Molecular and Cellular Pathobiology

Cancer Research

CtBP2 Modulates the Androgen Receptor to Promote Prostate Cancer Progression

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Abstract

The androgen receptor (AR) is the key driver of both early and advanced prostate cancer, making a complete understanding of its regulation important. Here, we report the identification of multiple AR-binding sites in the gene encoding the transcription factor CtBP2 (carboxyl terminal–binding protein), genetic variations of which have been associated with prostate cancer susceptibility. Notably, we found that SNPs in the human *CTBP2* gene that were associated with prostate cancer development were correlated with AR-enhancer activity. High CtBP2 expression levels correlated with poor prognosis in patients, whereas CtBP2 silencing reduced tumor growth in a mouse xenograft model of human prostate cancer. Consistent with its function as a transcriptional corepressor, CtBP2 repressed tumor-suppressor genes and AR corepressors in prostate cancer cells, such as *NCOR* and *RIP140*, by binding with AR to the promoter enhancers of these genes. Global gene-expression analyses revealed a positive effect on androgen-mediated gene expression, and CtBP2 silencing was found to increase AR interactions with corepressors that limit histone modification. Overall, our results show how CtBP2 contributes to prostate cancer progression by modulating AR and oncogenic signaling. *Cancer Res; 74(22); 6542–53. ©2014 AACR.*

Introduction

The actions of androgen receptor (AR) are essential for the proliferation of prostate cancer and its subsequent progression to castration-resistant prostate cancer (CRPC; refs. 1–3). Ligand-dependent dynamic changes in coregulator binding to AR are assumed to modulate AR-mediated signaling pathways by recruiting multiple histone–modifying enzymes (3, 4). Histone deacetylases (HDAC) and histone acetyltransferases are involved in the transcriptional regulation of nuclear receptors by regulating histone acetylation (5). Histone H3 lysine 9 (H3K9) demethylation is another representative histone marker for AR-mediated transcription (6). Various changes of AR-associated coregulators by androgen treatment affect cell characteristics and define prostate cancer progression (4).

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Recently, we have reported a novel AR transcriptional regulatory system involving the carboxyl terminal–binding protein (CtBP) family, namely *CTBP1*, and its antisense long noncoding RNA (*CTBP1-AS*; ref. 1). Ligand-dependent binding of CtBP1 to AR represses androgen-mediated gene induction at an early time point by inhibiting H3K9 demethylation. After androgen treatment, CtBP1 expression is reduced to activate androgen signaling by its antisense RNA. We also showed that low CtBP1 expression level is associated with poor prognosis and early PSA relapse after operation. However, the involvement of another CtBP family protein, CtBP2, in prostate cancer has not been fully investigated. Interestingly, the recent genome-wide association study (GWAS) using 50 K SNP arrays identified multiple prostate cancer risk loci, including the *CTBP2* locus (7–9).

In the present study, we investigated AR target genes by the genome-wide chromatin immunoprecipitation-sequence (ChIP-seq) study. We identified multiple AR-binding sites (ARBS) around the *CTBP2* locus. We also found that high expression level of CtBP2 is associated with poor prognosis in patients with prostate cancer. The results from functional experiments indicated that CtBP2 promotes prostate cancer cell growth and migration. In addition, our integrative transcriptional analysis indicated that CtBP2 has positive effects on AR signaling. Taken together, our findings raise the possibility that CtBP2 could be a promising therapeutic target in AR-activated prostate cancer.

Materials and Methods

Construction

For construction of the CtBP2 expression vector, *CTBP2* coding sequence (NM_001329) was amplified by PCR and then

inserted into the *EcoRI* and *XhoI* sites of pcDNA3.0, including the Flag-tag sequence at N-terminus. For construction of the ARBS–luciferase vector, the genomic region (chromosome 10: 126686612-126688021), including ARBS, was amplified by PCR. Cloned ARBS was inserted to the *MluI* and *XhoI* sites of the pGL3-promoter vector. The PCR primers were listed in Supplementary Table S1. To construct the *CTBP2*–ARBS–luciferase vector with mutation in SNPs or AREs (androgen-responsive elements), we amplified two separated regions of the ARBS by PCR using primers, including each mutation as shown in Fig. 1C, and then ligated two fragments by second PCR.

Luciferase assay

Cells were transfected with pGL3 vectors, including ARBSs and tk-pRL, by using the FuGENE HD reagent (Promega). The luciferase activities were determined as previously described (10).

Migration assay

Cellular migration assays were performed using Matrigel-coated invasion chambers (BD Biosciences). Briefly, cells were transferred into an insert with an 8-μm pore size in serum-free medium. Medium with 10% FBS was placed in the lower 24-well and cells were cultured for another 24 hours. Invading cells were fixed with methanol and stained with Giemsa. The number of invading cells in three random fields was counted using a microscope, and the average number of cells per field was calculated.

Western blot analysis and immunoprecipitation

For immunoprecipitation, cell lysate protein was incubated with anti-CtBP2, anti-CtBP1, anti-AR antibody, or normal rabbit IgG at 4°C overnight. Immunoblotting was performed as described previously (10).

siRNA

For siRNA experiments, we purchased a Stealth RNAi siRNA (Life Technologies)–targeting *CTBP2* (HSS175560; si*CTBP2* #0) and a negative control siRNA from Invitrogen. Cells were transfected with RNA using RNAi MAX (Life Technologies) 48 to 72 hours before each experiment. We also used two other siRNA-targeting *CTBP2* that were purchased from Sigma Genosys Japan. These two siRNA sequences were si*CTBP2* #1, 5′-GUGAUCGUGCGGAUAGGCAGU-3′ and si*CTBP2* #2, 5′-CACCCUGCUCUACAAUGUUGC-3′.

Patients and tissue samples

We obtained all prostate tissue samples from radical prostatectomy performed at the University of Tokyo Hospital (Tokyo, Japan). The prostate tissue sections submitted for this study contained 105 cancerous foci and 95 benign regions adjacent to tumors. The Tokyo University Ethics Committee approved this study and informed consent was obtained from each patient before surgery. The age of the patients ranged from 52 to 78 years (mean, 66.8 ± 6.0 years), and pretreatment serum PSA levels ranged from 2.2 to 136 ng/mL (mean, 16.9 ± 19.5 ng/mL). Other clinicopathologic parameters are shown in Supplementary Table S2.

Immunohistochemistry

Immunohistochemical analysis of CtBP2 and the antigen–antibody complex were visualized with 3,3′-diaminobenzidine solution (1 mmol/L 3,3′-diaminobenzidine, 50 mmol/L Tris-HCl buffer [pH 7.6], and 0.006% H_2O_2). In the immunohistochemical analysis, the immunoreactivity was evaluated in more than 1,000 carcinoma cells in each case and then we calculated the H-scoring system as described previously (11). Briefly, all CtBP2-positive carcinoma cells were classified into three groups according to immunointensity (i.e., strongly, moderately positive, or negative cells), and *H*-score was subsequently generated as follows: [2 × (percentage of strongly positive cells)] + [1 × (percentage of moderately positive cells)] + [0 × (percentage of negative cells)]. The percentage and immunointensity were determined by specialized pathologists and then we set the median value, *H*-score = 7 [average, 12 (range, 0–58)], as the cutoff point to classify all cases into two groups, higher and lower expression.

Statistical analyses

For the cell proliferation assay, we analyzed four wells. For the growth assay *in vitro* of stable cell lines, we performed two-way ANOVA at each time point. For other cell line experiments, statistical differences (*P* values) among groups were obtained using a two-sided Student *t* test. All experiments were performed at least twice and similar results were obtained. *P* values less than 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software) or MS Excel.

An association between immunoreactivity and clinicopathologic factors was evaluated using a Student *t* test, a cross-table using the χ^2 test, or a correlation coefficient (*r*) and regression equation. A cancer-specific survival curve was generated according to the Kaplan–Meier method, and the statistical significance was calculated using the log-rank test.

