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BRCA1 Expression Is Epigenetically Repressed in Sporadic Ovarian Cancer Cells by Overexpression of C-Terminal Binding Protein 2[1,2]

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Abstract

INTRODUCTION: Ovarian cancer is the leading cause of mortality from gynecological malignancy despite advancements in novel therapeutics. We have recently demonstrated that the transcriptional co-repressor C-terminal binding protein 2 (CtBP2) is overexpressed in epithelial ovarian carcinoma. *MATERIALS AND METHODS:* Reverse-transcribed cDNA from CtBP2 wild-type and knockdown ovarian cancer cell lines was hybridized to Affymetrix Gene 1.0 ST microarrays, and differentially expressed genes were studied. Immunohistochemical analysis of CtBP2 and BRCA1 staining of ovarian tissues was performed. Chromatin immunoprecipitation (ChIP) and luciferase assays were carried out. The effect of the drugs 4-methylthio-2-oxobutyric acid (MTOB) and poly(ADP-ribose) polymerase (PARP) inhibitor Olaparib on CtBP2 wild-type and knockdown cell lines was examined using methylthiazol tetrazolium assays and an xCELLigence System. *RESULTS:* Eighty-five genes involved in DNA repair, mitotic checkpoint, nucleosome assembly, and the BRCA1 network were differentially regulated by CtBP2 expression. ChIP and luciferase reporter assays using a *BRCA1* promoter–regulated luciferase construct indicated that the CtBP2 complex binds the *BRCA1* promoter and represses *BRCA1* transcription. Immunohistochemistry illustrated a significant inverse CtBP2 and BRCA1 expression in a panel of malignant ovarian tumor tissues. The CtBP2 inhibitor MTOB suppressed ovarian cancer cell survival in a CtBP2-dependent manner. Ovarian cancer cells with CtBP2 knockdown did not display increased sensitivity to the PARP inhibitor Olaparib. *CONCLUSION:* CtBP2 is an ovarian cancer oncogene that may play a significant role in epigenetically silencing BRCA1 function in sporadic epithelial ovarian cancer. CtBP2-specific inhibitors, such as MTOB, may be effective adjunct therapies in the management of patients with CtBP2-positive ovarian carcinoma.

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Introduction

Ovarian carcinoma has the highest fatality-to-case ratio of all gynecological malignancies. Thus, although ovarian cancer is the second most common gynecological malignancy, it accounts for the highest mortality rate among gynecological cancers. The majority of patients with epithelial serous cancer, the most common epithelial ovarian malignancy, present with metastatic disease, which has a 5-year survival rate of less than 25% and a 10-year survival rate approaching zero [1]. This alarming death rate is not only attributed to the advanced stage of disease at diagnosis but also to the lack of effective therapy for ovarian carcinoma. Therefore, what is urgently needed to impact survival in ovarian carcinoma are advances in development of disease-specific, targeted therapy. It is thus imperative to understand the biology of ovarian cancer and define the molecular events associated with malignant transformation and carcinogenesis.

C-terminal binding protein 2 (CtBP2) is a novel ovarian cancer tumor antigen that is overexpressed in most epithelial ovarian carcinoma [2,3]. Early studies suggested that CtBP2 may be a transcriptional co-repressor; however, recent data suggest that it may play a dual role in genetic transcription as it can act as both a transcriptional repressor and activator [4,5]. CtBP2 affects epithelial gene regulation and programmed cell death [6,7]. Survival analyses indicate worst survival in patients with positive CtBP2 expression [8]. Bergman and Blaydes demonstrated that CtBP proteins promote cell survival by suppressing the expression of several proapoptotic genes, thus acting as apoptotic transcriptional regulators [9]. Furthermore, CtBPs promote cell survival through the maintenance of mitotic fidelity [10]. Loss of CtBP expression suppresses cell proliferation through a combination of apoptosis, reduction in cell cycle progression, and aberrations in transit through mitosis [10].

Importantly, CtBP2 forms transcriptional complexes with more than 30 different transcriptional factors [2]. The CtBP2 complex regulates gene expression through epigenetic mechanisms. Epigenetic deregulations play a significant role in human cancer development. There are different mechanisms of epigenetic changes including acetylation and methylation of nucleosomal histones. CtBP2 protein is believed to be involved in chromatin remodeling and regulation of transcription programs in cancer cells through histone modification [4,11].

We have previously identified overexpression of CtBP2 in ovarian cancer and its function in regulating cell growth and chemoresponse [8,12]. Here, we designed a study aimed at examining the role of CtBP2 in epithelial ovarian carcinogenesis. Our objectives are to identify the gene expression profiles of ovarian cancer cells with knockdown of CtBP2, to highlight cellular pathways regulated or altered by CtBP2 in ovarian cancer, and to identify potential targeted therapy for ovarian carcinoma with elevated CtBP2 levels.

Materials and Methods

Establishment of Ovarian Cancer Cell Lines with Knockdown or Ectopic Expression of CtBP2

MCAS and SKOV3 ovarian cancer cells (1×10^5) were infected with 5×10^5 Mission lentiviral *CtBP2*-targeting shRNA and nontarget control shRNA transduction particles (Sigma-Aldrich, St Louis, MO) in the presence of 8 μg/ml hexadimethrine bromide (Sigma-Aldrich) and incubated overnight at 37°C and 5% CO_2. Puromycin (2 μg/ml) was added to the complete medium on the third day and changed every 3 days for 2 weeks. MCAS shRNA control cell lines were transfected with full-length *CtBP2* cDNA expression construct or empty vector (OriGene Technologies, Rockville, MD) using Lipofectamine 2000 transfection reagent (Invitrogen Corp, Carlsbad, CA). The transfected cells were selected using complete medium containing 500 μg/ml G418 (Invitrogen Corp). The cell lines were maintained in a 1:1 mixture of 199 medium and MCDB105 medium (Sigma, St Louis, MO) supplemented with 10% fetal calf serum (Invitrogen Corp). Knockdown of CtBP2 expression and stable clones with overexpression of CtBP2 were confirmed by Western blot analysis.

RNA Extraction and Gene Expression Profiling

RNA was extracted from SKOV3 and MCAS shRNA control and CtBP2 knockdown cell lines using TRIzol reagent (Invitrogen Corp). The quality and quantity of the RNA were tested using spectrophotometric analysis and Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA (1 μg) from each sample was used for gene expression profiling. cRNA was synthesized in a T7 polymerase–catalyzed reaction containing unmodified ribonucleoside triphosphates. cDNA was synthesized from the cRNA template using random primers and deoxyuridine triphosphate (dUTP), deoxythymidine triphosphate (dTTP), deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), and deoxyguanosine triphosphate (dGTP). Uracil DNA glycosylase and apurinic/apyrimidinic endonuclease I were used to fragment the cDNA at uracil-containing positions. The fragmented cDNA was end-labeled with a biotinylated nucleotide analog. The fragmented cDNA was added to a hybridization solution containing biotinylated control oligonucleotides and hybridized to the Human Gene 1.0 ST GeneChip microarrays overnight at 45°C. Unbound cDNA was then washed out using fluidics instrument and the remaining bound cDNA was fluorescently labeled using phycoerythrin-conjugated streptavidin. Additional fluorescents were added using biotinylated anti-streptavidin antibody and additional phycoerythrin-conjugated streptavidin. Each cDNA bound at its complementary oligonucleotide was excited using a confocal laser scanner and the positions and intensities of the fluorescent emissions were captured. The data were transferred to a file system ready for data analysis.

