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# *SMARCA4* Biology in Alveolar Rhabdomyosarcoma

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## Abstract

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and phenocopies a muscle precursor that fails to undergo terminal differentiation. The alveolar subtype (ARMS) has the poorest prognosis and represents the greatest unmet medical need for RMS. Emerging evidence supports the role of epigenetic dysregulation in RMS. Here we show that SMARCA4/BRG1, an ATP-dependent chromatin remodeling enzyme of the SWI/SNF complex, is prominently expressed in primary tumors from ARMS patients and cell cultures. Our validation

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AUTHOR CONTRIBUTIONS

NB, JD, WK, SB, AM, DS and CK designed the study; NB, JD, WK, EW, MER, TS, DS, KC and ANK performed experiments; NB, JD, WK, CK, DS, JD, CV and KN analyzed and interpreted data; NB and CK wrote the manuscript; CK directed studies.

CONFLICTS OF INTEREST

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studies for a CRISPR screen of 400 epigenetic targets identified SMARCA4 as a unique factor for long-term (but not short-term) tumor cell survival in ARMS. A SMARCA4/SMARCA2 protein degrader (ACBI-1) demonstrated similar long-term tumor cell dependence *in vitro* and *in vivo*. These results credential SMARCA4 as a tumor cell dependency factor and a therapeutic target in ARMS.

**Keywords**

epigenetics; SWI/SNF; chromatin remodeling complex; SMARCA4; SMARCA2; BRG1; BRM; rhabdomyosarcoma

## INTRODUCTION

Rhabdomyosarcoma (RMS) is a malignant tumor of mesenchymal origin phenocopying muscle precursor cells from as evidenced by gene expression and histological features of myogenic differentiation [1, 26]. RMS is the most common soft tissue sarcoma in childhood, accounting for 5–10% of all pediatric malignancies, with the embryonal subtype (ERMS) accounting for approximately half of cases [10]. However, the alveolar subtype of this tumor (ARMS) is known to have the poorest prognosis and greatest unmet medical need among rhabdomyosarcomas, attributed to a unique biology driven by the pathognomonic fusion genes PAX3:FOXO1 or PAX7:FOXO1 [33]. The 8% five-year progression-free survival for metastatic ARMS is dismal and has been unchanged for the last 49 years despite improvements in surgical technique, radiation delivery, and chemotherapy intensification [7, 19, 27, 34].

SWI/SNF is an evolutionarily conserved, multi-subunit ATP-dependent chromatin remodeling complex involved in regulation of gene transcription. At least three SWI/SNF complexes exist: BAF, PBAF and non-canonical ncBAF [23]. The unique feature of this family is the presence of a bromodomain motif in the ATPase subunit (either brahma (BRM) or brahma-related G1 (BRG1)) which facilitates recognition and binding with acetylated lysine residues within histone N-terminal tails *in vitro* [9]. Previous findings have shown that the interaction between BRG1, BRM and acetylated histones accounts for the stable binding of SWI/SNF to hyperacetylated chromatin within the regulatory regions of muscle genes [2]. SWI/SNF facilitates muscle-specific gene expression by altering chromatin structure at the regulatory regions of both early and late myogenic genes [2]. For example, Myogenin specifies muscle phenotype by cooperating with MEF2D to recruit SMARCA4 thereby altering chromatin structure at regulatory sequences and promote terminal differentiation [24].

Increasing evidence points to the role of the subunit of SWI/SNF complex in the initiation and progression of tumorigenesis. Typically, SWI/SNF function is abrogated in malignancies placing SMARCA4 in the role of a tumor suppressor [21]. SMARCA4 is shown to be mutated in 15% of Burkitt's lymphoma, 10–35% of non-small-cell-lung carcinoma, 5–10% of medulloblastoma and melanoma [13, 15, 18, 22, 25, 28].

In this study, we show that wildtype ATP-dependent chromatin remodeling enzyme SMARCA4 is more highly expressed in ARMS tumors than related protein SMARCA2,

a mutually exclusive catalytic subunit. Interestingly, SMARCA4 is virtually never mutated or deleted in ARMS [29], but SMARCA4 expression is requisite for PAX3:FOXO1 expression and thus chemotherapy resistance in ARMS, giving SMARCA4 an unusual oncogenic rather than tumor-suppressive role in ARMS [5]. Functionally, SMARCA4 is non-obligatory in the short-term but essential for the long-term ARMS tumor cell survival.

## RESULTS

### CRISPR screening identifies SMARCA4 as vital to ARMS survival

A pooled CRISPR screen of epigenetic genes necessary for tumor cell survival in murine RMS cell cultures yielded multiple gene candidates of interest (Figure 1). Sequencing data was used for quantification of CRISPR construct ability to impair cell viability was then compared for these selected genes between U66788 ARMS, U37125 ERMS, and 3T3 fibroblast cell lines (Figure 1A-C). Notably, *SMARCA4* was required for cell survival of U66788 (ARMS) more so than for U37125 (ERMS) or 3T3 fibroblasts (Figure 1A-B), whereas the other gene candidates impaired cell viability across all three cell cultures (Figure 1A-C). BRD4 was also a neighboring ARMS-specific screen hit (not labeled), but BRD4 has already been carefully addressed by in the literature [12]. In validation studies, depletion of *SMARCA4* significantly impaired cell survival in human ARMS cell lines Rh30 and Rh4 (Figure S1). These results draw attention to *SMARCA4* as a gene of importance in the context of ARMS. For mouse cell lines, CRISPR constructs against the ATPase and bromodomains of SMARCA4 both impaired cell viability. For human cell lines, CRISPR constructs also implicated DEXD domain and helicase domain loss for SMARCA4 as impairing cell viability.

### SMARCA4 expression is elevated in alveolar and embryonal rhabdomyosarcoma tumors

Thirty-nine archival ARMS tumor samples were analyzed for SMARCA2 and SMARCA4 expression using tissue microarray (TMA). Each usable sample represented in the TMA was given a score for the level of expression: 0 for no expression, 1 for positive expression that was weak or variable, and 2 for positive, strong, and uniform expression. SMARCA4 showed positive, strong, uniform expression in 46% of usable samples unlike SMARCA2 that showed 0% for stronger, uniform expression (Table 1). A representative IHC image and expression summary are shown for SMARCA2 and SMARCA4 expression of ARMS with scores of 1 and 2 in Figures 2A-B. Small cell carcinoma of the ovary, hypercalcemic type (SCCOHT) was used as a negative control since 90% of SCCOHT harbors inactive mutations in SMARCA4 [16, 36].

