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Cancer Res 2011;71:4866-4876. Published OnlineFirst May 18, 2011.

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CCI-779 Inhibits Cell-Cycle G₂-M Progression and Invasion of Castration-Resistant Prostate Cancer via Attenuation of UBE2C Transcription and mRNA Stability

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Abstract

The cell-cycle G₂-M phase gene *UBE2C* is overexpressed in various solid tumors including castration-resistant prostate cancer (CRPC). Our recent studies found *UBE2C* to be a CRPC-specific androgen receptor (AR) target gene that is necessary for CRPC growth, providing a potential novel target for therapeutic intervention. In this study, we showed that the G₁-S cell-cycle inhibitor-779 (CCI-779), an mTOR inhibitor, inhibited *UBE2C* mRNA and protein expression in AR-positive CRPC cell models abl and C4-2B. Treatment with CCI-779 significantly decreased abl cell proliferation *in vitro* and *in vivo* through inhibition of cell-cycle progression of both G₂-M and G₁-S phases. In addition, exposure of abl and C4-2B cells to CCI-779 also decreased *UBE2C*-dependent cell invasion. The molecular mechanisms for CCI-779 inhibition of *UBE2C* gene expression involved a decreased binding of AR coactivators SRC1, SRC3, p300, and MED1 to the *UBE2C* enhancers, leading to a reduction in RNA polymerase II loading to the *UBE2C* promoter, and attenuation of *UBE2C* mRNA stability. Our data suggest that, in addition to its ability to block cell-cycle G₁ to S-phase transition, CCI-779 causes a cell-cycle G₂-M accumulation and an inhibition of cell invasion through a novel *UBE2C*-dependent mechanism, which contributes to antitumor activities of CCI-779 in *UBE2C* overexpressed AR-positive CRPC. *Cancer Res*; 71(14); 4866-76. ©2011 AACR.

Introduction

The androgen receptor (AR), a ligand-dependent transcription factor, is expressed in both androgen-dependent prostate cancer (ADPC) and castration-resistant prostate cancer (CRPC; refs. 1, 2). One important function of AR in prostate cancer is to drive cell-cycle progression (3). Although it is well known that AR mainly regulates cell-cycle G₁ to S-phase transition in ADPC through AR-dependent regulation of CCND1, p21, and p27 (3), recent integrated analysis of AR cistrome and gene expression data in prostate cancer found that AR selectively binds to the enhancers of G₂-M phase genes in CRPC but not in ADPC, leading to higher G₂-M phase

gene expression and accelerated cell-cycle G₂-M progression in CRPC versus ADPC (4, 5).

One of such AR-regulated G₂-M phase genes in CRPC is *UBE2C*, a gene whose translation product is an anaphase-promoting complex/cyclosome (APC/C)-specific E2 ubiquitin-conjugating enzyme (6). Significantly, *UBE2C* mRNA and protein expression levels are overexpressed in CRPC cases (4, 7, 8). Consistent with the essential role of *UBE2C* in driving M-phase cell-cycle progression by inactivating the M phase checkpoint (9) or increasing the pool of active APC/C (10), silencing of *UBE2C* in CRPC cells arrests cell cycle in G₂-M phase and decreases CRPC cell proliferation, suggesting that *UBE2C* is a potential therapeutic target in CRPC (4).

In this study, we screened several clinically active compounds for their ability to decrease *UBE2C* expression. CCI-779 (cell-cycle inhibitor-779; temsirolimus), an ester analogue of mTOR inhibitor rapamycin currently under clinical evaluation (11), emerged from screening to have significant efficacy and potency in inhibition of *UBE2C* protein and mRNA expression in AR-positive CRPC cell lines abl and C4-2B. Although previous studies found that mTOR inhibitors, including CCI-779, decrease the growth of cancer cell lines (e.g., AR-negative CRPC cell lines PC-3 and DU-145) via G₁ to S-phase inhibition (12, 13), we show that CCI-779 inhibits the abl *in vitro* and *in vivo* growth by blocking both cell-cycle G₂-M and G₁-S transitions. Consistent with the newly identified role of *UBE2C* in promoting tumor invasion and metastasis

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-10-4576

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(14–16), we found that CCI-779 treatment decreases UBE2C-dependent cell invasion of abl and C4-2B cells. Finally, we found that the combined effects on attenuating UBE2C transcription and mRNA stability of CCI-779 lead to decreased mRNA levels of UBE2C. Collectively, this study identifies CCI-779 as a UBE2C inhibitor in CRPC.

Materials and Methods

Reagents and cell culture

CCI-779 (temsirolimus) was purchased from LC Laboratories. LNCaP cells were obtained from the American Type Culture Collection (ATCC) and C4-2B cells were purchased from ViroMed Laboratories. LNCaP and C4-2B cells were cultured in RPMI-1640 media (Invitrogen) supplemented with 10% FBS and authenticated by the suppliers. abl cells, an androgen-independent derivative of the LNCaP cell line, were kindly provided by Zoran Culig (Innsbruck Medical University, Innsbruck, Austria) and authenticated by Culig Laboratory, using AR sequence analysis, cytogenetic analysis, and comparative genome hybridization analysis (17). The abl cells were maintained in RPMI-1640 media containing 10% charcoal-stripped FBS. All 3 cell lines were passaged in our laboratory for less than 6 months after resuscitation.

Western blot

Cells or tumor tissues were collected and lysed as previously described (18). The total lysate sample (50 µg per lane) was resolved by SDS-PAGE and immunoblotted with primary antibodies. Antibodies against various proteins were purchased from the following sources: anti-UBE2C (A650) from Boston Biochem; anti-AR (441), anti-GATA2 (H116), anti-SRC1 (M341), anti-p300 (C20), and anti-MED1 (M255) from Santa Cruz Biotechnology; anti-CCND1 (ab24249) and anti-FoxA1 (ab23738) from Abcam; anti-calnexin from Stressgen, and anti-β-actin from Sigma-Aldrich. An anti-SRC3 antibody has been described previously (19).

Real-time reverse transcriptase PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen). Real-time reverse transcriptase PCR (RT-PCR) was conducted on 100 ng of RNA by using MultiScribe Reverse Transcriptase and SYBR Green PCR Kit (Applied Biosystems), according to the manufacturer's instructions. The following primers were used: UBE2C (5'-TGGTCTGCCCTGTATGATGT-3' and 5'-AAAAGCTGTGGGGTTTTTCC-3'; ref. 20); CCND1 (5'-TCCTCTCCAAATGCCAGAG-3' and 5'-GGCGGATTGGAAATGAACTT-3'), GAPDH (5'-TCCACCCATGGCAAATTC-3' and 5'-TCGCCCCACTTGATTTTGG-3'; ref. 19), and actin (5'-AGGCACCAGGGCGTGAT-3' and 5'-GCCACATAGGAATCCTTCTGAC-3'; ref. 21).

RNA interference

ON-TARGETplus siRNAs targeting CCND1 and UBE2C (siCCND1 and siUBE2C) and ON-TARGETplus control siRNA (siControl) were purchased from Dharmacon. siRNAs were transfected using Lipofectamine 2000 (Invitrogen).

Synchronization and fluorescence-activated cell-sorting analysis

Cells were arrested in G₂-M phase by using a thymidine-nocodazole block as previously described (22). Briefly, cells were first synchronized by arresting them at the G₁-S border with 2 mmol/L thymidine for 24 hours, followed by a 4-hour release and then cells were arrested at M phase with 100 ng/mL nocodazole for 12 hours. CCI-779 (50 nmol/L) or vehicle control was added at the same time as nocodazole. The cells were released from the nocodazole block with 2 washes of fresh medium and allowed to progress to G₁ phase. Cells were collected after the release (2 hours for abl cells, 1 hour for C4-2B cells, and 1.5 hours for LNCaP cells), stained with propidium iodide (PI; Sigma) and subjected to analysis by using a FACSCalibur cell flow cytometer (Becton Dickinson Biosciences). Fluorescence-activated cell-sorting (FACS) analysis was also done on unsynchronized cells after 13 hours of exposure to 50 nmol/L CCI-779.

