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Review

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Molecular Origin, Expression Regulation, and Biological Function of Androgen Receptor Splicing Variant 7 in Prostate Cancer

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Abstract
The problem of resistance to therapy in prostate cancer (PCa) is multifaceted. Key determinants of drug resistance include tumor burden and growth kinetics, tumor heterogeneity, physical barriers, immune system and microenvironment, undruggable cancer drivers, and consequences of therapeutic pressures. With regard to the fundamental importance of the androgen receptor (AR) in all stages of PCa from tumorigenesis to progression, AR is postulated to have a continued critical role in castration-resistant prostate cancer (CRPC). Suppression of AR signaling mediated by the full-length AR (AR-FL) is the therapeutic goal of all AR-directed therapies. However, AR-targeting agents ultimately lead to AR aberrations that promote PCa progression and drug resistance. Among these AR aberrations, androgen receptor variant 7 (AR-V7) is gaining attention as a potential predictive marker for as well as one of the resistance mechanisms to the most current anti-AR therapies in CRPC. Meanwhile, development of next-generation drugs that directly or indirectly target AR-V7 signaling is urgently needed. In the present review of the current literature, we have summarized the origin, alternative splicing, expression induction, protein conformation, interaction with coregulators, relationship with AR-FL, transcriptional activity, and biological function of AR-V7 in PCa development and therapeutic resistance. We hope this review will help further understand the molecular origin, expression regulation, and role of AR-V7 in the progression of PCa and provide insight into the design of novel selective inhibitors of AR-V7 in PCa treatment.

Introduction

Prostate cancer (PCa) is the second most frequently diagnosed cancer among men worldwide [1]. Conventional androgen deprivation therapy (ADT) has been the mainstay treatment for men with advanced PCa for many years [2]. However, disease progression and resistance to conventional ADT is inevitable, and the median overall survival for metastatic castration-resistant prostate cancer (CRPC) has remained <3 years [3]. With regard to the fundamental importance of the androgen receptor (AR) in all stages of PCa from tumorigenesis to progression, AR is postulated to have a continued critical role in CRPC. Mechanisms proposed for the continued transcriptional activity of the AR in spite of castrate levels of circulating testosterone include residual androgens from various sources that include adrenal glands and the tumor itself,

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increased levels of AR or altered expression of coregulators of AR within at least a subset of CRPC, gain-of-function mutations in the AR ligand-binding domain (LBD) that render antiandrogens agonistic, ligand-independent activation of the AR through its amino-terminal transcriptional domain (NTD) in the absence of ligand by alternative signaling pathways involving kinases and cytokines, and perhaps most importantly expression of constitutively active splice variants of AR that lack the LBD [4].

In 1994, Wilson and McPhaul [5] first described 2 forms, 110- and 87-kDa, of AR protein are present in human genital skin fibroblasts, they then revealed an 84-kDa AR forms in CWR-R1 PCa cell [6]. In 2002, Tepper et al. [7] reported a similar AR truncated variant occurring in 22Rv1 PCa cell which is characterized by a COOH-terminally truncated (CTD) AR isoform of 75–80 kDa. Six years later, Dehm et al. [8] described other AR isoforms are also expressed in 22Rv1 cells: a full-length version with duplicated exon 3 and 2 truncated versions lacking the CTD. Functionally, these AR isoforms are constitutively active and promote the expression of endogenous AR-dependent genes, as well as the proliferation of 22Rv1 cells in a ligand-independent manner. Since these initial reports, a number of AR splice variants (AR-Vs) have been identified in diverse cell lines, normal tissues, primary cancer, and metastases. In addition to the sequence features that distinguish the different variants, other features critical for clinical translation have been characterized, including their relative abundance, functional activity, and evidence for the corresponding protein product. On the basis of current studies, androgen receptor variant 7 (AR-V7) was determined to be the most important AR splice variant because it is the most abundant AR-Vs, its expression increases by ~20-fold in CRPC specimens, it is constitutively active in a ligand-independent manner, and it is detectable as a protein using variant-specific antibodies [9].

Identity of AR-V7

In 2009, Hu et al. [10] performed BLAST searches of AR intron sequences against the National Center for Biotechnology Information human expressed sequence tag database. High-quality hits were found in intron 1~3 but not in the remaining 4 introns. These transcribed "intronic" genomic fragments were considered as putative cryptic exons (CE). Hu et al. [10] focused on 3 putative CEs (CE1~3) in intron 3 to determine whether they were joined with the upstream exon 3 and 7 AR transcript variants, named AR-V1 to AR-V7, were constructed. Among these AR variants, RT-PCR analysis detected AR-V7 prevalently in CRPC samples. The full-length open reading frame of AR-V7 was then amplified from 2 clinical CRPC specimens and 22Rv1 cells. Hu et al. [10] further generated polyclonal antibodies, specifically against AR-V7. The antibodies recognized a single band of expected size (80 kDa) in VCaP and 22Rv1 cells. Moreover, AR-V7 protein was detected in human PCa xenografts, clinical CRPC specimens, and prostatectomy specimens. In the same year, AR-V7 was reported in another study in which AR-V7 was named as AR3. Using an antibody recognizing AR, Guo et al. [11] detected one 80-kDa band in the LNCaP derivative C-81, CWR-R1, and 22Rv1 cells. This short-form AR seemed to correspond to the truncated AR previously reported in CWR-R1 [6] and 22Rv1 cells [7]. They treated CWR-R1 and 22Rv1 cells with a panel of shRNAs targeting distinct regions of the AR gene. These shRNAs seemed to differentially knockdown AR and AR variants, suggesting that AR and AR variants may be translated from more than 1 transcript. These findings prompted Guo et al. [11] to clone possible alternative splice variants of AR by 3′RACE, and more than 20 splicing variants have been identified. Among them, AR-V7 which was predicted to encode a protein around 80 kDa was detected in a panel of human prostate tissues. They further developed a polyclonal antibody specific for AR-V7, specifically targeting the unique exon 3b of AR-V7, and AR-V7 protein could be detected in the androgen-insensitive cells, xenograft, and clinical specimens.

Origin of AR-V7

AR-V7 Presents in the Normal and Hyperplasia Prostate Tissues

Truncated AR mRNA and/or protein expression, in particular the AR-V7, has been shown to occur in normal and hyperplasia prostate [11–14], indicating that the mere presence of AR-V7 is unlikely to be carcinogenic. In benign prostate tissue, AR-V7 antibody mainly stained basal and stromal cells, but most of luminal epithelial cells were barely stained [11]. Other observations also suggest that there may be normal functions attributable to AR alternative splicing and synthesis of truncated AR isoforms [15–19]. The abundance of a specific splice variant is controlled by both gene transcription rate and splicing factor recruitment to the pre-mRNA during the alternative splicing process. One possible mechanism for the in-

crease in AR-V7 expression could be changes in expression or activity of factors that regulate AR splicing patterns. Current data suggested that ADT exerts a selective pressure favoring the expression of constitutively active AR-V7 in advanced PCa.

AR-V7 Expression Is Generally Linked with Gene Copy Number of Full-length AR

Full-length AR (AR-FL) and AR-V7 are both overexpressed in clinical CRPC specimens and induced in castrate conditions in CRPC xenografts, suggesting that increased AR-V7 levels may be coupled with enhanced transcription of the AR gene. Watson et al. [20] examined the AR-V7 expression in the VCaP and LuCaP35 xenograft models. Castration modestly increased both of AR-FL and AR-V7 mRNA, and quantitative analysis of AR-V7 mRNA levels relative to AR-FL revealed that AR-V7 levels range from 0.1 to 1.0% of AR-FL mRNA levels. They next addressed the same question in 10 clinical samples from patients with metastatic PCa and found similar AR-V7/AR-FL ratios ranging from 0.1 to 2.5%. These data indicate that AR-V7 expression is intimately linked to AR-FL levels, which is increased by approximately 10-fold in response to ADT, and, as such, AR-V7 expression is consequently also increased. AR gene copy number gain is considered an important determinant of AR-V7 mRNA levels in patients with CRPC metastases, although this observation alone does not explain why a proportion of encoded AR mRNAs become alternatively spliced.

