Alterations of BRMS1-ARID4A Interaction Modify Gene Expression but Still Suppress Metastasis in Human Breast Cancer Cells*

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The BRMS1 metastasis suppressor interacts with the protein AT-rich interactive domain 4A (ARID4A, RBBP1) as part of SIN3-histone deacetylase chromatin remodeling complexes. These transcriptional co-repressors regulate diverse cell phenotypes depending upon complex composition. To define BRMS1 complexes and their roles in metastasis suppression, we generated BRMS1 mutants (BRMS1mut) and mapped ARID4A interactions. BRMS1L174D disrupted direct interaction with ARID4A in yeast two-hybrid genetic screens but retained an indirect association with ARID4A in MDA-MB-231 and -435 human breast cancer cell lines by co-immunoprecipitation. Deletion of the first coiled-coil domain (BRMS1ΔCC1) did not disrupt direct interaction in yeast two-hybrid screens but did prevent association by co-immunoprecipitation. These results suggest altered complex composition with BRMS1mut. Although basal transcription repression was impaired and the pro-metastatic protein osteopontin was differentially down-regulated by BRMS1L174D and BRMS1ΔCC1, both down-regulated the epidermal growth factor receptor and suppressed metastasis in MDA-MB-231 and -435 breast cancer xenograft models. We conclude that BRMS1mut, which modifies the composition of a SIN3-histone deacetylase chromatin remodeling complex, leads to altered gene expression profiles. Because metastasis requires the coordinate expression of multiple genes, down-regulation of at least one important gene, such as the epidermal growth factor receptor, had the ability to suppress metastasis. Understanding which interactions are necessary for particular biochemical/cellular functions may prove important for future strategies targeting metastasis.

The ability of a cancer cell to complete all steps of the metastatic cascade requires diverse tumor-host interactions that are dependent on the coordinate expression of specific genes both intrinsically and extrinsically (1–3). The metastasis suppressor BRMS1 has been shown to regulate the expression of multiple genes leading to the suppression of metastasis in multiple model systems, including human breast carcinoma (4, 5), melanoma (6), and ovarian carcinoma (7), without preventing orthotopic tumor growth. Specifically, down-regulation of the pro-metastatic genes osteopontin (OPN) and urokinase-type plasminogen activator has been linked to BRMS1 expression (8, 9). Gap junctional intercellular communication is restored by BRMS1 through a change in connexin expression (10). Microarray and proteomic analyses have also been performed showing multiple changes in gene and protein expression when BRMS1 was introduced (11–13). Clinically, loss of BRMS1 protein has been correlated with progesterone receptor expression and inversely correlated with HER2 expression in breast cancer patients (14).

BRMS1 has been proposed to regulate transcription of genes by interaction with a large SIN3-HDAC chromatin remodeling complex through interaction with the protein AT-rich interactive domain 4A (ARID4A) that suppresses basal transcription in vivo using a Gal4-luciferase reporter assay (14). These findings have been confirmed by subsequent protein–protein interaction studies of other proteins known to be a part of this complex in addition to BRMS1 (15–18). A second mechanism identified for BRMS1 that may or may not be distinct from SIN3-HDAC involves the negative regulation of NFκB through interaction with RelA/p65 and inhibition of IκBα phosphorylation (8, 9, 19).

ARID4A is a part of multiple protein–protein complexes. In addition to the BRMS1-containing SIN3-HDAC complex, ARID4A interacts with the tumor suppressor retinoblastoma (20) to be recruited to E2F-dependent promoters (21, 22). Although these complexes share some of the same proteins as those identified with BRMS1, including SIN3 and HDAC1, distinct SIN3-HDAC complexes regulate particular transcription factor interactions, leading to activation or repression of specific genes (23). A model depicting how ARID4A regulates E2F-

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dependent transcriptional repression and that involves direct interaction of ARID4A with retinoblastoma and the 30-kDa SIN3-associated protein (SAP30) to recruit a SIN3-HDAC chromatin modifying complex to E2F-dependent promoters has been proposed by Branton and co-workers (24). Although multiple members of the SIN3-HDAC complexes have been described as tumor suppressors, there are no current reports of specific interactions necessary for or implicated in metastasis suppression.

