

# Expression of *SMARCB1* (*INI1*) mutations in familial schwannomatosis

Miriam J. Smith<sup>1,†</sup>, James A. Walker<sup>1,2,3</sup>, Yiping Shen<sup>3</sup>, Anat Stemmer-Rachamimov<sup>4</sup>, James F. Gusella<sup>3</sup> and Scott R. Plotkin<sup>1,5,\*</sup>

<sup>1</sup>Department of Neurology, <sup>2</sup>Massachusetts General Hospital Center for Cancer Research, <sup>3</sup>Center for Human Genetic Research, <sup>4</sup>Division of Neuropathology, <sup>5</sup>Pappas Center for Neuro-oncology, Massachusetts General Hospital, Boston, MA 02114, USA

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Genetic changes in the *SMARCB1* tumor suppressor gene have recently been reported in tumors and blood from families with schwannomatosis. Exon scanning of all nine *SMARCB1* exons in genomic DNA from our cohort of families meeting the criteria for ‘definite’ or ‘presumptive’ schwannomatosis previously revealed constitutional alterations in 13 of 19 families (68%). Screening of four new familial schwannomatosis probands identified one additional constitutional alteration. We confirmed the presence of mRNA transcripts for two missense alterations, four mutations of conserved splice motifs and two additional mutations, in less conserved sequences, which also affect splicing. Furthermore, we found that transcripts for a rare 3′-untranslated region (c.\*82C > T) alteration shared by four unrelated families did not produce splice variants but did show unequal allelic expression, suggesting that the alteration is either causative itself or linked to an unidentified causative mutation. Overexpression studies in cells lacking *SMARCB1* suggest that mutant *SMARCB1* proteins, like wild-type *SMARCB1* protein, retain the ability to suppress cyclin D1 activity. These data, together with the expression of *SMARCB1* protein in a proportion of cells from schwannomatosis-related schwannomas, suggest that these tumors develop through a mechanism that is distinct from that of rhabdoid tumors in which *SMARCB1* protein is completely absent in tumor cells.

## INTRODUCTION

Schwannomatosis (MIM#162091) is a third major form of neurofibromatosis, that is clinically and genetically distinct from NF1 (MIM#162200) and NF2 (MIM#101000) (1). The most common clinical symptom of schwannomatosis is intractable pain, although the mechanism by which this occurs is not well understood. Tumors from familial schwannomatosis patients frequently harbor somatic truncating mutations at the *NF2* (MIM#607379) gene on the long arm of chromosome 22 and loss of the wild-type *NF2* allele. However, they do not carry germline *NF2* mutations (2). Recently, a number of constitutional alterations have been reported in familial and some sporadic schwannomatosis patients in the *SMARCB1* (MIM#601607) gene (3–6), which is situated 5.8 Mb proximal to *NF2*.

Loss of *SMARCB1* has been linked previously to development of rhabdoid tumors (RTPS1; MIM#609322) (7). Rhabdoid tumors are highly malignant, appear in the first few years after birth, and are almost always lethal. Several RTPS1 families have been described (8) to include family members who are constitutional carriers of a *SMARCB1* mutation, but who never develop schwannomas. Recently, a multigenerational family was described with multiple members affected by either malignant rhabdoid tumors or by schwannomatosis, all of whom share a common germline *SMARCB1* exon 6 duplication mutation (9). A second family affected by RTPS1 and schwannoma has also been described with a c.472C > T *SMARCB1* mutation (10).

The existence of adult mutation carriers in RTPS1 families has led to the hypothesis that the risk of rhabdoid tumor development from these mutations is time dependent (6,11), in

\*To whom correspondence should be addressed at: Massachusetts General Hospital, 55 Fruit St, Yawkey 9E, Boston, MA 02114, USA. Tel: +1 6177263650; Fax: +1 6176432591; Email: splotkin@partners.org

†Present address: St Mary’s Hospital, University of Manchester, Manchester, M13 9WL, UK.

which case the development of schwannomas later in life becomes feasible. However, the majority of RTPS1 and schwannomatosis families remain distinct, making it more likely that the type or location of the mutation determines the resulting disease status.

Almost all of the constitutional *SMARCB1* alterations found in familial schwannomatosis patients are predicted to be non-truncating. In contrast, the mutations found in RTPS1 are mainly truncating mutations and large deletions, which lead to a complete loss of SMARCB1 protein. This difference in mutation type may underlie the difference in phenotype presented by these two conditions.

