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Molecular Pathways Driving Enzalutamide Resistance in Prostate Cancer: Roles of Notch Signaling and DNA Methyltransferases

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ABSTRACT

Prostate cancer is the second leading cause of cancer-related death among men in the United States. The androgen receptor (AR) antagonist enzalutamide is an FDA-approved therapy for patients with late-stage prostate cancer and is currently under clinical investigation for the treatment of early-stage disease. Although patients often show an initial favorable response to enzalutamide, tumors inevitably develop drug resistance. In this review, we summarized current knowledge of prostate cancer, with a particular focus on disease diagnosis, AR signaling, and available treatment options. We provided an in-depth discussion of enzalutamide, a major therapeutic agent for castration-resistant prostate cancer, emphasizing both AR-dependent and AR-independent mechanisms of resistance. We also reviewed the Notch signaling pathway, including Notch family members, mechanisms of pathway activation, its role in prostate cancer progression, and crosstalk between Notch and AR signaling. In addition, we discussed DNA methylation, covering the structure and function of DNA methyltransferases, the role of DNA methylation in development and adult tissues, its involvement in prostate cancer, its regulation of key cellular pathways and processes, and its contribution to drug resistance. Finally, we highlighted our recent findings on the roles of Notch signaling and DNA methyltransferases in enzalutamide resistance in prostate cancer.

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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). 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]. Neuroendocrine cells, a rare type of neurotransmitter cells located at the basement membrane, ensure signal transmission through secretion of neuropeptides and hormones [3]. 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 [4].

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 [5]. In the developed prostate, androgen ablation induces apoptotic death in the luminal cell population, leading to prostate shrinkage [6]. Re-introduction of testosterone to the environment rescues the lumen involution and induces a reconstruction of secretory structures by stimulation of luminal cell growth [7]. 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 [8]. 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 [9]. 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 [10]. The flexible hinge region, separating the DBD and LBD, plays a role in the transactivation and the intracellular localization of the receptor [11]. 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 [12].

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 [9]. 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 [12]. 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 [9]. 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 [13].

Prostate cancer is the most diagnosed cancer among men in the United States of America, with an estimated 313,780 new cases in 2025. It is the second leading cause of cancer-related deaths in men in the U.S., with an estimated 35,770 deaths in 2025 [14]. One in six 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. Other risk factors for developing prostate cancer are African ancestry, family history of the disease, and inherited genetic predispositions [15].

Most diagnosed prostate cancers are adenocarcinomas, originating from epithelial cells within the prostate gland. It is strongly believed that luminal cells are 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 form a tumor after the loss of Phosphatase and Tensin Homolog (PTEN) [16]. 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 [17]. Prostatic intraepithelial neoplasia (PIN) is a precursor lesion within the epithelium of the prostate that may progress to invasive prostate carcinoma. High-grade PIN is characterized by an overgrowth of luminal cells with enlarged, prominent nuclei within an intact prostate architecture [18]. These lesions are non-invasive, preserving a normal layer of basal cells and an intact basement membrane [19]. 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 [20].

In 1970, Wang and Valenzuela discovered the prostate-specific antigen (PSA), a glycoprotein encoded by the kallikrein-3 (KLK3) gene. PSA is exclusively expressed in the luminal cells within the prostate gland, playing a role in sperm motility and dissolving cervical mucus [21]. 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 or specific. High PSA levels can be detected when PSA production increases or when destruction of a tissue layer sep-

arating the duct from the bloodstream occurs [22]. This can happen in patients with benign prostatic hyperplasia, prostatitis, urinary retention, or injury [23]. Also, some subtypes of prostate cancer won't manifest any PSA increase, and it 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, family history questionnaires, and digital rectal examination (DRE) preceding a targeted biopsy are still the recommended guidelines for early detection of prostate cancer.

