Therapeutics, Targets, and Chemical Biology

Cancer Research

# UGT2B17 Expedites Progression of Castration-Resistant Prostate Cancers by Promoting Ligand-Independent AR Signaling

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## Abstract

Castration-resistant prostate cancer (CRPC) is characterized by a shift in androgen receptor (AR) signaling from androgen-dependent to androgen (ligand)-independent. UDP-glucuronosyltransferase 2B17 (UGT2B17) is a key enzyme that maintains androgen homeostasis by catabolizing AR agonists into inactive forms. Although enhanced UGT2B17 expression by antiandrogens has been reported in androgen-dependent prostate cancer, its roles in regulating AR signaling transformation and CRPC progression remain unknown. In this study, we show that higher UGT2B17 protein expression in prostate tumors is associated with higher Gleason score, metastasis, and CRPC progression. UGT2B17 expression and activity were higher in androgen-independent compared to androgen-dependent cell lines. UGT2B17 stimulated cancer cell proliferation, invasion, and xenograft progression to CRPC after prolonged androgen deprivation. Gene microarray analysis indicated that UGT2B17 suppressed androgen-dependent AR transcriptional activity and enhanced of ligand-independent transcriptional activity at genes associated with cell mitosis. These UGT2B17 actions were mainly mediated by activation of the c-Src kinase. In CRPC tumors, UGT2B17 expression was associated positively with c-Src activation. These results indicate that UGT2B17 expedites CRPC progression by enhancing ligand-independent AR signaling to activate cell mitosis in cancer cells. *Cancer Res; 76(22); 6701–11. ©2016 AACR.*



## Introduction

Androgen receptor (AR) signaling is critical for prostate cancer development as well as progression into castration-resistant prostate cancer (CRPC; refs. 1, 2). Blocking AR signaling delays tumor growth and prolongs patient survival (3, 4). These AR pathway inhibitors all target the ligand-binding domain (LBD) of the AR and prevent AR from being transcriptionally active. However, the benefit of these therapies is temporary and recurrence to CRPC is inevitable (3, 4). Several possible mechanisms that restore AR signaling have been defined, including AR gene overexpression and amplification (5), AR gain-of-function mutations in the LBD with a more promiscuous affinity to other ligands (6), aberrant AR gene splicing encoding constitutively active forms of AR (7, 8), and phosphorylation and subsequent activation of AR in the absence of androgen (9). These findings indicate that AR LBD inhibitors are insufficient to abolish AR signaling and that tumor cells can use ligand-independent AR signaling to enable progression under anti-AR therapies. This notion is further supported by genome-wide studies showing that AR remains functional in CRPC, but the AR transcriptome is shifted to one that predominantly regulates cell mitosis (10–12). These findings emphasize that most CRPC (a notable exception being neuroendocrine prostate cancer) still rely on AR, but mainly through ligand-independent AR transcriptional activity to drive tumor cell proliferation in an androgen depleted milieu. Defining molecular mechanisms that activate ligand-independent AR signaling would be important for developing effective therapies that prevent CRPC progression and maintain prostate cancer in an anti–AR-sensitive state.

The UGT2B17 gene encodes an enzyme that irreversibly catabolizes androgens into inactive forms by glucuronidation reactions, thereby maintaining androgen homeostasis in the prostate (13). Compared with benign prostate, prostate cancer expresses higher levels of UGT2B17 and its expression has been reported to further increase after antiandrogen treatment (14, 15). Using *in vitro* cell models, RNA silencing of UGT2B17 in LNCaP cells suppresses glucuronidation activities, but increases DHT levels in culture media and prostate cancer cell proliferation (16). These results suggest that enhanced UGT2B17 expression or activity would have suppressive effects on AR-driven tumor growth. However, high UGT2B17 expression levels in CRPC (17, 18) imply the opposite that UGT2B17 may facilitate to CRPC progression through mechanisms that are not fully characterized to date. Similar to AKR1C3, another critical enzyme in androgen steroidogenesis recently identified to possess novel functions as an AR coactivator (19) and an enhancer of AR function through the ubiquitin ligase Siah2 (20), UGT2B17 may also exert undefined actions to modulate AR signaling. As a membrane-bound enzyme localized in the endoplasmic reticulum of the cytosol (21), UGT2B17 may engage in ligand-independent activation of AR signaling by kinase pathways to drive CRPC progression.

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

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Through paracrine and autocrine regulations, cytokines and growth factors can stimulate AR phosphorylation and subsequent AR transactivation in ligand-independent manners (22–25). For example, c-Src kinase as a downstream effector of the EGF and IGF-1 pathways activates AR in the absence of androgens by using its SH3 domain to interact with and phosphorylate AR at the tyrosine 534 position (9). In fact, overactive c-Src was reported in CRPC (9, 26) and c-Src inhibition by shRNA or small molecules induced prostate tumor regression (27). These findings led us to hypothesize that c-Src may be activated by UGT2B17 to transform AR signaling into a ligand-independent mode, thereby enabling CRPC progression in the presence of therapies targeting the ligand-binding domain of the AR.

## Materials and Methods

### Tissue microarray and immunohistochemistry

Information on prostate cancer tissue microarrays (TMA), including Gleason score, duration of neoadjuvant hormone therapy (NHT), lymph node and distal organ metastasis was published previously (14, 26) and also presented in Supplementary Table S1. IHC was performed by Ventana Discovery XT (Ventana) using a DAB MAP kit as we reported (14). Antibody information was in Supplementary Table S2. Stained slides were scanned by a Leica SCN400 scanner and viewed by DIH Slide Path imaging system (Leica Microsystem). Pathological analysis was carried out by pathologist Dr. Ladan Fazli.

### Cell lines and reagents

LNCaP, VCaP, PC3, DU145, 22Rv1, and 293T cell lines were purchased from the ATCC. LNCaP95, LNCaP(AI), and MR49F cell lines were provided by Drs. Alan Meeker (Johns Hopkins Centre), Ralph Buttyan (Vancouver Prostate Centre), and Martin Gleave (Vancouver Prostate Centre). Cell culture conditions were described in our previous report (28). All cell lines were recently authenticated by STR or RNA sequencing assays. R1881, DHT, PP2, LY294002 and Stattic were purchased from Cedarlane. Enzalutamide (Enz) was from Haoyuan Chemexpress.

### Real-time PCR, Western blotting, and *in vitro* glucuronidation assays

Real-time PCR (28), Western blotting (28) and *in vitro* glucuronidation (15) assays were performed as described previously. PCR primer information was in Supplementary Table S3 and antibody information was in Supplementary Table S2. All experiments were repeated in triplicate for three independent times.

### RNA silencing and construction of cell lines with gain- and loss-of-function of UGT2B17

SiRNA against AR was purchased from Dharmacon. Lentiviral vectors encoding control, UGT2B17 and shRNA against UGT2B17 were purchased from Applied Biological Materials (Supplementary Table S4). Transient transfection was performed using Lipofectamine 3000 (Invitrogen). LNCaP cells overexpressing UGT2B17 and LNCaP95 cells with UGT2B17 knockdown were achieved by lentiviral approaches combined with puromycin selection as we reported (29).