In addition to ARMS, 39 archival ERMS tumor samples were analyzed for SMARCA2 and SMARCA4 expression using the TMA with SMARCA2 showing positive, strong, uniform expression in 54% of usable samples, whereas SMARCA4 exhibited positive, strong, uniform expression in 88% of usable samples (Table 1). A representative IHC image and expression summary are shown for SMARCA2 and SMARCA4 expression of ERMS with scores of 1 and 2 in Figures 2A-B.

Consistent with the higher expression of SMARCA4 in ARMS tumors, SMARCA4 protein expression was present at comparable or higher levels in murine and human ARMS cell lines or primary cultures (Rh30, CF-1, U23674, U66788 and U21494) compared to SMARCA2 expression (Figure 2B-C). In some mouse and human ERMS cell lines (U37125, U57810 and RD), SMARCA4 protein expression was higher than SMARCA2 expression (Figures 2B-C).

### ARMS prefer BAF canonical complexes

To narrow our focus of SWI/SNF complex type preference, we surveyed expression of BAF (ARID1A, SS-18), PBAF (BRD9) and ncBAF (PBRM) components in human ARMS and ERMS cell lines. Each component was present in all cell line except RD, which lacked BRD9 (Figure 2E). To determine which SWI-SNF complex contained SMARCA4, Rh30 was used as a representative human ARMS cell line. Pulldown of SMARCA4 by immunoprecipitation was associated with expression of the BAF complex component ARID1A, but not PBRM or BRD9 (Figure 2F-G).

### Loss of SMARCA4 expression caused modest impact on the viability of PAX3:FOXO1+ cells in the short term, but consistent effect in the long term.

Short-term (72hr) siRNA knockdown of SMARCA4 in human PAX3:FOXO1+ ARMS cell lines impaired cell viability to a variable degree (25–55%; Figures 3A-D). Results were comparable for a murine ARMS cell culture using a lentiviral-mediated stable, doxycycline-inducible shRNA system (Figure 3E). In complementary studies, SMARCA4 knockdown impaired cell migration in human or murine ARMS cell lines by 25–50% (Figure S2 A-C).

Given that these short-term cell viability results were comparatively lower in magnitude than the CRISPR cell viability screen, a long-term depletion assay, we evaluated long-term consequences of SMARCA4 in other long term validation experiments. In human ARMS cell lines, SMARCA4 knockdown by siRNA impaired anchorage-independent colony formation at 21 days by 30–70% (Figures 4A-D) as well as qualitative anchorage-dependent colony formation at 15 days (Figure 4E). Results were comparable for anchorage-independent and -dependent tumor growth for murine primary cell cultures harboring the inducible Smarca4 shRNA system (Supplemental Figure S3 A-B). These results are consistent with the CRISPR validation studies in human RMS cell lines (Figure S1) showing a clear, stepwise decrease in tumor cell viability from day 3 to day 18.

In an *in vivo* murine ARMS orthotopic allograft model of doxycycline dependent Smarca4 knockdown, Smarca4 knockdown delayed tumor growth (Figure 4F). The animals whose tumor had the greatest Smarca4 knockdown at the end of the study were also the tumors with the greatest progression delay (Figure 4G), further strengthening the hypothesis that SmarcA4 is a long term cell viability factor.

### Dual loss of SMARCA4 and SMARCA2 impacts tumor cell growth in PAX3:FOXO1+ ARMS

To translate our findings of long-term impaired cell viability of ARMS towards the clinic, we explored small molecule therapeutic approaches. Using the SMARCA4 bromodomain inhibitor PFI-3, short-term nor extended term, cell viability of human or murine ARMS

cultures was not impaired (Figure S4 A-D). We next turned to using the dual SMARCA4/SMARCA2 protein degrader ACBI-1. SMARCA4 and SMARCA2 protein expression was analyzed with ACBI-1 treatment. We observed decreased expression of both SMARCA4 and SMARCA2 (Figure 5A). We also assessed alteration in expression of PBRM1 because ACBI-1 potentially targets PBRM1 in addition to SMARCA4 and SMARCA2. PRBM1 expression was reduced by ACBI-1 notably at the higher concentration of 200 nM albeit with lower potency compared to the effect on SMARCA4 (Figure 5B). Cell viability was then analyzed with ACBI-1 treatment with no impaired cell viability at 3 days, but cell viability was increasingly impaired after 8 and 11 days of treatment in human Rh30 cells (Figures 5C).

To analyze whether the effect seen on cell viability with ACBI-1 treatment is due to degradation of SMARCA4 (and not SMARCA2 or PBRM1), we performed a rescue experiment over 5 days in Rh30 cells transiently transfected to rescue the expression of PBRM1 and SMARCA2 lost with ACBI-1treatment. No effect was seen on the cell viability with rescue of PBRM1 and SMARCA2 expression (Figure S6A-D), but the desired long-term rescue could not be performed with these transient transfection tools. Fortunately, however, this 5-day experiment coincides with results of 3-day studies (Figure 5C), showing no impairment of short-term cell viability with SMARCA4 degradation.

In an *in vivo* murine ARMS orthotopic allograft model, ACBI-1 delayed tumor formation and growth (Figure 5D) (Table S1).

### Higher expression of SMARCA4 correlates with increased patient survival

To correlate the results of our screening and validation studies in experimental model systems to human patients, we investigated *SMARCA4* expression at the time of diagnosis in relation to cell survival using a published microarray dataset [6]. Unexpectedly, high expression of *SMARCA4* was found to be correlated with an increased probability of RMS patient survival over time, whereas high expression of *SMARCA2* was associated with decreased survival probability (Figure S5). This observation remained consistent across alveolar, embryonal, and combined rhabdomyosarcoma patient groups. These results again should be taken in the context of *SMARCA4* expression in diagnostic biopsies at start of therapy and in the context of the chemotherapy administered (generally vincristine, actinomycin and cyclophosphamide for this group of patients). See Discussion.