Molecular and genotype profiling are being leveraged to improve screening, diagnosis, and prognosis of patients. Evaluating chromosomal abnormalities, sequence mutations, gene amplification, epigenetic changes, and metabolite 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 [24], the TMPRSS2:ERG gene fusion [25], activation of the PI3K/AKT pathway by loss of PTEN [26], germline mutations in HOXB13 [27], and hypermethylation of different gene promoters, including GSTP1 and AR [28]. 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, and metastasis in patients receiving or not receiving any treatments [29]. The Gleason score, established by the pathologist Dr. Donald Gleason following a study, is incorporated into all staging schemes currently used in the clinic to stage prostate cancer [30]. It is a grading score based on the histology and architectural patterns found in a prostate tumor [30]. The Gleason score is a formula combining two numbers depicting the grade of the two most common patterns within a biopsy, added together to 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 [30]. 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 [29]. According to this system, grade 1 includes all prostate cancers with Gleason scores of 6 or less, which are indolent cancers requiring only active surveillance. Grades 2 and 3 encompass Gleason scores 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 [28]. 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), clinicians 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 a 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 [31]. 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 [32]. 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 [33]. On the other hand, 3 other studies conducted in the U.S. and the U.K., comparing surgery, active surveillance, and radiotherapy, show no significant differences in mortality or metastatic lesions in the patient population [32]. The intermediate-risk group encompasses patients with clinical stages of T2b to T2c, a Gleason score of 7, or a 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 > 20 ng/mL, Gleason score ≥ 8, or a clinical examination ≥ 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 [34]. 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 [35]. 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 [36]. 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).

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 a castrate level of testosterone (< 50 ng/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 (mCRPC) 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 of mCRPC patients. In the TAX-327 trial, docetaxel, compared to the palliative agent mitoxantrone, demonstrated a survival advantage (18.9 months vs 16.5 months), a decrease in PSA levels, and improvements in the quality of life and pain in patients with mCRPC [37]. 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 [38]. 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 [39]. Other approaches have emerged as first and second-line chemotherapeutic treatments for mCRPC patients with substantial toxicity profiles [40].

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) [41]. 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 [42]. 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 [43].

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-hydroxy-progesterone, two critical precursors of testosterone [44]. Inhibiting this enzyme with the selective and irreversible inhibitor, Abiraterone acetate, blocks the synthesis of androgens and 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 [45]. Furthermore, abiraterone acetate was tested against a

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 [43].

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 a 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) [46].

Enzalutamide was developed to fill the need for an AR antagonist that has a 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 screening and testing in cell lines and tumor xenograft 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 mutation that confers resistance to bicalutamide [47]. 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 [48]. 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 [49]. These results prompted the FDA to approve the use of enzalutamide in men 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 the control group and a median overall survival of 35.3 months compared to 31.3 months, respectively [50]. 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 [51]. 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), and time to PSA progression [52]. Enzalutamide plus ADT was compared to placebo plus ADT in ARCHES, a phase III trial in men with metastatic castration-sensitive prostate cancer (mCSPC). Another phase III trial 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 [53]. This data resulted in the FDA-approval of enzalutamide for the treatment of mCSPC in December 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 [54]. These trials will shed 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 [55]. 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) [46]. Primary resistance to enzalutamide correlates with the expression of AR mutants or, more importantly, with the expression of AR-V7. 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) who 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 [46].

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.

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. 80% of CRPC patients exhibit overexpression of the AR protein; a significant portion of this observation is due to gene amplification [56]. Enzalutamide-treated patients have a higher frequency of AR overexpression, rendering it a potential resistance mechanism [57]. 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 [58]. 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 [59].

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 [60]. These mutations may play a role in the primary resistance to enzalutamide or may arise after enzalutamide treatment. Most of the observed mutations are 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). F877L and T878A are also mutations that affect the sensitivity of prostate cancer cells to enzalutamide in both in vivo and in vitro settings [61]. Furthermore, circulating free DNA from patients progressing on enzalutamide therapy has been found to harbor F877L/T878A and M896V/S889G double mutants [58].

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 ARv567es 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 [62]. ARv567es is characterized by the loss of exons 5, 6, and 7 in addition to a frameshift, early stop codon in exon 8 [63]. The expression of these 2 variants is associated with resistance to enzalutamide and abiraterone in patients and in research models [64]. Furthermore, AR-V7 levels were correlated with poorer survival and outcomes in patients with mCRPC [65]. In the 22RV1 cell line, which 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 [66].

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 induce an increase in GR expression and activation. GR shares common features with AR. Both receptors have homologous DBDs, which enable them to have similar binding sequences, resulting in overlap in their transcriptomes. 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 [67].

