IDENTIFYING AND TARGETING PATHWAYS INVOLVED IN ENZALUTAMIDE-RESISTANT PROSTATE CANCER

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Dedicated to my late father, Gerges Farah.

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ABSTRACT

Prostate cancer is the second leading cause of cancer death among men in the United States. The androgen receptor (AR) antagonist enzalutamide is an FDA-approved drug for treatment of patients with late-stage prostate cancer and is currently under clinical study for early-stage prostate cancer treatment. After a short positive response period to enzalutamide, tumors will develop drug resistance. In these studies, we uncovered that NOTCH signaling and DNA methylation are a deregulated in enzalutamide-resistant cells. *NOTCH2* and *c-MYC* gene expression positively correlated with *AR* expression in samples from patients with hormone refractory disease in which AR expression levels correspond to those typically observed in enzalutamide-resistance. The expression of Notch signaling components was upregulated in enzalutamide-resistant cells suggesting the activation of the pathway. Inhibition of this pathway *in vitro* and *in vivo* promoted an increase in the sensitivity to enzalutamide with an impact on AR expression. On the other hand, DNMT activity and DNMT3B expression were upregulated in resistant lines. Enzalutamide induced the expression of DNMT3A and DNMT3B in prostate cancer cells with a potential role for p53 and pRB in this process. The overexpression of DNMT3B3, a DNMT3B variant, promoted an enzalutamide-resistant phenotype in C4-2 cells. DNA methylation inhibition, using low-concentration decitabine, and *DNMT3B* knockdown induced a re-sensitization of resistant prostate cancer cells and tumors to enzalutamide. Decitabine treatment in enzalutamide-resistant induced a decrease in the expression of AR-V7 and changes in genes from the apoptosis, DNA repair and mRNA splicing pathways. Decitabine plus enzalutamide treatment of 22RV1 xenografts induced a decrease in tumor weight, KI-67 and AR-V7 expression and an increase in Cleaved-Caspase3 levels. All the above suggest that Notch signaling and DNA methylation pathways are deregulated after enzalutamide resistance onset, and targeting these pathways restores the sensitivity to enzalutamide.

CHAPTER 1. INTRODUCTION

1.1 Prostate cancer

1.1.1 The prostate gland and prostate cell types

The prostate is a male accessory sex gland, the size of a walnut, located around the urethra at the base of the bladder. According to John McNeal, the glandular portion of the human prostate is organized into 3 different zones; central, transition and peripheral zone, which is the main site of malignant tumor development. The glandular network is surrounded by a non-glandular fibromuscular stroma (1). At the cellular level, the gland ducts are lined with a luminal layer of polarized, columnar shaped cells. These epithelial secretory cells express the prostate specific antigen (PSA) and other markers such as cytokeratin 8 and 18 (CK8 and CK18) (2–4). Lining the basement membrane are basal cells characterized by the positive expression of CK5 and CK14 and a low expression of AR compared to luminal cells (2, 3, 5, 6). Neuroendocrine cells, a rare type of neurotransmitter cells located at the basement membrane, ensure signal transmission through secretion of neuropeptides and hormones (7). The fibromuscular stroma is composed of a layer of smooth muscle that helps excretion of fluids into the ejaculate, and mature fibroblasts that play a role in signaling and the extracellular matrix maintenance (8, 9).

1.1.2 Androgen signaling and the androgen receptor

Androgens are responsible for the proper development, differentiation and architectural maintenance of the prostate gland. During development, androgen ablation hinders the proper development of the prostate (10). In the developed prostate, androgen ablation induces apoptotic death in the luminal cell population leading to prostate shrinkage (11). Re-introduction of testosterone to the environment rescues the lumen involution and induces a reconstruction of secretory structures by stimulation of luminal cell growth (12, 13). Androgens, testosterone and the more potent dihydrotestosterone (DHT), mediate their functions through the AR signaling pathway. The *AR* gene, located on the X chromosome at the locus Xq11-Xq12, encodes a 919 amino acid protein that belongs to the superfamily of nuclear receptor transcription factors (14). The 110 KDa protein is composed of three functional domains: the N-terminal domain (NTD, amino acids 1-555) important for transactivation activity of the AR, the DNA binding domain

(DBD, amino acids 555-623) necessary for the binding function to promoters or enhancers of target genes and the ligand binding domain (LBD, amino acids 665–919) crucial for ligand binding and thus AR activation (15). Two transactivation domains, activation function 1 and 2 (AF1 and AF2), reside in the NTD and the LBD respectively. AF1 is constitutively active whereas AF2 is activated only upon ligand binding. These domains bind co-regulatory proteins and are indispensable for the full activity of AR (16–18). The flexible hinge region, separating the DBD and LBD, plays a role in the transactivation and the intracellular localization of the receptor (19, 20). The nuclear localization signal (NLS) (amino acids 617–633) is responsible for the active transport of the AR through the nuclear membrane upon its activation (21).

In its inactive state, the AR is bound through its LBD to heat-shock proteins (HSP90, HSP70 and p23) maintaining the receptor in a competent conformation to accept ligand binding (15). Upon androgen binding to the LBD, the AR becomes activated. Conformational changes occur in the LBD, detaching it from the heat-shock proteins, enabling dimerization and N/C-terminal interaction in AR. Following dimerization, AR is phosphorylated and translocated to the nucleus after exposing the NLS (21). In the nucleus, the receptor binds, through its DBD, to consensus sequences composed of inverted hexamers (5'-TGTTCT-3') separated by 3 random nucleotides named androgen response elements (AREs) or to more complex sequences (15, 22, 23). AREs are located within the promoter or enhancer regions of AR target genes. Upon binding to AREs, transactivation of androgen-regulated target genes occurs after recruitment of co-regulators and transcription machinery (24, 25)

1.1.3 Prostate cancer statistics

Prostate cancer is the top diagnosed cancer among men in the United States of America with an estimated 191,930 new cases in 2020. It is the second leading cause of cancer-related deaths in men in the U.S. with an estimate of 33,330 deaths in 2020 (26). 1 in 6 men will develop prostate cancer during their lifetime. Age is one of the main risk factors for prostate cancer and the probability of developing the malignancy significantly increases from 0.005% for men below 39 years old, to 2.2% for men between 40 and 59 years old, and to 13.7% for men aged 60 and above (27). Other risk factors for developing prostate cancer are African ancestry, family history of the disease and inherited genetic predispositions (27).

1.1.4 Prostate cancer originating cell and precursors

Most diagnosed prostate cancers are adenocarcinomas, originating from epithelial cells within the prostate gland. It is strongly believed that luminal cells are the tumor initiating cells due to the predominant expression of luminal cell markers in the tumor. However, several functional studies demonstrated that both basal and luminal cells may play the role of the prostate cancer cell of origin. Wang *et al.* showed that castration-resistant Nkx3.1-expressing cells can self-renew, regenerate prostate glandular structures and can form a tumor after the loss of Phosphatase and tensin homolog (PTEN) (28). Goldstein *et al.* show that basal cells, isolated from a normal prostate, generate tumors in immunodeficient mice upon overexpression of activated-AKT and -ERG in those cells (29). Prostatic intraepithelial neoplasia (PIN) are precursor lesions within the epithelium of the prostate that may progress to invasive prostate carcinoma. High-grade PIN are characterized by an overgrowth of luminal cells with enlarged, prominent nuclei within an intact prostate architecture (30). These lesions are non-invasive, preserving a normal layer of basal cells and an intact basement membrane (31). At the genetic and molecular levels, several events shared between prostate adenocarcinoma and high-grade PIN are believed to drive uncontrolled proliferation, leading to invasion and cancer development. Some of the changes include telomere shortening, allelic loss, loss of heterozygosity, gain of chromosomes, overexpression of oncogenes (such as c-Myc and c-Met), silencing of tumor suppressor genes by DNA methylation (such as RARB2 and APC) and presence of gene fusions (32, 33).

1.1.5 Diagnosis, staging and management of prostate cancer

In 1970, Wang and Valenzuela discovered the prostate specific antigen (PSA), a glycoprotein encoded by the kallikrein-3 (KLK3) gene (34). PSA is exclusively expressed in the luminal cells within the prostate gland playing a role in sperm motility and dissolving cervical mucus (35). In 1986, PSA testing was used to monitor treatment response, prostate cancer progression and recurrence. In 1994, the FDA approved PSA testing as a diagnostic tool in prostate cancer screening. Elevated PSA is detected in prostate cancer patients, however, it’s not exclusive and specific. High PSA levels can be detected when PSA production increases or when destruction of a tissue layer separating the duct from the blood stream occurs (36). This can happen in patients with benign prostatic hyperplasia, prostatitis, urinary retention or injury (37). Also, some subtypes

of prostate cancer won't manifest any PSA increase and would be hard to detect and diagnose using this screening method. Despite an ongoing dispute in the medical and scientific community on the importance and relevance of PSA testing, currently PSA screening alongside, family history questionnaires and digital rectal examination (DRE) proceeding a targeted biopsy is still the recommended guideline for early detection of prostate cancer.

Molecular and genotype profiling is being leveraged to improve screening, diagnosis and prognosis of patients. Evaluating chromosomal abnormalities, sequence mutations, gene amplification, epigenetic changes and metabolites concentrations are valuable insights into the characterization of an individual's disease. Alongside other screening and diagnostic methods, this will allow physicians to make more informed decisions regarding clinical management and treatment of patients. Some of the most common genomic markers of prostate cancer progression include *AR* amplification and slicing events (38, 39), the *TMPRSS2:ERG* gene fusion (40), activation of the PI3K/AKT pathway by loss of *PTEN* (41), germline mutations in *HOXB13* (42), and hypermethylation of different gene promoters including *GSTP1* and *AR* (43, 44). In addition to the genomic markers, a variety of blood and urine biomarkers can be used to improve decision-making regarding prostate cancer patients.

Grading of the biopsied tissue is essential after diagnosis to determine the prognosis and the preferred route for treatment. Evaluating the tumor grade in prostate cancer is a very strong predictive factor of biochemical failure, tumor recurrence, metastasis in patients receiving or not receiving any treatments (45). The Gleason score, established by the pathologist Dr. Donald Gleason following a study, is incorporate into all staging schemes currently used in the clinic to stage prostate cancer (46). It is a grading score based on the histology and architectural patterns found in a prostate tumor (46). The Gleason score is a formula combining two numbers depicting the grade of the two most common patterns within a biopsy, added together result in the final score. The patterns are scored from 1 to 5 based on their resemblance to the normal prostate gland architecture, 1 to 3 closely resembling an overall normal prostate, 4 and 5 representing an abnormal glandular structure (46, 47). Several modifications were amended on the grading of prostate tumors to keep up with the advances of screening, diagnosis and correlation with patient outcomes. Most recently in 2014, a 5-grade system encompassing the Gleason scoring system was accepted (45).

According to this system, grade group 1 includes all prostate cancers with Gleason scores 6 or less, which are indolent cancers requiring only active surveillance. Grade group 2 and 3 encompass Gleason score 3+4=7 and 4+3=7 respectively. Grade group 4 comprises all Gleason 8 scores and Grade group 5 includes Gleason scores of 9 and 10 (48). Patients with grade 1 tumors exhibit a very low chance of progression while grade 5 patients have a 25% chance of progression free 5-year survival. For those who experience recurrence, most of them will eventually succumb to the disease.

Upon screening and diagnosis, the next question to be asked is whether patients need to get immediate treatment. To answer that question, the clinical significance of the cancer at hand needs to be evaluated by looking at the tumor's biology and the patient's clinical situation. Patients are then classified into different groups depending on the aggressiveness of the tumor and the risk of cancer progression and recurrence. According to the latest risk stratification guidelines by the National Comprehensive Cancer Network (NCCN), clinician stratify the diagnosis into risk groups of low, intermediate and high risk based on the PSA level, the Gleason score and the clinical stage of the tumor. Patients with Gleason score ≤ 6, clinical stageT1 to T2a or PSA level < 10 ng/mL are in the low-risk group. They are usually recommended active surveillance (AS) over radical prostatectomy (RP) or Radiotherapy (RT) to avoid overtreatment and the effects of therapy on their quality of life (49). Studies comparing AS to RP and/or RT in patients with localized disease show conflicting results, drawing no definitive conclusions about the use of watchful waiting vs treatment in this population of patients (50–53). The study by Bill-Axelson *et al.* shows significant decreases in mortality and distant metastasis in patients subjected to surgical removal of the prostate compared to watchful waiting (52). On the other hand, 3 other studies conducted in the U.S. and the U.K. and comparing surgery, active surveillance and radiotherapy, show no significant differences in mortality or metastatic lesions in the patient population (50, 51, 53). The intermediate-risk group encompasses patients with clinical stage of T2b to T2c, Gleason score of 7, or PSA level of 10 to 20 ng/mL. Recommended treatment options for this group of patients include RP and RT. The American Urological Association, the European Association of Urology and the NCCN guidelines define high-risk prostate cancer in patients presenting any of the following criteria: PSA > 20ng/mL, Gleason score ≥ 8, or a clinical examination $\geq$ T2c. The standard of care for patients with localized high-risk prostate cancer is RP or RT with neoadjuvant

androgen deprivation therapy (ADT). In patients with locally advanced or metastatic disease, first line options include ADT or RT with neoadjuvant ADT.

ADT can be achieved surgically by physical castration or chemically, by inhibiting the production of testicular androgens in the aim of reducing testosterone levels in the organism. Prostate cancer is heavily reliant on androgens and androgen receptor signaling for growth and progression in different stages of the disease. In 1941, Huggins and Hudgins were the first to show that prostate cancer is hormone-dependent and can be treated by surgical castration or by administration of oral estrogen to patients (54). Several other ADTs were developed over the years to eliminate the need for surgical intervention and the side effects of estrogen treatment in men. Luteinizing hormone-releasing hormone (LHRH) agonists and antagonists are currently used as the preferred treatment for advanced prostate cancer patients (55, 56). These therapies are reversible, well tolerated and can achieve castrate testosterone levels in men. ADT has significant positive effects on overall survival, tumor regression, and presents with relief of urinary symptoms and bone pain (56). However, ADT is not curative in patients with advanced prostate cancer. After a positive response period, tumors become resistant to these therapies and develop a more deadly and aggressive form of the disease called castration resistant prostate cancer (CRPC).

1.1.6 Castration-resistant prostate cancer and its treatment

CRPC is characterized by a biochemical recurrence manifested by an increase in PSA serum levels and/or a metastatic progression detected by radiographic evidence, despite having castrate level of testosterone (<50ng/dL). Patients with CRPC will experience pain, discomfort and weakness from metastatic tumors in the bone, lymph nodes and soft tissues. Patients with metastatic CRPC (CRPC) will survive for 2 to 4 years and succumb to the disease that lacks curable therapies up to this point. The current standard of care for CRPC patients includes approaches that prolong life and offer some palliative relief including Docetaxel and second-line ADT such as Abiraterone and Enzalutamide.

Docetaxel was first approved by the FDA in 2005 for the treatment for mCRPC patients. In the TAX-327 trial, docetaxel compared to the palliative agent mitoxantrone, demonstrated survival advantage (18.9 months vs 16.5 moths), decrease in PSA levels and improvements in the quality

of life and pain in patients with mCRPC (57). Despite several efforts to find new regimens and drug combinations that would improve the effects of docetaxel on survival and toxicity, all attempts have failed (58–61). However, a new population of patients with no treatment options emerged post-docetaxel treatment. This patient population was resistant to docetaxel and was managed by mitoxantrone and prednisone treatment. Cabazitaxel, one of the therapies that showed improved overall survival in mCRPC docetaxel-resistant patients, presents with high toxicity events that need to be discussed with patients before administration (62). Other approaches have emerged as first and second-line chemotherapeutic treatments for mCRPC patients with substantial toxicity profiles (63–65).

Despite ADT resistance, continuation of the treatment approach is recommended due to its association with a brief survival benefit in patients (5 to 6 months) (66). Studies have shown that despite successful and prolonged ablation of testicular androgens, testosterone can still be produced at sufficient levels by the adrenal glands and in situ, enough to activate the AR in the tumor cells (67, 68). An alternative mechanism explaining this phenomenon is the hypersensitivity and overexpression of the AR protein in the tumor rendering it more sensitive to androgens or other ligands (69, 70).

To overcome the resistance mechanisms, different groups have designed approaches to target enzymes involved in the synthesis of androgens in all cells and to target the AR protein directly inhibiting its activation by androgens. Cytochrome P450 (17) alpha (CYP17) catalyzes the conversion of pregnenolone and progesterone to 17α hydroxypregnenolone and 17α hydroxyprogesterone, two critical precursors of testosterone (71, 72). Inhibiting this enzyme with the selective and irreversible inhibitor, Abiraterone acetate, blocks the synthesis of androgens thus, ligand-dependent AR signaling. In a phase III clinical trial, abiraterone acetate resulted in a significantly higher overall survival, time to PSA progression, radiologic progression-free survival and time to occurrence of first skeletal-related events in CRPC patients post docetaxel compared to the placebo group (73, 74). Furthermore, abiraterone acetate was tested against placebo in men with chemotherapy-naïve mCRPC patients. Results of the trial show an overall survival advantage in the abiraterone arm compared to the placebo group (73).

1.1.7 Enzalutamide

Enzalutamide is a second-generation anti-androgen, designed to bind in the LBD of the AR inhibiting its ability to bind androgens, translocate to the nucleus, bind AREs and recruit co-activators. In the AFFIRM trial, oral enzalutamide had significantly higher median overall survival compared to placebo in men with CRPC with prior exposure to docetaxel (18.4 months vs 13.6 months). Also, enzalutamide had a superior effect on the soft-tissue response rate (29% vs. 4%), quality of life response rate (43% vs. 18%), time to PSA progression (8.3 months vs 3 months), radiologic progression-free survival (8.3 months vs. 2.9 months) and time to occurrence of first skeletal events (16.7 months vs. 13.3 months) (75) .

Enzalutamide was developed to fill the need for an AR antagonist that has higher affinity than existing antagonists (flutamide, bicalutamide, and nilutamide) without being able to become an agonist and activate AR. MDV3100 (Enzalutamide) was selected for its superior bioavailability and half-life after a screening and testing in cell lines and tumor xenografts models overexpressing the AR mimicking a CRPC setting. Enzalutamide showed significant tumor regression in mice bearing AR-overexpression tumors. However, bicalutamide showed little to no effects and had agonist effects on others. Compared to bicalutamide, enzalutamide is unique in having a 4-fold higher affinity to AR, blocking AR translocation to the nucleus, abrogating AR complex binding to the DNA and inhibiting W741C *AR*, a point mutations that confers resistance to bicalutamide (76). Phase I and II clinical trials in patients with pre- and post-chemotherapy CRPC showed that 160 mg/d was the optimal dose due to having similar activity on limiting DHT binding as higher doses and having lower toxicity (77, 78). In 2012, after the AFFIRM phase III clinical trial, the FDA approved enzalutamide to treat CRPC patients as a second-line treatment after chemotherapy failure. In the PREVAIL study, 1717 chemotherapy-naïve men with mCRPC were enrolled, 85% of whom had received antiandrogens as ADT. Enzalutamide showed significant benefit in overall survival, progression-free survival and reducing PSA levels compared to placebo (79). These results prompted the FDA to approve the use of enzalutamide in mem with CRPC who have not yet received chemotherapy. PREVAIL, a phase II pre-chemotherapy trial, showed significant benefit in patients who had received bicalutamide as a 1st line treatment. Patients in the enzalutamide treated group had a median radiographic progression free survival of 20 months compared to 5.4 months in control and a median overall survival of 35.3 months compared to 31.3

months respectively (80). In a head-to-head comparison to bicalutamide, in the TERRAIN and STRIVE studies, enzalutamide showed its superiority by extending progression free survival and showing improvements in secondary end points (81, 82). PROSPER, a phase III trial in patients with non-metastatic CRPC at high risk of progression and with rapidly rising PSA levels, prompted the approval of enzalutamide for the treatment of non-metastatic CRPC patients by the FDA. Enzalutamide showed improvements in treatment duration (18.4 months vs 11.1 months in placebo), metastasis free survival (36.6 months vs 14.7 months in placebo), time to PSA progression (83). Enzalutamide plus ADT was compared to placebo plus ADT in ARCHES, a phase III trials in men with metastatic castration-sensitive prostate cancer (mCSPC). Another phase III trials in men with mCSPC, ENZAMET, compared the effect of enzalutamide to the standard of care (standard non-steroidal antiandrogen) with testosterone suppression. In both studies, patients in the enzalutamide groups had a reduced risk of metastatic progression or death with a similar safety profile as seen in previous trials (84, 85). These data resulted in the FDA-approval of enzalutamide for the treatment of mCSPC in December of 2019. EMBARK and ENZARAD are two phase III trials testing the effects of enzalutamide in non-mCSPC and in newly diagnosed localized prostate cancer respectively (86). These trials will shed the light on whether enzalutamide can be adopted as a therapy for prostate cancer patients at all stages of the disease.

Despite positive responses to enzalutamide treatment in populations of patients with m-CSPC and CRPC, a significant number of patients are primarily resistant to the therapy. Primary resistance to enzalutamide is characterized by worsening of clinical condition with or without radiological or biochemical progression after three months of exposure to enzalutamide (87). In the AFFIRM and PREVAIL trials 46 and 22% of patients respectively, did not respond to enzalutamide (PSA levels did not decline by more than 50% from baseline) (75, 79). Primary resistance to enzalutamide correlates with the expression of AR mutants or more importantly with the expression of AR-V7 (88–91). In contrast, the other portion of patients will eventually develop an acquired resistance to enzalutamide later during treatment. Patients from the AFFIRM and PREVAIL trials (54% and 78% respectively) that responded to enzalutamide initially had biochemical progression after a median time of 8.3 and 11.2 months respectively, suggesting the development of resistance in those patients (75, 79).

1.1.8 Enzalutamide resistance

Acquired resistance to enzalutamide in prostate cancer cells can originate from the alterations of AR-dependent and AR-independent pathways. At this time, primary and acquired enzalutamide-resistance mechanisms are not fully understood. A deep investigation of these underlying mechanisms is important to identify predictive factors of treatment, novel therapeutic approaches or combination therapies to overcome or delay resistance.

1.1.8.1 AR-dependent mechanisms

AR amplification and overexpression are two hallmarks of progression to m-CRPC and resistance to androgen and androgen receptor targeted therapies. It is usually manifested by AR gene amplification, AR mRNA overexpression, or AR protein overexpression (92). 80% of CRPC patients exhibit overexpression of the AR protein, a significant portion of this observation is due to gene amplification (92). Enzalutamide-treated patients have a higher frequency of AR overexpression, rendering it a potential resistance mechanism (93). In addition, a cohort of patients treated with enzalutamide in the PREMIERE trial had a shorter PSA progression free survival and a shorter overall survival that correlated with AR amplification in those patients (94). In the LNCaP cell line, acquired resistance to enzalutamide induces a significant increase in the expression of AR and AR variants compared to cells that are sensitive to the second generation antiandrogen (95).

Mutations in the AR are rare in untreated prostate cancer patients. However, in CRPC patients AR is mutated in 5% to 30% of patients after treatment with ADT (96–98). These mutations may play a role in the primary resistance to enzalutamide or may arise after enzalutamide treatment. Most of the observed mutations are located in the LBD, altering the promiscuity of the AR to ligands. H875Y and T878A are gain of function mutations leading to activation of the AR by alternative hormones (progesterone, estrogen and glucocorticoids) and first-generation antiandrogens (flutamide and bicalutamide) (99). F877L and T878A are also mutations that affect the sensitivity of prostate cancer cells to enzalutamide in both *in vivo* and *in vitro* settings (99). Furthermore, circulating free DNA from patients progressing on enzalutamide therapy have been found to harbor F877L/T878A and M896V/S889G double mutants (96).

Not all the AR identified variants in prostate cells are transcriptionally active factors. Around 20 alternatively spliced AR mRNA have been discovered, however, AR-V7 and AR^{v567es} are the most studied. While most AR variants lack the LBD, AR-V7 is characterized by the addition of a cryptic exon 3 to its sequence (100). AR^{v567es} is characterized by the loss of exons 5, 6, and 7 in addition to a frameshift, early stop codon in exon 8 (101). The expression of these 2 variants is associated with resistance to enzalutamide and abiraterone in patients and in research models (102, 103). Furthermore, *AR-V7* levels were correlated with poorer survival and outcomes in patients with mCRPC (104). In the 22RV1 cell line, that has a substantially high expression of AR-V7 compared to AR-FL, AR-V7 is believed to replace the function of AR-FL. Knockdown of *AR-V7* in 22RV1 cells induces a re-sensitization to enzalutamide, suggesting that the variant may play a prominent role in resistance (105).

1.1.8.2 **AR-independent mechanisms**

In preclinical studies, the Glucocorticoid receptor (GR) has been shown to play a role in ADT and enzalutamide resistance in prostate cancer. In cell lines, xenograft models and clinical samples, acquired enzalutamide resistance and treatment with AR inhibitors induces an increase in GR expression and activation (106–108). GR shares common features with AR. Both receptors have homologous DBDs which enables them to have similar binding sequences resulting in overlap in their transcriptomes (106, 107). In enzalutamide resistant prostate cancer, GR can drive growth and progression by the transactivation of androgen-responsive genes without any AR stimulation. Ablation of GR expression in the VCaP enzalutamide-resistant cells resulted in restoring the response to enzalutamide (106).

PD-L1 was found to be overexpressed in a cohort of mCRPC patients. Its expression correlated with Gleason score, biochemical recurrence and the expression of AR and KI-67 in prostatectomy specimens (109). To further establish its implication in enzalutamide resistance, in vitro and *in vivo* tested the expression of PD-L1 in enzalutamide resistant cell lines and found it to be upregulated (110). In addition, in mouse xenograft studies harboring tumors that are enzalutamide resistant, PD-L1 expression was detectable in circulation (110). These observations suggest that cells can overcome AR blockade by enzalutamide through the activation of the PD-L1 / PD1 axis. Immune checkpoint blockade, PD1 inhibition, is being tested in a clinical trial with patients that

have enzalutamide-resistant mCRPC (111). Preliminary results show response in a subset of patients (111), suggesting that immune checkpoint blockade may hold promise to patients with primary or acquired resistance to enzalutamide.

In a cohort of patients having either neuroendocrine prostate cancer (NEPC) or mCRPC it was shown that molecular and gene signature were very similar between both groups, suggesting that NEPC can directly arise from CRPC (112). Although NEPC makes up 1% of all diagnosed prostate cancers, 30% of mCRPC have an NEPC signature (113). NEPC has become more interesting to researchers as it is an aggressive form of cancer and no treatment options currently exist for it. It is believed that under pressure from the AR blockade, cells can switch to the NEPC phenotype (114). Repressor element 1 silencing transcription factor (REST) downregulation following enzalutamide exposure is believed to be one of the mechanisms by which NEPC differentiation is enabled in treated prostate cancer (115). Enzalutamide can also promote the overexpression of SOX2 that results in the loss of important tumor suppressor genes such as TP53 and RB1, facilitating lineage plasticity and NEPC differentiation (116). In addition, overexpression of NMYC is a known NEPC driver that is regulated by the activity of the AURKA (117). Targeting the pathway is under investigation in clinical trials for the treatment of NEPC patients (117).

Autophagy is the mechanism by which cellular components are targeted for lysosomal degradation promoting self-digestion. In prostate cancer, autophagy may play a pro-survival role as a consequence of stress caused by androgen ablation (118, 119). It has been shown that autophagy can be an effect of enzalutamide treatment of responsive prostate cancer lines leading to the activation of the AMPK pathway and inhibition of mTOR signaling (120). Direct targeting of AMPK promoted the inhibition of autophagy in cells treated with enzalutamide, leading to cell death. Furthermore, in an orthotopic enzalutamide-resistant mouse model targeting autophagy in combination with enzalutamide led to a significant decrease in tumor growth compared to the vehicle treated group (120). Thus, showing the potential impact of autophagy on enzalutamide sensitivity in prostate cancer.

c-Myc, a well-known oncogene, has been shown to contribute to prostate carcinogenesis and progression. c-Myc is overexpressed at the mRNA and protein level in prostate adenocarcinoma

(121). Multiple events such as, gene amplification, active Wnt-Beta Catenine signaling or deletion of FOXP3 can contribute to the upregulation in the levels of the oncogene in the prostate (117). In a study by Grad *et al.*, c-Myc regulates AR expression by direct binding to its regulatory region (122). In primary and CRPC samples, c-Myc levels positively correlate with AR signaling activity and AR-FL and AR-Vs mRNA levels (123). Furthermore, c-Myc positively regulates the expression of AR by promoting its stability. On the other hand, another study by Gao *et al.*, shows that androgen independent prostate cancer cells exhibit an overexpression of c-Myc, driven by ligand-independent AR (124). These data suggest the existence of a positive loop between AR and c-Myc that may affect response to AR targeting therapies in prostate cancer cells.

With increased clinical usage of enzalutamide and drugs with similar targeting approaches, it is extremely important to identify the resistance mechanisms to these agents. By investigating mechanisms of resistance, patients may potentially have better outcomes and prognosis by identifying a genomic or proteomic predisposition to enzalutamide-resistance and getting access to better targeted therapies or by suggesting a combination therapy that would be tested in clinical trials. Below we will review two pathways we identified to be involved in enzalutamide resistance in prostate cancer.

1.2 Notch signaling pathway

The canonical Notch signaling pathway is a highly conserved and essential pathway involved in embryonic development, determining cell fate and organogenesis. On a cellular level, Notch pathway is involved in cell apoptosis, survival, differentiation and proliferation. The role of this system has been studied extensively since the early 1900s in Drosophila and it was identified based on a notch that appeared in the wing of flies (125). In the 1990s Notch signaling was granted a higher importance in humans as it was shown that mutations in *NOTCH1*, *NOTCH3* and *JAG1* caused T-cell lymphoblastic leukemia, CADASIL and Alagille syndrome, respectively (126–128).

1.2.1 Notch family of proteins

In mammals, the Notch family is composed of 4 receptors (Notch1, 2, 3, 4), where Notch1 and 2 are the most homologous (129). In contrast, Notch 3 and 4 have significant differences in their

extra- and intra-cellular domains (ECD and ICD) (129). The receptors can bind to five different ligands; Jagged-1 and -2 and Death-like ligands 1, 3 and 4 (130). All receptors and ligands are single-pass, transmembrane proteins that mainly require receptor-ligand interaction ensured by cell to cell contact, followed by a series of proteolytic modifications leading to the activation of the signaling pathway (131). In the ECD, Notch receptors are composed of 29 – 36 homologous epidermal growth factor (EGF)-like tandem repeats enabling their binding to ligands (129). The EGF-like repeats are followed by the negative regulatory region (NRR), composed of the three cysteine-rich Lin12/Notch repeats (LNR) and the heterodimerization domain (HD) (132). The NRR alongside the transmembrane fragment (TMF) play a crucial role in the activation of the pathway (133). This region encompasses the S1, S2 and S3 cleavage sites. Mutations and destabilization in the sequence of this region may lead to alterations in the activation of the pathway, resulting in diseases (133). The intracellular portion of the receptor contains a CSL-binding domain RAM, a nuclear localization signal (NLS), a PEST sequence, and seven ankyrin-like repeat (ANK) (132, 133). On the other hand, in the ECD, ligands are composed of an N-terminal (NT) domain followed by the Delta/Serrate/LAG-2 (DSL) domain which is important to the binding with Notch receptors (133). Alagille syndrome is a result of mutations of the DSL domain in Jagged1 (134). Dll1, Jagged1 and Jagged2 possess a Delta and OSM-11-like proteins (DOS) motif, essential for canonical Notch binding, and EGF-like repeats following the DSL domain (130). However, Dll3 and Dll4 lacking the DOS motifs require a DOS-containing non-canonical ligand in order to ensure contact and activation of Notch receptors. Jagged1 and 2 contain a Cysteine-rich region following the EGF-like repeats (130).

1.2.2 Activation of Notch signaling

Prior to their trafficking to the membrane, the Notch heterodimeric receptors are cleaved at the S1 cleavage site by Furin proteases in the trans-golgi network (131)(135). The two peptides are then assembled at the cell membrane by a non-covalent, Calcium-dependent bond leading to the formation of the Notch receptor in its final state at the cell surface (131).

