Utilizing Catalytic Topo II Inhibitor to Target Reestablished Androgen Receptor Signaling in Castration-Resistant Prostate Cancer

by

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Abstract

Prostate cancer, the most common malignancy in Canadian men, is a leading cause of cancer-related male mortality. Androgen deprivation therapy is the first-line treatment for advanced prostate cancer. However, a fatal relapse to androgen deprivation therapy is inevitable, which is often characterized by the establishment of an androgen-independent AR signalling that drives the disease to the lethal castration-resistant prostate cancer (CRPC) stage. Defining the mechanisms that promote the reestablishment of AR signaling including the androgen independence is important for therapy development and disease control. UDP-glucuronosyltransferase 2B17 (UGT2B17) is a key enzyme that maintains androgen homeostasis by catabolizing AR agonists into inactive forms and its expression has been reported to increase after antiandrogen treatment. Whether UGT2B17 plays a role in the progression of CRPC is unclear.

In this work, we demonstrated that the higher expression of UGT2B17 protein is associated with higher Gleason scores, increased metastasis and CRPC progression in prostate tumors. The expression and activity of UGT2B17 were also higher in androgen-independent cell lines compared to androgen-dependent cell lines. Overexpression of UGT2B17 stimulated cancer cell proliferation, invasion, and xenograft progression to CRPC after prolonged androgen deprivation. Furthermore, UGT2B17 not only suppressed androgen-dependent AR transcriptional activity but also enhanced androgen-independent AR transcriptional activity, mainly through activating the c-Src kinase. These results indicate that the UGT2B17-Src-AR signaling contributes to the reestablished AR signaling and expedites CRPC progression and blocking the UGT2B17-Src-AR cascade will be beneficial for overcoming the resistance in

CRPC patients. Accordingly, pharmacological targeting of the catalytic domain of DNA topoisomerase II (Topo II), which is known to be essential for AR-mediated transcriptional control, can completely block the transcriptional activity of reestablished AR, mutant ARs and AR splicing variants. Targeting Topo II also strengthened the efficacy of current anti-androgens in suppressing wild type AR activities. Furthermore, catalytic Topo II inhibitors inhibited CRPC and enzalutamide-resistant prostate cancer cell growth and xenograft progression.

Overall, my doctoral thesis demonstrates that the UGT2B17-Src-AR signaling axis contributes to the reestablished AR signaling and expedites CRPC progression, and that applying catalytic Topo II inhibitors can block the transcriptional activity of reestablished AR signaling and suppress CRPC progression.

Preface

Animal care and experimental procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) under the approval of the Animal Care Committee of the University of British Columbia (protocol #: A14-0088). All other experiments were carried out in accordance with the University of British Columbia Biosafety Committee approved protocol number #B13-0187.

A version of Chapter 2 has been previously published. [Li H], Xie N, Chen R, Verreault M, Fazli L, Gleave ME, Barbier O, Dong X. *UGT2B17 Expedites Castration-Resistant Prostate Cancer Progression by Promoting Ligand-independent AR Signaling. Cancer Research. 2016 Sep 22. pii: canres.1518.2016.* Article and figures are adapted with permission from the AACR. As the lead investigator, I was responsible for the experimental design, data collection and analysis, as well as manuscript composition. Xie N was involved in data collection and analysis. Chen R was involved in revising the manuscript. Verreault M was involved in UGT enzyme activity measurement. Fazli L was involved in tissue microarray construction and evaluation. Gleave ME and Barbier O were involved in interpreting data and reviewing the article. Dong X was the supervisory author of this project and was involved throughout the project in concept formation and manuscript design.

A version of Chapter 3 has also been previously published. [Li H], Xie N, Gleave ME, Dong X. *Catalytic Inhibitors of DNA Topoisomerase II Suppress the Androgen Receptor Signaling and Prostate Cancer Progression. Oncotarget. 2015 May 12.* Articles and figures are adapted with permission from Oncotarget Impact Journals. As the lead investigator, I was responsible for the

experimental design, data collection and analysis, as well as manuscript composition. Xie N was involved in producing and analyzing data. Gleave ME was involved in reviewing the manuscript. Dong X was the supervisory author of this project and was involved throughout the project in concept formation and manuscript design.

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List of Abbreviations

ADT: androgen deprivation therapy

AR: androgen receptor

ARE: androgen response element

ARPI: androgen receptor pathway inhibitor

ARVs: AR splice variants

AD: androstenedione

ACTH: adrenocorticotropic hormone

AKR1C: aldo-keto reductase family 1, member C

AKT: protein kinase B

ATP: adenosine triphosphate

ASOs: antisense oligonucleotides

BPH: benign prostatic hyperplasia

Bic: bicalutamide

BrdU: bromodeoxyuridine

BRCA2: breast and ovarian cancer susceptibility protein 2

CRPC: castration-resistant prostate cancer

CYP17: cytochrome P450 isoform 17

CSS: charcoal stripped serum

c-Src: proto-oncogene tyrosine-protein kinase Src

DRE: digital rectal examination

DHT: dihydrotestosterone

DHEA: dehydroepiandrosterone

DBD: DNA-binding domain

DNA-PK: DNA-dependent protein kinase

DSBs: double strand breaks

DCC: deleted in colorectal cancer protein

ETS: erythroblast transformation-specific

ERG: ETS-related gene

ENZ: enzalutamide

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

FDA: food and drug administration

FOXA1: forkhead box protein A1

FBS: fetal bovine serum

FACS: fluorescence-activated cell sorting

FGF: fibroblast growth factor

Grb2: growth factor receptor bound protein 2

HGF: hepatocyte growth factor

H&E: haematoxylin and eosin staining

HSD3B: hydroxy-delta-5-steroid dehydrogenase 3 beta

HSD17B: hydroxysteroid 17-β dehydrogenase

HSP: heat shock protein

H-score: histology scores

IHC: immunohistochemistry

IGF-1: insulin-like growth factor-1

ICRF187, ICRF193: bisdioxopiperazines

LHRH: luteinizing hormone-releasing hormone

LDB: ligand binding domain

LC-MS/MS: liquid chromatography–mass spectrometry

LY294002: PI3K/Akt inhibitor

MAPK: mitogen-activated protein kinase

Mcl-1: induced myeloid leukemia cell differentiation protein

NKX3.1: homeobox protein

NTD: N-terminal domain

NR3C4: nuclear receptor subfamily 3, group C, gene 4

NHT: neoadjuvant hormone therapy

NEPC: neuroendocrine prostate cancer

ORPK1: opioid receptor kappa 1

PCa: prostate cancer

PSA: prostate-specific antigen

PTEN: phosphatase and tensin homolog

PI3K: phosphatidylinositol 3-kinase

PKA: cAMP-activated kinase A

PKC: protein kinase C

PARP1: poly [ADP-ribose] polymerase 1

PDX: patient-derived xenograft

PP2: src kinase inhibitor

R1881: 17α-methyltrenbolone

SPOP: speckle type POZ protein

SRD5A: 5α-reductase

SH3: src homology 3

SHC: SH3 domain containing transforming protein

siRNA: small interfering RNA

Stattic: STAT3 inhibitor

TMPRSS2: transmembrane protease serine 2

Topo II: DNA Topoisomerase II

TNM: Tumor, Node, Metastasis

T: testosterone

TAU: transcriptional activation units

TMA: tissue microarray

UGE: urogenital sinus epithelium

UGM: urogenital sinus mesenchyme

UGT: UDP-glucuronosyltransferase

UDP: uridine diphosphate

UDPGA: UDP-glucuronic acid

3α-DIOL: androstane-3α-diol

3-DIOL-17G: 3-DIOL-glucuronide

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Chapter 1: Introduction

1.1 Prostate cancer

1.1.1 Overview

Prostate cancer (PCa) is the most common malignancy in men and a leading cause of cancer-related male mortality [1]. According to Global Burden of Cancer Study (GLOBOCAN) 2012 statistics from the International Agency for Research on Cancer, PCa accounted for 1.1 million estimated new cases and 307,500 estimated deaths worldwide. Base on the Canadian Cancer Statistics estimated incidence rate as of 2016, 1 in 8 Canadian men will be diagnosed with PCa in their lifetime, and the most common age of diagnosis is 60-69 years (40%). It was estimated that 4,000 Canadian men will die from PCa in 2016. The mortality rate of PCa has declined since the mid-1990s, owing to the introduction of improved systemic therapies [2, 3].

Early stage PCa presents as an androgen-dependent tumor, which is generally slow progressing and treated with localized therapies including surgery or radiation therapy [4]. Around 30% of the patients will progress to an advanced disease state [5]. First line treatment of locally advanced or metastatic PCa is androgen deprivation therapy (ADT) through medical castration with luteinizing hormone-releasing hormone (LHRH) agonists/antagonists or anti-androgens [6]. Although ADT on average leads to remission lasting 2-3 years, the disease inevitably progresses to castration-resistant prostate cancer (CRPC) stage, which is associated with poor prognosis and poses considerable therapeutic challenges [7].

Currently, there are several major challenges pertaining to the management of PCa, including the understanding of the progression from hormone naïve PCa to CRPC and the lack of effective

therapeutic agents to block all reestablished AR signaling in CRPC (Figure 1.1). The historical median survival estimation of CRPC is 18 to 24 months [8]. Latest Food and Drug Administration (FDA) approved androgen receptor pathway inhibitors (ARPIs) such as enzalutamide and abiraterone successfully prolong the overall survival time by ~3 months in their double-blind, randomized phase III studies [9, 10]. These facts emphasized that AR signaling remains a mainstream therapeutic target to delay tumor progression and alternative ARPIs that thoroughly block the AR signaling are needed.

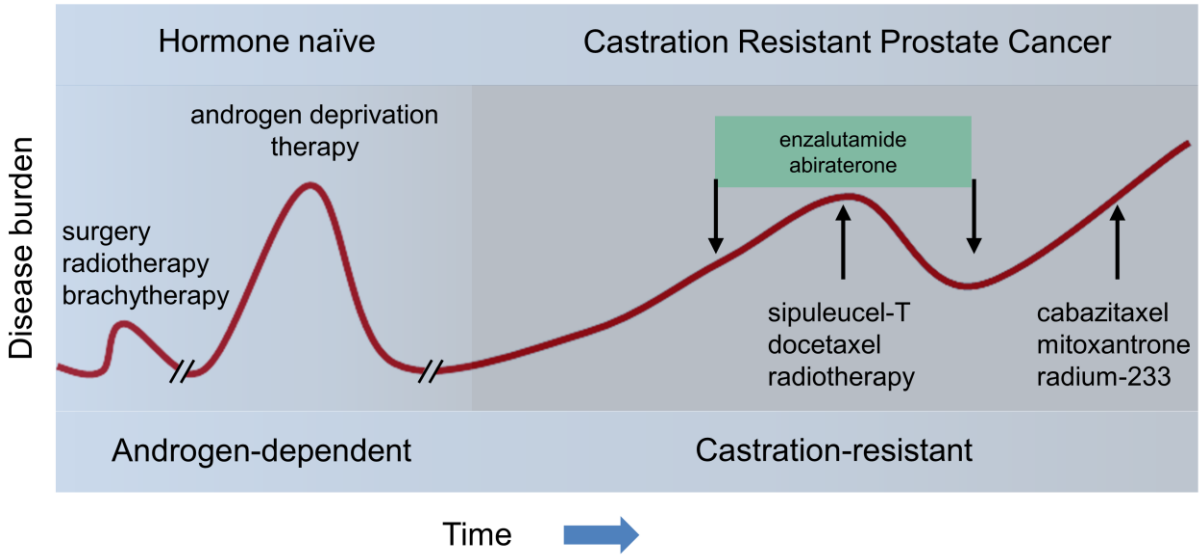

Figure 1.1 The disease progression and current treatment landscape of prostate cancer (PCa).

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1.1.2 The prostate

Human prostate development is initiated during the 10th week of gestation, when the prostatic buds begin to grow from the urogenital sinus epithelium (UGE) into the urogenital sinus mesenchyme (UGM) [11]. Androgens activate the AR in the UGM, generating the secretion of paracrine growth factors and cytokines that induce UGE budding, proliferation and

differentiation [12]. Growth factors such as hepatocyte growth factor (HGF) and fibroblast growth factors (FGF7 and FGF10) play a putative role in this stromal-epithelial signaling during prostate development [13]. While in the adult prostate, androgens act on the prostatic smooth muscle to maintain a growth-quiescent structure, involving the smooth muscle-derived protein PS20 and transforming growth factor beta (TGFβ) family members [14]. The UGE further differentiates into prostatic basal cells and luminal cells, while the UGM develops into prostatic smooth muscle cells and fibroblasts [15, 16]. Neuroendocrine cells are also scattered within the prostate. Together, these compartments form the epithelium and stromal structure of the prostate.

The human prostate is small in childhood. At puberty, it gradually grows into a walnut-sized gland (6-16 g) stimulated by elevated serum testosterone levels [12]. The adult prostate is an exocrine gland of the male reproductive and urinary systems [17]. It is located under the bladder, in front of the rectum and encircling the urethra [18]. Anatomically, the prostate is divided into three main areas: the central, peripheral, and transition zones [19]. The peripheral zone is the region from which adenocarcinoma and post-inflammatory atrophy commonly arise [19-21]. The transitional zone is the region from which benign prostatic hyperplasia (BPH) develops, a non-malignant overgrowth that causes urethra compression [21, 22]. The main functions of the prostate are to secrete the prostate fluid making up one part of the semen and to help expel semen during ejaculation [23]. The prostate secretion contains various enzymes that increase the mobility of sperm, such as prostate-specific antigen (PSA) [24]. The secretory function as well as the growth and differentiation functions of prostate have been shown to be regulated by androgens [25].

1.1.3 Prostate carcinogenesis

There are three major risk factors including age, race and family history that contribute to the development of PCa [26]. Age is the biggest factor as the majority of PCa cases were diagnosed after the age of 65 [27]. During the aging process, the human prostate is influenced by chronic inflammation induced by infections [28-30]. These abnormalities further lead to a number of cellular processes including hyper proliferation, prostatic cell de-differentiation, and disruption of the homeostatic microenvironment [31]. In addition to aging, AR gene polymorphism with shorter CAG repeats was associated with higher risk for PCa [32, 33]. Other risk factors include family history, germline genetic influences, race, and diet [2, 27, 34-36].

The genomic and transcriptomic profiling analyses of clinical samples provide a molecular pathology platform to identify genes involved in PCa carcinogenesis and progression [37]. Here we focus on the AR-related factors. Studies have demonstrated that AR-mediated transcriptional activity acts as a driver of genomic rearrangements in primary disease [38]. The gene fusion of the 5' untranslated end of an AR-regulated gene (transmembrane protease serine 2, TMPRSS2) and the ETS transcription family member (e.g. ERG) was identified in 57% of localized prostate tumors [39, 40]. Androgens and the AR have been suggested to promote the gene fusion of TMPRSS2: ERG through the recruitment of human DNA Topoisomerase II (Topo II) [41]. Non-ETS fusion PCa is often associated with FOXA1 mutations, SPOP mutations, CHD1 deletion, elevated SPINK1 gene expression, and RAF activation [42-45]. In particular, the AR interacts with forkhead box A1 (FOXA1) to modulate its transcriptional activity [46]. Mutations of FOXA1 increase cancer cell proliferation in primary and metastatic tumors [47]. SPOP and FOXA1 mutant tumors have the highest levels of AR-induced transcripts [48]. Androgen-

dependent AR regulates homeobox protein NKX3.1 (NKX3.1), and the latter's loss-of-function is regarded as an early event in prostate carcinogenesis [49]. Other common non-AR related mutations in PCa include loss-of-function mutations of phosphatase and tensin homolog (PTEN) and TP53 [47]. PTEN loss occurs in about 40% of primary tumors, and allows for the activation of the phosphatidylinositol 3-kinase (PI3K) pathway, which reciprocally interacts with the AR signaling [50].

A recent ground-breaking study on PCa evolution utilized whole-genome sequencing to characterize multiple metastases arising within patients [51]. Findings from this study revealed that the metastatic spread patterns of PCa were through *de novo* monoclonal seeding or transfer between metastatic sites. Moreover, lesions affecting tumor suppressor genes usually occurred as single events, whereas mutation in genes involved in AR signaling commonly involve multiple, convergent events in different metastases. Collectively, these genetic abnormalities associated with AR activities contribute to PCa initiation and progression.

1.1.4 Detection and diagnosis

Early stage PCa usually shows no symptoms because of its slow growth in the beginning, while invasive and metastatic PCa causes frequent and painful urination, weight loss, fatigue, and pelvic or lower back pain [52]. In the 1990s, PCa detection was achieved through findings on an abnormal digital rectal examination (DRE) and/or elevated PSA [53]. PSA is a member of the kallikrein gene family, also known as human kallikrein peptidase 3, and is regulated by androgen and the AR [54]. It is primarily produced by prostatic luminal epithelial cells, and acts as protease to liquefy semen [54]. Elevated serum PSA levels are probably a product of the

disruption of the cellular architecture of the prostate gland, diffusing PSA into wider circulation, thus reflecting the alterations made by prostate disease (PCa, benign prostatic hyperplasia, or prostatitis) [55].

To further detect the presence of PCa, patients are subjected to a prostate needle biopsy guided with ultrasound or magnetic resonance imaging for definitive diagnosis [56, 57]. This systematic biopsy procedure would include a minimum of 12 core samples, as recommended by the American Urological Association [58]. Tissue samples are then analyzed by haematoxylin and eosin (H&E) staining as well as immunohistochemistry (IHC), followed by histopathological assessment by pathologists [59]. Urological pathology defines the diagnosis of the patients with the Gleason grading system and Tumor, Node, Metastasis (TNM) stage [60]. The Gleason grading is based on the glandular pattern of the tumor, and is assigned a grade from 1 to 5, with 1 being the most differentiated, and 5 being undifferentiated tissue structure [61]. Gleason pattern 1: well-circumscribed nodule of closely packed glands, 2: nodule with more loosely arranged glands, 3: small glands with an infiltrative pattern between benign glands, 4: large irregular cribriform glands, 5: solid nests of tumor with central comedonecrosis. The most common and next most common grades observed from biopsies are combined to result in the Gleason score. A Gleason score of 6 or less refers to a low-grade cancer, whereas a Gleason score of 8-10 refers to a high-grade aggressive disease with poor patient prognosis.

In addition to grading, TNM and their sub-classifications indicate the carcinoma staging, location, and spread of the tumor [62]. Within the system, T (1-4) pertains to the tumor within

and immediately adjacent to the prostate tissue, N (0-1) describes the presence of lymph node metastasis, and M (0-1) denotes to the presence of distant metastasis.

1.1.5 Clinical treatments

Guidelines for PCa clinical management have been established in various countries and regions, and provide the general recommendation for localized, metastatic, or castration-resistant prostate cancer. Patients with localized, low-grade PCa (Gleason score of 6 or less) are evaluated for active surveillance [63]. Patients who opt for active surveillance are monitored with DRE and PSA testing quarterly or semiannually, and repeated prostate biopsy yearly [64].

Patients with intermediate to high-risk (Gleason score >7) PCa and a life expectancy of at least 10 years are recommended to receive therapies such as surgery (radical prostatectomy) with salvage radiation therapy and radiation therapy (external beam radiotherapy or brachytherapy) with neoadjuvant androgen-deprivation therapy [65]. Radical prostatectomy is the surgical removal of the prostate and adjacent tissue, including the seminal vesicle [66]. External beam radiotherapy most commonly involves the use of beams of gamma radiation, directed at the prostate and surrounding tissues through multiple fields [67]. Radiotherapy is usually recommended in combination with androgen deprivation therapy; as such adjuvant ADT could greatly benefit the overall survival rate [68].

Although initially effective, 20-30% of localized PCa cases after the surgery and radiation therapies will progress to advanced stages within 5 years [5]. Biochemical relapse after radical prostatectomy is indicated by rising PSA levels, and the subsequent metastasis of the disease is

detected by multiparametric magnetic resonance imaging (MRI) and positron emission tomography (PET) scanning [69]. First line treatment for locally advanced, recurrent, and metastatic PCa is androgen deprivation therapy, which reduces serum androgen levels through medical or surgical castration [70]. Medical castration interferes with the hypothalamic-pituitary-adrenal axis [70]. It involves the continuous administration of luteinizing hormone-releasing hormone (LHRH) agonists or antagonists [71]. Despite being effective initially, virtually all patients will develop biochemical and clinical treatment resistance in approximately 2 to 3 years, entering the lethal castration-resistant prostate cancer (CRPC) stage [71].

1.1.6 Castration-resistant prostate cancer

Castration-resistant prostate cancer (CRPC) is extremely life-threatening, giving the patient a 16-18 month median overall survival [72]. Patients who exhibit PSA recurrence following castration with no radiological evidence of metastases, are referred to as M0 CRPC subset [73]. Currently, there are no FDA-approved drugs specifically recommended for M0 CRPC [74]. Men with extensive metastatic CRPC will be offered several treatment options including androgen receptor pathway inhibitor (ARPI) and systemic chemotherapy [75].

Next generation ARPIs, including enzalutamide (Xtandi, approved in 2014 based on the PREVAIL trial [10]) and abiraterone acetate (Zytiga) with prednisone (approved in 2012 based on the COU-302 trial [69]), serve as the first-line therapies for men with asymptomatic or minimally symptomatic chemotherapy-naive M1 CRPC [9, 10]. Enzalutamide (ENZ) is a second generation antiandrogen and a potent oral nonsteroidal AR antagonist [10]. Abiraterone acetate inhibits cytochrome P450 isoform 17A1 (CYP17A1), which exhibits as two enzymes 17α-

hydroxylase and 17,20-lyase [9]. Abiraterone also inhibits 11β hydroxysteroid dehydroxylase, 5α reductase and 3β HSDII. The CYP17A1 catalyzes the conversion and formation reactions of extragonadal androgens dehydroepiandrosterone (DHEA) and androstenedione from their precursors [76]. Enzalutamide and abiraterone also prolonged overall survival among patients with metastatic CRPC who previously received chemotherapy in the AFFIRM [81] and COU-AA-301 [80] trials, respectively. Sipuleucel-T (Provenge) is the first FDA-approved therapeutic vaccine for the treatment of patients with asymptomatic or minimally symptomatic metastatic CRPC [77].

Men with symptomatic metastatic CRPC are recommended for treatment with docetaxel (first line chemotherapy), a taxane-based microtubule-depolymerizing agent that prolongs the progression-free and overall survival time than previous used mitoxantrone base on the TAX 327 phase III study in 2004 [78]. Cabazitaxel has emerged as a second-line chemotherapy option [79]. Radium-223 is recommended for mCRPC with symptomatic bone metastases after FDA approval in 2013 based on the ALSYMPCA trial [80]. Clinical evidence also indicates that 5-10% of patients develop neuroendocrine/anaplasitic phenotype with undetectable PSA, yet rapid disease progression [81]. No effective therapy is available for such tumors besides systemic chemotherapy including platinum-docetaxel combinations [80].

1.1.7 ARPI resistance

Even though recently approved ARPIs such as ENZ and abiraterone showed improvements in the survival of CRPC patients, resistance to these drugs eventually occurs. Clinical studies have revealed that the gain in median survival after docetaxel of ENZ is 4.8 months and abriaterone is

3.9 months. All patients' disease eventually progressed after 15-24 months [9, 10]. Thus, none of these drugs are curative. Additionally, incidences of resistance towards these agents, occurring shortly after treatment, have been increasingly reported. The resistance mechanism (Chapter 1.2) indicates that AR signaling still functions under maximum androgen blockade conditions. An improved understanding of the mechanism underlying ligand-independent AR signaling is crucial for the development of new strategies to suppress aggressive cancers or delay their recurrence.

1.2 AR signaling in CRPC

The AR is a transcription factor belonging to the steroid receptors superfamily [82]. Upon binding with androgens, the AR disassociates from chaperon proteins in the cytoplasm, dimerizes, and is transported to the nucleus. The ligand-activated AR recruits coregulators, including coactivators or corepressors, to the specific DNA sequences (androgen response element, ARE) within the targeted gene promoters, resulting in either enhanced or repressed gene transcription rates [83]. In addition, the AR can also form a protein complex with proto-oncogene tyrosine-protein kinase Src (c-Src) and trigger mitogen-activated protein kinase (MAPK) pathways to mediate AR non-genomic actions [84, 85]. Androgen homeostasis is critical to maintain the ligand-dependent AR signaling [70].

1.2.1 Androgen production

Testosterone (T) is the major circulating androgen. It is produced from cholesterol in the Leydig cells of the testis [86]. This synthesis is stimulated by luteinizing hormone and follicle-stimulating hormone, released from the pituitary gland, which is, in turn, regulated by luteinizing

hormone-releasing hormone (LHRH) from the hypothalamus [87]. In plasma, T is mainly bound to sex hormone-binding globulin [88]. Unbound testosterone diffuses through the cell membrane into the cytoplasm, and undergoes conversion to dihydrotestosterone (DHT) by the 5α-reductase 1 and 2 (SRD5A1 and SRD5A2) [89]. A 5α-reductase inhibitor, finasteride is used for the treatment of BPH [90]. DHT is the major form of androgen found within the prostate [91].

Adrenal glands also produces circulating androgens: dehydroepiandrosterone (DHEA) and androstenedione (AD) [92]. This production is stimulated by adrenocorticotropic hormone (ACTH), released by the pituitary gland in response to the corticotropin-releasing factor [93].

The molecular mechanisms of androgen biosynthesis are divided into classical and non-classical pathways. In the classical pathway, reactions begin with the cleavage of cholesterol into pregnenolone and progesterone [94]. These C21 precursors are then converted to the adrenal androgens (DHEA and AD) through 17α-hydroxylase (CYP17A) [94]. The adrenal androgens are converted to T through a series of reactions involving the activities of hydroxy-Δ-5-steroid dehydrogenase 3β- and steroid Δ-isomerase 2 (HSD3B), Hydroxysteroid 17-β dehydrogenase (HSD17B), and Aldo-keto reductase family 1, member C3 (AKR1C3) [95]. T is converted to potent DHT by SRD5A [95]. In the backdoor pathway, C21 precursors initially undergo reduction with SRD5A, then react with the AKR1C2, CYP17A1, and HSD17B3 to produce Androstandiol [96]. The product then undergoes a reversible reaction to form DHT [96]. Recent studies showed that *de novo* steroidogenesis of DHT from DHEA and AD provide the residual intra-prostatic androgens after ADT [97, 98]. Similar to the abiraterone targeting CYP17A1

enzyme [99], AKR1C3 [100] and HSD3B1 [101] are also involved in the DHT biosynthesis route in PCa, and could be targets for further androgen blockade.

1.2.2 Androgen catabolization

In the human prostate, the degradation of androgens occurs through Phase I androgen metabolism and Phase II glucuronidation [102]. Phase I is a reversible conversion between DHT to its metabolites androsterone or 3α-Androstanediol (3α-DIOL), and is mediated by HSD17B or HSD3B [103]. The metabolites are then completely inactivated by uridine diphosphate (UDP)-glucuronosyltransferase (UGT) enzymes [104]. The UGT enzymes transfer the glucuronosyl group from UDP-glucuronic acid (UDPGA) to hydrophobic molecules (DHT). The resulting glucuronide products, such as androsterone-glucuronide (androsterone-G) and 3α-DIOL-glucuronide (3α-DIOL-G) are water-soluble and easily excreted [102, 105]. This glucuronidation system provides intracrine hemostasis of androgen, keeping DHT active in the prostate while releasing minimal DHT into circulation [106].

In humans, UGT enzymes are classified as UGT1, UGT2, UGT3 and UGT8, based on their homology and structure (see the UGTs homepage: http://som.flinders.edu.au/FUSA/ClinPharm/UGT/index.html, Figure 1.2). The UGT1A gene is located on chromosome 2q37, consisting of 13 individual exons 1 and five common exons. Alternative splicing generates 13 mRNA isoforms, 9 of which are translated into functional proteins (UGT1A1, 1A3-10). UGT1A1, 1A3, 1A4, 1A6 and 1A9 are expressed in the human liver, and UGT1A7, 1A8, 1A10 are expressed in the gastrointestinal tract [107].

The UGT2 locus is present on chromosome 4q13 and is subdivided into UGT2A and UGT2B. UGT2A isoforms (UGT2A1-3) are expressed in the nasal mucosa and the lungs [108, 109]. In contrast to UGT1A, seven UGT2B functional isoforms (UGT2B4, 2B7, 2B10, 2B11, 2B15, 2B17, 2B28) and five pseudogenes are encoded by multiple unique genes consisting of six exons [110]. Studies also uncovered several other members (e.g. UGT3A, UGT8) and their potential functions [111, 112]. The first exons of the UGTs most probably encode the substrate-binding domains, while the remaining exons encode the co-substrate UDPGA binding and transmembrane domains [113]. The molecular structure of UGTs define their ability to catalyze the transfer of the glucuronic-acid moiety at multiple functional sites (e.g. carbonyl, carboxyl, sulfuryl, hydroxyl and amine groups) and further determine their substrates (e.g. bilirubin, steroid hormones, bile acid or fatty acid derivatives) with variable activities [114, 115]. Although several UGT1A members could conjugate estradiol and estrone, they have no significant activity on androgens [116]. Of the functional human UGT2B enzymes isolated to date, only four UGT2Bs have favorable kinetics for androgen glucuronidation, namely UGT2B7, 2B15, 2B17, and 2B28 [117].

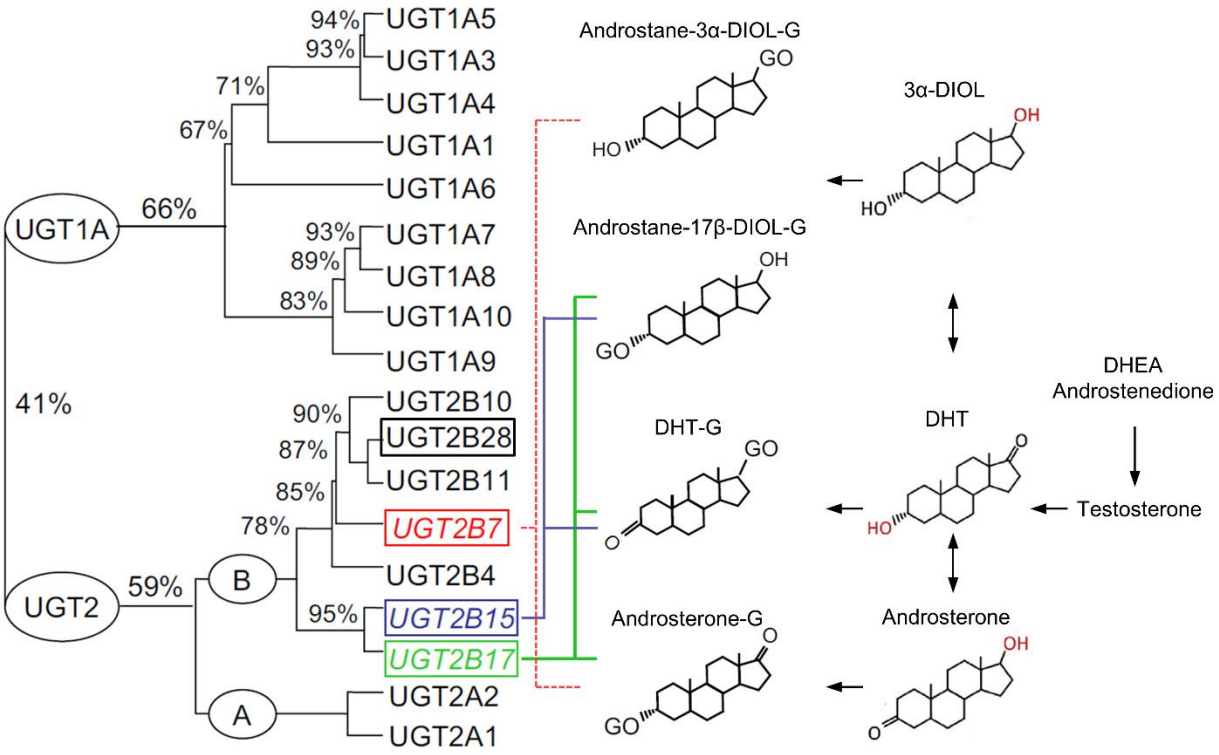


Figure 1.2 UGT superfamily and their androgen catabolization.

Modified from Barbier O, Bélanger A. Best Pract Res Clin Endocrinol Metab. 2008 Apr; 22 (2):259-70 with permission from Elsevier.

UGT2B7 conjugates the 3-hydroxyposition of 3α-DIOL and androsterone, but not the 17-hydroxyposition of DHT. UGT2B7 transcripts have been detected in several tissues but not in the prostate. UGT2B15 conjugates the 17-hydroxyposition of androgens and exerts moderate capacity for 3α-DIOL and DHT glucuronidation. Contrary to UGT2B7, UGT2B15 are abundant in the prostate gland, as well as the liver, kidney, skin, mammary gland and uterus [102]. The UGT2B15 and UGT2B17 amino acid sequences are remarkably high (96%), but their specificity toward androgens are different, since UGT2B17 glucuronidates both the 3- and 17-hydroxypositions of androgen molecules (Figure 1.2) [118]. The capacity of UGT2B17 to conjugate androsterone and DHT is highest among these three UGTs, and the efficiency of

UGT2B17 in conjugating 3α-DIOL is similar to UGT2B15 and 2B7 [104]. Since the capacity of UGT2B17 to conjugate androsterone is 4 times higher than that of UGT2B7, it is considered to be the major androsterone-conjugating enzyme [102]. UGT2B17 transcripts are detected in several androgen-sensitive tissues, such as the prostate gland, skin, brain, mammary gland, and uterus, in addition to the liver and kidney [102]. Recent studies reveal that a novel UGT2B member, UGT2B28, could conjugate testosterone with a modulated affinity [119]. UG2B28 is expressed in the liver and breast, as well as the prostate with relatively low levels [120]. Multiple studies identified the UGT2B15 and 2B17 isoforms as the major UGT enzymes for localized androgen glucuronidation in prostate cells [118, 121, 122].

Immunohistochemical analyses of UGT2B15 and UGT2B17 indicate that they are expressed in the normal human prostate [118]. On the cellular level, the glucuronidation reactions mediated by the UGTs may take place in the luminal aspect of endoplasmic reticulum (ER) [123]. These two UGTs' expression and function were confirmed in human prostate cancer cell line LNCaP [124]. Transient knockdown of UGT2B15/17 in LNCaP cells would increase the expression of androgen response genes and cell proliferation rates under the stimulation of DHT. These reports supported the idea that androgen sensitivity of PCa cells is influenced by UGT expression.

Polymorphisms in UGT2B15 and 2B17 were also investigated in multiple meta-analyses. The UGT2B15 polymorphisms (e.g. alias D85Y namely, rs1902023) were associated with an increased risk of PCa [125]. The UGT2B17 deletion genotype was linked with a decrease of circulating 3α-DIOL levels, yet not associated with Gleason score in Caucasians [119]. In addition to the genetic study, investigations into the alteration of UGT2B15 and UGT2B17

expression during PCa progression provide more constructive information. Firstly, researchers identified androgen-dependent AR signaling as a negative regulator of UGT2B15 and UGT2B17 genes expression [126, 127]. Using androgen antagonists flutamide and bicalutamide could reverse this suppression [128]. Therefore, the glucornodiation regulated by androgen is considered to affect PCa progression.

Further research evaluated UGT2B15 and UGT2B17 protein expression by immunohistochemistry (IHC) on tissue microarrays containing 179 tissue cores [129]. UGT2B15 expression levels were reduced in hormone naïve tumors and even lower in the CRPC patients' samples, when compared to benign prostatic hyperplasia (BPH), and eventually undetectable in lymph node metastases. By contrast, UGT2B17 was significantly more abundant in all Gleason-scored tumors compared to BPH. The protein levels of UGT2B17 were 5-fold more in metastases than in BPH. Enhanced UGT2B17 mRNA levels were found in PCa tumors expressing constitutively active AR splice variants, yet the potential regulating mechanism has not been elucidated [130]. The binding of forkhead box protein A1 (FOXA1) to the UGT2B17 promoter might contribute to its regulation [131]. In chronic lymphocytic leukemia, UGT2B17 has been identified as a disease accelerator [132], and knockdown of UGT2B17 in an endometrial carcinoma cell line downregulated the anti-apoptotic protein Mcl-1 [133]. A recent study reported that UGT2B28 expression was positively associated with high-grade PCa [134].

The functions of other androgen catabolizing enzymes in PCa were also studied. The expression of AKR1C2 involved in the bypass DHT catabolism (only in CRPC) was also increased in CRPC [135]. A member of the Aldo-keto reductase (AKR) family, AKR1C3 was recently

identified to possess novel functions as an AR coactivator [136] and an enhancer of AR function through the ubiquitin ligase Siah2 [137]. Together, these findings supported the idea that the catabolizing enzymes may have potential cancer-related functions.

1.2.3 AR structure and function

The AR is classified as NR3C4 (nuclear receptor subfamily 3, group C, gene 4), and belongs to the steroid hormone receptor superfamily [82]. The human AR gene is located on the X chromosome at Xq11-12 [138]. Eight exons of the AR encode a protein of 919 amino acids [139]. The N-terminal domain (NTD) is encoded by exon 1; the DNA-binding domain (DBD) is encoded by exons 2 and 3; a small hinge region and the ligand binding domain (LBD) are encoded by exons 4 to 8 (Figure 1.3).

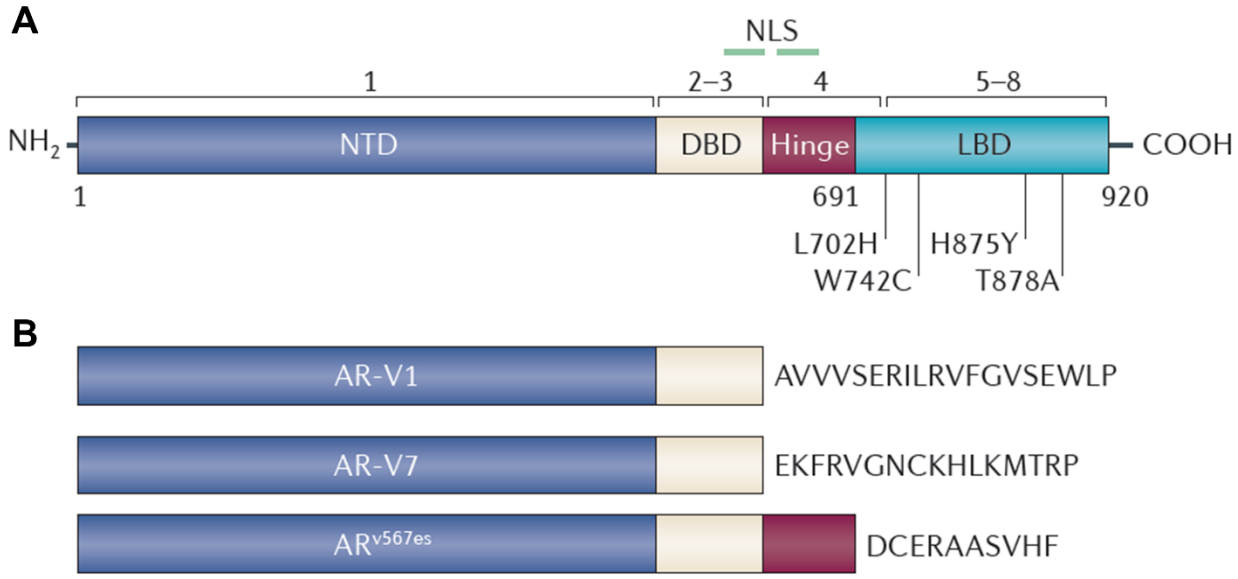


Figure 1.3 Domain structure of the AR, AR mutations and splice variants.

Adapted from Watson PA, Arora VK, Sawyers CL. Nat Rev Cancer. 2015 Dec; 15 (12):701-11 with permission from Nature Publishing Group.

The NTD of the AR accounts for over 60% (1 to 558 residues) of the AR protein [140]. The crystal structure of the NTD is not available due to the high flexibility of the NTD [141]. It contain two transcriptional activation units (TAU), termed TAU-1 (101-370 residues) and Tau-5 (360-485 residues) [140, 142]. There are two motifs, FxxLF (23-27 residues) and WxxLF (433-437 residues) for the interaction with the LBD [142]. This interaction, known as NH_2- and carboxyl-terminal (N/C) interaction, is critical for the androgens binding to the LBD [143]. The AR NTD mediates AR transcriptional activity through the recruitment of several transcription machinery components (e.g. transciption factor II) [144], coactivators (e.g. cAMP-response element-binding protein binding protein) [145] and co-repressors (e.g. nuclear receptor corepressor 2) [146]. The NTD contains poly-glutamine fragments, encoded by a polymorphic (CAG)nCAA repeat [147]. Studies have reported that any variation of the length of the poly-glutamine could result in x-linked spinal and bulbar muscular-atrophy (Kennedy disease) [148].

The DBD of the AR contains 66 amino acids with two zinc finger motifs [149]. The P box (GSCKV, 577-581 residues) interacts with the DNA, while the D box from the second zinc finger (ASRND, 596-600 residues) mediates AR dimerization [149]. The AR DBD recognizes classical androgen response elements (AREs), AGAACAnnnTGTTCT with two 5'-AGAACA-3' hexameric half-sites [150, 151]. A study found that the AR DBD is formed as two anti-parallel β-strands and one α-helix [152]. The structure makes the AR dimer bind to the DNA in a "head-to-head" way [152].

The hinge region (625-689 residues) contains a nuclear localization signal (617-RKCYEAGMTLGARKLKKL-634) [153]. This signal interacts with importin α/importin β complexes to facilitate the nuclear translocation of the AR [154]. The hinge region also contains multiple sites for posttranslational modifications including methylation and ubiquitination [155].

The most studied structure of the AR is the LBD, and is the target of current antiandrogens [156]. The AR LBD binds androgens and induces the conformation change allowing the formation of the AF-2 pocket [157]. AF-2 recruits a co-regulator (e.g. p160 steroid receptor co-activator SRC family) during transcriptional activation [158]. Compared to the co-activator LxxLL peptides, AF-2 shows a higher affinity with the NTD FxxLF peptide to form N/C interaction. These structures enhance the transcriptional activity [159].

The unbound protein of the AR in the cytoplasm forms complexes with chaperon proteins including heat shock protein (HSP) 90, HSP70, HSP40, FK506-binding proteins (FKBP51 and FKBP52), and other components to maintain its structural integrity [160]. Upon binding to DHT, the AR disassociates from chaperones, formats N/C terminal interaction, and interacts with co-factors including importin α, and transports into the nucleus. After dimerization, the AR binds to the ARE, and recruits co-activators, co-repressors and other transcriptional factors to lead the transcription of target genes [161]. The downstream genes involved in androgen-dependent AR transcription include gene groups involved in cell proliferation [39, 162, 163], cell cycling [164], cell apoptosis [165], and cell adhesion [166].

Aside from classical transcriptional activity, the AR also interacts with kinases to exert its non-genomic functions [167]. The AR has been found to interact with the Src homology 3 (SH3) domain of the tyrosine kinase c-Src [84]. This association of the AR with c-Src stimulates c-Src kinase activity in the LNCaP PCa cell line. A target of the c-Src is the SH3 domain containing transforming protein (SHC) [168]. The tyrosine phosphorylated SHC interacts with growth factor receptor-bound protein 2 (Grb2), which in turn activate Ras, leading a Ras/mitogen-activated protein kinase (MAPK) activation [169]. Thus, the AR and c-Src interaction further stimulates members of the MAPK signaling cascade [85]. Moreover, it is suggested that phosphorylation kinases including MAPK, protein kinase B (Akt) , cAMP-activated kinase A (PKA), protein kinase C (PKC), and c-Src could modulate the transcriptional activity of the AR by phosphorylating the serine or threonine residues of the AR or its co-activators (e.g. steroid hormone receptor co-activator SRC, transcriptional intermediary factor 2 TIF2) [170-173]. Among these kinases, c-Src was found to phosphorylate the AR at multiple sites, of which the AR Y534 is the major one [174]. It is also shown elevated levels of c-Src and AR Y534 activation in CRPC tumors and pAR Y534 positive tumors, were associated with high Gleason scores. These studies indicated that the non-genomic function of the AR is possibly related to its androgen-independent transcriptional activity.

1.2.3.1 AR inhibitor development

Based on the understanding of the AR structure and activation mechanism, various AR inhibitors have been developed to treat prostate cancer. Since the 1970s, several steroidal anti-androgens such as cyproterone acetate, oxendolone and spironolactone have been tested, yet showed clinical limitations, and are rarely used [175]. Non-steroidal anti-androgens were developed to

address these limitations [175]. The first generation of antiandrogens, including flutamide, nilutamide and bicalutamide (Bic) were used clinically. Among them, bicalutamide was widely used in PCa therapeutics due to its better pharmacokinetic profile [176].