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 [68]. To further establish its implication in enzalutamide resistance, in vitro and in vivo, the expression of PD-L1 was found to be upregulated. In addition, in mouse xenograft studies harboring tumors that are enzalutamide resistant, PD-L1 expression was detectable in circulation [69]. These observations suggest

that cells can overcome AR blockades 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 who have enzalutamide-resistant mCRPC [70]. Preliminary results show a response in a subset of patients, suggesting that immune checkpoint blockade may hold promise for patients with primary or acquired resistance to enzalutamide [70].

In a cohort of patients having either neuroendocrine prostate cancer (NEPC) or mCRPC, it was shown that molecular and gene signatures were very similar between both groups, suggesting that NEPC can directly arise from CRPC [71]. Although NEPC makes up 1% of all diagnosed prostate cancers, 30% of mCRPC have an NEPC signature [72]. 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 [73]. 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 [74]. 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 [75]. In addition, overexpression of NMYC is a known NEPC driver that is regulated by the activity of AURKA. Targeting the pathway is under investigation in clinical trials for the treatment of NEPC patients [76].

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 [77]. 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. 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 [78]. 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 [79]. Multiple events, such as gene amplification, active Wnt-β-Catenin signaling, or deletion of FOXP3, can contribute to the upregulation in the levels of the oncogene in the prostate [75]. In a study by Grad et al., c-Myc regulates AR expression by direct binding to its regulatory region. In primary and CRPC samples, c-Myc levels positively correlate with AR signaling activity and AR-FL and AR-Vs mRNA levels [80]. 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 [81]. 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.

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, the 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 wings of flies [82]. 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 [83].

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

significant differences in their extracellular and intracellular domains (ECD and ICD) [84]. The receptors can bind to five different ligands: Jagged-1 and -2 and Death-like ligands 1, 3, and 4 [85]. 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 [86]. In the ECD, Notch receptors are composed of 29 – 36 homologous epidermal growth factor (EGF)-like tandem repeats, enabling their binding to ligand. 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) [87]. The NRR, alongside the transmembrane fragment (TMF), plays a crucial role in the activation of the pathway. 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 [88]. The intracellular portion of the receptor contains a CSL-binding domain RAM, a nuclear localization signal (NLS), a PEST sequence, and seven ankyrin-like repeats (ANK) [87]. 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 [88]. Alagille syndrome is a result of mutations of the DSL domain in Jagged1 [89]. Dll1, Jagged1, and Jagged2 possess a Delta and OSM-11-like protein (DOS) motif, essential for canonical Notch binding, and EGF-like repeats following the DSL domain. However, Dll3 and Dll4, lacking the DOS motifs, require a DOS-containing non-canonical ligand to ensure contact and activation of Notch receptors. Jagged1 and 2 contain a Cysteine-rich region following the EGF-like repeats [85].

Before their trafficking to the membrane, the Notch heterodimeric receptors are cleaved at the S1 cleavage site by Furin proteases in the trans-Golgi network. 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 [86].

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 [90]. The release of the ECD induces a conformational change in the NNR region, leading to the exposure of the S2 cleavage site [91]. Studies in Drosophila have demonstrated the necessity of ADAM10/Kuz in the cleavage at the S2 site and thus the activation of the pathway [92]. In general, ADAM metalloproteinases mediate cellular interactions with neighboring cells or with other components of the microenvironment [93]. To ensure ADAM-mediated proteolysis, membrane proteins need to be co-expressed with the metalloproteases and adopt a specific conformational state, priming their cleavage by ADAM. Studies have shown that recognition of ADAM targets is dependent on specific conformations rather than consensus sequences, leading to juxta-membrane cleavage [94]. Importantly, ECD shedding by mechanical pull ensures the conformational change in the remaining extracellular portion of the receptor, leading to cleavage by the ADAM proteolytic enzymes. Several metalloproteases of the ADAM family play a crucial role in Notch signaling, making them an essential player within the signaling pathway [95]. Abrogation of ADAM10 in fruit flies by the introduction of a dominant negative allele or by RNA interference hindered the proteolysis of the Notch receptor and signaling activation. 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 [96]. ADAM10 deficiency in mammalian cells abrogated the cleavage of Notch, which was not rescued by ADAM17. Furthermore, dominant negative expression of ADAM17 did not hinder the activation of the ligand-dependent Notch signaling pathway. In contrast, under conditions where Notch ligand-dependent activation was defective, including EDTA chelation and NRR mutants, ADAM17 was essential to activate the pathway [97]. 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 before the release of its ICD, Notch is cleaved at the S3 cleavage site by the γ-Secretase complex. 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. γ-Secretase localizes to the plasma membrane, late endosomes, and lysosomes [98]. In addition to the plasma membrane, co-localization of Notch and γ-Secretase can occur in late endosomes after endocytosis of the receptor [99], 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 is more stable than NICD with a Leucine or Serine, which can impact the sustainability of the signal. It has been shown that plasma membrane processing of Notch receptor favors production of the NICD peptides with Valine at its N-terminal. Whereas γ-Secretase-induced cleavage at the endosome membranes generates the degradation-susceptible NICD fragments. 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 to inhibit 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 [100].

