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Original Article

Breast cancer metastasis suppressor 1 modulates SIRT1-dependent p53 deacetylation through interacting with DBC1

Xueni Liu*, Elphire Ehmed*, Boyao Li, Jianming Dou, Xiaojing Qiao, Wenyong Jiang, Xi Yang, Shouyi Qiao, Yanhua Wu

*State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, Shanghai 200438, P. R. China. *Equal contributors.*

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Abstract: Breast cancer metastasis suppressor 1 (BRMS1) is a specific tumor metastasis suppressor implicated in the regulation of chromatin modification and gene transcription. However, the molecular mechanism of BRMS1 remains to be elucidated. Here, we report that DBC1 (deleted in breast cancer 1), is a novel interacting protein of BRMS1. The imperfect leucine zipper motifs of BRMS1 and the N-terminal domain of DBC1 are required for the interaction. DBC1 is identified as an important negative regulator of SIRT1's activity and genotoxic stress response. We demonstrated that BRMS1 is able to interrupt endogenous DBC1-SIRT1 association. Consistently, SIRT1-dependent p53 acetylation under genotoxic stress is also affected by BRMS1. Overall, our results identify BRMS1 as a novel regulator of DBC1-SIRT1 complex and SIRT1-dependent p53 deacetylation.

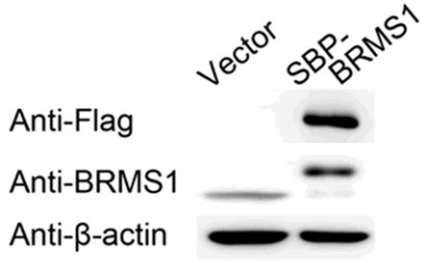
Keywords: BRMS1, DBC1, protein interaction, SIRT1, p53

Introduction

The *BRMS1* (Breast cancer metastasis suppressor 1) gene was initially cloned in the chromosome region 11q13, which exhibits a high frequency of deletion in late-stage, metastatic breast carcinoma [1]. BRMS1 is active in suppressing tumor metastasis in breast cancer, melanoma, ovarian carcinoma and non-small lung cancer, among others [1-4]. Protein structural analysis of BRMS1 protein discovered two coiled-coil motifs and several imperfect leucine zipper motifs, which are demonstrated to be important for the protein-protein interaction of BRMS1 [5, 6]. Additionally, two nuclear localization sequences (NLSs) inside BRMS1 protein are necessary for nuclear import [7, 8]. Further mechanism exploration characterized BRMS1 as an important component of mSin3a•HDAC complex involved in regulating histone status [6]. Moreover, NF-κB is an important substrate of BRMS1•HDAC complex [9, 10]. Multiple transcriptional targets of NF-κB, including OPN, uPA and Twist1, are identified as the main effectors of BRMS1 in suppressing tumor metastasis [9-13]. These effectors are involved in distinct cellular events, such as cell apoptosis, cell invasion and epithelial-mesenchymal transition. An E3 ligase function of BRMS1 on the histone acetyltransferase p300 has been reported recently, which suggests a novel mechanism of BRMS1 in suppressing tumor metastasis [14].

The silent information regulator2 (Sir2) is a NAD-dependent deacetylase that regulates chromatin silencing in yeast [15]. SIRT1 is the mammalian orthologue of yeast Sir2, which has emerged as an important regulator of aging [16], metabolism and cancer development [17]. Direct deacetylation of multiple important substrates such as p53, NF-κB and FOXO1, is the main mechanism of SIRT1 in regulating various physiological events [18-20]. DBC1 (Deleted in Breast Cancer 1) is a nuclear protein encoded by a gene initially cloned from chromosome 8p21 which was homozygously deleted in breast cancer [21]. Further studies demonstrated that DBC1 is a native inhibitor of SIRT1 [22, 23]. DBC1 promotes p53 acetylation and cell apoptosis through binding to SIRT1's catalytic domain. In this study, we provide evidence that SIRT1-DBC1 interaction can be regulated by

A



B

Protein	Coverage	Score	#Proteins	#Peptides
BRMS1	47.54	630.53	5	16
FOXK1	28.10	887.94	4	20
DBC1	18.74	328.79	9	12
Sin3a	16.10	434.14	10	20
HUWE1	15.86	2127.49	8	64
Hsp70	12.60	350.20	2	20
Hsp90	11.20	458.92	10	15
HDAC1	10.73	34.84	6	2

Figure 1. DBC1 is a potential BRMS1-interacting protein. A. Constructs encoding SBP-tagged BRMS1 was transfected into 293T cells and the expression of BRMS1 was confirmed by SDS-PAGE and visualized by immunoblotting. B. The pull-down products from tandem affinity purification using 293T cells expressing SBP-tagged BRMS1 were analyzed. Selected data from mass spectrometry analysis are shown in the table.

metastasis suppressor BRMS1. SIRT1-dependent p53 acetylation is also affected by BRMS1 expression.

