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Breast Cancer Metastasis Suppressor 1 Functions as a Corepressor by Enhancing Histone Deacetylase 1-Mediated Deacetylation of RelA/p65 and Promoting Apoptosis▽†

Yuan Liu, Philip W. Smith, and David R. Jones*

Department of Surgery, University of Virginia School of Medicine, Charlottesville, Virginia

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The antiapoptotic transcription factor NF-κB is constitutively activated in many cancers and is important for cytokine-mediated progression and metastatic movement of tumors. Breast cancer metastasis suppressor 1 (BRMS1) is a metastasis suppressor gene whose mechanisms of action are poorly understood. In this report, we demonstrate that BRMS1 decreases the transactivation potential of RelA/p65 and ameliorates the expression of NF-κB-regulated antiapoptotic gene products. BRMS1 immunoprecipitates with the RelA/p65 subunit of NF-κB with protein-protein interactions occurring at the C terminus region of the rel homology domain but not at its known transactivation domains. Moreover, BRMS1 functions as a corepressor by promoting binding of HDAC1 to RelA/p65, where it deacetylates lysine K310 on RelA/p65, which suppresses RelA/p65 transcriptional activity. Selective small interfering RNA knockdown of BRMS1 confirms that chromatin-bound BRMS1 is required for deacetylation of RelA/p65, while enhancing chromatin occupancy of HDAC1 onto the NF-κB-regulated promoters *cIAP2* and *Bfl-1/A1*. We observed in cells lacking BRMS1 a dramatic increase in cell viability after the loss of attachment from the extracellular matrix. Collectively, these results suggest that BRMS1 suppresses metastasis through its ability to function as a transcriptional corepressor of antiapoptotic genes regulated by NF-κB.

Aberrant or constitutive activation of the nuclear transcription factor NF-κB has been detected in many human malignant tumors (7, 14, 16, 46, 58, 61). We have previously shown that NF-κB plays an important role in the resistance of non-small-cell lung cancer (NSCLC) to chemotherapy and histone deacetylase (HDAC) inhibition (30, 31, 41). Recently, the importance of cytokine-mediated activation of IKKβ and subsequently NF-κB in the promotion and progression of inflammatory-associated epithelial cancers has been identified (23, 32, 48). Furthermore, low levels of the cytokine tumor necrosis factor (TNF) produced by tumor-associated macrophages have been suggested to actually increase tumor growth and metastatic progression through NF-κB-dependent signaling cascades (5, 32). Purported mechanisms through which NF-κB promotes tumor progression and metastatic movement include the upregulation of antiapoptotic gene products, as well as proangiogenic factors (1). Thus, there is increasing evidence that upregulation of cell survival signals via NF-κB is one of the initial critical events that must occur during the metastatic process.

Transcriptionally active NF-κB is typically composed of a heterodimeric protein complex, of which the best studied and most common form is the RelA/p65/p50 heterodimer, where RelA/p65 contains the transactivation domains. Classic activation of NF-κB occurs after IKK isoform phosphorylation of cytosolic IκB, which results in its proteasomal degradation and subsequent liberation of NF-κB which, in turn, translocates into the nucleus, where it enhances transcription (4, 21). Intranuclear NF-κB activity is regulated by transcriptional coregulators that function by connecting sequence-specific activators to the basal transcriptional machinery, in addition to modifying chromatin through their intrinsic histone acetyltransferase (HAT) or HDAC activity (50). RelA/p65 binds to the CBP/p300 transcriptional coactivator, as well as p/CAF, and overexpression of these coactivators enhances the transactivation potential of NF-κB (47, 56). The transcriptional activity of RelA/p65 is also modulated by several HDACs, including HDAC-1, -2, and -3, and SIRT1 (2, 10, 64). Post-translational modifications are pivotal for chromatin-associated activities of RelA/p65 in that acetylation of RelA/p65 is important for its transcriptional activity and DNA binding, as well as impairing its assembly with IκBα and nuclear exportation (11, 33).

In contrast to the prometastatic role of NF-κB, breast cancer metastasis suppressor 1 (BRMS1) is a gene that was mapped to chromosome 11q13 and was originally identified as a metastasis suppressor gene (55). The BRMS1 protein product is localized predominantly in the nucleus and contains an imperfect leucine zipper motif and various coiled-coil domains (43, 55). BRMS1 mRNA expression has been shown to be markedly reduced in melanoma and breast cancer cell lines, and stable overexpression of BRMS1 in these cell lines significantly inhibited their metastatic potential (13, 54). Interestingly, there is no evidence to date that BRMS1 regulates metastatic movement through alterations in matrix metalloproteinase levels, cellular adhesion profiles, or alterations in tumor cell invasion (52). Recent studies indicate that BRMS1 is a selective component of the mSin3a/HDAC corepressor complex resulting in

* Corresponding author. Mailing address: Department of Surgery, Box 800679, University of Virginia, Charlottesville, VA 22908-0679. Phone: (434) 243-6443. Fax: (434) 982-1026. E-mail: djones@virginia.edu.

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basal transcription repression as measured by a GAL4 luciferase reporter of the myelomonocytic growth factor minimal promoter (42). BRMS1 has also been shown to negatively regulate the nuclear translocation of NF-κB and urokinase-type plasminogen activator transcripts through processes involving inhibition of IκBα phosphorylation (13).

Given that BRMS1 functions as a metastasis suppressor and has been associated with a known corepressor complex and that NF-κB activation is associated with the development of metastases, we sought to determine whether BRMS1 may be regulating NF-κB transcriptional activity through effects on RelA/p65 acetylation. Using an NSCLC model system, we demonstrate that BRMS1 represses both the basal and inducible transactivation potential of RelA/p65 by functioning as a corepressor for HDAC1 in addition to decreasing HDAC1 binding to RelA/p65. Using site-directed mutagenesis and selective small interfering RNA (siRNA) knockdown of specific HDACs, we have identified that the BRMS1/HDAC1 corepressor complex reduces NF-κB-dependent transcription through deacetylation of RelA/p65 on lysine K310. Futhermore, siRNA knockdown of BRMS1 recruits endogenous acetyl-K310-RelA/p65 to the chromatin of NF-κB-dependent antiapoptotic genes *cIAP2* and *Bfl-1/A1* promoters while simultaneously inhibiting endogenous HDAC-1 chromatin occupancy. Finally, using an anoikis cell model of metastasis, we demonstrate that BRMS1 significantly increases apoptosis in suspended NSCLC cells after cytokine stimulation.

Collectively, these results indicate that BRMS1 functions as a corepressor that modulates NF-κB-dependent antiapoptotic transcription at the chromatin level. These observations suggest that BRMS1 expression may prevent metastases by the ability of this corepressor to regulate NF-κB transcription and cell survival after the loss of cellular adhesion.

MATERIALS AND METHODS

Cell culture, surgical specimens, reagents, and plasmid constructs. Human NSCLC lines (NCI-H157, NCI-H358, NCI-H460, NCI-A549, and NCI-H1299), a normal human bronchial epithelial cell line (NL-20), and tumorigenic but nonmetastatic human embryonic kidney cells (HEK 293T) (63) were obtained from the American Type Culture Collection (Manassas, VA). NSCLC cell lines and HEK 293T cells were grown as described previously (64). NL20 cells were cultured in Ham F-12 medium (Invitrogen, Carlsbad, CA) with supplements according to the manufacturer's protocol. Human NSCLC specimens and adjacent noncancerous lung were preserved according to standard surgical resection from four patients at the Division of Thoracic Surgery, University of Virginia, with informed consent and Human Investigations Committee approval. The 3x-κB luciferase reporter (3x-κB-Luc), Gal-4 luciferase construct (Gal4-Luc), expression vectors encoding Gal4-p65 fusion protein (1-286, 286-520, 286-551, 520-551, 1-551, and 286-551/K310R), expression vectors (pGEX) encoding GST-p65 fusion proteins (1-305, 245-455, and 354-551), and plasmids (pCMV) encoding Flag-tagged p65 were previously described (41, 51, 64). Human BRMS1 cDNA was cloned by PCR (the primers were 5′-GTATGAATTCGACCTGTC CAGCCTCCAAGC-3′ [forward] and 5′-GTATCTCGAGTCAAGGTCCATCC GATTTTC-3′ [reverse]; the restriction sites are underlined) and inserted into hemagglutinin (HA)-tagged pCMV vector (Clontech, Palo Alto, CA) and pcDNA3.1(+) vectors (Invitrogen, Carlsbad, CA) using the restriction enzymes EcoRI and XhoI (New England Biolabs, Beverly, MA). Human HDAC1 was cloned by PCR (the HDAC1 primers were 5′-CGGAATTCACGATGGCGCA GACGCAGGGCAC-3′ [forward] and 5′-CGGAATTCGGCCAACTTGACCT CCTCCTTG-3′ [reverse]) and inserted into pcDNA3.1(+) vectors using EcoRI sites. siRNA SMART pool human BRMS1, HDAC1, HDAC3, and siCONTROL nontargeting siRNA were purchased from Dharmacon (Chicago, IL). The antibodies used in the present study were as follows: BRMS1 (Abnova Corp., Taiwan); RelA/p65, mSin3A, p300, myc, and normal rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology, Santa Cruz, CA); α-acetyl-lysine, HDAC1, HDAC3, Ac-H3(Lys9/Lys14), and Ac-H4 (Lys8) (Cell Signaling Technology, Beverly, MA); M2 Flag-epitope tag, β-tubulin, and α-tubulin (Sigma Aldrich, St. Louis, MO); and HA-epitope tag (BD Biosciences, Palo Alto, CA). Acetyl-p65 (K310) antibody was kindly provided by Marty W. Mayo (Charlottesville, VA). Recombinant TNF was purchased from Sigma (St. Louis, MO). The TNT T7 Quick-Coupled transcription/translation system was obtained from Promega Biosciences (San Luis Obispo, CA).

