Breast Cancer Metastasis Suppressor 1 (BRMS1) Forms Complexes with Retinoblastoma-binding Protein 1 (RBP1) and the mSin3 Histone Deacetylase Complex and Represses Transcription*

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Breast cancer metastasis suppressor 1 (BRMS1) suppresses metastasis of multiple human and murine cancer cells without inhibiting tumorigenicity. By yeast two-hybrid and co-immunoprecipitation, BRMS1 interacts with retinoblastoma binding protein 1 and at least seven members of the mSin3 histone deacetylase (HDAC) complex in human breast and melanoma cell lines. BRMS1 co-immunoprecipitates enzymatically active HDAC proteins and represses transcription when recruited to a Gal4 promoter in vivo. BRMS1 exists in large mSin3 complex(es) of ~1.4–1.9 MDa, but also forms smaller complexes with HDAC1. Deletion analyses show that the carboxyl-terminal 42 amino acids of BRMS1 are not critical for interaction with much of the mSin3 complex and that BRMS1 appears to have more than one binding point to the complex. These results further show that BRMS1 may participate in transcriptional regulation via interaction with the mSin3-HDAC complex and suggest a novel mechanism by which BRMS1 might suppress cancer metastasis.

The complex process of cancer cell dissemination and the establishment of secondary foci involves the acquisition of multiple abilities by metastatic cells. For example, blood-borne metastasis requires cells to invade from the primary tumor, enter the circulation, survive transport, arrest at a secondary site, recruit a blood supply, and proliferate at that site (1). The ability to accomplish all of these steps likely involves changes in, and coordinated expression of, a large assortment of genes. Consistent with this notion, several genes, proteins, and pathways have been associated with metastatic progression, including oncogenes, motility factors, and matrix metalloproteinases (1, 2). In addition to metastasis-promoting genes, a new class of molecules called metastasis suppressors has been described (reviewed in Refs. 2–5). By definition, metastasis suppressors inhibit metastasis without blocking primary tumor growth, presumably by inhibiting one or more steps necessary for metastasis. To date, 13 metastasis suppressor genes have been identified that reduce the metastatic ability of cancer cell line(s) in vivo without affecting tumorigenicity, namely breast cancer metastasis suppressor 1 (BRMS1),1 CRSP3, DRG1, KAI1, KISS1, MKK4, NM23, RhoGDI2, RKIP, SSECKs, VDUPI, E-cadherin, and TIMPs (reviewed in Refs. 4 and 5).

We identified BRMS1 using differential display to compare highly metastatic breast carcinoma cells with related but metastasis-suppressed cells (6). Enforced expression of BRMS1 suppressed metastasis in three animal models, namely human breast (6), murine mammary (7), and human melanoma cells (8). Additionally, BRMS1 mapped to loci in murine (7) and human (6) genomes that had previously been implicated in metastasis control (9). The BRMS1 protein localized to nuclei and restored gap junctional intercellular communication in both breast and melanoma tumor cell lines (8, 10, 11), but its molecular functions remain to be elucidated.

One approach to determine a mechanism of action involves identifying which proteins interact with BRMS1. In this report, we utilized yeast two-hybrid and co-immunoprecipitation (co-IP) to demonstrate that BRMS1 interacts with retinoblastoma-binding protein 1 (RBP1). This association led to experiments to demonstrate that BRMS1 interacts with at least seven members of the mammalian Sin3 (mSin3) mSin3-histone deacetylase (HDAC) complexes, including HDAC1 and HDAC2.

Human HDACs exist in many large, multi-subunit protein complexes (12) that are recruited to specific regions by DNA-binding factors. As their name indicates, HDACs remove acetyl groups from lysine residues at the N-terminal tails of core

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1 The abbreviations used are: BRMS1, breast cancer metastasis suppressor gene 1; co-IP, coimmunoprecipitation; RBP1, retinoblastoma binding protein 1; HDAC, histone deacetylase; mSin3, mammalian Sin3 (suppressor of defective silencing 3); NuRD, nucleosomal remodeling and deacetylation; E2, retinoblastoma; NCoR, nuclear receptor corepressor; SMRT, silencing mediator for retinoic acids and thyroid hormone receptor.

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Antibodies recognizing E2F and retinoblastoma (Rb) were bought from LY11. Antibodies directed against Garth Nicolson. Anti-RBP1 (clone LY32 and initial aliquots of clone LY11) were gifts of Dr. Philip Branton. Antibodies directed against BRMS1 were passaged at 80% confluence using 2 mM EDTA in CMF-DPBS. C8161.9 and 66cl4 cells were passaged at 80% confluence using 2 mM EDTA in CMF-DPBS.

