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Silencing of SPRY1 Triggers Complete Regression of Rhabdomyosarcoma Tumors Carrying a Mutated *RAS* Gene

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Abstract

RAS oncogenes are among the most frequently mutated genes in human cancer, but effective strategies for therapeutic inhibition of the RAS pathway have been elusive. *Sprouty1* (*SPRY1*) is an upstream antagonist of RAS that is activated by extracellular signal-related kinase (ERK), providing a negative feedback loop for RAS signaling, and other evidence suggests that *SPRY1* may have a tumor suppressor function. Studies of RAS status in the human childhood tumor rhabdomyosarcoma (RMS) indicated mutations in approximately half of the tumors of the embryonal rhabdomyosarcoma subtype (ERMS) but not the alveolar subtype (ARMS). ERMS tumors also showed overexpression of *SPRY1*, which was indeed upregulated by mutant RAS. However, we found that, in the presence of mutant RAS, the function of SPRY1 was changed from an antagonist to an agonist of RAS signaling. Thus, SPRY1 supported formation of activated ERK and mitogen-activated protein/ERK kinase and was essential for ERMS cell proliferation and survival. Conversely, silencing of *SPRY1* in ERMS cells (but not ARMS cells) abolished their tumorigenicity in mice. Moreover, silencing of *SPRY1* caused regression of established ERMS tumors (but not ARMS tumors) formed in xenograft settings. Our findings argue that SPRY1 inhibition can offer a therapeutic strategy to treat childhood RMS and possibly other tumors carrying oncogenic *RAS* mutations. *Cancer Res; 70(2); 762–71. ©2010 AACR.*

Introduction

About one third of all human cancers are thought to carry a mutated *RAS* gene (1). Mutations are concentrated in codons 12, 13, and 61 of the three *RAS* family members (*HRAS, KRAS*, and *NRAS*) and result in activation of the RAS downstream pathway. Extensive research in this field has revealed much of the downstream signaling pathways that execute the programs initiated by mutant RAS, but therapeutic inhibition of the RAS pathway has not yet been successful (1, 2). This may indicate that much redundancy exists downstream of oncogenic RAS or vital targets have escaped our attention.

Sprouty proteins are among the downstream mediators of RAS signaling. The mammalian *SPRY* family has four members (*SPRY1, SPRY2, SPRY3*, and *SPRY4*), which differ in tissue distribution, activity, and interaction partners (3). Sprouty proteins antagonize RAS signaling during development and postnatal tissue growth and maintenance. *SPRY* expression is transcriptionally upregulated by increased extracellular signal-regulated kinase (ERK) activity, providing a negative feedback loop for RAS signaling (4). Decreased expression of *SPRY* genes in human cancer, including tumors from breast, prostate, and liver tissue, suggested a tumor suppressor function (5–7). Indeed, several studies showed that SPRYs, when overexpressed in cell lines, inhibit cell proliferation, migration, and anchorage-independent growth *in vitro* (8, 9) and limit tumor growth *in vivo* (10–12). The decreased *SPRY* expression in human cancer has been attributed to loss of heterozygosity or increased promoter hypermethylation (13, 14). However, *SPRY* genes are upregulated in some other cancers, suggesting alternative mechanisms at play. In some cases, gene mutation of SPRY targets may obliterate the tumor-suppressive effect of SPRY. For instance, SPRY2 has been shown to bind and inhibit wild-type, but not mutant, BRAF (15, 16). Consequently, melanoma cells, wild-type for BRAF, showed low SPRY2 levels, whereas melanoma cells with mutant BRAF had no decreased SPRY2 levels. Alternatively, phosphorylation of SPRY2 (i.e., by constitutive growth factor signaling) also prevents association with wild-type BRAF (15), allowing increased *SPRY* expression in these tumors. These latter studies describing elegant mechanisms of tumor escape further enforce the suggested tumor-suppressive role for SPRY proteins in diverse tissues.

Here, we have analyzed SPRY1 in rhabdomyosarcoma (RMS), a pediatric malignancy expressing markers of myogenic differentiation. We found that *SPRY1* levels were consistently higher in the embryonal subtype (ERMS) compared with the alveolar subtype (ARMS). We show that the

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elevated SPRY1 expression in ERMS is caused by oncogenic RAS mutations. They are frequent (~50%) in ERMS but absent in ARMS. We further show that in the presence of oncogenic RAS, the function of SPRY1 changed from an antagonist to an agonist of RAS/ERK signaling. In this context, SPRY1 is essential for proliferation and survival. RMS tumors grown in mice and harboring a RAS mutation completely regressed after SPRY1 silencing. Silencing SPRY1 in RMS tumors without RAS mutation had no effect.

Materials and Methods

Tumor specimen for microarray data and sequence analysis. Fresh tumor and normal skeletal muscle tissue were obtained and handled as previously described (17). The generation and processing of expression profile data is described elsewhere.[1] Informed consent was obtained from all volunteers, from each patient, or from the legal guardian of each patient.

Cell culture. RMS cell lines (see Supplementary Data) were cultured in DMEM containing 10% FCS (Life Technologies) supplemented with glutamine and penicillin/streptomycin (Life Technologies). Stably transfected cell lines were selected in medium containing blasticidin (200 μg/mL) and zeocin (2.5 μg/mL). Cells were cultured in a 5% CO_2 humidified atmosphere.

Sequence analysis. HRAS, KRAS, NRAS, BRAF, and SPRY1 were sequenced using BigDye Terminator v3.1 chemistry (Applied Biosystems). Sequencing was performed on an ABI 3100 capillary sequencer (Applied Biosystems). Forward and reverse sequences on electropherograms were analyzed using Codon Code aligner. Primer sequences and analysis of promoter methylation are described in Supplementary Data.

Generation of inducible cell lines. To generate RMS cells with doxycycline-inducible transgene expression, we used the Tet repressor system as previously described (18). Full-length human *SPRY1* cDNA was cloned into pCDNA4/TO/Myc-HisA (Invitrogen). *shSPRY1* oligonucleotides (Supplementary Table S1; Eurogentec Nederland b.v.) were cloned in the pTER vector followed by transfection into cells expressing the Tet repressor. To induce *SPRY1* or *shSPRY1* expression, 100 ng/mL doxycycline was added to the culture medium.