To address this issue, we have carried out analysis of *SMARCB1* transcripts from 14 schwannomatosis families, shown to harbor constitutional alterations (6), in order to verify expression of the non-truncated products predicted from each mutation.

## RESULTS

### Immunohistochemistry of schwannomas reveals mosaic pattern of SMARCB1 expression

Immunostaining for schwannomas from families 1, 5 and 10 which harbor the c.\*82C > T mutation and family 11 harboring the c.364G > T mutation have been published previously (12) and show a mosaic pattern of staining for SMARCB1 protein, consistent with loss of expression in a subset of tumor cells. Tumor sections from families 9 and 19 were subsequently analyzed by immunostaining for SMARCB1 and also revealed a mosaic pattern of mixed positive and negative nuclei in all four schwannomas from a member of family 19 (c.795 + 1G > T) and a single schwannoma from family 9 (c.158G > T). Representative staining is shown for a tumor from family 19 in Figure 1. No tumors were available for testing from other families in this study.

### cDNA analysis of familial schwannomatosis samples

To confirm the predicted effects of constitutional mutations identified previously (6), we analyzed cDNA derived from mRNA of lymphoblastoid cell lines carrying each of the 10 germline alterations found in 14 families. The results are summarized in Table 1. Representative chromatograms of the sequencing results for each of the seven mutant transcripts predicted to produce a viable protein and the three predicted to undergo nonsense-mediated decay are shown in Figure 2A–J.

#### Missense mutations

Each of the missense alterations, c.41C > A (p.Pro14His) and c.158G > T (p. Arg53Leu), found in family 4, and families 9 and PA-3, respectively, was detected in the cDNA transcript generated from its corresponding lymphoblast cell line (Fig. 2A and B), indicating that the mutant allele for both of these alterations is expressed at the mRNA level.

#### Splicing alterations

Four mutations were detected in conserved splice sites in four kindreds. An intron 3 mutation (c.362 + 1G > A) amplified a faint band containing a splice variant that results in an

in-frame deletion of the last 45 bp of exon 3 (Fig. 2C). An exon 4 alteration, c.364G > T, caused skipping of exon 4, without changing the reading frame (Fig. 2D). Another mutation at the ultimate base of exon 4, c.500G > A, produced altered splicing that resulted in the in-frame loss of the last 111 bp of the exon (c.390\_500del, Fig. 2E). The intron 6 change, c.795 + 1G > T produced two alternative splice variants. The first involved the in-frame inclusion of the first 45 bp of intron 6 (Fig. 2F) while the second, which was much less abundant, involved skipping of exon 6, with the resulting frame-shift predicted to lead to a premature stop codon and mRNA decay (Fig. 2G).

Sequence analysis of lymphocyte DNA from family 23 [family 07-367 in Eaton *et al.*, (10)] identified the nonsense mutation c.472C > T in exon 4. We isolated three alternately spliced cDNA amplicons derived from the corresponding lymphoblast cell line RNA for this family. First, amplification of exons 3–6 detected two different deletions: complete exon 4 loss (c.363\_500del) and partial exon 4 deletion (c.390\_500del). Amplicons for exons 5–9 then detected a deletion of the whole of exon 7 (Fig. 1H). The exon 4 changes in the transcript were the same as those found in other families and have the potential to produce non-truncated protein. However, the presence of the exon 7 deletion would lead to a premature stop codon and destabilize these mutant transcripts; therefore, they are predicted to undergo nonsense-mediated decay.

Two additional potential splice site alterations were identified further from the consensus splice sequence the intron 4 change, c.500 + 5G > T, and the intron 5 change, c.629-5T > G. Interestingly, the former leads to the same splicing alteration as the c.500G > A mutation, five bases away (Fig. 2E). The intron 5 alteration, c.629-5T > G, in family P/Qu, seems to disrupt the exon 6 splice acceptor sequence, leading to the insertion of 145 bp of the 3' end of intron 5 (Fig. 2I), using a cryptic splice acceptor, ag/TTT. This alteration results in a premature stop codon within the intronic sequence and is predicted to lead to mRNA decay.