## DISCUSSION

Emerging evidence supports pivotal roles of epigenetic aberrations in RMS. We found entinostat, a class I specific HDAC inhibitor, pharmacologically silences pathognomonic gene fusions PAX3:FOXO1 and PAX7:FOXO1 in ARMS [1, 5]. A related epigenetic study has shown that histone acetyltransferase P/CAF (KAT2B) is overexpressed in ARMS patient samples and cell lines and that silencing P/CAF or pharmacological inhibition of its acetyltransferase activity down-regulates PAX3:FOXO1 fusion protein levels with reduced proliferation and tumor burden in xenograft mouse models [4].

In this current study, CRISPR screening of 400 epigenetic targets identified SMARCA4 as a unique factor for cell survival in ARMS. In biopsies, a preference of SMARCA4 expression to SMARCA2 expression was observed, and SMARCA4 expression was associated with the classical SWI/SNF BAF complex in ARMS. While we have cleanly established that SMARCA4 depletion is a chemotherapy-sensitizer in the short term [5], SMARCA4 depletion had variable effect on cell viability in the short term. Longer term measures of SMARCA4 depletion delayed anchorage-independent and -dependent colony formation and *in vivo* tumor progression. Dual SMARCA4 and SMARCA2 depletion using the protein degrader ACBI-1 also demonstrated an inhibitory effect on tumor growth *in vitro* and *in vivo*. Dual SMARCA4 and SMARCA2 inhibition may be feasible and desirable, given that in SMARCA4 mutant cancers, SMARCA2 can be a synthetic lethal vulnerability [14] and that dual inactivation of SMARCA4 and SMARCA2 has only minimal effects on hematopoietic progenitors (although delivery to cardiac vascular endothelial cells would need to be avoided) [35]. Furthermore, small cell carcinoma of the ovary hypercalcemic type (SCCOHT), cell lines and xenografts deficient in both SMARCA4 and SMARCA2 are hypersensitive to EZH2 inhibition in other SWI/SNF mutant cancers [8, 17]. The EZH2-SMARCA4 interaction is a future avenue for our protein degrader studies.

A further question might be whether the ATPase or bromodomain are essential to the biology of ARMS. In a previously published study of lung cancer cell lines H1299 and A549 [32], anchorage-dependent colony formation was impaired by ATPase-mutation but not bromodomain mutation for both SMARCA4 and SMARCA2. For a murine *MLL-AF9/Nras*$^{G12D}$ RN2 AML cell line, proliferation was impaired by an ATPase-defective SMARCA4 mutant (K798R) but not a dominant-negative bromodomain mutant (N1506A) [30]. Similarly, in our CRISPR studies, ARMS cell viability was significantly more impaired by SMARCA4 ATPase mutants relative to the bromodomain mutants. We further investigated the effect of SMARCA4 bromodomain inhibition by PFI-3 in vitro for up to 10 days and observed negligible reduction of ARMS cell viability, supporting the notion that the ATPase domain is of greater relevance when targeting SMARCA4.

An apparent paradox exists with respect to SMARCA4 vs SMARCA2 expression in patient biopsies and the survival of those patients after therapy which is better for SMARCA4-high RMS patients, and worse for SMARCA2-high RMS patients. Two possible explanations could be that the dataset was gene expression microarray-based (imprecise) or that RNA expression does not reflect protein expression or protein activity (*e.g.*, incorporation in active BAF complexes). Alternatively, the associations could be taken in the specific context of chemotherapy treatment-related survival, and the interaction of SMARCA4 expression and the chemotherapy regimen given to most patients in this Intergroup Rhabdomyosarcoma Study Group IV cohort (vincristine, actinomycin and cyclophosphamide). In non-small cell lung cancer [3], high SMARCA4 expression was associated with sensitivity to cisplatin and vinorelbine and improved long term patient survival. For rhabdomyosarcoma, a similar phenomenon could be at play in the chemotherapy treatment of children who are treated with highly similar drugs. Given that five-year disease-free survival for high risk or relapsed/refractory ARMS is only 8% [27], implying intrinsic chemotherapy resistance of the target tumor cell subpopulation, one could take advantage of SMARCA4/SMARCA2

depletion approaches without concern that chemotherapy sensitivity is lost (because the target population of tumor cells is already intrinsically resistant).

In summary, we report that SMARCA4 is a pivotal susceptibility in ARMS tumor cell survival over the long-term as one might expect for an epigenetic factor. Translationally, SMARCA4 inhibition via protein degraders is one of the direct approaches to be developed; alternatively, indirect SMARCA4 inhibition through HDAC3 as we have reported [5] is a clinically amenable approach given that a Children's Oncology Group phase I clinical trial (ADVL1513, NCT02780804) shows entinostat is tolerable in children with relapsed or refractory solid tumors [20], and that pediatric patients have higher drug exposures and longer half-life versus adults ($C_{max}$ of 140.8 nM at a dose of 4 mg/m$^2$ and half-life 45 hours in children) – yet no dose-limiting toxicities (DLTs) for this narrow spectrum HDAC1–3 inhibitor. Thus, targeting SMARCA4 directly (*i.e.*, protein degradation) or indirectly (*i.e.*, entinostat) could provide a novel therapeutic breakthrough for children with PAX:FOXO1+ ARMS.

## MATERIALS AND METHODS

### Epigenome-wide CRISPR screen

A pooled CRISPR screen was conducted to identify genes of therapeutic interest using primary mouse tumor cell cultures. The methodology has been previously described [31]. Briefly, we previously cloned an epigenome-focused sgRNA library that targets 192 methyltransferase, demethylase, acetyltransferase, deacetylase, ATPase and bromodomain-containing proteins. This library was lentivirally-transduced into Cas9-expressing RMS cell lines, followed by quantification of sgRNA abundance over multiple passages using deep-sequencing. Cas9-expressing U66788, U37125 and NIH-3T3 cells were transduced with epigenome-wide sgRNA library at a multiplicity of infection of 0.3 and cultured for 18 days. Genomic DNA was isolated and amplicons around sgRNA sequences were PCR amplified. PCR product was used for library preparation and then finally sequenced. In total, the screen used ~1000 gRNA at 4–5 gRNA targeting per domain, and hits were defined for a gene target when at least 2 gRNA scored with over 5-fold change.