AR Genomic Architecture Alteration Accompanies Enhanced AR-V7 Expression in PCa Cells

It was demonstrated that 22Rv1 cell exhibits significantly increased mRNA expression of AR-V7. The androgen-dependent CWR22Pc cell, which was derived from the same original CWR22 xenograft model as 22Rv1, was found to express extremely low but detectable transcript expression of AR-V7. Interrogation of AR gene structure demonstrated that the region harboring exon 2b, 3, and CE1–3 was present in the genome at 2-fold higher copy number in 22Rv1, but not CWR22Pc [21]. Importantly, long-term culture of the lineage-related CWR22Pc in the absence of androgen resulted in the outgrowth of a castration-resistant population of cells that harbored the exact same break fusion junction and repair signature as 22Rv1, and displayed increased expression of truncated AR-V7 mRNAs and proteins. A 48-kb deletion located in intron 1 of the AR gene was further identified in a subpopulation of CWR-R1 which expresses high levels of AR-V7 [22]. In addition, single cell cloning demonstrated that high-level AR-V7 expression was restricted to cells positive for this deletion [23]. Together, these data indicate that distant changes in the AR genomic architecture can cause splice switches that favor the expression of AR-V7.

AR Gene Structural Rearrangement and AR-V7 Expression: Needs Clinicopathologic Correlation

The most common documented alterations in the AR gene, occurring in ~60% of CRPC, are AR gene amplification or mutation. Henzler et al. [24] reported diverse AR genomic structural rearrangements (AR-GSRs) as a class of molecular alterations occurred in one-third of CRPC-stage tumor tissues. AR-GSRs occur in the context of copy-neutral and amplified AR and display heterogeneity in breakpoint location, rearrangement class, and subclonal enrichment in tumors within and between patients. However, there did not appear to be differences in the relative levels of AR-V7 or AR-FL mRNA in tumors that were positive for an AR-GSR event (n = 10) compared with tumors that were negative (n = 20) for an AR-GSR event. Henzler et al. [24] considered previous work demonstrating that AR-GSR-positive cells in PCa cell lines and patient-derived xenografts are often subclonal but represent the tumor cell fractions expressing high levels of AR-Vs and displaying androgen-independent growth. Under this scenario, Henzler et al. [24] suggested that subclonality of AR-GSRs would confound efforts to link discrete AR-GSR events to gene expression data from bulk tumor samples, in particular for a broadly expressed mRNA such as AR-V7.

Constitutive Alternative Splicing of AR-V7

Polyadenylation Specificity Factor Complex Is Essential for Formation of AR-V7 mRNA 3′ End

Van Etten et al. [25] reported that the splicing of AR-V7 is regulated by a single polyadenylation signal (PAS) in AR intron 3. They queried public datasets for 6 core genes encoding the cleavage and polyadenylation specificity factor (CPSF) complex: CPSF1, CPSF2, CPSF3, CPSF4, WDR33, and FIP1L1. Upon recruitment of this complex to pre-mRNAs, CPSF4 and WDR33 contact the PAS and CPSF3 cleaves mRNA downstream of the PAS, triggering polyadenylation. They observed that CPSF1 and CPSF3 mRNA levels were upregulated in primary PCa versus matched normal prostate tissue. Depletion of CPSF1 and CPSF3 led to reduced expression of AR-V7 and increased expression of AR-FL mRNA in 22Rv1-un-

dup3 cells which was an AR gene corrected 22Rv1 cells. Next, they knocked down CPSF1 in LNCaP95 cells, which further confirmed the increase in AR-FL and decrease in AR-V7 mRNA expression were not restricted to the 22Rv1 genetic background. Blocking this signal with morpholino technology or silencing of the polyadenylation factor CPSF1 caused a splice switch that inhibited expression of AR variants and blocked androgen-independent growth of CRPC cells.

Increased Recruitment of Splicing Factors Contributes to AR-V7 Splicing

Liu et al. [26] chose a panel of splicing factors, including U1A, U2AF65, AFS/SF2, hnRNP I, PSF, and p54nrb that were demonstrated to play essential roles in mRNA splicing. No changes in protein levels of these splicing factors were observed under ADT among different cell lines. Liu et al. [26] next determined whether recruitment of splicing factors to the AR gene was altered following ADT conditions. Chromatin immunoprecipitation (ChIP) assays were performed with primers amplifying the P1–P3 regions, corresponding to the 5′ and 3′ splice regions for AR and AR-V7. Consistent with ADT-induced AR gene transcription, the recruitment of pol II to P1, P2, and P3 regions were significantly higher in MDV-treated VCaP cells. These changes were concurrent with increased recruitments of several RNA splicing factors (U1A, U2AF, ASF/SF2, and p54nrb) to P1, P2, and P3 regions. They further demonstrated that the splicing proteins splicing factor U2AF65 and SRSF1 acted as "pioneer" factors, directing the recruitment of the spliceosome to SREs located adjacent to the 3′ splice site of AR-V7, thus increasing the expression of AR-V7 mRNA. Together, these results suggested that spliceosome recruitment to the AR gene contributed to AR-V7 splicing.

Aberrant Expression of Splicing Factors Play Pivotal Role in AR-V7 Generation

The role of the spliceosome in PCa is currently a major area of clinical research. Alternatively spliced variants of the AR that remain constitutively active in the absence of circulating androgens are currently the best-described splicing aberrations in patients with PCa. hnRNPA1 is overexpressed in prostate tumors compared with benign prostates, and the levels of hnRNPA1 and AR-V7 are positively correlated with each other in PCa. Regulatory circuit involving hnRNPA1 plays a central role in the generation of AR-V7 [27]. Tummala et al. [28] further reported that Lin28 promotes the development of resistance to currently used targeted therapeutics by enhancing the expression of AR-V7. They demonstrated that the upregulation of splicing factors such as hnRNPA1 by Lin28 may mediate the enhanced generation of AR-V7 in Lin28-expressing cells.

Shiota et al. [29] found 156 of 180 kinase phosphorylation sites, including RSK, were activated in CRPC cells, leading to increased phosphorylation of YB-1, which is a key molecule in the progression to CRPC [30]. At the mRNA and protein levels, AR-FL was not affected with YB-1 knockdown, while the AR-V7 was significantly decreased in 22Rv1 and VCaP cells. Inversely, expression levels of AR-V7 were increased by YB-1 overexpression at both mRNA and protein level suggesting that YB-1 phosphorylation by RSK specifically regulates AR-V7 splicing. Moreover, Nakata et al. [31] investigate the dynamics of AR splice variant generation using the JDCaP model that expresses AR-Vs under androgen depletion. They identified DDX39B as a regulator of AR-V7 mRNA expression. Simultaneous knockdown of DDX39B and its paralog DDX39A drastically and selectively downregulated AR-V7 mRNA expression in multiple AR-V7-positive PCa cell lines.

SF3B1, is a core spliceosomal protein that binds upstream of the pre-mRNA branch site and is thought to be required for the recognition of most 3′ splice sites. While alternatively spliced versions of the AR spliced at cryptic exon 3 have been implicated in the development of treatment resistance and disease progression in patients with CRPC, with the reported incidence of SF3B1 mutations in patients with PCa being in the region of 1% [32, 33], the contribution of SF3B1 mutations to treatment resistance through this mechanism could prove to be limited. Kawamura et al. [34] reported that SF3B2 is a critical determinant of AR-V7 expression. SF3B2 bound to AR exon 1 and CE3, the inclusion of which would lead to AR-V7 transcript. SF3B2 overexpression increased AR-V7 protein expression in 22Rv1 and LNCaP95 cells, while the AR-FL protein was not significantly affected.

Recently, Jiménez-Vacas et al. [35] measured the expression levels of 43 key spliceosome components and splicing factors in 2 cohorts of PCa samples: clinically localized PCa samples (n = 84) and highly aggressive PCa samples (n = 42). A profound dysregulation in the expression of multiple components of the splicing machinery were found in PCa compared to their nontumor adjacent regions. Notably, overexpression of SNRNP200, SRSF3, and SRRM1 were associated with AR-V7 expression in aggressiveness in PCa. Functional and mechanistic assays were performed in normal prostate cells and PCa cells in response to SNRNP200, SRSF3, and/or SRRM1 silencing

revealed an overall decrease in proliferation/migration rate in PCa cells through the modulation of key oncogenic splicing variants expression levels (e.g., AR-V7) and alteration of oncogenic signaling pathways.