Cell proliferation assay

Cell proliferation was measured by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay according to the manufacturer's instruction (Roche). Briefly, this assay entails the addition of 10 µL WST-1 reagents per 100 µL cell cultures in a 96-well plate. These cultures were incubated for 30 minutes and the absorbance at 450 nm was determined by an ELISA Microplate Reader (Bio-Rad).

Xenograft model

Male, 6-week-old, Balb/c athymic nude mice were obtained from Charles River Laboratory and acclimated for 1 week in a pathogen-free enclosure before start of study. All experiments were conducted in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). abl cells (2 × 10⁶ cells/flank) were suspended in 50% Matrigel (Becton Dickinson) and subcutaneously inoculated bilaterally into the flanks of mice, monitored daily, and tumor size was quantified with calipers twice a week (17, 23). When tumors had grown to 100 mm³, treatments were initiated. Mice were randomly assigned into 2 cohorts with 10 mice per group. CCI-779 (treated group) or vehicle solution [5% Tween 80 (Sigma) and 5% polyethylene glycol 400 (Sigma); ref. 13; control group] was given intraperitoneally (i.p.) at the dose of 10 mg/kg for 4 consecutive days per week (24). The injection volume was 0.1 mL/10 g body weight. Tumor volume was calculated by using the standard formula: $V = \text{length} \times \text{width}^2 \times 0.5$. Body weight was also monitored biweekly. After 4 weeks, mice were euthanized and tumor tissues were weighed and subjected to Western blot analysis.

Transfection and invasion assay

Cells grown in 6-well plates were transfected with siUBE2C or siControl, or 2 µg of pCS2-myc-UBE2C (kindly provided by Michael Rape, University of California, Berkeley) or a control pCS2-myc vector (a gift from David Turner, University of Michigan), using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, 4 × 10⁵ cells were seeded on the

Matrigel-coated filters (BD BioCoat BD Matrigel Invasion Chamber; Becton Dickinson) in the upper chamber, which was filled with media containing 0.1% FBS. The lower chamber was filled with media supplemented with 10% FBS. Both chambers were treated with 50 nmol/L CCI-779 or vehicle. The cells were allowed to invade for 48 hours. The cells on the underside of the filter were then fixed with 80% methanol, stained with 0.3% crystal violet, and counted using a light microscope. The invasion results were normalized by cell proliferation under the same treatment conditions.

Chromatin immunoprecipitation and ReChIP

Chromatin immunoprecipitation (ChIP) was done as previously described (19). The antibodies used were: anti-AR (N20), anti-GATA2 (H116), anti-SRC1 (M341), anti-SRC3, anti-p300 (C20), anti-MED1 (M255) from Santa Cruz Biotechnology; anti-FoxA1 (ab23738) from Abcam; and anti-RNA pol II (8WG16) from Covance. SRC3 ChIP was done with SRC3 antibodies as previously described (19). The ChIP-enriched DNA was then quantified by quantitative PCR (qPCR) using specific primers for the *UBE2C* enhancers 1 and 2 (4) and the *UBE2C* promoter, respectively. Negative controls used were sequences containing androgen-responsive elements (ARE) that do not have actual binding to AR and FoxA1 (4). The primer sequences used in ChIP assay were as follows: *UBE2C* enhancer 1 (5'-TGCCTCTGAGTAGGAACAGGTAAGT-3' and 5'-TGCTTTTTCATCATGGCAG-3'; ref. 4); *UBE2C* enhancer 2 (5'-CCACAACTCTTCTCAGCTGGG-3' and 5'-TTCTTTCTTCCCTGTTACCCC-3'; ref. 4); *UBE2C* promoter (5'-GCCC-GAGGGAAATTGGAT-3' and 5'-TTACTCCGCGTGGGAACT-3'); control ARE region 1 (5'-CACAGAATCAGTCTAGGGTGCTCTT-3' and 5'-CTGCATGCTCAAGGAGTG-TGTT-3'; ref. 4), and control ARE region 2 (5'-GCTGATTCAATTACCTCCCAGAA-3' and AGTTTGGGACAGACGGGAAA-3'; ref. 4). ReChIP assays were carried out as previously described (25).

mRNA stability assay

abl cells were treated with 50 nmol/L CCI-779 or vehicle. Simultaneously, 5 µg/mL actinomycin D (Sigma) was used to block mRNA synthesis. Cells were collected at various time points (0, 6, 9, 12, 18, and 24 hours) after treatment and *UBE2C* mRNA level was quantified by quantitative reverse transcriptase PCR (qRT-PCR). The $t_{1/2}$ was calculated using the iterative curve-fitting software SigmaPlot (SPSS) by fitting 4-parameter exponential decay curves described by the formula $y = a * \exp [b / (c * x + d)]$. Estimations of c , designated as C , and corresponding standard errors, designated as $SE(C)$, were used to calculate standard error ($t_{1/2} = \log(2)/C$). $t_{1/2} \pm SE(t_{1/2})$ was calculated as follows: $\log(2)/[C + SE(C)]$, $\log(2)/[C - SE(C)]$.

Results

CCI-779 downregulates *UBE2C* protein and mRNA expression levels in CRPC and ADPC cells

Our initial compound screenings for identification of *UBE2C* inhibitors were carried out on abl cells. As a CRPC cell model, abl mimics the clinical properties of a significant

proportion of CRPC cases. For example, recent studies reporting that AR upregulates cell-cycle genes (e.g., *UBE2C*, *CDC20*, and *CDK1*) in abl cells mimic the pattern of upregulated genes observed in human CRPC versus ADPC cases (4, 7, 8). CCI-779 emerged from screening because it potentially decreased both protein and mRNA levels of *UBE2C* in abl cells (Fig. 1A and B). We further extended our study to another CRPC cell model C4-2B that overexpresses the AR (26). We confirmed that *UBE2C* protein and mRNA levels were significantly decreased in CCI-779-treated C4-2B cells (Fig. 1A and B). Interestingly, the inhibitory effect of CCI-779 on *UBE2C* protein and mRNA levels was also seen in an ADPC cell line LNCaP, although the effect was less effective than that observed in abl and C4-2B cells (Fig. 1A and B). Consistent with previous studies showing that the mTOR pathway is required for translation of mRNAs of critical G₁ phase cell-cycle genes such as *CCND1* (12), we found that treatment of abl, C4-2B, and LNCaP cells with CCI-779 significantly reduced *CCND1* protein, but not mRNA, expression level (Fig. 1A and B). Thus, CCI-779 decreases protein expression levels of both *UBE2C* and *CCND1*, as well as *UBE2C* mRNA expression level in prostate cancer cells.

To further investigate whether CCI-779-mediated decrease in *UBE2C* mRNA expression was dependent on CCI-779-induced reduction in *CCND1* protein expression, we examined the effect of CCI-779 on *UBE2C* mRNA level in *CCND1*-silenced and control-silenced abl cells. Silencing of *CCND1* caused a complete cell-cycle G₁ arrest, which was barely enhanced by CCI-779 treatment (Fig. 1C and D). Significantly, treatment of abl cells with CCI-779 decreased *UBE2C* mRNA level in abl cells already arrested in G₁ phase (Fig. 1D). These results indicate that CCI-779 can directly decrease *UBE2C* mRNA expression in a *CCND1* expression and G₁ arrest independent manner. We obtained essentially similar results in C4-2B cells (Supplementary Fig. S1).

CCI-779 blocks both G₂-M and G₁-S cell-cycle progression and decreases cell proliferation in CRPC and ADPC cells

Because *UBE2C* plays an essential role in promoting G₂-M phase cell-cycle progression in prostate cancer cells (4) and CCI-779 inhibited *UBE2C* expression (Fig. 1), we next examined the effect of CCI-779 on G₂-M phase cell-cycle progression. abl, C4-2B, and LNCaP cells were synchronized to G₂-M phase by using a thymidine-nocodazole block and then released for 1 to 2 hours. As shown in Figure 2A, whereas treatment of cells with CCI-779 had no effect on G₂-M synchronization, CCI-779 treatment led to an increase in the G₂-M phase and a decrease in the G₁ phase after releasing from G₂-M synchronization, suggesting that CCI-779 markedly delayed G₂-M to G₁ transition in all 3 cell lines. Consistent with the functional role of CCI-779 in decreasing *CCND1* protein expression level (Fig. 1A) and a recent study showing that CCI-779 arrests prostate and breast cancer cells in G₁ phase (24), CCI-779 blocked unsynchronized abl, C4-2B, and LNCaP cells in G₁ phase (Fig. 2B). The inhibition of CCI-779 on G₂-M and G₁-S cell-cycle progression was correlated with a significantly decreased cell proliferation of abl, C4-2B, and LNCaP (but more notably abl and C4-2B cells; Fig. 2C).

