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Deshmukh D, Yang X, **Xu J**, Qiu Y. RNF6 promotes ubiquitin-dependent degradation of the cyclin dependent kinase inhibitor p27Kip1 in prostate cancer. *(In preparation)*

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ABSTRACT

Title of Dissertation: Targeting the crosstalk between AR3 and E2F1 as a prospective therapy for drug-resistant prostate cancer

Jin Xu, Doctor of Philosophy, 2020

Dissertation Directed by: Yun Qiu, PhD, Professor of Pharmacology

Key words: prostate cancer, combination therapy, drug resistance, androgen receptor splice variants AR3 (a.k.a. AR-V7), E2F1, auranofin

Background: Drug resistance is one of the most prevalent causes of death in advanced prostate cancer patients. Combination therapies that target cancer cells via different mechanisms to overcome resistance have gained increased attention in recent years. However, the optimal drug combinations and the underlying mechanisms are yet to be fully explored.

Aim and methods: The aim of this study is to investigate drugs that inhibit the growth of cells that are resistant to standard chemo and androgen deprivation therapy, and determine the underlying mechanisms of their action. To achieve this aim, we established cell lines that are resistant to this standard combination drug treatment and tested new compounds to overcome this “double drug” resistance.

Results: Our results show that combination of enzalutamide (ENZ) and docetaxel (DTX) effectively inhibit the growth of prostate cancer cells that are resistant to either DTX or ENZ alone. The downregulation of transcription factor E2F1 plays a crucial role in cellular inhibition in response to the combined therapy. Notably, the androgen receptor (AR) variant AR3 (a.k.a. AR-V7), but not AR full length (AR-FL), positively regulates E2F1 expression in these cells. Specifically, E2F1 regulates AR3 and forms a positive regulatory feed-forward loop. Moreover, this drug combination treatment also results in DNA double strand break via the E2F1-AR3 signaling axis. Importantly, we established new drug-resistant cell lines that are resistant to ENZ+DTX combination therapy and found that the expression of both AR3 and E2F1 was restored in these double drug-resistant cells. Furthermore, we identified that auranofin, an FDA-approved drug for the treatment of rheumatoid arthritis, overcame the drug resistance and inhibited the growth of drug-resistant prostate cancer cells both *in vitro* and *in vivo*.

Conclusion and significance: This proof-of-principle study demonstrates that targeting the E2F1/AR3 feedforward loop via a combination therapy or a multi-targeting drug could circumvent castration resistance in prostate cancer.

Targeting the Crosstalk Between AR3 and E2F1 as A Prospective Therapy for Drug-Resistant Prostate Cancer

by

Jin Xu

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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Dedication

This thesis is dedicated to my friends and family who unconditionally supported me through this process. Thank you all for your love, encouragement and always believing in me over the years.

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Xu J, Qiu Y. Current opinion and mechanistic interpretation of combination therapy for castration-resistant prostate cancer. Asian journal of andrology. 2019 May;21(3):270.

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AA	Abiraterone
ACS	American Cancer Society
ADT	Androgen Deprivation Therapy
AR	Androgen Receptor
AR-FL	Androgen Receptor-Full Length
AR3	Androgen Receptor Splicing Variant 3 a.k.a. AR-V7
AR3Tg	AR3 transgenic
ARE	Androgen Response Element
ARV	Androgen Receptor Splicing Variant
BRD4	BET family protein bromodomain containing 4
CDK1	Cyclin-dependent kinase 1
CHAARTED	Chemo-Hormonal Therapy Versus Androgen Ablation Randomized Trial for Extensive Disease in Prostate Cancer
ChIP	Chromatin Immunoprecipitation
ChIP-seq	Chromatin Immunoprecipitation Sequencing
CLTA-4	anti-cytotoxic T-lymphocyte antigen-4

CRPC	Castration-Resistant Prostate Cancer
CSS	Charcoal-Stripped Serum
DBD	DNA-Binding Domain
DDR	DNA Damage Response
DHT	Dihydrotestosterone
DMEM	Dulbecco's modification of Eagle's medium
DREs	Digital Rectal Examinations
DTX	Docetaxel
E2F1-LUC	E2F1 promoter-driven luciferase reporter
EMT	Epithelial-Mesenchymal Transition
ENZ	Enzalutamide
FBS	Fetal Bovine Serum
FDA	U.S. Food and Drug Administration
FDR	False Discovery Rates
GSEA	Gene Set Enrichment Analysis
HER-2	Human Epidermal Growth Factor Receptor-2
HSP90	Heat Shock Protein 90

LBD	C-terminal Ligand Binding Domain
LHRH	Luteinizing Hormone Releasing Hormone
lncRNA	Long non-coding RNA
MDSCs	Myeloid-Derived Suppressor Cells
miRNAs	micro RNAs
NEPC	Neuroendocrine Prostate Cancer
NLS	Nuclear Localization Sequence
NTD	N-terminal Transactivation Domain
Pca	Prostate Cancer
PD-1	Anti-programmed cell death 1
PD-L1	Programmed cell death ligand-1
PIN	Prostate Intraepithelial Neoplasia
PSA	Prostate-Specific Antigen
qRT-PCR	Quantitative real-time PCR
RPMI	Roswell Park Memorial Institute
shRNA	Short hairpin RNA
SINT-1	Sintokamide A

siRNA	small-interfering RNA
STAMPEDE	Systemic Therapy in Advancing or Metastatic Prostate Cancer
TCGA	The Cancer Genome Atlas Program
TRUS	Transrectal Ultrasound
UPS	Ubiquitin-proteasome system

Chapter 1: Introduction

Prostate cancer

Prostate cancer (Pca) is one of the most lethal cancers in western countries. According to Cancer Facts & Figures estimates, 191,930 new cases will be diagnosed and 33,330 men will die from Pca in US in 2020. Nearly one in every nine men will be diagnosed with Pca in their lives. The most common risk factor of Pca is age. Other major risk factors include family history, hormones, race, diet and environmental agents (1). Patients with localized Pca do not develop a life-threatening syndrome and are curable by either surgery or radiation treatment. The 5-year survival rate in United States of an individual diagnosed with Pca is greater than 98% according to the American Cancer Society (ACS). However, the 5-year survival rate of those with more advanced metastases of Pca is only estimated to be 31%, with very limited treatment options. The androgen receptor (AR) signaling pathway plays a key role in the progression of Pca, and androgen deprivation therapy (ADT) is the standard treatment for advanced Pca. Unfortunately, a number of patients will relapse and inevitably develop more aggressive metastatic castration-resistant prostate cancer (CRPC). Although there are some U.S. Food and Drug Administration (FDA)-approved treatment options for CRPC (i.e., taxane compounds, abiraterone, enzalutamide, and sipuleucel-T), drug resistance will eventually develop. Currently, there is no effective treatment for relapsed CRPC (2). Therefore, there is an urgent need to elucidate the underlying molecular mechanisms of drug resistance of CRPC and develop new therapies for CRPC.

Epidemiology and diagnosis

Incidence rates for Pca increased in the late 1980s and early 1990s owing to the adoption of the prostate-specific antigen (PSA) test. Mortality rates attributed to Pca have declined from the peak in 1993 (3). According to Cancer Facts & Figures, the incidence of Pca decreased 3.8% per year on average from 2007 to 2016. However, estimated incidence rates are predicted to be elevated for the next 20 years and will be associated with increased life expectancy (4). In United States, both the incidence and mortality rates of Pca are the highest in the African American population. It has been suggested that racial disparities were the greatest in low-grade Pca, and that African American men were more likely to die from low-grade Pca than other populations (5). However, a recent study by Dess et al. (6) suggests that these disparities were attributed to nonbiological factors such as accessibility to high-quality care and PSA screening, as opposed to biological and intrinsic factors. This finding was further supported by several systematic studies, which demonstrated that there were no racial differences in the associations between Pca and lifestyle and family history (7, 8).

The primary diagnostic methods of Pca are digital rectal examinations (DREs), PSA blood tests, and transrectal ultrasound (TRUS) guided biopsy. DRE has been shown to have an average positive predictive value of 43.3% for Pca (9). While the PSA test has relatively high sensitivity and can detect quantities as low as 4 ng/ml, the specificity of PSA has not been optimized. Even for those who routinely receive PSA tests starting in their 50s, approximately 70% of men with elevated PSA levels do not have Pca (10). The TRUS biopsy verifies the results of abnormal DREs or elevated PSA levels. However, the accuracy of the TRUS biopsy is highly dependent on the number and the position of cores.

Thus, tumors could be undetected or over/under graded. In addition, the TRUS biopsy is an invasive method which can result in unfavorable side effects. Thus, a multi-parametric MRI is used as a triage test prior to TRUS to improve the detection accuracy and to reduce the unnecessary use of a primary biopsy. Multi-parametric MRIs can provide information on tissue anatomy and characteristics, and has allowed 27% of patients to avoid a primary biopsy and increased the detection rate by up to 18% if followed by a TRUS biopsy in comparison to a TRUS biopsy alone (11). In addition to traditional detection methods, the emergence of biomarkers can increase successful Pca diagnoses to new levels. Biomarkers, including androgen receptor splice variant (*ARV*)-7 (*ARV7* or *AR3*), which occurs in around 25.2 percent of CRPC patients and causes higher survival risks emergent recently (12). Other biomarkers are the *TMPRSS2-ERG* fusion gene, micro RNAs (miRNAs), and the *PTEN* gene, provide substantial prognostic value and a more accurate assessment of the stages of cancer progression (13).

Prostate cancer progression and treatment

Pca is a heterogenous disease that is characterized by heterogenous genomic alteration, morphology, and progression. Shen & Shen have previously characterized the stages of progression of Pca and the genetic and cellular events that drive the progression (Fig. 1) (14), and found that prostate intraepithelial neoplasia (PIN) is the precursor of Pca and is usually caused by inflammation and genetic aberrations. With the re-activation of developmental signaling pathways and an increase in abnormal genetic activity, PIN can further progress to adenocarcinoma, and eventually to metastatic cancer.

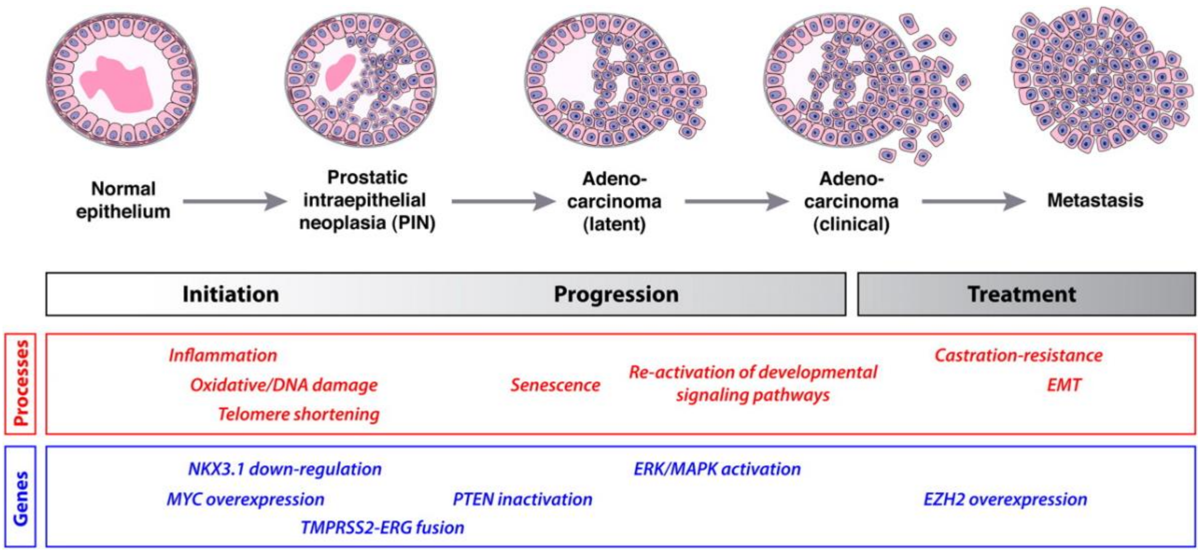


Figure 1. Progression pathway for human prostate cancer.

Stages of progression are shown, together with molecular processes and genes/pathways that are likely to be significant at each stage. (Figure adapted from Michael M. Shen, and Cory Abate-Shen *Genes Dev.* 2010;24:1967-2000 (14))

A recent molecular study on primary Pca suggests that the majority of cases of Pca can be categorized into seven subtypes based on gene fusion and mutation events (15). ETS-family fusions (*ETV1*, *ETV4*, *ERG*, and *FLI1*) are the most frequent molecular events found in Pca, as the ETS-family regulates a wide range of cellular events, including differentiation, cell cycle regulation, cell migration, cell proliferation, apoptosis, and angiogenesis. *TMPRSS2-ETS* gene fusion is one of the earliest discovered and most predominant types of *ETS* fusion. As T*MPRSS2* is an androgen-regulated gene, *TMPRSS2-ETS* fusion places *ETS* transcription factors under the control of androgen signaling and further contributes to the progression of Pca. Moreover, while Pca has a relatively lower rate of genetic mutation than other types of malignancy (16), this does not mean that genetic mutation is any less important in the progression of Pca, in which the aberrant genes are more concentrated. Given the role of genetic mutation in the progression of malignancies, the DNA damage response (DDR) pathway is critical to genomic integrity and normality. Deficiencies in the DDR can result in cell death or the abnormal growth of the cells. A recent systematic study showed that the DDR germline mutation rate is approximately 18.6%, while the somatic mutation rate is approximately 10.7% (17). The genes that code for the homologous recombination proteins ATM, BRCA2, and PALB2 exhibit the highest rates of mutation. These genes play critical roles in drug treatment and resistance. In addition, the progression of Pca can also be modulated by epigenetic events such as DNA methylation and histone modification. A total of 164 epigenetically-silenced genes have been discovered in primary tumors (15), and these silenced genes are likely to remain downregulated in metastatic Pca. Of these genes, *SHF*, *FAXDC2*, *GSTP1*, *ZNF154*, and *KLF8* have been shown to be epigenetically silenced in more than 85% of primary Pca tumors (15).

Cancer is a collection of diseases caused by abnormal cell growth and uncontrolled cell division. Malignant cancers cause mortality by invading functional vital organs and negatively impacting normal physiological processes. From this point of view, one of the most important strategy to combat cancer is to inhibit abnormal cell growth and proliferation. Radiotherapy and chemotherapy have been the main strategies used in the field of cancer treatment for nearly a century. Radiation and chemotherapy drugs affect cell growth and division via multiple pathways. As cancer cells divide much faster than normal cells, cancer cells are more vulnerable to radiation and chemotherapy drugs. It should also be noted that the damage to normal tissues is inevitable during these types of treatments.

Androgen signaling is one of the most important signaling pathways in the proper function and maintenance of the prostate gland, while abnormal androgen signaling is a main driving force for the progression of Pca. Thus, the primary goal of treating Pca is to decrease the levels of androgens. Hence, hormonal therapy has been the main focus of Pca treatments for nearly 8 decades. Aside from the radical prostatectomy, which uses surgery to remove prostate gland, numerous types of drugs with varying mechanisms of action have been developed to reduce levels of androgens. The initial attempt at a hormonal therapy drug was synthetic estrogen diethylstilbestrol, which acts by inhibiting luteinizing hormone releasing hormone (LHRH) via negative feedback on the hypothalamic-pituitary axis. LHRH agonists and gonadotropin releasing hormone antagonists were developed in the late 20th century in order to stop the production of testosterone by the testicles. More recently, new-generation drugs such as the nonsteroidal antiandrogen enzalutamide (ENZ) and the CYP17A1 inhibitor abiraterone (AA) have been approved by FDA. While these drugs prolong the survival of patients to some extent, the development of resistance to hormonal therapy is inevitable. Thus, it is important to further understand the mechanisms of drug-resistant metastatic Pca.

Androgen receptor splicing variant

Androgen receptor (AR) plays a key role in Pca progression. AR is a transcriptional regulator that belongs to the nuclear receptor family and is the major mediator of androgen signaling in prostate cells. AR consists of an N-terminal transactivation domain (NTD), a DNA-binding domain (DBD), a hinge region, and a C-terminal ligand binding domain (LBD) (18) and is known to be stabilized by the molecular chaperone heat shock protein 90 (HSP90) in the cytoplasm. Upon binding to androgens (i.e., testosterone and dihydrotestosterone), AR dissociates from HSP90, forms a homodimer and translocates into the nucleus. Once in the nucleus, AR binds to the androgen response element (ARE) in the regulatory region of its target genes and modulates their expression (19), (20). As one of target genes of AR, *PSA* is currently the most sensitive and widely used biomarker in monitoring the presence, progression, and therapeutic responses in Pca (19), (21). More importantly, it is well-known that the androgen-AR pathway contributes to cell proliferation in Pca, inhibits apoptosis, and promotes metastasis (19). Thus, the inhibition of AR function by ADT therapy is a first-line treatment for advanced Pca patients. In recent years, ARVs have emerged as potential important players in Pca progression and therapeutic resistance. Since 2008, more than a dozen of ARVs lacking the ligand binding domain have been identified from human Pca cell lines and xenografts (22), (23), (24), (25), (26), (27). The updated lists of ARVs are showed in Figure 2. These ARVs attracted much attention in the field due to their ligand-independent transcription activity.

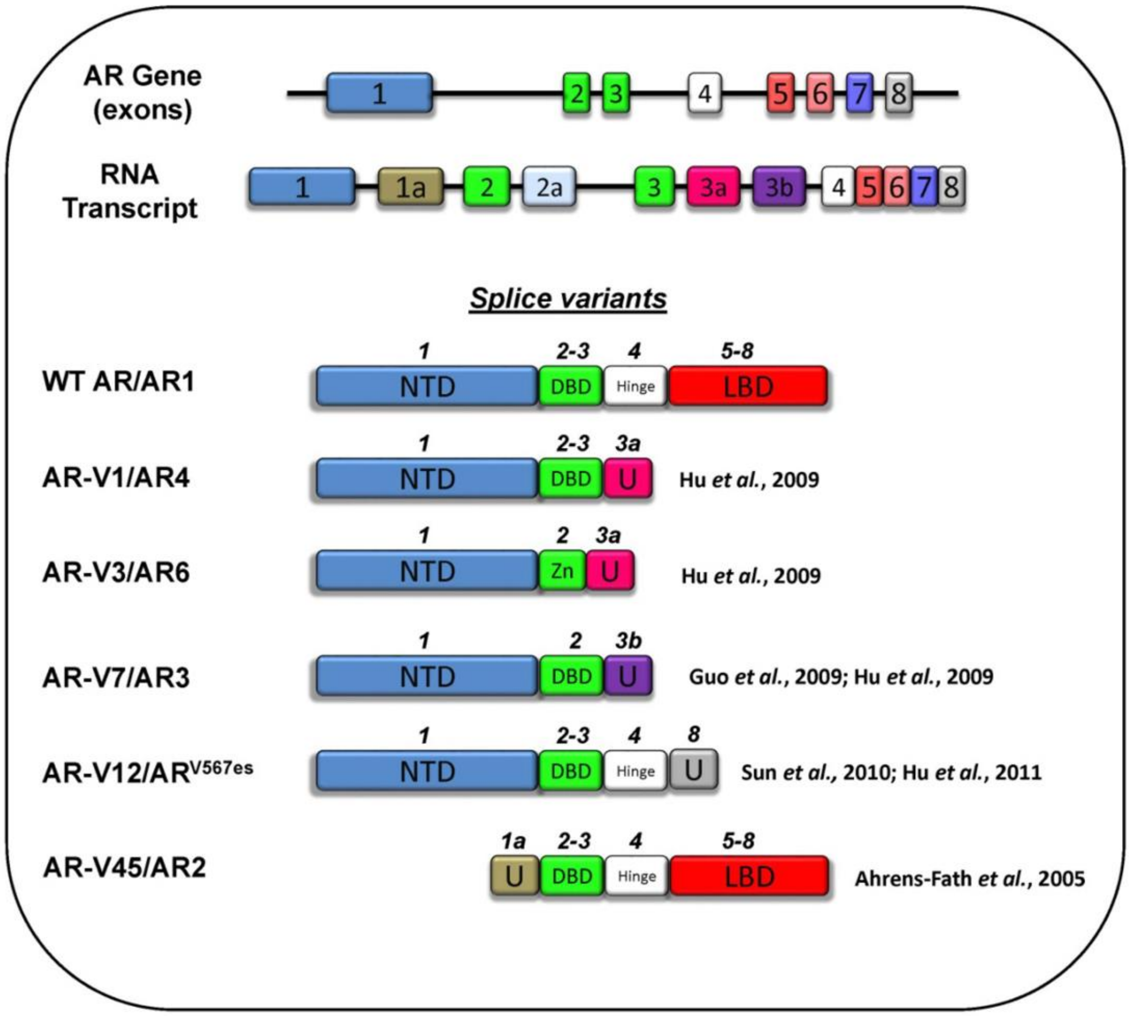


Figure 2. Common splice variants of the AR.

WT AR is organized as eight exons and consist of NTD, DBD, hinge region, and LBD. Each splice variant corresponding exons and cryptic exons are shown above. (Figure adapted from Lallous, N. et al., *International journal of molecular sciences*, *14*(6), 12496-12519 (28).)

Expression of ARVs

The presence of *ARVs* transcripts in human prostate tissues have been validated by RT-PCR and RNA-seq (23), (25), (29). AR3 protein has been detected in human prostate tissues using isoform specific antibodies (23), (25). The relative abundance of *AR3* transcript appears to be highest among various *ARVs* detected in human prostate tissues (23), (29). It should be noted that the cryptic exon utilized in *AR3* (Exon 3-CE3) is also detectable in benign tissues (23), (29), suggesting that this variant may also have a functional role in normal prostate. In general, *ARVs* have higher expression in more aggressive and metastatic tumor derived cell lines and xenograft models (23), (25), (26). Among *ARVs*, *AR3* and AR_{V567es} appear to have relatively higher expression levels than other isoforms. Hu et al.'s RT-PCR data showed that expression levels of *AR3* was significantly higher in CRPC patients than in hormone-naive Pca patients (25). To further characterize the expression profile of AR3, Guo et al. (23) developed a polyclonal antibody specific for AR3. In consistence with Hu et al.'s data, immunohistochemistry analysis revealed that AR3 expression is significantly increased in more malignant Pca tissues. Interestingly, it was observed that AR3 redistributed from basal and stromal cells to luminal epithelial cells with the increasing aggressiveness of Pca. In addition, the staining also showed that nuclear translocation of AR3 is significantly increased in CRPC patient samples compared with hormone-naive Pca samples (23). These studies suggested a potential functional activation of AR3 during Pca progression.

Bone is the primary site where metastatic Pca spreads. A recent clinical report showed that transcripts of *ARVs* (*ARV1*, *AR3*, and AR_{V567es}) were increased in Pca bone metastasis. The higher expression of *AR3* and AR_{V567es} in bone metastasis was associated with poorer prognosis (30). The correlation of ARVs and other important parameters in Pca were also monitored. It is well known that PSA level after castration is a prognostic biomarker for distal metastasis. Kaplan–Meier analysis indicated that patients with higher AR3 cytoplasmic staining usually have higher PSA recurrence and lower overall survival probability (23). A more recent study on a larger cohort of patients further validated the importance of ARVs, especially AR3, in metastatic Pca and CPRC (31). They compared AR3 expression among three patient groups (localized Pca $n = 100$, newly diagnosed metastatic Pca $n = 104$, and CRPC $n = 46$). The number of AR3 positive patients was significantly larger in newly diagnosed metastatic Pca group compared to localized Pca group. This rate increased more dramatically when comparing CRPC to localized Pca. More importantly, the data revealed that the presence of AR3 was associated with shorter survival (31). Thus, these studies suggest a strong link between ARVs and Pca progression. A recent study further supports that AR3 is related to and valuable for clinical application. It showed that AR3 can be detected in circulating tumor cells from CRPC patients. Patients with higher AR3 expression in circulating tumor cells had shorter PSA progression-free survival (32). These findings suggest that AR3 may serve as a prognostic and predictive biomarker for metastasis Pca patients.

It has been well documented that transcription of *AR* genes is elevated upon androgen derivation possibly due to relief of negative feed-back regulation by AR full length (AR-FL) (33), (34). The relative increase of *ARVs* compared to *AR–FL* in CRPC could have resulted from change of splicing factors, RNA binding proteins, micro RNAs (miRNAs) (34), (35), (36), (37). RNA splicing factors U2AF65 and ASF/SF2 were shown to be critical for AR3 splicing under androgen deprivation condition (34). RNA binding protein Sam68 could distinctly regulate *AR–FL* and *ARVs* transcription. Exogenous expression of SAM68 significantly elevated *AR3*, but not *AR–FL* transcript levels (36). In addition, epigenetic factors such as miRNAs are also involved in ARV transcript production. miR-124 directly targeted *ARV4* and *AR3* and downregulated their expression (37). Thus, the expression of *ARVs* are likely regulated at multiple levels including transcriptional, posttranscriptional as well as posttranslational.

Role of AR-FL in the regulation of ARVs activity

Although ARVs are shown to be able to regulate canonical and non-canonical AR target genes, it is still under debate whether ARVs have distinct functions independent of AR-FL (23), (25), (38). Some studies suggest that the presence of AR–FL is critical for activation of ARVs. It has been shown that expression of ARVs could be repressed by either targeting its ligand binding domain or small-interfering RNA (siRNA) knockdown of AR–FL (38). A recent mechanistic study also supports a role of AR–FL in ARVs' function (39). It was showed that AR–FL and ARVs formed a heterodimer mediated by DNA-binding domain and by N- and C-termini of ARV and AR–FL, respectively. The dimerization seemed to be required for the function of ARV. Mutants disrupting ARV and AR–FL interaction diminished ARV's transactivation and reduced growth rate of ARV positive androgen-independent Pca cells (39). This study suggests that though ARVs are constitutively active, they might still need the presence of AR–FL to exert their functions. However, this study could not exclude the possibility that mutation of the FxxLF motif of ARV, which regulates the recruitment of binding factors, may also compromise its ability to interact with other co-factors essential for its transcriptional activity. On the other hand, some studies suggest that ARVs may also exert its unique function independent of AR–FL in addition to their overlapping activity. Two studies have shown that selective knock-down of *ARVs* in CWR-R1 cells caused the changes of a unique subset of genes that are not affected by selective knockdown of *AR–FL* (23), (40). It is also reported that ARVs are resistant to pharmacological inhibition of ligand binding domain of AR–FL. Specific knocking down of *ARVs*, but not *AR–FL*, had dramatically effects on canonical AR pathway genes expression under pharmacological inhibition (40). A more recent report revealed that AR3 was capable of promoting Pca migration and sphere-formation under the condition that exogenous expression of AR3 alone in AR-negative Pca cells (PC3/DU145 cells) by upregulating genes involved in epithelial-mesenchymal transition (EMT) (41). This is consistent with a study in genetic modified mouse model in which AR3 is targeted expressed in mouse epithelial cells using the *ARR2PB* promoter (42). In this mouse model, overexpression of AR3 in luminal epithelial cells appeared to antagonize the terminal differentiation process driven by AR–FL by promoting EMT. Therefore, the requirement of ARVs for the presence AR–FL may very likely depend on cell context.