### Cell proliferation, invasion, and migration assays

Cell proliferation rates were measured by using the CellTitre 96 AqueousOne kit (Promega) and bromodeoxyuridine (BrdUrd) assay kit (Millipore) according to the manufacturer's protocol. Cell migration and invasion assays were performed using 6-well BD Control/Matrigel Chambers (BD Biosciences) as we reported (30). Cell invasion and migration rates were calibrated relative to the control group. Experiments were performed in triplicate and repeated three times.

### Human prostate cancer xenografts

A total of $1 \times 10^6$ control or LNCaP(UGT2B17) cells in 0.1 mL Matrigel (BD Labware) were inoculated subcutaneously in the bilateral flanks of 6- to 8-week-old male athymic nude mice ($n = 7$/group; Harlan Sprague Dawley Inc.). Tumor volume ($V = L \times W \times D \times 0.5236$) and body weight were measured weekly. Serum PSA levels were determined by ELISA. When tumor volumes reach 200 $mm^3$, mice were castrated. Mice were sacrificed when tumor volume > 10% of bodyweight (or >20% loss of bodyweight). Tumors were harvested for evaluation of mRNA levels of indicated genes by real-time PCR. All animal procedures were under the guidelines of the Canadian Council on Animal Care.

### Gene microarray studies

Gene microarray analyses were performed as described before (31). Total RNA was extracted by using the mirVana RNA Isolation Kit (Ambion) from three independently repeated experiments. The quality and quantity of RNA were assessed with an Agilent 2100 Bioanalyzer (Caliper Technologies Corp.). Amplified and Alexa Fluor 3–labeled RNA samples were hybridized onto the Human Agilent 8 × 60k (Agilent Technologies), along with Alexa Fluor 5 labeled human reference RNA. Statistical Analysis of Microarray (SAM) program (http://statweb.stanford.edu/~tibs/SAM/) was used to analyze expression differences between RNA samples. Unpaired *t* tests were calculated for all probes passing filters and *q*-values were estimated using the FDR multitest correction method. Gene expression data have been deposited in the Gene Expression Omnibus (GEO) under accession GSE82189. IPA software (Ingenuity Systems) was used to analyze UGT2B17 regulated gene groups and signaling pathways.

### Immunoprecipitation, immunofluorescence, and proximity ligation assay

Immunoprecipitation assays (32) and immunofluorescence (28) assays were performed as we previously reported. Proximity ligation assay (PLA) assays were performed using the Duolink *in situ* red starter kit mouse/rabbit (Sigma). LNCaP(UGT2B17) cells were fixed with 4% paraformaldehyde and permeabilized by 0.2% Triton-X100. Fixed cells were incubated with primary antibodies overnight at 4°C. Secondary probe, ligation and amplification reactions were performed following the manufacturer's instructions. Fluorescence images were captured by Zeiss fluorescent microscope (Carl Zeiss).

### Statistical analysis

Statistical analyses were carried out using GraphPad Prism (version 6) for one-way ANOVA and parametric statistics (two-tailed Student unpaired *t* test) with the level of significance set at $^*$, $P < 0.05$, $^{**}$, $P < 0.01$, and $^{***}$, $P < 0.001$, and SPSS Statistics (version 20.0) for the $\chi^2$ test with the level of significance set at $P < 0.05$.

## Results

### UGT2B17 is associated with CRPC progression and androgen insensitivity of prostate cancer cells

Previous studies showed that UGT2B17 mRNA expression was highly elevated in CRPC (17, 18). Here, we evaluated UGT2B17 protein expression by immunohistochemistry (IHC) on TMAs containing 604 tissue cores from 287 patients as described in Supplementary Table S1 as well as in (14). UGT2B17 IHC was scored according to the intensity with no/weak as 1, medium as 2 and strong as 3 histology scores (H-score). Higher UGT2B17 expression was more prevalent in tissue cores with higher Gleason scores (Fig. 1A), and UGT2B17 H-scores were positively correlated with the Gleason score ($r = 0.3034$, $P < 0.001$; Supplementary Table S5). Compared with benign prostate, UGT2B17 was highly expressed in prostate cancer and further elevated by NHT (Fig. 1B). Consistent with previous reports on UGT2B17 mRNA expression (17, 18), a larger proportion of CRPC tissue cores fell into the category of strong UGT2B17 protein expression (Fig. 1B). Higher UGT2B17 expression was also observed in tumors that were collected for initial diagnosis but were reported to have either lymph node or distal metastasis in follow-up examinations (Fig. 1C). These results indicate that increased UGT2B17 expression is associated with higher grade and CRPC progression.

In prostate cancer cell lines, we found that androgen-independent LNCaP(AI) and LNCaP95 cells expressed higher UGT2B17 RNA and protein levels than androgen-dependent LNCaP cells (Fig. 1D and E). Treatment with the AR agonist, R1881 (non-metabolizable androgen), suppressed UGT2B17 mRNA levels, consistent with previous reports showing that ligand-activated AR suppresses UGT2B17 transcription (14, 33). Interestingly, enzalutamide-resistant MR49F cells expressed low UGT2B17 mRNA but strong protein expression, suggesting that posttranslational mechanisms may enhance UGT2B17 protein stability in these cells. We further applied glucuronidation assays to show that elevated UGT2B17 expression in LNCaP(AI) and LNCaP95 cells was functional, since UGT2B17 metabolites including testosterone-G, DHT-G, 3a-diol-17G and the UGT2B17-specific vorinostat-G were all significantly increased in LNCaP(AI) and LNCaP95 cells, which levels were proportional to UGT2B17 expression (Fig. 1F). These results indicate that enhanced UGT2B17 glucuronidation is also associated with androgen insensitivity of prostate cancer cells.

### UGT2B17 enhances prostate cancer cell growth and invasion after prolonged androgen deprivation

To study the impact of UGT2B17 on prostate cancer cell growth, we used the lentiviral approach to construct LNCaP cells overexpressing UGT2B17 and LNCaP95 cells with UGT2B17 knockdown (Supplementary Fig. S1). Prolonged androgen deprivation to LNCaP cells enhanced UGT2B17 protein levels (Supplementary Fig. S2A). Upregulation of UGT2B17 expression was also accompanied by increased glucuronidation (Supplementary Fig. S2B). We found that altered