Transfection and transduction. For each transfection, 8 μg/dish of empty vector (pHAPAK1crib) or KRAS-G12D (KRASmt; based on NM_004985) were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. KRAS-G12D was cloned in *Bam*HI and *Pst*I of pHAPAK1crib. The lentiviral short hairpin RNA (shRNA) constructs (Supplementary Table S2) against *SPRY1* and *NRAS* were derived from the TRC library (Sigma-Aldrich Chemie BV). Replication-defective viral particles were produced in HEK293T cells as described (19).

Cell proliferation and viability assay. The modified NIH/3T3 proliferation protocol was described previously (18). Cell viability was determined using the MTT assay as previously described (20). The mitogen-activated protein/ERK kinase (MEK) inhibitor PD98059 was from Sigma.

Western blot analysis. Cells/tissues were processed for Western blot analysis as described (21). Primary antibodies raised against hemagglutinin (HA) were obtained from Santa Cruz Biotechnology; ERK1/2, phosphorylated ERK (P-ERK) 1/2 (Thr^{202}/Tyr^{204}), MEK, phosphorylated MEK (P-MEK; $Ser^{217/221}$), RAS, and poly(ADP-ribose) polymerase (PARP) antibodies were from Cell Signaling; and SPRY1 antibodies were from Abcam.

Xenograft experiments. RMS cells were harvested by trypsin treatment and counted. Cells were suspended at 1×10^6/20 μL in PBS supplemented with 10% mouse serum and injected in the tibialis anterior muscles of 4- to 8-wk-old nonobese diabetic/severe combined immunodeficient (NOD/SCID) male mice (Charles River). To silence *SPRY1*, animals were i.p. injected with doxycycline (40 μg/mL in PBS) and kept on 200 μg/mL doxycycline in drinking water, which was refreshed weekly. Control animals were injected with sterile PBS and received regular drinking water *ad libitum*. In total, eight pairs (treated and untreated) of animals were included in the experiments. Animals were screened regularly to follow tumor formation and sacrificed before tumor reached 2 cm^3.

Tumor formation was determined by measuring diameter (d) and tumor length (l). The average diameter of a healthy hind leg was 0.4 cm, so that the corrected tumor diameter (d_c) was d – 0.4. Tumor volume (V in mm^3) was calculated by $V = (d_c)^2 * (l/2)$ adapted from Jacob and colleagues (22).

To investigate protein expression in the xenografts, tumors were resected at indicated time points after adding doxycycline, snap frozen, and homogenized in radioimmunoprecipitation assay buffer (described above) using an Ultra-Turrax. Samples for gel electrophoresis were prepared as described above. All animal experiments were conducted under the institutional guidelines and according to the law and approved in DAG101025 by the local animal ethics committee.

Results

RAS mutations occur frequently in ERMS but are absent in ARMS. *RAS* genes are frequently mutated in RMS (6, 7), but it is unknown how these mutations are distributed over the ERMS and ARMS subtypes. We therefore sequenced *HRAS*, *KRAS*, and *NRAS* (codons 12, 13, and 61) and *BRAF* (codon 600) in 10 RMS cell lines and 23 primary RMS tumors. No *RAS* or *BRAF* mutations were found in 2 of 2 ARMS cell lines and in 10 of 10 tested primary ARMS samples (Table 1). In contrast, 3 of 8 (37.5%) ERMS cell lines and 6 of 13 (46%) primary ERMS samples harbored activating mutations in *HRAS*, *KRAS*, or *NRAS*. *RAS* mutations were therefore exclusively found in the ERMS subtype.

Elevated SPRY1 expression in ERMS is associated with hyperactive ERK signaling. We used gene expression profiles from ARMS and ERMS tumors (23) and cell lines to search for differential expression of RAS signaling–related genes and found that the levels of *SPRY1* and its homologues *SPRY2* and *SPRY4*, feedback inhibitors of ERK signaling (4, 24), were consistently higher in ERMS than in ARMS (Fig. 1*A*;

[1] G. Schaaf et al., in preparation.

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Table 1. ERMS-specific activating mutations in *HRAS, KRAS*, and *NRAS*

A

RMS cell line	RMS subtype	NRAS		KRAS		HRAS		BRAF	SPRY1	
		Mutation	Amino acid	Mutation	Amino acid	Mutation	Amino acid	Amino acid	Mutation	Amino acid
TE441.T	ERMS	wt		wt		wt		wt	wt	
TE617.T	ERMS	wt		wt		wt		wt	wt	
Hs.729.T	ERMS	wt		wt		wt		wt	wt	
RD	ERMS	CAA→CAT	Q61H	wt		wt		wt	wt	
T174	ERMS	CAA→CAT	Q61H	wt		wt		wt	wt	
TE381.T	ERMS	CAA→CAT	Q61H	wt		wt		wt	wt	
Ruch2	ERMS	wt		wt		wt		wt	wt	
Hs.926	ERMS	wt		wt		wt		wt	wt	
RH30	ARMS	wt		wt		wt		wt	wt	
RH4	ARMS	wt		wt		wt		wt	wt	

B

Tumor sample	RMS subtype	NRAS		KRAS		HRAS		BRAF	SPRY1	
		Mutation	Amino acid	Mutation	Amino acid	Mutation	Amino acid	Amino acid	Mutation	Amino acid
RMS1	ERMS	wt		wt		wt		wt	wt	
RMS2	ERMS	wt		wt		GGT→CGT	G13R	wt	wt	
RMS3	ERMS	wt		wt		wt		wt	wt	
RMS4	ERMS	wt		wt		wt		wt	wt	
RMS5	ERMS	wt		CAA→CTA	Q61L	wt		wt	wt	
RMS6	ERMS	wt		GGT→TGT	G12C	wt		wt	wt	
RMS7	ERMS	wt		GGT→GCT	G12A	wt		wt	wt	
RMS8	ERMS	GGT→GAT	G12D	wt		wt		wt	wt	
RMS9	ERMS	wt		wt		wt		wt	wt	
RMS10	ERMS	wt		wt		wt		wt	TAT→TGT	Y29C
RMS11	ERMS	wt		wt		wt		wt	wt	
RMS12	ERMS	GGT→GTT	G13V	wt		wt		wt	wt	
RMS13	ERMS	wt		wt		wt		wt	ND	
RMS14	ARMS	wt		wt		wt		wt	wt	
RMS15	ARMS	wt		wt		wt		wt	wt	
RMS16	ARMS	wt		wt		wt		wt	wt	
RMS17	ARMS	wt		wt		wt		wt	wt	
RMS18	ARMS	wt		wt		wt		wt	wt	
RMS19	ARMS	wt		wt		wt		wt	wt	
RMS20	ARMS	wt		wt		wt		wt	wt	
RMS21	ARMS	wt		wt		wt		wt	ND	
RMS22	ARMS	wt		wt		wt		wt	ND	
RMS23	ARMS	wt		wt		wt		wt	ND	

