A novel t(4;22)(q31;q12) produces an *EWSR1*–*SMARCA5* fusion in extraskeletal Ewing sarcoma/primitive neuroectodermal tumor

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Over 90% of Ewing sarcoma/primitive neuroectodermal tumors (PNETs) feature an 11;22 translocation leading to an *EWSR1*–*FLI1* fusion. Less commonly, a member of the ETS-transcription factor family other than *FLI1* is fused with *EWSR1*. In this study, cytogenetic analysis of an extraskeletal Ewing sarcoma/PNET revealed a novel chromosomal translocation t(4;22)(q31;q12) as the sole anomaly. Following confirmation of an *EWSR1* rearrangement by the use of *EWSR1* breakpoint flanking probes, a fluorescence *in situ* hybridization positional cloning strategy was used to further narrow the 4q31 breakpoint. These analyses identified the breakpoint within RP11-481K16, a bacterial artificial chromosome (BAC) clone containing two gene candidates *FREM* and *SMARCA5*. Subsequent RACE, RT-PCR, and sequencing studies were conducted to further characterize the fusion transcript. An in-frame fusion of the first 7 exons of *EWSR1* to the last 19 exons of *SMARCA5* was identified. *SMARCA5* encodes for hSNF2H, a chromatin-remodeling protein. Analogous to *EWSR1*–ETS-expressing NIH3T3 cells, NIH3T3 cells expressing *EWSR1*–hSNF2H exhibited anchorage-independent growth and formed colonies in soft agar, indicating chimeric protein tumorigenic potential. Conversely, expression of *EWSR1*–hSNF2H in NIH3T3 cells, unlike *EWSR1*–ETS fusions, did not induce EAT-2 expression. Mapping analysis demonstrated that deletion of the C-terminus (SLIDE or SANT motives) of hSNF2H impaired, and deletion of the SNF2_N domain fully abrogated NIH3T3 cell transformation by *EWSR1*–*SMARCA5*. It is proposed that *EWSR1*–hSNF2H may act as an oncogenic chromatin-remodeling factor and that its expression contributes to Ewing sarcoma/primitive neuroectodermal tumorigenesis. To the best of our knowledge, this is the first description of a fusion between *EWSR1* and a chromatin-reorganizing gene in Ewing sarcoma/PNET and thus expands the *EWSR1* functional partnership beyond transcription factor and zinc-finger gene families.

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The Ewing family of tumors including Ewing sarcoma and primitive neuroectodermal tumor (PNET) arise in bone or soft tissue of children and adolescents and have a high propensity for recur-

rence and distant metastases.^{1–3} Ewing sarcoma/PNETs are characterized by chromosomal translocations^{4–9} that fuse the *EWSR1* gene (22q12) to a subset of ETS-transcription factor gene family members, most commonly *FLI1* (11q24)¹⁰ and less frequently *ERG* (21q22),¹¹ *ETV1* (7p22),¹² *E1A-F* (17q21),^{13,14} or *FEV* (2q35–36).¹⁵ The ETS gene family members are defined by an approximately 85 amino acid (AA) long DNA-binding domain that binds to a core GGAA/T nucleotide sequence and further specificity in binding is defined by a flanking DNA core

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motif.^{16–18} *EWSR1* is an ubiquitously expressed protein with an RNA-binding domain within its C-terminal region and a strong transactivation domain at the N-terminal.^{19–21} The transactivation domain exerts its activity when juxtaposed to a DNA-binding domain. The fusion of the *EWSR1* N-terminal region to the C-terminus of an ETS protein results in an aberrant transcription factor that is presumed to be the initiating oncogenic event in an Ewing sarcoma/PNET.

Although most *EWSR1* rearrangements in Ewing sarcoma result in fusion with an *ETS*-transcription factor gene family member, there are also rare reports of *EWSR1* fusions with genes encoding a member of the zinc-finger family of proteins.^{22,23} In addition, a fusion between *EWSR1* and a gene from a transcription factor family other than *ETS*, the *NFATc2* gene (encodes for a member of the *NFAT*-transcription factor family), has recently been described.²⁴

In this study, cytogenetic analysis of an extra-skeletal Ewing sarcoma/PNET arising in the lumbosacral region of a 5-year-old female revealed a t(4;22)(q31;q12) resulting in a novel fusion between *EWSR1* and a member of the WSTF-SNF2h chromatin-remodeling complex family of genes, *SMARCA5*.

