuation functions of hSpry2 by phosphorylation of hSpry2 Y55. However, this is not a direct connection: we show that hSpry2 Y55 is directly phosphorylated by SRC following activation and recruitment to the FGFR signalling complex in a process that is FRS2-dependent. We also show that as a result SRC preferentially associates with the Y55 phosphorylated form of hSpry2. These results show that a subset of FGFR signalling functions are executed by the recruitment of non-receptor tyrosine kinases of the SRC family, that the signal activation is physically connected to signal attenuation by the recruitment of hSpry2 into the FGFR/FRS2-SRC signalling complex and that hSpry2 Y55 plays a pivotal role in signal attenuation.

The activity of SRC has been associated with several RTK-induced signal transduction systems (Thomas and Brugge, 1997; Abram and Courtneidge, 2000; Courtneidge, 2002). SRC has been implicated in FGFR-induced neurite outgrowth (Kremer et al., 1991), is required for cell migration and shape changes induced by FGFR (Liu et al., 1999) and is a necessary component for FGFR1 signal transduction in *Xenopus* (Browaeys-Poly et al., 2000). The SFKs, XFyn and Laloo, are required for FGF-induced mesoderm induction during *Xenopus* early development (Weinstein et al., 1998; Hama et al., 2001; Kusakabe et al., 2001; Weinstein and Hemmati-Srivanlou, 2001). Here we extend these observations by showing that, in mammalian cells, SRC kinase activity is induced by FGFR kinase activity in a process which is dependent upon FGFR-associated FRS2. FRS2 is a direct substrate for the FGFR kinase (Xu et al., 1998; Ong et al., 2000) undergoing phosphorylation at multiple tyrosine residues in the effector domain. Based upon the defined mechanism of SRC kinase activation following dephosphorylation of Y527 and subsequent release of the SRC-SH2 domain (Bjorge et al., 2000; Harrison, 2003), we propose that the mechanism of SRC activation by FGFRs involves interaction of one, or more, FRS2 phosphorylated tyrosine residues with the SRC SH2 domain. We note that Y^{306}ENI in FRS2 conforms to the consensus SRC SH2 binding site (Songyang et al., 1993) although the exact residue(s) involved in this process remain to be experimentally defined.

This study also shows that activation of SFKs is an intrinsic feature of FGFR signalling mediated via the phosphorylation of FRS2, which suggests that the activity of non-receptor tyrosine kinases may be more significant in the propagation of FGFR signals than has so far been appreciated. It has been well established, for example, that SRC plays a profound role in the regulation of actin cytoskeleton assembly/disassembly and cell adhesion (Frame, 2004). FGFR signalling has also been shown in a variety of models to elicit profound effects on cytoskeletal architecture and cell shape (Wilson and Leptin, 2000; Davidson et al., 2001; Rozenblatt-Rosen et al., 2002) and it will be important to determine the extent to which these issues are interconnected.

In this study we identify hSpry2 residue Y55 as a key target for the FGFR/FRS2-SRC kinase axis. This confirms previous reports (Sasaki et al., 2001; Hanafusa et al., 2002), which have implicated this residue in Sprouty-mediated attenuation of Ras-MAP kinase signalling. The phosphorylation of this conserved amino acid is also known to regulate the association of Sprouty with other key signalling intermediates: GRB2 (Hanafusa et al., 2002), FRS2 (Tefft et al., 2002) and CBL (Fong et al., 2003; Hall et al., 2003; Rubin et al., 2003). Other proteins that associate with Sprouty in a Y55-independent manner include Raf (Sasaki et al., 2003), caveolin-1 (Impagnatiello et al.,