NOTE: Genomic DNA from a panel of 10 human RMS cell lines (A) and 23 archival human primary RMS samples (B) was sequenced for mutations in coding exons containing codons 12, 13, and 61 of *HRAS, KRAS*, and *NRAS* and codon 600 of *BRAF* and for mutations in exons 1B and 2 of *SPRY1*.

Abbreviations: wt, wild-type; ND, not determined.

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Supplementary Fig. S1). We decided to study the role of SPRY1 in RMS.

SPRY1 is known as a tumor suppressor gene, and mutational inactivation might allow ERMS cells to tolerate elevated levels of SPRY1. Sequencing of the coding region of *SPRY1* in 29 RMS cell lines and tumors identified a single mutation (Y29C) in only one ERMS sample (Table 1). The significance of this mutation is currently unknown and requires additional study, but this shows that mutational inactivation of SPRY1 in RMS is unlikely.

Western blot analyses detected SPRY1 protein in four of six ERMS cell lines but not or only at low levels in ARMS cell lines (Fig. 1*B*). Sequence analysis excluded promoter hypermethylation as a major cause of the low *SPRY1* levels in ARMS (Supplementary Fig. S2). Alternatively, the differential *SPRY1* expression could be explained by different levels of RAS/ERK signaling because it is known that SPRY proteins are induced by activated ERK (25). Therefore, we analyzed ERK phosphorylation in RMS cells. Figure 1*B* shows that elevated SPRY1 expression correlated with increased levels of P-ERK. To directly test the association between ERK activity and SPRY1 expression in RMS cells, we inhibited ERK activity in the ERMS cell line RD with the MEK inhibitor PD98059. Consequently, phosphorylation of ERK was abrogated and SPRY1 levels were reduced (Fig. 1*C*). These results suggest that elevated SPRY1 levels in ERMS are caused by activated ERK signaling. Interestingly, three of four ERMS cell lines (indicated with an asterisk in Fig. 1*B*) with elevated P-ERK and SPRY1 levels contain a mutated *RAS* gene (Table 1). In the fourth ERMS cell line with high P-ERK and SPRY1 levels, TE617T, we could not detect *RAS* or *BRAF* mutations.

***SPRY1* levels are increased by oncogenic RAS.** To further substantiate the correlation between mutant RAS, ERK signaling, and SPRY1 expression, we transiently transfected the ERMS cell line RUCH2 (wild-type for all three *RAS* genes and low P-ERK and SPRY1 levels) with a construct expressing constitutively active KRAS (KRASmt). Mutant KRAS not only significantly increased P-ERK levels but also increased SPRY1 levels (Fig. 2*A*).

Inversely, we applied lentivirally mediated shRNA to silence the mutated *NRAS* gene in the ERMS cell line RD. As a control, wild-type *NRAS* was also silenced in the ARMS cell line RH30. Two of the five shRNA constructs were very efficient in silencing *NRAS* expression (A3 and A4; Fig. 2*B*). In RD cells, this resulted in reduced P-ERK and SPRY1 levels. In RH30, there was no correlation between *NRAS* knockdown and SPRY1/P-ERK levels (Fig. 2*B*).

Furthermore, we used panRAS oligonucleotides to knock down all three members of the *RAS* family (*HRAS, KRAS*, and *NRAS*). Transfection of these oligonucleotides into RD cells resulted in potent *NRAS* knockdown and concomitant