Materials and methods

Plasmid construction

Human BRMS1 cDNA were described previously [13] and was subcloned into different mammalian expression vectors and bacterial expression vectors. SBP vector carrying streptavidin-binding peptide tag and S tag was kindly gifted by Prof. Jiaxue Wu (State key laboratory of Genetic Engineering, Fudan University). Flag-tagged DBC1 was kindly gifted by Prof. Domenico Delia (Fondazione IRCCS Istituto Nazionale dei Tumori; Milan, Italy). HA-SIRT1 was kindly gifted by Prof. Shimin Zhao (State key laboratory of Genetic Engineering, Fudan University). Plasmid Lenti CRISPR was kindly gifted by Prof. Yongming Wang (State key laboratory of Genetic Engineering, Fudan University).

Cell lines, treatments

293T and A549 cells were maintained in DMEM (Dulbecco's modified Eagle's medium, Gibco), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were maintained at 37°C and 5% CO_2. Etoposide (Sigma) was used at 20 μM. Cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunoblotting

Cell lysates or immunoprecipitates were subjected to SDS-PAGE and proteins were transferred to Polyvinylidene fluoride (Millipore). The membrane was blocked, and incubated with primary antibody at 4°C overnight and followed by secondary antibody for 1 h at room temperature. Afterward, the proteins of interest were visualized using ECL chemiluminescence system (Bio-Rad). The related antibodies we used includes anti-p53 (FL-393; Sigma), anti-FLAG (F3165; Sigma), anti-β-actin (AC-74; Sigma), anti-acetyl-p53 (2525S; Cell Signaling Technology), anti-DBC1 (A300-432A; Bethyl Labrotories), anti-SIRT1 (74504; Santa Cruz), anti-Myc (05-724; Millipore), anti-HA (abmart), anti-BRMS1 (5702; abcam), Rabbit IgG (Proteintech) and anti-GST (CAB4169; Thermo).

Immunoprecipitations

Cells were lysed with 1× cell lysis buffer (Thermo), and the lysate was centrifuged. The supernatant was precleared with protein A/G beads (Thermo) and incubated with antibody overnight. The related antibodies used in immunoprecipitation include anti-myc, anti-flag and anti-DBC1 described above. Thereafter, protein A/G beads were applied, all at 4°C. After 2 h of incubation, pellets were washed five times with lysis buffer and resuspended in sample buffer and analyzed by immunoblotting.

GST-pull down

To generate recombinant proteins from bacteria, BRMS1 was cloned into pGEX-4T-1 and DBC1 was cloned into pET-28a, respectively. These plasmids were then transformed into E.*coli* BL21 to induce expression of recombinant protein. Purification of GST-BRMS1 recombinant protein was performed utilizing the