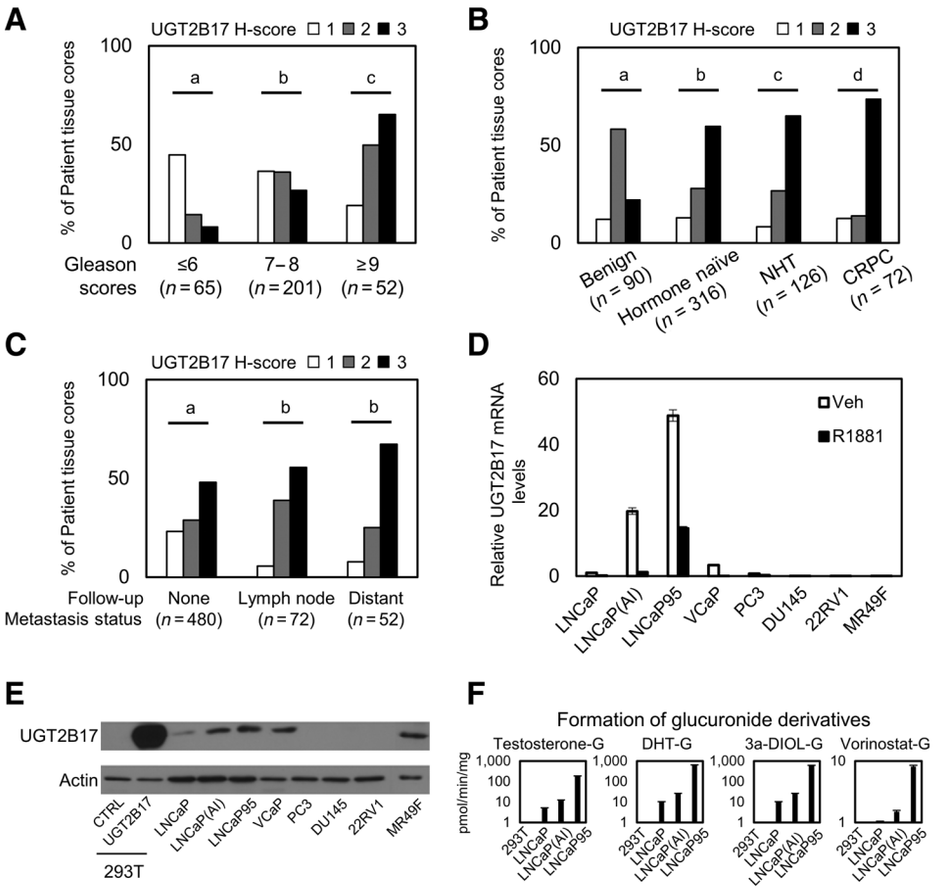


**Figure 1.**
UGT2B17 is associated with prostate tumor progression and androgen insensitivity of prostate cancer cells. **A–C,** immunohistochemistry was performed using the UGT2B17 antibody on prostate cancer TMAs. Histology score (H-score) of each tissue core was evaluated on a scale of 1 to 3 as described in Materials and Methods. The percentages of tissue cores in each H-score group sorted by Gleason score (**A**), prostate cancer progression (**B**), and metastasis status (**C**) were calculated. Comparison between the groups was analyzed using the $\chi^2$ test by SPSS Statistics. Values without a common letter were significantly different, $P < 0.05$. **D** and **E,** LNCaP, LNCaP(AI), LNCaP95, VCaP, PC3, DU145, 22RV1, and MR49F cells cultured under the condition of vehicle or 10 nmol/L R1881 treatment. Total RNA and protein lysates were collected for real-time PCR assays (**D**) and immunoblotting (**E**), respectively. **F,** cell homogenates from 293T, LNCaP, LNCaP(AI), LNCaP95 were collected for *in vitro* glucuronidation assays. The formation rates of glucuronidation derivatives including testosterone-G, DHT-G, 3a-diol-17G, and vorinostat-G were measured. Results were presented as the mean ± SEM ($n = 3$).

UGT2B17 expression changed LNCaP and LNCaP95 cell proliferation in response to androgens. Cells were cultured in medium containing charcoal-stripped serum (CSS) and treated with vehicle, 10 nmol/L of R1881, or 10 μmol/L of Enz. LNCaP cell proliferation was stimulated by R1881 by approximately 4 fold, but inhibited by enzalutamide or androgen deprivation (Fig. 2A, top). UGT2B17 overexpressing LNCaP cells slightly reduced their sensitivity to androgen. LNCaP95 cells did not respond to AR agonist or antagonist in cell growth, but became responsive to R1881 with regard to cell proliferation when UGT2B17 expression was depleted by RNA silencing. However, when cells were pre-treated with vehicle, R1881 or Enz for 28 days and then seeded for MTS assays, LNCaP cell proliferation rates were stimulated only approximately 1.5 fold by R1881 (Fig. 2A, bottom). After 28 days of culture, enhanced UGT2B17 expression rendered LNCaP cells insensitive to R1881 and Enz treatment. Cell growth in UGT2B17-depleted LNCaP95 cells was suppressed by R1881 but stimulated by R1881. Similarly, BrdUrd incorporation assays confirmed the findings from MTS assays that androgen-regulated DNA synthesis (reflecting cell proliferation rate) was also modulated by UGT2B17 in both LNCaP and LNCaP95 cells (Fig. 2B). These UGT2B17 effects on DNA synthesis appeared as early as 14 days of treatment. To determine whether UGT2B17 also regulates prostate cancer cell motility, we pre-treated LNCaP cells with vehicle, R1881 or Enz for 0 or 28 days, and then performed Transwell cell migration and invasion assays (Fig. 2C). Under prolonged androgen deprivation, LNCaP cell invasion and migration became insensitive to R1881 when UGT2B17 was overexpressed. These results together indicated that UGT2B17 modulates sensitivity of prostate cancer cells to androgens with regard to cell proliferation and invasion.