Materials and methods

Clinical

The patient was a 5-year-old female who presented with a 5-week history of low-back pain and increasing right lower extremity weakness. MRI studies with and without contrast revealed a large enhancing mass of the lumbosacral spinal canal (L4-5 through S2-3) with evidence of cortical destruction of the posterior sacral spine and anterior extension to the colonic wall at the rectosigmoid junction.

Following admission for pain management, the patient subsequently underwent an L4 through midsacral laminoplasty and excision of the epidural tumor. Intraoperatively, it was acknowledged that not all of the tumor could be removed (particularly anteriorly and laterally on the right side). Grossly, the excised specimen was composed of red to tan fragments of bone and soft tissue measuring 6.0 × 5.5 × 4.6 cm in aggregate.

Cytogenetic Analysis

Cytogenetic analysis was performed on a representative sample of the excised neoplasm using standard tissue culture and harvesting procedures. Briefly, the tissue was disaggregated mechanically and enzymatically, then cultured in RPMI 1640 media supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin-L-glutamine (Irvine Scientific, Santa Ana, CA, USA) for 3–8 days. Two to four hours before harvest, cells were exposed to

colcemid (0.02 µg/ml). Following hypotonic treatment (0.074 M KCl for 30 min for flasks and 0.8% Na citrate for 25 min for coverslips), the preparations were fixed three times with methanol:glacial acetic acid (3:1). Metaphase cells were banded with Giemsa trypsin, and the karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN, 2009).²⁵

Cosmid and Bacterial Artificial Chromosome Probes

For analysis of the *EWSR1* (22q12) and *FLI1* (11q24) gene loci, cosmid probes²⁶ (obtained from O Delattre, Institut Curie, Paris, France) flanking these two gene loci were selected in combination with an α -satellite probe for the centromeric region of chromosome 4 (Oncor, Gaithersburg, MD, USA). In an effort to further define the chromosomal breakpoint on chromosome 4, the following 4q31-specific bacterial artificial chromosome (BAC) clones were identified from the NCBI Map Viewer (<http://www.ncbi.nlm.nih.gov/mapview>) and obtained from BAC/PAC Resources Center (Children's Hospital Oakland Research Institute, Oakland, CA, USA): RP11-83A24, RP11-308D13, RP11-739C17, RP11-54P19, RP11-481K16, RP11-578N3, RP11-269F11, RP11-318C13, RP11-269F11, and RP11-557J10. In addition, RP11-222M10, a BAC clone spanning the *EWSR1* locus, was also used.

Fluorescence *In Situ* Hybridization

Bicolor fluorescence *in situ* hybridization (FISH) studies were performed on 4;22 translocation-positive metaphase cells and/or cytologic touch preparations of the tumor tissue. Cosmid and BAC probes were directly labeled by nick translation with either Spectrum Green or Spectrum Orange-dUTP utilizing a modified protocol of the manufacturer (Vysis, Downers Grove, IL, USA). Hybridization was conducted as previously described.²⁷ Hybridization signals were assessed in five metaphase cells or 200 interphase nuclei with strong, well-delineated signals by two different individuals. As a negative control, FISH studies were simultaneously conducted on karyotypically normal peripheral-blood lymphocytes. Images were acquired using the Cytovision Image Analysis System (Applied Imaging, Santa Clara, CA, USA).

Identification of *EWSR1*–*SMARCA5* Fusion Transcript

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). The 3' RACE (3'-rapid amplification of cDNA ends) was performed using the SMART-RACE cDNA amplification kit and protocol (Clontech, Palo Alto, CA, USA). Briefly, first-strand cDNA was reverse transcribed from 0.3 µg total RNA using Superscript II and the 3'-RACE

Table 1 Oligonucleotide primers used in this study*RT-PCR primers*

EWSR1-421	5'-CAACAAAGCTATGGAACCTATG-3'
EWSR1-615	5'-CAAGACCCGAGGATGGAA-3'
SMARCA5-1450	5'-ACTATTTCTGACAACTTAGATTTC-3'
SMARCA5-1793	5'-CCTCTCAACTAGTTTTTGATCCC-3'
SMARCA5-1954	5'-CAAAGAGATATGGATGATTACAACATT-3'