Gene Expression Profiling Data Analysis

Expression data in CEL file format were imported into R and Bioconductor software. All Affymetrix control genes were removed and the remaining Affymetrix probe clusters were imported into the Affymetrix Power Tools software (APT package). The data were normalized by robust multichip average (RMA) preprocessing followed by "per chip" normalization, i.e., data were normalized to the median of all probe sets on each chip, and lastly by "per gene" normalization, i.e., normalized to the median expression of each probe set across all samples. An analysis of variance with a Benjamini and Hochberg multiple testing correction of $P < .05$ was performed on the filtered data set to find differences between the wild-type shRNA control cell lines and the CtBP2 knockdown cell lines. To further look for differences between the various combinations of subtypes, *t* tests were performed with a Benjamini and Hochberg multiple testing correction cutoff of $P < .05$. Differentially expressed genes between the wild-type cell lines and the CtBP2 knockdown cell lines were imported into the Ingenuity Systems Pathway Analysis (www.ingenuity.com) and GeneGo MetaCore Pathway Analysis and were annotated with protein-protein interactions from the intact repository to identify differentially expressed networks. Twenty-seven differentially expressed genes were selected for validation studies using quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assays were performed on SKOV3 and MCAS ovarian cancer cells. The cells were grown in 199 and MCDB105 media (Sigma-Aldrich) supplemented with 10% fetal calf serum (Invitrogen Corp) and incubated at 37°C in a humidified 5% CO_2 incubator. Approximately 5×10^7 cells in suspension were cross-linked with 1% formaldehyde solution for 10 minutes at 37°C. The reaction was stopped with cold 1× phosphate-buffered saline (PBS) plus BSA. The cells were then washed with cold 1× PBS and then scraped into PBS plus complete protease inhibitors (Roche, Basel, Switzerland). They were lysed with lysis buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% sodium dodecyl sulfate (SDS)] supplemented with Complete protease inhibitors (Roche) for 10 minutes. The cells were then sonicated on a Vibra-Cell VCX 130 (Sonics, Newtown, CT) using a 3-mm tip with four 30-second pulses at 12% amplitude. Fractions of the clarified lysates were stored at −20°C as input samples. The remainder of the lysates were diluted with 10 volumes of ChIP dilution buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100] and incubated overnight at 4°C with various selected antibodies prebound to Dynal magnetic beads (Invitrogen Corp). The anti-CtBP2 antibody was from BD Biosciences (San Jose, CA). The anti–acetyl histone H3 (Lys9), anti–acetyl histone H3 (Lys14), and ChIPAb$^+$ dimethyl histone H3 (Lys9) antibodies were from EMD Millipore (Billerica, MA). The anti–polymerase II (Pol II) (N-20)X antibody was from Santa Cruz Biotechnology (Dallas, TX). The DNA-protein bead complexes were washed with RIPA buffer [50 mM Hepes (pH 7.6), 0.7% sodium deoxycholate, 1% NP-40, and 0.5 M lithium chloride] six times and then twice with TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. The DNA-protein cross-links were reversed in the ChIP and input samples by adding 100 μl of reverse buffer (1% SDS and 0.1 M $NaHCO_3$) and heating to 65°C for 6 hours. The ChIP-enriched DNA and input chromatin DNA were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol and stored at −20°C for further studies.

qRT-PCR and Quantitative Real-Time PCR

qRT-PCRs were performed to validate differentially expressed genes identified by gene expression profiling. Primer sets corresponding to the genes of interest were designed using the Primer Express Software (Applied Biosystems, Foster City, CA; Table W1). Quantitative real-time PCRs (qPCRs) were performed to quantify the gene-specific chromatin DNA obtained following ChIP assays and the corresponding input samples. Primer sequences corresponding to promoter sequences for *BRCA1* and *CDX1* ChIP qPCR were conducted as described by Di et al. and Joo et al. [13,14]. Twenty-microliter mixtures were prepared using the following components for each reaction: 10 μl of SYBER Green PCR Master Mix (Applied Biosystems), 1.6 μl of 5 μM forward and reverse primers, 1 μl of DNA template, and 7.4 μl of double distilled H_2O. The mixtures were placed in a 96-well plate (BrandTech Scientific, Essex, CT), and the reaction was carried out in a 7300 Real-Time PCR System (Applied Biosystems). A cycle threshold (C_T) value was recorded for each sample using a 7300 SDS software (Applied Biosystems). For qRT-PCR, the expression of each gene in each sample relative to the control was calculated using the $2^{-\Delta\Delta CT}$ method to relate the C_T values of gene expression to the C_T values for the housekeeping gene *cyclophilin A*. ChIP qPCR data were normalized against qPCR data of the input DNA. All qPCR reactions were repeated at least twice for mean values and SDs. Significance of differences in C_T values of the treatment group was determined using a two-tailed *t* test on testing whether the mean is different from the value of 1.0, which was set for controls. A difference was deemed significant when it reached the 5% level, i.e., $P \leq .05$.

Immunohistochemistry for BRCA1 and CtBP2 Expression Correlation Analysis

Immunohistochemistry (IHC) was performed on a panel of 48 archived formalin-fixed, paraffin-embedded malignant ovarian carcinoma tissues. The corresponding blocks were sectioned at a thickness of 7 μm, mounted on SuperFrost Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA), and dried at 50°C for 24 hours. Deparaffinization was achieved using xylene followed by rehydration with a descending series of ethanol solutions. Antigen retrieval was performed using antigen-unmasking solution (Vector Laboratories, Burlingame, CA) in a pressure cooker. Endogenous peroxidases were then blocked using 0.3% H_2O_2 in methanol for 15 minutes. Following a 30-minute blocking with the corresponding normal serum, sections were incubated with primary antibodies at 4°C overnight. The two primary antibodies used were CtBP2 mouse IgG1 antibody (BD Biosciences) and BRCA1 monoclonal mouse antibody (EMD Millipore). The reaction was visualized using VECTASTAIN Elite ABC Kit with DAB chromogen as a substrate (Vector Laboratories). Sections were lightly counterstained with hematoxylin and mounted. Immunohistochemical results were evaluated blindly by two trained investigators. Staining was graded semiquantitatively and the final histologic score was obtained by multiplying the proportion of the stained epithelial area (from 0 for absence to 3 for more than 95% of the total epithelial area) with the intensity of the stain (from 0 for no stain to 3 for highly intense stain). Pearson correlation analysis was performed to evaluate the correlation between BRCA1 and CtBP2 expression. A ceiling effect was observed for CtBP2 scores, and therefore, cases with highest CtBP2 scores of 9 were excluded in the analyses.

Luciferase Assay

Because our SKOV3 cells were integrated with an exogenous luciferase reporter gene, only the MCAS cell lines were used in this assay. The *BRCA1* promoter–firefly luciferase reporter plasmid has been described before [15]. MCAS cells with variable CtBP2 expression were co-transfected with this plasmid and a *Renilla* luciferase–encoding *pRL-CMV* plasmid (Promega, Madison, WI) as an internal control, using Lipofectamine 2000 reagent (Invitrogen Corp). Following a 48-hour incubation period, the cells were washed with 1× PBS and 1× passive lysis buffer (Promega) was added. Cells were lysed for 15 minutes at room temperature. The lysates were transferred to a microtube and were subjected to a single freeze-thaw cycle. The cell lysates were then centrifuged at 12,000*g* for 15 seconds and the supernatant was used for the assay. Firefly and *Renilla* luciferase activities of cell lysates were measured using the Dual Luciferase Assay System (Promega) in a Turner TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) according to the manufacturer's instruction. The relative firefly luciferase activity was normalized to *Renilla* luciferase activity for each cell line. The results were repeated three times and reported as means ± SEs. Significance of differences was determined using two-tailed *t* test. A difference was deemed significant when it reached the 5% level, i.e., $P \leq .05$.

In Vitro *Cell Survival Assessment Following Drug Treatment*

SKOV3 and MCAS ovarian cancer cells with various levels of CtBP2 expression were seeded in triplicates in 96-well plates and treated with

the poly(ADP-ribose) polymerase (PARP) inhibitor Olaparib (AZD221; Selleck, Houston, TX) in serial dilutions. After a 48-hour incubation period, 20 μl of 5 mg/ml methylthiazol tetrazolium solution was added to each well and incubated for 4 hours. The medium was removed and 100 μl of combined solubilization solutions (half of 10% SDS in 0.01 N HCl and half of 0.1 N HCl in 2-propanol) was added to each well. The plates were shaken to dissolve the blue formazan crystals and then read using a microplate reader at an absorbance of 562 nm. Ovarian cancer cells were also treated with serial dilutions of 4-methylthio-2-oxobutyric acid (MTOB; Sigma-Aldrich) and viability measured by methylthiazol tetrazolium assay. In addition, real-time cell viability was also determined for the MTOB treatments using an xCELLigence System (Roche Applied Science, Indianapolis, IN), which measured electrical impedance across interdigitated gold microelectrodes on the bottom of a tissue culture plate. The culture plate was assembled on the RTCA DP analyzer and data were gathered at 5-minute intervals for 10 hours at 37°C in 5% CO_2 chamber. The data obtained were analyzed using the RTCA software (Roche Applied Science).

Results

Establishment of Ovarian Cancer Cell Lines with Differential Expression of CtBP2

SKOV3 and MCAS ovarian cancer cell lines were successfully transduced with lentivirus containing control shRNA or *CtBP2* shRNA constructs yielding CtBP2 wild-type cell lines (SKOV3-CON and MCAS-CON) and CtBP2 knockdown cell lines (SKOV3-Sh2, SKOV-Sh3, and MCAS-Sh2; MCAS-Sh3 was lost during maintenance of cell lines and was reestablished only after the gene expression profiling experiment), respectively. We have also established MCAS ovarian cancer cell lines that stably harbored an exogenous copy of CtBP2 expression construct. Western blot analysis (Figure W1) showed that there was a specific reduction of CtBP2 expression, not the more ubiquitously expressing CtBP1 expression in the knockdown cell lines. The cell lines with exogenous copy of CtBP2 expression construct showed almost no increase of CtBP2 protein levels, even though qRT-PCR confirmed overexpression of the transcripts (data not shown). There has been report suggesting a post-transcriptional mechanism to maintain a threshold of CtBP2 protein level [16].