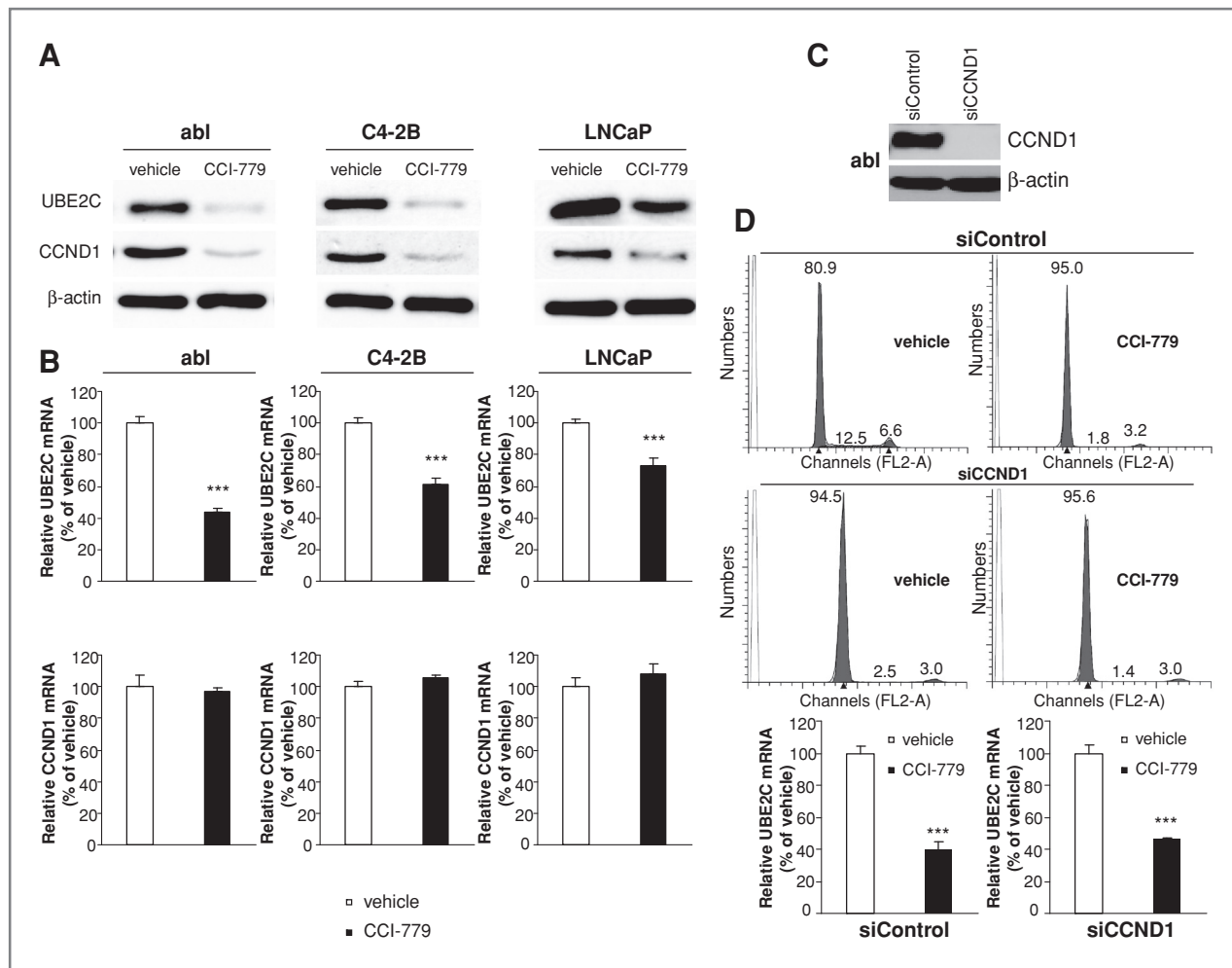


Figure 1. Effects of CCI-779 on UBE2C and CCND1 expression in CRPC and ADPC cells. **A**, CCI-779 downregulates protein expression levels of UBE2C and CCND1 in abl, C4-2B, and LNCaP cell lines. Cells were treated with 50 nmol/L CCI-779 or vehicle for 24 hours and Western blot analyses were carried out with indicated antibodies. **B**, CCI-779 decreases mRNA expression level of UBE2C but not CCND1. Cells were incubated with 50 nmol/L CCI-779 for 24 hours. Total RNA was isolated and analyzed by qRT-PCR, using gene-specific primers. The results represent mean $\pm$ SE of 3 experiments conducted in triplicate. ***, $P < 0.001$ as compared with the vehicle control. **C**, silencing of CCND1 decreases CCND1 protein expression in abl cells, which were transfected with siControl or siCCND1. Western blot analyses were carried out 48 hours after transfection, using the indicated antibodies. **D**, CCI-779 decreases UBE2C mRNA expression in both siControl- and siCCND1-transfected abl cells. Top, FACS analyses were carried out using siControl- or siCCND1-transfected abl cells treated with CCI-779 or vehicle for 24 hours. Cell number (%) in each cell-cycle phase is indicated in the graph. Bottom, total RNA was isolated from siControl- or siCCND1-transfected abl cells treated with CCI-779 or vehicle for 24 hours. qRT-PCR was then conducted using UBE2C-specific primers. ***, $P < 0.001$ as compared with the vehicle control.

To further delineate the role of UBE2C in CCI-779-mediated inhibition of CRPC cell proliferation, the effects of CCI-779 on cell proliferation of UBE2C-silenced or control-silenced abl and C4-2B cells were examined. The inhibitory effect of CCI-779 on abl cell proliferation was markedly decreased in UBE2C-silenced (28%) versus control-silenced (45%) abl cells (Fig. 2D), suggesting that UBE2C-silencing-mediated abl cell growth inhibition (Fig. 2D) significantly contributes to growth-inhibitory effect of CCI-779 on abl (Fig. 2C). In contrast, as UBE2C silencing only slightly decreased C4-2B cell proliferation (Fig. 2D), CCI-779-mediated inhibition of C4-2B cell proliferation (Fig. 2C) was presumed to be mostly due to CCI-779-induced decreased

expression of CCND1 rather than UBE2C in C4-2B cells (Figs. 1A and 2D).

CCI-779 inhibits *in vivo* growth of abl xenograft through downregulation of UBE2C and CCND1

We further extended our studies to an *in vivo* xenograft model to validate the significance of our *in vitro* findings. Approximately 2 weeks after the inoculation of abl cells, mice were treated with CCI-779 (10 mg/kg, i.p.) for 4 consecutive days weekly for 4 weeks. Mice generally tolerated CCI-779 without showing any apparent toxicity throughout the experiment. No significant difference in body weight was observed between groups after the 4-week period (data not shown).

