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SUMO1 negatively regulates BRCA1-mediated transcription, via modulation of promoter occupancy

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ABSTRACT

BRCA1, a tumor suppressor gene, is implicated in the repression and activation of transcription via interactions with a diverse range of proteins. The mechanisms regulating the action of BRCA1 are not fully understood. Here, we use the promoters of Gadd45α, $p27^{KIP1}$ and $p21^{WAF1/CIP1}$ to demonstrate that SUMO1 represses transactivation potential of BRCA1 by causing BRCA1 to be released from the promoters and augmenting histone deacetylation via recruitment of histone deacetylase (HDAC) activity. Consistently, silencing of SUMO1 led to recruitment of BRCA1 and release of HDAC1 at the BRCA1 target promoters, and subsequent transcriptional activation of the BRCA1 target genes. Furthermore, a sumoylation-incompetent mutant missing the sumoylation donor site suppressed BRCA1-induced activation of transcription, whereas E2 UBC9 or the dominant-negative mutant UBC9 had no effect, implying that repression of BRCA1-mediated activation of transcription by SUMO1 is independent of sumoylation. Repression of BRCA1-mediated activation of transcription by SUMO1 was reversed by DNA damage by inducing the release of SUMO1 from the Gadd45α promoter and the recruitment of BRCA1, along with increased histone acetylation, to enhance activation of transcription. Together, our data provide evidence that SUMO1 plays a role in the activation-repression switch of BRCA1-mediated transcription via modulation of promoter occupancy.

INTRODUCTION

Functional loss of BRCA1 causes defective transcription-coupled or recombination-mediated DNA repair, deregulated proliferation and predisposition to familial breast cancers (1–2), suggesting a role of BRCA1 in tumor suppression via a diversity of functions including transcription, DNA repair and cell cycle. Biochemical studies on proteins that interact with BRCA1 also provide evidence for the various roles of BRCA1 (2,3). BRCA1 interacts with transcription and chromatin-remodeling proteins, including CtIP/CtBP, RbAp46/48, RNA polymerase II, histone deacetylases (HDACs), histone acetyl transferases (HATs), c-myc, JunB, p53, Rb, estrogen receptor, androgen receptor and ZBRK1, suggesting that BRCA1 is involved in transcription and modulation of chromatic structure (2–7). The interaction of BRCA1 with a diversity of transcriptional regulators is consistent with the observed physiological actions of BRCA1 and supports the function of BRCA1 as a tumor suppressor via regulation of transcriptional activity (2,3).

BRCA1 forms both transcriptional activator- and repressor-complexes with a variety of proteins that regulate transcription, and activates or suppresses the transcription of genes involved in the cell cycle, control of growth and response to DNA damage (6,8–12). The interaction of BRCA1 with HDAC1 and 2 (13), RNA helicase (14), CBP and p300 (5,15), and the BRG1 subunit of the SWI/SNF complex (16,17), implies a critical role for BRCA1 in chromatin remodeling. It has been shown that the transactivation potential of BRCA1 is enhanced by the binding of CBP and p300 in a phosphorylation-independent manner (5). Together with RNA helicase A, BRCA1 is a component of the SWI/SNF complex, a large ATP-dependent chromatin remodeling complex, aiding the access of transcriptional machinery and transcription-coupled DNA repair proteins to DNA (17). The interaction of BRCA1 with HDAC1 and 2 mediates repression of transcription via the induction of histone deacetylation (4,6,18). In summary, results from these studies provide support for the involvement of BRCA1 in a variety of processes including transcription, DNA repair and recombination by control of chromatin remodeling.

The SUMO pathway is known to mediate repression of transcription by chromatin remodeling (19,20). Many transcription factors, including HDAC1 (21), p300/CBP (22), CtBP (23), STAT-1 (24) and BKLF (25), are subject

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to SUMO-mediated repression of transcription that is accompanied by histone deacetylation. Interestingly, histone 4 (H4) is sumoylated and leads to gene silencing through recruitment of the HDAC complex (19). Growing evidence highlights the widespread role of SUMO in repression of transcription.

In the present study, we have identified and characterized SUMO1 as a negative regulator of BRCA1-mediated activation of transcription. We find that SUMO1 induces the recruitment of HDAC activity to the BRCA1-regulated promoters of Gadd45α, $p27^{KIP1}$ and $p21^{WAF1/CIP1}$ genes, leading to reduced histone acetylation and subsequent repression of transcription. Furthermore, SUMO1 appears to suppress BRCA1-mediated activation of transcription by releasing BRCA1 and recruiting HDAC1, in a sumoylation-independent manner. Taken together, our findings suggest that SUMO1 modulates transcription by repressing BRCA1-mediated activation of transcription by chromatin remodeling involving deacetylation.

MATERIALS AND METHODS

Plasmid construction

To generate baits using BRCA1, four overlapping BRCA1 truncated fragments, #1 (1–324), #2 (260–553), #3 (502–802) and #4 (758–1064) were generated from pGex-BRCA1 vectors (26,27) and subcloned into pLexA (Clontech). BRCA1 V122A, V412A, V412A/V415A, I769A, V772A, I783A/V788A and V412A/I783A/V788A were constructed from the cloned BRCA1#1 to #4-pLexA plasmids and pcDNA-HA-BRCA1 (26,27) by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). To construct the SUMO-pLexA fusion bait vector, SUMO1 cDNA fragment was generated by RT-PCR from RNA obtained from 293T cells. The BRCA1 C-terminal #5 (1005–1313) and #6 (1314–1863) fragments were generated by PCR from pGex-BRCA1 vectors (26,27) and the fragments were inserted into pB42AD (Clontech). Recombinant histidine-tagged human SUMO1 (His-SUMO1) protein vector was generated by inserting the corresponding cDNA containing the entire open reading frame into a pET28a vector (Novagen, San Diego, CA). Mammalian expression vectors for SUMO1 and SUMO1ΔGG were generated by inserting full-length cDNA fragments, generated by PCR from pET28a-SUMO1 using the following primers: SUMO1 (5′-GGAAGATCTATGTCTGACCAGGAGGCAAA-3′ and 5′-TCCCCGCGGCTAAACCGTCGAGTGACCC C-3′) and SUMO1ΔGG (5′-GGAAGATCTATGTCTG ACCAGGAGGCAAA-3′ and 5′-CCGCTCGAGCTACG TTTGTTCCTGATAAACTTCAA-3′), into pDsRed (Clontech). To generate mammalian expression vectors for Ubc9, HDAC1/2, p300 and BARD1, cDNAs containing the entire open reading frame of each gene were produced by RT-PCR of 293T or MCF7 mRNAs. The fragments were inserted into a pCDNA3 vector (Invitrogen, Carlsbad, California) hooked to the HA or FLAG epitope. Double-stranded siRNA for BRCA1 and SUMO1 were generated using the pSUPER vector (28). The sequences used for siRNA are as follows: BRCA1 (5′-GATCCCCTCTGTCTGGAGTTGATCAATTCAAG AGATTGATCAACTCCAGACAGATTTTTGGAAA-3′ and 5′-AGT TTTTCCAAAAATCTGTCTGGAGTTG ATCAATCTC TTGAATTGATCAACTCCAGACAGA GGG-3′), SUMO1 (5′-GATCCCCTGGTGATAAATAA GATCGA TTCAAGAGATCGATCTTATTTATCACC ATTTTTG GAAA-3′ and 5′-AGCTTTTCCAAAAATG GTGATAA ATAAGATCGATCTCTTGAATCG ATCTTATTTATC ACCAGGG-3′), and HDAC1 (5′-GATCCCCTGTCA AGAGCTTTAACCTGTTCAA GAGACAGGTTAAAG CTCTTGACATTTTTGGAA A-3′ and 5′-AGCTTTT CCAAAAATGTCAAGAGCTT TAACCTGTCTCTTG AACAGGTTAAAGCTCTTGA CAGGG-3′).

Yeast two-hybrid assay

Yeast two-hybrid assays were carried out according to the manufacturer's protocol (Clontech). β-Galactosidase activity was measured in duplicate from three independent clones. This assay was performed using pLexA or pB42AD in the yeast strain EGY48 (Clontech). Sequencing of the library inserts of clones interacting with fragments #1 to #4 of BRCA1, was performed using Software (ABI. Foster City, CA).

Pull-down analysis

The histidine-tagged recombinant SUMO1 (His-SUMO1) and SUMO1ΔGG (His-SUMO1ΔGG) proteins were expressed and purified using pET-SUMO1 and SUMO1ΔGG in *Escherichia coli* BL21 (DE3) cells (Stratagene). The expression and purification of glutathione S-transferase (GST) fusion proteins and GST pull-down assays were carried out as described previously (27). Following incubation of approximately equal amounts of different purified GST fusion BRCA1 fragment proteins mixed with His-SUMO1 and His-SUMO1ΔGG proteins in the presence of glutathione-Sepharose 4B beads (Amersham Biosciences, Sweden), bound proteins were probed with anti-SUMO1 (Zymed, Carlsbad, California) antibody.

Cell cultures and transfection

U2OS, 293T and HeLa cells were grown in DMEM (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin–streptomycin (GIBCO, Gaithersgurg, MD). Transfection was performed with the Effectene transfection kit (Qiagen Inc, Valencia, CA).

Immunoblotting and coimmunoprecipitation

Cells were suspended in lysis buffer containing 17 mM Tris pH 8.0, 50 mM NaCl, 0.3% Triton X-100, 0.3% NP-40 and a protease inhibitor cocktail tablet (Roche, Switzerland). BRCA1, UBC9 and SUMO1 were detected with anti-BRCA1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-UBC9 (BD Biosciences), anti-SUMO1 (Santa Cruz Biotechnology and Zymed), anti-FLAG (Sigma, St. Louis, MO) and anti-LexA (Invitrogen) antibodies, respectively.

For coimmunoprecipitation experiments, cell lysates were subjected to immunoprecipitation and the resulting immunoprecipitates were analyzed by immunoblotting with the indicated antibodies.

Reporter assay

U2OS, 293T or HeLa cells were incubated in 24-well plates, 1 day before transfection. Transfections were performed in quadraplicate using 1–200 ng of expression vector for pcDNA-HA-BRCA1 (wild type or mutants, V122A, V412A, V412A/V415A, I769A, V772A, I783A/V788A and V412A/I783A/V788A), pEGFP-SUMO1, pDsRed-SUMO1, pDsRed-SUMO1ΔGG or pcDNA3.1 (Invitrogen) as a control. Cotransfection with 20 ng of pGadd45α-luciferase reporter (10) and 1 ng of pRL SV40 or pRL TK (Promega, Madison, WI) was performed to control for transfection efficiency. At 1 day after transfection, cells were treated with or without γ-irradiation (4 or 8 Gy) or 0.3 μM trichostatin A (TSA, Sigma), and incubated for another 24 h before being harvested and assayed using the Dual Luciferase Reporter Assay (Promega). The luciferase activity was standardized against the transfection efficiency for each sample. Values are the mean ± SEM. from 3 to 6 experiments ($^*P < 0.05$ compared with reporter alone).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays using antisera specific to acetylated histone 4 (H4) (Upstate Biotechnology, Lake Placid, NY), HDAC1 (Upstate Biotechnology), HDAC2 (Upstate Biotechnology), BRCA1 (Santa Cruz Biotechnology), SUMO1 (Santa Cruz Biotechnology), UBC9 (BD Biosciences), BARD1 (Santa Cruz Biotechnology), RNA polymerase II (Santa Cruz Biotechnology) and p300 (Santa Cruz Biotechnology) were performed according to the manufacturer's instructions (Upstate Biotechnology). The DNA representing either 0.1% of the total chromatin sample (input) or 5–10% of the immunoprecipitate DNA was amplified using the following promoter-specific primers: Gadd45α (5′-GCTG GGGTCAAATTGCTGG-3′ and 5′-GCTCGCTCGCTC CCCGGAC-3′), $p27^{KIP1}$ (5′-GCTTCCCGGGAGAGGA GCG-3′ and 5′-CGAGCGCGCCGCCTCCCCG-3′), $p21^{WAF1/CIP1}$ (5′-CCTTGCCTGCCAGAGGGG-3′ and 5′-CAGCTGCTCACACCTCAG-3′), ErbB2 (5′-CCCGG ACTCCGGGGGAGG-3′ and 5′-CCCGGGGGGCTCC CCTGG-3′), β-actin (5′-GAGGGGAGAGGGGGTAA AA-3′ and 5′-AGCCATAAAAGGCAACTTTCG-3′), and GAPDH (5′-TACTAGCGGTTTTACGGGCG-3′ and 5′-TCGAACAGGAGCAGAGAGCGA-3′). The PCR conditions consisted of 95°C for 2 min, followed by 30–35 cycles of 95°C for 30 sec, 58°C for 30 s and 72°C for 30–60 s.

DNA binding assays

Binding reactions were performed with 300 μg of nuclear extract in a binding buffer composed of 12% glycerol, 12 mM Hepes (pH 7.9), 4 mM Tris (pH 7.9), 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 10 μg of poly(dI-dC) (Amersham Biosciences). Probes were prepared by annealing of oligonucleotide containing biotin on the 5′-nucleotide of the sense strand (5′-GCAGGCTGATT TGCATAGCCCAATGGCCAAGCTGCATGCAAATG AGGCGGA, −107 to −57 of the human GADD45α promoter) to the respective complementary oligonucleotide. The binding reaction and electrophoresis were performed at room temperature. BRCA1 protein bound to the biotin-labeled Gadd45α promoter oligonucleotides was pulled down with streptavidin–agarose beads (Sigma) and detected as described previously (29).