Cell proliferation assay

The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was performed using the CellTiter 96 Aqueous (Promega), according to the protocol provided by the manufacturer.

In vivo tumor formation assay

LNCaP (3×10^6) cells were s.c. injected into each side of twenty 5-week-old male BALB/c nude mice. When the tumor volume reached 100 mm^3, injection of siRNA was started. Each tumor was transfected with 5 μg of si*CTBP2* or control RNA, three times a week by using the Lipofectamine RNAi MAX Transfection Reagent (Life Technologies). Tumor volume was determined using the formula $0.5 \times r^1 \times r^2 \times r^3$ ($r^1 < r^2 < r^3$).

Cell culture and reagents

VCaP cells were grown in DMEM medium supplemented with 10% FBS. LNCaP, DU145, and RWPE cells were grown in RPMI medium supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin. VCaP and LNCaP cells were purchased from the ATCC in 2009. In the ATCC, cells were authenticated by short tandem repeat analysis. DU145 and

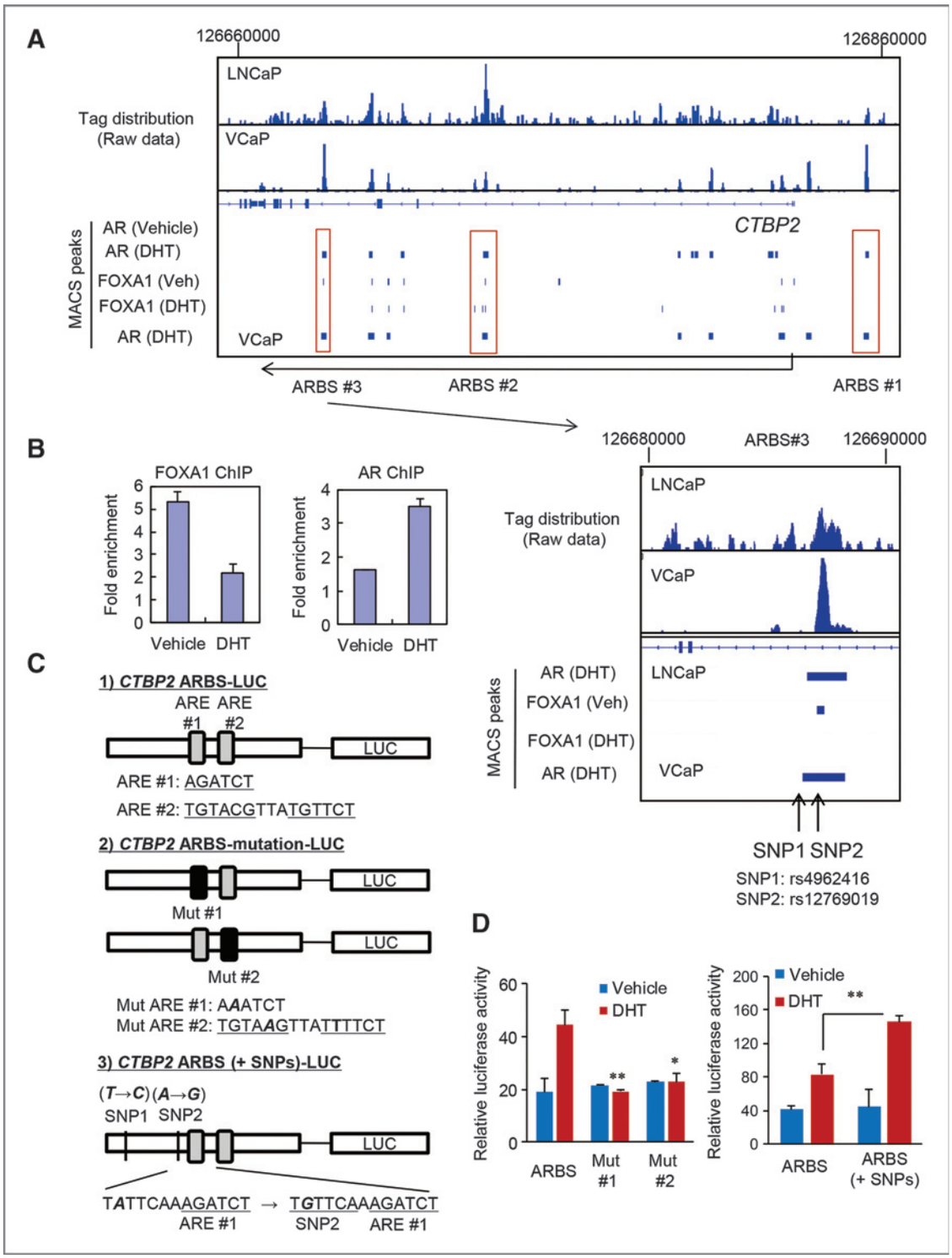

Figure 1. Alteration of androgen-mediated–enhancer activity by *CTBP2* genetic risk allele within ARBS identified by ChIP-seq. A, identification of multiple ARBSs around the *CTBP2* gene. AR ChIP-seq was performed in VCaP and LNCaP cells. Significant ARBSs ($P < 10^{-5}$) were determined by MACS. The loci of SNPs, prostate cancer risk alleles, are shown by arrows. ARBSs validated by ChIP–qPCR (ARBS #1–3) are shown by red boxes. B, validation of FOXA1 and ARBS (ARBS #3) by ChIP analysis. LNCaP cells were treated with DHT or vehicle for 24 hours. Fold enrichment over IgG control was determined by qPCR. C, the luciferase vector, including ARBS #3, was constructed. Mutated vectors [ARBS mutation LUC and ARBS (+SNPs)-LUC] were constructed as shown. D, ARBS-enhancer activity is dependent on AREs and genomic risk allele. Luciferase analysis was performed in LNCaP cells; bar, SD; $n = 3$ (B and D). *, $P < 0.05$; **, $P < 0.01$.

RWPE cells were obtained from Saitama Medical University (Saitama, Japan) in 2008. STR analysis was performed for authentication (last tested in 2014). The antibodies used in this study were FOXO1 (rabbit polyclonal; Sigma), RIP140 (ab42126; Abcam), SRC1 (ab84; Abcam), and NCOR (PA1-844A; Pierce). Other antibodies and reagents used have been previously described (1, 10, 12). Of note, 10 nmol/L DHT was used for androgen treatment.

ChIP and quantitative PCR

ChIP and qPCR (quantitative PCR) were performed as previously described (1, 10, 12). The primer sequences are listed in the Supplementary Table S1 or described in previous studies (1, 12, 13).

Quantitative RT-PCR

Total RNA was isolated using ISOGEN reagent. First-strand cDNA was generated using the PrimeScript RT Reagent Kit (Takara). The primer sequences are listed in the Supplementary Table S1 or previously described (1, 10, 13).

Microarray

For expression microarrays, the GeneChip Human Exon 1.0 ST Array (Affymetrix) was used according to the manufacturer's protocol (1). The data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE58309.

ChIP-seq

AR, AcH3, K4me3, and CtBP2 ChIP-sequence was performed using an Illumina Genome Analyzer GAIIx or Hiseq (Illumina). These sequence results have been deposited in NCBI's Gene Expression Omnibus (GSE58428). Libraries were prepared according to Illumina's instructions. FOXA1 sequence data in LNCaP cells were downloaded from NCBI (GSE27823). Unfiltered 36-bp sequence reads were aligned against the human reference genome (hg18) using CASAVA v1.8 (Illumina). Signal scores of AR bindings were calculated using Model-based analysis of ChIP-seq (MACS) and the threshold of ARBSs was a P value of $<1.0\ E^{-5}$. Integrative genomic viewer version 2.2 was used for visualization. Motif analysis of binding sequences was performed using HOMER (14).