RACE primers

SMARCA5-f: 1431	5'-GGTTTTGCGTCCATTCTCTTCGTCG-3'
SMARCA5-f: 2977	5'-GCTCAGATTGAAAGGGGAGAGGCGAG-3'
SMARCA5-r: 805	5'-CCCATTCTTACATTCTGCTGCCCGTAGC-3'
SMARCA5-r: 2374	5'-TGAACCTTCGCCCATCTTGGAGAGC-3'
SMARCA5-r: 3054	5'-CCGTCCAATCTTTGTGTCAAGTGCTTTC-3'

cDNA synthesis primer (5'-CDS) from the kit. For construction of 5'-RACE-Ready cDNA, 0.3 µg of the RNA sample was mixed with 5'-CDS primer A and SMART II A oligo, and reverse transcribed as recommended by the supplier. An aliquot of each first-strand cDNA reaction was then amplified using an *EWSR1* or *SMARCA5* gene-specific forward or reverse primer (Table 1) and a universal primer mix (SMART-RACE kit). A total of 5 µl of 100-fold dilution of the first PCR products was then reamplified using AmpliTaq in a second PCR reaction with heminested primers consisting of the universal anchor primer (SMART-RACE kit) as the reverse primer and EWSR1-2 (internal to EWSR1-1; Table 1) as the forward primer. Second-round PCR products were electrophoresed, purified, and subcloned into pCR4/TOPO vector (Invitrogen). Colonies with recombinant plasmids containing the RACE-PCR products were cleaved with *EcoRI* restriction enzyme and analyzed by gel electrophoresis to estimate the size of the insert. Plasmid clones from the 5' and 3' RACE reactions with the longest inserts were selected for sequencing. Two plasmids, EWSR1-RACE.52 and SMARCA5-RACE.33 overlapped with EWSR1–SMARCA5 (421–1793) and extended the nucleotide sequences into 5' and 3' direction yielding a cDNA contig of 3820 nucleotides.

Reverse Transcription–Polymerase Chain Reaction for *EWSR1*–*SMARCA5*

RT–PCR analysis was performed using the Advantage one-step RT–PCR kit (Clontech) with sense *EWSR1* and antisense *SMARCA5* primers (Table 1) in the following combination; EWSR1-421/SMARCA5-1793, EWSR1-615/SMARCA5-1450, and EWSR1-421/SMARCA5-1954. The PCR thermal cycling protocol was performed according to the manufacturer's instructions with an annealing temperature of 58°C. The RT–PCR products of all reactions were subcloned and sequenced.

Construction of Full-Length *EWSR1*–*SMARCA5* cDNA

The *EWSR1*–*SMARCA5* (421–1793) RT–PCR product in the PCR4/TOPO vector was cut with *Bam*HI

and *Mlu*I restriction enzymes, ligated to an *Not*II/*Bam*HI fragment of EWSR1–RACE.52 and an *Mlu*I/*Bsr*GI fragment of SMARCA5–RACE.33, and introduced into the *Eag*I/*Bsr*GI sites of LITMUS38i vector (New England Biolabs, Ipswich, MA, USA).

Generation of Retroviral Vectors and Expression in NIH3T3 Cells

The *EWSR1*–*SMARCA5* chimeric cDNA in the LITMUS38i vector was amplified by PCR to add the epitope tag FLAG and the Kozak consensus translation initiation sequences into the N-terminal region. The primers used in this construction step were 5'-CCACCATGGATTACAAGGATGACGACGA TAAGGCGTCCACGGATTACA GTACC-3' and 5'-TC ATAGTTTCAGCTTCTTTTTTCTTCTCGACCATCA GGTGCGCC-3'. The amplified fragments were then cloned into pCR4/TOPO plasmid (Invitrogen) and sequenced multiple times. Subsequently in another PCR amplification, the *Eag*I site was added to the 5' and the *Xho*I site was added to the 3' end of the *EWSR1*–*SMARCA5* fusion cDNA. The sequence-confirmed, FLAG-tagged *EWSR1*–*SMARCA5* was digested with *Eag*I and *Xho*I and then transferred into the mouse retroviral vector MIEG3²⁸ (obtained from DA Williams, Children's Hospital Medical Center, Cincinnati, USA). MIEG3 is a bicistronic murine stem cell virus-based retroviral vector, containing an encephalomyocarditis virus IRES element (internal ribosome entry site) immediately preceding the gene encoding eGFP (enhanced green fluorescent protein). The co-expression of eGFP with the gene of interest enables detection of infected cells by flow cytometry or fluorescent microscopy. Generation of retroviral supernatants from Phoenix Amphi (ATCC product# SD 3443) packaging cells was achieved as previously described.²⁹ The retroviral vector was transfected into the Phoenix-Amphi packaging cell line by calcium phosphate, and the viral supernatants were collected 48 h after transfection by centrifugation.³⁰ The supernatants, containing the *EWSR1*–*SMARCA5*–MIEG3 retrovirus particles, were used to infect NIH3T3 cells. NIH3T3 cells were infected twice with the MIEG3 retrovirus containing the fusion gene, wild-type *EWSR1*, *SMARCA5*, and the various deletion mutants of *EWSR1*–*SMARCA5*. GFP-positive cells were sorted using fluorescence-activated cell sorting (FACS), as described previously.²⁹