Activation of the signaling pathway occurs upon cell-to-cell contact that results in the interaction of a Notch receptor with one of its canonical ligands. A pulling force produced by the receptor-ligand interaction is necessary for the detachment of the Notch ECD (NECD) from the rest of the

receptor, allowing its internalization by the receiving cell via endocytosis (136). The release of the ECD induces a conformational change in the NNR region leading to the exposure of the S2 cleavage site (137). Studies in Drosophila have demonstrated the necessity of ADAM10/Kuz in the cleavage at the S2 site and thus the activation of the pathway (138). In general, ADAM metalloproteinases mediate cellular interactions with neighboring cells or with other components of the microenvironment (139). To ensure ADAM-mediated proteolysis, membrane proteins need to be co-expressed with the metalloproteases and adopt a specific conformational state, priming its cleavage by ADAM. Studies have shown that recognition of ADAM targets is dependent on specific conformations rather that consensus sequences, leading to juxta-membrane cleavage (140). Importantly, ECD shedding by mechanic pull ensures the conformational change in the remaining extracellular portion of the receptor leading to cleavage by the ADAM proteolytic enzymes (141). Several metalloproteases of the ADAM family play a crucial role in Notch signaling, making them an essential player within the signaling pathway (142). Abrogation of ADAM10 in fruit flies by introduction of a dominant negative allele or by RNA interference hindered the proteolysis of the Notch receptor and signaling activation (138). In mammals, ADAM17 (TACE) plays a role in the S2 cleavage of Notch receptors. Studies have demonstrated the requirement for ADAM10 to process ligand-dependent Notch activation whereas ADAM17 is required for ligand-independent cleavage of the receptors (143). ADAM10 deficiency in mammalian cells abrogated the cleavage of Notch, which was not rescued by ADAM17 (144). Furthermore, dominant negative expression of ADAM17 did not hinder the activation of ligand-dependent Notch signaling pathway (144). In contrast, under conditions where Notch ligand-dependent activation was defective, including EDTA chelation and NRR mutants, ADAM17 was essential to activate the pathway (144). These observations illustrate the importance of the S2 cleavage step and the role of ADAM10 and ADAM17 in the process.

After its S2 cleavage and prior to the release of its ICD, Notch is cleaved at the S3 cleavage site by the γ-Secretase complex (145). This proteolytic complex resides in the intramembrane space and contains multiple interchangeable subunits. The 170 KDa complex is composed of 4 subunits including the catalytic proteins presenilin1 or 2 (PS1 or PS2), presenilin enhancer 2 (Pen-2) involved in the maturation of the complex, Nicastrin implicated in substrate recognition and anterior pharynx defective-1 (Aph-1a or Aph-1b) serving as a stabilizing scaffold for other

subunits (146). γ-Secretase localizes to the plasma membrane, late endosomes and lysosomes (147–149). In addition to the plasma membrane, co-localization of Notch and γ-Secretase can occur at in late endosomes after endocytosis of the receptor (150), adding another layer to the complexity of this signaling pathway . Furthermore, cleavage of Notch by γ-Secretase can generate NICD fragments with different N-terminal amino acids that can affect their stability. It has been shown that NICD with an N-terminal Valine are more stable than NICD with a Leucine or Serine, which can impact the sustainability of the signal (151). It has been shown that plasma membrane processing of Notch receptor favors production of the NICD peptides with Valine at its N-terminal (151). Whereas γ-Secretase-induced cleavage at the endosome membranes generates the degradation susceptible NICD fragments (151). γ-Secretase cleavage of Notch at the S3 site is the critical last step before the release of the active form of the receptor. Thus, researchers have developed multiple approaches to block this terminal cleavage step in the processing of Notch in the aim of inhibiting this pathway. Inhibitors such as DAPT, compound E and PF-03084014 have been developed to target the catalytic components of the complex leading to an inhibition of conformational changes and limiting target recognition and processing (152).

After its S3 cleavage and release in the cytoplasm, the NICD translocates to the nucleus. Unbound CSL forms complexes with transcription repressors and occupies Notch target genes promoters (153). Upon its entry to the nucleus, NICD associates with CSL (153). The RAM-mediated association of NICD to CSL promotes the detachment of co-repressors. The RAM/ANK interface is recognized by Mastermind (MAM) (154). The NICD/CSL/MAM complex recruits co-activators, histone acetyltransferases and transcription machinery to promote the expression of Notch target genes (155). NICD stability and expression is essential for the maintenance of Notch signaling and promoting Notch target gene expression. Studies have shown that, aside from the N-terminal residues that can affect its stability, NICD is regulated by post-translational modifications including phosphorylation, ubiquitination, hydroxylation and acetylation (156). These modifications can influence the half-life of the cleaved protein and its ability to bind co-regulators (157). Furthermore, these modifications may play differential roles in different NICD isoforms promoting a diverse, non-redundant response by different Notch receptors.

Extensive transcriptomic and genomic studies in different cell lines and organisms revealed the diversity of Notch target gene output in different organisms, cell types, stages of the cell cycle and in different cell lineage stages. Hairy and enhancer of split-related (HESR) genes are among the most recognized and studied Notch target genes. This family includes Hes1, Hes5, Hes6 and hairy/enhancer-of-split related with YRPW motif 1 (Hey1) that are known to play transcription repressive roles (158). Other important Notch target genes include, c-Myc (159) and cyclin D1 (160).

On the other hand, studies have shown that Notch can be associated with non-canonical ligands influencing its activity. Furthermore, Notch can interact with alternative pathways, independent of ligand binding to induce alteration in the output of those signaling pathways.

Non-canonical Notch activation can occur in the absence of its conventional ligands and transcription factors. Notch can be stimulated by non-canonical ligands leading to the activation of the signaling pathway. Delta-like 1 homolog (Dlk1) is believed to be a repressive ligand to Notch signaling due to the absence of the DSL domain in its sequence (130). These observations demonstrate that Notch signaling is complex and different mechanisms and alternative pathways can contribute to its activation or inhibition.

1.2.3 Notch signaling in the prostate and prostate cancer

Notch plays a significant role in the development and homeostasis of most organs in mammals. During organogenesis Notch mainly contributes to lineage specification and differentiation by acting on progenitor cells. Notch has been shown to be involved in determining cell-fate in the heart, brain, liver, pancreas, breast and the prostate (161–165).

Prostate development is mainly regulated by androgens, however, pathways like Sonic hedgehog, Fibroblast Growth Factor 10, Bone morphogenic Protein 4 and 7 and Notch signaling have been shown to contribute (166).

In the developing prostate, Notch signaling is required for proper development and branching of the gland. In transgenic mice designed to selectively eliminate Notch1-expressing cells, Wang et

al showed selective apoptotic death in the basal layer accompanied by a defective branching morphogenesis and an inhibition of luminal cell differentiation (167). Also elimination of Notch1-expressing cells inhibited the regeneration of the castration-induced abrogation of the luminal cells in the prostate (167). Inhibition of the signaling pathway by targeting the γ-Secretase complex in ex-vivo-grown ventral prostates from rats promoted an abnormal development and architecture in the developing prostate and induced an increase in basal cell proliferation (168). Also, knockout of RBPJ (aka CSL) decreased basal cell proliferation and survival in the embryonic and postnatal prostate (169). In contrast, Wu et al showed that murine prostates with a constitutively expressed NICD exhibited a higher rate of progenitor cell proliferation due to the silencing of the tumor suppressor PTEN in the embryonic and postnatal stages (169). In a study by Kwon et al, exogenous expression of NICD promoted the proliferation and survival of luminal progenitor cells by the activation of NF-κB and PI3K-AKT signaling (170). These observations suggest that Notch signaling operates predominantly in progenitor cells of the embryonic and postnatal developing prostate. However, the contradicting roles of Notch grant a deeper investigation of its role in the different prostate cell types to better understand the functionality of the receptors and the underlying mechanisms involved in the regulation of prostate development and regeneration.

Contradictory observations were also noted in prostate tumorigenesis, where Notch can play both tumor suppressor and tumor promoter roles. Regarding the expression of Notch proteins in prostate adenocarcinoma, we will highlight results suggesting a pro and an anti-tumorigenic role. In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model expressing GFP under the Notch1 promoter it was shown that epithelial and metastatic prostate cancer cells exhibited a high expression of GFP indicating a high expression of Notch1 in those populations of cells (171). In humans, a study by Yu et al, and Zhu *et al.* show that Notch1 and Jagged1 are overexpressed in prostate cancer samples and metastatic tumor samples compared to normal tissue (172, 173). In contrast, Notch1-deficient murine prostates overexpress a multitude of prostate oncogenes such as c-Fos, c-Jun, PSCA, FGF18 and PTOV1 (168, 174).

Examining the proliferative effects of Notch signaling in prostate cancer, data have shown again a contradicting role of this pathway. The expression of Jagged1, a canonical Notch ligand, correlates with the proliferation of prostate cancer cells. Repressing Jagged1 in PC3, DU145, LNCaP and

LAPC4 induced a significant decrease in cell growth, whereas its upregulation promoted proliferation in LNCaP and LAPC4 cells (173). PC3 cells overexpressing ectopic Dll4 compared to unaltered PC3 cells showed an increase in growth rate of tumors xenografts (175). In contrast, overexpression of NICD in LNCaP, DU145 and PC3 prostate cancer cell lines halted the proliferation of these cells (171).

Signaling of Notch plays a role in regulating apoptosis in prostate cancer cells. RNA interference knockdown of Notch1 in PC3 cells induced transactivation of the pro-apoptotic Bax and silencing of anti-apoptotic protein Bcl-2. This resulted in a decrease in proliferation, increase in apoptosis and sensitivity to the chemotherapeutic docetaxel (176).

Prostate cancer stem cells are hypothesized to be a tumor-initiating population of cells, characterized by therapy-resistance and cancer regeneration. A subpopulation of DU145 cells having prostate cancer stem cells characteristic has a high expression of several Notch signaling pathway proteins such as Notch1, Jagge1, Dll1 and Dll3(177). Domingo-Domenech *et al.* investigated a docetaxel-resistant population of cells in tissue samples. Notch and Sonic Hedgehog signaling were activated in these cells and inhibition of these pathways ablated these cells (178). Apart from its involvement in prostate cancer cell proliferation, cell death and stem cells, the Notch pathway plays a pivotal role in other aspects of prostate cancer including hypoxia, angiogenesis, cell migration, cell invasion and metastasis. These studies are reviewed in (166).

1.2.4 Notch signaling in drug-resistant prostate cancer

More importantly for the purpose of our study, we will highlight the implication of Notch pathway in therapy resistance in prostate cancer. Cancer stem cells are believed to play a crucial role in therapy resistance due to their slow proliferation rate and their expression of several genes involved in drug resistance. The importance of Notch signaling to prostate cancer stem cells has shed the light on its involvement in therapy resistance. In breast cancer, another hormone driven cancer, studies have shown that therapies targeting the estrogen receptor induce an increase in the activity of Notch pathway, which sets a precedent for prostate cancer, being an androgen stimulated cancer with high similarities between the estrogen and androgen receptors (179). Furthermore, Notch1 signaling has been shown to promote chemoresistance through the abrogation of proper p53

signaling in prostate cancer cells through the alteration of the PI3K/AKT pathway (180). ABCC1 belongs to the ABC transporters family, inherently involved in resistance mechanisms by functioning as drug pumps to eliminate intracellular pools of drug molecules, is regulated by the Notch signaling pathway (181).

Other investigators have recently showed that Notch signaling can play a role in enzalutamide-resistant prostate cancer. Mohamed *et al.* show that AR and Notch signaling inhibition in ERG-positive prostate cancer cells induces a decrease in proliferation, increase in apoptosis and strong inhibition of AR and PSA (182). Cui *et al.* showed tat exogenous expression of NICD in prostate cancer cells induce resistance to ADT and treatment with γ-Secretase inhibitors induce promotes sensitivity to ADT by acting through the p38/MAPK pathway and Bcl-2/Bax axis (183).

1.2.5 Crosstalk between Notch signaling and androgen receptor signaling

Due to the involvement of Notch signaling in prostate cancer it is important to explore the crosstalk between this pathway and the AR signaling pathway, the master regulator of prostate cancer. Nantermet *et al.* found that upon activation of the AR signaling pathway, the expression of Notch signaling components like Notch1 and Jagged1 where downregulated and Sel-1L and presenilin-1 were upregulated (184). On the other hand, the downstream targets of Notch signaling, Hey1 and HEYL, have been shown to play a co-repressor role to AR by binding to its AF1 domain and inhibiting the expression of androgen-regulated genes in the prostate (185, 186). These observations suggest an interaction between AR signaling and Notch signaling in the context of the prostate gland. The crosstalk between Notch and AR and its role in prostate cancer stem cells and drug resistance poses the question of the involvement of Notch signaling in enzalutamide resistance.

1.3 DNA methylation

Eukaryotic DNA is characterized by the presence of methyl groups, predominantly on Cytosine molecules that are followed by a Guanine (CpG) (187). This modification is called DNA methylation and was identified as the first epigenetic mark that can influence gene expression without modifying the actual DNA sequence. Early studies have shown that this epigenetic

modification is repressive (188, 189) and involved in X chromosome silencing and gene imprinting (190, 191). Despite being conserved through evolution, some organisms lack DNA methylation in their genomes (192). The significantly lower than expected number of CpG sites in methylation-prone genomes led scientists to hypothesize that methylated CpG can be highly mutagenic. Studies have shown that the deamination of methylated Cytosines can result in aberrant conversion of this residue to a Thymine reducing overall CpG count and potentially affecting genomic outcomes (193). In general, promoter regions of genes have a high concentration of CpG dinucleotides called CpG islands. Those clusters of CpG dinucleotides are usually unmethylated to ensure proper expression of genes.

1.3.1 Functions and structure of DNA methyltransferases

The methyl group is deposited on the 5th carbon of the Cytosine (5mC) base by a group of enzymes named DNA methyltransferases (DNMTs) (194). Of note, other methylated bases exist such as N4-methylcytosine and N6-methyladenine (195). However, in mammals the predominant form of methylated DNA is 5mC. In humans, DNMTs are divided into 3 categories: maintenance methyltransferases, de novo methyltransferases and methyltransferases with no catalytic activity. Due to the symmetrical positioning of DNA methylation on the double stranded DNA, it was hypothesized that methylation patterns from the parental strand are copied to the daughter strand following its synthesis (196). This prompted scientists to raise the question about the presence of an enzyme catalyzing this reaction. DNMT1 is a maintenance methyltransferase in charge of faithfully copying the patterns of 5mC from the mother strand to the daughter strand after DNA replication (196). DNMT3A and DNMT3B are de novo methyltransferases responsible for adding new methylation marks on unmethylated cytosines (196). DNMT3L is a non-canonical methyltransferase that lacks catalytic motifs (197).

All DNMTs contain a N-terminal regulatory domain followed by a catalytic domain in the C-terminal. The regulatory portion of DNMT1 contains a DNMT1-associated protein 1 (DMAP1) binding domain, a replication foci targeting sequence (RFTS), a CXXC domain and two bromo-adjacent homology (BAH) domains (198). The DMAP1 binding domain ensures the binding of DNMT1 to DMAP1 and Histone deacetylase 2 (HDAC2) in the S-phase (199). The DNMT1-DMAP1-HDAC2 complex executes a triple-function characterized by the copying of 5mC into the

daughter strand, silencing gene expression and deacetylating histones resulting in a repressive chromatin at the replication site (199). Two smaller domains, the PCNA binding domain and the nuclear localization signal (NLS) are located between the DAMP1 binding domain and the RFTS domain (200). During the S-phase, RFTS targets DNMT1 to replication foci and deletion of RFTS abrogates this localization (201). Replication-independent localization of the maintenance methyltransferase to the chromatin, specifically during the G2/M phase is exhibited by its targeting sequence domain (TS) (202). Hemi-methylated DNA targeting of DNMT1 occurs through the interaction of its RFTS domain with the ubiquitin-like with PHD and ring finger domains 1 (UHRF1) (203). The CXXC and BAH domains coordinate the recognition of unmethylated and hemi-methylated DNA and autoinhibit the catalytic activity of DNMT1 (204, 205). CXXC-binding to unmethylated DNA protects the DNA from any de novo methylation while allowing exclusive enzymatic activity on bound hemi-methylated DNA (204, 205).

On the other hand, the N-terminal regulatory region of DNMT3 enzymes contains a Pro-Trp-Trp-Pro (PWWP) domain and an ATRX-DNMT3-DNMT3L (ADD) domain (206). Although its exact function is still unknown, it has been shown that the sequence upstream of the PWWP domain of DNMT3A and DNMT3B plays a role in the nuclear localization and DNA binding (207). Studies have also shown that the N-terminal sequence is required for the association of DNMT3 with intact nucleosome structures (208). The PWWP domain guides DNMT3 to the body of transcribed genes by its binding to the trimethylated H3K36 mark (209, 210). Furthermore, the PWWP domain is involved in DNA binding and in targeting the enzymes to satellite repeats and pericentromeric DNA (211, 212). The ADD domain enables the methylation of unmethylated H3K4-associated DNA by binding to the unmodified Lysine (213). This domain also has an autoinhibitory function by associating with the catalytic motifs of the enzyme, when not bound to Histones (214).

Prior to Cytosine methylation, the C-terminal domains of all DNMTs need to adopt a specific conformation to accept the methyl-group donor S-adenosylmethionine (AdoMet) (215) . The mechanism by which DNA is methylated requires the base to be flipped into the catalytic pocket of the enzyme following its synthesis (216) . Aside from its catalytic role, this C-terminal domain is involved in DNA recognition and binding. Although this family of methyltransferases shares highly homologous sequences and domains, the subtle difference in these sequences and domains

ensures the unique functions and roles of DNMTs *in vivo*. Further playing into the regulation and diverse functions of these enzymes in different cells and tissues are the post-translational modifications, protein interactions, expression patterns and alternative splicing.

1.3.2 DNA methylation in developing and adult tissues

DNA methylation and DNA methyltransferases play a pivotal role in embryogenesis, tissue development and differentiation of cells. This role is illustrated by early studies in which knockout of DNMT1 and DNMT3B induced embryonic lethality and knockout of DNMT3A caused death in mice at 4 weeks of age (217). DNA methylation-mediated regulation of genes in development starts in germ cells with the erasure of DNA methylation marks by passive demethylation (218). Following the replication-induced hypomethylation, DNMT3A and DNMT3L play an essential role in establishing de novo methylation sites on imprinted genes during female and male gametogenesis (219–221). It has been shown that knockout of DNMT3A or DNMT3L in mice impairs the de novo DNA methylation process and leads to biallelic expression of normally imprinted genes (219–221). Following fertilization and zygote formation, both pronuclei undergo asymmetrical DNA demethylation . After embryo implantation, the genome will be methylated again by de novo methyltransferases ensuring proper gene imprinting, chromosome X-inactivation and silencing of transposable elements in embryonic cells .

During prostate development in gestation, the urogenital sinus (UGS) gives rise to buds that will form the ductal network of the prostate gland under regulation of androgens and epithelial-mesenchymal interactions (222). The dynamic expression of DNMTs during the different stages of prostate development suggest that they play a role in the process (222). To ensure prostate bud outgrowth and proper adhesion of epithelial cells, DNMTs methylate the CHD1 gene promoter leading to its silencing and proper prostate branching morphogenesis (223). When treated with a DNA methylation inhibitor, CHD1 promoter is hypomethylated, the protein is highly expressed hindering prostate bud outgrowth (223). In the UGS mesenchyme, where both AR and DNMTs are expressed, the promoter of AR exhibits changes in DNA methylation during development (224, 225). Inhibition of DNA methylation results in an increased AR expression and increased sensitivity to androgens in the UGS mesenchyme (225). These observations illustrate a dynamic

role for DNA methylation by influencing the expression of key molecules involved in prostate development.

The expression of DNMT1, DNMT3A and DNMT3B is significantly reduced in adult differentiated tissues compared to their expression in embryonic stem cells (226). Different organs and tissues have different expression levels of DNMTs and their alternatively spliced variants (226). In adult somatic cells the predominant form of DNMT3B is DNMT3B3, a variant with no catalytic activity, in contrast to the catalytically active DNMT3B1 in embryonic stem cells (227). DNMT3B3 plays a role similar to DNMT3L as a binding partner to catalytically active DNMTs guiding them to genomic sites for DNA methylation (228). DNMT3B exist in more than 30 isoforms generated by alternative splicing events, mainly affecting the C-terminal domain rendering these isoforms inactive (229–231). In adult normal tissues, the essential role of DNA methylation is promoting genomic integrity and proper expression of inherited genes by silencing transposable and repetitive elements in addition to ensuring correct gene imprinting and X-chromosome silencing (232). In the normal differentiated prostate, the role of DNA methylation and DNMTs is restricted to maintain the silencing of unneeded genes and transposable and repetitive elements in the genome.

1.3.3 DNA methylation in prostate cancer

Although the exact mechanism behind the initiation of prostate cancer is still unknown, it is believed that the accumulation of genetic and epigenetic aberrations plays an important role in the onset and progression of the disease. DNA methylation has been well studied in the context of prostate cancer (233, 234). Studies have shown a relationship between DNA methylation and the initiation and the progression of prostate cancer (235). Aberrant hypermethylation of CpG islands in the promoter regions of tumor suppressor genes is one of the mechanisms believed to contribute to carcinogenesis (236). Normally unmethylated CpG-islands can become methylated by a deregulated DNA methylation pathway (237–239). This leads to the silencing of tumor suppressor genes which contributes to the malignant process (240). On the other hand, hypomethylation is more widespread within the genomes of cancer cells (241, 242). Hypomethylation of normally methylated promoter regions of oncogenes and imprinted genes are observed in many cancers, including prostate cancer (243, 244). Additionally, the hypomethylation of repetitive elements and

retrotransposons can contribute to genetic instability which can play a contributing role to malignancy (245, 246).

The combination of abrogated DNA methylation events with genetic mutations and aberrations are believed to play a role in driving the initiation of prostate cancer (247). The importance of tumor suppressors in prostate cancer is illustrated by several studies around the genetic loss of genes such as *PTEN*, *TP53* and *RB1* and their implication in the initiation and progression of the disease (248–250). These studies show that mutation or loss of these genes are correlated with prostate cancer. Additionally, researchers generated mouse models that can develop spontaneous tumors by combining deletions of such tumor suppressor genes (251)(252). Moreover, genetic events leading to the formation of fusion proteins have been shown to play a role in prostate cancer. ETS or the active fragment of the transcription factor ERG driven by the promoter of an AR-regulated gene, called TMPRSS2, has been shown to play a role in the cancer of the prostate (253). Studies by different groups have shown that active ERG promotes cell migration and invasiveness in prostate cancer (254–256). It also plays a role in restricting the epithelial differentiation profile of prostate cells by binding to AR-regulated downstream targets (257).

Promoter gene hypermethylation, the predominant epigenetic event in early prostate cancer, is believed to contribute to disease initiation. Lee *et al.* were the first to identify Glutathione S-transferase P1 (GSTP1) promoter hypermethylation in prostate cancer samples and cell lines (43). Silencing of GSTP1 by hypermethylation is widely observed in PIN and cancerous lesions compared to normal prostatic tissue (258). In normal conditions, GSTP1 plays a protective role against oxidative and xenobiotic stress-induced DNA damage in prostate cells, thus its loss is believed to play a tumor promoting role (259). MGMT, a protein involved in the DNA damage/repair pathway that removes O(6)-alkylguanine lesions from the DNA, is found hypermethylated in tumor and urine samples from prostate cancer patients (260, 261). Esteller *et al.* have shown that hypermethylation-mediated silencing of *MGMT* promotes mutagenic DNA damage phenotype to genes that are involved in cancer, such as *TP53* and *K-ras* (262). In addition, loss of MGMT expression affects the sensitivity of tumors to chemotherapeutic agents (262). These studies suggest that the expression of genes from the DNA damage and repair response

pathways may be compromised in the prostate as a result of DNA hypermethylation. Aberrant expression of such genes may contribute to cancer initiation and progression and therapy response.

1.3.4 DNA methylation regulates key cellular pathways and processes

DNA hypermethylation of genes involved in the hormonal response is commonly observed in hormone-driven cancers. Jarrad *et al.* showed that the methylation of the CpG island in the AR promoter region is associated with its silencing in several AR-negative cell lines. The treatment of theses cell lines with a demethylating agents reverses the loss of AR expression by methylation (44). Although the prevalence of AR hypermethylation is low in clinical samples (263, 264), it is believed that AR expression contributes to the onset and progression of androgen-independent prostate cancer. Immunohistochemical analyses show a significant decrease in the expression of both estrogen receptors Esr1 and Esr2 in prostate cancer samples (265, 266). The decrease in estrogen receptor expression correlates with poor prognosis (266). Hypermethylation of the estrogen receptor promoter region was identified as the main mechanism by which silencing of both homologues occurs (263, 267). The importance of nuclear hormone receptors in different hormone-driven cancers is well studied. The above studies suggest that DNA methylation can play a role in hormonal cancers by directly influencing the expression of receptors such as the androgen and estrogen receptors.

Cell cycle genes are deregulated in many cancers resulting in an increased proliferation and survival of cancer cells, promoting uncontrolled tumor growth. The balance between cyclin-dependent kinases (CDKs) and CDK inhibitors maintains a well-regulated cell cycle in normal cells. The loss of this balance, seen in multiple cancer types including prostate cancer (268), promotes the uncontrolled progression of the cell cycle that may contribute to carcinogenesis. Hypermethylation and silencing of CDK inhibitors such as CDKN1A (269), 1B (269), 1C (270) and 2A (271) have been observed in a subset of prostate tumor samples. RASSF1, a tumor suppressor gene within the oncogenic RAS signaling pathway, is silenced in more that 50% of prostate cancer samples (235). The loss of RASSF1 promotes the oncogenic potential and inhibits the apoptotic effect of Ras proteins leading to increased cell proliferation and survival (272, 273). These observations shed the light at the role of DNA methylation in promoting malignant potential by affecting the cell cycle.

Aberrant CpG island promoter methylation is also observed in a subset of pro-apoptotic genes. Hypermethylation and silencing of DAPK, FHIT, SLC5A8, SLC18A2, and TNFRSF10C was observed in up to 88% of prostate tumor samples compared to their normal controls (274). This pathway plays a pivotal role in cancer development and treatment. The deregulation of apoptosis by silencing pro-apoptotic proteins may promote drug resistance in prostate cancer. Hypermethylation-induced loss of SLC18A2 and TNFRSF10C is positively correlated with biochemical recurrence in prostate cancer samples (275).

DNA methylation is also involved in metastasis and invasion of prostate cancer cells. Tissue inhibitors of metalloproteinases (TIMPs) were originally identified as antagonists to the family of matrix metalloproteinases (MMPs) that promote tumor invasion and metastasis (276). MMPs are known to play a pivotal role in degrading the extracellular matrix allowing cells to migrate to metastatic sites. Promoter methylation of TMP-3 was observed in a high portion of urine and tumor samples collected from prostate cancer patients (260, 261). Adissu *et al.* showed that knockout of TIMP-3 increases the expression and activity of MMPs and promotes invasiveness in a prostate cancer mouse model (277). These studies illustrate the significant advancements made in the study of hypermethylated genes and their association with functional pathways and prostate cancer.
Methylation of large portions of the genome exists as a silencing mechanism for sequences that may cause genomic instability, chromosomal rearrangements and mutations. Repetitive elements, retrotransposons, and imprinted genes constitute a significant part of the genome that is silenced under normal conditions. Whenever the DNA methylation machinery is deregulated, regions with normally highly methylated DNA may lose the 5-mC mark rendering them hypomethylated. Hypomethylation of repetitive sequences and retrotransposons may have a detrimental effect on genomic integrity. In prostate cancer, both genome-wide hypomethylation and gene specific hypomethylation are observed in late stages of the disease (278). In terms of genome-wide hypomethylation, LINE-1 retrotransposons, which are normally methylated, are hypomethylated in up to 50% of human prostate cancer samples tested (279, 280). Global hypomethylation, illustrated by hypomethylation of LINE-1 was shown to be associated with development and progression of prostate cancer and increase in mortality in higher Gleason score patients (281, 282). On the other hand, targeted DNA hypomethylation to specific genes such as uPA, PLAU, CAGE, CYP1B1 and Ras oncogenes is observed in prostate cancer (274). These genes possess an

oncogenic potential, thus their overexpression following aberrant hypomethylation can drive prostate cancer progression and promote metastasis in later stages of the disease.

1.3.5 DNA methylation in drug resistance

Epigenetic mechanisms, especially DNA methylation, are believed to play an important role in drug resistance in cancer. The aim of cancer therapeutics is to halt the growth or induce death in the population of cancer cells by causing apoptosis, inducing cell cycle arrest and halting proliferation among other mechanisms. However, after prolonged treatment administration, tumors will develop resistance to cancer therapies and resume growth. The absence of mutations in drug targets, the silencing of cell death pathways and the over-activation of survival and proliferation pathways prompted scientists to investigate the epigenome in cells with acquired drug resistance. DNA methylation has been shown to contribute to acquired therapy resistance by acting on genes and pathways that may influence cell response to treatment in different cancer types (283–286). The ATP-binding cassette (ABC) transporters are a known family of proteins to play a pivotal role in multi-drug resistance (287). The expression of ABC proteins correlate with the resistance to cancer therapies in different cancers (288). The expression of ABC proteins is regulated epigenetically by DNA methylation in different tissue types. Hypomethylation in the promoter region of the multi-drug resistance protein ABCB1 was observed in samples from bladder cancer patients exposed to chemotherapy (289). The expression of ABCB1 correlated with hypomethylation of its promoter region and with the development of a drug resistant phenotype (289). ABC transporters are believed to pump drug molecules out of cells, lowering the intracellular concentration of therapy molecules leading to a hindered response. ABCG2 exhibited an increase in expression associated with hypomethylation of its promoter following treatment with sulfasalazine and topotecan in T-ALL and ovarian cancer cell lines (290). The increase of ABCG2 expression was associated with a resistant phenotype (290). In prostate cancer, Zhu *et al.* have shown that treatment of docetaxel resistant prostate cancer cell lines with an inhibitor of ABCB1 reverses the resistant phenotype and sensitizes the cells to docetaxel (291). Despite the extensive studies on ABC transporters, the involvement of DNA methylation in the regulation of these proteins in prostate cancer is understudied and their role in enzalutamide resistance is poorly understood.

Another mechanism by which DNA hypomethylation can affect drug resistance is abrogating the expression of cell cycle checkpoint protein which contributes to overriding the checkpoints leading to progression of the cell cycle and uncontrolled growth. Hypomethylation of PTPN6, a protein phosphatase that plays a role in mitotic progression among other cellular processes, leads to a marked increase in its expression (292). Overexpression of PTPN6 contributes to resistance in bortezomib, cisplatin, and melphalan-treated glioma cancer cells (292). The mechanism behind drug-induced hypomethylation of promoters is still poorly explored. Researchers hypothesize that active demethylation of promoter regions is occurring upon drug treatment leading to overexpression of hypomethylated genes (293). Another contributor may be the drug-induced expression of novel DNMT3B isoforms resulting in targeting of active DNA methyltransferases to a new set of CpG sites leading to hypomethylation of untargeted loci (294).

Moreover, hypermethylation of promoter regions of genes involved in apoptosis, cell cycle regulation and DNA repair have been observed in drug-resistant cancer lines (295). Silencing of pro-apoptotic genes such as DAPK1 and APAF-1 has been reported in multiple malignancies (296–300). DAPK1 is found silenced by genetic loss or by hypermethylation (301). Low expression of DAPK1 correlates with poor prognosis and tumor recurrence in multiple cancer types (300, 302–304). More importantly, DAPK1 loss has been shown to play a role in resistance to different drugs in cervical, endometrial, lung and pancreatic cancer and its expression can be restored by treatment with the demethylating agent 5-aza-cytidine (305–309). The loss of another pro-apoptotic factor, APAF-1, hindered the p53 activation cascade in melanoma cells leading to chemotherapy resistance (295). Chemotherapy sensitivity was restored after treatment with DNA methylation inhibitors that increased the expression of APAF-1 (310).