The second generation anti-androgens include the recently FDA-approved enzalutamide. One study found an IC_{50} of 21 nM for enzalutamide and 160 nM for bicalutamide at the AR in the LNCaP cell line (7.6-fold difference) [177]. The ARN509 showing enhanced efficacy than enzalutamide, is now in phase III clinical trials [178]. These drugs demonstrate enhanced anti-AR efficiency, preventing the nuclear translocation of the AR, and impairing AR binding to the ARE. ODM-201 emerged as a novel anti-androgen, using a similarly competitive antagonist strategy, and is now in Phase III clinical trials [179].

Another major type of ARPI targets androgen steroidogenesis. Abriaterone, blocking the androgen synthesis by the adrenal glands, testes, and within PCa, is used in clinical PCa treatment [180]. Another CYP17A1 inhibitor, Galeterone (TOK-001) is currently being held before going to Phase III clinical trial due to its lack of superiority compared to enzalutamide [181].

Based on the understanding of the AR DBD structure, researchers identified small-molecule inhibitors that disrupt the DBD-DNA interaction of the AR [182]. These DBD inhibitors were proved to block the transcriptional activity of the full length AR and its constitutively active variants, and further block CRPC and ENZ-resistant tumor progression [183]. Further

optimization of these compounds, with greater potency and stability, would contribute to the novel DBD inhibitors.

A small-molecule inhibitor of the AR NTD, EPI-001 has been identified [184]. EPI-001 covalently binds to the AF-1 region of the NTD [185]. Studies showed that EPI-001 reduced both full-length and AR splicing variants, and suppressed the growth of CRPC xenografts that expressed AR splicing variants [186]. The general thiol-alkylating activity and modulation of the peroxisome proliferator activated receptor-gamma of EPI001 raised concerns [187]. A derivative of EPI-001, EPI506 has now entered a phase I/II trial. Inhibition of bromodomain-containing protein 4, which interacts with the AR NTD, could be another potential strategy [188].

Other AR targeting strategies include targeting chaperone protein HSP90 using Alvespimycin [189] or Onalespib [190], and blocking deubiquitinating enzymes using ASC-J9 [191]. Targeting AR co-activators such as the P160 steroid receptor co-activator family (SRC1-3) also showed promising results [192]. These inhibitors result in the degradation of the AR protein and possess a potential therapeutic effect in CRPC.

1.2.3.2 Resistance mechanisms

Mechanisms contributing to the failure of anti-androgen therapies for AR-driven tumors include: **1**) AR gene amplification/overexpression, which re-activates the AR signaling in the androgen depletion milieu; **2)** generation of AR splice variants (AR-Vs) that are constitutively transcriptionally active and can drive CRPC regardless the presence of ARPIs [193-200]; and **3**)

accumulation of PCa cells carrying AR mutations in the LBD, which recognize ARPIs as AR agonists [201, 202].

AR gene amplification/overexpression: Studies from clinical cohorts, PCa xenograft models, and cell lines indicated increased transcription levels of AR after ADT [203]. Higher mRNA and protein levels of the AR represent a mechanism of resistance to ADT [204]. The X chromosome rearrangement and gain of copy numbers drive AR overexpression in 60% of CRPC [205]. The consequences of AR overexpression confer the hypersensitivity of tumor tissue to androgens and the aberrancy of the AR. It also should be mentioned that the AR regulates a distinct transciptiome in CRPC under ENZ treatment, compared to primary PCa [206].

AR splice variants: The truncated AR variants lacking the LBD are constitutively active and not affected under the AR LBD targeted agents [207]. These AR variants are recognized as one of the mechanisms supporting androgen-independent expression of AR target genes and androgen-independent growth of PCa cells [197]. Alternative splicing of cryptic exons in the AR locus or exon skipping generates C-terminal truncated AR mRNAs [200]. Although studies show that AR variant mRNA (e.g. AR45) was synthesized in normal prostate tissues, consistent findings revealed that there was an increased level of AR alternative splicing variants in CRPC cells, when compared to hormone naïve PCa cells [193, 194, 196]. Splicing of the AR exons 1/2/3/CE3 (AR-V7) and splicing of the AR exons 1/2/3/4/8 (AR_{v567ed}) are the most commonly expressed variants in PCa [208, 209]. Their expression levels correlated with an increased risk of biochemical relapse and resistance to ENZ and abiraterone in clinical patient samples [208]. Constitutively active AR-V7 regulates the mitotic form of the AR transciptome over and above

differentiation [210, 211]. Our group recently identified that enhanced AR gene transcription by ADT increases, and splicing factors recruitment to the AR pre-mRNA contributes to, the increased mRNA levels of AR-V7 in PCa cells [212]. Furthermore, the homodimer of AR-Vs and heterodimer between ARVs and AR-FL were found, revealing comprehensive transcription regulation of AR-Vs in CPRC [213, 214].

AR mutations: Several mutated forms of AR confer the ligand promiscuity to impede the efficacy of current ARPIs especially the mutations in the LBD (e.g. T878A, L702H, W742C, H875Y and F877L) [215]. The *in vitro* characterizations of T878A and H875Y show they could be activated rather than inhibited by nilutamide and flutamide [216, 217]. Based on the crystal structure analysis, AR could accommodate larger molecules with the extra space offered by the T878A mutation [218, 219]. This causes aberrant AR activation in response to hydroxyflutamide, cyproterone acetate and alternative steroids. The W742C mutations identified in human PCa cell line LNCaP led to enhanced AR transcriptional activity driven by anti-androgen bicalutamide [220]. The mutation F877L was initially found in LNCaP cells under the selective pressure of ENZ [201]. The cells and tumors with the mutation F877L acquire resistance to ENZ and ARN509, turning these anti-androgens into AR agonist. The reposition of the docking helix 12 from the F877L could be responsible for this drug resistance [221]. The F877L was identified in circulating cell-free DNA from one CRPC patient cohorts treated with ENZ or ARN-509 [222]. Interestingly, the L702H, not having been linked to anti-androgen resistance, could be activated by glucocorticoids [223]. The L702H mutations as well as the T878A and H875Y mutataions conferring the property to be activated by adrenal androgens and progesterone have been found in the CRPC patients receiving abiraterone [222, 224, 225]. It has

been proposed that these mutations might be a consequence under the ARPI pressure with the compensation benefit toward other steroid ligands [226].

Alternative Pathways: In addition to above mechanisms, deregulation of the AR chaperones (e.g. HSP27) [227] and genomic modification of AR co-activators (e.g. SRC [228], FOXA1 [228]) could indirectly activate the AR. Activation of kinase-dependent signaling pathways (e.g. PI3K/AKT [50]), stress response pathways (e.g. clusterin [50]), and deregulation of DNA repair and cell cycle (e.g. BRCA2 [229]) contribute to CRPC progression as well. Drugs including OGX-027 (antisense targeting HSP27), KX2-391 (src kinase inhibitor), and Olaparib (PARP inhibitor) [230] are currently in clinical trial [8]. Tumors can also adapt to an AR-independent disease termed neuroendocrine prostate cancer (NEPC) with minimal response to conventional CRPC therapies [231, 232]. Evidence suggests that prostatic adenocarcinoma transdifferentiates to NEPC [233, 234]. Currently, platinum-based chemotherapy is used to treat NEPC, meanwhile MLN8237 (an aurora kinase A inhibitor) is currently in clinical trial [235].

1.2.4 AR-mediated transcription initiation

One of the major limitations of current clinical ARPIs is that none of them can thoroughly inhibit the transcriptional activity of ARs, including AR-Vs and mutant ARs. Reestablished AR-mediated transcription controlled the emergence of resistance [236]. Proteomic analysis approaches found that the AR recruits several factors, including Ku70, Ku80, poly(ADP-ribose) polymerase 1 (PARP1), DNA-dependent protein kinase (DNA-PK), and DNA topoisomerase II (Topo II) to bind to the ARE of downstream genes initiating the transcription [237, 238]. Transient knockdown of these proteins impaired induction of AR-mediated transcription [41,

239]. Among these factors, Topo II is known to cooperate with multiple nuclear hormone receptors and transcription factors that are bound to promoters and enhancers of newly transcribed genes [41, 239]. Topo II enzymes induce transient, but fixable DNA double strand breaks (DSBs) to relax the topology of the DNA and allow the AR to initiate gene transcription [41]. Topo II catalytic activity is required for efficient AR-mediated transcriptional initiation, and is involved in the chromosomal conformational changes during the activation of AR transcription.

Transient silencing of Topo II impairs AR transcriptional activity and AR-driven cell proliferation [41]. Overexpression of Topo II has been reported in PCa and its levels were associated with poor prognosis of PCa patients [240, 241]. Together, these findings suggest Topo II as potential therapeutic target in PCa.

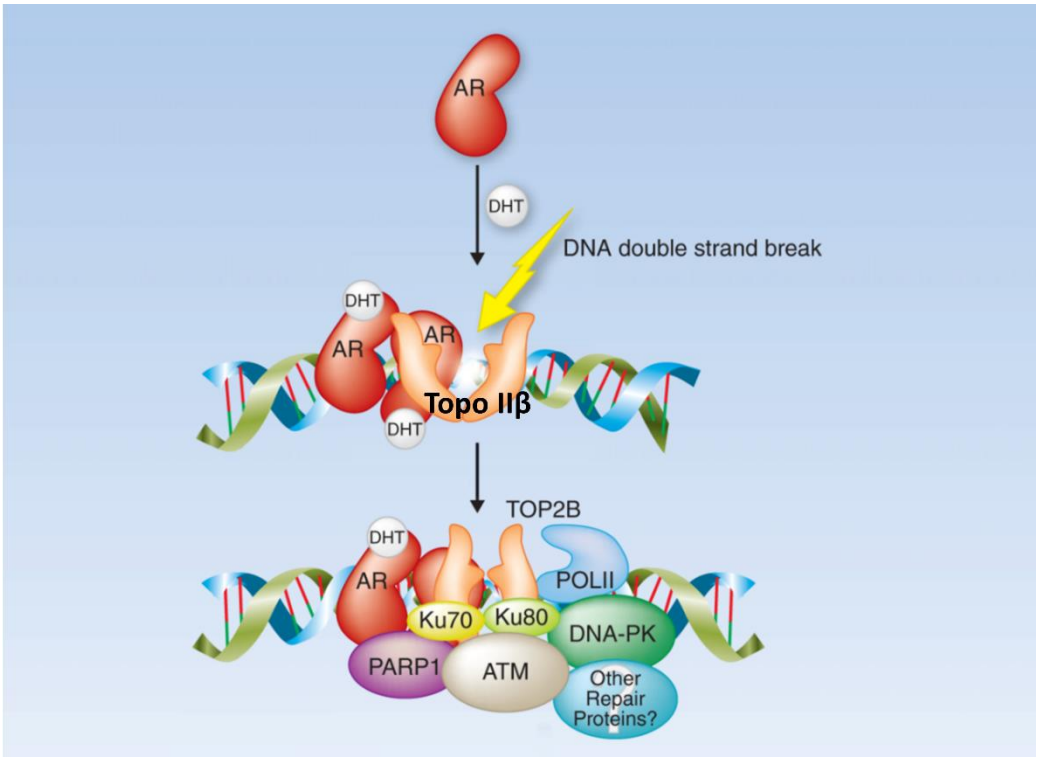


Figure 1.4 A hypothetical model of AR-mediated transcriptional programs.

Adapted from Haffner MC, De Marzo AM, Meeker AK et al. Clin Cancer Res. 2011 Jun 15; 17 (12):3858-64 with permission from AACR.

1.2.4.1 Topoisomerase II

The transcription process requires a separation of the two strands of the DNA double helix [242]. In order to solve these DNA topology problems, topoisomerases cleave DNA by nucleophilic attack on a phosphate of the phosphodiester DNA backbone, by using a catalytic tyrosine on the enzyme [243]. Topoisomerases are classified by the number of DNA strands they cleaved [244]. Type I topoisomerases cleave a single strand, while Type II topoisomerases cleave a double strand.

Human type II topoisomerases including Topo IIα and Topo IIβ, function as homodimers to relax the supercoils and to resolve catenanes and DNA knots [245]. Their catalytic cycles begin with binding to two DNA segments, cleaving one of the segments, creating a DSB, passing the intact DNA segment through the DSB, then relegating the DSB [244, 246, 247]. After closing the protein gate, the enzyme either dissociates from the DNA or goes through another round of catalysis. Both Mg^{2+} and ATP are required for this process [248]. In vitro studies have suggested that Topo II is required ahead of the recruitment of RNA polymerase II for promoters, thus playing an important role in transcription initiation [249]. Particularly, a recent study reported that Topo IIβ is recruited to the TMPRSS2 and ERG promoters, and is required for AR-dependent gene expression [41]. The recruitment of Topo II is thought to help mobilize other proteins to promoters [250]. Topo II activities are also required to de-catenate the intertwined chromosomes at the M phase of cell cycle during cell mitosis [251].

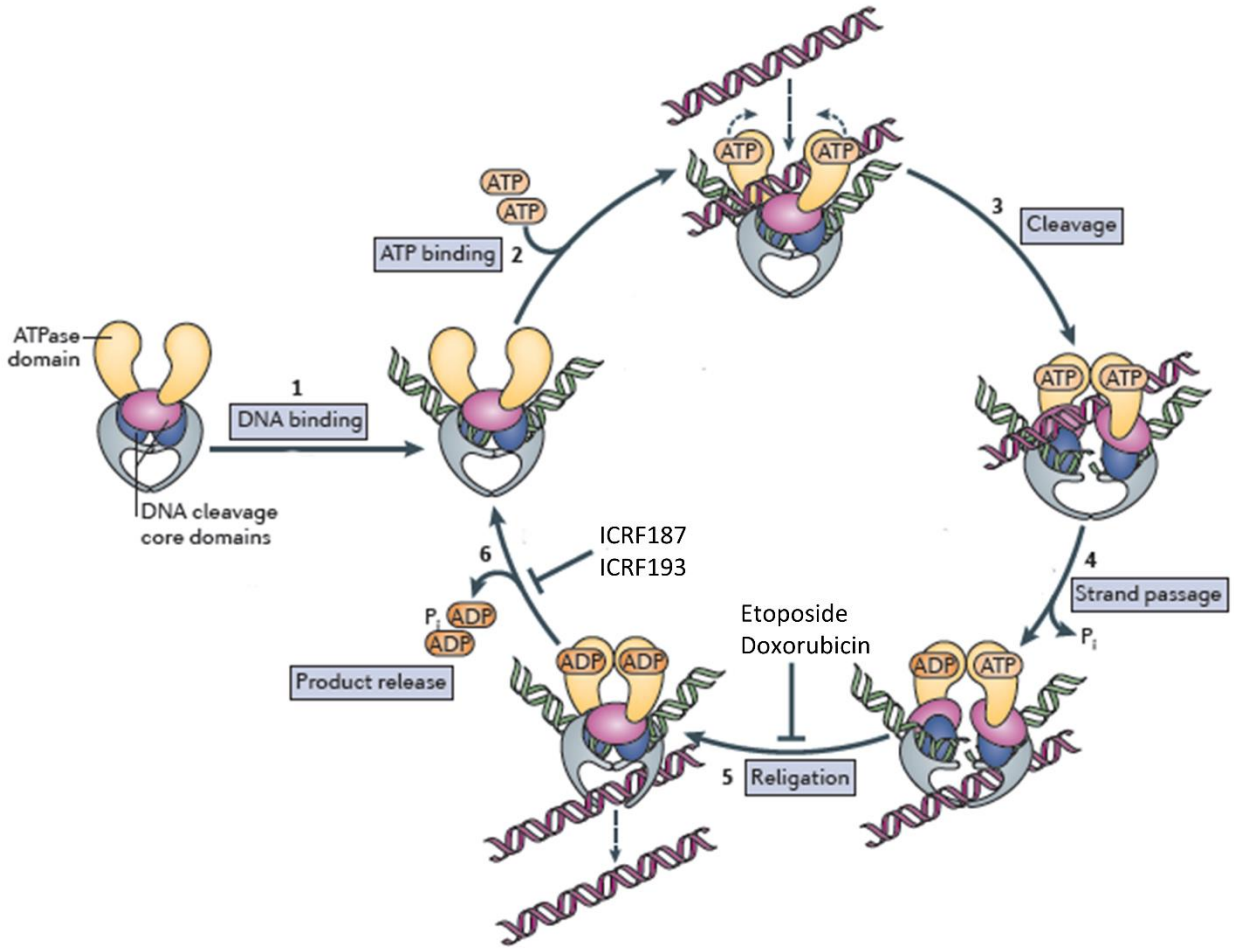


Figure 1.5 Topo II reaction cycle and the points at which agents can disrupt function.

Modified from Vos SM, Tretter EM, Schmidt BH et al. Nat Rev Mol Cell Biol. 2011 Nov 23; 12(12):827-41 with permission form Nature Publishing Group.

1.2.4.2 Topoisomerase II inhibitors

There are two categories of Topo II inhibitors, classified as poisons and catalytic Topo II inhibitors [252]. Though stabilizing the intermediate enzyme-DNA complex (or cleavage complex) [253], the poison Topo II inhibitors prevent the relegation or increase the cleavage reaction, resulting in an enhanced level of Topo-DNA covalent complexes and DNA DSBs [254]. The elevated DSBs further activate the DNA damage response, induce apoptosis and lead to cell cycle arrest [255]. Several poison Topo II inhibitors, i.e. daunorubicin, doxorubicin,

mitoxantrone, and etoposide are widely used as chemotherapeutic drugs clinically [256]. Etoposide, the major representative of the group, has been approved for the treatment of lung cancer, choriocarcinoma, ovarian and testicular cancers, lymphoma, and acute myeloid leukemia. The other member of this group, teniposide (VM-26), is approved for central nervous system tumors, malignant lymphoma, and bladder cancer [257]. Single-agent oral etoposide had minimal activity with 2 partial responses out of 22 patients in treating CRPC [258]. Mitoxantrone plus prednisone provided palliation for some symptomatic PCa patients with no difference in overall survival compared to prednisone alone [259]. Chemotherapy carboplatin plus etoposide induced partial responses in treating mCRPC, yet the benefit-risk ratio of this regimen seemed unfavorable due to high toxicity [260].

In contrast, the catalytic Topo II inhibitor regulates the activity of Topo II without interfering with the cleavage complex, thus causing fewer DSBs [261]. They either prevent the binding of Topo II to DNA (e.g. aclarubicin), block the ATP binding site (e.g. novobiocin) or inhibit the cleavage reaction (e.g. bisdioxopiperazines ICRF187, ICRF193) [262]. Some studies suggested that ICRF187 could suppress the proliferation and induce G_2/M arrest in small cell lung cancer and leukemia cell lines [263, 264]. Very few of the catalytic inhibitors has been approved as anticancer drugs, one exception is ICRF-187 (Dexrazoxane) [265]. Since ICRF187 could directly trap Topo II [266], it is used as a cardioprotective agent to antagonize the poison effect of doxorubicin [267].

1.3 Thesis theme and rationale

The lack of therapy for castration-resistant prostate cancer remains a major unmet clinical needs. The recent approvals of more potent ARPIs have shown encouraging results in delaying disease progression in CRPC patients. Unfortunately, resistance towards these ARPIs eventually occurs. Emerging evidences suggested that cancer cells escape ARPI through expressing constitutively active AR splice variants, or AR mutants that can recognize ARPIs as agonists. With these substitute routes, the recurrent cancer cells are able to reestablish AR activities even under maximum androgen blockade conditions. The mechanisms driving this transition from androgen-dependent to androgen-independent AR-mediated transcription is unknown.

Preliminary work from our collaborators, the Barbier group, has revealed an association between UGT2B17 and advanced PCa samples. Enhanced UGT2B17 expression by antiandrogens was reported in prostate cancers. Moreover, accumulating evidence supports the roles of UGT2B17 in disease progression in other cancers. Collectively, these findings suggest that UGT2B17 may play functional roles in the regulation of AR signaling transformation and CRPC progression. Therefore, the current study first sought to further validate the clinical association of UGT2B17 with PCa using clinical samples and to investigate the functional roles of UGT2B17 in regulating AR-mediated transcription in PCa cell lines and xenograft models. Next, based on previous knowledge of the AR-mediated transcription machinery, we further utilized catalytic Topo II inhibitors to block the AR-mediated transcription initiation. The effects of catalytic Topo II inhibitors on AR-FL, AR mutants, and AR splicing variants were tested in PCa cell line models. Finally, based on the *in vitro* results, we explored the therapeutic potential of the catalytic Topo II inhibitor in CRPC and Enz-resistant tumor xenograft models.

The overall goal of this study is to investigate the transformation of AR-mediated transcription, and to evaluate the potential therapeutic efficacy of targeting Topo II in CRPC and Enz-resistant tumor models.

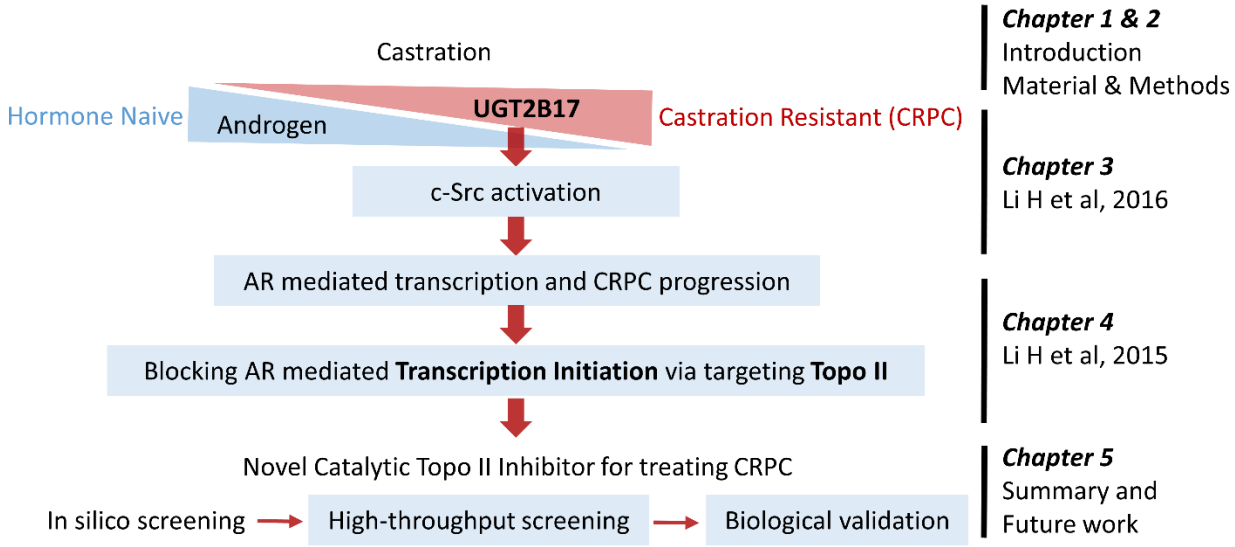


Figure 1.6 Diagram illustrating the outline of the thesis.

1.4 Hypotheses and specific aims

The main hypotheses of the study are as follows: (1) the reestablishment of AR signaling to the androgen-independent state is promoted by UGT2B17, and (2) blocking all forms of reestablished AR-mediated transcription initiation through the catalytic Topo II inhibitor will suppress CRPC tumor progression.

Main objective: To study the roles of UGT2B17 in promoting AR signaling in CRPC, and to assess the suppression effect of catalytic Topo II inhibitors on AR-mediated transcription initiation for treating the CRPC.

Specific Aims:

Specific aim 1. To investigate the AR signaling transformation by UGT2B17 in CRPC

Specific aim 2. To utilize the Topo II inhibitors to target AR signaling in PCa cell line models

Specific aim 3. To evaluate the inhibition efficacy of Topo II inhibitor using PCa xenograft models

Chapter 2: Materials and Methods

2.1 Materials

R1881, DHT, PP2, LY294002, Stattic, etoposide, ICRF187, ICRF193, merbarone, aclacinomycin A and genistein were purchased from Cedarlane (Burlington, Canada). Enzalutamide (ENZ) was obtained from Haoyuan Chemexpress (Shanghai, China). Chemicals, solvents and solutions were obtained from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise indicated.

2.2 Tissue microarray

This study was done on a total of 604 prostate cancer specimen cores from the Vancouver Prostate Centre Tissue Bank. All patients have signed an informed consent form, which adheres to a protocol that was reviewed and approved by the UBC Clinical Research Ethics Board (Certificate #: H09-01628). Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with haematoxylin and eosin (H&E). Pathologist Dr. Ladan Fazli reviewed the slides and marked desired areas on them and their correspondent blocks. Tissue microarrays (TMAs) were constructed using a manual tissue microarrayer (Beecher Instruments, Westminster, USA), by punching duplicate or triplicate cores of 1 mm diameter from each marked block. Information on PCa tissue microarrays (TMAs) including Gleason score, duration of neoadjuvant hormone therapy (NHT), lymph node and distal organ metastasis was published previously [268] and is also presented in Table 2.1.

Groups	# of patients	# of cores
Benign	27	90
Hormone Naïve	166	316
NHT treated	57	126
CRPC	37	72
Total	287	604

Table 2.1 Vancouver Prostate Centre tissue microarray information.

2.3 Immunohistochemistry

Immunohistochemistry (IHC) staining was conducted with the Ventana Discovery XT autostainer (Ventana, Tuscan, USA) and examined with an UltraMap DAB kit (Ventana). Anti-UGT2B17 (EL-95, provided by Dr. Barbier; 1:150 dilution), Anti-Src (Cell signaling #2109; 1:1500 dilution), Anti-pSrc Y416 (Cell signaling #2101; 1:25 dilution), and Anti-Ki67 (Thermo Fisher RM9106; 1:500 dilution) antibodies were used. Stained slides were scanned by a Leica SCN400 scanner and viewed with a DIH Slide Path imaging system (Leica Microsystem, Toronto, Canada). Values on a four-point scale were assigned to any tumor cells. Pathological analysis was carried out by Dr. Ladan Fazli. Descriptively, 0 represents no staining of any tumor cells, 1 represents faint or focal staining, 2 represents a strong signal in a minority of cells, and 3 represents a strong signal in the majority of cells. The scoring method is also described in other studies [129, 269].

2.4 Cell lines

Human PCa cell lines LNCaP, VCaP, PC3, DU145, 22Rv1, and embryonic kidney cell line 293T were purchased from ATCC (Manassas, USA). LNCaP95 is an androgen-independent cell line derived from a long-term continuous culture of LNCaP cells in androgen-depleted conditions since 1995. These were provided by Dr. Alan K. Meeker (Johns Hopkins University, Baltimore, USA). LNCaP(AI) was derived from parental LNCaP cells after more than one year's growth in androgen-depleted medium, and were provided by Dr. Ralph Buttyan (Vancouver Prostate Centre, Canada). The MR49F cell line is an ENZ-resistant PCa cell line generated from LNCaP CRPC xenografts treated with ENZ as described in other studies [270], provided by Dr. Martin Gleave (Vancouver Prostate Centre, Canada). PC3, DU145 and 293T cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose/L-glutamine (Hyclone, Mississauga, Canada) with 10% Fetal Bovine Serum (FBS) (Gibico, Mississauga, Canada). LNCaP and 22RV1 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI1640) medium with 10% FBS. LNCaP95 and LNCaP(AI) cells were maintained in RPMI1640 medium with 5% charcoal stripped serum (CSS) (Hyclone). MR49F cells were derived from LNCaP cells and cultured in medium containing 10 μM of ENZ. PC3(AR-V7) cells are PC3 cells induced with exogenous AR-V7 protein by lentiviral infection and selected by Blasticidin, provided by Dr. Liangliang Liu from our lab. The results were published in a previous report [271]. The LNCaP cell line that was stably expressing EGFP-AR was provided by Dr. Paul Rennie (Vancouver Prostate Centre). The AR status of the human PCa cell lines used in this study are listed in Table 2.2. The AR and AR-V7 protein levels in LNCaP, LNCaP(AI), LNCaP95, MR49F, PC3(mock), and PC3(AR-V7) cell lines are also shown in Figure 2.1. All cell lines were authenticated by short tandem repeat assays or RNA sequencing assays.

Name	AR gene amplification	AR	AR-V7	AR mutants
LNCaP	-	+	+/-	+
LNCaP(AI)	-	+	+	+
LNCaP95	-	+	+	+
22RV1	-	+	+	+
VCaP	+	+	+	-
MR49F	-	+	+	+
PC3	NA	-	-	-
DU145	NA	-	-	-

Table 2.2 The AR status of the human PCa cell lines used in this study.

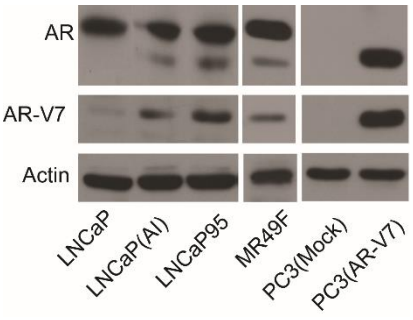


Figure 2.1 AR and AR-V7 protein levels.

AR and AR-V7 protein levels in LNCaP, LNCaP(AI), LNCaP95, MR49F, PC3(mock) and PC3(AR-V7) cell lines were detected by Western blotting with AR(N-20) and AR-V7 (Precision Antibody AG10008) antibodies. Beta actin was used as loading control.

2.5 Reverse-transcriptase PCR and real-time PCR

Total RNA was extracted using a Purelink RNA mini kit (Invitrogen, Burlington, Canada) and treated with DNase I (Qiagen, Mississauga, Canada) at room temperature for 15 min to eliminate any DNA contamination. The reverse transcription reaction was performed using random hexamers and SuperScript II (Invitrogen), after which the product was used as a template for

PCR. Real-time PCR was performed on the ABI PRISM 7900 HT system (Applied Biosystems, Burlington, Canada) using the FastStart Universal SYBR Green Master mix (Roche, Mississauga, Canada). Cycling was performed using default conditions of the 7900HT Software (Applied Biosystems): 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Relative quantification of gene transcription was calculated, GAPDH, β-actin or RPL13A were used as the internal control genes. The Ct values of these three housekeeping genes, under the different treatments, were also tested (Figure 2.2). All real-time PCR assays were carried out using three technical replicates and three independent cDNA syntheses. Primer information is listed in Table 2.3.

Name	**Primer sequences**
UGT2B17 F	5'-ACCAGCCAAACCCTTGCCTAA G -3'
UGT2B17 R	5'-GGCTGATGCAATCATGTTGGCAC -3'
AR F	5'-CCAGGGACCATGTTTTGCC -3'
AR R	5'-CGAAGACGACAAGATGGACAA -3'
AR-V7 F	5'- CAGGGATGACTCTGGGAGAA-3'
AR-V7 R	5'- GCCCTCTAGAGCCCTCATTT-3'
Topo IIβ F	5'-AGC CAT TGA CGC AGT TCA TGT-3'
Topo IIβ R	5'-CCT GGC ACA AAG GTA ACC TCC-3'
PSA F	5'- AGTGCGAGAAGCATTCCCAAC-3'
PSA R	5'- CCAGCAAGATCACGCTTTTGTT-3'
TMPRSS2 F	5'-GGACCTGACCTGCCGTCTAGAA-3'
TMPRSS2 R	5'-GGTGTCGCTGTTGAAGTCAGAG-3'
UBE2C F	5'-TGGTCTGCCCTGTATGATGT-3'
UBE2C R	5'-AAAAGCTGTGGGGTTTTTCC-3'
GAPDH F	5'-ATATGTTCTCCTGCCTACTGGAA-3'
GAPDH R	5'-GGTGTCGCTGTTGAAGTCAGAG-3'
Beta ACTIN F	5'-GGA CTT CGA GCA AGA GAT GG -3'

Name	Primer sequences
Beta ACTIN R	5'-AGC ACT GTG TTG GCG TAC AG -3'
RPL13A F	5'-GCCATCGTGGCTAAACAGGTA -3'
RPL13A R	5'-GTTGGTGTTCATCCGCTTGC -3'
ANAPC F	5'-CTGATGAAAGCTATACTCCAAGCA-3'
ANAPC R	5'-GGAACATGAATCCAGCCACT-3'
CDC20 F	5'-CCTCTGGTCTCCCCATTAC-3'
CDC20 R	5'-ATGTGTGACCTTTGAGTTCAG-3'
ID1 F	5'-CTACGACATGAACGGCTGTTACTC-3'
ID1 R	5'-CTTGCTCACCTTGCGGTTCT-3'
NDRG1 F	5'-GTGGAGAAAGGGGAGACCAT-3'
NDRG1 R	5'-ACAGCGTGACGTGAACAGAG-3'
CDK1 F	5'-CCTAGTACTGCAATTCGGGAAATT-3'
CDK1 R	5'-CCTGGAATCCTGCATAAGCAC-3'
PRDM4 F	5'-CACCTTCACTGCAAATGGAA-3'
PRDM4 R	5'-AAGTCACTGGTCCATGTTCG-3'
FKBP5 F	5'-GCGGAGAGTGACGGAGTC-3'
FKBP5 R	5'-TGGGGCTTTCTTCATTGTTC-3'
PDE9A F	5'-GATCCCAATGTTTGAAACAGTGAC-3'
PDE9A R	5'-TCCCAAAGTGGCTGCAGC-3'
NKX3.1 F	5'-CCCACACTCAGGTGATCGAG-3'
NKX3.1 R	5'-GAGCTGCTTTCGCTTAGTCTT-3'
DCC F	5'-GCCCCTACTTCTTCGCCTAC-3'
DCC R	5'-CACAGTCTCCAGCTCTGTGC-3'
OPRK1 F	5'-AACTCGCTGGTCATGTTCGT-3'
OPRK1 R	5'-CTCTGAAAGGGCATGGTTGT-3'

Table 2.3 Real-time PCR primer information.

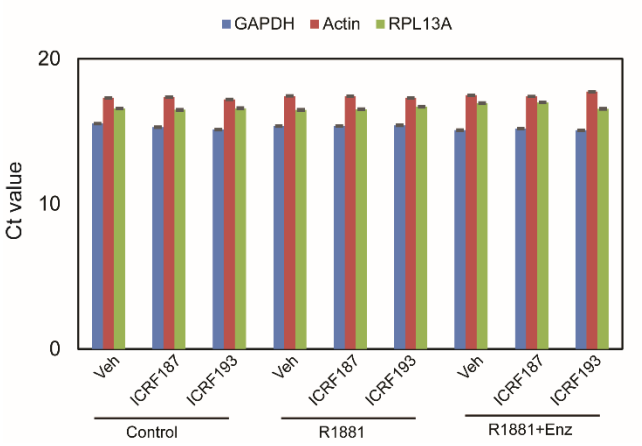


Figure 2.2 Ct values of housekeeping genes under treatment.

LNCaP cells were cultured in medium containing 5% CSS. Cells were treated with vehicle, 1 nM of R1881 or 1 nM of R1881 plus 5 μM of ENZ for 24 hours. Cells were also co-treated with vehicle, 1 μM of ICRF187 or 1 μM of ICRF193 as indicated. Ct values of GAPDH, beta actin and RPL13A were measured by real-time PCR.

2.6 Immunoblotting

Cells were collected and incubated with lysis buffer (50 mM Tris pH8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS) plus proteinase inhibitors (Roche) and PhosSTOP (Roche) followed by a brief sonication to extract protein lysate. The concentrations of the protein samples were measured using a Pierce BCA Protein Assay Kit (Thermo Fisher, Mississauga, Canada) according to the manufacturer's protocol. Protein samples were separated by electrophoresis on an 8%-15% SDS polyacrylamide gel, then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, USA) in transfer buffer (25 mM Tris-HCl, 250 mM glycine, 0.1% SDS, pH 8.3) for 40-60 min at 25 V in room temperature, as we reported previously [272]. Membranes were blocked with blocking buffer (TBST with 5% BSA), then immunoblotted with specific antibodies (1:500 or 1:1000 dilution) listed in Table 2.4. After being immunoblotted with anti-rabbit/mouse IgG-HRP secondary antibodies (Santa Cruz, Dallas, USA) (dilution factor 1:10,000), membranes were probed by Pierce ECL Western Blotting

Substrate (Thermo Fisher), and exposed to autoradiography film (Genesee Scientific, San Diego, US), followed by a film processor (EL-RAD, Vancouver, Canada). Experiments were repeated three independent times, and one representative blot was shown. Densitometry analyses of protein bands from three repeated assays were performed by the Image J software (NIH).

Name	Clone	Company(CAT#)
β-actin	Ac-15	Sigma Aldrich (A5441)
pSrc Y416		Cell signaling (#6943)
c-Src		Cell signaling (#2110)
pAKT		Cell signaling (#9271)
tAKT		Cell signaling (#9272)
pSTAT3		Cell signaling (#9138)
tSTAT3		Cell signaling (#9139)
Flag	M5	Sigma Aldrich (F4042)
AR (N-20)		Santa Cruz (sc-816)
pAR Y534		Established in Dr. Qiu lab
UGT2B17	EL95	Established in Dr. Barbier lab
AR-V7		Precision Antibody (AG10008)
Tubulin	11H10	Cell Signaling (#2125)
Histone H3		Abcam (Ab1791)
Topo IIβ	H-286	Santa Cruz (sc-13059)
pSTAT5		Cell signaling (#4322)
tSTAT5		Cell signaling (#9363)

Table 2.4 Antibodies information.

2.7 Transfection and RNA silencing

Plasmid DNA were transfected into cells using Lipofectmine 2000 (Invitrogen), while small interfering RNA (siRNA) was transfected by siLentFect reagent (Bio-Rad, Mississauga, Canada)

following the manufacturer's instructions. Transient transfection vectors pcDNA3.1 AR, pcDNA3.1 Flag-AR-V7, PSA-luciferase reporter, and renilla luciferase were provided by Dr. Xuesen Dong. Vector pcDNA3.1 UGT2B17 was provided by Dr. Olivier Barbier. Point mutations of the AR (F877L, F877L/T878A and W742C) were generated with a Q5 site-directed mutagenesis kit (NEB, Toronto, Canada) using pcDNA3.1 AR as a template, by research assistant Ms. Ning Xie from Dr. Dong's group. The information of the site-directed mutagenesis primers is listed in Table 2.5, and the siRNA are listed in Table 2.6.

Name	**Primer sequences**
AR F877L F	5'-CGAGAGAGCTGCATCAGTTAACTTTTG-3'
AR F877L R	5'-AGCAGGTCAAAAGTTAACTGATGCAG-3'
AR F877L/T878A F	5'-CAGCTCGCTTTTGACCTGCTAATCAAGTCACACATG-3'
AR F877L/T878A R	5'-ATGCAGCTCTCTCGCAATAGGCTGCACGGAG-3'
AR W742C F	5'-TCCTGCATGGGGCTCATGGTGTTTGCCATGGGCTGG-3'
AR W742C R	5'-GTACTGAATGACAGCCATCTGGTCGTCCAC-3'

Table 2.5 Site-directed mutagenesis primers for the AR.

Name	**Company(CAT#)**
Control siRNA (Dharmacon)	Dharmacon (D-001210-01-20)
siAR	Dharmacon (CAAGGGAGGUUACACCAAAUU)
Control siRNA (Santa Cruz)	Santa Cruz sc-37007
Topo IIβ	Santa Cruz sc-36697

Table 2.6 siRNA information.

2.8 Construction of cell lines with gain- and loss-of-function of UGT2B17

Lentiviral vectors encoding control, UGT2B17 (CAT#LV352009) and shRNA (CAT# iV026677) against UGT2B17 were purchased from Applied Biological Materials (Vancouver, Canada). LNCaP cells overexpressing UGT2B17 and LNCaP95 cells with UGT2B17 knockdown were achieved using lentiviral approaches, combined with puromycin selection, as we reported previously [273]. Briefly, lentiviral vector encoding either control, UGT2B17 or shRNA against the UGT2B17, together with 2nd Generation Packaging Mix (ABM) were transfected into 293T cells by Lipofectamine 2000 for 24 hours. Lentiviral particles were harvested by removing medium 48 hours after transfection, and were used to infect LNCaP or LNCaP95 cells. After 5 μg/ml puromycin selection for 3 weeks, polyclonal and antibiotic-resistant cells were pooled and labeled as LNCaP(mock), LNCaP(UGT2B17), VCaP(mock), VCaP(UGT2B17), LNCaP95(shCTRL) and LNCaP95(shUGT2B17) cell lines.

2.9 *In vitro* glucuronidation assays

In vitro glucuronidation assays were performed by the research assistant Ms. Mélanie Verreault from the Dr. Barbier group. The formation of glucuronide derivatives was measured using cell lysates (27.5 μg) or xenograft homogenates (75 μg) in 50 mM Tris-buffered saline (pH 7.4) with dithiothreitol (0.5 mM) in the presence of 1 mM UDPGA, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 100 μg/ml phosphatidylcholine, 8.5 mM saccharolactone (for negative controls), and 100 μM testosterone, DHT, 3α-DIOL or vorinostat (postivie control) substrates. Assays were incubated at 37 °C for 2 hours and terminated by adding 100 μl of methanol followed by centrifugation at 14,000g for 10min. The formation of testosterone-G, DHT-G, 3α-DIOL-G and vorinostat-G was measured by using liquid chromatography coupled with mass spectrometry

(LC-MS/MS) as previously described [124]. All experiments were repeated in triplicate for three independent times.

2.10 Luciferase reporter assay

Cells were transiently transfected with PSA-luciferase reporter and renilla luciferase vectors, treated with indicated treatments, and collected. Luciferase activities of the cell lysates were determined using the Dual-luciferase reporter assay system (Promega, Madison, USA) according to the manufacturer's protocol. Luminescent signals were measured by Infinite M200Pro (Tecan, San Jose, USA), transfection efficiency was normalized to renilla luciferase activities. Experiments were repeated in triplicate for three independent times.

2.11 Immunoprecipitation

Immunoprecipitation experiments were performed as previous described [274]. Briefly, cell lysates were extracted by the NETN buffer containing 0.5% NP40, 1 mM EDTA, 50 mM Tris, and 150 mM NaCl plus proteinase and phosphatase inhibitor (Roche). Pre-cleared lysates were incubated with anti-Flag antibody and the associated proteins were immunoblotted by indicated antibodies. Experiments were repeated at least three times, and one set of the representative blots is shown.

2.12 Chromatin immunoprecipitation (ChIP)

ChIP assays were performed by research assistant Ms. Ning Xie as previously described [275]. In brief, LNCaP cells were grown in phenol red-free RPMI 1640 medium with 10% CSS for 2 days before exposure to treatments. Chromatin was cross-linked with 1% formaldehyde for 10

min at 37°C and sonicated in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.0) plus protease inhibitor. After centrifugation, 10 µl of the supernatants was used as input, and the remaining lysate was subjected to a ChIP assay with anti-AR antibody. DNA templates retrieved from ChIP were analyzed by real-time PCR using the primers listed in Table 2.7. Enrichments of immunoprecipitated DNA fragments were determined by the threshold cycle (Ct) value. Data were calculated as a percentage of input and plotted as fold changes over control IgG. ChIP data were derived from five independent experiments with samples in triplicate. Data are presented as mean ± SEM.

Name	Primer sequences
PSA enhancer F	5'-TGGGACAACTTGCAAACCTG-3'
PSA enhancer R	5'-CCAGAGTAGGTCTGTTTTCAATCCA-3'
TMPRSS2 F	5'-TGGTCCTGGATGATAAAAAAAGTTT-3'
TMPRSS2 R	5'-GACATACGCCCCACAACAGA-3'

Table 2.7 ChIP primers.

2.13 MTS and BrdU incorporation assays

Cell proliferation rates were measured by using CellTitre 96 AqueousOne kit (Promega) and Bromodeoxyuridine (BrdU) assay kit (Millipore, Toronto, Canada) according to the manufacture's protocol. Each experiment contained six replicate wells and three independent experiments were performed.

2.14 Invasion and migration assays

Cell migration and invasion assays were performed using 6-well BD control/matrigel chambers (BD Biosciences, Mississauga, Canada) as we reported previously [276]. After treatment or

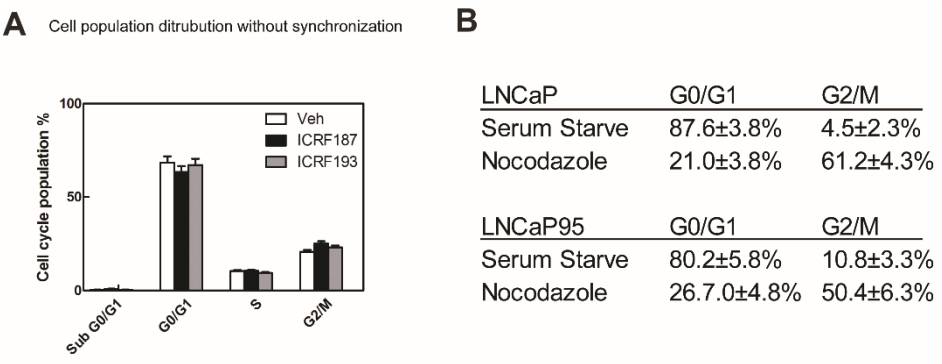
transfection, LNCaP or VCaP cells (10^5/well) were suspended in phenol red-free RPMI 1640 medium and seeded in BD Matrigel invasion chamber or control chamber without Matrigel. After incubation at 37°C with 5% CO_2 for 18 hours, non-invading or non-migratory cells in the upper chamber were gently removed by cotton swabs. Cells that reached to the lower chamber were fixed, stained with mounting medium containing DAPI (Vector Laboratories, Burlingame, USA), and photographed by an Axiovert 200 microscope (Carl Zeiss, Germany). Invaded or migrated cell numbers were counted by the Image J software. Cell invasion and migration rates were calibrated relative to the control group. Experiments were performed in triplicate and repeated three times.

