

Supplementary Data legends

Supplementary Figure S1: *SPRY2* and *SPRY4* are differentially expressed in RMS

(A) *SPRY2* and *SPRY4* are differentially expressed between ARMS and ERMS samples as determined by Affymetrix U133A analysis of 15 ERMS, 10 translocation-positive and four translocation-negative ARMS samples. The U133A Affymetrix Microarray dataset was generated by Wachtel and colleagues (1) and was obtained from www.ebi.ac.uk/arrayexpress under accession no. E-MEXP-121.

(B) Expression of *SPRY1-4* in proliferating RMS cells. The human embryonal RD, TE381T, TE617T, Hs729T, T174 (ATCC, Rockville MD), and RUCH2 (kind gift from Dr. Schäfer, Division of Clinical Chemistry and Biochemistry, Department of Pediatrics, University of Zürich, Switzerland), and alveolar RH30 and RH4 (kind gift from Dr. Lollini, Department of Experimental Pathology, University of Bologna, Italy). Affymetrix microarray profiling was performed for eight RMS cell lines during logarithmic growth (2). For Affymetrix microarray analysis, fragmentation of RNA, labeling, hybridization to HGU133 Plus 2.0 microarrays, and scanning were carried out according to the manufacturer's protocol (Affymetrix Inc.). The expression data were normalized with the MAS5.0 algorithm within the GCOS program of Affymetrix.

Supplementary Figure S2: Determination of hypermethylation of human *SPRY1* promoter

The human *SPRY1* locus generates two splice variants (3) that contain a different non-coding exon 1 (exon 1A and 1B in Figure; accession numbers: NM_005841 (TV1) and NM_199327 (TV2); Figure S1A). Three CpG islands were identified in the human *SPRY1* promoter in the regions containing exon 1A or 1B using MethPrimer (<http://www.urogene.org/methprimer/>).

To determine promoter hypermethylation of *SPRY1* in rhabdomyosarcomas, genomic DNA in two ARMS cell lines with low *SPRY1* expression and two ERMS cell lines with normal endogenous *SPRY1* expression was digested with *XbaI* and treated with ProtK. SSS1 methyltransferase-treated DNA was used as a positive control. Fragments sizing from 5-8 Kb were isolated. Digested DNA was treated with bisulphate using Zymo EZ-DNA Methylation kit (Baseclear, Leiden, The Netherlands) according to the manufacturer's instructions. PCR was carried out using C to T modified primers (Table S1). PCR products were ligated into pGEM-T easy cloning vector (Promega, Madison, WI, USA) and sequenced using T7a primers (9-10 per clones per target). The figure depicts the CpG-island rich regions in the two *SPRY1* transcript variants (3). The dashed bars indicate the regions that are amplified by the three C to T modified primer pairs. The figure shows the results for RMS cell lines (B) and primary tumors (C). For each primer pair a line with circles shows the result for an individual sample. The circles depict the consecutive CpG-rich islands of that region. SSS1 methyltransferase-treated DNA was used as a positive control. Filled circles indicate methylated DNA, open circles unmethylated DNA.

Supplementary Figure S3: *SPRY1* expression decreases after RAS knockdown in RMS cells

SPRY1 is transcriptionally regulated by RAS. Total RNA (20 µg per lane) was separated on a 1% agarose gel and blotted as described (4). To downregulate *RAS* in RMS cells antisense oligonucleotides were designed that target *H-*, *K-* and *N-RAS* (normal and mutant forms). To increase the stability of these panRAS oligonucleotides Locked Nucleid Acids (LNA) were incorporated during synthesis (5). The sequence of panRas LNA is 5'-TTGAtggcaaataCACa-3' and control LNA is 5'-ACCTatgtctacgCTGc-3' (LNAs are indicated in capitals). The LNA monomers were obtained from Exiqon A/S (Denmark) and were synthesized by Santaris

Pharma AS (Hørsholm, Denmark), as described previously (6, 7). 5-Methyl-C was used in all the LNAs. LNAs were used at a final concentration of 50 nM.

