Cancer Research

BRMS1 Suppresses Lung Cancer Metastases through an E3 Ligase Function on Histone Acetyltransferase p300

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Abstract

The mechanisms through which the metastasis suppressor gene *BRMS1* functions are poorly understood. Herein, we report the identification of a previously undescribed E3 ligase function of BRMS1 on the histone acetyltransferase p300. BRMS1 induces polyubiquitination of p300, resulting in its proteasome-mediated degradation. We identify BRMS1 as the first eukaryote structural mimic of the bacterial IpaH E3 ligase family and establish that the evolutionarily conserved CXD motif located in BRMS1 is responsible for its E3 ligase function. Mutation of this E3 ligase motif not only abolishes BRMS1-induced p300 polyubiquitination and degradation, but importantly, dramatically reduces the metastasis suppressor function of BRMS1 in both *in vitro* and *in vivo* models of lung cancer metastasis. *Cancer Res; 73(4); 1308–17. ©2013 AACR.*

Introduction

Breast cancer metastasis suppressor-1 (BRMS1) suppresses the development of metastasis in lung, melanoma, and breast cancers (1–3). Intratumoral levels of BRMS1 are severely reduced in non–small cell lung cancer (NSCLC) and this reduction correlates with tumor progression and poor prognosis (4). BRMS1 is primarily intranuclear with its regulation at the transcriptional level secondary to *BRMS1* promoter methylation (5, 6). In addition, BRMS1 is regulated through post-translational modifications involving Cul3-SPOP–mediated ubiquitination (7).

While the relevance of BRMS1 to metastasis biology and the processes governing its regulation are becoming clearer, the mechanisms through which BRMS1 suppresses metastases remain poorly characterized.

Previously, our group reported that BRMS1 functions as a corepressor by inhibiting NF-κB transactivation through deacetylation of RelA/p65 (1). Interestingly, BRMS1-mediated repression of NF-κB–dependent transcription was not completely rescued following histone deacetylase inhibitor treatment, suggesting that BRMS1 might also be affecting acetyltransferase(s) activity. In support of this theory, we noticed that both acetyl-H3 and -H4 loading across *cIPA2* and *Bfl1* promoters was markedly reduced following ectopic BRMS1 expression and robustly restored following siRNA knockdown of BRMS1 (1). This observation provided the first clue that in addition to its corepressor function, BRMS1 may have additional functions that govern its ability to act as a metastasis suppressor.

The transcriptional coactivator and acetyltransferase p300 acts as a scaffold to link the basal transcriptional machinery with DNA-binding transcription factors via direct protein–protein interaction (8) while also possessing intrinsic histone acetyltransferase activity (9). p300 is essential for a variety of biologic processes such as cell growth, proliferation, differentiation, cell-cycle regulation, DNA damage response, as well as embryonic organ development (10). Several studies have indicated that p300 is highly expressed in solid tumor malignancies and that overexpression of p300 is related to tumorigenesis and cancer progression (11–13).

In this study, we show that BRMS1 functions as an E3 ligase to specifically promote polyubiquitination and proteasome-mediated degradation of p300. Moreover, we identify a CXD motif in BRMS1 that is responsible for its E3 ligase activity. Mutation of this E3 ligase motif not only abolished BRMS1-induced p300 polyubiquitination and degradation, but importantly, dramatically reduced the metastasis suppressor function of BRMS1 in both *in vitro* and *in vivo* models of lung cancer metastasis. Collectively, this study identifies a previously undescribed E3 ligase function of BRMS1 that serves as an important mechanism through which BRMS1 suppresses metastases.

Materials and Methods

Cell culture, surgical specimens, antibodies, and reagents

Human NSCLC cell lines, normal human bronchial epithelial cell line (NHBE), and human embryonic kidney cells (HEK 293T) were obtained from the American Type Culture Collection and were grown as described (1). Human NSCLC specimens and adjacent noncancerous lung were preserved in liquid

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nitrogen following standard surgical resection from patients at the Division of Thoracic Surgery, University of Virginia (Charlottesville, VA) with individual informed consent and Human Investigations Committee approval. The antibodies used were: p300, β-actin, CBP, $His_{(6)}$-epitope tag, Gal4 (DBD), and normal rabbit immunoglobulin G (IgG, Santa Cruz Biotechnology); BRMS1 (Abnova Corp); ubiquitin, M2 Flag-epitope tag, and α-tubulin (Sigma Aldrich); HA-epitope tag (BD Biosciences); and Ac-H3K14 and Ac-H4K8 (Abcam). siRNA SMART pools: human BRMS1, human p300, and nontargeting siRNA (Control) were purchased from Thermo Scientific. Proteasome inhibitor MG132 was purchased from EMD Chemicals. FLAG-ubiquitin, E1, E2 (Ubc5a), and ATP were purchased from Boston Biochem.

Plasmid construction

The plasmids encoding hemaglutinnin (HA)-tagged full-length p300, HA-tagged BRMS1 wild-type, and Flag-tagged p/CAF were generated as previously described (1, 14). Serial deletions of HA-tagged BRMS1 cDNA were amplified by PCR and inserted into the HA-tagged pCMV vector (Clontech) using *Eco*RI/*Xho*I (New England Biolabs). The expression constructs) encoding glutathione *S*-transferase (GST) fusion BRMS1 proteins, GFP-fusion BRMS1, FLAG-tagged BRMS1, BRMS1 E3M (C126A/D128N), and BRMS1 L/IM (L105R/L108R/L112R/I114N/I116N) were generated as described in Supplementary Materials and Methods. The expression vectors encoding ubiquitin-KO, $His_{(6)}$-ubiquitin, and Gal4-p300 were provided by M.W. Mayo. The pBluescript-$His_{(6)}$-p300 plasmids were a kind gift from by Dr. James T. Kadonaga (University of California, San Diego, La Jolla, CA). The pCMV-HA-p300 ΔN (Δ1-616) plasmid was kindly provided by Dr. David M. Livingston (Dana-Farber Cancer Institute, Boston, MA).

In vitro protein expression and purification

GST-fusion BRMS1 protein was expressed and purified as described previously (1). $His_{(6)}$-p300 protein was expressed in HEK 293T cells and purified using Ni-NTA agarose beads according to the manufacturer's protocol (Qiagen).

In vitro and *in vivo* (cell culture–based) ubiquitination assays

In vitro ubiquitination assays were conducted as previously described with minor modification (15). In brief, ubiquitination assays were conducted in ubiquitin reaction buffer with 100 ng/mL Flag-ubiquitin, 100 nmol/L E1, 100 nmol/L E2 (Ubc5a), 100 nmol/L BRMS1 as E3, substrates GST/GST-fusion BRMS1 (autoubiquitination assays) or $His_{(6)}$-p300 and incubated at 37°C for 60 or 120 minutes, respectively.