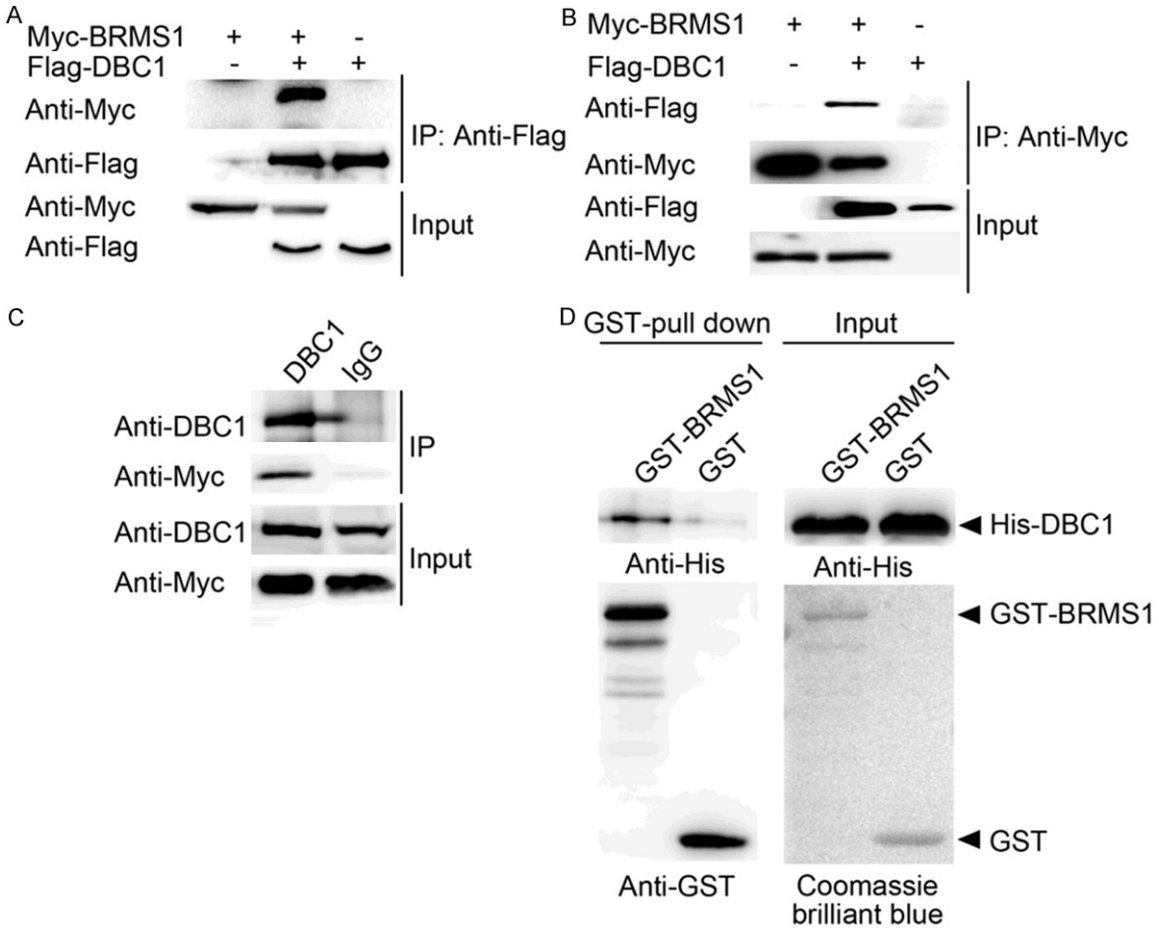


Figure 2. BRMS1 interacts with DBC1. (A, B) 293T cells were transfected with indicated plasmids. Cell lysates were prepared and subjected to immunoprecipitation with anti-Myc (A) or anti-Flag (B) antibody. The immunoprecipitates were detected by immunoblotting with anti-Myc and anti-Flag antibodies, respectively. (C) Co-immunoprecipitation of endogenous DBC1 and Myc-tagged BRMS1 proteins in A549 cells. The Myc-tagged BRMS1 protein was immunoprecipitated by DBC1 antibody, and rabbit IgG was used as a negative control. The immunoprecipitates were detected by immunoblotting with anti-DBC1 and anti-Myc antibodies, respectively. (D) Bacterially expressed GST fusion BRMS1 protein bound to glutathione-Sepharose beads and incubated with recombinant His-DBC1. Bound His-DBC1 was detected by immunoblotting with anti-His antibody.

Glutathione Agarose kit. Purified recombinant proteins were used in GST pull-down assays to verify their interaction. Glutathione Agarose were bound to GST-BRMS1 and incubated with bacteria lysates containing His-DBC1. After incubation, beads-bound protein complexes were flushed with wash buffer, and then subjected to immunoblotting analysis.

Establishment of BRMS1 knockout cell line

Human CRISPR/CAS9 guide RNA (gRNA) primers were: *BRMS1* gRNA #1 sense: 5'CACCGAGCCTCAAGATTCGCATTC3', *BRMS1* gRNA #1 antisense: 5'AAACGAATGCGAATCTTGAGGCTC'. *BRMS1* gRNA #2 sense: 5'CACCGAAGCAGTTCTCGGAGCTAA', *BRMS1* gRNA #2 antisense: 5'AAACTTAGCTCCGAGAACTGCTTC'. The primer oligonucleotides were annealed and cloned into the pLentiCRISPR vector. Recombinant pLenti CRISPR-BRMS1 were verified by sequencing analysis. 293T cells were transfected with pLenti CRISPR-BRMS1-sgRNA-#1, pLenti-CRISPR-BRMS1-sgRNA-#2 and empty vector separately, and then subjected to selection with puromycin. Independent colonies were isolated and subjected to anti-BRMS1 immunoblotting to select *BRMS1*-knockout clones and control clones. Genomic DNA of these clones was isolated and further subjected to mutation analysis through sequencing.

