NEOPLASIA
www.neoplasia.com

Volume 14 Number 10 October 2012 pp. 905–914 905

Role of Transcriptional Corepressor CtBP1 in Prostate Cancer Progression[1,2]

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Abstract

Transcriptional repressors and corepressors play a critical role in cellular homeostasis and are frequently altered in cancer. C-terminal binding protein 1 (CtBP1), a transcriptional corepressor that regulates the expression of tumor suppressors and genes involved in cell death, is known to play a role in multiple cancers. In this study, we observed the overexpression and mislocalization of CtBP1 in metastatic prostate cancer and demonstrated the functional significance of CtBP1 in prostate cancer progression. Transient and stable knockdown of CtBP1 in prostate cancer cells inhibited their proliferation and invasion. Expression profiling studies of prostate cancer cell lines revealed that multiple

Abbreviations: PCa, prostate cancer; CtBP1, C-terminal binding protein 1; HDAC, histone deacetylase; siRNA, small interfering RNA

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[1]Research reported in this publication was supported in part by grants from the Department of Defense to A.M.C. (PC020322) and the National Cancer Institute of the National Institutes of Health to S.V. (R01CA154980). S.V. is also supported by a grant from the National Cancer Institute of the National Institutes of Health (R01CA157845). This work was also supported in part by the Prostate Cancer Foundation (A.M.C.), NIH Prostate Specialized Program of Research Excellence P50CA69568, and Early Detection Research Network UO1 CA111275 (A.M.C.). A.M.C. is also supported by the Doris Duke Charitable Foundation Clinical Scientist Award and the Howard Hughes Medical Institute. A.M.C. is an American Cancer Society Research Professor and a Taubman Scholar of the University of Michigan.
[2]This article refers to supplementary materials, which are designated by Table W1 and Figures W1 to W4 and are available online at www.neoplasia.com.
[3]Share senior authorship.
Received 23 July 2012; Revised 22 August 2012; Accepted 24 August 2012

DOI 10.1593/neo.121192

tumor suppressor genes are repressed by CtBP1. Furthermore, our studies indicate a role for CtBP1 in conferring radiation resistance to prostate cancer cell lines. *In vivo* studies using chicken chorioallantoic membrane assay, xenograft studies, and murine metastasis models suggested a role for CtBP1 in prostate tumor growth and metastasis. Taken together, our studies demonstrated that dysregulated expression of CtBP1 plays an important role in prostate cancer progression and may serve as a viable therapeutic target.

Neoplasia (2012) 14, 905–914

Introduction

Transcriptional corepressor C-terminal binding protein 1 (CtBP1) is known to play a crucial role in cellular homeostasis by regulating the expression of numerous genes [1]. CtBP1 binds to and modulates the activities of several transcription factors such as BKLF, FOG 1 and 2, ZEB, EVI-1, and Zinc finger protein IKAROS among others [2]. DNA-binding proteins recruit CtBP1 through the PLDLS motif, originally identified in adenovirus early region 1A (E1A) protein [2,3]. Studies have shown that CtBP1 has NAD-dependent dehydrogenase activity and forms repressive complexes with other proteins [4,5]. The enzymatic function of CtBP1 makes it an attractive therapeutic target. Whereas the precise mechanism of CtBP1-mediated transcriptional repression is unclear, studies suggest that it may involve histone deacetylases (HDACs) that remove acetyl groups from histone tails, enabling chromatin condensation and repression of gene expression [6,7]. CtBP1 has also been shown to interact with the polycomb group (PcG) transcriptional repressor HPC2 [8]. In *Drosophila*, CtBP was reported to regulate the expression of intergenic transcripts that regulate DNA binding by PcG proteins [9], providing a link between CtBP1 and PcG member histone methyltransferase EZH2, which is overexpressed in a wide variety of aggressive tumors including prostate cancer [10]. A considerable amount of evidence suggests a critical role for CtBP1 in tumor growth and epithelial-mesenchymal transition [11]. One well-characterized target of CtBP1-mediated transcriptional repression is the tumor suppressor and cell adhesion molecule E-cadherin [12–15]. In myeloid leukemia cells, EVI-1–mediated transformation is abrogated when its CtBP1 binding motifs are mutated, implicating a role for CtBP1 in promoting oncogenesis in these cancers [16]. In breast cancer, CtBP1 suppresses apoptosis and promotes cell cycle progression [17], and a recent study indicated that most of the invasive ductal breast cancer cases were CtBP1-positive compared to normal breast tissue [18]. Furthermore, in pituitary tumor cells, knockdown of CtBP1 resulted in reduced cell proliferation [19]. Whereas these studies suggest an oncogenic role for CtBP1, a detailed molecular mechanism of CtBP1-mediated tumorigenesis has not been explored in prostate cancer.

In the present study, we validated the overexpression of CtBP1 and characterized its role in prostate cancer progression. Gene expression studies using RNA from CtBP1-modulated prostate cell lines identified targets of CtBP1-mediated repression. Knockdown studies demonstrated that CtBP1 expression is essential for prostate cancer cell growth and proliferation as well as reactivation of the target tumor suppressors. Removal of CtBP1 reduced cell survival and sensitized aggressive prostate cancer cells to radiation. Importantly, our *in vivo* studies uncovered a critical role for CtBP1 in prostate cancer growth and tumor metastasis. Overall, our investigations indicate that CtBP1 plays an essential role in prostate cancer progression and warrants consideration as a valuable therapeutic target.

Materials and Methods

Expression Analysis

CtBP1 gene expression data were procured from cDNA microarray analysis [20]. To measure the CtBP1 transcript levels, total RNA was isolated from prostate cell lines and prostate tissue samples using the RNeasy Mini Kit (Qiagen, Valencia, CA). Quantitative polymerase chain reaction (qPCR) was performed as described [21]. All primers were synthesized by Integrated DNA Technologies, Coralville, IA. PCR reactions were performed in triplicates. Primer sequences used in the present study include CtBP1: F, TCACAGGCCGGATCCCAGACAG and R, GGTACCTATAGGCAGCCCCATTGAGC and F, CCGTCAAGCAGATGAGACAA and R, GGCTAAAGCTGAAGGGTTCC; E cadherin: F, GGAGGAGAGCGGTGGTCAAA and R, TGTGCAGCTGGCTCAAGTCAA; ARHGDIB: F, ACAGGACTGGGGTGAAAGTG and R, GAGCCTCCTCAACTGGAGTG; LCN2: F, CAAGGAGCTGACTTCGGAAC and R, TACACTGGTCGATTGGGACA.

For immunoblot analysis, 10 μg of normal and prostate cancer tissues as well as prostate cancer cell line lysates was boiled in sample buffer, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ). The membrane was incubated for 1 hour in blocking buffer (TBS, 0.1% Tween, 5% nonfat dry milk) and incubated overnight at 4°C with respective primary antibodies, and signals were visualized after incubating with secondary antibody conjugated with HRP. Densitometric scan of the immunoblot was performed using ImageJ. The following antibodies and dilutions were used for the immunoblots: anti-CtBP1 (1:2000 in blocking buffer, BD Biosciences [San Jose, CA], Cat. No. 612042,), anti-LCN2 (1:10,000, R&D Systems [Minneapolis, MN], Cat. No. AF1757), phospho-H2AX (1:2000, Millipore [Billerica, MA], Cat. No. 16-202A), anti–β-actin mouse monoclonal antibody (1:20,000, Sigma [St Louis, MO], Cat. No. A5316-500ul), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:5000, Abcam [Cambridge, MA], Cat. No. ab8245), and β-tubulin (1:2000, Santa Cruz Biotechnology [Santa Cruz, CA], sc-9104).

Immunohistochemistry

Benign and prostate cancer tissues were obtained from the radical prostatectomy series at the University of Michigan and from the Rapid Autopsy Program, both part of the Michigan Prostate SPORE Tissue Core. Institutional Review Board approval was obtained to procure and analyze the tissues used in this study. Immunohistochemistry was carried out using standard biotin-avidin complex

to evaluate CtBP1 expression using mouse monoclonal antibody against CtBP1 (BD Biosciences) as well as a rabbit polyclonal antibody [22].