### CRISPR mediated depletion of SMARCA4 in human ARMS cell lines

Seven individual sgRNAs against SMARCA4 were cloned into lentiviral vector expressing GFP. Cas9-expressing Rh30 and Rh4 cells were transduced with lentiviruses harboring individual sgRNA in the presence of 8 μg/ml of polybrene. Fraction of GFP positive cells were determined at different time points from day 3 to day 18 post-transduction.

### Clinical significance of expression of *SMARCA4* and *SMARCA2*

The intergroup rhabdomyosarcoma study group (IRSG)-IV was queried for RMS patient survival data and tumor biopsy *SMARCA4* and *SMARCA2* expression levels. Kaplan-Meier estimates of survival probability were generated as previously described in order to investigate potential associations between patient prognosis and *SMARCA4* and *SMARCA2* expression.

## Cell culture

Human ARMS cell line Rh30, Rh41, ERMS cell lines RD and ARMS PDX culture CF-1 were cultured in growth medium (GM) RPMI 1640 (11875–093; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine Serum (FBS) (26140079; Thermo Fisher Scientific) and 1% penicillin/Streptomycin (15140–122; Thermo Fisher Scientific). Human ARMS cell line CW9019 cells, murine ARMS culture U23674, U66788 and murine ERMS culture U57810, U37125 and NH3–3T3 were cultured in DMEM supplemented with 10% FBS and 1% antibiotics. Primary human skeletal myoblasts (HSMM) (CC-2580; Lonza Inc, Allendale, NJ, USA) were cultured in GM (SKBM-2 CC-3244; Lonza Inc) supplemented with 10% FBS and 1% penicillin/streptomycin. HSMM was differentiated until day 4 in differentiation medium DM: F-12 (11320033; Thermo Fisher Scientific).

U66788 stable lines expressing shRNA against Smarca4 was generated by transducing cells with Dharmacon SMARTvector Inducible shRNA lentiviruses at a MOI of 0.3, in the presence of 10ug/ml polybrene. 48 hours after transduction, cells were exposed to growth media containing 2ug/ml puromycin. Selection was carried out for 4 days with single media change and then cells were expanded, archived and frozen down.

Cell cultures were confirmed free of mycoplasma contamination and authenticated via STR profiling by the University of Arizona Genetics Core (University of Arizona, Tucson, AZ, USA). Additional mycoplasma testing was performed using the MycoAlert™ PLUS Mycoplasma Detection Kit (LT07–705, Lonza Bioscience, Basel, Switzerland).

## Cell Viability Assay

Cells were plated in opaque 96-well plates in triplicates. Next day, transfection/dox treatment was performed, and then incubated for 72 hours. Cell viability was measured using CellTiter-Glo (Promega) assay according to manufacturer's protocol.

## Anchorage-independent colony forming assay

For soft agar assays, $2\times10^3$ cells were resuspended in 1 ml of 0.35% agarose solution (in growth medium) and layered over, already solidified 0.5ml of 0.7% agarose (in growth medium) in well of a 24 well plate. After 3 weeks, colonies were photographed with a brightfield microscope and counted manually for quantification.

## Anchorage-dependent colony forming assay

For anchorage-dependent cell culture colony formation assays, $10^3$ cells were plated per well of a 6-well plate in triplicate. Plates were incubated for 15 days with single media change. After 15 days, media was removed, colonies were fixed with paraformaldehyde and stained with 0.1% crystal violet for 20 minutes. Stained colonies were imaged using gel imager. For, U66788 cells, total incubation time was 7 days.

## Cell migration assay

An siRNA/Doxycycline treatment was carried out in 6 well plates as mentioned above. 24 hours after transfection, cells were serum starved for 24 hours and then trypsinized.

50000 (Rh30 and U66788 shSmarca4) or 100000 (Rh41) cells were plated onto Boyden chambers in serum-free media. In the lower chamber media containing 10% FBS was added. Cells were incubated for 24/48 hours and then stained with crystal violet. For quantification crystal violet was eluted with 10% acetic acid solution and OD was measured at 595nm.

### Immunoblotting

To prepare protein lysate, cells were lysed in radio immunoprecipitation (RIPA) buffer (89901, Thermo Fisher Scientific) containing both protease and phosphatase inhibitors (Sigma Aldrich, St. Louis, MO). Lysates were homogenized and clarified by centrifugation at 14,000 rpm for 10 minutes. Thirty μg of protein were electrophoresed in 7.5–10% mini protean polyacrylamide gel (4561024, Bio-Rad, Hercules, CA, USA) transferred to PVDF membranes (1620255, Bio-Rad) for immunoblot analysis with anti-SMARCA4/Brg-1 (sc-17796, Santa Cruz Biotechnology, Dallas, TX, USA), anti-SMARCA2 (ab15597, Abcam, San Francisco, CA, USA), anti-PBRM1 (A301–59A, Bethyl Laboratories, Montgomery, TX, USA), anti-β-actin (ab8227, Abcam, San Francisco, CA, USA), and anti-GAPDH (2118, Cell Signaling Technology, Danvers, MA, USA).

### Tissue microarrays

Tissue microarrays were obtained from the Children's Oncology Group Biorepository (ARMS TMA 3000–30-P8967 and ERMS 3000–30-P8968) and stained for BRG (rabbit monoclonal, Epitomics, 2822–1) or BRM (rabbit polyclonal, Sigma Prestige HPA029981) by coauthor ANK as previously described [16].

### Knockdown of *SMARCA4* in cell cultures

For silencing of SMARCA4, ARMS and ERMS cells were transfected with 100 nM of *SMARCA4* (L-010431–00-0005 5 nmol), or scrambled siRNA (Dharmacon) using Lipofectamine RNAimax (13778150, Invitrogen). 25pmol of siRNA was used per well of a 6-well plate. All downstream analysis was carried out after 72 hours after transfection. For all human cell lines, medium containing transfection mix was replaced with fresh media 2 hours after transfection to reduce cell death. (L-041135–00-0005 against mouse *Smarca4* and L-010431–00-0005 against human *SMARCA4*). Non-targeting siRNA pool was used as a control.