Total RNA isolation, quantitative reverse transcriptase PCR (RT-PCR), and NF-κB-regulated gene expression assays. Total RNA was extracted by using TRIzol (Invitrogen) according to the manufacturer's protocol. In brief, 10^6 cells or 100 mg of tissue was lysed with 1 ml of TRIzol, and the proteins were separated by chloroform. RNAs were precipitated with isopropanol, and cDNAs were synthesized by using an Advantage RT for PCR enzyme kit (Clontech, Palo Alto, CA). BRMS1 expression was determined by real-time PCR with an iCycler IQ (Bio-Rad, Hercules, CA). The human BRMS1 primers were TGCAGCGG AGCCTCAAG (forward) and TCACATCCAGACAGAAGCCCT (reverse). Human HPRT gene was amplified as a reference gene (38). Real-time PCR quantification was processed. The expression of BRMS1 (ΔT_C) was normalized by an endogenous reference gene (HPRT) (T_{CR}). The ΔT_C value was calculated by subtracting the T_C value of the reference (T_{CR}) from the T_C value of the sample (T_{CS}): $\Delta T_C = T_{CS} - T_{CR}$. The relative expression ($2^{-\Delta\Delta TC}$) to a calibrator (placenta RNA; Clontech, Palo Alto, CA) was determined by subtracting the ΔT_{CC} (calibrator, $\Delta T_{CC} = T_{CCS} - T_{CCR}$) from the ΔT_C value ($\Delta\Delta T_C = \Delta T_{CC\ (calibrator)} - \Delta T_C$).

For NF-κB-regulated gene expression assays, HEK 293T cells or H1299 cells were plated 24 h before transfection. For BRMS1 overexpression assays, BRMS1 expression vector or empty vector as a control was transiently transfected by using Polyfect reagent (QIAGEN, Valencia, CA) according to the manufacturer's instructions. For BRMS1 knockdown assay, siRNA BRMS1 or siRNA control (100 nM) was transfected by using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The day after transfection, an additional 100 nM siRNA was transfected into H1299 cells as described above. At 36 h after the first transfection, TNF (10 ng/ml) was added to the cells for an additional 12 h. Total RNA was extracted by using TRIzol, followed by real-time RT-PCR as described above. The PCR primers used were as follows: *cIAP-2*, the 5′-GCTGTGATGGTGGACTCAGG-3′ (forward) and 5′-TGGCTTGAACTT GACGGATG-3′ (reverse); *Bfl-1/A1*, 5′-TCATATTTTGTTGCGGAGTTCA-3′ (forward) and 5′-TCCAGCCAGATTTAGGTTCAAA-3′ (reverse); *Bcl-xL*, 5′-GCATTGTGGCCTTTTTCTCC-3′ (forward) and 5′-GCTGCTGCATTGTT CCCATA-3′ (reverse); *VCAM-1*, 5′-GCTGCTCAGATTGGAGACTCA-3′ (forward) and 5′-CGCTCAGAGGGCTGTCTATC-3′ (reverse); *ICAM-1*, 5′-C TGTGTCCCCCTCAAAAGTC-3′ (forward) and 5′-GGGGTCTCTATGCCCA ACAA-3′ (reverse); *E-Selectin*, 5′-TGAAGCTCCCACTGAGTCCAA-3′ (forward) and 5′-GGTGCTAATGTCAGGAGGGAGA-3′ (reverse); and *GAPDH*, 5′-CTACACCTTGCCTGTGAGCA-3′ (forward) and 5′-GACACGTGTGGCC ATTGTAG-3′ (reverse).

Transfection and luciferase reporter assays. Plasmids and reporters were transiently cotransfected to cells at 40 to 60% confluence by using Polyfect reagent as described above. Luciferase reporter activity assays were performed as previously described (41). β-Galactosidase activities were analyzed as control for the efficiency of transfection. Luminescence was normalized to protein concentrations, and all transfection data are means ± the standard deviations (SD) of three independent experiments performed in triplicate analyses.

Immunoprecipitation and Western blot. HEK 293T cells were transfected with expression vectors encoding Flag-tagged p65, HA-tagged BRMS1, or both. Cells were harvested 48 h after transfection. In select experiments, HEK 293T cells were transfected with siRNA BRMS1 or control. For immunoprecipitation assays, primary antibody (2 μg of anti-HA-epitope tag, 3.4 μg of anti-Flag-epitope tag, 3.1 μg of anti-β-tubulin, 4 μg of anti-myc, 4 μg of anti-p65, 4 μg of anti-BRMS1, 4 μg of anti-HDAC1, or 4 μg of anti-HDAC3) was mixed with precleared cell lysates for an hour at 4°C before the addition of 30 μl of protein agarose A/G (Santa Cruz, Santa Cruz, CA), and reactions were tumbled overnight at 4°C. The agarose beads were extensively washed, and the eluted protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblot analysis. All Western blots were performed according to standard procedures. Proteins were separated by SDS–12% PAGE. After transfer, nitrocellulose membranes (Bio-Rad, Hercules, CA) were probed with primary antibodies at a 1:1,000 dilution and secondary antibodies (Promega, Madison, WI) at a 1:5,000 dilution in blocking solution. Proteins were visualized with enhanced chemiluminescent substrate (Pierce Biotechnology, Rockford, IL).

GST binding assays. Glutathione *S*-transferase (GST)–p65 fusion proteins were expressed in *Escherichia coli* BL21 (Amersham, Piscataway, NJ) and puri-

fied as described previously (18). The relative abundance of the fusion protein to GST was evaluated by using an SDS–10% PAGE gel stained with Coomassie blue. pcDNA3.1(+)-BRMS1 expression vector was translated with the TNT reticulocyte system binding with ^{35}S-labeled methionine (Amersham, Piscataway, NJ) according to the manufacturer's protocol. Labeled BRMS1 was rocked for 2 h at room temperature with GST-p65 fusion proteins in a total volume of 500 μl of NETN buffer (20 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.7 mM EDTA, 0.05% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride). The reactions were extensively washed in NETN buffer, and the bound proteins were resolved on 15% denaturing SDS-PAGE gels for analysis by autoradiography.

RelA/p65 deacetylation assays. RelA/p65 deacetylation assays were performed as previously described (11). HEK 293T cells were transfected with expression vector encoding HA-tagged BRMS1 or empty vector. In select experiments, cells were cotransfected with siRNA HDAC1, HDAC3, or siRNA control as described above. After treatment with TNF (0, 20, or 40 ng/ml) for 1 h, cell lysates were resolved on SDS-PAGE gels and immunoblotted with a specific acetyl-p65(K310) antibody. For in vitro deacetylation assays, HEK 293T cells were cotransfected with expression vectors encoding Flag-tagged p65 with or without p300. Cell lysates were immunoprecipitated with 4 μg of Flag antibody. RelA/p65 deacetylation assays were performed by incubating 20 μl of immunoprecipitated Flag-RelA/p65 protein with 10 μl of in vitro-translated BRMS1 protein and/or increasing amounts of HDAC1 protein by using the TNT reticulocyte system in HDAC buffer (10 mM Tris-HCl [pH 8.0], 10 mM NaCl, 10% glycerol) for 2 h at room temperature. The reactions were washed three times in HDAC buffer and then analyzed by immunoblotting with a pan-acetyl-lysine antibody.

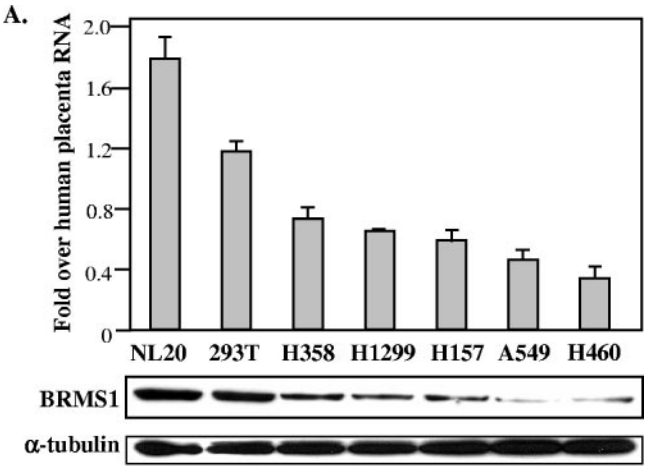
Caspase-3, DNA fragmentation, and cell death assays. HEK 293T cells or H1299 cells were transiently transfected with pCMV-BRMS1 expression vector or empty vector as control. Cells were left untreated or treated with TNF (50 ng/ml) 24 h after transfection. At 48 h posttransfection, apoptosis was quantified by quantitation of nucleosomes released into the cytoplasm using a cell death detection ELISA Plus kit (Roche Applied Science) according to the manufacturer's directions. Caspase-3 activity was determined by the addition of an APC-DEVD protein conjugate (Calbiochem, San Diego, CA) to cellular extracts containing 30 μg of protein. The fluorescence of caspase-3-cleaved protein conjugates was measured. For cell death assays, BRMS1 was overexpressed by transfecting pCMV-BRMS1 expression vector or empty vector as a control or suppressed by transfecting siRNA BRMS1 or siRNA control into HEK 293T and H1299 cells as described above. At 48 h after transfection, cells were suspended, respectively, for 0, 0.5, 1, 2, or 3 h. Cells were then replated, and the percentage of cell death was obtained by enumerating the trypan blue-positive cells versus the total number of cells after an additional 24 h of incubation.

