

BRD4-NUT Fusion Oncogene: A Novel Mechanism in Aggressive Carcinoma¹**Christopher A. French,² Isao Miyoshi, Ichiro Kubonishi, Holcombe E. Grier, Antonio R. Perez-Atayde, and Jonathan A. Fletcher***Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts 02115 [C. A. F., J. A. F.]; Departments of Pathology [A. R. P.-A.], and Medicine [H. E. G., J. A. F.], Children's Hospital, Boston, Massachusetts 02115; Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115 [H. E. G., J. A. F.]; and Kochi Medical School, Okohcho, Nankoku, Kochi, Japan 783-8505 [I. M., I. K.]***Abstract**

The poorly differentiated carcinoma with t(15;19)(q13, p13.1) is characterized by its highly aggressive, invariably lethal clinical course. The chromosome 19 translocation breakpoint targets the *BRD4* double bromodomain-containing gene, which functions in regulation of cell cycle progression. Herein we demonstrate that *BRD4* is fused with nearly the entire transcript of the novel 15q13 gene, *NUT* (nuclear protein in testis), forming a 6.4-kb fusion oncogene, *BRD4-NUT*. *NUT*, like *BRD4*, is predicted to encode a nuclear protein but, unlike the ubiquitous *BRD4* transcript, is expressed only in testis. These findings establish a model to elucidate the oncogenic consequences of unscheduled *NUT* expression and altered *BRD4* function. Very few fusion oncogenes have been identified in epithelial tumors, and *BRD4-NUT* is the first fusion oncogene mechanism identified in a highly lethal form of carcinoma.

Introduction

Translocation t(15;19)(q13, p13.1) characterizes a rare, aggressive, and lethal carcinoma arising in midline organs of young people. The translocation results in a heretofore uncharacterized fusion oncogene. As described previously (1), we have determined that the chromosome 19 translocation breakpoint targets the *BRD4* bromodomain gene, whereas the chromosome 15 breakpoint involves a 9-kb region on chromosome band 15q13. *BRD4* is expressed normally as two alternative transcripts with identical 5' ends; but the coding sequence of the longer *BRD4* transcript is approximately twice the length of the shorter transcript. Both *BRD4* transcripts encode the NH₂-terminal bromodomains, whereas the longer *BRD4* transcript encodes COOH-terminal lysine-rich regions that are not encoded by the shorter *BRD4* transcript. Notably, the t(15;19) translocation breakpoint transects the coding sequence of the longer *BRD4* gene, whereas the shorter *BRD4* transcript is unperturbed.

In vitro (2, 3) and *in vivo* (4) studies of the murine *BRD4* have revealed a critical role in the regulation of cell cycle progression and cellular proliferation. *BRD4* associates with chromatin (3) and binds replication factor C (RFC; Ref. 2). Notably, whereas *BRD4* expression regulates G₂-M transition, *BRD4* overexpression inhibits G₁-S phase transition. In addition, *in vivo* studies (4) suggest a pivotal role for *BRD4* in cellular proliferation during embryogenesis.

To fully characterize the molecular mechanism of oncogenesis in t(15;19)-associated carcinomas, we undertook mapping and cloning of the chromosome 15 translocation target. Herein, we demonstrate that the chromosome 15 translocation rearranges the novel gene, *NUT*, resulting in a *BRD4-NUT* fusion oncogene. We also report expression

profiles for *NUT* and *BRD4* in normal tissues. These studies reveal the first known fusion oncogene in a highly malignant form of epithelial neoplasia.

Materials and Methods

Electronic Sequence Analysis. Human ESTs³ in the 15q13 translocation breakpoint (*Nop10p* region) region were identified using the Human Genome Browser (UCSC). Sequence analyses were performed using BLAST [National Center for Biotechnology Information (NCBI)].