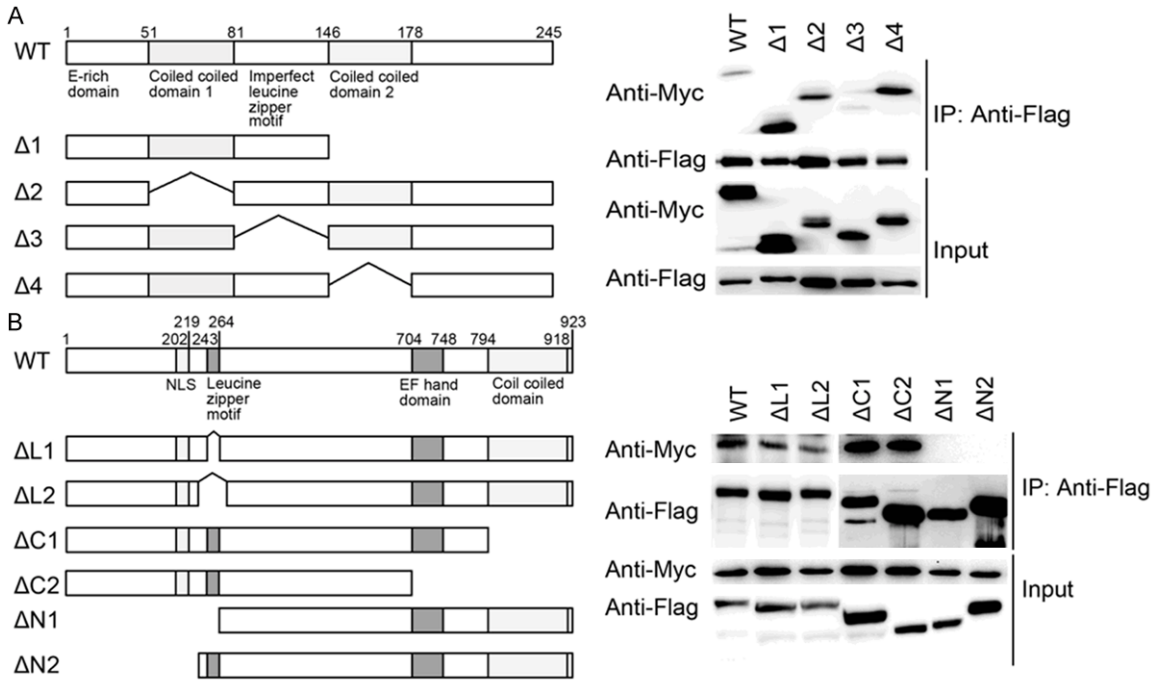


Figure 3. Delineation of the domains mediating the mutual interactions between BRMS1 and DBC1. A. Plasmids encoding Myc-tagged full-length or deletion mutants of BRMS1 were co-transfected with plasmids encoding Flag-tagged full-length DBC1 into A549 cells. Immunoprecipitation and immunoblotting were performed 36 h post-transfection as indicated. B. Plasmids encoding Flag-tagged full-length or deletion mutants of DBC1 was co-transfected with plasmids encoding Myc-tagged full-length BRMS1 into A549 cells. Immunoprecipitation and immunoblotting were performed 36 h post-transfection.

Results

Identification of DBC1 as a potential BRMS1-associated protein

To elucidate potential interacting proteins of BRMS1, 293T cells were used to express SBP-tagged BRMS1 (**Figure 1A**). Then a large-scale tandem affinity purification procedure was carried out according to F. Zhang's protocol [24]. As shown in **Figure 1B**, several previously identified BRMS1-associated proteins were found in this purification, including histone deacetylase complex components such as Sin3a and HDAC1 [5] and Hsp70 and Hsp90 chaperones [25]. More importantly, several novel BRMS1-associated proteins were identified, such as transcriptional factor Forkhead box protein K1 (FOXK1), E3 ubiquitin-protein ligase HUWE1, etc. To our interest, we reproducibly found DBC1, also known as KIAA1967 or CCAR2, is also a novel BRMS1-associated protein.