Gene Expression Profiling to Identify Genes Regulated by CtBP2

Normalized gene expression data over 30,000 genes in each of the SKOV3-CON, SKOV3-Sh2, SKOV3-Sh3, MCAS-CON, and MCAS-Sh2 cell lines were analyzed to yield an 85-gene expression signature (Figure W2), largely involved in mitotic checkpoint, nucleosome assembly, and DNA repair, to be differentially expressed between the CtBP2 wild-type and knockdown cell lines (Table W2). Pathway analyses using both GeneGo MetaCore and Ingenuity Pathway Analysis programs highlighted key functional networks regulated by CtBP2 including cell cycle regulation, DNA replication and repair, and chromatin assembly (Table W3). In particular, Ingenuity analysis highlighted an enrichment of differentially expressed genes including *BRCA1* in a DNA damage response pathway (Figure 1*A*). Selected differentially expressed genes were validated using qRT-PCR on cDNA from CtBP2 wild-type and knockdown cell lines (Figure W3).

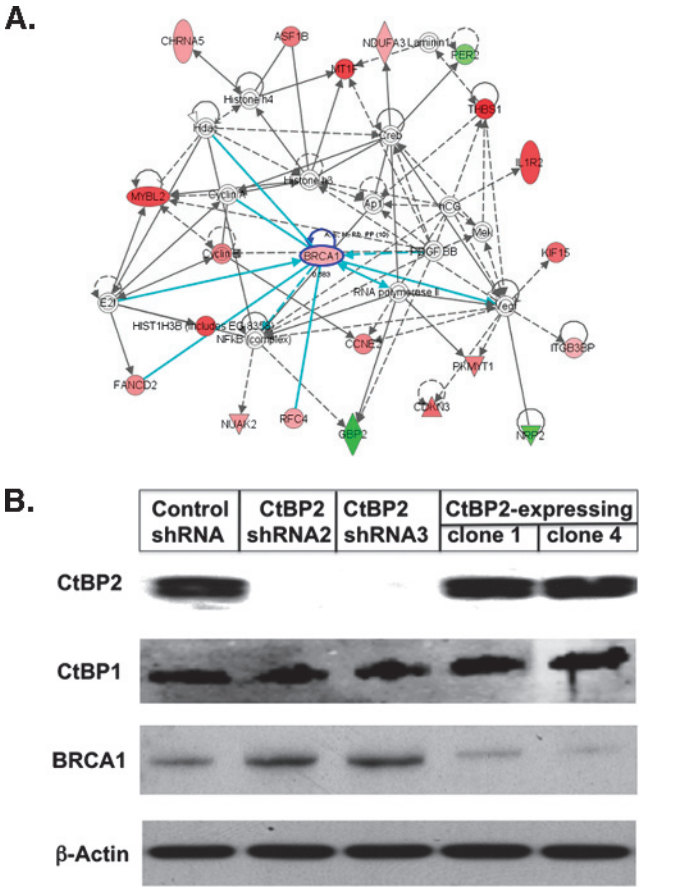

Figure 1. Gene expression profiling to identify differentially expressed gene signatures in the CtBP2 knockdown cell lines. (A) A novel BRCA1 DNA damage response pathway identified by Ingenuity Pathway Analysis program. (B) Western blot analysis to show that BRCA1 protein expression in the MCAS cells corresponds to cellular CtBP2 expression. β-Actin was used as a loading control.

CtBP2 Regulates BRCA1 Expression in Ovarian Cancer Cells

We were interested in the potential control of CtBP2 on BRCA1 expression in ovarian cancer cells. Western blot analysis showed that the CtBP2 knockdown cell lines had significantly increased levels of BRCA1 protein, whereas in ovarian cancer cells that stably harbored an exogenous copy of CtBP2 expression construct, there was significant repression of the BRCA1 expression (Figure 1*B*). To explore whether CtBP2 binds to *BRCA1* promoter and regulates *BRCA1* transcription in ovarian cancer cells, ChIP analysis was performed on the CtBP2 wild-type and knockdown cell lines using an anti-CtBP2 monoclonal antibody. qPCR on the ChIP DNA products using a *BRCA1* promoter primer set showed significant reduction of CtBP2 binding to *BRCA1* promoter in the CtBP2 knockdown cell lines compared to the CtBP2 wild-type cell lines (Figure 2*A*). As CtBP co-repressor complex was known to regulate gene expression through histone modifications at the bound sites, we evaluated the histone activation status at the *BRCA1* promoter in the ovarian cancer cell lines by employing the anti–acetyl histone H3 (Lys9) and anti–acetyl histone H3 (Lys14) antibodies in ChIP experiments on CtBP2 wild-type and knockdown cell lines. The results indicated that the knockdown cell lines had increased histone activation–associated acetylation at the *BRCA1* promoter (Figure 2, *B* and *C*). To confirm the results, anti–dimethyl histone H3 antibody was used in the ChIP assay and

the results showed that the knockdown cells had reduced level of suppression-associated histone methylation compared to the CtBP2 wild-type cell lines (Figure 2*D*). Lastly, use of an RNA Pol II–specific antibody in the ChIP assay also showed increased association of Pol II with the *BRCA1* promoter in the knockdown cell lines (Figure 2*E*).

To evaluate the *BRCA1* promoter activity in the cancer cell lines, transient transfection of a firefly luciferase cDNA plasmid under the control of the *BRCA1* promoter was performed with MCAS cell lines with, respectively, CtBP2 wild-type, CtBP2 knockdown, and expression of an exogenous CtBP2 expression construct. Results of the luciferase assays demonstrated that *BRCA1* promoter activity was inversely related to the expression level of CtBP2 in the ovarian cancer cell lines and corroborated Western blot analysis results (Figure 2*F*). Hence, all the results indicate that cellular *BRCA1* expression is negatively correlated with CtBP2 expression in ovarian cancer cells.

Di et al. have reported that estrogen induction resulted in CtBP loss from the *BRCA1* promoter and increased *BRCA1* transcription [13]. We investigated if estrogen stimulation could affect BRCA1 expression in ovarian cancer cells. Western blot analysis of cell lysates after estrogen stimulation did not result in changes of BRCA1 expression. Probing of the membrane for estrogen receptor expression showed that there was little to no expression of estrogen receptor in our panel of ovarian cancer cell lines (Figure W4).

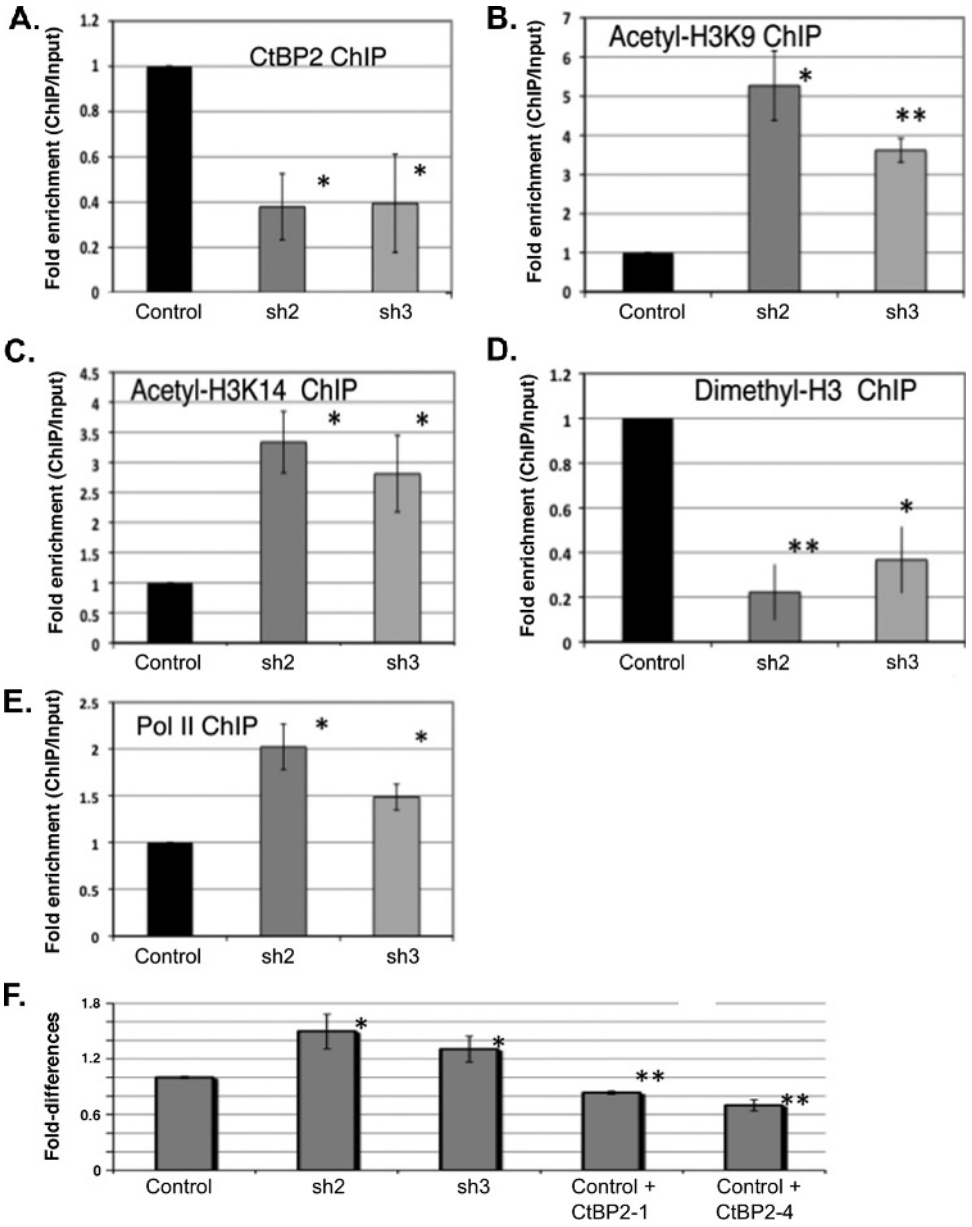

Figure 2. CtBP2 binds to *BRCA1* promoter and epigenetically represses *BRCA1* expression. (A) ChIP assay using anti-CtBP2 antibody. (B) ChIP assay using anti–acetyl histone H3K9 antibody. (C) ChIP assay using anti–acetyl histone H3K14 antibody. (D) ChIP assay using anti–dimethyl histone H3 antibody. (E) ChIP assay using anti–RNA Pol II antibody. All the ChIP data are shown relative to the controls (defined as 1). (F) Luciferase reporter assay to determine the *BRCA1* promoter activity. Results shown are averages of both MCAS and SKOV3 cancer cell lines and all significant values are relative to control. Significance of differences was determined using two-tailed *t* test. $*P < .01$; $**P < .05$.

