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Enhancer Detection and Developmental Expression of Zebrafish *sprouty1*, a Member of the *fgf8* Synexpression Group

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Signaling pathways mediated by receptor tyrosine kinases (RTKs) are under positive and negative regulation, and misregulation of RTK signaling results in developmental defects and malignancy. A major class of antagonists of Fgf and Egf signaling are the Sprouty proteins. Through an enhancer detection approach, we isolated the *sprouty1* (*spry1*) gene, expressed in multiple developing organs during embryogenesis. We analyzed expression of *spry1* between tail bud stage and 10 days postfertilization. From the tail bud stage on, transcript and reporter are detected in the craniofacial region and in the mid–hindbrain boundary, where expression persists until adulthood. Further expression domains are the telencephalon, hindbrain, dorsal diencephalon and epiphysis, branchial arches, pituitary, and the tubular gill epithelium. In the trunk *spry1* is also prominently expressed in pronephros, the lateral line and tail fin. Sprouty1 acts in Fgf signaling downstream of Fgfr1, as its expression is abrogated through the small molecule inhibitor of this receptor, SU5402. *Developmental Dynamics 237:2594–2603, 2008.*

Key words: rhombomere centers; radical fringe; optic nerve astrocytes; cranial ganglia; pax2

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INTRODUCTION

Morphogenesis through cell proliferation, migration, and survival is precisely orchestrated by a combination of signaling pathways. These are regulated at multiple levels of signal transduction, from transcriptional to posttranslational levels (Fernig and Gallagher, 1994), by both activators and inhibitors. A prominent class of inhibitors function through negative feedback loops, whereby the inhibitor is transcriptionally induced by the signaling pathway that it eventually inhibits. Members of this class include the Sprouty proteins, which function downstream of RTK signaling (Mason et al., 2006).

Drosophila Sprouty was identified in genetic screens for regulators of tracheal branching (Hacohen et al., 1998) and eye development (Casci et al., 1999). Sprouty is able to antagonize a wide range of RTKs (Gross et al., 2001), activated by FGF and EGF ligands (Kramer et al., 1999; Reich et al., 1999), but also inhibits the Torso and Sevenless RTKs (Casci et al., 1999). In the *Drosophila* airways, the expression of *spry* is induced through the FGF receptor Heartless upon binding its Fgf ligand Branchless. The *spry*-expressing cells assume a terminal cell fate in the *Drosophila* airways, whereas adjacent cells are inhibited from doing so (Hacohen et al., 1998). Similarly, in vertebrates expression of *spry* genes is activated in regions with Fgf signaling. Four vertebrate *sprouty* homologues have been identified in chicken (Minowada et al., 1999), human (Huebert et al., 2004;

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Wang et al., 2006), and mouse (de Maximy et al., 1999; Tefft et al., 1999; Mailleux et al., 2001).

Sprouty proteins have regulatory functions during development and maintenance of multiple structures during embryogenesis and in adulthood. For example, *SPRY1* misregulation is involved in human prostate cancer (Kwabi-Addo et al., 2004), and human congenital kidney malformations (Basson et al., 2005), *Spry2* overexpression causes craniofacial defects in mouse (Goodnough et al., 2007), and decreased activity of *SPRY2* is associated with lung cancer (Sutterluty et al., 2007), while *SPRY4* is downregulated in human prostate cancer (Wang et al., 2006).

Sprouty activity is tightly regulated at several levels, through posttranslational modification, regulatory proteins and localization in cellular compartments. Not only *spry* gene expression, but also the subcellular localization of Sprouty proteins, is directly modulated by growth factors. In the absence of stimulation by growth factors, endogenous Sprouty proteins are widely distributed throughout the cell, localized in the perinuclear regions, in vesicles and in the plasma membrane (Impagnatiello et al., 2001; Yigzaw et al., 2001). Upon RTK stimulation by growth factors, Sprouty proteins are translocated from the cytosol to the plasma membrane (Lim et al., 2000, 2002) by association with caveolin-1 and palmitoylation of C-terminal cysteine residues located in the highly conserved translocation domain of Sprouty (SpryTD; Lim et al., 2000, 2001; Impagnatiello et al., 2001). This recruitment of Sprouty protein to the membrane is important because several Sprouty binding partners are located at the inner surface of the membrane, such as Grb2 (Gross et al., 2001) and Raf (Sasaki et al., 2003). The SpryTD domain also contains a highly conserved motif responsible for binding to Raf1 (RBD, Raf1-binding domain; Sasaki et al., 2003). In addition, although more variable between species, the N-terminal domain of Sprouty proteins also harbors functional motifs. In particular, this area contains a highly conserved tyrosine residue, which becomes phosphorylated after growth factor stimulation. This phosphorylation is required for Sprouty to inhibit RTK signaling. The sequence surrounding the tyrosine residue in the N-terminal part of the protein resembles a c-*Src* autophosphorylation site (Smart et al., 1981).

Sprouty specifically inhibits the Ras/MAP/ERK signaling pathway (Shaw et al., 2007), but does neither affect the phosphoinositide 3-kinase (PI3K) nor other MAPK pathways (Yusoff et al., 2002). Although there are many studies on Sprouty proteins, their exact mechanism and place of action in the pathway remain controversial: Sprouty may act downstream of RTK and upstream of Ras (Hacohen et al., 1998; Gross et al., 2001; Leeksma et al., 2002), or at the level of Raf (Reich et al., 1999; Yusoff et al., 2002). Recent evidence suggests that Sprouty2 might act through inhibition of trafficking of activated EGFR from early to late endosomes (Kim et al., 2007).

Given the crucial functional roles of Sprouty proteins, in particular in modulating the activity of signaling centers in the vertebrate embryo, it would be important to complete our understanding of which pathways they regulate, and to generate live tracers of Sprouty activity. We report here the isolation through enhancer detection of zebrafish *sprouty1* gene (*spry1*), which on the basis of its expression pattern and its response to manipulation of Fgf8 signaling appears to be involved in the regulation of the Fgf8 pathway. This is the first description of this gene in zebrafish. In addition, comparing the expression of endogenous *spry1* messenger RNA to that of the fluorescent reporter in an enhancer trap line shows that this line is a faithful and sensitive reporter of *spry1* expression.

RESULTS AND DISCUSSION

Enhancer Detection Insertion Identifies *sprouty1* in the Zebrafish Genome

With the aim of identifying developmentally regulated genes, our lab has conducted a large-scale enhancer detection project (Ellingsen et al., 2005; Laplante et al., 2006). We recovered an enhancer detection line, CLGY786, where the YFP expression pattern overlapped with that of fibroblast growth factor 8a (*fgf8a*).