Guo et al. (23) performed microarray analysis to compare AR–FL and AR3 regulated genes in Pca cells selectively knocking down *ARV* or *AR–FL*. They showed that 71 genes were commonly regulated by both AR–FL and AR3 and 117 genes were preferentially regulated by AR3 but not AR–FL. Furthermore, they also identified novel ARE sites in the *AKT1* regulatory regions by chromatin immunoprecipitation (ChIP) assay. These ARE sites were preferentially occupied by AR3 but not AR–FL. Interestingly, a study to compare AR binding sites in CRPC and androgen-responsive Pca revealed a program shift of AR binding. Cell cycle regulator UBE2C is one of the top score targets of AR under androgen independent condition. In this study, the AR binding sites were identified by chromatin immunoprecipitation sequencing (ChIP-seq) analysis using an antibody recognizing the N-terminal part of AR which is common for both AR–FL and ARVs (43).

A follow-up study using AR3 specific antibody validated the specific binding of *UBE2C* promoter by AR3 but not AR–FL under androgen depleted conditions (44). Consistent with the ChIP assay, transient expression of exogenous AR3 preferentially modulated cell-cycle regulated genes. The expression of cell-cycle gene *UBE2C* appears to be correlated with *AR3*, but not *AR–FL* in both Pca cells and clinical CRPC specimens (45). However, in a later study, Li et al. (40) performed gene set enrichment analysis (GSEA) to examine the response of the identified "ARV-specific" M-phase-genes in CWR-R1 cells. They found that this ARV-regulated M-phase genes were positively-enriched in androgen-induced AR gene signature. They also performed GSEA with gene expression datasets derived from androgen-sensitive LNCaP cells in response to different doses of dihydrotestosterone (DHT). Interestingly, the ARV signature was positively enriched in the 1 nmol/L DHT dataset, but negatively enriched in the 100 nmol/L DHT dataset. They concluded that those mitotic genes such as *CDCA5*, *ZWINT*, and *CCNA2* appeared to be unique ARV targets under androgen depleted conditions may reflect the proliferative events driven either by ARVs or androgen-stimulated AR. In addition, it has been showed that ARVs may bind to its unique targets independently of AR–FL, but coordinate with AR–FL in regulating canonical AR target genes (44). In addition, distinct roles of ARVs might be attributed to their ability of binding of unique transcriptional mediators. It has been shown that MED1 can be co-immunoprecipitated with AR_{V567es} independently of AR–FL. AR_{V567es} and p-MED1 co-occupies on the regulatory regions of *UBE2C* genes and promotes *UBE2C* expression independently of androgen (46). Another report showed that RNA-binding protein SAM68 interacts with AR3 and enhances its transcriptional activity (36).

Nuclear localization of ARVs are regulated differently than AR-FL. It is well established that ligand binding promotes AR to dissociate from chaperone HSP90 in the cytoplasm and translocate to the nucleus. However, ARVs only contain a portion of nuclear localization sequence (NLS) and lack the C-terminal AR dimerization domain and HSP90 binding domain. It is conceivable that the nuclear translocation mechanism of ARVs might differ from that of AR–FL (23), (47), (48). It was showed that ARVs nuclear localization was independent on AR–FL and canonical HSP90 chaperone shuttling (49). Recent studies revealed that microtubule-stabilizing taxane compound was capable of inhibiting nuclear translocation and transcriptional activity of AR–FL (50), (51). The subcellular localization of AR–FL was associated with the patients' response to taxane chemotherapy (52). On the other hand, ARV was shown to be associated with microtubules to a much less extent than AR–FL. Several studies showed that nuclear translocation of AR3 was independent on microtubule motor protein dynein while AR–FL does (47), (52). Instead, a study has shown that nuclear translocation of AR3 can be inhibited by an import in β inhibitor (53). These data suggest that nuclear translocation and activation of ARVs might be modulated by different mechanisms. More importantly, because nuclear localization and activity of AR3 is not inhibited by standard taxane chemotherapy, it is likely that AR3 may play a greater role in CRPC after taxane treatment (47). Along the same line, nuclear localized AR3 is able to trans-active canonical and non-canonical AR pathway genes and promotes Pca growth under androgen depleted conditions (23), (25), (44), (49).

Function of AR variants in metastasis prostate cancer

It is conceivable that ARVs play important roles in Pca progression and promote growth in castration resistant cells. Overexpression of AR3 in ARVs negative Pca cells promoted cell growth under androgen depleted conditions. Knocking down of *AR3* in ARVs positive Pca cells significantly attenuated cell growth (23). *UBE2C* is one of the cell-cycle genes specifically regulated by AR3 under androgen depleted conditions. AR3, but not AR–FL, binds to the regulatory regions of UBE2C and promotes it expression (44), (45). The expression correlation between *UBE2C* and *AR3* has also been confirmed in clinical samples (45). On the other hand, genes regulated by AR–FL are primarily related to biosynthesis, metabolism and secretion (45). In parallel with this finding, knockdown of *AR3* significantly reduced DNA synthesis while apoptosis was unaffected. In contrast, knockdown of *AR–FL* promoted apoptosis (23). The effect of AR3 on cell growth is partially contributed by AR3 specific regulation of serine/threonine kinase AKT1 (23). A recent report also showed that AR3 transcriptional activity can be inhibited by multiple AKT inhibitor and restoration of PTEN activity (54). These data suggested that AR3 and PI3K-AKT pathway might form a positive regulation loop that contributes to Pca progression. This feedback loop is distinct from the negative feedback loop that AR–FL and PI3K-AKT repress the activity of each other (55), (56).

In addition to cell-cycle regulation, AR3 is a critical factor for Pca metastasis. The AR3 transgenic (AR3Tg) mouse generated by Sun and his colleague (42) provides a new tool to study the role of AR3 in prostate. Their microarray analysis revealed 414 genes that were differentially expressed in AR3Tg compared to wild-type animals. GSEA indicated that AR3 regulates multiple tumor-associated autocrine/paracrine factors including TGFβ2 and IGF1. TGFβ and IGF signaling are well-known positive contributors to Pca progression. Interestingly, a recent report showed that IGF-1R might be one of the upstream signaling of AR. IGF signaling modulates both AR–FL and constitutively active ARVs activity via receptor phosphorylation (57). Thus, it is possible that AR3 and TGFβ2/IGF1 form a positive regulatory loop in androgen-independent Pca. As demonstrated in another report, the link between AR3 and autocrine/endocrine signaling is that AR3 expression and activation is associated with TNF-NF-κB signaling. NF-κB activates AR3 modulates ENZ resistance (58). Down-regulation of NF-κB signaling decreased *AR3* expression and restored the growth inhibiting effect of anti-androgen drug (59). Autocrine/paracrine pathway signaling factors have been shown to have important function on the proliferation, survival, migration and maintenance of stem cell in various types of cancer including Pca (60), (61), (62), (63). Thus, these data suggest that the regulatory network of AR3 and autocrine/paracrine signaling is critical for CRPC progression.

Accompanied with the elevated autocrine/paracrine signaling, the expression of a number of EMT-associated genes including N-cadherin, vimentin, snail and twist were also increased in AR3 overexpressed Pca cells and AR3Tg animals (42). These finding were further validated by another group in multiple Pca cell lines. Overexpression of AR3 in LNCaP and DU145 cells led to upregulation of EMT markers fibronectin and ZEB1, respectively. Wound healing assays showed that AR3 is able to promote cell migration (41). The correlation of AR3 and EMT markers highly suggest a role of AR3 in Pca metastasis (41), (42), (64). In addition to EMT genes, stem cell signatures genes were also observed to be increased in AR3Tg prostate (42). Consistent with the observations in AR3Tg, knocking down of *AR3*, but not *AR–FL*, reduced the expression of stem cell marker genes *Nanog* and *Oct4* (41) and overexpression of AR3 in Pca cells promotes prostasphere formation (41). In summary, these data suggest that AR3 contributes to Pca metastasis and

CRPC progression through promoting the process of EMT and acquiring stem cell properties.

Epigenetic regulation refers to dynamic alterations of the molecular function without the change of DNA sequence. Epigenetic regulation including DNA methylation, histone modification, microRNA and long non-coding RNA and other non-DNA sequence alterations lead to molecular changes. The importance of epigenetics regulation in cancers including PCs has long been discovered (65). Average DNA methylation and histone methyltransferase EZH2 level are increased in CRPC (66), (67), (68). This up-regulation of epigenetic elements play critical roles in Pca progression and the development of CRPC (69). AR has recently been shown contribute to the epigenetic regulation in multiple aspects. AR can recruit epigenetic co-activator/repressor to modulate certain genes expression. Association of AR with histone demethylase LSD1 and JMJC is critical for downstream androgen responsive genes expression (70). Epigenetic factor EZH2 acts as a coactivator of AR and the interaction is critical for CRPC (68). AR is not only able to cooperate with protein. A recent finding showed that AR bound to long non-coding RNA (lncRNA) *PRNCR1* and *PCEGM1* which enhance both androgen dependent and androgen independent AR regulatory program and Pca proliferation (71). In addition to cooperate with epigenetic factors, AR is also able to regulate gene expression via modulating epigenetic factors expression. A study showed that AR is able to regulate *EZH2* expression through modulating miR-101 expression (72). Androgen induced miR-21 upregulation was shown to play important roles in hormone-dependent and hormone-independent Pca growth (73). The close relationship of AR and epigenetic factors opened a new window of how ARVs and epigenetic factors cooperate. Due to the lack of C-terminus ligand binding domain, ARVs may exert distinct roles in epigenetic regulation compared with AR–FL. Indeed, a few reports published in recent years gave us some clues of the role of ARVs in epigenetic regulation. Microarray and real-time PCR data in AR3Tg mouse model revealed that miR-29 expression was decreased when compared with the wild-type control (42). miR-29 is a critical tumor suppressor microRNA which was reported to be down-regulated in various types of cancer. MiR-29 serves as a tumor suppressor which is involved in multiple tumor related pathways including immune regulation, cell proliferation, cell senescence, apoptosis, and metastasis (74). In addition to target traditional tumor-related pathways, miR-29 is also able to target epigenetic factor DNA methyltransferase (DNMT) (74). DNMT is responsible for establishing and maintaining DNA methylation and the pattern of DNA methylation is critical for cancer progression. In consist with the hypothesis that AR3 may regulate *DNMT* expression via miR-29, a recent report showed that *DNMT* expression and *AR3* expression were shown cooperatively up-regulated in 22Rv1 androgen independent cells with long term bicalutamide treatment (75). Interestingly, this report also showed that DNMT activity was significantly decreased after addition of androgen, suggesting AR–FL and ARVs may have distinct roles in regulating DNMT activity.

The dynamic repertoire of AR–FL and ARVs may provide a sophistic regulatory system to orchestrate cellular responses to androgens and other extracellular stimuli. In summary, AR3 may regulate gene transcription independently, cooperatively or competitively with AR–FL and plays a critical role in progression of Pca. The proposed model of the role of AR3 in CRPC is on Figure 3.

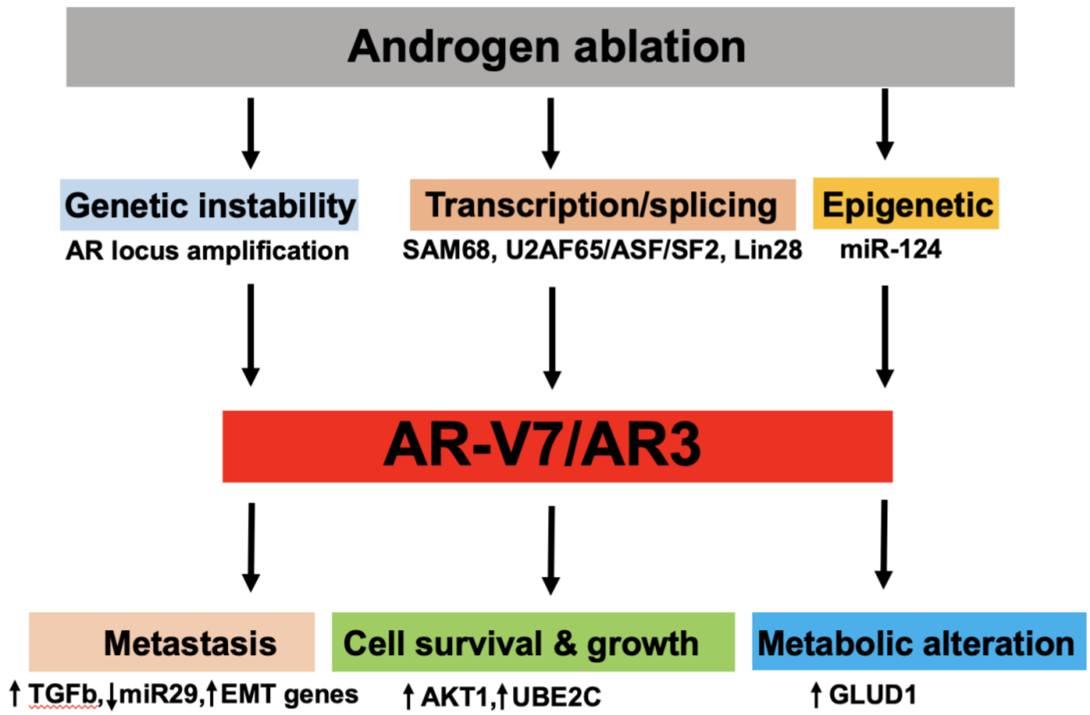


Figure 3. Proposed model of the role of AR3 in CRPC.

Upon androgen ablation, changes of genetic stability, transcription/splicing and epigenetic regulation alter the expression and activity of AR3. In addition to canonical AR–FL regulated genes, AR3 is able to regulate a subset of distinct target genes to promote metastasis, cell survival and growth, and metabolic alteration in Pca cells.

Mechanism of prostate cancer drug resistance

One of the main obstacles in the treatment of advanced Pca is drug resistance. Most patients with early-stage metastatic Pca benefit significantly from ADT. However, resistance to ADT develops rapidly. Chemotherapies and next generation anti-androgen therapies are currently used to treat CRPC (Fig. 4). Unfortunately, the development of resistance to these new treatments is inevitable. In Pca, mechanisms of resistance include persistent androgen signaling, AR bypass pathways, and alternative pathways that are independent of androgen

(76). Other general mechanisms of resistance include drug efflux alterations and the inhibition of cell death.

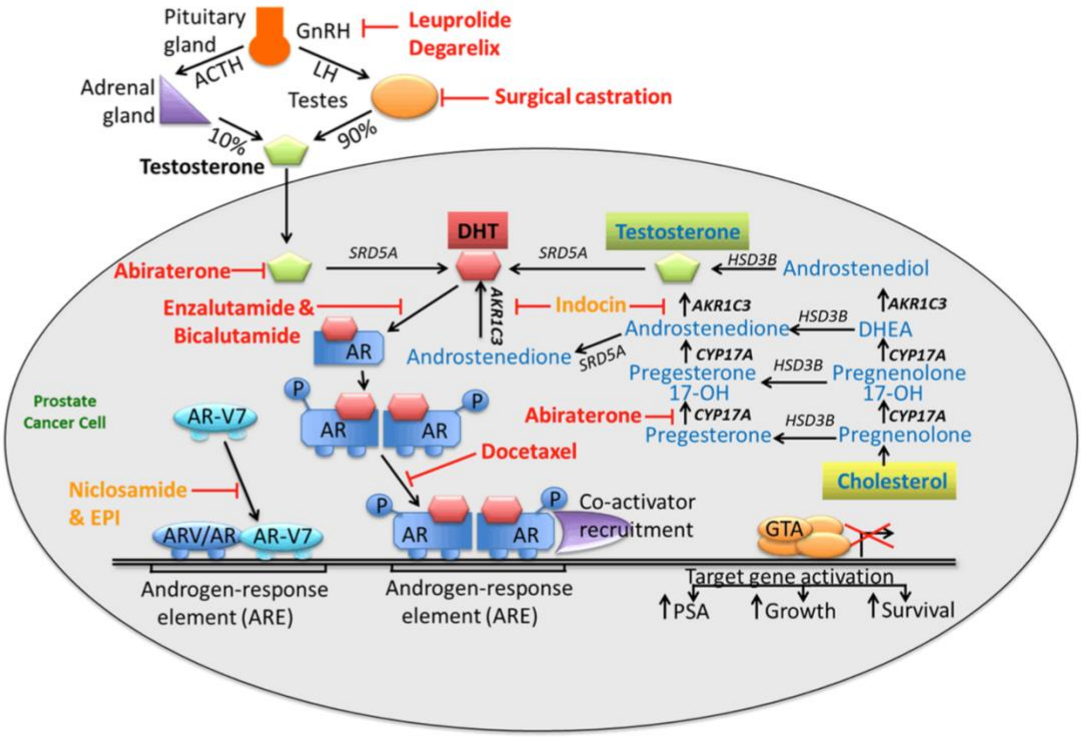


Figure 4. An overview of approved and experimental treatment strategies for CRPC targeting the androgen axis.

Currently approved therapies are written in red while experimental therapies are in orange. (Figure adapted from Cameron Armstrong and Allen Gao, *American journal of clinical and experimental urology*, *3*(2), 64. (77))

Resistance to anti-androgen therapy

ADT has been used as the first-line treatment for metastatic Pca for decades. ADT can reduce Pca growth by either lowering androgen levels or directly targeting AR. Unfortunately, most patients inevitably develop resistance to ADT and progress to CRPC, which is significantly more difficult to cure. During the transition to CRPC, tumor cells change from the androgen-dependent to the androgen-independent state. The new generation of anti-androgen drugs, AA, and ENZ, was approved for the treatment of CRPC by the FDA in 2011 and 2012, respectively. Both drugs increase the median survival time for more than 3 months compared with placebo (78-80). Despite the early success of AA and ENZ, clinicians have already noticed the limitations of these drugs. Approximately 20% of patients have no response to the treatment. Most patients who initially respond to the treatment rapidly develop resistance in <2 years (32, 78-81). Therefore, it is essential to investigate the mechanism by which resistance is acquired. The mechanisms of anti-androgen drug resistance can be categorized into two groups as follows: (1) restoration of AR signaling via upregulated steroidogenesis, AR mutation/amplification, ligand-independent activation of AR, AR crosstalk with other signaling pathways, and constitutively activated AR variants; and (2) activation of bypass pathways independent of AR signaling; and (3) androgen independent mechanisms (Fig. 5).

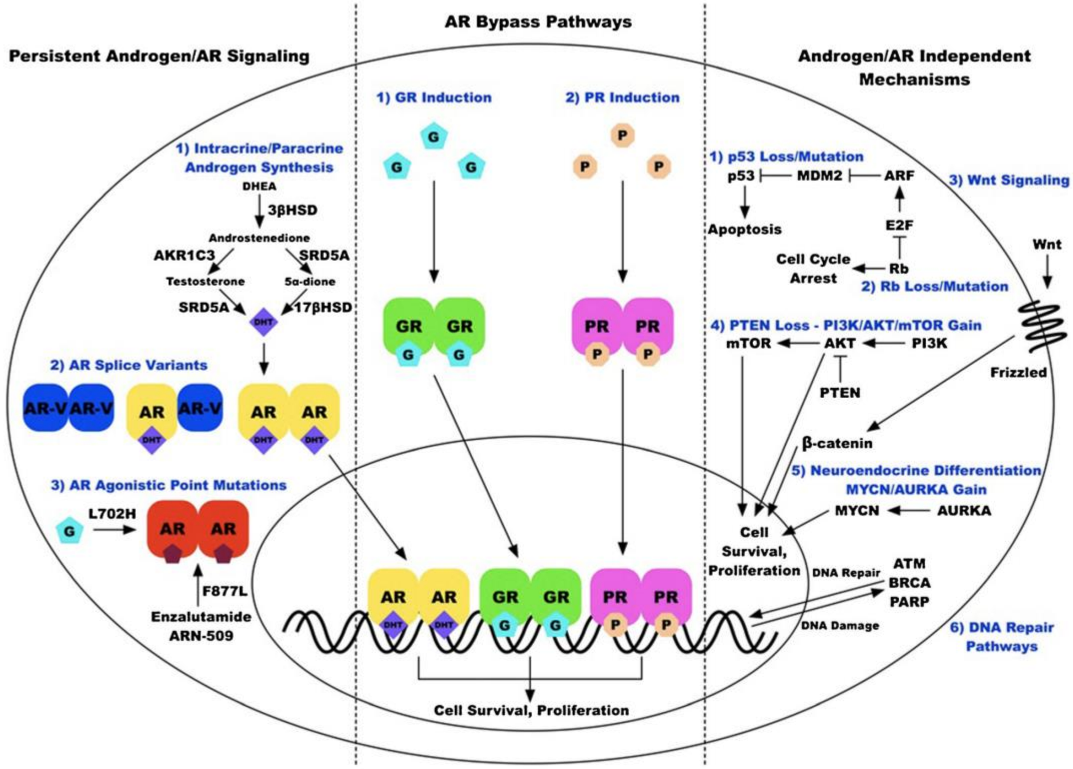


Figure 5. Resistant mechanisms to AR signaling targeted therapies.

(Figure adapted from Silberstein et. al., *Current urology reports* 17, no. 4 (2016): 29. (76))

Resistance to taxane

Taxanes are a class of mitotic inhibitors that function through stabilizing GDP-bound tubulin in the microtubule. The taxane compounds docetaxel (DTX) and cabazitaxel are FDA-approved chemotherapies for CRPC. DTX is widely used in various types of cancer, including Pca. More recent evidence suggests that DTX can reduce Pca progression through downregulating AR transcriptional activity in addition to its mitotic inhibitory function (52). The microtubule motor protein dynein may play an essential role in AR nuclear transportation. Taxane compounds can disrupt the microtubule structure, and thus, inhibit dynein motor function. AR nuclear translocation on ligand binding is a necessary process for the transcriptional activity of AR. Targeting AR nuclear transportation by the taxane compound reduces AR activity. In LNCaP cells, taxane treatment inhibits AR nuclear translocation and subsequently blocks AR signaling activation (52). DTX has also been shown to have roles in the regulation of antiapoptotic factors and cell cycle regulators (82). Thus, DTX affects multiple targets simultaneously and has served as a successful multi-target therapy for many years. Although DTX provides a significant overall survival benefit to CRPC patients, as compared to mitoxantrone treatment (17.5 months *vs* 15.6 months, $P = 0.02$ by the Log-rank test), it is noteworthy that, in the same study, a large portion of CRPC patient did not respond to DTX very well. Even among those who responded initially, the progression-free survival rate at 24 months after enrollment declined and was similar to those treated with mitoxantrone (83). Both prostate-cancer-specific and cancer-in-general mechanisms could be involved in taxane resistance. AR signaling re-activation is a prostate-cancer-specific mechanism that confers taxane resistance. There is evidence to suggest that the AR signaling pathway is restored in DTX-resistant cells, and AR activation could desensitize DTX treatment (84). The presence of ARVs play important roles in AR signaling re-activation-induced taxane resistance. A preclinical report has shown that taxane is unable to inhibit the transcriptional activity of AR3 and AR_{V567es} (53). This phenomenon can be explained by the observation that microtubule binding of AR is mediated by the C-terminal region of AR (47). Thus, AR3, which lacks the C-terminus, does not cosediment with microtubules. The nuclear localization and function of AR3 may not be impaired by microtubules targeting taxane drugs. Non-AR-driven taxane resistance is another major challenge. The complete AR-independent property makes anti-androgen agents nonfunctional. neuroendocrine prostate cancer (NEPC) is the major subtype of "complete AR-independent prostate cancer" (85). Although NEPC merely represents <2% of all Pca cases, NEPC potentially causes approximately 25% lethality in Pca (86, 87). A recent report has shown that ENZ treatment promotes Pca cell neuroendocrine differentiation through the recruitment of mast cells (88).

Immunotherapy-resistance

In the past few decades, researchers have started to reconsider taking advantage of the immune response in cancer treatment after the first wave of "immunotherapy" in the 19th century. Immunotherapy eliminates tumors using the host's own immune system. The advantages of immunotherapy over other therapeutic methods are increased safety, durability, and likely greater efficiency in overcoming diverse pro-survival mechanisms (89). However, tumors can utilize various mechanisms to escape immune targeting. Molecular events of acquired immunotherapy resistance include gene mutation and deletion, alternative splicing, and oncogenic signaling/epigenetic/microenvironment alterations (90). A recent study has reported that the *JAK1/2* and *B2M* mutation might lead to a lack of response to interferon (IFN)-γ and loss of surface expression of Class I MHC, respectively (91). It is also believed that gene deletion and alternative splicing are involved in immunotherapy resistance. Loss of CD19 antigen in response to CART-19 therapy through CD19 deletion and mRNA splicing make antitumor T cells unable to recognize targets (92). It is noteworthy that acquired resistance has been previously observed in metastatic melanoma patients who initially responded to immunotherapy (93). Sipuleucel-T is the only FDA-approved immunotherapy for the treatment of CRPC. In a placebo-controlled phase III trial, CRPC patients receiving sipuleucel-T treatment showed a significantly reduced risk of death compared with the placebo group (94, 95). In a more recent study involving 512 metastatic CRPC patients, the median survival advantage of sipuleucel-T-treated patients was 4.1 months longer than those in the placebo group (96). Although little is known about the acquired resistance of sipuleucel-T due to the lack of long-term observations, subgroups of patients in that study displayed primary resistance to this therapy with minimal response.