Induction of AR-V7

AR-V7 Expression Is Hormone-Dependent

Watson et al. [20] examined the kinetics of AR-V7 expression in the VCaP and LuCaP35 xenograft models. No AR-V7 protein expression was detected in VCaP tumors grown in intact mice. However, a substantial increase in AR-V7 expression was detected after castration. Remarkably, this increase was completely extinguished following testosterone replacement. In contrast to VCaP, LuCaP35 xenografts expressed AR-V7 when grown in intact mice. Castration modestly increased AR-V7 mRNA, but these increases did not result in obviously higher protein levels. As with VCaP, testosterone replacement downregulated both AR-FL and AR-V7 mRNA levels. Consistently, Liu et al. [26] demonstrated that AR-V7 expression was reversibly regulated by DHT and MDV treatments in VCaP cells, and AR and AR-V7 mRNA levels were maintained in relative high levels under maximum ADT conditions, but significantly decreased when DHT was added. Meanwhile, Yu et al. [36] reported that AR-V7 was readily detectable and consistently increased in the abiraterone-resistant VCaP xenografts relative to levels in biopsies from the matched CRPC xenografts before starting abiraterone. The mean increase of AR-V7 expression was 53-fold during the development of castration resistance, whereas AR-FL was increased to a lesser extent (10-fold). Similarly, AR-V7 was increased approximately 3-fold in abiraterone-resistant xenografts relative to levels in castration-resistant xenografts before abiraterone, whereas AR-FL was less increased (1.4-fold). These results indicated that mRNA splicing of AR-V7 was a dynamic and reversible process. Meanwhile, AR-V7 expression is hormone-dependent and likely to be an acute response to castration therapy rather than a driver of castration-resistant clonal expansion.

AR-V7 Expression Is Differently Modulated by Medicine Treatment in Different PCa Cells

Li et al. [37] chose LNCaP, LNCaP95, 22Rv1, and VCaP cells to study AR protein expression under castration conditions, because they have representative genetic aberrations in AR and PTEN genes in addition to many other mutations in their genome. LNCaP, 22Rv1, and VCaP cells were cultured and then switched to androgen-deprived medium plus ENZ for maximum androgen blockade (MAB). LNCaP95 cells were routinely maintained in androgen-deprived medium and were treated with ENZ. Both endogenous mRNA levels of AR-FL and AR-V7 increased in all cells. However, these cells responded to MAB differently in AR-V7 protein expression. MAB rapidly reduced AR-FL and AR-V7 protein levels but increased pSer(213) of AR-FL and AR-V7 in PTEN-deficient LNCaP and LNCaP95 cells. In PTEN-sufficient 22Rv1 cells, where undetectable pAkt were expressed, MAB increased AR-FL and AR-V7 but reduced AR pSer(213) protein levels. In PTEN-sufficient VCaP cells, MAB induced AR-FL and AR-V7 protein expression without alteration of pSer(213) levels, suggesting enhanced AR-FL and AR-V7 protein expression in VCaP cells (bearing AR gene amplification) by MAB was likely related to increased AR gene transcription.

Signaling Pathways Involved in AR-V7 Regulation

NF-Kb Signaling Pathway

LNCaP cells stably expressing NF-κB2/p52 exhibited higher survival rates when treated with enzalutamide [38]. Meanwhile, C4-2B and 22Rv1 cells chronically treated with enzalutamide were found to express higher levels of NF-κB2/p52. Moreover, LNCaP cells expressing p52 exhibit higher expression of AR-V7 and downregulation of NF-κB2/p52 in VCaP and 22Rv1 cells abolished expression of AR-V7. These results collectively demonstrate that resistance to enzalutamide may be mediated by NF-kB2/p52 via activation of AR and AR-V7. Jin et al. [39] further reported that the activation of classical NF-κB signaling increases the expression of AR-V7 mRNA in LNCaP cells and converts androgen-sensitive cells to become androgen insensitive. Downregulation of NF-κB signaling inhibits AR-V7 expression in C4-2B and 22Rv1 cells and restores responsiveness of CRPC to antiandrogen therapy. In addition, they demonstrated that combination of antiandrogen with NF-κB-targeted therapy inhibits efficiently tumor growth of human CRPC xenografts. These results indicate that induction of AR-V7 by activated NF-κB signaling is a critical mechanism of PCa progression.

TGF-β Signaling Pathway

In 22Rv1 cells, exogenous administration of TGF-β induced Twist1 expression. Shiota et al. [40] examined Twist1 and AR transcript levels after TGF-β stimulation in 22Rv1 cells. Twist1 and both full-length and AR-V7 transcript were induced by TGF-β stimulation in a time-

dependent manner. Similarly, at the protein level, the expression of Twist1 and both AR-FL and AR-V7 were induced by exposure to TGF-β. Twist1 silencing using Twist1-specific siRNA abolished the TGF-β-induced upregulation of Twist1 as well as the production of both AR-FL and AR-V7 transcripts. In addition, Twist1 knockdown suppressed AR expression of both full-length and V7 forms at the protein level. These results demonstrated AR-V7 can be regulated by TGF-β/Twist1 pathway.

IGF Signaling Pathways

Canonical AR signaling and insulin-like growth factor (IGF) signaling pathways are tightly interconnected. A clinical phase II study published encouraging results for metformin, yielding an objective PSA response and inducing disease stabilization in a relevant proportion of CRPC patients [41]. Zengerling et al. [42] analyzed the effects of NVP-AEW541 (IGF-R inhibitor) on AR-V7 levels in 22Rv1 cells. In 22Rv1 cells, NVP-AEW541 diminished endogenous AR/AR-V7 levels. They next analyzed the effects of NVP-AEW541 on PC-3 cells transfected with expression constructs for AR-V7. Inhibition of IGF-R with NVP-AEW541 dose-dependently diminished androgen-response element (ARE) reporter gene activity in AR-V7-transfected PC-3 cells. These data suggest that the IGF/IGF-R axis is a modulator of AR-V7 signaling and provide a rationale for developing growth factor receptor targeting therapies for CRPC.

Epigenetic Regulation of AR-V7

MicroRNAs

miR-124 binds AR-V7 in the 3′ untranslated region (UTRs), and transfection of 22Rv1 cells with miR-124 downregulated the expression of AR-V7 [43]. Kumar et al. [44] further screened a library of 810 miRNA mimics to identify miRNAs that alter AR-V7 activity. miRNA-binding sites were found within the AR 3′ UTR and within the AR-V7 coding regions. 10 miRNAs (miR-30b-3p, miR-30c-5p, miR-30d-5p, miR-488-5p, miR-9-5p, miR-541-3p, miR-411-3p, miR-654, miR-138-5p, and miR-646) were capable of reducing expression of AR-V7 protein in VCaP cells. In addition, 5 miRNAs were found within the coding regions of AR-V7, and 3 miRNAs (miR-646, miR-371-3p, and miR-193a-3p) notably decreased AR-V7-GFP protein levels in PC-3 cell. Naiki-Ito et al. [45] performed in silico analysis using an miRbase Sequence Database to detect miRNAs that directly interact with AR-V7. miR-8080 was identified as an miRNA that is able to bind the 3′ UTR of AR-V7. Functionally, forced transfection of miR-8080 caused a decrease in AR-V7 and significantly suppressed cell proliferation by the induction of caspase-dependent apoptosis of 22Rv1.

lncRNAs

PCGEM1 was identified as a PCa-specific lncRNA that is capable of promoting proliferation and inhibiting apoptosis. PCGEM1 interacts with splicing factors hnRNPA1 and U2AF65 [46]. Moreover, ADT induces PCGEM1 and causes its accumulation in nuclear speckles. ADT-induced PCGEM1 regulates the competition between hnRNPA1 and U2AF65 for AR pre-mRNA and promotes PCGEM1 to interact with both hnRNPA1 and U2AF65 with different consequences. While the interaction of PCGEM1 with hnRNPA1 suppresses AR-V7 by exon skipping, its interaction with U2AF65 promotes AR-V7 by exonization. Moreover, Wang et al. [47] observed increased AR-V7 and the lncRNA Malat1 expression in established EnzR-PCa cell lines and in some PCa patients who received Enz treatment. They reported that Malat1 is indispensable for Enz-induced AR-V7 production in VCaP and EnzR-C4-2 cells and may function via interacting with SF2 to splice the AR transcript. Targeting the Malat1/AR-V7 axis resulted in altering the PCa resistance to therapy.