RNA isolation and RT-PCR

Total RNA was isolated from cultured cells using TRIzol reagent (Life Technologies, Inc, Gaithersburg, MD) according to the manufacturer's instructions. RT-PCR was carried out using 2–3 μg of total RNA from cells overexpressing or silencing SUMO1, BRCA1 and/or HDAC1. The primer sequences for glyceraldehydes-6-phosphate dehydrogenase (GAPDH; used as an invariant housekeeping gene internal control), Gadd45α, $p27^{KIP1}$, $p21^{WAF1/CIP1}$, β actin, ErbB2 and Cyclin D1 genes are as follows: GAPDH (5′-GTCAACGGATTTGGTCTGTA TT-3′, 5′-AGTCTTCTGGGTGGCAGTGAT-3′), Gadd45α (5′-TG ACTTTGGAGGAATTCTCGGC-3′, 5′-ATGAATGTG GATTCGTCACCAGCACGCAGT-3′), $p27^{KIP1}$ (5′-CCT CTTCGGCCCGGTGGAC-3′, 5′-TCTGCTCCACAGA ACCGGC-3′), $p21^{WAF1/CIP1}$ (5′-CCTCCTCGGCCCGG TGGAC-3′, 5′-CCGTTTTC GACCCTGAGAG-3′), β-actin (5′-TGGATTCCTGTGG CATTCATGAAAC-3′ and 5′-TAAAACGCAGCTCAG TTACAGTCCG-3′), ErbB2 (5′-TGGCTGCAAGAAGA TCTTTG-3′, 5′-TGC AGTTGACACACTGGGTG-3′) and CyclinD1 (5′-CCT CTTGTGCCACAGATG-3′, 5′-GATGTCCACGTCCC GCAC-3′). RT-PCR was performed as follows: annealing at 55°C with 20–28 cycles for detected genes.

Real-time PCR

All reactions were performed in triplicate using SYBR Green PCR Master Mix kit (ABI) and an ABI PRISM 7000 Sequence Detector (ABI). Primers were used as described in RT-PCR and ChIP. The PCR levels were determined by normalization to that of GAPDH control for quantification of mRNA transcripts for RT-PCR or that of input DNA for quantitative ChIP assay. Quantities of immunoprecipitated DNA or input DNA were determined based on a standard curve.

RESULTS

Interaction of BRCA1 with SUMO1

SUMO1 was identified as a BRCA1-interacting protein by using four overlapping BRCA1 truncated fragments from 1 to 1064, which did not include the transactivation domains at the C-terminus, as baits in a yeast two-hybrid system (Figure 1A and B). The N-terminal (1–324) and central (758–1064) BRCA1 fragments interacted more strongly than the other two fragments (260–553

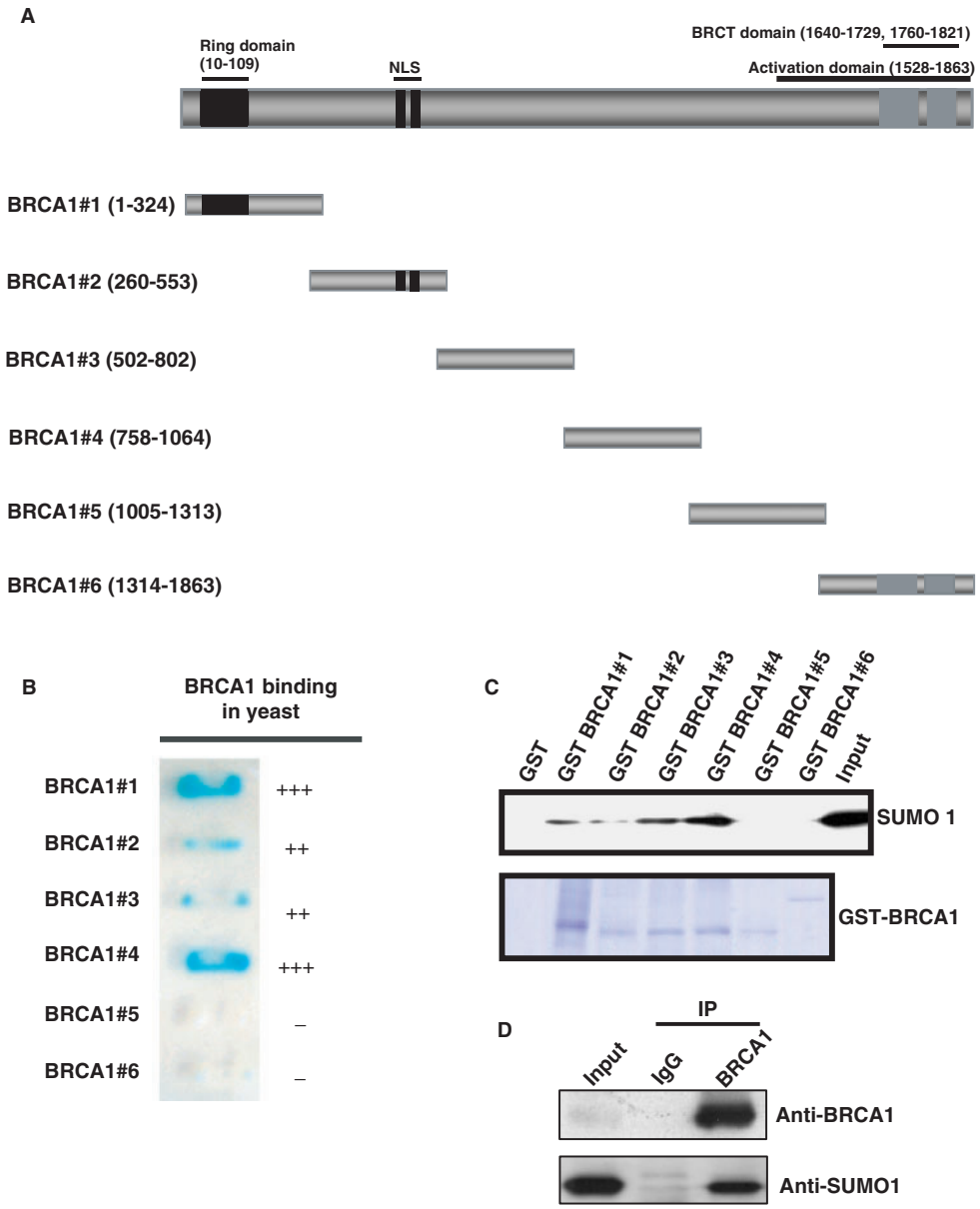


Figure 1. BRCA1 associates with SUMO1 in vitro and in vivo. (**A**) Schematic representation of BRCA1 constructs. The RING domain (RING), nuclear localization signal sequences (NLS), activation domain and two BRCT domains (BRCT) are indicated. Numbers above the BRCA1 constructs used in this study indicate the amino acid residues of the respective BRCA1 fragments. (**B**) Interaction of BRCA1 with SUMO1 in the yeast two-hybrid system. Yeast cells transformed with two-hybrid plasmids were grown under induced conditions for reporter gene activation. The streaks represent yeast cells cotransformed with either pLexA-BRCA1 (1–324, 260–553, 502–802 or 758–1064) and pB42AD-SUMO1. The ability of the transactivation domains of BRCA1 to interact with SUMO1 was measured following cotransformation of yeast cells with pB42AD-BRCA1 (1005–1313 or 1314–1864) and pLexA-SUMO1. (**C**) In vitro interaction of BRCA1 with SUMO1. The six GST-BRCA1 (1–324, 260–553, 502–802, 758–1064, 1005–1313 and 1314–1864, indicated as #1–#6, respectively) and GST proteins were immobilized on GST-Sepharose beads and incubated with His-SUMO1 proteins. His-SUMO1 proteins bound to immobilized GST-BRCA1 proteins were analyzed by immunoblotting with an anti-SUMO1 antibody (Upper). Twenty percent of the input SUMO1 proteins (input). SUMO1 did not bind to immobilized GST. An equivalent amount of GST-BRCA1 protein was used for immobilization (Lower). (**D**) *In vivo* interaction of BRCA1 with SUMO1. Extracts of 293T cells were immunoprecipitated with anti-BRCA1. Coimmunoprecipitated SUMO1 proteins were detected by immunoblots with anti-SUMO1 antibody.

and 502–802) (Figure 1B). Next, we examined whether SUMO1 interacted with BRCA1 transactivation domains (1005–1313 and 1314–1863) by using SUMO1 as the bait (Figure 1B). Unlike the fragments from 1–1064, the transactivation domains (1005–1863) did not associate with SUMO1 (Figure 1B).

To determine whether BRCA1 interacted directly with SUMO1, six GST-fusion BRCA1 protein fragments spanning the entire BRCA1 open reading frame (Figure 1A) were immobilized on GST-sepharose beads and incubated with recombinant His-SUMO1 proteins. Beads bound to His-SUMO1 protein were recovered from each GST-BRCA1 fusion protein and detected by immunoblotting after electrophoresis. His-SUMO1 bound strongly to the GST-BRCA1 fusion proteins containing all four N-terminal and internal

BRCA1 fragments (1–324, 260–553, 502–802 and 758–1064) but not to the two GST-BRCA1 fusion proteins containing the transactivation domains at the C-terminus (1005–1313 and 1314–1863) (Figure 1C). This result is consistent with the yeast two-hybrid results (Figure 1B) and reveals that BRCA1 interacts directly with SUMO1 *in vitro*.

We next tested whether BRCA1 interacted with SUMO1 *in vivo*. SUMO1 was coimmunoprecipitated with the anti-BRCA1 antibody from 293T cell lysates, indicating that endogenous BRCA1–SUMO1 interaction occurs in 293T cells (Figure 1D). Taken together, the interaction experiments show that BRCA1 interacts with SUMO1 in mammalian cells.

Repression of BRCA1-mediated transcription by SUMO1

To understand the role of the BRCA1–SUMO1 complex, we investigated the effect of SUMO1 on BRCA1-mediated transcription. Thus, we evaluated the transcriptional activity of BRCA1 by using a luciferase reporter driven by Gadd45α promoter, which is known to be regulated by BRCA1. BRCA1 alone induced transcription from the Gadd45α promoter by 2.6-fold, whereas SUMO1 alone reduced the Gadd45α transcription by ~30% of the basal level in U2OS cells (Figure 2A). Moreover, SUMO1 together with BRCA1 suppressed BRCA1-induced transcriptional activity from the Gadd45α promoter to level lower than the basal level in U2OS cells (Figure 2A). In contrast to the results with SUMO1, UBC9 failed to have an effect on Gadd45α transcription (Figure 2A). UBC9 did not enhance or suppress transcription from the Gadd45α promoter in the presence or absence of BRCA1 (Figure 2A). UBC9 did not exhibit any additive effects in combination with SUMO1 (Figure 2A), suggesting that UBC9 does not play a role in the regulation of BRCA1-induced transcriptional activity from the Gadd45α promoter.

Next, we examined whether SUMO1 affected BRCA1-induced transcriptional activity in other cell lines. Consistent with the observations in U2OS cells, SUMO1 repressed BRCA1-induced transcriptional activity in 293T and HeLa cells to the basal level (Supplementary Figure 1), suggesting that the repressive effect of SUMO1 on BRCA1-mediated transcription may be general.

To further examine whether the transcriptional repression of Gadd45α gene is a bona fide SUMO1 activity and not an artifact of reporter assay, we measured the total levels of Gadd45α transcript by RT-PCR analysis following ectopic expression of BRCA1 and/or SUMO1. As expected, BRCA1 alone induced transcription of Gadd45α gene (Figure 2B). However, SUMO1 alone had little effect on the Gadd45α transcription (Figure 2B). SUMO1 in the presence of exogenously expressed BRCA1 proteins repressed BRCA1-induced Gadd45α transcription to the comparable levels observed in cells without exogenously expressed BRCA1 and SUMO1 or in cells with exogenously expressed SUMO1 alone (Figure 2B). The RT-PCR results for Gadd45α mRNA (Figure 2B) is consistent with the observations from the luciferase reporter hooked with Gadd45α promoter (Figure 2A and

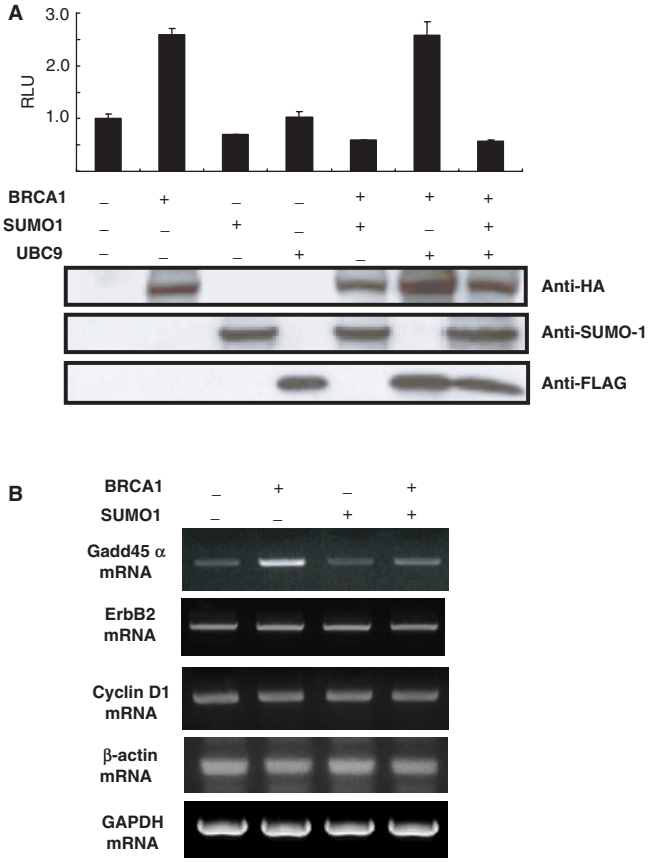


Figure 2. SUMO1 represses BRCA1-induced transcriptional activity from the Gadd45α promoter. (**A**) The induction of transcriptional activity by BRCA1 of the Gadd45α promoter, on the promoter-driven reporter gene, was monitored in the presence and absence of SUMO1 or UBC9, as indicated, in U2OS cells. The luciferase activity of the reporter gene alone was arbitrarily set to one. Results were obtained from six separate experiments, and standard deviations are shown ($^{*}P < 0.05$). Immunoblots for exogenously expressed HA-BRCA1, SUMO1 and FLAG-Ubc9 proteins were performed with anti-HA, anti-SUMO1 and anti-FLAG antibodies, respectively, to ensure that all levels are equivalent. (**B**) Expression of Gadd45α transcripts in the presence and absence of BRCA1 and SUMO1 was analyzed by RT-PCR. The amount of GAPDH transcripts is shown as a quantitative control for Gadd45α transcripts.

Supplementary Figure 1), suggesting that SUMO1 can physiologically regulate the BRCA1-mediated Gadd45α transcription.