2001) and PTP1B (Yigzaw et al., 2003). Many of these effectors have been implicated in SRC signalling: either as regulators of SRC activity [PTP1B, CBL (Bjorge et al., 2000; Sanjay et al., 2001)], as SRC substrates [Raf (Tran and Frost, 2003)] or SRC-associated proteins [caveolin (Li et al., 1996)]. This may be no coincidence because we show in this study that there is a pre-existing physical association between SRC and hSpry2 that is enhanced by phosphorylation of hSpry2 Y55. Some studies have shown that the C-terminal cysteine-rich membrane targeting region of Sprouty is required to confer its inhibitory activity on the ERK1/2 pathway (Yigzaw et al., 2001; Egan et al., 2002). A proline-rich sequence (P^{192}RPLP) in this region conforms to the SRC-homology 3 domain (SH3) target recognition sequence (Rickles et al., 1994). It is therefore possible that SRC also exhibits an association with Sprouty, via the C-terminal membrane-targeting domain that, upon association with FRS2, results in activation of SRC and concomitant phosphorylation of hSpry2 Y55. Where we demonstrate SRC-hSpry2 association (Fig. 6), the absence of C-terminal domain of hSpry2 does not eliminate any further possibility of its interaction with SRC. Since *sprouty* genes exhibit highly specific patterns of expression in development (Chambers and Mason, 2000), it will be important to test these hypotheses using genetic approaches in vivo. As we observed (Fig. 3C, Fig. 4B), there was no linear relationship between tyrosine-phosphorylation of hSpry2 and inhibition of ERK1/2, which suggests the inhibition of ERK1/2 by Sprouty may involve more domain(s) and/or molecules cooperated in this process aside from Y55.

Together, these data show that recruitment of SRC to the FGFR signalling machine via FRS2 acts as a signalling bifurcation that converts feed-forward processes, which are FRS2-dependent, into hSpry2 phosphorylation-dependent attenuation processes. SRC – often considered a feed-forward agent in signal transduction – is in this case an agent of signal attenuation.

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References

Abram, C. L. and Courtneidge, S. A. (2000). Src family tyrosine kinases and growth factor signalling. *Exp. Cell Res.* **254**, 1-13.

Anderson, J., Burns, H. D., Enriquez-Harris, P., Wilkie, A. O. and Heath, J. K. (1998). Apert syndrome mutations in fibroblast growth factor receptor 2 exhibit increased affinity for FGF ligand. *Hum. Mol. Genet.* **7**, 1475-1483.

Barton, V. A., Hudson, K. R. and Heath, J. K. (1999). Identification of three distinct receptor binding sites of murine interleukin-11. *J. Biol. Chem.* **274**, 5755-5761.

Blake, R. A., Broome, M. A., Liu, X., Wu, J., Gishizky, M., Sun, L. and Courtneidge, S. A. (2000). SU6656, a selective Src family kinase inhibitor, used to probe growth factor signaling. *Mol. Cell. Biol.* **20**, 9018-9027.

Bjorge, J. D., Jakymiw, A. and Fujita, D. J. (2000). Selected glimpses into the activation and function of Src kinase. *Oncogene* **19**, 5620-5635.

Browaeys-Poly, E., Cailliau, K. and Vilain, J. P. (2000). Signal transduction pathways triggered by fibroblast growth factor receptor 1 expressed in *Xenopus laevis* oocytes after fibroblast growth factor 1 addition. Role of Grb2, phosphatidylinositol 3-kinase, Src tyrosine kinase, and phospholipase Cgamma. *Eur. J. Biochem.* **267**, 6256-6263.

Burgar, H. R., Burns, H. D., Elsden, J. L., Lalioti, M. D. and Heath, J. K. (2002). Association of the signalling adaptor FRS2 with fibroblast growth factor receptor 1 (Fgfr1) is mediated by alternative splicing of the juxtamembrane domain. *J. Biol. Chem.* **277**, 4018-4023.

Cary, L. A., Klinghoffer, R. A., Sachsenmaier, C. and Cooper, J. A. (2002). Src catalytic but not scaffolding function is needed for integrin-regulated tyrosine phosphorylation, cell migration, and cell spreading. *Mol. Cell. Biol.* **22**, 2427-2440.

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

Chambers, D. and Mason, I. (2000). Expression of sprouty2 during early development of the chick embryo is coincident with known sites of FGF signalling. *Mech. Dev.* **91**, 361-364.

Christofori, G. (2003). Split personalities: the agonistic antagonist Sprouty. *Nat. Cell Biol.* **5**, 377-379.

Courtneidge, S. A. (2002). Role of Src in signal transduction pathways. The Jubilee Lecture. *Biochem. Soc. Trans.* **30**, 11-17.

Davidson, G., Dono, R. and Zeller, R. (2001). FGF signalling is required for differentiation-induced cytoskeletal reorganisation and formation of actin-based processes by podocytes. *J. Cell Sci.* **114**, 3359-3366.

de Maximy, A. A., Nakatake, Y., Moncada, S., Itoh, N., Thiery, J. P. and Bellusci, S. (1999). Cloning and expression pattern of a mouse homologue of drosophila Sprouty in the mouse embryo. *Mech. Dev.* **81**, 213-216.