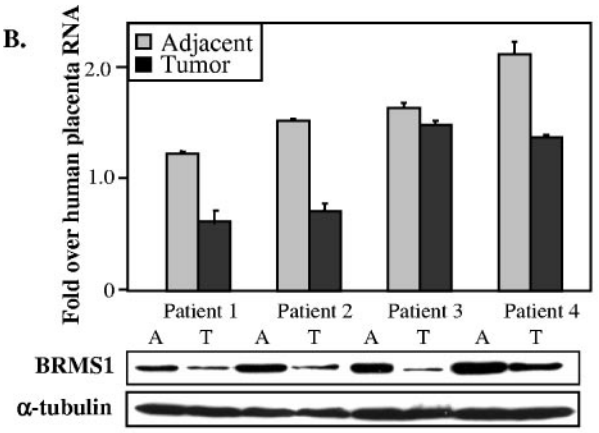
ChIP. For overexpression experiments, HEK 293T cells were transfected with pCMV-BRMS1 or empty vector. For RNA interference experiments, HEK 293T cells were transfected with siRNA BRMS1 or siRNA control as described above. After 36 h, the cells were left untreated or were treated with TNF (10 ng/ml) for an additional 12 h. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (34). DNA was immunoprecipitated with 4 μl of antibody (anti-BRMS1, anti-p65, anti-p50, anti-mSin3A, anti-acetyl-p65 K310, anti-HDAC1, anti-HDAC3, Ac-H3, Ac-H4, or normal rabbit IgG) and purified. The regions of the human *cIAP-2* and *Bfl-1/A1* promoters containing κB binding sites were targeted for amplification as described previously (38). PCR data were analyzed as previously described (8).

Statistical analysis. The results of all experiments represent the means ± the SD of three separate experiments performed in triplicate unless otherwise noted. Statistical differences between control and BRMS1 groups were determined by a two-tailed, unpaired Student *t* test when appropriate. *P* values of <0.05 were considered significant.

RESULTS

BRMS1 expression is decreased in NSCLC. NSCLC is a highly malignant cancer with a proclivity for metastasizing to both lymph nodes and distant organs. Since BRMS1 is a suppressor of metastasis, this suggests that the aggressiveness of NSCLC may be related to the intratumoral BRMS1 levels. To test this hypothesis, BRMS1 mRNA and protein expression levels were determined in five different human NSCLC cell lines by real-time RT-PCR and immunoblotting. Compared to the immortalized bronchial epithelial NL20 and HEK293T cells, BRMS1 mRNA levels are markedly decreased in all NSCLC cell lines examined. Parallel results were shown in BRMS1 protein expression (Fig. 1A).





Patient #	Histology	pStage	Nodal Metastasis
1	Bronchioloalveolar	IIIB	Yes
2	Adenocarcinoma	IIIB	Yes
3	Squamous	IIB	Yes
4	Adenocarcinoma	IA	No

FIG. 1. BRMS1 expression is reduced in NSCLC cells and tumor tissues. (A) BRMS1 mRNA and protein expression in tumorigenic but nonmetastatic cell lines (NL20 and HEK 293T) and NSCLC cell lines (H358, H1299, H157, A549, and H460). mRNA levels were evaluated by quantitative RT-PCR and calculated as a ratio to the human placenta control (top). Protein levels of BRMS1 were detected by Western blotting with α-tubulin as a loading control (bottom). Real-time RT-PCR data represent the mean ± the SD of three experiments performed in triplicate. (B) BRMS1 mRNA and protein expression in tumor tissues and patient-matched adjacent lung tissues. mRNA and protein levels for patient-matched samples were determined as in panel A. The pathological stage is presented using the international staging system for lung cancer (44). Stage IA NSCLC has limited local disease with no metastasis to lymph nodes. Stage IIB NSCLC includes spread to local lymph nodes. Stage IIIB disease includes metastasis to regional lymph nodes outside of the lung itself.

To determine whether this expression pattern was also present in surgically resected human NSCLC and adjacent noncancerous lung tissue, BRMS1 mRNA and protein levels were detected by using the same techniques. As shown in Fig.

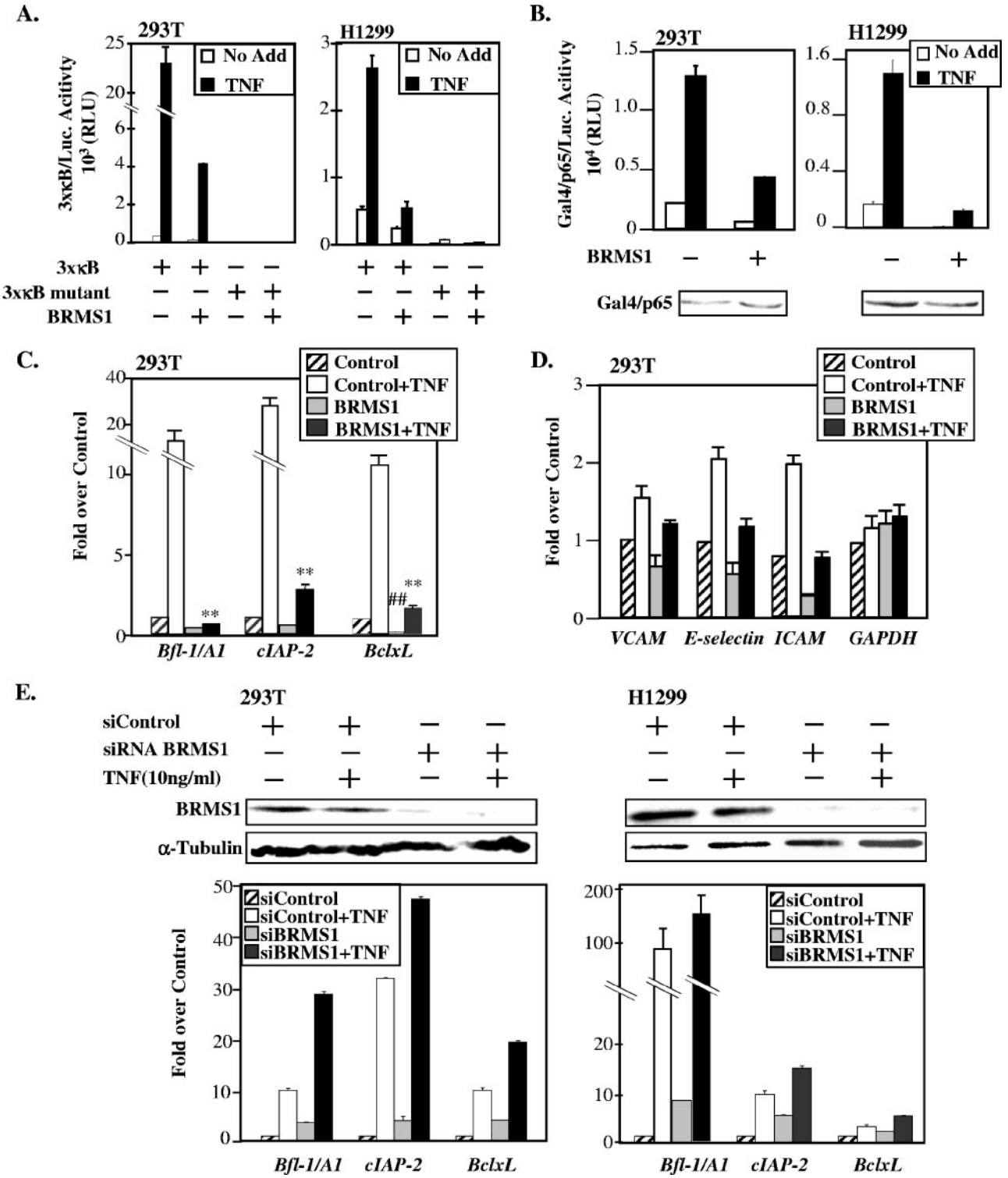


FIG. 2. BRMS1 inhibits constitutive and TNF-induced NF-κB transcriptional activity and NF-κB-dependent gene expression. (A) 293T and H1299 cells were transiently cotransfected with the NF-κB-responsive reporter (3x-κB-Luc) or mutant 3x-κB-Luc reporter and expression vector encoding BRMS1. At 18 h after transfection the cells were treated or not treated with TNF (10 ng/ml) for 12 h. The luciferase activity was determined. All transfections were normalized for cytomegalovirus (CMV)–β-galactosidase activity. (B) 293T cells and H1299 cells were transiently cotransfected with Gal4-Luc reporter and expression vector encoding the fusion protein Gal-4-p65 with or without BRMS1. At 18 h after transfection the cells were treated or not treated with TNF (10 ng/ml) for 12 h. The luciferase activity was determined. All transfections were normalized for CMV–β-galactosidase activity. (C) 293T cells were transiently transfected with BRMS1 or CMV-driven empty vector (Control). Cells were treated or not treated with TNF (10 ng/ml) for 12 h. Total RNA was extracted, and the NF-κB regulated antiapoptotic gene mRNA levels were analyzed by quantitative RT-PCR. The data are plotted as the fold over control, where results from cells transfected only with control vector were normalized to 1 for each gene. ##, $P < 0.01$ compared to control; **, $P < 0.01$ compared to control + TNF. (D) 293T cells were

1B, the expression of BRMS1 mRNA and protein is decreased in nearly all of the tumor tissues compared to adjacent noncancerous tissues. Interestingly, there was a reduction in BRMS1 protein and mRNA levels in the one patient (patient 4) with early-stage disease. Therefore, BRMS1 expression in both NSCLC cell lines and tumor tissues is markedly decreased compared to noncancerous cells and tissues, suggesting that the development and progression of NSCLC may involve the loss of BRMS1 expression.