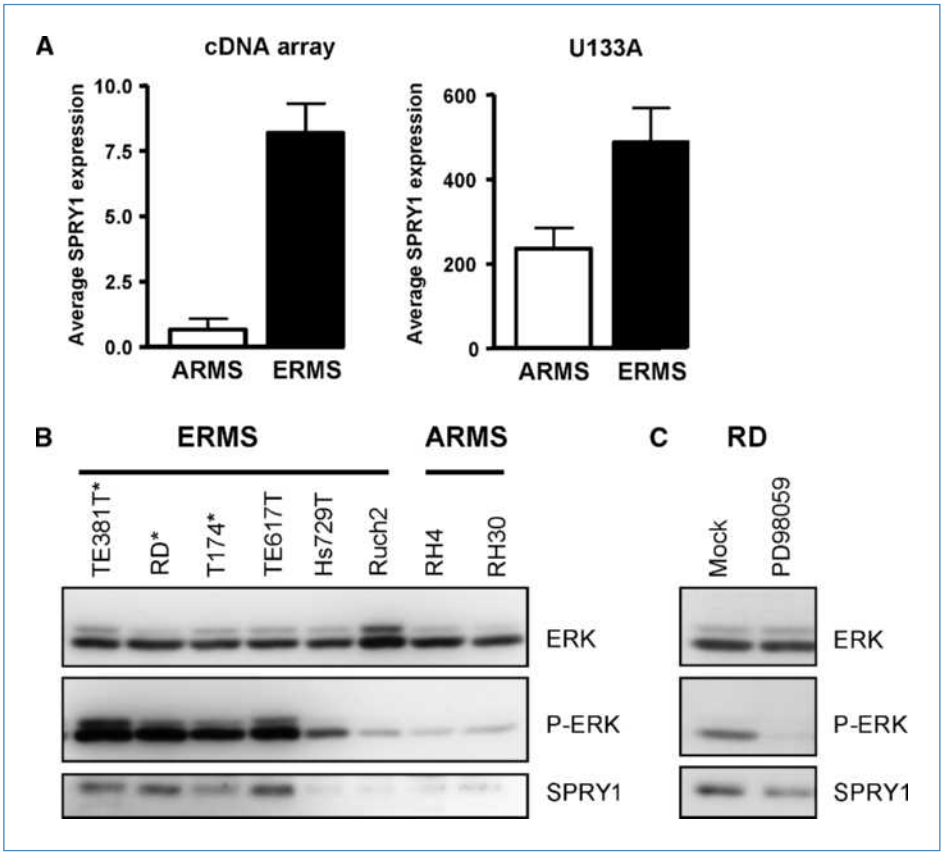

Figure 1. *SPRY1* is differentially expressed in RMS and is associated with hyperactive ERK signaling. *A, SPRY1* is differentially expressed between ARMS and ERMS samples as determined by a custom cDNA microarray analysis of 6 ARMS and 19 ERMS samples and Affymetrix U133A analysis of 15 ERMS, 10 translocation-positive ARMS samples, and 4 translocation-negative ARMS samples. The U133A Affymetrix microarray data set was generated by Wachtel and colleagues (23). *B,* Western blot analysis of P-ERK status in proliferating RMS cells. Eight RMS cell lines were harvested during logarithmic growth. Total cell lysates were analyzed. *Asterisk,* cell lines with a sequence-verified RAS mutation. Total ERK was used to show equal loading. *C,* Western blot analysis of ERK phosphorylation and SPRY1 expression in MEK inhibitor–treated RMS cells. Parental RD cells were incubated with 50 μmol/L PD98059 for 24 h to inhibit ERK phosphorylation. Total cell lysates were analyzed.

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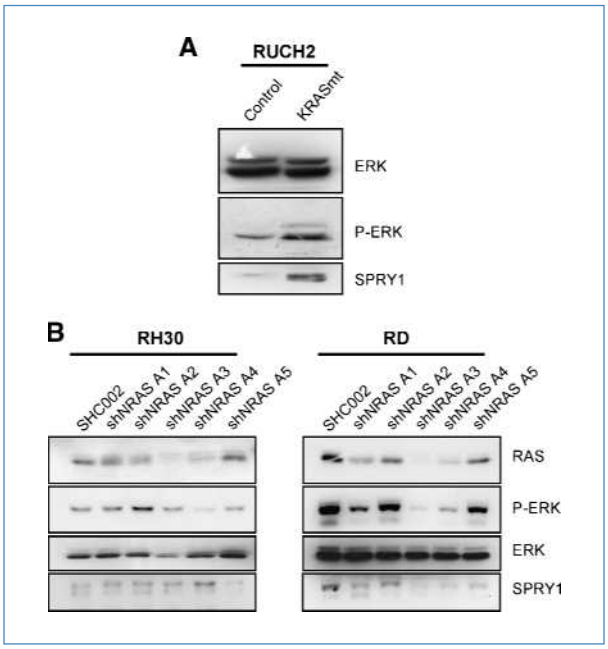

Figure 2. Oncogenic RAS induces SPRY1 expression. *A,* Western blot analysis of SPRY1 and P-ERK levels. RUCH2 (wild-type for RAS) ERMS cells were transfected with empty vector (*Control*) or KRAS-G12D (*KRASmt*) and harvested for Western blot analysis 48 h later. Total cell lysates were analyzed. *B,* Western blot analysis of RAS, P-ERK, and SPRY1 levels. RD and RH30 cells were transduced with a control lentivirus (*SHC002*) or with lentiviruses containing five different shRNAs against *NRAS*. Cell lysates were analyzed 72 h later by Western blotting.

decrease of *SPRY1* mRNA levels (Supplementary Fig. S3). Transfection into RH30 cells also reduced *NRAS* levels, but the low basal *SPRY1* levels did not change. In summary, oncogenic RAS induces ERK activity and SPRY1 expression levels in ERMS cells.

SPRY1 expression is a RAS/ERK agonist in ERMS cells. Literature data have dubbed *SPRY* genes as tumor suppressor genes (4). We therefore asked which role SPRY1 exerts in RMS cells. We silenced endogenous *SPRY1* expression in both RD and RH30 cells using four different lentivirally mediated shRNAs. In RH30 (ARMS cell line), silencing of *SPRY1* had no effect on MEK or ERK phosphorylation (Fig. 3*A*). In contrast, in RD (ERMS cell line), *SPRY1* knockdown resulted in an attenuation of both MEK and ERK phosphorylation (Fig. 3*A*). Similar results were found when *SPRY1* was silenced in TE381T cells (Fig. 3*B*), another ERMS cell line harboring oncogenic RAS (Table 1). In ERMS cells with an activated *RAS* gene, SPRY1 expression is apparently required to maintain high P-MEK and P-ERK levels. These data were confirmed when *SPRY1* was upregulated in the RD and RH30 cell lines. Doxycycline-induced overexpression of ectopic *SPRY1* resulted in decreased ERK phosphorylation in RH30 cells, but it strongly increased ERK phosphorylation in RD cells (Fig. 3*C*). The latter effect was attenuated by preincubation with the MEK inhibitor PD98059, indicating that SPRY1 acts upstream of MEK. The potent effects of *SPRY1* silencing on P-MEK levels (Fig. 3*A*) substantiate this conclusion. These data fully contradict a tumor suppressor role of SPRY1 in ERMS cells. To test whether the two RMS cell lines depend on RAS signaling, we have measured cell viability after incubation with increasing concentrations PD98059. RD cells seemed to be more sensitive to this MEK inhibitor than RH30 cells (Fig. 3*D*), showing that in RD ERMS cells RAS signaling is not only activated but the cells also depend on it.

***SPRY1 is required for proliferation and survival of ERMS cells harboring oncogenic* RAS.** To further investigate the role of SPRY1 in RMS cells *in vitro* and *in vivo*, we constructed RD and RH30 cell clones (two clones per cell line) expressing a doxycycline-inducible shRNA specific for *SPRY1* (RD-shSPRY1 and RH30-shSPRY1). The induction of shRNA resulted in both RD and RH30 cells in a strongly reduced SPRY1 expression (Fig. 4*A*). Strikingly, *SPRY1* knockdown *in vitro* resulted in a virtually complete growth arrest in RD cells but only slightly reduced proliferation in RH30 cells (Fig. 4*B* and *C*). Also in TE381T cells, another ERMS cell line harboring oncogenic RAS (Table 1), *SPRY1* silencing completely inhibited cell proliferation (Fig. 4*C*). However, silencing *SPRY1* in ERMS RUCH2 cells (wild-type RAS) did not affect cell proliferation (Fig. 4*C*). The same results for all these cell lines were also found with a second doxycycline-inducible *SPRY1* shRNA construct (Supplementary Fig. S4). Moreover, *SPRY1* silencing by the four different lentivirally mediated shRNAs used in Fig. 3 also resulted in a strongly decreased viability in ERMS cell lines harboring oncogenic RAS (RD and TE381T) but not in ARMS or ERMS cells without oncogenic RAS (RH30 and Hs729T) or in VH10 primary human fibroblasts (Supplementary Fig. S5).