Next, we investigated whether SUMO1 could down-regulate transcriptions from other promoters that are not regulated by BRCA1. BRCA1 did not alter the transcriptions from ErbB2, CyclinD1 and β-actin promoters (Figure 2B). Also, SUMO1 either alone or in the presence of BRCA1 had little effect on transcriptions of theses promoters (Figure 2B). These observations suggest that the negative transcriptional effect of SUMO1 may not be general to transcriptions from many other promoters.

Repression of BRCA1-induced transcriptional activity by SUMO1 in a sumoylation-independent manner

Sumoylation of H4 and several transcriptional regulators (30) is thought to be essential for repression of

transcription. For this reason, we investigated whether sumoylation was required for repression of BRCA1-induced transcription by SUMO1. The transcriptional activity from the Gadd45α promoter was monitored in the presence of SUMO1 that was unable to sumoylate substrates (SUMO1ΔGG) (Figure 3A). Inactive SUMO1ΔGG had glycine residues at 99 and 100 in the C-terminal region which were deleted. By comparison to wild-type SUMO1, sumoylation-incompetent SUMO1ΔGG exhibited a similar suppressive effect on transcription from the Gadd45α promoter by BRCA1 (Figure 3A). We also examined whether dominant-negative UBC9 (DN-UBC9) that is unable to conjugate SUMO1 with substrates, had an effect on SUMO1-mediated repression of BRCA1-induced transcriptional activity. DN-UBC9 did not alter BRCA1-mediated activation of Gadd45α transcription with or without SUMO1, consistent with the effect of wild-type UBC9 (Figure 2A). Together, these results indicate that sumoylation does not play a role in the repression of BRCA1-induced transcriptional activity by SUMO1.

Next, we investigated whether SUMO1ΔGG interacted with BRCA1 (Figure 3B and C). Purified His-SUMO1ΔGG was incubated with the six GST-tagged BRCA1 fragments immobilized on GST-agarose beads. SUMO1ΔGG interacted with four BRCA1 fragments (1–1064) (Figure 3B), in a manner consistent with the wild-type protein (Figure 1C). Interaction of BRCA1 with SUMO1ΔGG was also examined in 293T cells (Figure 3C). EGFP–SUMO1ΔGG was detected in anti-BRCA1 immunoprecipitates, showing that the BRCA1–SUMO1ΔGG interaction occurs *in vivo* (Figure 3C). These observations show that BRCA1 is able to interact with sumoylation-incompetent SUMO1ΔGG, and this interaction may lead to suppression of BRCA1-induced transcriptional activity without covalent modification by SUMO1.

BRCA1–SUMO1 interaction is required for repression of BRCA1-mediated transcription by SUMO1

We next tested whether BRCA1–SUMO1 interaction functionally links to SUMO1-induced repression of BRCA1-mediated transcription. To this end, we identified potential sumoylation-independent SUMO1 interaction motifs (SIMs, 31–34) in BRCA1 (Figure 4A). To determine whether the putative SIMs in BRCA1 directly mediated the interaction of BRCA1 with SUMO, we mutated the putative SIMs in four BRCA1-truncated fragments from the SUMO1 interacting region (1–1064, Figure 1A) and evaluated SUMO1 interacting activity by using the mutant BRCA1 fragments as baits in the yeast two-hybrid system (Figure 4B). Mutations of the SIMs, ^{122}VSII^{125} (V122A) from the N-terminal BRCA1 (1–324) and ^{769}ISLV^{772} (I769A and V772A) from the central BRCA1 (502–1064) displayed SUMO1 interaction activities almost equal to that observed from BRCA1 wild type (Figure 4B). In contrast, mutations of the SIMs, ^{412}VLDV^{415} (V412A) and ^{783}ISLLEV^{788} (^{783}ISLL^{786} and ^{785}LLEV^{788}, I783A/V788A) exhibited reduced SUMO1 interaction activities, compared with that of BRCA1 wild type (Figure 4B). Taken together, these interaction experiments suggest that the ^{412}VLDV^{415}, ^{783}ISLL^{786} and ^{785}LLEV^{788} SIMs in BRCA1 play a role in interacting with SUMO1.

We next tested whether the BRCA1–SUMO1 interaction is required for the SUMO1-induced repression of BRCA1 transcription. To do this, we generated full-length BRCA1 mutants containing amino acid changes within the SIMs and evaluated BRCA1-induced activation of Gadd45α transcription. The Gadd45α promoter-driven transcription was upregulated by approximately 3.8-fold (in the presence of BRCA1-containing mutations of one SIM; V412A, V412A/V415A or I783A/V788A) to 5.8-fold (in the presence of BRCA1-containing mutations of two SIMs; V412A/I783A/V788A) (Figure 4C), displaying higher transcriptional activation by comparison to BRCA1 wild type (∼2.2-fold) (Figure 4C). These findings indicate that SIMs in BRCA1 inhibit BRCA1's transcriptional activity possibly by interaction of SUMO1 with SIMs. To test this, we analyzed effects of SUMO1 on transcriptional activities of BRCA1 mutants (Figure 4D). The repression activity of SUMO1 decreased by ∼30% (in the presence of one-SIM BRCA1 mutants, V412A, V412A/V415A or I783A/V788A, from ∼3.8-fold to ∼1.7-fold) to 50% (in the presence of two-SIM BRCA1 mutant, V412A/I783A/V788A, from ∼5.8-fold to ∼3.9-fold) (Figure 4D and Supplementary Figure 2) compared to that seen with BRCA1 wild type (100%, from ∼2.2-fold to ∼0.65-fold), indicating that disruption of SIMs in BRCA1 attenuates repressive activity of SUMO1 against BRCA1-mediated transcription. Together, these analyses support that the SUMO1 interaction motifs in BRCA1 participate in BRCA1–SUMO1 interaction and sequentially exert influence of SUMO1 in the repression of BRCA1-induced transcription.

Repression of BRCA1-induced transcriptional activity by SUMO1 via histone deacetylation

Several recent observations indicate a functional link between the SUMO system and HDACs in mediating transcriptional repression via the formation of transcriptionally repressive chromatin. The possibility that HDACs may function together with SUMO1 to control BRCA1-mediated transcription is also supported by the association of BRCA1 with HDACs (13). Thus, we tested the effect of an HDAC inhibitor, TSA, on Gadd45α transcription (Figure 5A). TSA enhanced Gadd45α transcription without regard to expression of BRCA1 and SUMO1 (Figure 5A). By comparison to BRCA1 alone (∼2.5-fold) and TSA alone (∼2.0-fold), TSA together with BRCA1 synergistically increased Gadd45α transcription by ∼6.1-fold (Figure 5A). Moreover, TSA abrogated the suppressive effect of SUMO1 on BRCA1-induced Gadd45α activation (Figure 5A), suggesting that SUMO1 cannot repress BRCA1-induced transcription in the presence of TSA (Figure 5A). Together, these results show that HDAC activity contributes to the repression of BRCA1-induced transcription by SUMO1.

To further support the role of HDAC activity in repression of BRCA1-induced transcriptional activity

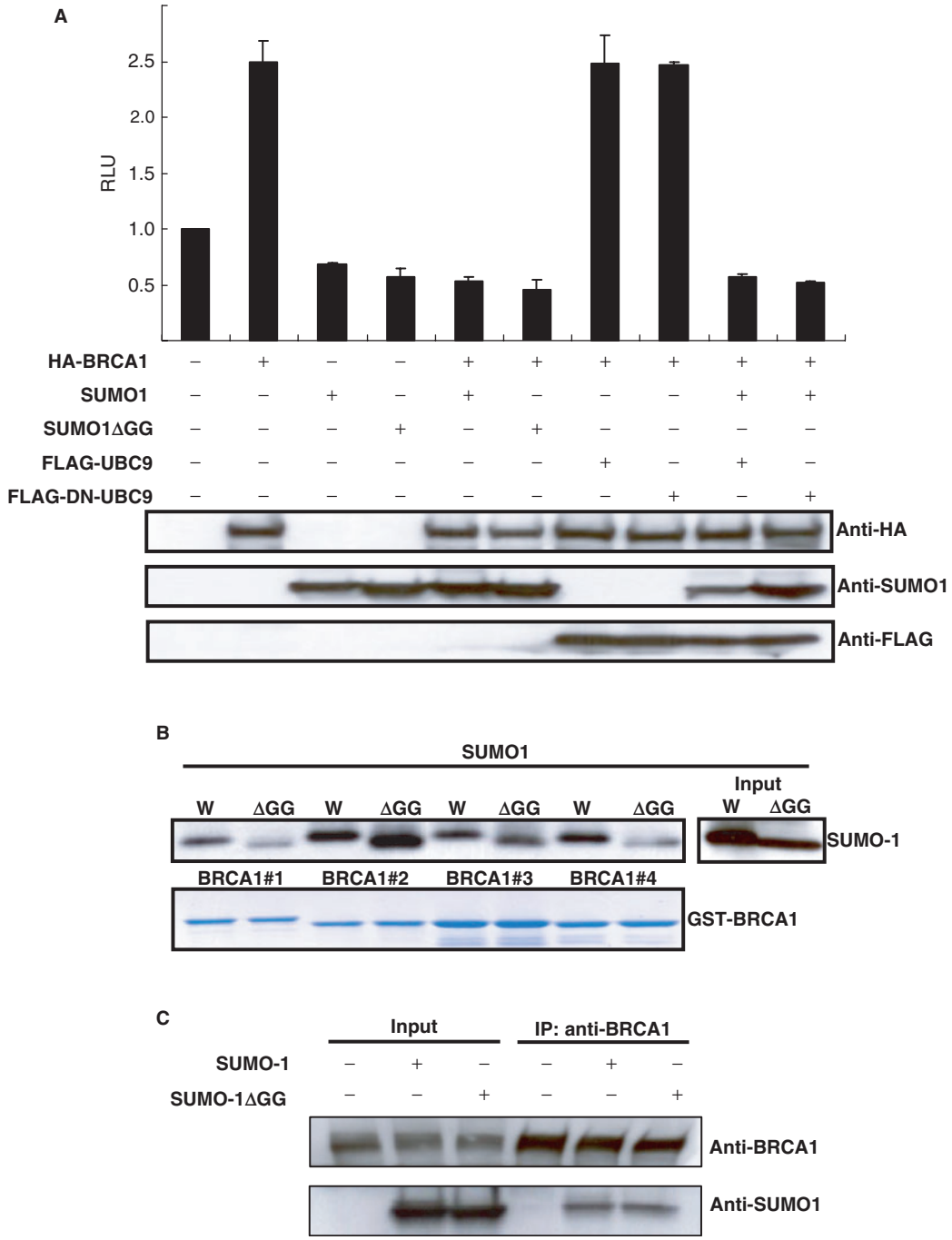


Figure 3. Sumoylation-independent repression of BRCA1-induced transcription of the Gadd45α promoter by SUMO1. (**A**) The activity of the Gadd45α promoter-driven reporter was analyzed in the presence of wild-type SUMO1 or its mutant derivative, SUMO1ΔGG, in U2OS cells cotransfected with BRCA1. The transcription from the Gadd45α promoter was also measured in the presence of the wild type (UBC9) or dominant-negative (DN-UBC9) UBC9. The results were analyzed as described in Figure 2. (**B**) *In vitro* association of BRCA1 with SUMO1ΔGG. The GST pull-down assay was performed as described in Figure 1, except that the SUMO1ΔGG protein was used. (**C**) *In vivo* association of BRCA1 with SUMO1ΔGG. Either wild-type SUMO1 or its mutant derivative SUMO1ΔGG, were transiently expressed in 293T cells. Equivalent amounts of total cellular protein were immunoprecipitated with anti-BRCA1 (IP: anti-BRCA1) antibody. Coimmunoprecipitated SUMO1 proteins were detected by an anti-SUMO1 immunoblot (Anti-SUMO1). The immunoprecipitated BRCA1 proteins were detected by an anti-BRCA1 antibody (Anti-BRCA1).

by SUMO1, we investigated the histone acetylation status at the Gadd45α promoter using chromatin immunoprecipitation (ChIP) with anti-acetyl H4. BRCA1 alone increased the level of acetylated H4 at the Gadd45α promoter. The addition of SUMO1 decreased the level of acetylated H4 at the Gadd45α promoter compared with the level in the presence of BRCA1 alone (Figure 5B). This result suggests a close relationship between Gadd45α transcription and histone acetylation. We next observed that TSA treatment increased the level of acetylated histones at the Gadd45α promoter compared with the level in non-treated cells (Figure 5B). TSA alone led to an