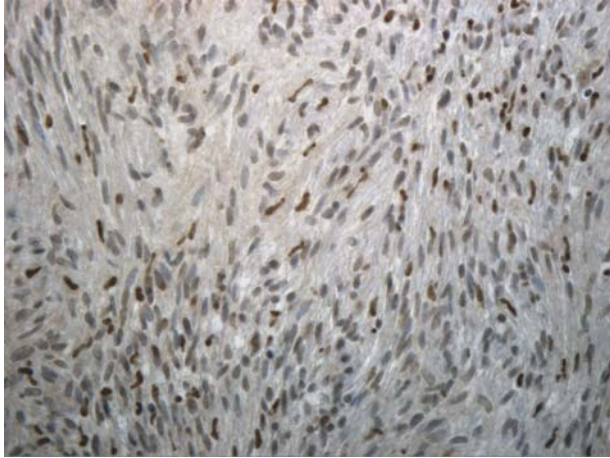
#### Variants of uncertain significance

Four unrelated families contained the same alteration, c.\*82C > T, within their 3'UTR (Fig. 2J). This alteration was not found in an unaffected panel of 100 alleles and is not recorded in the dbSNP database (6,13). None of the four families produced splice variants in the cDNA analysis screen, but the alteration was observed in the transcript.

### Allelic expression analysis of the c.\*82C > T alteration

Allelic expression analysis has previously been used to quantify under- or overexpression of mutant *NF2* transcripts from *NF2* patient-derived lymphoblast cell RNAs (14). We used this technique to measure expression levels of the c.\*82C > T alteration.

The full-length *SMARCB1* transcripts were amplified, sequenced and the level of expression of the mutant 'T' allele in cDNA was calculated, relative to the genomic sequence, using the Mutation Surveyor DNA analysis program (SoftGenetics, State College, PA, USA). The results revealed unequal allelic expression, with a decreased expression of



**Figure 1.** Immunostaining for SMARCB1 shows a mosaic pattern of SMARCB1 expression in a schwannoma from family 19 with a mixture of positive (brown) and negative (blue) nuclei in cells.

the mutant allele by  $\sim 27\%$  compared with the wild-type. To ensure that differences in expression for the c.\*82C > T change were not due to differential expression in lymphoblastoid cells, two known SNPs (rs34399789 and rs2229354), identified on an unaffected allele in two families, were tested for unequal expression in unaffected family members. These polymorphisms both showed equal expression in full-length *SMARCB1* cDNA. The results suggest that the c.\*82C > T alteration is associated with reduced expression and is either causative or linked to a causative mutation.

#### The *SMARCB1* mutant 3'UTR alters mRNA stability

To substantiate the effect of the mutant *SMARCB1* 3'UTR on RNA expression levels, we transfected HEK293T cells with a pGL3-promoter luciferase vector containing a wild-type *SMARCB1* 3'UTR, or a c.\*82C > T mutant 3'UTR. Forty-eight hours after transfection, the level of luciferase expression was significantly reduced under the regulation of the c.\*82C > T mutant 3'UTR compared with the wild-type 3'UTR (Fig. 3A). To distinguish between the possibilities of the c.\*82C > T mutation affecting either translational efficiency or stability of the mRNA, we performed qRT-PCR on RNA extracted from HEK293T cells transfected with luciferase constructs bearing the wild-type or the c.\*82C > T 3'UTRs, at 48 and 96 h post-transfection. At 48 and 96 h, the c.\*82C > T mutant mRNA levels were reduced by  $\sim 20$  and  $\sim 45\%$ , respectively, compared with the wild-type (Fig. 3B). Together, these results suggest that the c.\*82C > T mutant 3'UTR leads to a reduced expression of the *SMARCB1* transcript, possibly by destabilizing the mRNA.

#### Cyclin D1 reporter activity is appropriately suppressed by mutant SMARCB1

To begin to elucidate the mechanism by which mutations in *SMARCB1* affect its normal function in cells, we created expression constructs for three of the mutant *SMARCB1* transcripts found in our cohort, predicted to result in altered

**Table 1.** Germline mutations found in familial schwannomatosis kindreds and the effects observed in mRNA (family 23 has both schwannomatosis and RTPS1)