We mapped the genomic position of the retroviral insertion using inverse polymerase chain reaction (PCR). A 273-bp flanking sequence was isolated, sequenced and used to search the zebrafish genome assembly in the Ensemble database (http://www.ensembl.org/) using BLASTN. A single unique match with 99.27% identity was found, locating the proviral insertion 3,333 bp upstream of an uncharacterized gene in the zebrafish genome on chromosome 14 (Zv7). Sequence searches using BLAST indicated that this gene is a member of the zebrafish *spry* family. In agreement with this finding, known *spry* genes in vertebrates exhibit expression patterns similar to zebrafish *fgf8a*. Phylogenetic analyses and structural features (detailed below) further identified this gene as *spry1*, to date uncharacterized in zebrafish.

Phylogenetic Analysis of *Spry1*

We used phylogenetic analysis to determine the evolutionary relationship between the isolated cDNA and members of the *spry* family. Sequences were initially selected based on BLAST analysis from the NCBI database. For clarity of the diagram, not all available members of the Sprouty family were used in the analysis. As expected, Sprouty sequences segregated into four groups. The isolated zebrafish sprouty grouped with other vertebrate Sprouty1 orthologs (Fig. 1A), indicating that it is the *Danio rerio spry1* gene.

The Zebrafish Sprouty1 Predicted Amino Acid Sequence Harbors All Distinctive Features of Sprouty Proteins

The full-length cDNA of *spry1* was cloned by degenerate reverse transcriptase-PCR (RT-PCR) and Rapid Amplification of cDNA Ends (RACE) on RNA prepared from 24 hours postfertilization (hpf) zebrafish embryos. The *spry1* cDNA comprises 1,270 nucleotides, containing a single open reading frame of 879 bp encoding a predicted protein of 292 amino acid

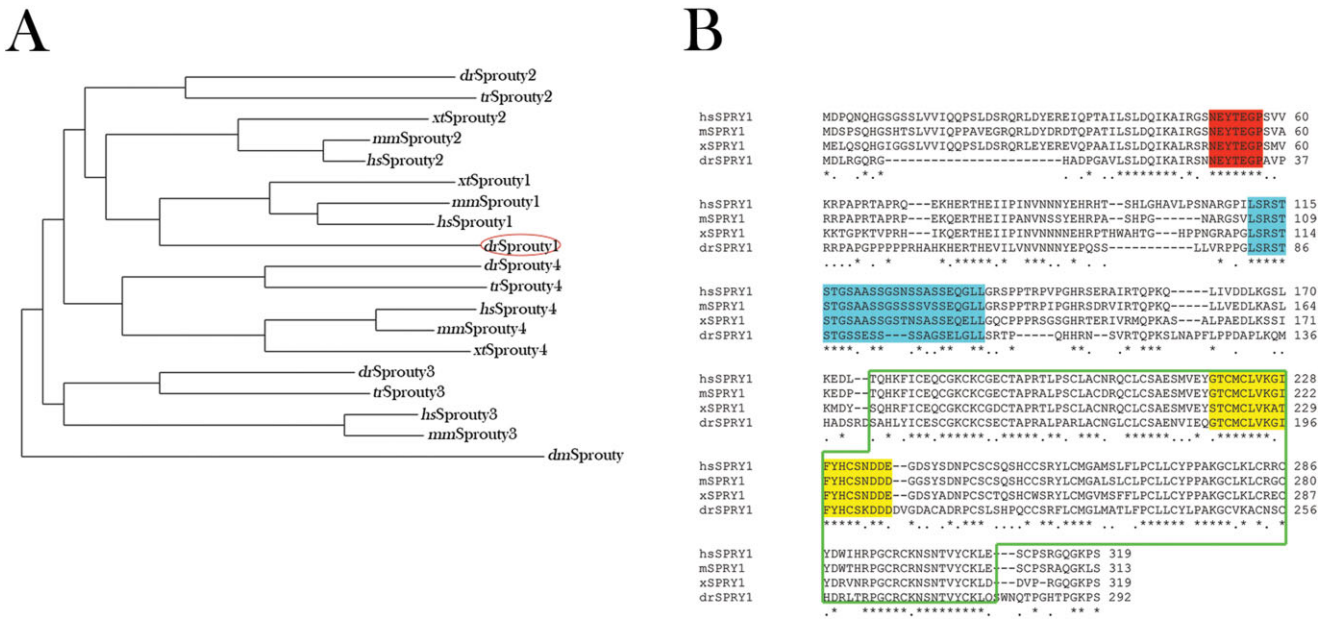

Fig. 1. Phylogenetic analysis of the Sprouty family and sequence analysis of zebrafish Sprouty1. **A:** sprouty1 of zebrafish grouped with Sprouty1 sequences of other species. The sequences of *Drosophila melanogaster* and vertebrate Sprouty proteins were aligned using Clustal W, and the tree was constructed using the Neighbor-Joining method. *Drosophila* Sprouty was used as an outgroup. *dr*: *Danio rerio*, *tr*: *Takifugu rubripes*, *hs*: *Homo sapiens*, *xt*: *Xenopus tropicalis*, *dm*: *Drosophila melanogaster*. The peptides were predicted by the Ensembl analysis pipeline from either a GeneWise or Genscan prediction followed by confirmation of the exons by comparison to protein, cDNA, and expressed sequence tag databases. **B:** Sequence alignment of the vertebrate Sprouty1 (SPRY) proteins. Stars and dots below the sequence alignment indicate identical or similar amino acids in all proteins, respectively. Sequence alignment of human (hsSPRY), mouse (mSPRY), *Xenopus* (xSPRY) and zebrafish (drSPRY) Sprouty1 proteins. Dashes indicate gaps introduced to optimize the alignment. Functional domains and motifs are marked: PY motif with conserved tyrosine Y30 (red block), highly conserved serine-rich region (blue block), RBD motif (Raf1-binding domain; yellow block), and Sprouty translocation domain (SpryTD) at the C-terminal end of the protein (green box).