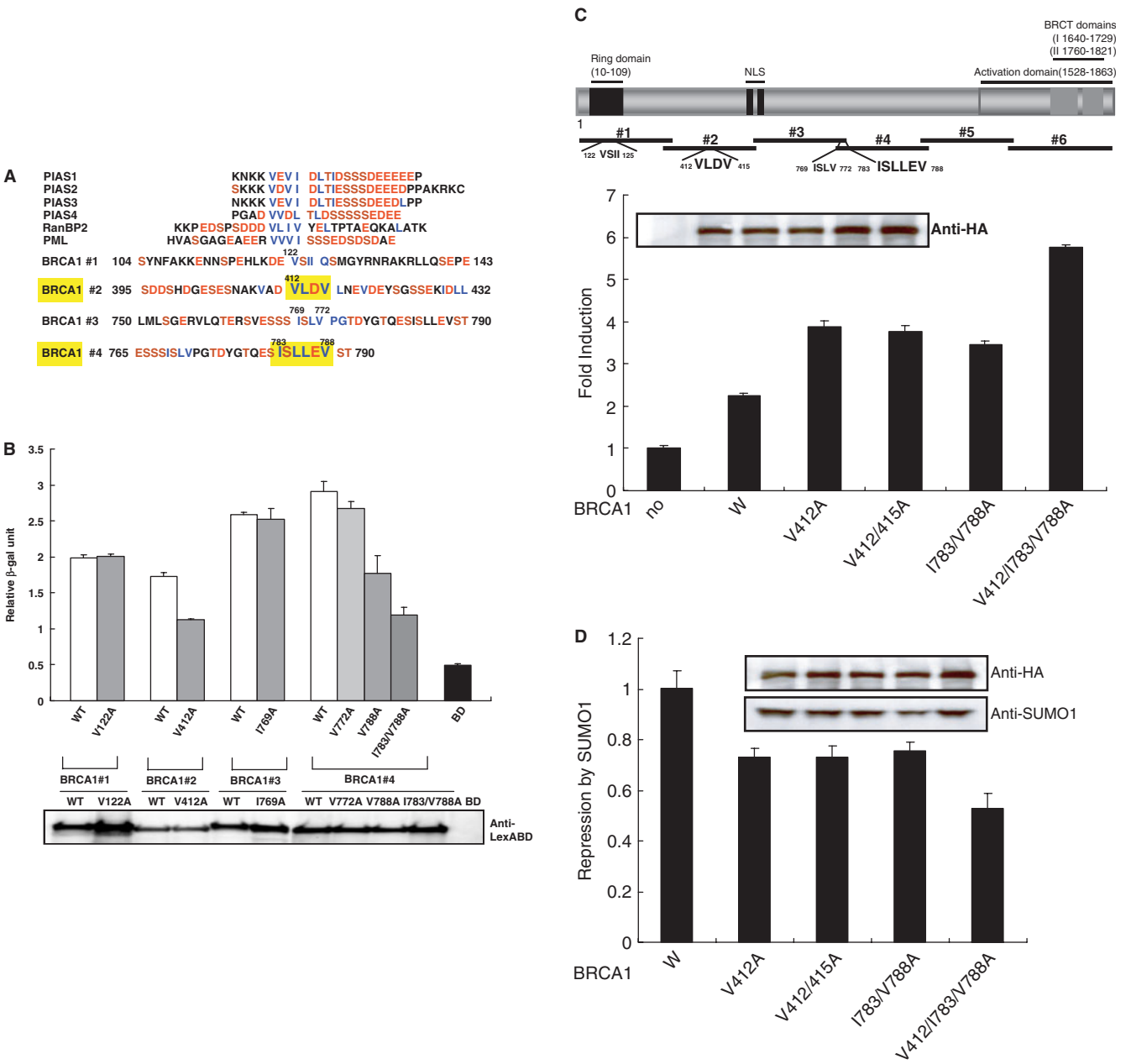


Figure 4. BRCA1 has SUMO interaction motifs. (**A**) The putative SUMO interaction motifs (SIMs) of BRCA1 along with that of other proteins (31–34) are aligned. (**B**) SIMs in BRCA1 mediates interaction of BRCA1 with SUMO1. The ability of putative SIMs of BRCA1 as described in (A) to interact with SUMO1 was measured by monitoring β-galactosidase activity, following cotransformation of yeast cells with pLexA-BRCA1 (1–324, 260–553, 502–802 and 758–1064; wild type or mutant fragments) and pB42AD-SUMO1. (Bottom) Equivalent protein expression of wild-type and mutant LexA-BRCA1 was examined by immunoblotting using anti-LexA antibodies. (**C**) Mutation of SIMs enhances BRCA1's transcriptional activity. The transcription from the Gadd45α promoter was measured in the presence of BRCA1 wild-type (W) or SIM mutants. The results were analyzed as described in Figure 2. (Inset) Immunoblotting for ensuring of equivalent protein expression of wild type and SIM mutant HA-BRCA1 was performed with anti-HA antibody. (**D**) SIMs are required for SUMO1-induced repression of BRCA1-mediated transcription. The repression of transcriptional activity of BRCA1 by SUMO1 was monitored in the presence of SUMO1. The repressive activity of SUMO1 on the wild type BRCA1-mediated Gadd45α transcription (W) was set to one. The relative repression of SIM mutant BRCA1-mediated transcription by SUMO1 to that of wild type BRCA1 was analyzed. Results were obtained from three separate experiments, and standard deviations are shown ($^{*}P < 0.05$). Immunoblots for exogenously expressed HA-BRCA1 and SUMO1 proteins were performed with anti-HA and SUMO1 antibodies, respectively, to ensure that all levels are equivalent.

induction of histone acetylation almost equal to the level in cells expressing BRCA1 in the absence of TSA (Figure 5B). The level of histone acetylation in cells expressing BRCA1 in the presence of TSA was higher than in cells expressing BRCA1 in the absence of TSA (Figure 5B). The level of histone acetylation in cells coexpressing BRCA1 and SUMO1 in the presence of TSA is similar to that observed in cells expressing BRCA1 alone in the presence of TSA (Figure 5B). Thus, TSA abrogated the reduction of histone acetylation by

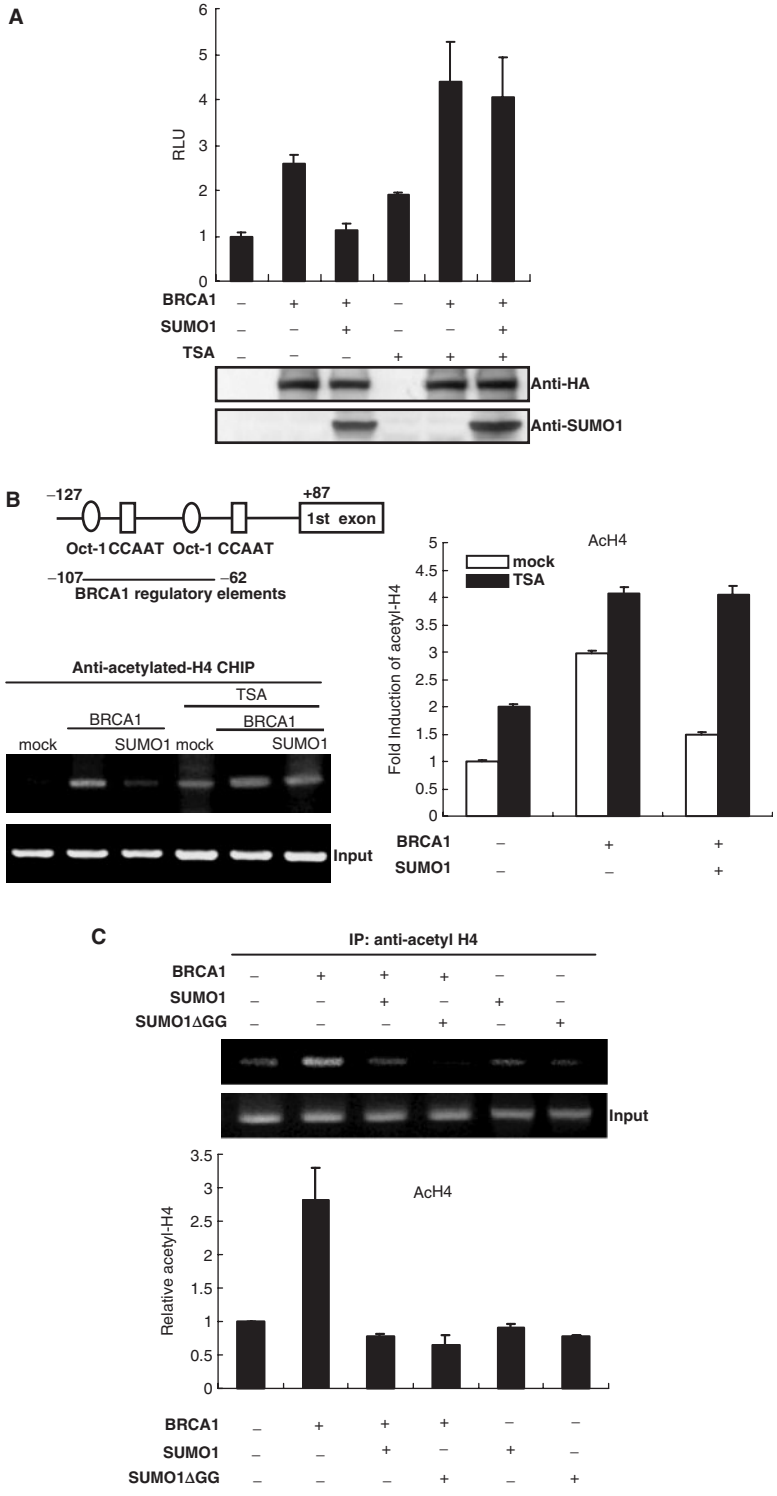


Figure 5. SUMO1-mediated repression of BRCA1-induced transcriptional activity occurs in a histone deacetylase-dependent manner. (**A**) TSA abolished the repressive activity of SUMO1. The activity of Gadd45α-luciferase reporter was measured in U2OS cells treated with or without TSA. The luciferase activity of the untreated cells transfected with reporter only was set to one. The results were analyzed as described in Figure 2. Equivalent protein expression of HA-BRCA1 and SUMO1 was examined by immunoblotting using antibodies to HA and SUMO1, respectively. (**B**) SUMO1 reduced the level of histone acetylation of the Gadd45α promoter. The acetylation status of histones at the Gadd45α promoter was monitored by chromatin immunoprecipitation (ChIP) with anti-acetyl H4 antibody. In 293T cells, ChIP assays were performed in the presence or absence of BRCA1 and SUMO1. In addition, changes in the acetylation status of histone were measured following treatment without or with TSA (TSA). The endogenous Gadd45α promoter DNA that coprecipitated with the anti-acetyl H4 antibody was detected by PCR (left) or replicate quantitative real time PCR (right). All PCRs and real-time PCRs were normalized to the input control. (Right) Quantity of immunoprecipitated DNA from the untransfected cells untreated with TSA was set to one. (**C**) SUMO1ΔGG induced a reduction in histone acetylation at the Gadd45α promoter. ChIP analysis of the endogenous Gadd45α promoter in 293T cells was performed in the presence of transfected wild-type SUMO1 or its mutant derivative SUMO1ΔGG. The results were analyzed as described in (B).

SUMO1, leading to increased histone acetylation at the Gadd45α promoter. TSA-induced histone acetylation was in good correlation with the induction of Gadd45α transcription following TSA treatment (Figure 5A and B). Together, these data provide further support for the involvement of HDACs in the repression of BRCA1-induced transcriptional activity by SUMO1.

To further investigate the sumoylation-independent repression of BRCA1-induced transcriptional activity by SUMO1 (Figures 3), we analyzed the histone acetylation status of the Gadd45α promoter following expression of SUMO1ΔGG (Figure 5C). Sumoylation-defective SUMO1ΔGG reduced the level of histone acetylation to almost equal level in cells expressing wild-type SUMO1 (Figure 5C), thereby providing further support for the observation that sumoylation is not important in the repression of BRCA1-induced transcriptional activity by SUMO1.

SUMO1 recruits HDAC1 to and releases BRCA1 from the Gadd45α promoter

As the repressive effect of SUMO1 is TSA-sensitive and inversely correlated with the level of histone acetylation at the Gadd45α promoter, we investigated the influence of SUMO1 on the recruitment of HDACs by performing chromatin immunoprecipitation (ChIP) with anti-HDAC1 and 2 in the presence or absence of SUMO1 (Figure 6A). Expression of SUMO1 induced the recruitment of HDAC1 but not 2 to the Gadd45α promoter, with or without the coexpression of BRCA1 (Figure 6A). In contrast, BRCA1 alone caused the release of HDAC1 from the Gadd45α promoter (Figure 6A). Together, these observations indicate that SUMO1 can recruit HDAC1 to the Gadd45α promoter, and consequently diminish the level of histone acetylation (Figures 5B and C). ChIP ssays were then performed to assess whether SUMO1 interferes with the association of BRCA1 with the Gadd45α promoter. Exogenously expressed BRCA1 was recruited to the Gadd45α promoter (Figure 6A and B). The addition of SUMO1 led to a reduction in the level of BRCA1 recruited to the Gadd45α promoter (Figure 6A and B). These observations indicate that SUMO1 induces the recruitment of HDAC1 to the Gadd45α promoter and the release of BRCA1 from the promoter.

Next, we examined whether SUMO1 affected recruitment of BARD1, which interacts with BRCA1, at the Gadd45α promoter. As shown in Figure 5A, BRCA1 expression induced recruitment of BARD1 and SUMO1 expression induced release of BARD1 at the Gadd45α promoter, similar to the BRCA1 result. Similarly, RNA polymerase II, another BRCA1-interacting protein, was recruited to the promoter following BRCA1 expression and it was released from the promoter following SUMO1 expression (Figure 6A). However, expression of BRCA1 or/and SUMO1 had no effect on recruitment of p300 at the promoter (Figure 6A). The level of p300 at the promoter was not altered in the presence of BRCA1 or/and SUMO1. These observations indicate that SUMO1 induced the release of BARD1 and RNA polymerase II but not p300 from the Gadd45α promoter.

ChIP experiments revealed the release of HDAC1 but not HDAC2 from the Gadd45α promoter in the absence of SUMO1 expression. In contrast, BRCA1, BARD1 and RNA polymerase II, but not p300, were released in the presence of SUMO1 expression. Hence, we assessed whether expression of SUMO1 might differentially affect protein interactions between BRCA1/HDAC1, BRCA1/HDAC2, and BRCA1/BARD1 and BRCA1/p300. The presence of SUMO1 expression had little effect on BRCA1 interactions with HDAC1 or HDAC2 (Supplementary Figure 3). Similarly, interactions of BRCA1/BARD1 or BRCA1/p300 were not changed by SUMO1 expression (Supplementary Figure 3).

As SUMO1ΔGG represses BRCA1-induced transcription (Figure 3A), we tested whether SUMO1ΔGG could also affect the association of HDAC1 or BRCA1 with the Gadd45α promoter. ChIP analysis showed that expression of SUMO1ΔGG induced the recruitment of HDAC1 to the Gadd45α promoter (Figure 6C). SUMO1ΔGG also induced the release of BRCA1 from the Gadd45α promoter (Figure 6C). These results suggests that the effect of SUMO1ΔGG on the association of HDAC1 and BRCA1 with the Gadd45α promoter is consistent with wild-type SUMO1 (Figure 6A and B) and further supports the finding that SUMO1-induced repression of BRCA1-induced transcription is not mediated by sumoylation.

Based on our results that SUMO1 induced recruitment of HDAC1 at the promoter in the sumoylation-independent manner, we tested whether HDAC1 is required for SUMO1-induced repression of BRCA1's transcriptional activity. Thus, we examined the effect of HDAC1 knockdown on the transcriptional repression by SUMO1 (Figure 6D). HDAC1 knockdown had little effects on Gadd45α transcription without regard to expression of BRCA1 (Figure 6D). By comparison to BRCA1 alone (~2.5-fold) and HDAC1 knockdown alone (~1.1-fold), HDAC1 knockdown together with expression of BRCA1 barely increased Gadd45α transcription (~2.7-fold) (Figure 6D). However, HDAC1 knockdown attenuated the suppressive effect of SUMO1 on BRCA1-induced Gadd45α activation by ~45% (Figure 6D), suggesting that SUMO1 is required for HDCA1 for efficient repression of BRCA1-induced transcription.