Construction of *EWSR1*–*SMARCA5* Deletion Mutants

EW-SMd270-351 deletion mutants in pCR4-TOPO were created to delete the SNF2_N region utilizing the QuickChange kit (Stratagene, La Jolla, CA, USA) with primers 5'-CTACGGGCAGCAGAATGTAAAA TGGGGTAAA-3' and 5'-CCCAAGAAGAGAAATTG TTTGAAGAGTCTTTCCTAGGCCC-3' in the first

step, and for the second step, primers 5'-GGG CCTAGGAAAGACTCTTCAAACAA TTTCTCTTCTT GGG-3' and 5'-ATAGGAGATAAAGAACAAAGAGC TGCTTTTG TCAGAGACGTTTTATTACCGGGAGAA TGGG-3'. Deletion mutants EW-SM1-870 and EW-SM1-989 were prepared by PCR using the following primer pairs: 5'-CCACCATGGATTACAAGGATGAC GACG-3' and 5'-TGGAG GAAAGAACTGGAAATCC TGAACATTGG-3', and 5'-CCACCATGGATTACAA GGATGACGACG-3' and 5'-AATATCATCACGACCC CACTTCTC ATTAGC-3', respectively.

Sequence Analysis

Plasmid clones were sequenced using ABI PRISM® BigDye™ Terminator Cycle Sequencing kit version 2 and ABI PRISM® 3730 DNA Analyzer, a capillary electrophoresis system (ABI). Sequence analysis was performed using the MacVector with Assembler Version 10.4.11 (MacVector, Inc, Cary, NC, USA) sequence analysis program.

Immunoprecipitation and Western Blotting

Forty-eight hours after infection, the transfected NIH3T3 cells were harvested. Cytoplasmic and nuclear extracts were prepared using NE-PER® reagents (Pierce Biotechnology, Inc, Rockford, IL, USA) according to the manufacturer's instructions. Precleared nuclear and cytoplasmic extracts were immunoprecipitated using *EWSR1* and hSNF2H antibodies. Antibody/protein complexes were collected by Protein-A Sepharose, and subsequently subjected to SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 3% bovine serum albumin, incubated with M2 anti-FLAG antibody (Sigma, St Louis, MO, USA), washed and treated by secondary antibodies conjugated with peroxidase, and visualized by enhanced chemiluminescence (Pierce Biotechnology, Inc). For immunoblotting, transduced cells were lysed in M-PER (Pierce Biotechnology, Inc). Protein concentrations were assessed utilizing the BCA Protein Assay kit (Pierce Biotechnology, Inc) and 25 µg of total protein lysates/lane were run on denaturing SDS-polyacrylamide gels, transferred to nitrocellulose membranes. Membranes were blocked with 3% bovine serum albumin, incubated with the indicated primary antibodies and horseradish peroxidase-conjugated secondary antibodies, and developed using enhanced chemiluminescence (Pierce Biotechnology, Inc).

Primary antibodies used in these studies were goat polyclonal anti-hSNF2H (sc-8760, C-16), goat polyclonal anti-*EWSR1* (sc-6533, N-18), or goat polyclonal anti-human EAT-2 (sc-21572), M2 anti-FLAG antibody (Sigma), and horseradish peroxidase-conjugated secondary antibodies obtained

from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Soft Agar Assay

Transformation of NIH3T3 cells by *EWSR1*, *SMARCA5*, *EWSR1-SMARCA5*, and *EWSR1-SMARCA5* deletion variants was examined using a colony formation in soft agar culture assay. SeaKem GTG agar in H₂O was prepared, autoclaved, and mixed 2 × DMEM with 20% FBS to reach a final concentration of 0.9% and allocated at 1 ml/35 mm dish. A second agar containing 0.34% agar was prepared in a similar process, but also included NIH3T3-transformed cells at a final concentration of 2 × 10⁴/ml. A total of 2 ml of this second agar mixture was layered on top of the base layer, and allowed to solidify for 30 min–1 h, before incubating at 37°C in 5% CO₂. Plates were cultured for 3–4 weeks or until colonies were visible. The colonies were stained with 1 ml/well of 0.05% nitroblue tetrazolium in PBS and colonies larger than 100 µm were counted for each plate.