Furthermore, silencing of genes involved in DNA repair mechanisms has been shown to affect sensitivity to cancer therapies. Hypermethylation-mediated silencing of MLH1 and SRBC has been shown to be associated with a chemoresistance phenotypes in different cancers (311–315). In contrast, hypermethylation-mediated silencing of MGMT and FANCF was found to correlate with chemosensitivity of cancer cells (316–319). In prostate cancer, DNA methylation contributes to cabazitaxel-resistance by deregulating genes involved in the regulation of the cell cycle and the cell death response. In DU145 cells, Ramachandran *et al.* show that pre-treatment with the DNA

methylation inhibitor 5-azacytidine leads to an improved response to cabazitaxel compared to cells with no pre-treatment (320). In another study by Garvina *et al.*, the authors show that treatment with the DNA methylation inhibitor 5-azacytidine reduces the bicalutamide-induced resistant phenotype in prostate cancer cells (321). However, the role of specific DNA methyltransferases and specific DNA methylation changes have not been studied in the widely used antiandrogen enzalutamide. A study of global methylation changes, its effect on global gene expression and the consequences on response to enzalutamide is warranted to explore the role of this epigenetic mark in enzalutamide-resistant prostate cancer.

CHAPTER 2. MATERIALS AND METHODS

2.1 Mammalian cell lines

LNCaP and C4-2 were used as enzalutamide-sensitive cell lines. Whereas, MR49F, C4-2R and 22RV1 were considered enzalutamide-resistant cell lines. For this study LNCaP and C4-2 were paired with their enzalutamide-resistant counterparts MR49F and C4-2R, respectively. 22RV1 was used as an independent cell line with no enzalutamide-sensitive pair. The LNCaP and 22RV1 lines were purchased from ATCC. The C4-2 line was acquired from the M.D. Anderson Cancer Center. Enzalutamide-resistant lines MR49F was gifted from Dr. Amina Zoubeidi at the Vancouver Prostate Cancer Center (322) and C4-2R was gifted from Dr. Allen Gao at the University of California Davis (323). All cell lines were maintained in RPMI 1640 medium supplemented with 10% (v/v) Fetal Bovine Serum at 37°C in a humidified incubator with 5% carbon dioxide. MR49F and C4-2R were constantly maintained in medium supplemented with 10 and 20 µM enzalutamide, respectively.

2.2 Inhibitors

PF-03084014, enzalutamide, decitabine were purchased from Selleckchem. For *in vitro* experiments, original stock solutions of PF-03084014, enzalutamide and decitabine was prepared by suspending the compounds in DMSO. Further diluted stocks were prepared as needed by diluting with DMSO for PF-03084014 and enzalutamide or PBS for decitabine. Decitabine solution were diluted from the original stock on a need basis. For *in vivo* experiments, PF-03084014 was suspended in 0.5% Methylcellulose, enzalutamide was formulated in 5% DMSO and 0.1% Tween80 suspended in 1% carboxymethyl cellulose and an original stock of decitabine was formulated in DMSO then diluted in PBS.

2.3 Western blot

Cells were harvested and washed with cold PBS once. Pellets were resuspended in TBSN buffer (20 mmol/L Tris-HCl, pH 8.0, 0.5% NP-40, 5 mM EGTA, 1.5 mmol/L EDTA, 0.5 mM sodium vanadate and 150 mM NaCl) or RIPA buffer supplemented with protease and phosphatase inhibitor cocktail. The Pierce BCA Protein Assay Kit was used to measure sample protein

concentrations at a wavelength of 570 nm. Equal amounts of proteins were mixed with 4x SDS loading buffer, boiled at 95°C for 5 minutes and loaded onto SDS-PAGE gels. SDS-PAGE gels were prepared at different percentages ranging from 6 to 12 % depending on investigated protein size. Upon protein separation, proteins were transferred to PVDF membranes and subjected to detection using appropriate primary and secondary antibodies. GAPDH and β-Actin were used as housekeeping genes. SuperSignal™ West Dura Extended Duration Substrate was used to expose blots on the ChemiDoc imaging system.

2.4 RNA-sequencing for experiment comparing enzalutamide-sensitive to -resistant cell lines

Total RNA was extracted from LNCaP, MR49F, C4-2, C4-2R after 4-hour treatment with Enzalutamide using RNeasy kit (QIAGEN). Libraries were prepared using TruSeq Stranded kit (Illumina) at the Purdue Genomics Facility. 2x100 bp reads were sequenced in 2 lanes using the HiSeq2500 on high-output mode. Before library preparation the dscDNA quality was checked using an Agilent Bioanalyzer with the High Sensitivity DNA Chip. All plots were checked to ensure that read quality for the reads that would be used in the remainder of the analysis were of high quality and with no problems. Tophat2 (324, 325) was used to align reads to the Ensembl Homo sapiens genome database version GRCh38.p5. The mitochondrial chromosome and the nonchromosomal sequences were excluded from the analysis. The htseq-count script in HTSeq v.0.6.1 (326) was run to count the number of reads mapping to each gene. DESeq2 (327), edgeR (328) and Cufflinks2 (329, 330) were used for differential expression analysis.

2.5 Gene Set Enrichment Analysis

Clinical data were collected from Level 3 (for Segmented or Interpreted Data, IllumninaHiSeq_RNASeqV2) of the TCGA database. Samples were divided into low-grade (Gleason score <8) and high-grade (Gleason score ≥8). Of the high-grade samples, we looked at 72 out of 497 cases that had been treated with anti-hormone therapy. We then separate the 72 patients into *AR* high and *AR* low based on the expression of the genes from the *AR* signaling pathway. *AR* high cases were considered to mimic Enzalutamide-resistant cells and *AR* low cases were considered to mimic Enzalutamide-sensitive cells. Using GSEA we detected pathway enrichment in *AR* high (26 samples) vs *AR* low (46 samples).

2.6 Colony formation assay

Cells were seeded at equal densities in 6-well plates, followed by treatment with DMSO as a control, PF-03084014, enzalutamide or the combination of both. After colonies became of size, cells were subjected to fixation with 4% Paraformaldehyde followed by staining with 0.5% crystal violet solution.

2.7 MTT Assay

To evaluate cell viability, plates were primed with Poly-D-lysine hydrobromide purchased from MilliporeSigma followed by seeding of cells at densities ranging between 1000 to 3000 cells/well. The following day cells were transfected or treated with respective drugs. At the end of incubation time, cells were then treated with 0.5 mg/ml MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) for an hour. Supernatant was removed and DMSO was added to dissolve crystals. Optical density was measured at a wavelength of 570 nm.

2.8 Lentivirus production and Notch1 knockdown in C4-2R cells

A lentivirus system encoding shRNA targeting *NOTCH1* mRNA was used for knockdown. sh*NOTCH1* carrying the coding sequence of *NOTCH1* 5'- GCATGTGTAACATCAACAT -3' was cloned in pLVshRNA-puro. shCtrl carrying a scrambled sequence was cloned in pLVshRNA-puro. Lentiviral particles were generated by transfecting shCtrl or sh*NOTCH1* with psPAX2 and PMD.2G plasmids at a ratio of 4:3:1 in 293T cells. Medium was collected and used to infect C4-2R cells with corresponding shRNA to generated C4-2R-shCtrl or C4-2R-sh*NOTCH1*.

2.9 22RV1 mouse xenografts

Castrated nu/nu mice were subjected to a right flank subcutaneous inoculation of 2 x 10^5 cells 22RV1 cells suspended in PBS and mixed with Matrigel (1:1). Tumor-bearing mice were randomly assigned into 4 groups where mice from different groups received through oral gavage a vehicle control, 20 mg/kg/day of Enzalutamide, 110 mg/kg of PF-03084014 daily on alternate weeks, or the combination of both. Upon necropsy tumor weight and body weight were measured. Castrated NRG mice were subjected to a right flank subcutaneous inoculation of 7.5 x 10^5 cells 22RV1 cells suspended in PBS and mixed with Matrigel (1:1). Tumor-bearing mice were

randomly assigned into 4 groups: vehicle, decitabine, enzalutamide and combination. Mice received treatment on a 4-day ON, 2-days OFF regimen for 4 cycles. Mice received the vehicle and the enzalutamide 20mg/kg/day injections through oral gavage and the vehicle and the decitabine 0.5 mg/kg/day injections through intraperitoneal injections. Upon necropsy tumor size, tumor weight and body weight were measured.

2.10 Tissue Processing and staining

Samples were fixed in 10% neutral buffered formalin. They were then placed in a Sakura Tissue-Tek VIP6 tissue processor for dehydration through graded ethanols, clearing in xylene and infiltration with Leica Paraplast Plus paraffin. After processing, tissues were embedded in Leica Paraplast Plus paraffin. Tissue sections were taken at a thickness of 4 micrometers using a Themo HM355S microtome. Sections were mounted on charged slides and dried for 30-60min in a 60C oven. After drying, all slides were deparaffinized through 3 changes of xylene and rehydrated through graded ethanols to water in a Leica Autostainer XL. Using the Leica Autostainer XL, slides are stained in Gill's II hematoxylin, blued, and counterstained in an eosin/phloxine B mixture. Finally, slides are dehydrated, cleared in xylene and coverslipped in a toluene-based mounting media (Leica MM24).

After deparaffinization, antigen retrieval was done with a TRIS/EDTA pH9 solution in a BioCare decloaking chamber at a temperature of 95C for 20 minutes. Slides were cooled for 20 minutes at room temperature and transferred to TRIS buffer with Tween 20 detergent (TBST). The rest of the staining was carried out at room temperature using a BioCare Intellipath stainer.

2.10.1 Immunohistochemistry

Slides were incubated with 3% hydrogen peroxide in water for 5 minutes. Slides were rinsed with TBST and incubated in 2.5% normal goat serum for 20 minutes. Excess reagent was blown off and either Ki67 (Cell Marque, 275R-16) at a dilution of 1:100 (0.364ug/mL) or Cleaved Caspase 3 (Cell Signal Tech, 9661) at 1:200 (2.6ug/mL) for 30 minutes. The negative control slide was stained with Rabbit IgG (Vector Labs, I-1000) at a concentration of 1:5000 (1ug/mL) for 30 minutes. Slides were rinsed twice in TBST and a goat anti-rabbit secondary (Vector Labs, MP-

7451) was applied for 30 minutes. Slides were rinsed twice in TBST and Vector ImmPACT DAB (Vector Labs, SK-4105) was applied for 5 minutes. Slides were rinsed in water and transferred to a Leica Autostainer XL for hematoxylin counterstain, dehydration and coverslipping.

2.10.2 Immunofluorescence

After deparaffinization and antigen retrieval, slides were stained using specific Cleaved-Caspase3, KI-67, AR and HES1 antibodies. DAPI staining was also done on all slides to visualize cell nuclei. Staining was accomplished with the M.O.M.TM kit from VECTOR LABORATORIES.

2.11 DNMT activity assay

LNCaP, MR49F, C4-2 and C4-2R cells were seeded after being removed from enzalutamide maintenance medium for one passage. LNCaP and C4-2 cells were then treated with DMSO, 5 μM and 10 μM enzalutamide. After 5 days, cells were collected and washed once with PBS. Cell pellets were subjected to the NE-PER™ Nuclear and Cytoplasmic Extraction kit where cytoplasmic and nuclear fractions were separately isolated. 10 μg of nuclear extracts were used as input amount for the EpiQuik™ DNMT Activity/Inhibition ELISA Easy Kit (Colorimetric). Samples were processed according to the manufacturer's instructions and optical density was measured at a wavelength of 450 nm.

2.12 DNA methylation quantification assay

LNCaP, MR49F, C4-2 and C4-2R cells were seeded after being removed from enzalutamide maintenance medium for one passage. LNCaP and C4-2 cells were then treated with DMSO, 5 μM and 10 μM enzalutamide. After 5 days, cells were collected and washed once with PBS. Cell pellets were subjected to the DNeasy Blood & Tissue kit where genomic DNA was extracted. 200 ng of genomic DNA were used as input amount for the MethylFlash™ Methylated DNA Quantification Kit (Colorimetric). Samples were processed according to the manufacturer's instructions and optical density was measured at a wavelength of 450 nm.

2.13 RNA isolation, cDNA synthesis and qRT-PCR

LNCaP, MR49F, C4-2 and C4-2R cells were seeded after being removed from enzalutamide maintenance medium for one passage. After 5 days, cells were collected and washed once with PBS. Cell pellets were subjected to the RNeasy Mini kit where RNA was extracted. cDNA synthesis reaction was prepared by mixing 500-1000 ng total RNA, 250µM dNTPs (Amresco), 0.5 µM each of random hexamer and oligo(dT)15 primers (Promega), and 200 units M-MLV reverse transcriptase with included reaction buffer (NEB). cDNA was amplified using RT² SYBR Green qPCR Mastermix according to the manufacturer's protocol on the LightCycler® 480. Primers used for these experiments are listed below: DNMT3B (pan): Fwd 5'-GACTTGGTGATTGGCGGAA-3' Rev 5'-GGCCCTGTGAGCAGCAGA-3'; DNMT3B1: Fwd 5'-ATAAGTCGAAGGTGCGTCGT-3' Rev 5'-GGCAACATCTGAAGCCATTT-3'; DNMT3B2: Fwd 5'-TACCCGGGATGAACAGGCCCG-3' Rev 5'-TAGTGCACAGGAAAGCCAAAGATC-3'; DNMT3B3: Fwd 5'-GATGAACAGGATCTTTGGCTTT-3' Rev 5'-GCCTGGCTGGAACTATTCACA-3'; DNMT3B4: Fwd 5'-CCCGGGATGAACAGTTAAAGAA-3' Rev 5'-TGGACACGTCTGTGTAGTGC-3'; DNMT3B5: Fwd 5'-TAGGATAGCCAAGGATCTTTGG-3' Rev 5'-GGCCTGGCTGGAACTATTCA-3'; DNMT3B7: Fwd 5'-AAAGCCCAGCTTCCCTGA-3' Rev 5'-GCCCTTGATCTTTCCCCACA-3'; DNMT3B8: Fwd 5'-TACACACAGTCCCTGAGACG-3' Rev 5'-GCCCTTGATCTTTCCCCACA-3'; DNMT3A (pan): Fwd 5'-CAATGACCTCTCCATCGTCAAC-3' Rev 5'-ATGCCAACGGCCTGTTCATA-3'; DNMT1: Fwd 5' CACCATTGGCAATGAGCGGTTC-3' Rev 5'-TGAAAGCTGCATGTCCTCAC-3'. Actin: Fwd 5'GTGGGGGACTGTGTCTCTGT-3' Rev 5'- AGGTCTTTGCGGATGTCCACGT-3' was used as a reference for relative transcript expression. Data was processed on the LightCycler 96 software.

2.14 RNA interference

For siRNA knockdown, siRNAs for AR, DNMT1, DNMT3A and DNMT3B were purchased from Santa Cruz Biotechnology. Non-targeting siRNA was also purchased from Santa Cruz Biotechnology. Transfection using Opti-MEM™ I Reduced Serum Medium (Gibco) and lipofectamine Lipo3000 reagent (Thermo Fisher), for all siRNA transfections, was used according

to the manufacturer's instructions. Quantities were scaled according to the plate-size and siRNA amount.

2.15 Overexpression of DNMT3B isoforms

pcDNA3/Myc-DNMT3B3 was a gift from Arthur Riggs (Addgene plasmid # 37546 ; http://n2t.net/addgene:37546 ; RRID:Addgene_37546) and pcDNA3/Myc-DNMT3B1 was a gift from Arthur Riggs (Addgene plasmid # 35522 ; http://n2t.net/addgene:35522 ; RRID:Addgene_35522) and were both acquired from Addgene. DNMT3B7 was a gift from Lucy Godley at the University of Chicago department of medicine. Empty vector pcDNA3 was used as a control. Cells were seeded and transfected the next day with the purified plasmids using the transfection reagent Lipo3000 (Thermo Fisher) according to the manufacturer's instructions. Cells were then subjected to selection with Geneticin ordered from Thermo Fisher. Experiment were conducted after at least 14 days of selection with Geneticin.

2.16 RNA-sequencing experiment comparing enzalutamide-resistant to enzalutamide sensitive cells and cells pre- and post-treatment with decitabine or decitabine plus enzalutamide

RNA extraction, library preparations, and sequencing reactions were conducted at GENEWIZ, LLC. (South Plainfield, NJ, USA) as follows:

Total RNA was extracted from cell pellet samples using Qiagen RNeasy Plus Universal mini kit following manufacturer's instructions (Qiagen, Hilden, Germany).

Extracted RNA samples were quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked using Agilent TapeStation 4200 (Agilent Technologies, Palo Alto, CA, USA).

RNA sequencing libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina following manufacturer's instructions (NEB, Ipswich, MA, USA). Briefly, mRNAs were first enriched with Oligo(dT) beads. Enriched mRNAs were fragmented for 15 minutes at 94 °C. First strand and second strand cDNAs were subsequently synthesized. cDNA fragments were end repaired and adenylated at 3'ends, and universal adapters were ligated to cDNA fragments, followed by index addition and library enrichment by limited-cycle PCR. The sequencing libraries

were validated on the Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA), and quantified by using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) as well as by quantitative PCR (KAPA Biosystems, Wilmington, MA, USA).

The sequencing libraries were pooled and clustered on 1 lane of a flowcell. After clustering, the flowcell was loaded on the Illumina HiSeq instrument according to manufacturer's instructions. The samples were sequenced using a 2x150bp Paired End (PE) configuration. Image analysis and base calling were conducted by the HiSeq Control Software (HCS). Raw sequence data (.bcl files) generated from Illumina HiSeq was converted into fastq files and de-multiplexed using Illumina's bcl2fastq 2.17 software. One mismatch was allowed for index sequence identification.

2.16.1 Quality control and read-mapping:

Raw Illumina reads were obtained from Purdue Genomics Core in FASTQ format. Sequence data quality was determined using FastQC (331) software (version 0.11.7). Quality trimming and filtering were performed using Fastp (332) (version 0.19.5) to remove the basecalls with a Phred33 score less than 30 and any reads shorter than 50 basepairs. Quality trimmed reads were mapped against the indexed Homo_sapiens.GRCh38.99 reference genome using STAR(333) aligner (version 2.5.4b). STAR derived mapping results and annotation (GTF) file were fed into the HTSeq (326) package (version 0.7.0) to obtain the read counts for each gene feature in each replicate. Counts from all replicates were merged together using a custom Perl script to generate a gene counts matrix for all samples.

2.16.2 Differential Expression (DE) analysis:

For DE analysis in each comparison, genes with 0 counts across all replicates (control and treatment) were discarded from the counts matrix. When genes have 0 counts in one sample but not in others, the counts were converted from 0 to 1 to avoid having infinite values being calculated for fold change. The final combined counts matrix was utilized for further differential gene expression analysis using 'R-Bioconductor' DESeq2 (327) (version 1.22.2) and edgeR (328) (version 3.24.3) packages. The quality of the counts matrix was verified by determining basic statistics such as data range and matrix size prior to statistical tests.

Both DESeq2 and edgeR methods use negative binomial distribution based statistical models and perform specific estimate variance-mean tests. Both methods determine DE genes with P-value and adjusted P-values of false discovery rate (FDR) to correct for multiple tests. In DESeq2, DE is determined by application of Empirical Bayes shrinkage for dispersion estimation and a Wald test was used for significance testing. In edgeR, an edgeR object was created using the counts matrix, library sizes (number of reads sequenced in each group) and experimental design. Normalization factors were calculated for the counts matrix, followed by estimation of common dispersion of counts and DE using an exact test.

2.16.3 Pathway analysis

Pathway analysis was performed using the ClusterProfiler (334) and QIAGEN Ingenuity Pathway Analysis (IPA) toolkit. From each comparison, the genes that are DE by both edgeR and DESeq2 methods at FDR $\leq$ 0.05 significance and lof2FC cutoff $\pm$2 was used as an input for pathway analysis. A list of enriched KEGG pathways and GO categories was obtained from the ClusterProfiler results. A list of proprietary canonical pathways, upstream regulators and diseases/functions associated with DE genes was obtained from the IPA.

2.16.4 Gene set enrichment analysis

Gene set enrichment analysis was performed using the GSEA (335) method. A list of all the genes expressed in current data were derived from the DESeq2 (336) results. A customized expression metric was calculated using the log2FoldChange and p-value for each gene as determined by the DESeq2 (336) package and the formulae signed fold-change * -log10pvalue. Ranking of the genes was performed in descending order by the calculated expression metric. This pre-ranked gene-list was used as an input to GSEA and enrichment was performed against the Hallmark, Curated and Oncogenic gene set collections with default parameters.

2.17 Band quantification on ImageJ

After data collection and analysis using Image Lab, blots were copied into ImageJ. Band intensity data of investigated proteins and its loading control were measured. Immunohistochemistry

staining was quantified on ImageJ according to the protocol created by Crowe *et al.* (337). Data was normalized and calculated on Excel.

2.18 Statistical analysis

Standard 2-tailed Student t tests and One-Way ANOVA were performed to analyze statistical significance of the results. Two-way ANOVA tests were performed to analyze statistical significance of datasets with grouped observations. A p-value of less than 0.05 indicates statistical significance. All graphs and statistical analyses were completed on Prism Graphpad.

CHAPTER 3. NOTCH SIGNALING IS ACTIVATED IN AND CONTRIBUTES TO RESISTANCE IN ENZALUTAMIDE-RESISTANT PROSTATE CANCER CELLS

3.1 Introduction

Prostate cancer is the most diagnosed cancer in men in the United States (338). Despite the high survival rate of prostate cancer patients, most develop resistance to therapies leading to a more aggressive form of the disease and ultimately death. Enzalutamide, initially approved as a second-line treatment of metastatic prostate cancer patients, has shown significant beneficial results when used as first-line therapy for patients with metastatic prostate cancer or even early stage prostate cancer in combination with hormonal therapy (339, 340). Shortly after a brief response period to enzalutamide patients will develop resistance leading to death (341). With enzalutamide in ongoing clinical trial on early stage prostate cancer patients, it is of high importance to understand the mechanism by which prostate cancer patients develop resistance to enzalutamide and identify novel approaches to overcome resistance. By understanding and identifying these mechanisms, targeted combination therapies can be used to treat patients who develop drug resistance.

In general, ADT resistance and specifically enzalutamide resistance mechanisms mainly highlight the role of the AR in resistance development, including AR amplification, activating mutations, and AR variants (322, 323). In this study, we hypothesized that genes and pathways outside of the AR signaling pathway contribute to enzalutamide-resistance in prostate cancer cells. To test our hypothesis, we used RNA-sequencing to compare previously developed enzalutamide-resistant cell lines to their parental cell lines to identify potential non-AR pathways that contribute to resistance. Our results show that the Notch signaling pathway is linked to enzalutamide-resistance. Abrogation of this pathway *in vitro* and *in vivo* restores sensitivity of prostate cancer cells to enzalutamide.

3.2 Results

3.2.1 Long-term enzalutamide treatment of prostate cancer cell lines induces global gene expression changes after acquiring resistance

Kuruma *et al.* and Liu *et al.* developed enzalutamide-resistant cell lines MR49F and C4-2R derived from the parental cell lines LNCaP and C4-2 respectively (322, 323). These cell lines were developed by extensive treatment with high concentrations of enzalutamide in a tissue culture setting or in an *in vivo* xenograft mouse model to serve as a model to study enzalutamide-resistance (322, 323). To compare enzalutamide-sensitive to enzalutamide-resistant cell lines, RNA was isolated from LNCaP, C4-2, MR49F and C4-2R after a 4-hour treatment with enzalutamide. Around 30000 features were detected in all cell lines after RNA-sequencing, excluding genes with zero counts. Sample-to-sample matrix heatmap reveals that the biological replicates are consistent within each sample group (Fig.3.1 A). In addition, principle component analysis (PCA) shows that our replicates cluster together within each group (Fig.3.1 B). Figures 3.1 A and 3.1 B show that LNCaP is the closest to MR49F. On the other hand, C4-2 has the most similarity with C4-2R. This validates our choice for pairing LNCaP with MR49F and C4-2 with C4-2R. For differentially expressed genes (DEG) analysis we used 3 different algorithms, DESeq2, EdgeR and Cufflinks. Using EdgeR with a 1% false discovery rate and 2-fold change cutoff we were able to identify a significant number of genes that are differentially expressed in LNCaP vs MR49F and C4-2 vs C4-2R (Fig.3.1 C). Overall, the intersection of the 3 algorithms showed 901 genes that have been differentially expressed in MR49F compared to LNCaP and 1266 genes differentially expressed in C4-2R compared to C4-2 (Fig.3.1 D). Finally, Table 3.1 shows the number of genes that are up- or down-regulated in LNCaP vs MR49F and C4-2 vs C4-2R discovered by the independent algorithms. Our data indicates that long-term enzalutamide treatment leads to alteration of global gene expression.

3.2.2 Notch signaling pathway is enriched in patient samples that mimic enzalutamide-resistant cell lines

Our bioinformatics data from comparing enzalutamide-resistant to enzalutamide-sensitive cell lines revealed that the normalized counts of *AR* were significantly and consistently increased in MR49F and C4-2R compared to LNCaP and C4-2 respectively (Fig.3.2 A). This indicates that cell lines with acquired resistance exhibit a higher expression of *AR* compared to cells that are still

sensitive the drug. Due to the lack of clinical samples from patients treated with enzalutamide, we analyzed a cohort of 72 patients with hormone-refractory prostate cancer (Fig.3.2 B). Of the 72 patients, 26 had a high *AR* gene expression mimicking cell lines that are resistant to enzalutamide and 46 had a low *AR* gene expression mimicking cell lines that are sensitive. After separating the samples into *AR* low vs. *AR* high, the dataset was subjected to gene set enrichment analysis (GSEA). Genes from the "pre-Notch transcription and translation signaling" and "pre-Notch expression and processing signaling" pathways are significantly enriched in the *AR* high group (Fig.3.2 C and D). These pathways are subgroups of the Notch signaling pathway from Reactome. Furthermore, we used Pearson correlation analysis to identify genes that correlate with *AR* expression in the dataset. *NOTCH2* expression positively correlated with *AR* expression, with a correlation coefficient of 0.4331 (Fig.3.2 E and G). In addition, one of the downstream targets of Notch signaling *c-MYC* positively correlated with *AR* expression, with a correlation coefficient of 0.4536 (Fig.3.2 F and H). These data indicate that Notch signaling pathway may play a role in resistance to enzalutamide in prostate cancer.

3.2.3 Expression of Notch signaling pathway genes is deregulated in enzalutamide-resistant cells

To further explore the implication of Notch signaling in resistance, we compared the expression of genes from this pathway in enzalutamide-resistant vs. enzalutamide-sensitive cell lines. As shown in Figures 3.3 A and 3.3 B, more than 30 genes from the Notch signaling pathway are either up- or down-regulated in MR49F and C4-2R compared to LNCaP and C4-2 respectively (Table 3.2).

To investigate the functional features of the Notch signaling pathway in enzalutamide-resistant cell lines we examined the protein expression of important genes from this pathway. Notch family of proteins is composed of 4 transmembrane receptors (NOTCH 1, 2, 3, and 4) that are cleaved upon ligand-binding on the cell surface rendering their activation and leading to downstream events (130, 342, 343). Our results show that cleaved-NOTCH1 has a higher expression in MR49F and C4-2R compared to their sensitive counterparts. However, the cleaved forms of NOTCH2 and NOTCH4 were consistently expressed among all cell lines. It is impossible to predict the status of

cleaved NOTCH3 expression in these cells due to the lack of a specific antibody to test it. This data suggests that NOTCH1 signaling is over-activated in the resistant cells.

Upon NOTCH-ligand binding, NOTCH proteins exhibit a conformational change leading to S2 cleavage in the NOTCH extracellular domain by disintegrin and metalloprotease (ADAM) proteins (344). NOTCH S2 cleavage leads to a S3 cleavage by the gamma-secretase complex releasing the NOTCH intracellular domain (NICD) into the cytoplasm (136). To explore the implication of the ADAM family of proteins in the activation of Notch signaling, especially NOTCH1, in enzalutamide-resistant cells we tested the protein expression of ADAM9, ADAM10 and ADAM17 (TACE). ADAM9 is similarly expressed in LNCaP vs. MR49F and downregulated in C4-2R compared to C4-2 (Fig.3.3 D). TACE is upregulated in both resistant cell lines compared to their sensitive counterparts. The active form of ADAM10 (lower band) is highly upregulated in MR49F and C4-2R compared to LNCaP and C4-2 respectively. These data indicate that upstream proteases, responsible for the activation of the Notch signaling pathway are predominantly upregulated in resistant cells, offering a potential explanation to the increased expression of cleaved-NOTCH1.

To explore a potential mechanism involved in the exclusive activation of NOTCH1, the highly activated ADAM10 was inhibited using GI254023X in MR49F and 22RV1 (another enzalutamide-resistant cell line) cells. Upon treatment with GI254023X, cleaved-NOTCH1 expression is significantly reduced in 22RV1 and MR49F (data not shown). However, the expression of cleaved NOTCH2 appears to be unaffected by GI254023X treatment. This offers a potential explanation to the exclusive overexpression of cleaved-NOTCH1 compared to the expression of cleaved-NOTCH2 and -NOTCH4 in resistant cells.

After its internalization, the NICD translocates to the nucleus and activates the expression of Notch signaling downstream targets such as hairy and enhancer of split (HES) genes, c-MYC, and others (345). To confirm that Notch signaling is further activated in enzalutamide-resistant cells compared to enzalutamide-sensitive cells we tested the protein expression of HES1 and c-MYC. Both proteins are upregulated in MR49F and C4-2R compared to LNCaP and C4-2 respectively (Fig.3.3 D). This indicates that Notch signaling is activated in enzalutamide-resistant cells.

3.2.4 Knockdown of *NOTCH1* in C4-2R cells increases sensitivity to enzalutamide.

To test whether Notch1 signaling is directly involved in resistance, we designed and generated shRNA vectors targeting *NOTCH1* and *NOTCH2* in C4-2R cells. Figure 3.4 A shows successful knockdown of *NOTCH1* in C4-2R cells compared to cells infected with shCtrl virus. Exposure of C4-2R-sh*NOTCH1* cells to enzalutamide induces an increase in apoptosis represented by cleaved-PARP expression compared to C4-2R-shCtrl cells (Fig.3.4 B). Furthermore, exposure of C4-2R-sh*NOTCH1* cells to increasing concentrations of enzalutamide induces a decrease in cell proliferation (Fig.3.4 C). In contrast, enzalutamide didn't induce the same effects on C4-2R-sh*NOTCH2* cells when compared to shCtrl (Fig.3.5 A, B and C). This suggests that knockdown of *NOTCH1* is sufficient to reduce cell proliferation and increase apoptosis indicating a re-sensitization of C4-2R to enzalutamide.

3.2.5 Inhibition of Notch signaling pathway re-sensitizes enzalutamide-resistant cells to enzalutamide and increases apoptosis and decreases colony formation ability

To test whether inhibiting Notch signaling in prostate cancer enzalutamide-resistant cells re-sensitizes these cells to enzalutamide we treated MR49F, C4-2R and 22RV1 cells with enzalutamide, PF03084014 or both. Treatment with PF-03084014 induced a decrease in HES1 and c-MYC expression, indicating an inhibition of Notch signaling in all cell lines (Fig.3.6 A, B and C). Exposure to both PF-03084014 and enzalutamide induced an increase in cleaved-PARP expression levels indicating an increased apoptosis after 72 hours (Fig.3.7 A, C and E). In addition, in the same groups we observed a decrease in AR and PSA levels in MR49F and C4-2R compared to enzalutamide exposure alone (Fig.3.7 A and C). In 22RV1, the combination of enzalutamide and PF-03084014 caused a reduction in AR and AR-V7 protein levels (Fig.3.7 E). Furthermore, Figures 3.7 B, D and F show a decrease in colony numbers and colony size in cells treated with both PF-03084014 and enzalutamide compared to cells treated with DMSO, enzalutamide or PF-03084014 alone. These results suggest that inhibition of Notch signaling re-sensitizes resistant cells to enzalutamide by blocking the AR signaling and reducing AR expression.

3.2.6 PF-03084014 and enzalutamide treatment induces a decrease in 22RV1 tumor xenografts

To further validate our *in vitro* data, we generated an enzalutamide-resistant mouse xenograft model using 22RV1 cells. Nude mice were castrated and inoculated subcutaneously with 22RV1 cells. Upon tumor formation mice were treated for 4 weeks with a vehicle, enzalutamide, PF-03084014 or the combination of enzalutamide and PF-03084014. Mice in the combination group showed a significant decrease in tumor weight compared to vehicle or single treatment groups (Figs.3.8 A and C). There were no significant signs of toxicity in mice. Mice from the combination group showed a significant decrease in body weight only when compared to mice treated with PF-03084014 alone (Fig.3.8 B).