Combination therapy

Combination therapy has proven to be very effective for the treatment of advanced cancers. The combination of the FDA-approved human epidermal growth factor receptor-2 (HER-2) antibody trastuzumab with chemotherapy has shown significant survival benefits compared to either therapy alone in HER-2-positive breast cancer (97). Given that androgen signaling is a major driver of Pca development and progression, Pca treatment is predominantly focused on targeting the androgen signaling pathway. Not much effort has been devoted to test therapies inhibiting multi-targets in the treatment of Pca in the past decades. In recent years, there has been an increasing interest in the development of optimal sequential and/or combination treatments using two or more drugs to overcome drug resistance in Pca. We searched the clinicaltrials.gov website and found that nearly a quarter (67 out of 293) of phase 1–4 clinical trials investigating Pca treatment (active, not recruiting, or completed studies with results by September 15, 2018) involve multiple drug combinations. The combination of anti-cancer drugs with distinct mechanisms of action can enhance drug efficacy and efficiency compared with monotherapy. Synthetic lethality caused by combinatorial therapies may also avoid the development of drug cross-resistance. In addition, combination therapy has the potential to lower the dose of each drug required for treatment. Therefore, it may reduce the cytotoxicity and adverse effects caused by the high doses used in monotherapy.

Concomitant targeting AR pathway

AR is the most important drug target in Pca treatment. Insufficient inhibition of AR and AR pathway reactivation are major causes of anti-androgen therapy resistance. *AR* amplification and mutation represent key molecular mechanisms of anti-androgen drug resistance. Somatic mutation of *AR* can be inherited or acquired in response to anti-AR therapies. Recent studies using next-generation sequencing technology have demonstrated that a mutation in C-terminal ligand binding domain of AR can cause an agonist switch, which results in anti-androgen therapy resistance (98, 99). The *AR* F876L mutation, which confers resistance to ENZ, was observed in circulating tumor DNA from anti-androgen therapy-resistant patients (98). Recent evidence suggests that the combination of anti-androgen therapies could have additive effects on androgen signaling. Systemic Therapy in Advancing or Metastatic Prostate Cancer (STAMPEDE) is a randomized controlled multi-group and multi-stage study on the effects of the combination of multiple anti-AR therapies. A recently published STAMPEDE report demonstrated an obvious survival benefit of the combination of AA and ADT. In a total of 1917 patients not previously treated with hormone therapy, the 3-year survival was 83% in the combination group compared with 76% in the ADT alone group (hazard ratio [HR]: 0.63, 95% confidence interval [CI]: 0.52–0.76, $P < 0.001$) (80). This encouraging finding warrants further studies of additional anti-androgen drugs in combination. However, not all anti-androgen combination studies have shown a positive outcome. Preliminary data from the PLATO trial (NCT01995513) on the combination of AA and ENZ did not show a benefit on progression-free survival of CRPC (100). Long-term observations are needed for a more objective evaluation.

In addition to conventional AR-targeted therapy, lipid nanoparticle-delivered antisense therapy represents a promising therapeutic direction. Antisense therapy is activated through binding to a specific RNA sequence and inhibiting the function of RNA. Lee et al. (101) demonstrated that siRNA targeting *AR* delivered through lipid nanoparticles was able to silence *AR* expression and decrease serum PSA levels in xenograft tumors. Further analysis suggested that AR-targeted siRNA could induce *AR* mRNA cleavage within the sequence recognized by *AR* siRNA. Antisense therapy is essentially used to inhibit targets that are undruggable or resistant to conventional therapy. AR reactivation causes anti-androgen therapy resistance, and thus, AR-targeted antisense therapy can potentially be applied in anti-androgen resistant Pca. Constrained-ethyl modified antisense oligonucleotide (Gene 2.5) is a newer generation antisense oligonucleotide with an improved tissue half-life *in vivo*. A study using Gene 2.5 oligonucleotide against both *AR-FL* and *AR3* demonstrated that this oligonucleotide decreased both *AR-FL* and *AR3* mRNA and inhibited the growth of the LNCaP-derived ENZ-resistant Pca cell line and patient-derived CRPC xenografts (102). The oligonucleotide delivery efficiency is a critical factor limiting the usage of this therapy. A recent study has shown that the efficiency of oligonucleotide delivery in the absence of carriers can be improved by a small molecule, 6BIO. Five-fold less *AR*-target antisense oligonucleotide was required to achieve 50% reduction of AR protein expression in the presence of 6BIO compared with *AR*-target antisense oligonucleotide alone (103). The augmenting effect of 6BIO may be caused by its GSK-3α/β inhibition activity. This finding supported by the data showed that the addition of the GSK-3α/β inhibitor CHIR99021 could increase oligonucleotide activity. Till date, the clinical efficiency of antisense therapy as monotherapy is still debated. Recently, investigators have examined the effects of the combined utilization of targeted drugs and antisense therapy. Combining conventional therapies with antisense therapy potentially exerts a higher degree of growth inhibition and lower toxicity. A recent study has shown that the combination of antisense oligonucleotides targeting histone lysine methyltransferase EZH2 with anti-androgen pathway drugs has a more profound effect on the inhibition of Pca growth *in vitro* and *in vivo* compared with the single agent (104). In this report, the authors also described an altered AR cistrome and enhanced AR dependence following inhibition of EZH2 in CRPC, suggesting a potentially increased efficiency of anti-androgen therapy in the presence of EZH2 inhibitor (104). The promising results from these preclinical studies provide a rationale to further investigate the clinical effects of the combination of antisense and targeted therapies.

The deregulation of ARV is another important mechanism of anti-androgen therapy resistance. ARVs lacking the C-terminal ligand binding domain exhibit nuclear localization and are constitutively active in the absence of androgens (23, 25, 26). AR3 is one of the most well-studied ARVs in preclinical and clinical studies. AR3 expression in circulating tumor cells is strongly associated with resistance to ENZ and AA therapies in Pca patients (32). The clinical findings are supported by evidence showing that knockdown of AR3 expression in an androgen-independent Pca cell line restores the response to anti-androgen therapy (40). Given that currently available anti-androgen drugs mainly target the C-terminal ligand binding domain of AR and are expected to be ineffective for inhibiting ARVs, how to block the activity of ARV has become an attractive topic in recent years. One possible strategy is to develop inhibitors targeting conserved regions of both AR and AR variants. The N-terminal activation function-1 domain of AR is essential for both AR-FL and AR variants. Thus, the N-terminal domain of AR is a promising drug target to overcome ARV-induced anti-androgen drug resistance. EPI belongs to a set of small molecules that target the N-terminal domain of AR. EPI-001 was found to bind to AR and block the protein-protein interaction of AR and coactivators, in turn inhibiting the transcriptional activity of both AR and ARVs (105, 106). However, the excessive high pill burden caused early termination of phase I/II trials of EPI-506 in CRPC with progression after ENZ or AA (NCT02606123). Notably, the excessive adverse effects may be solved by the addition of a secondary anti-androgen therapy. Theoretically, the dose of each drug can be lowered in combination to achieve a similar efficacy. The AR N-terminal targeting antagonist sintokamide A (SINT-1) is shown to block the protein-protein interactions with AR functional partners and inhibit the activity of both AR and ARVs (107). Although sintokamide A and EPI compound both targets the N-terminal part of AR, these drugs may exert anti-androgen functions via different mechanisms. EPI was observed to inhibit AR expression at both mRNA and protein levels, and it was able to block interleukin 6-induced transactivation of AR. In contrast, SINT-1 did not affect AR expression and was able to significantly inhibit forskolin-induced AR transactivation (107). Importantly, this report demonstrated an additive inhibitory effect on PSA luciferase activity in cells treated with the combination of EPI and SINT-1 compared with either drug alone.

The DNA-binding domain (DBD) is another conserved region in both AR and ARVs. Researchers have identified a surfaced exposed pocket on DBD, which can serve as a potential target site. DBD targeting compounds VPC-14228/14449 have been shown to diminish the interaction of AR with androgen response element; therefore, VPC-14228/14449 can effectively block the transcriptional activity of AR and ARVs at a low sub-micromolar concentration (108). Unlike ENZ, VPC-14228/14449 does not affect the nuclear localization of AR and ARVs, making it a promising agent for targeting drug-resistant nuclear-localized AR and ARVs. The proteasome-dependent protein degradation pathway has also been utilized to decrease the AR and ARV level. The AR degradation enhancer ASC-J9 and niclosamide have been shown selectively degrade ARVs, resulting in decreased ARV transcriptional activity (109-111). ASC-J9 disrupts the interaction between AR and the AR coregulators ARA55 and ARA70 and enhances the interaction between AR and phosphorylated MDM2 (109)). This AR-Mdm2 interaction causes AR ubiquitination and increases the susceptibility of AR to degradation. The combination of niclosamide and ENZ demonstrates a strong growth inhibitory effect on ENZ-resistant cells both *in vitro* and *in vivo* (110). The expression of the ARV is regulated by both gene transcription and splicing factor recruitment to pre-mRNA (34). Recently, researchers have discovered that the splicing factors hnRNP, ASF/SF2, and U2AF65 are important for *AR3* splicing (34). A natural compound, quercetin, has been shown to bind to and retain hnRNPA1 in the cytoplasm, thus decreasing the AR3 expression level (112). Specifically, the down-regulation of AR3 production may result in higher AR dependency and potentially increase the AR-targeted ENZ response. A recent study has shown that the combination of quercetin and ENZ synergistically reduces AR and AR3 expression in ENZ-resistant C4-2B-Enza-R and 22Rv1-Enza-R cell lines (112).

AR activity can be regulated by other pro-survival factors and pathways. Inhibition of the transcriptional activity of AR and ARVs can also be achieved through different routes other than direct targeting of AR. PIP5K1α is an essential coactivator for both AR and ARVs. According to a recent report, AR3, PIP5K1α, and cyclin-dependent kinase 1 (CDK1) physically form a complex and cooperatively promote tumor progression (113). This report demonstrated that the PIP5K1α inhibitor ISA-2011B disrupts AR and AR3 protein stability and suppresses tumor growth and invasiveness in an AR3 overexpression tumor xenograft model. The same study also showed that the combination of ISA-2011B and ENZ diminishes AR and AR3 protein levels in both the nucleus and cytoplasm in androgen-independent 22Rv1 cells more efficiently than either drug alone. STAT3 activation has been shown to enhance the recruitment of AR to androgen response elements and increase the androgen-independent activity of AR (114). IL-6-induced activation of STAT3 promotes androgen-independent growth and is one of the potential ENZ resistance mechanisms. Simultaneous targeting of AR and AR coactivator by ENZ and STAT3 inhibitor AG490 can reverse IL-6-induced ENZ resistance in an LNCaP-s17 IL-6-overexpressing cell model (114).

It is well-known that AR signaling is closely associated with epigenetic factors, including DNA methyltransferases, histone modulators, miRNAs, lncRNAs, and other epigenetic cofactors (115, 116). Bromodomain proteins (*e.g.,* the bromodomain and extraterminal [BET] family) can bind acetylated lysine residues in histone and associate with AR on chromatin, resulting in increased expression of AR-regulated pro-survival factors. It has been reported that elevated AR activity increases BET protein expression-enhanced BET-mediated chromatin opening in advanced Pca (117). Inhibition of the BET family protein bromodomain containing 4 (BRD4) by the small molecule inhibitor JQ1 decreases AR and ARV expression and disrupts the recruitment of AR to the target genes in chromatin (118, 119). A recent study has shown that a BET inhibitor could restore drug sensitivity in ENZ-resistant cells. In that study, the combination of ENZ and JQ1 blocked AR signaling and exhibited enhanced tumor growth inhibition in ENZ-resistant LNCaP-AR and VCaP cells (120). These findings are corroborated by the observation that the combination of ENZ and JQ1 is more effective in AR-overexpressed LNCaP cells than in parental LNCaP cells (117). Interestingly, a very recent study on BET inhibitor resistance has demonstrated that AR signaling is reactivated via CDK9-mediated phosphorylation in BET inhibitor-resistant cells (121). BET inhibitor-resistant cells exhibit enhanced sensitivity to ENZ treatment. These studies suggest that combining anti-androgen therapy and BET targeting treatment may potentially avoid the development of resistance to either therapy.

Taxane-based combination therapy

The combination of anti-androgen therapy and DTX has received increased attention in recent clinical studies. The synergistic effect of targeting microtubules and AR signaling is one advantage of the taxane plus ADT combination. The initial Chemo-Hormonal Therapy Versus Androgen Ablation Randomized Trial for Extensive Disease in Prostate Cancer (CHAARTED) trial demonstrated a promising outcome of combining DTX plus ADT for the treatment of CRPC. A recently reported long-term survival analysis from the CHAARTED trial results showed a median overall survival of patients with high-volume tumors of 51.2 months for DTX plus ADT versus 34.4 months for ADT alone (HR: 0.63, 95% CI: 0.50–0.79, $P < 0.001$) (122). The GETUG 12 study conducted in France also demonstrated that DTX plus ADT improved relapse-free survival compared with ADT alone in high-risk localized Pca (123). Recent studies provide a rationale for the addition of second-generation anti-androgen drugs to DTX treatment. A recent preclinical study has shown that the addition of DHT desensitizes AR-positive LAPC4 cells to DTX treatment, which suggests that the re-activation of AR signaling contributes to DTX resistance (84). The combination of ENZ with DTX dramatically reduced LAPC4 cell growth even in the presence of DHT, demonstrating that DTX sensitivity with DHT stimulation is mediated by the H3K4me3 histone demethylase, KDM5D. Knocking down KDM5D in LNCaP cells caused DTX insensitivity in the presence of DHT. Early phase Ib results for ENZ plus DTX have demonstrated manageable toxicity (124). Several clinical trials are currently examining the safety and efficacy of the ENZ and DTX combination (NCT03246347, NCT02685267, NCT01565928). In addition, preliminary results from a phase I/II trial of cabazitaxel plus AA have shown that 12 CRPC patients (46%) achieved a PSA response after receiving DTX and AA (125). This progress warrants further investigation of the anti-cancer mechanisms of the combination of taxane with new-generation anti-androgen agents in preclinical cell and animal models.

Additional cancer-in-general mechanisms underlying taxane resistance include tubulin isotype alterations, microtubules/cytoskeleton mutations, drug efflux pump overexpression, and upregulation of pro-survival pathways. The deregulation of β-tubulin isoforms has received accumulated attention in recent years. βIII-tubulin is a β-tubulin isoform that is predominantly expressed in neurons and testis. Although βIII-tubulin expression was rarely observed in early-stage Pca patients, the βIII-tubulin expression is elevated in CRPC patients and associated with taxane resistance (126, 127). A recent report has demonstrated that βIII-tubulin is a direct target of AR and the expression of βIII-tubulin can be regulated by androgen stimulation in mouse and rat Sertoli cells (128). Since constitutively activated AR3 can also regulate conventional AR signaling pathway targets, AR3 may very likely play a role in βIII-tubulin-induced DTX resistance. A recent study of high-content screening of a well-characterized clinical compound has demonstrated that the Src-Abl dual kinase inhibitor PD180970 can decrease AR3 expression and inhibit androgen-independent cell proliferation (129). More importantly, Src kinase may play a direct role in regulating the stability and function of βIII-tubulin. Our preliminary study found that βIII-tubulin protein levels can be reduced by the Src inhibitor SU6656 (unpublished data). These findings suggest that the Src inhibitor could be a potential complement to taxane therapy. Dasatinib is an FDA-approved Src family tyrosine kinase inhibitor for children with chronic myelogenous leukemia. Early results for dasatinib in metastatic CRPC have been disappointing. The benefit of dasatinib is minimal, but the adverse effects are not tolerable (130). A recent clinical trial using DTX plus dasatinib failed to show an overall survival benefit for CRPC compared with DTX monotherapy. The median overall survival was 21.5 months for DTX plus dasatinib versus 21.2 months for DTX alone (HR: 0.99, 95.5% CI: 0.87–1.13; P = 0.90) (131). Although the study did not demonstrate a statistically significant survival benefit, a case study in this project reported improved bone scans outcomes, a high rate of soft-tissue responses, and decreased bone turnover markers with prolonged dasatinib treatment after combination treatment in a subset of patients (132). In addition, the combination of dasatinib with DTX showed durable 50% PSA declines in 26 of 46 patients (57%) in an earlier phase I/II trials conducted in CRPC patients (133). In addition to Src family inhibitors, the well-known TK domain tyrosine kinase inhibitor imatinib is another promising therapy that may function together with DTX. In a preclinical study, the combination of DTX and imatinib enhanced cancer cell apoptosis through inhibition of DTX-induced NF-κB activation in anaplastic thyroid cancer cells

(134). Another study has reported that cancer cell growth and invasion ability are reduced in response to DTX plus imatinib treatment (135). Although the preliminary phase II trial of DTX plus imatinib in metastatic breast cancer patients shows a low response rate with toxicity issues, a subpopulation of patients (6 of 37) has shown a partial response (NCT00193180). Focal adhesion kinase plays an essential role in DTX-resistant CRPC (136). Cotreatment of DTX-resistant AR-negative Pca cell lines with the focal adhesion tyrosine kinase inhibitor PF-00562271 and DTX attenuates cell growth and induces apoptosis. There are also several ongoing trials investigating taxane plus other non-antiandrogen therapeutic methods such as anti-angiogenesis agents, mTOR inhibitor, and radiation therapy (137).

Combinatorial immunotherapy

The success of antibody-based (anti-programmed cell death 1 [PD-1]/programmed cell death ligand-1 [PD-L1] and anti-cytotoxic T-lymphocyte antigen-4 [CLTA-4]) immunotherapies in other cancers makes them extremely attractive in investigations of their efficacy in the treatment of CRPC. However, the preliminary outcome of immunotherapy as monotherapy is disappointing in Pca. Little or no response was observed in these studies (138, 139). The poor response of anti-PD-1/PD-L1 treatment in Pca patients may be due to the lack of antigen expression in prostate tumors. A recent comprehensive immunohistochemical analysis of PD-L1 in Pca specimens has shown that PD-L1 is not expressed in localized and benign prostate hyperplasia, and it is only expressed in a small subset of CRPC specimens (140). Recovery of antigen expression is required for immune checkpoint therapies in Pca. Fortunately, the antigen can be re-expressed under certain circumstances. According to a recent report, although PD-L1 is rarely expressed in primary tumors, its expression can be upregulated in response to IFN-γ in PC3 Pca cell line (141). Increases in PD-L1/2+ dendritic cells are observed in ENZ-resistant patients compared with responders (142). In the same study, PD-L1 upregulation in patients was verified in AR-positive/PSA-negative ENZ-resistant Pca cells compared with ENZ-sensitive cells. Increased immunotherapy efficacy was observed in anti-androgen-resistant patients. In a study of anti-PD-1 pembrolizumab with continued ENZ treatment for CRPC patients with ENZ resistance, researchers observed increased PD-L1 expression and the presence of CD3+, CD8+, and CD163+ leukocyte infiltration (143). Thus, the general concept is to utilize a secondary therapeutic method to reduce the immunosuppression-induced resistance or insensitivity and make CRPC re-targetable by immunotherapy.

Chemotherapy and radiation therapy are also feasible for enhancing the efficacy of immunotherapy. Both therapies are considered to cause a massive cytotoxic effect in rapidly dividing cancer cells. Tumor antigen and other components released from the dead cells may potentially affect the immune response. Radiation-induced cell death results in increased expression and release of tumor antigen and cell signaling proteins, including MHC-I, death receptors, immunomodulatory cytokines, adhesion molecules, and costimulatory molecules (144). The presence of these components may induce immunostimulatory effects and enhance the efficacy of immunotherapy. Metastatic urothelial cancer shares a similar low response rate to checkpoint inhibitor monotherapy. In an early clinical trial report, the immune-targeting drug pembrolizumab plus DTX showed encouraging anti-tumor activity with improved progression-free survival in metastatic urothelial cancer (NCT02437370) (145). A synergistic response to combinatorial immunotherapy and radiation/chemotherapy has also recently been observed in Pca. A clinical study showed that surviving-specific CD8+ T lymphocytes in peripheral blood were increased in colorectal cancer and Pca patients after radiation therapy (146). Another phase III clinical trial investigating the addition of CTLA-4-targeted ipilimumab to radiotherapy for DTX-resistant bone metastatic CRPC has shown promising results (147). Despite the absence of a statistically significant improvement in overall survival, *post hoc* subgroup analyses demonstrated that overall survival and progression-free survival were improved for patients with poor prognostic features. In early phase I/II results of this study, researchers also found a subset of patients with a PSA response in the ipilimumab plus radiotherapy group (148). In addition to promoting an immunostimulatory effect, chemotherapy and radiation therapy may enhance immunotherapy through inhibiting immune suppressors. Myeloid-derived suppressor cells (MDSCs) play an important role in immune evasion in Pca. An *in vivo* study has shown that DTX is able to suppress MDSCs while increasing the cytotoxic T lymphocyte response (149). The chemotherapy drug cyclophosphamide has been shown to suppress immune suppressor cells and stimulate an immune response at a proper dose (150). Repression of MDSCs can also be achieved through targeted drugs in addition to radiation and immunotherapy. BEZ234 is a multikinase inhibitor that can attenuate MDSC frequency and immunosuppressive activity. An immune checkpoint blocker plus BEZ234 can greatly reduce the tumor mass and metastasis in a novel nongermline CRPC mouse model (151). Mechanistically, BEZ234 suppresses the secretion of cytokines that promote MDSC activity through the inhibition of PI3K signaling. This process sensitizes immunotherapy-resistant CRPC to immune checkpoint blockers. More recently, a study conducted by the

National Institutes of Health is testing the efficacy of the combination of multiple immunotherapies for biochemically recurrent Pca (NCT03315871). In that study, the PSA-targeted immunotherapy PROSTVAC is combined with tumor-associated antigens. The carcinoembryonic antigen- and mucin-targeted immunotherapy CV301 PROSTVAC monotherapy has recently shown disappointing results in a phase III study. The bifunctional fusion protein MSB0011359C is also being tested in that study. This fusion protein is composed of two functional domains, a monoclonal antibody against PD-L1 and the extracellular part of TGF-β receptor II, which "trap" TGF-β. Therefore, MSB0011359C can block both PD-L1 and TGF-β pathways. TGF-β has an essential role in immune suppression in advanced cancers. TGF-β inhibits the function of T and natural killer cells. Thus, MSB0011359C can be considered as an immunotherapy antibody combined with anti-immune suppressor therapy. A very recent study has shown that MSB0011359C induces $CD8^+$ and natural killer cell activity and decreases the tumor volume in a breast and colon cancer preclinical model (152). In the same study, the authors also showed the synergistic activity of MSB0011359C when combined with another immunotherapy TWIST vaccine that targets the tumor-associated antigen TWIST, suggesting an advantage of targeting multiple immune factors. Together, these studies provide evidence of an objective benefit of adding a secondary therapy to improve the sensitivity of CRPC to immune therapy.

Chapter 2: Materials and Methods

Cell culture and drug treatment

R1/DTX was developed as previously described (153). R1-ADR (CWR-R1 derived cells resistant to DTX and androgen-deprivation therapy (ADT)) was developed from R1/DTX and cultured in charcoal-stripped serum (CSS) containing Roswell Park Memorial Institute (RPMI) 1640 medium for 3 months in the presence of 1nM DTX, and were maintained under the same conditions. AI, a LNCaP derivative cell line, was cultured long-term in CSS medium, and was a gift from Dr. Wenliang Li from the University of Texas Health Science Center at Houston. LN-ADR (LNCaP derived cells resistant to DTX and ADT) was developed by culturing the AI cells in the CSS-containing medium and treating with DTX. The concentration for selection was determined by treating cells with 0.1, 1, 2, 5, and 10nM DTX for 3 days. Based on the cell survival rate (about 50%) after treatment for 3 days, 1nM DTX was used. In 3 months, the cells could grow in the presence of 1nM DTX at the similar doubling time as AI cells, and they were then maintained under these conditions. ENZ-resistant R1-ENZR (CWR-R1 derived cells resistant to ENZ) cells was developed by culturing the CWR-R1 cells in the CSS-containing medium in the presence of 40μM ENZ. In 3 months, the cells could grow in the presence of 40μM ENZ at the similar doubling time as CWR-R1 cells, and they were then maintained under these conditions. R1-DDR (CWR-R1 derived cells resistant to DTX and ENZ) and LN-DDR (LNCaP derived cells resistant to DTX and ENZ) double drug resistant cell lines were developed from R1-ADR and LN-ADR, respectively. Double resistant cell lines were cultured in CSS containing RPMI 1640 medium in the presence of 1nM DTX and 10μM (R1-DDR) or 20μM (LN-DDR) ENZ. In 3 months, the cells could grow in the presence of 1nM DTX and 10µM (R1-DDR) or 20µM (LN-DDR) at the similar doubling time as R1-ADR or LN-ADR cells, respectively, and they were then maintained under these conditions. Drug resistant prostate cancer cell lines used in the present study were cultured in CSS-containing medium and were subcultured regularly when confluent unless otherwise mentioned. All other cell lines were initially purchased from American Tissue Culture Collections (ATCC). Prostate cancer cell lines used in this study were listed in Table 1.

	medium	serum	resistance	parental cell line
LNCaP	RPMI 1640	FBS	N/A	N/A
CWR-R1	RPMI 1640	FBS	ADT	CWR22
22Rv1	RPMI 1640	FBS	ADT	CWR22
R1-ADR	RPMI 1640 w/o phenol red	CSS	ADT+DTX	CWR-R1
LN-ADR	RPMI 1640 w/o phenol red	CSS	ADT+DTX	LNCaP
R1-ENZR	RPMI 1640 w/o phenol red	CSS	ADT+ENZ	CWR-R1
R1-DDR	RPMI 1640 w/o phenol red	CSS	DTX+ENZ	CWR-R1
LN-DDR	RPMI 1640 w/o phenol red	CSS	DTX+ENZ	LNCaP

Table 1. Prostate cancer cell lines used in this study.