Cell Lines. We established two rapidly growing, immortal cell lines from t(15;19) carcinomas, both of which have been reported previously (5, 1). The cell lines have been cytogenetically stable, with persistence of the t(15;19) translocation in all cells after more than 20 passages.

RNA Isolation and RT-PCR. Polyadenylate-enriched RNA was isolated using Micro FastTrack (Invitrogen Corporation, Carlsbad, CA). RT-PCR was performed using the SMART RACE Kit (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instructions, with first-strand synthesis using SMART adapter primers. *BRD4-NUT* fusion cDNA was then amplified by nested PCR using ExTaq (Takara Bio Inc., Otsu, Shiga, Japan). Primers were chosen using the Whitehead Genome Center Primer3 software, and all of the primers were numbered according to *BRD4* and *NUT* cDNA sequences (as per GenBank accession nos. AF386649 and AF482429, respectively). First-round primers were BR2276F (AAGTTGATGTGATGCCG-GCTCCTC) and NUT1194R (GAGGTCTCTGGGCTTTACGCTGACG), and second-round primers were BR2334F (GAGTCCAGTGAGTCCAGCTC-CTCTG) and NUT1132R (GGAATGTACTGGCTGCTGGCAAA). Gel-purified nested PCR products were cycle sequenced by incorporation of ABI PRISM Big Dye Terminators (Perkin-Elmer, Inc., Wellesley, MA) and analyzed on an ABI 310 sequencer.

Nested PCR-amplification of *NUT-BRD4* was performed as described for *BRD4-NUT* using several forward and reverse primer combinations. Details of the oligonucleotide primers are available by request.⁴

Northern Blot Analysis. Polyadenylate-enriched RNA was isolated from cultured cells using Micro FastTrack kit (Invitrogen), separated by electrophoresis through a formaldehyde-containing gel, and then transferred to a Hybond-N membrane (Amersham Biosciences, Piscataway, NJ). The blot was hybridized with a 400-bp *NUT* cDNA probe. Details of the oligonucleotide primers for all Northern blot probes are available by request.⁴ The blot was then stripped and hybridized with a 650-bp 5' *BRD4* cDNA probe, followed by additional rounds of stripping and hybridization with cDNA probes to 3' *BRD4* and β -Actin. All of the cDNA probes were labeled using a Prime-It II Random Primer Labeling kit (Stratagene, La Jolla, CA) and then purified by S-200HR spin column chromatography (Pharmacia Corporation, Peapack, NJ). Multipletissue Northern blots (Human MTN Blot II and III, Clontech Laboratories, Inc.) were hybridized with the *NUT*, 5' *BRD4*, and β -Actin cDNA probes, as above.

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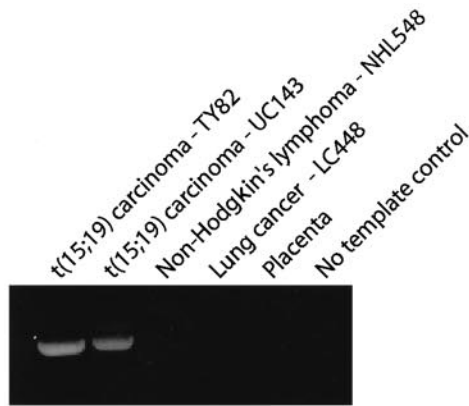
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³The abbreviations used are: EST, expressed sequence tag; MTN, multiple tissue Northern (blot); UCSC, University of California at Santa Cruz; BLAST, basic local alignment search tool; RT-PCR, reverse transcription-PCR; ET, extraterminal; CBP, Creb binding protein.