Mammalian SDS3 (mSD3; suppressor of defective silencing 3) was recently reported to be an integral component of the mSin3 complex and acts to stabilize HDAC1 within the complex (17). BRMS1 shares homology with mSD3, suggesting that BRMS1 belongs to a protein family (17). mSin3-associated proteins, SAP18 and SAP30, which are believed to serve as adapter molecules, complete the core complex as currently understood (18–20).

MATERIALS AND METHODS

Cell Lines, Cell Culture, and Transfections—MDA-MB-231 is a human estrogen receptor- and progesterone receptor-negative cell line derived from pleomorphic ductal breast carcinoma. C8161.9 and 66cl4 cells were passaged at 80% confluence using 2 mM EDTA in Ca2+/Mg2+-free Dulbecco’s modified Eagle’s medium and Ham’s F12 medium supplemented with 5% fetal bovine serum (Atlanta Biologicals, Atlanta, Georgia), 1% non-essential amino acids, and 1 mM sodium pyruvate. Transfected cells also received 500 μg/ml G418 (Genetecin; Invitrogen). All cells were maintained on 100-mm Corning tissue culture dishes at 37 °C with 5% CO2 in a humidified atmosphere. MDA-MB-231 cells were passaged at 80–90% confluence using a solution of 0.125% trypsin and 2 mM EDTA in Ca2+/Mg2+-free Dulbecco’s phosphate buffer saline (CMF-DPBS). C8161.9 and 66cl4 cells were passaged at 80–90% confluence using 2 mM EDTA in CMF-DPBS.

BRMS1 was cloned in the constitutive mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) under control of the cytomegalovirus promoter. No antibiotics or ampicillin were used. All cell lines were found to be negative for Mycoplasma spp. contamination using a PCR-based method (TaKaRa, Madison, WI).

To detect BRMS1 protein expression, a chimeric molecule was constructed with an N-terminal epitope tag (SV40T epitope 901, 24, 25). Epitope-tagged full-length BRMS1 and deletion mutants were cloned into pcDNA3 before introduction into cells by electroporation (Bio-Rad model Gene Pulsar; 220 V, 960 microfarads, ~8 ohms). Briefly, cells (0.8 ml; 1 × 106 cells/ml) from 80% confluent plates were detached, plasmid DNA (10–40 μg) was added to the cells, and the mixture was placed onto ice for 5 min before electroporation, followed by 10 min on ice prior to plating on 100-mm cell culture dishes. Transfectants were selected using G418 (Genetecin; 500 μg/ml). Single-cell clones were isolated by limiting dilution in 96-well plates. Stable transfectants were assessed for protein expression by immunoblotting.

Construnctions—Deletion mutants were created by unidirectional digestion with exonuclease III as described previously (26). Briefly, pcDNA3 901-BRMS1 was digested by Apal and Bsa36I in the 5′ multiple cloning site and then digested with 150 units of III (Hind) (0.5 M) at 37 °C. Reactions were stopped at different time points to create a nested set of C-terminal BRMS1 deletion mutants. Sequencing confirmed that the following 3′ deletion mutants were successfully created: 1) 901-BRMS1Δ204–246 + LYSVFT; 2) 901-BRMS1Δ164–246 + TIL; and 3) 901-BRMS1Δ191–246 + FYSVT. Additional amino acids were added before a short streptag for DNA was transcribed prior to encountering a stop codon. Hereafter, these constructs will be designated BRMS1Δ204–246, BRMS1Δ164–246, and BRMS1Δ191–246, respectively.

Antibodies—An antibody directed against the 901 epitope was generously provided by Dr. Satvinder Tehswa (anti-MTA1 was a gift from Dr. Garth Nicolson). Antibodies against HDAC1, HDAC3, NCoR, RBPP1 (clone LY11), SAP30, mSin3A, and SMRT were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies recognizing E2F and retinoblastoma (Rb) were bought from Pharmingen. Antibodies directed against HDAC2, Mad1, Max, Mi-2, p107, p130, RBpA46, RBpA48, SAP18, and mSin3B were obtained from Santa Cruz Biotechnology.