### Transient Transfection

RH30 was seeded at 10,000 cells in 100 μL media per well of a 96-well plate and incubated overnight. The next day, cells were transfected in triplicate wells with LMH-SMARCA2 or LMH-PBRM1 (GenScript BioTech, Leiden, Netherlands). Plasmid DNA was combined with FuGENE® 6 Transfection Reagent (E2691; Promega, Madison, WI) and Opti-MEM (11058021; Thermo Fisher, Waltham, MA) in a 3:1 reagent:DNA ratio following the FuGENE® 6 Transfection Reagent protocol. A volume of 5 μL containing 0.1 μg DNA was added per well. Control wells received transfection vehicle only. For western blot lysate preparation, RH30 was seeded at 300,000 cells in 1 mL media per well of 6-well plates and the concurrent transfection was scaled equivalently. Transfection efficiency in all plates was verified by checking GFP reporter expression using a ZEISS Axio Zoom.

### ACBI-1 protein degrader studies

The SMARCA4/A2 protein degrader ACBI1 and the control cis ACBI-1 developed by the Ciulli lab & Boehringer Ingelheim is previously described [11]. ACBI-1 and cis ACBI-1 were obtained and are amongst the compounds freely available from OpnMe (https://opnme.com/molecules/smarca2-4-acbi1).

### Animal studies

***PROTAC studies*:** Under institutional IACUC approval, $10^6$ Rh30 cells pre-tx for 3 days with ACBI-1 SmarcA4/A2 protac or cis-protac control then orthotopically xenografted into the right gastrocnemius of cardiotoxin pre-injured 8-week old SHO mice and tumor growth was followed by caliper measurement.

**shRNA studies:** Under institutional IACUC approval, $4\times10^5$ mouse aRMS U66788 primary tumor culture cells harboring stably inducible shSmarcA4 were orthotopically allografted into the right gastrocnemius of cardiotoxin pre-injured 8-week-old SHO mice and tumor growth was followed by caliper measurement. Mice were administered doxycycline water (0.2 mg/ml) with sucrose (0.5 mg/ml) beginning 3 days prior to injection. Injected cells had been pre-treated with doxycycline 5 days prior to injection as described in the *in vitro* studies.

### Statistical analysis

In most studies, significance was determined by a one-tailed Student's t test and $p$ values of <0.05 were considered statistically significant. Statistical significance was set at * $P < 0.05$ and **$P < 0.01$. Error bars indicate mean ± standard deviation (SD). For Figure. 5D, the statistical approach was based on a repeated measures linear model of tumor volume terms of group with an autoregressive order 1autocorrelation matrix.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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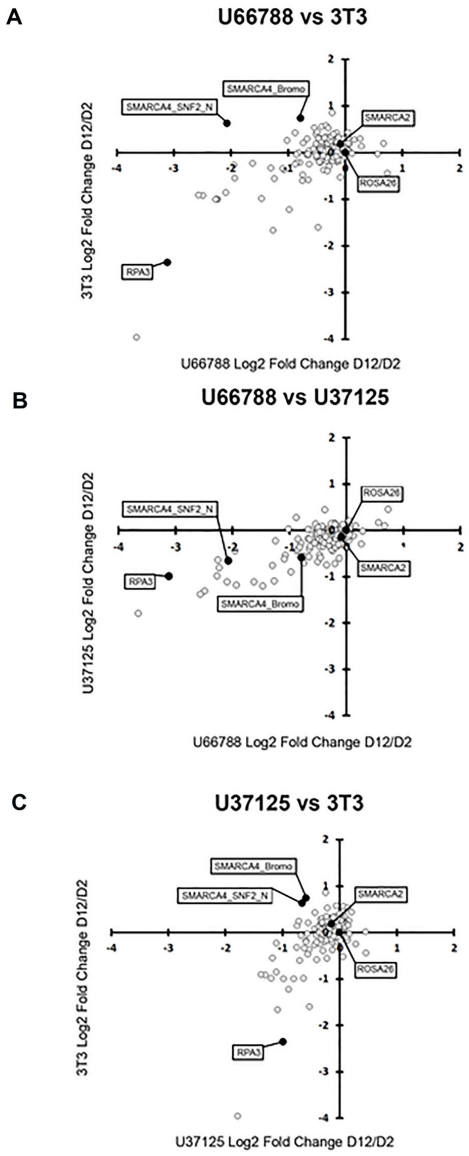



**Figure 1. CRISPR epigenome screen for mouse sarcoma cell cultures.**

(A) Comparison of Day12/Day2 raw depletion values (as log2 fold change values) between NIH-3T3 (mouse fibroblasts) and U66788 (mouse ARMS line), NIH3T3 and U37125 (mouse ERMS line) and U37125 and U66788 cell lines. Fold change of sgRNA is calculated between d2 and d12 after transduction with CRISPR sgRNA library. *Rosa* and *Rpa3* depletions are represented as negative and positive controls, respectively. (B) Comparison of U66788 ARMS cell line and U37125 ERMS cell line. Cell survival of U66788, but not U37125, was dependent on SMARCA4. (C) Comparison of NIH-3T3 cell line and U37125 ERMS cell line.