In the MDA-MB-231 and -435 metastatic human breast cancer cell lines, the BRMS1-SIN3-HDAC complexes are not active tumor suppressors. Orthotopic tumors are still able to grow at a similar rate when BRMS1 is re-expressed in these metastatic cells that have no detectable levels of endogenous BRMS1, but metastasis is suppressed by ~90%. Because we previously showed a direct yeast two-hybrid (Y2H) interaction of BRMS1 with ARID4A, we hypothesized that this interaction plays an important role in the ability of BRMS1 to suppress metastasis. To test this hypothesis, we generated a series of deletion mutants of BRMS1 protein that differentially interact with ARID4A. We tested their ability to suppress metastasis and evaluated metastasis-associated phenotypes. Understanding these protein-protein interactions and the intricate roles they play in the process of metastasis, distinct from tumorigenesis, is important to target this deadly disease.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture**—MDA-MB-231 and MDA-MB-435 are human estrogen receptor-negative and progesterone receptor-negative cell lines derived from metastatic infiltrating ductal breast carcinomas (25, 26). The origin of MDA-MB-435 has been questioned because the cells express melanoma-associated genes in cDNA microarray experiments (27, 28). However, the patient was reported to have only a breast carcinoma. MDA-MB-435 cells can be induced to secrete milk lipids (29) and have a propensity to metastasize from mammary gland, but not from subcutaneous sites (30). Both cell lines form progressively growing tumors when injected into the mammary fat pads of immunocompromised mice. MDA-MB-435 cells develop macroscopic metastases in the lungs and regional lymph nodes by 10–12 weeks postinoculation but rarely metastasize after direct injection into the lateral tail vein. The opposite pattern exists for MDA-MB-231 in athymic mice.

The cell lines were cultured in a mixture (1:1, v/v) of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (Invitrogen) supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 0.02 mM non-essential amino acids, and 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA) without antibiotics or antifungals. All cultures were confirmed negative for Mycoplasma spp. infection using a PCR-based test (TaKaRa, Shiga, Japan). Cells were maintained on 100-mm Corning tissue culture dishes at 37 °C with 5% CO₂ in a humidified atmosphere. When cultures reached 80–90% confluence they were passaged using a solution of 2 mM EDTA in Ca²⁺/Mg²⁺-free Dulbecco’s phosphate-buffered saline.

** Constructs and Transductions**—BRMS1 mutants were created by QuikChange II site-directed mutagenesis (Stratagene, La Jolla, CA) and were confirmed by DNA sequencing. The constructs were amplified by PCR with BamHI and XhoI restriction enzyme digestion sites at each end. After enzyme digestion, the products were ligated into the lentivirus vector vesicular stomatitis virus G (31). Packaging and transfection of the lentivirus in 293T cells and transduction of the MDA-MB-231 and -435 cells were described previously (17, 32, 33). Single cell clones were obtained and screened for expression of BRMS1 or BRMS1 mutants by Western blotting.

** Yeast Two-hybrid Analysis**—The Y2H screen was performed essentially as described (34, 35). Briefly, BRMS1 or BRMS1 mutant cDNA was cloned in-frame with the Gal4 DNA-binding domain into the pDEST32 vector (Invitrogen), and ARID4A corresponding to the C-terminal amino acids 1007–1257, amplified from human breast cDNA library, was cloned in-frame with the Gal4 activation domain into the pGAD424 vector (Clontech, Mountain View, CA). The yeast Y190 strain was co-transformed with these vectors and grown on synthetic defined/Trp⁻/Leu⁻ plates at 30 °C until colonies reached 1–3 mm in diameter. The colonies were lifted by filter paper, lysed in liquid N₂, and inverted onto another filter soaked with Zbuffer/5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) solution. β-Galactosidase activity was observed by color change and visually measured corresponding to time as follows: + + , blue within 4 h; + , blue within 4–8 h; and − , longer than 8 h.

**Antibodies, Co-immunoprecipitation, and Western Blotting**—The monoclonal antibody 1a5.7 directed against a peptide corresponding to the C terminus of BRMS1 (33) was generated and validated by Western blotting, immunoprecipitation, and mass spectroscopy. Other antibodies used in this study were purchased as indicated: anti-ARID4A clone LY11 (Upstate Biotechnology, Lake Placid, NY), anti-EGFR (Cell Signaling Technology, Danvers, MA). Co-immunoprecipitation with 1a5.7 was performed as described previously for other antibodies (17) except that protein L-agarose (Pierce) was used in place of protein A/G-agarose. Western blotting was also performed as described except that mouse TrueBlot (eBioscience, San Diego, CA) was used as the secondary antibody for the immunoprecipitated samples.