For *in vivo* ubiquitination assays, cells were lysed using denaturing lysis buffer with 6 mol/L guanidine. $His_{(6)}$-ubiquitin complexes were precipitated using Ni-NTA agarose beads. After extensively washing in the presence of 8 mol/L urea, the eluted protein was analyzed by immunoblot.

Transient transfection and stable transfection

NSCLCs were transfected using polyfect (Qiagen) as described previously (1). For stable transfections, single-cell clones were selected in the presence of G418 (800 μg/mL; Life Technologies) for 2 weeks.

Immunoprecipitation and Western blot

Immunoprecipitation and Western blotting were conducted as previously described (1). p300 antibody (10 μg/500 μg protein) was used in immunoprecipitation assays. For Western blotting, primary antibodies were used at 1:1000 dilution and secondary antibodies (Promega) were used at 1:5,000 dilution.

NSCLC tissue microarrays

We created a tissue microarray containing 194 NSCLCs and matched adjacent noncancerous tissue. The preparation, immunostaining, and scoring of the NSCLC specimen blocks were described previously (4, 5). The p300 polyclonal antibody was used at 1:200 dilution for 30 minutes.

Transwell invasion assays

Invasion of NSCLCs was assessed as previously described (4). Briefly, 2.5×10^4 cells suspension was plated in the invasion chamber (BD Biosciences). After incubation for 20 hours, cells were fixed and staining with 0.1% crystal violet.

NSCLC murine xenograft models

NSCLC xenografts were created and measured as previously described (16). All animal experiments were approved by the University of Virginia Animal Care and Use Committee. Briefly, the flanks of 4-week-old athymic nude mice (Taconic) were inoculated with 0.1 mL of media containing 2×10^6 cells (H157 Cont, BRMS1 WT, and E3M, $n = 10$/group). After the tumor diameter grew to approximately 0.5 cm, tumors were measured twice per week and volumes were calculated. Mice were euthanized when the diameter of tumors reached 1.5 cm. The lungs were examined under a dissecting microscope to count visible lung surface tumor metastatic deposits.

Total RNA isolation and reverse transcription PCR

Quantitative reverse transcription (RT)-PCR reactions were carried out as described previously (1). The p300 primers were 5′-GGGACTAACCAATGGTGGTG-3′ (forward) and 5′-GCCACCAACTCCCATATTGA-3′ (reverse).

Statistical analysis

The results of all experiments represent the means ± the SD of 3 separate experiments conducted in triplicate. Statistical differences were determined by Student *t* test when appropriate. $P < 0.05$ was considered statistically significant.

Results

BRMS1 and p300 protein expression levels are inversely correlated in human lung cancer

Given our previous observations (1, 4), we sought to determine whether there was a differential protein expression of p300 and BRMS1 in NSCLC specimens. Immunostaining for p300 protein was conducted using a tissue microarray with human NSCLC specimens ($n = 194$), as well as matched adjacent noncancerous tissue. Robust intranuclear staining

for p300 protein was observed in the majority of NSCLC samples compared with adjacent noncancerous tissue (about 7.1-fold increase; Fig. 1A). In addition, in contrast to BRMS1, we observed that p300 protein levels are relatively high in multiple NSCLC cell lines than in noncancerous cell lines (NHBE and HEK 293T; Fig. 1B). Furthermore, overexpression of p300 significantly promotes the invasion potential of NSCLC H157 cells, but not cell growth (Fig. 1C and Supplementary Fig. S1). BRMS1 inhibits the basal and p300-induced invasion of H157 cells (Fig. 1C). Interestingly, overexpression of BRMS1 dramatically inhibits the protein expression of both endogenous and ectopic p300 (Fig. 1C). Collectively, these data suggest that BRMS1 and p300 may function oppositely in NSCLCs and BRMS1 specifically blocks p300-induced invasion through regulating p300 expression.

BRMS1 regulates p300 protein expression through proteasome-mediated degradation

To confirm that BRMS1 directly affects the expression of p300, we artificially regulated the expression level of BRMS1 in NSCLC H1299 cells using siRNAs specific for BRMS1 knockdown or an ectopic expression plasmid for BRMS1 (HA-BRMS1). Knockdown of BRMS1 dramatically increased the protein level of p300, as well as the levels of histone acetylation (acetyl-H3 K14 and acetyl-H4 K8; ref. 17). In contrast, both the p300 expression and histone acetylation were reduced by ectopic expression of BRMS1 (Fig. 2A). Interestingly, the presence or absence of BRMS1 had no effect on the expression of the transcriptional coactivators CBP or p/CAF (Fig. 2A and Supplementary Fig. S2). To experimentally address whether BRMS1 inhibits *p300*