Yeas Two-hybrid Screen—A yeast two-hybrid screen was performed to isolate cDNAs encoding BRMS1-interacting proteins essentially as described in the manufacturer's instructions (Clontech MATCHMAKER LexA). Full-length BRMS1 was cloned in-frame with the GAL4 DNA binding domain (LexA) in the pDBTrp (Invitrogen) vector to obtain plasmid pBRMS1. This GAL4DBB3-RMS1 fusion (bait) construct was used to transform AH109 (MATa, trp-901, leu-2, 112, ura3–52, his3–200, gal4Δ, gal80Δ, lys2::GALUAS-GALI-TAT-ADE3, GALUAS-GAL2-TAT-ADK2, URA3::MEL1::MELI::DATA-lacZ, MEL1::). Human breast, prostate, and placenta cDNA libraries in pACT2 (MATCHMAKER, BD Biosciences Clontech) were screened in yeast drops plated on minimal medium containing 1% glucose, tryptophan, and leucine. His+ colonies were tested for growth on minimal medium lacking adenine, tryptophan, leucine, and β-galactosidase activity as described previously (27). cDNA plasmids were isolated from each positive yeast clone using Zymoprep (Zymo Research, Orange, CA) and sequenced. The interaction phenotype was lost when either the bait or prey plasmid was lost from the cell. Re-introduction of missing partners restored growth on minimal medium lacking histidine, tryptophan, and leucine, growth on medium lacking adenine, tryptophan and leucine, and restoration of β-galactosidase activity.

β-35S Protein Labeling—Cells were grown to 80–90% confluence in 100-mm tissue culture plates. Media were removed and replaced with 3 ml of cysteine- and methionine-free media (Invitrogen) containing 1% fetal bovine serum for 1 h. Media were removed and replaced with 3 ml of cysteine- and methionine-free media containing 5% fetal bovine serum and 100 μCi/ml 35S-express protein labeling mix (PerkinElmer Life Sciences). Cells were incubated for 18 h before protein was collected for co-IP.

Co-immunoprecipitation—Cells (90–95% confluence) were washed twice with ice-cold PBS and lysed with ice-cold lysis buffer (0.5% Igepal CA-630 (Sigma), 50 mM Tris, pH 8, 150 mM NaCl, and 2 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 50 mM NaF, 0.2 mM Na3VO4, and 10 μM of a protease inhibitor mixture containing 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF), pepstatin A, and benzamidine 4-glyoxalysylglycylglycine (BGG); bestatin, leupeptin, and aprotinin (Sigma). Lysate was kept at 4 °C during all subsequent steps. Lysate was passed through a 21-gauge needle several times, incubated on ice for 1 h, then centrifuged for 1 h at 12,000 × g in a Sorvall MC 12V microcentrifuge with an F12/M.18 rotor to remove insoluble debris. Lysates were then rocked gently in the presence of antibody for 1 h, followed by the addition of 20 μl of protein A PLUS agarose beads (Santa Cruz Biotechnology) and rocking overnight. Agarose beads were washed twice with ice-cold PBS, heated to 60 °C in 3 ml sample buffer, subjected to SDS-PAGE, and transferred to polyvinyldene difluoride membrane for immunoblotting. For 35S-labeled samples, films were exposed directly to polynvinyldene difluoride membrane. Immunoreactivity was detected by autoradiography and the immunoprecipitated protein to verify the quality of the immunoprecipitation and assess the equal loading of lanes.

Size Exclusion Chromatography—Whole cell protein lysate (pooled from 10 106-mm plates using 1 ml of lysis buffer each) was applied to a Superose 6 HR 10/30 size exclusion column (Amersham Biosciences). The column was run using lysis buffer with 1 mM phenylmethylsulfonyl fluoride and 0.5 mM ethylenediaminetetraacetic acid and 0.5 mM dithiothreitol at a flow rate of 0.2 ml/min. Fractions (500 μl) were collected, and 420 μl of each fraction were used for co-IP. The remaining 80 μl was used for immunoblotting.

HDAC Activity Assay—Following co-IP, agarose beads were combined with 400 μl of HDAC assay buffer (15 mM Tris, pH 7.9, 10 mM NaCl, 10 mM NaF, 10 mM sodium butyrate, 10 mM DTT, 100 μg/ml aprotinin, and 0.3 μg/ml containing 1.5 μg 3H-labeled chicken reticulocyte core histones (28) with or without 250 mM sodium butyrate (an HDAC inhibitor). Samples were inverted continuously on a rotating wheel for 3 h at 30 °C, and HDAC activity was measured as described previously (28). Briefly, the reaction was stopped by adding 100 μl of 1 × HCL/0.4 M acetic acid and 0.4 M sodium acetate. Samples were vortexed for 30 s and centrifuged at 8,000 × g for 5 min. An aliquot (0.6 ml) of the upper (organic) phase was then counted for radioactivity in a 5-ml scintillation mixture (Fisher).