2.15 Fluorescence-activated cell sorting (FACS)

Cell cycling was analyzed by FACS with 40 ug/ml propidium iodide staining following the protocol (http://www.meduniwien.ac.at/user/johannes.schmid/PIstaining3.htm). Relative DNA contents from 10,000 cells were analyzed by FACSCanto II flow cytometer and BD FACSDiva software v5.0.3 (BD) as we reported [277]. All experiments were carried out in triplicate.

For a pilot experiment, non-synchronized LNCaP and LNCaP95 cells were treated with vehicle, 10 μM of ICRF187 or 2 μM of ICRF193 for 12 hours, then replenished with culture medium containing serum. After 1.5 hours for LNCaP or 2 hours for LNCaP95, cells were collected and used for FACS assays to determine cell populations at G_0/G_1, S and G_2/M phases. The chosen time points were determined by the optimization protocols shown in our previous publications [276, 278], and also consistent with the publication from another group [279]. Topo II inhibition showed little impact on asynchronous LNCaP and LNCaP95 cells (Figure 2.3A). To synchronize

LNCaP and LNCaP95 cells, they were either serum starved, or treated with 100 ng/ml nocodazole for 12 hours, then replenished with culture medium containing serum. Percent of cells synchronized in G_0/G_1 phase after 12-hour serum starvation or G_2/M phase after nocodazole treatment are shown in Figure 2.3B.



LNCaP	G0/G1	G2/M
Serum Starve	87.6±3.8%	4.5±2.3%
Nocodazole	21.0±3.8%	61.2±4.3%

LNCaP95	G0/G1	G2/M
Serum Starve	80.2±5.8%	10.8±3.3%
Nocodazole	26.7.0±4.8%	50.4±6.3%

Figure 2.3 Non-synchronized cell population distribution of LNCaP and LNCaP95 treated with ICRF187 and ICRF193.

(**A**) LNCaP cells culturing in RMPI containing 5% CSS medium treated with vehicle, 10 μM of ICRF187 or 2 μM of ICRF193 were collected and used for FACS assays to determine cell populations at G_0/G_1, S and G_2/M phases. (**B**) Synchronized cell population over total cells percentage of LNCaP and LNCaP95 in Figure 4B were showed in the table.

2.16 Gene microarray studies

Gene microarray analyses were performed in Laboratory for Advanced Genome Analysis (Vancouver Prostate Centre) as described before [276]. Research assistant Ms. Anne Haegert performed the experiments, bioinformatics coordinator Mr. Robert Bell did the statistical analysis, and bioinformatics scientist Mr. Shawn Anderson helped with the GEO data deposit. Total RNA was extracted by using the mirVana RNA Isolation Kit (Ambion, Burlington, Canada) from three independently repeated experiments. The quality and quantity of RNA were

assessed with an Agilent 2100 Bioanalyzer (Caliper Technologies Corp., Canada). Amplified and Alexa Fluor 3 labeled RNA samples were hybridized onto the Human Agilent 8x60k (Agilent Technologies, Santa Clara, USA), along with Alexa Fluor 5 labeled human reference RNA. The Statistical Analysis of Microarray (SAM) program (http://www-stat.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 false discovery rate (FDR) multi-test 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.

2.17 Fluorescence microscopy immunofluorescence

Cells were counterstained by DAPI and images were captured by using a Zeiss LSM780 confocal microscope (Carl Zeiss Instruments) as we reported previously [272]. To determine AR subcellular localization under treatment, we first used AR (N-20) antibody to detect endogenous AR. Immunofluorescence signal diminished very rapidly under ICRF and ENZ treatment, possibly attributed to the degradation of endogenous AR under such conditions. In order to detect AR cytosol localization, we transiently transfected GFP-AR to boost AR expression, as described in other studies characterizing ENZ on AR localization [177, 178].

AR subcellular localization was also confirmed by Western blotting nuclear and cytoplasmic extracts from cells transfected with Flag-tagged AR, AR mutants and the AR-V7 splice variant, using a nuclear protein extraction kit (Sigma) according to the manufacturer's instructions.

2.18 Proximity ligation assay (PLA)

Protein-protein interactions were detected by Duolink in situ PLA red starter (Sigma, Oakville, Canada) as we reported [280]. Briefly, 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^{o}C. Secondary probe, ligation, and amplification reactions were performed following the manufacturer's instructions. Slides were mounted with DAPI, and fluorescence images were captured by Zeiss fluorescent microscope (Carl Zeiss, Toronto, Canada).

2.19 Human prostate cancer xenografts

Animal care and experimental procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) under the approval of the Animal Care Committee of the University of British Columbia (protocol #: A14-0088).

A total of 1×10^6 control or LNCaP(UGT2B17) cells in 0.1 ml Matrigel (BD Labware) were inoculated subcutaneously in the bilateral flanks of 6-8 week old male athymic nude mice (n=7/group) (Harlan Sprague Dawley Inc., Mississauga, Canada). Tumor volume (V= L*W*D*0.5236) and body weight were measured weekly. Serum PSA levels were determined by ELISA (ClinPro International, Union City, USA). 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.

Weight loss curves were estimated to demonstrate the tolerability of ICRF187. Maximum dosage was determined from previous studies [281, 282]. Increasing doses of ICRF187 were exposed to 5 mice/group for 2 months, activity score and body weight were measured to evaluate the toxicity effect. ICRF187 in our study did not cause weight loss (Figure 2.4).

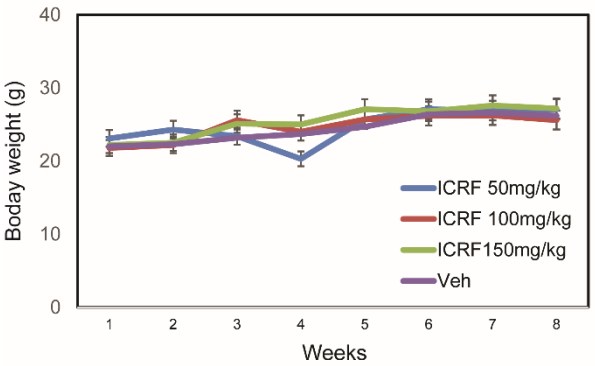


Figure 2.4 Mice weight loss curve of ICRF187 treatment.

Male nude mice were treated with vehicle, 50 mg/kg ICRF187, 100 mg/kg ICRF187 or 150 mg/kg ICRF187 (n=5/group) for 2 months. Body weight was measured weekly. Data was shown as mean ± SEM (n=5).

To construct CRPC LNCaP xenografts, a total of 1×10^6 LNCaP cells in 0.1 ml Matrigel were inoculated subcutaneously in bilateral flanks of 6-8 week old male athymic nude mice. Tumor volume, body weight and serum PSA levels were measured weekly. When serum PSA concentrations reached 50 ng/mL, mice were castrated. CRPC LNCaP tumors were defined when PSA levels relapsed to pre-castrated levels. Animals were randomly separated into four groups, treated daily with control, 10 mg/kg ENZ, 50 mg/kg ICRF187 or 5 mg/kg ENZ plus 25 mg/kg ICRF187. To establish ENZ-resistant MR49F xenografts, castrated mice were inoculated subcutaneously with MR49F cells and treated with 10 mg/kg ENZ daily till endpoint. When tumor volumes reached 100 mm^3, mice were also treated daily with vehicle, 25 mg/kg or 50

mg/kg of ICRF187. Tumor volumes and PSA levels were measured. CRPC 22RV1 xenografts were constructed by inoculating 22RV1 cells subcutaneously in bilateral flanks of nude mice. When mice were castrated and tumors reached 100 mm^3, mice were treated daily with vehicle or 50 mg/kg ICRF187. PC3 xenografts were constructed by inoculating PC3 cells subcutaneously in male athymic nude mice. When tumors reached 200 mm^3, mice were treated daily with vehicle or 50 mg/kg ICRF187. In all experiments, animals were sacrificed if tumor mass was ~10% of the body weight or any endpoint situation according to the animal protocol. At the endpoint, tumors were harvested for evaluations of mRNA levels of AR-targeted genes by real-time PCR. Tumor samples were also used to construct tissue microarrays by Ms. Estelle Li to measure Ki67 expression by immunohistochemistry (IHC).

2.20 Statistical analysis

Statistical analyses were carried out using GraphPad Prism (version 6, La Jolla, USA) for one-way ANOVA and parametric statistics (2-tailed Student's unpaired t-test) with the level of significance set at $P<0.05$ as *, $P<0.01$ as ** and $P<0.001$ as ***, and SPSS Statistics (version 20.0, Markham, Canada) for chi-square test with the level of significance set at $P<0.05$.

2.21 Technical Support

I would like to thank Mrs. Mary Bowden, Mr. Darrell Trendall and Ms. Estelle Li for their technical support of xenograft model and pathology analyses. We are also grateful to Dr. Yun Qiu for providing the pAR Y534 antibody. We thank Dr. Jocelyn Trottier for technical assistance in the LC-MS/MS quantification of glucuronides.

Chapter 3: Androgen-independent AR Signaling Transformed via UGT2B17 Expedites CRPC Progression

Note: Articles and figures are adapted with permission from AACR.

3.1 Introduction

Androgen receptor (AR) signaling is critical for prostate cancer (PCa) development as well as progression into castration-resistant PCa (CRPC) [236, 283]. Blocking AR signaling delays tumor growth and prolongs patient survival [180, 284]. These AR pathway inhibitors all target the ligand-binding domain (LBD) of the AR and prevent the AR from being transcriptionally active. However, the benefit of these therapies is temporary, and recurrence to CRPC is inevitable [180, 284]. Several possible mechanisms that reestablished AR signaling have been defined, including AR gene overexpression and amplification [203], AR gain-of-function mutations in the LBD with a more promiscuous affinity to other ligands [202], aberrant AR gene splicing, encoding constitutively active forms of the AR [193, 194], and the phosphorylation and subsequent activation of the AR in the absence of androgen [174]. These findings indicate that AR LBD inhibitors are insufficient to abolish AR signaling, and that tumor cells can utilize ligand-independent AR signaling to enable progression under anti-AR therapies. This notion is further supported by genome-wide studies showing that the AR remains functional in CRPC, but the AR transcriptome is shifted to one that predominantly regulates cell mitosis [206, 210, 285]. These findings emphasize the conclusion that most CRPC (a notable exception being neuroendocrine prostate cancer) still relies on the AR, but mainly through ligand-independent AR transcriptional activity that drives 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 PCa 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 [102]. Compared to benign prostate, PCa expresses higher levels of UGT2B17, and its expression has been reported to further increase after antiandrogen treatment [129, 269]. Using *in vitro* cell models, RNA silencing of UGT2B17 in LNCaP cells suppresses glucuronidation activity, but increases DHT levels in culture media and PCa cell proliferation [124]. 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 [97, 286] imply the opposite – that UGT2B17 may facilitate to CRPC progression through mechanisms that are not fully characterized to date. UGT2B17 may also exert undefined actions to modulate AR signaling similar to AKR1C3, which is another critical enzyme in androgen steroidogenesis that was recently identified to possess novel functions as an AR coactivator [136] and an enhancer of AR function through the ubiquitin ligase Siah2 [137]. As a membrane-bound enzyme localized in the endoplasmic reticulum of the cytosol [117], UGT2B17 may engage in ligand-independent activation of AR signaling by the kinase pathways that drive CRPC progression.

Through paracrine and autocrine regulation, cytokines and growth factors can stimulate AR phosphorylation and subsequent AR transactivation in a ligand-independent manner [172, 287-289]. For example, c-Src kinase, as a downstream effector of the EGF and IGF-1 pathways, activates the AR in the absence of androgens by using its SH3 domain to interact with, and

phosphorylate, the AR at the tyrosine 534 position [174]. In fact, overactive c-Src was reported in CRPC [174, 290] and c-Src inhibition by shRNA or small molecule-induced prostate tumor regression [291]. 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.

3.2 Results

3.2.1 UGT2B17 is associated with CRPC progression and androgen insensitivity of PCa cells

Previous studies showed that UGT2B17 mRNA expression was highly elevated in CRPC [97, 286]. Here, we evaluated UGT2B17 protein expression by immunohistochemistry (IHC) on tissue microarrays containing 604 tissue cores from 287 patients as described in Table 2.1 (Chapter 2.2) as well as in other studies [269]. UGT2B17's IHC was scored according to the intensity of the histology scores (H-scores): no response/weak as 1, medium as 2, and strong as 3. Higher UGT2B17 expression was more prevalent in tissue cores with higher Gleason scores (Figure 3.1A), and UGT2B17 H-scores were positively correlated with the Gleason score ($r=0.3034$, $p<0.001$) (Table 3.1). Compared with benign prostate, UGT2B17 was highly expressed in PCa, and further elevated by NHT (Figure 3.1B). Consistent with previous reports on UGT2B17 mRNA expression [97, 286], a larger proportion of CRPC tissue cores fell into the category of strong UGT2B17 protein expression (Figure 3.1B). 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 (Figure 3.1C). These results

indicate that increased UGT2B17 expression is associated with higher grade and CRPC progression.

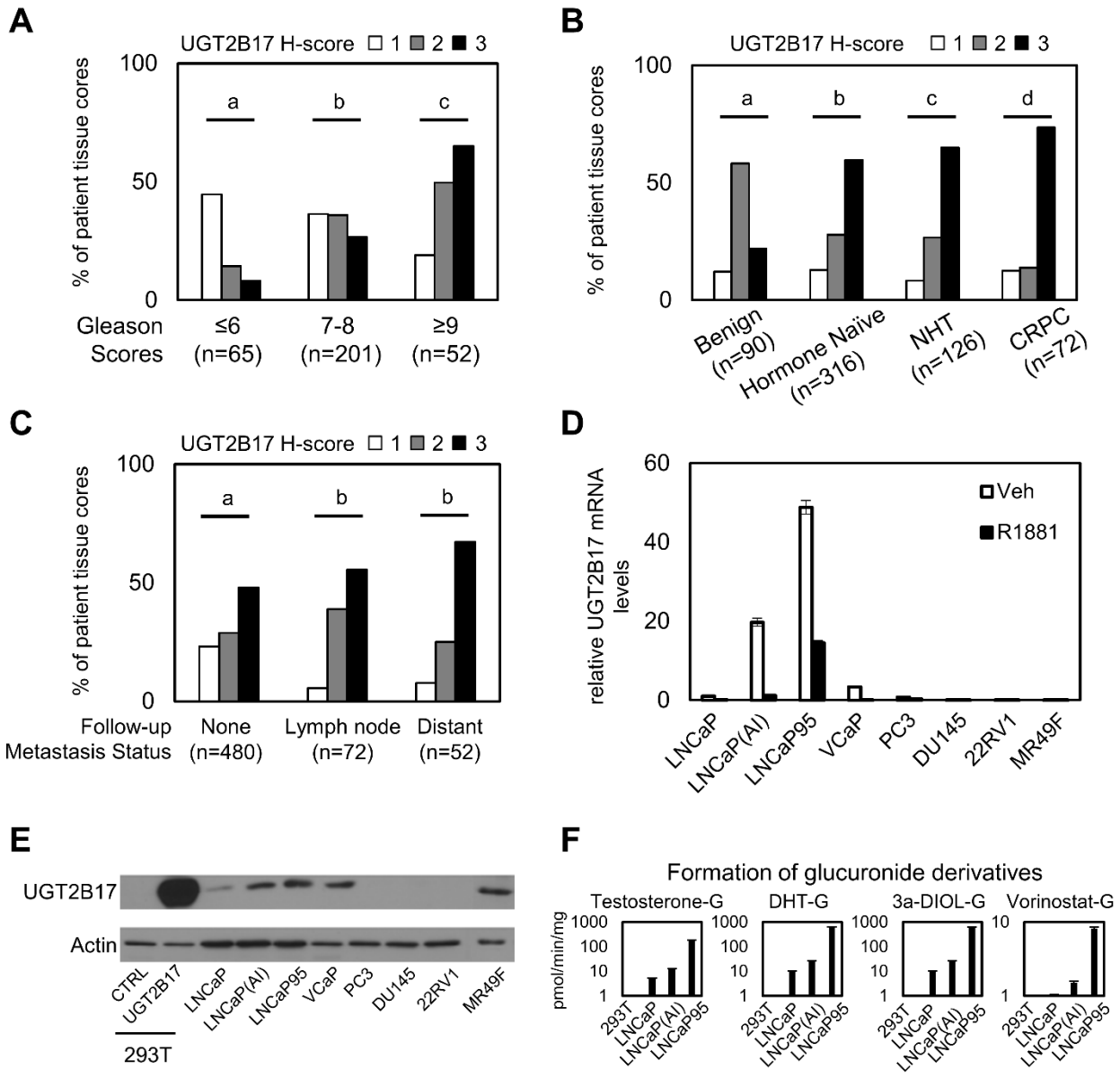


Figure 3.1 UGT2B17 is associated with prostate tumor progression and androgen insensitivity of prostate cancer cells.

(**A-C**) Immunohistochemistry was performed using the UGT2B17 antibody on PCa tissue microarrays. Histology scores (H-scores) of each tissue core were evaluated by Dr. Ladan Fazli on a scale of 1-3 as described in Chapter 2.3. The percentages of tissue cores in each H-score group were calculated and were sorted by (**A**) Gleason score, (**B**) PCa progression and (**C**) metastasis status. Comparison between the groups was analyzed using the chi-square

test by SPSS Statistics. Values without a common letter are significantly different, $p<0.05$. (**D**-**E**) LNCaP, LNCaP(AI), LNCaP95, VCaP, PC3, DU145, 22RV1 and MR49F cells cultured under the condition of vehicle or 10 nM 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, 3α-DIOL-17G and vorinostat-G were measured by Ms. Mélanie Verreault. Results were presented as the mean ± SEM (n=3).

Correlation	**UGT2B17 H-score vs Total Gleason Score**
r value	0.3043
95% confidence interval	0.1971 to 0.4043
P value	***< 0.001

Table 3.1 Correlation analysis of UGT2B17 H-score and Gleason score.

In PCa cell lines, we found that androgen-independent LNCaP(AI) and LNCaP95 cells expressed higher UGT2B17 RNA and protein levels than androgen-dependent LNCaP cells (Figure 3.1D-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 [126, 269]. 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 the UGT2B17 metabolites including testosterone-G, DHT-G, 3α-DIOL-17G and the UGT2B17-specific vorinostat-G were all significantly increased in LNCaP(AI) and LNCaP95 cells with an UGT2B17 dependent manner (Figure 3.1F). These

results indicate that enhanced UGT2B17 glucuronidation is also associated with androgen insensitivity of PCa cells.

3.2.2 UGT2B17 enhances PCa cell growth and invasion after prolonged androgen deprivation

To study the impact of UGT2B17 on PCa cell growth, we used the lentiviral approach to construct LNCaP cells overexpressing UGT2B17 and LNCaP95 cells with UGT2B17 knockdown (Figure 3.2). Prolonged androgen deprivation of LNCaP cells enhanced UGT2B17 protein levels (Figure 3.3A). Upregulation of UGT2B17 expression was also accompanied by increased glucuronidation (Figure 3.3B).

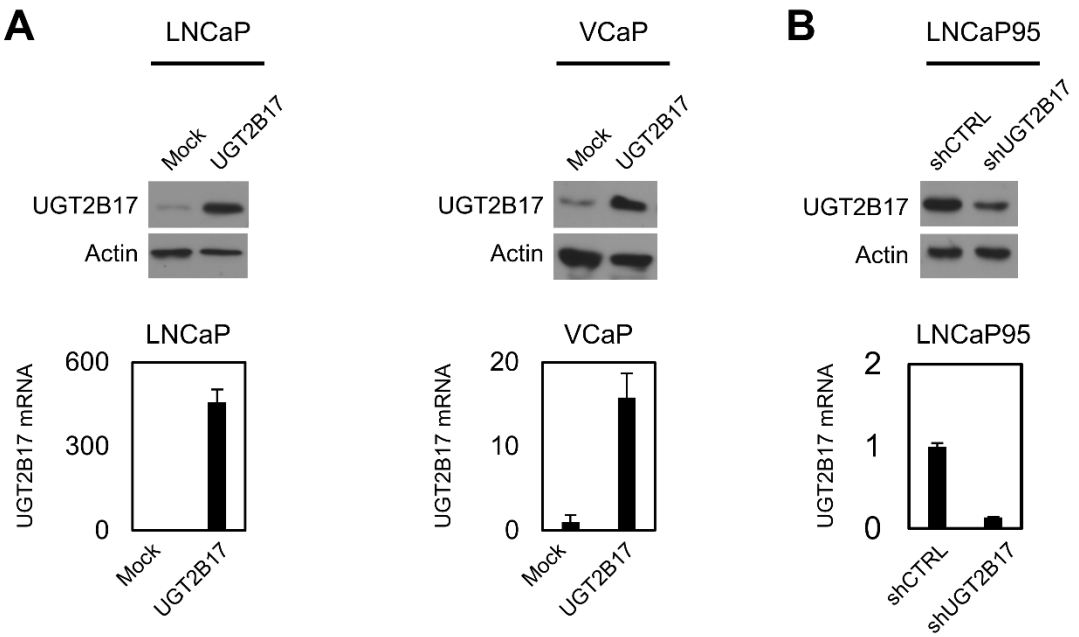


Figure 3.2 Construction of PCa cell lines using the lentiviral approach.

(**A**) LNCaP and VCaP cells were transduced by lentivirus encoding mock or UGT2B17. (**B**) LNCaP95 cells were transduced by lentivirus encoding control or shUGT2B17. Cells were cultured in RMPI1640 medium containing 10

μg/ml puromycin for selection. Immunoblotting and real-time PCR results confirmed UGT2B17 expression in these cell lines.

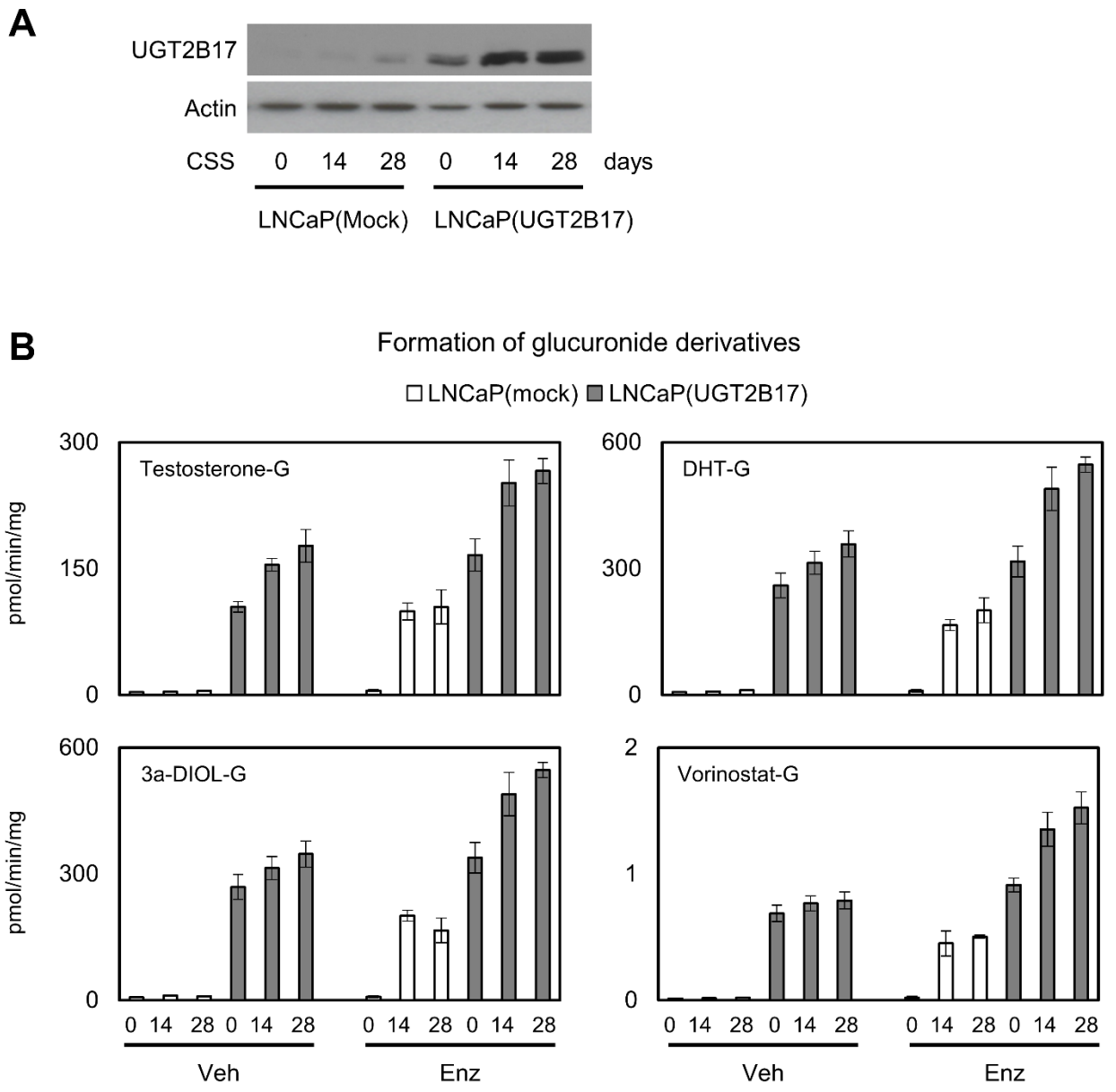


Figure 3.3 Androgen deprivation increases LNCaP(UGT2B17) glucuronidation activity.

(**A**) LNCaP(mock) and LNCaP(UGT2B17) cells were cultured in RPMI1640 medium containing 5% CSS for 0, 14 or 28 days. Protein levels of UGT2B17 and β-actin were measured by immunoblotting. (**B**) LNCaP(mock) and LNCaP(UGT2B17) cells were cultured in RPMI1640 medium containing 5% CSS plus vehicle or 10 μM of ENZ

for 0, 14 or 28 days. The formation of glucuronide derivatives was measured by Ms. Mélanie Verreault, and the results were shown as mean ± SEM from three repeated experiments.

We found that altered 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 nM of R1881, or 10 μM of ENZ. LNCaP cell proliferation was stimulated by R1881 approximately 4 fold, but inhibited by enzalutamide or androgen deprivation (Figure 3.4A, 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 (Figure 3.4A, 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 ENZ, but stimulated by R1881. Similarly, BrdU 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 (Figure 3.4B). These UGT2B17 effects on DNA synthesis appeared as early as 14 days of treatment.

To determine whether UGT2B17 also regulates PCa cell motility, we pre-treated LNCaP cells with vehicle, R1881 or ENZ for 0 or 28 days, and then performed trans-well cell migration and invasion assays (Figure 3.4C). 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 PCa cells to androgens with regard to cell proliferation and invasion.

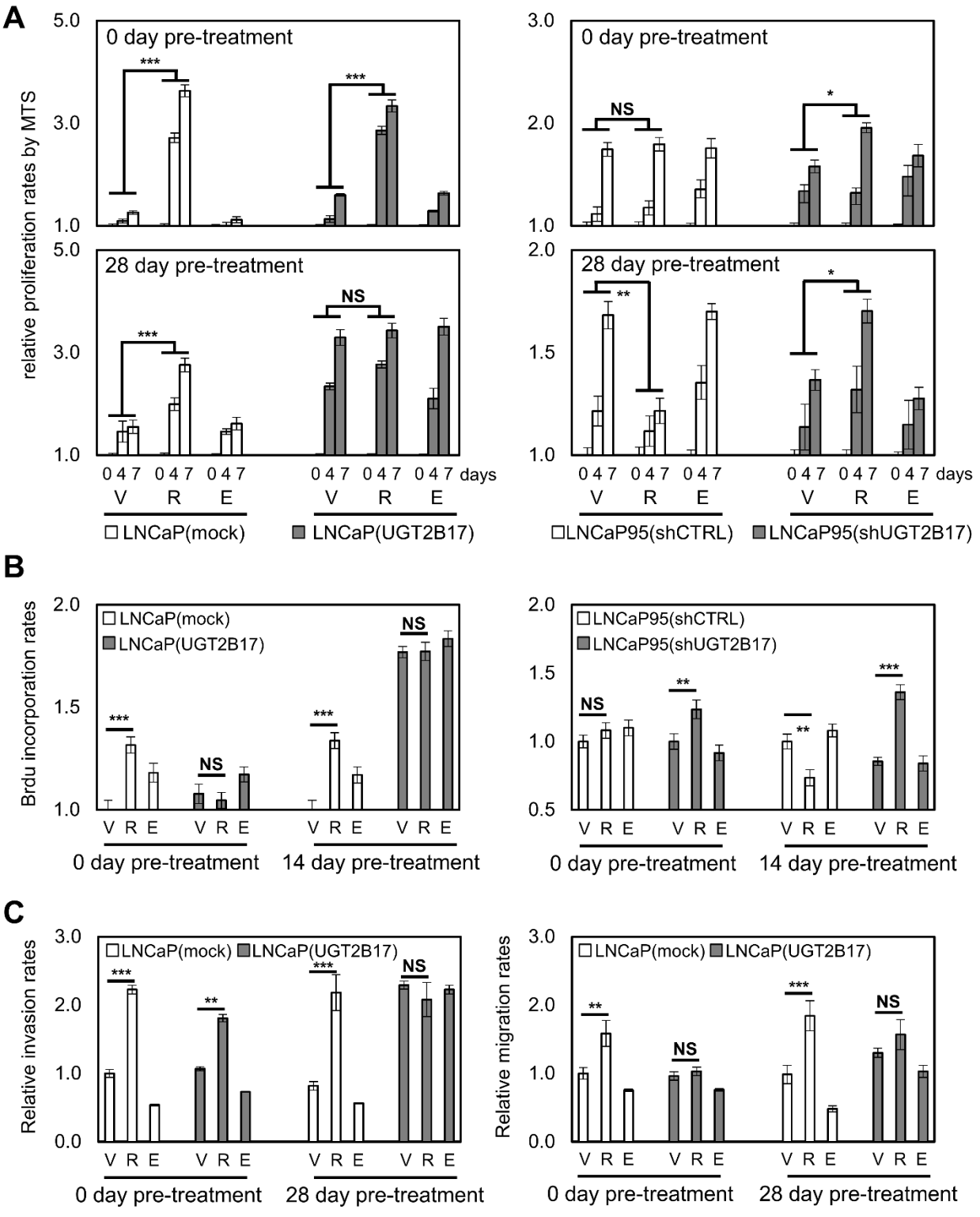


Figure 3.4 UGT2B17 enhances PCa cell growth and invasion after prolonged androgen deprivation.

(**A**-**B**) LNCaP(mock), LNCaP(UGT2B17), LNCaP95(shCTRL) and LNCaP95(shUGT2B17) cells were pre-treated in the RPMI1640 medium containing 5% CSS under the condition of vehicle (V), 10 nM of R1881 (R), or 10 μM of ENZ (E) for 0 (**A**, top), 28 (**A**, bottom) or 14 (**B**) days. Relative cell proliferation rates within 0-7 days were measured by MTS assays in (**A**) and by BrdU incorporation assays in (**B**). (**C**) LNCaP(mock) and LNCaP(UGT2B17) cells were cultured in RPMI1640 medium containing 5% CSS under the condition of vehicle, 10 nM of R1881, 10 μM 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 shown as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by student t-test with $P<0.05$ as *, $P<0.01$ as ** and $P<0.001$ as ***. NS, nonsignificant.

3.2.3 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 (Figure 3.5A), but lower serum PSA levels (Figure 3.5B), when compared to LNCaP control tumors. Tumor volume doubling time before (100-200 mm^3) and post castration (250-500 mm^3) was also calculated (Figure 3.5C). LNCaP control tumors showed delayed tumor growth with increased tumor doubling time after castration when compared to 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.

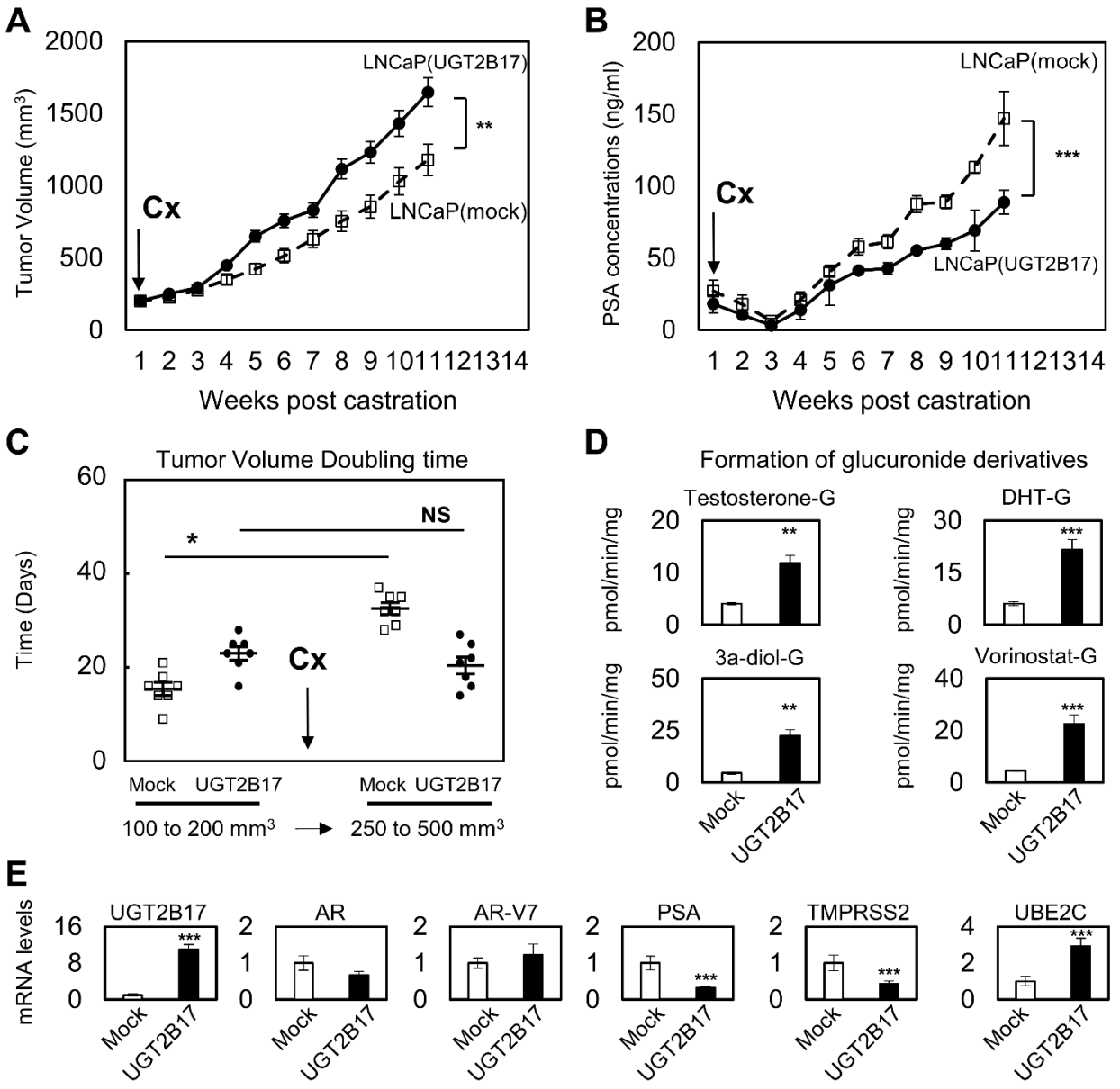


Figure 3.5 UGT2B17 accelerates CRPC progression of LNCaP xenografts.

LNCaP(mock) and LNCaP(UGT2B17) xenografts were established as described in Chapter 2.19. Mice were castrated when tumor volume reached 200 mm^3. (**A**) Tumor volume and (**B**) serum PSA levels 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 student t-test with $P < 0.01$ as ** and $P < 0.001$ as ***.

When tumors were harvested and used for glucuronidation assays, we found that increased UGT2B17 expression was associated with enhanced UGT2B17 activity in catabolizing androgens (Figure 3.5D). Since 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, while the expression of AR-regulated mitotic genes such as UBE2C was increased (Figure 3.5E). 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 AR mediated gene transcription that promotes cell mitosis.

3.2.4 UGT2B17 alters AR signaling under androgen deprivation

To decipher molecular mechanisms by which UGT2B17 enhances PCa cell growth independent of androgen, we performed gene microarray analysis 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 that the top ranked gene group (n=101 genes) was associated with cell differentiation (Figure 3.6A). 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 (Figure 3.6B), suggesting that UGT2B17-regulated transcriptome shifted from cell differentiation to cell cycle after prolonged androgen deprivation.

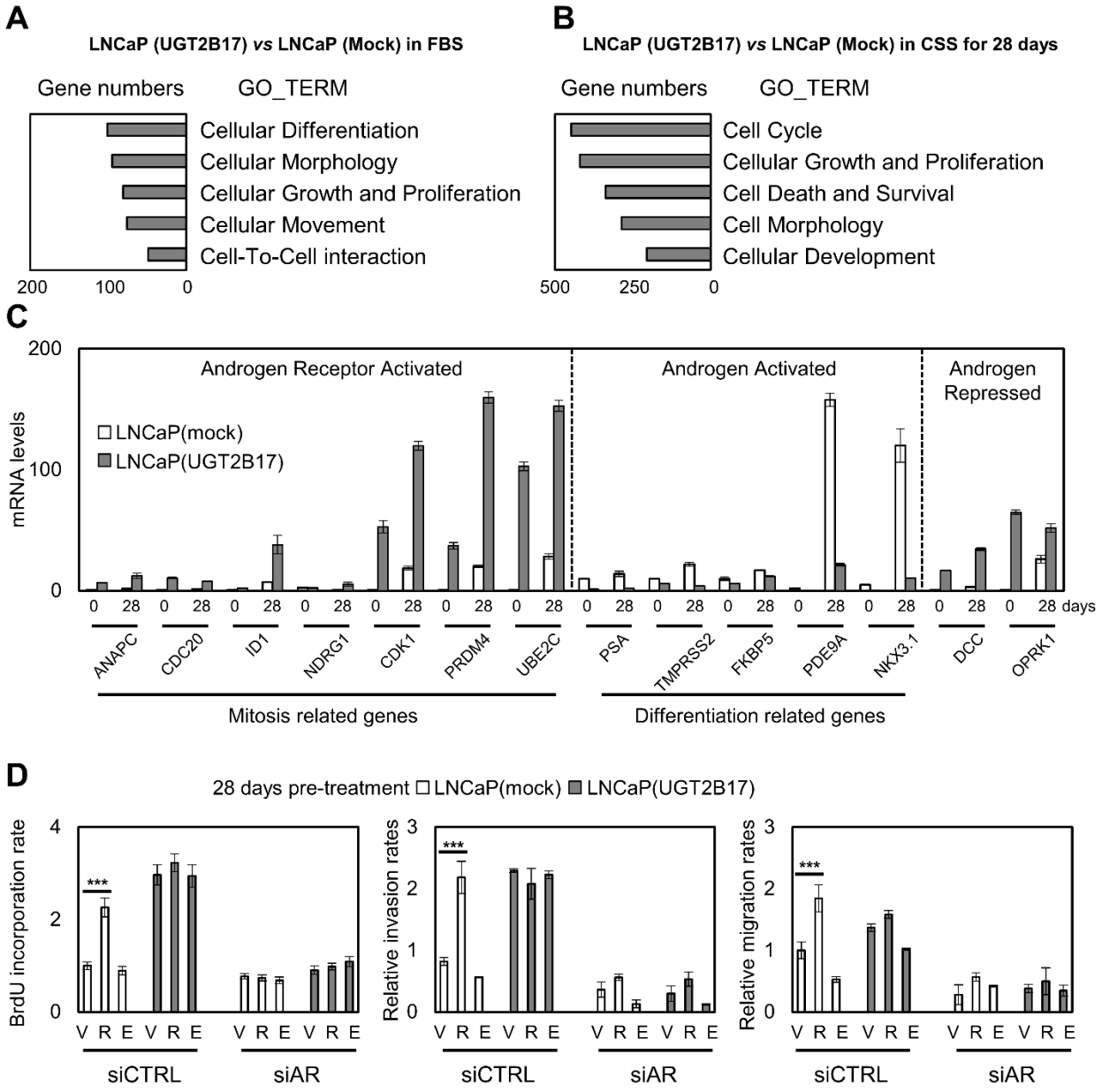


Figure 3.6 UGT2B17 alters AR signaling under prolonged androgen deprivation.

(**A-B**) LNCaP(mock) and LNCaP(UGT2B17) cells were cultured in RPMI1640 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 analysed 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 RPMI1640 medium containing 5% CSS under the condition of vehicle (V), 10 nM of R1881 (R), or 10 μM of ENZ (E) for 28 days. Cells were then

transfected with control siRNA or siRNA against AR. Relative BrdU incorporation, cell invasion and migration rates were measured. All data were repeated in three independent experiments and shown as mean ± SEM. Statistical analyses were performed by student t-test with $P<0.001$ as ***.

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 up-regulated by the AR, independent of androgen [285]. Enhanced UGT2B17 expression in LNCaP cells strongly stimulated the expression of these genes in a time-dependent manner (Figure 3.6C), suggesting that UGT2B17 enhanced ligand-independent AR transactivation. The expression of genes such as PSA, TMPRSS2, FKBP5, PDE9A and NKX3.1 was stimulated, while the expression of genes such as DDC and OPRK1 was repressed by androgens [292]. Overexpression of UGT2B17 in LNCaP cells impaired the androgen regulation of these genes. To exclude the possibility that the altered gene expression was caused by factors other than the AR, we depleted AR expression by RNA silencing (Figure 3.7). We confirmed that AR knockdown abolished UGT2B17 actions on genes regulated by both ligand-dependent and ligand-independent AR transcriptional activities. To confirm that UGT2B17 actions on cancer cell growth and invasion are mediated by the AR, we showed that UGT2B17-stimulated BrdU incorporation, cell invasion and migration were abolished by AR depletion (Figure 3.6D). These results show that UGT2B17 activates ligand-independent, but inhibits androgen-dependent, AR actions in regulating gene expression, PCa cell proliferation and invasion under prolonged androgen deprivation conditions.

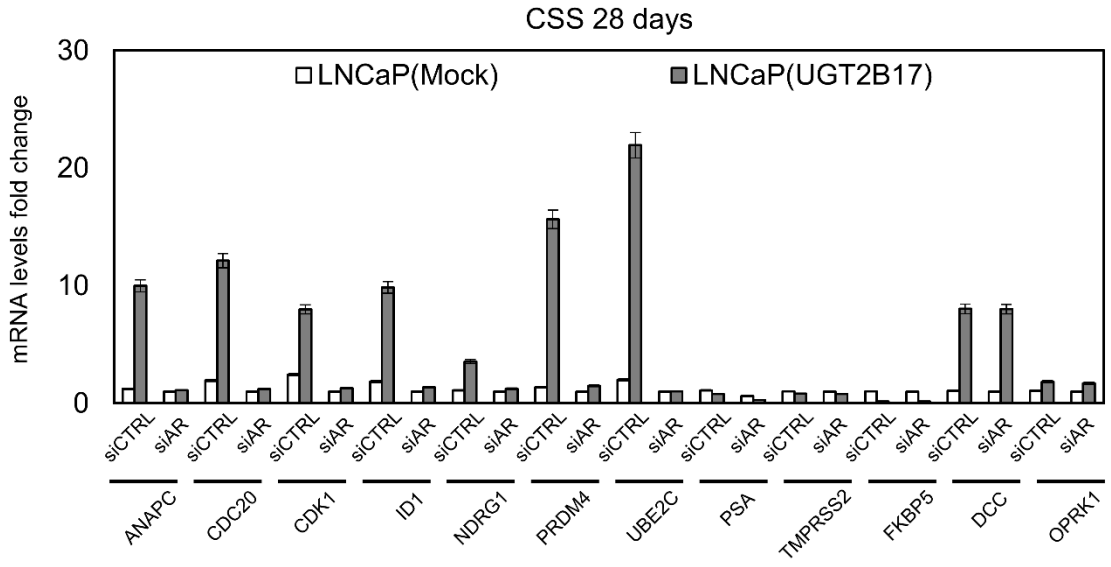


Figure 3.7 AR knockdown diminishes UGT2B17-stimulated gene upregulation.

LNCaP(mock) and LNCaP(UGT2B17) cells were cultured in RPMI160 medium containing 5% CSS for 28 days, and then transfected with control siRNA or siRNA against AR. Total RNA was extracted for real-time PCR assays. All data were repeated in three independent experiments and are shown as mean ± SEM.