We next analyzed status of histone acetylation at the Gadd45α promoter when the level of endogenous HDAC1 was reduced. In the presence of exogenous SUMO1, depletion of HDAC1 by silencing led to less reduction of the level of acetylated histones, compared with the reduced level of acetylated histones without depletion of HDAC1. However, HDAC1-depletion had little effect on the histone acetylation without exogenous SUMO1 proteins (Figure 6E). Thus, depletion of HDAC1 alleviated the reduction of histone acetylation by SUMO1, leading to the increased histone acetylation at the Gadd45α promoter. HDAC1 depletion-induced histone acetylation (Figure 6E) was in good correlation with the induction of Gadd45α transcription in HDAC1-depleted cells (Figure 6D). Together, these results show that HDAC1 activity functions in the repression of BRCA1-induced transcription by SUMO1, via induction of histone deacetylation at the promoter.

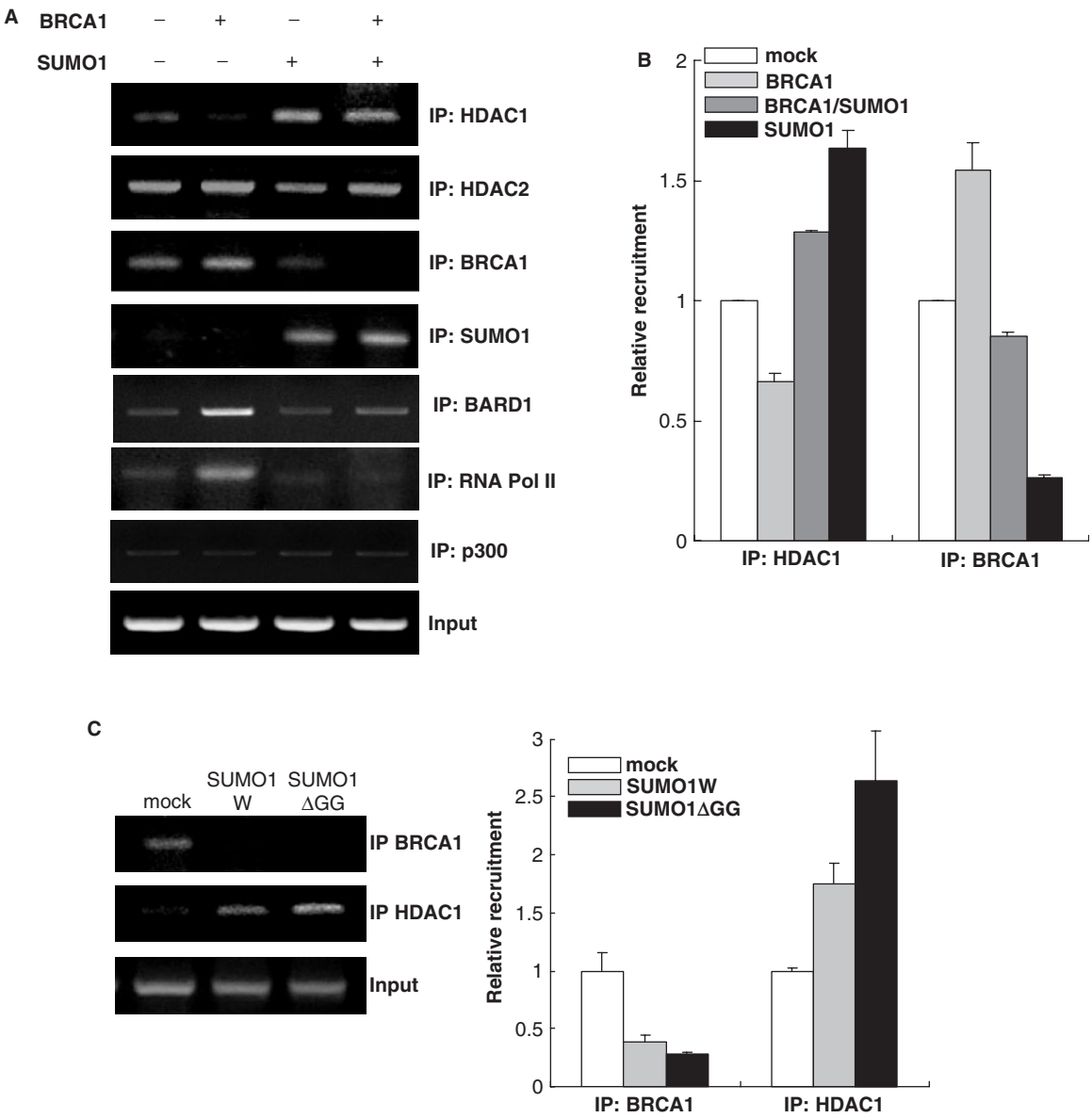
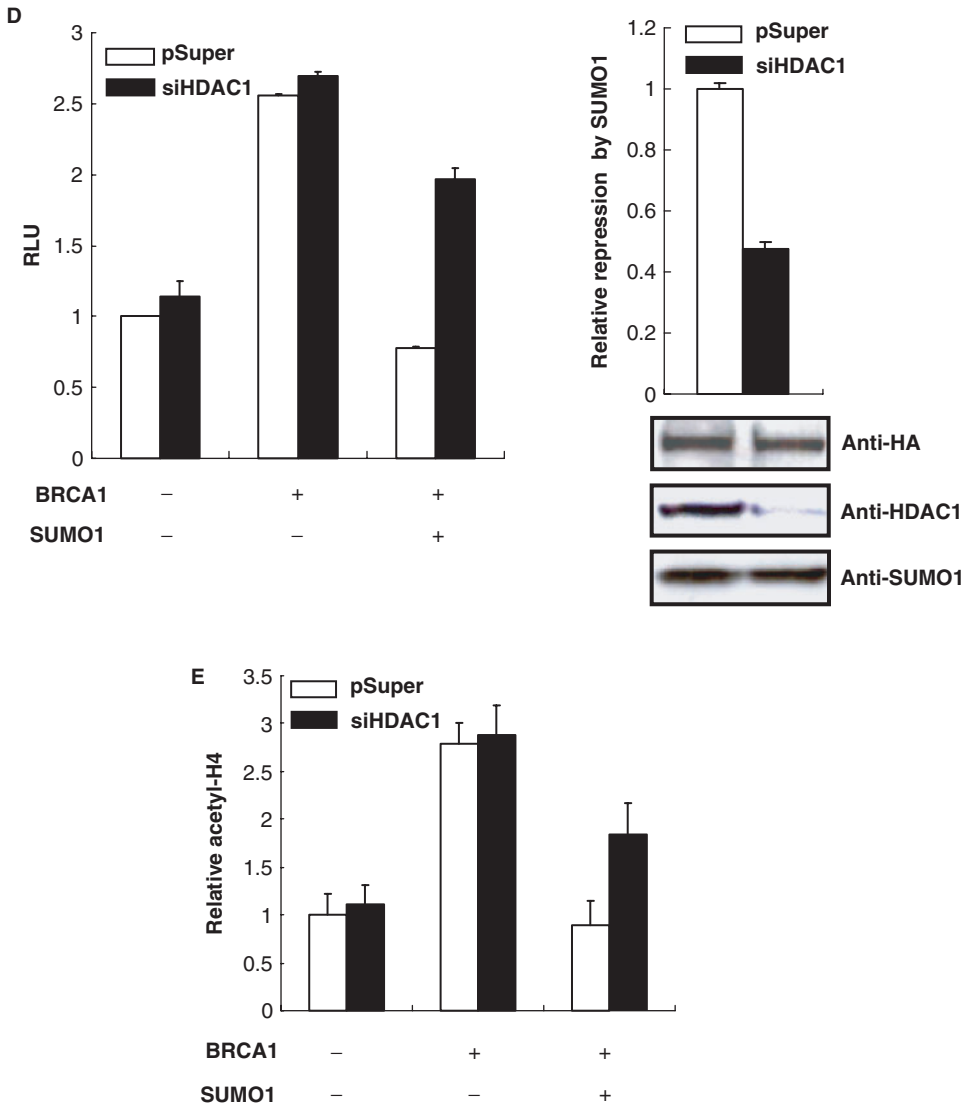


Figure 6. The Gadd45α promoter occupancy of BRCA1, SUMO1 and HDAC1. (**A**) SUMO1 induced recruitment of HDAC1 and release of BRCA1 at the Gadd45α promoter. ChIP analysis of Gadd45α promoter in 293T cells expressing BRCA1 and SUMO1 was performed using antisera specific to the indicated proteins. (**B**) Real-time PCRs were performed for quantitative ChIP analysis of Gadd45α promoter with anti-HDAC1 (IP: HDAC1) and anti-BRCA1(IP: BRCA1) antibodies, respectively. (**C**) ChIP analysis of the Gadd45α promoter in 293T cells in the presence of SUMO1ΔGG using antisera specific to BRCA1 (IP: BRCA1) and HDAC1 (IP: HDAC1). (Right) Quantitative ChIP analysis was performed using real-time PCR. (**D**) HDAC1 plays a role in SUMO1-induced repression of BRCA1-mediated transcription. Gadd45α-luciferase reporter gene activity was measured in U2OS cells transfected with plasmids as indicated, in the absence (white) or presence of siRNA against HDAC1 (black). The values represent the mean ± SEM from three experiments ($^{*}P < 0.05$). The relative induction of the Gadd45α promoter by BRCA1 to that of the reporter alone was measured in the presence or absence of HDAC1 silencing. (Right) Relative repressive activity of SUMO1 was measured in the HDAC1 knockdown cells (black), as indicated and analyzed in Figure 4 (D). Knockdown of HDAC1 protein and equivalent expression of BRCA1 and SUMO1 proteins were examined by immunoblotting using anti-HDAC1, anti-HA and anti-SUMO1 antibodies, respectively. (**E**) Depletion of HDAC1 attenuated the SUMO1-induced reduction in histone acetylation at the Gadd45α promoter. Quantitative ChIP analysis of the endogenous Gadd45α promoter in 293T cells was performed using real-time PCRs in the HDAC1-depleted cells and performed as described in Figure 5.

Silencing of SUMO1 stimulates BRCA1-mediated transcription and BRCA1–interaction with promoters

The reporter assay, RT-PCR and ChIP analysis of the Gadd45α promoter were repeated in SUMO1 knockdown cells to ascertain the physiological relevance of these findings (Figures 1–6). We first evaluated Gadd45α transcription by using the reporter construct driven by the Gadd45α promoter. As expected, Gadd45α promoter-driven transcription was upregulated by approximately 1.6-fold (in the presence of exogenous BRCA1 proteins) to 2-fold (in the absence of exogenous BRCA1 proteins), when the level of endogenous



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SUMO1 was reduced (Figure 7A). The induction of BRCA1-mediated transcription in SUMO1-depeleted cells (Figure 7A) was comparable to that observed in cells expressing BRCA1 SIM-mutants (Figure 4C), indicating that SUMO1 indeed plays a role in repression of BRCA1-induced transcription. In contrast, Gadd45α transcription was downregulated by ~20% (in the presence of exogenous BRCA1 and SUMO1 proteins or in the cells without either exogenous expression) to 35% (in the presence of exogenously expressed BRCA1 alone), when the level of BRCA1 was reduced by deletion of endogenous BRCA1 (Figure 7A).

We next analyzed expression of Gadd45α transcripts in SUMO1 knockdown cells by RT-PCR (Figure 7B). In consistent with the results obtained from the Gadd45α reporter (Figure 7A), the level of Gadd45α mRNAs increased in the SUMO1-depleted cells and decreased in the BRCA1-depleted cells (Figure 7B). In the presence of exogenous BRCA1, depletion of SUMO1 by silencing led to further induction of Gadd45α mRNA, compared with the induced level of Gadd45α mRNA without exogenous BRCA1 (Figure 7B). Together with Gadd45α promoter-driven reporter results in the SUMO1-depleted cells, the Gadd45α mRNA expression results indicate that SUMO1 may function as a physiological repressor for BRCA1 transcriptional activity.

Also, depletion of endogenous SUMO1 led to induction of the recruitment of BRCA1 to the Gadd45α promoter and the release of HDAC1 (Figure 7C). This result further supports the notion that SUMO1 represses BRCA1-induced transcription from the Gadd45α promoter by modulating protein association at the promoter. Also, silencing of BRCA1 induced recruitment of SUMO1 and HDAC1 to the promoter (Figure 7D), suggesting that assembly of SUMO1 and disassembly of BRCA1 at the Gadd45α promoter is important for repression of BRCA1-induced transcription by SUMO1.