Fluorescence-activated cell sorting analyses showed that *SPRY1* silencing in RD cells, but not in RH30 cells, resulted in a strong increase in the sub-G_1 fraction, suggesting apoptotic cell death (Supplementary Fig. S4*D*). To show that the shSPRY1-mediated loss of cell survival was indeed due to SPRY1 depletion and not caused by off-target effects, we used our doxycycline-inducible *SPRY1* RD cell line (see Fig. 3*C*). Silencing of *SPRY1* in these cells by three independent lentiviral shRNAs resulted in a strong inhibition of cell proliferation, which could in all three cases be rescued by the concomitant doxycycline-induced expression of ectopic *SPRY1* (Fig. 4*D*; Supplementary Fig. S6). Finally, to show that this SPRY1-specific effect on survival is indeed mediated at the level of or upstream from MEK, we transiently expressed constitutively active MEK1 (CA-MEK) in RD and RH30 cells before *SPRY1* knockdown. In RD cells, CA-MEK partially rescued the SPRY1-dependent loss of cell survival (Supplementary Fig. S7*A*). In contrast, in RH30 cells, *SPRY1* knockdown did not affect cell survival and no effect of ectopic CA-MEK expression was observed (data not shown). Western blot analysis showed that CA-MEK expression prevented attenuation of P-MEK following silencing of *SPRY1*, indicating that the effects of SPRY1 in RD ERMS cells are (partially) MEK dependent (Supplementary Fig. S7*B*). All these results show that RAS-mutated RMS cells not only strongly depend on MEK1 activity but that, in the context of oncogenic RAS, these cells require SPRY1 for proliferation and survival.

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SPRY1 is essential for RMS formation and maintenance in vivo. Next, we analyzed whether SPRY1 was also necessary for *in vivo* tumorigenicity of the RD and RH30 tumor cell lines. We grafted the RH30-shSPRY1 and RD-shSPRY1 cells orthotopically in the tibialis anterior muscle of the hind leg of immunodeficient (NOD/SCID) mice. Each mouse received the RH30 cells and RD grafts in opposite legs. Xenografts formed in all control animals (n = 5, not treated with doxycycline) both at the RH30-shSPRY1 and at the RD-shSPRY1 injection sites (Fig. 5*A*). Both cell lines formed large tumors of small round blue cells within 6 weeks (Fig. 5*A* and *B*). A second group of mice was treated with doxycycline 1 week after grafting to silence *SPRY1* expression in the xenografts. In the doxycycline-treated mice, RH30-shSPRY1 still formed tumors that were only slightly retarded in their growth (note that tumors in control mice were harvested 1 week before the tumors in the doxycycline-treated mice). Strikingly, RD-shSPRY1 cells completely failed to form tumors when mice were treated with doxycycline to silence *SPRY1* (Fig. 5*A*). Histologic analysis of the xenografts from the doxycycline-treated animals showed a small patch of cells remaining at the site of injection of RD-shSPRY1 cells, surrounded by otherwise normal skeletal muscle (Fig. 5*B, right, bottom row*).

As human tumors are, at diagnosis, usually well established, treatment is more difficult. We therefore also analyzed the effect of *SPRY1* silencing in established tumors. We injected RH30-shSPRY1 and RD-shSPRY1 as described above. However, in these experiments, doxycycline treatment was not initiated until clearly palpable tumors were formed (2–4 weeks after injection; note that RH30 tumors have a slightly slower take). Addition of doxycycline to the drinking water did not affect the growth kinetics of RH30 tumors (Fig. 5*C*). However, already a few days after adding doxycycline, RD tumors stopped growing and eventually completely regressed (Fig. 5*C*). The effect was observed in all five animals in the doxycycline group but not in any of the five control animals.

To verify *in vivo* the effects of *SPRY1* silencing, we repeated the xenograft experiment with RH30-shSPRY1 and RD-shSPRY1 cells and resected tumors before complete regression. At 8 and 12 days of treatment with doxycycline, RD tumors were regressing but were still at ~85% and ~50% of their maximal tumor size, respectively. Western blot analyses