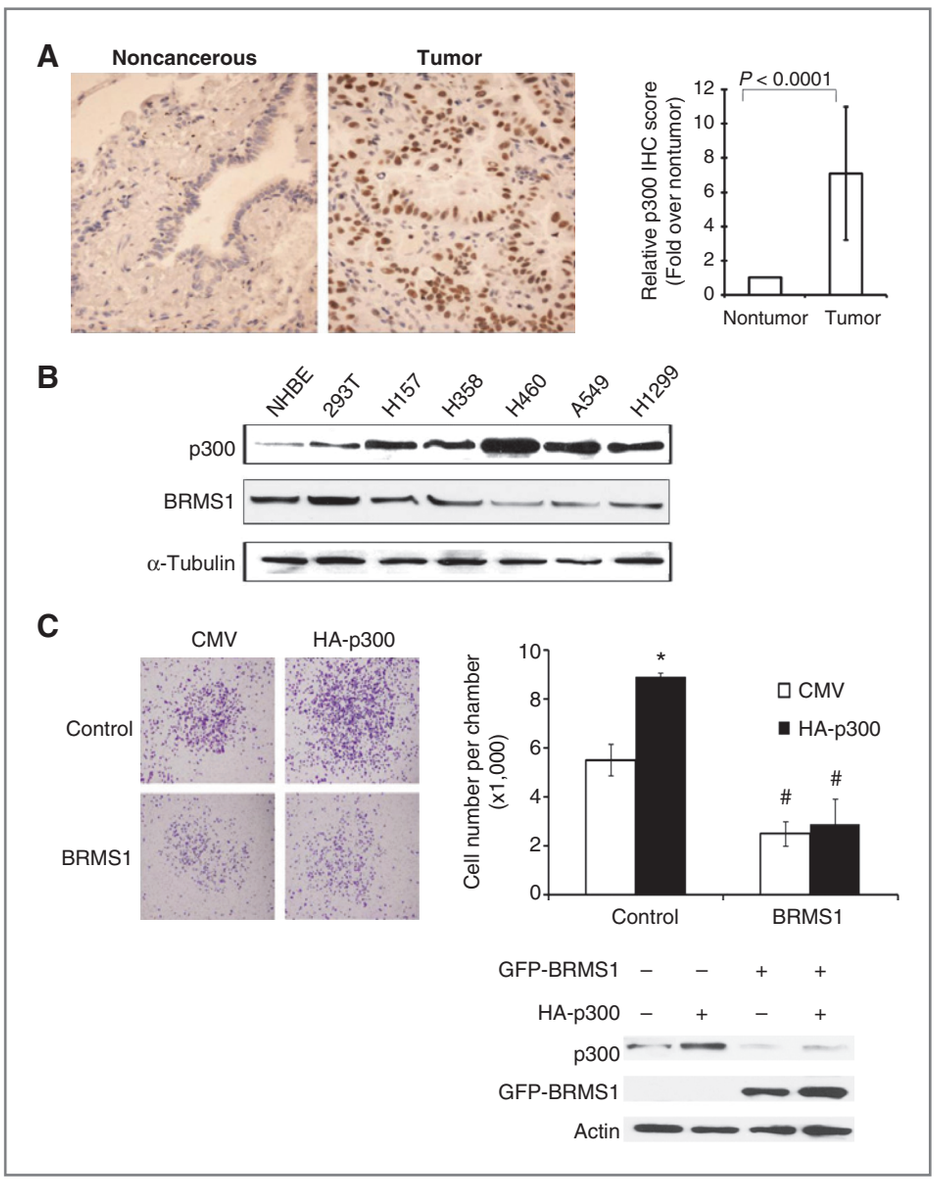

Figure 1. The protein level of p300 inversely correlates with BRMS1 expression. A, photomicrographs (left) and scoring index (right) showing nuclear staining for p300 in human NSCLCs and matched adjacent noncancerous lung tissue. B, the protein levels of p300 and BRMS1 were detected in the indicated cell lines. C, NSCLC H157 cells stably expressing BRMS1 or empty vector (Control) were transfected with HA-p300 or empty vector (CMV). The invasion assays were conducted. *, $P < 0.001$ compared with CMV; #, $P < 0.001$ compared with control group. Western blotting showed the expression of p300 and ectopic BRMS1.

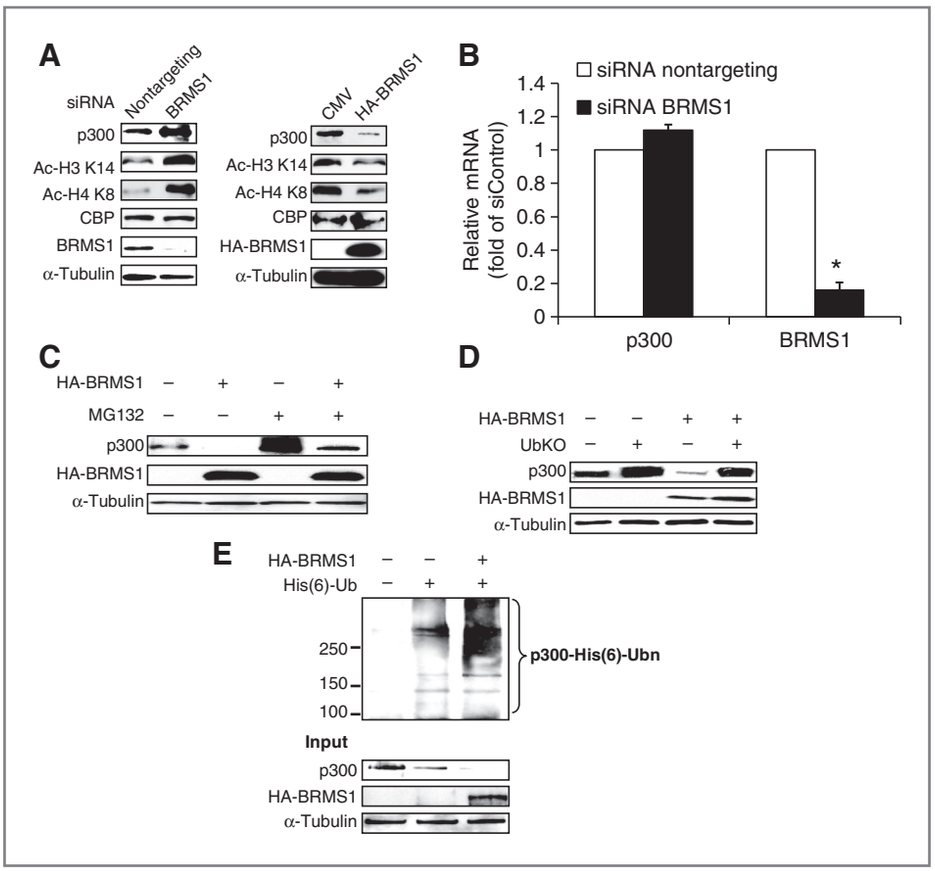

Figure 2. BRMS1 regulates the protein level of p300 through the proteasome pathway. A, H1299 cells were transfected with siRNA BRMS1 or siRNA nontargeting sequences (left) or HA-BRMS1 or empty vector (CMV, right). The expression of indicated proteins was assessed. B, H1299 cells were transfected with siRNA BRMS1 or siRNA nontargeting sequences, and quantitative RT-PCR was carried out. *, $P < 0.01$ compared with siRNA nontargeting sequences. C, H157 cells were transfected with HA-BRMS1 or empty vector and then treated with or without MG132 (5 μmol/L) for 12 hours. The expression of endogenous p300 and HA-BRMS1 was assessed. D, H1299 cells were transfected with HA-BRMS1 and/or UbKO. The expression of p300 and HA-BRMS1 was assessed. E, H1299 cells were cotransfected with $His_{(6)}$ ubiquitin and HA-BRMS1. $His_{(6)}$-ubiquitin complexes were precipitated using Ni-NTA agarose beads and immunoblot was conducted using anti-p300 antibody.

transcription, we carried out quantitative RT-PCR following siRNA knockdown of BRMS1 and found that reduction of *BRMS1* does not affect *p300* transcription (Fig. 2B). Collectively, these data suggest that BRMS1 inhibits p300 expression through posttranslational modification(s).