To confirm the relationship between BRMS1 and DBC1, Myc-tagged BRMS1 and Flag-tagged DBC1 were co-expressed in 293T cells and then cell lysis were subjected to co-immunoprecipitation. Myc-BRMS1 can be detected in anti-Flag immunoprecipitates (**Figure 2A**) and *vice versa* (**Figure 2B**), but no binding was detected in control cells. A549 cells were then utilized to detect the interaction between endogenous DBC1 and transfected BRMS1. As shown in **Figure 2C**, myc-tagged BRMS1 was readily immunoprecipitated with the anti-DBC1 antibody, but not with the IgG control. Furthermore, GST-pull down experiment was carried out to test whether BRMS1 binds to DBC1 *in vitro*. As shown in **Figure 2D**, the purified GST-BRMS1 recombinant protein, but not GST alone, efficiently pulled down the recombinant His-DBC1. All these results demonstrated that BRMS1 is able to interact with DBC1. In addition, we considered a possibility that BRMS1 may also interact with SIRT1 which is a major DBC1-associated protein to form a large protein complex. However, in a co-immunoprecipitation experiment using 293T cells co-expressing HA-SIRT1 and Myc-BRMS1, either transfected or endogenous SIRT1 could not be detected in anti-Myc immunoprecipitates (data not shown).