Results

Identification of an ARBS around the prostate cancer susceptibility locus

To investigate the AR transcriptional network in prostate cancer, we have mapped ARBSs in LNCaP cells by ChIP-chip in the previous study (13) . In this study, we performed ChIP-seq in AR-positive prostate cancer cell lines. The number of significant ARBSs ($P < 10^{-5}$ by MACS, ref. 15) in the whole genome was 10,974 (fold > 10) in LNCaP cells and 12,764 (fold > 15) in VCaP cells. Multiple prostate cancer susceptibility loci have been identified by a recent GWAS using 50 K SNP arrays (7). We compared the genomic loci of AR-binding genes with such susceptibility loci and then found that *CTBP2*, a gene with corepressor function, is an AR-targeted gene with SNPs highly associated with prostate cancer development.

Multiple ARBSs were found in the introns and 5′ upstream region of *CTBP2* (Fig. 1A). We also mapped FOXA1-binding sites and some of these overlap with ARBSs. First, we validated the androgen-dependent recruitment of AR and FOXA1 to these sites. By ChIP and qPCR analysis, we observed ligand-dependent AR recruitments and FOXA1 bindings at these ARBS#1–3 (Fig. 1B and Supplementary Fig. S1A). Next, we investigated AR transcriptional regulation at 1 ARBS (ARBS #3) situated in the fifth intron. In this region, two SNPs (these SNPs are in linkage disequilibrium, frequency: 0.27; ref. 7) have been shown to be associated with prostate cancer development by GWAS and its associated studies (7–9). Surprisingly, one of those SNPs (rs12769019) is located at the peak center of the ARBS (Fig. 1A). At this location, a half ARE site (ARE #1) was found. Interestingly, by changing the SNP to a nucleotide that was strongly associated with prostate cancer (A→G), this half-site ARE could be converted to a palindrome ARE with one spacer (noncanonical-type ARE; Fig. 1C; ref. 16). In addition, we also found noncanonical-type ARE #2 (TGTACGTTATGTTCT) in ARBS #3. To examine the effects of AREs and the sequence change by the SNP on AR-mediated–enhancer activity, we constructed a luciferase vector containing the ARBS sequence (Fig. 1C). We then confirmed that the SNPs of both LNCaP and VCaP cells are the major frequency alleles, which are not associated with prostate cancer. First, we demonstrated that androgen-mediated upregulation of transcriptional activity was depleted by inserting mutations among the AREs (ARE #1 and ARE #2; Fig. 1C and D). By introducing disease-associated SNPs to the ARBS–luciferase vector as mutations, we observed increased transcriptional response upon changing both SNPs, or changing 1 SNP corresponding to AR-binding peak (Fig. 1D and Supplementary Fig. S1B), which suggests that these SNPs were associated with the AR-mediated–enhancer activity of this location.

High CtBP2 expression is a novel prognostic factor of prostate cancer

Next, we analyzed the expression of CtBP2 in prostate cancer. We treated LNCaP cells with vehicle or DHT and then performed Western blot or quantitative RT-PCR (qRT-PCR) analysis. We observed the induction of *CTBP2* at the mRNA and protein levels by 24 hours androgen treatment (Fig. 2A and Supplementary Fig. S1C). In prostate cancer cell lines, we analyzed CtBP2 expression by Western blot analysis. In the presence of DHT, CtBP2 is highly expressed in AR-positive cells, suggesting that AR plays a role in inducing CtBP2 expression (Fig. 2B).

We also examined the protein levels of CtBP2 in clinical samples. By immunohistochemical analysis, we investigated CtBP2 protein expression in prostate cancer tumors ($n = 105$) and benign tissues (Supplementary Table S2; Fig. 2C, top) using a CtBP2-specific antibody not cross-reacting with CtBP1 (1). We observed strong expression of CtBP2 in the nucleus of prostate cancer tissue. Comparatively weak staining was observed in benign prostate tissues surrounding cancerous regions. Kaplan–Meier analysis showed poor

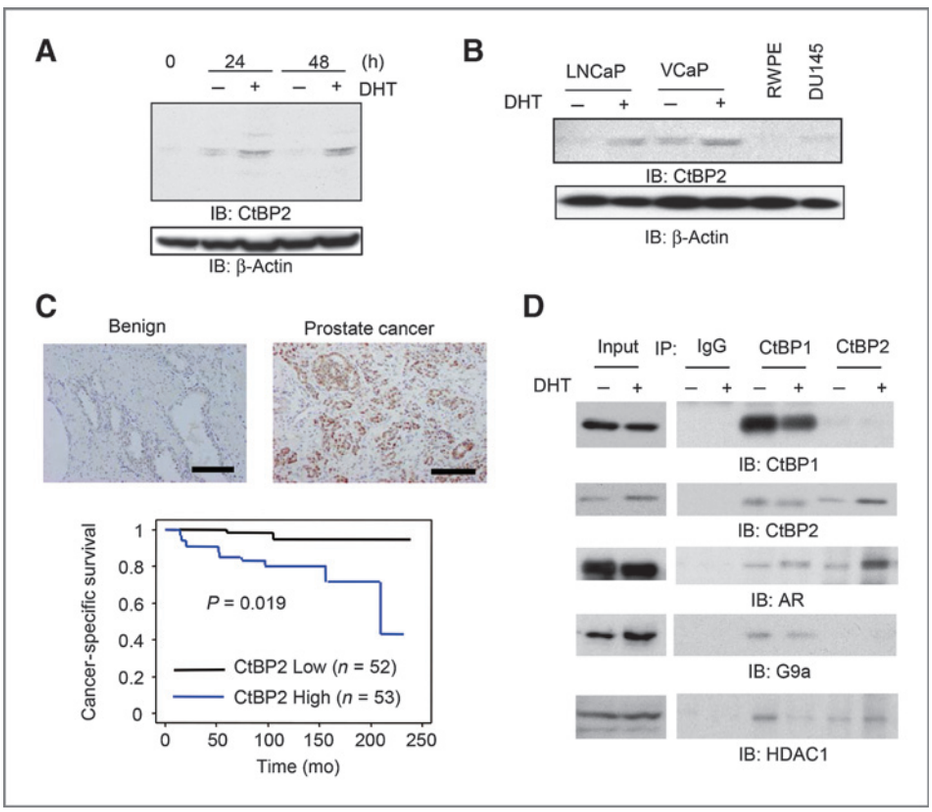

Figure 2. Androgen-responsive CtBP2 functions as a prognostic factor of prostate cancer. A, CtBP2 is induced by androgen treatment. LNCaP cells were treated with DHT or vehicle for 24 and 48 hours. Western blot analysis of CtBP2 was performed. B, expression of CtBP2 in prostate cancer cell lines. CtBP2 protein levels in LNCaP, VCaP, RWPE, and DU145 cells were analyzed by Western blot analysis. C, CtBP2 is upregulated in prostate cancer. Top, immunohistochemistry of CtBP2 was performed in prostate cancer; bar, 100 μm. Bottom, Kaplan–Meier analysis using the log-rank test was performed. D, CtBP2 interacts with AR androgen dependently . LNCaP cells were treated with vehicle or DHT for 24 hours. Cell lysates were immunoprecipitated by indicated antibodies. Western blot analysis of CtBP2, CtBP1, AR, HDAC1, and G9a was performed.

cancer-specific survival in patients with higher CtBP2 expression compared with patients with lower expression ($P = 0.019$; Fig. 2C, lower).