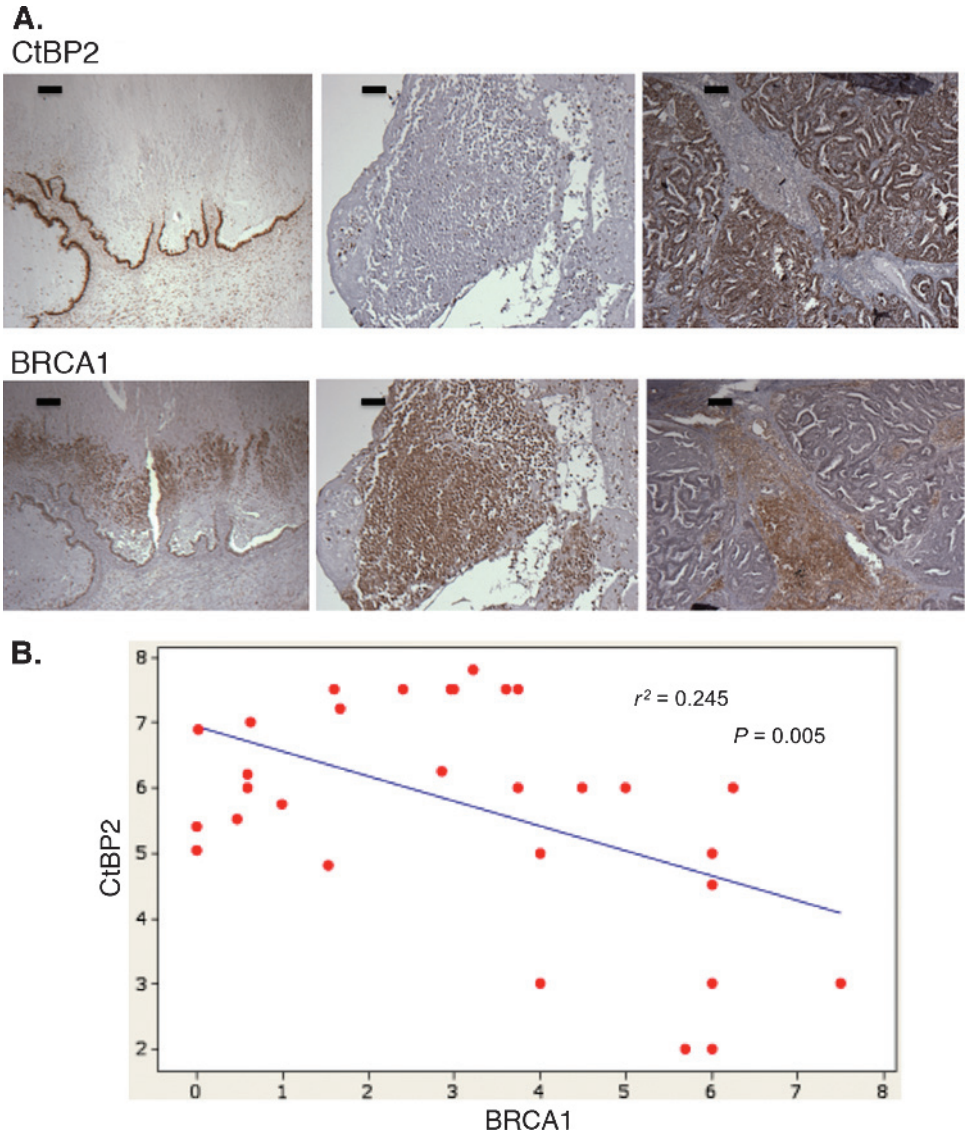

Figure 3. IHC to investigate the relationship between CtBP2 and BRCA1 protein expression in clinical ovarian tumors. (A) Representative images of malignant ovarian carcinomas that show negative association of CtBP2 and BRCA1 staining. Scale bar represents 50 μm. (B) A scatterplot to show the negative relationship between CtBP2 and BRCA1 protein staining.

Clinical Ovarian Tumors Showed Inverse Relationship between CtBP2 and BRCA1 Expression

To evaluate the clinical relevance of CtBP2 control on BRCA1 expression, we stained a panel of 48 malignant ovarian tumors for CtBP2 and BRCA1 proteins. Any tumor cases with known *BRCA1* mutations were excluded from this study. Forty-two malignant ovarian tumors stained strongly positive (score of 5 or above) for CtBP2, with 33 of them showing correspondingly weak or no BRCA1 signals (0 to 4). There were five samples that stained relatively weak (below 4) for CtBP2; four of them had BRCA1 scores higher than 5. Statistical analysis of the CtBP2 and BRCA1 staining in the clinical samples showed a significant Pearson correlation coefficient of −0.495 (r^2 = 0.245, P = .005). Representative IHC samples with this inverse expression relationship and a scatterplot of the data are shown in Figure 3. Hence, taken together, the *in vivo* data are consistent with the *in vitro* findings and support the notion of regulation of *BRCA1* expression by CtBP2 co-repressor complex in ovarian cancer cells.

Ovarian Cancer Cell Lines with Changes of CtBP2 Expression Showed Differential Response to CtBP2 Inhibitor but Similar Response to the PARP Inhibitor Olaparib

As patients with *BRCA* mutations showed positive response to DNA-damaging agents such as PARP inhibitors [17–19], MCAS cell lines with CtBP2 wild-type, knockdown, and an exogenous CtBP2 expression construct were treated with serial dilutions of the PARP inhibitor Olaparib. Cell survival at various Olaparib concentrations was similar among all the cell lines irrespective of the CtBP2 expression status (Figure 4*A*). Importantly, as CtBP2 is overexpressed in most of the malignant ovarian tumors and regulates BRCA1 function, CtBP2 may serve as an important target for chemotherapeutic treatment of ovarian cancer. As a pilot test to this hypothesis, we have treated the same cancer cell lines with MTOB, which inhibits CtBP2's dehydrogenase activity at high concentrations [20]. As shown in Figure 4*B*, even though MTOB exhibited modest cytotoxicity at low doses, the survival of cancer cell lines to MTOB was inversely related to cellular CtBP2 level at higher doses. We have also measured the MTOB

effects using the Roche xCELLigence System and similar results were obtained (Figure W5*A*). To evaluate if cellular BRCA1 level changes with the MTOB treatment, Western blot analysis of the cell lysates after MTOB treatments was performed. The results showed general down-regulation of BRCA1 levels with increasing doses of MTOB. The CtBP2-overexpressing line lost almost all the BRCA1 expression beginning with 1 mM MTOB, whereas cancer cell lines with CtBP2 knockdown maintained higher levels of BRCA1 (Figure W5*B*).

Discussion

In this study, ovarian cancer cell lines with knockdown of CtBP2 expression were established and gene expression profiling was performed. Gene signatures for the knockdown cell lines were identified and the various gene expression levels were compared to control cell lines with wild-type CtBP2 expression. This allowed for identification of critical pathways altered by suppressed CtBP2 expression in ovarian cancer. Cell cycle genes and DNA recombination and repair genes, including *BRCA1* and *Fanconi anemia, complementation group D2* (*FANCD2*) gene, were upregulated in CtBP2 knockdown cell lines compared to wild-type, control cell lines. This suggested that the CtBP2 co-repressor complex regulates *BRCA1* expression in ovarian cancer cells.