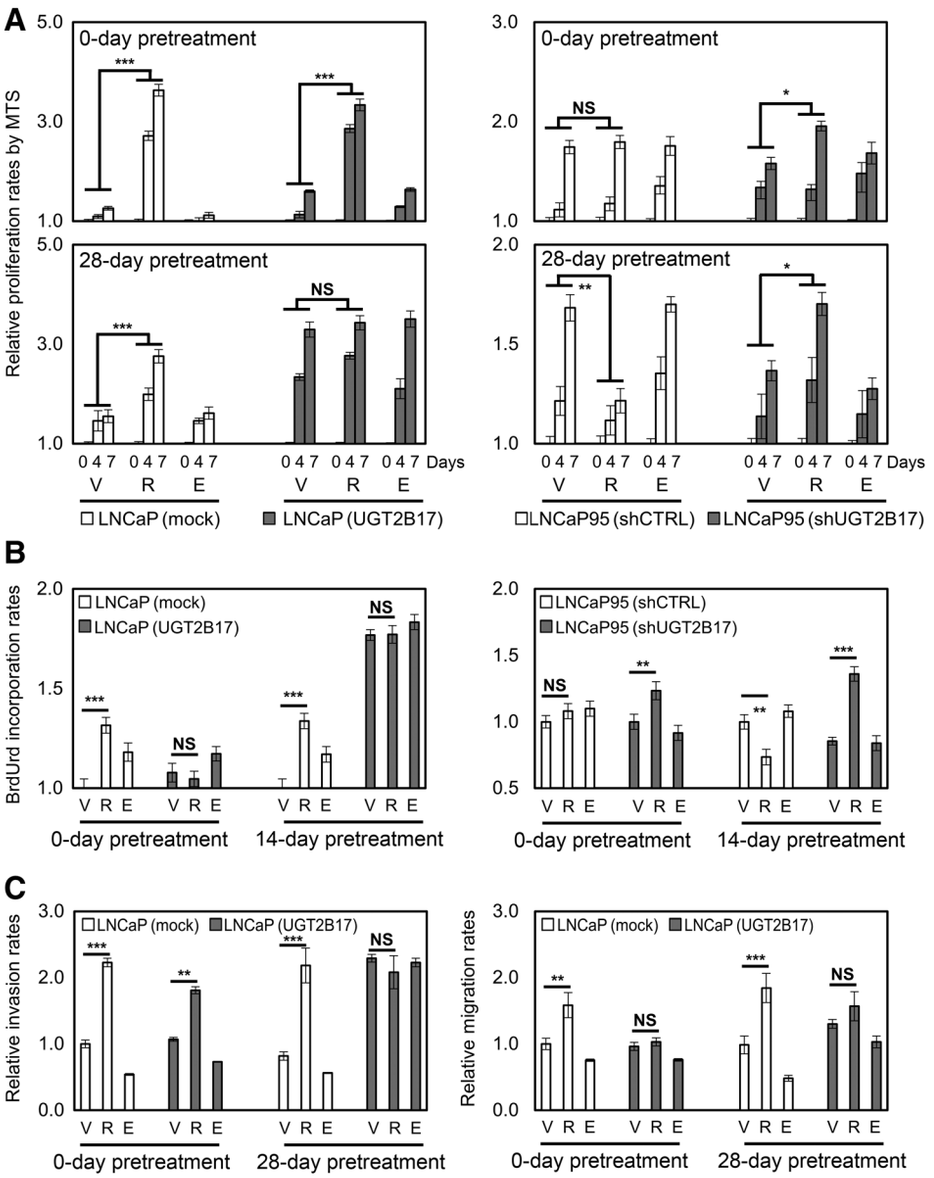


**Figure 2.**

UGT2B17 enhances prostate cancer cell growth and invasion after prolonged androgen deprivation. **A** and **B,** LNCaP (mock), LNCaP (UGT2B17), LNCaP95 (shCTRL), and LNCaP95 (shUGT2B17) cells were pre-treated in the RPMI-1640 medium containing 5% CSS under the condition of vehicle (V), 10 nmol/L of R1881 (R), or 10 μmol/L of Enz (E) for 0 (**A,** top), 28 (**A,** bottom), or 14 (**B**) days. Relative cell proliferation rates within 0 to 7 days were measured by MTS assays in **A** and by BrdUrd incorporation assays in **B**. **C,** LNCaP (mock) and LNCaP (UGT2B17) cells were cultured in RPMI-1640 medium containing 5% CSS under the condition of vehicle, 10 nmol/L of R1881, 10 μmol/L of Enz for 0 or 28 days. Relative cell invasion or migration rates were then measured. All data were repeated in three independent experiments and are shown as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by the Student *t* test with *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. NS, nonsignificant.

**UGT2B17 accelerates CRPC progression in LNCaP xenografts**

We used the LNCaP xenograft model to test the impact of UGT2B17 on prostate tumor growth *in vivo*. After 11 weeks of castration, tumors overexpressing UGT2B17 showed a significantly larger average tumor volume (Fig. 3A), but lower serum PSA levels (Fig. 3B), when compared with LNCaP control tumors. Tumor volume doubling time before (100-200 $mm^3$) and post castration (250-500 $mm^3$) was also calculated (Fig. 3C). LNCaP control tumors showed delayed tumor growth with increased tumor doubling time after castration when compared with that before castration. However, the growth of LNCaP (UGT2B17) tumors was not affected by castration since the tumor doubling time remained the same. These results indicate that enhanced UGT2B17 allows LNCaP xenografts to adapt to castration and more rapidly progress into CRPC.

When tumors were harvested and used for glucuronidation assays, we found that increased UGT2B17 expression was associated with enhanced UGT2B17 activity in catabolizing androgens (Fig. 3D). Because DHT can be catabolized at a rate of approximately 22 pmol/min/mg in LNCaP (UGT2B17) tumors, DHT is eliminated rapidly even considering it may be generated through *de novo* intratumoral steroidogenesis. Under such low androgen levels, the expression of androgen-dependent genes such as PSA and TMPRSS2 was decreased in LNCaP (UGT2B17) tumors, whereas the expression of AR-regulated mitotic genes such as UBE2C was increased (Fig. 3E). These studies suggest that AR-driven LNCaP (UGT2B17) xenograft growth is less reliant on androgens as ligands during CRPC progression, and is associated with shifts in gene transcription increasing cell mitosis.

**UGT2B17 alters AR signaling under androgen deprivation**

To decipher molecular mechanisms by which UGT2B17 enhances prostate cancer cell growth independent of androgen, we performed gene microarray using LNCaP (mock) and LNCaP(UGT2B17) cells cultured under the regular serum (FBS) or androgen depleted serum (CSS) conditions for 28 days. Under the FBS condition, 381 genes were significantly altered by UGT2B17 with fold change over 2. Gene ontology (GO) analysis by DAVID 6.7 (http://david.abcc.ncifcrf.gov/) further identified the top ranked gene group ($n = 101$ genes) was associated with cell differentiation (Fig. 4A). However, after 28 days of CSS treatment, the expression of 1581 genes was altered by UGT2B17, among which 359 genes were associated with cell cycle (Fig. 4B), suggesting that UGT2B17 regulated transcriptome shifted from cell differentiation to cell cycle after prolonged androgen deprivation.

Results from these GO analyses were further confirmed by real-time PCR. There are seven mitosis-related genes (ANAPC, CDC20, ID1, NDRG1, CDK1, PRDM4, and UBE2C) known to be upregulated by AR independent to androgen (10). Enhanced UGT2B17 expression in LNCaP cells strongly stimulated the expression of these genes in a time-dependent manner (Fig. 4C), suggesting that UGT2B17 enhanced ligand-independent AR transactivation. The expression of genes such as PSA,