RNA Interference

Small interfering RNA (siRNA) duplexes for RNA interference of CtBP1 was purchased from Dharmacon, Lafayette, CO (Thermo Scientific, Cat. No. LQ-008609-00-0002). Short hairpin RNA (shRNA) constructs were generated using pGreen-puro vector for two of the most efficient siRNA duplexes by SBI (System Biosciences, Mountain View, CA). Lentivirus for the stable knockdown of CtBP1 was generated by the University of Michigan Vector Core. To perform the siRNA knockdown, we plated prostate cancer cell lines DU145, PC3, and LNCaP at 2×10^5 cells per well in a 6-well plate for immunoblot analysis and cell proliferation analysis and at 1.5×10^3 cells per well in a

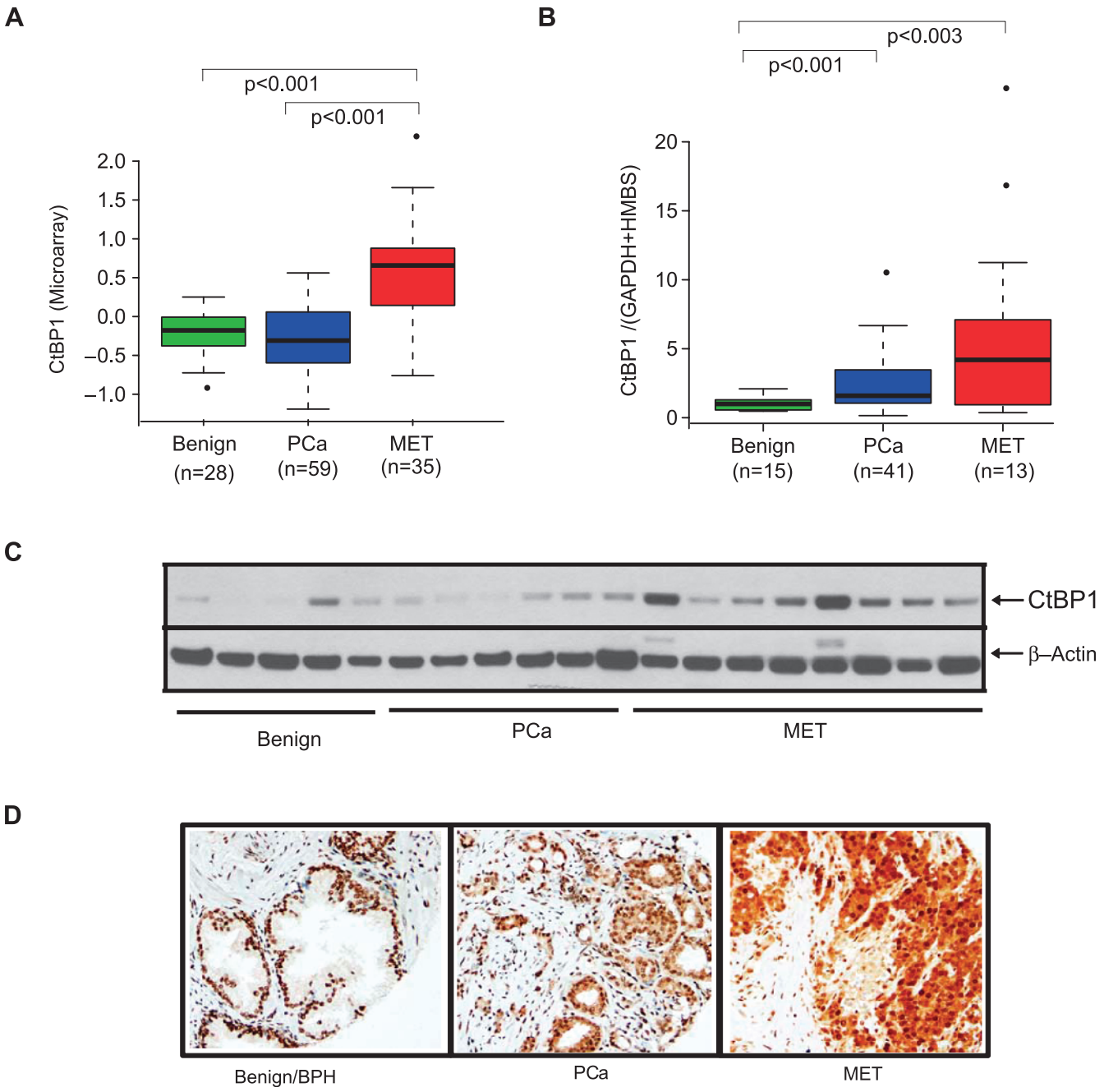


Figure 1. Identification of CtBP1 overexpression in prostate cancer (PCa). (A) CtBP1 expression in the prostate cancer gene expression profiling study. Data were obtained from benign, prostate cancer, and metastatic prostate cancer (MET) tissue expression profiling. (B) Real-time qPCR of CtBP1 transcripts in benign and prostate cancer. Ratio was calculated relative to GAPDH. The boxes extend from the lower to the upper quartile of the data, and whiskers extend to the most extreme data point that is no more than 1.5 times the interquartile range from the box. Points beyond this range are shown as black dots. (C) Expression of CtBP1 protein in prostate cancer. Extracts from prostate specimens were assessed for expression of CtBP1 by immunoblot analysis. β-Actin was used as a loading control. (D) Immunohistochemical analysis of CtBP1 in prostate cancer. Left, benign prostate epithelia exhibiting nuclear staining. Localized prostate cancer exhibiting nuclear and cytoplasmic staining. Metastatic prostate cancer shows increased cytoplasmic CtBP1 staining.