Immunofluorescent staining of tumors showed effective inhibition of the Notch signaling pathway by PF-03084014 represented by a significant decrease in the percentage of HES1 expressing cells in the PF-03084014 and combination treatment groups (Fig.3.6 D). In addition, a significant increase in cleaved-Caspase 3 expressing cells in the combination treatment group compared to the other groups indicated an increase in apoptosis (Fig.3.8 D and E). KI-67 staining was significantly decreased in tumors from the combination treatment group compared to the other groups, suggesting a reduced proliferation (Fig.3.8 D and F). Combination of enzalutamide and PF-03084014 induced a significant decrease in AR expressing cells within the tumor (Fig.3.8 D and G). These results suggest that blocking Notch signaling *in vivo* re-sensitizes 22RV1 cells to enzalutamide by increasing apoptosis, reducing cell proliferation and AR expression.

3.3 Discussion

The already FDA-approved enzalutamide is currently under investigation in clinical trials with early stage prostate cancer patients. If the outcome of these trials turns out to be a success, enzalutamide will become widely used for treatment of prostate cancer patients at all stages of the disease. However, one of the major obstacles is development of enzalutamide resistance in patients. Most of therapy-resistance studies in prostate cancer focus on the role of the AR signaling pathway in overcoming the resistance to ADT and enzalutamide (346). In this study, our aim was to identify pathways that are independent of the AR signaling pathway and may play a role enzalutamide resistance. We compared enzalutamide-resistant cells MR49F and C4-2R to their enzalutamide-

sensitive counterparts LNCaP and C4-2, respectively. Our analysis revealed hundreds of genes that are up- or down-regulated in the enzalutamide-resistant cells (Fig.3.1). The combination of our RNA-seq with patient sample bioinformatics analysis helped identify Notch signaling pathway as a potential deregulated pathway in enzalutamide-resistant cells.

In prostate cancer, studies have shown contradicting roles of NOTCH signaling where it either plays a tumor suppressive or a tumor promoting role (347, 348). Our results indicate that the Notch signaling pathway plays a tumor promoting role in prostate cancer by contributing to enzalutamide-resistance and promoting cell survival. Furthermore, NOTCH proteins have been shown to contribute to therapy resistance in prostate cancer (349). In addition, studies have shown synergistic positive results in prostate cancer cells when Notch signaling inhibitors were combined with androgen deprivation therapies (ADTs) (350, 351). Our data supports the observations above where we show that Notch signaling is activated in enzalutamide-resistant cells and inhibition of this pathway restores sensitivity to enzalutamide.

In addition, Notch signaling has been shown to be a driver of stemness and contributor to survival and maintenance of cancer stem cells, which are believed to a driver of cancer therapy resistance (348, 352). There is evidence that ADT and enzalutamide treatment promote the acquisition of stem cell-like features in prostate cancer cell (353, 354). Deregulation of Notch signaling in enzalutamide-resistant cells may and play a role in maintenance of stem-like features leading to therapy resistance.

To investigate whether Notch signaling is active in resistant cells, expression of cleaved-Notch isoforms was tested. Our data suggests that Notch1 signaling is active due to a high expression of cleaved-NOTCH1 in the resistant cells (Fig.3.3 B). Furthermore, downstream targets such as HES1 and c-MYC were overexpressed in resistant cells indicating an active pathway (Fig.3.3 C). However, due to the lack of a specific cleaved-NOTCH3 antibody we were unable to verify the expression of this protein. Thus, Notch3 may also be playing a role in the activation of downstream targets.

To further understand how Notch signaling is activated in enzalutamide-resistant cells we investigated the expression of upstream proteases involved in Notch receptor activation and downstream targets of the pathway. Our results show that ADAM10 is activated and TACE is upregulated in the resistant cells (Fig.3.3 C). ADAM10 and TACE overexpression contributes to therapy resistance and their inhibition leads to re-sensitizing tumor cells to existing therapies in different cancers (355–357). Here, we believe that the overexpression of the metalloproteases might be involved in the activation of the Notch1 signaling pathway. According to our data, inhibition of ADAM10 with GI254023X reduced the expression of cleaved-NOTCH1 exclusively (Fig.3.3 D), which suggests a higher sensitivity of Notch1 cleavage to changes in ADAM10 expression or activation. Other explanations may be attributed to selectivity in ligands towards Notch1 over other isoforms (358), and the elevated number of reported mutations in the heterodimerization domain of Notch1 that can affect its ligand independent cleavage compared to the other isoforms (359).

Notch1 signaling is shown to become activated and to play a role in cell death and drug-sensitivity after cells acquire resistance in other cancers (360, 361). To investigate whether Notch1 signaling plays a significant role in enzalutamide resistance we generated sh*NOTCH1* C4-2R cells. These cells have a better response to enzalutamide treatment compared to shCtrl cells (Fig.3.4). In contrast, knockdown of *NOTCH2* in C4-2R didn't yield the same results.

Inhibitors and blockers of Notch signaling are being investigated in clinical trials in multiple cancers. To investigate whether blockade of Notch signaling pathway reverses enzalutamide-resistance in MR49F, C4-2R and 22RV1, we used the gamma secretase inhibitor PF-03084014. Combination of enzalutamide and PF-03084014 showed increase in cleaved-PARP levels in all cell lines (Fig.3.8 A, C and E). One of the established mechanisms of resistance to ADT is increased AR and AR-variants expression in prostate cancer cells (322, 323). Our data indicate that exposure to enzalutamide and PF-03084014 decreased full-length AR, PSA and AR-V7 expression, potentially explaining the restoration of enzalutamide function in the resistant cells (Fig.3.8 A, C and E). Additionally, increased levels of c-MYC has been shown to promote ligand-independent prostate cancer survival (362). Our data show that combination of enzalutamide and

the gamma secretase inhibitor decreases c-MYC expression in the resistant cells offering another mechanism by which resistance is reversed in those cells (Fig.3.7 A, B and C).

Furthermore, the inhibition of AR and Notch signaling together induced a significant decrease in colony formation ability of MR49F, C4-2R and 22RV1 (Fig.3.8 B, D and F). In the xenograft model, the combination therapy showed growth inhibition in tumors compared to vehicle and single treatment groups, with no signs of significant toxicity. This observation was accompanied by an increase in cleaved-Caspase-3 expression, a decrease in KI-67 expression and a decrease in AR expressing cells which replicated our *in vitro* cell line data (Fig.3.8).

Finally, our study adds up to existing data on the oncogenic role of Notch signaling in prostate cancer. Notch1 signaling is activated in enzalutamide-resistant cells, and inhibition of this pathway may restore enzalutamide function *in vitro* and *in vivo*, leading to a better outcome. These data offer a precedent for the combination of Notch signaling inhibitors with enzalutamide and potentially other ADTs.

Figure 3.1. Long-term enzalutamide treatment of prostate cancer cell lines induces global gene expression changes after acquiring resistance.

A, Heatmap showing the Euclidian distances between samples, made with the DESeq2 transformed data after a regularized log transformation was performed. B, PCA plot of libraries (salmon colored points represent C4-2 samples, green represent C4-2R samples, teal represent LNCaP samples, and purple represent MR49F samples.). Overall 54% of the variance is captured by the first principle component (shown on the x-axis) and principle component two (shown on the y-axis) accounts for 28% of the variation. Data was normalized in DESeq2 and a regularized log transformation performed prior to doing the PCA. C, Smear plots from edgeR showing, in red, transcripts differentially expressed (FDR < 0.01) between, respectively, enzalutamide-resistant/enzalutamide-sensitive cells. Blue lines indicate 2-fold-change cutoff. The x-axis shows the average log(count per million) and the y-axis shows the log2(fold change). D, Venn diagrams of overlap between differential expression results amongst the three statistical packages Cufflinks, edgeR and DESeq2 for both comparisons.

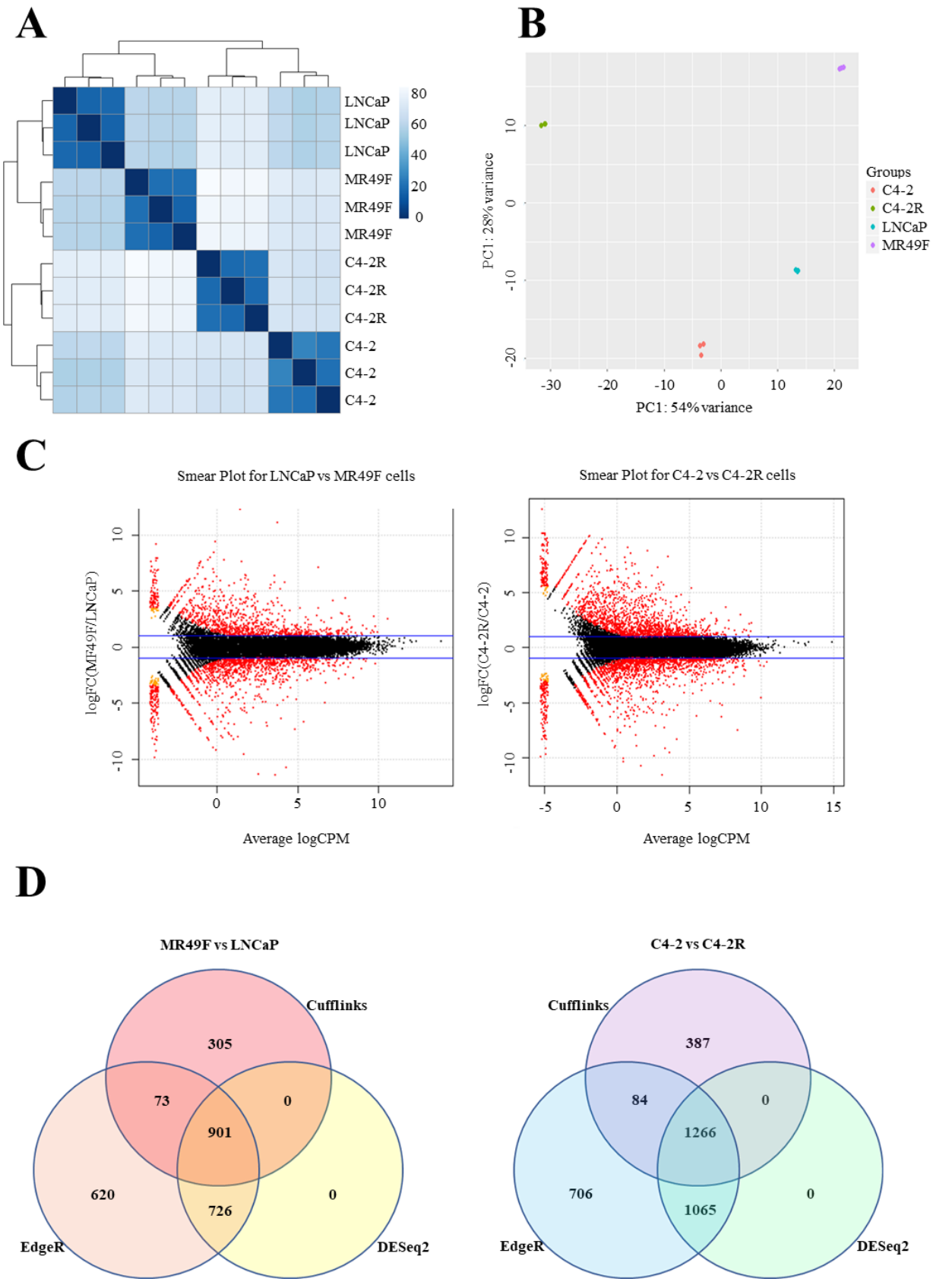

A
LNCaP
LNCaP
LNCaP
MR49F
MR49F
MR49F
C4-2R
C4-2R
C4-2R
C4-2
C4-2
C4-2
80
60
40
20
0
B
PC1: 28% variance
PC1: 54% variance
Groups
C4-2
C4-2R
LNCaP
MR49F
C
Smear Plot for LNCaP vs MR49F cells
logFC(MR49F/LNCaP)
Average logCPM
Smear Plot for C4-2 vs C4-2R cells
logFC(C4-2R/C4-2)
Average logCPM
D
MR49F vs LNCaP
Cufflinks
EdgeR
DESeq2
305
73
0
901
620
726
0
C4-2 vs C4-2R
Cufflinks
EdgeR
DESeq2
387
84
0
1266
706
1065
0

Figure 3.2. Notch signaling pathway is enriched in patient samples that mimic enzalutamide-resistant cell lines.

A, Boxplot of normalized *AR* counts for all samples using DESeq2. B, Heatmap representing a cohort of patients from the TCGA database, separated into samples with high *AR* expression (26 samples) and samples with low AR expression (46 samples). C, GSEA enrichment plot of the Reactome pre-Notch transcription and translation gene set in the cohort of patient with *AR* low vs. *AR* high. The plot indicates a negative enrichment, meaning that the queried gene set correlates with *AR* high samples. D, GSEA enrichment plot of the Reactome pre-Notch expression and processing gene set in the cohort of patient with *AR* low vs. *AR* high. The plot indicates a negative enrichment, meaning that the queried gene set correlates with *AR* high samples. E, *NOTCH2* gene expression analysis in the cohort of patients and its correlation with *AR* gene expression indicates a strong positive correlation with a correlation coefficient of r=0.4331. F, *c-MYC* gene expression analysis in the cohort of patients and its correlation with *AR* gene expression indicates a strong positive correlation with a correlation coefficient of r=0.4536. G and H, *NOTCH2* and *c-MYC counts* matched to the samples number from the cohort of patients.

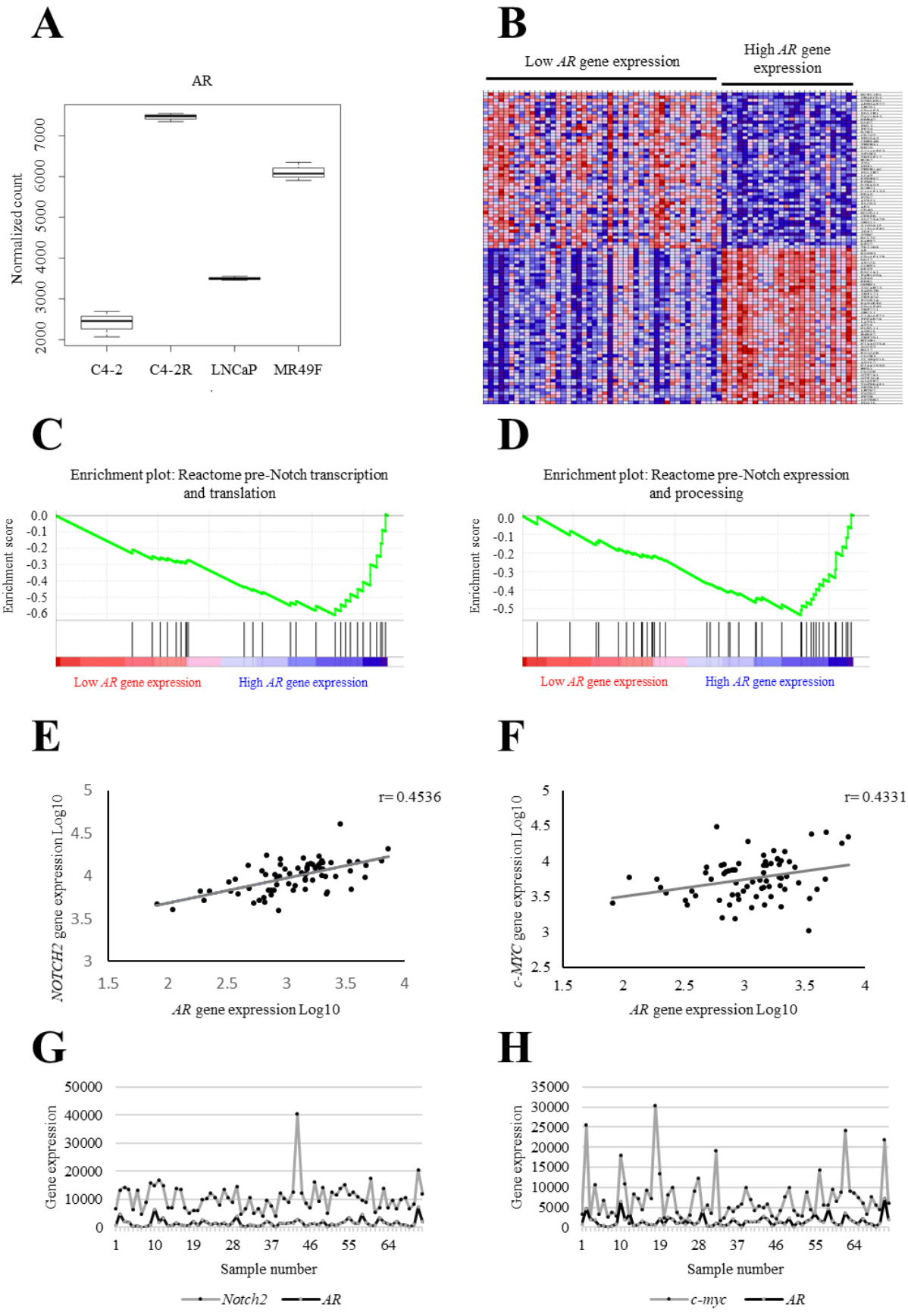
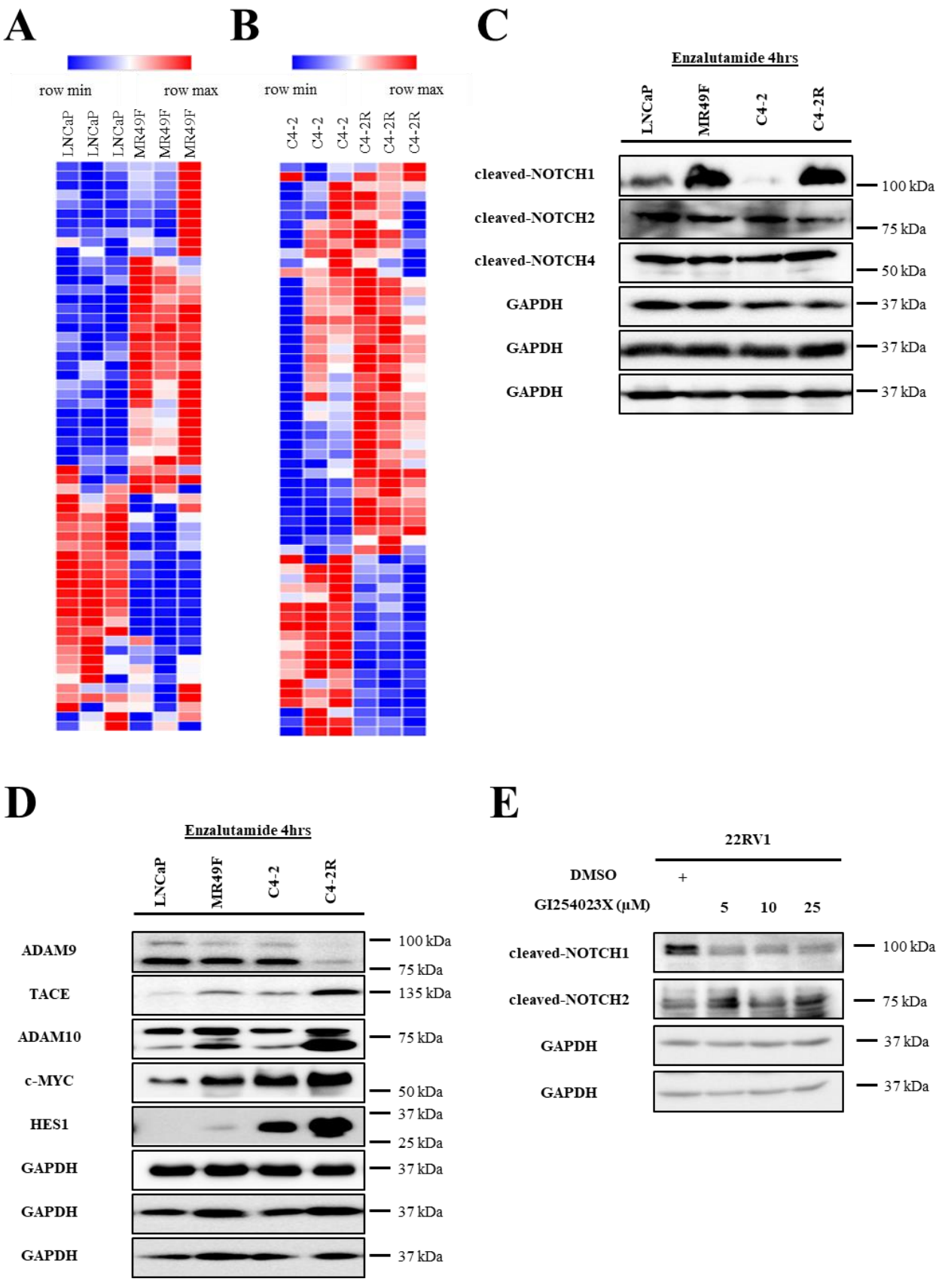

A
AR
Normalized count
2000 3000 4000 5000 6000 7000
C4-2
C4-2R
LNCaP
MR49F
B
Low AR gene expression
High AR gene expression
C
Enrichment plot: Reactome pre-Notch transcription and translation
Enrichment score
0.0 -0.1 -0.2 -0.3 -0.4 -0.5 -0.6
Low AR gene expression
High AR gene expression
D
Enrichment plot: Reactome pre-Notch expression and processing
Enrichment score
0.0 -0.1 -0.2 -0.3 -0.4 -0.5
Low AR gene expression
High AR gene expression
E
r= 0.4536
NOTCH2 gene expression Log10
5 4.5 4 3.5 3
1.5 2 2.5 3 3.5 4
AR gene expression Log10
F
r= 0.4331
c-MYC gene expression Log10
5 4.5 4 3.5 3 2.5
1.5 2 2.5 3 3.5 4
AR gene expression Log10
G
Gene expression
50000 40000 30000 20000 10000 0
1 10 19 28 37 46 55 64
Sample number
Notch2
AR
H
Gene expression
35000 30000 25000 20000 15000 10000 5000 0
1 10 19 28 37 46 55 64
Sample number
c-myc
AR

Figure 3.3. Expression of Notch signaling pathway genes is deregulated in enzalutamide-resistant cells.

A, Heatmap representing gene expression patterns of Notch signaling pathway genes in LNCaP vs MR49F cell lines. B, Heatmap representing gene expression patterns of Notch signaling pathway genes in C4-2 vs C4-2R cell lines. C and D, LNCaP, MR49F, C4-2 and C4-2R cells were treated with enzalutamide for 4 hours and subjected to western blot analysis. E, 22RV1 cells were treated with GI254023X for 48 hours and subjected to western blot analysis.


A
row min
row max
LNCaP
LNCaP
LNCaP
MR49F
MR49F
MR49F
B
row min
row max
C4-2
C4-2
C4-2
C4-2R
C4-2R
C4-2R
C
Enzalutamide 4hrs
LNCaP
MR49F
C4-2
C4-2R
cleaved-NOTCH1
cleaved-NOTCH2
cleaved-NOTCH4
GAPDH
GAPDH
GAPDH
100 kDa
75 kDa
50 kDa
37 kDa
37 kDa
37 kDa
D
Enzalutamide 4hrs
LNCaP
MR49F
C4-2
C4-2R
ADAM9
TACE
ADAM10
c-MYC
HES1
GAPDH
GAPDH
GAPDH
100 kDa
75 kDa
135 kDa
75 kDa
50 kDa
37 kDa
25 kDa
37 kDa
37 kDa
37 kDa
E
22RV1
DMSO
+
GI254023X (μM)
5
10
25
cleaved-NOTCH1
cleaved-NOTCH2
GAPDH
GAPDH
100 kDa
75 kDa
37 kDa
37 kDa

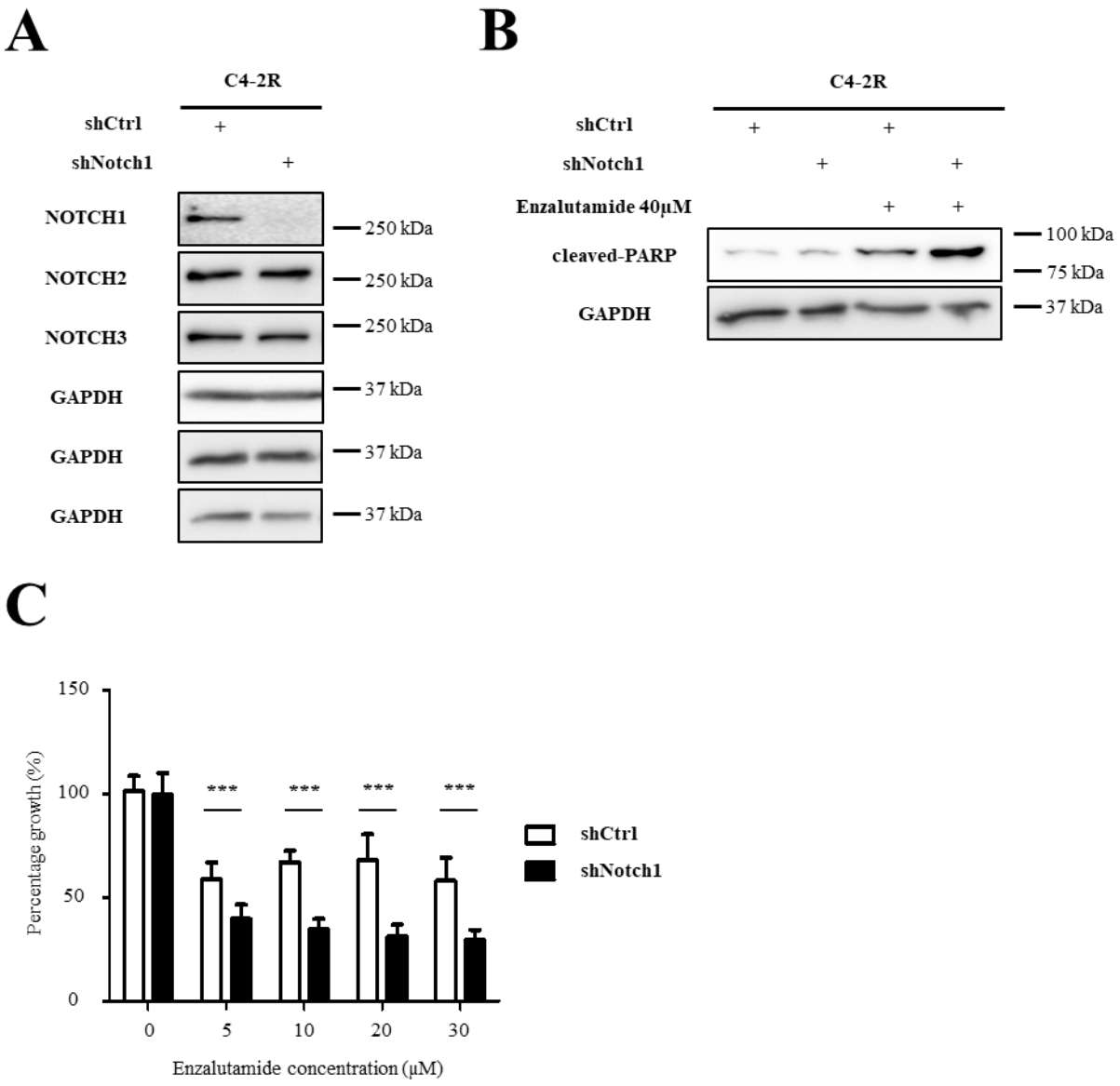


Figure 3.4. Knockdown of *NOTCH1* induces an increase in cell death and a decrease in cell proliferation.

A, C4-2R cells were infected with shCtrl or sh*NOCH1* virus particles and selected with puromycin. Cells were collected and subjected to western blot analysis with indicated antibodies. B, Stable C4-2R shCtrl and sh*NOTCH1* cells were treated with DMSO or 40 μM enzalutamide for 72 hours and subjected to western blot analysis. C, Stable C4-2R shCtrl and sh*NOTCH1* cells were treated with DMSO or increasing concentrations of enzalutamide and subjected to MTT assay. Optical densities were measured and normalized to DMSO-treated samples. *$p<0.05$ and **$p<0.01$.

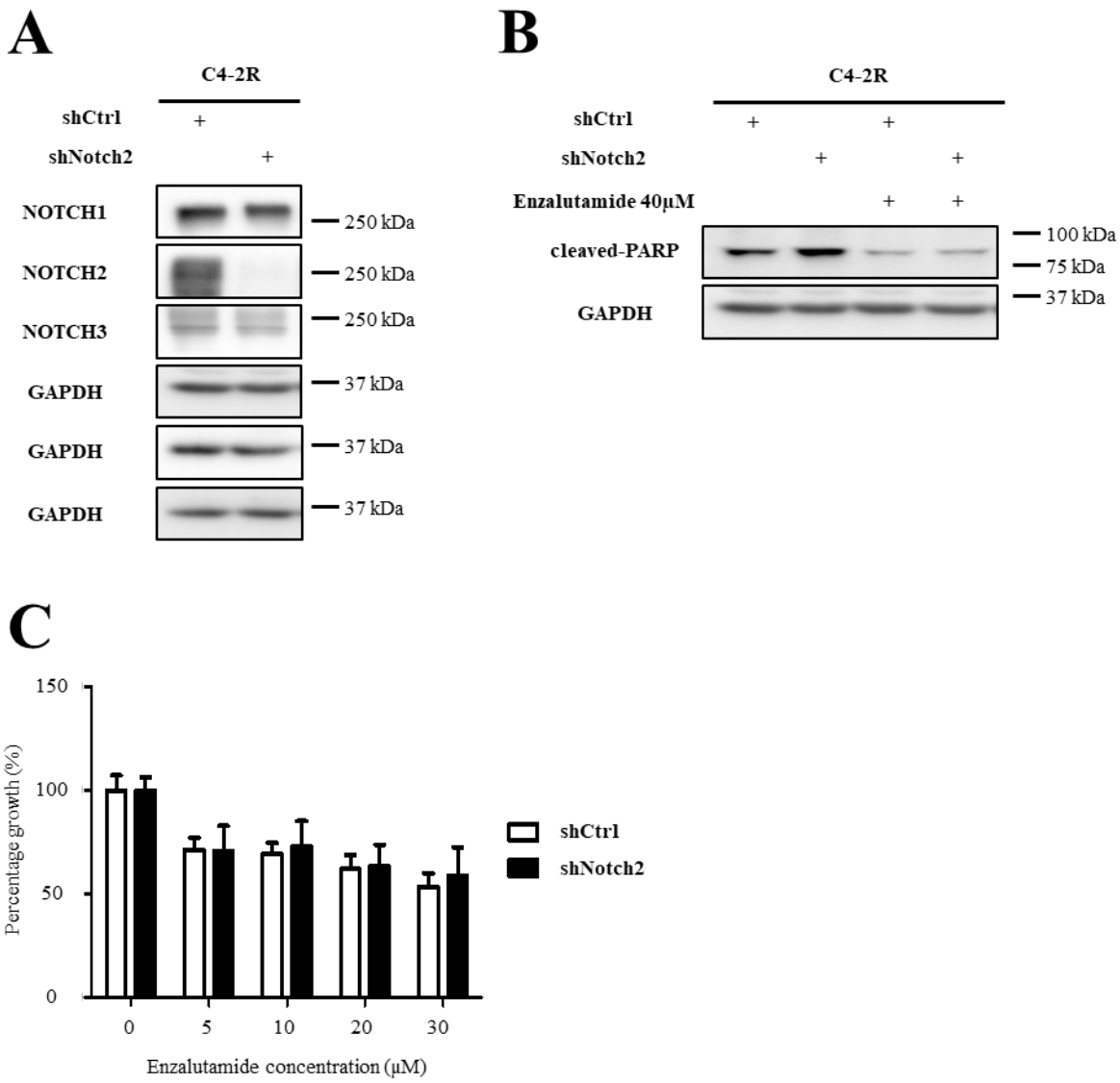


Figure 3.5. Knockdown of *NOTCH2* induces no changes in cleaved-PARP expression and in cell proliferation.