Since BRCA1 activates $p27^{Kip1}$ (35) and $p21^{WAF1/CIP1}$ (36) gene transcriptions, we questioned whether BRCA1-stimulated transcription of $p27^{Kip1}$ or $p21^{WAF1/CIP1}$ genes

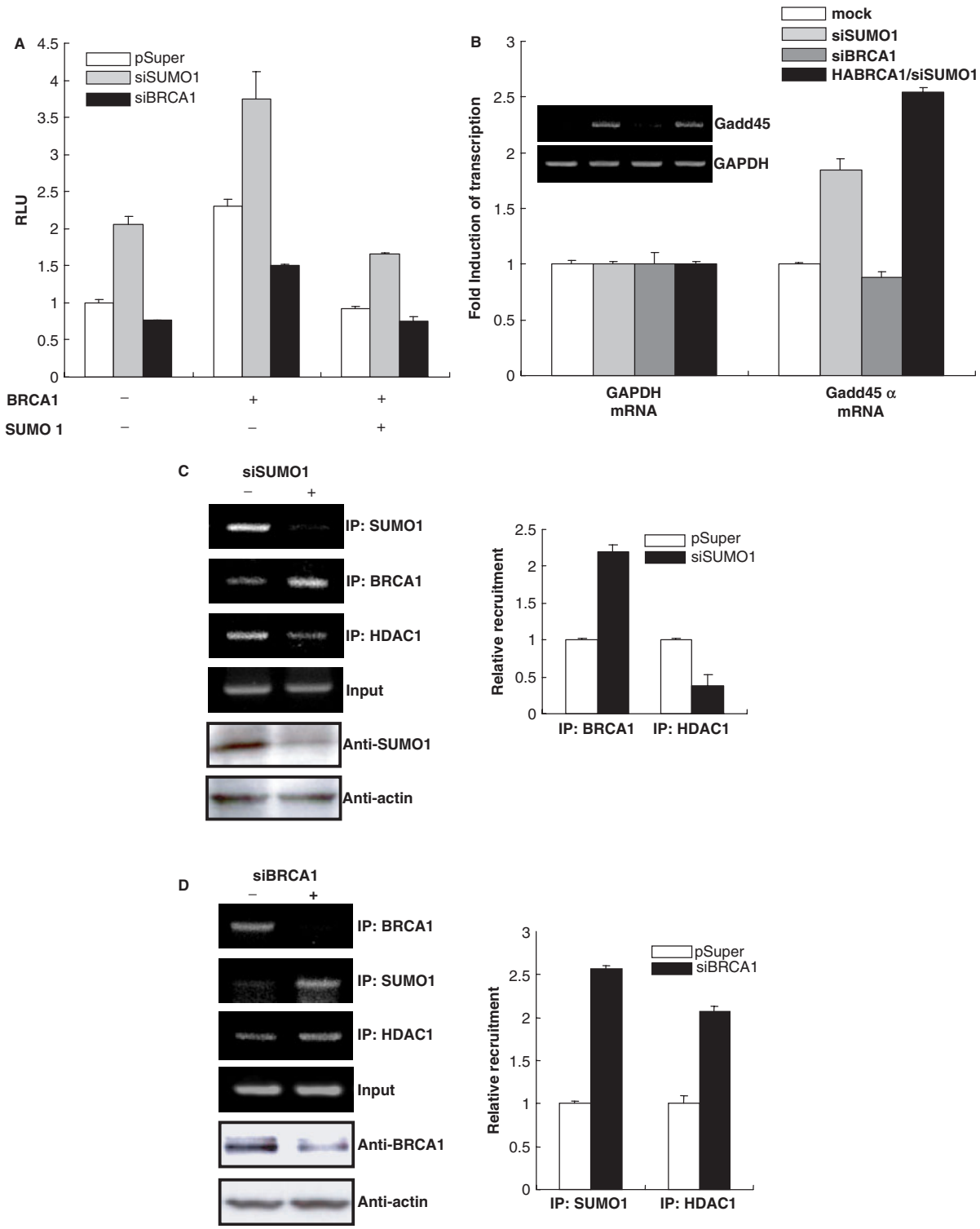


Figure 7. Analysis of transcription from the Gadd45α promoter following silencing of SUMO1 or BRCA1. (**A**) Gadd45α-luciferase reporter gene activity was measured in U2OS cells transfected with plasmids as indicated, in the absence (white) or presence of siRNA against SUMO1 (gray) or BRCA1 (black). The values represent the mean ± SEM from three experiments ($^{*}P < 0.05$). The relative induction of the Gadd45α promoter by BRCA1 to that of the reporter alone was measured in the presence or absence of SUMO1 (gray) or BRCA1 (black) silencing. (**B**) Expression of Gadd45α mRNAs was measured using real-time RT-PCR in U2OS cells transfected with plasmids as indicated, in the absence (−) or presence (+) of siRNA against SUMO1 (siSUMO1) or BRCA1 (siBRCA1). (Inset) A representative agarose gel analysis of RT- PCR products. (**C** and **D**) BRCA1 and SUMO1 compete for the Gadd45α promoter. ChIP analyses of the endogenous Gadd45α promoter in 293T cells transfected with siRNAs against SUMO1 (C) or BRCA1 (D) were performed with each indicated antibody. The DNA precipitated in the immunocomplexes was PCR (left) or real-time PCR (right) amplified using primers specific to the Gadd45α promoter. Immunoblots demonstrate the reduction of the indicated target protein level.

might be repressed by SUMO1. First, we measured levels of $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ mRNAs following silencing of SUMO1. Similar to the Gadd45α gene transcription, depletion of endogenous SUMO1 induced mRNA levels of $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ genes (Figure 8A), suggesting that SUMO1 can also repress transcription of $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ genes.

To see whether SUMO1 can inhibit association of BRCA1 with $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ promoters, we performed the ChIP experiment in the SUMO1-depleted cells. Silencing of SUMO1 led to induction of BRCA1 association with both $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ promoters (Figure 8B). In contrast, silencing of SUMO1 reduced the level of HDAC1 associated with both $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ promoters (Figure 8B). Next, we examined whether the level of promoter-associated SUMO1 and HDAC1 is induced in the BRCA1-depleted cells. Silencing of BRCA1 induced association of SUMO1 and HDAC1 with both $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ promoters (Figure 8B). Together, results from $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ experiments further support the molecular mechanisms by which SUMO1 can downregulate the BRCA1-activated transcriptions via inhibition of BRCA1 association with promoters.

Next, we tested whether SUMO1 can reduce the level of histone acetylation at $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ promoters. Depletion of SUMO1 by silencing led to an induction of the level of acetylated histones at $p21^{WAF1/CIP1}$ (~2.5-fold) and $p27^{Kip1}$ (~3.2-fold) promoters, consistent with the Gadd45α promoter result (~4.3-fold) (Figure 8C and D). In contrast, exogenously expressed SUMO1 diminished the level of histone acetylation at both promoters (~40 ~ 45%) (Figure 8D), supporting that reduction of histone acetylation at BRCA1 target promoters plays a role in SUMO1-induced repression. Also, both SUMO1 and SUMO1ΔGG inhibited association of BRCA1 with both $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ promoters (Figure 8D), suggesting that dissociation of BRCA1 from both promoters contributes to repression of BRCA1-induced transcription by SUMO1.

To see whether deletion of SUMO1 can affect expression of other genes rather than BRCA1 targets, we analyzed mRNA transcripts of ErbB2 and Cylin D1. In SUMO1-knockdown cells, transcription of ErbB2 and Cylin D1 genes was not altered (Figure 8E), suggesting that SUMO1-induced repression may not be general. In consistent with the transcription results, status of histone acetylation at the ErbB2 and Cylin D1 promoters was not changed following deletion of SUMO1 (Figure 8E), supporting that effects of SUMO1 on transcriptional repression is not general.

SUMO1 reduces binding of BRCA1 to the Gadd45α promoter

The ChIP (Figures 6–8) and coimmunoprecipitation (Supplementary Figure 3) results suggest that regulation of BRCA1-mediated transcription by SUMO1 seems to be largely dependent on modulation of protein–DNA interactions but not protein–protein interactions, for the control of promoter occupancy. Thus, we next examined whether SUMO1 affected the DNA–binding activity of BRCA1 at the Gadd45α promoter, using biotin-labeled oligonucleotides containing the BRCA1–binding region (29). When BRCA1 alone was exogenously expressed, the ability of BRCA1 to bind to the Gadd45α promoter was strengthened (Figure 9A). However, when SUMO1 and BRCA1 were expressed together, the binding ability of BRCA1 was diminished significantly (Figure 9A). In consistent with the results with exogenously expressed BRCA1 (Figure 9A), SUMO1 led to a dissociation of endogenous BRCA1 from DNA (Figure 9B). Similarly, SUMO1ΔGG also induced release of endogenous BRCA1 from Gadd45α promoter DNA (Figure 9B). SUMO1 also reduced DNA–binding activity of exogenous and endogenous BARD1 (Figure 9). These DNA–binding results were in good agreement with repression of Gadd45α transcription following exogenous SUMO1 expression (Figures 2–5). These observations indicate that SUMO1 can affect binding of BRCA1 to the Gadd45α promoter, resulting in reduced recruitment of BRCA1 into the Gadd45α promoter and repression of transcription of Gadd45α gene.

SUMO1 represses BRCA1-induced transcriptional activity stimulated by DNA damage

BRCA1-induced transcriptional activity is enhanced by DNA damaging genotoxic treatment. We investigated whether SUMO1 repressed BRCA1-induced transcriptional activity in the presence of DNA damage. Consistent with previous reports (10,27), the level of Gadd45α transcription increased (~2.5- to 3.5-fold) upon γ-irradiation when compared to the level in non-treated cells in the absence or presence of BRCA1 expression (Figure 10A). Expression of BRCA1 further enhanced Gadd45α gene expression in γ-irradiated cells (~2.5-fold) (Figure 10A), consistent with the induction of Gadd45α transcription in the non-treated cells (~2.5-fold) (Figures 2–5). SUMO1 markedly reduced the activation of transcription of Gadd45α by BRCA1 in γ-irradiated cells (Figure 10A), which is consistent with the effect of SUMO1 in non-treated cells (Figures 2–5). These observations indicate that SUMO1 can repress BRCA1-induced transcriptional activity in the presence and absence of DNA damage.

ChIP assays were performed to analyze the association of BRCA1 and HDAC1 with the Gadd45α promoter in γ-irradiated cells (Figure 10B). The recruitment of BRCA1 and the release of SUMO1 were stimulated significantly upon γ-irradiation. Moreover, HDAC1 was released from the Gadd45α promoter following γ-irradiation (Figure 10B), possibly leading to histone acetylation and induced transcription. These findings further support the observation that the release of SUMO1 from the Gadd45α promoter takes place simultaneously with the recruitment of BRCA1 to the Gadd45α promoter, and correlates with Gadd45α expression.

DISCUSSION

In this study we describe a novel function of SUMO1 in the negative regulation of BRCA1-mediated

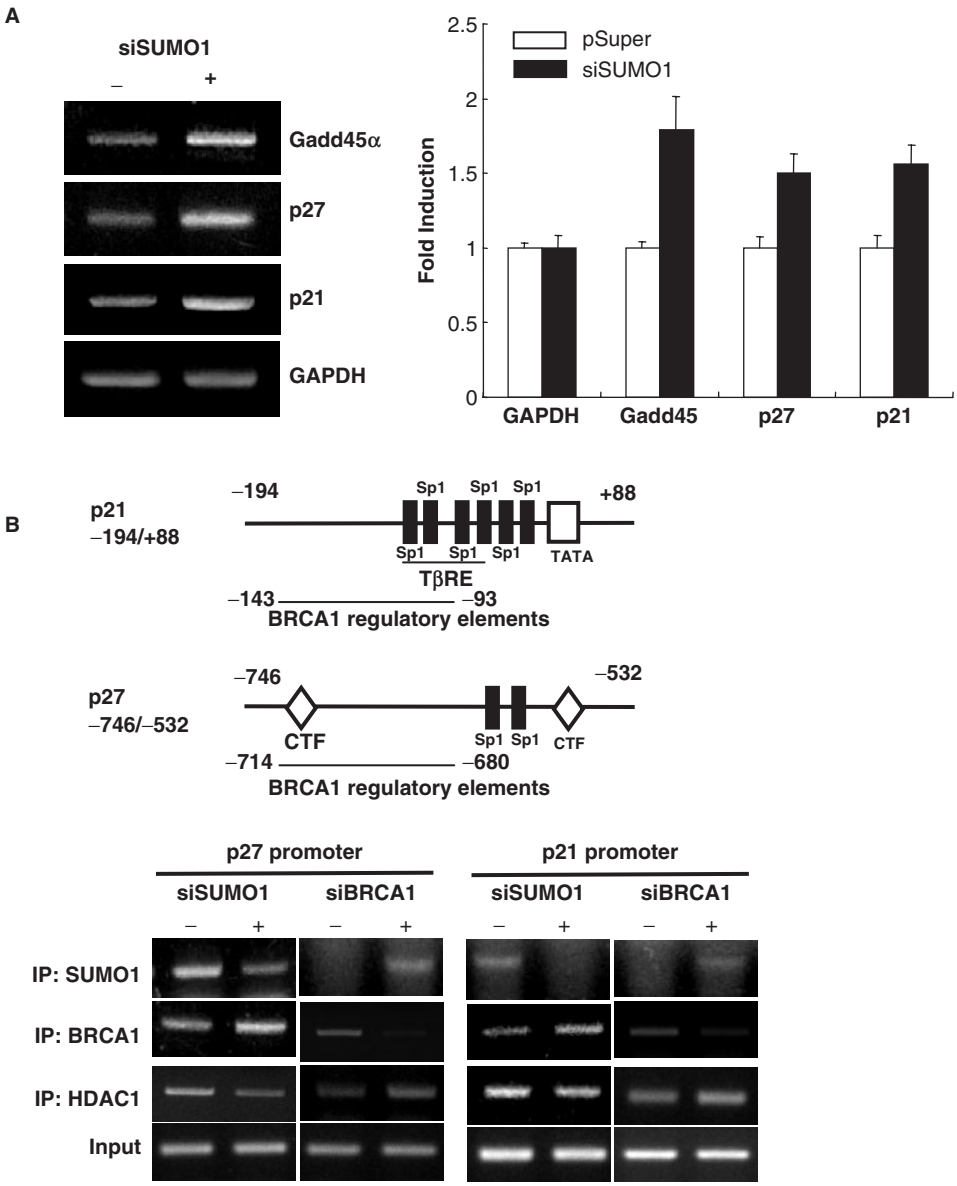
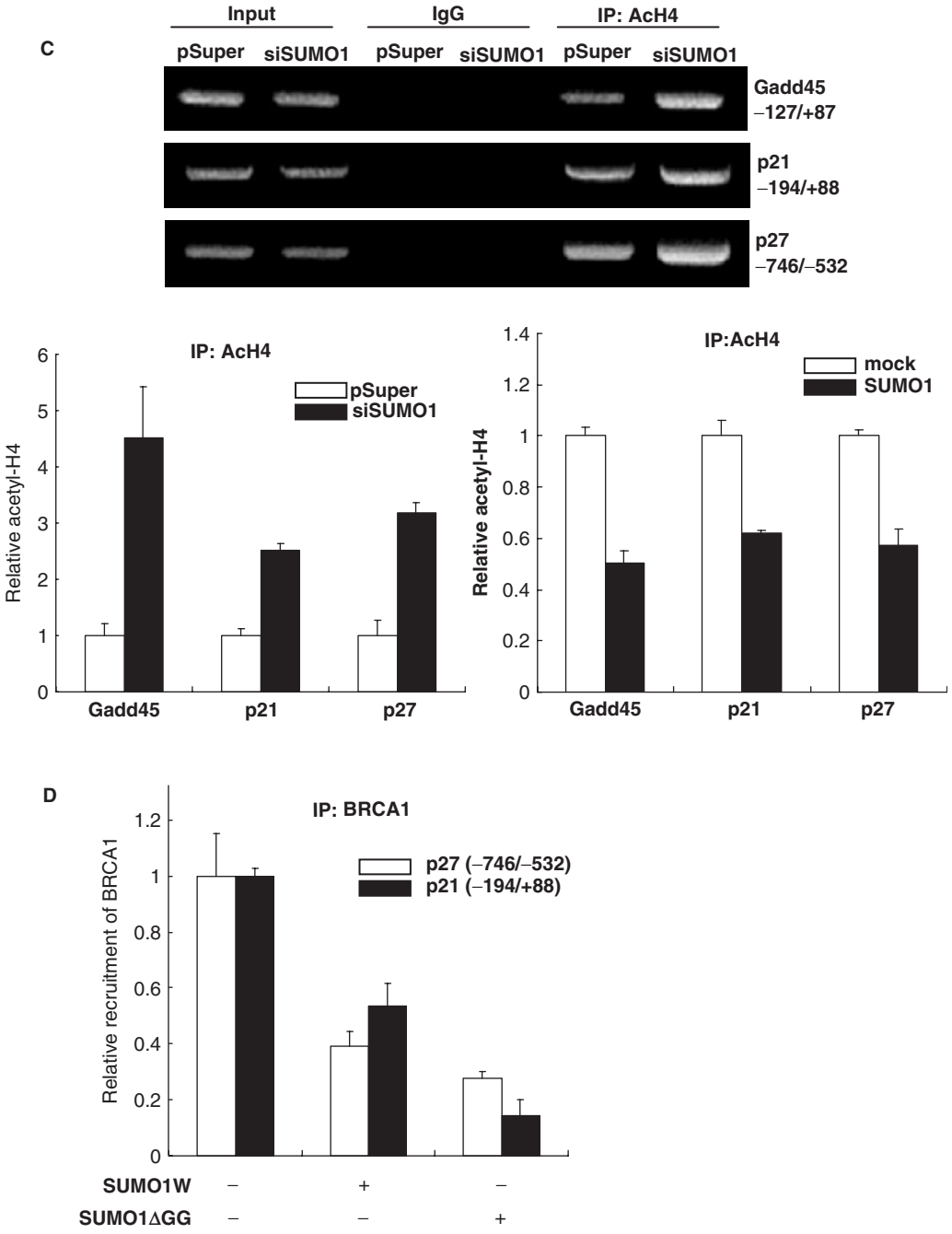