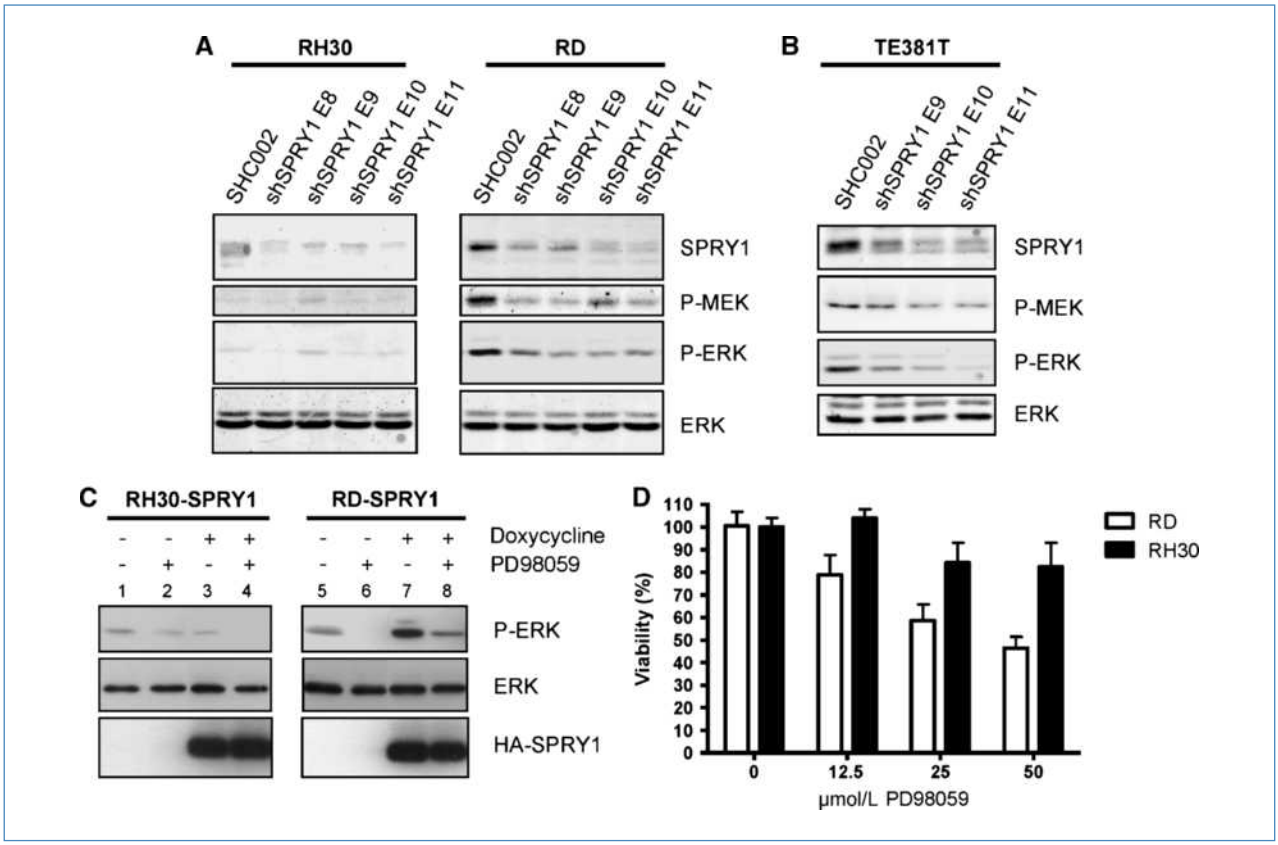

Figure 3. SPRY1 is a RAS/ERK agonist in ERMS cells. *A,* Western blot analysis of parental RH30 and RD cells transduced with four different lentiviral shRNAs against *SPRY1* or control virus (*SHC002*). In all blots, total ERK is shown as loading control. *B,* Western blot analysis of parental TE381T cells transduced with three different lentiviral shRNAs against *SPRY1* or control virus (*SHC002*). Total cell lysates were analyzed. *C,* Western blot analysis of ERK phosphorylation in RMS cells overexpressing HA-tagged SPRY1. RH30-SPRY1 and RD-SPRY1 cells were cultured for 3 d with or without doxycycline before replating the cells. One day later, cells were treated (24 h) in the absence/presence of MEK1/2 inhibitor PD98059 (50 µmol/L) before Western blot analysis. *D,* cell viability assay of RMS cells treated with PD98059. RH30 (*black columns*) and RD (*white columns*) cells were treated with indicated concentrations of PD98059 and viability was determined 72 h later.

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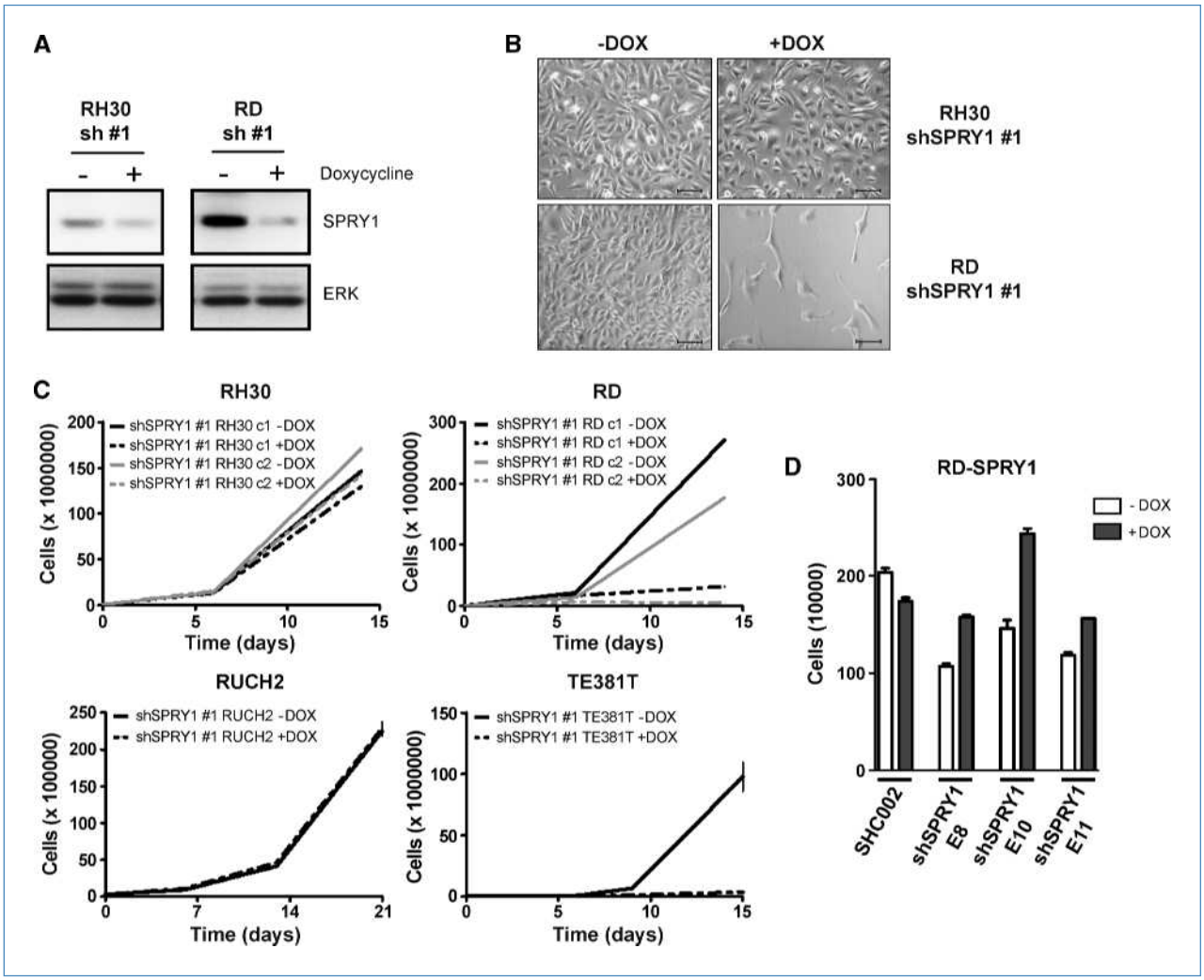