Reporter Assays—BRMS1 cDNA was cloned in-frame with the N-terminal Gal4-DNA binding domain in pBIND (Promega). Subconfluent (80–90%) COS7 cells were transfected using the FuGENE reagent (Roche Diagnostics) with indicated plasmid DNA and a luciferase reporter plasmid containing four GAL4 binding sites upstream of the myelomocytic growth factor minimal promoter, kindly provided by Dr. Ron Eisenman. pRLSV40 (Renilla luciferase) was used as a transfection control. Trichostatin A (50, 150, and 300 ng/ml, Sigma)
BRMS1 Interacts with the mSin3 HDAC Complex

RESULTS

RBP1 and mSds3 Were Identified as BRMS1-interacting Proteins by Yeast Two-hybrid Screen—A yeast two-hybrid screen was performed using prey libraries from three human tissues, breast, placenta, and prostate. Breast was chosen because BRMS1 was first identified as a metastasis suppressor in breast cancer. Placenta and prostate were chosen because BRMS1 mRNA is highly expressed in these tissues (6). Full-length BRMS1 was used as the “bait.” RBP1 was present in the majority of positive clones from breast and placenta libraries, so it was chosen for further studies (Fig. 1, A and B).

The FLJ00052 expressed tag was present as two independent positive clones in a prostate library. During the completion of the work reported here, FLJ00052 was identified as the mammalian ortholog (mSds3, GenBank™ accession number XM_045014 mapping to human chromosome 12q24.23) of the yeast Sds3 protein. There are other related genes according to the LocusLink (www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q = FLJ00052&ORG = &V = 0), suggesting the existence of additional mSds3 orthologs. mSds3 is an integral component of the mSin3-HDAC co-repressor complex, modulates HDAC activity, and stabilizes the complex (17). Antibodies recognizing mSds3 are not available commercially; thus, we have not yet been able to test whether BRMS1 pulled down mSds3.

BRMS1 and RBP1 Are Reciprocally Co-immunoprecipitated in Human Breast and Melanoma Cancer Cells—MDA-MB-231 human breast carcinoma cells and C8161.9 human melanoma cells were transfected with 901 epitope-tagged BRMS1. Immunoprecipitation of BRMS1 followed by immunoblot with two RBP1-specific antibodies (clones LY11 and LY32) (Fig. 1, C and D) showed that BRMS1 co-immunoprecipitates RBP1 (Fig. 1, C and D). Negative controls (co-IP using anti-901 in vector-transfected cells or co-IP using an irrelevant antibody, anti-Lamin A/C) did not pull down RBP1 (Fig. 1, C and D). Antibody directed against RBP1 co-immunoprecipitated BRMS1 in both breast carcinoma (Fig. 1E) and melanoma (Fig. 1F).

To begin defining the binding domains of BRMS1 responsible for interactions with RBP1, three C-terminal deletion mutants of 901-tagged BRMS1 were generated by exonuclease III digestion, designated BRMS1Δ204–246, BRMS1Δ164–246, and BRMS1Δ91–246 (Fig. 2C). Deletion constructs were transfected into both MDA-MB-231 and C8161.9. The latter expressing clones were experimentally more useful, because expression of all three deletion mutants was approximately equivalent to full-length protein (data not shown, but can be inferred from Fig. 2B). In MDA-MB-231, only BRMS1Δ204–246-expressing clones had protein levels approximating full-length BRMS1 (inferred from Fig. 2A). Anti-901 antibody was used to co-immunoprecipitate deletion mutants, and immunoblotting was used to detect RBP1 (Fig. 2, A and B). Loss of amino acids 204–246 did not decrease binding to RBP1 in either cell line (Fig. 2, A and B). Loss of amino acids 164–246 diminished binding (by ~90% by densitometry), and loss of amino acids 91–246 abrogated binding (Fig. 2B). Absence of binding by BRMS1Δ91–246 was controlled internally for nonspecific binding of RBP1 to the 901 epitope. Interestingly, in both MDA-MB-231 and C8161.9, BRMS1Δ204–246 co-immunoprecipitated RBP1 more effectively (~1.5-fold) than full-length BRMS1 (Fig. 2, A and B).