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A



B

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MSAESGPGTRLRNLPVMDGLETQSMSTTQAQAQPQANAASTNPPPPETSNPNPKRQNTNQLYLLRVV
BROMODOMAIN 1
LKT LWKHQFAWPFQFVDVAVKLNLPDYKIIKT PMDMGT I KKRLENNYVWNAQECIQDFNTMTNICY IYN
KPGDDIVLMAEAELEKFLQKINLELPTETEIMI VQAKGRGRGRKETGTAKPGVSTVPTTQASTPPQQTQ
PQNPFPVQATPHPPFAVTPDLIVQTPVMTVPPVQPIQT PPPVFPQFQPPPAPAPQVQSHPP IIAATPQ
PVKTKKGVKRRKADTTTPTTI DPEHEPSSLPEPEKTT KLQORRESSRPVKPKKDVDPDQSHQPAPEKSSKV
BROMODOMAIN 2
SEQLKCCSGI LKEMFAKHAAYAWPFYKPVVDEALGLHDYCDI I KHPMDMTI KSKLEAREYRDAQEFGA
DVRMLFSNCKYKYNPPDHEVAVAMARKLDQVEMRFAPKMPDEPEEPVAVSSPAVPPPTKVVAVPSSSDSS
DSSSDSDSST DSEERAQR LAELQEQ LKAVHEQLAALSQPQNKPKKKEKDKKKEKKEKHKRKEEVEEN
NLS
KSKAKAPEPPPKTKKNNSSNSVNSKEPEAPMKSKPPPT YESEEDKCKPMSYEEKRQLSLDINKLPGKEL
ET DOMAIN NLS
GRVVHIIQSREPSLKNSNPDEIBIDFETLKPSTLRELERVYVTSCLRRKKRKEQAEKVDVIAGSSKMKGFSS
SERINE RICH BRD4 NUT
SESSESSSSSSDSESETASALPGPDMSPKPSAALSFPALPFLPPTSDPPDHPPREPPFPQIMPVSVFS
PDNPLMLSAFPSSLLVTGDDGPCLSGAGAKVIVKVT EGGSAEPSQTNFI LTQTALNSTAGPTPCGGL
EGPAPPVFTASNKTI LPSKAVGVSQEGPGLPQPQPPVAVLQVPI VPLEKAWPGHGTGEGGPVATLS
KPSLGDRS KISKDVY ENFRQWRQYKALARRHLSQSPDTEALSCFLI PVLRSLARLKPMTLEBGLPLAVQ
EWEHTSNFDRMI FYEMAERFMEFEAEEMQIQNTQLMNGSQGLSPAT PLKLDPLGLASEVCQPPVYIPKK
AASKTRAPRRQRKAQRPPAPEAPKEIPEAVKEYVDIMEWLVGTHLATGESDGKQEEEGQQEEEGMYP
DPGLLSYINELCSQKVFVSKVEAVIHPQFLADLLSPEKQRDPLALI EEELEQEBGLTLAQVLQKRLMALEE
EEDAEAPPFSFGAQLDSSPSGSVEDEGDGRLRPSPGLQGAGAAACLGKVS SSGKRAREVHGGQEQALDS
PRGMHRDNTLPSSSWDLQPELAAPQGT PGPLGVERRSGKVINQVSLHQDHLGGAGPPGHCLVADRT
SEALPLCQGGQFPESTPSLDAGLAELAPLQGGLEKQVLGLQKQQQTGGRGVLPQGEPLAVPWEQSSG
AMWGDRTGPTMAQSYDQNPSPRAAGERDDVCLSPGVWLSSEMDAVGLELPVQIEEVI ESFQVEKCVTEYQ
EGCQGLSRGNISLGPGETLVPGDTESSVIPCQGTAAAALEKRNYSCLPGPLRANSPPLRSKENQEQSC
ETVGHPSDLWAEKCFPLLES GDSTLGSSKETLPPTCQGNLLIMGTEDASSLPEASQEAAGSRGNSFSPILLE
TIEFVNI LDVKKDCGLQLRVSEDTCP LNVHSYDPQGEGRVDPDL SKPNLAPLQESQSYTTGT PKATSS
HQGLGSLPRRGT RNAI VPRET SVSKTHRSADRAGKKEKKEEEDDEELS NFAYLLASKLSLSPREHPL
SPHHASGGQSGRASHLLPAGAKGKPSKLPYVAKSGKRALAGGPAPTEKTPHSGAQLGVPREKPLALGVVR
NLS
PSQPKRRKCDSFVTGRRKKRRRSQ