We then compared the functions of CtBP2 with CtBP1 in prostate cancer cells. Our immunoprecipitation results showed that as a result of CTBP2 being induced by androgen, it markedly interacts with AR after 24 hours DHT treatment (Fig. 2D). CtBP2 interaction with AR at 24 hours could be detected more clearly than CtBP1. We also used siRNA (si*CTBP2* #0) specifically targeted to *CTBP2* to create a knockdown to analyze the role of CtBP2 (Fig. 3A). In our previous result, *CTBP1* knockdown accelerates cell proliferation and gene induction at an early time point (4 hours) after androgen treatment (1). However, we observed *CTBP2* knockdown inhibited cell proliferation, although *CTBP1* knockdown enhanced cell proliferation (Fig. 3B). Androgen-mediated gene induction at an early time point was not observed by *CTBP2* knockdown (Supplementary Fig. S2). Therefore, CtBP2 may have a different role in prostate cancer than CTBP1.

CTBP2 expression is associated with tumor growth and invasion

To further examine the function of CtBP2, we generated LNCaP cells that stably overexpressed exogenous CtBP2 (Flag-CtBP2 #1 and Flag-CtBP2 #2; Fig. 3C). The results showed that CtBP2 overexpression enhanced prostate cancer cell growth and migration (Fig. 3D and E). To explore the potential of CtBP2 as a therapeutic target, we designed two siRNAs (si*CTBP2* #1 and #2) that effectively reduced CtBP2 expression at low concentrations (Fig. 4A and Supplementary Fig. S3A). We observed decreased cell migration following transfection of these siRNAs into AR-positive prostate cancer cells (Fig. 4B). In addition, we observed that androgen-dependent cell proliferation was repressed by *CTBP2* knockdown (Supplementary Fig. S3B and S3C). We then examined the potential tumor-promoting effects of CtBP2 and the therapeutic effect of siRNA *in vivo*. We subcutaneously transplanted nude mice with LNCaP cells and then injected si*CTBP2* or siControl into the tumors three times a week, after they formed. Tumor growth in the xenograft model was reduced by *CTBP2* knockdown (Fig. 4C and D).

CtBP2-binding sites overlap with ARBSs and are associated with androgen-mediated gene regulation

To identify the direct CtBP2-binding sites in prostate cancer cells,we performed ChIP-seq in LNCaP cells. In total 1,815 significant CtBP2-binding sites ($P < 10^{-4}$ by MACS) were identified in vehicle-treated condition and 9,917 binding sites in 24 hours DHT-treated condition (Fig. 5A) . To investigate the correlation between CtBP1 and CtBP2, we also performed CtBP1 ChIP-seq analysis. Because CtBP1 is repressed by androgen treatment and association with AR is peaked at 1 hour after androgen stimulation (1), we performed CtBP1 ChIP in LNCaP cells treated with vehicle or DHT treatment for 1 hour. We obtained 7,955 significant CtBP1-binding sites in DHT-treated condition (Fig. 5A). CtBP1-binding sites (3,511 sites) overlapped with CtBP2, suggesting colocalization of both isoforms (Fig. 5B). These binding sites were validated by

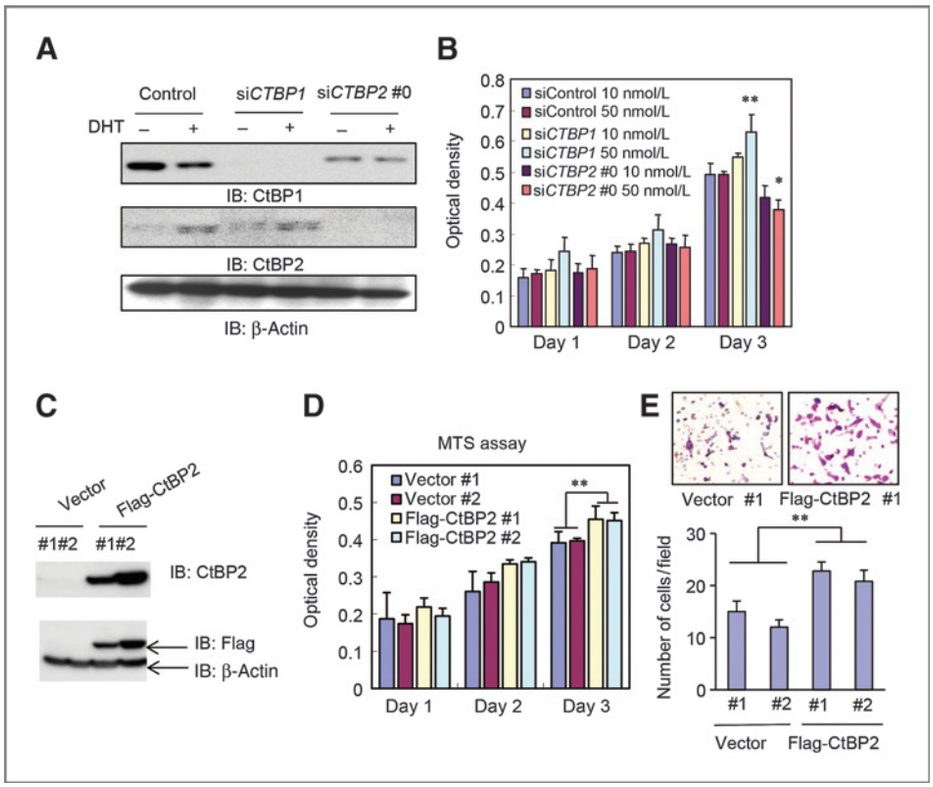

Figure 3. CtBP2 promotes cell proliferation and cell migration. A, validation of specific siRNA against *CTBP1/2*. LNCaP cells were transfected with si*CTBP1/2* (50 nmol/L). B, knockdown of *CTBP1/2* showed different effects on cell proliferation in prostate cancer. LNCaP cells were transfected with si*CTBP1/2* and then cell growth was assayed; $n = 4$. C, establishment of LNCaP cells stably expressing Flag–CtBP2. Western blot analysis was performed to analyze protein levels of CtBP2 in each clone. D, overexpression of CtBP2 promotes cell proliferation. Cell proliferation assay was performed in CtBP2-overexpressing cells; $n = 4$. E, overexpression of CtBP2 promotes cell migration. Control or CtBP2-overexpressing cells were seeded in Matrigel-coated top chamber. The average numbers of invading cells are shown; bar, SD; $n = 5$; *, $P < 0.05$; **, $P < 0.01$.

conventional ChIP assay and then we observed CtBP1/2 recruitments were comparable with ChIP-seq signals (Supplementary Figs. S4 and S5).

Next, we investigated the role of CtBP2 in AR transcriptional regulation. As expected by the androgen-dependent interaction between CtBP2 and AR, we also observed that 1,165 CtBP2-binding sites were overlapped with ARBSs (Fig. 5B). To investigate the correlation of CtBP2-binding sites with the nearest known genes, we calculated the distance between the transcriptional start site (TSS) of the nearest RefSeq gene and each binding peak (Fig. 5C). Approximately 50% of CtBP2-binding sites were enriched within 3 kb from TSS. In contrast, ARBSs were distributed widely across the whole genome as previously reported (2, 13). The representative AR target genes such as *APP* (12), *KLK3/PSA*, and *TMPRSS2* exhibited signal enrichments of CtBP2 bindings around ARBSs in ChIP-seq data (Fig. 5D and Supplementary Fig. S4). Moreover, by analyzing sequences of CtBP2-binding sites, we found motifs of AR and AR-collaborating factors such as FOXA1 (16) and ETS1/ERG (17) are significantly enriched around the peaks (Fig. 5E). Thus, these results suggest the involvement of CtBP2 in AR transcriptional regulation.

Furthermore, we evaluated the histone modification patterns in CtBP2-binding regions. To determine the histone-modified regions, we also performed ChIP-seq of activated histone marks (AcH3 and K4me3) in LNCaP cells. Although CtBP2 is known as transcriptional corepressor, CtBP2-binding sites overlapped with such activated histone modifications (61% for AcH3 and 71% for K4me3) androgen dependently (Fig. 5F), suggesting that CtBP2 bindings are highly correlated with activated genes. By combining ChIP-seq data and the gene-expression profiles (analyzed in Fig. 7C), we observed that CtBP2-binding genes were significantly enriched in both androgen-induced and -repressed genes, indicating the direct involvement of CtBP2 in both gene regulations (Fig. 5G).