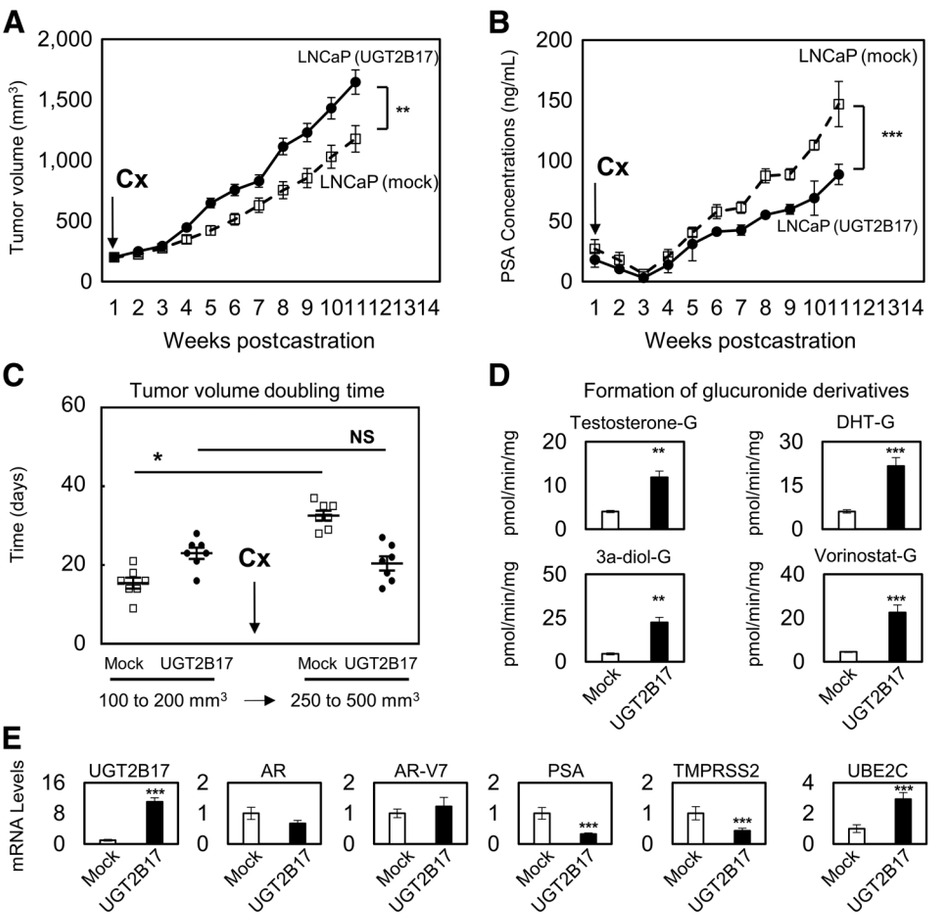


**Figure 3.**

UGT2B17 accelerates CRPC progression of LNCaP xenografts. LNCaP (mock) and LNCaP (UGT2B17) xenografts were established as described in Materials and Methods. Mice were castrated when tumor volume reached 200 $mm^3$. **A** and **B,** tumor volume (**A**) and serum PSA levels (**B**) were measured weekly. **C,** tumor doubling time was calculated during tumor growth from 100 to 200$mm^3$ and from 250 to 500 $mm^3$. **D,** xenograft tissues were collected and used for *in vitro* glucuronidation assays. **E,** total RNA from the xenograft tissues was extracted for real-time PCR assays. Statistical analyses were performed by one-way ANOVA, followed by the Student *t* test with **, $P < 0.01$ and ***, $P < 0.001$.

TMPRSS2, FKBP5, PDE9A, and NKX3.1 was stimulated, whereas the expression of genes such as DDC and OPRK1 was repressed by androgens (34). Overexpression of UGT2B17 in LNCaP cells impaired androgen regulation of these genes. To exclude the possibility that the altered gene expression was caused by factors other than AR, we depleted AR expression by RNA silencing (Supplementary Fig. S3). We confirmed that AR knockdown abolished UGT2B17 actions on gene regulated by both ligand-dependent and ligand-independent AR transcriptional activities. In contrast, AR-v7 siRNA did not affect mRNA levels of the mitosis genes, suggesting that AR-v7 does not mediate ligand-independent AR signaling in UGT2B17 overexpressing LNCaP cells (data not shown). To confirm that UGT2B17 actions on cancer cell growth and invasion

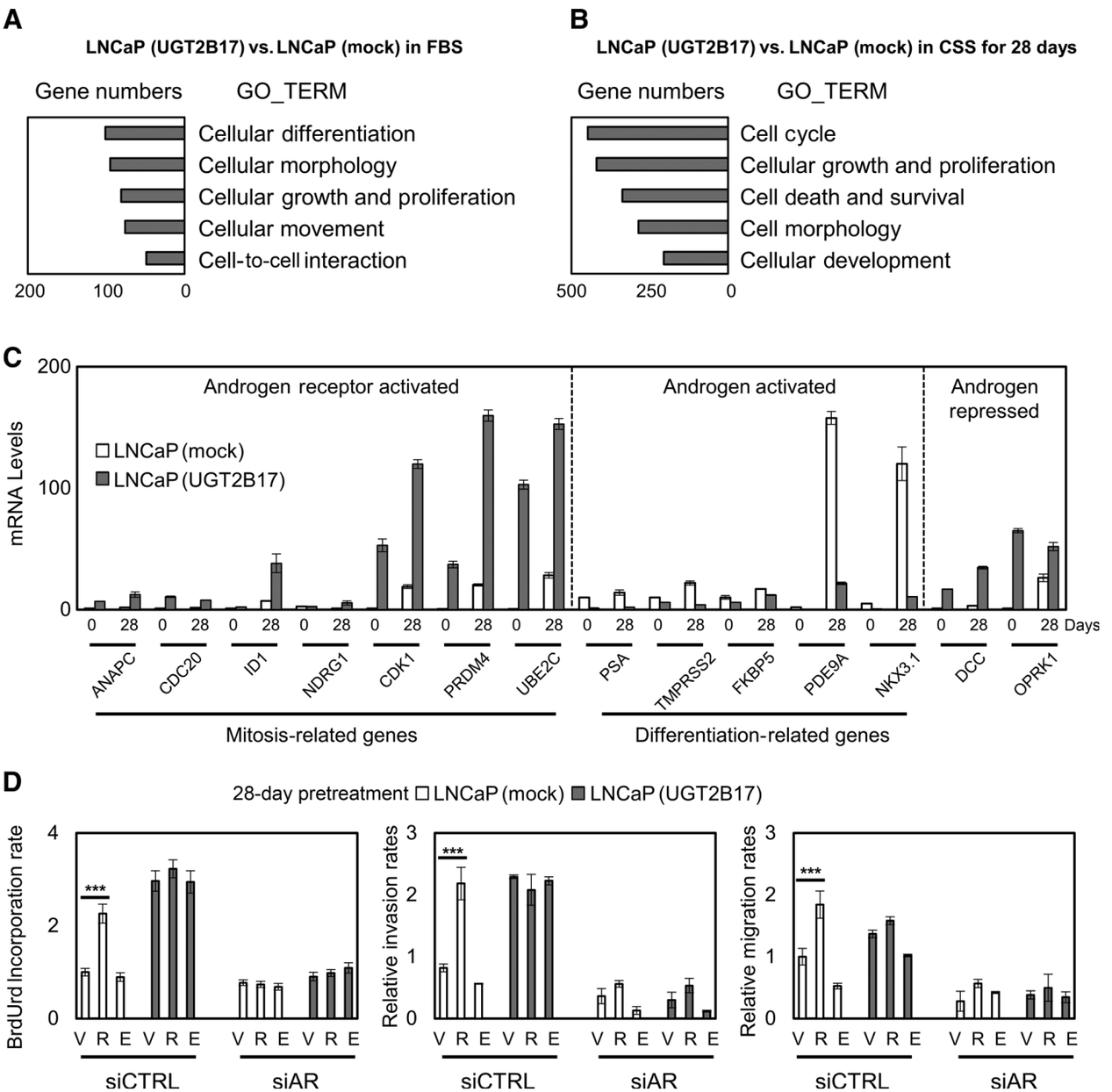


**Figure 4.**

UGT2B17 alters AR signaling under prolonged androgen deprivation. **A** and **B,** LNCaP (mock) and LNCaP (UGT2B17) cells were cultured in RPMI-1640 medium containing 5% FBS (**A**) or CSS (**B**) for 0 or 28 days. Total RNA was collected ($n = 3$ repeats/experimental condition) for microarray assays. Differentially expressed genes with fold change over 2 were analyzed by DAVID (version 6.7). Top 5 ranked GO_TERM sorted gene groups were listed. **C,** the mRNA levels of indicated genes were validated by real-time PCR assays. **D,** LNCaP (mock) and LNCaP (UGT2B17) cells were cultured in RPMI-1640 medium containing 5% CSS under the condition of vehicle (V), 10 nmol/L of R1881 (R), or 10 μmol/L of Enz (E) for 28 days. Cells were then transfected with control siRNA or siRNA against AR. Relative BrdUrd incorporation, cell invasion, and migration rates were measured. All data were repeated in three independent experiments and are shown as mean ± SEM. Statistical analyses were performed by the Student $t$ test with ***, $P < 0.001$.