It has been suggested that p300 is a substrate for the ubiquitin–proteasome proteolytic pathway (18, 19). To determine whether BRMS1 affects p300 expression through the proteasome pathway, we used the proteasome inhibitor MG132 to treat NSCLC H1299 cells. As shown in Fig. 2C, overexpression of HA-BRMS1 drastically decreased p300 protein levels. However, treatment with MG132 rescued p300 expression even in the presence of ectopic BRMS1 expression. To further confirm that the proteasome pathway is involved in this process, we used a cDNA encoding ubiquitin with the lysines replaced by arginines (UbKO; ref. 20). As shown in Fig. 2D, the presence of UbKO rescued the expression of p300 protein at both the basal level and during ectopic expression of HA-BRMS1. This strongly suggests that ubiquitin–proteasome–mediated proteolysis is involved in the BRMS1-induced decrease of p300 protein. Finally, as shown in Fig. 2E, BRMS1 promotes polyubiquitination of p300 compared with control in the NSCLC H1299 cells. In summary, BRMS1 decreases p300 protein expression by inducing polyubiquitination and proteasome-mediated degradation of p300.

BRMS1 functions as an E3 ligase to mediate p300 degradation

The E3 ligase is the key enzyme in the ubiquitination process and is capable of catalyzing auto-ubiquitination (15, 21). To test the hypothesis that BRMS1 may have E3 ligase activity, we first conducted an *in vitro* autoubiquination assay. A purified GST-BRMS1 fusion protein was used to avoid concerns about contamination from ubiquitination-related enzymes in eukaryotic cells. As shown in Fig. 3A, the E3 activity of BRMS1 was observed when all enzymes were present (Fig. 3A, lane 2). However, in the absence of either E1 or E2, BRMS1 failed to autoubiquitinate (Fig. 3A, lanes 4 and 6). In addition, BRMS1 autoubiquitination also occurs in cells (Fig. 3B). These data suggest that BRMS1 possesses intrinsic E3 ligase activity as evidenced by its ability to catalyze the formation of polyubiquitin chains.

To confirm that p300 is a substrate of BRMS1 E3 ligase, we repeated the *in vitro* ubiquitination assay using $His_{(6)}$-p300 as the substrate. BRMS1 dramatically enhanced the polyubiquitination of p300 in the presence of E1 and E2 (Fig. 3C). Interestingly, we observed that $His_{(6)}$-p300 was partially auto-ubiquitinated even in the absence of BRMS1. A previous report had showed that the N-terminus region of p300 contains intrinsic E3 ligase activity, which catalyzes the auto-ubiquitination of p300 (15). To clarify whether BRMS1 modulates polyubiquitination of p300 by enhancing the intrinsic E3 ligase activity of p300, we used a cDNA construct that encodes a p300

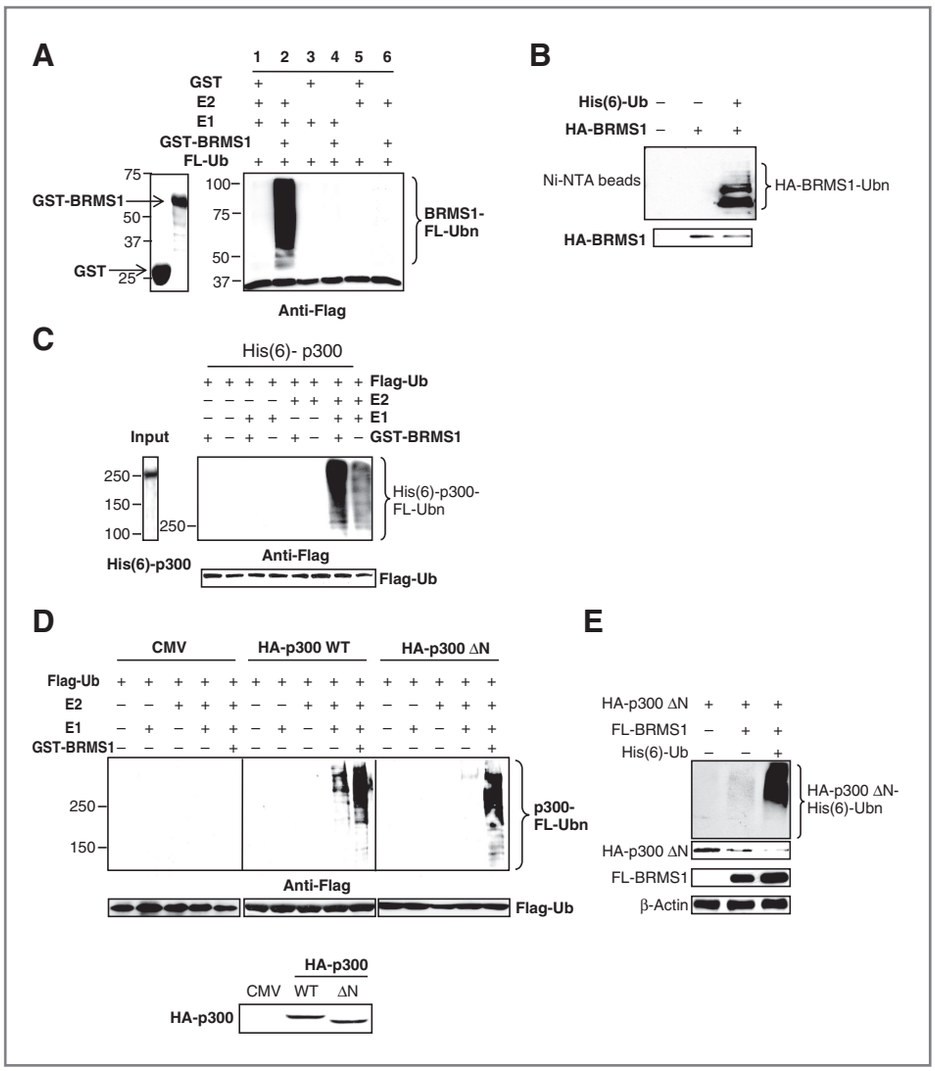

Figure 3. BRMS1 functions as an ubiquitin ligase to mediate p300 degradation. A, left, Coomassie blue–stained gel containing purified fusion proteins. Right, GST-BRMS1 or GST incubated with ubiquitination reactions. The products were analyzed by immunoblots using anti-Flag antibody. B, HEK 293T cells were transfected with HA-BRMS1 and $His_{(6)}$-ubiquitin. $His_{(6)}$-ubiquitin complexes were pulled down by Ni-NTA beads and Western blotting was conducted using anti-HA antibody. C, left, purified $His_{(6)}$-p300 protein from HEK 293T cells. Right, $His_{(6)}$-p300 protein was incubated with the ubiquitination reactions ± GST-BRMS1. The products were analyzed as described in A. D, HEK 293T cells were transfected with indicated HA-p300 or empty vector. The complexes were pulled down with anti-HA antibody and incubated in ubiquitination reactions. The products were analyzed using an anti-Flag antibody. E, H1299 cells were transfected with HA-p300 ΔN, $His_{(6)}$-ubiquitin, and Flag-BRMS1. Immunoprecipitations were conducted using anti-HA antibody, and the products were analyzed using anti-$His_{(6)}$ antibody.