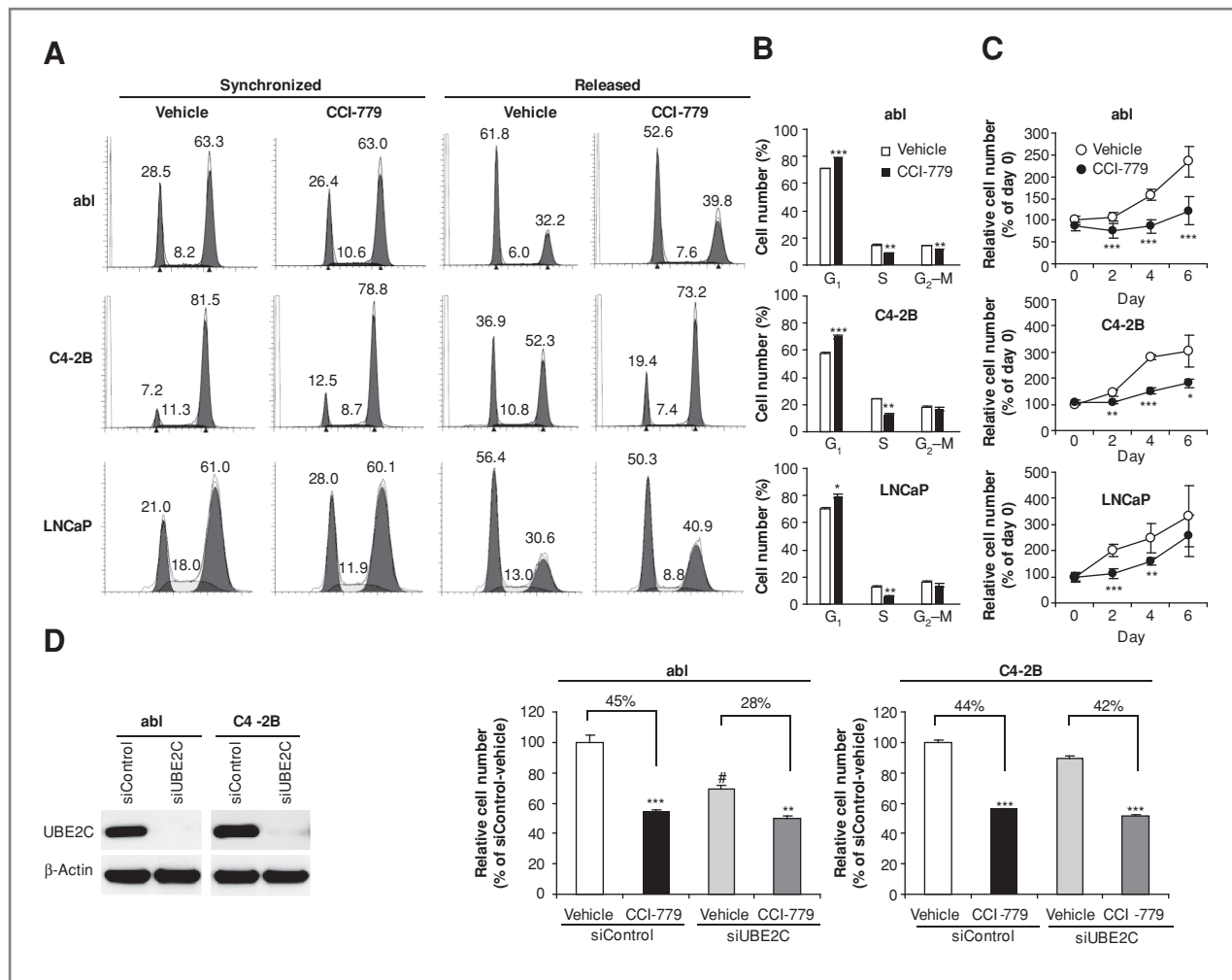


Figure 2. Effects of CCI-779 on CRPC and ADPC cell-cycle progression and cell proliferation. A, CCI-779 delays cell-cycle G₂-M to G₁ phase transition. FACS analyses were carried out using cells released from thymidine-nocodazole block in the presence or absence of CCI-779 (50 nmol/L). Cell number (%) in each cell-cycle phase is indicated in the graph. B, CCI-779 arrests unsynchronized cells in G₁ phase. Thirteen hours after exposure of cells to 50 nmol/L CCI-779, FACS analyses were carried out. C, CCI-779 inhibits cell proliferation. Cell proliferation was determined by WST-1 assay on the indicated days (0, 2, 4, and 6) in the absence or presence of CCI-779 (50 nmol/L). The data are presented as a percentage of the cell number on day 0. The results are mean $\pm$ SE of 2 to 3 independent experiments conducted in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ as compared with vehicle control. D, effects of CCI-779 treatment on cell proliferation of UBE2C-silenced or control-silenced abl and C4-2B cells. abl or C4-2B cells were transfected with siControl or siUBE2C. Eight hours posttransfection, cells were treated with CCI-779 or vehicle and cell proliferation assays were conducted on day 4. **, $P < 0.01$; ***, $P < 0.001$ as compared with corresponding vehicle control; #, $P < 0.001$ as compared with siControl-transfected vehicle control.

Remarkably, the tumor growth was inhibited by CCI-779 even after 1 week of treatment. By the end of the study, the tumor volume dramatically decreased from $234 \pm 33 \text{ mm}^3$ in control group to $57 \pm 4 \text{ mm}^3$ in CCI-779-treated group (Fig. 3A and B). In addition, there was no measurable tumor in 4 of 10 CCI-779-treated mice. Tumor weight measurement further supported our findings, as the average value was $78.6 \pm 15.5 \text{ mg}$ for control group as compared with $13.5 \pm 2.5 \text{ mg}$ for the CCI-779 group (Fig. 3C). More importantly, Western blot analysis confirmed that levels of both UBE2C and CCND1 protein were significantly decreased in tumor tissues following treatment with CCI-779 (Fig. 3D). These data suggest that CCI-779 significantly decreases CRPC cell *in vivo* growth through

inhibition of UBE2C and CCND1. Similar effect of CCI-779 on CRPC cell growth and protein expression of CCND1 and UBE2C was observed in castrated mice (Supplementary Fig. S2).

CCI-779 inhibits UBE2C-dependent CRPC cell invasion *in vitro*

The role of UBE2C is not limited to promoting cell growth. Recent studies have found that UBE2C expression is positively correlated with metastasis in patients with various cancer types, including colorectal cancer (14), breast cancer (15), and soft tissue tumors (16). Consistent with these clinical observations, an *in vitro* study has shown that UBE2C downregulation