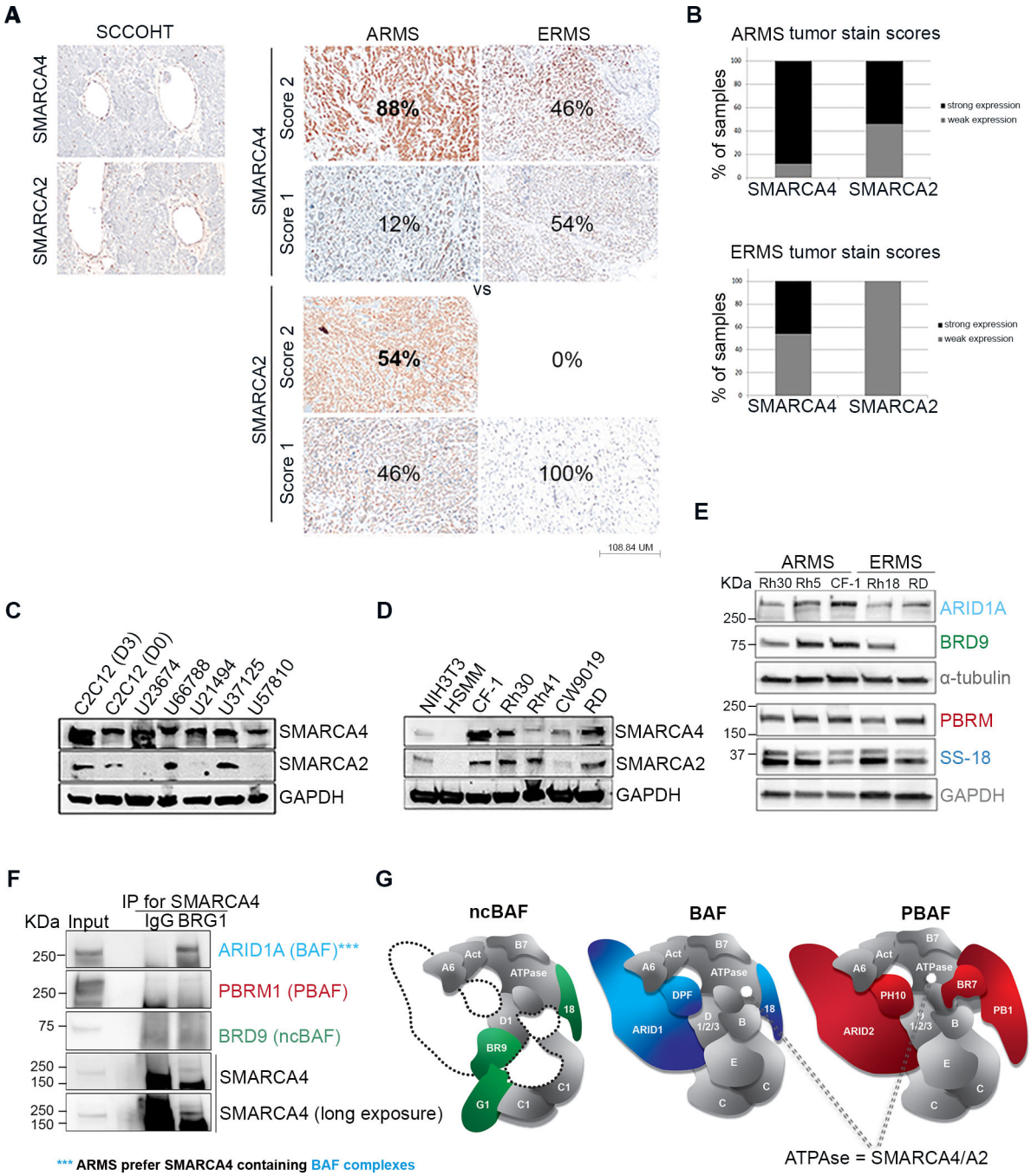



**Figure 2. SMARCA2/4 expression in ARMS, ERMS primary tumors and cell lines.**

(A) SMARCA2/SMARCA4 expression in ARMS/ERMS tumors accessed by immunohistochemistry (see Table 1 for expanded results and description). Scale bar, 100 μm. (B) Graphical representation of percentage of ARMS tumor samples expressing SMARCA4 and SMARCA2 (strong vs weak expression). (C) Expression of SMARCA4 and SMARCA2 in murine ARMS and ERMS cell lines assessed via western blotting. Proliferating and differentiated C2C12 cell line used as control. (D) Expression of SMARCA4 and SMARCA2 in human ARMS and ERMS cell line assessed via western

blotting. Proliferating HSMM and 3T3 cell lines were used as controls. (E) Analysis of the components of SWI-SNF complexes in ARMS cell lines. (F) Immunoprecipitation studies in Rh30 ARMS cell line to analyze the association of SMARCA4 with SWI-SNF BAF complex component ARID1A, SWI-SNF PBAF complex component PBRM1, and SWI-SNF ncBAF complex component BRD9. (G) Schematic of BAF complexes adapted from [23].