Figure 4. Silencing *SPRY1* induces a strong growth arrest and increased cell death in ERMS cells. *A,* Western blot analysis of *SPRY1* silencing following the doxycycline-inducible shSPRY1 expression. RH30-shSPRY1 and RD-shSPRY1 cells were treated with doxycycline for 3 d and prepared for Western blotting. Total ERK is shown as loading control. Two different *SPRY1* shRNA targets per cell line were used (see also Supplementary Fig. S4). *B,* phase-contrast photographs of RMS cells following *SPRY1* knockdown. RH30-shSPRY1 and RD-shSPRY1 cells were treated with or without doxycycline (*DOX*) for 3 d before replating the clones. Cells were grown for another 72 h in the presence of doxycycline. Photographs show representative wells. Experiment was performed at least twice with similar results. *C,* effect of shSPRY1 expression on the proliferation of RH30, RD, RUCH2, and TE381T cells. Cells were treated with doxycycline (*dotted lines*) to induce ectopic shSPRY1 as compared with nontreated cells (*solid lines*). For RD and RH30, two clones for each *SPRY1* target are shown (see also Supplementary Fig. S4). For RUCH2 and TE381T, the whole population of positive transfectants is shown for the two *SPRY1* targets (see also Supplementary Fig. S4). *D,* induced growth inhibition by silencing *SPRY1* in RD cells is rescued by overexpression of ectopic *SPRY1*. RD-SPRY1 cells (see Fig. 3*C*) were treated with doxycycline to induce ectopic HA-SPRY1; concomitantly, the cells were transduced with three different lentiviral shRNAs against *SPRY1* or control virus (*SHC002*). The data presented are for day 9 after treatment.

clearly showed that adding doxycycline indeed induced *SPRY1* silencing both in RH30 and in RD tumors. However, only in RD tumors, *SPRY1* silencing resulted in reduced P-MEK and P-ERK levels, which is in line with the *in vitro* experiments (Fig. 3*A*). The decrease of P-ERK was attenuated at the 12-day time point possibly due to apoptotic cells (26). Indeed, PARP levels were strongly reduced in the RD tumors, suggesting massive apoptosis (Fig. 5*D*). This was not observed for the RH30 tumors.

In conclusion, our results show that *SPRY1* expression in RD ERMS cells, which is increased by oncogenic RAS signaling, is required not only *in vitro* for cell proliferation and survival but also *in vivo* for formation and maintenance of the tumors.

Discussion

Data presented in this article show that endogenous levels of SPRY1 seem to protect oncogenic RAS-positive ERMS cells from the induction of cell death. Silencing *SPRY1* in these cells irreversibly and very potently inhibited cell proliferation and survival both *in vitro* and *in vivo*. Even well-established tumors grown in mice completely regressed when we

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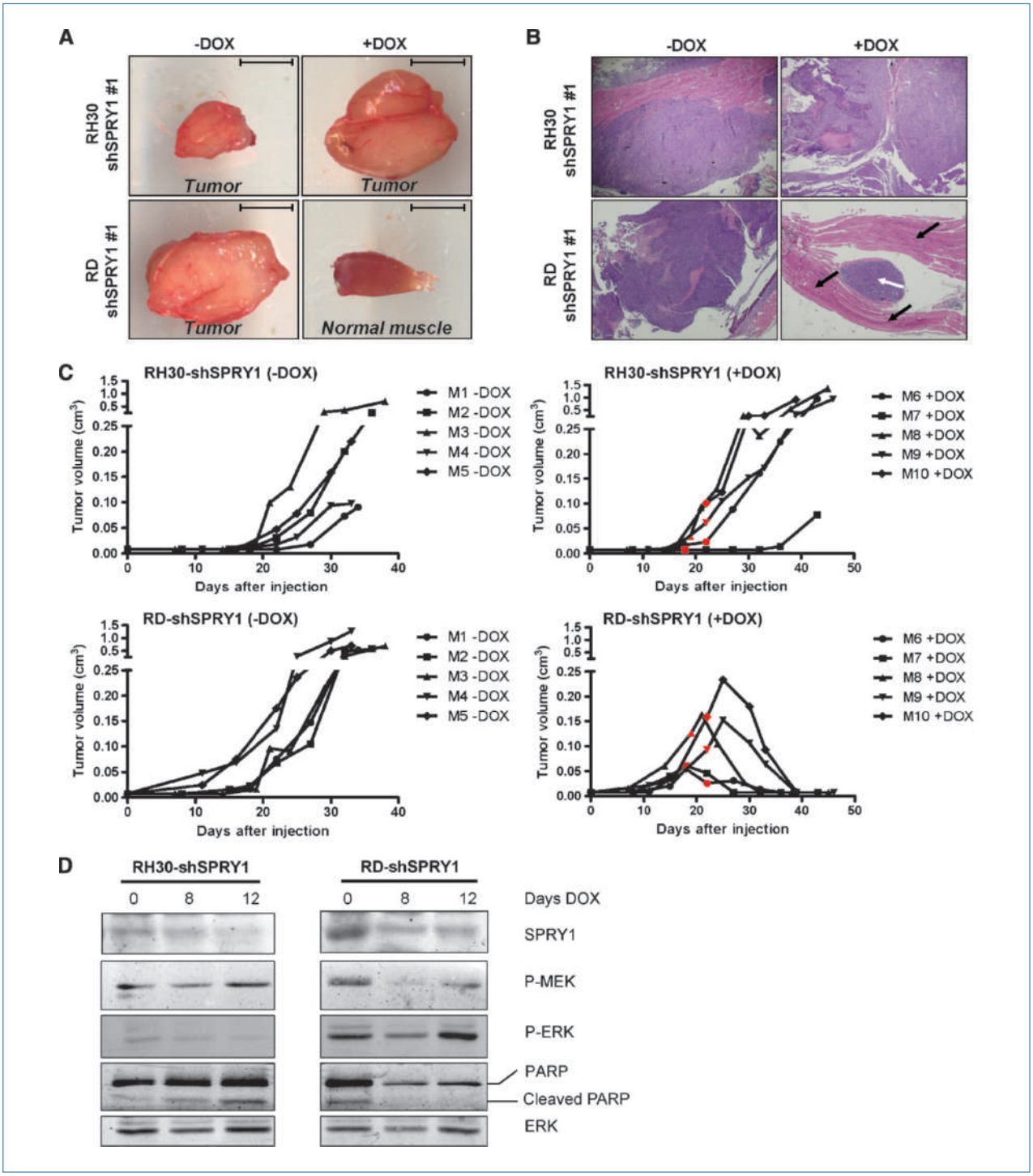