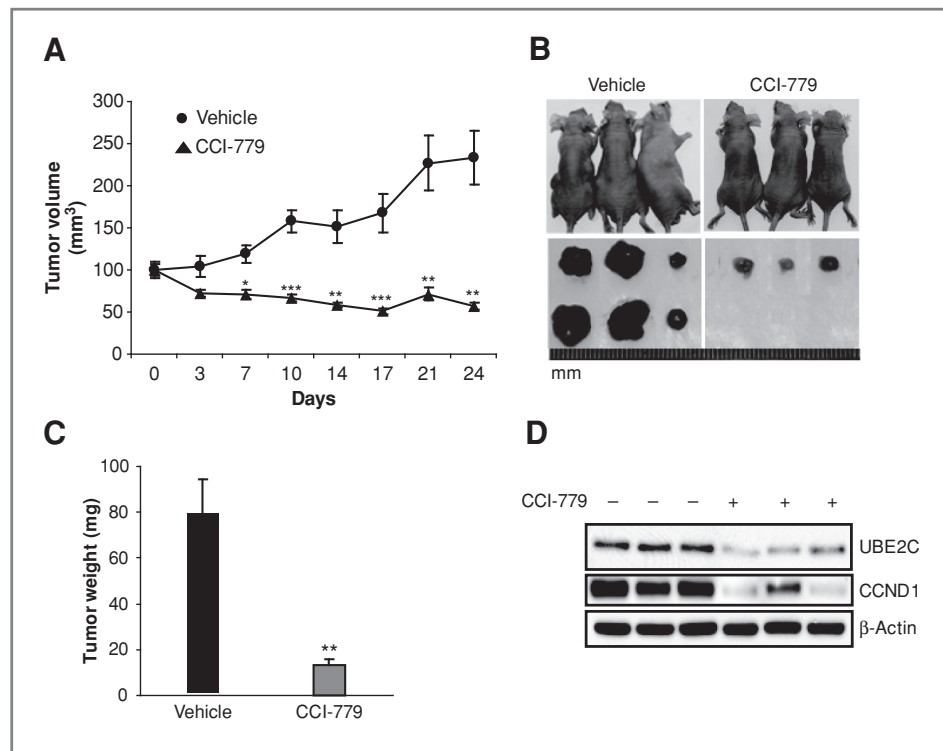


Figure 3. CCI-779 inhibits *in vivo* CRPC tumor growth through downregulation of UBE2C and CCND1. **A**, CCI-779 inhibits the growth of subcutaneous abl xenograft tumors in nude mice. abl cells (2×10^6 /flank) were inoculated into both flanks of male Balb/c nude mice, and the treatments were initiated when tumor size reached 100 mm³. The mice were i.p. injected with 10 mg/kg CCI-779 or the vehicle solution for 4 consecutive days every week for 4 weeks (days 0–3; days 7–10; days 14–17; and days 21–24). Tumor volume was measured twice per week and normalized to the percentage of the initial tumor size, which was assigned as 100%. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with vehicle group; $n = 10$ mice for each group. **B**, representative images of tumor-bearing mice and their tumors on day 25 after treatments. **C**, average tumor weight for control and CCI-779-treated groups; $n = 16$, for control group; $n = 8$, for CCI-779-treated group. **, $P < 0.01$. **D**, Western blot analysis shows that CCI-779 reduces protein expression of UBE2C and CCND1 in engrafted tumor tissues. β-Actin is a loading control. The blot is representative of 2 independent experiments.

and overexpression decreases and increases invasiveness of a human colon cancer cell line HT-29, respectively (14). To investigate whether UBE2C expression affects abl, C4-2B, and LNCaP cell invasion, we transfected a siRNA targeting UBE2C (siUBE2C), a control siRNA (siControl), UBE2C vector, or a control vector in all 3 cell lines (Fig. 4A) followed by Matrigel invasion assays. Control vector- or siControl-transfected abl and C4-2B cells were significantly more invasive than similarly transfected LNCaP cells (Fig. 4B and C; Supplementary Fig. S3). Interestingly, silencing of UBE2C significantly decreased, whereas overexpression of UBE2C significantly increased, invasiveness of abl and C4-2B but not LNCaP cells (Fig. 4B and C; Supplementary Fig. S3), suggesting that UBE2C is necessary for CRPC cell invasion but not sufficient for ADPC cell invasion. It is possible that additional invasion-related proteins are required for UBE2C to mediate ADPC cell invasion. We next examined the effect of CCI-779 on cell invasion. Exposure of control vector-transfected abl, C4-2B, and LNCaP cells to CCI-779 significantly reduced invasion of abl and C4-2B but not LNCaP cells (Fig. 4C; Supplementary Fig. S3). Importantly, UBE2C overexpression reversed most of this CCI-779-induced invasion inhibitory effect (Fig. 4C; Supplementary Fig. S3). These results

suggest that CCI-779-induced prevention of CRPC cell invasion is mediated mostly by UBE2C.

Mechanisms for CCI-779 inhibition on UBE2C mRNA expression in CRPC cells

To investigate the underlying mechanisms for UBE2C mRNA inhibition by CCI-779 in CRPC cells, we first examined the effect of CCI-779 on recruitment of AR, its collaborating transcription factors FoxA1 and GATA2 (25), and its coactivators histone acetyltransferases [(HAT); SRC1, SRC3, and p300] and Mediator subunit (MED1; ref. 19) to the 2 *UBE2C* enhancers located –32.8 and +41.6 kilobases (kb) away from the transcription start site (TSS) of *UBE2C* gene in abl cells (4). abl cells were treated with CCI-779, and ChIP assays were conducted using antibodies against AR, FoxA1, GATA2, SRC1, SRC3, p300, MED1, and RNA polymerase II (pol II). Although exposure to CCI-779 did not affect AR binding at the 2 *UBE2C* enhancers, CCI-779 treatment decreased and increased FoxA1/GATA2 recruitment to the *UBE2C* enhancers 1 and 2, respectively (Fig. 5A–Fig. 5C). Significantly, CCI-779 treatment attenuated the recruitment of AR coactivators SRC1, SRC3, p300, and MED1 to both *UBE2C* enhancers but not the 2 negative control regions (Fig. 5D–Fig. 5G). Consistent with