A, C4-2R cells were infected with shCtrl or shNotch2 virus particles and selected with puromycin. Cells were collected and subjected to western blot analysis with indicated antibodies. B, Stable C4-2R shCtrl and sh*NOTCH2* cells were treated with DMSO or 40 μM enzalutamide for 72 hours and subjected to western blot analysis with indicated antibodies. C, Stable C4-2R shCtrl and sh*NOTCH2* cells were treated with DMSO or increasing concentrations of enzalutamide and subjected to MTT assay. Optical densities were measured and normalized to DMSO-treated samples.

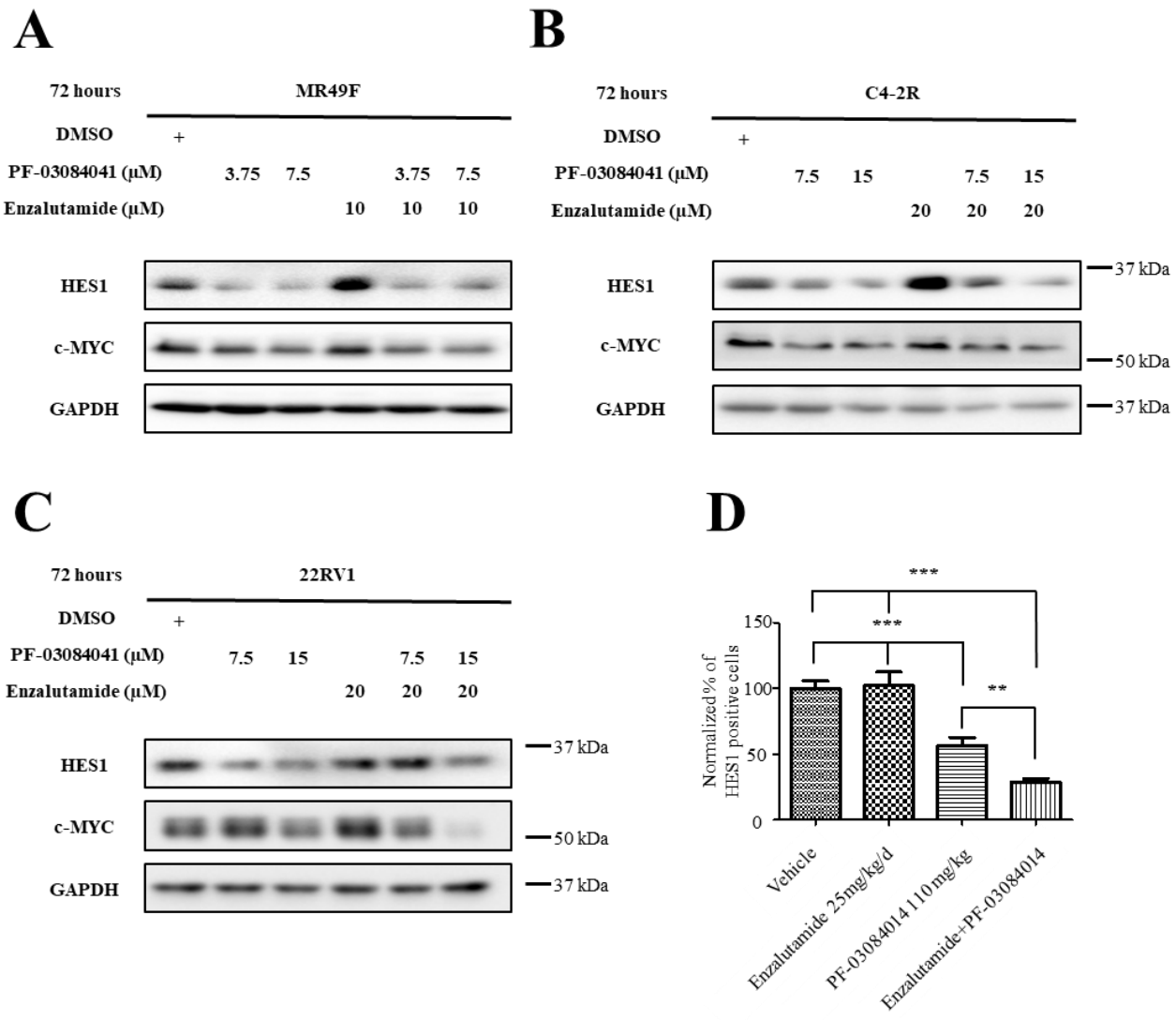


Figure 3.6. PF-03084014 reduces the expression of Notch signaling downstream targets in enzalutamide-resistant cell lines *in vitro* and *in vivo*.

A, B and C, MR49F, C4-2R and 22RV1 cells were treated with DMSO, PF-03084014, enzalutamide or both at the indicated concentrations for 72 hours. Cells were collected and subjected to western blot. D, Quantification of HES1 positively stained cells (n=3). All groups were normalized to the vehicle control group. *p<0.05, **p<0.01 and ***p<0.001.

Figure 3.7. Inhibition of Notch signaling pathway re-sensitizes enzalutamide-resistant cells to enzalutamide and causes cell death and decreased colony formation ability.

A, C and E, MR49F, C4-2R and 22RV1 cells were treated with DMSO, PF-03084014, enzalutamide or both at the indicated concentrations for 72 hours. Cells were collected and subjected to western blot analysis. B, D, and F, Low seeding density MR49F, C4-2R and 22RV1 cells were treated with DMSO, PF-03084014, enzalutamide or both at the indicated concentrations until colonies were formed. Cells were fixed with 4% Formaldehyde and stained with crystal violet.

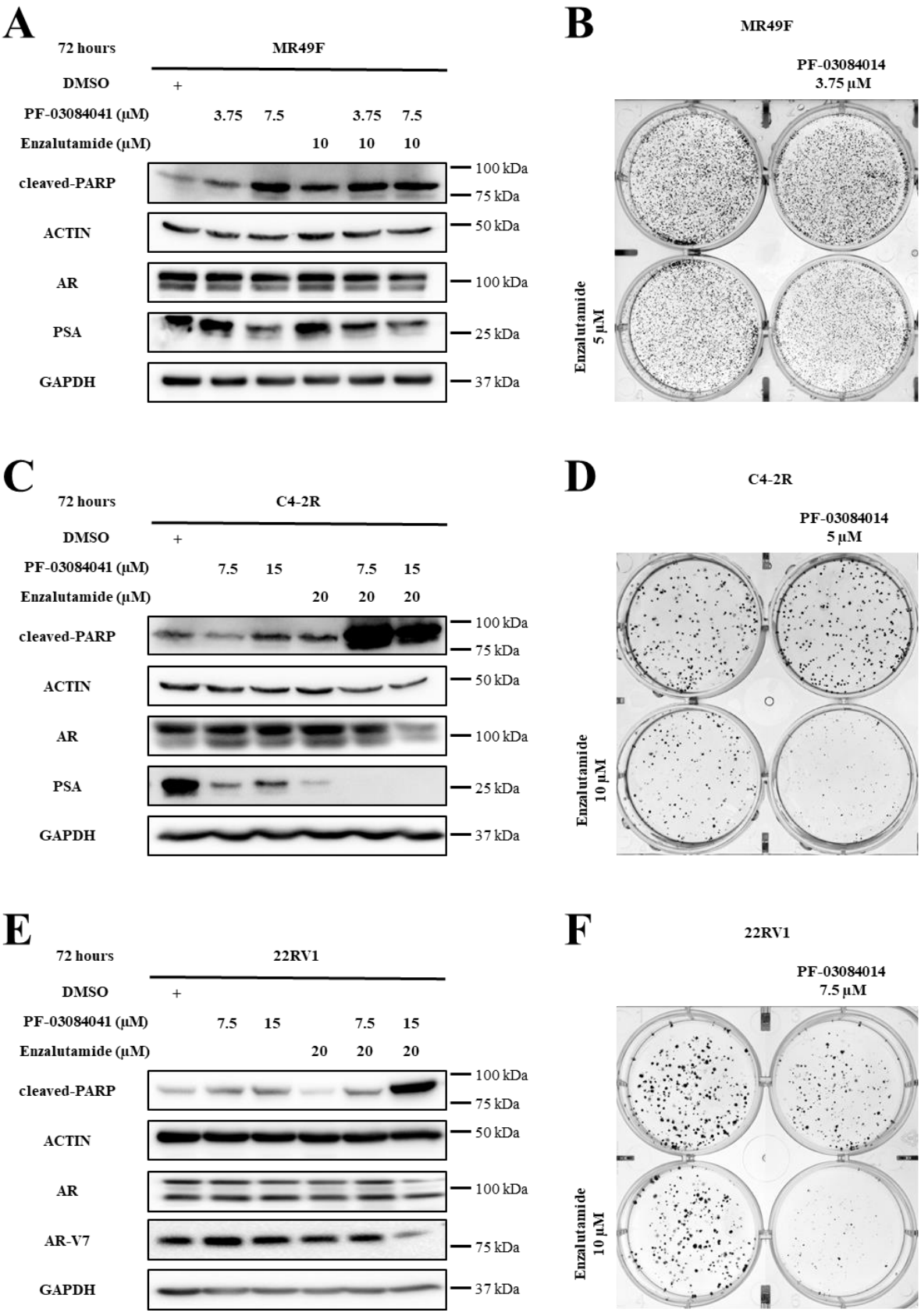

A
72 hours
MR49F
DMSO
+
PF-03084041 (μM)
3.75
7.5
3.75
7.5
Enzalutamide (μM)
10
10
10
cleaved-PARP
ACTIN
AR
PSA
GAPDH
100 kDa
75 kDa
50 kDa
100 kDa
25 kDa
37 kDa
B
MR49F
PF-03084014
3.75 μM
Enzalutamide
5 μM
C
72 hours
C4-2R
DMSO
+
PF-03084041 (μM)
7.5
15
7.5
15
Enzalutamide (μM)
20
20
20
cleaved-PARP
ACTIN
AR
PSA
GAPDH
100 kDa
75 kDa
50 kDa
100 kDa
25 kDa
37 kDa
D
C4-2R
PF-03084014
5 μM
Enzalutamide
10 μM
E
72 hours
22RV1
DMSO
+
PF-03084041 (μM)
7.5
15
7.5
15
Enzalutamide (μM)
20
20
20
cleaved-PARP
ACTIN
AR
AR-V7
GAPDH
100 kDa
75 kDa
50 kDa
100 kDa
75 kDa
37 kDa
F
22RV1
PF-03084014
7.5 μM
Enzalutamide
10 μM

Figure 3.8. PF-03084014 combined with enzalutamide treatment induces a decrease in enzalutamide-resistant tumor xenograft.

A and B, 22RV1 xenograft mice were treated with a vehicle control, enzalutamide, PF-03084014 or a combination of enzalutamide and PF-03084014. Upon completion, tumor weight and body weight were measured. (n=4 mice) C, Representative image of tumors from different treatment groups. D, Immunofluorescent images from tumor sections stained with cleaved-Caspase3, KI-67, AR and DAPI. E, F and G, Quantification of cleaved-Caspase3, KI-67 and AR positively stained cells (n=3). All groups were normalized to the vehicle control group. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

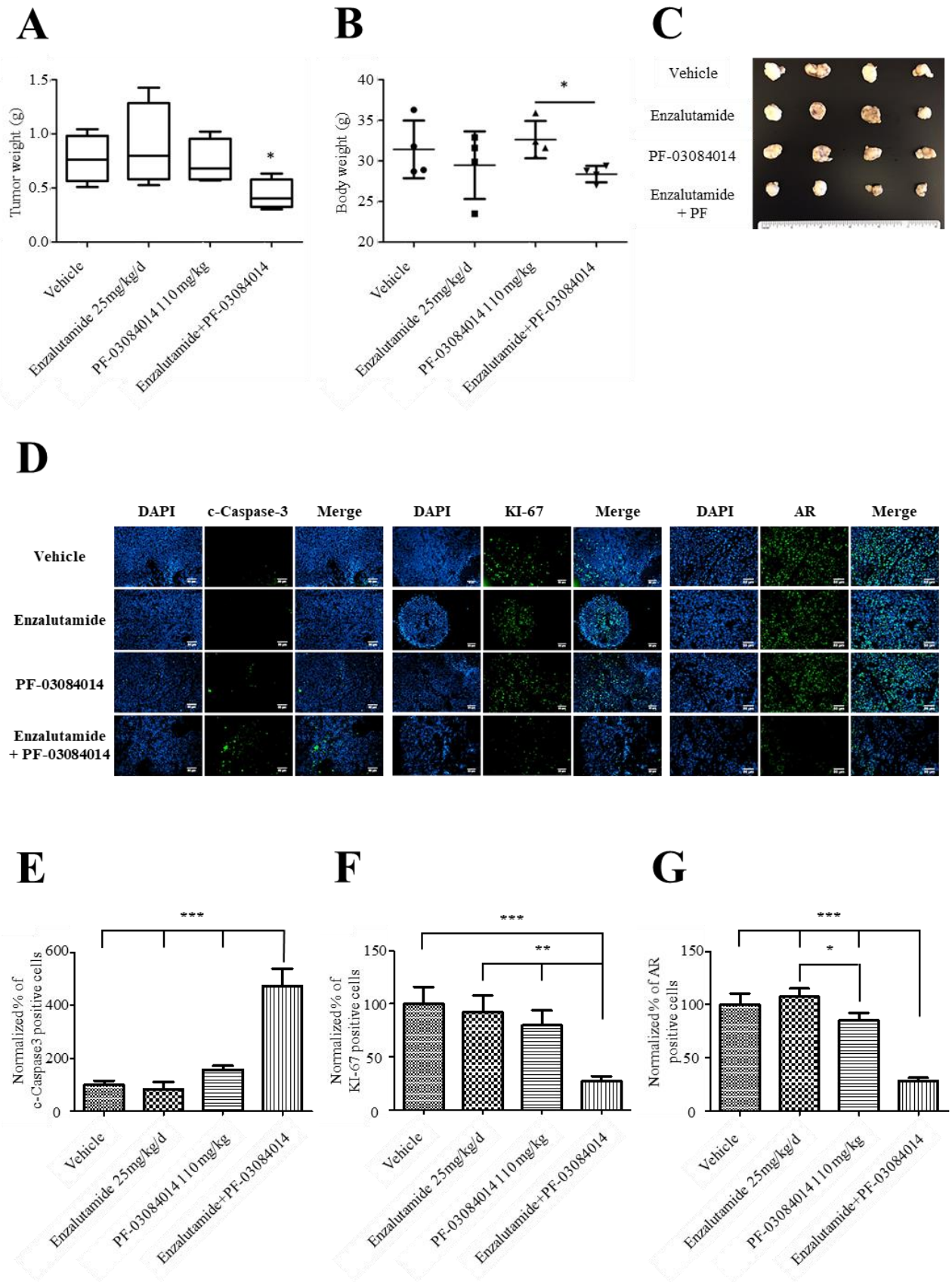

A
Tumor weight (g)
1.5
1.0
0.5
0.0
*
Vehicle
Enzalutamide 25mg/kg/d
PF-03084014 110mg/kg
Enzalutamide+PF-03084014
B
Body weight (g)
40
35
30
25
20
*
Vehicle
Enzalutamide 25mg/kg/d
PF-03084014 110mg/kg
Enzalutamide+PF-03084014
C
Vehicle
Enzalutamide
PF-03084014
Enzalutamide + PF
D
DAPI
c-Caspase-3
Merge
DAPI
KI-67
Merge
DAPI
AR
Merge
Vehicle
Enzalutamide
PF-03084014
Enzalutamide + PF-03084014
E
Normalized% of c-Caspase3 positive cells
600
400
200
0
***
Vehicle
Enzalutamide 25mg/kg/d
PF-03084014 110mg/kg
Enzalutamide+PF-03084014
F
Normalized% of KI-67 positive cells
150
100
50
0
***
**
Vehicle
Enzalutamide 25mg/kg/d
PF-03084014 110mg/kg
Enzalutamide+PF-03084014
G
Normalized% of AR positive cells
150
100
50
0
***
*
Vehicle
Enzalutamide 25mg/kg/d
PF-03084014 110mg/kg
Enzalutamide+PF-03084014

Table 3.1. Total, up-regulated and down-regulated transcripts differentially expressed in enzalutamide-resistant compared to sensitive cells.

Table 1

MR49F vs LNCaP, 1% FDR, 2FC cutoff

	Total	Up in Resistant Cells	Down in Resistant Cells
DESeq2	1,627	738	889
edgeR	2,320	1,026	1,294
Cufflinks	1,279	719	560

Table 2

C4-2R vs C4-2, 1% FDR, 2FC cutoff

	Total	Up in Resistant Cells	Down in Resistant Cells
DESeq2	2,331	1,206	1,125
edgeR	3,121	1,554	1,567
Cufflinks	1,737	871	866

Table 3.2 List of differentially expressed genes in LNCaP vs. MR49F and C4-2 vs. C4-2R. The list of genes corresponds to the heatmaps represented in Figures 3A and 3B. Genes are listed in an order matching the representative heatmap.

LNCaP vs. MR49F	C4-2 vs. C4-2R
SEL1L	UBB
FOXA1	RPS19
RPS27A	MYC
RBPJ	GALNT11
SNW1	APH1A
ADAM10	MIB2
IFT74	NCSTN
MIB1	APH1B
WDR12	MAML3
MAML1	PSEN1
RPS19	EPN1
PSEN2	PSEN2
NCSTN	SEL1L
UBB	CREBBP
SUSD5	POFUT1
MYC	ADAM17
APH1A	RBPJ
DTX4	ITCH
IFT172	NKAP
ADAM17	EP300
GMDS	MAML1
DTX3	IFT74
NEURL1B	SNW1
UBC	KAT2B
GALNT11	RBM15
APH1B	NOTCH2
CDKN1B	SPEN
NKAP	APP
ITGB1BP1	NOTCH3
KAT2B	IFT172
KRT19	ITGB1BP1
S1PR3	JAG2
GOT1	WDR12
MIB2	JAG1
NLE1	HES1
POFUT1	SUSD5
JAG2	DTX3
NOTCH2	KRT19
PSEN1	DTX4
PERP	HNF1B
NOTCH1	RPS27A
SPEN	MDK
CREBBP	FOXA1
EP300	ADAM10
APP	GOT1
MDK	NOTCH1
NOTCH3	UBC
SOX9	GMDS
MAML3	CEBPA
JAG1	PERP
UBA52	CDKN1B
ANXA4	S1PR3
EPN1	SOX9
DTX2	NEURL1B
CEBPA	UBA52
ITCH	PSENEN
PSENEN	DTX2
HNF1B	NLE1
RBM15	MIB1
HES1	ANXA4

CHAPTER 4. TARGETING THE ENZALUTAMIDE-MEDIATED CHANGES IN DNMTS AND DNA METHYLATION RESTORES THE RESPONSE TO ENZALUTAMIDE IN RESISTANT PROSTATE CANCER CELLS

4.1 Introduction

Prostate cancer is the top diagnosed cancer and the second leading death-causing cancer in American men (26). Patients with prostate cancer are well managed in the early stages of the disease. Prostate cancer is driven by the androgen receptor signaling pathway, that is activated upon the binding of the ligand, dihydrotestosterone, to the receptor resulting in regulation of downstream gene expression (363). Approaches targeting the androgen signaling pathway have been used in the clinic to treat prostate cancer patients (364). However, after a short-lived response to androgen deprivation therapies, patients become resistant to these approaches, leading to a more aggressive form of the disease ending in death. Enzalutamide, an AR antagonist, is FDA-approved for the treatment of prostate cancer patients across different stages of the disease (76). Although enzalutamide showed benefit in treating post-chemotherapy CRPC patients, almost all will stop responding to the antiandrogen, after several months of treatment, limiting their options (75, 99). It is important to study and identify the underlying mechanisms by which prostate cancer cells develop resistance to enzalutamide. These efforts will allow researchers to discover biomarkers of resistance predisposition, to develop advanced therapeutic molecules and to suggest combination approaches with enzalutamide to overcome resistance or prolong the response to this drug.

DNA methylation is an epigenetic mark, deposited on Cytosine molecules by a family of DNA methyltransferases. In promoter regions, methyl-Cytosines promote gene silencing by their interplay with transcription factors and components of the chromatin remodeling machinery (365–367). DNA methyltransferases are heavily spliced, resulting in a complicated network of proteins implicated in this process (229, 231, 294, 368, 369). In the prostate, DNA methylation changes are detected in prostate cancer samples compared to normal samples (261, 370, 371). Many tumor suppressor genes, such as *APC*, *RASSF1A*, *GSTP1*, and *INK4A*, have been found to be hypermethylated and silenced playing a role in prostate cancer initiation and progression (372–374). Clearly, DNA methylation plays a role in prostate cancer, however, its role in enzalutamide

resistance is unexplored and poorly understood. In this study, we aim to investigate the role of DNA methylation in enzalutamide resistance by exploring the transcriptome and methylome of enzalutamide-resistant prostate cancer cells. We also aim to explore the effect of enzalutamide on DNA methyltransferases and the role of these enzymes and their splice variants in the cellular response to enzalutamide.

4.2 Results

4.2.1 Enzalutamide treatment induces an increase in DNMT activity and DNA methylation in prostate cancer cell lines that becomes persistent after resistance onset

In our previous study, we compared gene expression in enzalutamide-responsive cells to enzalutamide-resistant cells using RNA sequencing (375). Differential gene expression revealed a high number of genes that are up or downregulated in the resistant cells compared to their sensitive counterparts. In comparing MR49F to LNCaP, we observed a higher number of downregulated genes associated with the resistant cell line. The data suggested that a repressive mechanism may be playing a role in promoting transcriptional silencing in several genes upon the onset of resistance. One of the best studied epigenetic gene silencing mechanisms is DNA methylation mediated by the family of DNA methyltransferases. To investigate whether DNA methylation is playing a role in enzalutamide resistance in prostate cancer cells, we first tested DNMT activity in a panel of prostate cancer enzalutamide-sensitive and -resistant cells. Nuclear extracts from LNCaP, MR49F, C4-2 and C4-2R were subjected to the EpiQuik DNMT Activity/Inhibition ELISA Easy Kit. Results show a significant and consistent increase in DNMT activity in MR49F (4.67 OD/h/mg) and C4-2R (9.35 OD/h/mg) compared to LNCaP (1.995 OD/h/mg) and C4-2 (2.51 OD/h/mg) cells, respectively (Fig.2.1 A). To investigate whether enzalutamide is directly involved in the increase of DNMT activity in prostate cancer cells, LNCaP and C4-2 cells were treated with DMSO, 5 µM or 10 µM of enzalutamide for 5 days. Enzalutamide treatment promoted a significant increase in DNMT activity in LNCaP cells at 5 µM (7.83 OD/h/mg) and 10 µM (9.56 OD/h/mg) and an increase in C4-2 cells at 10 µM (8.27 OD/h/mg) compared to DMSO-treated cells (LNCaP: 1.995 OD/h/mg; C4-2: 2.51 OD/h/mg) (Fig.2.1 B and C). These results suggest that enzalutamide treatment induces an increase in DNMT activity in prostate cancer cells. DNA methyltransferases catalyze the deposit of 5-mC marks on DNA molecules, thus changes in DNMT activity directly correlate with changes in DNA methylation. To investigate whether this is true in our cell lines,

we tested global DNA methylation by measuring the overall concentration of 5-mC in those cells. Cells were collected after a 5-day incubation and global 5-mC were measured using the MethylFlash Methylated DNA 5-mC Quantification Kit. Genomic DNA was extracted from LNCaP, MR49F, C4-2 and C4-2R cells and subjected to the kit. Our data shows that MR49F (2.18%) has a higher global DNA methylation compared to its parental line LNCaP (1.23%) (Fig.2.1 D). We also observed a very limited increase in global 5-mC concentration in C4-2 versus C4-2R, however, this increase is not significant (1.86% vs. 2.13%) (Fig.2.1 D). To test whether enzalutamide treatment can affect global DNA methylation, LNCaP and C4-2 were treated with DMSO, 5 µM or 10 µM of enzalutamide for 5 days followed by measurement of 5-mC concentrations. Our results indicate an increase in 5-mC concentration in LNCaP following treatment with enzalutamide 5 µM and 10 µM compared to DMSO. However, only treatment of LNCaP cells with 5 µM enzalutamide for 5 days produces a significant increase in DNA methylation compared to its control treatment (1.23% vs. 2.35%) (Fig.2.1 E). No significant changes were seen in global DNA methylation in C4-2 post-enzalutamide treatment at both concentrations (Fig.2.1 F). These results suggest that the direct correlation between DNMT activity changes and global DNA methylation are seen in the androgen-dependent cell line LNCaP. In the androgen independent cell lines C4-2, the increase in DNMT activity doesn't correlate with an increase in global DNA methylation which may implicate discrepancies in the regulation of DNMTs and DNA methylation between both cell lines. Although no significant effects of enzalutamide treatment on global methylation were seen in C4-2, this doesn't mean that the antiandrogen-induced DNMT activity increase has no impact on DNA methylation. The changes induced by the increase in DNMT activity in C4-2 cells following treatment with enzalutamide may occur at specific gene loci instead of at a global scale.

4.2.2 DNMT3B is the predominantly overexpressed DNA methyltransferase in enzalutamide-resistant cell lines

Changes in the expression of DNA methyltransferases directly correlate with changes in cellular DNMT activity and DNA methylation (376). DNMT activity directly correlates with the expression of DNMT1, DNMT3B or the combination of both in primary and metastatic TRAMP mice tumors (377). To understand the underlying mechanisms behind the increase in DNMT activity in enzalutamide resistant cell lines we assessed the expression of DNMTs in enzalutamide-

sensitive and –resistant cells. We used qRT-PCR and western blot to study the transcript and protein levels of these enzymes in all cell lines. LNCaP, MR49F, C4-2, C4-2R and 22RV1 cells were cultured for 5 days followed by an assessment of DNMT expression. Transcript levels of *DNMT1* and *DNMT3B* were significantly increased in MR49F compared to LNCaP cells (Fig.2.1 G and I). The increase in *DNMT3A* and *DNMT3B* expression is mild but not significant when comparing C4-2R to C4-2 cells (Fig.2.1 H and I). On the other hand, at the protein level, DNMT1 expression was not significantly changed between the different cell lines (Fig.2.1 J). However, DNMT3B was consistently and significantly upregulated in the resistant cells (Fig.2.1 J). DNMT3B expression in MR49F and C4-2R was 2-fold higher compared to LNCaP and C4-2 respectively. The DNMT3B variant, DNMT3B7, was also significantly upregulated in the resistant cells compared to their sensitive counterparts. The DNMT3A variant, DNMT3A1, was significantly overexpressed exclusively in MR49F compared to LNCaP cells (Fig.2.1 J). No significant changes were observed in the expression of DNMT3A1 in C4-2 versus C4-2R or in the expression of DNMT3A2 in both pairs. This data suggests that shows a correlation between enzalutamide resistant and the overexpression of DNMT3B. This may indicate that DNMT3B is playing a role in the increased DNMT activity observed in the resistant cells. The results also implicate that DNMT3A adds another layer of complexity in the comparison between LNCaP and MR49F and the impact the expression of DNMT3A and DNMT3B might have on DNMT activity and DNA methylation.

4.2.3 The expression of DNMT3A and DNMT3B is induced following enzalutamide treatment and AR knockdown in prostate cancer cells

The effect of androgen receptor signaling and enzalutamide on the expression of DNA methyltransferases is poorly studied. It has been shown that DNMT expression negatively correlates with AR status in different cell lines. Also, bicalutamide has been shown to promote increases in the expression of DNMT3A and DNMT3B. To better understand the mechanism behind the increase in DNMT activity following enzalutamide treatment, we assessed changes in the expression of DNMTs after AR knockdown and treatment with enzalutamide. LNCaP, MR49F, C4-2 and C4-2R cells were first transfected with siRNAs targeting a scrambled sequence as a control (siCtrl) or targeting the AR (siAR). Then, transfected cells were treated with DMSO or 5 µM enzalutamide for 3 days before being collected for western blot analysis. Our results show that

treating cells with enzalutamide alone induces an increase in the expression of DNMT3A and DNMT3B without having any effect on DNMT1 protein expression (Fig.2.2 A and B). Additionally, knocking down AR also induces an increase in the levels of DNMT3A and DNMT3B in the same cell lines (Fig.2.2 A and B). These results suggest that targeting AR signaling in prostate cancer cells induces the expression of DNMTs, specifically DNMT3A and DNMT3B. This increase in DNMT3A and DNMT3B expression following enzalutamide treatment correlates with the increase in DNMT activity observed in LNCaP and C4-2.

DNMT3B exists in more than 30 isoforms in mammalian cells (229, 231, 294). To investigate which of the DNMT3B variants are induced in prostate cancer cells after enzalutamide treatment, we used qRT-PCR to measure the transcript levels following enzalutamide treatment. 5 μM enzalutamide treatment in LNCaP and C4-2 cells induced an increase in the expression of *DNMT3A*, *DNMT3B1*, *DNMT3B3* and *DNMT3B7*. All the data from figures 2.1 and 2.2 suggest that enzalutamide treatment of prostate cancer cells results in an overexpression of different isoforms of de novo methyltransferases and the overexpression of these DNA methyltransferases correlates with enzalutamide resistance in prostate cancer.

Tumor suppressor proteins pRB and p53 are directly involved in the regulation of the expression of DNA methyltransferases in mammalian cells (378). pRB and p53 bind to and inhibit the activity of SP1/SP3 and E2F1, respectively (378). The expression of SP1/SP3 and E2F1 directly correlates with the expression of DNMTs (378). It has been shown that these transcription factors are directly involved in the transcriptional activation of DNA methyltransferases (378). To understand the mechanisms by which DNMT3A and DNMT3B expression is induced in enzalutamide-resistant cells and following enzalutamide treatment, we investigated the expression of E2F1, SP3 and MDM2, an E3 ubiquitin ligase involved in the degradation of p53 and pRB (378). Our data shows that MDM2 protein levels are elevated in C4-2R compared to C4-2 cells (Fig.2.3 A). Its expression in MR49F is lower than that in LNCaP cells (Fig.2.3 A). E2F1 expression is consistently increased in MR49F and C4-2R cells compared to LNCaP and C4-2, respectively (Fig.2.3 A). To test whether this pathway is directly involved in the enzalutamide-mediated overexpression of DNMT3B, we treated C4-2R cells with enzalutamide and looked for any changes in the expression of MDM2. Enzalutamide-treatment induced an increase in the expression of MDM2 accompanied

by an increase in the expression of DNMT3B (Fig.2.3 B). We then tested whether the increase in DNMT3B expression is mediated by the p53 pathway and regulated by MDM2. MR49F cells were incubated with DMSO, 5 µM enzalutamide followed by treatment with the MDM2-p53 interaction inhibitor Nutlin-3a. Our data shows that the increase in DNMT3B expression following enzalutamide treatment was abolished after the inhibition of MDM2 in MR49F (Fig.2.3 C). These results suggest that the enzalutamide-mediated increase in the expression of DNMT3B and the overall upregulation of DNMT3A and DNMT3B in enzalutamide-resistant cells are mediated through the deregulation in the expression of proteins from the p53 and pRb pathways.

4.2.4 Ectopic expression of DNMT3B3 promotes an enzalutamide-resistant phenotype in prostate cancer cells

The roles of DNMT3B variants are poorly understood in the context of prostate cancer. To understand the functional involvement of the different DNMT3B variants in the response of prostate cancer cells to enzalutamide treatment, we first compared the expression of DNMT1, 3, 4, 5, 7 and 8 in enzalutamide-resistant cells versus enzalutamide sensitive cells. The comparison of LNCaP to MR49F and C4-2 to C4-2R revealed similar observations regarding the expression of the different DNMT3B isoforms investigated. DNMT3B2 expression was reduced in both MR49F and C4-2R compared to LNCaP and C4-2, respectively (Fig.2.4 A and B). The decrease in DNMT3B2 expression in MR49F compared to LNCaP cells was significant (Fig.2.4 A). On the other hand, DNMT3B3 and DNMT3B7 both show a consistent and significant increase in their expression in the resistant lines compared to their sensitive counterparts (Fig.2.4 A and B). To test whether the overexpression of DNMT3B3 and DNMT3B7 is directly involved in the response to enzalutamide treatment, C4-2 cells were transfected with pcDNA, Myc-DNMT3B1, Myc-DNMT3B3, FL-DNMT3B7 and Myc-DNMT3B3 plus FL-DNMT3B7. Cells were then selected for more than 14 days to allow enough time for DNA methylation changes to occur in the genome (Fig.2.4 C). Transfected C4-2 cells were then exposed to increasing concentrations of enzalutamide and cell viability was assessed after 6 days of treatment. MTT assay results show that C4-2 cells overexpressing DNMT3B3 or DNMT3B3 plus DNMT3B7 exhibited a significant decrease in sensitivity to enzalutamide compared to cells transfected with the empty vector (Fig.2.4 D). The difference in cell viability was observed starting from a concentration of 1 µM enzalutamide. These data suggest that the overexpression of DNMT3B3 may play a direct role in

the response to enzalutamide and promoting an enzalutamide-resistant phenotype in prostate cancer cells.