3.2.5 UGT2B17 activates the 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 further investigate these findings, LNCaP cells overexpressing either control or UGT2B17 were treated with androgen deprivation for 0 or 28 days (Figure 3.8A). UGT2B17 increased c-Src activation, which was further increased by androgen deprivation. Additionally, STAT3 and AKT were also stimulated by UGT2B17 under androgen deprivation (Figure 3.8A). 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 (Figure 3.8B). Furthermore, PP2 but not LY or Stattic suppressed UGT2B17-induced cell proliferation after androgen deprivation (Figure 3.8C). These results indicate that c-Src is the

upstream regulator of AKT and STAT3, mediating UGT2B17 actions in regulating cell cycling under androgen deprivation.

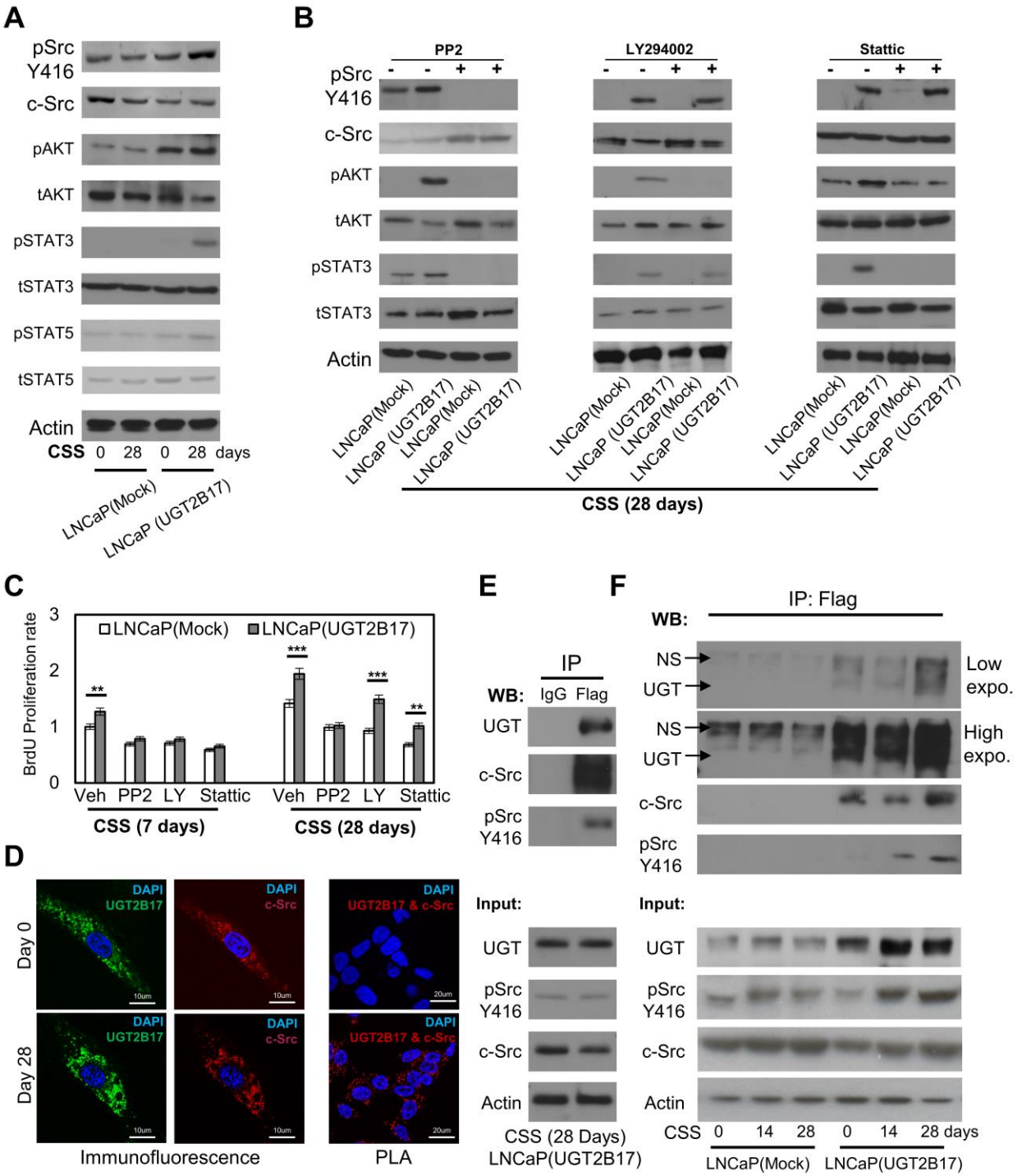


Figure 3.8 UGT2B17 activates the AR through c-Src kinase in CRPC progression.

(**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 RPMI1640 medium containing 5% CSS for 28 days. Cells were then treated with vehicle, or 10 μM of PP2, WP1066 or Stattic for another 12 hours. Protein levels of pSrc Y416, c-Src, pAKT, total AKT, pSTAT3, total STAT3, pSTAT5, total STAT5 and β-actin were measured by immunoblotting. (**C**) LNCaP(mock) and LNCaP (UGT2B17) cells were cultured in RPMI1640 medium containing 5% CSS for 7 and 28 days, and then treated with vehicle or 10 μM of PP2, WP1066 or Stattic for 48 hours. Relative BrdU incorporation rates were measured. (**D**) LNCaP(UGT2B17) cells were cultured in RMPI1640 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 the Materials and Methods section. Red dots represent UGT2B17 and c-Src interactions. (**E-F**) LNCaP(mock) and LNCaP(UGT2B17) cells were cultured in RPMI1640 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: non-specific (NS) proteins appeared when the UGT2B17 antibody was used to perform co-IP.

Because both UGT2B17 and c-Src are expressed in the cytosol, we performed PLA and co-immunoprecipitation (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 (Figure 3.8D). However, protein-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 (Figure 3.8E). Co-IP assays also indicated that UGT2B17 protein continuously accumulated after androgen deprivation in a time-dependent fashion, while robust c-Src association with UGT2B17 first occurred on day 14, and strong c-Src activation occurred on day 28 (Figure 3.8F). These results suggest that prolonged androgen

deprivation accumulates UGT2B17 protein expression, which in turn recruits and activates c-Src kinase.

In addition to LNCaP, we also exploited the VCaP cell line and observed similar results, that VCaP cells overexpressing UGT2B17 became insensitive to androgen deprivation or enzalutamide after 14-28 days of treatments, as exemplified by BrdU incorporation rates and cell invasion rates (Figure 3.9A-B). Expression of AR-activated genes as well as androgen-suppressed genes were also upregulated by UGT2B17, while androgen-activated gene expression remained unchanged during prolonged androgen deprivation (Figure 3.9C). Furthermore, activation of c-Src by UGT2B17 in VCaP cells is similar to that in LNCaP cells (Figure 3.9D). However, under androgen deprivation or enzalutamide treatment conditions, the constitutively active AR variants had been demonstrated to drive the AR signaling [193, 194, 197]. Results from VCaP cell lines are therefore difficult for us to distinguish ligand-independent AR-FL actions from constitutive transactivation of AR variants.

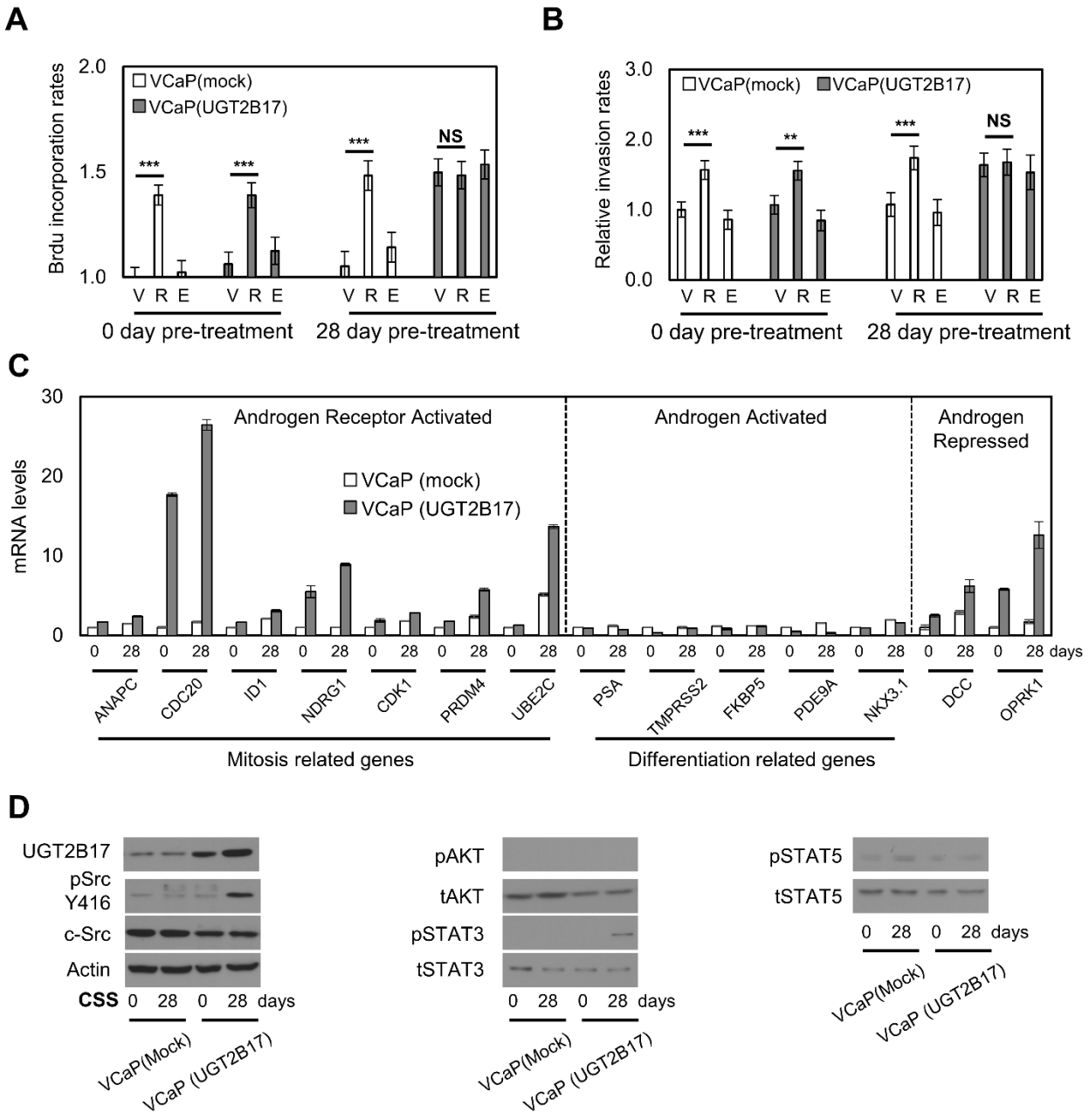


Figure 3.9 UGT2B17 enhances VCaP cell growth and invasion after prolonged androgen deprivation.

VCaP(mock) and VCaP(UGT2B17) cells were cultured in the RPMI1640 medium plus 5% CSS. Cells were treated with vehicle, 10 nM of R1881 or 10 μM of ENZ for 0 or 28 days. (**A**) Cell proliferation rates were determined by BrdU incorporation assays. (**B**) Cell invasion rates were determined by BD Matrigel Invasion assays. (**C**) Relative mRNA levels of androgen receptor activated, androgen activated and androgen repressed genes were determined by real-time PCR. (**D**) Protein levels of UGT2B17, pSrc, tSrc, pAKT, total AKT, pSTAT3, total STAT3, pSTAT5, total STAT5 and β-actin were determined by immunoblotting. All data were repeated in three independent experiments,

and are shown as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by student t-test with P< 0.05 as *, P<0.01 as ** and P<0.001 as ***.

3.2.6 Elevated UG2B17 expression is associated with c-Src activation

To define whether elevated UGT2B17 expression is associated with c-Src activation in PCa patients, we analyzed UGT2B17, c-Src, pSrcY416 protein levels in PCa TMAs (Table 3.2 and Figure 3.10A). 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 (Figure 3.11). Collectively, these results demonstrate that elevated UGT2B17 expression activates c-Src in CRPC tumors.

	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]

Table 3.2 Correlation analysis of H-score between UGT2B17 and c-Src or pSrc Y416.

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's coefficient tests. Statistical significance is indicated in bold font as [a] P < 0.05, [b] P < 0.01.

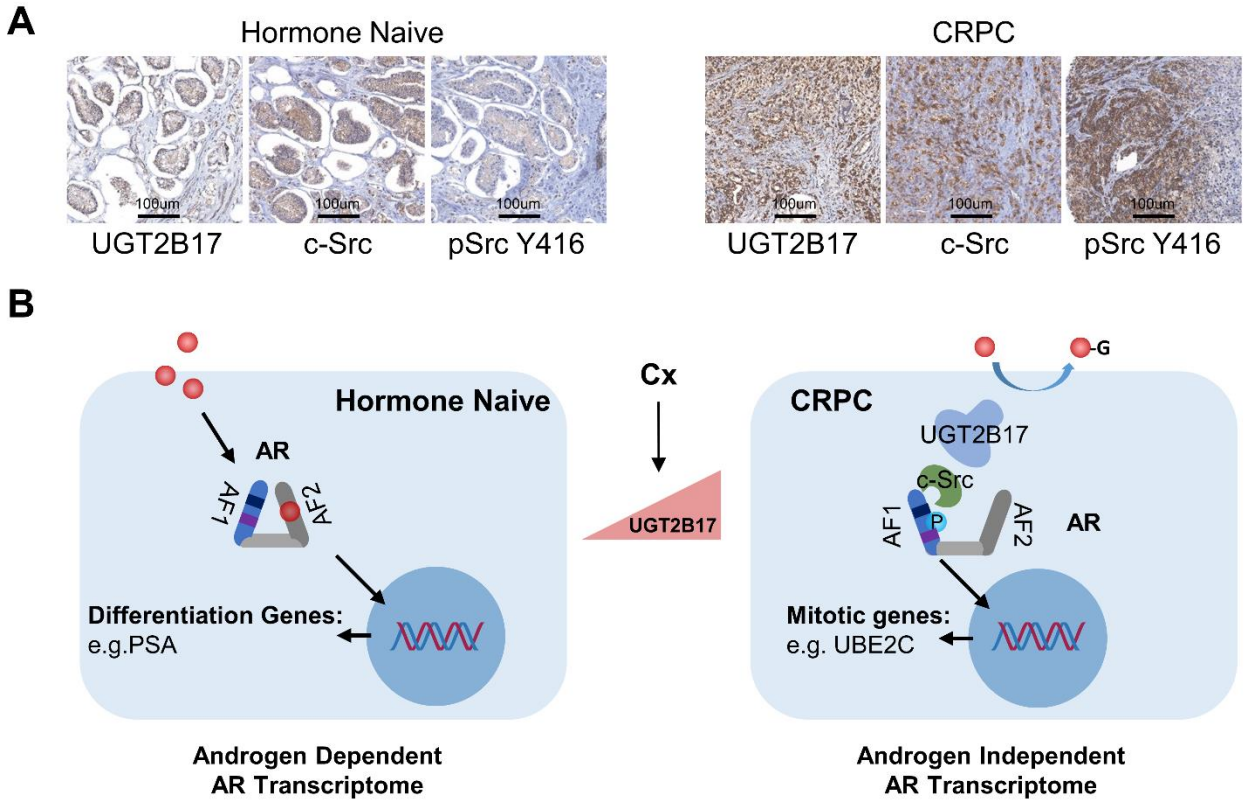


Figure 3.10 UGT2B17 expression is associated with c-Src activation in CRPC.

(**A**) Representative IHC images of UGT2B17, c-Src and pSrc Y416 in the hormone naïve and CRPC tissue groups were shown. (**B**) The mechanisms by which increased UGT2B17 by androgen deprivation in CRPC activates the AR through stimulating c-Src.

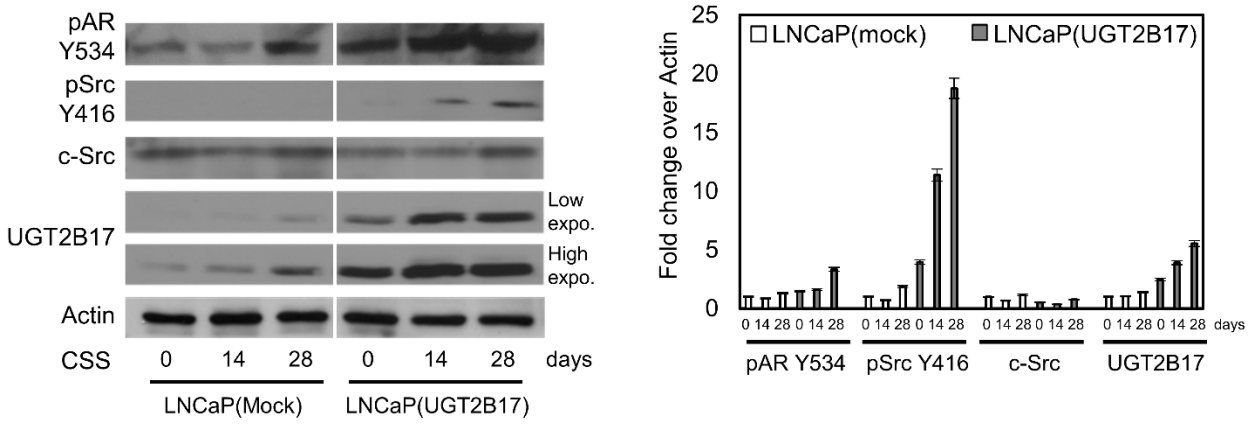


Figure 3.11 UGT2B17 protein expression is correlated with pAR Y534 and pSrc Y416 levels in PCa cell lines.

LNCaP(mock) and LNCaP(UGT2B17) cells were cultured in RPMI1640 medium containing 5% CSS for 0, 14 or 28 days. Cell lysates were collected for detection of pAR Y534, pSrc Y416, c-Src, UGT2B17, and β-actin by immunoblotting. Densitometry analyses of protein bands from three repeated assays were performed by the Image J software. All values normalized with data from LNCaP(mock) day 0 as 1.

3.3 Discussion

Our studies identified a novel function for UGT2B17 that promotes ligand-independent AR signaling to expedite CRPC progression. We demonstrated that elevated UGT2B17, under conditions of prolonged androgen deprivation, activated c-Src kinase and stimulated AR transactivation independent of androgens (Figure 3.10B). 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.

While PCa 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 [206, 210, 285]. Since 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 [143, 293, 294]. 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 [295, 296]. 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 the LBD [199]. By contrast, deletion of Tau5 in the AR inhibits androgen-independent AR activity in androgen-insensitive cells, but enhanced androgen-dependent AR activity in androgen-sensitive PCa cells [296]. 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. The AR is still recruited to chromatin in CRPC, but to different locations compared to that in castrate sensitive tumors [206]. The observation that low PSA tumors are often more aggressive and have poorer prognosis [297, 298] 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 the AR to act ligand-independently.

Although enhanced UGT2B17 expression has been previously reported to be associated with CRPC [97, 129, 299], 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 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. Since UGT2B17 protein is accumulated in PCa cells after prolonged androgen depletion (Figure 3.3 and Figure 3.8F), 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 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 (Figure 3.2-3.9). One possible explanation for this is that transient androgen depletion stimulates *de novo* steroidogenesis, while prolonged androgen depletion combined with UGT2B17 protein accumulation abolishes androgen exposure of the AR more thoroughly. In addition, shift of AR signaling to ligand-independent mode requires c-Src activation, resulting from UGT2B17 accumulation in PCa 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 [300]. Regardless of the 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, while antiandrogens enhanced the mRNA levels of UGT2B17 [97, 126]. AR recruitment to the UGT2B17 promoter was also reported to be responsible for suppressing UGT2B17 gene transcription [126]. It remains to be determined whether ligand-independent AR signaling induced by UGT2B17 in turn would enhance UGT2B17 transcription through a feed-forward mechanism and whether the AR regulates UGT2B17 protein stability.

Mechanisms that determine whether or not CRPC tumors develop resistance to new generation antiandrogens such as enzalutamide and abiraterone are not fully understood. Generations of LBD truncated AR splice variants and accumulation of gain-of-function of mutations in the LBD had been credited for the therapy resistance [193, 194, 202]. However, not all CRPC tumors express AR-V7 or mutant ARs. Since CRPC tumors predominantly express high levels of AR, our studies propose that anti-AR resistant tumors are mainly driven by reestablished AR signaling from being androgen-dependent to becoming androgen-independent, which in turn regulates the transcription of mitosis genes. Such AR functional reprogramming is manifested by a shift in AR dependence on AF-2 in the 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 [102]. Compared to the benign prostate, UGT2B15 protein levels were reduced in hormone naive tumors and CRPC, and became undetectable in lymph node metastases [129]. In contrast to UGT2B17, which can catabolize all three major androgens, UGT2B15 can only de-activate DHT with much lower efficacy than UGT2B17 [122]. Recently, UGT2B28 expression was reported to be positively associated with high-grade PCa, suggesting that it may play a similar role to UGT2B17 [134]. 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 supporting CRPC progression. We propose that a combination of AR pathway inhibitors with UGT2B17-Src-AR signaling axis inhibition could more potently suppress CRPC progression.

3.3.1 Targeting UGT2B17-Src-AR signaling axis

To target the UGT2B17-Src-AR signaling axis, we first considered targeting the UGT2B17 enzyme protein expression, enzyme activity and its interaction with c-Src. As we have shown in Figure 3.3, using lentiviral approaches to knockdown UGT2B17 suppressed the LNCaP95 cell proliferation rate. To enhance the knockdown effect, we further transiently transfected siRNA against UGT2B17 in LNCaP95, achieving improved suppression of cell proliferation and cell cycling. The antisense oligonucleotides (ASOs), which are synthetic polymers of chemically modified deoxyribonucleotides containing sequences designed to be complementary to the sense sequence of target mRNAs [301], provides one feasible strategy to target UGT2B17. One of the criteria of ASOs targeting is that the targeted gene should have higher expression in pathological tissues than normal tissues. As we mentioned in Chapter 1.2.2, and in a previous report [302], the UGT2B17 mRNA and protein are highly expressed in the gastrointestinal tract and liver. In prostate tissues, as well as in the liver, the UGT2B family also contribute to the metabolism of drugs, endobiotics, and bile constituents. The UGT2B family also interacts with the cytochrome c family, including CYP2A8 and CYP3C4 [303, 304], which are responsible for the metabolism of enzalutamide [305]. A comprehensive drug interaction investigation and tissue-specific ASOs are required before directly targeting UGT2B17 mRNA.

Factors regulating UGT2B17 are also potential targets for UGT2B17 inhibition. Studies showed that the Farnesoid X receptor (FXR) negatively regulated UGT2B17 expression [306]. The FXR activators, chenodeoxycholic acid, or GW4064 treatment could downregulate UGT2B17 mRNA and protein expression levels in LNCaP cells. The off-target effect of FXR activators and FXR/AR interaction is subject to further investigation. Several recent studies revealed more

regulation mechanisms of UGT2B17, including the regulation of UGT2B17 by the miR-376c in PCa cells [307], AR-V7 potentially regulates UGT2B17 through binding to its promoter [135], and FOXA1 assists the AR as a coregulator of UGT2B17 in breast cancer cells [131]. Taken together, these upstream regulators provide the second way to target UGT2B17.

A third strategy is to inhibit the enzyme activity of UGT2B17. Flavonoids contained in red wine could inhibit UGT2B17 glucuronidation activity in the *in vitro* assays [308]. Green tea extracts showed effective inhibition of PCa tumor growth [309], and were also reported to suppress UGT2B17 activity [310]. We have treated LNCaP, LNCaP95 and MR49F with the effective components from green tea extracts, including Catechin gallate, Epicatechin and Epigallocatechin gallate at their maximum dosage. A mild proliferation inhibition on LNCaP was found. While little was observed on LNCaP95 and MR49F (unpublished collaboration data). Above all, all UGT2B17 inhibition strategies including targeting drugs and natural products, need to consider potential drug interactions.

The mRNA and protein expression of UGT2B17 is repressed by androgen-dependent AR signaling, which further emphasize the importance of the UGT2B17 inhibition timing [124]. The ideal time for UGT2B17 inhibition would be at the onset of abiraterone relapse, which inhibits *de novo* androgen synthesis. Combination treatments of the UGT2B17 inhibition and the anti-androgen ENZ proceed afterwards to restrain and inactivate AR signaling.

Due to the lack of studies on UGT2B17 structures, the UGT2B17 and c-Src interaction mechanism remains to be elucidated. The tyrosine kinase c-Src has attracted a plethora of

attention in the PCa research field. In 2000, Dr. Auricchio's group found that the non-genomic function of the AR interacts with c-Src to trigger PCa carcinogenesis [84] and in 2006, Dr. Yun Qiu's group found that c-Src could phosphorylate the AR [174]. Numerous other studies suggested that c-Src is a potential target in CRPC (reviewed in [311]); two major inhibition methods were developed. The first one is targeting the SH3 domain of c-Src using small peptides mimicking the domain sequence in the AR [312]. These peptides prevented the S phase entry of LNCaP cells and the growth of LNCaP xenografts. However, there were no further follow-up studies done after this paper in 2007. More efforts were focused on the inactivation of c-Src, as it was found to mediate PCa cell growth, invasion, and metastasis in preclinical model systems. Preclinical observations suggest that raised c-Src activity in tumors of patients with prostate cancer is associated with decreased sensitivity to androgen ablation, increased bone metastasis, and shorter survival. One of the c-Src inhibitors, dasatinib (BMS-354825), inhibited prostate cancer growth and metastasis, and suppressed PCa cell-induced osteoclastic activity in the bone microenvironment in preclinical models [313, 314]. Additionally, the combination of dasatinib and docetaxel had greater activity than either agent alone in a mouse xenograft model of prostate cancer [315, 316]. Despite the successful clinical use of dasatinib in leukemia, the Phase III randomized trial of docetaxel and dasatinib in metastatic CRPC patient showed no significant improvement on overall survival [317]. In addition, most c-Src inhibitors were screened from high-throughput assays [318]. They are binding to the kinase domain with P-binding phosphate loop folding over the ATP binding site [319]. Several tyrosine kinases, including c-Src, BCR/Abl, and c-kit, shared the same binding machinery [320]. C-Src inhibition needs to be further improved until we find a more specific c-Src-AR binding inhibitor.

The final effector of this UGT2B17-Src-AR signaling is the classic central target AR. All FDA approved ARPIs (e.g. enzalutamide) follow the same mechanism of AR inhibition and, therefore, can suffer from the same limitation. They target AR LBD and prevent AR from becoming transcriptional active [180, 284]. However, none of these ARPIs can inhibit other transcriptionally active forms such as AR-Vs and mutant ARs. In fact, enzalutamide enhances the expression of AR-Vs [321] and enriches tumor cells with mutant ARs [201, 202]. Because of the emergence of resistance and the side effects of current ARPIs [322], there is an urgent need to develop entirely new types of anti-AR therapy to block the activities of not only the AR, but also AR-Vs and mutant ARs.

The transcriptome driven by the AR in hormone naïve tumors was significantly different from that in CRPC [285, 323]. Genome-wide studies showed that the AR signaling in CRPC shifts from one pathway primarily driving epithelial cell differentiation to a survival pathway, consequently accelerating cancer cell mitosis [210, 285, 323]. These findings together indicate a new perspective to block the AR signaling in CRPC, whereby targeting AR-regulated cell mitosis in addition to AR-mediated transcription initiation would suppress CRPC progression more efficiently.

3.3.2 DNA topoisomerase II is a potential drug target for CRPC

DNA Topoisomerase II **(**Topo II) is required for both AR-mediated transcription initiation and cell mitosis [41, 261]. It performs a two-step enzymatic reaction that cleaves and re-ligates double strand DNA breaks to relieve the super-helical state of DNA and disentangles interlinked chromosomes. AR-mediated transcription initiation requires Topo II to be recruited to target

androgen-responsive promoters for opening chromatin and allowing transcription initiation to proceed [41, 239, 324]. RNA silencing of Topo II impairs AR transcriptional activity and AR-driven cell proliferation [41, 239, 324]. Importantly, overexpression of Topo II was reported in PCa, and its levels were associated with poor prognosis of PCa patients [240, 241]. These findings together indicate that Topo II is an attractive therapeutic target for CRPC.

Topo II inhibitors can induce G_2/M cell cycle arrest [251, 325-328]. There are two types of Topo II inhibitors, Topo II poisons and catalytic inhibitors. Topo II poisons are exemplified by etoposide. DNA breaks created by Topo II cannot be re-ligated by etoposide, resulting in DNA damage. Such unfixable DNA breaks cause G_2/M cell cycle arrest and subsequently apoptosis [251, 325, 326]. The catalytic inhibitors are exemplified by bisdioxopiperazines (e.g. ICRF187 and 193). ICRF187 binds the catalytic domain of Topo II and prevents Topo II from creating DNA breaks [327]. ICRF187 inhibits chromosome condensation and segregation at the M phase of the cell cycle, when Topo II is required to de-catenate the intertwined chromosomes [328]. Unlike Topo II poisons, ICRF187 inhibits Topo II activity without creating DNA breaks, thereby exhibiting less cytotoxic effects. We hypothesize using catalytic Topo II inhibitors could thoroughly block all forms of AR signaling in CRPC tumors. Catalytic Topo II inhibitors might represent a novel class of AR signaling blocking reagents.

Chapter 4: Suppressed AR Signaling and CRPC Progression Utilizing Topo II Inhibitor

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4.1 Introduction

Despite frequently durable responses to ADT, progression to castration-resistant prostate cancer (CRPC) invariably occurs, most often driven by the reactivation of the AR pathway via mechanisms involving AR amplification, overexpression, mutations as well as intratumoral steroidogenesis [98, 204, 329]. Recently, more potent androgen receptor (AR) pathway inhibitors like abiraterone and enzalutamide (ENZ) improved survival in metastatic CRPC [180, 284]. However, resistance emerges even with the most potent AR pathway inhibitors [180, 284]. These observations emphasize that alternative approaches are required to thoroughly suppress AR signaling in patients with CRPC.

Genome-wide profiling studies demonstrated that the AR regulated transcriptome in CRPC is significantly different from that in ADT-naïve prostate cancers [285]. AR-activated genes in CRPC were dominated by cell cycle and mitosis genes such as UBE2C, CDC20 and CDK1 [285, 323]. These results suggest that targeting cell mitosis controlled by AR signaling in CRPC may inhibit tumor growth and progression more effectively.

The transcriptional activity of the AR requires DNA Topoisomerase II (Topo II) to be recruited to target promoters [41, 239, 324]. Topo II creates transient, but fixable DNA double strand breaks to relax the topology of the DNA, which is required for the AR to mediate transcription

initiation [239, 324]. Blocking Topo II expression or function impairs AR transcriptional activity and AR-driven cell proliferation [41]. Additionally, Topo II was also demonstrated to be responsible for gene fusion of TMPRSS2-ERG, one of the most common genomic alterations in prostate cancer [41, 324]. Increased expression of Topo II is associated with a higher Gleason score and relative ADT insensitivity of prostate tumors [240]. These findings suggest that inhibition of both Topo II and AR may cooperatively de-activate AR signaling and delay CRPC progression.

There are two types of Topo II inhibitors, Topo II poisons and catalytic inhibitors. Topo II poisons are exemplified by the cytotoxic chemotherapeutic, etoposide. When DNA breaks are created by Topo II, etoposide inhibits Topo II to re-ligate DNA breaks resulting in DNA damage. Such unfixable DNA damages cause cell cycle arrest and subsequently apoptosis [251, 325, 326]. Catalytic Topo II inhibitors are exemplified by ICRF187 and ICRF193, which target the catalytic domain of Topo II and prevent formation of DNA double strand breaks, resulting in unrelaxed DNA conformation[327]. Additionally, ICRF193 was reported to induce cell cycle arrest by inhibiting chromosome condensation and segregation at the M phase of the cell cycle, as Topo II is required to de-catenate the intertwined chromosomes [328]. Unlike Topo II poisons, catalytic inhibitors block Topo II activity without creating DNA breaks, and hence exhibit minimal cytotoxic effects. Together, these findings led us to hypothesize that catalytic Topo II inhibitors may block AR signaling and induce G_2/M cell cycle arrest in prostate cancer cells.

In this study, we demonstrate that catalytic Topo II inhibitors can block AR signaling and inhibit prostate cancer cell proliferation and CRPC growth, and provide proof-of-principle that co-

targeting Topo II in combination with AR pathway inhibitors may cooperatively de-activate the AR and delay CRPC progression.

4.2 Results

4.2.1 Topo II is required for the transcriptional activity of the AR

Two Topo II isoforms, alpha and beta, are widely expressed in prostate cancer cells [330]. We confirmed that Topo IIβ was highly expressed in our collection of human prostate cancer cell lines (Figure 4.1A). Androgen-independent LNCaP(AI) and LNCaP95 cell lines, as well as ENZ-resistant MR49F cell lines expressed higher levels of Topo IIβ than their parental LNCaP cells. Androgen treatment did not significantly alter Topo IIβ expression. In LNCaP cells, Topo IIβ silencing dramatically reduced mRNA levels of AR-regulated genes including PSA and TMPRSS2 (Figure 4.1B-C). These results were also confirmed in MR49F and LNCaP95 cells (Figure 4.1C). Reduced mRNA expression of PSA and TMPRSS2 by Topo II silencing was not due to reduced AR protein levels in these cells (Figure 4.1B).

ICRF187 and ICRF193 are catalytic inhibitors to both Topo II isoforms [331]. We treated prostate cancer cells with these inhibitors to determine their impacts on AR signaling by measuring AR-targeted transcription of PSA, TMPSS2 and FKBP5 genes (Figure 4.2A). Although LNCaP and LNCaP95 cells responded to 1 nM of R1881 by upregulating PSA, TMPSS2, and FKBP5 expression, both ICRF187 and ICRF193 caused 30-60% reduction in the mRNA levels of these genes. Similar results were also obtained in VCaP cells (Figure 4.2B). Moreover, ICRF187 and ICRF193 further inhibited PSA mRNA levels when LNCaP cells were

under 5 μM of ENZ (Figure 4.2A). Importantly, ICRF187 and ICRF193 suppressed PSA mRNA levels by 40-50% in ENZ-resistant MR49F cells (Figure 4.2D).

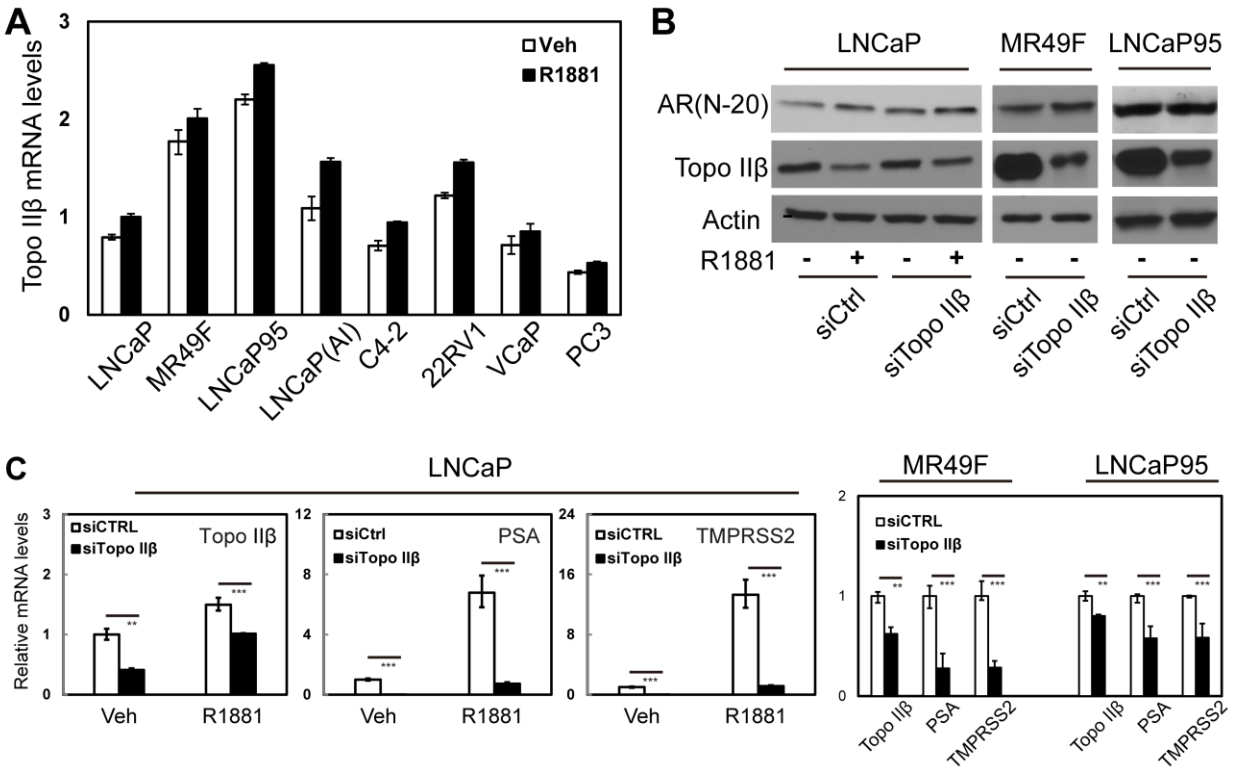


Figure 4.1 Blocking Topo II expression represses AR transcriptional activity.

(**A**) LNCaP, MR49F, LNCaP95, LNCaP(AI), C4-2, 22RV1, VCaP and PC3 cells were cultured in mediums containing 5% CSS for 48 hours and then treated with vehicle or 1 nM of R1881 for 24 hours. (**B-C**) Relative Topo IIβ mRNA levels to GAPDH were measured by real-time PCR. LNCaP, MR49F, and LNCaP95 cells were transfected with control or Topo IIβ siRNA, and then treated with vehicle or 1 nM of 1881 for 24 hours. AR and Topo IIβ proteins were detected by immunoblotting (**B**). (**C**) Relative mRNA levels of Topo IIβ, PSA and TMPRSS2 to GAPDH were measured by real-time PCR. Data represent mean ± SEM (n=3) with P<0.01 as ** and P<0.001 as *** (student's t-test).

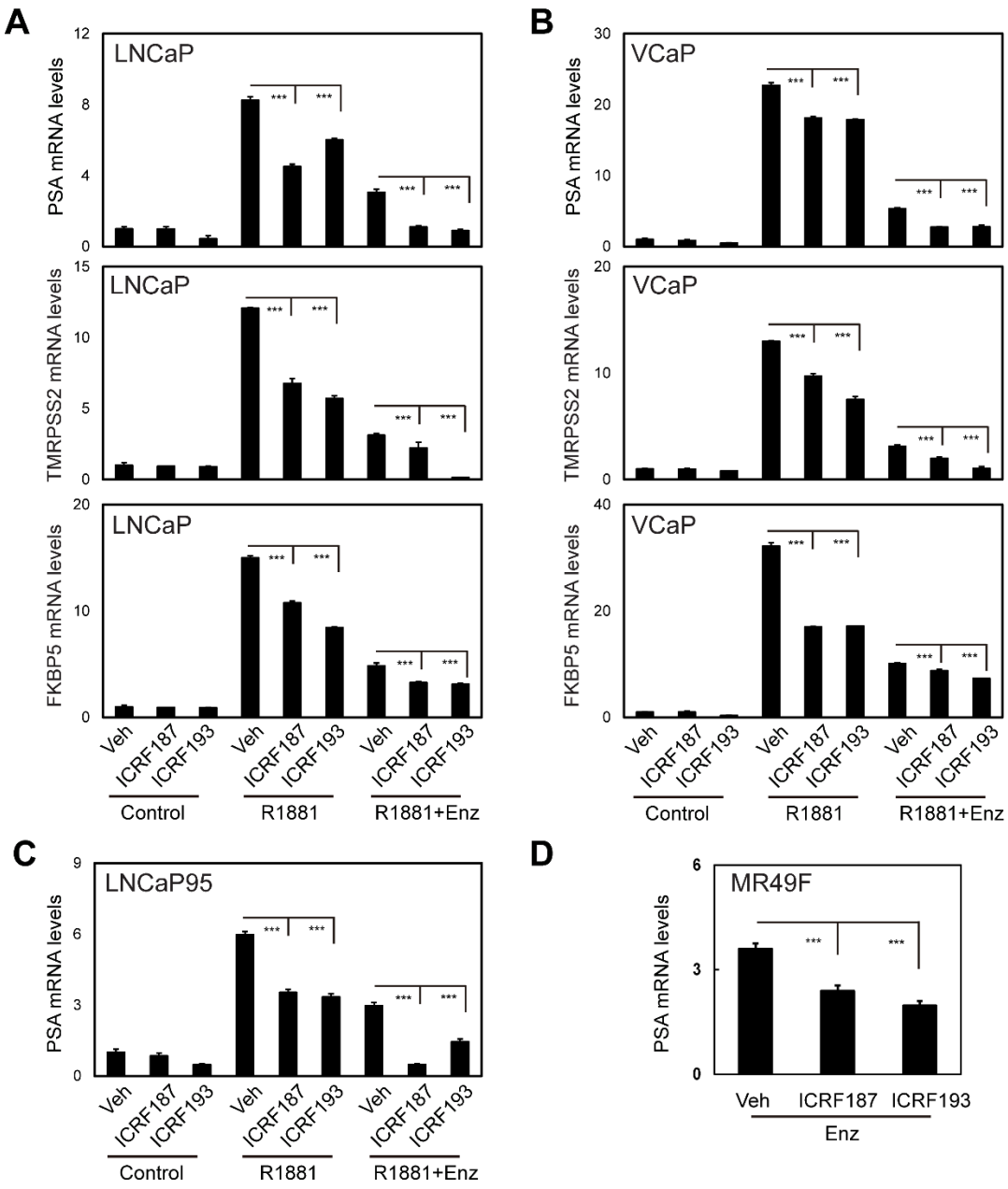


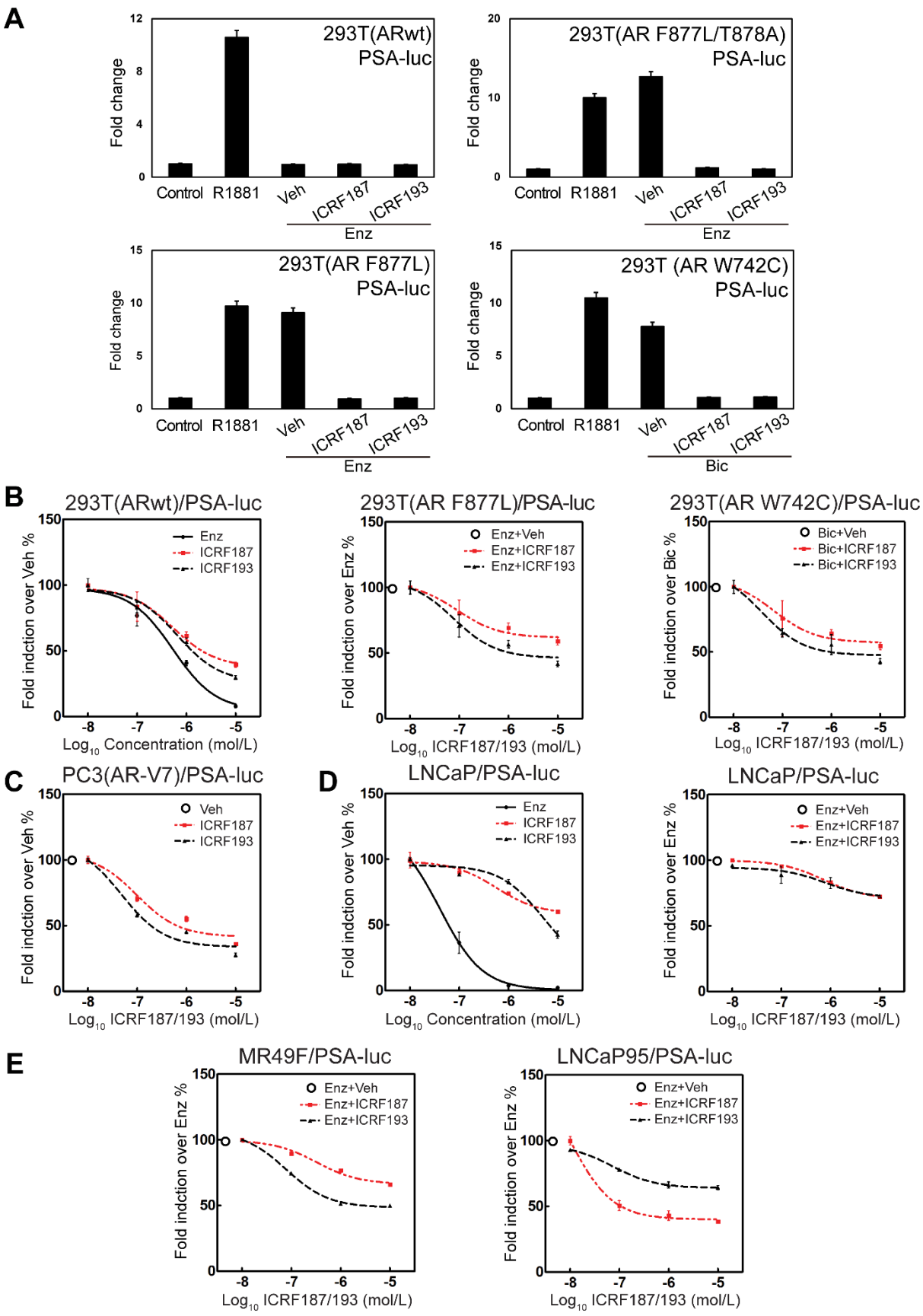
Figure 4.2 Inhibiting Topo II activity represses AR transcriptional activity.