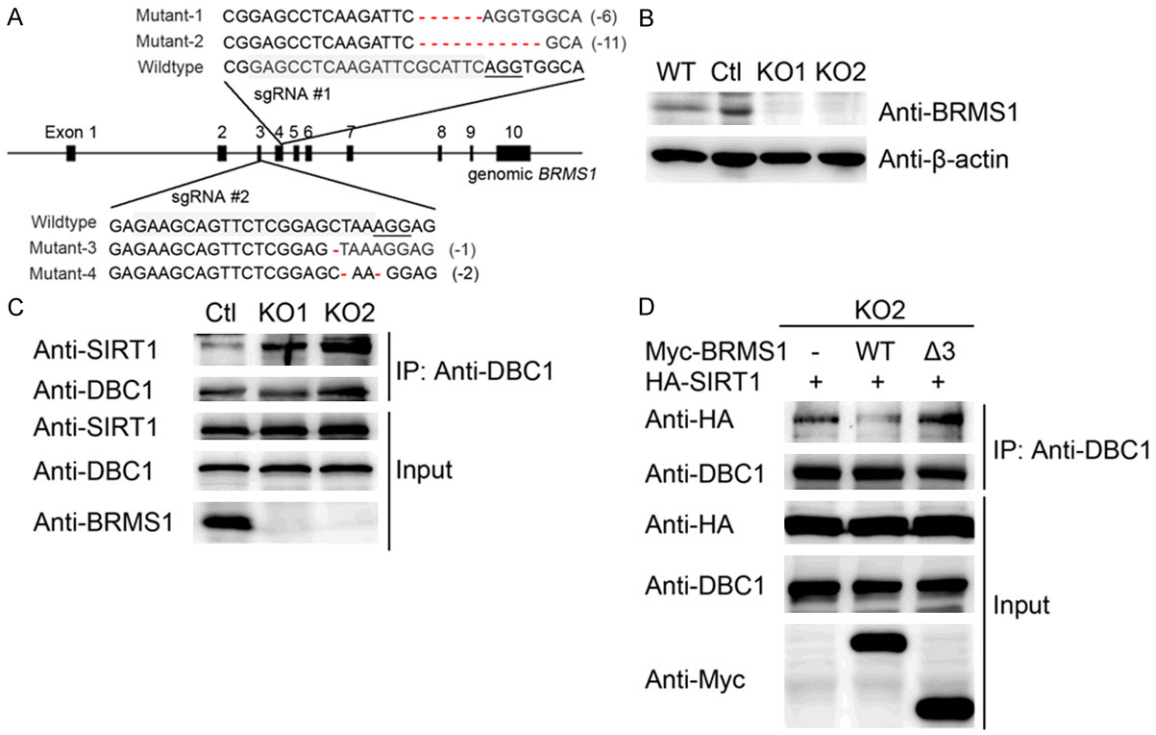


Figure 4. BRMS1 blocks the association between DBC1 and SIRT1. A. A schematic of the sgRNAs targeting the fourth (sgRNA #1) and the third (sgRNA #2) exon of the *BRMS1* gene. The sgRNA sequences are shaded in gray and the PAM motif is underlined. Mutant allele 1 and 2 were identified in *BRMS1* KO1 clone (using sgRNA #1) whereas mutant allele 3 and 4 were from *BRMS1* KO2 (using sgRNA #2). Deletions are indicated by a dashed line and the numbers of deleted basepairs are shown in the brackets following the sequences. B. The expression level of endogenous BRMS1 protein in wild-type, control, *BRMS1* KO1 and KO2 293T clones were detected by anti-BRMS1 immunoblotting. C. Co-immunoprecipitation of endogenous DBC1 and SIRT1 proteins in indicated cells. SIRT1 protein was immunoprecipitated by DBC1 antibody. The immunoprecipitates were detected by immunoblotting with anti-DBC1 and anti-SIRT1 antibodies, respectively. D. Plasmids encoding Myc-tagged full-length or Δ3 deletion mutant of BRMS1 were co-transfected with plasmids encoding HA-tagged full-length SIRT1 into 293T cells. Cell lysates were prepared and subjected to immunoprecipitation. HA-SIRT1 protein was immunoprecipitated by DBC1 antibody. The immunoprecipitates were detected by immunoblotting with anti-Myc and anti-HA antibodies, respectively.

Mapping the binding sites on BRMS1 and DBC1

BRMS1 contains two coiled-coil motifs and several imperfect leucine zipper motifs that are important for the protein-protein interaction [1]. To identify the region of BRMS1 that are responsible for the BRMS1-DBC1 interaction, we generated a series of deletion mutants of BRMS1. A549 cells were co-transfected with full-length Flag-DBC1 and individual deletion mutant of Myc-BRMS1, respectively. As shown in **Figure 3A**, we found that deletion of residue 82-145 of BRMS1 (Δ3), which contains the imperfect leucine zipper motifs, greatly diminished the binding of BRMS1 with DBC1. However, loss of either coiled-coil domain of BRMS1 exhibited no effect on BRMS1-DBC1 interaction.

To identify the domains of DBC1 that mediates the BRMS1-DBC1 interaction, a similar strategy was used. We generated a series of deletion mutants of DBC1 and then transfected A549 cells with full-length Myc-BRMS1 and each deletion mutants of Flag-DBC1 (**Figure 3B**). We found that deletion of the N-terminal region of DBC1 (ΔN1 and ΔN2), but not the leucine zipper motif (ΔL1 and ΔL2) or the C-terminal region (ΔC1 and ΔC2), abolished the binding between DBC1 and BRMS1. Therefore, the N-terminus of DBC1 is required for the interaction between DBC1 and SIRT1.

BRMS1 interrupts the association between DBC1 and SIRT1

To explore the potential function of BRMS1-DBC1 interaction, we utilized the CRISPR/Cas9