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 gene promoters [101]. Upon its entry to the nucleus, NICD associates with CSL. The RAM-mediated association of NICD to CSL promotes the detachment of co-repressors. The RAM/ANK interface is recognized by Mastermind (MAM) [102]. The NICD/CSL/MAM complex recruits co-activators, histone acetyltransferases, and transcription machinery to promote the expression of Notch target genes [103]. NICD stability and expression are essential for the maintenance of Notch signaling and for 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. These modifications can influence the half-life of the cleaved protein and its ability to bind co-regulators [104]. 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 to YRPW motif 1 (Hey1), which are known to play transcription repressive roles [105]. Other important Notch target genes include c-Myc and cyclin D1 [106].

On the other hand, studies have shown that Notch can be associated with non-canonical ligands, influencing their activity. Furthermore, Notch can interact with alternative pathways, independent of ligand binding, to induce alterations 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 [74]. These observations demonstrate that Notch signaling is complex and different mechanisms and alternative pathways can contribute to its activation or inhibition.

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 is involved in determining cell-fate in the heart, brain, liver, pancreas, breast, and prostate [107].

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 [108]. 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. Also, the elimination of Notch1-expressing cells inhibited the regeneration of the castration-induced abrogation of the luminal cells in the prostate. 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 [109]. Also, knockout of RBPJ (aka CSL) decreased basal cell proliferation and survival in the embryonic and postnatal prostate. 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 [110]. 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 [111]. 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 [112]. In humans, a study by Yu et al and Zhu et al. shows that Notch1 and Jagged1 are overexpressed in prostate cancer samples and metastatic tumor samples compared to normal tissue. In contrast, Notch1-deficient murine prostates overexpress a multitude of prostate oncogenes such as c-Fos, c-Jun, PSCA, FGF18, and PTOV1 [113].

Examining the proliferative effects of Notch signaling in prostate cancer, data have shown a contradictory 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 [114]. PC3 cells overexpressing ectopic Dll4, compared to unaltered PC3 cells, showed an increase in growth rate of tumor xenografts. In contrast, overexpression of NICD in LNCaP, DU145, and PC3 prostate cancer cell lines halted the proliferation of these cells [115].

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 the anti-apoptotic protein Bcl-2. This resulted in a decrease in proliferation, an increase in apoptosis, and sensitivity to the chemotherapeutic docetaxel (116). Prostate cancer stem cells are hypothesized to be a tumor-initiating population of cells, characterized by therapy-resistance and cancer regeneration. The subpopulation of DU145 cells having prostate cancer stem cell characteristics has a high expression of several Notch signaling pathway proteins, such as Notch1, Jagged1, Dll1, and Dll3. 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 [116]. 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 [117].

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 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 the Notch pathway, which sets a precedent for prostate cancer, being an androgen-stimulated cancer with high similarities between the estrogen and androgen receptors [118]. 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 [119]. ABCC1 belongs to the ABC transporter family, inherently involved in resistance mechanisms by functioning as

a drug pump to eliminate intracellular pools of drug molecules, and is regulated by the Notch signaling pathway [120].