* Fetal bovine serum (FBS), Charcoal-Stripped Serum (CSS)

HEK-293T cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% FBS. Transfection experiments were carried out using FuGENE HD (Roche Applied Science) according to the manufacturer's instructions. DTX was kindly provided by Sanofi Aventis. Enzalutamide (S1250) was purchased from Selleckchem and dissolved in DMSO (stock concentration: 50mM). Rucaparib (S1098) was purchased from Selleckchem and dissolved in DMSO (stock concentration: 10mM). Cabazitaxel (S3022) was purchased from Selleckchem and dissolved in DMSO (stock concentration: 10mM). Auranofin (BML-EI206-0100) was purchased from Enzo Life Sciences and dissolved in DMSO (stock concentration: 200mg/ml).

Constructs, Antibodies, and short hairpin RNA

The plasmids used in the present study are as follows: the E2F1 expression vector pCMVHA E2F1 was a gift from Kristian Helin (Addgene plasmid # 24225) (154), the E2F1 DNA binding mutant vector pCMV E2F1 E132 was a gift from Kristian Helin (Addgene plasmid # 24224) (155), the E2F1 promoter luciferase vector pGL2-AN was a gift from William Kaelin (Addgene plasmid # 20950) (156), and the AR expression vector and ARR2-Luc vector were generated as previously described (23, 157).

The antibodies used in this study include: Rabbit polyclonal anti-AR3 (23), Anti-AR3(Clone RM7; ReMab), Anti-AR-FL (sc-815; Santa Cruz), Anti-AR (sc-816; Santa Cruz), Anti-α-tubulin (clone DM1A, Sigma), Anti-E2F1 (clones KH20 and KH95; Millipore), Anti-E2F1 (A300-766A; Sigma), Anti-Rb (9309S; CST), Anti-PARP1(sc-8007; Santa Cruz), Anti-Caspase3 (9661S, CST), Anti-GAPDH (sc-365062; Santa Cruz), Anti-PSA (sc-7316; Santa Cruz), Anti-Ki67 (9449, CST), Anti- γ-H2AX (9718S, CST), Anti-H3 (9715S, CST)

The short hairpin RNAs (shRNA) used in this study include:

shAR-FL was purchased from Sigma.

shAR3:TAGGCTAATGAGGTTTATTTCTCAAGAGAAAATAAACCTCATTAGCCT TTTTTTTTC shE2F1-1:

CCGGCTACTCAGCCTGGAGCAAGAACTCGAGTTCTTGCTCCAGGCTGAGTAG
TTTTTG

shE2F1-2:

CCGGCGCTATGAGACCTCACTGAATCTCGAGATTCAGTGAGGTCTCATAGCG
TTTTTG

Cell growth, spheroid, and apoptosis assay

Cell growth was measured using a CCK8 assay (Vita Scientific). Cells were seeded in 96-well plates (Thermo Fisher Scientific) at a density of 2000 cells/well. Drugs were added the following day (D1), and then refreshed every two days for 3–6 days as indicated in each experiment. Then, 10 µl of the CCK8 solution was added to each well and cells were cultured for 1.5 h at 37°C. Absorbance was measured using a plate reader at 450nm wavelength. Five wells were used for each experimental condition. Spheroid formation was assessed using Ultra-low attachment multiple well plate (CLS7007-24EA, Corning/Costar). Spheroid size was evaluated using the free software ImageJ (NIH). 8,000 cells were suspended in a final volume of 100 µL/well and cultured for 4 days. Then, half of old medium was replaced by fresh medium containing the appropriate amounts of agents every two days and cultured for 14 days. Finally, a TUNEL apoptosis assay was performed using the DeadEnd™ Fluorometric TUNEL System (Promega) according to the manufacturer's instructions.

RNA-seq

Cells were lysed in QIAzol lysis reagent (Qiagen). Total RNA was extracted using a RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA-seq was performed by Arraystar, Inc. Briefly, total RNA from each sample was quantified using a NanoDrop ND-1000. Subsequently, 1–2 μg of total RNA was used to prepare the sequencing library according to the following steps: 1. Total RNA was enriched by oligo (dT) magnetic beads (rRNA removed); and 2. RNA-seq library was prepared using the KAPA Stranded RNA-Seq Library Prep Kit (Illumina), which incorporates dUTP into the second cDNA strand and renders the RNA-seq library strand-specific. The completed libraries were qualified using an Agilent 2100 Bioanalyzer and quantified using the absolute quantification qPCR method. To sequence the libraries, the barcoded libraries were mixed, denatured to single stranded DNA in NaOH, captured on Illumina flow cell, amplified *in situ*, and subsequently sequenced for 150 cycles at both ends using the Illumina HiSeq 4000. RNA-seq data was deposited in NCBI's Sequence Read Archive (BioProject Accession: PRJNA623560). Analyses were performed using BRB-ArrayTools, which was developed by Dr. Richard Simon and the BRB-ArrayTools Development Team. Changes in gene expression were examined by two-tailed two-sample t-test and the Benjamini-Hochberg procedure (158) was used to derive false discovery rates (FDR). Meanwhile, the fold changes of genes were calculated. We also acknowledge our use of GSEA software the and Molecular Signature Database (MSigDB) (159, 160).

Proteomic profiling analysis

Label free protein quantification were performed as described by Lo et al. (161). Differential protein expression were examined by two-tailed two-sample t-test and the

Benjamini-Hochberg procedure (158) was used to derive false discovery rates (FDR). Meanwhile, the fold changes of genes were calculated. Molecular and cellular function and upstream regulator data were analyzed through the use of IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis).

Quantitative real-time PCR and chromatin immunoprecipitation

Total RNA was extracted using a RNeasy Mini kit (Qiagen) and reverse transcribed using Transcriptor Reverse Transcriptase (Roche). Quantitative real-time PCR (qRT-PCR) was performed as previously described (42).

The qRT-PCR primer sequences used in this study include:

AR-FL:

F:CTACTCCGGACCTTACGGGGACATGCG

R:GGGCTGACATTCATAGCCTTCAATGTGTGAC

AR3:

F: CTACTCCGGACCTTACGGGGACATGCG

R: TGCCAACCCGGAATTTTTCTCCC

E2F1:

F: AGCTGGACCACCTGATGAAT

R: GTCCTGACACGTCACGTAGG

PCNA:

F: GCCACTCCGCCACCATGTTCG

R: GCCTAAGATCCTTCTTCATCCTC

CDC6:

F: CTGGAGTTTGCTGCTGCCGCT

R: GAGCACCAGAAAGGTAAAGGC

ATAD2:

F: AGTACGGTCAAGCAGGCAAG

R: TTTCCCTCTTGCTGTCCAATC

Actin:

F: GCTCGTCGTCGACAACGGCTC

R: CAAACATGATCTGGGTCATCT

Chromatin immunoprecipitation (ChIP) was performed as previously described (157). $1*10^{8}$ R1-ADR cells were used for each ChIP. The ChIP primer sequences used in this study include:

ChIP-E2F1-P:

F: GTTGGGGGCTACAGGTTGAG

R: AAGTCCCGGCCACTTTTACG

ChIP-FKBP5:

F: GCATGGTTTAGGGGTTCTTGC

R: AACACCCTGTTCTGAATGTGGC

ChIP-AR-siteA:

F: GACTCGCAAACTGTTGCATT

R: TACAGCACTGGAGCGGCTA

ChIP-AR-siteB:

F: CCTAGCAGGGCAGATCTTGT

R: TCCCCTTCTCTTGCTCAGAA

ChIP-AR-siteC:

F: GGTAGGAAGTGGCTGAATTCTGGATGA

R: CCCTGCCCATGCACCTGCTC

ChIP-ATAD2-distal:

F: TGTCTGTAATCCCAGCACTTCG

R: CCATATCGGCCAAGCTAGTCTC

Luciferase reporter assay

Luciferase assays were carried out as previously described (23). Briefly, HEK293T cells were transfected with AR-FL, AR3, E2F1, or the E2F1 E132 mutant expression plasmids in various combinations as indicated. Cells were then cultured in DMEM medium. Forty-eight hours after transfection, dual-luciferase assays were performed according to the manufacturer's instructions (Promega). The results are presented as the relative changes in luciferase activity.

Co-Immunoprecipitation and Immunofluorescence

For HEK-293T exogenous IP, cells were transfected with the appropriate plasmids and allowed to grow for 48 h in 100 mm dishes. For R1-ADR endogenous IP, cells were plated in P150 dishes for 3 days, 1nM Dihydrotestosterone (DHT) was added, and cells were incubated for 4 h before being collected as indicated. 3×10^8 cells were used for each IP. Immunoprecipitation was performed as previously described (24).

Immunofluorescence was performed as previously described (24).

Flow cytometry analysis

Histone H2AX phosphorylation were detected using LSR 2 flow cytometer. Briefly, 60% confluent R1-ADR cells were fixed and incubated with proper antibody. Antibody staining were performed as described by Huang et al. (162).

Animal model

The animal studies were performed according to protocol ##0917012 reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland School of Medicine, Baltimore, MD, USA. The R1-DDR xenograft was carried out as previously described (23). Animals were treated with solvent or DTX (5mg/kg once per week) + ENZ (10mg/kg per day, 5 days per week) or auranofin (5 mg/kg per day, 5 days per week), n = 5 per group. Tumor sizes and body weight were measured every Monday and Thursday. Tumor volumes were calculated according to the following formula: $0.52 \times r_1^2 \times r_2$ ($r_1 < r_2$). Results of tumor volumes were expressed as means ± SEM. The effect of treatment was analyzed by one-way ANOVA. Post-hoc analysis was performed using Tukey’s test. Relative body weight was analyzed by repeated measures ANOVA with group as between subject factor and time as within subject factor. Immunohistochemical staining of mouse xenograft tumors was carried out as previously described (157).

Chapter 3: Combined treatment with DTX and ENZ inhibits cell growth and induces a distinct gene expression pattern

Introduction

One of the major challenges in treating advanced Pca is therapeutic resistance. Current treatment options for CRPC patients include anti-androgenic therapies (ADT, AA, and ENZ), taxane drugs (docetaxel and cabazitaxel), and immunotherapy (sipuleucel-T). These treatments typically only prolong survival time by 2–8 months owing to the development of resistance to the treatments (163). The molecular mechanisms of drug resistance include AR pathway reactivation, ARVs, the activation of bypass pathways, impaired DNA repair pathways, and enhanced drug efflux. Thus, the development of next generation drugs would be an ideal long-term strategy to improving treatments for Pca. As multiple mechanisms could be involved in drug resistance, it is difficult to achieve the complete inhibition of survival pathways with monotherapy, as other alternative pathways could be activated in response to treatment and could continue to contribute to tumor progression. Thus, there is an inherent theoretical advantage to using combination therapies to enhance efficacy via the more complete inhibition of major pro-survival pathways through suppression of multiple pro-survival factors. Currently, the primary treatment options are to determine the optimal drug sequence and find effective combinations of currently available treatments. The pioneers of clinical studies, such as CHAARTED and STAMPEDE trials, have demonstrated that the overall survival of patients treated with multi-therapy combinations is significantly better than those treated with ADT alone (122, 164, 165).

Despite the early clinical success of multi-target therapies, the molecular mechanisms underlying more effective combination therapies are still poorly understood. The results of combination therapy may not merely reflect the additive effects of the two treatments, but may in fact be synergistic. For example, it has been shown that anti-androgen sensitized Pca cells to radiotherapy by enhancing DNA damage-induced apoptosis (166, 167).

Early clinical evidence from the REASSURE trial indicates that a combination of radium-223 and enzalutamide provides better overall survival rates without increasing the major side effects for CRPC patients (168). Likewise, radium-223 combined with DTX has exhibited the durable suppression of prostate-specific antigen in a drug trial (169). On the other hand, a phase 3 ERA 223 trail that combined radium-223 and AA acetate with prednisone or prednisolone observed an increase in side-effects and a lower overall survival in patients than in the placebo group (170). Thus, further studies regarding the mechanisms and safety of combination therapies would be worthwhile.

In this study, we generated ADT- and docetaxel-resistant Pca cell lines and tested various drug combinations to assess the effects of the drugs on cell growth and apoptosis. We found that a combined therapy using DTX and ENZ was the most optimal combination in inducing these above changes, and further analyzed alterations in the transcriptional program as induced by the treatment.

Results

Combined treatment with DTX and ENZ inhibited cell growth and induced apoptosis in monotherapy-resistant cells

To develop potential combined therapies for ADT- and DTX-resistant Pca cells, we first generated ADT- and DTX- resistant Pca cell models R1-ADR and LN-ADR, which were derived from CWR-R1 and LNCaP parental cells, respectively (Fig. 6A). It is widely accepted that switching to ENZ significantly prolonged the survival of patients after treatment with DTX (171). Thus, we examined the effect of ENZ on DTX-resistant R1-ADR and LN-ADR cells. Both cell lines exhibited very limited responses to ENZ treatment (Fig. 6B). However, in the presence of 1nM DTX, the efficacy of ENZ to inhibit cell growth was enhanced at least 20 fold in both cell lines, which exhibited much lower IC50 values (R1-ADR: IC50 ≈ 1.4 μM, LN-ADR: IC50 ≈ 2.6 μM) in comparison to ENZ treatment alone (R1-ADR: IC50 ≈ 53.4 μM, LN-ADR: IC50 > 50 μM). We also showed that the combined DTX+ENZ therapy exerted a better inhibitory effect than DTX alone in ENZ-resistant R1-ENZR cells (Fig. 6C). These results indicate that the combination effects of these two drugs are independent of the order of prior monotherapies. In addition, we also tested various other drug combinations, such as the PARP inhibitor rucaparib and the taxane compound cabazitaxel (Fig. 6D, E), CK1/2 inhibitors (D-4476/CX-4945), GSK3 inhibitors (CHIR-99021), and an AKT inhibitor (perifosine) (data not shown). However, we did not observe the dramatic enhancement of growth inhibition with these drugs combined with DTX or ENZ as we observed in DTX+ENZ combination.

Next, we tested this drug combination in CWR-R1 parental cells. Interestingly, DTX+ENZ does not further enhanced inhibitory effect on cell growth caused by either drug alone (Fig.

6F). Together, these data indicate that, of the drugs tested here, the combination effects only occurred when DTX+ENZ were combined in resistant cells. Next, we evaluated the inhibitory effect of these drugs in a tumor spheroid model. Similar to the effect in 2D cell culture, the combination of DTX and ENZ inhibited 3D-spheroid growth to a greater extent than did DTX or ENZ alone in R1-ADR and LN-ADR cells (Fig. 7A). To assess whether the inhibitory effect of the DTX+ENZ combination therapy was the result of apoptosis, we performed a TUNEL assay with different drug treatments. The combination of DTX and ENZ exhibited a significantly higher number of TUNEL-positive cells in comparison to either drug alone (Fig. 7B). To further confirm this finding, apoptosis markers for the levels of cleaved PARP and cleaved caspase-3 were measured by western blot analysis, and were consistently increased in the combined therapy group in both cell lines (Fig. 7C). Taken together, these results indicate that the combination of DTX and ENZ inhibited the growth and induced apoptosis of DTX-resistant Pca cells.

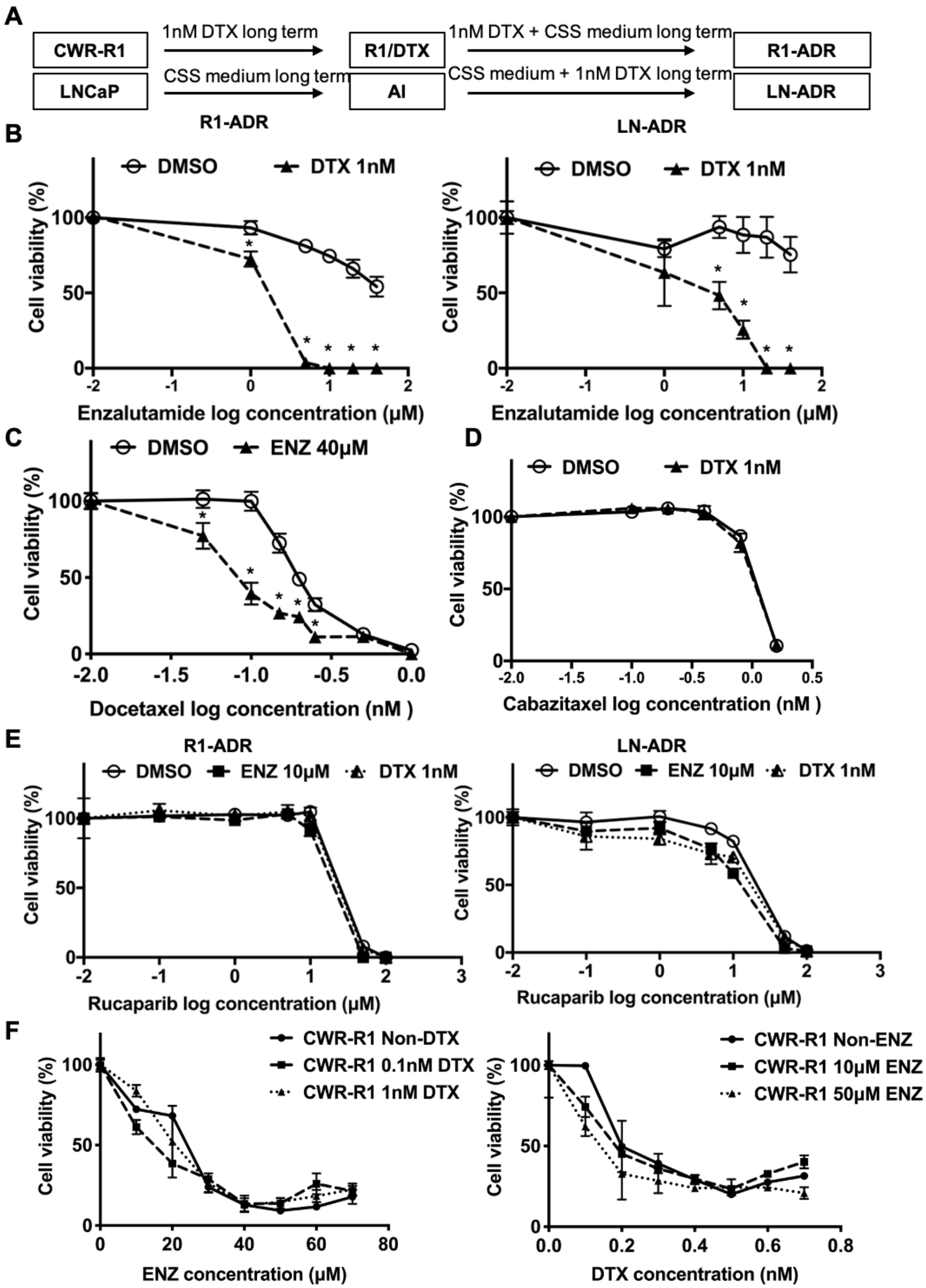


Figure 6. DTX-ENZ combined treatment inhibited growth of drug resistant cells.

A, Schematic diagram of the establishment of drug resistant cell lines. B, Cell growth was determined by CCK-8 assays. R1-ADR (left panel) and LN-ADR (right panel) cells were treated with different doses of ENZ for 6 days with or without 1nM DTX. C, The presence of ENZ enhanced the growth inhibition effect of DTX in ENZ-resistant R1-ENZR cells. R1-ENZR cells were treated with different doses of DTX for 3 days with the presence 40μM ENZ. Error bars, S.D. *, $p<0.05$. D, Combined DTX + cabazitaxel has little or no enhanced effect compared to cabazitaxel alone in R1-ADR cells. R1-ADR cells were treated with different doses of Cabazitaxel for 4 days with the presence 1nM DTX. E. ENZ or DTX has little or no enhanced effect in combination with PARP inhibitor Rucaparib compared to Rucaparib alone. R1-ADR (left panel) and LN-ADR (right panel) cells were treated with different doses of Rucaparib for 4 days with the presence of 10μM ENZ or 1nM DTX. F, Combined DTX and ENZ does not elevated the growth inhibition effect compared to either drug alone. CWR-R1 cells were treated with different doses of DTX and/or ENZ for 3 days.

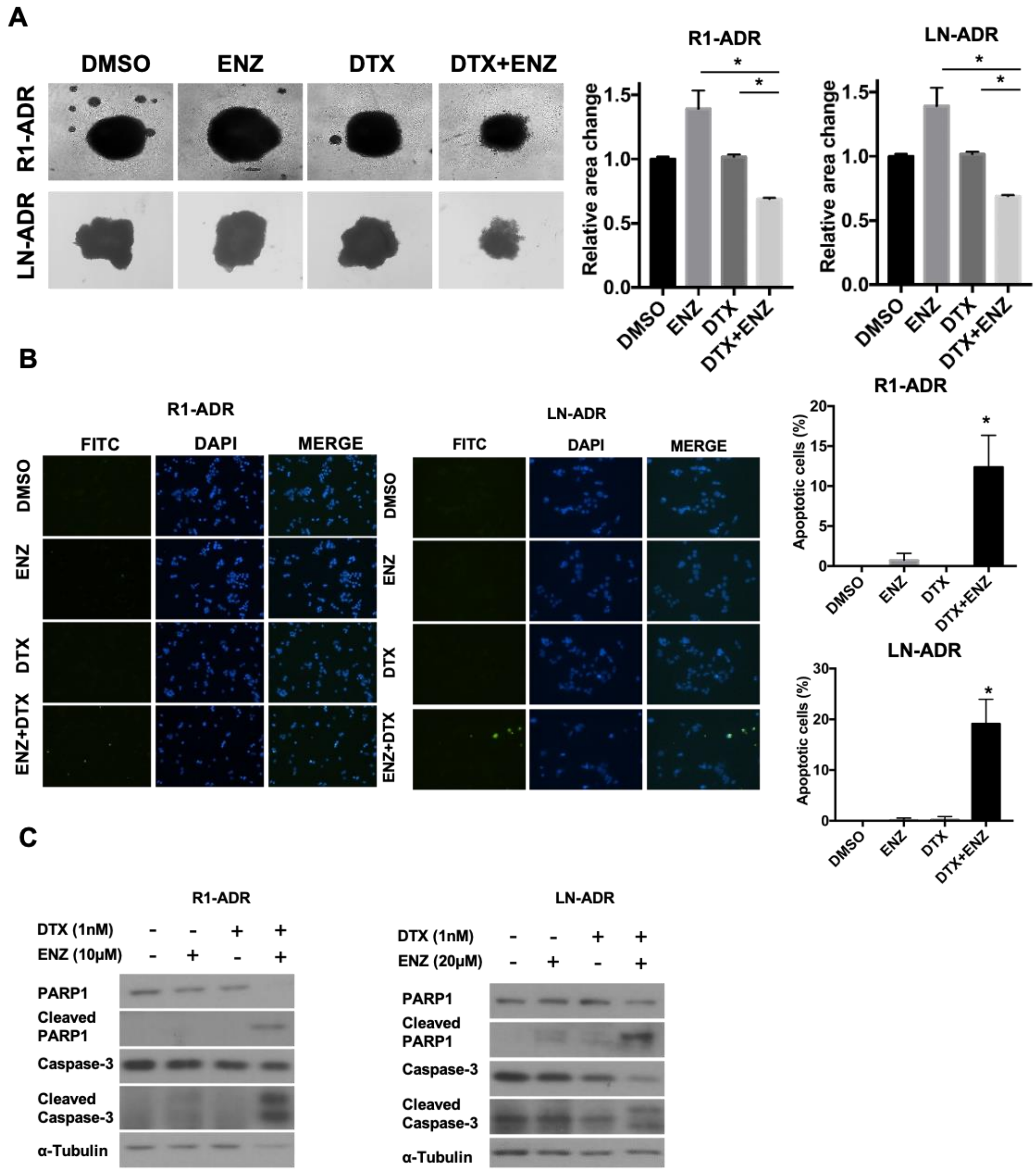


Figure 7. DTX-ENZ combined treatment inhibited 3D-spheroid growth and induced apoptosis of drug resistant cells.

A, Morphology (left panel) and quantification (right panel) of tumor spheroids after 14 days of treatment with 40μM ENZ or/and 2nM DTX (R1-ADR) or 4nM DTX (LN-ADR). B, TUNEL assays were performed to detect apoptosis under the indicated treatments in

R1-ADR and LN-ADR cells (left panel) after 48 hours treatment and quantification (right panel). Cells were treated with 1nM DTX or/and 10μM ENZ (R1-ADR) or 20μM ENZ (LN-ADR). Error bars, S.D. *, $p<0.05$. C, Protein level of cleaved PARP1 and cleaved caspase-3 in R1-ADR (left panel) and LN-ADR (right panel) cells was determined by western blot. Cells were treated with 1nM DTX or/and 10μM ENZ (R1-ADR) or 20μM ENZ (LN-ADR) for 48 hours.

Differential gene expression in prostate cancer cells in response to combined treatment with DTX and ENZ

To investigate the molecular mechanism and cellular pathways responsible for the inhibition of growth observed in response to the combined therapy, we performed RNA-seq to identify differentially-expressed genes in R1-ADR cells after 12 hours of drug treatments (Fig. 8A). We were specifically interested in changes in gene expression in the combined treatment, as neither DTX nor ENZ alone inhibited cell growth. First, we performed a GSEA to compare DTX+ENZ with the other groups (control, DTX alone, or ENZ alone). In agreement with the TUNEL assay result, which showed an increase in apoptosis in the combined therapy group, we found that apoptosis-related hallmarks were enriched in the DTX+ENZ combination group using GSEA (Fig. 8B). Next, we compared patterns of gene expression between DTX+ENZ and the control, DTX alone, or ENZ alone. An analysis of RNA-seq data identified 25 genes that were commonly upregulated and 17 genes that were commonly downregulated in DTX+ENZ treatment groups in comparison to other groups (Fig. 8C), many of which were found to be related to cell cycle processes, DNA replication, and DNA repair. To further narrow down the possible changes in gene expression responsible for the inhibition of growth as a result of the combined therapy, we analyzed altered genes in KEGG pathways in cancer. Our analysis demonstrated that several pathways—including *CREB5*, *WNT5A*, and *E2F1*—were all altered in response to the DTX+ENZ combined therapy in comparison to either drug alone (Fig. 8D). CREB5 is a cAMP response element-binding protein and function as a cAMP response element dependent trans-activator. A recent study showed that CREB5 promoted ENZ resistance in an AR-dependent manner (172). WNT5A is a Wnt secreted signaling protein family member. Lines of evidence have shown WNT5A was associated with prostate cancer aggressiveness (173-175). E2F1 is an important transcriptional mediator in cancer progression and has both oncogenic and tumor-suppressive properties. To further unravel the underlying mechanisms responsible for the inhibition of growth observed in response to the combined therapy, we performed proteomic profiling analysis in R1-ADR cells after 96 hours of drug treatments (Fig. 9A). Similarly, we compared proteomic profile between DTX+ENZ and the control, DTX alone, or ENZ alone. An analysis of proteomics data identified 630 genes that were commonly upregulated and 459 genes that were commonly downregulated in DTX+ENZ treatment groups in comparison to other groups (Fig. 9B). We were able to confirm CREB5 upregulation in response to double drug treatment. However, due to the low WNT5A and E2F1 protein levels in resistant cells and the technical limitation of the method, we could not detect WNT5A and E2F1 in this experiment. Next, we analyzed molecular and cellular functions using Ingenuity Pathway Analysis software. Consistent with the RNA-seq data, cell death and survival, DNA repair, as well as other pathways were altered (Fig. 9C). We further analyzed perturbed upstream regulators, and found E2F1 as one of the affected upstream regulators in response to double drug treatment (Fig. 9D). Together, these data suggest that R1-ADR cells has an altered and unique gene expression and proteomic profile in response to combined treatment.