Repressive effect of CtBP2 on AR-binding tumor-suppressor genes by direct recruitment to ARBSs

We further investigated the molecular mechanism by analyzing the downstream signals of CtBP2 bindings. Among AR- and CtBP2-binding genes that are repressed or induced weakly by androgen, we identified several tumor-suppressor genes such as *FOXO1* and *PTEN* (Supplementary Fig. S6). Thus, we examined the effect of CtBP2 overexpression on the gene expressions. First, FOXO1 expression levels were reduced both at the protein and mRNA levels in these cells (Fig. 6A and B). We identified ARBSs in the promoter and enhancer region of *FOXO1* using AR ChIP-seq data (Fig. 6C). ChIP and ChIP-seq analysis showed AR and CtBP2 recruitment to these ARBSs following androgen treatment, thereby suggesting the androgen-dependent–repressive role of CtBP2 on the promoter and enhancer (Fig. 6C and D and Supplementary Fig. S6A and S6D). Negative regulation of *FOXO1* by CtBP2 was also confirmed by knockdown of *CTBP2* in both LNCaP and VCaP cells (Fig. 6E and Supplementary Fig. S6B and S6C). We performed qRT-PCR analysis and then observed that *FOXO1* expression levels and androgen-dependent induction were enhanced by siRNA transfection.

Moreover, another tumor-suppressor gene, PTEN, has been reported to be repressed by DHT in AR-positive prostate

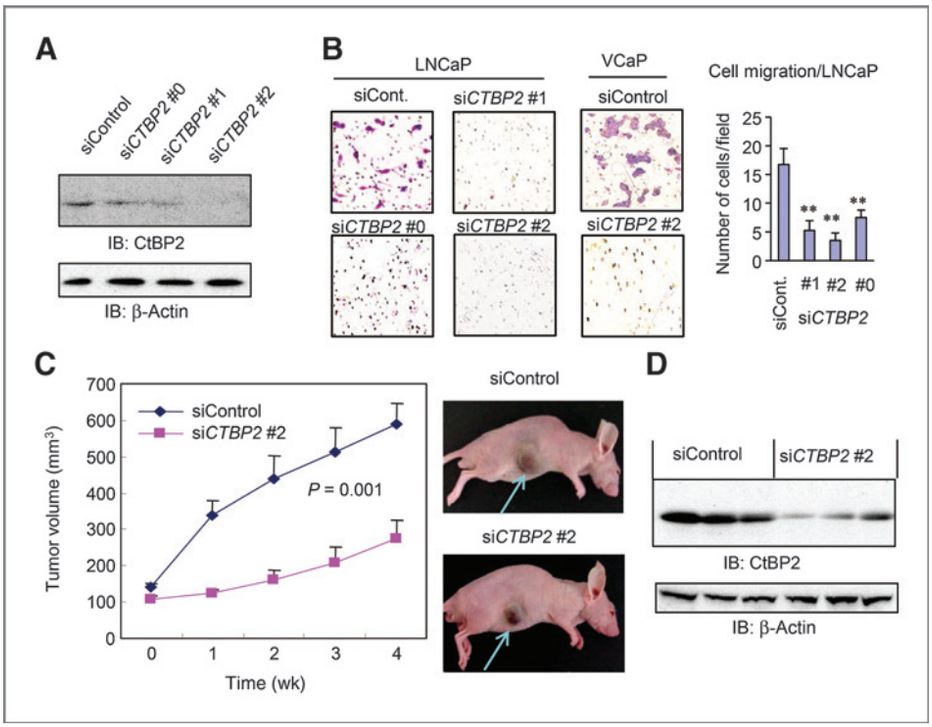

Figure 4. CtBP2 could be a therapeutic target of prostate cancer. A, knockdown of CtBP2 by selected siRNAs (si*CTBP2* #1 and #2). LNCaP cells were transfected with si*CTBP2* #0–2 (10 nmol/L) or siControl (10 nmol/L). B, knockdown of CtBP2 inhibits cell migration of prostate cancer cells. Cells were transfected with si*CTBP2* #0–2 (10 nmol/L) or siControl (10 nmol/L) and then migration assay was performed; $n = 5$; **, $P < 0.01$. C, left, nude mice were inoculated with LNCaP cells and growth curves of tumors in nude mice treated with siControl or si*CTBP2* #2 ($n = 7$); bar, SD (right). Right, representative view of LNCaP tumors. D, knockdown of CtBP2 in tumors transfected with si*CTBP2* ($n = 3$).

cancer cells (18, 19). We also observed AR and CtBP2 recruitment to the promoter of *PTEN* by ChIP and ChIP-seq analysis (Fig. 6D and Supplementary Fig. S7A–S7C). We demonstrated the involvement of CtBP2 in this androgen-dependent repression at transcriptional level in AR-positive prostate cancer cells by qRT-PCR analysis (Supplementary Fig. S7D–S7G). Therefore, these transcriptional-repressive effects of AR and CtBP2 may account for the enhanced cell migration and growth by CtBP2 in prostate cancer.

CtBP2 negatively regulated AR corepressors for transcriptional activation

AR ChIP-seq showed that AR corepressors such as *RIP140* (20) and *NCOR* (21) were also targeted by AR (Fig. 6F and Supplementary Fig. S8A). Our qRT-PCR analysis demonstrated that inductions of these genes were enhanced by CtBP2 knockdown (Fig. 6G and Supplementary Fig. S9A). In addition, ChIP and ChIP-seq data indicated the ligand-dependent recruitment of CtBP2 to the ARBSs of these genes (Fig. 6H and Supplementary Fig. S8B and S8C). We confirmed that decreased levels of corepressors such as *RIP140* and *NCOR* in CtBP2-overexpressing cells by qRT-PCR (Supplementary Fig. S9B). We expect that these negative effects of CtBP2 on AR corepressors may activate AR transcriptional activity.

Next, we investigated the effects of CtBP2 on androgen-mediated transcriptional activity by using two ARBS-containing luciferase vectors (Fig. 7A). Androgen-mediated upregulation of luciferase activity was higher in CtBP2-overexpressing cells than in vector control cells. We also observed upregulation of androgen-regulated genes such as *PSA* and *CTBP1-AS* using qRT-PCR (Fig. 7B), suggesting positive effects of CtBP2 overexpression on these AR-mediated gene inductions.

We investigated the global effects of CtBP2 knockdown on AR-binding genes by microarray analysis (Fig. 7C). Because of the repressive function of CtBP2, *CTBP2* depletion activated the genes that are weakly induced or repressed by DHT. Conversely, we observed inhibition of highly androgen-responsive gene inductions following *CTBP2* knockdown, thereby suggesting the positive effect of CtBP2 on these androgen signals. Positively androgen-regulated genes include CAMKK2 (22, 23), APP (12), and IGF1R (24), which are associated with prostate cancer progression. Thus, we assume that CtBP2 promotes tumor growth by activating such genes.

In addition, such CtBP2-regulated genes are significantly associated with CtBP2 bindings (Supplementary Fig. S10A). Interestingly, motif analysis of CtBP2-binding regions associated with androgen-induced or repressed genes showed differential patterns of enrichments of AR or its collaborative factor motifs (ETS1, ERG, and NF1), suggesting that the transcription complex patterns by such transcription factors may determine the role of CtBP2 in androgen-mediated gene regulation (Supplementary Fig. S10B).