(**A**) LNCaP, (**B**) VCaP, (**C**) LNCaP95 and (**D**) MR49F cells were cultured in medium containing 5% CSS. Cells were treated with vehicle, 1 nM of R1881 or 1 nM of R1881 plus 5 μM of ENZ for 24 hours. Cells were also co-treated with DMSO, 1 μM of ICRF187 or 1 μM of ICRF193 as indicated. Relative RNA levels of PSA, TMPSS2 and FKBP5 to GAPDH were measured by real-time PCR from three independent experiments. Data represent mean ± SEM (n=3) with $P<0.01$ as ** and $P<0.001$ as *** (student's t-test).

4.2.2 ICRF187 and ICRF193 block transcriptional activity of the AR, AR mutants, and AR-V7 in prostate cancer cells

We next tested the effect of catalytic Topo II inhibitors on the transcriptional activity of mutant AR and the AR-V7 splice variant. The AR carrying the F877L mutation can be transcriptionally activated by ENZ, while the W742C mutation can activate the AR by bicalutamide [201, 202]. In AR negative 293T cells, PSA-luciferase reporter activities driven by AR(F877L) or AR(F877L/T878A) in the presence of 10 μM of ENZ were significantly suppressed by ICRF187 or ICRF193 (Figure 4.3A). Similarly, ICRF187 and ICRF193 also inhibited the luciferase activity driven by AR(W742C) in the presence of bicalutamide. Additionally, luciferase activity driven by AR-V7 was also strongly repressed by ICRF187 and ICRF193 dose-dependently in AR negative PC3 cells (Figure 4.3C). When LNCaP cells were transfected with the PSA-luciferase reporter and treated with 1 nM of R1881 plus 0.01-10 μM of ENZ, ICRF187 or ICRF193, we observed that ENZ potently suppressed the transcriptional activity of AR-FL. ICRF87 and ICRF193 also showed dose-dependent inhibition of luciferase activity but to milder extents (Figure 4.3D). However, when LNCaP cells were co-treated with 5 μM of ENZ plus 0.01-10 μM of either ICRF187 or ICRF193, a further 20% deduction in luciferase activity was observed. These results suggest that Topo II inhibitors can increase the efficacy of ENZ in blocking the AR function. In ENZ-resistant MR49F, ICRF187 and ICRF193 resulted in 40% and 60% decrease in luciferase activity respectively (Figure 4.3E). LNCaP95 cells express not only full length AR, but also AR splice variants including AR-V7 and AR_{v567ed}. Co-treatment of ICRF187 or ICRF193 with 5 μM of ENZ induced a further 60% decrease in luciferase activity driven by the AR splice variants in LNCaP95 cells (Figure 4.3E). Interestingly, several other catalytic inhibitors of Topo II including merbarone [332], aclacinomycin A [333] and genistein

[325] all showed suppressive impacts to AR signaling in prostate cancer cells (Figure 4.4). By contrast, the Topo II poison, etoposide, increased AR transactivation dose-dependently (Figure 4.4). Together, these results demonstrate that catalytic Topo II inhibitors can repress AR transcriptional activity in prostate cancer cells.


A
293T(ARwt) PSA-luc
293T(AR F877L/T878A) PSA-luc
293T(AR F877L) PSA-luc
293T (AR W742C) PSA-luc
Fold change
Control R1881 Veh ICRF187 ICRF193
Enz
Bic
B
293T(ARwt)/PSA-luc
293T(AR F877L)/PSA-luc
293T(AR W742C)/PSA-luc
Fold indction over Veh %
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Enz
Enz+Veh
Bic+Veh
ICRF187
Enz+ICRF187
Bic+ICRF187
ICRF193
Enz+ICRF193
Bic+ICRF193
Log10 Concentration (mol/L)
Log10 ICRF187/193 (mol/L)
C
PC3(AR-V7)/PSA-luc
Veh
D
LNCaP/PSA-luc
E
MR49F/PSA-luc
LNCaP95/PSA-luc

Figure 4.3 Topo II catalytic inhibitors suppress AR mutant and AR-V7 transcriptional activities.

(**A**) 293T cells were transfected with a PSA-luciferase reporter plus expression plasmids encoding the wild type AR, AR(F877L), AR(W742C) or AR-V7. Cells were treated with DMSO, 1 nM of R1881, 10 μM of ENZ or bicalutamide, 10 μM of ENZ or bicalutamide plus 1 μM of ICRF187 or ICRF193 for 24 hours. (**B**) 293T cells were cultured in medium containing 10% FBS, transfected with wild type AR and treated with vehicle, 0.01-10 μM of ICRF187, ICRF193 or ENZ. 293T cells transfected with AR(F877L) were treated with 10 μM of ENZ plus vehicle, 0.01-10 μM of ICRF187 or ICRF193. 293T cells transfected with AR(W742C) were treated with 10 μM of bicalutamide plus vehicle, 0.01-10 μM of ICRF187 or ICRF193. (**C**) PC3 cells transfected with AR-V7 were treated with vehicle, 0.01-10 μM of ICRF187 or ICRF193. (**D**) LNCaP cells were transfected with a PSA-luciferase reporter and treated with 1 nM of R1881. Cells were also treated with 0.01-10 μM of ENZ, ICRF187 or ICRF193 (left panel). Cells were treated with 5 μM of ENZ plus 0.01-10 μM of ICRF187 or ICRF193 (right panel). (**E**) MR49F and LNCaP95 cells were transfected with a PSA-luciferase reporter. MR49F cells were treated with 10 μM of ENZ plus 0.01-10 μM of ICRF187 or ICRF193, while LNCaP95 cells were treated with 5 μM of ENZ plus 0.01-10 μM of ICRF187 or ICRF193 for 24 hours. Relative luciferase activities were calibrated with renilla from three independent experiments and were presented as mean ± SEM (n=3). Values from vehicle treatment were set as 100%.

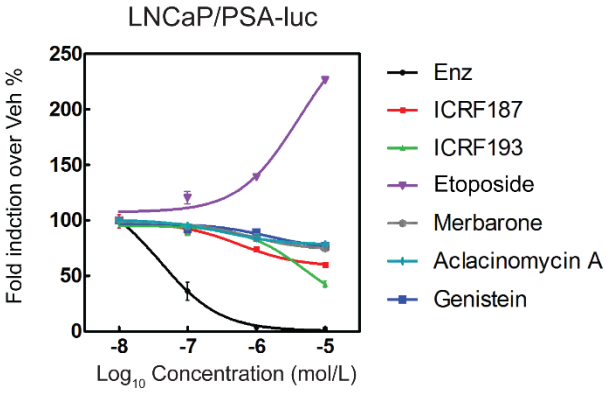

Figure 4.4 Catalytic inhibitors of Topo II show a suppressive impact on AR signaling.

LNCaP cells were transfected with a PSA-luciferase reporter and treated with 1 nM of R1881. Cells were also treated with vehicle or 0.01-10 μM of ENZ, ICRF187, ICRF193, etoposide, merbarone, aclacinomycin A or

genistein for 24 hours. Relative luciferase activities were calibrated with renilla from three independent experiments and were presented as mean ± SEM (n=3). Values from vehicle treatment were set as 100%.

4.2.3 ICRF187 and ICRF193 impair DNA binding and nuclear localization of the AR

To define mechanisms by which Topo II inhibitors repress AR transactivation, we performed ChIP assays (Figure 4.5A). Within 2 hours of R1881 treatment, the AR was recruited to the androgen-responsive elements in PSA and TMPRSS2 promoters. However, ICRF187 or ICRF193 resulted in 30-50% reduction of AR recruitment. These changes were not due to decreased AR protein levels within the 2-hour treatment. However, co-treatment of ICRF187 or ICRF193 with ENZ for 24 hours resulted in greater deduction in AR protein levels when compared with ENZ treatment alone. LNCaP cells expressing GFP-AR were next used to study the effects of ENZ and Topo II inhibitors on subcellular localization of AR-FL. As expected, R1881 induced, while 10 μM of ENZ blocked, nuclear localization of AR-FL (Figure 4.5B). Nuclear localization of AR-FL was reduced by 1 μM of ICRF187 or ICRF193, comparable with that of ENZ. In addition, we also study subcellular localizations of AR mutants and AR-V7 under catalytic Topo II inhibitor treatment by Western blotting assays (Figure 4.5C-D). 293T cells were transfected with plasmids of wild type AR, AR(F877L), AR(W742C) or AR-V7 and then treated with vehicle, ICRF187, or ICRF193 in the presence of 10 nM of R1881, 10 μM of ENZ or 10 μM of bicalutamide. ICRF187 and ICRF193 reduced protein levels of wild type AR, AR(F877L), AR(W742C) in the nuclear extracts, but increased their protein levels in cytosol fractions. However, AR-V7 protein was primarily localized in nuclear fraction. Together, these results suggest that Topo II catalytic inhibitors suppress AR recruitment to its target promoters and reduce AR protein nuclear localization.

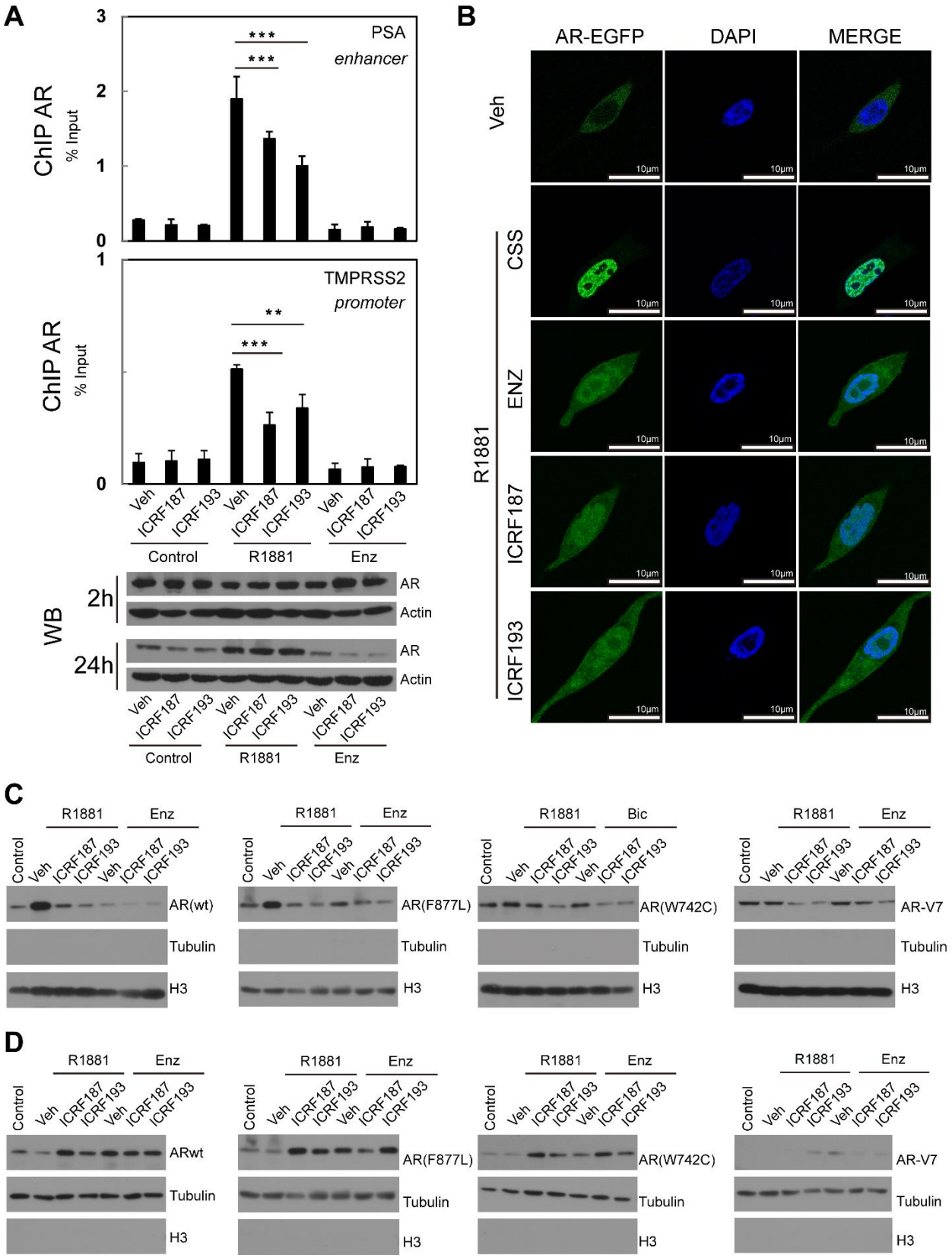

Figure 4.5 ICRF187 and ICRF193 inhibit AR recruitment to target promoters and AR nuclear localization.

(**A**) LNCaP cells were cultured in RPMI1640 medium containing 5% CSS and treated with vehicle, 1 μM of ICRF187 or 1 μM of ICRF193 in addition to vehicle, 10 nM of R1881 or 10 μM of ENZ treatment for 2 hours. Three independent ChIP experiments were performed using the AR antibody. Precipitated DNA fragment were used as templates to amplify the PSA enhancer and the TMPRSS2 promoter by real-time PCR. Data represented mean ± SEM (n=3) and plotted as percentage of input. $P < 0.01$ ** and $P < 0.001$ as *** (student's t-test). AR protein levels under 2 and 24-hour treatment were detected by Western blotting. (**B**) LNCaP cells expressing EGFP-AR were cultured in RPMI1640 medium containing 5% CSS. Cells were treated with vehicle, 10 nM of R1881, 10 nM of R1881 plus 10 μM of ENZ, 10 nM of R1881 plus 1 μM of ICRF187, or 10 nM of R1881 plus 1 μM of ICRF193 for 6 hours. Cells were then fixed with 4% paraformaldehyde and mounted with DAPI. Representative confocal microscopic images showed AR localization (Green) and nucleus (Blue). (**C**-**D**) 293T cells were transfected with plasmids encoding wild type AR, AR(F877L), AR(W742C) and AR-V7. Cells were treated with vehicle, 1 μM of ICRF187 or 1 μM of ICRF193 in addition to 10 nM of R1881, 10 μM of ENZ or 10 μM of bicalutamide for 24 hours. Nuclear (**C**) and cytosol (**D**) protein extracts were immunoblotted with AR, tubulin and Histone H3 antibodies. Three independent experiments were performed and one set of Western blotting images are presented.

4.2.4 ICRF187 and ICRF193 suppress prostate cancer cell growth and delay cell cycling at the G_2/M phase

MTS assays were used to study the effects of catalytic Topo II inhibitors on prostate cancer cell growth. Parental LNCaP cell growth was similarly inhibited by ENZ, ICRF187 or ICRF193 under androgen deprived conditions (Figure 4.6A). By contrast, ICRF187 and ICRF193, but not ENZ, suppressed LNCaP95, MR49F cell, and 22Rv1 cell growth rates. Additionally, neither ENZ nor ICRF187/ICRF193 had suppressive impacts on cell proliferation of AR negative PC3 and DU145 cells. We next performed FACS assays to study the effects of ICRF187 and ICRF193 on cell cycling of prostate cancer cells. The cell cycling of LNCaP and LNCaP95 cells were first synchronized at the G_0/G_1 stage by serum starving and then released by adding medium containing 10% serum. We observed that neither ICRF187 nor ICRF193 altered cell

population distributions during cell cycling (Figure 4.6B). However, when cell cycle was synchronized at the G_2/M phases by nocodazole and then released, ICRF187 and ICRF193 caused significant delays for the cells passing through the G_2/M and entering into the G_1 phase (Figure 4.6C). These results indicate that ICRF187 and ICRF193 inhibited cancer cell proliferation through impeding cell cycling.

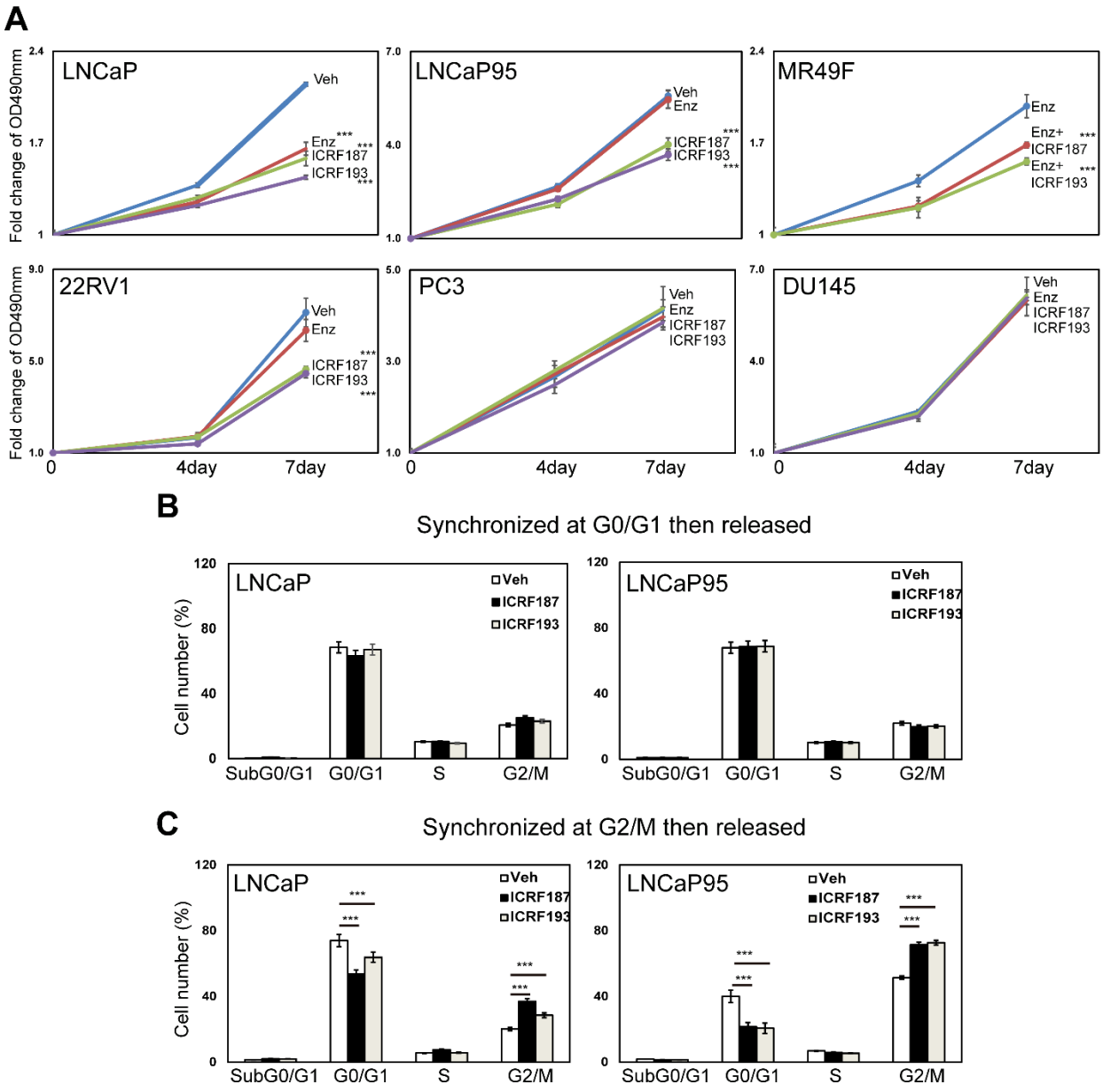

Figure 4.6 ICRF187 and ICRF193 inhibit prostate cancer cell growth and delay cell cycling in the G_2/M phase.

(**A**) LNCaP, LNCaP95, MR49F, 22RV1, PC3 and DU145 cells were cultured in mediums containing 5% CSS for 48 hours. Cells were treated with vehicle, ENZ, 10 μM of ICRF187 or 1 μM of ICRF193 for 0-7 days. The dose of ENZ was 1 μM in LNCaP, 5 μM in LNCaP95 and 22RV1 cells, and 10 μM in MR49F cells. MTS assays measured the relative cell growth rates to day 0. Results were from three independent experiments (n=6/repeat). (**B**) LNCaP and LNCaP95 cells were serum starved for 12 hours and then replenished with culture medium containing serum. Treatments of vehicle, 10 μM of ICRF187 or 2 μM of ICRF193 were also applied to LNCaP cells for 1.5 hours or to LNCaP95 cells for 2 hours. (**C**) LNCaP and LNCaP95 cells were cultured in growth medium containing 100 ng/ml nocodazole in addition to vehicle, 10 μM of ICRF187 or 2 μM of ICRF193 for 12 hours. Cells were then replenished with nocodazole medium containing vehicle, 10 μM of ICRF187 or 2 μM of ICRF193 for LNCaP cells for 1.5 hours or for LNCaP95 cells 2 hours. Cells were collected and used for FACS assays to determine cell populations at G_0/G_1, S and G_2/M phases (**B-C**). Results were repeated from two independent experiments (n=3/repeat). One-way ANOVA followed by student t-test was performed with $P < 0.001$ as ***.

4.2.5 ICRF187 inhibited CRPC xenograft tumor growth

The inhibitory effects of ICRF187 were tested in four CRPC xenograft models. After 8 weeks of treatment of CRPC LNCaP tumors, 10 mg/kg daily of ENZ reduced tumor growth by 45%, compared to 24% reduction by 50 mg/kg daily of ICRF187 (Figure 4.7A). However, combinational treatment using lower doses of ENZ (5 mg/kg) and ICRF187 (25 mg/kg) reduced tumor volume by 64%. Similar changes in serum PSA levels were also observed. The expression of AR-targeted genes including PSA, TMPRSS2 and UBE2C as well as the tumor proliferation index Ki67 were more strongly inhibited by ENZ plus ICRF187 (Figure 4.8). ICRF187 inhibited ENZ-resistant MR49F xenograft growth and PSA secretion dose-dependently (Figure 4.7B). ICRF187 suppressed AR-regulated gene expression and Ki67 index (Figure 4.8). Additionally, 50 mg/kg of ICRF187 inhibited CRPC 22RV1 but not AR negative PC3 xenograft growth

(Figure 4.7C-D). These results demonstrate that ICRF187 can enhance the effects of ENZ in ENZ-sensitive LNCaP CRPC xenografts. It can also inhibit ENZ-resistant CRPC xenograft growth as monotherapy.

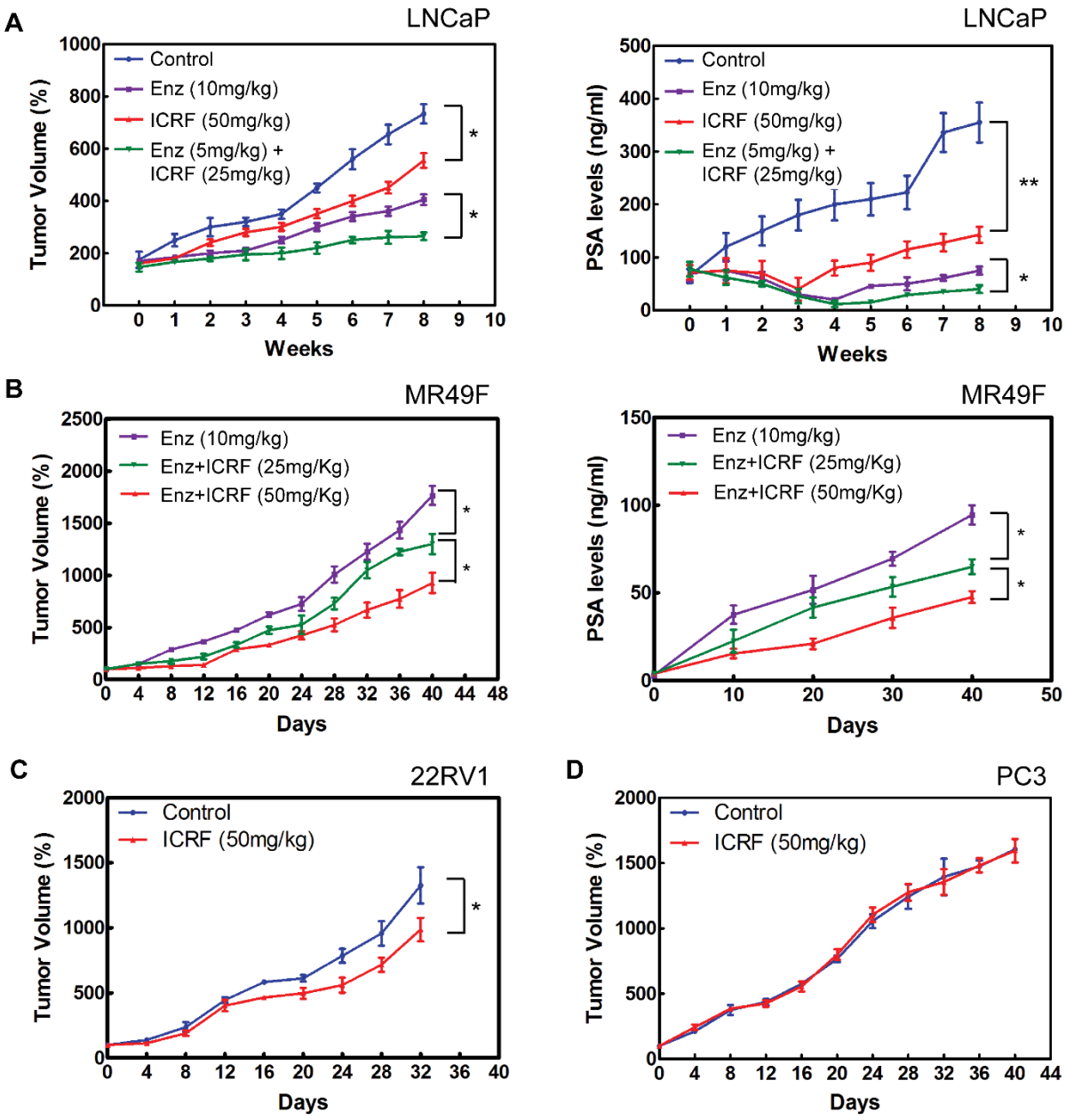

Figure 4.7 ICRF187 inhibits CRPC growth of human prostate cancer xenografts.

(**A**) CRPC LNCaP xenografts were treated with vehicle, 10 mg/kg ENZ, 50 mg/kg ICRF187 or 5 mg/kg ENZ plus 25 mg/kg ICRF187 (n=7/group). Tumor volume and serum PSA levels were measured weekly. (**B**) Mice bearing ENZ-resistant MR49F tumors were randomly divided into three groups (n=9/group) and treated with 10 mg/kg ENZ, 10 mg/kg ENZ plus 25 mg/kg ICRF187, or 10 mg/kg ENZ plus 50 mg/kg ICRF187. Tumor volume and serum PSA levels were measured. (**C**) CRPC 22RV1 and (**D**) PC3 xenografts were treated with control or 50 mg/kg or ICRF187. Tumor volumes were measured. Statistical analyses were performed by one-way ANOVA followed by student t-test with $P < 0.05$ as * and $P < 0.01$ as **.

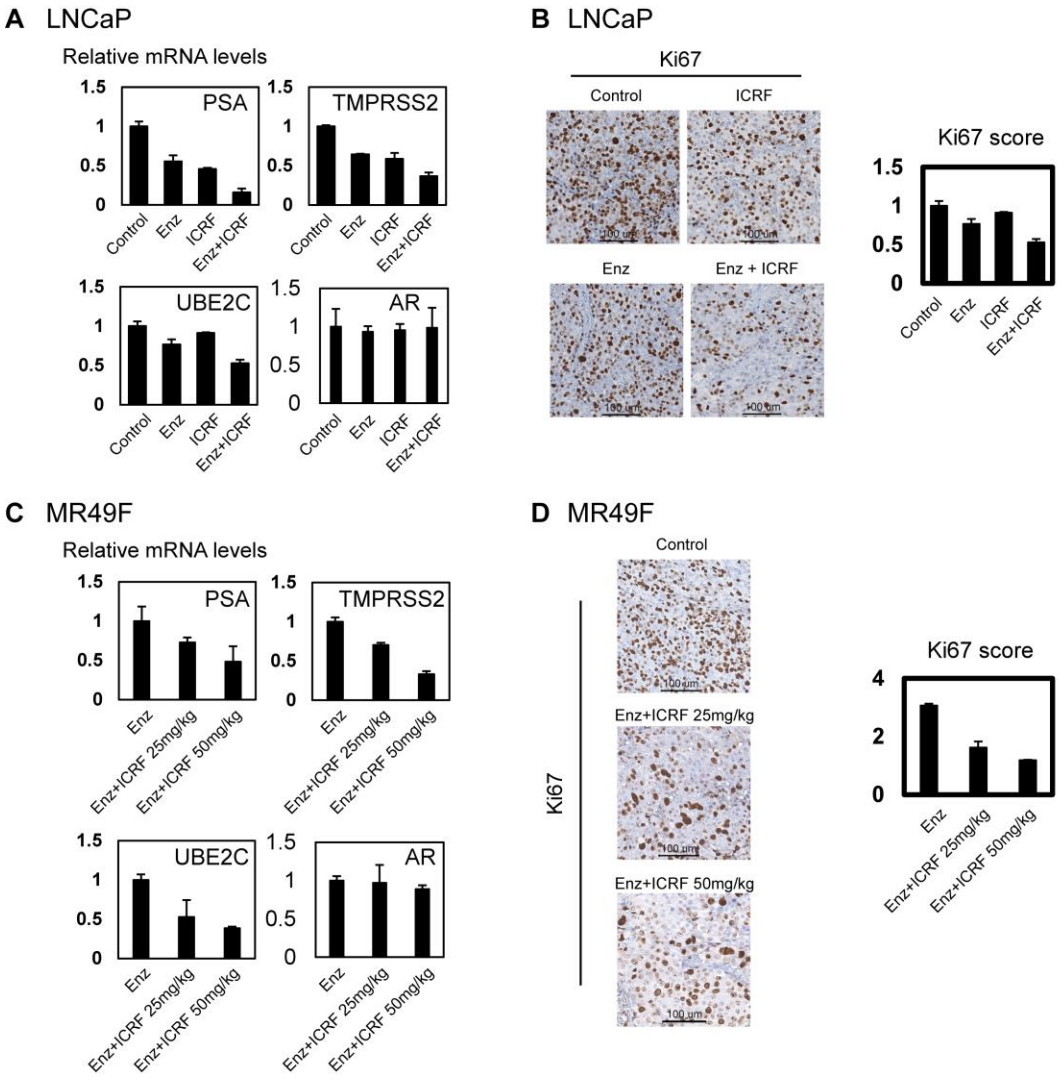

Figure 4.8 Relative mRNA levels from xenograft tissues and Ki67 IHC staining.

(**A**) PSA, TMPRSS2, UBE2C and AR mRNA levels from CRPC LNCaP xenografts treated with ENZ and or ICRF187 were measured by real-time PCR. (**B**) Ki67 histology score was determined. (**C**) PSA, TMPRSS2, UBE2C and AR mRNA levels from ENZ-resistant MR49F xenografts treated with ENZ and or ICRF187 were measured by real-time PCR. (**D**) Ki67 histology score was determined.

4.3 Discussion

Although it is well established that AR-mediated transcription initiation requires Topo II to create DNA double strand breaks at the target promoters, this fundamental knowledge has not yet been translated into effective therapies to inhibit AR signaling in CRPC patients. Our study indicated that catalytic Topo II inhibitors can block both the transcriptional activity of the AR and prostate cancer cell mitosis. While re-activation of AR signaling and mitosis of cancer cells are two major features of CRPC, our study identifies catalytic Topo II inhibitors as a potential co-targeting approach in combination with AR pathway inhibitors in CRPC.

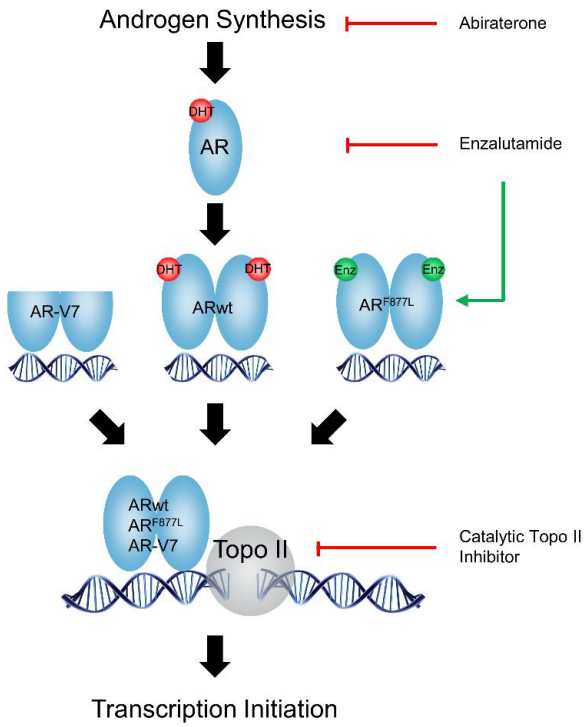

Figure 4.9 The mechanisms by which catalytic Topo II inhibitors and anti-AR agents block the AR pathway in prostate cancer cells.

ADT and newer AR pathway inhibitors such as abiraterone and ENZ aim to retain the AR in its transcriptionally inactive state, by either inhibiting androgen synthesis or antagonizing the ligand binding domain (LBD) of the AR. However, these drugs induce resistance via adaptive changes in the AR genome, including AR amplification, mutations, and constitutively active AR splice variants [201, 202, 321]. For example, ENZ and ARN-509 promote accumulation of the F877L point mutation in the AR, which converts antagonist to agonist function and confers anti-AR resistance [201, 202]. These studies highlight that targeting the LBD of the AR will have limited success due to activation of adaptive survival pathways that compromise the effectiveness of AR blockade. Our studies present an alternative strategy, whereby targeting AR-mediated gene transcription initiation may enhance castration therapy regardless of the transactivation status of the AR, AR mutants, or AR splice variants (Figure 4.9). Using the ENZ-resistant MR49F and androgen-independent LNCaP95 cell models, we show that ICRF187 and ICRF913 potently inhibit AR signaling (Figures 4.1-4.5), cancer cell proliferation (Figure 4.6), and CRPC xenograft growth (Figure 4.7). Catalytic Topo II inhibitors may therefore represent a novel class of non-LBD inhibitors of AR signaling, which may be beneficial for CRPC patients.

In contrast to AR pathway inhibitors, catalytic Topo II inhibitors target not only AR-mediated transcription initiation, but also suppress cancer cell mitosis. Since a downstream effector of re-activated AR signaling in CRPC tumors is acceleration of tumor cell mitosis [285, 323], targeting both cancer cell cycling and AR transcriptional activity may more strongly inhibit CRPC growth. In support of this hypothesis, inhibitors to cyclin-dependent kinases are effective

in delaying ENZ-resistance cancer cell growth [202]. Our results further indicated that co-treatment of Topo II inhibitors with ENZ results in stronger suppression of AR signaling (Figure 4.1-4.5), CRPC cell growth, and tumor growth (Figure 4.6-4.7).

To investigate the specificity of the catalytic Topo II inhibitor towards AR-directed transcription, we have also address the impact of catalytic Topo II inhibitors on transcription factors other than the AR. Blocking Topo II expression or function can also repress the transactivation of the estrogen receptor [239]. We measured E2-induced ERE-luciferase, P4-induced progesterone receptor element (PRE)-luciferase, and TNFα-induced NFkB-luciferase activity inhibited by ICRF187 and ICRF193 (Figure 4.10). We measured several house-keeping genes by real-time PCR and did not observe suppressive effects by ICRF187 and ICRF193 (Figure 2.2, Chapter 2). The effectiveness of the inhibitory effects to specific transcription factors is also cell-context dependent. Catalytic inhibitors would be more effective in targeting AR signaling in prostate cancer cells. AR negative PC3 and DU145 cells were included in this study, Topo II inhibitors did not suppress their cell proliferation rates (Figure 4.6).

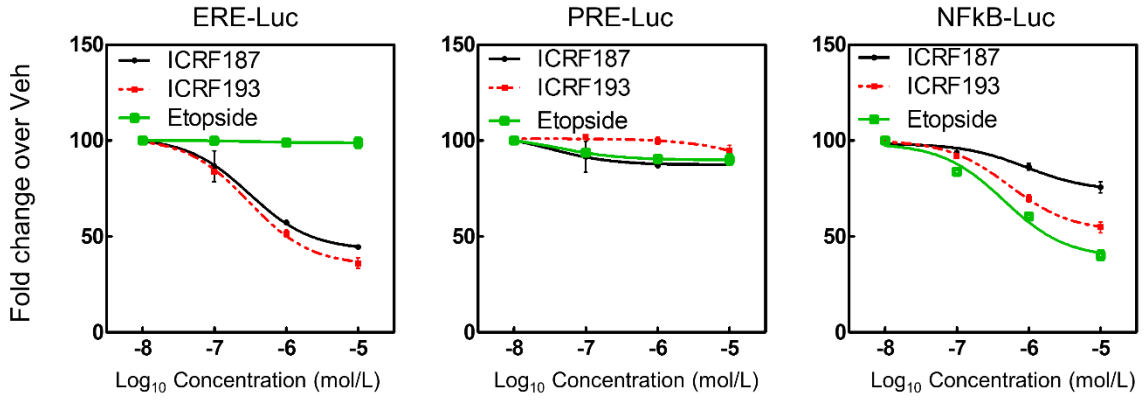

Figure 4.10 ICRF187 and ICRF193 inhibits the ERE-luciferase and NFkB-luciferase activities.

293T cells were transiently transfected with an ERα/3xERE-luc, PRβ/3xPRE-luc (provided by Ms. Ning Xie), or NFkB-luciferase reporter (Invitrogen). Cells were then treated with either control, 10 nM E2, 10 nM P4, or 100 ng/ml TNF-α (BD Biosciences) plus 0.01-10 μM of ICRF187, ICRF193, or etopside for 6 hours. Luciferase activities were calibrated with renilla from three independent experiments and presented as mean ± SEM (n=3). Values from vehicle treatment were set as 1.

The knowledge that Topo II is required for AR transcriptional activity may lead to two possible therapeutic strategies for prostate cancer patients. One possibility is to utilize super physiological doses of androgen in combination with Topo II poisons, as proposed previously [334]. This strategy hypothesized that the combination of supraphysiologic androgen and etoposide would produce DSBs and stabilize them, subsequently promote CRPC cell death [335]. However, due to the presence of Topo II poisons, AR-induced DNA double strand breaks cannot be fixed, thereby leading to G_2/M cell cycle arrest, followed by apoptotic cell death. The potential risk would be that surviving cells could accumulate complex genomic rearrangement, adaptive DNA repair systems, and greater heterogeneity. Additionally, AR transcriptional activity is enhanced by Topo II poisons (Figure 4.4). The other possible therapeutic strategy is applying AR pathway inhibitors with catalytic Topo II inhibitors to repress AR activation and AR-mediated transcription initiation. Since catalytic topo II inhibitors prevent DNA double strand breaks, they also cause cell cycle delay at the G_2/M phases by interfering with chromosome condensation and segregation during mitosis, and have a lower chance to induce further genomic rearrangement.

In summary, we demonstrated that catalytic Topo II inhibitors can block AR signaling and inhibit tumor growth of castration-resistant xenografts, identifying catalytic Topo II inhibitors as potentially novel drugs to treat patients with CRPC.

Chapter 5: Conclusions

5.1 Summary of findings

The lack of an effective treatment for lethal CRPC remains as a major unmet clinical need. Existing therapies such as ARPIs only serve to delay the inevitable disease progression, as resistance towards these agents will typically occur shortly following treatment. The increased ability of cancer cells to reestablish AR signaling driven tumor progression is a fundamental mechanism for treatment resistance.

The overall objective of this doctoral study was to investigate the roles of androgen-independent AR signaling, stimulated by the androgen catabolizing enzyme UGT2B17, and to assess the therapeutic efficacy of blocking AR-mediated transcription initiation using catalytic Topo II inhibitor. The main hypotheses are as follows: (1) UGT2B17 plays a functional role in promoting the androgen-independent AR signaling in CRPC, and (2) targeting of AR-mediated transcription initiation through catalytic Topo II inhibitor can suppress CRPC tumor growth.

In chapter 3, we first evaluated the UGT2B17 protein expression levels by IHC on Vancouver Prostate Centre tissue microarrays. We showed 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.

In chapter 4, for the purpose of exploring novel strategies in blocking the AR mediated transcription initiation, we began with treating CRPC and ENZ-resistant prostate cancer cells with increasing doses of catalytic Topo II inhibitors, ICRF187 and ICRF193. Luciferase reporter assays showed that both inhibitors suppressed the transcriptional activities of wild-type AR, mutant AR (F877L and W742C), and AR-V7 splice variant. ICRF187 and ICRF193 decreased AR recruitment to its target promoters and reduced AR nuclear localization. Both ICRF187 and ICRF193 inhibited androgen-dependent and ENZ-resistant cancer cell proliferation, and delayed cell cycling at the G_2/M stages. In castration-resistant LNCaP tumors, ICRF187 inhibited tumor growth, PSA secretion and the expression of several other AR-regulated genes. These repressive effects became stronger when tumors were co-treated with ENZ. Additionally, ICRF187 effectively inhibited tumor growth of ENZ-resistant MR49F as well as castration-resistant 22RV1 xenografts. Catalytic Topo II inhibitors can block AR signaling and inhibit tumor growth of castration-resistant xenografts, suggesting their potential applications in treating castration-resistant cancers in patients.

5.2 Strengths and limitations

Overall, this thesis work represented the first analysis functionally linking UGT2B17 to the reestablishment of AR signaling and established a proof-of-principle to block the reestablished

AR signaling through targeting Topoisomerase II. Our research began with the validation of the clinical relevance of UGT2B17, in addition to its functional importance in the stimulation of c-Src activating androgen-independent AR transcription, followed by design and validation of the blocking of AR-mediated transcription initiation through catalytic Topo II inhibitors, eventually tested preclinical treatment efficacy in multiple models. The results from the *in vivo* treatment tests (using CRPC and ENZ-resistant xenograft models), serve to further corroborate the initial hypothesis, which described a significant role of androgen-independent AR signaling in promoting PCa.

To investigate the AR signaling transformation by UGT2B17 in CRPC, we included 604 TMA cores cohorts in our study (Chapter 2). More PCa specimens from the tumor bank would further strengthen the findings from Chapter 3.2.1 on UGT2B17. Beyond basic IHC staining, the Duolink in situ PLA assay [336] for detecting the c-Src and UGT2B17 interaction directly on tumor tissue TMAs will be instrumental to enhance the clinical relevance of our conclusions. From the bioinformatics perspective, we have explored the databases from the cBioportal (http://www.cbioportal.org/) [337, 338], Oncomine (https://www.oncomine.org/) [339] and GEO databases [340]. While Topo II overexpression is strongly associated with poor prognosis, little information about UGT2B17 is existed, mainly due to the non-specific UGT2B17 probe on the previous Microarray ChIP. The accurate RNA-seq databases will provide us with the platform to integrate the UGT2B17 profiling with clinical prognosis. Also, analysis of RNA-seq databases from patient-derived xenograft (PDX) models will further provide more clinically relevant xenograft models for functional analysis and pre-clinical drug development [232].

There are multiple PCa cell line models to evaluate UGT2B17 functions and catalytic Topo II inhibitors. While gain-of-function of UGT2B17 was thoroughly investigated, significant shRNA or siRNA knockdown of UGT2B17 was not yet achieved. Going forward, we may apply the CRISPR-Cas9 system [341] to generate the UGT2B17 knockout in PCa cell lines such as LNCaP95 or MR49F cells. The impact of loss-of-function UGT2B17 on the androgen sensitivity, cellular function and molecular signaling of PCa cells will then be evaluated. Results from this technique would extend our *in vivo* xenograft model findings. The application of the genome editing technique combining with targeting delivery strategy will certainly broaden our understanding in clinical therapeutics [342].

The LNCaP(UTG2B17) tumor showed a faster growth speed compared to LNCaP(mock) in the CRPC stage (Chapter 3.2.3). Whether or not this enhanced growth speed is related to the resistance of xenograft models to anti-androgens warrants further investigation. We could test the tumor formation rates of LNCaP(UGT2B17) after prolonged androgen deprivation conditions in pre-castrated mice. Treating the established CRPC LNCaP(UGT2B17) tumors with ENZ would characterize the *in vivo* drug resistance regulated by UGT2B17. Analysis of collected tissues and primary cultures would help improve the understanding of the molecular mechanisms of the resistance. Moreover, c-Src is suggested to upregulate UGT2B15 glucuronidation activity through tyrosine phosphorylation [343]. Elucidating the protein interaction structure of c-Src/UGT2B17 would greatly improve our current understanding of the mutual positive feedback between c-Src and UGT2B17. These would also contribute to the interaction disruption strategy discussed in chapter 3.4. Since c-Src is also revealed to interact with the AF1 domain of the AR,

the activation of AR-V7 should be also considered. More specific AR-V7 downstream gene luciferase assays such as UBE2C-Luc should be included in future studies [279].