**Reporter Assays**—BRMS1 or BRMS1 mutant cDNA was cloned into the pBIND vector (Promega, Madison, WI). COS-7 cells were co-transfected with pBIND and pG5luc vectors using Lipofectamine 2000 (Invitrogen). Cells were rinsed once with phosphate-buffered saline and lysed with passive lysis buffer (Promega). Luciferase activity was measured with the Dual-Luciferase assay (Promega) using an AutoLumat LB 953 luminometer (Berthold Technologies, Oak Ridge, TN). Renilla luciferase (phRL-SV40; Promega) was used as a transfection control.

**Metastasis Assays**—Spontaneous and experimental metastasis assays were performed as described previously (4, 5). Ten mice per experimental group were used. The vector-only MDA-MB-231 and -435 cells were previously shown to have the same metastatic phenotype as the parental cells and thus were not included as a control in all experiments to limit the number of animals used (4, 5, 33). Animals were maintained under the guidelines of the National Institutes of Health and the University of Alabama at Birmingham. All protocols were...
approved by the Institutional Animal Care and Use Committee. Food and water were provided ad libitum.

Statistical Analyses—The number of lung metastases was compared for BRMS1- and BRMS1 mutant-transduced cell lines with the parental or vector-only transduced lines. A Kruskal-Wallis analysis of variance of ranks procedure was used with Dunn’s post hoc test. Calculations were performed using SigmaStat statistical analysis software (SPSS Inc., Chicago, IL). Statistical significance was defined as a probability $p \leq 0.05$.

RESULTS

Second Coiled-coil Domain of BRMS1 Interacts with ARID4A—The direct interaction of BRMS1 with ARID4A was previously identified by Y2H screening, but the region of BRMS1 necessary for interaction had not been determined. Mutants containing gross deletions of BRMS1 were generated and screened by Y2H analysis with ARID4A to map this interaction. Because of their roles in protein-protein interaction, we focused on disruption of the putative coiled-coil domains. Deletion of the first coiled-coil domain between residues 61 and 92, BRMS1CC1, had no impact on ARID4A binding (Fig. 1). However, deletion of the second coiled-coil domain between residues 130 and 187, BRMS1CC2, abolished this interaction. Truncation of BRMS1 at residue 137 also failed to elicit an interaction with ARID4A. Further analyses showed that the second coiled-coil domain alone, between residues 147 and 180, could interact with ARID4A, demonstrating that this domain was necessary and sufficient for interaction. Partial deletion of the N or C terminus of this domain, BRMS1CC2:N or BRMS1CC2:C, disrupted interaction with ARID4A.

Point Mutant BRMS1L174D Abolishes Direct Interaction with ARID4A—A series of BRMS1 point mutants were generated in the second coiled-coil domain and screened for interaction with ARID4A by Y2H analysis (Fig. 1). Because deletion of the last seven amino acids in this domain, BRMS1CC2:N, disrupted interaction with ARID4A, mutations were selected in this region between residues 174 and 180. Conservative mutations, BRMS1S177A and BRMS1L174A, maintained a strong interaction with ARID4A. Addition of a charged side chain, BRMS1L174D, also maintained a strong interaction. However, the same mutation two amino acids upstream, BRMS1L174F, abolished the interaction with ARID4A.

ARID4A Maintains Indirect Association with BRMS1L174D but Not BRMS1L174C—Stable cell lines were generated using two metastatic breast cancer cell lines, MDA-MB-231 and -435, with a BRMS1 mutant that maintains direct interaction with ARID4A, BRMS1L174C, and the point mutant BRMS1L174D, which disrupted ARID4A-BRMS1L174D interaction. Co-immunoprecipitation of BRMS1 or BRMS1 mutants with the BRMS1 monoclonal antibody, 1a5.7, demonstrated that BRMS1L174D indirectly associated with ARID4A, whereas BRMS1L174C was not able to associate with ARID4A in these breast carcinoma cell lines (Fig. 2).

BRMS1 Mutant Differentially Regulates Gene Expression—As part of a co-repressor complex, BRMS1 was previously found to repress basal transcription using a luciferase reporter assay (14). Disrupting the direct interaction of BRMS1 with the point mutant BRMS1L174D also inhibited suppression of basal transcription (Fig. 3A). Down-regulation of the pro-metastatic protein OPN by BRMS1 has been proposed to be important for BRMS1-mediated metastasis suppression (9). BRMS1L174D, but not BRMS1L174C, down-regulated OPN expression (Fig. 3B). More recently, BRMS1 was found to specifically down-regulate EGFR when BRMS1 was re-expressed in both cell lines. Both of the BRMS1 mutants, BRMS1L174D and BRMS1L174A, retained the ability to down-regulate EGFR (Fig. 3C).