are mediated by AR, we showed that UGT2B17-stimulated BrdUrd incorporation, cell invasion, and migration were abolished by AR depletion (Fig. 4D). These results evidently show that UGT2B17 activates ligand-independent but inhibits androgen-dependent AR actions in regulating gene expression, prostate cancer cell proliferation and invasion under prolonged androgen deprivation condition.

### UGT2B17 activates AR through c-Src kinase during CRPC progression

Among the cell-cycle gene group ($n = 359$ genes) regulated by UGT2B17, the IPA software predicted AR, c-Src, AKT and STAT3 as the upstream regulators. To validate these findings, LNCaP cells overexpressing either control or UGT2B17 were treated with androgen deprivation for 0 or 28 days (Fig. 5A). UGT2B17 increased c-Src activation, which was further increased by androgen deprivation. In addition, STAT3 and AKT were also stimulated by UGT2B17 under androgen deprivation (Supplementary Fig. S4). We showed that the c-Src inhibitor (PP2) not only abolished c-Src, but also AKT and STAT3 activation. In contrast, AKT and STAT3 inhibitors (LY and Stattic, respectively) had no impact on c-Src activation regulated by UGT2B17 (Fig. 5B). Furthermore, PP2 but not LY or Stattic suppressed UGT2B17-induced cell proliferation after androgen deprivation (Fig. 5C). These results indicate that c-Src is the upstream regulator of AKT and STAT3, mediating UGT2B17 actions in regulating cell cycling under androgen deprivation.

Because both UGT2B17 and c-Src are expressed in the cytosol, we performed PLA and coimmunoprecipitation (co-IP) assays to determine whether these two proteins interact with each other. Immunofluorescence assays first confirmed that both UGT2B17 (green) and c-Src (red) were localized in the cytosol after 0 or 28 days of androgen deprivation (Fig. 5D). Protein–interaction between UGT2B17 and c-Src was only observed after 28 days of androgen deprivation. This result was further confirmed by using flag antibody to precipitate flag-UGT2B17 and c-Src simultaneously (Fig. 5E). Co-IP assays indicated that UGT2B17 protein continuously accumulated after androgen deprivation in a time-dependent fashion. We also observed an increased UGT2B17 association with c-Src under prolonged androgen deprivation (Fig. 5F). These results suggest that prolonged androgen deprivation accumulates UGT2B17 protein expression, which in turn recruits and activates c-Src kinase.

To confirm whether elevated UGT2B17 expression is associated with c-Src activation in prostate cancer patients, we analyzed UGT2B17, c-Src, pSrcY416 protein levels in prostate cancer TMAs (Table 1; Fig. 6A). We found a positive association between UGT2B17 with c-Src only in CRPC tissue cores ($r = 0.3986$, $P = 0.0484$) and an even stronger association between UGT2B17 and pSrcY416 ($r = 0.5157$, $P = 0.0099$). Similar correlations were also observed in UGT2B17 overexpressed LNCaP cells under androgen deprivation (Supplementary Fig. S5). Collectively, these results demonstrate that elevated UGT2B17 expression activates c-Src in CRPC tumors.

## Discussion

Our studies identify a novel function for UGT2B17 that promotes ligand-independent AR signaling to expedite CRPC progression. We demonstrate that elevated UGT2B17, under conditions of prolonged androgen deprivation, activates c-Src kinase and stimulates AR transactivation independent of androgens (Fig. 6B). Because of the prominent role of the UGT2B17-Src-AR signaling pathway in contributing to CRPC progression, we propose a combination strategy of UGT2B17 inhibition and antiandrogens for more effective control of CRPC.

Although prostate cancer is initially androgen sensitive and responds to androgen deprivation therapies, adaptive survival pathways often culminate in CRPC. The AR remains transcriptionally active in CRPC, but its actions are shifted to regulate the mitotic transcriptome under anti-AR therapy (10–12). Because the AR possesses a ligand-dependent AF-2 domain and a ligand-independent AF-1 domain, AR antagonists facilitate transition to ligand-independent AR signaling likely through altering AR protein conformation and differential activation of AF-1 and AF-2 domains. In the presence of androgen, the $^{178}$LKDIL$^{182}$ motif in Tau1 and the $^{435}$WHTLF$^{439}$ motif in Tau5 of the AF-1 domain interact with the LBD, creating intramolecular interactions that are necessary for ligand-dependent AR transactivation (35–37). Androgen deprivation or AR antagonists not only block AF-2 activity but also disrupt AR intramolecular protein interactions, resulting in an opportunity for AF-1 to be exposed for phosphorylation and activation by kinases (38, 39). We show here that prolonged androgen deprivation enhances UGT2B17 to activate c-Src, which in turn stimulates AR functions to upregulate transcription of mitosis-related genes. Consistent with this hypothesis, C-terminus truncated AR splice variants present strong constitutive transactivation due to the loss of LBD (40). By contrast, deletion of Tau5 in AR inhibits androgen-independent AR activity in androgen-insensitive cells, but enhanced androgen-dependent AR activity in androgen-sensitive prostate cancer cells (39). These findings together emphasize that androgens and AF-2 may function as switches to modulate AF-1 activity. AR antagonists like enzalutamide do not entirely block AR function, but rather shift the AR function towards ligand-independence driven by AF-1. AR is still recruited to chromatin in CRPC, but to different locations compared with that in castrate sensitive tumors (12). The observation that low PSA tumors are often more aggressive and have poorer prognosis (41, 42) may reflect AR function mode in these tumors shifting away from regulating androgen (ligand)-dependent AR target genes such as PSA. Together, these results support that upregulation of UGT2B17 expression by prolonged androgen deprivation serves as a key facilitator for AR to act ligand independently.