Figure 8. Silencing of SUMO1 enhances transcription of $p27^{KIP1}$ and $p21^{WAF1/CIP1}$ genes. (**A**) Transcription of $p27^{KIP1}$ and $p21^{WAF1/CIP1}$ genes was analyzed following silencing of SUMO1. Induction of $p27^{KIP1}$ (p27 mRNA) and $p21^{WAF1/CIP1}$ (p21 mRNA) expression by silencing of SUMO1 was analyzed by RT-PCR. A representative agarose gel (left) and real-time PCR (right) analyzing RT-PCR products for Gadd45α, $p27^{KIP1}$ and $p21^{WAF1/CIP1}$ using total RNA isolated from 293T cells transfected with pSUPER (−) or pSUPER-siSUMO1 (+) is shown with GAPDH as a quantitative control. (**B**) Effect of SUMO1 on the association of BRCA1 with target promoters of $p27^{KIP1}$ and $p21^{WAF1/CIP1}$ genes. Following silencing of SUMO1 (siSUMO1) or BRCA1 (siBRCA1), ChIP assays were carried out using anti-SUMO1 (IP: SUMO1), anti-BRCA1 (IP: BRCA1) or anti-HDAC1 (IP: HDAC1) antibodies. The pulled-down chromatin pools were then PCR or real-time PCR amplified with primers for promoters of $p27^{KIP1}$ (p27 promoter) and $p21^{WAF1/CIP1}$ (p21 promoter) genes. As a positive control, 0.01% of the total chromatin sample before immunoprecipitation (Input) was used for PCR amplification. (**C**) Effect of SUMO1 on the acetylation status of histones at the Gadd45α (Gadd45), $p27^{KIP1}$ (p27) and $p21^{WAF1/CIP1}$ (p21) promoters was monitored by chromatin immunoprecipitation (ChIP) with anti-acetyl H4 antibody (IP: AcH4). In 293T cells, ChIP assays were performed in the presence or absence of siRNAs against SUMO1 and ectopic expression of SUMO1. The endogenous Gadd45α promoter DNA that coprecipitated with the anti-acetyl H4 antibody was detected by PCR (top) or real-time PCR (bottom). All PCRs and real-time PCRs were normalized to the input control. The ChIP using real time PCR was performed and analyzed as described in Figure 5. (**D**) Effect of SUMO1ΔGG on the association of BRCA1 with target promoters of $p27^{KIP1}$ (white) and $p21^{WAF1/CIP1}$ (black) genes. ChIP analysis of the endogenous $p27^{KIP1}$ and $p21^{WAF1/CIP1}$ promoters in 293T cells was performed in the presence of transfected wild-type SUMO1 or its mutant derivative SUMO1ΔGG. The results were analyzed as described in (C). (**E**) No effect of SUMO1 on transcription and histone acetylation of GAPDH, ErbB2 and CyclinD1 promoters, which are not under the control of BRCA1. (Top) Transcription of GAPDH, ErbB2 and CyclinD1 genes was analyzed by real-time RT-PCR as described in (A). (Bottom) The acetylation status of histones at the GAPDH, ErbB2 and CyclinD1 promoters was analyzed as described in (C).



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transcription. We have provided several lines of experimental evidence to suggest that the BRCA1-induced transcriptional activity is inhibited by SUMO1 in a sumoylation-independent manner (Figure 10C). First, expression of SUMO1 led to the recruitment of HDAC1 to target promoters of BRCA1, the release of BRCA1, and the subsequent repression of transcription of the BRCA1 target genes, Gadd45α, $p27^{KIP1}$ and $p21^{WAF/CIP1}$. Second, silencing of SUMO1 led to transcriptional induction of the BRCA1 target genes, possibly via recruitment of BRCA1 and release of HDAC1 at the BRCA1 target promoters, thereby supporting a repressive role of SUMO1 in the control of BRCA1-induced transcription. Third, disruption of sumoylation-independent SUMO interaction motifs (SIMs) in BRCA1 alleviated interaction of BRCA1 with SUMO1 and consequently attenuated repressive effects of SUMO1 on BRCA1-mediated transcription, suggesting that noncovalent interaction between BRCA1 and SUMO1 is required for the SUMO1-induced repression. Fourth, UBC9, or the dominant-negative mutant DN UBC9, had little effect on the Gadd45α trascription. Also, dominant-negative SUMO1ΔGG, that is missing the SUMO1 donor site essential for sumoylation, maintained the capacity to

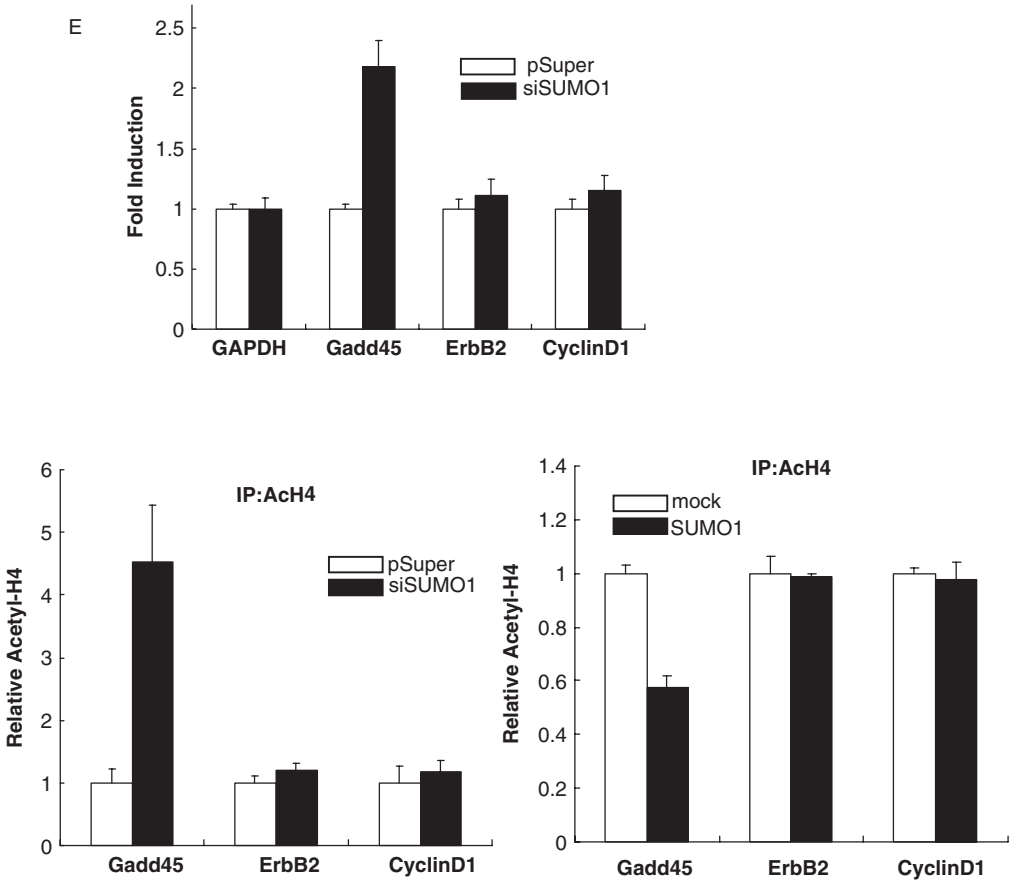

Figure 8. Continued.

repress BRCA1-mediated Gadd45α transcription, in a manner similar to that observed for wild-type SUMO1. This result provided support for the notion that sumoylation is not involved in the repression of BRCA1-induced transcription. In summary, these results strongly suggest that sumoylation is not closely linked to repression of BRCA1-induced transcriptional activation.

Transcriptional repression by SUMO1 is mostly mediated by the sumoylation of sequence-specific transcriptional factors, coactivators and corepressors, including STAT1, TCF, c-Jun, ARNT, CEBPα, c-myb, Sp3, IRF-1, SREBP, SRF, Elk, AP1/2, androgen receptor, progesterone receptor and Huntington (30). The majority of evidence has established the collaborative integration of sumoylation with histone deacetylation to achieve repression of transcription. The repression of BRCA1-mediated transcription by SUMO1 is different from the known mechanism of repression of transcription, as it does not involve sumoylation. Sumoylation-independent regulation by SUMO1 is observed in other cellular processes including Rad51-mediated homologous recombination (37) and the regulation of apoptosis mediated by ASK (38). In addition, sumoylation-independent regulation by other SUMO family members, SUMO2 and SUMO3, has been observed in the process activating androgen receptor-mediated transcription (39). Recently, a novel model is proposed that noncovalent SUMO interaction mediated by SUMO interaction motif (SIM) may represent a mechanism that could control many various pathways, including formation of PML nuclear body (34). These results, together with the present study, support sumoylation-independent physiological role of SUMO proteins. Despite the importance of SIMs in repression by SUMO1, SUMO1-mediated repressive activity was attenuated but remained in the presence of the double SIM BRCA1 mutant. This result suggests that there could be additional SIMs in BRCA1 (Supplementary Figure 4). Also, we cannot rule out the possibility that other transcriptional repressor(s) can bind to both SUMO1 and SIM-deleted BRCA1, and suppress partially BRCA1-mediated transcription in a BRCA1's SIM-independent way. Together, these findings provide evidence that the mechanisms controlling cellular processes regulated by SUMO1 and its paralogs, SUMO2 and 3, are diverse.

The present results suggest that HDAC1 but not 2 plays a role in the repression of BRCA1-mediated transcriptional activity by SUMO1 (Figure 10C). First, repression of BRCA1-mediated transcriptional activity by SUMO1 was TSA-sensitive. Second, SUMO1 overexpression enhanced histone deacetylation of the BRCA1 target promoters via the recruitment of HDAC1 but not 2. Conversely, the reduction of SUMO1 by siRNA led to a decrease in HDAC1 recruitment and histone deacetylation, and enhanced transcriptional activity of the BRCA1 target promoters. Third, the depletion of