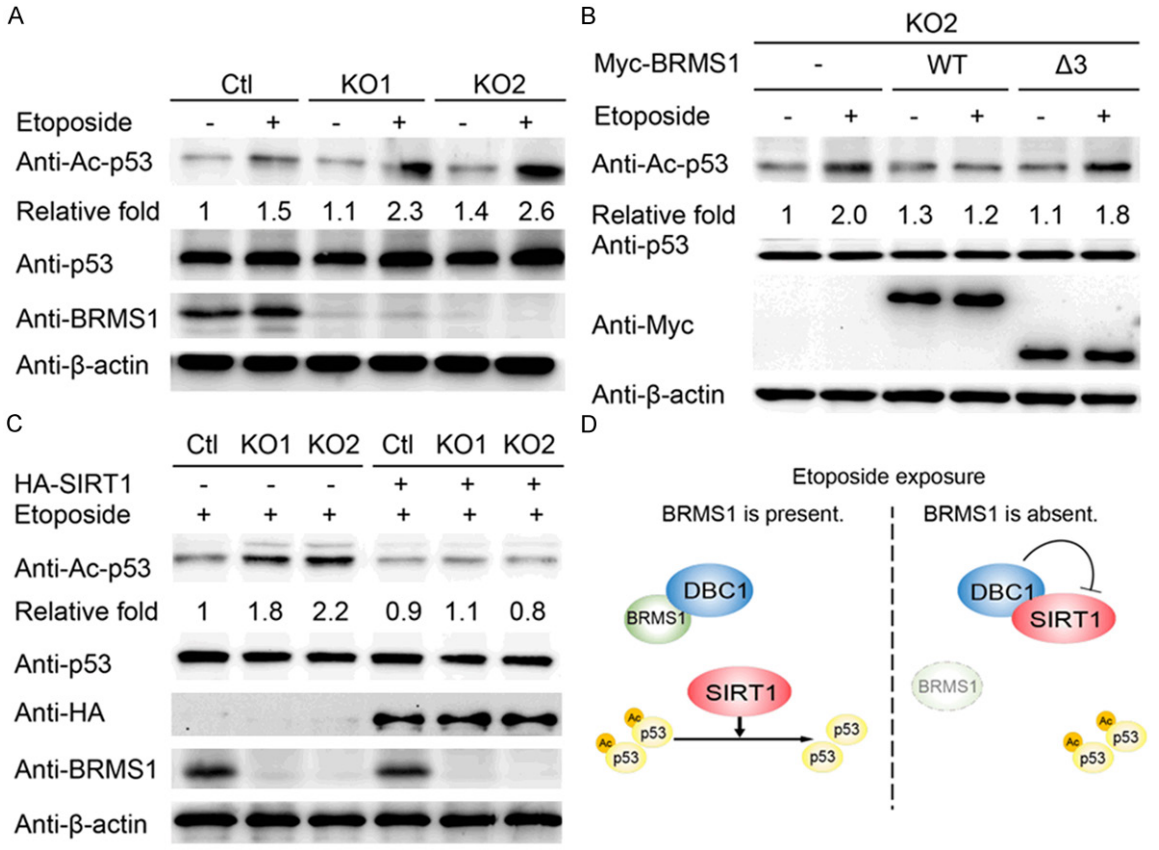


Figure 5. BRMS1 regulates p53 acetylation upon etoposide exposure. A. The acetylated p53 level and the protein levels of endogenous p53 were detected by immunoblotting with indicated antibodies from control or knockout cells before and after etoposide exposure. Densitometric analyses (relative fold) show the ratios of acetylated p53-K382/total p53 normalized to the value of Ctl cells. B. *BRMS1*-knockout KO2 clones were transfected with plasmids encoding Myc-tagged full-length BRMS1, *BRMS1* Δ3 mutant or vector, followed by etoposide exposure (20 μM, 6 h). The acetylated p53 level and total p53 level were detected with indicated antibodies. C. Plasmid encoding HA-tagged SIRT1 were transfected into indicated cells. Immunoblotting were performed after additional 6 h of etoposide exposure, the acetylated p53 level and the total p53 protein levels were detected with indicated antibodies. D. Graphical representation of DBC1 regulation in the presence and absence of BRMS1. DBC1 acts as a SIRT1 inhibitor and regulates SIRT1-dependent functions upon etoposide exposure. But this process can be abrogated by BRMS1, indicates that BRMS1 might be an upstream regulator of DBC1 in the DNA-damage response.

system to establish *BRMS1* knockout cell clones. 293T cells were selected due to relative high endogenous BRMS1 expression. Two different gRNAs (gRNA #1 and gRNA #2) targeting genomic *BRMS1* sequence were designed to induce DNA break and repair, leading to mutation and gene disruption (**Figure 4A**). KO1 (*BRMS1* knockout using gRNA #1) and KO2 (*BRMS1* knockout using gRNA #2) are two stable clones selected for further experiments. Genomic DNA was isolated from KO1 and KO2 clones for mutation analysis. As shown in **Figure 4A**, four different *BRMS1* alleles with indels were found, all of which caused frameshift mutations. Western blot analysis was further performed to confirm the efficiency of *BRMS1* knockdown in KO1 and KO2. As shown in **Figure 4B**, both KO1 and KO2 lost endogenous *BRMS1* expression as compared with wildtype 293T (WT) or control cells (Ctl) transfected with empty vector.

Given that BRMS1 binds to the N-terminus of DBC1, it is possible that BRMS1 might affect the association between DBC1 and SIRT1. To test this hypothesis, we carried out co-immunoprecipitation assay in *BRMS1* knockout 293T cells and control cells. As shown in **Figure 4C**, endogenous SIRT1 protein can be co-immunoprecipitated by anti-DBC1 antibody in all three clones, however, the binding ability of endogenous SIRT1 and DBC1 was significantly increased in both *BRMS1* knockout cells. To further confirm this finding, a rescue assay was

performed. Both HA-tagged SIRT1 and Myc-tagged BRMS1 were co-transfected into *BRMS1* deficient KO2 clone before performing the co-immunoprecipitation assay. As shown in **Figure 4D**, the binding of HA-SIRT1 and endogenous DBC1 was successfully reduced by overexpression of BRMS1 in KO2 clone. Moreover, when the BRMS1 Δ3 mutant without the binding ability with DBC1 was introduced into KO2 clone, no obvious change was observed in DBC1-SIRT1 association. Collectively, these data indicates that BRMS1 might compete the binding motif of DBC1, leading to a suppression of DBC1-SIRT1 association.