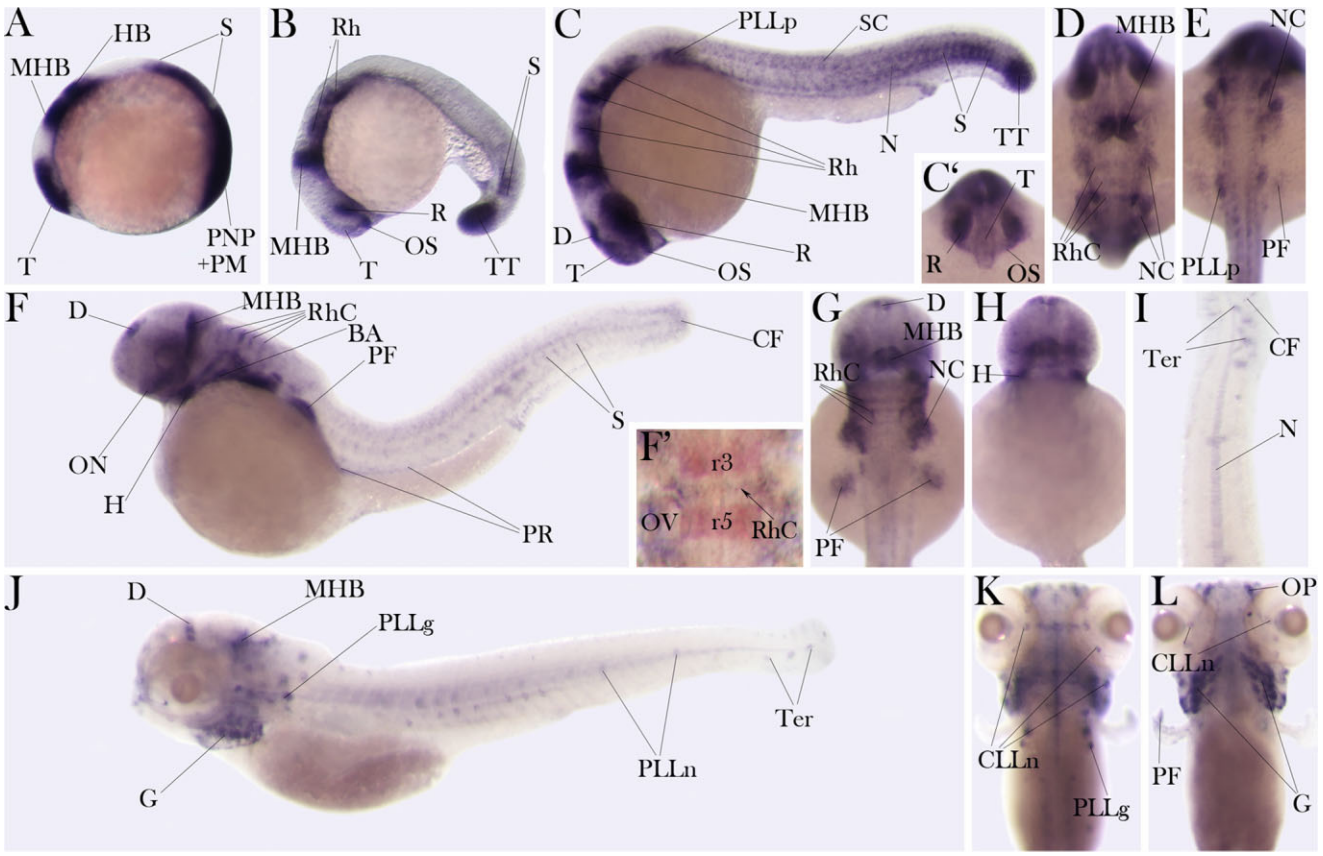

Fig. 2.

residues with a molecular weight of 27.7 kDa (bankit1053103 EU379656).

Sequence analyses showed that zebrafish sprouty1 protein is closely related to vertebrate orthologues, and an alignment using ClustalW software revealed an overall sequence consensus with 54% identity to human, 52% to mouse, and 50% to frog Sprouty1 (Fig. 1B). However, identified functional domains of the predicted protein display much higher amino acid conservation. The structure of zebrafish Sprouty1 is identical to the other vertebrate Sprouty proteins with three putative, highly conserved regions, namely a short N-terminal PY sequence, a C-terminal cysteine-rich "Sprouty translocation domain" (SpryTD), and a short Raf1-binding motif within SpryTD.

The PY motif (NEYTEGP in Sprouty 1 and 2 proteins) contains one invariable tyrosine residue (Y^{30}; Fig. 1B; red block). The inhibitory activity of Sprouty proteins is dependent upon this residue. Upon Fgf and Egf stimulation, Y^{30} is rapidly phosphorylated (Hanafusa et al., 2002; Mason et al., 2004) and directly bound by the SH2 domain of its partners. The residues located immediately C-terminal to phosphorylated Y^{30} are probably also crucial for the interaction between Sprouty and SH2 domains. For instance, this is the case for the interaction of Sprouty with the c-Cbl protein after induction by growth factors (Mason et al., 2004). All Sprouty proteins also contain a highly conserved serine-rich region, with a phosphorylated serine residue (Fig. 1B; blue block). This modification does not appear to be modulated by growth factor stimulation, and the functional role of the phosphoserine is unclear (Impagnatiello et al., 2001).

The similarity to the fly protein is restricted primarily to SpryTD (Fig. 1B; green box). This domain is located between amino acid position 143 and 278 in zebrafish Sprouty1 and shows a high level of homology to Sprouty1 proteins of other species: 69% identity to the human protein, and 67% to mouse and frog. The SpryTD domain of zebrafish Sprouty1 includes 23 cysteine residues and is presumably responsible for translocation of the protein to the cell membrane after activation by growth factor signaling. Cysteines can be subject to posttranscriptional modifications, for instance palmitoylation by means of labile thioester bonds (Veit and Schmidt, 1998), and this was shown to happen in Sprouty proteins (Impagnatiello et al., 2001). Another important motif of the SpryTD domain is the RBD motif (Raf1-binding domain), which is responsible for protein–protein interactions and mediates binding to Raf1 (Fig. 1B; yellow block).

Developmental Expression of *spry1*

In mammals, *spry1* expression was detected during development of the central nervous system, limbs, lungs, and teeth (Szebenyi and Fallon, 1999; Zhang et al., 2001).

We analyzed *spry1* expression by in situ hybridization to determine its transcription profile in zebrafish, to compare it with that of other species, and to assess whether the enhancer detection line CLGY786 is a faithful reporter of *spry1* expression. Observation of YFP expression in transgenic embryos of the CLGY786 line, both live and by immunodetection, revealed that YFP expression mimics the expression pattern of the *spry1* gene, at least during the time-frame of our analysis (i.e., up to day 10 of development). Both the *spry1* and YFP profiles are described below.