circRNAs

Greene et al. [48] assessed AR-V7 expression in an isogenic cell model of enzalutamide resistance and circRNA profiling was performed on the panel using a high throughout microarray assay. Of particular interest was circ0004870, which was downregulated in enzalutamide-resistant cells, decreased in cells that highly express AR, and decreased in malignant cells. The associated parental gene was identified as RBM39, a member of the U2AF65 family of proteins. RBM39 encodes a member of the U2AF65 family of proteins, and it has previously been shown that U2AF65 leads to expression of AR-V7 via the lncRNA and PCGEM1, binding to AR pre-mRNA38. These data suggest that hsa_circ_0004870, through RBM39, may play a critical role in the development of enzalutamide resistance through the regulation of AR-V7. Moreover, the expression of circZMIZ1 was higher in the plasma of human PCa than the paired benign prostatic hyperplasia (BPH) patients' plasma [49]. Moreover, in cultured PCa cells, knockdown of circZMIZ1 inhibited cell proliferation and caused cell cycle arrest at G1. Mechanistically, circZMIZ1 could increase the expres-

sion of AR and AR splice variant 7 (AR-V7), which may be partly contributed to the occurrence and development of PCa.

Histone Demethylation and Phosphorylation

KDM4B promotes AR-V7 expression and KDM4B is phosphorylated by protein kinase A under conditions that promote castration resistance, eliciting its binding to the splicing factor SF3B3 [50]. KDM4B binds RNA specifically near the 5′-CE3, upregulates the chromatin accessibility, and couples the spliceosome to the chromatin. KDM4B can function as a signal-responsive transacting splicing factor and scaffold that recruits and stabilizes the spliceosome near the alternative exon, thus promoting its inclusion indicating KDM4B-regulated alternative splicing as a pivotal mechanism for generating AR-V7.

ACK1 is a structurally unique NRTK (non-receptor tyrosine kinases) upregulated in primary PCa and CRPC [51]. Mahajan et al. [52] purified histones from 5 freshly frozen human CRPCs and subjected to mass spectrometry-based identification of posttranslational modifications. The tyrosine kinase ACK1 phosphorylated histone H4 at tyrosine 88 upstream of the AR transcription start site. WDR5/MLL2 complex reads the H4-Y88 phosphorylation marks and deposits the transcriptionally activating H3K4-trimethyl marks promoting AR transcription. Reversal of the pY88-H4 epigenetic marks by the ACK1 inhibitor (R)-9bMS sensitized naive and enzalutamide-resistant PCa cells and reduced AR and AR-V7 levels to mitigate CRPC tumor growth.

LSD1 substrates for demethylation are mono- and dimethylation at lysine 4 of histone H3 (H3K4me1 and H3K4me2). Regufe da Mota et al. [53] utilized cell line models of CRPC through overexpression of AR-V7 to test the impact of LSD1 inhibition on AR-V7 activation. Chemical inhibition of LSD1 resulted in reduced activation of AR-V7 in LNCaP and 22Rv1. Moreover, the histone demethylase JMJD1A functions as a key coactivator for AR by epigenetic regulation of H3K9 methylation. The AR-V7 protein level correlated positively with JMJD1A in a subset of human PCa specimens. JMJD1A knockdown reduced AR-V7 levels in PCa cells, while it had no effect on AR-FL [54]. Mechanistically, JMJD1A promoted alternative splicing of AR-V7 through HNRNPF, a splicing factor known to regulate exon inclusion. Knockdown of JMJD1A or HNRNPF inhibited splicing of AR-V7, but not AR-FL. Importantly, JMJD1A interacts with and promote the recruitment of HNRNPF to a CE3b on AR pre-mRNA for AR-V7 generation.

Conformation of AR-V7 Protein

AR-V7 Structure

AR comprises 4 discrete functional domains, namely, an NTD (encoded by exon 1), the sequence of which is highly variable and inherently disordered; a DNA-binding domain (DBD; exons 2, 3), which consists of a highly conserved 66-residue core made up of 2 zinc- nucleated modules, a hinge region (exon 4), and a carboxy-terminal LBD (exons 4–8), where the androgenic ligands testosterone and DHT bind. In the presence of circulating androgens, AR undergoes conformational changes and dimerizes with other ligand-bound AR subunits to form homodimers. Nuclear localization of the AR is dependent on the AR bipartite nuclear localization sequence (NLS), which is highly conserved between many nuclear receptors and contains 2 clusters of basic amino acids. The NLS is recognized by the transport adaptor proteins importin-α and importin-β, which regulate the shuttling of the AR homodimers into the cell nucleus. The importin-α–importin-β cargo import complex then moves through nuclear-pore complexes to the nucleus, where it is dissociated by the Ras family GTPase Ran, thus releasing the AR [55]. The NLS is also recognized and bound by dynein, a motor protein that interacts with cellular microtubules to enhance nuclear translocation of the AR via a cytoskeletal transport network. Once in the nucleus, the AR complex binds with DNA at specific sites known as AREs through its DBD to upregulate or downregulate the transcription of various genes. AR-V7 is a truncated isoform of the canonical AR-FL protein that lacks the LBD (encoded by exons 4~8) but retains the NTD (encoded by exons 1), which is responsible for the majority of AR transcriptional activity, the DBD (encoded by exons 2/3), which mediates AR dimerization and DNA interactions, and the CE3 (cryptic exons 3) which is a C-terminal sequence originating from introns 3. This confirmatory change has been shown to maintain AR-V7 in a constitutively active state in the absence of a ligand, resulting in persistent transcriptional activation and survival signaling in tumor cells. AR-V7 contains 2 clusters of basic amino acids in the COOH-terminal tail encoded by AR exon CE3, one of which aligns with the second basic amino acid cluster of the wild-type AR bipartite NLS.

Nuclear Imports of AR-V7 Are Microtubule Independent

AR-V7 was localized in the nuclei of 22Rv1 cells under androgen-depleted conditions, and the proportion of nuclear AR-V7 did not change on androgen stimula-

tion [10]. Using exogenously transfected AR-V7 in AR-negative PC-3 cells AR-V7 localized to the nucleus and induced PSA reporter gene expression in an androgen-independent manner [10]. Zhang et al. [56] investigated the nuclear translocation mechanisms of AR-V7. Enhanced green fluorescent protein (EGFP)-tagged AR-V7 were expressed in COS-7 cells and the localization of the fusion proteins was analyzed by fluorescence microscopy. Unlike EGFP-AR-FL, which required androgen stimulation for nuclear import, EGFP-AR-V7 spontaneously translocated to the nucleus. When docetaxel and paclitaxel were added to the culture medium following androgen stimulation, accumulation of AR-FL in the cytoplasm was observed after treatment. However, treatment with the taxanes had no effect on the subcellular distribution of AR-V7. They further performed fluorescence recovery after photobleaching (FRAP) assays with additional microtubule inhibitors. KX-01 is a novel peptidomimetic inhibitor of Src family of kinases, but also inhibits tubulin polymerization, and nocodazole causes microtubule disassembly. Once again, these drugs inhibited the nuclear import of AR-FL, but not that of AR-V7. Collectively, these results suggest the nuclear translocation of AR-V7 is not mediated by the microtubules. Moreover, FRAP assay was conducted in COS-7-transfected with EGFP-AR-V7 and treated with importazole, a specific inhibitor of importin β. AR-V7 was found to accumulate in the cytoplasm following importazole treatment suggesting that AR-V7 are imported to the nucleus by the importin α/β machinery.