Dhalluin, C., Yan, K. S., Plotnikova, O., Lee, K. W., Zeng, L., Kuti, M., Mujtaba, S., Goldfarb, M. P. and Zhou, M. M. (2000). Structural basis of SNT PTB domain interactions with distinct neurotrophic receptors. *Mol. Cell.* **6**, 921-929.

Egan, J. E., Hall, A. B., Yatsula, B. A. and Bar-Sagi, D. (2002). The bimodal regulation of epidermal growth factor signaling by human Sprouty proteins. *Proc. Natl. Acad. Sci. USA* **99**, 6041-6046.

Fong, C. W., Leong, H. F., Wong, E. S., Lim, J., Yusoff, P. and Guy, G. R. (2003). Tyrosine phosphorylation of Sprouty2 enhances its interaction with c-Cbl and is crucial for its function. *J. Biol. Chem.* **278**, 33456-33464.

Frame, M. C. (2004). Newest findings on the oldest oncogene; how activated Src does it. *J. Cell. Sci.* **117**, 989-998.

Gould, K. L. and Hunter, T. (1988). Platelet-derived growth factor induces multisite phosphorylation of pp60c-Src and increases its protein-tyrosine kinase activity. *Mol. Cell. Biol.* **8**, 3345-3356.

Guy, G. R., Wong, E. S., Yusoff, P., Chandramouli, S., Lo, T. L., Lim, J. and Fong, C. W. (2003). Sprouty: how does the branch manager work? *J. Cell Sci.* **116**, 3061-3068.

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.

Hadari, Y. R., Kouhara, H., Lax, I. and Schlessinger, J. (1998). Binding of Shp2 tyrosine phosphatase to FRS2 is essential for fibroblast growth factor-induced PC12 cell differentiation. *Mol. Cell. Biol.* **18**, 3966-3973.

Hajihosseini, M. K., Lalioti, M. D., Arthaud, S., Burgar, H. R., Brown, J. M., Twigg, S. R., Wilkie, A. O. and Heath, J. K. (2004). Skeletal development is regulated by fibroblast growth factor receptor 1 signalling dynamics. *Development* **13**, 325-335.

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.

Hama, J., Xu, H., Goldfarb, M. and Weinstein, D. C. (2001). SNT-1/FRS2alpha physically interacts with Laloo and mediates mesoderm induction by fibroblast growth factor. *Mech. Dev.* **109**, 195-204.

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.

Harrison, S. C. (2003). Variation on a Src-like theme. *Cell* **112**, 737-740.

Hudson, K. R., Vernallis, A. B. and Heath, J. K. (1996). Characterization of the receptor binding sites of human leukemia inhibitory factor and creation of antagonists. *J. Biol. Chem.* **271**, 11971-11978.

Impagnatiello, M. A., Weitzer, S., Gannon, G., Compagni, A., Cotton, 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.

Klint, P. and Claesson-Welsh, L. (1999). Signal transduction by fibroblast growth factor receptors. *Front Biosci.* **4**, D165-D177.

Kremer, N. E., D'Arcangelo, G., Thomas, S. M., DeMarco, M., Brugge, J. S. and Halegoua, S. (1991). Signal transduction by nerve growth factor and

fibroblast growth factor in PC12 cells requires a sequence of Src and Ras actions. *J. Cell Biol.* **115**, 809-819.

Kusakabe, M., Masuyama, N., Hanafusa, H. and Nishida, E. (2001). Xenopus FRS2 is involved in early embryogenesis in cooperation with the Src family kinase Laloo. *EMBO Rep.* **2**, 727-735.

Kypta, R. M., Goldberg, Y., Ulug, E. T. and Courtneidge, S. A. (1990). Association between the PDGF receptor and members of the Src family of tyrosine kinases. *Cell* **62**, 481-492.

Landgren, E., Blume-Jensen, P., Courtneidge, S. A. and Claesson-Welsh, L. (1995). Fibroblast growth factor receptor-1 regulation of Src family kinases. *Oncogene* **10**, 2027-2035.

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

Li, S., Couet, J. and Lisanti, M. P. (1996). Src tyrosine kinases, Galpha subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. *J. Biol. Chem.* **271**, 29182-29190.

Li, X., Wheldon, L. and Heath, J. K. (2003). Sprouty: a controversial role in receptor tyrosine kinase signalling pathways. *Biochem. Soc. Trans.* **31**, 1445-1446.