Figure 5. SPRY1 is essential for growth and maintenance of RD-ERMS xenografts. *A,* photographs of resected xenografts. RD-shSPRY1 and RH30-shSPRY1 cells were injected orthotopically in immunodeficient NOD/SCID mice. Tumors were harvested after 5 or 6 wk in control mice (*–DOX*) or doxycycline-treated mice (*+DOX*), respectively. In total, five mice per group (control and doxycycline-treated) were tested with similar results. Representative picture from one of the experiments. *B,* H&E stain of the paraffin-embedded tumors shown in *A*. *White arrow,* tumor area; *black arrows,* normal healthy muscle. *C,* growth curves from RH30-shSPRY1 (*top*) and RD-shSPRY1 (*bottom*) xenografts generated as described above. Note that each mouse received RH30-shSPRY1 and RD-shSPRY1 xenografts to allow comparison between the cell types. The figures depict tumor formation in time. After palpable tumors were formed, mice were treated with doxycycline (*red symbols* in graph) or PBS (control or *–DOX*). *D,* Western blot analysis of RH30-shSPRY1 and RD-shSPRY1 xenografts generated as described above. Tumors were resected and prepared for Western blot analysis at 8 and 12 d after doxycycline treatment. Total ERK is shown as loading control.

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silenced *SPRY1* expression. Silencing *SPRY1* in ERMS or ARMS cells or primary fibroblasts without RAS mutations had no or only minimal effects on proliferation.

SPRY proteins are transcriptionally upregulated by the activated RAS pathway, as we have also shown in this article. Most studies reporting on the function of SPRY proteins in normal and diseased tissue show an antagonistic role for SPRY proteins, including SPRY1 (27), in the RAS/ERK pathway. However, in a recent study, Pratilas and colleagues (28) showed that, in tumor cells with mutated BRAF, this negative feedback loop is disabled. Although the expression of SPRY or other RAS pathway feedback inhibitors, such as DUSP proteins, was elevated, they were no longer able to inhibit the RAS pathway. Brady and colleagues (15) showed that differences in, for instance, SPRY2 phosphorylation may play a role in this. However, it is unlikely that this is the case for SPRY1 in ERMS, as we did not detect any mobility shifts of SPRY1 compared with ARMS or other cell lines. Moreover, our data presented here show that SPRY1 in an oncogenic RAS background in fact supports ERK signaling and is even essential for proliferation and survival of cells. SPRY1 overexpression in RD ERMS cells harboring oncogenic NRAS increased P-ERK levels, whereas silencing *SPRY1* in these cells decreased P-ERK levels (Fig. 3). In RMS cells without RAS mutations, SPRY1 still antagonized RAS/ERK signaling.

Activating RAS mutations occur frequently in RMS, but we show that they are only present in ERMS but not in ARMS tumors. About a third of all human cancers are thought to carry a mutated *RAS* gene (29). Elevated levels of *SPRY1* or *SPRY2* have indeed also been reported in non–small cell lung cancer and melanoma (12, 30), tumors that frequently harbor *RAS* or *BRAF* mutations. In line with our observations, Sutterlüty and colleagues (12) showed that induction of SPRY2 was also unable to antagonize RAS/ERK signaling in non–small cell lung cancer cells expressing oncogenic *RAS*. Whether the other *SPRY* homologues, which we also found to be upregulated in ERMS versus ARMS tumors (Supplementary Fig. S1), behave similarly as SPRY1 remains to be investigated. Recent data from Lito and colleagues (31, 32) showed that SPRY2 expression was required for xenograft formation of HRAS-V12–transformed fibroblasts. However, silencing *SPRY2* in fibrosarcoma cells expressing oncogenic *NRAS* only slightly inhibited tumor formation (32). As SPRY2 was silenced in those experiments by stable expression of shRNAs in the cultured cell lines, selection for resistance to *SPRY2* silencing might have played a role in those results. In our study, we used doxycycline-induced silencing, avoiding selection of resistant clones. The use of doxycycline-inducible shRNA allowed us to study the role of SPRY1 even in established xenografts from RMS cell lines.

The striking results we obtained by silencing *SPRY1 in vivo* in ERMS cells harboring oncogenic RAS (i.e., complete tumor regression) strongly suggest that a significant population of ERMS patients (~50% of ERMS patients or ~40% of all RMS patients) might benefit from therapeutic approaches (small-molecule inhibitors, antibodies) that specifically target either SPRY1 or other members of the RAS/ERK pathway, particularly MEK. The study by Pratilas and colleagues (28) showed that tumor cells with mutational activation of the RAS pathway were sensitive to MEK inhibitors, whereas cells activated by upstream receptor tyrosine kinases were insensitive. We show by multiple approaches that in ERMS cells with oncogenic RAS, SPRY1 supports survival at the level of MEK. Moreover, we show that RD ERMS cells were indeed also sensitive for the growth-inhibiting effects of the MEK1/2 inhibitor PD98059, whereas the RH30 ARMS cells were not. However, clinical use of PD98059 is limited due to its toxicity. Other MEK inhibitors have now been developed that show inhibition of tumor growth of, for example, thyroid xenografts harboring mutant RAF or RAS (33, 34). Clinical trials with these inhibitors or similar agents are ongoing (35, 36). Until now, SPRY1 has not been reported to antagonize oncogenic RAS function. The tumor-supporting function described in this study may not be restricted to RMS but may represent a common event in human cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Silencing of SPRY1 Triggers Complete Regression of Rhabdomyosarcoma Tumors Carrying a Mutated *RAS* Gene

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