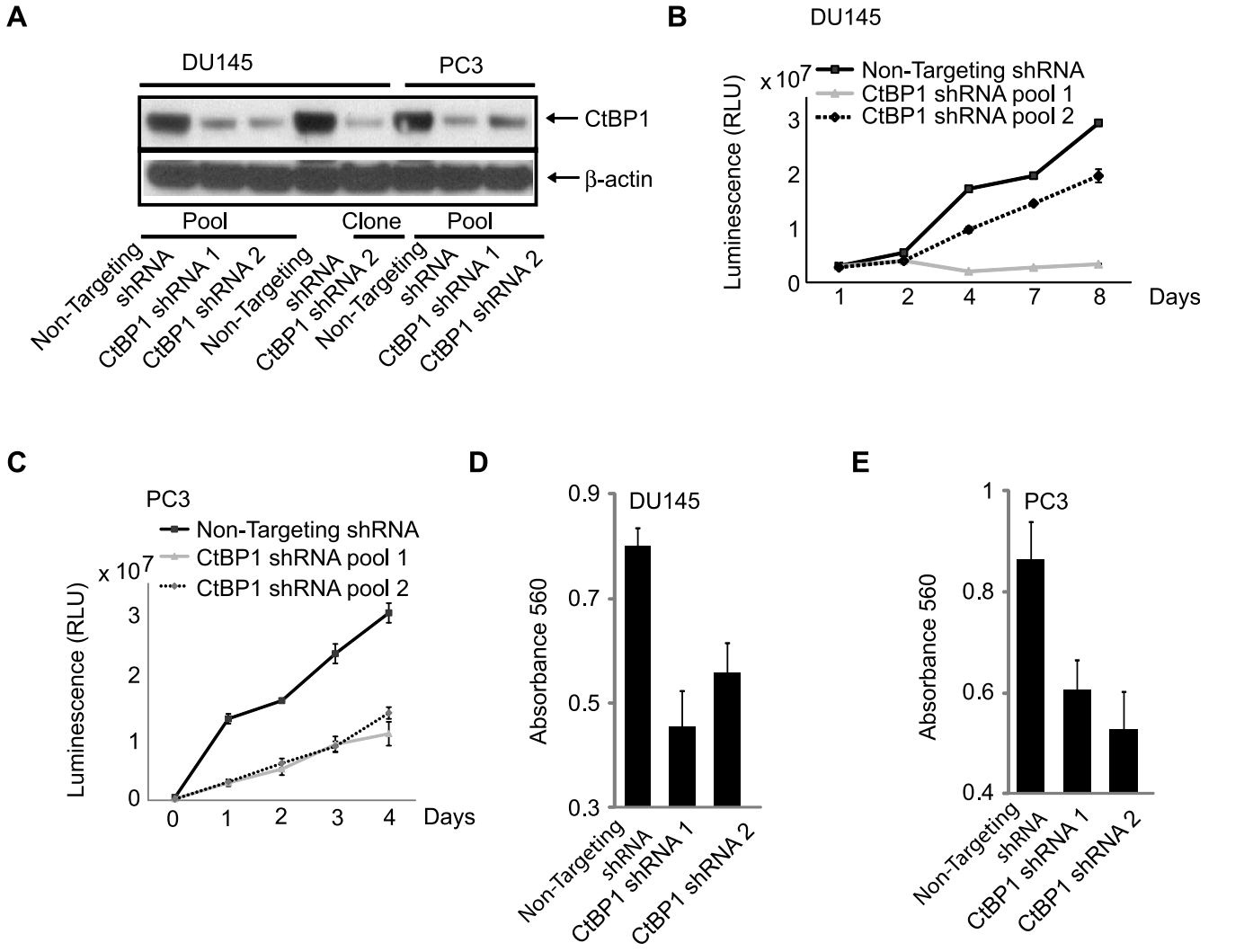
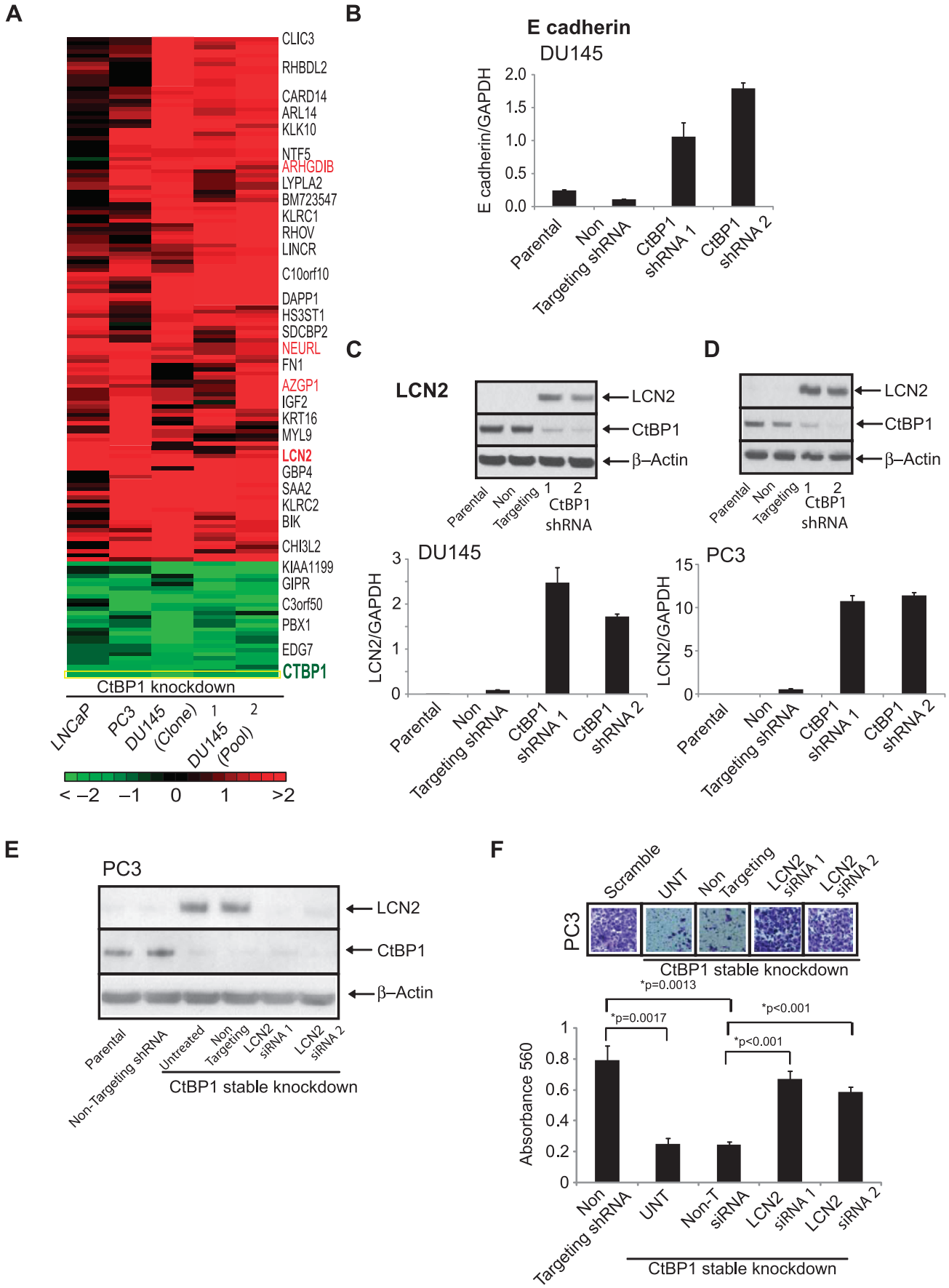


Figure 2. CtBP1 plays a role in cell proliferation and invasion. (A) Knockdown of CtBP1 in prostate cancer cell lines. Immunoblot analysis using lysates from aggressive prostate cell lines DU145 and PC3 treated with two CtBP1-specific shRNA lentivirus and nontargeting control lentivirus. β-Actin was used as control. (B, C) Knockdown of the CtBP1 reduces prostate cancer cell proliferation. Cell proliferation was measured using cells transduced with CtBP1 shRNA duplex or control nontargeting shRNA using DU145 and PC3 cells. (D, E) Knockdown of the CtBP1 reduces prostate cancer invasion. DU145 and PC3 cells in which CtBP1 was stably knocked down using two specific shRNA lentivirus against CtBP1 were used in Boyden chamber matrigel invasion assay. Nontargeting shRNA lentivirus served as control. Invaded cells were stained and absorbance was measured.

96-well plate for Cell Titer-Glo (Promega, Madison, WI) proliferation assays. Twelve hours after plating, the cells were transfected with siRNA duplex, using Oligofectamine (Invitrogen, Carlsbad, CA). A second identical transfection was performed 24 hours later. Sixty-four hours after the first transfection, the cells were harvested for RNA isolation or lysed for immunoblot analysis. For knockdown of LCN2 (NGAL), specific siRNA (Dharmacon, Cat. Nos J-003679-07 and J-003679-09) were used in DU145 and PC3 stable CtBP1 knockdown cells.

Figure 3. CtBP1 knockdown reactivate tumor suppressors in prostate cancer. (A) Heat map of genes that are significantly altered by CtBP1 stable knockdown in LNCaP, PC3, and DU145 cells. Log_2(Cy5/Cy3) ratios are shown for each expression array. Red and green represent upregulated and downregulated genes, respectively, in CtBP1 knockdown cells, relative to the median of the reference pool. Black signifies no change in expression. Known and putative tumor and metastasis suppressors are indicated in red letters. The color bar indicates the fold change; red denotes up-regulation and green represents down-regulation. (B) CtBP1 regulates expression of E-cadherin. E-cadherin expression was measured in CtBP1 stable knockdown DU145 cell lines and compared to control cell lines by qPCR. (C and D) CtBP1 regulates expression of LCN2. LCN2 expression was measured in CtBP1 stable knockdown DU145 and PC3 cell lines by qPCR and immunoblot analysis. (E) LCN2 knockdown in stable CtBP1 knockdown PC3 cells. Expression of LCN2 and CtBP1 were tested by immunoblot. (F) LCN2 knockdown enhances invasion. LCN2 was targeted by two independent specific siRNA in stable CtBP1 knockdown PC3 cell line, and invasion experiment was performed using Boyden chamber matrigel assay (photomicrographs are shown in the inset; blue staining represents invaded cells).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DAPP1
HS3ST1
SDCBP2
NEURL
FN1
AZGP1
IGF2
KRT16
MYL9
LCN2
GBP4
SAA2
KLRC2
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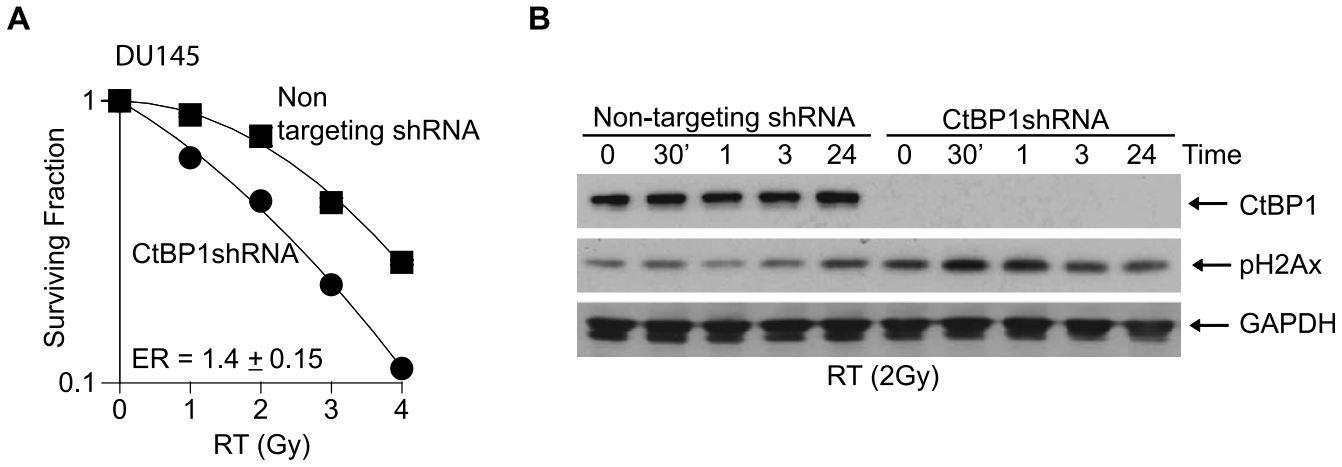