The breast and ovarian tumor suppressor BRCA1 is a well-recognized biologic agent in ovarian tumorigenesis. BRCA1 has important functions in cell cycle checkpoint control and DNA repair [21]. Mutations in the *BRCA1* gene can cause defective double-stranded DNA repair through homologous recombination [21]. Germ-line mutations in the *BRCA1* gene predispose females to ovarian cancer and account for approximately 8% of invasive epithelial ovarian carcinoma in the general population [22–24]. Notably, the BRCAness concept is an interesting trait found in many human cancers [25] including ovarian cancer [26,27]. Sporadic tumors exhibiting BRCAness behavior may exhibit deregulation of molecular pathways similar to those occurring in tumors with *BRCA* gene mutations. The inactivation of *BRCA1* in sporadic ovarian cancer may occur through a number of epigenetic mechanisms including aberrant methylation of the *BRCA1* promoter [28]. Cytosine methylation of the CpG islands in the *BRCA1* promoter leads to

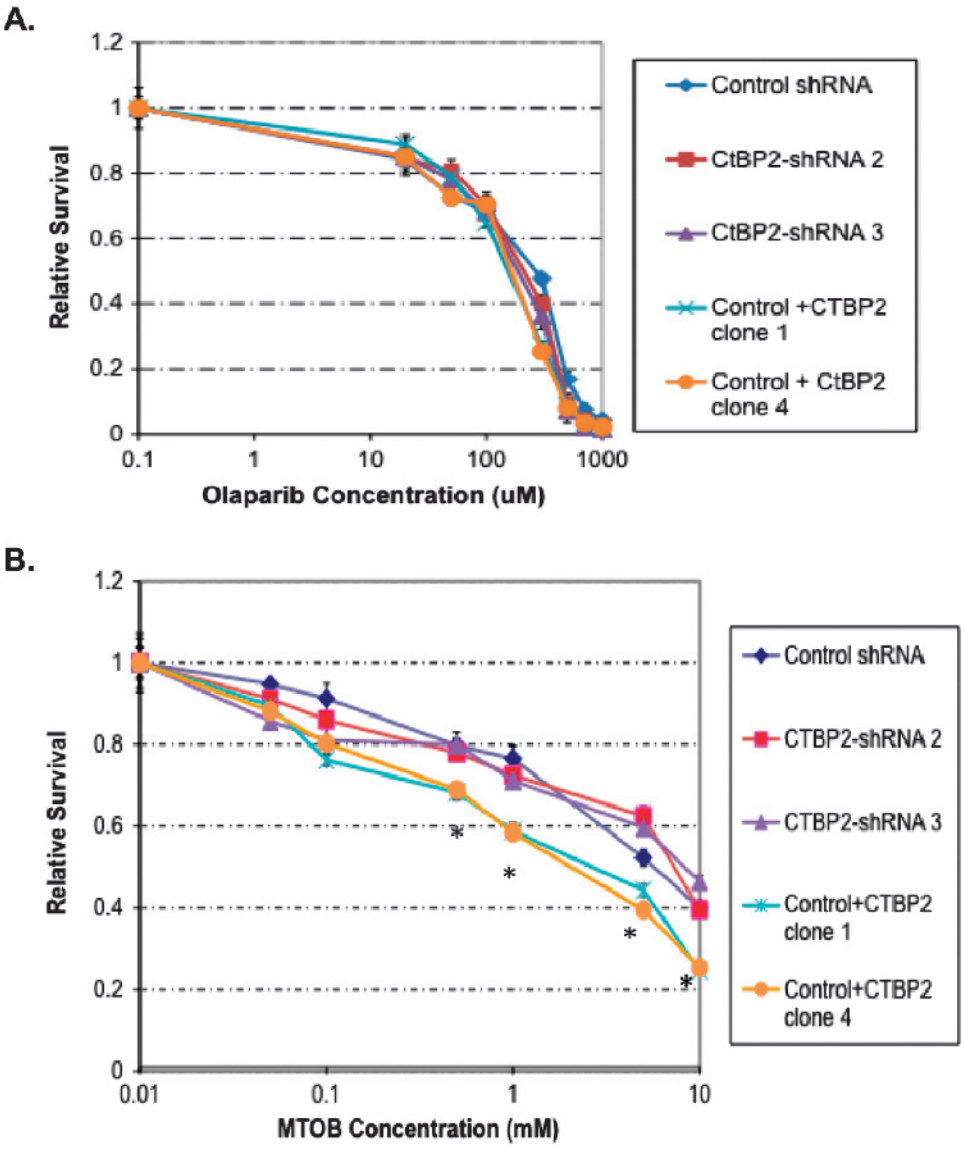

Figure 4. Sensitivity of ovarian cancer cell lines with changes of CtBP2 expression to (A) PARP inhibitor Olaparib and (B) CtBP2 inhibitor MTOB. *$P < .05$ relative to the control cell lines.

repressed expression and function of the BRCA1 protein. Ultimately, this leads to an ovarian cancer clinical presentation that appears similar to that encountered in *BRCA1* gene mutation carriers. Importantly, the most recent report of The Cancer Genome Atlas research network indicates that 8.5% of high-grade serous ovarian tumors have *BRCA1* germ-line mutations, 3% somatic *BRCA1* mutations, and 11.5% epigenetic *BRCA1* silencing due to DNA hypermethylation [29].

Our data show that knockdown of CtBP2 in ovarian cancer cells reverses the suppression of *BRCA1* expression. ChIP analyses using antibodies targeting CtBP2, RNA Pol II, and histone activation markers indicate that cell lines with CtBP2 knockdown have reduced binding of the CtBP2 co-repressor complex and increased activity of the *BRCA1* promoter compared to ovarian cancer cells with wild-type CtBP2 levels. These findings are further affirmed by the results of the luciferase assays using a *BRCA1* promoter–luciferase reporter construct. Hence, our data demonstrate that histone modification of the *BRCA1* promoter by the CtBP2 co-repressor complex may represent an important mechanism in *BRCA1* silencing in ovarian cancer cells. Moreover, IHC analysis of *BRCA1* and CtBP2 expression in malignant ovarian tumor tissues confirms the inverse association between BRCA1 and CtBP2 expression in clinical specimens and the clinical relevance of CtBP2 function. Di et al. showed that a "metabolic switch" by estrogen induction drove CtBP loss from the *BRCA1* promoter and increased *BRCA1* transcription, suggesting a molecular link between caloric intake and tumor suppressor expression in mammary cells [13]. To this end, we propose that this metabolic switch is disrupted in ovarian cancer, as we did not find changes of BRCA1 in response to estrogen stimulation (Figure W4). This lack of metabolic switch might be due to little or absence of estrogen receptor expression in the ovarian cancer cell lines.

The relation between CtBP proteins and chemoresistance in human cancers has been previously described [2,16]. Furthermore, ovarian cancer cells with *BRCA1* gene mutations have increased sensitivity to PARP inhibitors [17–19]. Our results indicate that ovarian cancer cells with differential CtBP2 expression and thus differential BRCA1 protein expression did not display a differential response to the PARP inhibitor Olaparib (Figure 4*A*). The most recent data of The Cancer Genome Atlas indicate that ovarian cancer patients with epigenetically silenced *BRCA1* exhibit treatment response and survival that is similar to patients with wild-type *BRCA* status and are worse than patients with *BRCA1* gene mutations [29]. As such, tumors with epigenetic *BRCA1* silencing appear to be phenotypically different from tumors with *BRCA1* gene mutations.

Our group has previously demonstrated that CtBP2 is overexpressed in ovarian carcinoma and down-regulation of CtBP2 impairs tumor growth and increases drug sensitivity [8,12]. Hence, targeting CtBP2 may have therapeutic implications in ovarian carcinoma. Straza et al. investigated the effect of the CtBP dehydrogenase inhibitor MTOB at high concentrations on human cancers [20]. This chemical was found to be cytotoxic to cancer cells and induced apoptosis in a p53-independent manner [20]. We tested the effect of MTOB treatment on ovarian cancer cell lines with differential CtBP2 expression. Indeed, MTOB suppressed cell survival in cell lines with CtBP2 overexpression compared to wild-type and CtBP2 knockdown cell lines. These results suggest that CtBP2 inhibition may provide a suitable therapeutic index for cancer therapy. Furthermore, CtBP2 inhibitors such as MTOB may represent effective targeted therapeutics for ovarian cancers with elevated CtBP2 levels. It is intriguing that BRCA1 proteins in general decreased with increasing doses of MTOB (Figure W5*B*). We hypothesize that the diminished BRCA1 protein levels reflect more the dynamics of cancer cell death, with the CtBP2-overexpressing cells showing the highest cell death and the greatest loss of BRCA1 expression, while the knockdown cell lines maintained higher levels of BRCA1 expression.

In conclusion, we propose that CtBP2 is an ovarian cancer oncogene that is elevated in the majority of epithelial ovarian cancers and represses *BRCA1* expression. Our data provide strong evidence that the CtBP2 co-repressor complex plays a significant role in the deregulation of *BRCA1* function in ovarian carcinomas, which can have significant clinical relevance. Moreover, as CtBP2 was found overexpressed in 83% of ovarian epithelial tumors [8,12] and it consistently downregulates the expression of many cell cycle and DNA repair genes in addition to *BRCA1* for accelerated cell growth, genetic instability, and tumorigenesis, this CtBP2-dependent epigenetic mechanism apparently plays a central role to affect a large number of clinical cases of sporadic ovarian carcinoma. Further studies employing ChIP coupled with genome tiling microarrays (ChIP-chip) and sequencing (ChIP-Seq) are warranted to delineate genome-wide *in vivo* binding sites and target genes regulated by CtBP2 complex. The CtBP2 inhibitor MTOB was lethal to ovarian cancer cells overexpressing CtBP2. Future studies may determine if the cytotoxic drug MTOB is an effective targeted therapy for patients with CtBP2-positive ovarian carcinoma.