4.2.5 Decitabine treatment reestablishes the response to enzalutamide in enzalutamide-resistant cells

Decitabine, a DNA methylation inhibitor, induces global hypomethylation by promoting the degradation of DNA methyltransferases in mammalian cells. Decitabine is an FDA approved compound for the treatment of patients with Myelodysplastic diseases. Decitabine has been historically investigated at high doses, where it has been shown to induce DNA damage leading to its cytotoxic effect in mammalian cells (379, 380). To eliminate some of the cytotoxic effects of decitabine and for the purpose of our study, we used decitabine at sub-micromolar concentrations. We first asked whether DNA methylation inhibition can restore the sensitivity to enzalutamide in the resistant cells. To test this question, we assessed the apoptotic response of a pre-treatment with decitabine followed by exposure to enzalutamide. LNCaP, MR49F, C4-2 and C4-2R were pre-incubated with 0, 250 or 1000 nM decitabine for 5 days followed by a treatment with DMSO, 10, 20 or 40 µM of enzalutamide for 3 days. Cells were then collected and examined for the protein expression of DNMTs and apoptotic markers by western blot. Pre-treatment with 250 and 1000 nM decitabine enhances the apoptotic response to enzalutamide starting at a concentration of 10 µM in C4-2R cells (Fig.2.5 A). This is demonstrated by a significant increase in Cleaved-PARP and Cleaved-Caspase 3 levels in the double-treatment groups compared to the single-treatment groups (Fig.2.5 A). In MR49F cells, a similar response was observed in the group with the highest concentration of decitabine. In contrast, LNCaP and C4-2 cells didn't exhibit increases in the levels of the cleaved apoptotic proteins assessed. Only the combination of 1000 nM decitabine and 40 µM enzalutamide in LNCaP cells showed an increase in Cleaved-PARP expression (Fig.2.5 A). Decitabine pre-treatment induced a significant decrease in the expression of DNMT1, DNMT3A and DNMT3B in all cell lines. These results suggest that the decrease in DNMT levels and inhibition of DNA methylation in enzalutamide-resistant cells induces an increase in the sensitivity to the enzalutamide-mediated apoptotic response.

To investigate the effect of decitabine treatment in combination with enzalutamide on cellular proliferation, LNCaP MR49F, C4-2 and C4-2R cells were subjected to MTT assays after treatment

with DMSO, decitabine, enzalutamide, and the combination of decitabine and enzalutamide. Our results show that the exposure of resistant cells to decitabine and enzalutamide induces a significant decrease in cell proliferation compared to exposure to single agents or DMSO (Fig.2.5 B). C4-2 cells exhibited a similar response to the enzalutamide-resistant cells. However, in LNCaP cells we didn't see any significant differences between the groups treated with the combination compared to the groups treated with a single agent (Fig.2.5 B). Overall, this data suggests that the combination of enzalutamide and DNA methylation inhibition promotes apoptosis and a decrease in cell proliferation in the enzalutamide-resistant cells.

4.2.6 Knockdown of DNMT3B reestablishes the response to enzalutamide in enzalutamide-resistant cells

Decitabine treatment promotes the degradation all DNA methyltransferases in prostate cancer cells as seen in Figure 2.5 B. To examine the involvement of the individual DNA methyltransferases in the response to enzalutamide, cells were transfected with siRNAs targeting a scrambled sequence (siCtrl), DNMT1, DNMT3A or DNMT3B followed by a treatment with enzalutamide. Knockdown of DNMT3B combined with enzalutamide treatment increased the levels of Cleaved-PARP exclusively in MR49F and C4-2R cells (Fig.2.6 A). The knockdown of DNMT1 and DNMT3A did not produce an increase in the levels of the cleaved apoptotic protein. In LNCaP and C4-2 cells, knockdown of DNMT3B alone, without enzalutamide, produced a significant increase in Cleaved-PARP levels (Fig.2.6 A). These results suggest that, among DNA methyltransferases, DNMT3B plays a role in promoting enzalutamide-mediated apoptosis resistance in MR49F and C4-2R. Also, in MTT assays examining the combination of DNMT knockdown plus enzalutamide treatment, we observed the most significant decrease in cell viability and proliferation in MR49F and C4-2R following the knockdown of DNMT3B and treatment with enzalutamide (Fig.2.6 B). We also observed a decrease in cell proliferation in the enzalutamide-resistant cells transfected with siDNMT1 and treated with enzalutamide and in C4-2R cells transfected with siDNMT3A and treated with enzalutamide. In LNCaP cells our data shows a decrease in cells proliferation following the knockdown of DNMT1 combined with enzalutamide (Fig.2.6 B). In C4-2 cells a significant decrease in cell proliferation was observed in siDNMT3A and siDNMT3B samples treated with enzalutamide.

To better understand which of the DNA methyltransferases plays a bigger role in the resistance to enzalutamide in prostate cancer cells, MR49F and C4-2R cells were transfected with siRNA targeting a scrambled sequence, DNMT1, DNMT3A and DNMT3B followed by treatment with increasing concentrations of enzalutamide. Following MTT assays, the data from each siRNA group was normalized to the group treated with DMSO (0 µM enzalutamide) to identify which of the siRNAs produces the highest decrease post-enzalutamide treatment compared to siCtrl. Our data in figure 2.7 showed that transfection of C4-2R and MR49F with siDNMT3B induced the most significant decrease in cell proliferation in response to increasing concentrations of enzalutamide. All the results suggest that DNMT3B plays a pivotal role in the response to enzalutamide in enzalutamide-resistant cells.

4.2.7 Decitabine treatment decreased AR-V7 levels in enzalutamide-resistant cells

Studies have previously shown that the promoter of the *AR* gene can be methylated and its methylation regulates its expression in different prostate cells (44, 264, 381). Due to the precedents connecting DNA methylation to *AR* we explored the effect of the combination treatment on the AR signaling pathway in our enzalutamide-resistant cells. We treated C4-2R and 22RV1, a cell line with primary resistance to enzalutamide and high AR-V7 expression, with decitabine and enzalutamide. After 5 days of treatment, protein expression of key players in the AR signaling pathway was examined. Decitabine treatment, at 500 nM induced a consistent decrease in AR-V7 expression in both C4-2R and 22RV1 cells (Fig.4.8 A and B). In addition, a decrease in AR expression was observed in both decitabine-treated groups in both cell lines. Decitabine also induced the expression of PSA in C4-2R and 22RV1 cells, which have very low detectable expression of the protein. These results suggest that the response seen after treatment of enzalutamide-resistant cells with decitabine may increase the response to enzalutamide by decreasing the expression of AR and its oncogenic variant AR-V7 in these cells. Increased AR and AR-V7 expression in prostate cancer cells are well-known mechanisms of resistance to enzalutamide, thus reducing the expression of both protein post-decitabine treatment indicates a role for DNA methylation in the process of resistance to the antiandrogen.

4.2.8 Decitabine promoted a decrease in AR-V7 levels accompanied by a decrease in tumor volume in 22RV1 xenograft mice treated with enzalutamide

To investigate the effect of DNA methylation inhibition combined with enzalutamide on tumor growth *in vivo*, castrated NRG mice were inoculated with 22RV1 cells and treated for 3 weeks with a vehicle control, 0.5 mg/kg decitabine, 20 mg/kg enzalutamide and the combination of both. After the completion of the experiment, we observed a significant decrease in the tumor volume in mice treated with enzalutamide and decitabine compared to the vehicle control and the single-treatment groups (Fig.4.9 A). The combination of 20 mg/kg of enzalutamide with 0.5 mg/kg decitabine induced a significant decrease in tumor weight compared to the vehicle control group at necropsy (Fig.4.9 B). There were no significant toxicities induced by the different treatments administered to the mice shown by their body weights, measured upon necropsy (Fig.4.9 D). Decitabine treatment induced a significant decrease in the expression of DNMT1, DNMT3A and DNMT3B, as expected. AR-V7 expression in the group treated with enzalutamide and decitabine was significantly lower than the vehicle-treated group (Fig.4.9 E). No significant changes were seen in AR full length expression based on the sample size we used (Fig.4.9 E). However, the expression AR was slightly lower in the combination-treated group compared to the vehicle-treated group.

Immunohistochemistry analysis of apoptosis and cellular proliferation markers was completed on the tumors. These results show an increase in the number of apoptotic cells, reflected by the increase in the intensity of Cleaved-Caspase3 staining, in the combination treatment group compared to the vehicle control, enzalutamide and decitabine groups (Fig.4.10 A and B). On the other hand, KI-67 staining was significantly decreased in the combination group compared to all other groups indicating a decrease in cellular proliferation within the tumors (Fig.4.10 A and C). Our *in vivo* data recapitulates our observations *in vitro* in other cell lines. These observations suggest that inhibiting the DNA methylation pathway with DNA methylation inhibitors, such as decitabine, in combination with enzalutamide can promote a slower tumor growth by increasing cell apoptosis and inhibiting cell proliferation.

4.2.9 Decitabine reverses the expression of key genes in the enzalutamide-resistant cell line C4-2R

DNA methylation inhibitors produce a global hypomethylation phenotype in the treated cells promoting changes in gene expression. To study the impact of the upregulation of DNMT3B in enzalutamide-resistant cells compared to enzalutamide-sensitive cells, to better understand the underlying mechanisms of resistance mediated by changes in DNA methylation and to investigate the impact of decitabine treatment on enzalutamide-resistant cells, we designed an RNA sequencing and Bisulfite sequencing experiment depicted in Figure 4.10. In this experiment we will compare enzalutamide-sensitive cells LNCaP and C4-2 to the enzalutamide-resistant cell line C4-2R, in comparisons C1 and C2 respectively (Table 1). Then we will compare untreated C4-2R cells to C4-2R cells treated with decitabine or decitabine plus enzalutamide, in comparisons C3 and C4 respectively (Table 1). In these comparisons we will explore the changes in gene expression following an RNA-sequencing experiment. This approach will allow us to identify high interest genes and pathway that are over or under-represented in C4-2R cells compared to LNCaP and C4-2. We will then investigate whether these high interest genes are methylated using the differentially methylated regions (DMR) analysis after Bisulfite sequencing. The expression of these high interest genes will then be explored in C3 and C4 to identify any reversal in their expression and DNA methylation pattern compared to C1 and C2. These analyses will allow us to identify a subset of genes that up or downregulated in C4-2R compared to sensitive cells that become down or upregulated following decitabine treatment. We believe that these genes mediate the response to enzalutamide seen after decitabine treatment in the resistant cells. The identification of these players is important to help us understand underlying mechanisms of resistance to enzalutamide in prostate cancer cells and mechanisms by which DNMTs and DNA methylation drive resistance in these cells.

We first did a Gene Set Enrichment Analysis and explored the hallmark gene set collection in C2 and C3. The graphs in Figures 4.11 A and B show GSEA with significant positive or negative enrichment scores on DEGs before and after enzalutamide resistance (Fig.4.12 A) and before and after treatment of resistant cells with decitabine (Fig.4.12 B). Our results show a reversal in the enrichment of several genes from different gene sets after treatment with decitabine. Genes that were positively enriched in enzalutamide-sensitive cells compared to enzalutamide-resistant cells,

such as HALLMARK_MYC_TARGETS_V1, HALLMARK_MYC_TARGETS_V2, HALLMARK_E2F_TARGETS and HALLMARK_G2M_CHECKPOINT, became negatively enriched in C4-2R cells following treatment with the DNA methylation inhibitor decitabine (Fig.4.12 A and B). In contrast, genes of the HALLMARK_ANDROGEN_RESPONSE gene set were found to be negatively enriched in C4-2R compared to C4-2. Decitabine treatment reversed its enrichment score and promoted an overexpression of androgen response genes following DNA methylation. This suggests that decitabine treatment may play a role in the activation of androgen receptor signaling in C4-2R cells, resulting in an increased sensitivity to enzalutamide in these cells. Moreover, gene sets such as HALLMARK_APOPTOSIS and HALLMARK_DNA_REPAIR were not significantly changed between C4-2 and C4-2R cells. However, decitabine treatment stimulated the overexpression of apoptotic genes in C4-2R cells and induced a decrease in the expression of DNA repair genes (Fig.4.12 B). These observations suggest that genes from these gene sets are involved in the decitabine-mediated response to enzalutamide in resistant cells.

To narrow down the list of genes we isolated 2 subsets of genes depicted in the heatmaps in Figure 4.13. In Figure 4.12 A, the heatmap represents 74 genes that had a Log2FC>1 in C1 and C2 accompanied by a Log2FC<-1 in C3. In Figure 4.12 B, the heatmap represents 307 genes that had a Log2FC<-1 in C1 and C2 accompanied by a Log2FC>1 in C3. Among genes in heatmap (A), we identified *ABCB11*, *MYB*, *WNT10B*, *WT1*, *MCM7*, *BRCA1* and *PAX1*. These genes are known to play a pro-survival and pro-resistance role in cancer. In contrast, in heatmap (B) we discovered *ALPK2* and *AIFM2*. Both genes play a pro-apoptotic role in cancer and may be potentially involved in promoting an enzalutamide-resistant phenotype in prostate cancer cells. Our bioinformatics data support our *in vitro* and *in vivo* results by demonstrating decitabine-mediated changes in pathways that reverse enzalutamide resistance.

4.3 Discussion

With the increasing use of enzalutamide for the treatment of prostate cancer patients across different stages of the disease and its clinical investigation for the treatment of early-stage prostate cancer patients and breast cancer patients, it is vital to understand the mechanisms by which cells develop resistance to this anti-androgen. In this study, we investigated the role of DNA

methylation and DNA methyltransferases in the resistance to enzalutamide and the effect of enzalutamide treatment on this pathway. It is known that Dynamic changes in DNA methylation and DNMT expression occur upon prostate carcinogenesis and with prostate cancer progression (274). It has been shown that bicalutamide, a first generation anti-androgen, induces changes in DNA methylation and an increase in the expression of DNMTs in prostate cancer cell lines (321). However, the effect of enzalutamide on DNA methylation and DNMT expression and the mechanism behind these changes are unknown. More importantly, the functional impact of changes in DNA methylation and in DNA methyltransferase expression are not studied in the context of established enzalutamide-resistant cells.

In untreated enzalutamide-resistant lines, MR49F and C4-2R, compared to their sensitive counterparts LNCaP and C4-2 cells, we found a significant increase in DNMT activity (Fig.4.1 A). The increase in DNMT activity translated to an increase in global DNA methylation only in MR49F compared to LNCaP cells (Fig.4.1 D). The low global DNA methylation in LNCaP cells compared to MR49F, C4-2 and C4-2R can be attributed to the fact that LNCaP is considered an androgen dependent line, whereas the others are considered androgen independent. Mishra *et al.* have previously shown that differences in the amount and pattern of global DNA methylation exist between the androgen-sensitive LNCaP cells and PC3 and DU145, two androgen-negative cell lines (382). The increase in DNMT activity was correlated with a significant increase in DNMT3B protein expression in MR49F and C4-2R (Fig.4.1 J). By testing the expression of different DNMT3B variants, we identified an overexpression of *DNMT3B3* and *DNMT3B7* in the enzalutamide-resistant lines. DNA methyltransferases are alternatively spliced or driven by alternative promoters in mammalian cells, leading to the identification of more than 30 different expressed isoforms (229, 231, 294). A simple bioinformatics analysis on **http://www.webgestalt.org/** using our differentially expressed gene-list from the C2 comparison, was able to identify "mRNA splicing" as the top 1 overrepresented REACTOME pathway. A deeper look into our DEG results helped us identify many genes involved in mRNA splicing. Vezf1 increases the expression of the alternatively spliced *DNMT3B* variants binding to the intronic regions of *DNMT3B* and promoting the accumulation of RNA-pol II (383, 384). *Vezf1* expression was increased in C4-2R cells compared to LNCaP and C4-2 (0.78623 and 0.74989 log2FC in C1 and C2, respectively). Also, RBM proteins involved in alternative splicing are deregulated in the

C1 and C2 comparisons. RBM10 regulates the expression of DNMT3B isoforms in mouse embryonic fibroblasts (385). Other splicing factors, such as Splicing Factor (SR) proteins and Serine and Arginine Rich Splicing Factor (SRSF) proteins are deregulated in our resistant cell line. These observations suggest that the deregulation in the mRNA splicing machinery in enzalutamide-resistant cells can account for the changes in the expression DNMT3B variants.
Studies have shown the involvement of the MDM2/p53/SP1-SP3 axis and the MDM2/pRB/E2F1 axis in the regulation of the expression of DNMT1, DNMT3A and DNMT3B in mammalian cells (378). MDM2, an E3 ubiquitin ligase, promotes the ubiquitination and proteasomal degradation of p53 and pRB (386, 387). p53 and pRB bind to and inhibit the transcriptional activation function of SP1/SP3 and E2F1, respectively (378). We investigated the involvement of these pathways in the increased expression of DNMT3A and DNMT3B. Our results showed an increase in the expression of E2F1 in the resistant lines compared to LNCaP and C4-2 (Fig.4.3 A). The increase in E2F1 expression in MR49F and C4-2R correlates with the expression of DNMT3B. Additionally, our bioinformatics results revealed an increase in Wilm's Tumor 1 (WT1) expression in C1 and C2 comparisons (Log2FC of 4.71972 and 1.35031, respectively). WT1 promotes the transcriptional activation of *DNMT3A* in mammalian cells (388). It's not clear whether WT1 has any role in the regulation of DNMT3B expression in prostate cancer cells.

The effect of enzalutamide on DNA methylation and DNMT expression and activity was investigated in enzalutamide-sensitive cell lines. Our findings suggest that enzalutamide promote an increase in the activity of DNMTs in LNCaP and C4-2 cells (Fig.4.1 B and C). The increase in DNMT activity in the androgen-independent cell lines, LNCaP, correlates with an increase in global 5-mC levels after enzalutamide treatment (Fig.4.1 E). In all cell lines, 5 μM enzalutamide treatment induced an increase in DNMT3B and DNMT3A protein and transcript levels (Fig.4.2 A, B and C). In LNCaP and C4-2 cells, enzalutamide induced an increase in the expression of *DNMT3B1*, *DNMT3B3* and *DNMT3B7* (Fig.4.2 C). These results suggest that long-term enzalutamide treatment induces an increase in the expression of DNMT3A and DNMT3B, of which DNMT3B becomes stably overexpressed upon the onset of enzalutamide resistance.

We investigated the mechanism by which the increase of DNA methyltransferase expression is mediated following treatment of prostate cancer cells with enzalutamide. Treatment of C4-2R cells

with enzalutamide promotes an increase in the expression of MDM2 which directly correlates with the expression of DNM3B. (Fig.4.3 B). Nutlin-3a inhibits the binding of MDM2 to p53, promoting the activity of the tumor suppressor (389). Treatment of MR49F cells with Nutlin-3a after enzalutamide treatment abolished the enzalutamide-mediated increase in DNMT3B expression in the cells. Collectively, these results suggest a role for p53 in the enzalutamide-mediate regulation of DNMTs in prostate cancer cells. However, additional experiments need to be done to confirm the direct involvement of p53 in this response and eliminate any alternative hypotheses and potential mechanisms.

The roles of DNMT3B variants in the context of prostate cancer and the effect of their expression on enzalutamide response are not studied. DNMT3B3 and DNMT3B7 are catalytically inactive DNA methyltransferase and their expression correlates with an aberrant DNA methylation profile (228, 231). The inactive DNMT3B isoforms, DNMT3B3, has been show to act as a binding partner of catalytically active de novo DNA methyltransferases, DNMT3A and DNMT3B (228). The accessory role of DNMT3B3 is believed to be associated with the regulation of the recruitment of active DNA methyltransferases to genomic sites for methylation (228). DNMT3B7 expression correlates with the tumorigenesis and cancer aggressiveness across different tissue types (390, 391). The functional significance of the increased expression of DNMT3B3 and DNMT3B7 was investigated in C4-2 cells. Specifically, the impact of DNMT3B3 and DNMT3B7 on enzalutamide response was assessed. Enzalutamide treatment of C4-2 cells overexpressing DNMT3B3 and DNMT3B3 plus DNMT3B7 had a significantly lesser impact on cell proliferation compared to control C4-2 cells. These results show that mostly DNMT3B3 plays a pro-tumor role in prostate cancer cells by promoting an enzalutamide resistance phenotype.

The increase in DNMT activity and DNMT expression in enzalutamide-resistant cell lines MR49F and C4-2R prompted us to investigate the involvement of this pathway in promoting resistance to the anti-androgen. DNA methylation and DNA methyltransferases have been shown to promote drug resistance in cancers including the cancer of the prostate (283, 320, 392). Researchers have previously shown that treatment of DNA methylation-mediated drug resistance with the cytidine analog, azacytidine, restores the sensitivity and reverses drug resistance in a number of cases (320, 393, 394). Here we assessed whether low concentrations of decitabine affect the sensitivity to

enzalutamide in MR49F and C4-2R. Our results showed that pre-treatment with decitabine followed by enzalutamide treatment induced an increase in Cleaved-PARP and Cleaved-Caspase3 which indicates an increase in apoptosis (Fig.4.5 A). These results correlate, with the findings from our RNA-sequencing data. In C3, the GSEA suggested an enrichment of apoptotic genes in C4-2R cells treated with decitabine (Fig.4.12 B). Furthermore, pro-apoptotic proteins *AIFM2* and *ALPK2* are among the genes that have exhibited a significant increase in their expression post-decitabine treatment after being downregulated in C4-2R cells compared to sensitive cells. We also found that decitabine treatment decreased the expression of the anti-apoptotic gene, *BCL2*, after it was upregulated in C4-2R cells. All the evidence supports a role for decitabine in reversing the expression of key apoptotic genes to promote apoptosis following combination with enzalutamide treatment in the resistant cells.

Cytidine analogs get incorporated into DNA molecules and form a covalent bond with all 3 DNA methyltransferases, promoting their proteasomal degradation (395). To test which of the DNMTs plays the most pivotal role we knocked down all 3 methyltransferases and tested the cells' responses to enzalutamide. Our results indicate that DNMT3B knockdown in combination with enzalutamide promotes an increase in apoptotic markers and the most significant decrease in cell proliferation in enzalutamide-resistant cells (Figures 4.6 and 4.7). These results indicate that DNMT3B is playing a significant role in the resistance to enzalutamide in prostate cancer cells, further supporting our findings from figure 4.1, where we found DNMT3B to be upregulated in MR49F and C4-2R cells.

Enzalutamide targets the androgen signaling pathway by binding to and inhibiting the androgen receptor (76). AR aberrations, including the amplification of the receptor and the expression of splice variants are among well-known mechanisms by which cells acquire resistance to the antiandrogen (396). We investigated the effect of DNA methylation inhibition and enzalutamide on AR and its signaling pathway. In figure 4.8, we showed that the exposure of enzalutamide-resistant cells to decitabine induced a significant decrease in the expression AR-V7 and AR. Also, our DEG results show that components of the mRNA splicing pathway, involved in the splicing of AR and AR-V7 expression, are deregulated in C4-2R cells. Factors such as *U2AF2*, *HNRNPU*, *NONO and HNRNPL* are found to be overrepresented in C4-2R cells and downregulated after

decitabine treatment (data not shown). These factors were shown to be involved in the alternative splicing of AR and generation of AR-V7 (397). In sum, the decrease in the expression of AR-V7 can be explained by the deregulation of the mRNA splicing machinery and can offer a potential mechanism to understand the effects of the combination treatment on the survival and apoptosis enzalutamide-resistant cells. Antonarakis *et al.* previously showed that AR-V7 expression correlates with the resistance to enzalutamide in patients (398) and Luna Velez *et al.* showed that AR-V7 silencing promotes apoptosis and inhibition of survival in androgen-independent prostate cancer cells (399). Moreover, previous studies have shown that *AR* promoter can be methylated, and this methylation-mediates its silencing in prostate cancer cells (44, 264, 381). Tian *et al.* showed that decitabine treatment increased the expression of *AR* (381), which is opposite to our observations in the enzalutamide resistant cell lines C4-2R and 22RV1. It would be interesting to investigate the methylation pattern on *AR* in enzalutamide-resistant prostate cancer cells and compare it to previously published data.

In our *in vivo* experiment, we used the primary enzalutamide-resistant cell line, 22RV1, to generate xenograft tumors in castrated mice to assess the combination of decitabine and enzalutamide. Results from figures 4.9 and 4.10 recapitulate our *in vitro* data in MR49F and C4-2R. The combination of enzalutamide and decitabine induced a decrease in tumor volume, tumor weight, AR-V7 expression and KI-67 staining and an increase in Cleaved-Caspase3 staining compared to vehicle-treated samples. To further confirm our findings, our future experiments should focus on repeating this experiment in a patient-derived xenograft model that mirrors our enzalutamide-resistant cell lines.

Our sequencing and bioinformatics data offer insights into possible mechanisms of resistance to enzalutamide and mechanisms of decitabine-mediated restoration of drug sensitivity. Our GSEA data showed a deregulation in key pathways that can be pursued in future experiments to identify individual genes that are involved in the resistance to enzalutamide or that can be targeted to restore enzalutamide sensitivity (Fig.4.12). We analyzed the upstream regulators using the list of DEGs we generated. The identified upstream regulators are accompanied with an activation z-score that indicates whether their function is activated (positive z-score) or inhibited (negative z-score). In this analysis, we observed that the upstream regulators AR and dihydrotestosterone are inhibited

in C4-2R compared to C4-2 cells with an activation z-score of -3.125 and -2.858, respectively. After decitabine treatment, AR inhibition was reversed. This suggests that AR signaling is activated, providing a rationale behind combining decitabine and enzalutamide in C4-2R cells (activation z-score 2.664). This observation is consistent with our *in vitro* experiments, further confirms the GSEA results and offers a rational behind using enzalutamide and decitabine as a combination treatment in enzalutamide-resistant cells.

Finally, we generated heatmaps to identify genes that exhibited a change in expression following enzalutamide resistance onset and a reversal of this change following decitabine treatment (Fig.4.13). We listed genes of interest that will be pursued in future experiment to investigate their involvement in enzalutamide resistance. A literature review allowed us to link these genes to prostate cancer progression or drug resistance which provides a rationale behind future investigation. MYB overexpression promotes survival of prostate cancer cell lines after androgen depletion (400). MYB depletion restores the apoptotic response induced by androgen depletion (400). WT1 has been shown to be involved in regulating the expression of genes in cooperation with the androgen receptor and in playing a role in prostate cancer progression (401–403). MCM7 overexpression is associated with tumor growth and poor survival in prostate cancer patients (404). Shi *et al.* have shown that MCM7 binds to AR and promotes survival after treatment with AR-targeting therapies (405). Germline mutations in homology recombination DNA repair genes, such as BRCA1, are associated with better outcomes in patients treated with enzalutamide(406). These genes and pathways will be investigated for their role in the response to enzalutamide in resistant cells.

In conclusion, this study shows that enzalutamide promotes epigenetic changes in prostate cancer cells leading to aberrations in gene expression and signaling pathways leading to the onset of drug resistance. DNA methylation and DNMT3B, specifically DNMT3B3, play a central role in this mechanism (Fig.4.15). Decitabine treatment restores the sensitivity to enzalutamide in resistant prostate cancer cells, *in vitro* and *in vivo*. Components of the apoptosis, DNA repair and AR signaling pathways are influenced by changes in DNA methylation in enzalutamide-resistant prostate cancer cells and future efforts will be made to elucidate their roles in resistance onset and in enzalutamide response.

Figure 4.1. DNA methyltransferase activity and DNMT3B are overrepresented in enzalutamide resistant cell lines

A, D, G-J, LNCaP, MR49F, C4-2, C4-2R and 22RV1 cells were seeded and cultured for 5 days in regular medium. B, C, E and F LNCaP and C4-2 cells were treated with DMSO, 5 or 10 μM Enzalutamide for 5 days. A-C, Nuclear extracts were collected from cells after 5 days and DNMT activity was measured with the EpiQuik DNMT Activity/Inhibition ELISA Easy Kit (Colorimetric) according to the manufacturer's protocol. (D-F) Genomic DNA was collected from cells after 5 days and DNA methylation was measured with the MethylFlash Methylated DNA 5-mC Quantification Kit (Colorimetric) according to manufacturer's protocol. G-I, RNA was extracted from cells after 5 days followed by cDNA synthesis. Transcript levels of DNMT1, DNMT3A and DNMT3B were measure using qRT-PCR. ACTIN was used as a reference gene. J, Cells were subjected to protein extraction after 5 days followed by western blot analysis of DNMT1, DNMT3B, DNMT3B7, DNMT3A1 and DNMT3A2. GAPDH was used as loading control. Band intensity was quantified using ImageJ. In all comparisons, MR49F was compared to LNCaP and C4-2R and 22RV1 were normalized to C4-2. Two-tailed student t-test was used to evaluate statistical significance *$p<0.05$ and **$p<0.01$.