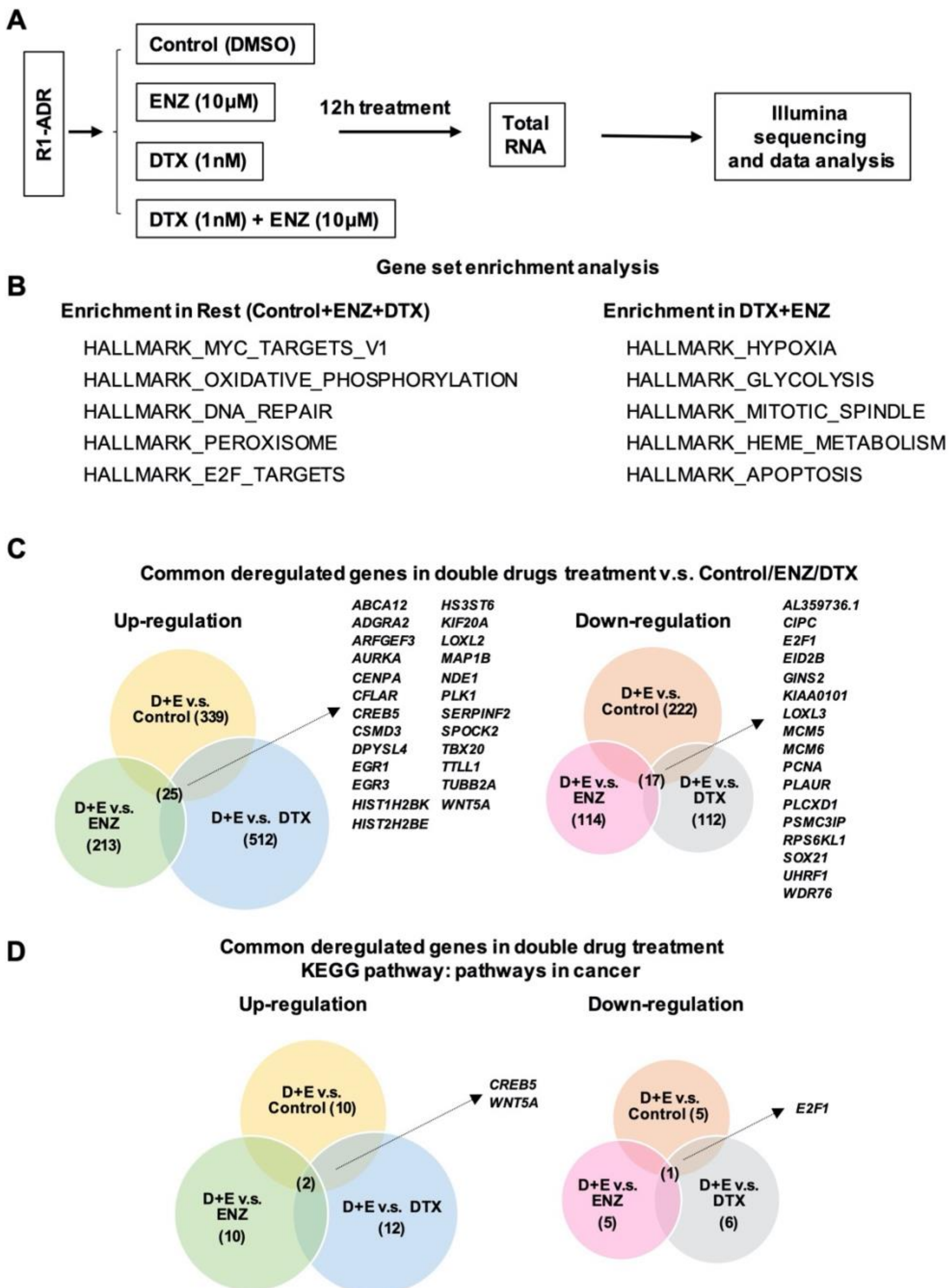
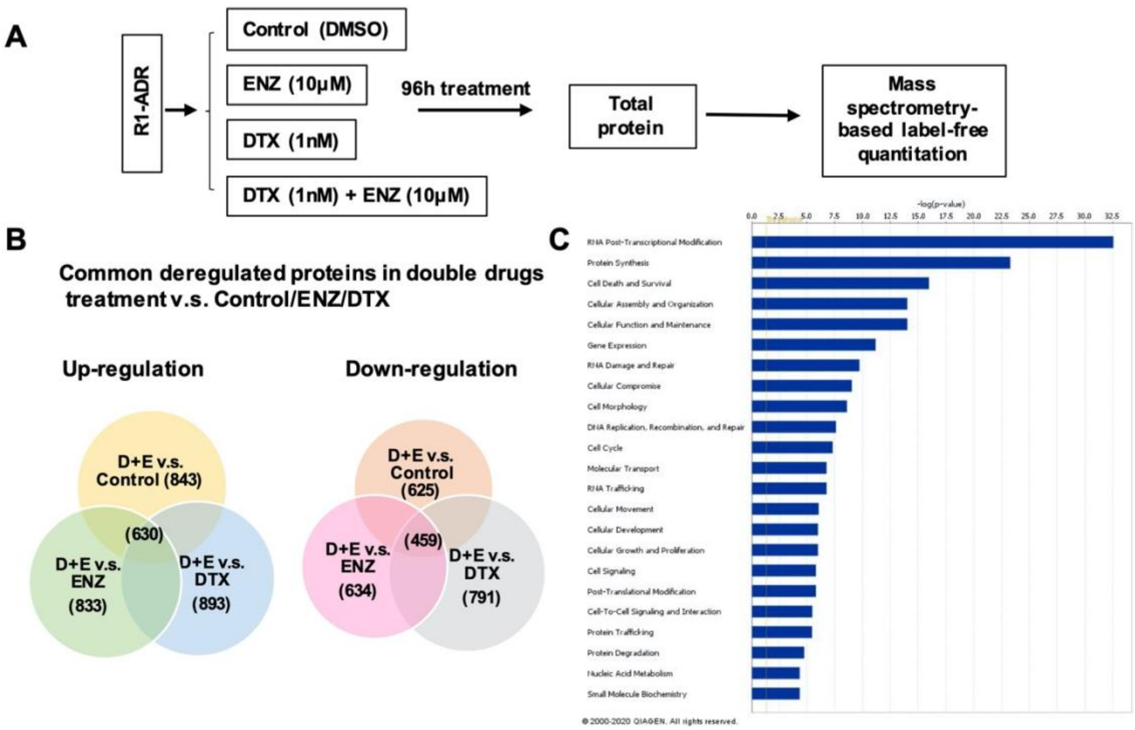


Figure 8. Differential gene expression in response to DTX-ENZ combined treatment.

A, Flowchart of RNA sample preparation and RNA-seq. B, Gene set enrichment analysis of DTX+ENZ v.s. Rest (Control+ENZ+DTX). Top 5 gene sets with lowest FWER p-value were presented. C, Analysis of common deregulated genes in double drugs v.s. Control or ENZ or DTX group. ($p<0.05$, $FDR<0.25$, fold change > 1.4) D, Analysis of common deregulated genes in double drugs v.s. Control or ENZ or DTX group in KEGG pathways in cancer gene list. ($p<0.05$, $FDR<0.25$, fold change > 1.4)



D

Upstream Regulator	Molecule Type	p-value of overlap
HNF4A	transcription regulator	5.17E-21
TP53	transcription regulator	3.61E-18
MYCN	transcription regulator	1.56E-11
ESR1	ligand-dependent nuclear receptor	5.56E-11
CST5	other	5.28E-10
MYC	transcription regulator	1.03E-09
5-fluorouracil	chemical drug	3.29E-09
miR-1-3p (and other miRNAs w/seed GGAAUGU)	mature microRNA	7.8E-09
arsenic trioxide	chemical drug	4.13E-08
ST1926	chemical drug	1.01E-07
MAPT	other	1.88E-07
ERBB2	kinase	1.92E-07
PHF12	transcription regulator	2.93E-07
miR-16-5p (and other miRNAs w/seed AGCAGCA)	mature microRNA	5.01E-07
RICTOR	other	9.15E-07
CDKN1A	kinase	1.22E-06
YAP1	transcription regulator	1.67E-06
methylselenic acid	chemical reagent	1.83E-06
metribolone	chemical reagent	2.2E-06
RPS15	other	2.82E-06
CCND1	transcription regulator	4.1E-06
E2F1	transcription regulator	4.18E-06
1,2-dithiol-3-thione	chemical reagent	4.71E-06
nitrofurantoin	chemical drug	5.12E-06
MLXIPL	transcription regulator	8.67E-06
CD 437	chemical drug	8.83E-06
IL4	cytokine	9.78E-06
napabucasin	chemical drug	1.34E-05
SYVN1	transporter	1.68E-05
methylprednisolone	chemical drug	2.04E-05
sirolimus	chemical drug	2.53E-05
PC-SPES	chemical drug	2.64E-05
CCNK	kinase	5.37E-05
PTP4A1	phosphatase	6.26E-05
TGFB1	growth factor	0.000074

Figure 9. Proteomics alteration in response to DTX-ENZ combined treatment.

A, Flowchart of protein sample preparation and label free quantitation. B, Analysis of common deregulated proteins in double drugs v.s. Control or ENZ or DTX group ($p<0.05$, FDR<0.05, fold change > 1.4). C, Ingenuity pathway analysis of molecular and cellular functions in disease function analysis. ($p<0.001$, fold change > 1.4) DTX+ENZ v.s. Control. D, Ingenuity pathway analysis of deregulated upstream regulator ($p<0.0001$, fold change > 1.4) DTX+ENZ v.s. Control.

Pro-survival role of E2F1 in drug resistant prostate cancer cells

Because CREB5 and WNT5A were considered to promote prostate cancer aggressiveness and drug resistance (172-175). The up-regulation of *CREB5* and *WNT5A* in response to DTX+ENZ could be counter reaction in response to DTX+ENZ acute treatment. Of the three commonly altered genes in KEGG pathways in cancer, E2F1 is an important transcriptional mediator in cancer progression and has both oncogenic and tumor-suppressive properties (Fig. 10). To assess whether E2F1 is critical in the progression of Pca, we analyzed publicly available clinical databases by cancer type using cBioPortal. We found a relatively high rate of genomic amplification or mutation of E2F1 in several types of cancers, including Pca (Fig. 11A). A further analysis of E2F1 in Pca showed that E2F1 was highly amplified in selective Pca studies (Fig. 11B). A Kaplan-Meier survival analysis of the The Cancer Genome Atlas Program (TCGA) prostate adenocarcinoma dataset using the UCSC Xena platform indicated a trend of survival disadvantage for patients with high E2F1 expression (top 25%) in comparison to those with low E2F1 expression (bottom 25%) (Fig. 11C). Thus, as an increase in E2F1 expression plays an important role in advanced Pca, the growth inhibition from the DTX+ENZ combined treatment could be caused by the suppression of E2F1 activity. The downregulation of *E2F1* expression in response to the combined treatment was validated by qRT-PCR (Fig. 12A). In agreement with the qRT-PCR results, E2F1 protein reduction was detected by western blot of prolonged combined treatment in R1-ADR and LN-ADR cells (Fig. 12B). To validate the pro-survival role of E2F1 in drug resistant cells, we knocked down *E2F1* in R1-ADR cells. The cell viability was decreased in *E2F1* knock-down cells and can be partially rescued by exogenously expressed E2F1 (Fig. 12C). Furthermore, growth inhibition as a result of the combined treatment can be partially rescued by exogenously expressed E2F1 in R1-ADR cells (Fig. 12D). Together, these data strongly suggest that E2F1 is an important pro-survival mediator in drug-resistant cells.

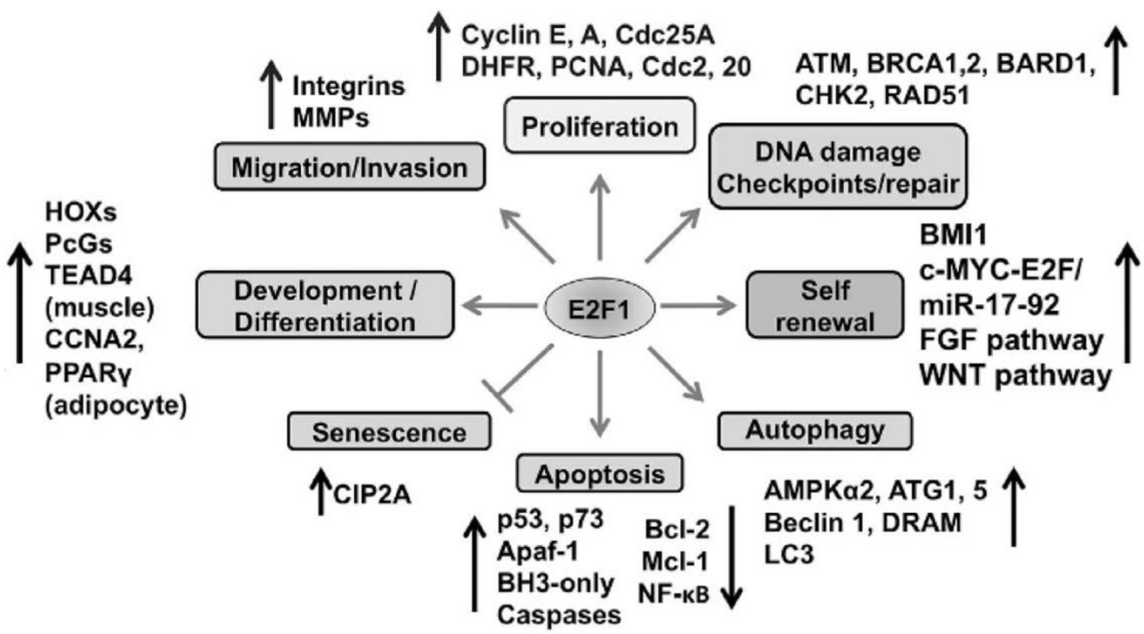


Figure 10. Biological functions of E2F1.

(Figure adapted from P Meng and R Ghosh, *Cell death & disease*, *5*(8), e1360-e1360. (176))

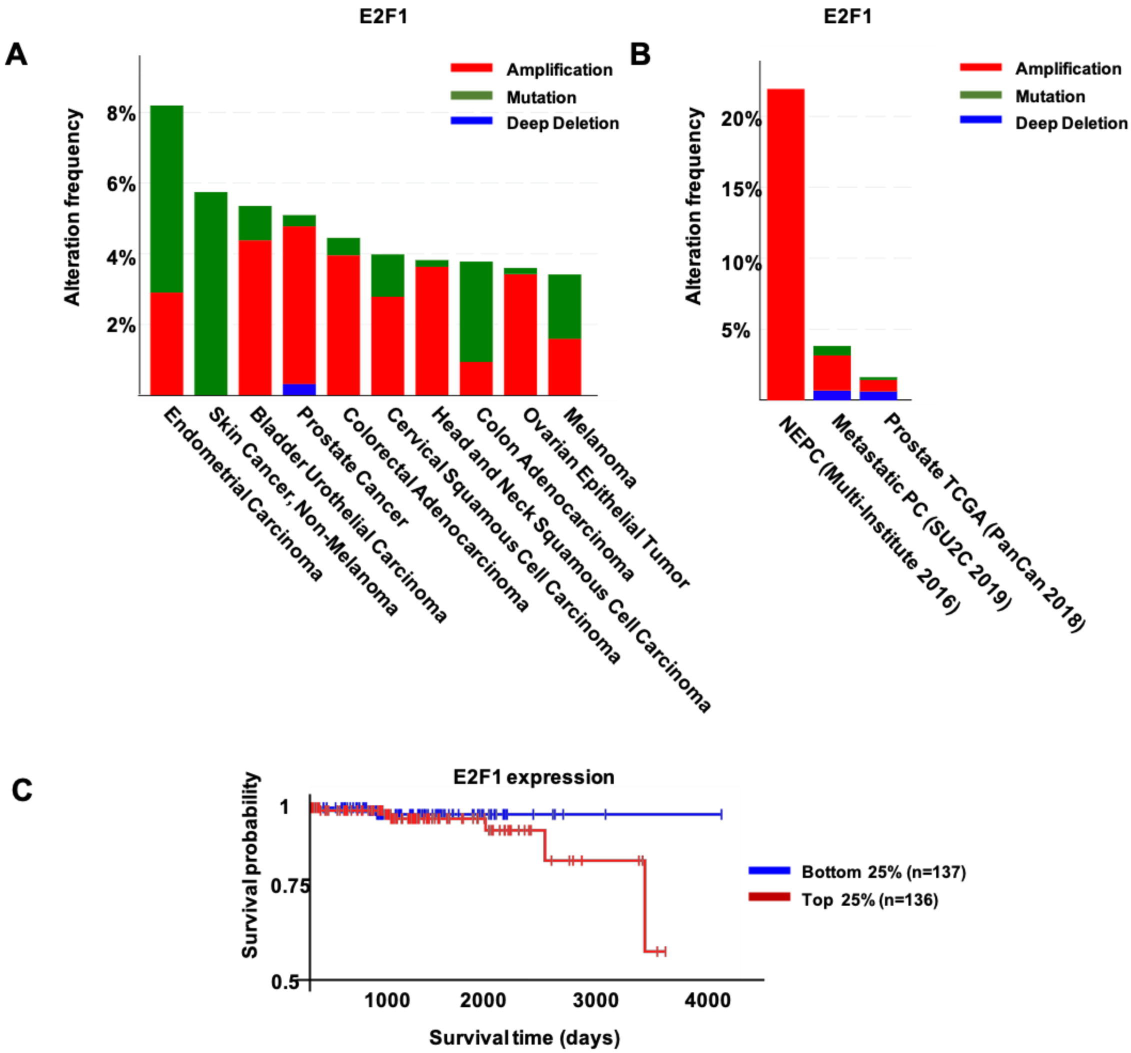


Figure 11. E2F1 expression is associated with prostate cancer progression.

A, Cancer types summary, E2F1 alteration frequency in various cancer types (Curated set of non-redundant studies, minimum 100 total cases per cancer type, minimum 3% altered cases). Data was acquired and analyzed from cBioPortal. B, E2F1 alteration frequency in selected Pca studies. Data was acquired and analyzed from cBioPortal. C, Kaplan-Meier overall survival analysis of TCGA prostate adenocarcinoma database. P-value = 0.2800, Log-rank test statistics = 1.167. Data was acquired and analyzed from UCSC xena.

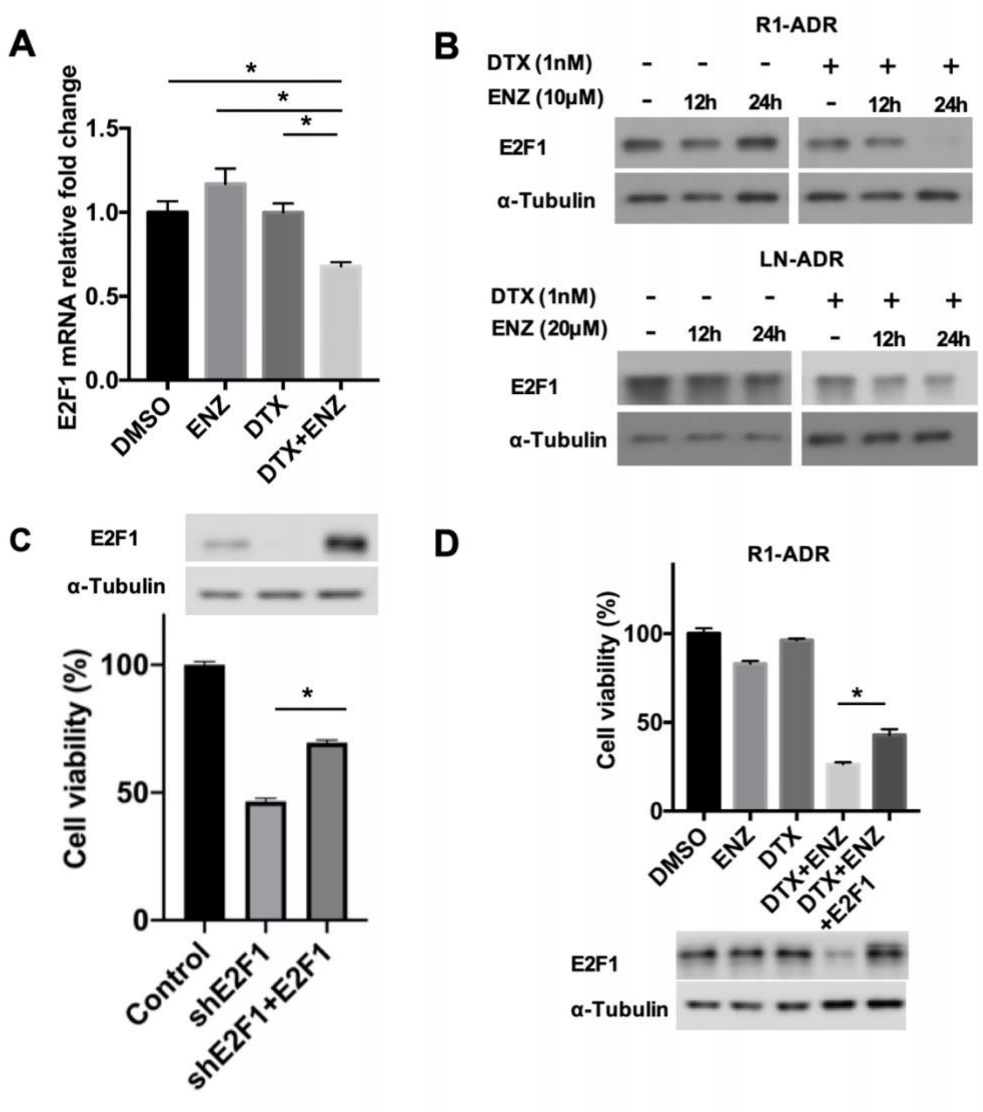


Figure 12. E2F1 has pro-survival function in drug resistant cells.

A, Expression of E2F1 in R1-ADR cells 12 hours after drug treatment were determined by qRT-PCR. Error bars, S.D. *, $p<0.05$. B, Protein level of E2F1 in R1-ADR (upper panel) and LN-ADR (lower panel) cells 12 and 24 hours after drug treatment were determined by western blot. C. Exogenously expressed E2F1 partially rescued E2F1 knock-down caused growth inhibition. Cell growth was determined by CCK-8 assays.

Error bars, S.D. *, p<0.05. D, Re-expressed E2F1 partially rescued DTX+ENZ induced growth

Discussion

In this study, we generated ADT- and DTX-resistant Pca cell lines and tested various combined drug therapies. We found DTX+ENZ effectively inhibited the growth of Pca cells and induced apoptosis in ADT- and DTX-resistant cells. An RNA-seq analysis suggests that E2F1 is one possible target of the combined treatment. We observed a decrease in E2F1 expression in response to the combined DTX+ENZ treatment. The E2F family of proteins plays a critical role in multiple cellular processes. There are six E2F family proteins, where the E2F1-3 transcription factors are generally considered to be gene expression activators and E2F4-6 are considered to be gene expression repressors. E2F1 plays a dual role in tumor progression, as it functions as both an oncogene and a tumor suppressor. It has been well-documented that E2F1 can both induce apoptosis in tumor cells and inhibit tumor growth. Various mechanisms—such as the p53-dependent pathway, the p73 pathway, and the Apaf1 pathway—have been attributed to E2F1-induced apoptosis. In response to DNA damage, E2F1 is stabilized and induces the expression of pro-apoptotic genes. Thus, DNA-damaging agents have been proposed in the treatment of tumors with high levels of E2F1 expression for an additive anti-tumor effect. It has been reported that the expression of E2F1 enhanced the cytotoxic effect of ionizing radiation *in vitro* and *in vivo* (177). On the other hand, E2F1 has also been shown to be oncogenic in various tumors. Here, we analyzed a TCGA Pca cohort and found that high levels of E2F1 expression is associated with worse overall survival. It has been shown that E2F1 activates multiple oncogenic pathways and promotes the survival, invasion, and migration of Pca cells (178, 179). E2F1 is also a critical regulator of the DNA-damage response via its role as a transcription factor, as well as various other roles. E2F1 regulates the expression of several DNA repair genes, involved in DNA double strand break (DSB) repair such as homogolous recombination (HR) protein *RAD51*, non homologous end-joining (NHEJ) protein *DNA-PKcs* and single strand break repair protein *XRCC1* (180-182), and can also directly localize to the DNA damage foci and promote the recruitment of DNA repair factors (183). In the present study, we found that the knockdown of E2F1 suppressed drug-resistant cell growth, indicating that E2F1 contributes to the survival of resistant cells.