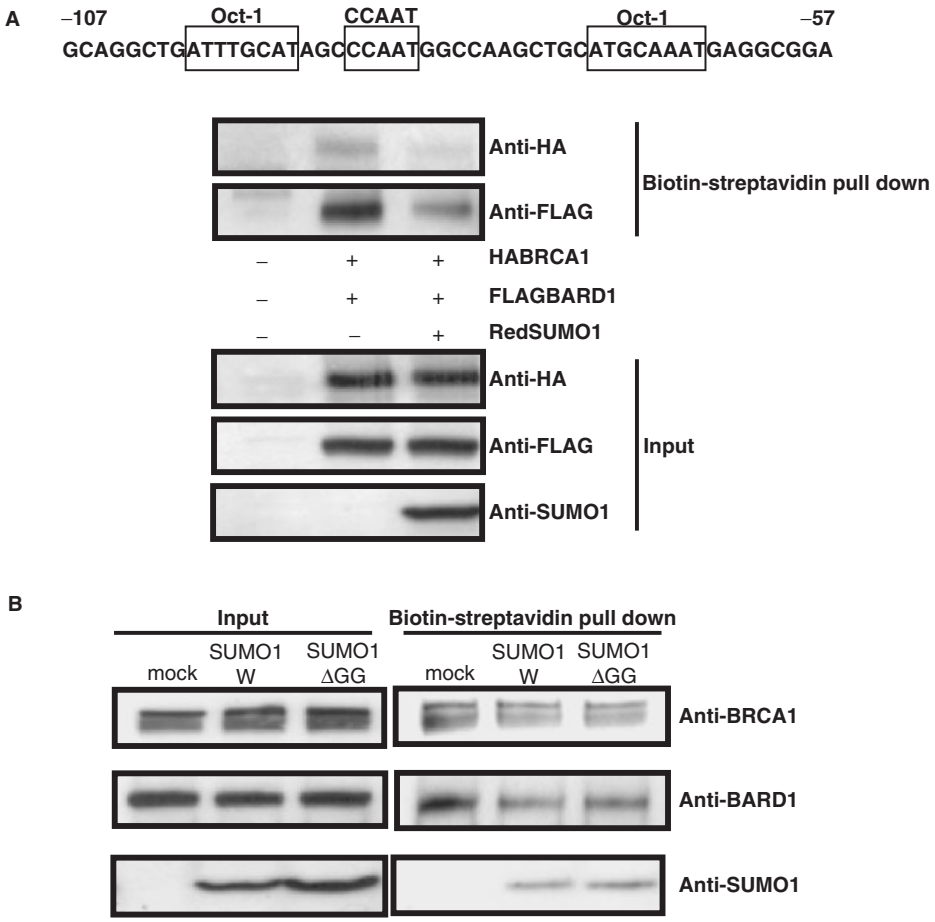

Figure 9. SUMO1 inhibits binding of BRCA1 to the Gadd45α promoter. (**A**) BRCA1-Gadd45α promoter-binding assay was performed with nuclear extract from cells transiently expressing HA-BRCA1, FLAG-BARD1 and/or SUMO1. Biotin-labeled Gadd45α promoter (−107 to −57) DNA was incubated with nuclear extract at room temperature in binding buffer. Following incubation, BRCA1 protein bound to biotin-labeled Gadd45α promoter DNA was isolated with streptavidin-agarose. The BRCA1–DNA–streptavidin–agarose complex was loaded onto a SDS gel. Proteins bound to DNA were detected by immunoblotting with anti-HA (for HA-BRCA1), anti-FLAG (for FLAG-BARD1) or anti-SUMO1 (for SUMO1) antibodies. The level of exogenous HA-BRCA1, FLAG-BARD1 and RFP-SUMO1 proteins were evaluated by immunoblotting with anti-HA, anti-FLAG and anti-SUMO1 antibodies, respectively. The representative figure was shown from seven separate experiments. (**B**) BRCA1-Gadd45α promoter-binding assay was performed as described in (A) with nuclear extract from cells transiently expressing either wild-type SUMO1 or its mutant derivative SUMO1ΔGG alone. Endogenous BRCA1 and BARD1 proteins or exogenous RFP-SUMO1 and RFP- SUMO1ΔGG bound to DNA were detected by immunoblotting with anti-BRCA1, anti-BARD1 and anti-SUMO1 antibodies, respectively. The representative figure was shown from three separate experiments.

HDAC1 abrogated the repressive effects of SUMO1 on BRCA1-mediated transcription. The results of the present study show that HDAC recruitment and subsequent histone deacetylation are common to the mechanism of sumoylation-independent repression and sumoylation-dependent transcriptional repression by SUMO1.

Although it is unclear that sumoylated transcriptional regulatory proteins are released from, or associated with promoters, growing evidence from investigations into sumoylation-dependent transcriptional repression suggest that SUMO1 is present at the repressed promoter. In the sumoylation of transcriptional proteins, the targeting of SUMO1 to a promoter can lead to the recruitment of other factors that repress transcription, including HDACs. The results of the present study for sumoylation-independent repression of BRCA1-induced transcriptional activity are consistent with the promoter occupancy of SUMO1 and HDAC1. Importantly, the presence of SUMO1 at the BRCA1 target promoters led to the release of BRCA1 from the promoters, suggesting that SUMO1 effectively reverses the promoter occupancy of BRCA1 in addition to HDAC1. Thus, sumoylation-independent repression of BRCA1-mediated transcriptional activity would affect the promoter occupancy of BRCA1 but not necessarily the associated sumoylated transcriptional proteins. Intriguingly, SUMO1 can downregulate DNA-binding activity of BRCA1, which subsequently results in the release of BRCA1 from the promoters. Taken together, those results from BRCA1-coimmunoprecipitation assays, ChIP analysis at the BRCA1 target promoters, and BRCA1–DNA-binding experiments point to a regulatory mechanism for BRCA1-mediated transcription, promoter occupancy through modulation of protein–DNA association but not protein–protein association by SUMO1.

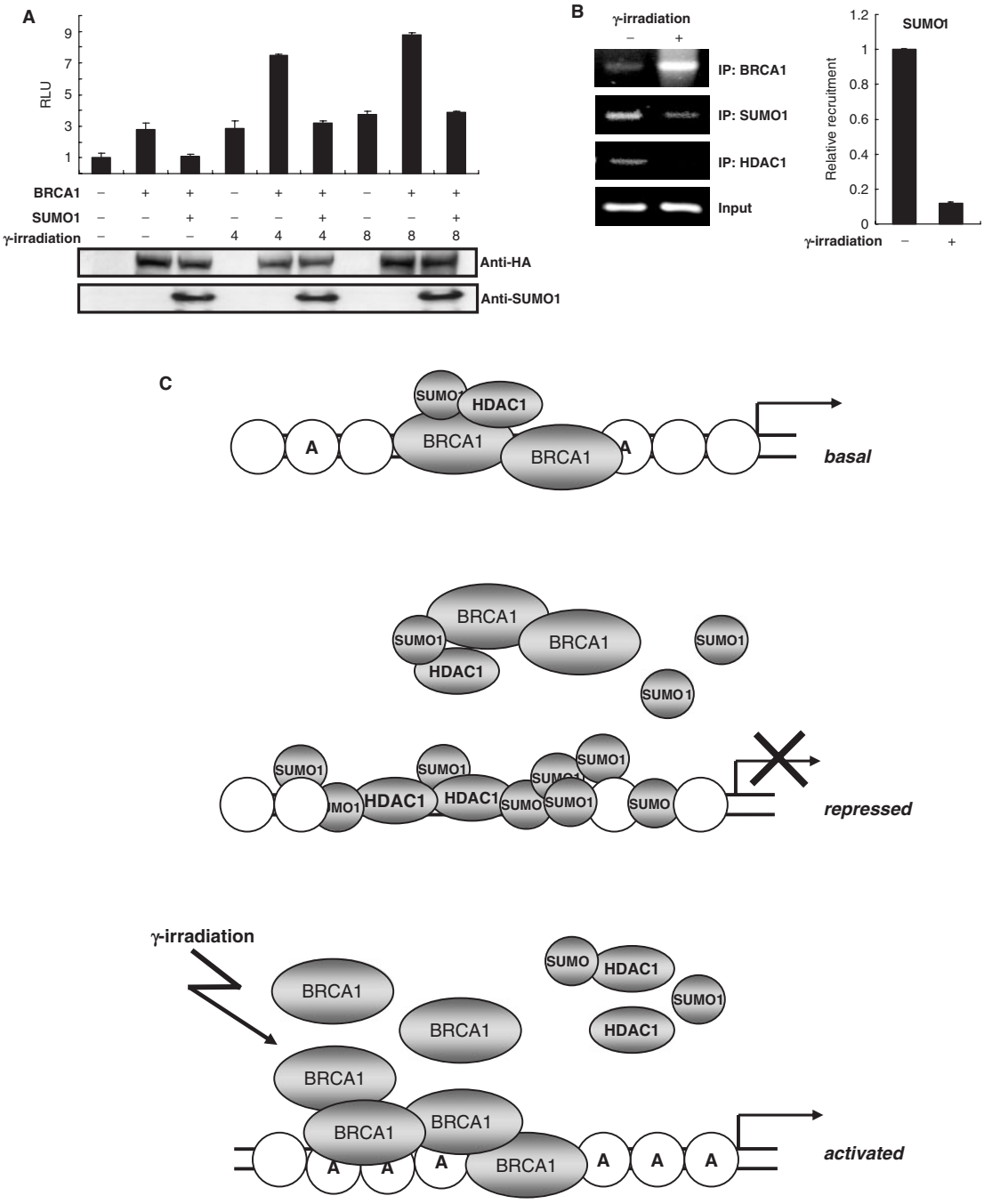

Figure 10. SUMO1 represses BRCA1-induced transcriptional activity following DNA damaging. (**A**) The activities of Gadd45α reporter gene was monitored upon γ-irradiation in U2OS cells cotransfected with the Gadd45α reporter together with constructs as indicated. One day after transfection, cells were treated with or without γ-irradiation at the indicated dose (4 or 8 Gy). Expression of HA-BRCA1 and SUMO1 was measured by immunoblotting with antibodies to HA and SUMO1. Data represent the mean ± SEM from four separate experiments ($^*P < 0.05$). (**B**) ChIP analysis of the endogenous Gadd45α promoter in 293T cells treated with (+) or without (−) γ-irradiation. (Left) Coprecipitated Gadd45α promoter DNA, by each indicated antibody, was detected by PCR. (Right) Release of SUMO1 from Gadd45α promoter was detected by real time ChIP analysis, in the presence of γ-irradiation. (**C**) A model of repression of BRCA1-mediated transcription by SUMO1. In repression, SUMO1 causes disassembly of BRCA1 and assembly of HDAC1 at BRCA1 target promoter. As a result, level of acetyl-histones is reduced. In activation, BRCA1 is recruited and associated with promoters. Also, SUMO1 and HDAC1 are released from the promoters. Subsequently, the level of acetyl-histones increases.

Depletion of endogenous SUMO1 caused enhanced BRCA1 and reduced HDAC1 presence at the BRCA1 target promoters, leading to transcriptional activation, while overexpression of SUMO1 or SUMO1ΔGG had the opposite effect. Moreover, the effect of SUMO1ΔGG was consistent with the effect of wild–type SUMO1 and both UBC9 and DN-UBC9 did not compromise the promoter occupancy and activation of transcription by BRCA1. These results further support a sumoylation-independent mechanism for the action of SUMO1 on

BRCA1-mediated activation of transcription. In contrast, depletion of endogenous BRCA1 promoted the recruitment of SUMO1 and HDAC1 to the BRCA1 target promoters, and overexpression of BRCA1 induced the release of SUMO1 and HDAC1 from the promoters. Collectively, these data are consistent with a model whereby SUMO1 recruits HDAC1 to the BRCA1 target promoter and induces the release of BRCA1, in a sumoylation-independent manner. It appears that recruitment of HDAC1 and the release of the transcriptional activator, BRCA1, are necessary for sumoylation-independent repression of transcription, whereas only recruitment of HDACs is required for sumoylation-dependent repression.

Promoter ChIP results for the BRCA1 target promoters suggest the importance of stoichiometry between BRCA1 and SUMO1 for promoter occupancy and regulation of transcription. This implies that BRCA1 and SUMO1 compete for promoter occupancy. This mechanism may explain the results for the interaction between BRCA1 and SUMO1 and their differential promoter occupancy. Initially, we observed an interaction between BRCA1 and SUMO1 in yeast with no homologous BRCA1 gene. However, coimmunoprecipitation experiments showed that the interaction in mammalian cells was weak, suggesting that the encounter between these proteins was transient, possibly as the proteins interacted at the promoter site.

DNA damage reversed the repression of BRCA1-mediated activation of transcription by SUMO1. Treatment with γ-irradiation led to the recruitment of BRCA1 to the Gadd45α promoter and the release of both SUMO1 and HDAC1, resulting in enhanced transcriptional activation by BRCA1. These results are consistent with the results generated from overexpression of BRCA1 or silencing SUMO1. Because DNA damage enhances the level of BRCA1, it is likely that an increased level of BRCA1 is recruited to the promoter due to DNA damage, possibly leading to the sequential release of SUMO1 and HDAC1. Taken together, these results further support a mechanism involving differential promoter occupancy and reverse transcriptional regulation by SUMO1 and BRCA1.

Our finding that BRCA1 domains with transactivational potential, possibly by association with transcriptional cofactors such as histone acetyl transferase p300/CBP, did not associate with SUMO1 or UBC9, suggest that repression of BRCA1-induced transcription by SUMO1 is not attributed to inhibition of the association with transcriptional coactivators in BRCA1. It appears that SUMO1 affects promoter occupancy by modulating the assembly and disassembly of proteins that regulate transcription and can remodel the protein context at a promoter by a sumoylation-independent mechanism that leads to repression of transcription. In addition, our data showing downregulation of BRCA1–DNA association by SUMO1 support the potential role of SUMO1 in modulation of protein complex at a promoter.

Mutations of the BRCA1 gene account for ~5% of breast and ovarian cancer cases, but the importance of BRCA1 in suppression of sporadic cancers may be far greater than what the frequency of BRCA1 mutations in sporadic cancers suggests. The level of BRCA1 is low in ~30% of sporadic breast cancers (40). Here, we have hypothesized that SUMO1 may serve as a negative regulator of BRCA1's tumor suppression functions in a part of the remainder sporadic breast cancers that express similar levels to normal breast tissues. To get insights into our suggestions that the negative regulator of BRCA1 is a bona fide SUMO1 activity and not an artifact of enforced expression of SUMO1, we have investigated whether SUMO1 level raises in physiological conditions in which activity of BRCA1 in tumor suppression has been compromised. We found that the level of SUMO1 protein is markedly increased in some breast, lung and ovarian cancer cell lines, compared with that in normal epithelial cells, despite comparable levels of BRCA1 protein (Supplementary Figure 5). However, γ-irradiation, a DNA damage signal, had little effect on induction of SUMO1 expression, in either normal or cancer cells. Consistently, SUMO1 mRNA level is high in cancer cells (data not shown). These results suggest that the level of SUMO1 might be rising in response to unknown carcinogen-induced stress or signal and subsequently attenuates BRCA1's activity. In carcinogenesis processes, SUMO1's function in downregulation of BRCA1 activity may have implications for development of sporadic breast and ovarian cancers without direct mutations or alterations in expression of BRCA1 gene.

BRCA1 exerts tumor suppression through multiple functions. Therefore, additional studies are required to evaluate whether and how SUMO1 antagonizes BRCA1 functions in maintaining genomic integrity via DNA repair, cell cycle checkpoint and other processes, besides its role in transcription. Due to rare mutations of BRCA1 in cancers, BRCA1 may be inactivated by alternative mechanisms. Therefore, we need to address the question whether SUMO1 can achieve functional inhibition of BRCA1 compromising the diverse roles of BRCA1 in tumor suppression.

In conclusion, we have examined the molecular mechanism underlying BRCA1-mediated activation of transcription. Although it is possible that a functional interplay between the SUMO system and HDAC1 plays a role in repression of transcription, the present study describes a novel sumoylation-independent mechanism whereby SUMO1 represses BRCA1-mediated activation of transcription via modulation of promoter occupancy.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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