The alternative way to block all forms of AR-mediated transcription is through the use of catalytic inhibitors, which achieved effective results. Yet there are several defects of current catalytic Topo II inhibitors that prevent them from becoming effective anticancer drugs. In addition to their metabolic instability, they have serious off-target and toxic effects [344-347]. For example, bisdioxopiperazines can be metabolized into ion chelating agents [344]. Merbarone can also bind to and inhibit other types of Topoisomerases [345, 346]. These off-target effects often lead to low anti-cancer effects, but serious toxicity, as observed during pre-clinical studies [347]. Even though several new catalytic Topo II inhibitors (e.g. NSC35866 and TSC24) were discovered lately, their activities are only effective at micro molar level to inhibit Topo II activity, indicating insufficient potency [348, 349]. Thus, a more potent and less cytotoxic effect catalytic Topo II inhibitor is needed for the ideal treatment of CRPC.

5.3 Overall significance

This study has identified a novel function for UGT2B17 that promotes ligand-independent AR signaling to expedite CRPC progression and indicated that catalytic Topo II inhibitors can block both the transcriptional activity of AR and prostate cancer cell mitosis. We demonstrated that elevated UGT2B17, under conditions of prolonged androgen deprivation, activates c-Src kinase and stimulates AR transactivation independent of androgens. The androgen-independent AR activation, including AR mutations and AR splicing variants, could be blocked by catalytic Topo II inhibitors. In short, we found a novel UGT2B17-Src-AR signaling axis, and their terminal

blocker catalytic Topo II inhibitor, as a novel therapeutic ARPI. This work may lead to the future clinical development of novel catalytic Topo II inhibitors, particularly for the treatment of late stage CRPC patients. In addition, since Topo II has been found to be highly up-regulated in numerous other cancers, these proof-of-principle findings may be useful in the development of therapies for cancer types other than prostate cancer.

The application of catalytic Topo II inhibitor not only blocked full-length AR, but current ARPI-resistant AR mutations and AR splicing variants could serve as an alternative solution to treat drug resistant tumors. Future drug screening work will bridge our established proof-of-principle data in tackling AR signaling, and lead to the development of drug candidates with improved potency against Topo II that thoroughly block the activities of all forms of AR protein in our established PCa cell and xenograft models. Such leads may further be tested for pharmacodynamics and pharmacokinetic behavior, and will be integrated with our existing academia-based drug development program. Since the emergence of castration resistance is the lethal end stage of the disease, we anticipate that the proposed research will eventually have a substantial impact on patient survival.

5.4 Ongoing and future research directions

To overcome the above mentioned Topo II inhibitors' defects, we propose employing structure-based drug design approaches to discover more potent, more selective, and more bioavailable drugs targeting the Topo II catalytic domain. Fortunately, the protein structures of the catalytic domain of Topo II (PDB: 4J3N & 1PVG) were solved. Since we have established a high-throughput screening system (Figure 5.2D), prostate cancer cell assays, CRPC xenograft models,

as well as an effective computer-aid drug design pipeline at the Vancouver Prostate Centre (VPC), we intend to identify and design next-generation catalytic Topo II inhibitors to thoroughly suppress the activities of all AR isoforms and their regulated mitosis transcriptomes in CRPC.

Through the iterative synergy between biochemical testing, synthesis, and computational chemistry, we will discover and optimize prototype catalytic inhibitors of Topo II to completely abolish AR signaling in CRPC (Figure 5.1).

Our ongoing and future directions are:

1. To identify new catalytic Topo II inhibitors using *in silico* and high-throughput screening systems.
2. To confirm the inhibitory impacts of lead compounds on the AR signaling in PCa cell models.
3. To evaluate the suppressive effects of optimized lead compounds in PCa xenograft models.

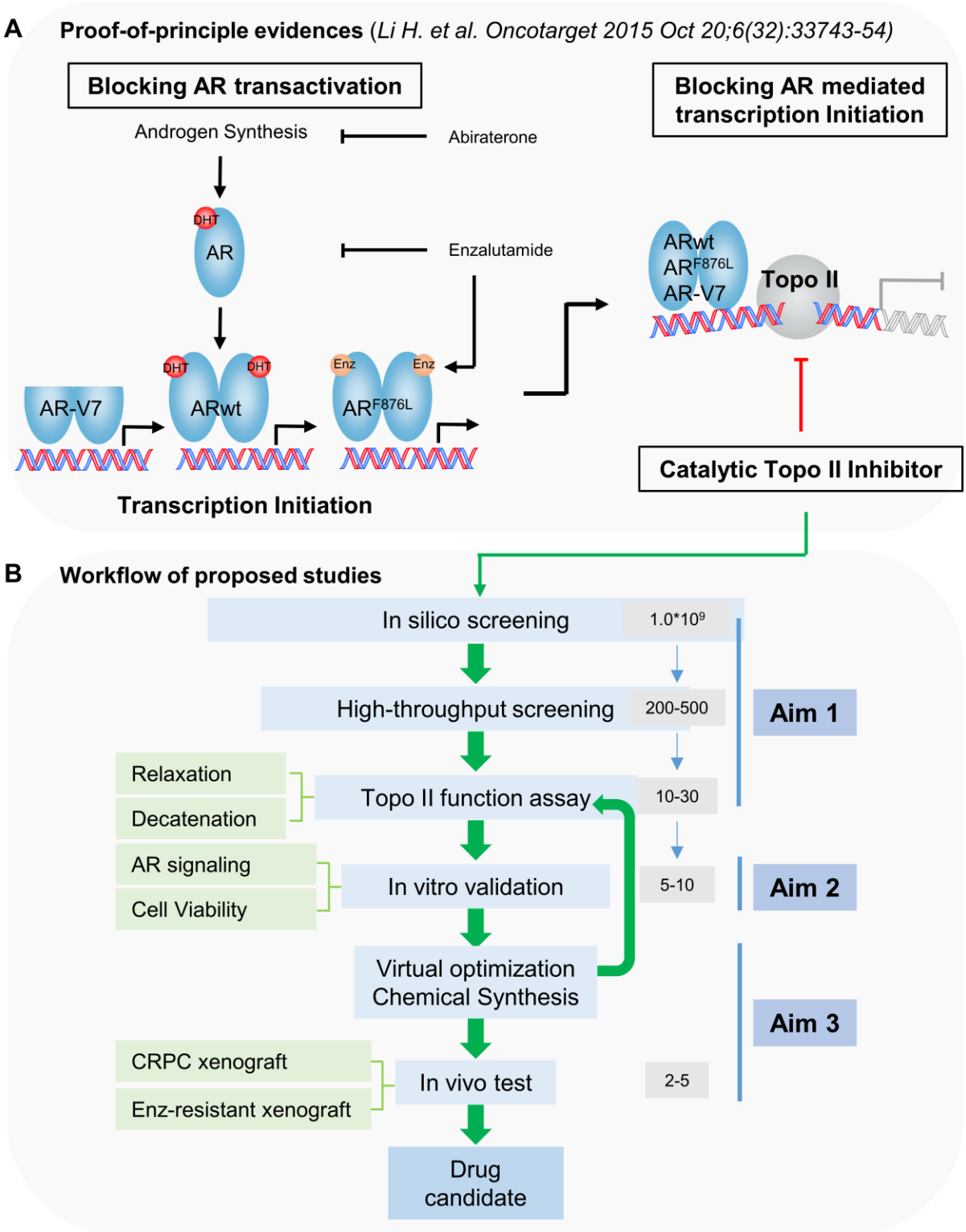

Figure 5.1 Workflow of future study.

(A) The mechanisms by which catalytic Topo II inhibitors and anti-AR agents block AR pathways in prostate cancer cells. (B) An illustration of the proposed specific aims 1-3.

5.4.1 High-throughput Screening System

In Silico Screening: Topo II DNA binding motifs (PDB: 4J3N & 1PVG) [350, 351] have been set in the docking platform Glide (collaboration with Dr. Cherkasov) for screening compounds from the ZINC database [182, 352, 353]. After filtration by docking score and physicochemical properties, top-ranked structures will be clustered through the fingerprint method. We have 50 candidates from 1st round screening. A final selection of 200-500 chemicals will be purchased.

Fluorescence anisotropy-based assay: We will use our established fluorescence anisotropy-based assay for high-throughput screening of the 200-500 compounds identified by *in silico* screening (Figure 5.2D). In this assay, the FITC labeled oligonucleotide 5'-TTCTTCTTC-3' detection probe preferentially binds the relaxed double-strand plasmids containing the triplex forming $(TTC)_9$ sequence in contrast to supercoiled plasmids. Control or increasing doses of identified lead compounds will be added to a 384-well-plate-based reaction system containing human Topo II enzyme and supercoiled triplex forming plasmids. Once the reactions stop, fluorescence anisotropy will be determined using Tecan infinite F500. Using this assay, we showed that ICRF187 reduced Topo II mediated fluorescence anisotropy values dose-dependently, approving that this high-throughput assay was ready to screen for potential catalytic Topo II inhibitors (Figure 5.2D).

Topo II relaxation and decatenation assays: Top 10-30 ranked chemicals from the high-throughput experiments will be tested by standard Topo II activity assays using commercial kits

(Figure 5.2E-F). Supercoiled plasmids and catenated kinetoplast DNA will be incubated with Topo II enzyme in the present of control or increasing doses of lead compounds. Substrate and product DNA molecules will then be separated by gel electrophoresis. The efficiency of DNA relaxation and decatenation will be determined by band analysis using Image J software.

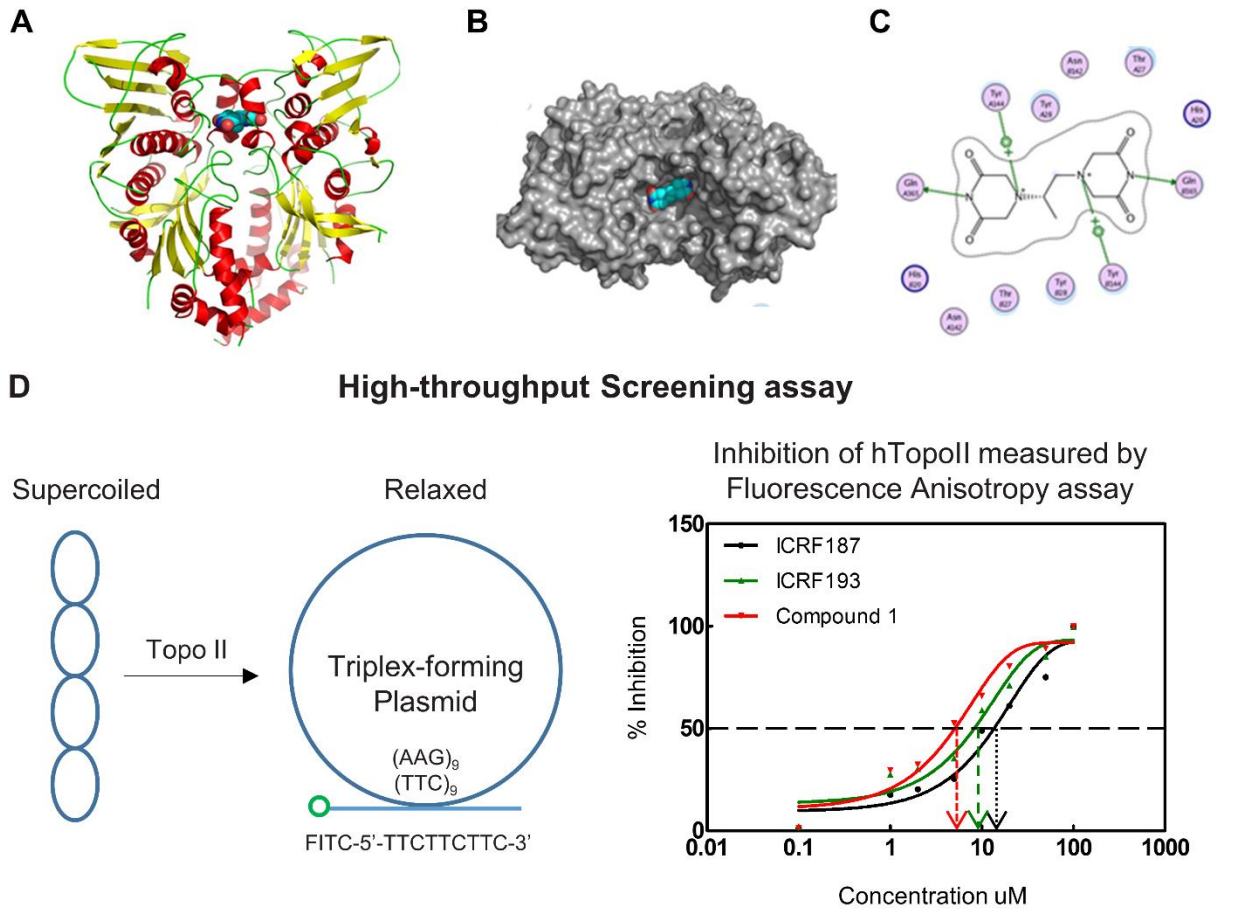

Figure 5.2 *In silico* and *in vitro* screening systems.

(A) Cartoon representation showing the ternary structure of topoisomerase II. The ligand, ICRF187, is shown in cyan color. (B) The binding site of ICRF-187, as revealed by X-ray crystallography (PDB ID: 1QZR). The structure shows that the ligand binds to a dimer interface between two ATPase protomers of topoisomerase II. (C) A network

of interactions is formed between topoisomerase II and ICRF187. Interactions are shown in green color. (D) High-throughput screening. Left: the mechanism of High-throughput screening assay. FITC labeled oligonucleotide 5'-TTCTTCTTC-3' detection probes preferentially bind the relaxed double-strand plasmids containing the triplex forming $(TTC)_9$ sequence compared to supercoiled ones. Right: 10 ng supercoiled pUC19-TFO triplex-forming plasmids were incubated with 0.6 µl of DMSO or 0.1-100 µM ICRF187, ICRF193 or Compound 1 in the presence of Topo II enzyme, and reaction buffers perform in 384 well plate (Total volume 30ul). Reactions were stopped after 30 minutes, followed by incubation with detection probe for 1 hour, fluorescence anisotropy was measured by Tecan infinite F500 (Excitation 485 nm, Emission 535 nm). The inhibition percentages were calibrated to the values from the Topo II group. (E) Relaxation assay was performed by treating 25 ng supercoiled pUC19 plasmids with vehicle, 10 µM of etopside, ICRF187 or Compound 1 in the presence of 5U Topo II enzyme and reaction buffer. EcoR I digested plasmids were used to indicate the linear form. Gel electrophoresis image was shown with EB staining. (F) Decatenation assay was carried out using kDNA-incubated 5U Topo II enzyme in the presence of vehicle, 10 µM etoposide, ICRF187, or Compound 1. Linear kDNA was also loaded; gel electrophoresis image was shown with EB staining.

5.4.2 Multiple PCa cell line models

Identified lead compounds will then be tested for their suppressive effects to AR signaling using our established assays in multiple PCa cell models described in Chapter 4.

AR transcriptional activities: Prostate cancer lines (LNCaP, LNCaP95, VCaP and MR49F) with known expression profile of AR, AR-Vs and mutant ARs will be treated with control or increasing doses of lead compounds under the conditions of vehicle, 1 nM of R1881 and or 10 µM of enzalutamide treatment (Chapter 4.2.2). Expression levels of AR targeted genes including PSA, TMPRSS2 and FKBP5 will be measured by real-time PCR. PSA-luciferase reporter assays will also be performed to measure AR transcriptional activity.

AR Recruitment to Target Promoters: LNCaP cells will be treated with control or lead compounds under the conditions of vehicle, R1881 and/or enzalutamide treatment. ChIP assays will be performed using the AR antibody to detect the occupancy of the AR onto PSA and TMPRSS2 promoters (Chapter 4.2.3).

AR Cellular Localization: LNCaP cells expressing GFP tagged AR, AR(F877L) or AR-V7 will be treated with control or lead compounds under the conditions of vehicle, R1881 and/or enzalutamide. Fluorescence image will be captured with confocal microscope (Chapter 4.2.3). To quantify the nuclear and cytosol distribution, 293T cells transfected wild type AR, AR (F877L) or AR-V7 and treated with control or lead compounds. Protein extractions from nuclear and cytosol will be immunoblotted with AR, tubulin and Histone H3 antibodies.

Cell viability: To assess the effects of lead compounds on cell viability, we will perform MTS assays in AR positive (specific) and AR negative (non-specific) cell lines. Compounds will be administered at a concentration of 0-50 μM for 0-8 days followed by evaluation for MTS cell viability as we reported in Chapter 4.2.4.

Cell cycling: To study the impacts of lead compounds on prostate cancer cell mitosis, fluorescence-activated cell sorting (FACS) assays will be used. Cells will be synchronized at the G_2/M phases by nocodazole and then released with growing medium containing vehicle or lead compounds. Fixed cells will be stained with propidium iodide and analyzed by BD FACS Canto

II flow cytometer as we shown in Chapter 4.2.4. Mitosis-index will be calculated with the method described in previous report [354].

5.4.3 Multiple PCa xenograft models

Optimization: Derivatives of the lead compounds will be re-analyzed to achieve enhanced affinity.

Toxicity test: Increasing doses of lead compounds will be exposed to 10 mice/group for 2 months, body weight, activity score, and Kaplan-Meier survival plots will be estimated.

PCa Xenografts: The most promising inhibitors will ultimately be evaluated in LNCaP, LNCaP95, MR49F and 22Rv1xenografts and patient derived xenografts. AR negative PC3 and DU145 xenografts will be used as controls. We will also carry out direct testing of synergistic or additive AR inhibitory effects with existing ARPIs for the most promising synthetic candidates.

5.4.4 Expectations and pitfalls

Targeting the DNA binding motifs of Topo II is a novel strategy. We have also prepared the ATPase and conformation transforming domains as the alternative target sites. Effects of the selected compounds on other transcription factors including ERα, PRβ, GR, NFkB and AP1 will also be determined to guarantee drug specificity to AR. AR negative prostate cancer cells, lacking the AR-driven mitosis, will also be used to control for the off-target toxicity.

5.4.5 Significance

Our proposed project will bridge our established proof-of-principle data in tackling AR signaling to the development of potent drug candidates thoroughly blocking the Topo II-mediated AR transcription initiation. Further pharmaceutics analysis will be integrated with our academia-based drug development program and eventually benefit patient survival.

Bibliography

1. Ferlay, J., I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, and F. Bray, *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012.* Int J Cancer, **2015**. 136(5): p. E359-86.
2. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2016.* CA Cancer J Clin, **2016**. 66(1): p. 7-30.
3. Torre, L.A., R.L. Siegel, E.M. Ward, and A. Jemal, *Global Cancer Incidence and Mortality Rates and Trends--An Update.* Cancer Epidemiol Biomarkers Prev, **2016**. 25(1): p. 16-27.
4. Resnick, M.J., T. Koyama, K.H. Fan, P.C. Albertsen, M. Goodman, A.S. Hamilton, R.M. Hoffman, A.L. Potosky, J.L. Stanford, A.M. Stroup, R.L. Van Horn, and D.F. Penson, *Long-term functional outcomes after treatment for localized prostate cancer.* N Engl J Med, **2013**. 368(5): p. 436-45.
5. Bubendorf, L., A. Schopfer, U. Wagner, G. Sauter, H. Moch, N. Willi, T.C. Gasser, and M.J. Mihatsch, *Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients.* Hum Pathol, **2000**. 31(5): p. 578-83.
6. Schroder, F., E.D. Crawford, K. Axcrona, H. Payne, and T.E. Keane, *Androgen deprivation therapy: past, present and future.* BJU Int, **2012**. 109 Suppl 6: p. 1-12.
7. Feldman, B.J. and D. Feldman, *The development of androgen-independent prostate cancer.* Nat Rev Cancer, **2001**. 1(1): p. 34-45.
8. Wyatt, A.W. and M.E. Gleave, *Targeting the adaptive molecular landscape of castration-resistant prostate cancer.* EMBO Mol Med, **2015**. 7(7): p. 878-94.
9. Ryan, C.J., M.R. Smith, J.S. de Bono, A. Molina, C.J. Logothetis, P. de Souza, K. Fizazi, P. Mainwaring, J.M. Piulats, S. Ng, J. Carles, P.F. Mulders, E. Basch, E.J. Small, F. Saad, D. Schrijvers, H. Van Poppel, S.D. Mukherjee, H. Suttmann, W.R. Gerritsen, T.W. Flaig, D.J. George, E.Y. Yu, E. Efstathiou, A. Pantuck, E. Winquist, C.S. Higano, M.E. Taplin, Y. Park, T. Kheoh, T. Griffin, H.I. Scher, D.E. Rathkopf, and C.-A.-. Investigators, *Abiraterone in metastatic prostate cancer without previous chemotherapy.* N Engl J Med, **2013**. 368(2): p. 138-48.
10. Beer, T.M., A.J. Armstrong, D.E. Rathkopf, Y. Loriot, C.N. Sternberg, C.S. Higano, P. Iversen, S. Bhattacharya, J. Carles, S. Chowdhury, I.D. Davis, J.S. de Bono, C.P. Evans, K. Fizazi, A.M. Joshua, C.S. Kim, G. Kimura, P. Mainwaring, H. Mansbach, K. Miller, S.B. Noonberg, F. Perabo, D. Phung, F. Saad, H.I. Scher, M.E. Taplin, P.M. Venner, B. Tombal, and P. Investigators, *Enzalutamide in metastatic prostate cancer before chemotherapy.* N Engl J Med, **2014**. 371(5): p. 424-33.
11. Marker, P.C., A.A. Donjacour, R. Dahiya, and G.R. Cunha, *Hormonal, cellular, and molecular control of prostatic development.* Dev Biol, **2003**. 253(2): p. 165-74.
12. Hayward, S.W. and G.R. Cunha, *The prostate: development and physiology.* Radiol Clin North Am, **2000**. 38(1): p. 1-14.
13. Peehl, D.M. and J.S. Rubin, *Keratinocyte growth factor: an androgen-regulated mediator of stromal-epithelial interactions in the prostate.* World J Urol, **1995**. 13(5): p. 312-7.
14. Gerdes, M.J., T.D. Dang, M. Larsen, and D.R. Rowley, *Transforming growth factor-beta1 induces nuclear to cytoplasmic distribution of androgen receptor and inhibits androgen response in prostate smooth muscle cells.* Endocrinology, **1998**. 139(8): p. 3569-77.

15. Wang, Y., S. Hayward, M. Cao, K. Thayer, and G. Cunha, *Cell differentiation lineage in the prostate.* Differentiation, **2001**. 68(4-5): p. 270-9.
16. Hayward, S.W., L.S. Baskin, P.C. Haughney, B.A. Foster, A.R. Cunha, R. Dahiya, G.S. Prins, and G.R. Cunha, *Stromal development in the ventral prostate, anterior prostate and seminal vesicle of the rat.* Acta Anat (Basel), **1996**. 155(2): p. 94-103.
17. Cunha, G.R., A.A. Donjacour, P.S. Cooke, S. Mee, R.M. Bigsby, S.J. Higgins, and Y. Sugimura, *The endocrinology and developmental biology of the prostate.* Endocr Rev, **1987**. 8(3): p. 338-62.
18. McNeal, J.E., *Anatomy of the prostate: an historical survey of divergent views.* Prostate, **1980**. 1(1): p. 3-13.
19. McNeal, J.E., *The zonal anatomy of the prostate.* Prostate, **1981**. 2(1): p. 35-49.
20. McNeal, J.E., *Origin and development of carcinoma in the prostate.* Cancer, **1969**. 23(1): p. 24-34.
21. McNeal, J.E., *Normal histology of the prostate.* Am J Surg Pathol, **1988**. 12(8): p. 619-33.
22. Abate-Shen, C. and M.M. Shen, *Molecular genetics of prostate cancer.* Genes Dev, **2000**. 14(19): p. 2410-34.
23. Elzanaty, S., J. Erenpreiss, and C. Becker, *Seminal plasma albumin: origin and relation to the male reproductive parameters.* Andrologia, **2007**. 39(2): p. 60-5.
24. Armbruster, D.A., *Prostate-specific antigen: biochemistry, analytical methods, and clinical application.* Clin Chem, **1993**. 39(2): p. 181-95.
25. Brinkmann, A.O., *Molecular mechanisms of androgen action--a historical perspective.* Methods Mol Biol, **2011**. 776: p. 3-24.
26. Heidenreich, A., P.J. Bastian, J. Bellmunt, M. Bolla, S. Joniau, T. van der Kwast, M. Mason, V. Matveev, T. Wiegel, F. Zattoni, N. Mottet, and U. European Association of, *EAU guidelines on prostate cancer. part 1: screening, diagnosis, and local treatment with curative intent-update 2013.* Eur Urol, **2014**. 65(1): p. 124-37.
27. Welch, H.G. and P.C. Albertsen, *Prostate cancer diagnosis and treatment after the introduction of prostate-specific antigen screening: 1986-2005.* J Natl Cancer Inst, **2009**. 101(19): p. 1325-9.
28. Kumar, B., S. Koul, L. Khandrika, R.B. Meacham, and H.K. Koul, *Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype.* Cancer Res, **2008**. 68(6): p. 1777-85.
29. Elkahwaji, J.E., R.J. Hauke, and C.M. Brawner, *Chronic bacterial inflammation induces prostatic intraepithelial neoplasia in mouse prostate.* Br J Cancer, **2009**. 101(10): p. 1740-8.
30. De Marzo, A.M., E.A. Platz, S. Sutcliffe, J. Xu, H. Gronberg, C.G. Drake, Y. Nakai, W.B. Isaacs, and W.G. Nelson, *Inflammation in prostate carcinogenesis.* Nat Rev Cancer, **2007**. 7(4): p. 256-69.
31. Cano, P., A. Godoy, R. Escamilla, R. Dhir, and S.A. Onate, *Stromal-epithelial cell interactions and androgen receptor-coregulator recruitment is altered in the tissue microenvironment of prostate cancer.* Cancer Res, **2007**. 67(2): p. 511-9.
32. Balic, I., S.T. Graham, D.A. Troyer, B.A. Higgins, B.H. Pollock, T.L. Johnson-Pais, I.M. Thompson, and R.J. Leach, *Androgen receptor length polymorphism associated with prostate cancer risk in Hispanic men.* J Urol, **2002**. 168(5): p. 2245-8.

33. Sun, J.H. and S.A. Lee, *Association between CAG repeat polymorphisms and the risk of prostate cancer: a meta-analysis by race, study design and the number of (CAG)n repeat polymorphisms.* Int J Mol Med, **2013**. 32(5): p. 1195-203.
34. Julin, B., A. Wolk, J.E. Johansson, S.O. Andersson, O. Andren, and A. Akesson, *Dietary cadmium exposure and prostate cancer incidence: a population-based prospective cohort study.* Br J Cancer, **2012**. 107(5): p. 895-900.
35. Castro, E., C. Goh, D. Olmos, E. Saunders, D. Leongamornlert, M. Tymrakiewicz, N. Mahmud, T. Dadaev, K. Govindasami, M. Guy, E. Sawyer, R. Wilkinson, A. Ardern-Jones, S. Ellis, D. Frost, S. Peock, D.G. Evans, M. Tischkowitz, T. Cole, R. Davidson, D. Eccles, C. Brewer, F. Douglas, M.E. Porteous, A. Donaldson, H. Dorkins, L. Izatt, J. Cook, S. Hodgson, M.J. Kennedy, L.E. Side, J. Eason, A. Murray, A.C. Antoniou, D.F. Easton, Z. Kote-Jarai, and R. Eeles, *Germline BRCA mutations are associated with higher risk of nodal involvement, distant metastasis, and poor survival outcomes in prostate cancer.* J Clin Oncol, **2013**. 31(14): p. 1748-57.
36. Masko, E.M., E.H. Allott, and S.J. Freedland, *The relationship between nutrition and prostate cancer: is more always better?* Eur Urol, **2013**. 63(5): p. 810-20.
37. Lapuk, A.V., C. Wu, A.W. Wyatt, A. McPherson, B.J. McConeghy, S. Brahmbhatt, F. Mo, A. Zoubeidi, S. Anderson, R.H. Bell, A. Haegert, R. Shukin, Y. Wang, L. Fazli, A. Hurtado-Coll, E.C. Jones, F. Hach, F. Hormozdiari, I. Hajirasouliha, P.C. Boutros, R.G. Bristow, Y. Zhao, M.A. Marra, A. Fanjul, C.A. Maher, A.M. Chinnaiyan, M.A. Rubin, H. Beltran, S.C. Sahinalp, M.E. Gleave, S.V. Volik, and C.C. Collins, *From sequence to molecular pathology, and a mechanism driving the neuroendocrine phenotype in prostate cancer.* J Pathol, **2012**. 227(3): p. 286-97.
38. Barbieri, C.E. and S.A. Tomlins, *The prostate cancer genome: perspectives and potential.* Urol Oncol, **2014**. 32(1): p. 53 e15-22.
39. Tomlins, S.A., D.R. Rhodes, S. Perner, S.M. Dhanasekaran, R. Mehra, X.W. Sun, S. Varambally, X. Cao, J. Tchinda, R. Kuefer, C. Lee, J.E. Montie, R.B. Shah, K.J. Pienta, M.A. Rubin, and A.M. Chinnaiyan, *Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer.* Science, **2005**. 310(5748): p. 644-8.
40. Tomlins, S.A., S.M. Aubin, J. Siddiqui, R.J. Lonigro, L. Sefton-Miller, S. Miick, S. Williamsen, P. Hodge, J. Meinke, A. Blase, Y. Penabella, J.R. Day, R. Varambally, B. Han, D. Wood, L. Wang, M.G. Sanda, M.A. Rubin, D.R. Rhodes, B. Hollenbeck, K. Sakamoto, J.L. Silberstein, Y. Fradet, J.B. Amberson, S. Meyers, N. Palanisamy, H. Rittenhouse, J.T. Wei, J. Groskopf, and A.M. Chinnaiyan, *Urine TMPRSS2:ERG fusion transcript stratifies prostate cancer risk in men with elevated serum PSA.* Sci Transl Med, **2011**. 3(94): p. 94ra72.
41. Haffner, M.C., M.J. Aryee, A. Toubaji, D.M. Esopi, R. Albadine, B. Gurel, W.B. Isaacs, G.S. Bova, W. Liu, J. Xu, A.K. Meeker, G. Netto, A.M. De Marzo, W.G. Nelson, and S. Yegnasubramanian, *Androgen-induced TOP2B-mediated double-strand breaks and prostate cancer gene rearrangements.* Nat Genet, **2010**. 42(8): p. 668-75.
42. Barbieri, C.E., S.C. Baca, M.S. Lawrence, F. Demichelis, M. Blattner, J.P. Theurillat, T.A. White, P. Stojanov, E. Van Allen, N. Stransky, E. Nickerson, S.S. Chae, G. Boysen, D. Auclair, R.C. Onofrio, K. Park, N. Kitabayashi, T.Y. MacDonald, K. Sheikh, T. Vuong, C. Guiducci, K. Cibulskis, A. Sivachenko, S.L. Carter, G. Saksena, D. Voet, W.M. Hussain, A.H. Ramos, W. Winckler, M.C. Redman, K. Ardlie, A.K. Tewari, J.M. Mosquera, N. Rupp, P.J. Wild, H. Moch, C. Morrissey, P.S. Nelson, P.W. Kantoff, S.B.

Gabriel, T.R. Golub, M. Meyerson, E.S. Lander, G. Getz, M.A. Rubin, and L.A. Garraway, *Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer.* Nat Genet, **2012**. 44(6): p. 685-9.
43. Rubin, M.A., C.A. Maher, and A.M. Chinnaiyan, *Common gene rearrangements in prostate cancer.* J Clin Oncol, **2011**. 29(27): p. 3659-68.
44. Liu, W., J. Lindberg, G. Sui, J. Luo, L. Egevad, T. Li, C. Xie, M. Wan, S.T. Kim, Z. Wang, A.R. Turner, Z. Zhang, J. Feng, Y. Yan, J. Sun, G.S. Bova, C.M. Ewing, G. Yan, M. Gielzak, S.D. Cramer, R.L. Vessella, S.L. Zheng, H. Gronberg, W.B. Isaacs, and J. Xu, *Identification of novel CHD1-associated collaborative alterations of genomic structure and functional assessment of CHD1 in prostate cancer.* Oncogene, **2012**. 31(35): p. 3939-48.
45. Palanisamy, N., B. Ateeq, S. Kalyana-Sundaram, D. Pflueger, K. Ramnarayanan, S. Shankar, B. Han, Q. Cao, X. Cao, K. Suleman, C. Kumar-Sinha, S.M. Dhanasekaran, Y.B. Chen, R. Esgueva, S. Banerjee, C.J. LaFargue, J. Siddiqui, F. Demichelis, P. Moeller, T.A. Bismar, R. Kuefer, D.R. Fullen, T.M. Johnson, J.K. Greenson, T.J. Giordano, P. Tan, S.A. Tomlins, S. Varambally, M.A. Rubin, C.A. Maher, and A.M. Chinnaiyan, *Rearrangements of the RAF kinase pathway in prostate cancer, gastric cancer and melanoma.* Nat Med, **2010**. 16(7): p. 793-8.
46. Lam, E.W., J.J. Brosens, A.R. Gomes, and C.Y. Koo, *Forkhead box proteins: tuning forks for transcriptional harmony.* Nat Rev Cancer, **2013**. 13(7): p. 482-95.
47. Barbieri, C.E., C.H. Bangma, A. Bjartell, J.W. Catto, Z. Culig, H. Gronberg, J. Luo, T. Visakorpi, and M.A. Rubin, *The mutational landscape of prostate cancer.* Eur Urol, **2013**. 64(4): p. 567-76.
48. Cancer Genome Atlas Research, N., *The Molecular Taxonomy of Primary Prostate Cancer.* Cell, **2015**. 163(4): p. 1011-25.
49. Bethel, C.R., D. Faith, X. Li, B. Guan, J.L. Hicks, F. Lan, R.B. Jenkins, C.J. Bieberich, and A.M. De Marzo, *Decreased NKX3.1 protein expression in focal prostatic atrophy, prostatic intraepithelial neoplasia, and adenocarcinoma: association with gleason score and chromosome 8p deletion.* Cancer Res, **2006**. 66(22): p. 10683-90.
50. Carver, B.S., C. Chapinski, J. Wongvipat, H. Hieronymus, Y. Chen, S. Chandarlapaty, V.K. Arora, C. Le, J. Koutcher, H. Scher, P.T. Scardino, N. Rosen, and C.L. Sawyers, *Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer.* Cancer Cell, **2011**. 19(5): p. 575-86.
51. Gundem, G., P. Van Loo, B. Kremeyer, L.B. Alexandrov, J.M. Tubio, E. Papaemmanuil, D.S. Brewer, H.M. Kallio, G. Hognas, M. Annala, K. Kivinummi, V. Goody, C. Latimer, S. O'Meara, K.J. Dawson, W. Isaacs, M.R. Emmert-Buck, M. Nykter, C. Foster, Z. Kote-Jarai, D. Easton, H.C. Whitaker, I.P.U. Group, D.E. Neal, C.S. Cooper, R.A. Eeles, T. Visakorpi, P.J. Campbell, U. McDermott, D.C. Wedge, and G.S. Bova, *The evolutionary history of lethal metastatic prostate cancer.* Nature, **2015**. 520(7547): p. 353-7.
52. Birtle, A.J., A. Freeman, J.R. Masters, H.A. Payne, S.J. Harland, and B.S.o.O.C. Registry, *Clinical features of patients who present with metastatic prostate carcinoma and serum prostate-specific antigen (PSA) levels < 10 ng/mL: the "PSA negative" patients.* Cancer, **2003**. 98(11): p. 2362-7.
53. Lilja, H., D. Ulmert, and A.J. Vickers, *Prostate-specific antigen and prostate cancer: prediction, detection and monitoring.* Nat Rev Cancer, **2008**. 8(4): p. 268-78.

54. Chan, D.W., D.J. Bruzek, J.E. Oesterling, R.C. Rock, and P.C. Walsh, *Prostate-specific antigen as a marker for prostatic cancer: a monoclonal and a polyclonal immunoassay compared.* Clin Chem, **1987**. 33(10): p. 1916-20.
55. Stamey, T.A., N. Yang, A.R. Hay, J.E. McNeal, F.S. Freiha, and E. Redwine, *Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate.* N Engl J Med, **1987**. 317(15): p. 909-16.
56. Deliveliotis, C., V. John, G. Louras, S. Andreas, E. Alargof, F. Sofras, and N. Goulandris, *Multiple transrectal ultrasound guided prostatic biopsies: morbidity and tolerance.* Int Urol Nephrol, **1999**. 31(5): p. 681-6.
57. Dickinson, L., H.U. Ahmed, C. Allen, J.O. Barentsz, B. Carey, J.J. Futterer, S.W. Heijmink, P.J. Hoskin, A. Kirkham, A.R. Padhani, R. Persad, P. Puech, S. Punwani, A.S. Sohaib, B. Tombal, A. Villers, J. van der Meulen, and M. Emberton, *Magnetic resonance imaging for the detection, localisation, and characterisation of prostate cancer: recommendations from a European consensus meeting.* Eur Urol, **2011**. 59(4): p. 477-94.
58. Carter, H.B., P.C. Albertsen, M.J. Barry, R. Etzioni, S.J. Freedland, K.L. Greene, L. Holmberg, P. Kantoff, B.R. Konety, M.H. Murad, D.F. Penson, and A.L. Zietman, *Early detection of prostate cancer: AUA Guideline.* J Urol, **2013**. 190(2): p. 419-26.
59. van der Kwast, T.H., C. Lopes, C. Santonja, C.G. Pihl, I. Neetens, P. Martikainen, S. Di Lollo, L. Bubendorf, R.F. Hoedemaeker, and C. Members of the pathology committee of the European Randomised Study of Screening for Prostate, *Guidelines for processing and reporting of prostatic needle biopsies.* J Clin Pathol, **2003**. 56(5): p. 336-40.
60. Humphrey, P.A., *Gleason grading and prognostic factors in carcinoma of the prostate.* Mod Pathol, **2004**. 17(3): p. 292-306.
61. Epstein, J.I., L. Egevad, M.B. Amin, B. Delahunt, J.R. Srigley, P.A. Humphrey, and C. Grading, *The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma: Definition of Grading Patterns and Proposal for a New Grading System.* Am J Surg Pathol, **2016**. 40(2): p. 244-52.
62. Schroder, F.H., P. Hermanek, L. Denis, W.R. Fair, M.K. Gospodarowicz, and M. Pavone-Macaluso, *The TNM classification of prostate cancer.* Prostate Suppl, **1992**. 4: p. 129-38.
63. Wilt, T.J., M.K. Brawer, K.M. Jones, M.J. Barry, W.J. Aronson, S. Fox, J.R. Gingrich, J.T. Wei, P. Gilhooly, B.M. Grob, I. Nsouli, P. Iyer, R. Cartagena, G. Snider, C. Roehrborn, R. Sharifi, W. Blank, P. Pandya, G.L. Andriole, D. Culkin, T. Wheeler, and G. Prostate Cancer Intervention versus Observation Trial Study, *Radical prostatectomy versus observation for localized prostate cancer.* N Engl J Med, **2012**. 367(3): p. 203-13.
64. Dall'Era, M.A., P.C. Albertsen, C. Bangma, P.R. Carroll, H.B. Carter, M.R. Cooperberg, S.J. Freedland, L.H. Klotz, C. Parker, and M.S. Soloway, *Active surveillance for prostate cancer: a systematic review of the literature.* Eur Urol, **2012**. 62(6): p. 976-83.
65. Akduman, B. and E.D. Crawford, *Treatment of localized prostate cancer.* Rev Urol, **2006**. 8 Suppl 2: p. S15-21.
66. Holmberg, L., A. Bill-Axelson, F. Helgesen, J.O. Salo, P. Folmerz, M. Haggman, S.O. Andersson, A. Spangberg, C. Busch, S. Nordling, J. Palmgren, H.O. Adami, J.E. Johansson, B.J. Norlen, and N. Scandinavian Prostatic Cancer Group Study, *A randomized trial comparing radical prostatectomy with watchful waiting in early prostate cancer.* N Engl J Med, **2002**. 347(11): p. 781-9.

67. Widmark, A., O. Klepp, A. Solberg, J.E. Damber, A. Angelsen, P. Fransson, J.A. Lund, I. Tasdemir, M. Hoyer, F. Wiklund, S.D. Fossa, S. Scandinavian Prostate Cancer Group, and O. Swedish Association for Urological, *Endocrine treatment, with or without radiotherapy, in locally advanced prostate cancer (SPCG-7/SFUO-3): an open randomised phase III trial.* Lancet, **2009**. 373(9660): p. 301-8.
68. Warde, P., M. Mason, K. Ding, P. Kirkbride, M. Brundage, R. Cowan, M. Gospodarowicz, K. Sanders, E. Kostashuk, G. Swanson, J. Barber, A. Hiltz, M.K. Parmar, J. Sathya, J. Anderson, C. Hayter, J. Hetherington, M.R. Sydes, W. Parulekar, and N.C.P.M.U.P. investigators, *Combined androgen deprivation therapy and radiation therapy for locally advanced prostate cancer: a randomised, phase 3 trial.* Lancet, **2011**. 378(9809): p. 2104-11.
69. Amling, C.L., M.L. Blute, E.J. Bergstralh, T.M. Seay, J. Slezak, and H. Zincke, *Long-term hazard of progression after radical prostatectomy for clinically localized prostate cancer: continued risk of biochemical failure after 5 years.* J Urol, **2000**. 164(1): p. 101-5.
70. Sharifi, N., J.L. Gulley, and W.L. Dahut, *Androgen deprivation therapy for prostate cancer.* JAMA, **2005**. 294(2): p. 238-44.
71. Schally, A.V., A. Arimura, Y. Baba, R.M. Nair, H. Matsuo, T.W. Redding, and L. Debeljuk, *Isolation and properties of the FSH and LH-releasing hormone.* Biochem Biophys Res Commun, **1971**. 43(2): p. 393-9.
72. Karantanos, T., P.G. Corn, and T.C. Thompson, *Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches.* Oncogene, **2013**. 32(49): p. 5501-11.
73. Smith, M.R., F. Kabbinavar, F. Saad, A. Hussain, M.C. Gittelman, D.L. Bilhartz, C. Wynne, R. Murray, N.R. Zinner, C. Schulman, R. Linnartz, M. Zheng, C. Goessl, Y.J. Hei, E.J. Small, R. Cook, and C.S. Higano, *Natural history of rising serum prostate-specific antigen in men with castrate nonmetastatic prostate cancer.* J Clin Oncol, **2005**. 23(13): p. 2918-25.
74. Cookson, M.S., B.J. Roth, P. Dahm, C. Engstrom, S.J. Freedland, M. Hussain, D.W. Lin, W.T. Lowrance, M.H. Murad, W.K. Oh, D.F. Penson, and A.S. Kibel, *Castration-resistant prostate cancer: AUA Guideline.* J Urol, **2013**. 190(2): p. 429-38.
75. Saad, F., K.N. Chi, A. Finelli, S.J. Hotte, J. Izawa, A. Kapoor, W. Kassouf, A. Loblaw, S. North, R. Rendon, A. So, N. Usmani, E. Vigneault, and N.E. Fleshner, *The 2015 CUA-CUOG Guidelines for the management of castration-resistant prostate cancer (CRPC).* Can Urol Assoc J, **2015**. 9(3-4): p. 90-6.
76. Attard, G., A.S. Belldegrun, and J.S. de Bono, *Selective blockade of androgenic steroid synthesis by novel lyase inhibitors as a therapeutic strategy for treating metastatic prostate cancer.* BJU Int, **2005**. 96(9): p. 1241-6.
77. Kantoff, P.W., C.S. Higano, N.D. Shore, E.R. Berger, E.J. Small, D.F. Penson, C.H. Redfern, A.C. Ferrari, R. Dreicer, R.B. Sims, Y. Xu, M.W. Frohlich, P.F. Schellhammer, and I.S. Investigators, *Sipuleucel-T immunotherapy for castration-resistant prostate cancer.* N Engl J Med, **2010**. 363(5): p. 411-22.
78. Tannock, I.F., R. de Wit, W.R. Berry, J. Horti, A. Pluzanska, K.N. Chi, S. Oudard, C. Theodore, N.D. James, I. Turesson, M.A. Rosenthal, M.A. Eisenberger, and T.A.X. Investigators, *Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer.* N Engl J Med, **2004**. 351(15): p. 1502-12.