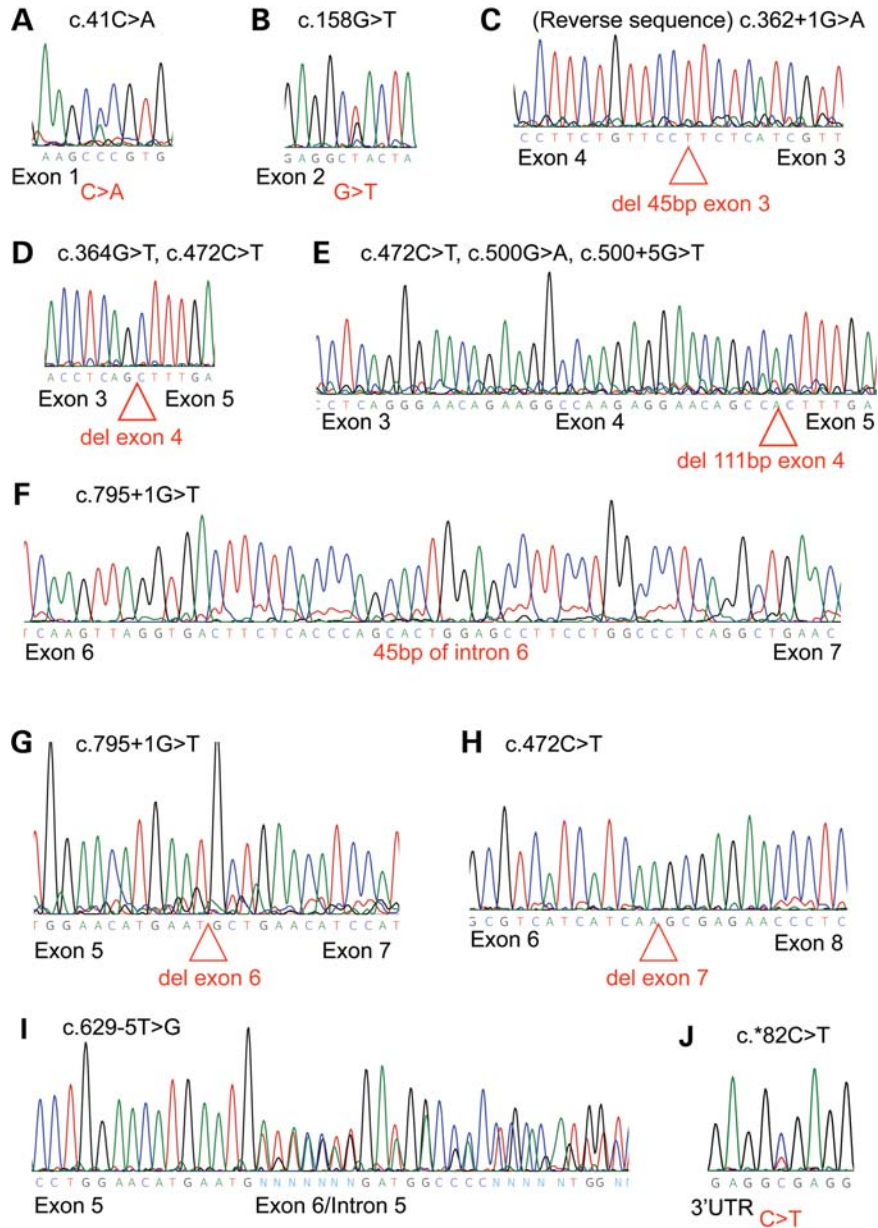
Family ID	Exon	Germline mutation	Mutant amplicons identified
4	1	c.41C > A	r.41C > A
9	2	c.158G > T	r.158G > U
PA-1	2	c.158G > T	r.158G > U
E	3	c.362 + 1G > A	r.318_362del
11	4	c.364G > T	r.363_500del
23	4	c.472C > T	r.363_500del, r.390_500del, r.796_986del
PA-3	4	c.500G > A	r.390_500del
V	4	c.500 + 5G > T	r.390_500del
P/Qu	6	c.629-5T > G	r.500ins501-145_501-1
19	6	c.795 + 1G > T	r.795_796ins795 + 1_795 + 45ins, r.628_795del
1	9	c.*82C > T	r.*82C > U
3	9	c.*82C > T	r.*82C > U
5	9	c.*82C > T	r.*82C > U
10	9	c.*82C > T	r.*82C > U

protein sequence. The exon 1 missense, c.41C > A, and exon 2 missense, c.158G > T, mutations were created by site-directed mutagenesis. The splice mutant lacking exon 4 was isolated from full-length cDNA derived from mRNA from a lymphoblastoid cell line with the c.364G > T mutation. These expression constructs were used to determine whether mutant SMARCB1 proteins are able to suppress elevated cyclin D1 expression, as has been shown previously for wild-type SMARCB1 (15). A luciferase reporter construct containing the cyclin D1 promoter region  $-1745$  to  $+35$  was used to transfect MON cells, which lack endogenous SMARCB1 and have elevated levels of cyclin D1 (15). This construct was co-transfected with either a wild-type SMARCB1 construct or a mutant transcript construct. When wild-type SMARCB1 was reintroduced into MON cells, luciferase expression was suppressed by  $\sim 60\%$  (Fig. 4) similar to previous reports (15). Similarly, when MON cells were transfected with mutant *SMARCB1* constructs, luciferase expression was suppressed by 53% (exon 1 missense), 74% (exon 2 missense) or 65% (exon 4 deletion). The results show that these schwannomatosis-related mutant SMARCB1 proteins retain the ability to repress cyclin D1 transcription.