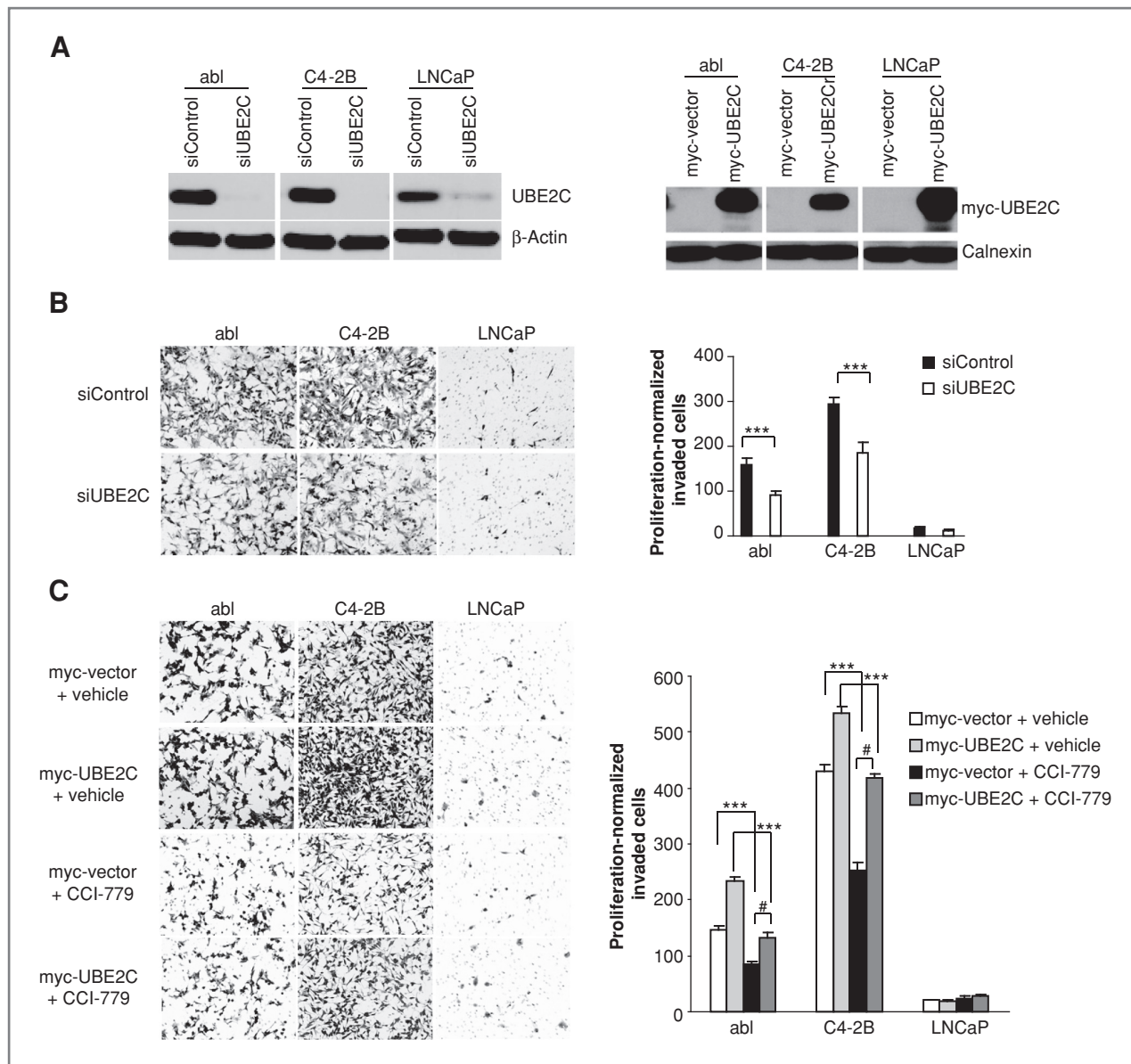


Figure 4. CCI-779 inhibits UBE2C-dependent CRPC cells invasion *in vitro*. **A**, left, UBE2C silencing decreases UBE2C protein expression. abl, C4-2B, and LNCaP cells were transfected with siControl or siUBE2C. Forty-eight hours posttransfection, Western blot analyses were carried out using the antibodies indicated. Right, UBE2C overexpression increases UBE2C protein expression. abl, C4-2B, and LNCaP cells were transiently transfected with pCS2-myc vector or pCS2-myc-UBE2C. Forty-eight hours later, cell lysates were analyzed by Western blotting, using the antibodies indicated. **B**, left, representative photomicrographs (100 $\times$ magnification) show that UBE2C silencing inhibits invasiveness of abl and C4-2B cells. The invaded cells were stained and photographed. Right, quantification of the invaded cells for each cell line after siRNA transfection. The stained cells were manually counted from 5 randomly chosen 100 $\times$ fields and normalized with cell proliferation (see Supplementary Fig. S3). ***, $P < 0.001$. **C**, left, representative photomicrographs (100 $\times$ magnification) show that CCI-779 inhibits invasion of abl and C4-2B cells, and this effect is mostly reversed by UBE2C overexpression. Right, quantification of the invaded cells for each cell line in the absence or presence of CCI-779 (50 nmol/L). The stained cells were manually counted from 5 randomly chosen 100 $\times$ fields and normalized with cell proliferation (see Supplementary Fig. S3). ***, $P < 0.001$ as compared with vehicle-treated groups; #, $P < 0.001$ as compared with CCI-779-treated group without UBE2C overexpression.

the notion that HAT modifies chromatin structure to allow Mediator facilitating pol II recruitment to target gene promoters (27–29), exposure to CCI-779 significantly reduced the pol II level at the *UBE2C* promoter (Fig. 5H).

Because exposure to CCI-779 had no effect on protein expression levels of these coactivators (Fig. 5I), ReChIP assays

were conducted to investigate whether CCI-779 treatment affected AR-coactivator interaction on chromatin. First-round ChIP was carried out with AR or MED1 antibodies, followed by second-round ChIP with p300 or AR antibodies. CCI-779 treatment significantly decreased interactions between AR and p300, and between AR and MED1 (Fig. 5J)

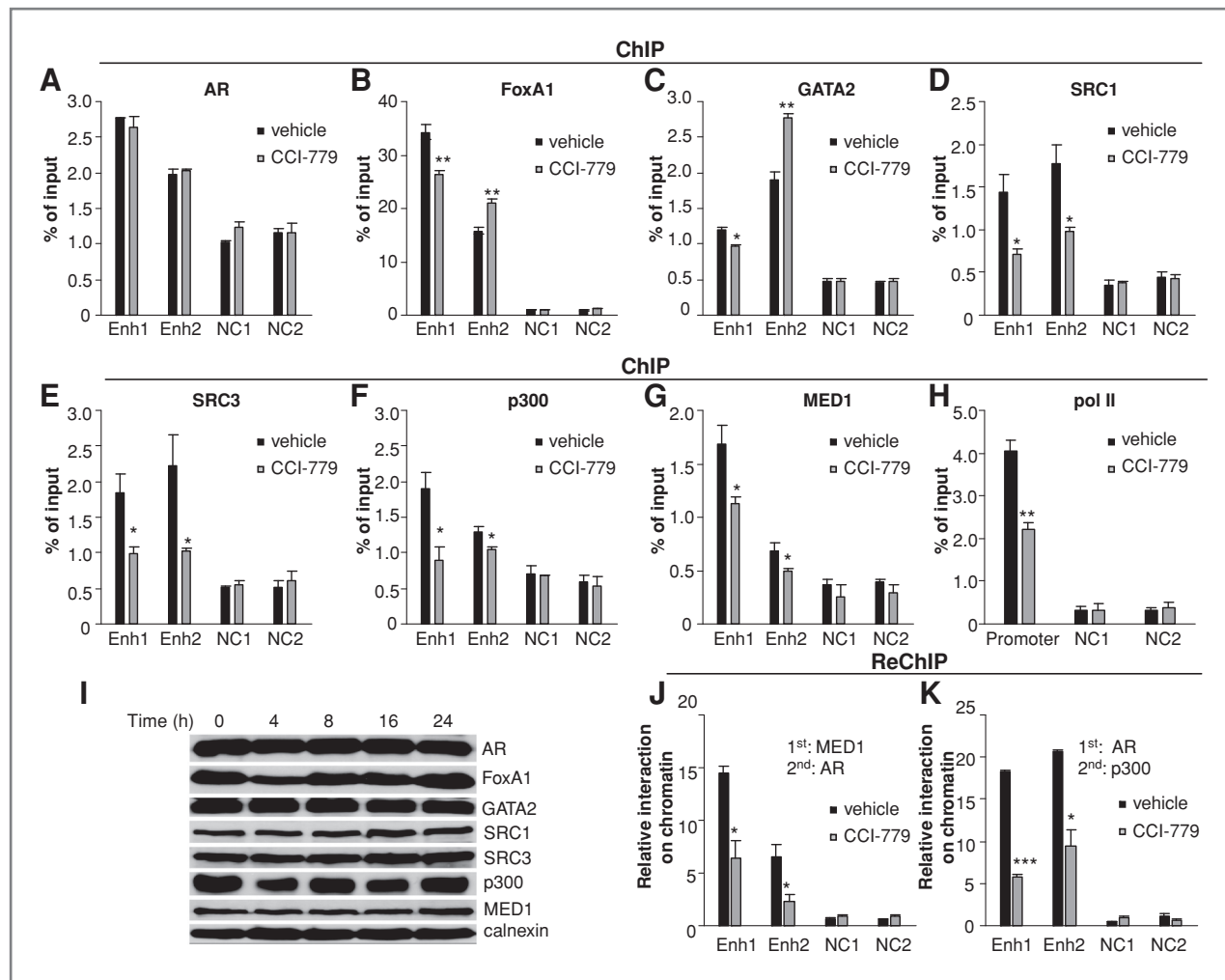


Figure 5. CCI-779 inhibits the recruitment of AR coactivators and pol II to *UBE2C* regulatory regions in CRPC cells. *abl* cells were treated with 50 nmol/L CCI-779 or vehicle for 16 hours and subjected to ChIP analysis with antibodies against AR (A), FoxA1 (B), GATA2 (C), SRC1 (D), SRC3 (E), p300 (F), MED1 (G), or pol II (H), respectively. The DNA precipitates were then quantified by qPCR, using primers for the *UBE2C* enhancers 1 and 2 (Enh1 and Enh2) and the *UBE2C* promoter [mean ($n = 3$) $\pm$ SE]. Negative controls (NC1 and NC2) were sequences containing AREs but without actual binding of AR and FoxA1. *, $P < 0.05$; **, $P < 0.01$ as compared with vehicle. I, protein levels of AR, FoxA1, GATA2, SRC1, SRC3, p300, and MED1 remained unchanged following treatment with 50 nmol/L CCI-779 for the indicated duration (0, 4, 8, 16, and 24 hours). J and K, ReChIP assays were conducted with antibodies against MED1 (J) and AR (K), for first ChIP, and AR (J) and p300 (K), for ReChIP [(mean ($n = 3$) $\pm$ SE)]. *, $P < 0.05$; ***, $P < 0.001$ as compared with vehicle.

and K), indicating that CCI-779 treatment reduces coactivator binding through disruption of AR-coactivator interactions. Taken together, these data suggest that CCI-779 decreases AR-transcription-complex loading on *UBE2C* regulatory regions, which may account, at least in part, for the decreased *UBE2C* mRNA expression after CCI-779 treatment.

We further addressed whether CCI-779 affects *UBE2C* mRNA stability in CRPC cells. *abl* cells were incubated in the presence of either actinomycin D (to block *de novo* transcription)/CCI-779 or actinomycin D/vehicle for 6 to 24 hours. As shown in Figure 6, the *UBE2C* mRNA was destabilized by CCI-779 with a $t_{1/2} = 8.52 \pm 0.19$ hours, as compared with the vehicle $t_{1/2} = 10.97 \pm 0.21$ hours. These data suggest that both attenuated gene transcription and mRNA stability contribute to CCI-779 inhibition of *UBE2C* mRNA level in CRPC cells.