Other investigators have recently shown 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, an increase in apoptosis, and strong inhibition of AR and PSA. Cui et al. showed that exogenous expression of NICD in prostate cancer cells induces resistance to ADT and treatment with γ-Secretase inhibitors promotes sensitivity to ADT by acting through the p38/MAPK pathway and Bcl-2/Bax axis [120].

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 was downregulated, and Sel-1L and presenilin-1 were upregulated [121]. 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 [122]. 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.

DNA methylation

Eukaryotic DNA is characterized by the presence of methyl groups, predominantly on Cytosine molecules that are followed by a Guanine (CpG) [123]. 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 [124] and involved in X chromosome silencing and gene imprinting [125]. Despite being conserved through evolution, some organisms lack DNA methylation in their genomes. 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 [126]. 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.

The methyl group is deposited on the 5th carbon of the Cytosine (5mC) base by a group of enzymes named DNA methyltransferases (DNMTs) [127]. Of note, other methylated bases exist, such as N4-methylcytosine and N6-methyladenine [128]. 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. 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. DNMT3A and DNMT3B are de novo methyltransferases responsible for adding new methylation marks on unmethylated cytosines. DNMT3L is a non-canonical methyltransferase that lacks catalytic motifs [129].

All DNMTs contain an 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 [130]. The DMAP1 binding domain ensures the binding of DNMT1 to DMAP1 and Histone deacetylase 2 (HDAC2) in the S-phase. 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 [131]. Two smaller domains, the PCNA binding domain and the nuclear localization signal (NLS), are located between the DAMP1 binding domain and the RFTS domain. During the S-phase, RFTS targets DNMT1 to replica-

tion foci and deletion of RFTS abrogates this localization [132]. Replication-independent localization of the maintenance methyltransferase to the chromatin, specifically during the G2/M phase, is exhibited by its targeting sequence domain (TS). 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) [133]. The CXXC and BAH domains coordinate the recognition of unmethylated and hemi-methylated DNA and autoinhibit the catalytic activity of DNMT1. CXXC-binding to unmethylated DNA protects the DNA from any de novo methylation while allowing exclusive enzymatic activity on bound hemi-methylated DNA [134].

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 [133]. 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. Studies have also shown that the N-terminal sequence is required for the association of DNMT3 with intact nucleosome structures [135]. The PWWP domain guides DNMT3 to the body of transcribed genes by its binding to the trimethylated H3K36 mark [136]. Furthermore, the PWWP domain is involved in DNA binding and in targeting the enzymes to satellite repeats and pericentromeric DNA. The ADD domain enables the methylation of unmethylated H3K4-associated DNA by binding to the unmodified Lysine. This domain also has an autoinhibitory function by associating with the catalytic motifs of the enzyme when not bound to Histones [137].

Before cytosine methylation, the C-terminal domains of all DNMTs need to adopt a specific conformation to accept the methyl-group donor S-adenosylmethionine (AdoMet) [138]. The mechanism by which DNA is methylated requires the base to be flipped into the catalytic pocket of the enzyme following its synthesis [139]. 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.

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 the knockout of DNMT1 and DNMT3B induced embryonic lethality, and the knockout of DNMT3A caused death in mice at 4 weeks of age [139]. DNA methylation-mediated regulation of genes in development starts in germ cells with the erasure of DNA methylation marks by passive demethylation [140]. 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. 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 [141]. 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 the regulation of androgens and epithelial-mesenchymal interactions. The dynamic expression of DNMTs during the different stages of prostate development suggests that they play a role in the process [142]. 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. When treated with a DNA methylation inhibitor, the CHD1 promoter is hypomethylated, the protein is highly expressed, hindering prostate bud outgrowth [143]. In the UGS mesenchyme, where both AR and DNMTs are expressed, the promoter of AR exhibits changes in DNA methylation during development. Inhibition of DNA methylation results in an increased AR expression and increased sensitivity to androgens in the UGS mesenchyme [144]. 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 [144]. Different organs and tissues have different expression levels of DNMTs and their alternatively spliced variants. 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. DNMT3B3 plays a role similar to DNMT3L as a binding partner to catalytically active DNMTs, guiding them to genomic sites for DNA methylation [145]. DNMT3B exists in more than 30 isoforms generated by alternative splicing events, mainly affecting the C-terminal domain, rendering these isoforms inactive. 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 [146]. 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.