N-terminal (1–616 aa) deletion protein (HA-p300ΔN), which lacks the intrinsic E3 ligase activity (15). We observed no autoubiquitination of HA-p300 ΔN in the absence of BRMS1. More importantly, GST-BRMS1 also induced the formation of polyubiquitin chains on HA-p300ΔN (Fig. 3D). A similar result was also observed in our *in vivo* ubiquitination assays (Fig. 3E). Collectively, these data show that BRMS1 is an E3 ligase and can catalyze p300 polyubiquitination, regardless of the intrinsic p300 E3 ligase activity.

The C-terminus region of p300 interacts with BRMS1 and is required for BRMS1-induced degradation

Confirmation that BRMS1 and p300 interact would further support our premise that BRMS1 functions as an E3 ligase for p300. As shown in Fig. 4A, BRMS1 endogenously interacts with p300. Next, we sought to map the BRMS1-binding region in p300 using Gal4-fused p300 proteins. As shown in Fig. 4B, BRMS1 binds p300 in the 1,905–2,414 aa region.

To further characterize the binding region of BRMS1 on p300, we used a series of p300 deletion expression constructs (22). As shown in Fig. 4C, deletion of amino acids 2,042–2,157 in p300 rescued BRMS1-induced degradation (Fig. 4C) and polyubiquitination (Fig. 4D) of p300. We next asked whether the binding region on p300 also contained the lysine residues that undergo BRMS1-induced ubiquitination and result in p300 proteasome degradation. Mutation of 3 lysines in the putative binding region (2,042–2,157), either individually or in combination, failed to completely rescue BRMS1-mediated p300 degradation (Supplementary Fig. S3A and S3B). This suggests that the lysine residues on p300 undergoing BRMS1-induced ubiquitination resulting in the proteasome-mediated degradation of p300 do not reside in the same region as the BRMS1: p300 protein interaction.

The linker region and a CLD motif in BRMS1 are required for its E3 ligase function

To map the p300 interaction region in BRMS1, we created a series of deletion constructs of the BRMS1 protein including expanded deletion of CC1 or CC2 domain (ref 23; Fig. 5A). Co-immunoprecipitation assays indicate that deleting the region

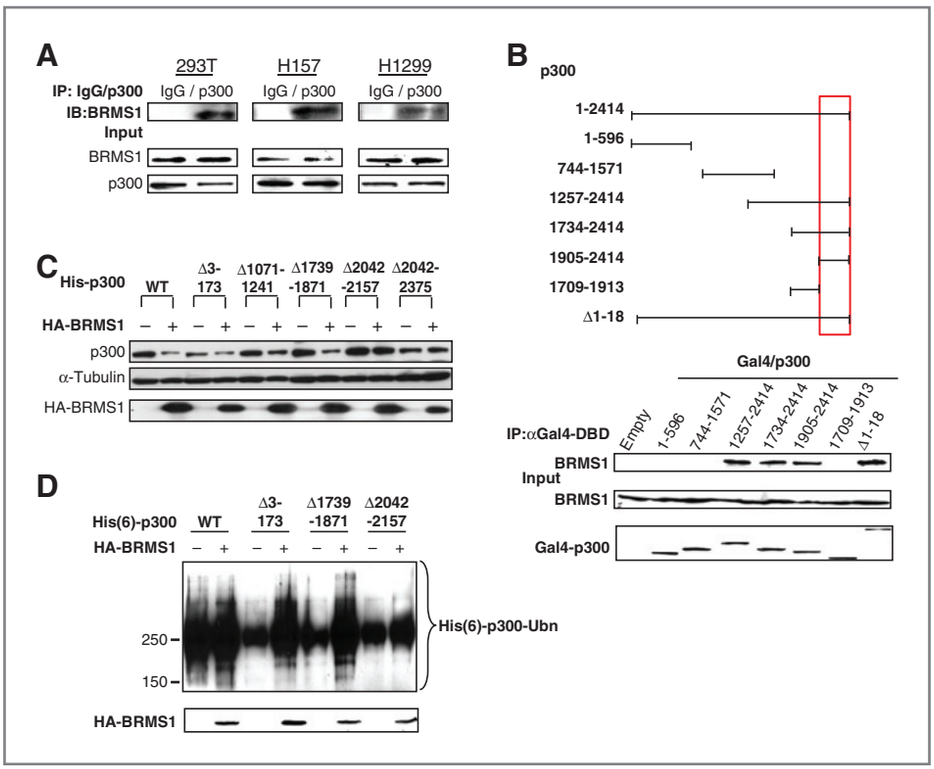

Figure 4. The C-terminal region of p300 is responsible for BRMS1 binding. A, coimmunoprecipitation was conducted using anti-p300 antibody or rabbit IgG followed by immunoblotting for the presence of endogenous BRMS1. B, top, schematic illustration of GAL4-fused p300 proteins. Bottom, HEK 293T cells were transfected with Gal4-p300 fusion proteins as indicated. Immunoprecipitations were conducted using anti-Gal4(DBD) antibody, and the presence of BRMS1 was analyzed. C and D, H1299 cells were cotransfected with indicated $His_{(6)}$-p300 and HA-BRMS1 or empty vector. C, the expression of $His_{(6)}$-p300 proteins was detected using anti-$His_{(6)}$ antibody. D, $His_{(6)}$-p300 complexes were pulled down by Ni-NTA beads and Western blotting was conducted using anti-ubiquitin antibody.

(84–124 aa) between 2 coiled-coil domains, BRMS1 fails to bind to endogenous p300. Furthermore, only wild-type or the first coiled-coil domain deletion (ΔCC1) BRMS1 constructs resulted in p300 degradation, suggesting that the E3 ligase function of BRMS1 is in the CC2 and/or linker region (Fig. 5A). A similar result was observed using p300 ΔN as the substrate of BRMS1 (Supplementary Fig. S4A). We hypothesized that the role of the linker region in BRMS1 is related to protein–protein interaction, whereas the CC2 domain may contain BRMS1 E3 ligase activity. In support of this hypothesis, we identified an imperfect LXXLL motif (105–116 aa) in this linker region, which is similar to the p300-interacting domain of SRC-1 (Supplementary Fig. S4B; ref. 24). Selective mutation of the conserved leucine/isoleucine residues in this linker region resulted in a robust decrease in BRMS1 and p300 interaction (Supplementary Fig. S4C). Next, to mitigate any interference related to endogenous BRMS1, we conducted *in vitro* GST pull-down assays. As shown in Fig. 5B, wild-type BRMS1 binds p300 whereas selected mutation of LXXLL leucine/isoleucine residues in BRMS1 fails to bind to p300. Thus, the LXXLL motif in the linker region of BRMS1 appears to be responsible for p300 recognition and binding.