BRMS1 regulates p53 acetylation upon etoposide exposure

It is well-studied that the binding of DBC1-SIRT1 exerts an inhibitory effect on SIRT1's deacetylase activity [22]. Our finding that BRMS1 can block DBC1-SIRT1 interaction led us to raise another question that whether BRMS1 can regulate SIRT1's deacetylase activity. To address this question, the acetylation of p53 was analyzed in *BRMS1* knockout clones and control clones upon etoposide exposure. As shown in **Figure 5A**, etoposide treatment induced hyperacetylation of p53 in control cells. Significant increases in the acetylation level of p53 upon etoposide exposure were observed in both KO1 and KO2 clones by comparison with control cells, which is consistent with the stronger DBC1-SIRT1 association in knockout clones.

To further confirm the regulatory role of BRMS1 on p53 acetylation, similar rescue assay was performed by introducing exogenous BRMS1 into KO2 clone. A significant reduction of p53 acetylation was observed in cells overexpressing full-length BRMS1 but not BRMS1 Δ3 mutant (**Figure 5B**). These results indicate that BRMS1 can suppress the acetylation of p53 and p53-dependent gene expression upon DNA damage stimuli.

To further determine whether the suppressive effect of BRMS1 on p53 acetylation in response to DNA damage is SIRT1 dependent, we investigated the p53 acetylation upon etoposide treatment after overexpression of HA-tagged SIRT1. SIRT1 expression reversed p53 hyperacetylation caused by *BRMS1* depletion (**Figure 5C**). Collectively, these results indicate that BRMS1 is able to regulate SIRT1-dependent p53 deacetylation upon DNA damage stress.

Discussion

Here, we reported for the first time that BRMS1 can interact with DBC1 and mediate SIRT1-dependent p53 deacetylation upon etoposide exposure. It has been well studied that following DNA damage, ATM phosphorylates DBC1, inducing DBC1-SIRT1 binding, leading to p53 acetylation [26]. We further demonstrated that the presence of BRMS1 can decrease acetylated p53 level by binding to DBC1 and abrogating the DBC1-mediated inhibition of SIRT1 deacetylase activity, whereas the absence of BRMS1 might facilitate DBC1-SIRT1 interaction, leading to high level of acetylated p53 (**Figure 5D**).

Our results identified BRMS1 as a natural SIRT1 agonist. SIRT1 is a multifaceted protein deacetylase that is involved in a wide variety of cellular processes, such as gene silencing, apoptosis, stress resistance and fat and glucose metabolism [17]. The combination of these cellular functions might contribute to an anti-aging effect in mammals. On the other hand, SIRT1 activity has also been linked to tumorigenesis, although the function of SIRT1 in cancer is rather complicated. SIRT1 can deacetylate the tumor suppressor protein p53, leading to inhibition of its transactivation potential, which somehow supports the idea that SIRT1 could be oncogenic. However, all the currently available data from animal models indicates that SIRT1 is tumor suppressor *in vivo*. It is conceivable that SIRT1 activity might be a 'double-edged sword' that requires tight regulation. Our findings that metastasis suppressor BRMS1 can potentiate SIRT1 activity led us to speculate that BRMS1 may participate in metastasis suppression by the synergistic effect between BRMS1 and SIRT1.

DBC1 has recently emerged as a master regulator of transcriptional processes through its regulation of both chromatin remodeling enzymes and transcription factors. The negative regulation of SIRT1 by DBC1 is an important finding with broad impact, as SIRT1 is known to have a multitude of substrates and is involved in modulating housekeeping cellular pathways as we discussed above. DBC1 also negatively regulates the enzymatic activity of HDAC3, another histone deacetylase [27]. Overexpression of DBC1 prevented HDAC3-mediated deacetylation of MEF2D, by competing with MEF2D for binding to HDAC3. The con-

nection between DBC1 and chromatin remodeling was also expanded to other enzymes, such as histone methyltransferase Suv39H1, which is responsible for heterochromatin formation [28]. Interestingly, SIRT1 also interacts with Suv39H1 and increases Suv39H1's methyltransferase activity [29]. The binding of DBC1 to Suv39H1 disrupted the SIRT1-Suv39H1 activated complex [28]. However, what needed to be noticed is that the N-terminal domain (amino acids 1-264) of DBC1 is indispensable for binding all of these proteins, Suv39H1, SIRT1, HDAC3, and BRMS1. Whether BRMS1 can also modulate other protein interactions of DBC1 needs to be further investigated.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Yanhua Wu, State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, 2005 Songhu Road, Shanghai 200438, P. R. China. Tel: +86-21-51630593; Fax: +86-21-51630593; E-mail: yanhuawu@fudan.edu.cn

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