Discussion

The AR is often expressed and functional in most CRPC patients, and current clinical studies on CRPC focus on targeting AR itself by using AR antagonists (e.g., MDV-3100; ref. 30) or inhibitors of androgen synthesis (e.g., abiraterone acetate; ref. 31). Although these agents decrease CRPC growth in some patients, elimination of all AR activity blocks some beneficial actions of AR (e.g., inhibition of some oncogenes such as *PCDH11*; ref. 32) and contributes to undesirable effects such as bone loss (33) and metabolic syndrome (34). An alternative approach for the inhibition of the cancer-promoting AR signaling pathway in CRPC is to target AR downstream target genes involved in CRPC growth. Given our recent findings showing that knocking down of CRPC-specific AR-target G₂-M phase

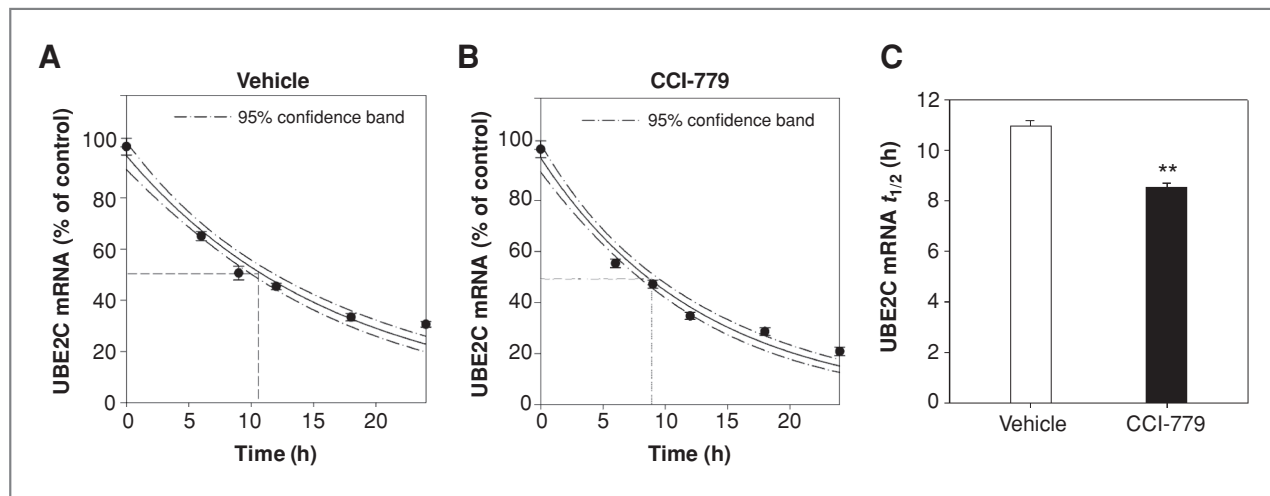


Figure 6. CCI-779 decreases mRNA stability of UBE2C in CRPC cells. A and B, degradation of UBE2C mRNA in abl cells in the absence or presence of CCI-779. C, CCI-779 shortens the half-life ($t_{1/2}$) of UBE2C mRNA. abl cells were treated with 50 nmol/L CCI-779 or vehicle with transcription was blocked by actinomycin D. UBE2C mRNA level was quantified at the indicated time points after actinomycin D treatment. Data are presented as the percentage of the mRNA level measured at time 0 (without adding actinomycin D). **, $P < 0.01$ as compared with vehicle.

genes (e.g., *UBE2C*, *CDK1*, and *CDC20*) significantly decreases CRPC cell growth (4), we propose that G₂–M phase genes could serve as new targets for therapeutic intervention.

In this study, we identified the mTOR inhibitor CCI-779 as an inhibitor for UBE2C in CRPC cells. We showed that CCI-779 treatment significantly decreases UBE2C mRNA and protein expression in CRPC cells at its pharmacologically attainable concentrations in clinical trials (refs. 35, 36; Fig. 1). Although it is well known that inhibition of mTOR decreases protein expression levels of some genes by dephosphorylation of p70 ribosomal S6 kinase (S6K1) and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1; refs. 12, 37), recent studies have found that mTOR inhibitors also decrease RNA levels of some genes (37, 38) through a variety of mechanisms. It has been shown that rapamycin inhibits mitochondrial gene transcription by disruption of protein–protein interactions between a transcription coactivator PGC-1 α and a transcription factor yin-yang 1 (YY1), resulting in decreased recruitment of PGC-1 α to the promoters of mitochondrial genes (39). Furthermore, rapamycin prevents sterol regulatory-element-binding protein 1 (*SREBP1*) target gene expression through inhibition of nuclear accumulation of *SREBP1* (40). Additionally, rapamycin blocks PPAR- γ protein expression leading to decreased PPAR- γ target gene expression (41). With regard to the mechanisms for CCI-779 inhibition of UBE2C mRNA level, while CCI-779 does not affect AR binding and protein expression levels of AR and its coactivators, CCI-779 inhibits the recruitment of AR coactivators to the *UBE2C* enhancers through disruption of AR–coactivator interactions, leading to decreased pol II loading on the *UBE2C* promoter (Fig. 5). Interestingly, we also found that CCI-779 attenuates UBE2C mRNA stability (Fig. 6), suggesting that CCI-779-induced direct downregulation of UBE2C mRNA levels is caused by decreased UBE2C mRNA transcription and stability.

Although mTOR inhibitors have shown great potential as antitumor agents and CCI-779 has been approved by the U.S. Food and Drug Administration (FDA) as the first-line treatment in patients with advanced refractory renal cell cancer (RCC; ref. 11), results from clinical studies on mTOR inhibitors in CRPC have been somewhat disappointing. For example, it was reported that that therapeutic response was observed in only 17% to 25% CRPC patients treated with rapamycin alone (42, 43). One of the explanations for such clinical observations is that rapamycin and CCI-779 may activate AR target genes such as *PSA* and *KLK4* in cultured CRPC cells and xenografts, leading to a decreased effect of mTOR inhibitors on inhibition of cell proliferation (42, 44). However, as the AR target genes examined in these studies (42, 44) are not directly relevant to cell growth and invasion, it is not very clear that the failure of mTOR inhibitors as monotherapy is caused by mTOR inhibitor-activated AR signaling. Interestingly, our studies found that CCI-779 significantly decreases the expression of a CRPC-specific AR target gene *UBE2C* in CRPC cell models abl and C4-2B (Fig. 1). The overexpressed UBE2C in abl and C4-2B cells, as compared with LNCaP cells (4), plays a critical role in cell proliferation and/or invasion (Figs. 2–4; ref. 4). CCI-779, acting partially through a UBE2C-dependent mechanism, significantly decreases abl cell growth *in vitro* and *in vivo* (Figs. 2 and 3). Importantly, we also found that CCI-779-induced inhibition of abl and C4-2B cell invasion is mediated mostly by UBE2C (Fig. 4). Although the average level of UBE2C expression in CRPC patients is significantly higher than that in ADPC patients, UBE2C expression in CRPC cases is highly variable (4). Thus, it is possible that those CRPC patients with high UBE2C expression will have better therapeutic response for CCI-779 than those with low UBE2C expression. Further studies are needed to investigate whether UBE2C is able to serve as a biomarker for predicting CCI-779 therapy response in CRPC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Gustavo Leone for helpful discussion and Hsueh-Li Tan for help with mice castration surgery.

Grant Support

This study was funded by NIH grant no. R00 CA126160 (to Q. Wang) and The Ohio State University Comprehensive Cancer Center (to Q. Wang and K.K. Chan).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 20, 2010; revised April 21, 2011; accepted May 10, 2011; published OnlineFirst May 18, 2011.