In order to develop a more effective combination therapy, it is first necessary to develop a more detailed understanding of the possible mechanisms of action of these combinations. It worth noting that the re-expression of E2F1 only partially rescued the inhibition induced by DTX+ENZ, indicating the possibility that additional mechanisms are at work to suppress cell growth. It has been previously reported that the ABCB1 drug efflux pump was overexpressed in DTX-resistant C4-2B-TaxR cells in comparison to DTX-sensitive C4-2B parental cells (184). The addition of the anti-androgenic agent bicalutamide inhibited the efflux activity of ABCB1 and reversed DTX resistance in C4-2B-TaxR cells *in vitro* and in a xenograft model. However, it has been also reported that androgen signaling negatively regulated ABC transporter expression in androgen-dependent Pca cells (185). In the present study, a proteomic analysis of whole cells via mass spectrometry showed that the levels of ABC transporter proteins were upregulated in response to treatment with DTX+ENZ. Thus, further studies will be necessary to determine the effect of anti-androgenic compounds on ABC transporters across different types of Pca cells. In our RNA-seq analysis, we also observed alterations in important genes involved in DNA repair, mitotic spindle formation, glycolysis. It is very interesting to study how these pathways are affected by double-drug treatments and whether these pathways are involved in drug resistance.

Chapter 4: AR3 regulates E2F1 expression and forms a positive feedback regulation loop with E2F1

Introduction

It is well accepted that ARVs pose one of the greatest challenges in Pca drug resistance. Constitutively-activated AR3 plays an important role in the progression and drug resistance of CRPC, and is associated with primary resistance to both AA and ENZ therapies (32). Higher levels of *AR3* transcripts in tumor cells circulating in the blood have been shown to be associated with shorter time to treatment failure and a shorter overall survival time with AA or ENZ treatment, indicating an unfavorable prognosis for patients (186, 187). Thus, there is an urgent need to study the molecular and biochemical mechanisms of AR3-mediated drug resistance.

One of the most hotly debated topics in the field is whether the presence of AR-FL is required for the function of AR3. Recent evidence suggests that AR3 functions independently of AR-FL. It has been shown that the overexpression of AR3 alone in AR-negative PC3 and Du145 cell lines promoted cell migration, clonogenic growth, and sphere formation (188). Chan et al. tested the ability of AR3 to bind to ARE sites using electrophoretic mobility shift assays in non-AR expressing COS-7 cells, and the results showed that AR3 was able to bind to canonical ARE via homodimerization (119). On the other hand, some reports have argued that the presence of AR-FL is mandatory for the growth-promoting function of AR3. It has been shown that the expression of AR3 was coupled to AR-FL expression and that the anti-androgenic or selective knock down of AR-FL decreased AR3-induced growth (38). Cao et al. (44) showed that ARV regulated canonical androgen-responsive genes by co-occupying the target gene promoter with AR-FL. Thus, it is likely that AR3 promotes cell growth via both AR-FL-dependent and AR-FL-independent mechanisms and that the process is cell-type and context dependent.

There is growing body of evidence that recognizes the unique functions of ARVs and that AR3s can regulate distinct transcriptional programs in comparison to the androgen-induced AR pathway. This gives rise to a new question: Do ARVs regulate target genes by binding to unique regulatory regions? A few recent studies that used genome-wide ChIP-seq analyses suggest that ARVs binds to DNA with AR-FL. A recent study investigated AR3 binding sites by performing ChIP-seq analyses using two different antibodies and showed that AR3 and AR-FL heterodimerized and bound to the same chromatin sites (189). The authors claimed that AR3 preferentially interacted with a transcriptional co-repressor and negatively regulated tumor suppressor genes. Notably, results from another genome-wide study supported the notion that ARVs and AR-FL bind to the same sites and showed that ARV preferentially bound to DNA at open chromatin that was pre-occupied by AR-FL, while AR-FL prefers to localize to closed chromatin regions (190). Consistent with this finding, chromatin pioneer factor HoxB13 was required for AR3 activity and universally colocalized with AR3 at open chromatin in CRPC genomes (191). However, this study also revealed that HoxB13 only colocalized with AR3, and not with AR-FL in both a CRPC cell model and in clinical specimens, indicating that AR3 binds chromatin independent of AR-FL. Another study that used ChIP-seq showed that a significant portion of AR3 binding sites lacked AR-FL binding. Furthermore, BRD4 and ZFX co-occupy with AR3 on AR3-unique binding sites at target gene promoters (192). Controversies could arise from a number of factors, such as antibody binding affinity and specificity, cell lines, and drug selection pressure. In the previous chapter, we showed that E2F1 is one of the most important survival factors in Pca cells, and is downregulated by DTX+ENZ. Interestingly, some studies have shown that AR3 preferentially regulates cell cycle-related genes, including the E2F family member E2F7 (45, 190, 192). Thus, it is possible that the loss of E2F1 in response to double drug treatment is caused by a decrease in either AR3 or the activity of AR3 cofactors. In this chapter, we sought to determine whether AR3 preferentially regulates a subset of genes, such as E2F1, independently of AR-FL in drug-resistant cells.

Results

ARV and AR-FL differentially regulates E2F1 expression in docetaxel and ADT-resistant cells

We observed AR3 and E2F1 protein levels increased in docetaxel and ADT-resistant R1-ADR and LN-ADR cells compared to their parental cells (Fig. 13A). It is very possible that AR3 and E2F1 are corporately up-regulated and contribute to Pca aggressiveness. As a result, we wondered whether AR or AR3 played a role in the regulation of E2F1 expression. To test this hypothesis, we knocked down *AR-FL* or *AR3* expression in R1-ADR cells using target-specific shRNAs. The level of E2F1 protein was decreased only in AR3 knock-down cells (Fig. 13B). To determine whether AR3 regulated E2F1 on the transcriptional level, we examined *E2F1* mRNA levels via qRT-PCR. In agreement with the western blot results, we found that the levels of *E2F1* mRNA were decreased in *AR3* knock-down cells (Fig. 13C). In addition, the treatment of R1-ADR cells with 1, 10, or 100 nM of DHT had little effect on E2F1 protein expression (Fig. 13D), suggesting that AR-FL and androgen signaling play a very limited role in regulating E2F1 in these cells. To further examine whether AR3 directly regulates E2F1 expression by binding to *E2F1* regulatory regions in chromatin, we analyzed the *E2F1* promoter sequence and identified several putative androgen response elements (AREs). We then performed a ChIP assay using anti-AR3 and anti-AR-FL antibodies and found that AR3, but not AR-FL, binds to the *E2F1* promoter. The specificity of AR3 binding was confirmed by knocking down *AR3*, but not *AR-FL*, which effectively diminished the recruitment of AR3 to the *E2F1* promoter (Fig. 13E). The *FKBP5* regulatory region was used as a positive control. Next, we examined the activity of AR-FL and AR3 using an *E2F1* promoter-driven luciferase reporter (E2F1-LUC). As shown in Figure 14A, only AR3, but not AR-FL, enhanced E2F1 reporter activity, which was not affected by addition of AR-FL or treatment with DHT or ENZ. Using the ARR2-LUC reporter, we also tested whether AR3 and AR-FL could enhance canonical androgen pathway target reporter activity. We found AR3 itself was sufficient to induce ARR2-LUC reporter activity (Fig. 14B). Interestingly, in the presence of AR-FL, ENZ effectively lowered the AR3-induced ARR2-LUC activity, which is in agreement with those of previous studies (38). Taken together, these data suggest that AR3 regulates E2F1 expression independent of AR-FL in R1-ADR cells, and that dependency on AR-FL may be context dependent.

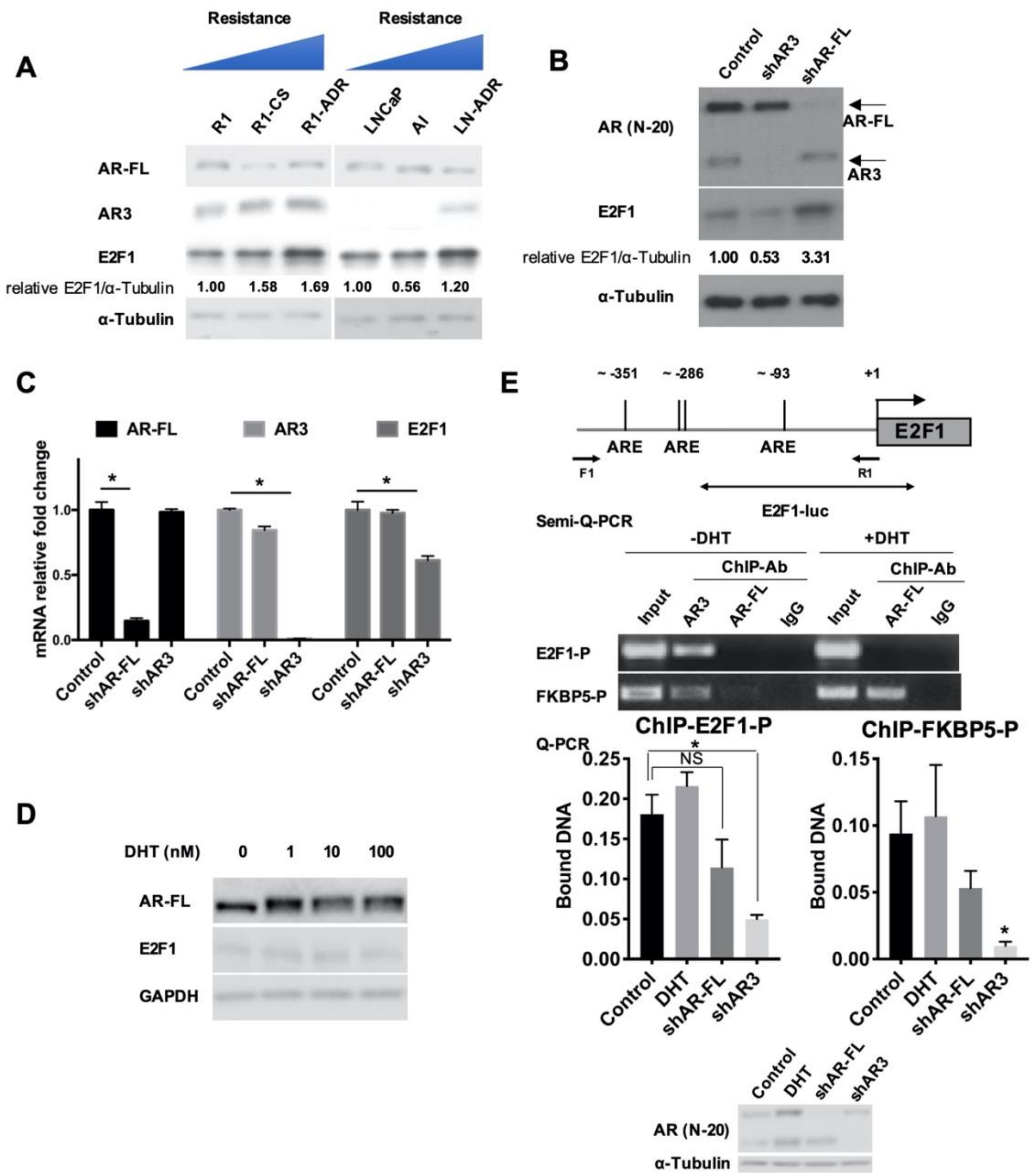


Figure 13. AR3 and AR-FL differentially regulate E2F1 expression.

A, AR-FL, AR3, and E2F1 expression in panels of cells were determined by western blot. B-C, R1-ADR cells were infected with the lentivirus encoding the control shRNA, AR3 shRNA (shAR3) or AR-FL shRNA (shAR-FL) for 48 hours. Protein level of E2F1, AR-

FL, and AR3 were determined by western blot (B) and mRNA level were determined by qRT-PCR (C), Error bars, S.D. *, p<0.05. D, E2F1 expression under different doses of DHT treatment for 24h was determined by western blot. E, Binding of AR3 or AR-FL to the putative ARE sites of human *E2F1* gene regulatory region was analyzed by ChIP assays. *FKBP5* promoter primer sets used as a positive control. Error bars, S.D. *, p<0.05.

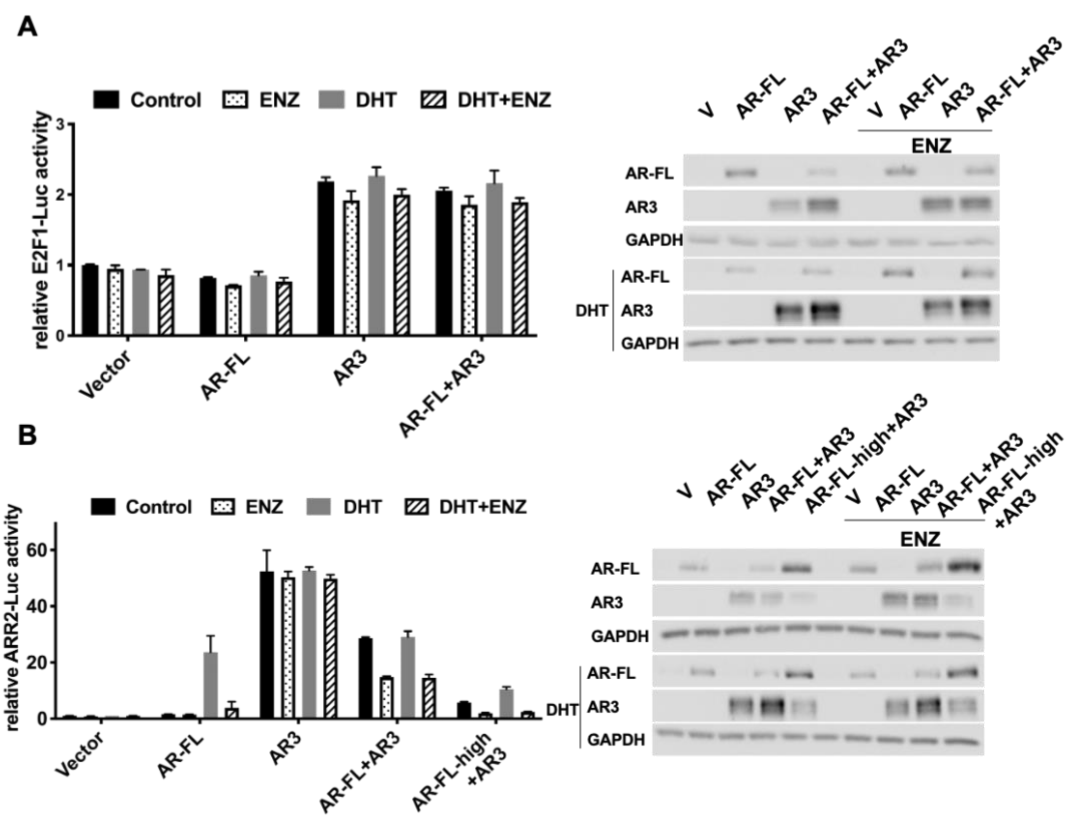


Figure 14. AR3 and AR-FL differentially regulated target gene expression.

A, The E2F1-promoter driven luciferase reporter (E2F1-LUC) activity was measured in HEK293T cells treated with 0.1nM DHT or/and 10µM ENZ (left panel). Expression of AR-FL, and AR3 were confirmed by western blot with GAPDH as a loading control (right panel). B, The ARR2-promoter driven luciferase reporter (ARR2-LUC) activity was measured in HEK293T cells treated with 0.1nM DHT or/and 10μM ENZ (left panel). Expression of AR-FL, and AR3 were confirmed by western blot with GAPDH as a loading control (right panel).

Differential regulation of E2F1 by AR3 and AR-FL is mediated by recruitment of the of co-factor Rb

Next, we aimed to examine how AR-FL and AR3 differentially regulate E2F1 in R1-ADR cells. It has been reported that E2F1 interacts with AR-FL directly, in either an androgen-dependent or androgen-independent manner (193, 194). Interestingly, both E2F1 activation signaling and AR activation signaling are positively correlated and overlap both in CRPC cell lines and patient samples (99, 195). Moreover, a recent report showed that AR can repress the expression of a subset of E2F-regulated genes by recruiting hypo-phosphorylated Rb upon DHT stimulation (196). Thus, it is possible that AR3 and E2F1 cooperatively regulate gene expression, including the regulation of E2F1 itself via the differential recruitment of transcriptional co-factors in comparison to AR-FL. To test this hypothesis, first, we performed co-immunoprecipitation assays to determine whether E2F1 was associated with AR-FL or AR3 in R1-ADR cells. Figure 15A shows that both endogenous AR-FL and AR3 were able to be co-immunoprecipitated with, and therefore interact with, E2F1 in R1-ADR cells. Such interactions were also confirmed when E2F1 was overexpressed with AR-FL or AR3 in 293T cells (Fig. 15B), suggesting a direct interaction between E2F1 and AR-FL or AR3. Previous studies have shown that AR-FL interacted with Rb, and that binding was enhanced by DHT treatment in exogenous overexpression HEK293T cells (196, 197). Next, we examined the interaction between Rb with AR-FL or AR3 in R1-ADR cells. In agreement with previous reports, AR-FL interacted with Rb and this interaction was enhanced by DHT (Fig. 15C). However, AR3 was not co-immunoprecipitated with Rb, suggesting that there is a selective interaction between ARVs and Rb in these cells. Furthermore, it has been shown that E2F1 can be positively self-regulated by binding to its own promoter (156). Thus, we next examined whether AR3 and E2F1 regulate E2F1 expression using E2F1-LUC. Indeed, AR3+E2F1 enhanced luciferase activity compared to E2F1 alone (Fig. 16A). Conversely, the addition of AR-FL did not further increase luciferase activity in comparison to E2F1 alone. Next, we co-expressed the mutant E2F1 plasmid with no DNA binding activity with AR-FL or AR3, examined luciferase activity using E2F1-LUC, and found that AR3+mutant E2F1 did not increase luciferase activity in this case (Fig. 16B). Taken together, these findings suggest that AR3 and AR-FL recruit different cofactors and that the AR3+E2F1 complex regulates E2F1 expression.

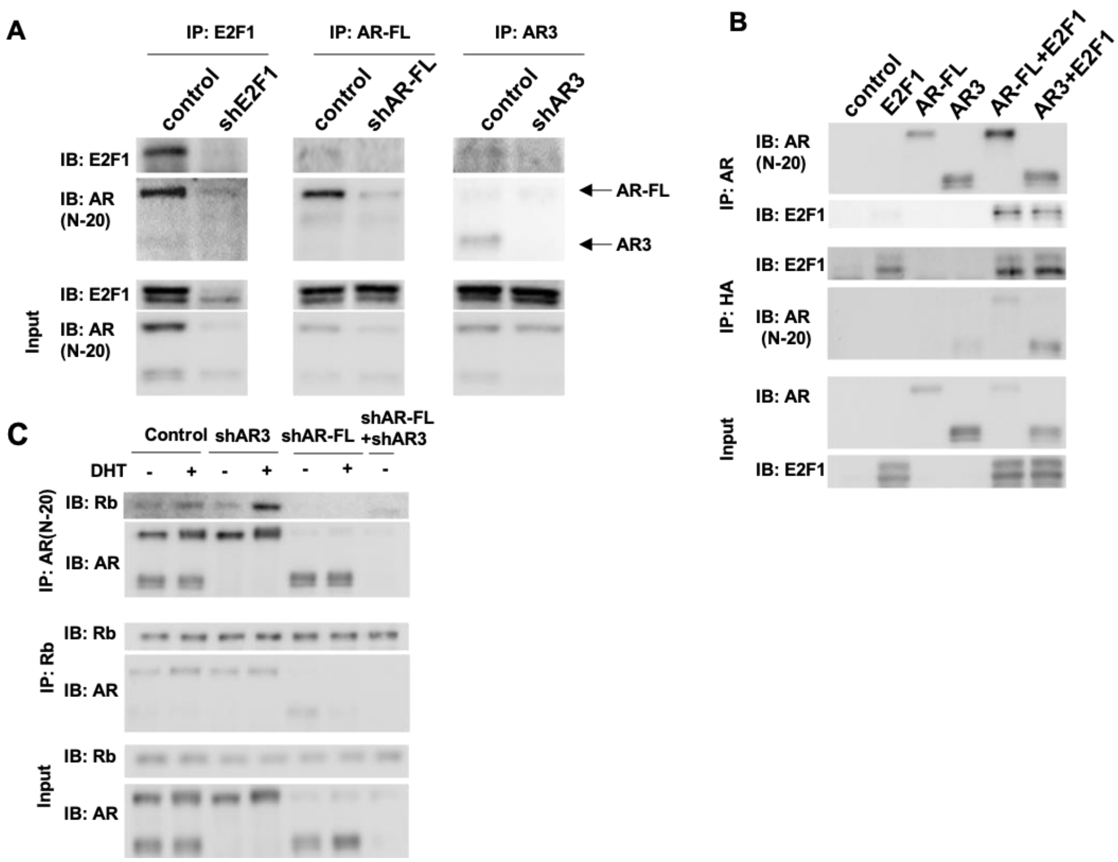


Figure 15. AR3 and AR-FL recruited different cofactors in drug resistant Pca cells.

A, R1-ADR cells were infected with the lentivirus encoding the control shRNA, AR3 shRNA (shAR3) or AR-FL shRNA (shAR-FL) for 48 hours. Cell lysates were then immunoprecipitated with an anti-AR or anti-E2F1 antibody followed by western blot. B, HEK293T cells were cotransfected with AR-FL, AR3, and/or E2F1-HA. Cell lysates were then immunoprecipitated with an anti-AR or anti-HA antibody followed by western blot. C, R1-ADR cells were infected with the lentivirus encoding the control shRNA or AR3 shRNA (shAR3) or AR-FL shRNA (shAR-FL) for 48 hours and then treated with 10nM DHT for 2 hours before collection. Cell lysates were then immunoprecipitated with an anti-AR or anti-Rb antibody followed by western blot.

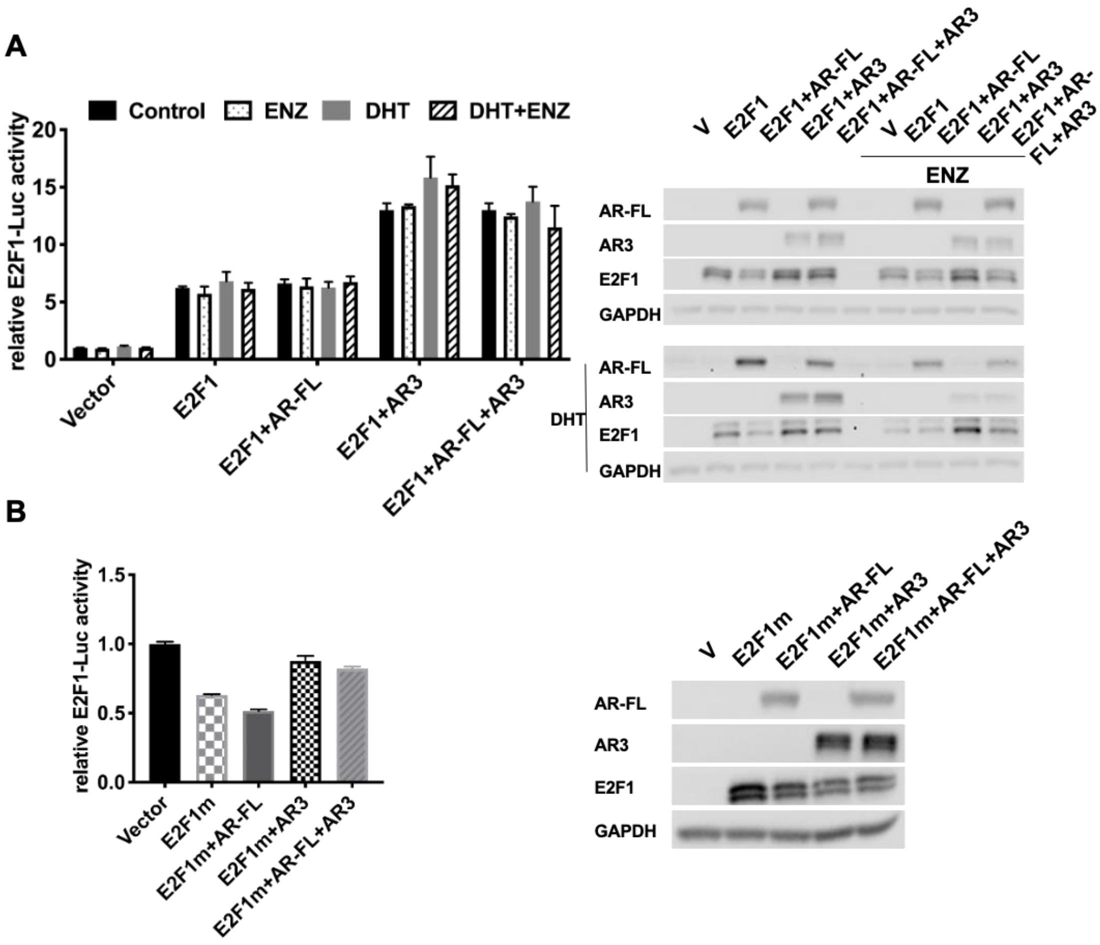


Figure 16. E2F1 and AR3 cooperatively regulated E2F1 expression.

A, E2F1-LUC activity was measured in HEK293T cells treated with 0.1nM DHT or/and 10μM ENZ (left panel). Expression of AR-FL, AR3, and E2F1 were confirmed by western blot with GAPDH as a loading control (right panel). B, E2F1-LUC assay with mutant E2F1 was performed in HEK293T cells (left panel). Expression of AR-FL, AR3, and E2F1m were confirmed by western blot with GAPDH as a loading control (right panel).

E2F1 regulated AR expression and forms a positive feedback loop with AR3

Because ENZ is known to target AR-FL, we also examined AR-FL and AR3 expression after treatment with DTX+ENZ for 4 days. Figure 17A shows that the protein level of AR-FL and AR3 decreased along with that of E2F1. As it has been previously shown that E2F1 regulated AR expression (198), it is possible that the combined treatment induced the downregulation of E2F1, which could in turn have caused a reduction in both AR-FL and AR3. To test this hypothesis, we knocked down *E2F1* using two E2F1-specific shRNAs. The level of AR-FL and AR3 were examined by qRT-PCR and western blot. The results show that both AR-FL and AR3 mRNA and protein expression decreased upon E2F1 knock-down in R1-ADR cells (Fig. 17B, C). We also examined the effect of *E2F1* knock-down in other cell lines. Similarly, we found that both AR-FL and AR3 protein level were decreased by *E2F1* knock-down (Fig. 17D). Controversially, Davis et al. (199) demonstrated that the overexpression of E2F1 inhibited AR mRNA and protein expression in LNCaP cells. A more recent study from the same group showed that E2F1 and DNMT1 co-occupied the AR promoter and repressed AR expression (198). To resolve the apparent discrepancy, we over-expressed E2F1 at various concentrations in R1-ADR cells and examined the effects of this on AR gene expression. In agreement with previous studies, E2F1 inhibited both AR-FL and AR3 expression at higher levels of expression (Fig. 17E). However, at relatively lower levels of expression, E2F1 was able to promote AR-FL and AR3 expression. This finding suggests that the role of E2F1 in regulating AR expression is closely associated with transcript abundance, and is possibly dictated by the availability of its co-factor(s). To further examine the endogenous binding of E2F1 to the *AR* promoter, we performed a ChIP assay, which tested the E2F1 binding sites on *AR* promoter using a primer set from Valdez et al. (sites A, B, and C) (198). In comparison to the *E2F1* knock-down group, the control exhibited much higher E2F1 ChIP binding affinity to sites A and B (Fig. 17F). While we also examined DNMT binding to these sites, there was no evidence of DNMT1 binding in R1-ADR cells (data not shown). Thus, these data indicate that E2F1 directly regulates the expression of AR in DTX-resistant Pca cells, and that this regulation is possibly context and concentration dependent.