Motif of Nuclear Localization

Alanine mutation of Lys-629 and Arg-631 in AR-V7, which both align to the second basic amino acid cluster in the AR-FL NLS, shifted AR-V7 expression from predominantly nuclear to a mixed nuclear/cytoplasmic pattern [57]. Conversely, alanine mutation of Lys-636 and Lys-639 in AR-V7 had no effect on nuclear localization. Chan et al. [57] explored whether dominant negative Ran Q69L, which prevents the carrier importin from releasing cargo into the nucleus, could alter subcellular distribution of AR-V7 variant. Indeed, dominant negative Ran Q69L caused a shift in AR-V7 expression to a mixed nuclear/cytoplasmic pattern. A similar effect of dominant negative Ran Q69L was observed on the nuclear K636A/K639A mutant version of AR-V7, but no effect was observed for the nuclear/cytoplasmic K629A/R631A mutant version, showing that the dominant negative effects of Ran Q69L were mediated through the Lys-629/Arg-631 motif. These data indicate that AR-V7 displays enhanced nuclear localization because amino acid residues Lys-629 and Arg-631 reconstitute the second half of the bipartite AR NLS. Notably, alanine mutations in these residues did not have the same magnitude of effect as for AR-FL, indicating alternate modes of nuclear import existed for truncated AR variants. Indeed, the K629A/R631A mutant version of AR-V7 displayed a paradoxical higher level of transcriptional activity on various AR-responsive promoters in AR-dependent LNCaP cells and AR-null DU145 cells, further confirming that the classical mode of AR nuclear import is not the main determinant of truncated AR-V7 activity.

AR-V7 Dimerization

AR-V7 and ARv567es, not only homodimerize and heterodimerize with each other but also heterodimerize with AR-FL [58]. Heterodimerization of AR-Vs and AR-FL was mediated by N- and C-terminal interactions and by the DBD of each molecule, whereas AR-V7 homodimerization was mediated only by DBD interactions. Notably, AR-V7 dimerization was required to transactivate target genes and to confer castration-resistant cell growth. Cato et al. [59] compared chromatin-binding sites shared by AR-FL and AR-V7 (n = 2,629) and sites occupied by AR-FL only (n = 4,737). They carried out sequential AR-FL/AR-V7 ChIP-re-ChIP experiments and detected positive signal enrich, suggesting a potential functional interaction of the 2 receptors. They next employed acceptor photobleaching fluorescence resonance energy transfer and observed strong signals for AR-FL/AR-FL and AR-FL/AR-V7 or AR-V7/AR-FL interactions, but not for AR-V7/AR-V7 homotypic interaction. Although these results reinforce a model of AR-FL and AR-V7 heterodimerization, they do not establish codependent binding on chromatin, as this is not required for the fluorescence resonance energy transfer signal. To further investigate chromatin binding, ChIP-seq of both AR variants was performed in shAR-V7 and shAR-FL LNCaP95 cells. KD of AR-V7 significantly reduced AR-FL chromatin binding in DHT condition. Concordantly, loss of AR-FL also reduced AR-V7 binding in both treatment conditions. Similar results were obtained in 22Rv1 cells, where codependent binding of AR-FL and AR-V7 was observed at ARE containing sites with high levels of both factors. Taken together, these results suggest that AR-FL and AR-V7 form heterodimers and can modulate their respective DNA-binding affinities.

AR-V7 Protein Degradation

Akt protein kinase phosphorylates serine 213 and 791 of AR-FL, activates Mdm2 ubiquitin ligase, and induces AR-FL protein degradation by proteasome pathway [60]. Protein phosphatase-1 (PP-1) was demonstrated to dephosphorylate only serine 650 and increase AR protein expression and activity [61]. However, AR-V7 does not have serine 650 and 791. Enzalutamide induces differential pSer(213) levels of AR-V7 protein depending on the gene context of cancer cells [37]. Independent to Akt activity, PP-1 regulates AR-v7 protein degradation through the proteasome pathway in both PTEN-sufficient 22Rv1 cells and PTEN-deficient LNCaP, LNCaP95, and PC-3 cells. Moreover, both PP-1 and Akt regulates AR-FL and AR-V7 protein stability through Mdm2 ubiquitin ligase. Mdm2 recognizes phosphorylated serine 213 of AR-V7 and induces AR-V7 ubiquitination and protein degradation. The balance between PP-1 and Akt activation governs AR-V7 phosphorylation status and activation of the Mdm2 ubiquitin ligase. These findings highlight the decisive roles of PP-1 and Akt for AR-V7 protein expression and activities when AR is functionally blocked. Meanwhile, the ubiquitin-proteasome system is suppressed in enzalutamide/abiraterone-resistant PCa [62]. AR/AR-V7 proteostasis requires the interaction of E3 ubiquitin ligase STUB1 and HSP70 complex. STUB1 disassociates AR/AR-V7 from HSP70, leading to AR/AR-V7 ubiquitination and degradation. Inhibition of HSP70 significantly inhibits prostate tumor growth and improves enzalutamide/abiraterone treatments through AR/AR-V7 suppression.

Aldo-keto reductase 1C3 (AKR1C3) was type 5 17 β-hydroxysteroid dehydrogenase and responsible for intratumoral androgen biosynthesis, contributing to the development of CRPC. Significant upregulation of AKR1C3 is observed in CRPC patient samples and derived CRPC cell lines. AKR1C3 increased AR-V7 expression in resistant PCa cells through enhancing protein stability via regulation the ubiquitin mediated proteasome pathway, and AKR1C3-reprogramed AR signaling in enzalutamide-resistant PCa cells [63]. Additionally, bioinformatical analysis of indomethacin-treated resistant cells revealed that indomethacin significantly activates the unfolded protein response (UPR) and suppresses AR/AR-V7 pathways. Targeting AKR1C3 with indomethacin significantly decreases AR/AR-V7 protein expression in vitro and in vivo through activation of the ubiquitin mediated proteasome pathway.

Coregulators Interacting with AR-V7

Coactivators Contribute to Transcriptional Activity of AR-V7

Vav3, a Rho GTPase guanine nucleotide exchange factor, is an AR coactivator that is upregulated in human PCa. Vav3 potently enhanced the transcriptional activity and nuclear levels of AR-V7 [64]. Coimmunoprecipitation revealed physical interaction between AR-V7 and Vav3. Magani et al. [65] further demonstrated that the Vav3 diffuse B-cell lymphoma homology domain interacted with the N-terminal region of AR-V7. FHL2 interacts with a variety of transcription factors known to be involved in tumor development. FHL2 and AR-V7 colocalized in the nucleus and 75% of CRPCs sections exhibited both nuclear FHL2 and AR-V7. AR-V7 bound to FHL2 in the 22Rv1 cell and ligand-independent coactivation of AR-V7 by FHL2 was constitutive [66]. FHL2 coactivation of AR-V7 following enzalutamide treatment was examined in DU145 cells that do not express endogenous AR. Stockley et al. [67] explored the role of the RNA-binding protein Sam68 on AR-V7 expression and transcription function. They show that Sam68 controls expression of exon 3b resulting in an increase in endogenous AR-V7 mRNA and protein expression in RNA-binding-dependent manner. They identify a novel protein-protein interaction between Sam68 and AR-V7 mediated by a common domain (NTD) shared with AR-FL. PIP5K1α is a lipid kinase similar to PI3K and acts on PI3K/AKT/PTEN pathways and thereby regulates cell survival and migration.

AR-V7 physically interacts with PIP5K1α and CDK1 through formation of protein–protein complexes in 22Rv1 cells [68]. Meanwhile, PIP5K1α and CDK1 influence AR-V7 expression also through AKT-associated mechanism-dependent on PTEN status. Aurora A kinase has been shown to be a coactivator of the AR, phosphorylating 2 residues within the N-terminal domain of the receptor. Knockdown of Aurora A reconfigures splicing of AR pre-mRNA to discriminately down-regulate synthesis of AR-V7, without effecting AR-FL mRNA; and as a consequence, AR-V-driven proliferation and survival of CRPC cells is markedly reduced [69]. DBC1 acts as a key regulator of AR-V7 transcriptional activity and stability in CRPC cells. DBC1 functions as a coactivator for AR-V7 and is required for the expression of AR-V7 target genes, including CDH2, a mesenchymal marker linked to CRPC progression [70]. DBC1 is required for recruitment of AR-V7 to its target enhancers and for long-range chromatin looping between the CDH2 enhancer and pro-

moter. Mechanistically, DBC1 enhances DNA binding activity of AR-V7 by direct interaction and inhibits CHIP E3 ligase-mediated ubiquitination and degradation of AR-V7 by competing with CHIP for AR-V7 binding, thereby stabilizing and activating AR-V7.