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Results

Identification of an EST Flanking the Chromosome 15q13 Breakpoint. Our previously reported Southern blotting analyses (1) narrowed the chromosome band 15q13 breakpoint to a <9-kb region. We searched for genes in this region using the Human Genome Browser (Human Genome Project Working Draft, UCSC). Thereby, we identified a spliced EST (GenBank accession no. AL040312) whose sequence appeared to span the <9-kb translocation breakpoint region. BLAST analysis revealed that this EST overlapped a 2134-bp cDNA clone (GenBank accession No. AL133071) that, as ascertained by 6-frame translation Baylor College of Medicine (BCM) Search Launcher), comprised the 3' coding sequence of a novel gene. Because the eight known ESTs for this gene (GenBank) were from testis cDNA libraries and were also based on Northern blot evidence for testis-restricted expression (see below) and for the presence of typical nuclear localization signals (see below), we have preliminarily named this gene *NUT* (Nuclear Protein in Testis).

Identification of the *BRD4-NUT* Fusion Transcript. RT-PCR was performed empirically using *NUT* reverse primers and *BRD4* forward primers (exon 10) predicted, based on our genomic localizations, to be 5' to the *BRD4* translocation breakpoint. A 1-kb *BRD4-NUT* fusion product was amplified readily and reproducibly from each of two t(15;19)-positive cancers, whereas *BRD4-NUT* RT-PCR products were not obtained from t(15;19)-negative control cDNAs (Fig. 1A). Sequencing revealed that the *BRD4-NUT* RT-PCR products in both of the t(15;19)-positive tumors were identical, containing in-frame fusion transcripts in which *BRD4* exon 10 is fused to *NUT* exon 2.

RT-PCR using numerous nested forward and reverse primer pairs failed to amplify a *NUT-BRD4* product from either of the t(15;19)-positive carcinoma cell lines.

***BRD4-NUT*, *BRD4*, and *NUT* Gene Structures.** The t(15;19) translocation breakpoint bisects the *BRD4* longer transcript into components encoding amino acids 1–720 and 721–1372. The NH₂-terminal component (Fig. 1B) contains the BRD4 bromodomains and, therefore, might contribute chromatin-binding and potential coactivation functions (6) to the BRD4-NUT fusion oncoprotein. Other BRD4 domains in BRD4-NUT are less well characterized. These include a potential kinase domain (3), an ET protein-protein interaction domain (7), and a serine-rich potential transactivation or corepressor domain (Ref. 8; Fig. 1B).

The chromosome 15 translocation breakpoint separates *NUT* exon 1 (potentially encoding amino acids 1–5) from exons 2–7 (encoding amino acids 6–1127). Hence, almost the entire *NUT* coding sequence is contained in the *BRD4-NUT* fusion transcript. *NUT* (GenBank accession no. AF482429) is predicted to encode a *M_r* 120,000 nuclear protein with 64% homology to a novel *M_r* 66,800 (GenBank accession no. AL132656) protein encoded by a locus on chromosome 10.

Expression of *BRD4-NUT*. *NUT* expression was evaluated by Northern blot analysis in a t(15;19)-positive carcinoma cell line, normal human testis, and in cell lines established from other malignant tumors (Fig. 2A). A 6.4-kb putative *BRD4-NUT* transcript was expressed in the t(15;19)-positive carcinoma cell line, and a 3.6-kb wild-type *NUT* transcript was expressed in normal testis. *NUT* ex-

Fig. 1. A, RT-PCR demonstrates identical *BRD4-NUT* fusion transcripts in t(15;19)-positive carcinoma cell lines, TY82 and UC143. Control reactions were performed using cDNAs from NHL548 (*non-Hodgkin's lymphoma*), LC448 (*lung carcinoma*), total placenta, and no template. B, predicted BRD4-NUT protein sequence includes the two bromodomains, ET domain, and serine-rich domain from BRD4, and almost the entire *NUT* sequence. The bipartite nuclear localization sequences (NLS) are preserved in the BRD4 component, and an additional putative NLS is contributed by *NUT*.