BRMS1 Mutants Suppress Metastasis—To determine whether the direct interaction of BRMS1-ARID4A is necessary for BRMS1-mediated metastasis suppression, two model systems were utilized representing spontaneous (MDA-MB-435, 4 Hedley, B. D., Welch, D. R., Allan, A. L., Al-Katib, W., Dales, D. W., Postenkova, C. O., Casey, G., MacDonald, L. C., and Chambers, A. F. (2008) Int. J. Cancer, in press.
5 K. S. Vaidya, S. Harihar, P. A. Phadke, L. J. Stafford, D. G. Hicks, G. Casey, D. B. Dewald, and D. R. Welch, submitted for publication.
from an orthotopic injection) and experimental (MDA-MB-231, following intravenous injection) metastasis. BRMS1L174D, which does not directly interact with ARID4A, and BRMS1/H9004CC1, which can still directly interact with ARID4A, were tested for their ability to suppress metastasis in these two systems. Both mutants in both of the assays suppressed metastasis comparable with suppression by wild-type BRMS1 (Fig. 4). These results demonstrate that direct interaction of BRMS1-ARID4A is not necessary for BRMS1-mediated metastasis suppression.

**DISCUSSION**

The coordinate expression of multiple genes is necessary to enable a cancer cell to complete all the steps of the metastatic cascade, and proteins that regulate transcription may dramatically impact this process (1–3). SIN3/HDAC complexes epigenetically regulate gene expression and may be critical determinants for cancer spread. Although many proteins associated with these complexes have been characterized as tumor suppressors, there are currently no reports of specific interactions necessary for metastasis suppression. In this study, we have extended our previous findings that BRMS1 is a member of a SIN3/HDAC complex(es), and we focused specifically on the direct interaction of BRMS1 with ARID4A. These studies are part of a long-term objective of characterizing how these complex proteins are interacting to understand the intricate roles they play in metastasis.

BRMS1 is a predominantly nuclear protein that suppresses metastasis in multiple xenograft model systems by inhibiting multiple steps of the metastatic cascade (36). BRMS1 protein contains several potential protein-binding domains including coiled-coil and imperfect leucine zippers, and previous reports demonstrated that BRMS1 interacts with ARID4A as part of large (~1.2 MDa) SIN3/HDAC complex(es) (15–18, 37). Because BRMS1 has been shown to affect the transcription of multiple genes that are known to play a role in metastasis and SIN3/HDAC complexes epigenetically regulate gene expression, we asked if the direct interaction of BRMS1-ARID4A was necessary for BRMS1-mediated metastasis suppression. To test this hypothesis, the domain of BRMS1 required for ARID4A interaction was mapped, and a point mutant that disrupted this
interaction was generated. Unexpectedly, we found that metastasis was still suppressed with BRMS1 mutants that did not associate with ARID4A either directly, BRMS1_L174D, or indirectly, BRMS1/CC1. These studies have enabled us to propose a model of how BRMS1 is associated with a SIN3/HDAC complex (Fig. 5).

Although Y2H screening is usually accepted as a measure of direct protein-protein interaction, there is a small possibility for other proteins to bridge interactions between the two proteins of interest. However, based on our data that the second coiled-coil domain of BRMS1 alone could interact with the C-terminal domain of ARID4A in a Y2H genetic screen, it is unlikely that the interaction is occurring through bridging proteins. The finding argues that CC2 is sufficient for ARID4A interactions. Furthermore, if bridging proteins were required, then BRMS1_L174D should have also interacted with ARID4A because ARID4A and BRMS1_L174D are still associated according to co-immunoprecipitation. Moreover, Y2H screening has been performed with SAP30 and HDAC1 (other members of the same complex). No interaction with BRMS1 was found (data not shown), further supporting the observation that a positive interaction in Y2H screening most likely reflects a direct protein-protein interaction.

Initially, the data between the Y2H screening and co-immunoprecipitation appear discordant. However, the findings are entirely internally consistent once one considers how the complex as a whole interacts. Previous studies have shown that suppressor of defective silencing 3 (SUDS3, mammalian SDS3) is in the same SIN3/HDAC complex (17, 37). Combined with recent Y2H preliminary data showing that BRMS1_L174D directly interacts with SUDS3, whereas BRMS1/CC1 does not, it is tempting to speculate that SUDS3-BRMS1 interaction is required as a tether for the ARID4A-BRMS1 interaction. This provides a rational explanation for why BRMS1/CC1 does not co-immunoprecipitate ARID4A in human breast cancer cells, whereas BRMS1_L174D does co-immunoprecipitate ARID4A.