To investigate the mechanism for activation of AR signaling by CtBP2, we analyzed the histone modifications in ARBSs of genes that were positively regulated by CtBP2. We performed ChIP analysis of activated histone markers, H3K4 methylation and AcH3, and repressive marker H3K9 methylation. The results showed the repressive effects of si*CTBP2* on androgen-mediated histone H3 acetylation (Fig. 7D and E) in AR- and CtBP2-colocalized–enhancer region (TMPRSS2 5′ upstream), suggesting the direct role of CtBP2 for activated histone acetylation. One of the positive enhancer markers, histone H3K4 methylation, was not modulated by si*CTBP2* treatment,

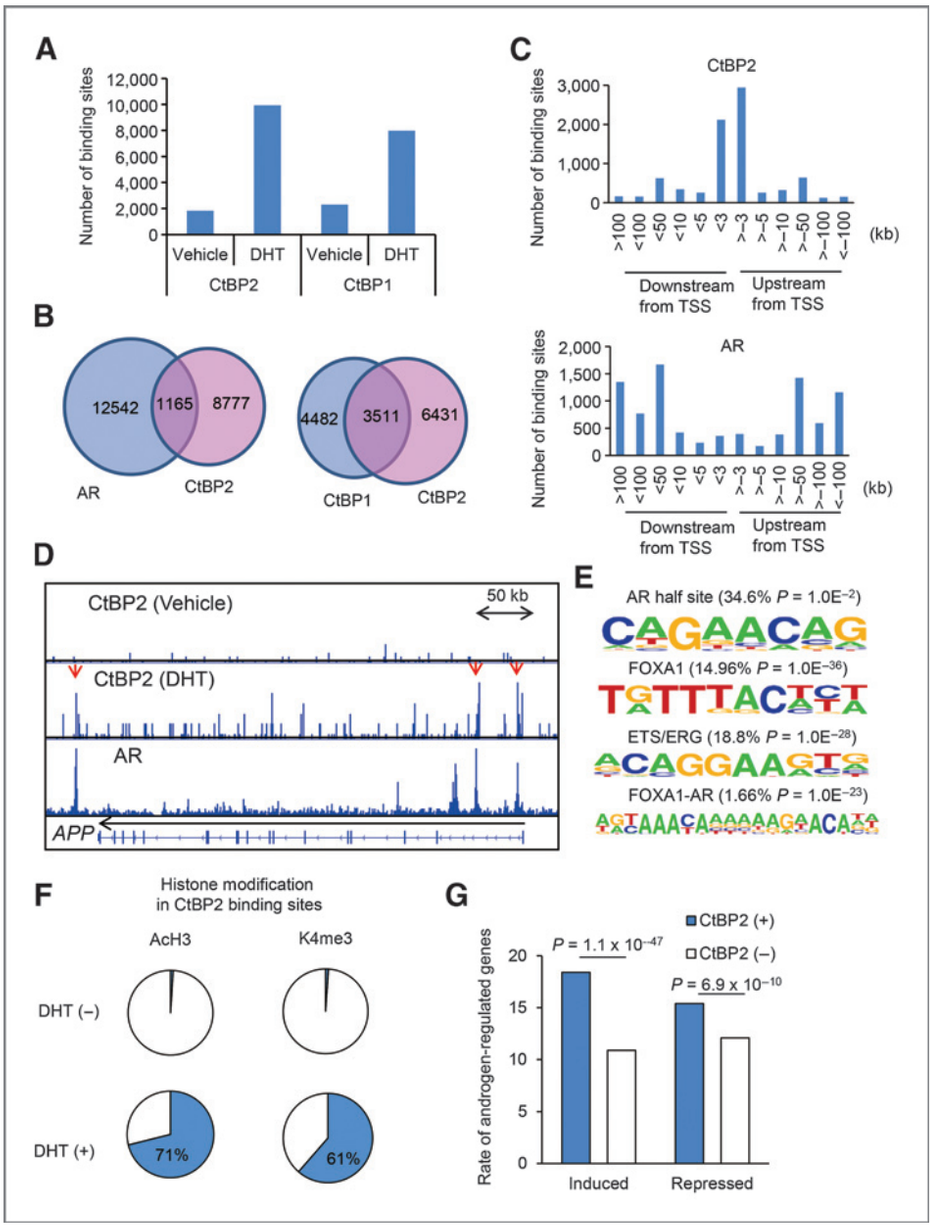

Figure 5. Genome-wide analysis of CtBP2-binding sites in prostate cancer cells by ChIP-seq. A, identification of CtBP1/2-binding sites by ChIP-seq. LNCaP cells were treated with vehicle or DHT for 1 hour in CtBP1 ChIP-seq or 24 hours in CtBP2. Binding sites ($P < 10^{-4}$) were determined by MACS. B, overlapping of CtBP2-binding sites with CtBP1- or ARBSs. Venn diagrams depict the overlap of significant AR-, CtBP1- and CtBP2-binding sites. C, CtBP2-binding sites are enriched around promoter regions. Distribution of AR/CtBP2–binding sites on a genome-wide scale around the TSSs of nearest RefSeq genes is shown. D, mapping of CtBP2- and ARBSs around an androgen-regulated gene, APP. Red arrows, colocalized loci of CtBP2 and AR. Black arrow, the direction of the gene. E, motif analysis of CtBP2-binding sequences. We analyzed 200-bp DNA sequences around CtBP2-binding peaks by using HOMER. Percentages of binding sites containing each motif are shown. F, activated histone modification in CtBP2-binding sites. Genome-wide analysis of histone modifications (H3K4me3 and AcH3) was performed by ChIP-seq. Histone-modified regions were determined by MACS (P value $< 10^{-5}$). Percentages of CtBP2-binding sites overlapped with regions with histone modification are shown. G, CtBP2-binding sites are enriched around androgen-regulated genes. Nearest genes to CtBP2-binding sites were selected as CtBP2 (+) genes. Androgen-regulated genes (induced, fold change > 1.25; repressed, fold change < 0.8) were selected by microarray analysis in Fig. 7C. Percentages of androgen-regulated genes in CtBP2-binding genes [CtBP2 (+)] and other genes were plotted. P values were calculated by the χ^2 test.

whereas H3K9 demethylation was inhibited by si*CTBP2* treatment.

To explore the mechanism for histone modifications by CtBP2 recruitments, we further analyzed the associations of AR with cofactors by immunoprecipitation assay. Interestingly, the results of immunoblots showed that AR interactions with the corepressors such as G9a, HDAC, RIP140, and NCOR were increased by si*CTBP2* treatment (Fig. 7F). Conversely, no