## DISCUSSION

Exon scanning analysis of *SMARCB1* in our cohort of familial schwannomatosis patients has now identified a total of 14/23 (61%) probands with a germline point mutation. The majority of these mutations are predicted to be non-truncating. This contrasts with the mutational spectrum of RTPS1 in which mutations are predicted to be truncating (7,16). We analyzed cDNA from this select group of familial schwannomatosis patients to confirm the predicted effects of constitutional mutations identified during initial *SMARCB1* exon scanning and found that almost all of the constitutional alterations in the *SMARCB1* gene produced a mutant transcript that is likely to produce a non-truncated protein.



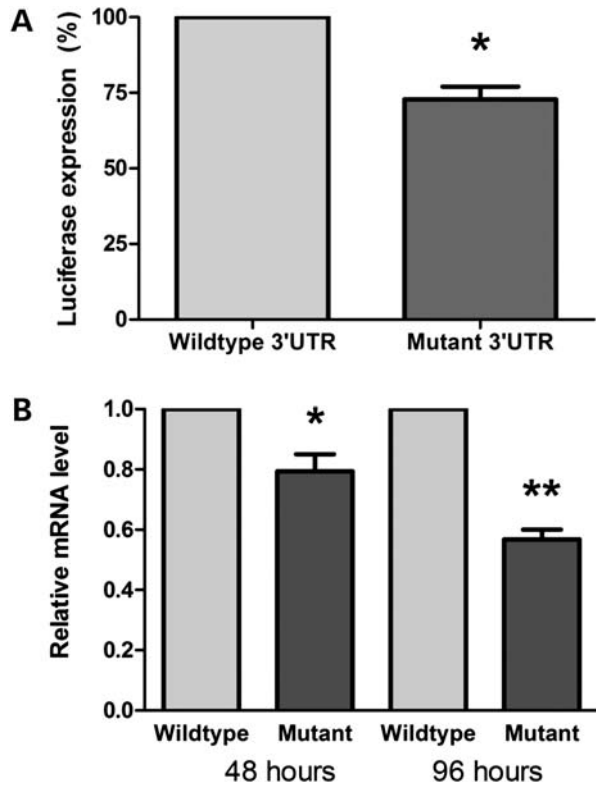


**Figure 2.** Chromatograms showing *SMARCB1* mutant transcripts identified in the study. (A) Exon 1 missense mutation; (B) exon 2 missense mutation found in families 9 and PA1; (C) exon 3 splice mutation that removes the first 45 bp of exon 3; (D) in-frame deletion of the entire exon 4 sequence found in families 11 and 23; (E) exon 4 splice mutation leading to the deletion of the last 111 bp of exon 4 in families PA-3, V and 23; (F) exon 6 splice mutation causing inclusion of the first 45 bp of intron 6; (G) family 19 transcript deleting exon 6, predicted to cause nonsense-mediated decay; (H) family 23 deletion of exon 7, predicted to cause nonsense-mediated decay; (I) family P/Qu mutant transcript including 145 bp of intron 5, predicted to cause nonsense-mediated decay; (J) 3'UTR change found in families 1, 3, 5 and 10.

Families P/Qu and 23 both produced mutant transcripts that are likely to be degraded by nonsense-mediated decay. Family 23 has a family history of both schwannomatosis and RTPS1 and, in addition to the truncating mutation, also has a somatic deletion of the wild-type *SMARCB1* allele in a tumor (10). The c.472C > T nonsense mutation is known to predispose to RTPS1, but it is unclear why some members of the same family developed schwannomas rather than rhabdoid tumors. It is possible that a modifier gene is involved. It is also unclear how the mutation, which occurs in exon 4, causes a

deletion of exon 7. It is possible that a second, unidentified, mutation exists within intron 6, leading to skipping of exon 7.