Figure 4. Role of CtBP1 in radiation resistance. Effects of CtBP1 knockdown on clonogenic survival of DU145 cells. (A) Stable clones of CtBP1 along with controls were radiated with clinically relevant doses of radiation and immediately plated for clonogenic survival. (B) Phospho-H2AX immunoblot after radiation treatment of control and stable CtBP1 knockdown cells. GAPDH was used as a loading control.

Gene Expression Analysis of CtBP1 Knockdown Cells

RNA isolated from shRNA knockdown DU145, PC3, and LNCaP as well as nontarget control cells were used for gene expression profiling. Expression profiling was performed using the Agilent Whole Human Genome Oligo Microarray (Agilent, Santa Clara, CA) according to the manufacturer's protocol. Statistical analysis of gene expression array was performed. Microarray probes were identified as differential on CtBP1 knockdown if the mean $\log_2$(Cy5/Cy3) ratio across cell lines was significantly different from zero as measured by one-sample two-sided Student's *t* tests, using a *P*-value cutoff of .05. The list of differentially expressed genes was additionally filtered such that the mean $\log_2$(Cy5/Cy3) ratio exceeded $\log_2$(2.5) in absolute value. The resulting list of 155 genes are shown in Figure 3*A* as a heat map and listed in Table W1. Statistical analysis was performed using R (www.r-project.org), version 2.15.0.

Cell Proliferation Assays

For cell counts at 96 and 120 hours, the cells were treated with trypsin and replated in six-well dishes 64 hours after the first transfection. Stable knockdown of CtBP1 was performed using shRNA strategy using lentiviral construct with specific duplex sequences targeting CtBP1. DU145 and PC3 cell lines were used for stable CtBP1 knockdown. LCN2 and ARHGDIB were knocked down in stable CtBP1 knockdown PC3 and DU145 cells. Sequence information of all the siRNA used in this study has been given in the Supplementary materials. Cell proliferation was determined using ATPase assay kit (Promega) as described [23]. Additionally, cell proliferation was measured by cell counting. For this, 10,000 cells/well (DU145 and PC3) were seeded in 24-well plates (n = 3), and cells were harvested and counted at specified time points by Coulter counter (Beckman Coulter, Fullerton, CA).

Basement Membrane Matrix Invasion Assay

For invasion assays, control shRNA stable cells or *CtBP1* stable knockdown cells as well as wild-type DU145 and PC3 cells were used. Equal numbers of the indicated cells were seeded onto the basement membrane matrix (BD Biosciences) present in the insert of a 24-well culture plate. RPMI medium supplemented with 10% FBS was added to the lower chamber as a chemoattractant. After 48 hours, noninvading cells and extracellular matrix were removed with a cotton swab. Invaded cells were stained with crystal violet and photographed. The inserts were treated with 10% acetic acid, and absorbance was measured at 560 nm.

Chromatin Immunoprecipitation Assay

The ChIP assays were performed as described [24]. Briefly, DU145 cells at 60% confluency were cross-linked with 1% formaldehyde for 10 minutes, followed by quenching with 0.125 M glycine for 5 minutes at room temperature. Cells were lysed and sonicated to fragment the chromatin to an average size of 500 bp. This was followed by overnight incubation with the antibodies and protein A or G magnetic beads. Cross-links were reversed by incubating chromatin at 62°C for 2 hours, and DNA was isolated. Tri-Methyl-Histone H3 (Lys4) antibody was obtained from Cell Signaling Technology, Danvers, MA (Cat. No. 9751S). Rabbit IgG (Diagenode [Denville, NJ], Cat. No. kch-504-250) was used as a control. Purified DNA was analyzed by qPCR to determine fold enrichment relative to input DNA. The primer sequences for the promoters analyzed are provided as follows. Primers used for ChIP assay are LCN2: F, TGCAGAAATCTTGCCAAGTG and R, GGGATCTAGGGTGGGTTGAT; ARHGDIB: F, CCCAGGGTTTCCTCTTCAA and R, TCAGTGCTTCACGTCTCTGTC; GAPDH: F, TACTAGCGGTTTTACGGGCG and R, TCGAACAGGAGGAGCAGAGAGCGA.

Clonogenic Survival Assay

Clonogenic survival assays were performed using standard techniques [25]. Cells were subcultured at clonal density immediately after irradiation. Cell survival curves were fitted using the linear quadratic equation, and the mean inactivation dose was calculated according to the method of Fertil and Malaise [26].

Chicken Embryo Chorioallantoic Membrane Assay

Chicken embryo chorioallantoic membrane (CAM) assay was performed as described previously [27]. To measure metastasis, we harvested lungs on day 18 of embryonic growth and analyzed for the