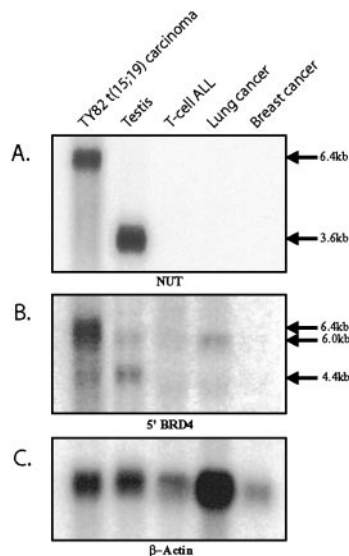


Fig. 2. Northern blot analysis of *NUT* (A), *BRD4* (B), and β -actin (C) expression in TY82 t(15;19)-positive cell line, normal testis, and three t(15;19)-negative cancer cell lines. A, a 6.4-kb aberrant *NUT* transcript is seen in TY82, and a normal 3.6-kb *NUT* transcript is demonstrated only in testis. B, *BRD4* probe hybridizes to the 6.4-kb aberrant transcript in TY82, corroborating a *BRD4-NUT* fusion transcript. 4.4- and 6.0-kb normal *BRD4* transcripts are expressed variably in all of the samples.

pression was not detected in t(15;19)-negative tumor cell lines. Re-hybridization of the blot with a *BRD4* 5'-end cDNA confirmed the fusion nature of the t(15;19)-positive carcinoma 6.4-kb *BRD4-NUT* transcript (Fig. 2B). Rehybridization with a *BRD4* 3'-end cDNA showed no evidence of a *NUT-BRD4* fusion transcript (data not shown), and was, therefore, consistent with the aforementioned RT-PCR evaluations. These findings indicate that *BRD4-NUT*, and not its reciprocal transcript, *NUT-BRD4*, is the functional oncogenic transcript in t(15;19)-positive carcinoma.

Expression of *BRD4* and *NUT* in Normal Tissues. *BRD4* and *NUT* expression were evaluated by MTN II and III (Clontech Laboratories, Inc.). *BRD4* 4.4- and 6.0-kb transcripts, which likely encode the short and long *BRD4* isoforms, respectively, were expressed ubiquitously (Fig. 3A). By contrast, *NUT* expression was highly restricted. A 3.6-kb *NUT* transcript was confirmed in normal testis,

whereas all other tissues lacked demonstrable *NUT* expression (Fig. 3B).

Discussion

The studies reported herein demonstrate that the t(15;19) translocation in poorly differentiated carcinoma results in fusion of the *BRD4* and *NUT* genes. *BRD4-NUT* transcripts were demonstrated by RT-PCR in two t(15;19)-positive cell lines, whereas *NUT-BRD4* reciprocal transcripts were not demonstrable in either cell line. Northern blotting confirmed these findings. Therefore, we conclude that *BRD4-NUT* is the functional fusion oncogene associated with the t(15;19) translocation. Although the oncogenic mechanisms of *BRD4-NUT* have not yet been determined, the t(15;19) is highly characteristic of a clinically distinctive cancer, and it is likely that *BRD4-NUT* plays a pivotal pathogenetic role in this extremely lethal form of carcinoma.