Results

Histology

Histopathologically, relatively solidly packed, uniformly small-sized neoplastic cells in a background of necrosis were identified (Figure 1a). Individual cells exhibited an increased nuclear to cytoplasmic ratio with hyperchromatic nuclei surrounded by smooth nuclear membranes and occasional clear to slightly vacuolated cytoplasms. Mitotic figures were readily identifiable. Immunohistochemical studies demonstrated that the neoplastic cells were reactive for vimentin, CD99 (membranous, Figure 1a inset), synaptophysin (focal), and neuron-specific enolase (focal). The neoplastic cells were negative for chromogranin, cytokeratin (AE1/3), muscle-specific actin, desmin, and leukocyte common antigen. The diagnosis of extraskeletal Ewing sarcoma/PNET was rendered.

Cytogenetic and FISH Data

A t(4;22)(q31;q22) was observed as the sole anomaly in nine cells (Figure 1b). One cell demonstrated extra copies of chromosomes 4, 8, and 22, in addition to the 4;22 translocation. Another cell was karyotypically normal. The complete chromosomal complement is 46,XX,t(4;22)(q31;q11.2)[9]/49,idem,+4,+8,+22[1]/46,XX [1].

FISH analysis of metaphase cells exhibiting the 4;22 translocation with an alphoid sequence probe specific for chromosome 4 and *EWSR1* breakpoint flanking cosmid probes confirmed the presence of a rearrangement of the *EWSR1* locus with translocation of the probe signal distal to *EWSR1* on the

Figure 1 (a) Representative histology of the tumor is showing a relatively monotonous round cell population with occasional mitotic figures. The neoplastic cells exhibit membranous immunoreactivity for CD99 (inset). (b) Partial G-banded karyotype demonstrating the $t(4;22)(q31;q12)$. (c) FISH analysis conducted on destained metaphase cells exhibiting the 4;22 translocation with an alphoid sequence probe specific for chromosome 4 and *EWSR1* breakpoint flanking cosmid probes confirm the presence of an *EWSR1* rearrangement with translocation of the green probe signal distal to *EWSR1* on the der(22) (red arrow) to the der(4) (white arrow). The green and yellow arrows indicate the normal chromosome 4 and normal chromosome 22 homologs, respectively. (d) FISH analysis with the RP11-418K16 BAC clone spanning the proximal (centromeric) portion of the *SMARCA5* locus in green and the RP11-222M10 BAC clone spanning the *EWSR1* locus in red confirmed the presence of a fusion between these two clones on the der(4), yellow arrow. The smaller red signal represents the remainder of the *EWSR1* spanning probe on the der(22) and the single green and larger red signals represent the normal chromosome 4 and 22 homologs, respectively.

derivative chromosome 22 homolog [der(22)] to the derivative chromosome 4 homolog [der(4)] (Figure 1c). However, FISH analysis of these abnormal metaphase cells with cosmid probes proximal and distal to *EWSR1* and *FLI1*, respectively, were negative for an *EWSR1*–*FLI1* fusion, suggesting involvement of a gene other than *FLI1* (data not shown).

FISH interphase cell positional cloning studies of the der(4) narrowed the breakpoint region to a single BAC clone, RP11-481K16. Bicolor FISH analysis using the RP11-481K16 and RP11-222M10 (spanning the *EWSR1* locus) BACs confirmed the presence of a fusion between these two clones in 25% of the cells analyzed (Figure 1d). Two genes located within the region covered by the RP-11481K16 BAC clone include *FREM* and *SMARCA5*. Note, only a

proximal portion (centromeric) of *SMARCA5* is covered by RP-11481K16.

Characterization of *EWSR1*–*SMARCA5* Transcript and Fusion Protein

Sequence analysis of the RACE products demonstrated an in-frame fusion of *EWSR1* and *SMARCA5*. *SMARCA5* maps to 4q31³¹ and like *EWSR1* is oriented with its 3' end telomeric. In contrast, the other gene overlapping with the RP-11481K16 BAC clone sequence, *FREM*, is in the opposite transcriptional orientation.