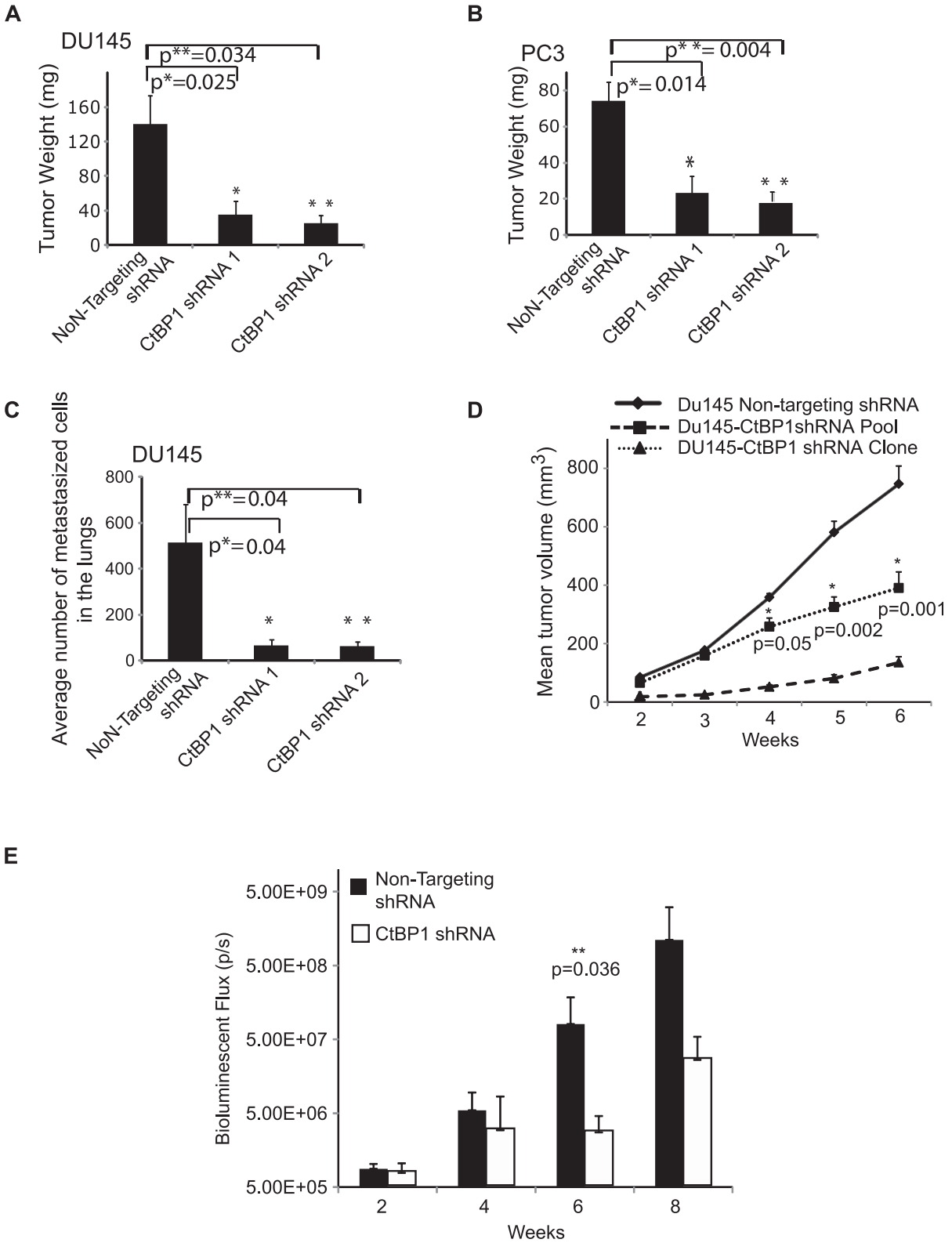

Figure 5. CtBP1 knockdown reduces prostate tumor growth *in vivo*. (A, B) Chicken CAM assay. Tumor growth was measured in CAM models using DU145 and PC3 stable CtBP1 knockdown cells or control nontargeting shRNA stable cells. Tumors were harvested and tumor weights were measured. (C) CtBP1 knockdown reduces metastasis of DU145 cells in CAM assay. Metastasized cells to the lungs of chicken embryos were quantified using human Alu–specific PCR. (D) CtBP1 knockdown inhibits tumor growth in a mouse xenograft model. Plot of mean tumor volume trajectories over time for the mice inoculated with CtBP1 stable knockdown pools (dotted line), CtBP1 stable knockdown clone (broken line), and control (solid line) cells. Error bars represent SEM. (E) CtBP1 knockdown inhibits tumor metastasis. PC3 luciferase stable CTBP1 knockdown or nontargeting control shRNA-treated cells were used in this study. At 2, 4, 6, and 8 weeks after transplantation, the establishment of metastases was followed by BLI. Data are representative of BLI of mice that had developed metastases.

presence of tumor cells by quantitative human Alu–specific PCR. Genomic DNA from lungs was prepared using the Puregene DNA purification system (Qiagen) and was quantified as previously described [28]. For measuring tumor growth, embryos were sacrificed on day 18 and extraembryonic xenografts were excised and weighed.

Prostate Tumor Xenograft Model

All procedures involving mice were approved by the University Committee on Use and Care of Animals at the University of Michigan and conform to their relevant regulatory standards. To evaluate the role of CtBP1 in tumor formation, we propagated stable CtBP1 knockdown DU145 pools, single clone, and vector control cells and inoculated 5×10^6 cells subcutaneously into the dorsal flank of 5-week-old male nude athymic BALB/c nu/nu mice (n = 10 for each group; Charles River Laboratory, Wilmington, MA). Tumor size was measured weekly, and tumor volumes were calculated using the formula $(\pi/6)$ $(L \times W^2)$, where L = length of tumor and W = width.

Murine Tumor Metastasis Models and Bioluminescent Imaging

Experimental procedures were approved by the University Committee on Use and Care of Animals. Male CB17 severe combined immunodeficient mice (4–6 weeks of age) were bred in-house. CtBP1 knockdown PC3-Luc cell pools or nontargeting shRNA-transduced control cells were used for the metastasis model. Animals underwent intracardiac injections of 200,000 cells and were imaged once weekly by bioluminescent imaging (BLI) using a Xenogen IVIS 200 System at the University of Michigan's Center for Molecular Imaging as previously described [29]. Mice were injected with luciferin (100 μl at 40 mg/ml) by intraperitoneal injections. Ventral images were acquired 13 minutes after injection under 1.5% isoflurane anesthesia. Tumor burden of each animal was determined with Living Image software using regions of interest encompassing the entire animal. Animals with no tumor take were defined as those with bioluminescent flux less than 1.151×10^6 p/s at week 8, and these animals were removed from subsequent analysis. Statistical significance was determined using one-sided two-sample t tests. Three animals closest in bioluminescent flux to each group's mean reading at week 8 were selected as representative images.

Results

CtBP1 Is Overexpressed in Metastatic Prostate Cancer

DNA microarray analysis indicated an up-regulation of CtBP1 in metastatic prostate cancer (Figure 1*A*). To validate this observation, we performed real-time qPCR using RNA from multiple prostate cancer and benign tissue samples. Real-time qPCR analysis confirmed the overexpression of CtBP1 in malignant prostate cancer tissues relative to benign prostate samples (Figure 1*B*). We next performed immunoblot analysis of prostate tissue using a CtBP1-specific mouse monoclonal antibody. Results indicated increased CtBP1 protein expression in metastatic prostate cancer relative to localized prostate cancer or benign prostate tissues (Figure 1*C*). Densitometric quantification of the immunoblot indicated significant overexpression of CtBP1 in metastatic prostate cancer tissues (Figure W1*A*). Additionally, CtBP1 was mainly localized to the nucleus (Figure 1*D*) in benign tissue. However in aggressive prostate cancers, increased CtBP1 staining was observed in the cytoplasm by immunohistochemistry analysis (Figure 1*D*). This observation was confirmed using an independent rabbit polyclonal antibody against CtBP (Figure W1*B*). Immunostaining using secondary antibody alone did not show any specific staining of the prostate tissue (Figure W1*C*).

CtBP1 Is Involved in Prostate Cancer Cell Proliferation and Invasion

Because CtBP1 is known to play an oncogenic role in other cancers, we examined the role of CtBP1 in prostate cancer cell proliferation and invasion. We used both transient RNA interference as well as stable knockdown strategy specifically targeting CtBP1 in aggressive prostate cell lines DU145 and PC3. The transient and stable knockdown of CtBP1 were confirmed by immunoblot analysis (Figures 2*A* and W2*A*). Using a commercially available cell proliferation assay reagent Cell Titer-Glo (Promega), we observed a decrease in cell proliferation on both transient and stable knockdown of CtBP1 relative to control cells (Figures 2, *B* and *C*, and W2*B*). Likewise, knockdown of CtBP1 in prostate cancer cells reduced the ability of these cancer cells to invade in a Boyden chamber matrigel invasion assay (Figures 2, *D* and *E*, and W2*C*). Together, these observations support the involvement of CtBP1 in the proliferation and invasion of prostate cancer cells *in vitro*.

Regulation of Gene Expression by CtBP1 in Prostate Cancer Cells

To dissect the functional role of CtBP1 in prostate cancer progression and transcriptional repression in prostate cancer cells, we performed global gene expression analysis using RNA from CtBP1 knockdown prostate cell lines. We generated stable CtBP1 knockdown DU145, PC3, and LNCaP prostate cancer cell lines using lentivirus-based shRNA. We identified multiple molecular targets of CtBP1 that became activated on CtBP1 knockdown including tumor suppressors and metastasis suppressors (Figure 3*A* and Table W1). We validated the reactivation of known CtBP1 repression target E-cadherin in CtBP1 knockdown cells (Figure 3*B*) as well as novel targets LCN2 and ARHGDIB (RhoGDI2) that are implicated in invasion and metastasis suppression [30,31] (Figures 3, *C* and *D*, and W3, *A* and *B*). Chromatin immunoprecipitation analysis at the promoter region of these genes in CtBP1 stable knockdown cells suggested an increase in the activating histone H3–trimethyl lysine 4 methylation mark compared to control GAPDH (Figure W3, *C*–*E*). Additionally, we performed the knockdown of the reactivated LCN2 in PC3-CtBP1 stable knockdown cell lines using two independent duplex targeting LCN2 (Figure 3*E*). Whereas knockdown of CtBP1 reduced the invasion along with reactivation of LCN2, subsequent knockdown of LCN2 in these cells led to reversion to invasive phenotype (Figure 3*F*), indicating a critical role for LCN2 in CtBP1-mediated invasion.