Analysis of BRMS1 failed to identify a motif characteristic of any of the classic E3 ligases (25). Recently, Shao and colleagues reported that a CXD catalytic motif in the bacterial pathogen *Shigella flexneri* that can function as an E3 ligase (26). Subsequent analysis of the amino acid sequence of BRMS1 revealed a CXD motif (C126/L127/D128) that we hypothesized may function as its E3 catalytic domain (Fig. 5C). To experimentally address this possibility, we created a cDNA construct to express a BRMS1 protein with C126A/D128N mutations. We then generated NSCLC H157 and H1299 cell lines, which stably express BRMS1 wild-type (WT), C126A/D128N mutant (E3M), or an empty vector (control). In contrast to NSCLC H157 and H1299 WT BRMS1 cells, E3M BRMS1 cells failed to induce endogenous p300 polyubiquitination and its subsequent degradation, as well as preserved the half-life time of p300 protein (Fig. 5C and Supplementary Fig. S4D). In addition, mutation of these 2 amino acids (C126A/D128N) had no effect the BRMS1: p300 interactions (Supplementary Fig. S4E). Finally, *in vitro* ubiquitination assays were conducted using purified GST-BRMS1 WT or E3M as the E3 ligase and $His_{(6)}$-p300 and BRMS1 (Fig. 5D and Supplementary Fig. S4F) as the substrates. BRMS1 WT induced p300 polyubiquitnation (Fig. 5D) and its own autoubiquitination (Supplementary Fig. S4F) in an ATP-dependent manner. However, BRMS1 E3M failed to induce either autoubiquitination or p300 polyubiquitination (Fig. 5D).

Collectively, our results indicate that the imperfect LXXLL motif in the linker region of BRMS1 (84–124 aa) is important for the protein–protein interaction between BRMS1 and p300. Furthermore, a CXD motif (126–128 aa) in BRMS1 is responsible for the E3 catalytic activity of BRMS1 as evidenced by the fact that mutation of this CXD motif results in the complete loss of BRMS1 E3 ligase function.

The E3 ligase function of BRMS1 is important for tumor growth and metastasis

To experimentally address the potential biologic significance of this novel E3 ligase function of BRMS1, cell invasion assays were conducted. BRMS1 WT–expressing cells have a

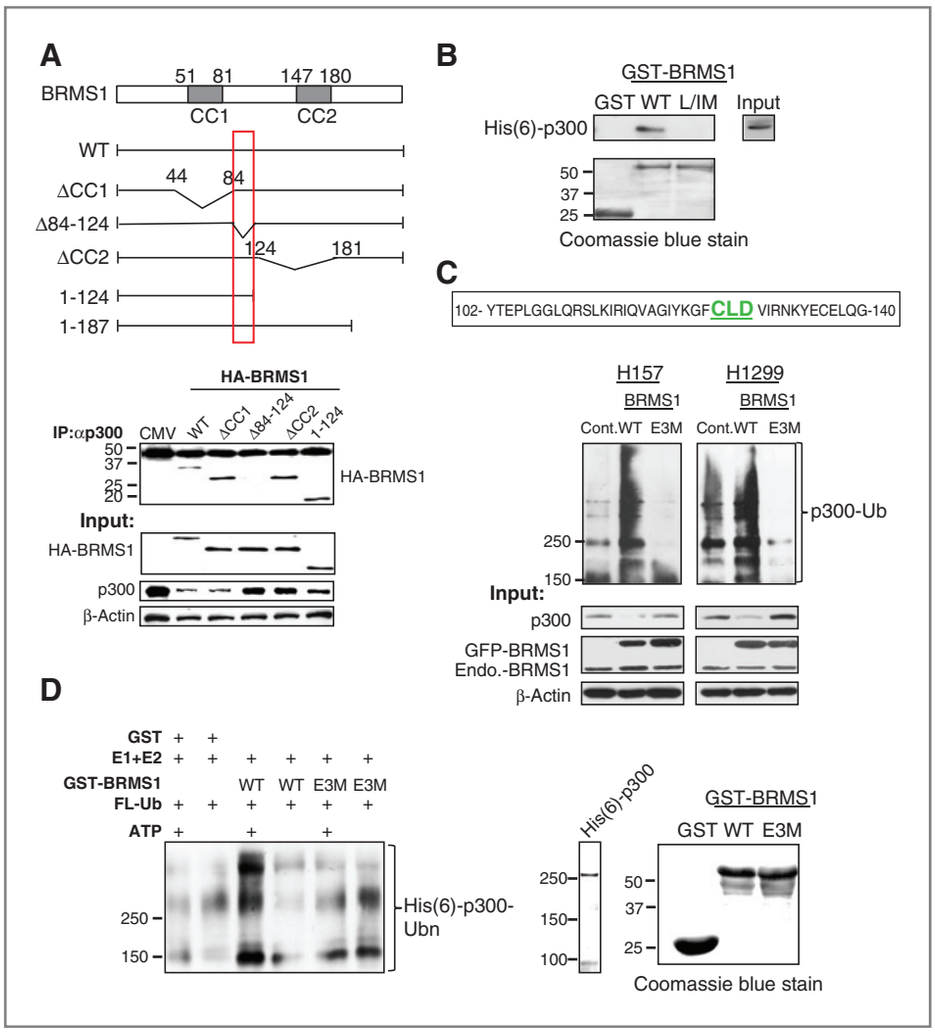

Figure 5. A CLD motif in BRMS1 is indispensable for its function as an ubiquitin ligase of p300. A, top, schematic illustration of HA-BRMS1 proteins. Gray boxes, coiled-coil domains. Bottom, HEK 293T cells were transfected with the indicated HA-BRMS1 proteins. Immunoprecipitations were conducted using anti-p300 antibody and immunoblots were conducted with anti-HA antibody. B, GST-fused BRMS1 WT or L/I M were incubated with purified $His_{(6)}$-p300. The pull-down complexes were analyzed by immunoblot with anti- $His_{(6)}$ antibody. C, top, schematic illustration of the CLD (C126/L127/D128) motif. Bottom, H1299 and H157 cells were stably transfected with BRMS1 WT, E3M (C126A/D128N), or empty vector (Control). Immunoprecipitations were conducted using anti-p300 antibody and Western blotting was conducted using anti-ubiquitin. D, purified $His_{(6)}$-p300 protein was incubated with ubiquitination reactions ± ATP and GST-BRMS1. Left, the polyubiquitinated $His_{(6)}$-p300 was assessed by anti-Flag antibody. Right, Coomassie blue–stained gels showed purified proteins.