BRMS1 Does Not Appear to Complex with Rb or p107 or to Modulate E2F-dependent Gene Expression—RB1 binds Rb family members p105 (RB) and p107 (30–32). Rb proteins, in turn, bind E2F and tether RB1 to E2F-responsive gene promoters. In this way, RB1 directly suppresses transcription. We tested the hypothesis that BRMS1 is part of an RB1-Rb-E2F complex; however, BRMS1 did not co-immunoprecipitate p105 or p107 in MDA-MB-231 (Fig. 2A) or C8161.9 cells (data not shown). Likewise, BRMS1 did not affect luciferase expression using an E2F-responsive promoter (data not shown). Taken together, these findings suggest that BRMS1 does not act as part of an RB1-Rb-E2F complex and that BRMS1 might be part of a previously undescribed RB1 complex that does not contain Rb.

BRMS1 Co-immunoprecipitates Several 35S-labeled Proteins in MDA-MB-231—Anti-901 was used to co-immunoprecipitate BRMS1 from 35S-labeled lysates from BRMS1-transfected MDA-MB-231. Vector-transfected cells were used as controls. In addition to BRMS1, several additional bands were evident, including prominent large proteins at ~200 kDa, ~160 kDa, and ~50 kDa as well as less intense bands just below 50 kDa and another at ~30 kDa. (Fig. 3). Parallel experiments were performed using BRMS1-transfected C8161.9 and BRMS1 (murine ortholog; Ref. 7)-transfected 66cl4. Similar 35S-labeled proteins were co-immunoprecipitated by anti-901 (data not shown). The pattern was reminiscent of previously published
results showing that RBP1 interacts with the mSin3 HDAC complex (31, 32). Specifically, HDAC1 and HDAC2 migrate at ~65/60 kDa. mSin3B and mSin3A migrate at ~160/150 kDa. These molecular mass proteins corresponded to the most prominent radiolabeled proteins co-immunoprecipitated with BRMS1 (Fig. 3). Therefore, we hypothesized that BRMS1 is a component of the mSin3-HDAC complex.

BRMS1 Is a Component of the mSin3-HDAC Complex in C8161.9 and MDA-MB-231—Immunoprecipitation of epitope-tagged BRMS1 followed by immunoblotting showed that BRMS1 pulled down seven proteins shown previously to be part of mSin3-HDAC complexes, namely mSin3A, mSin3B, HDAC1, HDAC2, SAP30, RbAp46, and RbAp48 (Fig. 4). The same proteins were not precipitated in vector-transfected cells (Fig. 4, lane 1), nor were they pulled down using an antibody to the nuclear protein Lamin A/C (Fig. 4, lane 2). Western blots demonstrated that BRMS1-associated proteins were present at comparable levels in both vector- and BRMS1-transfected cell lysates (data not shown), ruling out the possibility that vector-transfected cells had lower levels of mSin3-HDAC complex components. Interactions between BRMS1 and mSin3-HDAC were relatively strong, because they persisted in 0.5 M NaCl.

Antibodies recognizing mSin3B, HDAC1, HDAC2, and SAP30 "reverse" co-immunoprecipitated BRMS1 in C8161.9 cells as well (Fig. 6A).
mSin3-HDAC complex proteins exhibited the same general interaction pattern with BRMS1 deletion mutants as did RBP1, with some exceptions. BRMS1(Δ204–246) co-immunoprecipitated mSin3A, mSin3B, SAP30, and HDAC2 at levels comparable with full-length BRMS1 (Fig. 4). However, BRMS1(Δ204–246) co-immunoprecipitated HDAC1, RbAp46, and RbAp48 less efficiently than full-length BRMS1 (reduced ~40% by densitometry) (Fig. 4). This discrepancy is evident on co-IP/immunoblots simultaneously probed for HDAC1 and mSin3B, clearly demonstrating differential binding (data not shown). BRMS1(Δ164–246) co-immunoprecipitated all mSin3-HDAC complex components significantly less efficiently than full-length BRMS1 (reduced ~90% by densitometry), whereas BRMS(Δ91–246) did not co-immunoprecipitate any complex proteins (Fig. 4).