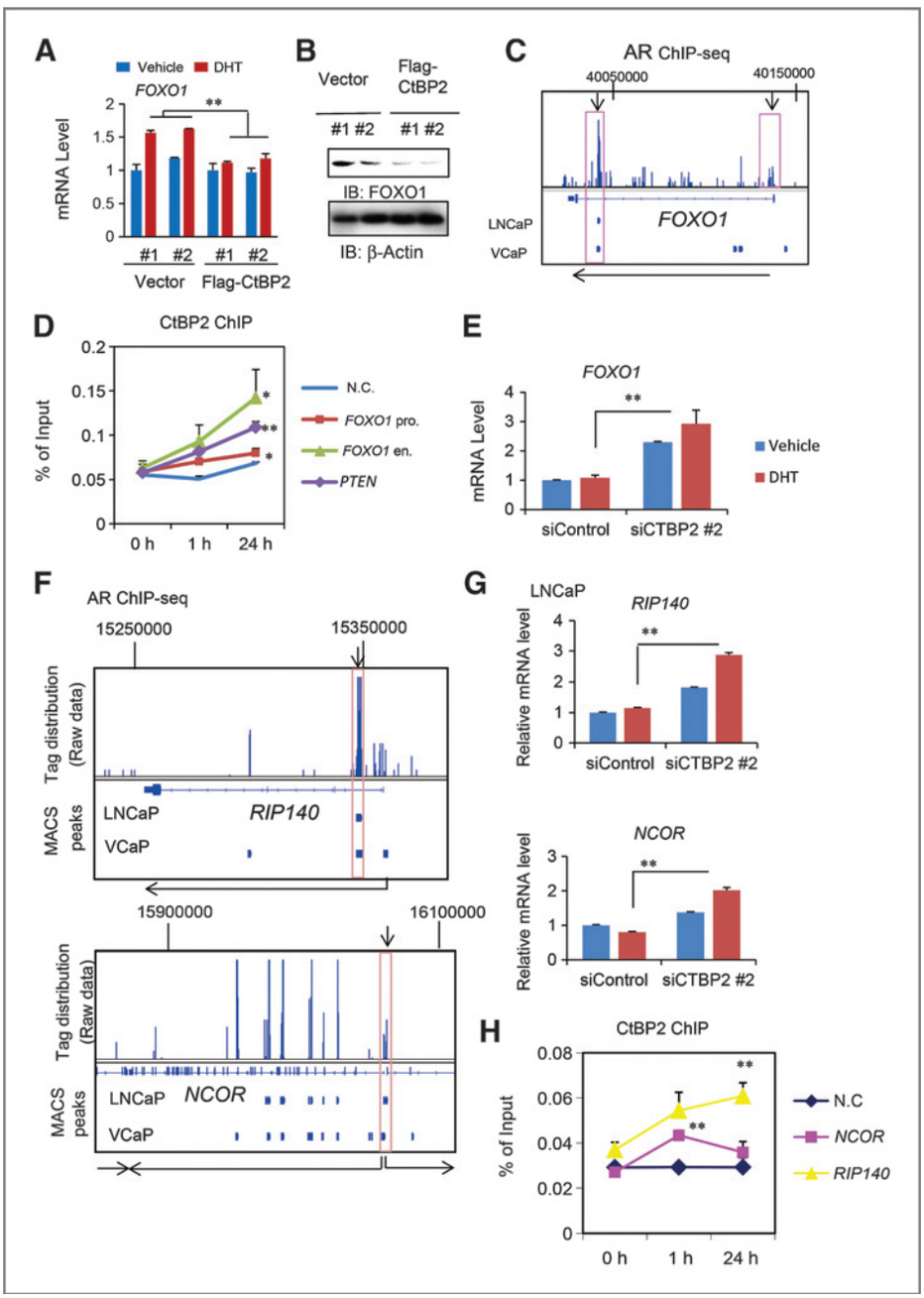

Figure 6. CtBP2 has a repressive effect on the transcription of AR corepressors and tumor-suppressor genes by direct recruitment to ARBS. A, repression of *FOXO1* mRNA by CtBP2 overexpression. Stable cell lines were treated with vehicle or DHT for 24 hours. Expression levels were measured by qRT-PCRs. B, Western blot analysis of *FOXO1* was performed in CtBP2-overexpressing cells or controls. C, AR ChIP-seq view in the *FOXO1* region. Significant ARBSs ($P < 10^{-5}$) were calculated by MACS. Gene direction and the location of primer used for ChIP assay are shown by arrows. D, recruitment of CtBP2 to the ARBSs of *FOXO1* and *PTEN*. LNCaP cells were treated with DHT for 1 and 24 hours and then CtBP2 ChIP analysis was performed. E, *CTBP2* knockdown induces *FOXO1*. LNCaP cells transfected with si*CTBP2* or siControl were treated with vehicle or DHT for 24 hours. Expression levels were determined by qRT-PCRs. F, AR ChIP-seq view in the *RIP140* and *NCOR1* region. Significant ARBSs (*P* value $< 10^{-5}$) were calculated by MACS. Significant ARBSs are shown (red box). Gene direction and the location of the primers used for the ChIP assay are shown by arrows. G, *CTBP2* knockdown induces AR corepressors. LNCaP cells transfected with si*CTBP2* or siControl were treated with vehicle or DHT for 24 hours. Expression levels were determined by qRT-PCRs. H, recruitment of CtBP2 to ARBSs of *RIP140* and *NCOR1*. LNCaP cells were treated with DHT for 0, 1, and 24 hours. CtBP2 ChIP analysis was performed; N.C., negative control locus; bar, SD; $n = 3$; *, $P < 0.05$; **, $P < 0.01$.

significant change was observed in the AR protein level and association of AR with a representative AR coactivator, SRC1. Therefore, AR transcriptional activation may be due to the activated histone patterns caused by CtBP2 inhibitory effect on AR interaction with these corepressors and histone methyltransferases or the transcription of AR-binding corepressors such as *NCOR* and *RIP140* (Supplementary Fig. S11).

Discussion

Members of the CtBP family act as corepressors and modulate gene regulatory networks by interacting with transcription factors (25, 26). CtBPs have PLDS domains that interact with histone-modifying enzymes, such as HDAC, LSD1, and G9a, to modulate histone acetylation and methylation levels. Although the *CTBP* family is associated with cancer, the roles of CtBPs in cancer vary depending on the cancer type (27–29).

CTBP2 was originally identified by GWAS as a candidate susceptibility gene of prostate cancer (7). However, it has been unclear how these SNPs affect the function or expression of CtBP2. In the present study, AR ChIP-seq showed that two SNPs are located in the ARBS within the *CTBP2* gene. We confirmed the lack of each ARE in the vicinity of the SNPs