decreased invasive potential compared with control. However, cells expressing BRMS1 E3M did not alter the invasion ability of control NSCLC H1299 (Fig. 6A and Supplementary Fig. S5A) and H157 cells (Supplementary Fig. S5A). Given that the only known substrate to date for BRMS1 E3 activity is p300, we also asked whether the decreased NSCLC cell invasion associated with the loss of BRMS1 E3 ligase function was a p300-dependent process. We used siRNAs specific to p300 to selectively knockdown its expression in NSCLC H1299 stable cells as described above. p300 knockdown decreased the invasive potential in the control cells and had very little impact in BRMS1 WT cells where p300 levels are already very low. Importantly, p300 knockdown resulted in a robust 3-fold reduction in NSCLC cell invasion in BRMS1 E3M cells (Fig. 6A). These data suggest that the E3 ligase activity of BRMS1 is essential for suppressing the invasion potential of NSCLCs, and the proinvasion phenotype exhibited following the loss of BRMS1 E3 ligase function is, at least in part, a p300-regulated process.

To assess the importance of BRMS1 E3 ligase activity on the rate of tumor growth and the development of metastases *in vivo*, we used an NSCLC xenograft model known to our laboratory (4). Similar to other reports (4, 27), BRMS1 WT tumors had no significant effect on tumor growth rates or tumor volumes compared with control tumors (Fig. 6B). Interestingly, BRMS1 E3M tumors grew significantly faster and their tumor volume increased more rapidly compared with BRMS1 WT and control tumors (Fig. 6B). Comparison of overall tumor burden at each time point reveals that ectopic expression of BRMS1 E3M had a higher rate of tumor formation than BRMS1 WT and control (Supplementary Fig. S5B). In agreement with our hypothesis, we found similar levels of ectopic BRMS1 WT and E3M proteins in the primary tumors with decreased endogenous p300 protein in tumors with ectopic BRMS1 WT protein, but not in tumors with ectopic BRMS1 E3M (Fig. 6C).

Consistent with our previous finding (4), we observed a decreased number of lung metastases in the BRMS1 WT xenografts compared qith control. However, there was a significant increase in lung metastases from BRMS1 E3M tumors compared with BRMS1 WT and control xenografts (Fig. 6D). These observations support the tumor and

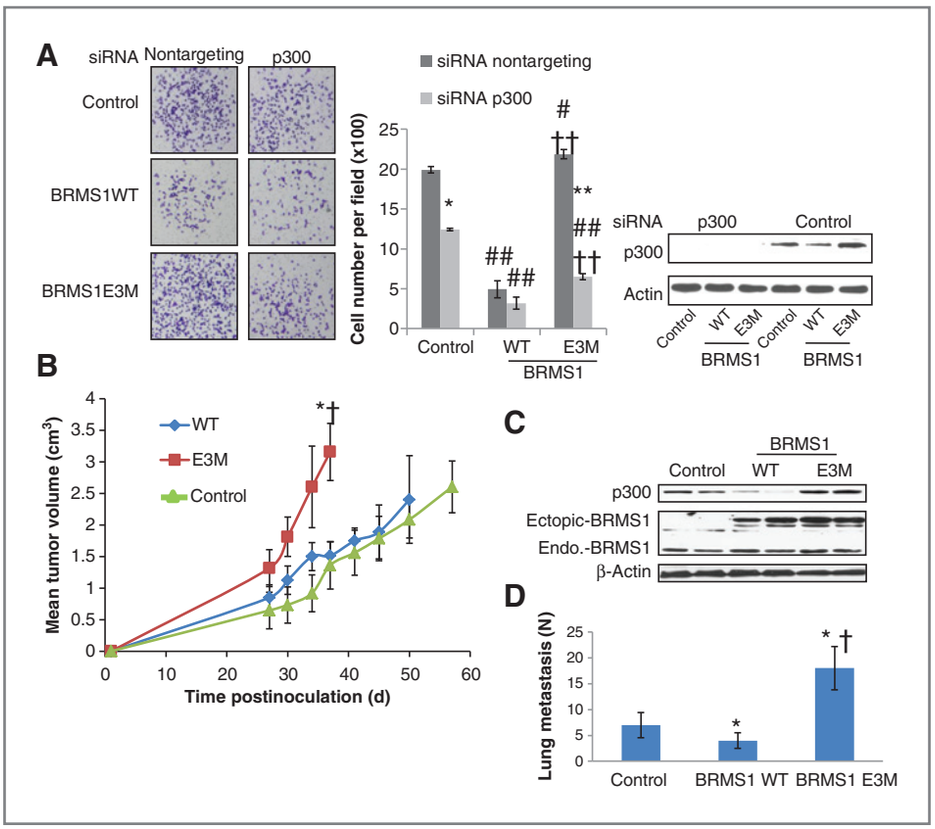

Figure 6. Mutation of CXD E3 ligase motif abolishes the function of BRMS1 as tumor metastasis suppressor. A, H1299 cells stably expressing BRMS1 were transfected with siRNA p300 or siRNA nontargeting sequences and the invasion ability was evaluated. *, $P < 0.05$; **, $P < 0.01$ compared with matched siRNA nontargeting sequences groups; #, $P < 0.05$; ##, $P < 0.01$ compared with control; ††, $P < 0.01$ compared with BRMS1 WT. B, BRMS1 E3M-mutant promotes tumor growth. Mice xenografts were generated using H157 empty vector (Control; $n = 6$), BRMS1 WT ($n = 9$), or E3M cells ($n = 7$). The graph showed the primary tumor growth rates and tumor volume. *, $P < 0.05$ compared with Control; †, $P < 0.05$ compared with BRMS1 WT. C, Western blotting indicate the expression of endogenous p300, ectopic BRMS1, and β-actin in the subcutaneous tumors. D, BRMS1 E3M-mutant increases pulmonary metastases. Graphic data are expressed as the mean ± SEM. *, $P < 0.05$ compared with control; †, $P < 0.05$ compared with BRMS1 WT.

metastasis biologic relevance of the E3 ligase function of BRMS1. In fact, loss of its E3 ligase activity not only produced the expected increase in numbers of metastases but also surprisingly resulted in enhanced and more rapid tumor growth—characteristics not commonly associated with metastasis suppressor genes.