Knockdown of CtBP1 Renders Prostate Cancer Cells Sensitive to Radiation

CtBP1 has been shown to play a role in chemoresistance in breast cancer cells [32]. Furthermore, homeodomain-interacting protein kinase 2 is known to phosphorylate CtBP1, leading to its degradation on UV stimulation and causing apoptosis of cells [33,34]. To determine if CtBP1 also plays a role in regulating radiation resistance and DNA damage repair processes, we assessed the effect of CtBP1 on radiation-induced cell death. CtBP1 knockdown DU145 cells showed considerable reduction in clonogenic survival fraction (enhancement ratio, 1.4 ± 0.15) (Figure 4*A*), which correlated with the slower resolution of H2AX phosphorylation (Figure 4*B*).

CtBP1 Is Essential for Prostate Tumor Growth and Metastasis

To study the effect of CtBP1 on tumor growth and metastasis *in vivo*, we employed a chicken CAM model. CAM was performed as described previously [27] using CtBP1 knockdown DU145 and PC3 prostate cancer cells. Depletion of CtBP1 results in significantly reduced tumor weight compared to nontarget transfected control cells in both DU145 and PC3 cells (Figure 5, *A* and *B*). Further, the lungs of chicken embryos displayed attenuated metastasis in the CtBP1 knockdown group compared to the control group (Figure 5*C*).

We next examined CtBP1-mediated tumorigenesis in a murine xenograft model. Single clone of CtBP1 knockdown cells as well as pooled CtBP1 knockdown DU145 cells showed significantly reduced tumor growth in mice (Figure 5*D*). We also employed a murine metastasis model using PC3 luciferase cells. CtBP1 stable knockdown in PC3 luciferase cells was confirmed by qPCR as well as immunoblot analysis (Figure W4*A*). Mice injected with CtBP1 knockdown prostate cancer cells showed reduced metastasis compared to nontargeting shRNA-transduced control PC3 luciferase cells (Figures 5*E* and W4*B*). These data provide compelling evidence for critical role of CtBP1 in prostate tumor growth and metastasis.

Discussion

In this study, we measured the expression level of transcriptional corepressor CtBP1 in prostate cancer and investigated the mechanism of its oncogenic action. It is becoming increasingly clear that dysregulated transcriptional repression plays a crucial role in tumorigenesis. Several epigenetic modifiers including HDACs and PcG proteins mediate transcriptional repression in cancer cells through posttranslational modification of histones. CtBP1 is known to act as a corepressor that binds to histone modifiers such as HDACs, G9A, as well as LSD1 [35] and recruits repressive complexes to promoters of tumor suppressors to inhibit their expression. Our study uncovers the role of CtBP1 in prostate cancer progression and its molecular targets. We demonstrated the overexpression and mislocalization of CtBP1 to cytoplasm in aggressive prostate cancers. The mechanism of CtBP1 mislocalization in aggressive cancer and the functional significance of cytoplasmic CtBP1 are yet to be elucidated. CtBP1 acts as a transcriptional repressor in prostate cancer, and gene expression profiling studies using multiple prostate cancer cell lines indicated derepression of numerous target genes on CtBP1 knockdown, many of which are tumor suppressors. Rescue experiments underscored the role of these transcriptional targets in prostate cancer progression. Moreover, LCN2, a target of CtBP1-mediated repression is known to function as an invasion and angiogenesis suppressor in pancreatic cancer [30]. Mice injected with MIAPaCa-2 pancreatic cells overexpressing LCN2 showed reduced tumor volume, local and distant metastasis, and angiogenesis. Our data show that LCN2 plays a similar role in prostate cancer cells. A recent study has shown that ARHGDIB, identified here as a CtBP1-mediated repression target, reduces tumor metastasis by altering inflammation in the tumor microenvironment [36]. Our *in vitro* and *in vivo* studies suggest that these targets are involved in CtBP1-mediated oncogenesis in prostate cancer cells. Knockdown of CtBP1 sensitized DU145 cells to radiation, suggesting an important role for CtBP1 in radiation-induced DNA repair. Interestingly, our preliminary mass spectrometry data suggest that CtBP1 binds to DNA damage repair pathway proteins (Varambally et al., unpublished observations). Importantly, these results have implications for patient overexpressing CtBP1 that undergo radiation therapy.

Here, we propose a model of CtBP1-mediated corepression and tumor suppressor axis in prostate cancer, wherein CtBP1 represses multiple tumor suppressors in prostate cancer. However, further studies are required to elucidate the mechanism of CtBP1-mediated radiation resistance to determine whether CtBP1 overexpression predicts aggressive disease and if it has a functional role in the cytoplasm. The NAD-dependent dehydrogenase activity of CtBP1 makes it an attractive therapeutic target. Small-molecule inhibitors targeting CtBP1 enzymatic activity or its interaction with downstream targets and binding proteins could potentially serve as an effective strategy to inhibit its oncogenic activity.

Acknowledgments

We thank Jyoti Athanikar for critical reading of the manuscript. We also thank John Prensner, Xuhong Cao, and Jacob Scherba for assistance in experiments; the University of Michigan Vector Core for generation of CtBP1 shRNA lentivirus; and Prof. G. Chinnadurai (Institute for Molecular Virology, Saint Louis University Health Sciences Center, Doisy Research Center, St Louis, MO) for helpful discussions.

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Table W1. List of Genes Altered by CtBP1 Knockdown in Prostate Cancer Cells.

Gene Name	Direction	Fold Change
LCN2	Up	11.03372089
SAA1	Up	10.62440645
SLC6A14	Up	10.3697991
THC2682885	Up	8.499569184
SAA4	Up	8.218110514
SAA2	Up	7.998343841
THC2650074	Up	6.757080752
THC2650501	Up	6.629116935
SERPINB3	Up	6.492061322
DAPP1	Up	6.312501496
UBD	Up	6.252205533
C10orf10	Up	6.20416844
ACTL8	Up	5.728690584
PDZK1IP1	Up	5.41349766
EDN1	Up	5.312535078
NOD2	Up	5.271836541
C10orf10	Up	5.010336999
ST6GALNAC5	Up	4.924610899
SLPI	Up	4.714816831
SLC6A12	Up	4.699978694
DENND2A	Up	4.695348901
SAA3P	Up	4.656360545
GBP4	Up	4.644158877
KLK10	Up	4.419614789
KLRC2	Up	4.399289221
SCGB2A1	Up	4.372932373
PDZK1IP1	Up	4.328325248
CHI3L2	Up	4.312129669
SLPI	Up	4.254349021
COP1	Up	4.222383184
FAM26B	Up	4.182196359
VNN1	Up	4.028550143
SERPINB4	Up	3.993168302
C3	Up	3.959253087
C1orf167	Up	3.949487904
A_23_P77389	Up	3.894306375
ENST00000372493	Up	3.77246666
ENST00000369783	Up	3.721224797
ETV7	Up	3.618856373
OASL	Up	3.592721037
KLRC1	Up	3.575376247
A_32_P167111	Up	3.549373992
CASP1	Up	3.53112085
EDN2	Up	3.518170121
AY927488	Up	3.506095338
CSPG4	Up	3.488841263
BMF	Up	3.419565273
BIK	Up	3.412660642
RHOV	Up	3.398891204
A_23_P247	Up	3.395778679
SERPINA3	Up	3.37938261
NEURL	Up	3.34920203
KLK10	Up	3.338074193
HS3ST1	Up	3.270192482
THC2582296	Up	3.227026308
FLJ13744	Up	3.223450915
CTSS	Up	3.213339698
LOH11CR2A	Up	3.183020595
C1QTNF5	Up	3.169958498
KRT16	Up	3.106029197
IL23A	Up	3.053823822
KRT16	Up	3.031365254
TMEM92	Up	2.99350621
APOL3	Up	2.990667894
RHOV	Up	2.965110679
SPRR1A	Up	2.963827972
S100A4	Up	2.96284402
BM723547	Up	2.95591769
PRICKLE2	Up	2.926055588
IGF2	Up	2.893507103
LOC254848	Up	2.866625949
AZGP1	Up	2.865917344
CARD6	Up	2.86345534

Table W1. (*continued*)