BRMS1 represses the transactivation potential of RelA/p65. Previous studies have indicated that the nuclear translocation and DNA-binding activity of NF-κB is suppressed in breast cancer MDA-MB-231 and melanoma C8161.9 cell lines that stably overexpress BRMS1 (13). To determine whether BRMS1 could abolish NF-κB transcriptional activity in our system, transient-transfection 3x-κB luciferase assays were performed after treatment with TNF. We chose TNF as a stimulus based its ability to enhance the metastatic progression of epithelial cancers in an NF-κB-dependent manner (32). As shown in Fig. 2A, BRMS1 dramatically diminished basal and TNF-induced NF-κB transcriptional activity in both cell lines. Failure of BRMS1 to affect transcription in cells expressing a mutant 3x-κB-Luc reporter gene confirms NF-κB specificity. Given that BRMS1 has been shown to affect NF-κB through decreased cytosolic IκB processing (13), we sought to specifically examine the ability of BRMS1 to regulate the transactivation potential of RelA/p65 only. To accomplish this, Gal4/p65-Luc reporter gene assays were performed (Fig. 2B). These demonstrated that BRMS1 significantly represses the transactivation potential of RelA/p65 after TNF stimulation.

In order to determine whether BRMS1 would also decrease the expression of classic NF-κB-dependent antiapoptotic genes (*cIAP-2*, *Bfl-1/A1*, and *BclxL*) (9, 60, 67), 293T cells were transfected with expression vector encoding BRMS1 and quantitative RT-PCR was performed (Fig. 2C). BRMS1 significantly decreased TNF-induced *cIAP-2*, *Bfl-1/A1*, and *Bcl-xL* transcripts. To determine whether BRMS1 also decreased other NF-κB-regulated genes known to be involved in metastasis, RT-PCR experiments were repeated for the intracellular adhesion proteins *ICAM*, *VCAM*, and *E-selectin* (36, 37, 59). As shown in Fig. 2D, there was no significant effect of BRMS1 overexpression on these transcripts, as well as no effect on the non-NF-κB regulated gene *GAPDH*. To confirm that regulation of NF-κB-regulated antiapoptotic genes is specific to BRMS1, selective silencing of BRMS1 expression using siRNA resulted in a robust rescue of all of these antiapoptotic genes (Fig. 2E). These results indicate that BRMS1 inhibits the transactivation potential of RelA/p65 and establishes the first evidence that BRMS1 may modulate cancer cell survival through transcriptional repression of NF-κB-regulated antiapoptotic genes.

BRMS1 coimmunoprecipitates with the RelA/p65 and p50 subunits of NF-κB. We next determined whether BRMS1 could coimmunoprecipitate with components of NF-κB that are known to have antiapoptotic function, RelA/p65 and p50. Exogenous RelA/p65 or BRMS1 were reciprocally immunoprecipitated after the overexpression of HA-tagged BRMS1 and Flag-tagged RelA/p65 (Fig. 3A). The negative control, β-tubulin monoclonal antibody, or myc polyclonal antibody failed to detect the immunoprecipitation protein complexes, confirming the specificity for the anti-Flag and anti-HA antibodies, respectively. Although coimmunoprecipitation of BRMS1 and RelA/p65 occurred in these overexpression assays, we wanted to determine whether these two proteins would interact in a similar fashion endogenously. As shown in the reciprocal coimmunoprecipitation assays in Fig. 3B, endogenous BRMS1 in nuclear extracts does coimmunoprecipitate with both endogenous RelA/p65 and p50. This association between BRMS1 and the nuclear RelA/p65 and p50 subunits of NF-κB implies that BRMS1 may affect NF-κB transcription by directly interacting with the RelA/p65-p50 heterodimer.

BRMS1 directly binds the RelA/p65 subunit of NF-κB. RelA/p65 contains an N-terminal Rel homology domain (RHD) consisting of approximately 286 amino acids which mediates DNA binding and dimerization (22). Three transactivation domains are present in the C terminus region of RelA/p65, which regulate the interaction of NF-κB with various basal transcriptional components and also associates with p300/CBP transcriptional coactivators (3, 20, 22, 47, 56). To further characterize the protein binding domains between BRMS1 and RelA/p65, we performed GST pull-down assays. GST and truncated GST-RelA/p65 fusion proteins (Fig. 3C) were used to examine their interaction with BRMS1 in an in vitro rabbit reticulocyte transcription/translation system. As shown in Fig. 3D, BRMS1 was found to strongly bind to GST-RelA/p65(245-455) fusion protein and to modestly interact with GST-RelA/p65(1-305) fusion protein but not to GST alone and the GST-RelA/p65(354-551) construct. This suggests that the region of RelA/p65 which directly interacts with BRMS1 is primarily located between amino acids 245 and 354 of RelA/p65. This region corresponds to the C-terminal end of the RHD but does not include the transactivation domains of RelA/p65.

To determine whether there was a correlation between the BRMS1-binding regions within the RelA/p65 protein and the transactivation potential of RelA/p65, Gal4/p65 constructs were ectopically expressed in the 293T system, and the Gal4/p65 luciferase activity was determined in the presence or absence of BRMS1. Maximal BRMS1-mediated repression of RelA/p65 transactivation occurred in cells overexpressing the Gal4-RelA/p65(286-551) and Gal4-RelA/p65(286-520) proteins (Fig. 3E). BRMS1 had little to no ability to repress

transiently transfected with BRMS1- or CMV-driven empty vector (control). Cells were treated or not treated with TNF (10 ng/ml) for 12 h. Total RNA was extracted, and NF-κB regulated gene and housekeeping gene (GAPDH) mRNA levels were analyzed by quantitative RT-PCR. The data were analyzed as described above. No statistical difference was found between control and BRMS1 groups. (E) 293T cells and H1299 cells were transiently transfected with siRNA BRMS1 or siRNA control. Cells were treated or not treated with TNF (10 ng/ml) for 12 h. The total RNA was extracted, and NF-κB regulated antiapoptotic gene mRNA levels were analyzed by quantitative RT-PCR. The data are plotted as described for panel C. Western blots of whole-cell lysates show effective knockdown by siRNA with α-tubulin as the loading control.