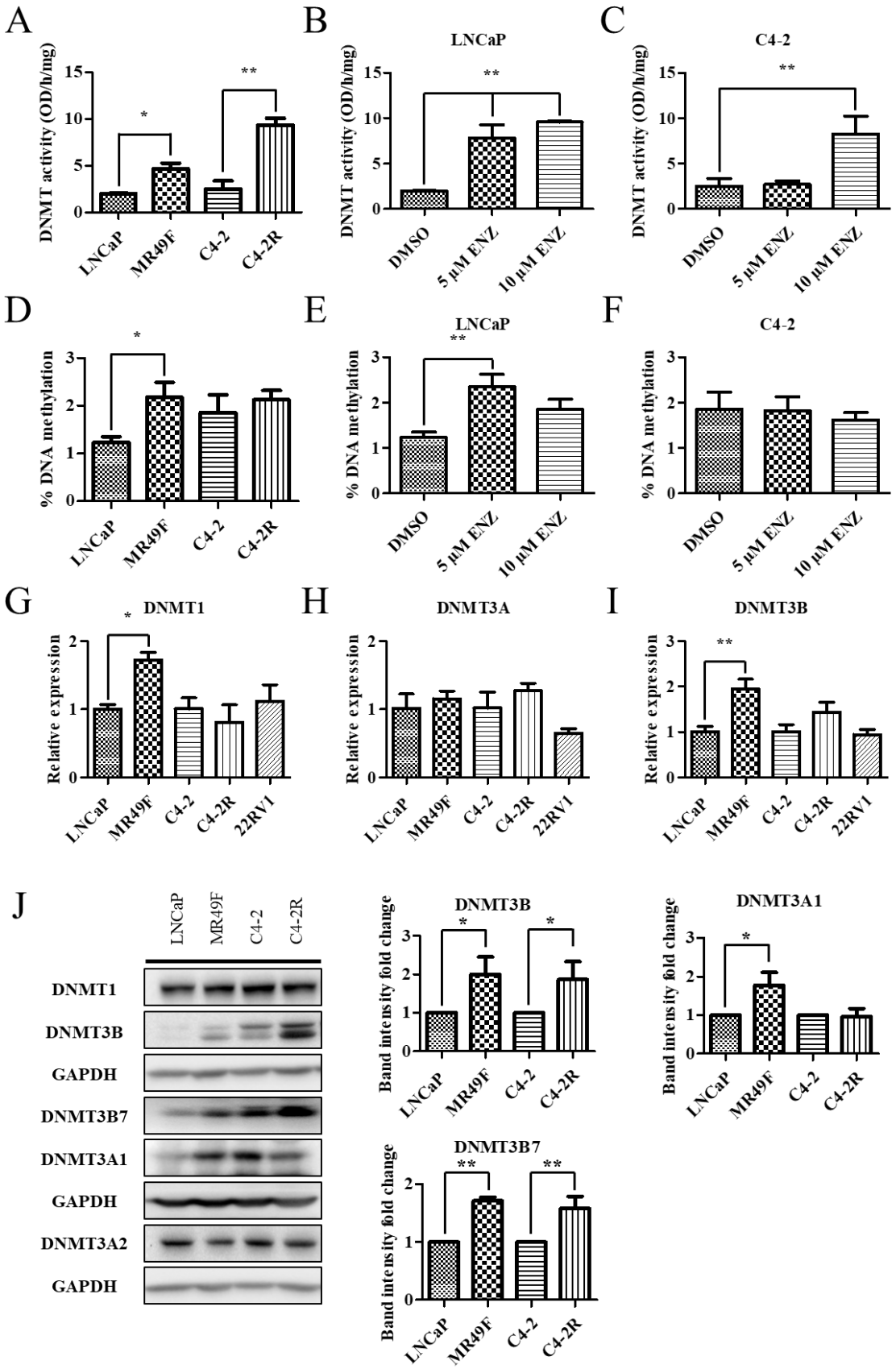
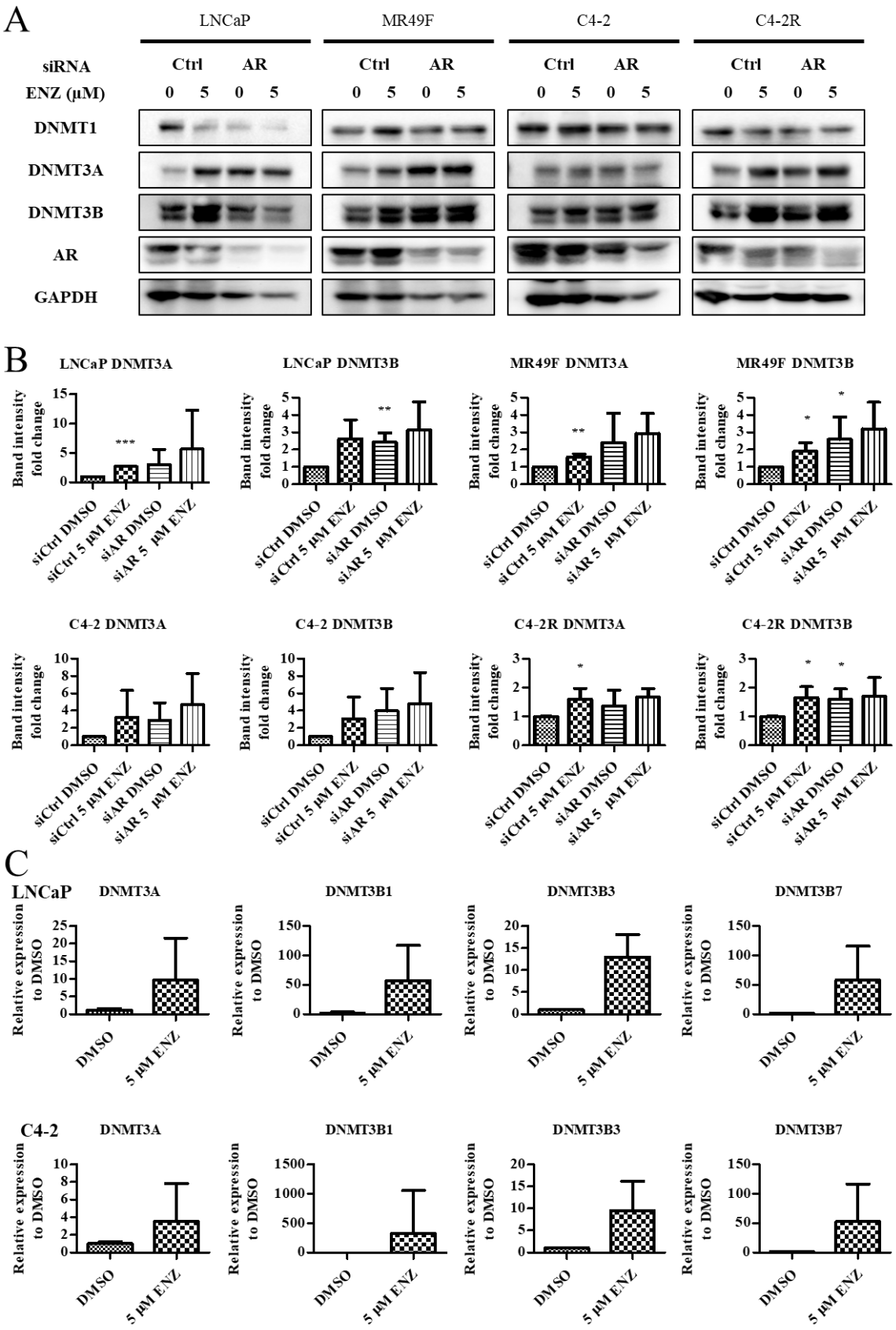

A
DNMT activity (OD/h/mg)
LNCaP
MR49F
C4-2
C4-2R
B
LNCaP
DMSO
5 μM ENZ
10 μM ENZ
C
C4-2
D
% DNA methylation
E
F
G
DNMT1
Relative expression
22RV1
H
DNMT3A
I
DNMT3B
J
DNMT1
DNMT3B
GAPDH
DNMT3B7
DNMT3A1
DNMT3A2
Band intensity fold change
DNMT3A1
DNMT3B7

Figure 4.2. Enzalutamide induces and increase in the expression of DNMT3B and DNMT3A in prostate cancer cells

A, LNCaP, MR49F, C4-2 and C4-2R cells were transfected with siCtrl or siAR followed by treatment with DMSO or 5 μM Enzalutamide for 3 days. Cells were subjected to protein extraction followed by western blot analysis of DNMT1, DNMT3A, DNMT3B and AR. GAPDH was used as a loading control. B, Band intensity of DNMT3A and DNMT3B was quantified using ImageJ. In all comparisons, band intensities of all groups were normalized to the siCtrl DMSO treated group. C, Total RNA was extracted from LNCaP and C4-2 cells treated with DMSO and 5 μM Enzalutamide. After cDNA synthesis, transcript levels of DNMT3A, DNMT3B1, DNMT3B3 and DNMT3B7 were measured by qRT-PCR. ACTIN was used as a reference gene. Two-tailed student t-test was used to evaluate statistical significance *$p<0.05$, **$p<0.01$ and ***$p<0.001$.


A
LNCaP
MR49F
C4-2
C4-2R
siRNA
Ctrl
AR
ENZ (μM)
0 5 0 5
DNMT1
DNMT3A
DNMT3B
AR
GAPDH
B
LNCaP DNMT3A
LNCaP DNMT3B
MR49F DNMT3A
MR49F DNMT3B
C4-2 DNMT3A
C4-2 DNMT3B
C4-2R DNMT3A
C4-2R DNMT3B
Band intensity fold change
siCtrl DMSO
siCtrl 5 μM ENZ
siAR DMSO
siAR 5 μM ENZ
C
LNCaP
C4-2
DNMT3A
DNMT3B1
DNMT3B3
DNMT3B7
Relative expression to DMSO
DMSO
5 μM ENZ

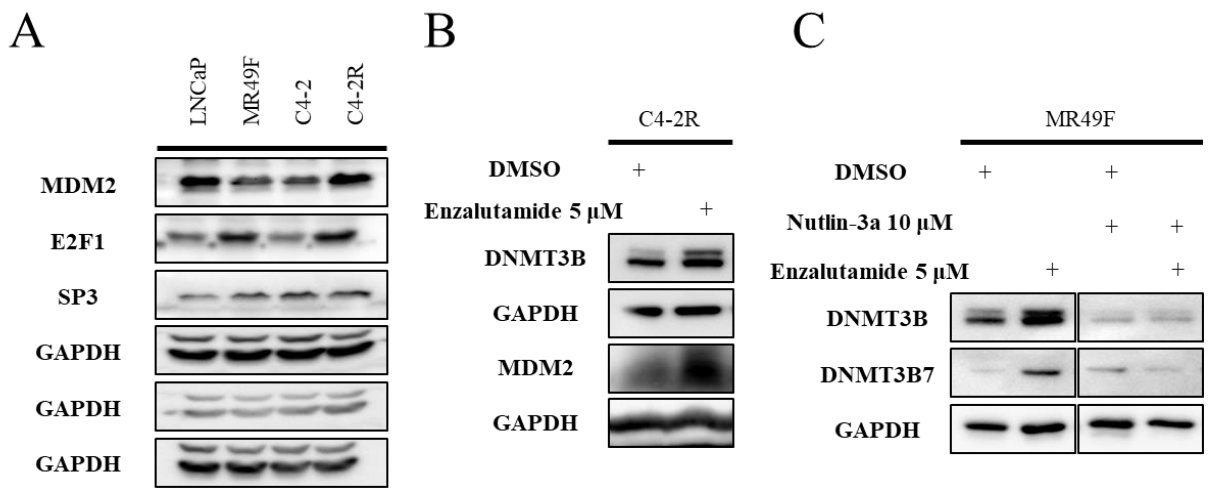


Figure 4.3. The p53 and pRB pathways are involved in the enzalutamide-mediate increase in DNMT3B in prostate cancer cells

A, LNCaP, MR49F, C4-2 and C4-2R cells seeded for 5 days followed by western blot analysis of MDM2, E2F1 and SP3. GAPDH was used as a loading control. B, C4-2R cells were treated with DMSO or 5 µM Enzalutamide for 3 days followed by western blot analysis of DNMT3B and MDM2. GAPDH was used as a loading control. C, MR49F cells were treated with DMSO or 5 µM Enzalutamide for 2 days followed by treatment with 10 µM of Nutlin-3a for 1 day. Samples were then subjected to western blot analysis of DNMT3B. GAPDH was used as a loading control.

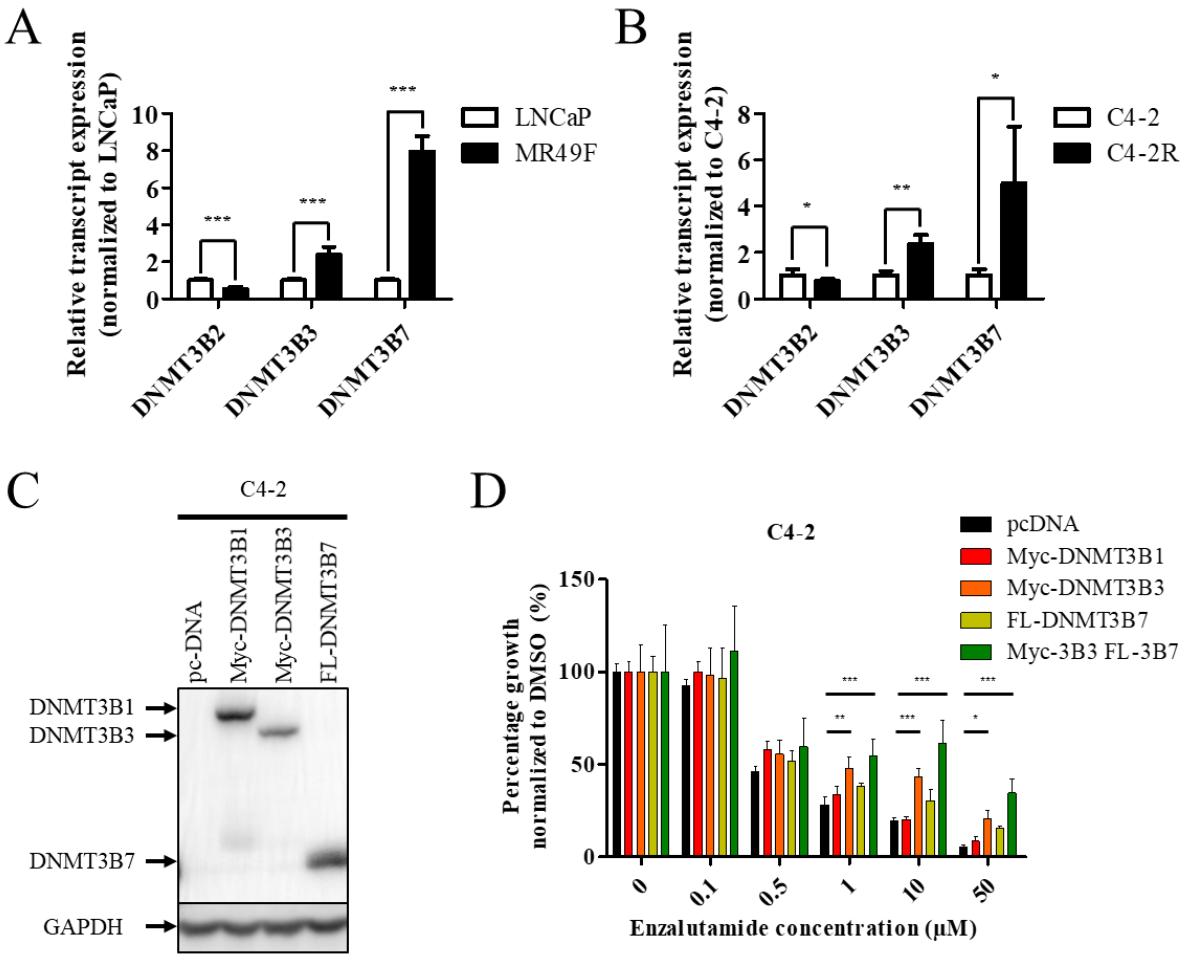


Figure 4.4. Overexpression of DNMT3B3 promotes an enzalutamide resistant phenotype in prostate cancer cells

A, B, LNCaP, MR49F, C4-2 and C4-2R cells seeded for 5 days followed by total RNA extraction. After cDNA synthesis, transcript levels of DNMT3B2, DNMT3B3 and DNMT3B7 were measured by qRT-PCR. ACTIN was used as a reference gene. C, C4-2 cells were transfected with pcDNA, Myc-DNMT3B1, Myc-DNMT3B3 and FL-DNMT3B7. Cells were then selected with Geneticin for at least 10 days followed by western blot analysis of DNMT3B. GAPDH was used as loading control. D, Stably transfected C4-2 cells were seeded in 96 well plates followed by treatment with DMSO (0) or 0.1, 0.5, 1, 10 or 50 µM Enzalutamide for 6 days. Cells were then subjected to MTT assay and O.D. was measured at 570 nm. Percentage growth was normalized to DMSO (0)-treated samples. Two-way ANOVA was used to evaluate statistical significance *p<0.05, **p<0.01 and ***p<0.001.

Figure 4.5. Inhibition of DNA methylation in enzalutamide-resistant cells restores response to enzalutamide

A, LNCaP, MR49F, C4-2 and C4-2R cells pre-treated for 5 days with 0, 250 and 1000 nM decitabine followed by treatment with DMSO (0) or 10, 20or 40 µM enzalutamide for 3 days. Proteins were then extracted followed by western blot analysis of Cleaved-PARP, DNMT1, DNMT3A DNMT3B, and Cleaved Caspase3. GAPDH was used as a loading control. B, LNCaP, MR49F, C4-2 and C4-2R cells were seeded in 96 well plates followed by treatment with DMSO, decitabine, enzalutamide or the combination of decitabine and enzalutamide. Cells were were subjected to MTT assay when control wells became confluent and O.D. was measured at 570 nm. Percentage growth was normalized to DMSO treated samples. Two-way ANOVA was used to evaluate statistical significance ***$p<0.001$.

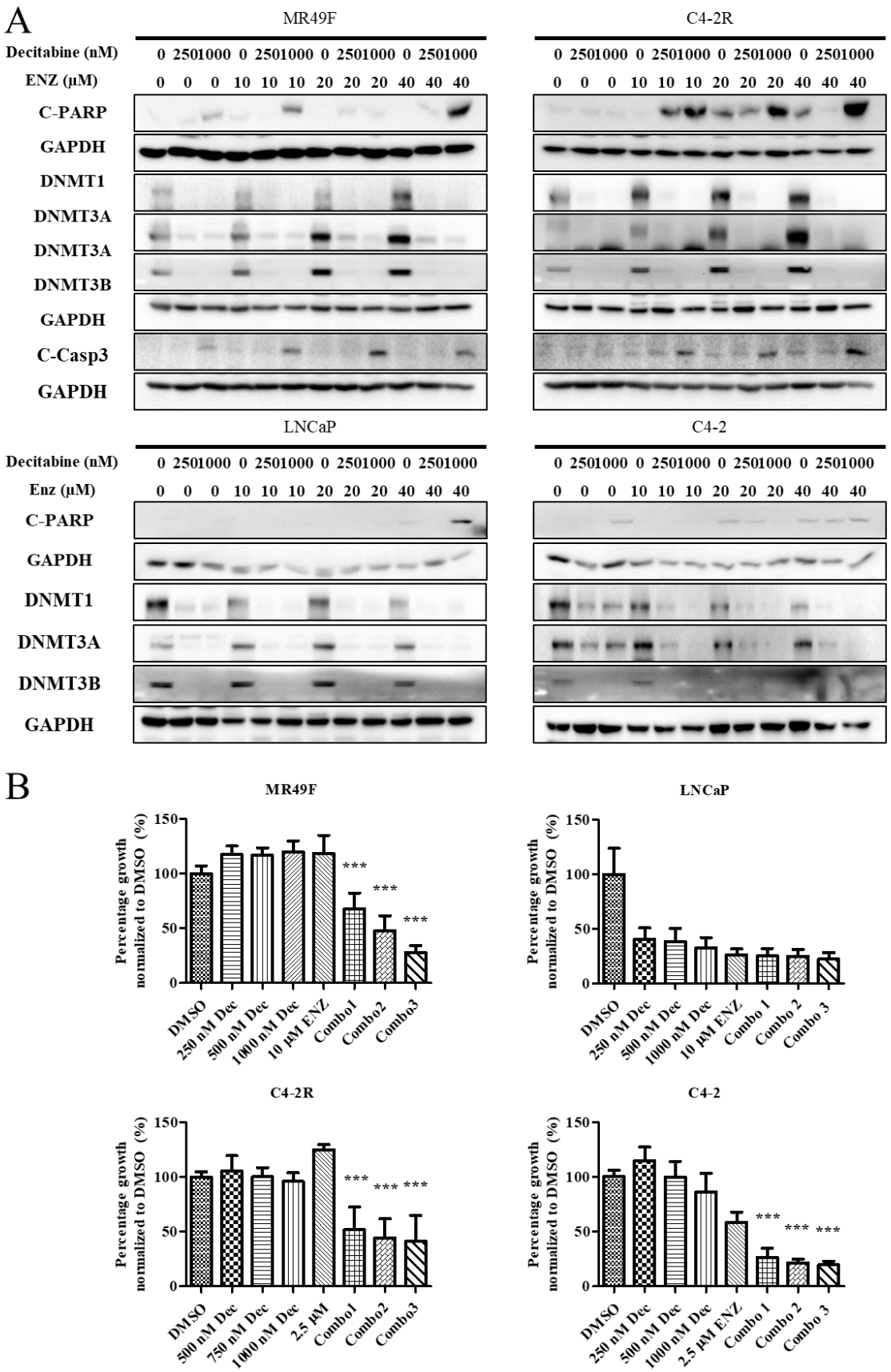
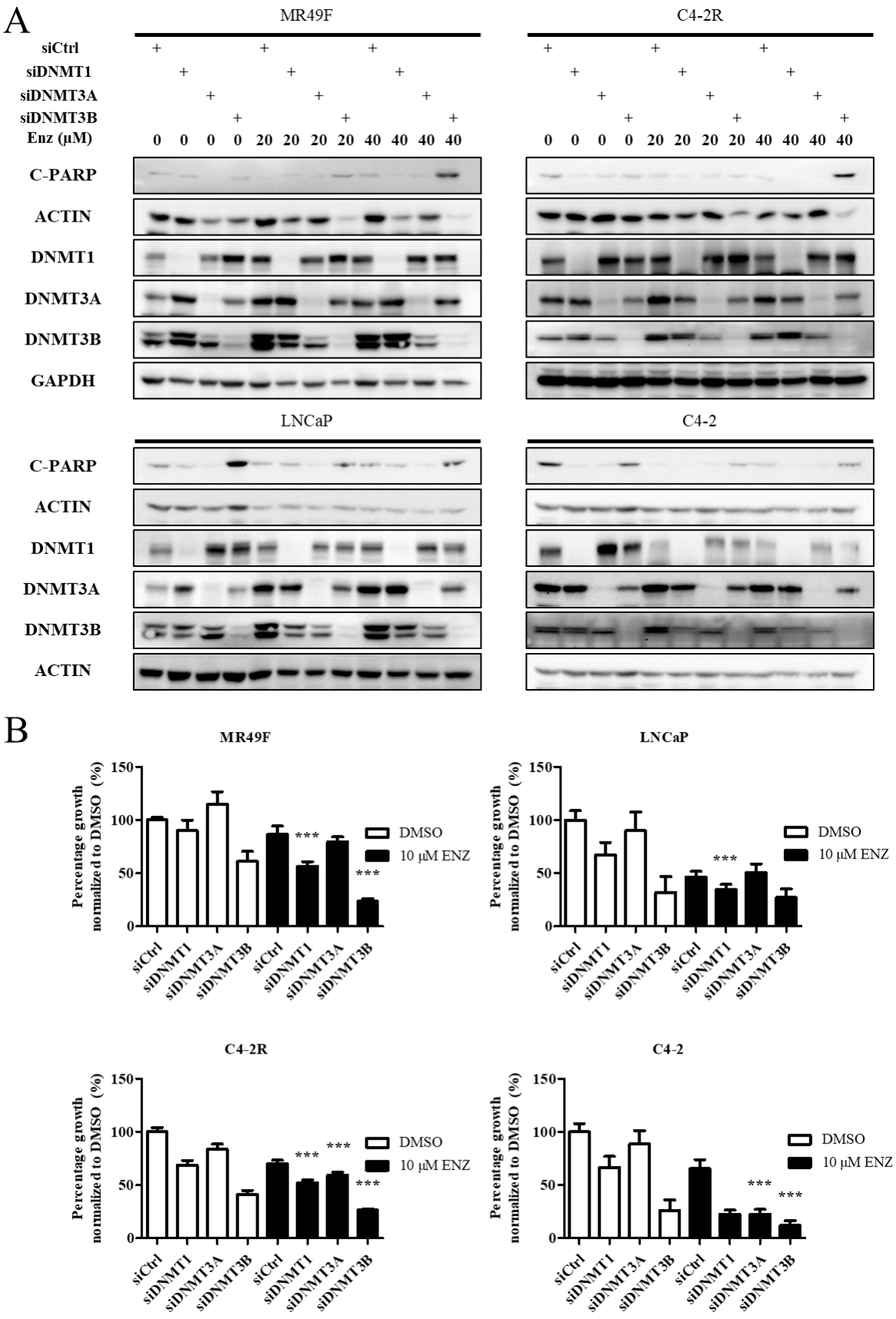

A
MR49F
C4-2R
Decitabine (nM)
0 2501000 0 2501000 0 2501000 0 2501000
ENZ (μM)
0 0 0 10 10 10 20 20 20 40 40 40
C-PARP
GAPDH
DNMT1
DNMT3A
DNMT3A
DNMT3B
GAPDH
C-Casp3
GAPDH
LNCaP
C4-2
Decitabine (nM)
0 2501000 0 2501000 0 2501000 0 2501000
Enz (μM)
0 0 0 10 10 10 20 20 20 40 40 40
C-PARP
GAPDH
DNMT1
DNMT3A
DNMT3B
GAPDH
B
MR49F
LNCaP
C4-2R
C4-2
Percentage growth normalized to DMSO (%)
150
100
50
0
***
DMSO
250 nM Dec
500 nM Dec
1000 nM Dec
10 μM ENZ
Combo1
Combo2
Combo3
DMSO
250 nM Dec
500 nM Dec
1000 nM Dec
10 μM ENZ
Combo 1
Combo 2
Combo 3
DMSO
500 nM Dec
750 nM Dec
1000 nM Dec
2.5 μM
Combo1
Combo2
Combo3
DMSO
250 nM Dec
500 nM Dec
1000 nM Dec
2.5 μM ENZ
Combo 1
Combo 2
Combo 3

Figure 4.6. The combination of DNMT3B knockdown and enzalutamide induces and increase in apoptosis and a decrease in cell growth in MR49F and C4-2R cells

A, LNCaP, MR49F, C4-2 and C4-2R cells were transfected with siCtrl, siDNMT1, siDNMT3A and siDNMT3B followed by treatment with DMSO (0) or 20 or 40 µM enzalutamide for 3 days. Cells were then collected, and proteins extracted followed by western blot analysis of Cleaved-PARP, DNMT1, DNMT3A and DNMT3B. GAPDH and ACTIN were used as loading controls. B, LNCaP, MR49F, C4-2 and C4-2R cells were seeded in 96 well plates followed by transfection with siCtrl, siDNMT1, siDNMT3A and siDNMT3B. Cells were then treated with treatment with 10 µM enzalutamide. Cells were subjected to MTT assay when control wells became confluent and O.D. was measured at 570 nm. Percentage growth was normalized to siCtrl/DMSO-treated samples. Two-way ANOVA was used to evaluate statistical significance ***$p<0.001$.


A
MR49F
C4-2R
siCtrl
siDNMT1
siDNMT3A
siDNMT3B
Enz (μM)
0 0 0 0 20 20 20 20 40 40 40 40
C-PARP
ACTIN
DNMT1
DNMT3A
DNMT3B
GAPDH
LNCaP
C4-2
B
MR49F
LNCaP
C4-2R
C4-2
Percentage growth normalized to DMSO (%)
DMSO
10 μM ENZ
siCtrl
siDNMT1
siDNMT3A
siDNMT3B
***

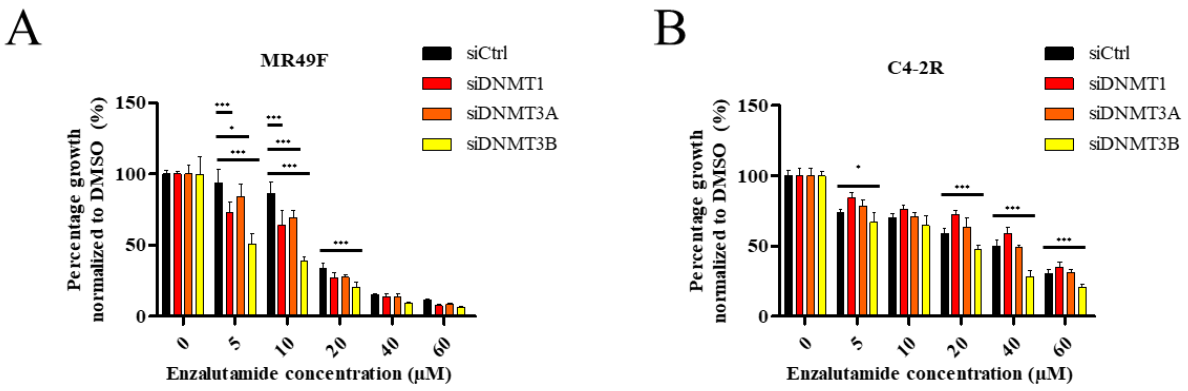


Figure 4.7. Knockdown of *DNMT3B* induces a decrease in cell growth in response to increasing concentrations of enzalutamide in resistant cells

MR49F (A) and C4-2R (B) cells were seeded in 96 well plates followed by transfection with siCtrl, siDNMT1, siDNMT3A and siDNMT3B. Cells were then treated with DMSO (0) or 5, 10, 20, 40 or 60 µM enzalutamide Cells were subjected to MTT assay when control wells became confluent and O.D. was measured at 570 nm. Percentage growth was normalized to DMSO-treated samples. Two-way ANOVA was used to evaluate statistical significance *p<0.05, **p<0.01 and ***p<0.001.

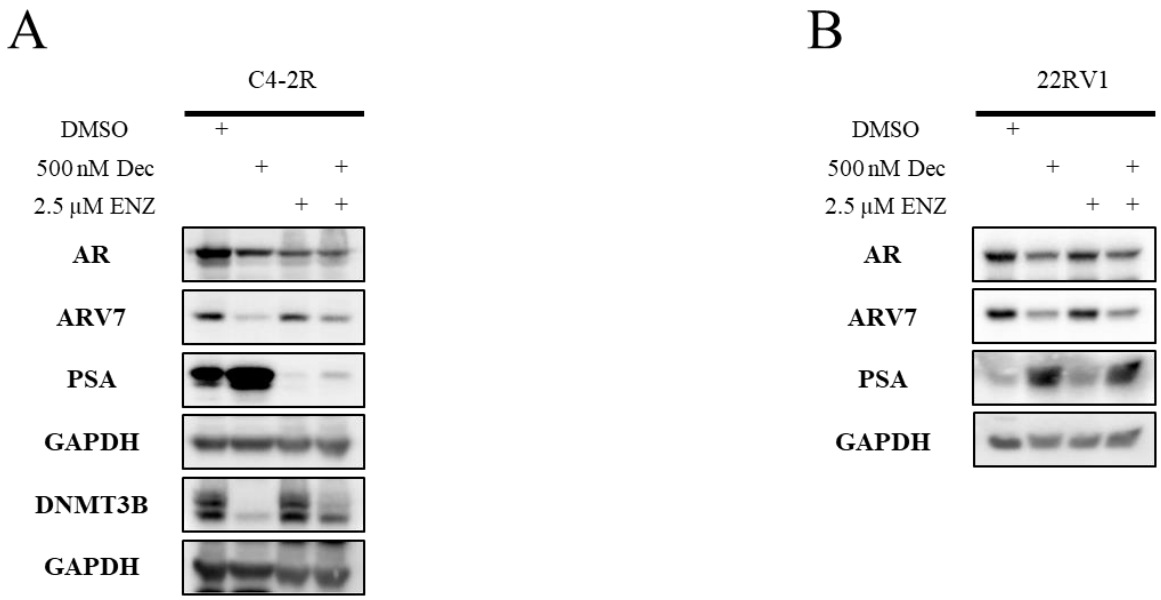
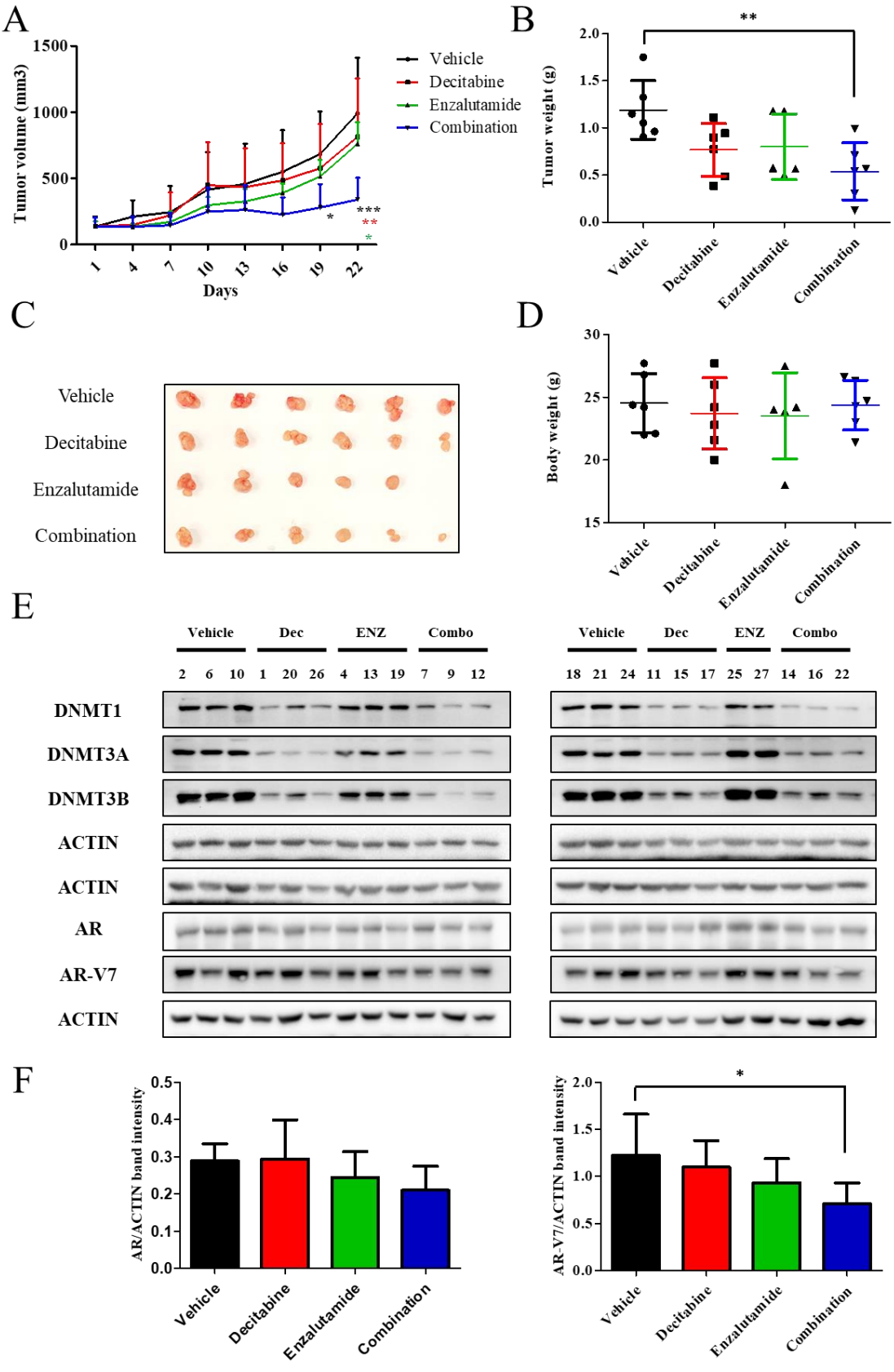


Figure 4.8. Decitabine treatment decreases AR and AR-V7 expression in prostate cancer cells

C4-2R (A) and 22RV1 (B) cells were treated with DMSO, 500 nM decitabine, 2.5 µM enzalutamide or both for 5 days. Cells were then collected, and proteins were extracted followed by western blot analysis for AR, AR-V7, PSA, DNMT3B. GAPDH was used as a loading control.