To determine whether BRMS1 interacted with mSin3-HDAC complex proteins in human breast cancer cells, proteins were co-immunoprecipitated from BRMS1-transfected MDA-MB-231. Six mSin3-HDAC complex proteins, mSin3A, mSin3B, HDAC1, HDAC2, SAP30, and RbAp48 (Fig. 5), were pulled down with BRMS1. Co-IP in vector-transfected cells did not co-immunoprecipitate these proteins (Fig. 5, lane 1) despite the proteins being present in both vector- and BRMS1-transfected lysates (Fig. 5, lanes 4 and 5). As above, interactions persisted in 0.5 M NaCl. RbAp46, a member of the core mSin3-HDAC complex, did not co-immunoprecipitate with BRMS1 in MDA-MB-231 cells (Fig. 5). Antibodies recognizing mSin3B, SAP30, HDAC1, and HDAC2 co-immunoprecipitated BRMS1 in MDA-MB-231 (Fig. 6B). BRMS1(Δ204–246) co-immunoprecipitated mSin3-HDAC proteins at levels comparable with full-length BRMS1 (Fig. 5). In both melanoma and breast carcinoma cells, it was not possible to definitively demonstrate that BRMS1 co-immunoprecipitates SAP18, because SAP18 anti-sera also recognized a band at ~18 kDa in vector- and BRMS1-transfected cells (Fig. 5).

BRMS1 Interacts with a Subset of mSin3-HDAC Complexes—Many proteins that bind HDAC complexes are responsible for recruiting complexes to specific promoters. However, BRMS1 does not have a predicted DNA-binding motif, suggesting that it might serve a different role as a member of subsets of mSin3-HDAC complexes.

As a first step to evaluate those potential roles, the ability of BRMS1 to co-immunoprecipitate selected HDAC complex components was tested. Mad and Max were the first proteins shown to recruit the mSin3-HDAC to a specific promoter (33–35), but BRMS1 did not co-immunoprecipitate Mad1 or Max (data not shown). The unliganded nuclear hormone co-receptors SMRT and NCoR have also been reported to recruit the mSin3 (36–39), but there are contradictory data (40). In our system, BRMS1 did not co-immunoprecipitate SMRT or NCoR (data not shown). mSin3-HDAC interaction with MeCP2, a methyl CpG-binding protein, has also suggested that repression associated with DNA methylation may be mediated, in part, by deacetylation (41). Yet, BRMS1 did not co-immunoprecipitate MeCP2 (data not shown). Because the core HDAC subunit (HDAC1, HDAC2, RAp46, and RbAp48) is also present in the NuRD HDAC complex (16), we asked whether BRMS1 complexed with NuRD. BRMS1 did not co-immunoprecipitate Mi-2 or MTA1, two members of the NuRD complex (data not shown). HDAC3, which is related to HDAC1 and HDAC2 and can complex with RBP1 (32), did not co-immunoprecipitate with BRMS1 (data not shown).
BRMS1 interacts with the mSin3 HDAC complex

To determine the size of BRMS1 complexes, whole cell lysates from BRMS1-transfected C8161.9 cells were subjected to Superose 6 size exclusion chromatography. Fractions were separated by PAGE, transferred to polyvinylidene difluoride, and immunoblotted for 901-BRMS1, HDAC1, SAP30, and mSin3B. These four proteins were chosen because they are core members of the complex. BRMS1 eluted in multiple peaks from the column with complex sizes ranging from ~100 to 2,000 kDa. BRMS1 elution was most prominent in peaks 5 and 6 (~1.7 MDa). HDAC1 also eluted in multiple peaks (fractions 4–22) with the majority present in fractions 8 and 9 (~1.4 MDa, Fig. 8A). SAP30 was detected in two peaks, one from fractions 4 through 14 and another from fractions 19 to 24, suggesting the existence of at least two complexes, the first ~1 MDa and the second ~<200 kDa (Fig. 8A). mSin3B is detected uniformly in fractions 3–17, indicating involvement in complexes ranging from ~2 MDa to hundreds of kDa (Fig. 8A).

BRMS1 was immunoprecipitated from 420 μl of each fraction followed by PAGE and immunoblot. The vast majority (>90%) of BRMS1 was present in complexes ranging in size between 1.4 and 1.9 MDa (fractions 5–9 shown in lanes 5–9, Fig. 8B). BRMS1 also precipitated in fractions 10–23. HDAC1, SAP30, and mSin3B co-immunoprecipitated with BRMS1 in fractions 5–9, although SAP30 is most abundant in fractions 8 and 9 (lanes 8 and 9, Fig. 8B). HDAC1, however, also co-immunoprecipitated with BRMS1 in fractions 10–21, suggesting that BRMS1 can be involved in smaller complexes with HDAC1 (lanes 10–21, Fig. 8B).