The only mutant transcript detected in family P/Qu contained an insertion of the last 145 bp of intron 5, which leads to a stop codon 26 codons into the insertion. This is predicted to lead to mRNA decay and no expression of the mutant copy of *SMARCB1*. This family did not show loss of heterozygosity (LOH) by microsatellite marker analysis at the *SMARCB1* locus, suggesting that the wild-type copy of *SMARCB1* is still present in tumors, although it remains

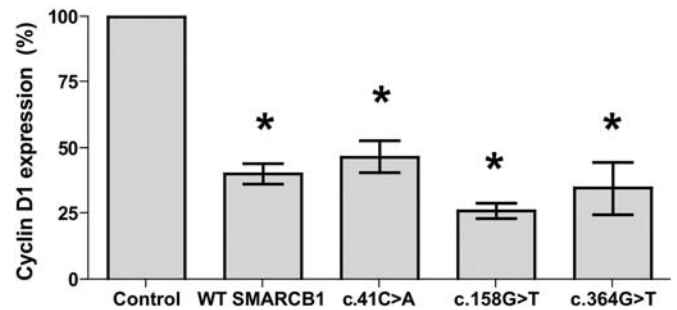


**Figure 3.** The *SMARCB1* mutant 3'UTR alters mRNA stability. Relative mRNA expression levels of wild-type and mutant *SMARCB1* 3'untranslated regions in HEK293T cells. (A) Luciferase levels indicate the relative expression under the control of wild-type and c.\*82C > T mutated *SMARCB1* 3'UTRs, normalized to transfection efficiency by GFP fluorescence \* $P < 0.01$ . (B) qRT-PCR of wild-type and mutant *SMARCB1* 3'UTR expression levels at 48 and 96 h post-transfection normalized to both GFP and GAPDH \* $P < 0.015$ ; \*\* $P < 0.001$ .

possible that other mechanisms may affect this remaining allele. LOH was, however, seen at the *NF2* locus—downstream of *SMARCB1*—in a tumor from family P/Qu, supporting the theory that co-mutation of these two genes is involved in schwannoma formation (4–6). Unfortunately, no tumor material was available for immunohistochemical analysis.

Tumors from schwannomatosis kindreds frequently carry *SMARCB1* alterations in conjunction with loss of the wild-type allele. The combination of a constitutional *SMARCB1* mutation with a somatically acquired truncating mutation in the *NF2* gene and loss of the wild-type copies of both genes indicates cross-talk between tumorigenic pathways and a complex mechanism of schwannoma formation in this disorder. Indeed, a multi-hit hypothesis has been suggested (3–6).

The alteration, c.\*82C > T, found in the 3' UTR of four unrelated kindreds (13) in our cohort, showed no splice variants, but was detected by sequencing of the full-length transcript from lymphoblast cell lines, suggesting that the mutant mRNA is expressed. Allelic expression analysis, and qRT-PCR on cells transfected with wild-type and mutant 3'UTRs, showed unequal expression with decreased levels of the mutant allele in comparison with the wild-type. Families 3 and 5, for which tumor DNA was available, showed LOH for both the *SMARCB1* and *NF2* loci and showed somatic



**Figure 4.** Cyclin D1 activity is appropriately suppressed by both wild-type and mutant *SMARCB1* proteins. Introduction of both wild-type and mutant *SMARCB1* transcripts (c.41C > A missense, c.158G > T missense or c.364G > T splice mutant) into MON cells which lack endogenous *SMARCB1* leads to suppression of luciferase reporter activity under the control of the cyclin D1 promoter. Normalized to transfection efficiency by GFP fluorescence \* $P < 0.05$ .

mutation of the remaining *NF2* allele (6). These results, together with the frequency of this alteration in unrelated families, support a pathogenic status for the c.\*82C > T mutation and implicate it in the occurrence of schwannomatosis disease.

#### SMARCB1 regulation of cyclin D1

Loss of *SMARCB1* in RPTP1 leads to upregulation of cyclin D1 and progression into the cell cycle. A cyclin D1 repression assay showed that schwannomatosis-related missense and splice mutants are capable of suppressing cyclin D1 activity in a similar way to that shown for the wild-type *SMARCB1* protein. This finding suggests that the downstream effects of *SMARCB1* alterations are different in schwannomatosis compared with RPTP1, with cyclin D1 and related cell cycle processes being affected primarily in the RPTP1 tumors.