Analysis of *spry1* expression revealed a striking similarity with the expression domains of *fgf8a*. At the beginning of segmentation, *spry1* is strongly expressed in the telencephalon as well as the tail bud and is continuously expressed at the midbrain–hindbrain boundary (MHB). It also displays transient expression in hindbrain. Posteriorly, *spry1* is expressed in the newly formed somites as well as in the unsegmented paraxial mesoderm and neural plate (Figs. 2A, 3A, 4A). At the 16/18-somite stage, expression is observed in the rhombomeres, the optic stalk, ventral retina, and telencephalon (Figs. 2B, 3B, 4B). Approximately 22 hpf, *spry1* displays strong expression in dorsal diencephalon and epiphysis, anterior and posterior lateral line primordia, pectoral fin primordia, neural crest, notochord, and in the tail tip. *spry1* is also expressed at low level in the spinal cord (Figs. 2C–E, 3C–E, 4C–E). Later *spry1* is detected in cranial and trunk lateral line neuromasts and in cells along the entire pronephros (Wingert et al., 2007). *spry1* has been shown to be a target of the Wilms tumor gene *Wt1* in the kidney (Gross et al., 2003). Expression is detectable in the MHB, rhombomere centers, dorsal diencephalon and epiphysis, pituitary, branchial arches, optic nerve, hyoid, lateral line neuromasts, pectoral fins, and in caudal fin at 48 hpf (Fig. 2F–H, 3F–H, 4F–H). Cross-sections at 3 and 6 days postfertilization (dpf) reveal *spry1* expression in optic nerve astrocytes, cells in the skin under the jaw and inside the mouth, the gill operculum, and several cranial ganglia, in particular the trigeminal ganglia, anterior and posterior lateral line ganglia, and otic ganglia (Fig. 5). In the ear *spry1* is expressed in the support cells (Fig. 5D). The expression in optic nerve astrocytes identifies a cell population that also express *pax2* (Macdonald et al., 1997), and it was recently was shown that fgf signaling is necessary for maintenance of *Pax2* expression in the chick optic nerve (Soukkarieh et al., 2007). *spry1* expression then becomes weaker, and at 5 and 6 dpf mRNA was detectable in lateral line neuromasts, gill epi-

Fig. 2. In situ hybridization with *spry1* antisense probe on wild-type embryos. **A–L:** At the 2-somite stage (A), 18-somite stage (B), 22 hours postfertilization (hpf; C–E,F′), 48 hpf (F–H), and 120 hpf (I–L). A–C,F,I,J: Lateral view, anterior to the left (I, anterior to the bottom); C′,H,L: ventral view, anterior to the top; D,E,F′,G,K: dorsal view, anterior to the top. *spry1* expression domains are similar to the *fgf8a* expression pattern. Note in F′ (*spry1*-blue, *ephA4*-red marks r3 and r5) that rhombomere expression is not in register with rhombomere boundaries, but rather corresponds to rhombomere centers. BA, branchial arches; CF, caudal fin fold; CLLn, cranial lateral line neuromasts; D, dorsal diencephalon; G, gills, H, hyoid; HB, hindbrain; MHB, midbrain–hindbrain boundary; N, notochord; NC, neural crest; ON, optic nerve; OP, olfactory placodes; OS, optic stalk; OV, otic vesicle; PF, pectoral fin; PLLg, posterior lateral line ganglion; PLLn, posterior lateral line neuromasts; PLLp, posterior lateral line primordium; PM, paraxial mesoderm; PNP, posterior neural plate; PR, pronephros; S, somites; SC, spinal cord; R, retina; Rh, rhombomeres; RhC, rhombomere centers; T, telencephalon; Ter, terminal neuromasts; TT, tail tip.

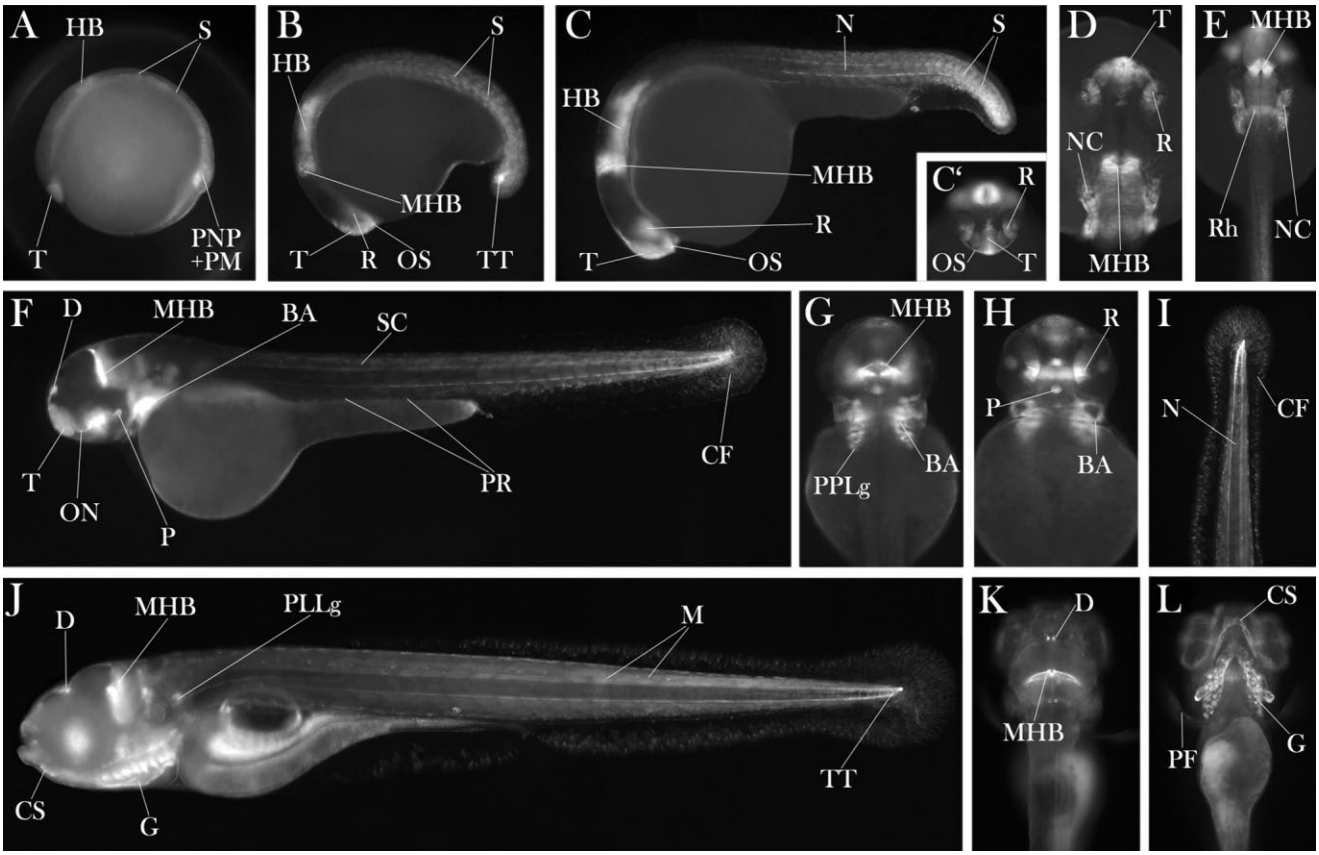

Fig. 3. YFP expression pattern in CLGY786 line mimics endogenous *spry1*. **A–L:** At the 2-somite stage (A), 16-somite stage (B), 22 hours postfertilization (hpf; C–E), 48 hpf (F–H), and 120 hpf (I–L). A–C,F,I,J: Lateral view, anterior to the left (I, anterior to the bottom); C′,H,L: ventral view, anterior to the top; D,E,G,K: dorsal view, anterior to the top. P, pituitary; CS, craniofacial skeleton; M, muscle. For other abbreviations, see Figure 2.

thelium, MHB, dorsal diencephalon, pituitary and epiphysis, and caudal fin (Fig. 2I–L, 3I–L, 4I–L, 5F–J). The expression in rhombomere centers was confirmed by double in situ staining with *ephA4* which is expressed in rhombomeres 3 and 5 (Fig. 2F′). At 10 dpf YFP in the transgenic line was still expressed in MHB and in the gill epithelium, where it persisted until adulthood (data not shown).