Several aspects of these findings are unique and intriguing. The t(15;19)-positive poorly differentiated carcinoma is one of very few epithelial neoplasms that harbor defining translocations (9). Among this limited group, the t(15;19)-positive carcinoma is by far the most clinically aggressive. Most patients die within 3 months (1, 10). Therefore, this cancer provides one of the few models with which to study (a) the role of fusion oncogene mechanisms in epithelial neoplasia, and (b) oncogenic mechanisms of clinically aggressive neoplasia. In particular, the transforming mechanisms of bromodomain oncoproteins, although studied recently in hematological neoplasia (11), have not been evaluated in solid tumors.

Although the clinical ramifications of *BRD4-NUT* have not been determined in systematic studies, all of the known t(15;19)-positive carcinomas were rapidly metastasizing and extremely lethal. Therefore, we expect that diagnostic recognition of *BRD4-NUT* will be useful in identifying this clinically aggressive subset of carcinomas. Presumably, individuals with t(15;19)-positive carcinoma require immediate treatment with intensive systemic therapies to attempt ablation of their rapidly progressive disease. Such therapeutic efforts will be enabled by prompt molecular diagnosis of the *BRD4-NUT* mechanism. The clinical diagnosis of *BRD4-NUT* oncogenes can be accomplished by several methods. The translocation t(15;19) can be demonstrated in fresh clinical specimens by conventional karyotyping, as has been reported by several groups (10, 12–15). *BRD4-NUT* genomic rearrangement can

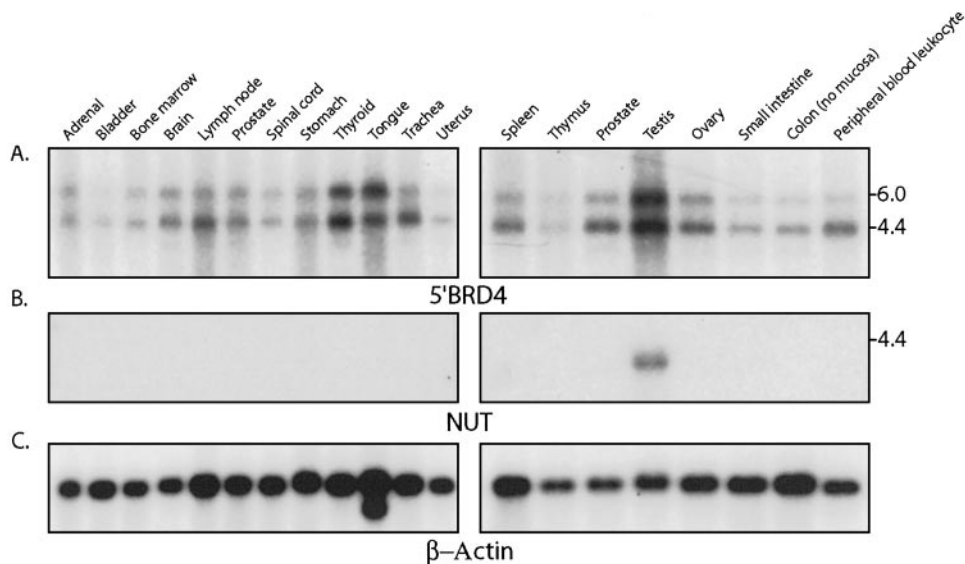


Fig. 3. Evaluation of *BRD4* and *NUT* expression in normal human tissues. Clontech MTNs (MTN II and III) were hybridized with cDNA probes to 5' *BRD4* (A), *NUT* (B), and β -actin (C). *BRD4* 4.4 and 6.0 kb transcripts are ubiquitously expressed (A), whereas *NUT* is expressed exclusively in testis (B).

also be demonstrated, including in frozen or formalin-fixed specimens, by fluorescence *in situ* hybridization (1). Although not yet evaluated in clinical specimens, we have shown herein that the *BRD4-NUT* genomic rearrangement can be determined at the transcript level by RT-PCR. Furthermore, it is conceivable that NUT immunohistochemistry might provide a simple alternative to molecular detection, given that *NUT* expression has been identified thus far only in normal testis and t(15;19)-positive carcinoma.