Discussion

The present study shows that the metastasis suppressor BRMS1 promotes proteasome-mediated degradation of the transcriptional coactivator p300 via a newly described E3 ligase function found in a unique CXD motif with catalytic activity located in the second coiled-coil domain of BRMS1. This observation has immediate implications including the ability of BRMS1 to affect targets of p300-dependent transcription as well as relevance to the metastasis biology of lung cancer and likely other solid tumor malignancies where BRMS1 has been shown to be important (4, 27). In addition, we found that BRMS1 binds the C-terminal region of p300 (2,042–2,157 aa), whereas the linker region (84–124 aa) in BRMS1 is required for p300 binding. Perhaps most importantly, the E3 ligase activity of BRMS1 is critical to its ability to function as a suppressor of metastases such that mutation of this E3 catalytic motif severely impaired the ability of BRMS1 to suppress lung cancer metastases.

It has been reported that high expression of p300 correlates with tumor aggressive feature and poor prognosis (11–13, 28, 29). As a ubiquitous transcriptional coactivator, it is not surprising that p300 activates a number of tumor-related transcription factors, such as Myc (30), androgen receptor (31), E2F1 (32), NF-κB (33), and STAT1 (34). In response to DNA damage, p300 promotes DNA replication and repair to induce tumor cell proliferation and chemo- and radioresistance via acetylation of histone H3/H4 in multiple types of cancer (35, 36). In support of the increasing appreciation of the importance of p300 in tumor biology, we also observed a robust increase in p300 expression in both cancer cell lines and human NSCLCs, compared with normal human bronchial epithelial cells and matched adjacent noncancerous lung tissues, respectively. Furthermore, overexpression of p300 significantly increases the invasion potential of NSCLCs. However, p300 does not appear to promote the growth of tumor cells in our 3-dimensional cell culture systems. Thus, based on our data, more likely p300 may function as a metastasis promoter but not a tumor enhancer in NSCLCs.

Despite the importance of p300 in tumor biology as well as a number of important cellular processes (37), there is surprisingly little known regarding how p300 protein is regulated. It has been shown that p300 protein is a target of the ubiquitin–proteasome degradation pathway (19, 38, 39). Our study shows that BRMS1 is a bona fide E3 ligase for p300 with the E3 catalytic activity of BRMS1 directly regulating the p300 protein level. Similar to several reported atypical E3 ligases (15, 40, 41), BRMS1 cannot be classified as a member of the known E3 ligase families. The E3 ligase function of the CXD motif was uncovered following the identification of ubiquitin ligase

activity in the bacterial pathogen *Shigella flexneri* type III effector IpaH9.8 (26). The cysteine in the CXD motif acts a thiol nucleophile to catalyze ubiquitin transfer through a transthiolation reaction, which is analogous to the catalytic cysteine in the HECT-type E3 ligases. Similar to the bacterial E3 IpaH family of E3 ligases, BRMS1 contains an evolutionarily conserved E3 catalytic motif (CXD, C126/L127/D128). Interestingly, examination of other metastasis suppressor proteins failed to identify a CXD motif, suggesting that this motif and E3 ligase function may be unique to BRMS1. As we show, double mutation of Cys126 and Asp128 resulted in complete abolishment of BRMS1 E3 ligase activity, inability of BRMS1 to polyubiquitinate p300, and immediate and complete restoration of p300 protein levels. This study provides the first evidence that this CXD motif has E3 ligase activity in eukaryote cells, in addition to the previously described prokaryote cells. This perhaps introduces BRMS1 as the first of potentially many new E3 ligases in eukaryotes that could be classified using this biochemical and structural distinction.

As previously noted, there are 2 coiled-coil domains in BRMS1 that are involved in protein–protein interaction (42). Unexpectedly, deletion of either coiled-coil domain failed to impair the interaction of BRMS1 with p300. Interestingly, deletion of the linker region (84–124 aa) between these 2 coiled-coil domains or mutation of the conserved leucine/isoleucine residues in this region completely disrupted the BRMS1-p300 interaction. While our data suggest that this linker region between the 2 coiled-coil domains is critical for BRMS1-binding p300, we cannot unequivocally prove this. It is possible that altering the linker region may induce conformational changes in BRMS1, resulting in a functional change of the coiled-coil domain(s), which are bridged by the linker region.

This study identifies a novel E3 ligase function of BRMS1 that explains its ability to suppress metastasis. Our data suggest that the E3 ligase activity is essential for BRMS1 suppressing the invasion potential of NSCLCs and p300 as an important substrate of BRMS1 is involved in this process. As an E3 ligase, it is plausible that BRMS1 has some other unidentified substrate(s) that also is/are related to BRMS1-mediated metastasis suppression, except for p300. In addition, our group and others previously reported that BRMS1 can function as a corepressor to inhibit tumor metastasis (1, 43). How the E3 ligase function and the corepressor function play together contributing to BRMS1-mediated metastasis suppression needs to be further clarified in the future research. Interestingly, loss of BRMS1 E3 ligase function via mutation of the CXD motif results in a robust increase of tumor invasiveness and the development of distant metastases in both cell culture and *in vivo* lung cancer model systems, although the protein levels of p300 do not have significant difference between control and E3-mutant groups. These data suggest that BRMS1 E3-mutant promoting lung cancer metastasis is not a completely p300-dependent process. It is possible that E3-mutant alters the corepressor function of BRMS1, resulting in the increase of metastasis. However, our *in vitro* deacetylation data do not support this postulate (data not shown). In addition, more likely E3 mutant, like mutant p53 (44), might result in BRMS1 gain-of-function and possessing some oncogenic properties to promote tumor growth and metastasis. Supporting this hypothesis, we surprisingly found that in contrast to *BRMS1* being solely classified as a metastasis suppressor gene without effect on the primary tumor growth (4, 27), lung cancer cells expressing E3-mutant BRMS1 exhibited a significant increase in NSCLC tumorigenesis as well as primary tumor growth. Thus, loss of the E3 catalytic activity of BRMS1 appears to induce a different tumor biology function not previously associated with BRMS1 and metastasis suppressor genes in general (4, 23, 27) and this BRMS1 E3 mutant gain-of-function is p300-independent.

In summary, this study identifies a novel E3 ligase function for the metastasis suppressor gene *BRMS1*. We show that BRMS1 is a bona fide E3 ligase for p300 and as such promotes polyubiquitination and proteasome-mediated degradation of p300. We identify BRMS1 as the first eukaryote structural mimic of the bacterial IpaH E3 ligase family and establish that the evolutionarily conserved CXD motif is responsible and critical for its E3 ligase function. Finally, this study offers important mechanistic and functional insights into how BRMS1 functions as a metastasis suppressor in NSCLCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Writing, review, and/or revision of the manuscript: Y. Liu, E.H. Hall, L.S. Shock, D.R. Jones
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