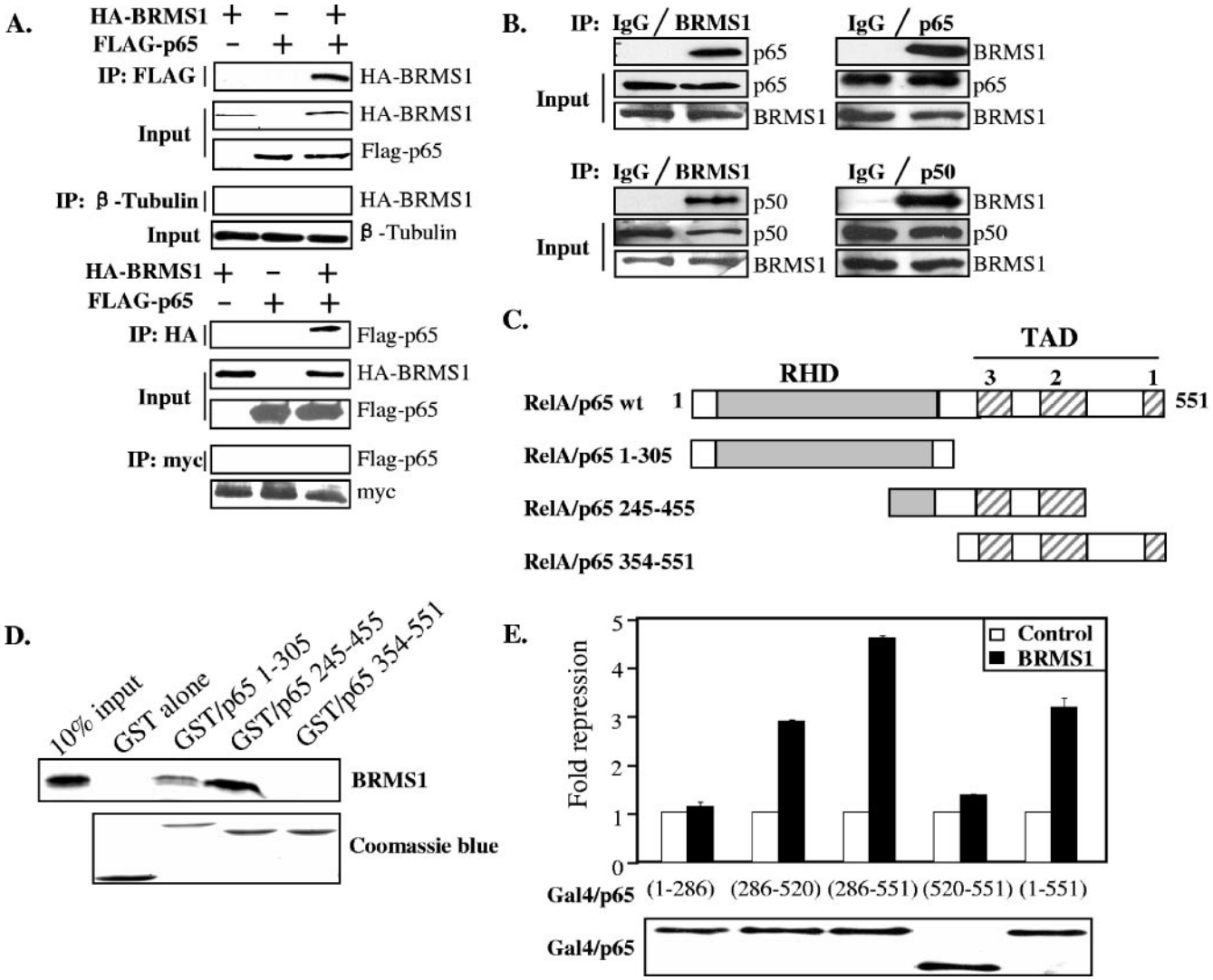


FIG. 3. BRMS1 colocalizes with RelA/p65 and p50 subunits and directly interacts with RelA/p65 subunit of NF-κB. (A) 293T cells were cotransfected with expression vector encoding HA-tagged BRMS1, Flag-tagged RelA/p65, or both. Cell lysates were immunoprecipitated with Flag polyclonal antibody or HA monoclonal antibody, followed by immunoblotting for the presence of BRMS1 or RelA/p65, respectively. Input protein showed that relatively equal amounts of HA-tagged BRMS1 and Flag-tagged RelA/p65 were expressed or immunoprecipitated. The same cell lysates were immunoprecipitated with β-tubulin monoclonal antibody or myc polyclonal antibody, respectively, as controls for nonspecific interference from antibody. (B) 293T cell isolated nuclear lysates were coimmunoprecipitated with negative control (IgG) or antibody to BRMS1, RelA/p65, or p50, followed by immunoblotting for the presence of endogenous RelA/p65, p50, or BRMS1. Input protein showed that relatively equal amounts of endogenous, RelA/p65, p50, and BRMS1 were expressed or immunoprecipitated. (C) Schematic illustration of RelA/p65 proteins. The 1-551 construct encodes the full-length Rel/p65 protein. RHD, Rel homology domain (▤); TAD, transactivation domains (▨). (D) In vitro-translated [^{35}S]methionine-labeled BRMS1 was incubated with GST-RelA/p65 fusion proteins (1-305, 245-455, and 354-551) or GST alone (negative control). Proteins retained by GST or GST-RelA/p65 fusion proteins were eluted and analyzed by SDS-PAGE and autoradiography (n = 3) (Top). GST-RelA/p65 fusion proteins separated on an SDS–15% PAGE gel and stained with Coomassie blue indicating the relative amounts of GST fusion protein. (E) 293T cells were transiently cotransfected with Gal4-Luc reporter and expression vector encoding Gal4-RelA/p65 fusion proteins (1-286, 286-520, 286-551, 520-551, and 1-551). In addition, cells were transfected with expression vector encoding BRMS1 or empty vector (control). All transfections were normalized with CMV–β-galactosidase activity. The fold repression was calculated where basal transcription values for each Gal4-RelA/p65 vector were normalized to 1. Western blot analysis confirms the expression of the various Gal4-p65 fusion proteins.

RelA/p65 transactivation at the N-terminus and C-terminus regions of the protein. Collectively, these data suggest that BRMS1-mediated repression of the transactivation potential of RelA/p65 could be the result of direct binding between the two proteins within the region located between amino acids 245 and 354 of RelA/p65.

BRMS1 deacetylates RelA/p65 on lysine residue 310. The transactivation potential of RelA/p65 is regulated in part through its reversible acetylation (11, 64). The RelA/p65 protein is known to be acetylated at five lysine (K) residues, 122, 123, 218, 221, and 310. K310 is known to be the most important for the full transactivation function of RelA/p65 (33, 64).

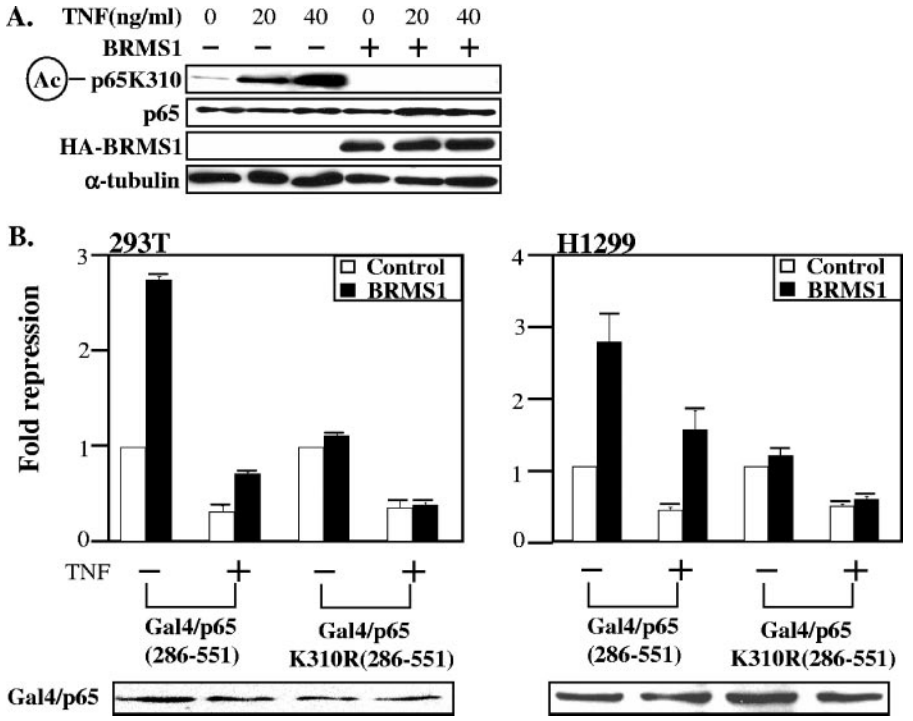


FIG. 4. BRMS1 abolishes TNF-induced NF-κB acetylation at lysine 310. (A) 293T cells were cotransfected with expression plasmid encoding HA-tagged BRMS1 or empty vector control. At 48 h posttransfection, cells were treated with TNF for 1 h at indicated concentrations. Western blots of whole-cell lysates demonstrate acetylation at lysine 310 on p65 by using specific acetyl-p65(K310) antibody and relatively constant total p65. Other blots confirmed the expression of HA-BRMS1 and α-tubulin as a loading control. (B) 293T and H1299 cells were transiently transfected with the Gal4-Luc reporter and plasmid encoding Gal4-p65 fusion protein (286-551) or a mutant Gal4-p65 fusion protein (286-551, K310R). In addition, plasmid encoding BRMS1 or empty vector (control) was cotransfected as indicated. At 18 h after transfection, the cells were treated or not treated with TNF (10 ng/ml) for 12 h. The luciferase activity was determined. All transfections were normalized with CMV–β-galactosidase activity. The fold repression was calculated where basal transcription values for either Gal4-RelA/p65 vector or K310R mutant vector were normalized to 1. Western blot analysis confirmed the expression of the various Gal4-p65 fusion proteins.

Based on our data to this point, we next hypothesized that BRMS1 may repress the transactivation potential of RelA/p65 by modulation of its acetylation state. Using our anti-acetyl RelA/p65 K310 antibody, deacetylation assays were performed which show that endogenous RelA/p65 is acetylated on K310 in dose-dependent manner after stimulation with TNF. Importantly, the addition of BRMS1 effectively deacetylated RelA/p65 on K310 despite escalating doses of TNF (Fig. 4A).

We next investigated whether deacetylation of RelA/p65 K310 was an important target in BRMS1-mediated repression of RelA/p65 activity. As shown in Fig. 4B, ectopic expression of BRMS1 again dramatically reduced basal and TNF-induced RelA/p65 transactivation potential, supporting our prior experiments (Fig. 2A and B). In addition, overexpression of a site-directed mutant of the Gal4-p65 (K310R) fusion protein resulted in ca. 50% repression of the RelA/p65 transactivation potential compared to the wild-type constructs, a finding that is in agreement with prior reports (11). Importantly, transient transfection of BRMS1 failed to repress both the basal and the TNF-induced transactivation potential of RelA/p65 after the loss of lysine K310 (Fig. 4B). These data strongly suggest that deacetylation of K310 on RelA/p65 is required for BRMS1-mediated repression of the transactivation potential of RelA/p65.

BRMS1 functions as a corepressor for RelA/p65 deacetylation. To determine which class of HDACs is primarily responsible for BRMS1-mediated deacetylation of RelA/p65, 3x-κB luciferase assays were repeated in the presence of HDAC inhibitors. Trichostatin A (TSA) is a typical inhibitor for class I and II HDACs but not for class III (40). Nicotinamide (NAM) is a specific negative regulator of class III HDACs (6). TSA significantly rescued the BRMS1 repression of basal and TNF-induced NF-κB-dependent transcription, but NAM did not (Fig. 5A). Thus, inhibition of class I/II HDACs, but not class III, is involved in BRMS1-mediated deacetylation of RelA/p65.