The presence of *EWSR1*–*SMARCA5* fusion transcripts was subsequently confirmed by RT–PCR analysis (Figure 2). A reciprocal *SMARCA5*–*EWSR1*

Figure 2 Ethidium-bromide agarose gel electrophoresis of the reverse-transcription polymerase chain reaction (RT-PCR) products. Lanes M1 and M2 represent the 1.0-kb and 100-bp DNA molecular weight marker ladders, respectively. Lanes 1, 2, and 3 demonstrate the *EWSR1*-*SMARCA5* fusion transcripts as detected by the gene-specific primer combinations indicated in the upper left hand corner (primer combinations also described in Table 1).

fusion product was not identified. The constructed full-length *EWSR1*-*SMARCA5* cDNA was found to result from the fusion of the first 7 exons of *EWSR1* to the last 19 exons of *SMARCA5* (Figure 3). This fusion transcript is 3986 nucleotides long and encodes a 1143 AA chimeric protein composed of the 264 NH2-terminal AAs of the transcriptional activation domain/regulatory domain of *EWSR1* and the 878 COOH-terminal AAs of hSNF2H (corresponding to AA position of hSNF2H from 175 to 1052) plus a single AA (N, Asn) at the breakpoint. The *EWSR1*-hSNF2H fusion protein, in addition to the serine-tyrosine-glutamine-glycine-rich (SNYG) N-terminal domain of *EWSR1*, contains five conserved domains, SNF2_N, SmB, Hand, Slide, and SANT of hSNF2H.^{31,32}

Western blot analyses conducted on the nuclear and cytosolic extracts of NIH3T3 cells transfected with wild-type *EWSR1*, *SMARCA5*, and *EWSR1*-*SMARCA5* cDNAs in MIEG3 vector revealed that only the nuclear extracts exhibited bands of the expected size for all of the proteins (Figure 4). This finding suggests that *EWSR1*-hSNF2H can be synthesized *in vitro* and that the chimeric protein enters the nucleus with a similar efficiency to *EWSR1* and hSNF2H.

Figure 3 Schematic diagrams of *EWSR1*, *SMARCA5*, and *EWSR1*-*SMARCA5* fusion gene (a) and fusion protein (b). The nucleotide and AA sequence of the fusion gene and its protein product around the breakpoint are illustrated in part (c).

Figure 4 Immunoblot analyses of *EWSR1*, hSNF2H and *EWSR1*-hSNF2H proteins demonstrate the subcellular localization of *EWSR1*-hSNF2H fusion protein (cytoplasmic (C) and nuclear (N) extracts).

Transforming Properties of *EWSR1*-hSNF2H

To assess for functional similarities or differences between *EWSR1*-FLI1 and *EWSR1*-hSNF2H, the ability for both fusions to modulate expression of a common target gene was investigated. EAT-2 (SH2 domain-containing 1B, SH2D1B) was selected because its expression is induced in *EWSR1*-FLI1, but not in *EWSR1* or FLI1-transfected NIH3T3 cells.³³

A comparison of NIH3T3 cells infected with MIEG3-*EWSR1*-FLI1 and MIEG3-*EWSR1*-*SMARCA5* fusion constructs revealed comparable levels of *EWSR1*-FLI1 and *EWSR1*-hSNF2H in cell lysates (Figure 5a). However, in contrast to *EWSR1*-FLI1-transformed NIH3T3 cells, EAT-2 expression was not induced in the NIH3T3 cells by the *EWSR1*-*SMARCA5* construct (Figure 5b), suggesting that *EWSR1*-FLI1 and *EWSR1*-hSNF2H may not regulate the same repertoire of target genes.

The failure of EWSR1-hSNF2H to induce EAT-2 expression implies that there might be functional differences between EWSR1-FLI1 and EWSR1-hSNF2H fusion proteins. To determine whether these differences might be explained by NIH3T3 cell transformation ability, NIH3T3 populations expressing either fusion were tested in liquid medium for anchorage-dependent growth. As illustrated in Figure 6, both EWSR1-FLI1 and EWSR1-hSNF2H support anchorage-independent growth of NIH3T3 cells.

For assessment of the role of the various hSNF2H motifs present in the chimeric EWSR1-hSNF2H protein and for determination of which region(s) is/are essential for transformation, epitope-tagged deletion mutants of EWSR1-hSNF2H producing

retrovirus were constructed in which three different motifs of the hSNF2H protein were deleted. The transforming properties of the mutated *EWSR1-SMARCA5* constructs were evaluated by infecting NIH3T3 cells with either the *EWSR1-SMARCA5* wild-type virus or one of the deletion mutated versions (Figure 7). Agar assays conducted on primary infectants revealed that NIH3T3 cells expressing any of the deleted fusion cDNA constructs displayed lowered colony forming activity or failed to form colonies in soft agar. Western blot analyses of the polyclonal primary infected cells of all three deleted constructs demonstrated the

Figure 5 Effect of the EWSR1-SMARCA5 on EAT-2 expression. (a) Immunoblot demonstrating equivalent levels of protein expression of the FLAG-tagged EWSR1-FLI1 and EWSR1-hSNF2H proteins in NIH3T3 cells. (b) Immunoblot analysis of EAT-2 in NIH3T3 cells expressing EWSR1-FLI1 or EWSR1-hSNF2H proteins.