Immunohistochemistry for *SMARCB1* revealed a mosaic pattern of mixed positive and negative nuclei in all tumor specimens. In a previous report, this finding was interpreted as loss of protein expression in a subset of tumor cells resulting in a mosaic of null and haploinsufficient cells (12). This could be as a result of transient or unstable expression. However, further work would be required to determine this. It is also unclear why the exon 2 mutation, which appears to produce a more highly expressed transcript, also leads to a mosaic pattern of protein production. It could be that the mutant DNA is transcribed normally, while the mutant mRNA is mis-folded, causing a reduction in translation efficiency.

This data, in conjunction with the different spectrum of mutations in comparison with that seen in RPTP1, suggests that in familial schwannomatosis kindreds, almost all constitutional alterations in the *SMARCB1* gene are capable of producing mutant, but viable transcripts, that yield *SMARCB1* protein with altered levels of functionality in schwannoma tumor cells. Further work is required to determine the precise mechanism by which mutant *SMARCB1* protein can promote the pathogenesis of schwannomas.

## MATERIALS AND METHODS

### Patient material

We studied 13 of 19 carefully characterized schwannomatosis kindreds described previously (6) and four additional familial probands. Lymphoblastoid cell lines were established from peripheral blood samples as described previously (17). High-molecular-weight DNA was extracted from peripheral blood leukocytes, cultured lymphoblasts, frozen pulverized tumor, cultured tumor and normal tissues obtained at autopsy using a PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). This study was approved by the Institutional Review Board of Partners HealthCare and informed consent was obtained from the patients participating in the study. For patients who had died, an autopsy permit was used as consent.

### Multiplex ligation-dependent probe amplification

Multiplex ligation-dependent probe amplification was carried out as described by Boyd *et al.* (6), using a SALSA multiplex ligation-dependent probe amplification P258 *SMARCB1* kit (MRC-Holland, Amsterdam, the Netherlands). Briefly, 20–500 ng DNA were used for hybridization, ligation and amplification of the *SMARCB1* exon probes according to the manufacturer's instructions. The amplification products were analyzed using an ABI 3730 DNA Analyzer, with Biomek FX robotics and with GeneScan 500 LIZ (Applied Biosystems, Foster City, CA, USA) as the internal size standard. Relative probe signals were calculated by dividing each measured peak by the sum of all peak areas for that sample. DNA from four unaffected individuals was used for control samples.

### cDNA analysis

mRNA was extracted from frozen cell pellets of established lymphoblastoid cell lines for each kindred, using the PolyA-Tract mRNA extraction kit (Promega, Madison, WI, USA). Poly(A)<sup>+</sup> mRNA was reverse transcribed to cDNA with oligo(d)T primers, using the Superscript III cDNA first strand synthesis kit (Invitrogen, Carlsbad, CA, USA). Full-length *SMARCB1* and three overlapping fragments of the *SMARCB1* transcript were PCR amplified from the cDNAs using nested primers. For the full-length transcript, the primers 5'-CAGCCCTCCTGATCCCT-3' and 5'-CCCAATCTTCTGAGATGCTC-3' were used. The reverse primer 5'-ACAAATGGAATGTGTGCCGG-3' was used when the 3'UTR SNPs were amplified. Exons 1 through 4 were amplified with primers 5'-CAGCCCTCCTGATCCCT-3' and 5'-TCACAGCTGGGTCATGGTC-3', exons 3–6 were amplified by 5'-CACGGATACACGACTCTAGC-3' and 5'-CACTCAAAGTGGTCCACC-3' and for exons 5–9, 5'-CCATGCTCCACAACCATC-3' and 5'-CCCAATCTTCTGAGATGCTC-3' were used. PCR products were electrophoresed on 2% agarose gels, with a normal control. Aberrantly sized fragments were excised and analyzed by direct sequencing on an ABI Prism 3730 DNA analyzer.

Quantitative analysis of the c.\*82C > T mutation (previously denoted c.1240C > T) was carried out using the Mutation Surveyor program v3.20 (Softgenetics LLC, State College,

PA, USA) by comparison of relative levels of C and T alleles in cDNA with reference to levels in genomic DNA.