Our analysis of *spry1* expression pattern reveals a striking correlation of domains with *fgf8a*, and with *spry2*, and *spry4*. The expression of *spry1* appeared to be somewhat more widespread than *fgf8a* in all domains where these genes overlap. Expression domains of *spry1*, *spry2*, and *spry4* in the majority overlap, however, in a few cases they are expressed in adjacent or complementary domains (Furthauer et al., 2001, 2004).

spry1 Expression Is Inhibited Upon Down-Regulation of Fgf Signaling

Sprouty proteins are known antagonists of Fgf signaling and operate by means of a negative feedback loop, their expression being induced by Fgf activity. To confirm that *spry1* in zebrafish follows the same functional scheme, we performed in situ hybridization for *spry1* expression in *fgf8a/acerebellar* mutants. We observed that *spry1* expression is abolished in mutant embryos at the MHB, rhombomeres, optic stalk, and dorsal diencephalon, all domains where *fgf8a* activity is lost in *ace* mutants (Fig. 6A,B). In contrast, *spry1* expression is maintained in the tail bud, suggesting that it might respond to other Fgfs active in this location, such as *fgf3*, *fgf4*, *fgf10*, or *fgf16* (Niswander and Martin, 1992; Mansour, 1994) and Zebrafish Information Network (ZFIN) http://zfin.org/.

To confirm a direct regulation of *spry1* expression by Fgf signaling, we performed a time-course analysis by incubating embryos at the 18-somite stage with the Fgf signaling antagonist SU5402 for various time periods (Fig. 6C–J). Embryos were then fixed immediately and *spry1* expression was visualized by in situ hybridization. We observed that *spry1* expression was reduced substantially after 1 hr of incubation but not in all expression domains: it was still detectable at low levels in the MHB, telencephalon, somites, and tail bud. The expression in the MHB and telencephalon disappeared after 6 hr of incubation, in the tail bud after 12 hr and in the somites after 16 hr of incubation. These results attest a mostly immediate regulation by Fgf activity, with slight variations in space that might

Fig. 4. Immunostaining of the CLGY786/*spry*1 line shows domains where YFP protein is expressed. **A–L:** At the 2-somite stage (A), 18-somite stage (B), 22 hours postfertilization (hpf; C–E), 48 hpf (F–H), and 120 hpf (I–L). A–C,F,I,J: Lateral view, anterior to the left (I, anterior to the bottom); C′,H,L: ventral view, anterior to the top; D,E,G,K: dorsal view, anterior to the top. For abbreviations, see Figures 2 and 3.

reflect differences in Fgf signaling levels.

Sprouty proteins, by inhibiting growth factor signaling, control multiple developmental processes and largely contribute to organ patterning. While the *Drosophila* genome contains only one *spry* gene (Hacohen et al., 1998), four *spry* genes are found in all vertebrates studied to date. It is interesting that, while many *fgf* genes have been kept in duplicate after the teleost-specific whole genome duplication, zebrafish, like tetrapods, have kept only the complement of four *spry* genes without any further duplication and subfunctionalization. It is possible that the different Sprouty proteins elicit different effects after being activated by particular growth factors. Alternatively, an efficient level of inhibitory activity required for proper organ patterning may only be achieved by the simultaneously expression of several *spry* genes and the synchronized expression of several Sprouty proteins.

In conclusion, we have identified, through enhancer detection, zebrafish *spry1*, suggesting that this gene is regulated through long-range enhancers, similar to other developmental regulatory genes (Sandelin et al., 2004; Kikuta et al., 2007a,b). We demonstrated that the expression of *spry1* is highly dynamic and is fitting with embryonic territories that are known *fgf8a* expression domains, and that Fgf signaling is both necessary and sufficient to induce *spry1* expression. Finally, our findings also provide a transgenic reporter line of *spry1* expression and Fgf activity during zebrafish embryonic development.

EXPERIMENTAL PROCEDURES

Embryos

Embryos were obtained from TAB wild-type fish, *acerebellar/fgf8a*$^{-/-}$ (Reifers et al., 1998) and CLGY786 homozygous enhancer detection transgenics maintained at 28°C on a 14 hr light/10 hr dark cycle. Embryos were staged according to Kimmel et al. (1995).

Mapping of the Proviral Insertion in the CLGY786 Line by Inverse PCR

Genomic DNA was isolated from tail-biopsies of six adult heterozygous transgenic CLGY786 fish and extracted with the DNase Tissue Kit (Qiagene). Each genomic DNA sample was divided into two separate reactions, digested with *Bgl*II or *Bam*HI. The flanking genomic sequences of the activated proviral vector were amplified using inverse PCR, with the following primers: 5′: 786R5′-TACCGACGCAGGCGCATAAAATC; 786F5′-TTTGTACACCCTAAGCCTCCGCC; 786nestR5′-ATAAGTTGCTGGCCAGCTTACCTC; 786nestF5′-TCCCCCTTGAACCTCCTCGTTC; and 3′: 786R3′-CGGTGGTGCAGATGAACTTCAGG; 786F3′-AAG-