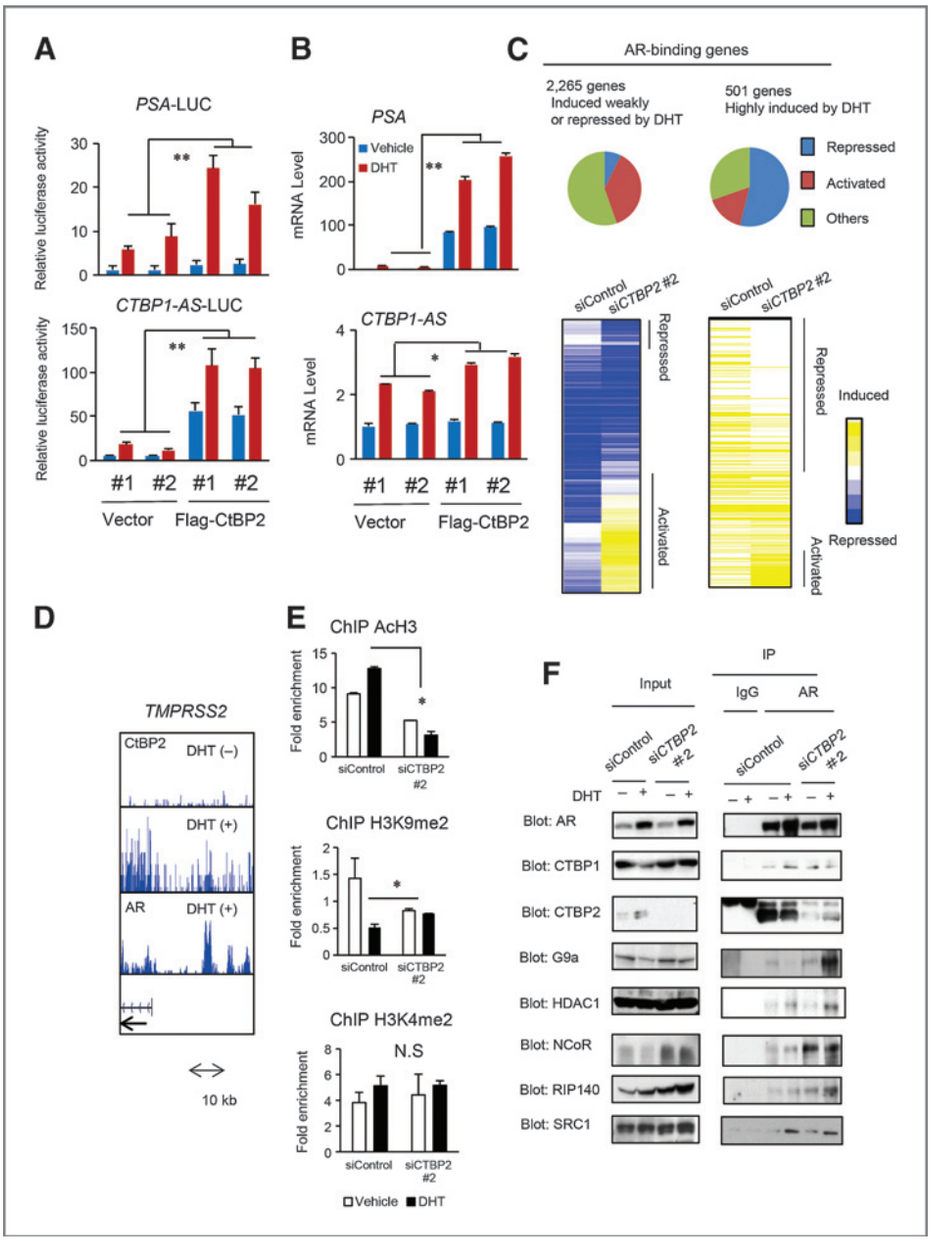

Figure 7. CtBP2 promotes AR-mediated gene activation by regulating corepressor bindings to AR. A, CtBP2 overexpression increased AR transcriptional activity. Luciferase assay (*PSA* and *CTBP1-AS* ARBS-LUC) was performed in CtBP2-overexpressing cells. B, CtBP2 overexpression promotes androgen-mediated transcription. LNCaP cells overexpressing CtBP2 or vector controls were treated with vehicle or DHT for 24 hours. Gene inductions were measured by qRT-PCRs. C, global analysis of CtBP2 effects on androgen regulation of AR-binding genes. LNCaP cells transfected with si*CTBP2* or siControl were treated with vehicle or DHT for 24 hours. AR-binding genes induced weakly (fold < 1.1) and highly (fold > 1.4) were selected by microarray analysis. Effect of knockdown of *CTBP2* was determined by changes of fold inductions. D, CtBP2 ChIP-seq view of TMPRSS2-enhancer region is shown. E, ChIP analysis of acetylated histone H3, dimethylated histone H3K9, and H3K4 was performed in TMPRSS2-enhancer region; bar, SD; $n = 3$ (A, B, and E); *, $P < 0.05$; **, $P < 0.01$. N.S., not significant. F, CtBP2 knockdown increased AR association with repressive histone modifiers. LNCaP cells transfected with si*CTBP2* #2 or siControl were treated with vehicle or DHT for 24 hours. Cell lysates were immunoprecipitated by AR antibodies or normal IgG. Western blot analysis of each cofactors or enzymes was performed.

reduced androgen-mediated–enhancer activity, suggesting the importance of both AREs probably by cooperative action. We demonstrated that these SNPs change increased the androgen-dependent–enhancer activity of the ARBS. Because the SNP change makes palindrome ARE from half ARE sequence, it is possible that this sequence variation may alter the AR-binding ability for activated transcription of the gene. Moreover, as indicated by our immunohistochemical analysis, increased expression may be associated with prostate cancer progression.

We presented a novel regulatory mechanism for AR transcriptional regulation by CtBPs. CtBP1 represses AR-regulated gene inductions in a ligand-dependent manner. *CTBP1* knockdown accelerates gene induction at an early time point after androgen treatment (1). However, androgen-mediated gene induction at an early time point was not observed by *CTBP2* knockdown. These differences may be due to the differential regulation of both isoforms by androgen. Androgen treatment represses CtBP1 by activating its antisense RNA, and the release of the repressor CtBP1 from the regulatory regions of AR-regulated genes leads to the transcriptional activation (1). In contrast, CtBP2 interacts with AR even at a later time point following androgen treatment because of AR-mediated induction of CtBP2. In addition, overexpression of CtBP2 did not repress AR-mediated transcriptional activity; rather it activated representative AR-binding promoters and enhancers. We suggest that the direct repressive effect of CtBP2 may be weak compared

with that of CtBP1 and be effective only on a subset of AR-binding genes.

Although many studies have revealed CtBP functions, only a few have shown the difference between CtBP1 and CtBP2 (30, 31) because both proteins have more than 80% similarity with respect to their amino acid sequences. CtBP2 has specific amino acids at the N-terminal region and *in vitro* analysis has revealed that this sequence may be involved in the functional difference between CtBP1 and CtBP2 (32, 33). Therefore, they have dissimilar interactions with repressive histone-modifying enzymes. For example, acetylation of CtBP2 by CBP released G9a from CtBP2 (34). Our experimental results demonstrated that CtBP2 interactions with G9a are weak compared with CtBP1 (Fig. 2D). We expect that such differential corepressor interactions may impact on the epigenetic effects by the CtBP family proteins.

As indicated by our genome-wide analysis using ChIP-seq and microarray analysis, CtBP2 promotes the androgen-dependent induction of a subset of AR- and CtBP2-binding genes. By *CTBP2* knockdown, AR interacted with more corepressors, such as HDACs, to repress histone acetylation. Therefore, CtBP2 recruitment may inhibit the AR interactions with corepressors at highly activated enhancers or promoters. Meanwhile, we observed that CtBP2 also has direct repressive effects on a subset of AR-binding genes, such as FOXO1 and PTEN. These genes are weakly induced or repressed by androgen. However, knockdown of *CTBP2* increased their expression levels or inductions by androgen, suggesting the involvement of CtBP2 in repression of these genes in prostate cancer. FOXO1 is a tumor-suppressor gene and its high expression is associated with a good prognosis of patients with prostate cancer (35). FOXO1 also interacts with AR to negatively regulate AR-mediated transcriptional activity (36, 37). In addition, as an indirect mechanism of transcriptional activation by CtBP2, we found that CtBP2 transcriptionally repressed negative regulators of AR, such as *NCOR* or *RIP140*. Taken together, these findings suggest that CtBP2 regulates the sensitivity to androgen by modulating corepressor complexes at AR-binding enhancers or promoters.

The concept of a feedback mechanism by which AR controls its transcriptional activity by androgen-regulated CtBP2 is in line with the past reports regarding other coactivators. For example, androgen-responsive FHL2 coactivates AR for robust AR activation in the progression to CRPC (38–40). In contrast, we showed a novel mechanism in which CtBP2 functions as corepressors to promote transcriptional repression by AR. Moreover, CtBP2 promotes AR-mediated gene induction by blocking AR association with other coregulator complexes. Such differential functions of CtBP2 as a repressor or activator may be dependent on the AR-centered complex by AR-collaborating transcription factors as suggested by our motif analysis. Taken together, our proposed mechanism suggests that AR transcriptional regulation is governed by such feedback loops for AR-mediated gene repression or induction.

We also presented the potential of CtBP2 for use as a novel therapeutic target. Injections of si*CTBP2* reduced tumor growth in a prostate cancer xenograft model of AR-positive prostate cancer cells. In addition, high expression of CtBP2 is associated with poor survival rates in patients with prostate cancer. Given that AR expression and activity is increased in CRPC, targeting CtBP2 would be a promising approach to treat CRPC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Takayama, S. Takahashi, Y. Homma, S. Inoue
Development of methodology: K. Takayama
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Takayama, T. Fujimura, S. Takahashi, S. Inoue
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Takayama, T. Suzuki, T. Urano, S. Inoue
Writing, review, and/or revision of the manuscript: K. Takayama, S. Takahashi, Y. Homma, S. Inoue
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Takayama, T. Fujimura, Y. Homma, S. Inoue
Study supervision: T. Urano, Y. Homma, S. Inoue

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