Gene Name	Direction	Fold Change
ARL14	Up	2.845714686
A_32_P20040	Up	2.824936081
RHBDL2	Up	2.824004231
TRAF1	Up	2.801123087
POU2F3	Up	2.787456539
CEACAM1	Up	2.781567452
GPR20	Up	2.742585123
KSR1	Up	2.73469618
ELF3	Up	2.723674882
FHAD1	Up	2.723599976
GBP5	Up	2.716484367
A_32_P40673	Up	2.715719853
MSTP9	Up	2.709864821
APOL3	Up	2.709100778
LINCR	Up	2.699925499
A_32_P153361	Up	2.69165116
YPEL2	Up	2.677090933
INCA	Up	2.668568056
ARHGDIB	Up	2.660968084
COL16A1	Up	2.659553097
CSTA	Up	2.65303983
LEF1	Up	2.651075658
ACVRL1	Up	2.647718605
NTF5	Up	2.644979171
SDCBP2	Up	2.643978408
C1orf167	Up	2.640432821
LY96	Up	2.627523781
FLJ22675	Up	2.625238807
SP8	Up	2.615780109
DENND2A	Up	2.609905282
MARCO	Up	2.60692239
ADRA1B	Up	2.602117819
CCL3	Up	2.598626493
TNFSF15	Up	2.597525144
NFE2	Up	2.595618858
DLL1	Up	2.594785429
HLA-DMA	Up	2.593241301
MYL9	Up	2.59106039
FN1	Up	2.587362245
MMP19	Up	2.585415317
SDC4	Up	2.58044802
KNDC1	Up	2.575092752
B3GALT4	Up	2.574645428
YPEL2	Up	2.574150346
CARD14	Up	2.57094572
CLIC3	Up	2.561894522
C1orf116	Up	2.55762971
ADORA2A	Up	2.54337386
AB014766	Up	2.531727802
LYPLA2	Up	2.530422216
KRT14	Up	2.521067012
ARG2	Up	2.511097922
AP1M2	Up	2.506939054
FOXO4	Up	2.503068888
AL080205	Down	0.399113121
ADAMTS15	Down	0.395735507
MBD5	Down	0.393249606
EDG7	Down	0.388723832
AA581414	Down	0.383696657
A_24_P915566	Down	0.382915063
AF090920	Down	0.381286744
PGA5	Down	0.376015457
ARL11	Down	0.365791354
CTBP1	Down	0.362998695
SLC7A11	Down	0.362424314
ENST00000327625	Down	0.361342653
ADAM7	Down	0.355771403
A_24_P922440	Down	0.345827631
KLF17	Down	0.333723905
CTBP1	Down	0.325783221
BMPR1B	Down	0.323357446
GIPR	Down	0.315843641
LOC348174	Down	0.299451578

Table W1. (*continued*)

Gene Name	Direction	Fold Change
C3orf50	Down	0.298466301
AF334588	Down	0.297229095
PBX1	Down	0.257104943
C13orf21	Down	0.255784914
ENST00000259289	Down	0.251085668
KIAA1199	Down	0.228711041
C12orf42	Down	0.217435692
LOC152573	Down	0.212965398
AK021467	Down	0.199419469

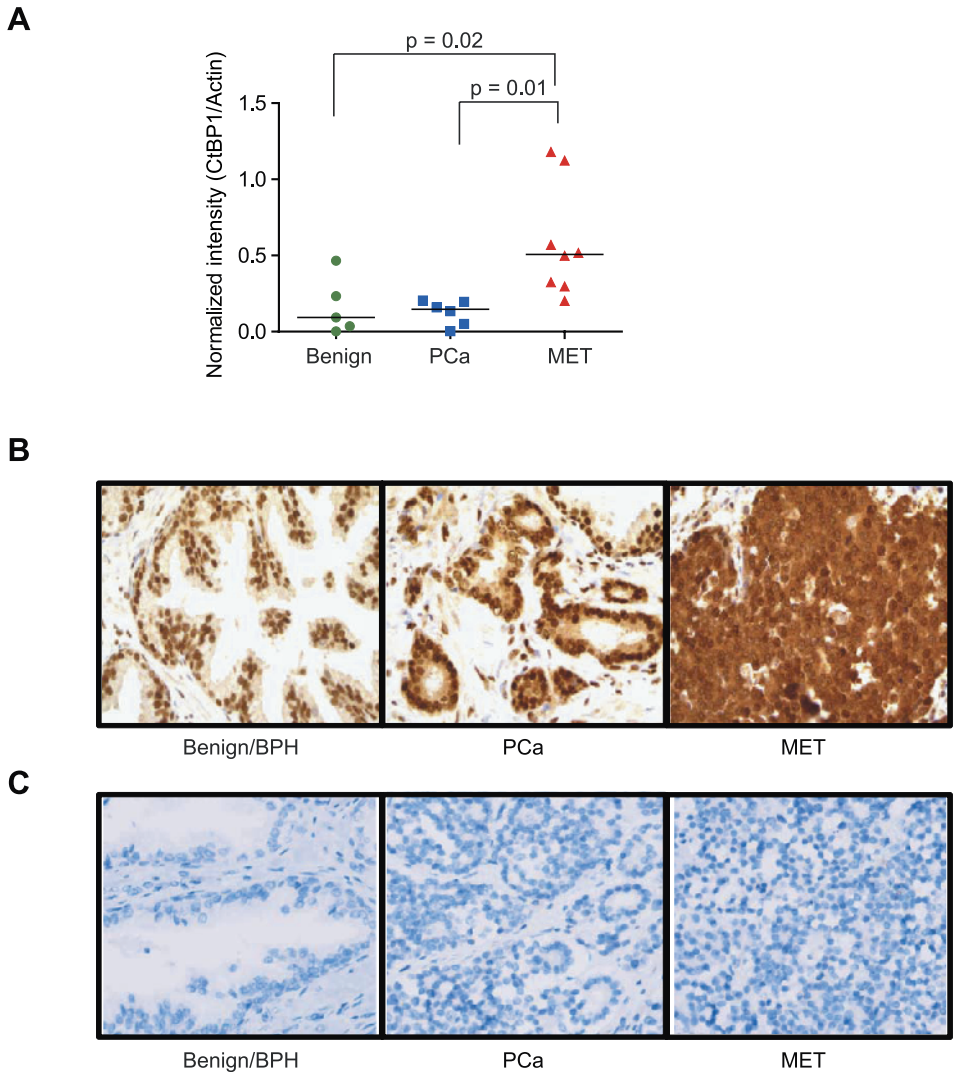

Figure W1. (A) Densitometric scanning analysis of prostate tissue CtBP1 expression. Each point represents a tissue sample. (B) Expression and localization of CtBP1 protein in prostate cancer. Immunohistochemical analysis of CtBP1 in prostate cancer. (C) Immunohistochemical staining of prostate tissues with secondary antibody alone.

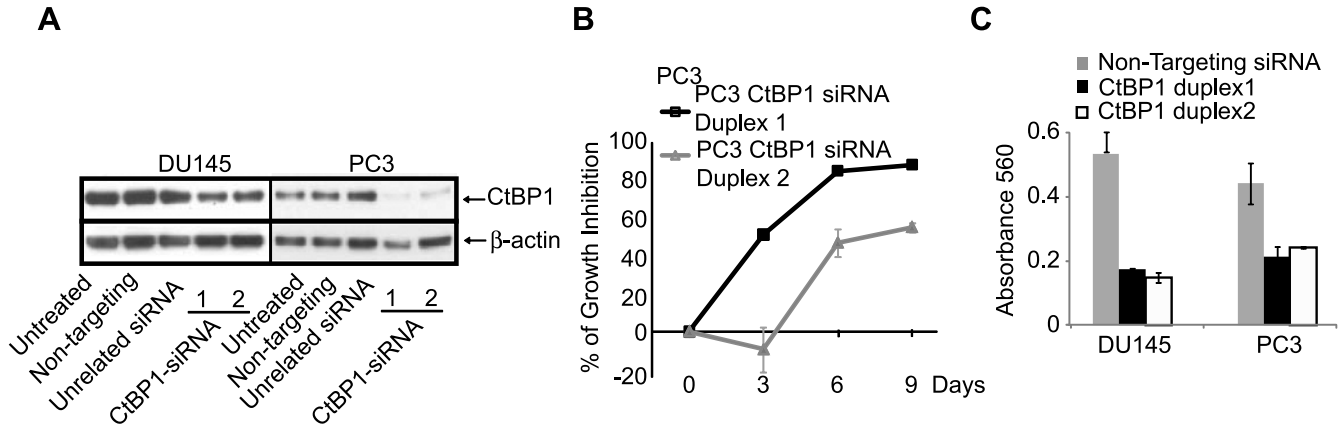

Figure W2. Transient knockdown of CtBP1 reduces cell proliferation and inhibits invasion. (A) Immunoblot analysis using lysates from prostate cell lines DU145 and PC3 treated with two independent CtBP1-specific siRNA duplex on nontarget control duplex. β-Actin was used as control. (B) Cell proliferation was measured using cells transfected with CtBP1 siRNA duplex or control nontargeting siRNA using PC3 cells. (C) DU145 and PC3 cells were treated with two CtBP1-specific siRNA duplex and control duplex, and Boyden chamber matrigel invasion assay was performed. Nontargeting siRNA served as control. Invaded cells were stained, and absorbance was measured.