Lim, J., Wong, E. S., Ong, S. H., Yusoff, P., Low, B. C. and Guy, G. R. (2000). Sprouty proteins are targeted to membrane ruffles upon growth factor receptor tyrosine kinase activation. Identification of a novel translocation domain. *J. Biol. Chem.* **275**, 32837-32845.

Lin, H., Xu, J., Ischenko, I., Ornitz, D. M., Halegoua, S. and Hayman, M. J. (1998). Identification of the cytoplasmic regions of Fibroblast growth factor (FGF) receptor 1 which play important roles in induction of neurite outgrowth in PC12 cells by FGF-1. *Mol. Cell. Biol.* **18**, 3762-3770.

Liu, J., Huang, C. and Zhan, X. (1999). Src is required for cell migration and shape changes induced by Fibroblast growth factor 1. *Oncogene* **18**, 6700-6706.

Mailleux, A. A., Tefft, D., Ndiaye, D., Itoh, N., Thiery, J. P., Warburton, D. and Bellusci, S. (2001). Evidence that Sprouty2 functions as an inhibitor of mouse embryonic lung growth and morphogenesis. *Mech. Dev.* **102**, 81-94.

Mason, I. J. (1994). The ins and outs of Fibroblast growth factors. *Cell* **78**, 547-552.

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 over-expressed. *Development* **126**, 4465-4475.

Mohammadi, M., Honegger, A. M., Rotin, D., Fischer, R., Bellot, F., Li, W., Dionne, C. A., Jaye, M., Rubinstein, M. and Schlessinger, J. (1991). A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. *Mol. Cell. Biol.* **11**, 5068-5078.

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.

Ong, S. H., Guy, G. R., Hadari, Y. R., Laks, S., Gotoh, N., Schlessinger, J. and Lax, I. (2000). FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. *Mol. Cell. Biol.* **20**, 979-989.

Ong, S. H., Hadari, Y. R., Gotoh, N., Guy, G. R., Schlessinger, J. and Lax, I. (2001). Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. *Proc. Natl. Acad. Sci. USA* **98**, 6074-6079.

Ralston, R. and Bishop, J. M. (1985). The product of the proto-oncogene c-Src is modified during the cellular response to platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA* **82**, 7845-7849.

Rickles, R. J., Botfield, M. C., Weng, Z., Taylor, J. A., Green, O. M., Brugge, J. S. and Zoller, M. J. (1994). Identification of Src, Fyn, Lyn, PI3K and Abl SH3 domain ligands using phage display libraries. *EMBO J.* **13**, 5598-5604.

Rozenblatt-Rosen, O., Mosonego-Ornan, E., Sadot, E., Madar-Shapiro, L., Sheinin, Y., Ginsberg, D. and Yayon, A. (2002). Induction of chondrocyte growth arrest by FGF: transcriptional and cytoskeletal alterations. *J. Cell Sci.* **115**, 553-562.

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.

Sanjay, A., Houghton, A., Neff, L., DiDomenico, E., Bardelay, C., Antoine, E., Levy, J., Gailit, J., Bowtell, D., Horne, W. C. et al. (2001). Cbl associates with Pyk2 and Src to regulate Src kinase activity, alpha(v)beta(3) integrin-mediated signaling, cell adhesion, and osteoclastmotility. *J. Cell Biol.* **152**, 181-195.

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.

Sasaki, A., Taketomi, T., Kato, R., Saeki, K., Nonami, A., Sasaki, M., Kuriyama, M., Saito, N., Shibuya, M. and Yoshimura, A. (2003). Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. *Nat. Cell Biol.* **5**, 427-432.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases. *Cell* **103**, 211-225.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J. et al. (1993). SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**, 767-778.

Stoletov, K. V., Ratcliffe, K. E. and Terman, B. I. (2002). Fibroblast growth factor receptor substrate 2 participates in vascular endothelial growth factor-induced signaling. *FASEB J.* **16**, 1283-1285.

Tefft, J. D., Lee, M., Smith, S., Leinwand, M., Zhao, J., Bringas, P., Jr, Crowe, D. L. and Warburton, D. (1999). Conserved function of mSpry-2, a murine homolog of Drosophila Sprouty, which negatively modulates respiratory organogenesis. *Curr. Biol.* **9**, 219-222.

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-L706.

Thomas, S. M. and Brugge, J. S. (1997). Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.* **13**, 513-609.