The COOH-terminal end of BRD4-NUT incorporates almost the entire *NUT* sequence. Given that *NUT* expression is restricted to the testis, it is likely that unscheduled *NUT* expression in the t(15;19)-positive carcinomas results from oncogenic juxtaposition to *BRD4*, with expression then being regulated by the *BRD4* promoter elements. *NUT* oncogenic function likely results both from ectopic expression in an epithelial cell lineage and from the structural consequences of fusion to *BRD4*. Expression of *NUT* protein, therefore, may be a specific marker of the t(15;19) carcinoma, as well as a potential therapeutic target.

BRD4 represents the first known oncogene from the BET family of bromodomain genes, which are defined by the presence of NH₂-terminal bromodomain(s) and an ET domain (7). The intact bromodomain regions and the ET domain are contained in the BRD4 component of the BRD4-NUT fusion oncoprotein. The second bromodomain of *BRD4* has been shown to directly bind replication factor C, a multi-subunit complex essential for DNA replication, and through this interaction, inhibits G₁-S phase transition when overexpressed (2).

Given the known functions of the longer *BRD4* isoform, and the ubiquitous presence of the biologically uncharacterized short isoform, the oncogenic *BRD4* mechanisms are likely to be complex in the t(15;19) carcinoma. One scenario that warrants evaluation is that the *BRD4* short and long isoforms might have qualitatively different, and even opposing, functions in cell cycle regulation. Such a mechanism could be relevant in the t(15;19) carcinomas, in which the translocation transects the coding sequence of the *BRD4* long isoform, without affecting that of the short isoform. These considerations suggest that the t(15;19) translocation breakpoint might abrogate *BRD4* long isoform function, and it is significant that the *BRD4* component in the BRD4-NUT fusion protein comprises virtually all of the predicted *BRD4* short isoform. Therefore, the BRD4-NUT oncoprotein could function as a dominant negative in relationship to the *BRD4* long isoform, and might also contribute a gain-of-function equivalent of the *BRD4* short isoform. Notably, the *BRD4* short and long isoforms are expected to compete for binding to chromatin, given that their bromodomain-containing NH₂-terminal regions are identical. Consequences of BRD4-NUT fusion that might contribute to transforming function could, therefore, include: (a) reduced expression of *BRD4* long isoform; (b) increased avidity of BRD4-NUT in chromatin binding, resulting in functional inhibition of the non-fusion *BRD4* isoforms; and (c) other altered *BRD4* functions, *e.g.*, perturbed interactions with corepressors/coactivators, possibly resulting from the *NUT* component of the fusion protein.

Although *BRD4-NUT* is the first example of a bromodomain-containing oncogene in a solid tumor, bromodomain-containing fusion oncoproteins have been characterized in several types of leukemia. For example, the CBP bromodomain-containing protein is rearranged in acute myelogenous leukemia with t(8;16) translocation (11), resulting in a fusion oncogene. MOZ is a transactivat-

ing component of the MOZ-CBP complex (16), which is thought to regulate transcription of genes that influence myeloid differentiation programs. In contrast, the MOZ-CBP fusion oncoprotein inhibits AML1-mediated transcription and thereby creates a leukemogenic myeloid differentiation block through dominant-negative effects on AML1 function (16). By analogy, the BRD4-NUT fusion oncoprotein might function in part via perturbed interactions between *NUT* and *NUT*-binding proteins. This possibility seems particularly likely in that nearly the entire *NUT* sequence is included in BRD4-NUT. However, the model of BRD4-NUT in t(15;19) carcinoma differs from that of MOZ-CBP in leukemia, because MOZ-CBP fusion alters the function of proteins that are normally expressed in the transformed cell lineage. In the case of BRD4-NUT, there are presently no clues (other than nuclear localization) as to the normal function of *NUT*, and there is no evidence that non-oncogenic *NUT* plays important functional roles, or is even expressed, in epithelial cell lineages.

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