References

- Heinlein CA, Chang C. Androgen receptor in prostate cancer. *Endocr Rev* 2004;25:276–308.
- Knudsen KE, Penning TM. Partners in crime: deregulation of AR activity and androgen synthesis in prostate cancer. *Trends Endocrinol Metab* 2010;21:315–24.
- Balk SP, Knudsen KE. AR, the cell cycle, and prostate cancer. *Nucl Recept Signal* 2008;6:e001.
- Wang Q, Li W, Zhang Y, Yuan X, Xu K, Yu J, et al. Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. *Cell* 2009;138:245–56.
- Sharma A, Yeow WS, Ertel A, Coleman I, Clegg N, Thangavel C, et al. The retinoblastoma tumor suppressor controls androgen signaling and human prostate cancer progression. *J Clin Invest* 2010;120:4478–92.
- Ye Y, Rape M. Building ubiquitin chains: E2 enzymes at work. *Nat Rev Mol Cell Biol* 2009;10:755–64.
- Varambally S, Yu J, Laxman B, Rhodes DR, Mehra R, Tomlins SA, et al. Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. *Cancer Cell* 2005;8:393–406.
- Stanbrough M, Bubley GJ, Ross K, Golub TR, Rubin MA, Penning TM, et al. Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. *Cancer Res* 2006;66:2815–25.
- Reddy SK, Rape M, Margansky WA, Kirschner MW. Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. *Nature* 2007;446:921–5.
- van Ree JH, Jeganathan KB, Malureanu L, van Deursen JM. Overexpression of the E2 ubiquitin-conjugating enzyme UbcH10 causes chromosome missegregation and tumor formation. *J Cell Biol* 2010;188:83–100.
- Konings IR, Verweij J, Wiemer EA, Sleijfer S. The applicability of mTOR inhibition in solid tumors. *Curr Cancer Drug Targets* 2009;9:439–50.
- Bjornsti MA, Houghton PJ. The TOR pathway: a target for cancer therapy. *Nat Rev Cancer* 2004;4:335–48.
- Wu L, Birlle DC, Tannock IF. Effects of the mammalian target of rapamycin inhibitor CCI-779 used alone or with chemotherapy on human prostate cancer cells and xenografts. *Cancer Res* 2005;65:2825–31.
- Chen S, Chen Y, Hu C, Jing H, Cao Y, Liu X. Association of clinicopathological features with UbcH10 expression in colorectal cancer. *J Cancer Res Clin Oncol* 2010;136:419–26.
- Loussouarn D, Campion L, Leclair F, Campone M, Charbonnel C, Ricolleau G, et al. Validation of UBE2C protein as a prognostic marker in node-positive breast cancer. *Br J Cancer* 2009;101:166–73.
- Cunha IW, Carvalho KC, Martins WK, Marques SM, Muto NH, Falzoni R, et al. Identification of genes associated with local aggressiveness and metastatic behavior in soft tissue tumors. *Transl Oncol* 2010;3:23–32.
- Culig Z, Hoffmann J, Erdel M, Eder IE, Hobisch A, Hittmair A, et al. Switch from antagonist to agonist of the androgen receptor bicalutamide is associated with prostate tumour progression in a new model system. *Br J Cancer* 1999;81:242–51.
- Guan YJ, Wang X, Wang HY, Kawagishi K, Ryu H, Huo CF, et al. Increased stem cell proliferation in the spinal cord of adult amyotrophic lateral sclerosis transgenic mice. *J Neurochem* 2007;102:1125–38.
- Wang Q, Carroll JS, Brown M. Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. *Mol Cell* 2005;19:631–42.
- Okamoto Y, Ozaki T, Miyazaki K, Aoyama M, Miyazaki M, Nakagawara A. UbcH10 is the cancer-related E2 ubiquitin-conjugating enzyme. *Cancer Res* 2003;63:4167–73.
- Chang TC, Zeitels LR, Hwang HW, Chivukula RR, Wentzel EA, Dews M, et al. Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation. *Proc Natl Acad Sci U S A* 2009;106:3384–9.
- Whitfield ML, Zheng LX, Baldwin A, Ohta T, Hurt MM, Marzluff WF. Stem-loop binding protein, the protein that binds the 3' end of histone mRNA, is cell cycle regulated by both translational and posttranslational mechanisms. *Mol Cell Biol* 2000;20:4188–98.
- Morton CL, Houghton PJ. Establishment of human tumor xenografts in immunodeficient mice. *Nat Protoc* 2007;2:247–50.
- Fung AS, Wu L, Tannock IF. Concurrent and sequential administration of chemotherapy and the mammalian target of rapamycin inhibitor temsirolimus in human cancer cells and xenografts. *Clin Cancer Res* 2009;15:5389–95.
- Wang Q, Li W, Liu XS, Carroll JS, Jänne OA, Keeton EK, et al. A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. *Mol Cell* 2007;27:380–92.
- Sobel RE, Sadar MD. Cell lines used in prostate cancer research: a compendium of old and new lines—part 1. *J Urol* 2005;173:342–59.
- Sharma D, Fondell JD. Ordered recruitment of histone acetyltransferases and the TRAP/Mediator complex to thyroid hormone-responsive promoters *in vivo*. *Proc Natl Acad Sci U S A* 2002;99:7934–9.
- Taatjes DJ. The human Mediator complex: a versatile, genome-wide regulator of transcription. *Trends Biochem Sci* 2010;35:315–22.
- Malik S, Roeder RG. The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. *Nat Rev Genet* 2010;11:761–72.
- Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, et al. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* 2009;324:787–90.
- Attard G, Reid AH, A'Hern R, Parker C, Oommen NB, Folkard E, et al. Selective inhibition of CYP17 with abiraterone acetate is highly active in the treatment of castration-resistant prostate cancer. *J Clin Oncol* 2009;27:3742–8.
- Agoulnik IU, Bingman WE 3rd, Nakka M, Li W, Wang Q, Liu XS, et al. Target gene-specific regulation of androgen receptor activity by p42/p44 mitogen-activated protein kinase. *Mol Endocrinol* 2008;22:2420–32.
- Vanderschueren D, Vandenput L, Boonen S, Lindberg MK, Bouillon R, Ohlsson C. Androgens and bone. *Endocr Rev* 2004;25:389–425.
- Braga-Basaria M, Dobs AS, Muller DC, Carducci MA, John M, Egan J, et al. Metabolic syndrome in men with prostate cancer undergoing long-term androgen-deprivation therapy. *J Clin Oncol* 2006;24:3979–83.
- Raymond E, Alexandre J, Faivre S, Vera K, Maternan E, Boni J, et al. Safety and pharmacokinetics of escalated doses of weekly intravenous infusion of CCI-779, a novel mTOR inhibitor, in patients with cancer. *J Clin Oncol* 2004;22:2336–47.
- Hidalgo M, Buckner JC, Erlichman C, Pollack MS, Boni JP, Dukart G, et al. A phase I and pharmacokinetic study of temsirolimus (CCI-779)

- administered intravenously daily for 5 days every 2 weeks to patients with advanced cancer. *Clin Cancer Res* 2006;12:5755–63.
37. Wullschlegel S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell* 2006;124:471–84.
 38. Peng T, Golub TR, Sabatini DM. The immunosuppressant rapamycin mimics a starvation-like signal distinct from amino acid and glucose deprivation. *Mol Cell Biol* 2002;22:5575–84.
 39. Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1 α transcriptional complex. *Nature* 2007;450:736–40.
 40. Porstmann T, Santos CR, Griffiths B, Cully M, Wu M, Leevers S, et al. SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. *Cell Metab* 2008;8:224–36.
 41. Kim JE, Chen J. regulation of peroxisome proliferator-activated receptor- γ activity by mammalian target of rapamycin and amino acids in adipogenesis. *Diabetes* 2004;53:2748–56.
 42. Wang Y, Mikhailova M, Bose S, Pan CX, deVere White RW, Ghosh PM. Regulation of androgen receptor transcriptional activity by rapamycin in prostate cancer cell proliferation and survival. *Oncogene* 2008;27:7106–17.
 43. Amato RJ, Jac J, Mohammad T, Saxena S. Pilot study of rapamycin in patients with hormone-refractory prostate cancer. *Clin Genitourin Cancer* 2008;6:97–102.
 44. Kaarbo M, Mikkelsen OL, Malerod L, Qu S, Lobert VH, Akgul G, et al. PI3K-AKT-mTOR pathway is dominant over androgen receptor signaling in prostate cancer cells. *Cell Oncol* 2010;32:11–27.