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Table W1. Primer Sequences Used in the qRT-PCRs.

Gene	Forward Primer	Reverse Primer
ATAD2	5′-GCGGAGGAAAAGGAGTCGTAA-3′	5′-AACATCGGCAAGGCTTGCT-3′
ASF1B	5′-TATGTTGGCTCGGCTGAGAGT-3′	5′-CGTCGGCCTGAAAGACAAA-3′
BRCA1	5′-TGTGAAGGCCCTTTCTTCTG-3′	5′-TCAAGGAACCTGTCTCCACA-3′
CCL2	5′-TCTCGCCTCCAGCATGAAAGT-3′	5′-GCATTGATTGCATCTGGCTGA-3′
CCNE2	5′-AAAGCCTCAGGTTTGGAGTGG-3′	5′-TGTCCCCCTTTTCTGAAGGTG-3′
CENP1	5′-CAAGATGATGCACTGTGCCCT-3′	5′-GCATTCCCATTTTGGCCTG-3′
CDKN3	5′-CCATCATCATCCAATCGCAG-3′	5′-TCTCCCAAGTCCTCCATAGCA-3′
DIAPH3	5′-TGCCAAAAGATGTTCGGCAG-3′	5′-TTAAGCTCCTTGGCGACTGGA-3′
ESCO2	5′-TCATCGACGCTGGTCAGAAAC-3′	5′-TCCAGAAACCTGTGGTGATGC-3′
FANCD2	5′-AAGCAATGTATGCCGCTCCT-3′	5′-TTGGAATGCCCACACAGGT-3′
FBXO5	5′-CTGAGGTTGCCAAGACATTGAA-3′	5′-CAAATCCACAGCCTTCTCGTTT-3′
HIST1H3B	5′-AGACCGATCTTCGCTTCCAGA-3′	5′-TAGCATGGATGGCGCAAAG-3′
KIF15	5′-TGAGCCCAAGACCTTCACGTT-3′	5′-CGCTCATGCAAGACTCCACAA-3′
MCM8	5′-TGCCCTGGTACTTGGTGATCA-3′	5′-TACTTTGCTGCTCCATGGCTT-3′
MND1	5′-GTGATCCGCAAGTTGTGGAAG-3′	5′-GGCCCAAGATTTTATTGCGA-3′
MNS1	5′-ATGAAGCAGCTGGAACACAGG-3′	5′-AAATCCTTGCCGCCTTTGC-3′
MYBL2	5′-AGTTTGGACAGCAGGACTGGA-3′	5′-CCCCTTGACAAGGTCTGGATT-3′
NCAPG2	5′-CTGCACTTTGTTGGATTGCCT-3′	5′-AAGCTGTGTTGCTCTTGGCCT-3′
PKMYT1	5′-ACAGCAGCGGATGTGTTCAGT-3′	5′-TCCAGCATCATGACAAGGACA-3′
RAD54L	5′-TCAAAGCCTTTCAAAGTCCCC-3′	5′-GGCTCATACAGAACCAAGGCA-3′
RFC4	5′-AGCGGAGAGAACAAGAAAGCC-3′	5′-AGCACTGCAACCACTTCTTCC-3′
SGOL1	5′-GCCAAGGAAAGATGCCTGA-3′	5′-CCTGCGTTTGCCAATCTCT-3′
SPC24	5′-CCGTGTACGTGGCTCAACTTT-3′	5′-TCGCTGATGAATTTCCTGGAG-3′
SPC25	5′-TTGAAATCGGAAAGTTGGCG-3′	5′-CGAAAAGTGCCAGTTCGTCCT-3′
TAF9B	5′-ATAGCAACCCCACAAACGGTG-3′	5′-TGCAGGAACTGGTTTGACAGG-3′
THBS1	5′-CATCCAAAGCGTCTTCACCAG-3′	5′-TGGAACCATTCACCACGTTGT-3′
ZWINT	5′-GCCATCAAAATTGGCCTCAC-3′	5′-GCCATTTGTTTCTTGGCCTG-3′
Cyclophilin A	5′-CTGGACCCAACACAAATGGTT-3′	5′-CATGCCTTCTTTCACTTTGCC-3′

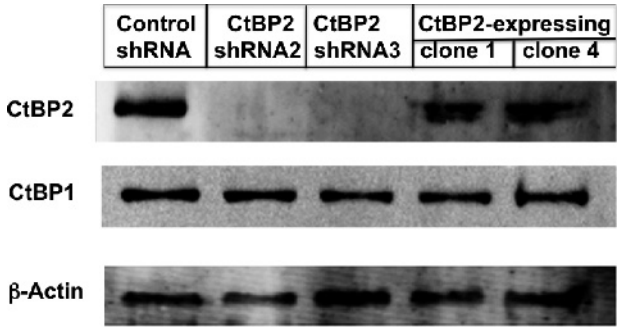

Figure W1. Expression of CtBP proteins in ovarian cancer cells. Western blot analysis to show the expression of CtBP1 and CtBP2 proteins in the MCAS cell lines harboring, respectively, CtBP2-targeting shRNA constructs or an extra copy of CtBP2 expression construct. β-Actin was used as a loading control.

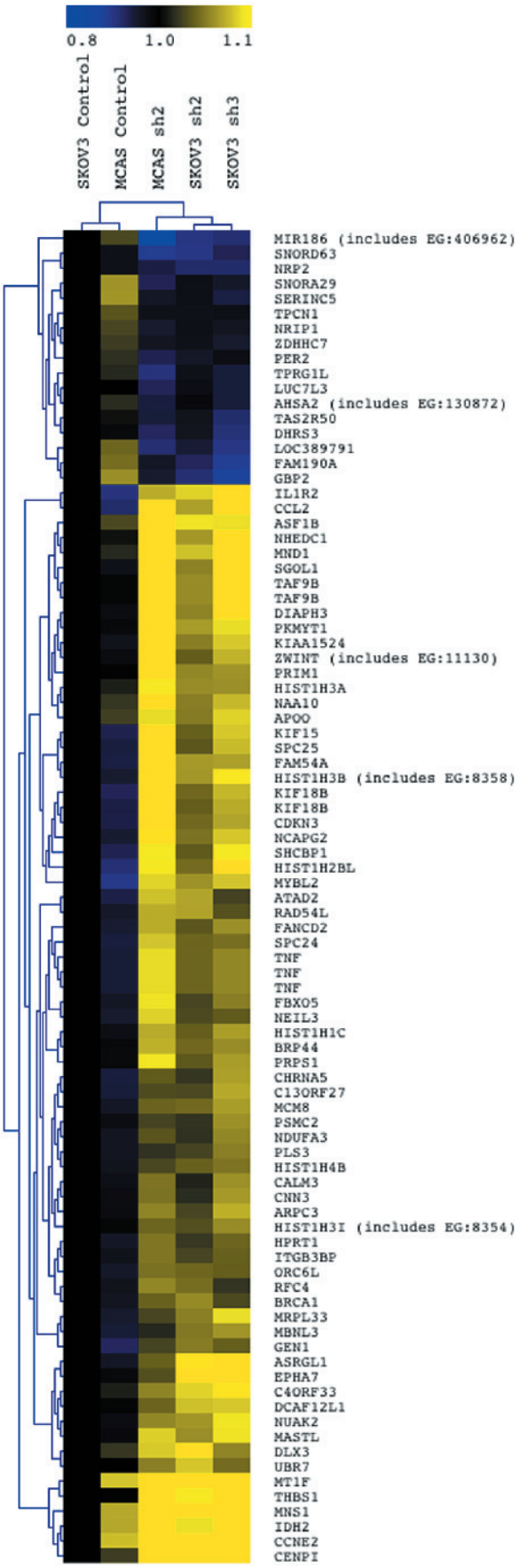

Figure W2. Heat map of the 85-gene expression signature in the control and CtBP2 knockdown MCAS and SKOV3 cell lines.

Table W2. The 85-Gene Signature for CtBP2 Function Identified by Gene Expression Profiling.