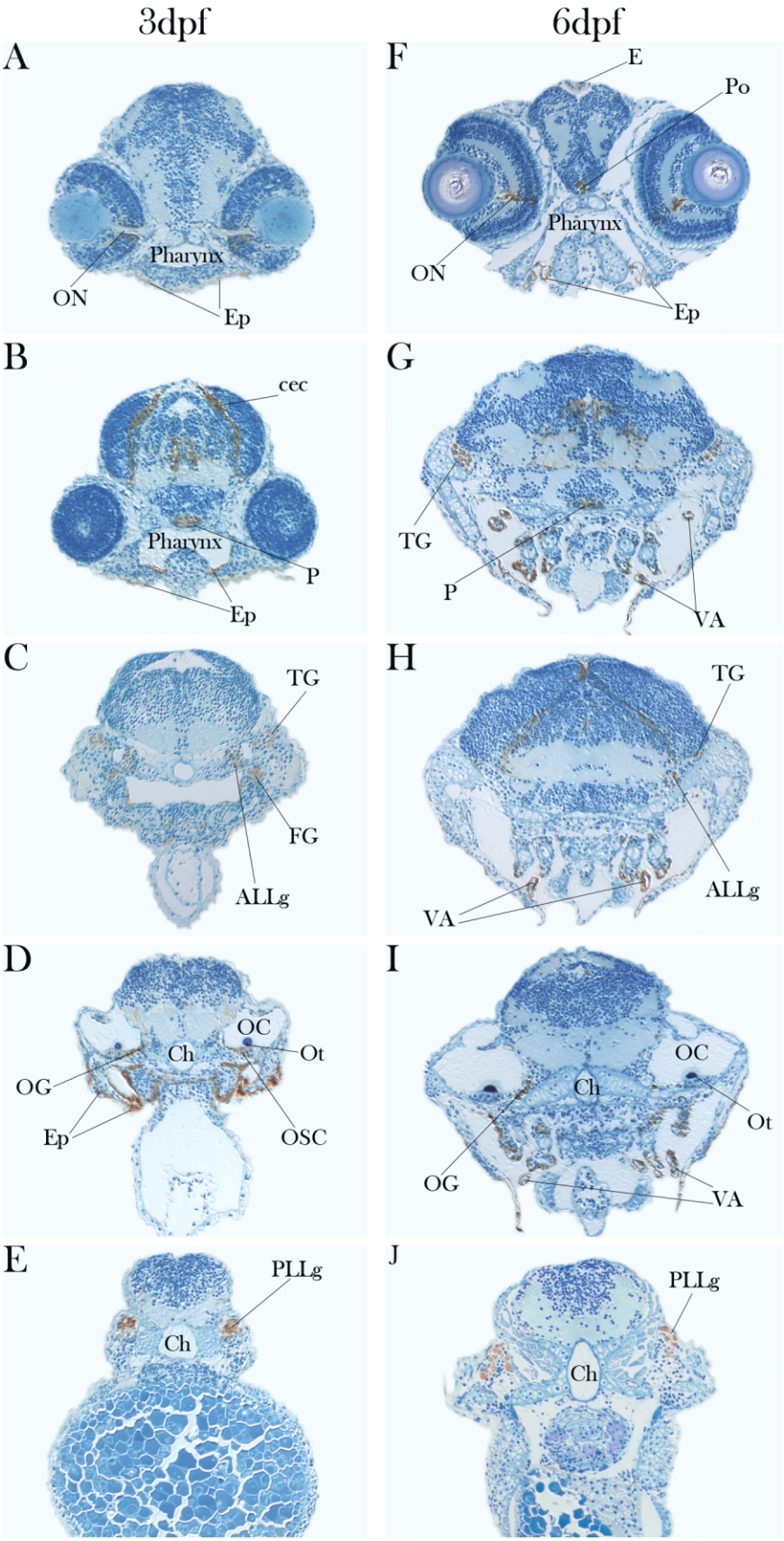

Fig. 5. Immunostaining of the CLGY786/*spry*1 line, cross-sections of anterior regions show domains where YFP protein is expressed. **A–E:** At 3 hours postfertilization (hpf). **F–J:** At 6 hpf. ALLg, anterior lateral line ganglion; cec, cerebellar commissure; Ch, chorda dorsalis; E, epiphysis; Ep, epithelium; FG, facial ganglion; mlf, medial longitudinal fascicle; OC, otic capsule; OG, otic ganglion; OSC, otic support cells; Ot, otolith; Po, preoptic region; TG, trigeminal ganglion; VA, visceral arches. For other abbreviations, see Figure 2 and 3.

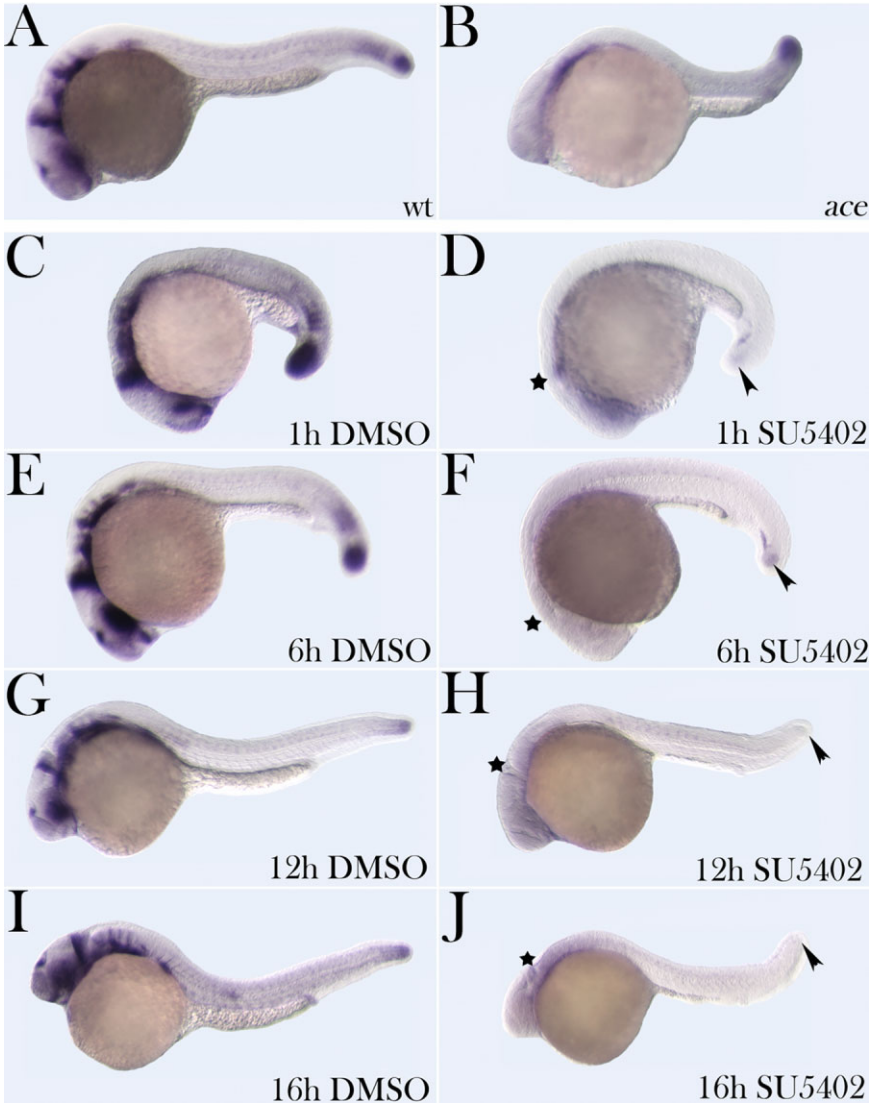

Fig. 6. Sprouty1 and the Fgf signaling pathway. **A,B:** Twenty-four hour postfertilization stage. Loss of *fgf8a* in *ace* inhibits *spry1* expression. In situ hybridization with *spry1* antisense probe in wild-type (wt) (A) and *ace* *fgf8a*$^{-/-}$ mutant (B) embryos. **C–J:** Wild-type embryos treatment with the inhibitor of FGF signaling SU5402 commenced at the18-somite stage. Incubation for 1 hr (C,D), 6 hr (E,F), 12 hr (G,H), and 16 hr (I,J). The asterisk indicates midbrain–hindbrain boundary (MHB) position and arrowhead the tail tip. Expression of *spry1* is inhibited after 1 hr of incubation in most of the domains, and after 16 hr of incubation there is no detectable expression of *spry1*.