79. Paller, C.J. and E.S. Antonarakis, *Cabazitaxel: a novel second-line treatment for metastatic castration-resistant prostate cancer.* Drug Des Devel Ther, **2011**. 5: p. 117-24.
80. Parker, C., S. Nilsson, D. Heinrich, S.I. Helle, J.M. O'Sullivan, S.D. Fossa, A. Chodacki, P. Wiechno, J. Logue, M. Seke, A. Widmark, D.C. Johannessen, P. Hoskin, D. Bottomley, N.D. James, A. Solberg, I. Syndikus, J. Kliment, S. Wedel, S. Boehmer, M. Dall'Oglio, L. Franzen, R. Coleman, N.J. Vogelzang, C.G. O'Bryan-Tear, K. Staudacher, J. Garcia-Vargas, M. Shan, O.S. Bruland, O. Sartor, and A. Investigators, *Alpha emitter radium-223 and survival in metastatic prostate cancer.* N Engl J Med, **2013**. 369(3): p. 213-23.
81. Nelson, E.C., A.J. Cambio, J.C. Yang, J.H. Ok, P.N. Lara, Jr., and C.P. Evans, *Clinical implications of neuroendocrine differentiation in prostate cancer.* Prostate Cancer Prostatic Dis, **2007**. 10(1): p. 6-14.
82. Nuclear Receptors Nomenclature, C., *A unified nomenclature system for the nuclear receptor superfamily.* Cell, **1999**. 97(2): p. 161-3.
83. Wang, D. and D.J. Tindall, *Androgen action during prostate carcinogenesis.* Methods Mol Biol, **2011**. 776: p. 25-44.
84. Migliaccio, A., G. Castoria, M. Di Domenico, A. de Falco, A. Bilancio, M. Lombardi, M.V. Barone, D. Ametrano, M.S. Zannini, C. Abbondanza, and F. Auricchio, *Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation.* EMBO J, **2000**. 19(20): p. 5406-17.
85. Kousteni, S., T. Bellido, L.I. Plotkin, C.A. O'Brien, D.L. Bodenner, L. Han, K. Han, G.B. DiGregorio, J.A. Katzenellenbogen, B.S. Katzenellenbogen, P.K. Roberson, R.S. Weinstein, R.L. Jilka, and S.C. Manolagas, *Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity.* Cell, **2001**. 104(5): p. 719-30.
86. Mendelson, C., M. Dufau, and K. Catt, *Gonadotropin binding and stimulation of cyclic adenosine 3':5'-monophosphate and testosterone production in isolated Leydig cells.* J Biol Chem, **1975**. 250(22): p. 8818-23.
87. Golsteyn, E.J. and M.J. Fritzler, *The role of the thymus-hypothalamus-pituitary-gonadal axis in normal immune processes and autoimmunity.* J Rheumatol, **1987**. 14(5): p. 982-90.
88. Anderson, D.C., *Sex-hormone-binding globulin.* Clin Endocrinol (Oxf), **1974**. 3(1): p. 69-96.
89. Russell, D.W. and J.D. Wilson, *Steroid 5 alpha-reductase: two genes/two enzymes.* Annu Rev Biochem, **1994**. 63: p. 25-61.
90. Bartsch, G., R.S. Rittmaster, and H. Klocker, *Dihydrotestosterone and the concept of 5alpha-reductase inhibition in human benign prostatic hyperplasia.* Eur Urol, **2000**. 37(4): p. 367-80.
91. Shiraishi, S., P.W. Lee, A. Leung, V.H. Goh, R.S. Swerdloff, and C. Wang, *Simultaneous measurement of serum testosterone and dihydrotestosterone by liquid chromatography-tandem mass spectrometry.* Clin Chem, **2008**. 54(11): p. 1855-63.
92. Rainey, W.E., B.R. Carr, H. Sasano, T. Suzuki, and J.I. Mason, *Dissecting human adrenal androgen production.* Trends Endocrinol Metab, **2002**. 13(6): p. 234-9.
93. Antoni, F.A., *Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor.* Endocr Rev, **1986**. 7(4): p. 351-78.

94. Luu-The, V., A. Belanger, and F. Labrie, *Androgen biosynthetic pathways in the human prostate.* Best Pract Res Clin Endocrinol Metab, **2008**. 22(2): p. 207-21.
95. Payne, A.H. and D.B. Hales, *Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones.* Endocr Rev, **2004**. 25(6): p. 947-70.
96. Auchus, R.J., *The backdoor pathway to dihydrotestosterone.* Trends Endocrinol Metab, **2004**. 15(9): p. 432-8.
97. Montgomery, R.B., E.A. Mostaghel, R. Vessella, D.L. Hess, T.F. Kalhorn, C.S. Higano, L.D. True, and P.S. Nelson, *Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth.* Cancer Res, **2008**. 68(11): p. 4447-54.
98. Locke, J.A., E.S. Guns, A.A. Lubik, H.H. Adomat, S.C. Hendy, C.A. Wood, S.L. Ettinger, M.E. Gleave, and C.C. Nelson, *Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer.* Cancer Res, **2008**. 68(15): p. 6407-15.
99. Cai, C., S. Chen, P. Ng, G.J. Bubley, P.S. Nelson, E.A. Mostaghel, B. Marck, A.M. Matsumoto, N.I. Simon, H. Wang, S. Chen, and S.P. Balk, *Intratumoral de novo steroid synthesis activates androgen receptor in castration-resistant prostate cancer and is upregulated by treatment with CYP17A1 inhibitors.* Cancer Res, **2011**. 71(20): p. 6503-13.
100. Liu, C., W. Lou, Y. Zhu, J.C. Yang, N. Nadiminty, N.W. Gaikwad, C.P. Evans, and A.C. Gao, *Intracrine Androgens and AKR1C3 Activation Confer Resistance to Enzalutamide in Prostate Cancer.* Cancer Res, **2015**. 75(7): p. 1413-22.
101. Chang, K.H., R. Li, B. Kuri, Y. Lotan, C.G. Roehrborn, J. Liu, R. Vessella, P.S. Nelson, P. Kapur, X. Guo, H. Mirzaei, R.J. Auchus, and N. Sharifi, *A gain-of-function mutation in DHT synthesis in castration-resistant prostate cancer.* Cell, **2013**. 154(5): p. 1074-84.
102. Barbier, O. and A. Belanger, *Inactivation of androgens by UDP-glucuronosyltransferases in the human prostate.* Best Pract Res Clin Endocrinol Metab, **2008**. 22(2): p. 259-70.
103. Labrie, F., V. Luu-The, A. Belanger, S.X. Lin, J. Simard, G. Pelletier, and C. Labrie, *Is dehydroepiandrosterone a hormone?* J Endocrinol, **2005**. 187(2): p. 169-96.
104. Belanger, A., G. Pelletier, F. Labrie, O. Barbier, and S. Chouinard, *Inactivation of androgens by UDP-glucuronosyltransferase enzymes in humans.* Trends Endocrinol Metab, **2003**. 14(10): p. 473-9.
105. Hsing, A.W., J.K. Reichardt, and F.Z. Stanczyk, *Hormones and prostate cancer: current perspectives and future directions.* Prostate, **2002**. 52(3): p. 213-35.
106. Labrie, F., *Intracrinology.* Mol Cell Endocrinol, **1991**. 78(3): p. C113-8.
107. Tukey, R.H. and C.P. Strassburg, *Human UDP-glucuronosyltransferases: metabolism, expression, and disease.* Annu Rev Pharmacol Toxicol, **2000**. 40: p. 581-616.
108. Sneitz, N., M.H. Court, X. Zhang, K. Laajanen, K.K. Yee, P. Dalton, X. Ding, and M. Finel, *Human UDP-glucuronosyltransferase UGT2A2: cDNA construction, expression, and functional characterization in comparison with UGT2A1 and UGT2A3.* Pharmacogenet Genomics, **2009**. 19(12): p. 923-34.
109. Bushey, R.T., D.F. Dluzen, and P. Lazarus, *Importance of UDP-glucuronosyltransferases 2A2 and 2A3 in tobacco carcinogen metabolism.* Drug Metab Dispos, **2013**. 41(1): p. 170-9.

110. Guillemette, C., E. Levesque, M. Harvey, J. Bellemare, and V. Menard, *UGT genomic diversity: beyond gene duplication.* Drug Metab Rev, **2010**. 42(1): p. 24-44.
111. Meech, R., N. Mubarokah, A. Shivasami, A. Rogers, P.C. Nair, D.G. Hu, R.A. McKinnon, and P.I. Mackenzie, *A novel function for UDP glycosyltransferase 8: galactosidation of bile acids.* Mol Pharmacol, **2015**. 87(3): p. 442-50.
112. Meech, R., A. Rogers, L. Zhuang, B.C. Lewis, J.O. Miners, and P.I. Mackenzie, *Identification of residues that confer sugar selectivity to UDP-glycosyltransferase 3A (UGT3A) enzymes.* J Biol Chem, **2012**. 287(29): p. 24122-30.
113. Riedy, M., J.Y. Wang, A.P. Miller, A. Buckler, J. Hall, and M. Guida, *Genomic organization of the UGT2b gene cluster on human chromosome 4q13.* Pharmacogenetics, **2000**. 10(3): p. 251-60.
114. Mackenzie, P.I., K.W. Bock, B. Burchell, C. Guillemette, S. Ikushiro, T. Iyanagi, J.O. Miners, I.S. Owens, and D.W. Nebert, *Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily.* Pharmacogenet Genomics, **2005**. 15(10): p. 677-85.
115. Bigo, C., S. Caron, A. Dallaire-Theroux, and O. Barbier, *Nuclear receptors and endobiotics glucuronidation: the good, the bad, and the UGT.* Drug Metab Rev, **2013**. 45(1): p. 34-47.
116. Guillemette, C., A. Belanger, and J. Lepine, *Metabolic inactivation of estrogens in breast tissue by UDP-glucuronosyltransferase enzymes: an overview.* Breast Cancer Res, **2004**. 6(6): p. 246-54.
117. Turgeon, D., J.S. Carrier, E. Levesque, D.W. Hum, and A. Belanger, *Relative enzymatic activity, protein stability, and tissue distribution of human steroid-metabolizing UGT2B subfamily members.* Endocrinology, **2001**. 142(2): p. 778-87.
118. Chouinard, S., G. Pelletier, A. Belanger, and O. Barbier, *Cellular specific expression of the androgen-conjugating enzymes UGT2B15 and UGT2B17 in the human prostate epithelium.* Endocr Res, **2004**. 30(4): p. 717-25.
119. Nadeau, G., J. Bellemare, E. Audet-Walsh, C. Flageole, S.P. Huang, B.Y. Bao, P. Douville, P. Caron, Y. Fradet, L. Lacombe, C. Guillemette, and E. Levesque, *Deletions of the androgen-metabolizing UGT2B genes have an effect on circulating steroid levels and biochemical recurrence after radical prostatectomy in localized prostate cancer.* J Clin Endocrinol Metab, **2011**. 96(9): p. E1550-7.
120. Levesque, E., D. Turgeon, J.S. Carrier, V. Montminy, M. Beaulieu, and A. Belanger, *Isolation and characterization of the UGT2B28 cDNA encoding a novel human steroid conjugating UDP-glucuronosyltransferase.* Biochemistry, **2001**. 40(13): p. 3869-81.
121. Barbier, O., H. Lapointe, M. El Alfy, D.W. Hum, and A. Belanger, *Cellular localization of uridine diphosphoglucuronosyltransferase 2B enzymes in the human prostate by in situ hybridization and immunohistochemistry.* J Clin Endocrinol Metab, **2000**. 85(12): p. 4819-26.
122. Gauthier-Landry, L., A. Belanger, and O. Barbier, *Multiple roles for UDP-glucuronosyltransferase (UGT)2B15 and UGT2B17 enzymes in androgen metabolism and prostate cancer evolution.* J Steroid Biochem Mol Biol, **2015**. 145: p. 187-92.
123. Miners, J.O., P.A. Smith, M.J. Sorich, R.A. McKinnon, and P.I. Mackenzie, *Predicting human drug glucuronidation parameters: application of in vitro and in silico modeling approaches.* Annu Rev Pharmacol Toxicol, **2004**. 44: p. 1-25.

124. Chouinard, S., O. Barbier, and A. Belanger, *UDP-glucuronosyltransferase 2B15 (UGT2B15) and UGT2B17 enzymes are major determinants of the androgen response in prostate cancer LNCaP cells.* J Biol Chem, **2007**. 282(46): p. 33466-74.
125. Hajdinjak, T. and B. Zagradisnik, *Prostate cancer and polymorphism D85Y in gene for dihydrotestosterone degrading enzyme UGT2B15: Frequency of DD homozygotes increases with Gleason Score.* Prostate, **2004**. 59(4): p. 436-9.
126. Bao, B.Y., B.F. Chuang, Q. Wang, O. Sartor, S.P. Balk, M. Brown, P.W. Kantoff, and G.S. Lee, *Androgen receptor mediates the expression of UDP-glucuronosyltransferase 2 B15 and B17 genes.* Prostate, **2008**. 68(8): p. 839-48.
127. Chouinard, S., G. Pelletier, A. Belanger, and O. Barbier, *Isoform-specific regulation of uridine diphosphate-glucuronosyltransferase 2B enzymes in the human prostate: differential consequences for androgen and bioactive lipid inactivation.* Endocrinology, **2006**. 147(11): p. 5431-42.
128. Guillemette, C., D.W. Hum, and A. Belanger, *Regulation of steroid glucuronosyltransferase activities and transcripts by androgen in the human prostatic cancer LNCaP cell line.* Endocrinology, **1996**. 137(7): p. 2872-9.
129. Paquet, S., L. Fazli, L. Grosse, M. Verreault, B. Tetu, P.S. Rennie, A. Belanger, and O. Barbier, *Differential expression of the androgen-conjugating UGT2B15 and UGT2B17 enzymes in prostate tumor cells during cancer progression.* J Clin Endocrinol Metab, **2012**. 97(3): p. E428-32.
130. Zhang, X., C. Morrissey, S. Sun, M. Ketchandji, P.S. Nelson, L.D. True, F. Vakar-Lopez, R.L. Vessella, and S.R. Plymate, *Androgen receptor variants occur frequently in castration resistant prostate cancer metastases.* PLoS One, **2011**. 6(11): p. e27970.
131. Hu, D.G., L.A. Selth, G.A. Tarulli, R. Meech, D. Wijayakumara, A. Chanawong, R. Russell, C. Caldas, J.L. Robinson, J.S. Carroll, W.D. Tilley, P.I. Mackenzie, and T.E. Hickey, *Androgen and Estrogen Receptors in Breast Cancer Coregulate Human UDP-Glucuronosyltransferases 2B15 and 2B17.* Cancer Res, **2016**. 76(19): p. 5881-5893.
132. Gruber, M., J. Bellemare, G. Hoermann, A. Gleiss, E. Porpaczy, M. Bilban, T. Le, S. Zehetmayer, C. Mannhalter, A. Gaiger, M. Shehata, K. Fleiss, C. Skrabs, E. Levesque, K. Vanura, C. Guillemette, and U. Jaeger, *Overexpression of uridine diphospho glucuronosyltransferase 2B17 in high-risk chronic lymphocytic leukemia.* Blood, **2013**. 121(7): p. 1175-83.
133. Hirata, H., Y. Hinoda, M.S. Zaman, Y. Chen, K. Ueno, S. Majid, C. Tripsas, M. Rubin, L.M. Chen, and R. Dahiya, *Function of UDP-glucuronosyltransferase 2B17 (UGT2B17) is involved in endometrial cancer.* Carcinogenesis, **2010**. 31(9): p. 1620-6.
134. Belledant, A., H. Hovington, L. Garcia, P. Caron, H. Brisson, L. Villeneuve, D. Simonyan, B. Tetu, Y. Fradet, L. Lacombe, C. Guillemette, and E. Levesque, *The UGT2B28 Sex-steroid Inactivation Pathway Is a Regulator of Steroidogenesis and Modifies the Risk of Prostate Cancer Progression.* Eur Urol, **2016**. 69(4): p. 601-9.
135. Zhang, A., J. Zhang, S. Plymate, and E.A. Mostaghel, *Classical and Non-Classical Roles for Pre-Receptor Control of DHT Metabolism in Prostate Cancer Progression.* Horm Cancer, **2016**. 7(2): p. 104-13.
136. Yepuru, M., Z. Wu, A. Kulkarni, F. Yin, C.M. Barrett, J. Kim, M.S. Steiner, D.D. Miller, J.T. Dalton, and R. Narayanan, *Steroidogenic enzyme AKR1C3 is a novel androgen receptor-selective coactivator that promotes prostate cancer growth.* Clin Cancer Res, **2013**. 19(20): p. 5613-25.

137. Fan, L., G. Peng, A. Hussain, L. Fazli, E. Guns, M. Gleave, and J. Qi, *The Steroidogenic Enzyme AKR1C3 Regulates Stability of the Ubiquitin Ligase Siah2 in Prostate Cancer Cells.* J Biol Chem, **2015**. 290(34): p. 20865-79.
138. Lubahn, D.B., D.R. Joseph, P.M. Sullivan, H.F. Willard, F.S. French, and E.M. Wilson, *Cloning of human androgen receptor complementary DNA and localization to the X chromosome.* Science, **1988**. 240(4850): p. 327-30.
139. Gottlieb, B., L.K. Beitel, A. Nadarajah, M. Paliouras, and M. Trifiro, *The androgen receptor gene mutations database: 2012 update.* Hum Mutat, **2012**. 33(5): p. 887-94.
140. Jenster, G., H.A. van der Korput, J. Trapman, and A.O. Brinkmann, *Identification of two transcription activation units in the N-terminal domain of the human androgen receptor.* J Biol Chem, **1995**. 270(13): p. 7341-6.
141. Lavery, D.N. and I.J. McEwan, *Structural characterization of the native NH2-terminal transactivation domain of the human androgen receptor: a collapsed disordered conformation underlies structural plasticity and protein-induced folding.* Biochemistry, **2008**. 47(11): p. 3360-9.
142. Claessens, F., S. Denayer, N. Van Tilborgh, S. Kerkhofs, C. Helsen, and A. Haelens, *Diverse roles of androgen receptor (AR) domains in AR-mediated signaling.* Nucl Recept Signal, **2008**. 6: p. e008.
143. He, B., J.A. Kemppainen, and E.M. Wilson, *FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor.* J Biol Chem, **2000**. 275(30): p. 22986-94.
144. McEwan, I.J. and J. Gustafsson, *Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF.* Proc Natl Acad Sci U S A, **1997**. 94(16): p. 8485-90.
145. Aarnisalo, P., J.J. Palvimo, and O.A. Janne, *CREB-binding protein in androgen receptor-mediated signaling.* Proc Natl Acad Sci U S A, **1998**. 95(5): p. 2122-7.
146. Dotzlaw, H., U. Moehren, S. Mink, A.C. Cato, J.A. Iniguez Lluhi, and A. Baniahmad, *The amino terminus of the human AR is target for corepressor action and antihormone agonism.* Mol Endocrinol, **2002**. 16(4): p. 661-73.
147. Sleddens, H.F., B.A. Oostra, A.O. Brinkmann, and J. Trapman, *Trinucleotide repeat polymorphism in the androgen receptor gene (AR).* Nucleic Acids Res, **1992**. 20(6): p. 1427.
148. Kazemi-Esfarjani, P., M.A. Trifiro, and L. Pinsky, *Evidence for a repressive function of the long polyglutamine tract in the human androgen receptor: possible pathogenetic relevance for the (CAG)n-expanded neuronopathies.* Hum Mol Genet, **1995**. 4(4): p. 523-7.
149. Umesono, K. and R.M. Evans, *Determinants of target gene specificity for steroid/thyroid hormone receptors.* Cell, **1989**. 57(7): p. 1139-46.
150. Claessens, F., P. Alen, A. Devos, B. Peeters, G. Verhoeven, and W. Rombauts, *The androgen-specific probasin response element 2 interacts differentially with androgen and glucocorticoid receptors.* J Biol Chem, **1996**. 271(32): p. 19013-6.
151. Denayer, S., C. Helsen, L. Thorrez, A. Haelens, and F. Claessens, *The rules of DNA recognition by the androgen receptor.* Mol Endocrinol, **2010**. 24(5): p. 898-913.
152. Shaffer, P.L., A. Jivan, D.E. Dollins, F. Claessens, and D.T. Gewirth, *Structural basis of androgen receptor binding to selective androgen response elements.* Proc Natl Acad Sci U S A, **2004**. 101(14): p. 4758-63.

153. Zhou, Z.X., M. Sar, J.A. Simental, M.V. Lane, and E.M. Wilson, *A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNA-binding domain and modulation by NH2-terminal and carboxyl-terminal sequences.* J Biol Chem, **1994**. 269(18): p. 13115-23.
154. Cutress, M.L., H.C. Whitaker, I.G. Mills, M. Stewart, and D.E. Neal, *Structural basis for the nuclear import of the human androgen receptor.* J Cell Sci, **2008**. 121(Pt 7): p. 957-68.
155. van der Steen, T., D.J. Tindall, and H. Huang, *Posttranslational modification of the androgen receptor in prostate cancer.* Int J Mol Sci, **2013**. 14(7): p. 14833-59.
156. Pereira de Jesus-Tran, K., P.L. Cote, L. Cantin, J. Blanchet, F. Labrie, and R. Breton, *Comparison of crystal structures of human androgen receptor ligand-binding domain complexed with various agonists reveals molecular determinants responsible for binding affinity.* Protein Sci, **2006**. 15(5): p. 987-99.
157. Berrevoets, C.A., P. Doesburg, K. Steketee, J. Trapman, and A.O. Brinkmann, *Functional interactions of the AF-2 activation domain core region of the human androgen receptor with the amino-terminal domain and with the transcriptional coactivator TIF2 (transcriptional intermediary factor2).* Mol Endocrinol, **1998**. 12(8): p. 1172-83.
158. Zhou, X.E., K.M. Suino-Powell, J. Li, Y. He, J.P. Mackeigan, K. Melcher, E.L. Yong, and H.E. Xu, *Identification of SRC3/AIB1 as a preferred coactivator for hormone-activated androgen receptor.* J Biol Chem, **2010**. 285(12): p. 9161-71.
159. He, B., J.A. Kemppainen, J.J. Voegel, H. Gronemeyer, and E.M. Wilson, *Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH(2)-terminal domain.* J Biol Chem, **1999**. 274(52): p. 37219-25.
160. Smith, D.F. and D.O. Toft, *Minireview: the intersection of steroid receptors with molecular chaperones: observations and questions.* Mol Endocrinol, **2008**. 22(10): p. 2229-40.
161. Heinlein, C.A. and C. Chang, *Androgen receptor in prostate cancer.* Endocr Rev, **2004**. 25(2): p. 276-308.
162. Wu, Y., W. Zhao, J. Zhao, J. Pan, Q. Wu, Y. Zhang, W.A. Bauman, and C.P. Cardozo, *Identification of androgen response elements in the insulin-like growth factor I upstream promoter.* Endocrinology, **2007**. 148(6): p. 2984-93.
163. Pignon, J.C., B. Koopmansch, G. Nolens, L. Delacroix, D. Waltregny, and R. Winkler, *Androgen receptor controls EGFR and ERBB2 gene expression at different levels in prostate cancer cell lines.* Cancer Res, **2009**. 69(7): p. 2941-9.
164. Lu, S., M. Liu, D.E. Epner, S.Y. Tsai, and M.J. Tsai, *Androgen regulation of the cyclin-dependent kinase inhibitor p21 gene through an androgen response element in the proximal promoter.* Mol Endocrinol, **1999**. 13(3): p. 376-84.
165. Gao, S., P. Lee, H. Wang, W. Gerald, M. Adler, L. Zhang, Y.F. Wang, and Z. Wang, *The androgen receptor directly targets the cellular Fas/FasL-associated death domain protein-like inhibitory protein gene to promote the androgen-independent growth of prostate cancer cells.* Mol Endocrinol, **2005**. 19(7): p. 1792-802.
166. Liu, Y.N., Y. Liu, H.J. Lee, Y.H. Hsu, and J.H. Chen, *Activated androgen receptor downregulates E-cadherin gene expression and promotes tumor metastasis.* Mol Cell Biol, **2008**. 28(23): p. 7096-108.

167. Foradori, C.D., M.J. Weiser, and R.J. Handa, *Non-genomic actions of androgens.* Front Neuroendocrinol, **2008**. 29(2): p. 169-81.
168. Unni, E., S. Sun, B. Nan, M.J. McPhaul, B. Cheskis, M.A. Mancini, and M. Marcelli, *Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence.* Cancer Res, **2004**. 64(19): p. 7156-68.
169. Gotoh, N., M. Toyoda, and M. Shibuya, *Tyrosine phosphorylation sites at amino acids 239 and 240 of Shc are involved in epidermal growth factor-induced mitogenic signaling that is distinct from Ras/mitogen-activated protein kinase activation.* Mol Cell Biol, **1997**. 17(4): p. 1824-31.
170. Culig, Z., A. Hobisch, M.V. Cronauer, C. Radmayr, J. Trapman, A. Hittmair, G. Bartsch, and H. Klocker, *Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor.* Cancer Res, **1994**. 54(20): p. 5474-8.
171. Lin, H.K., S. Yeh, H.Y. Kang, and C. Chang, *Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor.* Proc Natl Acad Sci U S A, **2001**. 98(13): p. 7200-5.
172. Ueda, T., N.R. Mawji, N. Bruchovsky, and M.D. Sadar, *Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells.* J Biol Chem, **2002**. 277(41): p. 38087-94.
173. Yeh, S., H.K. Lin, H.Y. Kang, T.H. Thin, M.F. Lin, and C. Chang, *From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells.* Proc Natl Acad Sci U S A, **1999**. 96(10): p. 5458-63.
174. Guo, Z., B. Dai, T. Jiang, K. Xu, Y. Xie, O. Kim, I. Nesheiwat, X. Kong, J. Melamed, V.D. Handratta, V.C. Njar, A.M. Brodie, L.R. Yu, T.D. Veenstra, H. Chen, and Y. Qiu, *Regulation of androgen receptor activity by tyrosine phosphorylation.* Cancer Cell, **2006**. 10(4): p. 309-19.
175. Fang, S. and S. Liao, *Antagonistic action of anti-androgens on the formation of a specific dihydrotestosterone-receptor protein complex in rat ventral prostate.* Mol Pharmacol, **1969**. 5(4): p. 428-31.
176. Blackledge, G.R., *Clinical progress with a new antiandrogen, Casodex (bicalutamide).* Eur Urol, **1996**. 29 Suppl 2: p. 96-104.
177. Tran, C., S. Ouk, N.J. Clegg, Y. Chen, P.A. Watson, V. Arora, J. Wongvipat, P.M. Smith-Jones, D. Yoo, A. Kwon, T. Wasielewska, D. Welsbie, C.D. Chen, C.S. Higano, T.M. Beer, D.T. Hung, H.I. Scher, M.E. Jung, and C.L. Sawyers, *Development of a second-generation antiandrogen for treatment of advanced prostate cancer.* Science, **2009**. 324(5928): p. 787-90.
178. Clegg, N.J., J. Wongvipat, J.D. Joseph, C. Tran, S. Ouk, A. Dilhas, Y. Chen, K. Grillot, E.D. Bischoff, L. Cai, A. Aparicio, S. Dorow, V. Arora, G. Shao, J. Qian, H. Zhao, G. Yang, C. Cao, J. Sensintaffar, T. Wasielewska, M.R. Herbert, C. Bonnefous, B. Darimont, H.I. Scher, P. Smith-Jones, M. Klang, N.D. Smith, E. De Stanchina, N. Wu, O. Ouerfelli, P.J. Rix, R.A. Heyman, M.E. Jung, C.L. Sawyers, and J.H. Hager, *ARN-509: a novel antiandrogen for prostate cancer treatment.* Cancer Res, **2012**. 72(6): p. 1494-503.
179. Fizazi, K., C. Massard, P. Bono, R. Jones, V. Kataja, N. James, J.A. Garcia, A. Protheroe, T.L. Tammela, T. Elliott, L. Mattila, J. Aspegren, A. Vuorela, P. Langmuir, M. Mustonen, and A.s. group, *Activity and safety of ODM-201 in patients with progressive*

metastatic castration-resistant prostate cancer (ARADES): an open-label phase 1 dose-escalation and randomised phase 2 dose expansion trial. Lancet Oncol, **2014**. 15(9): p. 975-85.

180. de Bono, J.S., C.J. Logothetis, A. Molina, K. Fizazi, S. North, L. Chu, K.N. Chi, R.J. Jones, O.B. Goodman, Jr., F. Saad, J.N. Staffurth, P. Mainwaring, S. Harland, T.W. Flaig, T.E. Hutson, T. Cheng, H. Patterson, J.D. Hainsworth, C.J. Ryan, C.N. Sternberg, S.L. Ellard, A. Flechon, M. Saleh, M. Scholz, E. Efstathiou, A. Zivi, D. Bianchini, Y. Loriot, N. Chieffo, T. Kheoh, C.M. Haqq, H.I. Scher, and C.-A.-. Investigators, *Abiraterone and increased survival in metastatic prostate cancer.* N Engl J Med, **2011**. 364(21): p. 1995-2005.
181. Yu, Z., C. Cai, S. Gao, N.I. Simon, H.C. Shen, and S.P. Balk, *Galeterone prevents androgen receptor binding to chromatin and enhances degradation of mutant androgen receptor.* Clin Cancer Res, **2014**. 20(15): p. 4075-85.
182. Li, H., F. Ban, K. Dalal, E. Leblanc, K. Frewin, D. Ma, H. Adomat, P.S. Rennie, and A. Cherkasov, *Discovery of small-molecule inhibitors selectively targeting the DNA-binding domain of the human androgen receptor.* J Med Chem, **2014**. 57(15): p. 6458-67.
183. Dalal, K., M. Roshan-Moniri, A. Sharma, H. Li, F. Ban, M.D. Hassona, M. Hsing, K. Singh, E. LeBlanc, S. Dehm, E.S. Tomlinson Guns, A. Cherkasov, and P.S. Rennie, *Selectively targeting the DNA-binding domain of the androgen receptor as a prospective therapy for prostate cancer.* J Biol Chem, **2014**. 289(38): p. 26417-29.
184. Sadar, M.D., *Small molecule inhibitors targeting the "achilles' heel" of androgen receptor activity.* Cancer Res, **2011**. 71(4): p. 1208-13.
185. Myung, J.K., C.A. Banuelos, J.G. Fernandez, N.R. Mawji, J. Wang, A.H. Tien, Y.C. Yang, I. Tavakoli, S. Haile, K. Watt, I.J. McEwan, S. Plymate, R.J. Andersen, and M.D. Sadar, *An androgen receptor N-terminal domain antagonist for treating prostate cancer.* J Clin Invest, **2013**. 123(7): p. 2948-60.
186. Yang, Y.C., C.A. Banuelos, N.R. Mawji, J. Wang, M. Kato, S. Haile, I.J. McEwan, S. Plymate, and M.D. Sadar, *Targeting Androgen Receptor Activation Function-1 with EPI to Overcome Resistance Mechanisms in Castration-Resistant Prostate Cancer.* Clin Cancer Res, **2016**. 22(17): p. 4466-77.
187. Brand, L.J., M.E. Olson, P. Ravindranathan, H. Guo, A.M. Kempema, T.E. Andrews, X. Chen, G.V. Raj, D.A. Harki, and S.M. Dehm, *EPI-001 is a selective peroxisome proliferator-activated receptor-gamma modulator with inhibitory effects on androgen receptor expression and activity in prostate cancer.* Oncotarget, **2015**. 6(6): p. 3811-24.
188. Asangani, I.A., V.L. Dommeti, X. Wang, R. Malik, M. Cieslik, R. Yang, J. Escara-Wilke, K. Wilder-Romans, S. Dhanireddy, C. Engelke, M.K. Iyer, X. Jing, Y.M. Wu, X. Cao, Z.S. Qin, S. Wang, F.Y. Feng, and A.M. Chinnaiyan, *Therapeutic targeting of BET bromodomain proteins in castration-resistant prostate cancer.* Nature, **2014**. 510(7504): p. 278-82.
189. Pacey, S., R.H. Wilson, M. Walton, M.M. Eatock, A. Hardcastle, A. Zetterlund, H.T. Arkenau, J. Moreno-Farre, U. Banerji, B. Roels, H. Peachey, W. Aherne, J.S. de Bono, F. Raynaud, P. Workman, and I. Judson, *A phase I study of the heat shock protein 90 inhibitor alvespimycin (17-DMAG) given intravenously to patients with advanced solid tumors.* Clin Cancer Res, **2011**. 17(6): p. 1561-70.
190. Ferraldeschi, R., J. Welti, M.V. Powers, W. Yuan, T. Smyth, G. Seed, R. Riisnaes, S. Hedayat, H. Wang, M. Crespo, D. Nava Rodrigues, I. Figueiredo, S. Miranda, S.

Carreira, J.F. Lyons, S. Sharp, S.R. Plymate, G. Attard, N. Wallis, P. Workman, and J.S. de Bono, *Second-Generation HSP90 Inhibitor Onalespib Blocks mRNA Splicing of Androgen Receptor Variant 7 in Prostate Cancer Cells.* Cancer Res, **2016**. 76(9): p. 2731-42.
191. Yamashita, S., K.P. Lai, K.L. Chuang, D. Xu, H. Miyamoto, T. Tochigi, S.T. Pang, L. Li, Y. Arai, H.J. Kung, S. Yeh, and C. Chang, *ASC-J9 suppresses castration-resistant prostate cancer growth through degradation of full-length and splice variant androgen receptors.* Neoplasia, **2012**. 14(1): p. 74-83.
192. Geng, C., B. He, L. Xu, C.E. Barbieri, V.K. Eedunuri, S.A. Chew, M. Zimmermann, R. Bond, J. Shou, C. Li, M. Blattner, D.M. Lonard, F. Demichelis, C. Coarfa, M.A. Rubin, P. Zhou, B.W. O'Malley, and N. Mitsiades, *Prostate cancer-associated mutations in speckle-type POZ protein (SPOP) regulate steroid receptor coactivator 3 protein turnover.* Proc Natl Acad Sci U S A, **2013**. 110(17): p. 6997-7002.
193. Hu, R., T.A. Dunn, S. Wei, S. Isharwal, R.W. Veltri, E. Humphreys, M. Han, A.W. Partin, R.L. Vessella, W.B. Isaacs, G.S. Bova, and J. Luo, *Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer.* Cancer Res, **2009**. 69(1): p. 16-22.
194. Guo, Z., X. Yang, F. Sun, R. Jiang, D.E. Linn, H. Chen, H. Chen, X. Kong, J. Melamed, C.G. Tepper, H.J. Kung, A.M. Brodie, J. Edwards, and Y. Qiu, *A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth.* Cancer Res, **2009**. 69(6): p. 2305-13.
195. Hu, R., W.B. Isaacs, and J. Luo, *A snapshot of the expression signature of androgen receptor splicing variants and their distinctive transcriptional activities.* Prostate, **2011**. 71(15): p. 1656-67.
196. Sun, S., C.C. Sprenger, R.L. Vessella, K. Haugk, K. Soriano, E.A. Mostaghel, S.T. Page, I.M. Coleman, H.M. Nguyen, H. Sun, P.S. Nelson, and S.R. Plymate, *Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant.* J Clin Invest, **2010**. 120(8): p. 2715-30.
197. Li, Y., S.C. Chan, L.J. Brand, T.H. Hwang, K.A. Silverstein, and S.M. Dehm, *Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant prostate cancer cell lines.* Cancer Res, **2013**. 73(2): p. 483-9.
198. Liu, L.L., N. Xie, S. Sun, S. Plymate, E. Mostaghel, and X. Dong, *Mechanisms of the androgen receptor splicing in prostate cancer cells.* Oncogene, **2013**.
199. Chan, S.C., Y. Li, and S.M. Dehm, *Androgen receptor splice variants activate androgen receptor target genes and support aberrant prostate cancer cell growth independent of canonical androgen receptor nuclear localization signal.* J Biol Chem, **2012**. 287(23): p. 19736-49.
200. Dehm, S.M., L.J. Schmidt, H.V. Heemers, R.L. Vessella, and D.J. Tindall, *Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance.* Cancer Res, **2008**. 68(13): p. 5469-77.
201. Joseph, J.D., N. Lu, J. Qian, J. Sensintaffar, G. Shao, D. Brigham, M. Moon, E.C. Maneval, I. Chen, B. Darimont, and J.H. Hager, *A clinically relevant androgen receptor mutation confers resistance to second-generation antiandrogens enzalutamide and ARN-509.* Cancer Discov, **2013**. 3(9): p. 1020-9.
202. Korpal, M., J.M. Korn, X. Gao, D.P. Rakiec, D.A. Ruddy, S. Doshi, J. Yuan, S.G. Kovats, S. Kim, V.G. Cooke, J.E. Monahan, F. Stegmeier, T.M. Roberts, W.R. Sellers,

W. Zhou, and P. Zhu, *An F876L mutation in androgen receptor confers genetic and phenotypic resistance to MDV3100 (enzalutamide).* Cancer Discov, **2013**. 3(9): p. 1030-43.
203. Chen, C.D., D.S. Welsbie, C. Tran, S.H. Baek, R. Chen, R. Vessella, M.G. Rosenfeld, and C.L. Sawyers, *Molecular determinants of resistance to antiandrogen therapy.* Nat Med, **2004**. 10(1): p. 33-9.
204. Visakorpi, T., E. Hyytinen, P. Koivisto, M. Tanner, R. Keinanen, C. Palmberg, A. Palotie, T. Tammela, J. Isola, and O.P. Kallioniemi, *In vivo amplification of the androgen receptor gene and progression of human prostate cancer.* Nat Genet, **1995**. 9(4): p. 401-6.
205. Wyatt, A.W., F. Mo, K. Wang, B. McConeghy, S. Brahmbhatt, L. Jong, D.M. Mitchell, R.L. Johnston, A. Haegert, E. Li, J. Liew, J. Yeung, R. Shrestha, A.V. Lapuk, A. McPherson, R. Shukin, R.H. Bell, S. Anderson, J. Bishop, A. Hurtado-Coll, H. Xiao, A.M. Chinnaiyan, R. Mehra, D. Lin, Y. Wang, L. Fazli, M.E. Gleave, S.V. Volik, and C.C. Collins, *Heterogeneity in the inter-tumor transcriptome of high risk prostate cancer.* Genome Biol, **2014**. 15(8): p. 426.
206. Sharma, N.L., C.E. Massie, A. Ramos-Montoya, V. Zecchini, H.E. Scott, A.D. Lamb, S. MacArthur, R. Stark, A.Y. Warren, I.G. Mills, and D.E. Neal, *The androgen receptor induces a distinct transcriptional program in castration-resistant prostate cancer in man.* Cancer Cell, **2013**. 23(1): p. 35-47.
207. Dehm, S.M. and D.J. Tindall, *Alternatively spliced androgen receptor variants.* Endocr Relat Cancer, **2011**. 18(5): p. R183-96.
208. Antonarakis, E.S., C. Lu, H. Wang, B. Luber, M. Nakazawa, J.C. Roeser, Y. Chen, T.A. Mohammad, Y. Chen, H.L. Fedor, T.L. Lotan, Q. Zheng, A.M. De Marzo, J.T. Isaacs, W.B. Isaacs, R. Nadal, C.J. Paller, S.R. Denmeade, M.A. Carducci, M.A. Eisenberger, and J. Luo, *AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer.* N Engl J Med, **2014**. 371(11): p. 1028-38.
209. Qu, Y., B. Dai, D. Ye, Y. Kong, K. Chang, Z. Jia, X. Yang, H. Zhang, Y. Zhu, and G. Shi, *Constitutively active AR-V7 plays an essential role in the development and progression of castration-resistant prostate cancer.* Sci Rep, **2015**. 5: p. 7654.
210. Cai, C., H.H. He, S. Chen, I. Coleman, H. Wang, Z. Fang, S. Chen, P.S. Nelson, X.S. Liu, M. Brown, and S.P. Balk, *Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1.* Cancer Cell, **2011**. 20(4): p. 457-71.
211. Hu, R., C. Lu, E.A. Mostaghel, S. Yegnasubramanian, M. Gurel, C. Tannahill, J. Edwards, W.B. Isaacs, P.S. Nelson, E. Bluemn, S.R. Plymate, and J. Luo, *Distinct transcriptional programs mediated by the ligand-dependent full-length androgen receptor and its splice variants in castration-resistant prostate cancer.* Cancer Res, **2012**. 72(14): p. 3457-62.
212. Liu, L.L., N. Xie, S. Sun, S. Plymate, E. Mostaghel, and X. Dong, *Mechanisms of the androgen receptor splicing in prostate cancer cells.* Oncogene, **2014**. 33(24): p. 3140-50.
213. Xu, D., Y. Zhan, Y. Qi, B. Cao, S. Bai, W. Xu, S.S. Gambhir, P. Lee, O. Sartor, E.K. Flemington, H. Zhang, C.D. Hu, and Y. Dong, *Androgen Receptor Splice Variants Dimerize to Transactivate Target Genes.* Cancer Res, **2015**. 75(17): p. 3663-71.

214. Zhan, Y., G. Zhang, X. Wang, Y. Qi, D. Li, S. Bai, T. Ma, E.K. Flemington, H. Zhang, O. Sartor, P. Lee, and Y. Dong, *Interplay Between Cytoplasmic and Nuclear Androgen Receptor Splice Variants Mediate Castration Resistance.* Mol Cancer Res, **2016**.
215. Robinson, D., E.M. Van Allen, Y.M. Wu, N. Schultz, R.J. Lonigro, J.M. Mosquera, B. Montgomery, M.E. Taplin, C.C. Pritchard, G. Attard, H. Beltran, W. Abida, R.K. Bradley, J. Vinson, X. Cao, P. Vats, L.P. Kunju, M. Hussain, F.Y. Feng, S.A. Tomlins, K.A. Cooney, D.C. Smith, C. Brennan, J. Siddiqui, R. Mehra, Y. Chen, D.E. Rathkopf, M.J. Morris, S.B. Solomon, J.C. Durack, V.E. Reuter, A. Gopalan, J. Gao, M. Loda, R.T. Lis, M. Bowden, S.P. Balk, G. Gaviola, C. Sougnez, M. Gupta, E.Y. Yu, E.A. Mostaghel, H.H. Cheng, H. Mulcahy, L.D. True, S.R. Plymate, H. Dvinge, R. Ferraldeschi, P. Flohr, S. Miranda, Z. Zafeiriou, N. Tunariu, J. Mateo, R. Perez-Lopez, F. Demichelis, B.D. Robinson, M. Schiffman, D.M. Nanus, S.T. Tagawa, A. Sigaras, K.W. Eng, O. Elemento, A. Sboner, E.I. Heath, H.I. Scher, K.J. Pienta, P. Kantoff, J.S. de Bono, M.A. Rubin, P.S. Nelson, L.A. Garraway, C.L. Sawyers, and A.M. Chinnaiyan, *Integrative clinical genomics of advanced prostate cancer.* Cell, **2015**. 161(5): p. 1215-28.
216. Veldscholte, J., C. Ris-Stalpers, G.G. Kuiper, G. Jenster, C. Berrevoets, E. Claassen, H.C. van Rooij, J. Trapman, A.O. Brinkmann, and E. Mulder, *A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens.* Biochem Biophys Res Commun, **1990**. 173(2): p. 534-40.
217. Tan, J., Y. Sharief, K.G. Hamil, C.W. Gregory, D.Y. Zang, M. Sar, P.H. Gumerlock, R.W. deVere White, T.G. Pretlow, S.E. Harris, E.M. Wilson, J.L. Mohler, and F.S. French, *Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells.* Mol Endocrinol, **1997**. 11(4): p. 450-9.
218. Bohl, C.E., Z. Wu, D.D. Miller, C.E. Bell, and J.T. Dalton, *Crystal structure of the T877A human androgen receptor ligand-binding domain complexed to cyproterone acetate provides insight for ligand-induced conformational changes and structure-based drug design.* J Biol Chem, **2007**. 282(18): p. 13648-55.
219. Bohl, C.E., W. Gao, D.D. Miller, C.E. Bell, and J.T. Dalton, *Structural basis for antagonism and resistance of bicalutamide in prostate cancer.* Proc Natl Acad Sci U S A, **2005**. 102(17): p. 6201-6.
220. Hara, T., J. Miyazaki, H. Araki, M. Yamaoka, N. Kanzaki, M. Kusaka, and M. Miyamoto, *Novel mutations of androgen receptor: a possible mechanism of bicalutamide withdrawal syndrome.* Cancer Res, **2003**. 63(1): p. 149-53.
221. Balbas, M.D., M.J. Evans, D.J. Hosfield, J. Wongvipat, V.K. Arora, P.A. Watson, Y. Chen, G.L. Greene, Y. Shen, and C.L. Sawyers, *Overcoming mutation-based resistance to antiandrogens with rational drug design.* Elife, **2013**. 2: p. e00499.
222. Azad, A.A., S.V. Volik, A.W. Wyatt, A. Haegert, S. Le Bihan, R.H. Bell, S.A. Anderson, B. McConeghy, R. Shukin, J. Bazov, J. Youngren, P. Paris, G. Thomas, E.J. Small, Y. Wang, M.E. Gleave, C.C. Collins, and K.N. Chi, *Androgen Receptor Gene Aberrations in Circulating Cell-Free DNA: Biomarkers of Therapeutic Resistance in Castration-Resistant Prostate Cancer.* Clin Cancer Res, **2015**. 21(10): p. 2315-24.
223. Zhao, X.Y., P.J. Malloy, A.V. Krishnan, S. Swami, N.M. Navone, D.M. Peehl, and D. Feldman, *Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor.* Nat Med, **2000**. 6(6): p. 703-6.