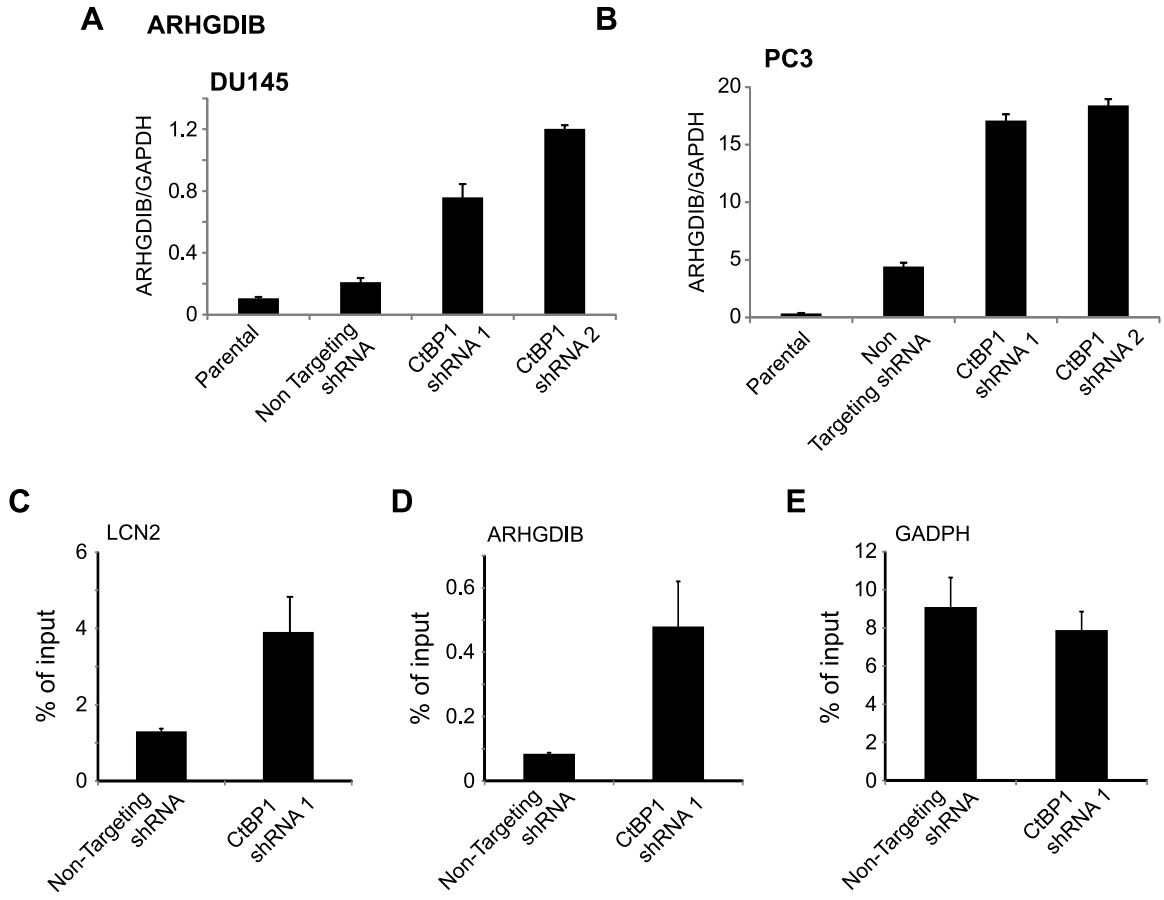

Figure W3. CtBP1 regulates gene expression. (A and B) ARHGDIB expression in stable CtBP1 knockdown DU145 and PC3 cells. (C and D) Chromatin immunoprecipitation experiments indicate increased HeK4me3 trimethylation mark at the promoters of LCN2 and ARHGDIB on knockdown of CtBP1. (E) GAPDH promoter did not show altered HeK4me3.

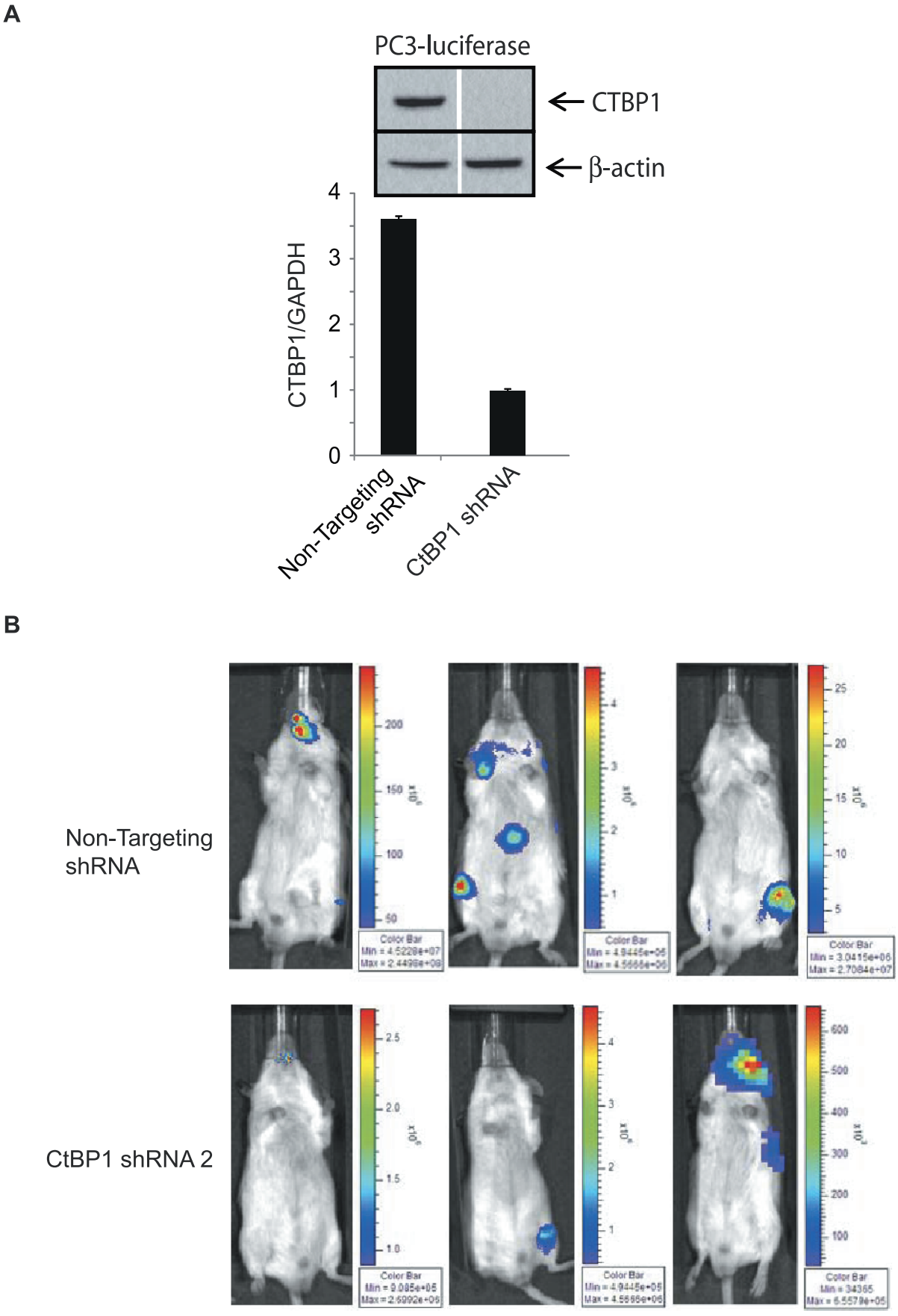

Figure W4. Role of CtBP1 metastasis. (A) Real-time qPCR and immunoblot of CtBP1 stable knockdown PC3 luciferase cells. (B) Representative bioluminescent images of mice inoculated with control PC3 luciferase cells and CtBP1 stable knockdown PC3 luciferase cells.