AR-V7 Interacts with Transcription Factors

Bohrer et al. [71] found that a putative transcription repression domain in the NH2 terminus of FOXO1 is dispensable for FOXO1 inhibition of the AR. Protein-binding assays reported that FOXO1 binds to the transcription activation unit 5 (TAU5) motif in the AR NTD, a region required for recruitment of p160 activators, including SRC-1. Forced expression of FOXO1 blocked the effect of SRC-1 on AR variants' transcriptional activity by decreasing the binding of SRC-1 to the AR NTD. Ectopic expression of FOXO1 inhibited expression of endogenous genes activated primarily by alternatively spliced AR variants in castration-resistant 22Rv1 cells. FOXO1 binds to the TAU5 motif in the AR NTD and inhibits ligand-independent activation of AR-Vs. In the same year, Mediwala et al. [72] analyzed potential signaling pathways associated with AR-V7 constitutive activation in PTEN(−) PC-3 and LNCaP cells. Analysis of the contributing mechanisms demonstrated the involvement of the PI3K/AKT/FOXO1 signaling pathway, and a significant reduction of AR-V7 constitutive activity under conditions of PTEN reactivation. These results suggested the PTEN/PI3K/AKT/FOXO1 was a potential pathway for inhibition of aberrant AR activation.

Forkhead box protein A1 (FOXA1 or HNF3A) recognizes specific chromatin sites and make them accessible to AR-FL [73]. This interaction has been localized to regions downstream of the DBD. FOXA1 may function to both facilitate and repress AR signaling at discriminate genomic loci. AR deletion mutants that are truncated after the DBD have diminished functional interaction with FOXA1. Thus, AR target genes that are sensitive to FOXA1 levels for regulation might be differentially responsive to AR-V7. Krause et al. [74] found that AR and AR-V7 have distinct activities on target genes that are coregulated by FOXA1. Using a motif search to identify cis-regulatory elements at AR-V7-solo sites, Cai et al. [75] reported AR-V7 binding and AR-V7-mediated activation at these unique targets do not require FOXA1 but rely on ZFX and BRD4. Knockdown of ZFX or BRD4 inhibition suppresses growth of CRPC cells.

Gli2, a transcription factor in the Hedgehog pathway, is overexpressed in CRPC. Li et al. [76] localized the regions of AR-Gli2 protein interaction and determined the domains within Gli2 needed for AR coactivation. Results showed Gli2 binds the tau5/AF5 ligand-independent activation domain in the AR N-terminus. Gli2 interaction with AR tau5/AF5 was further substantiated by the ability of Gli2/Gli2-CTD to co-activate truncated AR-V7. The gonadoblastoma gene, testis-specific protein Y-encoded (TSPY), on the Y chromosome and its X-homolog, TSPX, are cell cycle regulators and function as a proto-oncogene and a tumor suppressor, respectively, in human oncogenesis. TSPY and TSPX competitively bind to the AR and AR-V7, at their conserved SET/NAP domain, and exacerbate and repress the transactivation of the AR/AR-V7 target genes in ligand-dependent and ligand-independent manners, respectively [77]. Chen et al. [78] defined high-resolution HoxB13 cistromes by performing HoxB13 ChIP-exo. Remarkably, integrative analysis of HoxB13 and AR-V7 revealed a precise colocalization between HoxB13 and AR-V7 genomic binding in cell lines. They explored the physical association of AR-V7 and HoxB13 with coimmunoprecipitation of the endogenous proteins. HoxB13 interacted with AR-V7 in vivo. They next expressed the FL AR-V7 and 4 regions of AR-V7 as GST fusion proteins and tested their ability to interact with in vitro-translated HoxB13. AR-V7 reported a strong interaction with HoxB13 protein via its DNA-binding domain.

AR-V7 Interacts with Transcriptional Repressors

Cato et al. [59] employed coIP in LNCaP95 cells following AR-V7 or AR-FL KD and observed a small but reproducible increase in binding of AR-V7 to NCOR1 and NCOR2 upon AR-FL depletion. This supports a model whereby AR-V7 preferentially interacts with specific transcriptional corepressors, such as members of the NCOR family. They observed that the transcriptional responses to siNCOR1 or siNCOR2 were strongly attenuated within the shAR-V7 and shAR-FL cell lines, whereas this was not the case in the control cell line (shGFP) containing both AR-V7 and AR-FL. This suggests that NCOR-mediated transcription is, at least in part, dependent on the presence of AR-V7 and AR-FL. Taken together, these findings suggest that AR-V7 mediates its repressive function by preferentially interacting with corepressors NCOR1 and NCOR2. Cato et al. [59] further utilized ChIP-seq to assess levels of histone H3K27 acetylation (H3K27ac), a mark of active enhancers and transcriptional activity. To correlate H3K27ac levels with AR function, they stratified the averaged H3K27ac signals at the AR sites using Pearson correlation and found many signals are mostly differentially regulated by AR-FL and

AR-V7. These signals were biased toward shAR-V7 upregulated and shAR-FL downregulated genes indicating that AR-V7 dependent gene repression is a consequence of AR-V7-mediated inhibition of H3K27ac. These data suggest that the AR-V7 repressive function stems from binding NCOR corepressors, which results in negative regulation of H3K27ac.

AR-V7 and AR-FL: Cooperation and Competition

Feedback between AR Protein and AR/AR-V7 Transcripts

AR-V7-dependent genes were found to be induced at low AR levels but repressed at high AR levels, and this observation mirrors the canonical biphasic androgen-stimulated growth response observed in cell line models [23]. Ligand-bound AR binds to an ARE located in intron 1 of the AR gene and recruits repressive lysine-specific demethylase activity, leading to AR-mediated feedback inhibition of AR transcriptional activity. ADT relieves this feedback inhibition, increasing AR transcriptional output and expression of AR-V7 and possibly AR-V7 [79]. In addition, the existence of an androgenic downstream repressor element in intron 2 of the AR gene has been shown to control receptor expression in VCaP cells. By recruiting the histone methyltransferase LSD1 to these loci in response to DHT, the receptor down-regulates AR gene transcription through demethylating histone H3 lysine 4 at upstream cis-regulatory elements [79]. In line with this, a series of preclinical studies demonstrated that AR-V7 mRNA expression is rapidly upregulated subsequent to ADT and can be reversed when AR-FL signaling is reactivated [20]. These data indicate that AR-V7 expression is acutely and reversibly regulated, perhaps by the way of androgen-regulated changes in AR transcriptional output, AR alternative splicing, or both. The rapid reversibility of these reactions suggests that expression of AR-V7 in this case cannot be accounted by clonal selection of cell populations with AR gene rearrangements. Interestingly, Cao et al. [80] reported AR-V7 bound to the promoter of its specific target without AR-FL, but co-occupied the promoter of canonical AR target with AR-FL in a mutually dependent manner. AR-V7 expression attenuated both androgen and enzalutamide modulation of AR-FL activity/cell growth and mitigated the in vivo antitumor efficacy of enzalutamide. This study highlights a dual function of AR-Vs in mediating castration resistance. In addition to trans-activating target genes independent of AR-FL, AR-V7 can serve as a "rheostat" to control the degree of response of AR-FL to androgen-directed therapy via activating AR-FL in an androgen-independent manner.

Constitutive Transcription Activity of AR-V7 Is Not Entirely Dependent on AR-FL

The nuclear localization patterns of AR-V7 were the same in AR-null Cos-7 and DU145 cells and AR-dependent LNCaP cells, indicating that nuclear localization is not affected by cell type or presence of AR-FL [57]. AR-V7 overexpression in LNCaP that contains AR-FL confers anchorage-independent growth in the absence of androgens [20]. However, this phenotype was reversed upon treatment with the LBD-dependent AR inhibitor, implicating a functional requirement for AR-FL. If AR-V7 requires AR-FL for activity, one would predict that depleting AR-FL using siRNA specific to AR LBD in a cell line that expresses functional variants lacking LBD would lead to abrogation of constitutive activity associated with variant expression. Contrary to this prediction, AR-FL is not required for variant transcriptional activity as shown by the application of LBD-specific siRNA in LNCaP cells that ectopically expressed AR-V7 [81]. Consistent with the conclusion that at least AR-V7 does not require AR-FL, variant-associated growth of 22Rv1 cells under androgen deprivation conditions is not dependent on AR-FL, as shown by knocking down its expression using 2 independent siRNAs targeting exons encoding AR LBD [82].