RH30 and RD cells were transfected with panRAS LNA antisense oligonucleotides and total RNA was isolated 48 h later. *SPRY1* RNA levels were determined by Northern blot analysis as described above. 18S/28S RNA is shown as loading control. The full length cDNA sequences of human *NRAS* and *SPRY1* were used as specific probes.

Supplementary Figure S4: *SPRY1* silencing eliminates cell proliferation and induces apoptosis in ERMS cells

(A) Western blot analysis of *SPRY1* silencing following the doxycycline-inducible shSPRY1 expression. This figure shows the second target (see also Figure 4A).

(B-C) Effect of shSPRY1 expression on the proliferation of RH30 (B), RD (B), RUCH2 (C) and TE381T (C) cells. Cells were treated with DOX (dotted lines) to induce ectopic shSPRY1 as compared to non-treated cells (solid lines). For RD and RH30 two clones for each *SPRY1* target are shown (see also Figure 4). For RUCH2 and TE381T the whole population of positive transfectants is shown for the two *SPRY1* targets (see also Figure 4).

(D) SubG1 analysis in *SPRY1*-silenced RMS cells. RD-shSPRY1 and RH30-shSPRY1 cells were treated with DOX for 3 days and grown to confluency. Cells were replated w/o DOX and harvested for propidium iodide staining three days later. The subG1 fraction was analyzed on a BD LSRII flow cytometer (BD Franklin Lakes, NJ, USA). Data was analyzed by flowjo software (Treestart, Ashland, OR, USA). The figure shows determination of subG1 from a triplicate experiment. Experiment was repeated twice with similar results. Results from two different *SPRY1* targets and clones are shown.

Supplementary Figure S5: *SPRY1* silencing reduces viability in cells harboring oncogenic RAS

Cell viability assay of RMS cells and human primary fibroblasts. RD (oncogenic RAS), TE381T (oncogenic RAS), RH30 (wt RAS), Hs729T (wt RAS) and VH10 (wt RAS) cells were transduced with four different lentiviral shRNAs against *SPRY1* or control virus (SHC002). Viability was determined 6 days later.

Supplementary Figure S6: Silencing of *SPRY1* in RD cells and concomitant overexpression of ectopic *SPRY1*

Western blot analysis of *SPRY1* expression in RD-*SPRY1* cells (see Fig. 4D) after treatment with DOX to induce ectopic HA-*SPRY1* and concomitant transduction with three different lentiviral shRNAs against *SPRY1* or control virus (SHC002). The data presented is for day 9 after treatment. Total ERK is shown as loading control.

Supplementary Figure S7: *SPRY1* mediates cell survival at the level of MEK1 in RD cells

(A) RD and RH30 cells were plated at 2500 cells per well (96-well plate) and transfected with increasing amounts of CA-MEK1 (pCMV5-HA-delta-MEK1-EE; a kind gift from Roger Davis, Howard Hughes Medical Institute, University of Massachusetts Medical School, USA). 24h after transfection *SPRY1* was silenced by adding DOX. 72 h after the initial transfection, cells were re-transfected with CA-MEK (and kept with/without DOX). Cell viability was measured 6 days after start of DOX treatment.

(B) RD cells were transfected with 2 μ g/100.000 cells and DOX was added directly. Cells were harvested 4 days after transfection. Total cell lysates were analyzed by Western blotting.

Table S1: Table with primer sequences

The table shows the forward and reverse primer sequences used to determine *SPRY1* promoter methylation, for subcloning fragments into the expression vectors, sequencing of exons of *HRAS*, *KRAS*, *NRAS*, *BRAF* and *SPRY1* and for the generation of cDNA fragments used as probes in Northern blotting.

Table S2: Lentiviral constructs

The table shows the TRC library numbers for the shSPRY1 and shNRAS constructs used in this study. The TRC library SHC002 negative control sequence is also shown.

References Supplementary data

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