Figure 7 (a) Schematic structure of EWSR1-hSNF2H and the deletion mutants. (b) Immunoblot analyses of EWSR1-hSNF2H and the deletion mutants. (c) Soft agar colony assay of EWSR1-hSNF2H and the deletion mutants-expressing NIH3T3.

Figure 6 Cell morphology of NIH3T3 cells infected with MIEG3, MIEG3-EWSR1-FLI1, and MIEG3-EWSR1-SMARCA5 retroviruses. NIH3T3 cells infected by the MIEG3, MIEG3-EWSR1-FLI1, and MIEG3-EWSR1-SMARCA5 retroviruses were plated on six-well plates without prior FACS selection. Colonies shown are representative of the cell population.

presence of the mutant protein (Figure 7). The expression level of the deletion mutants was equal to or greater than those seen in full-length *EWSR1-SMARCA5*-transformed clones. Therefore, the lack of transforming activity by the mutated constructs appears to be due to loss of biologic function and not from underexpression or instability of the deleted products.

Discussion

A remarkable diversity in the *EWSR1* C-terminal fusion gene partners that provide essential DNA-binding capacity to the chimeric protein have been observed in Ewing sarcoma/PNET. *FLI1*, a member of the ETS family of transcription factors, dominates as the most common translocation gene partner in Ewing sarcoma/PNET, followed by *ERG*, and less commonly other *ETS* gene family members.³⁴ In all Ewing sarcoma/PNETs with *EWSR1-ETS* fusions, the DNA-binding domain of the ETS factor is included, resulting in aberrant transcription factors that can regulate genes mediating the oncogenic phenotype of this neoplasm.^{33,35} The *EWSR1* protein contains an N-terminal serine-tyrosine-glutamine-glycine-rich region that when fused to a heterologous DNA-binding domain, potentially stimulates gene transcription. The ETS factors bind purine-rich sequences with GGAA/T core consensus sequence, surrounded by sequences that contribute to the specificity of each *EWSR1-ETS* fusion variant.

In this study, the identification, cloning, and functional analysis of a novel fusion oncoprotein in an extraskeletal Ewing sarcoma/PNET is described. The t(4;22)(q31;q12) chromosomal translocation juxtaposes *EWSR1* to *SMARCA5*. *SMARCA5* encodes hSNF2H, a protein with remarkable similarity in AA sequence to ISWI, a key component of chromatin-remodeling factors in *Drosophila*.^{31,32} The packaging of DNA into chromatin limits the accessibility of transcription factors to regulatory regions of genes. Eukaryotic cells contain two principal modules of chromatin-modifying activities: histone-modifying complexes and ATP-dependent chromatin-remodeling complexes.³⁶ The protein, hSNF2H, is one of the components of various ATP-dependent chromatin-remodeling complexes³⁷ including ACF/BAZ-like^{32,38,39} WICH,⁴⁰ CHRAC,⁴¹ NoRC,⁴² RSF,⁴³ and NuRD complexes.⁴⁴ While there are some differences between complexes, all feature a conserved repositioning specificity *in vivo* and also the ability of hSNF2H to mediate DNA accessibility for the interacting DNA-binding factors by sliding the histone octamer. *In vitro*, hSNF2H is able to interact with DNA regardless of the presence of core histones.⁴⁵

Divergent biological functions of the chromatin-remodeling complexes may largely arise from other properties conferred by complex-specific subunits. hSNF2H, as a component of the WICH complex,

mobilizes nucleosomes, regulates the transcription of various genes, and has a function in RNA polymerase I and RNA polymerase III transcription.⁴⁶

In RSF, one of the chromatin remodeling and spacing factor systems, hSNF2H interacts with Rsf-1 and promotes the formation of competent RNA polymerase II transcription initiation complexes on chromatin, as a result of its ability to mobilize nucleosomes.⁴⁷ Recently, Rsf-1 has been linked to cancer-specific gene amplification in ovarian, breast, bladder, esophageal, and head and neck cancers.⁴⁸ It has been suggested that the interaction between Rsf-1 and hSNF2H may represent a survival signal for tumors, which overexpress Rsf-1.