Tran, N. H. and Frost, J. A. (2003). Phosphorylation of Raf-1 by p21-activated kinase 1 and Src regulates Raf-1 autoinhibition. *J. Biol. Chem.* **278**, 11221-11226.

Twigg, S. R., Burns, H. D., Oldridge, M., Heath, J. K. and Wilkie, A. O. (1998). Conserved use of a non-canonical 5′ splice site (/GA) in alternative splicing by fibroblast growth factor receptors 1, 2 and 3. *Hum. Mol. Genet.* **7**, 685-691.

Ulrich, S. M., Kenski, D. M. and Shokat, K. M. (2003). Engineering a "methionine clamp" into Src family kinases enhances specificity toward unnatural ATP analogues. *Biochemistry* **42**, 7915-7921.

Underhill-Day, N., McGovern, L. A., Karpovich, N., Mardon, H. J., Barton, V. A. and Heath, J. K. (2003). Functional characterization of W147A: a high-affinity interleukin-11antagonist. *Endocrinology* **144**, 3406-3414.

Weinstein, D. C. and Hemmati-Brivanlou, A. (2001). Src family kinase function during early Xenopus development. *Dev. Dyn.* **220**, 163-168.

Weinstein, D. C., Marden, J., Carnevali, F. and Hemmati-Brivanlou, A. (1998). FGF-mediated mesoderm induction involves the Src-family kinase Laloo. *Nature* **394**, 904-908.

Weiss, A. and Schlessinger, J. (1998). Switching signals on or off by receptor dimerization. *Cell* **94**, 277-280.

Wilkie, A. O. M., Morriss-Kay, G. M., Jones, E. Y. and Heath, J. K. (1995). Functions of Fibroblast growth factors and their receptors. *Curr. Biol.* **5**, 500-507.

Wilson, R. and Leptin, M. (2000). Fibroblast growth factor receptor-dependent morphogenesis of the Drosophila mesoderm. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **355**, 891-895.

Wong, A., Lamothe, B., Lee, A., Schlessinger, J., Lax, I. and Li, A. (2002a). FRS2 alpha attenuates FGF receptor signaling by Grb2-mediated recruitment of the ubiquitin ligase Cbl. *Proc. Natl. Acad. Sci. USA* **99**, 6684-6689.

Wong, E. S., Fong, C. W., Lim, J., Yusoff, P., Low, B. C., Langdon, W. Y. and Guy, G. R. (2002b). Sprouty2 attenuates epidermal growth factor receptor ubiquitylation and endocytosis, and consequently enhances Ras/ERK signalling. *EMBO J.* **21**, 4796-4808.

Xu, H. and Goldfarb, M. (2001). Multiple effector domains within SNT1 coordinate ERK activation and neuronal differentiation of PC12 cells. *J. Biol. Chem.* **276**, 13049-13056.

Xu, H., Lee, K. W. and Goldfarb, M. (1998). Novel recognition motif on

Fibroblast growth factor receptor mediates direct association and activation of SNT adapter proteins. *J. Biol. Chem.* **273**, 17987-17990.

Yamada, S., Taketomi, T. and Yoshimura, A. (2004). Model analysis of difference between EGF pathway and FGF pathway. *Biochem. Biophys. Res. Commun.* **314**, 1113-1120.

Yigzaw, Y., Cartin, L., Pierre, S., Scholich, K. and Patel, T. B. (2001). The C terminus of sprouty is important for modulation of cellular migration and proliferation. *J. Biol. Chem.* **276**, 22742-22747.

Yigzaw, Y., Poppleton, H. M., Sreejayan, N., Hassid, A. and Patel, T. B. (2003). Protein-tyrosine phosphatase-1B (PTP1B) mediates the anti-migratory actions of Sprouty. *J, Biol. Chem.* **278**, 284-288.

Yusoff, P., Lao, D. H., Ong, S. H., Wong, E. S., Lim, J., Lo, T. L., Leong, H. F., Fong, C. W. and Guy, G. R. (2002). Sprouty2 inhibits the Ras/MAP kinase pathway by inhibiting the activation of Raf. *J. Biol. Chem.* **277**, 3195-3201.

Zhan, X., Plourde, C., Hu, X., Friesel, R. and Maciag, T. (1994). Association of fibroblast growth factor receptor-1 with c-Src correlates with association between c-Src and cortactin. *J. Biol. Chem.* **269**, 20221-20224.