AR-V7 Has Few Long-Term DNA Interactions in the Absence of AR-FL

AR-V7 is reported to regulate a transcriptional program that is similar but not identical to that of AR. To further determine whether expression of the AR-V genes requires the presence of endogenous AR-FL, Hu et al. [83] generated stable LNCaP clones with or without endogenous AR-FL protein and transiently transfected these clones with AR-V7 in androgen-deprived conditions. In this independent series of expression profiles, the AR-V7 UP gene set remains as the top-ranked gene set induced by AR-V7 and the absence of endogenous AR-FL did not attenuate induction of the AR-V7 UP signature. Thus, the presence of AR-FL is not required for induction of cell cycle genes by constitutively active AR-V7. Cato et al. [59] further determined the AR isoform-specific chromatin-binding kinetics using FRAP. The experiment was performed using wild-type (WT; AR-FL or AR-V7) or DBD mutants of AR (AR-FL-R585K or AR-V7-R585K). Due to impaired DNA binding, the R585K mutant displays a

quicker recovery time after photobleaching compared with WT AR, as it does not form long-term interactions with DNA [84]. While this was the case for AR-FL, AR-V7WT and the R585K mutant FRAP signals were indistinguishable. This suggests that AR-V7, in the absence of AR-FL, has few long-term DNA interactions, further supported by the finding that AR-FL has a speckled nuclear distribution, which was not observed for AR-FL-R585K, AR-V7, or AR-V7-R585K.

AR-V7 Facilitated AR-FL Nuclear Localization

AR-V7 facilitated AR-FL nuclear localization in the absence of androgen and mitigated the ability of the antiandrogen enzalutamide to inhibit AR-FL nuclear trafficking [80]. Zhang et al. [56] further investigated whether AR-V7 mitigate the inhibitory effect of AR-FL nuclear translocation by docetaxel, they expressed EGFP-AR-FL with or without TurboFP635-tagged AR-V7 in the AR-null COS-7 cells. When co-expressed with TurboFP635, EGFP-AR-FL was retained in the cytoplasm following docetaxel treatment. However, in the presence of AR-V7-TurboFP635 or ARv567es-TurboFP635, the inhibitory effect of docetaxel was significantly attenuated. To further understand how AR-Vs circumvent docetaxel-mediated cytoplasmic sequestration of AR-FL, they conducted the microtubule-binding assay in COS-7 cells co-transfected with AR-FL and an AR-V7. The binding of AR-FL to the microtubules was markedly reduced when it was co-expressed with AR-V7. Taken together, these results suggest that the constitutively active AR-V7 could divert AR away from the microtubules and facilitate its nuclear translocation in a microtubule-independent manner.

Binding Sites in Chromatin of AR-FL and AR-V7 Largely Overlap

Cai et al. [75] mapped genomic binding of endogenous AR-V7 by ChIP-Seq in 22Rv1 cells. Without ligand, AR-V7 displayed significant chromatin occupancy across the genome. They found the AR-V7 binding in ligand-starved cells largely overlap that of DHT-stimulated AR-FL at 15,162 out of a total of 17,409 sites. These AR-FL/AR-V7 common sites were mainly at enhancers enriched with motifs of ARE and FOXA1. To further identify the direct chromatin targets of AR-FL and AR-V7, Cato et al. [59] examined their respective cistromes using ChIP-seq in LNCaP95 cells treated with and without DHT. They observed 3,497 binding sites for AR-V7 and 12,389 binding sites for AR-FL, in the absence of hormone. DHT treatment increased the AR-V7 cistrome 2-fold (n = 6,149) and the AR-FL cistrome 5-fold (>60,000 sites). Although a small number of sites were exclusive to the AR-V7 cistrome, most AR-V7 sites were contained within the AR-FL cistrome. In addition, they next compared the 2 AR cistromes (without DHT) with a cistrome using an N-terminal AR antibody that recognizes both AR isoforms. Peaks that were common between at least 2 of the 3 cistromes were defined as "high-confidence" AR-binding sites (n = 2,828). The remaining AR-V7-unique peaks (n = 595), unlike the "high-confidence" AR-V7 peaks, were only minimally affected by silencing of AR-V7, despite efficient protein reduction. Taken together, these results suggest that most of the exclusive AR-V7 peaks are in fact artifacts rather than bona fide AR-V7 chromatin-binding sites.

Transcriptional Activity of AR-V7

Transcription Activities of AR-V7 and AR-FL Are Similar but Not Identical

AR-V7 induced canonical androgen-responsive genes (KLK3, KLK2, NKX3-1,FKBP5, and TMPRSS2) in LNCaP cells [10]. Guo et al. [11] analyzed the differential gene expression resulted from AR-V7 knockdown by microarray analysis in CWR-R1 and 22Rv1 cells. A set of 188 genes was consistently detected in both cell lines when AR-V7 was specifically knocked down. Among them, 71 genes were commonly regulated by both AR and AR-V7. However, many classic AR-regulated genes, such as CLU, TMEPAI, PSA, and CLDN4 were not affected by AR-V7 knockdown. Meanwhile, the transcripts upregulated (PSA and TMPRSS2) or downregulated (PCDH11 and AR-FL) by AR-FL were also regulated by AR-V7 suggesting AR-V7 regulated canonical AR target genes [74]. However, AR-V7 was somewhat less effective than AR-FL in inducing TMPRSS2 and in repressing PCDH11. Moreover, R1881 did not induce expression of EDN2 or ETS2, and remarkably, both of these transcripts were upregulated by AR-V7 indicating that loss of the LBD was sufficient for the observed differences. Differential regulation of target genes correlated with preferential recruitment of AR or AR-V7 to specific cis-regulatory DNA sequences providing an explanation for some of the observed differences in target gene regulation.

AR-V7-Regulated Cistromes Are Heterogeneous in Different Cell Lines

To identify AR-V7-regulated genes contributing to CRPC progression, Chen et al. [78] performed an RNA-seq assay in hormone-depleted 22RV1 and LN95 cells

and found AR-V7 upregulated but not downregulated genes were involved in several cancer related processes and recurrence. Remarkably, while AR-V7 controlled similar biologic and disease pathways in 22RV1 and LN95 cell, it regulated distinct sets of genes in these 2 CRPC cell models reflecting the heterogeneity of AR-V7 driven CRPC. To investigate the underlying regulatory mechanisms for AR-V7-regulated gene expression in CRPC cells, they defined high-resolution AR-V7 cistromes in hormone-depleted 22RV1 and LN95 cells using ChIP-exo approach. Consistent with the distinct AR-V7 transcriptomes, AR-V7 cistromes were also heterogeneous between 22RV1 and LN95 cell. In addition to AR-V7-binding locations that overlapped with AR-FL-binding regions, AR-V7 preferred binding sites (63.2% for 22RV1 and 41.6% for LN95) were identified.

AR-V7 Endows Proprietary Transcription Fingerprints

Guo et al. [11] analyzed the differential gene expression resulted from AR-V7 knockdown by microarray analysis in CWR-R1 and 22Rv1 cells. A set of 188 genes was consistently detected when AR-V7 was specifically knocked down. Guo et al. [11] next identified at least 2 putative ARE sites in the AKT1 regulatory region and reported that AR-V7, but not AR, was able to bind to these ARE sites determined by ChIP assays, suggesting that AR-V7 may directly regulated AKT1 transcription. Hu et al. [83] further examined transcriptional changes driven by forced expression of AR-V7 in the presence or absence of AR-FL signaling by Gene Enrichment Analysis. Transient expression of exogenous AR-V7 in parental LNCaP cells induced expression of cell cycle genes, under both androgen-depleted and stimulated conditions. On the other hand, top gene sets increased by ligand-dependent AR-FL are dominated by those related to biosynthesis, metabolism, and secretion. Cai et al. [75] mapped genomic binding of endogenous AR-V7 by ChIP-Seq in 22Rv1 cells. They identified a significant portion of AR-V7 peaks (12.8%; 2,221 out of 17,409) lacking AR-FL binding. AR-V7-solo binding was mainly found at promoters, indicating a distinct recruitment mechanism. Transcriptome analysis identified 1,178 genes up and 648 downregulated by AR-V7. GSEA analysis support involvement of AR-V7 in activation of androgen-responsive, oncogenic, cell cycle progression, and cancer progression-associated genes. Integration of RNA-Seq and ChIP-Seq data identified 475 of AR-V7-activated genes as direct AR-V7 targets in 22Rv1 cells. They also found 329 transcripts uniquely or preferentially upregulated by AR-V7, compared to AR-FL. To better delineate AR-V7 from AR-FL function, Cato et al. [59] profiled gene expression in the inducible shRNA cells by RNA-seq. They observed a higher number of upregulated (n = 300) compared with downregulated genes (n = 129) in response to AR-V7 depletion, suggestive of a preferentially repressive AR-V7 transcriptional function. Some AR-FL-activated pathways overlapped with AR-V7-repressed ones, while others were unique, with an unclear discernible pattern. A direct comparison of AR-FL- and AR-V7-dependent transcriptomes in the absence of hormone revealed significant differences between AR-FL and AR-V7 transcriptomes, with no significant correlation between them, although expression of some genes, including the canonical AR targets KLK-2, KLK-3, and IGF-1, was activated by AR-V7 and AR-FL. This observation conflicts with the hypothesis that AR-V7 simply acts as a constitutively active form of AR-FL but instead suggests that AR-V7 and AR-FL have different transcriptional roles in CRPC.