BRMS1 co-immunoprecipitates HDAC activity—To determine whether BRMS1-associated HDAC1 and HDAC2 were enzymatically active, complexes were assessed for deacetylase activity in C8161.9. Full-length BRMS1 co-immunoprecipitated HDAC activity; BRMS1(Δ204–246) pulled down less HDAC activity. BRMS1(Δ164–246) co-immunoprecipitates still less HDAC activity, whereas BRMS1(Δ91–246) pulled down only background activity (Fig. 7). This pattern is reminiscent of the pattern of interaction with HDAC1 seen by immunoblot (Fig. 4). As a positive control, anti-HDAC1 antibodies were able to pull down HDAC activity (Fig. 7) proportionate to the amount of antibody used (i.e. when 2× anti-HDAC1 was used, double the HDAC activity was precipitated). These results show that only a small portion of the HDAC1
activity present in the protein lysate is being measured. Vector-transfected cells and co-IP with anti-Lamin A/C served as negative controls (Fig. 7).

**BRMS1 Interacts with the mSin3 HDAC Complex**

Using a yeast two-hybrid screen, two cDNA clones identified as FLJ00052 were identified in the prostate library. As studies were underway to follow-up RBP1, mSin3, and HDAC findings, FLJ00052 was re-designated by GenBank™ as mSds3, the mammalian ortholog of *Saccharomyces cerevisiae* Sds3. Sds3 has been implicated in gene silencing through a Sin3-Rpd3 pathway (Rpd3 in a yeast HDAC1 ortholog) and is an integral component of the yeast Sin3-Rpd3 complex that is required for histone deacetylase activity (17, 54). BRMS1 shares 18% identity and 49% similarity with a large region of yeast Sds3 and 23% identity and 49% similarity with mSds3, mSds3, analogous to its yeast ortholog, is a component of the mSin3-HDAC complex, stabilizes HDAC1 within the complex, and augments HDAC activity (17). Another predicted mammalian protein of unknown function (designated MGC11296) is homologous to both Sds3 and BRMS1. Homology to BRMS1 is particularly strong (58% identity; 79% similarity for the C-terminal 196 amino acids of BRMS1 and the N-terminal 196 amino acids of MGC11296). The high level of sequence similarity between these molecules, combined with their associations with mSin3-HDAC complexes, suggests the existence of a BRMS1 family of proteins that may play a crucial role in altering metastasis by regulating the so-called histone code (29, 49).

**DISCUSSION**

Epigenetic regulation of the metastatic phenotype was proposed in 1889 when Sir Stephen Paget recognized that tumor cells colonize certain organs preferentially based, in part, upon how they respond to signals from the microenvironment (42). Trainer and co-workers later showed that treatment of murine melanoma cells with the DNA de-methylating agent 5-azacytidine resulted in reversible reduction of metastatic lung colonization (43). Recent studies have shown that treatment of cells with 5-azacytidine can induce expression of the metastasis suppressor genes *Nm23* (44) and *KAI1* (45). Links between metastasis and HDAC activity first became apparent when the breast cancer metastasis promoting gene, *MTA1*, was identified as a component of the NuRD-HDAC complex (46, 47). *MTA1* has subsequently been shown to repress estrogen receptor-dependent transcription in an HDAC-dependent manner (48). Likewise, loss of expression of heterochromatin protein 1 (HP1) has been associated with acquisition of metastatic potential in human breast cancer (49). Together, these findings support the hypothesis that regulation of the transcriptome by a variety of mechanisms is a critical determinant of cancer spread. The findings reported here represent the first direct evidence that a metastasis suppressor gene is a component of an HDAC complex. It is possible that specialized HDAC complexes may promote (as implied by *MTA1*) or inhibit (as implied by BRMS1) cancer metastasis. The data compel the hypothesis that metastasis is regulated, at least in part, by histone deacetylase activity, chromatin remodeling, and/or transcriptional repression. Connections between HDAC activity and cancer have emerged in recent years, stemming from observations that HDAC inhibitors, such as trichostatin A and suberoylanilide hydroxamic acid (SAHA), can induce growth arrest, differentiation, and/or apoptosis in transformed cultured cells (50). In pre-clinical animal models, HDAC inhibitors have demonstrated impressive anti-tumor activity which, in turn, led to several ongoing HDAC inhibitor clinical trials (50–53). The data presented here, along with data regarding MTA1 and HP1 cited above, are consistent with the hypothesis that HDAC inhibitors may influence not only primary tumors but also distant metastases.