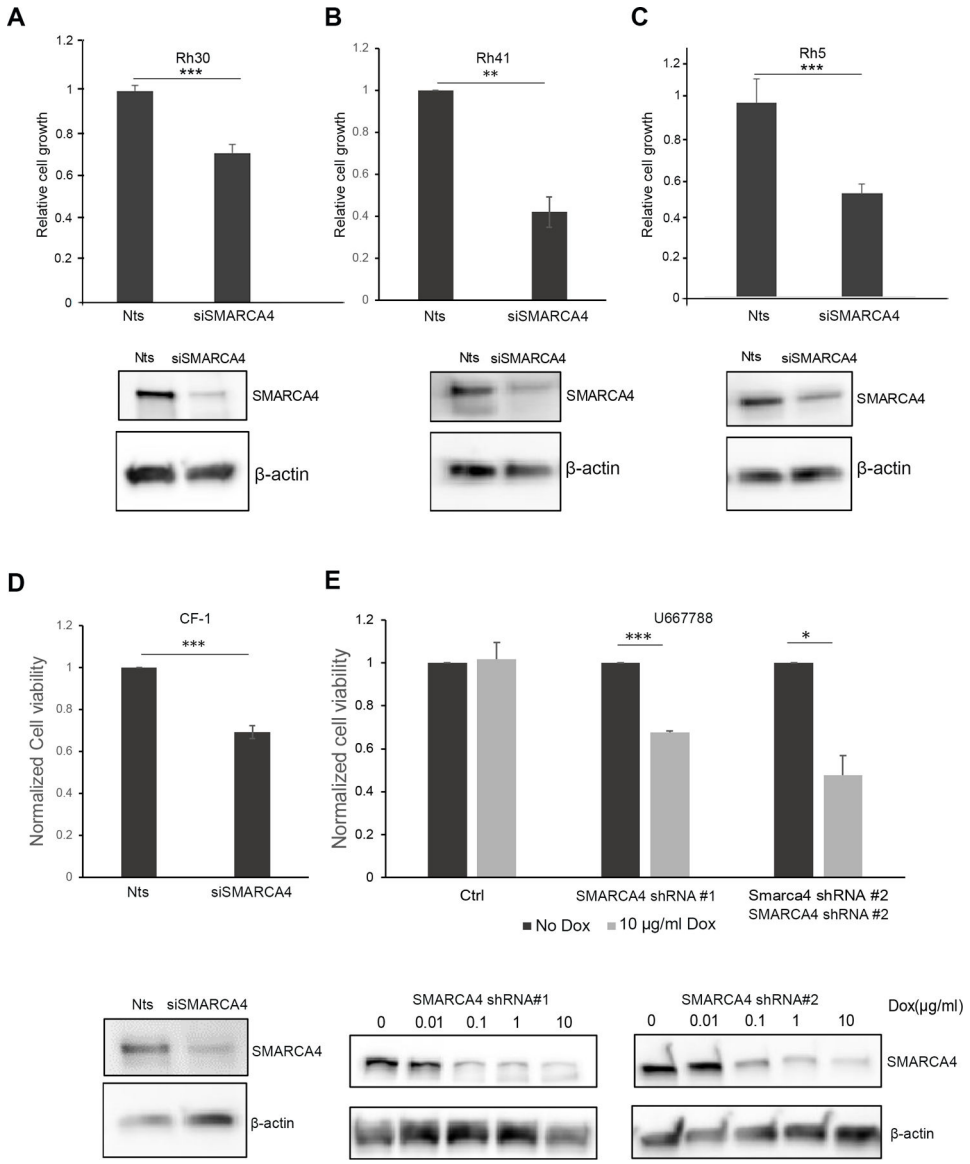



**Figure 3: Short-term knockdown of SMARCA4 using siRNA only partially compromises cell viability of human ARMS cell lines.**

(A-D) Relative cell viability at 72 hours as measured by CellTiterGlo assay of Rh30, Rh41, Rh5 and CF-1. Non-targeting siRNA (Nts) served as a control. (E) Relative cell viability at 72 hours of murine ARMS U667788 primary cell culture. Paired immunoblots show efficiency of SMARCA4 knockdown in each of the cultures. Error bars indicates mean± SE of biological replicates (n=3, p*≤ 0.05, p**≤ 0.01, p***≤ 0.001)

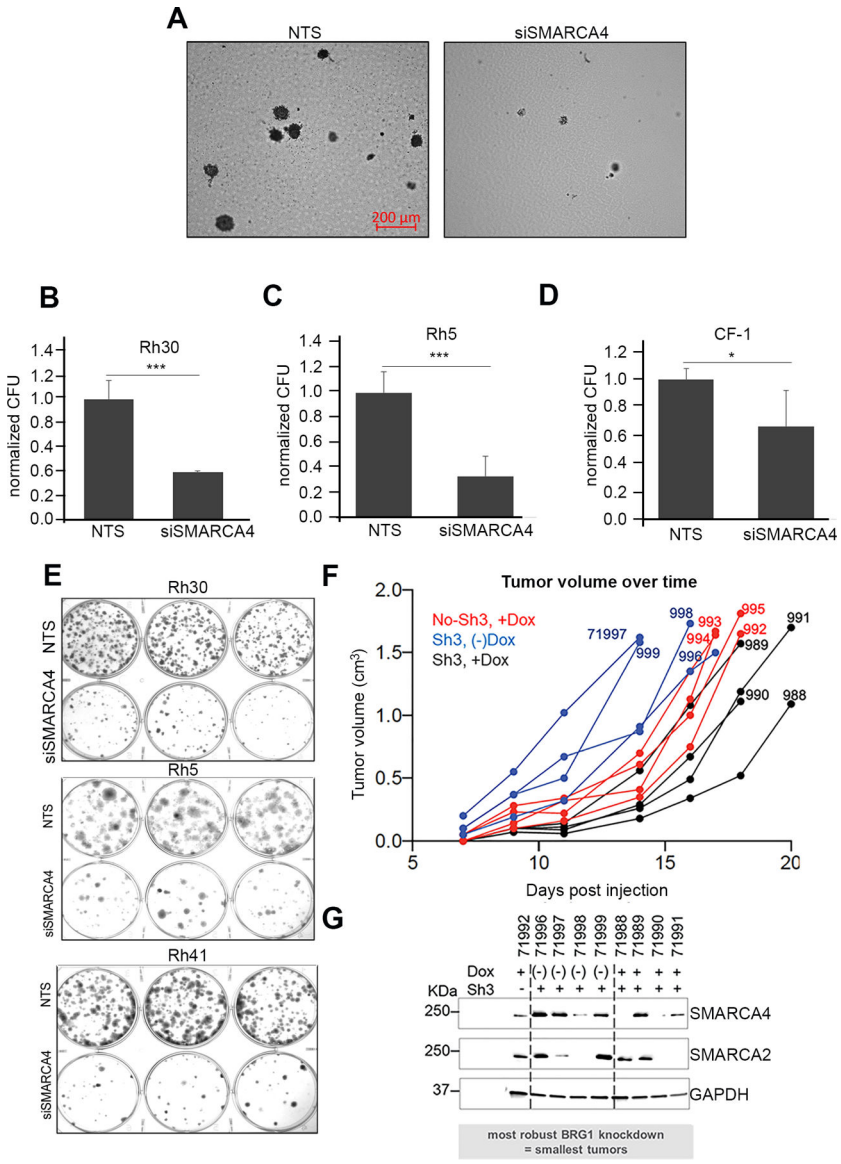
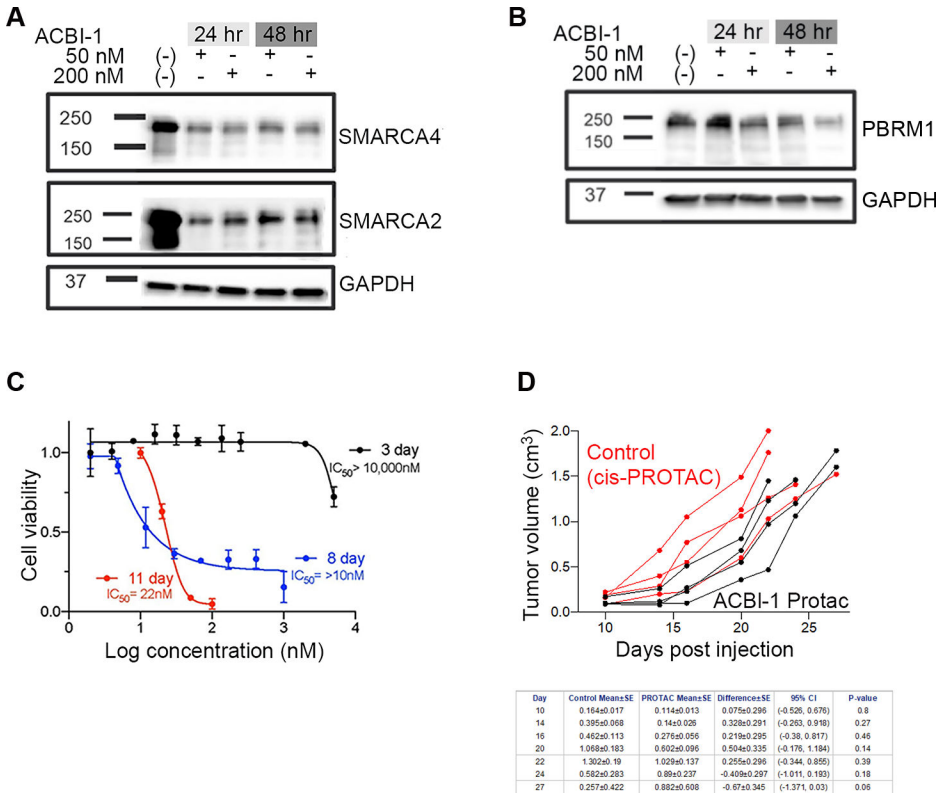