Roles of AR-V7

AR-V7 Acts as a Driver of PCa Initiation in Transgenic Mouse Model

Sun et al. [85] established a transgenic mouse model (AR3Tg) with targeted expression of the constitutively active and androgen-independent AR-V7 in prostate epithelium. They found that overexpression of AR-V7 modulated expression of a number of tumor-promoting autocrine/paracrine growth factors (including TGF-β2 and IGF-1) and expands prostatic progenitor cell population, leading to development of prostatic intraepithelial neoplasia. In addition, they reported that some epithelial-mesenchymal transition-associated genes are upregulated in AR3Tg prostates, suggesting that AR-V7 may antagonize AR activity and halt the differentiation process driven by AR and androgen. This notion was supported by the observations that the number of CK5(+)/CK8(+) intermediate cells was increased in AR3Tg prostates after castration, and expression of AR-V7 transgene in these intermediate cells compromises prostate epithelium regeneration upon androgen replacement. These results demonstrated that AR-V7 was a driver of PCa, at least in part, through modulating multiple tumor-promoting autocrine/paracrine factors.

AR-V7 Is a Driver of Progression in PCa Cell Models

Overexpression of AR-V7 was sufficient to confer ligand-independent growth of androgen-dependent LNCaP

cells [8, 11], whereas knockdown of AR-V7 in hormone-resistant 22Rv1 and CWR-R1 cells attenuated their growth under androgen-depleted conditions, suggesting an indispensable role of AR-V7 in ablation-independent growth of PCa cells [22, 23]. Importantly, it was known that AR-V7 can be acutely induced following suppression of AR-FL in 2 cell lines, VCaP and LNCaP95 [83]. Corroborating the functional distinctions between AR-FL and AR-V7, endogenous induction of AR-V7 was accompanied by genome-wide changes of gene expression consistent with a shift of AR signaling mediated by AR-V7 [83]. Therefore, constitutively active AR-V7 appeared to mediate a broader function than simply another mechanism to "rescue" canonical AR signaling. Collectively, these studies reported that although AR-V7 was expressed at levels that were substantially lower than those of AR-FL, it was sufficient to mediate a shift toward a transcriptional program mediated by AR-V7 when AR-FL was suppressed.

Represses Negative Regulators of Tumor Proliferation

Cato et al. [59] found that both AR-FL-dependent gene activation and AR-V7 dependent gene repression were prominent features of CRPC. To elucidate the function of AR-V7 repression, they analyzed 57 candidate genes from a genome-wide CRISPR knockout screen in LNCaP95 cells. They detected 4 AR-V7-repressed genes with a negative effect on CRPC cell proliferation (SLC30A7, B4GALT1, HIF1A, and SNX14), indicating that these genes may have antitumor functions. In support of the potential clinical importance of this finding, they observed that PCa patients with low expression of the 4 genes were at greater risk of disease recurrence than patients with high expression of all 4 genes. Moreover, expression of the 4 genes was lower in metastatic than in primary disease. Similarly, B4GALT1, SLC30A7, SNX14, and HIF1A expression was negatively correlated with metastasis development and PCa-specific mortality. Taken together, these results suggested that AR-V7 promoted CRPC progression by repressing genes that negatively regulate tumor growth, and were associated with poor PCa prognosis.

Differential Regulates Metabolic Signaling Pathways

Using LNCaP cells with inducible expression of AR-V7 as a model system, AR-V7 stimulated glycolysis measured by extracellular acidification rate was similar to AR [86]. However, further analyses using metabolomics and metabolic flux assays revealed several differences. Whereas AR increased citrate levels, AR-V7 reduced citrate mirroring metabolic shifts observed in CRPC patients. Flux analyses indicated that the low citrate was a result of enhanced utilization rather than a failure to synthesize citrate. Moreover, flux assays suggested that compared to AR, AR-V7 exhibited increased dependence on glutaminolysis and reductive carboxylation to produce some of the tricarboxylic acid cycle metabolites.

Promoting Metastatic Potential through NF-Kb/IL-6 Signaling Pathway

Several lines of evidence suggest that IL-6 is a key mediator in the acquisition of metastatic potential in PCa. In LNCaP and 22Rv1 PCa cells transiently overexpressing AR-V7, NF-κB was activated and could result in upregulated IL-6 gene expression, indicating a positive interaction between AR-V7 expression and activated NF-κB/IL-6 signaling in CRPC pathogenesis [87]. Interestingly, both AR-V7-induced NF-κB activation and IL-6 gene transcription in LNCaP and 22Rv1 cells could be inhibited by melatonin. Furthermore, stimulation of AR-V7 mRNA expression in LNCaP cells by betulinic acid, a pharmacological NF-κB activator, was reduced by melatonin treatment. These data support the presence of bidirectional positive interactions between AR-V7 expression and NF-κB activation in CRPC pathogenesis.

Promotes Epithelial-to-Mesenchymal Transition and Cancer Stemness

N-cadherin expression in LNCaP cells was increased in the presence of constitutively active AR-V7, and this result was confirmed in C4-2B cells [88]. In addition to the increased expression of N-cadherin, an upregulation of other mesenchymal markers expression such as VIMENTIN, SNAIL, and ZEB1 was observed in the presence of AR-V7 suggesting constitutively active AR-V7 on the regulation of mesenchymal markers in PCa. Overexpression of AR-V7 not only led to increased expression of fibronectin and ZEB1 in LNCaP and DU145 cells, respectively, but also increased the expression of stem cell markers such as Nanog in LNCaP cells and Lin28B in DU145 cells which were consistent with increased mRNA expressions of these markers [89]. In contrast, downregulation of AR-V7, but not AR through transfection with siRNA reduced the expression of Nanog, Oct4, and ZEB1 in 22RV1 cells, which normally express high levels of AR-V7.

Conclusion

AR-V7 has been implicated in the progression of advanced PCa. AR-V7 is a truncated isoform of AR, a subset of which lack a LBD and remain constitutively active in

the absence of circulating androgens, thus promoting cancer cell proliferation. Currently, AR-V7 have been proposed not only to contribute to the resistance to anti-androgen therapies but also to be associated with unfavorable clinical outcomes. However, our understanding of the biology of the AR-V7 and how it contributes to PCa remains incomplete, as reflected in the dearth of AR-V7 targeted therapeutic agents. To date, attempts to specifically inhibit the formation of AR-V7 have been unsuccessful and novel therapeutic strategies are needed to address the oncogenic effects of AR-V7, which can drive lethal forms of PCa. Nevertheless, disruption of alternative splicing of AR-V7 mRNA, functional conformation of AR-V7 protein, interaction between AR-V7 and co-regulators, and transcriptional activity of AR-V7 are potential therapeutic avenues of advanced PCa.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

All the authors contributed to the preparation of this manuscript. Ye Chen was responsible for searching the literature, collecting the data, analyzing article, and drafting the first copy. Tian Lan was responsible for confirming the topic; fine tuning the manuscript, figures, and legends, final editing; and preparation of the manuscript for submission. All authors read and approved the final manuscript.

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