CTTGAGCTCGAGATCCGGATTAG; 786nestR3′-TTCAGGGTCAGCTTGCCGTAG; 786nestF3′-TATCAGTGGTCCAGGCTCTAGTTTTG. The PCR products were cloned into pCR 2.1-TOPO TA vector (TOPO TA cloning kit, Invitrogen) and sequenced with M13 forward primer. The genomic localization of the proviral insertion was identified by BLAST searching against the zebrafish genome sequence in Ensembl database, as described previously (Ellingsen et al., 2005; Laplante et al., 2006).

Cloning of Zebrafish sprouty 1

spry1 was cloned by PCR from genomic DNA using the following primer set: 5′-GCACATCATCATCATCATCTTCACC and 5′-CACCATCAGTTTGTGCCTCAGGAT. The reaction was carried out at 94°C for 5 min, then 35 cycles at 94°C for 30 sec, 53°C for 30 sec, 72°C for 90 sec, and a final extension at 72°C for 5 min. The PCR product was directly processed for a nested PCR using the following primers: 5′-GCATAGGTGTTGGAATTGACATC and 5′-CAGTTTGTGCCTCAGGATGGTTTCC, under similar conditions as described above. The PCR product was subcloned into pCR II-Topo Vector (Invitrogen). The sequence of zebrafish *spry1* has been submitted to GenBank (accession no. bankit1053103 EU379656).

Whole-Mount In Situ Hybridization and Immunodetection

In situ hybridizations were carried out as described (Adolf et al., 2006). The digoxigenin- and fluorescein-labeled antisense RNA probe for *spry1* and *ephA4* (Xu et al., 1995) in situ hybridization was synthesized with T7 RNA polymerase (RNA labeling kit) after *Kpn*I digestion.

Embryos for immunodetection were obtained by mating homozygous fish of line CLGY786. Immunodetection was performed as described previously (Ellingsen et al., 2005). Sectioning and counterstaining were done according to Becker et al. (1998). Structures were identified in correlation with Mueller and Wullimann (2005).

Inhibition of Fgf Signaling by SU5402 Treatment

For incubation with SU5402, embryos were manually dechorionated and allowed to develop in a Petri dish with a layer of 1.5% agarose in E3 medium until the 18-somite stage. SU5402 (Mohammadi et al., 1997; 10 μM, Calbiochem) was added to the embryos at the desired stage, and embryos were incubated in the dark, with 0.75 mM phenylthiourea (PTU) to prevent pigmentation. Control embryos were incubated in an equivalent concentration of DMSO added to the E3 medium with PTU. The embryos were collected after 1, 6, 12, and 16 hr of incubation and fixed for in situ hybridization.

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REFERENCES

Adolf B, Chapouton P, Lam CS, Topp S, Tannhauser B, Strahle U, Gotz M, Bally-Cuif L. 2006. Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. Dev Biol 295: 278–293.

Basson MA, Akbulut S, Watson-Johnson J, Simon R, Carroll TJ, Shakya R, Gross I, Martin GR, Lufkin T, McMahon AP, Wilson PD, Costantini FD, Mason IJ, Licht JD. 2005. Sprouty1 is a critical regulator of GDNF/RET-mediated kidney induction. Dev Cell 8:229–239.

Becker TS, Burgess SM, Amsterdam AH, Allende ML, Hopkins N. 1998. not really finished is crucial for development of the zebrafish outer retina and encodes a transcription factor highly homologous to human Nuclear Respiratory Factor-1 and avian Initiation Binding Repressor. Development 125:4369–4378.

Casci T, Vinos J, Freeman M. 1999. Sprouty, an intracellular inhibitor of Ras signaling. Cell 96:655–665.

de Maximy AA, Nakatake Y, Moncada S, Itoh N, Thiery JP, Bellusci S. 1999. Cloning and expression pattern of a mouse homologue of drosophila sprouty in the mouse embryo. Mech Dev 81:213–216.

Ellingsen S, Laplante MA, Konig M, Kikuta H, Furmanek T, Hoivik EA, Becker TS. 2005. Large-scale enhancer detection in the zebrafish genome. Development 132:3799–3811.

Fernig DG, Gallagher JT. 1994. Fibroblast growth factors and their receptors: an information network controlling tissue growth, morphogenesis and repair. Prog Growth Factor Res 5:353–377.

Furthauer M, Reifers F, Brand M, Thisse B, Thisse C. 2001. sprouty4 acts in vivo as a feedback-induced antagonist of FGF signaling in zebrafish. Development 128: 2175–2186.

Furthauer M, Van Celst J, Thisse C, Thisse B. 2004. Fgf signalling controls the dorsoventral patterning of the zebrafish embryo. Development 131:2853–2864.

Goodnough LH, Brugmann SA, Hu D, Helms JA. 2007. Stage-dependent craniofacial defects resulting from Sprouty2 overexpression. Dev Dyn 236: 1918–1928.

Gross I, Bassit B, Benezra M, Licht JD. 2001. Mammalian sprouty proteins inhibit cell growth and differentiation by preventing ras activation. J Biol Chem 276:46460–46468.

Gross I, Morrison DJ, Hyink DP, Georgas K, English MA, Mericskay M, Hosono S, Sassoon D, Wilson PD, Little M, Licht JD. 2003. The receptor tyrosine kinase regulator Sprouty1 is a target of the tumor suppressor WT1 and important for kidney development. J Biol Chem 278: 41420–41430.

Hacohen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. 1998. sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. Cell 92:253–263.

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

Huebert RC, Li Q, Adhikari N, Charles NJ, Han X, Ezzat MK, Grindle S, Park S, Ormaza S, Fermin D, Miller LW, Hall JL. 2004. Identification and regulation of Sprouty1, a negative inhibitor of the ERK cascade, in the human heart. Physiol Genomics 18:284–289.

Impagnatiello MA, Weitzer S, Gannon G, Compagni A, Cotten M, 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.

Kikuta H, Fredman D, Rinkwitz S, Lenhard B, Becker TS. 2007a. Retroviral enhancer detection insertions in zebrafish combined with comparative genomics reveal genomic regulatory blocks - a fundamental feature of vertebrate genomes. Genome Biol 8(suppl 1):S4.

Kikuta H, Laplante M, Navratilova P, Komisarczuk AZ, Engstrom PG, Fredman D, Akalin A, Caccamo M, Sealy I, Howe K, Ghislain J, Pezeron G, Mourrain P, Ellingsen S, Oates AC, Thisse C, Thisse B, Foucher I, Adolf B, Geling A, Lenhard B, Becker TS. 2007b. Genomic regulatory blocks encompass multiple neighboring genes and maintain conserved synteny in vertebrates. Genome Res 17: 545–555.