The direct interaction of ARID4A with BRMS1 in Y2H screening required the second coiled-coil domain of BRMS1.

### Functional BRMS1 Protein Interactions

**Lung metastases**

| Cell line | Incidence | Mean ± SEM | P < 0.05 |
|-----------|-----------|------------|----------|
| MDA-MB-231 | 10/10 | 85 ± 8 | ** |
| -BRMS1 | 10/11 | 9 ± 2 | ** |
| -ΔCC1.1 | 9/10 | 3 ± 1 | ** |
| -ΔCC1.2 | 9/10 | 19 ± 8 | ** |
| -ΔCC1.3 | 9/9 | 6 ± 2 | ** |
| -L174D.3 | 9/9 | 4 ± 1 | ** |
| -L174D.4 | 10/10 | 20 ± 6 | ** |

| MDA-MB-435 | 10/10 | 23 ± 8 |
|------------|-------|--------|
| -BRMS1 | 9/10 | 4 ± 1 | ** |
| -ΔCC1.1 | 8/10 | 2 ± 1 | ** |
| -ΔCC1.2 | 7/10 | 2 ± 1 | ** |
| -ΔCC1.3 | 7/9 | 2 ± 1 | ** |
| -L174D.2 | 8/9 | 3 ± 1 | ** |
| -L174D.3 | 9/10 | 3 ± 1 | ** |
| -L174D.4 | 8/9 | 4 ± 1 | ** |

FIGURE 4. BRMS1 mutants suppress metastasis. The table on the left shows the incidence and the mean number of lung metastases for each group. The data are shown graphically on the right with black dots representing the number of lung metastases from each mouse; the box represents the 10th and 90th percentiles, and the black line is the mean for each group. The MDA-MB-231 parental cell line had seven mice that had too many metastases to count and were conservatively assigned a value of 100.

6 A. C. Silveira and D. R. Welch, manuscript in preparation.
Functional BRMS1 Protein Interactions

is required for the coordinate expression of genes necessary for metastasis. Changing the stoichiometric balance and/or the prevalence of the component proteins could significantly affect gene expression patterns that, in turn, regulate cancer metastasis. The latter hypothesis is supported by the finding that the BRMS1 mutants still maintain the ability to interact with (at least some) members of the SIN3-HDAC complexes. At present, it is not possible to determine whether BRMS1 re-expression has changed the overall equilibrium that leads to specific functional differences. By disrupting direct ARID4A-BRMS1 interactions using BRMS1<sup>L174D</sup>, basal transcription was no longer repressed as evidenced by the Gal4-luciferase reporter assay. Yet OPN was specifically down-regulated by BRMS1<sup>L174D</sup>, but not BRMS1<sup>ΔCC1</sup>, whereas both BRMS1<sup>L174D</sup> and BRMS1<sup>ΔCC1</sup> mutants maintained the ability to down-regulate EGFR. Together, these findings demonstrate how the downstream functions of BRMS1-based multiprotein complexes could change by modifying the equilibrium of individual proteins. We are cautious, however, because a definitive role for EGFR changes in BRMS1-mediated metastasis suppression has not yet been definitively established.

The possibility also exists that BRMS1<sup>L174D</sup> or BRMS1<sup>ΔCC1</sup> may suppress metastasis by affecting different steps in the metastatic cascade. Unfortunately, a robust in vitro assay that predicts metastasis suppression has not yet been identified. Therefore, it is not currently possible to directly test this hypothesis. Also, although unlikely, it is possible that the metastasis suppression caused by BRMS1<sub>mut</sub> may be via a different mechanism than BRMS1. Again, testing this possibility will require an in vitro surrogate assay.

Although the primary focus of this study was the interaction of BRMS1 with ARID4A, other BRMS1 protein interactions could have been affected by mutating BRMS1, including NFκB, BAF57, N-Myc interactor, and/or CCG1. All of these transcription complex components could affect the ability of BRMS1 to suppress metastasis. Whether these complexes are distinct from or associated with SIN3-HDAC is presently not known.

Clearly, future studies are necessary to characterize these BRMS1 interactions to understand how each complex is involved in metastasis so that it will be possible to identify specific transcriptional targets relevant to cancer metastasis. Nonetheless, without understanding the exact mechanism by which the BRMS1 mutants suppress metastasis, it is exciting to postulate that minimal domains of BRMS1 or perhaps small peptides or inhibitors that modify these protein-protein interactions could inhibit metastasis.

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Functional BRMS1 Protein Interactions

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