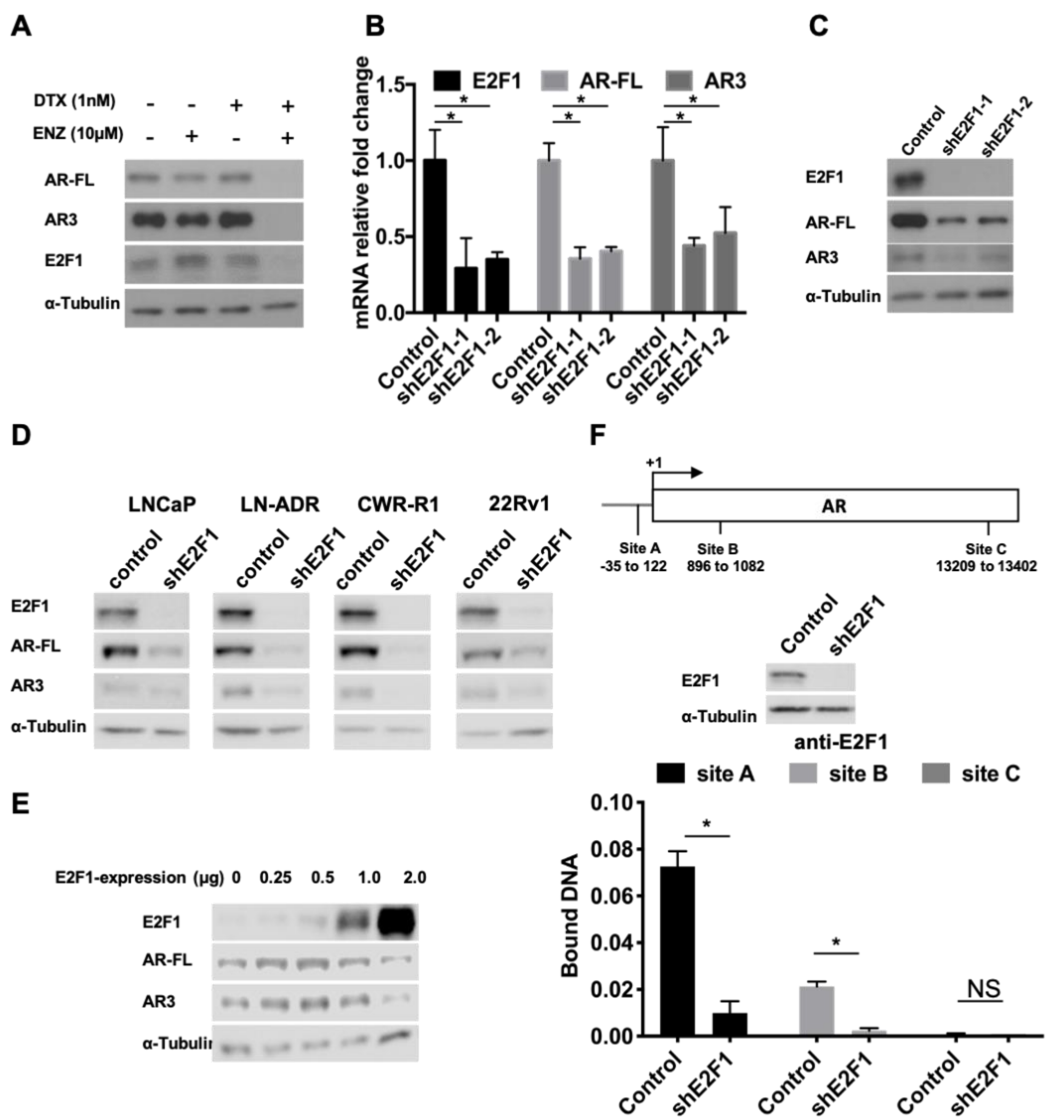


Figure 17. E2F1 regulated AR expression.

A, AR-FL, AR3, and E2F1 protein level 4 days after DTX+ENZ treatment were determined by western blot. B-C, R1-ADR cells were infected with the lentivirus encoding the control shRNA or E2F1 shRNA for 48 hours. Expression level of E2F1, AR-FL, and AR3 were determined by qRT-PCR (B), Error bars, S.D. *, $p<0.05$ and western blot (C). D. Cells were infected with the lentivirus encoding the control shRNA or E2F1 shRNA for 48 hours. Protein level of E2F1, AR-FL, and AR3 in multiple Pca cell lines were determined by western blot. E, Different concentration of E2F1 plasmids were transfected into R1-ADR cells and cultured for 48 hours. Protein level of E2F1, AR-FL, and AR3 were determined by western blot. F, Binding of E2F1 to the regulatory regions of *AR* was analyzed by ChIP assay.

Loss of AR3 and E2F1 caused DNA double strand break accumulation in DTX-resistant Pca cells

A number of studies have begun to examine the role of AR signaling and E2F1 signaling in DNA damage response. Previous studies have suggested that AR regulated a transcriptional program of DNA repair genes (200-202), and E2F1 regulated several major DNA repair pathways (180, 182). As a result, we questioned whether AR3 and E2F1 corporately regulate DNA damage response in drug-resistant Pca cells. To answer this question, we first examined the levels of the most lethal forms of DNA damage, DNA double strand breaks (DSBs) as a consequence of drug treatment by immunostaining for the established DSB marker, γ-H2AX (203). Figure 18A. shows that γ-H2AX was accumulated only under DTX+ENZ double drug treatment. The upregulation of γ-H2AX in response to the combined treatment was validated by immunofluorescent flow cytometry and western blot analysis (Fig. 18B, C). Next, to test whether AR3 and E2F1 play a role in DNA double strand break accumulation in drug-resistant cells, we knocked down *E2F1* or *AR3* using specific shRNAs. γ-H2AX level was increased upon *E2F1*, or *AR-FL*, or *AR3* knock-down in R1-ADR cells (Fig. 18D). ATAD2 is an is an E2F1 target and an important epigenetic reader during replication and mediates expression and activation of factors including, DNA repair factors BRCA1 and cell cycle checkpoint CHK1/2 (204, 205). Our qPCR of mRNA show that *ATAD2* and other E2F target genes *PCNA* and *CDC6* are decreased upon combined treatment (Fig. 18E). *ATAD2* knockdown sensitizes triple-negative breast cancer to chemotherapy and radiation (204). In addition, it has been shown AR-FL interacts with E2F1 and regulated *ATAD2* expression (194). We, thus performed a ChIP assay using anti-AR-FL, anti-AR3, and anti-E2F1 antibodies and found all these factors can binds to *ATAD2* regulatory region, and the specificity was confirmed by knocking down experiment. Knockdown of either *AR-FL* or *AR3* decreased AR antibody binding and knockdown of *E2F1* decreased E2F1 antibody binding to the regulatory regions of *ATAD2* (Fig. 18F). This finding is consistent with the previous ChIP-seq result that both AR and E2F1 can binding to *ATAD2* distal regulatory regions (194). Next, we examined the mRNA levels of *ATAD2* by qRT-PCR with respect to depletion of E2F, AR3 or AR-FL. We found *ATAD2* mRNA was decreased in *E2F1* and *AR3* knock-down cells, but not in AR-FL knock-down cells (Fig. 18G), indicating the distinct regulatory function of ARVs and AR-FL on ATAD2, and potentially with respects to DNA repair in drug-resistant Pca. Together, these results indicate AR3 and E2F1 play important roles in DNA double strand break accumulation.

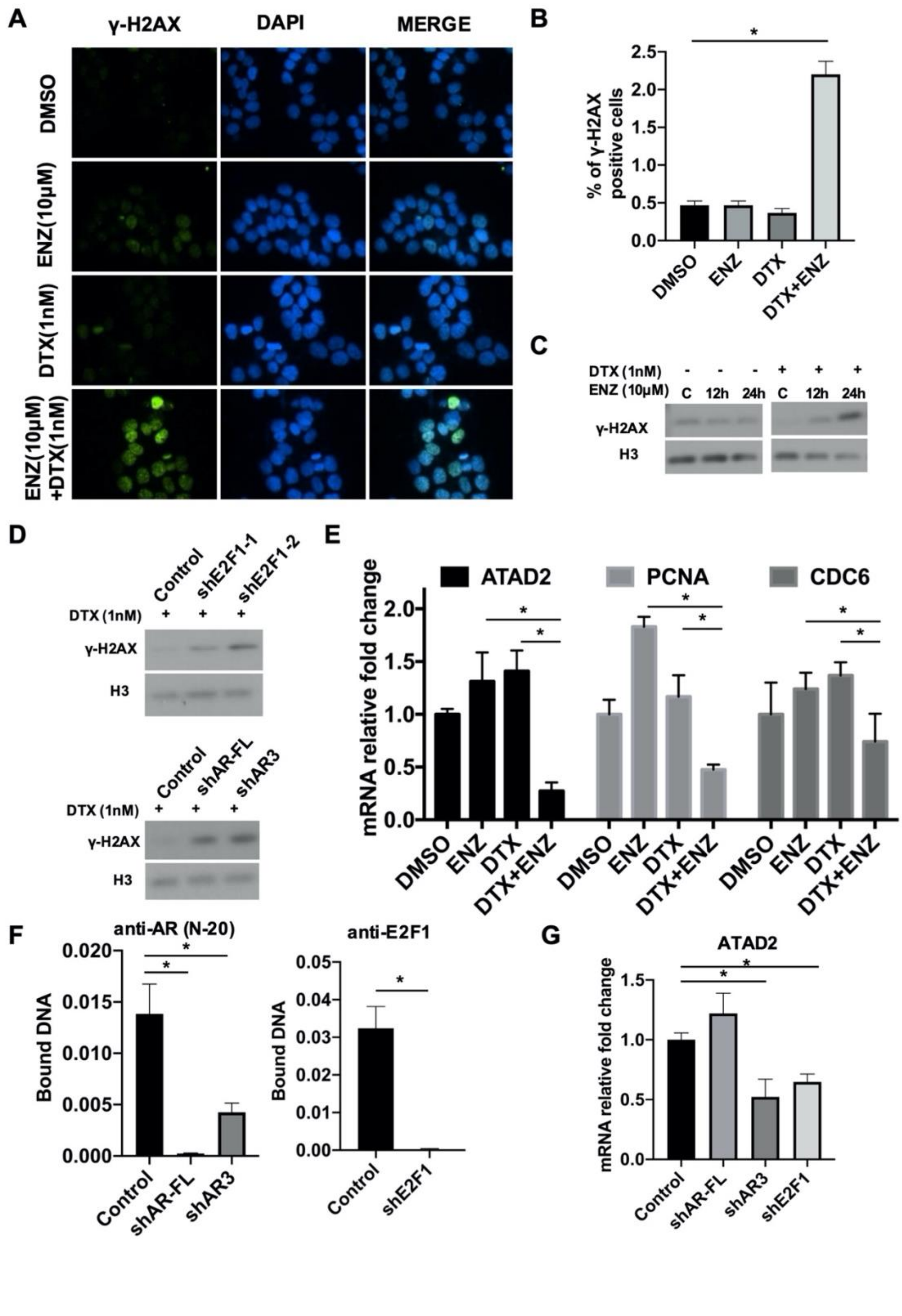


Figure 18. AR3 and E2F1 axis associated with DNA double strand break accumulation.

A, Immunofluorescent staining of γ-H2AX in response to drug treatment. B-C, Flow cytometric analysis (B) and western blot (C) of γ-H2AX in R1-ADR cells in response to drug treatment quantify graphically. D, R1-ADR cells were infected with the lentivirus encoding the control shRNA or *E2F1,* or *AR-FL,* or *AR3* shRNAs for 48 hours. γ-H2AX was determined by western blot. E, mRNA expression of *ATAD2*, *PCNA*, and *CDC6* were determined by qRT-PCR in response to drug treatment. Error bars, S.D. *, $p<0.05$ F, Binding of AR-FL and AR3 (left panel) and E2F1 (right panel) to the regulatory regions of *ATAD2* were analyzed by ChIP assay. G, R1-ADR cells were infected with the lentivirus encoding the control shRNA or *AR-FL* or *AR3* or *E2F1* shRNAs for 48 hours. Expression level of *ATAD2* were determined by qRT-PCR (B), Error bars, S.D. *, $p<0.05$

Discussion

In the present study, we showed that AR3 and E2F1 formed a positive feedback regulatory loop. The findings of previously published studies on the effects of E2F1 on AR regulation are not consistent. In agreement with our finding that E2F1 positively regulates AR3, Sharma et al. (206) showed that the overexpression of E2F1 increased levels of *AR-FL* mRNA, and that the knockdown of *E2F1* suppressed *AR-FL* expression. On the contrary, another study that examined the expression of E2F1 and AR in 667 prostate tissue cores, showed an inverse relationship between E2F1 and AR expression. Furthermore, the ectopic expression of E2F1 significantly downregulated AR mRNA and protein levels in prostate epithelial mPrE and androgen-responsive LNCaP cells (198, 199). Further studies demonstrated that transcription repressor DNMT1 bound to the E2F1 binding sites on the AR promoter and negatively regulated AR expression. Hence, we also tested the binding of DNMT1 to these sites via a ChIP assay but were unable to detect DNMT1 binding activity at these sites in drug-resistant cells. These contradictory findings may indicate an alteration in the transcriptome in drug-resistant cells. In addition, after carefully examining the results of previous studies, we found that the level of E2F1 overexpression observed in the present study was different from those of Sharma et al., Davis et al., and Valdez et al. (198, 199, 206). Moreover, the protein levels of overexpressed E2F1 were significantly lower in the study by Sharma et al. than in the latter two studies. As E2F1 is dual role regulator, the expression of E2F1 is tightly regulated, and may have distinct functions in response to different levels of E2F1 expression. In the present study, we ectopically expressed various levels of E2F1 and found that E2F1 induced AR3 expression at lower doses and inhibited E2F1 at higher does. Future research should focus on the effects of E2F1 expression levels on alterations to the transcriptome. In this study, we also showed that E2F1 is a unique target of AR3 but not AR-FL. The unique function of AR3 independent of AR-FL is also supported by several AR3-specific ChIP-seq experiments (189-191, 207). On the other hand, it also has been demonstrated AR3 binding on DNA can only be found together with AR-FL (189). The inconsistencies in findings may be a result of the different cell lines used in the present study. It has been previously shown that AR3 exhibits weaker binding activity on a subset of genes than AR-FL in response to androgen (208). In the present study, AR3 expression is increased in drug-resistant cells, and thus may play more important roles. It is very possible that the DNA binding activity of AR3 is enhanced in more aggressive and resistant cells. In addition, we carefully examined currently available ChIP-seq results in public databases, and found that the average read counts of AR3 ChIP-seq are very low. Thus, it is very possible that a substantial number of AR3 binding sites are missing as a result of insufficient reads. In the present study, we used the more traditional and more sensitive ChIP-PCR to detect AR3 binding, and succeeding in confirming AR3 transcriptional activity via luciferase assay. Another possible explanation for disparities in results could be the differences in antibodies used by the present study and previous studies. In the present study, we performed a ChIP assay using a customized rabbit anti-AR3 polyclonal antibody as previous described (23). We then performed a ChIP assay using commercially available anti-AR3 (clone RM7) monoclonal antibodies to test the AR3 binding activity of binding sites on the E2F1 promoter. Surprisingly, we could not detect AR3 binding on these sites using the anti-AR3 (clone RM7) monoclonal antibody. These results should strongly encourage researchers to re-examine AR3 binding by using different antibodies, especially polyclonal antibodies.

Recent studies suggest that AR and E2F1 cooperatively regulate downstream genes, including genes in the DNA repair pathway (194, 196). In addition, E2F1 transcriptionally regulates several DNA repair genes, such as *RAD51*, *XRCC1*, and *DNA-PKcs* (180-182). AR has also been shown to directly or indirectly regulate a large group of DNA repair genes (202). In the present study, we found that several genes associated with the DNA repair pathway were downregulated, and that the double drug treatment resulted in the accumulation of the DNA double strand break marker γ-H2AX. The knockdown of *AR3* or *E2F1* also increased levels of γ-H2AX. Further research will be necessary to determine the specific repair pathway that is regulated by AR3 and E2F1. Actually, we observed a possible increase in S and M phase of R1-ADR cells in response to double drug treatment by cell cycle analysis (data not shown). Homologous recombination preferentially repairs DNA double strand breaks and occurs predominantly in S phase. Thus, experiments to examine homologous recombination repair genes, such as *RAD51* and *BRCA2* and their impact on HR repair activity are warranted. More interestingly, it is possible that AR3 and E2F1 regulates DNA damage repair via a non-transcriptional mechanism. A recent study revealed that ARVs localized to loci with DNA damage and interacted with DNA damage repair factors under the combined treatment of ADT and radiation therapy (200). A similar non-transcriptional role for E2F1 in DNA damage repair has also been reported (183). Thus, it is very interesting to examine the subcellular localization of AR3 and E2F1 and test the possible interaction with DNA repair factors at the foci in response to the double drug-treatment.

Chapter 5: Auranofin inhibits AR3 and E2F1 expression and suppresses double drug-resistant cell growth *in vitro* and *in vivo*

Introduction

Cross-resistance between taxane drugs and ENZ are common in CRPC (209, 210). In the present study, we found that cells treated with DTX+ENZ eventually acquired resistance to both drugs after long-term culture. Thus, we sought to find a potential therapeutic agent to overcome the DTX+ENZ drug resistance. Auranofin is an FDA-approved drug that is typically used to treat rheumatoid arthritis. Auranofin was initially identified as a reduction/oxidation enzyme inhibitor that suppresses inflammation and stimulates cell-mediated immunity. Thioredoxin reductases (TrxRs) are the main target of auranofin, and TrxRs are closely associated with the ROS response and are involved in DNA repair. TrxRs are overexpressed in many types of cancers and are known to contribute to malignancies (211, 212). Auranofin induces oxidative stress and ultimately causes apoptosis. Thus, auranofin is promising candidate for cancer treatment. Auranofin works against cancer via multiple mechanisms. In addition to its anti-TrxRs actions, auranofin is a potent ubiquitin-proteasome system (UPS) inhibitor. UPS is involved in the regulation of many cellular processes, and plays an important role in cancer cell survival. One study showed that auranofin inhibited the proteasome-associated deubiquitinases UCHL5 and USP14 and induced apoptosis in cancer cell models (213). It also has been proposed that auranofin exerted anticancer activity via the inhibition of the PI3K/AKT/mTOR axis (214). These findings indicate that there are multiple mechanisms of action for the anticancer activity of auranofin. More importantly, Liu et al. reported that auranofin treatment lead to cell cycle arrest and apoptosis in Pca cells via the degradation of the androgen receptor, suggesting the potential usefulness of auranofin in the treatment of Pca (215).

In this study, we identified the potential anti-tumor drug auranofin in drug-resistant Pca cells and determined the anti-tumor effects of auranofin both *in vitro* and *in vivo*. We also explored the possibility that the transcriptional downregulation of AR3 and E2F1 may be partially responsible for the anti-tumor activity of auranofin.

Results

AR and E2F1 expression is recovered in DTX-ENZ resistant prostate cancer cells

Cross-resistance is common in CRPC patients (209, 210). We found cells treated with DTX+ENZ eventually acquired resistance to both drugs after approximately 3 months in culture. Thus, we established DTX-ENZ double drug-resistant Pca cell lines, R1-DDR and LN-DDR, by culturing R1-ADR and LN-ADR in medium containing both drugs. To determine the pathways altered in the drug resistant cells, we performed RNA-seq analysis and compared R1-DDR cells to R1-ADR cells treated with DTX+ENZ. Figure 19A shows that the expression of most of the commonly dysregulated genes in R1-ADR cells treated with DTX+ENZ, including *E2F1*, were restored in the double drug-resistant R1-DDR cells. We further validated the protein expression of AR/ARv and E2F1 by western blot and found that the protein levels of both AR/ARv and E2F1 were recovered in R1-DDR cells (Fig. 19B). To assess whether E2F1 and AR are critical in double drug-resistant cell growth, we knocked down AR-FL, AR3, or E2F1 by target-specific shRNA in R1-DDR. Cell growth was consequently inhibited by knocking down the target genes, especially with AF3 and E2F1 knockdown (Fig. 19C). Together, these data suggest that the recovery of E2F1 and AR signaling plays an important role in the survival of cells in the DTX-ENZ double drug-resistant cell lines.

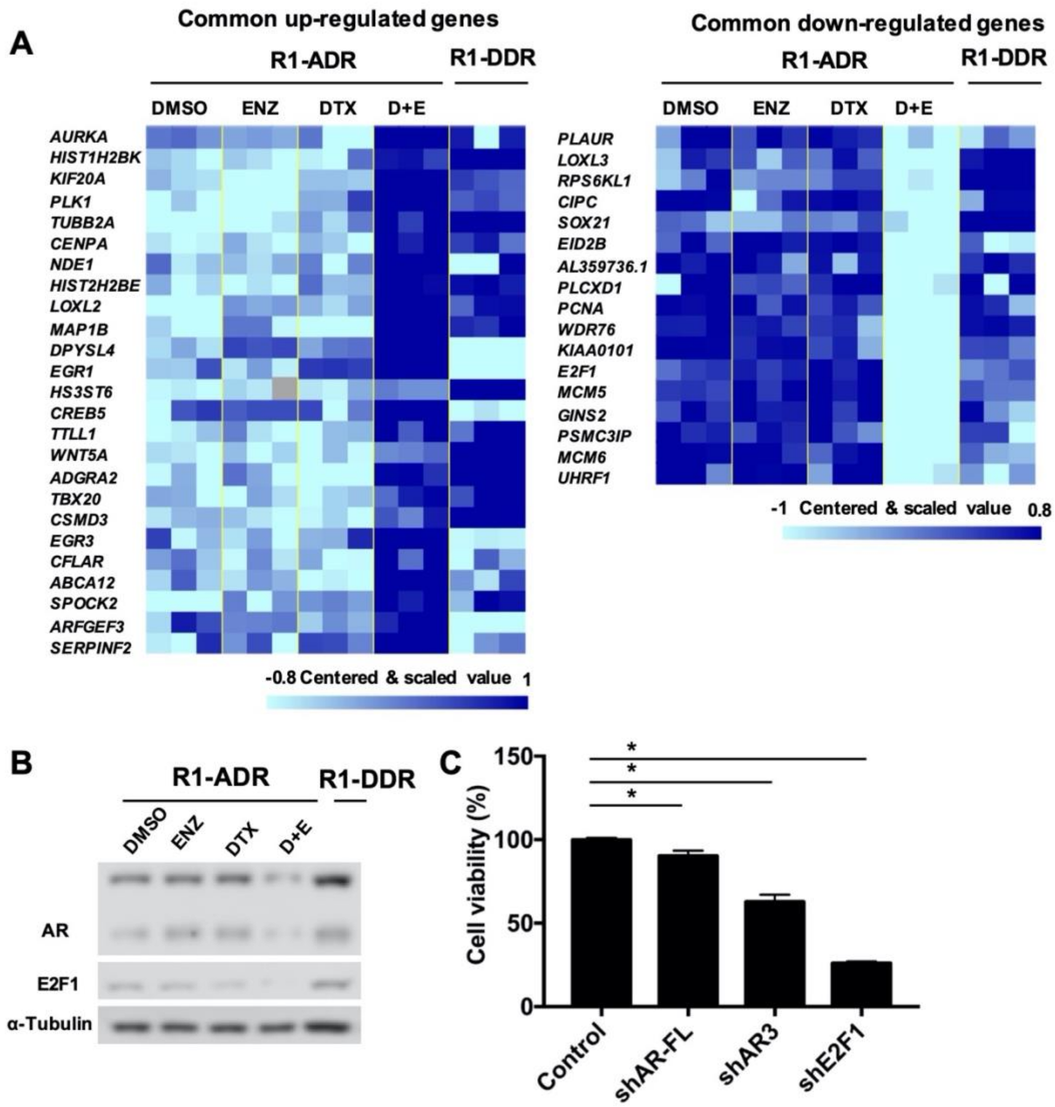


Figure 19. E2F1 and AR/ARv expression were recovered in R1-DDR cells.

A, RNA-seq analysis, heatmap of those commonly dysregulated genes in R1-ADR cells treated with DTX+ENZ, we compared R1-DDR cells (maintained in DTX+ENZ) to R1-ADR cells treated with DTX+ENZ (p<0.05, FDR<0.25, fold change > 1.4). Missing values are in color “gray”. B, protein level of AR/ARv and E2F1 in R1-DDR cells were determined by western blot. C, R1-DDR cells infected with lentivirus encoding control or *E2F1* or *AR-FL* or *AR3* shRNA. Cell growth was determined by CCK-8 assays 3 days after infection.