Figure 4.9. Inhibition of DNA methylation in combination with enzalutamide induces a decrease in tumor growth in a 22RV1 xenograft model.

24 castrated NRG mice were inoculated with 750,000 22RV1 cells with Matrigel. Mice were then randomized in 4 different groups. Mice received a vehicle control treatment, decitabine 0.5 mg/kg, enzalutamide 20 mg/kg and the combination of decitabine and enzalutamide. Mice received treatment 4 days on and 2 days off. A, Tumor volume was measured every 3 days. B, Tumor weight was measured using a standard scale upon necropsy. C, A representative picture of the tumors upon necropsy. D, General toxicity was assessed by body weight measurement at day 22 before necropsy. E, Proteins were extracted from tumor followed by western blot analysis for DMNT1, DNMT3A, DNMT3B, AR and AR-V7. ACTIN was used as a loading control. F, AR and AR-V7 bands were quantified using ImageJ software. Two-way ANOVA was used to evaluate statistical significance *$p<0.05$, **$p<0.01$ and ***$p<0.001$. Two-tailed student t-test was used to evaluate statistical significance *$p<0.05$ and **$p<0.01$.


A
Tumor volume (mm3)
1500
1000
500
0
1
4
7
10
13
16
19
22
Days
Vehicle
Decitabine
Enzalutamide
Combination
***
**
*
B
Tumor weight (g)
2.0
1.5
1.0
0.5
0.0
**
Vehicle
Decitabine
Enzalutamide
Combination
C
Vehicle
Decitabine
Enzalutamide
Combination
D
Body weight (g)
30
25
20
15
Vehicle
Decitabine
Enzalutamide
Combination
E
Vehicle
Dec
ENZ
Combo
2 6 10 1 20 26 4 13 19 7 9 12
Vehicle
Dec
ENZ
Combo
18 21 24 11 15 17 25 27 14 16 22
DNMT1
DNMT3A
DNMT3B
ACTIN
ACTIN
AR
AR-V7
ACTIN
F
AR/ACTIN band intensity
0.5
0.4
0.3
0.2
0.1
0.0
Vehicle
Decitabine
Enzalutamide
Combination
AR-V7/ACTIN band intensity
2.0
1.5
1.0
0.5
0.0
*
Vehicle
Decitabine
Enzalutamide
Combination

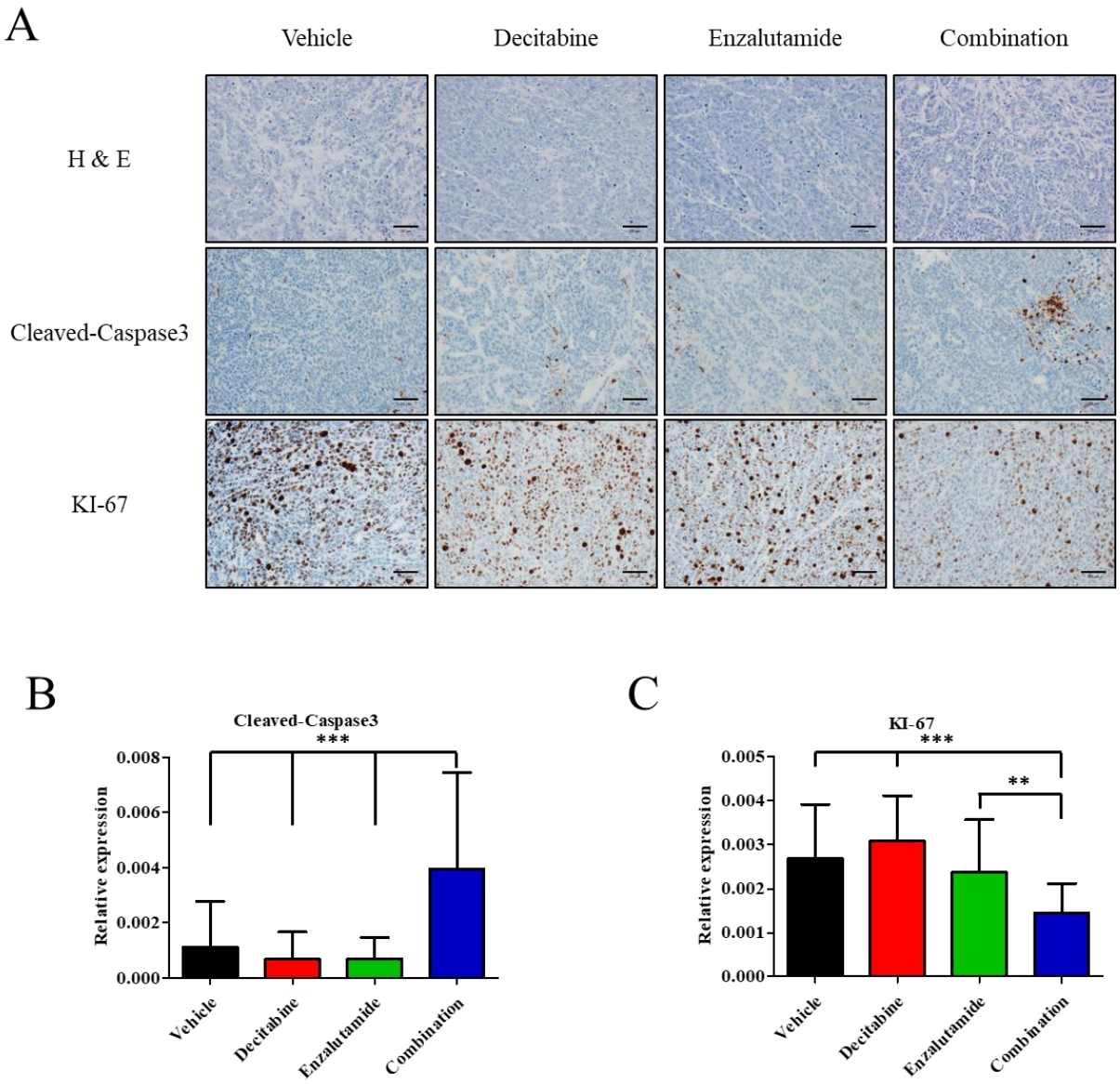


Figure 4.10. Decitabine and enzalutamide combination induces and increase in apoptosis and decrease in cell proliferation in 22RV1 xenograft tumors.

Tumors were sectioned at a thickness of 4 micrometer and stained with Cleaved-Caspase3 and KI-67. A, Representative images of slides stained with H & E, Cleaved-Caspase3 and KI-67 from each treatment group. B, Quantification of Cleaved-Caspase3 relative staining intensity. C, Quantification of KI-67 relative staining intensity. Staining intensity was quantified from at least 8 images per tumor slide. Two-tailed student t-test was used to evaluate statistical significance *$p<0.05$ and **$p<0.01$.

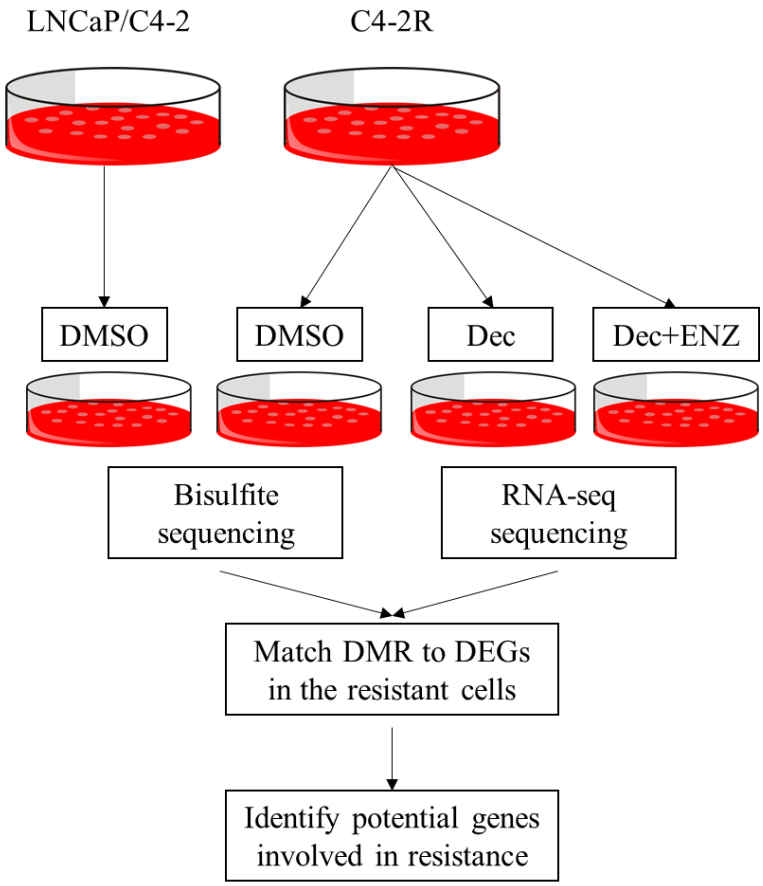


Figure 4.11. Scheme depicting the comparison between enzalutamide-sensitive and enzalutamide resistant cells and the comparison between enzalutamide-resistant cells pre and post decitabine treatment using RNA-sequencing and Bisulfite sequencing.

Table 4.1 Table illustrating the different comparison groups for the RNA-sequencing and Bisulfite sequencing studies.

In C1 we compared LNCaP DMSO to C4-2R DMSO, where LNCaP was the control group. In C2 we compared C4-2 DMSO to C4-2R DMSO, where LNCaP was the control group. In C3 we compared C4-2R DMSO to C4-2R 500 nM Dec, where C4-2R DMSO was the control group. In C4 we compared C4-2R DMSO to C4-2R Combo, where C4-2R DMSO was the control group.

Comparison ID	**Comparison info**	**Treatment group**	**Control group**
C1	C4-2R DMSO vs. LNCaP DMSO	C4-2R DMSO	LNCaP DMSO
C2	C4-2R DMSO vs. C4-2 DMSO	C4-2R DMSO	C4-2 DMSO
C3	C4-2R 500 nM Dec vs. C4-2R DMSO	C4-2R 500 nM Dec	C4-2R DMSO
C4	C4-2R Combo vs. C4-2R DMSO	C4-2R Combo	C4-2R DMSO

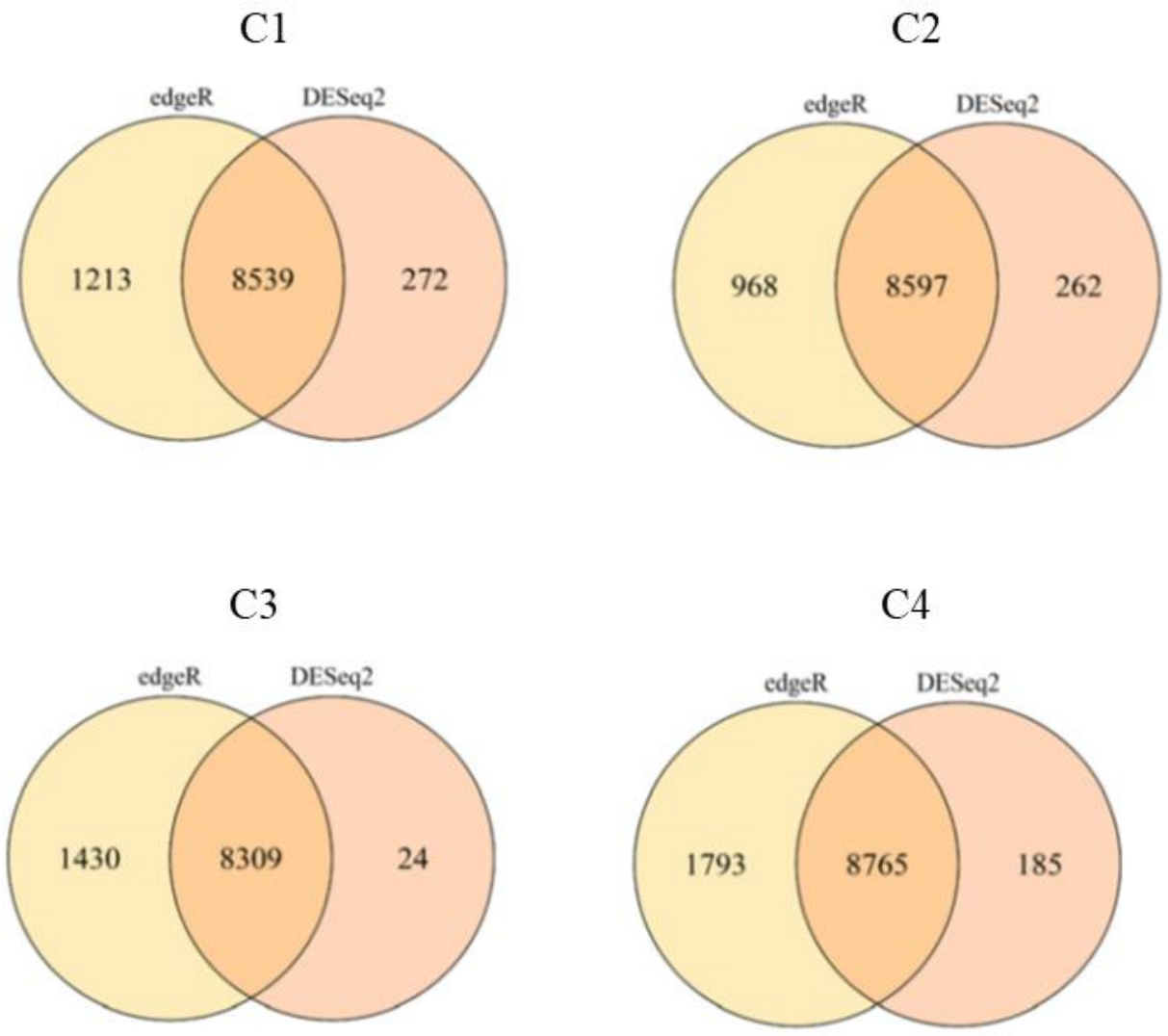


Figure 4.12. Venn diagram showing an overlap of DE (FDR ≤ 0.05) genes between DESeq2 and edgeR methods.

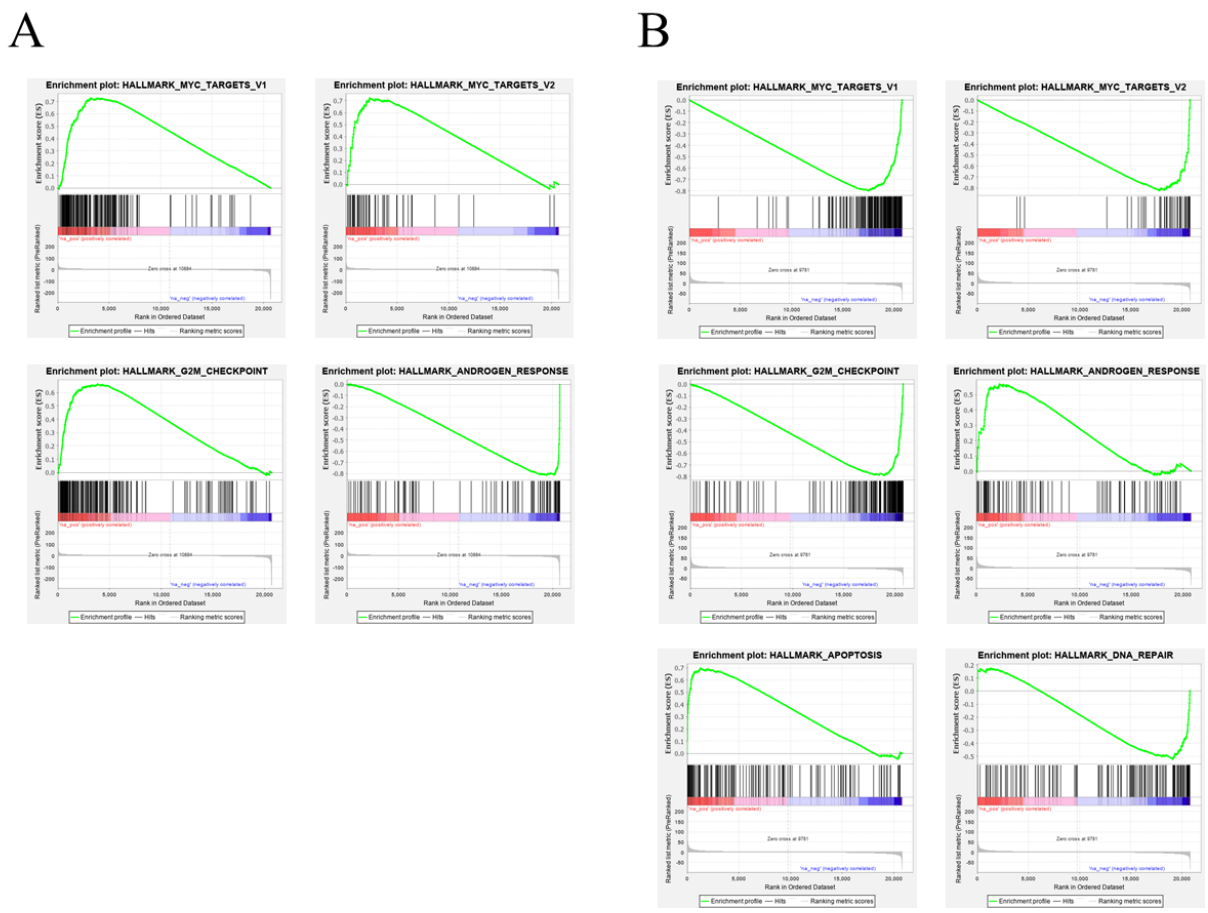


Figure 4.13. Decitabine treatment of C4-2R cells reverses the enrichment of critical gene sets within the HALLMARK set of genes.

A, GSEA from the HALLMARK gene sets in the C2 comparison (C4-2 vs. C4-2R). B, GSEA from the HALLMARK gene sets in the C3 comparison (C4-2R DMSO vs. C4-2R 500 nM Dec).

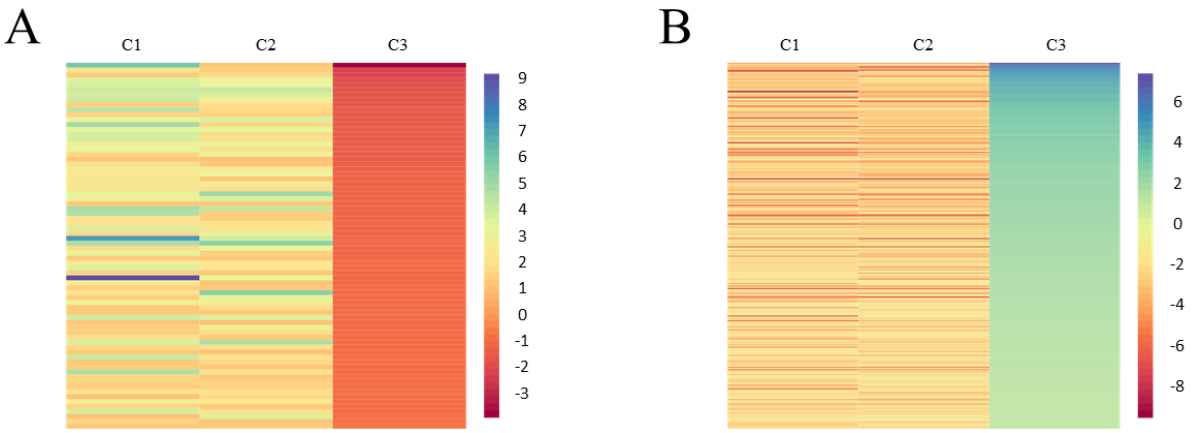


Figure 4.14. Decitabine treatment of C4-2R cells reverses the expression of key genes with potential role in enzalutamide resistance.

A, Heatmap depicting the expression of genes that have a log2FC>1 in C1 and C2 and a log2FC<-1 in C3. B, Heatmap depicting the expression of genes that have a log2FC<-1 in C1 and C2 and a log2FC>1 in C3. Gene sets were separated in Excel followed by heatmap generation on Diplayr.com.

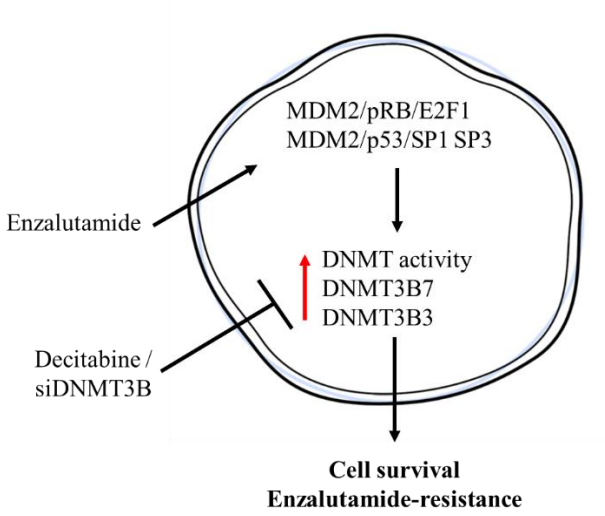


Figure 4.15. The effect of the enzalutamide-mediated increase in DNMT3B expression can be attenuated by the targeting of DNA methylation or DNMT3B in prostate cancer cells.

CHAPTER 5. FUTURE DIRECTIONS

5.1 Investigate the involvement of ADAM family members in enzalutamide resistance

In our first study, we show that ADAM10 and ADAM17 are overexpressed in prostate cancer enzalutamide-resistant cell lines compared to their sensitive counterparts (Fig.3.3). Additionally, our bioinformatics data shows significant changes in the expression of around 20 family members of the ADAM and ADAM with thrombospondin motifs (ADAMTS) in enzalutamide-resistant lines. Moreover, our results suggest that ADAM10 is directly promoting an increase in the expression of the active form of Notch in our resistant cells (Fig.3.3). ADAM10 and ADAM17 play a regulatory role in three pathways that we have identified to be involved in resistance to enzalutamide in prostate cancer; the Wnt/β-catenin pathway (354), the Notch signaling pathway (407), and the Ephrin signaling pathway (408). Maretzky *et al*. and Reiss *et al.* have shown that ADAM-10 mediated cleavage of E-Cadherin and N-Cadherin at the cell surface results in a change in the cellular localization of β-catenin (409, 410). These events play a role in promoting the activation of β-catenin signaling, which we found to be involved in enzalutamide resistance. As we have mentioned in Chapter 3, ADAM10 and ADAM17 are involved in the S2 cleavage step of Notch cleavage leading to the activation of the signaling pathway, which promotes an enzalutamide resistance phenotype in prostate cancer cell lines. Finally, ADAMs 10, 12, 13 and 17 have been shown to play a role in the cleavage of Ephrins leading to the regulation of the signaling pathway (411).

There is evidence suggesting an interplay between androgens and the expression and translocation of ADAM and ADAMTS proteins. These results show that androgen treatment promotes the nuclear translocation of ADAM10 to the nucleus. In the nucleus, ADAM10 is colocalized with the AR and present in an aggressive subset of prostate cancer tumors (412). On the other hand, ADAMTS15 expression correlates negatively with exposure of prostate cancer cells to androgens (413). All of the observations above warrant a deeper investigation of the effect of enzalutamide treatment on the different members of the ADAM and ADAMTS families. These results will shed light on the underlying mechanisms by which the expression of these metalloproteinases is changing after the onset of resistance. Also, further experiments will help us better understand the

involvement of the different ADAM proteins in promoting enzalutamide resistance, resulting in novel therapeutic approaches.

5.2 Investigate the mechanisms by which differentially methylated genes promote enzalutamide resistance in prostate cancer

In our second study, we show that multiple candidate genes may be playing a role in the onset or progression of enzalutamide-resistant prostate cancer in our cell lines (Fig.4.14). As future steps, we aim on identifying the role of differentially methylated genes in the survival of enzalutamide-resistant prostate cells. To do so, we will explore the DNA methylome data generated after the analysis of differentially methylated regions in the genome. These data combined with the RNA-sequencing data and basic functional experiments will help us uncover additional mechanisms by which prostate cancer cells can adapt after prolonged enzalutamide treatment.

As mentioned in our results in Chapter 4, we were able to identify several genes that may contribute to enzalutamide resistance, to increased cell survival and to decreased apoptotic response in prostate cancer cells. The change in the expression of these genes and its correlation with a better response to enzalutamide led us to believe that these genes are good candidates to pursue. For example, *MYB* expression increases in C4-2R cells compared to LNCaP and C4-2 cells. Following decitabine treatment of C4-2R cells the expression of *MYB* significantly decreases leading us to hypothesize that this gene may be linked to the onset or progression of enzalutamide resistance. In support of this data, Srivastava e*t al.* show that the overexpression of MYB supports a castration-resistant phenotype in prostate cancer cells (400). The knockdown to MYB in prostate cancer lines promotes a pro-apoptotic response and induces cell cycle arrest, responses like what was seen post-decitabine treatment in C4-2R cells and in accordance with our results. In another study, Li *et al.* show that c-MYB compensates to the loss or inhibition of AR by sharing an array or downstream target genes, with the nuclear receptor, involved in DNA damage repair (414). It is also shown that a synergy exists between the targeting of MYB or its downstream genes in combination with the inhibitors of other DNA damage response pathways leading to *in vitro* and *in vivo* cytotoxic response in CRPC lines. Since decitabine is well-known to induce significant negative regulation on DNA repair genes, silencing of MYB alongside other DNA damage response genes can potentially be another mechanism to targeting enzalutamide-resistant prostate cancer. To

investigate whether MYB is one of the drivers of enzalutamide resistance and one of the key genes to promote response to enzalutamide after decitabine exposure, a series of experiments need to be done. First, the methylation status and protein expression levels of MYB need to be investigated in C4-2R cells compared to LNCaP and C4-2 to confirm that DNA methylation is involved in the overexpression of the gene and to confirm that the overexpression is maintained at the protein level. Then, knockdown of MYB, using shRNA targeting, in C4-2R should be investigated with and without exposure to enzalutamide. If MYB overexpression promotes enzalutamide resistance, then knockdown of the gene in C4-2R should promote an increased response to enzalutamide in prostate cancer cells. Finally, the overexpression of MYB in combination with decitabine and enzalutamide treatment will uncover whether MYB is necessary to promote the synergistic effects of this combination in enzalutamide-resistant prostate cancer cell lines.

On the other hand, the change in the expression of *ALPK2* and *AIFM2* should be also investigated following enzalutamide-resistance onset as they can be key regulators of the apoptotic response to the antiandrogen in prostate cancer cells. ALPK2 has been shown to play a pro-apoptotic role in luminal colorectal cancer cells (415). Yoshida *et al.* observed that the knockdown of *ALPK2*, using RNA interference, decreased the levels of cleaved-caspase3 and reduced the expression of DNA repair genes. On the other hand, *AIFM2* is induced following stimulation with DHT in HPr-1AR prostate cancer cells (416). AIFM2 is known to promote apoptotic response in different cancer models, however, the apoptosis-inducing mechanism of this protein is still unknown (416, 417). To investigate the implication of ALPK2 and AIFM2 in the response to the combination therapy in C4-2R enzalutamide-resistant prostate cancer cells, it is important to investigate the methylation status of these genes and validate their protein expression in our cell lines. Following the validation experiments, functional assays should be performed to study the effect of the expression of these genes on enzalutamide response in prostate cancer. Knockdown of *ALPK2* and *AIFM2* will be executed in enzalutamide-sensitive cell lines LNCaP and C4-2 followed by the investigation of enzalutamide response in these lines. To study whether ALPK2 and AIFM2 play a direct role in the synergistic effects of the combination of decitabine and enzalutamide, the two proteins will be overexpressed in C4-2R cells followed by enzalutamide treatment and apoptotic response assessment. Also, *ALPK2* and *AIFM2* will be knocked down in C4-2R cells followed by decitabine and enzalutamide treatment and apoptosis will be tested to determine whether the expression of

these genes is necessary for the apoptotic response induced by the combination therapy. These experiments will help us uncover the specific mechanisms by which DNA methylation and DNMT3B overexpression play a role in promoting enzalutamide resistance and will also help us rationalize the combination of decitabine and enzalutamide in prostate cancer.

More experiment should be executed to investigate the mechanism by which enzalutamide promotes the overexpression of DNMTs in prostate cancer cell lines. Our results show that enzalutamide treatment promotes an increase in DNMT3B expression in prostate cancer cells through the p53 or pRB pathways, where we also highlighted a role for the MDM2 E3 ubiquitin ligase. In future experiments we will explore the role of MDM2, p53 and pRB in the enzalutamide-mediated increase of DNMTs by further understanding the impact of enzalutamide treatment on the expression of MDM2, p53 and pRB. The regulation of AR by MDM2-mediated ubiquitination is well documented and studied in several studies (418, 419). However, the role of the AR in the regulation of MDM2 and the implications on its downstream targets is poorly understood. Tovar *et al*. show that the treatment with the MDM2 inhibitor promotes an amplified response to androgen ablation therapy, which aligns with our model (420). The direct impact of enzalutamide treatment on the expression of the different players within this pathway and its effects on DNMTs expression is still poorly understood. In future experiments, we will investigate the role of androgen receptor, specifically enzalutamide treatment, in promoting the expression of MDM2 or inhibiting the expression of p53 and pRB. Chip-seq experiment can be done to investigate the direct binding of the AR on regulatory regions of these genes. If the regulation is not at the transcript level, functional assays to confirm that the regulation is at the protein level by influencing the degradation process of these proteins. Stability of MDM2, p53 and pRB can be assessed after enzalutamide exposure in prostate cancer cells. These experiments will better identify the mediator of the enzalutamide effect on the expression of the DNA methyltransferases.

Our data demonstrated that decitabine treatment produced a significant decrease in AR-V7 expression in enzalutamide resistant lines. However, the direct mechanism by which decitabine treatment is involved in this process is still unknown. In the next experiments we will test whether decitabine treatment directly impact AR-FL and AR-V7 transcript levels. This experiment will help us identify if decitabine treatment selectively affects the variant forms of AR or the overall

AR-FL mRNA, thus abrogating alternative splicing pathways. If AR-V7 is exclusively impacted by decitabine we will examine the DNA methylation patterns on the AR gene and test hypotheses linking DNA methylation to alternative splicing and AR-V7 levels. The increase in alternative splicing factors is remarkable according to our bioinformatics data. The reversal of this signature following treatment of C4-2R cells with decitabine led us to hypothesize that the splicing machinery might be a contributor to the changes in AR-V7 expression following DNA methylation inhibition. Identification of specific factors and proteins, within the alternative splicing pathway, may hold value in better understanding the regulation of AR-V7 in prostate cancer cells.

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