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. Studies have shown a relationship between DNA methylation and the initiation and progression of prostate cancer [147]. Aberrant hypermethylation of CpG islands in the promoter regions of tumor suppressor genes is one of the mechanisms believed to contribute to carcinogenesis. Normally, unmethylated CpG islands can become methylated by a deregulated DNA methylation pathway [148]. This leads to the silencing of tumor suppressor genes, which contributes to the malignant process. On the other hand, hypomethylation is more widespread within the genomes of cancer cells. Hypomethylation of normally methylated promoter regions of oncogenes and imprinted genes is observed in many cancers, including prostate cancer. Additionally, the hypomethylation of repetitive elements and retrotransposons can contribute to genetic instability, which can play a contributing role to malignancy [149].

The combination of abrogated DNA methylation events with genetic mutations and aberrations is believed to play a role in driving the initiation of prostate cancer [150]. 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 [151]. These studies show that mutation or loss of these genes is correlated with prostate cancer. Additionally, researchers generated mouse models that can develop spontaneous tumors by combining deletions of such tumor suppressor genes (151). 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 [152]. Studies by different groups have shown that active ERG promotes cell migration and invasiveness in prostate cancer [153]. It also plays a role in restricting the epithelial differentiation profile of prostate cells by binding to AR-regulated downstream targets [152].

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 [28]. Silencing of GSTP1 by hypermethylation is widely observed in PIN and cancerous lesions compared to normal prostatic tissue [153]. In normal conditions, GSTP1 plays a protective role against oxidative and xenobiotic stress-induced DNA damage in prostate cells; its loss is believed to play a tumor-promoting role [154]. 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. 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. In addition, loss of MGMT expression affects the sensitivity of tumors to chemotherapeutic agents [155]. These studies suggest that the expression of genes from DNA damage and repair response pathways may be compromised in the prostate because of DNA hypermethylation. Aberrant expressions of such genes may contribute to cancer initiation and progression, and therapy response.

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 these cell lines with a demethylating agent reverses the loss of AR expression by methylation [44]. Although the prevalence of AR hypermethylation is low in clinical samples, 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 [156]. The decrease in estrogen receptor expression correlates with poor prognosis [157]. Hypermethylation of the estrogen receptor promoter region was identified as the main mechanism by which silencing of both homologues occurs [154]. 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 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 [158], promotes the uncontrolled progression of the cell cycle that may contribute to carcinogenesis. Hypermethylation and silencing of CDK inhibitors such as CDKN1A, 1B, and 2A [159] 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 than 50% of prostate cancer samples [158]. The loss of RASSF1 promotes the oncogenic potential and inhibits the apoptotic effect of Ras proteins, leading to increased cell proliferation and survival [160]. These observations shed light on 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 were observed in up to 88% of prostate tumor samples compared to their normal controls [161]. 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 [162].

DNA methylation is also involved in the 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. 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 proportion of urine and tumor samples collected from prostate cancer patients [163]. 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 [164]. 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. 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 [165]. Global hypomethylation, illustrated by hypomethylation of LINE-1, was shown to be associated with the development and progression of prostate cancer and an increase in mortality in higher Gleason score patients [166]. On the other hand, targeted DNA hypomethylation to specific genes such as uPA, PLAU, CAGE, CYP1B1, and Ras oncogenes is observed in prostate cancer [140]. 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.

DNA methylation in drug resistance

Epigenetic mechanisms, especially DNA methylation, are believed to play an important role in drug resistance in cancer. Cancer therapeutics aim 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 overactivation 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 [167]. The ATP-binding cassette (ABC) transporters are a known family of proteins that play a pivotal role in multidrug resistance. The expression of ABC proteins correlates with the resistance to cancer therapies in different cancers [168]. 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. The expression of ABCB1 correlated with hypomethylation of its promoter region and with the development of a drug-resistant phenotype [169]. 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. The increase of ABCG2 expression was associated with a resistant phenotype [170]. 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 [168]. 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 by abrogating the expression of the 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. Overexpression of PTPN6 contributes to resistance in bortezomib, cisplatin, and melphalan-treated glioma cancer cells [171]. 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 [172]. 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 [173].