Although enhanced UGT2B17 expression has been previously reported associate with CRPC (15, 18, 43), our studies define several mechanisms whereby UGT2B17 enhances ligand-independent AR signaling for CRPC progression: 1) UGT2B17 enhances intratumoral androgen depletion to facilitate AR signaling shifts towards a ligand-independent mode. This is supported by our results showing that androgen-independent LNCaP95, LNCaP(AI) cells and enzalutamide-resistant MR49F cells have higher UGT2B17 expression and glucuronidation activity. UGT2B17-stimulated cell proliferation after prolonged androgen depletion and UGT2B17-driven CRPC xenograft growth are both associated with enhanced androgen catabolism activities. Intratumoral androgen depletion by UGT2B17 suppresses AF-2 activity, but encourages AF-1 to be phosphorylated by kinases and become transcriptionally active; 2) UGT2B17 also interacts with and activates c-Src kinase, which in turn stimulates AR phosphorylation and activation

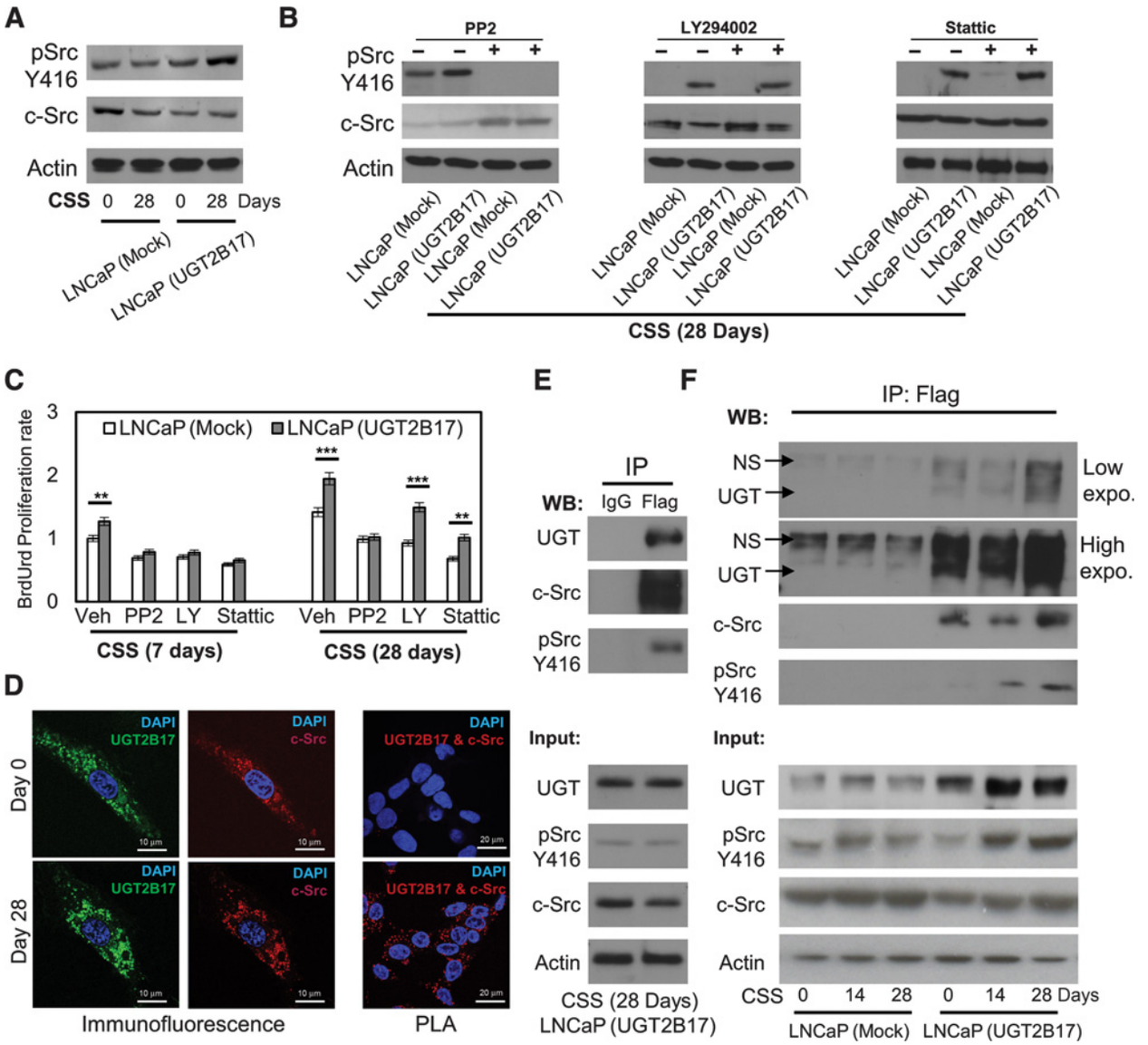


**Figure 5.**

UGT2B17 activates AR through c-Src kinase for CRPC progression. **A,** LNCaP (mock) and LNCaP (UGT2B17) cells were cultured in RPMI1640 medium containing 5% CSS for 0 or 28 days. **B,** LNCaP (mock) and LNCaP (UGT2B17) cells cultured in RPMI-1640 medium containing 5% CSS for 28 days. Cells were then treated with vehicle, or 10 μmol/L of PP2, WP1066 or Stattic for another 12 hours. Protein levels of pSrc Y416, c-Src, and actin were measured by immunoblotting. **C,** LNCaP (mock) and LNCaP (UGT2B17) cells were cultured in RPMI-1640 medium containing 5% CSS for 7 and 28 days, and then treated with vehicle or 10 μmol/L of PP2, WP1066 or Stattic for 48 hours. Relative BrdUrd incorporation rates were measured. **D,** LNCaP (UGT2B17) cells were cultured in RMPI-1640 medium containing 5% CSS for 0 or 28 days. Immunofluorescence assays (left) were performed with UGT2B17 (green) and c-Src (red) antibodies. PLA assays (right) using UGT2B17 and c-Src antibodies were performed as described in Materials and Methods. Red dots, UGT2B17 and c-Src interactions. **E** and **F,** LNCaP (mock) and LNCaP (UGT2B17) cells were cultured in RPMI-1640 medium containing 5% CSS for 0, 14, or 28 days. Whole-cell lysates were collected for immunoprecipitation using IgG or flag antibody (**E**) or using flag antibody (**F**). Eluent was then immunoblotted with UGT2B17, c-Src, or pSrc Y416 antibodies. Note: nonspecific (NS) proteins appeared when the UGT2B17 antibody was used to perform co-IP.

independent of androgens. As a non-receptor tyrosine kinase, c-Src needs to be recruited to the membrane-bound tyrosine kinase receptors to activate downstream signal pathways. However, UGT2B17 is an endoplasmic reticulum membrane-bound enzyme that may have a relatively weak affinity to c-Src. Because UGT2B17 protein is accumulated in prostate cancer cells after prolonged androgen depletion (Supplementary Fig. S2; Fig. 5F), we propose that when UGT2B17 expression reaches a threshold, the enzyme begins to interact with and activate c-Src kinase. This may explain why UGT2B17 can only activate c-Src after its expression reaches certain levels; and 3) it should also be noted that UGT2B17 activated c-Src can trigger downstream effectors in addition to AR such as MAPK, AKT and STAT3. These signal pathways may in turn regulate AR

**Table 1.** Correlation of UGT2B17 expression with c-Src and pSrc Y416 (Pearson coefficient test)

| | c-Src vs UGT2B17 | | pSrc Y416 vs UGT2B17 | |
|---|---|---|---|---|
| **Group** | ***r* value** | ***P* value** | ***r* value** | ***P* value** |
| Benign | 0.2988 | 0.1469 | 0 | >0.9999 |
| Hormone naïve | 0.1992 | 0.1896 | 0.1768 | 0.2509 |
| NHT | −0.1548 | 0.4805 | −0.2495 | 0.2396 |
| CRPC | 0.3986 | **0.0484**[a] | 0.5157 | **0.0099**[b] |

NOTE: Correlation analysis of H-scores between UGT2B17 and c-Src or pSrc Y416 in benign, hormone naïve, NHT and CRPC tissue groups were performed using Pearson coefficient tests. Statistical significance is indicated in bold font.
[a]$P < 0.05$.
[b]$P < 0.01$.

transactivation indirectly and modulate gene transcription associated with cell mitosis. Together, our results demonstrate that UGT2B17 can utilize multiple pathways to activate ligand-independent AR signaling to expedite CRPC progression.