Kim HJ, Taylor LJ, Bar-Sagi D. 2007. Spatial regulation of EGFR signaling by Sprouty2. Curr Biol 17:455–461.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310.

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

Kwabi-Addo B, Wang J, Erdem H, Vaid A, Castro P, Ayala G, Ittmann M. 2004. The expression of Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is decreased in human prostate cancer. Cancer Res 64:4728–4735.

Laplante M, Kikuta H, Konig M, Becker TS. 2006. Enhancer detection in the zebrafish using pseudotyped murine retroviruses. Methods 39:189–198.

Leeksma OC, Van Achterberg TA, Tsumura Y, Toshima J, Eldering E, Kroes WG, Mellink C, Spaargaren M, Mizuno K, Pannekoek H, de Vries CJ. 2002. Human sprouty 4, a new ras antagonist on 5q31, interacts with the dual specificity kinase TESK1. Eur J Biochem 269:2546–2556.

Lim J, Wong ES, Ong SH, Yusoff P, Low BC, Guy GR. 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.

Lim J, Yusoff P, Wong ES, Chandramouli S, Lao DH, Fong CW, Guy GR. 2002. The cysteine-rich sprouty translocation domain targets mitogen-activated protein kinase inhibitory proteins to phosphatidylinositol 4,5-bisphosphate in plasma membranes. Mol Cell Biol 22:7953–7966.

Macdonald R, Scholes J, Strahle U, Brennan C, Holder N, Brand M, Wilson SW. 1997. The Pax protein Noi is required for commissural axon pathway formation in the rostral forebrain. Development 124: 2397–2408.

Mailleux AA, Tefft D, Ndiaye D, Itoh N, Thiery JP, Warburton D, Bellusci S. 2001. Evidence that SPROUTY2 functions as an inhibitor of mouse embryonic lung growth and morphogenesis. Mech Dev 102:81–94.

Mansour SL. 1994. Targeted disruption of int-2 (fgf-3) causes developmental defects in the tail and inner ear. Mol Reprod Dev 39:62–67; discussion 67–68.

Mason JM, Morrison DJ, Bassit B, Dimri M, Band H, Licht JD, 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.

Mason JM, Morrison DJ, Basson MA, Licht JD. 2006. Sprouty proteins: multifaceted negative-feedback regulators of receptor tyrosine kinase signaling. Trends Cell Biol 16:45–54.

Minowada G, Jarvis LA, Chi CL, Neubuser A, Sun X, Hacohen N, Krasnow MA, Martin GR. 1999. Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. Development 126:4465–4475.

Mohammadi M, McMahon G, Sun L, Tang C, Hirth P, Yeh BK, Hubbard SR, Schlessinger J. 1997. Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science 276:955–960.

Mueller T, Wullimann MF. 2005. Atlas of early Zebrafish brain development. A tool for molecular neurogenetics. Amsterdam: Elsevier.

Niswander L, Martin GR. 1992. Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. Development 114:755–768.

Reich A, Sapir A, Shilo B. 1999. Sprouty is a general inhibitor of receptor tyrosine kinase signaling. Development 126: 4139–4147.

Reifers F, Bohli H, Walsh EC, Crossley PH, Stainier DY, Brand M. 1998. Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Development 125:2381–2395.

Sandelin A, Bailey P, Bruce S, Engstrom PG, Klos JM, Wasserman WW, Ericson J, Lenhard B. 2004. Arrays of ultraconserved non-coding regions span the loci of key developmental genes in vertebrate genomes. BMC Genomics 5:99.

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

Shaw AT, Meissner A, Dowdle JA, Crowley D, Magendantz M, Ouyang C, Parisi T, Rajagopal J, Blank LJ, Bronson RT, Stone JR, Tuveson DA, Jaenisch R, Jacks T. 2007. Sprouty-2 regulates oncogenic K-ras in lung development and tumorigenesis. Genes Dev 21:694–707.

Smart JE, Oppermann H, Czernilofsky AP, Purchio AF, Erikson RL, Bishop JM. 1981. Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus (pp60v-src) and its normal cellular homologue (pp60c-src). Proc Natl Acad Sci U S A 78:6013–6017.

Soukkarieh C, Agius E, Soula C, Cochard P. 2007. Pax2 regulates neuronal-glial cell fate choice in the embryonic optic nerve. Dev Biol 303:800–813.

Sutterluty H, Mayer CE, Setinek U, Attems J, Ovtcharov S, Mikula M, Mikulits W, Micksche M, Berger W. 2007. Down-regulation of Sprouty2 in non-small cell lung cancer contributes to tumor malignancy via extracellular signal-regulated kinase pathway-dependent and -independent mechanisms. Mol Cancer Res 5:509–520.

Szebenyi G, Fallon JF. 1999. Fibroblast growth factors as multifunctional signaling factors. Int Rev Cytol 185:45–106.

Tefft JD, Lee M, Smith S, Leinwand M, Zhao J, Bringas P Jr, Crowe DL, Warburton D. 1999. Conserved function of mSpry-2, a murine homolog of Drosophila sprouty, which negatively modulates respiratory organogenesis. Curr Biol 9:219–222.

Veit M, Schmidt MF. 1998. Membrane targeting via protein palmitoylation. Methods Mol Biol 88:227–239.

Wang J, Thompson B, Ren C, Ittmann M, Kwabi-Addo B. 2006. Sprouty4, a suppressor of tumor cell motility, is down regulated by DNA methylation in human prostate cancer. Prostate 66:613–624.

Wingert RA, Selleck R, Yu J, Song HD, Chen Z, Song A, Zhou Y, Thisse B, Thisse C, McMahon AP, Davidson AJ. 2007. The cdx genes and retinoic acid control the positioning and segmentation of the zebrafish pronephros. PLoS Genet 3:1922–1938.

Xu Q, Alldus G, Holder N, Wilkinson DG. 1995. Expression of truncated Sek-1 receptor tyrosine kinase disrupts the segmental restriction of gene expression in the Xenopus and zebrafish hindbrain. Development 121:4005–4016.

Yigzaw Y, Cartin L, Pierre S, Scholich K, Patel TB. 2001. The C terminus of sprouty is important for modulation of cellular migration and proliferation. J Biol Chem 276:22742–22747.

Yusoff P, Lao DH, Ong SH, Wong ES, Lim J, Lo TL, Leong HF, Fong CW, Guy GR. 2002. Sprouty2 inhibits the Ras/MAP kinase pathway by inhibiting the activation of Raf. J Biol Chem 277:3195–3201.

Zhang S, Lin Y, Itaranta P, Yagi A, Vainio S. 2001. Expression of Sprouty genes 1, 2 and 4 during mouse organogenesis. Mech Dev 109:367–370.

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