Log Ratio	ID	Symbol	Entrez Gene Name	Location	Type(s)
−0.501	8042195	*AHSA2*	*AHA1, activator of heat shock 90 kDa protein ATPase homolog 2 (yeast)*	Unknown	Other
0.658	8171823	*APOO*	*Apolipoprotein O*	Unknown	Other
0.655	7966315	*ARPC3*	*Actin-related protein 2/3 complex, subunit 3, 21 kDa*	Cytoplasm	Other
0.949	8034772	*ASF1B*	*ASF1 anti-silencing function 1 homolog B (S. cerevisiae)*	Nucleus	Other
0.831	7940643	*ASRGL1*	*Asparaginase-like 1*	Cytoplasm	Enzyme
0.879	8152668	*ATAD2*	*ATPase family, AAA domain containing 2*	Nucleus	Other
0.583	8015769	*BRCA1*	*Breast cancer 1, early onset*	Nucleus	Transcription regulator
0.606	7922095	*BRP44*	*Brain protein 44*	Plasma membrane	Other
0.618	7972674	*C13ORF27*	*Chromosome 13 open reading frame 27*	Unknown	Other
0.557	8097435	*C4ORF33*	*Chromosome 4 open reading frame 33*	Unknown	Other
0.630	8029831	*CALM3*	*Calmodulin 3 (phosphorylase kinase, delta)*	Plasma membrane	Other
1.300	8006433	*CCL2*	*Chemokine (C-C motif) ligand 2*	Extracellular space	Cytokine
0.863	8151871	*CCNE2*	*Cyclin E2*	Nucleus	Other
1.052	7974404	*CDKN3*	*Cyclin-dependent kinase inhibitor 3*	Nucleus	Phosphatase
1.274	8168794	*CENPI*	*Centromere protein I*	Nucleus	Other
0.683	7985213	*CHRNA5*	*Cholinergic receptor, nicotinic, alpha 5*	Plasma membrane	Transmembrane receptor
0.609	7917885	*CNN3*	*Calponin 3, acidic*	Cytoplasm	Other
0.527	8174972	*DCAF12L1*	*DDB1 and CUL4-associated factor 12–like 1*	Unknown	Other
−0.797	7912537	*DHRS3*	*Dehydrogenase/reductase (SDR family) member 3*	Cytoplasm	Enzyme
1.010	7971866	*DIAPH3*	*Diaphanous homolog 3 (Drosophila)*	Cytoplasm	Enzyme
0.510	8016609	*DLX3*	*Distal-less homeobox 3*	Nucleus	Transcription regulator
0.603	8128284	*EPHA7*	*EPH receptor A7*	Plasma membrane	Kinase
−0.761	8096425	*FAM190A*	*Family with sequence similarity 190, member A*	Unknown	Other
0.926	8129763	*FAM54A*	*Family with sequence similarity 54, member A*	Unknown	Other
0.800	8077731	*FANCD2*	*Fanconi anemia, complementation group D2*	Nucleus	Other
0.792	8130374	*FBXO5*	*F-box protein 5*	Nucleus	Enzyme
−0.903	7917530	*GBP2*	*Guanylate-binding protein 2, interferon-inducible*	Cytoplasm	Enzyme
0.745	8040440	*GEN1*	*Gen homolog 1, endonuclease (Drosophila)*	Unknown	Enzyme
0.709	8124397	*HIST1H1C*	*Histone cluster 1, H1c*	Nucleus	Other
1.053	8124510	*HIST1H2BL*	*Histone cluster 1, H2bl*	Nucleus	Other
0.575	8117330	*HIST1H3A*	*Histone cluster 1, H3a*	Unknown	Other
1.368	8124388	*HIST1H3B*	*Histone cluster 1, H3b*	Nucleus	Other
0.615	8124531	*HIST1H3I*	*Histone cluster 1, H3i*	Unknown	Other
0.519	8124385	*HIST1H4B*	*Histone cluster 1, H4b*	Nucleus	Other
0.640	8169984	*HPRT1*	*Hypoxanthine phosphoribosyltransferase 1*	Cytoplasm	Enzyme
0.927	7991374	*IDH2*	*Isocitrate dehydrogenase 2 (NADP+), mitochondrial*	Cytoplasm	Enzyme
1.395	8043981	*IL1R2*	*Interleukin-1 receptor, type II*	Plasma membrane	Transmembrane receptor
0.532	7916727	*ITGB3BP*	*Integrin beta 3 binding protein (beta 3-endonexin)*	Nucleus	Other
0.960	8089372	*KIAA1524*	*KIAA1524*	Cytoplasm	Other
1.066	8079237	*KIF15*	*Kinesin family member 15*	Nucleus	Other
1.037	8016147	*KIF18B*	*Kinesin family member 18B*	Unknown	Other
0.967	8016139	*KIF18B*	*Kinesin family member 18B*	Unknown	Other
−0.653	8158164	*LOC389791*	*Hypothetical LOC389791*	Unknown	Other
−0.507	8008493	*LUC7L3*	*LUC7-like 3 (S. cerevisiae)*	Nucleus	Other
0.872	7926821	*MASTL*	*Microtubule-associated serine/threonine kinase-like*	Unknown	Kinase
0.605	8175177	*MBNL3*	*Muscleblind-like 3 (Drosophila)*	Nucleus	Other
0.763	8060813	*MCM8*	*Minichromosome maintenance complex component 8*	Nucleus	Enzyme
−1.104	7916984	*MIR186*	*MicroRNA 186*	Unknown	MicroRNA
1.015	8097857	*MND1*	*Meiotic nuclear divisions 1 homolog (S. cerevisiae)*	Nucleus	Other
0.591	7989146	*MNS1*	*Meiosis-specific nuclear structural 1*	Unknown	Other
0.785	8041027	*MRPL33*	*Mitochondrial ribosomal protein L33*	Unknown	Other
1.444	7995825	*MT1F*	*Metallothionein 1F*	Cytoplasm	Other
1.406	8062766	*MYBL2*	*V-myb myeloblastosis viral oncogene homolog (avian)–like 2*	Nucleus	Transcription regulator
0.711	8175924	*NAA10*	*N(alpha)-acetyltransferase 10, NatA catalytic subunit*	Nucleus	Enzyme
0.948	8144153	*NCAPG2*	*Non-SMC condensin II complex, subunit G2*	Nucleus	Other
0.623	8031097	*NDUFA3*	*NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3, 9 kDa*	Cytoplasm	Enzyme
0.714	8098423	*NEIL3*	*Nei endonuclease VIII-like 3 (E. coli)*	Nucleus	Enzyme
0.657	8177130	*NHEDC1*	*Na^+/H^+ exchanger domain containing 1*	Unknown	Other
−0.598	8069553	*NRIP1*	*Nuclear receptor interacting protein 1*	Nucleus	Transcription regulator
−0.670	8047738	*NRP2*	*Neuropilin 2*	Plasma membrane	Kinase
0.701	7923753	*NUAK2*	*NUAK family, SNF1-like kinase, 2*	Unknown	Kinase
0.535	7995354	*ORC6L*	*Origin recognition complex, subunit 6 like (yeast)*	Nucleus	Other
−0.532	8059996	*PER2*	*Period homolog 2 (Drosophila)*	Nucleus	Other
0.895	7998886	*PKMYT1*	*Protein kinase, membrane-associated tyrosine/threonine 1*	Cytoplasm	Kinase
0.585	8169473	*PLS3*	*Plastin 3*	Cytoplasm	Other
0.770	7964271	*PRIM1*	*Primase, DNA, polypeptide 1 (49 kDa)*	Nucleus	Enzyme
0.698	8169240	*PRPS1*	*Phosphoribosyl pyrophosphate synthetase 1*	Unknown	Kinase
0.514	8135250	*PSMC2*	*Proteasome (prosome, macropain) 26S subunit, ATPase, 2*	Nucleus	Peptidase
0.665	7901192	*RAD54L*	*RAD54-like (S. cerevisiae)*	Nucleus	Enzyme
0.627	8092640	*RFC4*	*Replication factor C (activator 1) 4, 37 kDa*	Nucleus	Other
−0.814	8112865	*SERINC5*	*Serine incorporator 5*	Plasma membrane	Transporter
1.154	8085754	*SGOL1*	*Shugoshin-like 1 (S. pombe)*	Nucleus	Other
1.011	8001133	*SHCBP1*	*SHC SH2-domain binding protein 1*	Unknown	Other

Table W2. (*continued*)

Log Ratio	ID	Symbol	Entrez Gene Name	Location	Type(s)
−0.569	8130580	*SNORA29*	*Small nucleolar RNA, H/ACA box 29*	Unknown	Other
−0.530	8114468	*SNORD63*	*Small nucleolar RNA, C/D box 63*	Unknown	Other
0.743	8034122	*SPC24*	*SPC24, NDC80 kinetochore complex component, homolog* (*S. cerevisiae*)	Unknown	Other
0.931	8056572	*SPC25*	*SPC25, NDC80 kinetochore complex component, homolog* (*S. cerevisiae*)	Unknown	Other
1.058	8173732	*TAF9B*	*TAF9B RNA Pol II, TATA box binding protein* (*TBP*)*–associated factor, 31 kDa*	Nucleus	Transcription regulator
1.058	8176263	*TAF9B*	*TAF9B RNA Pol II, TATA box binding protein* (*TBP*)*–associated factor, 31 kDa*	Nucleus	Transcription regulator
−0.508	7961281	*TAS2R50*	*Taste receptor, type 2, member 50*	Unknown	Other
1.660	7982597	*THBS1*	*Thrombospondin 1*	Extracellular space	Other
0.873	8177983	*TNF*	*Tumor necrosis factor*	Extracellular space	Cytokine
0.873	8118142	*TNF*	*Tumor necrosis factor*	Extracellular space	Cytokine
0.873	8179263	*TNF*	*Tumor necrosis factor*	Extracellular space	Cytokine
−0.520	7958960	*TPCN1*	*Two pore segment channel 1*	Plasma membrane	ion channel
−0.724	7897172	*TPRG1L*	*Tumor protein p63 regulated 1-like*	Unknown	Other
0.507	7976336	*UBR7*	*Ubiquitin protein ligase E3 component n-recognin 7* (*putative*)	Unknown	Enzyme
−0.576	8003180	*ZDHHC7*	*Zinc finger, DHHC-type containing 7*	Cytoplasm	Enzyme
0.827	7933707	*ZWINT*	*ZW10 interactor*	Nucleus	Other

Table W3. Key Functional Networks of the 85-Gene Expression Signature Identified by GeneGo MetaCore (Top) and Ingenuity Pathway Analysis (Bottom) Programs.