Our results show that AR signaling transformation regulated by UGT2B17 only becomes prominent after prolonged androgen depletion using LNCaP, LNCaP95 and VCaP cell models (Fig. 2–5; Supplementary Fig. S6). One possible explanation for this is that transient androgen depletion stimulates *de novo* steroidogenesis, whereas prolonged androgen depletion combined with UGT2B17 protein accumulation abolish androgen exposure of AR more thoroughly. In addition, shift of AR signaling to ligand-independent mode requires c-Src activation resulting from UGT2B17 accumulation in prostate cancer cells. Although our co-IP and PLA assays demonstrate that UGT2B17 interacts with and activates c-Src, these protein interactions may be indirect, possibly through some endoplasmic reticulum-associated protein factors such as PTP1B (44). Regardless of direct or indirect protein interactions between UGT2B17 and c-Src, the UGT2B17/c-Src complex leads to the activation of the ligand-independent AR signaling under prolonged androgen deprivation conditions. Several previous studies showed that androgen inhibited, whereas antiandrogens enhanced UGT2B17 mRNA levels (18, 33). AR recruitment to the UGT2B17 promoter was also reported to be responsible for suppressing UGT2B17 gene transcription (33). It remains to be determined on whether ligand-independent AR signaling induced by UGT2B17 in turn would enhance UGT2B17 transcription through a feed-forward mechanism and whether AR regulates UGT2B17 protein stability.

Mechanisms that CRPC tumors develop resistance to new-generation antiandrogens such as enzalutamide and abiraterone are not fully understood. Generation of LBD truncated AR splice variants and accumulation of gain-of-function of mutations in LBD had been credited for these therapy resistance (6–8). However, not all CRPC tumors express AR-v7 or mutant ARs. Because CRPC tumors predominantly express high levels of AR, our studies propose that anti–AR-resistant tumors are mainly driven by a re-programmed AR signaling from being ligand-dependent to ligand-independent that regulates transcription of mitosis genes. Such AR functional reprogramming is manifested by a shift in AR dependence on AF-2 in LBD to AF-1 in AR N-terminus to regulate target gene transcription. This explanation is more broadly applicable to CRPC tumors resistant to AR pathway inhibitors like abiraterone and enzalutamide. In this context, UGT2B17, which usually mediates androgen catabolism, paradoxically supports the transition of AR signaling to be ligand independent by activating c-Src kinase.

UGT2B17 belongs to the UGT2B family, in which UGT2B15 and UGT2B28 are the other two members expressed in the prostate that regulate androgen metabolism (13). Compared with the benign prostate, UGT2B15 protein levels were reduced in hormone naive tumors and CRPC, and became undetectable in lymph node metastases (15). In contrast with UGT2B17 that can catabolize all three major androgens, UGT2B15 can only de-activate DHT with much lower efficacy than UGT2B17 (45). Recently, UGT2B28 expression was reported to be positively associated with high-grade prostate cancer, suggesting that it may play a similar role to UGT2B17 (46). It remains to be determined whether UGT2B28 expression is associated with anti-AR therapies, CRPC progression and tumor metastasis.

In summary, our studies demonstrate that UGT2B17 facilitates c-Src–activated ligand-independent AR signaling, thereby

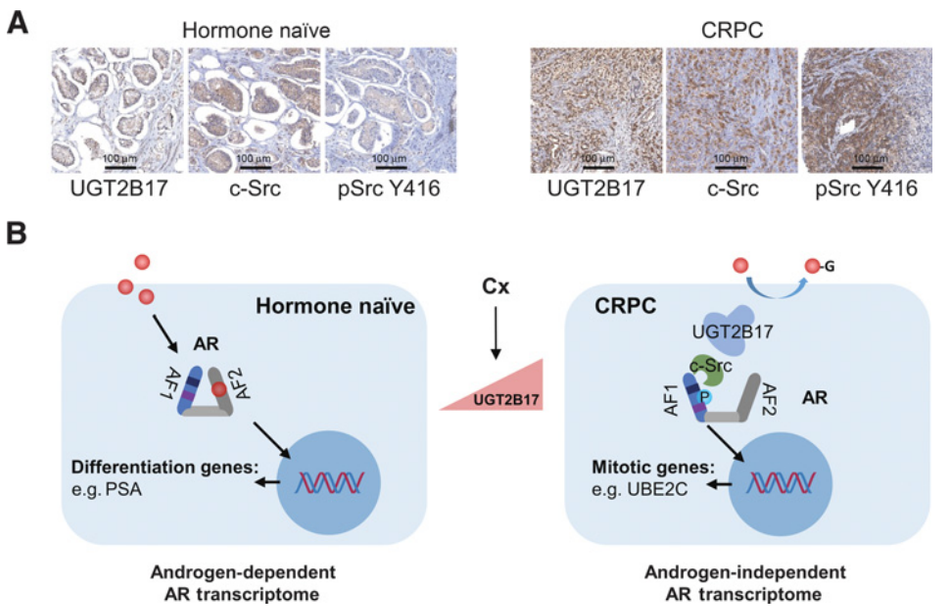


**Figure 6.**
UGT2B17 expression is associated with c-Src activation in CRPC. **A,** representative IHC images of UGT2B17, c-Src, and pSrc Y416 in the hormone naïve and CRPC tissue groups were shown. **B,** a diagram summarizes the mechanisms by which increased UGT2B17 by androgen deprivation in CRPC activates AR through stimulating c-Src.

supporting CRPC progression. We propose that combination of AR pathway inhibitors with UGT2B17 inhibition could more potently suppress CRPC progression.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

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**Development of methodology:** H. Li, L. Fazli, X. Dong
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** H. Li, N. Xie, M. Verreault, L. Fazli, M.E. Gleave, O. Barbier
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** H. Li, N. Xie, M. Verreault, L. Fazli, O. Barbier, X. Dong
**Writing, review, and/or revision of the manuscript:** H. Li, R. Chen, M. Verreault, L. Fazli, M.E. Gleave, O. Barbier, X. Dong
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** H. Li, R. Chen, L. Fazli
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