Previous studies have reported that BRMS1 forms complexes with retinoblastoma-binding protein 1 and the mSin3A histone deacetylase complex (42), with much of the corepressor function of mSin3A derived from associations with HDAC1 and -2 (24, 35, 45). In addition, HDAC3 is known to deacetylate RelA/p65 (10, 33) through its interactions with the corepressors NCoR and SMRT (25, 26). Coimmunoprecipitation assays were performed to identify which HDAC interacted with BRMS1. We found that HDAC1, but not HDAC3, associated with endogenous BRMS1 (Fig. 5B). Understanding that BRMS1 has a corepressor function, we next sought to determine whether BRMS1 could affect binding of HDAC1 to RelA/p65. As shown in Fig. 5C, siRNA to BRMS1 dramatically

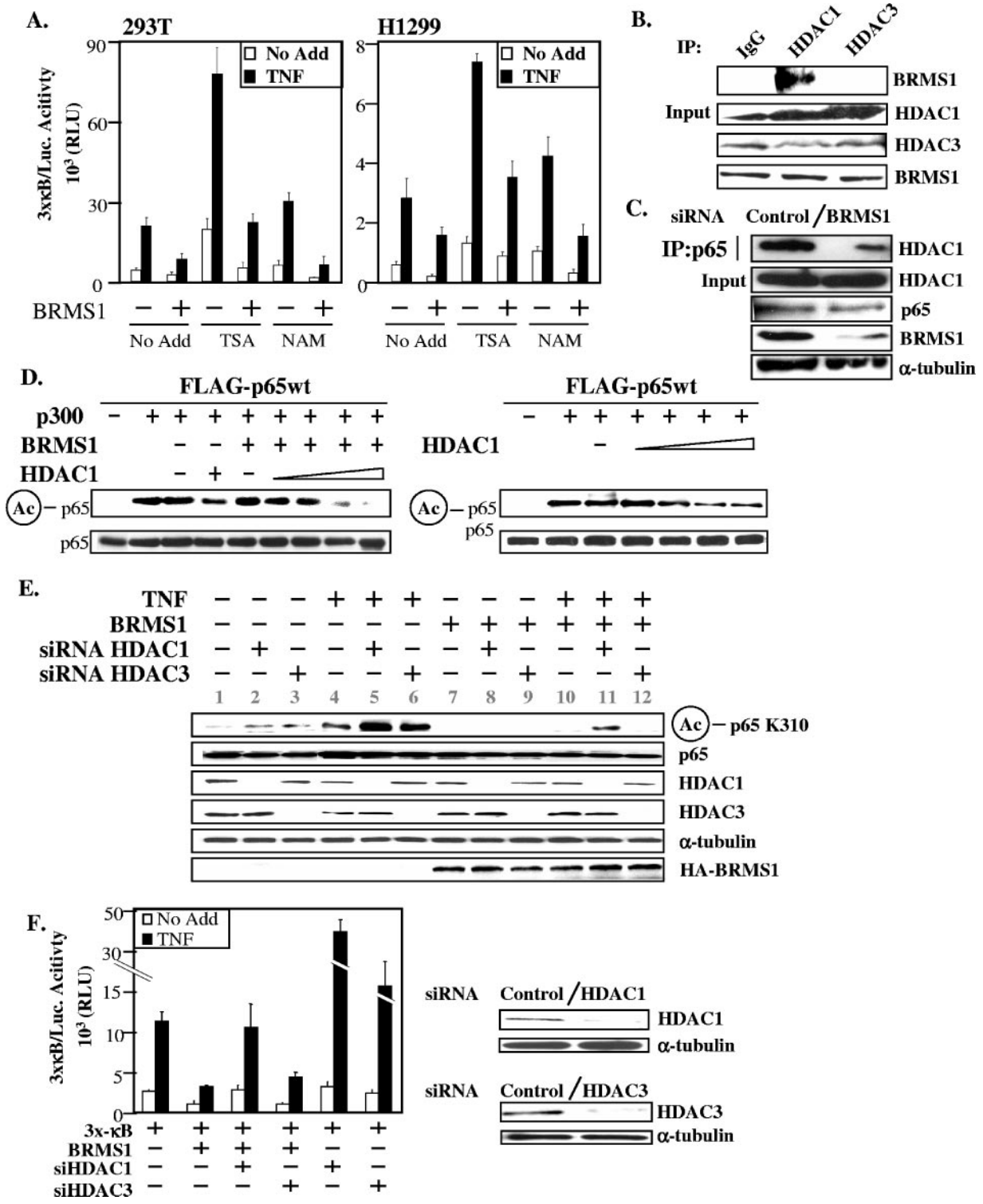


FIG. 5. HDAC1 is required for BRMS1-mediated RelA/p65 deacetylation. (A) 293T and H1299 cells were transiently cotransfected with 3x-κB-Luc reporter and plasmid encoding BRMS1 or empty vector control. At 18 h after transfection, cells were treated or not treated with TSA (500 nM) or NAM (500 μM) for an additional 12 h. Cells were simultaneously treated or not treated with TNF (10 ng/ml). The luciferase activity was determined. All transfections were normalized with CMV–β-galactosidase activity. The data represent a typical experiment performed in duplicate, with means ± the SD. (B) 293T whole-cell lysates were coimmunoprecipitated with control (IgG), anti-HDAC1, or anti-HDAC3 antibody, followed by immunoblotting for the presence of endogenous BRMS1. Input protein showed that relatively equal amounts of endogenous BRMS1, HDAC1, or HDAC3 were expressed or immunoprecipitated. (C) 293T cells were transfected with siRNA BRMS1 or siRNA control. Cell lysates were coimmunoprecipitated with anti-p65, and the presence of endogenous HDAC1 was probed by Western blotting. (D) 293T cells were transfected with expression vector encoding Flag-tagged RelA/p65 alone or with HA-tagged p300. Cell lysates were immunoprecipitated with anti-Flag antibody. On the left, the pellets were incubated with rabbit reticulocyte alone (lane 3), in vitro-translated HDAC1 (10 μl) alone, or

decreases binding of HDAC1 to RelA/p65, suggesting that BRMS1 is required for this process.

Although we have demonstrated that BRMS1 abolishes acetylation of RelA/p65 on K310, it is unclear whether this deacetylation is due to histone deacetylases known to associate with BRMS1 or, alternatively, whether BRMS1 itself has intrinsic deacetylase activity. To experimentally address this question, RelA/p65 was first acetylated using p300 in vivo and then incubated in vitro with translated BRMS1, increasing doses of HDAC1, or both. The addition of BRMS1 protein alone was unable to deacetylate RelA/p65. HDAC1 alone produced a modest deacetylation of RelA/p65, but when both recombinant proteins were added, significant deacetylation of RelA/p65 was observed (Fig. 5D). To confirm the requirement for BRMS1 in deacetylating RelA/p65, these in vitro deacetylation assays were repeated with increasing doses of HDAC1 alone and no BRMS1, and only modest deacetylation was observed (Fig. 5D). These results indicate that BRMS1 alone does not possess intrinsic HDAC activity; however, when expressed with HDAC1, together they effectively deacetylate RelA/p65.

In order to confirm that the ability of BRMS1 to deacetylate RelA/p65 was due to binding with HDAC1, we used RNA interference to separately knockdown HDAC1 or HDAC3 expression in the presence of BRMS1. As shown in Fig. 5E, selective HDAC1 knockdown in the presence of BRMS1 resulted in a partial rescue of endogenous RelA/p65 acetylation (compare lanes 10 and 11), but HDAC3 knockdown did not (compare lanes 10 and 12). In a similar experimental design, the transcriptional activity of NF-κB was partially rescued after the loss of HDAC1 even in the presence of BRMS1 overexpression with no demonstrable rescue following HDAC3 knockdown (Fig. 5F). Interestingly, in both deacetylation (Fig. 5E) and NF-κB transcriptional activity assays (Fig. 5F), knockdown of HDAC1 was unable to completely abolish BRMS1-mediated RelA/p65 deacetylation or repression of NF-κB transcriptional activity, suggesting that there are other HDACs that tether BRMS1. Despite these results, the data presented indicate that the BRMS1/HDAC1 complex functions to regulate the deacetylation of RelA/p65.

Silencing of chromatin-bound BRMS1 promotes acetylation of RelA/p65 and decreases HDAC1 loading to NF-κB-dependent promoters. To elucidate whether there was a direct correlation between BRMS1 deacetylation of RelA/p65 and repression of NF-κB mediated transcription, ChIP assays were performed across the promoter of two NF-κB-regulated antiapoptotic *cIAP-2* and *Bfl-1/A1* genes in the 293T and H1299 NSCLC cells. The H1299 cells were chosen because they have a reduced BRMS1 expression relative to the 293T cells. There are three putative NF-κB binding sites identified in *cIAP-2* promoter at positions −147, −197, and −210 and one putative NF-κB binding element in *Bfl-1/A1* promoter at position −823 (15, 27). As noted previously, unrelated downstream sequences in both of genes were also amplified as a control (see Fig. S1 in the supplemental material).

As shown in Fig. 6A and B, RNA interference inhibition of BRMS1 dramatically increases TNF-mediated endogenous acetylation of RelA/p65 (K310) on chromatin as demonstrated using our specific anti-K310 antibody. In addition, selective silencing of BRMS1 concomitantly decreases chromatin occupancy of HDAC1 without affecting HDAC3 loading. This occurs on both the *cIAP2* and the *Bfl-1/A1* promoters. In contrast, ectopic expression of BRMS1 resulted in enhanced recruitment of HDAC1 to the promoters and a significant reduction in acetylated RelA/p65 (Fig. 6C). Interestingly, RelA/p65 chromatin loading increased with either ectopic expression or silencing of BRMS1, suggesting that BRMS1 does not govern basal or inducible loading of RelA/p65 on chromatin. We also found that as a corepressor, BRMS1 can function as a more global regulator of chromatin structure, as evidenced by its ability to decrease promoter occupancy of Ac-H3 and Ac-H4 on both the *cIAP2* and the *Bfl-1/A1* promoters. These ChIP findings were not cell type specific since similar results were observed in the H1299 NSCLC cells (see Fig. S2 in the supplemental material).

These data provide compelling evidence that BRMS1 is a required corepressor that regulates NF-κB transcription by modulating the acetylation status of RelA/p65 in an HDAC1-dependent manner.