GO Process	*P* Value
Cell cycle	4.03105E − 12
Cell cycle process	8.58197E − 12
Cell cycle phase	1.03412E − 10
M phase	1.55910E − 10
DNA packaging	1.84488E − 10
DNA conformation change	1.01005E − 09
Protein-DNA complex assembly	1.06819E − 08
Protein-DNA complex subunit organization	1.81620E − 08
Nucleosome assembly	8.91502E − 08
Chromatin assembly	1.16916E − 07

ID	Score	Focus Molecules	Top Functions
1	35	17	Cell cycle, cancer, decreased levels of albumin
2	28	14	Cell cycle, cellular assembly and organization, DNA replication, recombination, and repair
3	25	13	Cellular development, cell death, cell-to-cell signaling and interaction
4	22	12	Cell cycle, cancer, hematological disease
5	14	8	Digestive system development and function, lipid metabolism, molecular transport

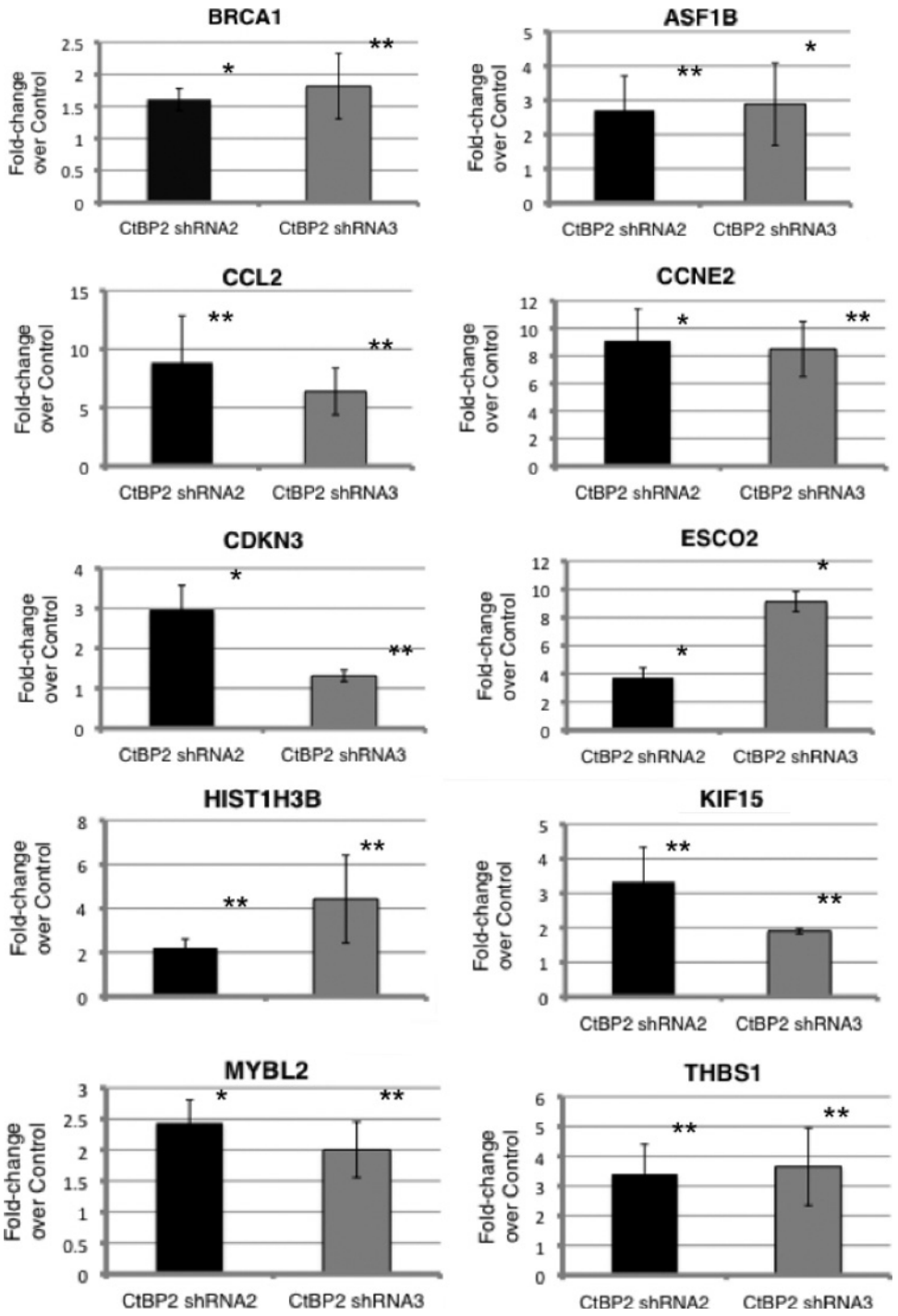

Figure W3. qRT-PCRs to verify the differential expression of identified genes of the *BRCA1* pathway in the control and CtBP2 knockdown cell lines. The expression in control cell lines is defined as 1. Significance of differences was determined using two-tailed *t* test. *$P < .01$; **$P < .05$.

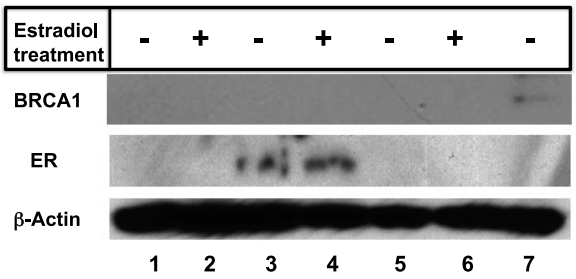

Figure W4. Estradiol treatment did not result in changes of BRCA1 level in ovarian cancer cells. Ovarian cancer cell lines MCAS (lanes 1 and 2), OVCA432 (lanes 3 and 4), and RMUGL (lanes 5 and 6) were treated with 100 nM estradiol for 30 minutes before harvesting for lysates and Western blot analysis. MCAS cancer cells with CtBP2 knockdown (lane 7) was used as reference. The estrogen receptor (ER) signal in lanes 3 and 4 was observed only after a long (1 hour) exposure.

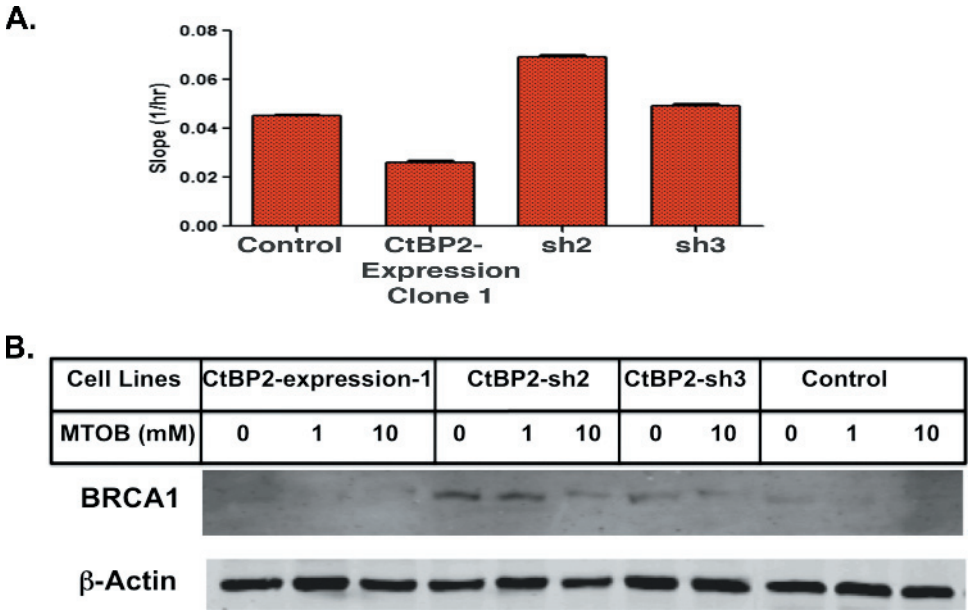

Figure W5. Sensitivity of ovarian cancer cell lines with changes of CtBP2 expression to CtBP2 inhibitor MTOB. (A) Measurement of cell viability using the xCELLigence System (Roche Applied Science). Cell viability kinetics are presented as linear slopes of the cell indices. (B) Western blot analysis of cell lysates for BRCA1 expression after MTOB treatment.