Auranofin inhibits AR and E2F1 expression and suppresses double drug-resistant cell growth *in vitro*

In a search for potential therapeutic agents to overcome double drug-resistance, we found that auranofin, a gold-based compound that is an FDA-approved drug for the treatment of rheumatoid arthritis, efficiently inhibited double drug-resistant cell growth (Fig. 20A). In recent years, the repurposing of auranofin has been investigated for number of diseases, including neurodegenerative disorders, HIV, infections, and cancer (216). A very recent study showed that auranofin promoted AR protein degradation and inhibited AR transcription, which in turn suppressed AR-positive Pca cell survival (215). Given the fact that AR-FL played a limited role in these cells, we speculate that there could be additional mechanisms of auranofin-induced growth inhibition. To further evaluate the effect of auranofin on monotherapy-resistant cells, we measured the growth inhibition of DTX-resistant R1-ADR cells and ENZ-resistant R1-ENZR cells. The results showed that auranofin inhibited growth in both cell lines in a similar manner (Fig. 20B). As we have already shown that DTX+ENZ suppressed drug-resistant cell growth via the downregulation of the AR3-E2F1 axis, we examined the expression of AR3 and E2F1 in response to auranofin treatment in double drug-resistant cells by qRT-PCR and western blot. The results show that levels of AR3 and E2F1 mRNA and protein were inhibited by auranofin in both R1-DDR and LN-DDR cells (Fig. 20C, D). We also observed increased apoptosis marker cleaved PARP and accumulation of DNA damage marker γ-H2AX in response to auranofin treatment (Fig. 20D). Moreover, qRT-PCR results revealed that mRNA level of previously mentioned E2F1 target genes *ATAD2*, *CDC6*, and *PCNA* were downregulated in response to auranofin (Fig. 20E). Together, these data suggest that auranofin is able to suppress the growth of resistant cells and decreased the AR3-E2F1 axis.

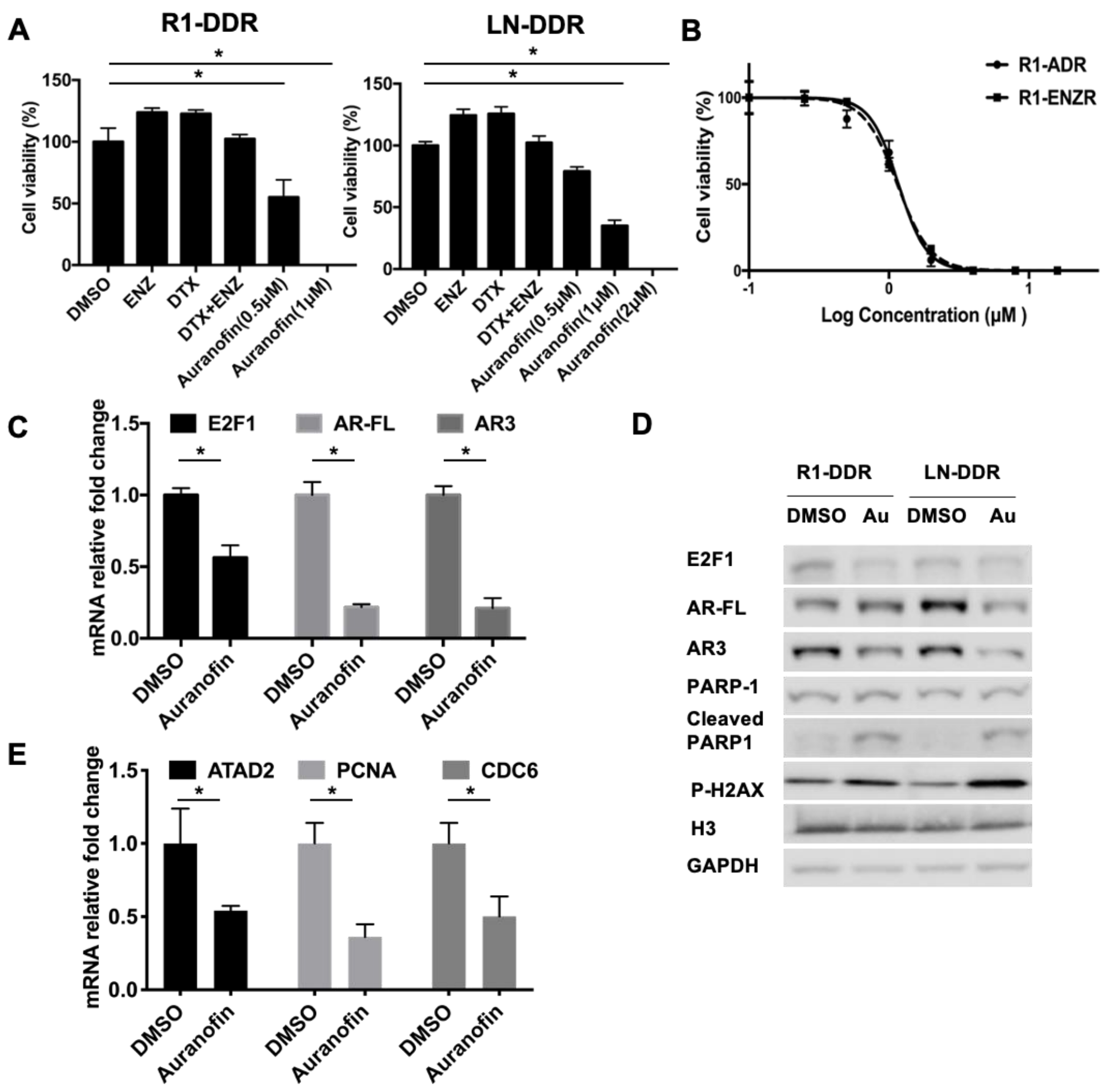


Figure 20. Auranofin inhibited the growth of double drug-resistant prostate cancer cells.

A, R1-DDR (left panel) and LN-DDR (right panel) cells were treated with different doses of auranofin for 72 hours. Cell growth was determined by CCK8 assay. B, R1-ADR and R1-ENZR cells were treated with different doses of auranofin for 72 hours. Cell growth was determined by CCK8 assay. C, R1-DDR cells were treated with 2μM auranofin for 12 hours, RNA expression of E2F1, AR-FL, and AR3 in R1-DDR cells were determined by qRT-PCR, Error bars, S.D. *, $p<0.05$ D, R1-DDR and LN-DDR cells were treated with 2μM auranofin for 12 hours, protein level of E2F1, AR-FL, AR3, cleaved PARP1, and γ-H2AX in R1-DDR and LN-DDR cells were determined by western blot. E, R1-DDR cells were treated with 2μM auranofin for 12 hours, RNA expression of *ARAD2*, *PCNA*, and *CDC6* in R1-DDR cells were determined by qRT-PCR, Error bars, S.D. *, $p<0.05$

Auranofin inhibits AR and E2F1 expression and suppresses double drug-resistant cell growth *in vivo*

To determine whether auranofin can inhibit tumor growth *in vivo*, we examined the effects of auranofin on double drug-resistant cells using tumor xenograft model in mouse. As shown in Figure. 21A and B, the volume and size of R1-DDR tumors were significantly smaller in auranofin treated mice compared to either vehicle control or DTX+ENZ treated group. Relative body weight did not change significantly within the groups, suggesting that the drugs were well tolerated (Fig. 21C). TUNEL assays showed that auranofin-treated xenograft tumors exhibited higher numbers of apoptotic-positive cells (Fig. 21D). Immunohistochemistry showed that the proliferation marker Ki67 was lower in response to treatment with auranofin (Fig. 21E). Western blotting showed that protein levels of E2F1 and AR tend to be lower in auranofin-treated tumors in comparison to controls or the DTX+ENZ group (Fig. 21F). Collectively, these findings suggest that auranofin decreased

E2F1 and AR3 expression and is a potent inhibitor of cell growth in double drug-resistant Pca cells *in vivo*.

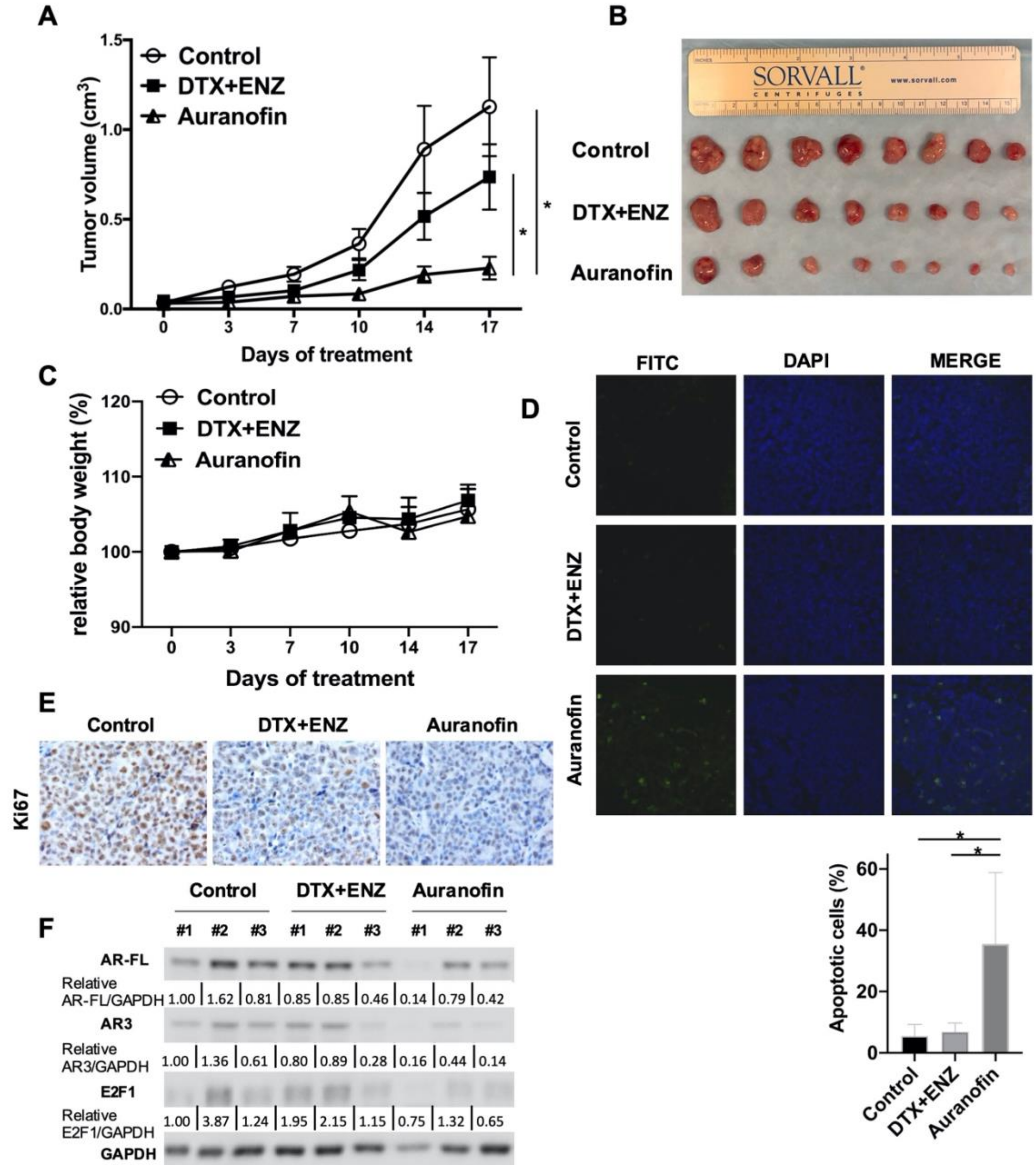


Figure 21. Auranofin inhibited the growth of double drug-resistant prostate cancer *in vivo*.

A, tumor volume. Animals were treated with solvent or DTX (5mg/kg once a week) + ENZ (10mg/kg per day, 5 days per week) or auranofin (5 mg/kg per day, 5 days per week), n = 5 per group. Error bars, S.E.M. *, $p<0.05$ B, Photograph of R1-DDR xenograft tumors. C, Relative body weight analysis by repeated measures ANOVA with group as between subject factor and time as within subject factor and no significant group difference was detected ($p = 0.889$). D, TUNEL assay was performed to detect apoptosis of R1-DDR xenograft. DAPI and TUNEL-FITC staining of R1-DDR xenograft tumors (upper panel) and Bar graph (lower panel) Error bars, S.D. *, $p<0.05$. E, Ki67 proliferation marker was examined by immunohistochemistry. F, AR/ARv and E2F1 protein level in R1-DDR xenograft were determined by western blot.

Discussion

The FDA-approved anti-rheumatoid arthritis agent auranofin has been shown to suppress the survival of many types of cancer cells in pre-clinical experiments. The anticancer activity of auranofin has been proposed to occur by way of several different mechanisms, including the inhibition of TrxRs, the inhibition of DUBs, and the inhibition of PI3K/AKT/mTOR (214, 215). In this study, our results provide evidence of the anticancer activity of auranofin in drug-resistant Pca *in vitro* and *in vivo*. Likewise, Liu et al. (215) has previously reported that auranofin can degrade AR and disrupt AR signaling in Pca cells. A detailed examination of this showed that auranofin suppressed the growth of LNCaP, 22Rv1, and PC3 cells, but not DU145 cells. This result indicates that the efficacy of auranofin is cell-type and context dependent. Interestingly, the inhibition of cell growth is more effective in androgen-independent 22Rv1 cells than in androgen-responsive LNCaP cells. PC3 and DU145 are AR-negative cells. However, auranofin induced the loss of cell viability in PC3 cells, but not in DU145 cells. These findings indicate that additional mechanisms may contribute to the suppression of cell growth by auranofin in Pca cells. We tested the effect of auranofin in various Pca cell lines and were able to replicate the findings of Liu et al. (215). We also examined the effect of auranofin in immortalized non-neoplastic human prostate epithelial RWPE-1 cells. Interestingly, auranofin enhanced the growth of RWPE-1 cells at relatively low doses and suppressed cell growth at high doses (data not shown). In addition, we found that auranofin inhibited the survival of neuroendocrine NE1.3 cells; androgen-independent C4-2, C4-2B, and CWR-R1 cells; ADT+DTX-resistant R1-ADR and LN-ADR cells; and DTX+ENZ-resistant R1-DDR and LN-DDR cells. We observed that auranofin tends to be more effective in more aggressive and resistant cell lines than in less aggressive cell lines. E2F1 plays important roles in multiple cellular processes, and is thus critical for the survival of almost all types of cells. Furthermore, E2F1 has become more important for tumor growth in more aggressive and drug-resistant types of cancer. Thus, aggressive cells are more vulnerable to the loss of E2F1. In this study, we demonstrated that auranofin resulted in the loss of AR3 and E2F1 expression in double drug-resistant cells, which partially explains the inhibition of the viability of cancer cells by auranofin. In the previous chapter, we discussed why AR3 and E2F1 are important to the DNA damage response. Auranofin may affect DNA damage repair by downregulating AR3 and E2F1.

Many studies suggest that auranofin increases the sensitivity of DNA damage agents. It has been shown that auranofin increased double strand breaks in DNA repair gene *BRCA1*-depleted cells, and increases the sensitivity of DNA-damaging radiation therapy (217, 218). In our preliminary study, we found that a portion of the DNA damage response was initiated before the downregulation of AR3 and E2F1 (data not shown). An increase in the phosphorylation of DNA damage response signaling factors ATM and CHK1/2 were observed as early as 15 mins after the addition of auranofin. This result suggests that auranofin may affect DNA repair process by directly targeting DNA repair genes in addition to indirectly regulating AR3 and E2F1. Future studies will be necessary to examine the direct targets of auranofin and the initial effects of auranofin treatment.

Chapter 6: Final result and discussion

The overall goal of this study was to find a potential therapeutic method to treat drug-resistant Pca and to understanding the underlying mechanisms of these new therapies. To achieve this goal, we first established DTX and ADT therapy-resistant cell lines and tested potential drugs and drugs combinations. We found that DTX+ENZ effectively inhibited drug-resistant cell growth. Furthermore, we found that DTX+ENZ effectively reduced both AR3 and E2F1 activities. AR3 is a constitutively-activated form of AR that can contribute to the progression of Pca and drug resistance, while E2F1 is a dual-role transcription factor that is important for cell cycle progression and the repair of DNA damage, and can either serve as an oncogene or tumor suppressor. In this study, we reported that AR3 directly bound to the regulatory regions of E2F1 and regulated E2F1 expression. On the other hand, we also found that E2F1 regulated AR3 expression. Thus, AR3 and E2F1 form a positive feedback loop in drug-resistant Pca cells. The drug combination DTX+ENZ both downregulated gene expression and resulted in growth suppression, DNA double strand break accumulation, and apoptosis. We also found that cells cultured in the presence of the DTX+ENZ combination treatment eventually acquired resistance to both drugs. The anti-inflammation compound auranofin was able to suppress the growth of double drug-resistant cells *in vitro* and *in vivo*. Our model summarized the major finding of this study (Fig. 22).

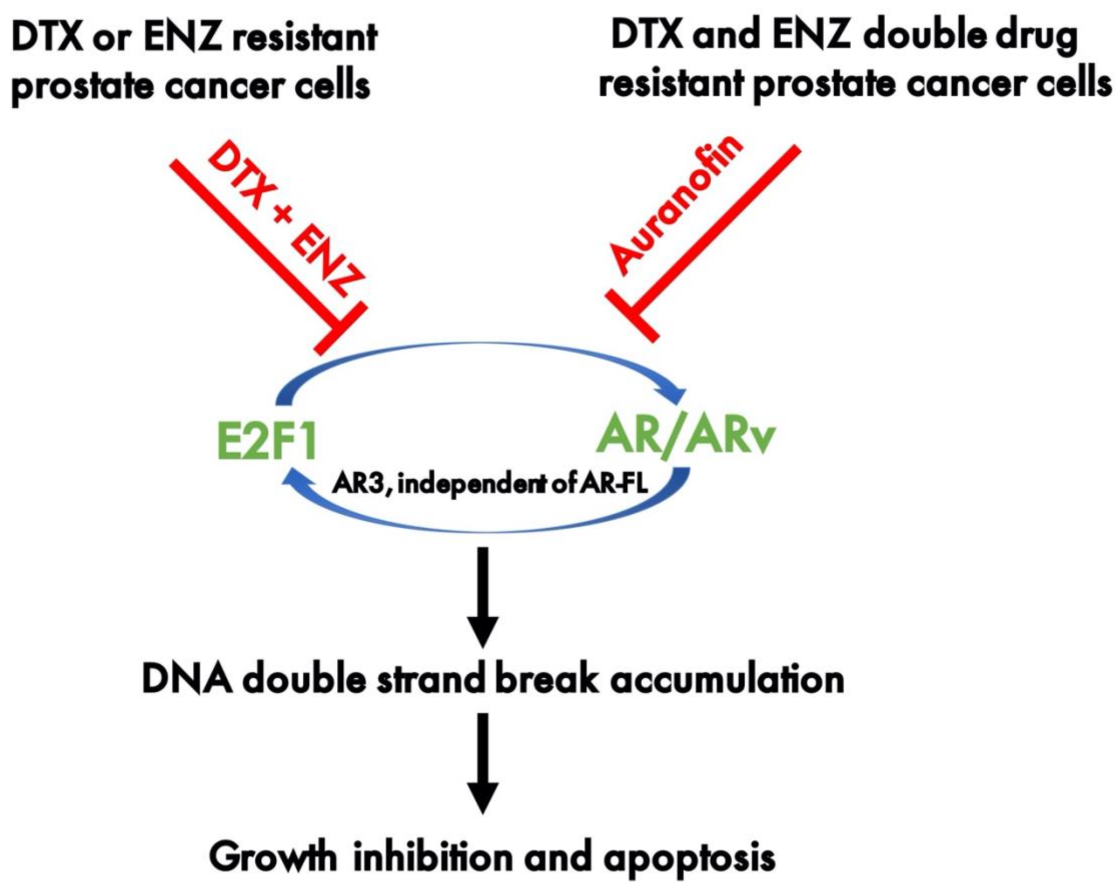


Figure 22. Graphical summary.

DTX+ENZ and Auranofin can induce growth inhibition and apoptosis of single drug resistant and double drug-resistant prostate cancer cells, respectively. Both treatments decreased AR3 activity and E2F1 activity. AR3 and E2F1 positively regulate each other at transcriptional level. Loss of AR3 and E2F1 activity further causes DNA double strand break accumulation and subsequently growth inhibition and cell death.

Aggressive CRPC is the leading cause of death for Pca patients, and the rapid development of drug resistance is inevitable. DTX is one of the first-line treatments for CRPC patients, while ENZ provides significant survival benefits for patients after chemotherapy (171). However, almost all patients developed resistance to ENZ in less than 2 years. To elucidate the molecular mechanisms and investigate new strategies for delaying and circumventing drug resistance, we developed cell models that were resistant to either DTX or ENZ. We tried several combinations of a variety of drugs and found that only DTX+ENZ effectively reduced the survival of the drug-resistant cells. This finding is in consistent with previous report showed antiandrogens inhibited drug efflux activity and reversed DTX resistance in advanced Pca (219). In order to further investigate the underlying mechanisms responsible for the efficacy of the combined treatment, we used RNA-Seq to detect the early-stage molecular events that were elicited in response to this combination. As a result, we found that E2F1 is a key regulator and was downregulated by the combined therapy. E2F1 is a known dual-function cancer progression mediator. It has been shown that while an excess amount of E2F1 triggers apoptosis, under certain signaling conditions, E2F1 can shift gears to pro-survival (220). The increased expression of E2F1 has been known to occur in many types of cancers, including Pca. We analyzed E2F1 profiling using two public databases (Trento/Cornell/Broad 2016 and the TCGA prostate adenocarcinoma, provisional), and found that E2F1 is either amplified or elevated in a significant portion of patients (221-223).

AR signaling, especially the ARV AR3-driven molecular events, are critical for therapeutic resistance in aggressive Pca. While the expression and activities of E2F1 and AR are often corelated, the details of this co-regulation are still not fully understood. It has been reported that a low dose of androgen induced E2F activity, while a high dose of androgen inhibited E2F activity (224). One explanation of this bidirectional regulation is that the high dose of androgen recruited Rb to common AR and E2F binding sites on the chromatin, and thus repressed the expression of genes regulated by the AR-E2F axis (including E2F1 itself)

(196). In the present study, we demonstrated that AR3 did not appear to be co-immunoprecipitated with Rb as was AR-FL. AR3 interacted with E2F1 and bound to the promoter region of E2F1. It is also worth noting that the AREs that we identified in the E2F1 regulatory region are half AREs, and therefore may only allow the ARV to bind in the presence of E2F1. This possibility is supported by our luciferase reporter assays, which show that AR3, but not AR-FL, can increase E2F1-luciferase activity. The addition of AR-FL to AR3 exerted no effect on E2F1-luciferase activity. These data could indicate that the activity of AR3 on E2F1 is not dependent on AR-FL. As expected, we also found that AR3 alone was able to induce ARR2-luciferase activity without the presence of AR-FL. This induction is not affected by treatment with either DHT or ENZ. However, ENZ does inhibit AR3 activity when AR-FL is co-expressed, and this inhibition is increased concomitantly with AR-FL expression. This finding is consistent with a previous study, which reported that the activity of constitutively active ARVs required AR-FL (38). A very recent study showed that AR3 and AR-FL bind to the same sites on chromatin and regulate H3K27ac (189). Another recent study showed that AR3, but not AR-FL, colocalized with HoxB13 on open chromatin and regulated target gene expression (191). The ChIP-seq results from another study showed that a significant portion of AR3 binding sites lacked AR-FL binding. BRD4 and ZFX co-occupies with AR3 on the AR3-unique binding sites of target gene promoters (192). Thus, it will be interesting to study whether the epigenetic modification or co-binding factors determine the distinct role of AR3 in different genetic contexts in the future. It should be noted that our study was mainly carried out in preclinical models. It has yet to be validated whether such mechanisms are applicable for predicting patients with acquired resistance.

Several recent reports have shown that ENZ is able to radiosensitize Pca *in vitro* and *in vivo* by impairing the DNA damage response (225, 226). Similarly, it is possible that there is a high level of DNA damage response in DTX and ENZ monotherapy-resistant cells. The combined treatment simultaneously diminished AR signaling and E2F1 expression. E2F1—a dual role regulator—is important in cell cycle progression, the DNA damage response, and apoptosis, and either the upregulation or loss of E2F1 can promote DNA damage-induced apoptosis (227, 228). Interestingly, the AR signaling pathway regulates a set of DNA repair genes, while anti-androgenic treatments cause increases in DNA damage (202). It has also been shown that ARVs interacted with DNA-PKs in response to DNA damage (200). Blocking the interaction of ARVs and DNA-PKs causes persistent DNA damage and cell death during radiation therapy. A recent report demonstrated that the loss of AR-mediated CDC6 expression alteration resulted in an accumulation of DNA damage and promoted apoptosis (201). More importantly, E2F1 and AR signaling have many common target genes, including genes associated with DNA damage repair and DNA replication (195, 196, 202). Thus, it is very likely that the loss of both E2F1 and AR3 causes DNA damage and an increase in apoptosis in the combined treatment. We will examine whether the AR3-E2F1 axis plays a role in the DNA damage response in a future study.

In this study, we generated DTX and ENZ double drug-resistant Pca cells, which were highly resistant to several compounds that we tested. However, we found that auranofin—an FDA-approved drug for the treatment of rheumatoid arthritis—effectively inhibited double drug-resistant Pca cell growth *in vitro* and *in vivo*. Furthermore, this effect is at least partially a result of the downregulation of the E2F1-AR3 axis. The main mechanism of action of auranofin is via the inhibition of redox enzymes. Increasing evidence shows that auranofin has anti-cancer effects (216). A very recent study showed that auranofin inhibited proteasomal deubiquitinases and facilitated the degradation of AR (215). In this study, we found that both the mRNA and protein levels of AR and E2F1 were reduced in response to treatment with auranofin, indicating an additional mechanism of action for auranofin. It has been shown that the auranofin target TrxR/Trx binds directly to PTEN and inhibits its lipid phosphatase activity (229). Another report showed that auranofin regulates the phosphorylation status of AKT and H2AX (214). Interestingly, we found the phosphorylation of both AKT and H2AX was altered in both DTX+ENZ and auranofin treatments (data not shown). Thus, it will be interesting to further investigate how these treatments affect the E2F1-AR3 axis in the future.

In summary, our proof-of-principle study demonstrates that simultaneously targeting E2F1 and AR3 via a combined therapy or a multi-targeting drug could circumvent castration resistance in Pca. Moreover, our studies showing the differential regulation of E2F1 expression by AR3, but not by AR-FL, in R1-ADR cells, suggests that AR3 may function independently of AR-FL in different genetic contexts via the differential recruitment of co-factors. These studies give us insight into future targeting strategies in castration-resistant Pca.

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