224. Carreira, S., A. Romanel, J. Goodall, E. Grist, R. Ferraldeschi, S. Miranda, D. Prandi, D. Lorente, J.S. Frenel, C. Pezaro, A. Omlin, D.N. Rodrigues, P. Flohr, N. Tunariu, S.d.B. J, F. Demichelis, and G. Attard, *Tumor clone dynamics in lethal prostate cancer.* Sci Transl Med, **2014**. 6(254): p. 254ra125.
225. Attard, G., A.H. Reid, R.J. Auchus, B.A. Hughes, A.M. Cassidy, E. Thompson, N.B. Oommen, E. Folkerd, M. Dowsett, W. Arlt, and J.S. de Bono, *Clinical and biochemical consequences of CYP17A1 inhibition with abiraterone given with and without exogenous glucocorticoids in castrate men with advanced prostate cancer.* J Clin Endocrinol Metab, **2012**. 97(2): p. 507-16.
226. Chen, E.J., A.G. Sowalsky, S. Gao, C. Cai, O. Voznesensky, R. Schaefer, M. Loda, L.D. True, H. Ye, P. Troncoso, R.L. Lis, P.W. Kantoff, R.B. Montgomery, P.S. Nelson, G.J. Bubley, S.P. Balk, and M.E. Taplin, *Abiraterone treatment in castration-resistant prostate cancer selects for progesterone responsive mutant androgen receptors.* Clin Cancer Res, **2015**. 21(6): p. 1273-80.
227. Zoubeidi, A., A. Zardan, E. Beraldi, L. Fazli, R. Sowery, P. Rennie, C. Nelson, and M. Gleave, *Cooperative interactions between androgen receptor (AR) and heat-shock protein 27 facilitate AR transcriptional activity.* Cancer Res, **2007**. 67(21): p. 10455-65.
228. Qin, J., H.J. Lee, S.P. Wu, S.C. Lin, R.B. Lanz, C.J. Creighton, F.J. DeMayo, S.Y. Tsai, and M.J. Tsai, *Androgen deprivation-induced NCoA2 promotes metastatic and castration-resistant prostate cancer.* J Clin Invest, **2014**. 124(11): p. 5013-26.
229. Grasso, C.S., Y.M. Wu, D.R. Robinson, X. Cao, S.M. Dhanasekaran, A.P. Khan, M.J. Quist, X. Jing, R.J. Lonigro, J.C. Brenner, I.A. Asangani, B. Ateeq, S.Y. Chun, J. Siddiqui, L. Sam, M. Anstett, R. Mehra, J.R. Prensner, N. Palanisamy, G.A. Ryslik, F. Vandin, B.J. Raphael, L.P. Kunju, D.R. Rhodes, K.J. Pienta, A.M. Chinnaiyan, and S.A. Tomlins, *The mutational landscape of lethal castration-resistant prostate cancer.* Nature, **2012**. 487(7406): p. 239-43.
230. Schiewer, M.J., J.F. Goodwin, S. Han, J.C. Brenner, M.A. Augello, J.L. Dean, F. Liu, J.L. Planck, P. Ravindranathan, A.M. Chinnaiyan, P. McCue, L.G. Gomella, G.V. Raj, A.P. Dicker, J.R. Brody, J.M. Pascal, M.M. Centenera, L.M. Butler, W.D. Tilley, F.Y. Feng, and K.E. Knudsen, *Dual roles of PARP-1 promote cancer growth and progression.* Cancer Discov, **2012**. 2(12): p. 1134-49.
231. Beltran, H., D.S. Rickman, K. Park, S.S. Chae, A. Sboner, T.Y. MacDonald, Y. Wang, K.L. Sheikh, S. Terry, S.T. Tagawa, R. Dhir, J.B. Nelson, A. de la Taille, Y. Allory, M.B. Gerstein, S. Perner, K.J. Pienta, A.M. Chinnaiyan, Y. Wang, C.C. Collins, M.E. Gleave, F. Demichelis, D.M. Nanus, and M.A. Rubin, *Molecular characterization of neuroendocrine prostate cancer and identification of new drug targets.* Cancer Discov, **2011**. 1(6): p. 487-95.
232. Lin, D., A.W. Wyatt, H. Xue, Y. Wang, X. Dong, A. Haegert, R. Wu, S. Brahmbhatt, F. Mo, L. Jong, R.H. Bell, S. Anderson, A. Hurtado-Coll, L. Fazli, M. Sharma, H. Beltran, M. Rubin, M. Cox, P.W. Gout, J. Morris, L. Goldenberg, S.V. Volik, M.E. Gleave, C.C. Collins, and Y. Wang, *High fidelity patient-derived xenografts for accelerating prostate cancer discovery and drug development.* Cancer Res, **2014**. 74(4): p. 1272-83.
233. Beltran, H., D. Prandi, J.M. Mosquera, M. Benelli, L. Puca, J. Cyrta, C. Marotz, E. Giannopoulou, B.V. Chakravarthi, S. Varambally, S.A. Tomlins, D.M. Nanus, S.T. Tagawa, E.M. Van Allen, O. Elemento, A. Sboner, L.A. Garraway, M.A. Rubin, and F.

Demichelis, *Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer.* Nat Med, **2016**. 22(3): p. 298-305.
234. Li, Y., N. Donmez, C. Sahinalp, N. Xie, Y. Wang, H. Xue, F. Mo, H. Beltran, M. Gleave, Y. Wang, C. Collins, and X. Dong, *SRRM4 Drives Neuroendocrine Transdifferentiation of Prostate Adenocarcinoma Under Androgen Receptor Pathway Inhibition.* Eur Urol, **2016**.
235. Lee, J.K., J.W. Phillips, B.A. Smith, J.W. Park, T. Stoyanova, E.F. McCaffrey, R. Baertsch, A. Sokolov, J.G. Meyerowitz, C. Mathis, D. Cheng, J.M. Stuart, K.M. Shokat, W.C. Gustafson, J. Huang, and O.N. Witte, *N-Myc Drives Neuroendocrine Prostate Cancer Initiated from Human Prostate Epithelial Cells.* Cancer Cell, **2016**. 29(4): p. 536-47.
236. Mills, I.G., *Maintaining and reprogramming genomic androgen receptor activity in prostate cancer.* Nat Rev Cancer, **2014**. 14(3): p. 187-98.
237. Mayeur, G.L., W.J. Kung, A. Martinez, C. Izumiya, D.J. Chen, and H.J. Kung, *Ku is a novel transcriptional recycling coactivator of the androgen receptor in prostate cancer cells.* J Biol Chem, **2005**. 280(11): p. 10827-33.
238. Wong, R.H., I. Chang, C.S. Hudak, S. Hyun, H.Y. Kwan, and H.S. Sul, *A role of DNA-PK for the metabolic gene regulation in response to insulin.* Cell, **2009**. 136(6): p. 1056-72.
239. Ju, B.G., V.V. Lunyak, V. Perissi, I. Garcia-Bassets, D.W. Rose, C.K. Glass, and M.G. Rosenfeld, *A topoisomerase IIbeta-mediated dsDNA break required for regulated transcription.* Science, **2006**. 312(5781): p. 1798-802.
240. Hughes, C., A. Murphy, C. Martin, E. Fox, M. Ring, O. Sheils, B. Loftus, and J. O'Leary, *Topoisomerase II-alpha expression increases with increasing Gleason score and with hormone insensitivity in prostate carcinoma.* J Clin Pathol, **2006**. 59(7): p. 721-4.
241. de Resende, M.F., S. Vieira, L.T. Chinen, F. Chiappelli, F.P. da Fonseca, G.C. Guimaraes, F.A. Soares, I. Neves, S. Pagotty, P.A. Pellionisz, A. Barkhordarian, X. Brant, and R.M. Rocha, *Prognostication of prostate cancer based on TOP2A protein and gene assessment: TOP2A in prostate cancer.* J Transl Med, **2013**. 11: p. 36.
242. Rhee, K.Y., M. Opel, E. Ito, S. Hung, S.M. Arfin, and G.W. Hatfield, *Transcriptional coupling between the divergent promoters of a prototypic LysR-type regulatory system, the ilvYC operon of Escherichia coli.* Proc Natl Acad Sci U S A, **1999**. 96(25): p. 14294-9.
243. Pommier, Y., E. Leo, H. Zhang, and C. Marchand, *DNA topoisomerases and their poisoning by anticancer and antibacterial drugs.* Chem Biol, **2010**. 17(5): p. 421-33.
244. Wang, J.C., *Cellular roles of DNA topoisomerases: a molecular perspective.* Nat Rev Mol Cell Biol, **2002**. 3(6): p. 430-40.
245. Champoux, J.J., *DNA topoisomerases: structure, function, and mechanism.* Annu Rev Biochem, **2001**. 70: p. 369-413.
246. Berger, J.M., S.J. Gamblin, S.C. Harrison, and J.C. Wang, *Structure and mechanism of DNA topoisomerase II.* Nature, **1996**. 379(6562): p. 225-32.
247. Wilstermann, A.M. and N. Osheroff, *Positioning the 3'-DNA terminus for topoisomerase II-mediated religation.* J Biol Chem, **2001**. 276(21): p. 17727-31.
248. Schmidt, B.H., A.B. Burgin, J.E. Deweese, N. Osheroff, and J.M. Berger, *A novel and unified two-metal mechanism for DNA cleavage by type II and IA topoisomerases.* Nature, **2010**. 465(7298): p. 641-4.

249. Joshi, R.S., B. Pina, and J. Roca, *Topoisomerase II is required for the production of long Pol II gene transcripts in yeast.* Nucleic Acids Res, **2012**. 40(16): p. 7907-15.
250. Pommier, Y., Y. Sun, S.N. Huang, and J.L. Nitiss, *Roles of eukaryotic topoisomerases in transcription, replication and genomic stability.* Nat Rev Mol Cell Biol, **2016**. 17(11): p. 703-721.
251. Munoz, P., F. Baus, and J. Piette, *Ku antigen is required to relieve G2 arrest caused by inhibition of DNA topoisomerase II activity by the bisdioxopiperazine ICRF-193.* Oncogene, **2001**. 20(16): p. 1990-9.
252. Bailly, C., *Contemporary challenges in the design of topoisomerase II inhibitors for cancer chemotherapy.* Chem Rev, **2012**. 112(7): p. 3611-40.
253. Fortune, J.M. and N. Osheroff, *Topoisomerase II as a target for anticancer drugs: when enzymes stop being nice.* Prog Nucleic Acid Res Mol Biol, **2000**. 64: p. 221-53.
254. D'Arpa, P., C. Beardmore, and L.F. Liu, *Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons.* Cancer Res, **1990**. 50(21): p. 6919-24.
255. Kaufmann, S.H., *Cell death induced by topoisomerase-targeted drugs: more questions than answers.* Biochim Biophys Acta, **1998**. 1400(1-3): p. 195-211.
256. Hartmann, J.T. and H.P. Lipp, *Camptothecin and podophyllotoxin derivatives: inhibitors of topoisomerase I and II - mechanisms of action, pharmacokinetics and toxicity profile.* Drug Saf, **2006**. 29(3): p. 209-30.
257. Baldwin, E.L. and N. Osheroff, *Etoposide, topoisomerase II and cancer.* Curr Med Chem Anticancer Agents, **2005**. 5(4): p. 363-72.
258. Hussain, M.H., K.J. Pienta, B.G. Redman, G.D. Cummings, and L.E. Flaherty, *Oral etoposide in the treatment of hormone-refractory prostate cancer.* Cancer, **1994**. 74(1): p. 100-3.
259. Tannock, I.F., D. Osoba, M.R. Stockler, D.S. Ernst, A.J. Neville, M.J. Moore, G.R. Armitage, J.J. Wilson, P.M. Venner, C.M. Coppin, and K.C. Murphy, *Chemotherapy with mitoxantrone plus prednisone or prednisone alone for symptomatic hormone-resistant prostate cancer: a Canadian randomized trial with palliative end points.* J Clin Oncol, **1996**. 14(6): p. 1756-64.
260. Flechon, A., D. Pouessel, C. Ferlay, D. Perol, P. Beuzeboc, G. Gravis, F. Joly, S. Oudard, G. Deplanque, S. Zanetta, P. Fargeot, F. Priou, J.P. Droz, and S. Culine, *Phase II study of carboplatin and etoposide in patients with anaplastic progressive metastatic castration-resistant prostate cancer (mCRPC) with or without neuroendocrine differentiation: results of the French Genito-Urinary Tumor Group (GETUG) P01 trial.* Ann Oncol, **2011**. 22(11): p. 2476-81.
261. Nitiss, J.L., *DNA topoisomerase II and its growing repertoire of biological functions.* Nat Rev Cancer, **2009**. 9(5): p. 327-37.
262. Nitiss, J.L., *Targeting DNA topoisomerase II in cancer chemotherapy.* Nat Rev Cancer, **2009**. 9(5): p. 338-50.
263. Sabisz, M. and A. Skladanowski, *Cancer stem cells and escape from drug-induced premature senescence in human lung tumor cells: implications for drug resistance and in vitro drug screening models.* Cell Cycle, **2009**. 8(19): p. 3208-17.
264. Lu, D.Y., M. Huang, C.H. Xu, W.Y. Yang, C.X. Hu, L.P. Lin, L.J. Tong, M.H. Li, W. Lu, X.W. Zhang, and J. Ding, *Anti-proliferative effects, cell cycle G2/M phase arrest and blocking of chromosome segregation by probimane and MST-16 in human tumor cell lines.* BMC Pharmacol, **2005**. 5: p. 11.

265. Creighton, A.M., K. Hellmann, and S. Whitecross, *Antitumour activity in a series of bisdiketopiperazines.* Nature, **1969**. 222(5191): p. 384-5.
266. Classen, S., S. Olland, and J.M. Berger, *Structure of the topoisomerase II ATPase region and its mechanism of inhibition by the chemotherapeutic agent ICRF-187.* Proc Natl Acad Sci U S A, **2003**. 100(19): p. 10629-34.
267. Xiao, H., Y. Mao, S.D. Desai, N. Zhou, C.Y. Ting, J. Hwang, and L.F. Liu, *The topoisomerase IIbeta circular clamp arrests transcription and signals a 26S proteasome pathway.* Proc Natl Acad Sci U S A, **2003**. 100(6): p. 3239-44.
268. Thomas, C., A. Zoubeidi, H. Kuruma, L. Fazli, F. Lamoureux, E. Beraldi, B.P. Monia, A.R. MacLeod, J.W. Thuroff, and M.E. Gleave, *Transcription factor Stat5 knockdown enhances androgen receptor degradation and delays castration-resistant prostate cancer progression in vivo.* Mol Cancer Ther, **2011**. 10(2): p. 347-59.
269. Grosse, L., S. Paquet, P. Caron, L. Fazli, P.S. Rennie, A. Belanger, and O. Barbier, *Androgen glucuronidation: an unexpected target for androgen deprivation therapy, with prognosis and diagnostic implications.* Cancer Res, **2013**. 73(23): p. 6963-71.
270. Yamamoto, Y., Y. Loriot, E. Beraldi, F. Zhang, A.W. Wyatt, N. Al Nakouzi, F. Mo, T. Zhou, Y. Kim, B.P. Monia, A.R. MacLeod, L. Fazli, Y. Wang, C.C. Collins, A. Zoubeidi, and M. Gleave, *Generation 2.5 antisense oligonucleotides targeting the androgen receptor and its splice variants suppress enzalutamide-resistant prostate cancer cell growth.* Clin Cancer Res, **2015**. 21(7): p. 1675-87.
271. Li, Y., N. Xie, M.E. Gleave, P.S. Rennie, and X. Dong, *AR-v7 protein expression is regulated by protein kinase and phosphatase.* Oncotarget, **2015**. 6(32): p. 33743-54.
272. Yu, Y., J.S. Lee, N. Xie, E. Li, A. Hurtado-Coll, L. Fazli, M. Cox, S. Plymate, M. Gleave, and X. Dong, *Prostate stromal cells express the progesterone receptor to control cancer cell mobility.* PLoS One, **2014**. 9(3): p. e92714.
273. Li, H., N. Xie, M.E. Gleave, and X. Dong, *Catalytic inhibitors of DNA topoisomerase II suppress the androgen receptor signaling and prostate cancer progression.* Oncotarget, **2015**. 6(24): p. 20474-84.
274. Xie, N., L. Liu, Y. Li, C. Yu, S. Lam, O. Shynlova, M. Gleave, J.R. Challis, S. Lye, and X. Dong, *Expression and function of myometrial PSF suggest a role in progesterone withdrawal and the initiation of labor.* Mol Endocrinol, **2012**. 26(8): p. 1370-9.
275. Dong, X., J. Sweet, J.R. Challis, T. Brown, and S.J. Lye, *Transcriptional activity of androgen receptor is modulated by two RNA splicing factors, PSF and p54nrb.* Mol Cell Biol, **2007**. 27(13): p. 4863-75.
276. Yu, Y., L. Liu, N. Xie, H. Xue, L. Fazli, R. Buttyan, Y. Wang, M. Gleave, and X. Dong, *Expression and function of the progesterone receptor in human prostate stroma provide novel insights to cell proliferation control.* J Clin Endocrinol Metab, **2013**. 98(7): p. 2887-96.
277. Li, H., Y. Li, D. Morin, S. Plymate, S. Lye, and X. Dong, *The androgen receptor mediates antiapoptotic function in myometrial cells.* Cell Death Dis, **2014**. 5: p. e1338.
278. Liu, L., Y. Li, N. Xie, O. Shynlova, J.R. Challis, D. Slater, S. Lye, and X. Dong, *Proliferative action of the androgen receptor in human uterine myometrial cells--a key regulator for myometrium phenotype programming.* J Clin Endocrinol Metab, **2013**. 98(1): p. 218-27.

279. Chen, Z., C. Zhang, D. Wu, H. Chen, A. Rorick, X. Zhang, and Q. Wang, *Phospho-MED1-enhanced UBE2C locus looping drives castration-resistant prostate cancer growth.* EMBO J, **2011**. 30(12): p. 2405-19.
280. Nadeem, L., O. Shynlova, E. Matysiak-Zablocki, S. Mesiano, X. Dong, and S. Lye, *Molecular evidence of functional progesterone withdrawal in human myometrium.* Nat Commun, **2016**. 7: p. 11565.
281. Kurz, T., D. Grant, R.G. Andersson, R. Towart, M. De Cesare, and J.O. Karlsson, *Effects of MnDPDP and ICRF-187 on Doxorubicin-Induced Cardiotoxicity and Anticancer Activity.* Transl Oncol, **2012**. 5(4): p. 252-9.
282. Martin, E., A.V. Thougaard, M. Grauslund, P.B. Jensen, F. Bjorkling, B.B. Hasinoff, J. Tjornelund, M. Sehested, and L.H. Jensen, *Evaluation of the topoisomerase II-inactive bisdioxopiperazine ICRF-161 as a protectant against doxorubicin-induced cardiomyopathy.* Toxicology, **2009**. 255(1-2): p. 72-9.
283. Lonergan, P.E. and D.J. Tindall, *Androgen receptor signaling in prostate cancer development and progression.* J Carcinog, **2011**. 10: p. 20.
284. Scher, H.I., K. Fizazi, F. Saad, M.E. Taplin, C.N. Sternberg, K. Miller, R. de Wit, P. Mulders, K.N. Chi, N.D. Shore, A.J. Armstrong, T.W. Flaig, A. Flechon, P. Mainwaring, M. Fleming, J.D. Hainsworth, M. Hirmand, B. Selby, L. Seely, J.S. de Bono, and A. Investigators, *Increased survival with enzalutamide in prostate cancer after chemotherapy.* N Engl J Med, **2012**. 367(13): p. 1187-97.
285. Wang, Q., W. Li, Y. Zhang, X. Yuan, K. Xu, J. Yu, Z. Chen, R. Beroukhim, H. Wang, M. Lupien, T. Wu, M.M. Regan, C.A. Meyer, J.S. Carroll, A.K. Manrai, O.A. Janne, S.P. Balk, R. Mehra, B. Han, A.M. Chinnaiyan, M.A. Rubin, L. True, M. Fiorentino, C. Fiore, M. Loda, P.W. Kantoff, X.S. Liu, and M. Brown, *Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer.* Cell, **2009**. 138(2): p. 245-56.
286. Stanbrough, M., G.J. Bubley, K. Ross, T.R. Golub, M.A. Rubin, T.M. Penning, P.G. Febbo, and S.P. Balk, *Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer.* Cancer Res, **2006**. 66(5): p. 2815-25.
287. Gregory, C.W., X. Fei, L.A. Ponguta, B. He, H.M. Bill, F.S. French, and E.M. Wilson, *Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer.* J Biol Chem, **2004**. 279(8): p. 7119-30.
288. Krueckl, S.L., R.A. Sikes, N.M. Edlund, R.H. Bell, A. Hurtado-Coll, L. Fazli, M.E. Gleave, and M.E. Cox, *Increased insulin-like growth factor I receptor expression and signaling are components of androgen-independent progression in a lineage-derived prostate cancer progression model.* Cancer Res, **2004**. 64(23): p. 8620-9.
289. Kraus, S., D. Gioeli, T. Vomastek, V. Gordon, and M.J. Weber, *Receptor for activated C kinase 1 (RACK1) and Src regulate the tyrosine phosphorylation and function of the androgen receptor.* Cancer Res, **2006**. 66(22): p. 11047-54.
290. Yang, C.C., L. Fazli, S. Loguercio, I. Zharkikh, P. Aza-Blanc, M.E. Gleave, and D.A. Wolf, *Downregulation of c-SRC kinase CSK promotes castration resistant prostate cancer and pinpoints a novel disease subclass.* Oncotarget, **2015**. 6(26): p. 22060-71.
291. Nam, S., D. Kim, J.Q. Cheng, S. Zhang, J.H. Lee, R. Buettner, J. Mirosevich, F.Y. Lee, and R. Jove, *Action of the Src family kinase inhibitor, dasatinib (BMS-354825), on human prostate cancer cells.* Cancer Res, **2005**. 65(20): p. 9185-9.

292. Zhao, J.C., J. Yu, C. Runkle, L. Wu, M. Hu, D. Wu, J.S. Liu, Q. Wang, Z.S. Qin, and J. Yu, *Cooperation between Polycomb and androgen receptor during oncogenic transformation.* Genome Res, **2012**. 22(2): p. 322-31.
293. He, B., R.T. Gampe, Jr., A.J. Kole, A.T. Hnat, T.B. Stanley, G. An, E.L. Stewart, R.I. Kalman, J.T. Minges, and E.M. Wilson, *Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance.* Mol Cell, **2004**. 16(3): p. 425-38.
294. Chamberlain, N.L., D.C. Whitacre, and R.L. Miesfeld, *Delineation of two distinct type 1 activation functions in the androgen receptor amino-terminal domain.* J Biol Chem, **1996**. 271(43): p. 26772-8.
295. Dehm, S.M. and D.J. Tindall, *Ligand-independent androgen receptor activity is activation function-2-independent and resistant to antiandrogens in androgen refractory prostate cancer cells.* J Biol Chem, **2006**. 281(38): p. 27882-93.
296. Dehm, S.M., K.M. Regan, L.J. Schmidt, and D.J. Tindall, *Selective role of an NH2-terminal WxxLF motif for aberrant androgen receptor activation in androgen depletion independent prostate cancer cells.* Cancer Res, **2007**. 67(20): p. 10067-77.
297. Qin, J., X. Liu, B. Laffin, X. Chen, G. Choy, C.R. Jeter, T. Calhoun-Davis, H. Li, G.S. Palapattu, S. Pang, K. Lin, J. Huang, I. Ivanov, W. Li, M.V. Suraneni, and D.G. Tang, *The PSA(-/lo) prostate cancer cell population harbors self-renewing long-term tumor-propagating cells that resist castration.* Cell Stem Cell, **2012**. 10(5): p. 556-69.
298. McGuire, B.B., B.T. Helfand, S. Loeb, Q. Hu, D. O'Brien, P. Cooper, X. Yang, and W.J. Catalona, *Outcomes in patients with Gleason score 8-10 prostate cancer: relation to preoperative PSA level.* BJU Int, **2012**. 109(12): p. 1764-9.
299. Mostaghel, E.A., B.T. Marck, S.R. Plymate, R.L. Vessella, S. Balk, A.M. Matsumoto, P.S. Nelson, and R.B. Montgomery, *Resistance to CYP17A1 inhibition with abiraterone in castration-resistant prostate cancer: induction of steroidogenesis and androgen receptor splice variants.* Clin Cancer Res, **2011**. 17(18): p. 5913-25.
300. Monteleone, M.C., A.E. Gonzalez Wusener, J.E. Burdisso, C. Conde, A. Caceres, and C.O. Arregui, *ER-bound protein tyrosine phosphatase PTP1B interacts with Src at the plasma membrane/substrate interface.* PLoS One, **2012**. 7(6): p. e38948.
301. Gleave, M.E. and B.P. Monia, *Antisense therapy for cancer.* Nat Rev Cancer, **2005**. 5(6): p. 468-79.
302. Beaulieu, M., E. Levesque, D.W. Hum, and A. Belanger, *Isolation and characterization of a novel cDNA encoding a human UDP-glucuronosyltransferase active on C19 steroids.* J Biol Chem, **1996**. 271(37): p. 22855-62.
303. Takeda, S., Y. Ishii, M. Iwanaga, P.I. Mackenzie, K. Nagata, Y. Yamazoe, K. Oguri, and H. Yamada, *Modulation of UDP-glucuronosyltransferase function by cytochrome P450: evidence for the alteration of UGT2B7-catalyzed glucuronidation of morphine by CYP3A4.* Mol Pharmacol, **2005**. 67(3): p. 665-72.
304. Kiang, T.K., M.H. Ensom, and T.K. Chang, *UDP-glucuronosyltransferases and clinical drug-drug interactions.* Pharmacol Ther, **2005**. 106(1): p. 97-132.
305. Gibbons, J.A., M. de Vries, W. Krauwinkel, Y. Ohtsu, J. Noukens, J.S. van der Walt, R. Mol, J. Mordenti, and T. Ouatas, *Pharmacokinetic Drug Interaction Studies with Enzalutamide.* Clin Pharmacokinet, **2015**. 54(10): p. 1057-69.
306. Kaeding, J., E. Bouchaert, J. Belanger, P. Caron, S. Chouinard, M. Verreault, O. Larouche, G. Pelletier, B. Staels, A. Belanger, and O. Barbier, *Activators of the farnesoid*

X receptor negatively regulate androgen glucuronidation in human prostate cancer LNCAP cells. Biochem J, **2008**. 410(2): p. 245-53.
307. Wijayakumara, D.D., D.G. Hu, R. Meech, R.A. McKinnon, and P.I. Mackenzie, *Regulation of Human UGT2B15 and UGT2B17 by miR-376c in Prostate Cancer Cell Lines.* J Pharmacol Exp Ther, **2015**. 354(3): p. 417-25.
308. Jenkinson, C., A. Petroczi, and D.P. Naughton, *Red wine and component flavonoids inhibit UGT2B17 in vitro.* Nutr J, **2012**. 11: p. 67.
309. Liao, S., Y. Umekita, J. Guo, J.M. Kokontis, and R.A. Hiipakka, *Growth inhibition and regression of human prostate and breast tumors in athymic mice by tea epigallocatechin gallate.* Cancer Lett, **1995**. 96(2): p. 239-43.
310. Jenkinson, C., A. Petroczi, J. Barker, and D.P. Naughton, *Dietary green and white teas suppress UDP-glucuronosyltransferase UGT2B17 mediated testosterone glucuronidation.* Steroids, **2012**. 77(6): p. 691-5.
311. Saad, F., *Src as a therapeutic target in men with prostate cancer and bone metastases.* BJU Int, **2009**. 103(4): p. 434-40.
312. Migliaccio, A., L. Varricchio, A. De Falco, G. Castoria, C. Arra, H. Yamaguchi, A. Ciociola, M. Lombardi, R. Di Stasio, A. Barbieri, A. Baldi, M.V. Barone, E. Appella, and F. Auricchio, *Inhibition of the SH3 domain-mediated binding of Src to the androgen receptor and its effect on tumor growth.* Oncogene, **2007**. 26(46): p. 6619-29.
313. Araujo, J.C., A. Poblenz, P. Corn, N.U. Parikh, M.W. Starbuck, J.T. Thompson, F. Lee, C.J. Logothetis, and B.G. Darnay, *Dasatinib inhibits both osteoclast activation and prostate cancer PC-3-cell-induced osteoclast formation.* Cancer Biol Ther, **2009**. 8(22): p. 2153-9.
314. Koreckij, T., H. Nguyen, L.G. Brown, E.Y. Yu, R.L. Vessella, and E. Corey, *Dasatinib inhibits the growth of prostate cancer in bone and provides additional protection from osteolysis.* Br J Cancer, **2009**. 101(2): p. 263-8.
315. Araujo, J.C., P. Mathew, A.J. Armstrong, E.L. Braud, E. Posadas, M. Lonberg, G.E. Gallick, G.C. Trudel, P. Paliwal, S. Agrawal, and C.J. Logothetis, *Dasatinib combined with docetaxel for castration-resistant prostate cancer: results from a phase 1-2 study.* Cancer, **2012**. 118(1): p. 63-71.
316. Yu, E.Y., G. Wilding, E. Posadas, M. Gross, S. Culine, C. Massard, M.J. Morris, G. Hudes, F. Calabro, S. Cheng, G.C. Trudel, P. Paliwal, and C.N. Sternberg, *Phase II study of dasatinib in patients with metastatic castration-resistant prostate cancer.* Clin Cancer Res, **2009**. 15(23): p. 7421-8.
317. Araujo, J.C., G.C. Trudel, F. Saad, A.J. Armstrong, E.Y. Yu, J. Bellmunt, G. Wilding, J. McCaffrey, S.V. Serrano, V.B. Matveev, E. Efstathiou, S. Oudard, M.J. Morris, B. Sizer, P.J. Goebell, A. Heidenreich, J.S. de Bono, S. Begbie, J.H. Hong, E. Richardet, E. Gallardo, P. Paliwal, S. Durham, S. Cheng, and C.J. Logothetis, *Docetaxel and dasatinib or placebo in men with metastatic castration-resistant prostate cancer (READY): a randomised, double-blind phase 3 trial.* Lancet Oncol, **2013**. 14(13): p. 1307-16.
318. Lombardo, L.J., F.Y. Lee, P. Chen, D. Norris, J.C. Barrish, K. Behnia, S. Castaneda, L.A. Cornelius, J. Das, A.M. Doweyko, C. Fairchild, J.T. Hunt, I. Inigo, K. Johnston, A. Kamath, D. Kan, H. Klei, P. Marathe, S. Pang, R. Peterson, S. Pitt, G.L. Schieven, R.J. Schmidt, J. Tokarski, M.L. Wen, J. Wityak, and R.M. Borzilleri, *Discovery of N-(2-chloro-6-methyl- phenyl)-2-(6-(4-(2-hydroxyethyl)- piperazin-1-yl)-2-methylpyrimidin-4-*

ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. J Med Chem, **2004**. 47(27): p. 6658-61.
319. Manley, P.W., S.W. Cowan-Jacob, and J. Mestan, *Advances in the structural biology, design and clinical development of Bcr-Abl kinase inhibitors for the treatment of chronic myeloid leukaemia.* Biochim Biophys Acta, **2005**. 1754(1-2): p. 3-13.
320. O'Hare, T., W.C. Shakespeare, X. Zhu, C.A. Eide, V.M. Rivera, F. Wang, L.T. Adrian, T. Zhou, W.S. Huang, Q. Xu, C.A. Metcalf, 3rd, J.W. Tyner, M.M. Loriaux, A.S. Corbin, S. Wardwell, Y. Ning, J.A. Keats, Y. Wang, R. Sundaramoorthi, M. Thomas, D. Zhou, J. Snodgrass, L. Commodore, T.K. Sawyer, D.C. Dalgarno, M.W. Deininger, B.J. Druker, and T. Clackson, *AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance.* Cancer Cell, **2009**. 16(5): p. 401-12.
321. Antonarakis, E.S., C. Lu, H. Wang, B. Luber, M. Nakazawa, J.C. Roeser, Y. Chen, T.A. Mohammad, H.L. Fedor, T.L. Lotan, Q. Zheng, A.M. De Marzo, J.T. Isaacs, W.B. Isaacs, R. Nadal, C.J. Paller, S.R. Denmeade, M.A. Carducci, M.A. Eisenberger, and J. Luo, *AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer.* N Engl J Med, **2014**. 371(11): p. 1028-38.
322. Nadal, R., M.E. Taplin, and J. Bellmunt, *Enzalutamide for the treatment of prostate cancer: results and implications of the AFFIRM trial.* Future Oncol, **2014**. 10(3): p. 351-62.
323. Cai, C., H.H. He, S. Chen, I. Coleman, H. Wang, Z. Fang, P.S. Nelson, X.S. Liu, M. Brown, and S.P. Balk, *Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1.* Cancer Cell, **2011**. 20(4): p. 457-71.
324. Lin, C., L. Yang, B. Tanasa, K. Hutt, B.G. Ju, K. Ohgi, J. Zhang, D.W. Rose, X.D. Fu, C.K. Glass, and M.G. Rosenfeld, *Nuclear receptor-induced chromosomal proximity and DNA breaks underlie specific translocations in cancer.* Cell, **2009**. 139(6): p. 1069-83.
325. Schmidt, F., C.B. Knobbe, B. Frank, H. Wolburg, and M. Weller, *The topoisomerase II inhibitor, genistein, induces G2/M arrest and apoptosis in human malignant glioma cell lines.* Oncol Rep, **2008**. 19(4): p. 1061-6.
326. Bower, J.J., Y. Zhou, T. Zhou, D.A. Simpson, S.J. Arlander, R.S. Paules, M. Cordeiro-Stone, and W.K. Kaufmann, *Revised genetic requirements for the decatenation G2 checkpoint: the role of ATM.* Cell Cycle, **2010**. 9(8): p. 1617-28.
327. Morris, S.K., C.L. Baird, and J.E. Lindsley, *Steady-state and rapid kinetic analysis of topoisomerase II trapped as the closed-clamp intermediate by ICRF-193.* J Biol Chem, **2000**. 275(4): p. 2613-8.
328. Ishida, R., M. Sato, T. Narita, K.R. Utsumi, T. Nishimoto, T. Morita, H. Nagata, and T. Andoh, *Inhibition of DNA topoisomerase II by ICRF-193 induces polyploidization by uncoupling chromosome dynamics from other cell cycle events.* J Cell Biol, **1994**. 126(6): p. 1341-51.
329. Taplin, M.E., G.J. Bubley, T.D. Shuster, M.E. Frantz, A.E. Spooner, G.K. Ogata, H.N. Keer, and S.P. Balk, *Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer.* N Engl J Med, **1995**. 332(21): p. 1393-8.
330. Taylor, B.S., N. Schultz, H. Hieronymus, A. Gopalan, Y. Xiao, B.S. Carver, V.K. Arora, P. Kaushik, E. Cerami, B. Reva, Y. Antipin, N. Mitsiades, T. Landers, I. Dolgalev, J.E. Major, M. Wilson, N.D. Socci, A.E. Lash, A. Heguy, J.A. Eastham, H.I. Scher, V.E.

Reuter, P.T. Scardino, C. Sander, C.L. Sawyers, and W.L. Gerald, *Integrative genomic profiling of human prostate cancer.* Cancer Cell, **2010**. 18(1): p. 11-22.
331. Shapiro, A.B. and C.A. Austin, *A high-throughput fluorescence anisotropy-based assay for human topoisomerase II beta-catalyzed ATP-dependent supercoiled DNA relaxation.* Anal Biochem, **2014**. 448: p. 23-9.
332. Fortune, J.M. and N. Osheroff, *Merbarone inhibits the catalytic activity of human topoisomerase IIalpha by blocking DNA cleavage.* J Biol Chem, **1998**. 273(28): p. 17643-50.
333. Larsen, A.K., A.E. Escargueil, and A. Skladanowski, *Catalytic topoisomerase II inhibitors in cancer therapy.* Pharmacol Ther, **2003**. 99(2): p. 167-81.
334. Denmeade, S.R. and J.T. Isaacs, *Bipolar androgen therapy: the rationale for rapid cycling of supraphysiologic androgen/ablation in men with castration resistant prostate cancer.* Prostate, **2010**. 70(14): p. 1600-7.
335. Schweizer, M.T., E.S. Antonarakis, H. Wang, A.S. Ajiboye, A. Spitz, H. Cao, J. Luo, M.C. Haffner, S. Yegnasubramanian, M.A. Carducci, M.A. Eisenberger, J.T. Isaacs, and S.R. Denmeade, *Effect of bipolar androgen therapy for asymptomatic men with castration-resistant prostate cancer: results from a pilot clinical study.* Sci Transl Med, **2015**. 7(269): p. 269ra2.
336. Jarvius, M., J. Paulsson, I. Weibrecht, K.J. Leuchowius, A.C. Andersson, C. Wahlby, M. Gullberg, J. Botling, T. Sjoblom, B. Markova, A. Ostman, U. Landegren, and O. Soderberg, *In situ detection of phosphorylated platelet-derived growth factor receptor beta using a generalized proximity ligation method.* Mol Cell Proteomics, **2007**. 6(9): p. 1500-9.
337. Gao, J., B.A. Aksoy, U. Dogrusoz, G. Dresdner, B. Gross, S.O. Sumer, Y. Sun, A. Jacobsen, R. Sinha, E. Larsson, E. Cerami, C. Sander, and N. Schultz, *Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal.* Sci Signal, **2013**. 6(269): p. pl1.
338. Cerami, E., J. Gao, U. Dogrusoz, B.E. Gross, S.O. Sumer, B.A. Aksoy, A. Jacobsen, C.J. Byrne, M.L. Heuer, E. Larsson, Y. Antipin, B. Reva, A.P. Goldberg, C. Sander, and N. Schultz, *The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data.* Cancer Discov, **2012**. 2(5): p. 401-4.
339. Rhodes, D.R., J. Yu, K. Shanker, N. Deshpande, R. Varambally, D. Ghosh, T. Barrette, A. Pandey, and A.M. Chinnaiyan, *ONCOMINE: a cancer microarray database and integrated data-mining platform.* Neoplasia, **2004**. 6(1): p. 1-6.
340. Barrett, T., S.E. Wilhite, P. Ledoux, C. Evangelista, I.F. Kim, M. Tomashevsky, K.A. Marshall, K.H. Phillippy, P.M. Sherman, M. Holko, A. Yefanov, H. Lee, N. Zhang, C.L. Robertson, N. Serova, S. Davis, and A. Soboleva, *NCBI GEO: archive for functional genomics data sets--update.* Nucleic Acids Res, **2013**. 41(Database issue): p. D991-5.
341. Ran, F.A., P.D. Hsu, C.Y. Lin, J.S. Gootenberg, S. Konermann, A.E. Trevino, D.A. Scott, A. Inoue, S. Matoba, Y. Zhang, and F. Zhang, *Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity.* Cell, **2013**. 154(6): p. 1380-9.
342. Cyranoski, D., *Chinese scientists to pioneer first human CRISPR trial.* Nature, **2016**. 535(7613): p. 476-7.
343. Chakraborty, S.K., N.K. Basu, S. Jana, M. Basu, A. Raychoudhuri, and I.S. Owens, *Protein kinase Calpha and Src kinase support human prostate-distributed*

dihydrotestosterone-metabolizing UDP-glucuronosyltransferase 2B15 activity. J Biol Chem, **2012**. 287(29): p. 24387-96.
344. Hasinoff, B.B. and E.H. Herman, *Dexrazoxane: how it works in cardiac and tumor cells. Is it a prodrug or is it a drug?* Cardiovasc Toxicol, **2007**. 7(2): p. 140-4.
345. Drake, F.H., G.A. Hofmann, H.F. Bartus, M.R. Mattern, S.T. Crooke, and C.K. Mirabelli, *Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II.* Biochemistry, **1989**. 28(20): p. 8154-60.
346. Drake, F.H., G.A. Hofmann, S.M. Mong, J.O. Bartus, R.P. Hertzberg, R.K. Johnson, M.R. Mattern, and C.K. Mirabelli, *In vitro and intracellular inhibition of topoisomerase II by the antitumor agent merbarone.* Cancer Res, **1989**. 49(10): p. 2578-83.
347. Slavik, M., P.Y. Liu, E.H. Kraut, R.B. Natale, L.E. Flaherty, and V.K. Sondak, *Evaluation of merbarone (NSC 336628) in disseminated malignant melanoma. A Southwest Oncology Group study.* Invest New Drugs, **1995**. 13(2): p. 143-7.
348. Jensen, L.H., A.V. Thougaard, M. Grauslund, B. Sokilde, E.V. Carstensen, H.K. Dvinge, D.A. Scudiero, P.B. Jensen, R.H. Shoemaker, and M. Sehested, *Substituted purine analogues define a novel structural class of catalytic topoisomerase II inhibitors.* Cancer Res, **2005**. 65(16): p. 7470-7.
349. Huang, H., Q. Chen, X. Ku, L. Meng, L. Lin, X. Wang, C. Zhu, Y. Wang, Z. Chen, M. Li, H. Jiang, K. Chen, J. Ding, and H. Liu, *A series of alpha-heterocyclic carboxaldehyde thiosemicarbazones inhibit topoisomerase IIalpha catalytic activity.* J Med Chem, **2010**. 53(8): p. 3048-64.
350. Tanabe, K., Y. Ikegami, R. Ishida, and T. Andoh, *Inhibition of topoisomerase II by antitumor agents bis(2,6-dioxopiperazine) derivatives.* Cancer Res, **1991**. 51(18): p. 4903-8.
351. Ishida, R., T. Miki, T. Narita, R. Yui, M. Sato, K.R. Utsumi, K. Tanabe, and T. Andoh, *Inhibition of intracellular topoisomerase II by antitumor bis(2,6-dioxopiperazine) derivatives: mode of cell growth inhibition distinct from that of cleavable complex-forming type inhibitors.* Cancer Res, **1991**. 51(18): p. 4909-16.
352. Ban, F., E. Leblanc, H. Li, R.S. Munuganti, K. Frewin, P.S. Rennie, and A. Cherkasov, *Discovery of 1H-indole-2-carboxamides as novel inhibitors of the androgen receptor binding function 3 (BF3).* J Med Chem, **2014**. 57(15): p. 6867-72.
353. Li, H., M.D. Hassona, N.A. Lack, P. Axerio-Cilies, E. Leblanc, P. Tavassoli, N. Kanaan, K. Frewin, K. Singh, H. Adomat, K.J. Bohm, H. Prinz, E.T. Guns, P.S. Rennie, and A. Cherkasov, *Characterization of a new class of androgen receptor antagonists with potential therapeutic application in advanced prostate cancer.* Mol Cancer Ther, **2013**. 12(11): p. 2425-35.
354. Rudolph, P., J. Peters, D. Lorenz, D. Schmidt, and R. Parwaresch, *Correlation between mitotic and Ki-67 labeling indices in paraffin-embedded carcinoma specimens.* Hum Pathol, **1998**. 29(11): p. 1216-22.

Appendix

I have the fortunate opportunity to participate in several projects during my doctoral studies.

Manuscripts from these projects are listed below.

List of Publications (during PhD study):

1. Li H, Xie N, Chen R, Verreault M, Fazli L, Gleave ME, Barbier O, Dong X. UGT2B17 Expedites Castration-Resistant Prostate Cancer Progression by Promoting Ligand-independent AR Signaling. ***Cancer Research***. 2016 Sep 22. pii: canres.1518.2016. PubMed PMID: 27659047.
2. Li H*, Yu Y*, Shi Y, Fazli L, Slater D, Lye S, Dong X. HoxA13 Stimulates Myometrial Cells to Secrete IL-1beta and Enhance the Expression of Contraction-Associated Proteins. ***Endocrinology***. 2016 May;157(5):2129-39. PubMed PMID: 26982635. (*co-first author)
3. Lan M, Li H, Bao L, Li M, Lye S, Dong X. *In vivo* Evidence of the Androgen Receptor in Association with Myometrial Cell Proliferation and Apoptosis. ***Reproductive Sciences***. 2016 Feb;23(2):264-71. PubMed PMID: 26342051.
4. Liu L*, Li H*, Dargahi D, Shynlova O, Slater D, Jones SJ, Dong X. HoxA13 Regulates Phenotype Regionalization of Human Pregnant Myometrium. ***The Journal of Clinical Endocrinology and Metabolism***. 2015 Dec;100(12):E1512-22. PubMed PMID: 26485220. (*co-first author)
5. Li H, Xie N, Gleave ME, Dong X. Catalytic inhibitors of DNA topoisomerase II suppress the androgen receptor signaling and prostate cancer progression. ***Oncotarget***. 2015 May 12. PubMed PMID: 26009876.
6. Li H, Li Y, Morin D, Plymate S, Lye S, Dong X. The androgen receptor mediates antiapoptotic function in myometrial cells. ***Cell Death & Disease***. 2014;5:e1338. PubMed PMID: 25032861.