### Construction of wild-type and mutant *SMARCB1* expression vectors

Mutagenic primers were designed to introduce the c.41C > A and c.158G > T point mutations identified in our cohort into the wild-type human *SMARCB1* sequence obtained from Origene (Rockville, MD, USA). Site-directed mutagenesis was carried out using the Quick-change site-directed mutagenesis kits (Stratagene, Carlsbad, CA, USA). Mutagenic primers for c.41C > A were GACCTTCGGGCAGAAGCACGTGAAGTTCAGCTGG and CCAGCTGGAAGTTCACGTGCTTCTGCCCGAAGGTC. Mutagenic primers used for c.158G > T were CCCTCACTCTGGAGGCTACTAGCCACTGTGGAAG and CTTCCACAGTGGCTAGTAGCCTCCA GAGTGAGGG.

The splice mutant lacking exon 4 was obtained by PCR amplification of full-length cDNA generated from mRNA of a lymphoblastoid cell line from family 11, using a forward primer containing an *NheI* restriction site, GCATGCTAGCATGATGATGATGGCGCTGAGC, and a reverse primer containing a *HindIII* restriction site, TTTAAGCTTCCAGGCCGGGGCCGTGTT. Each mutant transcript was sub-cloned into a pcDNA plasmid, containing a C-terminal GFP tag.

For the cyclin D1 repression experiment, the cyclin D1 promoter region –1745 to +35 was sub-cloned into the pGL3-basic plasmid, which contains a luciferase reporter gene (Promega, Madison, WI, USA).

For the 3'UTR experiment, site-directed mutagenesis was used to generate the c.\*82C > T mutant, with primers TGGCAAGGACAGAGGTGAGGGGACAGCCCA and TGGGCTGTCCCCTCACCTCTGTCTTGCCA. cDNAs representing the wild-type and c.\*82C > T mutant 3'UTRs were each sub-cloned into the pGL3-promoter vector, downstream of the luciferase coding region.

### Cell lines

HEK293T cells were purchased from ATCC (Manassas, VA, USA). MON tumor cells were obtained from the laboratory of Dr Olivier Delattre.

### Luciferase reporter assays

For experiments investigating *SMARCB1* 3'UTR regulation, using a luciferase reporter, HEK293T cells were co-transfected with the pGL3-promoter vector construct with either a wild-type or mutant *SMARCB1* 3'UTR and an eGFP vector, using Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA). Cells were harvested after 48 h and luciferase activity was measured using One-glow luciferase reagent (Promega, Madison, WI, USA).

For experiments investigating the regulation of cyclin D1, the wild-type *SMARCB1* transcript and three mutant transcripts [containing the exon 1 missense mutation (c.41C > A), the exon 2 missense mutation (c.158G > T) or the deletion of exon 4], were subcloned into the pcDNA vector with a



C-terminal GFP tag. The cyclin D1 luciferase vector was transfected into MON cells which lack endogenous SMARCB1 along with each of the *SMARCB1* constructs, or an empty GFP control vector. Cells were harvested after 48 h and luciferase activity was measured using One-glow luciferase reagent (Promega, Madison, WI, USA). Luciferase levels were normalized to transfection efficiency using GFP fluorescence.

#### qRT-PCR analysis of luciferase-3'UTR reporters

Total RNA was extracted from transfected cells using the RNeasy kit (Qiagen, Valencia, CA, USA) and treated with DNase (Ambion, Foster City, CA, USA). First-strand cDNA synthesis was performed using random hexamers (GE Healthcare, Piscataway, NJ, USA). Subsequently, mRNA expression levels of luciferase-3' UTR reporters were assessed using 1  $\mu$ l of the appropriate cDNA for real-time qRT-PCR using FastStart Universal SyberGreen and a LightCycler 480 machine (Roche, Indianapolis, IN, USA). The forward oligonucleotide primer (5'-GGTCTTACCGGAAACTCGAC-3') corresponds to a sequence at the 3' end of the luciferase cDNA; the reverse primer (5'-CTCTGTCCTTGCCAGAAGATG-3') is within the 3'UTR of *SMARCB1*. The results were analyzed using the comparative Ct method ( $\Delta\Delta$ Ct) and normalized to GAPDH and eGFP expression levels to ensure equal loading and transfection efficiencies.

#### Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections from four schwannomas resected from member of family 19 (c.795 + 1G > T) and a single schwannoma from family 9 were immunostained using a commercial SMARCB1 antibody (BD Transduction Laboratories, Franklin Lakes, NJ, USA) along with appropriate positive (normal cortex) and negative controls (RTPS1). Antigen retrieval was achieved by microwaving in a Borg Decloaker RTU for 45 min (primary antibody concentration 1:50).

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*Conflict of Interest statement.* None declared.

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