Moreover, hypermethylation of promoter regions of genes involved in apoptosis, cell cycle regulation, and DNA repair has been observed in drug-resistant cancer lines [173]. Silencing of pro-apoptotic genes such as DAPK1 and APAF-1 has been reported in multiple malignancies. DAPK1 is found silenced by genetic loss or by hypermethylation [174]. Low expression of DAPK1 correlates with poor prognosis and tumor recurrence in multiple cancer types [173, 174]. 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 [175]. The loss of another pro-apoptotic factor, APAF-1, hindered the p53 activation cascade in melanoma cells, leading to chemotherapy resistance (175). Chemotherapy sensitivity was restored after treatment with DNA methylation inhibitors that increased the expression of APAF-1 [176].

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 is associated with chemoresistance phenotypes in different cancers [177]. In contrast, hypermethylation-mediated silencing of MGMT and FANCF was found to correlate with chemosensitivity of cancer cells [178]. 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 com-

pared to cells with no pre-treatment [179]. 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 [180]. However, the role of specific DNA methyltransferases and specific DNA methylation changes has 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.

Our recent discoveries

In our studies, we discovered that NOTCH signaling and DNA methylation are 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 (181). 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 (182). 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.

Future Directions and Perspectives:

Despite substantial advances in understanding the individual roles of NOTCH signaling and DNA methyltransferases (DNMTs) in prostate cancer progression, their functional interplay, particularly in the context of enzalutamide resistance and mCRPC, remains incompletely defined. Future studies integrating these two epigenetic and developmental pathways are likely to yield important insights into therapy resistance, lineage plasticity, and tumor heterogeneity.

Emerging evidence suggests that NOTCH signaling promotes stem-like and basal/neuroendocrine features in advanced prostate cancer, phenotypes that are closely associated with resistance to AR–targeted therapies. DNMT-mediated DNA methylation, in parallel, contributes to stable transcriptional reprogramming by silencing luminal differentiation genes and reinforcing alternative lineage states. A key future direction will be to determine whether NOTCH activation directly reshapes the DNA methylation landscape through transcriptional or post-transcriptional regulation of DNMT1, DNMT3A, or DNMT3B.

Conversely, aberrant DNA methylation may regulate NOTCH pathway components themselves. Promoter methylation of NOTCH receptors, ligands (e.g., JAG1, DLL1), or downstream effectors (e.g., HES and HEY family genes) could establish epigenetically fixed NOTCH signaling states that persist even after AR pathway inhibition. Dissecting this bidirectional regulatory loop may explain the durable nature of enzalutamide resistance in CRPC.

Another important area for future investigation is whether DNMTs act as epigenetic "co-factors" for NOTCH-driven transcriptional programs. NOTCH intracellular domain (NICD)-mediated transcription may recruit DNMTs to specific genomic loci, leading to selective methylation and long-term repression of AR-regulated genes. Such a mechanism would provide a molecular basis for the irreversible loss of AR dependency observed in aggressive CRPC and neuroendocrine prostate cancer. Single-cell and spatial epigenomic approaches will be particularly powerful in defining how NOTCH-DNMT interactions contribute to intratumoral heterogeneity, enabling resistant subclones to coexist with AR-dependent cells under therapeutic pressure.

From a translational perspective, future studies should explore whether targeting DNMTs can disrupt NOTCH-driven resistance programs. DNMT inhibitors may re-sensitize tumors to enzalutamide by

reversing methylation-dependent silencing of luminal differentiation genes or by dampening NOTCH pathway activation. Conversely, combining NOTCH pathway inhibitors with epigenetic therapies could prevent or delay the emergence of therapy-resistant cell states. Importantly, identifying predictive biomarkers, such as NOTCH activation signatures coupled with DNMT expression or DNA methylation patterns, may help stratify patients who are most likely to benefit from such combination strategies.

Concluding Perspective

In summary, a deeper mechanistic understanding of NOTCH-DNMT crosstalk represents a critical future direction in prostate cancer research. Integrating developmental signaling pathways with epigenetic regulation offers a unifying framework to explain lineage plasticity, durable enzalutamide resistance, and disease progression in CRPC. Elucidating these interactions may ultimately lead to novel therapeutic strategies aimed at preventing or reversing resistance to AR-targeted therapies.

Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interest.

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