Translocations involving a chromatin-modifying gene have also been described in leukemia.⁴⁹ Specifically, in t(9;11)(p22;q23)⁴⁹ and t(11;19)(q23;p13.3)⁵⁰ leukemias, *MLL* (mixed-lineage leukemia gene; 11q23) is fused to *MLLT3* (*AF9*) and *MLLT1* (*ENL*), respectively. *MLLT3* and *MLLT1* are highly homologous with each other and are also homologous to proteins associated with the SWI/SNF complex. *MLLT1* encodes for one of the subunits of the SWI/SNF family of chromatin-remodeling complexes.⁵¹ The *MLL-MLLT1* fusion protein is associated with the chromatin-remodeling complex and synergistically activates transcription of various genes. *MLL-MLLT1* fusion protein recruits the chromatin-remodeling complex to genes, such as *HoxA7*, which are normally not controlled by *MLL*.⁵²

Our report is the first to describe an *EWSR1* fusion partner gene encoding for a chromatin-remodeling and spacing factor in Ewing sarcoma/PNET. The *EWSR1-hSNF2H* fusion protein, in addition to the serine-tyrosine-glutamine-glycine-rich (SNYG) N-terminal domain of *EWSR1*, contains five conserved domains and motifs, SNF2_N, SrmB, Hand, Sant, and Slide of hSNF2H. SNF2_N domain is found in proteins involved in a variety of processes including transcriptional regulation, DNA repair, DNA recombination, and chromatin unwinding. The SrmB domain is found in proteins with DNA and RNA helicase activity; it maintains one of the two ATP-binding sites of hSNF2H. The Hand domain confers DNA and nucleosome-binding properties to the protein. Sant and Slide have DNA-binding activity.

The *EWSR1-hSNF2H* fusion protein may function as part of a chromatin-remodeling complex. The *EWSR1-hSNF2H* chimeric protein could directly interact with DNA and regulate genes. The SNF2_N domain, maintained in the *EWSR1-hSNF2H* fusion protein, might serve as the ATPase component of the SNF2/SWI multi-subunit complex, and utilize energy derived from ATP hydrolysis to disrupt histone-DNA interactions, resulting in an increased accessibility of DNA to transcription factors and transcriptional regulation of genes not normally regulated by *EWSR1*. The SLIDE domain has a role in DNA binding, contacting DNA target sites similar

to c-Myb. The SANT domain is found in regulatory transcriptional repressor complexes in which it also binds DNA. The observation of deletion of the SNF2-N domain in *EWSR1-hSNF2H* in addition to loss of SLIDE and SANT domains with DNA-binding capacity suggests that the *hSNF2N* portion of the chimeric oncoprotein functions as an aberrant transcriptional regulator.

In spite of their structural differences, both *EWSR1-FLI1* and *EWSR1-ETV1* fusion proteins induce the expression of *SH2D1B* (*EAT-2*), an *EWSR1-FLI1* target gene.³³ Considering that many ETS proteins can bind to the same DNA sites suggests that *EWSR1-ETS* fusions promote oncogenesis via similar biologic pathways. In contrast, the *EWSR1-hSNF2H* fusion protein does not induce *SH2D1B* (*EAT-2*) expression in transfected NIH3T3 cells, suggesting that it may pursue a different biological pathway than *EWSR1-ETS* fusions in order to promote a similar Ewing sarcoma/PNET phenotype. Notably though, *EWSR1-hSNF2H*-expressing NIH3T3 cells do exhibit anchorage-independent growth and form colonies in soft agar, findings demonstrative of tumorigenic potential.

In summary, although most Ewing sarcoma/PNET *EWSR1* fusions have involved members of the *ETS* gene family, there are rare reports of *EWSR1* fusions with zinc-finger and *NFAT*-transcription factor family members.^{22–24} To date, however, there have been no reports of a fusion between *EWSR1* and a chromatin-remodeling gene. In this study, we cloned the fusion gene produced by a t(4;22) (q31;q12) in an extraskeletal Ewing sarcoma/PNET and demonstrated the tumorigenic activation of *SMARCA5*, a gene coding for a chromatin-reorganizing protein, by chimera formation with *EWSR1*. Future studies based on the structure–function relationship of the *EWSR1-hSNF2H* protein and functional analysis of the chimeric protein will provide insight into the mechanism of sarcomagenesis induced by this novel class of oncoproteins. Considering the structural and functional disparity between *ETS* genes and *SMARCA5*, the novel *EWSR1-hSNF2H* can serve as an excellent model system for examining alternative tumorigenic pathways in Ewing sarcoma/PNET.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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