Modulation of BRMS1 expression sensitizes cells to TNF-induced apoptosis. Understanding the importance of TNF-mediated tumor growth (32, 48) and our observation that ectopic expression of BRMS1 inhibits NF-κB-dependent antiapoptotic gene expression, we hypothesized that BRMS1 would sensitize cancer cells to apoptotic cell death. To experimentally address this hypothesis, apoptosis assays were performed on 293T cells and H1299 NSCLC cells that transiently overexpressed BRMS1. As shown in Fig. 7A and B, BRMS1 overexpression sensitized cells to TNF-induced apoptosis, as indicated by enhanced DNA fragmentation and caspase-3 activity, respectively.

After detachment from the cellular matrix, suspended normal endothelial and epithelial cells enter into programmed cell death, a process known as anoikis (19). Oncogenic transfor-

BRMS1 (10 μl) plus increasing doses of HDAC1 (0, 1, 5, 10, and 15 μl). On the right, the pellets were incubated with rabbit reticulocyte alone (lane 3) or increasing doses of in vitro-translated HDAC1 (0, 1, 5, 10, and 15 μl). A pan-acetyl-lysine antibody was probed by immunoblotting. RelA/p65 as a control was detected to show equal amounts of RelA/p65 in each IP. (E) 293T cells were transfected with expression vector encoding HA-tagged BRMS1or empty vector control. In addition, siRNA HDAC1, siRNA HDAC3, or siRNA control was cotransfected as indicated. At 48 h after transfection, cells were treated or not treated with TNF (20 ng/ml) for 1 h. Western blots of whole-cell lysates showed the relative differences in acetylation at K310 on p65 using specific acetyl-p65(K310) antibody and relatively constant levels of total p65 protein. Western blots for HDAC1 and HDAC3 show effective knockdown with siRNA. A Western blot for HA shows the expression of HA-BRMS1 and tubulin as a loading control. (F) 293T cells were transiently cotransfected with 3x-κB-Luc reporter and plasmid encoding BRMS1 or empty vector control. Cells were also cotransfected with siRNA HDAC1, siRNA HDAC3, or siRNA control. Cells were treated or not treated with TNF (10 ng/ml) for 12 h before harvesting for the luciferase activity assays. All transfections were normalized with CMV–β-galactosidase activity. Western blots indicate that siRNA to HDAC1 or HDAC3 specifically knocks down protein expression.

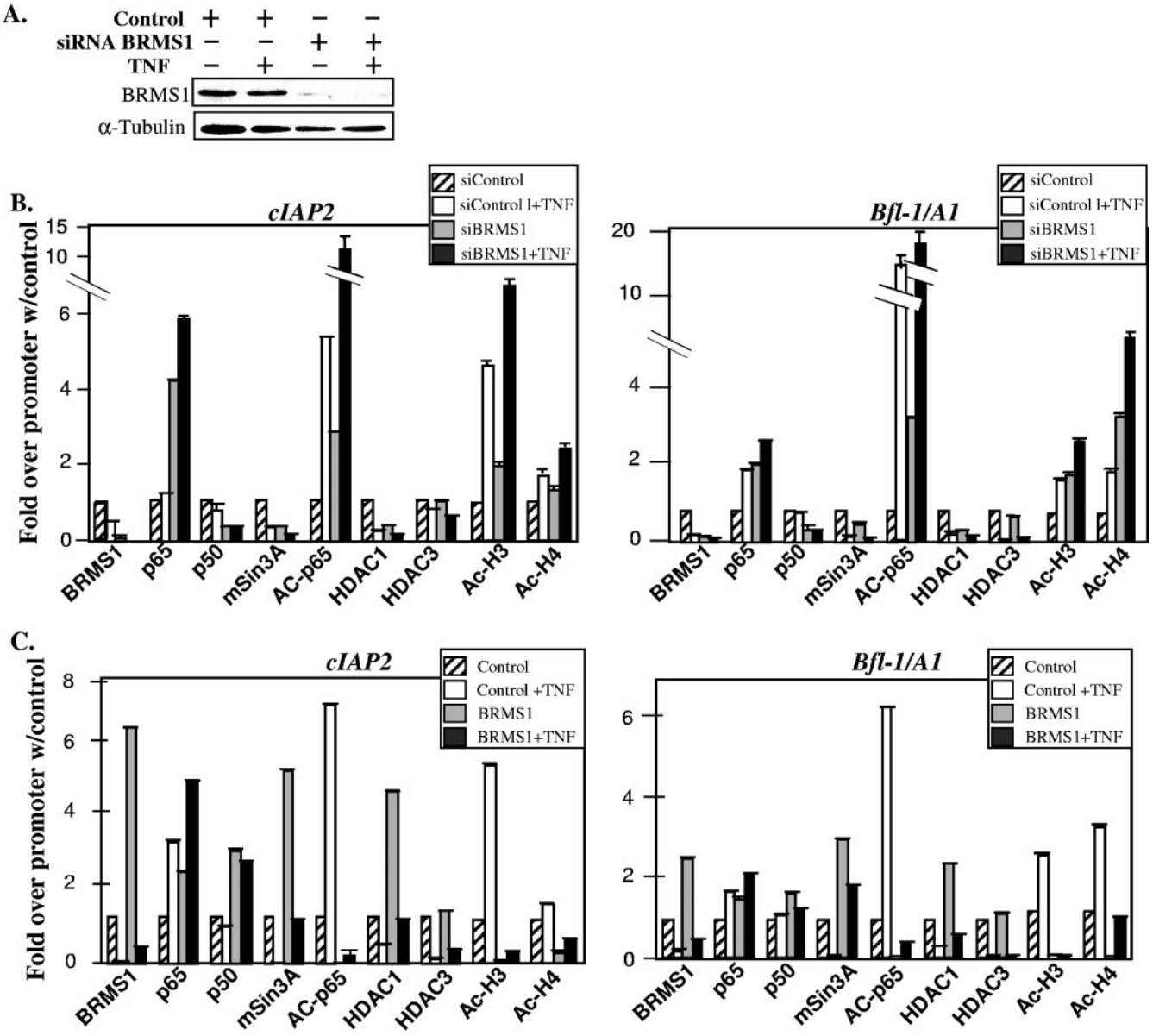


FIG. 6. BRMS1 potentiates recruitment of HDAC1 and diminishes chromatin-associated acetyl-p65 specific at K310 on both *cIAP-2* and *Bfl-1/A1* promoters. (A) Western blots indicate that siRNA to BRMS1 specifically knocks down BRMS1 expression. (B) 293T cells were transfected with siRNA BRMS1 or siRNA control (siControl). Cells were treated or not with TNF (10 ng/ml) for 12 h before harvesting. ChIP analysis was performed with the previously described antibodies. Chromatin-associated protein complexes were assessed by quantitative PCR using PCR primers specific to the *cIAP-2* and *Bfl-1/A1* promoters. The fold expression was calculated where the amplified promoter fragment with no treatment was normalized to 1. The results represent the means ± the SD of three separate experiments performed in triplicate. (C) Similar experiments were repeated as described above, with the exception that expression vector encoding BRMS1 or empty vector (Control) was ectopically expressed through transient transfection instead of siRNA.

mation is known to result in the ability of cells to survive in suspension. This ability of cancer cells to evade anoikis is required for the development of metastatic disease. To determine the relative contribution of BRMS1 to anoikis, a cell model of tumor cells in suspension was created. As shown in Fig. 7C, 293T and H1299 cells transiently expressing BRMS1 had significantly more cell death the longer they were in suspension compared to controls. Conversely, siRNA knockdown of BRMS1 resulted in little cell death despite being detached for over 3 h. This suggests that one mechanism of metastasis mediated by loss of BRMS1 expression may be through decreased tumor cell apoptosis or anoikis, potentially resulting in a larger number of viable cells for implantation at the metastatic site.

DISCUSSION

In order to explore signal transduction pathways related to BRMS1 and NF-κB, we chose to examine a lung cancer cell model system since lung cancer is a highly metastatic disease