**Figure 4. Suppression of SMARCA4 inhibits both anchorage-independent and dependent colony forming ability of ARMS cells.**

(A) Representative photographic images of anchorage-independent colony formation assay for Rh30 cells (B-D) Quantitative analysis of anchorage-independent soft agar colony formation assay for Rh30, Rh5 and CF-1 cells after 21 days of growth (CFU: Colony forming units). (E) Representative photographic images of anchorage-dependent colony formation assay for Rh30, Rh41 and Rh5 cells after staining with crystal violet at the end of 15 days of growth. Nts: Non-targeting siRNA. Error bars indicates mean± SE of biological replicates (n>=2, p*=< 0.05, p= 0.05–0.15). (F) Analysis of tumor growth in murine orthotopic allograft after a long-term DOX-inducible SMARCA4 knockdown. (G) Pharmacodynamic western blot analysis of SMARCA4 (BRG1) & SMARCA2 (BRM1).




| Day | Control Mean±SE | PROTAC Mean±SE | Difference±SE | 95% CI | P-value |
|---|---|---|---|---|---|
| 10 | 0.164±0.017 | 0.114±0.013 | 0.075±0.296 | (-0.526, 0.676) | 0.8 |
| 14 | 0.395±0.068 | 0.14±0.026 | 0.328±0.291 | (-0.263, 0.918) | 0.27 |
| 16 | 0.462±0.113 | 0.276±0.056 | 0.219±0.295 | (-0.38, 0.817) | 0.46 |
| 20 | 1.068±0.183 | 0.602±0.096 | 0.504±0.335 | (-0.176, 1.184) | 0.14 |
| 22 | 1.302±0.19 | 1.029±0.137 | 0.255±0.296 | (-0.344, 0.855) | 0.39 |
| 24 | 0.582±0.283 | 0.89±0.237 | -0.409±0.297 | (-1.011, 0.193) | 0.18 |
| 27 | 0.257±0.422 | 0.882±0.608 | -0.67±0.345 | (-1.371, 0.03) | 0.06 |

**Figure 5. A SMARCA4/A2 protein degrader requires long term exposure for cellular effect in RMS.**

(A) Treatment of ARMS cell line Rh30 with ACBI1 for 24–48 hours followed by analysis of biochemical degradation of SMARCA4 and SMARCA2 by western blot analysis. (B) Analysis of tumor cell viability in the short term (3 days) and long-term SMARCA4/A2 depletion (8 or 11 days). (C) *In vivo* studies for which $10^6$ Rh30 cells were injected into mouse gastrocnemius 24h after cardiotoxin pre-injury. Cells had been pre-treated for 3 days with 50 nM ACBI-1 SMARCA4/A2 PROTAC or cis- ACBI-1 (negative control) prior to injection and tumor initiation analyzed.

**Table 1.**

Scoring of Tissue Microarray Immunohistochemistry for SMARCA4 and SMARCA2.

| | Human ARMS | | Human ERMS | |
|---|---|---|---|---|
| | **SMARCA4** | **SMARCA2** | **SMARCA4** | **SMARCA2** |
| Total number of tumor samples | 39 | 39 | 38 | 39 |
| Number of useable samples | 33 | 30 | 24 | 30 |
| Number (%) useable samples with score = 0 | 0 (0%) | 0 (0%) | 0(0%) | 0(0%) |
| Number (%) useable samples with score = 1 | 4 (12%) | 14 (46%) | 13 (54%) | 12 (100%) |
| Number (%) useable samples with score = 2 | 29 (88%) | 16 (54%) | 11 (46%) | 0 (0%) |

A tissue microarray was performed for a variety of ERMS and ARMS tumor samples. Each sample was given a score for the level of expression: 0 for no expression, 1 for positive expression that was weak or variable, 2 for positive, strong, and uniform expression and a 9 if the sample was not useable. Each tumor was given multiple scores. For each tumor, a representative score was chosen for the expression of SMARCA4 and for the expression of SMARCA2. Of the useable samples, a percentage of tumors showing a 0, 1, or 2 is shown below.