Interestingly, BRMS1 appears to be part of a protein family in which all of the characterized members are components of the mSin3-HDAC complex. During the original yeast two-hybrid screen, two cDNA clones identified as FLJ00052 were identified in the prostate library. As studies were underway to follow-up RBP1, mSin3, and HDAC findings, FLJ00052 was re-designated by GenBank™ as mSds3, the mammalian ortholog of *Saccharomyces cerevisiae* Sds3. Sds3 has been implicated in gene silencing through a Sin3-Rpd3 pathway (Rpd3 in a yeast HDAC1 ortholog) and is an integral component of the yeast Sin3-Rpd3 complex that is required for histone deacetylase activity (17, 54). BRMS1 shares 18% identity and 49% similarity with a large region of yeast Sds3 and 23% identity and 49% similarity with mSds3, mSds3, analogous to its yeast ortholog, is a component of the mSin3-HDAC complex, stabilizes HDAC1 within the complex, and augments HDAC activity (17). Another predicted mammalian protein of unknown function (designated MGC11296) is homologous to both Sds3 and BRMS1. Homology to BRMS1 is particularly strong (58% identity; 79% similarity for the C-terminal 196 amino acids of BRMS1 and the N-terminal 196 amino acids of MGC11296). The high level of sequence similarity between these molecules, combined with their associations with mSin3-HDAC complexes, suggests the existence of a BRMS1 family of proteins that may play a crucial role in altering metastasis by regulating the so-called histone code (29, 49).

Although specific role(s) for BRMS1 within mSin3-HDAC complexes remain to be elucidated, the following lines of evidence suggest that the metastasis suppressor may be involved in recruiting and stabilizing HDAC1- (and/or modulating HDAC activity. 1) BRMS1 forms small complexes (100 kDa and greater) with HDAC1 but forms only large complexes (1.4 to 1.9 Mda) with Sin3B and SAP30 (Fig. 8B). 2) BRMS1 has distinct binding site(s) for the HDAC1-Rbpap46/48 core subunit as compared with the rest of the complex (mSin3A, mSin3B, SAP30, HDAC2, and RBP1) as demonstrated by BRMS1-(A204–246) binding less effectively to HDAC1-Rbpap46/48 than does full-length BRMS1; in contrast, BRMS1-(A204–246) binds the remaining complex components as effectively (Fig. 4). 3) The C-terminal 42 amino acids of BRMS1 appear to stabilize HDAC1-Rbpap46/48 within the complex, as deletion of these residues specifically compromises binding to these three components (Fig. 4). 4) Both characterized BRMS1 family members (Sds3 and mSds3) are required for optimal HDAC activity, and mSds3 specifically stabilizes HDAC1 within the mSin3 complex.

Although remarkably similar in breast carcinoma and melanoma cell lines, BRMS1-mSin3-HDAC complexes were distinct. Rbpap46 complexes with BRMS1 were not detected in MDA-MB-231 (Fig. 5), and the interaction with Rbpap48 appeared less robust than in C8161.9 (compare Figs. 4 and 5). Differential binding of BRMS1-(A204–246) to the HDAC1-Rbpap46/48 subunit in C8161.9 was not observed in MDA-MB-231 (compare Figs. 4 to 5). At this juncture, it is not possible to distinguish whether the differences are due to cell origin or presence of mutations that abrogate interactions of Rbpap46 with BRMS1-mSin3a. BRMS1-transfected MDA-MB-231 cells are suppressed for metastasis less than C8161.9 (40–90 versus 90–100%). It is tempting to speculate that differences in metastasis suppression may be related to a differential interaction between BRMS1 and the HDAC1-Rbpap46/48 subunit.

Preliminary data obtained with the BRMS1 deletion mu-
BRMS1 interacts with the mSin3 HDAC complex

...brms1 interacts with enzymatically active mSin3 HDAC complexes. BRMS1 is also shown to form smaller complexes with HDAC1 and to repress transcription when recruited to a promoter region. Besides defining a milieu in which BRMS1 works within cells, the data presented here imply that specific downstream mediators, regulated in part by HDAC activity, are critical to controlling metastatic behavior. Indeed, preliminary cDNA microarray and proteomic studies have identified a limited number of BRMS1-regulated genes. Understanding the role(s) of BRMS1-mSin3 HDAC complexes in the regulation of gene expression promises to provide insights into metastasis suppression, HDAC-mediated chromatin regulation, and BRMS1 physiology in noncancerous cells.

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Breast Cancer Metastasis Suppressor 1 (BRMS1) Forms Complexes with Retinoblastoma-binding Protein 1 (RBP1) and the mSin3 Histone Deacetylase Complex and Represses Transcription

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