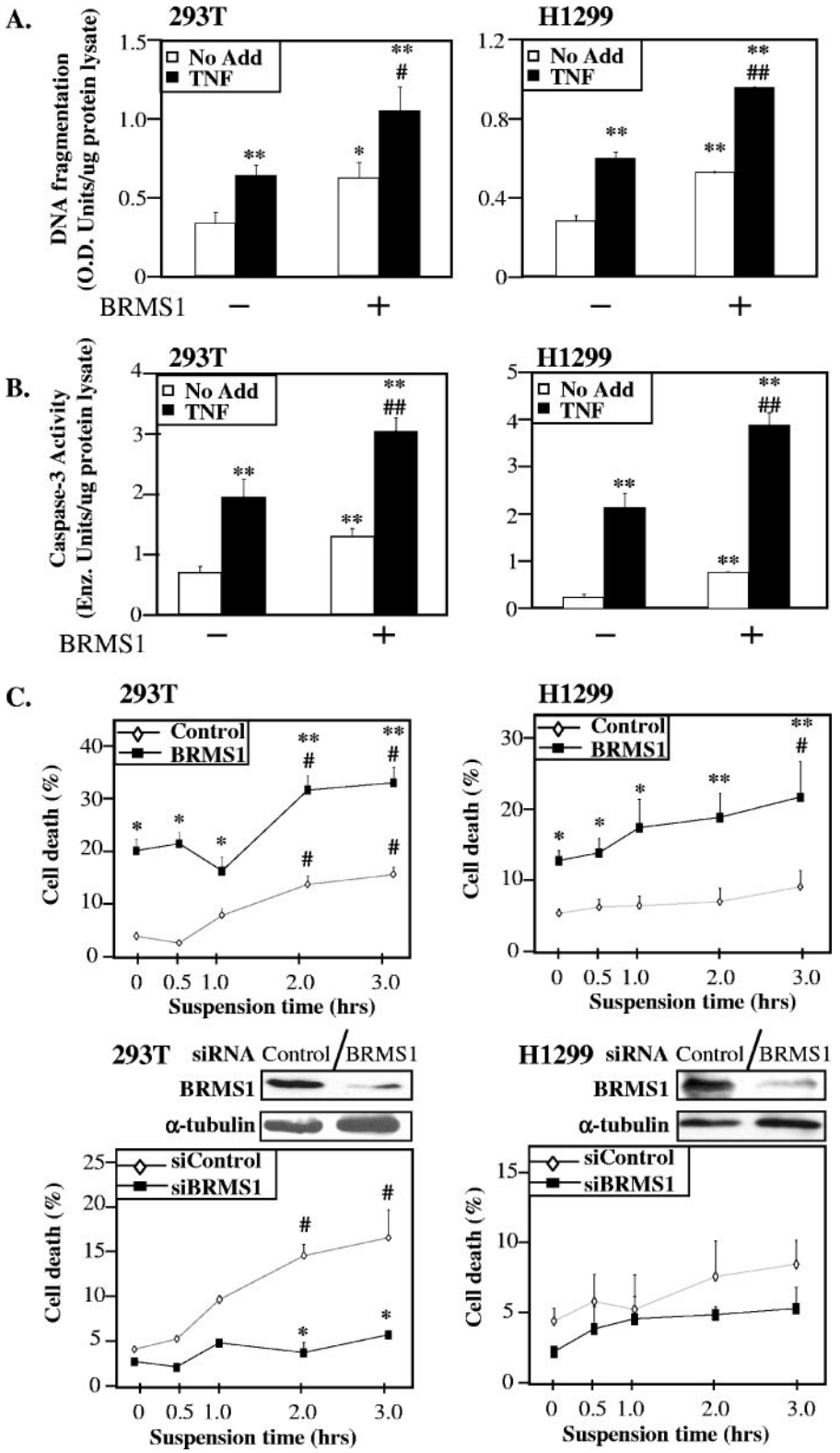


FIG. 7. BRMS1 sensitizes NSCLC cells to apoptosis. (A) 293T cells and H1299 cells were transiently transfected with expression vector encoding BRMS1 or empty vector control. At 24 h after transfection, cells were treated or not treated with TNF (50 ng/ml) for an additional 24 h. Cell extracts were analyzed for DNA fragmentation as a marker for apoptosis. * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to CMV plus No Add groups; # and ##, $P < 0.05$ $P < 0.01$, respectively, compared to CMV plus TNF groups. (B) 293T cells and H1299 cells were transfected and treated as described above. Cell extracts were analyzed for caspase-3 activation as a marker for apoptosis. (C) 293T and H1299 cells were transfected with pCMV-BRMS1 versus pCMV empty vector (Control) or with siRNA-BRMS1 versus siRNA-control as indicated. At 48 h after transfection, cells were suspended for various lengths of time. Cell death was measured by trypan blue exclusion assays. Western blots show that siRNA specifically knocks down BRMS1 protein expression. * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to CMV or siRNA control at each time point; #, $P < 0.05$ compared to time zero groups.

(28). One explanation for this is that during tumorigenesis there is a loss of metastasis suppressor function, thus priming the tumor to develop distant metastases. Evidence for this is shown in Fig. 1, where both BRMS1 mRNA and protein are decreased in NSCLC cell lines and human lung cancer tissues compared to normal epithelial lung cell line and adjacent noncancerous lung tissues.

The three separate steps required for a cancer to successfully metastasize are migration, invasion, and avoidance of cell death or apoptosis (17). Prior studies examining putative mechanisms of BRMS1-mediated suppression of metastasis have demonstrated no effect on cancer cell invasion properties, although breast cancer and melanoma cells overexpressing BRMS1 do show a modest decrease in motility compared to parental cells (52, 57). Other studies have also shown in breast cancer cells that BRMS1 restores gap junctional intercellular communication and increases mRNA expression of phosphorylated connexin 43, which is responsible for closing the gap junction communication (39, 53, 62). We have demonstrated here that an additional mechanism through which BRMS1 functions involves the ability of this corepressor to modulate NF-κB-dependent transcription of genes known to regulate cell survival. Casey and coworkers first reported that BRMS1 could modulate NF-κB nuclear translocation through an IKKβ-independent inhibition of phosphorylation of IκB, the cytosolic protein that sequesters NF-κB, thus preventing its nuclear translocation (13). We hypothesized that BRMS1, a predominantly nuclear and chromatin-associated protein, could also be regulating NF-κB transcriptional activity.

The present study demonstrates that BRMS1 significantly inhibits NF-κB-dependent transcription, specifically the transactivation potential of RelA/p65. Moreover, we show that selective silencing of BRMS1 restores expression of the NF-κB-dependent antiapoptotic genes *cIAP-2*, *Bfl-1/A1*, and *BclxL* in HEK 293T and NSCLC cells and sensitizes both cell lines to TNF-induced apoptosis (Fig. 2 and 7). Previous studies have shown that antiapoptotic proteins protect cancer cells from anoikis and prolong their survival, thus increasing the likelihood that they will survive following invasion through, and detachment from, the extracellular matrix (49, 66). The regulation of tumor cell viability as cancer cells migrate, invade, and then detach and enter the bloodstream is a key event in tumor metastatic progression. Thus, as demonstrated in the present study, BRMS1-mediated suppression of NF-κB-dependent antiapoptotic gene expression results in a proapoptotic state, priming the tumor for TNF-mediated cell death.

We show that BRMS1 coimmunoprecipitates endogenous RelA/p65 and p50 (Fig. 3). Moreover, BRMS1 directly interacts with the C-terminal region of the RHD of RelA/p65 (amino acids 254 to 354). The functional significance of BRMS1 binding to this region of RelA/p65 is shown in Fig. 3E, where maximal repression of the RelA/p65 transactivation potential occurs in constructs overexpressing this binding domain. Interestingly, HDAC1 has also been described to bind to this region of RelA/p65 as well (65).

While NF-κB transcription is regulated through interactions with its inhibitor IκB, more recent studies have identified the importance of acetylation in regulating its activity (10, 11, 64). The RelA/p65 subunit of NF-κB is acetylated by the coactivators p300, CBP, and p/CAF at five known lysine residues, with acetylation of K310 playing the prominent role in regulation of the full transcriptional activity of RelA/p65 (11, 33). In addition, TNF can also promote acetylation of K310 as demonstrated by us and others (12). Realizing that the binding domain of BRMS1 on RelA/p65 included K310, we wanted to determine whether BRMS1 was repressing RelA/p65 transcriptional activity by promoting deacetylation of this lysine residue. Our findings indicate that BRMS1 completely abolishes TNF stimulated acetylation of RelA/p65 on K310 and that this deacetylation results in a dramatic decrease in the transactivation potential of RelA/p65 after TNF treatment (Fig. 4). This observation is interesting given that TNF, along with interleukin-6 and other cytokines, released from tumor-associated macrophages has been shown to stimulate tumor progression through NF-κB-dependent pathways (32). Our data using siRNA knockdown of BRMS1 support the premise that in cancers lacking BRMS1 there is likely be a more robust response to TNF and other cytokine-mediated transcriptional activation of NF-κB-dependent antiapoptotic gene products, which are known to promote tumor cell survival.

While BRMS1 has been shown to associate with the mSin3a/HDAC complex (42), we demonstrate that BRMS1 itself functions as a corepressor by regulating binding of HDAC1 to RelA/p65 and promoting HDAC1-mediated deacetylation of RelA/p65. In contrast to NCoR and SMRT nuclear corepressors, there is no conserved nuclear receptor interaction motif in BRMS1. In addition, there are no classic hydrophobic regions on BRMS1 that have been associated with some corepressors (29). This observation, and the fact that BRMS1 tethers HDAC1 instead of HDAC3, provides a sharp contrast to other well described corepressors, such as NCoR and SMRT.

Our data demonstrate that chromatin-associated BRMS1 regulates the acetyl-K310 RelA/p65 status on the NF-κB-dependent *cIAP-2* and *Bfl-1/A1* promoters (Fig. 6). We cannot dismiss the role of mSin3a and its potential contribution to the inhibition of RelA/p65-dependent transcription since there is a concomitant decrease in mSin3a on chromatin after the loss of BRMS1. In addition, given the corepressor function of BRMS1, it is not surprising to see that it can also modify chromatin structure through enhanced deacetylation of histone tails H3 and H4. However, as noted in Fig. 5, in vitro coexpression of both BRMS1 and HDAC1 is sufficient to efficiently deacetylate RelA/65. Thus, BRMS1 enhances the repression of endogenous NF-κB-dependent antiapoptotic genes by promoting HDAC-1-mediated deacetylation of RelA/p65 directly on chromatin.

In summary, we demonstrate that the metastasis suppressor BRMS1 directly interacts with RelA/p65 and inhibits its transactivation potential, in part, by functioning as a corepressor for HDAC1-mediated deacetylation of RelA/p65. The loss of BRMS1 is likely to affect other transcription factors (i.e., AP1, STAT, etc.) that are regulated by HDAC1 and are involved in cancer cell survival signal transduction pathways. Future studies will focus on upstream events through which BRMS1 is regulated, particularly as they relate to the ability of BRMS1 to decrease tumor cell survival.

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